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# Yeast strains with antagonist activity against *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and their phenotypic characterization

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## Abstract

*Colletotrichum* is the causal agent of anthracnose in passion fruit crops, generating economic yield losses. Antagonistic microorganisms represent highly efficient alternatives for chemical compounds in controlling the anthracnose disease. In this research, 8 yeast strains (M1-M8) were isolated, characterized and their antagonistic activities against *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. were evaluated in vitro. The physiological assessments showed that potential antagonistic morphotypes (M2, M5 and M7) had optimal growth with distinct conditions: 3-5 pH, 30°C, 1-5% NaCl; and assimilated several carbon sources. These morphotypes exhibited low enzymatic activity on plant polymers. According to the physiological and inhibition tests, M2 appears to be a promising biocontrol agent against *C. gloeosporioides*. M2 was identified as the species *Meyerozyma caribbica* Kurtzman & M. Suzuki.

**Keywords:** Antagonism, Biocontrol, *Colletotrichum gloeosporioides*, *Meyerozyma caribbica*, Passion fruit

## Background

Passion fruits, *Passiflora edulis f. flavicarpa* Deneger, are widely grown in the tropical areas, especially in Brazil, Ecuador and Colombia. It is one of the most promissory crops in Colombia with approximately 7400 ha cultivated, with an average production of 94,217 t/year, and an average yield of 15 t/ha (Galeano et al. 2018). However, harvest and post-harvest problems, including fruit quality, generate important losses, mainly due to phytosanitary issues such as anthracnose, a fungal disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Pardo-De la Hoz et al. 2016).

This phytopathogen causes economic losses due to the necrotic tissue formation that affects the organoleptic properties of the fruit (Sharma and Kulshrestha 2015). Traditionally, chemical fungicides are widely used to control the anthracnose in passion fruit. Nevertheless, the repeated use of fungicides can induce resistance of *Colletotrichum* species to these compounds (Vieira et al. 2017). Moreover, recent control strategies are concerned with minimizing the use of chemical fungicides with their known hazards to the environment, fauna and human health (Alavanja et al. 2013). Thus, the use of biological control measures, especially antagonistic microorganisms, became an important alternative in the agricultural systems. Among these microorganisms, yeasts have proved to be very useful against different phytopathogens including the fungus *Colletotrichum* (Ruiz et al. 2016). They are considered promising biocontrol agents based on their genetic stability, simple nutritional requirements, survival under adverse conditions, resistance to agrochemicals and different mechanisms against phytopathogens such as antibiosis, production of lytic enzymes, parasitism, space and nutrients competition and induction of resistance in the plant (Ruiz et al. 2016; Pesce et al. 2017).

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Therefore, research on the diversity of yeast strains from their morphological, physiological and biochemical aspects is fundamental to understand their interactions with the environment, their optimal physiological conditions and their potential as biocontrol agents. In this context, the aim of this work was to isolate yeast strains with antagonistic activity against the fungus *C. gloeosporioides* that affects passion fruit crops in agro-ecosystems of the Colombian Orinoquia and identifying their phenotype characters.

## Materials and methods

### Sampling and isolation of yeasts

Sampling of apparently healthy passion fruits was carried out at a passion fruit agro-ecosystem (location, 4° 04′ 33.78″ N 73° 34′ 49.50″ W; average temperature, 25.5 °C; annual rainfall, 4384 mm; relative humidity, 67–83%; soil's pH, 4.5) from the municipality of Villavicencio, department of Meta, Colombia. Isolation of yeast strains was performed according to Castell Ochoa and Escallón Rodríguez (2009), where samples were washed with 0.2% sodium hypochlorite, 70% ethanol and sterile distilled water (SDW). The plant tissues were sectioned and soaked in 0.5% Tween 80 solution. The solutions were left in an orbital shaker at 150 rpm for 40 min, then were centrifuged at 1000 rpm for 10 min. The resulting pellets were suspended in 1 ml of SDW, and 300 μl aliquots were taken from each sample and plated on yeast extract agar. The macroscopic characterization of the colonies was performed according to Valencia Zapata (2004). Shape and reproductive state of the cells were described based on lactophenol blue coloured micropreparations, using an Olympus CX22 optical microscope (Kurtzman et al. 2011).

### Antagonistic activity of yeast strains against *C. gloeosporioides*, in vitro

Following the methodology used by Castell Ochoa and Escallón Rodríguez (2009), Petri dishes with PDA (potato dextrose agar) were used and 2 parallel striations at 3 cm from the centre were drawn. Each striation was inoculated using a loop covered with the isolated yeasts. A 0.5-cm diameter disc of the targeted *C. gloeosporioides* (from a strain plated in PDA and grown for 5 days) was placed in the centre of each Petri dish. The plates were incubated at 28–30 °C for 7 days. Later on, the fungus radial growth diameter (millimetres) was measured, and the percent reductions, compared to a control, were computed (Okigbo and Emeka 2010).

### Physiological characterization

#### *pH, thermotolerance and osmotolerance tests*

The physiological characterization consisted of analysing growth of the isolated yeasts under different stress conditions including pH, temperature and sodium chloride (NaCl) concentrations. These assays were performed, using yeast-peptone-dextrose (YPD) broth (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose), and modified according to the test. The values of the pH treatments were 3, 4, 5, 6 and 7 (adjusted, using 0.1 N HCl and 0.1 N NaOH). For the thermotolerance evaluation, the YPD broth was used without modifications and the growth was evaluated at 5, 30, 40 and 45 °C. Lastly, for the evaluation in different osmotic stress environments, dissimilar concentrations of sodium chloride were adjusted in the medium (1, 5, 10 and 15%). For all tests, 10 μl of the inoculum at a $1 \times 10^7$ cells/ml concentration was used and incubated for 48 h. Later on, biomass was measured based on the optical density (OD) at a 540 nm wavelength using a spectrophotometer (Mettler-Toledo). Absence of cell growth was considered negative.

#### *Assimilation of carbon sources and fermentative capacity*

Tests were carried out in modified YPD broth according to the carbon compound to be evaluated (glucose, fructose, galactose, xylose, sucrose, lactose, maltose) and incubated at 30 °C for 48 h. Three millilitres of each sample were used to determine OD at a 540 nm wavelength. Absence of cell growth was considered negative. To determine the fermentative capacity, 5 ml of 0.1% methyl red solution were added to the medium. Red colouration indicated positive and yellow colouration negative (Sanclemente 2015).

#### *Exoglucanase, endoglucanase and peroxidase tests*

The evaluation was carried out in a liquid culture medium with 0.8% carboxymethylcellulose (CMC) for endoglucanases, and 1 g/l of filter paper for exoglucanases; all of the media were inoculated with strains that are grown for 8 days at 28–30 °C. All treatments and a control (medium without yeast) were incubated for 6 days at 30 °C. Yeast growth was revealed by centrifuged 1 ml of 0.2% Congo red dye. OD was determined at 488 nm wavelength (OOrtiz Moreno and Uribe-Vélez 2011 and Montoya et al. 2014). For determining the peroxidases production, sawdust was used as a medium to replace the alkaline lignin. The yeast strains (grown for 8 days at 28–30 °C) were inoculated in 5 ml of sawdust medium. Again, the treatments and the control (sawdust medium without yeast) were incubated for 6 days at 30 °C. 0.4 ml of guaiacol was added to each medium and centrifuged. OD was measured at 488 nm wavelength (Ortiz Moreno and Uribe-Vélez 2011).

#### *Molecular identification of the potential antagonistic yeast strain*

Selected yeast isolate was cultured in YPD medium at 28–30 °C for 2–3 days. Taxonomic determination of the potential antagonistic yeast strain (M2) was carried out by the CorpoGen Research Centre (Colombia). Yeast DNA was extracted by the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The ribosomal DNA (rDNA) internal transcribed

spacer (ITS) regions 1 and 2 were amplified by polymerase chain reaction (PCR) with primers ITS4 and ITS5 (White et al. 1990), the PCR conditions were as described by Yurkov et al. (2012) and PCR fragments were purified. Sanger dideoxy sequencing of the ITS region was performed with ABI 3730 xl sequencer (Applied Biosystems, Foster City, CA, USA). For yeast identification, the nucleotide sequence obtained in this study was compared with the sequences deposited in the NCBI (www.ncbi.nih.gov), the UNITE (https://unite.ut.ee), and the Warcup Fungal ITS trainset 2 (from the Ribosomal Data Project) databases, using BLAST (Basic Local Alignment Search Tool). Sequences with at least 98% similarity were chosen.

### Experimental design and statistical analysis

For the antagonism evaluation and the physiological characterization tests, a complete randomized experimental design with 3 replicates per treatment was used. Each of the 3 isolated yeast constituted a treatment during the antagonism evaluation; and each condition of pH, temperature, NaCl concentration and carbon source was a treatment during the physiological characterization. A negative control in each case was considered. To estimate each yeast morphotype growth, the absorbance values of the replicates within each treatment were averaged to produce a mean value, and to this number, the blank value was subtracted. Data from the antagonism evaluation were analysed, using the Statistical Package for Social Sciences (SPSS Statistics® version 25), with a one-way ANOVA and a Tukey test (95%) for means separation (Gorgas et al. 2011). Data from the physiological characterization were analysed, using the R 3.6.0 software with a two-way ANOVA, a Shapiro-Wilk normality test and homogeneity of variance was assessed by Levene's test (Shapiro and Wilk 1965; O'Neill and Mathews 2002; R Core Team 2018).

## Results and discussion

### Sampling, isolation and characterization of yeast strains

Eight morphotypes of yeasts were obtained from the fruit and foliage samples of passion fruits. Five morphotypes were isolated from the phyllosphere of fruits (M1–M5) and 3 from the foliage (M6–M8). The isolated morphotypes exhibited similar characteristics at a macroscopic level. At the microscopic level, spherical, oblong and rectangular cellular forms were observed. The main form of asexual reproduction was budding, except for M5 that presented arthroconidia, which are produced by segmentation of pre-existing hyphae. Additionally, M5 exhibited a filamentous margin and a rough colony surface, meaning that this morphotype could belong to the genus *Geotrichum*.

### Antagonistic activity of yeast strains against *C. gloeosporioides*, in vitro

All of the 8 yeast strain treatments inhibited the mycelial growth of *C. gloeosporioides* by 27-54.8%. According to Okigbo and Emeka (2010), M1, M2, M3, M4, M6 and M8 showed moderately effective antagonism with inhibition rates of 27–39%. On the other hand, M5 showed the most effective antagonism against *C. gloeosporioides* (54.8% reduction of the mycelial growth) (Fig. 1). This was reflected in the ANOVA ($F = 10.599$; $P < 0.05$; df = 8), that indicated M1 to M6 and M8 had significant differences with the control and M5 inhibition activity.

### Physiological characterization

To analyse the potential of yeasts as biocontrol agents against *C. gloeosporioides*, a physiological characterization was carried out, using different criteria such as tolerance to distinct pH, temperature and NaCl concentration; growth and fermentative capacity in several carbon sources; and finally, enzymatic activity towards lignocellulosic substrates. From the inhibition percentages values, morphotypes M2, M5 and M7 were selected to continue with the physiological characterization.

#### *pH, thermotolerance and osmotolerance tests*

It was found that M2, M5 and M7 differed in their optimum pH, which ranged between 3 and 5. Yet all of the 3 morphotypes were able to grow in all the evaluated pH values (Fig. 2). These results are in agreement with previous studies that showed the yeast development in a wide range of pH, between 2 and 13.5, with an optimum of 4–6 (Medina et al. 2009; Zapata et al. 2010). The two-way ANOVA indicated significant effects for pH ($F = 3.47$; $P = 0.019$; df = 4) and for morphotype ($F = 203.98$; $P < 0.05$; df = 2) and a highly significant interaction effect of the two ($F = 4.74$; $P = 0.00077$; df = 8). M2 obtained its maximum growth at pH 3 with an absorbance of 0.891. This shows a preference for acidophilic environments, which represents an advantage as a biocontrol agent at harvest and post-harvest levels, since it can be used under conditions of passion fruit production and acidification processes originated by superficial tissue damage. The growth of this morphotype was inversely proportional to the increase in pH. Thus, increasing the pH value decreased the growth of yeast. On the other hand, the maximum growth for M5 was evidenced at pH 4 with an absorbance of 0.981. Finally, M7 showed its maximum growth with an absorbance of 0.133 at pH 5, however, a very low growth when compared with the other 2 morphotypes.

As shown in Fig. 3, growth of the 3 morphotypes was directly affected by temperature, obtaining an optimum growth at 30 °C. In previous studies, different antagonistic yeasts showed an optimal growth between 25 and 30 °C (Zapata et al. 2010). At 5 °C, M2 and M5 with absorbance values of 0.126 and 0.132, respectively, had a better

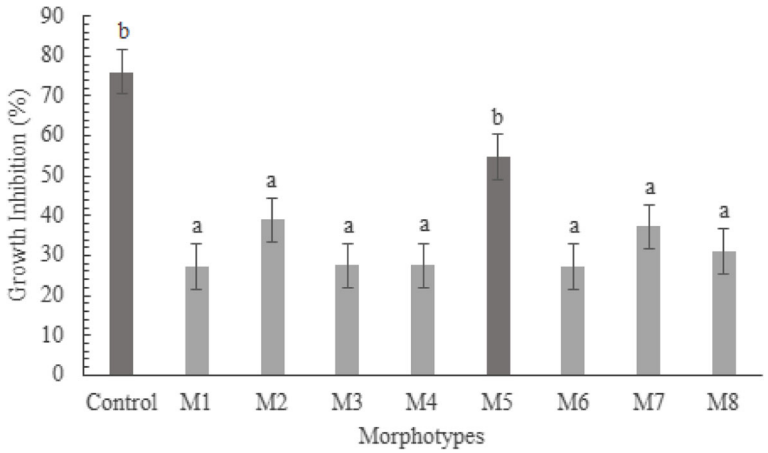


**Fig. 1** In vitro antagonistic activity of the isolated yeast morphotypes (M1 to M8), against *C. gloeosporioides*. The one-way ANOVA ($F = 10.599$; $P < 0.05$; df = 8) showed that M1 to M6 and M8 have significant differences with the control and M5 inhibition activity, which is specified with the letters "a" and "b." The error bars indicate standard error

development than M7 with an absorbance of 0.083. This suggests that the isolated morphotypes have adaptation mechanisms, at a biochemical level, that allow them to respond to environmental changes, since they are present in the phyllosphere and are constantly exposed to natural conditions (Medina et al. 2009). This feature is relevant to the biocontrol of quiescent diseases, such as anthracnose, that must be controlled under cold storage conditions (Lobell and Gourdji 2012). Conversely, at 40 and 45 °C, the M2 growth stood out, obtaining absorbance values of 0.694 and 0.234, respectively. The two-way ANOVA indicated highly significant effects for temperature ($F = 81.59$; $P < 0.05$; df = 3) and for morphotype ($F = 51.37$; $P < 0.05$; df = 2) and a highly significant interaction effect of the two ($F = 16.99$; $P < 0.05$; df = 6).

Moreover, the growth of the 3 morphotypes under different concentrations of NaCl showed that the best development was obtained at the concentrations 1 and 5%, where M2 achieved an absorbance of 1.013 and 0.301, respectively, followed by M5 and M7 in both concentrations (Fig. 4). The two-way ANOVA indicated highly significant effects for concentration ($F = 225.22$; $P < 0.05$; df = 4) and for morphotype ($F = 60.05$; $P < 0.05$; df = 2) and a significant interaction effect of the two ($F = 7.84$; $P = 0.000012$; df = 8), which indicated that osmotolerance is a key factor in yeast development (Pfannebecker et al. 2016). Under concentrations of 10 and 15%, the growth was limited. Previous studies have established that yeast use glycerol as the osmolyte to compensate for the increased external osmotic pressure; therefore, it is possible that the isolated morphotypes

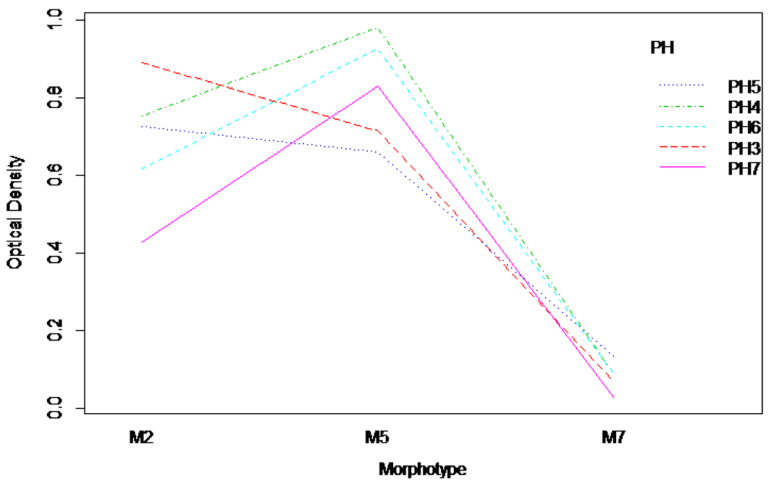


**Fig. 2** Growth of antagonistic yeast morphotypes: M2, M5 and M7, under different pH values. Growth was measured by optical density at 540 nm after 48 h under the evaluated pH. The two-way ANOVA indicated significant effects for pH ($F = 3.47$; $P = 0.019$; df = 4) and morphotype ($F = 203.98$; $P < 0.05$; df = 2) and a highly significant interaction effect ($F = 4.74$; $P = 0.00077$; df = 8)

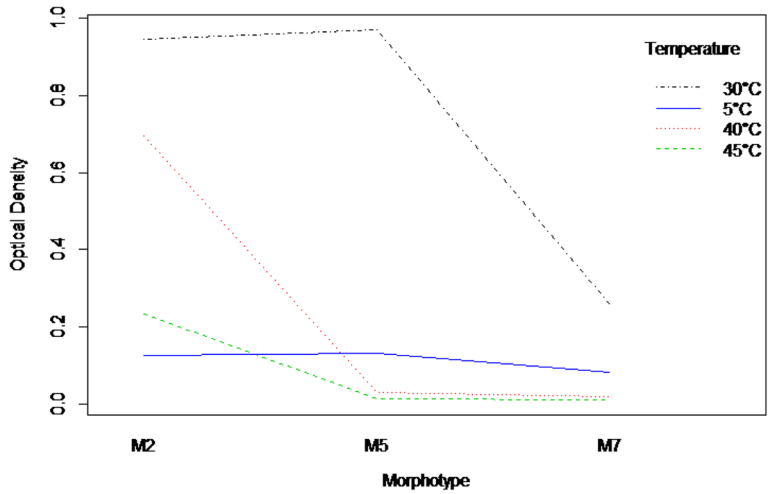


**Fig. 3** Growth of antagonistic yeast morphotypes: M2, M5 and M7, under different temperatures. Growth was measured by optical density at 540 nm after 48 h under the evaluated temperature. The two-way ANOVA indicated highly significant effects for temperature ($F = 81.59$; $P < 0.05$; df = 3) and morphotype ($F = 51.37$; $P < 0.05$; df = 2) and a highly significant interaction effect ($F = 16.99$; $P < 0.05$; df = 6)

had the ability to transport glycerol into cells (Bubnová et al. 2014). This adaptation to high osmotic pressures is crucial in yeast because plants face changing environments in terms of water accessibility, hence, determining the development and viability of the antagonists (Medina et al. 2009).

### *Assimilation of carbon sources and fermentative capacity*

As shown in Fig. 5, the 3 morphotypes were able to grow in all carbon sources, with a marked preference for disaccharide-type carbohydrates, especially sucrose. This was evidenced in the two-way ANOVA that indicated highly significant effects for carbon source ($F = 15.557$; $P < 0.05$; df = 6) and morphotype ($F = 13.488$; $P < 0.05$; df = 2) and a significant interaction effect ($F = 2.032$; $P = 0.0452$; df = 12). Out of the 3 morphotypes, M5 stood out for its efficient assimilation of all evaluated carbon sources, obtaining the highest growth when sucrose was used. The best growth when disaccharides sucrose, maltose and lactose were used was observed by M7, with an absorbance of 1.224, 0.904 and 0.747, respectively. On the contrary, the assimilation of the monosaccharides varied, finding a better performance, when using fructose and glucose. However, when galactose and xylose were used, the obtained absorbances were lower than 0.1. Furthermore, M2 grew best in sucrose, maltose and lactose with an absorbance of 1.005, 0.532 and 0.209, respectively. M2 growth was retarded in monosaccharides and completely stopped when xylose was used. As shown in Table 1, M2 had a positive fermentation process in glucose and sucrose. However, in xylose, fermentation could not be determined. M5 was positive for

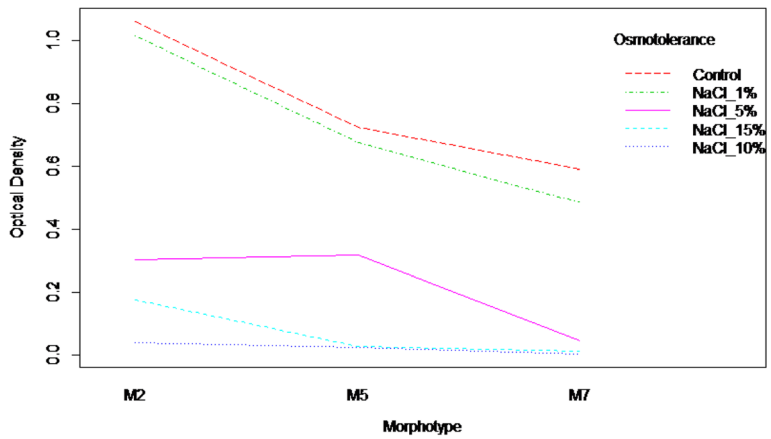


**Fig. 4** Growth of antagonistic yeast morphotypes: M2, M5 and M7, under different NaCl concentrations. Growth was measured by optical density at 540 nm after 48 h under the evaluated concentration. The two-way ANOVA indicated highly significant effects for concentration ($F = 225.22$; $P < 0.05$; df = 4) and morphotype ($F = 60.05$; $P < 0.05$; df = 2) and a significant interaction effect ($F = 7.84$; $P = 0.000012$; df = 8)

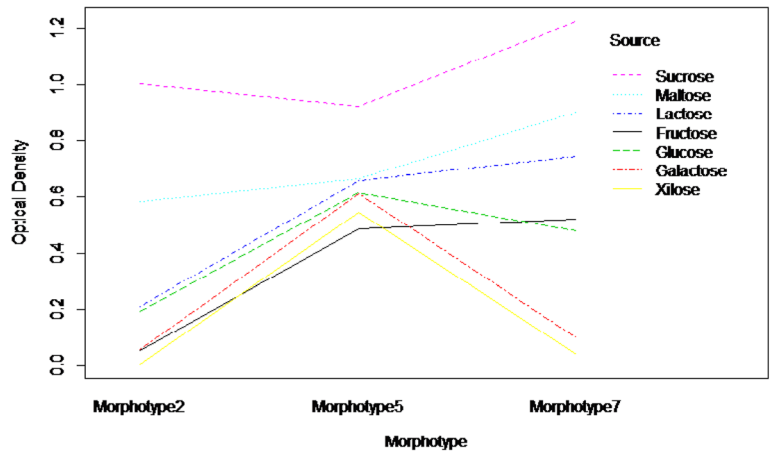


**Fig. 5** Growth of antagonistic yeast morphotypes: M2, M5 and M7, using different carbon sources. Growth was measured by optical density at 540 nm after 48 h under the evaluated source. The two-way ANOVA indicated highly significant effects for carbon source ($F = 15.557$; $P < 0.05$; df = 6) and morphotype ($F = 13.488$; $P < 0.05$; df = 2) and a significant interaction effect ($F = 2.032$; $P = 0.0452$; df = 12)

fermentation in glucose, fructose and sucrose. Lastly, M7 presented fermentation for glucose, fructose, xylose, sucrose and maltose.

### *Exoglucanase, endoglucanase and peroxidase tests*

Results from the detection of enzymatic activity towards lignocellulosic substrates showed that the 3 morphotypes grew in the media for endo and exoglucanases, but not in the peroxidase medium (Table 2). There was no complete hydrolysis of the carboxymethyl cellulose indicator and the filter paper. Therefore, the enzymatic activity was considered a low-positive response. Moreover, this low enzymatic activity on lignocellulosic substrates indicated that the morphotypes cannot cause damage and are innocuous for plants. Based on the colourimetric methods, Congo red dye (Montoya et al. 2014; Ortiz Moreno and Uribe-Vélez 2011) and guaiacol (Ortiz Moreno and Uribe-Vélez 2011), low enzymatic activity (Table 2) is indicated with (+). Absence of enzymatic activity is indicated with (–).

**Table 1** Fermentation profile using different carbon sources of the yeast morphotypes: M2, M5 and M7, based on the methyl red method (Sanclemente 2015). Positive fermentation is indicated with (+). Absence of fermentation is indicated with (–)

| Carbon source | Yeast morphotypes | | |
|---|---|---|---|
| | M2 | M5 | M7 |
| Glucose | (+) | (+) | (+) |
| Fructose | (–) | (+) | (+) |
| Galactose | (–) | (–) | (–) |
| Xylose | ND | (–) | (+) |
| Sucrose | (+) | (+) | (+) |
| Lactose | (–) | (–) | (–) |
| Maltose | (–) | (–) | (+) |

*ND* no data, it was not possible to perform this test

In the present study, the antagonist activity of M2, M5 and M7 yeast morphotypes against *C. gloeosporioides*, and the efficient growth of M2 when subjected to different stress conditions, were both highlighted. Based on the molecular analysis, M2 was identified as the species *Meyerozyma caribbica* Kurtzman & M. Suzuki. This species has previously exhibited high post-harvest antagonistic capability against this pathogen. Bautista-Rosales et al. (2013) reported that *M. caribbica* inhibited 86.7% of the anthracnose caused by *C. gloeosporioides* in *Mangifera indica* L. This antagonistic capacity was provided by several mechanisms such as competition for space and nutrients, production of hydrolytic enzymes, parasitism, biofilm formation and effective use of sucrose and fructose (Bautista-Rosales et al. 2013). This last trait is similar to what was obtained in this research. The species *M. caribbica* was also evaluated, as a biocontrol agent against *Rhizopus* sp. on peaches. Results showed that the decay incidence and lesion diameter were significantly reduced, and its modes of action were also based on the competition for space and nutrients and inducement of defence-related enzymes such as peroxidase, catalase and phenylalanine ammonia-lyase (Xu et al. 2013). Likewise, antagonistic activity of *M. caribbica* against *Rhizopus* sp. and *Botrytis* sp. that cause decay and grey mould decay in strawberries was documented before. The modes

**Table 2** Profile of enzymatic activity of exoglucanases, endoglucanases and peroxidases in the yeast morphotypes: M2, M5 and M7

| Enzymes | Yeast morphotypes | | |
|---|---|---|---|
| | M2 | M5 | M7 |
| Exoglucanases | (+) | (+) | (+) |
| Endoglucanases | (+) | (+) | (+) |
| Peroxidases | (–) | (–) | (–) |

of action involved inducing defence-related enzymes and pathogenesis-related proteins such as β-1, 3-glucanase that improved disease resistance (Zhao et al. 2012). Another species closely related to *M. caribbica*, *Meyerozyma guilliermondii* (Wickerham) Kurtzman & M. Suzuki (2010), has also shown its biocontrol efficacy against *C. gloeosporioides*, providing a significant reduction in mycelial growth and conidial germination (de Lima et al. 2013). Production of extracellular glucanase and chitinase enzymes was regarded as having a major role in the antagonistic action, since these enzymes act as depolymerases on the pathogen cell wall, causing lysis and death (Tseng et al. 2009). Also, emission of volatile compounds, such as acetic acid and hydrogen sulphide, and induction of several defence-related genes, have been reported as antagonistic mechanisms of this species (Cordero-Bueso et al. 2017; Yan et al. 2018b). Some other pathogens could be controlled, using *Meyerozyma* sp. as well, including *Penicillium* sp. that causes blue mould decay in pears and apples (Yan et al. 2018a), *Botrytis* sp. and *Aspergillus* sp. that affect grapevines (Kasfi et al. 2018), and *Fusarium* sp. (Fierro-Cruz et al. 2017).

Although M5 had a better performance during the physiological tests in the present study, this yeast strain had a disadvantage as a biocontrol agent. According to its morphological description, it belongs to the genus *Geotrichum*, which is related to acid rot, a common disease in citrus crops (Hernández-Montiel et al. 2011). Therefore, its production and commercial field release are not viable.

Furthermore, yeasts must be harmless for both plants and human beings in order to be used as biocontrol agents because they could become opportunistic phytopathogens of the crop or generate allergic reactions and residual mycotoxins in food (Ortiz Moreno and Uribe-Vélez 2011). *M. caribbica* has proved, in different toxicity and pathogenicity studies, a very low cytotoxicity and high biodegradability, which makes it a suitable product for sustainable agriculture (Ocampo-Suárez et al. 2017).

## Conclusions

The species *Meyerozyma caribbica* seems to be a promising biocontrol agent for the prevention of anthracnose in passion fruit crops of the Colombian Orinoquia; showing its antagonistic activity against *Colletotrichum gloeosporioides*. Its effective growth under different abiotic stress factors (acid pH, high temperature, osmotic pressure), aerobic and anaerobic development in distinct simple carbohydrates, and its low enzymatic activity on lignocellulosic substrates indicated that it is possibly innocuous for plants. These conditions are relevant when selecting biocontrol agents since they must have the capacity to develop under the same conditions of the pathogen, colonize surfaces of healthy and unhealthy fruits and grow in different storage conditions.

### Abbreviations

ANOVA: Analysis of variance; BLAST: Basic Local Alignment Search Tool; CMC: Carboxymethylcellulose; DNA: Deoxyribonucleotide acid; ITS: Internal transcribed spacer; OD: Optical density; PCR: Polymerase chain reaction; PDA: Potato dextrose agar; SDW: Sterile distilled water; YPD: Yeast-peptone-dextrose

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### Authors' contributions

The authors carried out all the experiments including the bioassays, analytical part, data analysis; wrote the manuscript; and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All data are available in the manuscript, and the materials used in this work are of high transparency and grade.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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