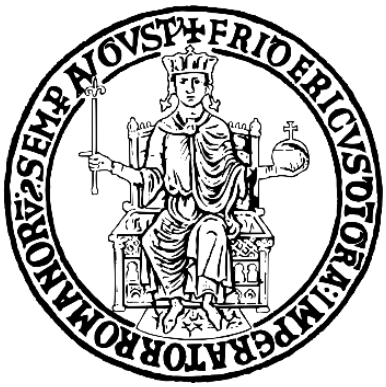
**UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II**



**DEPARTMENT OF AGRICULTURAL SCIENCES**

Ph.D. in Sustainable Agricultural and Forestry Systems and Food Security

Cycle XXXVI

# HOST SENSING IN THE FUNGAL PATHOGEN *FUSARIUM OXYSPORUM*

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# INDEX

# LIST OF FIGURES

# LIST OF TABLES

# ABSTRACT

*Fusarium oxysporum* (*Fo*) is a soil-borne fungal pathogen that causes vascular wilt disease in susceptible tomato plants, leading to significant annual yield losses. Despite the known chemical signals that attract *Fo* to host roots, its penetration mechanism and inducing signals remain elusive. To colonize plant root tissues, *Fo* hyphae have to penetrate sub-micrometric channels, such as those encountered in the plant's apoplast or symplast, a process likely requiring the generation of high physical pressure and the development of tiny cellular structures. The aim of this work has been to elucidate the physical and molecular mechanisms driving *Fo* penetration and virulence. In vitro assays showed that during the penetration process *Fo* hyphae are both able to penetrate pre-formed pores/channels of a fraction of a micron and to rapidly adapt to distinct osmotic environments. The latter process requires Hog1, a Mitogen-Activated Protein Kinase (MAPK) essential for osmoadaptation. We further demonstrate that hyphal compression and cell wall damage, likely outputs of fungal entrance into the host openings, trigger *Fo* penetration. Indeed, media supplementation with crude cell wall extracts or laminarin expedites *Fo* invasion in artificial membranes. Finally, we show that Ham7, Wsc1, and Mid1, putative mechanosensors and upstream components of the Mpk1 cell-wall integrity MAPK cascade, play significant roles in *Fo* virulence. Overall, this study unravels fundamental aspects of *Fo* pathogenicity, offering valuable insights for future disease management strategies.

# CHAPTER 1

## General introduction on *Fusarium* spp. complex

# CHAPTER 1 - General introduction on *Fusarium spp*. complex

The kingdom of fungi is evolutionarily distant from those of plants and animals and includes a high diversity of taxa characterized by different habitats, life cycles, morphologies, and nutrient demands. Most fungi are found in rich environments, where they assimilate organic molecules such as proteins, polysaccharides, and nucleotides, however few are also found in extreme environmental conditions. For example, *Aspergillus sydowii* inhabits hydrothermal fluids with temperatures that can reach 400 ºC (Burgaud *et al*., 2009), while *Penicillium chrysogenum* is found in the Atacama Desert, the driest non-polar desert in the world (Gonçalves *et al*., 2016), and *Nadsoniella nigra* var. *hesuelica* survives periodic freezing and thawing cycles in Antarctica (Lyakh *et al*., 1983). In general, the fungal kingdom constitutes a highly versatile group of eukaryotic carbon heterotrophic organisms that have successfully occupied most natural habitats (Knogge, 1996). Most known fungal species are strictly saprophytic, with less than 10% of the 100.000 known fungal species able to have an intimate interaction with plants. Just a fraction of these can infect and cause plant diseases (Knogge, 1996; Agrios, 1997). Fungal pathogens face unfavorable conditions during host colonization and thus need to be both resilient to immune defenses and able to obtain nutrients in conditions where they are difficult to extract. Fungal pathogens include plant pathogens that determine crop diseases and cause a serious economic impact on global agriculture, as well as human pathogens that cause life-threatening systemic infections in immunocompromised humans. Fungal phytopathogens are by far the most harmful class of plant pathogens. They cause over 70% of all crop diseases and each year destroy 15% of global agricultural production, which in terms of food calories is enough to feed half a billion people. Scheffer (1991) observed different levels of specialization in plant-fungal interactions. The first group includes the opportunistic parasites, which enter plants through wounds or require otherwise weakened plant hosts for colonization. These fungal species are usually characterized by a broad host range but a relatively low virulence. The second group involves true pathogens that rely on living plants to grow but can survive outside of their hosts. Most of these pathogens are highly virulent on only a limited number of host species. The third group contains obligate pathogens, for which living in plants is an absolute requirement to fulfill their complete life cycle. During infection, obligate fungal parasites engage in many sophisticated but poorly understood activities that redirect nutrient flow in plant tissues and alter the growth and morphology of the plant (Jackson

and Taylor, 1996). Based on relevance, the five most important fungal plant pathogens are *Magnaporthe oryzae*, *Botrytis cinerea*, *Puccinia* spp., *Fusarium graminearum,* and *Fusarium oxysporum* (Dean *et al*., 2012).

## 1.1 *FUSARIUM* SPECIES

The teleomorph of most *Fusarium* species belongs to the phylum *Ascomycota*, class *Ascomycetes*, order *Hypocreales*, genus *Gibberella*, while only a small number of *Fusarium* species have telomorphs in the *Hemanectria* and *Albonectria* genera (Leslie and Summerell, 2006). The *Fusarium* genus represents one of the most important groups in terms of fungal plant pathogens belonging to the *Ascomycota* phylum. The *Fusarium* genus is widely spread in nature and harbours both pathogenic and saprophytic species (Liddell, 1991). Leslie and Summerell (2006) reported that at least 80% of all cultivated plants are associated with one disease caused by a *Fusarium* species and reviewed all *Fusarium* species associated with plant diseases. *Fusarium* infection can occur at all of the plant developmental stages, from seed germination to mature vegetative tissues, depending on the *Fusarium* species involved and the host plant. Various *Fusarium* species can coexist in the same plant, causing diseases with a complex etiology and being able to produce secondary metabolites called mycotoxins (Logrieco *et al*., 2007). Early and precise identification of *Fusarium* spp. in every stage of infection is essential in predicting the potential toxicological risk to which the plants are exposed, other than preventing the formation of these metabolites, since most *Fusarium* species have specific mycotoxin profiles.

*Fusarium oxysporum* (*Fo*) is the most common species and includes 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). The ubiquitous soil-borne *Fo* causes vascular wilt diseases in a wide variety of economically important crops (Beckman, 1987). Vascular wilt has been a major limiting factor in the production of many agricultural and horticultural crops, including banana (*F. oxysporum* f. sp. *cubense*), cabbage (*F. oxysporum* f. sp. *conglutinans*), cotton (*F. oxysporum* f. sp. *vasinfectum*), flax (*F. oxysporum* f.sp *lini*), muskmelon (*F. oxysporum* f. sp. *melonis*), onion (*F. oxysporum* f. sp. *cepae*), pea (*F. oxysporum* f. sp. *pisi*), tomato (*F. oxysporum* f. sp. *lycopersici*), watermelon (*F. oxysporum* f. sp. *niveum*), china aster (*F. oxysporum* f.

sp. *callistephi*), carnation (*F. oxysporum* f. sp. *dianthi*), chrysanthemum (*F. oxysporum* f. sp. *chrysanthemi*), gladioli (*F. oxysporum* f. sp. *gladioli*) and tulip (*F. oxysporum* f. sp. *tulipae*) (Armstrong and Armstrong, 1981; MacHardy and Beckman, 1981).

## 1.2 BIOLOGY OF *FUSARIUM OXYSPORUM*

This species is provided with few morphological features useful for distinguishing the different species based on traditional methods, but with some experience and using the morphological characteristics (colony features, macroconidia, microconidia, chlamydospores, and other microscopic features), we can discriminate the most important pathogenic and toxigenic *Fusarium* species (Dongyou, 2009). Unfortunately, *F. oxysporum* changes its morphology and color depending on environmental conditions. The culture conditions affect the growth rate, shape, size, and abundance of conidia, as well as the number of septa and type of pigmentation (Booth, 1971). The mycelium appears first in white and then turns into a variety of colors that range from pink to dark purple. The species produces three different typologies of asexual spores: microconidia, macroconidia, and chlamydospores (Figure 1) (Agrios, 1997).

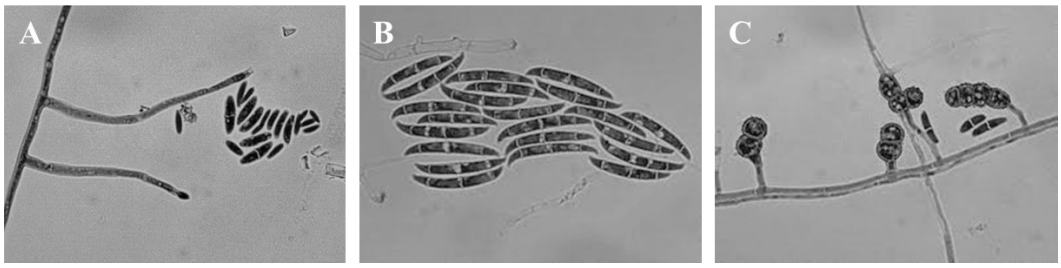


**Figure 1 - Different typologies of *F. oxysporum* conidia.**
(A) Microconidia; (B) Macroconidia; (C) Chlamydospores.

The septate macroconidia are produced in the aerial mycelium, particularly on mono and polyphialides, but often in specialised structures called sporodochia on short monophialides. The term "monophialide" refers to a conidiation cell with a unique pore from which the endoconidia are released, while a polyphialide can have multiple openings. Macroconidia of *Fusarium* species are sickle-shaped, with multiple septa, and contain three to five cells that are gradually pointed and curved toward the ends and resemble a banana or a canoe. They are commonly found on the surface of dead plants

killed by the pathogen. The other trait that is used is microconidia, single-cell dispersal structures that can vary in shape and size and are produced on the aerial mycelium, either in clumps or chains, both on monophialides and polyphialides, which are abundantly produced under most conditions. Finally, chlamydospores, the resistance structures with thick, high-lipid-content walls, can be formed on the middle or apex of the hyphae (Leslie and Summerell, 2006; Sen and Asan, 2009). Chlamydospores are generally developed through the modification of hyphal and conidial cells. Schippers and Van Eck (1981) proposed that chlamydospore formation depends on the nutrient status of the inoculum. Under field conditions, fungal inoculum may be subjected to much lower nutrient levels when compared to the "well-fed" macroconidia produced on rich agar media. Once carbohydrates are released from decaying plant tissue or from roots, chlamydospore germination is stimulated (Schippers and Van Eck, 1981). At high conidial densities, macroconidia do not germinate, but every conidium is converted into a chlamydospore. At low conidial densities, the conidia germinate but do not convert into chlamydospores (Schneider and Seaman, 1974). According to Griffin (1970), the inability of macroconidia to germinate at high conidial densities resulted from the presence of a self-inhibitor. Self-inhibitors are substances accumulating in the growth medium that suppress the germination of macroconidia at higher spore densities in the soil (Robinson and Park, 1966; Griffin, 1969; Robinson and Garett, 1969; Griffin, 1970). So, 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 periods of time in the soil during unfavorable periods with a saprophytic lifestyle (Kang *et al*., 2014).

Characteristic disease symptoms caused by *Fo* include vascular browning, leaf epinasty, stunting, progressive wilting, defoliation, and consequently plant death (Agrios, 2005). In addition to its remarkable plant pathogen activity, *Fo* can also cause a broad spectrum of diseases in both humans and mice, ranging from superficial or localized infections in healthy hosts to lethal fusarioses in immunocompromised individuals (Dignani and Anaissie, 2004; Nucci and Anaissie, 2007). In addition, an ecotoxicological study revealed the entomopathogenic activity of this fungus towards larvae of the greater wax moth *Galleria mellonella*, an invertebrate model host that is widely used for the study of microbial human pathogens (Navarro-Velasco *et al*., 2011). Thanks to all these

characteristics, *Fo* is considered a multi-host fungal pathogen and an excellent model system to study fungal pathogenicity mechanisms both in plants and mammals (Ortoneda *et al*., 2004).

## 1.3 CONIDIA-GERMINATION OF *F. OXYSPORUM*

Considering the significance of asexual spores in the success of *Fo* infection, it is of crucial importance to understand how *Fo* regulates conidia germination. The germination of spores is the preliminary step in fungal development, leading to the conversion of a dormant phase into a growing phase. It involves the breaking of dormancy by external signals such as chemical signals released from decaying plant tissues or from plant roots, whose composition is affected by the age of plants. Recently, Vitale and coworkers (2019), have reported that the autocrine pheromone signaling plays an important role in the breaking of dormancy of *Fo* conidia. In fact, the interaction between α-pheromone-Ste2 leads to repression of conidial germination while the a-pheromone-Ste3 interaction relieves germination repression. Dormancy break is followed by a pre-germination phase first and a germ-tube formation (GT) phase then, which 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 tubes. Generally, *Fo* presents 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 host colonization, a fundamental step for infection success (Ruiz-Roldán *et al*., 2010). Further, it has been demonstrated that the germ tubes of *Fo* stop their normal Brownian movements and rapidly re-orient their polarity axis towards closely located germ tubes from genetically identical *Fo* germlings or towards roots of host plants, chemotropic responses respectively leading to the generation of vegetative hyphal fusion (VHF) events or to host tissue adhesion, processes that contribute to the fungal development and invasive growth into the host (Prados-Rosales and Di Pietro, 2008; Turrà *et al.*, 2015).

## 1.4 VEGETATIVE HYPHAL FUSION IN *F. OXYSPORUM*

Vegetative hyphal fusion, also termed anastomosis, is a well-regulated process that determines the formation of a complex hyphal network and, in some conditions of growth, leads to the production of visible macroscopic fungal aggregates. After germination, during the colony initiation process, spores and germlings generate distinct cell protrusions termed conidial anastomosis tubes (CATs), cytoplasmatic interconnections between genetically identical or incompatible species. Germinating conidia of *Fo* frequently change the original axis of growth before fusing with each other through the formation of CATs, which differ from germ tubes (GTs) in 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, or side-to-side fusion of CATs formed from adjacent GTs (Kurian *et al*., 2018) (Figure 2).

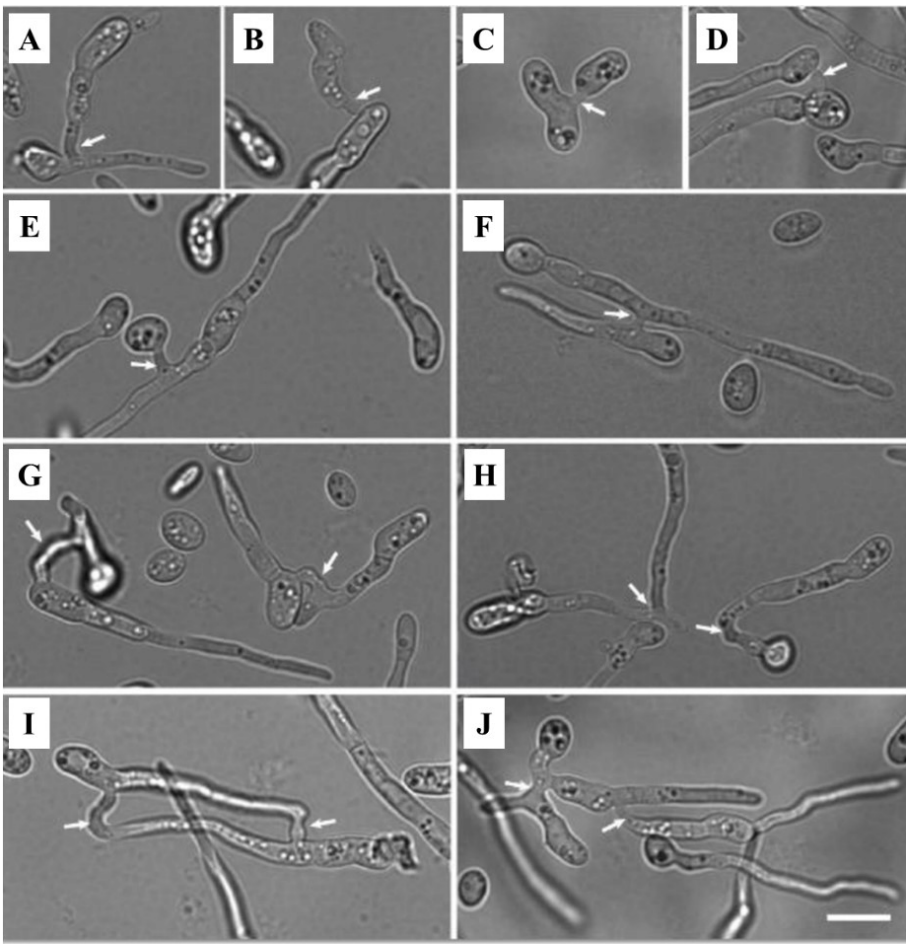


**Figure 2 - Different types of CAT fusions observed between *Fo* germlings.**

(A, F, H and I) CAT fusion between two GTs. (C) CAT fusion between two conidia that have not produced GTs. (D) CAT fusion between two conidia that have already produced long GTs. (B, E, G) CAT fusion between a GT and a conidium. CAT fusion resulted from tip-to-tip fusion of CATs formed from growing germ tubes (H), tip-to-side fusion of a CAT formed from a growing germ tube to a spore (A, B and I), and side-to-side fusion of CATs formed from adjacent germ tubes (F, G) (Kurian *et al*., 2018).

CAT fusion and its underlying signaling mechanisms have been extensively studied in *Neurospora crassa*. First, conidial anastomosis tubes emerge from conidia or germlings and exhibit chemotropic growth towards each other. Then, CAT tips make contact with the fusing partner, cell walls and plasma membranes dissolve and finally nuclei and organelles freely move in between fused partners. 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 pathogens that lack a known sexual stage, such as *Fo*, in which the occurrence of VHF represents a possible way to ensure variability through the transfer of genetic material (Prados-Rosales and Di Pietro, 2008; Ruiz-Roldán *et al*., 2010; Kurian *et al*., 2018).

## 1.5 LIFE CYCLE OF *F. OXYSPORUM*

Ascospores, hyphal fragments, macroconidia, or chlamydospores usually found in plant debris are considered the primary source of inoculum (Goswami and Kistler, 2004). Indeed, Shaner (2003) reported that the saprophytic survival of the pathogen in organic residues could transfer inoculum from one season to another, facilitating the capability of some *Fusarium* species to produce specific spore types (*i.e.* chlamydospores) able to survive for long periods of time in soil. Chlamydospores remain dormant and immobile in the remains of decayed plant tissues until stimulated to germinate after perception of nutrients that are released from the extending roots of a variety of plants (Beckman and Roberts, 1995). Following germination, a thallus is produced, from which conidia form in 6–8 hours and chlamydospores in 2-3 days if conditions are favorable. Hyphal chemotropism and invasion of the host roots is followed by the penetration of the epidermal root cells (Beckman and Roberts, 1995) 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, fungal hyphae grow inter- and intra-cellularly and

reach the xylem vessels that are used as a conduit 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 defense response based on a gene-for-gene recognition mechanism 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, which, combined with the increase in fungal burden, block water streaming through the vessels, leading to progressive wilting and death of the plant (Rep *et al*., 2004; Michielse and Rep, 2009). On the infected tissues and in the surrounding soil, all of the conidial forms usually produced by *Fo* develop and accumulate, concluding the fungal biological cycle (Figure 3).

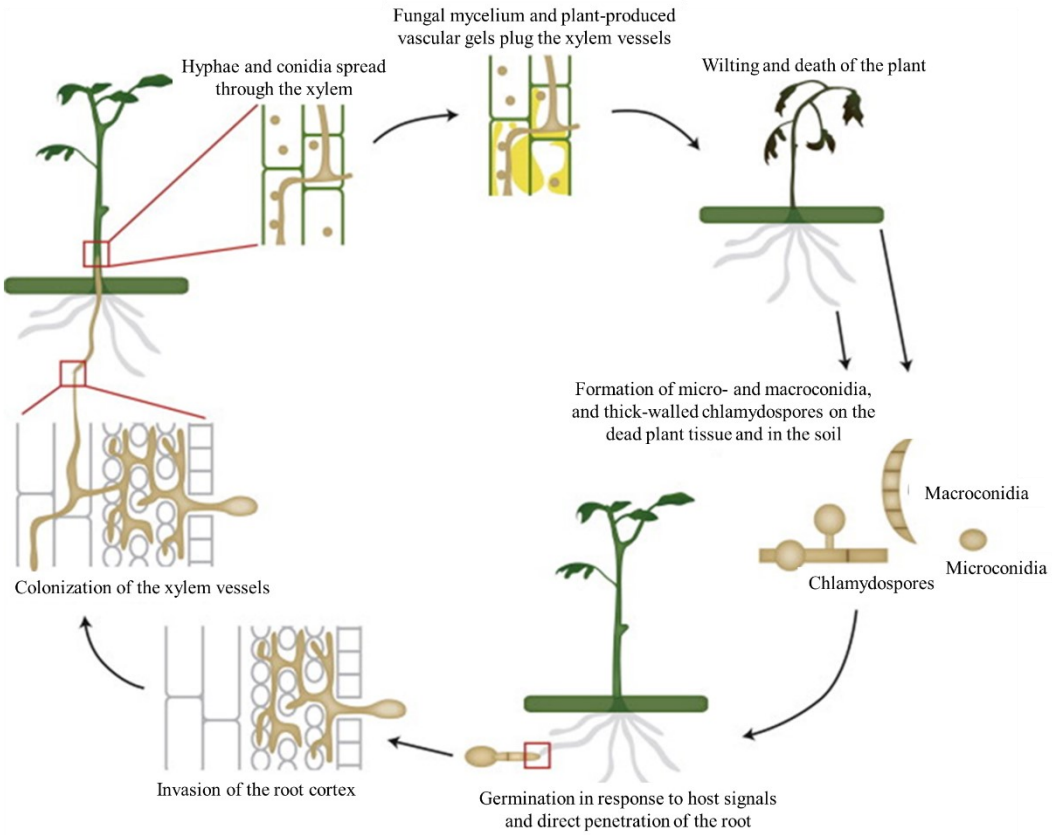


**Figure 3 - Life cycle of *Fusarium oxysporum*.**
(A) Germination in response to host signals and direct penetration of the root. (B) Invasion of the root cortex. (C) Colonization of the xylem vessels. (D) Hyphae and conidia spread through the xylem. (E) Fungal mycelium and plant produced vascular gels 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).

## 1.6 *F.* OXYSPORUM WILT: DISEASE DEVELOPMENT AND SYMPTOMS

*Fo* wilt is a warm-weather soilborne fungal disease that does optimally at temperatures of about 28 ºC. Temperatures above 34 ºC or below 17 ºC retard the disease's development. During the initial stages of the interaction, fungal pathogens must sense stimuli from the plant and respond with appropriate morphogenic and biochemical changes (Roncero *et al*., 2003). The signalling process represents the first and most critical step in defining the outcome of a fungal infection (Roncero *et al*., 2003). Griffin (1969) reported that soil-borne fungal pathogens, including *F. oxysporum*, are able to sense the presence of the plant even before physical contact, most likely through compounds present in root exudates. After the recognition of host, the fungal pathogen penetrating the plant roots and colonizing the vascular tissues (Bishop and Cooper, 1983a). To gain entrance into plant cells, fungi generally secrete a mixture of hydrolytic enzymes, including cutinases, cellulases, pectinases, and proteases. After penetration, the fungus often secretes toxins or plant hormone-like compounds that manipulate the plants' physiology to the benefit of the pathogen (Knogge, 1996).

*Fusarium* wilt becomes evident once the fungal pathogen has colonized and spread to various parts of the plant, causing blockage of the vessels as a result of mycelia growth. Vascular vessel blockage and nutrient absorption lead to acute disease development and symptoms. Effects caused by pathogens on plants are almost entirely the result of biochemical reactions taking place between substances secreted by the pathogen and those present in or produced by the plant. To gain entrance into plant cells, fungi generally secrete a mixture of hydrolytic enzymes, including cutinases, cellulases, pectinases, and proteases. After penetration, the fungus often secretes toxins or plant hormone-like compounds that manipulate the plants' physiology to the benefit of the pathogen (Knogge, 1996). *Fusarium* disease occurs in two forms, also called syndromes. The first form of the disease manifestation is acute wilt, a situation where the plants dry out very quickly and die while attached to the original position, while the second form of the syndrome is chronic wilt, where the plants live together with the pathogen for a longer

period of time, but remains stunted (Flood, 2006). This pathogen can affect plant physiology at any stage, and some of the symptoms that are exhibited include root rots and damping off in young plants, marginal yellowing, and sometimes full chlorosis on the leaves of mature plants (Zitter, 1998). Importantly, internal discoloration of the vascular bundles is the main symptom caused by *Fo* and that is also used as a diagnostic measure for *Fusarium* wilt.

## 1.7 *F. OXYSPORUM*-PLANT INTERACTION

*Fusarium oxysporum* is a parasitic pathogen that depends on plant tissues for nutrient supply and thus has developed specific strategies that rely on host penetration for its survival. Some of the penetration mechanisms described as being used by fungal pathogens include structural morphology adjustment, for instance, during hyphae development, or physiological changes that involve the production of specific sets of plant tissue degrading enzymes.

Vascular infection by *Fo* requires a series of highly regulated processes which are listed below in order:

- **Chemotropic growth**: Chemotropic growth is represented by the ability of fungal hyphae to redirect their growth axis towards a source of a chemical compound or an appropriate host. It thus represents one of the first processes that occur during fungal-plant interactions. Turrà *et al.,* (2015) demonstrated that *Fo* hyphae are able to redirect their growth to find food sources, an adequate mating partner or a proper host to infect. The latter process is triggered by the catalytic activity of root secreted class III peroxidases, a family of haem-containing enzymes present in all land plants.

- **Adhesion**: Fungal infection commences when infectious hyphae adhere to the host root surface (Bishop and Cooper, 1983a). Adhesion of fungi to the host surface is not a specific process, as they can adhere to the surfaces of both hosts and non-hosts plants (Vidhyasekaran, 1997). Site-specific binding may be important in anchoring the propagules at the root surface, after which other

processes required for host colonization can start (Recorbet and Alabouvette, 1997).

- **Penetration**: Penetration is likely to be controlled by a combination of different factors that include fungal compounds, plant surface structures, activators or inhibitors of fungal spore germination, and germ tube formation (Mendgen *et al*., 1996). The means by which wilt pathogens penetrate roots may differ, but there are two distinct types. Some pathogenic forms penetrate roots directly, whereas others must enter indirectly through wounds (Lucas, 1998). The most common sites of direct penetration are located at or near the root tips of both taproots and lateral roots (Lucas, 1998). The pathogen enters the apical region of the root, where the endodermis is not fully differentiated, and fungi are able to grow through and reach the developing protoxylem.

- **Colonization**: During the colonization phase, the mycelium advances intercellularly through the root cortex until it reaches the xylem vessels and enters them through the pits. The fungus then remains exclusively within the xylem vessels, using them to colonize the host (Bishop and Cooper, 1983b). Fungal colonization of the host's vascular system is often rapid and frequently facilitated by the formation of microconidia within the xylem vessel elements (Beckman *et al*., 1961) that are liberated and carried upward in the sap stream (Bishop and Cooper, 1983b). Mycelium growth in the vascular system leads to blockage of the vessels, resulting in water stress and the development of wilt symptoms. The process is accelerated when the plants try to react by producing defense factors such as tyloses, gels, and proliferation of the parenchyma cells, resulting in further blockage of the vessels (Ortiz *et al*., 2014).

## 1.8 GENETIC BASIS OF HOST SPECIFICITY IN *F. OXYSPORUM*

*Fusarium oxysporum* represents the most pervasive, anamorphic, and polytypic soil-borne pathogen (O'Donnell *et al*., 1998), capable of infecting more than 150 plant species. The host range of *Fo* varies from vegetables, flowers, field crops to plantation crops (Di Pietro *et al*., 2003; Rana *et al*., 2017; Edel-Hermann and Lecomte, 2019). Despite showing a broad host range, strains of *Fo* are highly host specific and are genetically and

morphologically distinct (Mandeel *et al*., 2005; Leslie and Summerell, 2008; Palmero *et al*., 2009; Edel-Hermann and Lecomte, 2019). Together, these host-specific forms constitute a consortium referred to as *F. oxysporum* species complex (FOSC) (Edel-Hermann and Lecomte, 2019). The host range and specificity of *Fo* are dictated by genes located on pathogenicity-associated genomic regions (Rep and Kistler, 2010; Ma *et al*., 2010; Ma *et al*., 2015; Williams *et al*., 2016). These pathogenicity-associated genes encode effector proteins, transcription factors (TFs), secreted enzymes, and proteins involved in secondary metabolism and signal transduction (Rep *et al*., 2002; Rep *et al*., 2004; Houterman *et al*., 2007; Van Der Does *et al*., 2008; Ma *et al*., 2010; Schmidt *et al*., 2013). A gene-for-gene relationship has been proposed for the interaction between *F. oxysporum* races and host cultivars since monogenic, dominant resistant traits against known races have been described for *F. oxysporum* f.sp. *lycopersici* (Roncero *et al*., 2003). In tomato, resistance gene I-2 confers resistance to *F. oxysporum* f.sp. *lycopersici* race 2 by expressing the avirulence gene avrI-2 (Ori *et al*., 1997; Mesterhazy *et al*., 1999). The gene-for-gene theory, however, does not include all *formae speciales* of *Fo*. For example, in the banana wilt pathogen, the grouping of *F. oxysporum* f.sp. *cubense* isolates into races is determined by their pathogenicity to a limited number of banana varieties in field conditions (Moore *et al*., 1993).

Since there is no known sexual stage in the life cycle of *Fo*, the most likely hypothesis is that the horizontal transfer of genetic information between isolates could generate the emergence of new pathogenic lineages. In fact, supernumerary chromosomes have been identified in *Fo* and are thought to have been acquired by genetic horizontal transfer through vegetative hyphal fusion (VHF) events leading to heterokaryon formation, and possibly the emergence of new pathogenic lineages. Indeed, genome sequence analysis of tomato pathogenic isolates of *F. oxysporum* f. sp. *lycopersici* (*Fol*) demonstrated the existence of 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). In both *F. oxysporum* and other pathogenic fungi some LS chromosomes carry effector genes associated with pathogenicity (Croll and McDonald, 2012; Raffaele and Kamoun, 2012). Effectors secreted by the pathogen facilitate its colonization by modulating immune responses in the host plant (Hogenhout *et al*., 2009). Secreted in Xylem (Six) proteins are one such example of effectors (Rep *et al*., 2002; Rep *et al*., 2004), two of them are mostly produced in xylem, and they are encoded by the

genes SIX1 and SIX2, which are positioned within 8 kb of each other and localized on one of the smallest chromosomes (Rep *et al*., 2004; Boix-Ruíz *et al*., 2015). The SIX1 product is a small protein rich in cysteine that has been revealed to be essential for *Fol* virulence (Selim *et al*., 2015). In another study, up to eight fungal proteins from the xylem sap of diseased plants were identified, and the genetic material encoding for these proteins was found to be is clustered in a similar region of the chromosome (Sasaki *et al*., 2015; Maldonado *et al*., 2018). SIX1, SIX2, SIX3, and SHH1 were unique to *Fol* isolates. Despite their polyphyletic origin, all the *Fol* isolates have a genomic region containing at least 8 kb of identical genes comprising SIX1, SIX2, and SHH1 that was lacking in other non-pathogenic isolates and *formae speciales*. This genomic region initially existed in ancestral *Fo* and subsequently vanished in all clonal lines except *Fol* (Jelinski *et al*., 2017; Maldonado *et al*., 2018; Debbi *et al*., 2018).

## 1.9 VIRULENCE MECHANISMS IN *F. OXYSPORUM*

During the initial stages of the interaction, fungal pathogens must sense stimuli from the plant and respond with appropriate morphogenic and biochemical changes (Roncero *et al*., 2003). The signalling process represents the first and most critical step in defining the outcome of fungal infection (Roncero *et al*., 2003). Effects caused by pathogens on plants are almost entirely the result of biochemical reactions taking place between substances secreted by the pathogen and those present in or produced by the plant. To gain entrance to plant cells, fungi generally secrete a mixture of hydrolytic enzymes including cutinases, cellulases, pectinases and proteases. After penetration, the fungus often secretes toxins or plant hormone-like compounds that manipulate the plants' physiology to the benefit of the pathogen (Knogge, 1996).

### 1.9.1 The role of signal transduction in pathogenesis

Studies from a wide range of fungi have converged to define two conserved signal transduction cascades regulating fungal development and virulence: a cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) signalling system (cAMPPKA) cascade, and a mitogen-activated protein kinase (MAPK) cascade (Lengeler *et al*., 2000). Using forward and reverse genetic approaches, several processes and genes have been identified or implicated as being important for pathogenesis. MAPK (FMK1) and G-

protein subunits α (FGA1) and β (FGB1) are required for (full) pathogenicity (reviewed in Di Pietro *et al*., 2003). More recently, a second gene encoding a G-protein α subunit (FGA2) has been identified and analyzed. In contrast with the reduced pathogenicity of *F. oxysporum* f. sp. *cucumerinum* FGA1 mutants, deletion of FGA2 led to a complete loss of pathogenicity on cucumber plants (Jain *et al*., 2005). In addition, the altered colony morphology and conidiation observed in FGA1 disruptants were not observed in FGA2 disruptants. These observations indicate that Fga1 and Fga2 have distinct functions, each of which is required for adaptation to the plant environment. The role of signaling pathways in vegetative hyphal fusion and the role of VHF in pathogenesis have recently been investigated (Rosales and Di Pietro, 2008). Although it was shown that VHF and pathogenesis share common signaling components, VHF is not required for pathogenesis, as a mutant disrupted for FSO1 – a gene required for efficient VHF—was fully virulent on tomato plants.

### 1.9.2 The genetics of *F. oxysporum* virulence

Soil-borne phytopathogenic fungi have appropriate signaling mechanisms that enable them to respond to external stimuli through variations in gene expression, leading to host recognition, root penetration, proliferation within host tissues or for overcoming the host defense mechanisms (Rep and Kistler, 2010). Fungal growth and virulence are mainly governed by two type of signal transduction pathways, namely mitogen-activated protein kinase cascades (MAPKs) and cyclic adenosine monophosphate (cAMP) (Liu *et al*., 2016). In *Fo*, mutations inactivating the gene encoding the Fmk1 mitogen-activated protein kinase made the pathogen incapable of penetrating tomato roots and cause disease symptoms on plants. Furthermore, use of fluorescence microscopy showed that they were unable to adhere to the root surface in contrast to the wild-type strain that was capable of firmly anchoring and piercing the root surface. Interestingly, pectate lyase and polygalacturonase enzymes, which are involved in plant cell wall degradation, were also downregulated in the mutant strain (Di Pietro *et al*., 2001; Guo *et al*., 2016; Pareek and Rajam, 2017). Cellular processes involving lipid metabolism and amino acid metabolism, protein translocation, cell wall integrity, and degradation of proteins seem to be crucial for the pathogenicity of *Fo* based on the functional categorization of its pathogenicity genes. For example, genes encoding for the developmental regulator *flbA*, the phosphomannose isomerase *Focpmi1* and the chitin synthase V *chsV* were identified to

play important roles in the virulence of *Fo*. In addition, gene knockout and complementation studies established that other proteins involved in cell wall integrity, such as the glycosylphosphatidylinositol-anchored protein ECM33, proteins involved in peroxisome biogenesis, and the transcriptional regulator CTI6, to be essential for *Fo* pathogenicity.

| **Gene** | **Product/function** | **Effect of gene inactivation/deletion** | **Reference** |
|---|---|---|---|
| *ARG1* | Argininosuccinate lyase | Strongly reduced virulence, arginine auxotrophy | Namiki *et al*., (2001) |
| *CTF1* | Transcription factor | Fully virulent | Rocha *et al*., (2008) |
| *DCW1* | Cell wall protein | Reduced virulence | Michielse *et al*., (2009) |
| *ECM33* | GPI-anchored protein | Regulates vegetative growth and virulence | Huang *et al*., (2022) |
| *FMK1* | Mitogen-activated protein kinase | Non-pathogenic, impaired in root attachment and invasive growth | Di Pietro *et al*., (2001a) |
| *CNA1/CNB1* | Calcium/calmodulin-dependent protein phosphatase | Regulates Conidiation, Chlamydospore Formation and Virulence | Hou *et al*., (2020) |
| *GAS1* | β-1,3 Glucanosyltransferase | Markedly reduced virulence, reduced growth on solid medium | Caracuel *et al*., (2005) |
| *STI35* | Functions in thiamine biosynthesis | Fully virulent | Ruiz-Roldan *et al*., (2008a) |
| *GCS* | Glucosylceramide synthase | Impairs the growth and virulence | Wang *et al*., (2022) |
| *YjeF* | Function in cellular metabolism damage-repair | Influences the sporulation and virulence | Wei *et al*., (2023) |

| | | | |
|---|---|---|---|
| *CTI6* | Transcription factor | Reduced virulence | Michielse *et al*., (2009) |
| *CHSV* | Class V chitin synthase | Strongly reduced virulence, hypersensitive to α-tomatine and $H_2O_2$ | Madrid *et al*., (2003) |
| *PMI1* | Phosphomannose Isomerase | Perturbs Cell Wall Integrity and reduces Virulence | Usman *et al*., (2023) |

**Table 1 - Gene knockouts in *Fusarium oxysporum* and their phenotypic effects.**

### 1.9.3 Cell Wall-Degrading Enzymes (CWDES)

*Fusarium oxysporum* produces several enzymes that act upon plant cell wall components (Agrios, 1997).

- **Pectinases**: These enzymes catalyze the degradation of pectic polysaccharides, the main component of the middle lamella, that is, the intercellular cement that holds in place the plant tissues (Rombouts and Pilnik, 1980). Pectic enzymes consist primarily of pectin methylesterase, polygalacturonase, and pectate lyase (Kawano *et al*., 1999; Verlent *et al*., 2004).

- **Pectin methylesterases**: The capacity to produce pectin methylesterase (PME) is a general feature among the vascular wilt *Fusarium* species (MacHardy and Beckman, 1981). PME is a pectin-degrading enzyme, and its enzymatic reaction results in partially demethoxylated pectin chains and methanol (Verlent *et al*., 2004). According to Deese and Stahman (1962), pectic depolymerase coupled with PME are important biochemical factors involved in producing the symptoms of *Fusarium* wilt in tomatoes.

- **Polygalacturonases**: Polygalacturonases (PGs) are important pectolytic enzymes produced by phytopathogenic fungi during the process of infection and colonization of host plants (Posada *et al*., 2001). PGs (poly[1,4-α-D-galacturonide] glucanhydrolases) are the first pectinases produced by pathogens

when cultured on isolated plant cell walls or during infection (Jones *et al*., 1972; Di Pietro and Roncero, 1998). According to Baayen *et al*. (1997), the development of wilt symptoms in inoculated carnations was accompanied by a quadratic increase in PG activity. At the same time, polygalacturonase activity is not closely related to virulence in *Fusarium*, in fact, the targeted inactivation of Pg1 and pg5 encoding endo-PG, and pgx4 encoding exo-PG, identified in *F. oxysporum* f.sp. *lycopersici*, had no effect on virulence (Di Pietro and Roncero, 1996; Di Pietro and Roncero, 1998; García-Maceira *et al*., 2000).

- **Pectate lyases**: Pectate lyases (PLs) catalyse the trans-elimination of pectate and have been suggested to play an important role in the development of vascular wilt (Linhardt *et al*., 1986; Beckman, 1987). A specific PL (designated PL1) has been purified and characterized from *F. oxysporum* f.sp. *lycopersici* and was found in infected tomato root and stem tissue (Di Pietro and Roncero, 1996b). However, according to Di Pietro and coworkers (2003), knockout of the encoding gene *pl1* had no effect on the virulence of the pathogen on tomato plants. The same result was obtained in the case of *F. solani* f.sp. *pisi*, where the inactivation of genes encoding for $PL_s$ had no detectable decrease in virulence (Rogers *et al*., 2000).

- **Cellulases**: Since cellulose could serve as a carbohydrate source for *F. oxysporum* following partial decomposition by cellulase enzymes, several studies have explored a possible role for cellulase in wilt pathogenesis. Cellulase enzymes, designated C1, C2 and Cx, are required to degrade cellulose. The C1 and C2 enzymes act upon native, insoluble cellulose to produce linear chains that are attacked by the Cx enzyme to produce cellobiose and glucose (MacHardy and Beckman, 1981). According to Husain and Dimond (1960), *Fusarium* produces both the Cx and C1 enzymes. No cellulase activity was detected in diseased stems of tomato plants during experiments performed by Matta and Dimond (1963). Husain and Dimond (1960) reported that the action of cellulase produced by the tomato wilt pathogen *F. oxysporum* f.sp. *lycopersici* may be conceived to act in pathogenesis in three ways. Firstly, it is involved in wilt induction. Secondly, hydrolytic products of cellulase activity may provide *Fusarium* with carbohydrates for its continued development in the host, and thirdly, it is involved

in the escape of the pathogen from vascular tissue in advanced stages of disease when the host is in a dying condition.

- **Hemicellulases**: Few studies have explored the involvement of hemicellulases in *Fusarium* wilt pathogenesis. These enzymes, however, could be of greater significance than pectinases and cellulase enzymes in that the greater part of the amorphous matrix in which cellulase microfibrils are embedded is composed of hemicellulose-type materials, such as glucans, xylans, and mannans (Zaroogian and Beckman, 1968; MacHardy and Beckman, 1981). No correlation was apparent between resistance to *F*. *oxysporum* f.sp. *cubense* and the amount of hemicellulose A in all varieties, but the resistant varieties had significantly more hemicellulose B than susceptible varieties (Zaroogian and Beckman, 1968). Several hemicellulases seem to be produced by many plant pathogenic fungi. Depending on the monomer released from the polymer on which they act, the enzymes are called xylanase, galactanase, glucanase, arabinase, mannase, and so on (Agrios, 1997).

### 1.9.4 Mycotoxins from *F. oxysporum*

As a typical vascular wilt fungus, *F. oxysporum* produces the characteristic xylem vessel clogging and the wilting of infected plants. Colonization and clogging of vessels in addition to secretion of several toxins by the fungus includes fusaric acid, lycomarasmin, dehydrofusaric acid, etc., play a major role in wilt symptoms development and progression. At least 11 species of *Fusarium*, such as plant pathogenic *F. oxysporum* produce the mycotoxin fumonisins (Desjardins, 2006). The toxigenic potential of *F. oxysporum* on plants and additional commodities and the extensive variety and frequent presence of fuminosin toxins has developed a major constraint in main food crops. Fusaric acid is another potential toxin produced by *F. oxysporum* including *Fol*. The production of fusaric acid has been related to the virulent strains of *Fusarium spp*. (Selim and Gammal, 2015). Fusaric acid is a potent toxin natural contaminating infected plants and cereals causing typical wilt symptoms in plants. Singh and coworkers, (2017) characterized the phytotoxic effects of fusaric acid in tomato leaves which revealed reduced photosynthesis, leaf wilting and necrosis, enormous lipid peroxidation and intracellular reactive oxygen species and cell death. In an attempt to demonstrate the role

of fusaric acid, Lopez-Diaz and coworkers (2018), found the gene fub1 was vital for the synthesis of the toxin and its derivatives in *Fo*. Targeted deletion of fub1 and consequently loss of fusaric acid production in *F. oxysporum* led to reduced severity of wilt symptoms and virulence in hosts.

## 1.10 *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* CAUSAL AGENT OF VASCULAR WILT DISEASE OF TOMATO PLANTS

The species of *Fo* cause wilt disease in more than 150 hosts and range with specific *formae speciales* (Bertoldo *et al*., 2015). Asha and coworkers, (2011) and Nirmaladevi and coworkers, (2016) reported *Fusarium oxysporum* Schlectend. Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen (*Fol*) cause vascular wilt of tomato disease and reduce the yield to the maximum extent (Asha *et al*., 2011). The family Solanaceae, which includes more than 3000 species, among them the cultivated tomato, is the only vegetable crop cultivated throughout the world. This crop is a vital component of daily food and is consumed as unprocessed fresh fruits as well as in various types of processed products (Brookie *et al*., 2018). Tomato wilt is one of the chief diseases caused by *Fol* (Borisade *et al*., 2017). The *Fol* enters the epidermis of the root, later spreads through the vascular tissue, and inhabits the plant xylem vessels, resulting in vessel clogging and severe water stress, as a result of which wilt-like symptoms appear (Singh *et al*., 2017). The disease is morphologically identified by wilted plants bearing yellow-colored leaves with minimal or absent crop yield. The progression of plant vascular infection by *Fol* is a complex phenomenon, and the sequential steps involved in the infection process are as follows: (I) root recognition through host-pathogen signals; (II) attachment to the surface of root hairs and hyphal propagation; (III) invasion of the root cortex and vascular tissue and differentiation within xylem vessels; and (IV) finally oozing of toxins and virulence factors.

### 1.10.1 Epidemiology

The overall distribution of *Fol* is known to be cosmopolitan and occurs predominantly as a soil saprophyte, which stands out amongst the most widely recognized and predominant fungi of cultivated soils. However, the different *formae speciales* of *Fo* often have varying degrees of distribution. This disease affects the tomato grown at a warm temperature (28

ºC) both in greenhouses and in the field (Bawa, 2016; Debbi *et al*., 2018). The disease is characterized by 70 to 60% fruit yield loss and wilted plants characterized by yellowed leaves (Ravindra *et al*., 2015). The three known F*ol* races (Races 1, 2, and 3) pathogens of tomato cultivars are distinguishable by their principal resistance genes. Most commercial tomato varieties grown across the world are resistant to races 1 and 2, and a few are resistant to race 3 (Biju *et al*., 2017). *Fol* spreads over short distances mainly through irrigation water and contaminated farm equipment, and it can spread over long distances through infected transplants, soils, etc. (Agrios, 2005). Certainly, once a region becomes contaminated with *Fol*, the fungus usually remains indefinitely (Animashaun *et al*., 2017; Prihatna *et al*., 2018).

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