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VOLATILE COMPOUNDS IN THE AROMA OF THREE SPECIES OF WOOD-ROTTING BASIDIOMYCETES AND THEIR ANTIFUNGAL POTENTIAL

Cristiana Virginia PETRE[1*], Alin Constantin DÎRȚU[2], Marius NICULAUA[3], Cătălin TĂNASE[4]

Abstract: This study aims to determine the volatile organic compounds synthesized by three species of wood-rotting basidiomycetes: *Coriolopsis gallica*, *Megacollybia platyphylla* and *Lentinus arcularius* and test their antifungal potential. The species were cultivated on liquid media and kept for 25 days at 25 °C. The surface cultures were then homogenized, filtrated and extracted using solid-phase extraction and analyzed by GC-MS. The volatile compounds identified were mainly alcohols, ketones, aldehydes and terpenes. The most common volatiles identified in the experiment are: 1-octen-3-ol, 3-hexanol, 3-methyl-1-butanol, 3-octanone, 2-hexanone, benzaldehyde, and limonene. The volatiles metabolites of these species were tested for their antifungal activity using the bi-compartmented Petri dishes method against two species of plant pathogenic fungi: *Fusarium solani* and *Sclerotinia sclerotiorum*, on three media. The volatiles produced by *Coriolopsis gallica* showed the highest antifungal potential against the phytopathogens. The results revealed the importance of media composition in the synthesis of antifungal volatile compounds.

Key words: antifungal compounds, *Coriolopsis gallica*, *Lentinus arcularius*, *Megacollybia platyphylla*, volatile metabolites.

Introduction

In their ecological niches, fungi interact with different organisms and during these interactions cooperation or competition relations are established. Fungi communicate intra- and inter-specifically using several chemical signals that can spread in the substrate and act in the near vicinity of the mycelia or are propagated through the air, generating a signal that can be perceived at longer distances [WHEATLEY, 2002].

Responsible for the long distance communication are the volatile molecules, secondary metabolites synthesized by fungi as mixtures of chemical compounds such as alcohols, ketones, aldehydes and aromatic compounds that have specific smells and can be perceived by other organisms. Within the ecosystems, the fungal volatile compounds are involved in various processes: intra- and inter-specific communication [MORATH & al. 2012], defense [HANSON, 2008] and signaling [FÄLDT & al. 1999; DRILLING & DETTNER, 2009].

[1] "Alexandru Ioan Cuza" University of Iași, "Anastasie Fătu" Botanical Garden, 7-9 Dumbrava Roșie, 700487, Iași – Romania.
[2] Romanian Academy – Iași Branch, Oenological Research Center, 8 Carol I., 700506, Iași – Romania.
[3] "Alexandru Ioan Cuza" University of Iași, Faculty of Chemistry, 11 Carol I, 700506, Iași – Romania.
[4] "Alexandru Ioan Cuza" University of Iași, Faculty of Biology, 20A Carol I, 700505, Iași – Romania.
[*] Corresponding author. E-mail: criss_petre@yahoo.com

Many studies proved the role of these volatile molecules as insect attractants or repellents [BORG-KARLSON & al. 1994; JONSELL & NORDLANDER, 1995; WOOD & al. 2001; DE BRUYNE & BAKER, 2008]. LARSEN & FRISVALD [1995] demonstrated that specific types of volatile compounds are sometimes produced by certain phylogenetic lines, in certain concentration and therefore can be used as taxonomical criteria. The quality and quantity of these volatile molecules vary depending on several factors such as: media composition and pH [BRUCE & al. 2000; WHEATLEY, 2002; EWEN & al. 2004], temperature and water content [TRONSMO & DENNIS, 1978; JELEŃ, 2002], genera, species and presence of other organisms [GRIFFITH & al. 1994; DE JONG & FIELD, 1997; HYNES & al. 2007], age and development stage of the mycelia [JELEŃ, 2002; WU & al. 2005]. FÄLDT & al. (1999) mention that during sporulation, *Fomes fomentarius* and *Fomitopsis pinicola* synthesize higher quantities of volatiles and when the water content is low and the temperature is high the concentration of these molecules is low. RÖSECKE & KÖNIG (2000) demonstrated that compared to the rest of the fruiting body, the mycelia from crust produces high quantities of terpenes.

The volatile compounds synthesized by the fruiting bodies differ from the ones produced by the *in vitro* cultures. Moreover, *in vitro* observations revealed that depending on the media composition, the volatiles produced by wood-rotting fungi vary both as aroma and as intensity [PETRE & TĂNASE, 2013a, 2013b], fact that underlines the importance of nutrients in the synthesis of volatile molecules [KAHLOS & al. 1994; BJURMAN, 1999].

Both *in vivo* and *in vitro* through volatile metabolites, the mycelia that colonize the same substrate influence each other, inducing morphological and physiological changes [WHEATLEY & al. 1997; HEILMANN-CLAUSEN & BODDY, 2005]. These molecules act either as inhibitors towards certain organisms (affecting the growth, biomass development and respiration process) or as metabolic stimulators [HUMPHRIES & al. 2001].

The specific properties of these secondary metabolites are used in various biotechnological processes, in the pharmaceutical industry as antibiotics, antivirals, antioxidants, immunostimulants [MUSILEK & al. 1969; ZHONG, 2004; LINDEQUIST & al. 2005; LUO & al. 2005; SMÂNIA & al. 2007; MORATH & al. 2012], cosmetic industry and perfumery - the volatiles with pleasant aromas [FRAATZ & ZORN, 2010; MORATH & al. 2012] or agriculture as antimicrobial compounds that can be used in the production of biopesticides [CLOUGH, 1993; LORENZEN & ANKE, 1998; PARK & al. 2003; MORATH & al. 2012].

This study aims to identify the volatile organic compounds synthesized by three species of wood-rotting fungi and to test their inhibitory activity towards two species of plant pathogenic.

Materials and methods

Fungal strains. The species *Coriolopsis gallica* (Fr.) Ryvarden, *Megacollybia platyphylla* (Pers.) Kotl. & Pouzar, *Lentinus arcularius* (Batsch) Zmitr. were collected from broadleaf forests from Iași county, Romania, and isolated on malt extract agar (MEA) within the Research Laboratory for Fungi with application in ecological reconstruction, Faculty of Biology, "Alexandru Ioan Cuza" University of Iași. The plant pathogenic species *Fusarium solani* (Mart.) Sacc. and *Sclerotinia sclerotiorum* (Lib.) de Bary were isolated from potato tubercles (*Solanum tuberosum*) and carrot roots (*Daucus carota* subsp. *sativus*). All fungi are maintained on MEA at a temperature of 4 ºC.

Solid-Phase Extraction. The wood-rotting basidiomycetes were cultivated on a liquid medium (KM), rich in macro- and microelements in order to stimulate the synthesis of volatile compounds, as described by KAWABE & MORITA (1993): 20 g×l^{-1} glucose, 2 g×l^{-1} peptone, 2 g×l^{-1} yeast extract, 0.25 g×l^{-1} KH_2PO_4; 0.25 g×l^{-1} $MgSO_4$×7 H_2O. After 25 days of incubation in the dark at 25 °C, the surface cultures were homogenized; 10 ml of homogenate were filtered and mixed with 20 ml of pure water and 1 µl of 4-hydroxy-4-methyl-2-pentanone was added as internal standard. The mixture was extracted on LiChrolut cartridges (Merck Millipore): EN (40-120 µm) 100 mg (bottom); RP-18 (40-63 µm) 200 mg (top). The cartridges were previously conditioned with 2×6 ml *n*-hexane, 2×6 ml dichloromethane, 2×6 ml acetone, 2×6 ml methanol and 2×6 ml pure water, allowing each solvent to pass completely before adding the next conditioning solvent. On the same filter, 10 ml of pure water mixed with 1 g NaCl, 1 g Na_2SO_4 and 1 g of KH_2PO_4 were passed to increase the ionic strength, in order to facilitate the extraction of the remaining compounds from the biomass. The filtrate was later passed over the same cartridges. The SPE cartridges were completely dried using compressed air and placed in a desiccator at 600 mbar for 24 h. Next, the cartridges were eluted with 1.5 ml of *n*-hexane, dichloromethane and acetone respectively and the eluate was dried on anhydrous sodium sulfate. Each extraction was performed in duplicate for every species. The eluents were collected in separate vials and analyzed by gas-chromatography with mass spectrometer detection (GC-MS).

GC-MS analysis. The GC-MS analysis of the samples was performed on a Shimadzu GC-MS 2010 equipped with a ZB WAXplus capillary column (10 m × 0.1 mm × 0.1 µm) operated in split mode injection (split ratio 1/10). The GC oven temperature was set from 35 ºC for 5 minutes, with an increase of 5 ºC/min to 220 ºC and hold for 5 minutes, with a total analysis time of 47 minutes. Helium was used as carried gas, with a total flow of 15.9 ml/min, column flow of 0.9 ml/min and a purge flow of 6 ml/min. The MS ionization source was operated in electron impact mode (EI) with the EI source temperature set at 200 ºC. The full scan mass-spectrums were acquired at every 0.1 seconds (equivalent with 5000 a.m.u), between 30-500 Da (m/z). The volatile and semi-volatile compounds from the extracts were identified by comparison with the NIST 2.0 database (applying a > 85% match as acceptance criteria), mass spectra and retention times from literature [KAWABE & MORITA, 1993; BREHERET & al. 1997; STROBEL & al. 2001].

Antifungal screening. The antifungal potential of the volatiles synthesized by the species of wood-rotting basidiomycetes was evaluated against *F. solani* and *S. sclerotiorum* using the bi-compartmented Petri dish method used by STROBEL & al. (2001) and SCHALCHLI & al. (2011, 2015), in such way that the two mycelia didn't come into contact and the inhibitory activity was only due to the volatile compounds.

The screening activity was carried out on three media with different compositions in order to test if and how various concentrations and sources of carbon, nitrogen and microelements influence the synthesis of antifungal volatiles: potato flakes malt extract agar (PFMEA): 20 g×l^{-1} potato flakes, 5 g×l^{-1} malt extract, 5 g×l^{-1} glucose, 15 g×l^{-1} agar (Merck); malt extract agar (MEA): 30 g×l^{-1} malt extract, 5 g×l^{-1} glucose and 15 g×l^{-1} agar (Merck) and KM medium: 20 g×l^{-1} glucose, 2 g×l^{-1} peptone, 2 g×l^{-1} yeast extract, 0.25 g×l^{-1} KH_2PO_4; 0.25 g×l^{-1} $MgSO_4$ × 7 H_2O and 15 g×l^{-1} agar.

The species of wood-rotting basidiomycetes were inoculated in one compartment of the plate and the plant pathogen in the other. The Petri dishes were wrapped in two layers of Parafilm and incubated in the dark at 25 ºC. Four replicates were made for every combination. The control plate contained only the plant pathogenic species inoculated in one of the

compartments. Measurements of the test and target colonies were made daily, until the pathogen's colony from the control plate completely covered the compartment. The inhibitory percentage was calculated for every plate: P = [C-T] × 100/C, where C represents the diameter of the control colony and T represents the diameter of the pathogen's colony exposed to the VOC synthesized by the test fungi [NIDIRY & BABU, 2005]. The medium inhibitory percentage (P_{med}) was calculated as the average value of all four replicates inhibitory percentages.

Results and discussion

The mass-chromatograms obtained for the three species of wood-rotting fungi contained large numbers of peaks and therefore it was necessary to establish several acceptance criteria. In order to identify the volatile compounds from the samples we compared the obtained results to data from NIST 2.0 library and data from literature regarding the retention times and mass spectra. Because for each species the sample was done in two replicates we subtracted the results obtained for the control sample from the chromatograms of each replicate. Also, the variability between the two replicates regarding the volatile compounds after the blank subtraction was expressed as relative standard deviation (RSD%) of the results from the two replicates and the results with the lowest variability (<30%) were accepted as positively identified compounds in the samples.

Tab. 1, 2 and 3 contain data regarding some of the volatile compounds identified in the *n*-hexane, dichloromethane (DCM) and acetone fractions and their retention times (RT, minutes).

Tab. 1. Volatile compounds identified in *Coriolopsis gallica* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	2.41	27,247.5	1.46	5-(2-methylpropyl)-nonane
	2.581	155,506	8.35	4,6-dimethyl-dodecane
	2.654	18,388.5	0.99	3-ethyl-3-methyl-heptane
	3.301	31,787	1.71	5-methyl-5-propyl-nonane
	5.259	118,889	6.39	limonene
	5.984	28,323	1.52	3-hexanol
	6.233	30,715	1.65	3-methyl-1-butanol
	7.064	36,246	1.94	2-heptanone
	9.309	80,301	4.31	4,6-dimethyl-dodecane
	10.115	46,800.5	2.51	2,6,11,15-tetramethyl-hexadecane
DCM	2.173	55,489	0.02	3-hexanone
	2.478	144,381	0.04	5-methyl-undecane
	2.533	98,938	0.03	2-hexanone
	3.156	23,808	0.01	2-methyl-2-pentanol
	3.502	38,824	0.01	4,4-dimethyl undecane
	7.074	21,810	0.01	4,6-dimethyl-2-heptanone
	9.101	121,688	0.04	4,6-dimethyl-dodecane
	16.633	85,612.5	0.02	4-hidroxy-3-methyl-2-butanone
Acetone	2.699	7,078.5	0.12	4-hidroxy-3-propyl-2-hexanone
	3.408	23,004	0.38	3-penten-2-one
	4.958	21,576	0.36	2,6-dimethyl-4-heptanone
	5.224	5,065,362	83.66	4-methyl-2-pentanol

Concerning the general aroma of the wood-rotting basidiomycetes, when cultivated on liquid KM media, *Megacollybia platyphylla* presented a sweet-almond, strong aroma that lasted for 8 weeks at 25 °C, the smell of *Coriolopsis gallica* culture was of rotting wood and lasted for 6 weeks, while for *Lentinus arcularius* we detected a mushroom-like odor that intensified with time and lasted for 8 weeks.

The mushroom-like aroma is given by 1-octen-3-ol, eight-carbon volatile produced *Megacollybia platyphylla* and *Lentinus arcularius* which is one of the most common volatile compounds synthesized by fungi [HANSON, 2008] and was mentioned as secondary metabolite in case of many species of basidiomycetes [BERGER & al. 1986a; GROSS & al. 1989; RAPIOR & al. 1996; GUEVARA & al. 2000a, 2000b; RÖSECKE & al. 2000; THAKEOW & al. 2008; ZIEGENBEIN & al. 2010].

Responsible for the almond aroma of *Megacollybia platyphylla in vitro* culture is the benzaldehyde also identified in the *Lentinus arcularius* samples, recorded by several authors in the volatile profile of other basidiomycetes [MAGA & al. 1976; BERGER & al. 1986a, 1987; GROSS & al. 1989; KAWABE & MORITA, 1993; FÄLDT & al. 1999; RÖSECKE & al. 2000; ZIEGENBEIN & al. 2006], volatile with different applications in biotechnology [MORATH & al. 2012]. Moreover, the sweet and fruity aroma of this species is also attributed to 3-octanone [BERGER & al. 1986b; COMBET & al. 2006] compounds identified in all three fractions.

Tab. 2. Volatile compounds identified in *Megacollybia platyphylla* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	5.28	84,761	18.19	limonene
	6.19	72,511.5	15.56	2-methyl-1-butanol
	7.07	6,870.5	1.47	4,6-dimethyl-2-heptanone
	7.28	119,694.5	25.69	3-octanone
	8.69	34,925.5	7.50	1-octen-3-one
	10.37	23,267.5	4.99	2,9-dimethyl-undecane
	14.20	57,599	12.36	benzaldehyde
DCM	2.83	38,091	9.62	2-methyl-1-propanol
	7.27	90,251	22.79	3-octanone
	8.70	37,740.5	9.53	1-octen-3-one
	13.41	130,521	32.96	1-octen-3-ol
	14.21	27,020.5	6.82	benzaldehyde
	15.79	27,499	6.94	linalool
	16.23	22,850.5	5.77	dihydro-4-methyl-2(3H)-furanone
Acetone	4.95	25,367	6.4	2-methyl-4-octanone
	6.88	26,613	6.72	2-hexanol
	7.33	25,952	6.55	3-octanone
	13.31	23,350	5.89	1-octen-3-ol

The ketone 3-penten-2-one from the extracts of *Lentinus arcularius* was mentioned by BERGER & al. (1986a, 1986b) in the volatile profile of *Bjerkandera adusta* and *Polyporus durus*. Also *Lentinus arcularius* synthesizes *in vitro* pantolactone, chemical

compound mentioned by GUEDES DE PINHO & al. (2008) as secondary metabolite produced by several edible fungi.

Few monoterpenes were identified in the samples, as BJURMANN (1999) noticed that on rich media fungi synthesize higher quantities of alcohols, while terpenes are produced on poor media.

Limonene, a monoterpene with a fresh, sweet-citrusy aroma [BREHERET & al. 1997] was produced by all three tested species and the literature mentions this compound as a metabolite synthesized by other species of wood-rotting basidiomycetes [RAPIOR & al. 1996; FÄLDT & al. 1999; RÖSECKE & al. 2000; ZIEGENBEIN & al. 2006, 2010].

Also linalool, another monoterpene was identified in the *Megacollybia platyphylla* samples this compounds contributing to the characteristic *in vitro* fruity aroma of this species.

Tab. 3. Volatile compounds identified in *Lentinus arcularius* extracts

Solvent	Retention times (min)	Average area	Contribution to the area	Compound
n-hexane	2.42	233,241	3.4	5-(2-methylpropyl)-nonane
	3.29	12,071.5	3.22	5-methyl-5-propyl-nonane
	5.28	183,381	2.68	limonene
	6.24	25,147	0.36	3-methyl-1-butanol
	6.82	13,582	3.62	2-hexanol
	7.07	33,384	0.48	2-heptanone
	11.4	21,045	0.3	nonanal
	12.7	15,929	4.25	2,6,10,15-tetramethyl-heptadecane
	21.9	30,505	8.13	hexadecane
DCM	2.45	61,971	0.02	3,7-dimethyl-decane
	2.49	56,280	0.02	2-hexanone
	5.39	30,955,358	12.43	4-methyl-2-pentanol
	5.99	249,271	3.63	3-hexanol
	6.23	27,627	0.4	3-methyl-1-butanol
	7.06	12,808.5	0.01	4,6-dimethyl-2-heptanone
	13.40	24,336	0.35	1-octen-3-ol
	14.2	33,293.5	0.01	benzaldehyde
	25.3	24,154	0.01	pantolactone
	30.94	22,039.5	0.01	phenol
	33.96	26,869.5	0.01	lactone
Acetone	2.61	9,555	0.12	4,6-dimethyl-dodecane
	2.7	26,350.5	0.34	2-hexanone
	3.4	19,056.5	0.25	3-penten-2-one
	3.54	87,211.5	1.12	4-methyl-3-penten-2-one
	5.24	7,632,185	98.17	4-methyl-2-pentanol
	6.88	31,781	0.46	2-hexanol
	26	116,622	1.7	3-buten-2-ol

Regarding *Megacollybia platyphylla*, STADLER & STERNER (1998) tested the antimicrobian activity of the fruiting bodies belonging to this species, obtaining positive results only against *Bacillus brevis*. PUJOL & al. (1990) successfully tested the antifungal

potential of the culture fluid against *Aspergillus fumigatus*, *Candida albicans* and *Candida tropicalis*. In our study, the antifungal activity of the volatiles emitted by this species was low, which means that the results obtained by PUJOL & al. (1990) were attributed to compounds other than volatiles.

As far as know there is no study that regarding the volatile profile of *Coriolopsis gallica* and no study that evaluates the antifungal potential of these secondary metabolites.

The antimicrobial potential of *Lentinus arcularius* extracts was successfully tested against several genera of pathogenic bacteria [SUAY & al. 2000; YAMAÇ & BILGILI, 2006] and fungi: *Aspergillus niger*, *Botryodiplodia theobroma*, *Alternaria brassica* and *Penicillium digitatus* [SRIVASTANA & SHARMA, 2011]. Regarding the metabolites produced by this species FLECK & al. (1996) isolated isodrimenediol, compound that showed a moderate antimicrobial activity, inhibiting the growth and development of several bacterial strains and yeasts, CABRERA & al. (2002) discovered the criptoporic and isocriptoporic acids, that tested negative for antimicrobial and antibiotic activities and OTAKA & ARAYA (2013) isolated two sesquiterpenes, but without testing their antimicrobial activity.

Tab. 4. Inhibitory percentages of the volatiles synthesized by the wood-rotting basidiomycetes against *Sclerotinia sclerotiorum*

Species	IP (%) on PFMEA	IP (%) on MEA	IP (%) on KM
Coriolopsis gallica	3.52%	4.41%	6.17%
Megacollybia platyphylla	2.94%	3.23%	3.52%
Lentinus arcularius	4.41%	5%	5.29%

The results obtained after the antifungal screening against *Fusarium solani* (Tab. 4) and *Sclerotinia sclerotiorum* (Tab. 5) showed that the higher inhibitory percentages were calculated on KM medium for all three species of wood-rotting basidiomycetes. The higher percentages against *Fusarium solani* makes this species more susceptible to the activity of the volatiles synthesized by the basidiomycetes.

Tab. 5. Inhibitory percentages of the volatiles synthesized by the wood-rotting basidiomycetes against *Fusarium solani*

Species	IP (%) on PFMEA	IP (%) on MEA	IP (%) on KM
Coriolopsis gallica	5.29%	6.76%	7.94%
Megacollybia platyphylla	4.11%	4.41%	4.7%
Lentinus arcularius	6.76%	6.76%	6.47%

The inhibitory activity of *Coriolopsis gallica* and *Lentinus arcularius* can be partially attributed to 3-methyl-1-butanol, alcohol characterized by a pungent alcoholic odor [GROSS & al. 1989; ABRAHAM & BERGER, 1994] which was identified in the volatile profile of several species of wood-rotting basidiomycetes [BERGER & al. 1986a; KAWABE & MORITA 1993; SCHLACHLI & al. 2011], but also in species such as *Saccharomyces cerevisiae* [FIALHO & al. 2010], *Muscodor albus* [STROBEL & al. 2001; EZRA & al. 2004] and *Trichoderma* spp. [WHEATLEY & al. 1997] known for their antifungal potential.

The antifungal screening was performed on three media, with different carbon and nitrogen sources, with or without microelements in order to determine the effect of media composition on the antifungal activity of the volatiles synthesized by the wood-rotting

basidiomycetes. The highest antifungal activity was measured on KM medium which is more complex than the other two media, having glucose as carbon source (20 g/l), peptone (2 g/l) and yeast extract (2 g/l) as nitrogen sources and several microelements such as: potassium and phosphorous (0.25 g/l KH_2PO_4), magnesium and sulphur (0.25 g/l $MgSO_4 \times 7H_2O$).

NORRMAN (1971) investigated the effect that different carbon sources have on the production of volatile compounds in yeasts, determining that glucose stimulated the synthesis of these molecules compared with fructose and glycerol. An increase in the glucose concentration also determined an increase in the quantity of volatiles produced by fungi. The results obtained in the case of the three species of wood-rotting fungi demonstrate that these conditions are valid also for these species of wood-rotting basidiomycetes.

WHEATLEY & al. (1997) monitored the influence of media composition on the synthesis of volatile compounds by species of *Trichoderma* and discovered a higher antifungal activity of these molecules on media with malt extracts (with the exact same composition as the one used in this study) compared with a minimal medium with 5 g/l glucose, asparagine and K, P, Mg, Cl, Fe, S, Mn, Zn, Cu and Ca mineral salts. Contrary, in our study, we recorded the highest inhibitory percentages against the two phytopathogens on the KM medium that had a higher concentration of glucose and mineral compounds (K, P, Mg and S).

Conclusions

The GC-MS analysis of the samples revealed the presence of VOCs such as alcohols, ketones, aldehydes, terpenes and other compounds. Some of the molecules are common fungal metabolites such as: 1-octen-3-ol, 3-octanone, 3-hexanone, 3-hexanol, 2-hexanol, 2-hexanone, 4,6-dimethyl-2-heptanone, 2-methyl-1-butanol, 3-methyl-1-butanol, benzaldehyde, linalool, limonene. These molecules are responsible for the aroma of the *in vitro* culture of the wood-rotting basidiomycetes and have biotechnological potential.

From our knowledge this is the first study that focuses on the volatile profile of *Megacollybia platyphylla* and *Coriolopsis gallica* grown on liquid media using SPE as the extraction method.

The volatile compounds synthesized by these species were tested for their antifungal potential against two plant pathogens: *Fusarium solani* and *Sclerotinia sclerotiorum*. The results of the screening revealed that the volatile metabolites synthesized by *Coriolopsis gallica* had the highest antifungal activity against the phytopathogenic fungi, fact that makes this basidiomycete a potential resource of bioactive compounds. Also the influence of the media composition was evaluated during this screening, highlighting the importance of the quantity and quality of the macro- and micro-elements in the synthesis of bioactive metabolites.

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