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Mestrado em Fisiologia Molecular de Plantas

Trabalho efectuado sob a orientação da
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e do
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e da
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Assessing the antifungal potential of *Hypholoma fasciculare* and its adherence to the roots of *Castanea sativa*

Abstract

In the region of Trás-os-Montes the chestnut has a high economic importance. In recent years, there has been a decrease of the area occupied by the chestnut, due to the emergence of diseases, like the ink disease, caused by *Phytophthora cinnamomi* and *P. cambivora*, or blight canker, caused by *C. parasitica*.

The presence of the fungus *Hypholoma fasciculare* was recently observed in the chestnut orchards of Trás-os-Montes (Bragança). Preliminary studies have suggested that this fungus causes serious damage to chestnut trees. *H. fasciculare* seems also to have an expressive antagonistic action against various fungi, some of which harmful and other beneficial, which could compromise the chestnut orchard sustainability. In order to evaluate the consequences arising from the use of *H. fasciculare* as a biological control agent, the antagonistic spectrum of this fungus was assessed against different fungi using the dual culture method. The results indicate that *H. fasciculare* exerts an antagonist action against distinct fungi, but also presents its growth affected by the interaction.

Keeping in mind the possible purification of compounds with antagonistic potential a fast and effective method to evaluate antimicrobial activity is desirable. Since the method of co-culture between filamentous fungi is relatively slow, requiring approximately 12 to 15 days for giving results, a new assay was optimized by using yeasts as indicators of antagonistic activity of *H. fasciculare*. Given the sensitivity of the tested yeasts and its high growth rate, this new assay not only proved to be suitable but also faster to evaluate antimicrobial activity of *H. fasciculare*. This method is proposed to be used in place of the traditional method of co-culture for assessing the antimicrobial potential. The same assay, performed with Gram-positive and Gram-negative bacteria, also revealed the antibacterial activity of compounds produced by *H. fasciculare*.

The interaction between *C. sativa* - *H. fasciculare* was evaluated by the macroscopic characterization of fungal adhesion to chestnut roots. Since the hydrophobin proteins could be involved in the adhesion process, the identification of the full sequence of the corresponding cDNA was attempted using RNA samples purified from samples collected during the process of interaction root-fungus. The prior identification of a genomic DNA sequence of *H. fasciculare* hydrophobin gene allowed starting the process of identifying the cDNA sequence of the same gene.

Keywords: *Hypholoma fasciculare*, *Castanea sativa*, antagonism, antimicrobial activity, interaction fungi-plant, interaction fungi-fungi

Avaliação do potencial antifúngico do *Hypholoma fasciculare* e sua adesão as raízes de *Castanea sativa*

Resumo

Na região de Trás-os-Montes o castanheiro possui uma elevada importância económica. Nos últimos anos, tem-se assistido a um decréscimo da área ocupada pelo castanheiro, devido ao aparecimento de doenças como a doença da tinta, causada por *Phytophthora cinnamomi* e *P. cambivora*; e o cancro cortical, causado por *C. parasitica*.

A presença do fungo *Hypholoma fasciculare* foi recentemente observada em soutos de Trás-os-Montes (Bragança). Estudos preliminares evidenciaram que este fungo causa sérios danos aos castanheiros. *H. fasciculare* possui ainda uma expressiva acção antagonista contra diversos fungos presentes no solo dos soutos, prejudiciais e benéficos, o que poderá comprometer a sustentabilidade dos soutos. Durante este projecto avaliou-se o espectro de acção antagonista de *H. fasciculare* contra diversos fungos, utilizando o método de cultura dupla, de forma a avaliar as consequências decorrentes da utilização deste fungo como agente de controlo biológico. Os resultados indicam que *H. fasciculare* exerce uma acção antagonista contra os fungos testados, mas também apresenta o seu crescimento afectado pela interacção.

Com vista a uma possível purificação dos compostos com potencial antagonista é desejável um método rápido e eficaz para avaliar a actividade antimicrobiana. Dado que o método de co-cultura entre fungos filamentosos é relativamente moroso, requerendo cerca de 12 a 15 dias para visualizar os resultados, um novo ensaio foi optimizado, utilizando leveduras como microrganismos indicadores de sensibilidade à actividade antagonista de *H. fasciculare*. Dada a sensibilidade das leveduras testadas e a sua elevada taxa de crescimento, este novo ensaio revelou-se não só adequado como mais rápido para avaliar a actividade antimicrobiana de *H. fasciculare*. Propõe-se assim a sua utilização em substituição do tradicional método de co-cultura para avaliar o potencial antimicrobiano do fungo em estudo. O mesmo ensaio, realizado com bactérias Gram-positivas e Gram-negativas, revelou ainda a actividade antibacteriana de compostos produzidos por *H. fasciculare.*

O estabelecimento da interacção entre *C. sativa - H. fasciculare* foi avaliado pela caracterização macroscópica da adesão do fungo às raízes de castanheiro. Dado que no processo de adesão poderão estar envolvidas hidrofobinas, a identificação da sequência completa do seu cDNA foi tentada recorrendo à purificação de RNA de amostras recolhidas durante o processo de interacção raíz-fungo. A partir da prévia identificação de uma sequência de DNA genómico de hidrofobina de *H. fasciculare* foi iniciado o processo de identificação da sequência de cDNA do mesmo gene.

Palavras-chave: *Hypholoma fasciculare*, *Castanea sativa*, antagonismo, actividade antimicrobiana, interacção fungo-planta, interacção fungo-fungo,

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Abbreviations list

Aa – Aa	Co-culture of *Alternaria arborsecens - Alternaria arborescens*
Bb-Bb	Co-culture of *Beauveria bassiana – Beauveria bassiana*
°C	Celsius degrees
cDNA	Complementary deoxyribonucleic acid
Cs-Cs	Co-culture of *Cordyceps sinensis – Cordyceps sinensis*
cm	centimeters
En-En	Co-culture of *Epicocum nigrum – Epicocum nigrum*
Fc-Fc	Co-culture of *Fusarium chlamydosporum – Fusarium chlamydosporum*
Fo-Fo	Co-culture of *Fusarium oxysporum – Fusarium oxysporum*
g	grams
Gm-Gm	Co-culture of *Gibberella moniliformis – Gibberella moniliformis*
h	hours
Hf- Aa	Co-culture of *Hypholoma fasciculare – Alternaria arborescens*
Hf-Bb	Co-culture of *Hypholoma fasciculare – Beauveria bassiana*
Hf-Cs	Co-culture of *Hypholoma fasciculare – Cordyceps sinensis*
Hf-En	Co-culture of *Hypholoma fasciculare – Epicocum nigrum*
Hf-Gm	Co-culture of *Hypholoma fasciculare – Gibberella moniliformis*
Hf-Fc	Co-culture of *Hypholoma fasciculare – Fusarium chlamydosporum*
Hf-Fo	Co-culture of *Hypholoma fasciculare – Fusarium oxysporum*
Hf-Hf	Co-culture of *Hypholoma fasciculare –Hypholoma fasciculare*
Hf-Mc	Co-culture of *Hypholoma fasciculare – Mucor circinelloides*
L	liter
LB	Luria- Bertani
Mc-Mc	Co-culture of *Mucor circinelloides – Mucor circinelloides*
Min	minutes
MMN	Melin-Norkrans
bp	base pairs
PDA	Potato Dextrose agar
PCR	Polymerase Chain Reaction
w/v	weight per volume
RNA	Ribonucleic acid
sec	seconds
YPD	Yeast Extract Peptone Dextrose

Chapter 1

General Introduction

The European chestnut (*Castanea sativa* Mill.) has high economic importance in countries of Mediterranean Europe, due to the wood quality and value of the fruit. Portugal is the second largest producer of chestnuts in the world, being the chestnut tree widely distributed through the north and center of the country, mainly in the interior region. One of the main producing areas is the north-eastern region (Trás-os-Montes), where this crop has a high economic interest. The wood is a raw-material used in timber construction, carpentry, cooperage, flooring or shipbuilding, being one of the preferred choices for making high quality furniture. In addition, the fruit is the most important export product of the fruit sector in Portugal. Along with the economic value, the tree has acquired a great importance at a cultural level, mainly in Trás-os-Montes region (Martins, 2004; Baptista *et al.*, 2007). Besides the use of chestnut fruits in the population diet, leaves and bark are also used to treat diseases due to their astringent, remineralizing, sedative and tonic properties (Carvalho, 2005). Associated with chestnut orchards found in this region, there are large quantities of edible mushrooms, some of which with high internationally commercial value (Baptista *et al*, 2007). Indeed, the mushroom harvesting represents an important source of income for the local population.

Soil microorganisms, such as fungi, bacteria, protozoa, algae or viruses, play relevant activities in plant development, nutrition and health (Kennedy, 1998; Bowen & Rovira, 1999; Barea *et al.*, 2005; Fageria & Stone, 2006). This action can be beneficial or harmful, depending on the interaction that occurs between microorganisms and plant roots.

Fungi are well known by their ability to decompose debris of plants and animals. Indeed, the fungal contribution on the decomposition and recycling of nutrients is very important since the soil acidity usually limits the growth of other groups of microorganisms (Deacon, 1997). But fungi can also cause reactions of antagonism to other organisms, being capable of parasite and/or pathogenic associations that cause disease and mutually favorable associations with the plant roots (Klein & Paschke, 2004). The fungal contribution on the decomposition and recycling of nutrients is important since the soil acidity usually limits the growth of other groups of microorganisms (Deacon, 1997).

The filamentous fungi are also associated with the translocation of nutrients within the soil. The penetration of hyphae into plant tissues could result in the release and incorporation of nutrients into the hyphae. Due to the fungal ability to

growth and colonize, the translocation of these nutrients to large areas will be promoted (Klein & Paschke, 2004).

Fungi may display a multitude of interactions with other soil microorganisms, including interactions with other fungi. The antagonistic action that some fungi have on others is due to the action of antibiotics or enzymes produced by the fungus, as well as the competition for food or parasitism (Brimner & Boland, 2003). As a result, the interaction may occur at a distance (antagonism at a distance) or after fungal contact ("hyphal interference" or parasitism). The most relevant type of antagonism that occurs in saprophytic fungi occurs through mycelial contact. Among the interactions that occur between fungus-fungus, the antagonism between filamentous fungi and yeast could be also included. A similar process happens when referring to antagonism between fungus and bacteria's.

In addition to the interactions with other microorganisms, soil fungi are also able to interact with root plants. These interactions can be also beneficial or harmful. Indeed, the most important plant pathogens are fungi. Chestnut trees could be strongly affected by diseases caused by fungi, such as ink disease or sudden oak death (caused by *Phytophthora cinnamomi* and *Phytophthora cambivora*), root disease (caused by *Armillaria mellea*) or chestnut blight (caused by *Cryphonectria parasitica*). These fungi are causing serious damage to chestnut groves, reducing the area occupied by this crop over the past decades, mainly in the region of Trás-os-Montes (Baptista, 2007).

The symbiotic association between fungi and plants, mycorrhization, is an important interaction occurring in the rhizosphere. This interaction cause physiological changes on host plant resulting in the improvement of their growth, as well as better resistence/tolerance to biotic and abiotic stresses (Jeffries *et al.*, 2003; Barea *et al.*, 2005). Despite the importance of mycorrhization for plant growth and productivity, the study of mycorrhizal fungi interaction with other microorganisms has been neglected. Most work has focused on the effect of inoculation of saprophytic fungi in mycorrhizal systems. The results indicate that, in general, saprophytic fungi reduce plant growth and inhibit root colonization by symbiotic fungus, impairing mycorrhization (Fracchia *et al.*, 1998; Godeas *et al.*, 1999; Murphy & Mitchell, 2001; Martinez *et al.*, 2004). However, this effect is not always observed and depends mainly on the species and strain of the saprophytic fungi or its symbiont (García-Romera *et al.*, 1998; Fracchia *et al.*, 2000, 2004; Werner *et al.*, 2002). The suppression of mycorrhiza by saprophytic fungi mainly occurs during the pre-colonization of symbiotic development, through inhibition of

spore germination or hyphal growth of mycorrhizal fungi. Results concerning the suppression of ectomycorrhizae formation by saprophytic fungi were also obtained through antagonism studies between ectomycorrhizal and saprophytic fungi (Martinez *et al.*, 2004).

The effect of the interaction between saprophytic fungi in the soil and ectomycorrhizal fungi can also have an impact on the mineral nutrition of host plants, mainly in nitrogen and phosphorus (Koide & Kabir, 2001; Wu *et al.*, 2003, 2005). When the levels of these nutrients in the soil are not limiting, saprophytic fungi increase their availability to ectomycorrhiza resulting in an improvement of the nutritional status of the host plant. This increase is especially important for the mineral nutrients that came from complex polymers, which could not be hydrolyzed by the majority of ectomycorrhizal fungi, but are hydrolyzed by the saprophytic fungi (Koide & Kabir, 2001; Wu *et al.*, 2005). When soils have limiting levels in these nutrients, there is a direct competition between saprophytic and ectomycorrhizal fungi, restricting the mineral nutrition of host plants (Koide & Kabir, 2001).

The presence of the fungus *Hypholoma fasciculare* was recently observed in the chestnut orchards of Trás-os-Montes (Baptista, 2007). Although this species is described as saprophytic, a preliminary study showed that *H. fasciculare* causes serious damage to chestnut trees and has a significant antagonistic action against several fungi present in the soil of the chestnut groves (P. Baptista, personal communication). Limiting the growth and spread of phytopathogenic fungi, *H. fasciculare* may give a significant contribution to the preservation of chestnut culture. However, as this fungus also restricts the growth of beneficial fungi, such as ectomycorrhizal fungus, *Pisolithus tinctorius* (Baptista, 2007), the sustainability of chestnut groves can also be compromised by the presence of *H. fasciculare*.

Hypholoma fasciculare belongs to the Basidiomycetes class, being frequent in northern Europe and North America. This fungus has been described as saprophytic, since its mushrooms are easily found on the dead wood of deciduous trees. The mycelial growth is morphologically characterized by the formation of strands (cords), which are aggregates of hyphae aligned along. This typical feature from cord-forming fungi, combined with the high growth rate, allows the occupation of large areas that can reach more than 100m in diameter (Boddy, 1993). As a result, cord-forming fungi present an ecological impact on the mobilization and translocation of biomass and nutrients within the soil, especially in forest ecosystems. As other fungal species that form mycelial cords, *H. fasciculare* has

been identified as a secondary colonizer of dead wood, showing ability to stay on the occupied site for many years (Dowson *et al.*, 1988c; Holmer & Stenlid, 1997).

Due to the high combative effect of *H. fasciculare* against other fungal species, its use as a biological agent against pathogenic fungi has been encouraged in recent years, such as the use in disease control of root rot cause by *Armillaria ssp*. (Chapman *et al.*, 2004). Antagonist actions recognized against other microorganism encourage several studies to understand the strategies of growth adopted by *H. fasciculare* in different resources (Dowson *et al.*, 1989), also inter-specific actions establish with other fungi species present in the same resource and its repercussions are being studied (Dowson *et al.*, 1988c, 1988a, 1988b; Nicolotti & Varese, 1996; Donnelly & Boddy, 2001; Wells & Boddy, 2002; Heilmann-Clausen & Boddy, 2005; Woods *et al.*, 2005, 2006; Cox & Scherm, 2006).

As mentioned earlier antagonism is not a mechanism unique of filamentous fungus. Antagonism can also occur in yeast and bacteria. In the XIX century, Pasteur, observe that when different bacteria were placed side by side the growth inhibition of one bacterium could be inhibited by the other. One bacterium produced substances that were lethal to other more sensitive strains of bacteria. These observations revolutionized the work on infectious diseases, leading to the characterization of a wide range of antimicrobial substances and promoting the production of antibiotics, bacteriolytic enzymes, and bacteriocins (Polonelli & Morace, 1986). A similar event in yeasts was first reported by Bevan and Makower (1963). Isolates of *Saccharomyces cerevisiae* secreted a substance that was lethal to other strains of the same species (Bevan & Makower, 1963). Using *S. cerevisiae* as killer yeast presents some advantages, since this yeast has been for the past two decades, the model system for much of the eukaryotic molecular genetics research. The conservation of the basic cellular mechanics of replication, recombination, cell division and metabolism between yeast and larger eukaryotes turned *S. cerevisiae* a good research model.

This project aims to evaluate the spectrum of antagonistic activity of *H. fasciculare* against other fungi present on the chestnut groves. The results would define the potential of this fungus as a biocontrol agent, and also determine the putative ecological consequences of that usage. Preliminary results have shown the antagonistic action of *H. fasciculare* against *Phytophtora* spp., which are extremely dangerous for chestnut groves (P. Baptista, personal communication). Keeping in mind the purification of a putative antagonistic compound, a simple and straightforward assay was optimized for testing the antagonistic activity of *H.*

fasciculare (Chapter 2). Because dual cultures with filamentous fungi will take too much time to give a result in a purification protocol, the assay optimization was focused on the application of yeasts (*S. cereviseae*) in the antagonistic assay. The spectrum of *H. fasciculare* action was also assessed co-culturing *H. fasciculare* and different strains of filamentous fungi (Chapter 3). To further evaluate the antagonistic spectrum of *H. fasciculare* and validate the use of the designed assay, this methodology was used for testing other yeasts and bacteria (Chapter 3).

Another objective of this project is to study the establishment of *C. sativa* - *H. fasciculare* interaction. Proteins with adhesion capacity, such as hydrophobins, were previously suggested to play an important role during the first hours of interaction (Baptista, 2007). The previous identification of a partial sequence of *H. fasciculare* hydrophobin gene will allow obtaining the complete sequence of the corresponding coding region by RACE (Chapter 4). The sequences obtained will be used to design new primers for cloning the coding region. Using various bioinformatics tools the DNA sequence and hydrophobin protein will be analyzed and compared with other homologous sequences (Chapter 4).

Chapter 2

Design of an assay for detection of *Hypholoma fasciculare* antimicrobial activity

2.1 Introduction

Antimicrobial activity refers to the ability of a microorganism to inhibit the growth of another microorganism and is normally due to the production and release of substances with that activity. An antimicrobial activity was probably observed for the first time by Pasteur and Joubert, in the XIX century, who described an inhibitory effect of bacteria isolated from urine against *Bacillus anthracis* in Ullmann, 2007. Since this first observation, a wide range of substances possessing antimicrobial activity has been discovered and characterized in many species of bacteria, yeast and fungi, either displaying activity against the same microbial group or effective against microbes of different groups. Penicillin, produced by the fungus *Penicillium notatum*, was discovered by Alexander Fleming in 1928 and was an important mark in medicine, triggering the search and the development of new antibiotics. However, fungi also interact with other fungi, either belonging to the same species or to different ones. Most of the times this interaction is a competition, with one organism usually inhibiting the growth of another fungus (Boddy, 1999).

In bacteria, the production of substances that are active against other bacteria - the bacteriocins – can be found in several species, either Gram-positive or Gram-negative, and different bacteriocins can be produced by a unique species. In yeasts a similar phenomenon is also found (Riley & Wertz, 2000; Savadogo *et al.,* 2006). Killer yeasts and their killer toxins were first reported by Bevan and Markower, in 1963, who described that an isolated *Saccharomyces cerevisiae* strain secreted a substance lethal to other strains of the same species. The occurrence of this phenotype also proved to be widely distributed in yeasts. Furthermore, some killer yeasts can produce more than one killer toxin type, as found for bacteriocins producing bacteria.

The antifungal potential of *Hypholoma fasciculare* is usually evaluated using a dual co-culture assay of *H. fasciculare* and other filamentous fungi which sensitivity is going to be tested. However, to see any interaction, it is necessary to wait for the growth of both fungi, which takes an average of 10-12 days.

Yeasts are also fungi, although unicellular, and they growth more rapidly than filamentous fungi. Taking this into consideration, attempts were made to design and develop a procedure to detect *H. fasciculare* antimicrobial activity using yeasts as sensitive indicator microorganisms. *Saccharomyces cerevisiae* was

chosen as the sensitive indicator yeast to develop this assay. This yeast is a model eukaryote microorganism in Biology due to its basic mechanism of DNA replication, chromosomal recombination, cell division, gene expression and metabolism, generally well conserved among yeasts and higher eukaryotes (Rose and Harrison, 1987, 1995; Sherman, 1998, 2002; Castrillo and Oliver, 2004). Several other properties make *S. cerevisiae* a particularly suitable organism for biological studies, such as its GRAS (Generally Regarded As Safe) status, the rapid growth, as well as the availability of simple methods of cultivation under controlled conditions. This yeast also represents a well defined system for which techniques of genetic manipulation have been extensively developed (Brown and Tuite, 1998; Sherman, 1998, 2002; Castrillo and Oliver, 2004). The most favorable features of this yeast is accessibility and its importance in the world economy, mainly in brewing and baking industry (Castrillo and Oliver, 2004).

A set of experiences was conducted in order to determine if yeasts – namely *S. cerevisiae* – could be used to evaluate the antimicrobial potential of *H. fasciculate* and, in such case, to define the best experimental conditions to detect and assess the fungal antagonist action. In particular, the effect of culture medium, inoculum concentration, *H. fasciculare* growth time or temperature and the temperature of the assay were studied. The aims of this work were essentially i) to reduce the time-response of the traditional methodology and ii) simultaneously evaluate the antimicrobial potential of *H. fasciculare* against other microorganisms, yeasts in particular.

2.2. Material & Methods

2.2.1. Culture and maintenance of microorganism

Saccharomyces cerevisiae used in this work belongs to the collection existent in the Biology Department in Universidade do Minho. They were transfer from the originals to new plates in YPDA medium and incubated, at their optimal growth temperature, at 30°C for 48h.

Hypholoma fasciculare was kindly provided by the Instituto Politécnico de Bragança. Axenic cultures of fungi were made on medium Melin-Norkrans (MMN) medium and Potato Dextrose Agar (PDA) medium. They were transferred to new plates and incubated, in the dark, at a temperature of 20°-23°C.

Medium YPDA: yeast extract 10 g/L; peptone 20 g/L; glucose 20 g/L; agar 20 g/L

Medium MMN: NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L

Medium PDA: 39 g/L

2.2.2. Evaluation of the anti-yeast activity of *Hypholoma fasciculare*

2.2.2.1. Culture of *Hypholoma fasciculare*

A small piece of *H. fasciculare* was inoculated in the centre of a plate containing an appropriate culture medium: MMN and PDA. In standard conditions, these plates were incubated in darkness, at room temperature for 6 days. After incubation, the plates were overlaid with the sensitive indicator strain (see section 2.2.2.2.).

The effect of temperature of growth and the effect of *H. fasciculare* growth time in fungal antimicrobial potential were determined culturing the fungus at different temperature values (20°C, 25°C, 30°C, 35°C and 40°C) or during different periods of time (2, 4, 6, 8, 10 and 12), respectively, according to the objectives of each experiment. These plates were then used to assay fungal antagonistic action against the chosen sensitive yeast indicator strain, as describe in the following section.

2.2.2.2. Detection of the anti-yeast activity of *Hypholoma fasciculare*

A suspension of *Saccharomyces cerevisiae* was prepared from an YPDA fresh culture in saline solution 0.85% (w/v) and mixed with agar 0.8% (w/v) in order to have 10^6 cells/ml (Figure 2.1 A, B, C). A volume of 8 ml of this mixture was then transferred to a plate previously inoculated with *H. fasciculare* (section 2.2.2.1) (Figure 2.1. D) and then incubated for 48h. The assay temperature was normally 25ºC unless in the experiments to evaluate the effect of temperature in fungal antagonistic action. In this case, plates were incubated at 20°C, 25°C, 30°C, 35°C and 40°C.

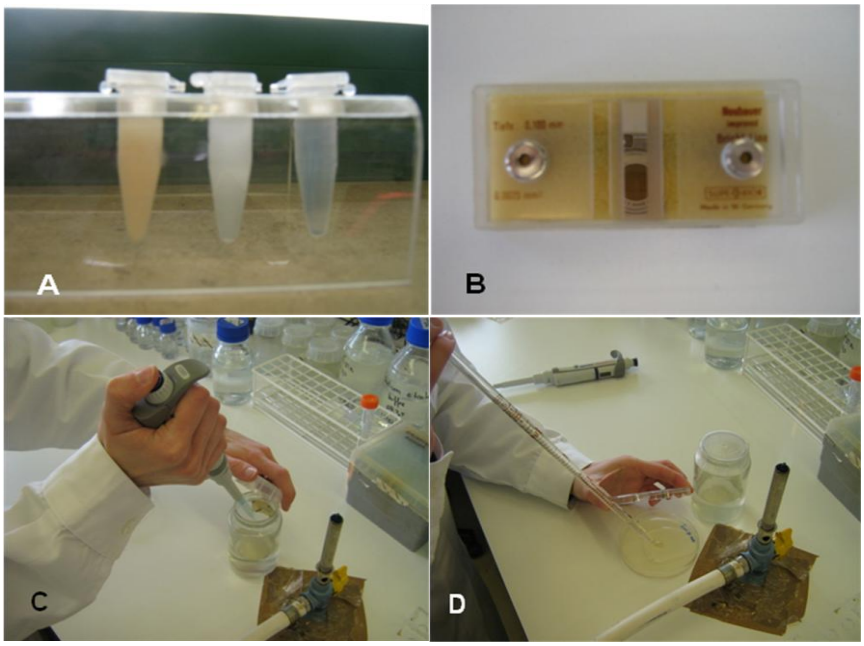


Figure 2.1 Assay to evaluate *H. fasciculare* antimicrobial activity (A) eppendorf tubes with different suspensions of *S. cerevisiae* in saline solution, (B) Neubauer chamber to count the number of cells in saline suspensions, (C) addition of cell suspension to agar 0.8%, and (D) making of the yeast overlay over *H. fasci*culare- inoculated plates.

The inoculum size used in the assay (10^6 cells/ml) was defined after a first experiment where different cell concentrations were tested (10^4 cells/ml, 10^6 cells/ml and 10^8 cells/ml)

Anti-yeast activity was evaluated after incubation and both the diameter of the fungus and the diameter formed by the limits of any inhibition zone were measured. These values were used to calculate the areas occupied by the fungus and the halo created by the fungus anti-yeast action.

2.3. Results and discussion

2.3.1. Choice of the assay medium to evaluate *Hypholoma fasciculare* antimicrobial activity

For this experiment *H. fasciculare* was cultivated in two different media - Mellin-Norkrans (MMN) and Potato Dextrose Agar (PDA) - for 6 days in the dark at room temperature. After this period, the evaluation of the fungus antimicrobial potential was performed according to the procedure above described (section 2.2.2.2). Figure 2.2 illustrates the type of results obtained.

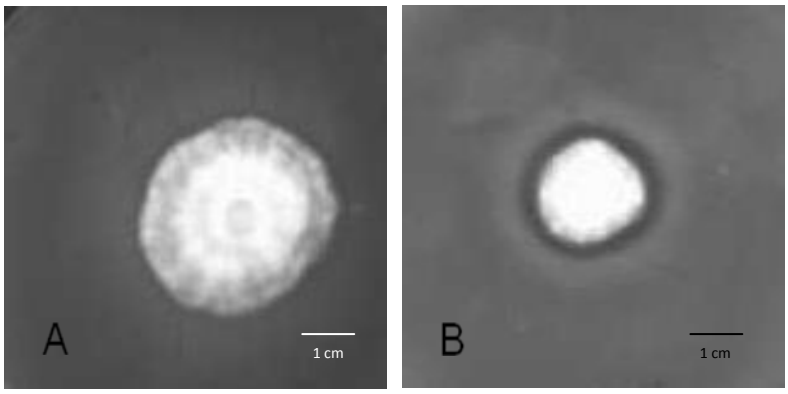


Figure 2.2. Assay for detection of the anti-yeast activity of *H. fasciculare* against *S. cerevisiae* in MMN (A) and PDA (B) media.

H. fasciculare displays better growth in MMN than in PDA. A *S. cerevisiae* growth inhibition zone or an inhibition halo was detected surrounding *H. fasciculare*, and corresponds to the fungus antagonistic action over the tested yeast. However, this antimicrobial activity is only observable in PDA, and not in MMN medium. The reasons that can lead to this result can be attributed to differences in pH as well as in the composition of each medium.

This first experience allowed to detect an antagonistic action of *H. fasciculare* against the yeast *S. cerevisiae*, never reported before. As such effect was only visible in PDA medium, the assay for antimicrobial activity was further designed using this medium.

2.3.2. Concentration of the sensitive indicator strain

To determine the inoculum size of the sensitive indicator strain to be used in the antimicrobial assay, *H. fasciculare* was cultured (section 2.3.2.1) for 6 days at 25°C and covered with an overlay of 10^4, 10^6 and 10^8 cells/mL of *S. cerevisiae*. Results were obtained after 48h incubation at 25ºC (Figure 2.3).

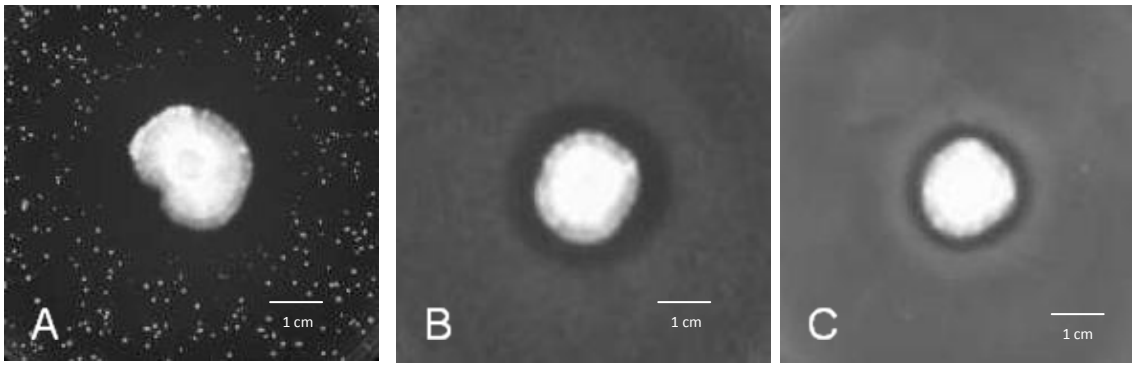


Figure 2.3. Effect of *S. cerevisiae* inoculum size on *H. fasciculare* anti-yeast activity (A) *S. cerevisiae* overlay consisting of 10^4 cell/mL. (B) 10^6 cell/mL and (C) 10^8 cell/mL. .

The *S. cerevisiae* growth inhibition zones become smaller with the increase of inoculum size, as would be expected. Therefore, the biggest halo size is seen in the assay with the smallest inoculum. However, the limit of this halo is not always clearly defined thus demanding special attention in its delimitation and measure. Therefore, the assay with an overlay with 10^6 cell/mL, although showing a smaller halo, seems to be the best experimental condition to detect and measure the *H. fasciculare* anti-yeast activity.

2.3.3. Effect of the time of *H. fasciculare* growth on antimicrobial activity

The effect of the time of growth of *H. fasciculare* on its anti-yeast activity was evaluated culturing the fungus for different periods of time - more precisely during 2, 4, 6, 8, 10 and 12 days – followed by the determination of its antagonistic potential against *S. cerevisiae*. Figure 2.4 represents the area occupied by the fungus and the area occupied by the fungus and its inhibition halo against the yeast *S. cerevisiae* for each time of growth. To better evaluate this anti-yeast activity, a ratio between both areas was also determined and represented in the same graph.

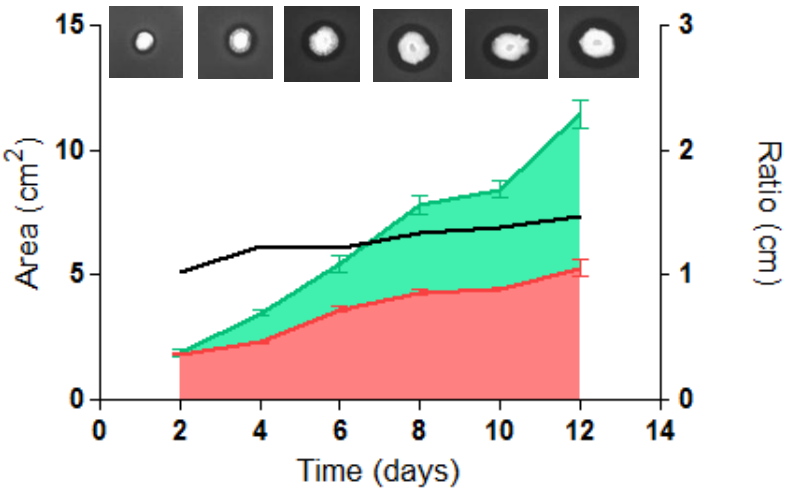


Figure 2.4. *Hypholoma fasciculare* antagonistic activity against *Saccharomyces cerevisiae* over time. The antimicrobial effect was evaluated after 2, 4, 6, 8, 10 and 12 days of fungal growth. The area occupied by the fungus is represented in red and the area occupied by the yeast growth inhibition zone in green. The line represents the ratio between both areas. From the left to the right, the images show the assay for detection of the antifungal activity of *H. fasciculare* in each experimental condition.

After 2 days of growth almost no inhibition halo was visible but the halo size increased with fungal growths. During growth, more cells are formed and probably more antagonistic substance, of a still unknown nature, is produced. The area occupied by the fungus is almost always larger than the area occupied by the respective yeast growth inhibition zones. However it seems that fungal growth is slowing and stabilizing over time whereas the halo size continues increasing, so after 12 days the area occupied by the halo was higher than the area occupied by the fungus. This experimental condition would thus be the best to see a higher antagonistic activity of *H. fasciculare* against *S. cerevisiae* but such antimicrobial activity is clearly detectable and already visible after 4 days of growth. In order to observe an even more evident phenotype, 6 days of fungal growth was the chosen time to perform the bioassay to evaluate *H. fasciculare* antimicrobial activity.

2.3.4. Effect of temperature on *H. fasciculare* antimicrobial activity

H. fasciculare was incubated for 6 days at the usual conditions - in darkness and at room temperature - and assayed for antimicrobial activity against *S. cerevisiae* at 20°C, 25°C, 30°C, 35°C and 40°C. The assay lasted 48h (Figure 2.5).

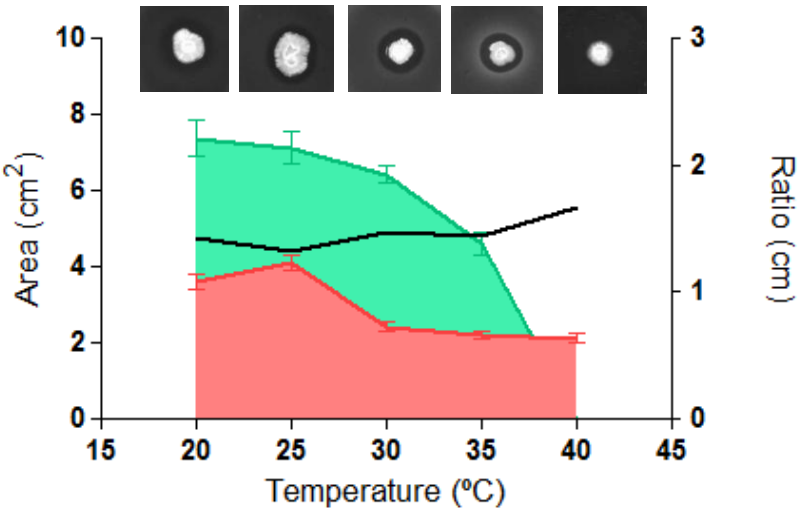


Figure 2.5. *Hypholoma fasciculare* antagonist activity against *Saccharomyces cerevisiae* when expose at different temperatures. The antimicrobial effect was evaluated at 20°C, 25°C, 30°C, 35°C and 40°C. The area occupied by the fungus is represented in red and the area occupied by the yeast growth inhibition zone in green. The line represents the ratio between both areas. From the left to the right, the images show the assay for detection of the antifungal activity of *H. fasciculare* in each experimental condition.

H. fasciculare displayed anti-yeast activity at all the temperature values tested, except at 40°C - temperature at which neither the fungus nor the yeast seemed to have grown - and the halo size decreased with the increase of temperature .The highest antimicrobial activity was observed at 20°C and 25ºC, although it was at 25°C that the fungus displayed better growth. As *S. cerevisiae* grows poorly at 20°C this can lead to an apparently higher anti-yeast activity: a bigger halo size is obtained due to a lower number of sensitive cells. Any increase in temperature above 25ºC led to a slower fungal growth.

Considering the ratio between both areas (Figure 2.4) it seems that the greatest *H. fasciculare* antimicrobial activity is displayed at 30ºC but it is also evident that fungal growth is negatively affected in this condition. In fact, when the same assay was performed in the presence of the vital dye methylene blue (Figure

2.6) the fungus acquired a blue coloration at 35ºC and was completely blue stained at 40ºC, indicating cell death.

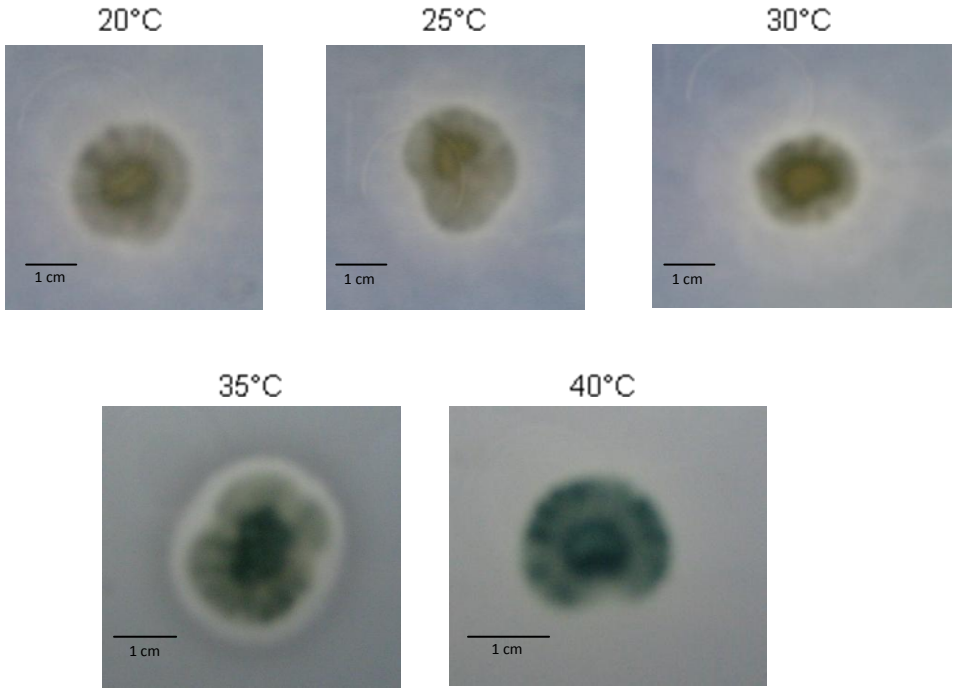


Figure 2.6. Assay to detect *H. fasciculare* anti-fungal activity against *S. cerevisiae* in the presence of the vital dye methylene blue.

Based on these results and considering that performing the assay at a temperature above 30ºC could have induced a heat stress, two new assays were performed. In one experiment, the fungus grew and was tested for antimicrobial potential at the temperature values tested in the above experiment (Figure 2.7). In another experience, *H. fasciculare* was grown at the same different temperature values during the 6 days-culturing period and then assessed for anti-yeast activity at 25°C (Figure 2.8).

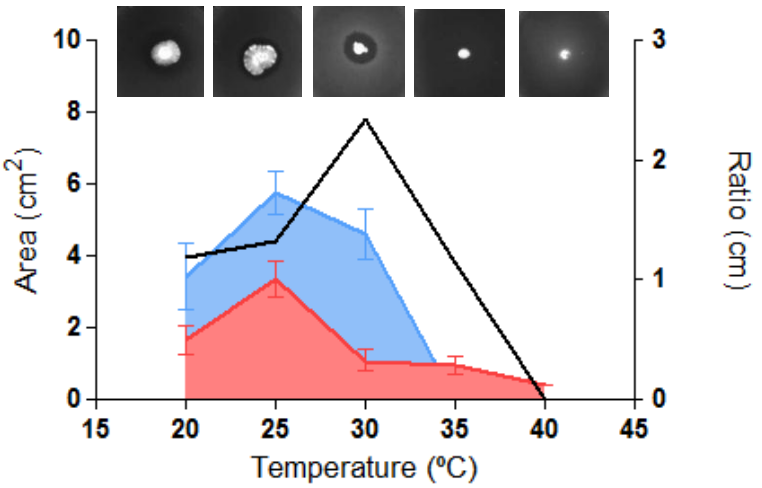


Figure 2.7. *Hypholoma fasciculare* antagonist activity against *Saccharomyces cerevisiae* when *H. fasciculare* is expose to different temperatures during its growth (20°C, 25°C, 30°C, 35°C and 40°C). The antimicrobial effect was evaluated at the same different temperatures. The area occupied by the fungus is represented in red and the area occupied by the yeast growth inhibition zone in green. The line represents the ratio between both areas. From the left to the right, the images show the assay for detection of the antifungal activity of *H. fasciculare* in each experimental condition.

Once again, 25ºC showed to be the optimal temperature for fungal growth and also to obtain the biggest total area, on the contrary to the previous assay. At 30°C the area occupied by the fungus was clearly lower but the ratio between both areas was the highest, which means a greater antimicrobial activity and hence the biggest halo. At 35°C and 40°C no inhibition halos were observed because the long exposure to these temperatures most probably has caused fungal death.

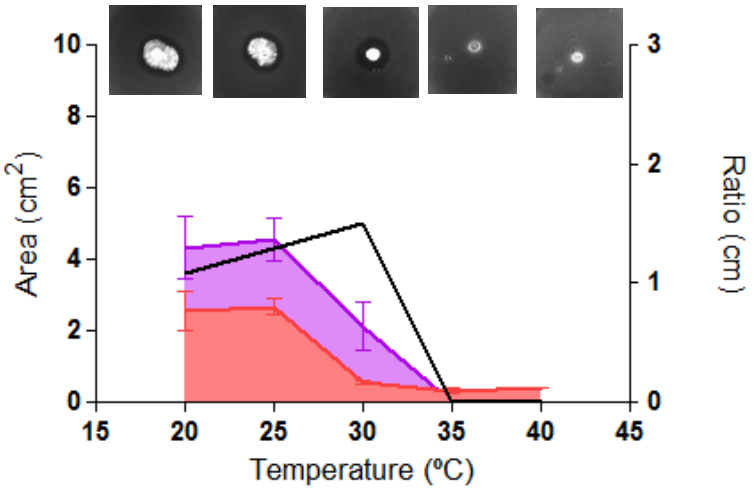


Figure 2.8. *Hypholoma fasciculare* antagonist activity against *Saccharomyces cerevisiae* when *H. fasciculare* is left to growth at is optimal temperature, 25°C. The antimicrobial effect was evaluated at 20°C, 25°C, 30°C, 35°C and 40°C. The area occupied by the fungus is represented in red and the area occupied by the yeast growth inhibition zone in green. The line represents the ratio between both areas. From the left to the right, the images show the assay for detection of the antifungal activity of *H. fasciculare* in each experimental condition.

When *H. fasciculare* was cultured at temperatures ranging from 20ºC to 40ºC followed by the evaluation of its antimicrobial potential (Figure 2.8), the same main observations were made. Thus, 20ºC - 25ºC showed to be the optimal temperature range for fungal growth, the best value being 25ºC (Figures 2.5, 2.7 and 2.8). These conditions were found in the laboratory throughout the working period, as room temperature values were confirmed to be in the same range, depending on the season of the year. The biggest total area is invariably obtained at 25ºC which is an optimal growth temperature as already referred, although the biggest halos were registered when the fungus was cultured and/or the assay was held at 30ºC. As this condition represents a stress to *H. fasciculare* growth – which however seems to favor the production of the antagonistic substance – the assay was decided to be conducted culturing the fungus (for 6 days) and evaluate its antagonistic potential at the same temperature: 25ºC. According to the other results here obtained, the bioassay for antimicrobial activity should be performed using PDA medium and an inoculum of 10^6 cell/ml of the sensitive indicator strain.

Chapter 3

Assessment of the antimicrobial activity of *Hypholoma fasciculare*

3.1. Introduction

In a biological community, the interaction between the various organisms that compose it is a constant phenomenon and has consequences in terms of its organization. These interactions can be permanent or temporary and are classified as positive, when there is mutual benefit for both living organisms, or just to one of them but without prejudice to the other; negative, if there is injury to both or to one of the interacting organisms in favor of the other and, finally, neutralism which is a type of interaction where none of the interacting species is affected. These forms of interaction can occur not only between individuals of different species but also between individuals of the same species (Baptista, 2007). The following table summarizes the main types of interaction in a biological community.

Table 3.1- Types of interaction between two species and their characteristics (adapted from Baptista, 2007)

Types of interaction	Species		Characteristics of the interaction
	A	B	
Neutralism	0	0	No species is affect by the other
Negative interactions			
Competition	-	-	Both species are negatively affected, because their competition leads to the exhaustion of a particular resource
Amensalism (antibiosis and allelopathy)	0	-	Secretion of substances by one of the species that are harmful to the other
Parasitism	+	-	One of the species (parasite) requires the presence of another species (host) to which is harmful
Predation	+	-	One of the species (predator) consumes other species (prey)
Positive interactions			
Commensalism	+	0	One of the species (commensal) benefits from the presence of other species, which is not affected
Protocooperation	+	+	Both species favor each other, but they survive without the presence of the other
Mutualism	+	+	Both species favor each other and require the presence of the other

0 Species not affected by the presence of the other
+ Species benefits with other or requires its presence
- Species hampered by the presence of the other

3.1.1. Types of interactions between saprophytic fungi

All the types of interaction described in table 3.1 can occur between saprophytic fungi; however competition is the most common type in this trophic group (Boddy, 2000). There is no single definition of competition but different definitions. The generally accepted one was proposed by Keddy (1989), who said that there is competition between two organisms when one of them carries a negative effect on the other, either consuming or controlling access to a resource whose availability is limited. Such interaction promotes a selective process that culminates usually with the preservation of the life forms best adapted to the environment, and the extinction of individuals with low adaptive power, thus constituting a key regulator of population density. We can define two types of competition: by interference (or direct) when an organism excludes the other directly on the availability of a resource usually through aggression (physical or chemical); and by exploitation (or indirect) if an organism uses a resource decreasing its availability or even making it unavailable for other organisms (Keddy, 1989). In saprophytic fungi, the competition may occur in two distinct phases, according to the state of colonization of the resource. In the first stage competition occurs by the obtention of resources not colonized by other fungal species. The success of this phase depends on several factors such as the effective dispersal and rapid germination of spores and the mycelium growth rate and ability to utilize the organic compounds available in the non-colonized substrate (Boddy, 2000). In the second phase there is a competition for resources already colonized by other fungal species. The achievement, maintenance and defense of the resource at this stage is done through the antagonistic mechanisms developed by the fungi. These mechanisms can operate at distance (distance antagonism) or after contact, resulting in reactions such as “hyphal interference” and parasitism (mycoparasitism). The result of contact establishment between opponent fungi can be the total or partial replacement of the mycelium of one of the fungal species, with the attacker gaining territory to the opponent fungi. Alternatively, the attacker fungus can grow on or through the mycelium of the opponent and there is no occupation of the territory occupied by the opponent fungi (Boddy, 2000).

In the **antagonism at a distance** the inhibition of fungal growth occurs in the absence of opposition, or prior to the establishment of physical contact between interacting fungal hyphae. Such antagonism is usually attributed to the production of volatile and/or diffusible compounds, such as antibiotics. The consequences vary

according to the combination of fungal species, the most common corresponding to the degeneration or replacement of the hyphae by the mycelium of the interacting species (Boddy, 2000). In contrast **hyphal interference** mechanism occurs when a hypha establishes contact with hypha or spores of other species which could result in the death of the interacting species with a series of scheduled events of cytoplasmic destruction. This type of antagonism is one of the most common found in saprophytic basidiomycete fungi. This mechanism is not fully understood, but is reported to be mediated by the production of diffusible metabolites, secreted only when the interacting hyphae make contact (Boddy, 2000).

The **mycoparasitism** happens when a fungus act as a parasite to the other. According to the general definition of parasite, a parasitic fungus obtains nutrients from other fungus (host) who lives in close association. Obtaining nutrients may occur after the death of host cells (necrotrophic parasite) or from its living cells (biotrophic parasite). Although not always existing in all parasitic – host interactions, the mycoparasitism process involves the following steps: perception of the host; growth of the parasite towards the host, contact and recognition of the host, and adhesion and penetration of the fungus parasite into the host (Whipps, 2001). After recognition, the parasitic hyphae adhere and roll gradually on the surface of the host hyphae eventually penetrating and growing inside them. In some cases it is possible to observe hyphal host bottleneck due to strong hyphal parasitic winding (Boddy, 2000). Penetration of parasitic hyphae into host cells is normally mediated by production of several extracellular enzymes. The mycoparasitism may also be associated with the production of antibiotics, toxins in quantities that cause the death or destruction of the host. The antagonistic activity of mycoparasite may also be associated with the production of antibiotics and toxins, in quantities that cause the death or destruction of their host (Inglis & Kawchuk, 2002; Bara *et al.*, 2003; Aggarwal *et al.*, 2004).

Mycelia contact is another mechanism of antagonism that has a great relevance to saprophytic fungi. It was named "mycelial interference" because of its similarity with the mechanism of “hyphal interference" (Dowson *et al.*, 1988c). It occurs after establishment of contact mycelia of two fungal species, resulting in morphological changes in the interacting fungi. In general, these morphological changes have the function to increase resistance to the penetration of mycelium invader fungus or enable the mycelial growth over or through the opponent fungal colony (Dowson *et al.*, 1988c). These morphological changes may specifically include the formation of dense mycelium which forms a "barrier" that prevents the

invasion of the interacting fungus; the formation an “invasive” mycelium by the creation of various growth fronts; the formation of a mycelium with aerial growth; or the formation of mycelial strands or rhizomorphs structures (Dowson *et al.*, 1988c; Boddy, 2000; Donnelly & Boddy, 2001; Wald *et al.*, 2004; Woods *et al.*, 2005). These changes are usually accompanied by a redistribution of the mycelium, which appears less dense in distal areas of the zone of interaction. This makes the colony more susceptible to the attacker fungus in the event of fragmentation of the zone of interaction (Boddy, 2000). In some interactions with fungi that form mycelial cords, discoloration can occur and induce a lytic response in one or both segments of mycelial cords.

3.1.2. Factors affecting the interaction between fungi

The effect of an interaction between fungi depends on various factors such as abiotic factors, which includes the temperature, the substrate, pH, water potential, atmospheric pressure and CO_2 levels; the physiological/nutritional state of interacting fungi species; the presence of a third species of fungi; and, most importantly, the intrinsic nature of the species, like its combativeness/aggressiveness (Boddy, 2000; Wald *et al.*, 2004; Cano & Bago, 2005).

Several studies have been undertaken in order to evaluate the effect of abiotic factors in establishing interactions between fungi. These abiotic factors may constrain the growth of interacting fungi in favor of another fungus. It was also demonstrated that most of the saprophytic species are greatly affected by water potential and even stop growing when the water potential reaches critical levels (Boddy, 2000; Wald *et al.*, 2004). The temperature and pH of the substrate also influence the growth rate of saprophytic fungi with consequences in terms of the effect on interaction (Schoeman *et al*, 2000; Wald *et al.*, 2004).

The availability of nutrients for both fungal species influences the interaction established by directly determining the nutritional status of interacting fungi (Holmer & Stenlid, 1997; Boddy, 2000; Wells & Boddy, 2002; Wald *et al.*, 2004). In general, fungi that occupy a larger volume of substrate have a greater combat capability compared to fungi that occupy lower volumes of substrate (Zakaria & Boddy, 2002; Wald *et al.*, 2004). However, most studies about the interaction between saprophytic fungal species were not performed using substrate but rather in axenic conditions, where the fungal growth occurs in culture medium. Although the results

provide valuable information, their direct extrapolation to the natural field conditions must be done carefully and with some reservation, since the results do not always coincide with that obtained in natural substrates (Dowson *et al.*, 1988c).

The combativeness/aggressiveness of both interacting species is what mostly determines the type of interaction established. There are some fungi that are unfit in attack or in defense of a resource, while others do it in a very effective way; others are effective in the attack but disqualified on defense or in contrary, they have bad features for the attack but can be excellent in defense of the resource holding. This multiplicity of responses allowed the establishment of a ranking of species in terms of its combativeness (Boddy, 2000). This hierarchy took into account the effect of combativeness against other fungi and also the response level of aggressiveness.

Interaction between saprophytic fungi may cause changes in the metabolic activity of interacting mycelia, which result mainly, from the defensive/combativeness ability against the opponent fungus (Freitag & Morrell, 1992). Evidence suggests that competition between two saprophytic fungi is associated with energy costs related to CO_2 changes during antagonistic interactions. In terms of functioning effects on the growth pattern and distribution of the mycelia in the translocation of nutrients or in the rates of CO_2 release could be observe (Boddy, 2000). Thus, competition among saprophytic fungi is an important regulator of population density.

The high aggressiveness displayed by saprophytic fungi suggests their potential use as biological control agent. Species like *Hypholoma fasciculare, Hypholoma australe*, *Phanerochaete filamentosa*, *Phanerochaete velutina*, *Coriolus versicolor*, *Stereum hirsutum*, *Ganoderma lucidum*, *Schizophyllum commune* and *Xylaria hypoxylon* have been describe for the biological control of *Armillaria spp* (Chapman *et al.,* 2004). This species is a root pathogen that attacks several forest species, fruit trees and some herbaceous plants in temperate and tropical regions (Pearce & Malajczuk, 1990; Pearce *et al.*, 1995; Chapman *et al.*, 2004; Cox & Scherm, 2006). A success, field tested, is the use of *Hypholoma fasciculare* against *Armillaria ostoyae* (Chapman, 2004). In this study, during a period of 3 to 5 years, a significant reduction in the mortality rate was observed for those plants exposed to *H. fasciculare*. Similar results were obtained with other fungal species, including *Hypholoma australe* and *Phanerochaete filamentosa* which were inoculated with a chemical treatment, reducing significantly the colonization of plants by the pathogenic fungus *Armillaria luteobubalina* (Pearce et al., 1995).

3.1.3. Interactions between filamentous fungi and yeast and between filamentous fungi and bacteria

Some reports refer the interaction between filamentous fungi and bacteria. Fermor and Wood (1981) describe a number of fungi that could degrade the cell wall of *B. subtilis*. Barron & Thorn (1987) reported that *Pleurotus* hyphae cause the lise of bacterial cells. Other more filamentous fungi that act in a similar way were then describing (Barron, 1988). Since the finding of this type of antagonism between microorganisms, several studies were conducted in order to discover/develop other antibiotics or other substances with antimicrobial activity. To our knowledge there are no relevant studies concerning interactions between filamentous fungi and yeast, both belonging to the fungi group. The literature available mainly refers to the action of filamentous fungi against filamentous fungi or to the interaction between different yeasts.

In *Castanea sativa* orchards present in Bragança, *Hypholoma fasciculare* carpophores are common. This fungus belongs to the trophic group of saprophytic fungi and has displays antagonistic activity against *Phytophthora spp*. (Baptista, 2007) and *Armillaria mellea* (Chapman & Xiao, 2000) two of the major chestnut tree pathogens. The antagonistic action of *H. fasciculare* against other fungi (mycorrhizal and phytopathogenic) needs to be evaluated to determine the interest of this fungus as a possible biocontrol agent. In this study the main goal was to assess the potential antagonistic activity of *H. fasciculare* on fungi present in chestnut orchards.

In chapter 2 an assay for the detection of antimicrobial activity of *H. fasciculare* cultures against *S. cerevisiae* was optimized. Given this anti-yeast activity other yeast were tested in this chapter. The assays were perform against different pathogenic species of the genus *Candida*. In the past decades infections caused by *Candida spp*. have been increasing. These infections are caused not only by the most common pathogen, *Candida albicans* but also by other *Candida* species. This generates some concerns since *Candida glabrata*, *Candida krusei* and *Candida guilliermondi* are highly resistant to antifungics. As certain filamentous fungi are known to have an antagonistic effect against bacteria, the *H. fasciculare* antibacterial action was also evaluated. The bacteria chosen were *Bacillus subtilis*, an example of a Gram-positive bacteria and *Escherichia coli*, an example of a Gram-negative one.

3.2. Material

3.2.1. Biological material

The fungi, *Hypholoma fasciculare, Alternaria arborecens, Beauveria bassiana, Cordyceps sinensis, Epicocum nigrum, Fusarium chlamydosporum, Fusarium oxysporum, Gibberella moniliformes and Mucor circinelloides,* used in this work, was kindly provided by the Instituto Politécnico de Bragança. The yeast and bacteria used in this work belong to the existing collections of yeast and bacteria at the University of Minho.

3.3. Methods

3.3.1. Culture and maintenance of filamentous fungi

Axenic cultures of fungi were made on medium Melin-Norkrans (MMN) medium and Potato Dextrose Agar (PDA) medium. The filamentous fungi were transferred to new plates and incubated, in the dark, at a temperature of 20°-23°C. Sub-cultures of fungi were carried out in intervals of 3 weeks.

Medium MMN: NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L

Medium PDA: 39 g/L

3.3.2. Co-culture assays for testing *Hypholoma fasciculare* antagonist activity against other filamentous fungi

3.3.2.1. Establishment of co-cultures

The potential antagonism of the saprophytic fungi *H. fasciculare* against several fungi was assessed in co-culture. Co-cultures were established using petri dishes (9 cm diameter) containing 10 ml of MMN medium, pH 6.6 or containing PDA medium. Inocula (with 0.7 cm diameter) of *H. fasciculare* and other fungi were collected from the peripheralic regions of active growing cultures and placed at a distance of 3 cm from each other, onto MMN or PDA medium. Incubation of cultures was carried out in the dark at room temperature. Unless otherwise stated, 3 different experiments were performed in which 6 replicas were used.

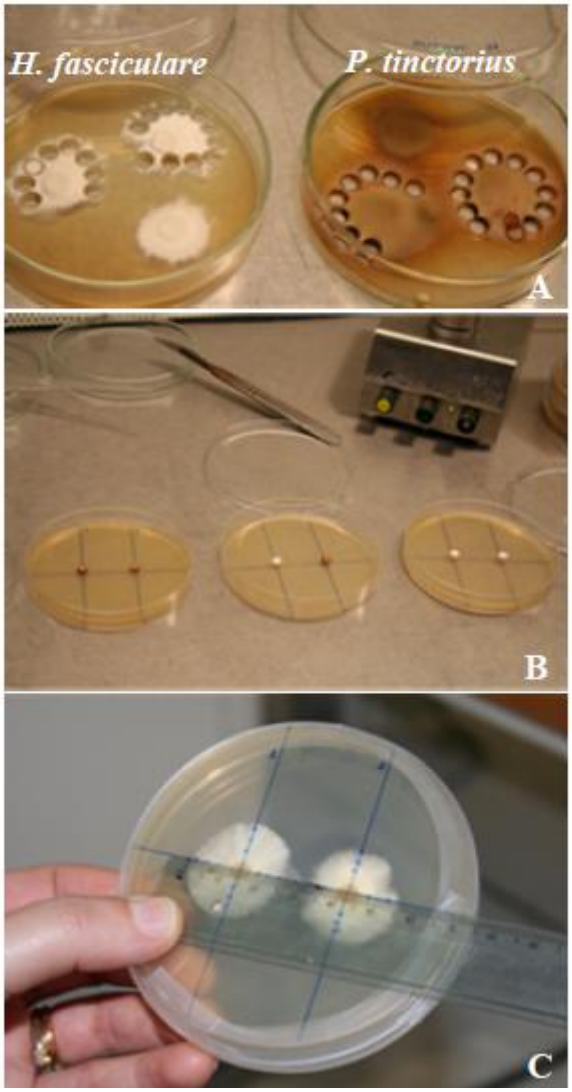


Figure 3.1. Establishment of co-culture assays. Sections of agar with 0.7 cm of diameter were removed from the actively growing peripheric regions of *H. fasciculare* cultures and from the cultures of the fungus to be tested (A) The inocula were transferred to MMN or PDA media and placed at distance of 3 cm between them (B) Three fungal combinations were made for each medium: *H. fasciculare*-interacting fungus; *H. fasciculare-H. fasciculare* and interacting fungus-interacting fungus. The radial growth of fungal colonies was measured (C) at intervals of 2 days, during 20 days after inoculation (Baptista, 2007).

3.3.2.2. Parameters assessed in co-cultures

The radial growth of fungal colonies was measured by determination of internal and external radius (Figure 3.2), during 20 days and results were expressed in mm/day. The morphological characterization of fungal colonies was also made, noting the changes in shape and color of the colony and color changes in the culture medium.

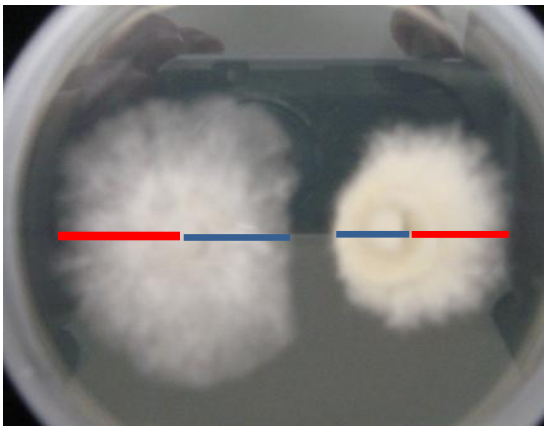


Figure 3.2. Determination of the radial growth of fungal colonies. The internal radius was measured in inter-seeding in the region (blue) and the external radiius in diametrical opposed regions (red).

3.3.3. Culture and maintenance of microorganisms

Yeasts were cultured in yeast extract peptone dextrose agar medium (YPDA) and incubated a 30°C for 48h. Sub-cultures were carried out every week. The yeast tested in this work were *Candida albicans* (IGC 3436T), *Candida dublinienesis* (CIPO 70), *Candida glabrata* (IGC 2418T), *Candida guilliermondii* (26D), *Candida krusei* (IGC 3341T), *Candida tropicalis* (IGC 3097T), *Candida parapsilosis* (28B) and *Saccharomyces cerevisiae* (IGC 4455NT). Bacteria, cultures were made in solidified Luria-Bertani medium (LB), and incubated for 24h at 37°C. Sub-cultures were carried out weekly. The bacteria tested in this work were *Bacillus subtilis* (48886) and *Escherichia coli* (CECT 423).

Medium YPDA: yeast extract 10 g/L; peptone 20 g/L; glucose 20 g/L; agar 20 g/L

Medium LB: yeast extract 5 g/L; triptone 10g/L; NaCl 10 g/L; agar 15g/L

3.3.4. Evaluation of the anti-yeast activity of *Hypholoma fasciculare*

The assay to evaluate the anti-*Candida* activity of *H. fasciculare* was performed by a modified agar diffusion assay, basically as described in Chapter 2. A small piece of the fungus with 0.7 cm diameter (inocula) was collected from the peripheral regions of an active growing culture, placed at the center of PDA plates and cultured in darkness at room temperature. After 3, 8 and 14 days of growth, these plates were overlaid with a suspension containing 10^6 cells/mL of the yeast to be tested, prepared in agar 0.8% (w/v). Anti-yeast activity was evaluated after 48h incubation at 25ºC and both the diameter of the fungus, and the diameter of the inhibition zone plus the diameter of the fungus were measured. These values were used to calculate the area occupied by the fungus and the area occupied by the inhibition halo created by the fungus action.

3.3.5. Evaluation of the anti-bacteria activity of *Hypholoma fasciculare*

The assay to evaluate the anti-bacterial activity of *H. fasciculare* was performed as essentially described in the previous chapter to evaluate the fungal anti-yeast activity, with some modifications. A small piece of the fungus (inocula) was placed in the middle of PDA plates which were incubated in darkness at room temperature. After 6 days of growth these plates were overlaid with a bacterial suspension containing 10^6 cells/mL, prepared in agar 0.8% (w/v). Anti-bacterial activity was evaluated after 48h incubation at 25ºC, 30°C and 35°C. The diameter of the inhibition zone and the diameter of the fungus were measured and both areas were determined.

3.4. Results and discussion

3.4.1. Interaction between *Hypholoma fasciculare* and filamentous fungi

The interaction between *H. fasciculare* and other filamentous fungi was evaluated by co-culture assays, in MMN or PDA media. As controls, co-cultures were performed using the same fungi, either a co-culture of *H. fasciculare* - *H. fasciculare* or interacting fungus – interacting fungus. Mycelial growth of each fungus was followed by the radial growth of fungal colonies, up to 20 days of culture (Figure 3.3 and appendixes). The morphological aspect of interaction was also followed during the same period (Figures 3.4, 3.5 and appendixes).

The assays were performed in Petri dishes of 9 cm diameter. Each fungal inoculum was placed 3 cm apart from each other.

An initial understanding about the behavior of *H. fasciculare* toward the interacting fungus could be perceived by the analysis of the growth curves of internal radius during the assays and their comparison with the corresponding controls (external radius and control plates). However, in this analysis is important to keep in mind that the fungus is growing in a restricted space. Between inocula, both fungi only have a shared space of 3 cm to growth, limiting not only their growth but also compromising the full interaction between fungi.

The effect of *H. fasciculare* over the interacting fungi is determined by comparison of interacting fungus growth in the presence of *H. fasciculare* and in the control (without *H. fasciculare*). In the same way, the effect of the interacting fungus on the *H. fasciculare* is determined by comparison of *H. fasciculare* growth in the presence of the interacting fungus and in the control (*H. fasciculare-H. fasciculare*).

As an example, the growth curves obtained during the interaction between *H. fasciculare* and *A. arborescens* will be fully discussed (Figure 3.3). The remaining results will be only displayed in the annexes and discussed all together.

Alternaria arborecens

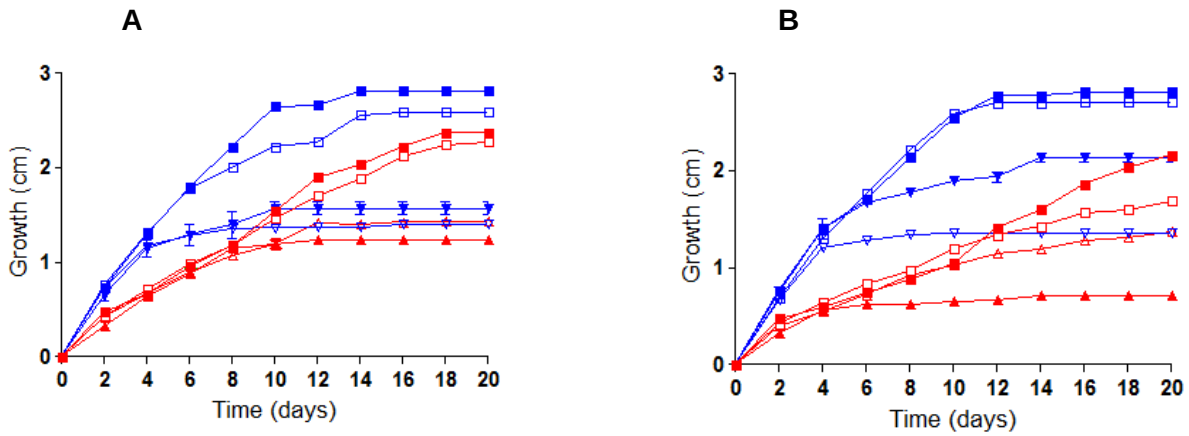


Figure 3.3. Variation of radial growth of *H. fasciculare* and interacting fungi during the co-culture assay in MMN (A) or PDA (B) media. After co-culture establishment, radial growth was measured for *H. fasciculare* (▲,△,■,□) and interacting fungus (▼,▽,■,□), every two days, during 20 days. The internal (triangles) and external (squares) radial growths correspond to the distance from the center of the inoculum to the outside edge of the fungal colony between both inocula or in the opposing region, respectively. The results obtained in the co-culture assay of *H. fasciculare* – *A. arborescens* are represented in open symbols, whereas the corresponding controls are displayed in full symbols. All the assays comprised six replicates, from which mean and SEM were derived.

A. arborecens in MMN

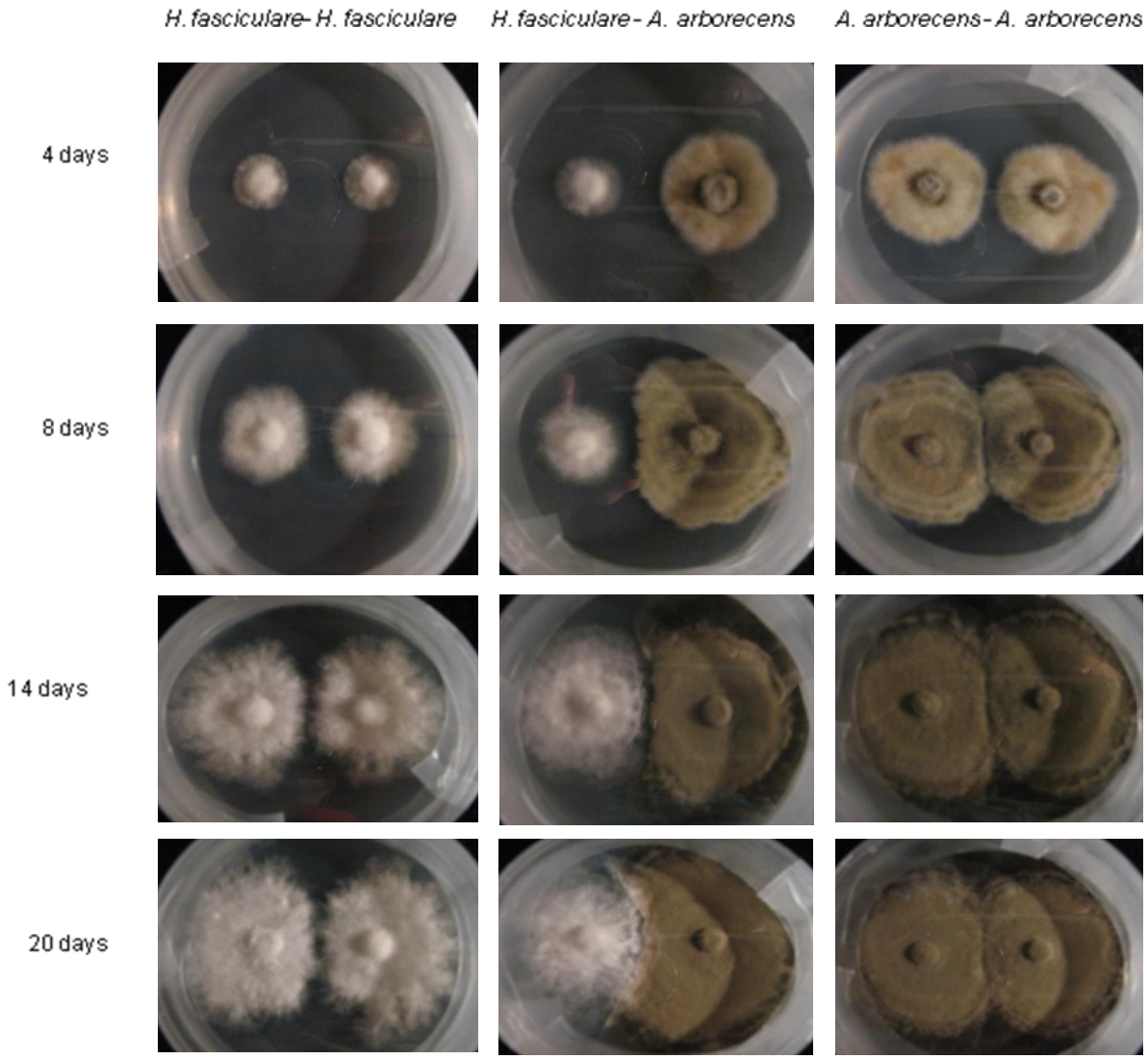


Figure 3.4. Morphological aspect of co-cultures established between *H. fasciculare-H. fasciculare* (Hf-Hf) in PDA medium; *H. fasciculare – A. arborescens* (Hf-Aa); *A. arborescens – A. arborescens* (Aa-Aa), during 20 days after inoculation.

A. arborecens in PDA

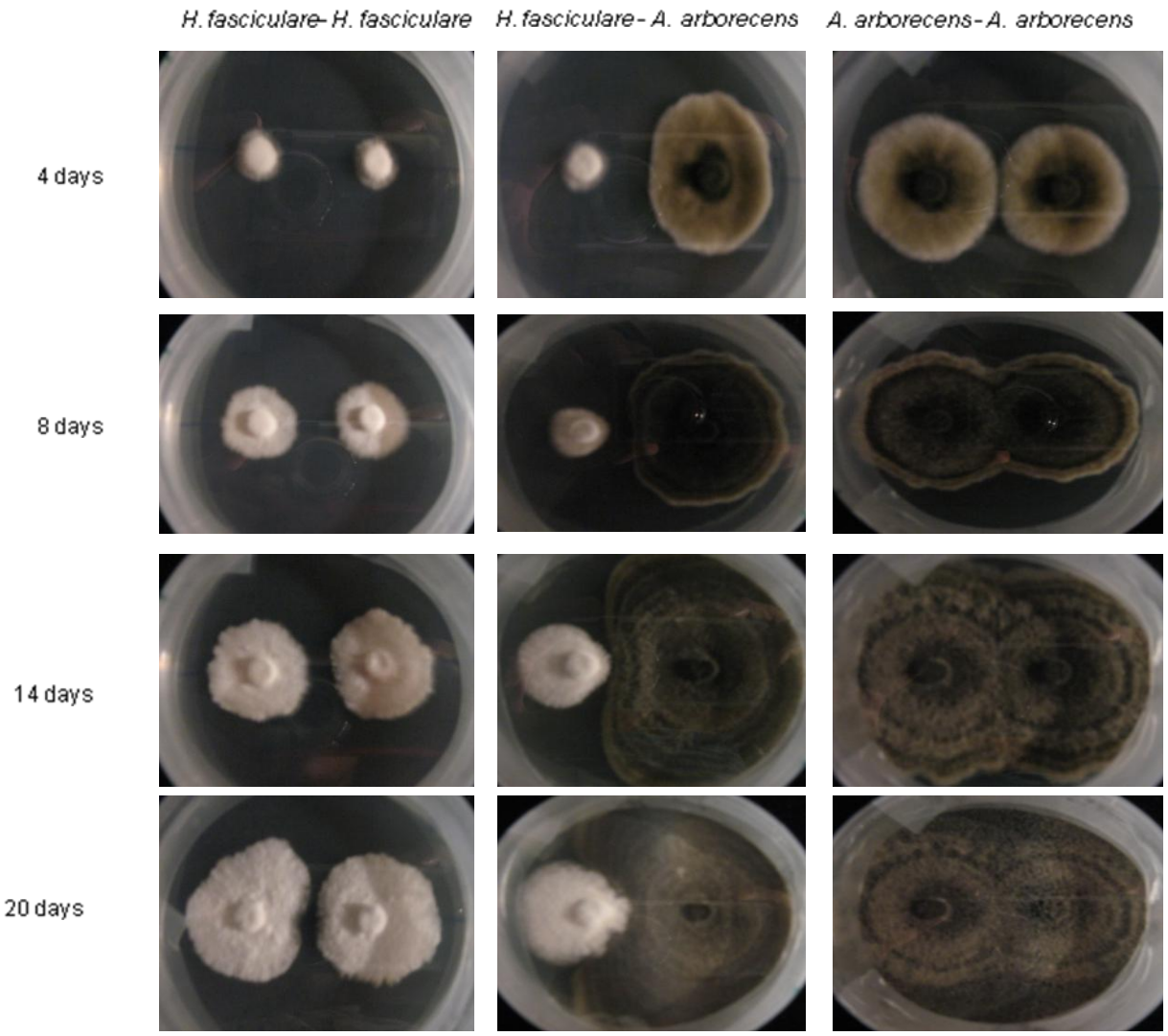


Figure 3.5. Morphological aspect of co-cultures established between *H. fasciculare-H. fasciculare* (Hf-Hf) in PDA medium; *H. fasciculare – A. arborescens* (Hf-Aa); *A. arborescens – A. arborescens* (Aa-Aa), during 20 days after inoculation.

Considering the *H. fasciculare* and *A. arborescens* internal growth curves (triangles, Figure 3.3), the growth of *H. fasciculare* seems to stabilize by the 10th day of co-culture, whereas the growth of *A. arborescens* stabilizes by the 4th day. As *A. arborecens* (▼,▽) as a stepper growth curve compared with *H. fasciculare* (▲,△), the space available for both fungi to grow is soon occupied by *A. arborescens*. These results indicate that this co-culture assay could be seriously compromised when testing fungi with very different growth rates. Differences on fungal growth rates will be also evident when analyzing the external growth curves (squares, Figure 3.3). The number of days that *A. arborescens* takes to reach the board of Petri dish (2.5-2.7 cm) are fewer when compared to *H. fasciculare.*

In MMN medium (Figure 3.3 A), the *H. fasciculare* internal growth of the control (△) is slightly higher than of the assay (▲). In contrast, the *A. arborescens* internal growth of the assay (▼) is higher than in the control (▽). Differences in the internal growth rates could indicate the presence of growth inhibitory or promoting substances that makes the interacting fungi to grow less or faster. Therefore, the results suggest that both fungi are affected by the presence of the other. However, these results should be interpreted with caution, since the differences could only reflect the higher growth rate of *A. arborescens* when compared to *H. fasciculare.*

In MMN medium (Figure 3.3 A), the *A. arborescens* external growth in the assay (■) is slightly higher than in the control (□). However, no differences were detected between the external growth rate of *H. fasciculare* in the control (□) and in the assay (■). The results suggest that *H. fasciculare* is not affected by the presence of *A. arborescens*, but this fungus could be positively affected by *H. fasciculare* compounds. Promoting substances to *A. arborescens* growth could be produced by *H. fasciculare*, leading to an increase of *A. arborescens* external growth in the assay. Although these substances could be either diffusible in the media or volatile, as they are affecting the external growth and do not have a major effect in the internal growth, they most probably are volatile substances. This analysis can only be made up to 14 days of growth, since from that day the fungus reaches the limit of the plate and their growth is restricted by the lack of space.

In PDA medium (Figure 3.3 B), the growth rate differences for both fungi are retained. Indeed, *A. arborecens* (▼,▽) also has a much higher growth rate compared to *H. fasciculare* (▲,△). The internal growth of *A. arborecens* in the control (▽) is interrupted by the 4th day, in contrast to the growth in the assay (▼) that continues up to the 14th day. This result seems to be mainly due to the more available space when *A. arborescens* is growing in the presence of the slow

growing fungus *H. fasciculare.* In contrast, the growth of *H. fasciculare* is negatively affected by the presence of *A. arborescens* (▲), which could be also due to the restriction of growth space. However, for both fungi other phenomenon seems to be contributing for the differences in the internal growth rates. By the 6th up to 12th days of interaction both fungi still have space to growth but they are growing slower. This result is evident from the morphological aspect of the co-cultures (Figure 3.5). The production of inhibitory substances by both fungi could be reducing the growth of the interacting fungus.

In PDA medium (Figure 3.3B), the *H. fasciculare* external growth in the control (□) is much lower than in the assay (■). This difference could be attributed to a growth promoting compound produced by *A. arborescens* that could be either volatile or diffusible in the medium. However, as this effect was not detected when considering the internal growth curves, the result could be also attributed to the irregular fungal colony growth. When comparing the external growth of *A. arborecens* in control (□) and on the assay (■) no differences are detected.

Altogether the results indicate that the interaction process between *H. fasciculare* and *A. arborescens* is strongly dependent on the growing media. In MMN medium, *H. fasciculare* seems to produce a substance that could promote *A. arborescens* growth. In PDA, this effect was not detected, but the production of inhibitory substances from both fungi is suggested. Different media could contribute for a differential production of growth inhibitory/promoting substances, leading to distinct antagonist reactions. In addition, different media lead to distinct fungal growth rates, which could interfere with this co-culture assay.

Following the same analysis for all the fungal interactions tested (growth curves and morphological aspects in appendixes) the results were all summarized in table 3.2.

Table 3.2. Effect of interacting fungi on the growth of *Hypholoma fasciculare* and effect of this fungus on the growth of the interacting fungi. This analysis was performed in MMN or PDA media. The symbols ↑ (or ↓) represents a promotion (or reduction) of growth in the presence of interacting fungus compared to control. The symbol = represents no effect.

MMN	Radial	*Hf-Aa*	*Hf-Bb*	*Hf-Cs*	*Hf-En*	*Hf-Gm*	*Hf-Fc*	*Hf-Fo*	*Hf-Mc*
Hypholoma fasciculare	External growth	↑	=	↑	=	↓	↓	=	↓
	Internal growth	↓	=	↓	↓	↓	↓	↓	↓
Interacting fungus	External growth	↑	=	=	=	=	↓	↓	↑
	Internal growth	↑	↓	↑	↑	↑	↑	↓	↑
PDA									
Hypholoma fasciculare	External growth	↑	=	↑	↑	↓	↓	=	↓
	Internal growth	↓	=	↑	↓	↓	↓	↓	↓
Interacting fungus	External growth	=	=	↑	=	↑	↑	=	↑
	Internal growth	↑	↑	↑	↑	↑	↑	↑	↑

Note: *Hf - Hypholoma fasciculare; Aa – Alternaria arborescens; Bb – Beauveria bassiana; Cs – Cordyceps sinensis; En – Epicocum nigrum; Gm – Gibberella moniliformis; Fc – Fusarium chlamydosporum; Fo – Fusarium oxysporum; Mc – Mucor circinelloides*

In MMN medium, in interactions between *H. fasciculare* and *C. sinensis, E. nigrum, G. moniliformis, F. chlamydosporum, F. oxysporum* and *M. circinelloides*, in internal growth the results suggest that both fungi are affected by the presence of the other. In external growth the results obtain to *C. sinensis*, suggest that *H. fasciculare* is not affected by the presence of this fungi, but the fungi could be positively affected by *H. fasciculare* compounds. Promoting substances to the fungi growth could be produced by *H. fasciculare*, leading to an increase of fungi external growth in the assay. To the interactions between *G. moniliformis*, *F. chlamydosporum* and *M. circinelloides* the results suggested reduction substances to the fungi growth could be produced by *H. fasciculare*, leading to an reduction of fungi external growth in the assay. In the case of *B. bassiana*, *E. nigrum* and *F. oxysporum*, the external growth to both fungi is similar in assays and in control to the external growth of *H. fasciculare*. Suggesting that, probably, there's no

interaction between the fungi. In contrast *B. bassiana* internal growth also suggests that *B. bassiana* is affected by the presence *H. fasciculare*. Reduction substances to *B. bassiana* growth could be produced by *H. fasciculare*, leading to an reduction of *B. bassiana* external growth in the assay.

In PDA medium in the interactions between *H. fasciculare* and *B. bassiana*, *C. sinensis, E. nigrum, G. moniliformis, F. chlamydosporum, F. oxysporum* and *M. circinelloides* in internal growth suggest that *H. fasciculare* is negatively affected by the presence of these fungi. At the external growth the results that *E. nigrum* and *G. moniliformis*, seems to be affected by *H. fasciculare*. This difference could be attributed to a growth promoting compound produced by *the* fungi that could be either volatile or diffusible in the medium. *G. moniliformis, F. chlamydosporum* and *M. circinelloides* seems to affect *H. fasciculare*. This result coul be attributed to a inhibiting compound produced by the fungi hat could be either volatile or diffusible in the medium. In the case of *F. oxysporum* the external growth to both fungi is similar in assays and in control to the external growth of *H. fasciculare*. Suggesting that, probably, there's no interaction between the fungi.

However these results (MMN and PDA) should be interpreted with caution, since the differences could only reflect the higher or lower growth rate of fungi when compared to *H. fasciculare.*

As the co-culture assay could be seriously compromised when testing fungi with very different growth rates, another approach was used to evaluate the interference of one fungus in the growth of another. Growth rates determined by external radius were compared in the absence (control) or in the presence of interfering fungi (table 3.3).

Table 3.3 Fungal growth rates determined from the external radius obtained in the assay on MMN (left) and PDA (right) for the interaction between *H. fasciculare* and each of the interacting fungus. The days correspond to the period of time from which growth rates were determined. The values in brackets correspond to the growth rates obtained in the corresponding controls.

	MMN medium			PDA medium		
	Growth rate (cm/day)			Growth rate (cm/day)		
	H. fasciculare (0.17)	Interacting fungi	Day	*H. fasciculare* (0.11)	Interacting fungi	Day
Hf-Aa	0,20	0,40 (0.30)	6	0,10	0,30 (0.40)	6
Hf-Bb	0.16	0.16 (0.16)	12	0,05	0,17 (0.16)	10
Hf-Cs	0.13	0.18 (0.19)	10	0,06	0,19 (0.18)	10
Hf- En	0.15	0.30 (0.30)	8	0,08	0,22 (0.34)	8
Hf-Gm	0.10	0.30 (0.30)	6	0,10	0,30 (0.30)	6
Hf- Fc	0.20	0.40 (0.30)	6	0,20	0,40 (0.30)	6
Hf-Fo	0.20	0.10 (0.20)	6	0,20	0,10 (0.20)	6
Hf- Mc	0.10	0.50 (0.40)	6	0,10	0,50 (0.40)	6

Note: *Hf - Hypholoma fasciculare; Aa – Alternaria arborescens; Bb – Beauveria bassiana; Cs – Cordyceps sinensis; En – Epicocum nigrum; Gm – Gibberella moniliformis; Fc – Fusarium chlamydosporum; Fo – Fusarium oxysporum; Mc – Mucor circinelloides*

In MMN medium, the external growth rates of *H. fasciculare* are greater in the presence of *A. arborecens, F. chlamydosporum* and *F. oxysporum* than in the control. These values may be the result of the release of growth promoting compounds from the interacting fungus or could only reflect the irregular growth of *H. fasciculare* colony. In contrast, in the presence of *B. bassiana, C. sinenesis, E. nigrum, G. moniliformis* e *M. circinelloides*, the external growth rate of *H. fasciculare* is greater in the control than in the presence of interacting fungi. In this case there should be no liberation of compounds by interacting fungus.

H. fasciculare has a slower growth in PDA medium than in MMN medium. While in the presence of *F. chlamydosporum* and *F. oxysporum, H. fasciculare* presents a higher growth rate than in the control assay, in the presence of *B. bassiana, C. sinensis* and *E. nigrum*, the growth rate of *H. fasciculare* is more reduced. The remaining fungi (*A. arborescens, G. moniliformis* and *M. circinelloides*) do not interfere with *H. fasciculare* growth. This difference between MMN and PDA media suggest that *H. fasciculare* display a different behavior from one medium to another when interacting with certain fungi.

Table 3.4 Fungal growth rates determined from the internal radius obtained in the assay on MMN (left) and PDA (right) for the interaction between *H. fasciculare* and each of the interacting fungus. The days correspond to the period of time from which growth rates were determined. The values in brackets correspond to the growth rates obtained in the corresponding controls.

	MMN medium			PDA medium		
	Growth rate (cm/day)			Growth rate (cm/day)		
	H. fasciculare (0.13)	Interacting fungi	Day	*H. fasciculare* (0.09)	Interacting fungi	Day
Hf-Aa	0.12	0.17 (0.14)	10	0.07	0.19 (0.14)	10
Hf-Bb	0.11	0.09 (0.10)	12	0.04	0.16 (0.15)	10
Hf-Cs	0.11	0.17 (0.14)	10	0.07	0.16 (0.14)	10
Hf- En	0.10	0.23 (0.21)	8	0.05	0.30 (0.18)	8
Hf-Gm	0.09	0.27 (0.18)	8	0.06	0.18 (0.18)	12
Hf- Fc	0.11	0.20 (0.15)	10	0.11	0.21 (0.19)	10
Hf-Fo	0.09	0.12 (0.13)	10	0.06	0.14 (0.12)	10
Hf- Mc	0.10	0.25 (0.75)	10	0.05	0.25 (0.38)	10

Note: *Hf - Hypholoma fasciculare; Aa – Alternaria arborescens; Bb – Beauveria bassiana; Cs – Cordyceps sinensis; En – Epicocum nigrum; Gm – Gibberella moniliformis; Fc – Fusarium chlamydosporum; Fo – Fusarium oxysporum; Mc – Mucor circinelloides*

Internal radius values were also used for determining growth rates, in the absence (control) or in the presence of interfering fungi (table 3.4). In MMN medium, *H. fasciculare* always display a more reduced growth rate in the presence of *A. arborecens, B. bassiana, C. sinenesis, E. nigrum, G. moniliformis, F. chlamydosporum, F. oxysporum* and *M. circinelloides* than in the control assay. This result suggests that all fungi that have been in contact with the *H. fasciculare* could have produced compounds that led to the growth inhibition of *H. fasciculare*.

In contrast with this result, in PDA medium only in the presence of *F. oxysporum,* the growth rate of *H. fasciculare* is greater in the assay than in the control. In the remaining interactions (*A. arborecens, B. bassiana, C. sinensis, E. nigrum, G. moniliformis, F. chlamydosporum* and *M. circinelloides*) the result obtained is identical to that achieved with MMN medium.

Until now has been discussed the effect of interacting fungi on *H. fasciculare,* now will be discussed the effect of *H. fasciculare* on the interacting fungi. In MMN medium *H. fasciculare,* at internal growth, lead to a reduce in the growth of *B. bassiana* and *F. oxysporum*. This effect also happens in the external

growth of *F. oxysporum* and *C. sinensis*. *H. fasciculare* don't affect the growth of *G. moniliformis*. On the other fungi *H. fasciculare* doesn't seem to affect their growth on the contraire seems to promote it. In PDA medium, different results are obtain. *H. fasciculare,* at internal growth, only reduces the growth of M. *circinelloides,* to the remaining fungi *H. fasciculare* doesn't display any effect, once again it seems promoting the growth. At external growth *H. fasciculare* effect *A. arborescens, E. nigrum* and *F. oxysporum*, the remaining fungi are not affected by *H. fasciculare*. Like in MMN *H. fasciculare* don't affect the growth of *G. moniliformis*.

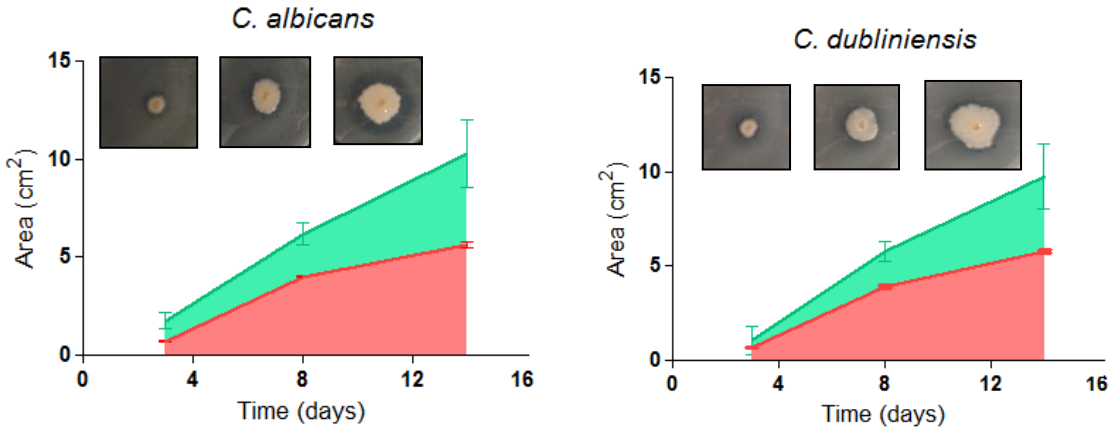
Taken all the results together, in MMN medium, *H. fasciculare* displays an antagonistic action against *A. arborecens*, *B. bassiana*, *E. nigrum* and *F. oxysporum*. However, *H. fasciculare* also suffers from an antagonizing effect promoted by the interacting fungi. The mycelial contact in MMN medium was observed during *H. fasciculare* interaction with *C. sinensis*, *G. moniliformis*, *F. chlamydosporum* and *M. circinelloides.* In this case, besides having mycelial contact between *H. fasciculare* and *M. circinelloides, H. fasciculare* appears to continue to growth beneath *M. circinelloides* mycelium (Figure 2 g; Appendix). In PDA medium, the behavior of *H. fasciculare* and the interacting fungi is slightly different. *H. fasciculare* in the presence of *A. arborecens*, *F. chlamydosporum*, *G. moniliformis* and *M. circinelloides* presents an antagonism by mycelial contact, whereas in the presence of *B. bassiana*, *E. nigrum* and *F. oxysporum* presents and antagonism at distance. For *C. sinensis - H. fasciculare* interaction the occurrence of antagonism is not obvious by analyzing the growth curves (Figure 4 b; Appendix). But the morphological aspect of interaction assays suggests a dual distance antagonism, because both fungi avoid mycelial contact (Figure 4 b; Appendix). Indeed, *C. sinensis* grows faster than *H. fasciculare,* and completely surrounds *H. fasciculare* colony without mycelial contact. Can also be observe (Figure 3 b, c, d, e; Appendix) that *C. sinensis, E. nigrum, G. moniliformis* and *F. chlamydosporum* suffers a discoloration.

Previous studies had demonstrated that the *H. fasciculare* antagonist ability could be used as a biological control agent against different fungal species belonging to the genus *Armillaria*. With the present work the antagonist effect of *H. fasciculare* was confirmed for all tested fungi, corroborating its potential use as a biological control agent against other fungal species.

3.4.2. Interaction between *Hypholoma fasciculare* and yeasts

The interaction between *H. fasciculare* and different yeasts was evaluated *in vitro* through the ability of this fungus to inhibit, or not, the growth of yeasts. After fungal growth for 3, 8 and 14 days, the plates were overlaid with a lawn of the yeast to be tested and the presence of an anti-yeast activity was detected after 48h growth at 25ºC as a growth inhibition zone or inhibition halo surrounding *H. fasciculare*. To determine and express this antifungal activity the area occupied by the fungus and the area occupied by the halo created by the fungus action were calculated.

H. fasciculare has the ability to inhibit the growth of all the tested yeasts being its antifungal activity more notorious against some of the species, as showed in Figure 3.7, which represents the areas occupied by the fungus and by the growth inhibition zones observed in each assay. *C. albicans*, *C. dubliniensis* and *C. parapsilosis* as well as *S. cerevisiae* are the most sensitive species after 3 days of fungal growth. The relatively large standard deviations values observed can possibly be explained by fungal sensitivity to temperature: it has grown at room temperature during the assays and these were performed during late winter and spring, when temperature fluxes were registered, ranging between 20ºC and 25ºC. On the contrary, *C. glabrata*, *C. guilliermondii*, *C. krusei* and *C. tropicalis* were more resistant to antifungal action, either after 3, 8 and even 14 days of fungal growth.



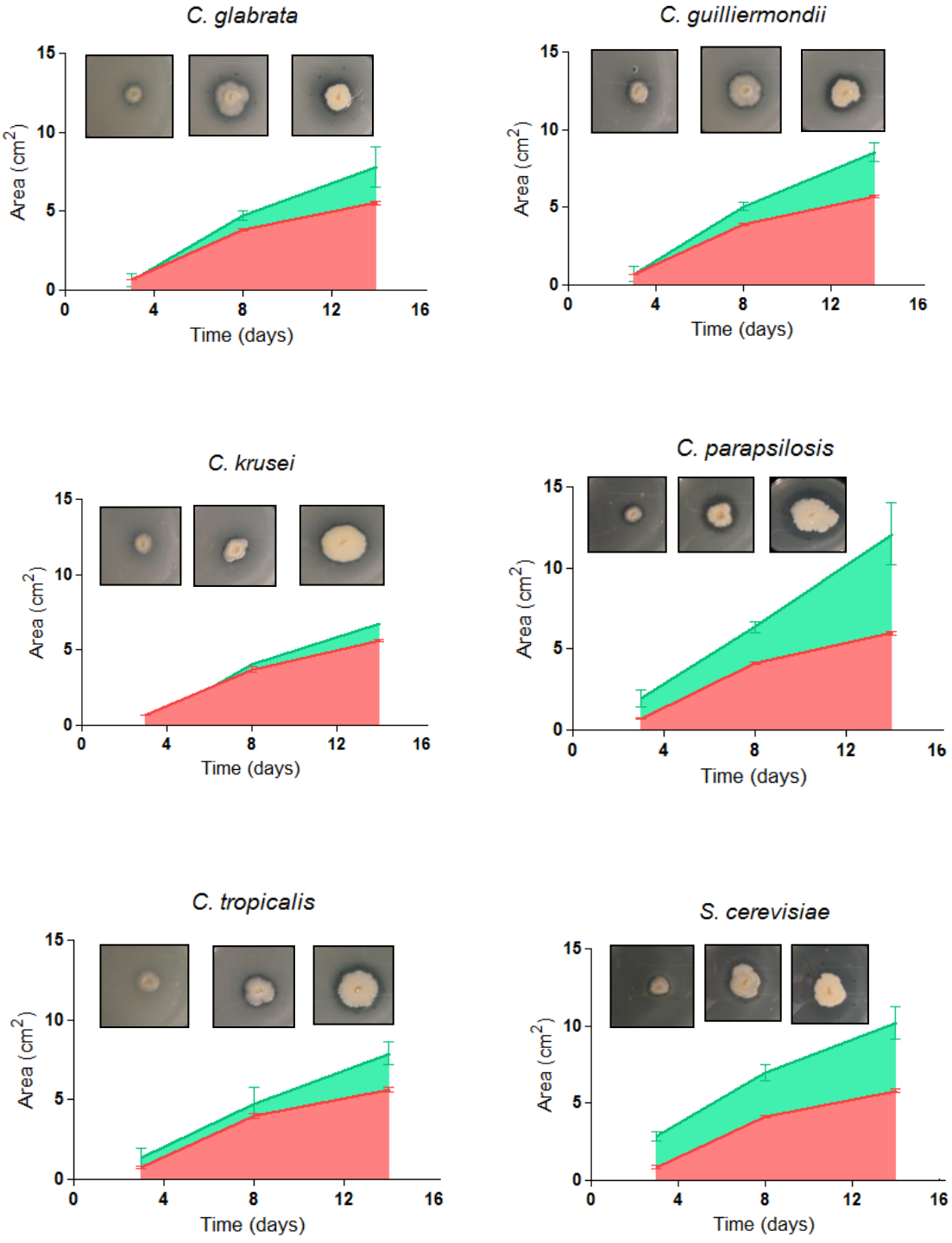


Figure 3.7 Influence of the Anti-fungal activity of *H. fasciculare* against *C. albicans, C. dublinienesis, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis and S. cerevisiae*. The antimicrobial effect was evaluated after 3, 8 and 14 days of fungal growth at 25ºC and was detected as the occurrence of yeast growth inhibition zones surrounding the fungus, as illustrated in the figures at the top of each chart. The areas occupied by the fungus and by the inhibition halos are respectively

represented in red and green. And morphological aspect of anti-fungal activity established between *H. fasciculare* and the different yeast.

In spite of the fact that the area occupied by the fungus is almost always higher than the area occupied by the inhibition halos, different patterns of yeast sensitivity could be observed. Thus, *H. fasciculare* antagonist action was stronger against *S. cerevisiae* and *C. parapsilosis,* and not so expressive but also very good against *C. albicans* and *C. dublinienesis*. *C. krusei* is apparently the most resistant yeast to fungal antimicrobial activity.

The observation that *H. fasciculare* displays an antagonistic action against yeasts is particularly interesting considering the panel of sensitive indicator strains used. As already referred, the majority of yeasts belongs to the genus *Candida* and is associated to drug-resistant opportunistic infections, which demand an urgent clinical alternative treatment. In this context, the isolation and identification of the fungal antimicrobial substance(s) can lead to the discovery and/or design of new antifungal drugs.

3.4.3. Interaction between *Hypholoma fasciculare* and bacteria

H. fasciculare exhibited a strong antagonist action against both bacteria tested, but its activity was higher against *Bacillus subtilis* than against *Escherichia coli* (Figure 3.8). As the optimal temperature for growth is 25°C for the fungus and 37°C for bacteria, the assays for antibacterial activity were performed at 25°C, 30°C and 35°C.

The halo size was always bigger in the case of *B. subtilis* than the observed for *E. coli* and the area occupied by the halo was constantly higher than the area occupied by the fungus, independently of the temperature of the assay. The lowest value was observed at 30°C and the highest when the temperature of the assay was raised to 35°C. In *E. coli* the halo size was slightly bigger at 30°C than the inhibition zone formed at 25°C. The apparent decrease at 35°C was accompanied by a simultaneous decrease of fungal growth. The increased susceptibility of the Gram positive bacteria, like *B. subtilis*, over the Gram negative, such as *E. coli*, to several compounds has been frequently supported by their differences in cell wall composition and structure. The presence of a lipid outer membrane external to the peptidoglycan layer in Gram negative bacteria can therefore constitute a barrier to the entrance of substances produced by *H. fasciculare*.

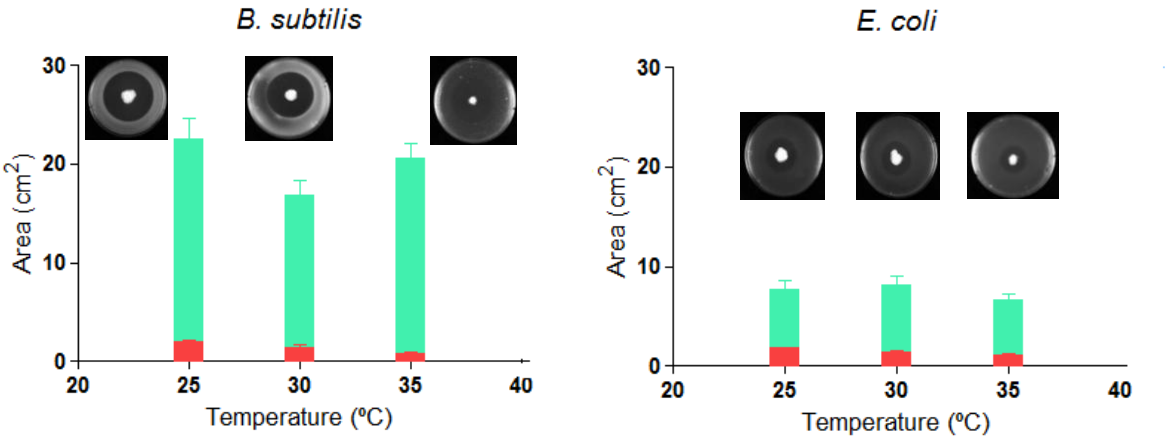


Figure 3.8. Influence of the temperature assay in *H. fasciculare* anti-bacterial activity against *B. subtilis* and *E. coli*. The areas occupied by the fungus and by the inhibition halos are respectively represented in red and green. And morphological aspect of anti-fungal activity established between *H. fasciculare* and *B. subtilis* and *E. coli*.

Contrary to what happens in yeasts, several studies were carried out in order to evaluate and elucidate the interactions between filamentous fungi and bacteria. Between 1987-88, Barron, described that filamentous fungi could cause the lyses of bacterial cells. However most fungi, from the different groups are not capable of causing the bacterial lyses.

These results could be due, in yeast and bacteria, to the release of compounds/substances by *H. fasciculare* resulting in the yeast/bacteria death. But to confirm this would be necessary to perform biochemical assays to determine the presence of possible compounds/substances.

Chapter 4

Adhesion of the *Hypholoma fasciculare* to the roots of *Castanea sativa*

4.1. Introduction

Plants have developed many strategies in order to survive stresses caused by biotic and abiotic factors. One of the most successful strategies is the mutualistic association formed with fungi, referred to as mycorrhiza (Smith & Read, 1997). In this relation the host plant receives mineral nutrients through the fungus and in turn the fungus obtains carbon from photosynthetic compounds. The majority of angiospermi and all gimnospermi plants are able to form mycorrhizal (Marschner, 1995).

Based on morphofunctional characteristics, mycorrhizae can be classified into tree distinct groups: endomycorrhizae, ectomycorrhizae and ectendomycorrhizae. The main characteristics of endomycorrhizae are the absence of fungal mantle at the root surface and the inter-intracellular growth of hyphae in root cortical cells (Smith & Read, 1997). In ectomycorrhizae hyphae develop in the intercellular spaces, penetrating between epidermal root cells and endodermal roots cells, forming a structure designated as Harting net. Characteristic of this association is also the development of a dense tangle of hyphae on the root surface, forming the so-called mantle (Smith & Read, 1997). The ectendomycorrhizae, as the name implies, are characterized by presenting fungal structures of the endo- and ectomycorrhizae, such as a poorly developed mantle, Hartig net and a slight penetration of hyphae within root cortical cells (Yu *et al.,* 2001).

Besides mycorrhiza, plants and fungi could interact through parasitic relations. Pathogenic fungi can develop strategies to penetrate into the plants cells and then obtain an abundant source of nutrients. In spite of all plants efforts to avoid fungal attack, pathogenic fungi seem to always develop a strategy to penetrate into the plant (Mendgen *et al.,* 1996).

4.1.1. Involvement of hydrophobins in the adhesion of *H. fasciculare* to the roots of *C. sativa*

The initiation, development and maintenance of a functional symbiosis involve a series of events that occur between soil fungi and root. After plant-fungus contact the plant has the capacity to recognize and interact with the fungus. In turn the fungus can adhere and penetrate into the root, bypassing the defense mechanisms of the host plant (Tagu *et al.,* 2002). In this process, the occurrence is

essential occurred the exchange of signals between the symbionts, to be recognized for their compatibility and to occur the preparation of both partners for the key morphological and physiological changes in the formation of mycorrhizal organs (Martin *et al.*, 2001b; Voiblet *et al.*, 2001; Podila, 2002). It was proposed that the initiation and maintenance of communication established between the symbionts is due to the fact that both have developed sensing molecules responsible for rapid adaptation to environmental change in the immediate immediacy of the root cells, they may transfer intracellular signals to the nucleus through signaling pathways, inducing or repressing specific genes responsible for phenotype mycorrhizal. Thus the formation of mycorrhizae appears as a process of interaction, marked by a sequence of events highly regulated and coordinated (Tagu *et al.*, 2002; Martin *et al.*, 2001; Voiblet *et al.*, 2001). Until now the nature of signals and the mechanisms of recognition and interactions are not clear.

Hydrophobins are proteins produced by filamentous fungus and are one of the most surface active proteins known. They were first described by Wessels and co-workers when studying highly expressed genes during fruit body formation of *Schizophyllum commum* (Wessels *et al.,* 1991). Hydrophobins are amphiphile proteins, comprising a hydrophilic and a hydrophobic part. These proteins can be found in liquid media of fungal cultures. They gather towards the fungal cell walls, cover fungal spores and coat the surface and cavities in fruiting bodies (Linder *et al.,* 2004; Sunde *et al.,* 2007; Szilvay *et al.,* 2006).

As a consequence, hydrophobins seem to perform different roles in fungi survival and adaptation to the environment. The most functional feature of these proteins seems to be their ability to interact with surfaces, coating surfaces and lowering the surface tension (Linder *et al.,* 2004; Martin *et al.,* 1999). Although all the work developed for several years on hydrophobin features, the biological role of these proteins still remains a challenge. Hydrophobins were suggested to play a role in protection, adhesion, growth and also may be involved in pathogenesis (Linder *et al.,* 2004). Indeed, hydrophobins are usually found as structural proteins located on surfaces of aerial structures, being already established a link between the hydrophobin expression and the ability of the fungus to develop aerial structures (Linder *et al.,* 2004). It was then proposed that the hydrophobic coating had a role in protecting structures against dissection and wetting. Hydrophobins can also mediate the attachment of fungal infection structures to their targets, either by acting as structural components of the appressorium cell wall or by binding and modifying host surfaces (Linder *et al.,* 2004). Hydrophobin genes have

been found in filamentous fungi belonging to Ascomycetes and Basidiomycetes (Linder *et al.,* 2004).

The amino acid sequence alignment of hydrophobins put in evidence a conserved sequence of 8 Cys residues, which is common to all hydrophobin. This feature suggests that all hydrophobins share a common disulfide network and a common fold (Linder *et al.,* 2004). Cys residues are critical for structural reasons, while other residues can vary substantially in different hydrophobin variants (Linder *et al.,* 2004). Two different classes of hydrophobins (class I and II) can be distinguished based on the patterns of clustering of hydrophobic and hydrophilic groups (Wösten, 2001, Linder *et al.,* 2004; Askolin *et al.,* 2006). Until now, class II hydrophobins were only observed in Ascomycetes, whereas class I hydrophobins were observed in both Ascomycetes and Basidiomycetes (Linder *et al.,* 2004). Class II forms the most uniform group, which turns easier to compare how conserved residues are related. In contrast, class I hydrophobins have more sequence variation and a wider range of sequence lengths. After comparing sequences from class I hydrophobins, it was convenient to further divide the class I in the sub-groups Ia and Ib, representing the hydrophobins of Ascomycetes and Basidiomycetes respectively (Linder *et al.,* 2004). Because of the similarity between hydrophobins class I and II, it was speculated that class II have evolved independently of class I, thus representing a case of convergent evolution (Linder *et al.,* 2004). Class Ia shows high divergence but distinct sub-groups are easily identified. The hydrophobicity of the amino acid sequence after the first Cys is higher in most class I hydrophobins than in class Ia or class II proteins (Linder *et al.,* 2004). While over 60% of the class I proteins have a high hydropathy level in that region, only 20% of class Ia and class II hydrophobins have high overall hydrophobicity.

Hydrophobins are found as multigene families. This multiple presence can be explained by two ways. Different genes could be differentially expressed as a response to development stages or environmental conditions, but are largely able to functional complement each other. Alternatively, different genes could fulfill different functional roles that are reflected in structural differences (Linder *et al.,* 2004).

Indeed, while class I hydrophobins generate highly insoluble aggregates that are only able to dissolve in the presence of a strong acid, the aggregates formed by class II hydrophobins are amphipathic and much more easier to dissolve (Hektor and Scholtmeijer, 2005). Different applications of hydrophobin proteins are

now being studied, such as those for industry (materials technology), food additives or medical devices (Linder, 2009).

Similarly to plant-pathogen interaction, response defense mechanisms have been suggested to occur in host plant. Clarification of these processes will allow ascertaining the role of saprophytic fungi as stress agents on the plant.

The study of host-fungus interactions requires the establishment of mycorrhizae in axenic conditions, since in natural conditions there are many abiotic and biotic factors that may interfere (Smith & Read, 1997). In these conditions, the pre-colonization and hyphae adhesion to the root surface typically occurs within the first 24 hours, whereas the mantle formation and Hartig net development occurs between 24 hours and 7 days of mycorrhization (Martin & Tagu, 1995; Le Quéré *et al.*, 2005).

In the adhesion process between fungi and host plant are involve several classes of proteins with specific properties at cellular adhesion level secreted by fungus. The search for genes differentially expressed during these interactions has greatly contributed to the identification of other classes of proteins with adhesion functions. Among these proteins are the hydrophobins, describe as being involve in other process of fungi development such as aerial growth of hyphae, hyphal aggregation during the formation of fruiting bodies (carpophores), sporulation and dispersal of spores in filamentous fungi, and in the infection of some fungal pathogens of plants and insects (Wessels, 1996; Kershaw & Talbot, 1998; Wösten, 2001). In the infection of fungal pathogens of plants, hydrophobins, play a key role, since the penetration and subsequent infection depend on the adherence of the pathogen to the host hydrophobic surface (Wessels, 1996; Wösten, 2001). Paula Baptista (2007) began develop a work aiming evaluated the involvement of hydrophobins in the adhesion of the saprophytic fungus *H. fasciculare* the roots of chestnut was initiated to study the expression of genes encoding hydrophobins H. fasciculare during the fungus-root contact. With this work was obtain the nucleotide sequence putatively coding for a hydrophobin of *H. fasciculare* as well the respective amino acid sequence deduced.

In this present work, using the nucleotide sequence obtain previously, keeping in mind the possible involvement of hydrophobins in the process of adhesion of *H. fasciculare* to the roots of *C. sativa*, the complete sequencing of the hydrophobin cDNA was aimed. Using biological samples, in which high hydrophobin gene expression is expected, a RNA fraction was purified from fungus-root interaction. After synthesizing the corresponding cDNAs, the use of

specific primers would allow the amplification of cDNA 3' ends. The complete sequence of hydrophobin coding region will allow phylogenetic and structural studies, as well the cloning of the complete coding region.

4.2. Material

4.2.1. Biological material

Chestnut seedlings were harvested at Bragança region (Trás-os-montes). *Hypholoma fasciculare* was provided by the Instituto Politécnico de Bragança (Paula Baptista).

4.3. Methods

4.3.1. Culture and maintenance of the fungi

Axenic cultures of *Hypholoma fasciculare* were cultured on MMN medium, in the dark, at a temperature of 23°-25°C. Sub-cultures were carried out in intervals of 3 weeks. For promoting the fungal interaction in chestnut root seedlings, *Hypholoma fasciculare* mycelium was on modified MMN liquid medium. Ten inocula, from the peripheral region of actively growing cultures of Hypholoma fasciculare on MMN medium were used to inoculated 250ml of MMN liquid medium. The cultures were kept in the dark, without agitation, at 23°-25°C, until dense mycelium was evident.

Medium MMN: NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acid 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L

MMN liquid medium modified: NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4.7H_2O$ 0.15 g/L; thiamine 0.10 g/L; glucose 5 g/L

4.3.2. *Castanea sativa* hydroponic culture

All the procedure for the hydroponic culture of chestnut seedlings is displayed in Figure 4.1. Chestnut seeds were washed in running tap water and disinfected with a solution of sodium hypochlorite (commercial solution, containing 3.5% (w/v) effective chloride) for 1 hour, with shaking (100 rpm). After five washes with sterile distilled water, seeds were sowed in sterile layers of moist sand and sealed within sterile plastic bags. Seed germination occurred in the dark, at a temperature of 4ºC during the first month, followed by an 18ºC incubation in the next two months.

Germinated seeds displaying an evident radicle were washed in running tap water to remove the sand and disinfected with sodium hypochlorite (commercial solution, containing 3.5% (w/v) effective chloride) for 15 minutes. Disinfected seeds were then rinsed in sterile water (at least three washes) and root tips were cut to promote the branching of the root system. Finally, two-three seeds were deposited in previously prepared sterile growing flasks (1.5L), which contained plastic nets suspended by wires and were filled with 250 ml of tap water. Flasks were kept in the dark at 23°C, until the emergence of the first leaves. Afterwards, the seedlings were incubated at same temperature, under a photoperiod of 16 h light (60 $\mu E.m^{-2}.s^{-1}$ light intensity) /8 h dark, for two months.

Figure 4.3. Hydroponic culture of *Castanea sativa* seedlings. Chestnut seeds were superficially disinfected with sodium hypochlorite [3.5% (w/v) effective chloride] under agitation (A). Seed stratification occurred at 4ºC in moist sand (B). After germination, seeds were again superficially disinfected in sodium hypochlorite [3.5% (w/v) effective chloride] (C) and the apical 4 cm of radicle was removed. Seeds were then deposited on a plastic net into the culture flasks (D) and cultures were kept in the dark at 23°C (E). After leaf emergence, seedlings were further incubated under a 16 h light/8 h dark photoperiod at 23ºC (F). (Baptista, 2007).

4.3.3. Inoculation of *Castanea sativa* root seedlings with *Hypholoma fasciculare*

Four-month-old chestnut seedlings with well-developed roots were used for studying the interaction of *H. fasciculare* with plant roots. The mycelium was prepared from axenic cultures in liquid modified MMN medium. After being extensively washed with sterile water, *H. fasciculare* mycelium was transferred to the culture flasks, containing the chestnut seedlings about four months. The culture flasks were then gently agitated to promote an even distribution of the mycelium. Seedlings incubation proceeded in the same conditions (photoperiod of 16 h light (60 $\mu E.m^{-2}.s^{-1}$ light intensity) /8 h dark, 23ºC).Root samples were collected after 24 or 48 hours of root-fungus contact. After being homogenized to a fine powder, using liquid nitrogen and a mortar and pestle, the biological material was kept at 80ºC until use.

4.3.4. Identification of the cDNA complete sequence of *Hypholoma fasciculare* hydrophobin

Using the nucleotide sequence obtain previously, aims to obtain the complete sequencing of the hydrophobin cDNA. To accomplish this aim was use biological samples, in which high hydrophobin gene expression is expected, a RNA fraction was purified from fungus-root interaction, for that the RNA was extracted using the Hot-Borat method (4.3.4.1). After synthesizing the corresponding cDNAs, the use of specific primers would allow the amplification of cDNA 3' ends, at this step the 3` RACE kit will be use (4.3.4.2). The complete sequence of hydrophobin coding region will allow phylogenetic and structural studies, as well the cloning of the complete coding region.

4.3.4.1 RNA extraction

The RNA extraction was performed using the hot-borate method (Wan and Wilkins, 1994).

The extraction buffer XT (10 ml) was previously incubated at 80°C, for 10min. After adding 1g of homogenized biological material, the solution was quickly homogenized, and 600µl proteinase K (20 mg/ml) was added. The mixture was then incubated at 42°C, 150 rpm, for 1.5h. The supernatant was placed into a new centrifuge tube and 800µl of KCl 2M was added. After vortexing, the solution was incubated on ice for 1h and centrifuged at 10,000 rpm, for 25 min, at 4°C (Avanti J-

25, Beckman; JA-25.50 rotor, Beckman). After supernatant recovering to a new corex tube, LiCl concentration was adjusted to 2 M using a LiCl 8 M solution. After mixing, the final solution was incubated overnight, at 4°C.

On the next day, the solution containing the precipitated RNA was centrifuged at 10,000 rpm, for 20 min, at 4°C (Avanti J-25, Beckman; JA-25.50 rotor, Beckman). The pellet was washed twice with 3 ml of cold LiCl 2M. For the washing steps, the solution was vortexed and centrifuged at 10,000rpm, for 15 min, at 4°C. The supernatant was solubilized in sterile ultrapure water (150 µl) and the insoluble material was precipitated by centrifugation at maximum speed in a microcentrifuge.

Hot – Borate extraction buffer (XT): 0.2 M sodium borate decahydrate (Borax); 30mM EDTA; 1% (p/v) SDS; 1% (p/v) sodium deoxycholate; 10mM DTT; 1% (v/v) NP-40; 2% (p/v) PVP-40
Note: DTT, NP-40 and PVP-40 were added just before use.

4.3.4.2. Analysis and spectrophotometric quantification of RNA

The amount and quality of purified RNA was determined by spectrophotometry (Nanodrop ND 1000). The analysis was performed by the determination of the value A_{230}, A_{260} and A_{280} of the sample solution. The quality of the sample was calculated based on the reasons A_{260}/A_{280} (protein contamination) and A_{260}/A_{230} (contamination by other compounds), is expected pure samples of RNA from a value 2 and 2.2 for both reasons. In order to assess the RNA integrity an electrophoretic analysis was performed, as described in section 4.3.4.4.

4.3.4.3 Primer design

The primers were designed for the previously identified *H. fasciculare* hydrophobin gene sequence (Baptista, 2007). For designing the PCR primers the program Oligo 6 was used. The main criteria considered were the size (21 bases), the melting temperature (Tm), the difference in Tm between primer pairs (less than 5ºC) and the difference between primers Tm and template (below 20ºC). The designed primers were *HydSP1* (5´- GTTGCCAGAGGCGCCGATCAC), *HydSP2* (5´- GGACGACGACGCCCAAGATGC) and *HydSP5* (5´-CCTCTGGCAACTCCTG

CTCCG), being distributed along the already identified sequence as depicted in Figure 4.4.

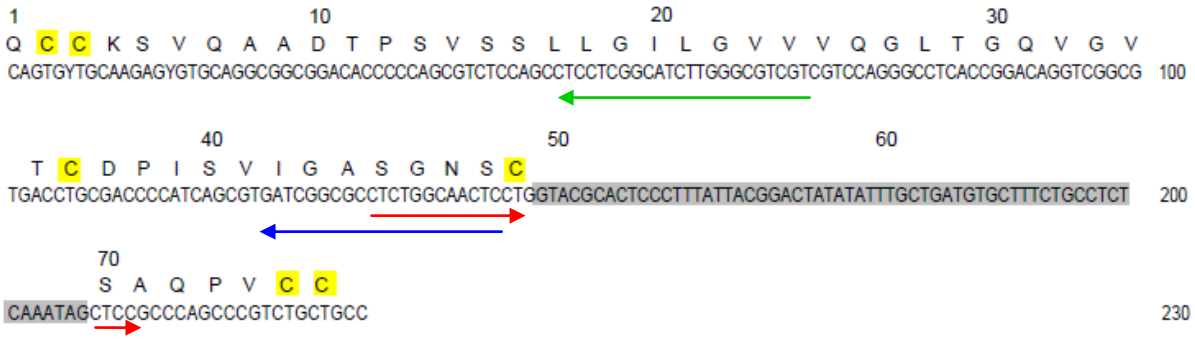


Figure 4.4. Nucleotide and deduced amino acid sequences of *HydHf* fragment of *Hypholoma fasciculare* (Baptista, 2007). The deduced amino acid sequence is shown in one letter code. The numbers on the right refer to nucleotides and numbers above refer to amino acids. The gray shaded nucleotide sequence represents the untranslated sequence (intron). The cysteine residues conserved in all hydrophobins are highlighted in yellow. The arrows represent the primers used in the work. Forward primer at red *(HydSP5*), reverse primers at blue (*HydSP1*) and green (*HydSP2*).

4.3.4.4. Agarose gel electrophoresis

RNA and DNA fragments were resolved by electrophoretic separation through an agarose gel in horizontal system.

RNA fragments were resolved by electrophoretic separation through an agarose gel in horizontal system. To prepare the gel, agarose (1% p/v) was prepared in TAE 0.5x buffer, that was also used as gel running buffer. The samples were mixed with 1µl of Gel Red (Biotarget). The molecular weight marker used was *MassRuler DNA Ladder Mix ready-to-use* (Fermentas). Electrophoresis occurred at 100 V until the front dye migrated two thirds of gel length.

DNA fragments were resolved by electrophoretic separation through an agarose gel in horizontal system. To prepare the gel, agarose (1% p/v) was prepared in TAE 0.5x buffer that was also used as gel running buffer. The samples were mixed with 1µl of Gel Red. The molecular weight marker used was *MassRuler DNA Ladder Mix ready-to-use*. Electrophoresis occurred at 50 V until the front dye migrated two thirds of gel length.

4.3.4.5. cDNA preparation and 3' RACE reaction

For determining the 3'-terminal sequence of hydrophobin transcript, cDNA will be prepared from mRNA, using the RACE kit of Roche. The reaction mixture (20 µl) was prepared according to the manufacturer instructions, using 4 µl of cDNA synthesis buffer, 2 µl of deoxynucleotide mixture, 1 µl oligo dT-anchor primer, 10 µl of total RNA extracted from chestnut roots associated with *H. fasciculare*, and 2 µl of sterile ultrapure water. After addition of 1 µl of transcript reverse transcriptase, the mixture was gently mixed and briefly spun down. The reaction proceeded at 55°C, for 1.5h, being further incubated at 85°C, for 5 min.

According to the RACE kit instructions, the cDNA was then directly amplified by PCR without a prior purification. A PCR reaction mixture (50 µl) was prepared containing 10µl of previously prepared cDNA product, 1µl of PCR anchor primer, 1µl of specific primer *HydSP5*, 1µl of dexyonucleotide mixture, 5 µl reaction buffer and 40.5 µl of sterile ultrapure water. After adding 0.5 µl of Taq Polimerase, the mixture was spun down and the PCR was initiated in a thermocycler (MJ-mini - BioRad), comprising the following steps:

Denaturation for 5min at 94°C
35 cicles of
    Denaturation for 45sec a 94°C
    Pairing for 45sec between 50°C and 60°C
    Extension for 30sec at 72°C
Final extension for 5min at 72°C

4.4 Results and Discussion

4.4.1. Adhesion ability of *Hypholoma fasciculare* to the roots of *Castanea sativa*

The adhesion capacity of *H. fasciculare* to the roots of *C. sativa* was evaluated macroscopically. As soon as the fungus enters into contact with chestnut roots, it becomes adherent to the plant tissue. The adhesion to the host surface is the initial process of infection of pathogenic fungi, from which the success of pathogenicity is largely dependent (Epstein & Nicholson, 1997). The adhesion of *H. fasciculare* to the roots of chestnut tree was already described by Baptista (2007).

Observations by scanning electron microscopy revealed a high adhesion of hyphae to the root surface, just after 48h hours of inoculation. The hyphae adhesion increased along the contact time between root and fungus. After 25 days, a compact coating of hyphae enveloping the roots was observed and secondary root cross sections revealed the xylem obstructed after 60 days of contact (Baptista, 2007).

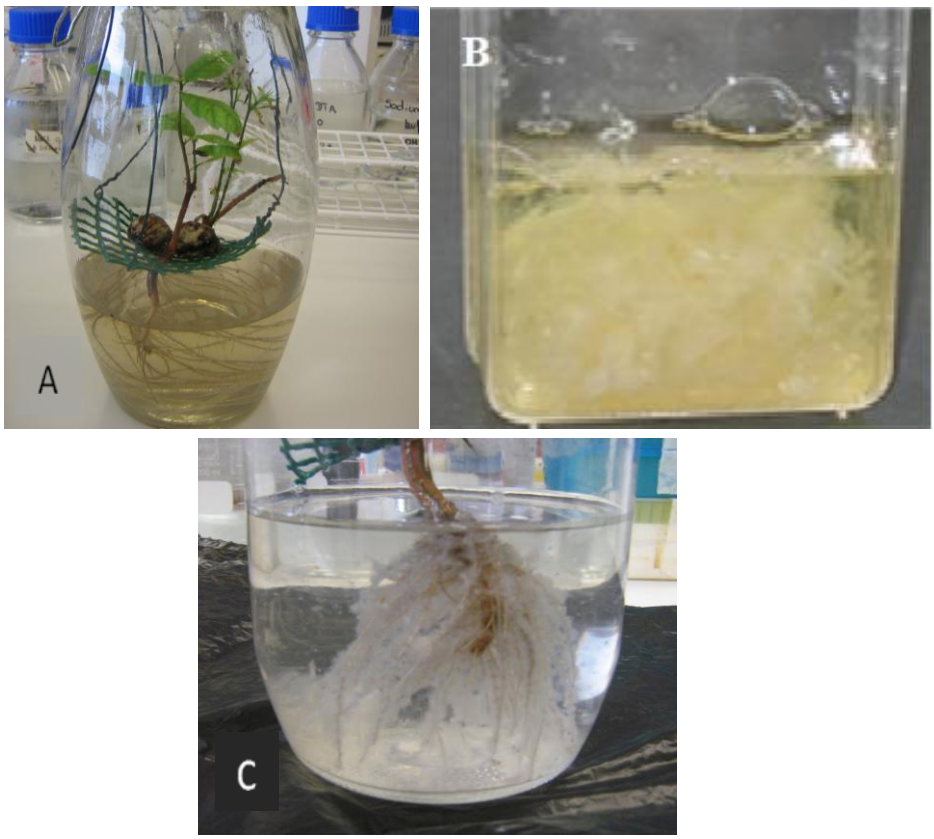


Figure 4.5. Inoculation of chestnut seedlings with *Hypholoma fasciculare*. Chestnut seedlings were cultivated in hydroponic system, under a 16 h light/8 h dark photoperiod, at 23°C (A). *H. fasciculare* was grown in liquid MMN medium, in darkness, at room temperature (B). After transferring the mycelium into the seedling culture flasks, the seedlings were further incubated under the same conditions (C).

Buffer TAE 50x: 2 M Tris; 0.95 M acetic acid; 50 mM EDTANa2 (pH 8.0) from 0.25 M EDTA (pH 8.0).

4.4.2. Identification of the complete sequence of hydrophobin transcript

In the work carried out by Paula Baptista, the response of chestnut seedlings to the saprophytic fungus *H. fasciculare* was studied during the first 48 h of root-fungus contact. Among other features, the adhesion ability of fungus to the chesnut root was evaluated, as well as the involvement of hydrophobins in this process. Using degenerated primers, a short genomic sequence from a *H. fasciculare* hydrophobin gene was identified. The objective of the present work is to obtain the full cDNA sequence of this hydrophobin gene. In order to guarantee the expression of this gene that has been described as being induced by the presence of plant roots, the interaction between chestnut roots and *H. fasciculare* was promoted.

The interaction between *H. fasciculare – C. sativa* roots occurred for 24 h and for 48 h. A mixture of RNAs purified from both samples will be used to synthetize the cDNA and to perform the RACE reactions. Following the RNA extraction, the determination of RNA quantity was determined by spectrophotometry and RNA integrity by agarose gel electrophoresis (Figure 4.6).

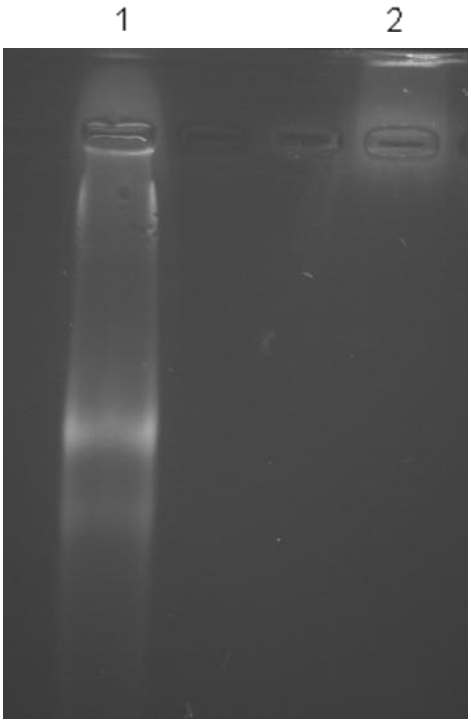


Figure 4.6. Electrophoretic analysis of RNA existent in the samples with 24h of root-fungus contact (1) and 48h of root-fungus contact (2). Electrophoresis was performed in a 1% (w/v) agarose gel, in which 10µl of each sample were applied.

The purification of an adequate amount of RNA for downstream reactions was only successful using the samples obtained after 24 h of chestnut root – *H. fasciculare* interaction. For this reason, the cDNA synthesis proceeded using only this single sample. For obtaining the complete cDNA sequence of hydrophobin gene, the 3` RACE kit of Roche will be used. The kit enables the transcription of specific mRNA sequences into first-strand cDNA as describe in section 4.3.4.5. This Kit was design for the rapid amplification of either 5`or 3` cDNA ends, in this step only the protocol of 3`will be used. For amplifying the 3` cDNA transcript, a PCR step will be performed using 3 different annealing temperatures. In the PCR was use the specific primer HydSP5.

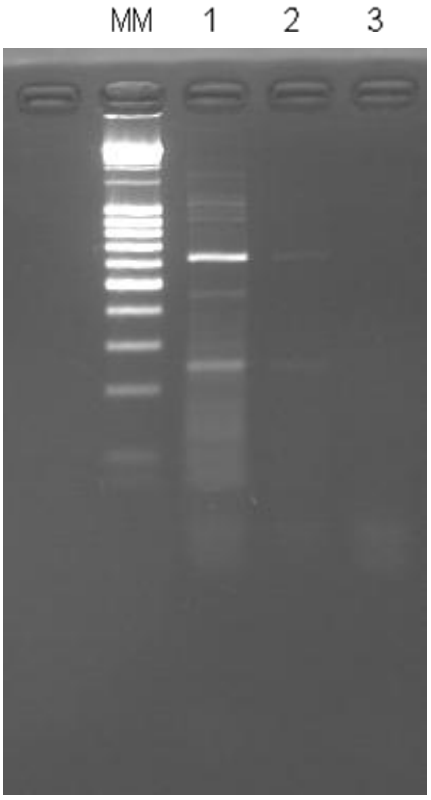


Figure 4.7. Electrophoretic analysis of the resulting products of the cDNA amplification with HydSP5 primer. The amplification was performed at different temperatures, in the figure are represented the electrophoretic result when subjected to 55°C (1), 60°C (2) and 65°C (3). MM: molecular height marker

The amplification of the putative 3'-terminal sequence of hydrophobin cDNA only occurred at an annealing temperature of 55°C (Figure 4.7). This result suggests that at least at 24 h after chestnut root and *H. fasciculare* contact the production of hydrophobins occurs. This product will be soon re-amplified, in order to allow its sequencing. Only the complete sequencing of this fragment will confirm

if this PCR product is from a hydrophobin transcript. When the success of 3' RACE is confirmed, the 5' RACE will be followed by using *HydSP1* and *HydSP2* primers.

Chapter 5

Conclusions and future perspectives

In the course of this work, the antimicrobial potential of the saprophytic fungus *H. fasciculare* against filamentous fungi was studied. The fungal interactions were established in two different culture media (MMN and PDA), using the dual culture method. In both media *H. fasciculare* displays an antagonistic action against all fungi tested. The antagonism was evidenced by growth reduction of interacting fungi in the presence of *H. fasciculare*. Besides this effect the interaction assays also suggest a high susceptibility of *H. fasciculare* to the presence of other fungi. Previous works suggested that the aggressiveness of saprophytic fungi, like *Hypholoma fasciculare, Hypholoma australe*, *Phanerochaete filamentosa*, *Phanerochaete velutina*, *Coriolus versicolor*, *Stereum hirsutum*, *Ganoderma lucidum*, *Schizophyllum commune* and *Xylaria hypoxylon* could be used as a biocontrol strategy for controlling *Armillaria spp* (Chapman, 2004). In this work it is shown that *H. fasciculare* not only displays an antagonistic action against all the tested fungi, but it also suffers from the antagonistic action of the interacting fungi. Face to these results, the use of *H. fasciculare* as a biocontrol agent could be restricted.

The antagonism mechanism adopted by *H. fasciculare*, in MMN medium, against *F. oxysporum* and *B. bassiana* was an antagonism at distance. *C. sinensis*, *G. moniliformis, F. chlamydosporum* and *M. circinelloides* presents an antagonism by mycelial contact. During the first days of interaction in MMN medium, *A. arborescens* and *E. nigrum* displayed antagonism at a distance, but later on presented an antagonism by mycelial contact. In PDA medium *H. fasciculare* display an antagonism at a distance for *F. oxysporum, B. bassiana* and *E. nigrum*. For *A. arborescens, F. chlamydosporum, G. moniliformis* and *M. circinelloides; H. fasciculare* display an antagonism by mycelial contact. In the case of *C. sinensis* the occurance of antagonism is not obvious, however the morphological aspect suggest a dual distance antagonism. The antagonism at a distance could be the result of compounds release by the antagonistic fungi. When studying the interaction between *H. fasciculare* and *Pisolithus tinctorius*, the volatile fraction was analyzed during the time course of the interaction (P. Baptista, personal communication). From this analysis, several volatile compounds (like linalool, *β*-pinene, *β*-elemene, valencene, among others) were identified that display an antifungal activity against fungi, leading to a fungal growth inhibition.

Keeping in mind a future project to purify the substance that confers the antagonistic activity, a faster and reliable method for determining the antimicrobial activity would be desirable. Using filamentous fungi in co-culture is a procedure that

requires 12 to 15 days to give an evident result, due to fungal slow growing rates, making this methodology a rather lengthy one. A bioassay using yeasts as indicators of *H. fasciculare* antagonistic activity was implemented. As *H. fasciculare* grown in MMN medium did not present as high anti-yeast activity as the one grown in PDA medium, all the assay design and optimization was performed in PDA. A set of experiments were then conducted using variable number of *Saccharomyces cerevisiae* cells and studying the influence of fungal growth time as well as the effect of temperature in *H. fasciculare* antimicrobial activity. The optimized bioassay should be performed with 10^6 cells/ml of the sensitive yeast and the antagonist effect is more evident when the fungus has grown for longer periods, although it could be distinguished after four days of fungal growth. Regarding the assay temperature, and considering all the variables, the best results were obtained at 25ºC. The design of this bioassay allows assessing the antimicrobial activity of fungi in a quick way and seems to be an innovative method since it was never performed.

The kinetics of antimicrobial compound production was also followed by incubating *H. fasciculare* at different temperatures for distinct periods and using the optimized assay for evaluating the antimicrobial activity. The ability to inhibit the yeast growth increases with *H. fasciculare* incubation temperature. Although fungal growth is restricted at high temperatures (30ºC), the antimicrobial activity is also higher, suggesting that in stressful conditions the fungus reacts by producing more antimicrobial compounds in an effort to survive, leading to the inhibition of *S. cerevisiae*. At higher temperatures (35°C or 40°C) the fungus seems to not grow.

In order to further confirm the antimicrobial activity of *H. fasciculare* against yeasts, the optimized bioassay was performed using different species of *Candida*, including *C. albicans, C. dublinienesis, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis*. The bioassay was performed with *H. fasciculare* grown for 3, 8 and 14 days. This fungus displayed antimicrobial activity against all the tested yeast species, although the results obtained with *S. cerevisiae, C. parapsilosis, C. albicans* and *C. dubliniensis* were more remarkable. From all the tested yeasts, *C. krusei* seemed to be the most resistant against *H. fasciculare* anti-yeast compounds, and its inhibition only became evident after 14 days of *H. fasciculare* growth. This experiment suggests that *H. fasciculare* displays a strong antagonist action against some *Candida* species that are pathogenic. These results could lead to the isolation and identification of new fungal antimicrobial substance(s) that could lead to the design of new antifungal drugs of interest, due

to the growing resistance of *Candida* opportunistic isolates to conventional antifungal agents.

H. fasciculare antibacterial activity was also evaluated using Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria. Both bacteria are susceptible to fungal action at all the tested temperatures, being *B. subtilis* more sensitive than *E. coli*. This difference could be related to the distinct cell wall structures. As Gram-positive bacteria do not have an outer membrane, they can be more vulnerable to *H. fasciculare* antimicrobial compounds. The identification and isolation of the compound(s) with antimicrobial activity, either against yeast or against bacteria, are future perspectives of this work.

When *H. fasciculare* was put in contact with *Castanea sativa* roots, in a liquid medium, a strong adhesion of the fungus to the root surface was clearly observed. In other plant root-fungus interactions this process has been associated to the production of hydrophobins, fungal proteins that play several important roles in the survival and adaptation of fungi (Linder *et al,* 2004). The previous identification of a genomic hydrophobin sequence from *H. fasciculare* allowed the design of RACE strategy to identify the complete sequence of hydrophobin cDNA. Following this approach, the detection of a putative PCR hydrophobin fragment obtained from RNA root-fungus samples suggests that the expression of hydrophobins only occurs after 24h of fungal inoculation. The final objective of this project was to infer the involvement of this protein in the adhesion process of the fungus to the roots. With the achieved results a re-amplification of the PCR product will be necessary in order to identify the hydrophobin 3' sequence. When the success of 3' RACE is confirmed, the 5' RACE will be followed. After the sequence is completed the analysis of gene expression in the situation of interaction between the fungus and the plant will be evaluated by qRT-PCR.

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Appendix

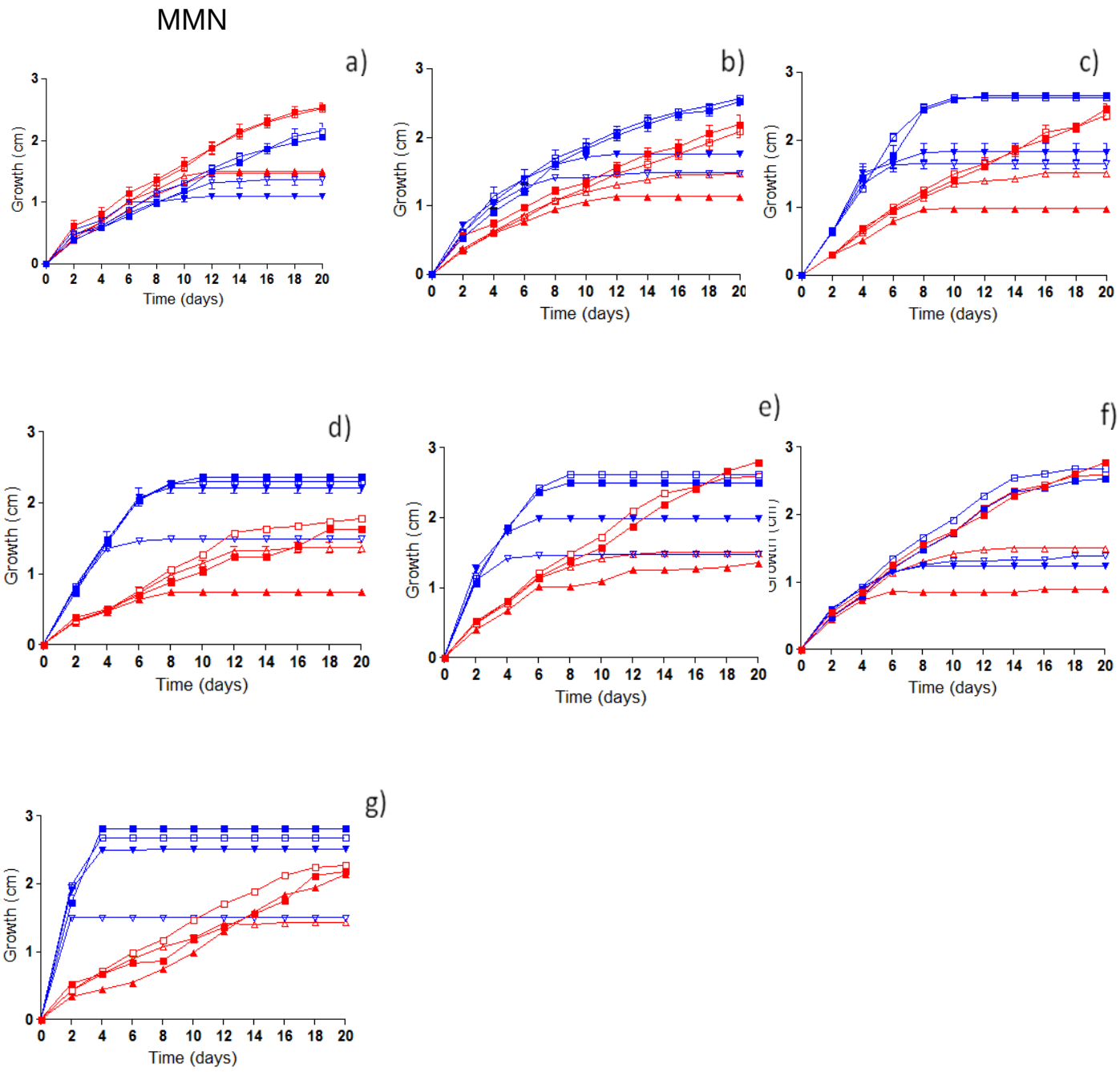
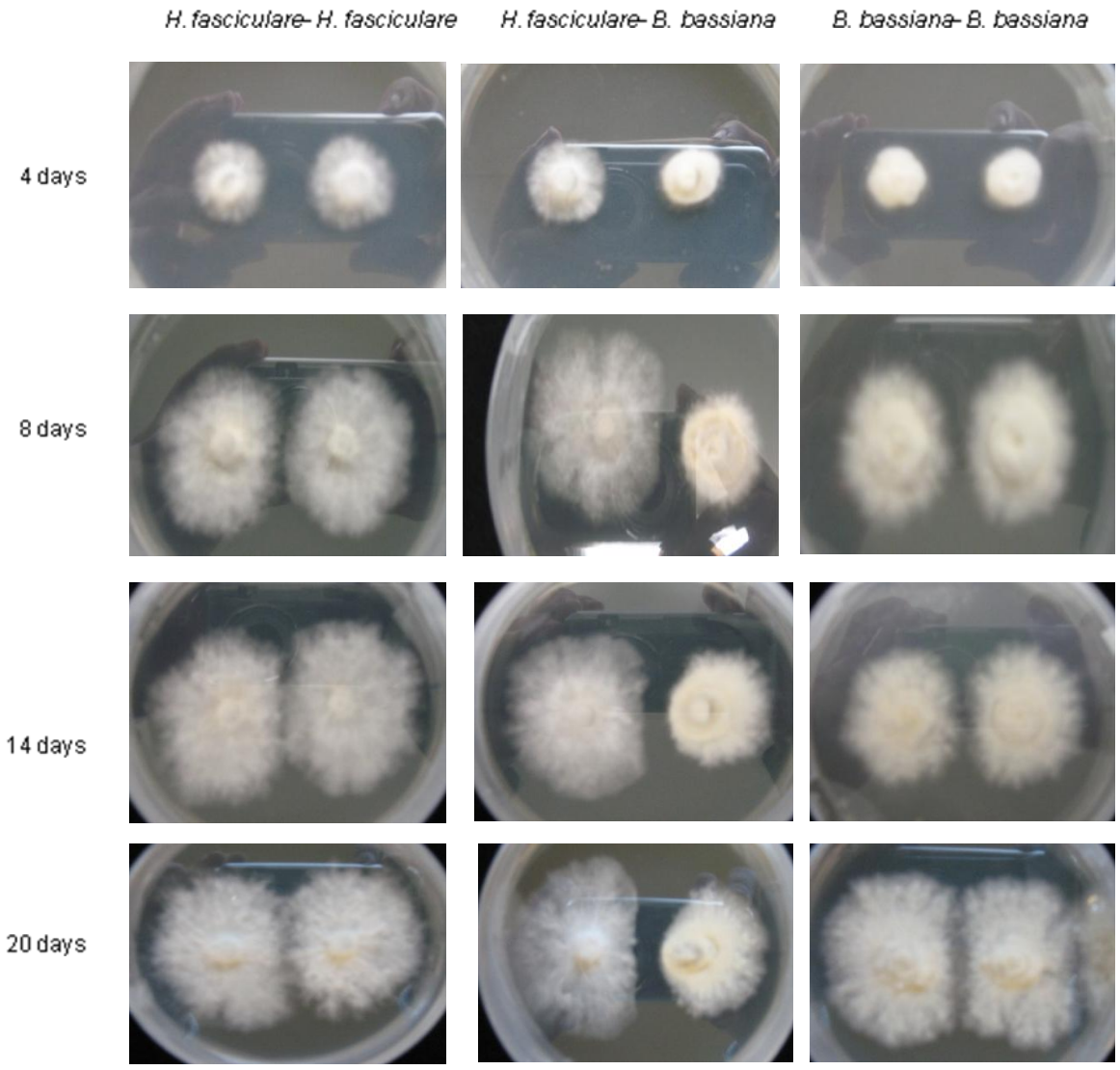


Figure 1. Variation of radial growth of *H. fasciculare* and interacting fungi during the co-culture assay in MMN (A) or PDA (B) media. After co-culture establishment, radial growth was measured for *H. fasciculare* (red ▲,△,■,□) and interacting fungus (blue ▼,▽,■,□), every two days, during 20 days. The internal (triangles) and external (squares) radial growths correspond to the distance from the center of the inoculum to the outside edge of the fungal colony between both inocula or in the opposing region, respectively. The results obtained in the co-culture assay of (a) *H. fasciculare – B. bassiana;* (b) *H. fasciculare – C. sinensis;* (c) *H. fasciculare – E. nigrum;* (d) *H. fasciculare – G. moniliformis;* (e) *H. fasciculare – F. chlamydosporum;* (f) *H. fasciculare – F. oxysporum;* (g) *H. fasciculare – M.*

circinelloides are represented in open symbols, whereas the corresponding controls are displayed in full symbols. All the assays comprised six replicates, from which mean and SEM were derived.

a)



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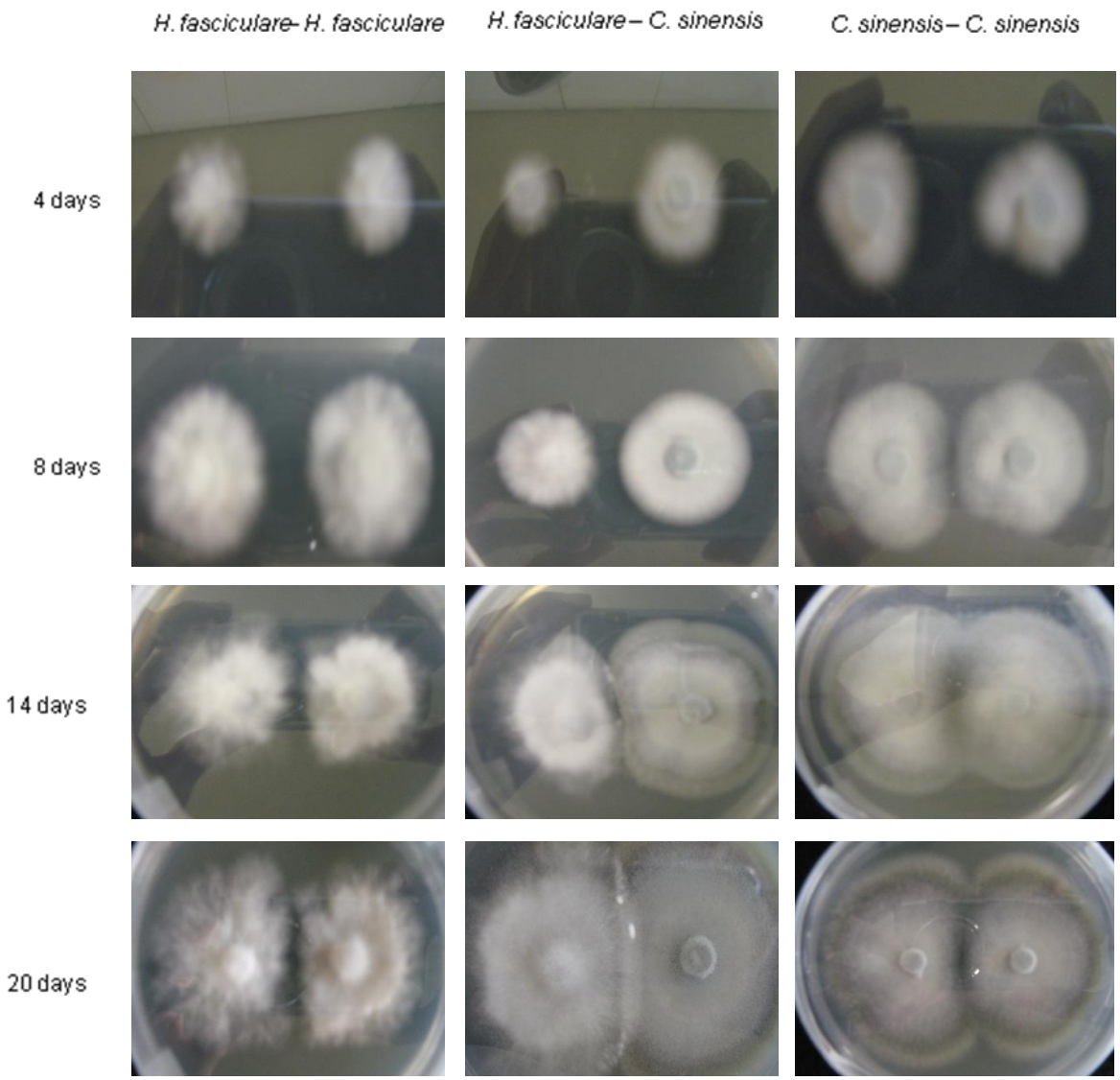
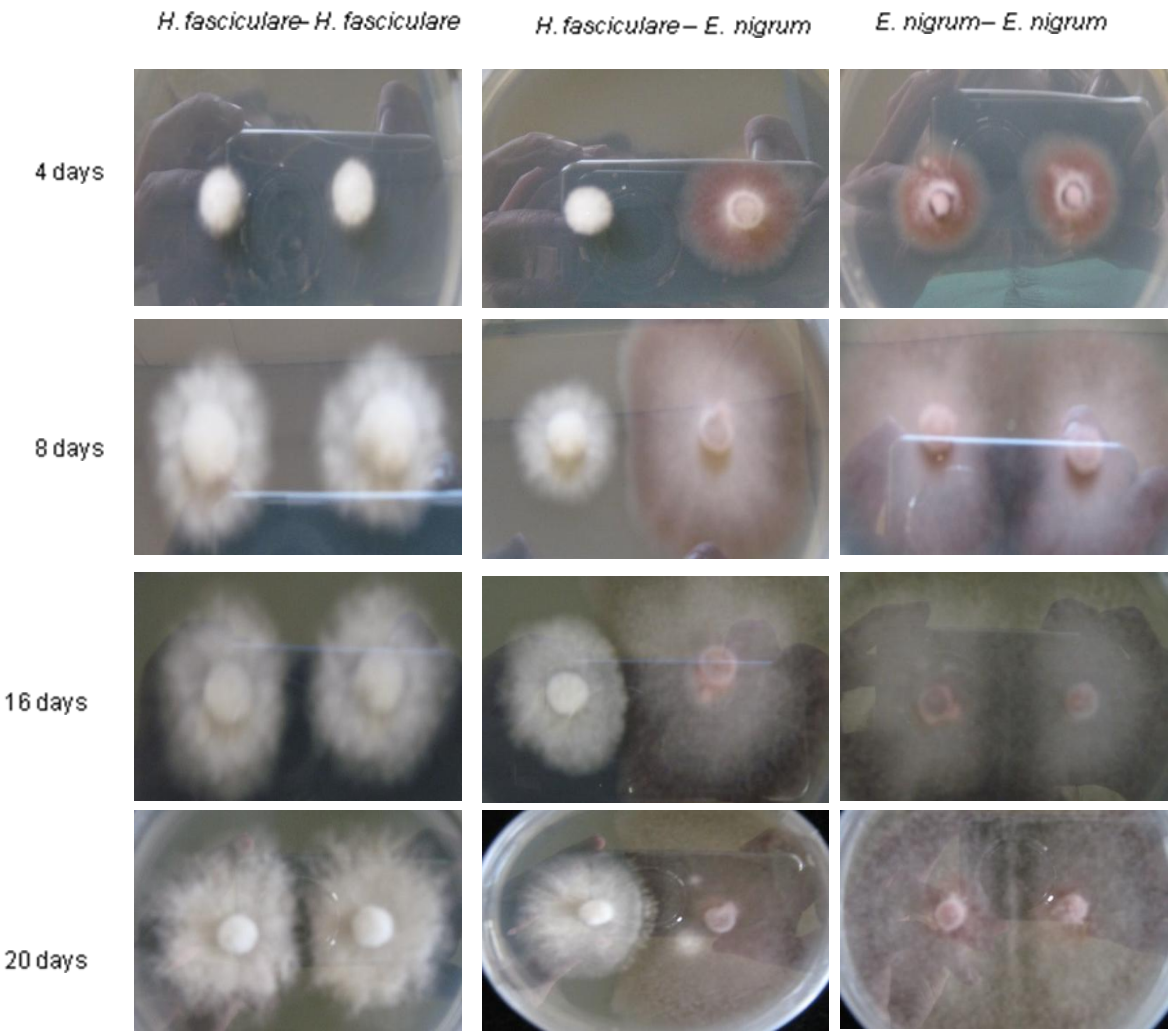
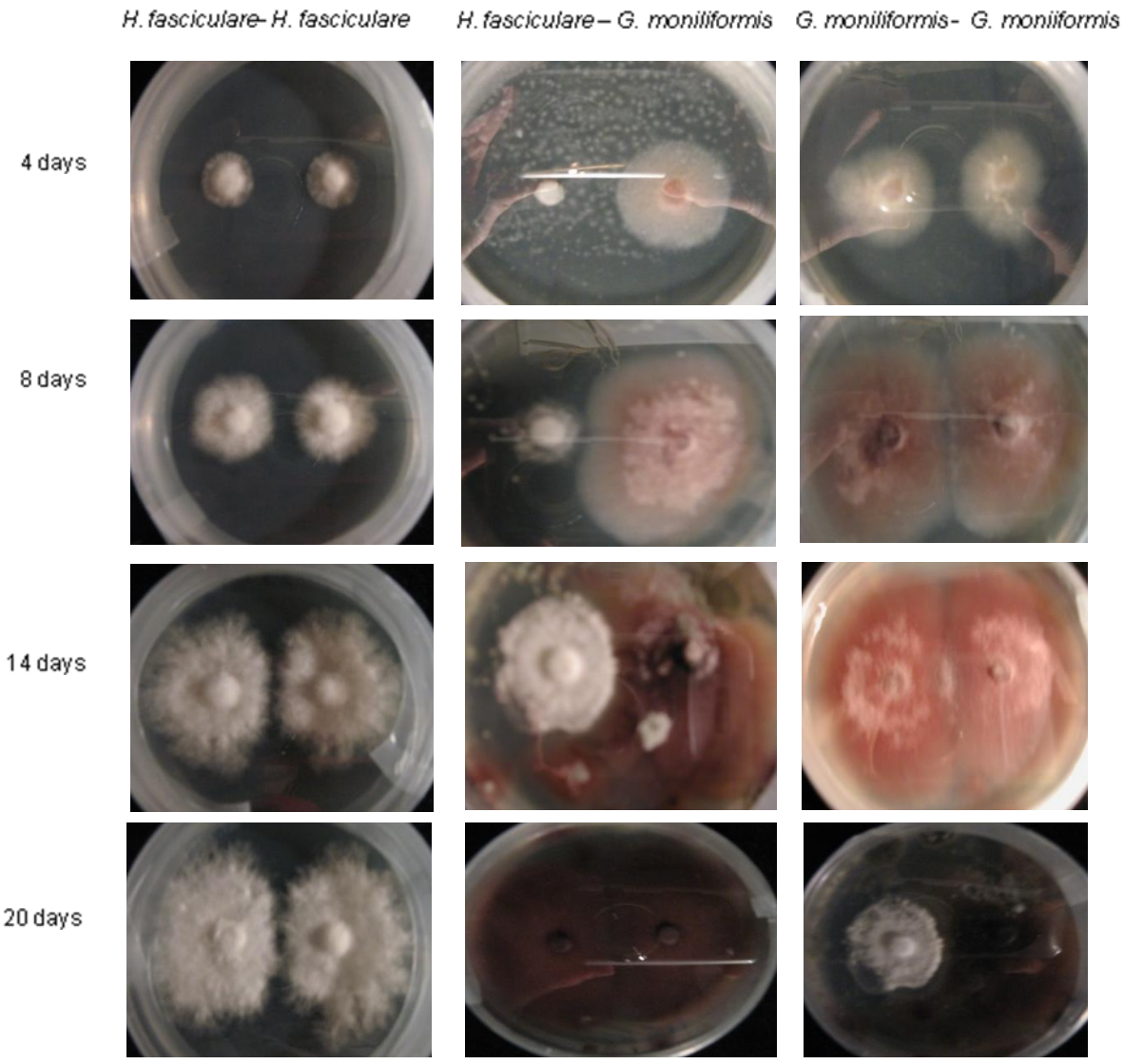

H. fasciculare- H. fasciculare
H. fasciculare – C. sinensis
C. sinensis – C. sinensis
4 days
8 days
14 days
20 days

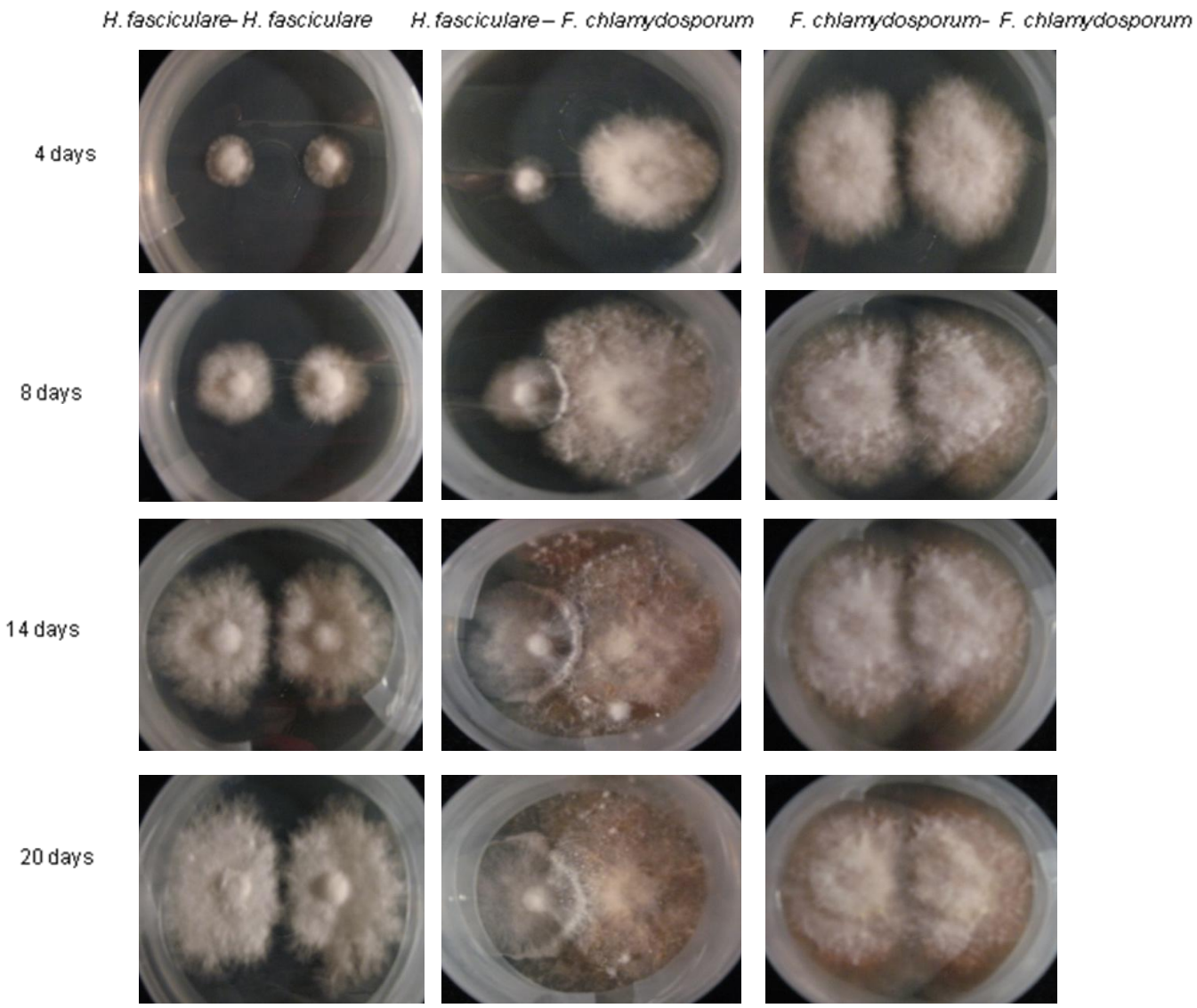
c)


H. fasciculare- H. fasciculare
H. fasciculare – E. nigrum
E. nigrum– E. nigrum
4 days
8 days
16 days
20 days

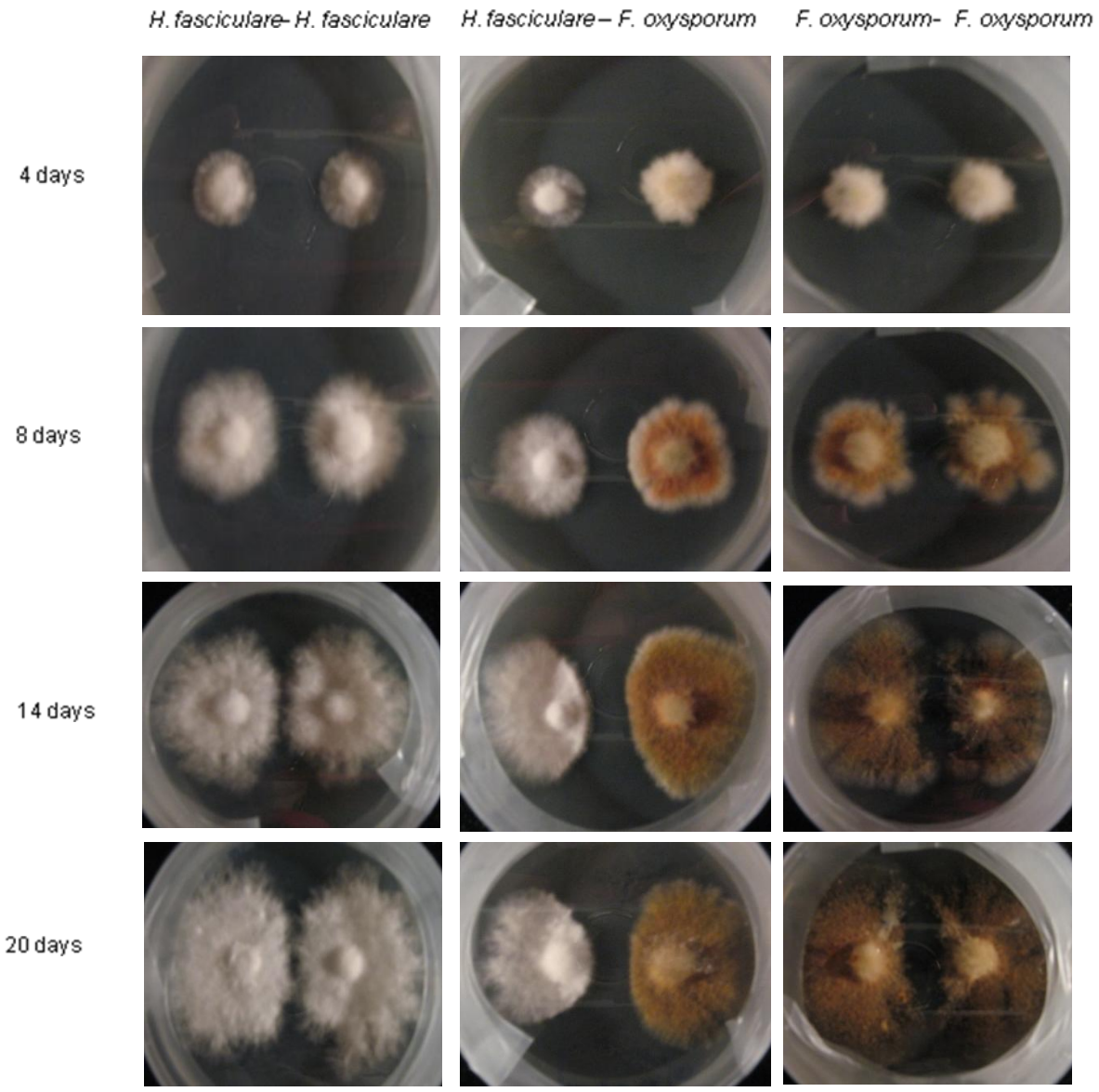
d)


H. fasciculare- H. fasciculare
H. fasciculare – G. moniliformis
G. moniliformis- G. moniliformis
4 days
8 days
14 days
20 days

e)


H. fasciculare- H. fasciculare
H. fasciculare – F. chlamydosporum
F. chlamydosporum- F. chlamydosporum
4 days
8 days
14 days
20 days

f)


H. fasciculare- H. fasciculare
H. fasciculare – F. oxysporum
F. oxysporum- F. oxysporum
4 days
8 days
14 days
20 days

g)

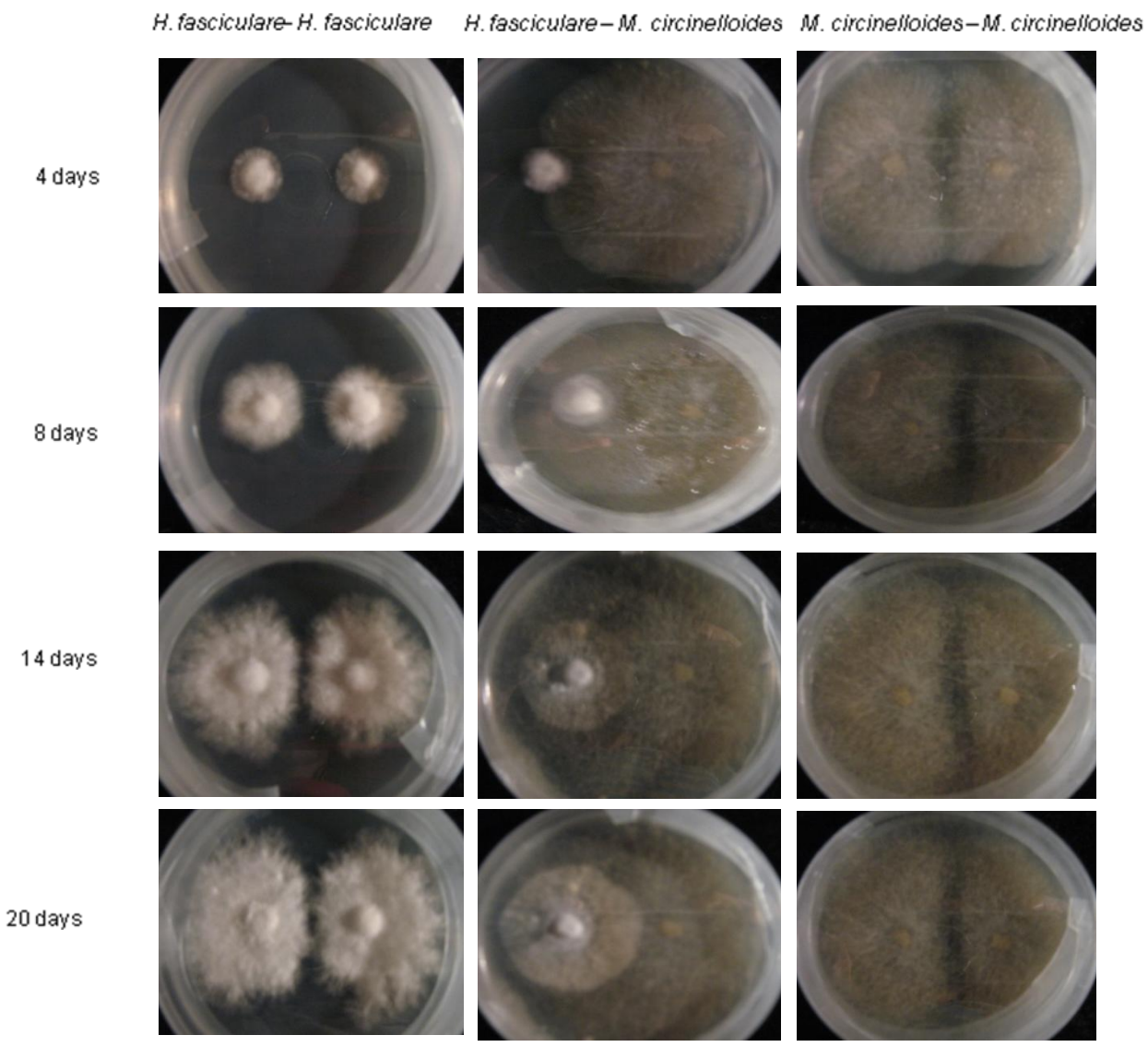


Figure 3. Morphological aspect of co-cultures established between *H. fasciculare-H. fasciculare* (Hf-Hf) in MMN medium; *H. fasciculare*-Interacting fungus (Hf-If); Interacting fungus-Interacting fungus (If-If), during 20 days after inoculation. a) *B. bassiana* (b) *C. sinensis* (c) *E. nigrum* (d) *G. moniliformis* (e) *F. chlamydosporum* (f) *F. oxysporum* (g) *M. circinelloides*

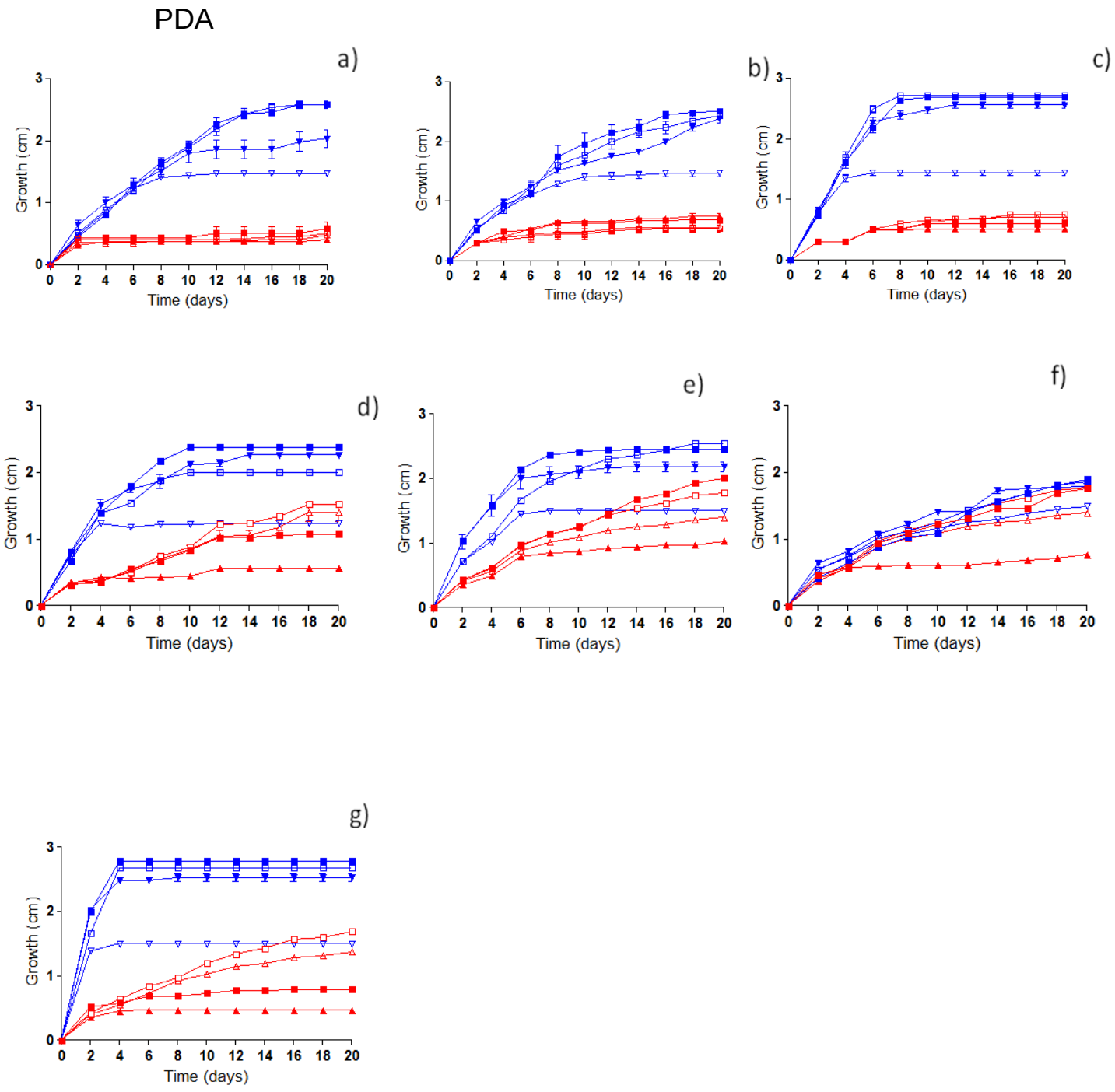
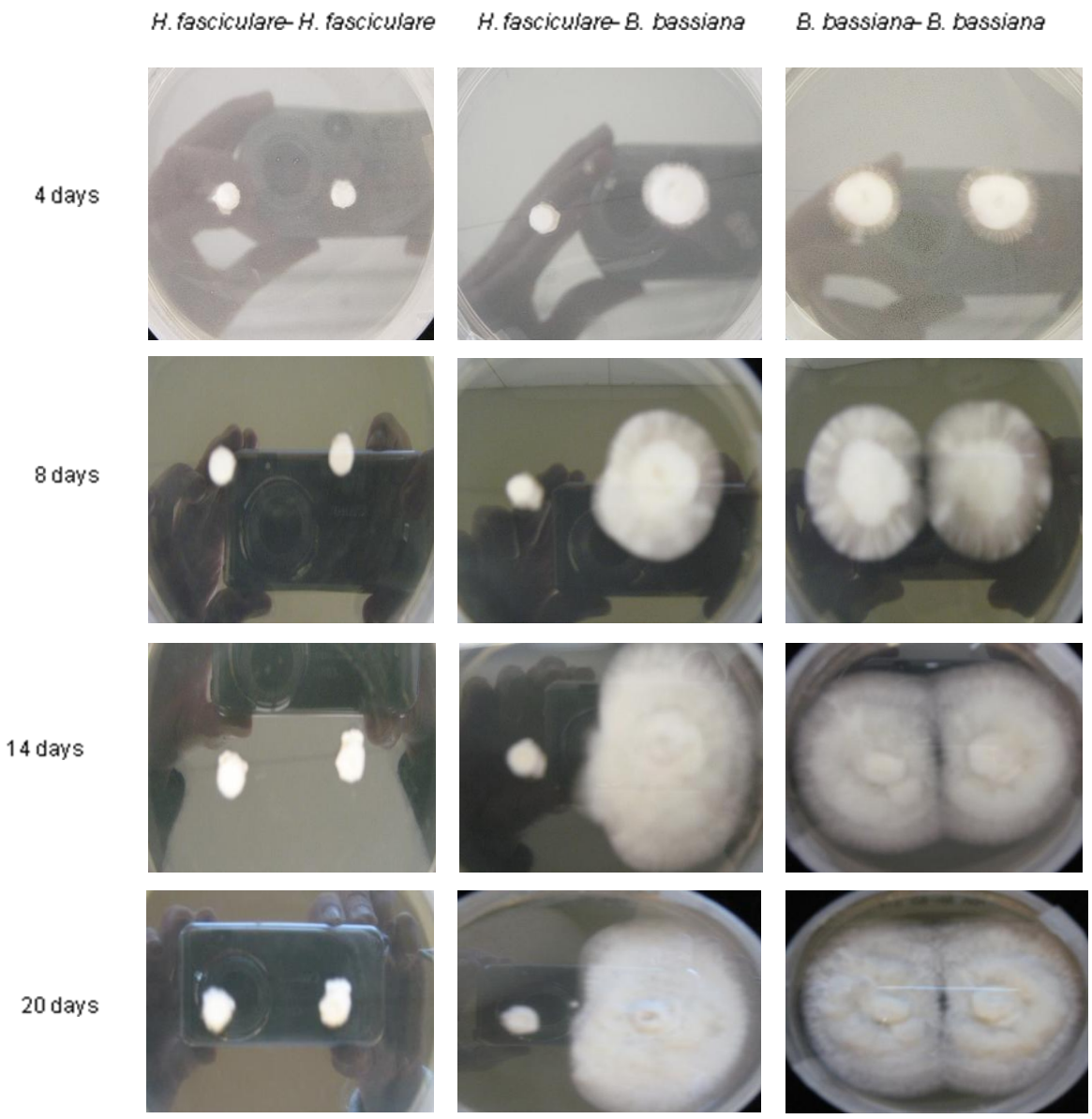


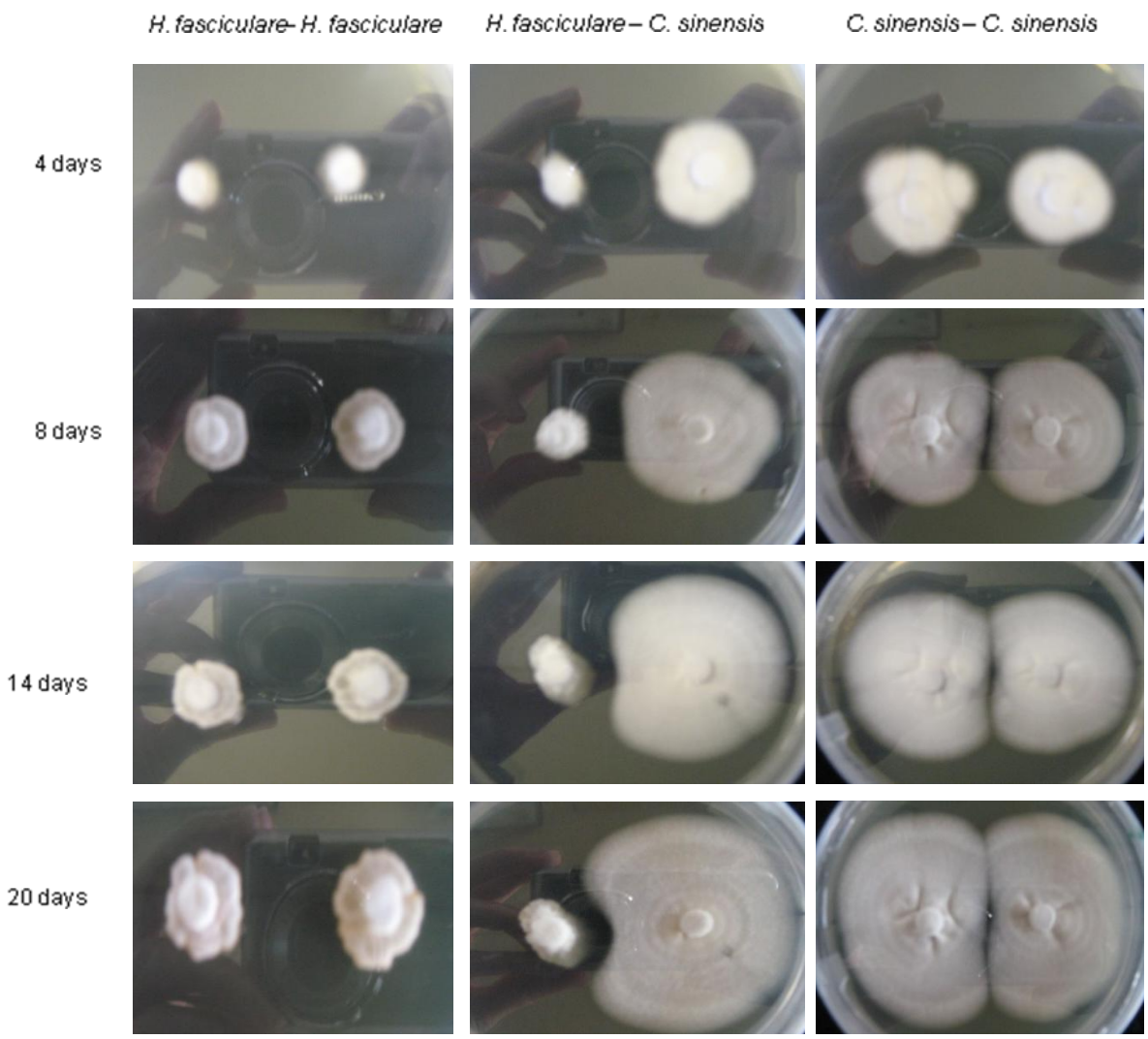
Figure 2. Variation of radial growth of *H. fasciculare* and interacting fungi during the co-culture assay in MMN (A) or PDA (B) media. After co-culture establishment, radial growth was measured for *H. fasciculare* (red ▲,△,■,□) and interacting fungus (blue ▼,▽,■,□), every two days, during 20 days. The internal (triangles) and external (squares) radial growths correspond to the distance from the center of the inoculum to the outside edge of the fungal colony between both inocula or in the opposing region, respectively. The results obtained in the co-culture assay of (a) *H. fasciculare – B. bassiana;* (b) *H. fasciculare – C.*

sinensis; (c) *H. fasciculare – E. nigrum;* (d) *H. fasciculare – G. moniliformis;* (e) *H. fasciculare – F. chlamydosporum;* (f) *H. fasciculare – F. oxysporum;* (g) *H. fasciculare – M. circinelloides* are represented in open symbols, whereas the corresponding controls are displayed in full symbols. All the assays comprised six replicates, from which mean and SEM were derived.

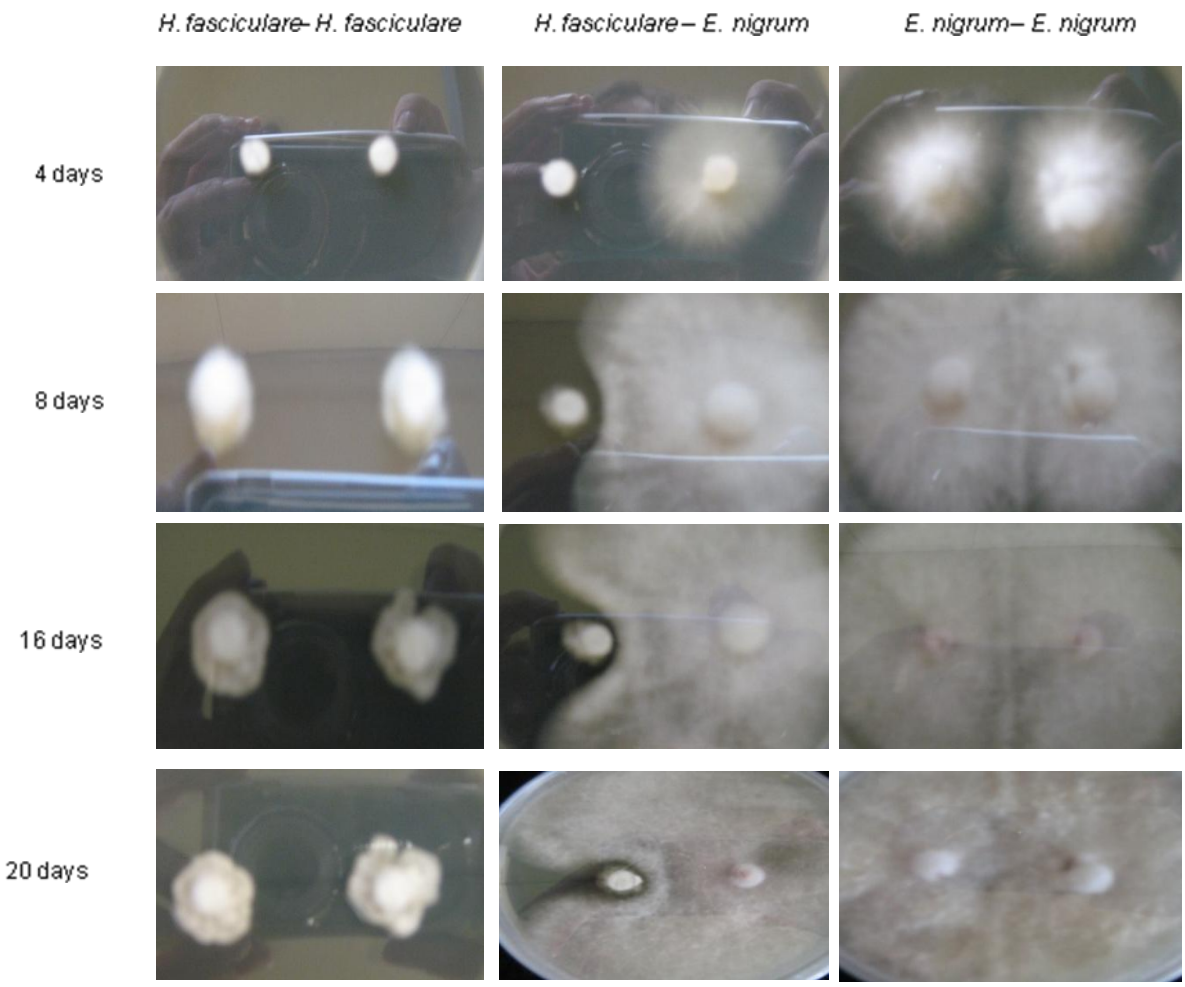
a)



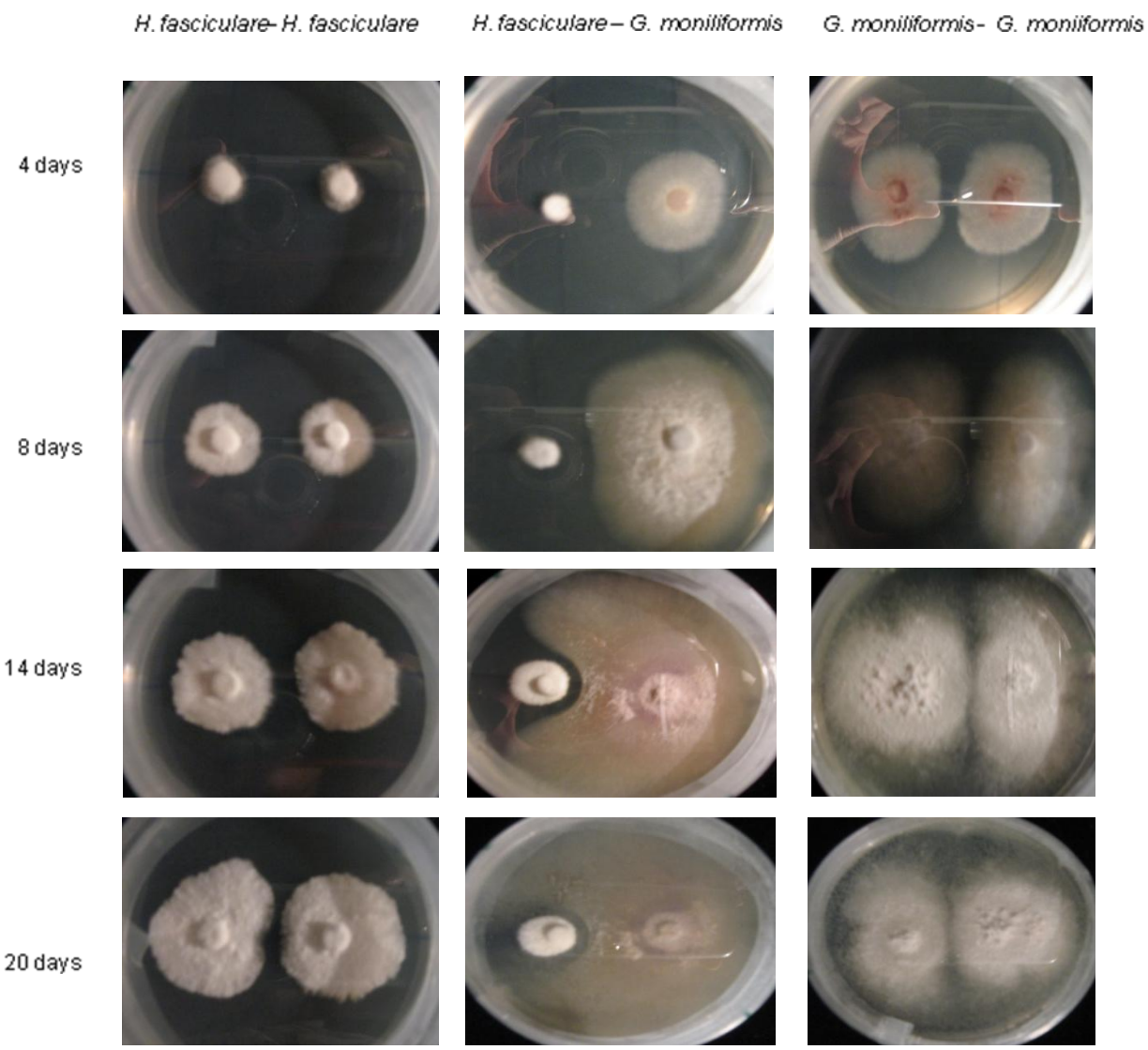
b)


H. fasciculare- H. fasciculare
H. fasciculare – C. sinensis
C. sinensis – C. sinensis
4 days
8 days
14 days
20 days

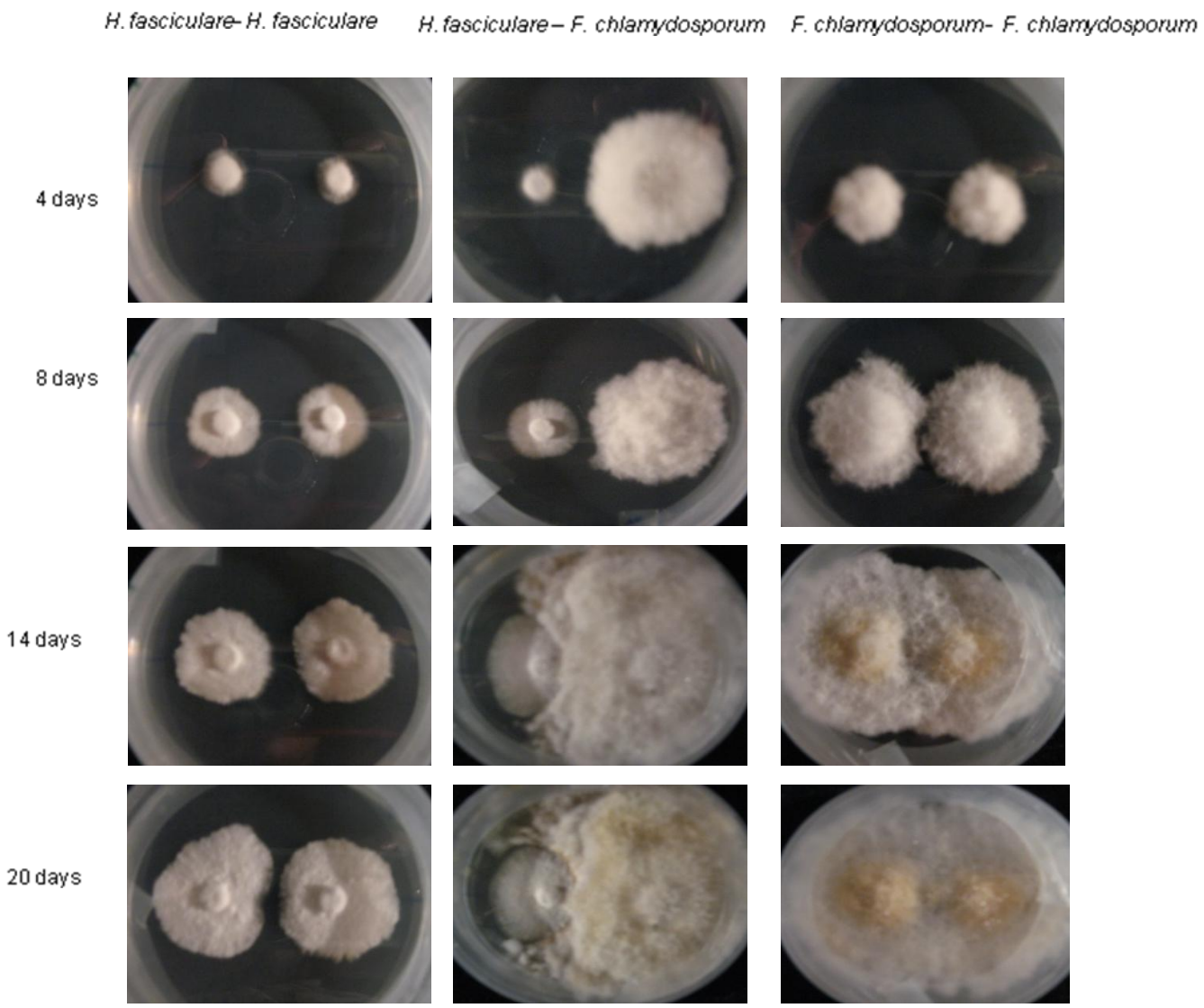
c)


H. fasciculare– H. fasciculare
H. fasciculare – E. nigrum
E. nigrum– E. nigrum
4 days
8 days
16 days
20 days

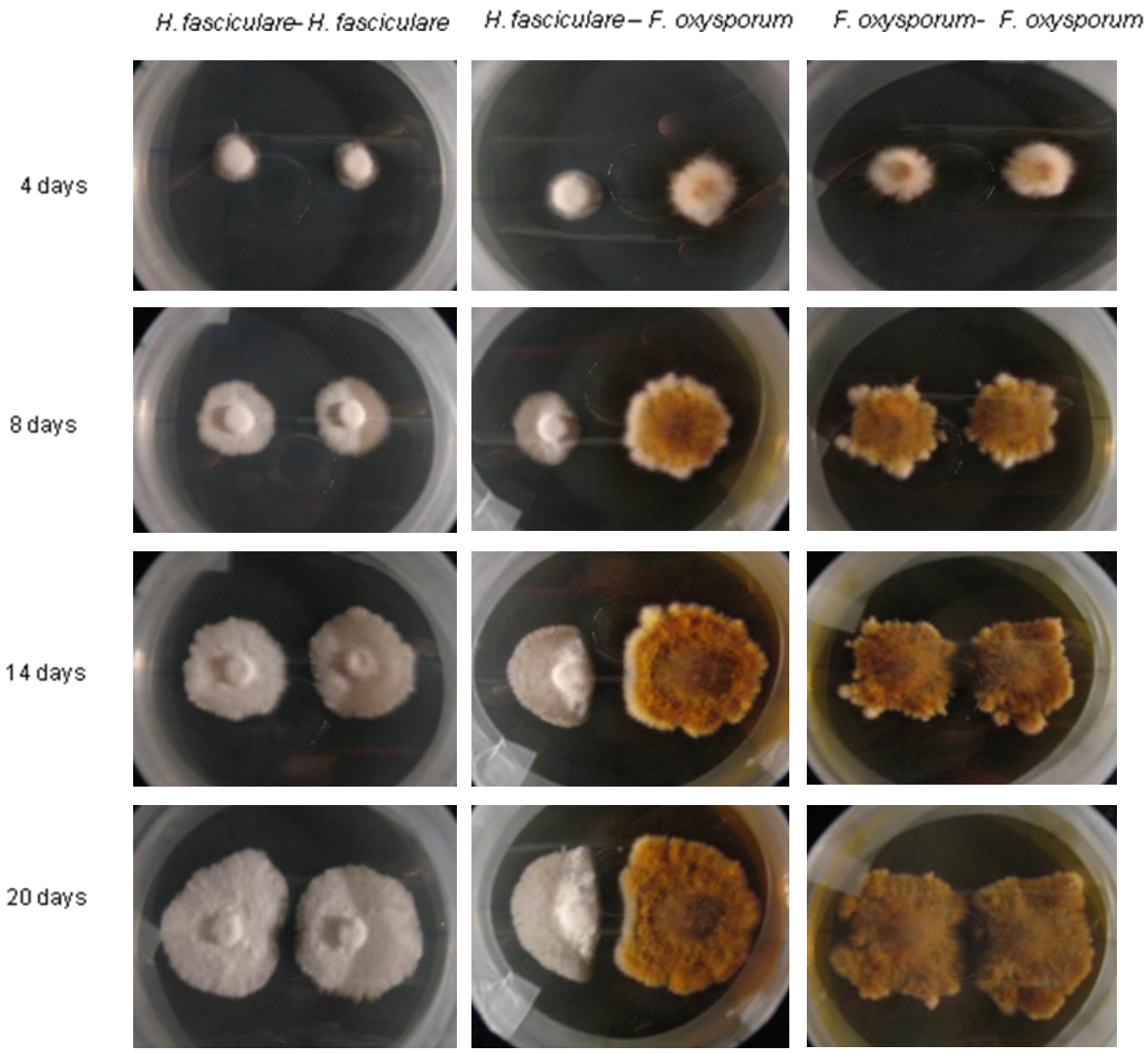
d)


H. fasciculare- H. fasciculare
H. fasciculare – G. moniliformis
G. moniliformis - G. moniliformis
4 days
8 days
14 days
20 days

e)


H. fasciculare- H. fasciculare
H. fasciculare – F. chlamydosporum
F. chlamydosporum- F. chlamydosporum
4 days
8 days
14 days
20 days

f)


H. fasciculare- H. fasciculare
H. fasciculare – F. oxysporum
F. oxysporum- F. oxysporum
4 days
8 days
14 days
20 days

g)

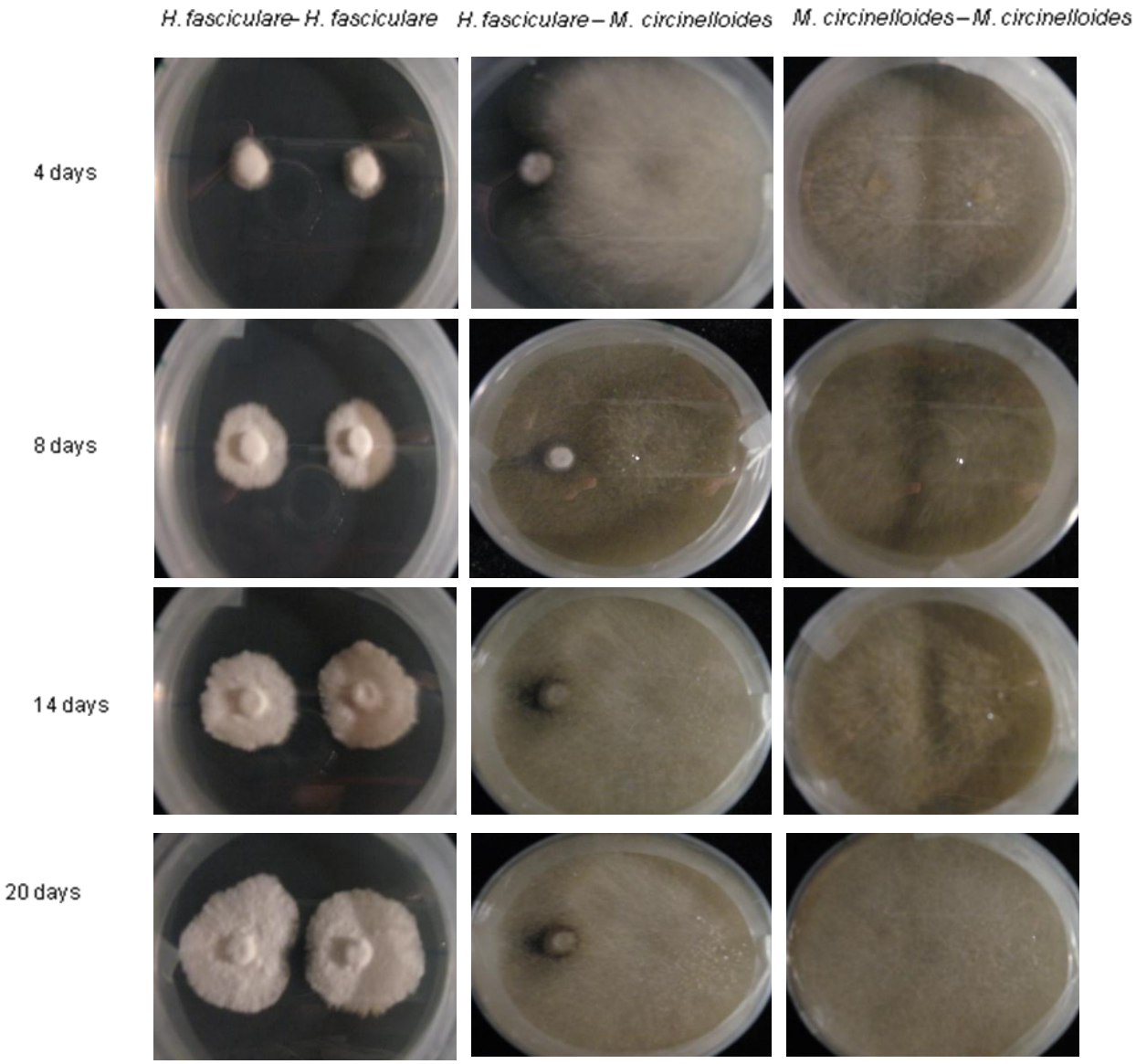


Figure 4. Morphological aspect of co-cultures established between *H. fasciculare-H. fasciculare* (Hf-Hf) in PDA medium; *H. fasciculare*-Interacting fungus (Hf-If); Interacting fungus-Interacting fungus (If-If), during 20 days after inoculation. (a) *B. bassiana* (b) *C. sinensis* (c) *E. nigrum* (d) *G. moniliformis* (e) *F. chlamydosporum* (f) *F. oxysporum* (g) *M. circinelloides*