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SHORT COMMUNICATION

Growth Inhibition of Phytopathogenic Fungi and Oomycetes by Basidiomycete *Irpex lacteus* and Identification of its Antimicrobial Extracellular Metabolites

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Abstract

In dual culture confrontation assays, basidiomycete *Irpex lacteus* efficiently antagonized *Fusarium* spp., *Colletotrichum* spp., and *Phytophthora* spp. phytopathogenic strains, with growth inhibition percentages between 16.7–46.3%. Antibiosis assays evaluating the inhibitory effect of soluble extracellular metabolites indicated *I. lacteus* strain inhibited phytopathogens growth between 32.0–86.7%. Metabolites in the extracellular broth filtrate, identified by UPLC-QTOF mass spectrometer, included nine terpenes, two aldehydes, and derivatives of a polyketide, a quinazoline, and a xanthone, several of which had antifungal activity. *I. lacteus* strain and its extracellular metabolites might be valuable tools for phytopathogenic fungi and oomycete biocontrol of agricultural relevance.

Keywords: antagonism, antifungal, extracellular metabolites, mycelium, terpenes

Phytopathogenic fungi and oomycetes have caused significant losses in several crop production around the world (Dean et al. 2012; Kamoun et al. 2015). Disease control caused by these pathogens depends to a large extent on agrochemicals, which use has increased worldwide (Carvalho 2017); however, they have accumulated in the trophic chains, affecting wildlife and livestock and causing public health problems (Bourguet and Guillemaud 2016). On the other hand, phytopathogenic microorganisms have increased their resistance to several agrochemicals used to fight them (Sparks and Lorsbach 2017), so they have become less effective. Therefore, the use of alternative plant protection biocontrol methods has been intensively explored during the last decades. Biocontrol methods include both preparations containing living microorganisms and bioactive metabolites obtained from organic or aqueous extracts of different taxa, which may be specific to those that need to be controlled and have low toxicity to wild and human life (Loiseleur 2017).

Previous studies have documented efficient *in vitro* antagonism of basidiomycete species against phytopathogenic microorganisms, through the use of more than one mechanism (White and Traquair 2006; Gholami et al. 2019). A wide variety of extracts and metabolites obtained from basidiomycete fungi have shown antimicrobial activity, sufficient for growth inhibition of pathogenic microorganisms of medical and agricultural relevance (Shen et al. 2017). Due to the intrinsic ecophysiological role, metabolite production with antimicrobial activity by vegetative mycelium is relevant for species competition; hence, not only antimicrobial activity of biomass extracts but also extracellular filtrates obtained from liquid cultures of vegetative mycelium has been evaluated (Shen et al. 2017). Here we analyzed a wild *I. lacteus* strain antagonist activity against worldwide relevant phytopathogenic fungi and oomycete species. We also conducted a chemical characterization of extracellular filtrates of such strain aiming to identify putative metabolites with antifungal/antimicrobial activity. The

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I. lacteus strain CMU-8413 was isolated from basidiocarps collected in September 2013 from the community of Atécuaro, Michoacán, Mexico. Both data on collection and phylogenetic identification of the strain have been previously reported (Damián-Robles et al. 2017). The strain has been deposited in the Laboratorio de Conservación Microbiana y Biotecnología del Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia, de la Universidad Michoacana de San Nicolás de Hidalgo, and it may be available for research purposes upon request. The phytopathogenic strains tested correspond to fungi *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, and oomycetes *Phytophthora capsici* and *Phytophthora cinnamomi*. All these phytopathogens have been isolated from crop fields in Michoacán, and they have been properly identified and kindly provided by Dr. Sylvia P. Fernández-Pavía, of the Universidad Michoacana.

Antagonism in dual culture and antibiosis assays were studied according to Steyaert et al. (2016) in three independent replicas for each phytopathogen. The incubation time was adjusted as described below, and incubation temperature to 28°C. The inoculum of *I. lacteus* and phytopathogens for these assays consisted of a 6 mm plug obtained from the edge of an actively growing colony in PDA at 28°C. For antagonism assays, phytopathogens were inoculated at one extreme of a 90 mm Petri plate containing potato dextrose agar (PDA) medium, and they were incubated until the colony reached a 2 cm radius. At this stage, the PDA Petri plate opposite extreme was inoculated in the same way with the *I. lacteus* strain, and then incubation was resumed. The fungi colony diameter was measured every 24 h. A respective control of axenic cultures for each phytopathogenic strain was incubated in the same condition. For antibiosis assays, Petri plates containing PDA were covered with sterilized cellophane sheets, and they were inoculated with *I. lacteus* strain in the center. After a 4-day incubation, the cellophane sheets with the mycelium were aseptically removed. This same PDA plate, on which the *I. lacteus* strain previously grew, was used for phytopathogens inoculation and incubation in independent assays, and colony diameter was measured every 24 h. Each phytopathogenic strain was inoculated in a fresh PDA medium and was incubated under the same conditions as a control.

For antagonism and antibiosis, assays were finished when the mycelium of each phytopathogen in the control plate had covered 2/3 of the surface area; then, the colony diameter was measured expressing the growth inhibition percentage according to the formula:

$$\% \text{ inhibition} = [(D1 - D2)/D1 \times 100]$$

where D1 = the phytopathogen colony diameter growing in PDA (fresh/axenic), and D2 = the phytopathogen colony diameter growing in dual culture or in the medium that contained *I. lacteus* which had been previously incubated.

In antagonism in dual culture assays, *I. lacteus* showed significant growth inhibition against *F. pseudocircinatum* (46.3%), *F. mexicanum* (16.7%), *C. coccodes* (22.8%), *P. cinnamomi* (35.0%) and *P. capsici* (22.9%) (Fig. 1), and it was not able to antagonize *C. gloeosporioides*. Using the same test, White and Traquair (2006) found that *I. lacteus* showed efficient growth inhibition of *Botrytis cinerea*, and this previous work is the only antagonism study between *I. lacteus* and phytopathogenic fungi, in addition to results reported here. Antibiosis assays showed even better *I. lacteus* growth inhibitory activity against the six phytopathogenic strains tested, with growth inhibition percentages fluctuating between 32.0 and 86.7%. *C. coccodes* was the most susceptible species (Fig. 1). The antibiosis assay is used to determine if a fungal strain produces non-volatile metabolites that diffuse into the medium and affect phytopathogenic fungi growth (Steyaert et al. 2016). As far as we could document, there has been no previously published work on antibiosis assays that used basidiomycetes to test their *in vitro* activity against phytopathogenic fungi/oomycetes.

Antifungal activity of aqueous and organic extracts from basidiocarp and vegetative mycelium of *I. lacteus* (Shen et al. 2017) had been documented. However, reports evaluating the antifungal activity of extracellular broth filtrates from *I. lacteus* are scarce; consequently, in this work, it was of particular interest to assess such effect and identifying metabolites secreted by studied strain. The studied strain growth kinetic was conducted in 250 ml Erlenmeyer flasks with 50 ml of potato dextrose broth (PDB) medium, inoculated with six inocula obtained as previously described. The inoculated flasks were incubated at 125 rpm of orbital shaking and 28°C for 14 days. The mycelium dry weight was determined every 24 hours, and the CMU-8413 strain reached the stationary phase after a seven-day incubation (data not shown). Extracellular filtrates were recovered three days after the studied strain reached the stationary phase at the end of 10 days of incubation. Broth culture at the stationary phase was filtered through Whatman No. 1 paper. The filtrate broth was recovered and concentrated by evaporation to dryness in a rotary evaporator at 70°C, without adding any solvent. Concentrates obtained were stored for no more than one week in 1 ml vials at –4°C until biological assays were carried out. Compounds identification in culture filtrate of independent samples for each assay was performed by UPLC (Acquity™ Class I, Waters Corporation) coupled to an orthogonal QTOF mass spectrometer (Synapt™ G1, Waters Corporation), as described elsewhere (Varela-Rodríguez et al. 2019). MS data were

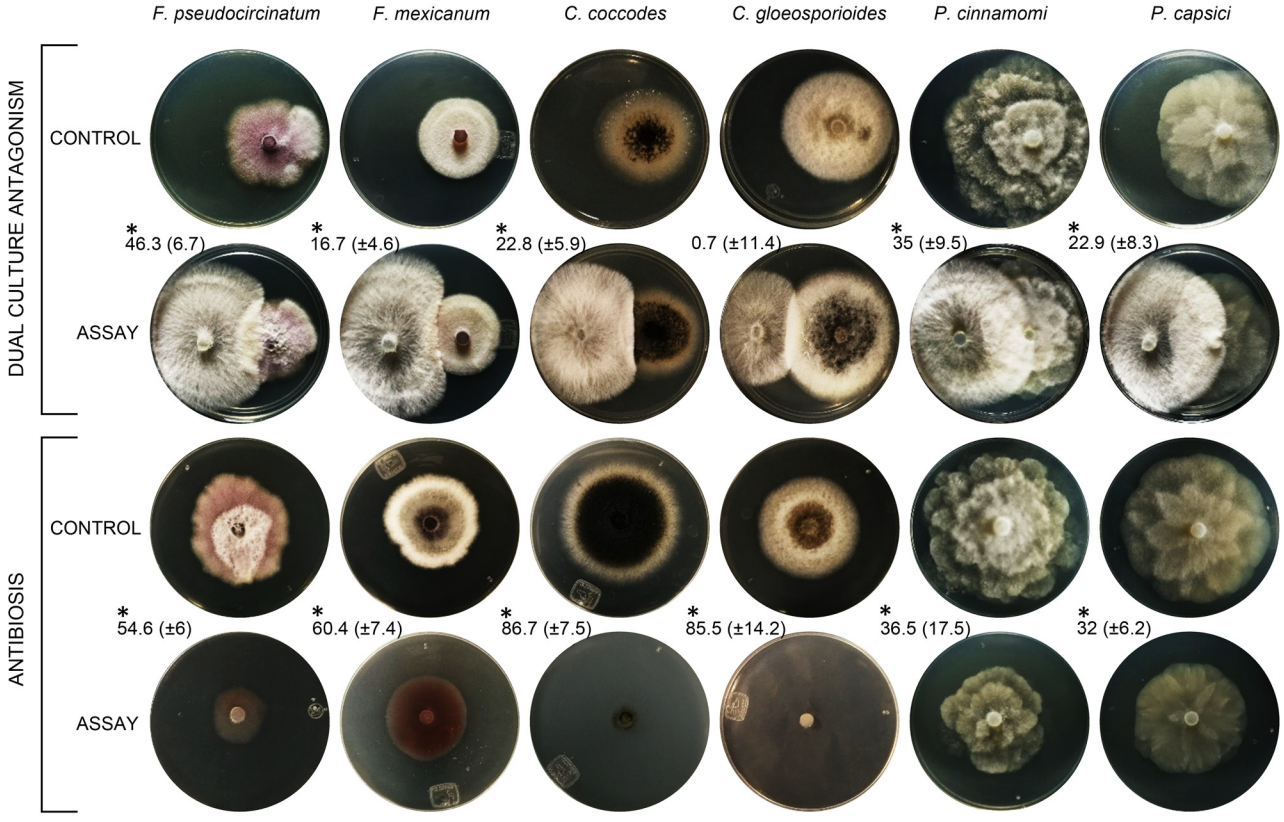

Fig 1. Phytopathogens growth inhibition in dual culture antagonism and antibiosis assays by *I. lacteus* (CMU-8413). In dual culture antagonism assays, *I. lacteus* was inoculated at the left. Tested phytopathogens were *Fusarium pseudocircinatum*, *Fusarium mexicanum*, *Colletotrichum coccodes*, *Colletotrichum gloeosporioides*, *Phytophthora capsici*, and *Phytophthora cinnamomi*. Assays were conducted in potato dextrose agar (PDA) medium at 28°C. Growth inhibition percentages are the mean of three independent assays and standard deviation (S.D.) is shown in parenthesis. Statistically significant ($p < 0.01$) growth inhibition values when compared with their respective control are indicated with an asterisk. Significance was determined by Student's *t*-tests, independent by groups, and they were carried out using STATISTICA data analysis software system (StatSoft, Inc. 2007, version 7. http://www.statsoft.com).

continuously acquired and processed with MassLynx® (version 4.1, Waters Corporation), and metabolites were putatively identified with Progenesis® QI for small molecules (Nonlinear Dynamics version 2.3, Waters Corporation) using Chemspider and Progenesis MetaScope as identification methods. The Progenesis QI software for small molecules considers precursor mass accuracy < than 5 ppm, fragmentation pattern, and isotopic similarities, each accounting for 20%. The maximum score is 60% (20 + 20 + 20), pre-identified metabolites have at least 50% of the total score.

A total of 14 extracellular metabolites (Table I) belonging to five chemical groups were found in the *I. lacteus* broth filtrate. The largest chemical group found was terpenes with nine metabolites, followed by aldehydes with two metabolites. The remaining metabolites found belong to polyketides, quinazolines, and xanthone derivatives, with one metabolite each. All metabolites found in *I. lacteus* extracellular broths were previously described in other basidiomycete or ascomycete species (Table II). Eight have been described as extracellular in broth media, like here. Terpenes produced by *I. lacteus* showed antifungal activity against *Nigrospora oryzae*, *C. gloeosporioides,* and *Didymella glomerata* (Wu et al. 2019). It has been previously documented among aldehydes that *I. lacteus* produces 5-pentenyl-2-furaldehyde, a potent antifungal volatile compound tested against the phytopathogens *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum fragariae,* and *B. cinerea* (Koitabashi et al. 2004). Also, a frequentin identified here in the *I. lacteus* broth is an aldehyde derivative that has been previously described as antifungal efficiently inhibiting spore germination in *Botrytis allii*, *Penicillium gladioli*, *Stachybotrys atra,* and *Mucor mucedo* (Curtis et al. 1951). Finally, microdiplodiasol is a xanthone derivative inhibiting the growth of fungal species *Microbotryum violaceum* (Siddiqui et al. 2011). Both, previous studies and extracellular metabolites secreted to the broth by the CMU-8413 *I. lacteus* strain allowed us to anticipate its efficient antagonism and growth inhibition of phytopathogenic fungi and oomycetes.

Phytopathogens growth inhibition assays conducted in solid PDA medium were used as indicative of extracellular inhibitory metabolites by CMU-8413

Table I
Extracellular metabolites produced by *Irpex lacteus* (strain CMU-8413) at stationary phase.

Compound name	Molecular formula	Observed m/z	Adduct	Main fragment ions m/z	Compound class
Apotrichodiol	$C_{15}H_{24}O_3$	251.1652	M-H	233.1547	Sesquiterpene
Apotrichothecene	$C_{15}H_{24}O_2$	201.1637	M+H-2H_2O	157.1481, 186.1403, 173.1348, 159.1185, 145.1029, 128.0639, 115.0558, 105.0711	Sesquiterpenoid epoxide
Blennin D	$C_{15}H_{22}O_4$	265.1436	M-H	237.1386, 221.1418, 205.1473, 191.1441, 187.1406, 175.1412	Sesquiterpene
Collybial	$C_{15}H_{20}O_2$	215.1424	M+H-H_2O/ M+H	187.1512, 173.1325, 159.0804, 157.1008, 142.0792, 128.0869, 115.0534	Sesquiterpene
Cyclocalopin A	$C_{15}H_{20}O_6$	295.1168	M-H	251.1160, 233.1149, 221.1123, 215.0924, 203.1027, 189.1142, 173.0818, 167.0634, 157.0526	Sesquiterpene
Dehydrooreadone	$C_{14}H_{18}O_3$	279.1218	M+FA-H	261.0987, 233.1046, 219.0902, 201.0804, 189.1178, 185.0502, 183.0663, 173.0874	Sesquiterpene
Dictyoquinazol A	$C_{17}H_{16}N_2O_4$	311.1090	M-H	267.1079, 237.0366, 205.1134, 193.1114, 187.1005, 175.1036, 159.0740, 151.0638, 149.0482	Quinazoline
Dihydromarasmone	$C_{15}H_{20}O_5$	279.1216	M-H	235.0976, 219.1390, 217.1131, 207.1277, 191.1342, 173.1250, 163.0871	Sesquiterpene
Frequentin	$C_{14}H_{20}O_4$	233.1181	M-H_2O-H	205.1234, 196.8934, 189.1195, 173.0864	Cyclohexanecarbaldehyde
Ganodermic acid Jb	$C_{30}H_{46}O_4$	453.3436	M+H-H_20	322.2571, 208.3768, 119.0945	Triterpene
Geosmin	$C_{12}H_{22}O$	203.1431	M+Na-2H	201.1177, 188.1100, 187.1010, 175.1426, 147.0792	Sesquiterpene
Microdiplodiasol	$C_{15}H_{18}O_7$	309.0948	M-H	265.0858, 203.0963, 193.1139, 187.0612, 175.1035	Xanthone derivative
Pandangolide 1	$C_{12}H_{20}O_5$	243.1242	M-H	203.6930, 181.1234	Polyketide
Piperdial	$C_{15}H_{22}O_3$	251.1617	M+H	233.1537, 215.1455, 205.1587, 191.1067, 187.1503, 177.0810, 159.1189, 145.1029	Unsaturated dialdehyde

Table II
The previous reports on extracellular metabolites produced by *Irpex lacteus* (strain CMU-8413) at stationary phase.

Metabolite	Fungal species	Source[a]	Bioactivity/Comment[a]	Reference
Apotrichodiol	*Fusarium* spp.	EM	mycotoxin	(Lebrun et al. 2015)
Apotrichothecene	*Fusarium* spp.	EM	mycotoxin	(Lebrun et al. 2015)
Blennin D	*Lentinellus cochleatus*	BE	inhibitor of leukotriene biosynthesis	(Wunder et al. 1996)
Collybial	*Collybia confluens*	EM	antibacterial, antiviral	(Simon et al. 1995)
Cyclocalopin A	*Caloboletus radicans*	BE	antioxidant, antibacterial	(Tareq et al. 2018)
Dehydrooreadone	*Marasmius oreades*	EM	NT	(Ayer and Craw 1989)
Dictyoquinazol A	*Dictyophora indusiata*/other basidiomycetes	BE	neuroprotective	(Lee et al. 2002)
Dihydromarasmone	*Marasmius oreades*	EM	antimicrobial	(Ayer and Craw 1989)
Frequentin	*Penicillium frequentans*/*Penicillium* spp.	EM	antifungal, antibacterial	(Curtis et al. 1951)
Ganodermic acid Jb	*Ganoderma lucidum*	ME	NT	(Shiao et al. 1988)
Geosmin	*Cortinarius herculeus*/*Cystoderma* spp.	BE	musty-earthy odor	(Breheret et al. 1999)
Microdiplodiasol	*Microdiplodia* sp.	EM	antifungal antibacterial	(Siddiqui et al. 2011)
Pandangolide 1	*Cladosporium marine*	EM	NA	(Gesner et al. 2005)
Piperdial	*Lactarius* spp./*Russula queletii*	BE	produced by damage	(Sterner et al. 1985)

BE – basidiocarp extract;
EM – extracellular metabolite produced by mycelium growing in broth;
ME – mycelium extract; NT – not tested as far as we know;
NA – not biological activity detected in conducted assays

I. lacteus strain. Based on such results, the identification of the extracellular metabolites was conducted in broth culture to ensure metabolites production in sufficient quantity to be identified by MS. However, it should be noted that the antagonistic and antibiosis assays performed in a solid medium might induce specific metabolite production, which was not produced in broth. In this regard, it has been previously documented that *I. lacteus* produces the antifungal terpenes conocenol B, 5-demethyl conocenol C, irpenigirin B, as well as 4-(4-dihydroxymethylphenoxy)benzaldehyde only by fermentation in the solid substrate but not in broth culture (Wu et al. 2019). Furthermore, while *I. lacteus* only produced the first compound in co-culture with the phytopathogen *N. oryzae*, the remaining three metabolites were produced both in axenic culture and in co-culture. Therefore, extracellular metabolites identified here were not necessarily the same as induced by antagonism in dual culture and antibiosis assays. Co-culture induction of specific metabolites may explain high percentages of fungal/oomycete growth inhibition found here in antagonism/antibiosis assays; however, these findings require further study. Hence, extracellular metabolites produced by the CMU-8413 strain described in this investigation may be considered basal, given that no induction conditions to increase its secretion were evaluated. Based on culture conditions described here, it may be suggested that gene clusters responsible for the synthesis of the identified extracellular metabolites were constitutively expressed or they were not subject to carbon catabolite repression (CCR). CCR and transcription factors associated with secondary metabolism were molecular and metabolic issues scarcely studied in basidiomycetes (Adnan et al. 2018; García-Estrada et al. 2018). Necessary physiological and metabolic studies, like those conducted here, could help to know what kind of metabolites may not be subject to this metabolic control.

It should be noted that extracellular metabolites produced by the CMU-8413 strain have a wide variety of pharmacological activities, besides antimicrobials previously described (Table II). For instance, apotrichodiol and apotrichothecene are neurotoxic mycotoxins (Lebrun et al. 2015), whereas dictyoquinazol A has been described as neuroprotective (Lee et al. 2002) and blennins are inhibitors of leukotriene biosynthesis (Wunder et al. 1996). So, it will be necessary to select favorable culture conditions to develop an enrichment protocol or use heterologous expression systems (Qiao et al. 2019) to obtain the desirable metabolites and avoid toxic molecules' synthesis. Further studies incubating the CMU-8413 strain in different induction conditions could show its potential to secrete a chemical diversity of metabolites useful for agriculture or other biotechnological applications.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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