INSTITUTO POLITÉCNICO DE BEJA

Escola Superior Agrária

Mestrado em Agronomia

Adjusting cultural practices to promote tomato mycorrhization and bio-protection against Fusarium diseases

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Beja,

2022

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Dissertação de Mestrado a apresentar na Escola Superior Agrária do Instituto Politécnico de Beja

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Beja,

2022

Acknowledgements

I would like to acknowledge and express my thanks to my supervisor, Maria Isabel Patanita, who has supported and guided me throughout this work.

Moreover, I am extremely thankful to my co-supervisor, Isabel Brito, responsible for the research project that provided the results explored in this work and in which I was included as a researcher. I would like to express my appreciation for all the knowledge, support and guidance developing this work and for the opportunity to be a part of such an interesting project in which I was able to learn so many fascinating things.

This endeavor would not have been possible without Professor Ana Alexandre, who helped in all things related to the research project from beginning to end, and was always available to teach and aid me whenever I needed.

To Professor Maria do Rosário Félix, words cannot express my gratitude for all the kindness, friendship, knowledge and unconditional guidance provided.

I wish to extend my special thanks to all participants that took part in the study and enabled this research to be possible. It was extremely important to have all of you as part of this work, either helping in the collection, processing or analysis of the field samples, analyzing data or giving brilliant comments and suggestions.

This work was funded by National Funds through Regional Operational Program Alentejo 2020 and co-founded by European Regional Development Fund (ERDF) under the Project ALT20-03-0246-FEDER-000056.

Finally, my warmest thanks to all my friends and family, who couldn't have been more supportive and were able to give all the encouragement I needed.

Abstract

The production of tomato for processing is in need of new cultural practices that allow lower environmental impacts and more efficient use of resources. The main goal of this work is to test the effect of anticipating soil loosening and plant-beds preparation together with the use of cover crops on soil biological parameters including the early mycorrhization of tomato roots for the control of Fusarium diseases. Soil enzymatic activity, cover crop dry matter production, incidence of nematode and Fusarium diseases and mycorrhizal colonization rate of the cover crops and tomato plants, were the parameters assessed in two locations – Pancas and Salvaterra. Two different cover crops were grown over winter and controlled in spring. Sampling and analysis were performed throughout the year. The results showed no significant influence of the treatments (oat cover crops, oat + rapeseed cover crops and test field control) in most of the parameters analyzed, which was not entirely surprising because of the intensive management of these fields over the past years. However, in Pancas, the oat + rapeseed treatment presented significantly lower nematode incidence. This proves the nematocidal potential of rapeseed, and suggests its use as an effective and economically attractive cover crop. Persisting with the implementation of this strategy is fundamental for more tangible results.

Keywords:

Solanum lycopersicum, cover crops, cultural practices, arbuscular mycorrhizae colonization, enzymatic activity, nematode incidence, *Fusarium* spp.

Resumo

A produção de tomate de indústria requer novas práticas culturais que permitam um menor impacto ambiental e uma utilização mais eficiente dos recursos. O principal objetivo deste trabalho é testar o efeito da antecipação da mobilização do solo e preparação dos camalhões, juntamente com a utilização de culturas de cobertura, em parâmetros biológicos do solo, incluindo a micorrização precoce das raízes de tomate para controlo de fusarioses. Os parâmetros avaliados, em dois locais – Pancas e Salvaterra –, foram a atividade enzimática do solo, a produção de matéria seca das culturas de cobertura, a incidência de nemátodos e *Fusarium* spp. e a taxa de colonização micorrízica das culturas de cobertura e tomate. As culturas de cobertura foram semeadas durante o inverno e controladas na primavera. As amostragens e análises foram realizadas ao longo de todo o ano. Os resultados apresentados não mostraram influência significativa dos tratamentos (culturas de cobertura de aveia, cultura de cobertura de aveia + colza e testemunha) na maioria dos parâmetros analisados, o que não foi totalmente surpreendente devido à gestão intensiva destes campos nos últimos anos. No entanto, em Pancas, o tratamento com aveia + colza apresentou uma incidência significativamente mais baixa de nemátodos. Isto prova o potencial nematodicida da colza, e sugere a sua utilização como uma cultura de cobertura eficaz e economicamente atrativa. Persistir com a implementação desta estratégia é fundamental para a obtenção de resultados mais tangíveis.

Palavras-chave:

Solanum lycopersicum, culturas de cobertura, práticas culturais, colonização micorrízica, atividade enzimática, incidência de nemátodos, *Fusarium* spp.

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List of Abbreviations

AC – Arbuscular Colonization

AM – Arbuscular Mycorrhizae

AMF – Arbuscular Mycorrhizal Fungi

ANOVA – Analysis of Variance

Ct – Threshold Cycle

CTAB - Hexadecyltrimethylammoniumbromide

DM – Dry Matter

DNA - deoxyribonucleic acid

ERM – Extraradical Mycelium

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

FORL – *Fusarium oxysporum* f. sp. *radicis lycopersici*

gDNA – Genomic DNA

HC – Hyphae Colonization

HSD – Honest Significant Difference

ITS – Internal Transcribed Spacer

MANOVA – Multivariate ANOVA

O – oat

O + R – oat + rapeseed

pb – Base pairs

PCR – Polymerase Chain Reaction

PNP - paranitrophenol

qPCR – quantitative PCR

T – test field control

TSWV – Tomato Spotted Wilt Virus

1. Introduction

The production of tomato for processing is facing the great challenge of finding cultural practices that allow lower environmental impacts and more efficient use of resources. Early mycorrhization of tomato plants by native soil arbuscular mycorrhizal fungi (AMF) provides several benefits, including bio-protection against biotic and abiotic stresses. Combining the beneficial management of the functional diversity of native AMF through the use of a cover crops, together with the practice of reduced or no-till techniques to keep the extraradical mycelium (ERM) intact it is possible to promote early arbuscular mycorrhizae (AM) colonization and allow bio-protection of the crop (Brito, Carvalho, & Goss, 2021).

By combining changes in the schedule of cultural practices such as soil loosening and plant-beds preparation with the use of cover crops during winter, the main goal of the present work was to promote tomato early mycorrhization allowing protection against Fusarium deseases. Furthermore, the aim was to investigate the role of cover crops, cultural practices and location, on the levels of soil enzymatic activity, dry matter production, and nematode and Fusarium diseases incidence.

Test fields were set in two different locations, Pancas and Salvaterra, both in the Ribatejo region of Portugal. Two cover crops were investigated, using a no cover crop treatment as a test field control. One of the cover crops used was oat, *Avena sativa,* and the other one was a consociation of oat and rapeseed, *Brassica napus*. Mycorrhization rates of the cover crops and tomato plants, dry matter production of the cover crops, soil enzymatic activity and nematode and Fusarium disease incidence were evaluated in the three different treatments to assess their influence.

The present work includes a literature review, in the first chapter, that comprises a state of the art of tomato production and its main problems and challenges, an assessment about AMF and its role in agronomic systems and, lastly, a review about soil enzymatic activity. Second chapter presents materials and methods, including a description of the study areas, the treatments and experimental design used, the cultural practices carried out and the sampling methods and data analysis performed. Results are presented in chapter three including dry matter production, AMF colonization rates of the cover crops and tomato, enzymatic activity of the soil, nematode and Fusarium diseases incidence, as well as statistical analysis. Discussion of the results is in chapter four where results are

analyzed according to the objectives set for this work and considering previous scientific knowledge. Main conclusions are described in chapter five.

2. Literature Review

2.1. Production of tomato (*Solanum lycopersicum*)

Solanum lycopersicum belongs to a diverse family, the *Solanaceae,* which includes over 3000 species scattered throughout a wide variety of habitats. Most of these species are of economic use, such as food (tomatoes, potatoes, peppers and eggplants), medicine (deadly nightshade, henbane, datura) and ornamental purposes (petunias) (Knapp & Peralta, 2016). Tomato is native to the western coastal area of South America, and its domestication occurred in Mexico, where it was widely cultivated by the Aztecs. Its introduction into Europe took place in the middle of the 16th century, starting in Spain and Italy. The great worldwide expansion of tomato cultivation occurred in the first decades of the 20th century (Almeida, 2014). The cultivated tomato has about 12 wild relatives which have a large genetic diversity that contributed to the breeding of modern tomato cultivars. This has resulted in tomato varieties with the markets desired characteristics, usually with resistance to certain diseases, which are now widely grown (Gatahi, 2020).

Tomato is considered one of the world's most important and widespread horticultural crops. This is an herbaceous plant of shrubby stature, cultivated as an annual, being a warm-season crop. The crop can be propagated by direct sowing, although transplanting is normally used. There are different cultivars which can be of determinate or indeterminate growth and vertical or horizontal growth. The shape of the fruit can be round, pyriform, elongated, or others, and the color of the ripe fruit can also vary between yellow, pink, orange or red (Almeida, 2014). The fruits have high-value properties and can be used for fresh consumption in salads or cooked as a vegetable, or processed into tomato paste, tomato sauce, ketchup, juice or dried tomato (Bawa, 2016; Motamedzadegan & Tabarestani, 2018).

In 2020, the world's area of harvested tomato was 5 015 983 ha, producing a total of 186 821 216 tons of tomatoes. The world's area of harvested tomato and its production has been growing over the last decades and the Asian continent presents the higher share of the world's production (54,5%), followed by America (17,5%) and Europe (15,7%).

Africa is responsible for 11,9% of the production and Oceania for only 0,3%. China is the main producer of tomatoes in the world, having an average production of 37 649 822 tons/year (between 1994 and 2020). The United States of America, India, Turkey, Egypt, Italy, Iran, Spain, Basil and Mexico also belong to the top ten producers of tomatoes in the world (FAO, 2022).

In Portugal, tomato is one of the main vegetable crops for fresh consumption and the main crop for horticultural industry. In 2020, production of tomato in Portugal was about 1 399 210 tons from a harvested area of 15 040 ha (FAO, 2022). The cultivation of tomatoes for industry is mostly distributed throughout Ribatejo, Douro Valley, Sorraia Valley and some irrigated areas of Alentejo, while tomatoes for fresh consumption are mostly produced in Ribatejo e Oeste, Algarve and Entre-Douro-e-Minho (Almeida, 2014; Ribeiro *et al.*, 2022).

Tomato is a warm-season crop, requiring day temperatures of 25ºC to 30ºC and night temperatures of 16ºC to 20ºC for optimal growth. For fruit development optimum temperatures are set at 18-24ºC, the night temperatures being more critical than day temperatures (Garg & Cheema, 2011; Motamedzadegan & Tabarestani, 2018). Although it grows better under warm conditions and low humidity, this is a fairly adaptable crop. Nevertheless, temperatures above 30ºC reduce fruit set, lycopene development and flavor, as well as yields. Low temperatures delay color formation and ripening. High humidity may increase disease attacks and also affect fruit ripening and that is why tomato thrives best in low to medium rainfall with supplementary irrigation during the off season (Bawa, 2016). Well drained and highly amended soils with good moisture retaining capacity, high organic matter and a pH range of 5-7,5 are ideal to grow tomato.

Productivity and quality of tomato is affected by many different factors and field crop management is critical for the success of the crop. Soil preparation with pre-planting water, fertilizers, chemical treatments and favorable plant bed conditions are some of the first concerns. Weed control, mineral nutrition, water economy and plant protection against pests and diseases during crop growth cycle are the basis for a good productivity and fruit quality (Atherton & Rudich, 1986).

2.2. Problems and challenges of tomato production

The production of industrial tomato is facing the great challenge of finding cultural practices that allow lower environmental impacts and more efficient use of resources. The

major concern is the excessive and increased use of agrochemicals, particularly in pest and disease management, that has raised a number of economic, ecological and health concerns. Economic concerns arise from the over reliance and increased costs of production of these inputs. Additionally, indiscriminate use of agrochemicals has resulted in ecological problems such as common pests and pathogenic agents developing resistance, elimination of natural enemies and heavy accumulation of chemical residues in the soil and water. Human health concerns focus on workers welfare, deviations from recommended doses and excessive run-off into soil and water sources. In a world where people are conscious about environmental impacts of agrochemical and all the health issues connected to their excessive use, finding sustainable cultural practices is a worldwide priority (Gatahi, 2020; Karungi, Kyamanywa, Adipala, & Erbaugh, 2011).

Among the many constraints affecting productivity and quality of tomato crop, diseases play a salient role and can be caused by many different agents including fungi, fungus-like organisms, bacteria, viruses and phytoplasmas, as well as physiological disorders, responsible for symptoms that include fruit spots, rots, wilts, and leaf spots/blights (Jones, 2014; Pritesh & Subramanian, 2011). Some of the most common diseases consist in late and early blight, caused by *Phytopthora infestans* and *Alternaria solani*, respectively, anthracnose (*Colletotrichum* spp.), bacterial wilt (*Ralstonia solanacearum*), bacterial canker (*Clavibacter michiganensis*), tomato spotted wilt virus (TSWV), verticillium wilt (*Verticillium* spp.), fusarium crown and root rot (*Fusarium oxysporum* sp. *radicis lycopersici*) and fusarium wilt (*Fusarium oxysporumsp. lycopersici*) (Agrios, 2005; Bawa, 2016; Gatahi, 2020). Regarding pests, some of the most important and common ones are root knot nematodes (*Meloidogyne* spp.), white fly (*Bemisia tabaci*), spider mites, thrips that can also transmit the TSWV, caterpillar american boll worm (*Helicoverpa* spp, *Heliothis punctigera* and *H. armigera*), aphids and leaf miners (*Tuta absoluta*) (Agrios, 2005; Gatahi, 2020).

This review will focus on Fusarium wilt and Fusarium crown and root rot, as well as on root knot nematodes, considering these are the problems addressed by the objectives of the present dissertation.

2.2.1. Fusarium wilt and Fusarium crown and root rot

Fungal diseases have high impact on tomato productions and, as mentioned before, fusarium wilt and fusarium crown and root rot are some of the most important ones, because they are responsible for severe yield losses throughout the world. These diseases

are caused by *Fusarium* spp., soilborne fungi, that are able to infect tomato plants by spore germination or mycelium, resulting in higher plant transpiration and lower nutrient translocation, causing wilting, crown and root rot and, ultimately, death of the plant (Akbar, Hussain, & Ali, 2018; Manikandan, Harish, Karthikeyan, & Raguchander, 2018).

Fusarium oxysporum is a worldwide spread and phylogenetically diverse species, well known as a mycotoxin producer (Irzykowska *et al*., 2012), and is considered as the most frequent species causing wilts, as well as crown and root rot, in different crops. Nevertheless, other Fusarium species have been constantly evolving and increasingly associated with many wilt diseases affecting different vegetables including bell pepper, chili pepper, cauliflower, sweet pepper, onion, potato, tomato and many others (Jamiołkowska, 2008; Li *et al*., 2017; Ramdial, Hosein, & Rampersad, 2016).

Considering *F. oxysporum* species, the two main formae speciales are *F. oxysporum f. sp. lycopersici* (FOL) and *F. oxysporum f. sp. radicis-lycopersici* (FORL). These formae speciales display genetic, epidemiological and symptomatologic differences. However, they are very difficult to discriminate by morphological and physiological features (Nelson, Toussoun, & Marasas, 1983; Rowe, 1980). FOL is responsible for Fusarium wilt, and FORL causes Fusarium crown and root rot, which are among the most intensively studied plant diseases. Both formae speciales cause extensive production losses in tomato fields and greenhouses, being considered as limiting factors for tomato production, despite the current management techniques available (McGovern, 2015).

Even though there are many management strategies that can prevent or reduce Fusarium diseases, most of them are harmful to the environment or not effective (Paul *et al.*, 2021). Once these pathogens spread in the field, their elimination is very difficult. Currently, the methods used to control these diseases are soil disinfection using fumigants, hot water or solarization, or using resistant cultivars, one of the most popular management strategies used as an alternative to agrochemical products. In order to solve these problems, research of other techniques to control Fusarium diseases, such as biological control and induced disease resistance, has been advanced (Arie, 2019).

2.2.2. Root knot nematodes

Root knot nematodes belong to the genus *Meloidogyne*, with more than 90 species described, although only 23 were found in Europe. *Meloidogyne* is considered worldwide as the most important genus of plant-parasitic nematodes, however information in the scientific literature on the economic impact of root-knot nematodes in Europe is scarce

(Wesemael, Viaene, & Moens, 2011). In tomato, *M. javanica* and *M. incognita*, are the prevalent species, found specially in warmer conditions of southern Europe, with 100% damage potential (Mekete, Mandefro, & Greco, 2004; Vito, Cianciotta, & Zaccheo, 1991; Wesemael *et al*., 2011).

In general, *Meloidogyne* spp. can occur in a wide range of soil types but their association with crop damage is markedly evident on sandy soil or sandy patches within fields (Van Gundy, 1985). However, damage from root knot nematodes is often overlooked, aboveground field symptoms may include stunning, chlorosis, wilting under sufficient soil moisture, and increased susceptibility of plants to other diseases, ultimately causing reduced yields (Ploeg, 2002). Symptoms on host roots infested are usually easily recognizable, although they can also be very small and hardly visible, and consist of galls ranging, encompassing the entire root (Moens, Perry, & Starr, 2009; Ploeg, 2002).

In addition to the direct losses due to nematode attacks, many indirect losses can occur. Nematode-damaged roots do not utilize water and fertilizers as efficiently as healthy roots do, and so there is a waste of resources. Additionally, root knot nematodes can be involved in disease complexes with other plant pathogens and their attacks can lower or break down plant resistance mechanisms (Wesemael *et al*., 2011). For instance, interactions between *Meloidogyne* spp. and Fusarium wilt have frequently been reported in many host crops, as well as interactions with *Rhizoctonia solani* and *Thielaviopsis basicola* (Back, Haydock, & Jenkinson, 2002; Castillo, Navas-Cortés, Gomar-Tinoco, Di Vito, & Jiménez-Díaz, 2003).

Management strategies to prevent and control root knot nematodes infestations are mostly based on chemical treatments. However, they have been restricted over the past few years in Europe due to environmental and health issues, limiting management options or obliging growers to apply more expensive control measures. Prevention measures, such as the use of certificated and healthy plants, decontamination of the soil (solarization or fumigation) and cleaning of machinery, are some of the most important ones to avoid spreading of nematodes. Crop rotations with non-host, immune or resistant crops can be used to control root knot nematodes, although the choices are limited due to the wide host range of several important species of nematodes (Wesemael *et al*., 2011). The use of marigold (*Tagetes* spp.) and rapeseed (*Brassica spp.*), both with nematocidal potential, has been studied with successful results in suppressing a wide range of nematode species, in both greenhouses and field conditions (Mojtahedi, Santo, Hang, & Wilson, 1991; Wang, Jooks, & Ploeg, 2007). Weed control is also very important since many weeds are

hosts to *Meloidogyne* spp. and can act as reservoir for these pests. Biological control using nematophagous fungi and bacteria have also been subject of many studies in Europe, however these agents generally provide too little control to be effective alone and their successful use in sustainable management strategies will depend on their integration with other control measures (Wesemael *et al.*, 2011).

2.3. Promoting tomato mycorrhization and bio-protection against biotic and abiotic stresses

2.3.1. Arbuscular mycorrhizal fungi (AMF): taxonomy, biology and morphology

AMF are endophytic fungi that grow within the root cortical zone and are obligate symbionts that display mutualist interactions with plants (Goss, Carvalho, & Brito, 2017). These interactions are based on bidirectional nutrient exchange, in which the fungi benefit from photosynthetic products of the plant and the plant takes advantage of an enhanced nutrient uptake and bio-protection against biotic and abiotic stresses (Brito, 2008).

AMF constitute a phylum of their one, the *Glomeromycota*, which is divided into four orders, namely *Glomerales, Diversisporales, Archaeosporales* and *Paraglomerales* (Schüβler, Schwarzott, & Walker, 2001), eleven families and twenty-two genera, which incorporate more than 220 different species (Krüger, Krüger, Walker, Stockinger, & Schüßler, 2012), identified based on the morphological characteristics of the spores (Krüger *et al*., 2012; Öpik *et al*., 2013). Species diversity of AMF in a specific location is enormous and varies with several environmental factors, among which are biotic factors (host plant species available) or abiotic factors (pH, nutrient availability, salinity, soil aggregation) (An, Hendrix, Hershman, Ferriss, & Henson, 1993). Each plant can establish symbioses with more than one AMF species, since several species of these fungi can coexist on the same root (McGonigle & Fitter, 1990). However, although they do not have strong host specificity, recent ecological studies on the diversity of AMF associated with different plants have confirmed that there is a strong host plant preference for specific AMF genotypes (Croll *et al*., 2008; Öpik, Metsis, Daniell, Zobel, & Moora, 2009; Scheublin, Ridgway, Young, & Van Der Heijden, 2004).

Any root system exhibiting arbuscular mycorrhizae has three important components: the host plant root itself and two associated mycelial systems, one in the soil, the extraradical mycelium (ERM), and the other within the root apoplast, formed by hyphae

that develop intercellularly within the plant. ERM, which grows in the soil and can extend several centimeters from the root surface, allows large volumes of soil to be exploited enhancing the uptake of many mineral nutrients, including phosphorus, allows the structural stability of the soil to be improved, colonizes other plants, supports and interacts with other soil organisms, and acts as a communication channel between plants (Brito, 2008; Goss, Carvalho, & Brito, 2017; Smith, & Read, 2008). In the internal component of the mycelium, within the root apoplast, AMF have distinctive structures called arbuscules, which are intracellular structures that have tree-like branching. Arbuscules are essential sites for the plant-fungus exchanges, where plants provide carbon compounds and the fungus provides mineral nutrients acquired from the soil (Goss, Carvalho, & Brito, 2017; Patanita, 2018) (Figure 1).

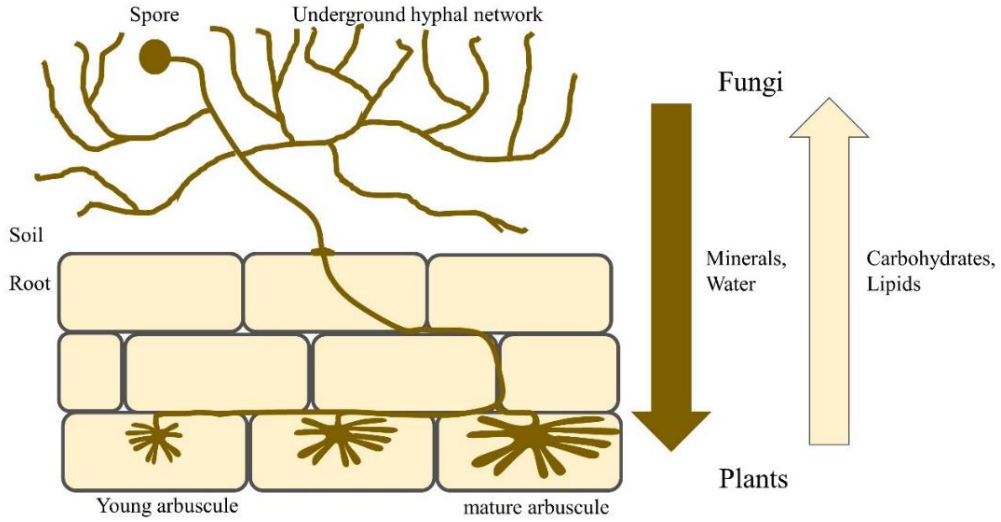

Figure 1 *- Schematic representation of AMF establishment inside a host plant and the known exchange between the two partners. Source: Florence Sessoms, in https://turf.umn.edu/news/arbuscular-mycorrhizal-fungi-tiny-friends-big-impact.*

The only known form of reproduction of this type of fungi is asexual reproduction, and is ensured by spores (long-term resistance structures that remain viable even when the host plant is not present) (Kabir, 2005). In most species, these develop from the ERM, although in some cases their development may occur intra-radicularly. Colonization of non-colonized tissues by AMF may be initiated by spores, previously colonized root fragments or ERM, although ERM is the preferential source of inoculum, allowing an early colonization and higher efficiency than the remaining propagules [36, 47,49].

2.3.2. AMF in agronomic systems and bio-protection against biotic and abiotic stresses

There is considerable evidence about the benefits of AMF association with plants in agronomic systems. Most of these benefits are related to the increase in soil volume that can be exploited by means of ERM (Bedini *et al*., 2009; Caravaca, Alguacil, Azcón, & Roldán, 2006), which absorbs nutrients found in the soil and is able to translocate them from long distances to the root of the host plant. Because they are thin, the hyphae of AMF can explore soil volumes unreachable by root hairs, and are able to access a greater amount of water and soil mineral elements more efficiently (Barley, 1970). This results in one of the most obvious and widely recognized advantages of mycorrhiza formation: the ability to absorb more water and nutrients (including nitrogen, phosphorus, potassium, calcium and magnesium), especially those that have low mobility in the soil and can't be absorbed as easily as others, such as phosphorus (Clark & Zeto, 2000; George, Marschner, & Jakobsen, 1995; Patanita, 2018).

In addition, AMF hyphae stabilize soil aggregates by 'enmeshing' their particles (Rillig & Mummey, 2006) and, as a result of the production of substances that bind them together, there is increased stability of soil structure (Goss & Kay, 2015).

Moreover, AMF are well known for increasing plants protection against abiotic and biotic stresses. Regarding abiotic stresses, AMF are recognized for helping plants thrive in hostile environments (Barea *et al*., 2011), which is of utmost importance given that adverse conditions, particularly exacerbated by global climate change, generate a wide variety of stresses that affect the stability of natural and agricultural ecosystems (Compant, Van Der Heijden, & Sessitsch, 2010). There are numerous studies about the benefits to plants under abiotic stresses provided by AMF, including tolerance to drought (Augé, 2001; Augé, Toler, & Saxton, 2015), salinity (Evelin, Kapoor, & Giri, 2009; Porcel, Aroca, & Ruiz-Lozano, 2012), heavy metal toxicity (Alho, Carvalho, Brito, & Goss, 2015; Brito, Carvalho, Alho, & Goss, 2014; Meier *et al.*, 2015), heat (Compant *et al*., 2010), cold (Charest, Dalpé, & Brown, 1993), industrial effluents (Oliveira, Dodd, & Castro, 2001), or osmotic stress (Ruiz-Lozano, 2003). Likewise, AMF can also improve performance after transplant shock (Meddad-Hamza *et al*., 2010).

When considering protection given by AMF to biotic stress, they have been studied in various crop-enemy combinations (Whipps, 2004) and are generally considered good allies in meeting the challenges for protecting host plants against biotic stresses. These fungi can provide host plants with some level of bio-protection against many soil

pathogens (Harrier & Watson, 2004), and this is possibly the most important role of AMF in natural ecosystems (Garg & Chandel, 2010). Plants colonized by arbuscular mycorrhizae have been observed to receive protection against pathogens, which is not observed in non-mycotrophic plants (Filion, St-Arnaud, & Jabaji-Hare, 2003). Many studies have reported that AMF can protect plants against a wide range of pathogens, including soil-borne fungal and bacterial pathogens, nematodes, phytopathogenic insects, and parasitic plants (Patanita, 2018; Pozo & Azcón-Aguilar, 2007; Whipps, 2004).

Some of these studies have proven that there is a functional complementarity, meaning that protection against pathogens is higher when there is a higher diversity of AMF species (Hu *et al*., 2010; Wehner, Antunes, Powell, Mazukatow, & Rillig, 2010).

Considering all the benefits stated above, the use of commercial inoculums of AMF on agricultural fields has the potential to improve sustainable management of crops. However, considering the obligate nature of the symbiosis and the diversity of species needed to accomplish functional complementarity, the production of commercial inoculum entails high costs, making its acquisition uncompetitive compared to the acquisition of fertilizers (Munkvold, Kjøller, Vestberg, Rosendahl, & Jakobsen, 2004; Saito & Marumoto, 2002). Thus, in the agronomic context, native populations of AMF represent a better alternative, because they are more abundant, diverse and well adapted than commercial inoculum (Goss, Carvalho, & Brito, 2017; Patanita, 2018).

The expression of the mechanisms that mediate AMF and host-plant interactions and can cumulatively grant plant bio-protection is certainly more effective in a well-established symbiotic mycorrhizal association (Garg & Chandel, 2010). Therefore, the extent of AMF colonization of the plant roots when confronted with the stress-causing agent is directly related to the level of bio-protection achieved (Diedhiou, Hallmann, Oerke, & Dehne, 2003; Khaosaad, García-Garrido, Steinkellner, & Vierheilig, 2007; Sikora *et al*., 2008). The symbiotic association must be created and well established before contact with the stress-causing agent to provide a high level of protection (Nogales, Aguirreolea, Santa María, Camprubí, & Calvet, 2009; Petit & Gubler, 2006; Rufyikiri, Declerck, Dufey, & Delvaux, 2000).

Considering the described above, it is important to highlight the imperative role of the ERM as a particularly effective source of propagules, since it allows early colonization of the host plant and develops faster than other types of propagules (Brito, Carvalho, Alho, Caseirio, & Goss, 2013; Martins & Read, 1997; McGonigle, Miller, Evans, Fairchild, & Swan, 1990). Accordingly, it is crucial to maintain the ERM intact to

overcome the limitations associated with the use of AMF in bio-protection (Brito *et al.*, 2014; Sikora *et al*., 2008) and allow early and rapid colonization of the host crop to provide adequate protection (Garg & Chandel, 2010; Khaosaad *et al*., 2007). Conversely, if the integrity of the ERM is affected, notably through the use of soil tillage, the colonization of the plant by AMF decreases and consequently leads to less efficient crop protection (Lendzemo & Kuyper, 2001).

There has been a growing interest in sustainable cropping systems that reduce environmental degradation. Cover crops can be an important component of these systems because of its contributions to soil conservation, quality and natural fertility, and crop performance. A cover crop can reduce soil erosion, increase water infiltration rate, organic matter content and nutrient availability in the soil, and control certain weeds (Munawar, Blevins, Frye, & Saul, 1990; Shepherd & Webb, 1999). Some cover crops are also hosts of AMF (Kabir & Koide, 2000) and can help maintain or increase the native mycorrhizal inoculum present in the soil by forming a network of extra-radicular mycelium (Kabir & Koide, 2002)

Accordingly, the strategy in study by the present work combines the beneficial management of the functional diversity of native AMF through the use of a cover crop that precedes the crop and acts as a developer for AMF, together with the use of reduced or no-till techniques to keep ERM intact and promote early AM colonization. This strategy has been studied and developed by researchers from University of Évora that already conducted pot trials and field trials proving its effectiveness in the bio-protection of the crop for instance, against *Fusarium oxysporum* infection in tomato or *Cephalosporium maydis* infection in maize (Brito *et al*., 2019; Brito *et al*., 2021; Carvalho, Brito, Alho, & Goss, 2015; Goss, Carvalho, & Brito, 2017; Patanita, 2018).

2.4. Soil enzymatic activity

Soil is a dynamic, living, natural and complex system due to the interactions between its biotic and abiotic compounds, including all physical, chemical and biological factors. Soil quality is one of the essential needs for increased agricultural productivity in various land uses and managements (Almeida, Naves, & Pinheiro, 2015; Bulletin *et al.*, 2017). Therefore, soil quality is defined by its capacity to function, within land use and ecosystem boundaries, to sustain biological productivity, maintain environmental quality

and promote plant, animal and human health (Carter, Gregorich, Anderson, Doran, Janzen, 1997).

In order to maintain soil quality, a balance between the parameters that define it is needed. Physical parameters are based on texture, root depth, infiltration rate, bulk density and water retention capacity. Chemical parameters are pH, total carbon, electrical conductivity and nutrient level. Biological and biochemical components are the primary indicators of soil health because they are dynamic, meaning they are sensitive to the changes that may occur in the presence of any degrading agent and may respond to changes in soil management more quickly than other soil variables. These components are C and N microbial biomass, potentially mineralizable N, soil respiration and enzymatic activity (Fazekašová, 2012; Seoane & Leiro, 2005).

Soil enzymes are often used as soil quality indicators because they are involved in energy transfer, release of inorganic nutrients for plant growth (C, N, P and S), organic matter decomposition, transformation of native soil organic matter, nitrogen fixation, detoxification of xenobiotics and the stabilization of soil structure (Bulletin *et al*., 2017; Utobo & Tewari, 2014). Therefore, enzymes are used as an index of soil microbial activity and fertility and, consequently, affect environmental quality and crop production (Tang *et al*., 2014). There are several representative enzymes used to study soil quality (Bulletin *et al*., 2017) and the present study focuses on phosphatase, ß-glucosidase, arylsulfatase and urease.

In recent years, many studies have shown that the enzyme and microbial activities of the soil are affected by soil tillage, cover crops and residue management (Ekenler & Tabatabai, 2003; Tang *et al*., 2014), application of fertilizer and organic matter (Crecchio, Curci, Pizzigallo, Ricciuti, & Ruggiero, 2004), crop rotations (Hamido & Kpomblekou-A, 2009), and other field management strategies (Bandick & Dick, 1999). For instance, no-till systems with winter cover crops, which are grown during an otherwise fallow period, already proved to be efficient in increasing phosphatase, ß-glucosidase and arylsulfatase enzyme activities (Chavarría *et al*., 2016; Mullen, Melhorn, Tyler, & Duck, 1998; Tang *et al*., 2014; Tyler, 2020). However, studies on urease activity present more controversial results, while some authors proved that living cover crops stimulated the activity of urease (Adetunji *et al.*, 2021), others obtain results that showed no effect of the cover crops in its activity (Hamido & Kpomblekou-A, 2009).

Phosphatases are a broad group of enzymes that play a key role in phosphorous cycle and are correlated to phosphorous stress and plant growth, being good indicators of soil fertility (Nannipieri, Giagnoni, Landi, & Renella, 2014; Riah, Laval, & Trinsoutrot-gattin, 2014). These enzymes can transform organic phosphorous into inorganic forms that are suitable for plants (Tang *et al.*, 2014).

ß-glucosidase is one of the most predominant enzymes in soils that catalyzes the hydrolysis of various ß-glucosides present in plant debris decomposing in the soil and resulting in glucose. Therefore, these are very important enzymes involved in the transformation/decomposition of organic matter in soil and its final product, glucose, is an important carbon energy source for soil microorganisms (Bulletin *et al*., 2017; Riah *et al.*, 2014; Tang *et al*., 2014).

Arylsulfatases are typically widespread in soils and are responsible for the sulfur cycling because of its involvement in the mineralization of organic sulfur compounds into inorganic forms for plant uptake. These enzymes can be secreted by bacteria into the external environment as a response to sulfur limitations. Thus, their presence in different soil systems is often correlated with the rate of microbial biomass, soil organic carbon content and rate of sulfur immobilization (Bulletin *et al.*, 2017; Mirleau, Wogelius, Smith, & Kertesz, 2005).

Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia, being a key component in the nitrogen cycle in soils, a process considered vital in the regulation of nitrogen supply to plants after fertilization (Adetunji *et al*., 2021; Hamido & Kpomblekou-A, 2009).

3. Materials and Methods

3.1. Study area

Study areas, Salvaterra and Pancas industry tomato fields, are located in the Ribatejo region of Portugal, both being intensively cropped with tomato and known to be infested with Fusarium diseases for many years. Salvaterra field is located in Salvaterra de Magos (39°02′10.6″ N, 8°47′54.5″ W) and Pancas field is located in Herdade de Pancas, Samora Correia (38º51′48.1″ N, 8º54′46.1″ W). Both study areas can be observed in Figure 2, as well as the test field locations within those areas. Salvaterra test field is, approximately

2.09 ha, each line being 696 m long. Pancas test field is, approximately 1,41 ha, each line being 460 m long.

In this region, during 2021, maximum temperatures varied from 13°C to 37°C, and minimum temperatures varied from -2°C to 16°C (Meteoblue, 2022).Composition of the soil from Salvaterra field is silt loam, with silt as a dominant component, however Pancas has clay in higher proportion, having a clay loam soil (information obtained by soil analysis performed by the producer). High moisture was found on the study areas, since this was a drip irrigated tomato field.

3.2. Treatments and experimental design

Salvaterra and Pancas industry tomato fields, have been intensively cropped with tomato for decades, which includes high input of pesticides, especially sodium metam treatments and successive and profound soil tillage.

To study the influence of adjusting cultural practices, namely the presence of cover crops, in the promotion of tomato mycorrhization and bio-protection against biotic and abiotic stresses, in each of the locations, test fields with three different treatments were installed. First treatment used was an oat, *Avena sativa,* cover crop, using oat as a developer for ERM. Second treatment was an association of oat and rapeseed, *Brassica napus*, using oat as a developer for ERM and rapeseed, which is a non-mycotrophic species, for its nematicide characteristics. The third treatment used was a no cover crop so that it would be possible to compare the results with a test field control. Experimental design used to study the influence of the treatment (independent factor) on the mycorrhizal colonization rate, soil enzymatic activity, nematode incidence and *Fusarium* spp. infection (dependent factors) was two randomized blocks (two replicates) with three treatments (two different cover crops and one control treatment), to sample 4 replicates per treatment and per block (random points along the block).

Figure 2 *- Study areas and test field locations. Source: Google earth, accessed on 5th of July 2022.*

3.3. Cultural practices

In order to allow the implementation of a cover crop to promote AMF, soil loosening and plant-beds preparation was anticipated in relation to the usual season and done immediately after tomato harvest, in September 2020, instead of before tomato plantation next year, as traditionally done. Cover crops were sown on the previously prepared ground during October 2020, using 60 kg/ha for the oat cover crop, and 30 kg/ha and 2 kg/ha, respectively, for the oat and rapeseed consociation cover crop (Figure 3). This change in the cultural practices schedule allows the development of an ERM associated to the cover crops over winter, the integrity of which must be maintained, especially by preventing profound tillage. Therefore, on February 2021, cover crops were controlled using 3,2 L of glyphosate and 1,5L of MCPA in 150 L of water per hectare. Figure 4 shows Salvaterra field including the test fields after cover crop control and the rest of the farm.

Figure 3 *- Cover crops of oat and consociation of oat and rapeseed during winter. Source: https://www.bioprotomate.uevora.pt/, accessed on the 5th of July 2022.*

Figure 4 *- Comparison between test fields and the rest of the farm after cover crop control. Source: https://www.bioprotomate.uevora.pt/, accessed on the 5th of July 2022.*

During April 2021, a surface shredder was used on the cover crop treatments to cut the remaining straw of the cover crops and plant-beds were rebuild to prepare the soil for tomato plantation. Additionally, fertilizer application was done before plantation. Plantation was carried out on May 2021 for the Salvaterra field and on June 2021 for the Pancas field (Figure 5). The plants used in Salvaterra were from the H1534 variety, from Heinzseed (Stockton, California, USA), which has a crop cycle that lasts between 110 to 115 days with mid-season maturity and can grow under humid or arid conditions (Heinz, 2019). The plants used in Pancas were from the Olivenza variety, with a crop cycle that lasts about 125 days and has high production and a great adaptability for different uses ("Tomato - Guidelines for the conduct of tests for distinctness, uniformity and stability," 2002). These varieties are highly tolerant to several diseases including to Fusarium wilt caused by FOL races 1, 2 and 3 (Heinz, 2019; *Tomate de industria - España*, n.d.). Plantation density was 33 000 plants/ha for both locations.

Figure 5 *- Planter. Source: https://www.bioprotomate.uevora.pt/ , accessed on the 5th of July 2022.*

3.4. Sampling and Data analysis

3.4.1. Dry Matter Production of the cover crop

To determine dry matter (DM) production of the cover crop, three random points per block and per cover crop treatment were sampled, on February 2021 in Salvaterra, using a wooden square with 1 m side that was randomly released in each of the points. All plants inside the square were cut off, placed in identified paper bags and oven-dried at 60ºC for 42h. After this period, the material contained in each paper bag was weighed using a semi-analytical balance and the DM production results noted.

3.4.2. Mycorrhizal colonization rate

Mycorrhizal colonization rate was measured using roots from the cover crops, sampled on February 2021, and from tomato plants, sampled on May and June 2021, respectively, for Salvaterra and Pancas. Four random points per block and per treatment were sampled, with the exception of the no cover crop treatment for mycorrhization rate of the cover crop. For the cover crops and tomato crop, in each point, respectively, three oat and tomato plant roots were carefully sampled to avoid damaging the smallest and thinnest roots.

For the analysis of the mycorrhizal colonization rate, it is necessary that roots are stained, in order to highlight the fungal structures, immediately after sampling and washing. Accordingly, trypan blue dye was used to stain these structures since it binds to

chitin components of the fungal cell wall. The trypan blue staining procedure consisted of the following steps:

a. Placing about 0,7g of roots from each composite sample in a histology cassette;
b. Soaking all cassettes in 10% potassium hydroxide (KOH);
c. Autoclaving for 15 minutes at 121 ºC to degrade and eliminate cellular constituents/cytoplasmic contents;
d. Washing thoroughly with running water to remove the excess of KOH;
e. Staining in a solution containing 0,1% Trypan Blue in lactoglycerol at a ratio of 1:1:1 (glycerol, 80% lactic acid, and water) for about 10 minutes at 70 ºC in a water bath. In this step, trypan blue will bind to chitin of the fungus cell wall;
f. Removing the cassettes containing the stained roots from the solution described above and store them in a 50% glycerol solution, which dissolves the trypan blue that is not bonded to fungal structures to allow a better contrast between these structures and others. Stained roots can be stored in this solution for long periods of time without compromising the results and they must only be observed after 48h.

To determine the rate of colonization by AMF, the intersection method described by McGonigle *et al*. (1990) was used. This method consists of preparing a microscopic slide, containing the stained roots and 50% (v/v) glycerol solution, which is covered with a 24 x 60 mm coverslip. On the slide, the roots are aligned parallel to the longest axis, and observed under an optical microscope at 200x magnification. For each sample, two slides were made and observed, and the two were treated as a single unit (McGonigle *et al*., 1990).

Quantification of mycorrhizal colonization was done by going through the whole slide perpendicularly to its longitudinal axis, with a constant change in the field of view, and counting the number of root intersections with the vertical crosshair. Arbuscules, hyphae or non-colonized root can be intersected (negative) and were counted separately. Arbuscular colonization (AC) was calculated by dividing the counts of this category by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections, because hyphae were only accounted for when arbuscules were observed close by. This shows the effort that was made not to

account for other fungi when quantifying hyphae, as arbuscules are characteristic of the fungal type under study (McGonigle *et al.*, 1990). All data obtained using this method were examined in random order with the identity of the roots unknown to the observer.

3.4.3. Soil enzymatic activity

Soil enzymatic activity was assessed based on the activity of important enzymes, such as arylsulfatase, β-glucosidase, phosphatase and urease. Soil samples were collected when cover crops were sampled, on February 2021 for both locations. Four random points per block and per treatment were sampled and in each point three sections from the first 10 cm of soil were taken using a soil probe, to make a composite homogenized sample. Soil samples were transported do the laboratory, sieved through a 2mm mesh and stored, separately, at -80 ºC until further analysis.

To estimate soil enzymatic activity of the previously mentioned enzymes, soil moisture, was assessed. To determine soil moisture, 10g of sieved soil was oven dry at 105 ºC for 24 hours, and then the dry weight was determined. Soil moisture is the difference between the 10g of wet soil and the dry weight of the soil. The value of a g of dry soil per g of wet soil is the dry weight divided by the wet weight.

Protocol to determine soil enzymatic activity was based on ISO 20130:2018(E), which allowed the measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates. This protocol included the establishment of calibration curves using several concentrations of para-nitro phenol (PNP), for the arylsulfatase, β-glucosidase, phosphatase enzymes, and ammonium chloride (NH_4Cl) for the urease enzyme. Soil solutions were prepared for each sample, in triplicate, using 4g of the sieved soil sample and 25 ml of deionized water, for each replicate, in flat bottom flasks and homogenized on an orbital agitator. Multichannel micropipettes were used to distribute soil suspensions in micro-well plates in four replicate wells (three wells were analytical point and one was the blank) with specific volumes according to the enzyme. Substrates used were different for each enzyme: potassium 4-nitrophenyl sulfate was used to determine arylsulfatase activity, 4-nitrophenyl β-D-glucopyranoside was used to determine β-glucosidase activity, 4-nitro-phenylphosphate disodium salt hexahydrate was used to determine phosphatase activity and urea was used to determine urease activity. Incubation time and temperature was also different for each enzyme: 4 hours at 37 ºC for arylsulfatase, 1 hour at 37 ºC for β-glucosidase, 30 minutes at 37 ºC for phosphatase and 3 hours at 25 ºC for urease. After incubation, reactions were stopped,

using Tris base 100 mmol/l pH 12 ± 0,1 and Calcium chloride dihydrate 0,5 mol/l for arylsulfatase, β-glucosidase, phosphatase enzymes and salicylate and cyanurate reagents for urease enzyme. Micro-well plates were centrifuged, the supernatants were transferred into new plates and the absorbance was measured on a spectrophotometer for microplates, using filter 405nm for arylsulfatase, β-glucosidase, phosphatase enzymes, and filter 650nm for urease enzyme. Results were obtained by subtracting measurement of blank from triplicate of sample and multiplying the difference with dilution factor (D) and soil volume (Vss), and dividing with reaction time and dry mass of sample (Wds). Additionally, results were expressed as milliunit for one gram of dry soil corresponding to nmole of PNP or ammonium chloride released per hour and g soil dry mass of sample (nmolPNP . g^{-1} dry soil . h^{-1} or nmolNH_aCl . g^{-1} dry soil . h^{-1}) (ISO, 2018).

$$A = \frac{(C_s - C_b) \times D \times V_{ss}}{RT \times W_{ds}}$$

where:

A - is the enzymatic activity in mU/g of dry sample (nmol/min/g of dry sample);

Cs - is the concentration of product formed in sample (nmol/ml);

Cb - is the concentration of product formed in blank (nmol/ml);

D - is the dilution of sample in microplate;

Vss - is the volume of sample solution (ml);

RT - is the reaction time (min);

Wds - is the mass of dry sample (g).

3.4.4. Nematode incidence

Soil samples to estimate nematode population were collected when tomato plants were sampled, May and June 2021, respectively, for Salvaterra and Pancas. Four random points from each treatment and each block were randomly chosen and soil was collected, for a total of 24 samples per study area. Samples were immediately transported to the laboratory where they were processed as promptly as possible.

Analysis of root gall nematodes (*Meloidogyne* spp.) in soil samples was carried out by Laboratório de Nematologia from Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento da Universidade de Évora, as a rendering service. The method used was a modification of Cobb's decanting and sieving technique using two

replicates with 250g from each sample, followed by an extraction on a Baermann funnel for 48 h and morphological observation with a binocular stereoscopic magnifier (Cobb, 1918). Results were obtained in number of nematodes per 250 cm^3 of soil.

3.4.5. *Fusarium* spp. infection in tomato plants

To evaluate *Fusarium* spp. infection in tomato plants, four plants from each treatment and each block were randomly collected, for a total of 24 samples per study area. Sampling happened eight weeks after plantation, on May and June 2021, respectively, for Salvaterra and Pancas. during the early morning, to minimize abiotic stress conditions, and samples were immediately transported to the laboratory where they were processed as promptly as possible. Plant crowns were detached and grounded into powder, separately for each sample, using sterile mortars and pestles, aiding the process with liquid nitrogen, and were stored at -80 ºC until further analysis. Ground plant material (plant crowns) was used for genomic DNA (gDNA) extraction.

gDNA extraction was performed from approximately 500 mg of material powder for each sample, using the CTAB (hexadecyltrimethylammoniumbromide) method (Doyle & Doyle, 1987) with some adaptations (Varanda et al., 2016). The quantification of gDNA and the evaluation of its purity were determined in a Quawell Q9000 micro spectrophotometer (Quawell Technology, Beijing, China). All DNA samples were diluted to a final concentration of 100 ng/µL.

The detection and quantification of *Fusarium* spp. in the samples was carried out by real-time quantitative PCR (qPCR), using a set of primers designed in the ribosomal internal transcribed spacer (ITS) region (Fw: 5'-AAAACCCTCGTTACTGGTAATCGT-3'; Rv: 5'-CCGAGGTCAACATTCAGAAGTTG-3', amplicon size 69 base pairs) (Campos et al., 2019).

qPCR was performed using 200 ng of gDNA per sample, 10 µL of NZY qPCR Green Master Mix (2x) (Nzytech, Lisbon, Portugal) and 40 nM of each primer, for a total volume of 20 µL, on a LineGene9600Plus system (BIOER, Hangzhou, China). Threshold cycle (Ct) values were attained, for each sample, with the following cycling conditions: 20 s at 95 ºC 168 for an initial denaturation, followed by an amplification program of 40 cycles of 15 s denaturation at 95ºC and 20 s at 60ºC. Additionally, a final step was added to the program to test PCR specificity, a dissociation curve, featuring a single cycle at 95ºC for 15 s, 60ºC for 1 min and rump-up 0.2ºC/s to 95ºC for 15ºC. Three technical

replicates were considered for each sample and *Fusarium* spp. isolates (including F. *oxyporum f. sp. radicis-lycopersici*, *F. oxysporum f.sp. lycopersici*, *F. oxysporum f. sp. cubense*, *F. incarnatum, F. equiseti*, *F. graminearum, F. verticillioides, F. subglutinans, F. proliferatum, F. sachari* and *F. clavum*) from the collection of the Mycology Laboratory, Mediterranean Institute for Agriculture, Environment and Development (MED), University of Évora, Portugal, were used as positive controls. The identity of the amplicon of the samples was confirmed by Sanger sequencing and specificity of qPCR reactions was evaluated by melting curve analysis.

3.4.6. Statistical Analysis

Statistical analysis was performed on IBM SPSS Statistics 24.0. Normality, homogeneity of variance and multivariate outlier tests allowed the determination of the appropriate test for mean comparison between groups. Kolmogorov-Smirnov and Shapiro Wilk were used to assess normality, Levene's test to evaluate equality of variances and Mahalanobis distance to determine the presence or absence of multivariate outliers.

Dry matter production of the cover crop in Salvaterra was analyzed using two randomized blocks (two replicates) with two treatments (oat cover crop and oat + rapeseed cover crop), to sample 3 replicates per treatment and per block. DM production means were compared using T-test for independent samples and a 95% confidence interval.

Influence of the treatment (independent factor) on the mycorrhization rate, enzymatic activity, nematode incidence and Fusarium infection (dependent factors) was analyzed using two randomized blocks (two replicates) with three treatments (two different cover crops and one control treatment), to sample 4 replicates per treatment and per block. Multivariate ANOVA (MANOVA) was performed to compare means and analyze the influence of the independent factors, treatment and location, on the dependent factors mycorrhization rate and enzymatic activity, using Pillai's Trace and univariate ANOVAs, performing Tukey post-hoc test when needed. Univariate ANOVA was also used to analyze the influence of the treatment and location on nematode incidence, performing Tukey post-hoc test when needed. Fusarium infection was classified in four different classes, according to the level of infection: no infection (ct mean > 29); low infection (29 < ct mean > 24); medium infection (22 < ct mean < 24) and high infection (ct mean < 22).

4. Results

4.1. Dry matter production of the cover crop

Dry matter production of the cover crop was high for both treatments, presenting mean values of 4489 kg/ha for the oat treatment and 4674 kg/ha for the oat + rapeseed treatment (Figure 6). A higher value was found for the consociation treatment however, no significant differences were found between means ($t(10) = 0{,}492$; $p > 0{,}05$).

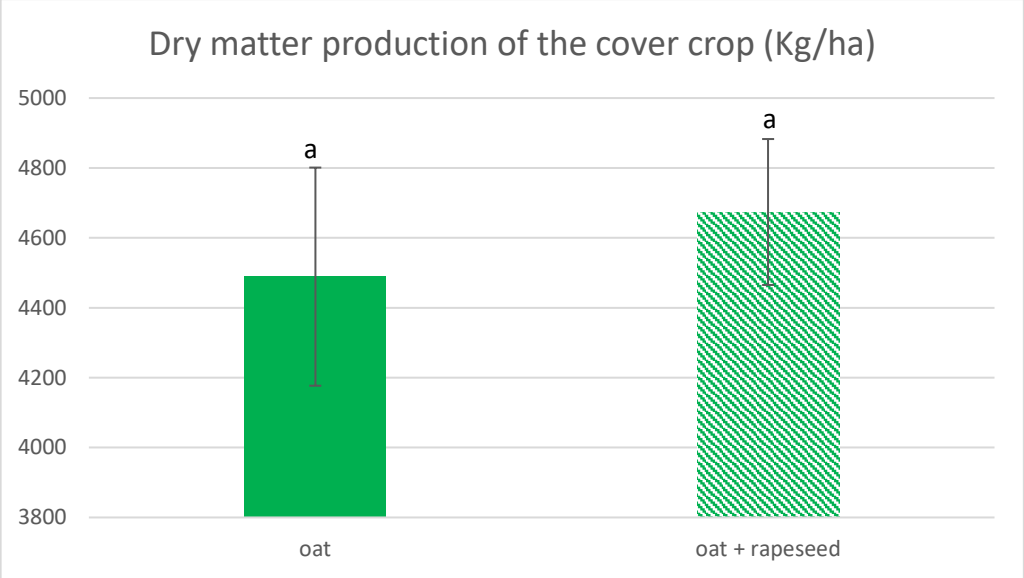

Figure 6 *- Dry matter production of the cover crop in Salvaterra. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other ($p > 0{,}05$).*

4.2. AMF colonization rate of the cover crop and tomato plants

AMF colonization rates of the cover crop were generally low for both treatments and locations, although it was possible to observe some oat roots with developed arbuscular colonization. Figure 7 shows an oat root from the consociation treatment with several AMF structures highlighted by the trypan blue.

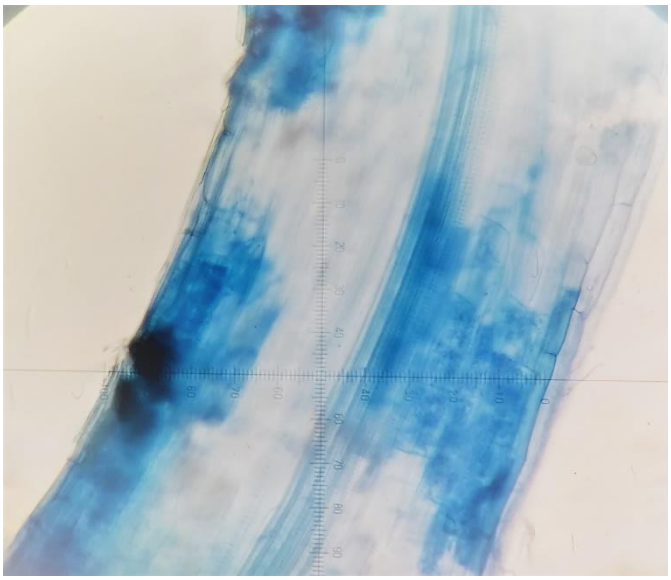

***Figure 7** - AMF colonized oat plant root. Source: Joana Ribeiro, August 2021.*

Cover crop AMF colonization rate showed the same pattern either estimated by hyphae counting or arbuscules counting. Mean hyphae colonization (HC) rate in Pancas was 3,77% ± 0,86% for the oat treatment and 6,28% ± 2,44% for the oat + rapeseed treatment. In Salvaterra, mean HC rate was 3,85% ± 1,70% and 1,63% ± 0,59% for the oat and oat + rapeseed treatments, respectively (Figure 8). Mean arbuscules colonization (AC) rate in Pancas was 1,68% ± 0,50% for the oat treatment and 2,82% ± 1,12% for the oat + rapeseed treatment. In Salvaterra, mean AC rate was 2,00% ± 1,08% and 0,78% ± 0,31% for the oat and oat + rapeseed treatments, respectively (Figure 9).

In Pancas, the oat + rapeseed treatment showed a tendency to favor AMF colonization in contrast to the oat treatment that present lower rates for HC and AC. However, MANOVA showed no effect of the treatment on the AMF colonization rate [Pillai's Trace = 0,064; F (2, 13) = 0,447; p > 0,05], considering HC and AC together. Subsequent univariate ANOVAs showed no effect of the treatment on the HC rate [F (1, 14) = 0,940; p> 0.05] and AC rate [F (1, 14) = 0,858; p > 0.05]. In Salvaterra, the oat treatment showed a tendency to favor AMF colonization in contrast to the oat + rapeseed treatment that present lower rates for HC and AC. Nevertheless, MANOVA showed no effect of the treatment on the AMF colonization rate [Pillai's Trace = 0,132; F (2, 13) = 0,987; p > 0,05], considering HC and AC together. Subsequent univariate ANOVAs showed no effect of the treatment on the HC rate [F (1, 14) =1, 521; p> 0,05] and AC rate [F (1, 14) = 1,163; p > 0,05] (Figures 8 and 9).

Comparing the two locations, similar colonization rates of oat roots were found for the oat treatment however, for the oat + rapeseed treatment slightly higher values were found in Pancas. Nevertheless, MANOVA showed no effect of the location on the AMF colonization rate neither for the oat treatment [Pillai's Trace = 0,093; F (2, 13) = 0,664; p > 0,05] nor for the oat + rapeseed treatment [Pillai's Trace = 0,210; F (2, 13) = 1,724; p > 0,05], considering HC and AC together. Accordingly, subsequent univariate ANOVAs showed no effect of the location on the HC rate [F (1, 14) =0,002; p> 0,05] and AC rate [F (1, 14) = 0,071; p > 0,05] for the oat treatment, and on the HC rate [F (1, 14) =3,429; p> 0,05] and AC rate [F (1, 14) = 3,057; p > 0,05] for the oat + rapeseed treatment (Figures 8 and 9).

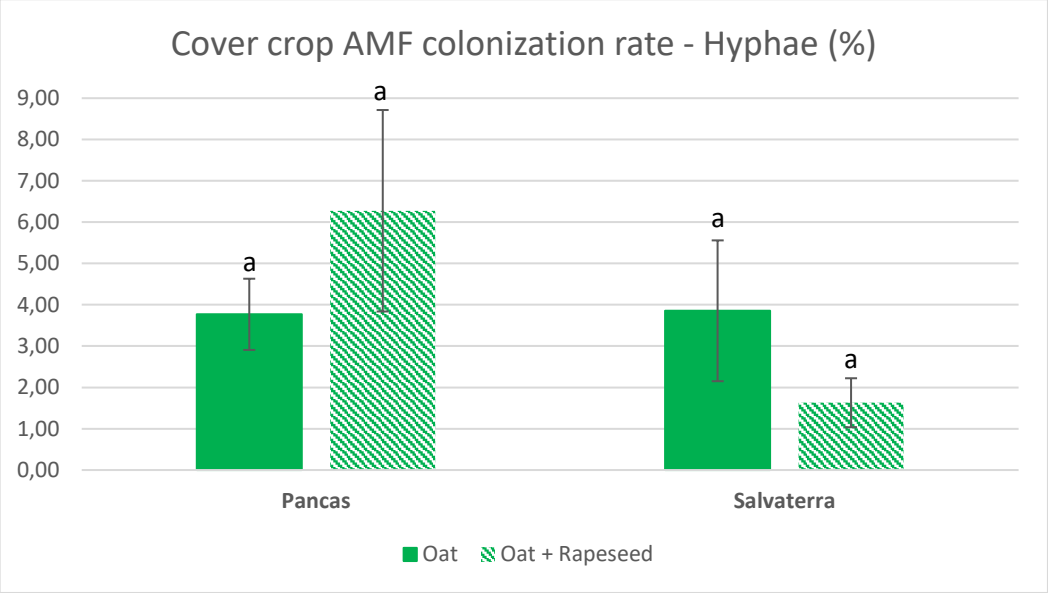

Figure 8 *- AMF colonization rate (hyphae counting) for oat in the cover crop treatments. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

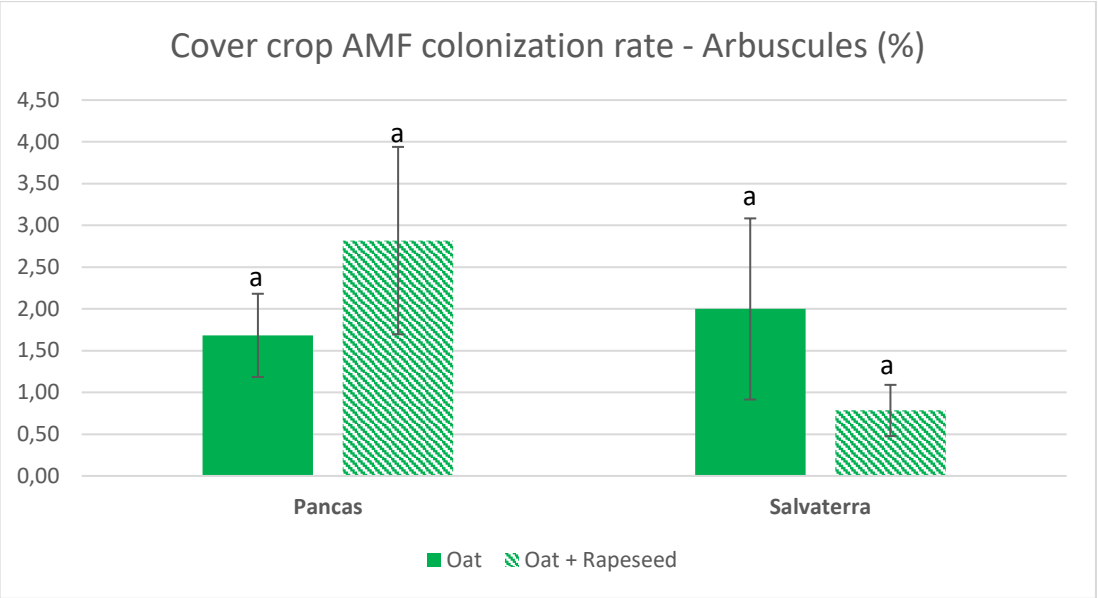

Figure 9 *- AMF colonization rate (arbuscules counting) for oat in the cover crop treatments. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other ($p > 0,05$).*

Tomato AMF colonization rates were generally low for all treatments and locations, similarly to AMF colonization rate of the cover crop, although it was possible to observe some tomato roots with developed arbuscular colonization. Figure 10 shows a tomato root from the consociation treatment with several AMF structures highlighted by the trypan blue.

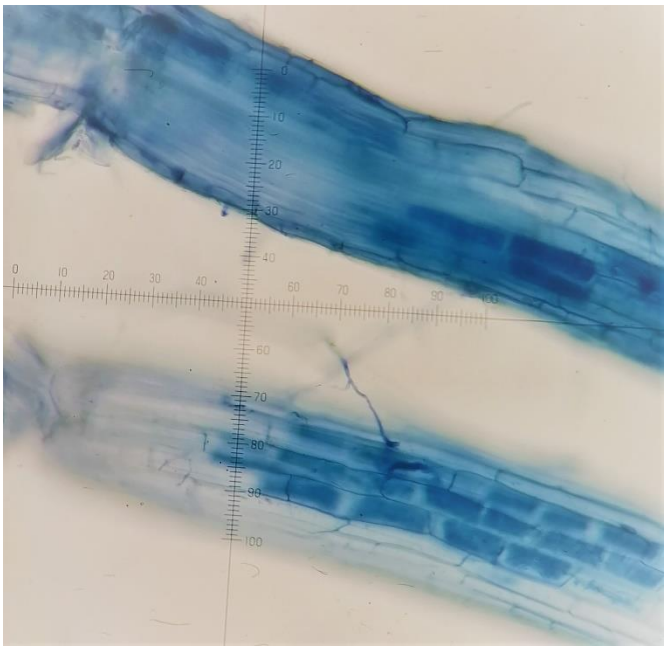

Figure 10 *- AMF colonized tomato plant root. Source: Joana Ribeiro, September 2021.*

Tomato AMF colonization rate showed the same pattern either estimated by hyphae counting or arbuscules counting. Mean HC rate in Pancas was 5,76% ± 1,12% for the test field control, 2,64% ± 0,89% for the oat treatment and 5,63% ± 2,03% for the oat + rapeseed treatment. In Salvaterra, mean HC rate was 0,19% ± 0,12% for the test field control and 0,49% ± 0,25% and 0,14% ± 0,07% for the oat and oat + rapeseed treatments, respectively (Figure 11). Mean AC rate in Pancas was 3,81% ± 0,78% for the test field control, 1,60% ± 0,57% for the oat treatment and 3,75% ± 1,45% for the oat + rapeseed treatment. In Salvaterra, mean AC rate was 0,14% ± 0,10% for the test field control, 0,31% ± 0,21% for the oat treatment and 0,05% ± 0,05% oat + rapeseed treatment (Figure 12)

In Pancas, the test field control and oat + rapeseed treatments showed a tendency to have higher AMF colonization than the oat treatment that present lower rates for HC and AC. However, MANOVA showed no effect of the treatment on the AMF colonization rate [Pillai's Trace = 0,131; F (4, 42) = 0,735; p > 0,05], considering HC and AC together. Subsequent univariate ANOVAs showed no effect of the treatment on the HC rate [F (2, 21) = 1,522; p> 0.05] and AC rate [F (2, 21) = 1,574; p > 0.05]. In Salvaterra, the oat treatment showed a tendency to favor AMF colonization in contrast to the test field control and oat + rapeseed treatments that presented lower rates for HC and AC. Nevertheless, MANOVA showed no effect of the treatment on the AMF colonization rate [Pillai's Trace = 0,128; F (4, 42) = 0,715; p > 0,05], considering HC and AC together. Subsequent univariate ANOVAs showed no effect of the treatment on the HC rate [F (2, 21) =1,317; p> 0,05] and AC rate [F (2, 21) = 0,950; p > 0,05] (Figures 11 and 12).

Comparing the two locations, higher colonization rates of the tomato roots were found for all treatments in Pancas comparing to Salvaterra. MANOVA showed effect of the location on the AMF colonization rate in the test field control [Pillai's Trace = 0,639, F (2, 13) = 11,487, p < 0,05], considering HC and AC together. Accordingly, subsequent univariate ANOVAs showed effect of the location on the HC rate [F (1, 14) = 24,573; p < 0,05] and AC rate [F (1, 14) = 21,571; p < 0,05]. Regarding the oat treatment, MANOVA showed no effect of the location on the AMF colonization rate [Pillai's Trace = 0,287, F (2, 13) = 2,611, p > 0,05], considering HC and AC together. However, univariate ANOVAs showed effect of the location on the HC rate [F (1, 14) = 5,376; p < 0,05], but no effect of the location on the AC rate [F (1, 14) = 4,492; p > 0,05]. For the oat + rapeseed treatment, MANOVA showed effect of the location on the AMF colonization rate [Pillai's Trace = 0,370; F (2, 13) = 3,817; p < 0,05], considering HC and

AC together. Accordingly, subsequent univariate ANOVAs showed effect of the location on the HC rate [F (1, 14) = 7,325; p < 0,05] and AC rate [F (1, 14) = 6,541; p < 0,05] (Figures 11 and 12).

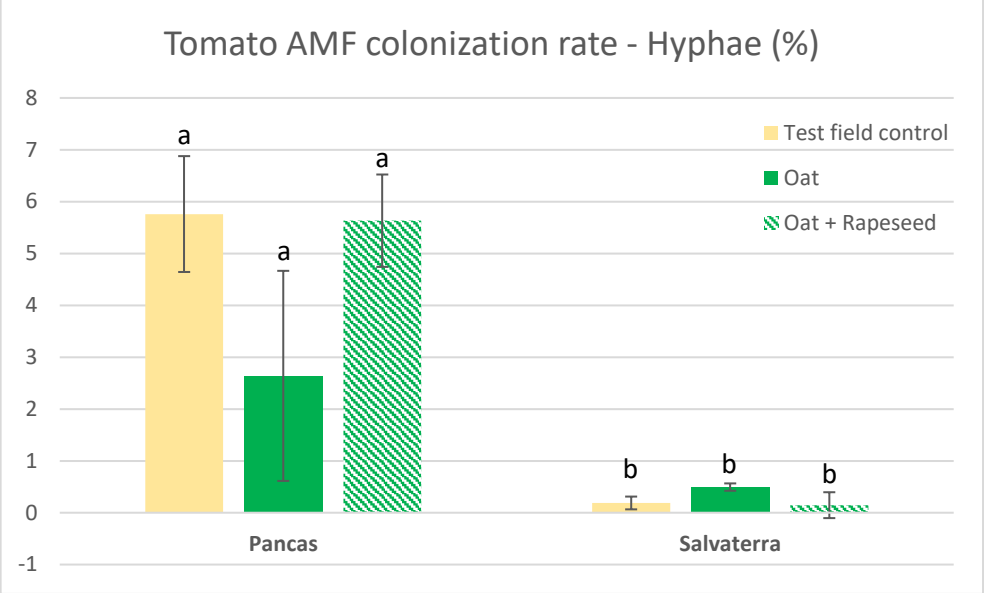

Figure 11 *- AMF colonization rate (hyphae counting) for tomato plants in the test field control and cover crop treatments. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

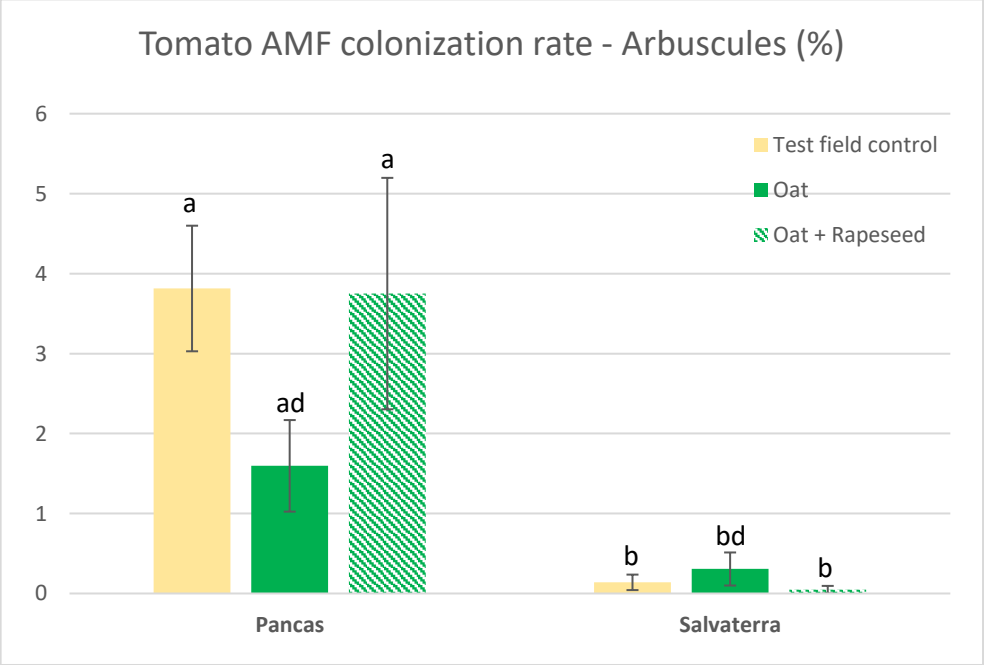

Figure 12 *- AMF colonization rate (arbuscules counting) for tomato plants in the test field control and cover crop treatments. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

4.3. Soil enzymatic activity

Soil enzymatic activity was quantified for all treatments in the two different locations. Considering all enzymes – arylsulfatase, phosphatase, ß-glucosidase and urease – MANOVA showed no effect of the treatment in their activity in Pancas [Pillai's Trace = 0,353, F (8, 38) = 1,020, $p > 0,05$]. However, in Salvaterra, there was influence of the treatment in enzymatic activity [Pillai's Trace = 0,839, F (8, 38) = 3,431, $p < 0,05$]. Soil enzymatic activity was generally higher in Pancas than in Salvaterra. Accordingly, and considering all enzymes, MANOVA showed effect of the location in enzymatic activity for the test field control [Pillai's Trace = 0,864, F (4, 11) = 17,445, $p < 0,05$], the oat treatment [Pillai's Trace = 0,830, F (4, 11) = 13,417, $p < 0,05$] and the oat + rapeseed treatment [Pillai's Trace = 0,885, F (4, 11) = 21,256, $p < 0,05$].

Arylsulfatase activity presented minimum mean values of 15,99 ± 1,07 µgPNP/ g of dry soil /h, for the oat treatment in Salvaterra, and maximum mean values of 38,84 ± 2,16 µgPNP/ g of dry soil /h, for the oat + rapeseed treatment in Pancas. In Pancas, higher activity mean values for this enzyme were found for the oat treatment (38,19 ± 2,73 µgPNP/ g of dry soil /h) and the consociation treatment (38,84 ± 2,16 µgPNP/ g of dry soil /h). Lower activity was found, in Pancas, for the test field control (35,64 ± 0,92 µgPNP/ g of dry soil /h). Nevertheless, univariate ANOVA showed no influence of the treatment in the level of activity of arylsulfatase in Pancas [F (2, 21) = 0,658; $p > 0,05$]. In Salvaterra, higher arylsulfatase activity was found for the test field control (21,08 ± 1,32 µgPNP/ g of dry soil /h), then for the oat + rapeseed treatment (19,37 ± 1,63 µgPNP/ g of dry soil /h) and lower mean values were found for the oat treatment (15,99 ± 1,07 µgPNP/ g of dry soil /h). In this case, univariate ANOVA showed influence of the treatment in the level of activity of arylsulfatase [F (2, 21) = 3,616; $p < 0,05$] and post-hoc Tukey test found significant differences between the test field control and the oat treatment (HSD = 5,09; $p < 0,05$). However, post-hoc Tukey test did not find significant differences between neither the test field control and the oat + rapeseed treatment (HSD = 1,71; $p > 0,05$) nor the oat + rapeseed and the oat treatment (HSD = 3,38; $p > 0,05$) (Figure 13).

Comparing the two locations, arylsulfatase activity was higher in Pancas for all treatments. Univariate ANOVA proved the influence of the location in the arylsulfatase activity for the test field control [F (1, 14) = 81,445; $p < 0,05$], for the oat treatment [F (1,

14) = 57,40; p < 0,05] and for the oat + rapeseed treatment [F (1, 14) = 51,55; p < 0,05] (Figure 13).

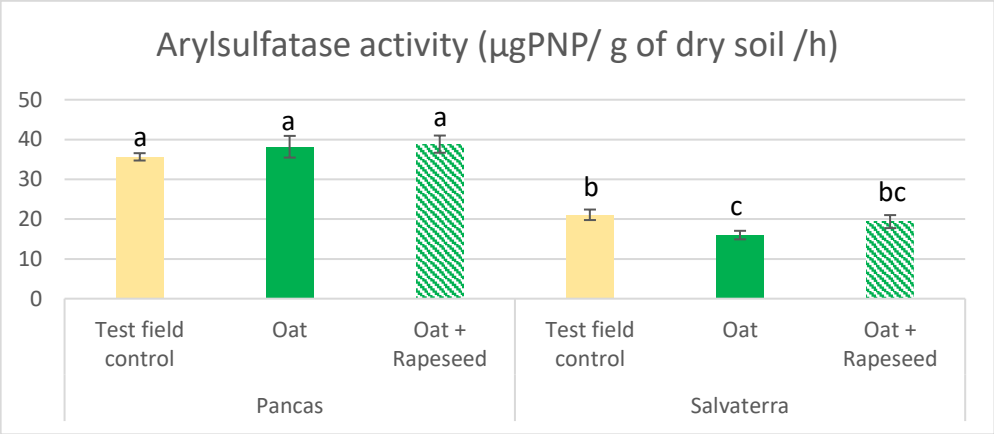

Figure 13 *- Arylsulfatase activity in µgPNP/ g of dry soil /h in each treatment and both locations. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

Phosphatase activity presented minimum mean values of 118,71 ± 10,47 µgPNP/ g of dry soil /h, for the test fiedl control treatment in Salvaterra, and maximum mean values of 202,76 ± 11,82 µgPNP/ g of dry soil /h, for the oat + rapeseed treatment in Pancas. In Pancas, higher activity mean values for this enzyme were found for the oat + rapeseed treatment (202,76 ± 11,82 µgPNP/ g of dry soil /h), then for the test field control (191,30 ± 9,22 µgPNP/ g of dry soil /h) and lower mean values for the oat treatment (177,37 ± 9,12 µgPNP/ g of dry soil /h). However, univariate ANOVA showed no influence of the treatment in the level of activity of phosphatase in Pancas [F (2, 21) = 1,575; p > 0,05]. In Salvaterra, higher phosphatase activity was found for the oat treatment (164,04 ± 14,68 µgPNP/ g of dry soil /h) and lower mean values were found for the test field control (118,71 ± 10,47 µgPNP/ g of dry soil /h) and oat + rapeseed treatment (125,30 ± 7,73 µgPNP/ g of dry soil /h). In this case, univariate ANOVA showed influence of the treatment in the level of activity of phosphatase [F (2, 21) = 4,68; p < 0,05] and post-hoc Tukey test found significant differences between the test field control and the oat treatment (HSD = 45,33; p < 0,05). However, post-hoc Tukey test did not find significant differences between neither the test field control and the oat + rapeseed treatment (HSD = 6,58; p > 0,05) nor the oat + rapeseed and the oat treatment (HSD = 38,75; p > 0,05) (Figure 14).

Comparing the two locations, phosphatase activity was higher in Pancas for all treatments. Univariate ANOVA proved the influence of the location in the phosphatase activity for the test field control [F (1, 14) = 27,06; p < 0,05] and the oat + rapeseed treatment [F (1, 14) = 30,06; p < 0,05], but not for the oat treatment [F (1, 14) = 0,595; p > 0,05] (Figure 14).

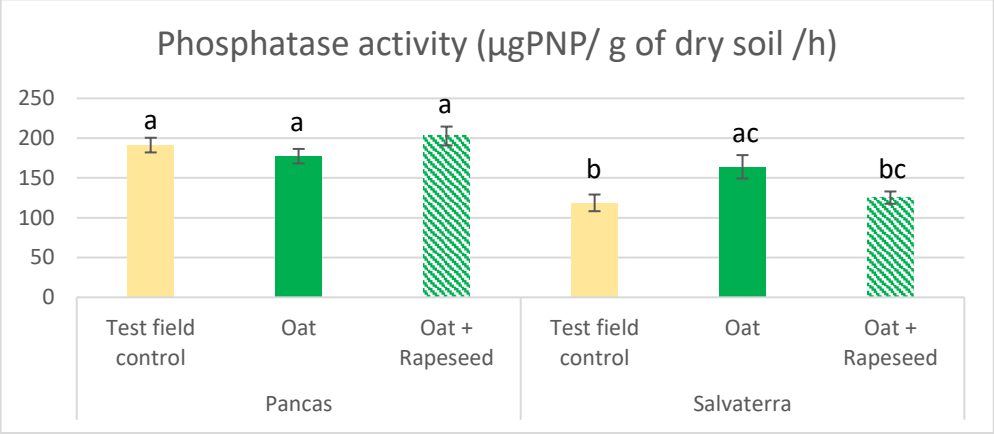

Figure 14 *- Phosphatase activity in µgPNP/ g of dry soil /h in each treatment and both locations. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

Activity of ß-glucosidase presented minimum mean values of 91,63 ± 8,56 µgPNP/ g of dry soil /h, for the test field control in Salvaterra, and maximum mean values of 167,70 ± 5,76 µgPNP/ g of dry soil /h, for the oat + rapeseed treatment in Pancas. In Pancas, higher activity mean values for this enzyme were found for the consociation treatment (167,70 ± 5,76 µgPNP/ g of dry soil /h), then for the oat treatment (164,54 ± 9,08 µgPNP/ g of dry soil /h) and lower activity was found for the test field control (160,10 ± 7,58 µgPNP/ g of dry soil /h). Nevertheless, univariate ANOVA showed no influence of the treatment in the level of activity of ß-glucosidase in Pancas [F (2, 21) = 0,253; p > 0,05]. In Salvaterra, higher activity mean values for this enzyme were found for the consociation treatment (106,27 ± 8,21 µgPNP/ g of dry soil /h), then for the oat treatment (99,87 ± 9,17 µgPNP/ g of dry soil /h) and lower activity was found for the test field control (91,63 ± 8,56 µgPNP/ g of dry soil /h). In this case, similarly to Pancas, univariate ANOVA showed no influence of the treatment in the level of activity of ß-glucosidase [F (2, 21) = 0,718; p > 0,05] (Figure 15).

Comparing the two locations, ß-glucosidase activity was higher in Pancas for all treatments. Univariate ANOVA proved the influence of the location in the ß-glucosidase

activity for the test field control [F (1, 14) = 35,84; p < 0,05], for the oat treatment [F (1, 14) = 25,09; p < 0,05] and for the oat + rapeseed treatment [F (1, 14) = 37,52; p < 0,05] (Figure 15).

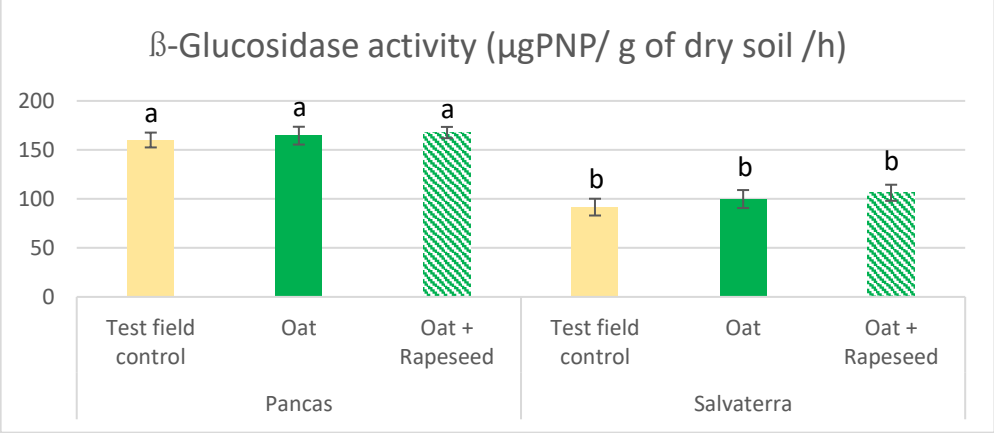

***Figure 15** - ß-Glucosidase activity in µgPNP/ g of dry soil /h in each treatment and both locations. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

Urease activity presented minimum mean values of 5,78 ± 0,48 µgNH_4Cl/ g of dry soil /h, for the test field control in Salvaterra, and maximum mean values of 12,19 ± 0,62 µgNH_4Cl/ g of dry soil /h, for the oat treatment in Pancas. In Pancas, higher activity mean values for this enzyme were found for the oat treatment (12,19 ± 0,62 µgNH_4Cl/ g of dry soil /h), then for the consociation treatment (11,60 ± 0,73 µgNH_4Cl/ g of dry soil /h) and lower activity was found for the test field control (10,38 ± 0,67 µgNH_4Cl/ g of dry soil /h). However, univariate ANOVA showed no influence of the treatment in the level of activity of urease in Pancas [F (2, 21) = 1,855; p > 0,05]. In Salvaterra, higher activity mean values for this enzyme were found for the consociation treatment (7,00 ± 0,42 µgNH_4Cl/ g of dry soil /h), then for the oat treatment (6,29 ± 1,30 µgNH_4Cl/ g of dry soil /h) and lower activity was found for the test field control (5,78 ± 0,48 µgNH_4Cl/ g of dry soil /h). In this case, similarly to Pancas, univariate ANOVA showed no influence of the treatment in the level of activity of urease [F (2, 21) = 0,533; p > 0,05] (Figure 16).

Comparing the two locations, urease activity was higher in Pancas for all treatments. Univariate ANOVA proved the influence of the location in the urease activity for the test field control [F (1, 14) = 31,17; p < 0,05], for the oat treatment [F (1, 14) = 16,71; p < 0,05] and for the oat + rapeseed treatment [F (1, 14) = 30,10; p < 0,05] (Figure 16).

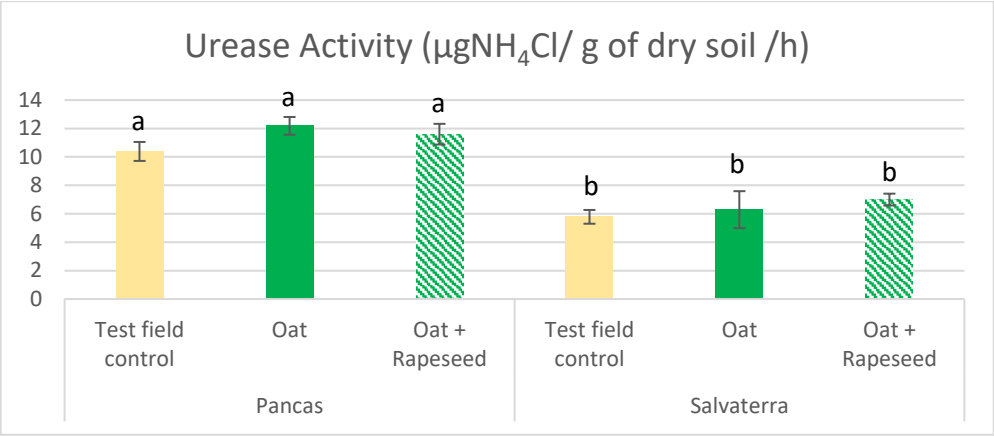

Figure 16 *- Urease activity in µgNH4Cl/ g of dry soil /h in each treatment and both locations. Error bars show the standard error associated to each mean value. Means sharing the same letter are not significantly different from each other (p > 0,05).*

4.4. Nematode incidence

Soil nematode counting showed minimum number of nematodes per 250cm^3 of soil in the oat + rapeseed treatment in Pancas and maximum number of nematodes per 250cm^3 of soil in the oat treatment in Salvaterra. In Pancas, mean number of nematodes per 250cm^3 of soil was higher for the test field control (6,75 ± 2,08 nematodes/250cm^3), then for the oat treatment (3,0 ± 0,73 nematodes/250cm^3) and lower for the consociation treatment (0,5 ± 0,5 nematodes/250cm^3). Univariate ANOVA showed influence of the treatment in the incidence of nematodes in Pancas [F (2, 21) = 5,78; p < 0,05] and post-hoc Tukey test found significant differences between the test field control and the oat + rapeseed treatment (HSD = 6,25; p < 0,05). However, post-hoc Tukey test did not find significant differences between neither the test field control and the oat treatment (HSD = 3,75; p > 0,05) nor the oat + rapeseed and the oat treatment (HSD = 2,50; p > 0,05). In Salvaterra, mean number of nematodes per 250cm^3 of soil was higher for the oat treatment (22,5 ± 6,20 nematodes/250cm^3), then for the test field control (16,25 ± 8,17 nematodes/250cm^3) and lower for the consociation treatment (17,52 ± 6,20 nematodes/250cm^3). However, univariate ANOVA showed no influence of the treatment in the incidence of nematodes in Salvaterra [F (2, 21) = 0,40; p >0,05] (Figure 17).

Comparing the two locations, univariate ANOVA showed no influence of the locations for the test field control [F (1, 14) = 1,27; p >0,05] and consociation treatment [F (1, 14) = 3,57; p >0,05], but significant differences were found for the oat treatment between locations [F (1, 14) = 9,77; p <0,05] (Figure 17).

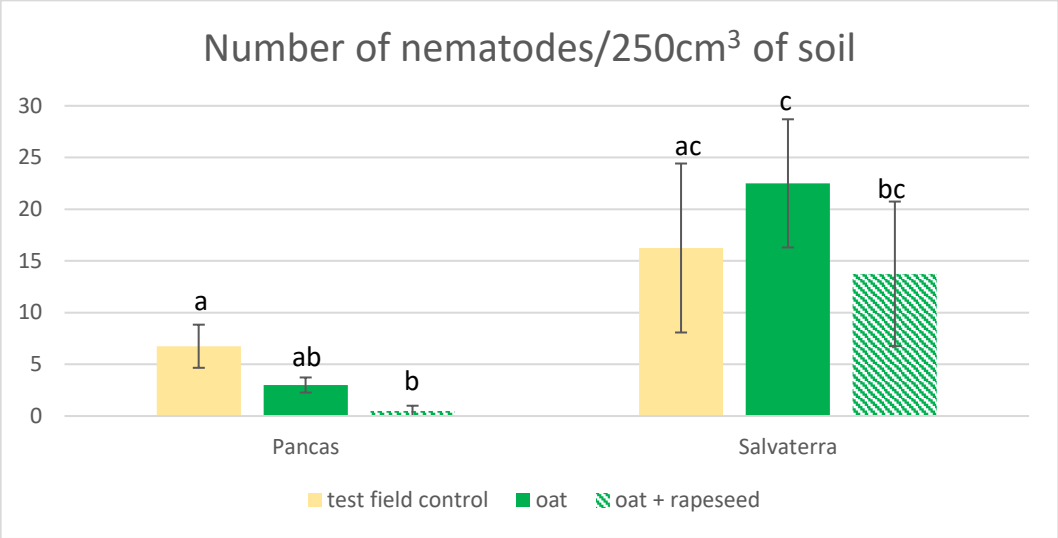

***Figure 17** - Number of nematodes per 250cm³ of soil in each treatment and both locations. Error bars show the standard error associated to each mean value. Means sharing the same letters are not significantly different from each other ($p > 0,05$).*

4.5. *Fusarium* spp. infection in tomato plants

Infection level of *Fusarium spp*. was quantified for all sampled plants. In Pancas, as well as in Salvaterra, 22 of the total plants sampled (n = 24 per location) were infected with *Fusarium* spp. In Pancas, most of the plants sampled showed medium or high levels of infection in total and for each treatment. A total of 15 plants showed high levels of infection: four plants in the test field control, six plants in the oat treatment and five plants in the oat + rapeseed treatment. Medium levels of infection were found in six plants in total: three plants in the test field control, two plants in the oat treatment and one plant in the oat + rapeseed treatment. Only one plant sampled in Pancas showed low levels of infection and it was found in the test field control. Plants with no infection were a total of two: one found in the oat treatment and one found the consociation treatment.

In Salvaterra, a total of six plants showed high levels of infection: two plants in the test field control, two plants in the oat treatment and two plants in the oat + rapeseed treatment. Medium levels of infection were found in six plants, similarly to high levels: two plants in the test field control, two plants in the oat treatment and two plants in the oat + rapeseed treatment. A total of ten plants showed low levels of infection: three plants in the test field control, four plants in the oat treatment and three plants in the oat + rapeseed treatment. Plants with no infection were a total of two: one found in the test field control and one found the consociation treatment.

These results may be observed in Table 1, per location, treatment and class of infection. Mean CTs for *Fusarium* spp. detection and quantification in each treatment and location and infection level is also presented in Appendix I – Table 1.

Table 1 *– Number of samples per infection level of Fusarium spp. in each treatment and both locations. No infection: ct mean > 29; low infection: 29 < ct mean > 24; medium infection: 22 < ct mean < 24; high infection: ct mean < 22.*

		Infection level			
		No infection	**Low**	**Medium**	**High**
Pancas	Test field control	0	1	3	4
	Oat	1	0	1	6
	Oat + Rapeseed	1	0	2	5
	Total	2	1	6	15
Salvaterra	Test field Control	1	3	2	2
	Oat	0	4	2	2
	Oat + Rapeseed	1	3	2	2
	Total	2	10	6	6

5. Discussion

Currently, there is a growing concern about the large areas of monoculture in major agricultural regions, since these systems are characterized by loss of fertility, increased soil erosion and surface runoff (Triplett & Dick, 2008). Furthermore, monoculture-based agro-ecosystems show little soil coverage, low aggregate stability and subsequent compaction and poor water infiltration (Sasal, Andriulo, & Taboada, 2006). Tomato crop is one of the most important and widespread horticultural crops in the world, one of the main crops for fresh consumption in Portugal and the main one for horticultural industry in the country (Almeida, 2014). This crop is often repeatedly grown in monoculture rather than being rotated with other crops, thus tomato fields are being severely affected by all the consequences of monocultures-based agro-ecosystems (Atherton & Rudich, 1986; Sasal *et al*., 2006; Triplett & Dick, 2008).

Therefore, it is crucial to find cultural practices that allow lower environmental impacts and more efficient use of resources for the production of industrial tomato, being the major concern the excessive use of agrochemicals, particularly in pest and disease management (Gatahi, 2020). This is the major issue that supports the importance, relevance and actuality of the present work. Although awareness is important and people seem to be more and more conscious about environmental impacts of agrochemicals and all of the health issues connected to their excessive use, finding sustainable cultural

practices is a worldwide priority (Karungi *et al*., 2011). This is why we investigate the effect of winter cover crops combined with changing the schedule of cultural practices such as soil loosening and plant-beds preparation to avoid disrupting the integrity of the soil structure, on dry matter production of the cover crop and its mycorrhizal colonization rate, tomato early mycorrhization, soil enzymatic activity and nematode and Fusarium diseases incidence.

Analysis of dry matter production of the cover crops in Salvaterra (Figure 6) showed high values for both treatments, within minimum and maximum values (3000–6000 kg/ha) found previously for above-ground DM produced by cover crops in mild climates (Weil & Kremen, 2007). These values allowed a nearly complete ground cover before the termination of cover crops and account for high nutrient concentration in the soil. Although no significant differences were found between the dry matter production of the oat cover crop and the oat + rapeseed cover crop, a tendency for higher values in the consociation treatment was observed. Probably, since standard error values are high, if sampling effort was improved, and more samples were collected, significant differences could be found. This is because rapeseed has been reported to be one of the most promising cover crops that produces a large amount of biomass and can offer numerous benefits and be economically attractive to farmers (AgMRC, 2022; Weil & Kremen, 2007).

AMF colonization of oat in cover crops (Figures 8 and 9) showed extremely low rates for both treatments and locations. However, as expected, AM colonization rates presented the same pattern either estimated by AC or HC. These two AM colonization rate estimations are obviously positively correlated since AMF infection always involve both hyphae and arbuscules. Initially, the hyphae adhere to the epidermis or root hairs and form infecting structures, called *appressorium*, from which the fungus will colonize the cortical zone of the plant root. Through mechanical and enzymatic action, the hyphae invade the cortex inter-cellularly becoming, subsequently, intracellular through the penetration of the epidermis cells, thus forming an "infection unit" including arbuscules (Souza, Silva, Cardoso, & Barreto, 2006).

In Pancas, oat + rapeseed treatment seemed to favor AM colonization, whereas in Salvaterra the oat treatment was where AM colonization rates were higher. However, these differences were not significant and, therefore, no effect of the treatment was observed in AM colonization rate of the cover crops. When comparing between locations, oat treatment showed approximate values for Pancas and Salvaterra. Nevertheless, the

consociation treatment showed higher AM colonization rates for Pancas, but no significant differences were found. Thus, no effect of the location was found for the AM colonization rate of the cover crops. It is important to notice that the evaluation of AM colonization rate of the cover crops was performed by quantifying arbuscules and hyphae in oat roots, in both the oat treatment and the consociation treatment, since rapeseed is a non-mycotrophic species.

The extremely low rates of AMF colonization combined with the fact that no influence was found neither for the treatment nor for the location in AM colonization might be related to the fact that both Pancas and Salvaterra fields have been monoculture-based agro-ecosystems with high inputs of pesticides, including metam sodium in large amounts, over the past decades. As mentioned before, monoculture-based agro-ecosystems systems are characterized by loss of fertility, increased soil erosion and surface runoff, meaning that microbial activity in the soil is compromised (Triplett & Dick, 2008). Additionally, excessive use of pesticides, including metam sodium, results in persistent changes in the microbial community of the soil, severely affecting microbial biomass and has the potential to alter important microbially mediated functions, including nutrient cycling and pollutant degradation (Macalady, Fuller, & Scow, 1998). Therefore, soil fertility and microbial activity in the soils of Pancas and Salvaterra should be heavily affected, thus it is not entirely surprising that AM colonization had such low rates, since the recovering of soil fertility and microbial activity is a complex and long process and AMF inoculum was probably very low.

Additionally, AM colonization efficiency depends on the type of propagules in the soil. These propagules may be originated from spores, previously colonized root fragments or ERM, even though intact ERM is the source of inoculum that grants an early and faster colonization (Klironomos & Hart, 2002). Conversely, the integrity of ERM is crucial to allow early and effective mycorrhization (I. Brito et al., 2013; Martins & Read, 1997; McGonigle *et al.*, 1990). Considering that the integrity of the ERM is severely affected by soil tillage and the colonization of plants by AMF decreases substantially (Lendzemo & Kuyper, 2001), it was not expected that cover crop colonization rates would be high since soil tillage have been intensively practiced over the past few years.

AMF colonization of tomato roots (Figures 11 and 12) showed low rates for all treatments and both locations. This result was not surprising since AM colonization of the cover crops also showed low rates, meaning that AMF inoculum was possibly still

very low. Similar to what was shown for the cover crops, and as expected and explained above, AM colonization rates presented the same pattern either estimated by AC or HC.

No influence of the treatments on AM colonization rates of tomato roots was found neither in Pancas nor in Salvaterra. However, once again this result was not entirely surprising because no influence of the treatments had been found for the cover crops.

Lower AM colonization of tomato roots in the test field control than in the cover crop treatments was expected, since no cover crop was there to develop ERM, however that was not the case. It is important to point out that the test field control plots had spontaneous vegetation that also includes mycotrophic plant species and was probably enough to develop ERM that allowed the colonization of tomato roots as efficiently as in the other treatments. This way, it is important to keep in mind that not only cover crops but also weeds and other elements of the crop rotation, if mycotrophic, can act as host plants and play this role (Brito *et al*., 2019).

Furthermore, the tendencies presented by cover crop AM colonization were confirmed by tomato AM colonization rates. Although no significant differences were found, in Pancas, similar to the cover crop results, the oat treatments seemed to favor colonization rates. Also, in Salvaterra, AM colonization appeared to be favored by the consociation treatment, similar to the cover crop results. These results confirm that cover crops can play a role in terms of developing the native AMF inoculum and its extensive ERM that, when kept intact, grants a more efficient and faster AM colonization of the crop that follows (Klironomos & Hart, 2002).

When comparing between locations, in contrast to the results observed for AM colonization of the cover crops, effect of the location on the HC and AC rates of tomato roots was found for all treatments except for AC of the consociation treatment. Pancas presented significantly higher AM colonization than Salvaterra, confirming the tendency showed by AM colonization of the cover crops. This might indicate that, although no differences were found for AM colonization of the cover crops between locations, the cover crops were probably more effective in recovering soil fertility and microbial activity in Pancas than in Salvaterra.

Regarding AMF colonization rates found in oat roots for both cover crops and in tomato roots in the different treatments, standard error values were considerably high, which might prevent significant differences between treatments to show. Future studies should consider improving the sampling effort, in order to collect more samples and decrease standard error values.

Soil enzymatic activity was estimated as an indicator of soil quality because of their involvement in innumerous processes connected to microbial activity and soil fertility, as explored before (Figures 13, 14, 15 and 16) (Bulletin *et al*., 2017; Tang *et al*., 2014; Utobo & Tewari, 2014). Enzyme activity was influenced by the location, since significantly higher enzymatic activity was found for all enzymes in Pancas than in Salvaterra. This supports AM colonization rates observed for tomato roots, which were also significantly higher in Pancas in contrast to Salvaterra. In view of these results, it is possible to conclude that soil quality in Pancas is better than in Salvaterra, and soil fertility and microbial activity was less compromised.

Considering all enzymes together (phosphatase, ß-glucosidase, arylsulfatase and urease), no effect of the treatment was found in Pancas but, on the contrary, in Salvaterra different treatments showed significantly different enzyme activity. However, even in Salvaterra, only arylsulfatase and phosphatase activities were influenced by the treatment, whereas ß-glucosidase and urease did not present significantly different values for different treatments.

Arylsulfatase activity in Salvaterra was significantly higher in the test field control than in the oat treatment, but no differences were found between the test field control and the oat + rapeseed treatment or between the oat treatment and the consociation. On the contrary, phosphatase activity in Salvaterra was significantly higher in the oat treatment than in the test field control, but no differences were found between the oat treatment and the consociation or between the test field control and the oat + rapeseed treatment.

These results do not support previous studies on the effect of cover crops in enzymatic activity in the soil, which might be related to the previous highly intensive management of the soil from both locations in this study, showing that soil fertility might need more time to recover. Scientific knowledge generally presents no-till and cover crops as efficient management strategies to improve enzymatic activity (Adetunji *et al.*, 2021; Chavarría *et al.*, 2016; Mullen *et al*., 1998; Tang *et al*., 2014; Tyler, 2020). However, these studies usually use as control a no cover crop treatment with tillage, whereas the test field treatment here present suffers the same tillage as the other treatments (done immediately after tomato harvest, in September 2020, instead of before tomato plantation). Additionally, and as mentioned before, test field control had spontaneous vegetation which might also contribute to improve enzymatic activity. These might be the reasons why no influence of the treatments was found for all enzymes in Pancas and for ß-glucosidase and urease in Salvaterra.

Moreover, enzyme activity levels are not constant over time (Liang, Grossman, & Shi, 2014; Weerasekara *et al*., 2017) and future studies might consider sampling in different time points to analyze the response of different enzymes over time.

Regarding nematode incidence (Figure 17), the treatment influenced the number of nematodes per 250m^3 of soil in Pancas, although no differences were found between treatments in Salvaterra. In Pancas, significant differences were found between the test field control and the oat + rapeseed treatment, and the number of nematodes was lower for the consociation treatment and higher for the test field control. In Salvaterra, even though no influence of the treatment was observed, tendency showed lower incidence of nematodes in the oat + rapeseed treatment. Comparing the incidence of nematodes between locations, higher values were found in Salvaterra, however, no influence of the location was observed, except for the oat treatment.

Results observed support the nematocidal potential of rapeseed and the results found by other researchers about the successful suppression of a wide range of nematode species by *Brassica* spp. (Mojtahedi *et al.*, 1991; Wang *et al*., 2007). The benefits of using various species of *Brassicaceae* for cover crops are well known and described and mostly come from glucosinolates, which are sulfur-rich compounds that brassica crops contain in large quantities. When broken down, glucosinolates form bio-toxic side products, including isothiocyanates, whose potential to control weeds, diseases, insects, and nematodes has been widely reviewed (Brown & Morra, 1996; Weil & Kremen, 2007). Therefore, these species seem to offer the most potential for providing farmers with new cover crop options that might have sufficient benefits to make them economically attractive.

Concerning *Fusarium* spp. infection, generally mild symptoms were presented by tomato plants in both Pancas and Salvaterra. However, *Fusarium* spp. was detected in most of the plants for both locations (Table 1). This might be related to the fact that the varieties used are highly tolerant to several diseases including Fusarium wilt caused by FOL races 1, 2 and 3 (Heinz, 2019), which means that although this specie might be present, it does not cause visible symptoms or it is not present in enough quantity to cause them. Additionally, the primers used in rtPCR are specific for *Fusarium* spp., however, there are some *Fusarium* species that are not pathogenic and which might be present but not causing disease. Future studies, that are already in course, will improve the specificity of primers to allow the detection of pathogenic *Fusarium* species only, particularly FOL and FORL.

Regarding the fact that *Fusarium* spp. was detected in most plants it is important to point out that these fungi are very well adapted and can survive under extreme conditions, disseminating by conidia, tomato seeds and seedlings, soil and other media, and even irrigated water. Pancas and Salvaterra tomato fields are intensively cropped and known to be infested with Fusarium diseases for many years which makes the dissemination of *Fusarium* spp. easy and its management very difficult (McGovern, 2015).

The present results show that the treatment did not influence the level of infection by *Fusarium* spp. neither in Pancas nor in Salvaterra. Nevertheless, this was not surprising because AM colonization rates of tomato roots were low and, similarly, not influenced by the treatment. Thus, it was not expected that the treatment could have any effect on *Fusarium* spp. incidence since AM colonization was not well establish and no bio-protection effect was given to the tomato plants. The symbiotic association must be created and be well established before contact with the stress-causing agent to provide a high level of protection. However, under field conditions, when stress is already present in the soil or plant, the role of AMF in bio-protection is challenged by the time required to reach an adequate colonization level (Garg & Chandel, 2010). Persisting with the implementation of this strategy is fundamental for more tangible results, not showed yet due to the initial soil conditions, with evident low levels of native AMF population and no influence of the treatments in most of the analysis carried out.

6. Conclusion

Tomato fields are being severely affected by all the consequences of monocultures-based agro-ecosystems. Therefore, it is crucial to find cultural practices that allow lower environmental impacts and more efficient use of resources, particularly in pest and disease management. The present results showed no influence of the treatments (oat cover crops, oat + rapeseed cover crops and test field control) in most of the analysis carried out, including dry matter production, AM colonization of the cover crop and tomato crop, ß-glucosidase and urease activity and *Fusarium* spp. infection. However, it is important to notice that the intensive use of these fields for tomato production over the last 15 years, the recurrent soil mobilization and excess of nutrients (accounting for high cover crop dry matter production) contributed to a low AMF native inoculum. Under these circumstances, the results were not entirely surprising knowing that the mycorrhization level was low and therefore little protection was given to tomato plants. Additionally,

changes in cultural practices do not cause immediate effects on soil biological parameters and the more degraded the system is, in terms of microbial activity, the longer it takes to recover.

Nevertheless, influence of the treatment was found for arylsulfatase and phosphatase enzymes, in Salvaterra, in accordance to previous studies. Additionally, in Pancas the oat + rapeseed treatment presented significantly lower nematode incidence and in Salvaterra the same pattern was found, although the difference was not significant yet. This proves the nematocidal potential of rapeseed, and suggests its use as an effective and economically attractive cover crop.

It is important to highlight the fact that persisting with the implementation of this strategy is fundamental for more tangible results, not showed yet due to the soil degradation.

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Appendix I

Table 1 *– Mean CTs for Fusarium spp. detection and quantification in each treatment and location and infection level.*

Location	Treatment	CTs mean	±	SD	Infection level
Pancas	T	22,57	±	0,158745	Medium infection
Pancas	T	21,60333	±	0,325167	High infection
Pancas	T	23,83	±	0,042426	Medium infection
Pancas	T	25,20333	±	0,110151	Low infection
Pancas	T	20,89667	±	0,176163	High infection
Pancas	T	23,96667	±	0,138684	Medium infection
Pancas	T	21,59667	±	0,098658	High infection
Pancas	T	21,09333	±	0,145029	High infection
Pancas	O	21,51333	±	0,661085	High infection
Pancas	O	20,24667	±	0,183394	High infection
Pancas	O	21,44	±	0,115326	High infection
Pancas	O	19,83	±	0,138924	High infection
Pancas	O	30	±		No infection
Pancas	O	20,00333	±	0,075719	High infection
Pancas	O	22,38	±	0,278747	Medium infection
Pancas	O	21,34667	±	0,282902	High infection
Pancas	O+R	30	±		No infection
Pancas	O+R	20,35	±	0,130767	High infection
Pancas	O+R	23,00667	±	0,133167	Medium infection
Pancas	O+R	22,19	±	1,338395	Medium infection
Pancas	O+R	21,6	±	0,355387	High infection
Pancas	O+R	20,93	±	0,36756	High infection
Pancas	O+R	20,50667	±	0,321455	High infection
Pancas	O+R	20,10667	±	0,497024	High infection
Salvaterra	T	21,335	±	0,13435	High infection
Salvaterra	T	26,38333	±	0,037859	Low infection
Salvaterra	T	23,155	±	0,021213	Medium infection
Salvaterra	T	30	±		No infection
Salvaterra	T	23,77	±	0,212132	Medium infection
Salvaterra	T	24,62	±	0,084853	Low infection
Salvaterra	T	25,005	±	0,601041	Low infection
Salvaterra	T	21,675	±	0,289914	High infection
Salvaterra	O	25,95	±	0,226274	Low infection
Salvaterra	O	21,465	±	0,077782	High infection
Salvaterra	O	25,13	±	0,183848	Low infection
Salvaterra	O	23,38	±	0,240416	Medium infection
Salvaterra	O	26,97	±	0,042426	Low infection
Salvaterra	O	22,28	±	0,014142	Medium infection
Salvaterra	O	17,8	±	0,127279	High infection
Salvaterra	O	24,04	±	0,155563	Low infection
Salvaterra	O+R	26,905	±	0,007071	Low infection

Salvaterra	O+R	29,24	±	0,438406	No infection
Salvaterra	O+R	23,36	±	0	Medium infection
Salvaterra	O+R	21,235	±	0,007071	High infection
Salvaterra	O+R	22,37	±	0,282843	Medium infection
Salvaterra	O+R	24,105	±	0,120208	Low infection
Salvaterra	O+R	21,975	±	0,205061	High infection
Salvaterra	O+R	26,555	±	0,007071	Low infection

SD: standard deviation; T: test field control; O: oat treatment; O+R: oat + rapeseed treatment