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# ROLE OF THE *MAT* LOCI IN THE DEVELOPMENT AND VIRULENCE OF *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI*

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**RIASSUNTO**

L'identità e la compatibilità sessuale nei funghi sono controllate da due *Mating-type* (*MAT*) loci, chiamati *MAT1-1* e *MAT1-2*, che regolano in maniera differente l'espressione di feromoni sessuali e recettori (α-feromone-Ste3 recettore o a-feromone-Ste2 recettore, rispettivamente). I funghi che presentano i *MAT* loci nel nucleo di due differenti individui della stessa specie sono eterotallici (auto-sterili), mentre quelli che presentano entrambi i *MAT* loci nello stesso nucleo sono omotallici (auto-fertili). *F. oxysporum* f. sp. *lycopersici* (Fol) è un fungo eterotallico che causa l'avvizzimento vascolare delle piante di pomodoro; Fol attraverso ferite o aperture naturali entra nelle radici della pianta, cresce intra- e inter-cellularmente raggiungendo i vasi xilematici per colonizzare la pianta. Sebbene il genoma di Fol contenga i *MAT* loci (*MAT1-1* nel 4287 e *MAT1-2* nell'isolato 54003, rispettivamente), non è stato ancora descritto alcun ciclo sessuale in questa specie fungina (Yun *et al.,* 2000). Recentemente, è stato dimostrato che feromoni e recettori in Fol 4287 regolano funzioni indipendenti dall'accoppiamento come il rilevamento chemiotropico dei segnali delle piante ospiti e la germinazione in dipendenza della densità conidica attraverso la generazione di un ciclo autocrino, (APS) (Turrà *et al.,* 2015; Vitale *et al.,* 2019). Con l'obiettivo di capire come i *MAT* loci regolano i processi indipendenti dall'accoppiamento, abbiamo generato una serie di mutanti in entrambi gli isolati Fol sia privi del *MAT1-1* che contenenti entrambi i *MAT* loci nello stesso genoma. Saggi fenotipici condotti con questi mutanti hanno rivelato che il *MAT1-1* locus inibisce, mentre *MAT1-2* promuove la germinazione conidiale ad alta densità cellulare. Inoltre, il *MAT1-1* locus ha mostrato essere un induttore della formazione di aggregati ifali e il *MAT1-2* locus un repressore della fusione ifale vegetativa in Fol. È interessante notare che, analizzando l'espressione dei geni correlati all'APS in Fol ad alte concentrazioni di inoculo, sia i livelli di trascrizione di *Bar1,* proteasi dell'α-feromone, che quelli dei geni dei feromoni e dei recettori sono più alti nei mutanti MAT1-1Δ e MAT1-1Δ+MAT1-2. Questi mutanti hanno mostrato una derepressione della germinazione conidiale, molto probabilmente dovuta ad un aumento della segnalazione a-feromone/Ste3 e dell'attività di *Bar1*, due meccanismi, già precedentemente descritti, che promuovono la germinazione ad alta densità cellulare (Vitale *et al.,* 2019). Poiché Fol è un patogeno vegetale, è stata infine valutata la patogenicità di tutti i mutanti verso piantine di pomodoro, per capire se i *MAT* loci avessero un ruolo nella regolazione di questo processo. Una riduzione parziale ma significativa della virulenza è stata riscontrata per il ceppo mutante MAT1-1Δ confrontato con il ceppo Fol 4287, suggerendo che i *MAT* loci potrebbero influenzare il successo dell'infezione in questo patogeno fungino.

Nel complesso, i risultati ottenuti rappresentano le prime evidenze che mostrano il ruolo dei *MAT* loci in funzioni indipendenti dall'accoppiamento quali germinazione conidica, fusione vegetativa e aggregazione ifale ad alta densità di inoculo e nella patogenicità del fungo asessuato *F. oxysporum.*

**ABSTRACT**

Sexual identity and compatibility in fungi are controlled by two *Mating-type* (*MAT*) loci, named *MAT1-1* or *MAT1-2,* differentially regulating the expression of one pheromone/receptor pair (α-pheromone-Ste3 receptor or a-pheromone-Ste2 receptor, respectively) over the other. Fungi that present *MAT* loci in different nuclei of two individuals of the same specie are heterothallic (self-sterile), whereas those carrying them in the same nucleus are homothallic (self-fertile). *F. oxysporum* f. sp. *lycopersici* (Fol) is a heterothallic soil-borne pathogen causing vascular wilt disease on tomato plants; it enters in the plant roots mainly through natural openings or wounds and grows inter- and intra-cellularly until it reaches the xylem vessels to colonize the entire plant. Although the genome of Fol contains conserved *MAT* loci (*MAT1-1* in the 4287 and *MAT1-2* in the 54003 isolates, respectively), no sexual cycle has been described yet in this fungal specie (Yun *et al*., 2000). Recently, sexual pheromones and receptors from the Fol 4287 isolate have been shown to regulate mating-independent functions such as chemotropic sensing of host plant signals and density-dependent conidial germination via autocrine pheromone signalling (APS) (Turrà *et al.,* 2015; Vitale *et al.,* 2019). With the aim to understand how *MAT* loci regulate mating-independent processes we generated a set of mutants in both Fol isolates either lacking the *MAT1-1* or containing both *MAT* loci in the same genome. Phenotypic assays carried out with these mutants revealed that the *MAT1-1* locus inhibits, while *MAT1-2* promotes conidial germination at high cell-density. Additionally, the *MAT1-1* locus showed to be an inducer of hyphal aggregate formation and the *MAT1-2* locus a repressor of vegetative hyphal fusion in Fol. Interestingly, by analysing the expression of APS related-genes in Fol at high concentrations of inoculum, we observed that the transcript levels of the α-pheromone specific protease *Bar1* and of both pheromone and pheromone-receptor genes are higher in the *MAT1-1Δ* and *MAT1-1Δ+MAT1-2* mutant strains. Noticeably, these mutants showed a derepression of conidial germination, most likely due to an increase in a-pheromone/Ste3 signalling and *Bar1* activity, two mechanisms previously described to enhance germination under high cell densities (Vitale *et al.,* 2019). Because Fol is a plant-pathogen, we finally tested the pathogenicity of all of the obtained mutants on tomato seedlings to understand if *MAT* loci could play a role in the regulation of this process. We observed only a partial but significant decrease in virulence for the *MAT1-1Δ* mutant strain in comparison to the wt Fol 4287 strain, suggesting that specific *MAT* loci might determine the pathogenic success in this destructive soil-borne fungal pathogen.

Collectively, our findings provide the first evidence for a role of *MAT* loci in mating independent processes such as quorum sensing, cell fusion and pathogenicity of the asexual fungus *F.oxysporum.*

# 1 INTRODUCTION

# 1 INTRODUCTION

## *1.1* FUSARIUM OXYSPORUM *SPECIES COMPLEX*

*Fusarium* genus represents the most important group of plant fungal pathogen belonging to the Ascomycota phylum. *Fusarium oxysporum* (Fo) is the most common species and comprises both pathogenic and non-pathogenic strains adapted to a wide range of geographical areas, climate conditions, ecological habitats and host plants (Di Pietro *et al.*, 2003; Ma *et al.*, 2013).

Fo is a soil-borne ascomycete and the causal agent of vascular wilt, a devasting disease that affect a large variety of economically important crops worldwide including banana, cabbage, carnation, chrysanthemum, cotton, flax, gladiolus, muskmelon, onion, pea, tomato, tulip and watermelon (Armstrong and Armstrong, 1981). In addition to its remarkable plant pathogen activity, it can cause a broad spectrum of diseases both in humans and mice, ranging from superficial or localized infections in healthy hosts to lethal disseminated fusarioses in immunocompromised individuals (Dignani and Anaissie, 2004; Nucci and Anaissie, 2007). In addition, ecotoxicological study revealed the entomopathogenic activity of this fungus toward the greater wax moth *Galleria mellonella* larvae, an invertebrate model host that is widely used for the study of microbial human pathogens (Navarro-Velasco *et al.,* 2011). For all these characteristics, Fo is considered a multi-host fungal pathogen and an excellent model system to study fungal pathogenicity mechanism both in plants and mammals (Ortoneda *et al*., 2004).

In Fo, no sexual stage has been described, for this reason the biological concept of species cannot be applied and we speak more properly of the Fo species complex. Phylogenetic studies revealed that Fo comprises over 120 *formae speciales* which infect different plant species. In most of the cases they are named as f. sp. (*i.e. F. oxysporum* f.sp. *ciceris*; *F. oxysporum* f.sp. *cubense; F. oxysporum* f.sp. *lycopersici*; *F. oxysporum* f.sp. *melonis*). Further, it has been demonstrated that different isolates of a given *forma specialis* have originated independently during evolution (O'Donnell *et al*., 2004; Michielse and Rep, 2009; Arie *et al.*, 2010). Indeed, complete genome sequence analysis of the tomato pathogenic form Fol indicated the existence of certain lineage-specific (LS) genomic regions, including four entire chromosomes that are absent in other *Fusarium* species such as *F. graminearum* and *F. verticillioides* (Ma *et al.*, 2010). Since no sexual stage has been described so far in Fo, the most likely hypothesis is that these supernumerary chromosomes have been acquired by horizontal transfer of genetic information, through vegetative hyphal fusion (VHS) with heterokaryon formation thus could

leading to the emergence of new pathogenic lineages. According to their strict host specificity pathogenic strains of *F. oxysporum* f.sp. *lycopersici* (Fol) can be further divided into three physiological races, named, according to their discovery, race 1, race 2 and race 3 (Srinivas *et al*., 2019).

### 1.1.1 Biology of *Fusarium oxysporum*

*F. oxysporum* changes its morphology and colour depending on the environmental conditions. The culture conditions affect growth rate, shape, size and abundance of conidia as well as number of septa and pigmentation (Booth, 1971). The aerial mycelium appears first in white and then turns to a variety of colours that range from pink to dark purple. The specie produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Fig. 1) (Agrios, 1997)**.**

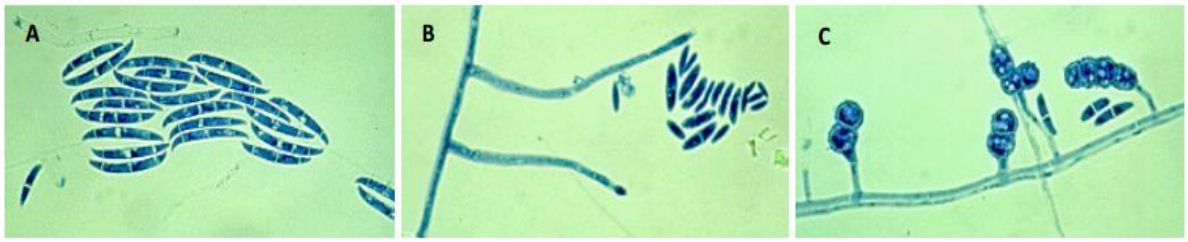


**Fig. 1.** Type of *F. oxysporum* conidia. (**A).** Macroconidia. (**B).** Microconidia. (**C).** Chlamydospores.

Microconidia are single-cell dispersal structures that are abundantly produced under most conditions, whereas macroconidia contain three to five cells that are gradually pointed and curved toward the ends and that are commonly found on the surface of dead plants killed by the pathogen. Chlamydospores are thick-walled cells generally developed through the modification of hyphal and conidial cells. Chlamydospore formation is induced by aging or unfavorable environmental conditions such as low temperatures or carbon starvation. They play an important role as primary inoculum for plant root infection and represent the principal structure by which the fungus can live for long-time in the soil during unfavorable periods with a saprophytic lifestyle (Kang *et al.,* 2014).

Considering the significance of asexual spores in the success of Fo infection, it is of crucial importance the understanding of how Fo regulates conidia germination. The successive steps

that determine a successful Fo infection include hyphal development, a prerequisite for vegetative hyphal fusion to occur, and the differentiation of infection hyphae.

### 1.1.2 Spore-germination of *Fusarium oxysporum* f.sp. *lycopersici*

The germination of spores is a fundamental step in fungal development leading to the conversion of a dormant cell into growing hyphae. It involves breaking of dormancy by external signals such as nutrients released from decaying plant tissue or from roots exudate, whose composition is affected by the age of plants, and then is followed by a pre-germination phase and germ-tube formation (GT) that marks the establishment of polar growth (Steinkellner *et al.,* 2005; Sharma *et al.,* 2016; Askun 2018). In certain fungi, germ tube emergence and septum formation are subject to precise spatial controls and are tightly coordinated with nuclear division, whereas in other filamentous fungi nuclear division is not required for the emergence of germ tube. Generally, Fo presents a unipolar conidial germination, in which most conidia produce a single germ tube characterized by the formation of a septum through which it is separated from microconidia that adhering to the root surface initiate the host colonization, a fundamental step for the infection success (Ruiz-Roldán *et al.,* 2010). Further, it has been demonstrated that microconidia and germ tubes of Fol stop their normal Brownian movements and rapidly orient their growth in presence of plant roots, subsequently adhering and colonizing them, showing not only specific chemotropic response toward plants but also between germlings toward each other, with the generation of hyphal fusion events contributing to the fungal development and invasive growth in the host (Prados-Rosales and Di Pietro, 2008; Turrà *et al,* 2015).

### 1.1.3 Vegetative hyphal fusion of *Fusarium oxysporum* f.sp. *lycopersici*

Vegetative hyphal fusion (VHF), also termed anastomosis, results in a well-regulated process which determines the formation of a complex hyphal network and, in some conditions of growth leads to the production of visible macroscopically fungal aggregates. After germination during the initiation colony, spore germlings generate distinct cell protrusions termed conidial anastomosis tubes (CATs), cytoplasmatic interconnections between genetically identical or incompatible species in a mature fungal colony. Germinating conidia of Fol frequently change the original axis of growth before fusing with each other through the formation of CATs which differ from germ tubes (GTs) to being thinner, shorter and without branches. As reported

previously, CAT fusion occurs either from tip-to-tip fusion of CATs formed from growing GTs, tip-to-tip fusion between spores, tip-to-side fusion of CATs formed from a growing GT to a spore, and side-to-side fusion of CATs formed from adjacent GTs (Kurian *et al.,* 2018) (Fig. 2).

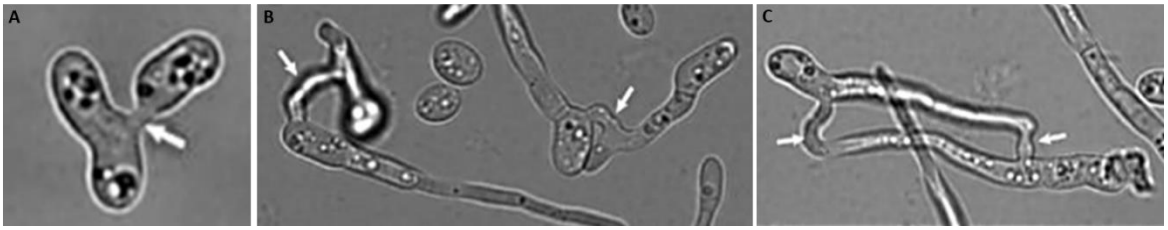


**Fig. 2.** Some different types of CATs fusion in Fol. **(A)** CAT fusion between two GTs; **(B)** CAT fusion between two conidia that have already produced long GTs; **(C)** tip-to-side fusion of a CAT formed from a growing germ tube to a spore (adapted from: Kurian *et al.,* 2018)

CAT fusion and its underlying signalling mechanisms have been extensively studied in *N. crassa*. First, conidial anastomosis tube emerges from conidia or germlings that subsequently, exhibits chemotropic growth towards each other. CAT tips make contact among them with the degradation of cell walls and plasma membrane through which nuclei and organelles move. Although fusion among incompatible strains usually results in the death of the fused cells, VHF represents a fundamental mechanism by which fungi ensure the transport of water and nutrients. Further this process is particularly important in the pathogens that lacks a known sexual stage as Fol, in which the occurrence of VHF is a way to ensure the variability through the transfer of genetic material among different strains or even species (Prados-Rosales and Di Pietro, 2008; Ruiz-Roldán *et al.,* 2010; Kurian *et al.,* 2018) .

### 1.1.4 Plant infection cycle of *Fusarium oxysporum* f.sp. *lycopersici* and management of vascular wilt

Generally, the infection cycle of Fo starts with microconidia or chlamydospore germination. Once the appropriate conditions for infection are found, the infectious hyphae initially perceive secreted compounds from the host root and then grow towards and adhere to them. Afterwards, infectious hyphae enter in the host plant through wounds or the natural openings at the intercellular junctions of cortical cells (Pérez-Nadales and Di Pietro, 2011; Turrà *et al.,* 2015). Once inside the plant roots, they grow inter- and intra-cellularly and reach the xylem vessels

used as a conduct to colonize the entire plant. Symptoms of wilt disease are variable and include combinations of vein clearing, leaf epinasty, wilting, chlorosis, necrosis and abscission. Severely infected plants wilt and die, whereas plants affected to a lesser degree become stunted and significantly lose productivity. Further, it has been demonstrated that a defence response based on a gene-for-gene is induced in the xylem contact cells (Rep *et al.*, 2004). Unlike the classical hypersensitive response (HR), this reaction mainly involves 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 (Rep *et al.*, 2004; Michielse & Rep, 2009) The three conidial forms develop and accumulate on the infected tissues and in the surrounding soil, concluding the fungal biological cycle (Fig. 3).

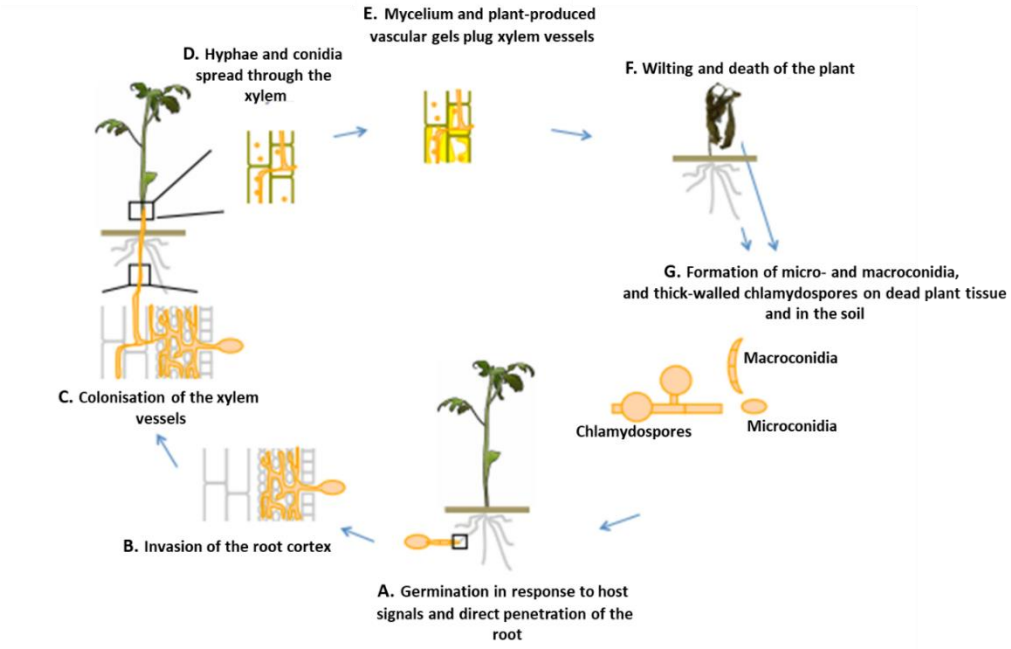


**Fig. 3.** Life cycle of *Fusarium oxysporum* f.sp. *lycopersici*. **A**. Germination in response to host signals and direct penetration of the root. **B**. Invasion of the root cortex. **C.** Colonization of the xylem vessels. **D.** Hyphae and conidia spread through the xylem. **E.** Fungal mycelium and plant-produced vascular gels plug the xylem vessels. **F.** Wilting and death of the plant. **G**. Formation of micro- and macroconidia, and thick-walled chlamydospores on the dead plant tissue and in the soil (Pérez-Nadales *et al.*, 2014).

The control of vascular wilt becomes very complicated in the contaminated regions because the chlamydospore can survive in infested soil indefinitely in the absence of host for several years (Cha *et al.*, 2016; Khan *et al.*, 2017). The management of fusariosis has evolved over the years.

Chemical treatment with methyl bromide was applied as early as in the 1930s (Rosskopf *et al.*, 2005). However, because of its adverse effects on human health and the environment, its use was gradually reduced from the late 90s until its final ban in most countries (Fravel *et al.*, 2003). For this reason, new methods for disease control have been developed and focused mainly on the generation of resistant cultivars towards certain races of Fol. In cases where is no resistance against *Fusarium* wilt the disease can only be controlled by preventing the introduction of the pathogen through destruction of diseased plants. In the last years the application of antagonistic bacteria or non-pathogenic strains of *F. oxysporum* represents a valid alternative strategy to contrast the fungal development through different mechanisms such as antibiosis, competition for nutrients, reduction of chlamydospore germination, competition on the host root and induction of plant defense and systemic resistance (Larena *et al.,* 2002; Larkin *et al.,* 2002; Khan *et al.,* 2006).

## 1.2 SEXUAL STRATEGIES IN FUNGI

Sexual reproduction represents a fundamental process in which eukaryotic organisms ensure genetic recombination, elimination of deleterious mutations and production of a better-adapted recombinant progeny. To achieve a successful sexual reproduction these three steps are required:

- **pre-fertilisation**: the formation of gametes by the same or distinct individuals;
- **fertilisation**: the fusion of gametes and zygote generation;
- **post-fertilisation**: the formation of a new gametes through meiosis and ploidy changes also via homologous recombination.

Thus, in higher eukaryotes, meiosis and the production of gametes with allelic combinations different from parental type provide the side effect of increased genetic variation in each new generation by ensure the survival of the species in response to environmental changing (Beukeboom *et al.,* 2014; Wallen *et al.,* 2018).

Fungi represent the most diverse among the eukaryotic kingdoms with an incredible variety of known life cycles. Two alternative reproductive strategies (sexual and asexual reproduction) have conferred to them a series of advantages during their evolution. For instance, asexual multiplication seems to be most suited for individuals that have adapted to a particular environment, such as plant pathogens capable of infecting specific genotypes of their host. Once established in such a niche, sexual recombination would lead to changes in the gene-pool with potential distortion of the expression profile required for optimal infection and spread in the host. Alternatively, with changing environmental conditions, asexual propagation would become a disadvantage as a novel make-up of the gene-pool resulting from a sexual cycle is required to adapt to these changes (Waalwijk *et al.,* 2006).

Sexual reproduction in fungi is a well-controlled process regulated by mating-type genes harboured in a single genetic mating type locus (*MAT*), whose expression regulates the sexual identity and mating partner recognition. *MAT* loci can exist as two genetic dissimilar structures flanked by large homologous regions. The genetic variability of *MAT* genes between the opposite *MAT* loci suggests that they don't share a common evolutionary origin and for this reason they have been named idiomorphs instead of alleles (Metzenberg and Glass, 1990; Heitman *et al.,* 2013).

Fungal species have evolved two main sexual reproductive strategies: heterothallism and homothallism. Heterothallic species (self-incompatible) possess *MAT* idiomorphs in two different nuclei and they need to mate of a compatible partner, morphologically identical but genetically different at the mating type locus. Homothallic species (self-compatible), carry both *MAT* idiomorphs in a single nucleus, usually closely linked or fused and are able to self-reproduce (Bennett and Dunny, 2010).

## 1.3 MATING TYPE DETERMINATION IN ASCOMYCETOUS FUNGI

### 1.3.1 *MAT* locus in the fungal model system of *Saccharomyces cerevisiae*

The genetic structure of *MAT* loci was first characterized in the fungal model *Saccharomyces cerevisiae* where the two idiomorphs are named *MAT*α or *MAT*a. The idiomorph *MAT*α expresses two proteins: **α1** (containing an α-box motif) and **α2** (containing a homeodomain motif), whereas *MAT*a encodes for only one protein **a1** with a homeodomain motif.

These proteins typically possess DNA binding domains that regulate the expression of α-specific (αsg's) or a-specific genes (asg's). The expression of α-specific or a-specific genes determine the formation of three cell types: haploid *MAT*α cells; haploid *MAT*a cells; diploid cells (Tsong *et al.*, 2003).

In haploid *MAT*α cells, **α1** in conjunction with a constitutively expressed protein, Mcm1, activates a set of α-specific genes, while the **α2**-Mcm1 complex represses a-specific genes that would otherwise be expressed constitutively. Deletion of the bidirectional promoter controlling α1 and α2 or of the entire *MAT*α locus results in haploid cells having an a-like mating behavior, due to the absence of α2 and the transcription of αsg's that is not induced in the absence of α1. For this reason, *MAT*a is not required for a-mating and the a1 gene is required, along with α2, only to repress haploid-specific gene expression in diploids. Moreover, *S. cerevisiae* has the ability to switch mating-type. This ability is related to the presence of two additional copies of the mating-type cassette, *HMLα* and *HMRa*, located at the telomeric region of the same chromosome harbouring the *MAT* locus and normally transcriptionally repressed by a process known as silencing. Mating-type switching occurs only in cells that have budded at least once (mother cells), through the activity of a functional endonuclease (HO) presents in the genome and that cleaves *MAT* locus promoting the recombination between the active and silent cassettes (Haber *et al.*, 2012; Hanson *et al.*, 2017).

Although *S. cerevisiae* is a non-pathogenic fungus, the molecular organization of its *MAT* loci is similar to the one of the most important human fungal pathogen *Candida albicans*, in which α1, α2 and a1 proteins also coordinate the expression of a- and α-specific genes. Nevertheless, the transcriptional circuits that regulate mating in *S. cerevisiae* and *C. albicans* present several important differences. In *S. cerevisiae* the identity of α-cells type is determined by the presence of **α1**, the activator of αsg's, and **α2**, the repressor of asg's. While in haploid a-cells, the *MAT* locus contains only the *MATa1* gene coding for the homeodomain protein **a1**, but this protein

is not required for a cell-type identity. The identity of a-cells is instead defined by the absence of both **α1** and **α2** proteins. The circuit is different in *C. albicans*: **a2** protein encoded by *MATa* idiomorph activates the a-specific genes, and **α1** induces the expression the α-specific genes. In both organisms, the expression of **a1** and **α2** act together to repress mating-type reaction (Tsong *et al.*, 2006; Hanson *et al.*, 2017) (Fig. 4).

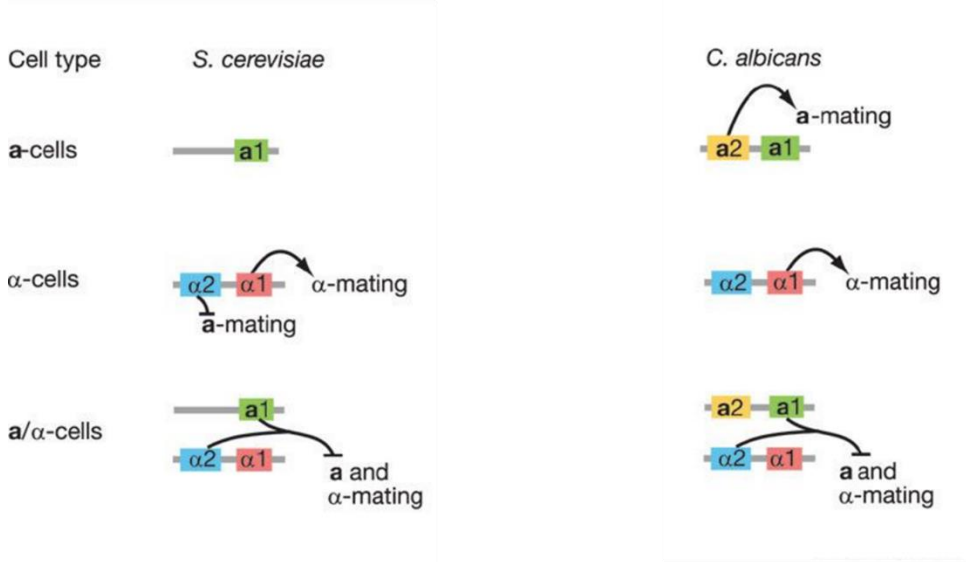


**Fig. 4.** The transcriptional circuits regulating mating type in *S. cerevisiae* and *C. albicans* (adapted from: Tsong *et al.*, 2006)

### 1.3.2 *MAT* locus in filamentous fungi

*MAT* locus composition has been characterized in several homothallic and heterothallic filamentous ascomycetes, including members of Dothideomycetes (*Cochliobolus*), Eurotiomycetes (*Aspergillus*, *Coccidioides* and *Histoplasma*), Leotiomycetes (*Pyrenopeziza* and *Rhynchosporium*) and Sordariomycetes (*Fusarium*, *Gibberella*, *Neurospora*, *Podospora*, and *Magnaporthe*), (Debuchy and Coppin, 1992; Glass *et al.*, 1988; Yun *et al.*, 2000; Foster and Fitt, 2003; Paoletti *et al.*, 2005; Fraser and Heitman, 2006). The nomenclature used differs greatly from species to species, but the general structure is similar. Turgeon and Yoder (2000) proposed to designate the *MAT* locus of heterothallic fungi as *MAT*1 and the two idiomorphs as *MAT*1-1 and *MAT*1-2, and the genes inside them as the symbol of the idiomorph, followed by a hyphen and a number (i.e. *MAT1-1-1*). The specific gene organization of the *MAT* locus can be variable among fungal species, but despite this, *MAT* idiomorphs encode always for two key transcriptional factors: the *MAT1-1-1* and the *MAT1-2-1* genes.

The *MAT1-1-1* gene is located in the *MAT1-1* locus and encodes for a protein with an α-box motif, equivalent to that of *S.cerevisiae*, while the *MAT1-2-1* is located in the *MAT1-2* locus and encodes a homeodomain-transcription protein equivalent to the ***a1*** protein of *S. cerevisiae*. The *MAT1-1* locus contains other two genes: *MAT1-1-2* [PPF domain (which contains conserved proline, proline, and phenylalanine residues) gene] and *MAT1-1-3* [an HMG (high-mobility group) domain gene], while the idiomorph *MAT1-2* includes another small open reading frame (ORF) of unknown function. Although the order of the gene inside the *MAT* locus can differ among fungal species, the genomic region in which they are placed is well-conserved and is flanked by the *APN2* gene (encoding for an endonuclease/DNA lyase) and *SLA2* gene (encoding for a protein that binds actin) (Fig. 5) (Heitman *et al.*, 2007; Ni *et al.*, 2011, Palmer *et al.*, 2014).

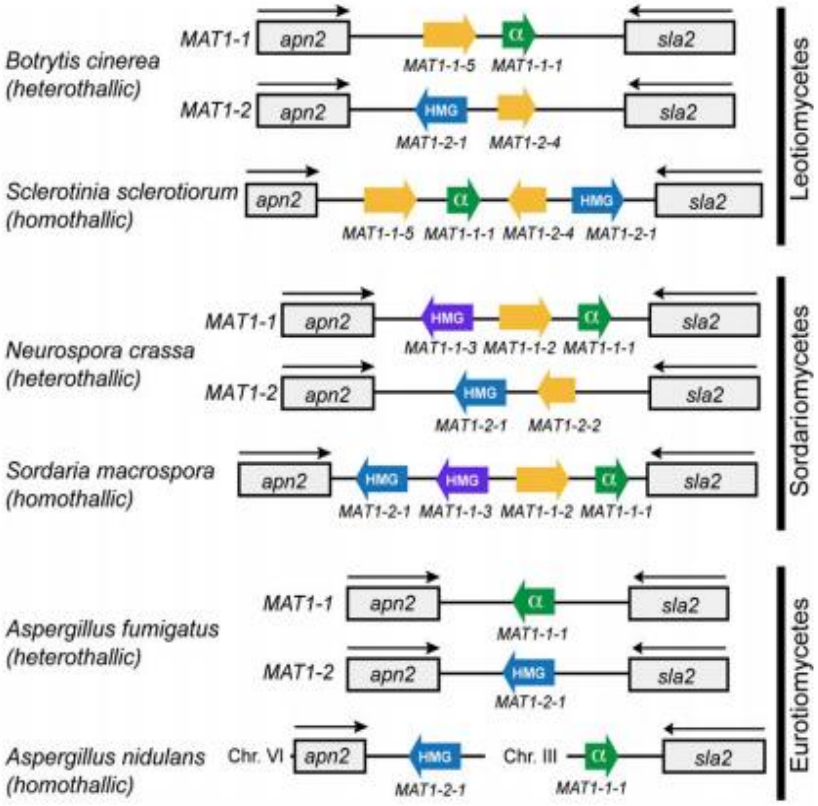


**Fig. 5.** *MAT* loci organization in some homothallic and heterothallic fungal species (Palmer *et al.*, 2014).

The two key transcriptional factors *MAT1-1-1* and *MAT1-2-1* define *MAT* identity and sexual development, whereas *MAT1-1-2* and *MAT1-1-3* genes are required at later stages during sexual development (Heitman *et al.*, 2007). Contrarily, homothallic species carry the genes of both

idiomorphs on the same chromosome, and both are indispensable for self-fertility (Lee *et al.*, 2003). For example, in the homothallic specie *Gibberella zeae*, also named as its anamorph *Fusarium graminearum*, *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, *MAT1-2-1* and *MAT1-2-3* are in the same chromosomal location, while in the closely related heterothallic fungus *Fusarium fujikuroi*, also named *Gibberella fujikuroi*, the same genes are dislocated into the two distinct idiomorphs (Martin *et al.*, 2011). Interestingly, genomic analysis demonstrated that the molecular organization of *MAT* loci in *Fusarium oxysporum* presents high similarity with the one of *Gibberella fujikuroi* and with those found in closely related sexually reproducing heterothallic species such as *Fusarium proliferatum* and *Fusarium verticillioides*. Indeed, the position of the idiomorphs, the size of the coding regions as well as the direction of transcription among these three related species are identical (Fig. 6) (Waalwijk *et al.*, 2006).

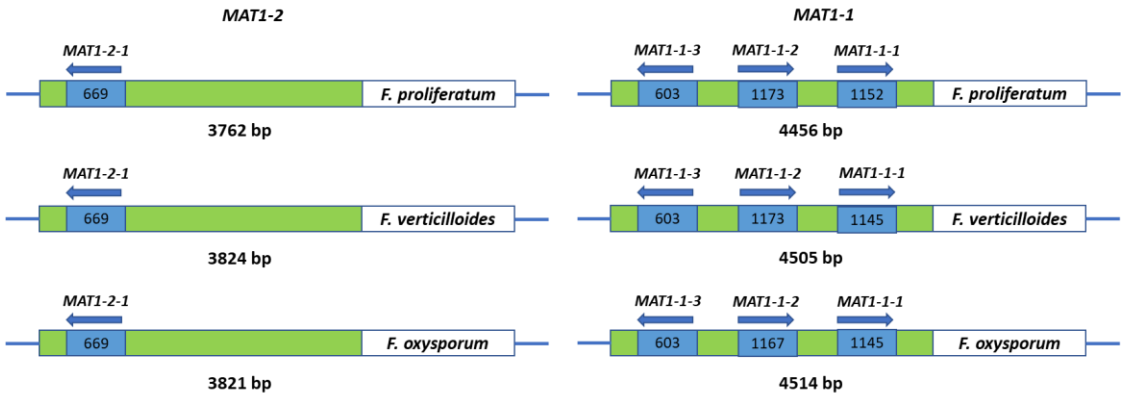


**Fig. 6.** Comparison of the related heterothallic species *F. proliferatum*, *F. verticillioides* and the asexual species *F. oxysporum*. Numbers in the boxes indicate the sizes of the coding sequences (adapted from: Waalwijk *et al.*, 2006)

In Fo the *MAT1-1* idiomorph is composed of three genes including a gene coding for a protein with an alpha-box motif *MAT1-1-1* (motif found in all ascomycete *MAT1-1* idiomorph), whereas the *MAT1-2* idiomorph contains a single gene *MAT1-2-1* encoding for a transcriptional factor with a high-mobility-group (HMG) DNA binding domain (motif found in all ascomycete *MAT1-2* idiomorph). *F. oxysporum MAT* genes are expressed and all the predicted introns are correctly processed (Yun *et al.*, 2000). These observations might indicate either that a cryptic sexual cycle may remain to be discovered in *F. oxysporum* or that *MAT* loci might regulate additional functions beyond sexual reproduction in this important class of fungal pathogens.

## 1.4 *MAT* LOCUS REGULATION OF SEXUAL AND ASEXUAL PROGRAMS

### *1.4.1 MAT* locus regulation in model fungal system *Saccharomyces cerevisiae* and *Candida albicans*

Generally, key transcriptional factors encoded by the *MAT* idiomorphs regulate the expression of genes involved in the mate recognition, cellular differentiation and meiosis (Klix, *et al.,* 2010). In the ascomycota fungi mating partner recognition is mediated by the interaction of diffusible pheromone peptides with their cognate G protein-coupled receptors on the cell surface. In *Saccharomyces cerevisiae* the two different *MAT* loci affect the expression of pheromones, receptors and other signalling proteins required for cell-cell recognition. The α-type cells secrete the α-pheromone peptide and possess the cell surface receptor Ste3, which binds and responds to a-factor. Similarly, a-type cells secrete a-factor, a lipid-modified peptide pheromone, and produce the cell surface receptor Ste2, which binds α-factor (Ni *et al*., 2011). Detection of a-pheromone gradient from the opposite mating type cells leads to a range of cellular responses that assure the coordinated control of the cell fusion and subsequent karyogamy (Arkowitz, 2009). Such responses include the arrest of the cell cycle at the G1 phase previous to the DNA synthesis. This process occurs to prepare haploid cells for the conjugation and induce change of the expression levels in a variety of genes. As a result, haploid cells change growth polarity to encounter the mating partner. Finally, they form a projection of their cell wall and cytoplasm, named "shmoo", in the direction of the of opposite mating type cell. Such protrusions undergo plasmogamy first and karyogamy then that through meiosis produces ascospores, each containing a haploid nucleus that can start a new reproduction cycle (van Drogen *et al.,* 2001; Wallen *et al.,* 2018). In some *C. albicans* strains *MAT* locus regulates a morphological switch from the predominant white form to the rarer and less stable opaque form of cells that is mating competent. When exposed to α-pheromone *C. albicans* cells form long polarized projections, similar to the "shmoo" projections observed in *S. cerevisiae* cells (Miller and Johnson, 2002; Bennett *et al.,* 2010) (Fig. 7).

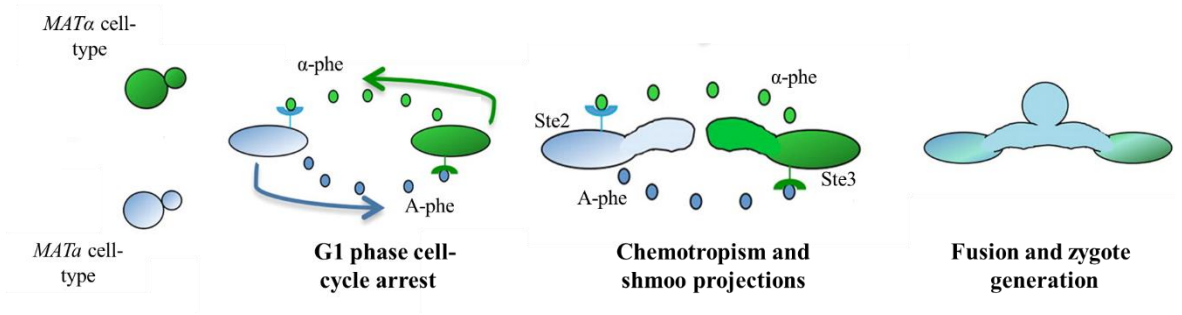


**Fig. 7.** Schematic representation of mating-type reaction in fungal model system (adapted from: Bennett *et al.,* 2010).

Signal transduction has been well-characterized in *S. cerevisiae* and involves the activation of a conserved MAPK (Mitogen Activated Protein kinase) that transduces the pheromone signal from outside of the cell into a transcriptional response in the nucleus (Elion, 2000; Lengeler *et al.*, 2000). Briefly, *S. cerevisiae* G-protein coupled receptors (GPCRs), Ste2 and Ste3, mediate mating responses through binding of opposite mating type pheromones (Ladds *et al.*, 2005; Xue *et al.*, 2008). The heterotrimeric G-protein complex consists of Gα (Gpa1) linked to the Gβγ dimer (Ste4- Ste18) subunits. The binding of pheromone to its cognate receptor leads to the activation of the heterotrimeric G-protein complex, with the dissociation of its subunits in Gα (Gpa1) and the Gβγ dimer, which binds and activates a downstream mitogen-activated protein kinase (MAPK) cascades (Wang and Dohlman, 2004). The cascade involves activation of Ste20, which in turn leads to successive phosphorylation of Ste11, Ste7, and finally of the Fus3 MAPK. The components of MAPK cascade are linked among them by Ste5 protein that promotes through conformational changes the phosphorylation process. However, this scaffold protein is less conserved than other proteins of the MAPK cascade and results limited to the *Saccharomycotina* fungi (Rispail *et al.*, 2009). Once phosphorylated, Fus3 activates the sequence specific DNA binding protein Ste12, which together with Mcm1, is responsible for activating the pheromone-responsive genes in *S. cerevisiae* (Fig. 8) (Bennett *et al.*, 2005; Choudhary *et al.*, 2015).

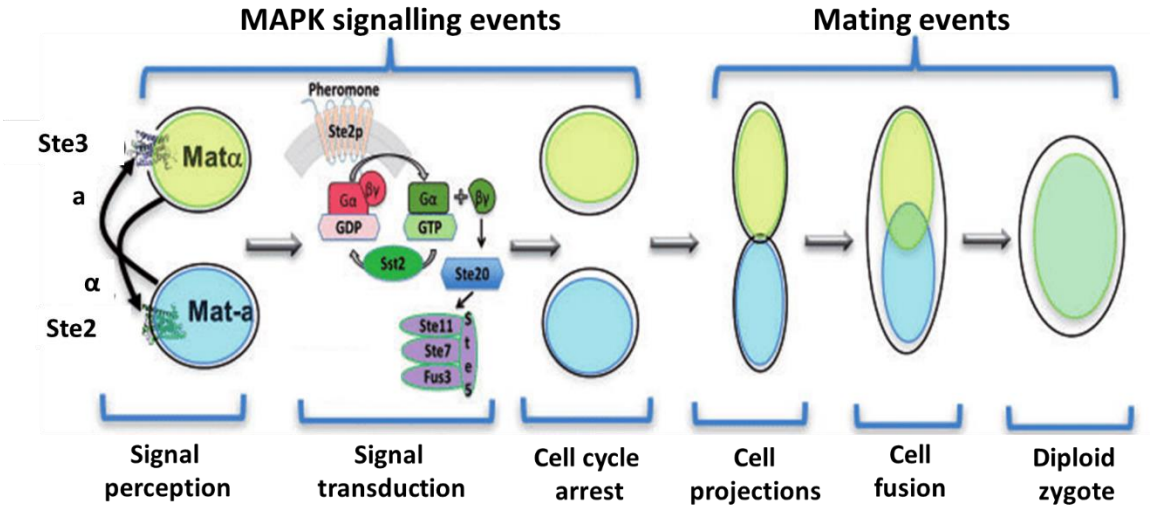


**Fig. 8.** *Saccharomyces cerevisiae* pheromone mating response (adapted from: Choudhary *et al.*, 2015)

It has been described that in both *S. cerevisiae* and *C. albicans MAT* genes play a role in regulating non-sex related functions. Indeed, in *S. cerevisiae* it has been demonstrated that the *a1* and *α2* genes are involved in cell wall maintenance during stress responses and invasive growth (Birkaya *et al.*, 2009). In *C. albicans* there are also indications that genes in the *MTL* locus play non mating roles in mating-incompetent α/a cells, in which *MTLa1* and *MTLα2* with other three apparently unrelated ‘‘nonsex’’ genes (NSGs), *PIK*, *PAP* and *OBP*, placed in the *C. albicans MAT* locus regulate biofilm formation and virulence (Srikantha *et al.*, 2012)

### 1.4.2 *MAT* locus regulation in filamentous fungi

Several studies reported that *MAT* genes regulate also the expression of pheromone and receptor genes in many ascomycetous fungi. The *MAT1-1-1* gene controls the expression of the α-factor, while the *MAT1-2-1* gene is responsible for a-factor expression. As a direct result, *MAT1-1* individuals express only the α-factor and *MAT1-2* individuals express only the a-factor (Bobrowicz *et al.*, 2002; Wilson *et al.*, 2018). Homothallic species, which typically possess both the *MAT1-1-1* and *MAT1-2-1* genes, are often able to express both pheromones (Pöggeler *et al.*, 2000; Lee *et al.*, 2008). In some ascomycetous heterothallic fungi pheromones and receptors play an essential role in their sexual fertility. In the heterothallic *Neurospora crassa*, the deletion of *pre1*, orthologs of Ste2 receptor of *S. cerevisiae*, leads to a female sterility

whereas deleting either of pheromones precursor genes generate male sterile isolates (Kim and Borkovich, 2006).

Similar results were found in heterothallic *Podospora anserina* and *Cryphonectria parasitica*, in which the α-factor is essential for male fertility (Turina *et al.,* 2003; Coppin *et al.,* 2005). Genes encoding both pheromones and their receptors have been identified in homothallic species such as *S. macrospora* and *F. graminearum* in which they mediate female fertility or male sterility and the development of mature fruiting bodies (Mayrhofer *et al.,* 2006; Lee *et al.,* 2008). The presence of pheromone and receptor genes have been found also in the homothallic *G. zeae* in which it has been evaluated the expression level of such genes. Moreover, many fungal species, without a sexual stage in their life cycle, possess a functional *MAT* locus supporting the hypothesis that, not only pheromones and receptors but the same genes inside the mating type sequences might have a biological role beyond sexual reproduction. Recent genome-wide profiling of DNA-binding proteins study findings, related with mating-type transcription factor functions, have shown , that *MAT1-1-1* of heterothallic *Penicillium chrysogenum* might be implicated in additional functions apart from those related to transcriptional regulation of sexual development genes, such as amino-acid, iron, and secondary metabolism (Becker *et al.,* 2015). Recently, also in the heterothallic model cellulolytic fungus *Trichoderma reseii* it has been suggested that the mating-type locus protein *MAT1-2-1* plays an important role in the cellulase production. The disruption of *MAT1-2-1* gene resulted in a compromised induced expression of cellulase genes in response to light or lactose. It has been demonstrated that this protein directly interacts with the key transcriptional activator *Xyr1* of *T. reseii* cellulase genes, regulating the cellulase gene expression in response to light (Zheng *et al.,* 2017).

## *1.5* THE PHEROMONE SYSTEM IN *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI* AND DETECTION OF PLANT SIGNALS

Despite its asexual lifestyle, Fo exists in two idiomorphic states (*MAT1-1* or *MAT1-2)*, with each *MAT* locus possessing a conserved genetic structure, in which all *MAT* genes are constitutively expressed (Yun *et al.,* 2000). However, the biological function of *MAT* loci in asexual fungal species remains largely unknown. Recently it has been demonstrated that the genome of the Fol isolate 4287, carrying the *MAT1-1* locus, possesses and encodes for a conserved pheromones-receptor system (Turrà *et al.*, 2015; Vitale *et al.,* 2019). The gene of the α-pheromone encodes for 10 highly homologous repeats of an active decapeptide (WCTWRGQPCW); whereas the gene of the a-pheromone encodes for an a-pheromone precursor containing three repeats of a 19/21-mer peptide with the consensus motif QTPGYPLSC(S/T)VM and a carboxy (C)-terminal CAAX domain—a hallmark of fungal a-pheromones. Moreover, the genome of Fol contains a predicted Ste2 and Ste3 orthologue genes that encode for seven-transmembrane receptors coupled to heterotrimeric G proteins (GPCR). Interestingly, it has been shown that, in contrast to other heterothallic fungi, the *MAT1-1* Fol isolate 4287, which should express only α-pheromone and the Ste3 receptor, co-expresses both pheromone/receptor pairs (Vitale *et al.,* 2019). In 2015 Turrà et al., already demonstrated that unisexual population of *MAT1-1* cells of Fol exhibit chemotropic hyphal growth towards α-pheromone gradients via the cognate receptor Ste2 and the conserved cell wall integrity (CWI) mitogen-activated protein kinase (MAPK) cascade MPK1. In a follow-up study it was demonstrated that the same pool of cells is also able to chemotropically respond to a-pheromone gradients via the cognate receptor Ste3 and the same MAPK cascade. Interestingly α- and a -pheromone are sensed by the cells in a competitive manner. Indeed, when the hyphae of Fol were exposed simultaneously to α- and a-pheromone gradients, in a competing gradient chemotropic assay, cells show preferential growth towards a-factor. This occurs because of the enzymatic activity of *Bar1*, a secreted protease that in *S. cerevisiae* and *C. albicans* specifically cleaves α -pheromone. Additionally, Fol is also able to secrete active a- and α -pheromone, which are used by cells to secrete and sense the same pheromones, undergoing autocrine pheromone signalling (APS). The biological role of APS in Fol is related to the induction of the expression of both pheromone and receptor genes at high cell density. The most important biological process determined by population density is spore germination. Indeed, similarly to many filamentous fungi, in Fo the germination of asexual spores (called conidia) is repressed at high cell densities. It has been discovered by generating mutant strains either lack pheromone,

receptor and (CWI) MAPK cascade downstream components genes that α-pheromone triggers cell-density-dependent repression of conidial germination via Ste2 and the (CWI) MAPK cascade. In contrast it has been demonstrated that a-pheromone/Ste3 signalling promotes germination in the presence of α-pheromone at low cell-density, possibly by competing with α/Ste2 triggered repression, but not at high cell-density in which α-pheromone degradation by *Bar1* and a/Ste3-mediated competitive recruitment of the CWI MAPK cascade are insufficient to counter α/Ste2 signalling and repression of germination (Vitale *et al.*, 2019). This finding reveals a novel mechanism to regulate fungal growth and development via APS.

Turrà et al. 2015 showed that in Fol Ste2 is not only specifically functional toward α-pheromone but that it also mediates the chemotropic response toward host plant secreted compounds. Fol infectious hyphae were able to perceive and re-orient their growth toward gradient of class III peroxidases, a family of haem-containing enzymes present in all land plants secreted from wounds and found in root exudate (Passardi *et al.*, 2004) (Fig. 9).

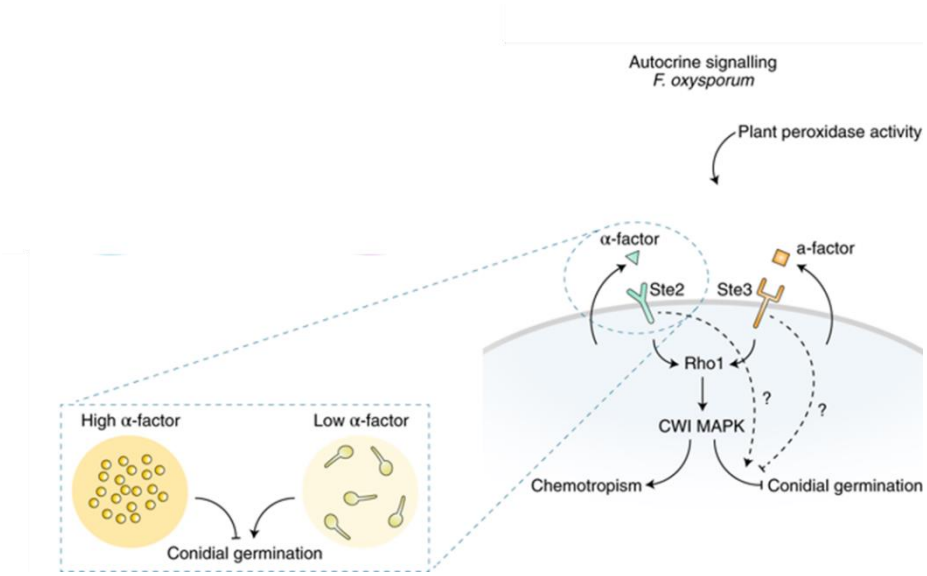


**Fig. 9.** Autocrine signalling in *F. oxysporum* (adapted from: Martin 2019)

These results revealed the existence of a regulatory mating-independent function for peptide pheromones that could also interest other filamentous fungi. Moreover, given that the expression of sexual pheromones and receptors is mediated by the *MAT* loci, these results suggest that the latters could regulate fungal biology beyond sexual development.

# 2 AIM OF THE RESEARCH

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*Fusarium oxysporum* f.sp. *lycopersici* is a soil-borne fungal pathogen that is the causal agent of vascular wilt in susceptible tomato plants (and other species), resulting in significant yield losses every year. The development of the disease starts with the germination of asexual spores considered the major source of inoculum in the fungal infection. Despite no sexual cycle has been described in Fol, its genome contains functional mating-type locus (*MAT*), genetic regions which exist as two idiomorphs, *MAT1-1* and *MAT1-2*, controlling the sexual identity and reproduction in many ascomycetous fungi (Yun *et al.,* 2000).

The molecular structure of *MAT* loci was characterized in many ascomycetous fungi; they encode for transcriptional factors that regulate the expression of pheromones and receptor genes, involving the mating-type reaction. The interaction between diffusible peptide pheromones and its cognate receptors mediates the mating partner recognition, the first step of sexual reproduction. *MAT1-1* locus regulates the expression of α-pheromone and Ste3, the cognate receptor of a-pheromone whereas *MAT1-2* locus regulates the expression of a-pheromone and Ste2, the receptor of α-pheromone.

Two different Fol races, Fol 4287 and Fol 54003 possess respectively the *MAT1-1* and the *MAT1-2* locus. Recent studies have demonstrated that unisexual *MAT1-1* population of Fol 4287 possess predicted pheromone and receptor genes encoding functional diffusible α- and a-pheromones that interact with their cognate Ste2 and Ste3 receptors, respectively. The interaction between pheromones and their cognate receptor regulate mating-independent functions such as conidial germination (Turrà *et al.,* 2015; Vitale *et al.,* 2019).

Currently it is unknown whether *MAT* loci have a role beyond sexual development. Here we investigated the role of *MAT* loci in the Fol 4287 and 54003 races by generating a set of mutant strains that either lack a *MAT* locus or contain both *MAT* loci in the same genome. We studied their role in regulating fundamental biological processes in the fungal development such as conidial germination, vegetative hyphal fusion and hyphal aggregation. Subsequently, in order to better understand how *MAT* loci regulate the expression of pheromones and receptors genes and how this regulation might affect the tested phenotypes, we analysed the expression level of such genes by quantitative real-time PCR (RT-qPCR) in all mutant strains. Finally, considering that spore-germination, hyphal fusion and aggregation are infection-related processes, we also

decided to explore the role of *MAT* loci in the fungal virulence by performing a plant infection assay with all mutant strains obtained in this study.

# 3 MATERIALS AND METHODS

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## 3.1 FUNGAL STRAINS

### 3.1.1 *Fusarium oxysporum* f. sp. *lycopersici* strains

The fungal strains used in this study are listed in detail in [Table 1]. All are derived from *F. oxysporum* f. sp. *lycopersici* 4287 or 54003.

**Table 1-** Strains of *Fusarium oxysporum* f. sp. *lycopersici* used in this study.

| Strain name | FOXG | Genetic Background | Genotype | Resistance | Reference |
|---|---|---|---|---|---|
| **4287** | | 4287 | *wild type race2* | | FGS[1] |
| **54003** | | 54003 | *wild type race3* | | FGS[1] |
| **MAT1-1Δ** | FOXG_03616/ FOXG_03615/ FOXG_17746 | 4287 | *Knockout mutant of the MAT 1-1 locus* | *Hyg* | *this study* |
| **MAT1-1Δ+ MAT 1-1** | FOXG_03616/ FOXG_03615/ FOXG_17746 | 4287 | *Complement strain of the MAT1-1Δ locus* | *hyg::neo* | *this study* |
| **MAT1-1Δ+ MAT1-2** | FOWG_12730 / FOWG_12731 | 4287 | *Insertional mutant obtained by inserting the MAT1-2 locus (containing one copy or two copy inserted)* | *hyg::neo* | *this study* |
| **MAT1-1+ MAT1-2** | FOWG_12730 / FOWG_12731 | 4287 | *Insertional mutant obtained by inserting the MAT1-2 locus (containing one copy or two copy inserted)* | *hyg::neo* | *this study* |
| **54003+ MAT1-1** | FOXG_03616/ FOXG_03615/ FOXG_17746 | 54003 | *Insertional mutant obtained by inserting the MAT1-1 locus (containing one copy or two copy inserted)* | *Hyg* | *this study* |

(1) *Fusarium* genetic stock center

## 3.2 PLASMIDS

The plasmids used in this study are listed in [Table 2]

**Table 2 -** Plasmids used in this study

| PLASMID | ORIGIN/FEATURES | REFERENCE |
|---|---|---|
| pAN7-1 | Derived from pUC18; *A.nidulans GpdA* promoter; phosphotransferase hygromycin B(*Hyg*$^{R}$) gene from *Streptomyces spp*.; *A.nidulans TrpC* terminator | (Punt *et al.*, 1987) |
| Neo$^{R}$pGemT-2 | Derived from the pAc5-GFPE2F1$_{1–230}$-T2A-mRFP1-NLS-CycB$_{1–266}$-T2A-neo plasmid with the primers NeoF and NeoR with the *A.nidulans GpdA* promoter and *A. nidulans TrpC* terminator | (Yang *et al.*, 2004; Zielke *et al.*, 2014) |

## 3.3 MEDIA AND BUFFER SOLUTIONS

The liquid and agar culture substrates used in this study were prepared with Reverse Osmosis (RO) deionized water and sterilized in an autoclave at 1.2 atmospheres and 120 °C for 20 minutes, or by filtration using filters with a porosity of 0.22 μm in diameter. Solid culture media were poured into Petri dishes before solidification. Buffer solution were prepared with RO ultrapure water and sterilized by filtration (0.22 μm pore size, Millipore). RO deionized and RO ultrapure water were obtained through the Direct-Q® 8 UV remote water purification system (Merck Millipore). The buffers and solutions used for each experimental protocol, will be described in the respective section. Below are listed the media used for cell growth and their preparation:

- **Potato dextrose broth (PDB):** boil 200 g of peeled potatoes in 0.6 L of water for 60 min. Filter and add 20 g of glucose and deionized water up to 1 L. Sterilize by autoclaving.

- **Potato dextrose agar (PDA):** dissolve 3.9% potato dextrose agar (w/v) (Scharlau Microbiology). For culturing *F. oxysporum* transformants add hygromycin B (55 µg/ml), neomycin (100 µg/ml), after autoclaving.
- **Regeneration minimal medium (RMM):** dissolve 0.15 g of $MgSO_4$ x 7·$H_2O$, 0.3 g of $KH_2PO_4$, 0.15g of KCl, 0.6g of $NaNO_3$, 60 g of sucrose and 6 g of glucose in 0.3 L. Add oxoid agar (3 g) for 0.2 L of Petri dishes (1.5% agar) and 0.5 g for top Agar (0.5%) for 0.1 L. Sterilize by autoclaving.
- **Minimal medium for conidial germination:** dissolve in 1L of water, 1 g of sucrose, 0.5 g of KCl, 0.5 g of $MgSO_4$ x $7H_2O$, 1 g of $KH_2PO_4$ and 2 g of $NaNO_3$. Adjust to pH 5.0 with NaOH and sterilize by filtration.
- **Minimal medium for hyphal fusion:** water agar (2%, w/v) supplemented with 25 mM $NaNO_3$. Sterilize by autoclaving.
- **Minimal medium for hyphal aggregation:** minimal medium with 3% of sucrose and 50 mM of $NaNO_3$. Sterilize by autoclaving.
- **Hepes (1M) for germination minimal medium:** dissolve 11.9 g of hepes (MW 238.3 g/mol) in 0.05 L of water and adjust to pH 7.4
- **Germination minimal medium with 25mM of sodium glutamate (GMM 25mM):** dissolve 30 g of sucrose, 0.5 g of KCl, 0.5 g of $MgSO_4$ x $7H_2O$, 1 g of $KH_2PO_4$, 2 g of $NaNO_3$ in 1L of water supplemented with *25* mM of sodium glutamate, 2 mL of 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) 1M and adjust to pH 7.4.
- **Germination minimal medium with 5mM of sodium glutamate (GMM 5mM):** dissolve 30 g of sucrose, 0.5 g of KCl, 0.5 g of $MgSO_4$ x $7H_2O$, 1 g of $KH_2PO_4$, 2 g of $NaNO_3$ in 1L of water supplemented with 5 mM of sodium glutamate, 2 mL of 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) 1M and adjust to pH 7.4.

## 3.4 GROWTH CONDITIONS

### 3.4.1 *Fusarium oxysporum* f.sp. *lycopersici* culture

*F. oxysporum* f.sp. *lycopersici* strains were cultured in PDB medium at 28°C with orbital shaking at 170 rpm. When needed the following antibiotics were added to the culture medium: hygromycin B (55 µg/ml) and neomycin (100 µg/ml). After 3 to 5 days cultures were collected by filtration through a nylon filter (Monodur; mesh size 10 µm). Filtrates were centrifuged at 10000 rpm for 10 min, the pellet containing the spores was washed using deionized water, resuspended in water to reach the desired concentration.

For long-term storage of the different strains 3-5 days old culture were collected as before and then resuspended in a solution of water and 30% glycerol (v/v), and stored at -80˚C. These suspensions were used for later inoculation to obtain fresh microconidia for each assay.

### 3.4.2 *Fusarium oxysporum* f.sp. *lycopersici* cultures for the expression analysis of RT-qPCR.

For gene expression analysis fresh microconidia were germinated in potato dextrose broth (PDB) and $5*10^6$ spores were transferred in 200 mL of germlings minimal medium (GMM 25mM) for 14 h. The germlings were then harvested by vacuum filtration and concentrated by resuspending them in a fivefold lower volume of the same medium (GMM 5mM). After 1 hour of incubation at 28°C at 80 rpm germlings were again harvested by vacuum filtration and stored at -80°C.

### 3.4.3 Tomato plant culture

Tomato seeds surface of Monika cultivar were sterilized in 20% (v/v) bleach. After 20 minutes, seeds were washed three times for 10 minutes with sterilized water. Seeds were planted on wet vermiculite and incubated in plant growth chambers at 28°C. Infection assays with *F. oxysporum* were performed with 14 days-old tomato plants.

## 3.5 MOLECULAR METHODOLOGY

### 3.5.1 DNA extraction and quantification

#### 3.5.1.1 Nucleic acid gDNA extraction from *Fusarium oxysporum* f.sp. *lycopersici*

Genomic DNA was extracted from Fol mycelium using CTAB[1] method (Di Pietro *et al.* 2001). Briefly, the mycelium of 3 to 5 days-old cultures were collected, frozen at -80°C and lyophilized. A glass-bead (Sigma Aldrich) of 5 mm diameter was added to each sample in 2 ml Eppendorf centrifuge tube and the mycelium was ground to a fine powder using a FastPrep-24™ 5G Instrument (MP Biomedicals) using one cycle of 30 seconds. To each sample, 1 ml of CTAB extraction buffer was added and quickly vortexed. Then, 4 µl of β-mercaptoethanol (Merck) and 500 µl of a chloroform:octanol 24:1 (v/v) solution were added and the mix was incubated firstly at 65°C for 30 minutes and then left at room temperature (RT) for other 15 minutes. Next, the samples were centrifuged for 5 minutes at 7000 rpm and the DNA recovered (500 µl) was precipitated by incubating with 100 % ice-cold ethanol at -20˚C overnight (ON). The day after, precipitated DNA was centrifuged for 10 min at 13400 rpm and the pellet was washed with 1 ml of ethanol 70 %. Finally, the pellet was dried and resuspended in 50-70 µl of deionized water, depending on the pellet size, and 4 µl RNase were added (10 mg/ml; Roche Life Science) and the samples incubated at 37˚C for 1 hour. Afterward the tubes can be used or stored at -20˚C.

**[1]CTAB extraction buffer:** 12.1 g/l Trizma base, 7.44 g/l EDTA, 81.8 g/l NaCl and 20 g/l Cetyltrimethylammonium bromide. Heat to 60ºC to dissolve and adjust to pH 8.0 with NaOH 10 N. Store at 37ºC to avoid precipitation.

#### 3.5.1.2 Nucleic acid gDNA quantification

DNA was quantified using a Nanodrop® ND-1000 spectrophotometer at 260 nm and 280 nm wavelengths respectively. The quality of the DNA was monitored by electrophoresis in a 0.7 % agarose gel (w/v).

### 3.5.1.3 DNA amplification reactions

After extraction and quantification, plasmid and genomic DNA were used to amplify fragments of interest. Standard and Fusion PCR reactions were employed using synthetic oligonucleotides that were designed according to the experimental purpose.

### 3.5.1.4 Standard PCR

PCR amplification reactions were performed in a thermocycler using the thermostable DNA polymerase of the Expand High Fidelity PCR System (Roche Life Sciences) for Fusion PCR reactions. Each PCR reaction contained 10X PCR buffer, 300 nM of each primers, 2 mM $MgCl_2$, 0.2 mM of dNTPs mix and 0.01 U/µl of polymerase. Genomic DNA was added at 50 to 200 ng/µl. PCR cycling conditions were defined by an initial step of denaturation (5 minutes, 94°C) followed by 35 cycles of 35 seconds at 94°C, 35 seconds at the calculated primer annealing temperature and 1 minute/1.5 Kb was stablished for DNA amplification at 72°C (or 68°C for templates larger than 3 kb), and a final extension step at 72°C (or 68°C) for 10 minutes. For PCR screening the thermostable BioTaq™ DNA Polymerase and Velocity DNA polymerase (Bioline) were used. For BioTaq™ DNA Polymerase (Bioline) each PCR reaction contained 300 nM of each primers, 2,5 mM $MgCl_2$, 0.2 mM of dNTPs mix and 0.01 U/µl of polymerase. Genomic DNA was added at 50 to 200 ng/µl**.** PCR cycling conditions were defined by an initial step of denaturation of 5 minutes, 94°C followed by 35 cycles of 35 seconds at 94°C, 35 seconds at the calculated primer annealing temperature and 1 minutes/1 Kb was established for DNA amplification at 72°C, and a final extension step at 72°C for 10 minutes. For Velocity DNA polymerase (Bioline), each reaction contained 5X Hi-Fi Reaction buffer, 300 nM of each primers, 0.2 mM of dNTPs mix and 0.01 U/ µl. Genomic DNA was added at 50 to 200 ng/µl. PCR cycling conditions were defined by an initial step of denaturation (2 minutes, 98°C) followed by 35 cycles of 35 seconds at 98°C, 35 seconds at the calculated primer annealing temperature and 1 minut/2 Kb was stablished for DNA amplification at 72°C, and a final extension step at 72°C for 10 minutes.

### 3.5.1.5 Fusion PCR for the generation of knockout mutants

Fusion PCR or overlap extension is a method used to fuse two or more PCR products (Fig. 10) (Yang *et al.*, 2004). Complementary oligonucleotides and polymerase chain reaction are used to generate two DNA fragments with overlapping ends. In this PCR reaction (PCR I) an approximately 2 Kb fragment upstream (promoter) and downstream (terminator) of the Open Reading Frame (ORF) of the target gene were amplified with oligonucleotides containing a tail homologous to the selectable marker cassette. The amplified fragments are then combined in a 'fusion' reaction without oligonucleotides in which the overlapping ends anneal. In this reaction, the 3'overlap of each strand serves as a primer for the 3' extension of the complementary strand and the promoter and terminator regions are fused with the selectable marker cassette (PCR II). The resulting fusion products are used as template for further amplification by PCR (PCR III) using the primers of interest. In this work, the main purpose of fusion PCR was the generation of linear DNA consisting of a selectable marker gene conferring hygromycin resistance, flanked by 1.5 kb stretches of DNA target gene of interest, for application of the "split-marker" deletion strategy for knockout mutant generation.

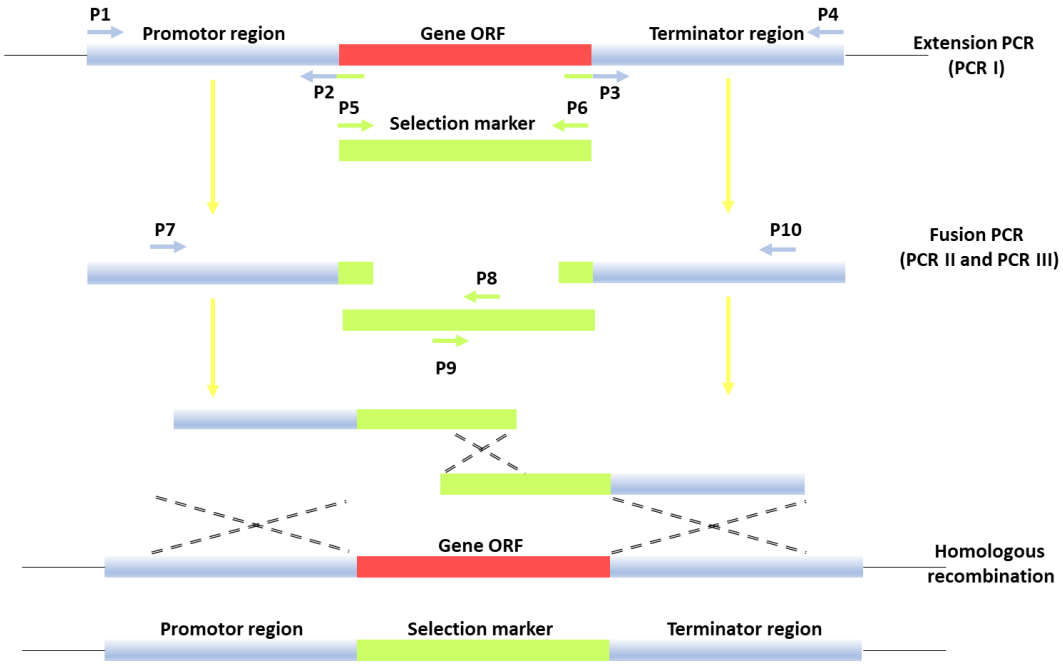



**Fig. 10. Schematic representation of the split-marker deletion strategy for gene knockout in *Fusarium oxysporum* f.sp. *lycopersici*.** Amplification of the upstream (promotor; P1-P2) and downstream (terminator; P3-P4) regions of the target gene with primers that contain overlapping ends to the antibiotic resistance cassette. The obtained PCR products are used as templates for the fusion PCR reactions between each flank and overlapping parts of the resistance cassette. Pairs of primers P7-P8 and P9-P10 were used to amplify the fragments which were subsequently used to transform *Fusarium oxysporum* f.sp. *lycopersici* protoplasts for gene ORF replacement by the resistance cassette through homologous recombination.

We found that fusion of two PCR products was best achieved by precipitation of the extension PCR products following the protocol described in 3.5.1.7 section and then using equimolar quantities of the purified products as templates for subsequent PCR reactions.

### 3.5.1.6 Synthetic oligonucleotides

Synthetic oligonucleotides used to generate PCR fragments for gene replacement, complementation or identification of mutants are listed in [Table 3]. Each oligonucleotide was designed with the Primer Analysis Software Oligo version 7.0 (Molecular Biology Insights, Inc. – Colorado, USA). Internal stability, duplex and hairpin formation and different physicochemical parameters, such as temperature of melting (Tm; calculated through the [2(A+T)° +4(G+C)°] method) were determined in each case. Oligonucleotides were synthesized by Isogen Life Science, Netherlands.

**Table 3 –** Synthetic oligonucleotides used in this study

| ***NAME*** | ***SEQUENCE 5'-3'*** | ***USE*** |
|---|---|---|
| MAT 1-5 FOR | *ATGACTACACACCCACCCAC* | Knockout MAT1-1 locus Fusion PCR |
| MAT 1-5 REV | ***TTTACCCAGAATGCACAGGTACACTTGTTT****AATGGCGTAAAACAATGTC AAGG** | |
| MAT 1-3 FOR | ***TGGTCGTTGTAGGGGCTGTATTAGGTCTCG****AGAAATAGCCAAACCTCTC TCTG** | |
| MAT 1-3 REV | *TGATACCAGAATTTGACCGCC* | |
| MAT 1-5 FORNEST | *TCTCTCCAAGCTACCTCCCT* | |
| MAT 1-3 REVNEST | *GCCGACGAGGACAGACACA* | |
| HYG-G | *CGTTGCAAGACCTGCCTGAA* | *Hyg*$^R$ cassette |
| HYG-Y | *GGATACCTCCGCTCGAAGTA* | |
| GPDA15B | *CGAGACCTAATACAGCCCCT* | *Hyg*$^R$ cassette/*Neo*$^R$ cassette |
| TRPTER15B | *GGATCCAAACAAGTGTACCTGTGCATTC* | |
| MAT 1-1COMPLFOR | *CAGAGAGAGGTTTGGCTATTTC* | MAT1-1 Complementation |
| MAT1-1 COMPLREV | *CCTTGACATTGTTTTACGCCAT* | |
| MAT 1-2 FORNEST | *ATCCACAAATACATCGCTTCCT* | MAT1-2 Insertional mutant |
| MAT1-2 REVNEST | *AGGAACACAGAAGATTGGCAC* | |
| MAT1-1 COMPLREV | *CCTTGACATTGTTTTACGCCAT* | MAT1-1 probe |
| MAT1-1 SEQ1 | *GGTAGTCAAATCAATGCGGTC* | |
| MAT1-2 SPLITFOR | *TTTGGTGTTGTGAGGAGGAGA* | MAT1-2 probe |
| MAT1-2 SPLITREV | *TGCTAGATTGACTGTGGTGGT* | |
| MAT1-2p2A | *ATAAAGGGCTGAAGCTGAACGAG* | Knockout MAT1-1 probe |
| MAT1-2 FOR | *CCTCTGTCGTTGCTTCCACC* | |
| ACTq7 | *ATGTCACCACCTTCAACTCCA* | Real time qPCR primer *(actin1)* |
| ACTq8 | *CTCTCGTCGTACTCCTGCTT* | |
| α-FOR | *AACGCCCTCCACGCAACT* | Real time qPCR primer *(MFα)* |
| α-REV | *AGCATCGGGGAAGAAGGTTT* | |
| A-FOR | *CCTTCCACCAAGAACACCAC* | Real time qPCR primer *(MFa)* |
| A-REV2 | *AGGGGGTAGCCGGGGGTTT* | |
| STE2-FOR2 | *ACATCACGTTCTTAGCAGCAG* | Real time qPCR primer *(ste2)* |
| STE2-REV2 | *TACACGAGCAGATGAAGAGAC* | |
| STE3-FOR | *TATGGTCTTCTTCGTCCTGG* | Real time qPCR primer *(ste3)* |
| STE3-REV | *CAAAGGGCTGAAGAGGAAATG* | |
| BAR1-FOR | *AAGAGAGACGGGACAATAGAC* | Real time qPCR primer *(bar1)* |
| BAR1-REV | *AAGAGAGACGGGACAATAGAC* | |
| AR17_qP_ppi_Fol-F | *AAGGGTGACCAGTTCGATAG* | Real time qPCR primer *(ppi)* |
| AR17_qP_ppi_Fol-F | *TTCTCGCCGAGCTTCATTTG* | |

* The sequence shown in bold type corresponds to the complementary region of the GpdA15B (MAT1-*5* REV) or Trpter8B (MAT1-3 FOR) primers. Synthetic oligonucleotides used in this study. All primers were designed with an annealing temperature of 62 - 64°C.

#### 3.5.1.7 Precipitation of DNA fragments and Southern blot probes

DNA fragments and probes for Southern blot were purified and concentrated before using. Briefly, 10% of volume of 3M sodium acetate at pH 5.0 was added and carefully mixed. 2.5 volume of 100% cold-ethanol were added to sample, mixed and incubated overnight at -20°C. Precipitated products were centrifuged at 13400 rpm for 30 minutes at RT. Pelleted products were washed with ethanol 70% (1 ml), centrifuged for 10 minutes again at 13400 rpm. Finally, the pellet was dried and resuspended with appropriate amount of sterile water.

### 3.5.2 Southern blot analysis

Southern blot analysis was performed digesting the transformants gDNA with BamHI restriction enzyme using the following reaction mix: 3 µl of BamHI 10 µl/ µl, 3 µl Buffer H 10X, 1 µg of gDNA. Digestion was incubated overnight at 37°C and the digested samples were run on agarose gel at 0,8% at a maximum speed of 70V. Prior to transferring the membrane DNA (Blot), it was necessary to prepare the electrophoretic gel, denaturing the DNA to pass to the next hybridization step. For this reason, the gel was slowly agitated under the following protocol, repeating each step twice at room temperature:

- Acid hydrolysis: 15 minutes with HCL 0.25M;
- Alkaline denaturation: 15 minutes with NaOH 0.5M + NaCl 1.5M;
- Neutralization: 30 minutes with Tris-HCl 0.5M pH 7.2 + NaCl 1.5M;

After denaturation the DNA was transferred, by capillary, on a positively charged nylon membrane (Roche). The transfer was carried out in a container with SSC buffer (10X) by placing inverted cuvette, 3 sheets of gel size filter paper absorbed into the SSC (10X) solution, 3 sheets of Whatman 3mm soaked in the SSC solution (10X), 3 Whatman tabs soaked in the SSC solution (3X), 3 Whatman 3MM dry papers, a stack of about 10 cm of paper napkins, a glass plate and a weight of about 0.5 kg, to avoid the formation of air bubbles between the Whatman 3mm sheets and the gel, or between gel and nylon membrane. The transfer took place at about 4°C overnight, then the nylon membrane was dried in a stove at 80°C for 90 minutes in order to definitively fix the DNA. To hybridize the probe, the membrane was pre-hybridized by incubating for at least 1 hour at 50°C with (20 ml) of pre-hybridized solution DIG Easy Hyb blocking agent (Roche). For the preparation of the blocking agent, the manufacturer's

instructions have been followed. Hybridization occurred, overnight, at 50 °C in slow-moving membrane, with 10 ml pre-hybridization solution in which about 20 ng/ml of probe were added. Prior to the hybridization it was necessary to denature the probe at 100°C for 10 minutes and cool in ice for 5 minutes. After the hybridization, the membrane was subjected to the following washings:

- 5 minutes in 2X SSC + 0.1% SDS (w/v) at room temperature (2 times);
- 15 minutes in SSC 0.5x + 0.1% SDS (v/v) at 50 °C (2 times);
- 2 minutes in Buffer 1 at room temperature;
- 30 minutes in Buffer 2 at room temperature;
- 30 minutes in Buffer 2 + anti-digoxigenin antibody conjugated with alkaline phosphatase (750 $Uml^{-1}$, Roche) at room temperature;
- 15 minutes in 50 ml Buffer 1 + 0.3% Tween-20 at room temperature (2 times);
- 5 minutes in Buffer 3 at room temperature.

The membrane was placed in 2 sheets of acetate, adding 500 µl of 1% (v/v) CDP-Star® solution in Buffer 3 and incubated for 5 minutes in the dark (the reaction is photosensitive) at room temperature. The action of alkaline phosphatase on CDP-Star® * substrate (Roche) originates an easily visible light signal with autoradiographic films, that was analysed exposing the membrane treated to X-ray using a dedicated scanner (Kodak X-OMAT AR).

### 3.5.3 RNA extraction and quantification

#### 3.5.3.1 Nucleic acid RNA extraction

Approximately 100 mg of frozen mycelium mycelia collected as described above were lyophilized and ground to fine powder using a FastPrep-24™ 5G Instrument (MP Biomedicals) (1 cycle of 10 seconds) in 2 ml Eppendorf centrifuge tube with a glass-bead (Sigma Aldrich) of 5 mm diameter. The powder was transferred on ice in a pre-chilled Eppendorf tube. TRIzol Isolation Reagent (1 mL) was added to the tube and the sample vortexed and incubated 5 minutes on ice. The supernatant obtained after 10 min of centrifugation (13400 rpm) at 4°C was transferred to a new vial and chloroform (1/5 of TRIzol volume) was added. The mix was gently vortexed by hand for 15 seconds and incubated 3 min at RT. Three phases were obtained after 20 min centrifugation (13400 rpm) at 4°C; the upper clean phase was taken and transferred to

a clean Eppendorf tube pre-chilled containing 750 µl of isopropanol. The tube was mixed well and incubated on ice for 5 min, then centrifuged 20 min (13400 rpm) at 4°C to precipitate RNA. The pellet was washed with 1 ml of 75 % ethanol (v/v) and centrifuged 5 min at 7500 g at 4°C. The pellet was then air dried for 5-10 min and resuspended in 50 µl RNAse free water.

#### 3.5.3.2 Nucleic acid RNA quantification

RNA was quantified in a Nanodrop® ND-1000 spectrophotometer at 260 nm and 280 nm wavelengths respectively. The quality of the RNA was monitored by electrophoresis in a 1.5 % agarose gel (w/v).

#### 3.5.3.3 Generation of cDNA from RNA samples

Prior to cDNA synthesis through reverse transcriptase enzyme (Roche), RNA was treated with DNAseI (Roche). Briefly, 2 µg of RNA sample was used in a final reaction volume of 20 µl containing 2 µl of 10X Buffer (Roche), 0.4 µl of DNAseI (Roche), incubated in a thermocycler PCR for 20 minutes at 37°C. EDTA 50 mM was added in the solution that was mixed well and incubated again for 10 minutes at 75°C. Next, a mix of 20 µl containing 8 µl of 5X Buffer (Roche), 2 µl of reverse transcriptase enzyme (Roche) and 10 µl of RNAse free water was added to the solution and mixed well. In order to obtain cDNA product this solution was putted in a thermocycler PCR at these conditions: an initial step of 5 min incubation step at 25°C followed by 10 minutes incubation step at 55°C and a final step of 5 minutes at 85°C to inactivate the enzyme. Efficacy of reverse transcription was verified by amplifying the housekeeping gene actin both from cDNA fungal mutant product and wild-type fungal genomic DNA in a standard PCR reaction using the primer pairs ACTq7/ACTq8 at these conditions: an initial denaturation step of 5 minutes at 94°C, followed by 30 cycles of 35 s at 94°C,35 s at 62°C, 30 s at 72°C and a final extension step for 7 minutes at 72°C. PCR products were run on agarose gel (1.5%).

### 3.5.4 RT-qPCR analysis

Quantitative real-time PCR reactions (RT-qPCR) were performed in an iCycler apparatus (BioRad, USA) using 7.5 µl of iQ SYBR Green Supermix (BioRad, USA), 400 ng of cDNA template and 300 nM of each gene-specific primer in a final reaction volume of 15 µl in RNAse water free. The following PCR program was used for all reactions: an initial denaturation step of 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C, and 20 sec at 80°C for measurement of fluorescence emission. Relative transcript levels were calculated by comparative threshold cycle (ΔΔCt) and normalized to the peptidyl- prolyl isomerase gene (ppi) (Livak and Schmittgen 2001; Pfaffl 2001).

## 3.6 GENERATION OF *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI* TRANSFORMANTS

### 3.6.1 Generation of *Fusarium oxysporum* f.sp. *lycopersici* protoplasts

Protoplasts were obtained following the protocol described by (Di Pietro *et al.,* 2001) with minor modifications. Briefly, to obtain protoplasts from Fol wild-type 4287, knockout of MAT 1-1 and 54003 strains, $5*10^8$ microconidia were inoculated in 200 ml of PDB for 13 hours at 28ºC and orbital shaking at 170 rpm. After O.N. incubation, germlings were harvested by filtration with a Monodur and carefully washed with OM[2]. Washed germlings were then transferred to a sterile 50 ml Falcon tube, containing 0.5% (w/v) of the enzyme EXTRALYSE® (Laffort) dissolved in OM. The mix of germlings and enzyme was incubated for, at least, 45 minutes at 30 ºC with slow shaking (60 rpm), and protoplasts accumulation was monitored under the microscope. When optimal number and quantity of protoplasts were achieved, the sample was filtered through a double layer of Monodur nylon filters and washed with two volumes of STC solution. The flow-through containing the protoplasts was collected in pre-chilled Corex (Pyrex) centrifuge tubes. Samples were centrifuged at 3000 rpm for 15 minutes at 4 ºC. Pelleted protoplasts were then carefully resuspended in 1 ml STC[3] and counted. The protoplast suspension was adjusted to a final concentration of $4*10^7$ protoplasts/ml and stored as 100 µl aliquots in Eppendorf tubes to be used for transformation. For long-term storage at -80 ºC, 10% (v/v) of PEG[4] and 1% (v/v) of DMSO (Merck) were added.

[2]OM: dissolve 1.2 M of $MgSO_4$ in 180 ml of RO deionized water and 0.1 M of $Na_2HPO_4$ in 100 ml of RO deionized water. Mix 20 ml of $Na_2HPO_4$ solution with all $MgSO_4$ solution. Adjust to pH 5.8 – 6.0 with orthophosphoric acid. Sterilize by filtration.

[3]STC: dissolve 14.52 g of sorbitol in 94 ml of RO deionized water and sterilize by autoclaving. Mix 5 ml of 1M $CaCl_2$ solution and 1 ml of Tris-HCl 1M at pH 7.5 with sorbitol solution. Sterilize by filtration.

[4]PEG: dissolve 6.279 g of MOPS (Sigma) in 50 ml of RO deionized water. Mix MOPS solution with 30 g of PEG 4000 and keep at 50 ºC to dissolve. Adjust final volume to 50 ml with MOPS and sterilize by filtration.

### 3.6.2 Transformation of *Fusarium oxysporum* f.sp. *lycopersici*

Transformation was performed as described in Di Pietro *et al.* (2001), with minor modifications. Briefly, in a pre-chilled Eppendorf tube were combined: 2-3 µg of transforming DNA with 10 µl of 0.1 M aurintricarboxylic acid (ATA; Sigma Aldrich), a potent inhibitor of nucleases, in a final volume of 60 µl with STC solution. For negative transformation control 50 µl of $H_2O$ were mixed with 10 µl of ATA. Mixes were incubated on ice for 20 minutes. In simultaneous, 100 µl of protoplasts ($4*10^7$ protoplasts/ml) generated as described above, were incubated on ice for 20 minutes. Next, protoplasts and DNA solutions were carefully mixed and incubated a further 20 minutes on ice. Then, 160 µl of PEG solution were added and mixed carefully, followed by 15 minutes of incubation at room temperature. STC (1 ml) was added to the transformed cells and samples were centrifuged 5 minutes at 3000 rpm. Next, protoplasts were resuspended in 200 µl of STC and 50 µl aliquots were mixed with 3 ml of top agar and spread onto plates containing 20 ml of solid regeneration medium (RMM). Plates were incubated at 28°C for 14 hours before addition of 3 ml of top agar containing 200 µg of hygromycin B or 100 µg of geneticin. Incubation at 28°C was prolonged for 4-5 days until transformant colonies became visible. Colonies were transferred to PDA plates supplemented with the respective selective marker, and transformants were submitted to two consecutive rounds of single monoconidial purification on selective PDA plates.

### 3.6.3 Identification of mutant strains

To identify mutant strains, genomic DNA of each of them was extracted and analyzed by PCR and Southern blot. PCR was performed using primer pairs located inside the selective marker and in the flanked region upstream or downstream the insertion. Southern blot analysis with gene-specific probes was performed as described by Di Pietro and Roncero (1998) using the non-isotopic digoxigenin labelling kit for DNA labelling and detection (Roche Life Sciences).

## 3.7 PHENOTYPIC ASSAYS

### 3.7.1 Conidial germination assay

1 ml of freshly collected microconidia at inhibitory concentration of inoculum (ICI: $8.6*10^7$ conidia/ml) and optimal concentration of inoculum (OCI: $3.19*10^6$ conidia/ml) were inoculated in minimal medium for conidial germination and incubated at 28°C and 170 rpm for 15h for samples at ICI and 13h for samples at OCI. The number of total and germinated conidia was then counted in an Olympus BH-2 microscope using differential interference contrast imaging (400 X magnification). At least 300 events were examined for each isolate and each experiment was repeated at least three times. The number of germinated conidia was scored and expressed as a percentage of germinated over the total number of counted conidia (Vitale *et al.*, 2019).

### 3.7.2 Vegetative hyphal fusion assay

Freshly collected microconidia (45 µl of a $7.0*10^7$ conidia/ml suspension) were spread on plates containing 5 ml of minimal medium for hyphal fusion by using sterile glass beads and incubated for 14h at 28°C. Vegetative hyphal fusion events were counted in an Olympus BH-2 microscope using differential interference contrast imaging (400 X magnification). Three hundred germlings were examined for each isolate and each experiment was repeated at least three times. The number of germ tubes involved in vegetative hyphal fusion was scored and expressed as a percentage of fusing hyphae over the total number of counted hyphae.

### 3.7.3 Hyphal aggregation assay

The ability to create mycelium aggregates was tested inoculating 100 µl of freshly collected microconidia at a concentration of $1*10^8$ conidia/ml in 2 ml of minimal medium for hyphal aggregation. After 14h of incubation at 28°C and 170 rpm, samples were transferred in multiwell plates and observed at Zeiss Axio Imager M2 microscope equipped with a Photometrics Evolve EMCCD camera.

### 3.7.4 Tomato plant infection assay

Tomato root infection assays were performed in a growth chamber as described by Di Pietro and Roncero (1998), using the susceptible cultivar to the race 0, 2 and 3 of Fol, Monika. Briefly, 2-week old tomato seedlings were inoculated with *F. oxysporum* strains by immersing the roots in a micro-conidial suspension containing $5*10^6$ spores/ml, planted in vermiculite, and maintained in a growth chamber. Ten days after inoculation, the severity of disease symptoms was evaluated using a disease index for *Fusarium* vascular wilt (Fig. 11) (Pérez-Nadales *et al.*, 2014) and survival percentage were recorded daily for 30 days as previously described in López-Berges *et al.* (2012). Fifteen plants were used for each treatment; plants survival was estimated by the Kaplan-Meier method and compared among groups using the log-rank test. The results were analyzed with the software GraphPad Prism 6.0.

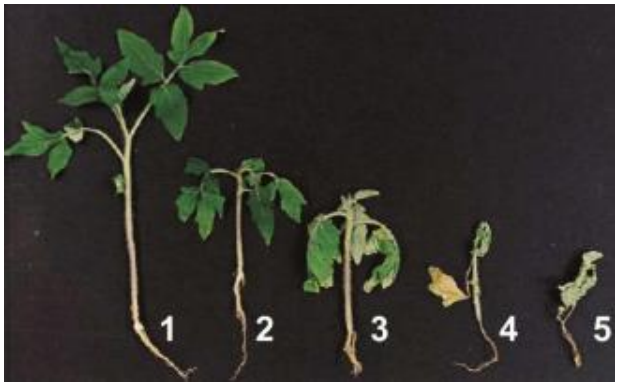



**Fig. 11.** Distinct stages of vascular wilt on tomato plants (adapted from: Pérez-Nadales *et al.*, 2014)

## 3.8 BIOINFORMATIC AND STATISTICAL ANALYSIS

Bioinformatic analysis were carried out using: BROAD *Fusarium* Database (http://www.broadinstitute.org/), NCBI (https://www.ncbi.nlm.nih.gov/) and EnsemblFungi website (www.fungi.ensembl.org) and the BLAST search in the genomes of *F. oxysporum* f. sp. *lycopersici* 4287 and 54003 isolates with *MAT* genes identified in different *Fusarium* species (Martin *et al.*, 2011).

Snapgene software (version 2.3.2, from GSL Biotech; available at www.snapgene.com) was used for the alignments of the *MAT1-1* and *MAT1-2* sequences from the same species.

Statistical analysis of the data obtained from the spore-germination and vegetative hyphal fusion experiments was conducted using Yates' corrected chi-squared test (two-sided), whereas in the pathogenicity assays the results were analysed with the software GraphPad Prism 6.0.

The plants survival was estimated by the Kaplan-Meyer method and the statistical significance of differences among groups was assessed by using the log-rank (Mantel-Cox) test. Differences indicated with a *P* value of less than 0.05 were considered significant.

The data obtained from the expression analysis were evaluated by "*t*" Students test and differences showed a *P* value < 0.05 were considered significant.

# 4 RESULTS

# 4 RESULTS

## 4.1 *MAT1-1* AND *MAT1-2* LOCUS ALIGNMENTS

Prior to proceeding with transformation of *F. oxysporum* f.sp. *lycopersici* protoplasts with the appropriate transformation constructs, genomic regions alignments and searches were carried out in several repositories of Fusarium genomic sequences. Sequence data can be found in the Fusarium Genome Database under the following accession numbers: *MAT1-1-1* (FOXG_03616), *MAT1-1-2* (FOXG_03615), *MAT1-1-3* (FOXG_17746), *MAT1-2-1* (FOWG_12730), *MAT1-2-3* (FOWG_12731). Complete *MAT1-1* and *MAT1-2* sequences of *F. oxysporum* f. sp. *lycopersici* 4287 and 54003 isolates, respectively, including flanking regions were obtained from the NCBI genome Database. The alignments of the *MAT* loci sequences from the same species, obtained by using Snapgene software, allowed us to identify both flanking homologous regions of *MAT* loci and the non-homologous regions (*MAT* idiomorphs) (Fig. 12).

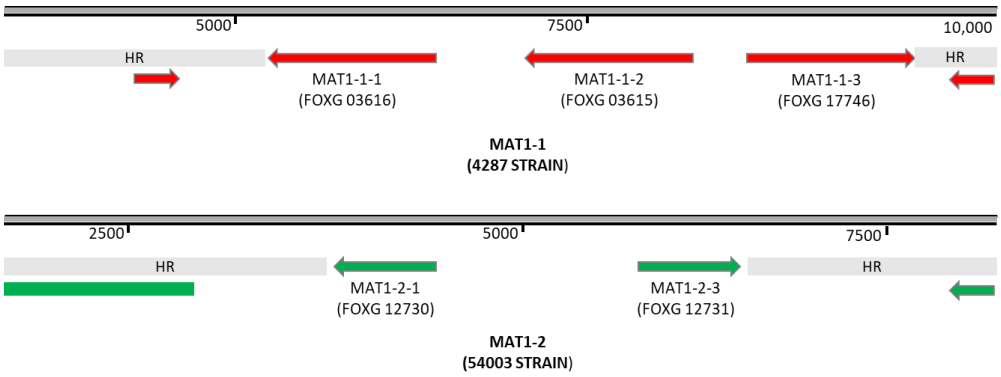



Fig. 12. *MAT1-1 and MAT1-2 alignment* (HR): homologous region.

## 4.2 *GENERATION OF MUTANTS STRAINS IN FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI*

### 4.2.1 Targeted deletion of *MAT1-1* locus in *Fusarium oxysporum* f.sp. *lycopersici* 4287

To obtain the knockout mutant of the entire *MAT1-1* locus in the Fol 4287, simultaneous replacement of the three genes *FOXG-03616, FOXG_03615* and *FOXG_17746* with a hygromycin resistance cassette under control of the *Aspergillus nidulans* GpdA promoter (PgpdA) and TrpC terminator (Ttrpc) was performed using the split-marker method (Catlett *et al*., 2003) following the protocol previously described by Rispail and Di Pietro (2009). DNA fragments flanking the *MAT1-1* locus were amplified from genomic DNA of *F. oxysporum* isolate 4287 with primer pairs MAT 1-5 FOR + MAT 1-5 REV (1150 bp) and MAT 1-3 REV + MAT 1-3 FOR (1436 bp), respectively, and PCR fused with partially overlapping truncated versions of the hygromycin B resistance cassette ($Hyg^R$) using primer combinations MAT 1-5 FORNEST + HYG-G and MAT 1-3 REVNEST + HYG-Y, respectively. The obtained split-marker fragments were quantified through nanodrop and used directly to co-transform protoplasts of the *F. oxysporum* 4287 isolate with hygromycin resistance. Transformants were then purified by monoconidial isolation as described by Di Pietro and Roncero (1998). Transformants carrying a homologous insertion at the *MAT1-1* locus were first identified by PCR analysis using pair of primers MAT1-2 FOR/MAT1-2p2a (2952 bp) that hybridize outside and inside of the fragment replaced and four putative transformants were found. Further analysis of the selected candidate transformants was performed by Southern blot to verify the correct insertion of the resistance cassette. Southern blot analysis of these transformants confirmed the replacement of a 5 kb BamHI fragment, corresponding to the wild-type *MAT1-1* allele, with a fragment of 7 kb in three of them (Fig. 13).

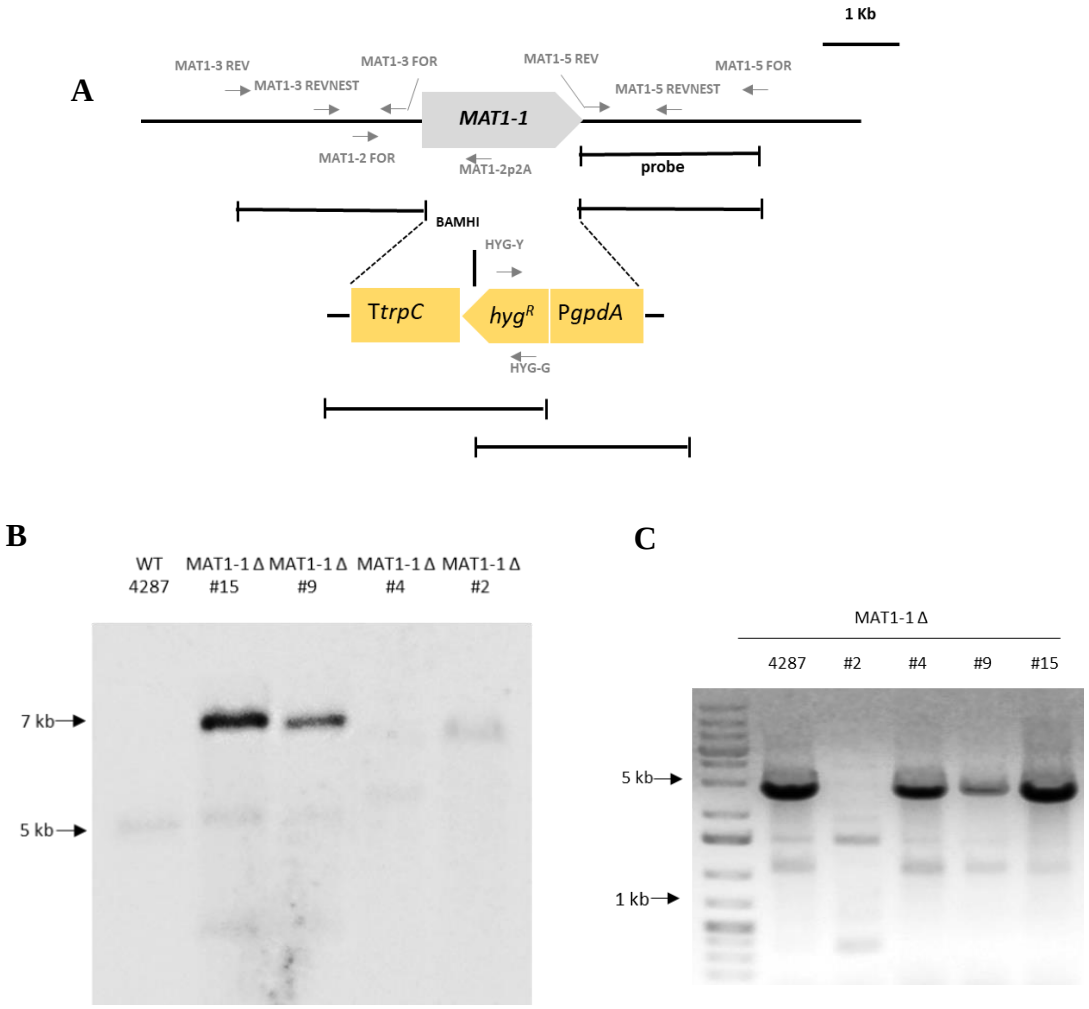



**Fig. 13. Targeted deletion of *MAT1-1* knockout in *Fusarium oxysporum* f.sp. *lycopersici* (A)** Physical maps of the *MAT1-1* locus and the split-marker gene replacement construct obtained by fusion PCR (MAT1-1Δ locus). Relative positions of the primers used for generation of the gene disruption construct and PCR analysis of the transformants, as well as the probe used for Southern blot are indicated. **(B)** Genomic DNA of the wild type 4287 isolate and four individual transformants digested with the BamHI restriction enzyme, separated on an agarose gel, were transferred to a nylon membrane and hybridized with the DIG-labelled DNA probe. **(C)** PCR analysis performed with MAT1-2p2a/MAT1-2 FOR primer pairs located inside *MAT1-1* locus.

### 4.2.2 *MAT1-1* complemented strain

To obtain the complemented strain of the *MAT1-1* knockout mutants in the Fol 4287 genetic background, we initially generate a PCR product of 4.2 Kb by using the following primer pairs: MAT1-1 COMPLFOR/MAT1-1 COMPLREV, encompassing the entire *MAT1-1* locus, from the Fol 4287 gDNA. Subsequently we amplified the neomycin resistance cassette gene from the $Neo^R$pGemT-2 plasmid using the GPDA15B/ TRPTER15B primers (2.7 Kb). The PCR fragments thus obtained were used to generate the complemented strain of the MAT1-1 Δ mutant by co-transformation. Co-transformation was performed using 7 µg of the *MAT1-1* amplicon and 2 µg of the $Neo^R$ cassette used for selection. Several neomycin-resistant transformants were found and analysed by PCR using the primer pair MAT1-1 COMPLFOR/MAT1-1 COMPLREV. After this PCR- based screening, we found that at least three transformants showed a banding pattern similar to that obtained with the Fol 4287 wild-type strain, whereas as expected no amplification was found in the Fol 54003 and MAT1-1 Δ strains. These results indicate that these strains, named MAT1-1 Δ + MAT1-1, are the complemented strains of the MAT1-1 Δ (Fig. 14).

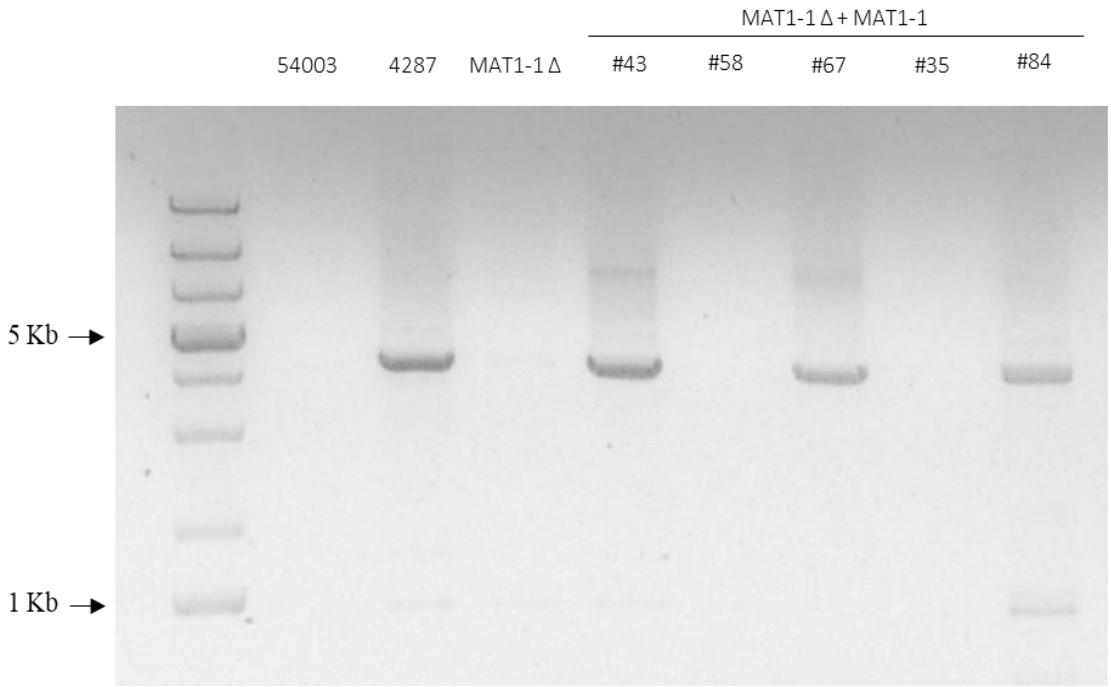



**Fig. 14. Identification of complemented strains of the *MAT1-1* locus in the MAT1-1 Δ.** PCR amplification of genomic DNA of *Fusarium oxysporum* f.sp. *lycopersici* 54003, *Fusarium oxysporum* f.sp. *lycopersici* 4287, MAT1-1 Δ and 5 putative complemented strains. PCR analysis was performed with MAT1-1 COMPLFOR/MAT1-1 COMPLREV primer pairs amplifying the entire region of *MAT1-1* locus (4.2 Kb).

### 4.2.3 Generation of MAT1-1 Δ insertional mutants containing only the *MAT 1-2* locus

To investigate the biological role of the *MAT1-2* locus in *F. oxysporum* f.sp. *lycopersici* we decided to generate insertional mutants in the MAT1-1 Δ genetic background by inserting the entire *MAT1-2* locus. The entire *MAT1-2* locus was amplified from the gDNA of the Fol 54003 strain by using the following primers pair: MAT1-2 FORNEST + MAT 1-2 REVNEST (3.8 Kb). MAT1-1 Δ insertional mutants containing the MAT 1-2 locus (MAT1-1Δ+MAT1-2) were obtained by co-transforming MAT1-1 Δ protoplasts with 2 μg of the $Neo^R$ cassette used as selective marker and 7 μg of the above PCR product. To identify MAT1-1Δ+MAT1-2 mutant strains we performed a PCR based screening with two different pairs of primer. The MAT1-2 FORNEST/MAT1-2 REVNEST primers (3.8 Kb) were used to define the presence/absence of the *MAT1-2* locus, while the MAT1-1 COMPLFOR/MAT1-1 COMPLREV primers (4.2 Kb) to confirm the absence of the *MAT1-1* locus in the genome of the obtained transformants. Four of the MAT1-1Δ+MAT1-2 transformants possess the expected PCR pattern (i.e. presence of the *MAT1-2* and absence of the *MAT1-1* locus) (Fig. 15A). Furthermore, to assess the number of copies of the *MAT1-2* locus in the MAT1-1Δ+MAT1-2 mutant strains, a Southern blot analysis was performed on two of them by constructing a MAT1-2 specific probe using the MAT1-2 SPLITFOR/MAT1-2 SPLITREV primer pair (1658 bp) (Fig. 15B). The Southern blot analysis revealed that the MAT1-1Δ+MAT1-2 # 18 mutant strain contains only one copy of the *MAT1-2* locus whereas the MAT1-1Δ+MAT1-2 # 28 mutant strain contains two copies of it (Fig. 15C). Both of them were tested in different phenotypic assays to study the effect of multiple copies of the *MAT1-2* locus on *Fusarium oxysporum* f.sp. *lycopersici* physiology.

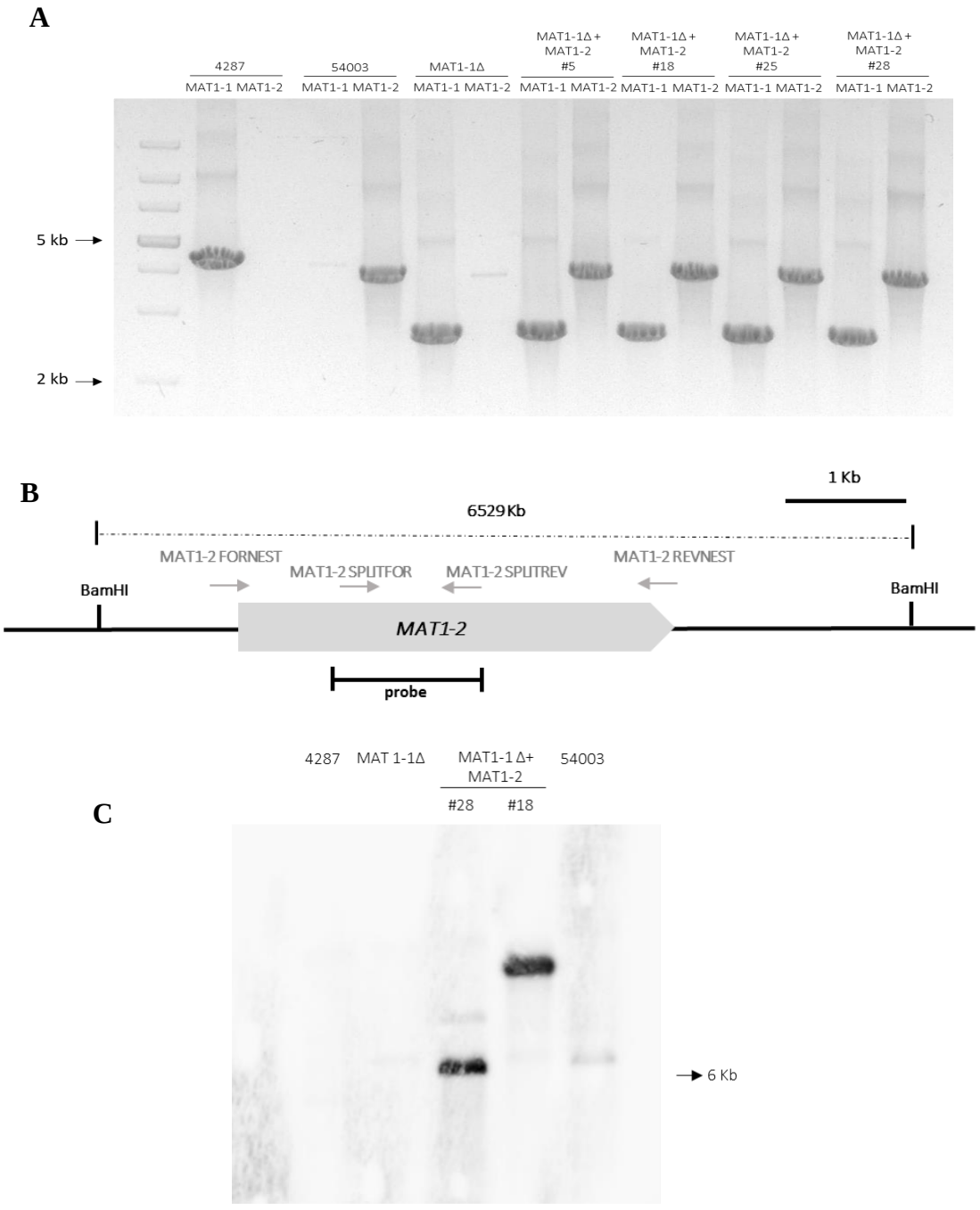



**Fig. 15. Insertional mutant strains containing *MAT1-2* locus. (A)** PCR analysis were performed to amplifying both *MAT* loci with MAT1-2 FORNEST/MAT1-2 REVNEST and MAT1-1 COMPLFOR/MAT1-1 COMPLREV primer pairs. **(B)** Physical maps of the *MAT1-2* locus and with relative positions of the primers used for the amplification of the locus and PCR analysis of the transformants, as well as the probe located inside the locus and used for Southern blot. **(C)** Genomic DNA of the wild type 4287 and 54003 isolates and *Fusarium oxysporum* f.sp. *lycopersici* transformants digested with the BamHI restriction enzyme, separated on an agarose gel, were transferred to a nylon membrane and hybridized with the DIG-labelled DNA probe.

### 4.2.4 Generation of *Fusarium oxysporum* f.sp. *lycopersici* 4287 insertional mutants containing both the *MAT1-1* and *MAT1-2* loci

To investigate what occurs when both *MAT* idiomorphs are present in the same genome, we generated insertional mutants containing both *MAT* loci in in the Fol 4287 strain. First, we amplified the entire *MAT1-2* locus from the Fol 54003 gDNA using the MAT 1-2 FORNEST + MAT 1-2 REVNEST primer pair (3.8 Kb). Then was amplified the $Hyg^R$ resistance gene cassette from the pAN7-1 plasmid using the GPDA15B/ TRPTER15B primers (2.7 Kb). Co-transformation was performed by using 2µg of the *MAT1-2* amplicon and 1µg of the $Hyg^R$ resistance cassette. Hygromycin resistance co-transformants were analysed for the presence of *MAT* loci amplifying *MAT1-2* locus with MAT1-2 FORNEST + MAT1-2 REVNEST (3.8 Kb) and *MAT1-1* locus with MAT1-5 FOR + MAT1-3 REV (6.8 Kb) primers pairs. Both a 3.8 Kb and 6.8 Kb amplification products were detected in four out of six selected hygromycin-resistant co-transformants but not in the 4287 or 54003 isolates. These co-transformants were purified by monoconidial isolation as described by Di Pietro and Roncero (1998). To assess the number of copies of the MAT1-2 locus in the Fol 4287 insertional mutants containing both the *MAT1-1* and *MAT1-2* loci (4287+MAT1-2), a Southern blot analysis was performed on three of them by constructing a MAT1-2 specific probe as described in the section 4.2.3. The Southern blot analysis showed that the 4287+MAT1-2#13 contains two copies of the *MAT1-2,* the 4287+MAT1-2#7 contains a single copy while the 4287+MAT1-2#14 contains multiple copies of the MAT1-2 (Fig. 16). 4287+MAT1-2 strains containing ono or two copies of the *MAT1-2* locus were tested in different phenotypic assays to study the effect of multiple copies of the *MAT1-2* locus on *Fusarium oxysporum* physiology.

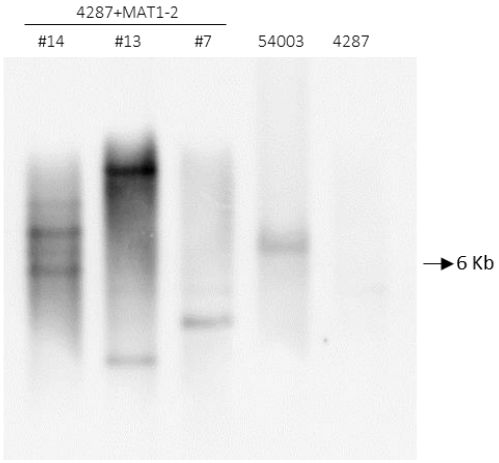



**Fig. 16. Identification of the number of copies of *MAT1-2* locus in *Fusarium oxysporum* f. sp. *lycopersici* 4287 insertional mutants containing both *MAT* loci.** Genomic DNA of the wild type 4287 and 54003 isolates and *Fusarium oxysporum* f.sp. *lycopersici* transformants digested with the BamHI restriction enzyme, separated on an agarose gel, were transferred to a nylon membrane and hybridized with the DIG-labelled DNA probe.

### 4.2.5 Generation of *Fusarium oxysporum* f.sp. *lycopersici* 54003 insertional mutants containing both the *MAT1-1* and *MAT1-2* loci

The role of *MAT* loci in *F.oxysporum* f. sp. *lycopersici* 54003 isolate was investigated by generating in this strain insertional transformants containing both idiomorphs in its genome. First the entire *MAT1-1* locus was amplified from the gDNA of *F.oxysporum* f. sp. *lycopersici* 4287 by using the MAT 1-1 COMPLREV + MAT 1-1 COMPLFOR primer pairs ( 4.2 Kb) and the $Hyg^R$ cassette as described in the 4.2.1 section. Both fragments were used to co-transform protoplasts obtained from the *F.oxysporum* f. sp. *lycopersici* 54003. Co-transformation was performed using 4.5 µg of *MAT1-1* amplicon and 1.5 µg of hygromycin resistance cassette. Several hygromycin resistance transformants were analysed through a PCR based screening using the MAT1-1 COMPLFOR/MAT1-1 COMPLREV primer pairs (Fig. 17) and then purified by monoconidial isolation described by Di Pietro and Roncero, (1998). To identify the number of copies of the *MAT1-1* locus in the putative Fol 54003 insertional mutants containing both the *MAT 1-1* and *MAT1-2* loci (54003+MAT1-1), a Southern blot analysis was performed using a MAT1-1 specific probe. The MAT1-1 specific probe was obtained by using Mat1-1Seq1/ Mat1-1 COMPLREV primer pair (1620 bp) (Fig. 18A). The Southern blot analysis

reveals that the 54003+MAT1-1#17 contains only one copy of the *MAT1-2* locus while the 54003+MAT1-1#27 contains two copies of it (Fig. 18B). Further to verify the presence of both *MAT* loci in the genome of 54003+MAT1-1 mutants another Southern blot analysis was performed by using a *MAT1-2* locus specific probe obtained with the MAT1-2 SPLITFOR/ MAT1-2 SPLITREV (1658 bp) (Fig. 19A). This last Southern blot analysis confirmed the presence of both *MAT* loci in the genome of the 54003+MAT1-1 mutants selected (Fig. 19B).

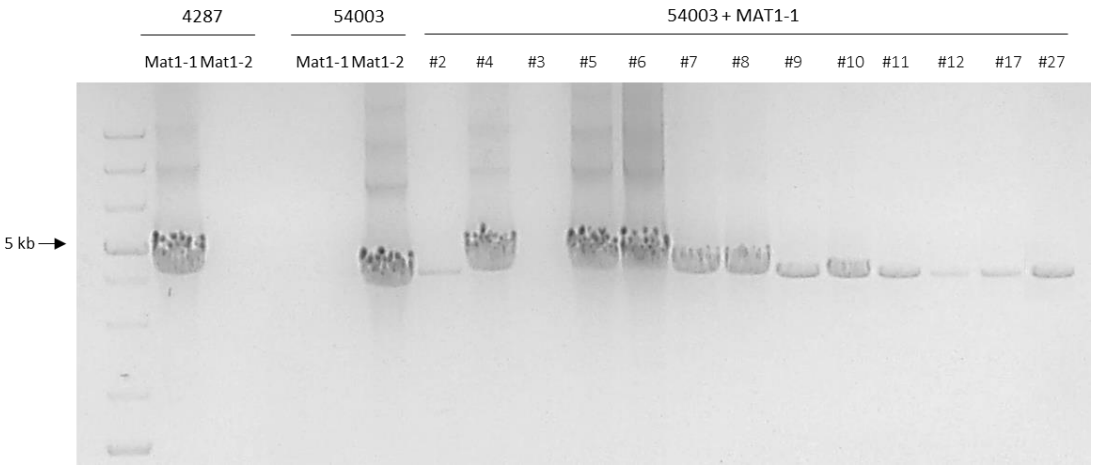



**Fig. 17. 54003+MAT1-1 insertional mutant strains containing *MAT1-1* locus**. PCR analysis was performed to amplifying *MAT1-1* locus with MAT1-1 COMPLFOR/MAT1-1 COMPLREV primer pairs.

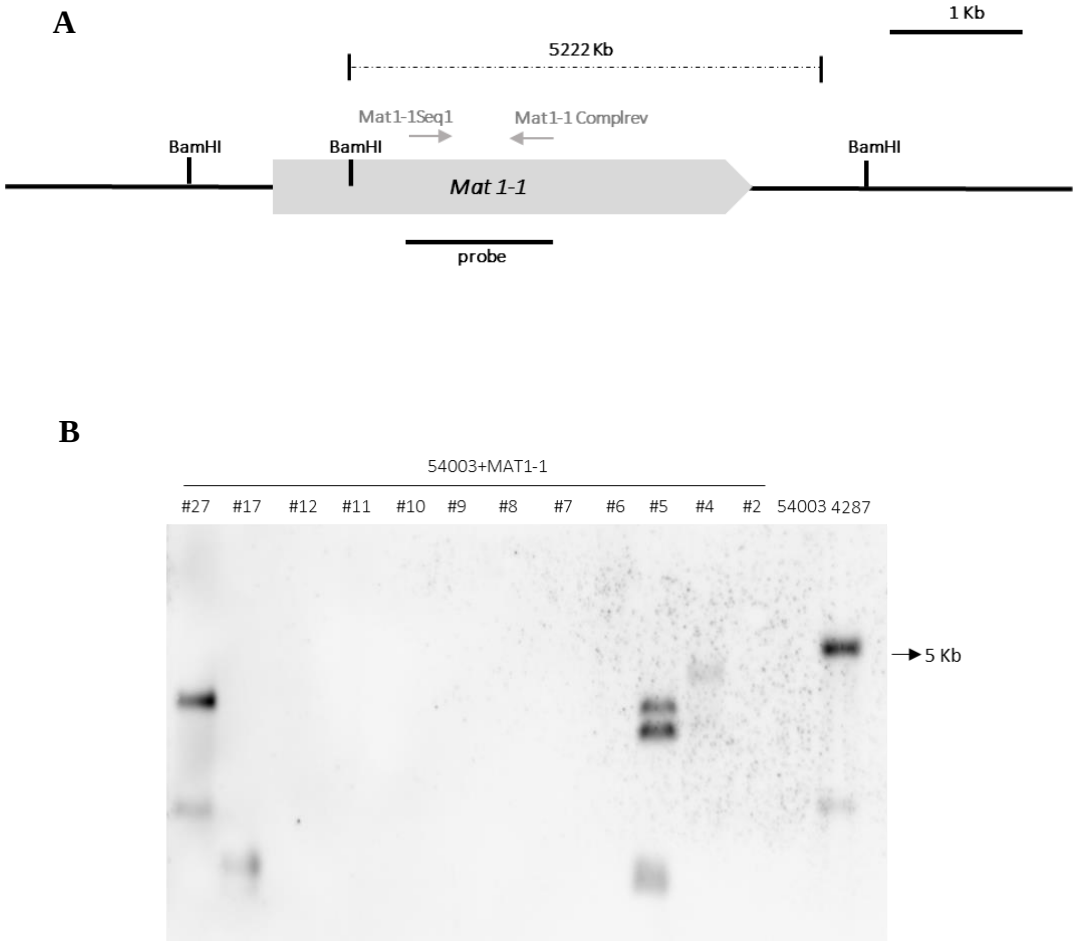



**Fig. 18. Identification of the number of copies of the *MAT1-1* locus in the 54003+MAT1-1 locus. (A)** Schematic representation of specific probe construction to check *MAT1-1* locus. **(B)** Genomic DNA of the wild type 4287 and 54003 isolates and 54003+MAT1-1 mutants digested with the BamHI restriction enzyme, separated on an agarose gel, were transferred to a nylon membrane and hybridized with the DIG-labelled DNA probe

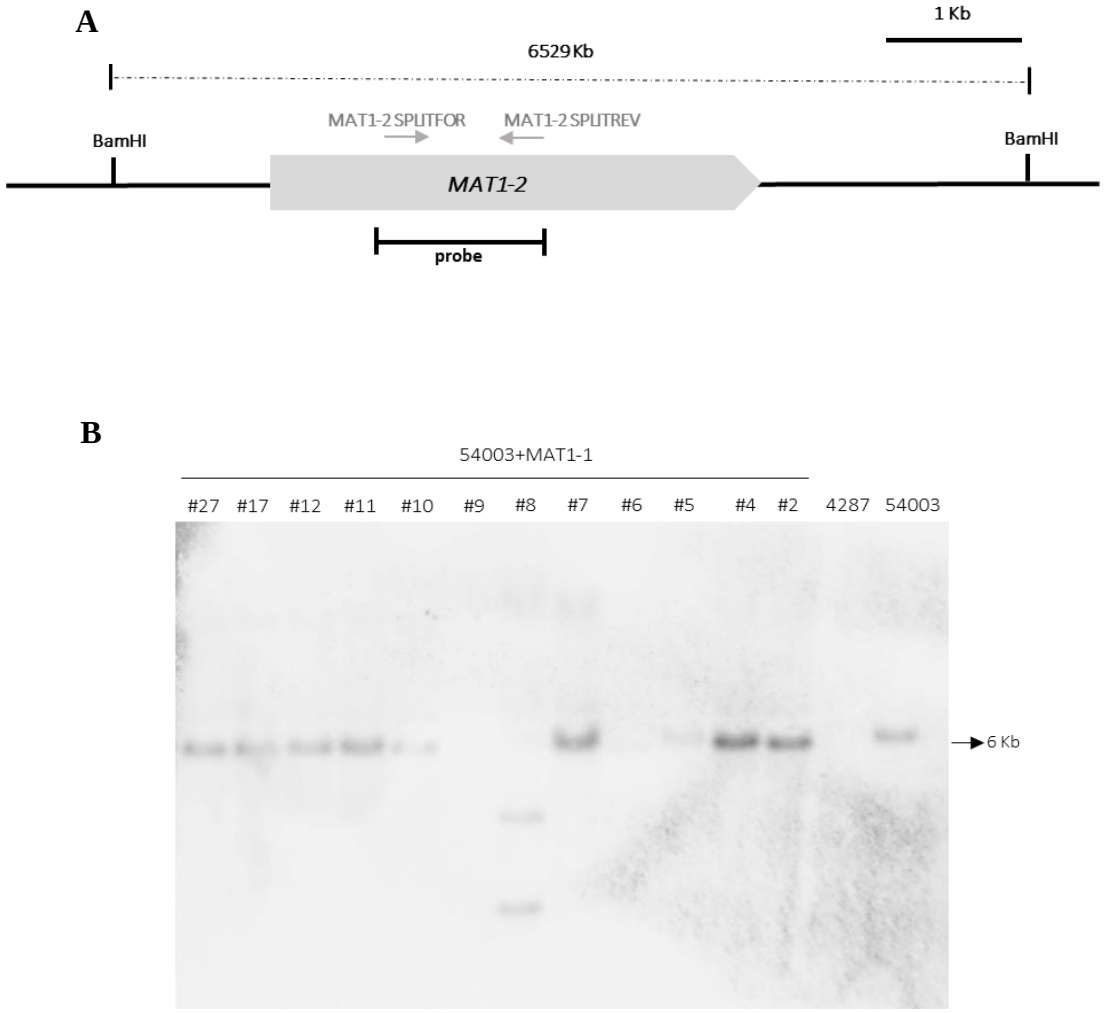



**Fig. 19. Identification of *MAT1-2* locus in the *Fusarium oxysporum* f. sp. *lycopersici* 54003 insertional mutant with both loci**. **(A)** Schematic representation of specific probe construction to identify *MAT1-2* locus. **(B)** gDNA of the wild type 4287 and 54003 isolates and 54003+MAT1-1 digested with the BamHI restriction enzyme, separated on an agarose gel, were transferred to a nylon membrane and hybridized with the DIG-labelled DNA probe.

## 4.3 PHENOTYPIC ASSAYS

### 4.3.1 Conidial germination is not inhibited in the *Fusarium oxysporum* f.sp. *lycopersici* 54003 (MAT1-2 mating type strain) at high cell density

Conidial germination represents a fundamental step in fungal development, mainly in asexual fungi where microconidia are efficient fungal propagules for the host infection. Here we studied the biological role of *MAT* loci, that regulate the expression of pheromone and cognate receptor genes, in conidial germination at two different concentrations of inoculum: optimal concentration of inoculum (OCI; $3.19*10^6$ conidia/ml) and inhibitory concentration of inoculum (ICI; $8.6*10^7$ conidia/ml) as already reported by Vitale and collaborators (2019). In order to investigate the role of *MAT* loci in this biological process we first evaluated conidial germination at OCI and ICI in both Fol 4287 (*MAT1-1* mating type) and 54003 (*MAT1-2* mating type) isolates. At OCI the germination rate oscillates around 90 % and 80% in the 4287 and 54003 Fol isolates, respectively (Fig. 20A). This indicates that both strains are fully able to germinate. In accordance with previous results we found that at ICI the 4287 strain shows approximately 15% of conidial germination. However, in the same condition the 54003 strain exhibits only a modest inhibition of germination (Fig. 20B). Collectively these results indicate a possible role of *MAT* loci in inhibition or derepression of conidial germination at high concentrations of inoculum.

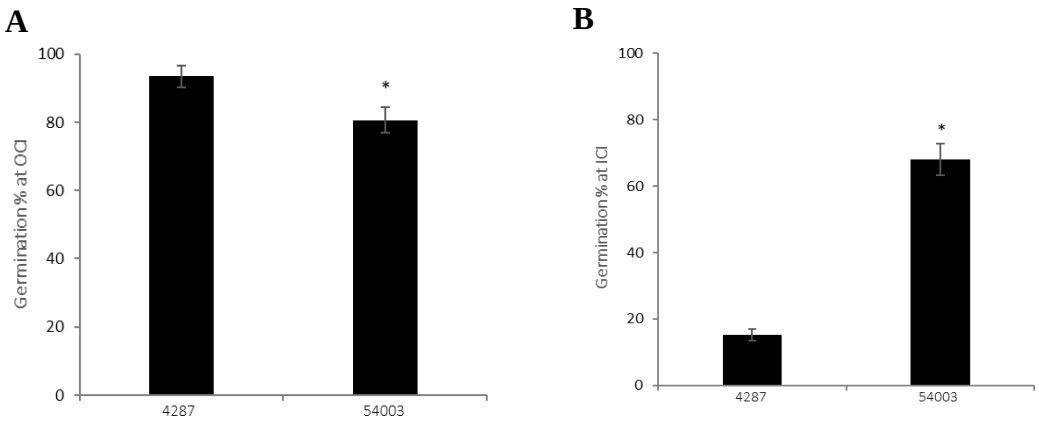



**Fig. 20. Conidial germination is not inhibited at ICI in the 54003 *Fusarium oxysporum* f.sp. *lycopersici* strain**. Microconidia of the indicated strains were inoculated at a concentration of $3,19*10^6$ conidia/ml **(A)** or of $8,6*10^7$ conidia/ml **(B)** in minimal medium with 0,1% of sucrose. After 13 h and 15 h of growth fungal cultures for OCI and ICI, samples were vortexed to dissociate weakly adherent hyphae, transferred to a microscope slide and observed using a binocular microscope (400X magnification) (*, $P < 0.0001$, versus 4287 strain). Data are presented as the mean ± standard deviations from three experiments. n=100 conidia.

#### 4.3.1.1 *MAT1-1* locus inhibits conidial germination at high concentrations of inoculum

To further investigate the role of the *MAT1-1* locus in conidial germination we studied this process in all of the strains in which we had deleted or inserted one or more copies of the entire MAT1-1 locus.

Initially we recorded the rates of conidial gemination at OCI and ICI in the MAT1-1Δ mutant and in its complemented strain (MAT1-1Δ + MAT1-1). At OCI, all strains showed similar percentages of spore-germination (90%) (Fig. 21A). Importantly conidial germination was fully restored in the complemented strain of the MAT1-1Δ, suggesting that the observed phenotype is due to the presence/absence of the *MAT1-1* locus. (Fig. 21B).

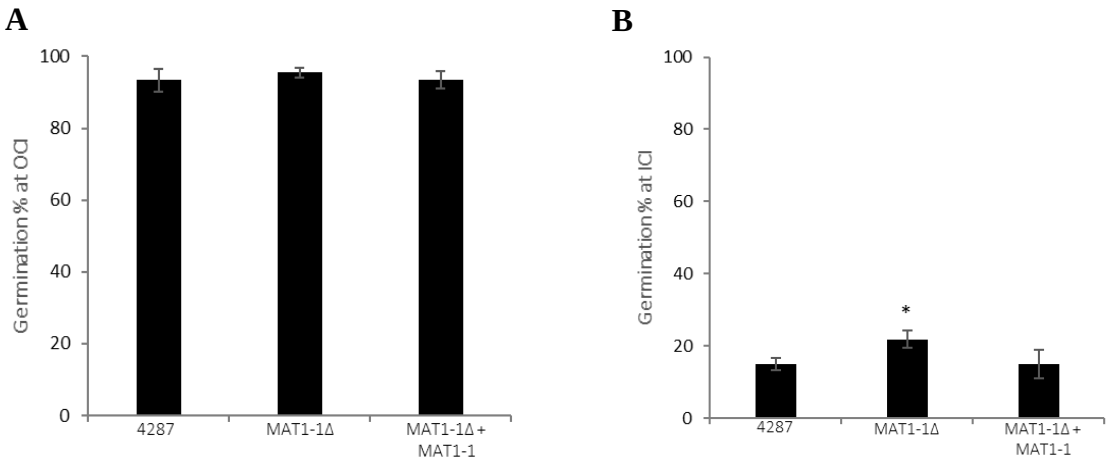



**Fig. 21. Quantification of conidial germination in the MAT1-1Δ and its complemented strain.** Microconidia of the indicated strains were inoculated at a concentration of $3{,}19*10^6$ conidia/ml **(A)** or of $8{,}6*10^7$ conidia/ml **(B)** in minimal medium with 0.1% of sucrose. After 13 h and 15 h of growth fungal cultures for OCI and ICI, samples were vortexed to dissociate weakly adherent hyphae, transferred to a microscope slide and observed using a binocular microscope (400X magnification) (*, $P < 0.05$, versus 4287 strain). Data are presented as the mean ± standard deviations from three experiments. n=100 conidia.

To better understand the role of the *MAT1-1* locus in this biological process we decided to examine its effect in the Fol 54003 insertional mutants containing both the *MAT1-1* and *MAT1-2* loci. The germination rate of the 54003+MAT 1-1 mutants containing one or two copies of the *MAT1-1* locus was assessed and compared to that of the 54003 wild-type strain. At OCI no significant differences were detected between the conidial germination rates of all the tested strains (Fig. 22A). While at ICI, both 54003+MAT 1-1 mutants tested showed a reduction of conidia germination percentage respect to those of the 54003 strain. This reduction is higher and statistically significant only in the 54003+MAT 1-1 mutant containing two copies of the *MAT1-1* locus, indicating also a putative additive effect of the latter on conidia germination (Fig. 22B).

Taken together, these findings provide the evidence that *MAT1-1* locus is involved in the repression of the spore germination in a density dependent manner.

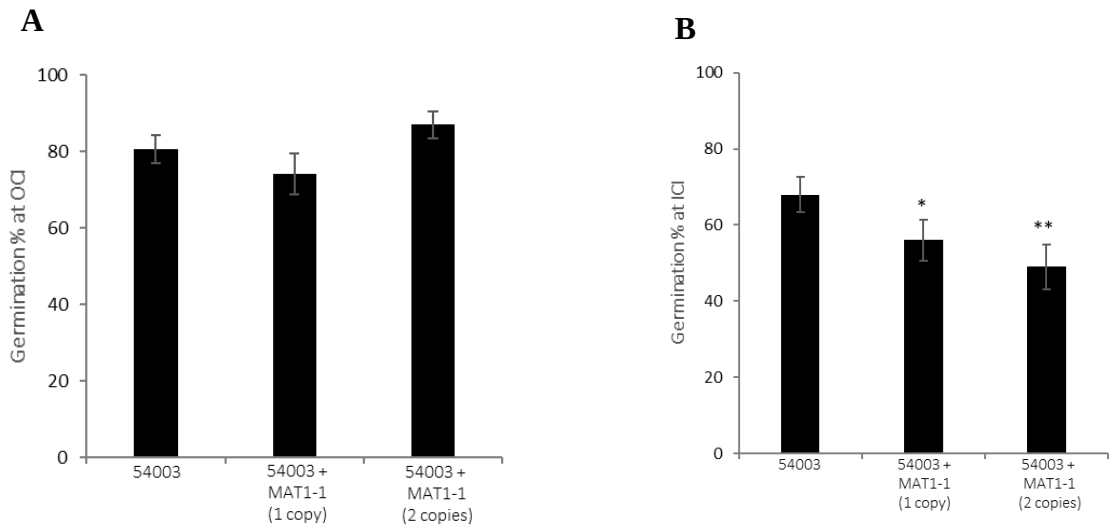



**Fig. 22. The addition of the *MAT1-1* locus in the *Fusarium oxysporum* f.sp. *lycopersici* 54003 strain represses conidial germination at ICI**. Microconidia of the indicated strains were inoculated at a concentration of $3{,}19*10^6$ conidia/ml **(A)** or of $8{,}6*10^7$ conidia/ml **(B)** in minimal medium with 0,1% of sucrose. After 13 h and 15 h of growth fungal cultures for OCI and ICI, samples were vortexed to dissociate weakly adherent hyphae, transferred to a microscope slide and observed using a binocular microscope (400X magnification) (*, $P < 0.05$; **, $P < 0.0001$, versus 54003 strain). Data are presented as the mean ± standard deviations from two experiments. n=100 conidia.

#### 4.3.1.2 *MAT1-2* locus derepresses conidial germination at high concentration of inoculum.

As for the *MAT1-1* idiomorph, in order to investigate a putative effect of the *MAT1-2* locus in conidia germination , we compared the percentage of germination of the MAT1-1 Δ mutants in which we inserted one or two copies of the entire *MAT1-2* locus (MAT1-1Δ + MAT1-2) with the 4287 wild type strain and the MAT1-1Δ mutant.

At OCI all the tested strains showed a similar germination percentage (Fig. 23A), whereas at ICI a significant germination increase was observed for both MAT1-1Δ + MAT1-2 insertional mutants when compared to the 4287 and the MAT1-1Δ strains. Strikingly, an additive effect of the number of *MAT1-2* copies on the increase of conidia germination at ICI was observed. Indeed, the MAT1-1Δ + MAT1-2 insertional mutant carrying a single copy of the *MAT1-2* locus showed a germination rate lower to that of the one carrying two copies of the *MAT1-2* locus (Fig. 23B).

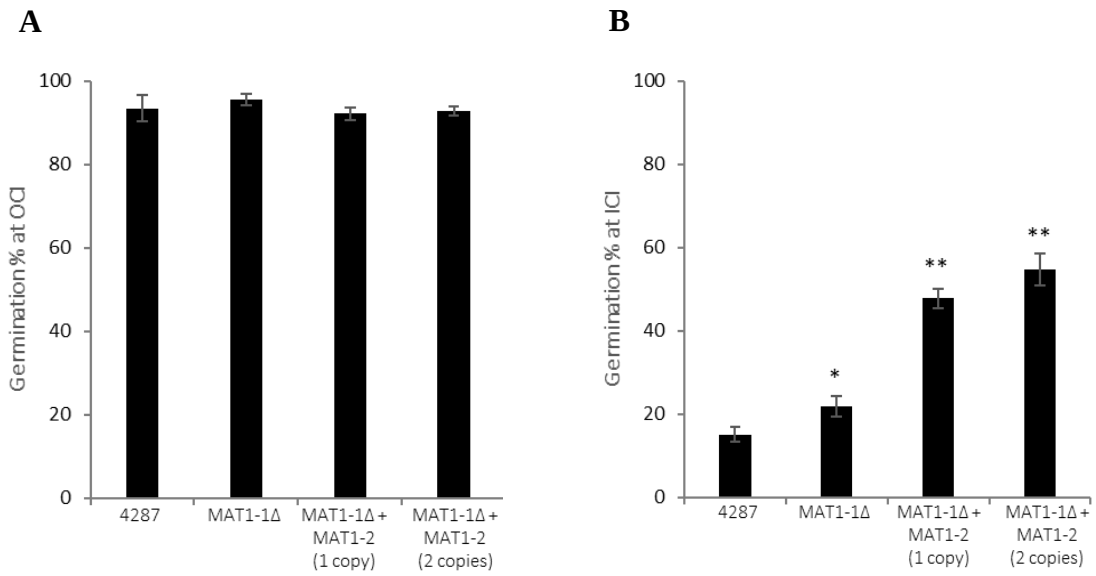



**Fig. 23. The addition of the *MAT1-2* locus in the MAT1-1 Δ strain derepresses conidial germination at ICI**. Microconidia of the indicated strains were inoculated at a concentration of $3{,}19*10^6$ conidia/ml (OCI) **(A)** or of $8{,}6*10^7$ conidia/ml (ICI) **(B)** in minimal medium with 0,1% of sucrose. After 13 h and 15 h of growth fungal cultures for OCI and ICI, samples were vortexed to dissociate weakly adherent hyphae, transferred to a microscope slide and observed using a binocular microscope (400X magnification) (*, $P < 0.05$, versus 4287; **, $P < 0.0001$, versus 4287 and MAT1-1Δ isolates). Data are presented as the mean ± standard deviations from three experiments. n=100 conidia.

To further corroborate the evidence that the *MAT1-2* locus might promote the germination of spores at ICI, we decided to investigate what would happen if both *MAT* loci were present in the genome of the 4287 strain. Thus, 4287 insertional mutants carrying both *MAT* loci were generated and the germination rate of those carrying a single or a double copy of *MAT1-2* locus was compared to that of 4287 wild-type strain at both OCI and ICI. While as expected at OCI the percentage of germination was similar among all strains (90%) (Fig. 24A), at ICI strains carrying also the *MAT1-2* locus showed a partial derepression. Again, an additive effect linked to the number of *MAT1-2* copies present in the 4287 genome was observed, similarly to that found in the 54003+MAT1-1 mutants (data showed in 4.3.1.1 section). Indeed, the germination rate recorded in the 54003+MAT1-1 strains containing a single copy of *MAT1-2* locus was lower than the one observed for those carrying two copies of it. All together these results demonstrate that the *MAT1-2* locus derepresses conidial germination at ICI (Fig. 24B).

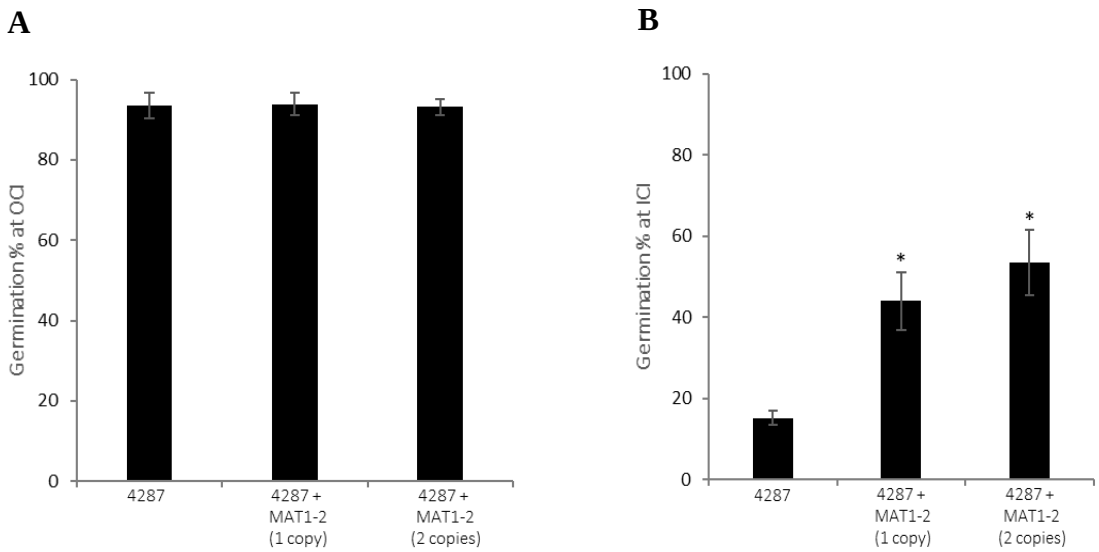



**Fig. 24. The simultaneous presence of *MAT1-1* and *MAT1-2* loci derepresses conidial germination at ICI.** Microconidia of the indicated strains were inoculated at a concentration of $3.19*10^6$ conidia/ml (OCI) **(A)** or of $8.6*10^7$ conidia/ml (ICI) **(B)** in minimal medium with 0,1% of sucrose. After 13 h and 15 h of growth fungal cultures for OCI and ICI, samples were vortexed to dissociate weakly adherent hyphae, transferred to a microscope slide and observed using a binocular microscope (400X magnification) (*, $P < 0.0001$, versus 4287). Data are presented as the mean ± standard deviations from three experiments n=100 conidia.

### 4.3.2 *Fusarium oxysporum* f.sp. *lycopersici* 54003 isolate is impaired in vegetative hyphal fusion

Vegetative hyphal fusion (VHF), or conidial anastomosis tube fusion (CAT) represents a crucial process required for the formation of hyphal networks and optimal fungal mycelium. Several studies on the VHF reported the presence of hyphal fusion events in Fol 4287 isolate and demonstrated that the fusion between germlings or fungal hyphae is affected by several environmental factors, such as nutrients, pH, temperature, but also it depends from cell-adhesion to the surface that is requires for the success of fusion events and from the density of fungal cells, in which a high cell-density can reduce hyphal fusion (Kurian *et al*., 2018). Previous results obtained in this study showed as the *MAT* loci affect the conidial germination process in Fol 4287 and 54003 strains; here we decided to further investigate their role on this other biological process that occurs after GT (germ-tube) formation during the colony initiation.

First, in order to investigate the role of the *MAT* loci on the VHF process, hyphal fusion efficiency of the two Fol strains wild-type 4287 and 54003 was recovered at high cell-density on minimal medium reported in the section 3.3. As expected, Fol 4287 wild-type strain presented hyphal fusion events, while surprisingly Fol 54003 strain failed to undergo hyphal fusion in the conditions tested (Fig. 25)

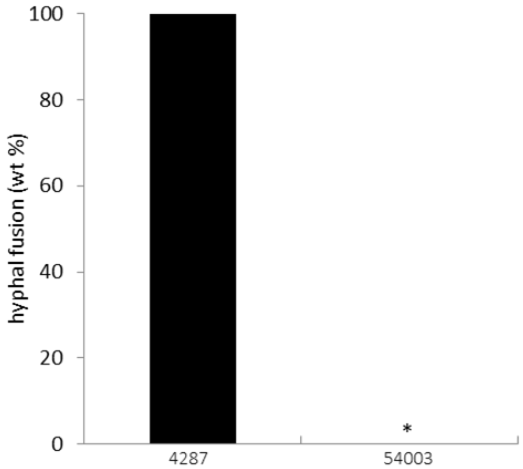



**Fig. 25. *Fusarium oxysporum* f.sp. *lycopersici* isolate 54003 is impaired in hyphal fusion.** Microconidia of the indicated strains were germinated for 14 hours on minimal medium supplemented with agar (2% w/V) and $NaNO_3$ (25 mM) and percentage of vegetative hyphal fusion events counted (*, $P < 0.0001$, versus 4287). Data are presented as the mean ± standard deviations from three experiments.

These findings suggested that *MAT* loci not only play a role in the germination process but also in other density-dependent processes such as in the occurrence of fusion events between fungal germlings or hyphae.

#### 4.3.2.1 The *MAT1-1* locus is required for efficient vegetative hyphal fusion in the *Fusarium oxysporum* f.sp. *lycopersici* 4287 isolate

To explore the importance of the *MAT1-1* locus on the VHF process in the Fol 4287 isolate, we tested VHF in the *MAT1-1*Δ and *MAT1-1*Δ + *MAT1-1* mutant strains.

Interestingly, *MAT1-1* locus deletion led to a partial but significant reduction in hyphal fusion events, while the genetically complemented strain showed a fully restored fusion ability, thus confirming that the *MAT1-1* locus plays a role in the regulation of this phenotypic trait in the Fol 4287 isolate (Fig. 26).

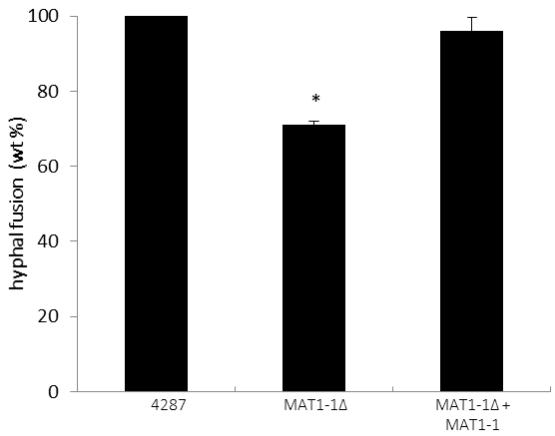



**Fig. 26. The *MAT1-1* locus is required for efficient vegetative hyphal fusion in the *Fusarium oxysporum* f.sp. *lycopersici* isolate 4287.** Microconidia of the indicated strains were germinated for 14 hours on minimal medium supplemented with agar (2% w/V) and $NaNO_3$ (25 mM) and percentage of vegetative hyphal fusion events counted (*, $P < 0.0001$, versus 4287). Data are presented as the mean ± standard deviations from three experiments.

Further, to understand if the presence of the *MAT1-1* locus alone was sufficient to restore hyphal fusion in the VHF-deficient Fol isolate 54003, we examined the occurrence of VHF events in Fol 54003 mutants carrying one or two insertional copies of the *MAT1-1* locus. Importantly, similarly to the 54003 wild-type strain, no hyphal fusion events were observed in any of the tested mutants (data not showed). These results suggest that the *MAT1-1* locus might regulate hyphal fusion in the Fol 4287 but not in the 54003 strain, likely due to the presence of additional regulation layers which depend on the Fol genetic background.

#### 4.3.2.2 The *MAT1-2* locus represses hyphal fusion in the *Fusarium oxysporum* f.sp. *lycopersici* 4287 isolate

In the section 4.3.2.1 we have found that the *MAT1-1* locus promotes vegetative hyphal fusion in the Fol 4287 isolate. However, whether the *MAT1-2* locus plays a similar or an opposite effect on VHF in this isolate is not clear. To study this aspect, we compared the number of VHF events observed in the Fol 4287 wild type strain with those of the mutants containing one or two extra copies of the *MAT1-2* locus. Interestingly, mutants containing both *MAT* loci in their genome showed a significant reduction in VHF, pointing out for a repressive function of the *MAT1-2* locus. This repression was further exacerbated by the absence of the *MAT1-1* locus in the 4287 genome, thus suggesting for a competitive effect of the different *MAT* loci on the VHF process. Furthermore, a dose-dependent effect could also be observed with higher repression of VHF in mutants with two copies of the *MAT1-2* locus in respect to those harboring only one copy of it (Fig. 27).

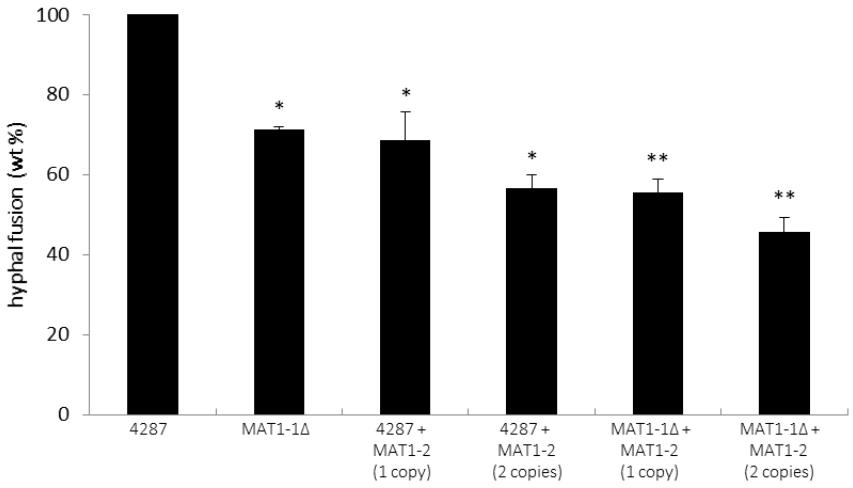



**Fig. 27. The *MAT1-2* locus represses hyphal fusion in the *Fusarium oxysporum* f.sp. *lycoperisici* 4287 isolate.** Microconidia of the indicated strains were germinated for 14 hours on minimal medium supplemented agar (2% w/V) and $NaNO_3$ (25 mM) and percentage of vegetative hyphal fusion events counted (*, $P < 0.0001$, versus 4287; **, $P < 0.0001$, versus 4287 and MAT1-1Δ strains). Data are presented as the mean ± standard deviations from three experiments.

### 4.3.3 *MAT1-1* and *MAT1-2* loci play a role in hyphal aggregation

The results showed before demonstrated that *MAT* loci play a role in both conidial germination and VHF in Fol, two phenotypes that depend upon cell density. Another important biological process already reported in Fol and that occurs at high cell density is the formation of fungal aggregates in liquid medium. (Segorbe *et al.,* 2017; Prados-Rosales and Di Pietro, 2008).

Here, to explore the role of *MAT* loci in hyphal aggregation both Fol 4287 and 54003 wild-type strains and all the mutant strains generated in this study were tested for their ability to form hyphal aggregates as described in the section 3.3. Our results showed that both 4287 and 54003 Fol isolates are able to form hyphal aggregates even though with different morphological characteristics (Fig. 28A). Interestingly, the knockout mutant of the entire *MAT1-1* locus obtained in the 4287 genetic background was impaired in the formation of hyphal aggregates, whereas as expected its complemented strain restored this phenotype. Moreover, all the insertional mutants containing additional copies of the *MAT1-2* locus in their genome (either in the MAT1-1 Δ or in the 4287 wild-type genetic background) were impaired to form hyphal aggregates. Strikingly, mutant strains obtained in the 54003 genetic background and carrying additional copies of the *MAT1-1* locus are partially able to form hyphal aggregates that were morphologically similar to the ones observed in the 4287 wild type strain (Fig. 28B). Collectively these results indicate that both *MAT* loci might regulate hyphal aggregation.

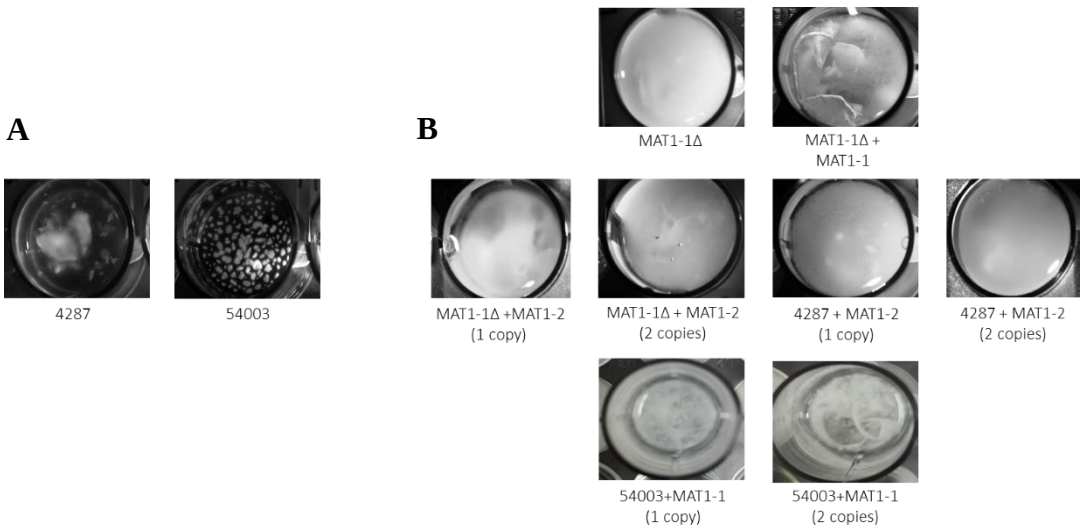



**Fig. 28. *MAT1-1* and *MAT1-2* loci play a role in hyphal aggregation.** Hyphal aggregates forming after 48 h of growth in minimal medium with 3 % of sucrose and 50mM $NaNo_3$. **(A)** Hyphal aggregates of wild-type 4287 and 54003. **(B)** Hyphal aggregates of mutant strains. Samples were vortexed to dissociate weakly adhered hyphae, transferred in a multiwell plate and observed using at Zeiss Axio Imager M2 microscope.

### 4.3.4 RT-qPCR Expression analysis

#### 4.3.4.1 Non-canonical regulation of pheromone/receptor genes occurs in 4287 and 54003 *Fusarium oxysporum* f.sp. *lycopersici* isolates

In order to understand how *MAT* loci regulate the expression of pheromones and their cognate receptors and how their regulation affects the phenotypes tested in our study, the transcriptional levels of such genes were analysed at high concentration of inoculum in all of the strains used in this work .

First, the expression of pheromones and their receptors was studied in the two 4287 (MAT1-1 mating type) and 54003 (MAT1-2 mating type) wild-type isolates. As already reported in Vitale et al. 2019 in the 4287 isolate both *a-phe/ste3* and *α-phe/ste2* gene pairs are expressed. Interestingly, we noticed that the same occurs in the 54003 wild-type isolate indicating that the regulation of pheromone/receptor genes also in this strain is different from that reported in other heterothallic ascomycetes (Fig. 29). By comparing the transcriptional levels of such genes in the two Fol isolates we observed that in the 54003 there is a five-fold higher expression of the *α-phe* precursor gene (Fig. 29).

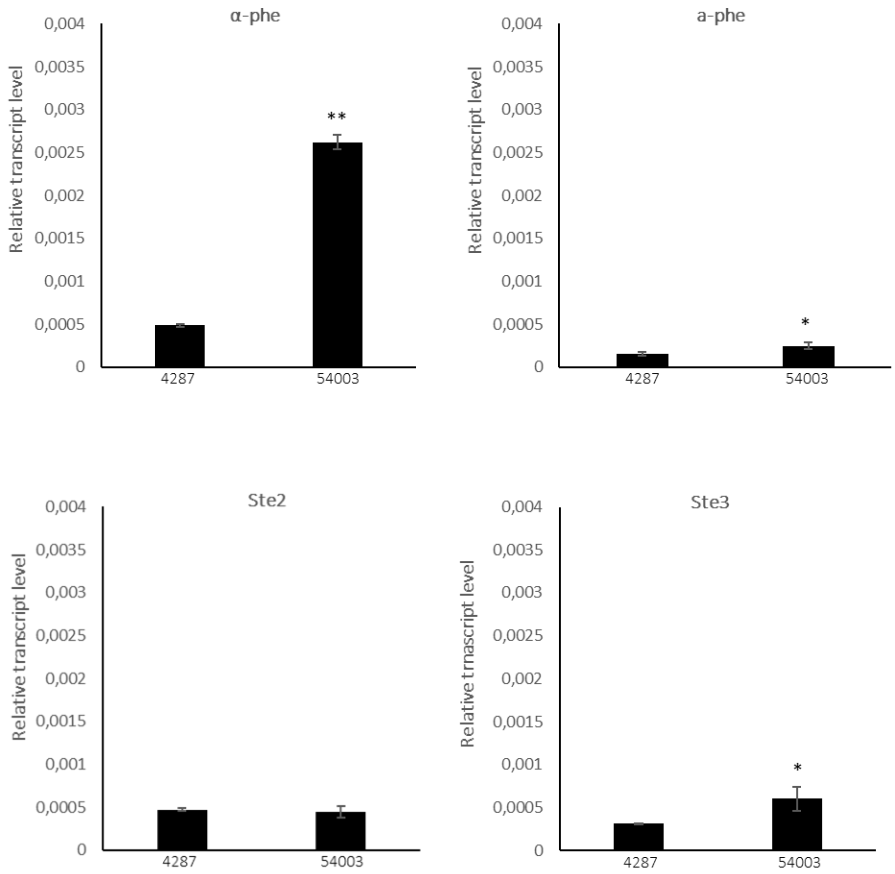



**Fig. 29. Both pheromone/receptors pair of genes are expressed at high concentration of inoculum in the *Fusarium oxysporum* f.sp. *lycopersici* 4287 and 54003 isolates.** Quantitative real-time RT-PCR analysis of pheromone and receptor genes performed in the 4287 (MAT1-1) and 54003 (MAT1-2) wild-type strains. Bars represent means ± standard deviations; n = 3 biological replicates from one representative experiment were prepared. Data were evaluated by "*t*" Students test (*, $P < 0.05$; **, $P < 0.0001$, versus 4287).

#### 4.3.4.2 The expression of the BAR1 gene is induced in the 54003 (MAT1-2 mating type) isolate

The *BAR1* gene encodes for a secreted protease that specifically cleaves the *α-phe* and regulates APS in the 4287 Fol isolate (Vitale *et al.*, 2019). Given its importance in regulating *α-phe* signalling, we decided to study how its expression is regulated by *MAT* loci. Our results indicate that as already reported by Vitale et al. (2019) for the 4287 isolate, the *BAR1* gene has a ten-fold higher expression level when compared to that of pheromones and receptors genes both in the 4287 and 54003 isolates. Interestingly, a ten-fold difference in the expression was detected for this gene between the two Fol isolates, suggesting a MAT locus- dependent regulation of APS genes (Fig. 30).

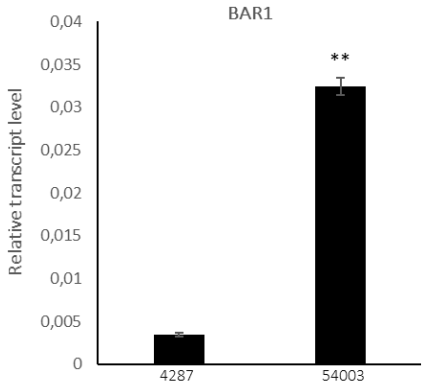



**Fig. 30. The expression of the *BAR1* gene is induced in the *Fusarium oxysporum* f.sp. *lycopersici* 54003 (MAT1-2 mating type) isolate** Quantitative real-time RT-PCR analysis of BAR1 gene performed in the 4287 (MAT1-1) and 54003 (MAT1-2) wild-type strains. Bars represent means ± standard deviations; n = 3 biological replicates from one representative experiment were prepared. Data were evaluated by "*t*" Students test (**, $P < 0.0001$, versus 4287).

#### 4.3.4.3 *MAT* loci have an opposite effect on the APS-related genes expression

To better understand the role of *MAT1-1* and *MAT1-2* loci in the regulation of APS -related genes expression, a RT-qPCR analysis was performed initially in the MAT1-1Δ mutant strain and in the insertional MAT1-1Δ+ MAT1-2 mutant strains carrying a single or a double copy of the *MAT1-2* locus. In the MAT1-1Δ mutant strain we observed an increase of the transcriptional levels of all of the analysed genes in comparison to the 4287 wild type strain (Fig. 31). These results suggest that the *MAT1-1* locus might act as a general repressor of sex-pheromone signalling. Interestingly, by comparing the transcriptional levels of these genes between the MAT1-1Δ+ MAT1-2 (1 copy) and the MAT1-1Δ mutant strains we found that *MAT1-2* insertion results in a further increase in APS-related genes expression, indicating that the *MAT1-2* locus might act as a positive regulator of APS gene regulation. In line with this, the insertion of two copies of the *MAT1-2* locus in the MAT1-1Δ genome results in a still higher expression of all APS related genes (except for S*te3*) (Fig. 31). Altogether our results are suggestive for a role of the *MAT1-1* locus acting as a repressor and of the *MAT1-2* locus as an inducer of APS-related genes in Fol.

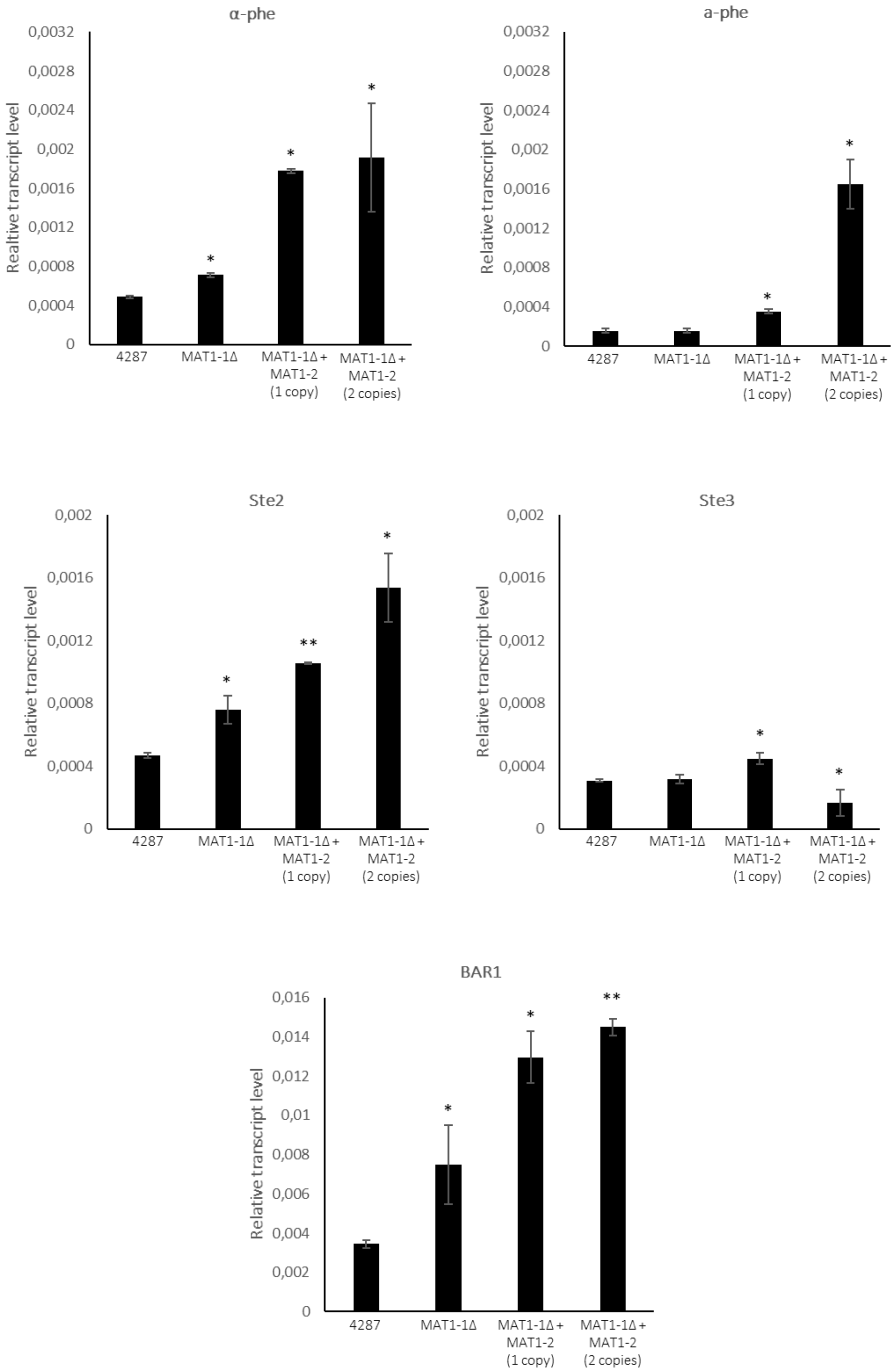



**Fig. 31. *MAT1-1* locus represses while *MAT1-2* locus induces APS-related genes expression** Quantitative real-time RT-PCR analysis of APS-related genes performed in the indicated mutant strains. Bars represent means ± standard deviations; n = 3 biological replicates from one representative experiment were prepared. Data were evaluated by "*t*" Students test (*, $P < 0.05$; **, $P < 0.0001$, versus 4287).

#### 4.3.4.4 The simultaneous presence of both *MAT* loci in *Fusarium oxysporum* f.sp. *lycopersici* alters APS-related gene expression

To understand how the simultaneous presence of the two opposite *Fusarium MAT* loci could influence the expression of APS-related genes in Fol we performed quantitative real-time RT-PCR experiments in the two Fol isolates 4287 and 54003, and in the 4287+MAT1-2 (1 copy or two copies of *MAT1-2*) and 54003 + MAT1-1 (1 copy or two copies of *MAT1-1*) insertional mutants. We then compared the expression of all APS-related genes from each insertional mutant with the Fol isolate used as a genetic background to generate the specific mutant. Addition of one copy of the *MAT1-2* locus in the 4287 genome resulted in a higher expression of both pheromone genes and of *Ste2*, but not of *Ste3* and *Bar1*, suggesting a steep increase in α-pheromone self-signalling in this mutant (Fig. 32). Interestingly, the addition of two copies of the *MAT1-2* locus in the 4287 genome resulted in a even higher expression of both pheromone genes, however the expression of the *Ste2* pheromone receptor and of the *Bar1* gene lowered down to wild type levels (Fig. 32), indicating the existence in Fol of a compensatory mechanism acting on the expression of pheromone, receptor and regulator genes to avoid over-signalling.

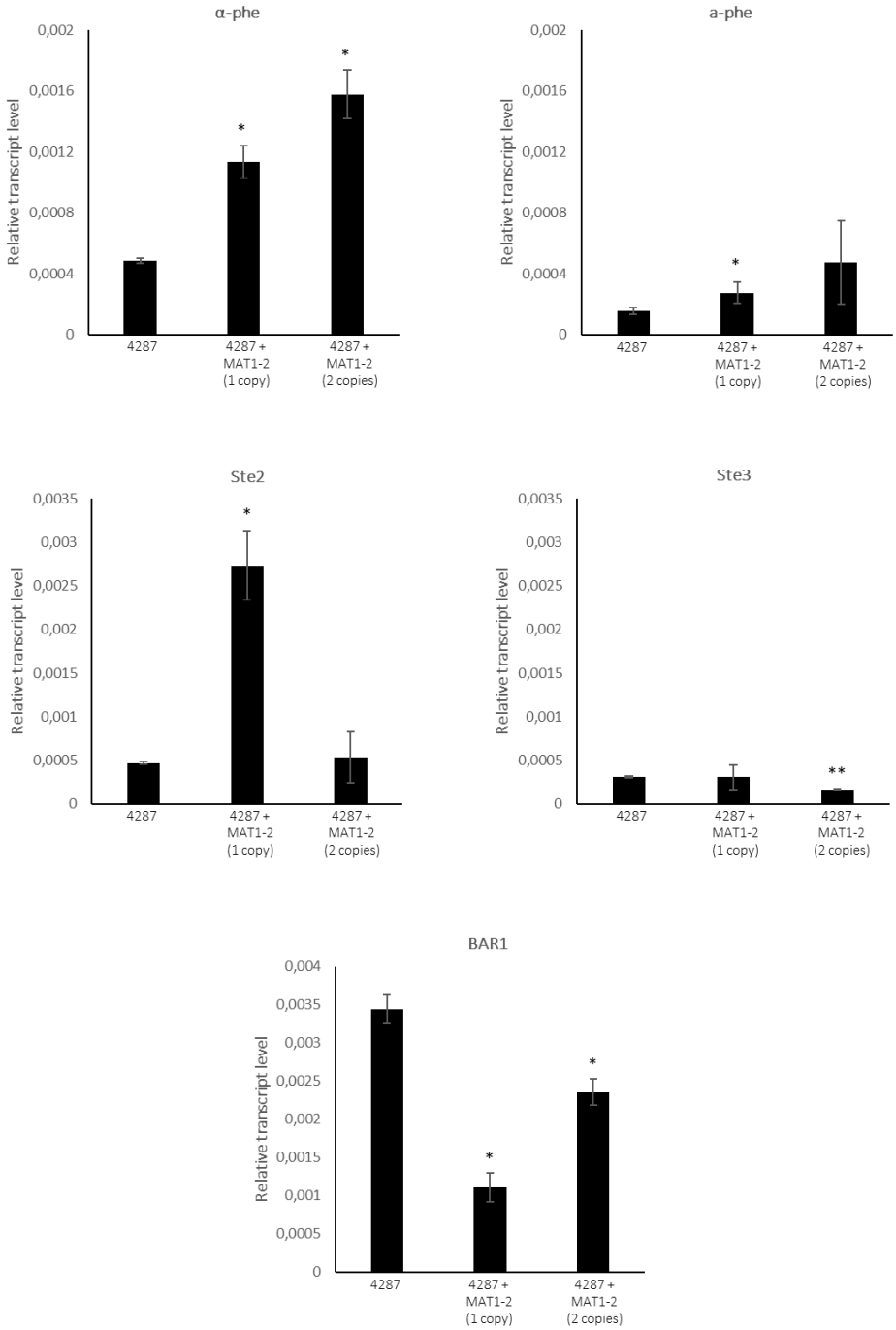



**Fig. 32. An increment of α-pheromone self-signalling in the *Fusarium oxysporum* f.sp. *lycopersici* 4287+MAT1-2 (1 copy) insertional mutants.** Quantitative real-time RT-PCR analysis of APS-related genes performed in the indicated mutant strains. Bars represent means ± standard deviations; n = 3 biological replicates from one representative experiment were prepared. Data were evaluated by "*t*" Students test (*, $P < 0.05$; **, $P < 0.0001$, versus 4287).

Remarkably, the insertion of one copy of the *MAT1-1* locus in the 54003 genome leaded to an increase in expression of all of the APS-related genes. However, as for the 4287 + MAT1-2 transformants the presence of supernumerary copies of the opposite *MAT* locus in the 54003 background resulted in an attenuation of *Ste2* and *Bar1*. In general, the expression of all APS-related genes was lower in this mutant in comparison to the transformants harbouring a single copy of the *MAT1-1* locus (Fig. 33).

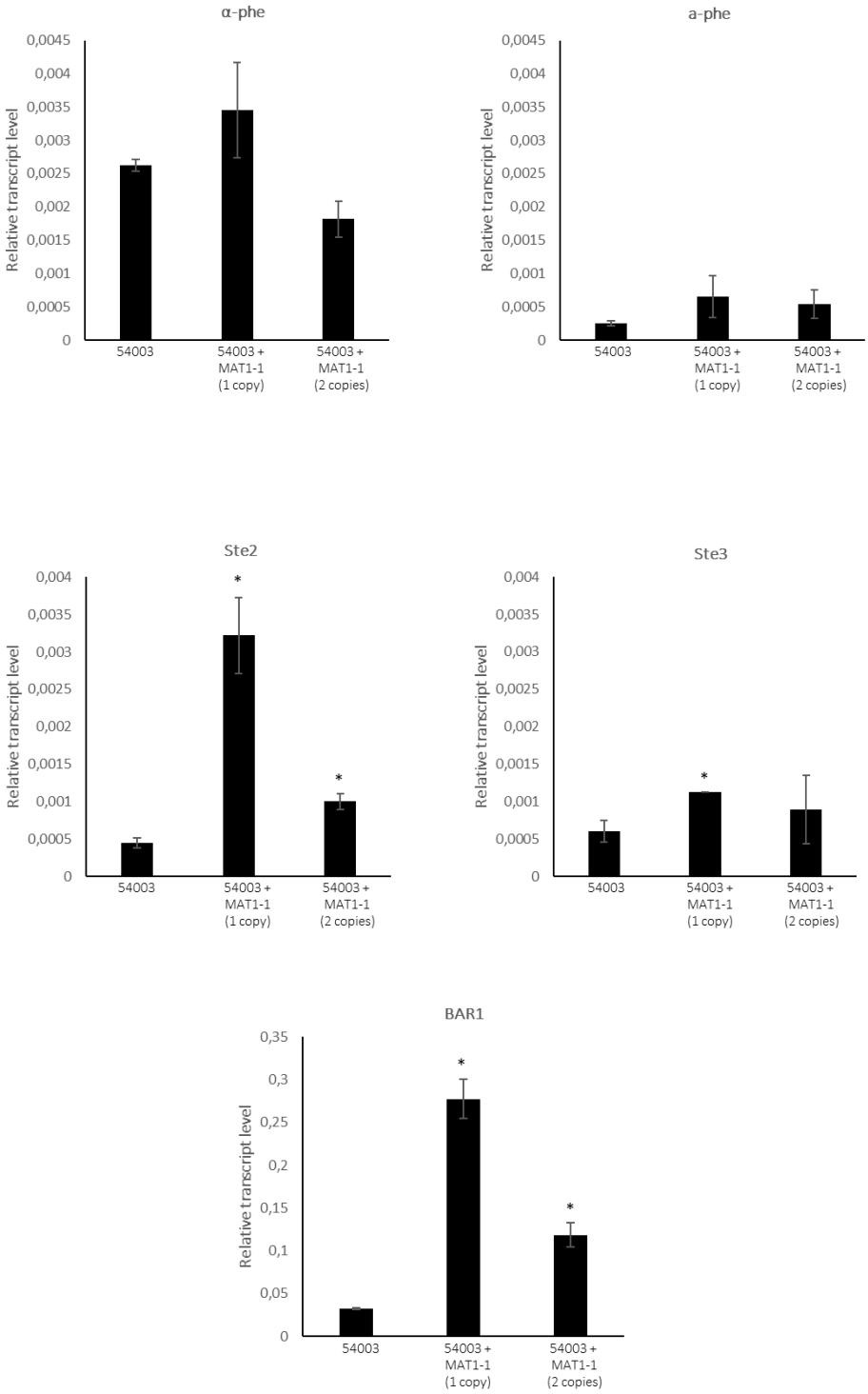



**Fig. 33. In the *Fusarium oxysporum* f.sp. *lycopersici* 54003 wild-type strain the insertion of one copy of the *MAT1-1* locus induce an increase of APS genes expression while the insertion of two copies of *MAT1-1* locus has on opposite effect.** Quantitative real-time RT-PCR analysis of APS-related genes performed in the indicated mutant strains. Bars represent means ± standard deviations; n = 2 biological replicates from one representative experiment were prepared. Data were evaluated by "*t*" Students test (*, $P < 0.05$, versus 54003).

### 4.3.5 Plant infection experiments

#### 4.3.5.1 *MAT1-1* locus promotes fungal virulence

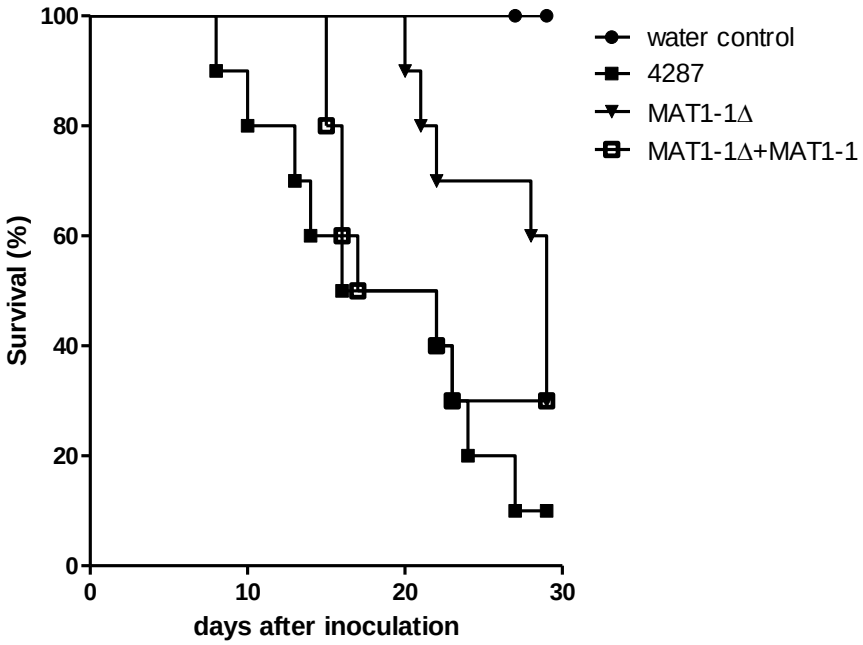
Spore germination, hyphal fusion and aggregation are essential processes for fungal development and adhesion on the plant roots contributing, directly or indirectly, to the success of host infection.

Because the previously obtained results have shown that *MAT* loci can affect these infected-related processes, we decided to explore the role of these genetic loci in Fol virulence on tomato plants. To this aim, the virulence of both wild-type isolates and of all of the generated mutant strains obtained in this study was assayed by the use of a root-dip infection protocol.

Interestingly, the two Fol isolates (4287 and 54003) exhibited differential virulence on tomato seedlings, with the Fol 54003 wild-type strain killing tomato plants slightly slower (Fig. 34).

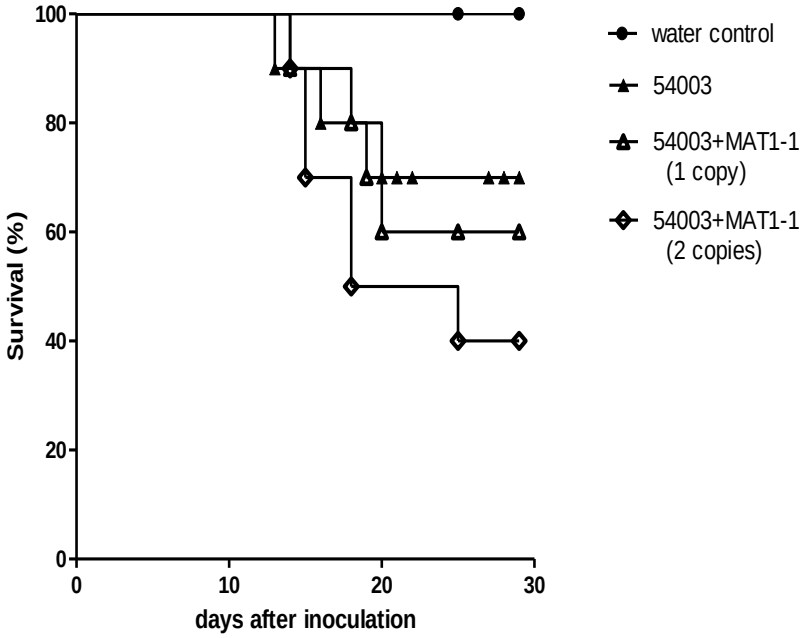
Strikingly, the knockout mutant of the entire *MAT1-1* locus in the Fol 4287 genetic background, but not its complemented strain, showed a slight but significant reduction of plant mortality respect to that observed by the Fol 4287 wildtype isolate (Fig. 34).

However, the introduction of a *MAT1-1* locus in the genome of the Fol 54003 wild-type strain didn't lead significant differences in the plant mortality although the disease symptoms appeared earlier in the plants infected with these co-transformants than in the 54003 isolate (Fig. 35).




| | log-rank test (Pvalue) |
|---|---|
| water vs water | |
| water vs 4287 | <0.0001 |
| 4287 vs MAT1-1Δ | 0.0264 |
| MAT1-1Δ + MAT1-1 | 0.3521 |

**Fig. 34. The deletion of the *MAT1-1* locus reduces virulence in *Fusarium oxysporum* f.sp. *lycopersici* 4287.** Kaplan–Meier plot shows the survival of tomato plants infected with the indicated *F. oxysporum* f.sp. *lycopersici* strains. Pathogenicity of MAT1-1Δ and its complemented strain MAT1-1Δ + MAT1-1 compared to that of the wild-type strains. Statistically differences among the groups were determined by log-rank statistics with the software GraphPad Prism 6.0. Differences indicated with a P value of less than 0.05 were considered significant.

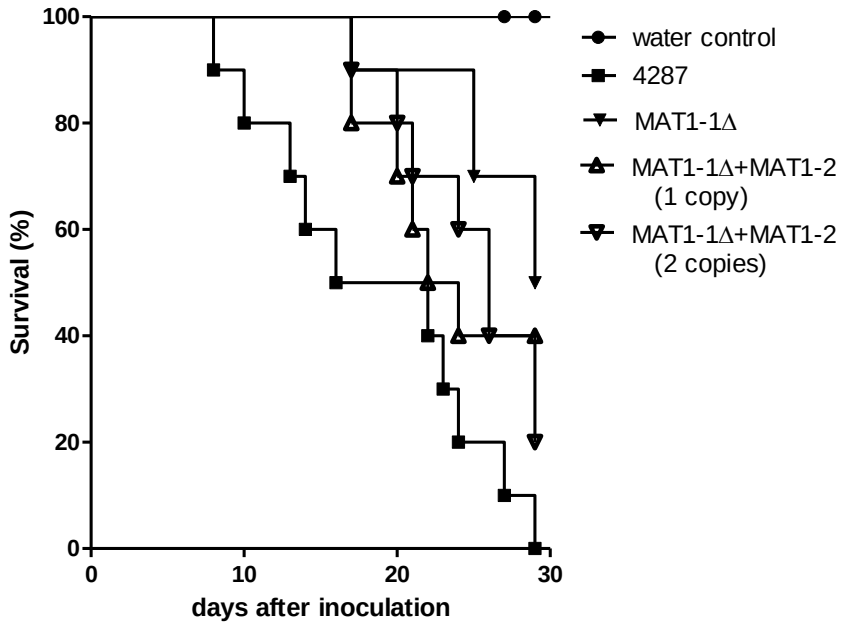



| | log-rank test (Pvalue) |
|---|---|
| water vs water | |
| water vs 54003 | 0.0671 |
| 54003 vs 54003 + MAT1-1 | 0.6961 |
| 54003 vs 54003 + MAT1-1 | 0.2429 |

**Fig. 35. The insertion of *MAT1-1* locus in *Fusarium oxysporum* f.sp. *lycopersici* 54003 isolate didn't lead significant differences in the plant mortality.** Kaplan–Meier plot shows the survival of tomato plants infected with the indicated *F. oxysporum* f.sp. *lycopersici* strains. Pathogenicity of 54003+MAT1-1 co-transformants compared to that of 54003 wild-type strain. Statistically differences among the groups were determined by log-rank statistics with the software GraphPad Prism 6.0. Differences indicated with a P value of less than 0.05 were considered significant.

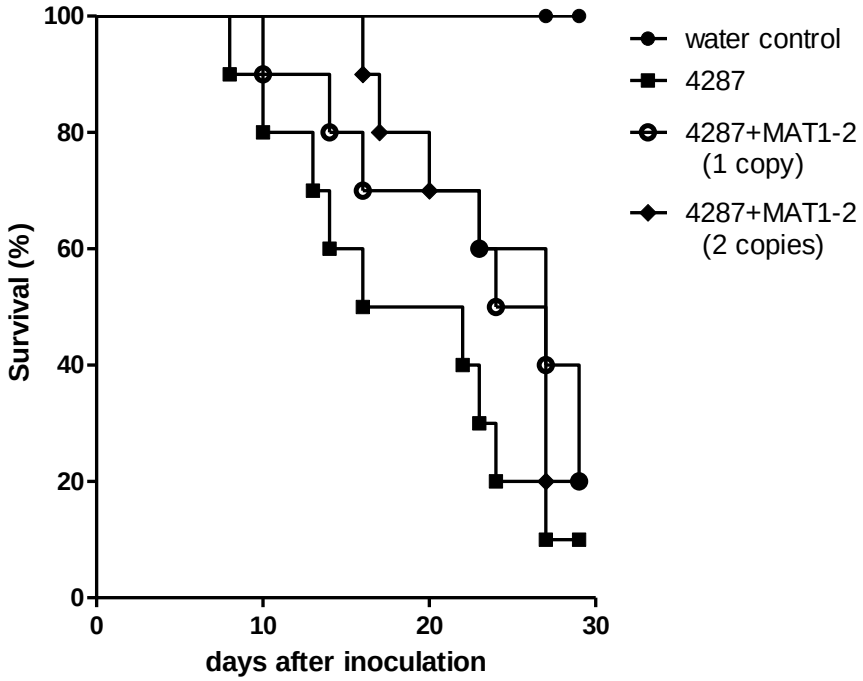
The insertion of one or two copies of the *MAT1-2* locus either in the 4287 or in the MAT1-1Δ background slightly, but not significantly, retarded fungal pathogenicity when compared to the parental strains (Fig. 36-37).

Collectively, these results indicate that *MAT* loci play a partial role in the regulation of Fol infective potential. While the *MAT1-1* locus promotes the *MAT1-2* slightly reduces fungal virulence.




| | log-rank test (Pvalue) |
|---|---|
| water vs water | |
| water vs 4287 | <0.0001 |
| 4287 vs MAT1-1Δ | 0.0051 |
| MAT1-1Δ vs MAT1-1Δ + MAT1-2 (1 copy) | 0.3398 |
| MAT1-1Δ vs MAT1-1Δ + MAT1-2 (2 copies) | 0.1468 |

**Fig. 36. The insertion of one or two copies of the *MAT1-2* locus in MAT1-1Δ retarded plant infection in *Fusarium oxysporum* f.sp. *lycopersici*.** Kaplan–Meier plot shows the survival of tomato plants infected with the indicated *F. oxysporum* f.sp. *lycopersici* strains. Pathogenicity of MAT1-1Δ + MAT1-2 co-transformants compared to that of wild-type strains. Statistically differences among the groups were determined by log-rank statistics with the software GraphPad Prism 6.0. Differences indicated with a P value of less than 0.05 were considered significant.




| | log-rank test (Pvalue) |
|---|---|
| water vs water | |
| water vs 4287 | <0.0001 |
| 4287 vs 4287 + MAT1-2 (1 copy) | 0.0742 |
| 4287 vs 4287 + MAT1-2 (2 copies) | 0,1151 |

**Fig. 37. The insertion of one or two copies of the *MAT1-2* locus retarded the plant infection in *Fusarium oxysporum* f.sp. *lycopersici.*** Kaplan–Meier plot shows the survival of tomato plants infected with the indicated *F. oxysporum* f.sp. *lycopersici* strains. Pathogenicity of 4287+MAT1-2 co-transformants compared to that of wild-type strains. Statistically differences among the groups were determined by log-rank statistics with the software GraphPad Prism 6.0. Differences indicated with a P value of less than 0.05 were considered significant.

# 5 DISCUSSION

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In heterothallic ascomycete fungi, sexual identity is under the control of two genomic regions named *MAT* loci whose structure and sequence are broadly conserved among fungal species. Normally, *MAT* loci contain either two or three genes. These genes generally encode for transcriptional factors carrying conserved DNA-binding motifs such as the α-box domain (*MAT 1-1-1*), the HMG domain (*MAT 1-1-3*, *MAT 1-2-1*) and the PPF domain (*MAT 1-1-2*). The only exception is the *MAT 1-2-3* gene whose function remains to be elucidated (Yun *et al.,* 2000; Debuchy *et al.,* 2006). The expression of these genes is induced during different stages of sexual reproduction to fine tune the abundance of opposite pheromone-receptor pairs and regulate sexual identity (Kim *et al.,* 2012). The function of *MAT* loci and pheromones in sexual development appears to be broadly conserved in ascomycetes and has been experimentally demonstrated for a number of species (Pöggeler, 2011). Interestingly, in the genome of the plant pathogen *F. oxysporum* two functional mating type idiomorphs are present and expressed, despite the fact that a sexual stage has not yet been described in this specie (Yun *et al.,* 2000). Thus, in order to investigate putative additional functions of *MAT* loci beyond sexual reproduction we generated a set of mutant strains in two Fol isolates differing for mating type locus composition. In the *MAT1-1* (also referred as *MAT*α) Fol 4287 strain we generated the knockout mutant of the entire locus *MAT1-1*, its complemented strain and a set of insertional mutants containing either the opposite *MAT* locus or both *MAT* loci in the same genome. Additionally, for the *MAT1-2* (also referred as *MAT*a) Fol 54003 isolate, we obtained insertional mutants carrying both *MAT* loci in its genome. Although Fol is a heterothallic fungus, in a previous study it was shown that the Fol 4287 *MAT1-1* strain, which should express only *α-pheromone* and *Ste3* genes, expresses both pheromone/receptor pairs (Vitale *et al.,* 2019). Interestingly, here we found that a similar scenario holds true also for the *MAT1-2* Fol 54003 strain. Furthermore, despite this strain should only express the *a-pheromone* and *Ste2* genes, *α-pheromone* and *Ste3* genes are more expressed than their counterparts.

Importantly, Vitale et al. (2019) demonstrated that this unusual transcriptional regulation of pheromone and receptor genes in the Fol 4287 strain is responsible for autocrine-mediated regulation of conidial germination. Conidial germination in filamentous fungi is a fundamental biological process required for both colony initiation and host–pathogen interaction. Conidial germination requires specific environmental conditions to occur and that might vary from

species to species (Turgeman *et al.,* 2016). Recent works demonstrated that many external factors can affect conidial-germination, hyphal growth and sporulation in *Fusarium* spp. For example, external pH and temperature represent key factors regulating these processes. Interestingly, self-inhibition of conidial germination has been described in a number of fungal species and several germination inhibitors have been identified (Leeder *et al.,* 2011; Ugalde *et al.,* 2014). In the recent work by Vitale et al. (2019) it was shown that conidial germination is inhibited in the Fol 4287 isolate at high cell-density, where the interaction between *α-pheromone* and *Ste2* activates the downstream CWI MAPK cascade and blocks germination. Contrarily, at lower concentrations of inoculum the *a-pheromone*/*Ste3* interaction promotes germination by counteracting *α-pheromone*-mediated signalling. Since *MAT* loci are known from the yeast model *Saccharomyces cerevisiae* (Tsong *et al.,* 2003; Ni *et al.,* 2011) to regulate the expression of both pheromone and pheromone receptors genes, here we decided to study if they could regulate conidial germination in the Fol in a cell-density manner. At this regard, we first noticed that despite germination at high concentrations of inoculum is inhibited in the 4287 (*MAT1-1*) strain, it is not inhibited in the 54003 (*MAT1-2*) isolate. Interestingly, RT-qPCR analysis performed on Fol germlings growing at high concentration of inoculum showed that the transcriptional level of the *a-pheromone*, *Ste3* and *Bar1* genes, the latter encoding for an α-pheromone specific protease exclusively secreted by a-type cells in the model yeasts *Candida albicans* and *S. cerevisiae* (Bennett *et al.,* 2010; Jin *et al.,* 2011), is higher in the 54003 in respect to the 4287 isolate. It is, thus, possible that both and increase in *a-pheromone/Ste3* signalling and the high expression of *Bar1* in the 54003 isolate might lead to a more efficient deregulation of the *α-pheromone/Ste2* signalling and of the block of conidial germination at cell densities that normally inhibit the germination of spores in the 4287 isolate. In line with the hypothesis that the *MAT1-2* locus might have a positive effect on conidial germination by repressing α-pheromone autocrine signalling, *MAT* locus substitution in the 4287 Fol isolate led to higher expression not only of the complete set of pheromone and receptor genes but also of the *Bar1* gene and consequent derepression of conidial germination at ICI. Interestingly, both *Bar1* gene induction and germination could be further increased by inserting two copies of the *MAT1-2* locus in the *MAT1-1*Δ knock-out mutant, leading to the conclusion that *MAT1-2* locus presence has a dose-dependent effect on *α-pheromone/Ste2* signalling repression. Reversely, the *MAT1-1* locus enhances *α-pheromone*-mediated signalling as its presence in the 4287 genome represses both *Bar1* gene expression and conidial germination at ICI. Surprisingly, the addition of the *MAT1-2* locus to the 4287 genome, resulted in an overall alteration of APS-

related gene expression, which differed upon the number of copies of the *MAT1-2* locus inserted in the genome. However, the expression of both pheromone genes but not of *Bar1* or of pheromone receptor genes increased as a consequence of the number of copies of the *MAT1-2* locus present in the genome suggesting that an alternative transcriptional scenario might occur when both *MAT* loci are present in the same genome. This is in line with what has been observed in the human pathogenic fungus *C. albicans*. Indeed, while in haploid *MAT*α and *MAT*a cells of this ascomycete the expression of the *α-Ste3* and *a-Ste2* pheromone-receptor genes are induced respectively, in diploids ones the coexistence of the two *MAT* loci represses the transcription of all pheromone and pheromone receptor genes (Alby and Bennett, 2010). Interestingly, Fol 4287 conidia transformed with additional copies of the *MAT1-2* locus were derepressed in germination at ICI, further confirming a positive role of the *MAT1-2* locus in the germination at high cell densities. However, because *MAT1-2* locus abundance in these mutants is linearly related with an increase of *a-pheromone* rather than *Bar1* gene expression, it can be postulated that germination derepression here might be a result of an increase in *a-pheromone/Ste3* signalling, quenching of the *α-pheromone/Ste2* signalling via the expression of inactive or non-processed versions of the *α-pheromone* peptide or of the *Ste2* receptor, or a result of both mechanisms. Quenching of α-pheromone perception has indeed been demonstrated to occur selectively in *S. cerevisiae* α-cell types but not in a-cells, due to a fine-tuned post-transcriptional regulation process delivering incomplete *Ste2* proteins thanks to the presence of a cryptic polyadenylation site in the coding region of the α-pheromone receptor (Di Segni *et al.,* 2011). Collectively our results show that while the *MAT1-1* inhibits the *MAT1-2* locus induces conidial germination at high cell density in the Fol 4287 isolate. A repressive activity of the *MAT1-1* locus on conidial germination at the cell densities mentioned above was further confirmed by inserting this genetic locus in the genome of the Fol (*MAT1-2*) isolate 54003. Indeed, *MAT1-1* locus abundance in this strain is linearly related with an increase in germination inhibition at ICI. However, in this case as for the 4287 mutants carrying both *MAT* loci in their genome co-presence of the two *MAT* loci in the 54003 genome resulted in an overall alteration of APS-related gene expression, suggestive again for the existence of an alternative transcriptional scenario. Noteworthy, despite these mutants were more repressed in conidial germination in comparison to the parental strain, no clear evidence of an increase of *α-pheromone/Ste2* or quenching of the *a-pheromone/Ste3* signalling could be observed through our RT-qPCR studies, thus suggesting the possibility that additional layers of regulation of the quorum-sensing might exist in a Fol isolate-dependent manner.

At different stages of colony development fungal hyphae can engage in hyphal fusion events that can occurs within the same mycelium or between hyphae from genetically different individuals. Generally, this latter case triggers the vegetative incompatibility response with the death of fused cells (Glass *et al.,* 2000). However, in certain pairings or conditions the vegetative incompatibility response can be suppressed and fused cells may occasionally survive and harbour both parental nuclei in the same cytoplasm, a possibility for horizontal transfer of genetic material between fungi (Shoji *et al.,* 2015). Vegetative hyphal fusion (VHF) is a complex regulated process which has been well-characterized in *N. crassa* and is divided into three steps: mutual recognition, hyphal homing and cell fusion (Read *et al.,* 2012). In the first two steps, hyphae initiate and maintain positive chemotropic growth, a process that requires tight regulation of the amount of secreted chemo-attractants. Low or excessive, receptor-saturating doses of chemoattractant might result in weak tropic responses, either due to receptor wandering or cellular "confusion" (McClure *et al.,* 2015; Turrà *et al.,* 2015; Turrà *et al.,* 2016, Vitale *et al.,* 2017). Interestingly, here we found that *MAT* loci also affect the vegetative hyphal fusion process in the *Fol* 4287 isolate. Indeed, *MAT1-1* locus deletion leads to a significant reduction in the number of observed hyphal fusion events. Interestingly, a stronger reduction in the efficiency of VHF is obtained in Fol 4287 transformants containing either the *MAT1-2* locus or both *MAT* loci in their genome. In contrast to what was previously observed for germination, VHF assays suggest that the *MAT1-1* locus might positively regulate, whereas the *MAT1-2* locus represses VHF. Strikingly, the wild-type Fol isolate 54003 failed at all to fuse in the tested conditions, and this also occurred after inserting the *MAT1-1* locus in its genome, suggesting that VHF impairment in this strain might depend upon additional, yet unknown factors, which are independent of *MAT* locus organization. Accordingly, differences in the fusion abilities of different isolates of the same species have already been reported in wild populations of the model ascomycete *N. crassa* and have been demonstrated to rely on additional players independent of *MAT* locus composition (Palma-Guerrero *et al.*, 2013).

Both hyphal fusion and adhesion are important processes in filamentous fungi regulating the ability to form hyphal aggregates, macroscopic structures often produced by Fol isolates when growing in liquid medium or in presence of plant roots. However, while hyphal aggregation has already been reported for the Fol 4287 isolate to occur in nutrient poor conditions (López-Berges *et al.*, 2010; Segorbe *et al.,* 2017), no evidence exists yet for the ability of the 54003 Fol isolates to form these multicellular structures. Here, we tested and compared hyphal aggregation in both Fol isolates (4287 and 54003). Interestingly, both of them formed visible

hyphal aggregates in liquid culture, though with different morphologies. In fact, aggregates resembled large sheets or small spheres in the 4287 or the 54003 isolates, respectively. Importantly, a 4287 mutant strain lacking the *MAT1-1* locus, but not its complemented strain, failed to produce hyphal aggregates likely due to its reduced ability to undergo hyphal fusion. On the other hand, Fol 54003 mutant strains carrying one or two additional *MAT1-1* loci formed hyphal aggregates morphologically similar to those observed in the 4287 Fol strain despite being VHF incompetent. These findings suggest that the aggregation process in *F. oxysporum* may involve both fusion-dependent and fusion-independent mechanisms and that the morphology of the aggregates might be a result of their relative contribution. While, a correlation between hyphal fusion competence and aggregation has been clearly established in *F. oxysporum* (López-Berges *et al.,* 2010; Segorbe *et al.,* 2017), fusion-independent mechanisms have remained elusive. One possibility is that they rely on cell surface adhesiveness, as shown in *C. albicans*, where aggregation of yeast cells during the formation of biofilms is mediated by agglutinins and glycosylphosphatidylinositol-anchored glycoproteins (Chandra *et al.,* 2001; Granger *et al.,* 2005; Segorbe *et al.,* 2017).

Genetic studies in Fol identified two signalling proteins, Fmk1 and Fso1, which play a crucial role in both vegetative hyphal fusion and aggregation. Importantly, both biological processes contribute to the establishment of colonizing hyphal networks around host roots, thus optimizing adhesion to host surfaces and exploitation of the limited nutrient resources encountered during infection (Prados-Rosales and Di Pietro, 2008; Kurian *et al*., 2018). However, while Fmk1 is fundamental for VHF and virulence-related functions such as adhesion, aggregation and invasive growth, Fso1 is essential for VHF but contributes only marginally to virulence (Prados-Rosales and Di Pietro, 2008). Because *MAT* loci seem to play a role in the regulation of VHF in Fol, we decided to test their importance in fungal virulence too. Strikingly, plants treated with 54003 Fol mutants carrying an extra *MAT1-1* locus died earlier, while those infected with 4287 Fol mutants lacking the entire *MAT1-1* locus lived longer, suggesting that the *MAT1-1* locus plays a minor but significant role in Fol virulence. By contrast *MAT1-2* locus insertion in the Fol 4287 genome didn't significantly alter fungal infectivity despite the 54003 Fol isolate is slightly less virulent than the 4287 one. The evidence that *MAT* loci might contribute to fungal virulence is in line with the previous findings that the deletion of either *MAT1-1-1* or *MAT1-2-1* genes from the homothallic fungus *F. graminearum*, leads to a reduction in virulence of corn stalk rot (Zheng *et al.,* 2013).

Further studies will be required to better understand how and which individual genes of *MAT* loci regulate fungal development and virulence in Fol. It will also be of interest to study how *MAT* gene expression is regulated depending on the different genetic context: background genome, presence of one or of two opposite *MAT* loci in the same genome. This information will likely give new insights on *MAT* locus dependent regulation of APS-related genes in Fol.

# 6 CONCLUSIONS

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This study provides the first evidence that *MAT* loci have mating-independent functions in the asexual fungus *F.oxysporum* f.sp. *lycopersici* (Fol). Our results demonstrated that *MAT* loci regulate fungal virulence and virulence-related processes such as spore-germination, vegetative hyphal fusion and aggregation.

In summary the results obtained indicate that:

- ***MAT* loci regulate conidial-germination in a cell-density manner in Fol.** In particular, the *MAT1-1* locus inhibits conidial germination while *MAT1-2* derepresses it at high concentration of inoculum.

- ***MAT* loci affect the vegetative hyphal fusion.** *MAT1-1* locus is required for an efficient vegetative hyphal fusion of Fol 4287 isolate. In contrast, Fol 54003 isolate is impaired to fuse and the *MAT1-2* locus shows to repress the VHF of Fol 4287 isolate.

- ***MAT* loci play a role in the formation of hyphal aggregates.** The two Fol 4287 and 54003 isolates aggregate with different morphological characteristics. All mutant strains obtained in this work failed to aggregate, except for the insertional mutants carrying additional copies of the *MAT1-1* locus in the 54003 genetic background in which hyphal aggregation seems to be similar to the one observed Fol 4287.

- ***MAT* loci regulate APS-related genes expression.** The two Fol 4287 and 54003 isolates express both pheromone and pheromones receptor genes and the expression of BAR1 is induced in Fol 54003 at high concentrations of inoculum. *MAT* loci have an opposite effect on the expression of APS related- genes. *MAT1-1* acts as a repressor while *MAT1-2* locus as an inducer of the expression of such genes. Surprisingly, the simultaneous presence of *MAT1-1* and *MAT1-2* loci alters the expression of these genes in the genome of both Fol 4287 and 54003 wild-type strains.

- ***MAT* loci affect the fungal virulence.** The Fol 4287 and 54003 isolates showed a different level of virulence toward tomato seedlings, with Fol 4287 which is more virulent than the Fol 54003. The deletion of *MAT1-1* locus reduces the virulence of 4287

wild-type strain , whereas the insertion of it in the genome of Fol 54003 as the insertion of one or two copies of *MAT1-2* locus in the genome of Fol 4287 strain don't lead significantly differences of virulence.

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