Trabajo Fin de Grado

Identificación de señales autogeneradas que afectan a la patogenicidad de *Fusarium oxysporum*

Identification of self-generated signals affecting fungal pathogenicity in *Fusarium oxysporum*

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Resumen

Fusarium oxysporum (Fo) es uno de los hongos fitopatógenos más destructivos a nivel mundial afectando a una gran variedad de cultivos vegetales, utilizados tanto como para alimentación humana, como animal. Por ello es esencial conocer cómo funciona su ciclo de reproducción y de infección, y así poder desarrollar estrategias apropiadas de contención. Ambos ciclos están regulados por una señalización autocrina de feromonas y la α-feromona, que modulan diferentes aspectos de la fisiología y patología de los hongos, entre ellos, la germinación, el quimiotropismo y el quorum sensing.

En el hongo modelo *S. cerevisae*, Bar1 es una aspartil proteasa secretada que actúa como "barrera" y antagonista de la señalización de la α-feromona. Dado que las proteasas Bar1 de diferentes hongos (*C. albicans* y *S. cerevisae*) reconocen y tienen un sitos de corte diferentes en la α-feromona, es difícil predecir dónde podría cortar Bar1 de *F. oxysporum* la α-feromona en esta especie fúngica. Aquí mostramos que la proteasa Bar1 de *F. oxysporum*, a diferencia de las de otros hongos (C. albicans y S. cerevisae), no es secretada, tal y como se predijo a través de un estudio bioinformático y por otras evidencias experimentales.

En efecto, utilizando exudados fúngicos de *F. oxysporum* y mutantes de *ΔBar1* pudimos identificar la presencia de una proteasa secretada hasta ahora desconocida, que corta la α-feromona de *F. oxysporum* entre Cys^2 y Thr^3, pero la presencia de Bar1 no fue identificada. No se dieron los mismos cortes en la α-feromona de *F. oxysporum* cuando se incubó directamente con la germínula del hongo. En este caso se detectó un sitio de corte adicional entre Thr^3 y Trp^4 cuando se utilizó germínula wt pero no en germínula de ΔBar1.

Es probable que *F. oxysporum*, al igual que otros hongos filamentosos y a diferencia de los hongos tipo levadura, necesite anclar Bar1 a su membrana por

alguna razón aún desconocida. Para entender mejor esto y el papel que desempeña una proteasa secretada aún no descrita en la señalización autocrina de *F. oxysporum*, serán necesarios más experimentos.

Summary

Fusarium oxysporum (Fo) is one of the most destructive plant pathogenic fungi worldwide, affecting a wide variety of plant crops used for both human food and animal feed. It is therefore essential to gain a better understanding of its reproduction and infection cycles in order to develop appropriate containment strategies. Both are regulated by α-pheromone and autocrine pheromone signalling that modulate different aspects of fungal physiology and pathology, including germination, chemotropism and quorum sensing.

In the model fungus S. cerevisiae, Bar1 is a secreted aspartyl protease that acts as a "barrier" and antagonist of α-pheromone signalling. Given the different recognition and cleavage sites of Bar1 proteases from different fungi (C. albicans and S. cerevisae) on their cognate α-pheromone peptides, it is difficult to predict where Bar1 from F. oxysporum might cleave α-pheromone in this fungal specie. Here we show that the Bar1 protease from F. oxysporum, differently from those from other fungi (C. albicans and S. cerevisae), is not secreted, as predicted through a bioinformatic approach and by further experimental evidences.

Indeed, by using F. oxysporum fungal exudates and ΔBar1 mutants we could identify the presence of a hitherto unknown secreted protease, which cuts F. oxysporum α-pheromone between Cys^2 and Thr^3, but not of Bar1. The same did not hold true when cuts F. oxysporum α-pheromone was incubated directly with fungal germling. In this case an additional cleavage site between Thr^3 and Trp^4 was detected when using wt but not ΔBar1 germlings.

It is probable that F. oxysporum, like other filamentous fungi and differently from yeast-like fungi, needs to anchor Bar1 to its membrane for some yet unknown reason. To better understand this and the role that a yet undescribed secreted protease plays in F. oxysporum autocrine signalling, further experiments will be required.

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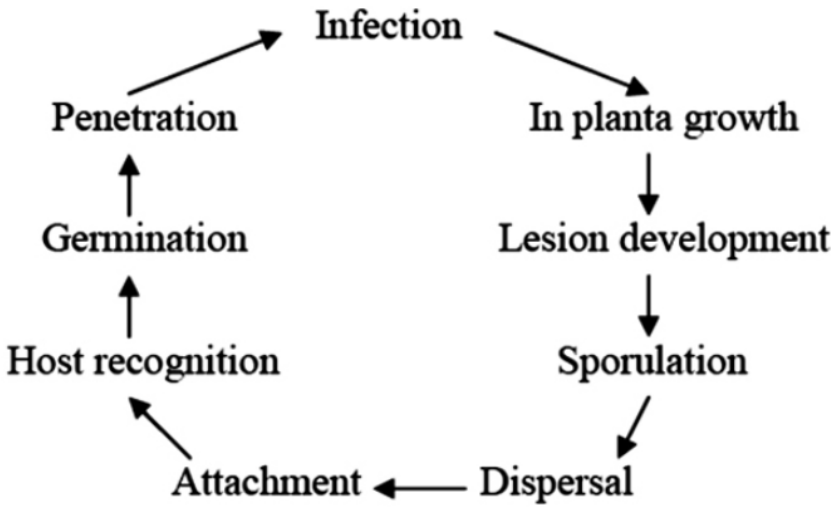
1. INTRODUCTION

PHYTOPATHOGENIC FUNGI

Plant pathogenic fungi represent a major cause of pre- and post-harvest vegetable, cereal and fruit crop losses worldwide. The damage they cause does not only refer to losses in biological production, i.e., to the alteration of the growth and development of the host plants under attack, but also to economic production losses. (Agrios G.N. 2005)

Plant pathogenic fungi are eukaryotic organisms, most of them possessing a defined cell wall, composed of an outer layer of glycoprotein and an inner skeletal layer of β-glucans or α-glucans and chitin (Plaza, Moreno and Castillo 2020), however, unlike plants, they do not contain chlorophyll. They mainly reproduce through spores, dispersal structures of the fungus, that can be sexual, asexual, or both. The basic structures of these fungi are called hyphae, tubular filaments surrounded by a cell wall, that together form an interconnected network termed mycelium. In some cases, hyphae might have specialised functions, i.e. nutrient uptake from the host or penetration (Figure 1) (Juárez-Becerra, Sosa-Morales and López-Malo 2010).

Figure 1. Life cycle of plant pathogenic fungi.



Phytopathogenic fungal interaction with the host plant can be summarised as follows:

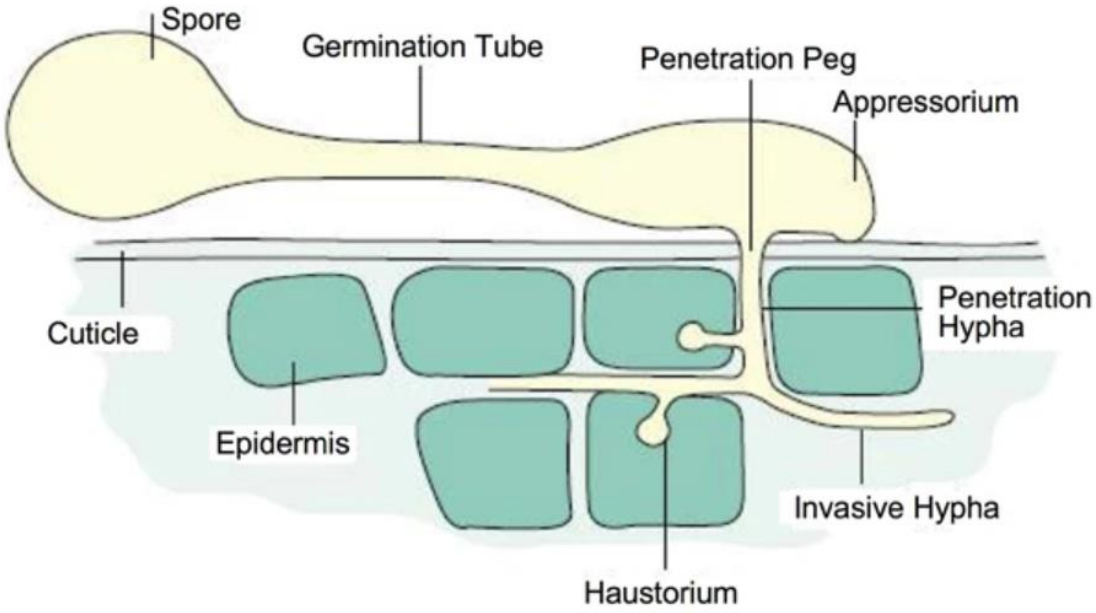
1. Attachment and spore germination on the host plant surface.
2. Formation of specialized infection structures (only for some plant pathogens).
3. Penetration into the external host tissues.
4. Intra- or intercellular colonisation of host tissues.

Importantly, most pathogenic fungi show specificity towards the plant organ to which they attach and very seldomly can attack all organs of the host plant; while some preferentially colonise aerial plant parts others mainly infect roots. Pathogenic fungi can employ different mechanisms to attach to the host plant surface. In either case, penetration of the fungus into the plant requires contact and adherence of the spores and/or the first hyphae resulting from the spore germination process (germination tube) to the plant surface. The mechanisms by which this process is achieved have not been fully understood yet.

In most fungi, spore germination occurs directly, by emitting one or more germ tubes. The process of fungal spore germination is initiated, as in plant seeds, by hydration and enlargement of the spore, hydrolysis of endogenous energy reserves, and synthesis of proteins, membrane and cell wall structural materials necessary for germ tube emergence and elongation. Several endogenous and exogenous factors are known to regulate spore germination. Normally, spores remain in a dormant or metabolically quiescent state, where germination is prevented by various physical or biochemical factors (*i.e.*, water, temperature, light, microbial activity and inhibitors and stimulants) and that influence this process in a species-specific manner. Soon after germination, elongating germ tubes emerging from the spores can undergo changes in the orientation axis in response to both chemically- (chemotropism) or surface-generated (thigmotropism) stimuli, processes that are either important to finely localizing

host cells or to penetrate into appropriate entry portals present on the surface of the host. In some cases the oriented growth of germ tubes requires their adherence to plant surfaces, which takes place through the production of a dense extracellular matrix composed of polysaccharides and/or glycoproteins (Rivera and Codina 1997). Pathogenic fungi have developed efficient strategies to invade and grow within plant hosts. Indeed, in contrast to bacteria, multicellular fungi do not only penetrate through the natural openings present on the surface of plant tissues but can also actively penetrate via the use of specialized structures (Figure 2) (Schäfer 2014).

Figure 2. Example of penetration via appressoria.



FUSARIUM OXYSPORUM

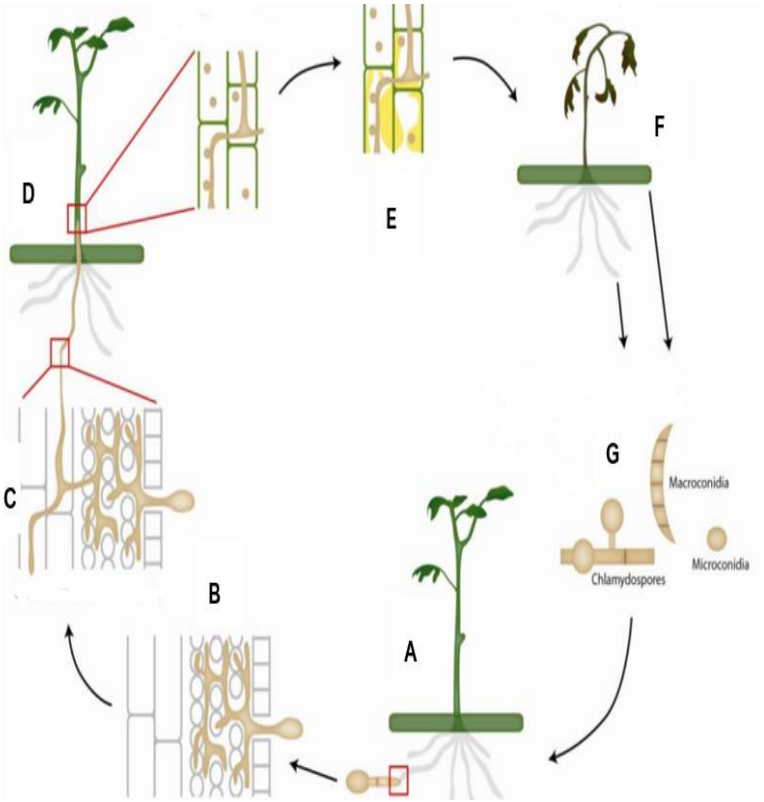
Fungi belonging to the genus *Fusarium* are ascomycetous plant pathogens that are distributed worldwide and adapted to a wide variety of geographical areas, climate conditions, ecological habitats and host plants. *Fusarium* wilt pathogens show a high level of host specificity and, based on the plant species and plant cultivars they can infect, they are classified into more than 120 *formae speciales* and races. (Fravel, Oliviain and Alabouvette 2002). These ascomycetes are

normally found in soil, water and decaying organic matter; the widespread distribution of the genus stemming from its ability to grow on a wide range of substrates and to efficiently persist in the soil for long period of time. In addition to this, Fusaria can cause a broad spectrum of diseases in humans, ranging from superficial or localized infections in healthy hosts to lethal disseminated fusarioses in immunocompromised patients.

Different isolates of *F. oxysporum* attack a wide range of economically important crops leading to Fusarium crown and root rot, damping-off and, most commonly, vascular wilts. Importantly, vascular wilt represents a major cause of production losses in several agricultural crops. (Jones *et al.* 2015)

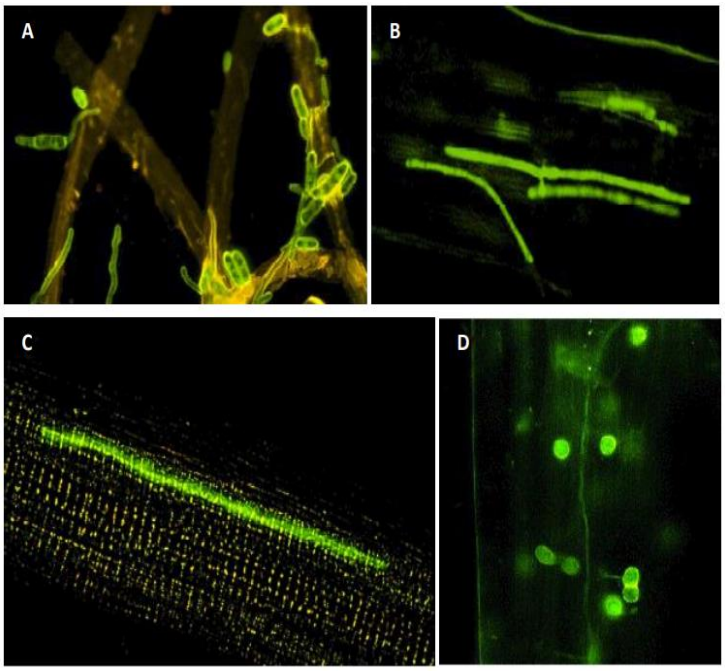
F. oxysporum infectious cycle (Figure 3) starts with conidial germination in the soil, directional growth of fungal hyphae towards the host root and root penetration (Figure 4A). Subsequently, hyphae enter the root cortex, in most cases through the apex, where the endodermis is not completely differentiated. Once inside the root, hyphae grow inter- and intra-cellularly to invade the cortex tissues and cross the endodermis, until they reach the xylem vessels (Figure 4B). The fungus then uses the xylem as an avenue to colonize the host (Figure 4C) (Pérez-Nadales and Di Pietro 2011), where it spreads among cells through the release of microconidia, which are massively produced in this stage of the infectious process. Plant defense responses happen mostly in the vasculature, mainly involving callose deposition, accumulation of phenolics and formation of tyloses and gels in the infected vessels, that combined with the increase of fungal burden, block water streaming through the vessels, leading to progressive wilting and death of the plant.

Figure 3*. F.oxysporum* life cycle.



(A) Germination in response to host signals and direct penetration of the root. **(B)** Invasion of the root cortex. **(C)** Colonisation of the xylem vessels. **(D)** Hyphae and conidia spread through the xylem. **(E)** Fungal mycelium and plant-produced vascular gels plug the xylem vessels. **(F)** Wilting and death of the plant**. (G)** Formation of macro-microconidia and thick-walled chlamydospores on the dead plant tissue and in the soil. (Pérez-Nadales and Di Pietro 2014)

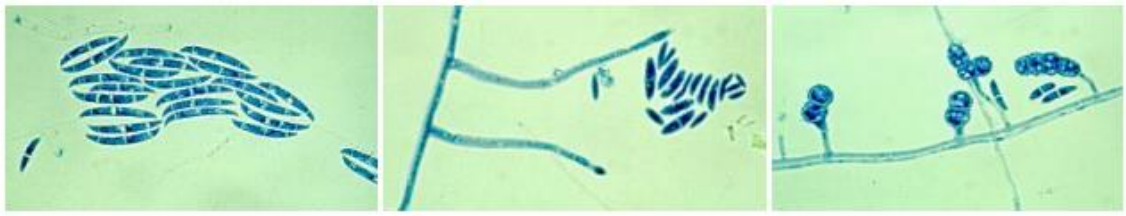
Figure 4. Penetration and colonization of tomato roots by *F. oxysporum.*



A. Germinated microconidia and penetration hyphae of *F. oxysporum* attaching to the root surface 24 h after inoculation. **B**. and **C**. Infection hyphae of *F. oxysporum* growing in the root cortex, 5 days after inoculation **(B);** and in a root xylem vessel, 7 days after inoculation **(C).** **D.** Chlamydospores of *F. oxysporum* wildtype strain produced on dying plant tissue. (Di Pietro, García-Maceira *et al.* 2001)

F. oxysporum spp. lacks a known sexual cycle. Despite this, vegetative hyphal fusion and heterokaryon formation have been suggested to ensure genetic variability through horizontal gene transfer (Kistler 1997). Asexual reproduction occurs through three types of conidia: microconidia, macroconidia and chlamydospores (Figure 5). Microconidia are single-cell dispersal structures that are abundantly produced under most conditions. Macroconidia contain three to five cells and are gradually pointed and curved toward the ends and are commonly found on the surface of dead plants killed by the pathogen (Pérez-Nadales and Di Pietro 2011). Chlamydospores are thick-walled cells generally developed through the modification of hyphal and conidial cells. Their formation is induced by aging or unfavorable environmental conditions such as low temperature or carbon starvation. Chlamydospores represent the principal structure for long-time survival during unfavorable periods in the soil and play an important role as primary inoculum for plant root infection. (Schäfer 2014)

Figure 5. Types of *F.oxysporum* asexual spores.



Macroconidia Microconidia Chlamydospores.

PHEROMONES AND SEXUAL REPRODUCTION IN ASCOMYCETOUS FUNGI

In nature, sexual reproduction is a fundamental mechanism by which eukaryotic organisms produce better-adapted recombinant progeny. Ascomycetous fungi have evolved two main reproductive styles, heterothallism (self-incompatibility) and homothallism (self-compatibility). Fungi requiring a compatible partner (genetically different for the composition of a sexual identity locus, but

morphologically identical individuals from the same species) to mate are called heterothallic, whereas those not requiring a mating partner and carrying both sexual identity loci in their genome are homothallic. A few other fungal species are pseudohomotallic, i.e. they are self-fertile, but the fusing nuclei must be genetically different at their sexual identity locus (Bennett and Dunny 2010). Importantly, in order to fuse and mate, fungal cells secrete and sense species-specific chemicals (pheromones) to coordinate cell behaviour and cell-to-cell attraction.

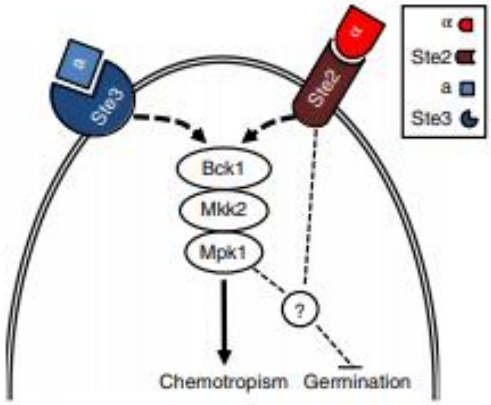
Fungi have been intensively studied because of their use of sexual pheromones to regulate intercellular (paracrine) signalling and conjugation. In ascomycetes, pheromones are small peptides or lipopeptides that are secreted into the extracellular milieu and able to induce morphological and transcriptional responses in target cells, where mating specificity is determined by the sets of pheromones and pheromone receptors expressed by each different cell type (Jones, *et al*. 2015).

Synthesis and secretion of pheromones and their receptors is controlled by a single genetic locus named mating type (*MAT*) locus. Heterothallic species have two opposite *MAT* loci (usually indicated as *MATα* and *MATa*) in different nuclei in contrast to homothallic species, whose carry both *MAT* loci in a single nucleus, usually closely linked or fused (Bennett and Dunny 2010). In the model ascomycetous fungus *Saccharomyces cerevisiae*, haploids cells of a specific *MAT* locus secrete each a peptide pheromone which is recognized by a cognate G-protein coupled receptor (GPCR) on a cell from the opposite mating type (*MATα* cells secrete α-pheromone which is sensed by Ste2 in *MATa* cells, while *MATa* cells secrete a-factor which is sensed by Ste3 in *MATα* cells) (Wang and Dohlman 2004).

Despite *F. oxysporum* is a heterothallic fungal specie either existing as *MATα* or *MATa* populations, recent studies have shown that *F. oxysporum MATα* cells can

actively produce both pheromone peptides, a- and α-pheromone, and their receptors, respectively Ste3 and Ste2 (Vitale *et al.* 2019). Importantly, in *F. oxysporum* cells secretion and sensing of their own pheromone peptides leads to an autocrine signalling mechanism (Figure 6) regulating different physiological outputs .

Figure 6. Pheromone signalling in *F. oxysporum*.

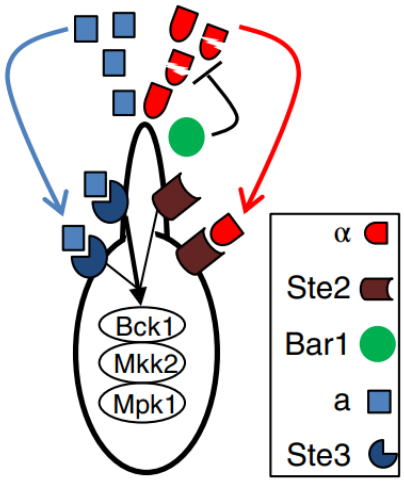


ROLE OF α-PHEROMONE IN QUORUM SENSING

Autocrine self-signalling via secreted peptides and cognate receptors regulates cell development in a variety of eukaryotes (Doğaner, Yan and Youk 2016). However, secreted peptides from higher fungi have been traditionally associated with paracrine non-self-signalling during sexual reproduction. In the model fungus *S. cerevisiae*, cells fall into two distinct mating types (MAT), which produce either a- or α-pheromone and the cognate receptors Ste2 or Ste3, respectively (Lee *et al.* 2010).

In yeast cells, down-regulation of the self-pheromone receptor and a-cell-specific cleavage of α-pheromone through the pheromone-specific aspartic protease Bar1 prevents inappropriate autocrine pheromone signalling (APS) during mating (Figure 7)(Alby, Schaefer and Bennett 2009). While APS can be artificially induced by manipulation of the pheromone secrete-and-sense circuit, its natural occurrence in ascomycete fungi has been described only recently (Dean, et al. 2012) (Vitale, Di Pletro and Turrá 2019).

Figure 7. Model for APS in a population of *F. oxysporum* cells.



Autocrine pheromone signalling (APS) in *F. oxysporum* MAT α cells is mediated by simultaneous secretion of a- and α-pheromone, with a-pheromone signalling prevailing as a result of Bar1-mediated depletion of α-pheromone pools. (Vitale, Di Pletro and Turrá 2019)

Indeed, despite *F. oxysporum* sexual cycle has not been described yet, its cells are able to co-express simultaneously both pheromone–receptor pairs (a-Ste3 and α-Ste2), resulting in autocrine regulation of physiological programs other than mating.

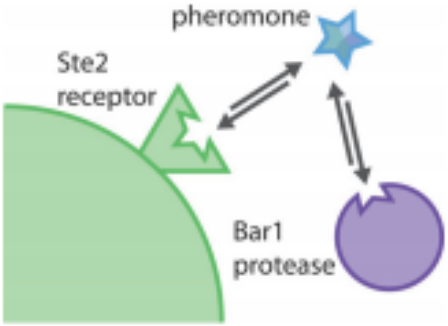
F. oxysporum unisexual cells express respond to α- and a-pheromone through conserved elements of the cell wall integrity mitogen-activated protein kinase (MAPK) cascade. While α-pheromone signalling inhibits conidial germination via Ste2, a-pheromone signalling activates it via Ste3. Importantly, at high concentrations of α-pheromone such as those found at high but not low inoculum densities, Ste2- prevails over Ste3-signalling thus inhibiting conidial germination. Of note, Ste2 in *F. oxysporum* also responds to the activity of plant peroxidases to promote chemotropic growth and infection (Martín 2019). Thus, this phytopathogenic fungus uses the same signalling pathway for both chemotropic sensing of mating factors and host cues or to regulate fungal development via quorum sensing (Vitale, Turrá and Di Pletro, 2019).

α-PHEROMONE AND BAR1 PROTEASE

Bar1 is a conserved aspartyl protease acting as a "barrier" and antagonist to α-pheromone signaling in multiple ascomycetous fungal species. There, the presence of Bar1 has been correlated with the ability of fungal cells to prevent undesired therefore prevents α-pheromone autocrine signaling events (Figure 8) (Jones *et al.* 2015).

Previous studies in *F. oxysporum* have demonstrated that an increase in the concentration of fungal germlings (up to five folds) causes an appreciable rise in both Bar1 and α-pheromone gene expression (Vitale, Di Pletro and Turrá 2019).

Figure 8. Interrelationships between Ste2, α-pheromone, and the Bar1protease.



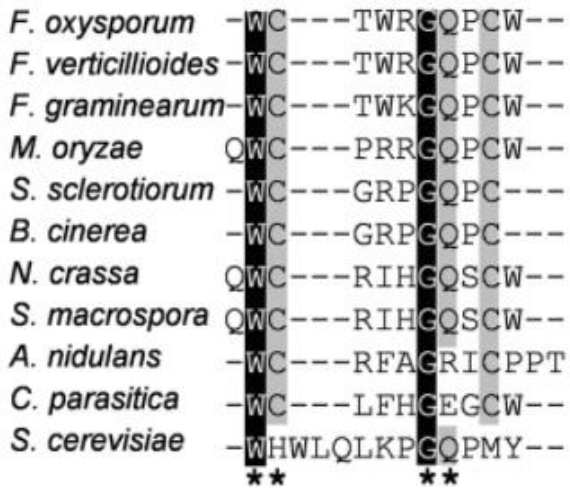
Model of interactions between Bar1, Ste2, and α-pheromone. Both Bar1 and Ste2 recognize the same or overlapping regions of the α-pheromone peptide, thereby facilitating coevolution of their substrate specificities together with pheromone divergence during speciation (Jones *et al.* 2015).

The α-pheromone from *F.oxysporum* is a small peptide of 10 aminoacids (WCTWRGQPCW) exerting different biological functions as it acts as a growth regulator, a chemoattractant and also as a quorum-sensing signal (Turrà *et al.* 2015) (Turrá and Di Pietro 2015) (Vitale, Di Pletro and Turrá 2019). Interaction of α-pheromone with its plasma membrane cognate receptor, Ste2, leads in turn to the activation of a conserved mitogen activated protein kinase (MAPK) pathway (Turrà *et al.* 2015), which triggers chemotropic growth and regulates fungal community behavior via inhibition of spore germination (Vitale, Di Pletro and Turrá 2019). In addition, α-pheromone also inhibits *F.oxysporum* growth and cell

division in a Ste2-independent manner. However, how α-pheromone-Ste2 interaction induces such a large set of cellular responses is currently unknown (Partida-Hanon *et al.* 2020).

Heterologously expressed Bar1 proteases from *S. cerevisae* and *Candida albicans* have been purified and biochemically characterized. Notably, the results of their interaction with the cognate pheromone peptide from the same fungal specie occurs at different amino acidic residues. In fact, while α-pheromone cleavage by Bar1 occurrs between residues Thr^{5} and Asn^{6} in the *C. albicans* tridecapeptide (GFRLTNFGYFEPG), *S.cerevisae* Bar1 cleaves the α-pheromone tridecapeptide (WHWLQLKPGQPMY) between Leu^{6} and Lys^{7} (Jones *et al.* 2015) (Naider and Becker 2003). Thus, given the difference in length and primary structure among the α-pheromone from *F.oxysporum* and those from *S.cerevisae* and *C. albicans* (Figure 9), it is difficult to predict potential Bar1 cleavage sites in the *F.oxysporum* pheromone peptide. Importantly, the α-pheromone from *F.oxysporum* and from few other filamentous fungal ascomycetes presents a couple of highly conserved amino acids at both its N- and C-termini, respectively Trp^{1}-Cys^{2} and Cys^{9}-Trp^{10} (Figure 9).

Figure 9. Amino acid alignment of predicted mature α-pheromone of *F. oxysporum* with orthologues from other ascomycete fungi.



Absolutely and highly conserved residues are shaded in black and in grey, respectively (Turrà *et al.* 2015).

2. OBJECTIVES

A. Bioinformatic analysis of Bar1 similarities/differences among different fungal species

B. Identify potential Bar1 cleavage sites in *F. oxysporum* α-pheromone

C. Identify potential Bar1-independent cleavage sites in *F. oxysporum* α-pheromone

3. MATERIALS Y METHODS

FUSARIUM OXYSPORUM GROWTH CONDITIONS

To collect fresh *F. oxysporum* microconidia to be used in the experiments, *F. oxysporum* conidia were inoculated in YPD medium supplemented with the required selective agent (Table 1-3), orbitally shacking at 28°C for 3-4 days and 170 rpm. Cultures were collected by filtration through a nylon filter (Monodur; mesh size 10 µm). Filtrates were centrifuged at 5000 rpm for 10 min, microconidia were collected and resuspended in sterile deionized water, then counted using a haemocytometer.

Freshly recovered microconidia were resuspended in sterile deionized water with 45% glycerol (v/v) and stored at - 80ºC. These suspensions were used for later inoculation to obtain fresh microconidia. A complete list of *F. oxysporum* strains used in this study is reported in Table 2. All media solutions used throughout this work are described in Table 1. Synthetic *F. oxysporum* α-pheromone (WCTWRGQPCW) or an N-terminally fluorescein isothiocyanate (FITC) labelled version thereof were obtained from GenScript.

Table 1. Media solutions used in this work.

Media solution	Ingredients and preparation
Minimum medium with 25 mM glutamate (Mm 25 mM)	Dissolve in 0,5L of water, 0,25g of MgSO4 x 7·H2O, 0,5g of KH2PO4, 0,25g of KCl, 1g of NaNO3, 15g of sucrose and 2,1g of glutamate. Add NaOH until the solution is adjusted to pH=7,4. Sterilize by autoclaving.
Minimum medium with 5 mM glutamate (Mm 5 mM)	Dissolve in 0,5L of water, 0,25g of MgSO4 x 7·H2O, 0,5g of KH2PO4, 0,25g of KCl, 1g of NaNO3, 15g of sucrose and 0,42g of glutamate. Add NaOH until the solution is adjusted to pH=7,4. Sterilize by autoclaving.
Yeast extract peptone dextrose medium (YPD)	Dissolve in 0,5L of water, 5g of yeast extract, 10g of glucose and 10g of tryptone. Sterilize by autoclaving. Add NaOH until the solution is adjusted to pH=8, for mutants to be treated with phleomycin.

Table 2. Strains of *Fusarium oxysporum* used in this study.

Strain	Genotype	Selective agent	Reference
4287	Wild type	-	(Di Pietro and Roncero 1998)
*Bar1*Δ	Knockout mutant of the *Bar1* locus	Phleomycin	(Vitale, Di Pletro and Turrá 2019)
Bar1Δ+Bar1	Insertional mutant obtained by reinserting the native *Bar1* locus	Phleomycin Hygromycin	(Vitale, Di Pletro and Turrá 2019)

αΔ	Knockout mutant of the *α* locus	Hygromycin	(Vitale, Di Pletro and Turrá 2019)
aΔ	Knockout mutant of the *a* locus	Hygromycin	(Vitale, Di Pletro and Turrá 2019)
αΔaΔ	Knockout mutant of the *a* and *α* loci	Phleomycin Hygromycin	(Vitale, Di Pletro and Turrá 2019)
Ste2Δ	Knockout mutant of the *Ste2* locus	Hygromycin	(Turrà *et al.* 2015)
Ste2ΔSte3Δ	Knockout mutant of the *Ste2* and *Ste3* loci	Hygromycin Phleomycin	(Vitale, Di Pletro and Turrá 2019)

Table 3. Selective agents used in this work.

Selective agent	
Hygromycin (50mg/ml)	40 µl in 100 ml of YPD medium
Phleomycin (20mg/ml)	20 µl in 100 ml of YPD medium at pH=8

F. OXYSPORUM α-PHEROMONE TREATMENT AND SAMPLE PREPARATION

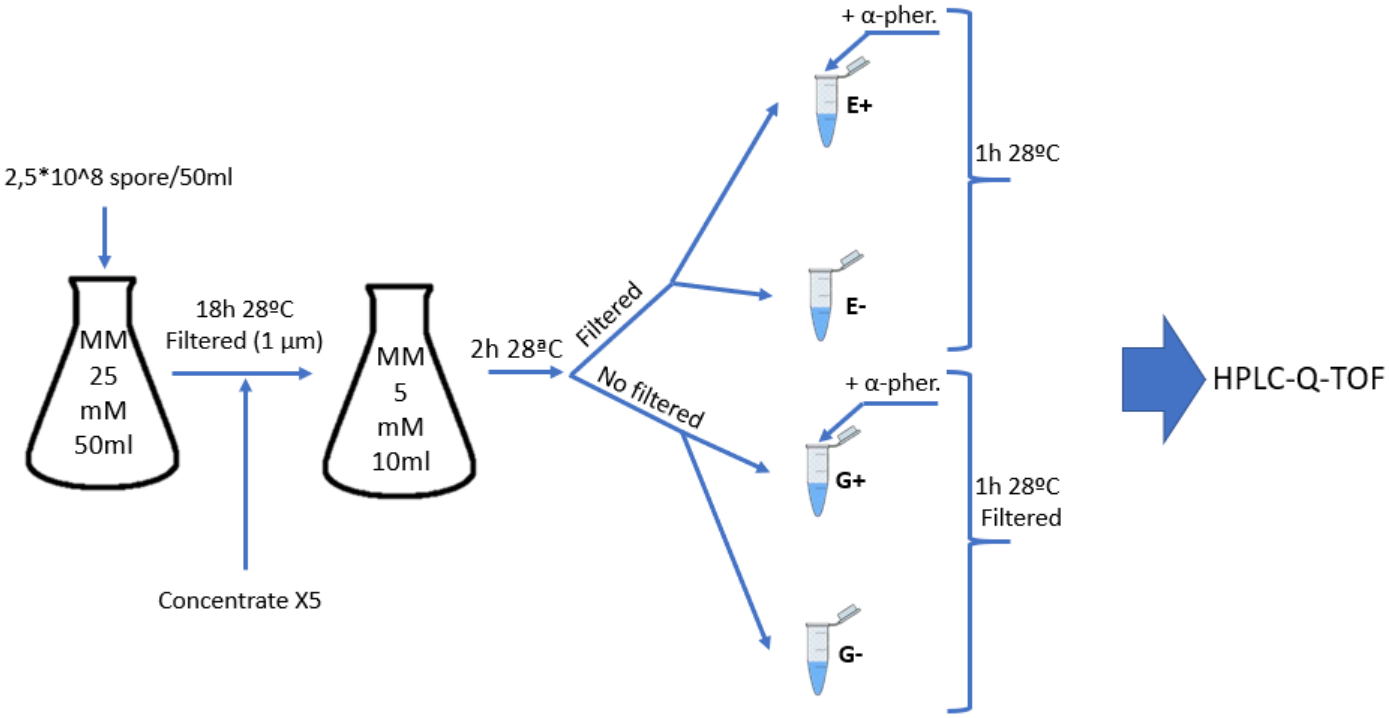
To incubate synthetic α-pheromone (GenScript) from *F. oxysporum* with either fungal germlings or their exudates, a total of 2,5 *10^8 *F. oxysporum* microconidia were germinated in 50 ml of minimal medium supplemented with 25 mM glutamate (MM 25 mM) at 28°C and 170 rpm for 18h in an orbital shaker. Fungal germlings were filtered through a 1-µm PluriStrainer mini (PluriSelect), concentrated five times by resuspending them in five-fold less volume of minimal medium supplemented with 5 mM glutamate (MM 5 mM) and incubated again at 28°C and 170 rpm for 2h.

To test potential Bar1 activity associated with *F. oxysporum* germlings (G), an aliquot (0,5 ml) of the fungal culture grown in MM 5 mM was either treated with

synthetic α-pheromone (200 µM final concentration) or not (negative control) and incubated for 1h at 28°C and 170 rpm. Finally, cultures were first filtered through a custom-made filtering device (composed of a P5000 pipette tip filled with a piece of cotton wool) and then through a 0.2 µm pore-size syringe filter (Sartorious) prior to injection into a HPLC-Q-TOF system (Figure 10).

To investigate if *F. oxysporum* germlings might secrete the Bar1 protease in the extracellular milieu, an aliquot (9 ml) of the fungal culture grown in MM 5 mM was first filtered through a custom-made filtering device (see above) and then through a 0.2 µm pore-size syringe filter to remove fungal propagules and obtain fungal exudates (E). Exudates (0,5 ml) were then either treated with synthetic α-pheromone (200 µM final concentration) or not (negative control) and incubated for 1h at 28°C and 170 rpm. Finally, exudates were injected into a HPLC-Q-TOF system (Figure 10).

Figure 10. Diagram of incubation and sample preparation.



ANALYSIS OF PHEROMONE PEPTIDE CLEAVAGE BY HIGH PERFONMANCE LIQUID CHROMATOGRAPHY – QUADRUPOLE TIME-OF-FLIGHT (HPLC-Q-TOF)

All samples were analyzed using an Agilent HP 1260 Infinity Series (Agilent 2020) liquid chromato-graph coupled to a Q-TOF mass spectrometer (Agilent 6540 UHD Quadrupole Time-of-Flight Accurate-Mass Mass Spectrometer (Creative proteomics 2015), and equipped with a DAD system (Agilent Technologies).

For chromatographic separation, a Sepachrom Adamas C-18-X-Bond (4.6 x 50 mm, 3.5 µm) (Sepachrom Srl) was used, maintained at a constant temperature of 37°C. Analyses were conducted at a flow rate of 0.6 mL/min, using a linear gradient system consisting of 0.1% (v/v) formic acid in water (Solvent A), and 0.1% (v/v) formic acid in acetonitrile (Solvent B). The gradient program was as follows: from 5% to 70% solvent B in 4 min, isocratic to 70% solvent B from 4 to 5 min; from 70% to 80% solvent B from 5 to 8 min and from 80% to 100% solvent B from 8 to 10 min; finally down to the initial condition (5% solvent B) from 10 to 15 min and after returning to the initial conditions, after 1 min, equilibrium was achieved. The injection volume was 10 µl. UV spectra were collected by DAD every 0.4 s from 190 to 750 nm, with a resolution of 2 nm. The MS system was equipped with a dual electrospray ionisation (ESI) source and operated in both positive and negative modes. The capillary was maintained at 2000 V, fragmenter voltage at 180 V, cone 1 (skimmer 1) at 45 V, Oct RFV at 750 V. The gas flow was set at 11 L/min, at 350 °C, and the nebuliser was set at 45 psig.

SEQUENCE RETRIEVAL

The protein sequence of *F.oxysporum* Bar1 (XP_018246670.1) was retrieved from a previous study (Vitale, Di Pletro and Turrá 2019). *F.oxysporum* Bar1 homologoues protein from the different fungal species used in the bioinformatics study (Table 4) were identified by the BLASTp algorithm through comparison with

the *F.oxysporum* Bar1 protein. A complete list of sequences corresponding to the identified Bar1 homologues in different fungal species is shown in “Supplements".

Table 4. Fungal species against which Bar1 orthologs were searched.

Human pathogens	Plants pathogens	Non-pathogenic
Aspergillus fumigatus	*Fusarium oxysporum*	*Neurospora crassa*
Candida glabrata	*Fusarium verticilloides*	*Sordaria macrospora*
Cryptococcus neoformans	*Fusarium solani*	*Trichoderma harzianum*
Candida albicans	*Verticillium dahliae*	*Saccharomyces cerevisiae*
	Magnaportha grisea	

BIOINFORMATIC TOPOLOGY PREDICTION AND PHYLOGENETIC ANALYSIS

Full-length sequences were aligned with Clustal Omega (Sievers and Higgins 2017), and manually inspected. Only fully aligned parts of the multiple sequence alignment were used. Presence of a signal peptide was determined with SignalP version 5.0 (Almagro *et al.* 2019). Prediction of transmembrane helices in proteins was done with Phobius (Käll, Krogh and Sonnhammer 2007). The phylogenetic trees were obtained from MAB Lirmm (Dereeper *et al.* 2010), with a phylogram tree style.

The m/z ratios of the different α-pheromone fragments were obtained with ExPASy (Compute pl/Mw) (Bjellqvist *et al.* 1993).

4. RESULTS AND DISCUSSION

IDENTIFICATION OF α-PHEROMONE PROTEOLYTIC ACTIVITY IN *F. OXYSPORUM* EXUDATES

The α-pheromone cleaving protease Bar1 from the fungal model specie *S. cerevisiae* has been shown to be largely secreted (95% of Bar1 activity found extracellularly) with only a limited activity of this enzyme associated to fungal cell walls or the periplasmic space (Ciejek and Thorner 1979) (Ballensiefen and Schmitt 1997) (Moukadiri, Jaafar and Zueco 1999) (Ballensiefen and Schmitt 2004). Thus, to identify potential cleavage sites of the *F. oxysporum* Bar1 orthologue into the α-pheromone peptide (WCTWRGQPCW) from the same fungus we incubated the decapeptide with fungal germling exudates (E). In order to maximize Bar1 activity in fungal cultures, *F. oxysporum* propagules were concentrated 5 times, as *Bar1* gene expression is known to be enhanced in high cell density conditions (Vitale *et al*. 2019). Importantly, HPLC-Q-TOF analysis of the α-pheromone peptide after incubation with fungal exudates from the wt strain yielded an M/Z ratio corresponding to the entire pheromone sequence (M/Z 1322), two complementary fragments (M/Z 1033 and 308) and a third fragment (M/Z 205) which is likely to be a proteolysis product of fragments M/Z 308 (Tables 5-6).

Table 5. Potential α-pheromone cleavage products. M/Z ratios were calculated by using the Compute pI/Mw tool of the ExPasy software

Sequence	M/Z	Sequence	M/Z
WCTWRGQPCW	1322		
WCTWRGQPC-	1136	**-W**	205
WCTWRGQP-	1033	**-CW**	308
WCTWRGQ-	936	**-PCW**	405
WCTWRG-	808	**-QPCW**	533
WCTWR-	751	**-GQPCW**	590
WCTW-	595	**-RGQPCW**	746
WCT-	409	**-WRGQPCW**	932
WC-	308	**-TWRGQPCW**	1033
W-	205	**-CTWRGQPCW**	1136

Table 6. Identified M/Z ratios from α-pheromone samples incubated with the germling exudates from the indicated *F.oxysporum* strains.

P, treated with α-pheromone; -P, no treated.

Exuadate	WT		Bar1Δ		Bar1Δ+Bar1	
M/Z	**+P**	**-P**	**+P**	**-P**	**+P**	**-P**
1322	**+**	**-**	**+**	**-**	**+**	**-**
1136	**-**	**-**	**-**	**-**	**-**	**-**
1033	**+**	**-**	**+**	**-**	**+**	**-**
936	**-**	**-**	**-**	**-**	**-**	**-**
932	**-**	**-**	**-**	**-**	**-**	**-**
808	**-**	**-**	**-**	**-**	**-**	**-**
751	**-**	**-**	**-**	**-**	**-**	**-**
746	**-**	**-**	**-**	**-**	**-**	**-**
595	**-**	**-**	**-**	**-**	**-**	**-**
590	**-**	**-**	**-**	**-**	**-**	**-**
533	**-**	**-**	**-**	**-**	**-**	**-**
409	**-**	**-**	**-**	**-**	**-**	**-**
405	**-**	**-**	**-**	**-**	**-**	**-**
308	**+**	**-**	**+**	**-**	**+**	**-**
205	**+**	**-**	**+**	**-**	**+**	**-**

In order to understand if at least one of the obtained fragments could derive from Bar1 proteolytic cleavage, the *F. oxysporum* α-pheromone peptide was incubated with fungal exudates from a *Bar1*Δ mutant or its complemented strain

*Bar1*Δ+*Bar1*. Noteworthy, HPLC-Q-TOF analysis did not show any difference in the between α-pheromone samples treated with wt or the *Bar1*Δ exudates, suggesting that *F. oxysporum* secretes an additional, yet unknown, extracellular protease able to cleave either the N-terminal Trp^{1}-Cys^{2} or the C-terminal Cys^{9}-Trp^{10} dipeptide (Tables 6). Importantly, gene deletion of any of the upstream components of the F. oxysporum pheromone signalling pathways did not affect the activity of this extracellular protease as α-pheromone samples treated with the exudates of *α*Δ, *a*Δ, *α*Δ*a*Δ, *Ste2*Δ and *Ste2*Δ*Ste3*Δ mutant strains produced the same peptide fragments (M/Z 1322, 1033, 308 and 205; Table 7).

Table 7. Identified M/Z ratios from α-pheromone samples incubated with the germling exudates from the indicated *F.oxysporum* strains.

P, treated with α-pheromone; -P, no treated.

Exudate	WT		αΔ		aΔ		αΔaΔ		Ste2Δ		Ste2ΔSte3Δ	
M/Z	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
1322	+	-	+	-	+	-	+	-	+	-	+	-
1136	-	-	-	-	-	-	-	-	-	-	-	-
1033	+	-	+	-	+	-	+	-	+	-	+	-
936	-	-	-	-	-	-	-	-	-	-	-	-
932	-	-	-	-	-	-	-	-	-	-	-	-
808	-	-	-	-	-	-	-	-	-	-	-	-
751	-	-	-	-	-	-	-	-	-	-	-	-
746	-	-	-	-	-	-	-	-	-	-	-	-
595	-	-	-	-	-	-	-	-	-	-	-	-
590	-	-	-	-	-	-	-	-	-	-	-	-
533	-	-	-	-	-	-	-	-	-	-	-	-
409	-	-	-	-	-	-	-	-	-	-	-	-
405	-	-	-	-	-	-	-	-	-	-	-	-
308	+	-	+	-	+	-	+	-	+	-	+	-
205	+	-	+	-	+	-	+	-	+	-	+	-

To further understand if the extracellular protease found in *F. oxysporum* exudates specifically cleaves the Trp-Cys peptide at the N- or the C-terminus of

or at both, we incubated an N-terminally FITC-labelled version of α-pheromone (Figure 11) with fungal exudates from the wt strain.

Figure 11. Representation of the N-terminally FITC-labelled version of *F. oxysporum* α-pheromone (FITC+Ahx+Peptide).

The obtained M/Z peaks correspond to the entire FITC+Ahx+Peptide and the FITC+Ahx fluorophore alone (M/Z 913 and 504), and two complementary fragments (M/Z 1033 and 811) matching respectively the M/Z values of FITC+Ahx+Trp^1-Cys^2 and of the remaining C-terminal peptide TWRGQPCW (Tables 8-9). Differently from the experiments performed with the unlabelled version of the α-pheromone, cleavage at Trp^1 was not observed, likely as a result of the bulky FITC+Ahx conjugating molecule which might prevent appropriate interaction between the peptide N-terminus and the cleaving protease.

Altogether these results indicate that *F. oxysporum* cells secrete a specific protease cleaving off the Trp^1-Cys^2 dipeptide from the α-pheromone N-terminus. Because Trp^1-Cys^2 have been previously shown to regulate the growth inhibitory effect of *F. oxysporum* α-pheromone in a Ste2-independent manner (Vitale, Partida-Hanon, et al. 2016), fungal regulation of Trp^1-Cys^2 cleavage might represent a novel signalling mechanism used by filamentous fungi to modulate their growth at high cell densities.

Table 8. Potential cleavage products of the FITC-labelled version of *F. oxysporum* α-pheromone. M/Z ratios were calculated by using the Compute pI/Mw tool of the ExPasy software.

Sequence	M/Z	Sequence	M/Z
***WCTWRGQPCW**	913 (Z=2)	**FITC+Ahx (*)**	504
***WCTWRGQPC-**	1639	**-W**	205
***WCTWRGQP-**	1536	**-CW**	308
***WCTWRGQ-**	1439	**-PCW**	405
***WCTWRG-**	1311	**-QPCW**	533
***WCTWR-**	1254	**-GQPCW**	590
***WCTW-**	1098	**-RGQPCW**	746
***WCT-**	911	**-WRGQPCW**	932
***WC-**	811	**-TWRGQPCW**	1033
***W-**	708	**-CTWRGQPCW**	1136

Table 9. Identified M/Z ratios from the FITC-labelled version of *F. oxysporum* α-pheromone sample incubated with the germling exudates from the wt F.oxysporum strain.

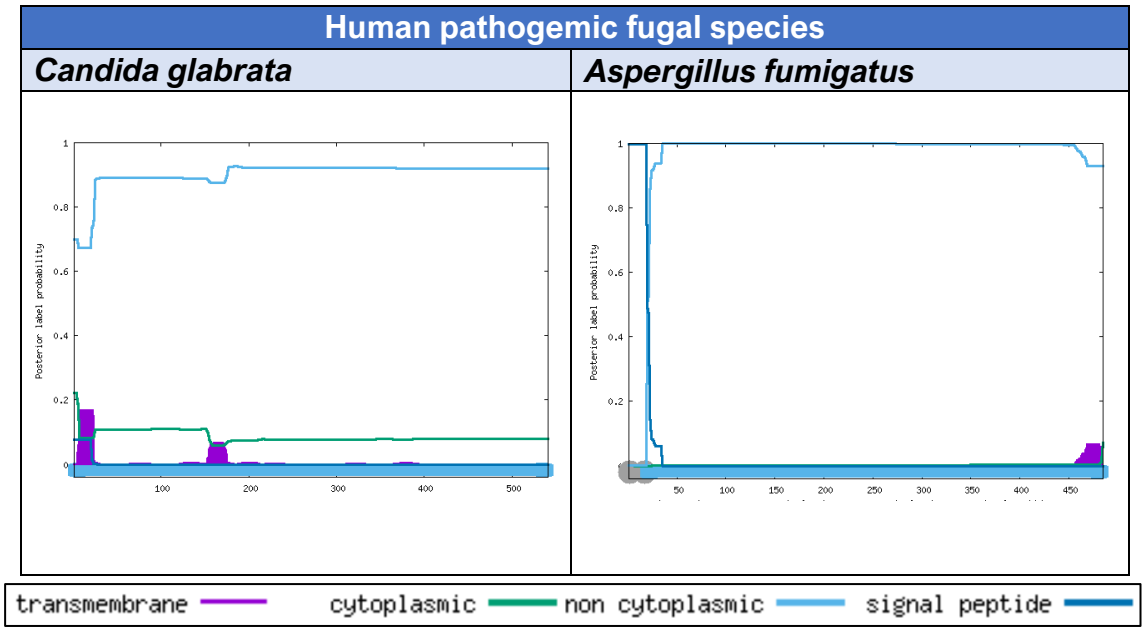
P, treated with α-pheromone; -P, no treated.

WT Exudate					
M/Z	**+P**	**-P**	**M/Z**	**+P**	**-P**
913	+	-	**504**	+	-
1639	-	-	**205**	-	-
1536	-	-	**308**	-	-
1439	-	-	**405**	-	-
1311	-	-	**533**	-	-
1254	-	-	**590**	-	-
1098	-	-	**746**	-	-
911	-	-	**932**	-	-
811	+	-	**1033**	+	-
708	-	-	**1036**	-	-

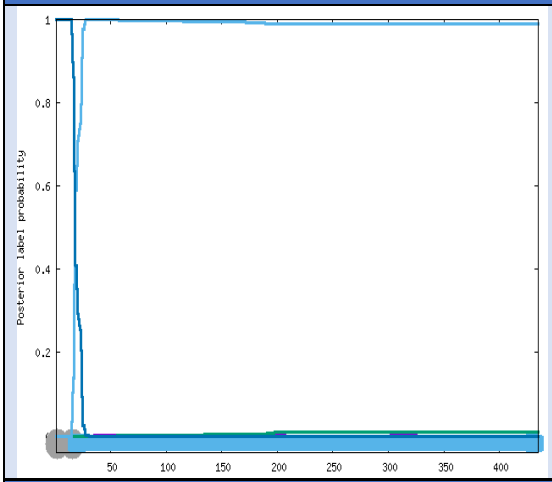
BIOINFORMATICS ANALYSIS OF BAR1 PROTEASE IN DIFFERENT FUNGAL SPECIES

Since HPLC-Q-TOF analysis of α-pheromone samples treated with fungal exudates did not allow for potential Bar1 cleavage site identification, we questioned whether Bar1 from *F. oxysporum* and other filamentous fungi could be not secreted in contrast to yeast (*S. cerevisiae*) or yeast-like (*C. albicans*) fungi. To this aim we performed an *in silico* analysis of Bar1 orthologues from different plant pathogenic, human pathogenic and non-pathogenic fungal species (Table 4) in order to identify potential transmembrane domain (Figure 12).

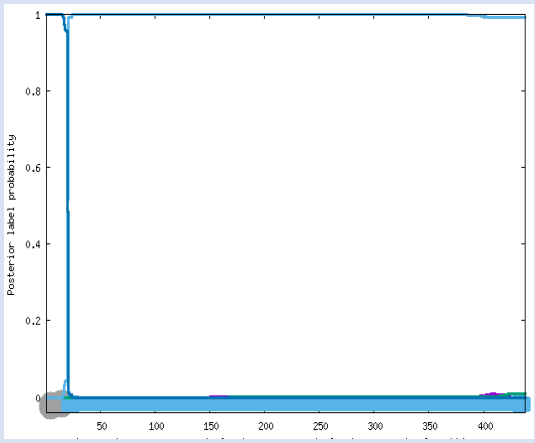
Figure 12. In silico prediction of potential transmembrane domains in Bar1 orthologues from the indicated fungal species.



Candida albicans

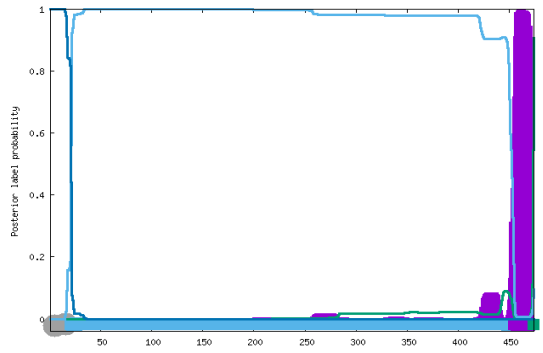


Cryptococcus neoformans

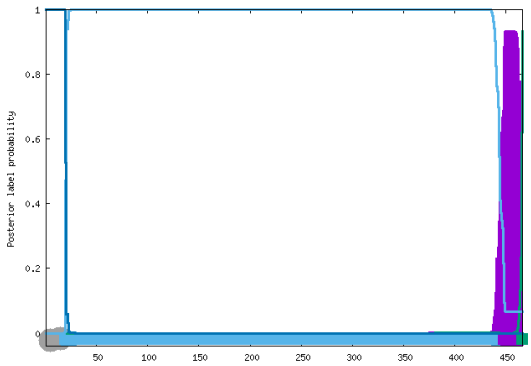


Plant pathogeic fungal species

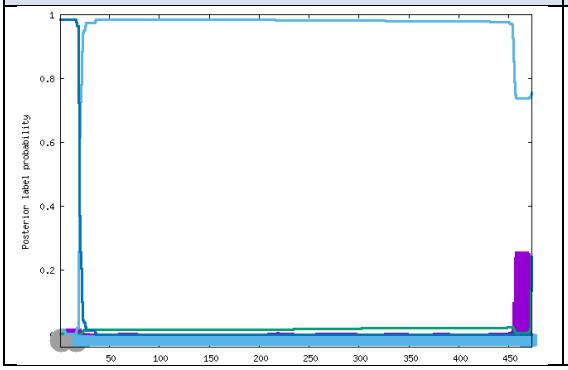
Magnaportha grisea



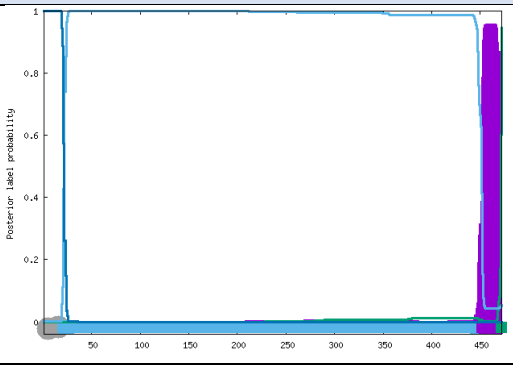
Fusarium verticilloides



Fusarium solani

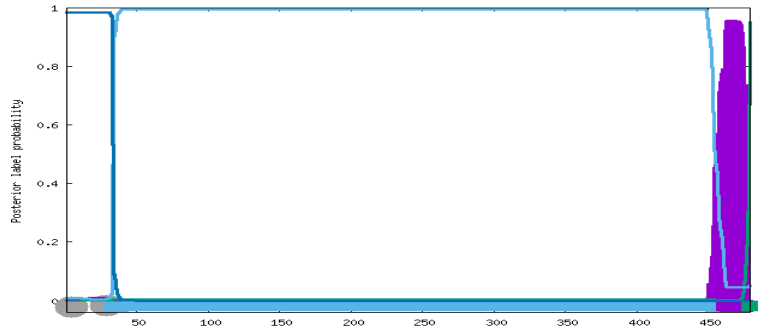


Verticillium dahliae



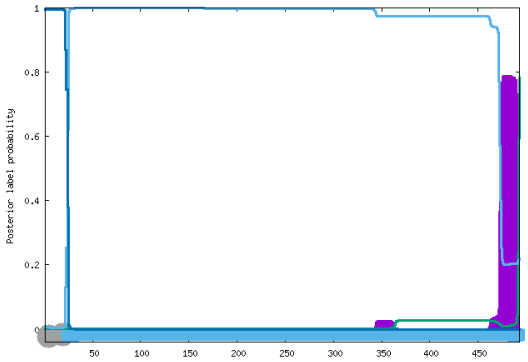
transmembrane — cytoplasmic — non cytoplasmic — signal peptide

Fusarium oxysporum

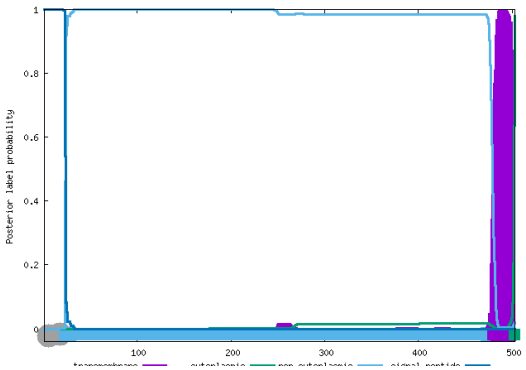


Non-pathogenic fungal species

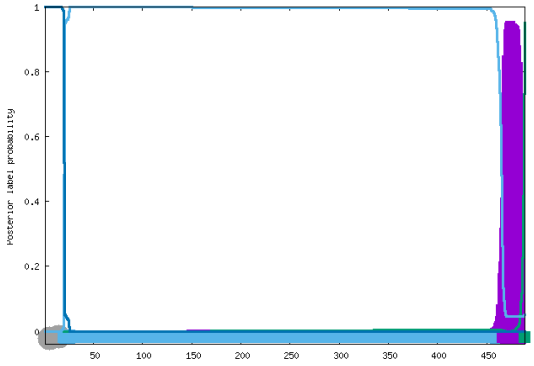
Neurospora crassa



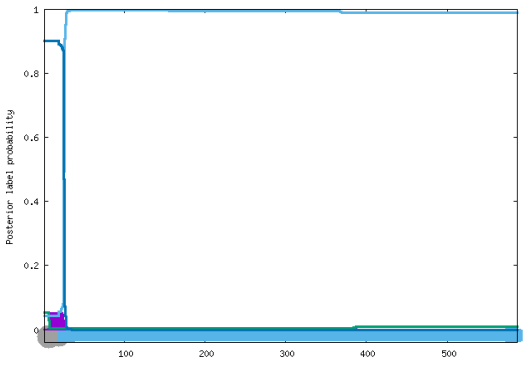
Sordaria macrospora



Trichoderma harzianum



Saccharomyces cerevisiae

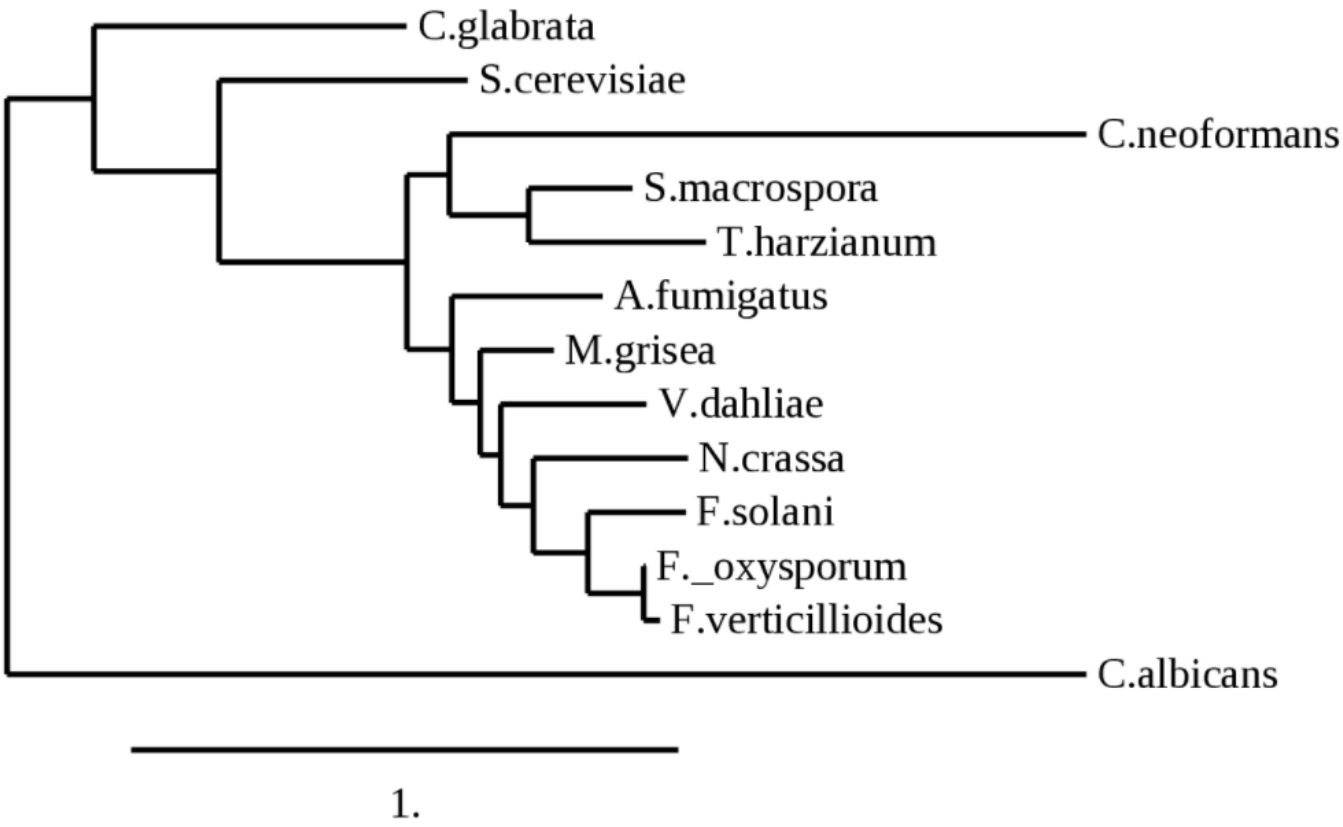


In all studied human pathogens, the Bar1 protein is predicted to be secreted, in contrast to all analysed plant pathogenic fungi, except for *F. solani*, where the presence of a C-terminal transmembrane domain was identified to be slightly

probable. In the case of Bar1 from non-pathogenic fungi a potential C-terminal transmembrane domain was identified in all of them except *S. cerevisiae*. Interestingly, while all filamenting fungi appeared to have a predicted C-terminal transmembrane domain in their Bar1 orthologue, yeast growing fungi (*i.e. Saccharomyces* spp, *Candida* spp, *Cryptococcus* spp.) did not.

To further corroborate the obtained results, a phylogenetic tree of Bar1 orthologues from the different fungal species was generated confirming that the presence of transmembrane domains coincides with the distance and branching of the phylogenetic tree (Figure 13).

Figure 13. Phylogenetic tree of Bar1 proteins from different fungal species.



IDENTIFICATION OF α-PHEROMONE PROTEOLYTIC ACTIVITY ON *F.OXYSPORUM* CULTURES

Because Bar1 from *F. oxysporum* and other filamentous fungi was predicted to harbour a transmembrane domain, we decided to incubate the α-pheromone peptide directly with fungal propagules rather than with their exudates to identify potential Bar1 cleavage sites. HPLC-Q-TOF analysis showed that α-pheromone incubation with fungal germlings from the wt strain yielded an M/Z ratio corresponding to two non-complementary fragments (1033 and 409) (Tables 5, 10), however not to the entire pheromone sequence (M/Z 1322). To understand if at least one of the obtained fragments could derive from Bar1 activity we incubated the *F. oxysporum* α-pheromone peptide with fungal germlings from a *Bar1Δ* mutant or its complemented strain *Bar1Δ+Bar1*. Interestingly, HPLC-Q-TOF analysis of these samples showed the presence of the 1033 but not of the 409 M/Z fragment. In addition to this, one (M/Z 205) and two (M/Z 205 and 308) peptide fragments were also detected in samples treated with exudates of the *Bar1Δ and Bar1Δ+Bar1* strains, respectively (Table 10). While the latter two fragments were also detected upon α-pheromone treatment with fungal exudates and thus correspond to the cleavage products of an extracellular protease, the N-terminal tripeptide WCT (M/Z 409) might represent a specific sub product of Bar1 activity. Further experiments will be needed to corroborate this hypothesis and to understand why the same fragment is not detectable upon α-pheromone treatment with the fungal germlings from the *Bar1Δ+Bar1* strain.

Table 10. Identified M/Z ratios from α-pheromone samples incubated with fungal germling from the indicated *F. oxysporum* strains.

P, treated with α-pheromone; -P, no treated

Germling	WT		Bar1Δ		Bar1Δ+Bar1	
M/Z	+P	-P	+P	-P	+P	-P
1322	-	-	-	-	-	-
1136	-	-	-	-	-	-
1033	+	-	+	-	+	-
936	-	-	-	-	-	-
932	-	-	-	-	-	-
808	-	-	-	-	-	-
751	-	-	-	-	-	-
746	-	-	-	-	-	-
595	-	-	-	-	-	-
590	-	-	-	-	-	-
533	-	-	-	-	-	-
409	+	-	-	-	-	-
405	-	-	-	-	-	-
308	-	-	-	-	+	-
205	-	-	+	-	+	-

5. CONCLUSIONS

F. oxysporum secretes an α-pheromone specific, yet unknown, protease cleaving the Trp1-Cys2 dipeptide from the α-pheromone N-terminus (Figure 14A).

The activity of this extracellular protease is independent of *F. oxysporum* pheromone signalling pathway activation.

Trp1-Cys2 cleavage might represent a novel signalling mechanism used by filamentous fungi to modulate their growth at high cell densities.

Bar1 from *F. oxysporum* and other filamentous fungi have a predicted C-terminal transmembrane domain, in contrast to yeast growing fungi.

The N-terminal tripeptide WCT might represent a cleavage product of Bar1 activity (Figure 14B). Further experiments are required to corroborate this hypothesis.

Figure 14. α-pheromone aminoacidic sequence and potential protease cleavage sites.

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7. SUPPLEMENTS

BAR1 PROTEIN SEQUENCES

Signalling peptide highlighted in green, transmembrane domains highlighted in yellow and signalling peptide and transmembrane domains highlighted in blue.

Human pathogens

Aspergillus fumigatus

>XP_747542.1 Aspartic-type endopeptidase (OpsB), putative [Aspergillus fumigatus Af293]

MRHIFSLLSIVCLMVKHGACLTLHQRDVPAVVSLDIKRSIVSDPVVRDRVRRKRDKTIGQTLDNAETLYFCNVTL
GTPGQALRLVLDTGSSDLWCNAANSTLCSDSNDSCNISGSYDPSSSSTYAYVSSDFNISYADGTGAVGDYAT
DILHIGGSTLRNLQFGIGYSSTSSEGVLGIGYPSNEVQVGQYGKDTYPNLPRAMVDQGLINSNAYSLWLNDLES
NTGSILFGGVNTGKYLGELQTLPIQKVNGRYSEFVIALTGVAFDSESHHKTYSSDALPAAVLLDSGSSLTYLPDS
IVENIYRDLNVAYEPSSGVGYLPCKLAGNNINITYTFSSPNITVMIDELLLDAGDLRFRDGARACIFGIVPAGDSTA
VLGDTFLRSAYVVYDIANNEISIANTNFNSTEDNILEIGVGPDSVPSATQVSHPVTSVVADGSGARIGAPTGASS
TTVPSISSAGALSAGVARADKQYLAIALIAVWFVLGL

Candida glabrata

>QNG14967.1 BAR1 [[Candida] glabrata]

MIQFINCYFIFNLVTVVNCLRLTIEKRIVSSHASLSKRSAVDLQFRRFNNLYYESVLEFGTPPQSIPLVLDTGSSDL
WVTLIGNPLCYNKGSGKPPKGLIDCSGLVFYDMDKSQTFDYLKNVTFKIGYADTTYTSGIWIVDIITLPEGHLKDL
QFGMAFATNATFSGVLGIGFPAMESVNGYEFAPGKFYPNFPLALKNAGFTNIAAYSIEYNDESKKGSILFGSIDT
SRFIGPLYTFPMINEFPGIADEPSTLSLTLDGIGIDGSCEQWVISNTKLPALLDTGSTLIELPHPITQSIASYLNATW
SDDHGLFVLACPSDDFLTTTDLAFTFGELHMKIPLRSFILLPDEESNGLCGLGITSSKGRVILGDSFLTHVYTVFD
LDNYMISLAPMAKSSLPGKVIEIPANGLIENAVISKSEKWTHFNDITVNEYGFRNYCDGELQDIHSYSSKLNSSSL
VYSSNFSSDEEQNSSESVSPSIFNTDSSIKEIYPSLSQKEHLSALLTTISTSEIHKPVTATITDSITTTIRVTKILTVL
QCAT

Cryptococcus neoformans

>OXB39612.1Ssaccharopepsin [Cryptococcus neoformans var. grubii]

MKTSAILIAALSAAASVEAGIHRMKLEKQAPPSTSLTGAFSPPPELEAKWLASKYLGQEYTEQMPFGGFGGAG
KKFKSGNKHTKHPEQEDEDRYWAQMVDQTAHSQMIDVLKGGHGVPLSNFMNAQYFTTVELGTPFQTFKVVL
DTGSSNLWVPSVKCTSIACFLHNKYDSSQSSTYKANGSDFEIHYGSGSLEGFISQDTLSIGDLVVKKQDFAEAT
KEPGLAFAFGKFDGILGLGYDTISVNHIVPPFYNMLNQHLLDEPVFSFRLGSSDENGGEAIFGGIDDSAYSGKLA
YVPVRRKGYWEVELESISFGDEELELENTGAAIDTGTSLIVMPTDVAELLNKEIGAEKSWNGQYTVDCNTVSSLP
KLAFTFGGKDYTLSADDYILNAGGTCISSFTGMDIPAPIGPLWIVGDVFLRKYYTVYDLGRNAVGFAESK

Candida albicans

>orf19.2082|SAP30 COORDS:Ca21chr2_C_albicans_SC5314:69105-70412W, Translated using codon table 12 (435 amino acids)

MLFTILSLLVPSLAVAVSNTGGAVKLDLTISDEHMYYIENLALGTPPQFISNVIVDSGSSDLMIVDSIYNFSASSSF
YNSNQTAIMKYGYGGQFPVYFINETIRSNDWKLSNLSMGLANISDMDSFSGILGIGFTRQELFKTNYSNFPYLLK
DQGYTKSVLFSFNGQDQNPSIIFGGIITNIIDGPLVRAPFIKVISFINQLDYWLMPTFTVNQIKLGDTIVSNQKTLYQ
IDSGTNGFVPPTPVLNNILKILGDDYIQDDNGNIYFDIKYIEGLNITFSVQGYDIGFQLVDIVGDTIERNSTTFVALN
VASCDIGYNAYEGLLPNLIFKYHYAIFDYDNAQIYFGKYKNSNGEANVVAVENGYQLPVPTVDVPDVEDTYSVIY
IAESETTVTVAPTEVQSTYSSFSISEGTSSNLTSGSTSDDKITSVTSNPQYC*

Plants pathogens

Fusarium oxysporum

>XP_018246670.1 Hypothetical protein FOXG_09428 [Fusarium oxysporum f. sp. lycopersici 4287]

MDELDRSTYKVSIMKSIQLSSLLLSLLSLTEGISLNKRDNGLEPRVMSVEIQRRTISDPISNDRIRLRKRDGTIDIGI
DNEQSLYFLNASLGTPPQDFRLHLDTGSSDLWVNAQGSKLCSTHANICSESGLYSPNKSSTYEYLNSDFNISY
ADGSGASGDYATETFRMGSVKLEDLQFGIGYVTSDNEGVLGIGYKSNEAQVGQLNRDAYDNLPAKLASKGLIA
SNAYSLYLNDLESATGTILFGGVDQEQYTGDLVTLPINKINGEFAELSITLQSVSADSETIADNLDLAVILDSGST
LSYLPATLTSDIYDIVGAQYEEGESVAYVPCDLGNDSGNLTFKFKDPAEISVPLSELVLDFTDVTGRQLSFDNGQ
AACTFGIAPTTGDISILGDTFLRSAYVVFDLENNEISLAQSNFDATKSHILEIGTGKHPVPTATGSGSSDNKENAA
ASLAPLGGDAAISMVAGAFALGFAWMLI

Fusarium verticilloides

>XP_018752852.1 Hypothetical protein FVEG_07058 [Fusarium verticillioides 7600]

MKSIQLSSLLLSLLPLTQAISLNKRDNGLEPRVMSVEIQRRTISDPISNDRRRLRRRDGTVDIDIDNEQSLYFLNA
SLGTPAQDFRLHLDTGSSDLWVNAEGSKLCSTHANICSESGLYSPNKSSTYEYLNSDFNISYADGSGASGDY
ATETFRMGSVKLEDLQFGIGYVTSDNEGVLGIGYKSNEAQVGQLNRDAYDNLPAKLASKGLIASNAYSLYLNDL
ESATGTILFGGVDQEQYIGDLVTLSINKINGGYSEFSITLQSVSADSETIVDNLDLTVILDSGSTLSYLPASLTSDIY
DIVGAQYEEGQSVAYVPCDIGNDSGNFTFKFKDSAEISVSLSEMVLDFTDVTGNQLSFDNGQAACTFGIAPTTG
DFSILGDTFLRSAYVVFDLDNNEISLAQSNFNATKSHILEIGTGKNAVPTVTGSGSSDNKENAAASLSPLGGDAA
VSMVAGAFALGFAWMLI

Fusarium solani

MKATRLFYAILSLASATGAISLHKRQDGLEPRVLALDLQRSTIRDPVGNDRKRLARRSGSINVGIDNLQSLYFFN
ASLGTPAQDFRLHLDTGSSDLWVNSNNSTLCSTPADVCATSGLYNASNSSTYKFVSSEFNITYADGSGSAGD
YATETFRVGKTVIKELQFGIGYESSSDQGVLGIGYSSNEAQVAEFGGEPYDNLPAKMAADGLIASNAYSLWLND
LDAASGTILFGGVDRKRYTGDLITVPVQKFGGRFSQFYITLTGLSVGSDTVDDSLALGVILDSGSTLTYLPTSLA
EAVFEIVGADYEEGASTAYVPCDLANTSGNLTFSFSSPAEITVPLSELVLDFTDVTGRRLRFDDGEPACMFGIAP

SPSGTNILGDTFLRSAYVVFDLGNNEVSLAQSNFDADGSDIVEIGSGDGAVPAATSADEPVTASGVAVSSANG
AGSLSPLGKDRAASLFVGALTLGFVWPLV

Verticillium dahliae

>RXG46419.1 Hypothetical protein VDGE_05848 [Verticillium dahliae]

MRTIPIWVLSSLLLSSTGEAVALRKRDGSPARVIGFDLESKIIQAPNDKNGMRRRNTVTASLDNLQTLYFVNATL
GNPPQQFRLHLDTGSSDLWVNTANSQLCQEVPNPCALSGMYAANQSSSYNYLGSYFNISYVDGSGATGDYV
TDDFNIGGTVLTDFQFGIGYESSAAQGILGVGYPMNEVQVGRAGMDPYDNIAAKMKAQGLIQSNAFSLYLNSL
SASTGSILFGGVDTEHFVGELQTLPIQMHADIHSEFLVTLTDVTLGSTTMGSDLAIAVLLDSGSSLSYLPDAMVK
EIYSMVGAVYQEEQAVAFVPCALRQSPANMTFTFSKPKITVPISELVLDLFKITGRQPTFSNGAPACLFGLAPAG
AGTHVLGDTFMRSAYIVYDMENNEISLAQTRFNATRSNILEIGTGRSSVPSAITVAEPVAATHGLRGGGAAAAH
STAHVLTPLAGPFLAGVISGILGFVSFFI

Magnaporthe grisea

>XP_030978115.1 Uncharacterized protein PgNI_09918 [Pyricularia grisea]

MRLIASLALLASLASSVTNGSSIPRRDSSAPAALRVVSMPMERQHISNPLERDRLRRRSSITATLDNEETLYFINV
SIGTPAQKLRLHLDTGSSDLWVNSPDSKLCSVSSQPCKYAGTYSANSSSTYQYVSSVFNISYVDGSGAQGDY
VSDTISLGNTKIDSLQFGIGYTSSSTQGILGVGYEANEVQVGRAGLKAYRNLPSRMVELGLIASNAYSLYLNDLQ
SNKGSILFGGVDTEQYTGTLQTVPIQANGGRMAEFLITLTSVTLTSASIGGDNLALAVLLDSGSSLTYLPDDIVK
STYSAVDAQYDSNEGAAYVPCSLANDQSKKMTFTFSGIKIDVSMNELVLDLVTSSGRRPSFRNGASACLFGIA
PAGKGTNVLGDTFLRSAYVVYDLDNNAISLAQTSFNATKTNVKEIGKGSNSVPGAVAVSSPVAATSGLSLSSS
GKSGSGASAPAIPMLLLVAGLFSGSLLVLL

Non-pathogenic

Neurospora crassa

>XP_957809.3 Aspartic proteinase [Neurospora crassa OR74A]

MKRTTIWEWILTASLLSTTEAFAIRQKQDADTPKMVSLQTERLSVPKPAARDKLQRRGMNDVALDNVIGGYYV
NVTIGTPGRNLSLHLDTGSSDTWVNSPSSILCQDEDKPCEYSGTYLANDSSTYEYISNHFDIKYVDGSGARGDY
ASDTFTIGNTKLNRLQFGIGYSSTNAQGLLGIGYTLSEVQTRAGLPAYNNLPAQMVADGLINSNAYSIWLNDLD
ALTGTILFGGVDAAKYEGDLLTLPVQTPEKGTYKNLMVTMTGLSLSQSQSSSSDKGNGDDTTQISKDNLALAV
LLDTGSTLSYLPSELVKPLYDAIGIEYITDPDGKVDGYAPCHLMSSSQSVMFSFSSPLQIAVPMNELIVNRTFHG
KLPRMPDGVTDACIFGIQERNGTGANTLGDTFLRSAYVVFDLDNNEISMAQTRFNATATDLKEIKKGKGGVPGA
KAVENPVEATSGLSGNEGGIYVNGAACELNVGMGMAWGLLVGATMVVLGL

Sordaria macrospora

>XP_003351945.1 Uncharacterized protein SMAC_00494 [Sordaria macrospora k-hell]

MKGCTPSALFLGPVLFSQLALAQQAPSGVVQWDIQKRPVPNVAPNRLLRRAGSTYQQIIQNEQARGGYFATC
EMGTPGQKVTLQLDTGSSDVWVPDSTASVCNQGACDLGSFDTSRSKTYKVVGKNEFDISYVDGSSSKGDYF

TDVFKIGGATVTNLTMGLGAKTDIAYGLVGVGYANNEAIVSNAQSLDAQYPNLPVTMVDDGLINTIAYSLWLND
LDSSEGNILFGGIDTKKYKGDLTRIKIYPSNNGYYFSFIVAMTQLQAISPSGNDTLTSDEFPIPVVLDSGTTLSYLP
QDLVEQVWHEVGADYSSRLQLAVIPCSKKTSKGYFSFQFAGPDGPRINVRMDELVLDLTSGNPPKYTSGPHK
GQDVCEFGIQNFTSAPYLLGDTFLRSAYVVYDLVNNEIALAETDFNSTQSNIVAFASMSAPIPSATQAPNQAAV
TNRPAVTSPAFSASPGFSDGSGNGGEDENASQGMPRAFGVAQMSVMAVSMVVAMIGSGIFALL

Trichoderma harzianum

>KKP01949.1 Aspartic proteinase [Trichoderma harzianum]

MKASPLAVAGVVLASAAQAQVVQFDIEKRHDAPRLRKRDATIDATLSNQKVQGGYFINVEVGTPGQNITLQLD
TGSSDVWVPASTAAICTQTSQRNPGCTFGSFNSDDSSTFEVVGENLFDITYVDGSSSKGDYIEDTFRINGINIHN
LTMGLGLDTSIANGLVGVGYINDEASLGTTRQTYPNLPVVLQQQKLINTVAFSLWLNDLDASTGSILFGGIDTEK
YHGDLTRINIISPNGGKTFTEFAVNLYQVQASSPSGTDTLSTSQETLTAVLDSGTTLTYLPQDMAEQAWNEVG
ATFSDELGIAVVPCSVGNINGHFAFTFAGADGPTINVTLSELVLDLFSGGPPPQFSSGPNKGQSICEFGIQNTTD
APYLLGDTFLRSAFVAYDLVNNEIGIAPTNFNSTQTNVVAFASSGAPIPSSTSAPNQSNTGHSSSTQSGMSAA
SGFHDGGDDNAASLTGAFSGPGMIVVGLTICYTLLGSAVFGVGWL

Saccharomyces cerevisiae

>BAR1 YIL015W SGDID:S000001277 Sccaromices

MSAINHLCLKLILASFAIINTITALTNDGTGHLEFLLQHEEEMYYATTLDIGTPSQSLTVLFDTGSADFWVMDSSN
PFCLPNSNTSSYSNATYNGEEVKPSIDCRSMSTYNEHRSSTYQYLENGRFYITYADGTFADGSWGTETVSINGI
DIPNIQFGVAKYATTPVSGVLGIGFPRRESVKGYEGAPNEYYPNFPQILKSEKIIDVVAYSLFLNSPDSGTGSIVFG
AIDESKFSGDLFTFPMVNEYPTIVDAPATLAMTIQGLGAQNKSSCEHETFTTTKYPVLLDSGTSLLNAPKVIADK
MASFVNASYSEEEGIYILDCPVSVGDVEYNFDFGDLQISVPLSSLILSPETEGSYCGFAVQPTNDSMVLGDVFL
SSAYVVFDLDNYKISLAQANWNASEVSKKLVNIQTDGSISGAKIATAEPWSTNEPFTVTSDIYSSTGCKSRPFLQ
SSTASSLIAETNVQSRNCSTKMPGTRSTTVLSKPTQNSAMHQSTGAVTQTSNETKLELSSTMANSGSVSLPT
SNSIDKEFEHSKSQTTSDPSVAEHSTFNQTFVHETKYRPTHKTVITETVTKYSTVLINVCKPTY*

BAR1 HOMOLOGOUS PROTEIN ALIGNMENTS

Figure 15. Fusarium alignments of Bar1.

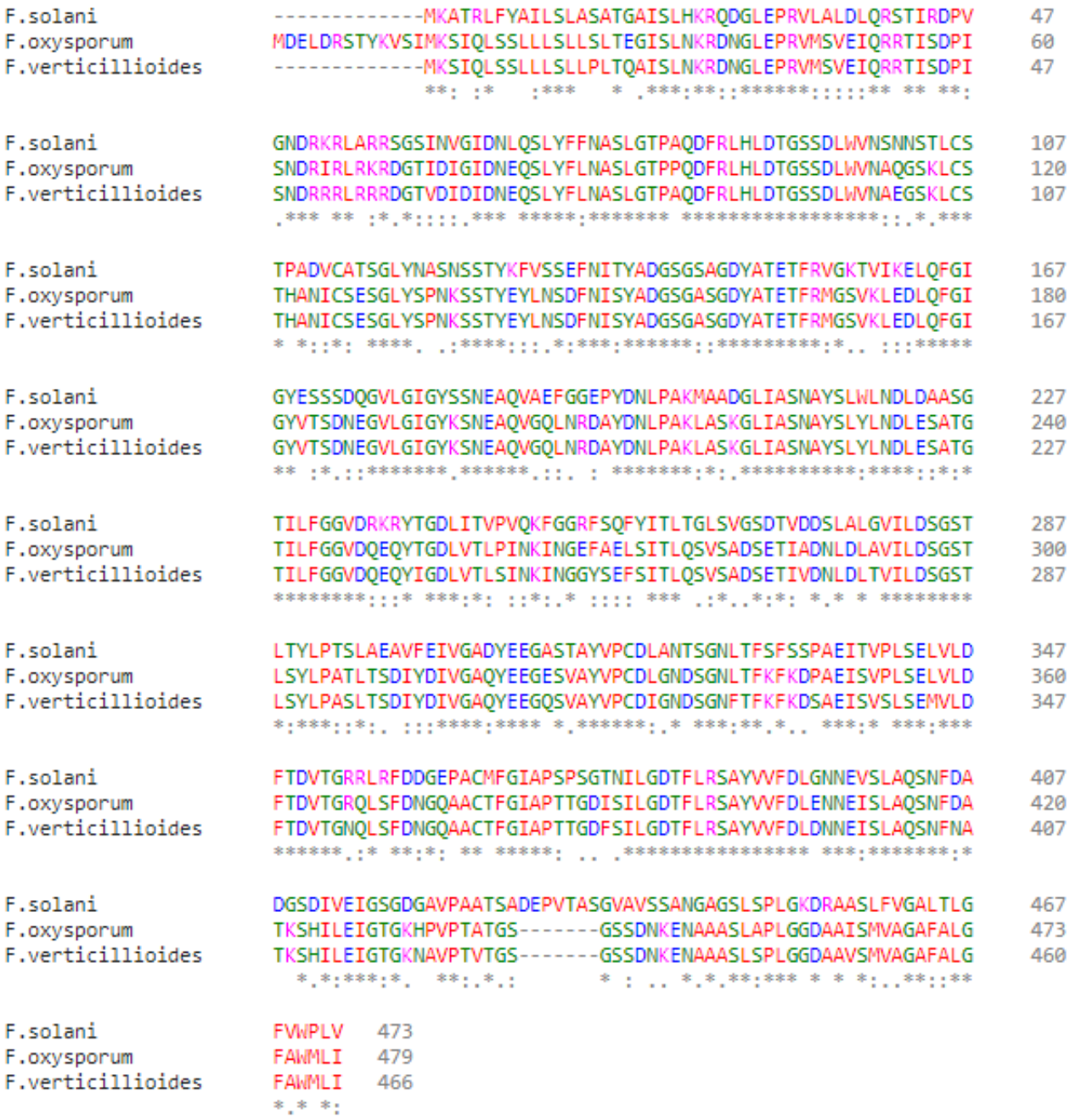

Figure 16. *S. cerevisiae, T. harzianum, V. dahliae* and *M. grisea* aligments of Bar1 protease.

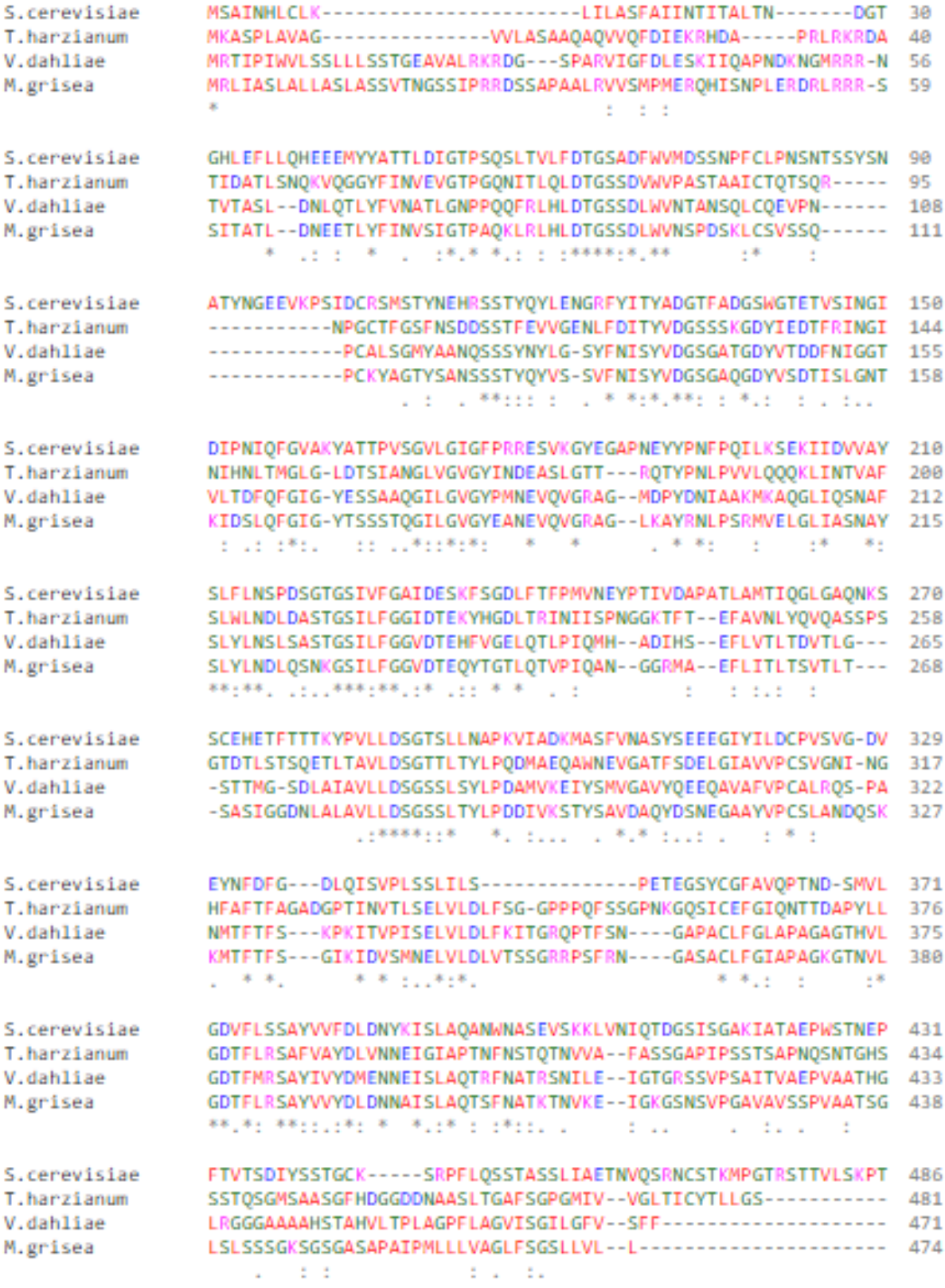

Figure 17. ***F. oxysporum, S. cerevisiae and C. glabrata*** **aligments of Bar1 protease.**