CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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"**Implicaciones de la simbiosis hongo-bacteria en el desarrollo sexual y adecuación de la especie *Rhizopus microsporus***"

Tesis que presenta

B.E. José Francisco Cabrera Rangel

para obtener el grado de

Maestro en Ciencias en Biología Integrativa

Directora de Tesis

Dra. Laila Pamela Partida Martínez

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CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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"Implications of the fungus-bacteria symbiosis in the sexual development and fitness of the species *Rhizopus microsporus*"

Thesis presented by
B.E. José Francisco Cabrera Rangel

In partial fulfillment of the requirements for the degree of
Master of Science in Integrative Biology

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DEDICATION

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CONTENTS

ABBREVIATIONS

°C	degree Celsius	DNA	deoxyribonucleic acid
*µ*g	microgram	DsRed	red fluorescent protein from *Discosoma* sp.
*µ*L	microliter	e.g	*exempli gratia* (for example)
(m+)	mating type plus	MRE	Mycoplasma-related endobacteria
(m-)	mating type minus	*et al.*	*et alii*
A	Adenine	F1	Fili 1
AcaA	carotenoid cleavage dioxygenase	FBI	Fungal-bacteria interactions
ATCC	American Type Culture Collection	Fig	Figure
atm	atmosphere	FPP	farnesyl pyrophosphate
b+	host with bacteria	Gm	Gram
b-	host without bacteria (cured strain)	G	Guanine
Blast	Basic Local Aligment Search Tool	GFP	green fluorescent protein
BLOS	bacteria-like organelles	GGPP	geranylgeranyl pyrophosphate
C	Cytosine	GPP	geranyl pyrophosphate
CarA	phytoene synthase	HMG	high mobility genes
CarB	phytoene dehydrogenase	HMG-CoA	β-Hydroxy β-methylglutaryl-CoA
CarG	geranylgeranyl pyrophosphate synthase	IPP	isopentenyl pyrophosphate
CarR	lycopene cyclase	isoA	farnesyl pyrophosphate synthase
CarS	carotenoid cleavage dioxygenase	KEGG	Kyoto Encyclopedia of Genes and Genomes

LIM	Laboratorio de Interacciones Microbianas
MEP	mevalonate-5-pyrophosphate
mg	Milligram
min	Minute
mL	Milliliter
nh	non-host
PCR	polymerase chain reaction
pH	potential of hydrogen
RNA	ribonucleic acid
RnhA	RNA helicase
T	Thymine
T2SS	type 2 secretion system
T3SS	type 3 secretion system
Tab	Table
TDH	4-dihydromethyltrisporate dehydrogenase
T_m	melting temperature
™	trade mark
TPT	triose-phosphate transporter
U	Uracil
WT	wild-type
YFP	yellow fluorescent protein

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABSTRACT

Endosymbiosis is a hallmark in the evolution of eukaryotes. Associated microbiota can affect the morphology, physiology, development, and fitness of their hosts. The assembly of these interacting species is considered an ecological and evolutionary functional unit, conceptualized as holobiont. Several fungal strains of *Rhizopus microsporus* live in symbiosis with members of the bacterial genus *Mycetohabitans (Burkholderia* sensu lato*)*. This symbiosis has profound implications in fungal biology, and thus, *R. microsporus* has been proposed as a model of fungal holobiont. In this symbiosis, endofungal bacteria are responsible for the production of toxins that favor the pathogenicity of *R. microsporus*. Moreover, *Mycetohabitans* are vertically transmitted through fungal spores and influence host reproduction, being critical for asexual reproduction and affecting the number of sexual zygospores produced by sexually compatible mating partners (m+ and m-). However, it is uncertain if symbiosis with *Mycetohabitans* represents a barrier for the sexual reproduction in the species *R. microsporus*, if the sexual-derived progeny is viable, if the sexual loci are equally distributed, and if inherited bacteria are uni or biparental. I evaluated sexual compatibility and the viability of produced zygospores from different mating combinations of *R. microsporus* strains: host (b+), natural non-host (nh) and cured host (b-) strains. Additionally, I quantified the segregation of bacteria in the sexual progeny (F1) through the identification of strain-specific bacterial genes through PCR. My results suggest that only host fungi can reproduce sexually, and that the absence of *Mycetohabitans* spp. in both host fungal partners compromises the development and viability of zygospores. In addition, I found that endobacteria stimulate β-carotene synthesis and the formation of trisporic acid, the sexual hormone of Mucorales. I also discovered that in the sexual progeny (F1), the bacteria and the sexual locus from the mating type minus (m-) are inherited. The observed incompatibility between host and non-host strains, and the sexual stimulation by the bacteria in host strains support the notion that symbiosis with *Mycetohabitans* spp. promotes speciation in reproductive-related traits in *R. microsporus*.

RESUMEN

La endosimbiosis es un punto clave en la evolución de los eucariontes. La microbiota asociada puede afectar la morfología, la fisiología, el desarrollo y la adecuación de sus anfitriones. El ensamblaje de estas especies interactuantes se considera una unidad funcional ecológica y evolutiva, conceptualizada como holobionte. Varias cepas fúngicas de la especie *Rhizopus microsporus* viven en simbiosis con miembros del género bacteriano *Mycetohabitans* (*Burkholderia* sensu lato). Esta simbiosis tiene profundas implicaciones en la biología de los hongos y, por lo tanto, *R. microsporus* se ha propuesto como un modelo de holobionte fúngico. En esta simbiosis, las bacterias endofúngicas son responsables de la producción de toxinas que favorecen la patogenicidad de *R. microsporus*. Además, *Mycetohabitans* spp. se transmiten verticalmente a través de las esporas de los hongos e influyen en la reproducción del huésped, siendo críticas para la reproducción asexual y afectando la cantidad de zigosporas producidas por parejas de apareamiento sexualmente compatibles (m+ y m-). Sin embargo, no está claro si la simbiosis con *Mycetohabitans* spp. representa una barrera para la reproducción sexual en la especie *R. microsporus,* si la progenie derivada de la reproducción sexual es viable, si el loci sexual se distribuye de forma equitativa, y si las bacterias heredadas son uni o biparentales. Evalué la compatibilidad sexual y la viabilidad de las zigosporas producidas a partir de diferentes combinaciones de apareamiento de cepas de *R. microsporus*: cepas hospedadoras (b+), no hospedadoras naturales (nh) y curadas (b-). Además, cuantifiqué la segregación de bacterias en la progenie sexual (F1) mediante la identificación de genes bacterianos específicos de cepa mediante PCR. Mis resultados sugieren que solo los hongos huésped pueden reproducirse sexualmente y que la ausencia de *Mycetohabitans* spp. en ambos hongos hospedadores compromete el desarrollo y la viabilidad de las zigosporas. Además encontré que las endobacterias estimulan la biosíntesis de β-carotenos y la formación de ácido trispórico, la hormona sexual de los Mucorales. También descubrí que en la progenie sexual (F1), se heredan las bacterias y el locus sexual del tipo de apareamiento negativo (m-).

La incompatibilidad observada entre las cepas hospederas y no hospederas, y la estimulación sexual por las bacterias en las cepas hospederas apoyan la idea de que la simbiosis con *Mycetohabitans* spp. promueve la especiación en rasgos relacionados con la reproducción en *R. microsporus*.

INTRODUCTION

In 1879 Anton de Bary proposed the term symbiosis to refer to interspecific associations, *i.e.*, interactions between two or more individuals of different species. Symbiosis can be brief, long-lasting, or even last the whole life. It can be classified based on the beneficial, harmful, or neutral effects on the fitness of each symbiont (Fig. 1). In addition, the symbiosis can be called "obligatory", when it is essential for the survival of one or both species, or "facultative", when it is not essential for survival (de Bary, 1879; Martin *et al.*, 2012).

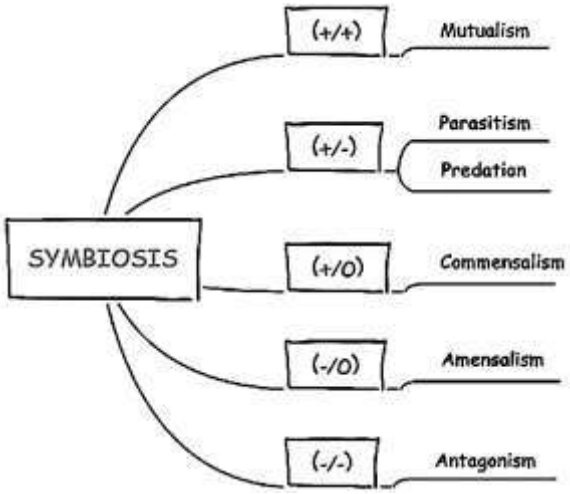


Figure 1. Symbiosis classification (Based on Martin *et al.*, 2012)**.** (+) Benefited species; (-) Harmed species; (0) Neutral effect on the species.

Symbiosis can be further defined by the type of spatial interaction, if a species (guest) lives inside cells of other species (host), or in the space formed between the cells of the host species, the interaction is called endosymbiosis. Otherwise, if it lives on the body or exterior of the host organism, it is defined as ectosymbiosis (Martin *et al.,* 2012).

Microorganisms can affect the morphology, physiology, development, and fitness of their hosts. The assembly of these interacting species can be considered an ecological and evolutionary functional unit, conceptualized as holobiont (Fig. 2). This term distinguishes itself by not only recognizing a host and its obligate symbionts, but also emphasizes the diversity of

facultative symbionts and their dynamic associations with a host. Host-associated microbiota and their genomes can be stable or labile components, and can be vertically or horizontally transmitted, allowing to propagate unique properties to the holobiont. The ensemble of genomes of the host and its symbionts is conceptualized as the hologenome. The traits encoded by this hologenome depend on its context and may result in benefit, damage or no effects (Margulis, 1991; Jefferson 1994; Zilber-Rosenberg & Rosenberg, 2008; Bordenstein & Theis, 2015; Casadevall *et al.*, 2015; Rosenberg & Zilber-Rosenberg 2016; Theis *et al.*, 2016; Partida-Martínez, 2017).

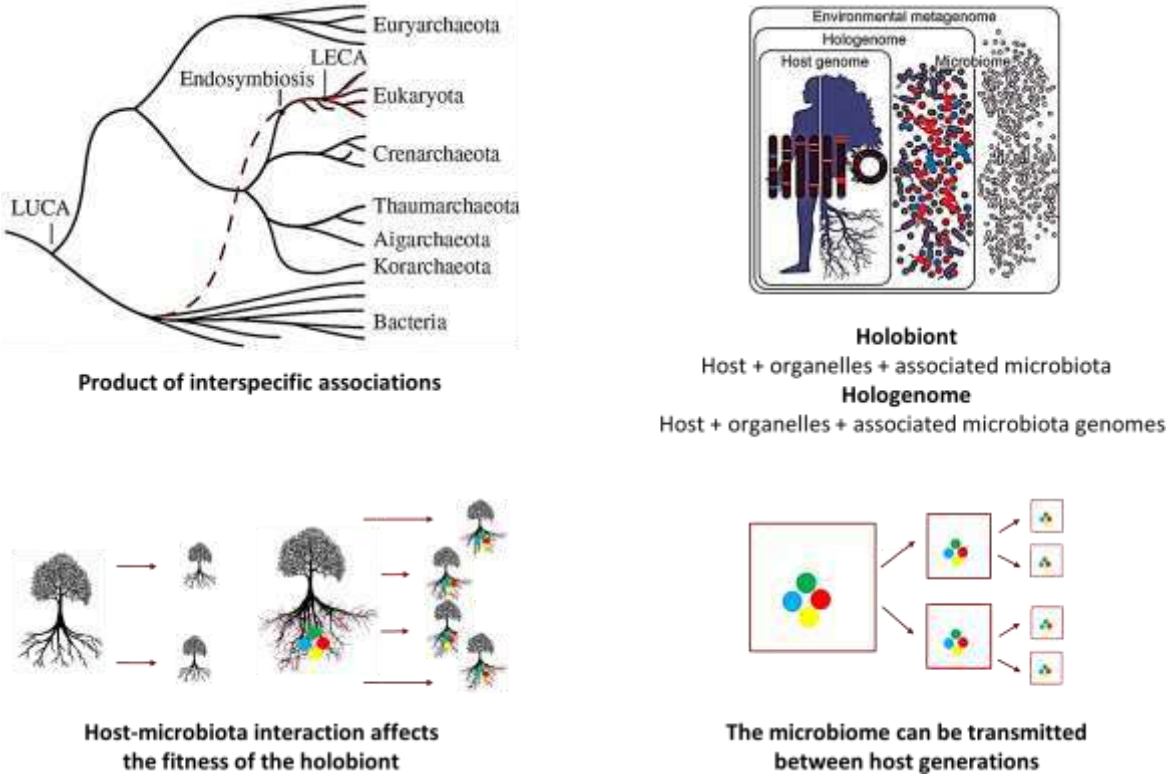


Figure 2. Holobiont: a functional biological unit (Based in Casadevall *et al.*, 2015; Bordenstein & Theis, 2015; Theis *et al.*, 2016).

The terms holobiont and hologenome have been mostly used in animals and plants, however, several reports that study associations wherein microorganisms reside within the hyphae of fungi have been published (Tab.1), expanding the use of this concept to the fungal kingdom.

Host	Guest	Effects in Host/Guest	Reference
Geosiphon pyriformis	*Nostoc punctiforme*	The fungus supplies inorganic nutrients such as phosphate, trace elements, and water. Cyanobacteria supplies carbohydrates by photosynthesis and nitrogen compounds by N2 fixation.	Von Fritz v. Wettstein, 1915; Kluge *et al.*, 1991; Kluge *et al.*, 1992; Shüβler & Kluge, 2001.
Endogone spp.	Bacteria-like organelles (BLOS)	Not analyzed.	Mosse, 1970.
Scutellinia spp.	BLOS	Not analyzed.	Schrantz, *et al.*, 1973
Glomus caledonius	BLOS	Not analyzed.	MacDonald & Chandler, 1981
Gigaspora margarita	*Candidatus* Glomeribacter gigasporarum	Changes in the metabolic profile of both symbionts.	Bianciotto *et al.*, 1996; Ruiz & Bonfante, 1999; Dearth *et al.*, 2018.
Tuber borchii	*Cytophaga* sp.	Not analyzed.	Barbieri *et al.*, 2000.
Laccaria bicolor	*Paenibacillus sp.*	Not analyzed.	Bertaux *et al.*, 2003.
Rhizopus microsporus	*Mycetohabitans rhizoxinica*	Production of toxins that increase the pathogenicity of the fungus. Fungal reproduction is highly modulated by bacteria.	Partida-Martínez & Hertweck, 2005; Partida-Martínez *et al.*, 2007; Partida-Martínez *et al.*, 2017; Mondo *et al.*, 2017.
Piriformospora indica	*Rhizobium radiobacter*	Promoting tripartite relationships with plants.	Sharma et al., 2008; Guo *et al.*, 2017.
Arbuscular mycorrhizal fungi (Glomeromycota)	*Candidatus* Moeniiplasma glomeromycotorum	Bacteria have reduced gene content, indicating metabolic dependence on the host.	Naumann M., *et al.*, 2010; Naito *et al.*, 2015.
Ascomycota fungal endophytes	Eight families of facultative bacteria	Not analyzed.	Hoffman *et al.*, 2010
Ustilago maydis	*Bacillus* sp.	Endosymbiotic N2-fixing association.	Ruiz-Herrera *et al.*, 2015.
Mortierella elongata	*Mycoavidus cysteinexigens*	Fungal host metabolism is highly modulated by bacteria.	Sato *et al.*, 2010; Ohshima *et al.*, 2016; Uehling *et al.*, 2017.
Mortierella elongata.	*Nannochloropsis oceanica*	Carbon and nitrogen transfer between fungus and algae.	Du *et al.*, 2019.

Table 1. Bacterial endosymbionts in fungi.

The fungal holobiont can become a model system for the assessment of conserved molecular interactions between eukaryotic cells and bacteria, and will allow us to understand the roles of endobacteria in fungal ecology and evolution (Partida-Martínez, 2017).

The diversity of associated microbiota can vary depending on the fungus and the organ considered. Endofungal bacteria reported in the Ascomycota and Basidiomycota appear to be transient in nature, can influence the host phenotype or fitness, and serve as environmental reservoirs or refuges for bacteria (Hoffman *et al.*, 2010). The best-studied endofungal bacteria belong to the family Burkholderiaceae, and are associated with early-diverging lineages of terrestrial fungi of the phylum Mucoromycota. These associations appear to be specific and have tightly coevolved over millions of years. This has resulted in host dependency and significant genome reductions for several bacterial endosymbionts (Mondo *et al.*, 2012; Araldi-Brondolo *et al.* 2016; Partida-Martínez, 2017; Deveau *et al.*, 2018).

The *Rhizopus microsporus* and *Mycetohabitans rhizoxinica* duo, discovered by Partida-Martínez & Hertweck in 2005, has become the most explored model thus far since it has been possible to isolate, cultivate and transform the guest outside the host. In addition to the possibility of generating fungal strains free of endosymbionts, and its possible subsequent reinfection. (Partida-Martínez & Hertweck, 2005 y 2007; Partida-Martínez *et al.*, 2007b; Partida-Martínez, 2013).

The genus *Rhizopus*

Species of the mucoralean genus *Rhizopus*, first described by Ehrenb in 1820, have a high impact on the food, medical, agricultural, and ecological sectors. *Rhizopus* species share morphological characteristics (Fig. 3), and display few detectable differences at the molecular level. The most recent phylogeny, where 192 orthologous genes were used, defined a monophyletic genus, with four species: *R. microsporus, R. stolonifer, R. arrhizus,* and *R. delemar* (Zygomycetes.org; Dolatabadi *et al.* 2014; Gryganskyi *et al.,* 2018).

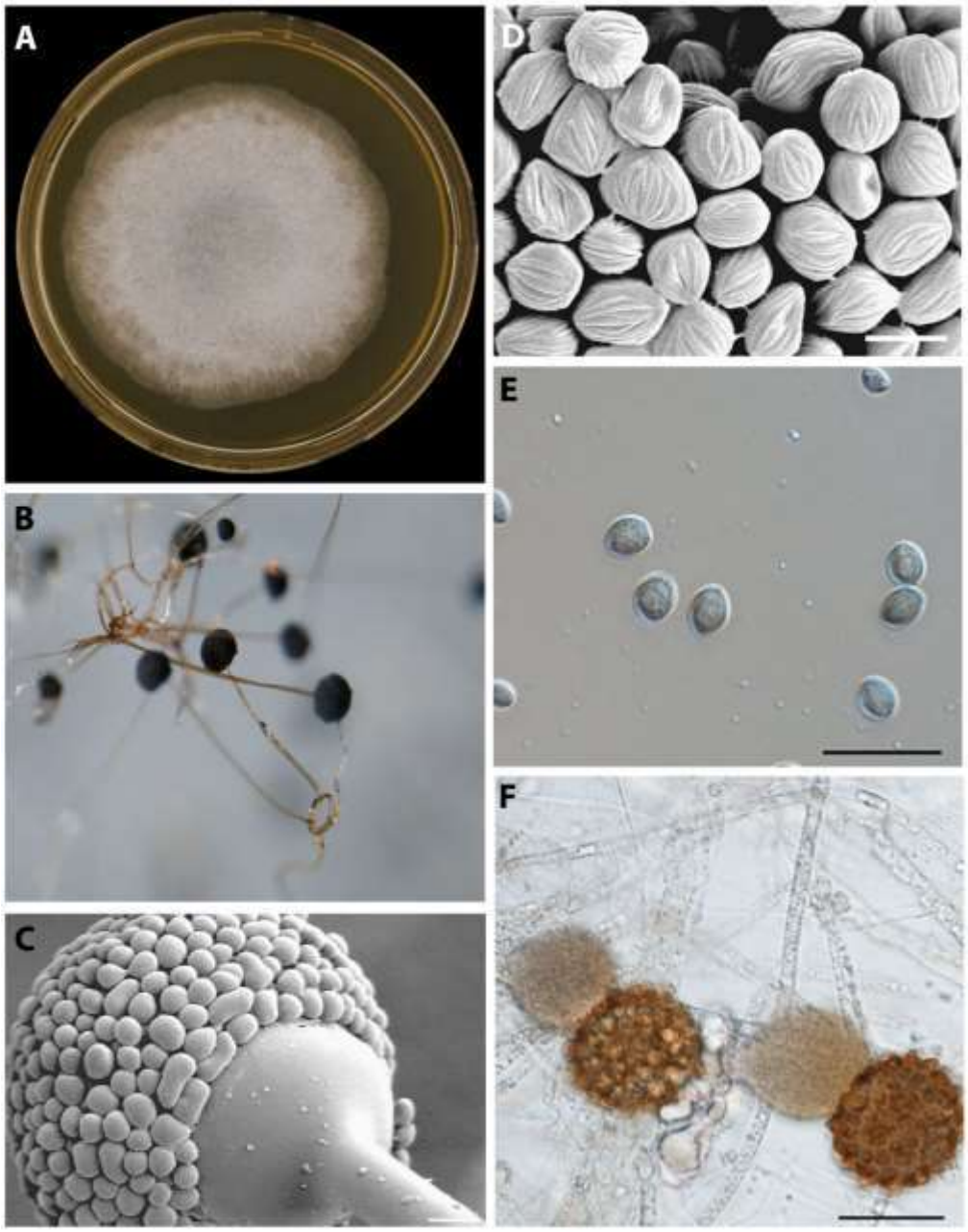


Figure 3. Morphology of the genus *Rhizopus*.

(A) *R. microsporus*. Colony on MEA 2 days incubation at 30 °C (Dolatabadi *et al.*, 2014).
(B) *R. microsporus*. Sporangia and rhizoids (Dolatabadi *et al.*, 2014).
(C) *R. microsporus* var. *oligosporus*. Sporangiophore (Jennessen *et al.*, 2008).
(D) *R. oryzae.* Sporangiospores (Jennessen *et al.*, 2008).
(E) *R. microsporus*. Sporangiospores (Dolatabadi *et al.*, 2014).
(F) *R. microsporus*. Zygospores (Gryganskyi *et al.*, 2018).

Rhizopus spp. are indispensable in the fermentation of a large diversity of foodstuffs, e.g., Tempeh, a traditional food dish of Indonesia based on yellow soybeans with meat flavor, and Meju, a block of dried soybeans in Korean cuisine used as condiments (Rohm *et al.,* 2010; Dolatabadi *et al.*, 2014).

Rhizopus spp. are also known as agents of human and animal diseases. Mucoromycosis is a fungal infection in severely immunocompromised patients, being *R. microsporus* and *R. arrhizus* the most common infective agents. The most frequent symptoms are invasive necrotic lesions in the nose and palate, orbital cellulitis, proptosis, and purulent rhinorrhea. The treatment consists of Amphotericin B (Porter & Kaplan, 2014).

The species *R. microsporus* represents a threat to the agricultural sector for causing rice seedling blight. This disease is initiated by an abnormal swelling of the seedling roots without any sign of infection by the pathogen. This characteristic symptom is in fact caused by the macrocyclic polyketide metabolite rhizoxin (Fig. 4a) that has been isolated from cultures of *Rhizopus* sp. The phytotoxin exerts its destructive effect by binding to rice β-tubulin, which results in inhibition of mitosis and cell cycle arrest. (Furuya *et al.*, 1974; Ibaragi *et al.*, 1973; Gho *et al.*, 1978; Noda *et al.*, 1980; Iwasaki *et al.*, 1984 and 1986; Koga-Ban *et al.*, 1995; Takahashi *et al.*, 1987; Partida-Martínez, 2005).

The symbiosis *Rhizopus microsporus* – *Mycetohabitans* spp.

The endofungal bacteria are the real producers of many specialized metabolites that were initially reported as *R. microsporus* products. The first one elucidated was rhizoxin (Fig. 4a), the potent antimitotic toxin that causes rice seedling blight; the second was rhizonin (Fig. 4b), a hepatotoxic cyclopeptide; followed recently by holrhizin A (Fig. 4c) and heptarhizin (Fig. 4d). Despite high similarity among all bacterial symbionts, only rhizoxins and holrhizin A are produced by all endofungal bacteria strains characterized to date from *R. microsporus*, while rhizonin and heptarhizin only are distributed in endofungal bacteria. (Partida-Martínez *et al.*, 2005; Partida-Martínez *et al.*, 2007, Lackner *et al.*, 2009; Niehs *et al.*, 2018 and 2018b).

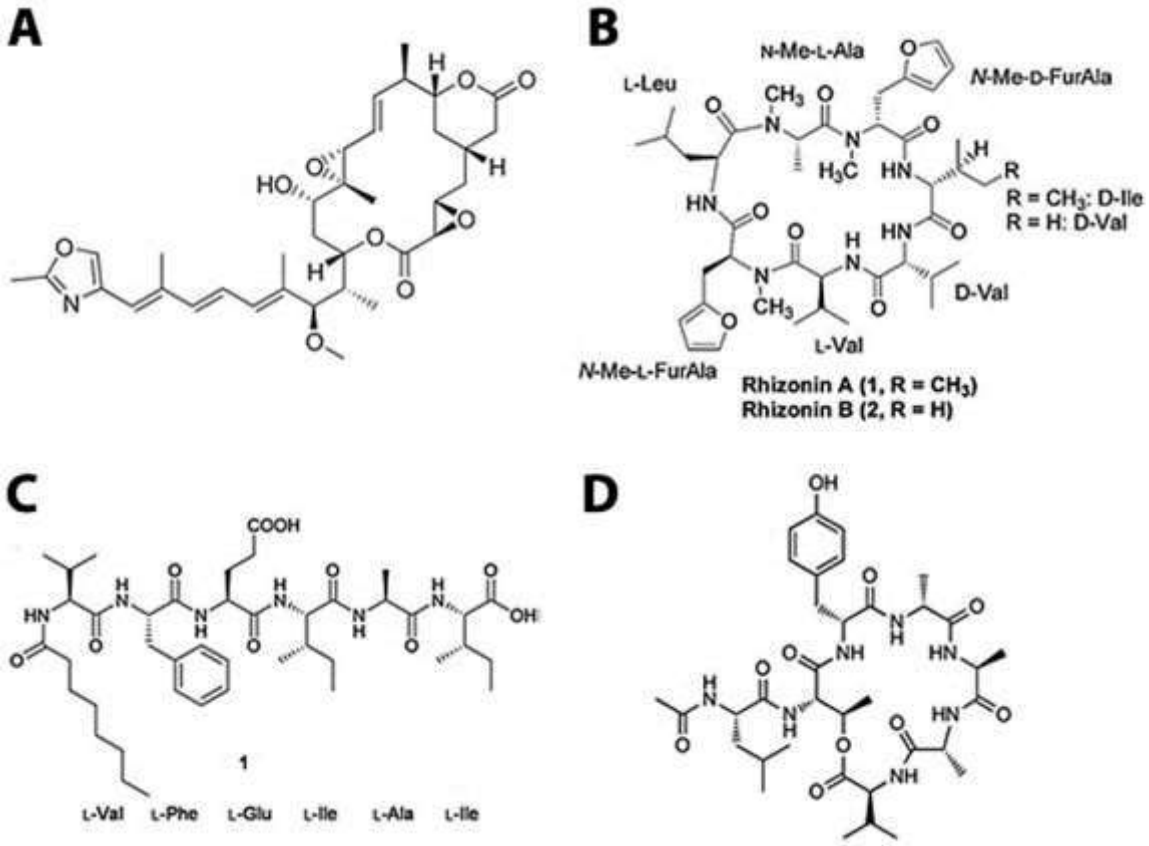


Figure 4. Chemical structures of (A) rhizonin, (B) rhizoxin, (C) holrhizin A and (D) heptarhizin (Partida-Martínez *et al.*, 2005; Partida-Martínez *et al.*, 2007, Niehs *et al.*, 2018 and 2018b).

Lackner and collaborators isolated eigth toxinogenic endobacteria from *Rhizopus microsporus* of highly diverse geographic origins and ecological niches (Tab. 2). The bacterial endosymbionts shared characteristic phenotypic traits, like secondary metabolite production and protein profile. The phylogenetic analyses provided strong evidence that all symbiont strains originated from a common ancestor and they form a new complex within the genus *Burkholderia,* which includes *B. rhizoxinica* and *B. endofungorum* species, now denominated as the novel genus *Mycetohabitans* (includes *M. rhizoxinica* and *M. endofungorum* species) within *Burkholderia* sensu lato. (Partida-Martínez et al., 2005; Partida-Martínez *et al.*, 2007; Lackner *et al.*, 2009 and 2009b; Partida-Martínez *et al.*, 2013; Estrada-de los Santos *et al.*, 2018).

Taxon	Strain designation	Origin	Bacterial endosymbiont (isolate)
Rhizopus microsporus van Tieghem	ATCC 62417	Rice seedlings, Japan	*Burkholderia rhizoxinica* Strain HKI-0454 (B1)
Rhizopus sp. strain F-1360	ATCC 20577	Soil, Japan	*Burkholderia* sp. Strain HKI-0512 (B2)
Rhizopus microsporus Tieghem var. *microsporus*	CBS 111563	Sufu starter culture, rice wine tablet, Vietnam	*Burkholderia* sp. Strain HKI-0455 (B3)
Rhizopus microsporus Tieghem var. *microsporus*	ATCC 52813 CBS 699.68	Soil, Ukraine	*Burkholderia* sp. Strain HKI-402 (B4)
Rhizopus microsporus Tieghem	CBS 112285	Ground nuts, Mozambique	*Burkholderia endofungorum* HKI-0456 (B5)
Rhizopus microsporus var. *chinensis* (Saito)	ATCC 52811 CBS 261.28	Not specified, United States of America	*Burkholderia* sp. strain HKI-0513 (B6)
Rhizopus microsporus Tieghem var. m*icrosporus*	ATCC 52814 CBS 700.68	Forest soil, Georgia	*Burkholderia* sp. strain HKI-0403 (B7)
Rhizopus microsporus Tieghem var. *microsporus*	CBS 308.87	Man, from deep necrotic tissue within the hand following a spider bite, Australia	*Burkholderia* sp. strain HKI-0404 (B8)

Table 2. *Rhizopus microsporus* strains and their bacterial endosymbionts. ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. (Lackner *et al.*, 2009).

During the initial studies on the true biogenetic source of rhizoxin, it was found that a symbiont-free host strain of *R. microsporus,* obtained by antibiotic treatment, can be reinfected with cultured endosymbionts by cocultivation, this observation provided strong evidence for a horizontal mode of transmission. The vertically transmission has been assessed through reinfection of fungal mycelia and it has been observed that *Mycetohabitans rhizoxinica* (Fig. 5), formerly known as *Burkholderia rhizoxinica* (*Burkholderia sensu lato*), is vertically transmitted through spores of its fungal host. Unexpectedly, *R. microsporus* spores are only formed when their symbionts are present, therefore, asexual reproduction of the fungal host is strictly dependent on their guests. Apparently, the fungus might have lost its ability to produce endogenous sporulation factors and became reliant on endobacteria for reproduction. (Partida-Martínez *et al.*, 2007).

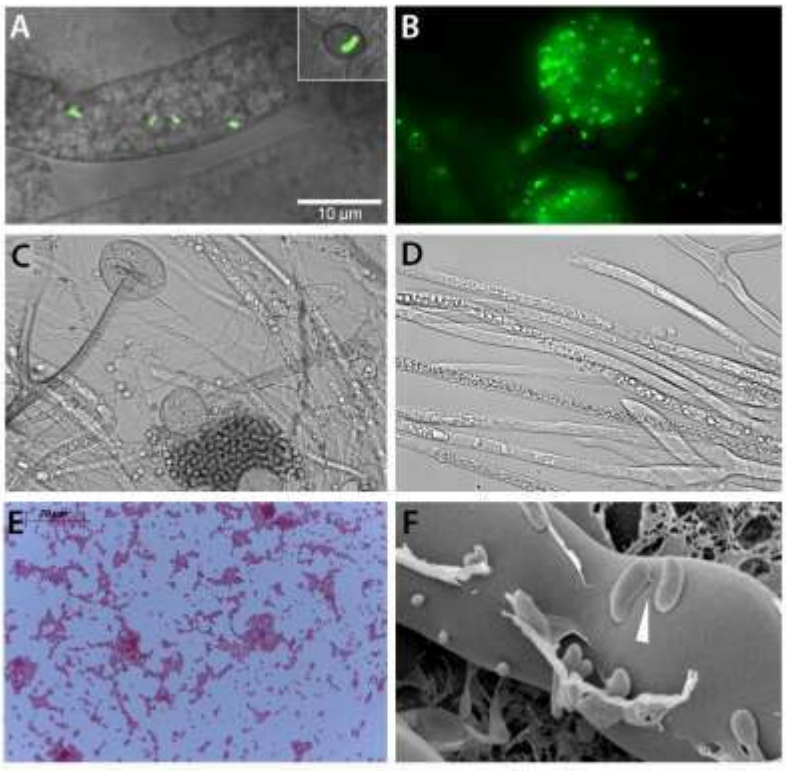


Figure 5. *Mycetohabitans rhizoxinica*, an endofungal symbiont.

A y B. *Mycetohabitans rhizoxinica* within *Rhizopus microsporus* (Moebius *et al.*, 2014; Partida-Martínez *et al.*, 2007 and 2007b).
C. *Rhizopus microsporus*, wt strain (Partida-Martínez *et al.*, 2007).
D. *Rhizopus microsporus*, cured strain (Partida-Martínez *et al.*, 2007).
E. *Mycetohabitans rhizoxinica,* Gram stain (This project).
F. Bacterial colonization of host *Rhizopus microsporus* (Moebius *et al.*, 2014).

Moebius and collaborators (2014) showed through comparative proteome analyses that the type 2 secretion system of the bacteria (T2SS) releases chitinolytic enzymes and chitin-binding proteins essential for bacteria to enter fungal hypha. Lackner and collaborators (2011) saw that a functional type III secretion system (T3SS), which is assumed to deliver effector proteins to the fungal cytosol, is also required for effective fungal colonization and elicitation of asexual reproduction. In 2016, Lastovetsky and collaborators reported that specific changes in fungal lipid metabolism, mediated by diacylglycerol kinase enzymes, are required to maintain

a mutualistic outcome of the interaction with bacteria. In Petri dishes, Lastovetsky saw that non-host *R. microsporus*, as well as other mucoralean fungi, interact antagonistically with *Mycetohabitans* derived from the host *R. microsporus,* and that these fungi were not invaded by them.

Sexual reproduction in Mucorales

Mucorales members have a bipolar mating system. Compatible sex plus (m+) and sex minus (m-) partners are recognizable among them in co-growth conditions. They have an exchange of chemical signals that induce the fusion of hypha, and later production of meiospores. This chemical communication among the complementary mating types of fungi induces the development of wide hyphae named zygophores, which grow one towards the other. These are composed of a distal region called progametangia, and a transverse wall that divide the apices thus separating a cell called gametangio. The remaining part that communicates with the hyphae is called suspensor. Gametangios fusion originate a single cell, the zygospore, which contains cytoplasm and haploid nuclei of both parentals. When germinating, each zygospore produces a sporangiophore very similar to the one produced during the asexual cycle (Fig. 6). Zygospore germination and progeny development within the genus *Rhizopus* have only been documented in a few cases, and it is not always consistent among different strains. The mating of *Rhizopus* represents a distinct pattern of zygospore production among Mucoralean fungi known as the "*Rhizopus* pattern", in which meiosis is delayed until zygospore germination. (Gryganskyi *et al.*, 2010; Lee & Idnurm *et al.*, 2017).

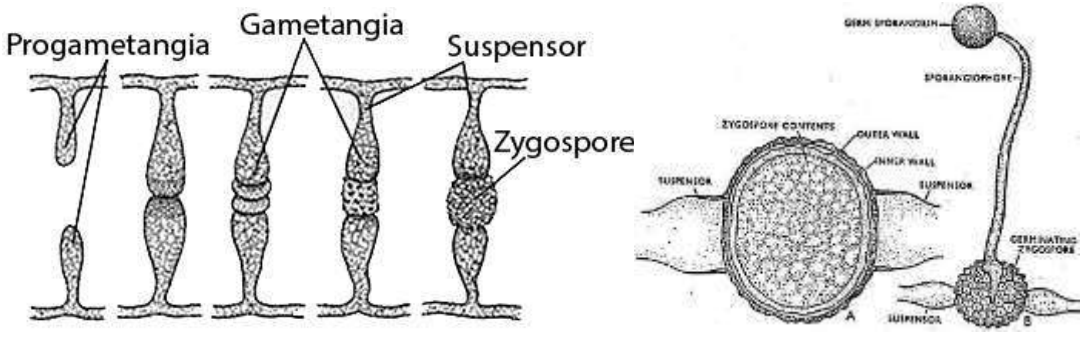


Figure 6. Zygospore development in Mucorales (Gauger, 1961; Agrios, 2005).

In Mucorales, partner recognition and progression of mating are mediated by trisporic acids and their precursors, which act as sex pheromones and are synthesized in a cooperative manner from intermediates provided by the complementary mating partner. Trisporic acid is a product of trisporoids pathway that derives of the carotenoids pathway which in turn derives from the mevalonate pathway. Mucoralean fungi synthesize carotenoids from the products of the mevalonate pathway (Fig. 7).

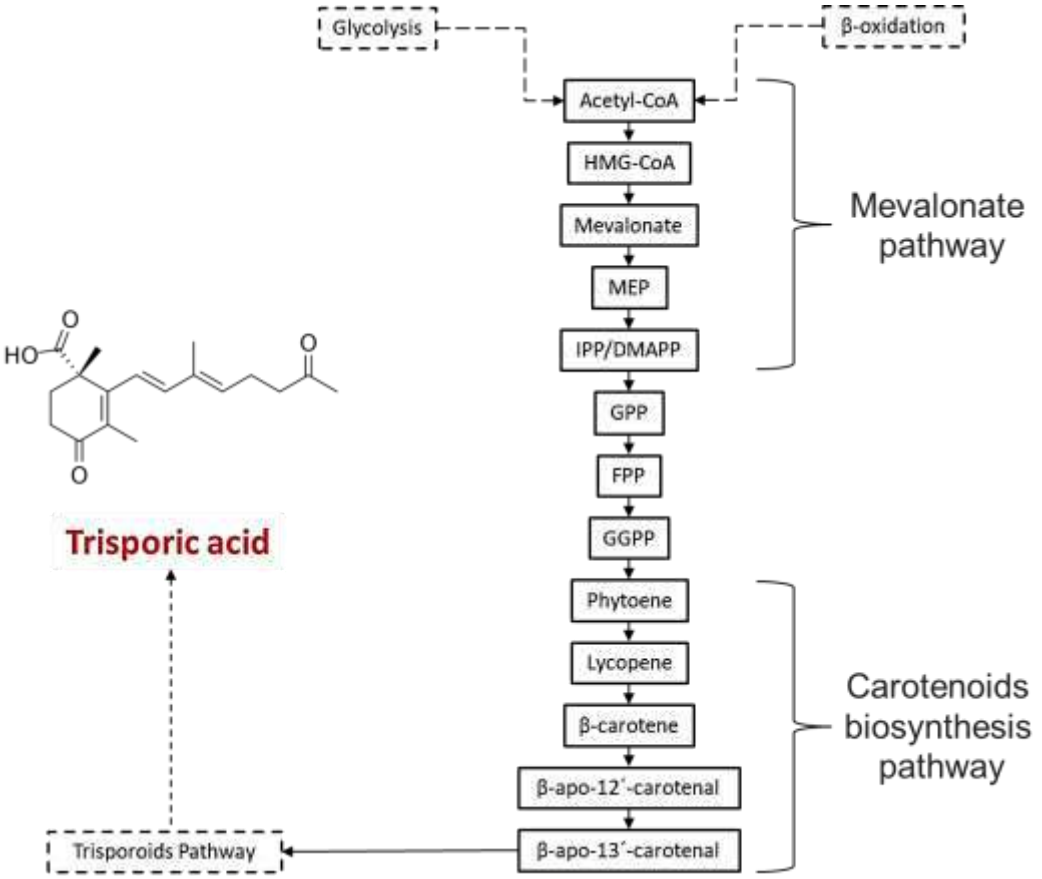


Figure 7. Mevalonate and carotenoids biosynthetic pathways are needed for the biosynthesis of Trisporic acid (Based on Arrach *et al.,* 2001, Almeida & Cerda 2008 and Alcalde & Fraser, 2016; Alcalde *et al.*, 2019).

The synthesis and cleavage of β-carotene are crucial for the production of trisporoids. The synthesis is performed by the protein products of *isoA*, *carG*, *carRA* and *carB* genes and the cleavage by *carS* and, *acaA* genes (Fig. 8).

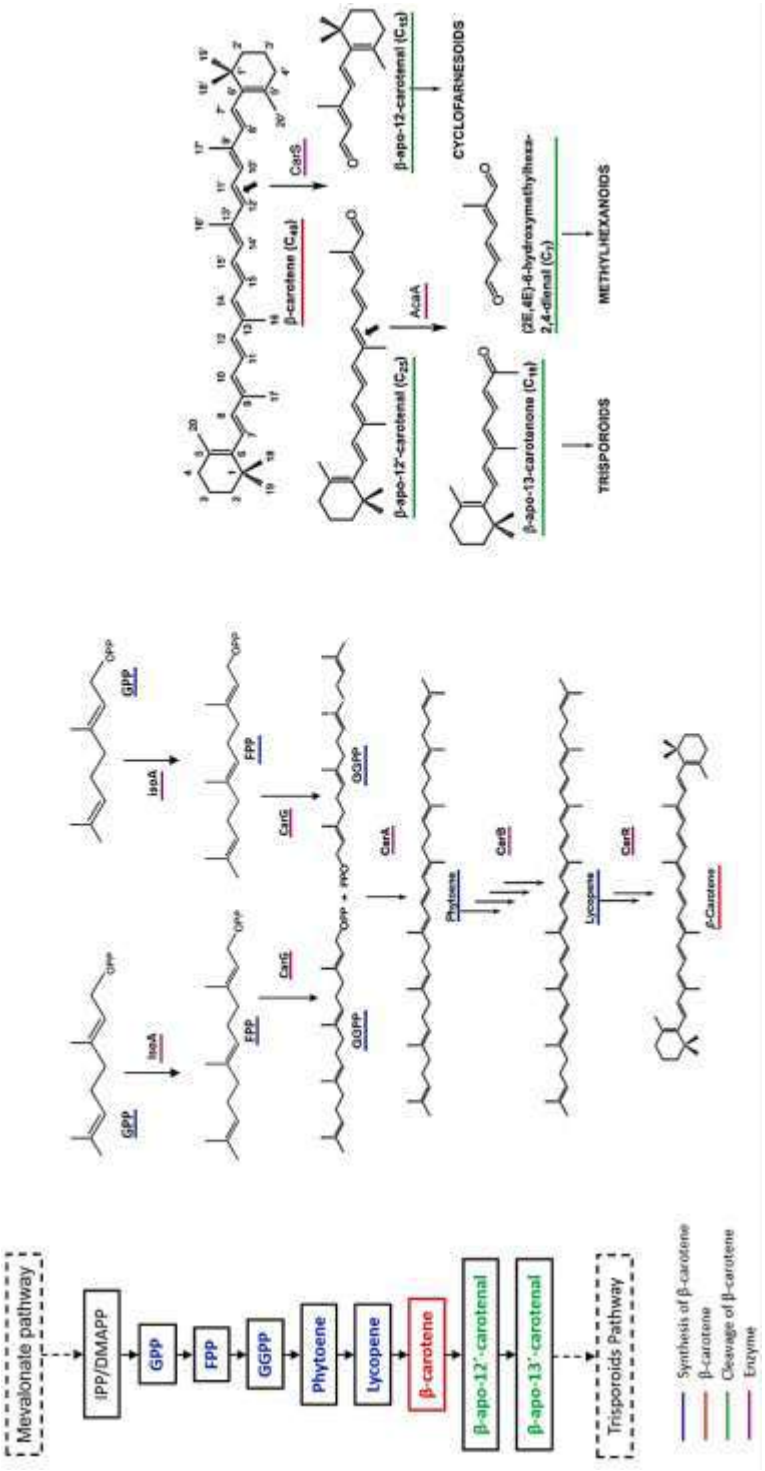


Figure 8. Synthesis and cleavage of β-carotene (Based on Arrach *et al.*, 2001; Almeida & Cerda 2008, Velayos *et al*. 2013, Alcalde & Fraser, 2016; Alcalde *et al.*, 2019).

A farnesyl pyrophosphate synthase (isoA) acts on a geranyl pyrophosphate molecule (GGP) to produce one farnesyl pyrophosphate molecule (FPP), that is later transformed to a geranylgeranyl pyrophosphate molecule (GGPP) by CarG enzyme. *carRA* gene has two domains: "R" and "A", which are translated as a single polypeptide, but with two functional independent enzymes. *carA* encodes a phytoene synthase that acts on 2 molecules of GGPP to synthesize 1 molecule of phytoene. Then, 4 units of CarB, wichs encode a phytoene dehydrogenase, acts to produce 1 lycopene molecule. Finally, 2 units of CarR are necessary for the cyclization of lycopene to produce β-carotene (Fig. 8) (Arrach *et al.*, 2001; Almeida & Cerda 2008; Velayos *et al.* 2013; Alcalde & Fraser, 2016).

The β-carotene molecules are cleavage by the action of two dioxygenase enzymes, CarS and AcaA, and the produced trisporoids (C18 molecules) are transformed to 4-dihydrotrisporin, which is the last common precursor for the biosynthesis of trisporic acid in the Mucorales (Fig. 9) (Alcalde *et al.*, 2019).

In the (m+), 4-dihydrotrisporin is converted into 4-dihydromethyltrisporate, difussed at the (m-) cells, and transformed in methyltrisporate to then complete the synthesis of trisporic acid. On the other hand, in (m-) cells, 4-dihydrotrisporin is converted into trisporin, difussed at (m+) and transformed in trisporol to then complete synthesis of trisporic acid (Fig. 9) (Medina *et al.*, 2011; Schimek *et al.*, 2012; Lee *et al.*, 2017).

The 4-Dihydromethyltrisporate dehydrogenase (TDH), encoded by the *tsp1* gene, mediates the conversion of the 4-dihydromethyl trisporate into methyltrisporate. The activity of TDH is differentially regulated in (m+) and (m–) fungi. TDH is activated in sexually stimulated (m–) cells, but not in non-stimulated (m–) or (m+) hyphae, indicating that this conversion only occurs in (m–) cells (Schimek *et al.*, 2005).

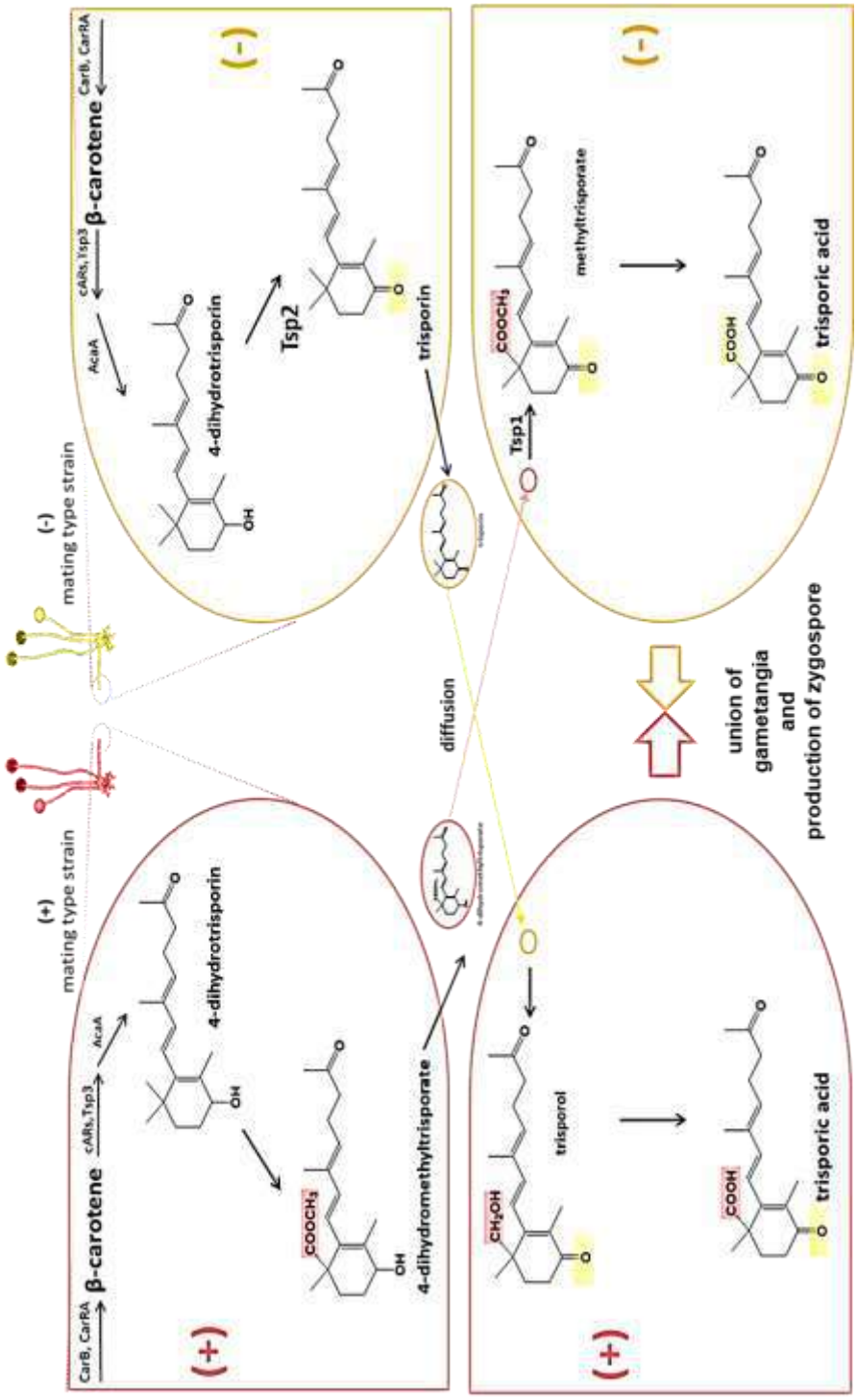


Figure 9. Trisporic acid synthesis in Mucorales (Based on Lee *et al.*, 2017).

The mating system of Mucoralean fungi is regulated by divergent alleles of a single gene: *SexP* and *SexM*. The sex gene is a member of the high mobility genes (HMG) family and is located between two flanking genes that code for a putative triose-phosphate transporter homolog (TPT) and an RNA helicase (RnhA) (Fig. 10) (Lee *et al.*, 2017).

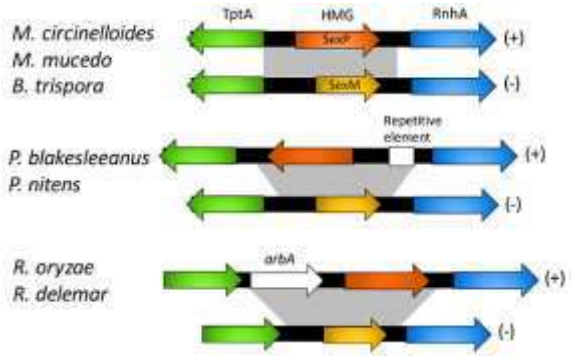


Figure 10. Sexual loci in Mucorales (Lee *et al.*, 2017).

How sexM and sexP control the sexual process is currently unknown. In several Mucorales, sexP gene is expressed during vegetative growth and mating; on the other hand, the *sexM* transcripts are expressed during mating. Supplementation of trisporic acid to the growing media during vegetative growth also induces the expression of *sexM*. Mutation of the c*ar* genes that are required for the synthesis of the trisporoids blocks the induction of the *sexM* and *sexP* genes. These observations suggest a connection between the sex genes and the pheromone trisporic acid, but the direct connection has yet to be revealed. There is the potential for an interaction between *sexP* and *sexM* to govern sexual development. Alternatively, each *s*exP or *s*exM transcription factor may have its own separate target genes that are specific for mating (Lee *et al.*, 2017).

Effects of *Mycetohabitans* on the sexual reproduction of *Rhizopus microsporus.*

Mondo and collaborators described that the effects of endobacteria to fungus are extended to sexual reproduction. They showed that the absence of endobacteria negatively influences the number of zygospores produced by sexually compatible host strains (Fig. 11). They proposed that the sexual

reproduction of *R. microsporus* is mediated by the symbiont gaining transcriptional control of the fungal *ras2* gene, which encodes a GTPase (Mondo *et al.*, 2017).

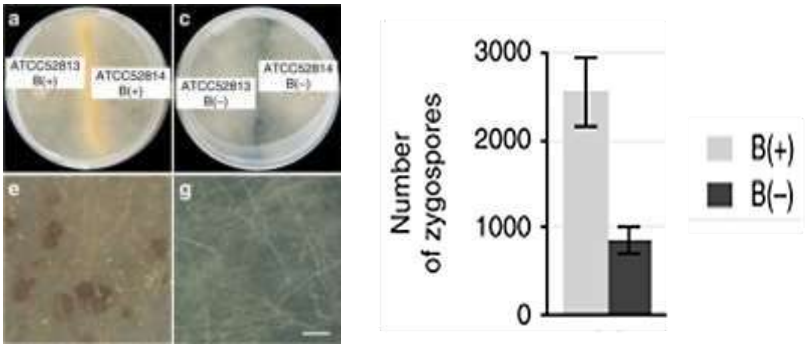


Figure 11. Impact of *Mycetohabitans rhizoxinica* presence on the sexual reproduction of *Rhizopus microsporus*. B (+) Presence of endobacteria. B (-) Absence of endobacteria (Modified of Mondo *et al.*, 2017).

In 2014, Dolatabadi and collaborators performed identification experiment of mating types of 48 strains of *R. microsporus* that belong to the CBS collection. They used the host strains CBS 699.68 (ATCC 52813) (m+) and CBS 700.68 (ATCC 52814) (m-) as control of mating. The goal of these authors was to define if the different varieties of *R. microsporus* were real or not. They reported 31 strains of (m+), 7 of (m-), and 8 that did not reproduce sexually under the tested conditions. Importantly, these authors concluded that *R. microsporus* is only one species. This would imply that a sexual compatibility exists, independently of whether some strains have bacterial symbionts and the majority of strains do not have (Partida-Martínez, 2007). In a later analysis (2016) they showed that 9 of the *R. microsporus* in the CBS collection harbored symbiotic bacteria, which is congruent with the previous report of Lackner, *et al.*, 2009.

All these previous results open an interesting series of questions: Does the symbiosis with *Mycetohabitans* spp. represent a reproductive barrier for sexual reproduction between host and non-host strains of *Rhizopus microsporus*? Does the presence of endobacteria enhance the sexual reproduction success of host *Rhizopus microsporus*? Does the absence of *Mycetohabitans* spp. compromise the viability of zygospores? Is the bacteria heritability biparental or uniparental?

HYPOTHESES

In the species *Rhizopus microsporus*:

1. The endosymbiont *Mycetohabitans* sp. represents a barrier for sexual reproduction between host and non-host strains of *Rhizopus microsporus*.

In host *Rhizopus microsporus* strains:

2. The presence of *Mycetohabitans* spp. enhances sexual reproduction success.

3. The absence of *Mycetohabitans* spp. compromises the viability of zygospores.

4. *Mycetohabitans* spp. are transmitted vertically through zygospores and their transmission is biparental and the sexual loci is equally distributed.

GENERAL AIM

Determine the effects of the symbiosis between *Mycetohabitans* spp. and *Rhizopus microsporus* on the sexual reproduction of the latter.

PARTICULAR AIMS

1. Determine if zygospores are produced in matings between host and non-host *R. microsporus* strains.

2. Determine if the presence/absence of *Mycetohabitans* spp. have effects on the formation, development, and viability of *R. microsporus* zygospores.

3. Determine if the production of β-carotene and/or trisporic acid are affected by the presence/absence of *Mycetohabitans* spp.

4. Determine if the segregation of *Mycetohabitans* spp. is biparental.

5. Determine the segregation of the sex locus in the progeny of *Rhizopus microsporus*.

MATERIALS AND METHODS

Species / ID Strain	Guest	Mating type (This work)	Mating type (Reported)	Source	Reference
Rhizopus microsporus 52813	*Mycetohabitans* sp. B4	(m+)	(m+) [1]	Soil, Ukraine	ATCC
R. microsporus 52813 b-	cured strain (b-)	(m+)	(m+) [2]	.	LIM
R. microsporus 52814	*Mycetohabitans* sp. B7	(m-)	(m-) [1]	Forest soil, Georgia	ATCC
R. microsporus 52814 b-	(b-)	(m-)	(m-) [2]	.	LIM
R. microsporus 52814	*M. rhizoxinica B7 - YFP*	(m-)	.	.	LIM
R. microsporus 52811	*Mycetohabitans* sp. B6	(m-)	(m+) [1] (m-) [2]	Not specified, USA	ATCC
R. microsporus 20577	*Mycetohabitans* sp. B2	(m+)	.	Soil, Japan	ATCC
R. microsporus 62417	*Mycetohabitans* sp. B1	(m+)	.	Rice seedlings, Japan	ATCC
R. microsporus 11559	Non-host (Nh)	(m+)	Not defined [2]	Rusia	ATCC
R. microsporus 52807	(Nh)	Not defined	(m-) [1]	Bread, China	ATCC
R. microsporus 22959	(Nh)	Not defined	(m+) [1]	Not specified	ATCC
R. microsporus 48010	(Nh)	Not defined	.	Tempeh, Indonesia	ATCC
R. microsporus 46348	(Nh)	Not defined	Not defined [1]	Tempeh, Indonesia	ATCC
R. microsporus 56018	(Nh)	Not defined	.	Not specified	ATCC
R. microsporus HPY99	*Mycetohabitans* sp.	(m+)	-	Papaya, Mexico	[3]
Rhizopus sp. HP475	*Mycetohabitans* sp.	Not defined	-	Tobacco, México.	[3]
Escherichia coli DH5α			.	.	Invitrogen
E. coli Juniper	.	.	.	.	BF-UG
E. coli DsRed	.	.	.	.	BF-UG

Table 3. Strains used in this project. ATCC: American Type Culture Collection; LIM: Laboratory of Microbial Interactions; BF-UG: Dr. Bernardo Franco – Universidad de Guanajuato. [1] Dolatabadi *et al.*, 2014 [2] Mondo *et al.*, 2017 [3] Cruz-Lachica *et al.*, 2018.

GROWTH MEDIA

To keep viable the organism stock throughout the experiments, the strains were grown in:

- *Rhizopus microsporus* - Potato Dextrose Agar at 30° C.
- *Mycetohabitans* species - Supplemented Tryptic Soy Broth at 30 °C.
- *Escherichia coli* - Luria-Bertani Agar at 37° C.

All the growth media were sterilized in an autoclave for 15 min, 121° C at 2 atm of pressure.

Potato Dextrose Agar (PDA)

4 g of potato extract, 20 g of dextrose, and 15 g of agar were suspended in 1 L of distilled water.

Supplemented Tryptic Soy Agar (Supplemented TSA)

15 g of casein peptone, 5 g of soy peptone, 5 g of sodium chloride, 20 mL of glycerol, 4.8 g of magnesium sulfate, and 15 g of agar were suspended in 1 L of distilled water.

Supplemented Tryptic Soy Broth (Supplemented TSB)

15 g of casein peptone, 5 g of soy peptone, 5 g of sodium chloride, 20 mL of glycerol, and 4.8 g of magnesium sulfate were suspended in 1 L of distilled water.

Luria-Bertani Agar (LB Agar)

10 g of Peptone, 5 g of yeast extract, 5 g of sodium chloride, and 15 g of agar were suspended in 1 L of distilled water.

Luria-Bertani Broth (LB Broth)

10 g of Peptone, 5 g of yeast extract, and 5 g of sodium chloride were suspended in 1 L of distilled water.

Malt Extract Agar (MEA)

12.75 g maltose, 2.75 g dextrose, 0.78 g peptone, 2.25 g glycerol, and 15 g agar were suspended in 1 L of distilled water.

PRIMERS AND PCR CONDITIONS

Primer ID	Sequence (5'- 3´)	Tm °C	Gene
ITS-1F	CTTGGTCATTTAGAGGAAGTAA	53	Internal transcribed spacer
ITS-4R	TCCTCCGCTTATTGATATGC	53	Internal transcribed spacer
16S-F27	AGAGGTTTGATCCTGGCTCAG	55	16S ribosomal RNA
16S-R1494	CTACGGRTACCTTGTTACGAC	55	16S ribosomal RNA
B4AMP1 F	GTGTCGAATCGCAGCAACC	64	cAMP signaling in bacteria
B4AMP1 R	TTTCCGGTCGATGTCTGCC	64	cAMP signaling in bacteria
B7Purin1 F	CAACAGTTTCGGCACAGGC	64	Purine utilization
B7Purin1 R	CACGGCCAACAACATGAGC	64	Purine utilization

Table 4. Primers used in this project.

Reagent	Volume µL
Buffer 10x Taq with KCL [100 mM Tris-HCl (pH 8.8 at 25°C),500 mM KCl, 0.8% (v/v) Nonidet P40]	1.0
DMSO	0.25
$MgCl_2$ (25 mM)	0.75
dNTPs (10 mM)	0.2
Primer R (10 µM) (Tab. 3)	0.5
Primer F (10 µM) (Tab. 3)	0.5
H_2O milli-Q	6.175
Taq DNA Polymerase, recombinant (5 u/µL)	0.125
DNA (40 ng/ µL)	0.5
Total volume of the PCR reaction	10 µL

Table 5. PCR reagents and concentration.

Temperature °C	Time min	Rx	Cycles
94	3:00	Denaturation	1 X
94	0:45	Denaturation	35 X
53	0:45	Annealing	
72	1:00	Extension	
72	7:00	Extension	1 X
4	∞	Cooling	1 X

Table 6.- PCR Conditions of the *ITS* gene.

Temperature °C	Time min	Rx	Cycles
95	2:00	Denaturation	1 X
95	0:40	Denaturation	35 X
55	0:30	Annealing	
72	2:00	Extension	
72	7:00	Extension	1 X
4	∞	Cooling	1 X

Table 7.- PCR conditions of the 16*S* rRNA gene.

Temperature °C	Time min	Rx	Cycles
95	3:00	Denaturation	1 X
95	0:45	Denaturation	35 X
64	0:45	Annealing	
72	7:00	Extension	1 X
4	∞	Cooling	1 X

Table 8.- PCR conditions of the *B4AMP1* gene.

Temperature °C	Time min	Rx	Cycles
95	3:00	Denaturation	1 X
95	0:45	Denaturation	35 X
64	0:45	Annealing	
72	7:00	Extension	1 X
4	∞	Cooling	1 X

Table 9.- PCR conditions of the *B7Purin1* gene.

STRAINS CHARACTERIZATION, MATINGS AND POST-ANALYSES

Preinocula

Mycelium from PDA plates incubated at 30°C for 2 days was used as inocula for all experiments.

Phenotyping and genotyping of strains of *Rhizopus microsporus*

Tested strains

Batch 1

- ATCC 52813 – Control: endobacteria (b+) and plus mating type (m+).
- ATCC 52814 – Control: endobacteria (b-) and minus mating type (m-).
- ATCC 11559 – Control: Non-host strain.
- ATCC 20577
- ATCC 62417
- ATCC 52811
- ATCC 52807
- ATCC 22959
- ATCC 48010

Batch 2: ATCC 46348, ATCC 56018, HPY99 and *Rhizopus* sp. HP475.

Morphological description (Batch 1)

In eight-fold, a disk of mycelium (~5 mm) was inoculated and grown for 96 h at 30 °C on plates of PDA. The diameter growth was measured every 24 h. The morphological structures were observed.

DNA extraction and amplification of the *ITS* and 16s *rRNA* genes

The gDNA was extracted of mycelium from PDA plates incubated at 30 °C for 72 h by Nicholson's method (Annex A). The *ITS* (Tab. 6) and *16s rRNA* (Tab. 7) genes were amplified by PCR (Tab. 5) from total gDNA.

Test of mating type (Batch 1 + 46348 + 56018)

In eight-fold, sexual reproduction was assessed on MEA plates co-inoculating disks of mycelia from tested strains at 5 mm distance for 7 days at 30 °C in darkness:

MATING	52813	52814	11559	20577	62417	52811	52807	22959	48010
52813	813x 813	813x814	813x599	813x577	813x417	813x811	813x807	813x959	813x010
52814	-	814x814	814x559	814x577	814x417	814x811	814x807	814x959	814x010
11559	-	-	599x559	599x577	599x417	599x811	599x807	599x959	599x010
20577	-	-	-	577x577	577x417	577x811	577x807	577x959	577x010
62417	-	-	-	-	417x417	417x811	417x807	417x959	417x010
52811	-	-	-	-	-	811x811	811x807	811x959	811x010
52807	-	-	-	-	-	-	807x807	807x959	807x010
22959	-	-	-	-	-	-	-	959x959	959x010
48010	-	-	-	-	-	-	-	-	010x010
46348	348x813	348x814	348x599	348x577	348x417	348x811	348x807	348x959	348x010
56018	018x813	018x814	018x559	018x577	018x417	018x811	018x807	018x959	018x010

Isolation and quantification of zygospores (Batch 1)

In four-fold, sexual spores produced were detached from agar plates in 15 mL 0.01% Triton X-100 with the aid of a glass spatula. The zygospores were separated from aggregates and cell debris by filtration using a polyester fiber. The zygospores were quantified and their diameter was measured. The average dry weight was calculated using the biomass of the remaining 4 Petri plates.

Phenotyping and genotyping of cured strains

Strains

- ATCC 52813 wt, with endobacteria (b+) and plus mating type (m+).
- ATCC 52814 wt, with endobacteria (b+) and minus mating type (m-).
- ATCC 52813 cured*, without endobacteria (b-).
- ATCC 52814 cured*, without endobacteria (b-).

(*) The bacteria-free strains (b−) were generated previously in the LIM by Dra. Partida-Martínez growing mycelia constantly in PDB supplemented with 50 µg/mL ciprofloxacin until the lack of sporulation.

Morphological description

In eight-fold, a disk of mycelium (~5 mm) was inoculated and grown for 96 h at 30°C on plates of PDA. The diameter growth was measured every 24 hrs. The morphological structures were observed.

DNA extraction and amplification of the *ITS* and *16s rRNA* genes

The gDNA was extracted of mycelium from PDA plates at 30°C for 72 h by Nicholson's method (Annex A). The *ITS* (Tab. 6) and *16s rRNA* (Tab. 7) genes were amplified by PCR (Tab. 5) from total gDNA.

Test of mating

In twenty-five-fold, sexual reproduction was assessed on MEA plates co-inoculating disks of mycelia from tested strains at 5 mm distance for 7 days at 30 °C in darkness:

Mating	52813 wt	52814 wt	52813 b-	52814 b-
52813 wt	813wt x 813wt	813wt x 814wt	813wt x 813b-	813wt x 814b-
52814 wt	-	814wt x 814wt	814wt x 813wt	814wt x 814b-
52813 b-	-	-	813b- x 813b-	813b- x 814b-
52814 b-	-	-	-	814b- x 814b-

Isolation and quantification of zygospores

In twenty-fold, sexual spores produced were detached from agar plates in 15 mL 0.01% Triton X-100 with the aid of a glass spatula. The zygospores were separated from aggregates and cell debris by filtration using a polyester fiber. The zygospores were quantified and their diameter were measured. The average dry weight was calculated using the biomass of the remaining 5 Petri plates.

Test of viability of zygospores

The zygospores were centrifuged in a 10–80% sucrose gradient at 4500 rcf for 60 min at 4 °C. The zygospores were harvested from 40% and 50% sucrose fraction and were observed on the microscope to corroborate their integrity. Monozygosporic cultures were incubated on 96-well plate PDA for 48 hours at 30 °C.

Extraction and quantification of β-carotene

Matings samples analyzed:

- ATCC 52813 wt x ATCC 52814 wt
- ATCC 52813 b- x ATCC52814 b-

Biomass of matings of 7 days was detached and mixed with 15 mL of ethyl acetate in 25 mL Erlenmeyer flask with a cap on magnetic stirrer overnight. The supernatant was decanted into a previously weighed 10 mL glass beaker and left exposed 24 h until ethyl acetate had evaporated. The product was weighed and used to prepare a solution 1 mg of extract / 100 µL of methanol. The samples were analyzed by HPLC in the Chromatography Laboratory of Unidad Irapuato of CINVESTAV.

Reconstruction of the metabolic pathway of carotenoids of *R. microsporus*

A bibliographic search of isoprenoids, carotenoids, and trisporoids synthesis pathways and the genes involved on Mucoralean fungi was performed (Fig. 8-9). The orthologous genes were searched on the *R. microsporus* genomes (Tab. 9) by Basic Local Alignment Search Tool (BLAST) and the OrthoDB database.

STRAIN	Symbiont	Genome Size (MB)	# Protein Genes	# Scaffolds	N50 Kb	Sequenced by
ATCC 52813	B4	26	10905	131	111	JGI
ATCC 52814	B7	25	11502	560	105	JGI
ATCC 62417	B1	48	18869	1386	198	HKI
ATCC 11559	-	48	19563	1554	53	HKI
CBS 344.29	-	49	20088	1554	53	HKI

Table 10. Information of genomes of *Rhizopus microsporus*.

The candidate genes (Annex D) were analyzed with the Conserved Domain Database (CDD), Pfam, and InterPro to identify the functional annotation of proteins. Clustal Omega was used to highlight areas of similarity between sequences.

Reconstruction of the metabolic pathway of *Mycetohabitans* spp.

To identify a possible pathway of isoprenoids and carotenoids synthesis I used Rapid Annotation using Subsystem Technology (RAST) to do a metabolic reconstruction of the endobacterial symbionts via KEGG map metabolic pathways:

STRAIN	Host	Genome Size (MB)	# Contigs
B4	ATCC 52813	3.57	2
B7	ATCC 52814	3.65	3
B1	ATCC 62417	3.75	5

Table 11. Information of genomes of associated *Mycetohabitans* spp. with *Rhizopus microsporus*.

Fungal comparative expression of isoprenoids and carotenoids genes

Based on the identified genes of the isoprenoids and carotenoids synthesis pathways of *R. microsporus*, I made an expression data comparison of the gene libraries processed by Bermúdez-Barrientos (LIM, 2016 - Annex B), between fungi mating with endosymbionts (ATCC 52813 wt x ATCC 52814 wt) and mating of cured fungi (ATCC 52813 b- x ATCC 52814 b-).

Bacterial comparative expression of isoprenoids and carotenoids genes

Based on the identified genes of the metabolic reconstruction of the isoprenoids, and carotenoids synthesis pathways of *M. rhizoxinica*, I made an expression data comparison of the gene libraries processed by Bermúdez-Barrientos (LIM, 2016; Annex B), between fungi mating with endosymbionts (ATCC 52813 wt x ATCC 52814 wt) and the strains grown independently (ATCC 52813 wt and ATCC 52814 wt).

Segregation analysis of *M. rhizoxinica*

Strain-specific genes were identified with RAST and OrthoVenn2. The gDNA was extracted of 12 monozygosporic cultures from PDA plates incubated for 72 h at 30 °C. Mycelial disks from each monozygosporic culture for the characterization of their mating type were conserved. The Strain-specific genes, *B4AMP1* (*Mycetohabitans* sp. B4, Tab. 8) and *B7Purin1* (*Mycetohabitans* sp. B4, Tab. 9), were amplified by PCR.

Segregation analysis of mating type

The mating type of the 12 monozygotic cultures was assessed on MEA plates by co-inoculating disks of their mycelia and mycelia of ATCC 52813 (m+) or ATCC 52814 (m-) strains at 5 mm distance for 12 days at 30 °C in darkness. The cultures were observed for 14 days to evaluated the generation of zygospores.

HARDWARE AND SOFTWARE

All statistical analyses and graphs were performed and generated in R 3.5.1. and Statistica 8.0.

For the reconstruction of the metabolic pathways and the analyses of sequences, the following resources were used:

- Blast - https://blast.ncbi.nlm.nih.gov/Blast.cgi
- Conserved Domain Database - https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
- Pfam - https://pfam.xfam.org/
- InterPro - https://www.ebi.ac.uk/interpro/
- Clustal Omega - https://www.ebi.ac.uk/Tools/msa/clustalo/
- RAST - https://rast.nmpdr.org/

For the analysis of unique genes and the design of oligos, the following programs were used:

- OrthoVeen2 - https://orthovenn2.bioinfotoolkits.net
- Primer-Blast - https://www.ncbi.nlm.nih.gov/tools/primer-blast/
- OligoAnalysis - https://www.eurofinsgenomics.eu/en/ecom/tools/oligo-analysis.aspx

The iconographies were designed in Photoshop CS6 and PowerPoint 2016.

The photographs were made with a Canon Eos 4000d camera.

Microscopic observations were done using the Leica DM750, Nikon Eclipse Ti, and Olympus microscopes.

RESULTS

Identification of characteristic structures of *Rhizopus microsporus*

All the wild type strains of *Rhizopus microsporus* used in this work showed the capacity to grow on the PDA and MEA culture media, as well as to form the characteristic structures of the species for its asexual reproduction, regardless of its origin (Fig. 12 A-D and 13).

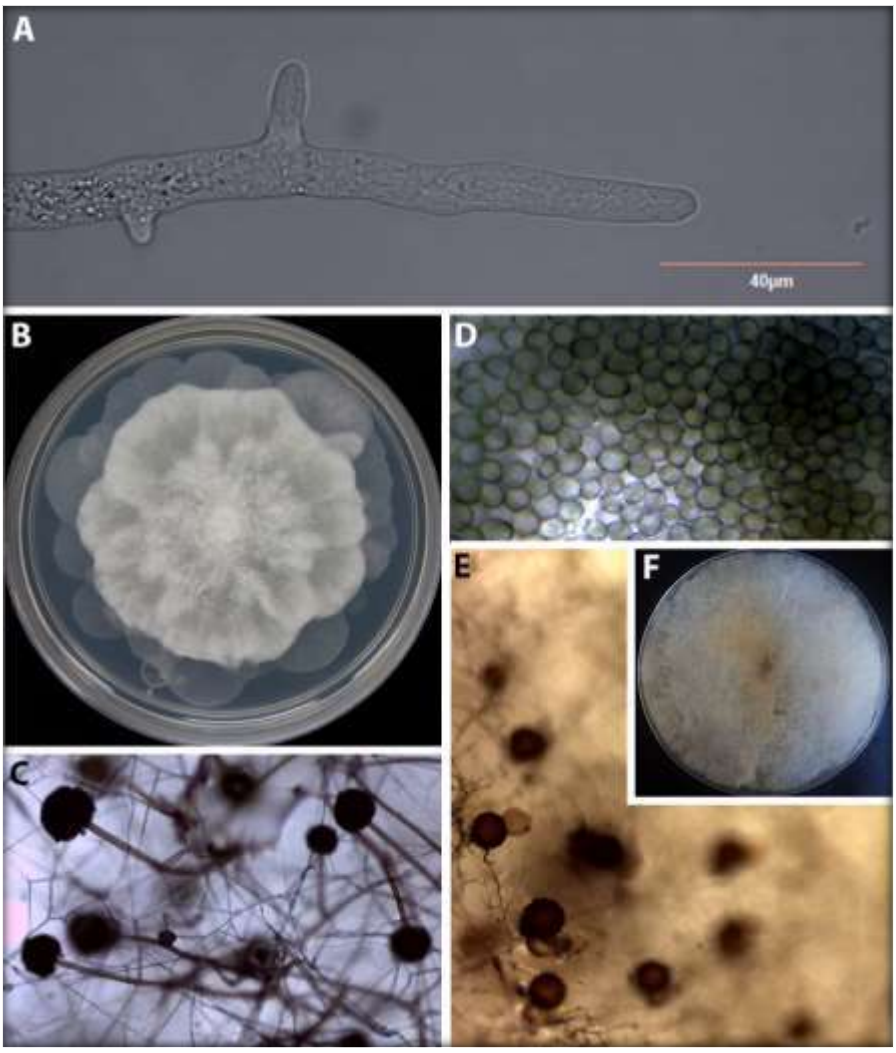


Figure 12. Morphological structures of *Rhizopus microsporus*.
(A) Hypha grown on PDA at 30 °C. Scale bar: 40µm. **(B)** Colony grown on PDA at 30 °C for 72 h. **(C)** Sporangia and rhizoids. **(D)** Sporangiospores. **(E)** Zygospores produced during the sexual reproduction. **(F)** Mating between plus and minus partners on MEA at 30°C for 7 d.

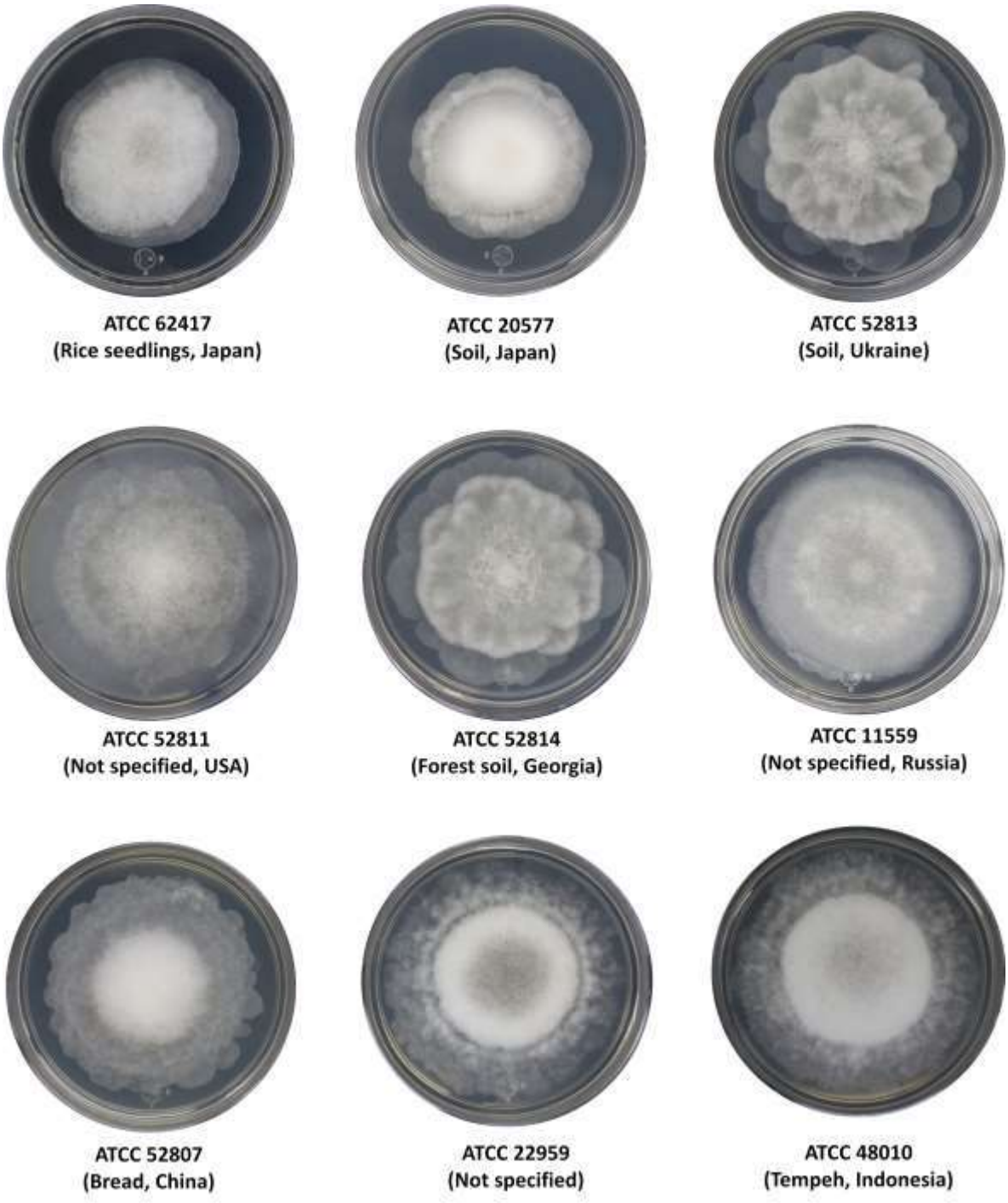


Figure 13. Morphology of the *Rhizopus microsporus* colonies. ATCC Strain (source). 72 h of growth at 30°C on PDA plates.

Identification of natural host and non-host strains

In order to evaluate all hypotheses, I corroborated in the first place the strains that have bacterial endosymbionts. In each strain, I amplified by PCR the fungal genetic marker *ITS,* as an amplification control, and the bacterial genetic marker *16s rRNA*. In my first batch of ATCC strains (Fig. 13) there are 4 non-host strains (11559, 52807, 22959, 48010; See annex 2A), and 5 strains with bacterial endosymbionts (52813, 20577, 62417, 52814 and 52811) in which the bacterial genetic marker *16s rRNA* amplified (~1400 pb):

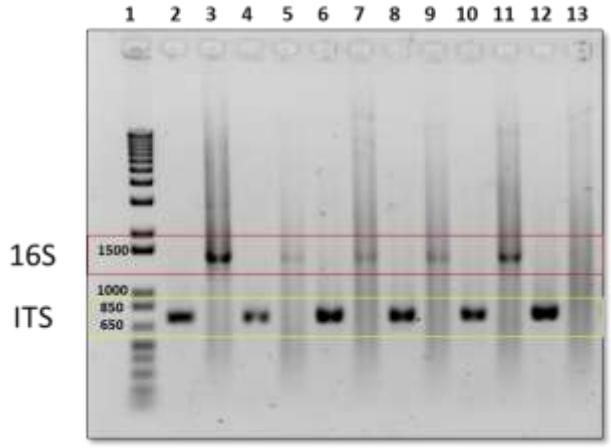
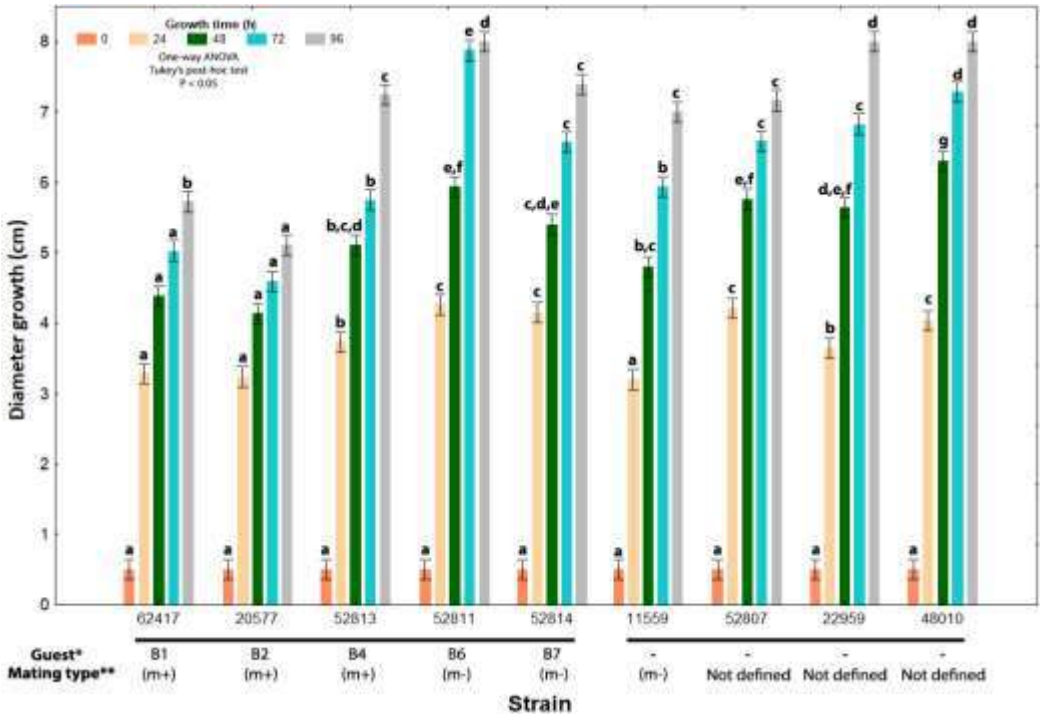


Figure 14. Identification of natural host strains of *Rhizopus microsporus*. Amplification by PCR of the fungal genetic marker *ITS* and the bacterial genetic marker *16s rRNA* from total gDNA. **(1)** DNA ladder, **(2)** ATCC 52813 *ITS*, **(3)** ATCC 52813 *16s rRNA*, **(4)** ATCC 20577 *ITS*, **(5)** ATCC 20577 *16s rRNA*, **(6)** ATCC 62417 *ITS*, **(7)** ATCC 62417 *16s rRNA*, **(8)** ATCC 52814 *ITS*, **(9)** ATCC 52814 *16s rRNA*, **(10)** ATCC 52811 *ITS*, **(11)** ATCC 52811 *16s rRNA*, **(12)** ATCC 11559 *ITS*, **(13)** ATCC 11559 1*6s rRNA* (Control -).

In the second batch of fungal strains, I identified 2 non-host (ATCC 46348, ATCC 56018; see annex 2B), and 2 with bacterial endosymbiont (HPY99 and *Rhizopus* sp. HP475; see annex 2B). The sequencing of the bacterial genetic marker 16s rRNA and their subsequent BLAST analysis, suggest that both bacteria belong to the *Mycetohabitans rhizoxinica* species. This would be the first report of *Mycetohabitans* spp. of a *Rhizopus microsporus* isolated from Mexican soils.

Differences in the growth between natural host and non-host strains

Subsequently, I monitored the growth on PDA plates of all strains for 96 h. I measured the diameter of the colony every 24 h to compare growth differences between the host and non-host strains (Fig. 15). Natural non-host strains tended grow more compared with host strains (Factorial ANOVA followed by Tukey's post-hoc test, $P < 0.05$). After 48 h, the growth differences were noticeable among all the strains (One-way ANOVA followed by Tukey's post-hoc test, $P < 0.05$), regardless of their isolation source.



***** Strains of *Mycetohabitans* spp. reported by Partida-Martínez 2007.

****** Mating type determined in this work: Mating type plus (m+) and minus (m-) (see the next section).

Figure 15. Differences in the growth of natural host and non-host strains. Growth curve on plates of PDA for 96 h at 30°C. The letters group the strains by significant differences in the diameter growth at a time point on the curve. One-way ANOVA followed Tukey's post-hoc test, $P < 0.05$, $n = 8$ in each point. Host strain (source): ATCC 62417 (Rice seedlings, Japan), ATCC 20577 (Soil, Japan), ATCC 52813 (Soil, Ukraine), ATCC 52811 (Not specified, USA) and ATCC 52814 (Forest soil, Georgia). Non-host strain (source): ATCC 11559 (Not specified, USSR), ATCC 52807 (Bread, China), ATCC 22959 (Not specified), and ATCC 48010 (Tempeh, Indonesia).

Identification of the mating type of the natural host and non-host strains of *Rhizopus microsporus*

The experiments of mating employing 11 *Rhizopus microsporus* strains (Batch 1 + ATCC 46348 and ATCC 56018) revealed that sexual reproduction is only successful when partners were both host strains (Figure 16). Lack of sexual reproduction was observed for all matings of non-host strains, and also for those that involved a host and a non-host partner, independently of the sex locus. The mating type of natural non-host strains can be determined by amplifying the HMG region of their sexual locus and comparing it by alignment analysis against the control sequences from ATCC 52813 (m +) and ATCC 52814 (m-) strains. There is a probability that the non-host strains used in this work are of the same sex.

MATING TEST			Host					Non-host					
			(m+)			(m-)		(m?)					
			52813	20577	62417	52814	52811	11559	52807	22959	48010	46348	56018
Host	(m+)	52813	x	x	x	✓	✓	x	x	x	x	x	x
		20577	x	x	x	✓	✓	x	x	x	x	x	x
		62417	x	x	x	✓	✓	x	x	x	x	x	x
	(m-)	52814	✓	✓	✓	x	x	x	x	x	x	x	x
		52811	✓	✓	✓	x	x	x	x	x	x	x	x
Non-host	(m?)	11559	x	x	x	x	x	x	x	x	x	x	x
		52807	x	x	x	x	x	x	x	x	x	x	x
		22959	x	x	x	x	x	x	x	x	x	x	x
		48010	x	x	x	x	x	x	x	x	x	x	x
		46348	x	x	x	x	x	x	x	x	x	x	x
		56018	x	x	x	x	x	x	x	x	x	x	x

✓ Zygospores production x No zygospores production

Figure 16. Summary of results of matings test of *Rhizopus microsporus* strains.
(m+) mating type plus. (m-) mating type minus. (m?) mating type not defined yet.

Phenotypic characterization of the zygospores produced in the successful matings between host strains.

Regardless of the fungal parents involved in the matings, the produced zygospores have a very similar phenotype. Zygospores have a characteristic dark brown color and morphology (Fig. 17). They have an average diameter of 55.66 ± 10.86 µm at 7 d after the inoculation of the strains (Fig. 17).

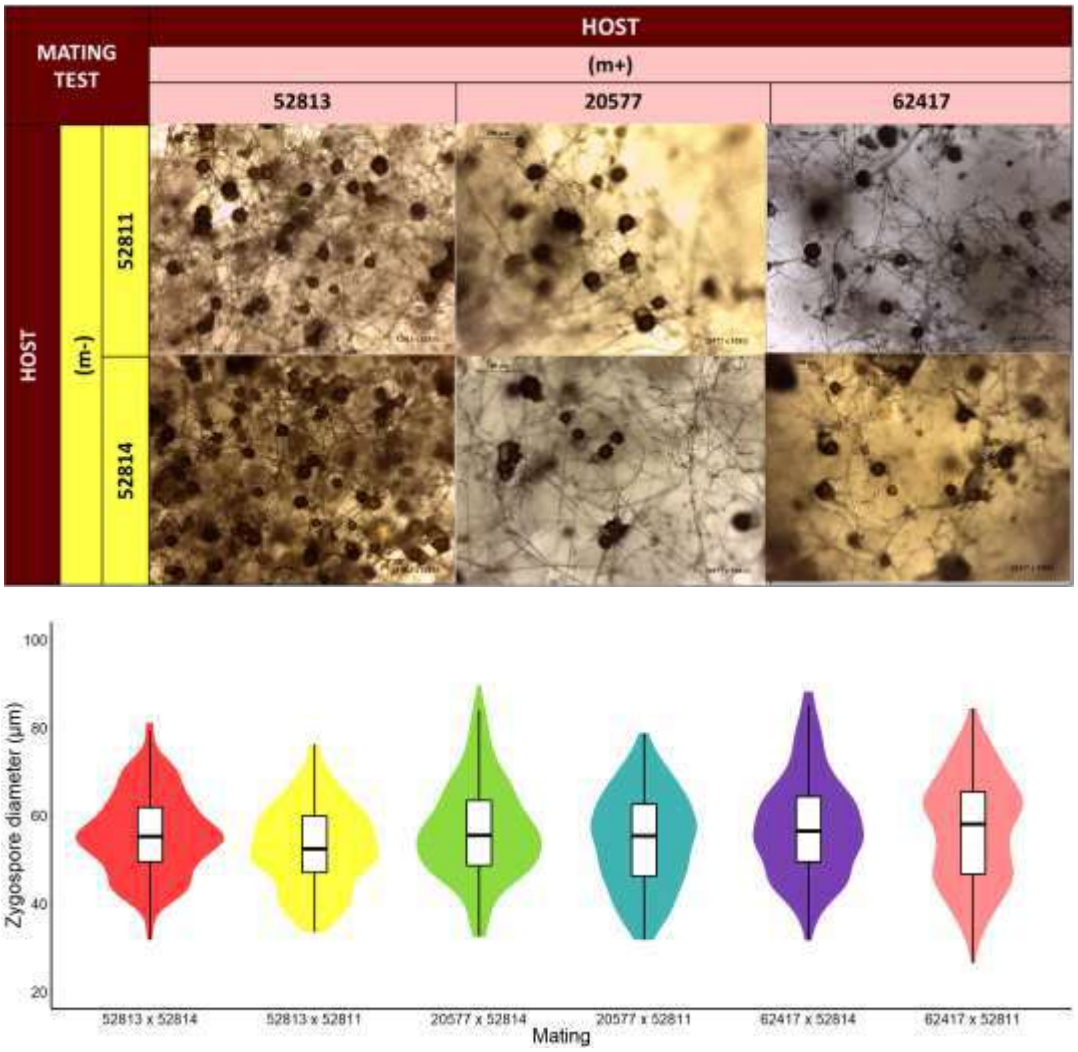


Figure 17. Zygospores produced by matings between host strains. Micrographs show zygospores produced after 7 days of the inoculation of strains. Scale bar represents 200 µm. The average diameter size for all zygospores produced is 55.66 ± 10.86 µm. Kruskal-Wallis test: H (5, N= 1200) =20.99742 p =.0008 (Annex C: Multiple comparisons of mean ranks for all group).

The produced zygospores were held between two unequal suspensors (Fig.12-E). In all successful matings, a straight line of zygospores at the interface between the two compatible strains was formed (Fig. 12-F), as well as brown coloration throughout that area.

On the other hand, the number of zygospores produced in these matings was influenced by the fungal parents involved (Factorial ANOVA followed by Tukey's post-hoc test; Fig. 18).

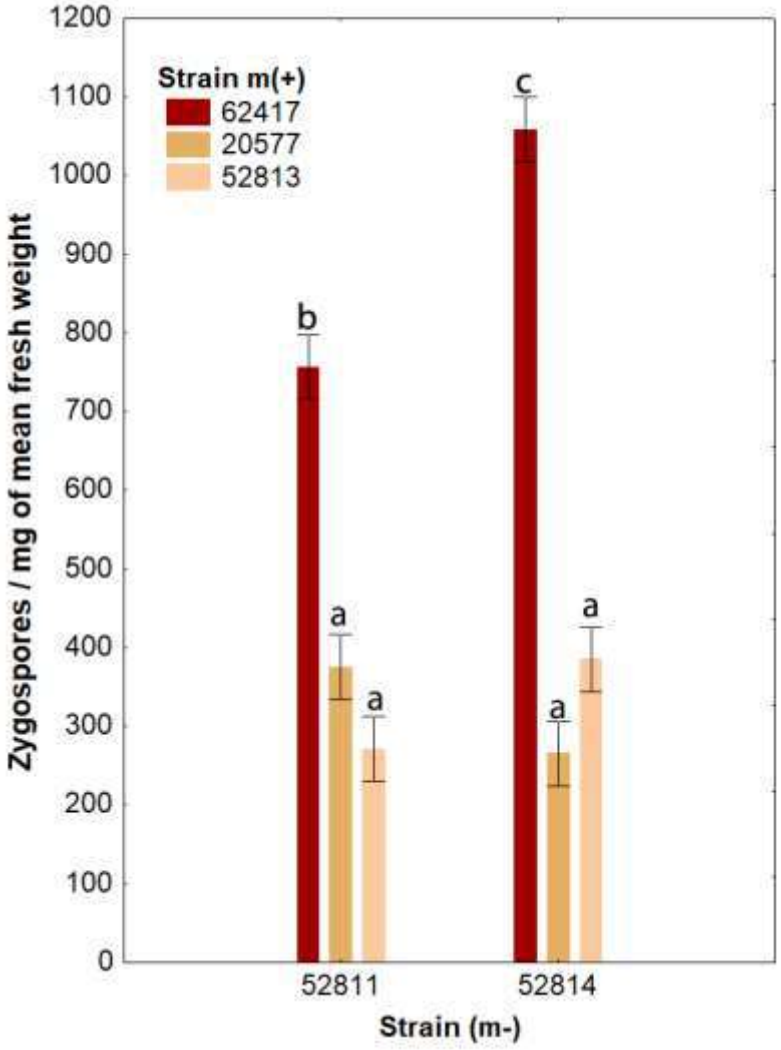


Figure 18. Quantification of zygospores produced by matings between host strains of *Rhizopus microsporus*. Factorial ANOVA followed by Tukey's post-hoc test, P <0.05, n = 4 in each mating combination.

Influence of the presence of *Mycetohabitans* on sexual reproduction between host strains.

In order to determine the influence of the symbiosis with *Mycetohabitans* on the sexual reproduction of *R. microsporus*, I made use of the "cured" strains (b-). To this end, I first verified the phenotype and genotype of all host strains that were previously treated with an antibiotic by Partida-Martínez.

To explain these experiments and their results, the following iconography will be used to differentiate between host and cured strains:

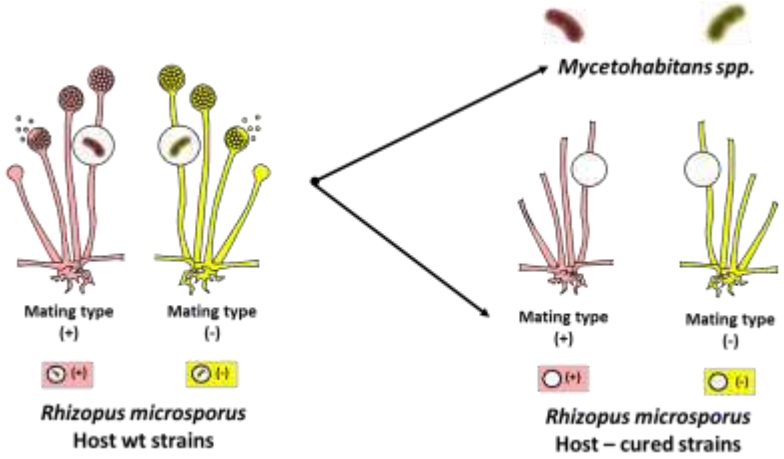


Figure 19. The Iconography of host and cured strains of *Rhizopus microsporus*.

The cured strains of *Rhizopus microsporus* used in this work showed the capacity to grow on the PDA (Fig. 20) and MEA culture media. The cured strains lack of the sporangiophore and sporangiospores production, as described by Partida-Martínez *et al.*, 2007.

Figure 20. Morphology of cured strains of *Rhizopus microsporus*. 72 h of growth at 30°C on plates of PDA. **(b-)** host without bacteria (cured strain).

These phenotypic observations were complemented with the genotypic characterization. As shown in Fig. 21, cured strains did not amplify the *16S rRNA* gene.

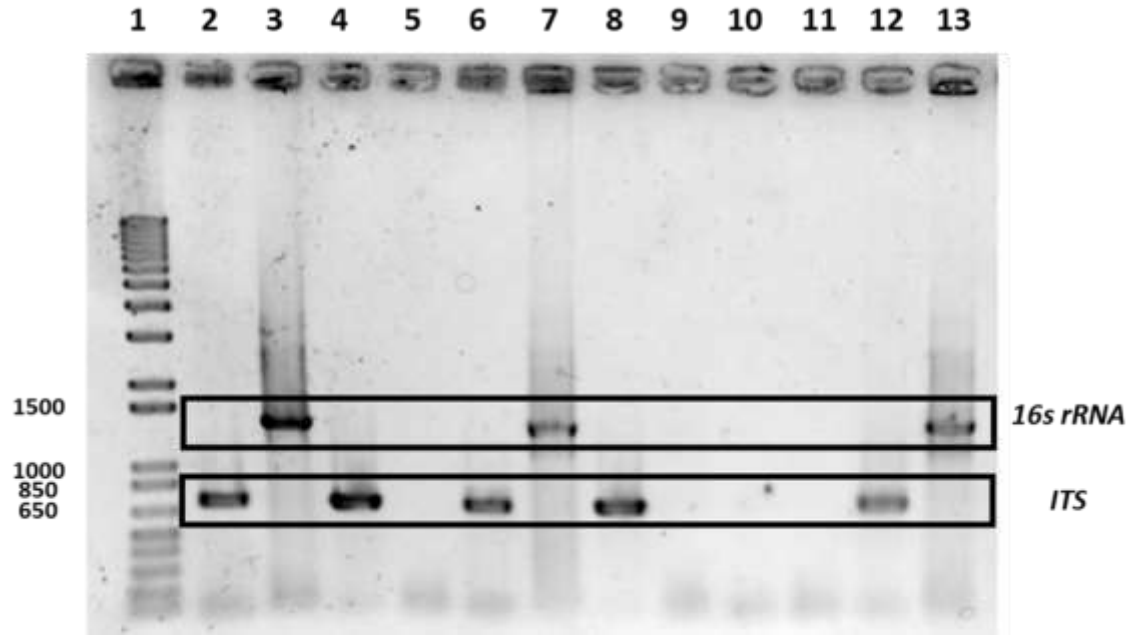


Figure 21. Molecular genotyping of cured strains of *Rhizopus microsporus*. Amplification by PCR of the fungal genetic marker *ITS* and the bacterial genetic marker *16s rRNA* from total gDNA. **(1)** DNA ladder, **(2)** ATCC 52813 *ITS*, **(3)** ATCC 52813 *16s rRNA*, **(4)** ATCC 52813 b- *ITS*, **(5)** ATCC 52813 b- *16s rRNA*, **(6)** ATCC 52814 *ITS*, **(7)** ATCC 52814 *16s rRNA*, **(8)** ATCC 52814 b- *ITS*, **(9)** ATCC 52814 b- *16s rRNA*, **(10)** Control (-) *ITS*, **(11)** Control (-) *16s rRNA*, **(12)** Control (+) *ITS*, **(13)** Control (+) *16s rRNA*.

Differences in the growth between host and cured host strains

I evaluated the growth differences between wild host and cured strains after 24 hr. These experiments showed that lack of *Mycetohabitans* had significant effects on growth, reducing it in strain ATCC 52813, but increasing it in strain ATCC 52814 (One-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$; Fig. 22).

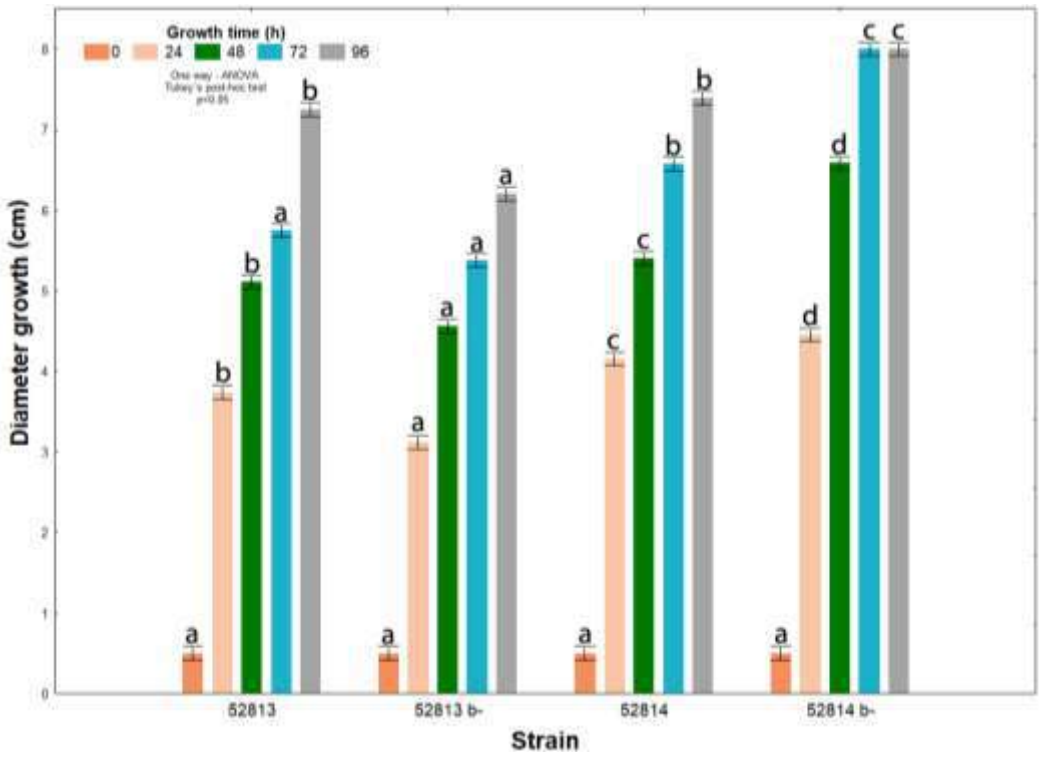


Figure 22. Growth curve of host and cured strains of *Rhizopus microsporus*. Growth on plates of PDA for 96 h at 30°C. One-way ANOVA followed Tukey's post-hoc test, P <0.05, n = 8 in each point. **(b-)** host without bacteria (cured strain). The letters group the strains by significant differences in the diameter growth at a time point on the curve. One-way ANOVA followed Tukey's post-hoc test, P <0.05, n = 8 in each point.

Impact on the zygospores production due to the absence of *Mycetohabitans* spp. in the host strains of *Rhizopus microsporus*.

Later, I did tests of mating on these strains to evaluate the effects caused by the absence of *Mycetohabitans* spp. (Fig. 23). These experiments revealed that sexual reproduction is 100% successful when at least one of the host parents keeps their natural bacteria (Fig. 23). I observed a reduction in the number of successful mating events (Fig. 23), as well as in the number of zygospores produced (Fig. 23) when both parents have been cleared of their natural bacteria. The colouring patterns associated with the production of carotenoinds/trisporic acids during the mating events seem to be different in all the treatments used (Fig. 23).

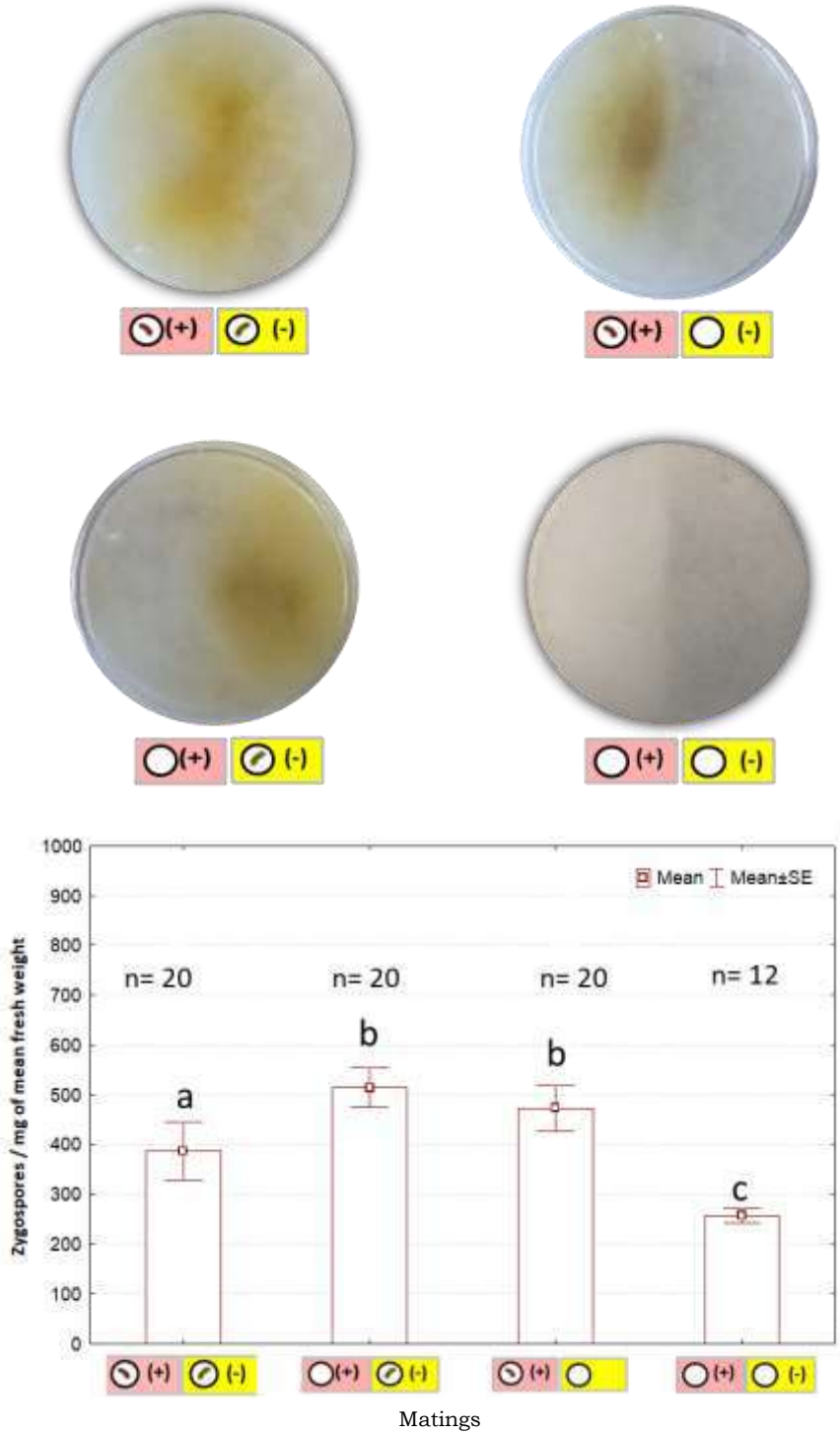
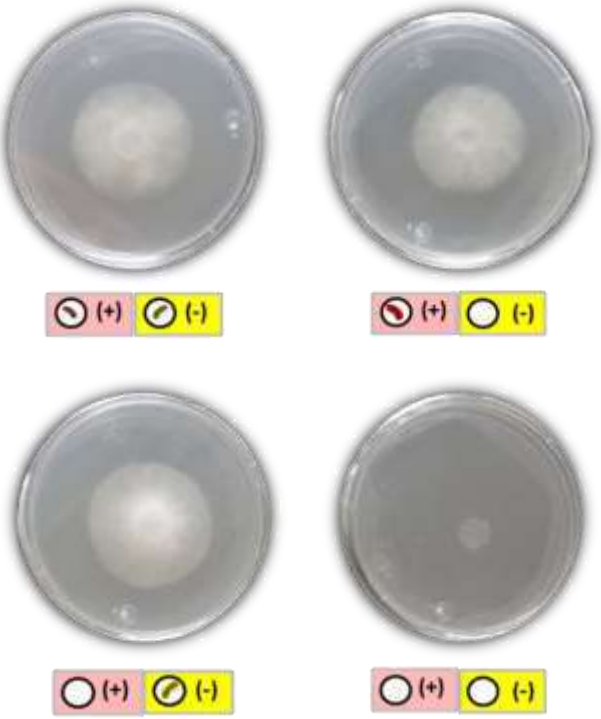


Figure 23. Production of zygospores on matings of host and cured strains of *Rhizopus microsporus*. Growth on plates of MEA for 7d at 30°C. One-way ANOVA followed Tukey's post-hoc test, P <0.05, n = 12 to 20 in each point.

Effects on zygospores viability of *Rhizopus microsporus* due to the absence of *Mycetohabitans*

The zygospores produced in the treatments with both cured parents lack viability (Fig.24).

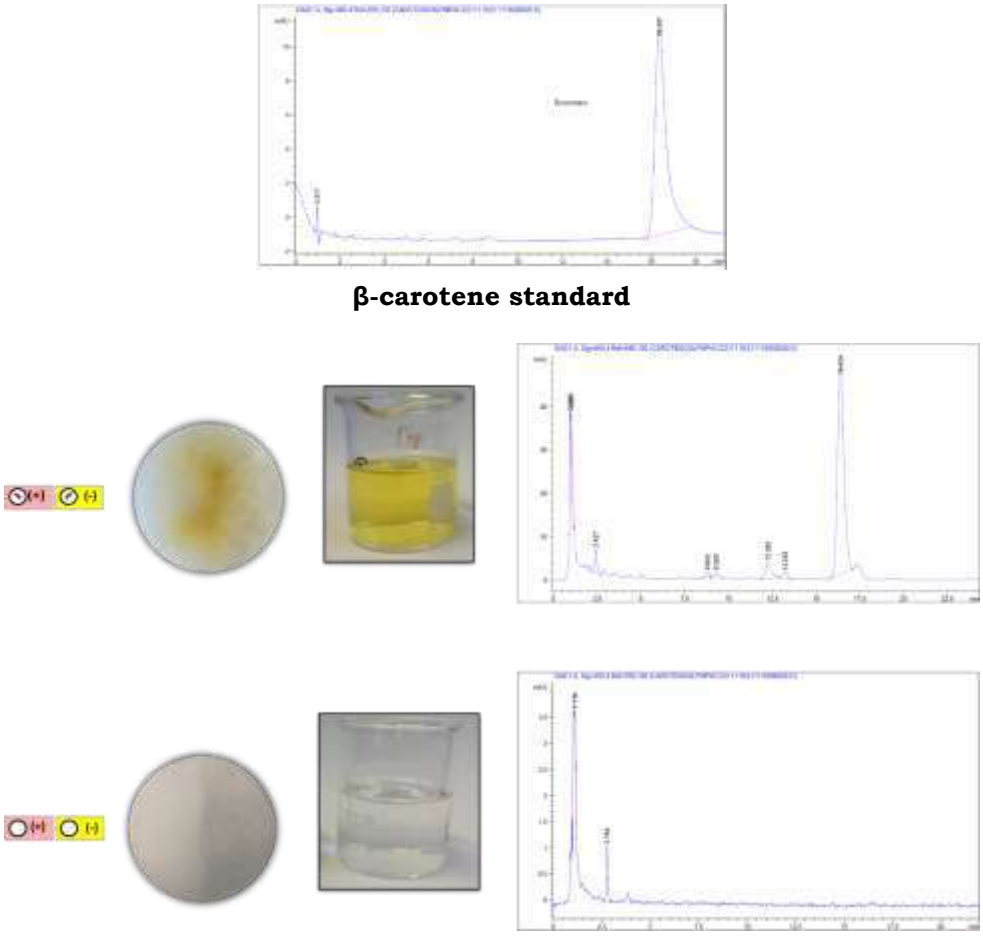


Mating [m+ x m-]	Zygospores tested	Germinated zygospores
(+) (-)	96	94
(+) (-)	96	92
(+) (-)	96	93
(+) (-)	96	0

Figure 24. Effects on zygospores viability of *Rhizopus microsporus* due to the absence of *Mycetohabitans* in one or both parental strains. Monozygosporic cultures incubated for 48 hours at 30 °C on PDA.

Effects on β-carotene production due to the absence of *Mycetohabitans*.

In addition to the decrease or absence of zygospores production, the lack of brown pigmentation was evident in the matings between both cured parents (Fig. 23). This coloration is potentially associated with the production of β-carotene, precursor of the sex hormone in Mucoralean fungi. The HPLC analysis showed the absence of 5 candidate compounds potentially involved on the synthesis of trisporic acid, including β-carotene, confirmed with the standard (Fig. 25).



β-carotene standard

Figure 25. Effects on β-carotene production due to the absence of Mycetohabitans spp. β-carotene identification for HPLC analyses (Zorbax C-18 LPM; Solvent: MeOH: CAN: DICLORO: BHT 55:40:5:0.1). The y-axis of the chromatogram is a measure of the intensity of absorbance (in units of mAU, or milli-Absorbance Units). The x-axis is in units of time (typically minutes), and is used to determine the retention time (tR) for each peak.

Reconstruction of the metabolic pathway of isoprenoids, terpenoids, and carotenoids genes of *Rhizopus microsporus* and *Mycetohabitans* spp.

In order to evaluate a possible metabolic integration between *Rhizopus microsporus* and its bacterial symbiont on the production of trisporic acid, I analyzed the metabolic pathway for carotenoids in fungi (Mucorales) (Fig. 26), as well as in *Mycetohabitans* spp. (Fig. 27). These analyses allowed me to identify the genes coding for the enzymes responsible for the synthesis and cleavage of β-carotene in the mating control strains ATCC 52813 (m+) and ATCC 52814 (m-)(Annex D and E), as well as the candidate genes for the biosynthesis of isoprenoids (MEP pathway) and terpenoids in *Mycetohabitans* sp. B4 and B7 (Annex F and G).

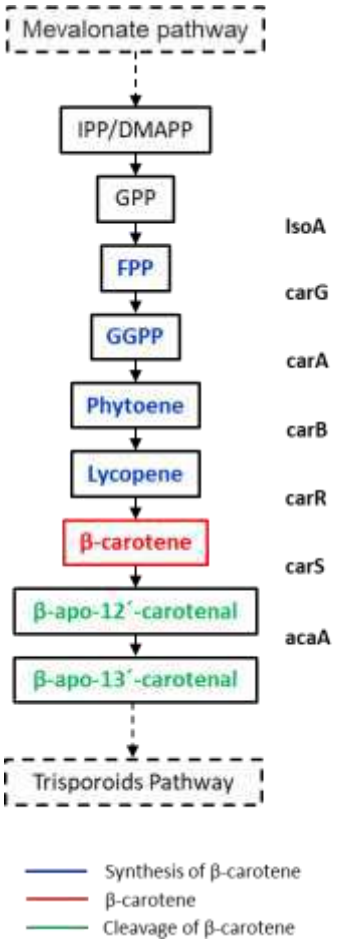


Figure 26. Carotenoids pathway in Mucoralean Fungi. Genes that encoded enzymes for the synthesis of β-carotene*: IsoA, carG, carA, CarB, and carR.* Genes that encoded enzymes for cleavage of β-carotene: *carS* and *acaA*.

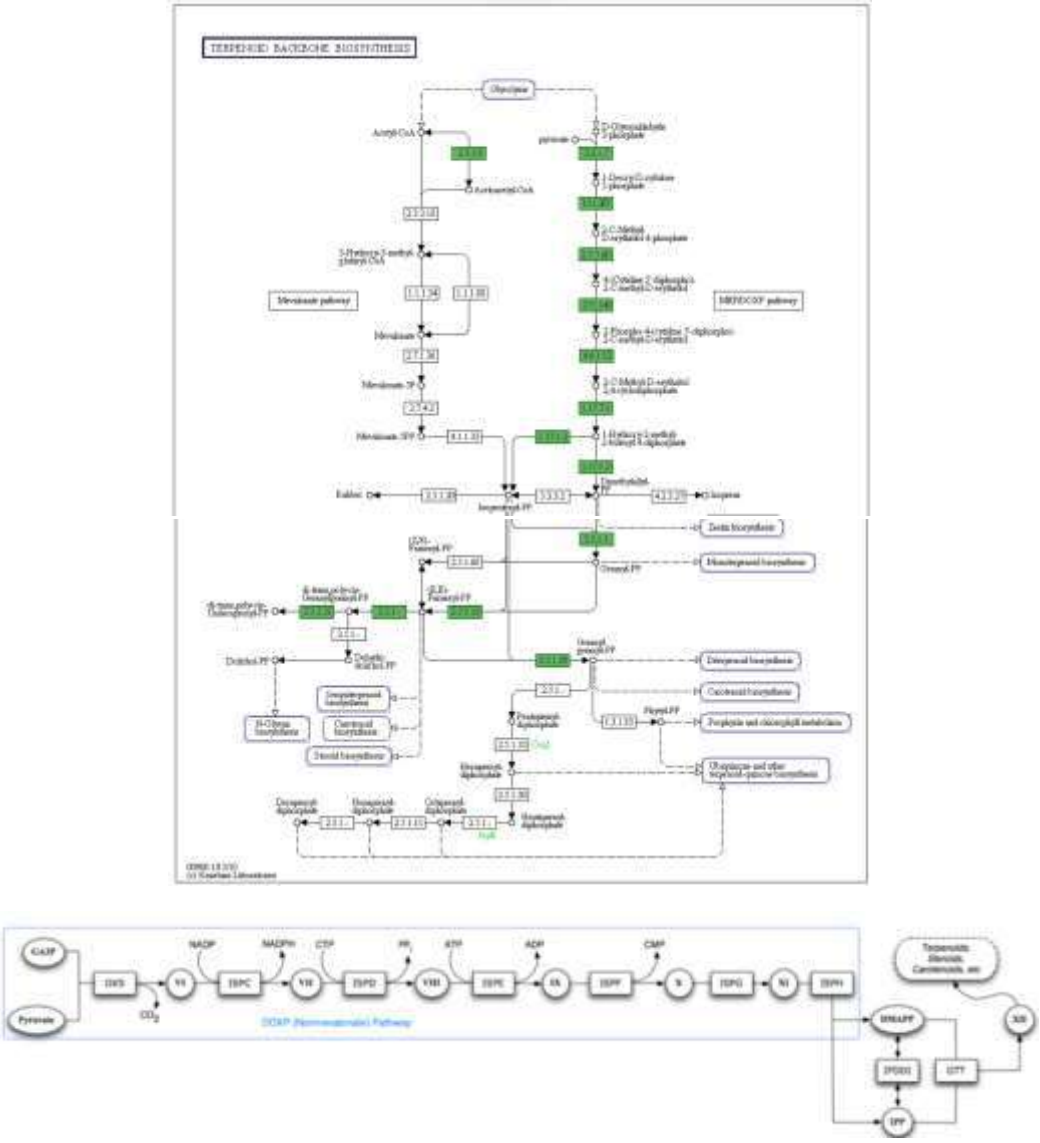


Figure 27. Reconstruction of the isoprenoids and carotenoids metabolic pathway of *Mycetohabitans* spp. Green boxes indicate the presence of genes in the genome with functional annotation. **VI** (1-Deoxy-D-xylulose-5-phosphate), **VII** (2-C-Methyl-D-erythritol 4-phosphate), **VIII** (2-C-Methyl-D-erythritol 4-phosphate), **IX** (4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol), **X** (2-C-methyl-D-erythritol 2,4-cyclodiphosphate), **XI** (1-Hydroxy-2-methyl-2-butenyl 4-diphosphate), **XII** (Geranyl diphosphate), **DMAPP** (Dimethylallyl diphosphate), and **IPP (**Isopentenyl diphosphate).

Mycetohabitans spp. have the genes necessary to produce Farnesyl pyrophosphate (FPP), but not for the synthesis of carotenoids. *Mycetohabitans* use the FPP molecule to synthesize UPP molecule, which enters to the Lipid cycle to synthesize a peptidoglycan molecule, an important component of the bacterial wall.

Fungal comparative expression of isoprenoids and carotenoids genes in matings of host and cured strains of *Rhizopus microsporus*.

Roberto-Bermudez (2016; Annex H), in the Microbial Interactions laboratory, analyzed RNA-Seq data of two *R. microsporus* host strains growing alone (asexual reproduction) or together (sexual reproduction), and with or without their corresponding endosymbionts. Once the genes had been identified in each genome (previous section), I used the processed RNA-Seq data to make a comparison of the expression of genes of synthesis and cleavage β-carotene, between matings with and without endosymbionts.

Libraries: (A) Mating of ATCC52813 with ATCC52814, formation of zygospores, both strains harbor endosymbionts. (B) Co-culture of ATCC52813 and ATCC52814 without their endosymbionts, both strains grow as sterile mycelia.

The comparison of data shows a decrement in the expression of candidate fungal genes when the fungi are mating in the absence of their endosymbionts (Fig.28).

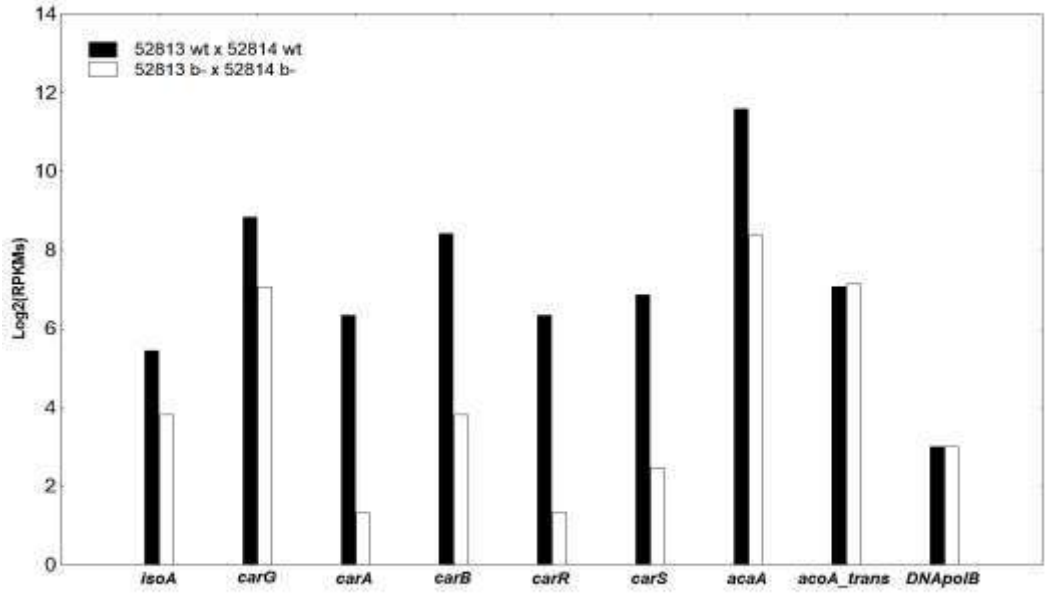


Figure 28. Fungal comparative expression of isoprenoids and carotenoids genes in matings of host and cured strains. (*IsoA*) Farnesyl pyrophosphate synthase**, (*carG*)** Geranylgeranyl pyrophosphate synthase, **(*carA*)** Phytoene synthase, ***(carB)*** Phytoene dehydrogenase synthase, ***(carR)*** Lycopene cyclase, ***(carS)*** Carotene dioxygenase, **(acaA)** Carotene dioxygenase, **(AcoAtrans)** Acetyl-coenzyme A transporter 1 (housekeeping genes of high expression level), **(DNApolB)** DNA polymerase family B (housekeeping genes of medium expression level).

Bacterial comparative expression of isoprenoids and carotenoids genes

In the case of the bacterial candidate genes, I used the processed RNA-Seq data to identify if there is an increase in the expression levels of the enzymes genes when the fungi are in mating conditions vs the expression under asexual reproduction (Fig 29)

Libraries: (A) Mating of ATCC52813 with ATCC52814, formation of zygospores, both strains harbor endosymbionts (B4+B7). (B) ATCC52813 with endosymbionts (B4), asexual sporulation via sporangia. (C) ATCC52814 with endosymbionts (B7), asexual sporulation via sporangia.

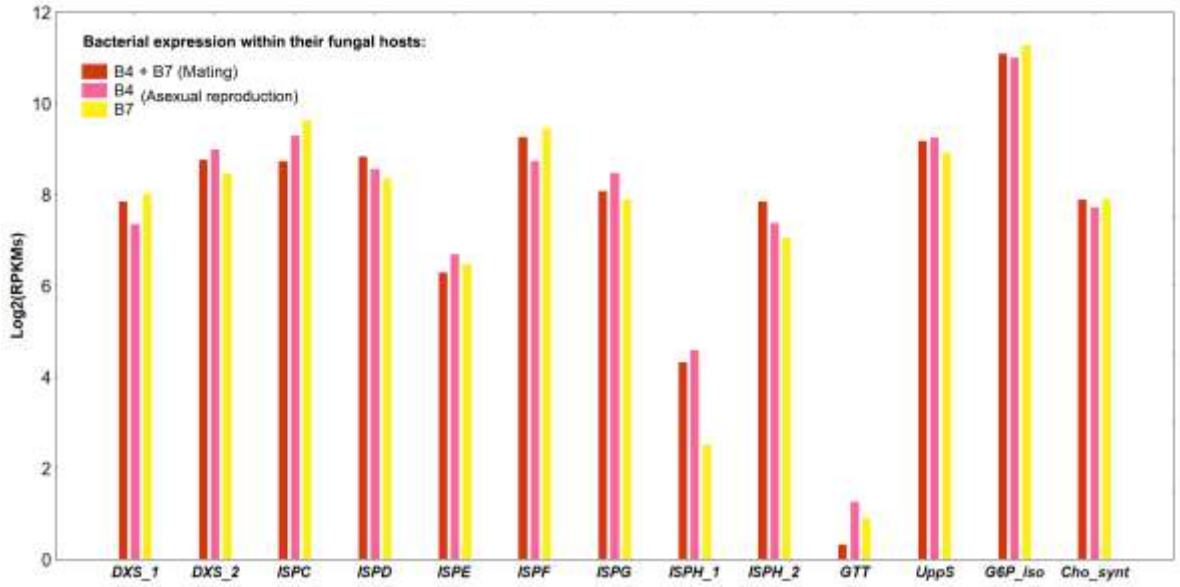


Figure 29. Bacterial comparative expression of isoprenoids and carotenoids genes. B4+B7 (mating A813wt vs A814wt), **B4** (A813wt), and **B7** (A813wt). **DXS** (1-deoxy-D-xylulose 5-phosphate synthase, **ISPC** (1-deoxy-D-xylulose 5-phosphate reducto- isomerase, **ISPD** (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, **ISPE** (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, **ISPF** (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), **ISPG** (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), **ISPH** (4-hydroxy-3-methylbut-2-enyl diphosphate reductase), **GTT** ((2E,6E)-farnesyl diphosphate synthase), and **UppS** (Undecaprenyl diphosphate synthase). **6GP_iso (**Glucose-6-phosphate isomerase) and **Cho_synt (**Chorismate synthase) are housekeeping genes of high and medium expression level, respectively.

The comparison of data shows a constitutive expression of the candidate bacterial genes in all treatments (Fig.28). Therefore, this expression is not regulated by the mating conditions of their host.

Inheritance of *Mycetohabitans* spp. in the sexual progeny (F1) of *Rhizopus microsporus*

To identify if bacterial inheritance is uniparental or biparental, strain-specific genes were amplified by PCR from extracted gDNA of 12 monozygosporic cultures.

***Mycetohabitans* sp. B4**; Host: ATCC 52813 (m +); Specific marker: cAMP signaling in bacteria (B4AMP1).

***Mycetohabitans* sp. B7**; Host: ATCC 52814 (m -); Specific marker: Purine utilization gene (B7Purin).

The Inheritance analyzes indicated that the inheritance of the bacterium in the sexual fungal progeny is uniparental and that it comes from the parent (m-) (Fig. 30).

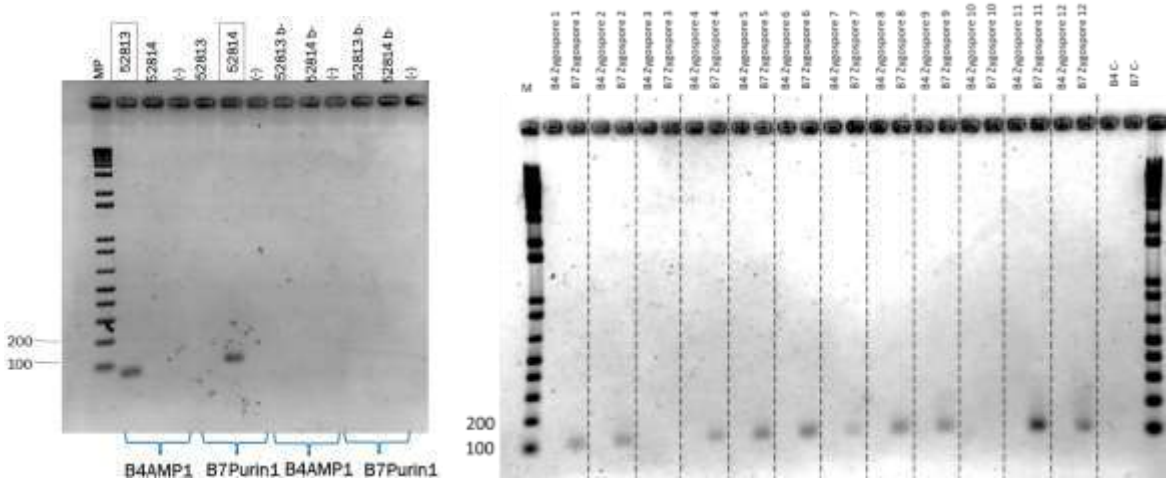


Figure 30. Segregation analysis of *Mycetohabitans* spp. in the sexual progeny (F1) of *R microsporus*. Strain-specific genes amplified by PCR from extracted gDNA of 12 monozygosporic cultures. Specific marker of *Mycetohabitans* sp. B4: cAMP signaling in bacteria (B4AMP1; 75pb). Specific marker of *Mycetohabitans* sp. B7: Purine utilization gene (B7Purin,109pb).

Segregation analysis of the mating type in the sexual progeny (F1) of *Rhizopus microsporus*

To identify the segregation of the mating type, I cultivated 12 isolated zygospores (F1) under the following mating conditions:

(A) Independently growth: I did not observe the production of zygospores (Fig. 31).
(B) Mating with ATCC 52813 (m+): I observed production of zygospores (Fig. 33).
(C) Mating with ATCC 52814 (m-): I did not observe the production of zygospores (Fig. 32).

Therefore, all the F1 fungal individuals analyzed have (m-) phenotypes.

(A)

(B)

Figure 31. F1 Individual of *Rhizopus microsporus*. (A) 24 h of growth at 30°C on plates of MEA. **(B)** 72 h of growth at 30°C on plates of MEA.

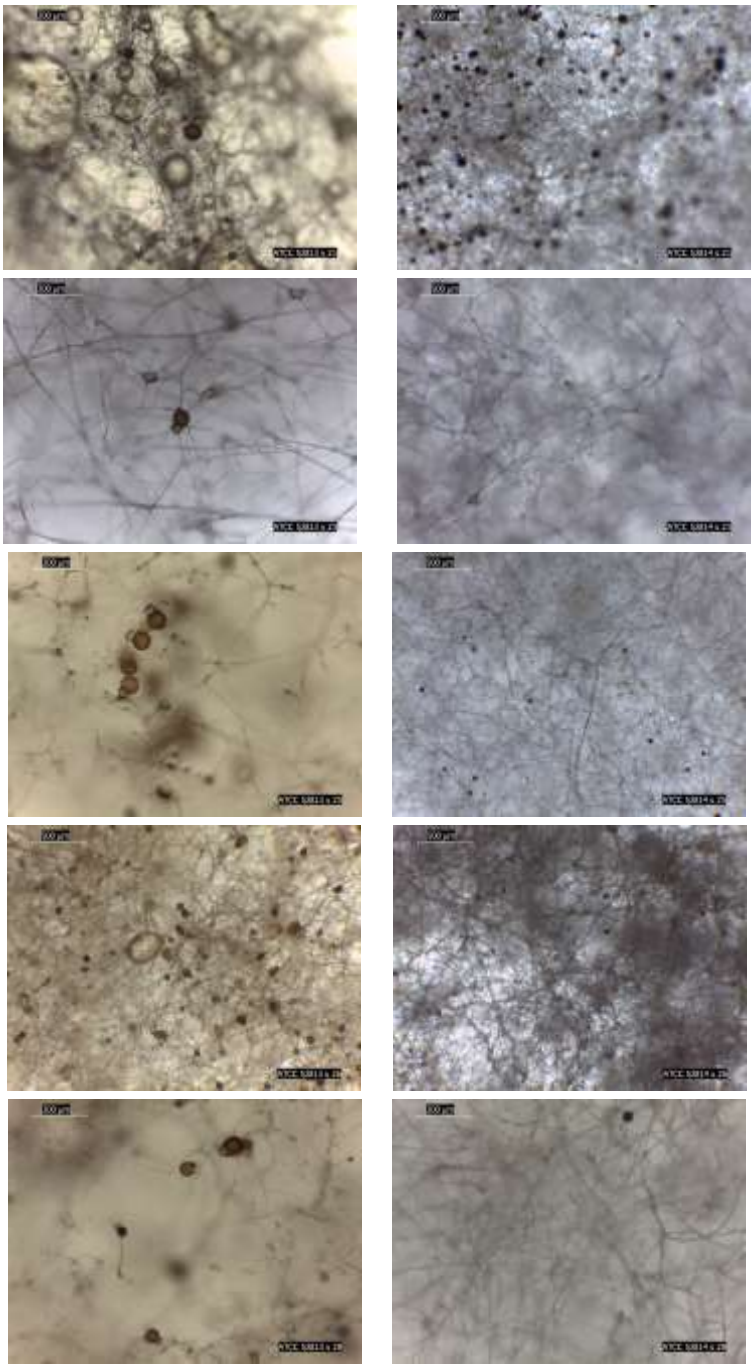


Figure 32. Segregation analysis of the mating type in F1 of *R. microsporus*. Production of zygospores in the mating between ATCC 5813 (+) m.t. and the zygospores #1 #3, #5, #6, and #8.

DISCUSSION

The Fungi as study models in the hologenomic theory of evolution (HTE), proposed by Rosenberg and Zilber-Rosenberg (2008) and based on the Margulis (Holobiont, 1990) and Jefferson (Hologenome, 1994) ideas, are possible thanks to reports of endohyphal interactions with bacteria and viruses in the last 30 years, and to the novel advances of the "omics" techniques. The HTE proposes that the set of dynamic interactions within the holobiont can modify the morphology, physiology, development, and fitness of the organisms.

The symbiosis between the Mucoralean fungus *Rhizopus microsporus* and the proteobacteria of the genus *Mycetohabitans* was discovered by Partida-Martínez and Hertweck in 2005, and was proposed as a fungal holobiont model a few years later (2017). This model is the most studied so far due to its advantageous capacity to evaluate Koch's postulates, being that both partners can be grown and potentially transformed independently, and the symbiosis can be reestablished through cocultivation. The research developed in the last 15 years has given a global panorama on the implications of this symbiosis. Specialized bacterial metabolites that increase the pathogenicity of the fungal host have been reported; bacterial enzymes that degrade the cell wall of the fungal host to invade it; as well as bacterial secretion systems that modulate an effective host colonization and elicitation of the asexual reproduction of their host.

Mondo *et al.* (2016) published the only report to date that studied the implications of this symbiosis on the sexual reproduction of host *R. microsporus*. They observed a decrease in the number of zygospores produced, and in the number of successful mating events when they eliminated endobacteria in both parents (Fig. 33). They suggested that the sexual reproduction is mediated by the symbiont gaining transcriptional control of the fungal *ras2* gene, which encodes a GTPase central to fungal reproductive development. On the other hand, there is an indirect study by Dolatabadi *et al.* (2014), in which these authors concluded that there is not a reproductive barrier between all varieties of *Rhizopus microsporus* regardless of their origin of isolation and variety (var. chinensis, var.

oligosporus, etc.) (Fig. 33). In this study, the ATCC 52813 (m +) and ATCC 52814 m (-) strains were used as controls for the mating in the species. These strains were previously identified as host strains by Partida-Martínez (2007). Additionally, Lastovetsky *et al.* (2016) reported that specific changes in the fungal lipid metabolism, mediated by diacylglycerol kinase enzymes, are required to maintain a mutualistic outcome of the interaction with bacteria. In Petri dishes, Lastovetsky saw that non-host *R. microsporus*, as well as other mucoralean fungi, interact antagonistically with *Mycetohabitans* derived from the host *R. microsporus,* and that these fungi were not invaded by them (Fig. 33).

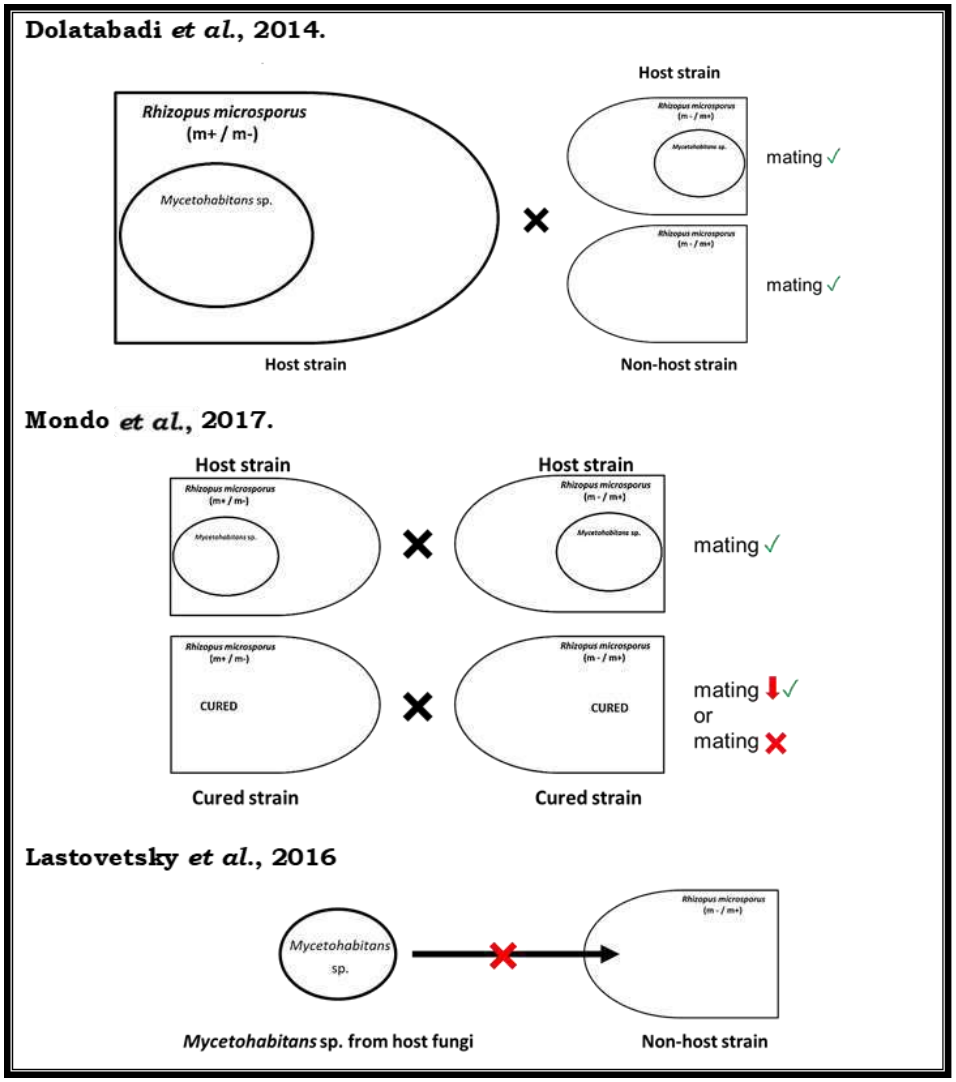


Figure 33. Reports summary of the sexual reproduction of *Rhizopus microsporus*.

Does the symbiosis between *R. microsporus* and *Mycetohabitans* spp. represent a reproductive barrier for the fungal species?

Dolatabadi *et al.* (2014 and 2016) reported at least 30 non-host strains of *R. microsporus* with the ability to form zygospores on conditions of growing with the complementary mating control strain [ATCC 52813 (m +) or ATCC 52814 (m-)]. They did not quantify the % success matings events; neither the production of zygospores. They used even a minimal production of zygospores to identify the sex of the strains. Assuming that the number of spores produced was less in the matings between host x non-host strains, we would have the next correlation:

I hypothesize that the species *R. microsporus* has normally a low sexual reproductive success, and that *Mycetohabitans* is a type of sexual stimulator for all strains, but at the same time, there is a rejection with non-host strains (trade-off) as reported by Lastovetsky (2016), which would result in few and inefficient reproductive events for the combination non-host x host strains; and that in the case of non-host x non-host strains, as the stimulation is basically lacking, the sexual reproduction is lost. I conducted a pilot experiment to test this hypothesis (Data not shown in the results section). I evaluate the mating between the cured host strain 52814 b- (m-) and the non-host strain 11559, genotyped as (m +) by BLAST of the HMG gene from its sexual locus (Appendix K), and I observed a very low production of zygospores (Fig. 34), which invites to test this hypothesis in the future.

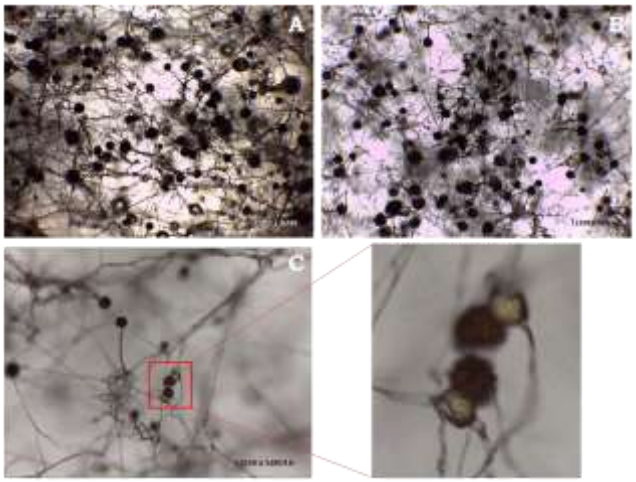


Figure 34. Mating between cured host and non-host strains. (A) 52813 (m+) x 11559 **(B)** 11559 x 52814 (m-) **(C)** 11559 x 52814 b- (m-).

Does the presence of endobacteria enhance the sexual reproduction success of host *Rhizopus microsporus*?

I did tests of mating to evaluate the effects caused by the absence of *Mycetohabitans* spp. (host x host, host x cured-host, and cured-host x cured-host strains). These experiments revealed that sexual reproduction is 100% successful when at least one of the host parents keeps their natural bacteria, suggesting that the presence of a single strain of bacteria in one of the parents has the ability to stimulate the sexual reproduction in both parents. Like Mondo *et al.*, I observed a reduction in the number of successful mating events, as well as in the number of zygospores produced (Fig. 23) when both parents do not have their symbionts - they lack the bacterial stimulant.

In addition to the decrease or absence of zygospores production, the lack of brown pigmentation was evident in the matings between both cured parents (Fig. 23). This coloration is potentially associated with the production of β-carotene, precursor of the sex hormone in Mucoralean fungi (Lee & Idnurm, 2017). The HPLC analysis showed the absence of β-carotene (Fig. 25). This indicates to me that *Mycetohabitans* could be producing β-carotene or some precursor of its biosynthesis, or stimulating its production by feeding it enzymatically. In order to evaluate a possible metabolic integration between *R. microsporus* and its guest on the production of trisporic acid, I analyzed the metabolic pathway for carotenoids in Mucorales (Fig. 26), as well as in *Mycetohabitans* spp. (Fig. 27).

These analyses allowed me to identify:

- The genes coding for the enzymes responsible of the synthesis and cleavage of β-carotene in the mating control strains ATCC 52813 (m+) and ATCC 52814 (m-) (Fig. 46; Appendix D and E).
- That *Mycetohabitans* does not have the necessary genes that encode enzymes that synthesize and cleavage the β-carotene in the mating control strains ATCC 52813 (m+) and ATCC 52814 (m-) (Fig. 27).
- *Mycetohabitans* synthesizes isoprenoids through the metabolic pathway of MEP/DOXP, and *Rhizopus microsporus* through the Mevalonate pathway:

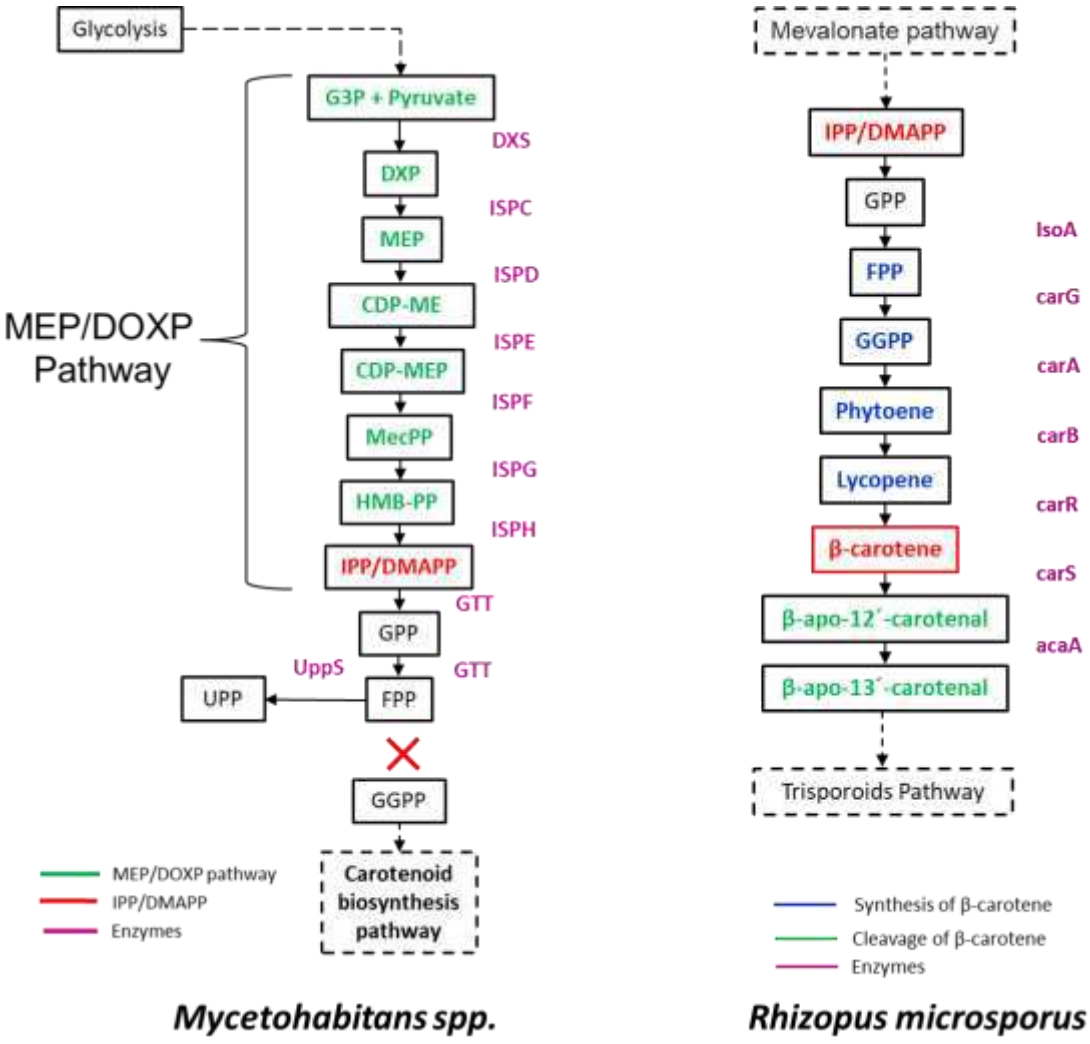


Figure 35. MEP/DOXP pathway of *Mycetohabitans* spp. and Mevalonate pathway of *Rhizopus microsporus*.

Once the genes had been identified in each genome, I used the processed RNA-Seq data generated by Roberto-Bermudez in his master's thesis (2016, Annex H) to make a comparison of the expression on the candidate genes on the libraries of two *R. microsporus* strains growing alone or together, and with or without their corresponding endosymbionts. The comparison of data shows:

- An increase in the expression of candidate fungal genes when the fungi are mating in presence of their endosymbionts (Fig.28).

- The bacterial candidate genes are expressed similarly in conditions of fungal mating and independent growing (Fig. 29).

- The *GTT* bacterial gene ((2E,6E)-farnesyl diphosphate synthase) showed a low expression (Fig. 29).

Doing an integrative analysis of the observed phenotypes, the identification of genes, and the analysis of expression; everything seems to indicate that *Mycetohabitans* provides the GPP and FPP molecules to the host *Rhizopus*. *Mycetohabitans* uses these molecules to synthesize UPP, which is a precursor to the compounds of the bacterial cell wall (Appendix L), so it makes sense that all the genes analyzed have a constitutive expression independently of the conditions in which the fungus is grown (sexual or asexual reproduction).

Possibly the GTT enzyme has a low expression because the fungus is taking the target molecules, or because the fungal GPP synthase and IsoA enzymes are being used for both organisms. So, the bacterial GPP and FPP molecules would take the role of the feeder of the β-carotene pathway which is integrated to the synthesis of trisporoids. Observations of the mating fungal phenotype support this idea since the production of β-carotene and the zygospores only takes place on the side of the plate where wild host is found, and not in the cured partner (Fig. 23).

The zygospores produced by matings between cured-host x cured-host strains lack viability (Fig.24). The bacterium could be producing other important compounds that influence the development of zygospores and/or their germination.

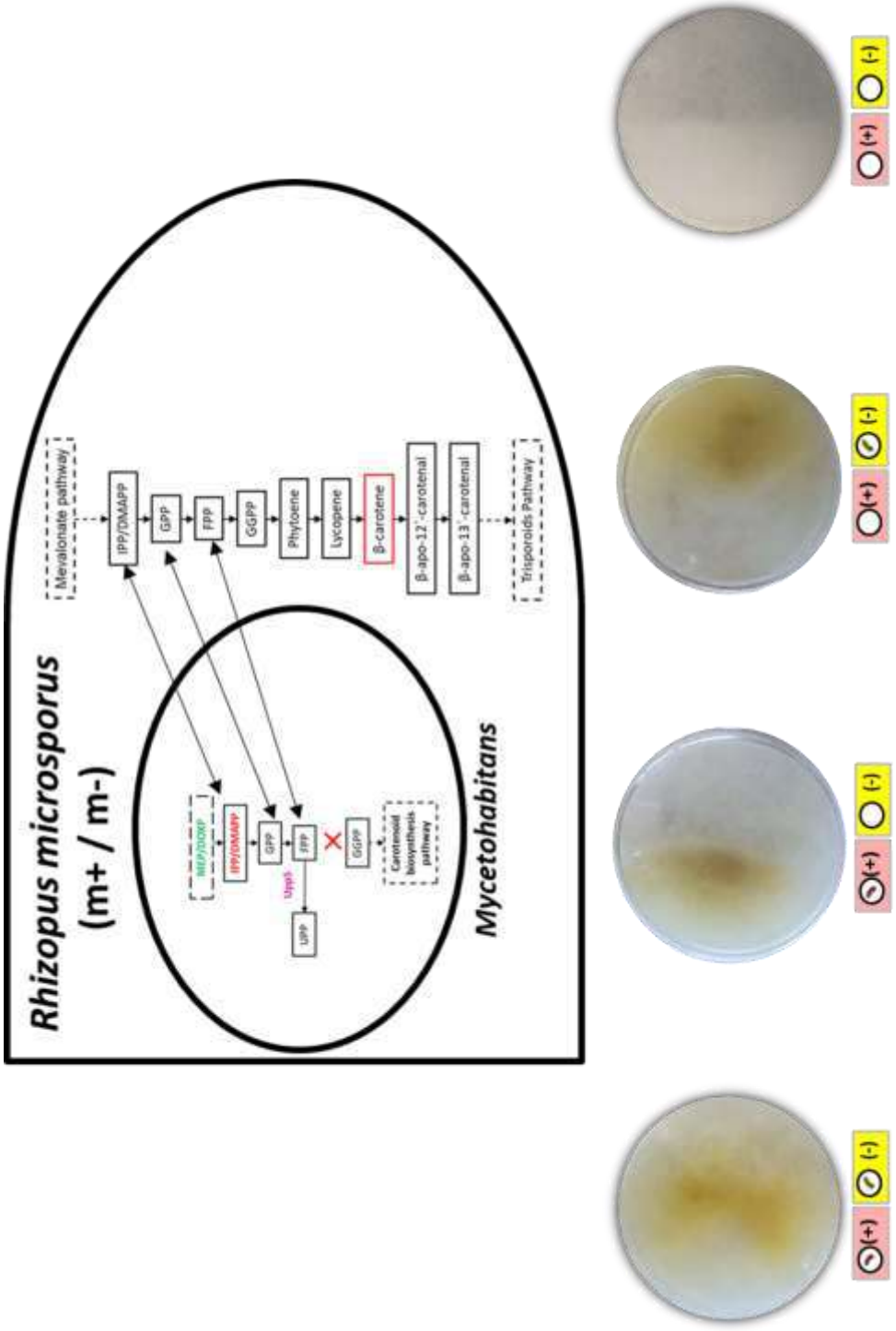


Figure 36. ***Mycetohabitans*** **provides the GPP and FPP molecules to the host** ***Rhizopus microsporus.***

Are bacteria inherited in the offspring? Is their heritability biparental or uniparental?

I used strain-specific markers to identify which bacterium was present in the F1 of *R. microsporus*. Only *Mycetohabitans* sp. B7, the native symbiont of strain ATCC 52814 (m-) was detected. This indicated that bacterial inheritance is uniparental.

These F1 individuals were grown on mating conditions. I tested the F1 zygospores against 52813 (m+) and 52814 (m-), and I only observed production of zygospores in all matings with the parental (m+) (Fig. 32). Therefore, all the fungal F1 individuals analyzed have phenotypes (m-).

I must point out that there are no reports so far of the development of a meiotic sporangiophore in the *Rhizopus microsporus* species. So, one possibility is that the zygospores are not performing meiosis and despite eliminating the mycelium where the zygospore develops, one of the suspensors, the largest (Fig. 12-E and 3-F), is able to emerge of the zygospore continuing its basal growth and preserving its bacterial endosymbiont.

This could be corroborated with the transformation of *Mycetohabitans* with fluorescent labeled proteins (Appendix J), cytometric analysis (Appendix I), which I have already standardized in this species; and by PCR analyzes of unique genes of each strain.

CONCLUSIONS

- The symbiosis with *Mycetohabitans* spp. seems to represent a barrier for the sexual reproduction between host and non-host strains of *Rhizopus microsporus.*

- The presence of *Mycetohabitans* spp. have effects on the formation, development, and viability of *Rhizopus microsporus* zygospores.

- The production of β-carotene in host strains is stimulated by the presence of *Mycetohabitans* spp.

- The segregation of *Mycetohabitans* spp. is uniparental. The inherited bacteria derive from the fungal partner with the locus SexM (m -).

- The sexual progeny of *Rhizopus microsporus* is only (m-).

PERSPECTIVES

- Finish quantitative and qualitative analyses of carotenoids and trisporoids and their genes (qRT-PCR) in matings of *Rhizopus microsporus* to corroborate the involvement of *Mycetohabitans* in their production.

- Verify that during sexual reproduction in host strains, the sexM (m-) is always inherited and also bacteria associated with the m- parental.

- Evaluate sexual reproduction in cured host strains and non-host strains.

REFERENCE

Alabid I., Glaeser S. P. & Kogel K. H. (2018). Endofungal bacteria increase fitness of their host fungi and impact their association with crop plants. *Current issues in molecular biology*, 30:59-74.

Alcalde E., Cerdá-Olmedo E. & Al-Babili S. (2019). Apocarotenoids produced from b-carotene by dioxygenases from Mucor circinelloides. Microbiology, 165:433–438. Doi: 10.1099/mic.0.000762.

Almeida E. & Cerdá-Olmedo. (2008). Gene expression in the regulation of carotene biosynthesis in Phycomyces. Curr Genet, 53, 129–137. Doi: 10.1007/s00294-007-0170-x.

Araldi-Brondolo S.J., Spraker J., Shaffer J.P., Woytenko E.H., Baltrus D.A., Gallery R.E., Arnold A.E. (2017). Bacterial endosymbionts: master modulators of fungal phenotypes. *Microbiol Spectrum*, 5(5).

Barbieri E., Potenza L., Rossi I., Sisti D., Giomaro G., Rossetti S., Beimfohr C. & Stocchi V. (2000). Phylogenetic characterization and *in situ* detection of a *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad. ectomycorrhizal mycelium. *Applied and Environmental Microbiology*, p.5035–5042.

Bertaux J., Schmid M., Prevost-Boure N. C., Churin J. L., Hartmann A., Garbaye J., & Frey-Klett P. (2003). In situ identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Applied and environmental microbiology*, 69(7), 4243–4248. Doi:10.1128/aem.69.7.4243-4248.2003.

de Bary H.A. (1879). Die erscheinung der symbios. *Vortrag auf der Versammlung der Deutschen Naturforscher und Aertze zu Cassel, Strasburg: Velag von Karl J. Trubner*, 1-30.

Bianciotto V., Bandi C., Minerdi D., Sironi M., Tichy H. V., & Bonfante P. (1996). An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. *Applied and environmental microbiology*, 62(8), 3005–3010.

Bordenstein S.R & Theis K.R. (2015). Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *Plos Biology*. 13: e1002226.

Casadevall A. & Pirofski L.A. (2015). What is a host? Incorporating the microbiota into the damage-response framework. *Infection and Immunity,* 83:2–7.

Dearth S. P., Castro H. F., Venice F., Tague E. D., Novero M., Bonfante P. & Campagna S. R. (2018). Metabolome changes are induced in the arbuscular mycorrhizal fungus *Gigaspora margarita* by germination and by its bacterial endosymbiont. *Mycorrhiza*, volume 28, pages 421–433.

Deveau A., Bonito G., Uehling J., Paoletti M., Becker M., Bindschedler S., Hacquard S., Hervé V., Labbé J., Lastovetsky O. A., Mieszkin S., Millet L.J., Vajna B., Junier P. & Bonfante P., Krom B. P., Olsson S., Elsas J. & Wick L. Y. (2018). Bacterial–fungal interactions: ecology, mechanisms and challenges. *FEMS Microbiology Reviews*, fuy008, 42, 2018, 335–352.

Dolatabadi S., Scherlach K., Figge M., Hertweck., Dijksterhuis J., Menken S. & de Hoog G. (2016). Food preparation with mucoralean fungi: A potential biosafety issue? *Fungal Biol*, 120(3):393-401. Doi: 10.1016/j.funbio.2015.12.001.

Dolatabadi S., Walther G., Van Den Ende G. & De Hoog G. S. (2014). Diversity and delimitation of *Rhizopus microsporus*. *Fungal Diversity*, 64(1): 145–63.

Du Z. Y., Zienkiewicz K., Vande Pol N., Ostrom N. E., Benning C., & Bonito G. M. (2019). Algal-fungal symbiosis leads to photosynthetic mycelium. *eLife*, 8, e47815. Doi:10.7554/eLife.47815.

Estrada de los Santos P., Palmer M., Chávez-Ramírez B., Beukes C., Steenkamp E. T., Briscoe L., Khan N., Maluk M., Lafos M., Humm E., Arrabit M., Crook M., Gross E., Simon M. F., Bueno dos Reis Junior F., Whitman W. B., Shapiro N., Poole P.N., Hirsch A. M., Venter S. N. & James E. K. (2018). Whole genome analyses suggest that *Burkholderia* sensu lato contains two additional novel genera (*Mycetohabitans* gen. nov., and *Trinickia* gen. nov.): Implications for the evolution of diazotrophy and nodulation in the *Burkholderiaceae*. *Genes*, 9, 389.

Furuya S., Kurata M. & Saito, (1974). T. Studies on *Rhizopus* sp. causing growth injury of young rice seedlings and its chemical control. *Proc. Assoc. Plant Protect. Shikoku* 9, 49–-55).

Gho N., Sato Z., Yaoita T. & Aoyagi, K. (1978). Studies on the control of *Rhizopus* in the nursery cases of rice seedlings. 5. Influence of a phytotoxic substance produced by *Rhizopus* on growth of rice. *Proc. Assoc. Plant Protect. Shikoku* 26,90–-94.

Gryganskyi A., Golan J., Dolatabadi S., Mondo S., Robb S., Idnurm A., Muszewska A., Steczkiewicz K., Masonjones S., Liao H., Gajdeczka M. T., Anike F., Vuek A., Anishchenko I. M., Voigt K., de Hoog G. S., Smith M. E., Heitman J., Vilgalys R. & Stajich J. E. (2018). Phylogenetic and phylogenomic definition of *Rhizopus* Species. *G3: Genes, Genomes, Genetics*, vol. 8 no. 6.

Gryganskyi A. P., Lee S. C., Litvintseva A. P., Smith M. E., Bonito G., Porter T. M., Anishchenko I. M., Heitman J., & Vilgalys R. (2010). Structure, function, and phylogeny of the mating locus in the *Rhizopus oryzae* complex. *PloS one*, 5(12), e15273.Doi: 10.1371/journal.pone.0015273.

Guo H., Glaeser S. P., Alabid I., Imani J., Haghighi H., Kampfer P. & Kogel K. H. (2017). The abundance of endofungal bacterium *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) increases in its fungal host *Piriformospora indica* during the tripartite sebacinalean symbiosis with higher plants. *Front Microbiol.*, 8:629.

Hoffman M.T. & Arnold E. (2010). Diverse Bacteria Inhabit Living Hyphae of Phylogenetically Diverse Fungal Endophytes. *Applied and Environmental Microbiology Journal*, p. 4063–4075.

Ibaragi T. (1973). Studies on rice seedling blight. I. Growth injury caused by *Rhizopus* sp. under high temperature. *Ann. Phytopathol. Soc. Jpn* 39, 141–-144.

Iwasaki S., *et al*. (1984). Studies on macrocyclic lactone antibiotics. VII. Structure of a phytotoxin "rhizoxin" produced by *Rhizopus chinensis*. *J. Antibiot.* 37, 354–-362.

Iwasaki S., *et al*. (1986). Studies on macrocyclic lactone antibiotics. VIII. Absolute structures of rhizoxin and a related compound. *J. Antibiot.* 39, 424–-429.

Kluge M., Mollenhauer D. & Mollenhauer R. (1991). Photosynthetic carbon assimilation in *Geosiphon pyriforme* (Kutzing) F.v. Wettstein, an endosymbiotic association of fungus and cyanobacterium. *Planta*, 185:311-315.

Kluge M., Mollenhauer D., Mollenhauer R. & Kape R. (1992). *Geosiphon pyriforme*, an endosymbiotic consortium of a fungus and a cyanobacterium (*Nostoc*), fixes nitrogen. *Bot Acta*, 105:343-344.

Koga-Ban Y., Niki T., Nagamura Y., Sasaki T. & Minobe Y. (1995) cDNA sequences of three kinds of beta-tubulins from rice. *DNA Res.* 2, 21–-26.

Lackner G., Möbius N., Partida-Martínez L.P., Boland S. & Hertweck C. (2011). Evolution of an endofungal lifestyle: Deductions from the *Burkholderia rhizoxinica* genome. *BMC Genomics*, 12, 210.

Lastovetsky O. A., Gaspar M. L., Mondo S. J., LaButti K. M., Sandor L., Grigoriev I. V., Henry S. A. & Pawlowska T. E. (2016). Lipid metabolic changes in an early divergent fungus govern the establishment of a mutualistic symbiosis with endobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 113(52), 15102–15107. Doi: 10.1073/pnas.1615148113.

Lee S. & c A. (2017). Fungal Sex: The Mucoromycota. *Microbiol Spectrum,* 5(2): FUNK-0041-2017. Doi:10.1128/microbiolspec.FUNK-0041-2017.

Macdonald R. M. & Chandler M. R. (1981). Bacterium-like organelles in the vesicular-arbuscular mycorrhizal ungus *Glomus caledonius*. *The New Phytologist*, 2:89, pp. 241-246.

Martin B.M & Schwab E. (2012). Symbiosis: "Living together" in chaos. *Studies in the History of Biology*, 4 (4): 7–25.

Margulis L. (1991). Symbiogenesis and Symbionticism. Symbiosis as a source of evolutionary innovation: Speciation and Morphogenesis. *MIT Press. Cambridge*, MA. pp. 1–13.

Moebius, N., Üzüm, Z., Dijksterhuis, J., Lackner, G., & Hertweck, C. (2014). Active invasion of bacteria into living fungal cells. *eLife*, 3, e03007. Doi:10.7554/eLife.03007.

Mondo S.J., Toomer K.H., Morton J.B., Lekberg Y. & Pawlowska T.E. (2012). Evolutionary stability in a 400-million-year-old heritable facultative mutualism. *Evolution*, 66(8):2564-76.

Mosse B. (1970). Honey-coloured, sessile *Endogone* spores: II. Changes in fine structure during spore development. *Arch. Mikrobiologie*, 74(2), 129–145. Doi:10.1007/bf00446901.

Naito M., Morton J. B. & Pawlowska T. E. (2015). Minimal genomes of mycoplasma-related endobacteria are plastic and contain host-derived genes for sustained life within Glomeromycota. *PNAS,* 112 (25) 7791-7796.

Naumann M., SchüBler & Bonfante P. (2010). The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*. *The ISME Journal*, 4, 862–871.

Niehs S.P, Dose B., Scherlach K., Roth M. & Hertweck C. (2018). Genomics-Driven Discovery of a Symbiont-Specific Cyclopeptide from Bacteria Residing in the Rice Seedling Blight Fungus. ChemBioChem, 9(20):2167-2172. DOI: 10.1002/cbic.201800400.

Niehs S.P., Scherlacha K. & Hertweck C. (2018). Genomics-driven discovery of a linear lipopeptide promoting host colonization by endofungal bacteria. Org. Biomol. Chem., 16, 6967. DOI: 10.1039/c8ob01515e.

Noda T., Hashiba T. & Sato Z. The structural changes in young swollen roots of rice seedlings infected with *Rhizopus chinensis* Saito. *Ann. Phytopathol. Soc. Jpn* 46, 40–-45.

Ohshima S., Sato Y., Fujimura R., Takashima Y., Hamada M., Nishizawa T., Narisawa K. & Ohta H. (2016). *Mycoavidus cysteinexigens* gen. nov., sp. nov., an endohyphal bacterium isolated from a soil isolate of the fungus *Mortierella elongate*. *International Journal of Systematic and Evolutionary Microbiology*, 66:5.

Partida-Martínez L.P. & Hertweck C. (2005). Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature*, 437(7060), pp. 884-8.

Partida-Martínez L.P. (2007). Discovery of Endofungal Bacteria: New Insights Into Toxin Biosynthesis and Bacterial-fungal Symbiosis. Thesis of PhD.

Partida-Martínez L.P. (2013) A model for bacterial-fungal interactions. LAP LAMBERT Academic Publishing. pp. 109. ISBN: 978-3-659-40707-9.

Partida-Martínez L.P. (2017). The fungal holobiont: Evidence from early diverging fungi. *Environmental Microbiology*, 19(8), 2919-23.

Partida-Martínez L.P., Flores de Looß C., Ishida K., Ishida M., Roth M., Buder K. & Hertweck C. (2007a). Rhizonin, the first mycotoxin isolated from the zygomycota, is not a fungal metabolite but is produced by bacterial endosymbionts. *Applied and Environmental Microbiology*, 73(3), pp. 793-7.

Partida-Martinez L.P., Groth I., Schmitt I., Richter W., Roth M. & Hertweck C. (2007b). *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. *International Journal of Systematic and Evolutionary Microbiology*. 57(Pt 11):2583-2590. doi:10.1099/ijs.0.64660-0.

Partida-Martinez L.P., Monajembashi S., Greulich K.O. & Hertweck C. (2007c). Endosymbiont-dependent host reproduction maintains bacterial-fungal mutualism. *Curr Biol*. 17(9):773-777. DOI: 10.1016/j.cub.2007.03.039.

Partida-Martínez, L.P. & Hertweck, C. (2007). A gene cluster encoding rhizoxin biosynthesis in "*Burkholderia rhizoxina*", the bacterial endosymbiont of the fungus *Rhizopus microsporus*. *ChemBioChem*, 8(1), pp. 41-5.

Porter R. & Kaplan J. (2014). El Manual Merck. *Editorial Medica Panamericana*. 19° edition.

Riedlinger J., Schrey S.D., Tarkka M.T., Hampp R., Kapur M. and Fiedler H.P. (2006). Auxofuran, a novel metabolite that stimulates the growth of fly agaric, is produced by the mycorrhiza helper bacterium *Streptomyces* strain AcH 505. *Appl Environ Microbiol.*, 72(5):3550-7.

Rosenberg E. & Zilber-Rosenberg I. (2016). Microbes drive evolution of animals and plants: the hologenome concept. *mBio* 31; 7: e01395.

Rohm B., Scherlach K., Möbius N., Partida-Martínez L.P. & Hertweck C. (2010). Toxin production by bacterial endosymbionts of a *Rhizopus microsporus* strain used for tempe/sufu processing. *International Journal of Food Microbiology,* 136, pp. 368-71.

Ruiz-Herrera J., León-Ramírez C., Vera-Nuñez A., Sánchez-Arreguín A., Ruiz-Medrano R., Salgado-Lugo H., Sánchez-Segura L. & Peña-Cabriales J. J. (2015). A novel intracellular nitrogen-fixing symbiosis made by *Ustilago maydis* and *Bacillus* spp. New Phytol, 207: 769-777. Doi:10.1111/nph.13359.

Ruiz-Lozano J. M. & Bonfante P. (1999) Identification of a putative P-transporter operon in the genome of a *Burkholderia* strain living inside the arbuscular mycorrhizal fungus *Gigaspora margarita*. *J. Bacteriol*, 181: 4106–4109.

Sato Y., Narisawa K., Tsuruta K., Umezu M., Nishizawa T., Tanaka K., Yamaguchi K., Komatsuzaki M. & Ohta H. (2010). Detection of betaproteobacteria inside the mycelium of the fungus *Mortierella elongate*. *Microbes Environ.*, Vol. 25, No. 4, 321–324.

Scherlach K., Partida-Martínez L.P., Dahse H.M. & Hertweck C. (2006). Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus*. *Journal of the American Chemical Society*, 128(35), pp. 11529-36.

Schimek C., Petzold A., Schultze K., Wetzel J., Wolschendorf F., Burmester A. & Wöstemeyer J. (2005). 4-Dihydromethyltrisporate dehydrogenase, an enzyme of the sex hormone pathway in Mucor mucedo, is constitutively transcribed but its activity is differently regulated in (+) and (−) mating types. *Fungal Genetics and Biology*, Vol. 42, Issue 9. DOI: 10.1016/j.fgb.2005.05.003.

Schüßler A., & Kluge M. (2001). *Geosiphon pyriforme*, an endocytosymbiosis between fungus and cyanobacteria, and its meaning as a model system for arbuscular mycorrhizal research. *Fungal Associations*, 151–161. Doi:10.1007/978-3-662-07334-6_9.

Schrantz, J. P. (1973). Presence de micro-organismes intra-cytoplasmiques probablement bacteriens, chez un Discomycete du genre *Scutellinia*. *Comptes Rendus Hebdomadaires des Séances de l'Academie des Sciences*, Paris, Serie D, 276,1541-1544.

Sharma M., Schmid M., Rothballer M., Hause G., Zuccaro A., Imani J., Kämpfer P., Domann E., Schäfer P., Hartmann A. & Kogel K. (2008). Detection and identification of bacteria intimately associated with fungi of the order *Sebacinales. Cellular Microbiology*, 10(11), 2235–2246.

Spraker J.E., Sanchez L.M., Lowe T.M., Dorrestein P.C. & Keller N.P. (2016). *Ralstonia solanacearum* lipopeptide induces chlamydospore development in fungi and facilitates bacterial entry into fungal tissues. *ISME*, 10:2317–30.

Sun X., Chen W., Ivanov S., MacLean A. M., Wight H., Ramaraj T., Mudge J., Harrison M. J. & Fei Z. (2019). Genome and evolution of the arbuscular mycorrhizal fungus *Diversispora epigaea* (formerly *Glomus versiforme*) and its bacterial endosymbionts. *New Phytol.*, 221 (3): 1556-1573.

Takahashi M., e*t al.* (1987). Studies on macrocyclic lactone antibiotics. XI. Anti-mitotic and anti-tubulin activity of new antitumor antibiotics, rhizoxin and its homologues. *J. Antibiot.* 40, 66–-72.

Theis K.R., Dheilly N.M., Klassen J.L., Brucker R.M., Baines J.F., Bosch T.C.G., Cryan J.F., Gilbert S.F., Goodnight C.J., Lloyd E.A., Sapp J., Vandenkoornhuyse P., Zilber-Rosenberg I., Rosenberg E. & Bordenstein S.R. (2016). Getting the hologenome concept right: an ecoevolutionary framework for hosts and their microbiomes. *mSystems* 1(2):e00028-16.

Uehling J., Gryganskyi A., Hameed K., Tschaplinski T., Misztal P. K., Wu S., Desirò A., Vande Pol N., Du Z., Zienkiewicz A., Zienkiewicz K., Morin E., Tisserant E., Splivallo R., Hainaut M., Henrissat B., Ohm R, Kuo A., Yan J., Lipzen A., Nolan M., LaButti K., Barry K., Goldstein A. H., Labbé J., Schadt C., Tuskan G., Grigoriev I., Martin F., Vilgalys R. & Bonito G. (2017). Comparative genomics of Mortierella elongate and its bacterial endosymbiont Mycoavidus cysteinexigens. *Environ Microbiol.*, 19:2964-2983.

Velayos A., Papp T., Aguilar R., Fuentes M., Eslava A., Iturriaga E., Álvarez M. (2003). Expression of the *carG* gene, encoding geranylgeranyl pyrophosphate synthase, is up-regulated by blue light in *Mucor circinelloides*. *Curr Genet.*, 43: 112–120.

von Wettstein F. (1915). Geosiphon Fr. v. Wettst., eine neue, interessante Siphonee. *Osterr Bot Z*, 65:145-156.

Zilber-Rosenberg I. & Rosenberg E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol Rev.*, 2008;32(5):723–735.

CURRICULUM VITAE

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Áreas de investigación

Elucidar las implicaciones de las interacciones hongo-endobacteria-virus a nivel de desarrollo sexual, adecuación y promoción de la especiación del hospedante, haciendo uso de herramientas moleculares y químicas para evaluar la complejidad biológica desde un enfoque integrativo.

Proyectos de Tesis

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"Implicaciones de la simbiosis hongo-bacteria en el desarrollo sexual y adecuación de la especie *Rhizopus microsporus*"
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Proyecto de Tesis de Licenciatura (enero 2015 – Julio 2016)
"Identificación de fitohormonas producidas por aislados mexicanos del hongo *Metarhizium* spp."
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Publicaciones

Espino-Vázquez A. N., Bermúdez-Barrientos J. R., Cabrera-Rangel J. Francisco., *et al*., Partida-Martínez L. P. 2020. Narnaviruses: novel players in fungal–bacterial symbioses. *The ISME Journal* vol. 14, p. 743–1754. DOI: 10.1038/s41396-020-0638-y

Servicio Social Profesional

Servicio Social Profesional (marzo-septiembre 2014)
"Fermentación de jugo de agave con una cepa tequilera de Saccharomyces cerevisiae modificada"
Laboratorio de Genética Molecular de Hongos, Universidad de Guanajuato.
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Estancias y Veranos de Investigación

Estancia de Investigación (agosto 2018)
"Localización de Mycetohabitans rhizoxinica (Burkholderia rhizoxinica) en las diferentes estructuras del hongo fitopatógeno Rhizopus microsporus"
Laboratorio Nacional de Microscopía Avanzada, CICESE.
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Tutor: Dra. Rosa Mouriño-Pérez.

8° Verano Estatal de Investigación CONCYTEG (junio a Julio 2015)
Laboratorio de Genética Molecular de Hongos, Departamento de Biología, UG.
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Tutor: Dr. Israel Enrique Padilla-Guerrero.

Estancia Profesional (septiembre-diciembre 2014)
"Selección Sexual en Tenebrio molitor"
Laboratorio de Ecología Evolutiva, Universidad de Guanajuato.
Guanajuato, Guanajuato, México.
Tutor: Dr. Jorge Alberto Contreras Garduño.

Ponencias de Trabajo

XIII Congreso Nacional de Biología Molecular y Celular de Hongos (3 de octubre de 2019)
"More than just sex: Implication of the fungal-bacterial symbiosis in the partner choice of *Rhizopus microsporus*"
José Francisco Cabrera-Rangel, Espino-Vázquez, Martínez-Vázquez y Laila P. Partida-Martínez.
Ensenada, Baja California, México.

Clase de Micología y Líquenes (28 de agosto de 2018)
"Implication of the fungal-bacterial symbiosis in the sexual development and fitness of *Rhizopus microsporus"*
José Francisco Cabrera-Rangel y Laila P. Partida-Martínez.
Facultad de Ciencias, Universidad Autónoma de Baja California. Ensenada, B.C., México.

Simposio Internacional: Enviromental *Metarhizium* Biotechnology (4 de agosto de 2017)
Effects in *Arabidopsis thaliana* by the fungus *Metarhizium*
José Francisco Cabrera-Rangel, González-Hernández, Torres-Guzmán y Padilla Guerrero I.
Guanajuato, Guanajuato, México.

Presentación de Carteles

XXIV Congreso Latinoamericano de Microbiología 2018 (13 al 16 de noviembre)
"Implication of the fungal-bacterial symbiosis in the sexual development and fitness of *Rhizopus microsporus*"
José Francisco Cabrera-Rangel, Espino-Vázquez, Martínez-Vázquez, Valadez-Cano y Laila Partida-Martínez.
Región Metropolitana de Santiago, Santiago, Chile.

XI Congreso Nacional de Biología Molecular y Celular de Hongos (octubre 2015)
"Volatile organic compounds produced for the genus *Metarhizium* and its effect during the interaction with the model plant *Arabidopsis thaliana"*
José Francisco Cabrera-Rangel, González-Hernández, Torres-Guzmán, Olmedo-Monfil, Wrobel, Salazar-Solís e Israel E. Padilla-Guerrero.
Puebla, Puebla, México.

Actividades de Divulgación

Día Abierto CINVESTAV (20 de octubre 2018)
XXV Semana Nacional de Ciencia y Tecnología en Guanajuato
Actividad: Holograma 3D de la evolución.
CINVESTAV
Irapuato, Guanajuato, México.

BEER TALKS: Divulgación Científica.
Actividad: Fundador y Organizador
Centro Regional de Expresión Artística (CREA Centro Cultural)
Irapuato, Guanajuato, México.
Sesión 01: "Más que solo sexo" – 16 de marzo de 2019
Sesión 02: "Alcohol, mitos y realidades" – 8 de junio de 2019
Sesión 03: "Haciendo visible lo invisible" – 10 de agosto de 2019
Sesión 04: "Entre la vida y la muerte" – 9 de noviembre de 2019
Sesión 05: "Ciencia Olvidada" – 8 de febrero de 2020
Sesión 06: "TIEMPOS DE COVID: Retos y Oportunidades para la Ciencia Mexicana" Seminario Web 23 de mayo de 2020.

THE BEER PITCH: Ciencia en 60 segundos
Actividad: Organizador y Maestro de ceremonias
Centro Regional de Expresión Artística (CREA Centro Cultural)
Irapuato, Guanajuato, México.
TBP 01– 10 de agosto de 2019
TBP 02 – 9 de noviembre de 2019
TBP 03 – 8 de febrero de 2020

DECLARACIÓN DE INDEPENDENCIA

Por este medio declaro que yo he preparado este trabajo de tesis de forma independiente y sin ayuda externa. Especialmente declaro que he citado de forma correcta y explícita a los autores y trabajos en los que esta tesis se apoya, así como las contribuciones de las personas que coadyuvaron en su desarrollo.

Irapuato, Guanajuato, México. A 17 de agosto de 2020

José Francisco Cabrera Rangel

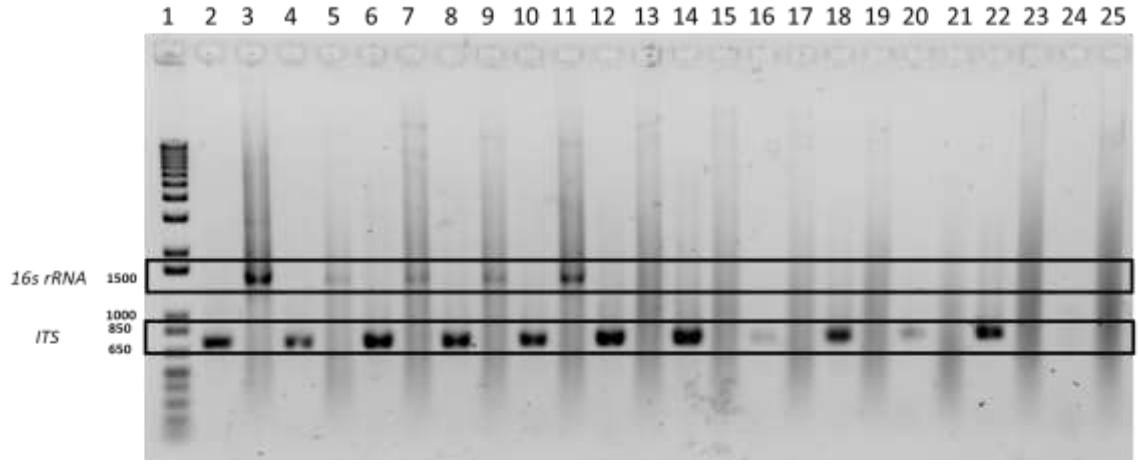
APPENDICES

APPENDIX A - Nicholson's method

1. Place fungal biomass in a mortar, add liquid nitrogen and grind.
2. Add 750 µL of Nicholson solution and macerate until a liquid solution be obtained.
3. Transfer 750 µL of the solution to a 2 mL microcentrifuge tube with 750 µL of volume of micropearls and shake at 12,000 rpm x 15 min in vortexer.
4. Add 750 µL of phenol-chloroform solution, mix in vortexer, and centrifuge the tubes at 12,000 rpm for 10 min.
5. Transfer aqueous phase to 1.5 mL microcentrifuge tubes.
6. Add 1 µL of RNAse-A (20 ng / mL) and incubate with shaking for 30 min at 37 ° C.
7. Add 200 µL of chloroform solution, vortex and centrifuge at 12,000 rpm x 10 min.
8. Transfer the top phase to new 1.5 mL microcentrifuge tube and add 200 µL of isopropanol solution (-20 ° C) and mix gently.
9. Incubate for at least 30 min at -20 °C.
10. Centrifuge the tubes at 12,000 rpm for 10 min. Remove the supernatant.
11. Add 50 µL of 70% ethanol solution (-20 ° C) to wash the pellet. Centrifuge at 12,000 rpm for 10 min.
12. Dissolve the pellet in 50 µL of T.E. solution. Quantify on NanoDrop and qualify in a 1% agarose gel (60 min at 90V).

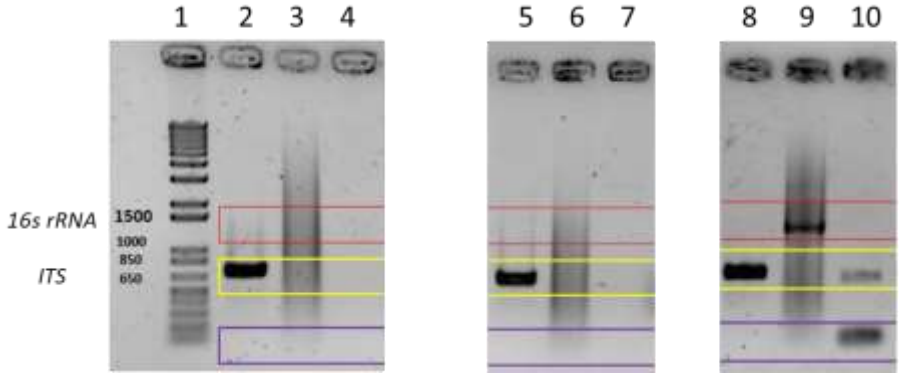
APPENDIX B. Molecular genotyping of *R. microsporus* strains.

A) Batch 1

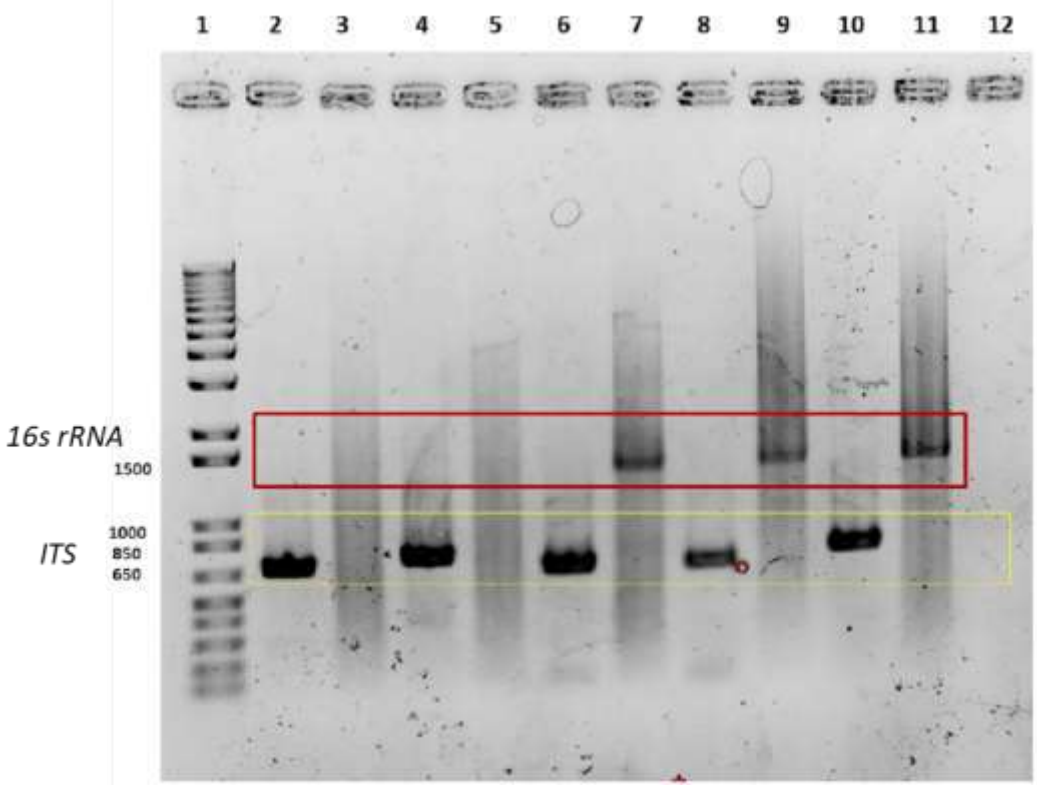


(1) DNA ladder, **(2)** ATCC 52813 *ITS*, **(3)** ATCC 52813 *16s rRNA*, **(4)** ATCC 20577 *ITS*, **(5)** ATCC 20577 *16s rRNA*, **(6)** ATCC 62417 *ITS*, **(7)** ATCC 62417 *16s rRNA*, **(8)** ATCC 52814 *ITS*, **(9)** ATCC 52814 *16s rRNA*, **(10)** ATCC 52811 *ITS*, **(11)** ATCC 52811 *16s rRNA*, **(12)** ATCC 11559 *ITS*, **(13)** ATCC 11559 *16s rRNA*, **(14)** ATCC 52807 *ITS*, **(15)** ATCC 52807 *16s rRNA*, **(16)** ATCC 52807-B ITS, **(17)** ATCC 52807-B *16s rRNA*, **(18)** ATCC 22959 *ITS*, **(19)** ATCC 22959 *16s rRNA*, **(20)** ATCC 22959-B *ITS*, **(21)** ATCC 22959-B *16s rRNA*, **(22)** ATCC 48010 *ITS*, **(23)** ATCC 48010 *16s rRNA*, **(24)** Control (-) *ITS*, **(25)** Control (-) *16s rRNA*.

B) Batch 2



(1) DNA ladder, **(2)** ATCC 46348 *ITS*, **(3)** ATCC 46348 *16s rRNA*, **(4)** ATCC 46348 *rhA* gene of rhizoxin cluster, **(5)** ATCC 56018 *ITS*, **(6)** ATCC 56018 *16s rRNA*, **(7)** ATCC 56018 *rhA*, **(8)** ATCC 62417 *ITS*, **(9)** ATCC 62417 *16s rRNA*, **(10)** ATCC 62417 *rhA*.



(1) DNA ladder , **(2)** ATCC 52807 *ITS*, **(3)** ATCC 52807 *16s rRNA*, **(4)** *R. oryzae ITS*, **(5)** *R. oryzae 16s rRNA*, **(6)** *Rhizopus* sp. HP475 *ITS*, **(7)** *Rhizopus* sp. HP475 *16s rRNA*, **(8)** HPY99 *ITS*, **(9)** HPY99 *16s rRNA*, **(10)** Control (+) *ITS*, **(11)** Control (+) *16s rRNA.*

The bacterial endosymbionts of HP475 and HPY99 strains were identified by sequencing of the *16s rRNA* gene:

- **HP475_16srRNA sequence cloned in plasmid pGEM-T-Easy:**

GCTGGCGGCATGCCTTACACATGCAAGTCGGACGGCAGCGCGGGTTTCGGCCTGGCGGCGAGTGGCGAACGGGTGA
GTAAGACATCGGAACGTGTCCTGGAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGCTCTGAGGAG
GAAAGCGGGGGACCTTCGGGCCTCGCGCTCAAGGGGCGGCCGATGGCAGATTAGCTAGTTGGTAGGGTAAAGGCCT
ACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTTGTCCGGAAAGAAAACGGCCTGGTTAATACCTGGGCTGGATGACGGTACCGGAAGAATAAG
CACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATGACTGGGCGTAAAGCG
TGCGCAGGCGGTTTGCTAAGACCGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTGGTGACTGGCAGGCTAGA
GTATGGCAGAGGGAGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGG
CAGCCTCCTGGGCCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCATGAAGTTGACCGCCTGGGGA
GTACGGTCGCAAGATTAGAACTCAAAGGAATTGACGGGGACCCGCACAGGCGGTGGATGATGTGATTAATTCGATGC
ACGCGAAAACCTTACCTACCCTTGACATGGTCGGAACCTGCTGAGAGGTAGGGGTGCCTCGAAAGAAGAACCGGC

Job Title: HP475_16srRNA
Program: Blastn
Molecule type: DNA
Query Length: 1000

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Uncultured Burkholderiaceae bacterium clone RM 16S ribosomal RNA gene, partial sequence	1777	1777	100%	0.0	98.71%	MT002708.1
Uncultured Burkholderiaceae bacterium clone 15-138 16S ribosomal RNA gene, partial sequence	1777	1777	100%	0.0	98.71%	MT002691.1
Burkholderia rhizoxinica strain H500 16S ribosomal RNA gene, partial sequence	1777	1777	100%	0.0	98.71%	HQ005407.1
Burkholderia rhizoxinica HKI 454, complete genome	1777	5327	100%	0.0	98.71%	FR687359.1
Uncultured bacterium clone S2-5-CL11 16S ribosomal RNA gene, partial sequence	1777	1777	100%	0.0	98.71%	EU769146.1
Burkholderia sp. LSB62417b 16S ribosomal RNA gene, partial sequence	1777	1777	100%	0.0	98.71%	DQ988984.1
Burkholderia rhizoxinica strain G7344 16S ribosomal RNA gene, partial sequence	1773	1773	100%	0.0	98.61%	HQ005405.1
Paraburkholderia rhizoxinica HKI 454 16S ribosomal RNA, partial sequence	1772	1772	100%	0.0	98.61%	NR_102769.1
Burkholderia rhizoxinica strain H3620 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0.0	98.61%	HQ005410.1
Burkholderia rhizoxinica strain H2199 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0.0	98.61%	HQ005408.1
Burkholderia rhizoxinica strain G8810 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0.0	98.61%	HQ005406.1
Burkholderia sp. LSB62417a 16S ribosomal RNA gene, partial sequence	1772	1772	100%	0.0	98.61%	DQ988983.1
Burkholderia sp. 20577 16S rRNA gene	1772	1772	100%	0.0	98.61%	AJ938141.1

- **HPY99_16srRNA sequence from PCR product:**

ACTTCCTGACTCCAAGGTGCACATTGCACTCCGGACTACGATCGGTTTTCTGGGATTAGCTCCCCCTCGCGGGTTGGCA
ACCCTCTGTTCCGACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCTCCCTAGAGTGCTCTTGCGTAGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTT
AACCCTCCATCTCAGGACCCAAGCTGACGACAGTCTTGCCTCTTTCTGTGCGCCGGTTCTTTTTCCAGCACCC

Job Title: HPY99_16srRNA
Program: Blastn
Molecule type: DNA
Query Length: 311

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Uncultured Burkholderiaceae bacterium clone 15-138 16S ribosomal RNA gene, partial sequence	449	449	94%	3e-122	94.22%	MT002691.1
Uncultured Burkholderiaceae bacterium clone RM 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	MT002708.1
Burkholderia rhizoxinica strain H3972 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005411.1
Burkholderia rhizoxinica strain H3620 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005410.1
Burkholderia rhizoxinica strain H2199 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005408.1
Burkholderia rhizoxinica strain H500 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005407.1
Burkholderia rhizoxinica strain G8810 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005406.1
Burkholderia rhizoxinica strain G7344 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	HQ005405.1
Uncultured bacterium clone S2-5-CL11 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	EU769146.1
Uncultured bacterium clone S1-1-CL5 16S ribosomal RNA gene, partial sequence	448	448	94%	1e-121	94.22%	AY725248.1
Burkholderia rhizoxina isolate TCB3 16S ribosomal RNA gene, partial sequence	448	448	93%	1e-121	92.86%	AY677091.1
Burkholderia ambifaria GT1022 gene for 16S ribosomal RNA, partial sequence	446	446	89%	4e-121	92.86%	LC496395.1
Burkholderia ambifaria strain B31 16S ribosomal RNA gene, partial sequence	446	446	89%	4e-121	92.86%	MH279778.1
Burkholderia cepacia strain PAC 2 16S ribosomal RNA gene, partial sequence	446	446	89%	4e-121	92.86%	MF984403.1

APPENDIX C - Statistical analysis of the *zygospore* diameter

Kruskal-Wallis test: H (5, N= 1200) =20.99742 p =.0008

Multiple comparisons of mean ranks for all group:

	52813 x 52814	52813 x 52811	20577 x 52814	20577 x 52811	62417 x 52814	62417 x 52811
52813 x 52814	-	0.125346	1.000000	1.000000	1.000000	1.000000
52813 x 52811	0.125346	-	**0.021724**	1.000000	**0.002190**	**0.005074**
20577 x 52814	1.000000	**0.021724**	-	1.000000	1.000000	1.000000
20577 x 52811	1.000000	1.000000	1.000000	-	0.364194	0.621857
62417 x 52814	1.000000	**0.002190**	1.000000	0.364194	-	1.000000
62417 x 52811	1.000000	**0.005074**	1.000000	0.621857	1.000000	-

APPENDIX D - IsoA, CarG, CarRA, CarB, CarS, AcaA protein IDs.

IsoA

>CEG66116.1 Putative Farnesyl pyrophosphate synthase [Rhizopus microsporus ATCC 62417]

>CEI88549.1 Putative Farnesyl pyrophosphate synthase [Rhizopus microsporus CBS 344.29]

>tr|A0A167NPH3|A0A167NPH3_PHYB8 Farnesyl diphosphate synthase OS=Phycomyces blakesleeanus (strain ATCC 8743b / DSM 1359 / FGSC 10004 / NBRC 33097 / NRRL 1555) OX=763407 GN=PHYBLDRAFT_109964 PE=3 SV=1

>tr|A0A1X2J049|A0A1X2J049_9FUNG Farnesyl pyrophosphate synthase OS=Absidia repens OX=90262 GN=BCR42DRAFT_399653 PE=3 SV=1

>tr|A0A1C7NP81|A0A1C7NP81_9FUNG Farnesyl pyrophosphate synthase OS=Choanephora cucurbitarum OX=101091 GN=ERG20 PE=3 SV=1

>tr|A0A1X2GX71|A0A1X2GX71_9FUNG Farnesyl pyrophosphate synthase OS=Hesseltinella vesiculosa OX=101127 GN=DM01DRAFT_1403146 PE=3 SV=1

>tr|A0A068S1L5|A0A068S1L5_9FUNG Farnesyl pyrophosphate synthase OS=Lichtheimia corymbifera JMRC: FSU:9682 OX=1263082 GN=LCOR_07346.1 PE=3 SV=1

>tr|A0A0C9MTL9|A0A0C9MTL9_9FUNG Farnesyl pyrophosphate synthase OS=Mucor ambiguus OX=91626 GN=MAM1_0134c06236 PE=3 SV=1

>tr|S2JNS8|S2JNS8_MUCC1 Farnesyl pyrophosphate synthase OS=Mucor circinelloides f. circinelloides (strain 1006PhL) OX=1220926 GN=HMPREF1544_08993 PE=3 SV=1

>CAD42869.1 farnesyl pyrophosphate synthase [Mucor lusitanicus]

>tr|I1CNE1|I1CNE1_RHIO9 Farnesyl pyrophosphate synthase OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880) OX=246409 GN=RO3G_14682 PE=3 SV=1

IsoA (cont.)

>tr|A0A1X2H5V2|A0A1X2H5V2_SYNRA Farnesyl pyrophosphate synthase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_495401 PE=3 SV=1

>ORE08396.1 farnesyl pyrophosphate synthase [Rhizopus microsporus var. microsporus ATCC 52814]

>ORE17266.1 farnesyl pyrophosphate synthase [Rhizopus microsporus ATCC 11559]

>tr|A0A077WI13|A0A077WI13_9FUNG Putative Farnesyl pyrophosphate synthase OS=Lichtheimia ramosa OX=688394 GN=LRAMOSA09205 PE=3 SV=1

>tr|A0A0B7NLL9|A0A0B7NLL9_9FUNG Uncharacterized protein OS=Parasitella parasitica OX=35722 GN=PARPA_13781.1 scaffold 47024 PE=3 SV=1

>XP_023466420.1 farnesyl pyrophosphate synthase [Rhizopus microsporus ATCC 52813]

CarG

>tr|A0A168QAA8|A0A168QAA8_ABSGL Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_09937.1 scaffold 11783 PE=3 SV=1

>tr|A0A1X2I5C2|A0A1X2I5C2_9FUNG Geranylgeranyl pyrophosphate synthase OS=Absidia repens OX=90262 GN=BCR42DRAFT_381286 PE=3 SV=1

>tr|A0A1C7NG66|A0A1C7NG66_9FUNG Geranylgeranyl pyrophosphate synthase OS=Choanephora cucurbitarum OX=101091 GN=carG_0 PE=3 SV=1

>tr|A0A068RWC2|A0A068RWC2_9FUNG Geranylgeranyl pyrophosphate synthetase OS=Lichtheimia corymbifera JMRC:FSU:9682 OX=1263082 GN=LCOR_04646.1 PE=3 SV=1

>tr|A0A077WH03|A0A077WH03_9FUNG Uncharacterized protein OS=Lichtheimia ramosa OX=688394 GN=LRAMOSA08460 PE=3 SV=1

>tr|S2JUI9|S2JUI9_MUCC1 Farnesyltranstransferase OS=Mucor circinelloides f. circinelloides (strain 1006PhL) OX=1220926 GN=HMPREF1544_00937 PE=3 SV=1

>tr|A0A168IR68|A0A168IR68_MUCCL Uncharacterized protein OS=Mucor lusitanicus CBS 277.49 OX=747725 GN=MUCCIDRAFT_180692 PE=3 SV=1

>tr|A0A0B7NN96|A0A0B7NN96_9FUNG Uncharacterized protein OS=Parasitella parasitica OX=35722 GN=PARPA_11276.1 scaffold 43311 PE=3 SV=1

>tr|A0A167JQZ7|A0A167JQZ7_PHYB8 Geranylgeranyl pyrophosphate synthetase OS=Phycomyces blakesleeanus (strain ATCC 8743b / DSM 1359 / FGSC 10004 / NBRC 33097 / NRRL 1555) OX=763407 GN=CarG2 PE=3 SV=1

>tr|I1BHQ6|I1BHQ6_RHIO9 Uncharacterized protein OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880) OX=246409 GN=RO3G_00440 PE=3 SV=1

>tr|A0A1X2HL85|A0A1X2HL85_SYNRA Geranylgeranyl pyrophosphate synthase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_529350 PE=3 SV=1

>CEG71184.1 Putative Farnesyltranstransferase [Rhizopus microsporus ATCC 62417]

CarG (Cont.)

>CEI87752.1 Putative Geranylgeranyl pyrophosphate synthase [Rhizopus microsporus CBS 344.29]

>ORE07226.1 terpenoid synthase Rhizopus microsporus ATCC 52814

>ORE16898.1 terpenoid synthase [Rhizopus microsporus ATCC11559]

>XP_023463652.1 geranylgeranyl pyrophosphate synthase [Rhizopus microsporus ATCC 52813]

CarRA

>tr|S2JTE4|S2JTE4_MUCC1 Bifunctional enzyme CarRP OS=Mucor circinelloides f. circinelloides (strain 1006PhL) OX=1220926 GN=HMPREF1544_10223 PE=4 SV=1

>tr|A0A0C9MMT4|A0A0C9MMT4_9FUNG Bifunctional enzyme CarRP-like OS=Mucor ambiguus OX=91626 GN=MAM1_0065c03952 PE=4 SV=1

>CEG75108.1 hypothetical protein RMATCC62417_10213 [Rhizopus microsporus ATCC 62417]

>CEI98584.1 hypothetical protein RMCBS344292_12689 [Rhizopus microsporus CBS 344.29]

>tr|A0A1X2IZU6|A0A1X2IZU6_9FUNG Lycopene beta-cyclase OS=Absidia repens OX=90262 GN=BCR42DRAFT_363009 PE=4 SV=1

>tr|A0A1X2GTC4|A0A1X2GTC4_9FUNG Lycopene beta-cyclase OS=Hesseltinella vesiculosa OX=101127 GN=DM01DRAFT_1397861 PE=4 SV=1

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>ORE02406.1 terpenoid synthase [Rhizopus microsporus var. microsporus ATCC 52814]

>ORE13622.1 terpenoid synthase [Rhizopus microsporus ATCC11559]

>tr|A0A1X2H2H5|A0A1X2H2H5_SYNRA Phytoene synthase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_532564 PE=4 SV=1

>tr|A0A162UN96|A0A162UN96_PHYB8 Phytoene Synthase/Lycopene cyclase OS=Phycomyces blakesleeanus (strain ATCC 8743b / DSM 1359 / FGSC 10004 / NBRC 33097 / NRRL 1555) OX=763407 GN=CarRA PE=4 SV=1

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>tr|A0A0B7NB96|A0A0B7NB96_9FUNG Uncharacterized protein OS=Parasitella parasitica OX=35722 GN=PARPA_10010.1 scaffold 39144 PE=4 SV=1

>tr|I1BJX8|I1BJX8_RHIO9 Uncharacterized protein OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880) OX=246409 GN=RO3G_01212 PE=4 SV=1

>XP_023461361.1 lycopene cyclase [Rhizopus microsporus ATCC 52813]

CarB

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>tr|A0A1X2IZQ9|A0A1X2IZQ9_9FUNG Phytoene dehydrogenase OS=Absidia repens OX=90262 GN=BCR42DRAFT_485306 PE=3 SV=1

>tr|A0A1C7N0F9|A0A1C7N0F9_9FUNG Phytoene desaturase OS=Choanephora cucurbitarum OX=101091 GN=carB PE=3 SV=1

>tr|A0A1X2GT92|A0A1X2GT92_9FUNG Phytoene dehydrogenase OS=Hesseltinella vesiculosa OX=101127 GN=DM01DRAFT_1397859 PE=3 SV=1

>tr|A0A068RLK8|A0A068RLK8_9FUNG Phytoene dehydrogenase OS=Lichtheimia corymbifera JMRC:FSU:9682 OX=1263082 GN=LCOR_02572.1 PE=3 SV=1

>tr|A0A077X0D9|A0A077X0D9_9FUNG Amino_oxidase domain-containing protein OS=Lichtheimia ramosa OX=688394 GN=LRAMOSA05214 PE=3 SV=1

>tr|A0A0C9M4Y3|A0A0C9M4Y3_9FUNG Phytoene dehydrogenase-like OS=Mucor ambiguus OX=91626 GN=MAM1_0065d03951 PE=3 SV=1

>tr|S2J0C7|S2J0C7_MUCC1 Phytoene dehydrogenase OS=Mucor circinelloides f. circinelloides (strain 1006PhL) OX=1220926 GN=HMPREF1544_10222 PE=3 SV=1

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>tr|A0A0B7NEC9|A0A0B7NEC9_9FUNG Amino_oxidase domain-containing protein OS=Parasitella parasitica OX=35722 GN=PARPA_10011.1 scaffold 39144 PE=3 SV=1

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>tr|I1CU28|I1CU28_RHIO9 Amino_oxidase domain-containing protein OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880) OX=246409 GN=RO3G_16669 PE=3 SV=1

>tr|A0A1X2H2F7|A0A1X2H2F7_SYNRA Phytoene dehydrogenase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_532563 PE=3 SV=1

>CEG75107.1 Putative Phytoene dehydrogenase [Rhizopus microsporus 62417]

>CEI93204.1 Putative Phytoene dehydrogenase [Rhizopus microsporus CBS 344.29]

>ORE02407.1 phytoene dehydrogenase [Rhizopus microsporus ATCC52814]

>ORE13623.1 phytoene dehydrogenase [Rhizopus microsporus ATCC11559]

>XP_023461362.1 phytoene dehydrogenase [Rhizopus microsporus ATCC 52813]

CarS

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>tr|A0A077WV29|A0A077WV29_9FUNG Uncharacterized protein OS=Lichtheimia ramosa OX=688394 GN=LRAMOSA11033 PE=3 SV=1

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>tr|A0A0B7NG16|A0A0B7NG16_9FUNG Uncharacterized protein OS=Parasitella parasitica OX=35722 GN=PARPA_08632.1 scaffold 33405 PE=3 SV=1

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>tr|A0A1X2HS21|A0A1X2HS21_SYNRA Carotene dioxygenase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_533527 PE=3 SV=1

>CEG67474.1 hypothetical protein RMATCC62417_03900 [Rhizopus microsporus ATCC 62417]

>CEJ04660.1 hypothetical protein RMCBS344292_18615 [Rhizopus microsporus CBS 344.29]

>ORE03982.1 putative carotene-dioxygenase [Rhizopus microsporus ATCC52814]

>ORE16808.1 putative carotene-dioxygenase [Rhizopus microsporus ATCC11559]

>XP_023467847.1 putative carotene-dioxygenase [Rhizopus microsporus ATCC 52813]

AcaA

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>tr|A0A1X2G8A6|A0A1X2G8A6_9FUNG Uncharacterized protein OS=Hesseltinella vesiculosa OX=101127 GN=DM01DRAFT_1293510 PE=3 SV=1

>tr|A0A0C9MVC4|A0A0C9MVC4_9FUNG Carotenoid cleavage dioxygenase 1 OS=Mucor ambiguus OX=91626 GN=MAM1_0161c06934 PE=3 SV=1

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>tr|I1BKS3|I1BKS3_RHIO9 Uncharacterized protein OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880) OX=246409 GN=RO3G_01507 PE=3 SV=1

>tr|A0A1X2HBU2|A0A1X2HBU2_SYNRA Carotenoid oxygenase OS=Syncephalastrum racemosum OX=13706 GN=BCR43DRAFT_557199 PE=3 SV=1

>CEG76724.1 hypothetical protein RMATCC62417_11584 [Rhizopus microsporus ATCC 62417]

>CEI90210.1 hypothetical protein RMCBS344292_04539 [Rhizopus microsporus CBS 344.29]

>ORE05316.1 putative carotene-dioxygenase [Rhizopus microsporus var. microsporus ATCC 52814]

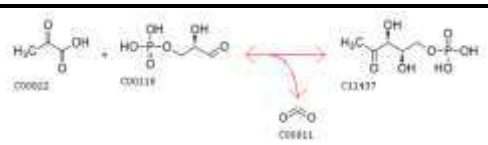
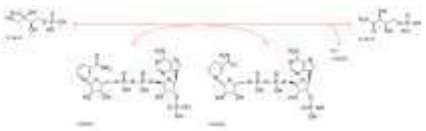
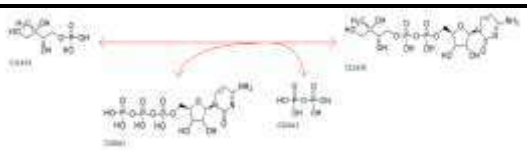
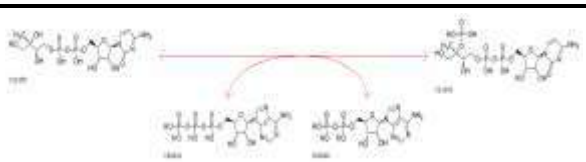
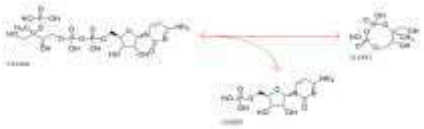
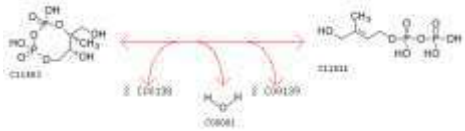
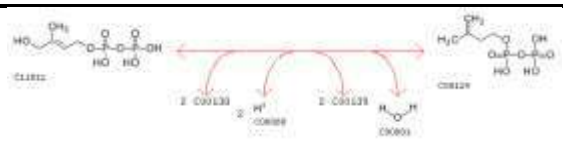
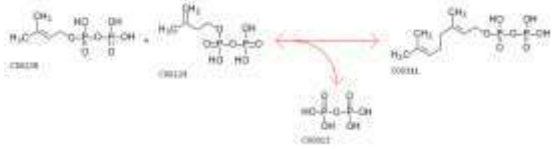
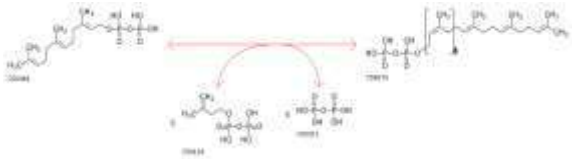
>ORE12734.1 hypothetical protein BCV71DRAFT_207174, partial [Rhizopus microsporus ATCC 11559]

>XP_023464643.1 putative carotene-dioxygenase [Rhizopus microsporus ATCC 52813]

APPENDIX F. Homologous genes of *Mycetohabitans* spp. in the terpenoids pathway.

ENZIME		STRAIN		
		Burkholderia rhizoxinica B1 (6666666.329271) *Mycetohabitans* sp. B1	*Burkholderia* sp. b13 FTPM01 (6666666.326653) *Mycetohabitans* sp. B4	*Burkholderia* sp. b14 FTPJ01 (6666666.326651) *Mycetohabitans* sp. B7
		ID	ID	ID
1-deoxy-D-xylulose 5-phosphate synthase	DXS	1680, 551	3189, 466	1000, 940, 941
1-deoxy-D-xylulose 5-phosphate reductoisomerase	ISPC	2191	127	1335
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	ISPD	2361	2574	1561
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	ISPE	3254	1743	2394
2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	ISPF	2360	2575	1560
1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	ISPG	2032	294	1162
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	ISPH	1281, 544	3183, 788	3325, 934
(2E,6E)-farnesyl diphosphate synthase	GTT	1679	467	3652, 3653
Undecaprenyl diphosphate synthase	UppS	2189	129	1333
Dimethylallyltransferase	DMAT	1679	467	3652, 3653
Farnesy diphosphate synthase	IspA	1679	467	3652, 3653
Octaprenyl diphosphate synthase	IspB	1679	467	3652, 3653

APPENDIX G – Hypothetical reaction mechanism of terpenoids biosynthesis in *Mycetohabitans* spp.

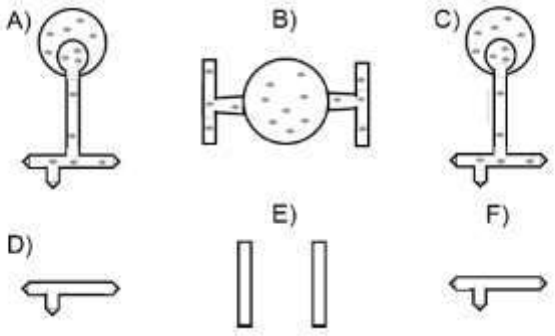
Pyruvate + D-Glyceraldehyde 3-phosphate <=> 1-Deoxy-D-xylulose 5-phosphate + CO2	
2-C-Methyl-D-erythritol 4-phosphate + $NADP^+$ <=> 1-Deoxy-D-xylulose 5-phosphate + NADPH + H^+	
2-C-Methyl-D-erythritol 4-phosphate + CTP <=> 4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol + Diphosphate	
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol + ATP <=> 2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol + ADP	
2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol <=> 2-C-Methyl-D-erythritol 2,4-Cyclodiphosphate + CMP	
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate + 2 Reduced ferredoxin <=> 1-Hydroxy-2-methyl-2-butenyl-4-diphosphate + H2O + 2 Oxidized ferredoxin	
1-Hydroxy-2-methyl-2-butenyl-4-diphosphate + 2 Reduced ferredoxin + 2 H+ <=> Isopentenyl diphosphate + 2 Oxidized ferredoxin + H2O	
Dimethylallyl diphosphate + Isopentenyl diphosphate <=> Diphosphate + Geranyl diphosphate	
trans,trans-Farnesyl diphosphate + 8 Isopentenyl diphosphate <=> di-trans,poly-cis-Undecaprenyl diphosphate + 8 Diphosphate	

APPENDIX H. José Roberto Bermúdez Barrientos, 2016.

Thesis to obtain the grade of Master in Sciences in Integrative Biology: "Exploring the molecular mechanisms maintaining the *R. microsporus* – *B. rhizoxinica* symbiosis"

Thesis directors: Dra. Laila Pamela Partida Martínez and Dr. Cei Leander Gastón Abreu Goodger

Bermúdez-Barrientos analyzed RNA-Seq data of two *R. microsporus* host strains growing alone or together, and with or without their corresponding endosymbionts:



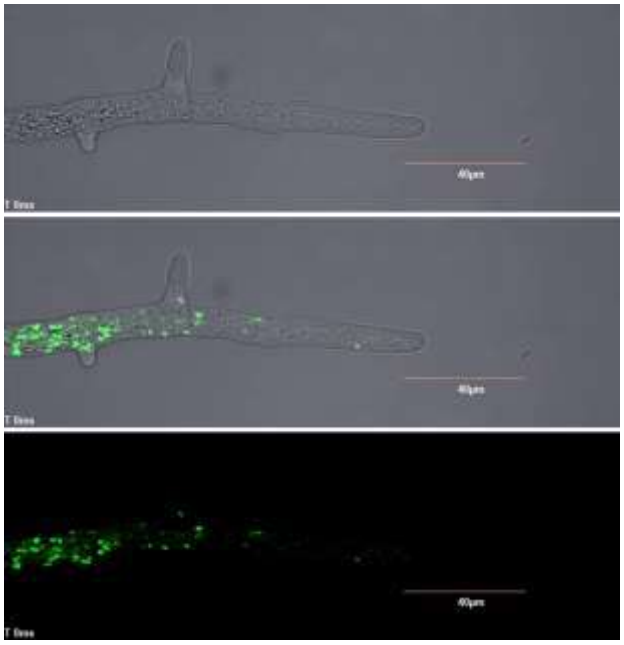
Experimental design and physiological state of RNA-Seq samples. A) ATCC52813 with endosymbionts, asexual sporulation via sporangia. B) Mating of ATCC52813 with ATCC52814, formation of zygospores, both strains harbor endosymbionts. C) ATCC52814 with endosymbionts, asexual sporulation via sporangia. D) ATCC52813 without its endosymbionts growing as sterile mycelium. E) Co-culture of ATCC52813 and ATCC52814 without their endosymbionts, both strains grow as sterile mycelia. F) ATCC52814 without its endosymbionts growing as sterile mycelium.

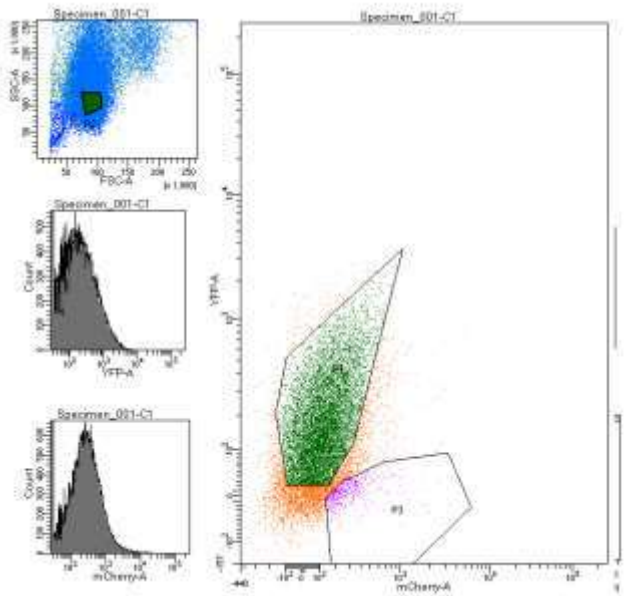
RNA-Seq libraries:

Library name	ATCC52813	ATCC52814	Bacterial endosymbiont
A13b	+	-	+ (B4)
A13c	+	-	-
A14b	-	+	+ (B7)
A14c	-	+	-
A13bA14b	+	+	+ (B4 & B7)
A13cA14c	+	+	-

APPENDIX I. – Cytometric analysis and microscopy of fluorescent-labelled bacteria

The transformed bacteria can be trackable within the mycelium by microscopy, and the sporangiospores by cytometric.

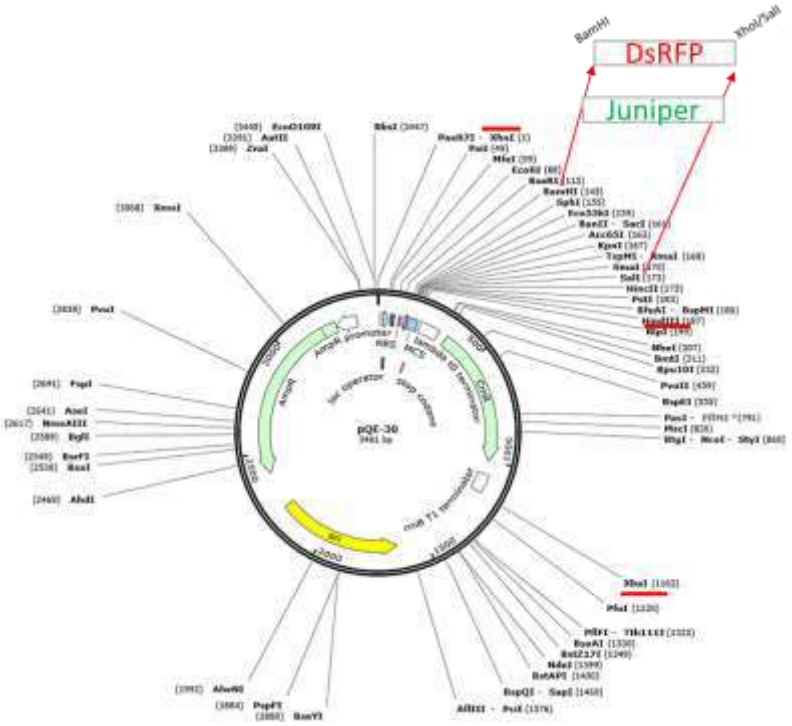


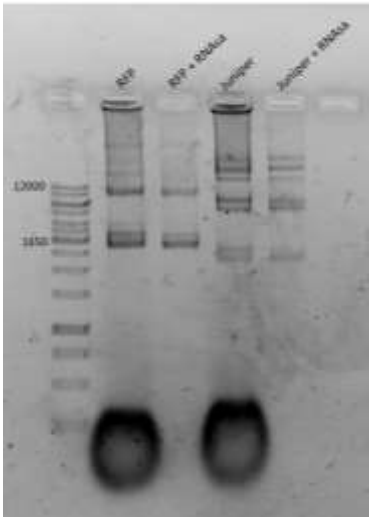


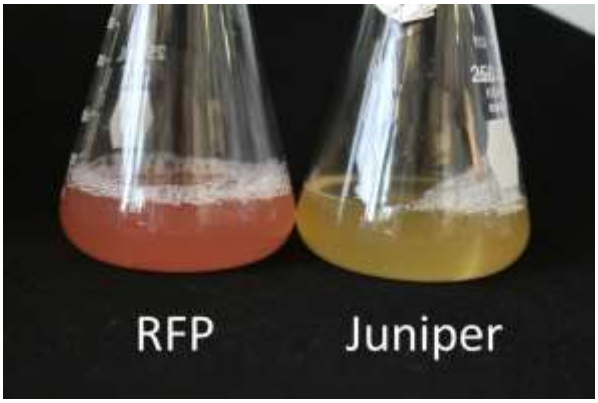
Mycelia and sporangiospores of *Rhizopus microsporus* ATCC 52814 (m-) with its endobacteria labelled with the Yellow Fluorescent Protein (YFP).

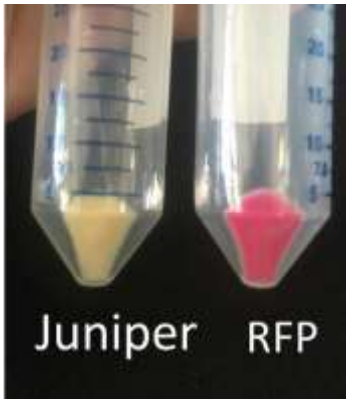
APPENDIX J.- Construction of plasmids with fluorescent proteins.

In collaboration with Dr. Bernardo Franco Barcenas from Departamento de Biología, Universidad de Guanajuato.









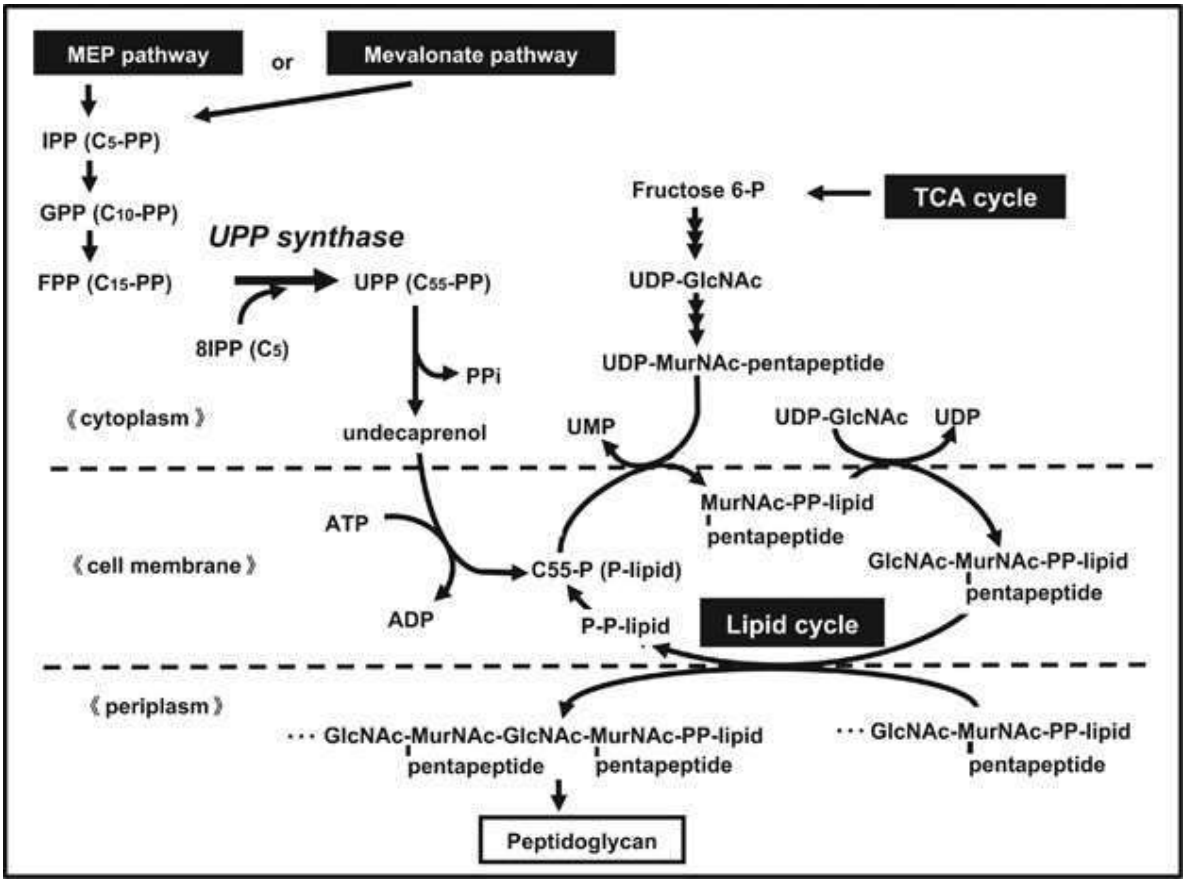
***Escherichia coli* DH5α transformed with the plasmids *PQe-30BFFC-RFP* (Red protein) and *PQe-30BFFC-Juniper* (Green protein).**

Appendix K. HMG gene of *R. microsporus* ATCC 11559

```
Rm_52814    ------------------------------------------------------------  0
Rm_11559    MKQQVEKSPISPRRQLKPKVSDNSSKILPVDDLLLLQEQGATSILISPSNVRIKAKDILC  60
Rm_82813    MKQQVEKGPMSPRRLLKPKISDDSSKVLPVDDLLLLQEQGETSILISPSNVRIKAKDILN  60

Rm_52814    ------------------------------------------------------MNA   3
Rm_11559    MVDNRQDFITLPDNGYCILRNDIFSALHAHGDRQADEHTAKLFLMDINSDVSTSKRPTNA  120
Rm_82813    MIDNRQDFITLPDNDYCILRNDIFSVLHAYGDRQADQHTVELFLMDIDFDVSTSKRPTNA  120
                                                                   **

Rm_52814    FLLYRQTKQKNLQF-KEKILSKDFSKAVAEMWRSEKEEVRSYYHRLAEEEKLKHRNKYPN  62
Rm_11559    FILYRTAWGKTVKSMFPEFNNSQISKILGAMWKWSDNQVKDKYIQRANEYRKVHKEKCPN  180
Rm_82813    FILYRTAWGKTVRSMFPEFNNSQISKILGAMWKWSGNQVKDKYIQRANEYRKIHKEKYPN  180
            *:*** :  *.::    :: ..::** :. **: . ::*:. * : *:* :  *::* **

Rm_52814    YKYSPRQKNKQSLTEGLDRRFVSTSVNQI-----ESNQPIHQEAQIDFEFNIATPPSLM-  116
Rm_11559    FVYNAKRVDKNKQVSATDDHHFEYNYPDLELQGSMVNQLVHSGTTNDNQTAYVSSSNFCS  240
Rm_82813    FVYSTKRVDKNKQVSATDDHHFECNYADLGLQGSMVNQLVHSGTADDNKSADALGNNFCS  240
            : *. :: :*:. ... * :... .  ::        ** :*. :  * :   .   .:

Rm_52814    IHDLQEFHDGSGL--------DILSLDCDLYT--LLSFLPEY----------------  148
Rm_11559    SNNYSGFTQKQNIATASDTSNSEWQKVCDLVLDILSSGISEDSVVDDQYWDILQNVDLEQ  300
Rm_82813    NNNYSGFTQKQNFATVSDISNSEWQKVCDLVLDILSSGIPEDSVIDDQYWDILQNVDLEQ  300
             :: . * : ..:        .  .  ***    * * : *

Rm_52814    ----------------  148
Rm_11559    SFFCSDWAAAVNLNQS  316
Rm_82813    SFFYNDWAAAVNLSQP  316
```

HMG gene of ATCC 11559 have 86% of Identity with ATCC 52813 (Needleman-Wunsch alignment of two sequences).

Appendix L. Cell wall synthesis pathway in bacteria.



Inokoshi, J., Nakamura, Y., Komada, S. et al. Inhibition of bacterial undecaprenyl pyrophosphate synthase by small fungal molecules. J Antibiot 69, 798–805 (2016). https://doi.org/10.1038/ja.2016.35

CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

Cinvestav

2019, "AÑO DEL CAUDILLO DEL SUR, EMILIANO ZAPATA"

Subdirección de Posgrado
SP/230/19
Ciudad de México, a 18 de marzo de 2019.

Dr. Alexander de Luna Fors
Coordinador Académico del
Programa de Biología Integrativa
Unidad Irapuato
P r e s e n t e

Por instrucciones del Dr. Gabriel López Castro, Secretario Académico, y en respuesta a la solicitud recibida el día 13 de marzo del presente año, le informo que de acuerdo al Capítulo XV, Artículo 75 del *Reglamento General de Estudios de Posgrado*, se permite la escritura en inglés de las tesis de grado, previo aval del Colegio de Profesores.

En la solicitud presentada, usted especifica la situación del estudiante, razón por la cual se autoriza su petición para que la tesis de José Francisco Cabrera Rangel, sea presentada en el idioma inglés.

Sin otro particular de momento, aprovecho la oportunidad para enviarle un cordial saludo.

Atentamente,

Arq. Hugo Flores Félix
Subdirector de Posgrado

HFF'cic

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