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Candida (Pichia) guilliermondii CMGB 44 WITH VERSATILE ANTIMICROBIAL ACTIVITY

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Abstract

The yeast Candida (teleomorph Pichia) guilliermondii is a biocontrol agent intensively studied as an ecological alternative for fungicides in preventing decay of plants. The strain C. (P.) guilliermondii CMGB 44, characterized using morpho-physiological tests, was accurately identified using PCR-RFLP of the ITS1-5.8S rRNA-ITS2 region. The phylogenetic genotyping studies confirmed the molecular analysis. The antifungal tests using seven phytopathogenic strains from natural isolates, showed that C. (P.) guilliermondii CMGB 44 had high inhibitory activity against Sclerotium sp., Rhizoctonia solani and Botrytis cinerea. The antimicrobial activity was also tested at 25, respectively, 28°C against four potential pathogenic Candida strains, with relative low results. The activity against Candida krusei and Candida tropicalis was augmented by adding sodium bicarbonate solution in a final concentration of 2%.
C. (P.) guilliermondii CMGB 44 presented versatile antimicrobial activity against plant and human pathogenic microbial strains. Future research will aim improvement of the antagonistic activity using an extended range of species/strains.

Key words*: antimicrobial activity, Candida, Candida (Pichia) guilliermondii, fungi, improvement.*

INTRODUCTION

The yeast species *Candida guilliermondii* is largely distributed in nature, on plants, in soil and water, comprising also human pathogenic strains involved in candidemias, especially in immunocompromised patients. The species represents the asporogenic (asexual) state of the teleomorph yeast *Pichia* (*Meyerozyma*) *guilliermondii*, of high interest for its antifungal abilities against phytopathogenic fungi and also for its ability to overproduce riboflavin (Sibirny & Boretsky, 2009; Papon et al., 2013). Although during decades *C. guilliermondii* and *P. (M.) guilliermondii* were considered as genetically heterogeneous being composed of phenotypically undistinguishable taxa, molecular biology analyses and genome sequencing techniques showed that *P. guilliermondii* represents a separate clade formed by sporogenous strains previously classified in *C. guilliermondii*. Thus, phylogenetic studies place *P. (M.) guilliermondii* in the same clade as many *Candida* species (*C. albicans*, *C. tropicalis, C. dubliniensis* and the *C. parapsilosis* branch) (Pryszcz et al., 2015; Mixão et al., 2019).
The present research focuses on the molecular identification of a new *C. (P.) guilliermondii* strain, its antagonistic interactions with fungi from natural isolates and improvement of antimicrobial activity against potential pathogenic strains of *Candida* sp.

MATERIALS AND METHODS

1. PCR-RFLP of ITS1-5.8S rDNA-ITS2 region

For the genomic DNA extraction, 100 µl of 10^8 cells/ml from an overnight culture grown in YPG medium (5 g/l yeast extract, 10 g/l peptone, 2 g/l glucose) at 28°C, 150 rpm, were centrifuged 6 min at 6500 rpm and the cell pellet was resuspended in 100 µl solution of 1% SDS supplemented with 0.8 M lithium chloride (LiCl). The suspension was incubated for 15 min at 70°C and 300 µl of 95% ethanol was added. After vortexing briefly, the suspension was centrifuged for 5 min at 13000 rpm, the pellet was washed with 500 µl of 70% ethanol, centrifuged, and the sediment was resuspended

in 40 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with RNase A (40 µg/ml). After a new centrifugation, the debris was removed, the supernatant containing genomic DNA was preserved for 24 hours at 4ºC and used for PCR amplification. The DNA extracts were analysed by gel electrophoresis using 0.8% agarose and Tris-Borate-EDTA buffer [0.5X].

The PCR amplification of the ITS1-5.8S rDNA-ITS2 was performed in a final volume of 50 µl comprising: 2 µl genomic DNA samples, 1.2 µM of each primer ITS1 (5'TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) and GoTaq Green Master Mix 2X (Promega). The amplification program was: 5 min at 94ºC, 40 cycles of 1 min at 94ºC, 30 sec at 55ºC, 2 min at 72ºC, and a final extension 5 min at 72ºC. The amplicon was digested for 90 min with 0.5 µl of *Cfo* I (5'-GCG/C-3'), *Hae* III (5'-GG/CC-3'), *Hinf* I (5'-G/ANTC-3') and *Msp* I (5'-C/CGG-3') (izoschisomere *Hpa* II) (10 U/µl, Promega). The amplicon and the restriction fragments were analysed by gel electrophoresis using 1.7% agarose and 0.5X Tris-Borate-EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide, visualized under UV light (UV-VIS Spectrophotometer), digitalized and the size of the amplicon and restriction fragments was determined using the Quantity One program (Bio-Rad).

2. Phylogenetic genotyping

For the phylogenetic studies, reference strains used were: *Candida tropicalis* CMGB 165 (Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest), *Rhodotorula glutinis* CMGB 189, *Ogatea* (*Hansenula*) *polymorpha* CMGB 85, *Pichia pastoris* CMGB 167, *Yarrowia lipolytica* CMGB 32 and *Candida albicans* ATCC 10231. The ITS1-5.8S rDNA-ITS2 amplicons were obtained and then digested with *Hinf* I according to the methods described above. The phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA) from the Quantity One program (Bio-Rad).

3. Antifungal activity

For testing the antifungal activity of *C.* (*P.*) *guilliermondii* CMGB 44, we used seven fungal strains of *Alternaria mali, Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium* sp., *Botrytis cinerea, Aspergillus carbonarius, Monilinia* sp. from natural isolates and preserved in the Fungal Collection of the Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine of Bucharest. The fungi were grown for 48 hours on PDA medium (400 g/l potato infusion, 20 g/l glucose and 15 g/l agar). Blocks of about 7 mm of fungal cultures were then spoted in the middle of Petri plates covered with PDA medium. The yeast strains were grown for 48 hours on YPGA medium (YPG medium supplemented with 20 g/l agar-agar), then streaked at 10 mm from the edges of the fungal blocks. The plates were incubated at 28ºC for seven days and checked daily. The antifungal activity of the yeasts was evaluated by measuring the inhibition zones using as reference plates without yeast cultures.

4. Screening and improving the anti-*Candida* activity

The *C.* (*P.*) *guilliermondii* CMGB 44 culture grown overnight on YPG medium at 28ºC, 150 rpm was centrifuged for 6 min at 6500 rpm. Equal aliquots of cell suspensions (10^8 cells/ml) separate (reference for screening tests) and, respectively, in mixtures with sodium bicarbonate ($NaHCO_3$) in final concentrations of 1, respectively, 2%, were plated on YMA medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, 20 g/l agar-agar) Petri plates floated with 10^6 cells/ml of: *Candida albicans* ATCC 10231, *Candida parapsilosis* CBS 604, *Candida tropicalis* CMGB 165 and *Candida krusei* CMGB 94. The results were recorded after five days of incubation at 25ºC, respectively, 28ºC.

RESULTS AND DISCUSSIONS

1. Molecular and phylogenetic identification

Previous morpho-physiological tests allowed the classification of the yeast strain CMGB 44 as belonging to *C. (P.) guilliermondii*, forming white-yellow, smooth colonies on YPGA medium after 48 hours of incubation and pseudohyphae with small blastoconidia (Figure 1).

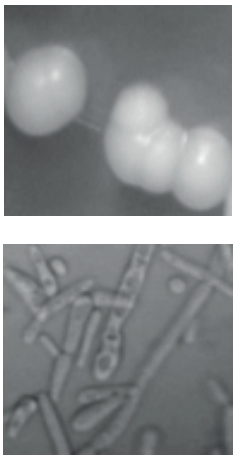

Figure 1. *C. (P.) guilliermondii* CMGB 44 (a) colonies and (b) cells and pseudohyphae

Molecular identification of the yeast strain *C.* (*P.*) *guilliermondii* CMGB 44 was based on the PCR-RFLP analysis of the ITS1-5.8S rDNA-ITS2 region, a technique intensively used in yeast taxonomy. The amplicon had 620 bp and the restriction fragments obtained using four restriction endonucleases, *Cfo* I, *Hae* III, *Hinf* I and *Msp* I, were determined and compared with those described in similar studies on *C. guilliermondii*, respectively, *Pichia (Meyerozyma) guilliermondii* strains (Table 1). The analysis of the restriction patterns revealed a high degree of similarity, confirming thus the taxonomic classification of our strain in the *C.* (*Pichia, Meyerozyma) guilliermondii* species. The variability of the size of the restriction fragments is most probable due to the different origin of the strains. Also, the *Msp* I endonuclease is not frequently used in PCR-RFLP studies and, therefore, there is limited information regarding the generated restriction profiles.

Table 1. Comparative analysis of the amplicons and restriction fragments of the ITS1-5.8S rDNA-ITS2 regions from *Candida (Pichia, Meyerozyma) guilliermondii* strains

Strain	Amplicon (bp)	Restriction fragments size (bp)				Reference
		Cfo I	*Hae* III	*Hinf* I	*Msp* I	
P. guilliermondii CMGB44	620	290, 240	370, 120, 70	320, 300	350, 140, 80	this study
P. guilliermondii	650	300, 270	400, 120, 70	310, 290	-	Bezerra-Bussoli et al., 2013
P. guilliermondii CECT 1019; 1021; 1438; 1456	625	300, 265, 60	400, 115, 90	320, 300	-	Esteve-Zarzoso et al., 1999
M. guilliermondii UAF214	607	293, 255, 49, 10	390, 116, 79, 17, 5	314, 285, 8	-	Pham et al., 2011
M. guilliermondii CECT 1019	625	300, 265, 60	400, 110, 90	320, 300	-	
M. guilliermondii NCYC 443	625	300, 265, 60	400, 115, 90	320, 300	-	
M. guilliermondii CBS 2030	605	320, 270	380, 120, 80	340, 300	-	
C. guilliermnondii	625	300, 265, 60	400, 115, 90	320, 300	-	Valles et al., 2007
P. guilliermondii	650	-	-	-	460, 90	Basílio et al., 2008
P. guilliermondii	630	260, 220, 50	450, 90	265	-	Jeyaram et al., 2008
C. guilliermondii ATCC 9058	608	-	-	-	371, 155, 82	Mirhendi et al., 2006

For a more accurate identification, we performed a phylogenetic genotyping analysis. On this purpose, the PCR-RFLP patterns obtained for *C.* (*P.*) *guilliermondii* CMGB 44 using the endonuclease *Hinf* I were integrated in a study comprising data from related *Candida* species (*C. tropicalis*, *C. albicans*) and, also, other yeast species/genera, some considered as phylogenetically distant.

According to our analysis (Figure 2), the strain *C.* (*P.*) *guilliermondii* CMGB 44 is placed near *C. tropicalis* and *C. albicans* forming a separate branch, far from *Y. lipolytica*, which conserves its place as an out-group.

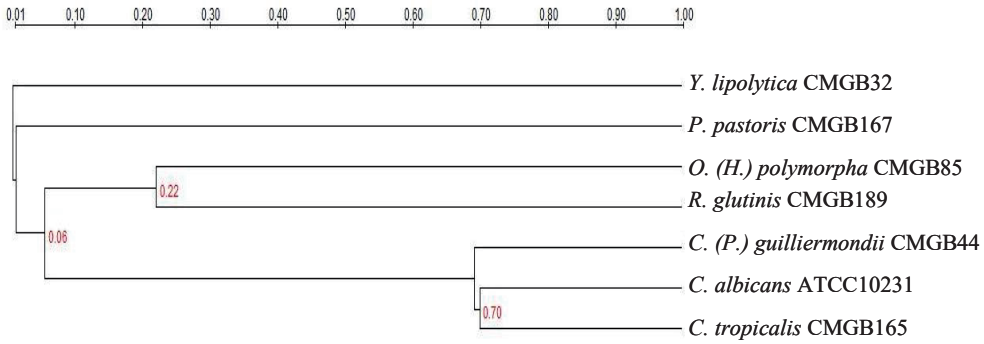

Figure 2. Dendrogram (UPGMA) representing the phylogenetic position of *C.* (*P.*) *guilliermondii* CMGB 44

Similar phylogenetic relationships were presented by Diezmann et al., (2004) during their analysis of the nuclear 18S rDNA and 26S rDNA of 73 ascomycetous taxa. Fitzpatrick et al. (2006) used the genome data for over 345.000 protein-coding genes from 42 fungal genomes, and described the close relation between the three *Candida* species. Moreover, their study explains the close position of *C. albicans* and *C. tropicalis* separately from *C.* (*P.*) *guilliermondii*, fact that might be also observed in our dendrogram. Thus, the CTG clade (organisms that translate CTG codon as serine instead of leucine) within *Saccharomycotina*, is divided in two branches in which *C.* (*P.*) *guilliermondii* represents *Candida* species with sexual reproduction. The position of the three *Candida* species on one side, and *P. pastoris* and *Y. lipolytica*, on the other side, also derived from the analysis of the variation of the GC content of all protein coding genes (Louis et al., 2012). The phylogenetic tree resulted from the analysis of 79 highly conserved sequence-specific DNA-binding proteins from 31 fungal species, also confirmed our results (Lohse et al., 2013).

2. Antifungal activity

In present, *P. guilliermondii* is one of the most promising biocontrol agents used for preventing the action of phytopathogenic fungi. The antifungal tests using seven phytopathogenic fungi from natural isolates, showed that the strain *C.* (*P.*) *guilliermondii* CMGB 44 totally inhibited the growth of *Sclerotium* sp. (Figure 3a) and had high activity against *R. solani* (Figure 3b). The growth of *B. cinerea* mycelium was also inhibited (Figure 3c), while low activity was observed in the case of *Sclerotinia* sp. (Figure 3d). On the contrary, no results were recorded against *A. mali*, *Aspergillus carbonarius* and *Monilinia* sp.

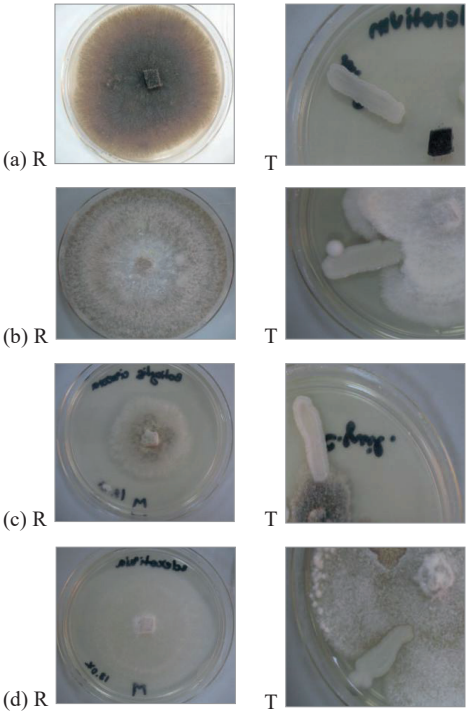

Figure 3. Antifungal activity of *C.* (*P.*) *guilliermondii* CMGB 44 against: (a) *Sclerotium* sp., (b) *R. solani*, (c) *B. cinerea* and (d) *Sclerotinia* sp. (R - reference; T - test plates)

Similar results were recorded against *B. cinerea* during other studies. Thus, *in vitro* and *in vivo* studies regarding the antifungal mechanism of action of *P. guilliermondii* showed that the yeast

was able to protect apples from *B. cinerea* by attaching to the fungal hyphae most probably due to a lectin link and by producing β-(1-3) glucanases (Wisniewski et al., 1991). The attachment of yeast cells also restricted the proliferation of *Colletotrichum capsici* causing chilli anthracnose. Moreover, the yeast competed with the fungus for the carbon (glucose, sucrose) and nitrate substrates (Chanchaichaovivat et al., 2008). Production of β-(1-3) glucanases and exochitinase was also responsible for *P. guilliermondii* activity against *Aureobasidium pullulans* from apple wounds. Furthermore, it seems that *P. guilliermondii* can trigger the production of ethylene in plant cells, which activates the phenylalaninammonium-lyase involved in the synthesis of phenols, phytoalexins and lignins wiht role in plant defense (Spadaro & Gullino, 2004). Also, a *P. guilliermondii* strain isolated from Moroccan citrus Valencia-Late oranges was effective against green mold produced by *Penicillium italicum* (Lahlali et al., 2014).

On the other hand, *C. guilliermondii* strains were proved as having antifungal activity. The biomass obtained from a *C. guilliermondii* strain isolated from heterograft tomato crop stoped the rotting of tomatoes produced by *Rhizopus stolonifer* (Zambrano et al., 2014). Other strains isolated from figs (*Ficus carica* L.) and cactus pear (*Opuntia ficus-indica*) fruits were active against *Penicillium expansum*, the mechanism being based on the competition for nitrogen sources (Scherm et al., 2003).

Several studies mentioned *Saccharomyces cerevisiae* as having growth promoting effect and biocontrol activity against *Sclerotium* or *Rhizoctonia* (Freimoser et al., 2019). The negative effect of *R. solani* was reduced by strains of *Candida saopaulonensis*, *Cryptococcus laurentii* and *Bullera sinensis* in cowpea plants (de Tenorio et al., 2019), respectively, by strains of *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* in suger beet (El-Tarabily, 2004). However, until present, we could not find any data on the antifungal activity of *C.* (*P.*) *guilliermondii* against *Sclerotium*, *Rhizoctonia* or *Sclerotinia* strains.

3. Anti-*Candida* activity

P. guilliermondii and its asexual form, *C. guilliermondii*, are studied mainly for their biocontrol potential for plant protection. Therefore, little is known regarding their antimicrobial activity against human pathogenic microbial strains. Moreover, although less pathogenic and invasive than other *Candida* species, such as *C. albicans*, *C. guilliermondii* strains were isolated in hospitals from immunocompromised patients with cancer and hematology diseases, or from various samples of skin, urine, blood or genital tract tissue and, therefore, might represent risk factors (Papon et al., 2013). Nevertheless, Acuña-Fontecilla et al. (2017) isolated from wine a *P. guilliermondii* strain with killer activity against bacterial strains of *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli*.

The strain *C.* (*P.*) *guilliermondii* CMGB 44 was tested against four *Candida* species with pathogenic potential, at 25, respectively, 28ºC. The two different temperatures were chosen since higher temperatures are favorable to pseudohyphal / hyphal transition in *Candida* cells which expose β (1,3)-glucan easier than the yeast-form cells making them thus more succeptible to the attack of the β-(1-3) glucanases (Chen et al., 2019). The screening tests revealed no antimicrobial activity of our strain for none of the tested temperatures (Figure 4aR, 4bR; Figure 5R).

Numerous studies mentioned using sodium bicarbonate for improving the biocontrol potential of various yeast species, based on its ability to inhibit spore germination (Karabulut et al., 2003; Yao et al., 2004). Moreover, previous research (Csutak et al., 2013) showed that adding sodium bicarbonate in final concentrations of 1 or 2% to *Metschnikowia pulcherrima* cells enhanced the antimicrobial activity of the yeast against pathogenic *Candida* strains. Therefore, for further tests, we used mixtures of *C.* (*P.*) *guilliermondii* CMGB 44 cells and sodium bicarbonate.

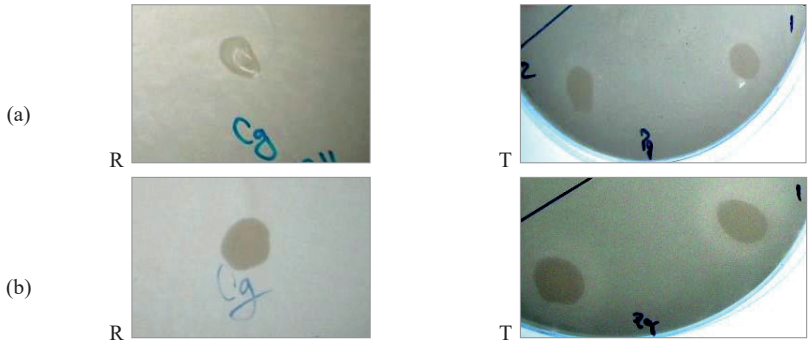

Figure 4. Antimicrobial activity of *C.* (*P.*) *guilliermondii* CMGB 44 after two days against *C. krusei* CMGB 94 at (a) 25ºC and (b) 28ºC (R - reference; T - test plates)

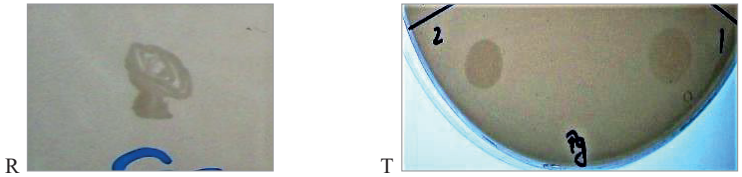

Figure 5. Antimicrobial activity of *C.* (*P.*) *guilliermondii* CMGB 44 after five days against *C. tropicalis* CMGB 165 at 28ºC (R - reference; T - test plates)

Positive results were recorded after two days of incubation against *C. krusei* CMGB 94 and *C. tropicalis* CMGB 165, even though the difference between the inhibition halos obtained using sodium bicarbonate 1%, respectively, 2%, were not significant (Figure 4T; Figure 5T). An interesting fact is that, while for *C. krusei* the temperature influenced visibly the antimicrobial activity, with a rapid response at 28ºC *vs*. 25ºC (Figure 4aT and bT) after two days of incubation, in the case of *C. tropicalis* an enhancement of the antimicrobial activity was recorded only at 28ºC after five days of incubation (Figure 5T). A possible explanation might reside in the variation of β-glucans content of the cell wall of different *Candida* species (Csutak et al., 2017).

CONCLUSIONS

Recent molecular approaches demonstrated the identity of *Candida guilliermondii*, a rare pathogenic species, and its sexual state, *Pichia guilliermondii*, intensively studied for its antimicrobial potential used in plant biocontrol. The strain *C.* (*P.*) *guilliermondii* CMGB 44 presented high antifungal activity against phytopathogenic fungi from natural isolates. Also, to our knowledge, this is the first reported case of a *C.* (*P.*) *guilliermondii* strain able to inhibit *Sclerotium*, *Rhizoctonia* or *Sclerotinia* sp. Moreover, the addition of sodium bicarbonate augmented its antimicrobial activity against *Candida* potential pathogenic strains, which represents the basis for new biomedical applications of *C.* (*P.*) *guilliermondii*. Further research will aim understanding the antagonistic mechanism of action using a wider range of plant and human pathogenic strains for developing applications both in biocontrol and biomedicine.

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