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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Isabel da Silva Henriques, Professora Auxiliar do Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra, e sob coorientação da Doutora Glória Catarina Cintra da Costa Pinto, Investigadora Doutorada do Departamento de Biologia da Universidade de Aveiro.

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FCT Fundação para a Ciência e a Tecnologia

“In science, as in the playing card experiment, novelty emerges only with difficulty, manifested by resistance, against a background provided by expectation.”

— Thomas S. Kuhn, The Structure of Scientific Revolutions

“When we are tired, we are attacked by ideas we conquered long ago.”

— Friedrich Nietzsche

o júri

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agradecimentos

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palavras-chave

Rizobioma; Pinheiro; *Fusarium*; priming; fosfito; ensaios de infeção; estrutura de comunidades bacterianas; resistência a fitopatógenos;

resumo

O rizobioma (i.e. o microbioma da rizosfera) tem um papel importante no crescimento da planta e na sua defesa contra agentes patogénicos. O cancro resinoso, causado pelo fungo *Fusarium circinatum*, afeta várias espécies de pinheiro. Estas têm diferentes graus de suscetibilidade à doença, desde *Pinus radiata* que é mais suscetível a *Pinus pinea* que é resistente. Esta doença está associada a elevadas perdas económicas no setor florestal, não tendo ainda sido identificadas estratégias eficazes para o seu controlo. O *priming* é um método ecológico que permite melhorar as defesas da planta. O fosfito tem sido usado com este fim, apresentando atividade antifúngica. No entanto, a interação do rizobioma, hospedeiro e priming nunca foi estudada neste patossistema. O principal objetivo deste trabalho é elucidar a dinâmica do rizobioma na interação entre *Pinus* spp. e *F. circinatum*. Os objetivos específicos são: 1) elucidar a dinâmica do rizobioma numa espécie de pinheiro suscetível e numa espécie resistente em resposta à inoculação de *F. circinatum*; 2) avaliar o impacto do fosfito e do seu modo de aplicação (foliar e rega) no rizobioma de uma espécie sensível.

Para atingir estes objetivos, foram efetuadas duas experiências com plântulas de pinheiro com 8 meses de ambas as espécies (*P. radiata* e *P. pinea*). As plantas foram inoculadas artificialmente no caule com $1x10^6$ esporos do fungo. Plantas não inoculadas constituíram o grupo controlo. O efeito do fosfito (3%) foi analisado em *P. radiata*, testando dois modos de aplicação (foliar e rega), assim como a resposta de plantas inoculadas e não inoculadas. Para ambas as experiências, as plantas foram monitorizadas e amostradas após 10 dias. No momento da amostragem a performance das plantas foi analisada, usando parâmetros fisiológicos, de trocas gasosas e capacidade antioxidante. O ADN foi purificado da rizosfera e foi utilizado para avaliar a estrutura do rizobioma através de PCR-DGGE e sequenciação massiva paralela do gene 16S rRNA. A diversidade funcional foi inferida usando o software Piphillin.

Comparando as duas espécies, apenas *P. radiata* demonstrou sintomas visíveis, assim como alterações significativas de parâmetros relacionados com trocas gasosas. Os rizobiomas das duas espécies revelaram ser significativamente diferentes. No rizobioma de *P. pinea* verificou-se uma maior abundância relativa de bactérias de famílias com funções específicas de promoção de crescimento em plantas (ex: *Nocardioidaceae, Burkholderiaceae*, *Xanthomonadaceae*). Para este rizobioma também foi estimada uma maior abundância de genes relacionados com a produção de monoterpenos, compostos com atividade antimicrobiana. A inoculação com *F. circinatum* teve um baixo impacto no rizobioma de ambas as espécies. Contudo, em *P. radiata* verificou-se uma maior abundância de *Kofleriaceae* após inoculação. Esta família tem sido associada a tecidos necróticos vegetais. A utilização de fosfito resultou numa redução de plantas sintomáticas após 10 dias, sendo esta redução mais evidente quando o fosfito foi aplicado por irrigação (30% de plantas sintomáticas) em comparação com a aplicação foliar (50%). Este efeito verificou-se também nos parâmetros de trocas gasosas, embora pouco acentuado. Em termos do rizobioma, o fosfito alterou significativamente a abundância de diversas famílias, especialmente quando foi aplicado por irrigação. Esta resposta parece estar relacionada com o decréscimo do pH do solo, que seleciona bactérias adaptadas a solos mais ácidos, como *Acidimicrobiaceae*, ou com o aumento da biodisponibilidade de fósforo que seleciona famílias adaptadas a essa condição como *Polyangiaceae*.

Em suma, este trabalho compara pela primeira vez a composição do rizobioma de *P. pinea* e *P. radiata,* tendo sido detetadas diferenças significantes que podem ter um papel importante na suscetibilidade destas espécies à infeção por *F. circinatum*. Apesar do fosfito ter um efeito de priming, o impacto deste composto no rizobioma pode ter implicações no desenvolvimento da planta, o que deve ser investigado.

keywords

Rhizobiome; Pine; *Fusarium*; priming; phosphite; infection trials; structure of bacterial communities; Phytopathogen resistance;

abstract

The rhizobiome (i.e. the rhizosphere microbiome) may play an important role in plant growth and defence against pathogens. Pitch canker, caused by the fungus *Fusarium circinatum*, infects a wide range of Pine species with different degrees of susceptibility, *Pinus radiata* is highly sensitive while *Pinus pinea* is resistant. To date, there are no suitable approaches available to control this threat, being associated with elevated economic losses to the forestry sector. Priming by chemical compounds such as phosphite, a priming agent with antifungal activity, is pointed as environmental-friendly approach to boost plant immune system. However, the relation between microbiome and host behaviour and priming was never studied in this pathosystem.

Therefore, the aim of this study is to unveil the dynamics of the rhizobiome in *Pinus* spp. -*F. circinatum* interaction. Specific aims are to elucidate rhizobiome dynamics: 1) in a susceptible and in a resistant Pine species in response to *F. circinatum* inoculation; and 2) after phosphite application (foliarly or irrigation) in of the susceptible species. To attain these aims, two experiments were set up using 8 month-old *Pinus* seedlings of both species. Plants were artificially stem inoculated with $1x10^6$ spores of the fungus. A non-inoculated control group was set up. The effect of phosphite (3%) was analyzed in *P. radiata*, testing two application modes (foliarly and irrigation) and included inoculated and non-inoculated plants (control).

For both experiments, plant symptoms were monitorized over time and sampled after 10 days, then physiological performance was assessed in needle by gas-exchange parameters and antioxidant capacity. DNA was purified from the rhizosphere and used to evaluate the rhizobiome structure by 16S rRNA gene PCR-DGGE and massive parallel sequencing. The functional diversity of the community was inferred using the Piphillin software.

When the two species were compared, visible symptoms were observed only in *P. radiata*, in parallel with significant alteration of gas-exchange parameters. Looking at species level, both rhizobiomes were significantly different, with a higher relative abundance of families known for their specific plant growth promoting traits (e.g. *Nocardioidaceae, Burkholderiaceae*, *Xanthomonadaceae*) in *P. pinea*. In *P. pinea* rhizobiome a higher abundance of genes related to the synthesis of monoterpenes, compounds with antimicrobial activity, was also estimated. However, *F. circinatum* inoculation had low impact on both species rhizobiome. Yet in *P. radiata* a higher abundance of *Kofleriaceae*, a family associated with plant necrotic tissues was evident after inoculation.

The use of phosphite resulted in a priming effect resulting in a clear reduction in of symptomatic plants after 10 days, particularly when applied by irrigation (30%) in comparison to foliar application mode (50%). Moreover, a slight alleviation of the negative impacts on gas exchange parameters comparing to non- priming inoculated plants were observed. At rhizobiome level, phosphite significantly affected the abundance of several families, especially when applied through irrigation. This response is probably related to soil pH decrease, selecting bacteria adapted to acidic soils such as *Acidimicrobiaceae*, while *Polyangiaceae* abundance may be related to an increase in phosphorus bioavailability.

In conclusion, our study compared for the first time the *P. radiata* and *P. pinea* rhizobiome, detecting significant differences that may play a role in the differential host susceptibility to *F. circinatum* infection. Despite the observed priming effect of phosphite, the strong impact on the plants' rhizobiome may has further implications on plant development, which deserve further investigation.

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1.Introduction

1.1 Rhizosphere - A blooming Oasis for diversity

The soil area affected by the roots is variable from plant to plant and is called rhizosphere. The rhizosphere's habitat is defined as the part of the soil that is influenced by the root's exudates, respiration, nutrient circulation as well as water circulation (Hinsinger et al., 2009). Due to these different aspects of root activity, particularly the water-soil interaction, alongside the natural extension of the root and the different metabolites produced by the surrounding microbiota, the soil particles around root are normally more tightly aggregated and less unstable (Czarnes et al., 2000). This facilitates the establishment of microbial communities. Specifically, bacteria can be found around the root in quantities up to 100 times higher than those observed in bulk soil (da Silva, 2006).

Bacterial communities are ubiquitously distributed throughout the environment. They can be found free-living or in association with a host. When attached to a host, microorganisms can provide beneficial advantages. In humans, the gut-associated microbiota has an important role in shaping the immune system and, consequently, it impacts the health of the human individual (Nicholson et al., 2012). Regarding other hosts, there are numerous studies that clarify the interactions between bacteria and plants (reviewed in Whipps, 2001). Even though bacteria can be found in a ubiquitous manner throughout the soil, in plants' rhizosphere is where they predominate (Jones et al., 2008).

Rhizosphere colonizing bacteria are commonly known as rhizobacteria (Schroth et al., 1982). As of recently, the microbiome inhabiting the rhizosphere has been defined as rhizobiome (Cúcio et al., 2016). The term rhizobiome is still not widely used as a substitute for rhizosphere microbiome, but, since 2018 it has gained popularity (figure 1). Throughout this document, rhizobiome will be used as a synonymous of the rhizosphere microbiome.

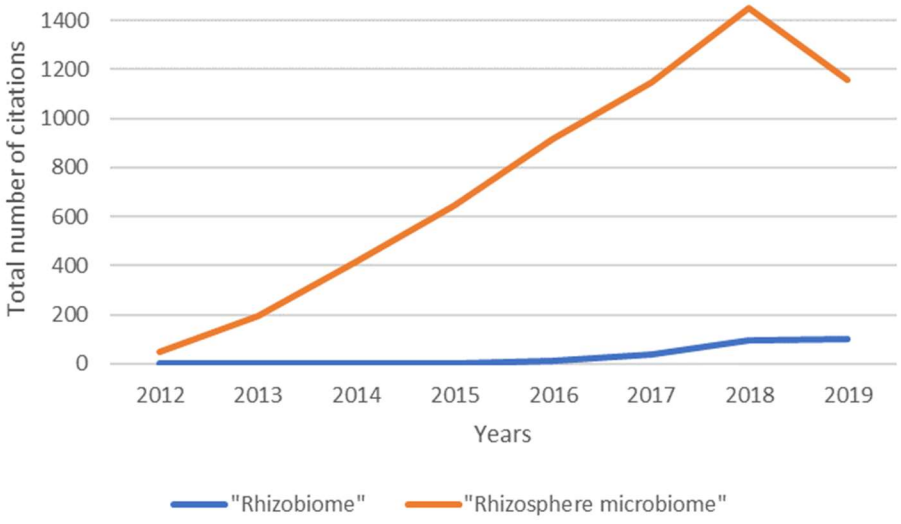

Figure 1: Total number of paper citations of the word "Rhizobiome" or "Rhizosphere microbiome" in published and unpublished articles from 2012 to 2019 (taken from the analysis tool of Web of Science by searching the respective key words) (September, 2019).

1.1.1 The different layers of the rhizosphere and main influential abiotic factors

The rhizosphere can be divided in three areas:

1) the endorhizosphere, the innermost part of the rhizosphere, which includes the cortex and the endodermis of the plant. This is where most microbial organisms colonize the vegetal tissue and directly benefit from exudate compounds (Van Peer et al., 1990).

2) the rhizoplane, the middle section of the rhizosphere that is composed of the root's surface, respective mucilage, along with soil particles attached to it (Foster, 1986).

3) the ectorhizosphere, the outermost layer of soil surrounding the root system.

By these definitions, it is implied that the rhizosphere is not static since it varies as time passes and from one species to another. Furthermore, the diversity of rhizosphere-

associated bacterial communities is affected by the plant species and soil characteristics (Marschner et al., 2001).

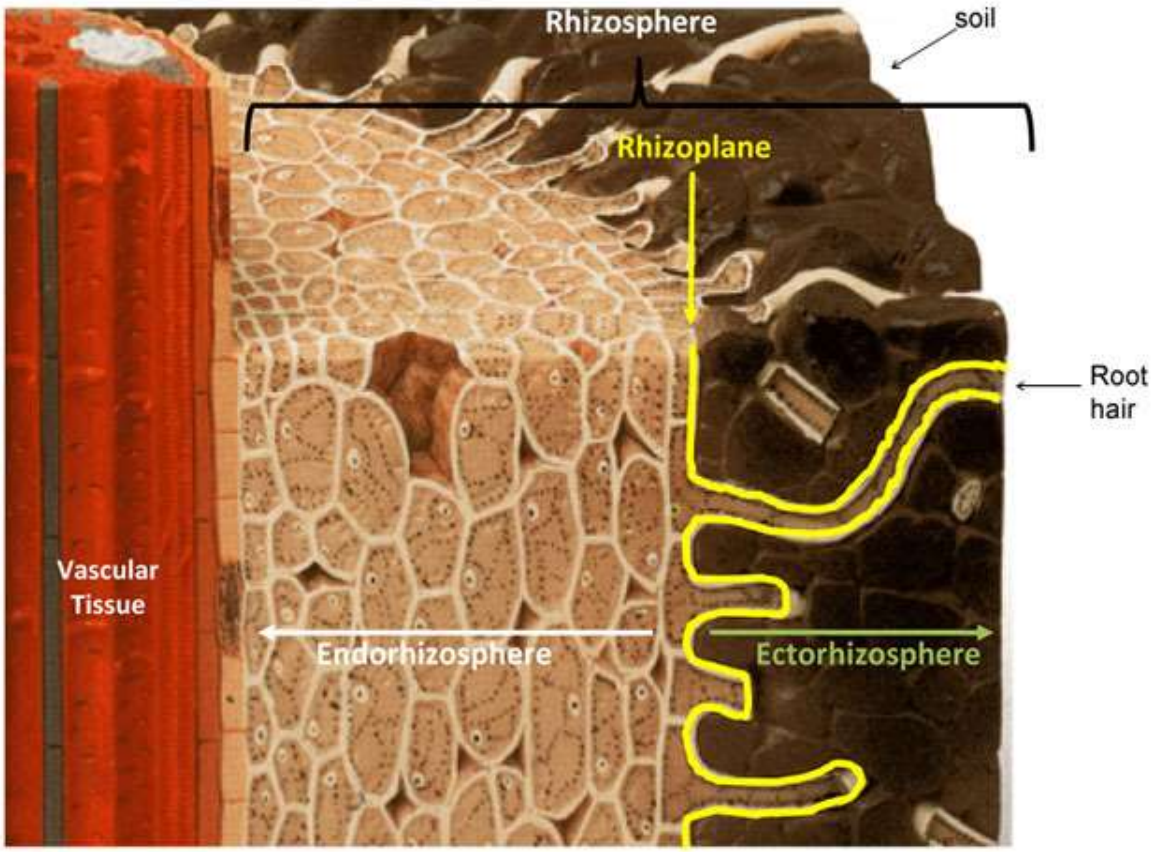

Figure 2: Rhizosphere: schematic representation displaying a cut of the 3 layers of the rhizosphere (in McNear, 2013).

One of the most abiotic influent factors that modulates the rhizosphere characteristics is the water flow derived from the root activity. This is due to the fact that water is the nature's most common vessel, which facilitates the transport of nutrients and enhances the transport system of the root. Water also enables the root soil colonization by applying turgidity pressure to each plant's cells (Sharp et al., 2004). On the other hand, pH is one the factors that impacts microbial communities in soil (Fierer et al., 2006). Extreme values of pH can cause ion toxicity to the root and, indirectly, adversely affect microbial communities (Marschner, 1995). It is also known that bacteria, in general, prefer a level of pH closer to neutral (Madigan et al., 1998). This preference is also related to the fact that the pH can influence nutrient availability, cationic metal solubility and organic carbon characteristics. Thus, pH values can be used as a predictor of bacterial diversity in a soil community (Brady et al., 1990; Lauber et al., 2009).

1.1.2 Classification of rhizosphere colonizing microorganisms

Rhizosphere microorganisms can be divided in root colonizers (endorhizosphere) and soil colonizers (ectorhizosphere and, to a certain degree, rhizoplane). The main difference among these groups is that the endophytes have characteristics that enable them to colonize the vegetal tissue (Rosenblueth et al., 2006). This competence to colonize the endorhizosphere, as described by Compant et. al. (2010), depends on the following bacterial traits: flagella, *nod* genes, cell-wall degrading enzymes, detoxification enzymes, type IV pili, twitching motility and LPS (Lipopolysaccharides). These characteristics facilitate bacterial movement through the vegetal tissue and enable bacteria to overcome the challenges imposed by the plant, such as bactericidal metabolites.

On the other hand, depending on their impact on plant growth, rhizobacteria can be classified as deleterious, neutral or beneficial. Deleterious RhizoBacteria (DRB) (Nehl et al., 1997) have a deleterious effect towards the plant, impairing its normal development. This antagonistic effect is achieved by the bacteria that aggressively colonize the plant's tissues, promptly metabolizing the plant's metabolites. It is important to note that DRB differ from phytopathogenic bacteria in the sense that the affected plants do not show symptoms of disease. This is due to the fact that DRB do not overpopulate and overmultiply in the vascular tissues of the plant, like what is normally observed in phytopathogenic bacteria (Hallmann, 2001). Therefore, the outcome of DRB colonization is more subtle (Kremer, 2007). Neutral groups do not pose any benefit or damage to the plant's development. Rhizosphere bacteria that have a beneficial effect on the plant's growth are usually called Plant Growth Promoting Rhizobacteria (PGPR). This group is very important to the development of PGPR may be used in order to enhance the growth of the plant (Beneduzi et al., 2012).

Beneficial conditions provided by the plants impact positively and in a synergistic way the microbial diversity of the rhizosphere habitat. It also attributes specific characteristics to this smaller yet richer portion of the Earth's soil, representing a plant-made oasis in below ground environments (Ranjard et al., 2001).

1.1.2.1. Indirect benefits provided by PGPR

Regarding indirect interactions by PGPR, the benefit provided to the plant is achieved by impairing the growth of phytopathogens, consequently diminishing any potential damage caused by these pathogenic organisms (Beneduzi et al., 2012). Such reduction can be achieved by

producing compounds that are toxic to the aforementioned pathogens. These by-products can be chitinases, proteases, glucanases, or lipases, enzymes that can damage fungal cells (Maksimov et al., 2011).

It is also possible for PGPR to directly compete for nutrients or specific minerals/iron in the soil, leading to an antagonistic response towards phytopathogenic organisms (Hernandez et al., 2004). Lastly, PGPR can also elicit an induced systemic resistance response from the plant, culminating with a plant with less susceptibility to disease (Kloepper et al., 2004). It is also important to note that sometimes root colonization is needed for a more effective symbiosis (Beneduzi et al., 2012).

1.1.3.2 Direct benefits provided by PGPR

The direct benefits provided by PGPR to their plant host imply an interaction between PGPR and its host. In this case, the activity of PGPR will directly enhance the development of the plant. Normally, these direct effects concern nitrogen fixation, increasing nutrient availability such as phosphate, production of phytohormones and increasing the root surface area. PGPR can also interact with other microorganisms and synergistically improve their activity and, consequently, the growth of the plant. All these mechanisms of control can be exerted in a combined way (Vessey, 2003). For instance phosphorous, along with nitrogen, has a very preponderant role in plant development. It is the second most limiting nutrient for plants. Even though there is plenty of phosphorous in the soil, it is not readily available to the plants (Paul, 2014). Its concentration can amount to 400/1 200 mg per Kg of soil (Rodríguez et al., 1999). Plants need the mono- or dibasic form of phosphate so that they can absorb it (Jha et al., 2015). PGPR solubilize phosphate in a number of ways. One of these ways is by releasing organic acids into the soil. Such acids include malic, tartaric, citric, succinic, lactic, gluconic and 2-ketogluconic acid (Prijambada et al., 2009). These acids will chelate divalent cations of Ca^{2+}, which will aid in the release of the phosphate from its insoluble forms. Together, these mechanisms will render phosphate usable by plants (Bolan et al., 1994). PGPR can also release extracellular phosphatases which will convert the inorganic phosphate into organic forms (Gnanamanickam, 2006).

1.1.3 *Pinus* associated rhizobacteria

The rhizobiome is species specific (Agler et al., 2016). Furthermore, it varies according to the developmental stage of the plant, e.g. vegetative or flowering (Chaparro et al., 2014). Normally, plants tend to require higher quantities of nitrogen at later stages of development, which, in turn, will

make the plant favor nitrogen fixing rhizobacteria (Nazoa et al., 2003). Additionally, the plant will also control its rhizobiome by exudation of specific compounds (Badri et al., 2009).

Studies regarding the bacterial rhizobiome of *Pinus* spp. are still very lackluster. Due to methodological limitations, early studies of Pines bacterial rhizobiome provided only a very partial view of the diversity of these communities (Fierer, 2017). For example, in a study conducted in 2002 by Krave and coworkers, bacteria belonging to the *Burkholderia, Nocardioides, Bradyrhizobium, Microbacterium, Rhizobium, Sphingobium,* and *Pseudomonas* genera, were found in soil associated with pine trees. These authors reported a dominance of alpha-Proteobacteria in their samples.

Another study performed in 2001 in rhizospheric soils from *Pinus contorta*, reported Proteobacteria and Acidobacteria as the prevalent phyla, regardless of the sampling location (Chow et al. 2002). Actinobacteria and Verrucomicrobia were also prominent phyla in the analysed samples. Authors also indicated that families such as *Sphingomonadaceae*, *Acetobacteraceae Rhizobiaceae*, *Caulobactereaceae, Burkholderiaceae, Comamonadaceae, Pseudomonadaceae* and *Acidimicrobiaceae* had high levels of abundance (Chow et al. 2002).

In 2010, Lottman and her colleagues studied the bacterial and fungal rhizobiome of *Pinus radiata*. Authors used denaturing gradient gel electrophoresis analysis and Amplified rDNA (Ribosomal DNA) Restriction Analysis. Even though these methods have limitations when compared to next generation sequencing of phylogenetic markers (Rastogi et al., 2011), the results allowed to identify the following bacterial families (listed by decreasing abundance): *Burkholderiaceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Mycobacteriaceae, Alicyclobacillaceae.* In accordance with other studies, Proteobaceria and Acidobateria are predominant phyla in Pine rhizobiomes.

A detailed study concerning *Pinus roxburghii* and its rhizobiome was performed by Naz et al., 2018. Culture-independent methods were used, namely 454 pyrosequencing. A clear predominance of Acidobacteria, followed by Proteobacteria, Firmicutes and Actinobacteria was observed. Furthermore, the most predominant families by phylum are displayed in table 1.

Table 1: Predominant bacteria in the rhizobiome of a *Pinus* species (*P. roxburghii*), adapted from Naz et al., 2018.

Phylum (Class)	**Family**
Acidobacteria (27% of the reads)	*Solibacteraceae*
Proteobacteria (20% of the reads)	*Hyphomicrobiaceae, Rhizobiaceae, Phyllobacteriaceae*

(Alphaproteobacteria)	
	Bradyrhizobiaceae, Rhodobiaceae, Sphingomonadaceae
	Rhodospirillaceae, Acetobacteraceae, Rhodobacteraceae
	Caulobactereaceae
Proteobacteria	
(Betaproteobacteria)	*Comamonadaceae*
Proteobacteria	
(Gammaproteobacteria)	*Xanthomonadaceae, Sinobacteraceae*
Proteobacteria	
(Deltaproteobacteria)	*Pseudomonadaceae, Haliangiaceae, Entotheonellaceae*
Firmicutes	*Lactobacillaceae, Bacillaceae, Paenibacillaceae*
(19% of the reads)	*Planococcaceae, Halanaerobiaceae*
Actinobacteria	*Symbiobacteriaceae, Clostridiaceae, Actinosynnemataceae*
(11% of the reads)	*Nocardioidaceae, Streptomycetaceae, Micrococcaceae*
	Micromonosporaceae, Thermomonosporaceae, Iamiaceae
	Geodermatophilaceae, Nocardiaceae, Solirubrobacteraceae

When taking into account the whole rhizobiome of the plant, it is relevant to target potential PGPR, due to their relevance for plant development. Examples of potential PGPR genera in the rhizobiome of *Pinus* species will be mentioned, in order to elucidate their importance in the plant's rhizobiome.

In 2005, Barriuso and his co-workers screened the rhizobiome of *Pinus pinea* and *Pinus pinaster* for the presence of PGPR. They were able to find bacteria that synthesized auxins, siderophores, phosphate solubilizing enzymes and 1-Aminocyclopropane-1-Carboxylate (ACC) degradation enzymes. In total 147 bacterial isolates displayed at least one PGPR trait. These were included in genera *Bacillus, Burkholderia, Curtobacterium, Staphylococcus* and *Arthrobacter*. Of all these genera, *Bacillus* was the predominant one. Moreover, this study was followed by another trial conducted by Barriuso and his co-workers in 2008, where they verified that 8 isolates were able to successfully promote the growth of *P. pinea*, suggesting their possible use as biofertilizers.

There are a few studies that elucidate the role of other genera of PGPR in *Pinus* (Bent et al., 2001, de Vasconcellos et al., 2009), such as *Pseudomonas* spp. and *Streptomyces* spp..

All in all, the structure of the rhizobiome of *Pinus* trees is still poorly characterized, with only a few species having their rhizobiomes analysed. Nonetheless, these studies highlight the 3

most prevalent phyla in the rhizobiome of pine trees: Proteobacteria, Acidobacteria and Actinobacteria and highlight their prevalence in the rhizobiome of Pine trees.

1.2 *Pine pitch Canker (PPC): the case study*

The pine pitch canker (PPC) disease is caused by *F. circinatum* and is responsible for the destruction of many Pine trees worldwide (Wingfield et al., 2008). In order to better understand the PPC, it is necessary to understand the two principal intervenient in PPC, the main host (Pine species) and the phytopathogen (*F. circinatum*), whose role in PPC will be reviewed.

1.2.1. The main Host: *Pinus* spp.

Pine trees belong to phylum Coniferophyta, commonly known as conifers. These trees produce ovule that turn into seeds. Seeds are normally found lying in a scale, which is found in a structure denominated cone, hence the designation Coniferophyta. Furthermore, these plants are gymnosperms, as their seeds are not enveloped in an ovule. Most Coniferophyta are evergreen trees, with perennial leaves (Radford et al., 2010). This phylum is subdivided in 5 families: *Pinaceae, Podocarpaceae, Cupressaceae*, *Araucariaceae* and *Taxaceae* counting from the largest to the smallest group (Farjon, 2010). *Pinaceae*, which consists of 231 species, is mainly distributed in the northern hemisphere. This family includes 6 genera: *Abies, Cedrus, Larix, Picea, Pinus*, and *Pseudotsuga* (Farjon, 1990). Genus *Pinus* contains 113 species (September, 2019), which classifies it as the largest genus in conifers (Debreczy, 2011). In accordance with the family distribution, *Pinus* is fairly widespread throughout the northern hemisphere as can be seen in figure 3 (Farjon et al., 2013).

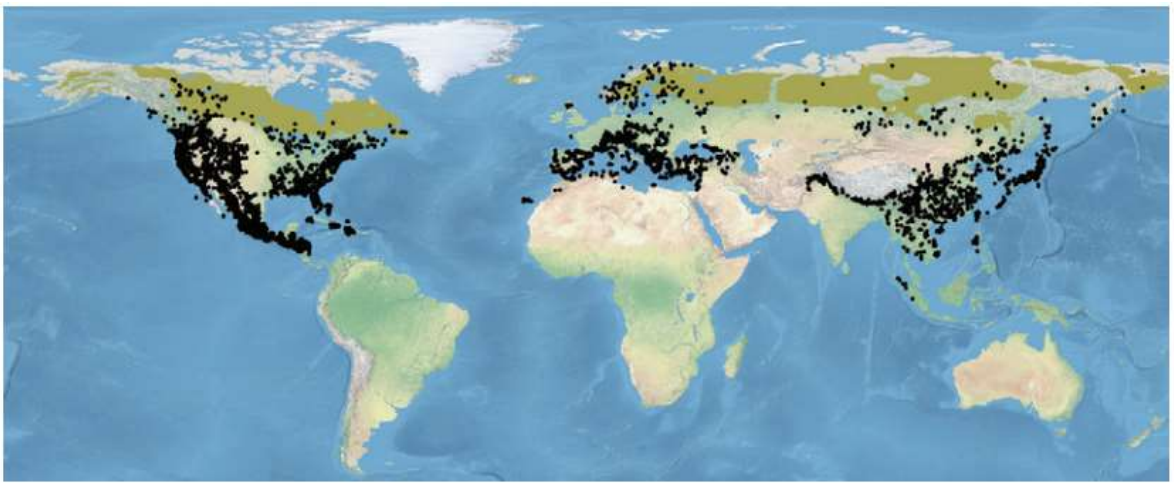

Figure 3: The worldwide distribution of the genus *Pinus* (in Farjon et al., 2003)

This genus most typical traits are the iconic perennial needle-like leaves, resin canals and seed cone, which has different shapes according to the species (Farjon et al., 1997). This genus is

very important to global timber production. Second only to *Eucalyptus, Pinus* represent the largest plantation area in the tropics (Lintunen et al., 2016). *Pinus* trees possess a light wood, with sturdy fibers, which makes them suitable to use for paper production (Ververis et al., 2004). Additionally, they are important for the production of resins, essential oils, medicinal compounds and decorative ornaments (Farjon et al., 2013).

In Portugal, 30% of its forest area is comprised of *Pinus* species. The most planted pine tree species include *P. pinaster* (22%) followed by *P. pinea* with 6% (ICNF, 2019). They represent an important part of Portugal's economy, about 3% of its domestic gross domestic product (ICNF, 2017). The principal commercially valuable products derived from pine trees are: timber, wood pulps, charcoal, pinions (pine seeds) and resins (Richardson, 2000).

Due to its very multipurpose wood, alongside its fast-growing capabilities, *P. radiata* is one the most important pine species in the world (Mead, 2013). It has very adaptable timber and is commonly used worldwide due its malleable characteristics (Kumar et al., 2004). It is also mainly grown in plantations, due to being a pine species that is very well-studied (Matheson et al., 1997; Gapare et al., 2006; Wu et al., 2006). The biggest plantations of *P. radiata* or Monterey Pine are in Australia, Chile, Ecuador, New Zealand, Italy, South Africa, Spain and also Uruguay, which represent about 4.2 million ha in plantations (Mead, 2013). Concerning Portugal, *P. radiata* is included in the group of "Another resinous trees", which represents 2% of the total forest area (ICNF, 2019).

The pine trees have different susceptibilities to phytopathogens (Bingham, 1971), including to Pine pitch canker (Amaral, et al., 2019; Martín-García et al., 2019). Some species such as: *Pinus tecunumanii, Pinus oocarpa, Pinus canariensis, Pinus pinea,* and *Pinus thunbergii* are very resistant to the pine pitch canker caused by *Fusarium circinatum,* while others such as *Pinus radiata, Pinus patula,* and *Pinus elliottii* are very susceptible to the same disease (reviewed in Martín-García et al., 2019)

According to The International Union for Conservation of Nature's Red List of Threatened Species (IUCN 2019), there are 12 species of *Pinus* species that at least endangered, on a global scale. This list includes *P. radiata,* which is known to be vulnerable to *F. circinatum.*

1.2.2. The phytopathogen: *Fusarium circinatum*

As of recent years, there have been increasing reports of pine diseases caused by *F. circinatum* (Quesada et al., 2019). This species of fungi is known to cause pitch canker in *Pinus* and is one of the most important pathogens of this plant genus (Nelson, 1981). The first report of this

disease was observed in the United States of America in 1946 (Hepting et al., 1946). In the following years, all throughout California, different types of *Pinus* species were affected, namely *Pinus radiata, P. muricata, P. pinea* and *P. halepensis* (Smith et al., 2007). Afterwards, in the late nineties, *F. circinatum* was reported in South Africa causing a root disease in *P. patula* (Viljoen et al., 1997). It has now spread throughout the continent and represents a major threat to the pine tree nurseries (Wingfield et al., 2008). Further along the timeline, in 2002, *F. circinatum* was reported in Chile in *P. radiata* (Wingfield et al., 2002). Regarding Europe, there have also been reports throughout the coastal border countries such as Portugal, Spain and Italy (Landeras et al., 2005; Carlucci et al., 2007; Bragança et al, 2009, respectively). The occurrences were reported in 2009, 2005 and 2007, respectively. The trees on which the fungus was detected were *P. pinaster*, *P. pinea*, *P. radiata* and *P. halapensis.* The fungus distribution can be seen in figure 4.

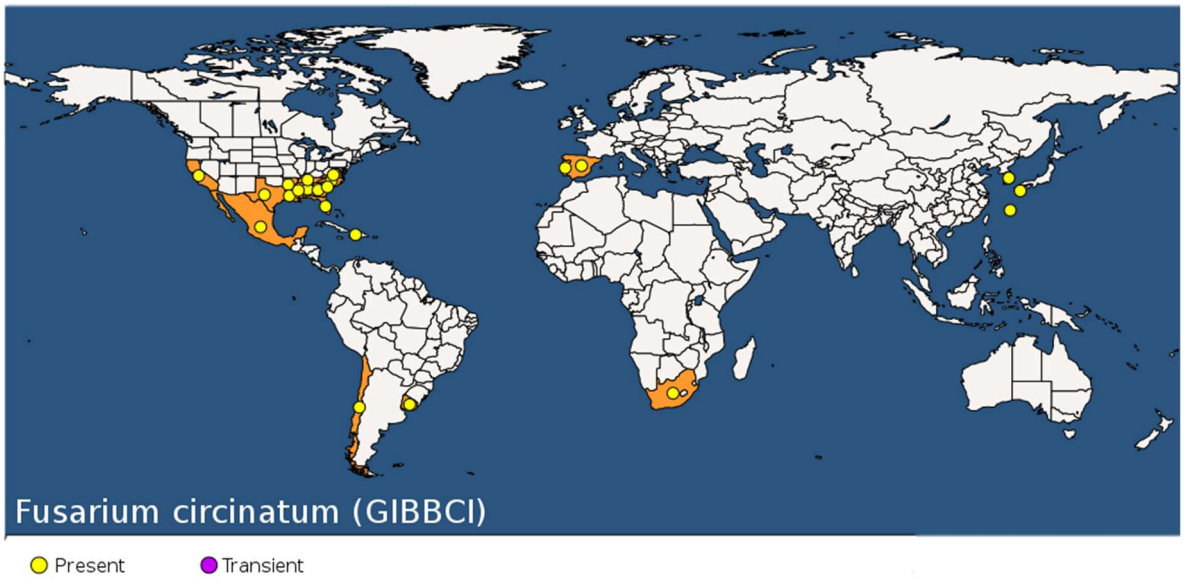

Figure 4: Global presence of *F. circinatum* (codename GIBBCI) (Yellow denotes presence; Purple denotes transience, when a pathogen is not established; There are no transient locations in the map); taken from https://gd.eppo.int (database last updated on 02/09/2019).

***Fusarium circinatum* - pathogenicity**

F. circinatum causes Pine pitch canker (PPC), which affects *Pinus* species and *Pseudotsuga menziesiiworldwide* (Wingfield et al., 2008; European Food Safety Authority [EFSA], 2010). Common symptoms in nurseries include damping of and wilting of the seed, branch die-back, stem cankers, pitch formation and mortality (Wingfield et al., 2008). There are more than 10 million ha potentially threatened by PPC in Europe (European Food Safety Authority [EFSA], 2010), where it is considered as a quarantine pathogen (Decision 2007/433/EC of 18 June 2007)

Fusarium circinatum is capable of infecting pine trees of all ages (Dwinell et al., 1985). Its spores can be spread through wind, soil movement, vegetal tissue movement, water splash and insect vectors (Martín-Rodrigues et al., 2013). Depending on the regions of the globe, the mechanisms of infection may vary (Dwinell et al., 1985). It can enter the plant through artificial wounds, caused by anthropogenic activities, as well as infecting the plant through the action of insect vectors that feed off the plant (Gordon et al., 2001). Nevertheless, the main method of dispersal of this fungus is by insect vectors, namely *Conophthorus radiatae* (Monterey pine cone beetles), *Pityophthorus spp.* (twig beetles) and *Ernobius punctulatus* (death-watch beetles) (Mcnee et al., 2002).

When the fungus is able to enter a tree, it generally multiplies near the infection site, where it produces spores which will induce the pitch canker in the host (Dwinell et al., 2001). Pitch canker is characterized by the obstruction of the water and resin flow. Hence, it is common to observe infected shoots bleeding resin, which is due to the girdling effect of this canker (Gordon, 2006). The canker functions as a "belt" that impedes any traversal trough its ring of infection. Additionally, infected trees also manifest symptoms such as wilting, discoloration of the leaves and branch dieback (James, 1987). In *P. radiata*, the impairment of the natural flow of water, constricts susceptible plant into closing their stomata to prevent water loss (reviewed by Amaral et al., 2019). In seedlings, a common symptom is damping off. The tissues infected range from roots to branches, seeds, cones and shoots (Wingfield et al., 2008). In *P. pinea*, which exhibits a greater resistance (Iturritxa et al., 2017), stomatal opening followed by an increase in transpiration was verified (Amaral et al., 2019)

***F. circinatum*, *Pinus* and its rhizobiome**

As of lately there are starting to appear more studies related to possible interactions between *F. circinatum,* rhizospheric bacteria (particularly PGPR) and *Pinus*. Due to the known beneficial effects of PGPR, some trials have been made to better understand the potential biocontrol of the fungus by PGPR. Regarding *Pinus radiata* and pitch canker, Iturritxa et al., in 2017, inoculated *Pseudomonas fluorescens*, *Erwinia billingiae*, and *Bacillus simplex* into the apical tip of the radiata pine. The plants were left to recover for 1 week, allowing proliferation of the bacteria. Then, by carving small wounds on the plants, the fungus was inoculated. Six weeks after this inoculation, the trees were sampled. The key aspect assessed in this study was the size of the lesion caused by fungus. It was observed that these PGPR strains were able to reduce the lesion length by 22%.

This study is very recent and is one of the few that looks into the possibility of manipulating the *Pinus* rhizobiome (in this case by adding PGPR) to mitigate the effects of *Fusarium infection*, or even to eliminate these effects. The promising results reported in this study suggest that it is urgent to explore this possibility by conducting further studies focusing on the interaction between plant, rhizobiome and fungus. First and foremost, it is urgent to characterize *Pinus* rhizobiome in structural and functional terms and to understand how this community responds or is affected by the development of the fungal infection.

1.2.3. Strategies used to control pine pitch canker

Many strategies are used throughout the world to combat *Fusarium*-caused infections, such as the pine pitch canker. Most of them opt for fungicides, such as azole-based compounds (reviewed in Martín-García et al., 2019) or biocontrol agents, such as other bacteria or fungi (e.g. *Trichoderma* spp., *Pseudomonas fluorescens*) (Larkin et al., 1998). Other methods of counteraction against this fungal infection comprise the usage of bioorganic fertilizer products, coupling microorganisms with antagonistic effects to a fertilizer, in order to maximize the antifungal effects of the compound (Shen et al., 2015). These biocontrol agents are often mixed with fertilizers rich in nitrogen, phosphate and potassium (Zhang et al., 2008). However, it was also proven that these biocontrol agents can be coupled with organic fertilizers (derived from manure) and still display antifungal effects (Ling et al., 2010).

Additionally, environmentally conscious methods can be applied to the treatment of the pine pitch canker (reviewed in Martín-García et al., 2019). These include immersion of pine seeds in hot water, which can reduce the number of infected seedlings without significant damage to the seeds (Bennett et al., 2010). Other less intrusive methods and crucial for forest sustainability include the selection of resistant species. The act of planting resistant species to eventually substitute plantations with susceptible pine trees is being considered by some countries (Porter et al., 2009; Amaral et al., 2019). However, one of the main limitations is the capacity of resistant species to adapt to the local climate (Martín-García et al., 2019). Recently, an emergence of potential PRIMING strategies such as chemical-only fertilizers is being observed, with phosphite representing an interesting compound for delaying the fungal progression (Thao et al., 2009, Cerqueira et al., 2017).

In Portugal, the ICNF (Instituto da Conservação da Natureza e das Florestas) has issued a plan of action for controlling the diffusion of this fungus (ICNF, 2016). Buffer zones were defined so

as to quarantine the pathogen inside a specific area. Currently, the only mode of action recommended by Portuguese authorities against *F. circinatum*, other than quarantining, is the burning of any plant material that clearly contains the fungus.

1.2.4. Priming

Throughout the recent years, a new concept of inducing resistance in plants has been proposed. It is referred as priming and consists on the increase of the basal defenses of the plant (Mauch-Mani et al., 2017). Priming occurs when there is an intensification in the activation of induced defense mechanisms. It can occur by biotic factors such as: pathogen stimuli, beneficial microorganisms stimuli and arthropods (Martinez-Medina et al., 2014;); On the other hand, priming can also be induced by abiotic factors, namely: small exposure to heat, cold, salt or specific chemical compounds (Singh et al., 2014). Essentially, the plant's exposure to these factors will trigger a higher defense response to stress (in the future) and will make the plant more resistant and stress tolerant (Martinez-Medina, 2016). Priming is potentiated by the fact that the plant memorizes the stress it was subjected to, which leaves the plant in an alert state (primed stated), where its defense responses have an above normal potential expression (Conrath et al., 2001). This is intertwined directly with the one of the key components of the priming response: the NPR1 gene (Non-expressor of pathogenesis resistance), which is a fundamental component of plant signaling during disease (Zhang et al., 2005).

A commonly used priming agent is the plant hormone, salicylic acid, which is capable of conferring resistance to specific abiotic stresses such as: chilling temperatures and salinity stress (Afzal et al., 2016). This and other commonly used hormones have regulatory effects on NPR1, which induce the primed state onto the plant (Conrath et al., 2002). Other commonly used compounds include: β-aminobutyric acid, probenazole, benzothiadiazole, jasmonic acid, azelaic acid and pipecolic acid (Oostendorp, 2001; Dempsey et al., 2012).

In pine trees, priming has been experimented on for years (Taylor et al., 1988¸Guo et al., 2012). The main objective of pine priming approaches was to increase the seed survival rate, as well as improving the seed germination (Wu et al., 2001). This priming mainly consisted on applying abiotic stress factors (e.g. short hot water bath, short exposure to heat or cold) to the seeds, which resulted in an increase in stress response elements, eventually leading to a better stress tolerance by the seed (Chen et al., 2013). Nonetheless, recently other compounds such as Phosphite have been suggested to prime plants in the *Pinus-F. circinatum* pathosystem (Cerqueira et al., 2017).

Phosphite priming

The usage of phosphate as a fertilizer in agriculture is very well documented. However, in recent years, the usage of the more interesting molecule of phosphite has started to grow (Achary et al., 2017). Phosphite (Phi) is a reduced form of Phosphate (Pi), where, one oxygen atom is replaced by an hydrogen one. In addition, there is a major difference between Phi and Pi. Pi is a macronutrient that can be absorbed by the plants, while Phi is a compound that does not provide any nutrition to the plant. Actually, in high dosages, Phi is commonly designated as a phytotoxic compound, while in low doses it can act as a fungicide (Thao et al., 2009). Several studies document the ability of phosphite to serve as a chemical control of phytopathogens.

On the long term, these fertilizers increase the biomass of each plant, as well as the internal concentration of phosphorous inside the plant (Turner et al., 2015). Turner and its coworkers (2015), in a trial that lasted for more than 30 years, noticed that fields where pine trees were fertilized with phosphate had better concentrations of other nutrients, such as nitrogen potassium. However, the usage of such fertilizers can increase the quantity of other toxic compounds on the soil, such as cadmium, lead and arsenic (Chen et al., 2008). These adverse effects led to the search for similar compounds with the same effect as phosphate. Since Phi is a similar composition to phosphate, it began being introduced in fertilizers in order to aid the resistance of plants against phytopathogens (Rickard, 2000).

Regular foliar application of phosphite has shown to be impactful towards the resistance against fungi. For example, a dosage of Phi diluted in water (10 g/L) resulted in a successful control of *Phytophthora cinnamomi* in *Eucalyptus marginata* (Tynan et al., 2001). However, it was also noticed that environmental abiotic factors also had an impact in the resistance conferred by Phi. Characteristics such as pH and water dispersed in the soil affected the results, often not granting the protection needed for the plants.

Regarding *Pinus radiata*, there have been a few studies (reviewed in Martín-García et al., 2019) that evaluated the effect that phosphite has on the interaction between the pine tree and respective phytopathogens. It has been suggested that phosphite is able to delay *F. circinatum* progression in a dose response manner, in vivo and in vitro (Cerqueira et al., 2017). This is due to fungicide capabilities of this compound, that have been already verified (Gentle et al., 1998; Percival et al., 2015). An important question that remains is regarding the impact of phosphite in the rhizobiome of these trees.

1.3 Aims of the study

As shown above, there is an increasing interest regarding the role of rhizobiome community in plant-pathogen interaction and further disease control. This community may play a relevant role during the infection caused by *Fusarium circinatum* in the plant. Depending on the composition of this community and its dynamics during infection, this role may be beneficial to the plant by promoting its resistance to the fungus (e.g. by the action of PGPR); On the other hand, this role may also be detrimental if the infection promotes the proliferation of opportunistic bacteria in the rhizobiome that may contribute to disease progression. As proven to combat other diseases (reviewed in del Carmen Orozco-Mosqueda et al., 2018), the manipulation of the rhizobiome may be an effective strategy to counter the effect of *F.* circinatum. However, manipulation strategies can only be designed if the rhizobiome of these trees is characterized in detail, exploring control and inoculated plants, and using model species that have a differential susceptibility to the pathogen. Nonetheless, studies regarding the rhizobiome of pine trees are lacking.

Therefore, the main aim of this study is to decipher the rhizobiome of pine species under their interaction with *F. circinatum*. To attain this aim we intend: 1) to elucidate the rhizobiome dynamics in a susceptible and in a resistant *Pinus* species comparing non-inoculated and inoculated plants (asymptomatic); 2) to evaluate if the susceptible species rhizobiome changes after the application of phosphite and if these changes depend on the application mode. Moreover, physiological markers including gas-exchange and antioxidant capacity were evaluated to assess plant performance under both *F. circinatum* inoculation and priming.

2. Materials and Methods

2.1 Plant material

Pine seedlings of 2 different species (*Pinus radiata, Pinus pinea*), with 15±2 cm height, were obtained from Sociedade Agrícola Pecuária Melo & Cancela Lda (Anadia, Portugal). Species were selected according to their susceptibility to *F. circinatum* infection: *P. pinea* is resistant and *P. radiata* is susceptible according to literature and to previous results obtained by our team (Amaral et al., 2019a; Iturrixa et al., 2013).

Seven months-old plants (23 *P. pinea* and 66 *P. radiata*), were kept under a greenhouse (Fitoclima D1200, Aralab, Portugal) at defined settings and were acclimatized for 60 days. The plants were subjected to a cycle of 16 hours of light and 8 hours of darkness (day/night cycle), with mean temperatures of 25ºC and 15ºC, respectively. Relative humidity was kept at 60%. The photosynthetic photon flux density (PPFD) was 500 µmol m^2 s^{-1}, during the day. The seedlings were watered daily and fertilized weekly (Frutifol, Nufarm, Portugal).

2.2 Fungal material

The *Fusarium circinatum* isolate (FcCa6) was obtained from the collection of the Forest Entomology and Pathology Lab at the University of Valladolid. The isolate was grown in Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany), at room temperature for 5 days. Afterwards, small square incisions were made on the border of the isolate (1-2 cm) and subsequently, immersed in Potato Dextrose Broth (PDB; Merck, Darmstadt, Germany), incubated at 20±2ºC for 1 day. In order to obtain a sample with $1x10^6$ spores, liquid medium with the fungus was filtrated through a 2µm gauze, removing most of the hyphae, and the concentration of spores was verified through counting on Neubauer-plates on a optical microscope.

2.3 Experimental design

Experiment 1: in this experiment, the impact of plant inoculation on the rhizobiome of two differently resistant species (*P. pinea* and *P. radiata*) was assessed. Two groups of plants were set up: 1) the control group (C), with an n of 8 individuals per species; and 2) the fungus-inoculated group (F), where the plants were inoculated with *F. circinatum* (with an n of 16 individuals, per species) (figure 5).

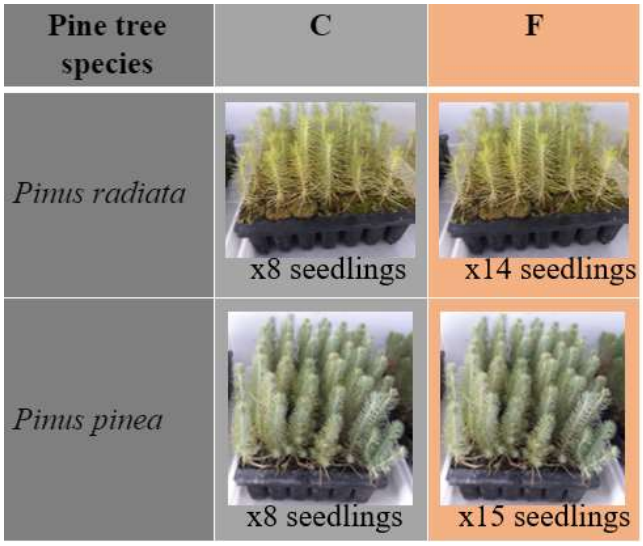

Figure 5: Schematic representation of the plant groups analyzed in Experiment 1, indicating the number of seedlings considered in each treatment. (C): Control group; (F): Inoculated with *F. circinatum.*

Experiment 2: in this experiment the effect of phosphite and of its application mode on the rhizobiome of *P. radiata* was analysed. Six treatments were applied: 1) the control group (C), with an n of 8 individuals; 2) the fungus-inoculated group (F), where the plants were inoculated with *F. circinatum*, with an n of 14/15 individuals; 3) the phosphite-treated group (PC(fol)) where phosphite was foliarly applied to the plants, with an *n* of 8 individuals)*;* 4) the group inoculated with the fungus and subjected to foliar phosphite treatment (PF(fol)), where both phosphite and fungus were applied to the plants at set intervals, with an *n* of 14 individuals; 5) the group to which phosphite was applied through irrigation (PCW), with an *n* of 8 individuals; and 6) the group inoculated with the fungus and to which phosphite was applied through irrigation at set intervals (PFW), with an *n* of 16 individuals (figure 6).

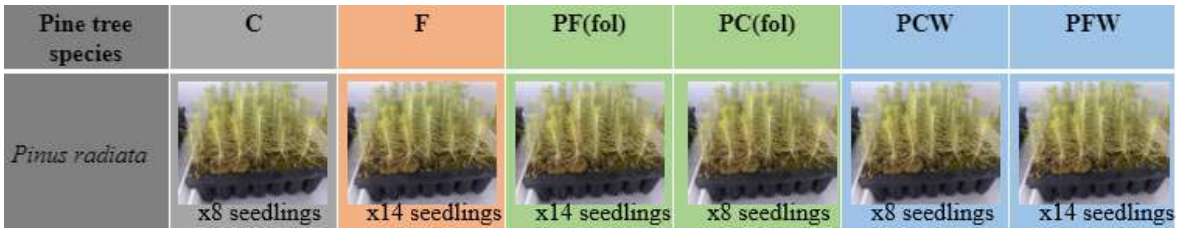

Figure 6: Schematic representation of treatments in Experiment 2, indicating the number of seedlings considered in each treatment. (C): Control group; (F): Inoculated with *F. circinatum*; (PF(fol)): Treated with foliar application of phosphite and inoculated with *F. circinatum*; (PC(fol)): Treated with foliar application of phosphite; (PCW): Treated with phosphite through irrigation; PFW): Treated with phosphite through irrigation and inoculated with *F. circinatum*.

The application of a commercial phosphite solution (Trafos Sinergy, Nutrisapec, Portugal) was performed on two intervals. Phosphite was diluted in water at 3% (v/v). The plants were

sprayed or irrigated with phosphite ($Phi_{3\%}$) 14 days and 7 days prior to fungal inoculation. A control group was kept with plants being sprayed or irrigated with water with the same pH as $Phi_{3\%}$ (5.8 pH). The phosphite application protocol was carried out according to Cerqueira et al., 2017.

Fungal inoculation and plants evaluation: For both the experiment 1 and experiment 2, fungal inoculation was performed by wounding the plants' stems using a sterile scalpel, followed by the inoculation of 10 µL of PDB with *F. circinatum* spores (with a concentration of $1x10^6$ spores/ml). The wounded site was sealed using Parafilm®. For control groups, the same procedure was followed but using 10 µL of PDB without the fungus.

Seedlings were kept under the same conditions described above for the acclimatization period. Plants were verified daily for symptoms of fungal infection, such as apical tip dieback and foliage discoloration. All symptoms were registered daily. The trial was conducted until 10 days after fungal inoculation.

2.4 Physiological parameters

Needle gas exchange results were evaluated to assess the plant performance under fungal inoculation, for *Pinus pinea* and *Pinus radiata*. These plant parameters were measured at the time of the sampling, before plants were processed. As stated above, only plants that did not show symptoms were selected. Net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1} DW^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1} DW^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1} DW^{-1}), and intercellular CO_2 concentration content (C_i, vpm) were measured with a gas-exchange system (LCpro-SD, ADC BioScientific Limited, Hertfordshire, UK) with a conifer type chamber. Inside the chamber, the following conditions were maintained during all the measurements: C_a (ambient CO_2 concentration): 404 µmol m^2 s^{-1} (in average); air flux: 201 µmol s^{-1}; block temperature: 21.5 °C. To find out the saturation light intensity A/PPFD (light response curves of CO_2 assimilation) curves were performed with the following photosynthetic photon flux density (PPFD): 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 µmol m^{-2} s^{-1}. Measurements at saturation light intensity were performed at 1000 µmol m^{-2} s^{-1}. Data was recorded when the measured parameters were stable (2–6 min).

2.5 Sample processing

Five non-symptomatic individuals of each treatment were selected for further processing. The plants' needles were immediately flash frozen with liquid nitrogen and kept at -80ºC for antioxidant capacity analysis. The plant was then divided in stem and root. The stem was cut into

small pieces and the Koch postulates were tested on the inoculated stems, by plating these stems in Potato Dextrose Agar (PDA) and subsequent *F. circinatum* growth verification. The remaining stem leftovers were flash frozen.

The roots were shaken vigorously, to keep only the soil adherent to the roots. Then, they were placed in falcon tubes filled with 35 mL of phosphate buffered saline (PBS). The tubes were agitated for 25 minutes, at 35 rpm. The root portion was separated from the soil (ectorhizosphere) and then washed several times, with the ectorhizospheric soil released being added to the rhizosphere tube. Between washes, this tube was centrifuged at 4ºC, during 10 min, at 4,000 g. Then, both tubes were flash frozen and kept at -80ºC. The rhizospheric soil present in these tubes was used for DNA extraction.

2.6 Antioxidant capacity analysis

Forty mg of needles previously frozen at -80ºC were grinded to a powder and added to a 1.5 mL solution of MetOH 70% (v/v). Additionally, the resulting solution was put in an orbital shaker at 700 rpm, at 25 ºC for 1 hour, followed by 5 sec in the vortex at maximum capacity and then were centrifuged at 10 000 g at 4 ºC for 15 min. The supernatant was collected and stored in a 13 mL tube. This process was repeated 3 additional times to obtain 6 mL of plant extract.

2.7.1 Total phenolic compounds

Total phenolic content was estimated by the Folin Ciocalteu's method (Singleton et al., 1999). To construct a calibration curve dilutions of the gallic acid standard were prepared in MetOH 70%, with the following concentrations: 1; 0.500; 0.250; 0.125; 0.063; 0.031; 0.016; 0.008; 0.004 (mg/mL).Then, 20 µL of gallic acid standards and samples were loaded in triplicate in each microplate well. Afterwards, 90 µL of dH_2O and 10 µL of Folin-Ciocalteau reagent solution were added to each well and the microplate was kept in the dark for 6 minutes. Subsequently, 80 µL of 7% Na_2CO_3 were added and the microplate was kept in the dark for 2 hours. After this period, absorbance was measured at 750 nm. The extracts were performed in triplicates. The blank was performed using Folin-Ciocalteau reagent diluted in MetOH 70%. The data for total phenolic contents of polyherbal formulation were expressed as mg of gallic acid equivalents / g of fresh weight.

2.7.2 Ortho-phenols content

Ortho-phenolic content was estimated by the Folin Ciocalteu's method (Singleton et al., 1999). The calibration curve was constructed with different dilutions of standard gallic acid in MetOH 70% with the following concentrations: 1; 0.500; 0.250; 0.125; 0.063; 0.031; 0.016; 0.008; 0.004 (mg/mL) were diluted in MetOH 70%. Then, 160 µL of gallic acid standards and samples were loaded in each microplate well. Afterwards, 40 µL of 5% sodium molybdate solution was added to each microplate well. The microplate was kept in the dark for 15 minutes. After this period, absorbance was measured at 370 nm. The extracts were performed in triplicates. The blank was performed using Folin-Ciocalteau reagent diluted in MetOH 70%. The data for total phenolic contents of polyherbal formulation were expressed as mg of gallic acid equivalents weight / g of fresh weight.

2.7.3 Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl)

DPPH was measured with the Trolox equivalent antioxidant capacity method (van den Berg, 1999). To build the calibration curve, different quantities of Trolox were diluted in MetOH 70%, with the following concentrations: 1; 0.500; 0.250; 0.125; 0.063; 0.031; 0.016; 0.008; 0.004 (mg/mL). Trolox aliquots and samples (22 µL) were loaded in a microplate in triplicate. Then, 200 µL of DPPH solution was added to each microplate well, which was kept in the dark for 30 minutes. After this period, absorbance was measured at 517 nm. The blank was performed using Trolox diluted in MetOH 70%. The data for total phenolic contents of polyherbal formulation were expressed as mg of Trolox equivalents / g of fresh weight.

2.7.4 Total flavonoid content

Total flavonoid content was measured with the aluminium chloride colorimetric assay. The calibration curve was built using standard catechin, diluted in MetOH 70%., with the following concentrations: 0.500; 0.250; 0.125; 0.063; 0.031; 0.016; 0.008; 0.004 (mg/mL). Then, 60 µL of plant extract was loaded in triplicate into a microplate. Then 28 µL of 5% $NaNO_2$ solution were added to each microplate well and kept in the dark for 6 minutes, followed by the addition of 28 µL of 10% $AlCl_3$ solution to each microplate well, being also kept in the dark for 6 minutes. Lastly, 120 µL of 4% NaOH solution was added to the samples and aliquots and the microplate was shaken for 20 s using the "option soft" of the microplate reader, and the absorbance was read at 510 nm. Aluminium chloride was diluted in MetOH 70% and served as the blank. The calibration curve was

plotted using standard catechin. The data for total phenolic contents of polyherbal formulation were expressed as mg of catechin equivalent / g of fresh weight.

2.7 PCR-DGGE

Samples resulting from the rhizospheric soil sample processing were weighted (0.25 gr). The respective DNA was purified using an extraction kit "Dneasy Powersoil Kit" (MoBio, Qiagen), as described by the manufacturer.

The 16S rRNA gene was amplified through PCR, using the following set of primers 27F (5'-AGAGTTTGATCCTGGCTAG-3') and 1492R (5' -GGTTACCTTGTTACGACTT-3') (Lane et al., 1992). For the PCR experiment, the reaction mixture contained: 6.25 µL of NZYTaq II 2x Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 1.25U DNA polymerase; NZYtech, Portugal), 0.75 µL of each aforementioned primer (0.3 µM), 1 µL of the template DNA (50-100 ng) and 16.25 µL of dH_2O. The temperature profile was performed as follows: an initial 3 min denaturation step at 94 ºC, followed by 30 cycles with a denaturing step of 60 sec at 92 ºC, an annealing step of 60 sec at 52 ºC and an extension step of 60 sec at 72 ºC, lastly, there was a final extension for 10 min at 72 ºC. The resulting amplicons were subsequently used as template in a Nested-PCR, with the intent to amplify the V3 region of the 16S rRNA gene. This amplification reaction was performed using the set of primers 338F with a GC clamp (5'-GACTCCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). For the PCR targeting the V3 region the mixture composition was: 12.5 µL of NZYTaq II 2x Green Master Mix 0.75 µL of each aforementioned primer (0,3 µM), 1 µL of the template DNA (50-100 ng) and 10 µL of dH_2O. The temperature model used was as follows: an initial 5 min denaturation step at 94 ºC, followed by 35 cycles with a denaturing step of 30 sec at 92 ºC, an annealing step of 30 sec at 55 ºC and an extension step of 30 sec at 72 ºC, lastly, there was a final extension for 30 min at 72 ºC.

The DGGE gel was prepared according to Muyzer et al., 1993, in which PCR products were loaded into an 8 % polyacrylamide (37.5:1, acrylamide/bisacrylamide) gel with a linear denaturing gradient from 35 % (low) to 60 % (high). The concentration for the 100% denaturing solution is 7 M Urea and 40 % formamide. Twenty µL of each PCR product plus 5 µL of DNA Gel Loading Dye (Thermofisher) were loaded in the gel. A DNA ladder (Henriques et al., 2004; Henriques et al., 2006) was loaded twice on each gel. The electrophoresis was carried out on a D-Code Universal Mutation Detection System (Bio-Rad), filled with 7 L of 1X TAE buffer (Sigma-Aldrich, Germany) at 60ºC. There were two steps to this electrophoresis: the first one lasted 15 min and was performed at 20 V,

followed by a second step that lasted 16h performed at 75 V. The resulting gel was stained in an Ethidium bromide solution (0.5 µg/mL) for 5 minutes and rinsed in distilled water for 20 minutes. The gel images were captured with a Molecular Imager FX system (Bio-Rad), with UV transillumination.

DGGE results were analysed using the software package GelCompar II (Applied Maths, Belgium). Dendrograms were constructed based on Pearson correlation between the densitometric curves and clustered according to the unweight pair group mean average (UPGMA) algorithm.

2.8 Massive parallel sequencing

Twenty-four samples were selected to be sequenced: 6 samples from control groups (*P. pinea* and *P. radiata)*, 6 samples inoculated with *F. circinatum* (*P. pinea* and *P. radiata)*. Regarding the impact of phosphite: 6 samples where phosphite was applied foliarly (3 samples where only phosphite was applied and 3 other where both fungus and phosphite were applied); 6 samples where phosphite was applied through irrigation (3 samples where only phosphite was applied and 3 other where both fungus and phosphite were applied).

Microbiome Profiling with Illumina MiSeq was performed by Eurofins Genomics (Ebersberg, Germany). Data processing and taxonomical classification consisted of removing all reads with errors, processing the remaining reads using minimum entropy decomposition (MED), which partitioned the marker gene dataset into OTUs (Operational Taxonomic Unit). Taxonomic data was assigned to each OTU by BLAST alignments of representative cluster sequences to the NCBI database. Only reference sequences with an 80 % sequence identity across at least 80% of the sequence were considered for reference purposes. Sequences were not assigned if they were considered as noise (which includes potential chimeric sequences and singletons). Normalization of the number of reads was achieved by taking into account the estimated number 16S gene copy number per phylogenetic (Angly et al., 2014).

2.9 *In silico* functional analysis

Data regarding the sample's OTU abundance table alongside the representative sequence file (fasta format) was provided to Piphillin (http://secondgenome.com/Piphillin) to obtain an inferred functional profile. For this, the OTUs were matched against the KEGG database (option: "KEGG Oct2018") using an identity cutoff of 97%. A list of KEGG orthologs (KOs) and their abundance was obtained for each sample.

The pathways selected for analysis were: Bacterial chemotaxis (ko02030), fatty acid metabolism (ko01212), carbon metabolism (ko00720), nitrogen metabolism (ko00910), amino acid metabolism (ko00330), sulfur metabolism (ko00920) and monoterpene biosynthesis (ko00902). A table consisting of the relative abundance of each pathway was built, using the Piphillin abundance table output.

2.10 Statistical analysis

Statistical analysis was conducted in R (R Foundation for Statistical Computing, R Core Team, Vienna, Austria), along with the "vegan" package, the software package PRIMER v6 (Primer-E Ltd., Plymouth, UK) and SPSS (version 25.0). All samples were tested for their normality (Shapiro-Wilk test), and, consequently, compared accordingly with parametric tests such as ANOVA or non-parametric tests such as the Kruskall-Wallis test.

Alpha diversity metrics were calculated based on OTUs abundance tables resulting from massive parallel sequencing analysis, transformed by log(x+1) in order to lessen the importance of samples with lower number of reads. These indexes were calculated from the means of each group. All groups were tested for their normality (Shapiro-Wilk test), and compared accordingly with ANOVA. Alpha diversity metrics analysed included Shannon-Wienner index (H), Pielou's evenness (J) and richness (S), which indicate the diversity, distribution of the diversity and species quantity, respectively. These indexes were calculated in PRIMER v6 software (Primer-E Ltd., Plymouth, UK).

Bray-Curtis dissimilarity (beta diversity) was also based on the transformation of sequencing data using the formula log(x+1). The results were analysed using a Permanova, as well as a Monte-Carlo test, using PRIMER v6 software (Primer-E Ltd., Plymouth, UK).

Rarefaction curves were constructed using R software package. The number of OTUs was plotted as a function of the number of reads (sampled size).

Heatmaps were constructed on Excel along with R software package. In each heatmap, the number of OTU counts was assigned to a bacterial family and a colour gradient was assigned to each value according to a scale of colours. Only the top 30 families assigned OTUs of each sample were taken into account. Results for each experimental (experiment 1 and 2) groups consisted of the mean values of the 3 samples resulting from sequencing. Bar charts were also derived from the same set of data and represented only samples with a value superior to 2% of the total number of reads.

Principal coordinate analysis (PCoA) was performed using PRIMER v6 software (Primer-E Ltd., Plymouth, UK) based on a Bray-Curtis dissimilarity matrix.

3. Results

3.1 Rhizobiome dynamics in response to inoculation with *F. circinatum*

Plant symptomatology

Two seedlings of *Pinus radiata* inoculated with *F. circinatum* displayed symptoms 7 days post inoculation, which corresponded to an 85% asymptomatic percentage rate for the F group (figure 7). These plants (n=2) exhibited wilting and apical damp-off. Koch postulates corroborated the *F. circinatum* infection, for the symptomatic plants in the inoculated group in *P. radiata*. The control group and *P. pinea* plants (both control and inoculated) displayed no symptoms of fungal inoculation, therefore are not presented in the graph. Ten days after inoculation, asymptomatic seedlings of the F group (*P. radiata*) lowered to 30%, while *P. pinea* inoculated group displayed no symptoms (fig. 7).

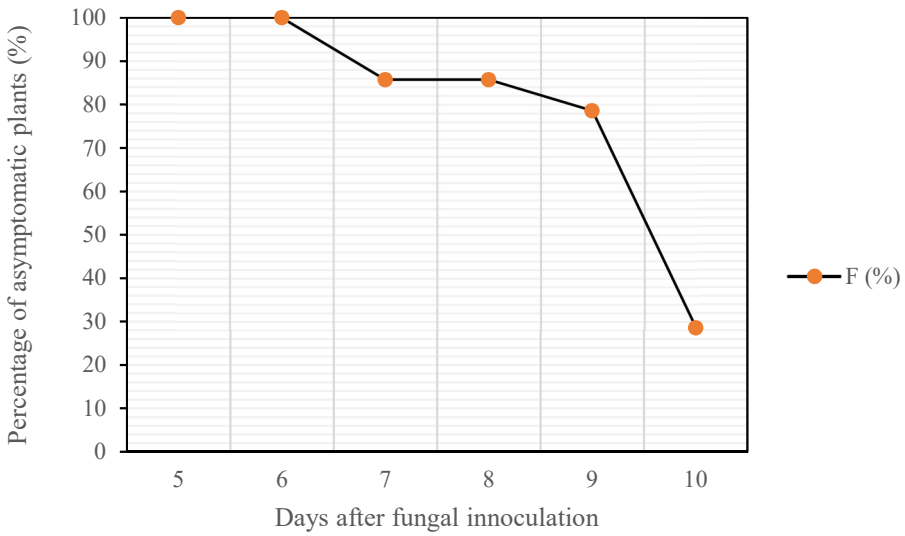

Figure 7: Percentage of asymptomatic seedlings in *P. radiata.* F: *P. radiata* plants inoculated with *F. circinatum*.

Needle gas exchange

The results of the gas exchange parameters presented in figure 8, showed a clear distinction among the inoculated plants of *P. radiata* and its respective control in all four parameters analyzed, showing lower rates of transpiration, stomatal conductance, no CO_2 assimilation rate but high levels of internal CO_2 concentration in inoculated plants (fig.8). On the other hand, *P. pinea* seedlings displayed no changes in needle gas exchange related parameters, comparing control groups to inoculated ones (fig. 8).

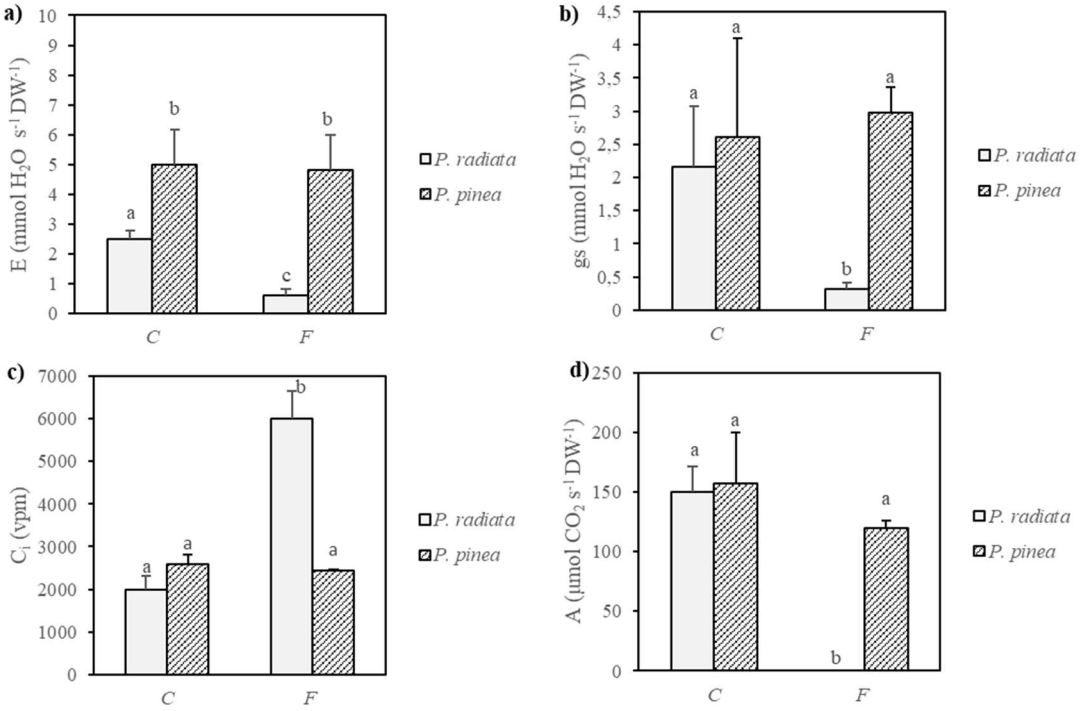

Figure 8: Needle gas exchange parameters: (a) transpiration rate (E), (b) stomatal conductance (gs), (c) intercellular CO_2 concentration (C_i) and (d) net CO_2 assimilation rate of *P. radiata* and *P. pinea* plants after *F. circinatum* inoculation. Data is presented as mean ± SD. Statistically similar data is grouped by similar lower-case letters.

Antioxidant Capacity

Needles resulting from the sampling process were grinded and subjected to different antioxidant assays, in order to determine the: Phenol (gallic acid equivalents), Ortho-phenols (gallic acid equivalents), 2,2-diphenyl-1-picrylhydrazyl DPPH (Trolox equivalents), Flavonoids (Catechin equivalents). Results are shown in figure 9. No significant difference in the levels of phenols, ortho-phenols, DPPH and flavonoids between non-inoculated and inoculated plants was registered, for both species (fig. 9). However, looking at control plants of both species, *P. pinea* samples displayed a significantly higher basal level of phenols, ortho-phenols and flavonoids compared to *P. radiata* while DPPH levels remained similar.

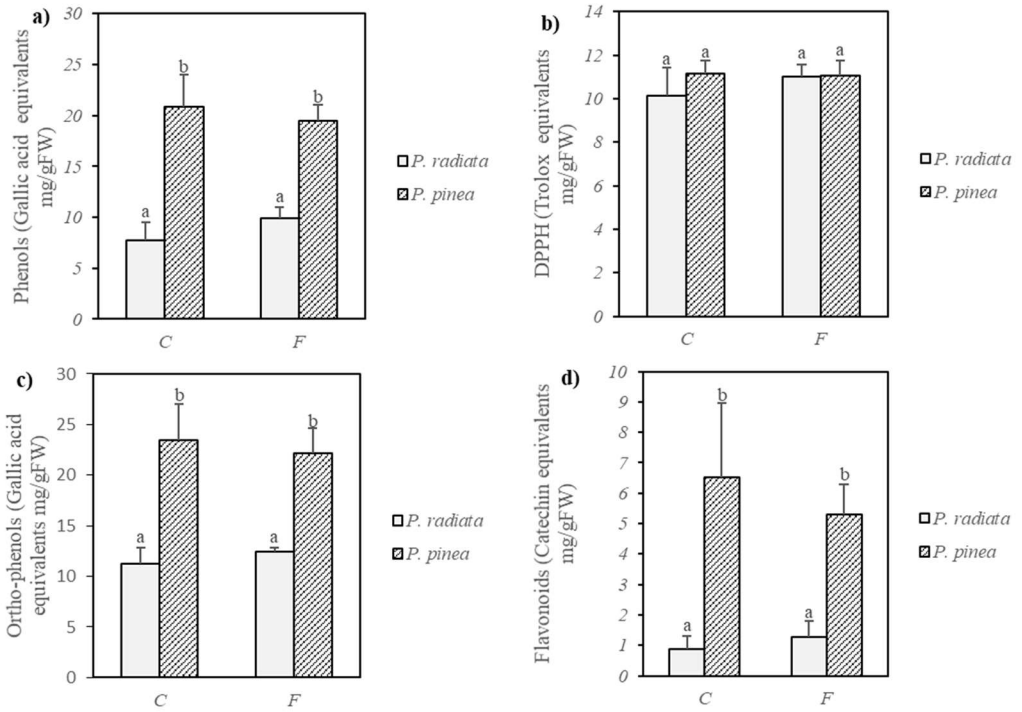

Figure 9: Antioxidant capacity plots: Phenol content (a), DPPH content (b), Ortho-phenols content (c), Flavonoids content (d) of *P. radiata* and *P. pinea* plants after *F. circinatum* inoculation. Data is presented as mean +- SD**.** Statistically similar data is grouped by similar lower-case letters.

3.1.1 Rhizobiome structure

A PCR-DGGE analysis was used to investigate the dynamics of the rhizobiome after fungal inoculation. Complex DGGE profiles were obtained for each sample analysed. A clustering analysis revealed a compositional shift after fungal inoculation for both species, with control samples sharing less than 60% similarity with samples from fungal-inoculated plants for *P. radiata* (figure 10).

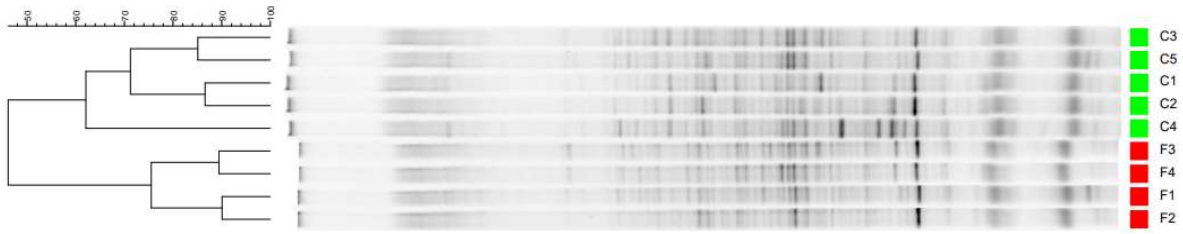

Figure 10: Dendrogram resulting from DGGE gel analyses, with C1 to C5 representing control group replicates and F1 to F4 representing *F. circinatum* inoculated plants of *P. radiata*. Dendrogram constructed based on the UPGMA clustering method, alongside a Pearson momentum correlation for the similarity clustering. Values presented in the scale represent % similarity.

A similar result was obtained for *P. pinea*, though the similarity between control communities and communities from inoculated plants was slightly higher (62%; figure 11).

Figure 11: Dendrogram resulting from DGGE gel, with C1 to C5 representing control group replicates and F1 to F5 representing *F. circinatum* inoculated plants of *P. pinea*. Dendrogram constructed based on the UPGMA clustering method, alongside a Pearson momentum correlation for the similarity clustering. Values presented in the scale represent % similarity.

Twelve samples were selected for massive parallel sequencing analysis: 6 samples from control groups (3 per species*)* and 6 samples inoculated with *F. circinatum* (3 per species*)*. Samples were selected in order to represent the different groups identified by clustering analysis of the DGGE profiles.

In total, 3 728 214 raw reads were obtained. After filtering the reads, removing chimeras and singletons, a total of 2 187 902 reads were assigned to OTUs. A copy-number correction was applied by dividing the reads by the known or assumed copy-number of 16S rRNA genes for each phylogenetic group. After this process, reads were assigned to 912 OTUs by BLAST alignments of representative cluster sequences to the NCBI database. On average, 42772±5753 reads were obtained per sample, which were assigned to 266 OTUs (234±18 per sample), as in table 2.

Table 2: Number of processed reads and OTUs per sample. C1 to C3 represent replicates of control groups; F1 to F3 represent replicates of the inoculated groups; Plant species is indicated before the replicate number.

Sample	Number of processed reads	Number of OTUs
P. radiata (F1)	37136	240
P. radiata (F2)	36169	243
P. radiata (F3)	37079	253
P. radiata (C1)	36747	266
P. radiata (C2)	40491	248
P. radiata (C3)	41423	245
P. pinea (F1)	41723	212
P. pinea (F2)	54638	208
P. pinea (F3)	47418	228
P. pinea (C1)	46204	234
P. pinea (C2)	47056	208
P. pinea (C3)	47185	228
TOTAL	513269	-

The rarefaction curve for each sample reached a point of full saturation (fig. 12).

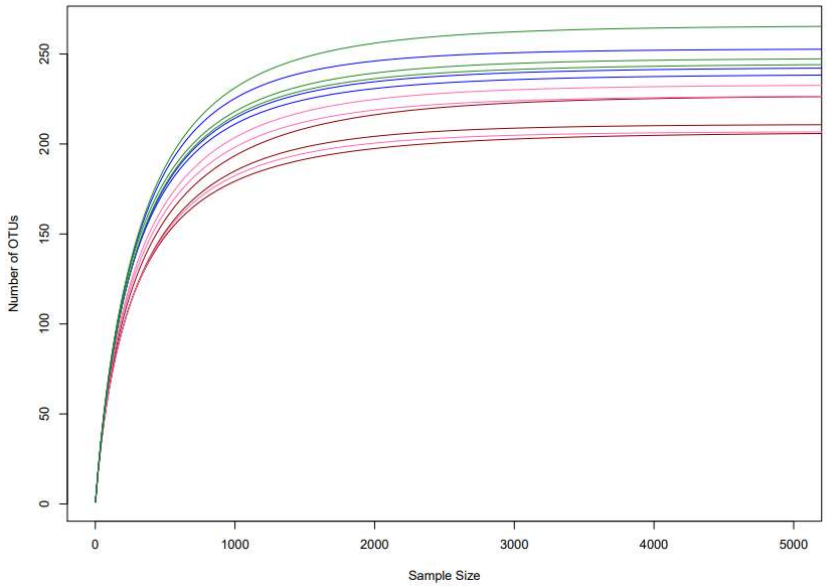

Figure 12: Rarefaction curves of the number of OTUs in function of the number of reads (maximum x value was shortened to 5000 to better view the graph). Replicates of *P. pinea* are represented in dark red (control) and hot pink (fungal inoculated); Replicates of *P. radiata* are represented in forest green (control) and blue (fungal inoculated)

Alpha diversity metrics (diversity, richness and evenness) were calculated based on the massive parallel sequencing results. Concerning these indexes, there were no significant differences between control and inoculated plants from the same species. On the other hand, the rhizobiome of *P. radiata* (control group) displayed a non significant higher richness and diversity than the rhizobiome of *P. pinea*, (Table 2).

Table 3: Alpha-diversity metrics (H, S and J) calculated for *P. pinea* and *P. radiata*. *(F): Fusarium* inoculated plants*; (C): Control*.

		Alpha-diversity metrics	
Treatments	**Richness S**	**Shannon-Wiener H**	**Pielou J**
P. pinea (F)	216±10.58	5.36±0.048	0.997±0.0002
P. pinea (C)	223±3.61	5.39±0.062	0.997±0.0001)
P. radiata (F)	245±6.81	5.49±0.029	0.998±0.0003
P. radiata (C)	253±11.36	5.52±0.044	0.997±0.0000

To calculate beta-diversity, the transformed [log(x+1)] abundance data was converted in a similarity matrix by using the Bray-Curtis dissimilarity measure. PCoA analysis revealed a distinct rhizobiome structure in the two pine species. *P. pinea* and *P. radiata* samples (control and inoculated) clustered with 60% similarity. *P. pinea* samples displayed less variance when compared to *P. radiata.* The two main axis in the PCoA plot explained 72.9% of the data variation (fig. 13). Species are clearly separated along PCO1, which explains 66.7% of the variation.

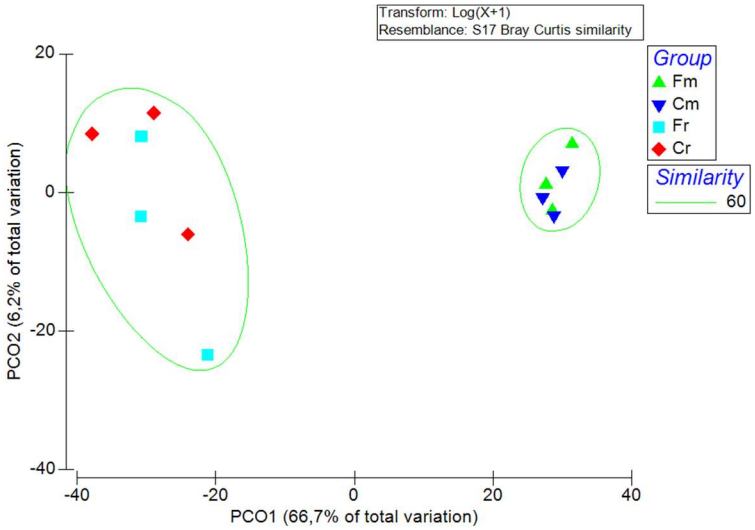

Figure 13: PCoA of the rhizobiome structure of the control groups of *P. radiata* (Cr) and *P. pinea* (Cm) and inoculated groups of *P. radiata* (Fr) and *P. pinea* (Fm). Samples were clustered according to their similarity.

A PERMANOVA, followed by a Monte-Carlo analysis showed a significant difference between rhizobiomes of both species, while fungal inoculation did not result in a significant impact on the rhizobiome structure of *P. pinea* nor *P. radiata.*

Table 4: Pair-wise permanova (perm) and Monte Carlo (MC) test results regarding beta diversity. Significant p-values are indicated with a * symbol; (F): *Fusarium*-inoculated plants; (C): Control plants.

Groups	**T**	**Unique P(perm)**	**perms**	**P(MC)**
F(radiata), C(radiata)	0.95198	0.634	10	0.499
C(pinea), C(radiata)	3.5541	0.108	10	0.005*
F(pinea), C(pinea)	1.307	0.107	10	0.188

Family diversity

Bar charts were used to represent the composition of the rhizobiomes in terms of bacterial families (Figure 14). Relative abundances lower than 2% were not considered. In *P. pinea* rhizobiome (of both control and inoculated plants), the most abundant families were *Acidobacteriaceae* (28±2.7% of the total number of reads for these samples), *Acidimicrobiaceae* (11±1.5%), *Acetobacteraceae* (7±0.9%) and *Conexibacteraceae* (5±0.7%). In *P. radiata* rhizobiome (of both control and inoculated plants), the community was dominated by *Acidobacteriaceae* (18±1.8%), *Micropepsaceae* (9±1.3%), *Acidimicrobiaceae* (7±1.4%), *Caulobacteraceae* (4±0.9%) and *Chitinophagaceae* (4±0.7%).

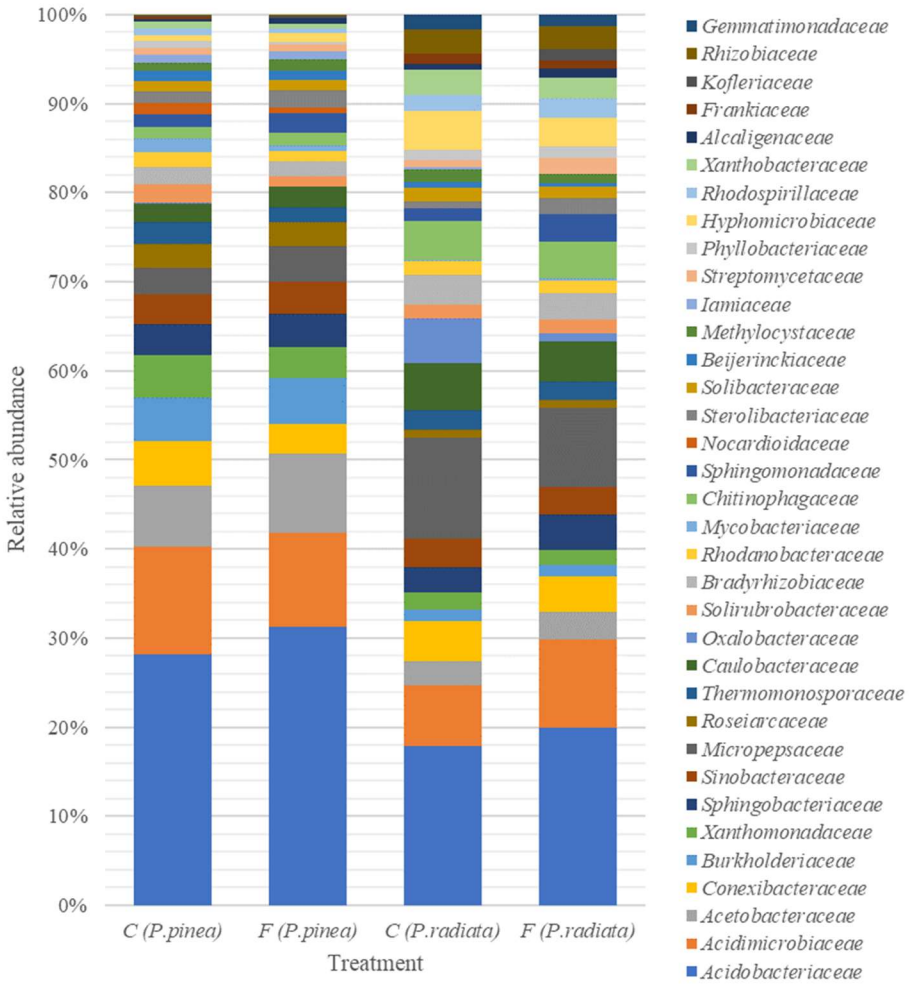

Figure 14: Relative abundance of each family per plant group. Bar chart only accounted for families with more 2% of total abundance of reads. (C): Control; (F): *Fusarium* inoculated plants**.**

Heatmaps were created based on the number of normalized read counts per sample, and representing the top 30 families of each group (control and inoculated plants, for both *P. radiata* and *P. pinea*). Results are shown in figure 15.

Significant differences in relative abundance of specific families between species and between control and inoculated plants' rhizobiomes (for both species) were observed. In the inoculated *P. radiata* plants' rhizobiome the family *Kofleriaceae* was detected being absent from the control plants. Differences between the rhizobiome of both species were also evident from this analysis. In fact, some bacterial families were highly abundant in *P. radiata* samples, being rare or absent in the *P. pinea* rhizobiome, e.g., *Micropepsaceae*, *Xanthobacteraceae* and *Rhizobiaceae*. On the other hand, some bacterial families were highly abundant in *P. pinea* samples, being rare or absent in *P. radiata*, e.g. *Nocardioidaceae*, *Burkholderiaceae*, *Roseiarcaceae,*

Gemmatimonadaceae, Xanthomonadaceae, Mycobacteriaceae. *Acidobacteriaceae* exhibited significantly higher values in *P. pinea* compared with the *P. radiata* rhizobiome.

	C *(P.pinea)*	F *(P.pinea)*	C (*P.radiata*)	F (*P.radiata*)
Acidobacteriaceae	**12662±(1486) [a]**	**13714±(1066)[a]**	**5834±(168)[b]**	**6507±(1166)[b]**
Acidimicrobiaceae	5435±(839) [a]	4588±(592)[a]	2202±(374)[a]	3254±(625)[a]
Acetobacteraceae	3068±(377) [a]	3897±(516)[a]	897±(251)[b]	991±(294)[b]
Conexibacteraceae	2220±(209) [a]	1452±(409)[a]	1482±(586)[a]	1315±(288)[a]
Burkholderiaceae	**2203±(263) [a]**	**2282±(783)[[a]**	**386±(167)[b]**	**448±(205)[b]**
Xanthomonadaceae	**2156±(225) [a]**	**1499±(563)[a],[b]**	**621±(303)[b]**	**520±(157)[b]**
Sphingobacteriaceae	1561±(118) [a]	1651±(261)[a]	947±(145)[a]	1321±(229)[a]
Sinobacteraceae	1476±(327) [a]	1600±(263)[a]	1029±(232)[a]	980±(46)[a]
Micropepsaceae	**1349±(272) [a]**	**1731±(275)[a]**	**3715±(808)[b]**	**2915±(145)[b]**
Roseiarcaceae	**1170±(217) [a]**	**1206±(105)[a]**	**287±(47)[b]**	**285±(30)[b]**
Thermomonosporaceae	1105±(168) [a]	729±(206)[a]	701±(163)[a]	698±(249)[a]
Caulobacteraceae	922±(120) [a]	990±(360)[a]	1730±(508)[a]	1467±(170)[a]
Oxalobacteraceae	82±(57) [a]	0±(123)[a]	1629±(1214)[a]	277±(211)[a]
Solirubrobacteraceae	903±(83) [a]	506±(499)[a]	495±(248)[a]	527±(233)[a]
Bradyrhizobiaceae	879±(195) [a]	731±(256)[a]	1074±(231)[a]	958±(203)[a]
Rhodanobacteraceae	784±(68) [a]	529±(429)[a]	526±(289)[a]	457±(109)[a]
Mycobacteriaceae	**689±(102) [a]**	**294±(59)[a]**	**52±(74)[b]**	**81±(74)[b]**
Chitinophagaceae	567±(277) [a]	596±(95)[a]	1420±(377)[a]	1340±(585)[a]
Sphingomonadaceae	623±(223) [a]	937±(263)[a]	468±(38)[a]	1021±(811)[a]
Nocardioidaceae	**570(38)±[a]**	**331(28)±[a]**	**0±(0)[b]**	**0±(0)[b]**
Sterolibacteriaceae	569±(570) [a]	807±(563)[a]	220±(91)[a]	573±(158)[a]
Solibacteraceae	525±(145) [a]	509±(164)[a]	515±(95)[a]	426±(155)[a]
Beijerinckiaceae	520±(14) [a]	479±(116)[a]	226±(118)[a]	161±(190)[a]
Methylocystaceae	427±(66) [a]	572±(62)[a]	456±(92)[a]	308±(79)[a]
Iamiaceae	392±(192) [a]	360±(117)[a]	58±(28)[a]	20±(42)[a]
Streptomycetaceae	343±(43) [a]	328±(214)[a]	274±(236=[a]	592±(315)[a]
Phyllobacteriaceae	340±(41) [a]	150±(167)[a]	366±(94)[a]	405±(277)[a]
Hyphomicrobiaceae	330±(143) [a]	407±(45)[a]	1415±(200)[b]	1068±(299)[a],[b]
Rhodospirillaceae	327±(38) [a]	227±(95)[a]	588±(92)[a]	688±(133)[a]
Xanthobacteraceae	317±(24) [a]	240±(88)[a]	950±(161)[a]	788±(198)[a]
Alcaligenaceae	161±(99) [a]	281±(227)[a]	193±(116)[a]	325±(136)[a]
Frankiaceae	117±(18) [a]	13±(100)[a]	382±(541)[a]	293±(229)[a]
Kofleriaceae	**43±(91) [a]**	**12±(31) [a]**	**0±(0)[b]**	**436±(122)[a]**
Rhizobiaceae	**51±(37) [a]**	**50±(105)[a]**	**876±(82)[b]**	**838±(72)[b]**
Gemmatimonadaceae	**0±(0) [a]**	**0±(0)[a]**	**550±(482)[a]**	**408±(226)[a]**

Figure 15: Heatmap of the number of reads per family in control groups and plants inoculated with *F. circinatum* of the species *P. radiata* and *P. pinea*. (C): Control; (F): *Fusarium* inoculated plants. Statistically similar groups are paired with the same lower-case letter. Standard deviation is presented in parenthesis. Bold indicates significant differences.

Functional diversity

Functional metagenomic inference of the rhizobiome was achieved using Piphillin, based on massive parallel sequencing results. In total, 135 genomes were predicted for this experiment. A total of 208 OTUs had more than 97% similarity with the corresponding region of the 16S rRNA genes in database genomes. A total of 6341 KOs were predicted for both rhizobiomes of *P. pinea* and *P. radiata*, followed by an inference of 321 family of genes. The selection of the displayed families of genes in figure 14, was based on relevant functions pertaining the rhizobiome and the plant. Other gene families were analyzed, as well as its specific KEGG orthologs. However, only statistically relevant data was presented, such as the monoterpene synthesis, displayed in fig. 16.

For bacterial chemotaxis, there were no differences verified in the respective rhizobiome of inoculated and control groups, in both species. However, there was a slightly higher number of genes related with bacterial chemotaxis in *P. radiata* than in *P. pinea*.. For the remaining gene families represented in figure 17, no differences were observed between species and/or experimental groups

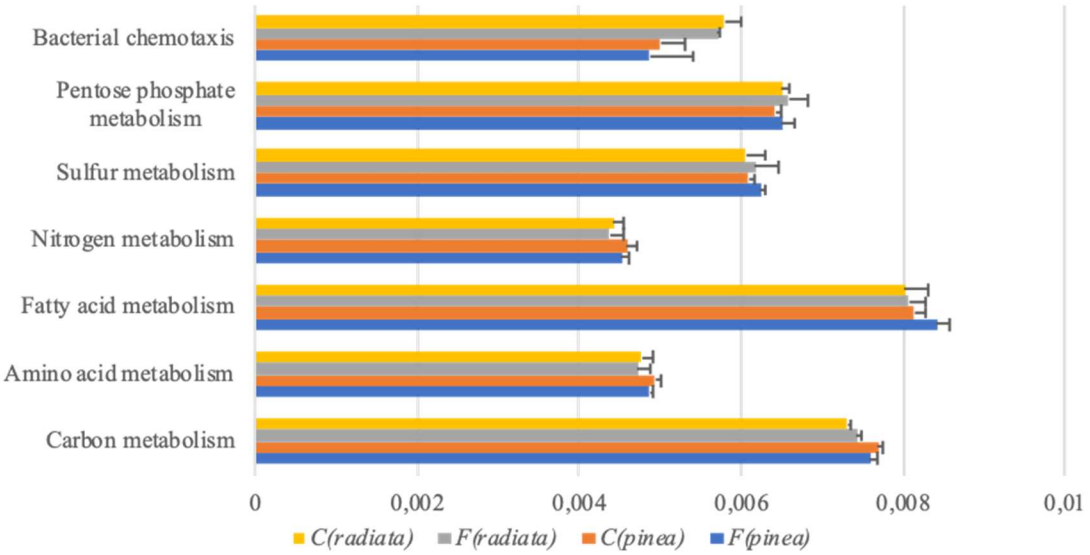

Figure 16: Meta-analysis of the inferred potential functions of the microbiome. (C): Control; (F): *Fusarium* inoculated plants

The analysis of monoterpene synthesis estimated a significantly higher abundance of this family of genes in *P. pinea* control samples when compared to *P. radiata* control samples (figure 17).

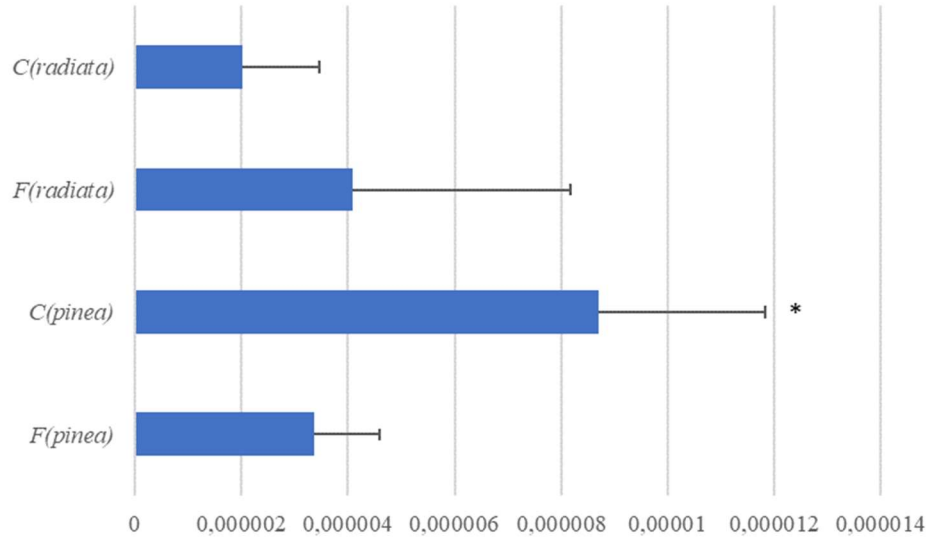

Figure 17: Relative abundance of the monoterpene synthesis functions of the microbiome. (C): Control; (F): *Fusarium* inoculated plants. Significantly different groups are marked with an asterisk.

3.2 Effect of phosphite and application mode on the *Pinus* rhizobiome

Plant symptomatology

A graph displaying plant survivability (number of symptomatic plants / total number of plants in the respective group) as a function of days after inoculation is presented in Figure 18. The seedlings of *Pinus radiata* inoculated with *F. circinatum* were the first to display symptoms (apical damp-off; Group F), within 7 days of fungal inoculations, with a percentage of asymptomatic plants of 85%, followed by seedlings inoculated with the fungus and treated with phosphite through irrigation (group PFW) with 93% of asymptomatic plants. At the end of the experiment, 10 days, the percentage of asymptomatic seedlings in the F group was 30%, while seedlings belonging to PFW had 70% and the seedling inoculated with the fungus and treated with phosphite through foliar application [PF(fol)] displayed 50% of percentage of asymptomatic plants. A phytotoxic effect of Phi was suggested by foliar discoloration of treated plants.

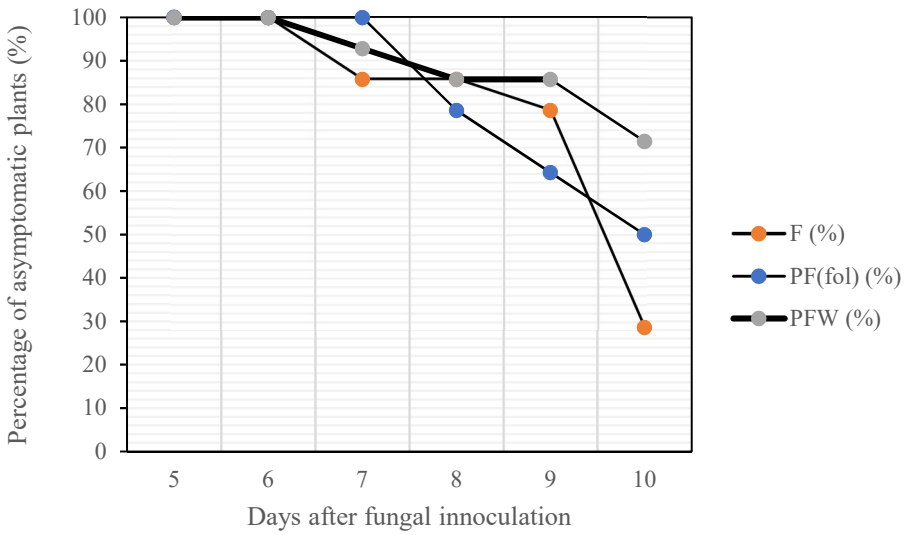

Figure 18: Percentage of asymptomatic seedlings for *P. radiata* plants throughout the experiment. F: *F. circinatum* inoculated plants; PF(fol): *F. circinatum* inoculated plants with Phi foliar application; PFW: *F. circinatum* inoculated plants watered with Phi.

Needle gas exchange

The graphs displaying the needle gas exchange parameters (figure 19) showed significantly lower values on the transpiration parameter for the F and PFW groups, when compared to other groups. On stomatal conductance, the inoculated plant group (F) exhibited lower values than the rest of the samples being significantly lower than control samples, while PF(fol) and PFW displayed intermediary levels of gs. Referring to internal CO_2, the F group displayed significantly higher values, followed by PC(fol) and PFW and lastly the rest of the samples. Concerning the

photosynthetic rate, the F group showed no net CO_2 assimilation rate/photosynthetic rate, followed by PFW, PF(fol) and the rest of the samples.

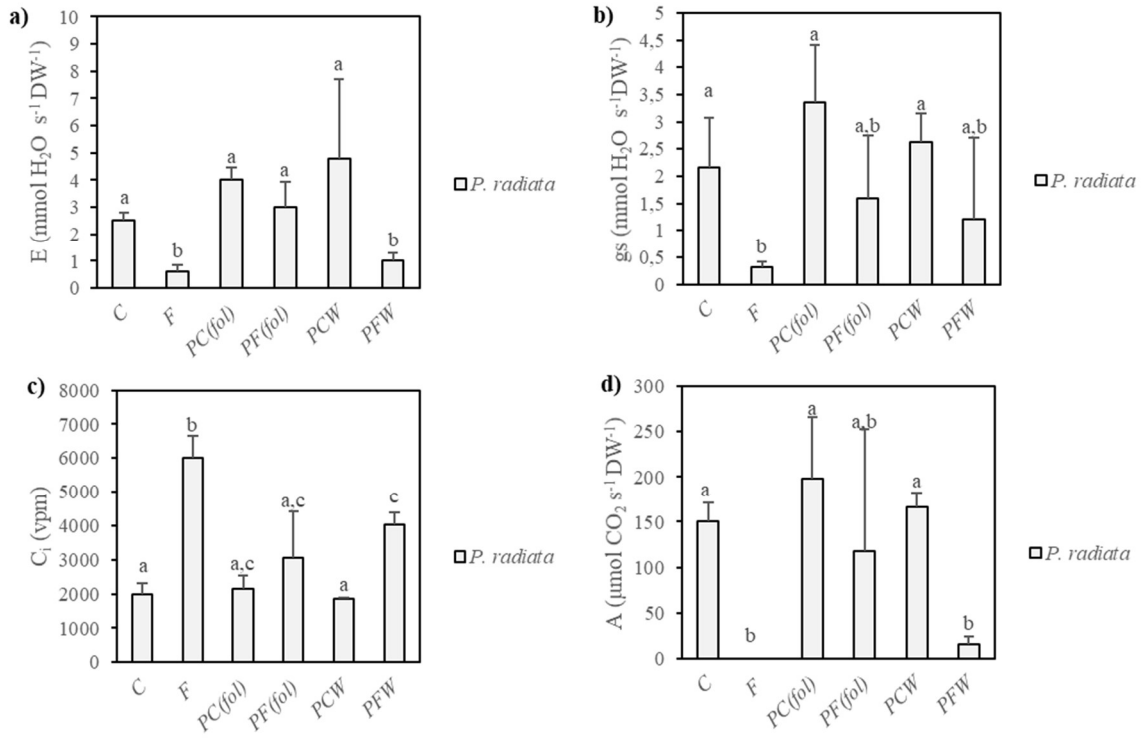

Figure 19: Needle gas exchange parameters: (a) transpiration rate (E), (b) content stomatal conductance (gs), (c) intercellular CO_2 concentration (Ci) and (d) net CO_2 assimilation rate (A) of *P. radiata* (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi

Antioxidant Capacity

In all parameters evaluated, the graphs (fig 20) show no significant differences in terms of phenolic compounds content for the plants' groups evaluated.

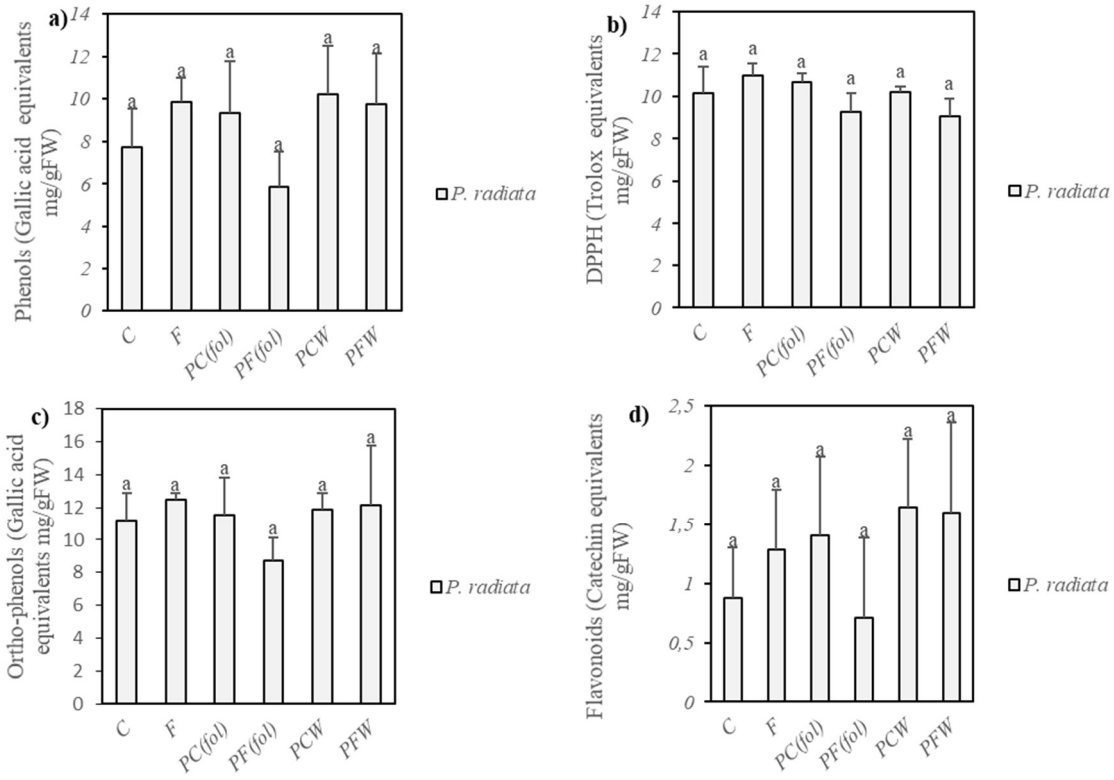

Figure 20: Antioxidant capacity plots: Phenol content (a), DPPH content (b), Ortho-phenols content (c), Flavonoids content (d) of *P. radiata*. Data is presented as mean ± SD. Statistically similar data is grouped by similar lower case letters. (C): control group; (F): Plants inoculated with *F. circinatum* group**; (**PFW): Plants inoculated with *F. circinatum* and treated with Phi through irrigation; (PCW): Non-inoculated plants treated with Phi through irrigation; PF(fol): Plants inoculated with *F. circinatum* and treated with Phi through foliar application; PC(fol): Non-inoculated plants treated with Phi through foliar application.

3.2.1 Rhizobiome Structure

A PCR-DGGE analysis of the 16S rRNA gene fragments was used to investigate changes in the structure of the rhizobiome under the different modes of phosphite application. Concerning the foliar application of Phi, clustering analysis revealed structural distinct rhizobiomes in plants treated with Phi and/or inoculated with the fungus, when compared to control plants (Figure 21). In fact the rhizobiome of control plants (group C; with only one replicate exception) shared less than 50% similarity with other plants' rhizobiomes On the other hand, a separation between samples from *Fusarium*-inoculated plants was not observed, regardless of phosphite application. Those samples were also similar in terms of their DGGE profiles to non-inoculated plants treated with Phi [group PC(fol)].

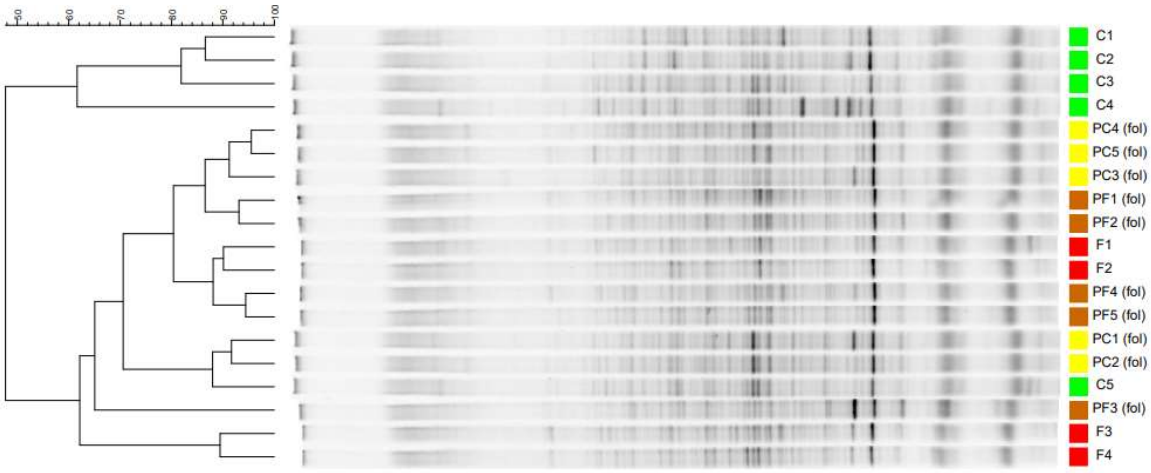

Figure 21: Dendrogram resulting from DGGE gel, with C1 to C5 representing control group replicates ; F1 to F4 representing *Fusarium* inoculated plants group replicates; PC(fol)1 to PC(fol)5 represent replicates of plant group with foliarly applied Phi; and PF(fol)1 to PF(fol)5 represent replicate of inoculated plants where foliar Phi was applied, *P. radiata* . Dendrogram constructed based on the UPGMA clustering method, alongside a Pearson momentum correlation for the similarity clustering. Values presented in the scale represent % similarity.

Concerning the treatment with Phi through irrigation, a similar pattern was observed with control samples (group C) sharing about 60% similarity with the remaining samples. Within the remaining samples, a separation between *Fusarium*-inoculated samples (group F) and samples treated with phosphite (groups PFW and PCW) was also evident, displaying about 80% similarity between these two groups. Profiles from phosphite treated plants (both control and *Fusarium* inoculated plants) clustered together (fig. 22).

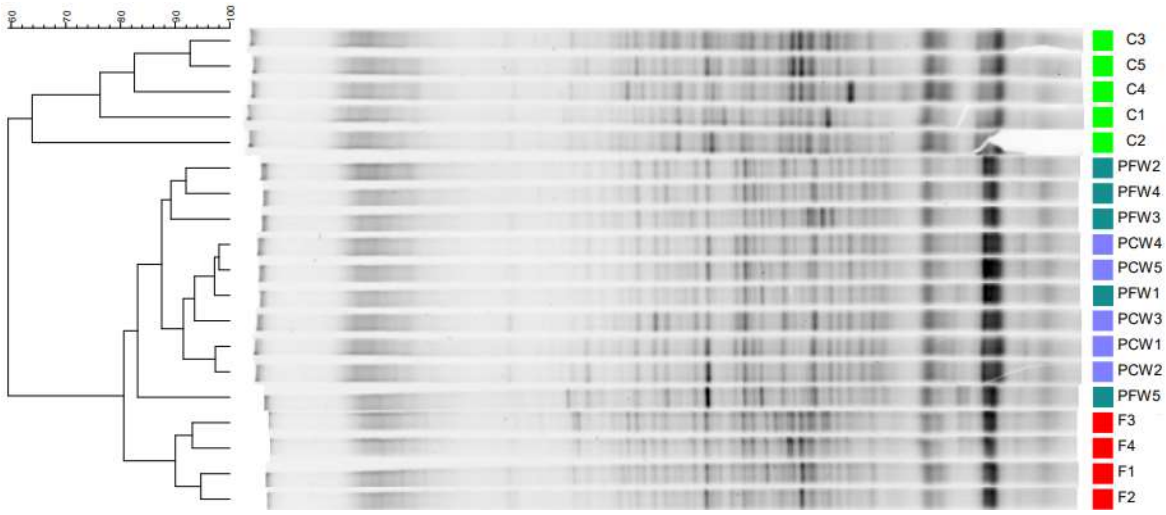

Figure 22: Dendrogram resulting from DGGE gel analysis, with C1 to C5 representing control group replicates; F1 to F4 representing *Fusarium*-inoculated plants; PCW1 to PCW5 representing plants irrigated with Phi and PFW1 to PFW5 representing *Fusarium*-inoculated plants irrigated with Phi. Dendrogram constructed based on the UPGMA clustering method, alongside a Pearson correlation for the similarity clustering. Values presented in the scale represent % similarity.

According to DGGE clustering analysis, eighteen samples were selected to be sequenced (Table 5). After quality filtering, a total of 749166 reads were obtained for these samples, with an average of 41620±9285 reads per sample, which were clustered in 240 OTUs (240±13 per sample).

Table 5: Number of processed reads and OTUs per sample. C1 to C3 represent replicates of control groups; F1 to F3 represent replicates of the inoculated groups; PC(fol)1 to PC(fol)3 represent replicates of plants with foliarly applied Phi; and PF(fol)1 to PF(fol)3 represent *Fusarium*-inoculated plants to which Phi was applied foliarly; PCW1 to PCW3 represent plants treated with Phi through irrigation; PFW1 to PFW3 *Fusarium*-inoculated plants treated with Phi through irrigation.

Sample	Number of processed reads	Number of OTUs
PFW1	31776	234
PFW2	40415	246
PFW3	37298	221
PCW1	64191	230
PCW2	34517	228
PCW3	63103	225
PF(fol)1	41989	257
PF(fol)2	30496	256
PF(fol)3	44689	230
PC(fol)1	48743	260
PC(fol)2	36562	240
PC(fol)3	46342	252
F3	37136	240
F2	36169	243
F1	37079	253
C1	36747	266
C2	40491	248
C3	41423	245
TOTAL	749166	-

The rarefaction curve for each sample reached a point of full saturation (fig. 23).

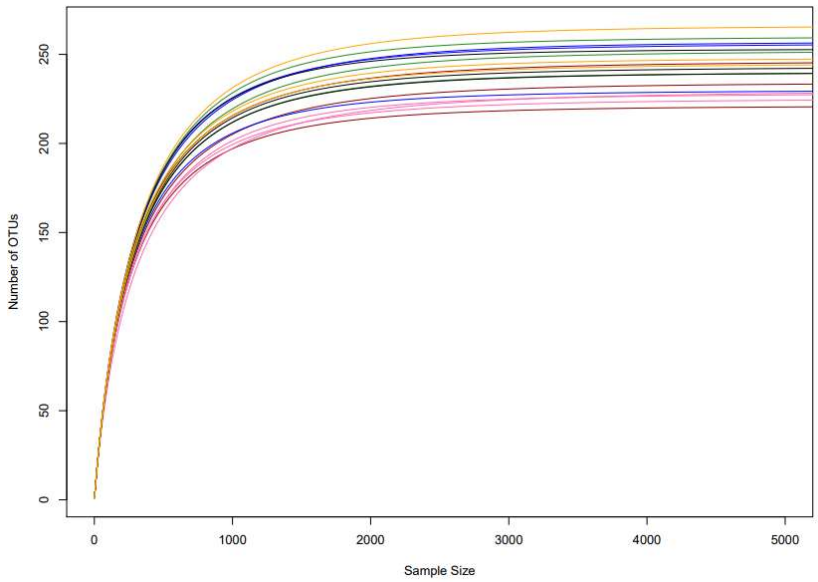

Figure 23: Rarefaction curves of the number of OTUs in function of the number of reads (maximum x value was shortened to 5000 to better view the graph). PFW, *F. circinatum*-inoculated plants, treated with Phi through irrigation, represented in dark red; PCW, non-inoculated plants treated with Phi through irrigation, represented in hot pink; PF(fol), *F. circinatum*-inoculated plants treated with Phi through foliar application, represented in green; PC(fol), non-inoculated plants treated with Phi through foliar application, represented in forest green; F: *Fusarium*-inoculated plants, represented in black; C: control plants, represented in orange;

A comparison of the alpha diversity parameters (i.e. Shannon-Wiener index, OTUs richness and eveness) between plants in the control group (C), plants inoculated with F. *circinatum* (F) and plants treated with phosphite [PC(fol); PF(fol);PFW;PCW] revealed no significant differences.

Table 6: Alpha-diversity metrics: OTUs Richness; Shannon Wiener index of diversity and community evenness. *radiata* (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi

	Alpha-diversity metrics		
Treatments	**Richness S**	**Shannon-Wiener H**	**Pielou J**
P. radiata (C)	253±11.36	5.52±0.044	0.997±0.0000
P. radiata (F)	245±6.81	5.49±0.029	0.998±0.0003
P. radiata (PFW)	234±12.50	5.44±0.054	0.997±0.0003
P. radiata (PCW)	228±2.52	5.41±0.010	0.997±0.0003
P. radiata PF(fol)	248±15.31	5.50±0.062	0.998±0.0001
P. radiata PC(fol)	251±10.07	5.51±0.042	0.998±0.0003

The PCoA analysis documents a separation among the rhizobiome between plants to which Phi was applied trough irrigation and the remaining plants included in this analysis. The two main axis in this plot accounted for 40% of the data variation (fig. 24), 29.3% assigned to PCO1 along which the separation of Phi-irrigated plants and the other groups occurred.

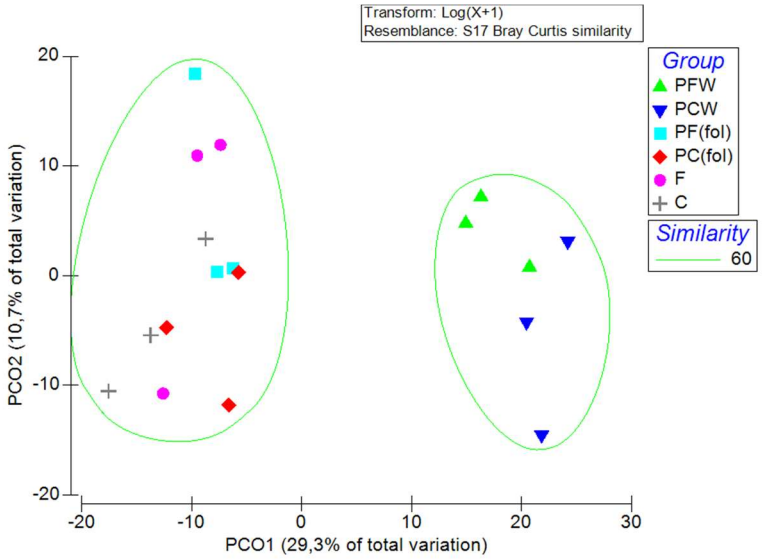

Figure 24: PCoA of the rhizobiome structure of *P. radiata*. (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi

The PERMANOVA analysis and Monte-Carlo test presented in Table 7, showed significant differences between the rhizobiome structure of PFW plants versus control plants (C), as well as between PW plants versus plants inoculated with *F. circinatum* (F), control group (C), PC(fol) and PF(fol). There were also significant differences between PFW and PF(fol).

Table 7: Pair-wise permanova (perm) and Monte Carlo (MC) tests regarding beta diversity. Significant p-values are indicated with a * symbol. PFW: Plants inoculated with *F. circinatum* and treated with Phi through irrigation; PCW: Plants treated with Phi through irrigation; PF(fol): Plants inoculated with *F. circinatum* and treated with Phi through foliar application; PC(fol): Plants treated with foliar application of Phi; (F): *Fusarium*-inoculated plants; (C): control plants.

Groups	t	Unique P (perm)	perms	P (MC)
PFW, PW	1.0434	0.314	10	0.376
PFW, PF(fol)	1.6744	0.113	10	0.076
PFW, PC(fol)	1.6297	0.097	10	0.081
PFW, C	1.7854	0.097	10	0.047*
PCW, PF(fol)	1.8643	0.099	10	0.043*
PCW, PC(fol)	1.7221	0.099	10	0.041*
PCW, C (radiata)	1.9451	0.101	10	0.029*
PF(fol), PC(fol)	0.99037	0.5	10	0.441
PF(fol), C (radiata)	1.2001	0.103	10	0.248
PC(fol), C (radiata)	1.029	0.383	10	0.416
PFW, F (radiata)	1.5566	0.089	10	0.084
PCW, F (radiata)	1.7325	0.109	10	0.049*
PF(fol), F (radiata)	0.89303	0.727	10	0.534
PC(fol), F (radiata)	0.97641	0.707	10	0.454
F (radiata), C (radiata)	0.95198	0.634	10	0.499

Family diversity

Bar charts were used to assess the relative abundance of each bacterial family in the rhizobiome of *P. radiata* treated with phosphite applied in different modes. Families representing less than 2% of the total reads were not included in the charts.

The analysis of the bar charts revealed a high relative abundance of *Acidobacteriaceae*, *Micropepsaceae*, *Acidimicrobiaceae*, in all samples. These 3 families comprised 30% to 40% of the relative abundance in all samples (fig. 25). Comparing plants to which phosphite was added through different aplication modes (irrigation vs. foliarly), the relative abundance of *Acidimicrobiaceae*, increased in PCW plants when compared to the control, while in foliarly application, only *Polyangiaceae* displayed higher abundance when compared to other samples.

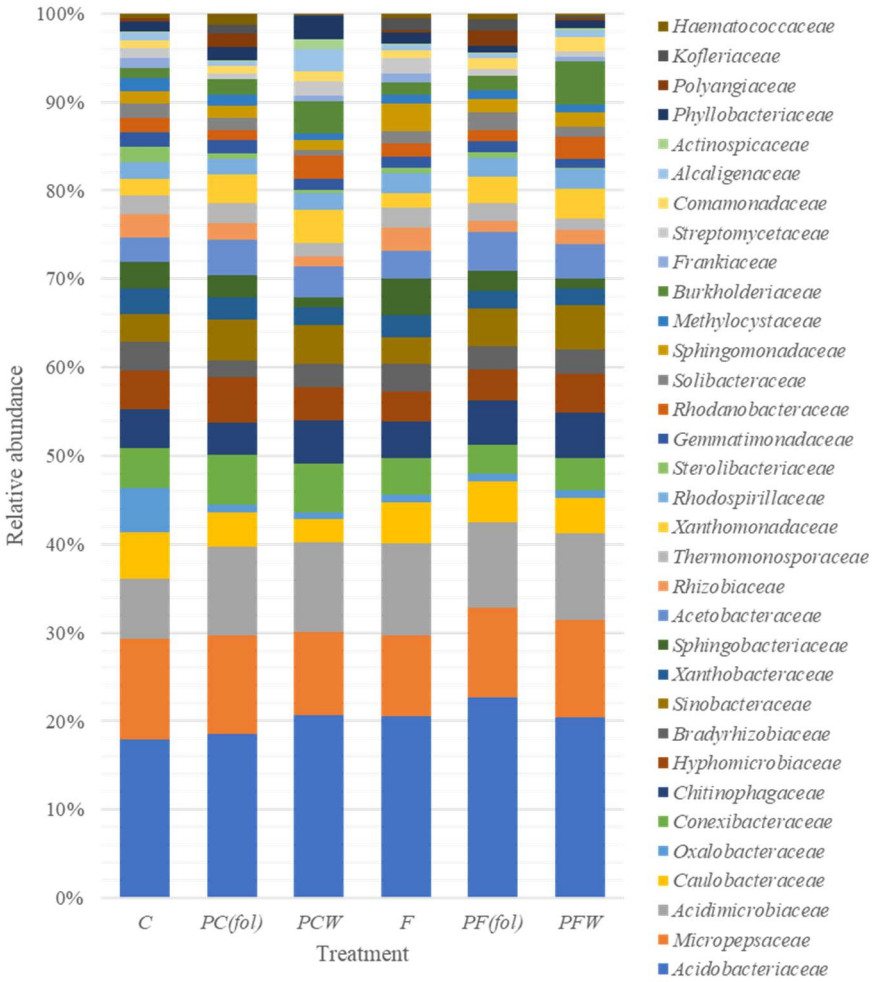

Figure 25: Relative abundance of bacterial families in each experimental group. (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi. Bar chart only accounted for families with more 2% of total abundance of reads.

An heatmap representing relative abundances of the 30 most represented families in each group is shown in Fig. 26. Families such as *Acidimicrobiaceae, Sinobacteraceae, Rhodanobacteraceae, Comamonadaceae,* had higher relative abundances in the rhizobiome of plants treated with phosphite, particularly in plants to which phosphite was added through irrigation (PCW). *Rhodanobacteraceae* and *Burkholderiaceae* were more prevalent in plants treated with phosphite through irrigation (PCW, PFW). *Polyangiaceae* only exhibits high abundance in foliar phosphite groups.

	C	PC(fol)	PCW	F	PF(fol)	PFW
Acidobacteriaceae	**5834±(168) [a]**	**7062±(733) [a,b]**	**10470±(718) [b]**	**6507±(1166) [a,b]**	**7792±(1199) [a,b]**	**6798±(1564) [a,b]**
Micropepsaceae	3715±(968) [a]	4221±(183) [a]	4735±(1084) [a]	2915±(145) [a]	3472±(880) [a]	3627±(308) [a]
Acidimicrobiaceae	**2202±(374) [a]**	**3818±(503) [a,b]**	**5073±(1550) [b]**	**3254±(625) [a,b]**	**3311±(293) [a,b]**	**3271±(696) [a,b]**
Caulobacteraceae	1730±(508) [a]	1454±(148) [a]	1333±(101) [a]	1467±(170) [a]	1578±(219) [a]	1314±(39) [a]
Oxalobacteraceae	1629±(1214) [a]	322±(278) [a]	411±(398) [a]	277±(211) [a]	322±(57) [a]	305±(400) [a]
Conexibacteraceae	1482±(586) [a]	2153±(1218) [a]	2760±(1136) [a]	1315±(288) [a]	1125±(461) [a]	1205±(398) [a]
Chitinophagaceae	1420±(377) [a]	1403±(474) [a]	2509±(860) [a]	1340±(185) [a]	1694±(215) [a]	1680±(258) [a]
Hyphomicrobiaceae	1415±(260) [a]	1919±(557) [a]	1861±(490) [a]	1068±(299) [a]	1226±(277) [a]	1487±(189) [a]
Bradyrhizobiaceae	1074±(231) [a]	730±(321) [a]	1338±(294) [a]	958±(203) [a]	879±(153) [a]	907±(66) [a]
Sinobacteraceae	**1029±(232) [a]**	**1764±(136) [a,b]**	**2191±(457) [b]**	**980±(46) [a]**	**1460±(345) [a,b]**	**1640±(395) [a,b]**
Xanthobacteraceae	950±(61) [a]	950±(240) [a]	1056±(549) [a]	788±(88) [a]	693±(83) [a]	627±(139) [a]
Sphingobacteriaceae	947±(145) [a]	962±(313) [a]	540±(200) [a]	1321±(229) [b]	795±(199) [a]	367±(126) [c]
Acetobacteraceae	897±(251) [a]	1487±(62) [a]	1776±(599) [a]	991±(294) [a]	1487±(240) [a]	1296±(451) [a]
Rhizobiaceae	876±(86) [a]	746±(119) [a]	580±(349) [a]	838±(472) [a]	437±(298) [a]	536±(427) [a]
Thermomonosporaceae	701±(163) [a]	840±(325) [a]	752±(254) [a]	698±(249) [a]	685±(277) [a]	425±(125) [a]
Xanthomonadaceae	621±(303) [a]	1247±(291) [a]	1890±(756) [a]	520±(157) [a]	1045±(443) [a]	1118±(174) [a]
Rhodospirillaceae	588±(92) [a]	671±(162) [a]	929±(157) [a]	688±(133) [a]	700±(97) [a]	746±(84) [a]
Sterolibacteriaceae	573±(91) [a]	218±0 [a]	218±(318) [a]	220±(158) [a]	218±(309) [a]	52±(74) [a]
Gemmatimonadaceae	550±(432) [a]	560±(21) [a]	653±(251) [a]	408±(126) [a]	432±(200) [a]	347±(254) [a]
Rhodanobacteraceae	**526±(86) [a]**	**458±(32) [a]**	**1321±(163) [b]**	**457±(54) [a]**	**456±(64) [a]**	**821±(259) [a]**
Solibacteraceae	515±(95) [a]	507±(126) [a]	297±(132) [a]	426±(155) [a]	665±(54) [a]	369±(137) [a]
Sphingomonadaceae	468±(38) [a]	518±(313) [a]	564±(331) [a]	1021±(811) [a]	513±(439) [a]	530±(347) [a]
Methylocystaceae	456±(92) [a]	475±(207) [a]	396±(157) [a]	307±(19) [a]	353±(24) [a]	289±(143) [a]
Burkholderiaceae	**386±(167) [a]**	**663±(345) [a]**	**1824±(512) [b]**	**448±(205) [a]**	**579±(57) [a]**	**1647±(300) [b]**
Frankiaceae	382±(541) [a]	100±(132) [a]	294±(318) [a]	292±(229) [a]	0±(0) [a]	151±(110) [a]
Streptomycetaceae	335±(236) [a]	238±(142) [a]	822±(1485) [a]	566±(315) [a]	239±(50) [a]	226±(166) [a]
Comamonadaceae	**325±(84) [a]**	**360±(75) [a]**	**600±(112) [b]**	**267±(95) [a]**	**410±(81) [a]**	**540±(69) [a]**
Alcaligenaceae	274±(116) [a]	183±(89) [a]	1259±(279) [b]	193±(136) [a]	183±(134) [a]	244±(140) [a]
Actinospicaceae	37±(53) [a]	32±(79) [a]	541±(765 [a])	60±(48) [a]	33±(46) [a]	98±(0) [a]
Phyllobacteriaceae	366±(94) [a]	559±(217) [a]	1339±(300) [a]	405±(277) [a]	286±(120) [a]	282±(99) [a]
Polyangiaceae	**118±(65) [a]**	**601±(126) [b]**	**32±(40) [a]**	**87±(61 [a])**	**612±(180) [b]**	**62±(45) [a]**
Kofleriaceae	0±(0) [a]	370±(301) [a,b]	66±(60) [a]	436±(122) [b]	417±(105) [b]	103±(102) [a,b]
Haematococcaceae	176±(115) [a]	486±(489) [a]	64±(86) [a]	150±(107) [a]	219±(59) [a]	87±(62) [a]

Figure 26: Heatmap of the counts of reads per family in control groups. (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi. Standard deviation is presented in parenthesis. Significantly different groups are presented with an different lower case letter. Bold indicates significant differences.

Functional diversity

Functional metagenomic inference of the rhizobiome sequencing results was applied using Piphillin. The selection of the displayed pathways in figure 27, was based on relevant functions pertaining the rhizobiome and the plant. Further analysis was made of KEGG orthologs belonging to other gene families. However, no significant differences were found (data not presented).

When analyzing the inferred gene families related with carbon metabolism, a significant difference between the control group (C) and the phosphite-irrigated group (PCW) was observed, with these gene family being enriched in the PCW plants. No other significant differences were observed.

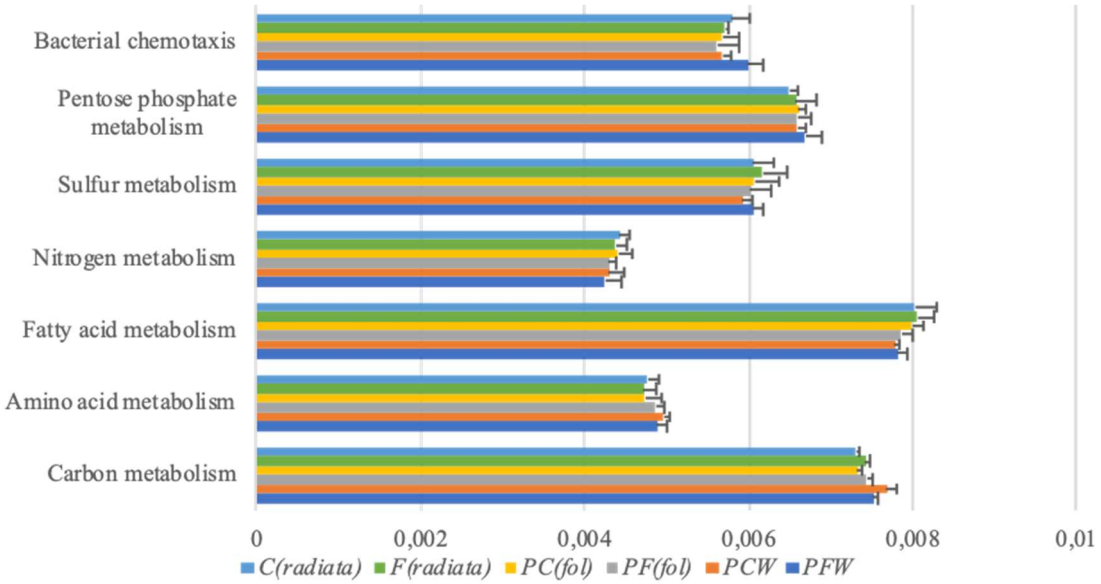

Figure 27: Meta-analysis of the inferred potential functions of the microbiome. (C): control group; (F): Plants inoculated with *F. circinatum* group; (PFW): Treated with irrigation of Phi, inoculated with *F. circinatum*; (PCW): Treated with irrigation of Phi; PF(fol): Treated with foliar application of Phi and inoculated with *F. circinatum*; PC(fol): Treated with foliar application of Phi

4. Discussion

The main goal of this study was to assess the dynamics of the rhizobiome, after inoculation of *Pinus* seedlings with *Fusarium circinatum*, considering different species and the impact of a priming agent. Given the importance of the rhizobiome to the nutrition, pathogen resistance and overall fitness of the plant, studying this community is an important step to fully understand the impact of phytopathogens and ideally to design new strategies to reduce this impact. Due to their distinct susceptibility to *F. circinatum* infection, two species of *Pinus* were chosen to investigate in this work: *Pinus radiata* (susceptible) and *Pinus pinea* (resistant) (Amaral et al., 2019). To our knowledge, this is the first study analyzing the rhizobiome of these species under the *F. circinatum* pathosystem. Besides comparing the responses of both species' rhizobiomes to the fungus inoculation, the effect of a priming agent, phosphite, on the rhizobiome of the susceptible species was also evaluated. The addition of phosphite to this trial was suggested by the recent reports of its capability to slow down fungal infections, an effect probably related with the antifungal properties of this compound (Cerqueira et al., 2017; Pereira et al., 2012). Two commonly used modes of application of phosphite were evaluated: foliar application and irrigation (Percival et al., 2015).

4.1 Rhizobiome dynamics in response to inoculation with *F. circinatum*

In contrast to what was observed for *P. pinea*, *P. radiata* displayed symptoms of infection throughout the experiment. This pattern of response was observed in other studies (Iturrixa et al., 2013). For example, Bragança et al., in 2009, performed a similar experiment and verified that *P. radiata* displayed symptoms after 8 days. Furthermore, authors also attested that *P. pinea* displayed no symptoms during the first weeks (reviewed in Amaral et al., 2019).

Regarding gas-exchange related parameters, significant differences were observed between the inoculated *P. radiata* plants and its respective control. Inoculated *P. radiata* plants displayed higher values of C_i (internal CO_2 concentration), lower gs and an impaired A (photosynthetic rate). These results are in line with previous studies (Cerqueira et al., 2017; Maoela et al., 2018). These changes occur, in part, due to the water stress imposed by the fungus to the plant (Amaral et al., 2019). The girdling effect of the fungus causes the plant to close the stomata, leading to lower transpiration rates, accumulation of CO_2 and a lower photosynthetic rate (Martín-Rodrigues et al., 2013; Li et al., 2013). In *P. pinea*, no differences were observed in these parameters, again confirming plants resistance.

Analyzing the antioxidant capability, there were no significant differences between inoculated plants and respective controls for both species. However, several studies point that plants usually accumulate phenolic compounds in response to a phytopathogenic attack (Beimen et al., 1992; Petkovšek et al., 2009). The lack of increase in phenolic compounds observed in our study might be related to the type of analysed plant material. Several studies suggested that there is an increase in phenolic accumulation after a fungal infection, but that increase only occurs in areas nearby the fungus entrance point (Gayoso et al., 2004). Between the two species, significantly higher levels of phenolic compounds were determined for *P. pinea*, the resistant species. This might affect the resistance of the plant to the fungal infection, given that these compounds have an essential role in the antioxidant response, triggered by fungal infections (Kubalt et al., 2016).

Rhizobiome structure

When analyzing the rhizobiome, the most abundant phyla were common to all analysed plants: *Proteobacteria*, followed by *Acidobacteria* and *Actinobacteria*, the three having a total relative abundance superior to 80% in each plant. These results are in accordance with previous studies of the rhizobiome in pine trees (Chow et al., 2002; Lottman et al., 2010). The most significant differences were observed between the two pine species (i.e. by comparing control plants), clearly indicating the presence of species-specific rhizobiomes already suggested for other plant species (Berendsen et al., 2011; Agler et al., 2016). This specificity is linked to the type of exudates the plant releases through the roots into the rhizosphere, which can modulate the rhizobiome and can vary among species and throughout the lifespan of the plant (Chaparro et al., 2014). Families more prevalent in *P. pinea* (with low abundance in *P. radiata*) included *Nocardioidaceae*; *Burkholderiaceae*; *Xanthomonadaceae* and *Mycobacteriaceae*. *Nocardioidaceae* and *Xanthomonadaceae* have been found in high abundance in disease suppressive soils (Rosenzweig et al., 2012), which might indicate potential PGP-traits of these bacteria. Some genera of *Xanthomonadaceae* found within our samples (e.g. *Lysobacter*) are capable of expressing anti-fungal compounds (de Bruijn et al., 2015). In Japan, reports indicate that soils are enriched with *Xanthomonadaceae* along with other *Gammaproteobacteria* in order to create a disease suppressive soil against Common scab (caused by a small set of *Streptomyces* species) (Kobayashi et al., 2015). *Burkholderiaceae* is a family commonly found in soils (Spain et al., 2009), having a widespread capability of producing ACC (1-aminocyclopropane-1-carboxylate) deaminase (Onofre-Lemus et al., 2009), which is a common PGP trait. ACC deaminase is the precursor of an important phytohormone, ethylene (Van de Poel et al., 2014). This molecule can often contribute to

ameliorate plant stress responses to drought and infection, consequently, promoting growth (Pandey et al., 2019).

On the other hand, in the rhizobiome of *P. radiata*, families *Micropepsaceae; Xanthobacteraceae* and *Rhizobiaceae* were more abundant in comparison to the *P. pinea* rhizobiome. *Xanthobacteraceae* and *Rhizobiaceae* are also known for their PGPR characteristics (Kannaiyan et al,. 2004). Their main PGP trait is related with nitrogen fixation (Requena et al., 1997) and there are many studies indicating the immediate plant benefits, such as the increase in root length and fruit yield (in non-legumes) (García-Fraile et al., 2012). However, while *Rhizobiaceae* have PGP traits, these were not related directly with fungal interactions. *Micropepsaceae* members are commonly found in soils or rhizospheric soils and to the best of our knowledge there are no reports of PGP activity for members of this family (Jorquera et al., 2012; Ofek et al., 2012; Bräuer, 2017). In summary, the traits exhibited by the aforementioned bacterial families in the rhizobiome of *P. radiata* were mainly related to growth promotion, while the ones more abundant in the rhizobiome of *P. pinea* exhibit not only direct growth promotion traits, but also indirect effects by showing antagonistic effects against phytopathogens. This distinct composition of the rhizobiome of the two species might affect the plants resistance to the fungus. In fact, supported by the fact that each rhizobiome is species-specific, the presence, in a resistant pine species (*P. pinea*), of PGPR families that are used in disease suppressive soils, might suggest a potential role of the rhizobiome in the defense of the pine species against *F. circinatum.*

When comparing the rhizobiomes of inoculated and non-inoculated plants, differences were observed in terms of the 16S rRNA gene DGGE profiles. These differences were slightly more accentuated for *P. radiata* than for *P. pinea*, suggesting a stronger impact of the fungus in the rhizobiome of *P. radiata* plants. However, a significant impact in the rhizobiome was not observed in terms of general alpha metrics such as diversity or richness, calculated based on massive parallel sequencing results. This apparent contradiction might be explained by the fact that PCR-DGGE accesses the dominant bacterial groups, each representing at least 1% of the community cells (Muyzer et al., 1993), which might include some of the phylotypes affected by the fungus inoculation.

Some studies in other plants also corroborate the hypothesis that above-ground infections do not impact significantly the rhizobiome of the respective plant (De Tender et al., 2016). The most prominent factors that can affect the rhizobiome (in the context of a plant-pathogen interaction) are the geographic origin of the soil (Mendes et al., 2014) and plant excreted compounds as well as other added chemical compounds (Chaparro et al., 2014). Even though significative differences

were not observed when analyzing the community as a whole, it is still possible to pinpoint differences by comparing the prevalence of specific groups. In *P. radiata*, *Kofleriaceae* members were only present in inoculated plants, while absent in control plants. This family is commonly associated with plant necrotic tissues (Garrity et al., 2006), suggesting that its abundance might be a consequence of early effects of the fungus on the plant even though the plants were asymptomatic. This is supported by the fact that plants of *P. radiata* displayed an impaired photosynthetic rate and lower stomatal conductance, characteristics of a non-healthy plant (reviewed in Amaral et al., 2019). Even though the seedlings did not show typical wilting symptoms, they could be stressed by the *F. circinatum* inoculation and therefore exhibit necrotic tissues in the roots.

The analysis of the predicted functional profiles suggested that no major pathways were affected by the fungus inoculation. Although the rhizobiome's amino acid metabolism and bacterial chemotaxis have been reported to decrease when a plant is inoculated with a phytopathogen (Wu et al., 2019), this was not verified in our study. On the other hand, a significant impact on the abundance of genes related with monoterpenes biosynthesis was inferred from our results. Research around monoterpene production by rhizobacteria is scarce, commonly being focused on the rhizobacterial improvement of monoterpene synthesis by the plant host (Santoro et al., 2015). However, monoterpenes have been shown to exhibit antibacterial behavior against *Staphylococcus aureus* and *Escherichia coli* (Marmulla et al., 2014).

4.2 Effect of phosphite and application mode on the *Pinus* rhizobiome

The use of Phi had a significant effect on the percentage of asymptomatic seedlings after 10 days, increasing from 30% for the Fungus-inoculated plants, to 50% for inoculated plants treated foliarly with Phi and 70% when Phi was added trough irrigation. The susceptibility of *P. radiata* to pine pitch canker (PPC) disease is known, taking about 8 days for the pine seedlings to show symptoms (Bragança et al., 2009; Iturrixa et al., 2013). As stated in Cerqueira et al., 2017, Phi can be an effective priming agent for the plant in PPC. Our results show that irrigating plants with phosphite, yielded the best results. According to some studies, these two modes of application can trigger different responses: foliar application exerts a more direct effect on the phytopathogen, while root irrigation might trigger an induced systemic resistance in plants (Graham, 2011; Percival et al., 2014). This type of induced systemic resistance leads to a better level of the plant's basal defenses, namely defenses against phytopathogens (Choudhary et al., 2007). This primed state of

the irrigated samples might justify an higher percentage of asymptomatic seedlings, when compared to foliarly treated plants.

When assessing the overall needle gas exchange parameters of the plants, inoculated plants presented a typical stress status, with lower values of photosynthetic rate, transpiration rate and stomatal conductance, regardless of Phi application. This decrease in values observed in inoculated plants are in accordance with previous studies carried out in *P. radiata* (Dalio et al., 2018; Cerqueira et al., 2017). Contrary to other results (Cerqueira et al., 2017), when analyzing control groups and Phi inoculated groups, there were no significant differences in the parameters analyzed. Phi can exhibit phytotoxic behavior (Achary et al., 2017), however, our samples, even though they displayed foliar discoloration, showed no significant stress status. This might be due to a lower concentration of Phi in comparison to Cerqueira et al., 2017.

When analyzing antioxidant response, no significant differences between samples were observed. For other plant species Phi application has been reported to increase total phenolic compounds (Ávila et al., 2011; Araujo et al., 2015), which might result in an increase in plant's defense response.

Rhizobiome structure

A 16S rRNA gene-targeted DGGE analysis revealed a compositional shift between control plants and plants treated with Phi and/or inoculated with the fungus. This effect might be related with an acidification of the soil resulting from phosphite application, favoring the growth of acidophilic bacteria (Jones et al., 2011). The analysis of massive parallel sequencing results also revealed a significant impact of phosphite in the rhizobiome, when the compound was added through irrigation. This stronger impact of the phosphite directly applied on the soil, in comparison to the foliarly-applied phosphite, might occur due to the fact that Phi tends to accumulate preferably on the root tips (Fairbanks et al., 2000), directly exposing the rhizobiome to higher concentrations of Phi (Olanrewaju et al., 2019).

Considering the most abundant phyla, there was an increase in relative abundance for *Acidobacteria, Actinobacteria* and *Proteobacteria* in the rhizobiome of plants treated with Phi through irrigation (PCW), when comparing to control plants. A slight increase of these phyla in the rhizobiome of plants treated with Phi through foliar application [PC(fol)] was also observed. Similar patterns were observed for plants inoculated with *F. circinatum* and treated with phosphite.

Acidimicrobiaceae, Sinobacteraceae, Rhodanobacteraceae and *Comamonadaceae* were significantly more abundant in plants treated with phosphite, irrespective of its mode of

application. *Acidimicrobiaceae* is commonly associated with acidic soils and iron oxidation (Stackebrandt, 2014). It is commonly detected in extreme conditions (Li et al., 2018) and is responsible for exerting a phytoremediation effect (Yadav et al., 2018). This bacterial family is also found in association with metal-accumulating plants (Guo et al., 2019). The addition of irrigated phosphite might have caused soil acidization, consequently selecting for members of this bacterial family. *Sinobacteraceae* has been found before in pine rhizospheric soils (Naz et al., 2018). There have been reports that this family is positively influenced by the addition of phosphorous to soils (Trabelsi et al., 2017). Furthermore, the species from family *Rhodanobacteraceae* have mostly been isolated from forest soils (Dahal et al., 2018; Cho et al., 2017). Additionally, some of the genera belonging to this family might also display acidophilic characteristics (Koh et al., 2015).

The comparison between the rhizobiomes exposed to Phi through the two modes of application, suggested the selection of distinct families. For example, an increase in the abundance of *Rhodanobacteraceae* and *Burkholderiaceae* was verified after irrigation with phosphite, while a foliar application increased the abundance of *Polyangiaceae*. *Burkholderiaceae* have a thriving relationship with pine mycorrhiza, furthermore having a two pump phosphate transport system, which was proposed to shunt the phosphorous transfer from the fungus to the plant (Ruiz-Lozano et al., 1999). When fertilizers rich in Phi are used, an increase on the amount of available phosphorous is expected and some species of *Burkholderiaceae* might be able to take advantage of this beneficial effect to grow, by shunting the phosphorous uptake from its host mycorrhiza (Adesemoye et al., 2009). The family *Comamonadaceae* can be commonly found in marine and terrestrial environments (Ma et al., 2015), and it has been hypothesized that these bacteria regulate (alongside mycorrhizae) the cycling of sulfonate in the soil (Kertesz et a., 2007). *Polyangiaceae* are commonly found in terrestrial soil, mainly associated to decaying plant material (Garcia et al., 2014). This family depends on available phosphorous to proliferate (Lee et al., 2019).

All in all, this experiment revealed that the addition of phosphite favors the proliferation of acidophilic bacteria in the plant's rhizobiome as well as bacterial families with phosphorous related dependencies. However, the *in silico* analysis to assess the predicted abundance of genes related to phosphite and phosphate metabolism did not reveal differences . Furthermore, the direct application of Phi through irrigation had a more prominent impact on the rhizobiome than the foliar application.

5. Final remarks and future perspectives

This is the first time that the rhizobiome of *P. pinea* and *P. radiata* was studied, in such detail, under the scope of the Pine pitch canker pathosystem. Regarding host susceptibility to pitch canker and the link to its rhizobiome, a higher prevalence of bacterial families with potential anti-fungal traits was observed in the rhizobiome of *P. pinea.* Families more abundant in *P. radiata*, although being associated to PGP traits,were not directly related to fungal suppression. Bacteria with anti-fungal traits in *P. pinea* rhizobiome may play a rolein this pine species' resistance to the pathogen. This opens the possibility of using such bacterial families in biofertilizers in order to confer resistance to susceptible trees. The fungus-inoculation had low impact on the rhizobiome of both species. However, *Kofleriaceae* were found in inoculated *P. radiata* samples and have been associated to necrotic plant tissues, which indicates a potential effect of the fungal inoculation on the rhizobiome of *P. radiata*.

In order to build upon the findings here presented it is necessary to further understand the nature of the interaction between specific members of the highlighted bacterial families, the plant and the fungus. Using selective media to isolate specific bacteria so as to verify their phenotypic traits would clarify which expressed metabolites could influence the pine tree's resistance (Da Costa et al., 2013). If the expressed compounds showed an antagonistic effect against *F. circinatum, in vitro* tests would have to be performed, in which the bacterial-produced compound would be tested against the fungus (Pandya et al., 2014). This would enable the potential production of a fertilizer with the added compound that would suppress the pitch canker. In case the expressed compounds would affect the plant systemic resistance, they could also be tested in plants and added to fertilizers to induce systemic resistance (Barka et al., 2000). These studies would elucidate the true nature of the potential bestowing of resistance by the rhizobiome to the respective pine host. Moreover, they would make possible the creation of alternative measures to combat the pine pitch canker.

The Phi priming also had an effect on the rhizobiome, by favoring and selecting the growth of specific acidophilic bacteria in the rhizosphere. The effect was more pronounced when phosphite was applied through irrigation. Additional studies could be made taking into account these Phi induced rhizobiomic changes, namely the corroboration whether the growth of Phi-induced acidophilic bacteria could further amplify the decrease of the pH, subsequently allowing the proliferation of new PGPR that require lowers levels of pH to grow (Tamreihao et al., 2018). This would enable farmers to take advantage of the soil modifications induced by the usage of Phi. Furthermore, further studies are needed to assess the impact of the soil acidification on the overall development of the plant.

All in all, the detailed overview of the rhizobiome of each *Pinus* species presented in this study highlights the importance and role of the rhizobiome in plant susceptibility to pathogens. The study of the impact of fertilizers and antifungal compounds used on plants should also take into account the rhizobiome structure, which can be affected and, ultimately, significantly impact the plant's development.

6. References

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