Evaluation of Plant Growth-Promoting Rhizobacteria associated with *Quercus suber*

Ana João Pereira

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**Universidade do Minho**
Escola de Ciências

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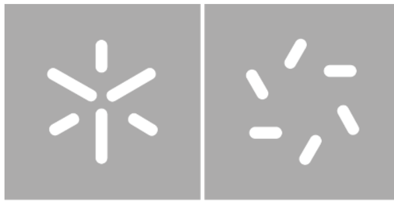
March 2021



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# Evaluation of Plant Growth-Promoting Rhizobacteria associated with *Quercus suber*

**Master Thesis**

Master's Degree in Molecular Biology, Biotechnology and Bioentrepreneurship in Plants

Work developed under supervision of

**Doctor Francisca Rodrigues dos Reis**

**Professor Doctor Maria Teresa Correia Guedes Lino Neto**



March 2021

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**Statement of integrity**

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Ana João Pereira

(Signature)

**Avaliação de Rizobactérias Promotoras de Crescimento Vegetal associadas com *Quercus suber***


## Resumo

O sobreiro (*Quercus suber* L.) é uma espécie florestal nativa da região Mediterrânica, que detém uma grande importância socioeconómica e ecológica, formando ecossistemas únicos. Apesar de estarem bem adaptadas as condições de seca e de altas temperaturas, a mitigação dos efeitos adversos das alterações climáticas tem sido desafiante para as florestas de sobreiro. Novas abordagens biológicas, como a utilização de Rizobactérias promotoras do crescimento vegetal (PGPR), podem auxiliar o desenvolvimento sustentável do sobreiro. No presente trabalho, as PGPR de três florestas de sobreiros portuguesas, com diferentes bioclimas (semiárido, sub-húmido e húmido), foram isoladas e os seus mecanismos de promoção de crescimento avaliados. PGPR isoladas de florestas mais húmidas apresentaram maiores capacidades de mobilização de um único nutriente, nomeadamente de fósforo e ferro. Porém, a floresta semiárida detinha um maior número de PGPR com combinações de metabolização de diferentes nutrientes. Estas PGPR mais promissoras, com características combinadas, foram posteriormente avaliadas quanto à sua capacidade de modular a arquitetura da raiz de *Arabidopsis thaliana*. Alterações morfológicas nas raízes de *A. thaliana* foram observadas, principalmente na presença de estirpes de *Bacillus*, *Serratia*, *Klebsiella*, *Cedecea*, *Rouxiella* e *Unknown* e *Unidentified*. PGPR da floresta semiárida demonstraram maior potencial para induzir a formação de pelos radiculares, possivelmente devido à síntese de compostos bacterianos capazes de gerar uma arquitetura radicular mais adequada a condições de *stress* abiótico. O potencial inibitório destas PGPR contra patógenos de sobreiro (*Biscogniauxia mediterranea* e *Diplodia corticola*) foi, também, estudado em ensaios de placa dupla. Um isolado bacteriano pertencente ao género *Serratia* apresentou efeitos supressores contra *B. mediterranea*, presumivelmente devido à produção de HCN, um composto volátil antifúngico. Por outro lado, isolados de *Bacillus* e *Unknown* agiram como possíveis agentes de biocontrolo de *D. corticola*. Embora mais estudos sejam necessários, esta pesquisa forneceu evidências sobre o potencial que as PGPR possuem para serem utilizadas como potencializadoras do crescimento vegetal e agentes de biocontrolo. Além disso, este trabalho pode ser a base para a seleção de bactérias resistentes, capazes de melhorar o vigor das plantas, mesmo em condições ambientais hostis.



**Palavras-chave:** Sobreiro; Rizobactérias promotoras de crescimento vegetal; Solo florestal; Agentes de bio-controlo; *Biscogniauxia mediterranea*; *Diplodia corticola*;

**Evaluation of Plant Growth-promoting Rhizobacteria associated with *Quercus suber***

## Abstract

Cork oak (*Quercus suber* L.) is a forest tree species native to the Mediterranean region that holds great socioeconomic and ecological importance, forming unique ecosystems. Despite being well adapted to drought and high temperature conditions, these forests have been forced to mitigate the adverse effects of climate change. New biological approaches, such as the use of plant growth promoting bacteria (PGPR), can assist the sustainable development of cork oak. In the present work, PGPR communities isolated from three Portuguese cork oak forests with different bioclimates (semi-arid, sub-humid and humid) were selected and their growth-promoting traits evaluated. PGPR isolated from the most humid forests presented traits related with higher single nutrient-mobilization, namely for phosphorus and iron. However, the semi-arid forest presented a higher number of PGPR displaying a combination of several PGPR traits. These selected PGPR with most promising combined traits were further tested for their ability to modulate the root-architecture of *Arabidopsis thaliana*. Morphologic changes of *A. thaliana* roots were particularly observed in the presence of *Bacillus, Serratia, Klebsiella, Cedecea, Rouxiella,* and *Unknown* and *Unidentified* strains. Due to the highest potential of PGPR from the semi-arid forest to induce root-hairs, these PGPR were suggested to produce compounds that induce a root-architecture more suitable for abiotic stressed environments. PGPR ability to inhibit antagonistic widespread cork oak phytopathogens (*Biscogniauxia mediterranea* and *Diplodia corticola*) was also evaluated by dual plate assays. A *Serratia* isolate presented a suppressive effect against *B. mediterranea*, probably due to the production of HCN, an antifungal volatile. On the other hand, a *Bacillus* and an *Unknown* isolate displayed a potential biocontrol role against *D. corticola*. Although more studies are required, this work provided evidences about the potential of PGPR to be used as plant growth enhancers and biocontrol agents. Furthermore, this work could be the basis for the selection of resistant bacteria capable of improving plant health, even under hostile environmental conditions.

**Keywords:** Cork oak; Plant Growth Promoting Rhizobacteria; Forest soil; Biocontrol Agent; *Biscogniauxia mediterranea*; *Diplodia corticola*.

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**Abbreviations List**

**°C –** Degree Celsius

**ANOVA** - Analysis of Variance

**APCOR** - Associação Patronal do Setor Corticeiro

**BLAST –** Basic Local Alignment Search

**CAS** - Chrome Azurol Sulphonate

**CFU –** Colony Forming Unit

**DNA –** Deoxyribonucleic Acid

**dpi –** Days Post Inoculation

**ER** - Ermida

**FAO** - Food and Agriculture Organization

**g** - Grame

**GPS** - Basic Local Alignment Search

**GR** - Grândola

**h** - Hours

**ha** - Hectare

**HCN** - Hydrogen Cyanide

**HDTMA** - Hexadecyltrimethylammonium Bromide

**IPMA** - Instituto Português do Mar e da Atmosfera

**ISR –** Induced Systemic Resistance

**L –** Litre

**LB** - Luria-Bertani

**LI** - Limãos

**m** - Metres

**min** - Minute

**MS** - Murashige and Skoog

**NASA** - National Aeronautics and Space Administration

**NCBI** - National Center for Biotechnology Information

**ns** – Non Significant

**OTU** - Operational Taxonomic Units

**PDA** - Potato Dextrose Agar

**PGI** - Pathogen Growth Inhibition

**PGPR** – Plant Growth Promoting Rhizobacteria

**PIPES** - Piperazine-N,N'-bis2-(ethanesulfonic acid)

***Q*** - Emberger Index

**rDNA** - Ribossomal Deoxyribonucleic Acid

**RNA** - Ribonucleic Acid

**rRNA** – Ribossomal Ribonucleic Acid

**s** – Second

**SDS** - Sodium Lauryl Sulfate

***Taq*** - *Thermus aquaticus*

**UV** - Ultraviolet

**v:v** – Volume/volume

**VOC** - Volatile Organic Compounds

**w:v** – Weight/volume

**YMA** – Yeast Extract Manittol Agar

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# Chapter I: General Introduction

### 1.1. Cork oak forests– a Mediterranean heritage

Cork oak (*Quercus suber* L.) is a slow-growing and long-lived evergreen tree, native to western and central Mediterranean region. The species is mainly distributed along coastal regions of southwest Europe (France, Italy, Portugal, and Spain) and northwest Africa (Algeria, Morocco, and Tunisia; Kim *et al.*, 2017). These woodlands are adapted to the Mediterranean climate, where precipitation is expected to decrease, and temperatures are expected to rise during warm season, with annual rainfall ranging between 400 mm to 1700 mm (Mendes *et al.*, 2018; APCOR, 2019). Mediterranean landscapes are typically mixed forest habitat types, comprising woodlands, scrub communities, pastures, and extensive agriculture fields (FAO, 2013). Extensive agro-silvo-pastoral systems – *montados* - are the most common organization for cork oak forests in Portugal and are typically found in the southern region of the country (Alentejo; Figure 1.1B). *Montados* present a low density of trees with 60–100 trees/ha, whereas high density cork oak forests – *sobreirais* - have at least 400 trees/ha and are typically found in central and northern Portugal (Figure 1.1A; Reis *et al.*, 2018).

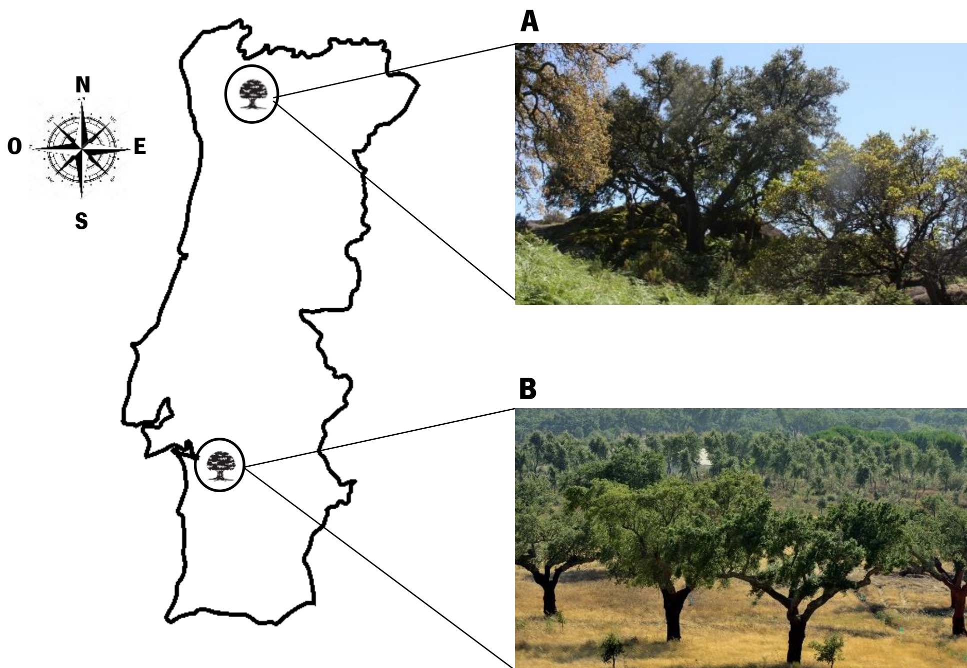


**Figure 1.1** Portuguese cork oak forests: *sobreirais* from Ermida (A) and *montados* from Grândola (B; APCOR, 2019).

### 1.1.1. Distribution and importance

Cork oak forests cover almost 1.5 million ha in Europe and 700,000 ha in North Africa (Figure 1.2A). Portugal has the largest distribution of cork oak Mediterranean forests with nearly 740,000 ha (34%), followed by Spain (574,248 ha; 27%), Morocco (383,120 ha; 18%), Argelia (230,000 ha; 11%), Tunisia (85,771 ha; 4%), France (65,228 ha; 3%), and Italy (64,800 ha; 3%; Figure 1.2A; APCOR, 2019).

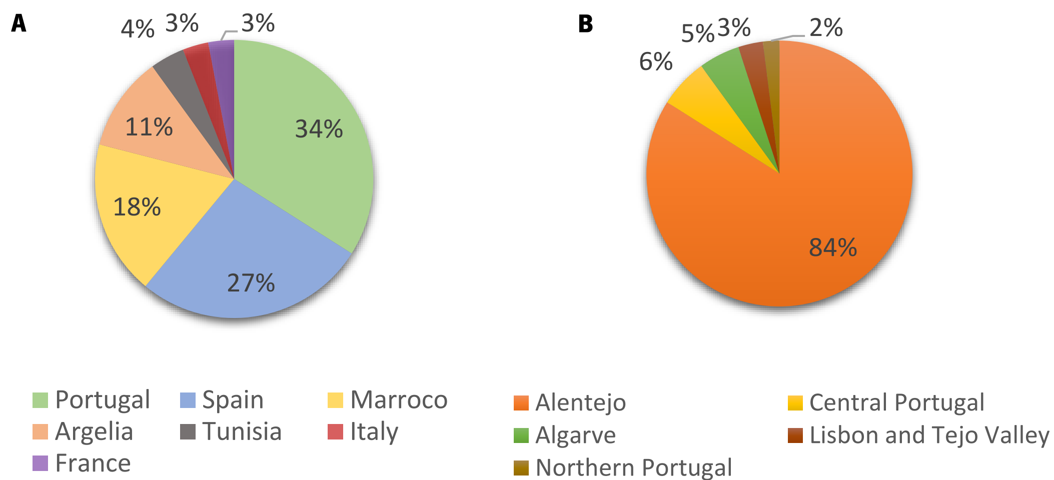


**Figure 1.2**. Cork oak forest area in Mediterranean countries (A; APCOR, 2019) and in different Portuguese regions (B; FAO, 2013).

Cork oak presents an outer bark of suberized dead cells that form a compact, elastic, impermeable, and thermally insulating tissue – the cork (FAO, 2013). Portugal owns 50% of the global cork market, producing about 100,000 tonnes of raw cork annually (Figure 1.3; APCOR, 2019). Cork industry main product are cork stoppers for wine industry, which represents 44% of annual Portuguese production. Other appliances of the cork have increased economic value, such as pavement and insolation material (*e.g.* the external fuel tanks of NASA's space shuttle), clothing, accessories, and decorative items (Kim *et al.*, 2017). But besides cork, cork oak forests also provide a broad range of goods and services, such as wood fuel, pasturage, forage, aromatic herbs, mushrooms, beekeeping, and leisure activities associated with rural areas (FAO, 2013). Beyond their economic importance, *montados* host a remarkable biodiversity and are unique ecosystems recognized for their ecological value and classified as protected habitats in the framework of the Natura 2000 Network, established by the European Union (Directive no. 92/43/EEC) since 1993 (Bugalho *et al.*, 2011).

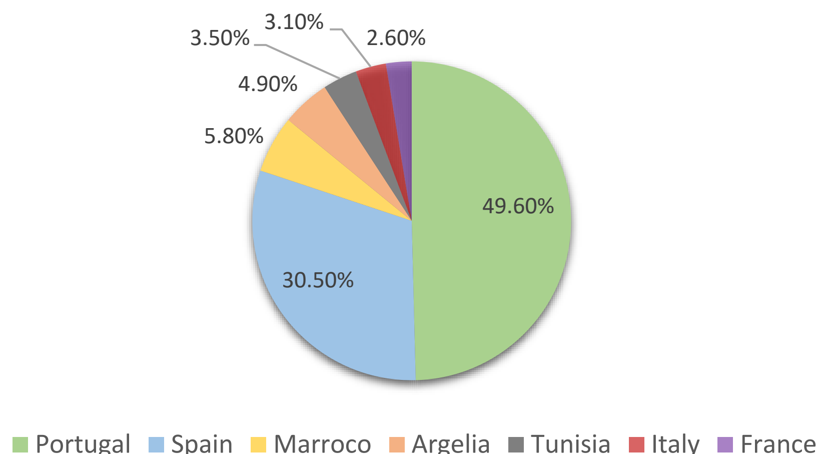


**Figure 1.3**. Annual cork production percentages in Mediterranean countries (APCOR, 2019).

### 1.1.2. Cork oak decline: drought and pathogens

Over the last decades, a severe reduction in cork oak forests area has been observed, due to the increasing temperatures and drought events, as well as changes in land use (such as overgrazing, extraction of firewood). These changes could lead to both physiological decline of trees and increase of tree vulnerability to insects and pathogens (Kim *et al.*, 2017). For example, the consequences of water deficits are known to enhance fungal diseases (Moricca *et al.*, 2016). Accordingly, disease incidence and drought both play the most significant role on forestry ecosystems and tree health (Desprez-Loustau *et al.*, 2006). Although well adapted to drought, the upcoming increase of the frequency and severity of dry periods could influence cork oak growth patterns and functional processes (Mendes *et al.*, 2018). During the past years, it has been widely reported an increase in cork oak diseases, including charcoal disease [caused by *Biscogniauxia mediterranea* (De Not.) Kuntze; Xylariales] and bot canker (caused by *Diplodia corticola* A.J.L. Phillips, A. Alves and J. Luque; Botryosphaeriales; Moricca *et al.*, 2016).

The endophytic fungus *Biscogniauxia mediterranea* is responsible for the charcoal canker. This disease is one of the main causes of cork oak decline, particularly in weakened trees, stressed by environmental conditions, such as drought (Evidente *et al.*, 2005; Henriques *et al.*, 2016). *B. mediterranea* has a long latent endophytic phase in oak trees aerial organs in which the infected trees are asymptomatic, and thus behaving as an opportunistic pathogen (Evidente *et al.*, 2005; Safaee *et al.*, 2017). When the host tree faces extended periods of drought and/or high temperatures, *B. mediterranea* spreads rapidly and many black stromata erupt from the dead bark, inducing discoloration of the woody

tissues, dieback, stem, and branch cankers (Henriques *et al.*, 2016). Atypical symptoms, such as discoloured and dried foliage, viscous liquid exudates on the trunk, and evident presence of a brown powdery mass in the liber, were also identified on young trees (Evidente *et al.*, 2005; Henriques *et al.*, 2012).

Furthermore, the infection of *Diplodia corticola* (anamorph of *Botryosphaeria corticola*) is causing serious and negative impacts on cork oak stress-declined forests, being the main pathogenic agent of bot canker (Fernandes *et al.*, 2014; reviewed by Félix *et al.*, 2017). *D. corticola* has been reported as more virulent than *B. mediterranea,* causing significant ecological and economic issues (Linaldeddu *et al.*, 2009). In cork oak trees, *D. corticola* causes progressive bark necrosis, growth of cambium and epicormic shoots, and leaves discoloration (Campanile *et al.*, 2007). The pathogenesis mechanism is still not fully understood, but secondary metabolites, such as phytotoxins, degradative and oxidative enzymes, and cytotoxic proteins, are suggested to play a role in infection (Fernandes *et al.*, 2014; Masi *et al.*, 2015; Félix *et al.*, 2017).

*B. mediterranea* and *D. corticola* cause severe economic losses and limited preventive measures for cork oak charcoal disease and bot canker are currently known, and thus good phytosanitary practices are the main mechanism adopted. Thiophanate-methyl and carbedazim fungicides have also been used against *D. corticola* but their potential harmful impact on human and environmental health is restricting their use (Luque *et al.*, 2008; Serrano *et al.*, 2015). Hence, new approaches are important for conserving the biodiversity and multifunctionality of these fragile, human-shaped ecosystems. In the era of sustainable crop production, the use of biological practices is becoming more common. Strategies like sustainable management practices, use of microbes or genetically engineered microbes to promote plant growth and use of biofertilizers have been currently adopted (Gouda *et al.*, 2018). Among these, soil microorganisms could be the way to achieve a sustainable agro-silvo-production.

### 1.2. Microbial communities associated with forest soils

A plant is not an individual. A well-regulated community of microorganisms is associated with the plant, which together comprise the "holobiont" (Rosenberg & Zilber-Rosenberg, 2016; Backer *et al.*, 2018). Microbial communities (archaea, bacteria, fungi, and protists) inhabiting the plant form the "plant microbiota" (Hassani *et al.*, 2018). Plants actively recruit these microorganisms, particularly from the nearby surrounding soil, which is a highly dynamic environment (Vandenkoornhuyse *et al.*, 2015; Compant *et al.*, 2019). In the soil-root interface, three different layers can be distinguished: (1)

rhizosphere - soil zone regulated by roots through the release of exudates that affect microbial activity; (2) rhizoplane - root surface that strongly binds soil particles; and (3) endosphere – the root internal tissues (Edwards *et al.*, 2018; Gouda *et al.*, 2018).

The plant–bacterial interactions occurring in the rhizosphere have an important role for plant growth and health (Compant *et al.*, 2019). Plant roots exude a huge diversity of organic nutrients and signals that attract different microbial populations, especially those able to metabolize plant-exuded compounds that will proliferate in this microbial habitat (Vacheron *et al.*, 2013). Consequently, the spatial heterogeneity in soil micro-environments can be explained by this dissimilar nutrient content or physical properties between different soil layers (Lladó & Baldrian, 2017). When compared to bulk soil, rhizosphere contains richer microbial populations (up to $10^{11}$ microbial cells per gram of root), due to the availability of large amounts of organic carbon released by plant roots (Berendsen *et al.*, 2012; Bakker *et al.*, 2013). This modulates the bacterial community composition, making the plant rhizosphere a preferred ecological niche for certain types of soil microorganisms, such as copiotrophic bacteria (Kachhap *et al.*, 2015; Ho *et al.*, 2017). These are fast growing microorganisms that prefer rich nutrient substrates and are sensitive to low moisture contents (Reis *et al.*, 2019). On the other hand, oligotrophic bacteria are mostly present in bulk soil, since they are well-adapted to lower substrate concentrations and low moisture contents (Ho *et al.*, 2017). Therefore, the plant can manage their microbiome, influencing the composition and function of the microbial community in the rhizosphere through root exudation, water and nutrient uptake by roots, respiration, and physicochemical changes in soil (Lladó *et al.*, 2017).

Undeniably, decoding the rhizosphere microbiome and related functions is essential for understanding the efficiency and dynamic of ecosystems. With the unquestionable upcoming climate changes, strategies to mitigate drought stress on forests have become crucial. Beneficial microorganisms have been used as important partners to prevent and tolerate plant drought stress (Backer *et al.*, 2018). Therefore, one of the strategies to mitigate cork oak's climatic stress could be the shaping of microbial communities, which has been reported to be very important for cork oak forests sustainability. Indeed, forest tree species, like cork oak, can create biotic interactions with a wide range of microorganisms from the rhizosphere, notably bacteria, and among these, Plant Growth-Promoting Rhizobacteria (PGPR; Mendes *et al.*, 2013; Maghnia *et al.*, 2019). When compared to mycorrhizal fungi, the bacterial communities from the Mediterranean cork oak forests are still poorly studied (Maghnia *et al.*, 2019), and studies on PGPR communities are scarce. As a matter of fact, PGPR studies are mainly focused on improving the plant production for feeding an increasing population while decreasing fertilizers use in

agriculture rather than in forestry. The lack of studies on forest PGPR can also be attributed to the longer life cycles that forest species hold against agriculture/horticulture species (García *et al.*, 2004). In any case, PGPRs are able to produce more vigorous forest plants. Recent studies concerning forest soils detected the regular presence of certain bacteria belonging to Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Acidobacteria phyla (Bevivino *et al.*, 2014; Lladó *et al.*, 2017; Reis *et al.*, 2019).

### 1.3. Importance and action mechanisms of Plant Growth-Promoting Rhizobacteria

Plant-associated bacterial communities comprise beneficial, neutral, or pathogenic microorganisms (Rout, 2014). PGPR are established in the rhizosphere and can benefit plant growth (Bhattacharyya & Jha, 2011). PGPR can commonly be divided into extracellular (ePGPR) and intracellular (iPGPR), according to their localization inside the plant root. The ePGPR are present inside root tissue, between cells of the root cortex spaces, but not inside the cells. On the other hand, iPGPR are present inside the root cells, and are generally able to produce specialized structures, called nodules (Gray & Smith, 2005). In general, PGPR are influenced by both biotic (plant genotypes, stage of plant development, plant defence mechanisms and other members of microbial community) and abiotic factors (soil composition, soil management and climatic conditions; Vacheron *et al.*, 2013).

The use of PGPR could play an important role in developing sustainable systems to promote plant growth, offering an attractive alternative of environmentally friendly control of plant diseases (Hayat *et al.*, 2010; Beneduzi *et al.*, 2012). PGPR mechanisms to promote plant growth are not fully understood, but include plant growth stimulation through plant hormones synthesis, action as biocontrol agents through inhibition of plant pathogens activity, plant nutrient supply, soil structure improvement and bioaccumulation (Figure 1.4; Hayat *et al.*, 2010). In general, plant growth promotion can be facilitated both through direct and indirect mechanisms (Figueiredo *et al.*, 2016).

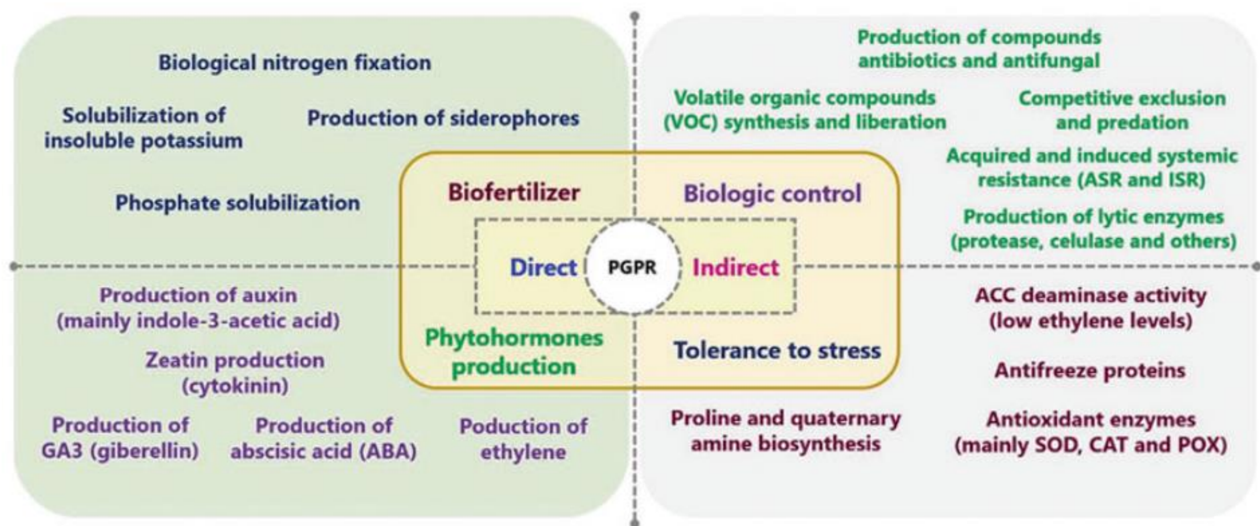


**Figure 1.4.** Schematic representation of the direct and indirect mechanisms used by PGPR (Figueiredo *et al.*, 2016).

### 1.3.1. Direct PGPR Mechanisms

PGPR directly promote plant development through the production of active compounds or by assisting plant nutrient supply (Beneduzi *et al.*, 2012). Bacterial important direct mechanisms include the production of phytohormones (such as auxins, gibberellins, cytokinins, ethylene, and abscisic acid), but also by increasing nutrient availability (Hayat *et al.*, 2010; Kaushal & Wani, 2015; Figueiredo *et al.*, 2016; Gouda *et al.*, 2018).

Phosphate is one of the major essential macronutrients required by plants for growth and development (Hayat *et al.*, 2010). This nutrient plays an important role in metabolic processes, such as in energy transfer, signal transduction, respiration, macromolecular biosynthesis, and photosynthesis (Gouda *et al.*, 2018). Soils generally contain a large amount of phosphate, but soluble phosphate concentration in soil is very low, usually 1 ppm or less, and only a small portion can be absorbed by plants (Vacheron *et al.,* 2013). Therefore, bacteria capable of dissolving this nutrient are crucial in soil enrichment (Rodríguez & Fraga, 1999; Sari & Fitri, 2019). PGPR can convert insoluble phosphates into monobasic ($H_2PO_4^-$) and dibasic ($HPO_4^{-2}$) phosphate ions, and thus enable plants to absorb this nutrient (Rodríguez & Fraga, 1999; Hayat *et al.*, 2010). Low molecular weight organic acids, synthesized by various soil bacteria, solubilize phosphates by chelating the cations bound to phosphate (Gouda *et al.*, 2018). Additionally, as a result of acidification of the medium through exudation of these compounds, PGPR can promote the mineralization of other crucial micro- and macronutrients that otherwise would

not be available for the plant (Sayyed *et al.*, 2012). PGPR can also solubilize phosphates through the production of phosphatases or phytases that hydrolyse organic forms of phosphate compounds (Vacheron *et al.*, 2013). Accordingly, many PGPR species – such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Microccocus*, *Aerobacter*, *Flavobacterium,* and *Erwinia* – can solubilize insoluble phosphate compounds, for instance, tricalcium phosphate, dicalcium phosphate, hydroxyl apatite, and rock phosphate (Gügi *et al.*, 1991; Skraly & Cameron, 1998; Rodríguez & Fraga, 1999; Hayat *et al.*, 2010).

Iron (Fe) is another essential micronutrient for almost all living organisms, but its availability is frequently limited, being commonly present in nature in its insoluble form – the ferric ion ($Fe^{3+}$; Ferreira *et al.*, 2019). To counteract this, PGPR can secrete siderophores, which are low-molecular weight secondary metabolites involved in chelating ferric iron from the environment (Arora & Verma, 2017; Gouda *et al.*, 2018). Hence, a potent siderophore, such as the ferric-siderophore complex, is reported to play an important role in iron uptake by plants (Beneduzi *et al.*, 2012). In addition, siderophore production may also be considered as an indirect defensive mechanism, protecting the plant from phytopathogens (Beneduzi *et al.*, 2012). Indeed, deleterious microorganisms are inhibited in rhizosphere by siderophore-producing PGPR due to iron starvation or competitive exclusion (Arora & Verma, 2017). Siderophore production is very common among *Pseudomonads*, *Frankia*, and *Streptomyces* spp. (Hayat *et al.*, 2010).

### 1.3.2. Indirect PGPR Mechanisms

PGPR are also indirectly involved in plant growth promotion by reducing or preventing harmful effects from phytopathogenic organisms and by producing repressive compounds that increase natural host resistance (Beneduzi *et al.*, 2012; Gouda *et al.*, 2018). When under attack, plants actively select specific microorganisms capable of supressing diseases, either through the production of enzymes or compounds with antimicrobial activity, or by promoting the so-called Induced Systemic Resistance (ISR) against various pathogens and pests, among other mechanisms (Bakker *et al.*, 2013; Gouda *et al.*, 2018). Undeniably, there are PGPR capable of acting as biocontrol agents. PGPR can produce chemical compounds, such as hydrogen cyanide (HCN), which is significantly toxic against phytopathogens (Rijavec & Lapanje, 2016), through inhibition of many metalloenzymes (Haas & Défago, 2005; Rijavec & Lapanje, 2016). PGPR are also involved in controlling fungal growth by producing fungal cell wall-degrading enzymes, such as chitinase and ß-1,3-glucanase (Hayat *et al.*, 2010; Gouda *et al.*, 2018). Moreover, antibiotics production by PGPR against several plant pathogens is currently considered one of the most

effective and most studied biocontrol mechanisms (Gouda *et al.*, 2018). Their activity is dose-dependent and, if present in high concentrations, antibiotics are known to eliminate other bacteria. However, when present in sub-lethal concentrations, they can have other effects, such as control of bacterial biofilm formation, motility, and biosynthetic pathways (Besset-Manzoni *et al.*, 2018). PGPR can also produce bacteriocins, which differ from traditional antibiotics by their relatively particular killing range, being only toxic to bacteria closely related to the producing strain (Beneduzi *et al.*, 2012). Therefore, PGPR can act as biological control agents by producing the previous mentioned growth inhibitors (*e.g.* antibiotics, bacteriocins, siderophores, and lytic enzymes; Jetiyanon & Kloepper, 2002). On the other hand, PGPR can additionally induce an indirect defensive approach by stimulating the plant biosynthesis of active pathogen suppressive compounds (Ongena *et al.*, 2000; Yang *et al.*, 2009). Rhizobacteria belonging to the genera *Arthrobacter, Azospirillum, Azotobacter, Bacillus, Serratia, Burkholderia, Enterobacter, Klebsiella, Pseudomonas, Rhodococcus* and *Streptomyces*, have been reported to antagonize plant pathogens (Tariq *et al.*, 2017).

Besides soluble molecules, PGPR also secret Volatile Organic Compounds (VOCs) that can shape microbial community around them, as well as influence the plant root development. These small molecules, with low molecular mass and high vapor pressure, have been described to improve plant growth, being able to inhibit pathogens and induce ISR (Vejan *et al.*, 2016; Besset-Manzoni *et al.*, 2018). VOCs may belong to different chemical families, such as alkenes, alcohols, benzenoids, aldehydes, ketones, or terpenes, and can directly or indirectly mediate disease resistance, abiotic stress tolerance, and plant biomass (Besset-Manzoni *et al.*, 2018; Gouda *et al.*, 2018). Approximately 350 bacterial species are known to produce around 846 different potential VOCs (Lemfack *et al.*, 2014). Several bacterial species from diverse genera, including *Stenotrophomonas*, *Serratia*, *Pseudomonas, Bacillus, Burkholderia, Erwinia, Agrobacterium, Staphylococcus* and *Xanthomonas,* have been reported to release VOCs (Santoro *et al.*, 2015). Moreover, VOCs produced by *Bacillus* spp. promote plant growth and health by preventing phytopathogens infection in *A. thaliana* seedlings (Ryu *et al.*, 2003; Xie *et al.*, 2009; Gutiérrez-Luna *et al.*, 2010). In the particular case of *Bacillus cereus, Bacillus simplex* and *Bacillus megaterium,* differential VOC emission has modulated both *A. thaliana* growth and root-system architecture, including morphologic root parameters, such as primary root length and incidence and lateral roots length and density (Gutiérrez-Luna *et al.*, 2010; Zou *et al.*, 2010).

### 1.4. Aims

PGPR are found to benefit plant fitness under biotic and abiotic stresses and thus could be a sustainable helpful tool in forestry systems. The main goal of this research was to select PGPR isolates presenting the best agronomic and forestry features that could enhance cork oak growth, even during environmental stressed events. PGPR from three distinct cork oak forests (Grândola, Limãos, and Ermida) displaying three different bioclimates (semi-arid, sub-humid, and humid, respectively) were isolated. To assess their potential interest, several biochemical features were evaluated, namely those associated with the availability of soil nutrients, and furtherly, their growth promotion and antagonistic/synergistic features (*i.e.,* PGPR skills as biofertilizers and biocontrol).

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# Chapter II: Isolation, biochemical characterization, and molecular identification of Plant Growth-Promoting Rhizobacteria

In this chapter, the biochemical characteristics displayed by bacteria isolated from three different cork oak forests are analysed. This characterization includes assessments for siderophores production, phosphate solubilisation, organic acids and HCN production. The most promising bacterial isolates presenting combined PGPR qualities were molecularly identified by sequencing of 16S rRNA gene and differences among bacterial communities between forests are discussed.

## 2.1. Methodology

### 2.1.1. Study sites and sample collection

Sampling occurred during the autumn season (November and December 2019) in Portugal, at three different geographic locations – Grândola (GR), Limãos (LI), and Ermida (ER) - presenting three distinct bioclimates (semi-arid, sub-humid, and humid, respectively; Figure 2.1; Table 2.1). Bioclimates were defined based on weather conditions, water availability levels, and local Emberger indexes (Rego & Rocha, 2014; Reis *et al.*, 2019; Table 2.1). The climatic parameter of Emberger ($Q$) accounts for the annual precipitation (P), maximal (M) and minimal (m) temperatures of the hottest and coldest months during the sampling year (Reis *et al.*, 2018).

From each sampled forest, soils were collected from three different trees, under the middle of cork oak canopy. The uppermost layer of soil that consists in plant litter and other organic material was removed before sampling and equal amounts three soil cores (with 5 cm in diameter and 10 cm in depth) were thoroughly mixed. Samples from the three trees were combined, resulting in a single sample from each forest. Soil samples were kept at 4 °C until processing.

**Figure 2.1**. Geographic distribution of sampled cork oak forests. Grândola (GR), Limãos (LI) and Ermida (ER) were selected based on the climatic parameter of Emberger (*Q*) and water availability conditions. Number of water drops means the bioclimate and water availability content – one drop (semi-arid), two drops (sub-humid) and three drops (humid) according to *Q*.

**Table 2.1**. Characterization of the geographic locations and environmental features of the cork oak sampling sites (adapted from Reis *et al.*, 2018). Averages of annual precipitation (P annual) over the past 30 years (1986–2016), precipitation in the months with the lowest (P min) and highest (P max) precipitation levels, annual temperature (T annual), and temperature of the coldest (T min) and hottest months (T max) were used to determine *Q*.

| **Location** | **Grândola (GR)** | **Limãos (LI)** | **Ermida (ER)** |
|---|---|---|---|
| **GPS location** | 38° 11´ 32.37" N<br>8° 37´ 11.41" W | 41° 31´ 51.54" N<br>6° 49´ 56.56" W | 41° 42´ 39.76" N<br>8° 6´ 14.87" W |
| **P annual (mm)** | 735.6 | 772.8 | 1448.4 |
| **P min (mm)** | 3.7 (July/August) | 15.4 (July) | 22 (July) |
| **P max (mm)** | 124.7 (December) | 121.6 (December) | 220.2 (December) |
| **T annual (° C)** | 16.6 | 15.0 | 12.7 |
| **T min (m) (° C)** | 10.1 (January) | 4.5 (January) | 9 (January) |
| **T max (M) (° C)** | 23.2 (August) | 21.7 (July/August) | 21.4 (July/August) |
| ***Q*** | 77.5<br>(semi-arid) | 88.9<br>(sub-humid) | 186.6<br>(humid) |
| **Forest system** | Montado | Sobreiral | Sobreiral |
| **Soil pH** | 6.01 | 5.10 | 4.97 |

### 2.1.2. Isolation of cork oak bacterial communities

To isolate cork oak soil PGPR, 1 g of each soil sample was transferred to a flask containing 10 mL of deionized water and subsequently stirred (Figure 2.2). Serial soil dilutions were prepared and 100 µL aliquots (from $10^0$ to $10^{-3}$ dilutions) were spread in triplicate onto YMA-CR (Yeast Extract Mannitol Agar-Congo Red) selective medium. Bacterial colonies were grown at 30 °C, 37 °C and 45 °C for 24 h and the number of CFUs (Colony Forming Units) were counted. Bacterial isolates obtained from each different temperature were subsequently grown on YMA-CR medium, at their respective growth temperature (30 °C, 37 °C or 45 °C) for 24 h and stored at 4 °C. In order to increase bacterial diversity of isolates, the bacterial colonies were selected based on different morphological characteristics (form, colour and elevation).

YMA-CR (per litre): 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $K_2HPO_4$, 1 g yeast extract, 10 g mannitol, 15 g agar, and 10 mL Congo red (Sobti *et al.*, 2015).

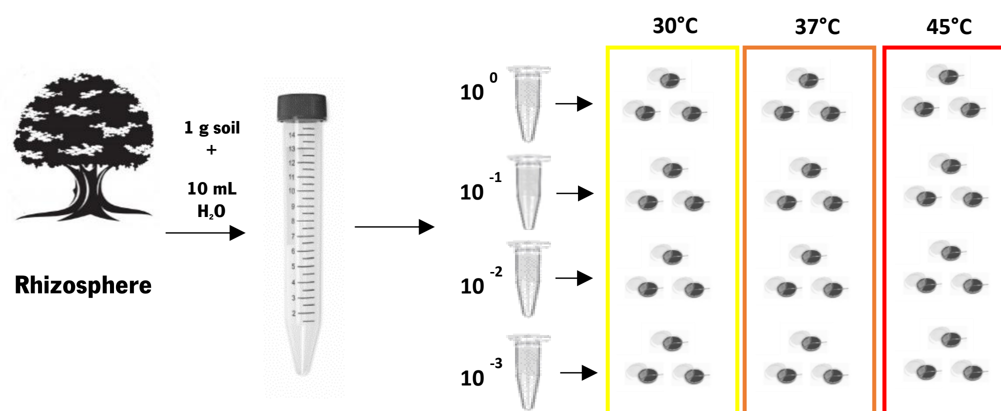



**Figure 2.2**. Experimental design for bacterial isolation. This experimental method was applied to samples from GR, LI and ER forests.

### 2.1.3. Biochemical characterization of bacterial isolates

Bacterial isolates were screened for siderophores production [using Chrome Azurol Sulphonate (CAS) agar medium], for phosphate solubilizing properties [using two different insoluble phosphate sources; Tricalcium Phosphate ($Ca_3PO_4$) and Aluminium Phosphate ($AlPO_4$) agar media], and for organic acids production [using Yeast Extract Manittol-Bromothymol blue (YMA-BB) agar medium]. Those bacterial

isolates that tested positive for these three biochemical assays were further screened for HCN producing ability [using Luria-Bertani (LB) agar medium supplemented with glycine].

#### 2.1.3.1. Siderophores Production

PGPR capability to scavenge iron using siderophores was evaluated by using CAS selective medium (Alexander & Zuberer, 1991). Bacterial isolates were streaked onto CAS agar medium. After 96 h of incubation at 30 °C, results were considered positive when bacteria induced an orange halo zone on CAS agar medium (bluish purple).

CAS agar medium was prepared as described by Alexander and Zuberer (1991). Four solutions were made. Solution 1 was obtained by a) Mixing 10 mL $FeCl_3 \cdot 6H_2O$ (1 mM) in HCl (10 mM); b) Preparing 50 mL of an aqueous solution of CAS (1.21 mg/mL); c) Preparing 40 mL of an aqueous solution of HDTMA (1.82 mg/mL); d) Mixing 10 mL of solution obtained in (a) with 50 mL of solution obtained in (b); e) Mixing solution (d) with 40 mL solution (c), gently and with constant stir; f) Autoclaving the final solution for 10 minutes at 121 °C, which was then cooled to 50 °C. Solution 2 was prepared by a) Preparing 750 mL of a salt solution containing 0.3 g $KH_2PO_4$, 0.5 g NaCl, and 1.0 g $NH_4Cl$; b) Dissolving 30.24 g of PIPES in the solution obtained in the previous step; c) pH was adjusted to 6.8, d) Adjusting volume to 800 mL with water; e) Adding 15 g of agar; f) Autoclaving for 10 minutes at 121 °C, which was then cooled to 50 °C. Solution 3 contained (per 70 mL): 2 g glucose, 2 g mannitol, 493 mg $MgSO_4 \cdot 7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4 \cdot H2O$, 1.4 mg $H_3BO_3$, 0.04 mg $CuSO_4 \cdot 5H_2O$, 1.2 mg $ZnSO_4 \cdot 7H_2O$ and 1.0 mg $Na_2MoO_4 \cdot 2H_2O$ and, lastly was autoclaved for 10 minutes at 121 °C, and then cooled to 50°C. Solution 4 comprised 30 mL filter-sterilized 10% (w:v) casamino acids; CAS agar medium was prepared by adding solution 3 to solution 2 along with solution 4, and lastly, with solution 1.

#### 2.1.3.2. Phosphate Solubilisation

Phosphate solubilizing microorganisms were screened using Pikovskaya (PVK) agar medium, supplemented with an insoluble phosphate complex ($Ca_3PO_4$ or $AlPO_4$) and Bromophenol blue (BPB; Pikovskaya, 1948; Mehta & Nautiyal, 2001). Bacterial isolates were streaked onto PBK-$Ca_3PO_4$ and PVK-$AlPO_4$ agar media, and after 96 h of incubation at 30 °C, results were positive when isolated PGPR strains induced a halo/clear zone on the selective media (greenish blue).

PVK-$Ca_3PO_4$ agar medium (per litre): 5 g $Ca_3PO_4$, 10.0 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.025 g BPB and 15 g agar. The pH of the medium was adjusted to 7 before autoclaving.

PVK-$AlPO_4$ agar medium (per litre): 5.0 g $AlPO_4$, 10.0 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.025 g BPB, and 15.0 g agar. The pH of the media was adjusted to 7.0 before autoclaving.

#### 2.1.3.3. Organic acids production

To detect organic acids production, bacterial isolates were streaked onto YMA-BB (Yeast Extract Mannitol Agar-Bromothymol Blue), and after 96 h of incubation at 30 °C, bacteria capable of medium acidification were identified when orange halos formed around colonies, against a blue coloured media (Sobti *et al.*, 2015).

> YMA-BB agar medium (per litre): 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $K_2HPO_4$, 1 g yeast extract, 10 g mannitol, 15 g agar and 5 mL BB.

### 2.1.4. HCN production assay

Bacterial isolates, which tested positive for all three previous biochemical tests (siderophores production, phosphate solubilizing properties and organic acids production), were streaked on LB agar medium supplemented with glycine, as described by Joseph *et al.* (2012). A filter paper soaked in picric acid (0.5%; w:v), and sodium carbonate (2%; w:v) was placed in the upper lid of the petri plate. Plates were incubated at 28 °C for 5 days. After this time changes in filter paper colour from yellow to orange, red or brown were considered positive.

> LB-GLY agar medium (per litre): 10 g NaCl, 10 g tryptone, 5 g yeast extract and 15 g agar and 4.4 g per litre glycine.

### 2.1.5. Molecular Identification of PGPR

#### 2.1.5.1. DNA extraction

PGPR isolates that tested positive for siderophores production, phosphate solubilizing properties, and organic acids production, were identified through molecular methods. These isolates were grown in liquid LB medium, at 30 °C for 24 h, and genomic DNA was extracted using the method described by Pitcher *et al.* (1989). DNA concentration was determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

> LB liquid medium (per litre): 10 g NaCl, 10 g tryptone, 5 g yeast extract.

#### 2.1.5.2. DNA amplification

Bacterial ribosomal subunit 16S gene was amplified using universal primers (*27F*: 5'-AGAGTTTGATCCTGGCTCAG-3' and *1492R*: 5'-GGTTACCTTGTTACGACTT-3'). Amplification of 16S region was performed using DFS-*Taq* DNA Polymerase (Bioron) and the thermocycling program: 94 °C for 7

min; 30 cycles of 94 °C (30 s), 50 °C (30 s) 72 °C (90 s), and a final extension step at 72 °C for 10 min. Amplification products were sequenced using *1492R* primer, at Macrogen (Amsterdam, The Netherlands). Obtained sequences were then blasted against available sequences (NCBI), using the BLAST algorithm. Identification was based on *e*-value, higher similarity identity and on ecological considerations.

#### 2.1.6. Data and statistical analyses

Differences between bacterial communities obtained from distinct forests, isolation temperatures and bioclimates were determined by Two-way ANOVA tests and Tukey's multiple comparison tests, using the Windows GraphPad Prism 6.01 program (GraphPad Software, La Jolla, CAUSE). Evolutionary distances between identified species and phylogenetic tree construction were based on the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). Comparisons were made using the Neighbor-Joining method (Saitou & Nei, 1987).

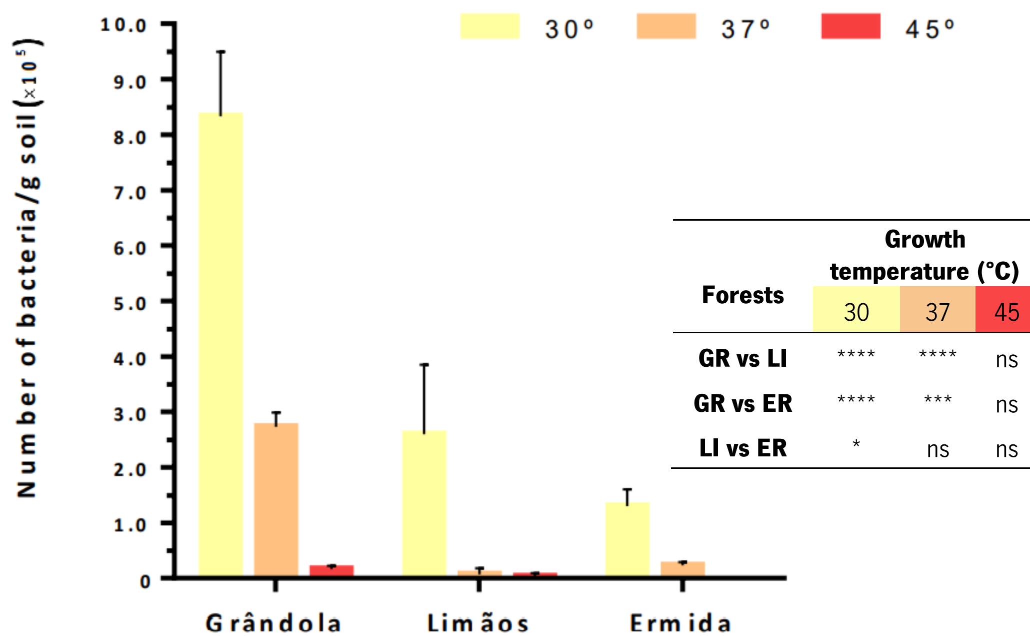
### 2.2. Results and Discussion

#### 2.2.1. Isolation of cork oak communities

Based on the number of CFUs, the titer (number of bacteria/g soil) of each forest soil was determined (Figure 2.3). Regardless of the temperature used for bacterial growth, soils obtained from GR forest always resulted in higher bacterial titers when compared to more humid forests. When comparing rainiest forests, LI revealed a significant higher titer at 30 °C than ER. No statistical significant differences between forests were observed when using a 45 °C incubation temperature.

For all sampled forests, the temperature that allowed a higher isolation of bacteria was 30 °C. In GR soils, when compared to the titer detected at 30 °C, there were 3-fold and 50-fold less bacteria that when using 37 °C and 45 °C incubation temperatures, respectively. The same trend was detected for LI and ER soils (13-fold and 26-fold less using 37 °C and 45 °C, respectively, for LI soils; and 6-fold less and no bacterial growth when using 37 °C and 45 °C, respectively, for ER soils). Therefore, most bacteria found in cork oak forests displayed higher growth at 30 °C. This agrees with the fact that most soil microorganisms are mesophilic, presenting the maximal growth temperatures between 25 °C and 35 °C (Brock *et al.*, 1994). In addition, GR had significantly increased bacterial growth at 30 °C and 37 °C when compared to the rainiest forests, which could suggest a morphological, anatomical, physiological, and molecular adaptation of microorganisms from these semi-arid lands to such stressful environmental

conditions. As a matter of fact, bacteria exposed to higher stress levels, caused by drought and/or high temperatures, have additional mechanisms to alleviate these pressures. Bacteria native from areas with limited water conditions are more qualified to deal with stress when compared to bacteria from irrigated areas (Marulanda-Aguirre *et al.*, 2008). Indeed, resistance to higher temperatures has resulted in adaptations of particular interest, such as the production of heat-stable enzymes, synthesis of heat shock proteins (known for their ability to lessen and protect cellular damage from increased temperatures), or even endospores formation (Tan *et al.*, 2013; Lladó *et al.*, 2017; Salazar-Badillo *et al.*, 2017). The presence of resistance spores (endospores) could have resulted in the higher titers found in GR soils.




| Forests | Growth temperature (°C) 30 | 37 | 45 |
|---|---|---|---|
| GR vs LI | **** | **** | ns |
| GR vs ER | **** | *** | ns |
| LI vs ER | * | ns | ns |

**Figure 2.3**. Titer of bacterial communities detected in each forest soil, according to bacterial growth temperatures used. Statistically significant differences are displayed on the side-table and significance levels are represented by * ($p \leq 0.05$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$); Non-significant differences are displayed with (ns).

In drier soils, motile bacteria are less likely to exploit nutrient resources, preventing less motile species from competition and supporting bacterial coexistence (Vos *et al.*, 2013; Carson *et al.*, 2010). Accordingly, studies conducted on *Eucalyptus maculata* forests soils describe that bacterial diversity and richness increases when water potential decreases (Carson *et al.*, 2010). Additionally, in the particular case of cork oak forests, drier bioclimates have been associated with more diverse and homogeneous soil bacterial communities (Reis *et al.*, 2019). Even though it was not possible to quantify diversity, this work also detected a higher bacteria abundance on driest soils.

To proceed to bacterial characterization, a subsample of bacteria was isolated for further studies. The isolation of distinct bacteria was promoted by selecting colonies with different morphological traits (visual colour, shape, and texture). A total of 324 single colonies were isolated, taking into consideration that, if possible, a maximum number of 50 bacterial isolates should be obtained from each soil and each isolation temperature (Table 2.2).

**Table 2.2.** Total number of isolates from each forest and each isolation temperature, selected to be used in subsequent studies.

| | **30 °** | **37 °** | **45 °** | **TOTAL** |
|---|---|---|---|---|
| **Grândola** | 40 | 40 | 24 | **104** |
| **Limãos** | 50 | 50 | 20 | **120** |
| **Ermida** | 50 | 40 | 10 | **100** |
| **TOTAL** | **140** | **130** | **54** | **324** |

### 2.2.2. Biochemical characterization of bacterial isolates

From a total of 7634 isolated colonies, 324 bacteria were isolated to be screened by biochemical assays (104 from GR, 120 from LI, and 100 from ER; Table 2.2). Detailed results for all tested bacterial isolates can be found in Annexes 1, 2 and 3.

Siderophores production was accomplished by most LI and ER isolated soil bacteria (55.70% and 55%, respectively), whereas only 46.15% GR bacteria were able to produce them (Figure 2.4A). Similar results were obtained for phosphate solubilisation with LI and ER soil bacteria presenting higher solubilising ability [55% and 57% for $Ca_3(PO_4)_2$, 65.83% and 52% for $AlPO_4$, in LI and ER, respectively] than GR bacterial isolates [42.31% for $Ca_3(PO_4)_2$ and 50.96% for $AlPO_4$]. When it comes to phosphate solubilizing properties, about 47% of all tested bacteria could solubilize at least one of the two forms of insoluble phosphate sources. Among these, 75% were able to solubilize both phosphate forms, whereas 17% and 8% only solubilized either $Ca_3(PO_4)_2$ or $AlPO_4$, respectively. Previous studies conducted with fungal strains have found that the phosphate solubilizing ability of microorganisms varied with phosphate substrates, and the strongest phosphate solubilisation was presented with $Ca_3(PO_4)_2$ as a source of insoluble phosphate, followed by $AlPO_4$. This could be attributed to the more complex structure of $AlPO_4$ when compared to $Ca_3(PO_4)_2$ (Majumder *et al.*, 2019). Acidification of media by $H^+$ production was a characteristic of the majority of GR soil bacteria (56.70%); however, it was not so evident in LI and ER

bacterial groups (42.50% and 46%, respectively). Concluding, both LI and ER soil bacterial groups presented the highest percentage of bacteria with siderophores production and phosphate solubilizing abilities, while the highest percentage of organic acids producing bacteria was registered in GR isolates (Figure 2.4A).

Bacterial isolates displaying the combination of these tested PGPR traits (51 isolates; 15.70% of total tested isolates; Figure 2.4B) were selected for further studies. Among these, GR forest contributed with half of selected isolates (25; 24% of total GR isolates), followed by ER and LI, that shared almost the same isolated number of isolates – 14 (14% of total ER isolates) and 12 isolates (10% of total LI isolates), respectively. Interestingly, as the water content decreases, there is an increase of bacterial isolates displaying all tested PGPR traits. From these 51 tested isolates, only one (8.3%; AJ11), isolated from LI forest soils, was capable of HCN production. Hence, besides siderophores production, phosphate solubilisation and organic acids production, this isolate also displayed a PGPR trait that suggests an antagonistic role.

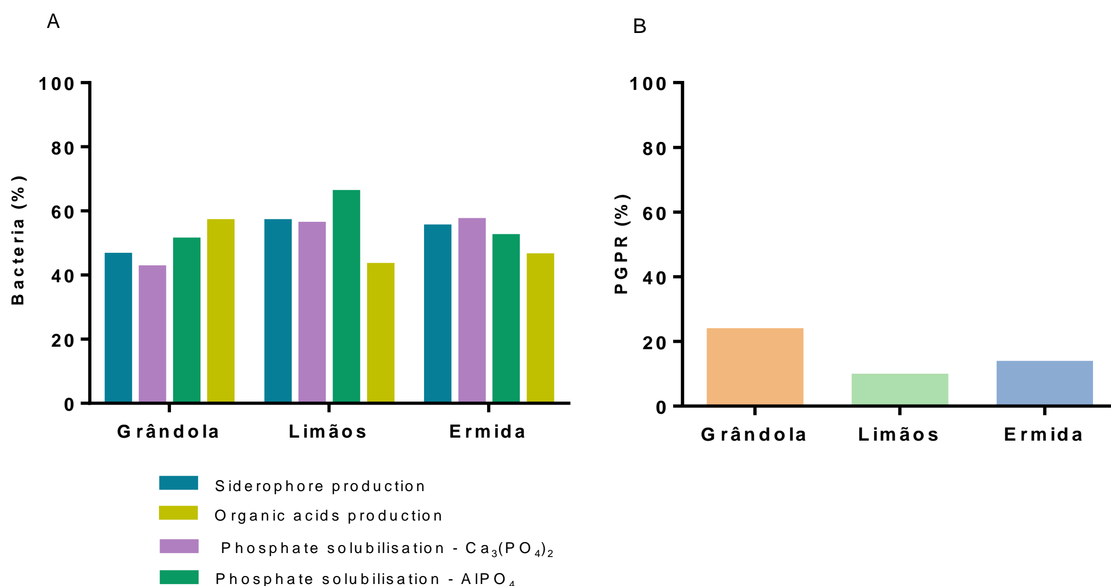



**Figure 2.4.** Percentage of bacteria, isolated from cork oak forests soils, displaying (A) individual or (B) all combined PGPR traits.

The dissimilar metabolic results observed in bacterial communities from different forests may be explained by their distinct forest systems, forest use, different human disturbances and cover vegetation, and different soil characteristics like pH and texture (Reis *et al.*, 2018). LI and ER have more similar forest systems – *sobreirais* – with high tree density and wild forest organization, whereas GR forests – *montados*

– are typically extensive agro-silvo-pastoral systems used for cork production and pasture. Conversely, the bioavailability of certain micronutrients in soils has been associated with stimulation of certain bacterial taxa capable of optimizing specific nutrient metabolizers (Ahmed & Holmström, 2014). This agrees with the so-called "cry-for-help" hypothesis, based on root exudation chemistry for the recruitment of required plant beneficial microbiomes (Rolfe *et al.*, 2019). Undeniably, plants have the ability to release primary and secondary metabolites, such as carbohydrates, amino acids, organic acids and membrane lipids through their roots. The concentration and composition of these compounds in root exudates varies upon exposure to stress and, ultimately, modulates the soil microbiome.

The obtained results raised several questions. Could the differences detected in the bacterial biochemical traits be correlated with the bioavailability of certain nutrients in those forest soils? Are the lower levels of bacterial siderophores production and phosphate solubilisation detected in GR forests indicative of less bioavailability of iron and phosphate in those soils? Are the environmental-stressed cork oaks from GR modulating their microbiome, explaining why these forests revealed a higher combination of tested PGPR traits? Additional studies are required to answer these queries.

#### 2.2.3. Molecular Identification of the Isolates

All 51 bacterial isolates were identified using molecular methods. Therefore, DNA was extracted from all isolates and presented high quantity and good quality for further PCR amplification. In order to identify each bacterial isolate, 16S region of bacterial DNA was amplified. From 51 initial bacterial DNAs, only 43 resulted in good amplification patterns and were sent to sequencing services. The remaining bacterial isolates will be further named *Unidentified*. From the 43 samples sent to sequencing services, 42 were successfully identified up to at least genera level (Table 2.3). One 16S sequence (AJ46) did not present any result when blasted against public database (hereafter named *Unknown*).

Sequenced Operational Taxonomic Units (OTUs) mainly belonged to *Firmicutes* phylum (24 OTUs; 55.81%), followed by *Proteobacteria* (18 OTUs; 41.86%). *Firmicutes* was only represented by *Bacillaceae* family, while *Proteobacteria* phylum registered three bacterial families [*Enterobacteriaceae* (11 OTUs), *Yersiniaceae* (6 OTUs) and *Pseudomonadaceae* (1 OTU)]. *Bacillaceae* was exclusively represented by the *Bacillus* genus (24 OTUs) belonging to five species, most of them identified as *Bacillus megaterium* (18 OTUs; 75.00% of *Bacillus* OTUs). *Enterobacteriaceae* (11 OTUs) was represented by three genera (*Cedecea, Klebsiella,* and *Ewingella*), *Yersiniaceae* (6 OTUs) by two genera (*Rouxiella* and *Serratia*) and *Pseudomonadacae* (1 OTU) by *Pseudomonas mohnii*.

**Table 2.3.** Identification of the 43 sequenced bacterial isolates. Identification was performed based on sequencing of bacterial barcode 16S. Obtained sequences were blasted against available sequences (NCBI), using the BLAST algorithm. The best BLAST-hit was used for OTU identification which was based on *e*-value, higher similarity identity and on ecological considerations. Information regarding isolation forest, bacterial isolation temperature and respective code is given.

| Forest | Isolation temperature (°C) | Code | Family | BLAST-HIT | *e*-value | % Identity |
|---|---|---|---|---|---|---|
| GR | 30 | AJ40 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 95.80 |
| | | AJ41 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 92.53 |
| | 37 | AJ42 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.69 |
| | | AJ43 | *Enterobacteriaceae* | *Klebsiella aerogenes* | 0.0 | 97.20 |
| | | AJ44 | *Enterobacteriaceae* | *Klebsiella aerogenes* | 0.0 | 98.02 |
| | | AJ45 | *Enterobacteriaceae* | *Klebsiella aerogenes* | 0.0 | 96.19 |
| | | AJ46 | *Unknown* | *Unknown* | - | - |
| | | AJ47 | *Bacillaceae* | *Bacillus megaterium* | 6.00e-139 | 79.63 |
| | | AJ48 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 93.26 |
| | | AJ49 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 98.96 |
| | | AJ50 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.45 |
| | | AJ51 | *Unidentified* | *Unidentified* | - | - |
| | | AJ52 | *Bacillaceae* | *Bacillus* sp. | 0.0 | 98.15 |
| | | AJ53 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 98.01 |
| | 45 | AJ54 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.94 |
| | | AJ55 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 96.49 |
| | | AJ56 | *Unidentified* | *Unidentified* | - | - |
| | | AJ57 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.69 |
| | | AJ58 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.47 |
| | | AJ59 | *Unidentified* | *Unidentified* | - | - |
| | | AJ60 | *Unidentified* | *Unidentified* | - | - |
| | | AJ61 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 85.52 |
| | | AJ62 | *Unidentified* | *Unidentified* | - | - |
| | | AJ63 | *Unidentified* | *Unidentified* | - | - |
| | | AJ64 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 96.35 |
| LI | 30 | AJ10 | *Unidentified* | *Unidentified* | - | - |
| | | AJ11 | *Yersiniaceae* | *Serratia quinivorans* | 0.0 | 97.07 |
| | | AJ14 | *Enterobacteriaceae* | *Cedecea neteri* | 0.0 | 97.47 |
| | 37 | AJ8 | *Enterobacteriaceae* | *Cedecea* sp. | 0.0 | 92.73 |
| | | AJ9 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 95.10 |
| | | AJ12 | *Enterobacteriaceae* | *Cedecea neteri* | 0.0 | 97.07 |
| | | AJ13 | *Unidentified* | *Unidentified* | - | - |
| | | AJ15 | *Enterobacteriaceae* | *Cedecea neteri* | 0.0 | 98.13 |
| | | AJ16 | *Enterobacteriaceae* | *Cedecea neteri* | 0.0 | 98.44 |
| | | AJ17 | *Bacillaceae* | *Bacillus megaterium* | 6.00e-70 | 80.21 |
| | | AJ18 | *Bacillaceae* | *Bacillus simplex* | 0.0 | 96.37 |
| | 45 | AJ19 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 95.32 |

**Table 2.3 (cont.)**

| Forest | Isolation temperature (°C) | Code | Family | BLAST-HIT | *e*-value | % Identity |
|---|---|---|---|---|---|---|
| **ER** | **30** | AJ21 | *Yersiniaceae* | *Rouxiella badensis* | 5.00e-171 | 85.15 |
| | | AJ22 | *Yersiniaceae* | *Rouxiella sp.* | 0.0 | 97.31 |
| | | AJ23 | *Bacillaceae* | *Bacillus cereus* | 0.0 | 97.99 |
| | | AJ24 | *Bacillaceae* | *Bacillus cereus* | 0.0 | 95.28 |
| | | AJ25 | *Pseudomonadaceae* | *Pseudomonas mohnii* | 0.0 | 96.34 |
| | | AJ26 | *Bacillaceae* | *Bacillus cereus* | 0.0 | 87.80 |
| | | AJ27 | *Enterobacteriaceae* | *Ewingella americana* | 0.0 | 98.38 |
| | | AJ28 | *Enterobacteriaceae* | *Ewingella americana* | 0.0 | 98.07 |
| | | AJ29 | *Yersiniaceae* | *Rouxiella* sp. | 0.0 | 96.18 |
| | **37** | AJ30 | *Yersiniaceae* | *Rouxiella sp.* | 0.0 | 97.71 |
| | | AJ31 | *Enterobacteriaceae* | *Klebsiella oxytoca* | 0.0 | 91.68 |
| | | AJ32 | *Yersiniaceae* | *Serratia* sp. | 3.00e-123 | 77.50 |
| | **45** | AJ33 | *Bacillaceae* | *Bacillus megaterium* | 0.0 | 97.18 |
| | | AJ34 | *Bacillaceae* | *Bacillus nakamurai* | 0.0 | 98.48 |

*Bacillus* was the only genus present in all sampled forests (Figure 3.5), which is corroborated by the ubiquitous presence of *Firmicutes* species in forestry systems (Bevivino *et al.*, 2014). Contrary to LI and ER, which presented exclusive genera, GR did not register any exclusive genus. However, *Bacillus* genus was recurrently found in all three sampled forest (Table 2.3). As discussed before, microbial communities from semi-arid environments could have been selected to tolerate low water availability by displaying characteristics that turns them more resistant to drought (*e.g.* endospores production). Indeed, *Bacillus* is regularly isolated from arid, semi-arid and desert bioclimates (Egamberdiyeva, 2005; Hernandez *et al.*, 2009; Moreno *et al.*, 2012; Hanna *et al.*, 2013). As Gram-positive bacteria, *Bacillus* species can produce endospores that increase its resilience in extreme conditions, such as water deficiency, high temperatures and high levels of UV radiation (Tan *et al.*, 2013). Interestingly, *Bacillus megaterium* was the most frequently species identified in GR forest (14 OTUs; Table 2.3).

More identified OTUs were detected across an increasing water availability gradient, with more humid forests presenting more bacterial genera identified. Although some studies suggest that bacterial richness and diversity increases with increase of drought stress (Carson *et al.*, 2010; Reis *et al.*, 2019), others describe that water availability is positively correlated with richness, diversity, and abundance of communities (Bachar *et al.*, 2010). Bachar *et al.* (2010) also considered that bacterial diversity is independent of precipitation gradient and community composition was found to be unique to each ecosystem in Mediterranean environments. However, in the present work, the initial bacterial communities were put through a series of biochemical selection processes that, by assembling a specific bacterial niche, do not allow to quantify the forest natural diversity.

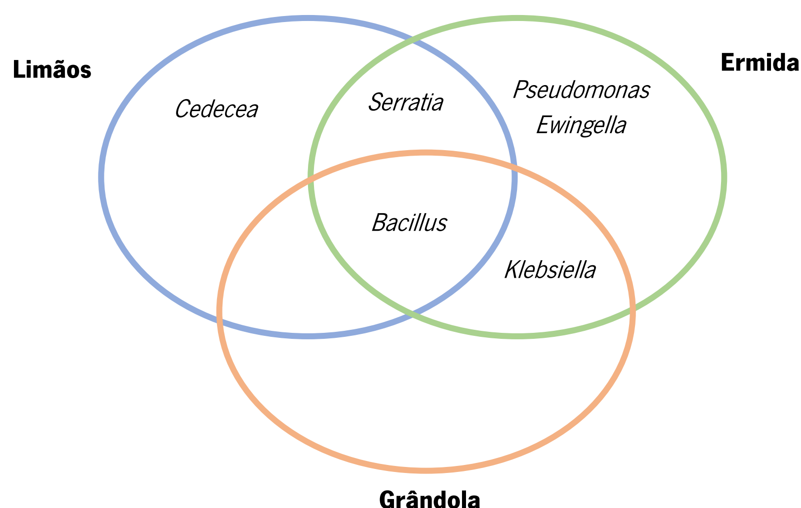



**Figure 2.5.** Venn diagram depicting the unique and overlapping genera present in the studied cork oak forests.

Phylogenetic analysis of identified bacterial species was performed to analyse the pattern of bacterial distribution according to cork oak forests (Figure 2.6). Phylogenetic tree presented two clades, one with similarities to species from *Bacillus* genus (clade 1), and the other with similarities to species from other genera (clade 2). However, isolate AJ61, *Bacillus megaterium*, oddly clustered in clade 2, probably due to misleading data from the ends of sequencing fragments. Hence, twenty-four OTUs were considered within the *Bacillus* clade, which was sub-divided into three subclades. Furthermore, AJ46 isolate, previously named as *Unknown*, clustered into subclade 1.2, making it a potential member of *Bacillus* genus, closely related to *B. megaterium* species. This may be in line with previous information reporting that *Bacillus* species are known for frequently exchange of genetic material in natural environments (Donnarumma *et al.*, 2010).

Phylogenetic results revealed that clustering patterns do not seem to be related with forest geographic location from where bacteria were isolated. In previous studies, performed in the Mediterranean region, forests with distinct land uses and soil management displayed different microbial communities (Bevivino *et al.*, 2014), contradicting these results. To ensure better discriminant genetic features, additional phylogenetic analysis regarding other genomic data, such as 23S rRNA gene or RNA polymerase β-subunit encoding gene (*rpo*B; Ludwig & Schleifer, 1994; Ko *et al.*, 2007) could be performed.

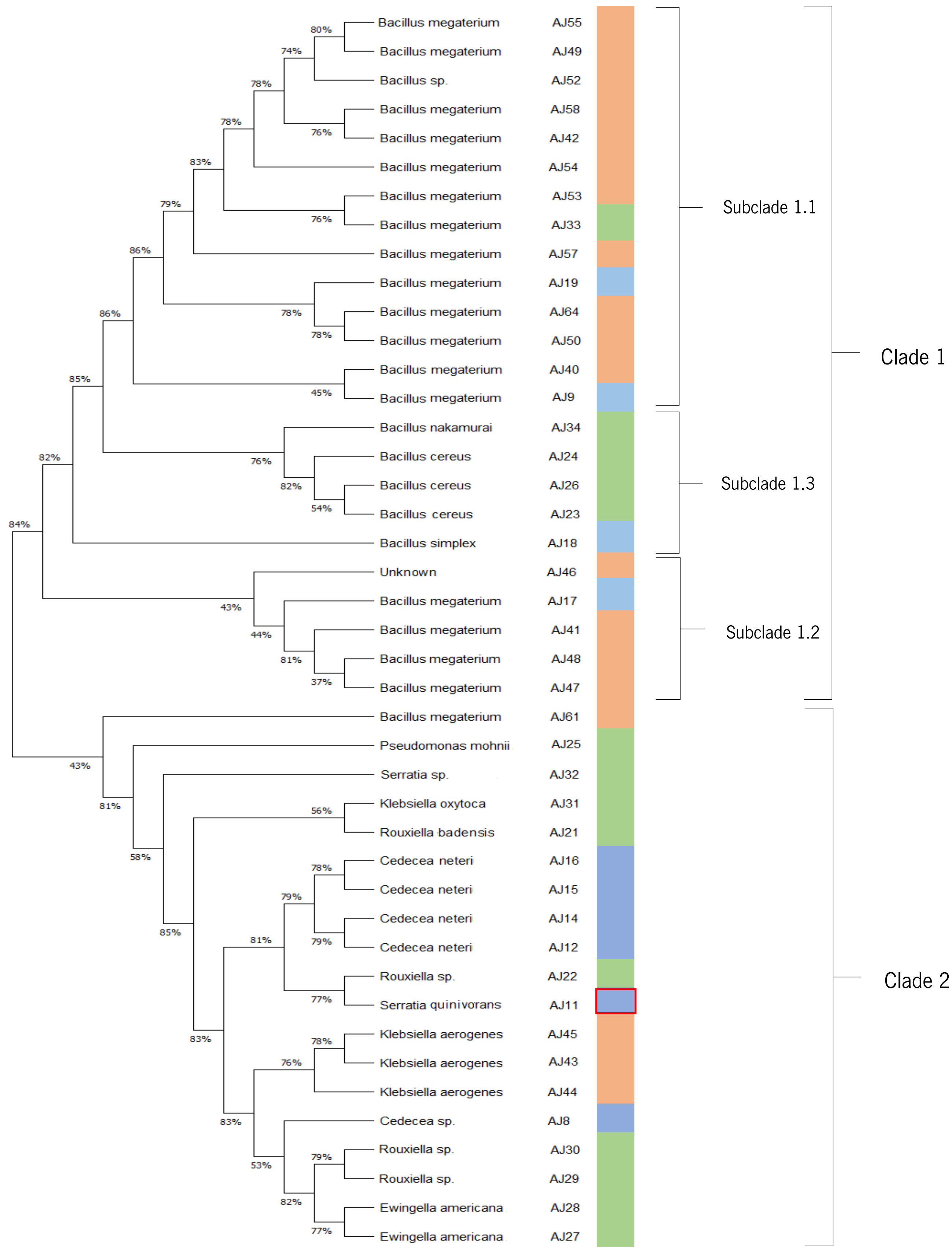



**Figure 2.6.** Phylogenetic tree of identified OTUs. Isolates obtained from GR are highlighted in orange, whereas isolates from LI and ER are highlighted in blue and green, respectively. All these isolates are capable of siderophores production, phosphate solubilisation and organic acids production. The only isolate with HCN production ability is highlighted with a red square.

## 2.3. Conclusion

This chapter allowed us to understand that PGPR communities are different in each forest. Beyond distinct climatic parameters, the studied forests also presented distinct forest systems, forest use, human disturbances and vegetation covers, as well as different soil physical-chemical features (Reis *et al.*, 2018). Bacteria from semi-arid forest GR seemed to be better adapted to higher temperatures and low nutrient availability conditions. Soils from this forest resulted in higher number of bacteria when using high incubation. Moreover, GR soil bacteria also revealed an increased number of PGPR traits. Even though there was a differential bacterial presence within forests, the *Bacillaceae* family was the most abundant among sampled forests. At the end, it was possible to hypothesise that microbial communities from semi-arid environments are adapted to better cope with abiotic stresses. Bacteria belonging to *Bacillus* genus could hold promising results at enhancing plant development even under stressful environmental circumstances. *Serratia quinivorans* isolate could be used for studying antagonistic behaviour within cork oak ecosystems.

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# Chapter III: Effects of PGPR inoculation on root-system architecture of *Arabidopsis thaliana*

In this chapter, the effects of the previous 51 selected PGPR on *Arabidopsis thaliana* root-system architecture will be evaluated. PGPR can modify root-architecture and the structure of root tissues mainly through their ability to interfere with the plant hormonal balance and VOC production. Co-inoculation with PGPR has been connected with the reduction of primary root growth, while accentuating other root morphologic parameters, such as thickening of root and increasing lateral roots and root hairs (reviewed by Vacheron *et al.*, 2013).

## 3.1. Methodology

### 3.1.1. PGPR isolates

Those bacterial isolates (51 isolates) that were previously found to be capable of siderophores production, phosphate solubilisation and organic acids production were selected and grown in liquid LB medium, at 30°C, for 24 h. PGPR growth was evaluated by densitometry ($OD_{600}$) and adjusted to 1.0.

### 3.1.2. PGPR co-inoculation with *A. thaliana*

*A. thaliana* (ecotype Columbia; Col-0) seeds were sterilized by submersion on solution of ethanol (70%; v:v) with Sodium Lauryl Sulfate (SDS; 0.05%; w:v) for 3-5 minutes, followed by immersion in ethanol (100%) for 10-20 s (adapted from Gutiérrez-Luna *et al.*, 2010). Eight seeds were plated on a straight line in Murashige and Skoog (MS) agar medium, 1 cm from the upper end of the plate. Seeds were stratified for 48 h at 4 °C. Plates were then vertically incubated at 21 °C, with a fixed photoperiod of 16 h of light and 8 h of darkness, for 96 h. A bacterial suspension (200 µL; $OD_{600}$ = 1) was placed in a parallel line, about 5 cm away from the seeds. Plates with no bacterial inoculation were used as control. Each treatment and control were performed in quadruplicate (51 PGPR x 4 plates x 8 seeds).

> MS agar medium (per liter): 4.302 g MS, 0.5 g 2-(N-morpholino) ethanesulfonic acid (MES), 15 g sucrose and 12 g agar. pH was adjusted to 5.7 before agar addition. Solution was autoclaved.

### 3.1.3. Evaluation of root morphological parameters

Root morphological parameters, including primary root length, number of lateral roots and root hair presence, were evaluated and data collected for statistical analysis at 3, 6 and 9 days post inoculation (dpi).

#### 3.1.4. Data and statistical analyses

Differences in primary root length and number of lateral roots were determined along time, considering each PGPR, forest and temperature used for bacterial isolation by One-way ANOVA tests, followed by Dunnett's multiple comparison tests, using the Windows GraphPad Prism 6.01 program (GraphPad Software, La Jolla, CAUSE). Statistical analysis of root hair presence (nominal variable) was performed using analysis tools in the Microsoft Excel program.

### 3.2. Results and Discussion

The effect of the 51 bacterial isolates on *A. thaliana* primary root architecture was evaluated by a dual culture method. To better understand the dynamics along time, root parameters were measured at 3, 6 and 9 days after PGPR inoculation. The specific effects of each strain (51 isolates) on root structure was evaluated individually and combined by forest and isolation temperature and compared to controls over time.

#### 3.2.1. PGPR effects on primary root length

In the presence of the majority of PGPR (70.59%), *A. thaliana* primary root length was significantly lower after 3 dpi ($p \leq 0.05$; Table 3.1; Annex 4). PGPR capable of inducing higher differences ($p \leq 0.0001$) on primary root length were identified as *Bacillus* (45.46%), *Unidentified* (18.18%), *Cedecea* (18.18%), among others (4.55%). Interestingly, all tested *Cedecea neteri* significantly ($p \leq 0.0001$) decreased primary root length at 3 dpi. Along time, there was a substantial decrease of PGPR significant suppressive effects on root length (41.17% at 6 dpi and 45.10% at 9 dpi; $p \leq 0.05$). At 6 dpi, PGPR with greater significant effects ($p \leq 0.001$) belonged to *Bacillus* (19.05%), *Unidentified* (19.05%), *Cedecea* (9.52%) or *Serratia* (4.76%) genera. A similar profile was observed at 9 dpi.

About 41% of total tested PGPR displayed a consistent suppressive effect during the full inoculation period ($p > 0.05$). These selected PGPR were mainly isolated from GR (47.62%), followed by LI (28.57%) and ER (23.81%), and are highlighted in bold in Table 3.1. These isolates mainly belonged to *Bacillus*, *Unidentified* and *Cedecea* genera (38.09%, 23.80% and 19.05%, respectively). Other PGRP that presented similar behaviour belonged to *Serratia*, *Rouxiella* and *Ewingella* genera, comprising all together 19.06% of total tested isolates. Among all tested bacteria, those with the better outcomes

**Table 3.1.** PGPR effect on *A. thaliana* primary root length development over time - 3 dpi, 6 dpi and 9 dpi. Values for primary root length represent the mean of all replica (cm). Primary root growth induction (PRGI %) represents the percentage of primary root length (cm) when in co-inoculation with PGPR in relation to control. The effect of each PGPR on primary root length is visualized using a heat map, where the most inhibitory effects are displayed in red and the less inhibitory effects are displayed in green. Bacteria presenting a consistent and significant inhibitory behaviour are depicted in bold, and those with the better outcomes are highlighted with a black box. Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

| Forest | Temp (°C) | Code | Identification | Primary root length (cm) | | | PRGI (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 3 dpi | 6 dpi | 9 dpi | 3 dpi | 6 dpi | 9 dpi | Mean |
| **Control** | | | | 1.40 | 1.48 | 1.92 | 100 | 100 | 100 | 100 |
| GR | 30 | AJ40 | *Bacillus megaterium* | 1.30 | 1.41 | 1.84 | 92,86 | 95,27 | 95,83 | 94,65 |
| | | **AJ41** | ***Bacillus megaterium*** | **0.86****** | **0.75**** | **1.56**** | 61,43 | 50,68 | 81,25 | 64,45 |
| | 37 | AJ42 | *Bacillus megaterium* | 0.93** | 0.94 | 1.23 | 66,43 | 63,51 | 64,06 | 64,67 |
| | | AJ43 | *Klebsiella aerogenes* | 1.01* | 1.15 | 1.30 | 72,14 | 77,70 | 67,71 | 72,52 |
| | | AJ44 | *Klebsiella aerogenes* | 1.19 | 1.20 | 1.73 | 85,00 | 81,08 | 90,10 | 85,40 |
| | | AJ45 | *Klebsiella aerogenes* | 1.20 | 1.23 | 1.70 | 85,71 | 83,11 | 88,54 | 85,79 |
| | | AJ46 | *Unknown* | 1.45 | 1.36 | 2.04 | 103,57 | 91,89 | 106,25 | 100,57 |
| | | AJ47 | *Bacillus megaterium* | 0.92**** | 1.95 | 1.27 | 65,71 | 131,76 | 66,15 | 87,87 |
| | | AJ48 | *Bacillus megaterium* | 0.98* | 0.88 | 1.21 | 70,00 | 59,46 | 63,02 | 64,16 |
| | | AJ49 | *Bacillus megaterium* | 0.97** | 1.07 | 1.37 | 69,29 | 72,30 | 71,35 | 70,98 |
| | | **AJ50** | ***Bacillus megaterium*** | **0.97**** | **0.83*** | **1.23*** | 69,29 | 56,08 | 64,06 | 63,14 |
| | | **AJ51** | ***Unidentified*** | **1.04*** | **0.81**** | **1.24*** | 74,29 | 54,73 | 64,58 | 64,53 |
| | | AJ52 | *Bacillus* sp. | 1.06 | 1.06 | 1.30 | 75,71 | 71,62 | 67,71 | 71,68 |
| | | AJ53 | *Bacillus megaterium* | 0.86**** | 0.90 | 1.13** | 61,43 | 60,81 | 58,85 | 60,36 |
| | 45 | **AJ54** | ***Bacillus megaterium*** | **0.72****** | **0.55****** | **1.11*** | 51,43 | 37,16 | 57,81 | 48,80 |
| | | AJ55 | *Bacillus megaterium* | 0.98**** | 1.00 | 1.29 | 70,00 | 67,57 | 67,19 | 68,25 |
| | | AJ56 | *Unidentified* | 0.95** | 1.01 | 1.24 | 67,86 | 68,24 | 64,58 | 66,89 |
| | | **AJ57** | ***Bacillus megaterium*** | **0.77****** | **0.74***** | **1.06***** | 55,00 | 50,00 | 55,21 | 53,40 |
| | | **AJ58** | ***Bacillus megaterium*** | **0.69****** | **0.56****** | **1.10***** | 49,29 | 37,84 | 57,29 | 48,14 |
| | | **AJ59** | ***Unidentified*** | **0.76****** | **0.74***** | **1.17**** | 54,29 | 50,00 | 60,94 | 55,07 |
| | | **AJ60** | ***Unidentified*** | **0.74****** | **0.59****** | **1.09***** | 52,86 | 39,86 | 56,77 | 49,83 |
| | | AJ61 | *Bacillus megaterium* | 1.16 | 1.33 | 1.70 | 82,86 | 89,86 | 88,54 | 87,09 |
| | | AJ62 | *Unidentified* | 1.01* | 0.96 | 1.40 | 72,14 | 64,86 | 72,92 | 69,97 |
| | | **AJ63** | ***Unidentified*** | **0.73****** | **0.69***** | **1.09***** | 52,14 | 46,62 | 56,77 | 51,85 |
| | | **AJ64** | ***Bacillus megaterium*** | **0.79****** | **0.56****** | **1.10***** | 56,43 | 37,84 | 57,29 | 50,52 |
| LI | 30 | AJ10 | *Unidentified* | 1.11 | 1.13 | 1.54 | 79,29 | 76,35 | 80,21 | 78,62 |
| | | **AJ11** | ***Serratia quinivorans*** | **0.94***** | **0.90*** | **1.23*** | 67,14 | 60,81 | 64,06 | 64,01 |
| | | **AJ14** | ***Cedecea neteri*** | **0.87****** | **0.83**** | **1.15**** | 62,14 | 56,08 | 59,90 | 59,37 |
| | 37 | AJ8 | *Cedecea* sp. | 0.97 | 1.09 | 1.16 | 69,29 | 73,65 | 60,42 | 67,78 |
| | | AJ9 | *Bacillus megaterium* | 1.05 | 1.08 | 1.38 | 75,00 | 72,97 | 71,88 | 73,28 |
| | | **AJ12** | ***Cedecea neteri*** | **0.82****** | **0.79***** | **1.09***** | 58,57 | 53,38 | 56,77 | 56,24 |
| | | **AJ13** | ***Unidentified*** | **0.83****** | **0.77***** | **1.13**** | 59,29 | 52,03 | 58,85 | 56,72 |
| | | **AJ15** | ***Cedecea neteri*** | **0.90****** | **0.85**** | **1.22*** | 64,29 | 57,43 | 63,54 | 61,75 |
| | | **AJ16** | ***Cedecea neteri*** | **0.84****** | **0.77***** | **1.15**** | 60,00 | 52,03 | 59,90 | 57,31 |
| | | AJ17 | *Bacillus megaterium* | 1.16 | 1.18 | 1.61 | 82,86 | 79,73 | 83,85 | 82,15 |
| | | AJ18 | *Bacillus simplex* | 0.97** | 1.01 | 1.28 | 69,29 | 68,24 | 66,67 | 68,07 |
| | 45 | AJ19 | *Bacillus megaterium* | 1.06 | 1.09 | 1.47 | 75,71 | 73,65 | 76,56 | 75,31 |
| ER | 30 | AJ21 | *Rouxiella badensis* | 1.08 | 1.10 | 1.47 | 77,14 | 74,32 | 76,56 | 76,01 |
| | | **AJ22** | ***Rouxiella* sp.** | **0.91****** | **0.86**** | **1.24*** | 65,00 | 58,11 | 64,58 | 62,56 |
| | | AJ23 | *Bacillus mycoides* | 0.95*** | 0.94 | 1.29 | 67,86 | 63,51 | 67,19 | 66,19 |
| | | AJ24 | *Bacillus cereus* | 1.22 | 1.25 | 1.68 | 87,14 | 84,46 | 87,50 | 86,37 |
| | | AJ25 | *Pseudomonas mohnii* | 1.60 | 1.66 | 2.25 | 114,29 | 112,16 | 117,19 | 114,55 |
| | | AJ26 | *Bacillus cereus* | 0.91** | 0.88 | 1.43 | 65,00 | 59,46 | 74,48 | 66,31 |
| | | **AJ27** | ***Ewingella americana*** | **0.83****** | **0.89*** | **1.20*** | 59,29 | 60,14 | 62,50 | 60,64 |
| | | AJ28 | *Ewingella americana* | 1.11 | 1.23 | 1.41 | 79,29 | 83,11 | 73,44 | 78,61 |
| | | AJ29 | *Rouxiella* sp. | 0.90*** | 0.9 | 1.27 | 64,29 | 60,81 | 66,15 | 63,75 |
| | 37 | AJ30 | *Rouxiella* sp. | 0.91*** | 0.95 | 1.36 | 65,00 | 64,19 | 70,83 | 66,67 |
| | | AJ31 | *Klebsiella oxytoca* | 0.78**** | 0.86 | 0.96** | 55,71 | 58,11 | 50,00 | 54,61 |
| | | **AJ32** | ***Serratia* sp.** | **0.69****** | **0.75***** | **1.01***** | 49,29 | 50,68 | 52,60 | 50,86 |
| | 45 | **AJ33** | ***Bacillus megaterium*** | **0.51****** | **0.33****** | **0.94****** | 36,43 | 22,30 | 48,96 | 35,89 |
| | | **AJ34** | ***Bacillus nakamurai*** | **0.57****** | **0.48****** | **1.03****** | 40,71 | 32,43 | 53,65 | 42,26 |

were identified as *B. megaterium* (50%), *Unidentified* (20%), *B. nakamurai* (10%), *Serratia* sp. (10%) and *Klebsiella oxytoca* (10%). The majority of these (80%) shared a common feature: the isolation temperature of 45 °C. These isolates reduced more than 55% the length of the primary root and are highlighted with a black box in Table 3.1.

The reduction of primary root length is in line with previous reports indicating that many PGPR may reduce the growth of primary root (Dobbelaere *et al.*, 1999), where the auxin indole-3-acetic acid (IAA) is mainly pointed as the reason behind these effects. Normally, different IAA concentrations result in different types of root formations (primary or lateral roots, or root hairs; Meuwley & Pilet, 1991; Dobbelaere *et al.*, 1999). In the particular case of primary root, relatively low levels of IAA are required to induce primary root growth. The combination of IAA produced by the plant and the bacteria should be optimum to promote plant growth and will determine whether bacteria will stimulate or suppress plant growth. Therefore, according to the IAA availability in the plant, the influence of bacterial IAA on plant roots ranges from positive to negative (Etesami *et al.*, 2015). The synthesis of high quantities of this auxin by PGPR has been found to inhibit the growth of roots rather than to promote it (reviewed by Etesami *et al.*, 2015). Several bacteria from plant rhizosphere possess the ability to produce IAA (reviewed by Cohen *et al.*, 2015), including bacteria from *Bacillus* and *Pseudomonas* genera (Swain *et al.*, 2007; Hariprasad & Niranjana, 2009). This agrees with the high number of *Bacillus* spp. with inhibitory effects on primary root growth, 33.33% of total bacteria, and 47.06% of which with a strong suppressive effect. However, the production of IAA alone does not explain the growth-supressing abilities by PGPR (Xie *et al.*, 1996). Ethylene levels could also modulate root development and, when its concentration remains high after germination, root elongation was found to be inhibited (Etesami *et al.*, 2015). Indeed, IAA and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase are believed to work in combination to modulate root-architecture, where the bacterial IAA induces the synthesis of ACC, the immediate precursor of ethylene, ultimately resulting in higher ethylene levels (Riov & Yang, 1989). However, those PGPR that have an enhanced activity of ACC deaminase present lower ethylene levels by hydrolysing ACC, thus promoting primary root growth by IAA. Indeed, co-inoculation with PGPR that could both secrete IAA and synthesize ACC deaminase resulted in longer roots when compared to inoculation with bacteria that only secrete IAA (Etesami *et al.*, 2015). This may explain why bacteria, such as *Pseudomonas mohnii* AJ25, could induce non-significant primary root growth, with opposite effects when compared to the majority of tested PGPR. As a matter of fact, there have been several reports associating *Pseudomonas* spp. with ACC deaminase production (Gamalero *et al.*, 2008; Jalili *et al.*, 2009; Saikia *et al.*, 2018).

The *A. thaliana* root-system architecture was found to be modified in co-culture with different PGPR species, such as in the presence of *Bacillus* spp. *(B. megaterium, B. subtilis, B. amyloliquefaciens, B. cereus, and B. simplex*), *Pseudomonas fluorescens, Serratia odorifera* and *Enterobacter cloacae* (Ryu *et al*., 2003; López-Bucio *et al*., 2007; Vespermann *et al*., 2007; Gutiérrez-Luna *et al*., 2010). However, the *Bacillus* influence on primary root growth is still ambiguous, with some studies registering either a decrease (Dobbelaere *et al*., 1999; López-Bucio *et al*., 2007) or no effect on primary root growth (Gutiérrez-Luna *et al*., 2010). This suggests that the growth promoting effect of individual strains or isolates is specific (Gutiérrez-Luna *et al*., 2010), as it was observed particularly with *Bacillus* spp. in this study.

### 3.2.2. PGPR effects on lateral roots formation

The results revealed that there was a global positive effect of PGPR on *A. thaliana* lateral roots formation. At 3 dpi, induction of the number of lateral roots by PGPR was not meaningful, where 88.23% of PGPR showed no significant differences with control ($p > 0.05$; Table 3.2; Annex 5). Among the 6 PGPR isolates (11.77%) presenting significant differences ($p \leq 0.05$), the highest induction was registered by *Serratia* sp. (AJ32; $p \leq 0.001$). At 6 dpi, there was an increase of the number of PGPR capable of inducing lateral roots (78.43%; $p \leq 0.05$), where an *Unknown* isolate (AJ46; $p \leq 0.001$) revealed to be the best lateral root inducer. By 9 dpi, the percentage of PGPR able to induce significant ($p \leq 0.05$) number of lateral roots remained the same (78.43%), and *Unknown* isolate (AJ46) remained the PGPR with the greatest stimulating results. This isolate was previously found to be a potential member of *Bacillus* genus, closely related to *B. megaterium* species (Chapter II). Indeed, several *Bacillus* strains, including *B. megaterium* isolates have been found to mainly promote lateral root growth in *A. thaliana* (reviewed by Fincheira & Quiroz, 2018).

Although there were highly significant differences ($p \leq 0.0001$) registered during the entire assay, time was found to be a key factor, presenting an increasingly positive effect towards stimulation of lateral roots number. About 78.43% of PGPR isolates stimulated lateral root formation over time, but few (7.84%) induced the number of lateral roots since early stage of seedling development (3 dpi).

**Table 3.2.** PGPR effect on *A. thaliana* lateral roots development over time - 3 dpi, 6 dpi and 9 dpi. Values for number of lateral roots represent the mean of all replica. Lateral roots induction (LRI %) represents the percentage of seedlings with lateral roots when in co-inoculation with PGPR in relation to control. The effect of each PGPR on number of lateral roots is visualized using a heat map, where the most stimulating effects are displayed in green and the less stimulating effects are displayed in red. Bacteria presenting a consistent and significant promoting behaviour are depicted in bold, and those with the better outcomes are highlighted with a black box. Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

| Forest | Temp. (°C) | Code | Identification | Number of Lateral roots | | | LRI (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 3 dpi | 6 dpi | 9 dpi | 3 dpi | 6 dpi | 9 dpi | Mean |
| **Control** | | | | 0,01 | 0.25 | 0.94 | 100 | 100 | 100 | 100 |
| GR | 30 | AJ40 | *Bacillus megaterium* | 0.22 | 2.47**** | 5.66**** | 2200 | 988,00 | 602,13 | 1263,38 |
| | | AJ41 | *Bacillus megaterium* | 0.34 | 1.97* | 4.16** | 3400 | 788,00 | 442,55 | 1543,52 |
| | 37 | AJ42 | *Bacillus megaterium* | 0.25 | 2.33** | 4.96**** | 2500 | 932,00 | 527,66 | 1319,89 |
| | | AJ43 | *Klebsiella aerogenes* | 0.31 | 2.28*** | 4.19** | 3100 | 912,00 | 445,74 | 1485,91 |
| | | AJ44 | *Klebsiella aerogenes* | 0.13 | 2.47**** | 4.28*** | 1300 | 988,00 | 455,32 | 914,44 |
| | | AJ45 | *Klebsiella aerogenes* | 0.66 | 2.97**** | 6.88**** | 6600 | 1188,00 | 731,91 | 2839,97 |
| | | AJ46 | *Unknown* | 0.63 | 3.33**** | 7.80**** | 6300 | 1332,00 | 829,79 | 2820,60 |
| | | **AJ47** | ***Bacillus megaterium*** | **0.84**** | **2.47***** | **5.06****** | 8400 | 988,00 | 538,30 | 3308,77 |
| | | AJ48 | *Bacillus megaterium* | 0.21 | 1.96* | 3.71 | 2100 | 784,00 | 394,68 | 1092,89 |
| | | AJ49 | *Bacillus megaterium* | 0.4 | 1.72 | 4.0* | 4000 | 688,00 | 425,53 | 1704,51 |
| | | AJ50 | *Bacillus megaterium* | 0.47 | 2.16** | 5.0**** | 4700 | 864,00 | 531,91 | 2031,97 |
| | | AJ51 | *Unidentified* | 0.59 | 2.69**** | 6.19**** | 5900 | 1076,00 | 658,51 | 2544,84 |
| | | AJ52 | *Bacillus* sp. | 0.41 | 2.72**** | 5.09**** | 4100 | 1088,00 | 541,49 | 1909,83 |
| | | AJ53 | *Bacillus megaterium* | 0.78* | 2.31*** | 3.63 | 7800 | 924,00 | 386,17 | 3036,72 |
| | 45 | AJ54 | *Bacillus megaterium* | 0.08 | 2.50*** | 4.92**** | 800 | 1000,00 | 523,40 | 774,47 |
| | | AJ55 | *Bacillus megaterium* | 0.5 | 2.53**** | 5.03**** | 5000 | 1012,00 | 535,11 | 2182,37 |
| | | AJ56 | *Unidentified* | 0.36 | 1.60 | 3.80 | 3600 | 640,00 | 404,26 | 1548,09 |
| | | AJ57 | *Bacillus megaterium* | 0.21 | 1.92 | 4.29** | 2100 | 768,00 | 456,38 | 1108,13 |
| | | AJ58 | *Bacillus megaterium* | 0.19 | 2.94**** | 5.19**** | 1900 | 1176,00 | 552,13 | 1209,38 |
| | | AJ59 | *Unidentified* | 0.09 | 2.81**** | 4.94**** | 900 | 1124,00 | 525,53 | 849,84 |
| | | AJ60 | *Unidentified* | 0.03 | 2.69*** | 4.88**** | 300 | 1076,00 | 519,15 | 631,72 |
| | | AJ61 | *Bacillus megaterium* | 0.09 | 2.97**** | 6.31**** | 900 | 1188,00 | 671,28 | 919,76 |
| | | AJ62 | *Unidentified* | 0.67 | 2.63**** | 5.88**** | 6700 | 1052,00 | 625,53 | 2792,51 |
| | | AJ63 | *Unidentified* | 0.09 | 2.53**** | 4.97**** | 900 | 1012,00 | 528,72 | 813,57 |
| | | AJ64 | *Bacillus megaterium* | 0.28 | 2.66**** | 4.69**** | 2800 | 1064,00 | 498,94 | 1454,31 |
| LI | 30 | AJ10 | *Unidentified* | 0.125 | 1.69 | 4.13** | 1250 | 676,00 | 439,36 | 788,45 |
| | | AJ11 | *Serratia quinivorans* | 0.63 | 2.84**** | 5.59**** | 6300 | 1136,00 | 594,68 | 2676,89 |
| | | AJ14 | *Cedecea neteri* | 0.53 | 1.91* | 4.09** | 5300 | 764,00 | 435,11 | 2166,37 |
| | 37 | AJ8 | *Cedecea* sp. | 0.125 | 2.69*** | 4.0 | 1250 | 1076,00 | 425,53 | 917,18 |
| | | AJ9 | *Bacillus megaterium* | 0.06 | 1.25 | 4.19 | 600 | 500,00 | 445,74 | 515,25 |
| | | AJ12 | *Cedecea neteri* | 0.69 | 2.22*** | 4.66**** | 6900 | 888,00 | 495,74 | 2761,25 |
| | | **AJ13** | ***Unidentified*** | **0.81**** | **2.44****** | **4.66****** | 8100 | 976,00 | 495,74 | 3190,58 |
| | | **AJ15** | ***Cedecea neteri*** | **0.75*** | **1.91*** | **4.41***** | 7500 | 764,00 | 469,15 | 2911,05 |
| | | AJ16 | *Cedecea neteri* | 0.41 | 1.72 | 3.88* | 4100 | 688,00 | 412,77 | 1733,59 |
| | | AJ17 | *Bacillus megaterium* | 0.34 | 2.97**** | 5.31**** | 3400 | 1188,00 | 564,89 | 1717,63 |
| | | AJ18 | *Bacillus simplex* | 0.13 | 1.42 | 3.67 | 1300 | 568,00 | 390,43 | 752,81 |
| | 45 | AJ19 | *Bacillus megaterium* | 0.31 | 2.25* | 4.56* | 3100 | 900,00 | 485,11 | 1495,04 |
| ER | 30 | AJ21 | *Rouxiella badensis* | 0.09 | 1.53 | 4.16** | 900 | 612,00 | 442,55 | 651,52 |
| | | **AJ22** | ***Rouxiella* sp.** | **0.75*** | **2.75****** | **5.47****** | 7500 | 1100,00 | 581,91 | 3060,64 |
| | | AJ23 | *Bacillus mycoides* | 0.44 | 2.34*** | 4.88**** | 4400 | 936,00 | 519,15 | 1951,72 |
| | | AJ24 | *Bacillus cereus* | 0.28 | 3.25**** | 5.53**** | 2800 | 1300,00 | 588,30 | 1562,77 |
| | | AJ25 | *Pseudomonas mohnii* | 0.50 | 2.88**** | 7.41**** | 5000 | 1152,00 | 788,30 | 2313,43 |
| | | AJ26 | *Bacillus cereus* | 0.44 | 2.31* | 3.69 | 4400 | 924,00 | 392,55 | 1905,52 |
| | | AJ27 | *Ewingella americana* | 0.38 | 2.38*** | 3.42 | 3800 | 952,00 | 363,83 | 1705,28 |
| | | AJ28 | *Ewingella americana* | 0.19 | 2.13 | 4.94** | 1900 | 852,00 | 525,53 | 1092,51 |
| | | AJ29 | *Rouxiella sp.* | 0.67 | 2.54**** | 5.25**** | 6700 | 1016,00 | 558,51 | 2758,17 |
| | 37 | AJ30 | *Rouxiella sp.* | 0.38 | 2.21** | 4.25** | 3800 | 884,00 | 452,13 | 1712,04 |
| | | AJ31 | *Klebsiella oxytoca* | 0.50 | 1.25 | 2.06 | 5000 | 500,00 | 219,15 | 1906,38 |
| | | AJ32 | *Serratia* sp. | 1.0*** | 2.04* | 2.75 | 10000 | 816,00 | 292,55 | 3702,85 |
| | 45 | AJ33 | *Bacillus megaterium* | 0.0 | 0.25 | 3.38 | 0 | 100,00 | 359,57 | 153,19 |
| | | AJ34 | *Bacillus nakamurai* | 0.0 | 2.44**** | 4.47*** | 0 | 976,00 | 475,53 | 483,84 |

*B. megaterium* (AJ47), *C. neteri* (AJ15), *Rouxiella* sp. (AJ22) and *Unidentified* (AJ13) were those PGPR isolates presenting lateral root induction during all the experiment (presented in Table 3.2 in bold). In order to not underestimate PGPR with non-significant differences in the early stages of inoculation, but that still presented very favourable results by the end of the assay, lateral roots induction (LRI %) was calculated along time and in relation to control. It was possible to understand that besides the previous selected PGPR, *Klebsiella aerogenes* (AJ45), *Unknown* (AJ46), *Unidentified* (AJ51), *B. megaterium* (AJ53 and AJ61), *S. quinivorans* (AJ11), *C. neteri* (AJ12), *Rouxiella* sp. (AJ29) and *Serratia* sp. (AJ32) also highly induced lateral roots formation, promoting a mean percentage of more than 25-fold more lateral roots than control (highlighted with a black box in Table 3.2).

PGPR community is reported to optimize the plant root surface through the stimulation of *A. thaliana* lateral roots production (Contesto *et al.*, 2010). Indeed, the *in vitro* inoculation with efficient PGPR strains is known to induce morphogenetic responses of the root system, mainly by increasing the number and/or the length of plant lateral roots (Desbrosses *et al.*, 2009). The less evident results at earlier stages of plant development (3 dpi) may be explained by the small length of the primary root, which may difficult the induction of lateral root formation, as reported by Beemster & Baskin (1998). Moreover, at this phase of plant development and growth, gene expression is found to be mainly directed towards primary root growth, and only afterwards genes responsible for hormonal regulation are expressed, resulting in lateral roots formation (Beemster & Baskin, 1998). Also, the production of bacterial exogenous IAA controls a wide variety of processes in plant development and plant growth, and unlike primary root, lateral root development is stimulated through high IAA levels (Dobbelaere *et al.*, 1999; Patten & Glick, 2002; Perrig *et al.*, 2007; Vacheron *et al.*, 2013). This agrees with the bacterial production of an antimicrobial compound 2,4-diacetylphloroglucinol (DAPG), which was reported to interfere with an auxin-dependent signalling pathway, stimulating the lateral root production (Brazelton *et al.*, 2008). In the present work, the results have, in fact, established an association between fluctuating concentrations of IAA and different root responses, where the same strains of *C. neteri*, *Rouxiella* sp. and *Unidentified* (AJ13) that supressed primary root elongation, possibly due to high IAA concentrations, could significantly promote lateral roots formation.

### 3.2.3. PGPR effects on root hairs presence

The effects of PGPR on root hairs incidence were positive and, over time, these outcomes became more evident against a control situation, where no root hairs ever developed during the entire assay (Table 3.3; Annex 6). By 3 dpi, 45.10% of the PGPR induced root hairs formation, and among these, about half presented above average results. At this stage, PGPR that induced the highest percentages of seedlings with root hairs development were *B. megaterium* (AJ50 and AJ53) and an *Unidentified* isolate (AJ56). At 6 and 9 dpi, the presence of almost every PGPR (94.12% and 98.04%, respectively) promoted root hairs development. Among these, 45.09% and 47.06% of the isolates, respectively, resulted in above average proportions of the evaluated root parameter. At the end of the experiment (9 dpi), from the PGPR isolates that promoted root hairs formation in more than 52% of *A. thaliana* seedlings (47.06% of the PGPR; highlighted with a black box), 41.67% were identified as *B. megaterium.*

Root hairs form a considerable portion of the root surface area and have been pointed as the main mechanical support to the plant, since they play an important role for soil anchorage during the seedling-stage of development. This role tends to shift to lateral roots when plants reach maturity (Choi & Cho, 2019). The process of root hair initiation was found to be modulated by ethylene or auxin treatments (Masucci & Schiefelbein, 1994), suggesting that these hormones are critical regulators of root hairs formation (Gilroy & Jones, 2000). This may explain why almost half of the tested PGPR could induce root hairs formation by the 3rd day of inoculation. Accordingly, bacterial hormones impact on root morphogenesis usually includes overproduction of root hairs and lateral roots (Persello-Cartieaux *et al.*, 2003) and exogenous bacterial phytostimulators, such as IAA, cytokinins and ethylene, have been also reported to induce root hairs formation (reviewed by Vacheron *et al.*, 2013). Additionally, the formation of root hairs has been shown to have developmental plasticity in response to nutrient stresses, where the number and density of root hairs was demonstrated to increase under such conditions (Gilroy & Jones, 2000). As previously stated, *Bacillus* species are particularly resilient to extreme conditions (Tan *et al.*, 2013), and in the present work, *Bacillus* spp., mainly *B. megaterium*, were the most frequent species presenting significant positive induction of root hairs formation. This supports previous findings which found that *B. megaterium* promotes root hairs development through the production of IAA and ethylene (López-Bucio *et al.*, 2007).

**Table 3.3.** PGPR effect on *A. thaliana*'s root hairs presence over time - 3 dpi, 6 dpi and 9 dpi - compared to control. Root hairs induction (RHI %) represents the percentage of *A. thaliana* seedlings that developed root hairs when in co-inoculation with PGPR. The effect of each PGPR on *A. thaliana* seedlings that developed root hairs is visualized using a heat map, where the most stimulating effects are displayed in green and the less stimulating effects are displayed in red. Bacteria that induced root hairs on more than 52% of *A. thaliana* seedlings by 9 dpi are depicted with a black box.

| Forest | Temp. (°C) | Code | Identification | RHI (%) 3 dpi | 6 dpi | 9 dpi |
|---|---|---|---|---|---|---|
| **Control** | | | | 0.00 | 0.00 | 0.00 |
| GR | 30 | AJ40 | *Bacillus megaterium* | 0,00 | 46,88 | 59,38 |
| | | AJ41 | *Bacillus megaterium* | 0,00 | 25,00 | 50,00 |
| | 37 | AJ42 | *Bacillus megaterium* | 0,00 | 66,67 | 75,00 |
| | | AJ43 | *Klebsiella aerogenes* | 0,00 | 65,63 | 71,88 |
| | | AJ44 | *Klebsiella aerogenes* | 0,00 | 93,75 | 93,75 |
| | | AJ45 | *Klebsiella aerogenes* | 3,13 | 28,13 | 43,75 |
| | | AJ46 | *Unknown* | 4,17 | 45,83 | 66,67 |
| | | AJ47 | *Bacillus megaterium* | 12,50 | 34,38 | 37,50 |
| | | AJ48 | *Bacillus megaterium* | 0,00 | 41,67 | 41,67 |
| | | AJ49 | *Bacillus megaterium* | 0,00 | 37,50 | 41,67 |
| | | AJ50 | *Bacillus megaterium* | 50,00 | 87,50 | 96,88 |
| | | AJ51 | *Unidentified* | 3,13 | 28,13 | 40,63 |
| | | AJ52 | *Bacillus* sp. | 12,50 | 68,75 | 68,75 |
| | | AJ53 | *Bacillus megaterium* | 15,63 | 84,38 | 96,88 |
| | 45 | AJ54 | *Bacillus megaterium* | 12,50 | 37,50 | 54,17 |
| | | AJ55 | *Bacillus megaterium* | 6,25 | 71,88 | 78,13 |
| | | AJ56 | *Unidentified* | 16,67 | 25,00 | 58,33 |
| | | AJ57 | *Bacillus megaterium* | 0,00 | 62,50 | 87,50 |
| | | AJ58 | *Bacillus megaterium* | 12,50 | 75,00 | 90,63 |
| | | AJ59 | *Unidentified* | 3,13 | 43,75 | 59,38 |
| | | AJ60 | *Unidentified* | 6,25 | 40,63 | 50,00 |
| | | AJ61 | *Bacillus megaterium* | 0,00 | 37,50 | 50,00 |
| | | AJ62 | *Unidentified* | 4,17 | 37,50 | 58,33 |
| | | AJ63 | *Unidentified* | 0,00 | 40,63 | 56,25 |
| | | AJ64 | *Bacillus megaterium* | 3,13 | 53,13 | 68,75 |
| LI | 30 | AJ10 | *Unidentified* | 0,00 | 18,75 | 21,88 |
| | | AJ11 | *Serratia quinivorans* | 3,13 | 31,25 | 34,38 |
| | | AJ14 | *Cedecea neteri* | 6,25 | 25,00 | 53,13 |
| | 37 | AJ8 | *Cedecea* sp. | 0,00 | 43,75 | 43,75 |
| | | AJ9 | *Bacillus megaterium* | 0,00 | 25,00 | 37,50 |
| | | AJ12 | *Cedecea neteri* | 12,50 | 31,25 | 34,38 |
| | | AJ 13 | *Unidentified* | 3,13 | 21,88 | 37,50 |
| | | AJ15 | *Cedecea neteri* | 9,38 | 34,38 | 53,13 |
| | | AJ16 | *Cedecea neteri* | 0,00 | 9,38 | 31,25 |
| | | AJ17 | *Bacillus megaterium* | 0,00 | 93,75 | 94.00 |
| | | AJ18 | *Bacillus simplex* | 0,00 | 29,17 | 33,33 |
| | 45 | AJ19 | *Bacillus megaterium* | 0,00 | 43,75 | 43,75 |
| ER | 30 | AJ21 | *Rouxiella badensis* | 0,00 | 28,13 | 31,25 |
| | | AJ22 | *Rouxiella* sp. | 3,13 | 43,75 | 46,88 |
| | | AJ23 | *Bacillus mycoides* | 0,00 | 46,88 | 53,13 |
| | | AJ24 | *Bacillus cereus* | 0,00 | 65,63 | 65,63 |
| | | AJ25 | *Pseudomonas mohnii* | 9,38 | 40,63 | 71,88 |
| | | AJ26 | *Bacillus cereus* | 0,00 | 12,50 | 25,00 |
| | | AJ27 | *Ewingella americana* | 0,00 | 16,67 | 41,67 |
| | | AJ28 | *Ewingella americana* | 0,00 | 0,00 | 56,25 |
| | | AJ29 | *Rouxiella* sp. | 0,00 | 8,33 | 29,17 |
| | 37 | AJ30 | *Rouxiella* sp. | 0,00 | 12,50 | 45,83 |
| | | AJ31 | *Klebsiella oxytoca* | 0,00 | 0,00 | 0,00 |
| | | AJ32 | *Serratia sp.* | 0,00 | 8,33 | 20,83 |
| | 45 | AJ33 | *Bacillus megaterium* | 0,00 | 0,00 | 21,88 |
| | | AJ34 | *Bacillus nakamurai* | 6,25 | 21,88 | 28,13 |
| | | | **Mean** | 4.29 | 39.05 | 51.99 |

### 3.2.4. Combination of PGPR effects

So far, the effects of each bacterial strain on specific evaluated root parameter were discussed. However, the selection of a PGPR that could simultaneously induced several stress-helpful root-architecture features would be appropriate for future work. The overall effects of each tested PGPR on primary root length, number of lateral roots and induction of root hairs presence are provided in Table 3.4.

The majority of PGPR (52.94%) could induce great outcomes on a single root parameter, while only about 19.61% could simultaneously modulate two root parameters. None of them displayed promising results on all three evaluated root parameters. From PGPR with combined effects, 50% (5 isolates; *B. megaterium* AJ64, AJ57, AJ58, AJ54 and *Unidentified* AJ63) could supress primary root growth, while inducing root hairs formation. Interestingly, they were all obtained from GR forest and four out of five were identified as *B. megaterium* strains. On the other hand, 40% (4 isolates; *Unknown* AJ46, *B. megaterium*, *Unidentified* AJ53, AJ62 and *C. neteri* AJ15) of PGPR isolates with combined traits simultaneously induced lateral roots and root hairs formation. Only one isolate (10%; AJ32) could inhibit primary root growth while promoting lateral roots formation, being identified as a *Serratia* sp. from the humid ER forest.

The most commonly occurring root-modulating phytohormone is IAA and the production of this auxin is well-known among PGPR. Various indigenous IAA-producing bacterial genera were found to synthesise different concentrations of IAA, inducing different responses plants (reviewed by Maheshwari *et al.*, 2015). These bacteria have been reported to have different IAA biosynthesis pathways and even a single bacterial strain could exhibit more than one pathway (Patten & Glick, 1996). Since different IAA concentrations results in different types of root formations (Meuwley & Pilet, 1991; Dobbelaere *et al.*, 1999), a very specific timing and/or balance between the IAA levels that each PGPR strain produces and those produced by the plant should occur (Etesami *et al.*, 2015) to result in a specific combination of root effects. Otherwise, the low levels of IAA required to promote primary root growth might not be sufficient to induce lateral roots or root hairs formation, or the other way around. This may be in agreement with the results obtained in the present work, where the majority of PGPR could considerably affect one single root-parameter and thus being the reason why PGPR promoting all root development were difficult to find.

**Table 3.4.** PGPR combined effects on *A. thaliana*'s root- architecture in relation to control. Primary root growth induction (PRGI %) and lateral roots induction (LRI %) represents the mean of the values registered throughout the full inoculation time and determined in Tables 3.1 and 3.2, respectively. Induction of root hair presence (IRH %) represents the percentage of *A. thaliana* seedlings that developed root hairs after 9 dpi. The effect of each PGPR on *A. thaliana* evaluated root parameters is visualized using a heat map, where the most and the less prominent PGPR effects are displayed in green and in red, respectively. Bacteria previously selected for having the greatest outcomes for each parameter are depicted with a black box.

| Forest | Temp (°C) | Code | Identification | PRGI (%) Mean | LRI (%) Mean | IRH (%) Mean |
|---|---|---|---|---|---|---|
| GR | 30 | AJ40 | *Bacillus megaterium* | 94,65 | 1263,38 | 59,38 |
| | | AJ41 | *Bacillus megaterium* | 64,45 | 1543,52 | 50,00 |
| | 37 | AJ42 | *Bacillus megaterium* | 64,67 | 1319,89 | 75,00 |
| | | AJ43 | *Klebsiella aerogenes* | 72,52 | 1485,91 | 71,88 |
| | | AJ44 | *Klebsiella aerogenes* | 85,40 | 914,44 | 93,75 |
| | | AJ45 | *Klebsiella aerogenes* | 85,79 | 2839,97 | 43,75 |
| | | AJ46 | *Unknown* | 100,57 | 2820,60 | 66,67 |
| | | AJ47 | *Bacillus megaterium* | 87,87 | 3308,77 | 37,50 |
| | | AJ48 | *Bacillus megaterium* | 64,16 | 1092,89 | 41,67 |
| | | AJ49 | *Bacillus megaterium* | 70,98 | 1704,51 | 41,67 |
| | | AJ50 | *Bacillus megaterium* | 63,14 | 2031,97 | 96,88 |
| | | AJ51 | *Unidentified* | 64,53 | 2544,84 | 40,63 |
| | | AJ52 | *Bacillus* sp. | 71,68 | 1909,83 | 68,75 |
| | | AJ53 | *Bacillus megaterium* | 60,36 | 3036,72 | 96,88 |
| | 45 | AJ54 | *Bacillus megaterium* | 48,80 | 774,47 | 54,17 |
| | | AJ55 | *Bacillus megaterium* | 68,25 | 2182,37 | 78,13 |
| | | AJ56 | *Unidentified* | 66,89 | 1548,09 | 58,33 |
| | | AJ57 | *Bacillus megaterium* | 53,40 | 1108,13 | 87,50 |
| | | AJ58 | *Bacillus megaterium* | 48,14 | 1209,38 | 90,63 |
| | | AJ59 | *Unidentified* | 55,07 | 849,84 | 59,38 |
| | | AJ60 | *Unidentified* | 49,83 | 631,72 | 50,00 |
| | | AJ61 | *Bacillus megaterium* | 87,09 | 919,76 | 50,00 |
| | | AJ62 | *Unidentified* | 69,97 | 2792,51 | 58,33 |
| | | AJ63 | *Unidentified* | 51,85 | 813,57 | 56,25 |
| | | AJ64 | *Bacillus megaterium* | 50,52 | 1454,31 | 68,75 |
| LI | 30 | AJ10 | *Unidentified* | 78,62 | 788,45 | 21,88 |
| | | AJ11 | *Serratia quinivorans* | 64,01 | 2676,89 | 34,38 |
| | | AJ14 | *Cedecea neteri* | 59,37 | 2166,37 | 53,13 |
| | 37 | AJ8 | *Cedecea* sp. | 67,78 | 917,18 | 43,75 |
| | | AJ9 | *Bacillus megaterium* | 73,28 | 515,25 | 37,50 |
| | | AJ12 | *Cedecea neteri* | 56,24 | 2761,25 | 34,38 |
| | | AJ13 | *Unidentified* | 56,72 | 3190,58 | 37,50 |
| | | AJ15 | *Cedecea neteri* | 61,75 | 2911,05 | 53,13 |
| | | AJ16 | *Cedecea neteri* | 57,31 | 1733,59 | 31,25 |
| | | AJ17 | *Bacillus megaterium* | 82,15 | 1717,63 | 94.00 |
| | | AJ18 | *Bacillus simplex* | 68,07 | 752,81 | 33,33 |
| | 45 | AJ19 | *Bacillus megaterium* | 75,31 | 1495,04 | 43,75 |
| ER | 30 | AJ21 | *Rouxiella badensis* | 76,01 | 651,52 | 31,25 |
| | | AJ22 | *Rouxiella* sp. | 62,56 | 3060,64 | 46,88 |
| | | AJ23 | *Bacillus mycoides* | 66,19 | 1951,72 | 53,13 |
| | | AJ24 | *Bacillus cereus* | 86,37 | 1562,77 | 65,63 |
| | | AJ25 | *Pseudomonas mohnii* | 114,55 | 2313,43 | 71,88 |
| | | AJ26 | *Bacillus cereus* | 66,31 | 1905,52 | 25,00 |
| | | AJ27 | *Ewingella americana* | 60,64 | 1705,28 | 41,67 |
| | | AJ28 | *Ewingella americana* | 78,61 | 1092,51 | 56,25 |
| | | AJ29 | *Rouxiella* sp. | 63,75 | 2758,17 | 29,17 |
| | 37 | AJ30 | *Rouxiella* sp. | 66,67 | 1712,04 | 45,83 |
| | | AJ31 | *Klebsiella oxytoca* | 54,61 | 1906,38 | 0,00 |
| | | AJ32 | *Serratia* sp. | 50,86 | 3702,85 | 20,83 |
| | 45 | AJ33 | *Bacillus megaterium* | 35,89 | 153,19 | 21,88 |
| | | AJ34 | *Bacillus nakamurai* | 42,26 | 483,84 | 28,13 |

### 3.2.5. Long-term effect of PGPR by forest and isolation temperature

Due to a desired long-term effect, differences between PGPR effects at 9 dpi was compared taking into consideration the sampled forest and temperature used for bacterial isolation (Figure 3.1). As previously discussed, overall results revealed that, on the long-term, the presence of PGPR from every forest significantly decreased primary root length in relation to control ($p \leq 0.0001$; Figure 3.1A), with no significant differences being detected among forests. Regarding differences between temperatures used for bacterial isolation, PGPR isolated at every temperature displayed significant inhibitory behaviour compared to control ($p \leq 0.0001$). However, differences among temperatures were detected, with PGPR isolated at 45 °C displaying a more prominent suppressive effect on primary root length than PGPR isolated at 30 °C or 37 °C. When discussing the effects of PGPR combined by different forests on lateral roots, the results revealed a similar significant improvement in the number of lateral roots of *A. thaliana* seedlings ($p \leq 0.0001$; Figure 3.1B). When comparing the temperatures used for bacterial isolation, all PGPR groups displayed similar behaviours ($p \leq 0.0001$). Hence, even though overall differences were observed, none of the PGPR features were discriminant for increased lateral roots production, since differences within each group were not significant ($p > 0.05$). Lastly, analysing the PGPR effects on root hairs formation, an increase of root hairs presence seemed to be positively correlated with low water availability based on forest bioclimate (Figure 3.1C). PGPR isolated from semi-arid forest GR stimulated the highest percentage (63.83%) of root hair presence, followed by sub-humid forest LI (43.14%) and humid forest ER (38.39%). When comparing temperatures used for bacterial isolation, PGPR isolated from all three isolation temperatures stimulated root hair presence. However, a relation between higher isolation temperatures and root hair incidence was registered, where the PGPR isolates from 45 °C were the greatest root hair inducers (57.51%) when compared to 30 °C and 37 °C (45.68% and 52.45%, respectively).

Out of the three evaluated root-parameters, the development of root hairs seemed to be the most affected by PGPR isolation backgrounds (isolation forest and temperature used for bacterial isolation). Moreover, PGPR isolated at 45 °C could also cause more prominent effects on the primary root growth. Interestingly, the development of root hairs was also found to be an adaptive response to stressful environmental conditions, as it allowed an improved root surface area, resulting in an intensification of

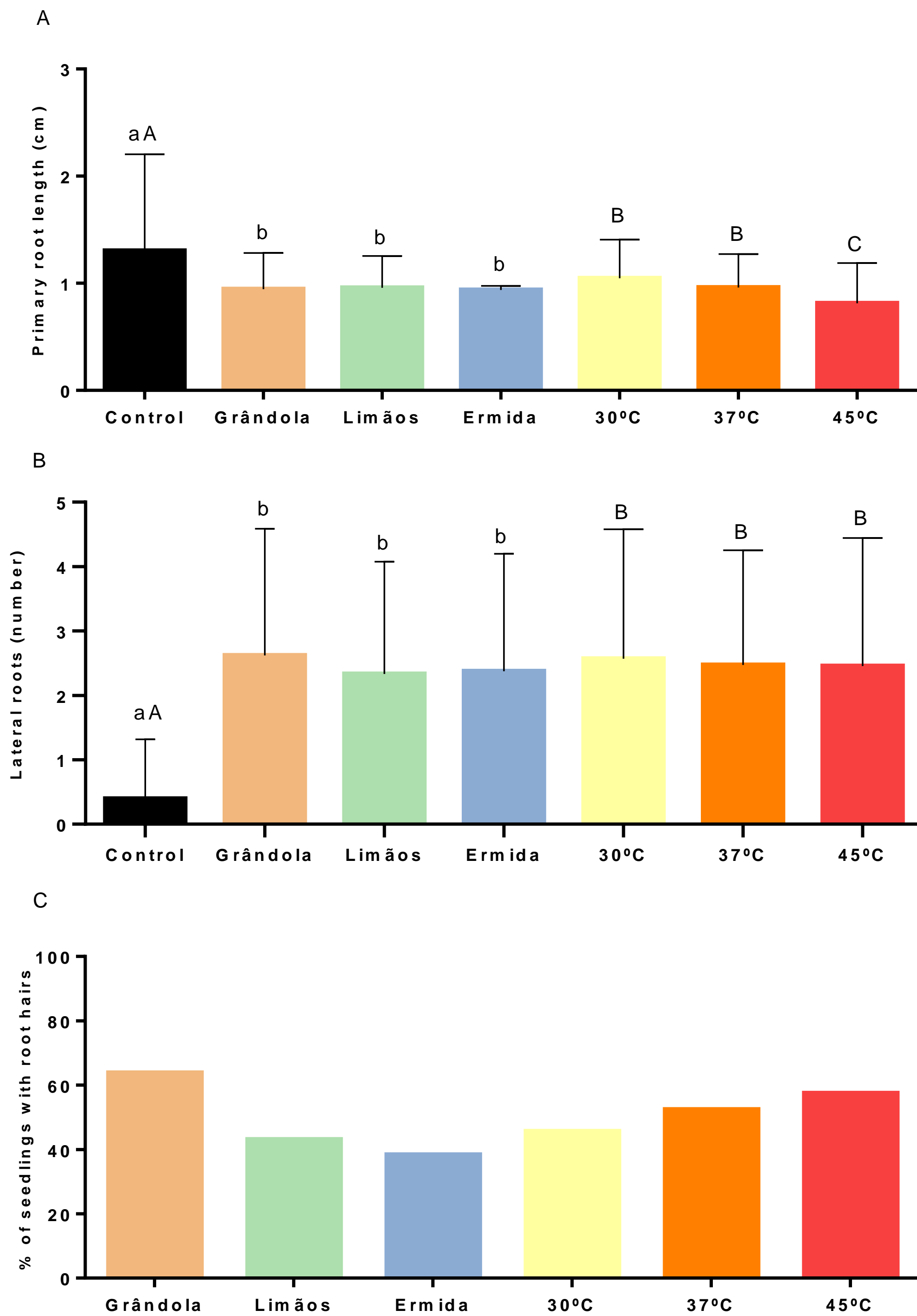


**Figure 3.1.** Effect of all isolated PGPR combined by forest and temperature used for PGPR isolation on *A. thaliana* (A) primary root length, (B) lateral roots development, and (C) root hairs presence, at 9 dpi. Different letters denote statistically significant differences (at $p \leq 0.05$), where lowercase letters refer to differences among forests and capital letters refer to differences among isolation temperatures.

nutrient and/or water uptake (Gilroy & Jones, 2000; Michael, 2001). Indeed, root hairs development is particularly sensitive to biotic and abiotic stimuli (López-Bucio *et al.*, 2007). In agreement, those PGPR isolated from drier and warmer environments seemed to induce higher percentage of root hairs frequency. These results support previous information associating bacteria exposed to drier environments to the promotion of a more suitable root architecture for the plant requirements (Lynch & Ho, 2005). Indeed, bacteria isolated from semi-arid areas or from moisture stressed conditions were found to increase plant growth and resistance to soil water deficits, through alteration of root-architecture of the plant (Ilyas & Bano, 2010; Yasmin *et al.*, 2013). PGPR from stressed soils or stressed host plants are believed to promote tolerance against abiotic and biotic stresses to plants, modulating the production of phytohormones and communicating between them through quorum sensing molecules, which also regulate gene expression and phytohormone production (Khan *et al.*, 2020). Additionally, accordingly to Ilyas & Bano (2010), PGPR isolates from water unstressed condition presented less production of several phytohormones (IAA, GA, and t-zr) when compared to isolates from water-stressed/arid areas.

### 3.3. Conclusion

The majority the studied PGPR induced changes on the root-architecture of *A. thaliana*. Many PGPR were able to repress primary root length, probably due to phytohormone production (IAA, ethylene), and among these, bacteria from the *Bacillus*, *Serratia*, *Klebsiella* and *Unidentified* genera, mainly isolated at 45°C, presented the most inhibitory effects. The most prominent results on the number of lateral roots were achieved by *Bacillus*, *Serratia*, *Klebsiella*, *Cedecea*, *Rouxiella*, *Unidentified* and *Unknown* strains. The time after inoculation seemed to be a key-factor for this root-parameter, where a strong enhanced number of lateral roots was observed by the 6$^{th}$ day forward. When accessing the effects of PGPR on root hairs, it was found that more than 98% of the tested bacteria could encourage the presence of root hairs by 9 dpi, and the most evident results were essentially induced with *B. megaterium* strains.

The isolation forest, with each associated bioclimate and isolation temperature, revealed to be important features for the promotion ability of root hairs occurrence by bacteria, where a positive correlation was registered between root hairs development and both water availability and higher temperatures. Besides phytohormones, PGPR have also been found to induce a suitable root morphology for abiotic stressed environments through the emission of organic volatile compounds (VOCs; Fincheira & Andrés, 2018), namely 2,3-butanediol, which increase the tolerance against abiotic stress, such as against drought (Liu & Zhang, 2015). Accordingly, *Bacillus* species have been reported as 2,3-butanediol

VOC producers (Wu *et al.*, 2018). However, only further studies to identify the bacterial compounds produced by each bacteria would be needed to provide more information.

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# Chapter IV: Antagonistic activity of selected PGPR against cork oak pathogens *Biscogniauxia mediterranea* and *Diplodia corticola*

Both *B. mediterranea* and *D. corticola* cause severe economic losses to cork oak production, as they cause cork oak charcoal disease and bot canker, respectively. Limited preventive measures are currently known, and fungicides represent a potential negative impact on human and environmental health (Moricca *et al.*, 2016). For these reasons, more sustainable strategies are being developed, where selected PGPR could play a potential role as biocontrol agents. In this chapter, PGPR isolates were screened for their antimicrobial effect on the cork oak pathogens, *B. mediterranea* and *D. corticola*.

## 4.1. Methodology

### 4.1.1. PGPR isolates

The previously selected 51 PGPR isolates characterized by displaying multiple PGPR traits (Chapter II) were grown in liquid LB medium, at 30 °C, for 24 h. PGPR growth was evaluated by densitometry ($OD_{600}$) and adjusted to 1.0. These isolates will be pre-screened *in vitro* for antifungal activity against cork oak phytopathogens *B. mediterranea* and *D. corticola*. Both endophytic phytopathogens were isolated from cork oak trees showing mild symptoms in Grândola region and subsequently identified (Costa *et al.*, 2020). Fungal cultures were maintained in Potato Dextrose agar (PDA) medium, at 25 °C in darkness. The most promising PGPR will be then selected for further studying antagonistic interactions using *in vitro* bioassays.

### 4.1.2. Protocol optimization for assessment of antifungal activity of PGPR

The protocol for assessment of antifungal activity of PGPR was improved by optimizing four different parameters: inoculant volume (1.0 µL, 2.5 µL, 5.0 µL or 10.0 µL), inoculant method - agar well diffusion or suspension drop diffusion (as described by Suaréz-Moreno *et al.*, 2019 and Kanini *et al.*, 2013, respectively), incubation temperature (25 °C or 28 °C) and incubation period (3 or 5 days). About four random PGPR from each forest were grown for 24 h in LB medium. Bacterial density was adjusted to 1.0 ($OD_{600}$). PDA plates were divided into four quadrants and a 5 mm agar plug from actively growing mycelia (*D. corticola* or *B. mediterranea*) was placed on the centre using an adaptation of the procedure described by Fusaro (1972; Figure 4.1). In each quadrant, different volumes of a single bacterial suspension (1.0 µL, 2.5 µL, 5.0 µL and 10.0 µL) was inoculated, 1 cm from the plate edge, using either the agar well diffusion or the suspension drop method (Figure 4.1). PDA plates were incubated in the dark at two different temperatures (25 °C and 28 °C) and the results were evaluated at 3 and 5 days for

each temperature and fungal species. Plates where no bacterial inoculation occurred were used as control.

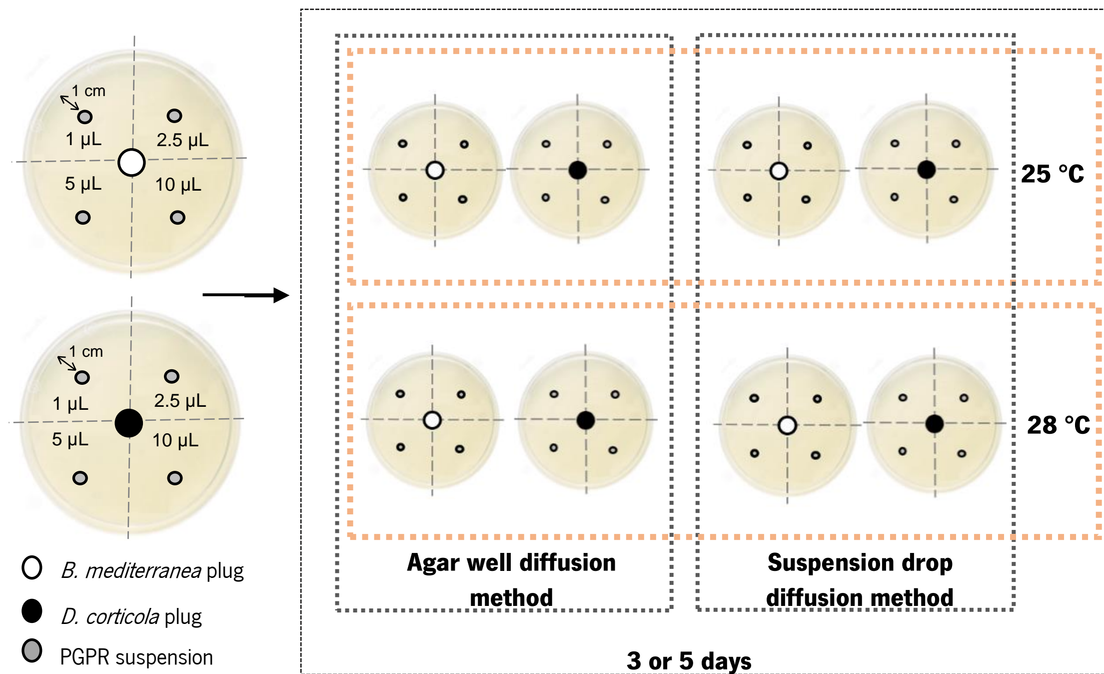


**Figure 4.1.** Experimental design for optimizing the evaluation of PGPR antifungal activity against cork oak fungal pathogens *B. mediterranea* and *D. corticola*. Four different parameters were optimized: inoculant volume (1.0 µL, 2.5 µL, 5.0 µL and 10.0 µL), inoculant method (wells perforation and bacterial suspension drop), incubation temperature (25 °C and 28 °C) and incubation period (3 or 5 days). As control, plates where no PGPR inoculation occurred were used.

### 4.1.3. *In vitro* PGPR screening for antifungal activity against *B. mediterranea* and *D. corticola*

The selected PGPR (51 isolates) were grown for 24 h in LB medium and their $OD_{600}$ was adjusted to 1. PDA plates were divided into four quadrants and a fungal plug was placed in the centre, using the previously mentioned method. In each PDA plate, four different PGPR were simultaneously tested. A drop of 2.5 µL of each PGPR suspensions was inoculated onto the agar surface, on each quadrant, 1 cm way from the edge of the plate. PDA plates were incubated at 25 °C, in the dark, and results were evaluated after 3 days for *D. corticola* and 5 days for *B. mediterranea*.

#### 4.1.4. *In vitro* PGPR antagonism assay against *B. mediterranea* and *D. corticola*

Selected PGPR strains (15 isolates) that promoted visible fungal growth inhibition were further studied through *in vitro* antagonism assays against the phytopathogenic fungi *B. mediterranea* and *D. corticola,* using a dual culture method described by Idris *et al.* (2007). PGPR isolates were grown for 24 h in LB medium and its $OD_{600}$ was adjusted to 1.0. A drop of 2.5 µL of bacterial suspension was positioned opposed to a fungal plug, and both were placed 2.5 cm away from the edges of the plate (Figure 4.2). Plates were incubated at 25 °C, in the dark, for 7 days for *D. corticola* and 9 days for *B. mediterranea.* Incubation periods were determined according to the requirement for each phytopathogen to reach the plate edge in controls. All *in vitro* antagonism assays were done in triplicate.

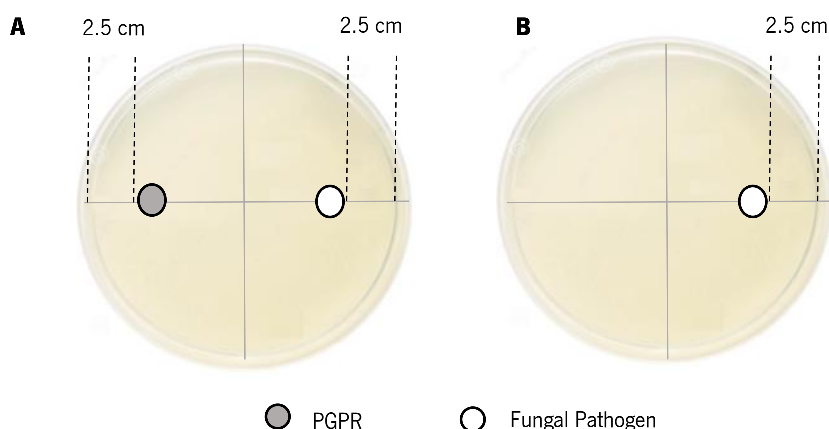


**Figure 4.2.** Schematic diagram of PGPR antifungal assays using (A) a dual culture method and (B) corresponding control.

Fungal mycelia area was measured at 3, 5 and 7 days for *D. corticola* and 5, 7 and 9 days for *B. mediterranea* using ImageJ software (Copyright 1993, 2016, Oracle; Annex 7), and the percentage of growth inhibition (PGI) was calculated using the following formula:

$$PGI = \frac{AC - AB}{AC} \times 100$$

Where, PGI: Pathogen Growth Inhibition (%);

AC: Area of pathogenic fungal growth in the control plate;

AB: Area of pathogenic fungus growing in the presence of PGPR.

#### 4.1.5. Data and statistical analyses

PGI (%) was determined and statistical analysis was performed by One-way ANOVA tests (Dunnett's multiple comparison tests) using the Windows GraphPad Prism 6.01 program (GraphPad Software, La Jolla, CAUSE).

### 4.2. Results and Discussion

#### 4.2.1. Protocol optimization for assessment of antifungal activity of PGPR

Four different parameters were tested to assess optimal conditions for assaying antifungal activity, namely the bacterial inoculant volume and method, temperature, and period of incubation. A more controlled behaviour for both bacteria and fungi was observed at 25 °C, when 2.5 µL of a bacterial suspension was used in a drop diffusion method. When agar was perforated to form wells, bacterial suspensions tended to grow underneath the PDA, preluding the assay. In addition, 5.0 µL and 10.0 µL bacterial inoculant volumes displayed excessive growth, whereas 1.0 µL resulted in reduced development of the culture. At 28 °C, bacteria were able to grow at a faster rate and even overlay the fungal pathogen at early stages of incubation. Accordingly, the bacterial isolates had shown the skill to easily grow at temperatures above 28°C in Chapter II. Regarding incubation periods, *B. mediterranea* needed five days to reach the bacterial drop, while *D. corticola* interacted after three days. These results are in agreement with those reported by Costa *et al.* (2020) that revealed that both fungal pathogens exhibited differential growth rates at 28 °C ± 2 °C (0.71 $cm^2/h$ for *D. corticola* and 0.34 $cm^2/h$ for *B. mediterranea*). Hence, co-culture of PGPR and fungal isolates was optimized by using an inoculation 2.5 µL of bacterial suspension drop, at 25°C, and the fungal growth observed after three and five days for *D.corticola* and *B. mediterranea*, respectively.

#### 4.2.2. *In vitro* PGPR screening for antifungal activity against *B. mediterranea* and *D. corticola*

All 51 isolates displaying multiple PGPR traits were screened for antifungal activity against *B. mediterranea* and *D. corticola* using the optimized protocol. About 30% (15 isolates) were able to inhibit fungal growth when compared to control. From these, eight isolates presented antifungal activity against *B. mediterranea*, twelve against *D. corticola*, and five inhibited both phytopathogens growth (Table 4.1).

**Table 4.1.** Evaluation of PGPR antifungal activity against *B. mediterranea* and *D. corticola*. Bacterial isolates were considered fungal suppressors (+) when an inhibitory halo of fungal growth was formed around the bacterial colony. Different colours represent PGPR with (+; green) and without (-; red) antifungal activity against *D. corticola* after three days and against *B. mediterranea* after five days. Information regarding bacterial isolation forest and temperature is also provided.

| Forest | Temp. (°C) | PGPR code | Identification | Antifungal activity against: | |
|---|---|---|---|---|---|
| | | | | ***B. mediterranea*** | ***D. corticola*** |
| GR | 30 | **AJ40** | ***Bacillus megaterium*** | + | - |
| | | AJ41 | *Bacillus megaterium* | - | - |
| | 37 | AJ42 | *Bacillus megaterium* | - | - |
| | | **AJ43** | ***Klebsiella aerogenes*** | - | + |
| | | AJ44 | *Klebsiella aerogenes* | - | - |
| | | AJ45 | *Klebsiella aerogenes* | - | - |
| | | **AJ46** | ***Unknown*** | - | + |
| | | AJ47 | *Bacillus megaterium* | - | - |
| | | AJ48 | *Bacillus megaterium* | - | - |
| | | **AJ49** | ***Bacillus megaterium*** | - | + |
| | | AJ50 | *Bacillus megaterium* | - | - |
| | | **AJ51** | ***Unidentified*** | - | + |
| | | AJ52 | *Bacillus* sp. | - | - |
| | | AJ53 | *Bacillus megaterium* | - | - |
| | 45 | **AJ54** | ***Bacillus megaterium*** | - | + |
| | | AJ55 | *Bacillus megaterium* | - | - |
| | | AJ56 | *Unidentified* | - | - |
| | | AJ57 | *Bacillus megaterium* | - | - |
| | | AJ58 | *Bacillus megaterium* | - | - |
| | | AJ59 | *Unidentified* | - | - |
| | | AJ60 | *Unidentified* | - | - |
| | | AJ61 | *Bacillus megaterium* | - | - |
| | | AJ62 | *Unidentified* | - | - |
| | | AJ63 | *Unidentified* | - | - |
| | | AJ64 | *Bacillus megaterium* | - | - |
| LI | 30 | AJ10 | *Unidentified* | - | - |
| | | **AJ11** | ***Serratia quinivorans*** | + | + |
| | | AJ14 | *Cedecea neteri* | - | - |
| | 37 | AJ8 | *Cedecea sp.* | - | - |
| | | AJ9 | *Bacillus megaterium* | - | - |
| | | AJ12 | *Cedecea neteri* | - | - |
| | | AJ13 | *Unidentified* | - | - |
| | | AJ15 | *Cedecea neteri* | - | - |
| | | **AJ16** | ***Cedecea neteri*** | + | + |
| | | AJ17 | *Bacillus megaterium* | - | - |
| | | AJ18 | *Bacillus simplex* | - | - |
| | 45 | AJ19 | *Bacillus megaterium* | - | - |
| ER | 30 | **AJ21** | ***Rouxiella badensis*** | + | + |
| | | **AJ22** | ***Rouxiella* sp.** | + | + |
| | | AJ23 | *Bacillus mycoides* | - | - |
| | | **AJ24** | ***Bacillus cereus*** | + | + |
| | | AJ25 | *Pseudomonas mohnii* | - | - |
| | | AJ26 | *Bacillus cereus* | - | - |
| | | AJ27 | *Ewingella americana* | - | - |
| | | AJ28 | *Ewingella americana* | - | - |
| | | AJ29 | ***Rouxiella sp.*** | - | + |
| | 37 | AJ30 | ***Rouxiella sp.*** | - | + |
| | | AJ31 | ***Klebsiella oxytoca*** | + | - |
| | | AJ32 | ***Serratia* sp.** | + | - |
| | 45 | AJ33 | *Bacillus megaterium* | - | - |
| | | AJ34 | *Bacillus nakamurai* | - | - |

The eight PGPR capable to inhibit *B. mediterranea* growth belonged to six different genera, namely *Bacillus* (*B. megaterium* and *B. cereus*), *Rouxiella* (*Rouxiella* sp. and *R. badensis*), *Serratia* (*Serratia* sp. and *S. quinivorans*), *Cedecea* (*C. neteri*) and *Klebsiella (K. oxytoca*). The same genera also presented antifungal activity against *D. corticola,* namely *Bacillus* (two *B. megaterium* and one *B. cereus*), *Rouxiella* (*Rouxiella* sp. and *R. badensis*), *Serratia* (*Serratia* sp. and *S. quinivorans*), *Cedecea (C. neteri*), *Klebsiella* (*K. aerogenes* and *K. oxytoca*), as well as two isolates without identification known - *Unidentified* AJ51 and *Unknown* isolate AJ46 (which clustered among *B. megaterium* strains, Chapter II). From these, five PGPR could simultaneously inhibit both phytopathogens, including two *Rouxiella* (*Rouxiella* sp. and *R. badensis*), one *B. cereus*, one *S. quinivorans* and one *C. neteri*. All these isolates will be further studied concerning their antagonistic activity. Regarding the isolation forest of these PGPR, 50% of ER tested isolates seemed to display antifungal activity, whereas GR and LI forests had 24% and 16.67%, respectively. Even though every forest possessed PGPR with antifungal activities, only the most humid forests presented PGPR capable of simultaneously control *B. mediterranea* and *D. corticola*. Indeed, these preliminary results could suggest a direct correlation between water availability and multiple antifungal PGPR traits. However, these should be taken with caution and further qualitative assessment should be performed.

### 4.2.3. *In vitro* PGPR antagonism assay

#### 4.2.3.1. *In vitro* PGPR antagonistic activity against *B. mediterranea*

The eight PGPR isolates presenting antifungal activity against *B. mediterranea* in the previous screening assay were qualitatively evaluated for their antagonistic activity along time. PGPR isolates presented a reduction of inhibitory activity against *B. mediterranea* with time (Figure 4.3). Accordingly, about 75% of the tested PGPR (6 isolates) significantly inhibited mycelial growth ($p \leq 0.05$) at 5 dpi, but from the 7$^{th}$ day forward, only *S. quinivorans* was able to maintain a significant ($p \leq 0.01$) antagonistic activity, holding PGI% values of 14.86% and 10.76% at 7 and 9 dpi, respectively. Indeed, PGPR from the *Serratia* genus (*Serratia* sp. and *S. quinivorans*) presented the highest PGI% at 5 dpi (26.23% and 23.07%; $p \leq 0.001$, respectively). On the other hand, *B. megaterium* and *Rouxiella* sp. never significantly repressed *B. mediterranea* growth during the full assay. Even though, to our knowledge, no information regarding the effects of PGPR on *B. mediterranea* has been previously reported, these results suggest that time was an important factor for fungal growth inhibition. The mycelial growth inhibition of *B. mediterranea* by bacterial isolates reveal the production of bacterial active toxic compounds against other fungi as

suggested by Bhattacharyya & Jha (2011). Most common PGPR suppressive mechanisms correspond to the inhibition of spore or mycelial growth, stimulation of hyphal wall degradation with lytic enzymes (β-1,3-glucanase, protease, and lipase), and production of HCN (Zhang *et al.*, 2015; Keswani *et al.*, 2016). In fact, bacteria from the *Serratia* genus have been reported as active producers of different lytic enzymes and several antibiotics (Ali *et al.*, 2019; Fernando *et al.*, 2005), as well as HCN producers (Dastager *et al.*, 2010). Interestingly, from all screened bacteria, *S. quinivorans* revealed to be the single HCN synthesizer, as described in Chapter II. Hydrogen cyanide is described as a volatile antifungal compound capable of inhibiting growth of several plant pathogens (Fernando *et al.*, 2005), and Tabli *et al.* (2018) have associated *S. quinivorans* with high HCN production.

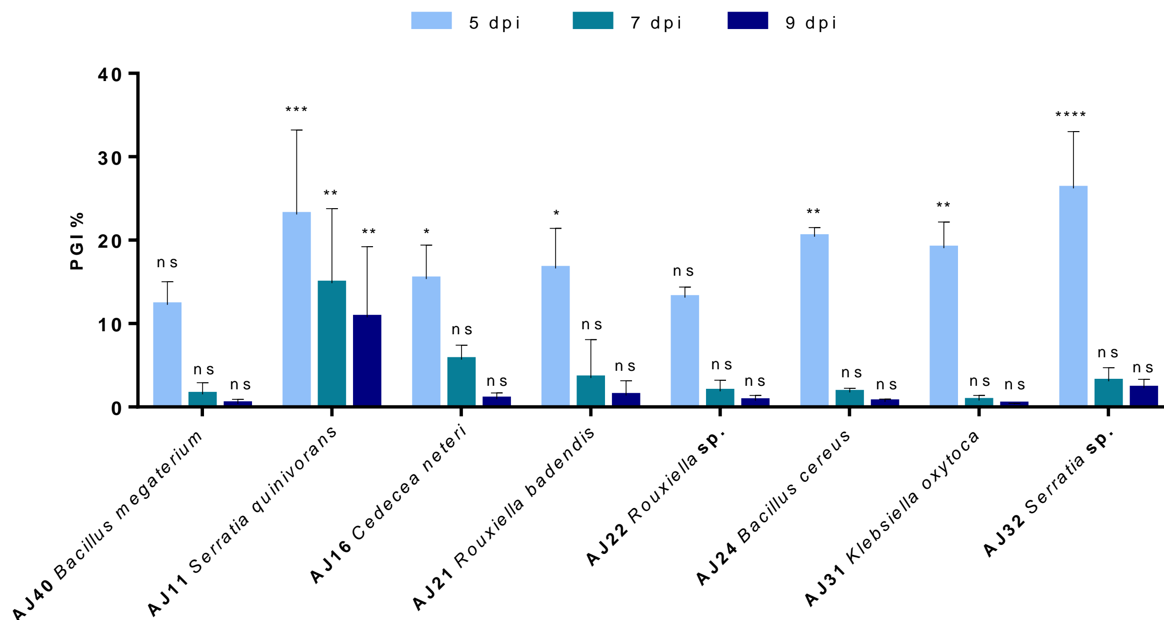


**Figure 4.3**. PGPR isolates effect on *B. mediterranea* growth inhibition (PGI%) over time - 5 dpi, 7 dpi and 9 dpi. Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Non-significant differences are displayed with (ns).

The antifungal activity of *S. quinivorans* was further studied by following the cultural features over time (Figure 4.4). As previously detected, the bacterial antagonistic behaviour was significantly more evident at early stages of inoculation ($p \leq 0.001$), where the highest antagonistic activity was displayed 5 days after incubation (Figure 4.4A). This could indicate that the production of bacterial antifungal compounds changed or *B. mediterranea* may become tolerant to bacterial activity over time, developing

protection mechanisms. Indeed, accumulation of a yellowish pigment at the contact front of *B. mediterranea* mycelium became evident at 7 dpi (Figure 4.4B). The production of pigments has been reported to be a defence mechanism adopted by several fungal species, providing them protection against adverse conditions (Gupta *et al.*, 2015).

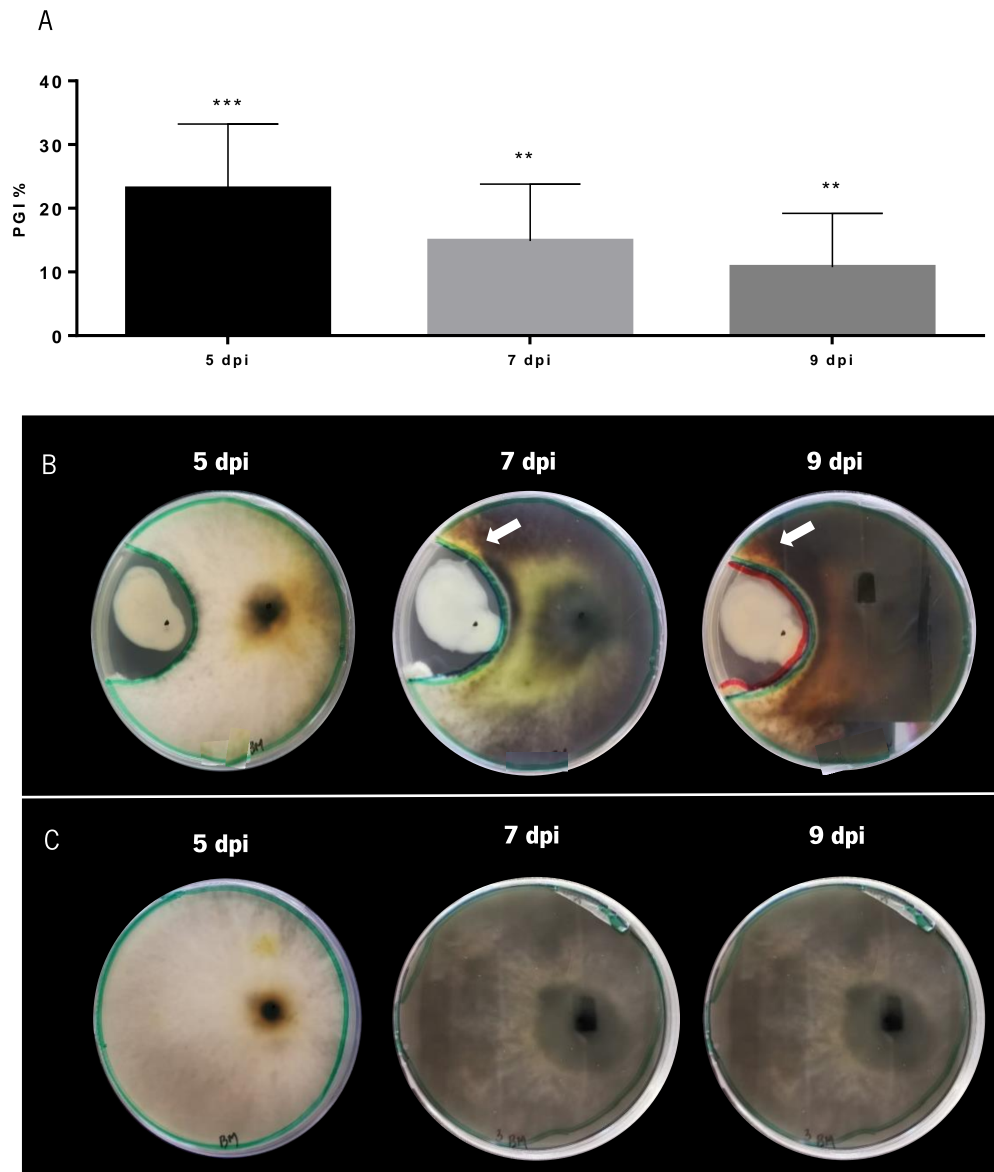


**Figure 4.4.** Inhibition of B. mediterranea by *S. quinivorans* over time - 5 dpi, 7 dpi and 9 dpi. (A) Growth inhibition (PGI%); asterisks represent statistically significant differences to control at $p \leq 0.01$ (**), $p \leq 0.001$ (***). (B) Cultural features from *B. mediterranea* during inoculation with *S. quinivorans*; white arrows point to a region where the pigment develops. (C) Cultural features from control, where no PGPR inoculation occurred.

#### 4.2.3.2. *In vitro* PGPR antagonistic activity against *D. corticola*

The potential antagonistic effects of the previously selected twelve PGPR against D*. corticola* were assessed over time (Figure 4.5). None of them revealed significant inhibitory activity by 3 dpi, when compared to control. Only *Unknown* isolate (AJ46) significantly inhibited *D. corticola* mycelial growth (*p* ≤0.0001) at 5 dpi, displaying a strong earlier antifungal effect on the pathogen. However, at 7 dpi, about 58% of tested PGPR presented significant antagonistic activity (*p* ≤0.05). Among these, *Rouxiella badensis*, *B. cereus* and *Unknown* AJ46 isolates revealed the highest inhibitions (*p* ≤0.01). On the other hand, *B. megaterium* (AJ49 and AJ54), *Rouxiella sp.* (AJ29 and AJ30) and *C. neteri* did not present significant antagonistic effects during all inoculation time.

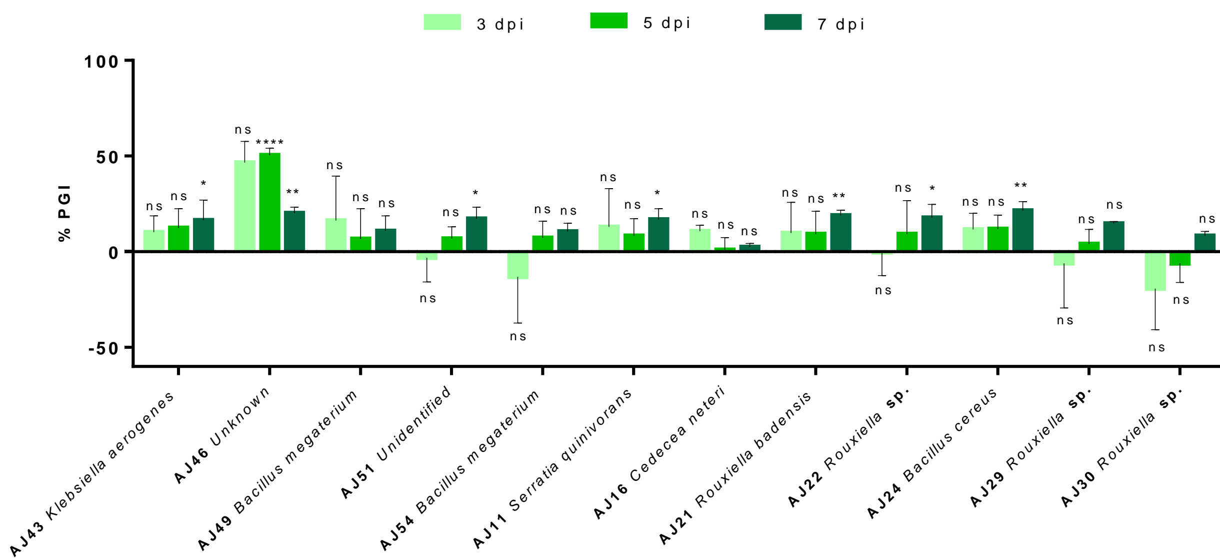


**Figure 4.5.** PGPR isolates effect on *D. corticola* growth inhibition (PGI%) over time - 3 dpi, 5 dpi and 7 dpi. Asterisks represent statistically significant differences to control at p ≤0.05 (*), p ≤0.01 (**) and p ≤0.0001 (****). Non-significant differences are displayed with (ns).

For further studying the antagonistic activity against *D. corticola*, the most promising PGPR (*Unknown* AJ46 and *B. cereus*) were further studied regarding their cultural features throughout time. An interaction period over seven days was needed for discriminating the PGRP antagonist effect against *D. corticola* (Figure 4.6A and B). These results suggested a late antagonistic effect that could be explained by a dose-dependent effect of the antimicrobial compound. Several antifungal compounds have been described to have a minimum inhibitory concentration against pathogens (MIC; Kathiravan *et al.*, 2012). After 7 dpi, D*. corticola* hyphae began to suffer morphological alterations during interaction with *Unknown*

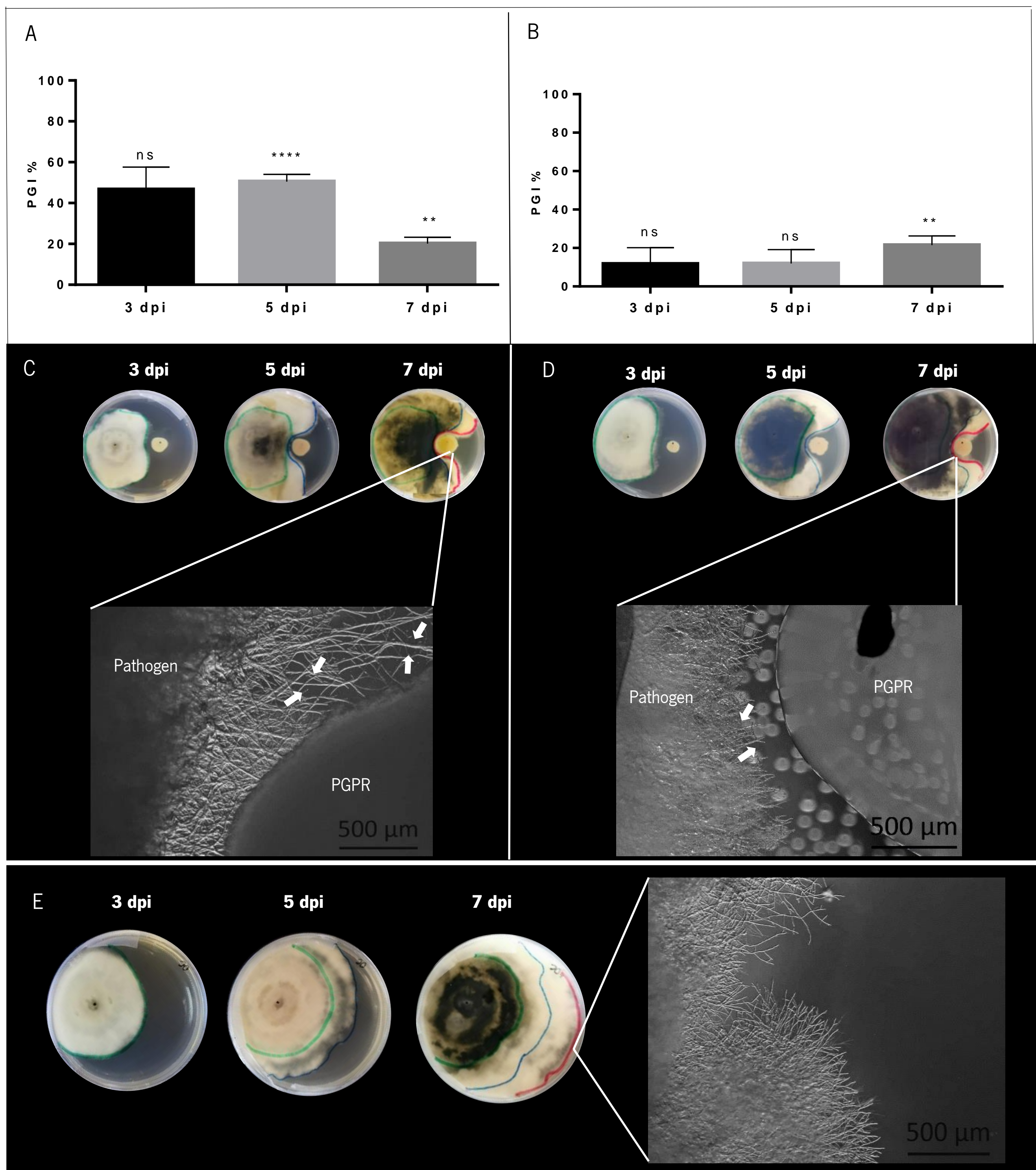


**Figure 4.6.** Growth inhibition (PGI%) of *D. corticola* by *Unknown* AJ46 (A) and *B. cereus* (B) over time - 5 dpi, 7 dpi and 9 dpi. Asterisks represent statistically significant differences to control at $p \leq 0.01$ (**) and $p \leq 0.0001$ (****). Non-significant differences are displayed with (ns). The cultural features of *B. mediterranea* when interacting with *Unknown* AJ46 (C) and *B. cereus* (D). Hyphal modifications in the interaction region revealed increased diameter and irregular growth directions of *D. corticola* hyphae (white arrows). For comparison, cultural features from control (E), where no PGPR inoculation occurred, are shown.

AJ46 and *B. cereus* (Figure 4.6C and D). The irregular branching, increased diameter, and irregular growth directions of *D. corticola* hyphae were among the most noticeable observed modifications. Until recently, little was known about whether antagonistic bacteria could alter the morphology of fungal hyphae during growth inhibition (Kang *et al.*, 2001). However, synthetic antimicrobial compounds were found to induce morphological alterations in the hyphae of fungal pathogens along with radial growth inhibition (Deora *et al.*, 2005). These authors also reported that bacterial strains belonging to *Bacillus*, *Pseudomonas*, *Klebsiella, Stenotrophomonas* and *Delftia* genera could induce *Peronosporomycete* pathogen hyphae morphologic alterations in a similar way to synthetic antimicrobial compounds (Deora *et al.*, 2005). More recently, other authors have experienced similar results with several *Pseudomonas, Delftia* and *Lysobacter* strains against the pathogenic agent *Phytophthora capsici* (Kamruzzaman *et al.*, 2015). Morphological changes were comparable to the alterations induced by the known antimicrobial compounds viscosinamide, phenazine-1-carboximide, methanol, xanthobaccin and zarilamide (Deora *et al.*, 2005). Some of these antimicrobial compounds have been reported to have a MIC against pathogens, including viscosinamide (Thrane *et al.*, 2000), phenazine-1-carboximide (Simionato *et al.*, 2017) and xanthobaccin (Yasuyuki *et al.*, 1999). To ensure better discriminant antimicrobial features, an extended inoculation period that could provide more information about how the microorganisms interact in long-term conditions would be relevant.

#### 4.2.4. Summary of the most promising antagonistic PGPR

After a qualitative analysis of those PGPR that presented possible antifungal traits, six isolates ultimately resulted in non-significant antagonistic activity towards the fungal pathogens (activity highlighted in grey in Table 4.2). Among these, all *B. megaterium* strains revealed to not hold antagonistic features against either *D. corticola* or *B. mediterranea.* In addition, two *Rouxiella* sp. isolates also revealed non-significant inhibitory activity, in contrast with other *Rouxiella* isolates. Although different isolates can belong to different species, the inhibitory activity can also be strain-specific, as already reported by Grossart *et al.* (2004). *B. mediterranea* was strongly supressed by *Serratia* isolates *(Serratia* sp. and *S. quinivorans*). Even though both resulted in a significant initial inhibition ($p \leq 0.001$), *S. quinivorans* stood out for being the only PGPR with constant antagonistic activity throughout the full inoculation time and inducing pigment formation by the fungi. These outcomes could be related to its ability of HCN production. On the other hand, PGPR with the most promising inhibitory effects against *D. corticola* were *B. cereus*, *Unknown* (AJ46; clustered among *Bacillus* spp. in Chapter II), and *R. badensis*, displaying similar statistical differences ($p \leq 0.01$) at 7 dpi. Among these, *Unknown* isolate AJ46 was able to present

a powerful earlier antagonistic effect ($p \leq 0.0001$), and along with *Bacillus cereus*, induce hyphal modifications in *D. corticola*. Three isolates – *S. quinivorans*, *R. badensis* and *B. cereus* - could simultaneously supress both fungal pathogens, but none presented combined outstanding results. Given that, the choice of PGPR would depend on the sought effect.

**Table 4.2.** Summary of the selected PGPR exhibiting antifungal activity against *B. mediterranea* and/or *D. corticola*. An initial detected and undetected antifungal activity is depicted with (+) and (-), respectively. Different colours represent PGPR with antifungal activity (green) and without antifungal activity (red) after seven days for *D. corticola* and nine days for *B. mediterranea*. PGPR with the best inhibitory results are presented in bold. Those PGPR isolates that did not hold significant antifungal activity in all antagonistic assays are highlighted in grey. Information regarding bacterial isolation forest and temperature are also provided.

| Forest | Temp. (°C) | PGPR code | Identification | Antifungal activity against: | |
|---|---|---|---|---|---|
| | | | | ***B. mediterranea*** | ***D. corticola*** |
| GR | 30 | AJ40 | *Bacillus megaterium* | + | - |
| | 37 | AJ43 | *Klebsiella aerogenes* | - | + |
| | | **AJ46** | ***Unknown*** | - | **+** |
| | | AJ49 | *Bacillus megaterium* | - | + |
| | | AJ51 | *Unidentified* | - | + |
| | 45 | AJ54 | *Bacillus megaterium* | - | + |
| LI | 30 | **AJ11** | ***Serratia quinivorans*** | **+** | + |
| | 37 | AJ16 | *Cedecea neteri* | + | + |
| ER | 30 | **AJ21** | ***Rouxiella badensis*** | + | **+** |
| | | AJ22 | *Rouxiella* sp. | + | + |
| | | **AJ24** | **Bacillus cereus** | + | **+** |
| | | AJ29 | *Rouxiella sp.* | - | + |
| | 37 | AJ30 | *Rouxiella sp.* | - | + |
| | | AJ31 | *Klebsiella oxytoca* | + | - |
| | | **AJ32** | ***Serratia* sp.** | **+** | - |

## 4.3. Conclusion

In this work, PGPR isolates exhibited different antagonistic effects against the cork oak fungal pathogens *B. mediterranea* and *D. corticola*. PGPR presented an *in vitro* inhibitory effect against *B. mediterranea* that decreased along time, probably due to the lapse production of antifungal compounds or to the defensive mechanisms created by the fungus. Nevertheless, *S. quinivorans* revealed an inhibitory activity that lasted throughout the full antagonist assay, possibly due to the production of the volatile HCN. Indeed, volatiles from the beneficial endophytes *Coniothyrium carteri* and *Fusarium oxysporum* also exhibited high inhibitory activity against *B. mediterranea* (Costa *et al.*, 2020). Accordingly, *S. quinivorans*

has the potential to be further explored in a biocontrol strategy to prevent *B. mediterranea* incidence. Regarding PGPR antagonistic effects against *D. corticola*, the isolates *Unknown* (AJ46; clustered among *Bacillus* strains), *B. cereus* (AJ24) and *Rouxiella* sp. (AJ22) presented promising results by inhibiting pathogen growth. Among these, the interactions with *Unknown* isolate and *B. cereus* ultimately resulted in *D. corticola* hyphal modifications, similar to those reported with known synthetic antimicrobial compounds (Deora *et al.*, 2005). However, these PGPR revealed a late effect, and extended inoculation periods could be further explored. Therefore, according to present results, PGPR from cork oak forests soil have the potential to biocontrol the cork oak diseases that these pathogens cause.

## 4.4. References

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# Chapter V: Conclusions

## 5.1. Conclusion and Future Perspectives

Natural occurring plant beneficial microorganisms, like PGPR, are in increasing demand due to their role in promoting more sustainable practices, not only in agriculture and crop production, but also in forestry. Their ecological significance is further enhanced as a more aware society is highly concerned about sustainability, health, and care of plant ecosystems. Studies that investigate how PGPR may act as development inducers relies not only in the biochemical characterization of these microorganisms, but also in the understanding of particular biological properties (*e.g.* phytohormone and/or VOCs production, antagonistic activities, among others), which could be potentially useful for applications in every plant-based systems. All these concerns were taken into consideration in the present study, which focused on the PGPR community existent in the rhizosphere of cork oak forests under three different bioclimates: semi-arid Grândola (GR), sub-humid Limãos (LI), and humid Ermida (ER). Hence, the scope of this research was to select PGPR isolates presenting the best agronomic and forestry features that could improve plant development, even during environmental stressed events.

In **Chapter II**, PGPR isolates were characterized, and those with the most encouraging nutrient-supply traits were further selected to be molecularly identified. Those PGPR isolated from forests exposed to overall greater abiotic stresses (drought, increased temperatures) displayed an enhanced adaptation to high temperatures and low nutrient availability conditions. Even though humid forests evidenced more PGPR that mainly presented a single nutrient-supply trait, PGPR with combined traits were mainly found in the semi-arid GR forest. *Bacillaceae* was the most frequently present family among sampled forests and displayed a greater incidence in GR forest. This corroborates previous information regarding the regular presence of *Firmicutes* phylum in forest soils (Bevivino *et al.*, 2014) and the regular isolation of bacteria from the *Bacillus* genus from dry bioclimates (Egamberdiyeva, 2005; Hernandez *et al.,* 2009; Moreno *et al.*, 2012; Hanna *et al.*, 2013). This is mainly due to its resilience to extreme conditions, such as water deficiency, high temperatures and high levels of UV radiation (Tan *et al.*, 2013). Therefore, PGPR communities from semi-arid environments (and specifically, bacteria belonging to the *Bacillus* genus) could hold a promising tool to enhance plant development, even under stressful environmental circumstances.

From Chapter II, we have selected those PGPR that displayed the combination of several nutrient-supply traits. In **Chapter III**, the same selected PGPR isolates were then screened for its ability to induce morphological changes in *A. thaliana* root architecture. Specifically, PGPR effects on primary root length, number of lateral roots, and root hairs presence were assessed. Results revealed that bacteria from the

*Bacillus*, *Serratia, Klebsiella,* and *Unidentified* genera strongly inhibited primary root length, supporting previous information that PGPR causes a reduction of primary root length, while accentuating other root morphologic parameters (Dobbelaere *et al.*, 1999; Vacheron *et al.*, 2013). Accordingly, highest number of lateral roots were achieved by *Bacillus*, *Serratia*, *Klebsiella*, *Cedecea*, *Rouxiella*, *Unidentified*, and *Unknown* strains. The time after inoculation seemed to be a key-factor when determining this root-parameter, as a strong enhanced number of lateral roots was observed at the 6$^{th}$ day and forward. Lastly, the presence of root hairs was induced by more than 98% of the tested PGPR, where the greater outcomes were mainly induced by *B. megaterium* strains. The presence of root hairs was strongly affected by the provenience of bacterial isolates. PGPR isolated from the semi-arid forest GR and using 45 °C temperature for bacterial isolation resulted in more root hairs presence. Several reports revealed that PGPR isolated from stressed soils are able to promote plant tolerance against abiotic and biotic stresses through the production of phytohormones or other bacterial compounds, which ultimately may end in the modulation of root-architecture of the plant (Ilyas & Bano, 2010; Yasmin *et al.*, 2013; Khan *et al.*, 2020). The presented results on the root are in agreement with the described effects of production of IAA by PGPR. However, other bacterial compounds, like VOCs, are also believed to be involved in plant growth promotion and in root architecture remodelling (Fincheira & Andrés, 2018). Classes of VOCs, such as aldehydes and ketones, may be involved in plant growth promotion (Gutiérrez-Luna *et al.*, 2010). Interestingly, the particular case of 2,3-butanediol, which was found to increase tolerance against drought (Liu & Zhang, 2015) has been associated with *Bacillus* species (Ryu *et al.*, 2003; Wu *et al.*, 2018). This may suggest a relevant contribution of VOCs on supporting plants to thrive in stressful circumstances.

The use of naturally adapted PGPR in biocontrol strategies has increased the interest in finding bacteria with antagonistic features against recognized widespread phytopathogens. For this reason, antagonistic studies against cork oak fungal endophytic pathogens (*B. mediterranea* and *D. corticola*) were conducted on **Chapter IV**. *B. mediterranea* infection is associated with cork oaks weakened by abiotic stress factors, such as water deficiency and/or high temperatures (Desprez-Loustau *et al.*, 2006). *D. corticola* is recognized for its endophytic lifestyle and opportunistic behaviour, persisting in a latent phase in asymptomatic tissues until cork the plant is under environmental stresses, like drought (Linaldeddu *et al.*, 2010). Both studied pathogens are positively correlated with high temperatures and drought, and forests exposed to water deficits have been shown to be associated with higher pathogen infection rates (Henriques *et al.*, 2016). All selected PGPR isolates (51) exhibited different antagonistic effects against cork oak pathogens. PGPR from stressed forests (such as GR) were expected to display more prominent antagonistic features when compared to other forests, as plants have been proved to be

able to manage their microbiota, influencing the composition and function of the microbial community in the rhizosphere (Lladó *et al.*, 2017). When under attack, plants actively select specific microorganisms that are more likely of supressing the disease (Bakker *et al.*, 2013; Gouda *et al.*, 2018). However, Costa *et al.* (2020) suggested the high potential that beneficial endophytic fungi obtained from cork oak humid forests (namely *Alternaria alternata* and *Fusarium oxysporum*) had at inhibiting *B. mediterranea* and *D. corticola.* These reports suggested that microorganisms from diverse bioclimates can hold potential antifungal activity. Indeed, *B. mediterranea* growth was significantly suppressed by the majority of tested PGPR, and *S. quinivorans* from LI forest, stood out for holding a consistent inhibitory activity during the full assay. Interestingly, this isolate was also found to be the only HCN producer, a volatile compound known for its antifungal features. These results are in line with those experienced by Costa *et al.* (2020), where volatiles from the beneficial endophytes *Coniothyrium carteri* and *Fusarium oxysporum* also exhibited high inhibitory activity against *B. mediterranea* (Costa *et al.*, 2020). Thus, *S. quinivorans* isolate could be pointed to be further explored as a biocontrol strategy to prevent *B. mediterranea* infection. Considering *D. corticola* development, *Unknown* AJ46, *B. cereus* AJ24 and *Rouxiella* sp. AJ22 isolates presented relevant inhibitory activity. Moreover, hyphal modifications similar to those induced by known synthetic antibiotics were observed by the end of the assay, when using *Unknown* and *Bacillus cereus* isolates. This suggests a late inhibitory effect and an extended inoculation period could be further explored. As far as we know, this is the first report on PGPR antagonistic potential against *D. corticola*. However, beneficial endophytic fungi, such as *Trichoderma viride*, *Epicoccum nigrum*, *Fusarium tricinctum*, *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Cytospora* sp. (Campanile *et al.*, 2007), and more recently, *Simplicillium aogashimaense*, *Coniothyrium carteri*, *Diaporthe passiflorae*, *Fimetariella rabenhorstii*, *Fusarium oxysporum, Chaetomium* sp., *Alternaria alternata* and *Penicillium olsonii* (Costa *et al.*, 2020) have been reported as *D. corticola* antagonists. This group of root-colonizing beneficial fungi have been recognized to control *D. corticola* growth through the induction of plant systematic resistance and production of antimicrobial compounds and lytic enzymes (Eriksson & Hawksworth, 2003; Halleen *et al.*, 2004; Djonović *et al.*, 2007; Costa *et al.*, 2020). In the same way, many PGPR are able to adopt such mechanisms, which could explain their antifungal features (Fernando *et al.*, 2005; Annapurna *et al.*, 2013; Kenneth *et al.*, 2019). Up to our knowledge, this chapter is the first known report of PGPR effects on cork oak pathogens. This information suggested that PGPR from cork oak forests should be further studied, as they may hold a potential biocontrol mechanism against fungi-induced cork oak diseases.

Altogether, this work increased the knowledge about how the environmental conditions affect PGPR traits, and ultimately provided new information about biocontrol mechanisms against cork oak severe pathogens. A subsequent experiment, where three-month old cork oak plantlets would be treated with the most promising selected PGPR (single or in a consortium) would have given us more information about the *in vivo* performance of PGPR. Several studies can be conducted for a better understanding of PGPR ecological roles. Among these, the identification of possible phytohormones and/or VOCs or other possible compounds produced by PGPR could be performed. For example, VOCs could be identified using solid phase microextraction (SPME) coupled to gas chromatography–mass spectrometry (GC-MS). Additionally, antifungal metabolites produced by PGPR could be identified through Liquid Chromatography Mass Spectrometry (LC-MS) and, finally, studies of synergy between these antimicrobial substances.

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# Annexes

**Annex 1.** Results regarding biochemical characterization of all GR bacterial isolates tested. Information about temperature used for bacterial isolation are provided, along with the respective bacteria and PGPR codes.

| Temp. (°C) | Bacterial Code | Biochemical Assay: Organic acids production | Biochemical Assay: Phosphate solubilisation $Ca_3PO_4$ | Biochemical Assay: Phosphate solubilisation $AlPO_4$ | Biochemical Assay: Siderophores production | PGPR Code |
|---|---|---|---|---|---|---|
| 30 | GR 1 | | | | | |
| | GR 2 | | | | | |
| | GR 3 | | | | | |
| | GR 4 | | | | | |
| | GR 5 | | | | | |
| | GR 6 | | | | | |
| | GR 7 | | | | | |
| | GR 8 | | | | | |
| | GR 9 | | | | | |
| | GR 10 | | | | | AJ40 |
| | GR 11 | | | | | |
| | GR 12 | | | | | |
| | GR 13 | | | | | |
| | GR 14 | | | | | |
| | GR 15 | | | | | |
| | GR 16 | | | | | |
| | GR 17 | | | | | AJ41 |
| | GR 18 | | | | | |
| | GR 19 | | | | | |
| | GR 20 | | | | | |
| | GR 21 | | | | | |
| | GR 22 | | | | | |
| | GR 23 | | | | | |
| | GR 24 | | | | | |
| | GR 25 | | | | | |
| | GR 26 | | | | | |
| | GR 27 | | | | | |
| | GR 28 | | | | | |
| | GR 29 | | | | | |
| | GR 30 | | | | | |
| | GR 31 | | | | | |
| | GR 32 | | | | | |
| 37 | GR 33 | | | | | |
| | GR 34 | | | | | |
| | GR 35 | | | | | |
| | GR 36 | | | | | |
| | GR 37 | | | | | |
| | GR 38 | | | | | |
| | GR 39 | | | | | |
| | GR 40 | | | | | |
| | GR 41 | | | | | |
| | GR 42 | | | | | AJ42 |
| | GR 43 | | | | | |
| | GR 44 | | | | | AJ43 |
| | GR 45 | | | | | |
| | GR 46 | | | | | |
| | GR 47 | | | | | |
| | GR 48 | | | | | |
| | GR 49 | | | | | |
| | GR 50 | | | | | |
| | GR 51 | | | | | |
| | GR 52 | | | | | |
| | GR 53 | | | | | |
| | GR 54 | | | | | |
| | GR 55 | | | | | AJ44 |
| | GR 56 | | | | | AJ45 |
| 37 | GR 57 | | | | | |
| | GR 58 | | | | | AJ46 |
| | GR 59 | | | | | |
| | GR 60 | | | | | |
| | GR 61 | | | | | AJ47 |
| | GR 62 | | | | | |
| | GR 63 | | | | | AJ48 |
| | GR 64 | | | | | AJ49 |
| | GR 65 | | | | | |
| | GR 66 | | | | | |
| | GR 67 | | | | | |
| | GR 68 | | | | | |
| | GR 69 | | | | | |
| | GR 70 | | | | | |
| | GR 71 | | | | | |
| | GR 72 | | | | | AJ50 |
| | GR 73 | | | | | |
| | GR 74 | | | | | |
| | GR 75 | | | | | |
| | GR 76 | | | | | AJ51 |
| | GR 77 | | | | | |
| | GR 78 | | | | | AJ52 |
| | GR 79 | | | | | |
| | GR 80 | | | | | AJ53 |
| 45 | GR 81 | | | | | |
| | GR 82 | | | | | AJ54 |
| | GR 83 | | | | | |
| | GR 84 | | | | | AJ55 |
| | GR 85 | | | | | AJ56 |
| | GR 86 | | | | | |
| | GR 87 | | | | | |
| | GR 88 | | | | | |
| | GR 89 | | | | | |
| | GR 90 | | | | | |
| | GR 91 | | | | | AJ57 |
| | GR 92 | | | | | AJ58 |
| | GR 93 | | | | | |
| | GR 94 | | | | | |
| | GR 95 | | | | | AJ59 |
| | GR 96 | | | | | AJ60 |
| | GR 97 | | | | | AJ61 |
| | GR 98 | | | | | AJ62 |
| | GR 99 | | | | | |
| | GR 100 | | | | | AJ63 |
| | GR 101 | | | | | AJ64 |
| | GR 102 | | | | | |
| | GR 103 | | | | | |
| | GR 104 | | | | | |

**Annex 2.** Results regarding biochemical characterization of all LI bacterial isolates tested. Information about temperature used for bacterial isolation are provided, along with the respective bacteria and PGPR codes.

| Temp (°C) | Bacteria Code | Biochemical Assay: Organic acids production | Phosphate solubilisation | | Siderophores production | PGPR code |
|---|---|---|---|---|---|---|
| | | | Ca2PO4 | AlPO4 | | |
| | LI 1 | | | | | |
| | LI 2 | | | | | |
| | LI 3 | | | | | |
| | LI 4 | | | | | |
| | LI 5 | | | | | |
| | LI 6 | | | | | |
| | LI 7 | | | | | |
| | LI 8 | | | | | |
| | LI 9 | | | | | |
| | LI 10 | | | | | |
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| | LI 22 | | | | | |
| | LI 23 | | | | | |
| | LI 24 | | | | | |
| 30 | LI 25 | | | | | |
| | LI 26 | | | | | |
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| | LI 30 | | | | | |
| | LI 31 | | | | | |
| | LI 32 | | | | | |
| | LI 33 | | | | | |
| | LI 34 | | | | | |
| | LI 35 | | | | | |
| | LI 36 | | | | | AJ14 |
| | LI 37 | | | | | |
| | LI 38 | | | | | AJ10 |
| | LI 39 | | | | | |
| | LI 40 | | | | | |
| | LI 41 | | | | | |
| | LI 42 | | | | | |
| | LI 43 | | | | | |
| | LI 44 | | | | | |
| | LI 45 | | | | | |
| | LI 46 | | | | | |
| | LI 47 | | | | | |
| | LI 48 | | | | | |
| | LI 49 | | | | | |
| | LI 50 | | | | | AJ11 |
| | LI 51 | | | | | |
| | LI 52 | | | | | |
| | LI 53 | | | | | |
| | LI 54 | | | | | |
| | LI 55 | | | | | |
| 37 | LI 56 | | | | | |
| | LI 57 | | | | | AJ12 |
| | LI 58 | | | | | |
| | LI 59 | | | | | |
| | LI 60 | | | | | |

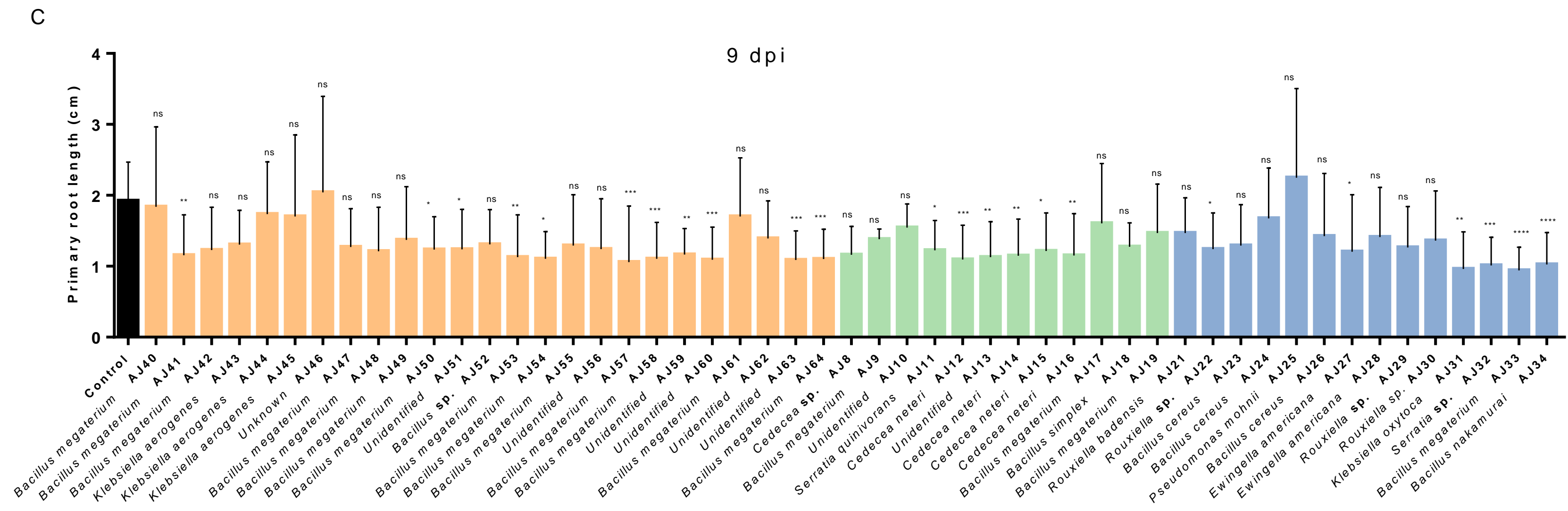
| Temp. (°C) | Bacteria Code | Biochemical Assay: Organic acids production | Phosphate solubilisation | | Siderophores production | PGPR Code |
|---|---|---|---|---|---|---|
| | | | Ca2PO4 | AlPO4 | | |
| | LI 61 | | | | | |
| | LI 62 | | | | | |
| | LI 63 | | | | | |
| | LI 64 | | | | | |
| | LI 65 | | | | | |
| | LI 66 | | | | | |
| | LI 67 | | | | | |
| | LI 68 | | | | | |
| | LI 69 | | | | | |
| | LI 70 | | | | | |
| | LI 71 | | | | | |
| | LI 72 | | | | | |
| | LI 74 | | | | | |
| | LI 75 | | | | | |
| | LI 76 | | | | | |
| | LI 77 | | | | | |
| | LI 78 | | | | | |
| | LI 79 | | | | | AJ18 |
| | LI 80 | | | | | AJ15 |
| 37 | LI 81 | | | | | AJ16 |
| | LI 82 | | | | | |
| | LI 83 | | | | | |
| | LI 84 | | | | | |
| | LI 85 | | | | | |
| | LI 86 | | | | | |
| | LI 87 | | | | | |
| | LI 88 | | | | | |
| | LI 89 | | | | | AJ13 |
| | LI 90 | | | | | |
| | LI 91 | | | | | AJ8 |
| | LI 92 | | | | | |
| | LI 93 | | | | | |
| | LI 94 | | | | | |
| | LI 95 | | | | | |
| | LI 96 | | | | | AJ9 |
| | LI 97 | | | | | |
| | LI 98 | | | | | AJ17 |
| | LI 99 | | | | | |
| | LI 100 | | | | | |
| | LI 101 | | | | | AJ19 |
| | LI 102 | | | | | |
| | LI 103 | | | | | |
| | LI 104 | | | | | |
| | LI 105 | | | | | |
| | LI 106 | | | | | |
| | LI 107 | | | | | |
| | LI 108 | | | | | |
| | LI 109 | | | | | |
| 45 | LI 110 | | | | | |
| | LI 111 | | | | | |
| | LI 112 | | | | | |
| | LI 113 | | | | | |
| | LI 114 | | | | | |
| | LI 115 | | | | | |
| | LI 116 | | | | | |
| | LI 117 | | | | | |
| | LI 118 | | | | | |
| | LI 119 | | | | | |
| | LI 120 | | | | | |

**Annex 3.** Results regarding biochemical characterization of all ER bacterial isolates tested. Information about temperature used for bacterial isolation are provided, along with the respective bacteria and PGPR codes.

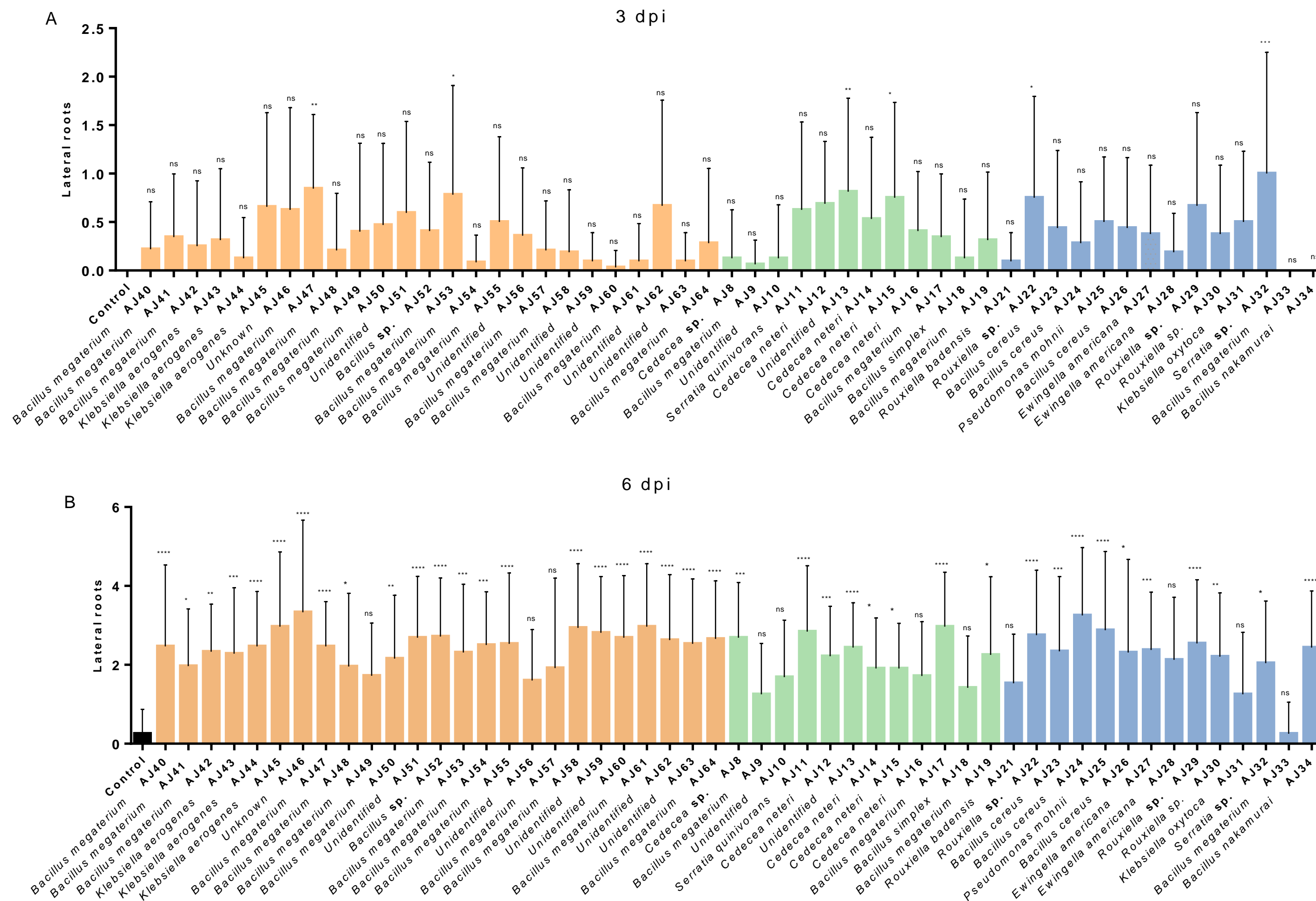
| Temp. (°C) | Bacteria code | Biochemical Assay | | | | PGPR code |
|---|---|---|---|---|---|---|
| | | Organic acids | Phosphate solubilisation | | Siderophores production | |
| | | | $Ca_2PO_4$ | $AlPO_4$ | | |
| 30 | ER 1 | | | | | |
| 30 | ER 2 | | | | | |
| 30 | ER 3 | | | | | |
| 30 | ER 4 | | | | | |
| 30 | ER 5 | | | | | |
| 30 | ER 6 | | | | | |
| 30 | ER 7 | | | | | AJ21 |
| 30 | ER 8 | | | | | AJ22 |
| 30 | ER 9 | | | | | |
| 30 | ER 10 | | | | | AJ23 |
| 30 | ER 11 | | | | | |
| 30 | ER 12 | | | | | |
| 30 | ER 13 | | | | | |
| 30 | ER 14 | | | | | |
| 30 | ER 15 | | | | | |
| 30 | ER 16 | | | | | AJ24 |
| 30 | ER 17 | | | | | |
| 30 | ER 18 | | | | | |
| 30 | ER 19 | | | | | |
| 30 | ER 20 | | | | | |
| 30 | ER 21 | | | | | AJ25 |
| 30 | ER 22 | | | | | |
| 30 | ER 23 | | | | | |
| 30 | ER 24 | | | | | |
| 30 | ER 25 | | | | | |
| 30 | ER 26 | | | | | |
| 30 | ER 27 | | | | | AJ26 |
| 30 | ER 28 | | | | | |
| 30 | ER 29 | | | | | |
| 30 | ER 30 | | | | | |
| 30 | ER 31 | | | | | |
| 30 | ER 32 | | | | | |
| 30 | ER 33 | | | | | |
| 30 | ER 34 | | | | | |
| 30 | ER 35 | | | | | |
| 30 | ER 36 | | | | | |
| 30 | ER 37 | | | | | |
| 30 | ER 38 | | | | | |
| 30 | ER 39 | | | | | |
| 30 | ER 40 | | | | | |
| 30 | ER 41 | | | | | |
| 30 | ER 42 | | | | | |
| 30 | ER 43 | | | | | AJ27 |
| 30 | ER 44 | | | | | AJ28 |
| 30 | ER 45 | | | | | |
| 30 | ER 46 | | | | | |
| 30 | ER 47 | | | | | AJ29 |
| 30 | ER 48 | | | | | |
| 30 | ER 49 | | | | | |
| 30 | ER 50 | | | | | |
| 37 | ER 51 | | | | | |
| 37 | ER 52 | | | | | AJ30 |
| 37 | ER 53 | | | | | AJ31 |
| 37 | ER 54 | | | | | |
| 37 | ER 55 | | | | | |
| 37 | ER 56 | | | | | |
| 37 | ER 57 | | | | | |
| 37 | ER 58 | | | | | AJ32 |
| 37 | ER 59 | | | | | |
| 37 | ER 60 | | | | | |
| 37 | ER 61 | | | | | |
| 37 | ER 62 | | | | | |
| 37 | ER 63 | | | | | |
| 37 | ER 64 | | | | | |
| 37 | ER 65 | | | | | |
| 37 | ER 66 | | | | | |
| 37 | ER 67 | | | | | |
| 37 | ER 68 | | | | | |
| 37 | ER 69 | | | | | |
| 37 | ER 70 | | | | | |
| 37 | ER 71 | | | | | |
| 37 | ER 72 | | | | | |
| 37 | ER 73 | | | | | |
| 37 | ER 74 | | | | | |
| 37 | ER 75 | | | | | |
| 37 | ER 76 | | | | | |
| 37 | ER 77 | | | | | |
| 37 | ER 78 | | | | | |
| 37 | ER 79 | | | | | |
| 37 | ER 80 | | | | | |
| 37 | ER 81 | | | | | |
| 37 | ER 82 | | | | | |
| 37 | ER 83 | | | | | |
| 37 | ER 84 | | | | | |
| 37 | ER 85 | | | | | |
| 37 | ER 86 | | | | | |
| 37 | ER 87 | | | | | |
| 37 | ER 88 | | | | | |
| 37 | ER 89 | | | | | |
| 37 | ER 90 | | | | | |
| 45 | ER 91 | | | | | |
| 45 | ER 92 | | | | | AJ33 |
| 45 | ER 93 | | | | | AJ34 |
| 45 | ER 94 | | | | | |
| 45 | ER 95 | | | | | |
| 45 | ER 96 | | | | | |
| 45 | ER 97 | | | | | |
| 45 | ER 98 | | | | | |
| 45 | ER 99 | | | | | |
| 45 | ER 100 | | | | | |

**Annex 4.** PGPR effect on *A. thaliana* primary root length development over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Asterisks represent statistically significant differences to control at *p* ≤0.05 (*), *p* ≤0.01 (**), *p* ≤0.001 (***) and *p* ≤0.0001 (****). Non-significant differences are displayed with (ns). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours –orange (GR), green (LI) and blue (ER).

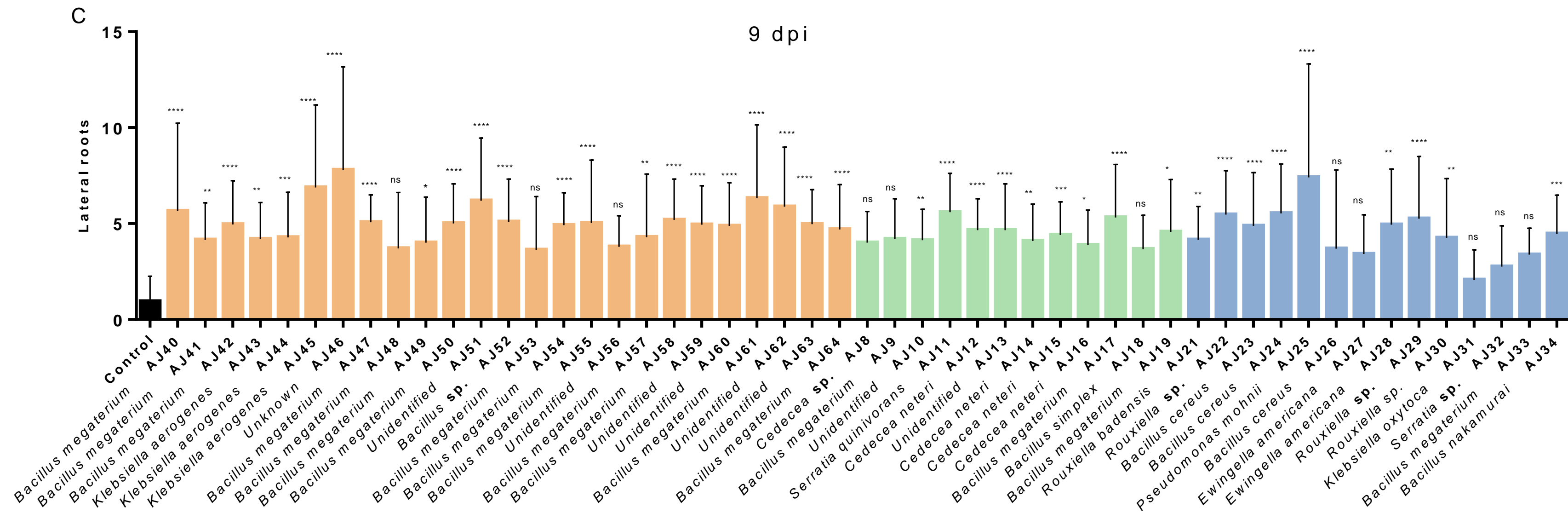
**Annex 4 (cont.).** PGPR effect on *A. thaliana* primary root length development over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Non-significant differences are displayed with (ns). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours – orange (GR), green (LI) and blue (ER).



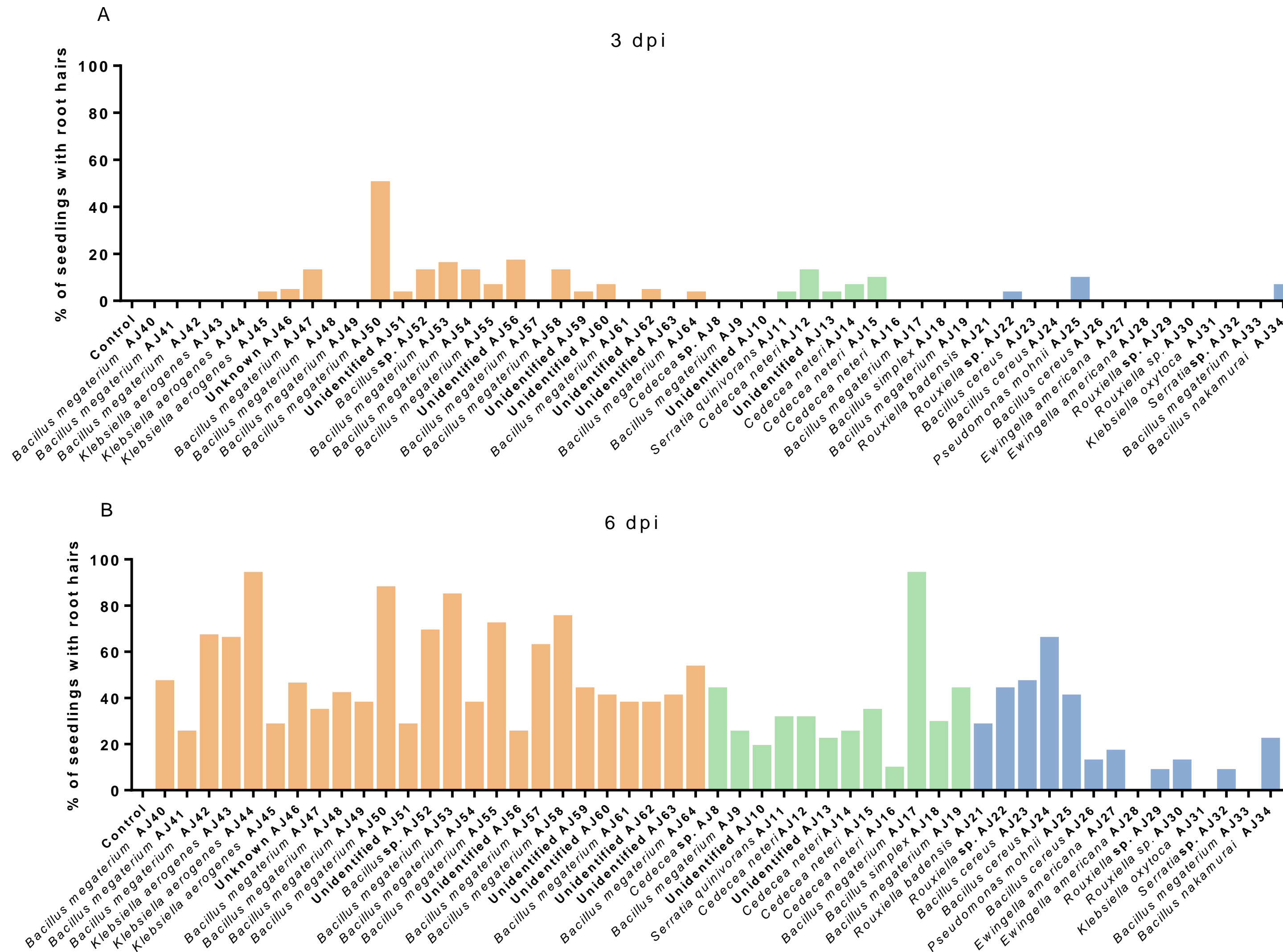
**Annex 5.** PGPR effect on *A. thaliana*'s lateral roots development over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Non-significant differences are displayed with (ns). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours – orange (GR), green (LI) and blue (ER).



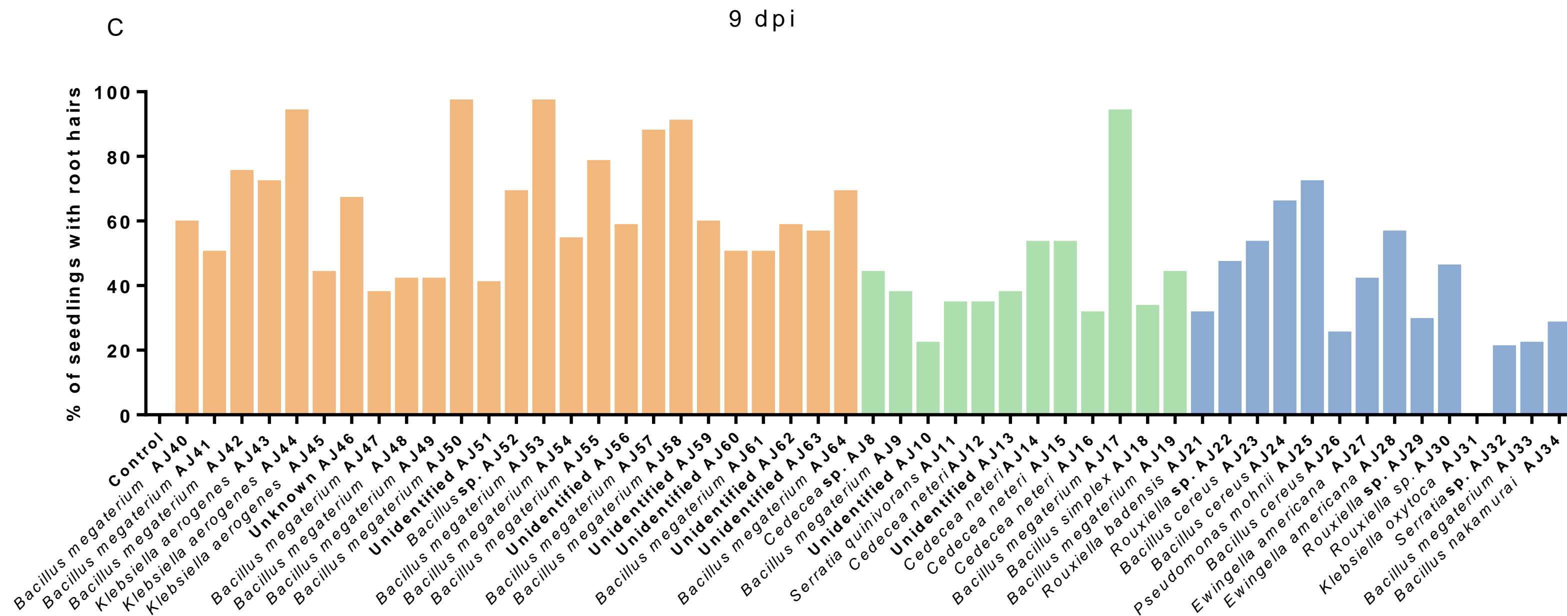
**Annex 5 (cont.).** PGPR effect on *A. thaliana*'s lateral roots development over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Asterisks represent statistically significant differences to control at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Non-significant differences are displayed with (ns). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours – orange (GR), green (LI) and blue (ER).



**Annex 6.** PGPR effect on *A. thaliana*'s root hairs presence over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours – orange (GR), green (LI) and blue (ER).



**Annex 6 (cont.).** PGPR effect on *A. thaliana's* root hairs presence over time - 3 dpi (A), 6 dpi (B) and 9 dpi (C). Isolates highlighted in different colour corresponds to isolation forest of PGPR, where isolate colours – orange (GR), green (LI) and blue (ER).



**Annex 7.** (A) *Biscogniauxia mediterranea* and (B) *Diplodia corticola* radial growth ($cm^2$) over time of inoculation with different PGPR. Data were obtained with ImageJ software.

| A | | ***Biscogniauxia mediterranea* radial growth ($cm^2$)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Forest** | **Isolate** | 5 dpi | | | 7 dpi | | | 9 dpi | | |
| | | R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 |
| **LI** | **AJ11** ***Serratia quinivorans*** | 44,786 | 55,488 | 44,898 | 54,821 | 59,023 | 47,839 | 59,477 | 59,685 | 50,310 |
| | **AJ16** ***Cedecea neteri*** | 55,223 | 54,080 | 50,469 | 59,707 | 60,764 | 58,608 | 63,21 | 62,507 | 62,367 |
| **ER** | **AJ21** ***Rouxiella badensis*** | 52,663 | 55,163 | 49,554 | 62,901 | 62,663 | 57,745 | 63,005 | 63,146 | 61,140 |
| | **AJ22 Rouxiella sp.** | 55,646 | 53,639 | 54,740 | 62,948 | 62,112 | 61,273 | 63,101 | 62,989 | 62,369 |
| | **AJ24** ***Bacillus cereus*** | 49,516 | 50,475 | 50,231 | 62,438 | 62,296 | 61,841 | 63,112 | 62,899 | 62,713 |
| | **AJ31** ***Klebsiella oxytoca*** | 51,711 | 48,458 | 52,666 | 63,014 | 62,372 | 63,034 | 63,014 | 63,094 | 63,131 |
| | **AJ32** ***Serratia*** **sp.** | 43,972 | 51,083 | 44,174 | 62,286 | 61,548 | 60,223 | 62,286 | 62,225 | 61,101 |
| **GR** | **AJ40** ***Bacillus megaterium*** | 55,464 | 53,153 | 57,073 | 62,378 | 63,209 | 61,424 | 63,274 | 63,209 | 62,671 |
| | **C-** | 63,094 | 62,591 | 63,099 | 63,300 | 63,300 | 63,300 | 63,300 | 63,300 | 63,300 |

| B | | ***Diplodia corticola* radial growth ($cm^2$)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Forest** | **Isolate** | 3 dpi | | | 5 dpi | | | 7 dpi | | |
| | | R1 | R2 | R3 | R1 | R2 | R3 | R1 | R2 | R3 |
| **LI** | **AJ11** ***Serratia quinivorans*** | 22,042 | 25,478 | 22,616 | 42,128 | 44,804 | 45,447 | 56,56 | 49,845 | 51,163 |
| | **AJ16** ***Cedecea neteri*** | 27,169 | 19,994 | 26,458 | 51,107 | 42,778 | 49,615 | 62,267 | 60,201 | 62,231 |
| **ER** | **AJ21** ***Rouxiella badensis*** | 24,986 | 25,007 | 23,065 | 40,179 | 46,500 | 44,174 | 50,541 | 53,03 | 49,804 |
| | **AJ22** ***Rouxiella*** **sp.** | 25,837 | 25,952 | 30,090 | 36,573 | 45,947 | 47,872 | 47,324 | 52,353 | 56,076 |
| | **AJ24** ***Bacilus cereus*** | 27,310 | 21,614 | 23,421 | 47,234 | 36,78 | 43,863 | 53,034 | 47,873 | 47,813 |
| | **AJ29** ***Rouxiella*** **sp.** | 25,271 | 30,443 | 29,994 | 46,220 | 43,546 | 48,957 | 53,266 | 54,584 | 53,645 |
| | **AJ30** ***Rouxiella*** **sp.** | 29,689 | 33,026 | 34,028 | 48,943 | 51,057 | 53,077 | 57,445 | 59,441 | 56,777 |
| **GR** | **AJ43** ***Klebsiella aerogenes*** | 23,509 | 22,130 | 27,906 | 42,713 | 37,455 | 46,592 | 53,219 | 45,783 | 59,180 |
| | **AJ46** ***Unknown*** | 12,729 | 15,100 | 15,411 | 23,963 | 22,530 | 25,079 | 51,977 | 48,220 | 50,954 |
| | **AJ49** ***Bacillus megaterium*** | 17,678 | 24,647 | 25,145 | 38,900 | 46,652 | 48,747 | 50,649 | 58,026 | 60,111 |
| | **AJ51** ***Unidentified*** | 32,231 | 25,720 | 26,429 | 50,747 | 43,798 | 40,58 | 52,387 | 55,799 | 48,405 |
| | **AJ54** ***Bacillus megaterium*** | 27,661 | 32,426 | 31,360 | 44,519 | 41,415 | 48,100 | 59,481 | 54,979 | 54,768 |
| | **C-** | 29,391 | 23,130 | 29,805 | 51,811 | 46,109 | 47,003 | 63,110 | 63,100 | 63,177 |