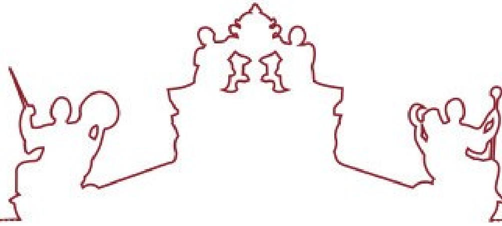


**Universidade de Évora - Instituto de Investigação e Formação Avançada**

Programa de Doutoramento em Biologia

Tese de Doutoramento

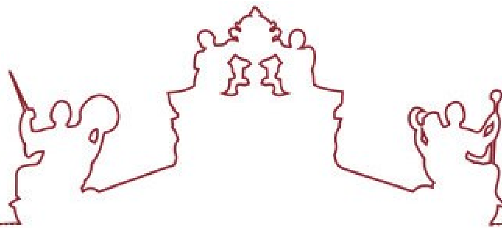
# Microbial functional response to soil disturbance and plant sequence. The case study of arbuscular mycorrhiza and the bioprotective effect of wheat against Mn.

Taiana de Araújo Conceição

Orientador(es) | Isabel Brito

Galdino Andrade Filho

Évora 2022



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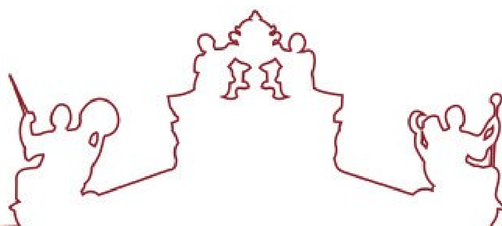

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Évora 2022

I dedicate this thesis to my uncle, Dr. Herbet Conceição, (tio Binho) thank you for making me believe it was possible.

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**Abstract**

Indigenous arbuscular mycorrhizal fungi (AMF) have been proved an important ally in plant growth, and in the current agronomic context their contribution may be essential. When plant colonization by AMF is initiated from an intact extra radicular mycelium (ERM), the infection develops earlier and faster and results in an optimization of the potential benefits for the host plant, specially providing protection against abiotic stress such as metal toxicity. This work studied, in a two-phase greenhouse pot experiment, the effect of wheat (*Triticum aestivum* L.) growth on the soil microbial functional profile, under Mn stress, in relation to wheat antecedent plant mycotrophy (ERM development) and soil disturbance (ERM intact). In the first phase was evaluated the effect of four different plants with different levels of mycotrophy on several parameters of soil biological activity as well as the effect of soil disturbance after each plant. Mycotrophic plants exhibited higher soil biological, enzymatic and metabolic activities. In turn, the soil disturbance decreased most parameters analysed while increased the count of culturable microbial functional groups and C metabolism. In the second phase of the experiment, the effect of wheat growth on the same parameters of soil biological activity was assessed, taking into consideration different antecedent plants and whether the soil was previously disturbed or not. Wheat plants grown after mycotrophic plants in undisturbed soils presented higher shoot dry weight, enzymatic activity, photosynthetic parameters and mycorrhizal colonization. These findings suggest that soil biological activity is highly affected by the preceding plant and soil disturbance, and greatly impacted on wheat growth under Mn toxicity. The improvement in wheat dry weight after mycotrophic plants in the undisturbed soil may rely on shifts in microbial functional profile associated with a well-established AMF colonization and the intact mycelium network.

**Resposta funcional microbiana à perturbação do solo e à sequência de plantas. O estudo de caso de fungos micorrízicos arbusculares e seu efeito bioprotetor no trigo contra a toxicidade do manganês.**

**Resumo**

Os fungos micorrízicos arbusculares (FMA), principalmente os autóctones, são um importante aliado no crescimento de plantas e no atual contexto agronómico o seu contributo pode ser essencial. Quando a colonização da planta por FMA é iniciada a partir de um micélio extra radicular (MER) intacto, a infecção desenvolve-se mais cedo e rapidamente, resultando numa otimização dos benefícios para a planta hospedeira, especialmente no que se refere à proteção contra estresses abióticos, como a toxicidade de metais. Este trabalho, desenvolvido numa experiência em vasos e em condições controladas (estufa), teve por objetivo estudar o efeito do crescimento do trigo (*Triticum aestivum* L.) sob estresse de manganês no perfil funcional do microbioma do solo, levando em consideração a micotrofia das plantas que o antecederam (desenvolvimento do MER) e a perturbação do solo (MER intacto ou perturbado). Numa primeira fase foi avaliado o efeito de quatro espécies de plantas com diferentes níveis de micotrofia em vários parâmetros de atividade biológica do solo, bem como o efeito da perturbação do solo após cada planta. Os resultados mostraram que no solo onde cresceram plantas micotróficas se observaram os maiores valores de atividade biológica, enzimática e metabólica. Por sua vez, a perturbação do solo causou uma diminuição na maioria dos parâmetros analisados, tendo aumentado o metabolismo de fontes de carbono e a contagem de grupos funcionais cultiváveis. Na segunda fase do experimento, avaliou-se o efeito do crescimento do trigo na atividade biológica do solo levando em consideração as diferentes plantas antecedentes e o facto de o solo ter sido previamente perturbado ou não. O trigo que cresceu após plantas micotróficas e em solo não perturbado apresentou maiores valores de matéria seca da parte aérea, de atividade enzimática, parâmetros fotossintéticos e colonização por FMA. Estes resultados sugerem que a

atividade biológica do solo é altamente afetada pelo tipo de planta antecedente e pela perturbação do solo, condicionando de forma significativa o crescimento do trigo em condições de toxicidade do Mn. Além disso, o aumento no peso seco do trigo crescido após plantas micotróficas e solo não perturbado parece depender das mudanças do perfil funcional microbiano do solo associado a uma colonização por FMA bem estabelecida na planta hospedeira e à integridade da rede de micélio desenvolvida no solo.

**List of abbreviations**

| | |
|---|---|
| OECD | Organization for Economic Cooperation and Development |
| FAO | Food and Agriculture Organization |
| SOC | Soil organic carbon |
| SOM | Soil organic matter |
| ROS | Reactive oxygen species |
| PGPB | Plant growth-promoting bacteria |
| AMF | Arbuscular mycorrhizal fungi |
| ERM | Extra radicular mycelium |
| CLPP | Community-level physiological profiling |
| RNA | Ribonucleic acid |
| DNA | Deoxyribonucleic acid |
| SBR | Soil basal respiration |
| MBC | Biomass microbial carbon |
| $qCO_2$ | Metabolic quotient |
| MPN | Most probable number |
| CFU | Colony forming units |
| TTC | 2,3,5-triphenyltetrazolium chloride |
| TPF | Triphenyl formazan |
| ISO | International Organization for Standardization |
| PSII | Photosystem II |
| ΦPSII | Quantum yield of photosystem II |
| Fv/Fm | Maximum yield efficiency of photosystem II |
| A | Photosynthetic rate |
| *gs* | Stomatal conductance to water vapor |
| ETR | Electron transport chain rate |

| | |
|---|---|
| qP | Photochemical quenching |
| AWCD | Average well color development |
| TSA | Tryptic soy agar |
| H’ | Shannon-Wiener diversity index |
| E | Shannon evenness index |
| PCA | Principal component analysis |
| PC | Principal component |
| nm | nanometer |

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Chapter 1

# State of the Art

### 1.1. Current challenges in agriculture – acidic soils

The global food system is expected to provide food for a population that will likely grow from 7.5 billion people now to nearly 10 billion by 2050. The key challenge in this scenario is to increase food production while at the same time reducing environmental impact, preserving the natural resources for future generations (OECD, 2021). Improving agricultural productivity in a sustainable way to meet increasing demand, entered the 2030 agenda of the United Nations and a key concern is how this additional production is going to be achieved (FAO, 2017) .

To meet the food demand, the solution relied on increasing the area of cropped fields. However, several studies have demonstrated the great impact on biodiversity losses in several ecosystems by conversing native forest and native grassland into crop fields (FAO, 2016; Tsiafouli et al., 2015). In addition to traditional agronomical practices as tillage, fertilizers and pesticides inputs, monoculture and irrigation can cause land desertification over time, soil erosion, acidification, nutrient deficiency and compaction, thus reducing cropping harvesting (Liu et al., 2021; Madarász et al., 2021). Both agricultural expansion and intensification are also major contributors to climate change. Agriculture is responsible for 30–35% of global greenhouse gas emissions, methane emissions from livestock and rice cultivation, and nitrous oxide emissions from fertilized soils (Foley et al., 2011).

In the last decades, influenced by the effects of *Green Revolution*, a growing trend towards the adoption of conservation agriculture has been observed, challenged by the need of a more efficient land use (Pingali, 2012). Conservation agriculture seeks to reduce soil disturbance by minimizing mechanical tillage, maintain a protective organic cover on the soil surface, and cultivate a wider range of plant species in associations, sequences and rotations (Goss et al., 2017a). In fact, many studies relate conservation or reduced tillage or even no tillage to the improvement of soil quality and the preservation of soil organic carbon (SOC) content (Krauss et al., 2020; Liu et al.,

2021; Madarász et al., 2021; Page et al., 2020). A reduction in tillage practices, associated with conservation agriculture, reduces soil organic matter (SOM) loss and in last instance reduces C emissions (Haddaway et al., 2017). A summary of benefits of this agricultural approached is presented in the figure 1.

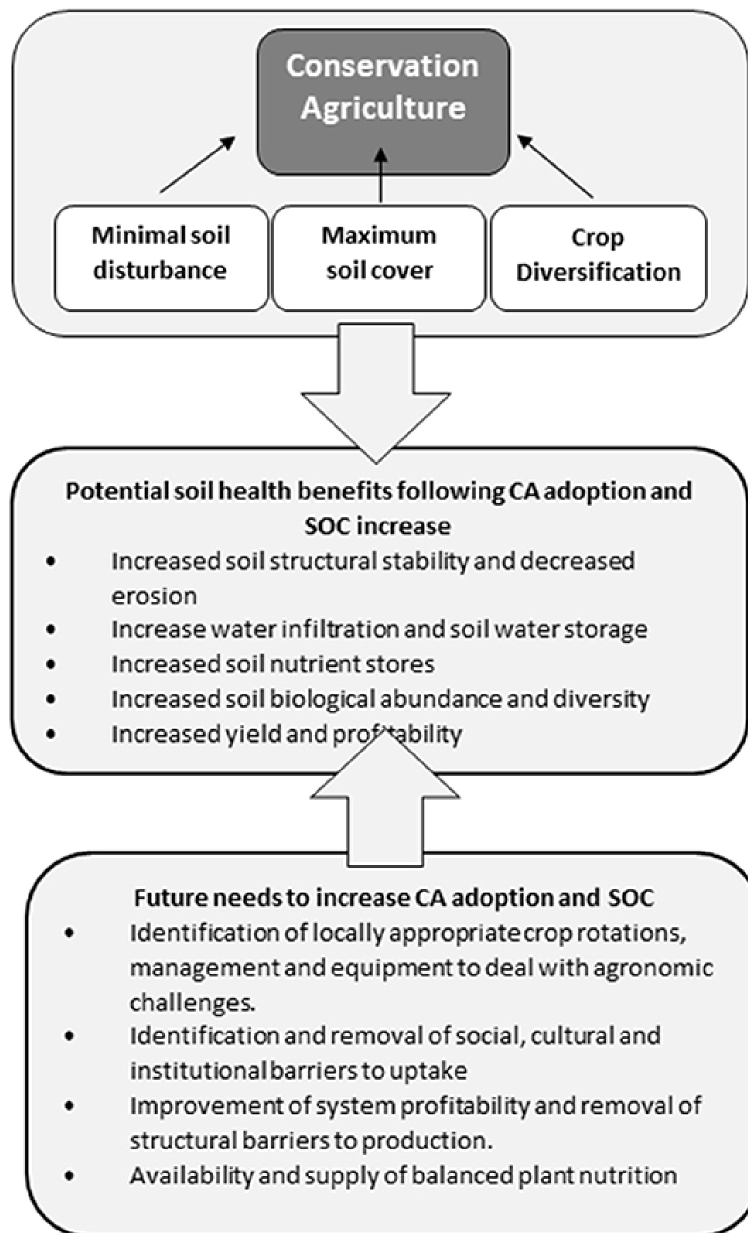


**Figure 1:** Summary of benefits of increased soil organic carbon (SOC) under conservation agriculture and the future needs to increase its adoption. Source: Page et al. 2020.

These benefits have led the adoption of conservation agriculture as an important tool to help ensure food production and agricultural productivity on this scenario of global climate change. However, it requires that this conservation approach is well

adapted to individual regions and environments, considering the specifics of each ecosystem. To successfully identify practices appropriate for different cropping systems, adequate research is required to develop the best strategy of effective management (Garbach et al., 2017; Kassam et al., 2019).

The most significant cause of yield loss varies within and between different regions in the world. In Portugal, Cambisols (FAO) are the major reference soil group. Ecosystems based on Cambisols that is originated from a granitic bedrock, result in soils with low SOM and very high acidity (Serrano et al., 2020). Soil acidity restricts agricultural production mainly due to nutrient deficiency and toxicity by metals such as manganese (Mn) and due to the different tolerance of botanical species, with significant impact on the culturable plant composition (Carvalho et al., 2015; Serrano et al., 2021).

In soil, Mn occurs in three oxidation states, i.e., the phytoavailable form $Mn^{2+}$, as well as the insoluble yet easily reducible forms $Mn^{3+}$ and $Mn^{4+}$ as Mn-oxides (Millaleo et al., 2010) . The redox status of Mn and therefore its bioavailability is largely influenced by soil pH. With decreasing pH, the amount of exchangeable manganese - mainly $Mn^{2+}$ form - increases in the soil solution (Fernando & Lynch, 2015). Another key factor in the Mn dynamics in soil is SOM. Given that SOM is negatively charged, it has a great Mn adsorption capacity, forming Mn complexes which decrease the amount of exchangeable Mn (Millaleo et al., 2010). Mn is also an essential element for plant development and mainly have two different functions: acting as an enzyme cofactor or as a metal with catalytic activity in biological clusters thus affecting many physiological processes. However, by far, its major involvement is in oxygen evolution in photosynthesis. Manganese plays at least two roles in this process, being involved both in the water-splitting reaction in the photosystem II (PSII) which provides the necessary electrons for photosynthesis, and in acting as cofactor of enzymes involved in isoprenoid biosynthesis, such as chlorophyll, therefore maintaining the structure of the stacking of the chloroplast lamellae (Alejandro et al., 2020).

When in excess, Mn cause plant toxicity and act as an important factor limiting plant growth on acid soils (Le Bot et al., 1990), thereby compromising productivity of several agronomic systems. Plant overexposure to Mn manifests most obviously as leaf chlorosis, but dark inclusions and/or crinkling symptoms are also generally interpreted as Mn stress. According to Alejandro et al. (2020), Mn stress in plants can be explained by two main hypotheses based on either symplastic or apoplastic via. The symplastic hypothesis proposes that Mn toxicity acts via photo-oxidative stress in the chloroplast that causes chlorosis. Conversely, in the apoplastic hypothesis, Mn stress damage is mainly due to the accumulation of Mn oxides, oxidized phenolic compounds, and reactive oxygen species (ROS), in the cell wall, leading to necrosis. The effects of Mn toxicity are likely to vary among plant species and genotypes (Jifu Li et al., 2019). In fact, a studied carried out in acidic soil of wheat growth under Mn toxicity have been demonstrated that Mn toxicity did alter the antioxidant enzymes activity, element uptake and subcellular distribution leading a reduce to shoot fresh weight, leaf extension, nodal root growth and early senescence of leaves (Faria et al., 2020).

Several strategies have been studied to mitigate the problem of acidity in soils. The amendment of dolomitic lime has showed a positive impact on pasture and cereal productivity (Serrano et al., 2020). The adoption of reduced tillage or no-tillage rather than conventional tillage also helped to improve soil stability and wheat yield over time (Carvalho, 2006, 2013). In addition, if crop residues are left on the soil surface in no-till management faster increases in soil SOM are observed (Carvalho et al., 2010). This is particularly important due the poor levels of SOM found in acidic soils in Alentejo. Another strategy employed to improve soil fertility is crop rotation. Preceding crops before wheat could help amendments input managements and water use efficiency (Carvalho et al., 1998; Carvalho & Basch, 1999). Nevertheless, crop yield increase in degraded acidic soils is a great challenge due to manganese toxicity. Among soil properties, its biological component is by far the most important since the soil microbiome directly affect nutrient cycling, soil organic matter content and plant nutrient

uptake (Goss et al., 2011). Therefore, alternative agricultural practices that resort preferentially in endogenous resources of the ecosystem itself should be taken in perspective to aid overcoming this issue.

### 1.2. Plant-microbe interaction

Plant-microbe interactions are a complex, dynamic and continuous process that took place with Earth's plant colonization. In both natural and agricultural systems, plants are frequently "invaded" by beneficial and pathogenic microorganisms, mainly bacteria and fungi. Even though these microorganisms may cause harmful interactions, they are also involved in many beneficial traits to plants (Dolatabadian 2020). Plant-microbe symbiosis can provide nutrients, direct stimulation of growth through phytohormones, antagonism to other soil pathogenic microorganisms and mitigation of biotic and abiotic stresses (Harman et al., 2021). Therefore, research on plant–microbe interaction is a key component in recognizing the soil functional status and thus the impacts of microbes on plants.

Soil is a closed system with a finite supply of essential elements responsible for plant development such as carbon (C), nitrogen (N), sulfur (S) and phosphorus (P), recycling of these elements is fundamental to avoid soil exhaustion. In soils, most of these nutrients are bounded to organic molecules unavailable for plants. Thereby, the microbial metabolic machinery is critical in the process of breaking down organic material into forms that can be reused by other organisms (Jacoby et al., 2017). In agrosystems, an appropriate management of beneficial microbes is vital for a sustainable crop production, as it improves the health and quality of the soil and aid in recycling crop residues (Dubey et al., 2019).

Additionally, plants can communicate with soil microbiome through rhizodeposition by root litter and exudation (Lynch & Whipps, 1990). The rhizodeposition process refers to the release of a wide range of compounds that takes place in the rhizosphere (Figure 2), which is defined as a region that is influenced by

these exudates and is home to a microbial community that is crucial for biochemical transformations of SOM. Because it is a carbon-rich environment, the rhizosphere has more micro-organisms than “bulk” soil (Marschner, 2012). The exudates not only shape the microbiome, but also are involved in the chemical signaling of beneficial root symbiosis with rhizobia and arbuscular mycorrhizal fungi (AMF), and in associations with plant growth-promoting bacteria (PGPB) (Bais et al., 2006).

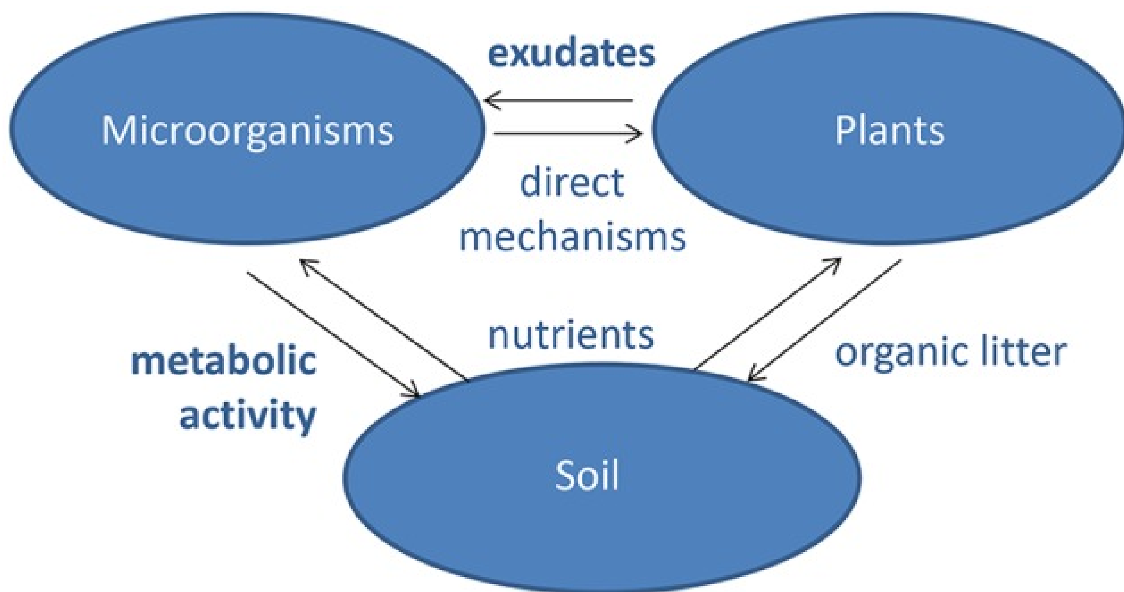


**Figure 2:** Interactions between plants, microbiota, and soil. Both plants and microorganisms obtain their nutrients from soil and change soil properties by organic litter deposition and metabolic activities, respectively. Microorganisms have a range of direct effects on plants and plants communicate with the microorganisms through metabolites exuded by the roots. The major knowledge gaps for understanding the mechanisms of plant–microbe interactions in the rhizosphere are shown in bold. Source: Jacoby et al., 2017.

The quantity and composition of root exudates are influenced by several factors and vary in time and space according to the position on the root. Genotype and plant age along with different environmental factors, grazing activities and anthropometric habits can modify quantitatively and qualitatively the amount of root exudation. Therefore the plant-microbiome interaction is known to be plant-species specific (Haldar & Sengupta, 2015). For example, legume plants, due to symbiotic interaction with nitrogen-fixing rhizobia, present a root exudation that differs, in quantity and

quality, from non-leguminous plants which generates the distinctive assembly of the rhizospheric microbiome (Santos & Olivares, 2021). This is also found comparing grass and forb species (Dietz et al., 2020). Plants that form symbiotic association with fungi may also have their root exudation altered, shifting the root microbiome composition. The extraradical mycelium (ERM) extending from colonized roots into the soil also serves as an additional niche for bacterial growth (Yuan et al., 2021). Consequently, the use of crop rotations or cover crops represents an interesting option for modifying soil microbial communities. Higher diversity of plant eco-functional groups creates heterogeneity of the favorable niches for different soil functional microbes. Therefore, crop diversification management with more plant functional groups could improve the soil condition, enhance soil fertility in long-term, and affect crop yield (Yang et al., 2020).

Besides responding to biotic factors, root metabolites are also released as a reaction to the abiotic environment. The influence of particular minerals or toxic metals in the soil affects the composition of root exudation. For example, organic acids (citric, malic and oxalic acids) are secreted and act to chelate soil metals (such as aluminum and manganese) therefore reducing their toxicity (Chen et al., 2015). Secretion of phenolic compounds is increased in P-deficient soils and secretion of signaling molecules like flavanones and flavones is enhanced in N-limiting conditions (Santos & Olivares, 2021). In case of Mn toxicity, the modification of root exudation in the rhizosphere also affected the composition of microorganisms involved in Mn oxidation and reduction (Marschner, 1991). Mn-oxidizing bacteria can decrease Mn availability in aerated or in poorly aerated soils.

In cropping systems, the use of specific agricultural practices impose strong selections in the rhizosphere composition community. Conventional tillage is known to cause physical disruption of the upper soil horizon, creating a homogeneous layer of soil with relatively uniform physical characteristics and nutrient distribution. This management practice also affects soil chemical properties and may result in soil

aggregate disruption, soil erosion, decreased amounts of SOM, shifted composition of microbiome and lower biodiversity of soil microbial species in long-term of soil use (Guo et al., 2016; Kraut-Cohen et al., 2020). Conversely, no-till practices with crop residue retention were shown to enrich the soil microbial community, to favor slower organic matter degradation and to enhance soil microbial diversity (Srour et al., 2020). However, metagenomics studies have shown that no-till is responsible to establish in some cases a less diverse but more oligotrophic, complex, and stable microbial communities. On a long-term basis, this can promote a higher abundance of microbes degrading more complex organic compounds, which enhances soil fertility (Delitte et al., 2021).

The loss of soil biodiversity is a major problem in current agrosystems since soil microorganisms participate in major biogeochemical cycles of nutrients. Traditional soil management could affect crop development by a loss of microbe interactions, shift the microbe functional complementarity and prevalence of other groups leading to a loss of redundancy (Griffiths & Philippot, 2013; Yin et al., 2000). An appropriate management of soil microbes can affect agricultural productivity, for instance by assisting and controlling nutrient availability or acquisition (Goss et al., 2011), promoting stress tolerance (Brito et al., 2019; Harman et al., 2021), increasing SOM content (Murphy et al., 2011), preventing soil erosion (Rillig & Mummey, 2006; Wilpiszeski et al., 2019), improving water retention (Timmusk & Zucca, 2019; Zheng et al., 2018), protecting against plant pathogen (Andreote et al., 2014) and driving the carbon dynamics and stocking (Witzgall et al., 2021). Therefore, contributing to a more sustainable agriculture in the current climate change scenario (Dubey et al., 2019).

### 1.3. The role of arbuscular mycorrhizal fungi (AMF) in plant protection

One of the most important plant-microbe association is the root colonization by arbuscular mycorrhizal fungi (AMF). This symbiosis likely made possible the conquest of land by the first plants about ~470 million years ago and currently colonizes more

than 80% of terrestrial plants (Goss et al., 2011). As highlighted by Goss et al. (2017b) this symbiont is present across all soil types and biomes, comprising natural and anthropogenic ecosystems. These features make them unique among other mutualistic symbionts underlying its great importance in almost all terrestrial ecosystems.

Inside the root, the AMF form arbuscules and hyphae that expands out of the root forming spores and hyphae beyond the rhizosphere. The formation of this hyphal network by the AMF significantly enhances the access to a large soil volume, generally causing improvement in plant nutrition. This is achieved due to an extra radicular mycelium (ERM) that expand the nutrient uptake outside the root depletion zone, increasing the nutrient translocation (Begum et al., 2019). Besides improving nutrition, several benefits to plants are accrued to AMF symbiosis: alleviation of water stress (Junqin Li et al., 2019), increased efficacy of N-fixation by legumes (Meng et al., 2015), tolerance to toxic heavy metals and soilborne pathogens (Brito et al., 2019; Hildebrandt et al., 2007), tolerance to adverse environmental conditions such as temperature, salinity and pH (Z. Li et al., 2020; Zhu et al., 2017), outlining protection against several biotic and abiotic stresses (Goss et al., 2017b). AMF also improve the quality of soil by enmeshing soil particles shaping its structure, and thus plant development (Verbruggen et al., 2016).

The microbial communities surrounding mycorrhizal roots and ERM are different from those of the rhizosphere of non-mycorrhizal plants since AMF colonization can affect root exudates (Basu et al., 2018). Furthermore, the ERM forms an additional niche that shape an entirely diverse microbiome in the rhizosphere and influence interactions with other beneficial microorganisms such as plant growth-promoting bacteria (PGPB) and rhizobia (Duponnois et al., 2008). PGPB are linked to induce changes in plant hormones and its release to the soil; produce volatile organic compounds which promote plant growth; improve nutrient availability and its uptake by plants and enhance abiotic stress tolerance (Efthimiadou et al., 2020). AMF also take part in a tripartite association with rhizobia. The AMF are known to enhance nodulation

in legumes, and the tripartite symbiosis often acts synergistically on root infection rate, mineral nutrition and plant growth (Checcucci & Marchetti, 2020).

Despite the generally recognized importance of AMF and plant symbiosis under natural systems for nutrient acquisition and bioprotection, its intentional use in agricultural systems has been marginal (Brito, Carvalho, Alho, et al., 2013). Most of studies have focused on commercial inoculants rather than management of indigenous organisms. Additionally, large-scale inoculation of AMF is generally impractical in most regions due to an increase of the cost of production and the consequences of using inocula are still poorly understood. Alternatively, cultivating plants that are natural hosts can increase the AMF population while maintaining mycorrhizal activity in soil. Therefore, studies that focus on native AMF management could help to maximize the benefits of this naturally occurring symbiosis and contribute to a more sustainable agricultural practice (Brito et al., 2012). However, before a host plant can benefit from mycorrhizal association, the colonization must be well stablished and therefore the timeliness can be more crucial than the extent of colonization (Goss & De Varennes, 2002). Despite different types of propagules, they may not be equally effective at producing new infection units. Among the different forms of AMF inoculum, an intact extraradical mycelium (ERM) has been shown to promote an earlier and faster colonization than the others types of propagules (Brito, Carvalho, & Goss, 2013; Fairchild & Miller, 1990; Klironomos & Hart, 2002). Consequently, managing native mycotrophic plants growth that induces ERM development of indigenous AMF associated with a soil with minimum disturbance can lead to a faster colonization of the subsequent crop that in turn can benefit from AMF bioprotection (Brito et al., 2019).

In the opposite, the soil disturbance caused by conventional soil management disrupts the established ERM, compromising its ability to start new AMF colorizations and also directly impacts in its associated microbiome. The soil disturbance can interfere with the AMF diversity as promoting or impairing specific AMF groups based on their life cycle and colonization strategies (Brígido et al., 2017; Brito et al., 2012;

Campos et al., 2018). Additionally, many mechanisms of bioprotection mediated by AMF can be altered by soil disturbance such as oxidative enzymes activation and Mn allocation (Faria et al., 2021a) and element uptake and cellular distribution (Faria et al., 2021b).

Several studies have linked AMF with metal toxicity alleviation in many ecosystems (Bothe et al., 2010; Cardoso et al., 2017; Gupta et al., 2019; Hildebrandt et al., 2007). Especially the manganese toxicity in acid soils (Brito et al., 2019; Garcia et al., 2020; Nogueira et al., 2004). However, despite the successful management of indigenous AMF to overcome abiotic stresses have been established in terms of crop growth (Goss et al., 2017b), the changes in the soil functional metabolism in wheat growth under Mn toxicity are still scarcely described.

### 1.4. Assessing soil functional microbial status

Soil management have strongly developed in last decades by adapting or innovating farming practices. But the development of these systems has generally become independent of soil biology and currently there is still a low database on microbiome metabolism dynamics under different management conditions (Basu et al., 2018). Soil functionality can be characterized by integrity of nutrient cycles and energy flows, stability, and resilience to disturbance or stress (van Bruggen & Semenov, 2000). Agronomic practices and soil management such as crop rotation and reduced tillage affect the biological properties of the soil. In fact, some of these biological properties can be used as valuable indicators of soil functional status (Srour et al., 2020). Microorganisms are critically important to maintaining the physical structure and many functions of soil, and there are still a lot more to discover about how cover cropping and tillage impact soil microbial community composition and the services they provide in agroecosystems (Schmidt et al., 2018).

Since microbes are critical in the process of breaking down and transforming dead organic material into forms that can be reused by other organisms, the microbial

enzyme systems involved in SOM transformation can be viewed as key ‘engines’ that drive the Earth's biogeochemical cycles (Gougoulias et al., 2014). Rhizosphere, the soil zone in contact with the root and influenced by root activities and root exudates, is a unique environment where the microorganisms are selectively enriched. As a result, the enzyme activities are more dominant in the rhizosphere zone compared with bulk soil mediating the biogeochemistry of minerals (Pandey et al., 2015). Important soil enzymes include those involved in C, N, P, and S cycling. The enzymes involved in C cycling may not directly provide nutrients for plant growth but are necessary for the proliferation of soil microorganisms that promote plant growth by other means. They degrade complex organic carbon compounds to release simple utilizable C compounds such as sugars, organic acids, etc. The enzymes of N, P, and S cycles mineralize compounds of respective nutrients from the soil organic compounds, which can be utilized by both microorganisms and plants (Dotaniya et al., 2019).

The most studied soil enzymes belong to the classes of oxidoreductases and hydrolases (Dotaniya et al., 2019). Dehydrogenase (EC 1.1.1.) is a group of oxidoreductase isoenzymes that is used as an indicator of general soil microbial activity because it occurs intracellularly in all living microbial cells. It plays a significant role in the biological oxidation of soil organic matter and can be assumed as proportional to the biomass of the microorganisms in soil (Wolinska et al., 2012). Arylsulfatase (arylsulfate sulfohydrolase, EC. 3.1.6.1), β-glucosidase (1,4 - D- glucosidase, EC 3.2.1.21) and phosphatase (phosphoric monoester hydrolases, EC 3.1.3) are key hydrolase enzymes involved in the mineralization of organic forms of S, C and P (Deng & Tabatabai, 1996; Klose et al., 1999). Therefore, soil enzymatic activity could be used as an indicator of functional profile and microbial status of soil management (Dick & Burns, 2011; Gianfreda & Ruggiero, 2006). However, due to their substrate specificity, enzymatic activity should not be assessed as an individual parameter to determine microbial activity indices (Alkorta et al., 2003).

Some other parameters have been generally accepted for evaluating changes in soil functional activity. Soil microbial biomass (carbon and nitrogen) and soil respiration have been wildly used as indicators of soil biological status (Vogel et al., 2019). Soil microbial biomass carbon has been commonly recognized as an important indicator of soil microbial properties. It represents the size of microbial pool which reflect soil organic matter changes such as carbon cycling (Hsieh et al., 2020). Soil respiration is also closely related to several functions of organisms. The measurement of soil basal respiration has been applied across a variety of studies and could be used to assess changes imposed to agricultural practices (Creamer et al., 2014). Metabolic quotient represents the metabolic status of soil microorganisms, in which larger values indicate greater stress conditions, but it has to be interpreted with caution, because an increase could also indicate an input of easily degradable carbon that stimulates microbial activity (Cardoso et al., 2017).

Heterotrophic microorganisms are dominant drivers of biogeochemical cycles and rapidly respond even to small soil interventions. Even though bacterial counting is considered a laborious methodology, it still could be used as the initial point of a study due to its relatively inexpensive cost and could assess the gross diversity of functional culturable microorganism (Nannipieri et al., 2017; Tate, 2020). Though only a small part of soil microorganisms is culturable, the effects of soil management on the soil functional microbiome still could be observed, since it leads to changes in the organic matter content that in turn influences microbial activity (Albino & Andrade, 2007). In functionally complex soil ecosystems, biogeochemical processes associated with cycling of C and N are the major energy flow systems, but other biogeochemical cycles, such as P, S and Mn are also important for plant nutrition, especially in acidic soils.

Nitrogen (N) is one of the most important nutrients in terrestrial ecosystems and its transformations in agro-ecosystems is indispensable to sustain crop production. Ammonification is one step of the nitrogen cycle during which microorganisms

mineralize small organic molecules containing an amine group in order to release ammonium which can be easily taken up by plants and thus play a vital role in plant growth (Wolińska et al., 2016). Phosphorus (P) is the second most limiting plant nutrient after N. Total P content in soil is usually high, but most of this soil P pool is not available for plant uptake. Bacteria that can mobilize P from unavailable soil pools and increase P availability to plants are of great importance (Alori et al., 2017).

Sulfur (S) oxidation and reduction are key biogeochemical processes to the energy metabolism in soils. Sulfur, although considered a secondary macronutrient, is vital for life, and biogeochemistry of S can affect agricultural productivity (Tourna et al., 2014). The form of biologically active sulfur for plants is the sulfate ion and heterotrophic microorganisms in soil can oxidize the elemental sulfur and produce sulfite or thiosulfate, that in turn can be transformed into sulfate (Albino & Andrade, 2007). The oxidation of Mn in acidic soils is almost entirely a microbial process. Mn is an essential element in plant nutrition and the influx from soil to plants relies on its oxidation state. When in excess, as in acidic soils, this element can be oxidized by bacteria into Mn oxides (Sparrow & Uren, 2014).

Thus, the ability of microbial communities to respond rapidly to the changes in land use can be employed to compare the effects of agroecological and conventional management. Since agroecological practices include an integration of several agricultural tools, such as reduced tillage and crop diversification, it would be expected that changes in microbial dynamics compared with conventional management would be observed. Therefore, considering that the higher microbial diversity in ecosystems could establish a functional equilibrium which may enable sustainability to be preserved, it is important to generate knowledge about the effect of management interventions on soil functional microbial communities (Chavarria et al., 2018).

An additional effective parameter to assess changes in the functional microbiome caused by agronomic practices is the community-level physiological profiling (CLPP) analysis. It indicates patterns of potential C source utilization by soil microbial

communities that may have effects on ecosystem processes. The Biolog® system has been used widely for environmental research, allowing the monitoring of changes in the soil microbial communities in the soil under the influence of various factors (Gryta et al., 2020). The advantages of CLPP over cell culture and molecular level RNA/DNA amplification-based techniques are the simplicity of the protocol and the reduced cost. Although CLPP involves inoculating plates with mixed cultures of microbes, where only a small percentage are culturable, this analysis could be effective at detecting spatial and temporal changes in soil communities and provides information regarding functional aspects of soil communities (Adams et al., 2017). The metabolic study of soil microbiomes regarding different carbon sources could be an indicator of changes in soil status or shifts caused by biotic and abiotic effects. Thus, based on carbon source metabolization by soil microbial communities, significative differences in the soil metabolic diversity can be detected (Gajda et al., 2019).

More broadly, the study of AMF and soil functional microbiome interaction as a response to abiotic stress, such as Mn toxicity, regarding the growth of specific host plants and soil management could be evaluated when the role of AMF in every scenario is better understood. This includes clearer view on the ways by which AMF interact with soil biota and can be achieved by measuring the microorganisms which are important in soil nutrient cycling (such as total bacteria and fungi) and organic matter mineralization (functional groups and enzymatic activity), as well as assessing other factors that influence their overall contributions in crop yield. Little is known about what makes soil microbial communities vulnerable to abrupt changes taking into consideration their functional state. These represent important gaps in knowledge given the sensitivity of soil microbial communities to crop management and their importance for ecosystem functioning.

### 1.5. Objectives

The main objective was to assess the differences in soil functional profiles and biological activity of wheat growth associated with the bio protective effect of arbuscular mycorrhiza under Mn toxicity, surveying the impacts imposed by growing anteceding plants with different levels of mycotrophy and soil disturbance and ERM integrity

Specifically, we aimed to:

1. Implement and adapt techniques for assessment of soil biological activity, soil enzymatic activity and soil functional profile in the Soil Microbiology Laboratory of Mediterranean Institute for Agriculture, Environment and Development (MED).
2. Assess the pattern of soil biological activity, enzymatic activity, functional profile and microbial diversity at different stages of plant sequences with different levels of mycotrophy and soil disturbance (extra radicular mycelium integrity)

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# The effect of plant mycotrophy and soil disturbance on soil microbial activity

## 2.1. Abstract

In cropping systems, the choices adopted for tillage system and plants cultivated can strongly influence the soil microbial population and its functionality. This work aimed to assess the effect of plant species, its level of mycotrophy, and soil disturbance, on the profile of microbial functional groups, enzymatic activity and general soil biological attributes in soil presenting Mn toxicity. We used two highly-mycotrophic plants (the grass *Lolium rigidum* and the legume *Ornithopus compressus*), one scarcely-mycotrophic (*Rumex bucephalophorus*) and a non-mycotrophic plant *Silene gallica* (control). Soil disturbance after plant growth was imposed by sieving the soil through a 4-mm screen. Soil biological parameters were evaluated before and after plant growth, and 10 days after soil disturbance. The soil under mycotrophic plants showed higher activity of dehydrogenase, soil basal respiration and microbial biomass carbon than non-mycotrophic plants, which in turn showed an increased metabolic quotient. Soil disturbance greatly affected the groups of Mn oxidizers and P solubilizers under mycotrophic plants. Enzymatic activity assessment showed that mineralization of organic matter was different among treatments and was linked to plant mycotrophy. Both plant species and soil disturbance altered biological activity, but the impact of Mn stress among mycotrophic and non-mycotrophic plants appeared to be different, suggesting that the presence of mycorrhiza and their associated microbiome play an important role in establishing differences in functional profile of soil microbes.

## 2.2. Introduction

Soil microbiomes are essential for the maintenance of biogeochemical processes and have a decisive role in nutrient cycling and therefore on the availability of nutrients for plant nutrition. As a response to different types of disturbance and stresses, the microbial community could be used as functional indicators of agricultural practice management (Zilli et al., 2003). Functional diversity includes the multiplicity of microbial activity in soils and is closely linked to ecosystem stability (Hampp & Tarkka, 2009). Among soil microorganisms, the most ubiquitous are mycorrhizal fungi that form an ancient and widespread symbiosis with vascular plants. Within that group, the most important for agricultural crops are the arbuscular mycorrhizal fungi (AMF). The AMF symbiosis consists of intercellular hyphal colonization of the root cortical zone and form arbuscules between cell wall and plasmalemma of some cells. In soil the AMF develop a net of extraradical mycelium (ERM) that extends beyond the rhizosphere exploiting a larger amount of soil and therefore enhancing acquisition of nutrients (Duponnois et al., 2008; Goss et al., 2017). Other benefits accrued by the host plant is the tolerance to biotic and abiotic stresses (Begum et al., 2019; Brito et al., 2014, 2019), modification of plant gene expression (Balestrini & Lanfranco, 2006), modulation of host defense system (García-Garrido & Ocampo, 2002) and the hormonal balance to improve plant growth (Chanclud & Morel, 2016). AMF also enhance soil stability due to formation of microaggregates important for soil structure and necessary for root development (Barea et al., 2011).

As plants start to establish in the soil, roots exudate carbon compounds which activates microbial populations. The part of soil directly influenced by plant roots is called the rhizosphere, where most biological events occur and contribute to the rhizosphere effect. Thus, the rhizosphere is greatly different from root-free zones in its physical, chemical and biological properties (Priyadharsini et al., 2016). Biological activity in the rhizosphere is modified when AMF are present, since it is well known that

these fungi alter root exudation, both quantitatively and qualitatively, and consequently the surrounding microbial communities (Andrade et al., 1997). They also represent an additional habitat for others soil microorganisms (Timonen & Marschner, 2006). Therefore, AMF can change the bacterial composition of the rhizosphere by stimulating certain functional groups and depressing others (Vestergård et al., 2008). The zone influenced by mycorrhizal roots and the extraradical hyphae is called mycorrhizosphere (Linderman, 2008).

Factors such as soil type, plant growth stage, plant type and plant species might also influence the rhizosphere effect (Dotaniya & Meena, 2015; Li et al., 2019; Manoharachary & Mukerji, 2006; Martínez-Espinosa et al., 2011). Thus, the microbial activity that occurs in the rhizosphere of mycorrhizal and non-mycorrhizal plants is completely different (Akyol et al., 2019; Garbaye, 1991; Offre et al., 2007). Studies comparing rhizosphere microbiome between mycotrophic and non-mycotrophic plants showed differences in population and functional groups of bacteria (Marschner & Timonen, 2005; Meyer & Linderman, 1986; Secilia & Bagyaraj, 1987). Even among mycotrophic plants, the rhizosphere and mycorrhizosphere effect of Gramineae and Leguminosae show differences in biological properties and microbial diversity (Dotaniya & Meena, 2015; Zhou et al., 2017).

Soil microbiomes are also greatly influenced by various abiotic factors, such as pH, soil moisture, oxygen availability, and soil texture. These parameters are likely to change with tillage and crop residue management, and therefore influence soil microbial communities (Degrune et al., 2017). Cropping practices can alter soil organic matter (SOM) content and therefore could shift the balance of rhizosphere and mycorrhizosphere communities in biodiversity and function. Soil tillage affects the amount of SOM and alters the physical and chemical properties of soil environment by affecting water content and aeration. Conventional cropping practices with intensive tillage lower the diversity of community genetic structure, disrupts nutrient cycling and result in less stability or resilience of soil functional status (Smith & Collins, 2007).

Some parameters have been generally accepted for evaluating changes in soil functional activity. Soil microbial biomass (carbon and nitrogen) and soil respiration have been widely used as indicators of soil biological status (Vogel et al., 2019). Metabolic quotient represents the metabolic status of soil microorganisms, in which larger values indicate greater stress conditions, but it has to be interpreted with caution, because an increase could also indicate an input of easily degradable carbon sources that stimulates microbial activity (Cardoso et al., 2013). Soil enzyme activities are considered indicative of specific biochemical reactions of the entire soil microbial community involved in SOM mineralization (Klose & Tabatabai, 1999). In consequence, soil enzyme activity has become an increasingly common tool for indicating microbial response to soil use and management (Xiao et al., 2018). The most studied soil enzymes belong to the classes of oxide reductases and hydrolases (Dotaniya et al., 2019). Dehydrogenase (EC 1.1.1.) form a group of oxide reductase isoenzymes that is used as an indicator of general soil microbial activity because it occurs intracellularly in all living microbial cells. It plays a significant role in the biological oxidation of soil organic matter and can be assumed as proportional to the microbial biomass in soil (Wolinska et al., 2012). Arylsulfatase (arylsulfate sulfohydrolase, EC. 3.1.6.1), β-glucosidase (1,4 - D- glucosidase, EC 3.2.1.21) and phosphatase (phosphoric monoester hydrolases, EC 3.1.3) are key hydrolase enzymes involved in SOM (S, C and P respectively) by hydrolyzation of organic compounds in inorganic forms (Deng & Tabatabai, 1996; Klose et al., 1999). Therefore, the soil enzymatic measurement could be used as an indicator of functional profile and microbial status driven by soil management (Dick & Burns, 2011; Gianfreda & Ruggiero, 2006). However, due to its substrate specificity, the enzymatic activity should not be assessed as an individual parameter to determine microbiological activity indices (Alkorta et al., 2003).

Brito et al. (2014) proposed a strategy for managing AMF based on selecting host plants for the intentional development of an extensive ERM, which, when kept intact by the adoption of appropriate tillage techniques, acts as the preferential source of

inoculum for the following crop. Colonization from ERM occurs earlier and faster than from spores, so protecting the new crop against biotic and abiotic stresses existing in the soil. An understanding of how microbial communities respond to different agricultural practices and perturbations is important to maximize the sustainability of soil resources (Bissett et al., 2013). Therefore, the present work aims to understand the effect of the strategy proposed by Brito et al. (2014) on the growth of the ERM developer plant and on the functional activities of the remaining soil microbiota. We assessed the effect of plant type, according to their level of mycotrophy and soil disturbance, on the profile of (i) microbial functional groups, (ii) enzymatic activity and (iii) general soil microbiological attributes.

## 2.3. Materials and Methods

### *2.3.1. Experimental design*

A pot experiment was performed in a greenhouse under controlled conditions from January to April, 2019. We used a sandy, acidic soil (sandy loam Eutric Cambisol - FAO) collected from the top 20 cm of a natural pasture at Herdade da Mitra-University of Évora, Alentejo, Portugal (38° 32´ N; 08° 00´ W), having an organic C content of 10.5 $g.kg^{-1}$, a pH of 4.8 in water, the ammonium acetate exchangeable manganese content at pH 7 was 29 ± 4 $\mu g.g^{-1}$, and previously described by Goss & Carvalho (1992) as causing Mn toxicity in wheat. This soil was characterized having a high AMF diversity (Alho et al., 2015; Brígido et al., 2017; Brito et al., 2014). It was homogenized by sieving to ensure that all treatments had the same initial conditions and then packed into 8 kg pots. Four common arable plants species, widespread in the Mediterranean basin, were sown in 8 replicate pots, with 5 plants per pot. Two species, *Ornithopus compressus* L. (a legume) and *Lolium rigidum* Gaudin (a grass) known to be highly-mycotrophic; one (*Rumex bucephalophorus* L.) known as scarcely-mycotrophic; the fourth species (*Silene gallica* L.) was non-mycotrophic and served as the negative

control. To avoid confounding effects, weeds were controlled by hand on a daily basis and all pots were watered approximately to field capacity (0.17 $g.g^{-1}$) by weight. The plants grew for 11 weeks, after which their aerial parts were severed from the roots in all pots. For the Disturbed treatment, the soil in half of the pots of each species was subjected to mechanical disturbance by passing through a 4 mm sieve to disrupt the extra radicular mycelium. Roots were collected during this process and their colonization by AMF determined after staining with trypan blue, according to the magnified intersections method (McGonigle et al., 1990). The soil was mixed, repacked into the same pots and shoot material was returned to the soil surface. The remainder of the pots of each species formed the Undisturbed treatment and shoot material was also returned to the soil surface. All pots were then left for 10 days. Soil was sampled to assess biological activity at three phases of the experiment: the first before planting (bulk soil), the second at 11 weeks after plant growth to check the effect of plant type and the third sampling at 10 days after soil disturbance to check the effects of soil disturbance. Sampled soil was passed through a 2 mm sieve and the functional activity was measured in terms of soil microbial activity, functional groups of culturable microorganisms count and enzymatic activity related to organic matter cycling.

#### ***2.3.2. Soil Microbial Activity***

Water holding capacity and water content were determined (Monteiro & Frighetto, 2000) and used in calculating the parameters. Soil basal respiration (SBR) was measured in a closed jar incubated for 7 days at 26º C (Silva et al., 2007). The $CO_2$ released was adsorbed in NaOH and determined by HCl titration. The results were reported as milligrams of $CO_2$ released per kilogram of soil per hour ($mgCO_2.kg\ soil^{-1}.h^{-1}$). The proportion of microbial biomass carbon (MBC) relative to total organic carbon followed the fumigation-extraction method suggested by Vance et al. (1987) in which the soil is fumigated with chloroform in a desiccator and the carbon released is estimated by an oxidation reaction with potassium permanganate. The values of MBC

are given by the carbon content of fumigated soil minus that of the non-fumigated soils divided by the proportion of microbial C recovered (*kc*). A value of 0.45 was used for *kc* in MBC calculation, as recommended by Joergensen (1996). Results were expressed as milligrams of carbon per kilogram of soil (mgC.kg soil$^{-1}$). The metabolic quotient ($qCO_2$), the ratio between SBR and MBC (Anderson & Domsch, 1990), was used to estimate the efficiency of substrate consumption by microorganisms as a stress indicator when the microbial biomass is affected.

#### 2.3.3. Functional groups of culturable microorganisms

Six functional groups of culturable soil microorganisms were evaluated: total bacteria, fungi, ammonifiers, sulfur (S) oxidizers, manganese (Mn) oxidizers and phosphorus (P) solubilizers. For bacteria, fungi, ammonifiers, and P solubilizers the protocols are described in Albino and Andrade (2007). Mn-oxidizing microorganisms were counted accordingly to Nogueira et al. (2007) in Garretesen's medium. S oxidizers were counted in thiosulfate broth as suggested by Vidyalakshmi and Sridar (2007) using bromothymol blue as indicator of pH acidity instead of bromocresol purple. Ammonifiers and sulfur oxidizers were expressed as the logarithm of most probable number per gram of soil (logMPN.g$^{-1}$) and the others as the logarithm of colony forming units per gram of soil (logCFU.g$^{-1}$).

#### 2.3.4. Enzymatic Activity

Dehydrogenase was measured according to Casida et al. (1964) with modifications. Soil (5 g) was incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (5 ml) for 24 h at 37º C. Triphenyl formazan (TPF) formed by the reduction of TTC under dehydrogenase activity during incubation, was extracted from the soil with 20 ml of methanol and left to decant for about 10 min. The supernatant was centrifugated at 5000 rpm for 5 min and then 3 ml were transferred to cuvettes and determined by spectrophotometry ($\lambda$ = 485nm) in triplicate (Monteiro & Frighetto, 2000). The arylsulfatase, β-glucosidase and phosphatase activities were measured

according to ISO 20130:2018 (ISO, 2018) in 96-well microplates. After the incubation time appropriate to each enzyme (240 min for arylsulphatase, 120 min for β-glucosidase and 30 min for phosphatase), their respective substrates (potassium ρ-nitrophenyl-sulphate, ρ-nitrophenyl-β-D-glucopyranoside, and ρ-nitrophenyl-phosphate) were hydrolyzed into a yellow colored ρ-nitrophenol and all determined by spectrophotometry (λ = 405nm).

#### *2.3.5. Statistical analysis*

The experimental design was a complete randomized block with four replicates. The treatments were in factorial arrange and consisted of two factors: plant type (4 levels) and soil disturbance (3 levels). ANOVA was performed based on the two factors using a generalized linear model and Tukey's test at 5% level was used to compare the means using the software Minitab 21® (Minitab, 2021).

### 2.4. Results

#### *2.4.1. Root colonization rate by AMF*

To confirm the mycotrophic level of the plants used in this study, the AMF root colonization rate was assessed. The AMF colonization rate was 84% and 75% for *O. compressus* and *L. rigidum*, respectively. The colonization rate of the former was significantly higher than to the latter. No root colonization by AMF was observed in *R. bucephalophorus* and *S. gallica* roots.

#### *2.4.2. Soil microbial activity*

The plant mycotrophy and the soil disturbance strongly affected the soil microbiological activity. The values of the soil sampling (Table 1), showed a great increase of the soil basal respiration (SBR) and microbial biomass carbon (MBC) after plant growth, especially compared to the before plant sampling (bulk soil). On the other hand, soil disturbance greatly affected SBR and MBC decreasing them. Among the plants, the largest value of SBR was observed under *O. compressus*. The greatest

value of MBC was found under *L. rigidum*. The results for the metabolic quotient ($qCO_2$), which is the ratio between basal respiration and microbial biomass carbon, indicated that the smallest value was under *L. rigidum*, and that soil disturbance did not significantly interfere with the metabolic status.

The analysis of the effect of soil disturbance associated with each plant showed that SBR was notably greater under *O. compressus* after plant growth. The soil disturbance strongly reduced SBR in all treatments and differences between plant types were lost. The MBC decreased greatly in the non-mycotrophic plant (*S. gallica*) after soil disturbance, whereas $qCO_2$ markedly increased.

**Table 1:** Effect of the plant species and soil disturbance on soil basal respiration (SBR), microbial biomass carbon (MBC) and metabolic quotient ($qCO_2$).

| | **SBR** (mg $CO_2$.kg$^{-1}$soil.h$^{-1}$) | | | | **MBC** (mg C. kg$^{-1}$soil) | | | | **$qCO_2$** (µg $CO_2$.µg$^{-1}$ MBC.h$^{-1}$)$10^{-3}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** |
| **Plants** | | | | | | | | | | | | |
| *O. compressus* | 0.71 cd | 1.39 a | 0.46 de | 0.85 A | 40,40 de | 131.93 ab | 57,87 cd | 76.73 B | 17.6 ab | 11.9 bc | 07.9 bc | 12.5 AB |
| *L. rigidum* | | 1.10 b | 0.29 e | 0.70 B | | 150.66 a | 94.90 bc | 95.32 A | | 07.6 bc | 03.0 c | 09.4 B |
| *R. bucephalophorus* | | 0.90 bc | 0.40 e | 0.67 B | | 117.10 ab | 54.39 d | 70.63 BC | | 07.7 bc | 10.6 bc | 12.0 AB |
| *S. gallica* | | 0.84 bc | 0.31 e | 0.62 B | | 118.48 ab | 13.88 e | 57.59 C | | 07.1 bc | 24.8 a | 16.5 A |
| ***Mean soil sampling*** | 0.71 B | 1.06 A | 0.36 C | | 40.40 C | 129.55 A | 55.26 B | | 17.6 A | 8.6 B | 11.6 B | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). BP: Before Planting; AP: After Plant growth; AD: After Soil disturbance.

### *2.4.3. Functional groups of culturable microorganisms*

In general, all microbial counts were increased after plants growth compared to before plant sampling (bulk soil), but the effect of the disturbance varied within the functional groups (Table 2). The mean of plant type and therefore its mycotrophy was not statistically different among the functional groups count except for Mn oxidizers. For this group, the lowest count was observed under the scarcely-mycotrophic plant (*R. bucephalophorus*). At the same time, soil disturbance affected all functional groups, except the S oxidizers and fungi. Plant growth increased fungi count but the amount of colony forming units remained the same after soil disturbance. The soil disturbance increased most of the microbial counts but decreased P solubilizers.

The interaction of plant type and soil disturbance was only significant for bacteria, P solubilizers and Mn oxidizers. Soil disturbance caused a significant increase in bacteria count after *O. compressus* and *R. bucephalophorus* whereas decreased P solubilizers under highly-mycotrophic and scarcely-mycotrophic plants. For Mn oxidizers, the soil disturbance strongly increased them after *O. compressus* and *R. bucephalophorus*.

**Table 2:** Effect of the plant species and soil disturbance on soil microbial functional groups counting of bacteria, Mn oxidizers, P solubilizers, fungi, ammonifiers and S oxidizers.

| | **bacteria** | | | | **Mn oxidizers** (Log CFU.g$^{-1}$) | | | | **P solubilizers** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** |
| **Plants** | | | | | | | | | | | | |
| *O. compressus* | | 6.17 c | 6.49 ab | 6.16 | | 5.27 cd | 5.98 a | 5.35 A | | 5.55 ab | 5.30 cd | 5.33 |
| *L. rigidum* | 5.84 d | 6.27 bc | 6.37 abc | 6.16 | 4.79 e | 5.56 bc | 5.80 ab | 5.38 A | 5.13 de | 5.71 a | 5.25 cde | 5.36 |
| *R. bucephalophorus* | | 6.11 cd | 6.57 a | 6.17 | | 4.98 de | 5.65 abc | 5.14 B | | 5.63 a | 5.08 e | 5.28 |
| *S. gallica* | | 6.23 bc | 6.11 cd | 6.06 | | 5.49 bc | 5.61 abc | 5.29 AB | | 5.41 bc | 5.29 cde | 5.28 |
| ***Mean soil sampling*** | 5.84 C | 6.20 B | 6.38 A | | 4.79 C | 5.32 B | 5.76 A | | 5.13 C | 5.57 A | 5.23 B | |
| | **fungi** (Log CFU.g$^{-1}$) | | | | **ammonifiers** | | | | **S oxidizers** (Log MPN.g$^{-1}$) | | | |
| | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** |
| **Plants** | | | | | | | | | | | | |
| *O. compressus* | | 5.02 | 5.12 | 5.02 | | 5.39 | 7.09 | 5.66 | | 2.97 | 3.33 | 2.83 |
| *L. rigidum* | 4.91 | 5.17 | 5.15 | 5.08 | 4.49 | 5.20 | 6.68 | 5.46 | 3.06 | 2.94 | 3.33 | 3.11 |
| *R. bucephalophorus* | | 5.05 | 5.20 | 5.05 | | 5.43 | 6.71 | 5.54 | | 3.18 | 3.38 | 3.21 |
| *S. gallica* | | 5.14 | 5.03 | 5.03 | | 4.89 | 6.13 | 5.17 | | 2.91 | 2.11 | 2.69 |
| ***Mean soil sampling*** | 4.91 B | 5.09 A | 5.12 A | | 4.49 C | 5.23 B | 6.65 A | | 3.06 | 3.00 | 2.82 | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). BP: Before Planting; AP: After Plant growth; AD: After Soil disturbance; CFU: colony-forming units; MPN: most probable number.

### *2.4.4. Enzymatic activity*

There was a great increase in enzymatic activity comparing the soil before sowing (bulk soil) and the soil after-plant growth (Table 3). In general, soil disturbance led to a decrease in the enzymatic activities measured in this study. The mean plant differed statistically throughout the sampling times in all enzymatic activities, except in β-glucosidase. The greater dehydrogenase activity was observed under the grass (*L. rigidum*), and the lowest under the non-mycotrophic plant. The values of arylsulfatase and phosphatase activities were least in *R. bucephalophorus* but did not differ between highly-mycotrophic (*O. compressus* and *L. rigidum*) and non-mycotrophic (*S. gallica*) plants.

The effect of soil disturbance after each plant type showed differences between mycotrophic and non-mycotrophic plants. The lowest dehydrogenase activity was found under the non-mycotrophic plant (*S. gallica*) after soil disturbance and the highest under *L. rigidum* after plant growth. Within plants in the after-plant sampling, the mycotrophic plants also exhibited the highest activity of arylsulfatase. The soil disturbance greatly affected arylsulfatase activity by decreasing it after the mycotrophic plants, except after the non-mycotrophic *S. gallica* which significantly increased. Similarly, phosphatase activity increased with the growth of highly-mycotrophic plants that was also greatly decreased by soil disturbance. Again, the exception was observed for the non-mycotrophic plant in which the soil disturbance significantly increased the phosphatase activity. Additionally, phosphatase and arylsulfatase activities did not change under the scarcely-mycotrophic plant (*R. bucephalophorus*) neither after planting nor after soil disturbance. The β-glucosidase activity did not differ statistically within plants in the after-plant sampling, but the soil disturbance greatly decreased it after the mycotrophic plants.

**Table 3:** Effect of the type of plant and soil disturbance on enzymatic activity of dehydrogenase, arylsulfatase, β-glucosidase and phosphatase.

| | **dehydrogenase** (μgTPF.g$^{-1}$ dry soil.h$^{-1}$) | | | | **phosphatase** (nmolp-nitrophenol.g$^{-1}$ dry soil.h$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** |
| **Plants** | | | | | | | | |
| *O. compressus* | 0.10 d | 0.35 bc | 0.14 d | 0.19 BC | 1160.12 b | 2020.98 a | 1296.96 b | 1492.69 A |
| *L. rigidum* | | 0.49 a | 0.14 d | 0.25 A | | 1765.93 a | 1314.35 b | 1413.47 A |
| *R. bucephalophorus* | | 0.42 ab | 0.11 d | 0.21 AB | | 1246.28 b | 1220.91 b | 1209.11 B |
| *S. gallica* | | 0.31 c | 0.06 d | 0.16 C | | 1277.99 b | 1946.45 a | 1461.52 A |
| ***Mean soil sampling*** | 0.10 B | 0.39 A | 0.11 B | | 1160.12 C | 1577.80 A | 1444.67 B | |
| | **arylsulfatase** (nmolp-nitrophenol.g$^{-1}$ dry soil.h$^{-1}$) | | | | **β-glucosidase** (nmolp-nitrophenol.g$^{-1}$ dry soil.h$^{-1}$) | | | |
| | BP | AP | AD | ***Mean plant*** | BP | AP | AD | ***Mean plant*** |
| **Plants** | | | | | | | | |
| *O. compressus* | 45.08 ab | 54.78 a | 39.90 bc | 46.59 A | 460.27 bcd | 618.94 abc | 369.10 d | 482.75 |
| *L. rigidum* | | 52.85 a | 40.72 b | 46.22 A | | 712.21 a | 409.34 cd | 527.26 |
| *R. bucephalophorus* | | 38.20 bc | 29.45 c | 37.58 B | | 559.65 abcd | 398.86 d | 472.91 |
| *S. gallica* | | 38.21 bc | 54.07 a | 45.78 A | | 567.76 abcd | 639.28 ab | 555.76 |
| ***Mean soil sampling*** | 45.08 A | 46.01 A | 41.03 B | | 460.27 B | 614.64 A | 454.15 B | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). BP: Before Planting; AP: After Plant growth; AD: After Soil disturbance.

## 2.5. Discussion

The level of mycotrophy of the plants used in this experiment was confirmed, being *O. compressus* and *L. rigidum* the highly-mycotrophic ones and no root colonization by AMF was found in *S. gallica*. These results are in agreement with the ones found by Brito et al. (2014) and Alho et al. (2015). Also, no arbuscular colonization was found in *R. bucephalophorus.* However, root colonization was observed in other studies, when *R. bucephalophorus* was grown following a previous mycotrophic plant and intact ERM was the preferential inoculum source (Goss et al., 2017) or having a residual colonization as in Brito et al. (2014), showing its ability to be colonized and therefore attesting its weak mycotrophy. This last study indicates a weak mycotrophy that is likely to be residual in less favorable conditions, as it was probably the case of the present study.

Mycotrophic plants differed from non-mycotrophic ones in some of parameters. Strongly mycotrophic plants led to greater SBR, MBC, P solubilizers and dehydrogenase, phosphatase and arylsulfatase activity when compared with non-mycotrophic. The root microbiome of plants grown in the same soil has been found to differ between plant species (Haldar & Sengupta, 2017). The microbial structure in the rhizosphere of mycotrophic plants are different from the non-mycotrophic (Andrade et al., 1998). The rhizosphere of mycorrhizal plants is microbiologically and biochemically more active compared with that of non-mycorrhizal plants due to greater deposition of carbohydrates from mycorrhizal roots to the soil (Andrade et al., 1998). The SBR and MBC clearly reflected the effect of plant with different levels of mycotrophy on soil functionality (related to SOM modifications). The great SBR seen in the soil under *O. compressus* (Table 1) could be due to an occurrence of considerable microbial activity. Legume plants provide more readily decomposable materials that stimulate soil microorganisms and therefore enhance biological activity in the rhizosphere (Koné et al., 2008). In contrast, the great MBC found under *L. rigidum* may result from the

considerable density of their roots. This grass has an extensive root system with abundant root hairs (Caradus, 1980). Thus would exudate larger amounts of organic compounds and therefore favor an increase of soil microbial biomass (Rocha et al., 2016). Significantly, the greater microbial activity observed in the soil after the growth of mycotrophic plants was not accompanied by an increased growth of total bacteria and fungi, indicating the presence of unculturable or recalcitrant organisms to the generalist culture media used (Hill et al., 2000). Our results showed differential influence of soil disturbance in the count of several microbial functional groups. With respect to these parameters, the effect of soil disturbance varied within groups. In some cases, there was increase, as seen for total bacteria, ammonifiers and Mn oxidizers, and in other cases decrease, as for P solubilizers. The differences in total bacteria after soil disturbance could be influenced by the rhizosphere effect and SOM mineralization. The overall proportion of aerobic bacteria is relatively lower in the rhizosphere because of low level of oxygen due to root and microbial respiration (Haldar & Sengupta, 2017). On the other hand, soil disturbance exposes SOM making it easily degradable and therefore available for microbial consumption (Mehra et al., 2018). The increase of available organic matter sources right after soil disruption promotes an increase in microbial communities (Janušauskaite et al., 2013). Another aspect to be considered is the probable decrease in the recalcitrant bacteria explained by the overall decrease in biological activity parameters such as SBR and MBC (Youseif et al., 2021).

The general increase in the ammonifiers and Mn oxidizers groups after soil disturbance could be explained by the decrease of redox potential observed in dehydrogenase activity and soil respiration. As suggested by Marschner and Timonen (2005), when the redox potential decreases, nitrate is used by microorganisms as alternative electron acceptor, followed by manganese oxides. Sparrow and Uren (2014) found that even small changes in water potential, that in turn could be influenced by tillage, can shift this balance to tip in favor of soil manganese oxidation process.

In fact, the soil disturbance did not strongly impact the functional groups in the non-mycotrophic plant as it did in the mycotrophic ones. The response between highly-mycotrophic and non-mycotrophic plants after soil disturbance were significantly different regarding Mn oxidizers and P solubilizers groups (Table 2). The interactions between AMF and plants could change the composition of Mn oxidizers communities in the rhizosphere. The ERM formed in the rhizosphere of the mycotrophic plants *per se* release compounds responsible for stimulation or inhibition of that functional group (Nogueira, 2002). The solubilization of organic phosphate in the soil is linked to its microbiome. This solubilization is closely associated with pH reduction and chelation that occur due to the release of organic and inorganic acids produced by bacteria and fungi metabolism (Kalayu, 2019). In that process, AMF play a key role by their ability of extending their widespread hyphae from the P depletion zone to explore a greater soil volume for inorganic P (Bolduc, 2011). AMF can also directly stimulate plant growth promoting bacteria involved in P transformations and associated with the ERM (Taktek et al., 2015). This could explain the great number of P solubilizers after the growth of both mycotrophic plants. Since agricultural practices could affect SOM and therefore P solubilizers (Alori et al., 2017), the decrease of this group could be a result of the rupture of the ERM caused by soil disturbance. Further evidence could be seen in the non-mycotrophic plant, in which the shifts on P solubilizers and Mn oxidizers were not significantly different between after plant sampling and after soil disturbance. These results clearly indicate the relevance of ERM and its integrity as a survival niche for some soil microbes.

Cropping systems strongly affect the soil biological activity, mainly by the choice of the cultivated plants but also by the tillage regime adopted (Degrune et al., 2017). Soil disturbance, such as caused by tillage, affected the soil microbiome and the biological processes they mediate, by changing the soil microaggregates that in turn affect water content and aeration, leading to modifications in soil function, stability and resilience (Smith & Collins, 2007). In the present study, these changes were reflected

in almost all assessed parameters. Our results showed a great decrease in most of microbiological attributes and enzymes activities 10 days after soil disturbance along with many studies showing that tillage reduces soil organic matter and in turn the microbial biomass (Francioli et al., 2014; Laudicina et al., 2011; Madejón et al., 2009; Martin-Lammerding et al., 2013; Vazquez et al., 2017).

Conventional tillage systems can decrease enzymatic activity in contrast with no-till or reduced tillage (Adetunji et al., 2017; Deng & Tabatabai, 1996, 1997; Li & Sarah, 2003). Our results showed that the soil disturbance led to a decrease in the mean of all enzymatic activities. A great decrease of dehydrogenase activity after soil disturbance was noted in which all differences previously observed between plants (Table 3) were lost and the activity fell to the range observed when no plants were growing in the soil. Tillage is known to strongly affect the activity of dehydrogenase by decreasing it over time due to a rapid mineralization of SOM and subsequent decrease of oxidative biological activity (Malik et al., 2013). Increases in SBR during the first day of soil disturbance have been reported in Kainiemi (2014) in which after one week the rate decreased to base-rate. This may explain our results, suggesting that after soil disruption a flush of readily available organic matter mineralization occurs and by the time of the last soil sampling (ten days after disturbance) the rates of SBR and MBC are again equivalent to the ones in soil before planting, or even lower. In agronomic context and under Mediterranean climate conditions, our results show the negative impact of tillage that often leads to soil organic matter impoverishment.

The effect of plant growth and soil disturbance (Table 1) on soil microbial parameters suggests that plants with different levels of mycotrophy responded differently and strongly influenced the biological activity. Soil disturbance strongly increased the $qCO_2$ in the non-mycotrophic plant, *S. gallica*, when compared to the mycotrophic ones which could be explained by differences in accessibility of C substrates, changes in metabolic rates (SBR and MBC) and changes in microbial community composition. This metabolic quotient is usually low when the environment is

more stable, or high when a stress occurs (Gajda, 2008; Guimarães et al., 2017) suggesting that mineralization process after soil disturbance in the soil under *S. gallica* was different from the mycotrophic plants. Even though total culturable bacterial did not change much under these circumstances, general dehydrogenase activity was also strongly affected by soil disturbance under *S. gallica*, confirming the severe impact of soil disturbance on soil biota after a non-mycotrophic plant.

In addition, our results showed that the effect of soil disturbance after each plant also can distinguish different patterns of enzymatic activity between mycotrophic and non-mycotrophic plants (Table 3). A decrease in the enzymatic activities among strongly mycotrophic plants was observed after soil disturbance. Conversely, for the non-mycotrophic plant, soil disturbance did not affect the activity of β-glucosidase, whereas the activities of phosphatase and arylsulfatase were significantly increased. The increase of phosphatase activity after disturbance in the soil under the non-mycotrophic plant may be due to synergistic activity of this enzyme released by plant roots (and accumulated in soil matrix) and its microbiome (Nannipieri et al., 2011) but further studies are required to confirm this hypothesis. Different families of plants have developed various strategies for P acquisition during their evolution (Hallama et al., 2019). As suggested by Kunze et al. (2011), high phosphatase activity in the soil under the non-mycotrophic plant seems to be more related to the plant species effect than to tillage practice. In the mycotrophic plants, the P uptake and phosphatase production are highly improved by the presence of AMF and its net of ERM (Dodd et al., 1987). Soil disturbance affects the AMF by disrupting the ERM (Brito et al., 2012) and therefore could lead to a decrease in the phosphatase activity (Sato et al., 2015). Additionally, soil enzymes are directly related to availability of nutrients and this could reflect in different rates of soil organic matter mineralization over time (Deng et al., 2019; Zarea et al., 2011). The mineralization rate in the soil under the non-mycotrophic plant based on enzymatic activities seems to be different from the mycotrophic plants.

*R. bucephalophorus*, considered as weakly mycotrophic plant, although no root colonization was observed in our study, presented contrasting results. For most parameters, results were similar to those obtained for strongly mycotrophic plants rather than to those of the non-mycotrophic one. However, it differed completely from all other plants in some parameters, notably having lower activities of arylsulfatase and phosphatase, and Mn oxidizers bacteria. Also, neither the presence of the plant nor soil disturbance seemed to affect the phosphatase activity under this plant when compared with the values before planting.

### 2.6. Conclusions

Plant growth, irrespectively of mycotrophy, increased all the soil microbial parameters and particularly dehydrogenase activity, more than tripled after plant growth when compared with bulk soil. Considering the dehydrogenase a key enzyme to evaluate the general microbial activity, it unquestionably illustrates the importance of plants on the soil microbial activity.

ERM and its specific associated microbiome have been related to a differential biological dynamic, and our results confirm a greater microbial activity associated with mycotrophic plants in the mean values of almost all parameters. Particularly, some metabolic differences between the grass and the legume can be highlighted regarding SBR, MBC, and dehydrogenase. Also, the biological activity and functional profile of microbiota associated with mycotrophic and non-mycotrophic plant roots are differentially affected by the stress induced by soil disturbance.

Soil disturbance had a negative effect on the general parameters of soil microbial activity, as in MBC, dehydrogenase and SBR, particularly for non-mycotrophic plants, and altered microbial communities promoting an increase in specific functional groups of microorganisms such as ammonifiers and Mn oxidizers. Regarding enzymatic activity, mineralization of SOM after soil disturbance under non-mycotrophic plants seemed to be different than under mycotrophic plants. This is supported by the results

of metabolic quotient, which indicates different rates of biological activities under the non-mycotrophic *S. gallica*. Mycotrophic plants have a diverse bacterial population associated with their ERM that inevitably suffers the impacts of ERM disruption caused by soil disturbance, as illustrated by the decrease of P solubilizers and phosphatase activity. In contrast, under the non-mycotrophic plant, the P solubilizers did not change and phosphatase activity increased after soil disturbance, indicating different strategies for P acquisition for these plants.

The activities of arylsulfatase and β-glucosidase were more affected after the growth of mycotrophic plants followed by soil disturbance, indicating the importance of ERM as niche and the functional implications of its disturbance. The specific results for enzyme activities of arylsulfatase, phosphatase and β-glucosidase together with the count of P solubilizers and Mn oxidizers reflect the differential effects of soil disturbance on mycotrophic and non-mycotrophic plants.

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# Changes induced by wheat on soil functional profile according to previous plant mycotrophy and soil disturbance under Mn toxicity

## 3.1. Abstract:

In the current agronomic context, the adoption of alternative forms of soil management is essential to increase crop yield. Agricultural sustainability requires practices that generate positive impacts and promote an increase in microbiome diversity as a tool to overcome adverse environmental conditions. An important ally are the indigenous arbuscular mycorrhizal fungi (AMF) that can improve plant growth and provide protection against abiotic stress such as metal toxicity. This work studied, in a greenhouse experiment, the effect of wheat (*Triticum aestivum* L.) growth on several parameters of biological activity and functional microbiome, under Mn stress, in relation to wheat antecedent plant mycotrophy and soil disturbance. When the wheat grew after highly-mycotrophic plants and the soil was not disturbed, the results showed an increase in wheat root AMF colonization and an increase of 2.5-fold in dry weight along with greater photosynthetic parameters and dehydrogenase activity. Conversely, soil disturbance before wheat planting increased the β-glucosidase activity and the count of functional groups of culturable microorganisms, irrespectively of antecedent plant, and decreased drastically the wheat dry weight, the AMF colonization and the chlorophyll content. These findings suggest that not only wheat growth, but the soil functional microbiome associated is affected by the antecedent type of plant and previous soil disturbance imposed. In addition, the improvement in wheat dry weight despite Mn toxicity may rely on shifts in biological activity associated with a well-established AMF colonization induced by the previous treatments.

### 3.2. Introduction

Over the past decades, the great challenges of agricultural systems to overcome the problem of food production is how to increase the crop yield without increasing the area of harvested land. Agricultural management with minimum soil disturbance together with crop diversification have been linked with higher soil quality and crop yield. This conservation management approach affects soil microorganisms and creates a favourable environment that will affect plant nutrition and may protect crops against abiotic stress (Goss et al. 2017; Page et al. 2020). Several strategies for the effective exploitation of indigenous microorganisms have been proposed to optimize the role of root-associated microbiome in nutrient supply and plant protection (Barea, 2015).

Extensive areas of soil in the south region of Portugal are characterized by its acidic properties that promote an increase in Mn ions bioavailability and cause great toxicity to the crops (Goss and Carvalho 1992). The excessive Mn availability can impact plant growth by affecting chlorophyll biosynthesis, replacing cofactors in enzymes involved in photosynthetic pathway and interfering in early reactions in photosystem II (PSII), causing a decline in photosynthetic rate and plant development (Alejandro et al. 2020). Mycorrhizal associations can bring several benefits to host plants, not only improving nutritional state but also helping them to overcome metal stress, particularly in well-established colonizations (Brito et al., 2014; Alho et al., 2015; Begum et al. 2019). Among the different propagules of AMF inoculum, the intact extra radicular mycelium (ERM), results in an earlier and fast root colonization and therefore leads to tolerance of metal stress (Brito et al. 2019).

A bioprotection strategy was developed by Brito et al. (2014) to overcome the problem of Mn toxicity in acidic soils by introducing a mycotrophic antecedent plant to develop an ERM that, when not disrupted, promotes an early and effective wheat (*Triticum aestivum*) root colonization. These antecedent plants are the instrument to

develop the ERM in the soil. In that study, it was reported that the wheat that grew after the mycotrophic plants, *Ornithopus compressus* (legume) and *Lolium rigidum* (grass), with an intact ERM presented a reduce in the shoot Mn content by 47% and 36%, respectively, doubled shoot phosphorus (P) content and an increase of 1.5-fold in wheat dry weight compared to the same mycotrophic plants with ERM disrupted. This approach takes advantage of an early root colonization by well adapted indigenous AMF developed by Mn tolerant plant species present in natural vegetation to promote bioprotection in the subsequent crop. Mycorrhizal associations affect not only plant development, but also act as determinants of the microbial community dynamics. The large surface area of the ERM provides nutrient-rich niches for colonization and growth of other soil microorganisms, especially bacteria, and it seems to have a specific selection pressure on the microbial composition (Andrade et al. 1997). In fact, the differences observed in the bioprotection strategy are accrued to the functional microbiome shaped by the AMF diversity managed by each antecedent plant (Brito et al. 2021).

The changes in functional microbiome induced by the ERM developer plants strategy can be assessed by measuring the key microbial and biochemical processes, in addition to other biological attributes, to evaluate the influence of crop practices on soil function (Bini et al. 2014). Soil microbial biomass carbon (MBC) has been commonly recognized as an important indicator of soil microbial properties. It represents the size of microbial pool which reflect soil organic matter changes such as carbon cycling (Hsieh et al. 2020). Soil respiration (SBR) is also closely related to several functions of organisms. The measurement of SBR, which originates from the mineralization of soil organic matter (SOM), has been applied across a variety of studies and could be used to assess changes imposed to agricultural practices (Creamer et al. 2014).

Soil enzymatic activities are also considered useful indicators of soil status because of their involvement in decomposition of SOM and rapid response to changes

in soil management. Major groups of enzymes have been used to evaluate the soil status. Dehydrogenases are an oxidoreductase group correlated to activity of viable cells. Arylsulfatase, phosphatase and β-glucosidase are hydrolases involved in the cycling of S, P and C from organic compounds (Nogueira et al. 2006). Due to substrate specificity, a group of enzymatic activities is necessary to infer the general status of the soil or microbiological activity index. However, there may not be a simple correlation between enzymatic activity and microbial functional diversity, due to the complexity of metabolic reactions and interactions of soil microbiota (Alkorta et al. 2003). Since relationships between soil enzymes and other parameters of soil biological activity are not direct, they need to be analyzed carefully (Hsieh et al. 2020).

Even though bacterial counting is considered a laborious methodology, still could be used as the initial point of a study due to its relatively inexpensive cost and could assess the gross diversity and function of culturable microorganisms (Nannipieri et al. 2017; Tate 2020). Therefore, since heterotrophic microorganisms are dominant drivers of biogeochemical cycles, shifts in count of functional microbes could be used to a preliminary assess of agronomic management impact. Additionally, several studies have demonstrated the impact of agricultural practices on soil microbial counts (Liu et al. 2017; Niewiadomska et al. 2020; Bolo et al. 2021).

Studies that links microbial communities response to different agricultural practices and perturbations could help predicting the outcome of specific managements interventions and maximize the sustainability of soil resources (Bissett et al. 2013). Therefore, this work aims to build on the knowledge involved in the strategy of AMF bioprotection against Mn toxicity in wheat proposed by Brito et al. (2014) studying the changes on soil functional profile induced by wheat growth in relation to antecedent plant mycotrophy and soil disturbance in the perspective of soil biological activity and microbial functional response.

### 3.3. Materials and Methods

### *3.2.1. Experimental design*

A pot experiment was performed from January to May, 2019 in a greenhouse. We used a sandy, acidic soil (sandy loam Eutric Cambisol - FAO) collected from the top 20 cm of a natural pasture at Herdade da Mitra-University of Évora, Alentejo, Portugal (38° 32´ N; 08° 00´ W), having an organic C content of 10.5 $g.kg^{-1}$, a pH of 4.8 in water, the ammonium acetate exchangeable manganese content at pH 7 was 29 ± 4 $\mu g.g^{-1}$, and previously described by Goss & Carvalho (1992) as causing Mn toxicity in wheat. This soil is characterized by a high AMF diversity (Brígido et al. 2017) and was used in previous experiments (Brito et al. 2014; Alho et al. 2015). To guarantee initial identical conditions in all treatments the soil was homogenized by sieving and packed into 8 kg pots. The experiment consisted of two phases. In the first one, four common arable plants species, widespread in areas exhibiting soil Mn toxicity, were sown in 8 replicate 8 kg pots, with 5 plants per pot to develop different levels of ERM. Two species, *Ornithopus compressus* L. (a legume) and *Lolium rigidum* Gaudin (a grass) are known to be highly-mycotrophic; one (*Rumex bucephalophorus* L.) is known as scarcely-mycotrophic; the fourth species (*Silene gallica* L.) is non-mycotrophic. After 11 weeks of growth, plants were excised. For the Disturbed treatment, the soil of half of the pots for each species was subjected to mechanical disturbance by passing through a 4 mm sieve. The soil and roots were mixed, repacked into the same pots and shoot material was returned to the soil surface. The remainder of the pots of each species formed the Undisturbed treatment; the shoot material was also returned to the soil surface. Ten days later, in the second phase of the experiment, wheat (*Triticum aestivum* L., var. Ardila) pre-germinated seeds were planted in all the 32 pots from the first phase plus 4 additional pots that did not received any plants in the first phase and allowed to grow for 21 days (Figure 1). After that period, wheat photosynthetic parameters, shoot dry weight, mycorrhizal colonization and soil biological activity from all replicates with were assessed.

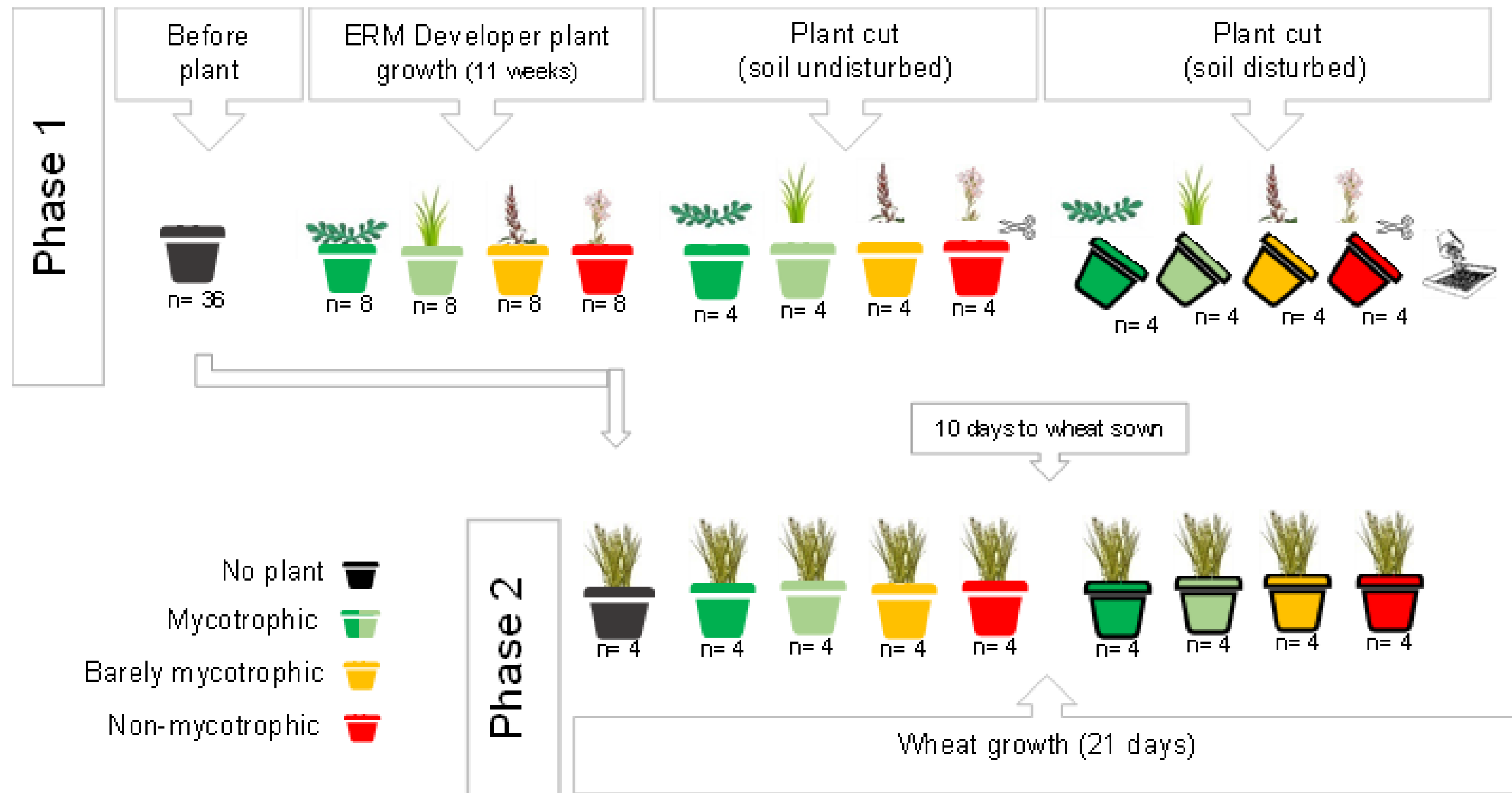


**Figure 1:** Experimental design. n=number of biological replicates. ERM: extra radicular mycelium

### *3.2.2. Soil Microbial Activity*

Water holding capacity and water content were determined (Monteiro and Frighetto 2000) and the information used to express the assessed attributes in soil dry basis. Soil basal respiration (SBR) was measured accordingly to Silva et al. (2007), in a closed jar and incubated for 7 days at 26º C. The $CO_2$ released was adsorbed in NaOH and determined by HCl titration. The results were reported as milligrams of $CO_2$ per kilograms of soil released per hour ($mgCO_2.kg\ soil^{-1}.h^{-1}$). The determination of total microbial biomass carbon (MBC) was performed by fumigating the soil with chloroform in a desiccator and the carbon content calculated following an oxidation reaction with potassium permanganate (Vance et al. (1987). The values of MBC were given by the carbon content of fumigated soil minus that of the non-fumigated soils, all divided by the proportion of microbial C recovered (*kc*). A value of 0.45 was used for *kc* in MBC calculation (Joergensen, 1996). Results were expressed as milligrams of carbon per kilograms of soil ($mgC.kg\ soil^{-1}$). The metabolic quotient ($qCO_2$) was used to estimate the efficiency of substrate consuming by microorganisms as a stress indicator and were calculated as the ratio between soil basal respiration and microbial biomass carbon (Anderson and Domsch 1990).

### *3.2.3. Functional groups of culturable microorganisms*

Six functional culturable groups of soil microorganisms were evaluated: bacteria, fungi, ammonifiers, S oxidizers, Mn oxidizers and P solubilizers. For bacteria, fungi, ammonifiers and P solubilizers the protocols were described in Albino and Andrade (2007). Mn-oxidizing microorganisms were counted in Garretesen's medium as suggested by Nogueira et al. (2007). Sulfur oxidizers were counted in thiosulfate broth (Vidyalakshmi and Sridar, 2007) using bromothymol blue as an indicator of pH acidity instead of bromocresol purple. Ammonifiers and sulfur oxidizers are presented as the logarithm of most probable number per gram of soil ($logMPN.g^{-1}$) and the others as the logarithm of colony forming units per gram of soil ($logCFU.g^{-1}$).

### *3.2.4. Enzyme Activity*

Dehydrogenase was measured according to Casida et al. (1964) with modifications. Soil (5 g) was incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (5 ml) for 24 h at 37º C. Triphenyl formazan (TPF) formed by the reduction of TTC under dehydrogenase activity during incubation was extracted from the soil with 20 ml of methanol and left to decant for about 10 min. The supernatant was centrifugated at 5000 rpm for 5 min and then 3 ml were transferred to cuvettes and determined by spectrophotometry ($\lambda$ = 485 nm) in triplicate (Monteiro & Frighetto, 2000). The arylsulfatase, β-glucosidase and phosphatase activities were measured according to ISO 20130:2018 (ISO 2018) in 96-wells microplates. During the incubation time indicated for each enzyme, their respective substrates (potassium ρ-nitrophenyl-sulphate, ρ-nitrophenyl-β-D-glucopyranoside and ρ-nitrophenyl-phosphate) were hydrolyzed into a yellow colored ρ-nitrophenol and determined by spectrophotometry thereafter ($\lambda$ = 405 nm).

### *3.2.5. Photosynthetic parameters*

The photosynthetic rate (A), stomatal conductance to water vapour (*gs*), electron transfer rate (ETR) and chlorophyll fluorescence parameters (photochemical quenching, qP) were measured in four leaves, of all the replicates, for each treatments using a leaf chamber fluorometer (LI-COR 6400-40, LI-COR and Lincoln, NE, USA). The total chlorophyll content was estimated *in vivo* using a portable SPAD meter (CL01 Chlorophyll content system, Hansatech Instruments, Pentney, King’s Lynn, United Kingdom). Maximum quantum efficiency of PSII was measured with a Pocket-PEA (Plant Efficiency Analyzer, Hansatech Instruments, Pentney King’s Lynn, United Kingdom), $F_v/F_m$ ratio was calculated using $(F_m - F_0)/F_m$, where $F_m$ is maximal fluorescence yield of the dark-adapted state and $F_0$ is minimum fluorescence yield (Fernandez-Göbel et al. 2019). All measurements were taken between 9 and 12 h in the morning.

### *3.2.6. Statistical analysis*

The experimental design was a complete randomized block with four replicates. The treatments were in factorial combination and consisted of two factors: plant type (with 5 levels) and soil disturbance (with 2 levels). ANOVA was performed based on the two factors using a generalized linear model and Tukey's test at 5% level was used to compare the means using the software Minitab 21® (Minitab 2021).

## 3.3. Results

### *3.3.1. Wheat root colonization, photosynthetic parameters, and shoot development*

Wheat plants that grew after the highly-mycotrophic plants (*O. compressus* and *L. rigidum*) reported the greatest means of root colonization by AMF, particularly in undisturbed soil (Table 1). In the disturbed treatment, no statistical differences were observed in relation to the plant mycotrophy and wheat AMF colonization rate. Precisely, the same pattern of results was observed regarding the wheat shoot dry weight. The greatest shoot development was found when wheat grew after the highly-mycotrophic plants but only when the soil was kept undisturbed. No previous plant or the growth of poorly or non-mycotrophic plants before wheat, irrespectively of soil disturbance, led to significantly lower AMF colonization rates of wheat. In contrast, the soil disturbance led to a great decrease in the wheat shoot dry weight even grown after scarcely- and non-mycotrophic plants (Figure 2).

**Table 1:** Effect of plant species and soil disturbance on wheat arbuscular colonization and shoot dry weight.

| | Arbuscular colonization (%) | | | Shoot dry weight (g/plant) | | |
|---|---|---|---|---|---|---|
| | Root Sampling | | ***Mean plant*** | Plant Sampling | | ***Mean plant*** |
| **Plants** | U | D | | U | D | |
| *O. compressus* | 65 a | 40 b | 53 A | 0.81 a | 0.19 cd | 0.50 A |
| *L. rigidum* | 64 a | 39 b | 51 A | 0.84 a | 0.27 bcd | 0.56 A |
| *R. bucephalophorus* | 40 b | 32 b | 36 B | 0.40 bc | 0.12 d | 0.26 B |
| *S. gallica* | 39 b | 30 b | 35 B | 0.46 b | 0.15 d | 0.30 B |
| *No Plant* | 27 b | | 27 B | 0.29 bcd | | 0.29 B |
| ***Mean sampling*** | 47 A | 34 B | | 0.56 A | 0.21 B | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). Means with no letters are no significantly different. U: undisturbed treatment and D: disturbed treatment.

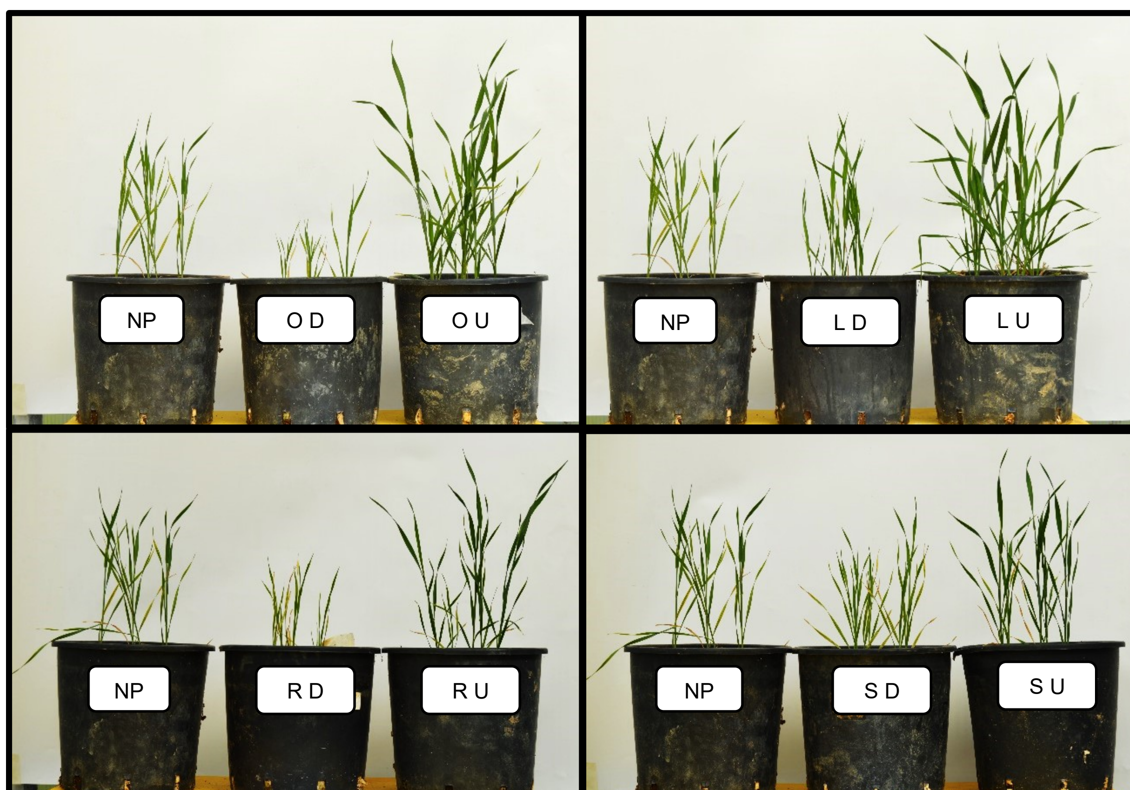


**Figure 2:** Comparison of wheat growth 22 days after planting. O D: after *O. compressus* under disturbed treatment, O U: after *O. compressus* under undisturbed treatment, L D: after *L. rigidum* under disturbed treatment, L U: after *L. rigidum* under undisturbed treatment, R D: after *R. bucephalophorus* under disturbed treatment, R U: after *R. bucephalophorus* under undisturbed treatment, S D: after *S. gallica* under disturbed treatment, S U: after *S. gallica* under undisturbed treatment, NP: after no plant.

For photosynthetic parameters (Table 2), the wheat that grew after mycotrophic plants showed higher of chlorophyll content, electron transport chain rate (ETR) and photochemical quenching (qP). Globally, the wheat that grew after no previous plant exhibited the lower photosynthetic rate (A) and maximum quantum of efficiency of

photosystem II (Fv/Fm). Soil disturbance promoted a general decreased in all photosynthetic, and these results depended on the different plants that grew before wheat. Therefore, wheat that grew after highly-mycotrophic plants showed higher photosynthetic activity (denoting a greater resistance to physiological damage in photosynthetic apparatus, face soil disturbance) than wheat grown after non-mycotrophic plants. Additionally, wheat plants that grew after the highly-mycotrophic plants (*O. compressus* and *L. rigidum*) showed high total chlorophyll content, particularly in undisturbed soil. In contrast, the ETR and qP significantly decreased with soil disturbance in wheat plants that followed the scarcely- or non-mycotrophic plants. In wheat that grew after *R. bucephalophorus*, the soil disturbance strongly decreased the photosynthetic rate (A) and the stomatal conductance (gs). Notably, the disturbance decreased the chlorophyll content in all the treatments, except after *R. bucephalophorus.*

**Table 2:** Effect of previous plant species and soil disturbance on wheat photosynthetic parameters: total chlorophyll content, photosynthetic rate (A), electron transfer rate (ETR), photochemical quenching (qP), stomatal conductance (gs) and maximum quantum efficiency of PSII (Fv/Fm)

| | **Total chlorophyll** ($\mu g/cm^2$) | | | **A** ($\mu molCO_2.m^{-2}.s^{-1}$) | | | **ETR** ($\mu mol.m^{-2}.s^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Plant Sampling** | | ***Mean plant*** | **Plant Sampling** | | ***Mean plant*** | **Plant Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 15.50 a | 1.18 c | 8.34 A | 16.61 abc | 13.52 bcd | 15.06 AB | 136.70 a | 135.24 a | 135.97 A |
| *L. rigidum* | 13.82 a | 2.05 c | 7.93 A | 16.44 abc | 16.33 abcd | 16.39 A | 147.70 a | 144.22 a | 145.96 A |
| *R. bucephalophorus* | 3.68 bc | 1.55 c | 2.61 B | 21.47 a | 10.46 d | 15.96 A | 145.55 a | 83.76 b | 114.66 BC |
| *S. gallica* | 5.40 b | 1.08 c | 3.24 B | 17.48 abc | 18.97 ab | 18.22 A | 136.01 a | 64.38 b | 100.19 C |
| *No Plant* | 2.60 bc | | 2.60 B | 11.76 cd | | 11.76 B | 133.40 a | | 133.40 AB |
| ***Mean soil sampling*** | 8.20 A | 1.69 B | | 16.75 A | 14.20 B | | 139.87 A | 112.20 B | |

| | **qP** | | | ***gs*** ($molH_2O.m^{-2}.s^{-1}$) | | | **Fv/Fm** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Plant Sampling** | | ***Mean plant*** | **Plant Sampling** | | ***Mean plant*** | **Plant Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 0.33 a | 0.30 a | 0.32 A | 0.20 a | 0.14 abc | 0.17 | 0.77 | 0.77 | 0.77 A |
| *L. rigidum* | 0.34 a | 0.30 a | 0.32 A | 0.15 ab | 0.16 ab | 0.16 | 0.78 | 0.77 | 0.77 A |
| *R. bucephalophorus* | 0.33 a | 0.18 b | 0.26 BC | 0.20 a | 0.04 c | 0.12 | 0.77 | 0.75 | 0.76 A |
| *S. gallica* | 0.30 a | 0.13 b | 0.22 C | 0.17 ab | 0.08 bc | 0.12 | 0.76 | 0.74 | 0.75 A |
| *No Plant* | 0.30 a | | 0.30 AB | 0.18 a | | 0.18 | 0.70 | | 0.70 B |
| ***Mean soil sampling*** | 0.32 A | 0.24 B | | 0.18 A | 0.12 B | | 0.76 | 0.74 | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). Means with no letters are no significantly different. U: undisturbed treatment and D: disturbed treatment

### 3.3.2. Soil microbial activity

Even though soil biological activity after wheat growth was not affected by the previous plants nor soil disturbance (Table 3), when wheat grew following no previous plants, lower values of basal respiration (SBR) and consequently lower metabolic quotient ($qCO_2$) were observed. There were no significant differences between the undisturbed and disturbed treatment regarding the general biological activity measured by MBC, SBR and $qCO_2$.

| | **SBR** ($mgCO_2.Kg^{-1}soil.h^{-1}$) | | | **MBC** ($mgC.Kg^{-1}soil$) | | | ***q*CO2** ($mgCO_2.mg^{-1}MBC.\ h^{-1}).10^{-3}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 1.19 | 1.13 | 1.16 A | 137.73 | 106.48 | 122.10 | 8.8 | 10.6 | 9.7 A |
| *L. rigidum* | 1.09 | 1.08 | 1.09 A | 113.42 | 114.58 | 114.00 | 9.9 | 9.7 | 9.8 A |
| *R. bucephalophorus* | 1.23 | 1.06 | 1.14 A | 109.95 | 112.26 | 111.11 | 11.3 | 9.8 | 10.5 A |
| *S. gallica* | 0.86 | 1.04 | 0.95 A | 129.63 | 118.05 | 123.84 | 6.9 | 9.3 | 8.1 AB |
| *No Plant* | 0.54 | | 0.54 B | 99.53 | | 99.53 | 5.5 | | 5.5 B |
| ***Mean soil sampling*** | 0.98 | 0.97 | | 118.05 | 110.18 | | 8.5 | 9.0 | |

**Table 3**: Effect of previous plant species and soil disturbance on soil basal respiration (SBR), microbial biomass carbon (MBC) and metabolic quotient ($qCO_2$) after wheat growth.

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). Means with no letters are no significantly different. U: undisturbed treatment and D: disturbed treatment.

### 3.3.3. Functional groups of culturable microorganisms

With respect to soil microbial counts after wheat growth, previous plant growth and soil disturbance both affected the groups differently (Table 4). The antecedent plant did not affect the mean counts of fungi, S oxidizers and ammonifiers groups whereas the soil disturbance did not affect the mean of total bacteria, P solubilizers nor fungi after wheat growth. However, the previous plant growth affected the mean of total bacteria, Mn oxidizers and P solubilizers, but not related to plant mycotrophy. The soil

disturbance affected the mean of Mn oxidizers, S oxidizers and ammonifiers, increasing these microbial counts when compared with the undisturbed treatment.

Considering the antecedent plant mycotrophy for the mean of total bacteria, the greatest count was found in the soil where wheat grew after the antecedent non-mycotrophic (*S. gallica*) and scarcely-mycotrophic (*R. bucephalophorus*) plants and the lowest when wheat came following the mycotrophic legume plant (*O. compressus*). For Mn oxidizers, the lowest mean was found in the soil where wheat grew with no previous plant. However, within the soil disturbed, Mn oxidizers count was higher irrespectively of the wheat antecedent plant, but in undisturbed soil significant differences were identified between the mycotrophic *L. rigidum* and the non-mycotrophic *S. gallica* as antecedent plants, with a higher value for the latter. For P solubilizers, the greatest mean was found in the soil in which wheat grew after the highly-mycotrophic *L. rigidum*, and the lowest mean after the non-mycotrophic *S. gallica*.

**Table 4:** Effect of previous plant species and soil disturbance on soil microbial functional group counting of total bacteria, fungi, Mn oxidizers, P solubilizers, ammonifiers and S oxidizers after wheat growth.

| | **Total bacteria** | | | **Fungi** (Log CFU.g$^{-1}$) | | | **Mn oxidizers** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 6.1 | 6.3 | 6.2 B | 5.2 | 5.0 | 5.1 | 5.6 cde | 6.0 abc | 5.8 A |
| *L. rigidum* | 6.2 | 6.6 | 6.4 AB | 5.0 | 5.2 | 5.1 | 5.4 de | 6.2 a | 5.8 A |
| *R. bucephalophorus* | 6.5 | 6.4 | 6.5 A | 5.1 | 5.3 | 5.2 | 5.7 bcd | 6.2 a | 6.0 A |
| *S. gallica* | 6.5 | 6.5 | 6.5 A | 5.1 | 5.2 | 5.2 | 5.9 abc | 6.1 ab | 6.0 A |
| *No Plant* | 6.2 | | 6.2 B | 5.0 | | 5.0 | 5.3 e | | 5.3 B |
| ***Mean soil sampling*** | 6.3 | 6.4 | | 5.1 | 5.1 | | 5.6 B | 5.9 A | |

| | **P solubilizers** (Log CFU.g$^{-1}$) | | | **Ammonifiers** | | | **S oxidizers** (Log MPN.g$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 5.4 | 5.3 | 5.4 AB | 7.3 | 8.9 | 8.1 | 3.1 | 4.3 | 3.7 |
| *L. rigidum* | 5.5 | 5.9 | 5.7 A | 6.9 | 8.2 | 7.5 | 3.4 | 4.2 | 3.8 |
| *R. bucephalophorus* | 5.5 | 5.1 | 5.3 AB | 7.7 | 8.7 | 8.2 | 3.6 | 4.2 | 3.9 |
| *S. gallica* | 5.1 | 5.3 | 5.2 B | 7.4 | 8.0 | 7.7 | 3.6 | 4.2 | 3.9 |
| *No Plant* | 5.6 | | 5.6 AB | 7.3 | | 7.3 | 3.6 | | 3.6 |
| ***Mean soil sampling*** | 5.4 | 5.4 | | 7.3 B | 8.2 A | | 3.5 B | 4.1 A | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). Means with no letters are no significantly different. U: undisturbed treatment and D: disturbed treatment.

### *3.3.4. Enzymatic activity*

The enzymatic activity was significantly affected by the type of plant that grew before wheat and by soil disturbance (Table 5). Considering the antecedent plant mycotrophy, the mean of higher enzymatic activity was observed in the soil where wheat grew after the highly-mycotrophic *L. rigidum*, except for phosphatase. Conversely, the greatest mean of phosphatase activity was registered in the soil in which wheat grew after the scarcely- and non-mycotrophic plants. Previous soil disturbance affected the mean of dehydrogenase and the β-glucosidase activities, by decreasing the first and increasing the last after wheat growth.

For dehydrogenase activity, there was a significant interaction (Table 5) between the antecedent plant and soil integrity. The greatest activity was observed in the soil where wheat grew after *L. rigidum* in the undisturbed treatment. Dehydrogenase activity strongly decreased when wheat grew after the highly-mycotrophic plants in the disturbed soil.

**Table 5**: Effect of previous plant species and soil disturbance on soil on enzymatic activity of dehydrogenase, arylsulfatase, β-glucosidase and phosphatase after wheat growth.

| | **dehydrogenase** (μgTPF.$g^{-1}$ dry soil.$h^{-1}$) | | | **arylsulfatase** | | | **phosphatase** (nmol ρ-nitrophenol. $g^{-1}$ dry soil.$h^{-1}$) | | | **β-glucosidase** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | | U | D | |
| *O. compressus* | 1.23b | 0.43c | 0.83B | 37.50 | 41.22 | 39.36AB | 2091.81 | 2250.25 | 2171.03BC | 499.09 | 621.40 | 560.24AB |
| *L. rigidum* | 2.33a | 1.25b | 1.79A | 42.86 | 41.61 | 42.24A | 2255.14 | 2391.39 | 2323.27AB | 538.91 | 687.76 | 613.34A |
| *R. bucephalophorus* | 0.91bc | 0.39c | 0.65B | 36.36 | 38.11 | 37.23AB | 2452.76 | 2723.72 | 2588.24A | 456.15 | 524.52 | 490.33AB |
| *S. gallica* | 0.80bc | 0.79bc | 0.79B | 37.66 | 36.84 | 37.25AB | 2430.72 | 2600.24 | 2515.48A | 476.75 | 483.34 | 480.04B |
| *No Plant* | 0.53 c | | 0.53B | 33.56 | | 33.56B | 1908.00 | | 1908.00C | 548.69 | | 548.69AB |
| ***Mean soil sampling*** | 1.16A | 0.68B | | 37.59 | 38.27 | | 2227.69 | 2374.72 | | 503.92B | 573.14A | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). Means with no letters are no significantly different. U: undisturbed treatment and D: disturbed treatment.

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### 3.4. Discussion

In this experiment we used a bi-factorial design to evaluate the effect of wheat growth under Mn toxicity on soil microbial activity and functional diversity taking in consideration the influence of antecedent plant mycotrophy and soil disturbance. We found that the plant mycotrophy and therefore the ERM formed in the soil, when kept intact (the undisturbed treatment) and thus granting an early and faster AM colonization, reflected in a great improvement in wheat dry weight and AMF colonization, compared to scarcely- and non-mycotrophic antecedent plants in agreement with the results found by Brito et al. (2014). Although they found that AMF colonization rate was statistically higher in wheat that grew after the legume (*O. compressus*) than the wheat that grew after the grass (*L. rigidum*), our results showed no statistical differences. We believe that could be attributed to the time that the antecedent plant grew before wheat was sown, 11 weeks in our study instead of 7 weeks in theirs.

Following the considerable AMF colonization and shoot dry weight, the soil where wheat grew after highly-mycotrophic plants in the undisturbed treatment (ERM intact) showed a higher dehydrogenase activity indicating a more active microbiome. Although no significant differences were detected, also SBR was higher where highly- or scarcely-mycotrophic plants in the undisturbed treatment (ERM intact), corroborating the idea of a more active microbiome in these treatments. Our results clearly show the importance of ERM integrity formed by the antecedent plant as an established and active niche for soil microorganisms that reflects in Mn toxicity alleviation and wheat growth improvement under stress.

The toxicity alleviation and the increase in wheat dry weight when wheat grew after mycotrophic plants is accrued to the development of the extra radicular mycelium (ERM) from the soil native AMF by ERM developer plants that, when kept intact, promotes an early wheat root colonization and could lower Mn in shoot and increase P

concentration (Brito et al. 2014). This effect was clearly observed in the photosynthetic parameters. Wheat that grew after scarcely- and non-mycotrophic plants exhibited lower rates of electron transfer rate (ETR), photochemical quenching (qP) and chlorophyll content. Toxic effects of Mn affecting photosynthetic parameters have been reported (Liang et al. 2019; Liu et al. 2019; Takagi et al. 2021). Mn toxicity is associated with photosynthetic enzymes alterations that can affect the biosynthesis of chlorophyll. Moreover, total chlorophyll content emerges as an efficient physiological indicator of functional microbiome induced by the ERM developer plants strategy in undisturbed soil.

High concentrations of Mn in soil also can induce oxidative stress response, indirectly decreasing the photosynthetic activity (Ribera et al. 2013). The generated reactive oxygen species (ROS) caused by the Mn excess are responsible for damages in photosystem II (PSII) (Guidi et al. 2019). Changes in qP likely influences ETR and PSII yield (Shao et al. 2014). In a similar experiment, Faria et al. (2021b) observed that AMF symbiosis can induce biochemical alterations that helped wheat counteract metal stress by reducing Mn ion uptake, altering the subcellular Mn allocation and increasing the activity of enzymes involved in stress response. Additionally, the soil disturbance and consequently ERM disruption also led to a great decrease of these parameters after the mycotrophic plants, confirming the importance of an intact ERM and early AMF colonisation in Mn toxicity alleviation.

The great impact of soil disturbance on the shoot dry weight of wheat which grew after the scarcely and non-mycotrophic plants, when compared to the undisturbed soil was statistically significant, even if the magnitude of the difference was much lower than for the wheat that grew after highly-mycotrophic plants. Irrespectively the antecedent plant mycotrophy, in the disturbed soil wheat shoot dry weight was lower when compared with undisturbed soil, and these results are in agreement with Brito et al. (2014).

We hypothesized that a high SOM consumption might have occurred during the 10 days between soil disturbance and wheat sowing (Figure 1). A lowered nutrient availability combined with the intense effect of the Mn toxicity, could also have influenced the poor wheat growth under these circumstances. It is known that soil disturbance causes a rapid loss in SOM content and therefore could reduce crop productivity (Madejón et al. 2007). The increase in SOM consumption is supported by the higher functional microorganisms counting (Mn oxidizers, S oxidizers and ammonifiers) observed in the disturbed treatment. In addition, the soil where wheat grew after the disturbed treatment, regardless antecedent plant mycotrophy, showed higher β-glucosidase activity after wheat growth. The short-term effects of soil mobilization are generally related to changes in extracellular soil enzyme activity (Pandey et al. 2015) and an increase in β-glucosidase activity has been reported after soil disturbance induced by conventional soil management (Deng and Tabatabai 1996).

The increase in the microbial functional groups was not surprizing considering the increased aeration associated with soil disturbance. Particularly, the wheat that grew after *L. rigidum* in the disturbed treatment (with ERM disrupted) presented lower AMF colonization and it was highly affected by Mn toxicity while Mn oxidizers group were significantly increased in the soil. Microorganisms that oxidize Mn are linked to decreases in its availability to plants (Marschner et al. 2003). The damage to the ERM caused by the disturbed treatment and consequent lower AMF colonization of wheat seems to activate other mechanisms of Mn alleviation, by stimulating this functional group as an attempt to mitigate the Mn toxicity. Although not significant, the impact of soil disturbance and ERM disruption on the increase of this functional group followed the same trend in wheat that grew after the other highly-mycotrophic plant *O. compressus* or the scarcely-mycotrophic *R. bucephalophorus*, but not after the non-mycotrophic *S. gallica*.

In addition, although the soil disturbance led to a strong increase in Mn oxidizers count in soil where wheat grew after highly- and scarcely-mycotrophic plants in our

study, in similar conditions, Brito et al. (2014) observed a relevant increase in Mn shoot content on these plants. Increase in Mn oxidizers population could reduce Mn toxicity by immobilizing this bioavailable ion as oxides (Yang et al. 2013) and lead to a greater growth of wheat under this circumstance, but that was not case. Mn concentration of wheat plants grown after *L. rigidum* in disturbed soil were 1.6 times higher (Faria et al. unpublished data) and shoot dry weight 3 times lower than in undisturbed soil. Our results indicate that changes in the wheat microbiome concerning Mn oxidizers are not associated with the detoxifying mechanisms of bioprotection when wheat grows after a mycotrophic plant and is promptly colonized by an intact ERM. In contrast, the high Mn oxidizers count was associated with an increase in wheat Mn shoot concentration and antioxidant enzymatic activity found by Faria et al. (2021) in the disturbed soil. Moreover, the soil disturbance and associated disruption or ERM, could lead to a loss of microbe's interactions, shift the microbe functional complementarity and prevalence of other groups leading to a loss of redundancy (Yin et al. 2000; Griffiths and Philippot 2013). Further research is required to study the differences in the rhizosphere microbiome of these plants.

### 3.5. Conclusion

The results suggest that wheat growth and its functional microbiome was affected by both treatments, antecedent plant and soil perturbation, imposed previously. Wheat that grew after highly-mycotrophic plants and in undisturbed soil had a higher shoot dry weight, AMF colonization and chlorophyll content, and the soil exhibited higher dehydrogenase activity, indicating the importance of the ERM as an active niche for microbial survival and maintenance of biological activity. Conversely, the wheat that grew after no previous plants and after non-mycotrophic plant jointly presented lower shoot dry weights, the lower rates of photosynthetic parameters, enzymatic activities, Mn oxidizers counts and SBR. Therefore, highlighting the importance of AMF in crop bioprotection strategies and its influence on the remaining soil microbiome.

Soil disturbance affected the rates of SOM mineralization, increasing the β-glucosidase activity and the count of the functional groups of Mn oxidizers, ammonifiers and S oxidizers while impacting differentially wheat growth. Even in the absence of ERM (after non- and scarcely-mycotrophic plants), the rates of the biological parameters may indicate that the soil disturbance highly impacted wheat development and soil nutrient dynamics, probably due synergistically to a high SOM consumption and nutrient depletion, increasing the stress caused by Mn toxicity.

When kept intact (in the undisturbed treatment), the ERM developed by the antecedent mycotrophic plants, *O. compressus* and *L. rigidum*, promoted an increase of almost 2.5-fold in wheat growth and made the photosynthetic rates greatly increase despite the Mn toxicity. Not only the AMF but the microbiome they shape could be involved in the stress alleviation. Most of studies on the influence of plant mycorrhization are performed in laboratory conditions with a single fungal symbiont species, which seldom represents the responses in natural conditions. Unraveling the biological changes induced by a naturally assembled AMF consortiums under toxic Mn levels could help the decision on the appropriate soil management, such as the choice of crop sequence or the tillage strategies. Further studies are required that permit a more holistic view of the effect of the different type of plants and soil disturbance on other parameters of soil functional profiling.

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# C metabolic profiling as response to previous plant mycotrophy and soil disturbance in wheat growth

## 4.1. Abstract:

Soil microorganisms play important role in the dynamic regulation of organic matter in soils. To assess the influence of agricultural practices on soil functional profiling we examined the effect of soil disturbance and plant sequence with different levels of mycotrophy in wheat rhizospheric microbiome. Soil samples were analyzed with community-level physiological profiles (CLPP) using Biolog™ Ecoplates. The results of average well color development (AWCD) showed that both factors (degree of mycotrophy of preceding crop and soil disturbance) affected the soil microbiome in the two phases, although no impact on Shannon Evenness Index was observed during the experiment. The Shannon-Wiener Diversity Index showed variations among the different preceding plants, but not in wheat analysis. The pattern of the C sources metabolism also changed differentially regarding plant type and soil disturbance during the experiment, being different even within the highly-mycotrophic plants (legume and grass). In the legume, an increase in amine/amides and phenolic acids metabolism was observed whilst in the grass an increase in phosphate-carbons and carbohydrates metabolism was more evident. Principal component analysis showed a grouping in the different phases of the experiment correlated to widening the metabolism of amino acids, carboxylic acids and carbohydrates. The results indicate that soil functional community structure reflects the soil agricultural practices conditions. Previous plant type and soil disturbance impacted the soil microbiome metabolic response (AWCD) in wheat generating different patterns of carbon metabolism related to previous plant mycotrophy.

## 4.2. Introduction

Soil is a complex and dynamic ecosystem, composed of several abiotic and biotic components that constantly interact with each other. Microorganisms regulate and influence important soil ecosystem processes and properties, playing a crucial role in maintaining and facilitating the geochemical cycles. Abiotic and biotic factors can change microbial community structure and also their ecosystem function. Soil microbial communities are affected by inherent soil properties and conditions, crop management approaches, and aboveground vegetation presence and type. (Adams et al., 2017; Gałązka et al., 2017; Insam & Goberna, 2008). Carbon is a key factor driving microbial growth in soil, and functional aspects related to this substrate utilization can provide important information of soil functional diversity (Lan et al., 2019).

Root exudation is a major source of soil organic carbon released by plant roots. These exudates are known to build a network of interactions between plant roots and their surrounding rhizospheric microbes. The composition of root exudates, which is under host-genetic control, likely defines the assembly of plant-specific root and rhizosphere microbial communities. The exudation of bioactive metabolites varies substantially between different plant species, as do their microbial communities (Hu et al., 2018). The exudates mainly consist of carbon-based compounds that can often be separated into two classes: low-molecular weight compounds, which include amino acids, organic acids, sugars, phenolics and an array of secondary metabolites, and high-molecular weight compounds like mucilage and proteins (Badri & Vivanco, 2009; Swamy et al., 2016). Root exudates mediate plant-microbe interactions and thereby regulate the plant growth, development, and yield. Symbiotic associations enhance plant growth by increased uptake of nutrients among other beneficial effects. Some of these beneficial interactions include fixation of atmospheric nitrogen through root nodule formation by rhizobia in legume plants, providing tolerance against biotic as well as abiotic stresses. Another symbiotic interaction with soil arbuscular mycorrhizal fungi

(AMF) can be accrued to have positive impacts in plants by improving P and other nutrients uptake, and providing bioprotection against biotic and abiotic stresses (Canarini et al., 2019; Goss et al., 2017a). Mycotrophic plants have been shown to impact AMF symbiosis establishment and the growth and productivity of succeeding crops, particularly when the soil is kept undisturbed (Brito et al., 2018; Carvalho et al., 2015). The extra radicular mycelium (ERM) network formed during the preceding culture, when kept intact, acts as preferential source of propagules for the succeeding crop allowing an early and faster root colonization and improving crop yield (Goss et al., 2017b). In addition, it has been hypothesized that fungal hyphae also can exude labile C sources that may stimulate decomposition of organic matter by free-living soil saprotrophs and consequently increases nutrient availability. In other words, hyphal exudation can trigger (analogous to the rhizosphere priming effect of root exudates) a hyphosphere priming effect (Jansa et al., 2013; Toljander et al., 2007).

Agronomic soil management practices are a critical factor in determining short- and long-term soil functional status. The microbial community structure may be also changed under different soil cultivation practices and residue management (Rachwał et al., 2021). Soil community diversity and response to disturbance are highly nuanced and vary with the type and severity of disturbance, the timescale studied and on the starting identity of the initial community (Smith et al., 2016). Shifts in soil microbial diversity after conventional tillage regimes have been documented where the soil mobilization can decrease (Guo et al., 2016) or increase (Hartman et al., 2018) this diversity. A possible explanation for an increase in diversity may involve the impacts of soil disturbance in short term, where tillage may increase nutrient availability and open niches for colonization that may otherwise have been inaccessible due to competitive exclusion (Wipf et al., 2021).

The Biolog® system has been used widely for environmental research, allowing the monitoring of changes in the soil microbial metabolism under influence of various factors (Gryta et al., 2020). The advantages of CLPP over cell culture and molecular

level RNA and DNA amplification-based techniques are the simplicity of the protocol and the greatly reduced cost. However, many limitations in the use of this approach for complex environmental samples have been previously reported. These problems include the potential preference for fast-growing bacteria in the assay, the need to ensure equivalence of inoculum sample size, the incubation time, the data analysis and the interpretation of the CLPP results (Lladó & Baldrian, 2017). Although this community-level physiological profiling (CLPP) involves inoculating plates with mixed samples of microbes, where only a small percentage are culturable, this analysis could be effective at detecting spatial and temporal changes in soil communities and provides information regarding functional aspects of soil communities (Adams et al., 2017).

The metabolic study of soil microbiome could indicate changes in soil status or shifts caused by biotic and abiotic effects. Thus, based on carbon source metabolization by soil microbial communities, significative differences in the soil metabolic diversity can be detected (Gajda et al., 2019). Therefore, the aim of this study was to assess the shifts in carbon metabolic profiling during a greenhouse pot experiment analyzing the effect of plant sequence with different levels of mycotrophy combined with or without soil disturbance in the wheat rhizospheric microbiome.

## 4.3. Materials and Methods

### *4.3.1. Experiment design*

A Greenhouse pot experiment was performed under controlled conditions from January to April, 2019. We used a sandy, acidic soil (sandy loam Eutric Cambisol - FAO) collected from the top 20 cm of a natural pasture at Herdade da Mitra-University of Évora, Alentejo, Portugal (38° 32´ N; 08° 00´ W), having an organic C content of 10.5 $g.kg^{-1}$, a pH of 4.8 in water, the ammonium acetate exchangeable manganese content at pH 7 was 29 ± 4 $\mu g.g^{-1}$, and previously described by Goss & Carvalho (1992) as causing Mn toxicity in wheat. This soil has been used in previous

experiments and is characterized by a high AMF diversity (Brígido et al., 2017) and manganese toxicity (Brito et al., 2014). The soil was homogenized by sieving to guarantee initial identical conditions in all treatments and packed into 8 kg pots and a two-phase experiment was conducted (Figure 1). In the first phase, four plants, which occurred naturally in the Montado system, were grown, two of them being non-and scarcely-mycotrophic (*Silene gallica* and *Rumex bucephalophorus*) and two highly-mycotrophic (*Ornithopus compressus*, a legume and *Lolium rigidum*, a grass). These plants were considered as ERM developer plants. Daily control of weeds to avoid any confounding effects was carried out by hand and all the pots were watered approximately to field capacity (0.17 $g.g^{-1}$) by weight. The plants grew for 11 weeks, after which their aerial parts were severed from the roots in all pots. For the Disturbed treatment, the soil of half of the pots of each species was subjected to mechanical disturbance by passing through a 4mm sieve. The soil and roots were mixed, repacked into the same pots and shoot material was returned to the soil surface. The remainder of the pots of each species formed the Undisturbed treatment and shoot material was also returned to the soil surface. All pots were then left for 10 days. Soil was sampled at three stages in this phase: the first before planting (bulk soil), the second, 11 weeks after plant growth to see the effect of plant mycotrophy and the third sampling 10 days after soil sieving to see the effects of soil disturbance. In the second phase, wheat (*Triticum aestivum* L., var. Ardila) was planted in all the 32 pots from the first phase plus 4 control pots that did not received any plants in first phase and allowed to grow for 21 days. The soil was sampled from all the pots to evaluate differences among the disturbed and undisturbed treatment compared with the control pots and the effect of the previous plant on metabolic profiling of wheat microbiome.

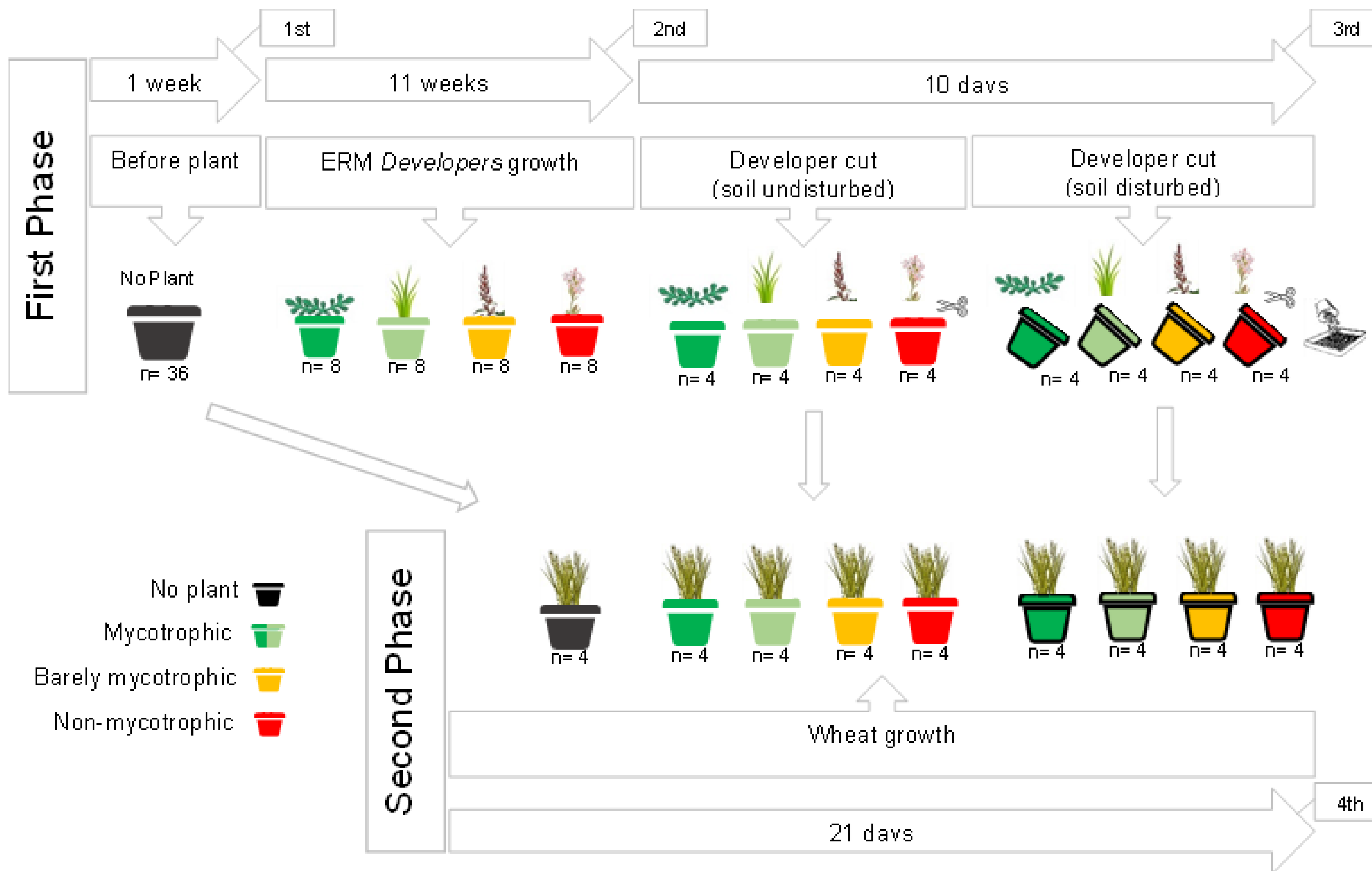


**Figure 1:** Experimental design. n=number of biological replicates; ERM: extra radicular mycelium; 1st to 4th = sampling time points.

### *4.3.2. Bacterial counting estimation*

The heterotrophic bacteria were estimated according to the protocol described in Albino & Andrade (2007). Briefly, 10 g of soil from each treatment were suspended in 90 mL of ¼ Ringer solution. A tenfold dilution was prepared and 0.1 mL of each tube was inoculated in TSA (Tryptic Soy Agar) in duplicate. The plates were incubated at 28º C for 48 hours and then the colony forming units (CFU) were determined.

### *4.3.3. BIOLOG™ Ecoplates*

The BIOLOG™ Ecoplates consists of a plate containing 96 wells, having 31 different carbon sources and a blank arranged in triplicate. The assay was conducted by adding 2.5 g of each of four replicates per treatment (10 g in total) in 90 mL of ¼ Ringer solution. We used the same Ringer solution used in the microbial counting to assure the same conditions in all the experiment. The soil suspensions were agitated for 30 minutes, 220 rpm, at room temperature and let it rest for 1 hour to decant. Then 1 mL of the supernatant was diluted to $10^{-3}$ according to the results found in the bacterial counting estimation (~$10^5$ CFU/g) and 120 µL were inoculated in each well. The plates were incubated at 28º C and the absorbance (λ= 590 nm) was read every 24 hours for 4 days (Goulart, 2013; Souza et al., 2012). The capability of microorganisms to utilize different carbon sources was measured by average well color development (AWCD) and treatments with larger rates were thought to have higher carbon source utilization. The calculation formula for the AWCD is:

$$AWCD = \sum_{i=1}^{n} \frac{Ci - R}{31} \quad 1$$

Where, Ci is the absorbance value of each reaction well at 590 nm, R is the absorbance value of the control well.

Shannon-Wiener diversity index (H´) was used as functional diversity index to investigate the diversity communities and Shannon evenness index (E) to characterize

the utilization patterns of carbon source by microorganisms. The formulas to calculate Shannon diversity and Shannon evenness are respectively:

Shannon-Wiener diversity index:

$$\mathbf{H'} = -\sum Pi\ lnPi \quad \mathbf{2}$$

$$\mathbf{Pi} = \frac{Ci - R}{\sum(Ci - R)} \quad \mathbf{3}$$

In the formula, the Pi represents the ratio of the absorbance value in the $i^{th}$ (1 to 31) well to the total absorbance values of all wells.

Shannon evenness index:

$$E = {H'}/{lnS} \quad 4$$

Considering S as the number of wells with positive activity within the replica.

The time point of 72 hours was chosen to calculate the average well color development (AWCD) and Shannon diversity (H´) and evenness (E) indices. A threshold was set in which the AWCD less than 0.06 was considered zero (Ge et al., 2018; Xu et al., 2015). To perform the analysis, the 31 carbon sources were also grouped into 7 carbon types (Goulart, 2013): amines and amides (phenylethylamine and putrescine), amino acids (L-arginine, L-asparagine, L-serine, L-threonine, L-phenylalanine and glycyl L-glutamic acid), carboxylic acids (pyruvic acid methyl ester, D-glucosaminic acid, D-galactonic acid γ-lactone, D-galacturonic acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid and D-malic acid), phenolic acids (2-hydroxy benzoic acid and 4-hydroxy benzoic acid), P-carbon (glucose-1-phosphate and D,L-α-glycerol phosphate), carbohydrates (D-cellobiose, α-D-lactose, β-methyl-D-glucoside, D-xylose, i-erythritol, D-mannitol and N-acetyl-D-glucosamine) and polymers (tween 40, tween 80, α-cyclodextrin and glycogen).

#### ***4.3.4. Statistical analysis***

The experiments were organized in a randomized block design with fourfold replication and factorial combination. ANOVA was performed based on the two factors

of the study using a generalized linear model. The ERM developer plants present in the first phase of the experiment were considered as one factor (with four levels) and the status of the ERM (bulk soil, after plant and after disturbance – three levels) as the second factor. In the second phase, the first factor was also ERM developer plants but with five levels (including control without any previous plants) and the second factor was soil disturbance (with two levels - disturbed and undisturbed). Tukey's test was applied to mean comparisons at a $p \leq 0.05$ significance level.

The AWCD values were used in the principal component analysis (PCA) and to construct the heat maps for an overall metabolic view of the experiment. All the analyses were conducted using Minitab 21® software statistic.

## 4.4. Results

### *4.4.1. Metabolic differences in the first phase*

In the first phase of the experiment, the average well color development (AWCD) and Shannon Diversity Index (H') were differentially affected by the treatments (Table 1). The AWCD and H' were smaller in the bulk soil and greatly increased after planting and after disturbance. AWCD was also greater for mycotrophic plants compared with the non-mycotrophic one. Among the mycotrophic plants, the legume (*O. compressus*) showed the highest AWCD and H' in after planting and after disturbance. In general, disturbance of the soil caused an increase in AWCD, except in relation to the non-mycotrophic plant (*S. gallica*). Although functional diversity (H') increased after disturbance, it was only significant in the soil under the highly-mycotrophic *L. rigidum*. The Shannon Evenness Index (E) was statistically different when comparing bulk soil and after disturbance sampling but did not differ at the after-plant sampling time.

**Table 1:** Effect of plant species and soil disturbance on the AWCD, Shannon Diversity and Shannon Evenness Indices in the first phase of the experiment

| | AWCD | | | | Shannon Diversity (H') | | | | Shannon Evenness (E) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Soil Sampling | | | *Mean plant* | Soil Sampling | | | *Mean plant* | Soil Sampling | | | *Mean plant* |
| **Plants** | BP | AP | AD | | BP | AP | AD | | BP | AP | AD | |
| *O. compressus* | 0.20 i | 0.76 d | 1.09 a | 0.68 A | 2.61 d | 3.22 a | 3.29 a | 3.04 A | 0.92 | 0.98 | 0.97 | 0.96 |
| *L. rigidum* | | 0.47 g | 1.06 b | 0.58 B | | 2.79 cd | 3.22 a | 2.87 BC | | 0.92 | 0.97 | 0.93 |
| *R. bucephalophorus* | | 0.55 f | 0.83 c | 0.53 C | | 3.11 ab | 3.21 a | 2.98 AB | | 0.92 | 0.95 | 0.93 |
| *S. gallica* | | 0.62 e | 0.43 h | 0.42 D | | 2.82 bcd | 3.03 abc | 2.82 C | | 0.94 | 0.94 | 0.93 |
| ***Mean soil sampling*** | 0.20 C | 0.60 B | 0.85 A | | 2.61 C | 2.98 B | 3.19 A | | 0.92 B | 0.94 AB | 0.96 A | |

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). AWCD: Average well color development; BP: Before Planting; AP: After Plant growth; AD: After Soil disturbance.

The soil disturbance also affected the metabolism of the different carbon types differently, depending on the plants (Figure 2). Among the highly-mycotrophic plants, it increased greatly the metabolism of carbohydrates, carboxylic acids, phenolic acids and polymers in *O. compressus* and amino acids, carbohydrates, phosphate-carbon and carboxylic acids in *L. rigidum*. In the scarcely-mycotrophic *R. bucephalophorus*, that increase was only great in the metabolism of phosphate-carbon, whereas for the other C-types the metabolism was lowered. In the non-mycotrophic plant *S. gallica*, the effect of the disturbance caused a decrease in the metabolism of most C-types, especially in amines and amides, amino acids and polymers. Also, in *S. gallica*, there was no metabolism of phosphate-carbon, neither after plant nor after disturbance and similarly with *R. bucephalophorus* the disturbance greatly decreased metabolism of polymers.

With respect to the metabolism of different C-sources, the soil disturbance highly increased the metabolism of pyruvic acid methyl ester for all plants. In *O. compressus*, *L. rigidum* and *R. bucephalophorus*, the soil disturbance caused an increase in the metabolism of L-asparagine, L-threonine and β-methyl-D-glucoside. Among the highly-mycotrophic plants, an increase in D-cellobiose, D-mannitol and N-acetyl-D-glucosamine also observed. In particularly, a shift in the metabolism of D-malic acid and tween 80 was found in *O. compressus*. In *L. rigidum*, an increase in D-galactonic

acid γ-lactone, D-galacturonic acid and glucose-1-phosphate was noted. The latter was also founded in *R. bucephalophorus* (Figure 3).

### *4.4.2. Metabolic differences in the second phase*

In the second phase of the experiment, the results of carbon metabolism found 21 days after wheat planting showed statistically significant differences in AWCD regarding previous plant and soil disturbance (Table 2). In general, the soil disturbance caused an increase in AWCD, except in the soil under wheat that followed *O. compressus*. The Indices of Diversity and Evenness were not affected either by the previous plant or soil disturbance.

| | **AWCD** | | | **Shannon Diversity (H')** | | | **Shannon Evenness (E)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** | **Soil Sampling** | | ***Mean plant*** |
| **Plants** | U | D | | U | D | | U | D | |
| *O. compressus* | 1.07 a | 0.90 d | 0.99 A | 3.44 | 3.36 | 3.40 | 1.00 | 0.98 | 0.99 |
| *L. rigidum* | 0.65 f | 1.02 b | 0.83 E | 3.36 | 3.42 | 3.39 | 0.99 | 1.00 | 0.99 |
| *R. bucephalophorus* | 0.78 e | 0.98 c | 0.88 D | 3.27 | 3.42 | 3.35 | 0.97 | 1.00 | 0.98 |
| *S. gallica* | 0.78 e | 1.02 b | 0.90 C | 3.31 | 3.42 | 3.36 | 0.97 | 1.00 | 0.98 |
| No Plant | 0,91 d | | 0.91 B | 3.38 | | 3.38 | 0,99 | | 0.99 |
| ***Mean soil sampling*** | 0.84 B | 0.97 A | | 3.35 | 3.40 | | 0.99 | 0.99 | |

**Table 2:** Effect of previous plant species and soil disturbance on the AWCD, Shannon Diversity and Shannon Evenness Indices in the second phase of the experiment (after wheat growth).

Means sharing different letters indicate significant differences between treatments at 5% level (Tukey's test). AWCD: Average well color development; U: undisturbed; D disturbed.

The previous soil disturbance differentially affected the metabolism of the different C-types regarding the plants the grew before the wheat. Soil samples in which wheat grew after the non-mycotrophic plant, the disturbance increased the metabolism of all c-types except the phosphate-carbons (Figure 2). The same effect was observed after *R. bucephalophorus*, with exception of phenolic acids and polymers that lowered. The effect was diverse between the highly-mycotrophic plants. The disturbance increased the metabolism of c-types except amines and amides and polymers in *L. rigidum*. However, in *O. compressus* a decrease in the metabolism of amines and amides, amino acids, carbohydrates and phosphate-carbons was observed in the

disturbed treatment. Significantly, carboxylic acids, phenolic acids and polymers seemed not to be affected by disturbance.

The wheat soil microbiome developed after highly-mycotrophic plants in the undisturbed treatment seemed to be more metabolically active under the various C-sources when compared with the disturbed treatment. In the wheat that grew after *O. compressus* with ERM disrupted, a highly increased metabolism of tween 40 and glycogen was observed. For *L. rigidum*, that increase was observed for L-serine, L-threonine and 4-hydroxy benzoic acid. Differently, in the soil under wheat that grew after the scarcely and non-mycotrophic plants, the disturbed treatment showed higher rates of metabolism of the C-sources (Figure 3).

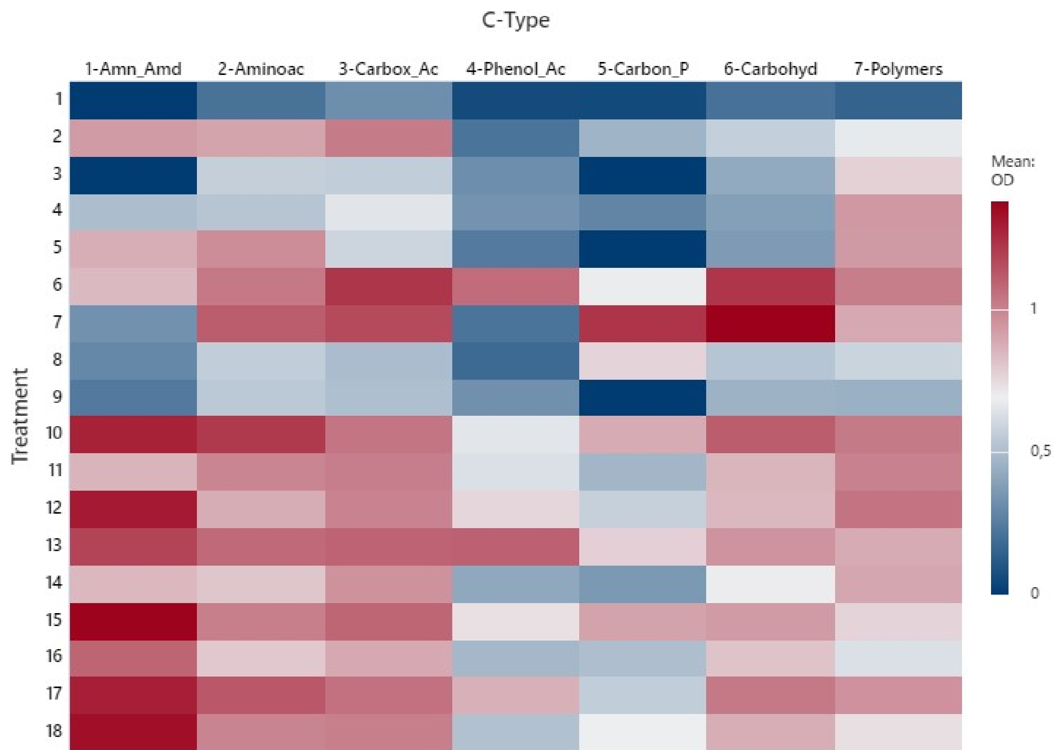


**Figure 2:** Heat map of optical density (O.D.) of C-type. First phase 1-9: 1-Bulk soil, 2- *O. compressus* after plant, 3- *L. rigidum* after plant, 4- *R. bucephalophorus* after plant, 5- *S. gallica* after plant, 6- *O. compressus* after disturbance, 7- *L. rigidum* after disturbance, 8- *R. bucephalophorus* after disturbance and 9- *S. gallica* after disturbance. Second phase 10-18: 10- wheat after *O. compressus* undisturbed, 11- wheat after *O. compressus* disturbed, 12- wheat after *L. rigidum* undisturbed, 13- wheat after *L. rigidum* disturbed, 14- wheat after *R. bucephalophorus* undisturbed, 15- wheat after *R. bucephalophorus* disturbed, 16- wheat after *S. gallica* undisturbed, 17- wheat after *S. gallica* disturbed and 18- wheat with no previous plants.

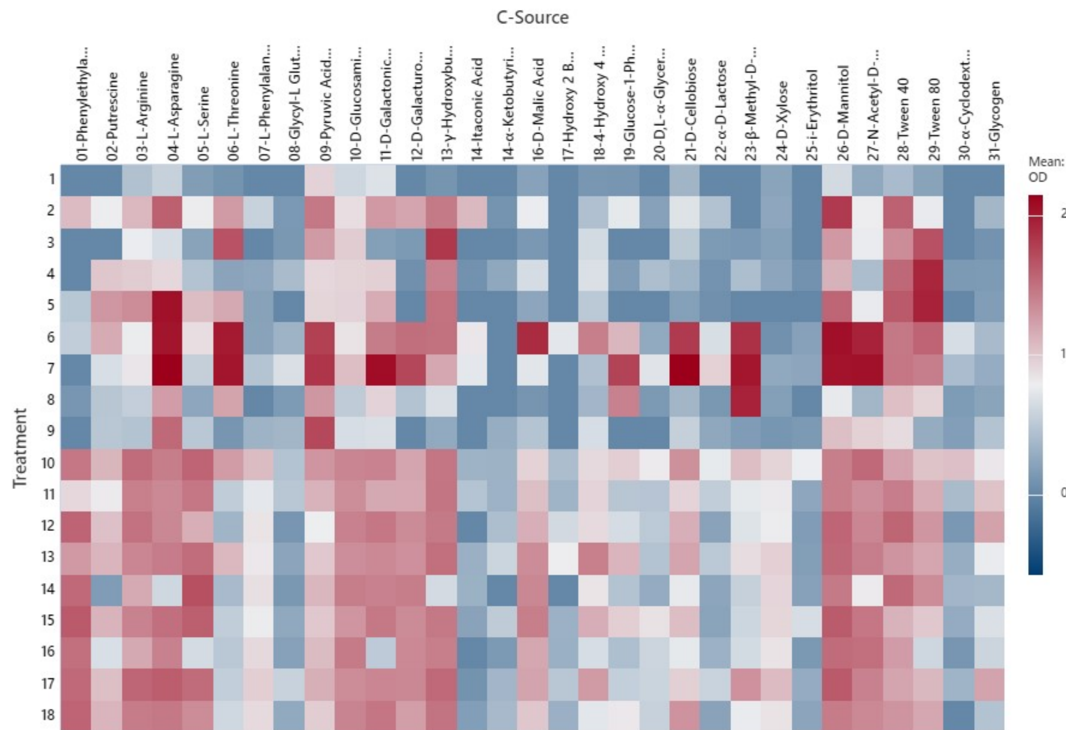


**Figure 3:** Heat map of optical density (O.D.) of C-source. First phase 1-9: 1-Bulk soil, 2- *O. compressus* after plant, 3- *L. rigidum* after plant, 4- *R. bucephalophorus* after plant, 5- *S. gallica* after plant, 6- *O. compressus* after disturbance, 7- *L. rigidum* after disturbance, 8- *R. bucephalophorus* after disturbance and 9- *S. gallica* after disturbance. Second phase 10-18: 10- wheat after *O. compressus* undisturbed, 11- wheat after *O. compressus* disturbed, 12- wheat after *L. rigidum* undisturbed, 13- wheat after *L. rigidum* disturbed, 14- wheat after *R. bucephalophorus* undisturbed, 15- wheat after *R. bucephalophorus* disturbed, 16- wheat after *S. gallica* undisturbed, 17- wheat after *S. gallica* disturbed and 18- wheat with no previous plants.

### *4.4.3. Overall metabolic profile*

Comparatively, the carbon metabolic activity associated with different C-types was broadened in the second phase of the experiment after wheat growth (Figure 2). The C-types with less metabolic activity were the phenolic acids and phosphate-carbons. The high metabolism of phosphate-carbon, specifically glucose-1-phosphate, observed in *L. rigidum* and *R. bucephalophorus* after disturbance in the first phase of the experiment remained in wheat that grew after these conditions in the second phase (Figure 3). Comparatively, *S. gallica* had the lowest metabolism of phosphate-carbons, it was absent in the first phase and low after wheat regardless the soil treatment. The higher rates of phenolic acids metabolism were found in the *O. compressus* after soil

disturbance in the first phase. However, in the second phase (after wheat growth), the greatest rates of phenolic acids metabolism were after *L. rigidum* in the disturbed soil, but only for 4-hydroxy benzoic acid.

The different mycotrophic plants showed differential metabolism during the experiment regarding carboxylic acids (Figure 2). The plants having none- or scarcely-level of mycotrophy were associated with the AWCD of D-malic, D-galacturonic and D-glucosaminic acids (Figure 3) increasing in the second phase. Additionally, the highly-mycotrophic plants maintained the highest rates of metabolism regardless the soil disturbance in all phases for γ-hydroxybutyric acid. Despite the second phase of the experiment enhanced the metabolism of carbohydrates, the highest AWCD of most carbon sources, such as D-cellobiose, D-mannitol, N-acetyl-D-glucosamine and β-methyl-D-glucoside, were observed associated with disturbed soil under highly-mycotrophic plants in the first phase (Figure 3). Significantly, some carbon sources that were not active in stimulating microbial metabolism among the treatments in the second phase showed some activity in the control, such as itaconic acid, α-D-lactose and α-cyclodextrin.

In the undisturbed treatment, the metabolism of the amino acids (Figure 2) increased in the soil under wheat that grew after the mycotrophic plants but decreased in the soil under wheat following the growth of the non-mycotrophic plant. Differently, for L-asparagine and L-threonine (Figure 3) the AWCD decreased in the soil under wheat that followed highly-mycotrophic plants and soil disturbed. After wheat growth, the metabolism of the amines and amides, notably the phenylethylamine increased. Additionally for this carbon source, the highest metabolism was found in wheat that grew after mycotrophic plants in the undisturbed soil; in opposite for the wheat that grew after scarcely and non-mycotrophic plants. In this case, the high metabolism was observed in the disturbed treatment (Figure 3). The same result could be observed for polymers. In the mycotrophic plants there was an increase of metabolism from the first

phase to the second in the undisturbed soil but following the scarcely and non-mycotrophic plants the increase only occurred in the disturbed soil (Figure 2).

Principal components analysis using all 31 carbon sources revealed a separation of substrate samples, indicating the different patterns of potential C use and different microbial communities. The time selected for analysis was 72 h and two types of C metabolization were analyzed. The first one was related to 7 types of carbon (C-type) and the second to the 31 sources of carbon (C-source) in the Ecoplate. Two principal components (PCs) were selected to be retained from a scree plot. For C-type, the first principal component (PC1) explained 70.5% and the second 11,6% of the total variance of the data and are plotted against each other (Figure 4). For C-source, the PC1 explained 43,9% and PC2 18,9% of the total variance of the data and are plotted against each other (Figure 5).

The principal component analysis (PCA) regarding C-source and C-type indicated that the carbon metabolism profile between first and second phases was different. Regarding the C-type, the PCA confirmed the higher rates of C metabolism at the wheat phase compared with initial phase of the experiment (Figure 4). The PC1 showed a positive relation with an increase in amino acids, carboxylic acids and carbohydrates metabolism. Exceptionally, in the disturbed treatment, the mycotrophic plants (*L. rigidum* and *O. compressus*) followed by *R. bucephalophorus* showed higher AWCD in the first phase. Microbiomes of *L. rigidum* and *R. bucephalophorus* differed from that of the wheat which grew after these same treatments metabolically in the PC2, having higher P-carbons metabolism. Conversely, the second phase of the experiment, after wheat growth, showed greater phenolic acids and amines and amides metabolism (Table 3A). According to the PCA, the microbiome metabolism of the mycotrophic *O. compressus* in the disturbed soil was metabolically similar to the wheat that grew after this treatment. In this analysis, the C metabolism of the wheat that grew after non- and scarcely-mycotrophic plants in the undisturbed soil was less

intense than in the disturbed soil even compared with the wheat that grew after no previous plants.

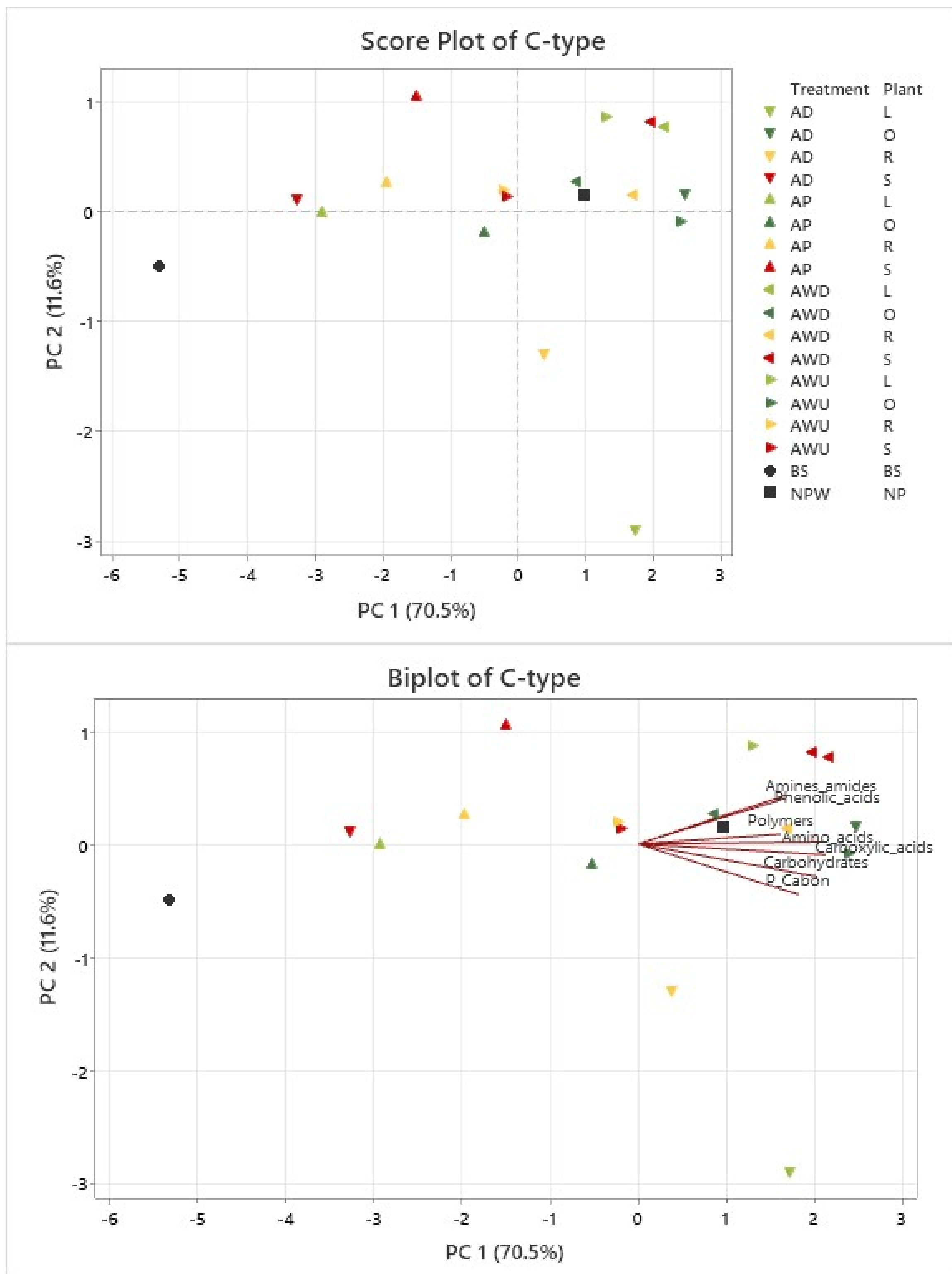


**Figure 4:** Principal Component Analysis (PCA) of C-type. BS: bulk soil; AP: after plant; AD: after disturbance; AWU: after wheat undisturbed treatment; AWD: after wheat disturbed treatment and NPW: no plant before wheat.

In the PCA of C-source, the PC1 also showed that in the first phase of the experiment, there was less metabolic activity than in the second (Figure 5). Significantly, there was an exception again for the mycotrophic plants (*L rigidum* and *O. compressus*) after disturbance, indicating higher C-sources metabolism comparatively similar to the microbiome after wheat growth. The increase in C-source metabolism regarding the PC1 was mostly in L-arginine, L-serine and L-phenylalanine (amino acids); D-glucosaminic, D-galacturonic and D-malic acids (carboxylic acids); 4-hydroxy benzoic acid (phenolic acid); D,L-α-glycerol phosphate (P-carbons); D-cellobiose, D-mannitol and N-acetyl-D-glucosamine (carbohydrates) (Table 3A). The metabolic differences among the microbiomes of mycotrophic plants after disturbance and the microbiomes after wheat growth mentioned previously are more evident in the PC2 and it is positively related to the metabolism of L-threonine and pyruvic acid methyl ester, and negatively to phenylethylamine, α-ketobutyric acid and D-xylose (Table 3B).

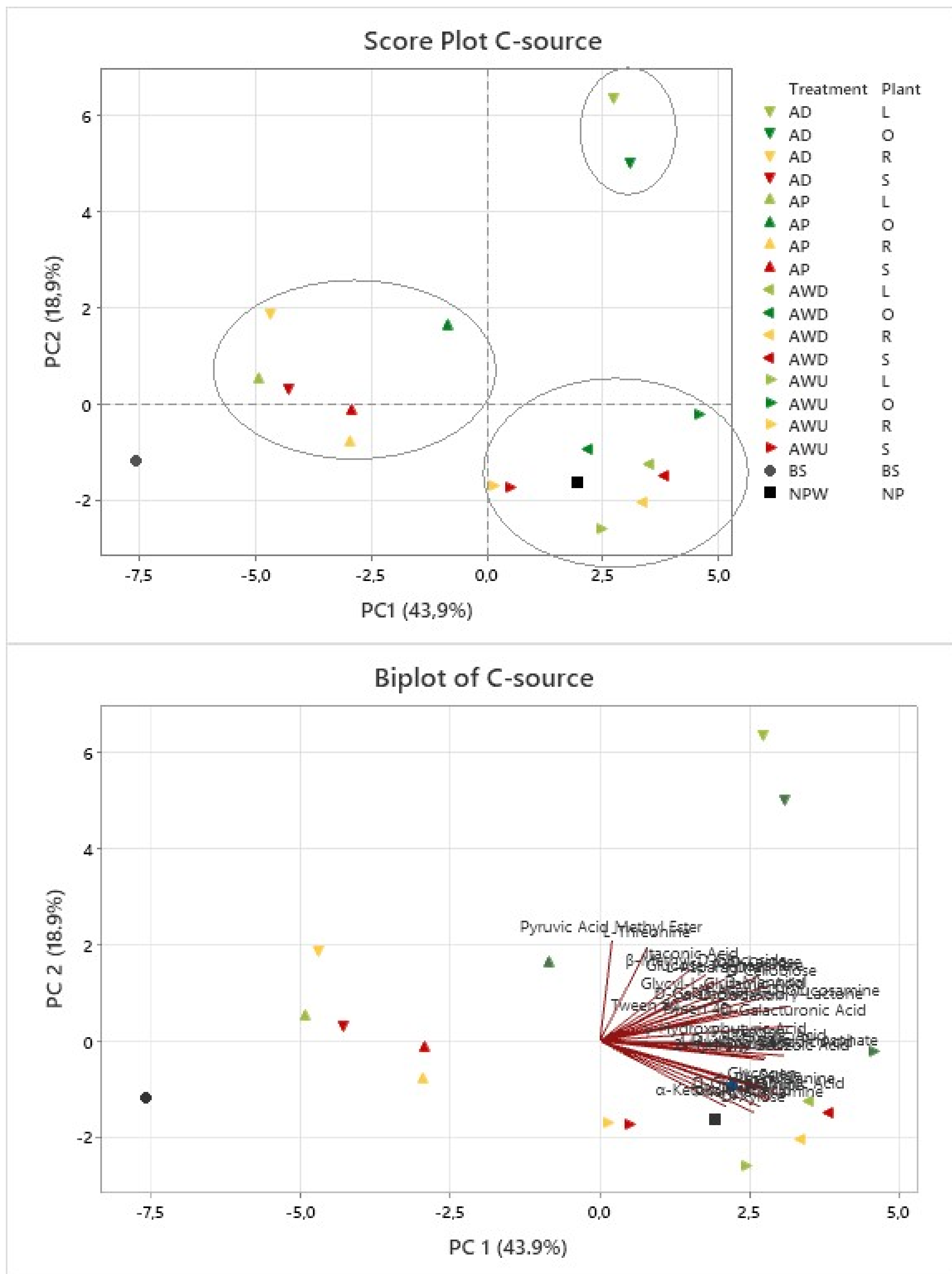


**Figure 5:** Principal Component Analysis (PCA) of C-source. BS: bulk soil; AP: after plant; AD: after disturbance; AWU: after wheat undisturbed treatment; AWD: after wheat disturbed treatment and NPW: no plant before wheat.

**Table 3:** Correlation between substrate utilization and two principal components (PC)

| A | Variable C-type | PC1 | PC2 |
|---|---|---|---|
| 1 | Amines and amides | 0,341 | **0,534** |
| 2 | Amino acids | **0,418** | 0,025 |
| 3 | Carboxylic acids | **0,430** | -0,121 |
| 4 | Phenolic acids | 0,339 | **0,505** |
| 5 | P-Carbons | 0,366 | **-0,555** |
| 6 | Carbohydrates | **0,409** | -0,353 |
| 7 | Polymers | 0,328 | 0,113 |

| B | Variable C-source | PC1 | PC2 |
|---|---|---|---|
| 1 | Phenylethylamine | 0,195 | **-0,231** |
| 1 | Putrescine | 0,182 | -0,018 |
| 2 | L-Arginine | **0,204** | -0,207 |
| 2 | L-Asparagine | 0,138 | 0,211 |
| 2 | L-Serine | **0,213** | -0,172 |
| 2 | L-Threonine | 0,058 | **0,331** |
| 2 | L-Phenylalanine | **0,218** | -0,188 |
| 2 | Glycyl-L Glutamic Acid | 0,150 | 0,150 |
| 3 | Pyruvic Acid Methyl Ester | 0,015 | **0,352** |
| 3 | D-Glucosaminic Acid | **0,207** | -0,202 |
| 3 | D-Galactonic Acid γ-Lactone | 0,196 | 0,118 |
| 3 | D-Galacturonic Acid | **0,238** | 0,055 |
| 3 | γ-Hydroxybutyric Acid | 0,155 | -0,008 |
| 3 | Itaconic Acid | 0,111 | **0,254** |
| 3 | α-Ketobutyric Acid | 0,153 | **-0,229** |
| 3 | D-Malic Acid | **0,222** | -0,031 |
| 4 | 2-Hydroxy Benzoic Acid | 0,199 | -0,065 |
| 4 | 4-Hydroxy Benzoic Acid | **0,203** | -0,058 |
| 5 | Glucose-1-Phosphate | 0,152 | 0,218 |
| 5 | D,L-α-Glycerol Phosphate | **0,225** | -0,052 |
| 6 | D-Cellobiose | **0,209** | 0,199 |
| 6 | α-D-Lactose | 0,194 | **0,229** |
| 6 | β-Methyl-D-Glucoside | 0,130 | **0,235** |
| 6 | D-Xylose | 0,188 | **-0,253** |
| 6 | i-Erythritol | 0,187 | -0,060 |
| 6 | D-Mannitol | **0,203** | 0,157 |
| 6 | N-Acetyl-D-Glucosamine | **0,232** | 0,125 |
| 7 | Tween 40 | 0,116 | 0,065 |
| 7 | Tween 80 | 0,054 | 0,077 |
| 7 | α-Cyclodextrin | 0,168 | 0,109 |
| 7 | Glycogen | 0,199 | -0,165 |

from EcoPlate analysis. A: C-type metabolism; B: C-source metabolism.

## 4.5. Discussion

Plant development more than doubled microbial activity and significantly increased functional diversity when compared with bulk soil (soil before planting). The greatest microbial activity (AWCD) and functional diversity (H') were associated with the plant that performs both mutualist symbioses, mycorrhiza and rhizobia (*O. compressus*), highlighting the importance of favorable niches for microbial diversity and interaction. The non-mycotrophic plant (*S. gallica*) exhibited a poorer activity and functional diversity (Table 1). Upon soil disturbance and the loss of habitat niches, differences in the functional diversity among the different plants was lost. Our results suggest that the soil disturbance affected differentially the microbiome metabolism linked to the different plants in the first phase of the experiment. Changes on soil organic matter (SOM) pools could reflect the balance between synthesis and degradation of that pool by the microbial biomass in relation with the species-specific root exudates. The rate at which each SOM metabolism responds to changes in management or other perturbation is likely to vary considerably between plant types and therefore to the nature of organic inputs (Bending et al., 2000; P. Marschner et al., 2001). The results also indicated that soil disturbance impacted differently *O. compressus* and *L. rigidum* in the first phase (Table 1). Legume plant residues decompose rapidly due to their low C/N ratio, as they have high nitrogen and water-soluble carbon contents. Grasses, on the other hand, are characterized by a high C/N ratio in plant residues and longer persistence on the soil surface as a result of low decomposition rate (Bending et al., 2000; Teixeira et al., 2014). In addition, soil disturbance impacts on soil microaggregates, water content and aeration, exposing the SOM and making it easily degradable and available to microbial consumption (Janušauskaite et al., 2013; Young & Ritz, 2000). This may explain the low AWCD in the disturbed treatment of the second phase (Table 2) after *O. compressus*. The SOM

content from the disturbed treatment seemed to be decomposed sooner than the other previous plants and therefore having a lower metabolic activity.

Biolog® indices are related to the metabolic activity, number, variety and diversity of bacteria, including diversity within and between functional groups, being affected by agricultural practices and C-inputs in the agro-ecosystem (Sofo et al., 2019). From the results of the second phase (Table 2), the significant increase in bacterial metabolic activity (AWCD) observed after soil disturbance in the first phase persisted after wheat growth, indicating that the mineralization process was still taking place at a faster rate than in undisturbed soil, with exception of the legume in which it decreased (as discussed above). The soil disturbance treatment homogenized the functional diversity (H´) created by the antecedent plant and wheat development did not affect it, nor to the predominance of few metabolic groups of bacteria (E). This suggests that the metabolic differences between the undisturbed and disturbed treatments in wheat likely resides in the functional activity rather than the abundance of specific bacterial taxonomic groups or the regularity with which the taxonomic groups are distributed. Substrate diversity indices may be used to initially assess functional diversity of soil (Bucher & Lanyon, 2005). However, this tool was not sensitive enough to establish differences of microbial CLPPs from the disturbed treatments in the second phase, where confounding effects of soil management may hinder the emergence of clear differences. Studies of soil microbiome metagenomic are in course to assess the qualitative differences within the treatments.

The ERM formed by mycotrophic plants in the first phase seemed to serve as an additional niche of metabolic activity for soil microbiome. The disruption of this ERM imposed by soil disturbance did change the soil functional profile of some carbon sources linked to biological process involving mycotrophic plants response to stress such as malic acid and glucose-1-phosphate. The disturbance increased the malic acid metabolism and the malate synthesis is hypothesized to be involved in soil Mn detoxification by some plants, including legumes and grass (Bi et al., 2019; De La Luz

Mora et al., 2009). In addition, glucose-1-phosphate can be correlated with mechanisms of grass to phosphorus deficiency (Byrne et al., 2011). The soil disturbance also increased the metabolism of N-acetyl-D-glucosamine and mannitol, carbon sources related to fungal metabolism. N-acetyl-D-glucosamine is a chitin monomer, an abundant polysaccharide found in the cell walls of fungi (Liu et al., 2017), and mannitol have been reported as cell storage for carbohydrates translocated into the mycelia (Meena et al., 2015).

Comparing the results of C metabolism with functional microbiome counts for this experiment, the higher metabolic activity of amines and amides (Fig 3) observed in the wheat phase and in *O. compressus* after disturbance (from the first phase) is in agreement with higher counts of ammonifiers bacteria (data presented in Chapter 2 and 3). As indicated by Marschner (1991), in acid soils the nitrification rate is lower than in neutral soils, and plants adapted to acid soils may either prefer $NH_4$ as source of N assimilation. In turn, ammonium ion is a direct product of bacterial degradation of nitrogen compounds, such as amines and amides (Arora, 2015; González-Moro et al., 2021). Significantly, phenolic acids metabolism also contributes to this clustering in the PCA (Table 3A). The primary sources of phenolic acids in soil are root exudates and decomposition of lignin in plant residues (Wilhelm et al., 2021). Consequently, its higher metabolism could be a direct effect of shoot and root remains and organic matter input of the first phase in addition to wheat exudates. Phenolic acids are also known to play multifunctional roles in rhizospheric plant-microbe interactions acting as signaling molecules in the initiation of legume-rhizobia symbioses (Mandal et al., 2010), hence the presence of *O. compressus* (from the first phase) in that cluster.

Soil disturbance may increase microbial oxidation process, while contributing to reduced microbial biomass (and microbial functional diversity) due to insufficient substrates for anabolic and catabolic functions (Bucher 2005). Generally, in long term field experiments, soil mobilization is known to impact microbial diversity by decreasing it (Lupwayi et al., 1998; Sofo et al., 2014). However, our results shows that the soil

disturbance increased the diversity, particularly after *L. rigidum* at 10 days after de disturbance. This result can be interpreted in the context of the Intermediate Disturbance Hypothesis (IDH), which predicts that disturbance can increase community diversity up to a certain level of disturbance strength or frequency, after which diversity will decrease. From that perspective, higher levels of labile organic carbon due the disturbance were available, increasing the level of C source reachability for microorganisms and therefore promoting functional diversity (Bongiorno et al., 2020; Zhang et al., 2018). Further studies, including more time sampling beyond 10 days after soil disturbance, is needed to verify that hypothesis.

The scarcely-mycotrophic *R. bucephalophorus* exhibited an intermediate metabolic profile during the experiment. Even though no root arbuscular mycorrhizal colonization were found in this experiment (data presented in Chapter 2 and 3), this plant has the ability to form AM symbiosis if intact ERM is the inoculum source (Goss et al., 2017b). Therefore, the ability to form AM symbiosis could have interfered in the metabolic processes that took place during the experiment.

### 4.6. Conclusion

The carbon metabolism in the soil was greatly stimulated after plant growth. The level of plant mycotrophy strongly conditioned the microbial activity and microbial metabolic profile in the rhizosphere after plant growth, and it was associated with a higher metabolic activity, particularly in the legume. This pattern remained after soil disturbance. The different AWCD profiles among the plant species seemed to be a direct effect of plant exudate release, that is species-specific. Mycotrophic and non-mycotrophic plants have different enhanced metabolic profiles especially regarding carboxylic acids and carbohydrates.

The soil disturbance also affected differently the C metabolism profile. Following soil disturbance, more C sources were available to be readily consumed and not only the intensity of metabolism increased but also the diversity of substrates used. After

soil disturbance, plants exhibiting the tripartite symbiosis showed a soil microbiome metabolic profile more related to amines and phenolic acids whilst the grass were more related to carbohydrates and P carbon, especially glucose-1-phosphate. The non-mycotrophic plant exhibited the lower metabolic activity profile whereas the scarcely-mycotrophic plant exhibited an intermediate profile.

The differences in the microbial metabolism (AWCD) associated with the different previous plants observed in the first phase persisted in the soil after the wheat growth, indicating that the previous treatments greatly affect wheat microbiome metabolism. As expected, due to the effects on substrate availability, plant mycotrophy, soil disturbance and plant sequence were important factors that influenced the microbial metabolic performance. With respect to C metabolism, the main differences in the disturbed soil compared with the undisturbed ones was the increase in the AWCD, except after the legume. The wheat that grew after the legume showed a different metabolism profile in which the disturbance led to a decrease of the AWCD. Meanwhile, the growth of the wheat seemed to dilute the differences in microbiome functional diversity (H´) irrespectively of previous soil disturbance.

Further research is underway to determine the changes in soil microbial community composition under the same conditions. The present results demonstrated that changes in patterns of substrate utilization and metabolic diversity by the Biolog-culturable soil microbial community are sensitive indicators of management-induced effects on soil biological properties, and hence changes in soil status. Agronomic decisions like the choice of crop sequence or the tillage techniques used have important practical implications in the soil microbial community and therefore should be taken into consideration.

## 4.7. References


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Chapter 5

# General discussion and future perspectives

## 5.1. General discussion and future perspectives

The benefits of AMF symbiosis in crop bioprotection are well reported (Goss et al., 2017). A successful strategy to overcome the stress caused by Mn and improve wheat growth in acidic soils have been proposed by Brito et al. (2014) and was further adapted for use in the presence of other stresses and host plants (Brito et al., 2021). When crops are challenged by biotic or abiotic stresses existing in the soil, the extent of AMF colonization when the host plant comes into contact with the stressor agent is directly related to the level of bioprotection achieved (Sikora et al., 2008). The strategy proposed consists of the intentional use of selected plants to develop an extensive extraradical mycelium, which, when kept intact by the adoption of reduced or no-tillage techniques, acts as preferential source of inoculum of the following plant, leading to the earlier and faster colonization of the crop by AM fungi, providing its bioprotection against biotic or abiotic stresses from the beginning of the vegetation cycle.

However, along with the changes in AMF, the changes in soil microbiome functionality that took place in every phase of the strategy proposed by Brito et al. (2014) are yet fully elucidated and were the main purpose of this work. Thus, a greenhouse pot experiment was conducted from January to April of 2019 to evaluate the effect of ERM development or not (plants with different levels of mycotrophy) and soil disturbance (ERM integrity) on wheat growth under Mn stress. I assessed the impact of mycorrhizal associations on shaping the soil functional microbiome and its relationship with wheat bioprotection against Mn toxicity. The parameters evaluated were related to biological activity of soil microorganisms and plant development in order to identify differences in soil functional profile within the treatments.

The results presented in this study agreed with the results found by Brito et al. (2014), demonstrating the importance of maintaining intact the ERM developed by the antecedent mycotrophic plant in wheat bioprotection against Mn stress. In the perspective of soil functional profiling, the results indicated that, in general, different

anteceding plant species and soil disturbance significantly affected the soil biological activity throughout the experiment. Moreover, the highly-mycotrophic plants differentially developed soil microbial communities and the disturbance of this microbiome interfered directly in wheat growth, which in turn also modified several biological processes and altered the soil functional profile.

Our results suggests that plant growth was able to affect the soil biological activity by strongly increase SBR, MBC, almost all functional microbial counting, enzymatic activity, metabolic activity and microbial diversity parameters compared to the soil before planting (bulk soil). As a plant starts to establish in the soil, the roots start to exude carbon compounds which activates microbial populations (Hu et al., 2018). After plant growth, the soil microbial activity was significantly greater among the highly-mycotrophic plants compared to the non-mycotrophic one, presenting increased SBR, MBC, enzymatic activities (dehydrogenase, phosphatase and arylsulfatase), C metabolic activity and microbial diversity.

Among the mycotrophic plants, the soil in which the legume (*O. compressus*) grew exhibited higher values of SBR and microbial diversity while the grass (*L. rigidum*) showed a more dehydrogenase activity. Some differences among the mycotrophic plants can also be evidenced regarding the C-type metabolism measured with the Biolog® Ecoplates. Though no differences were found in the polymers and carbohydrate metabolism, the legume presented higher metabolism of phenolic acids, carboxylic acids, amino acids and amines and amides compared to the grass. The grass, in turn, presented a high P-carbon metabolism. Plant mycotrophy and plant species might influence differently the soil microbiome. Thus, the microbial activity that occurs in the rhizosphere of mycorrhizal and non-mycorrhizal plants is completely different (Akyol et al., 2019). Studies comparing rhizosphere microbiome between mycotrophic and non-mycotrophic plants show differences in population and functional groups of bacteria (Marschner & Timonen, 2005). Even among mycotrophic plants the

rhizosphere and mycorrhizosphere effect of Gramineae and Leguminosae show differences in biological properties and microbial diversity (Dotaniya & Meena, 2015).

Irrespectively of the plant mycotrophy, the soil disturbance decreased the SBR, MBC and dehydrogenase activity while increased the C metabolic activity and microbial diversity. It has already established that soil disturbance, such as caused by tillage, affects the soil microbiome and the biological processes they mediate, by changing the soil microaggregates that in turn affect water content and aeration, leading to modifications in soil function, stability and resilience (Smith & Collins, 2007). The results presented were consistent with the view that after soil disruption, mineralization of a flush of readily available organic matter took place but by the time of the last soil sampling (10 days after the disturbance) the rates of SBR and C-Mic had moderated to values equivalent to those present at planting, or even less. In agronomic context and under Mediterranean temperature conditions, these results showed the negative impact of tillage practices that often leads to soil organic matter impoverishment. Further studies would be required, including different sampling times, to assess the rate of evolution of SOM consumption within the treatments.

The soil disturbance affected differentially the functional soil microbiome in relation with plant mycotrophy. The soil in which the non-mycotrophic plant grew exhibited the higher $qCO_2$ after soil disturbance. The non-mycotrophic plant showed the smallest value of MBC within the plants and consequently its greater metabolic quotient could indicate a more stressed environment or at least different patterns of SOM consumption with a greater carbon loss (Ferreira et al., 2010). Soil disturbance greatly decreased the enzymatic activity in the soil in which the highly-mycotrophic plants grew but was not affected in the presence of non-mycotrophic plant. The same pattern was observed in the P solubilizers counts. Additionally, the soil disturbance increased the microbial functional counts of total bacteria and Mn oxidizers where mycotrophic plants grew but again it was not observed under the non-mycotrophic plant. These results indicate that the interactions between AMF and plants could change the composition of

microbial communities in the rhizosphere. The ERM formed in the rhizosphere of the mycotrophic plants *per se* release compounds responsible for stimulation or inhibition of functional groups such as Mn oxidizers (Nogueira, 2002), highlighting the importance of the ERM and its integrity as an additional niche for some soil functional microbes.

In other aspects, the impact of soil disturbance was less severe for soil microbial activity associated with mycotrophic plants. A shift in C-sources linked to microbial metabolism associated with plant mechanisms involved in soil Mn detoxification, such as D-cellobiose, D-mannitol, N-acetyl-D-glucosamine, D-malic acid and glucose-1-phosphate were also observed under the highly-mycotrophic (Liu et al., 2017; Meena et al., 2015). Among the mycotrophic plants, the legume showed the highest value of microbial diversity (H') in terms of substrate use, and this parameter was not affected by soil disturbance. The legumes count with a tripartite symbiosis with rhizobia that shape a diverse functional microbiome able to consume other sources of carbon and therefore impacting on the metabolism profile (Checcucci & Marchetti, 2020).

After wheat growth, several parameters of soil biological activity were still highly influenced by the previous treatments imposed (plant mycotrophy and soil disturbance), except SBR, MBC and $qCO_2$. The wheat that grew after mycotrophic plants not only showed higher AMF colonization and shoot dry weight but also higher photosynthetic activity parameters (chlorophyll content, ETR and q*P*) despite Mn toxicity. Additionally, the soil where wheat grew after mycotrophic plants exhibited great enzymatic activities (dehydrogenase, arylsulfatase and β-glucosidase) and C metabolic activity. In general, the previous soil disturbance was associated with an increase in the microbial functional counts (Mn oxidizers, ammonifiers and S oxidizers) and β-glucosidase activity. Though the previous soil disturbance led to a strongly decrease in wheat dry weight irrespective the antecedent plant mycotrophy, the root AMF colonization markedly decreased (about 40%) when wheat grew after mycotrophic plants and the ERM was disrupted (soil disturbed treatment). The same pattern was observed to chlorophyll content measured in the wheat leaf and in soil dehydrogenase

activity. These results highlight that the biological processes induced by a well stablished ERM formed by the anteceding mycotrophic plant had a relevant impact on wheat yield under Mn toxicity. Specifically, the soil where wheat grew after the legume (*O. compressus*) with ERM intact (undisturbed treatment) exhibited the higher metabolism of carbohydrates, P-carbon, amino acids and polymers. This could indicate a different pattern in the SOM transformations occurring in the soil of wheat that grew after the legume.

The results that link shifts in patterns of substrate utilization and metabolic diversity by the Biolog-culturable soil microbial community and the effect of the different treatments proposed in this study were sensitive indicators of management-induced effects to soil biological properties, and hence changes in soil microbiome. Agronomic decisions like the choice of crop sequence or the tillage techniques used have important practical implications in the soil microbial community and therefore should be taken into consideration. Further research involving metagenomics is underway to determine the changes in soil microbial community composition under the same conditions.

The importance of an intact ERM to promote a fast AMF root colonization that will lead to an improvement of wheat development despite the adverse Mn toxicity effect in acidic soils was confirmed in this study. The ERM developer strategy proposed not only is associated with changes in the plant physiological process such as oxidative enzymes activation (Faria et al., 2021) and lower Mn shoot accumulation (Brito et al., 2021), but the wheat that grew after an intact ERM preserved an active functional microbiome more resilient to soil disturbance. Moreover, even though no differences in wheat dry weight were observed regarding the antecedent mycotrophic, clearly differences between the soil microbiome associated with wheat that grew after the legume and grass is presented in this work in relation to functional microbiome profiling. The results of Biolog® Ecoplates overall and specific C groups metabolism

showed a great metabolic activity and microbial diversity associated with legume plant in both phases of the experiment.

*R. bucephalophorus*, considered as scarcely-mycotrophic plant, although no colonization rate was observed in our study, presented contrasting results throughout the study. For most parameters, results were similar to those obtained for highly-mycotrophic plants rather than to those of the non-mycotrophic one. However, it differed completely from all other plants in some parameters, notably having the smallest values for enzymatic activity and Mn oxidizers count in the first phase of the experiment. In the second phase of the experiment, the wheat that grew after *R. bucephalophorus* exhibit most results of microbial activity similar to the non-mycotrophic plant and also presenting the smallest dry weight.

From a methodological perspective, important achievements were made to implement the protocols of biological activity used in this work. All the methodologies used had to be prior standardized and adapted to the Microbial Soil Laboratory (University of Évora) for the first time. Undoubtedly, a key achievement of this study included the establishment of soil enzymatic analysis in the according to ISO 2030:2018. This high throughput method allowed to increase the number of samples while reduce the cost and time of the analysis. The routine was then well standardized to β-glucosidase, arylsulfatase and phosphatase activity, and in a later stage to the nitrogen-related enzymes. Additionally, the standardization of the ISO 20130:2018 for soil enzymatic activity along with the protocols of several parameters of biological activity that resulted from this work will allow the laboratory to provide external services with a reduced cost. This could help the surrounding farmers to understand the impact of their agronomic management on soil microbial activity.

As pointed by Andreote et al. (2014), if plant microbiomes are better described and understood, the information will be available for the development of a better agriculture fields management. More precisely, it might be possible to alter the microbial community structure of a crop, for instance, taking advantage of indigenous

microorganism that are adapted to a specific environmental condition, leading to an increase in plant resistance, or harnessing of efficiency in the uptake of specific nutrients, or coping with biotic and abiotic stresses. In this way, the development of plant-microbes interaction strategies might result in the next revolution in agriculture, resulting in a more sustainable system for plant production.

## 5.2. References

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