Isolation of epiphytic yeasts from *Eugenia dysenterica* DC. fruits and evaluation of their antimicrobial activity against phytopathogenic fungi

Isolamento de leveduras epifíticas de frutos de *Eugenia dysenterica* DC. e avaliação de atividade antimicrobiana contra fungos fitopatogênicos

Camilla Martins Malta[I] | Eskálath Morganna Silva Ferreira[I] |
Thamar Holanda da Silva[I] | Divina Anne Batista Oliveira[I] | Filipe Miguel Pereira da Silva[I] |
Juliana Fonseca Moreira da Silva[I] | Raphael Sanzio Pimenta[I]

[I]Universidade Federal do Tocantins. Palmas, Tocantins, Brasil

Abstract: Plants commonly interact with microorganisms that may influence their physiology and performance. Epiphytic yeasts are microorganisms that can be found in the phylosphere, in significantly larger numbers in fruits than in other plant tissues due to their higher nutritional content. The present study aimed to contribute to knowledge of epiphytic yeasts associated with *Eugenia dysenterica* DC. fruits and to evaluate their antimicrobial activity against phytopathogens. *E. dysenterica* fruits were collected, washed in saline solution, and sonicated. Each fruit solution was plated in three Petri dishes with NYDA medium. Yeast identification was performed through morphological and physiological criteria, and richness evaluation was performed using the Jackknife 1 estimator. All isolated yeasts were tested for diffusible substances against three phytopathogenic fungi. Only four of 42 isolates were inhibited sporulation of *Aspergillus parasiticus*, but none was able to inhibit or diminish mycelium growth of any tested phytopathogen. The present study contributes to the characterization of the *E. dysenterica* microbiome, presenting the first report of *in vitro A. parasiticus* sporulation inhibition by epiphytic yeasts and suggesting their promising use in biological control of this phytopathogen.

Keywords: Antagonist yeasts. Biological control. *Aspergillus parasiticus*. Sporulation inhibition.

Resumo: As plantas interagem com microrganismos que podem influenciar na fisiologia e no desempenho das espécies. Leveduras epifíticas são microrganismos encontrados no filosfera, com maiores populações em frutos do que em outros tecidos vegetais, por haver maior disponibilidade de conteúdo nutricional. Os objetivos deste estudo foram contribuir para o conhecimento sobre leveduras epifíticas associadas com frutos de *Eugenia dysenterica* DC. e avaliar sua atividade antimicrobiana contra fitopatógenos. Os frutos foram coletados, lavados em solução salina e sonicados. Uma alíquota de cada solução obtida foi semeada em triplicata em placas de Petri contendo meio NYDA. Fez-se a identificação das leveduras por critérios morfofisiológicos e a avaliação da riqueza de espécies por estimador de riqueza Jackknife 1. Todas as leveduras isoladas foram testadas quanto à produção de substâncias difusíveis contra três fungos fitopatogênicos. Somente quatro dos 42 isolados foram capazes de inibir a esporulação de *Aspergillus parasiticus*, mas nenhum inibiu ou reduziu o crescimento micelial de fitopatógenos avaliados. Este estudo contribuiu para a caracterização do microbioma associado a *E. dysenterica* e consistiu na primeira observação da inibição *in vitro* da esporulação de *A. parasiticus* por leveduras epifíticas, o que sugere ser um método promissor para aplicação como controle biológico deste fitopatógeno.

Palavras-chave: Leveduras antagonistas. Controle biológico. *Aspergillus parasiticus*. Inibição da esporulação.

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Autor para correspondência: Raphael Sanzio Pimenta. Universidade Federal do Tocantins. Laboratório de Microbiologia Geral e Aplicada. ALC NO14 – Campus Universitário. Av. NS 15, Bloco II, Sala 5. Palmas, TO, Brasil. CEP 77001-090 (pimentars@uft.edu.br).
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INTRODUCTION

Plant-associated microorganisms have been shown to affect significantly their hosts' physiology and environmental adaptation, suggesting that evolution and ecology of plants and animals with their associated microorganisms can be understood as a holobiont organism context (Agler *et al.*, 2016). Specialized nutritional communities found on the surface of living plants, particularly on leaves, are known as epiphytes (Hongsanan *et al.*, 2016).

Fungal epiphytes are a polyphyletic group that colonize all known plant species and have a worldwide distribution (Schoch *et al.*, 2009; Wu *et al.*, 2011; Hyde *et al.*, 2013; Hongsanan *et al.*, 2014, 2015a, 2015b, 2015c; Li *et al.*, 2016). Many fungal epiphytes are obligate parasites, others are opportunists or symbionts (Wu *et al.*, 2011; Hongsanan *et al.*, 2015a), and some are saprobes (Chomnunti *et al.*, 2014; Hongsanan *et al.*, 2015c). On the other hand, plants are commonly engaged in neutral or mutualistic interactions with epiphytes that may have a positive contribution for their hosts (Partida-Martínez & Heil, 2011), such as influencing plants growth (Ludwig-Müller, 2015; Panke-Buisse *et al.*, 2015; Agler *et al.*, 2016), water economy of host plants (Stanton *et al.*, 2014), producing bioactive substances used by hosts as protective antifungal agents (Newman & Cragg, 2015), and conferring host plant resistance to insects and herbivores (Hansen & Moran, 2014).

However, there is a hypothesis that the interaction fungi-host plant must be balanced between the virulence of the fungi and the plant defences. If such a balance shifts, through plant defence deficiency or fungi virulence increase, a symptomatic manifestation may arise (Partida-Martínez & Heil, 2011).

The search for new microorganisms with potential use in biological control has become a priority worldwide (Berg *et al.*, 2014; Mahamuni *et al.*, 2017; van Lenteren *et al.*, 2018). Recent studies from tropical forests, savannas, and other biomes suggest that fungal diversity is greater in the tropics than in other regions (Nisa *et al*., 2015). The tropical environment can provide to these organisms' good conditions for growth and reproduction. Furthermore, a rich diversity of tropical plants can provide many nutrients, especially in their fruits to furnish excellent habitats for many microorganism communities and populations, including yeasts (Barriga *et al.*, 2014; Grondin *et al.*, 2015).

One of the most important steps in biological control research is the identification of species to be used as biocontrol agents and the role of antagonistic microorganisms in pathogen control (Köhl *et al.*, 2011). Microorganisms (bacteria, yeasts, and filamentous fungi) naturally present on fruits and plant surfaces, may inhibit the growth of other microorganisms, including plant pathogenic fungi (Sharma & Awasthi, 2010). Harvested fruits, leafs, nuts, grains, and other vegetable foods contaminated with pathogens have reduced shelf-life, quality and can be dangerous for human consumption. Products that are harvested and consumed fresh can be more readily decayed by fungal or bacterial pathogens. However, certain beneficial microorganisms can be used as biological control agents against postharvest diseases (Lugtenberg *et al.*, 2017). A good understanding of the relationships between pathogens, antagonistic microorganisms, fruits, plants and the environment is essential for the successful implementation of biological control in the postharvest phase (Talibi *et al.*, 2014).

Screening of new microorganisms for use as biological control agents against postharvest diseases is a difficult process (Köhl *et al.*, 2011). The selection of antagonistic yeasts from among the yeasts already present as resident in fruits can reduce the chance of failure, mainly due their natural adaptation to this environment, especially when compared with antagonists casually deposited on the plant surface or in the soil. Nevertheless, the selection of antagonist microorganisms should take into consideration that it must be genetically stable, have no complex nutrient requirements, be efficient at low population levels, be able to survive for long time periods under different

environmental conditions, be effective in the control different pathogen species in different plant species, be able to grow in simple culture media, be easy to inoculate in the substrate, not produce dangerous substances to the host plant or to the human consumer, be resistant industrial processing procedures, not grow at 37 °C, and not be associated with disease in humans, other animals or the host plant (Sharma & Awasthi, 2010).

Since the middle of the 20th century, several lineages of antagonist yeasts have been evaluated and used as efficient biocontrol agents of postharvest diseases of fruits and plants (Meng *et al.*, 2010; Manso & Nunes, 2011). Normally, the more frequent mechanisms related to the yeasts' capacity to control phytopathogens are competition for space and nutrients, mycoparasitism, induction of plant resistance, predation, and oxidative response (Pimenta *et al*., 2010; Zhang *et al.*, 2017).

Several yeasts, such as *Candida oleophila* Montrocher 1967, *Candida sake* (Saito & M. Ota) Uden & H.R. Buckley ex S.A. Mey. & Ahearn 1983, and *Cryptococcus albidus* (Saito) C.E. Skinner 1950 have been commercialized as biocontrol agents of postharvest diseases in the United States of America, Israel, South Africa, and Europe (Chen *et al.*, 2012).

The present study aims to contribute to knowledge of the epiphytic yeast community associated with the Cerrado native tree *Eugenia dysenterica* (Mart.) DC. and to evaluate the possibility that these microorganisms synthetize metabolites with antimicrobial activity.

MATERIALS AND METHODS

Ripe and healthy fruits of *E. dysenterica* were collected randomly from 15 plants in October 2011, from the Fazenda Suécia ranch near to Porto Nacional, Tocantins, Brazil (10° 68' S; 48° 37' W). The material was herborized and identification was made using identification keys and comparison with exicates in the UFT herbarium (HTO-9571). The fruits were aseptically collected, placed in sterile plastic bags, and transported to the Laboratory of Environmental Microbiology and Biotechnology of the Federal University of Tocantins where they were immediately processed.

The fruit samples were immersed in 0.85% saline solution and shaken at 75 rpm for 3 min. The solution was discarded, and new 0.85% saline solution was added. The samples were then sonicated at an ultrasonic bath for 1 min. 100μl of the solution were plated in Petri dishes with Nutrient Yeast Dextrose Agar medium (NYDA) (0.3% Meat extract, 0.5% Yeast extract, 0.5% Peptone, 1% Glycose, and 2% Agar) and incubated at 25-28 °C for 48 hours (Janisiewicz *et al.*, 2010). Three plates were used per each fruit.

The identification of yeasts was done through morphological and physiological criteria. All isolated yeasts were identified with taxonomic keys (Kurtzman *et al.*, 2011). Reproductive characteristics, sexual spores formation, physiological and biochemical profiles through the fermentative and assimilative capacity of different carbon sources were analysed.

The isolated yeasts were stored in cryogenic vials with GYMP medium (0.1% Yeast extract, 0.5% Peptone, 1% Glycose, and 0.02% Monobasic Sodium Phosphate) supplemented with 15% glycerol, at -80 °C in the microorganism collection of the Laboratory of General and Applied Microbiology of the Universidade Federal do Tocantins.

The evaluation of observed richness was performed using species data and the richness estimator Jackknife 1 available with EstimateS version 8.20 for Windows (Colwell, 2009). In order to prove the sufficiency of sampling, a species accumulation curve was produced per collected plant using Mao-Tau values with 200 randomizations, also with EstimateS software.

In order to perform antagonism essays, all isolated epiphytic yeasts were tested for diffusible substances against *Aspergillus parasiticus* Speare 1912 (IMI 2426989), provided by the International Mycological Institute, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. 1884 (CG – INCOPER 02), and *Monilinia fructicola* (G. Winter)

Honey 1928 (MFA 3635). The yeasts were transferred to a new plate with NYDA medium, inoculated linearly on one side of the plate, and incubated at 25-28 °C for 48 hours. A 6 mm diameter agar disk with each phytopathogen mycelium was inoculated by the side of the yeasts inoculum, and the plates were incubated at 25-28 °C for 72 hours. The yeasts' efficiency to control phytopathogen growth was classified as (A) = no inhibition, (B) = no growth inhibition but with sporulation inhibition, and (C) = growth inhibition. A negative control was prepared with a phytopathogen fragment inoculated alone in NYDA plates. The epiphytic yeasts were considered phytopathogen antagonists when they inhibited 50% or more of the phytopathogen mycelium growth.

RESULTS

Forty-five inoculated plates were obtained from plating the 15 *E. dysenterica* aqueous fruit solutions. After selection, isolation, and purification, 42 epiphytic yeasts were obtained. These epiphytic yeasts were grouped in 11 species (Table 1) according to taxonomic keys (Kurtzman *et al.*, 2011) and were considered to have ascomycete affinity since none of them reacted to DBB (Diazonium Blue B).

Considering species richness increase per sampled plant, it is possible to observe continuous increase in yeast species number with increrasing sample size, approaching a stability (straight line)and curve stabilization (Figure 1).

All 42 epiphytic yeasts obtained were used for direct antagonism essays against the phytopathogens *A. parasiticus*, *C. gloeosporioides* and *M. fructicola*. Only 4 yeasts [*Candida xylopsoci* Kurtzman 2001 (isolates 17 and 18), *C. sake* (isolate 23), and *Saccharomycopsis crataegensis* Kurtzman & Wick. 1973 (isolate 33)] were able to inhibit sporulation of *A. parasiticus*. The remaining 38 epiphytic yeasts were unable to inhibit or to reduce mycelium growth of any other tested phytopathogen (Table 2).

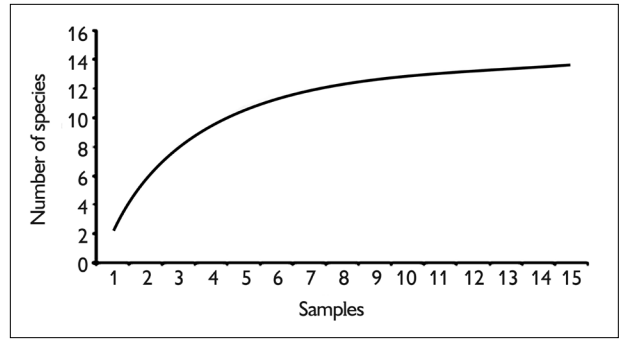

Figure 1. Yeast species richness curve per plant estimated with Jackknife 1 (software EstimateS) for *E. dysenterica*.

Table 1. Identified epiphytic yeasts isolated from *E. dysenterica* fruits.

N	Species	Number of isolates
1	*Candida boidinii* C. Ramirez 1953	2
2	*Candida oleophila* Montrocher 1967	1
3	*Candida sake* (Saito & M. Ota) Uden & H.R. Buckley ex S.A. Mey. & Ahearn 1983	9
4	*Candida xylopsoci* Kurtzman 2001	11
5	*Candida vartiovaarae* (Capr.) Uden & H.R. Buckley 1983	2
6	*Candida wyomingensis* Kurtzman 2000	1
7	*Hanseniaspora uvarum* (Niehaus) Shehata, Mrak & Phaff ex M.T. Sm. 1984	3
8	*Ogataea dorogensis* (G. Péter, Tornai-Leh., Fülöp & Dlauchy) Nagats., S. Saito & Sugiy. 2008	5
9	*Pichia membranifaciens* (E.C.Hansen) E.C. Hansen 1904	1
10	*Saccharomycopsis crataegensis* Kurtzman & Wick. 1973	1
11	*Wickerhamomyces silvicola* (Wick.) Kurtzman, Robnett & Basehoar-Powers 2008	4
Total		42

Table 2. Antagonism essays for diffusible substances with all the epiphytic yeasts isolated from *E. dysenterica* fruits against phytopathogens *Aspergillus parasiticus*, *Colletotrichum gloeosporioides* and *Monilinia fructicola*. Legends: A = no inhibition; B = no growth inhibition but with sporulation inhibition.

Isolated yeasts	*Aspergillus parasiticus*	*Colletotrichum gloeosporioides*	*Monilinia fructicola*	Isolated yeasts	*Aspergillus parasiticus*	*Colletotrichum gloeosporioides*	*Monilinia fructicola*
1	A	A	A	22	A	A	A
2	A	A	A	23	B	A	A
3	A	A	A	24	A	A	A
4	A	A	A	25	A	A	A
5	A	A	A	26	A	A	A
6	A	A	A	27	A	A	A
7	A	A	A	28	A	A	A
8	A	A	A	29	A	A	A
9	A	A	A	30	A	A	A
10	A	A	A	31	A	A	A
11	A	A	A	32	A	A	A
12	A	A	A	33	B	A	A
13	A	A	A	34	A	A	A
14	A	A	A	35	A	A	A
15	A	A	A	36	A	A	A
16	A	A	A	37	A	A	A
17	B	A	A	38	A	A	A
18	B	A	A	39	A	A	A
19	A	A	A	40	A	A	A
20	A	A	A	41	A	A	A
21	A	A	A	42	A	A	A

DISCUSSION

In the present study, 42 epiphytic yeasts were isolated from 45 inoculated plates with *E. dysenterica* aqueous fruit solutions. From these isolated yeasts, it was possible to identify 11 yeast species (Table 1) with ascomycete affinity, according to taxonomic keys (Kurtzman *et al.*, 2011). In a study of yeast communities associated to fruits, mushrooms, tree exudates, and *Drosophila* fruit flies, in two places of the Atlantic Forest of Minas Gerais, Brazil, 608 strains were identified as belonging to the genus *Candida*, *Cryptococcus*, *Debaryomyces*, *Galactomyces*, *Geotrichum*, *Issatchenkia*, *Kloeckera*, *Kodamaea*, *Metschnikowia*, *Myxozyma*, *Pichia*, *Pseudozyma*, *Saccharomyces*, *Saccharomycopsis*, *Torulaspora* and *Zygoascos* (Pimenta *et al.*, 2009). Even considering all the different characteristics related with the ecosystems and host plants, it is notable that in both studies the same 3 yeast species (*C. boidinii*, *P. membranifasciens* and *S. crataegensis*) were isolated from fruits. Surprisingly, the only other published report on yeast species isolated from the Cerrado native *E. dysenterica* fruits do not share a single yeast species with the present study. The 9 isolated yeast species were identified as belonging to the genera *Pseudozyma* and *Aureobasidium* (Sperandio *et al.*, 2015). This may be explained by the geographical and

environmental differences between the three collection locations (*Fazenda Suécia*, Tocantins; Olympic Center of the University of Brasília, Federal District and National Park of Brasília, Federal District), even if all of them are included in the Cerrado biome.

The species richness curve showed a proportional increase in species number and sample size, approaching as asymptote and curve stabilization (Figure 1). This suggests that the methodology used in this study was effective in isolating and identifying the yeast species present in *E. dysenterica*, which was confirmed with the richness estimator Jackknife 1 (Figure 1). Furthermore, the number of yeast species that may be found in fruits is significantly larger than the ones that may be found in other plant tissues, due to the higher nutritional value of the fruits. The species richness curve shows a stabilization tendency even with a small number of identified species because the methodology employed discarded all the transitory microbiota, leaving only the microbiota intimately associated with *E. dysenterica* fruits.

In this study, only 4 (two *C. xylopsoci*, one *C. sake*, and one *S crataegensis*) of the 42 isolated epiphytic yeasts inhibited sporulation of *A. parasiticus* and none were effective against *C. gloeosporioides* and *M. fructicola* (Table 2). As far as known, this is the first report of sporulation inhibition in *A. parasiticus* by *C. xylopsoci*, *C. sake*, and *S crataegensis*. However, a study using *Saccharomyces* sp. isolated from "ragi", a food ingredient traditionally used in Indonesia and usually composed of mixtures of moulds and yeasts, was able to significantly inhibit *A. parasiticus* growth, as shown by the decrease in mould growth, smaller vesicles, and lower number of phyallids (Dewanti-Hariyadi *et al.*, 2014). This result was reinforced by another report demonstrating the antagonistic effects of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen 1883 on the growth of *A. parasiticus* (Persons *et al.*, 2013). Moreover, a similar result to the present study reported an *A. parasiticus* sporulation inhibition, but did not interfere in the mycelium growth, using *Debaryomyces hansenii* (Zopf) Lodder & Kreger 1984 and *Pichia anomala* (E.C. Hansen) Kurtzman 1984 as fungi antagonists. The *D. hansenii* isolated yeasts were more effective, inhibiting in average 32% of sporulation while *P. anomala* inhibited in average 27% (Ramos *et al.*, 2010). Considering that phytopathogen sporulation reduction is a significant issue in control of fruit post-harvest diseases and shelf-life extended time, then *C. xylopsoci*, *C. sake,* and *S. crataegensis* isolates may, in the future, be used in biological control protocols against *A. parasiticus*.

CONCLUSION

The present study contributes to the characterization and identification of epiphytic yeasts in *E. dysenterica* fruits. Additionally, this is the first report of *in vitro A. parasiticus* sporulation inhibition by *C. xylopsoci*, *C. sake*, and *S crataegensis*. However, *A. parasiticus* mycelium growth was not affected, neither was *C. gloeosporioides* and *M. fructicola* sporulation or mycelium growth, by any of the 42 isolated epiphytic yeasts. Finally, *C. xylopsoci*, *C. sake*, and S. crataegensis belong to a group of epiphytic yeasts with promise for use in future biological control of fruit phytopathogens.

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