Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Engenharia Agronómica

Dissertação

The role of arbuscular mycorrhiza in the biological control of Fusarium oxysporum in tomato

José Tomás Madeira Pinto

Orientador(es) | Isabel Brito

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

Presidente	|	Vasco Fitas da Cruz (Universidade de Évora)
Vogais	|	Ana Alexandre (Universidade de Évora) (Arguente) Isabel Brito (Universidade de Évora) (Orientador)

Évora 2020

Acknowledgements

I would like to express my sincere gratitude to those that accompanied and guided me throughout this master's degree journey.

Most deserving acknowledgment are my dissertation supervisors: Prof. Isabel Brito, for her responsible, reliable and compassionate support, whose knowledge on the subject not only made this work possible but also a great privilege; and Prof. Maria do Rosário Félix, whose incredible encouragement and passion for plant pathology inspired my own interest in the subject, leading me up to this step in education. Both teachers of excellence, that make me proud and grateful to have attended the University of Évora.

My gratitude to Prof. Mário de Carvalho, for helping take the first steps in the dissertation, revealing the possible subject and work paths, and connecting me to my supervisors.

To the teams working in the Laboratories of Soil Microbiology and Plant Virology, for their friendly support and guidance throughout the entire lab work, highlighting the kind help of Doroteia Campos, who assisted in the qPCR assay and revised its written methodology. My special thanks to the technicians, Filipa Santos and Manuel Figo, who helped to set up and maintain the pot experiment.

Lastly, I would like to express gratitude to my parents, whose hard work and sacrifice that enabled my education I hope to one day match and honour with pride. Praise to my dear friend, Sofia Almeida, for always supporting me through hardships and for helping me better my writing.

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List of abbreviations

AC - Arbuscular Colonization

AM - Arbuscular Mycorrhiza

AMF - Arbuscular Mycorrhizal Fungi

Ct - Cycle Threshold

CTAB - Cetyl Trimethyl Ammonium Bromide

DI - Disease Incidence

DNA - Deoxyribonucleic acid

DW - Dry Weight

EDTA - Ethylenediaminetetraacetic acid

ERM - Extraradical mycelium

EUFIC - European Food Information Council

f. sp - *Forma specialis,* meaning “special form”.

FAO - Food and Agriculture Organization

FOL - *Fusarium oxysporum* f. sp. *lycopersici*

FORL - *Fusarium oxysporum* f. sp. r*adicis-lycopersici*

HC - Hyphal Colonization

INE - Instituto Nacional de Estatística

MED - Mediterranean Institute for Agriculture, Environment and Development

qPCR - Quantitative Polymerase Chain Reaction

RH - Root hair

TSWV - tomato spotted wilt virus

TYLCV - Tomato yellow leaf curl virus

UNEP - United Nations Environment Programme

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The role of arbuscular mycorrhiza in the biological control of *Fusarium oxysporum* in tomato

Abstract

The functional diversity amongst arbuscular mycorrhizal fungi (AMF) is scientifically recognized, yet not practically exploited as the understanding of the interactions between different crops and their rhizosphere microbiome is still very narrow.

Following the strategy developed by the team that guided this dissertation, this work was aimed to test different plants (*Lolium rigidum* and *Ornithopus compressus*) as hosts to develop AMF extraradical mycelium (ERM) for an early colonization of tomato plants and to evaluate their role in the bioprotection against *Fusarium oxysporum* f. sp *radicis-lycopersici*.

Although *L. rigidum* promoted a greater AM colonization of tomato plants, *O. compressus* was most beneficial, resulting in a reduced disease incidence and a higher dry weight in the tomato plants. The disease incidence was also lower in undisturbed soil (intact ERM) conditions, confirming the importance of an early AM colonization, accomplished through an intact ERM, in granting bioprotection to the tomato plants.

Keywords: Arbuscular mycorrhizal fungi, *Fusarium oxysporum* f. sp *radicis-lycopersici*, Bioprotection, Extraradical mycelium

O papel das micorrizas arbusculares no controlo biológico de *Fusarium oxysporum* em tomate

Resumo

Apesar da diversidade funcional entre fungos micorrízicos arbusculares (AMF) estar cientificamente descrita, o conhecimento do microbioma rizosférico das culturas é ainda bastante limitado e a sua utilização em termos práticos está ainda longe de ser uma realidade.

Seguindo a estratégia desenvolvida pela equipa que acolheu esta dissertação, este trabalho visou testar plantas (*Lolium rigidum* e *Ornithopus compressus*) como hospedeiros para desenvolver micélio extraradicular (ERM) de AMF, para obter uma colonização precoce nos tomateiros e avaliar o seu papel na bioprotecção contra *Fusarium oxysporum* f. sp. *radicis-lycopersici*.

Apesar do *L. rigidum* ter promovido uma colonização micorrízica superior nos tomateiros, o *O. compressus* foi mais benéfico, resultando numa redução de incidência da doença e num aumento do peso seco dos tomateiros. A incidência da doença foi menor em condições de solo não perturbado (ERM intacto), confirmando a importância da colonização micorrízica precoce, obtida a partir de ERM intacto, no efeito bioprotector a tomateiros.

Palavras-chave: Fungos micorrízicos arbusculares, *Fusarsium oxysporum* f. sp *radicis-lycopersici*, Bioprotecção, Micélio extrarradicular

Chapter 1

Literature review

1.1 Tomato origin and production worldwide

The tomato (*Lycopersicon esculentum* M.), belonging to the *Solanaceae* family, is one of the most important vegetable crops in the world, achieving worldwide popularity over the last century. Although the species is native to the lower Andes, between Ecuador and Chile, tomatoes were domesticated and widely cultivated by the Aztecs in ancient Mexico. In the 16th century they were introduced to Europe along with potatoes, maize and chili peppers by the Spanish conquerors. Initially regarded as poisonous and dangerous for consumption in Europe, tomatoes were eventually accepted as edible in Italy, where they still thrive in traditional cooking (EUFIC, 2001). In the first decades of the 20th century the processing industries were further developed, resulting in the worldwide expansion of the tomato crop. Nowadays, tomatoes are grown in a variety of climates all across the world in outdoor fields, greenhouses and nethouses.

Tomatoes are marketed in two distinct categories: Fresh market tomatoes, that are usually grown in a controlled environment for direct consumption, and can be eaten raw or cooked; and processing tomatoes, which are grown outdoors and mechanically harvested for the paste and canning industry. The concentrated paste is the main industry product and a common ingredient in sauces and ketchup, juices and soups. Tomatoes are considered a healthy food, being rich in provitamin A, vitamin C and the carotenoid Lycopene, responsible for its red color, and antioxidant properties.

On a global scale, the annual production of tomatoes amounts to approximately 180 million tons (FAO, 2018) and about a quarter of the total production is grown for the processing industry, making it the world's most processed vegetable. In Portugal, however, processing tomatoes makes up most of the national tomato production, with 1.65 million tons being produced in 2017, as opposed to the 97 thousand tons produced for fresh consumption (INE, 2017). Portugal is the third biggest producing country of processing tomatoes in Europe, just after Italy and Spain.

1.2 The Tomato Crop

The tomato crop is an herbaceous, bushy plant that exhibits a perennial type of growth, although it's usually cultivated as an annual crop. The plants can develop a deep root system, reaching up to 1.5m long, if no soil limitations are present in site. However, the root system of transplanted plants consists mainly of lateral and adventitious roots that are overall closer to the soil surface. Modern varieties have inflorescences with 5 to 12 complete and hermaphroditic flowers with yellow petals. The flowers do not produce nectar and the pollination type is mainly autogamous (about 95%), favored by insects like bees or bumblebees. The resulting fruit is a plurilocular berry that comes in many different sizes, shapes and colors, with a weight varying between 5 and 500g. The shape is usually round, piriform or elongated, and the skin color of the ripened fruit can be yellow, orange pinkish or, more commonly, red. Cherry-tomato cultivars bear smaller and generally bilocular fruits (Almeida, 2006).

It is a fast growing crop with a growing period of 90 to 150 days (FAO, nd), depending on the variety, climate, and crop management. It is also a very demanding plant that requires plenty of sunlight and mild temperatures. Minimal light intensity for flowering and fruit setting is 0.85 MJ/m^2, although the photoperiod and light quality have a relatively lower importance. The optimal temperature for growth ranges between 18 and 25ºC, with night temperatures between 10 and 20ºC, and relative humidity between 70 and 80%. Temperatures higher than 30ºC inhibit lycopene synthesis, and reduce the fruit quality. Higher humidity (>90%) leads to a greater incidence of pests, diseases and fruit rotting, while lower humidity followed by high temperatures may lead to blossom drop or fruit cracking. The crop is also very sensitive to frosts, and should not be grown outdoors in regions with less than 110 days free of frost (Almeida, 2006). Warm and dry temperate climates with plenty of sunlight are therefore preferred for tomato production. However, there is a large amount of cultivars available today, adapted to a wide range of temperatures and climates.

Their growth pattern distinguishes cultivars into two main groups: Indeterminate and determinate growth. Indeterminate growth cultivars grow continuously, indefinitely forming new inflorescences between a roughly constant number of leaves, which allow for multiple harvest opportunities. Indeterminate growth cultivars are usually grown vertically in greenhouses, with trellises for support, and harvested manually for the fresh consumption market. Determinate growth cultivars cease their growth after a specific number of inflorescences that depends on the cultivar. These cultivars bear

fruit faster and more intensively than indeterminate growth type cultivars, and are usually grown outdoors as annual processing tomatoes (Haifa Group, 2018).

1.2.1 Soil requirements and preparation

Tomatoes can be grown on many different soils, although deep, well-drained sandy-loam or loamy soils are preferred. The plants are not tolerant to soil ponding, especially in the early stages of seed emergence and fruit ripening. Although the crop is moderately sensitive to soil salinity, with a critical value of 2.5 dS/m, higher values may increase fruit quality in its soluble solids quantity (Almeida, 2006). The soil should be slightly acidic and should be limed if necessary to raise the pH to the ideal range of 6.0 to 6.5. Caution should be exercised when applying lime, as an excess can be just as serious as a deficiency. When the soil is low in organic matter it becomes hard and crusts badly during the summer months. This may be corrected by applying manure or organic matter to loosen the soil, and by planting a green cover crop such as rye or rye grass on the plot the winter before the tomatoes (Gould, 1992).

In Portugal tomatoes are usually planted after an extensive soil preparation, which is considered essential for the crop's success. Proper terrain leveling is also necessary to ease the mechanical harvest. The soil is tilled up to 30-45cm deep, as many times as needed to leave the terrain regulated and free of large clods. Necessary nutrients and other soil correctives are also applied and incorporated during this stage (Almeida, 2006).

Processing tomatoes are usually planted in raised beds with machinery help using containerized plants with 4 true leaves and about 15cm tall. The planting is done from late March to June, with densities between 30000 to 70000 plants per hectare, depending on soil fertility and water availability. Fresh market tomatoes are planted outdoors between July and August, or January to March if grown in a controlled environment, with plant densities indoors reaching 30000 to 35000 plants per hectare. Although the plantation densities are reduced in greenhouses, the vertical growing systems achieve a higher productivity that may compensate for the increased investment (Almeida, 2006).

1.2.2 Irrigation

Water availability is the decisive factor when it comes to productivity in the tomato crop. An adequate supply of water is necessary during the early plant growth, fruit set, and fruit enlargement periods. Water deficit effects are variable, depending on the

current phenological stage. During flowering and fruit setting it reduces fruit quantity, but in fruit growth and ripening stages it promotes an increase in soluble solids, at the cost of an overall reduced weight. The benefits of deficit irrigation in enhancing water use efficiency in the tomato crop is well regarded, although it requires careful planning as plants subjected beyond a certain level of water deficit may show adverse effects on marketable fruit yield. Tomato crop water requirements in the Mediterranean climate amounts to 4000 to 6000 m^3/ha and the most commonly used irrigation system is subsurface drip irrigation (Almeida, 2006). This type of irrigation system represents a large commitment in investment costs, as it usually covers an extensive area and requires additional equipment to install when the soil is being prepared for planting. Furthermore, the systems are usually designed for a specific crop and have a fixed spacing. These costs are particularly relevant when the fields become infected with soil-borne diseases such as Fusarium Wilt, forcing farmers to either switch to non-susceptible crops, which may require an irrigation system overhaul, or abandon the field altogether.

1.2.3 Main pests and diseases

Like all cultivated plants, the tomato crop is host to many different insect pests. These can cause unthrifty growth and damage the fruit in the form of scarring, tissue damage and aberrations in shape or color (Lange & Bronson, 1981).

The main pest targeting processing tomatoes is the African bollworm (*Helicoverpa armigera*), a fruit borer moth whose larvae feed on the inner parts of the fruits, causing extensive fruit damage and promoting decay caused by secondary infections. The potato-aphid (*Macrosiphum euphorbiae*) and the cotton-aphid (*Aphis gossypii*) are the most common aphids in outdoor tomato plantations. These feed on leaves and shoots by sucking its sap, reducing plant vigor and distorting its growth. In greenhouses, other pests such as leafminers (*Liriomyza spp.*), whiteflies (*Bemisia tabaci; Trialeurodes vaporariorum),* mites (*Tetranychus spp.*) and thrips (*Thrips tabaci; Frankliniella occidentalis*) are also commonly problematic (Almeida, 2006).

Regarding the diseases, the main and most destructive air-borne fungal diseases affecting tomato are Alternaria early blight caused by *Alternaria solani*, mainly affecting field crops and colder greenhouses; Gray mold caused by *Botrytis cinerea*, common in both field and greenhouse crops; Cladosporium leaf mold caused by *Cladosporium fulvum*, affecting mainly poorly ventilated greenhouses but also field crops in wet and warm areas; Powdery Mildew caused by *Leveillula taurica*, found both in field and

protected crops; and Late Blight caused by the pseudo-fungus *Phytophthora infestans* affecting field crops but also poorly ventilated greenhouses (Blancard, 2017).

Soil-borne fungal diseases are generally harder to control and cause significantly more damage than air-borne diseases, since the root and vascular areas are the most targeted and there is a lack of available fungicides. The main soil-borne fungal diseases are Anthracnose caused by *Colletotrichum coccodes*, affecting all crop systems; Fusarium crown and root rot caused by *Fusarium oxysporum* f. sp*. radicis lycopersici*, targeting both field and greenhouse crops; Damping-off caused by *Pythium spp.*, both in the field and soil or soilless greenhouse crops; Corky root caused by *Pyrenochaeta lycopersici*, typical in intensive crop systems; Sclerotinia drop caused by *Sclerotinia sclerotiorum*, affecting all production types. Soil fungi such as *Fusarium oxysporum f. sp. lycopersici* and *Verticillium dahliae are* particularly damaging since they mainly colonize vascular tissue (Blancard, 2017).

Bacterial diseases such as the Bacterial Speck caused by *Pseudomonas syringae pv. tomato,* Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* and Bacterial stem/fruit rot caused by *Erwinia* spp. are the most damaging for the tomato crop, and no effective control methods are available once the disease is established (Blancard, 2017).

Viruses such as the Tomato spotted wilt virus (TSWV) and the Tomato yellow leaf curl virus (TYLCV) are the most damaging viruses commonly found affecting tomatoes, since they can be spread by common insect vectors like thrips and whiteflies respectively (Blancard, 2017).

1.3 *Fusarium oxysporum*

The *Fusarium oxysporum* species complex includes many strains of pathogenic and non-pathogenic fungi, commonly found in the soil growing saprophytically on organic matter, or lying dormant as chlamydospores (Olivain *et al*., 2006). Its wide range of plant hosts and the high economic damage associated with crop diseases makes it one of the most relevant and well-studied pathogens of the century. *F. oxysporum* strains are grouped in *Formae speciales* (ff. spp.), according to their host range specificity, and at least 106 different ff. spp. have been well characterized and recorded (Edel-Hermann & Lecomte, 2019).

There are two main diseases caused by *F. oxysporum* affecting tomato: Fusarium crown and root rot, and Fusarium wilt.

The Fusarium Crown and Root Rot disease is caused by *Fusarium oxysporum* f. sp *Radicis Lycopersici* (*Forl),* which has become one of the most harmful soilborne pathogens of tomato worldwide (Shenashen *et al*., 2016). This Fusarium Root Rot was first described in Japan in 1969 (with yield losses of up to 40%), and is now present in many production areas worldwide (Canada, Mexico, Korea and several Mediterranean countries) occurring both in the field and in greenhouses, in soil and soilless crops (Blancard, 2017). Yield losses have been recorded to reach levels up to 90% in Tunisia (Hibar *et al*., 2007) and 95% in Canada (Jarvis *et al*., 1983), but severity varies widely by site and season.

The Tomato Fusarium Wilt disease is caused by *Fusarium oxysporum* f. sp *lycopersici (Fol*) and was first described over 100 years ago in the UK (Massee, 1895). Nowadays it is also present in many production areas on every continent, where its damage varies depending on the race and crop variety produced (Blancard, 2017). Resistance to Fusarium wilt has been challenged by the rising aggressiveness of many races in some production areas. Currently, three races are known to affect tomato: Race 1, the oldest one, is now widely present in many production areas; Race 2, first reported in 1945 in Ohio, has become serious since the 1960s in several countries like USA, Mexico, Brazil, Venezuela, Australia, The United Kingdom, Netherlands, Israel, Morocco, Iraq, Taiwan, and China; and Race 3, the most recent race, first described in 1978 in Australia, now occurs in Brazil, in some states of the USA, in Mexico and in Japan (Blancard, 2017). Economic damage from Fusarium Wilt can be very high with yield losses of up to 45% recently reported in India (Ramyabharathi *et al*., 2012).

1.3.1 Symptoms, life cycle and damage

Both *Forl* and *Fol* survive in the soil through crop residue harboring mycelium, micro and macro conidia, and chlamydospores with stronger walls. These can also be found in the dust of glasshouses and its irrigation system, various organic compounds and other plants from different botanical families (Blancard, 2017). Although *Fol and Forl* are not morphologically distinguishable, they can be differentiated by the symptoms in tomato, their host range, optimal disease environment and molecular techniques (McGovern, 2015).

The fungi can invade susceptible plants through wounds and natural openings formed by emerging new roots. Symptoms are variable according to the growth stage of the plants and weather conditions. Both *Forl* and *Fol* can cause damping-off symptoms in tomato seedlings which include yellowing, stunting, wilting and, in the case of *Forl*, premature loss of cotyledons and lower developing leaves, and basal stem necrosis (McGovern, 2015). Fusarium Crown and Root Rot and Fusarium Wilt symptoms in mature plants also include yellowing and wilting occurring around the time of the first harvest, and when day temperatures are highest (Roberts *et al*., 2001).

Regarding *Forl*, the primary symptoms are found on the roots and stem base of plants. Discoloration in vascular tissue is visible and generally limited 20-30 cm above the soil line, but also substantially in cortical tissue in the lower stem (McGovern, 2015). The lesions are reddish brown and moist, progressing quickly into rot and decomposition, especially in smaller diameter roots. Dark brown cankers frequently form on the stem base, usually developing longitudinally on one side of the stem, taking the form of a flame with a central pinkish color and mucous appearance (Figure1) (Blancard, 2017). Fusarium Crown and Root Rot growth is favored by cool temperatures, between 10º and 20ºC, while low soil pH, ammoniacal nitrogen and water-logged soils also aggravate the disease (McGovern, 2015).

Figure 1 - Root rot and vessel browning, characteristic of *F. oxysporum* f. sp *radicis-lycopersici* (Blancard, 2017).

Wilt symptoms caused by *Fol* in mature tomato plants are similar to the ones described for seedlings, and include yellowing and wilting of foliage, most noticeable after flowering and fruit set and during the hottest time of the day. However, Fusarium wilt symptoms can have a one-sided appearance caused by the blockage of discrete sectors of vascular tissue, and vascular discoloration that can extend up the entire stem length even into the vascular tissue of petioles (McGovern, 2015). Eventually, these symptoms spread to other leaflets and the plant becomes more generally affected, often leading to desiccation and death. In contrast with *Forl,* Fusarium wilt produces a more extensive discoloration in the plant's water-conducting tissue (Roberts *et al*., 2001). Although symptoms of *Fol* are also exacerbated by a low soil pH and the use of ammonium-based fertilizers, they are further enhanced by warmer temperatures, around 28ºC, as opposed to *Forl* which is favored by cooler temperatures (McGovern, 2015).

On diseased plants, an unusual growth of adventitious roots sometimes occurs above the infected regions. On dying or dead plants, the fungi produce masses of white mycelium, and yellow to orange conidia may appear in the necrotic tissues. These are spread by air currents and can reinfect soil previously sterilized by heat or biocide fumigants (Roberts *et al*., 2001). Chlamydospores arise from the modification of hyphal or conidial cells, and their induction is related to stress factors such as nutrient depletion (lack of host) and unfavorable environmental conditions (Smith, 2007). Chlamydospores have thicker walls and enable the fungus to survive long periods in

the soil and wooden stakes, until favorable conditions return. Long-range dissemination can occur through the transport of infected plants and soil or debris in shoes, machinery and other equipment (Roberts *et al*., 2001). More so, soil dust containing chlamydospores is easily dispersed by air currents and splashing water (Blancard, 2017).

1.3.2 Control of *Fusarium oxysporum*

At present, it is difficult to control Fusarium Wilt and Crown and Root Rot given their survivability and ability to quickly reinfect sterilized soil. Furthermore, pre-plant fumigation through the use of methyl bromide was a standard treatment for soilborne diseases in Europe and in the USA (McGovern, 2015), before being phased out by the *Montreal Protocol on Substances that Deplete the Ozone Layer*, adopted in 1987 (UNEP, 2019). No control method is currently effective when these diseases set during cropping (Blancard, 2017). Therefore efforts to control these diseases should be oriented towards their prevention and avoiding known infected areas in the first place.

1.3.2.1 Plant resistance

The use of resistant varieties is viewed as the most economic and ecological method of disease management. Many hundreds of tomato cultivars are resistant to *Fol* races 1 and 2, while some also have combined resistance to *Fol* races 1 and 2, and *Forl*. Fewer cultivars possess resistance to all *Fol* races, and the rarest type found is the combined resistance to both *Forl* and all races of *Fol* (McGovern, 2015). However, the host-pathogen interactions involving resistances are not simple, and constant effort on research and creation of new cultivars is needed to meet the pathogens' evolutionary mechanisms to overcome plant resistance.

1.3.2.2 Cultural practices in the field

Some cultural practices and prevention measures can reduce the chance of infection or prevent an outbreak, before and during cropping. It is essential to use healthy seedlings or certified seeds free from contamination, and in the nursery plants produced should not come in contact with the soil, especially if it has not been disinfected (Blancard, 2017). Injuries to the plants should be avoided when they are set in the field, as damage to the root system may make plants more susceptible to infections. Nitrate fertilizers are preferable, since they are less favorable to *Forl* and *Fol* than the ammonium type. The irrigation and nutrient solution water should be used with caution, especially if it comes from possibly contaminated sources. Also, stakes and

trellises should be disinfected before use, as well as other tools and equipment that contact infected plants.

Crop rotations with long periods of at least 3-4 years should be implemented to prevent the onset of the disease or reduce its impact, although farmers are usually focused on a single crop due to large investments in the irrigation system. Non-susceptible crops such as maize should be used in the rotation, and host plants such as peppers or eggplants should be avoided. Infected plants must not be buried but instead removed with its root system, which reduces the amount of inoculum in the soil and in the plot (Blancard, 2017).

1.3.2.3 Soil disinfestation

Soil disinfestation, through the use of steam or a fumigant, is a partially effective option. In the past, steam was commonly used in protected horticultural crops of high value, and was eventually switched to the cheaper soil fumigation due to fuel costs (McGovern, 2015). Alternatives to Methyl bromide have been the focus of research on agricultural fumigants, yet these materials often have a narrower spectrum of control, less predictable efficacy and may have their own environmental risks (Stapleton, 2000). Soil disinfestation using solarization is a non-chemical alternative and may be an effective tool in sunnier regions of the world, since it depends on solar energy to heat the soil to temperatures that are lethal to most pathogenic organisms (Ozbay and Newman, 2004). However, the effectiveness of soil disinfestation will depend on the precautions taken to avoid early recontamination, as *Fusarium oxysporum* is well known to quickly recolonize disinfected soil (Blancard, 2017).

1.3.2.4 Biological control

Effective management of Fusarium Wilt and Fusarium Crown and Root Rot using biological (as opposed to chemical) control would be ideal, considering the fungi's persistence in the field, and the environmental costs associated with soil disinfestation. It encompasses the use of isolates of bacterial and fungi species from many different genera, alone or combined with different modes of action. Some biocontrols directly reduce spore production, germination and survival through antibiosis, while competition and induced resistance were also described in recent studies (McGovern, 2015). Mycorrhizal fungi are an important part of ecosystems, and their mutualistic symbiosis with plants have an important role in nutrient cycling while it can also protect them from soilborne pathogens. Regarding the tomato crop, the presence of intact extraradicular arbuscular mycorrhizal mycelium has been demonstrated to increase the growth and

reduce the disease incidence in tomato plants inoculated with *Fusarium oxysporum.* f. sp *radicis-lycopersici*, both in greenhouse and field conditions (Brito *et al*., 2019), which hints towards their possible intentional use as a biocontrol.

1.4 Arbuscular mycorrhiza

The term “mycorrhiza”, meaning “fungus root” in Greek, was coined in 1855 by the German botanist Albert Bernard Frank in his study of relationships between plants and microorganisms present in the soil (Siddiqui *et al*., 2008). Mycorrhizal fungi are specialized members of the vast population of microorganisms that colonize the rhizosphere, creating symbiotic relationships with plant roots which they depend on to obtain organic carbon (Smith & Read, 2008). Mycorrhizal associations refer a wide range of interactions, and should not be universally categorized as mutualistic associations. Mutualistic associations include direct and indirect, or symbiotic and nonsymbiotic associations, many of which function by means other than nutrient transfer (Boucher *et al*., 1982; Paracer & Ahmadjian, 2000). In that sense, all Mycorrhizal associations are symbiotic, but some are not mutualistic (Brundrett, 2004).

Mycorrhizal associations are categorized based on the taxonomic group of fungi and plants involved, and there are currently seven types known: Arbuscular mycorrhiza, ectomycorrhiza, ectendomycorrhiza, arbutoid, monotropoid, ericoid and orchidoid mycorrhizae (Gupta, 2000). Arbuscular mycorrhizal fungi (AMF) together with over 80% of all vascular plants (Brundrett, 2002), form the most common type of mutualistic mycorrhiza, the arbuscular mycorrhiza (AM). Through their role in nutrient uptake, symbiotic arbuscular mycorrhiza are likely to have made possible the colonization of land by the first bryophyte-like plants, around 470 million years ago (Selosse *et al*., 2015). Their role in the ecosystems, including agroecosystems, has led to increasing studies regarding their ecology and geography (Fitter, 2005; Chaudhary *et al*., 2008), and has become a crucial research area in the last twenty years (Goss *et al*., 2017).

1.4.1 Taxonomic organization

Arbuscular mycorrhizae are formed in an enormously wide variety of host plants by obligatory symbiotic fungi, which belong to the fungal phylum *Glomeromycota* (Schüβler *et al*., 2001)

Currently, four orders are recognized within *Glomeromycota*: *Glomerales*, *Diversisporales*, *Archaeosporales* and *Paraglomerales*. More than 220 different species of AMF are encompassed in these orders, which include 11 families and 22 genera (Schüβler & Walker., 2010), although most of the species described belong to the genera *Glomus*, *Acaulospora*, *Scutellospora*, and *Gigaspora* (*Goss et al*., 2017). Nearly all herbaceous plants, shrubs and trees of temperate and tropical climates can form AM. Although AMF are able to associate with a wide range of host-plants, there is

increasing evidence for specificity or selectivity of some plant species for particular fungal symbionts (Smith & Read, 2008).

1.4.2 Structures and interactions

AMF are obligate biotrophs and although spores can germinate in the absence of host plants, they depend on them to complete their life cycle and produce the next generation of spores (Parinske, 2008). This type of mycorrhiza has three essential components: the host-plant root itself, and two types of fungal mycelia that develop outside, and inside the roots. Inside the root, the AMF colonize the apoplast, both in the intercellular space and in compartments formed by invagination of cortical cell plasma membranes (Smith & Smith, 2012). The fungi form highly branching structures resembling trees within the cortical cells, called Arbuscules (Figure 2), that are considered to be the essential locations for the exchange of carbon compounds from the plant for mineral nutrients from the fungus. These structures within the cells sometimes also appear as coiled hyphae. Some of these fungi also form large vesicles in the intercellular spaces, and accumulate storage products such as lipids and cytoplasm that serve as an energy source (Goss *et al*., 2017).

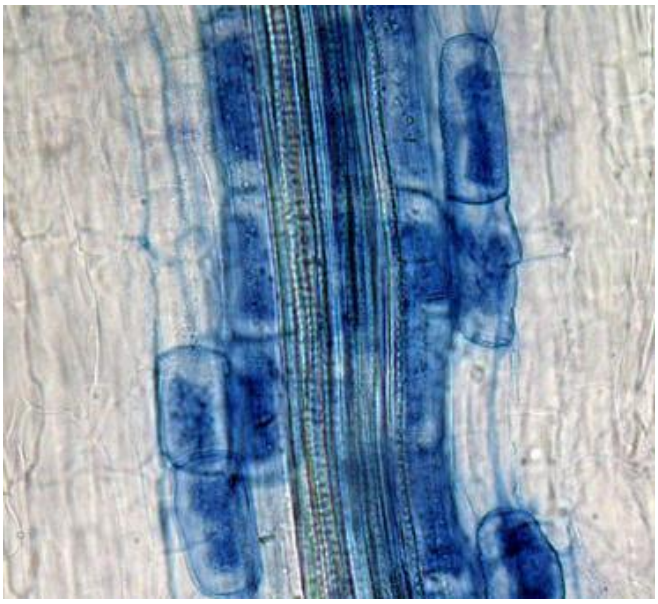

Figure 2 - Magnified arbuscular mycorrhizal structures, stained in blue. (Max Planck Institute of Molecular Plant Physiology, 2014)

The mycelium developing outside the root, known as Extraradical mycelium (ERM), grows extensively in the soil from the root surface, allowing further exploitation of soil reserves, colonization of new host-plants, enmeshment of soil particles, and acting as a link between plants. This link is achieved by the AMF's ability to form a common mycorrhizal network, even in host plants from different species, which allows the

transfer and re-allocation of nutrients between connected plants. Induced defense signals can also be transferred between pathogen-infected and healthy neighboring plants, suggesting that plants can perceive the signals and activate defenses before being affected themselves (Goss *et al*., 2017).

The process of root colonization by AMF is the result of a complex biochemical dialogue between the plant roots and the fungus. The dialogue is based on the emission and recognition of chemical signals by both symbionts, preventing the full expression of the plant defense mechanisms and allowing the symbiosis to become established (Harrison, 2005). The dialogue starts with the stimulation of the fungi by strigolactones present in root exudates (Harrison, 2005; Akiyama *et al*., 2005). These can stimulate the germination of spores, induce hyphal branching (on ERM or colonized root fragments) and switch on genes responsible for the signaling system of the AMF. The fungi then produce Myc Factors (chitin oligomers and lipochitooligosaccharide) that can interact with receptors in the epidermal cells of the host roots, starting processes in the host-plant that allow the symbiosis to be established (Goss *et al*., 2017).

1.4.3 Benefits to the host plants

The symbiosis with AMF is often beneficial to host-plants in different ways. Most benefits to the plans are due to the increased capacity to explore the soil through the extraradical mycelium. Estimates by Sieverding (1991) indicate an increase of 15cm^3 (up to 200 cm^3, depending on the environmental conditions) in the volume of soil explored for each centimeter of colonized root. The hyphae secrete enzymes into the soil and efficiently absorb available nutrients that are then carried through the hyphal network to the host plant root system, where they are exchanged for carbon.

Furthermore, hyphae from AMF are longer and thinner than root hairs (Figure 3), enabling them to reach greater depths and volume, extracting resources from soil pores inaccessible to plants. The enhanced soil volume explored and the ability to absorb and transport nutrients, especially those of low mobility in the soil, such as Phosphorus**,** are the most widely recognized advantages of the symbiosis (Goss *et al*., 2017).

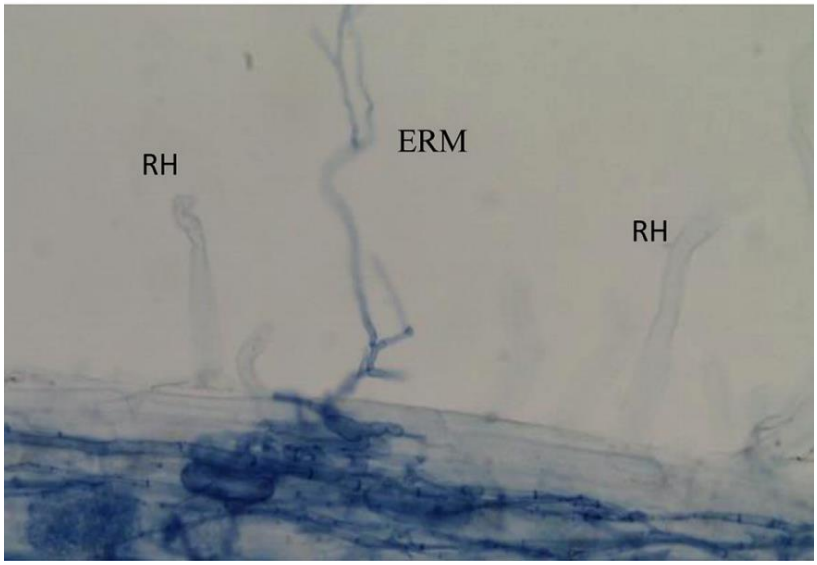

Figure 3 - Colonized root with extraradical mycelium (ERM) next to root hairs (RH) (Goss *et al*., 2017)

Yet there are many other studied benefits associated with AMF, not only related to greater resistance to biotic and abiotic stresses such as enhanced water intake and drought resistance, improved soil structure, protection against heavy metals and other pollutants, increased pathogen resistance (Quoreshi, 2008), increased hormonal production (Fernández *et al*., 2014; Cameron *et al*., 2013; Foo *et al*., 2013) and differential gene expression (Harrison, 1999a; Poulsen *et al*., 2005).

1.4.4 Protection against abiotic stresses

Abiotic stresses, such as drought, salinity, extreme temperatures and different types of soil toxicity present serious threats to agriculture because they are considered the primary cause of crop yield loss worldwide (Wang *et al*., 2003).

Mycorrhiza associations have been described to improve drought tolerance in many plant species such as maize, wheat, barley, soybean, onion, lettuce and tomato (Augé, 2001). This tolerance is mainly attributed to mycorrhiza induced changes in plant phenology (Augé, 2004), root morphology and the capacity of widespread extraradical mycelium to access smaller pores that root hairs are unable to reach (Smith & Read, 2008). More so, stabilization of soil aggregates and improvement of soil structure caused by ERM also indirectly increases soil moisture retention and water absorption (Bethlenfalvay & Shuepp, 1994). By increasing water use efficiency and water retention in the soil, AMF may protect plants against high temperature stress by improving photosynthesis and water status (Zhu *et al*., 2011). Enhanced tolerance to low temperatures by AMF was also demonstrated in tomato plants, achieved by a reduction of membrane lipid peroxidation, increased photosynthetic pigments, accumulation of

osmotic compounds and antioxidant enzyme activity (Latef & Chaoxing, 2010) The adjustment of osmotic potential by AM, through the higher accumulation of organic compounds such as proline, glycine betaine, carbohydrates such as sucrose and mannitol and non-organic ions including K and Cl, mycorrhizal plants can also improve production under osmotic stress relative to nonmycorrhizal plants (Azcón *et al*. 1996; Goicoechea *et al*. 1998; Ruiz-Lozano 2003; Ruiz-Lozano *et al*. 2006).

In contaminated soils under heavy metal stress, the unfavorable oxidative effects adversely influence plant growth. However, AMF are able to enhance production of antioxidant enzymes, which can alleviate the stress of such contaminants (Avery 2001; Ruiz-Lozano 2003). AMF can also protect host plants by improving nutrient absorption and by influencing the fate of the metal and metalloid ions in the plant and soil (Goss *et al*,. 2017). Their ability to negate the unfavourable effects of aluminium, manganese and iron (Nogueira *et al*., 2004; Brito *et al*., 2014; Davies *et al*., 2005; Cardoso & Kuyper, 2006; Miransari, 2010) on plant growth has also been demonstrated, especially at high concentrations. According to Joner & Leyval (1997), the uptake and immobilization of cadmium by extraradical hyphae of *Glomus mosseae*, caused a reduction in cadmium transfer to the plant. Khan *et al*. (2000) observed similar results for zinc, and stated that zinc absorbed by AM hyphae is crystallized in these hyphae and cortical cells of mycorrhizal roots. This process is called phytostabilization, by which AM increase plant ability to immobilize heavy metals in the soil, through absorption in their hyphae and consequently decreasing translocation from plant roots to shoots (Leyval *et al*. 2002).

1.4.5 Protection against biotic stresses

The roles of AMF in protecting their host against pathogens have been studied in many different combinations of host and pathogen species (Whipps, 2004), and are generally considered effective in facing the challenges of plant protection. Their association with host plants can provide them with bioprotection against many agronomically relevant soilborne pathogens, such as *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotinium*, *Verticillium* and also nematodes like *Heterodera*, *Meloidogyne*, *Pratylenchus*, or *Radopholus* (Harrier & Watson, 2004)

The AMF protection of its host plants against soil pathogens consists of many mechanisms which may be operating simultaneously at multiple levels (Azcón-Aguilar & Barea, 1997). In addition to the improved plant nutrition and damage compensation, competition for photosynthates or colonization sites between AMF and pathogens has

been reported (Pozo *et al*., 2010). Other mechanisms such as changes in the amount of carbon compounds released from the root system, changes in the microbial community of the rhizosphere, and the activation of local and systemic plant defenses. Resistance to plant pathogens can also be induced by priming of jasmonic acid-dependent defenses of AMF colonized plant roots (Cameron *et al*., 2013), comparable to the supply of a vaccine that enhances the immunological response of the host plant (Goss *et al*., 2017). Furthermore, these changes can be communicated from one plant to another by the transmission of signals through the common mycorrhizal networks, meaning disease resistance and induced defense signals can be transferred between healthy and pathogen-infected neighboring plants. This suggests that plants can be informed through the mycorrhizal network by afflicted neighboring plants and activate defense mechanisms before they are attacked themselves (Song *et al*., 2010).

1.4.6 The importance of an early and effective colonization

Early root colonization is crucial, if the potential benefits from AM are to be optimized in the host plants. Evidently, benefits such as a better nutrient supply or the ability to withstand biotic and abiotic stresses (in part due to precocious activation of the plant's local and systemic defenses) are optimally enhanced if they start at the beginning of the plant growing cycle. (Cameron *et al*., 2013; Khaosaad *et al*., 2007). Moreover, the amount of AMF infection sites on a root system can determine the extent of soil pathogen's ingress, since soilborne fungi and plant-parasitic nematodes occupy similar tissues as AM and are bound to compete for space (Harrier and Watson, 2004). However, early colonization is a more important factor than the level of colonization, if plants are to take full advantage of the AM symbiosis (Garg & Chandel, 2010). Vierheilig (2004) observed that high levels of AMF root colonization resulted in a strong suppression of further root invasion, after inoculating barley with two different AMF species at 4 days interval, which confirms an equivalent effect that only a well-established symbiosis could protect plants against soilborne pathogens. Brito *et al*. (2014) reported a strong positive relationship between dry matter production of wheat plants under adverse conditions of Manganese toxicity in the soil and the timing of AM colonization, comparing plants with an early and late colonization. However, in field conditions with the pathogen already present, the protecting role of AMF is challenged by the time required to achieve an adequate AM colonization level and the cost of large-scale application of commercial inoculum (Sikora *et al*., 2008).

1.4.7 AMF propagules and the importance of an intact ERM

The AMF can colonize host plants through different inoculum sources, called propagules. There are three such sources: Spores, fragments of AM colonized roots and ERM (Smith & Read, 2008). Although they are all able to start colonization of plant roots, the different propagule forms exhibit varying colonization capabilities (Requena *et al*., 1996; Klironomos & Hart, 2002).

Spores are structures produced by the hyphae in the soil, and the only plant-independent phase of the AMF. They can be very large (up to 500 µm in diameter), with lipid and carbohydrate storages and a thick resistant chitin wall that allows them to survive until conditions are favorable (Smith & Read, 2008). When adequate conditions of temperature and water are met, spores germinate producing a germination tube. This coenocytic hypha grows with marked apical dominance and can reach a few centimeters in length. If a host plant root is not contacted, the growth ceases after 2 to 4 weeks, and the hyphae septate apically and the protoplasm retract towards the spore (Mosse 1988; Logi *et al*., 1998) allowing its survival until conditions are favorable again.

Colonized fragments of root, dead or living, are also able to colonize hostplants. Root tissues protect the fungus from environmental hazards until the time when new hyphae can grow out from the roots and colonize other plants (Requena *et al*, 1996). Their survival is possible due to the structures called vesicles, which are swellings in the root cortex where lipids and cytoplasm are accumulated for storage, to be used as an energy source. Plants are colonized more rapidly when inoculated with AM colonized root fragments than when inoculated with spores (Abbot & Robson, 1981).

The extraradical mycelium network, developed extensively in the soil by mycotrophic plants, is perhaps the most important inoculum source for new AM colonization. This ERM consists of mainly two types of hyphae: runner and absorptive hyphae. Runner hyphae are capable and responsible for infecting new root segments, while absorptive hyphae are classified as structures primarily involved in the acquisition of soil resources (Friese & Allen, 1991). In undisturbed soil conditions, with supporting native vegetation, the hyphal network is more important than spores or colonized root fragments as an inoculum source (McGee *et al*., 1997; Kabir, 2005). Not only is the probability of a root intersecting an infection unit resulting from an ERM network greater, but runner hyphae from a well-developed ERM are quicker to make contact with roots than a germinating spore (Jasper *et al*., 1989; Kliromonos & Hart, 2002). Moreover, an earlier and faster colonization has also been observed in the presence of

ERM (Brito *et al*., 2012a, 2013b; Goss and de Varennes, 2002). In a pot experiment, Brito (unpublished data, 2014) observed that a scarcely mycotrophic plant (*Rumex bucephalophorus*) was four times more colonized when grown after the highly mycotrophic plant *Ornithopus compressus* and in the presence of its ERM, than when it was colonized by spores in the sequence of a nonmycothropic plant (*Silene galica*). Similar results were observed by Püschel *et al*. (2007), who argued that because ERM is such a powerful form of inoculum, even plant species usually not hosting mycorrhizal fungi could be colonized.

However, the integrity of the ERM is essential for its efficacy as the propagule for an early AM formation. Brito *et al*. (2014) and Alho *et al*. (2015) studied the colonization of wheat and clover by a native AMF population, following different ERM Developer plants. Greater AM colonization of wheat and clover was observed when these plants were grown after mycotrophic host developer plants (*Ornithopus. compressus* or *Lolium rigidum*), than after the scarcely mycotrophic host plant (*Rumex bucephalophorus*), when the soil was kept undisturbed. Yet if the soil was disturbed and the ERM disrupted, the beneficial effect of the previous plant on the development of the ERM network was lost, and the initial colonization rates of wheat or clover declined to the levels observed when the previous plant was the scarcely mycotrophic host *R. bucephalophorus.* These findings clearly establish the importance of the host plant in the development of an infective ERM in the soil and the need to keep it intact to ensure an early colonization of the subsequent plants.

1.4.8 Strategy for an early AMF colonization

Even though the benefits to host plants from their symbiosis with AMF are well studied, their intentional use within agriculture cropping systems has not been fully exploited. There are three key aspects that hinder their potential use in agricultural ecosystems (Goss *et al*., 2017): The functional diversity between AMF species and between isolates of the same taxonomic unit; The time required to achieve an effective colonization, which has been demonstrated to be crucial in the expression of bioprotection benefits (Brito *et al*., 2014) and also in nutrient acquisition (Miller, 2000); and the high cost of commercial inoculum, which usually lacks biological diversity and may not provide the benefits that an indigenous population would offer. These hindrances highlight the need to develop a strategy that manages the indigenous AMF within different cropping systems, focused on improving AMF biological diversity in the rhizosphere and enhancing early colonization.

The general strategy advocated by Goss *et al*. (2017) is based on the establishment of an ERM granted by a specific host plant (Developer), which acts as the dominant form of AMF propagule, rapidly colonizing the roots of the subsequent plant as they grow and providing the benefits of an early and effective colonization. In order to maintain the integrity of the ERM in the cropping system, appropriate tillage techniques need to be adopted, with little or no soil mobilization.

Resorting to indigenous AMF communities avoids the problems associated with cost and low biodiversity of commercial inoculum, while also benefiting from their higher resilience and adaptation to local soil and climate. Setting a crop rotation system, prioritizing mycotrophic plants adapted to the region, is an opportunity to develop the ERM of indigenous AMF. In a crop rotation consisting entirely of mycotrophic elements, considering that the ERM can survive hot and dry periods, the AMF biodiversity should improve and its benefits enhanced. Since it is known that preferential associations exist between AMF and plant species, the AMF diversity present in the roots of the Developer will depend on the plant functional group, which can be found at different levels of botanical affinity. In a succession of two plants from different functional groups, assuming a colonization of the second plant preferentially initiated by an intact ERM, the diversity of AMF present in its roots will have been influenced by the first plant in the sequence (Brígido *et al*. 2017). The choice of the host plant to develop the ERM can therefore be used as a tool to manipulate and benefit from specific AMF within the available functional diversity.

Cover crops can be useful to enhance the role of AMF, particularly after nonmycotrophic plants in the rotation, such as rapeseed (*Brassica napus* L.), or long bare fallows and hot temperatures (Kabir & Koide, 2002; Lehman *et al*., 2012). Mycotrophic weeds that germinate after the first rains could also be relevant in restoring the ERM in the soil and increase the role of the indigenous AMF in enhancing nutrient uptake and crop growth (Brito *et al*., 2013b).

1.4.9 Developer plant strategy for pot experiments

The studies mentioned in the previous sections highlight the role of the ERM network developing plant (Developer), the importance of an intact ERM and an early colonization in benefiting the following crop. Most of these studies were conducted through pot experiments in controlled environments at a greenhouse scale, allowing the identification of key requirements for successful field applications. The strategy involved in these experiments consists of two distinct phases. In the first phase, the

Developer plants are grown in pots under controlled conditions, where the chosen plant species becomes a study factor. Phase one ends after a significant plant growth, and all the Developer plants are killed with a systemic herbicide to ensure that the means of ending the phase doesn't become a factor in the experiment. In the second phase of the experiment, crops or other studied plants are then grown on the pots where the ERM had been developed previously. The level of integrity of the ERM can be varied by mechanically disturbing the soil through a sieve, simulating tilled soil conditions. If the chosen Developers are mycotrophic, AMF will be present but the colonization may start from different types of propagule, depending if the soil was disturbed or not. In experiments reported by Brito *et al*. (2014) and Alho *et al*. (2015), in disturbed and undisturbed treatments following the growth of nonmycotrophic developers, the predominant propagules were spores, as no mycorrhizal development was expected from nonmycotrophic plants. In the Disturbed soil treatment after mycotrophic developers, the propagules could be disrupted ERM together with spores and colonized root fragments. Only in the Undisturbed soil treatment, following the growth of mycotrophic developers, were intact ERM, spores and colonized root fragments all present as propagules.

In another study described by Brito *et al*. (2019), again on a two-phase pot experiment in controlled conditions, *L. rigidum* was grown for 8 weeks in unsterilized soil to create an ERM formed by indigenous AMF. After being treated with a systemic herbicide, half of the pots were sieved to disrupt the ERM and the remaining pots were left untouched. Tomato seedlings were then planted in the pots and *F. oxysporum* f. sp. *radicis-lycopersici* was inoculated to the roots of each plant. After a 3 week growth period, shoot dry weight, arbuscular colonization and disease incidence were measured. In inoculated plants, the presence of intact ERM significantly increased the growth of tomato plants and reduced the disease incidence.

The strategy used in these experiments makes it possible to evaluate the significance of the source of propagules on AMF development and on the benefits accrued to the plants. By choosing a specific Developer, manipulating the AMF biological diversity present in the roots of the studied plant, it is possible to further study their intentional use in agriculture. Given the functional diversity observed in AMF, it is safe to assume that there is an optimal Developer for a given crop and goal, whether it is to improve resource efficiency or to protect against a biotic or abiotic stress.

1.5 Specific objectives

This work is intended to validate and enhance the knowledge obtained in similar experiments on the intentional use of AMF. The strategy based on an early colonization from a previously developed intact ERM has proven to be effective in the bioprotection of various crops against biotic and abiotic stresses (Brito *et al*., 2019), and the chosen Developer species is very significant. Following the previously described strategy in a two-phase pot experiment, the present work specific objectives are: to test different plants as hosts to develop the ERM (*Lolium rigidum* and *Ornithopus compressus*); and to evaluate the role of an early AMF colonization in the bioprotection of tomato against *Fusarium oxysporum* f. sp*. radicis-lycopersici.*

Chapter 2

Materials and Methods

2.1 Experimental design

This work experiment was conducted in pots on the semi-controlled environment of a greenhouse in the University of Évora, Portugal. Regarding the experimental outline, the experiment followed a randomized block design with 4 repetitions. The studied factors were the Developer species (*Lolium rigidum* or *Ornithopus compressus*), soil disturbance level (Undisturbed or Disturbed) and *Fusarium oxysporum f. sp radicis-lycopersici* presence (Uninoculated and Inoculated). Considering the number of repetitions and treatments, the total number of pots used was 32.

2.2 Establishment of the pot experiment

In the first phase of the experiment (on February 7th), unsterilized soil from the experiment site was collected and sieved, removing plant residue and other debris. The prepared soil was then weighed and 8 kilograms were placed into each pot, slightly compressing it to accommodate the total amount. Given the acidic nature of the soil, 6 grams of dolomite powder ($CaMg.2CO_3$) were also weighed and added to each pot to avoid nutrient toxicity issues.

The Developers were then planted in each pot (*L. rigidum* in one half and *O. compressus* in the other) and allowed to grow in order to develop different AMF communities in the soil (February 13th). The plants were watered daily with 200 ml of distilled water, and checked for weeds and pests throughout the growing period. After a significant growth period of about three months, the plants' aerial part was manually removed, leaving the intact ERM network behind in the pots (May 5th). To create a negative control where the ERM was broken, with a weaker AM colonization expected in the following tomato plants, half of the pots' soil was run through a sieve, cutting and fragmenting roots and ERM in the process. The remaining half of the pots was left untouched, representing an undisturbed soil where the intact ERM would be the preferential inoculum, granting a greater AM colonization from the beginning of the tomato plant cycle.

In the second phase of the experiment, five pregerminated tomato seedlings were planted in each of the Disturbed and Undisturbed pots (May 23rd). At this stage, half of

the plants were also inoculated with *Fusarium oxysporum* f. sp. *radicis lycopersici* by applying 1 ml of a spore suspension (10^6 conidia/ml) at the roots of the plants (May 24th). Afterwards they were each fertilized with a solution that contained 610 µl Solubor 5% (2 kg B/ha), 764 µl $ZnSO_4.7H_2O$ 1M (16 kg Zn/ha), 16 ml K_2SO_4 0,2M (100 kg of K_2O/ha), 3 ml $NH_4H_2PO_4$ 1M (30 kg P/ha) and 13 ml NH_4NO_3 (157 kg N/ha), to mimic standard fertilization practices for tomato production (May 29th).

The tomato plants were watered daily and allowed to grow for 3 weeks, before being examined and collected to evaluate the studied parameters (June 14th).

2.3 *Fusarium oxysporum* f. sp *radicis lycopersici* - propagation and preparation

The phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis lycopersici* used in this experiment belongs to the collection of the Mycology Laboratory, Mediterranean Institute for Agriculture, Environment and Development (MED), University of Évora. It was multiplicated and cultivated in Petri dishes with potato dextrose agar (PDA) (Oxoid) at room temperature (25–28^{o}C) for 12 days. After that, the spores produced by the fungus were collected using milliQ water and the suspension was adjusted to a concentration of $1x10^6$ conidia per ml of water. The spore counting was performed using a Neubauer's chamber in an optical microscope (Olympus BX-50) with a 40X objective.

2.4 Sampling and processing

In order to study and evaluate the different parameters, samples were collected at different stages of the experiment. At the end of the first phase, when the Developers had established the ERM network, root samples from each Developer of the Disturbed treatment pots were collected as the soil was sieved. These were carefully removed of soil and stained (see below) to confirm their AM colonization.

After the second phase, the tomato plants were visually checked for Fusarium disease symptoms and photographed for record and comparison. The cross section of the stem base of the plant was examined for symptoms such as necrosis, and a disease incidence (DI) level was assigned and recorded. Afterwards, the plants were cut a few centimeters above the soil and the aerial part was stored in identified paper bags. A portion of the stem base of each plant (of about 1 cm) was cut and stored in Falcon

tubes and kept in an ice box. Finally, the root systems were removed from the pots, cleaned and placed in identified plastic bags.

After being cleaned, the tomato root systems were stored in sample closed vials at 4^{o}C, until being further processed for examination. The paper bags containing the tomato aerial parts were placed in a lab drying oven at 50^{o}C for about 72 hours, in order to obtain dry plant material and the Falcon tubes with the stem base fragments were transferred from the icebox to freezers at -80^{o}C, until they were used for total DNA extraction.

2.5 Root preparation for AM quantification

In order to observe and study the mycorrhizal colonization, the roots have to be stained to highlight the fungal structures. The developer and tomato roots were rinsed and thoroughly cleaned and a small sample (of about a gram) for each treatment was cut and placed in an identified histology cassette. The cassettes were then immersed in a beaker with potassium hydroxide (KOH at 10% w/v) and autoclaved for about 15 minutes in order to clear the cells of cytoplasmic content. Afterwards, the cassettes were removed from the solution and rinsed thoroughly to remove any remaining KOH. To actually stain the roots, the cassettes were then immersed in another beaker with a 0,1% trypan blue solution containing water, glycerin and lactic acid in proportions of 1:1:1 (v/v/v). To allow the trypan blue to connect to the fungi's chitin, the beaker was placed in a water bath at about 50^{o}C and heated for 10 to 15 minutes. The root cassettes were then removed from the trypan blue solution and stored in a glycerol solution (50% v/v), where they remained until examination.

2.6 Stem sample processing for DNA extraction

To extract the total DNA from the stem base fragments, the CTAB (cetyltrimethylammonium bromide) method, described by Doyle & Doyle (1987), was followed after some preparation beforehand.

First, the stem fragments samples were removed from storage and subject to disinfection in order to eliminate epiphytic microorganisms. The disinfection was achieved by immersing the samples in a sequence of solutions, with 1 minute passing before switching them to another solution. The solutions used were ethanol (96% v/v), sodium hypochlorite (3% v/v) and ethanol (70% v/v), in this order. Afterwards, the stem samples were immersed in pure water for 2 minutes to remove any remains of the

previous disinfecting solutions. The samples were then macerated using a pestle and mortar with the help of liquid nitrogen, and placed in identified 1,5 ml microtubes.

The CTAB method itself was accomplished with the following steps: A small amount (of about 100 mg) of macerated sample was added to another 1,5ml microtube containing 600 µl of CTAB extraction buffer at 3% (10% CTAB, 5M NaCl, 0,5M EDTA pH 8.0, 1M Tris-HCl pH 8.0, as well as 4% PVP and 0,1% β-mercaptoethanol, added immediately before its use). This suspension was incubated at 55^{o}C for 90 minutes and mixed by shaking and inverting the microtubes every 15 minutes. After the incubation process, 600 µl of chloroform:isoamyl alcohol (24:1) was added to each microtube and mixed by shaking and inverting for 10 minutes. The samples were then centrifuged at 12000 rpm for 10 minutes, and their supernatant was transferred to another 1,5 ml microtube. 800 µl of freezing absolute ethanol (-20^{o}C) was then added to the new microtubes and again mixed by inversion, before being centrifuged at 13000 rpm for 20 minutes. The supernatant liquid was discarded and 500 µl of ethanol (70%) was added to the microtubes to centrifuge once more at 13000 rpm for 15 minutes. Finally, the supernatant was discarded and the remaining pellet was dried in a speed-vacuum centrifuge for about 30 minutes at 55^{o}C. The dried pellets of DNA were then resuspended in 30 µl of ultra-pure water and stored in freezers at -20^{o}C, where they remained until the next stage of the quantification process through real time qPCR.

2.7 Evaluated parameters

2.7.1 Disease incidence

The inoculation of *Fusarium oxysporum* after the planting naturally prompted the appearance of symptoms in the tomato plants. In order to evaluate the disease incidence (DI) in the studied plants, their stem sections were visually examined when the tomato plants were harvested and an assessment was made regarding the severity of symptoms. The observed symptoms, which range from a slight chlorosis to heavy necrosis and tissue collapse, were then graded from No visible symptoms scored as 1, to stem fully affected scored as 4 (Figure 4). In some extreme cases, the plant's vigor and occasional deaths were also recorded in order to draw further conclusions.

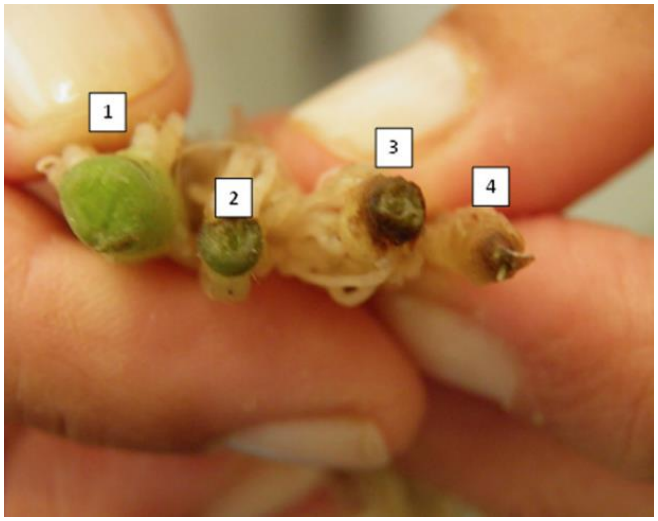

Figure 4 - Reference of *Fusarium oxysporum* f. sp *radicis-lycopersici* symptoms used to assess the disease incidence (Goss *et al*., 2017)

2.7.2 Dry weight

After the 71 hours drying, the plant parts were weighed in an analytical balance and the measurements recorded. The recorded values are effectively the Dry Weight (DW) of the 5 plants of each pot, excluding the root system, which is a useful tool to compare effective plant growth and production in such experiments.

2.7.3 Mycorrhizal colonization

To determine the mycorrhizal colonization rate of the root samples, the intersection method described by McGonigle *et al*. (1990) was applied. The method required the preparation of microscope slides, where the previously stained root samples were carefully placed and aligned parallelly with the slide's long axis. The roots were then covered with the glycerol (50% v/v) solution and a 24 x 60 mm coverslip, allowing its observation under the microscope lens. Each sample was assembled in two slide preparations, to reduce the associated counting error of the following steps. The mycorrhizal colonization rate was quantified using an optical microscope with a 200x magnification with a vertical eyepiece crosshair. The observation was made moving the field of view perpendicular to the long axis of the slide, with a turn width roughly equal to the size of the optical field. Any interception of the vertical crosshair with a root, except ones where the cortex part was missing, was considered and the root part was examined for mycorrhizal arbuscules or hyphae. Each interception throughout the samples was classified as "arbuscules", "hyphae" or "negative" in such manner. Observed hyphae were carefully examined for clues hinting towards their AMF nature, as many other fungi were also present in the samples and are of no interest to this study. The arbuscular colonization rate (AC) was calculated by dividing the

“arbuscules” category count by the total amount of intersections examined. In a similar way, the hyphal colonization rate (HC) was calculated by dividing the non-negative categories (arbuscules + hyphae) count by the total amount. These rates are representative of a plant’s mycorrhizal colonization and their value is an estimate of the proportion of the plant’s root containing arbuscules and hyphae.

2.7.4 *Fusarium* quantification - Ct values

To quantify and determine the DNA purity, the samples’ absorbance was measured on a NanoDrop-2000C spectrophotometer (Thermo Scientific). The DNA solutions were then either diluted by adding small volumes of ultra-pure water or dried in a CentriVap micro IR vacuum concentrator (LABCONCO), in order to obtain a final concentration of 20 ng/µL. To proceed with the qPCR, a mixture was prepared for each sample in a lab flow chamber, to avoid cross contamination, using 100 ng of gDNA as template, 2x NZY qPCR Probe Master Mix (Nzytech), 400 nM of each primer and 100 nM of probe (Nzytech) for a total volume of 20 µl. Two technical replicates of each sample were included in the qPCR plate, as well as *Fusarium oxysporum* f. sp*. radicis-lycopersici* DNA as a positive control and no-template solutions as a negative control. The primers and probe used for the TaqMan qPCR assay were described by Campos *et al* (2019) to specifically target *Fusarium spp*..

The qPCRs were carried out on a 7500 Real Time PCR System (Applied Biosystems) and the cycle threshold (Ct) values were acquired for each sample with the Applied Biosystems 7500 software v2.0.6 (Applied Biosystems) with the following cycling conditions: 10 min at 95 ^{o}C for the initial denaturation, an amplification program of 40 cycles at 95 ^{o}C for 15 s and 60 ^{o}C for 1 min. The fluorescence threshold was manually set above the background level. The Ct value of each sample is equal to the number of cycles required for the fluorescent signal to cross the defined threshold and is inversely proportional to the amount of target nucleic acid in the sample. This means that a lower Ct value represents a greater amount of *Fusarium* DNA present in the sample, and vice versa.

2.8 Statistical analysis

The collected data was processed using the MSTAT-C (version 1.42, Michigan State University) statistical package, following a variance analysis (ANOVA) with a Three Factor Randomized Complete Block design. The tri factorial ANOVA was carried out with 2 levels for the factor A (Lolium, Ornithopus), 2 levels for the factor B (Dist. and Undist.), 2 levels for the factor C (Uninoc., Inoc.) and 4 replicates. Following the ANOVA, the Fisher's Least Significant Difference (LSD) multiple range test was used to compare the means with a $p \leq 0.05$.

Chapter 3

Results

The analysis of the measured parameters and of the interactions between factors allows the understanding and demonstration of cause-effect relationships. In this work, the results will provide further knowledge about the best plant to develop the ERM for an early AM colonization of the tomato crop, in improving its overall growth and in granting bioprotection against *Fusarium oxysporum* f. sp. *radicis-lycopersici.*

The collected data concerning the evaluated parameters (Dry weight, Hyphal and Arbuscular colonization, Disease Incidence and Ct values) was statistically processed in order to determine the effect of the studied factors (Developer species, ERM integrity and *F. oxysporum* inoculation). The resulting values for each treatment and developer are resumed and presented in Table 1.

Table 1 - Obtained values of hyphal colonization (HC), arbuscular colonization (AC), dry weight (DW), disease incidence (DI) and cycle treshold (Ct, nº of cycles) for each treatment. Different lower-case letters highlight significant differences between treatments ($p<0,05$).

		HC (%)	AC (%)	DW (g/pot)	DI (1-4)	Ct
L. rigidum	UD, Uni	38,11	34,78	2,7 a	1	30,5
	UD, I	29,64	27,47	1,5 b	1,6	31,4
	D, Uni	19,35	15,92	1,1 b	1	30,5
	D, I	13,45	11,19	1 b	2,3	38,2
O. compressus	UD, Uni	25,24	22,29	1,7 b	1	28,3
	UD, I	28,13	23,37	2,7 a	1,1	32,8
	D, Uni	3,27	2,69	1,4 b	1	32,6
	D, I	5,54	4,66	1 b	1,6	29,5

Legend: UD - undisturbed soil; D - disturbed soil; Uni - uninoculated; I - inoculated

There was a statistically significant three-way interaction between the developer choice, soil disturbance and inoculation on the dry weight parameter, with the highest

values recorded in the "*L. rigidum,* UD, Uni" and "*O. compressus,* UD, I" treatments. Even though the dry weight did not demonstrate significant changes with the inoculation factor, its values were still higher in uninoculated plants. The disease incidence also showed no significant changes with a three-way interaction of the factors, yet inoculated plants displayed a lower incidence in undisturbed soil whose developer was *O. compressus.*

Analysing the mean values for each factor, the arbuscular and hyphal colonization rates were clearly conditioned by the soil disturbance, being significantly higher when the soil was not disturbed and the ERM remained intact. Soil disturbance significantly influenced the dry weight of the tomato plants, with higher values being recorded in undisturbed soil pots (Figure 5).

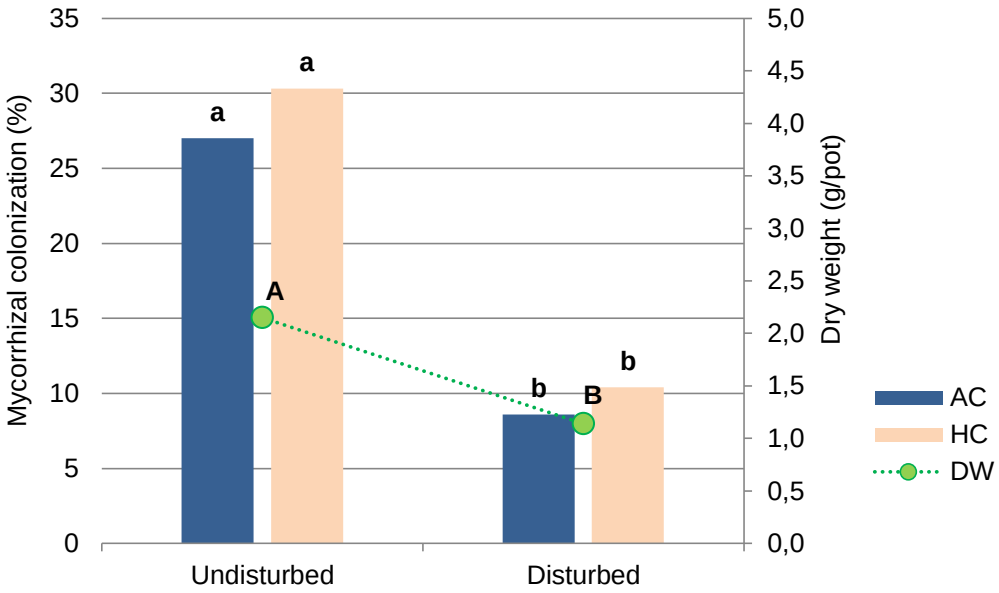

Figure 5 - The effect of soil disturbance (ERM disruption) on the arbuscular colonization (AC), hyphal colonization (HC) and dry weight (DW) of tomatoes. Different lower-case letters highlight significant differences between treatments for Mycorrhizal Colonization and capital letters for Dry weight ($p<0,05$).

Regarding the developer plant, *L. rigidum* granted a significantly higher arbuscular and hyphal mycorrhizal colonization to the subsequent tomato plants. The dry weight of the plants did not significantly vary with the different Developer plants, yet it was slightly greater after *O. compressus* (Figure 6 and 7).

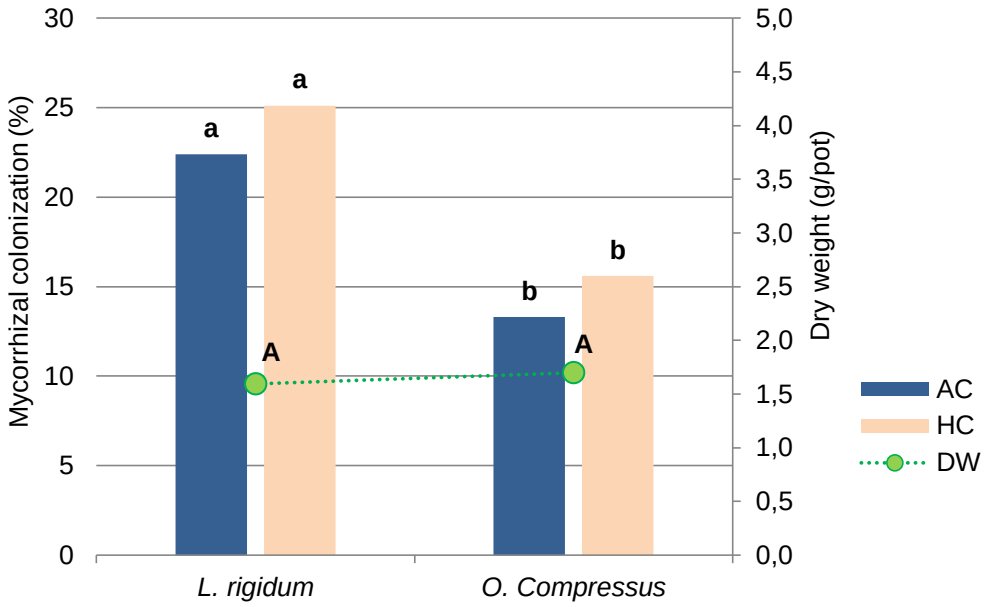

Figure 6 - The effect of the Developer plant on arbuscular colonization (AC), hyphal colonization (HC) and dry weight (DW) of tomato plants. Different lower-case letters highlight significant differences between treatments for Mycorrhizal colonization and capital letters for Dry weight ($p<0,05$).

Figure 7 - Growth of tomato inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici* in undisturbed soil (intact ERM). *Lollium rigidum* (A) and *Ornithopus compressus* (B).

The Ct values did not significantly vary with the factors under study, not even between inoculated and uninoculated plants, although it appears that the presence of *Fusarium* was generally lower after *O. compressus* and in undisturbed soil. However, the disease incidence was significantly affected by soil disturbance and inoculation. When

comparing inoculated plants, the undisturbed soil treatment scored a significantly lower Disease Incidence than the disturbed soil treatment (Figure 8 and 9).

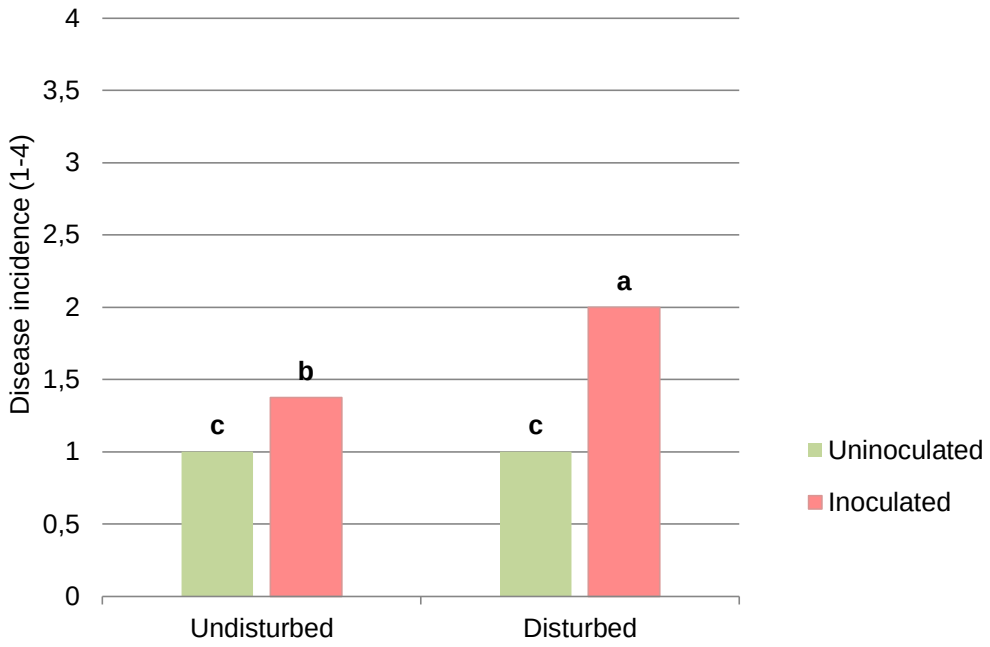

Figure 8 - Effect of the interaction between soil disturbance and inoculation on the disease incidence. Different lower-case letters highlight significant differences between treatments ($p<0,05$).

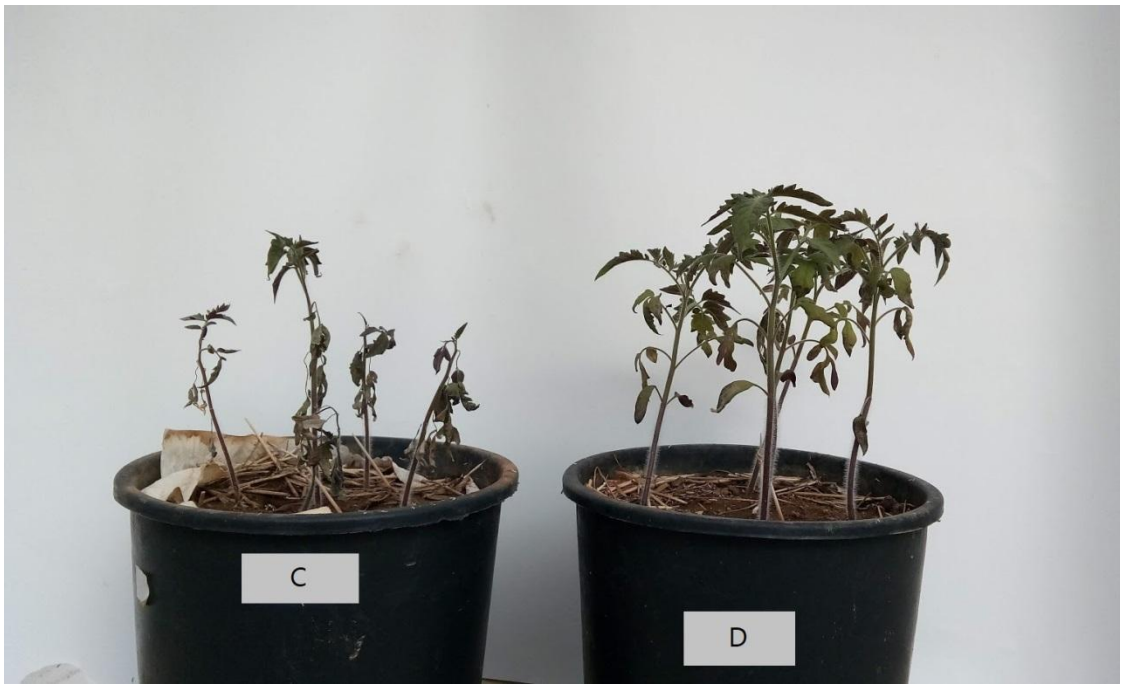

Figure 9 - Soil disturbance effect on the Disease Incidence in tomato plants inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*, after *L. rigidum* as a developer plant. Disturbed soil - ERM disrupted (C) and undisturbed soil - intact ERM (D).

Similarly, the Developer and inoculation factor interaction also significantly impacted the disease incidence. In inoculated plants whose developer was *L. rigidum,* the disease incidence was significantly higher when compared to plants preceded by *O. compressus* (Figure 10).

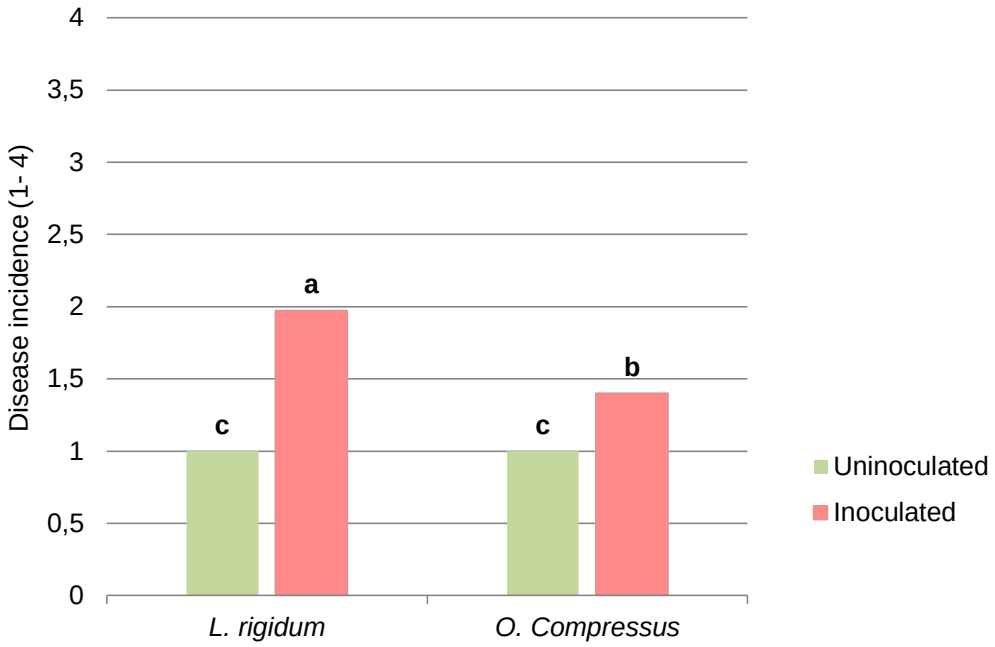

Figure 10 - Effect of the interaction between Developer plant and inoculation with *Fusarium oxysporum* f. sp. *radicis-lycopersici* on the disease incidence. Different lower-case letters highlight significant differences between treatments ($p<0,05$).

Chapter 4

Discussion

Considering that the AMF diversity associated with the roots of the Developer will influence the AMF assemblage colonizing the roots of the following crop, when the colonization of the second plant is preferentially initiated by an intact ERM (Brígido *et al*. 2017), the choice of a host plant to develop the ERM can be used as a tool to select and take advantage of a more beneficial AMF consortia from the available functional diversity in a certain soil. In that sense, the purpose of this work was to improve the knowledge of the effect of two mycotrophic plant species used in previous similar experiments (*Lolium rigidum* and *Ornithopus compressus*) on the development of the ERM, assessing their role and that of an early colonization in the bioprotection of the tomato crop against *Fusarium oxysporum* f. sp *radicis-lycopersici.*

The main findings of this experiment suggest that the *L. rigidum* Developer (and its associated ERM) grants a better AM colonization of tomato plants, leading to significantly higher arbuscular and hyphal colonization rates over *O. compressus*. However, *O. compressus* resulted in a significantly lower disease incidence in Fusarium inoculated tomato plants, which could make it a more adequate choice for a bioprotector role. The importance of an intact ERM and the benefits of an early colonization were also acknowledged, as the disease incidence was clearly and significantly higher in disturbed soils with a disrupted ERM.

As far as is known, no studies have been published comparing the effect of these Developer species on the mycorrhizal colonization of tomato plants. Similar experiments with the two Developers described by Brito *et al*. (2014) with wheat and Alho *et al*. (2015) with subterranean clover did not reveal significant differences in arbuscular colonization at the end of the crop growing cycle, being only slightly greater after O. compressus. A higher AM root colonization rate, however, should not be unquestionably regarded as preferable to a lower one, since it can be misleading about the extent of the symbiosis by not taking into account the relative growth of both organisms (Carvalho *et al*., 2015). In fact, good colonizers are sometimes inferior mutualists when compared to less infective species or isolates of AMF (Hetrick *et al*., 1993). Therefore, even though it delivered an inferior mycorrhizal colonization, it is not unreasonable to consider *O. compressus* a preferable Developer, as its associated AMF diversity resulted in a significantly reduced disease incidence and slightly greater dry weight of the following tomato plants, when compared to *L. rigidum*. Although non-

significant, the Ct values also appear to be generally higher after *O. compressus*, meaning a lower presence of *F. oxysporum,* which is consistent with the previous claim. The slightly increased dry weight likely derived from the improved bioprotection that allowed the plants to grow under a lesser disease stress.

The findings of this study are also consistent with the premises in literature about the importance of an early AM colonization achieved through an intact ERM (Goss *et al*., 2017). In this experiment, the disturbance of the soil and fragmentation of ERM before planting significantly decreased the mycorrhizal colonization of the tomato plants. Consequently, the inoculated tomato plants displayed a significantly higher disease incidence in these disturbed soil conditions, where the ERM had been disrupted, and their dry weight was significantly lower. These results were expected and match the ones described by Brito *et al* (2019), where the presence of an intact ERM developed by *L. rigidum* also significantly increased the growth and reduced disease incidence of tomato plants inoculated with *F. oxysporum* f. sp*. radicis-lycopersici*. Such findings corroborate the importance of a well-established AMF colonization of the crop in dealing with a stressing agent (Cordier *et al*., 1998; Slezack *et al*., 2000). The AMF protection against soilborne pathogens consists of many mechanisms mediated by AMF-host plant interactions that may operate simultaneously at multiple levels (Azcón-Aguilar & Barea, 1997) such as improved plant nutrition, competition for colonization sites, changes to the root system (Harrier & Watson, 2004; Wehner *et al*., 2010), general immunological priming (Clay, 2014) and priming of jasmonic acid-dependent defenses (Cameron *et al*., 2013). Enhanced by the presence of an intact ERM, the early colonization of AMF allows a precocious and more effective expression of these protecting mechanisms and, in this experiment, granted bioprotection to tomato plants, reducing the disease incidence of *F. oxysporum* f. sp *radicis-lycopersici.*

It is also worth acknowledging the different impact of the soil disturbance on the arbuscular colonization after the two Developers. Disturbing the soil reduced the AC of tomato plants to less than 50% after *L. rigidum,* whereas after *O. compressus* the rates were reduced to less than 20% of their undisturbed counterparts. The basis for this contrast is likely associated with different colonization strategies of their associated AMF species, as hyphae are more sensitive to soil disturbance than spores and subsequent colonization of additional roots is affected to a greater extent (Schalamuk & Cabello, 2010). This difference suggests that the AMF species associated with *O. compressus* that colonized the tomato plants are particularly sensible to the disruption of ERM, likely relying more on hyphal colonization than those associated with *L. rigidum.*

However, the results and reasonings of this work are limited by the output values produced by the qPCR. The absence of significant variance between Ct values and factors such as inoculation with *F. oxysporum* was certainly unpredicted, as was their low correlation with the disease incidence parameter. It was expected that the Ct values would be inversely proportional to the disease incidence and as such, that they would be generally lower in samples from inoculated tomato plants; yet they didn't significantly vary between inoculated and uninoculated plants.

Considering this outcome, and the fact that the experiment was conducted in a greenhouse with a semi-controlled environment, it is possible that another factor may have affected the results. The used soil was not sterilized, the pots were relatively close together and the greenhouse was being used for other experiments at the time, all of which hint towards the possibility of plant contamination. Furthermore, when assessing the disease incidence, some uninoculated plants were noted to irregularly display patterns of wilting on the leaves, which further consistent with this explanation. It isn't unlikely that *F. oxysporum* spores could have been disseminated through the air current or through the surplus irrigation water that accumulated at the bottom of the pots (Roberts *et al*., 2001; Blancard, 2017), and it is also possible that some other pathogen might have infected the plants through the collected soil or some other sources in the greenhouse environment.

Contamination with another soilborne pathogen might also explain unpredicted values such as the generally higher dry weight of inoculated plants from the *O. compressus* treatment, which was expected to be lower compared to uninoculated plants. In uninoculated plants, the soilborne pathogen would not be competing with *F. oxysporum* f. sp *radicis-lycopersici* and its free growth could result in a higher disease severity, crippling the plant growth and thus reducing the dry weight. Since the presence of *Fusarium* was detected across the majority of samples, it also seems likely that the inoculated *Fusarium* was disseminated in some way and contaminated the other uninoculated plants. However, considering that the primers and probes used in the TaqMan qPCR assay were designed to generally target *Fusarium* spp. (Campos *et al*., 2019), if the unknown pathogen belonged to the *Fusarium* genus it would be amplified in the procedure and influence the resulting Ct values. Given this lack of specificity in the qPCR procedure, it is impossible to confirm how exactly the results have been compromised.

Regardless of the problems and limitations of this work concerning the qPCR procedure and the possibility of contamination, the results still serve their purpose on

further clarifying the Developer choice of tomato plants. It was clear that there was a different performance of the two Developers in granting bioprotection against the present pathogen (whether or not the presence of *F. oxysporum* f. sp. *radicis-lycopersici* was proved with certainty) and in improving dry-matter production of tomato plants, where *O. compressus* stood out positively. The mentioned limitations also didn't invalidate the results regarding the ERM integrity, as it was visible the effect of disturbing the soil (and disrupting ERM) on the growth and disease incidence in the tomato plants, which was later supported by the mycorrhizal colonization assessment in the lab.

Chapter 5

Conclusions and future prospects

This dissertation aimed to assess the role of an early AMF colonization, granted by ERM previously developed in the soil by two different host plants (*L. rigidum* and *O. compressus*), in the bioprotection of tomato plants against *Fusarium oxysporum* f. sp. *radicis-lycopersici.* The findings indicate that although *L. rigidum* resulted in a superior AM colonization of tomato plants, *O. compressus* would be more suitable for a bioprotection role since it was significantly more effective in reducing the disease incidence of *F. oxysporum*. The early AM colonization, achieved through an intact ERM, proved critical in granting bioprotection to tomato plants, as the disease incidence was significantly lower in undisturbed soil (and undisrupted ERM) conditions.

Considering the contamination that occurred during the experiment, future works may benefit from its prevention by conducting the experiment in a more controlled environment, spatially isolating inoculated plants from uninoculated ones and using plates to contain irrigation water that would exit at the bottom of the pots.

The use of primers and probes in the TaqMan qPCR assay designed to specifically target *Fusarium oxysporum* f. sp. *radicis-lycopersici* would also have clarified the results and might be useful to future experiments. By using more specific primers and probes, the nature of contaminations could be determined since the Ct values would either be consistent with the inoculation factor, meaning another pathogen or factor had affected the results, or inconsistent, indicating a contamination between inoculated and uninoculated plants.

However, this work holds useful information for the prospect of managing indigenous AMF in agricultural systems, as the interactions between different crops and their rhizosphere microbiomes are still unfamiliar. The bioprotection strategy followed in this work is particularly relevant in the current agronomic situation, as there are yet no approved products for the chemical control of *F. oxysporum* and there is an increasing pressure for more ecologically friendly solutions. A bioprotection solution resorting to AMF seems promising, although further experimentation with other plant species is required in order to determine the optimal Developer for a single issue.

Ultimately, the right choice of the Developer could improve the chances of getting functional advantages from AMF in a crop sequence, such as bioprotection against

soilborne pathogens, and the knowledge presented in this work brings us a step forward towards the possibility of their reliable use in agriculture.

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