

Characterization and Biotechnological Applications of Plant Growth Promoting Bacteria

Marino Costa Santos

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Department of Biology, Faculty of Science, University of Porto (FCUP)
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Orientador

Maria da Conceição Lopes Vieira dos Santos, PhD

Full Professor, Faculty of Science, University of Porto (FCUP)

Coorientador

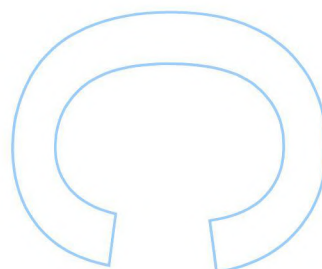
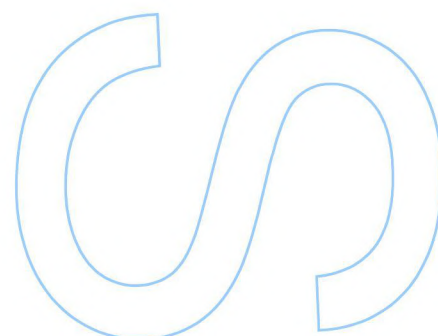
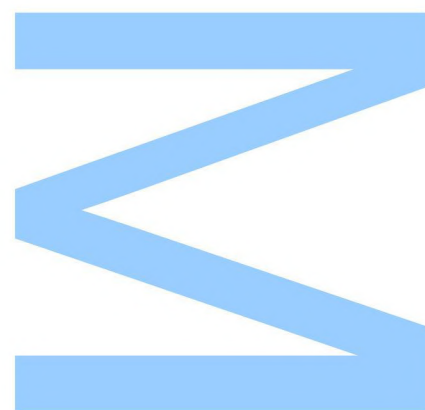
Guilhermina Miguel da Silva Marques, PhD

Assistant Professor, University of Trás-os-Montes e Alto Douro (UTAD)

Co-supervisor

Nuno Mariz Ponte, MSc

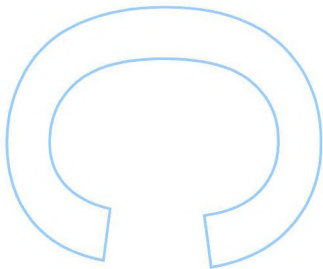
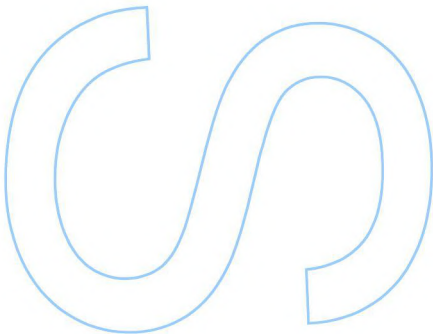
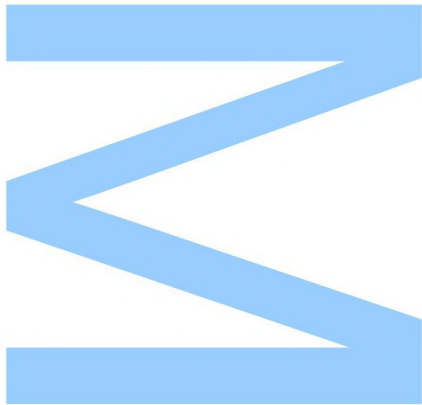
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Todas as correções determinadas
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Abstract

Sustainability in agriculture remains as one of the biggest current challenges, due to the fast-growing population and increasing demand for agricultural products. The use of fertilizers and pesticides allows high productivity, but their application has a huge negative impact on the environment. It is thus important that new sustainable alternatives to agro-chemicals are developed. One of the tools biotechnology proposes as an alternative is the manipulation of soil and plants microbiomes, through the application of beneficial bacteria, that have the ability to promote growth and protect plants from pathogens, through a variety of mechanisms such as the production of phytohormones and antimicrobial compounds. Currently, there are several formulations of biofertilizers and biopesticides commercially available, which shows that there is an increasing interest and a market for such products. Hence, it is important that plant microbiomes are studied, in order to isolate and characterize new microorganisms of interest. In this work, 21 isolates from the iB₂ and UTAD collections were characterized and tested for: a) plant growth promoting bacteria biochemical characteristics; b) growth inhibition of a relevant pathogen in Portugal; c) biofortification potential and influence on the photosynthetic metabolism of tomato plants. Results show that these isolates present plant growth promoting traits such as indole acetic acid production and nutrient solubilization, as well as the potential to antagonize a phytopathogen (*Pseudomonas syringae* pv. *actinidiae*), and some isolates showed a positive influence on the photosynthetic performance, sugar production and non-enzymatic defenses of tomato plants. Based on this work, the isolates from this collection are potential candidates as biofertilizers and biopesticides.

Keywords

Sustainable agriculture, Plant growth promoting bacteria (PGPB), Biocontrol, Biofertilizers

Resumo

A sustentabilidade na agricultura permanece como um dos maiores desafios atualmente, devido à tendência de crescimento rápido da população e consequente necessidade de fazer face à crescente demanda de produtos agrícolas. O uso de fertilizantes e pesticidas permite uma elevada produtividade, mas a sua aplicação tem um grande impacto negativo a nível ambiental, sendo por isso importante que se desenvolvam novas alternativas sustentáveis ao uso excessivo dos agroquímicos. Uma das ferramentas que a biotecnologia propõe como alternativa é a manipulação do microbioma dos solos e das plantas, através da aplicação de bactérias benéficas, com capacidade de promover o crescimento das plantas e protegê-las contra o ataque de agentes patogénicos, através de uma variedade de mecanismos, como a produção de fitohormonas e compostos antimicrobianos. Atualmente já existem várias formulações de biofertilizantes e biopesticidas, demonstrando o interesse crescente e mercado para estes produtos. Importa, assim, estudar os microbiomas das plantas, para isolar e caracterizar novos organismos de interesse. Neste trabalho, 21 isolados das coleções iB₂ e UTAD foram caracterizados e testados para: a) características bioquímicas de bactérias promotoras do crescimento de plantas; b) inibição do crescimento de um patogénico de relevância em Portugal; c) potencial de biofortificação e influência no metabolismo fotossintético, em tomateiro. Os resultados deste trabalho mostram que estes isolados apresentam características que podem promover o crescimento de plantas, como a produção de ácido indol acético e a solubilização de nutrientes, que têm o potencial de antagonizar um agente fitopatogénico (*Pseudomonas syringae* pv. *actinidiae*), e alguns mostraram ter uma influência positiva na performance fotossintética, produção de açúcares e defesas não enzimáticas em tomateiro. Com base nestes resultados, os isolados desta coleção são potenciais agentes biofertilizantes e biopesticidas.

Palavras-chave

Agricultura sustentável, Bactérias Promotoras do Crescimento de Plantas, Biocontrolo, Biofertilizantes

Publications/Presentations

ISI-JCR Papers

Santos, M., Ponte, N., Dias, M. C., Marques, G., Santos, C. (2020). Effect of *Bacillus* spp. and *Brevibacillus* sp. on the physiological status of *Solanum lycopersicum*. (under submission) – **Chapter IV**

Publications on Technical-Scientific Journals

Santos, M., Ponte, N., Marques, G. (2020). Bactérias Promotoras do Crescimento de Plantas como uma alternativa sustentável aos agroquímicos. Agrotec - Revista Técnico-Científica Agrícola, Ano Internacional da Sanidade Vegetal – **Chapter I**

Oral Communications

Santos, M.*, Silva M., Ponte, N., Santos, C. (2019) Characterization of a *Bacillus* sp. Isolate and possible biotechnological applications. Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal (18/12/2019). IV Jornadas de Engenharia Agronómica (*speaker) – **Chapter II**

Poster Presentations

Santos, M., Ponte, N., Marques, G., Santos, C. (2020). Biocontrol Potential of Plant Growth Promoting Bacteria Isolates Against *Pseudomonas syringae* pv. *actinidiae*. Faculdade de Ciências da Universidade do Porto, Portugal (02/12/2020). 4th Meeting (Online Edition) – Functional Biology and Biotechnology of Plants – **Chapter III**

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Abbreviations

ABA – Absciscic acid

ACC – 1-Aminocyclopropane-1-carboxylate

CAT – Catalase

Chl *a* – Chlorophyll *a*

Chl *b* – Chlorophyll *b*

C_i – Internal CO₂

CK – Cytokinins

DAPG – 2,4-Diacetyl phloroglucinol

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

DW – Dry weight

E – Transpiration rate

ET – Ethylene

ETR – Electron transport rate

F'_m – Maximum fluorescence in light

F_m – Maximum fluorescence

F_v'/F_m' – Maximum efficiency of photosystem II

F_v/F_m – Maximum quantum efficiency of photosystem II

FW – Fresh weight

GA – Gibberellins

gs – Stomatal conductance

h – hour(s)

IAA – Indole acetic acid

iWUE – Intrinsic water usage efficiency

LB – Luria-Bertani

min – minute(s)

NPQ – Nonphotochemical quenching

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OXI – Cytochrome c oxidase

PCA – Principal component analysis

PCR – Polymerase chain reaction

PGP – Plant growth promoting

PGPB – Plant growth promoting bacteria

P_N – Net carbon assimilation

PSII – Photosystem II

PVK – Pikovskaya's agar

qP – Photochemical quenching

QQ – Quorum quenching

QS – Quorum sensing

s – second(s)

SD – Standard deviation

SE – Solubilization efficiency

TPC – Total phenolic content

TSS – Total soluble sugars

VOCs – Volatile organic compounds

WUE – Water usage efficiency

ΦPSII – Quantum yield of photosystem II

I. Chapter 1: A Review on Plant Growth Promoting Bacteria as Sustainable Alternatives to Agrochemicals

Feeding a growing population while aiming for sustainability is one of the biggest challenges agricultural production faces (Calicioglu *et al.*, 2019; Singh *et al.*, 2019). Increasing global food demand has made agricultural practices more industrialized, with the use of agrochemicals such as fertilizers and pesticides (Carvalho, 2017). Although the application of agrochemicals allows food production in quantity and quality, its continuous and excessive use has a negative ecological impact (Warra *et al.*, 2020) and increases the risk of food chain contamination (Sharma and Singhvi, 2017; Sankhla *et al.*, 2018), both by not attending the needed safety periods prior consumption but also because agrochemicals end up contaminating soil and leaching into water reservoirs (Blaas and Kroeze, 2016), having a direct impact on ecosystems (Bruhl and Zaller, 2019) and, ultimately, entering into untargeted food chains.

Crop growth depends on abiotic factors such as soil nutrients, irrigation and temperature, but plant health is also strongly influenced by biotic factors such as soil microorganisms (Berendsen *et al.*, 2012). The rhizosphere, the soil region that surrounds plants' roots, is known for being a hotspot of a variety of microorganisms. Depending on their interactions with plants, these microorganisms can be beneficial, neutral or pathogenic (Mendes *et al.*, 2013; Xiong *et al.*, 2020).

Amongst the beneficial microorganisms that colonize plants and soil, there is a wide group of bacteria known as plant growth promoting bacteria (PGPB) (Glick, 2020), involved in stimulating plants' growth and health. PGPB can be either endophytic or symbiotic, when they are able to colonize plant tissues; rhizospheric, when they colonize the roots surface; or free living, if they colonize soil without directly being associated with plants (Souza *et al.*, 2015). PGPB can promote plant growth through a diversity of mechanisms, in a complex network of influence, which is widely dependant on factors such as the environment, bacterial and plant species (Figure I.1).

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Besides promoting growth, PGPB allow plants to withstand both biotic and environmental stresses, which is of great interest given the current scenario of climate changes (Ramakrishna *et al.*, 2019) that, besides being a threat to crop production, might be implicated in the emergence of new diseases (Elad and Pertot, 2014). In this chapter, some of the most important mechanisms of action of PGPB reported in literature and current perspectives regarding PGPB-products are briefly explained.

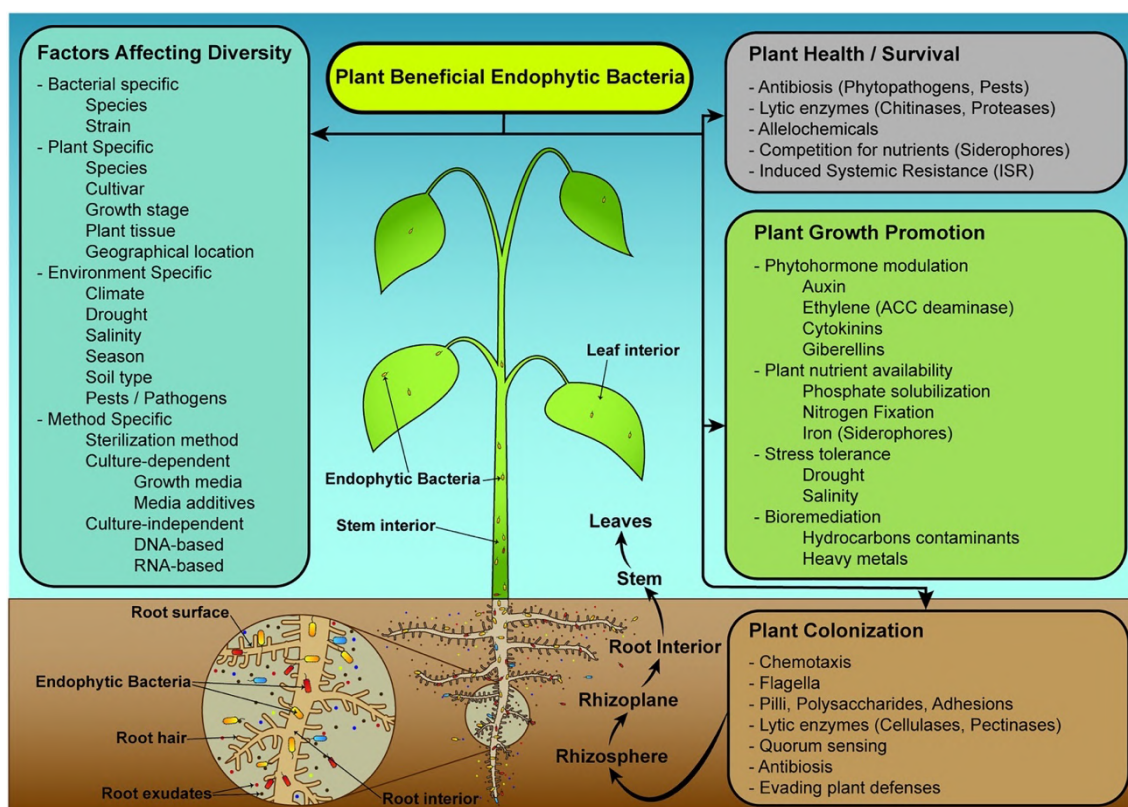


Figure I.1. Proposed summary of the mechanisms of action, colonization and factors affecting the bacteria-host system of endophytic PGPB (Retrieved from Afzal *et al.*, 2019).

1. Mechanisms of action of Plant Growth Promoting Bacteria

1.1. Modulation of Plant Hormone Levels

PGPB can promote growth directly by regulating various plant hormones like Indole-3-acetic acid (IAA), abscisic acid (ABA), Gibberellins (GA), cytokinins (CK) or

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ethylene (ET), which all act together in orchestrating plant performance, including health/survival, growth and cellular processes (Ali *et al.*, 2017).

Auxin production, mainly IAA, is a common feature among PGPB species, and one of the most reported mechanisms that explain plant growth promotion (Grobela *et al.*, 2015). IAA functions as an important signal molecule in the regulation of plant development including organogenesis, root initiation and elongation, cell division, stem elongation, and gene regulation (Perrot-Rechenmann, 2010; Takatsuka and Umeda, 2014). Bacterial produced IAA changes the naturally produced IAA levels in plants, ultimately resulting in enhanced growth and better root systems, with a higher surface area that allows better nutrient and water uptake. However, species, cultivars or plant characteristics (e.g. age, life cycle, phytosanitary status) condition plant's sensitivity to different auxin balance/levels. Depending if the plant's endogenous auxin levels are suboptimal or optimal, bacterial-produced IAA can have a stimulant or an inhibitory effect, by bringing those levels to optimal, or exceeding them, respectively (Vejan *et al.*, 2016; Olanrewaju *et al.*, 2017; Kudoyarova *et al.*, 2019). In literature, several studies show that IAA-producing bacteria are beneficial to several important crops. For instance, two IAA-producing PGPB, *Pseudomonas fluorescens* and *Bacillus subtilis*, increased root length, shoot length, root and shoot fresh and dry weight of onion seedlings (Reetha *et al.*, 2014). Similarly, a study on groundnut seeds showed that inoculation with IAA-producing *B. licheniformis* MML2501 improved seed germination rate, root length, shoot length, number of lateral roots and dry weight (Prashanth and Mathivanan, 2010). Additionally, inoculation of potato plants with two different IAA-producing *Bacillus* sp. increased auxin levels, and stimulated shoot and root length, the number of leaves and pigments' content (Ahmed and Hasnain, 2010).

Although bacterial IAA is most accountable for plant growth promotion, gibberellins (GA) are also known for stimulating and activating important growth and reproductive processes, such as stem elongation, seed germination, flowering and fruit setting (Hedden and Thomas, 2016; Vejan *et al.*, 2016). GA production has been observed in several different PGPB. For instance, two GA-producing isolates of *B. pumilus* and *B. licheniformis* promoted stem elongation and increased leaf area as well as leaf formation on developing lateral shoots of *Alnus* sp. plants (Gutierrez-Manero *et al.*, 2001). Similarly, GA-producing *B. cereus*, *B. macroides* and *B. pumilus* increased fresh weight of roots and shoots of pepper plants seedlings (Joo *et al.*, 2005).

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Besides modulating plant growth, phytohormones also have a fundamental influence on plant's response to stresses, such as drought, soil salinity, extreme temperatures, heavy metals, mechanical wounds and even pathogen attacks. In this sense, PGPB that produce these hormones might help plants withstanding different types of environmental stresses (Egamberdieva *et al.*, 2017).

One of those hormones is cytokinin (CK), which, besides controlling plant growth processes (e.g. cell division/elongation, stem elongation, leaf expansion and flowering), also promotes stomatal opening or root growth inhibition (Arkhipova *et al.*, 2007). When plants are stressed, for instance due to drought, CK levels usually drop, with consequences such as stomatal closing, reducing water losses, and promoting root growth, allowing a more efficient water uptake. However, since CK participate in processes such as cell division, cell enlargement, and tissue expansion, maintaining CK levels is important for plant growth and yield (Arkhipova *et al.*, 2007; Olanrewaju *et al.*, 2017). PGPB that produce CK can compensate the reduced CK level in response to stress, avoiding growth inhibition, thus maintaining yield. For instance, *Platanus orientalis* seedlings inoculated with CK-producing *B. subtilis* presented higher tissue water content and leaf water potential comparatively to non-inoculated plants under drought stress conditions (Liu *et al.*, 2013). Similarly, inoculation of water-deprived lettuce plants with CK-producing *Bacillus* sp. strain IB-22 led to higher dry weight as well as longer and wider leaves, comparatively to non-inoculated plants (Arkhipova *et al.*, 2007).

Ethylene (ET) is another plant hormone with an important role in environmental stress resistance. When stressed, plants synthesize 1-Aminocyclopropane-1-carboxylate (ACC) which is the ET precursor (Glick, 2014). High ET concentrations induce defoliation and inhibit root and stem growth, leading to premature senescence (Islam *et al.*, 2020). PGPB that produce ACC deaminase, which converts ACC to ammonia and α -ketobutyrate, have the ability of reducing stress ET levels, allowing plants to better withstand stress and avoid premature senescence, restoring normal development. It is important to mention that IAA can both facilitate plant growth and activate the transcription of ACC synthase, leading to high levels of ACC, and consequently ET, which down-regulates auxin response factors, so bacteria that produce IAA can promote plant growth, but also inhibit it. However, PGPB that produce ACC deaminase will decrease ACC levels, enabling IAA to promote plant growth without ET production (Glick, 2014; Olanrewaju *et al.*, 2017). Inoculation of rice plants

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with *Bacillus* sp., *Alcaligenes* sp. and *Ochrobactrum* sp. isolates, all presenting PGP traits such as ACC deaminase and IAA production, alleviated salt stress and promoted root and shoot growth, among other parameters, comparatively to non-inoculated plants (Bal *et al.*, 2012). Zafar-UI-Hye *et al.* (2018) demonstrated that inoculation of ACC deaminase producing *Stenotrophomonas maltophilia* and *Agrobacterium fabrum* on wheat seedlings promoted growth and increased shoot and root lengths, under cadmium toxicity conditions.

Absciscic Acid (ABA) is another plant hormone that plays a crucial role in several developmental processes, including seed and bud dormancy, as well as stress responses, such as stomatal closure in response to decreased soil water potential (Tardieu *et al.*, 1992; Tao *et al.*, 2011). PGPB can regulate ABA levels either by producing ABA or metabolizing plant produced ABA, ultimately altering ABA-mediated processes in plants. For instance, an ABA consuming PGPB can metabolize excessive ABA and prevent ABA-induced stomatal closure and inhibition of photosynthesis, which means that those PGPB can maintain plant cooling under high temperature and productivity under mild drought conditions. On the other hand, ABA producing PGPB can increase the ABA signal coming from roots to shoots, inducing ABA-dependent drought tolerance, and therefore can help plants to economize water usage by closing stomata under severe drought conditions (Kudoyarova *et al.*, 2019). A study in *Arabidopsis thaliana* showed that inoculation with *B. subtilis* GB03 augmented photosynthetic machinery and photosynthetic efficiency by decreasing the levels of ABA (Zhang *et al.*, 2008). Similarly, Shazhad *et al.* (2017) showed that inoculation of *Oryza sativa* with *B. amyloliquefaciens* RWL-1 reduced the levels of ABA in salt stressed plants, resulting in enhanced shoot and root growth, fresh and dry weight as well as higher aminoacid contents comparatively to non-inoculated plants.

1.2. PGPB Facilitate the Acquisition of Soil Nutrients

Another way by which PGPB can improve plant growth is by improving the uptake of essential nutrients that plants usually lack, such as nitrogen, phosphorous and iron, whether because soils are poor, or because some nutrients are not very soluble or in a biologically active form, and therefore cannot be properly absorbed and used by plants (Gamalero and Glick, 2011; Chhabra and Dowling, 2017).

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Nitrogen (N) availability is the most limiting factor to plant development, since it is the nutrient that is required in highest amounts, and the most yield-limiting in agricultural soils (Mantelin, 2013; Olanrewaju *et al.*, 2017). Diazotrophic PGPB have the ability of converting N in the molecular or atmospheric form (N_2) into biologically useful forms to plants such as ammonia (NH_3), since they express the nitrogenase enzyme (Dobbelaere *et al.*, 2003). Inoculation of four different N-fixing PGPB, *Azospirillum lipoferum* N-4, *A. brasilense* Wb-3, *Azoarcus* sp. K-1 and *Zoogloea* sp. Ky-1 in rice plants increased fixed nitrogen comparatively to non-inoculated control plants (Malik *et al.*, 1997). Similarly, inoculation of canola seedlings with *Paenibacillus polymyxa* P2b-2R resulted in plants with higher nitrogen content, due to atmospheric N fixation (Puri *et al.*, 2015).

Phosphorous (P) is also one of the most important nutrients to plants. P is usually present in high amounts in soil, but only a small portion of this element is soluble and able to be absorbed by plant roots (Pradhan and Sukla, 2006). PGPB that have the ability to solubilize phosphates, e.g. through the production of organic acids, can increase phosphorous availability in soil, hence facilitating its intake by plants. Inoculation of black pepper (*Piper nigrum* L.) cuttings with a phosphate-solubilizing bacteria resulted in an increase in phosphate availability and uptake by plants, as well as higher root and shoot length (Ramachandran *et al.*, 2013). Another study in maize plants showed that inoculation with four phosphate solubilizing PGPB, *E. asburiae* PSI3, *B. coagulans*, *S. marcescens* EB 67 and *Pseudomonas* sp. CBD 35 resulted in greater P uptake in shoots and grain tissues (Hameeda *et al.*, 2008).

Iron is also among the most limiting micronutrients for plant growth, since it has a very important role in plant development, including chlorophyll synthesis, thylakoid synthesis and chloroplast development (Imsand, 1998). Although it is usually abundant in soils, the oxidized ferric form has low solubility, and cannot be properly utilized by plants (Sharma *et al.*, 2013; Olanrewaju *et al.*, 2017). It is known that PGPB can solubilize ferric iron by producing a variety of low molecular weight compounds called siderophores (Scavino *et al.*, 2013; Gosh *et al.*, 2020), such as putrebactin, avaroferrin, bisucaberin, and alcaligin (Codd *et al.*, 2018) that have a very high affinity to Fe_3^+ . Sequestered iron can be both used by the bacteria or by plants, in that case, improving plant nutrition (Sharma *et al.*, 2003; Sah *et al.*, 2017). Sharma *et al.* (2013) demonstrated that field grown rice plants inoculated with eight different PGPB, including *Pseudomonas* spp., *Azospirillum lipoferum* and others belonging to

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Enterobacteriaceae species, lead to an increase of the iron content in plants, with some isolates almost doubling iron grain content. Similarly, Rana *et al.* (2012) demonstrated that inoculation of wheat plants with a PGPB increased iron content of grains, as well as other micronutrients such as Zn, Mn and Cu. Similarly, a study conducted on maize plants grown under iron-limiting conditions showed that inoculation with siderophore-producing *Pseudomonas* spp. GRP3A and *P. chlororaphis* ATCC 9446 reduced chlorosis appearance and improved root and shoot growth, as well as germination rates, comparatively to the control group (Sahrma and Johri, 2003).

1.3. PGPB Protect Plants from Pathogens

Besides modulating plant growth through the production of compounds like phytohormones and improving plant nutrient uptake by increasing the availability of essential nutrients in soils, as well as increasing resistance/resilience to some abiotic factors like drought, PGPB also play an important role in protecting plants from pathogens, by competing and suppressing their growth in a variety of ways (Scavino *et al.*, 2013).

Siderophores are implicated in both direct and indirect plant growth promotion by microorganisms. Besides facilitating iron uptake by plants, siderophores play a very important role in pathogen protection (Scavino *et al.*, 2013). Iron is essential for all living organisms, including phytopathogenic fungi and bacteria. Under limiting iron conditions, competition amongst organisms for bioavailable iron is high. PGPB are known for producing a variety of siderophores, to competitively chelate ferric iron, forming soluble Fe_3^+ complexes that are then taken up by active transport mechanisms (Gupta and Gopal, 2008; Kramer *et al.*, 2019). However, siderophore production is not exclusive to PGPB, since many pathogens can also use this mechanism to sequester iron. Nonetheless, there are many different types of siderophores, and some have better affinity to Iron than others. For instance, fungal siderophores have lower affinity to Iron than bacterial siderophores, so PGPB could limit or deprive pathogenic fungi from this essential element, hence limiting its growth. In this sense, high-affinity siderophore-producing PGPB can serve the important role of protecting plants from such pathogens. For instance, *Bacillus subtilis* CAS 15, a bacillibactin producing bacteria, inhibited the growth of 15 fungal phytopathogens *in vitro*, and it reduced the

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incidence of *Fusarium* wilt in pepper plants (Yu *et al.*, 2011). Similarly, siderophore-producing *Pseudomonas putida*, *P. putida* biovar B, *P. marginalis*, *Burkholderia cepacia*, *P. fluorescens* and *P. aeruginosa* showed *in vitro* antagonistic activity against *R. solani* and *B. cinerea* (Paéz *et al.*, 2005).

Another known mechanism by which PGPB can antagonize fungal pathogens is the synthesis of fungal cell wall degrading enzymes, such as chitinase, glucanase, protease and lipase, that facilitate the degradation of cell wall components such as chitin, glucans, proteins and lipids, respectively (Glick *et al.*, 2007; Olanrewaju *et al.*, 2017). For instance, Kumar *et al.* (2012) showed that a *Bacillus* sp. strain that produced chitinase, β -1,3-Glucanase (laminarinase) and β -1,4-Glucanase inhibited the growth of six different fungal pathogens, namely *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, *R. solani* and *Colletotrichum* sp. Another study conducted by Nielsen and Sorensen (2006) showed that two *P. polymyxa* and fourteen *B. pumilus* isolates, which demonstrated degradation of cellulose, mannan, xylan and casein, also exhibited antagonistic activity towards *R. solani*, *A. cochleoides* and *P. ultimum*.

Antibiotic production by PGPB is also one of the most important mechanisms of protecting plants from pathogens. Bacteria produce several metabolites that can act as antibacterial and antifungal compounds. Some examples of PGPB produced antibiotic compounds include subtilin, bacilysin, surfactin, iturin, fengycin, produced by *Bacillus* spp. and ecomycin, aerugine, azomycin and, one of the most efficient in plant pathogen control, 2,4-diacetyl phloroglucinol (DAPG), produced by *Pseudomonas* spp. (Martínez-viveros *et al.*, 2010; Olanrewaju *et al.*, 2017). It is known that PGPB exhibit a wide range of variety and a number of antibiotics produced which can affect the growth and metabolic activities of other microorganisms, even at low concentrations (Raaijmakers, 2002). For instance, *Bacillus subtilis*, the most representative species of the *Bacillus* genus, is known for having 4 to 5% of its genome dedicated to the production of antibiotics (Stein, 2005). There are several studies linking antibiotic production with the inhibition pathogens' growth. For instance, Romero *et al.* (2007) demonstrated that Iturin and Fengycin production are a key factor in antagonism of *B. subtilis* against *Podosphaera fusca*, a pathogen of cucurbit plants. Rai *et al.* (2017) showed that DAPG is the main compound responsible for the antagonistic activity of a *P. protegens* strain against *Ralstonia solanacearum*.

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Volatile organic compounds (VOCs), such as 2, 3-butanediol and 3-hydroxy-2-butanone released by PGPB as secondary metabolites, are also accountable for inhibiting pathogens growth (Jain *et al.*, 2017). VOCs are small organic molecules that have a high vapour pressure and therefore are easily volatilized. They are very interesting compounds since they can easily diffuse at long distances and are even able to penetrate soil pores (Kai *et al.*, 2006). An *in vitro* study utilizing two-chamber petri dishes showed that seven VOCs-producing bacterial strains including *Pseudomonas* spp. and *Serratia* spp. inhibited the growth of *R. solani* (Kai *et al.*, 2007). Méndez-Bravo *et al.* (2018) demonstrated that exposure of the pathogen *Phytophthora cinnamomi* to bacterial volatiles produced by the *B. acidiceler* strain A8a lead to degenerative alterations in hyphal morphology, and consequently growth inhibition.

Induced systemic resistance is another way by which PGPB can improve plant defenses against a broad spectrum of pathogens (Choudhary and Varma, 2016). A primary pathogen infection triggers a long-lasting and broad-spectrum disease resistance, in a process that is designed as Systemic Acquired Resistance (SAR). This type of resistance is not only induced in the site of infection, but instead it is activated systemically, meaning that it is also activated in the most distal parts of the plant, not exposed directly to the primary infection. Similarly, plant systemic resistance can also be elicited by PGPB colonization, but is regulated in a different way, and it is designated as Induced Systemic Resistance (ISR). The main protein involved in the regulation of both ISR and SAR is the redox-regulated protein nonexpressor of pathogenic-related genes 1 – *npr1*. Contrarily to SAR, that is activated by the accumulation of salicylic acid which in turn activates the *npr1* gene, encoding pathogenic-related proteins (e.g. chitinase and glucanase), ISR is dependent on pathways regulated by jasmonic acid and ethylene signaling, which also activate the *npr1* gene, acting as a “priming” for defense genes, leading to a physiological state of enhanced defensive capacity (Jain *et al.*, 2016; Olanrewaju *et al.*, 2017; Parray *et al.*, 2016). Figure I.2. summarizes these mechanisms of induced resistance.

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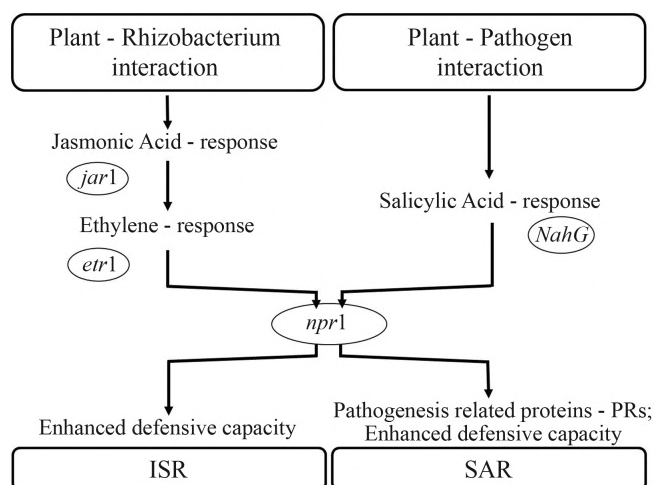


Figure I.2. Schematic representation of the interaction between plant host – pathogens and PGPB, involving SAR signalling and *npr1* (Retrieved from Beneduzi *et al.*, 2012).

There are several studies in literature reporting PGPB-mediated resistance priming of a huge variety of plant species. Chithrashree *et al.* (2011) demonstrated that *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens* and *Brevibacillus brevis* isolates led to an increase of the synthesis of defense enzymes in rice plants and were effective in protecting plants from the pathogen *Xanthomonas oryzae* pv. *oryzae*. Similarly, Sharma *et al.* (2019) showed that inoculation of a PGPB, *Klebsiella* spp. MBE02, activated several genes related to plant defense and ISR in peanut plants. Moreover, inoculation with this strain lead to increased growth and yield of plants in the presence of 4 different fungal pathogens, in field experiments.

Quorum Quenching (QQ) can also be one of the mechanisms of pathogen growth control. Quorum Sensing (QS) is a system of communication between bacterial cells that involves gene expression and is mediated by the diffusion of specific signaling molecules (auto-inducers) such as N-acrylhomoserine lactones (AHLs), which is dependent on cell concentration (Rodríguez *et al.*, 2020). Bacteria use this mechanism to detect the presence of other bacteria, so when they sense high cell density, a set of different genes are turned on, making them act in a coordinate manner. At a certain cell density, if the production of auto-inducers exceeds a certain threshold, pathogens may use QS to become more virulent (Olanrewaju *et al.*, 2017). One of the strategies PGPB can use to interrupt pathogen QS communication is QQ, which involves the production of enzymes such as lactonases, acylases and oxidoreductases, that degrade auto-inducers such as AHLs, preventing the pathogens

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from becoming more virulent (Rodríguez *et al.*, 2020). In fact, Rodríguez *et al.* (2020) demonstrated that a *P. segetis* strain fully degraded the AHLs of the pathogens *Dickeya solani*, *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum* and *Pseudomonas syringae* pv. *tomato*. Besides, QS mechanisms can coordinate processes such as antibiotic production or the secretion of enzymes (Altaf *et al.*, 2017), which could directly impair pathogens' metabolism, hence inhibiting its growth.

Compounds like VOCs, quorum sensing molecules (QSM), ET and methylsalicylic acid (MeSA), methyljasmonic acid (MeJA) are all involved in the complex mutual relationship between plant and microbiome species (e.g. rhizobiome), in a field that is emerging as 'signalomics' (Figure I.3). Plants and its beneficial microbiome species secrete molecules to the benefit of the other species. Perception of the priming stimulus activates signaling molecules, leading to the regulation of primary and secondary metabolism, including the control of gene expression towards promoting some pathways for secondary defense metabolites. Thus, when a secondary stimulus is perceived, the same process takes place but at an enhanced level to minimize impact on the plant (Mhlongo *et al.*, 2018). Interestingly, recent data suggests that plants are able to pass this acquired primed state to their progeny (trans-generational priming) (Mhlongo *et al.*, 2018).

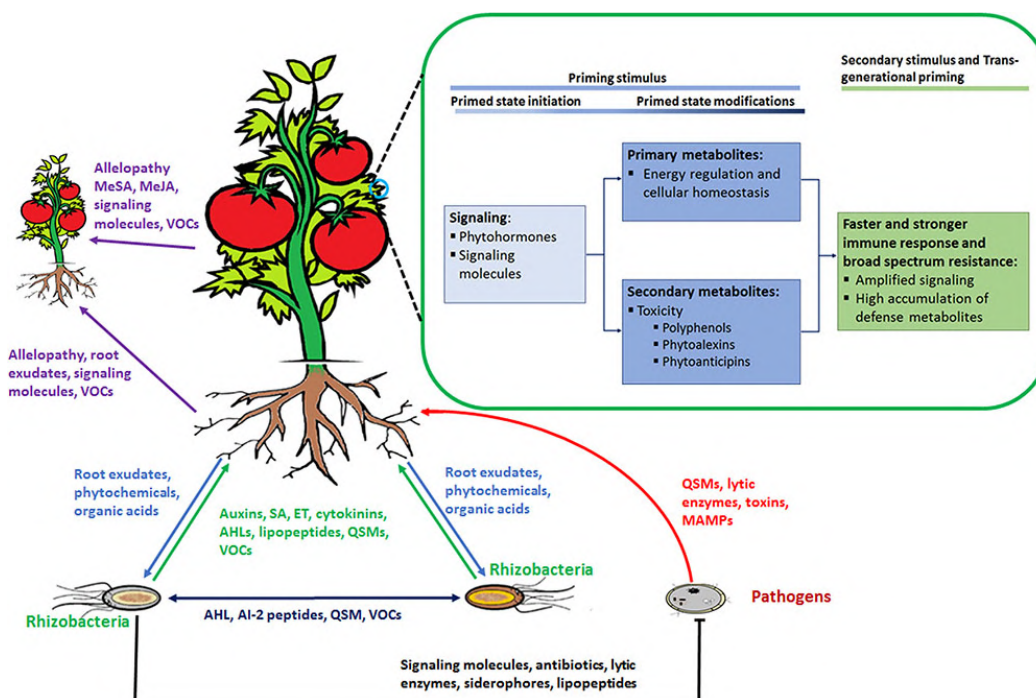


Figure I.3. Schematic summary of the rhizosphere plant and microbial 'signalomics' (Retrieved from Mhlongo *et al.*, 2018).

2. Can PGPB Replace Agrochemicals?

In late years, the use of biofertilizers and biopesticides, that are PGPB-based has been largely studied. Due to its multiple plant growth promoting characteristics and protection against pathogens, they are a promising alternative to current fertilizers and pesticides. In fact, there are multiple biofertilizers and biocontrol products regulated and available commercially in several countries, including Portugal (Table I), which demonstrates that there is interest and a market for such kind of products. In fact, studies reveal that its annual market demand is increasing globally (Maheshwari, 2011; Keswani *et al.*, 2019). However, although PGPB are a promising and growing greener alternative or additive to fertilizers and pesticides, there are a few obstacles in terms of environmental impact, biosafety and product formulation.

Table I. Examples of PGPB-based biocontrol products that are commercially available in Portugal, as of 2020 (Retrieved from ‘Listagem de produtos fitofarmacêuticos com autorização de venda em Portugal’, DGAV, 2020).

Commercial name	PGPB strain	Manufacturer
Botector	<i>Aureobasidium pullulans</i> DSM 14940 and 14941	Bioferm
Serenade Max	<i>Bacillus subtilis</i> QST 713	Bayer Crop Science
Serenade Aso	<i>B. amyloliquefaciens</i> QST 713	Bayer Crop Science
Amylo-X WG	<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> D747	Mitsui
Belthirul	<i>B. thuringiensis</i>	Probelte
Cedomon	<i>Pseudomonas chlororaphis</i> MA 342	Koppert
Costar WG	<i>B. thuringiensis</i> Kurstaki SA12	Mitsui
Dipel DF	<i>B. thuringiensis</i> Kurstaki ABTS 351	Kenogard
Flocter	<i>B. firmus</i> I-1582	Bayer Crop Science
Proradix	<i>Pseudomonas</i> sp. DSMZ 13134	Sourcon
Serifel	<i>B. amyloliquefaciens</i> MBI 600	Basf

2.1. Challenges and Perspectives

Artificial introduction of PGPB inevitably alters the microbial community balance and, in the case of foreign species, the autochthonous microbial community. Temporary, spatially limited and transient alterations have been observed (Castro-Sowinski *et al.*, 2007). These microbiome alterations might end up being benefic to plants, if the introduced PGPB has a synergistic or redundant effect with other benefic microorganisms, or it might have a deleterious effect if the introduced bacteria end up antagonizing already present beneficial organisms (Sharma *et al.*, 2017). Besides that, pre-established microflora might compete with the introduced PGPB, inhibiting its beneficial effects on plant growth (Sharma *et al.*, 2012).

Another relevant aspect of PGPB that could pose serious public health issues is that most PGPB species have antibiotic resistance genes (Ramakrishna *et al.*, 2019). It is known that antibiotic resistance is a worldwide serious problem nowadays, with predictions indicating that approximately 10 million people will lose their lives by 2050, due to antibiotic-resistant infections (Dadgostar, 2019). Soil microbiome is known for being a reservoir of such resistance genes against many classes of antibiotics, usually present in broad host range plasmids, which are easily transferred to other bacteria, through processes such as horizontal gene transfer. Besides that, there are many PGPB species that, even though are excellent plant growth promoters, are known to be opportunistic human pathogens (Berg *et al.*, 2005; Keswani *et al.*, 2019). Hence, biosafety of PGPB is one of the main obstacles to its application in field and at large-scale, and it certainly deserves further attention and study.

Another obstacle to PGPB-based products is transportation and storage. A PGPB product must remain stable and reliable for long periods of time, which implicates that it is in a form that allows it to maintain its characteristics after being stored. The use of carriers such as nanoparticles, biochar or alginate microbeads may also improve shelf life and survival rate of PGPB by protecting them from desiccation. Besides that, such carriers provide PGPB an effective environment for their rapid growth after releasing them to the environment, as well as a certain protection, ensuring its long-lasting survival and benefic effects (Bashan *et al.*, 2002; Bashan *et al.*, 2014). Endospore-forming PGPB such as *Bacillus* spp. are great candidates when

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it comes to longevity, since endospores can remain viable for many years (Nicholson, 2002; Marasco *et al.*, 2012).

Additionally, PGPB performance depends on the plant host and the environment. PGPB usually have a certain host affinity/specificity, and plants usually recruit specific organisms, which means that some PGPB might only promote growth of a group of similar plant species (Vaikuntapo *et al.*, 2014; Iyer and Rajkumar, 2017; Tovi *et al.*, 2019). Besides that, environmental conditions, such as temperature and soil properties are a key factor on the performance of a PGPB. A PGPB isolated from a hot and arid environment could work in a cold environment, but probably not in an optimal way (Glick, 2020).

Considering this, there are a few challenges that might hold off producers and need to be overcome in order to make PGPB competitive with high standards of current chemicals. For a PGPB product to succeed, it has to be at least similar to currently available agrochemicals in some aspects such as reasonable price, long-shelf life, reliability and consistency, ease of use and compatibility with farmers equipment and the ability to work under different and uncontrolled field conditions. Ideally, it should also have a broad-spectrum of action against pathogens while simultaneously be compatible with beneficial organisms, be compatible with many different crops and safe for the environment and animal and human health (Bashan *et al.*, 2014; Ramakrishna *et al.*, 2019). In this sense, the search for new PGPB isolates that combine the best characteristics and the study of their interaction with plants is extremely important, so that biofertilizers and biocontrol products are optimized and become competitive with agrochemicals.

It is also important to add that, even if biofertilizers are not able to fully substitute agrochemicals, its combination with lower doses of fertilizers may provide similar yields as those resulting from the use of fertilizers alone (Adesemoye and Kloepper, 2009; Nosratabad *et al.*, 2017). For example, combining PGPB with fertilizers can improve nutrient uptake and growth (Masood *et al.*, 2020). Besides that, pathogen control might be improved if pesticides are combined with antagonistic PGPB strains, as shown by Wu *et al.* (2020).

3. Aims

The main goal of this work is to characterize bacterial isolates from the iB₂ and UTAD collections, retrieved from different sources, and test them as biofortification agents as well as antagonists of a plant pathogen, in order to evaluate their potential as a sustainable alternative to current agrochemicals (fertilizers/pesticides). Aiming to achieve this goal, this work was divided into three main specific objectives, corresponding to chapters II, III and IV of this thesis.

First aim was to characterize a total of 21 isolates from the iB₂ and UTAD collections, all presumably PGPB. On a first approach, biochemical tests such as Gram stain, endospore stain, catalase production and cytochrome c oxidase production were performed. In addition, common PGPB features such as siderophore production, phosphate solubilization and IAA production were tested. Ultimately, to achieve accurate identification, molecular characterization was done, by amplification and sequencing of the 16s rRNA genes of all isolates.

Following the characterization, the second objective was to assess the 21 isolates antibacterial potential against the causing agent of kiwifruit bacterial canker. In order to do so, a reference strain of *Pseudomonas syringae* pv. *actinidiae* (Psa) was co-inoculated with the PGPB isolates, to evaluate if they had any inhibitory effect on the pathogen's growth.

To accomplish the third goal, selected isolates were screened for their biofortification potential. For this, and considering the previous characterization and preliminary screening tests, four isolates were selected and tested for their biofortification potential, using *Solanum lycopersicum* var. MicroTom, a widely used model plant. In order to better understand the effect of the inoculation with the selected PGPB on the physiological status of tomato plants, parameters such as photosynthetic pigments, carbohydrate contents, photosynthesis and gas exchange were measured.

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II. Chapter 2: Biochemical and Molecular Characterization of Plant Growth Promoting Bacteria Isolates

Abstract

The use of plant growth promoting bacteria (PGPB), or derived bioproducts, as a sustainable alternative to industrial fertilizers and pesticides is increasing. PGPB can promote plant growth and protect plants against pathogens, through a variety of mechanisms such as the production of phytohormones and antimicrobial compounds. PGPB includes a wide and heterogeneous group of bacteria, with *Bacillus* spp. and *Pseudomonas* spp. being the most studied and representative. Thus, there is an increasing interest to screen and characterize isolates with the potential to act as PGPB. In search for novel local PGPB, 21 bacterial strains previously isolated from chestnut roots, mushrooms, kiwifruit plants and walnut leaves (mainly from the North region of Portugal) were characterized and screened for growth promoting traits *in vitro*. Results showed that all isolates presented at least one plant growth promoting trait, since all produced Indole-3-acetic acid (IAA). Fourteen of the isolates were able to solubilize inorganic phosphate and five of the isolates produced siderophores. Molecular identification through the amplification and sequencing of 16S rRNA genes revealed that the isolates were predominantly *Bacillus* spp., three were *Alcaligenes* spp., one *Brevibacillus* sp. and one *Pseudomonas* sp., thus genera that are well described in literature as PGPB.

Keywords

Plant growth promoting bacteria (PGPB), Biofertilizers, Biocontrol, Sustainable agriculture

1. Introduction

Plant growth promoting bacteria (PGPB) are a group of beneficial bacteria that are able to promote growth and protect plants from biotic and abiotic stresses, through a diversity of mechanisms (Olanrewaju *et al.*, 2017; Etesami and Maheshwari, 2018). The main mechanism is the production of several phytohormones, such as auxins (mainly Indole-3-acetic acid), abscisic acid, cytokinins and ethylene, which act sinnergetically to modulate plant growth and metabolism (Olanrewaju *et al.*, 2017; Kudoyarova *et al.*, 2019). Another way in which PGPB can affect plant growth is by facilitating the uptake of nutrients, *viz.* by fixing nitrogen (Puri *et al.*, 2015), by producing and releasing Fe-chelating compounds called siderophores (Sharma *et al.*, 2013) or by solubilizing phosphate through the production and release of organic acids (Ramachandran *et al.*, 2013). Besides regulating plant hormones and nutrition, PGPB are also known for protecting plants against several different pathogens, through the competition for nutrients, in which siderophores may also be involved (Scavino *et al.*, 2013), the production of antibiotics, such as 2, 4-diacetylphloroglucinol (DAPG) (Rai *et al.*, 2017), and of volatile organic compounds, such as 2,3-butanediol (Jain *et al.*, 2017), cell wall degrading enzymes like chitinases (Kumar *et al.*, 2012), or by inducing plant systemic resistance (ISR) (Sharma *et al.*, 2019).

The PGPB group is extraordinarily diverse and it includes genus such as *Pseudomonas* (David *et al.*, 2018), *Bacillus* (Kashyap *et al.*, 2019), *Azospirillum* (Coniglio *et al.*, 2019), *Alcaligenes* (Hussain *et al.*, 2020), *Serratia* (Singh *et al.*, 2016), amongst others (Bhattacharyya and Jha, 2011; Yadav *et al.*, 2017). Despite not new, the manipulation of crop rhizosphere microbes gained interest in late years, following the need for sustainability in agricultural practices and the reduction of the use of agrochemicals such as fertilizers and pesticides (Chandra *et al.*, 2018; Tiwari *et al.*, 2019). Plant growth promoting inoculants have been used for years to ensure efficient nitrogen fixation in legumes, and currently there is already a variety of commercial products containing one or more PGPB strains, known as biofertilizers and biopesticides (Menhaz, 2016; Kumar, 2018).

The ideal PGPB must be able to colonize plants and be compatible with the already established natural microbial community, enhance plant yield, antagonize pathogens, have a broad spectrum of action, while being safe to the environment as

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well as human and animal health. Besides that, capacity for easy mass multiplication, ease of transportation and storage, as well as tolerance to adverse environmental conditions, are also desirable (Bashan *et al.*, 2014; Ramakrishna *et al.*, 2019).

Since PGPB are considered a gateway to sustainability in agricultural practices, it is important to search for new PGPB. This demands the characterization of new isolates, and the better understanding of its action mechanisms and interaction with plants, in order to find isolates that combine the most beneficial effects, with potential to use as new alternatives or complements to common agrochemicals. The aim of this work was to characterize 21 bacterial isolates, presumably PGPB, through the screening of common PGPB traits and molecular identification.

2. Material and Methods

2.1. Bacterial isolates

Bacterial isolates (21) were collected in the North region of Portugal, which is the main producing region of kiwifruit and chestnut, from stands of chestnut (rhizosphere), from mushrooms and from kiwifruit and walnut tree phyllosphere (Table II.1). The isolates have been stored in Luria-Bertani (LB) medium at -80 °C in 30% glycerol.

Table II.1. Source and Date of isolation of the collection bacterial isolates used in this work.

Isolate	Source	Origin and Date of Isolation	Collection
10PC	Chestnut roots	Vila Real, 2019	UTAD
11PC	Chestnut roots		
1PB1	Chestnut roots		
1PB2	Chestnut roots		
2P2	Mushroom		
2PB1	Chestnut roots		
3	Chestnut roots		
5BB2	Chestnut roots		
5PB1	Chestnut roots		
7PB2	Chestnut roots		
8BB2	Chestnut roots		
9BB1	Chestnut roots		
C9F	Chestnut roots		
EA1	Mushroom		
EA3	Mushroom		

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Isolate	Source	Origin and Date of Isolation	Collection
EF1	Mushroom	Vila Real, 2019	UTAD
EF2	Mushroom		
EF3	Mushroom		
UFG44	Chestnut roots		
PI34	Walnut phyllosphere	Alenquer, 2014	iB ₂
FV46	Kiwifruit phyllosphere	Felgueiras, 2014	

2.2. Microscopic Characterization

2.2.1. Gram staining

Bacterial cell membrane properties were analysed at a light microscope (Nikon Eclipse Ci-L, Japan), using Gram stained samples. Gram staining procedure was based on the protocol proposed by Moyes *et al.* (2009). A single colony of each bacterial culture was smeared onto a microscope slide, in circular motions using a sterile loop. Smears were fixed by heat and slides were then submerged in a crystal violet solution (0.125 g crystal violet, 10 mL ethanol 95% (v/v), 40 mL water) for 1 min and washed with distilled water. After being washed, slides were covered with a lugol solution. Decolorization of the slides was done by using an alcohol:acetone (5:1) solution. After decolorization, counterstain was performed by flooding the slides in a fuchsin solution [0.125 g fuchsin, 10 mL ethanol 95% (v/v), 40 mL water] for 1 min, and then all the slides were rinsed with distilled water. Samples were left to air-dry and then were observed with a light microscope (Nikon Eclipse Ci-L, Japan), using the 100x objective lens.

2.2.2. Endospore staining

Endospore formation was assessed by the Schaeffer–Fulton staining technique (Schaeffer and Fulton, 1993). A single colony of two-day old bacterial cultures was smeared onto a microscope slide, in circular motions using a sterile loop. Smears were fixated by heat, and covered with a filter paper, cut to cover the smear. The filter paper was saturated with a 0.5% (w/v) malachite green aqueous solution and the slides were steamed for 5 min, without letting the paper dry, adding more dye if necessary. Slides were then washed with water and counterstained with a 0.5% (w/v) alcoholic safranin

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solution, for 30 s. Samples were allowed to air-dry and were then observed in a light microscope (Nikon Eclipse Ci-L, Japan), using the 100x objective lens.

2.3. Biochemical Characterization

2.3.1. Cytochrome c oxidase Production

Cytochrome c oxidase production test was done following the protocol of Shields and Cathcart (2010). A sterile paper filter was soaked with a 1% (w/v) N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride (Wurster's blue) solution in different spots, one for each bacterial isolate. A single colony of each bacterial culture was smeared onto the soaked paper filter using sterile 10 μ L loops. Intense colour change within 30 s was considered a positive test.

2.3.2. Catalase Production

Catalase production test was done according to Reiner (2010). A drop of a 15% (v/v) hydrogen peroxide solution was placed into sterile microscope slides. A single colony of each bacterial isolate was smeared onto the solution using sterile 10 μ L loops. Oxygen production/Bubble formation within 30 s was considered a positive test.

2.3.3. Indole-3-acetic acid Production

To quantify the IAA produced by each bacterial isolate, a colorimetric technique was performed with the Van Urk Salkowski reagent using the Salkowski's method (Mohite, 2013; Flores *et al.*, 2018). The isolates were grown overnight in Luria-Bertani (LB) broth medium (Liofilchem, Italy) and incubated at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, at 150 rpm. Optical density at 600 nm (OD_{600}) was adjusted to 0.100 ± 0.005 , and 20 μ L of each diluted culture were transferred to 4 mL of LB broth medium supplemented with L-Tryptophan (2 g L^{-1}) and incubated overnight at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, at 150 rpm. Cultures were centrifuged after incubation (3600 rpm for 10 min), and 1 mL of each supernatant was transferred to a new tube. A volume of 0.5 mL of freshly prepared Salkowski's reagent (12 g L^{-1} of

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FeCl₃ in 7.9 M H₂SO₄) was mixed with each supernatant, and tubes were kept in the dark for 30 min. Optical densities were measured at 530 nm on the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA).

2.3.4. Phosphate Solubilization

The phosphate solubilizing ability of the isolates was tested on Pikovskaya's agar (PVK) medium (Rao, 1963;1997) containing an insoluble phosphate source (Tricalcium Phosphate). PVK medium contained 10 g L⁻¹ glucose, 5 g L⁻¹ (NH₄)₂SO₄, 0.5 g L⁻¹ (NH₄)₂SO₄, 0.2 g L⁻¹ NaCl, 0.1 g L⁻¹ MgSO₄·7H₂O, 0.2 g L⁻¹ KCl, 0.2 g L⁻¹ yeast extract, 0.002 g L⁻¹ MnSO₄·H₂O and 0.002 g L⁻¹ FeSO₄·7H₂O. Bacterial isolates were grown overnight at 25 °C ± 1 °C, at 160 rpm, in LB broth medium (Liofilchem, Italy). The OD₆₀₀ was adjusted to 0.100 ± 0.005, and 2 µL of each diluted culture were inoculated on the PVK plates. Solubilization halos were measured after 48 h and 6 days, using ImageJ software (NIH, USA). For comparison, phosphate solubilization capacity was expressed as Solubilization Efficiency (% SE), calculated as $Z-C/C \times 100$, where Z is the diameter of solubilization zone, and C is the colony diameter (Sridevi and Mallaiah, 2009).

2.3.5. Siderophore Production

For siderophore production detection, chrome azurol sulfonate (CAS) agar medium was prepared, according to Loudon *et al.* (2011). Four solutions were separately prepared and sterilized before they were mixed together. Solution 1, the Fe-CAS indicator, was prepared by mixing 10 mL of FeCl₃·6H₂O (in 10 mM HCl) with 50 mL of CAS solution (1.21 mg mL⁻¹) and 40 mL of Hexadecyltrimethylammonium bromide (HDTMA) (1.82 mg mL⁻¹). Solution 2, the buffer solution, was prepared by dissolving 30.24 g of Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) in 750 mL of a salt solution containing 0.3 g KH₂PO₄, 0.5 g NaCl and 1.0 g NH₄Cl. pH was adjusted to 6.8, using 50% (v/v) KOH. Water was added to bring the volume to 800 mL. Agar (15 g) was added. Solution 3 was prepared by mixing 28.5714 g of glucose, 28.5714 g of mannitol, 7.0428 g of MgSO₄·7H₂O, 0.2081 g of CaCl₂·2H₂O, 0.0167 g of MnSO₄·H₂O, 0.02 g of H₃BO₃, 0.0006 g of CuSO₄·5H₂O, 0.0171 g of ZnSO₄·7H₂O and 0.0143 g of

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$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Solutions 1, 2 and 3 were autoclaved at 121 °C for 10 min. Finally, 30 mL of a 10% (w/v) casamino acid solution was prepared and sterilized by filtration. To prepare the CAS agar medium, Solution 3 (70 mL) was added to the casamino acid solution (30 mL) and then to the Solution 2 (800 mL). At last, Solution 1 (100 mL) was added, with gentle stirring to prevent bubble formation. Bacterial isolates were grown overnight at 25 °C $\pm$ 1 °C, at 160 rpm in LB broth medium (Liofilchem, Italy). OD_{600} was adjusted to 0.100 ± 0.005 , and 10 μL of each diluted culture were inoculated on the CAS agar plates. Siderophore production was estimated after 24 h and 6 days of incubation at 25 °C $\pm$ 1 °C, based on the diameter of the halo zone, according to Park *et al.* (2019).

2.3.6. Heat Map Analysis

Heat map was constructed using all biochemical characterization data. Data relative to Gram staining, endospore staining, catalase production and cytochrome c oxidase production is qualitative and presented as positive or negative. Data relative to IAA production, siderophore production and phosphate solubilization were normalized and presented in percentage (%). 100% was defined as the largest mean in each data set. Graphpad™ Prism 7 software was used for both data treatment and heat map design.

2.4. Bacterial Identification by 16S rRNA Gene Sequencing

2.4.1. DNA extraction

Bacterial DNA was extracted following the method of Minas *et al.* (2011). Bacterial isolates were grown overnight in LB broth medium (Liofilchem, Italy), at 25 °C $\pm$ 1 °C. 1.4 mL of each culture were transferred to 2 mL centrifuge tubes and were centrifuged. Supernatants were discarded and pellets were resuspended with 900 μL of CTAB lysis buffer. Samples were incubated at 65 °C for 60 min before being centrifuged at 12000 $\times g$ for 5 min at 4 °C. Supernatants were transferred, and 900 μL of phenol:chloroform:isoamyl alcohol (25:24:1, pH= 6.7) were added for each

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extraction. Samples were vortexed and incubated for 10 min at room temperature, before being centrifuged at 12000 xg for 15 min at 4 °C, allowing for phase separation to occur. Upper aqueous phase was re-extracted adding another 900 μ L of phenol:chloroform:isoamyl alcohol. Next, samples were centrifuged at 12000 xg for 10 min at 4 °C and the upper aqueous phases were transferred to 2 mL tubes. Final extraction was performed with 900 μ L of chloroform:isoamyl alcohol (24:1) and layer separation occurred by centrifugation at 12000 xg for 15 min at 4 °C. Precipitation of the extracted DNA was achieved by adding 450 μ L of isopropanol. Samples were incubated at -20 °C for 1 hour, after which they were centrifuged at 7500 xg for 10 min at 4 °C. Supernatants were discarded and DNA pellets were washed three times in 1 mL of 70% (v/v) ethanol. Pellets were allowed to air-dry and then were resuspended in 50 μ L of 75 mM TE buffer (pH= 8.0). Suspended DNA samples were then stored at -20 °C. The extracted DNA quality and quantity were evaluated on a 1% (w/v) agarose gel and by measuring the absorption ratios 260 nm/280 nm and 260 nm/230 nm on the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA), using a μ Drop™ Plate (Thermo Scientific™, USA).

2.4.2. Amplification and Sequencing of 16S rRNA Genes

Degenerate primers 27-F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492-R 5'-TACGGYTACCTTGTTACGACTT-3' (Lane, 1985) were used for the amplification of the 16S ribosomal genes of all the bacterial isolates. Polymerase Chain Reaction (PCR) mixture consisted of 20 μ L of *Taq* Master Mix (Bioron), 6 μ L of PCR water (Bioron), 2 μ L of primer 27-F, 2 μ L of primer 1492-R and 10 μ L of DNA (1 μ g mL⁻¹). The PCR amplification program comprised of one cycle of initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min and 20 s, and one final elongation cycle at 72 °C for 7 minutes. Amplified PCR products were confirmed on a 1% (w/v) agarose gel and purified using the NZYGelpure kit (NZYtech, Portugal), according to manufacturer's instructions. Sequences were obtained by Sanger sequencing, performed by STABvida, Lda. (Lisbon, Portugal) and Eurofins Genomics (Ebersberg, Germany).

2.4.3. Phylogenetic Analysis based on 16S rRNA Genes

After receiving raw sequencing data, forward and reverse sequences were analysed in Geneious Prime software (USA). Consensus sequences of the partial 16S rRNA genes, were generated from high quality pairwise alignments.

Based on sequence similarity, using the Blastn algorithm in the National Center for Biotechnology Information (NCBI), type strains of the best matches (Table II.3) were selected for phylogenetic analysis (Table II.4). All sequences were aligned and adjusted for the same length (up to 1209 bp). Phylogenetic tree was built using the neighbour-joining method (bootstrap: 1,000 replicates) on Geneious Prime software (USA).

16S ribosomal RNA gene partial sequences of the 21 isolates were deposited in the National Center for Biotechnology Information (NCBI) database with the accession numbers presented on Table II.2.

Table II.2. GenBank accession number of the 16S ribosomal RNA gene partial sequences.

Isolate	GenBank Accession Number
10PC	MW160947
11PC	MW160948
1PB1	MW160938
1PB2	MW160939
2P2	MW160940
2PB1	MW160941
3	MW160942
5BB2	MW160943
5PB1	MW160944
7PB2	MW160945
8BB2	MW165065
9BB1	MW160946
C9F	MW160949
EA1	MW160950
EA3	MW160951
EF1	MW160952
EF2	MW160953
EF3	MW160954
UFG44	MW160955
PI34	MW160956
FV46	MW160957

2.5. Statistical Analysis

Biochemical characterization experiments were performed using 3 independent technical replicates for each isolate, except for the IAA production quantification assay, in which three individual replicates for each isolate were performed. Gram staining, cytochrome c oxidase production, catalase production and endospore formation related data is qualitative.

Phosphate solubilization and siderophore production data is semi-quantitative. IAA production data is quantitative. For IAA production, phosphate solubilization and siderophore production, presented values are the mean $\pm$ standard deviation (SD). Comparisons between treatments and the control were made using a one-way ANOVA test. When data were statistically different, the Dunnett Comparison Test ($p < 0.05$) was applied. GraphpadTM Prism 7 software was used.

3. Results

3.1. Microscopic Characterization

3.1.1. Gram Staining

The Gram stained bacterial samples were observed using a light microscope, and results are presented in Figures II.1, II.2 and II.3. Gram staining showed that 17 of the isolates were Gram-positive, with only isolates 3, 5BB2, 8BB2 and EA1 being Gram-negative (Figure II.1. g, h, and Figure II.2. k, n, respectively).

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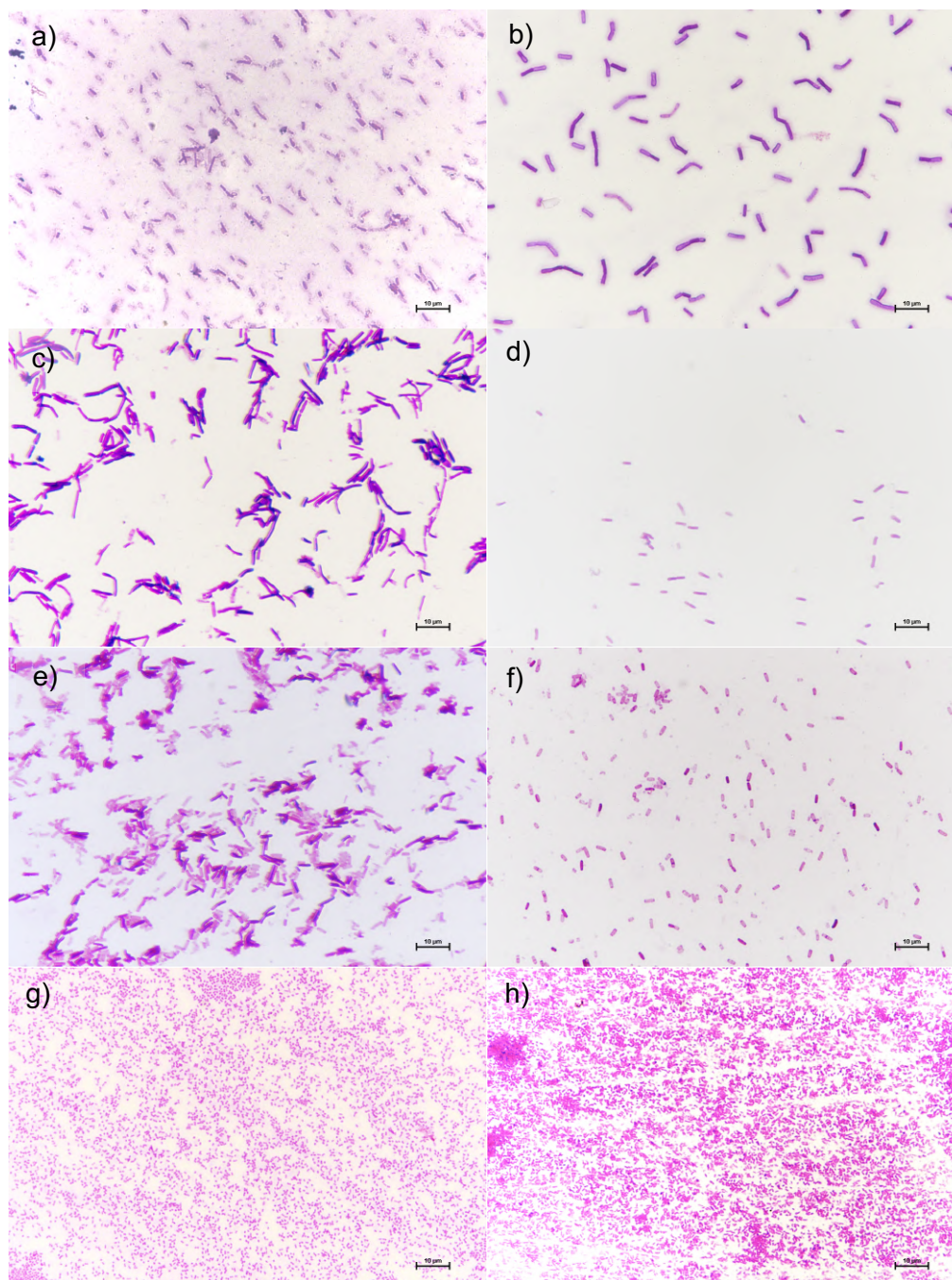


Figure II.1. Microscopical observation of bacterial smears colored by the Gram method. a) 10PC; b) 11PC; c) 1PB1; d) 1PB2; e) 2P2; f) 2PB1; g) 3; h) 5BB2. Bar = 10 µm.

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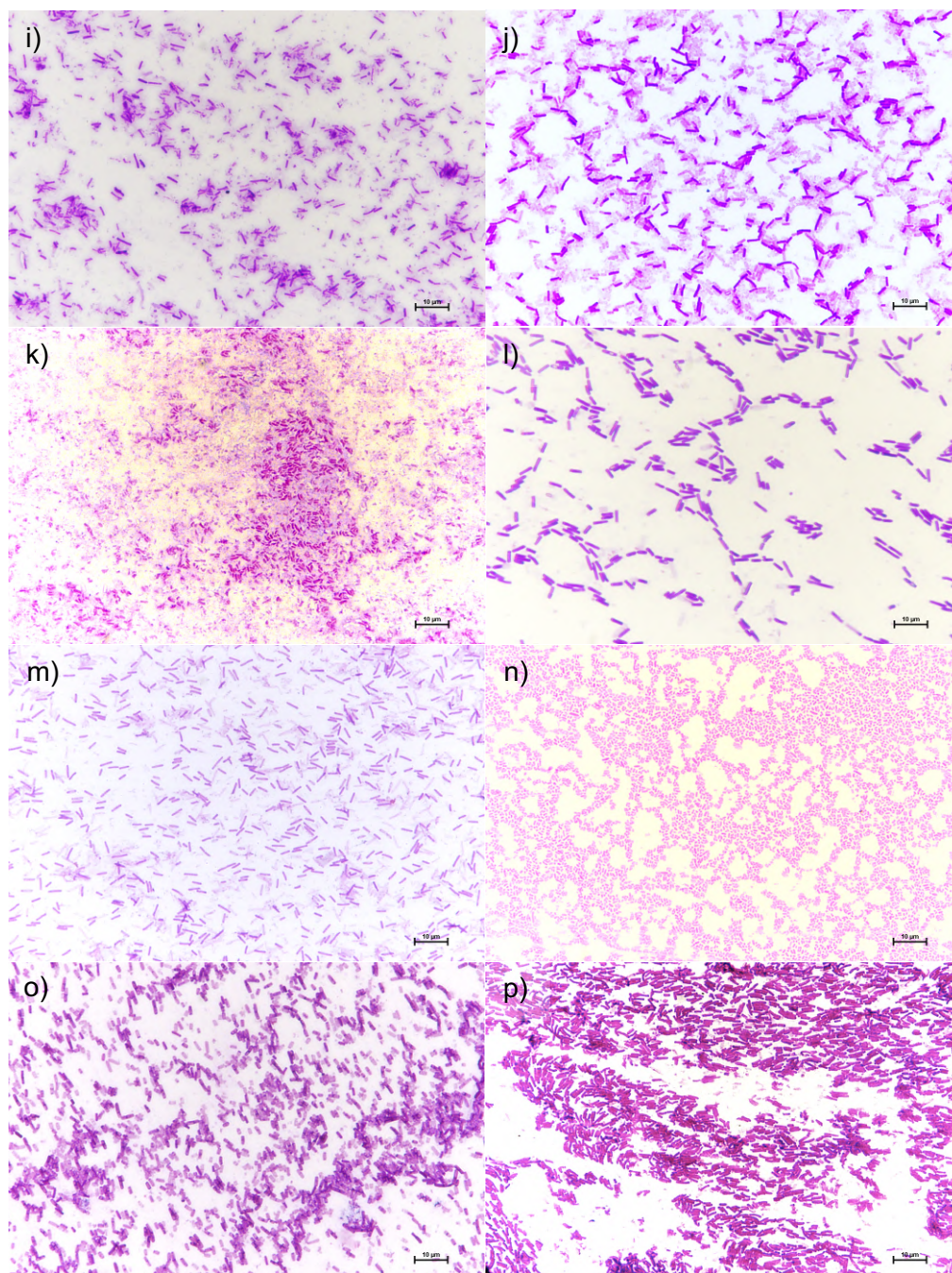


Figure II.2. Microscopical observation of bacterial smears colored by the Gram method. 5PB1; j) 7PB2; k) 8BB2; l) 9BB1; m) C9F; n) EA1; o) EA3; p) EF1. Bar = 10 µm.

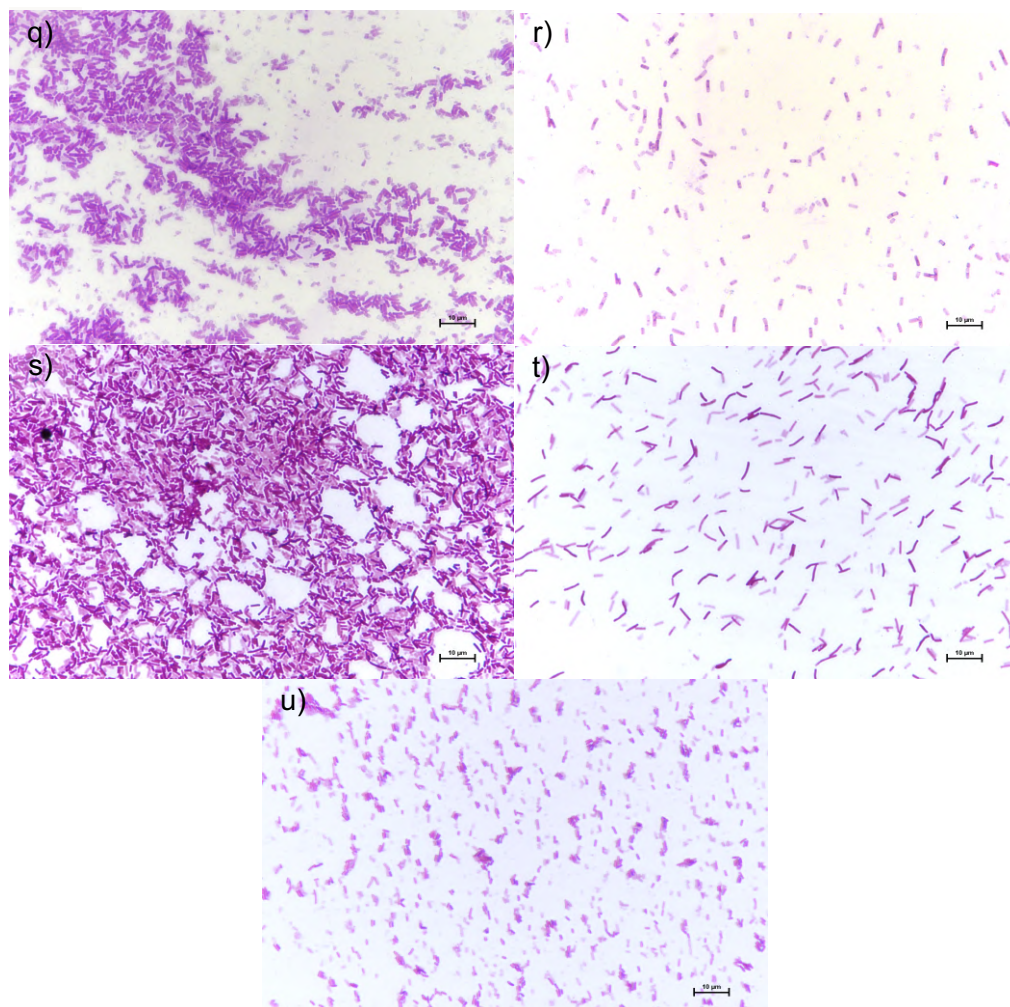


Figure II.3. Microscopical observation of bacterial smears colored by the Gram method. q) EF2; r) EF3; s) UFG44; t) PI34; u) FV46. Bar = 10 µm.

3.1.2. Endospore Staining

Endospore staining (Figure II.4) shows up as green, contrasting with vegetative cells, that show up as red. These structures were visible in isolates 10PC, 1PB2, 2P2, 2PB1, 5PB1, C9F, EF2, PI34 and 9PB1, showing that they were able to form endospores after 2 days of growth at room temperature.

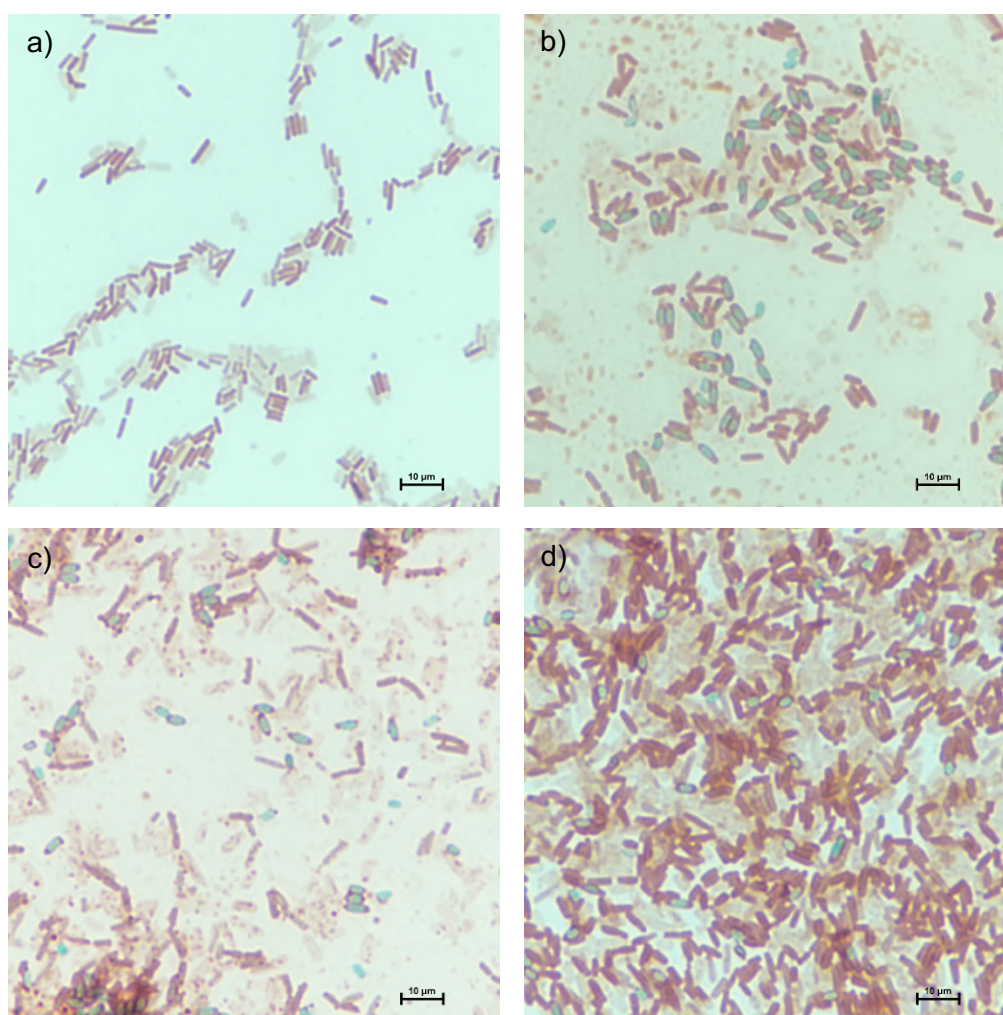


Figure II.4. Endospore staining of two-day old colonies of FV46 (a), 2P2 (b), 10PC (c) and 1PB2 (d). Endospores appear stained green, while vegetative cells appear red. FV46 did not form endospores, while 2P2, 10PC and 1PB2 showed endospore formation. Bar = 10 µm.

3.2. Catalase and cytochrome c oxidase Production

Bubble formation was not observed for isolates 1PB1 and C9F (Figure II.5.a), meaning that these isolates did not produce catalase, contrarily to the other 19 isolates, which were all catalase-positive (Figure II.5.b). Immediate and intense purple-color formation (Figure II.5.c) was observed for isolates 3, 5BB2, 7PB2 and 8BB2, meaning they were positive for cytochrome c oxidase production while the other isolates did not produce this enzyme.

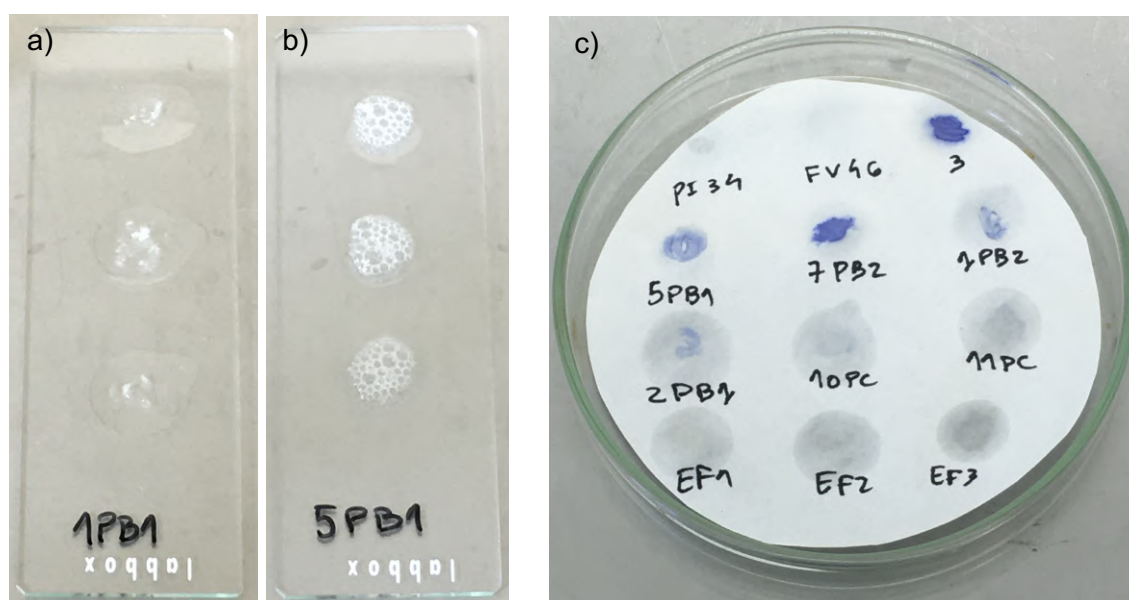


Figure II.5. Catalase and cytochrome c oxidase production tests. 1PB1 (a), a catalase-negative isolate, and 5PB1 (b), a catalase-positive isolate. 3 and 7PB2 (c) tested positive for cytochrome c oxidase production.

3.3. Plant Growth Promoting Traits

3.3.1. Indole-3-acetic Acid Production

All of the 21 isolates used in this work produced IAA in the presence of tryptophan, although significant differences in production were observed (Figure II.6). Isolate 1PB1 significantly produced more IAA than the other 20 isolates. More than half of the tested isolates produced more than $100 \mu\text{g mL}^{-1}$ IAA. 7PB2, 8BB2, 3, 5BB2,

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UFG44, FV46 and PI34 produced the least IAA, under $50 \mu\text{g mL}^{-1}$, with 7PB2 being the isolate that produced the least IAA.

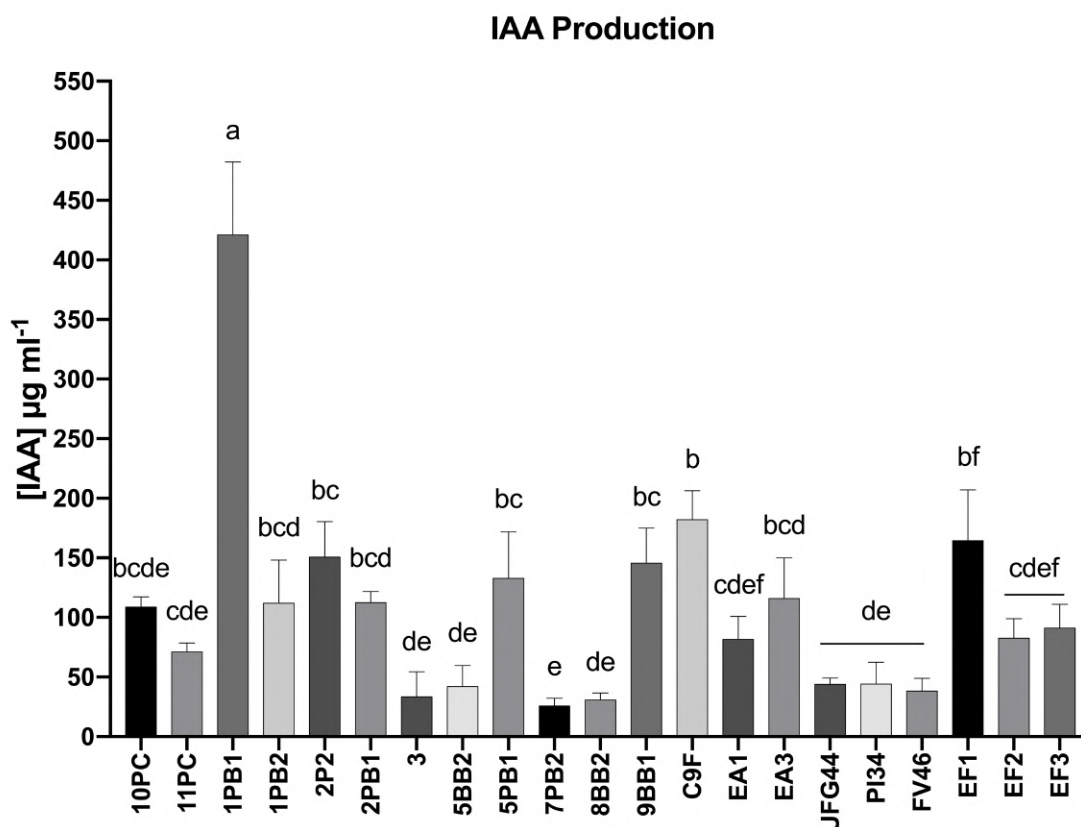


Figure II.6. Production of IAA in the presence of L-Tryptophan ($\mu\text{g mL}^{-1}$). Presented values are means $\pm$ SD ($n = 3$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.3.2. Phosphate Solubilization

Of the 21 isolates tested for phosphate solubilization, only 8BB2, EF1, EF3, UFG44, PI34 and FV46 formed clear halo zones on the PVK medium, after 48 h of incubation (Figure II.7.a). After 6 days of incubation (Figure II.7.b), a total of 13 isolates, 10PC, 1PB2, 2P2, 2PB1, 5PB1, 8BB2, EA1, EA3, EF1, EF2, EF3, UFG44, PI34 and FV46 were able to solubilize phosphate, showing clear halo zones around the colonies. Halo and colony diameter were measured and solubilization efficiency was calculated (Figure II.8). Out of the 13 phosphate solubilizing isolates, 11 of them showed a low solubilization efficiency, ranging from 7.6% (1PB2) to 50.1% (EF3), with no significant differences between them. PI34 and EF1 showed a statistically significant

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higher SE, of 107.1% and 157.7%. 8BB2 had the highest SE of all the of phosphate solubilizing isolates, of 375.4%.

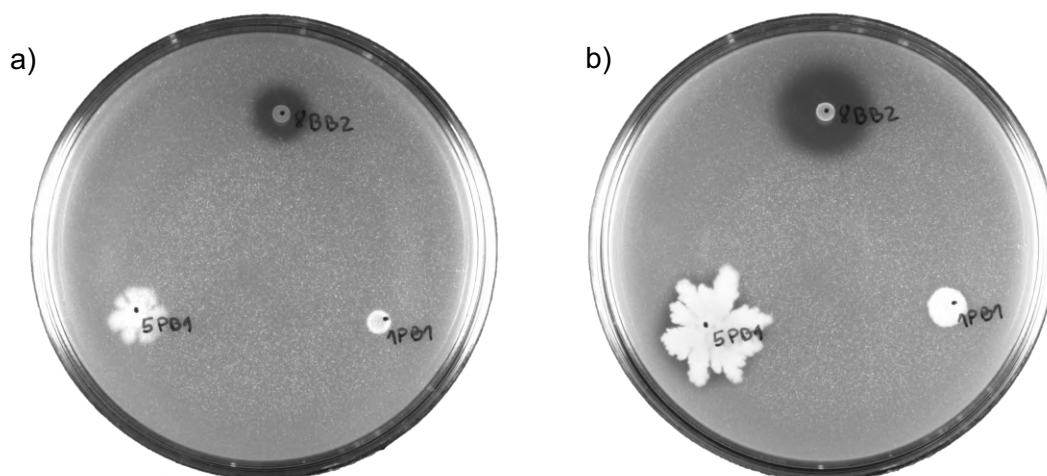


Figure II.7. Formation of clear solubilization zones on the PVK agar medium by isolate 8BB2, after 48 h of incubation (a), and isolates 8BB2 and 5PB1, after 6 days of incubation (b).

Phosphate Solubilization Efficiency (SE)

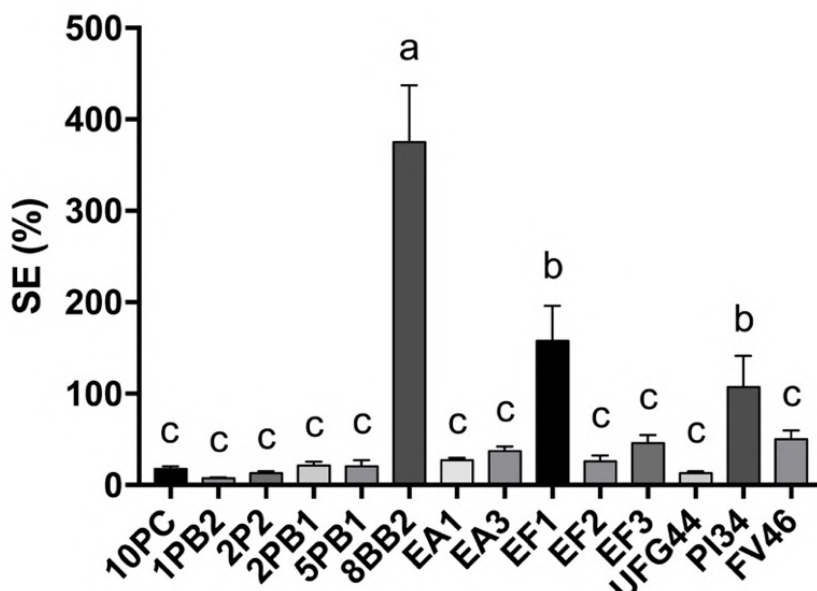


Figure II.8. Phosphate Solubilization Efficiency (% SE) of isolates 10PC, 1PB2, 2P2, 2PB1, 5PB1, 8BB2, EA1, EA3, EF1, EF2, EF3, UFG44, PI34 and FV46 after 6 days of incubation. Presented values are means $\pm$ SD ($n = 3$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.3.3. Siderophore Production

After 24 h of incubation (Figure II.9.a), isolates 3, 5BB2, 7PB2, 8BB2 and EF1 produced orange-yellow halos on the CAS agar medium, indicating siderophore production. Plates were further incubated, for 6 days (Figure II.8.b), but no other isolate showed siderophore formation. Halos were measured and results showed that 8BB2 was able to significantly produce more siderophores than the other isolates, which produced about the same amount, having no significant differences between them (Figure II.10).

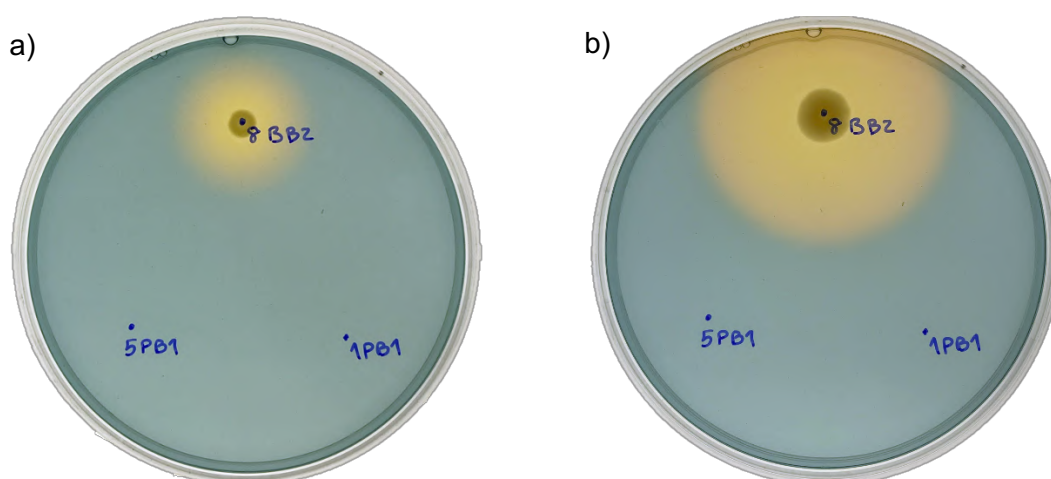


Figure II.9. Siderophore production by isolate 8BB2 on CAS Agar medium, after 24 h of incubation (a) and 6 days of incubation (b).

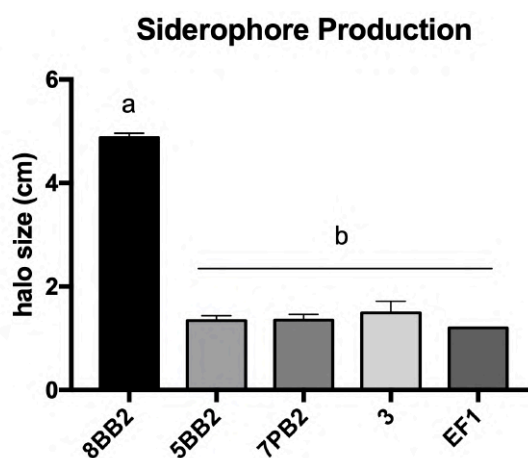


Figure II.10. Diameter of the halo zone formed on the CAS agar medium plates (cm), by isolates 8BB2, 5BB2, 7PB2, 3 and EF1 after 6 days of incubation. Presented values are means $\pm$ SD ($n = 3$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.4. Heat Map Analysis

Heat map analysis (Figure II.12) shows that 14 of the tested isolates presented more than one PGP trait. Of all the isolates, 8BB2 and EF1 were the only that presented the three PGP traits tested in this work, although 8BB2 produced small amounts of IAA comparatively to the other isolates. Isolates 10PC, 1PB2, 2P2, 2PB1, 5PB1, EA1, EF2, EF3, UFG44, PI34 and FV46 were both IAA producers and phosphate solubilizers. Isolates 3, 5BB2 and 7PB2 produced IAA and siderophores. Isolates 11PC, 1PB1, 9BB1 and C9F only produced IAA. Moreover, cytochrome c oxidase positive isolates were the best siderophore producers, while the isolates that did not produce oxidases were the best IAA producers. Among these, catalase negative isolates 1PB1 and C9F were the two best IAA producers. There seems to be no correlation between cell wall characteristics and the PGP traits tested here.

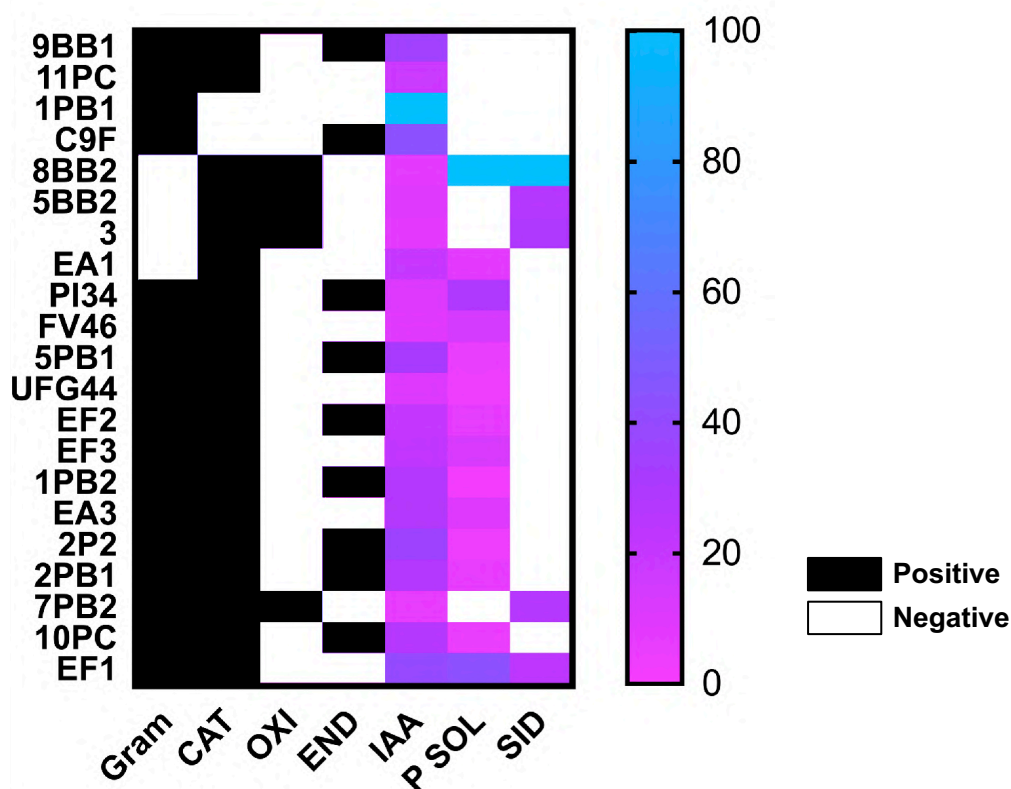


Figure II.11. Heat map showing an integrated analysis of the biochemical characteristics and normalized data (%) regarding plant growth promoting traits of the 21 PGPB isolates. Gram – Gram staining; CAT – catalase production; OXI – cytochrome c oxidase production; END – endospore formation after 2 days; IAA – IAA production; P SOL – phosphate solubilization efficiency; SID P – siderophore production based on halo diameter.

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3.5. Bacterial Identification by 16S rRNA Gene Sequencing

Molecular identification through sequencing of the 16S rRNA genes and comparison with the GenBank database using Blastn (Table II.3) allowed the identification of the isolates 10PC, 11PC, 1PB1, 2P2, 2PB1, 5PB1, 7PB2, 9BB1, EA3, EF1, EF2, EF3, FV46, PI34 and UFG44 as *Bacillus* spp., 3, 5BB2 and EA1 as *Alcaligenes* spp. Moreover, C9F was identified as a *Brevibacillus* sp. and 8BB2 as *Pseudomonas* sp.

Table II.3. Similarity of the isolates to species in the GenBank database, based on the 16S rRNA gene sequences.

Isolate	Species to which the isolate was most similar to in GenBank	NCBI Accession Number(s)	Query cover (%)	Identity (%)	E-value	Length (bp)
1PB1	<i>Bacillus aryabhattai</i> <i>B. megaterium</i>	MT184818 MK474947	100	100.00	0.0	1419
1PB2	<i>Bacillus</i> sp. <i>B. vallismortis</i>	KP795915 MT538262	100 99	99.86	0.0	1400
2P2	<i>B. velezensis</i> <i>B. siamensis</i> <i>B. subtilis</i> <i>B. amyloliquefaciens</i>	MT626060 MT605505 MT605412 MT585518	100	100.00	0.0	1251
2PB1	<i>B. subtilis</i> <i>B. velezensis</i> <i>B. siamensis</i> <i>B. amyloliquefaciens</i>	MT605412 CP054714 MN826567 MT487669	100	100.00	0.0	1380
3	<i>Alcaligenes aquatilis</i> <i>A. faecalis</i>	MT572474 MT378145	100	100.00	0.0	1375
5BB2	<i>A. faecalis</i> <i>Alcaligenes</i> sp.	MT378145 MN493925	100	100.00	0.0	1398
5PB1	<i>B. safensis</i> <i>B. pumilus</i> <i>B. zhangzhouensis</i>	MT501806 MN581181 MT081069	100	100.00	0.0	1398
7PB2	<i>B. amyloliquefaciens</i> <i>B. velezensis</i> <i>Bacillus</i> sp.	MT613661 MT611666 MN620386	100	99.57	0.0	1393

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Isolate	Species to which the isolate was most similar to in GenBank	NCBI Accession Number(s)	Query cover (%)	Identity (%)	E-value	Length (bp)
8BB2	<i>Pseudomonas protegens</i> <i>P. fluorescens</i> <i>Pseudomonas</i> sp.	MT505104 KY006471 MT255223	100	100.00	0.0	1332
9BB1	<i>Bacillus</i> sp. <i>B. cereus</i> <i>B. proteolyticus</i> <i>B. thuringiensis</i>	JX897934 MT611946 MT573794 CP050183	100	99.85 99.78 99.78 99.78	0.0	1376
10PC	<i>B. velezensis</i> <i>B. amyloliquefaciens</i> <i>B. subtilis</i> <i>B. siamensis</i>	MT626060 MT613661 MT110640 MN462850	100	100.00	0.0	1381
11PC	<i>B. simplex</i> <i>Bacillus</i> sp.	MT081080 KU513788	100	100.00	0.0	1365
C9F	<i>Brevibacillus</i> sp. <i>B. laterosporus</i>	MG851721 KT363762	99 100	100.00 99.93	0.0	1399
EA1	<i>A. aquatilis</i> <i>A. faecalis</i> <i>Alcaligenes</i> sp.	MT572474 MT378145 MN493925	100	99.84	0.0	1314
EA3	<i>B. vallismortis</i> <i>B. subtilis</i> <i>Bacillus</i> sp.	MT538262 KU847912 MH627232	100	99.78	0.0	1371
EF1	<i>B. amyloliquefaciens</i> <i>B. velezensis</i> <i>B. siamensis</i> <i>B. subtilis</i>	MT613661 MT611652 MT605505 MT373810	100	99.93	0.0	1397
EF2	<i>B. subtilis</i> <i>B. tequilensis</i>	MN696247 MH373539	100 99	100.00	0.0	1417
EF3	<i>B. vallismortis</i> <i>B. subtilis</i> <i>Bacillus</i> sp.	MT538262 KU847912 MH627232	100	99.85	0.0	1371
FV46	<i>B. velezensis</i> <i>B. pumilus</i> <i>B. safensis</i> <i>B. wiedmannii</i>	MT492019 MT367713 MN519460 MT239515	100	100.00	0.0	1418
PI34	<i>B. altitudinis</i> <i>B. xiamensis</i> <i>B. subtilis</i> <i>B. pumilus</i>	MT627439 MT611424 MT509759 MK603127	100	100.00	0.0	1383

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Isolate	Species to which the isolate was most similar to in GenBank	NCBI Accession Number(s)	Query cover (%)	Identity (%)	E-value	Length (bp)
UFG44	<i>B. zhangzhouensis</i> <i>B. pumilus</i> <i>B. safensis</i>	MN826587 CP054310 MT501806	100	100.00	0.0	1367

Based on the similarity of the 16s rRNA gene sequences of the 21 isolates with sequences deposited on the GenBank database, type and reference strains were selected, and their 16S rRNA gene sequences (Table II.4) were used to build a phylogenetic tree, in order to accurately identify the isolates, according to the type sequences they cluster with.

Table II.4. Type and Reference strains selected to build the phylogenetic tree, based on the 16S rRNA genes. [†] – Type strain; < – before; unk – unknown.

Type/Reference Strains	Strain	Date of isolation	Source	Origin	GenBank Assession Number
[†] <i>Bacillus safensis</i>	DSM 19292	<2007	Spacecraft assembly facility	Pasadena, USA	AF234854
<i>B. pumilus</i>	SH-B11	2012	Sugar beet rhizosphere	Wageningen, Netherlands	EU138517
[†] <i>B. amyloliquefaciens</i>	DSM 7	<1990	Soil	unk	AB006920
<i>B. subtilis</i>	RS10	2018	Soil	Karak, Pakistan	NZ_CP046860
[†] <i>B. vallismortis</i>	DSM 11031	<1996	Soil	Death Valley, USA	EU138463
[†] <i>B. velezensis</i>	DSM 28326	2004	Rice rhizosphere	Republic of Korea	NZ_CP011937
[†] <i>B. nakamurai</i>	NRRL B-41091	unk	Soil	Tierra del Fuego, Argentina	KU836854
[†] <i>B. proteolyticus</i>	LMG 28870	unk	Sediments	Pacific Ocean	KJ812418
[†] <i>B. wiedmannii</i>	DSM 102050	<2012	Raw milk	USA	KU198626
[†] <i>B. zhangzhouensis</i>	LMG 27144	unk	Aquaculture water	Zhangzhou, China	JX680133
[†] <i>Brevibacillus brevis</i>	DSM 30	<1990	unk	unk	X60612
[†] <i>B. agri</i>	DSM 6348	<1991	unk	unk	D78454

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Type/Reference Strains	Strain	Date of isolation	Source	Origin	GenBank Assession Number
[†] <i>B. laterosporus</i>	DSM 25	<1990	unk	unk	D78461
[†] <i>Alcaligenes faecalis</i>	DSM 30030	<1990	unk	unk	AB680368
[†] <i>A. faecalis subsp. phenolicus</i>	ATCC 8750	unk	Bioprocessor	unk	NR_043445
[†] <i>B. subtilis</i>	DSM 23778	unk	unk	France	NC_000964
[†] <i>B. thuringiensis</i>	Bt407	unk	unk	unk	NZ_CM000747
[†] <i>B. halotolerans</i>	ACCC 06527	unk	Saline soil	China	NR_156834
[†] <i>B. simplex</i>	DSM 1321	1989	Soil	unk	CP017704
[†] <i>B. dafuensis</i>	KCTC 43120	2019	Forest soil	China	CP042593
[†] <i>Pseudomonas protegens</i>	DSM 19095	2007	Soil	Switzerland	CP003190
[†] <i>B. safensis subsp. osmophilus</i>	LMG 30124	unk	Condensed milk	Spain	KY990920
[†] <i>B. megaterium</i>	DSM 32	<1990	unk	unk	NR_118962
[†] <i>B. megaterium</i>	DSM 21047	unk	Cryogenic tube	India	NR_115953
[†] <i>P. fluorescens</i>	DSM 50090	<1990	Pre-filter tanks	United Kingdom	MT605329

The neighbor-joining phylogenetic tree (Figure II.12) based on 1209 bp fragments of the 16S rRNA genes showed that there are 7 clusters: Cluster I) *Bacillus thuringiensis*/ *wiedmannii*/ *proteolyticus*; Cluster II) *Brevibacillus brevis*/ *agri*/ *halotolerans* *lateosporus*; Cluster III) *Bacillus megaterium*/ *simplex*; Cluster IV) *Pseudomonas protegens*/ *fluorescens*; Cluster V) *Alcaligenes faecalis*; Cluster VI) *Bacillus pumilus*/ *safensis*/ *zhangzhouensis*; Cluster VII) *Bacillus amyloliquefaciens* *s*/ *subtilis*/ *nakamurai*/ *velezensis*/ *vallismortis*.

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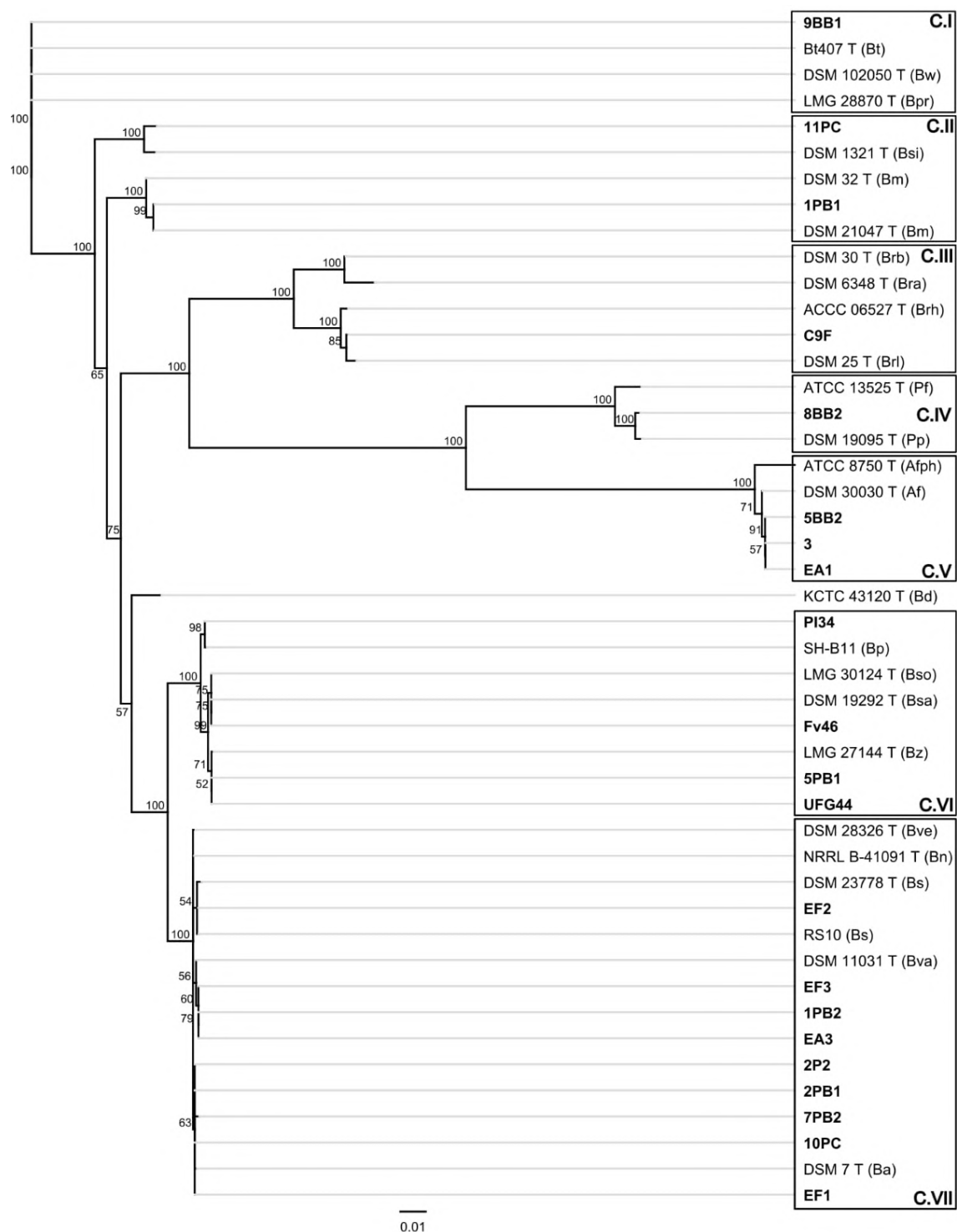


Figure II.12. Phylogenetic tree based on 1209 bp fragments of 16S rRNA gene sequences of the collection isolates and selected type and reference strains retrieved from GenBank (Table II.4). Neighbour-joining method (bootstrap: 1,000 replicates) was used to construct the phylogenetic tree on Geneious Prime software. Scale bar represents the number of the nucleotide substitutions per site. Bsa – *Bacillus safensis*; Bp – *Bacillus pumilus*; Ba – *Bacillus amyloliquefaciens*; Bs – *Bacillus subtilis*; Bva – *Bacillus*

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vallismortis; Be – *Bacillus velezensis*; Bn – *Bacillus nakamurai*; Bpn – *Bacillus proteolyticus*; Bw – *Bacillus wiedmannii*; Bz – *Bacillus zhangzhouensis*; Brb – *Brevibacillus brevis*; Bra – *Brevibacillus agri*; Bre – *Brevibacillus laterosporus*; Af – *Alcaligenes faecalis*; Afph – *Alcaligenes faecalis* subsp. *phenolicus*; Bs – *Bacillus subtilis*; Bt – *Bacillus thuringiensis*; Brh – *Brevibacillus halotolerans*; Bsi – *Bacillus simplex*; Bd – *Bacillus dafuensis*; Pp – *Pseudomonas protegens*; Bso – *Bacillus safensis*; Bm – *Bacillus megaterium*; Pf – *Pseudomonas fluorescens*; C.I – Cluster I; C.II – Cluster II; C.III – Cluster III; C.IV – Cluster IV; C.V – Cluster V; C.VI – Cluster VI; C.VII – Cluster VII.

Based on the analysis of the dendrogram (Figure II.12), 10PC, 2P2, 2PB1, 7PB2 and EF1 (Cluster VII) were identified as *Bacillus amyloliquefaciens*; EF2 (Cluster VII) was identified as *Bacillus subtilis*; 5PB1 and UFG44 (Cluster VI) as *Bacillus zhangzhouensis*; FV46 (Cluster VI) as *Bacillus safensis*; PI34 (Cluster VI) as *Bacillus pumilus*; 3, 5BB2 and EA1 (Cluster V) as *Alcaligenes faecalis*; C9F (Cluster III) as *Brevibacillus laterosporus*; 1PB1 (Cluster II) as *Bacillus megaterium*; 11PC (Cluster II) as *Bacillus simplex*; 1PB2, 9BB1, EA3 and EF3 and as *Bacillus* spp.

4. Discussion

PGPB are known for having a variety of mechanisms by which they promote plant growth and offer protection against pathogens. In this work, 21 bacterial isolates were characterized and tested for some common PGPB characteristics.

PGPB usually belong to genus such as *Pseudomonas*, *Serratia*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Acinetobacter* and *Alcaligenes* (Rodríguez and Fraga, 1999; Gupta *et al.*, 2015; Yadav *et al.*, 2017), which are all rod-shaped, and predominantly Gram-negative, except for *Bacillus* spp. which are Gram-positive rods. All of the 21 isolates tested were rod-shaped, 17 of them are Gram-positive while 4 are Gram-negative (Figures II.1, II.2 and II.3). Moreover, some *Bacillus* spp. are known for producing endospores (Nicholson, 2002; Fritze, 2004; Logan *et al.*, 2011), which was the case for isolates 10PC, 1PB2, 2P2, 2PB1, 5PB1, 9BB1, C9F, EF2 and PI34 (Figure II.4). Endospore production is desirable in PGPB, since it makes these bacteria resistant to adverse environmental conditions, allowing them to survive for long periods of time under harsh conditions such as extreme heat, cold and drought (Nicholson, 2002; Marasco *et al.*, 2012).

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All of the isolates produced IAA (Figure II.6), which was expected since it is a very common feature amongst soil bacteria and is considered in literature as the main mechanism that PGPB use to improve plant growth (Olanrewaju *et al.*, 2017). IAA production has been reported for species such as *Bacillus* spp. (Wahyudi *et al.*, 2011; Kumar *et al.*, 2012; Bhutani *et al.*, 2018), *Pseudomonas* spp. (Karnwal, 2009; Bharucha *et al.*, 2013; Marathe *et al.*, 2017) and *Alcaligenes* spp. (Ndeddy and Babalola, 2015; Hussain *et al.*, 2020), among several others. Of the isolates tested in this work, 1PB1 was the one that produced the most IAA in the presence of L-Tryptophan, followed by C9F, EF1, 2P2, 9BB1, 5PB1, EA3, 1PB2, 2PB1 and 10PC, which all produced more than 100 $\mu\text{g mL}^{-1}$ IAA, meaning that based on this analysis, these isolates could potentiate plant growth.

Another common PGPB characteristic is the ability of solubilizing inorganic phosphates. Phosphate solubilization is commonly observed in several different PGPB species such as *Bacillus* spp. (Prakash and Arora, 2019; Bahadir *et al.*, 2018), *Pseudomonas* spp. (Linu *et al.*, 2018; Yadav *et al.*, 2016) and *Alcaligenes* spp. (Shahid *et al.*, 2015; Behera *et al.*, 2017). Out of the 21 isolates, 10PC, 1PB2, 2P2, 2PB1, 5PB1, 8BB2, EA1, EA3, EF1, EF2, EF3, UFG44, PI34 and FV46 tested positive for phosphate solubilization (Figures II.7 and II.8). 8BB2, EF1 and PI34 were able to significantly solubilize more phosphate than the other isolates, which means that these isolates could possibly benefit plant nutrition, by increasing P uptake.

PGPB species are also known for producing siderophores, which can have both direct and indirect impact on plant growth and health. There are many studies in literature showing the ability of several PGPB genus of producing siderophores, including *Bacillus* (Wilson *et al.*, 2006; Yu *et al.*, 2011), *Pseudomonas* (Tripathi *et al.*, 2005; Páez *et al.*, 2005; Subramaniam and Sundaram, 2020), *Azotobacter* (Viscardi *et al.*, 2016; Shirinbayan *et al.*, 2019) and *Alcaligenes* (Sayyed and Chincholkar, 2009). Out of the isolates tested, 3, 5BB2, 7PB2, 8BB2 and EF1 were able to produce siderophores (Figures II.9 and II.10), suggesting that these isolates could compete for iron with plant pathogens, inhibiting their growth, or providing iron to plants, improving its uptake.

Most of the tested isolates presented more than one mechanism of action, as commonly observed in literature (De Souza *et al.*, 2014; ALKahtani *et al.*, 2020). The heat map analysis (Figure II.11) shows that 14 of the isolates possess at least two

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PGPB traits, which makes these isolates promising candidates to be used as biofertilizers and biocontrol agents. For instance, isolates 8BB2 and EF1 were capable of producing IAA, siderophores, and solubilize inorganic phosphate. Having a variety of mechanisms of action is key when searching for and selecting PGPB strains.

Molecular identification and phylogenetic analysis (Figure II.12) allowed the identification of the isolates as *Bacillus* spp. (1PB2, 9BB1, EA3 and EF3), *B. amyloliquefaciens* (10PC, 2P2, 2PB1, 7PB2 and EF1), *B. zhangzhouensis* (5PB1 and UFG44), *B. simplex* (11PC), *B. megaterium* (1PB1), *B. pumilus* (PI34), *B. safensis* (FV46), *B. subtilis* (EF2), *A. faecalis* (3, 5BB2 and EA1), *Brevibacillus laterosporus* (C9F) and *Pseudomonas protegens* (8BB2). In a general way, isolates grouped in clusters originated from the phylogenetic study of the 16s rRNA genes share similar biochemical traits, although with some variations. For instance, isolates in Clusters VI and VII (Figure II.12) are all IAA producers and phosphate solubilizers, with the exception of 7PB2, which did not solubilize phosphate. Nonetheless, these are all species described in literature as plant growth promoting bacteria (Ramette *et al.*, 2011; Lateef *et al.*, 2015; Tiwari *et al.*, 2019), which corroborates the biotechnological potential of these isolates.

5. Conclusions

Searching for new PGPB that have the best and the most growth promoting traits is very important in order to develop alternatives to chemicals that are both effective and sustainable. This work suggests that the iB₂ and UTAD collections' isolates exhibit very important growth promoting traits, such as IAA production and solubilization of nutrients such as phosphorous and iron, that are usually in sub-optimal levels in agricultural soils and hence are compensated with the application of chemical fertilizers. Based on the biochemical characterization and molecular identification, the collection isolates are potential candidates to be used as biofertilizers and/or biocontrol agents.

Although these isolates seem promising, further biochemical characterization is needed, namely regarding the ability of nitrogen fixation and VOCs production,

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pathogenicity, rhizobial competence, and environmental stress tolerance, such as salinity and extreme temperatures.

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III. Chapter 3: Plant Growth Promoting Bacteria as Biocontrol Agents Against *Pseudomonas syringae* pv. *actinidiea* (Psa)

Abstract

Plant pathogens are a major limiting factor of crop production, and they end up causing huge yield and quality losses. The recurrent use of pesticides remains the most used disease-control method, even though their use has negative impacts on the environment and public health. In late years, the interest in new safer biotechnological tools to control the spread and severity of crop diseases has increased, and one of the proposed alternatives to these agrochemicals is the use of plant growth promoting bacteria (PGPB). PGPB can improve plant health through a diversity of mechanisms, which include protection against pathogens, mainly due to competition for nutrients, the synthesis of antimicrobial compounds and induced systemic resistance (ISR). *Pseudomonas syringae* pv. *actinidiea* (Psa), the causal agent of bacterial canker of kiwifruit plants (*Actinidia* spp.), is currently the most threatening pathogen of this crop. In this work, a group of 21 PGPB isolates including *Bacillus* spp., *Alcaligenes faecalis*, *Pseudomonas protegens* and *Brevibacillus laterosporus*, were tested *in vitro* for their antagonism capacity against this pathogen. Results show that a total of 13 isolates inhibited the growth of Psa, with some of them being more effective than others. The group of most efficient Psa antagonists includes *Alcaligenes faecalis* isolates 5BB2 and 3, as well as *Bacillus amyloliquefaciens* isolate 7PB2. Thus, these isolates deserve further studies with the host and pathogen for further evaluation of their efficacy as antagonists in real scenarios.

Keywords

Antagonism, Biocontrol, Plant growth promoting bacteria (PGPB), *Pseudomonas syringae* pv. *actinidiea* (Psa), Sustainable agriculture

1. Introduction

Crop pathogens and pests are one of the biggest threats to yield and quality of food production, although pesticides are widely used in current agricultural practices. Plants and plant pathogens are in constant co-evolution, which allows pathogens to adapt and displace existing strains, leading to the emergence of new resilient strains (McDonald and Stuckenberg, 2016). This constant evolution makes pathogens a relevant and continuous threat to production, and ultimately leads to huge economical losses to producers (Savary *et al.*, 2019).

Whilst commercial chemical pesticides persist the most effective and used tool to control crop pathogens, their intensive use, and sometimes poorly regulated application, come with downsides. Besides being a gateway for pathogen resistance and consequently the emergence of new resilient strains, it also has many negative impacts on the environment, as well as animal and human health (Sharma and Singhvi, 2017; Bruhl and Zaller, 2019). Besides, authorized pesticides often show ineffective against emerging diseases that threaten many cultures worldwide (Gilardi *et al.*, 2018).

With sustainability being a priority in agricultural practices, new ways of controlling plant pathogens that have a reduced environmental impact are being explored. One of the alternatives could be the use of plant growth promoting bacteria (PGPB) (Yadav *et al.*, 2017; Chandra *et al.*, 2018). These bacteria, found in soil and mainly on the rhizosphere surroundings of plants, are known for promoting growth through the production of hormones (e.g. Indole-3-acetic acid) and by facilitating soil nutrient uptake (e.g. by fixing nitrogen and solubilizing phosphate and iron) as well as protecting plants from pathogens, such as fungi and bacteria, through a diversity of mechanisms.

This capacity of inhibiting pathogen growth is often referred to as antagonism, which is essentially the reduction of severity of damage caused by pathogens. Competition for nutrients and root niches is one of the ways by which PGPB can protect plants. For example, PGPB that produce siderophores can competitively acquire iron, making it less available for pathogens (Scavino *et al.*, 2013). PGPB can also inhibit pathogen growth by producing a variety of hydrolytic enzymes (such as chitinases, proteases, and lipases) which can lyse pathogens' cell walls (Kumar *et al.*, 2012). Production of antibiotics (such as DAPG, Iturin and Fengycin) is also a common

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feature of PGPB (Romero *et al.*, 2007; Rai *et al.*, 2017). Additionally, certain PGPB can trigger Induced Systemic Resistance (ISR), which is a non-specific systemic defense mechanism activated in response of an infection by a pathogen, or in this case, triggered by PGPB, that makes plants more resistant to future infections, against a variety of pathogens (Jain *et al.*, 2016; Olanrewaju *et al.*, 2017; Parray *et al.*, 2016). Regulation of plant ethylene levels through the production of 1-Aminocyclopropane-1-carboxylate (ACC) deaminase can also alleviate stress caused by the infection of pathogens (Van Loon, 2007; Beneduzi *et al.*, 2012). Several studies show that PGPB such as *Pseudomonas* spp., *Bacillus* spp. and others can inhibit the growth of many different phytopathogens (Sivasakthi *et al.*, 2014; Tariq *et al.*, 2017).

Pseudomonas syringae pv. *actinidiae* (Psa) is the etiological agent of bacterial canker of kiwifruit plants (*Actinidia deliciosa* and *A. chinensis*). Although this disease is originary from Japan and China (1980s), a more severe form of Psa broke out in Italy in 2008 and spread to many other countries, including Portugal, where it was detected in 2010. Psa represents a threat to the global kiwi fruit production. In 2009, it was included on the EPPO (European and Mediterranean Plant Protection Organization) A2 alert list. There are five genetically different Psa populations, or biovars, 1, 2, 3, 5 and 6 (the latter, present in a restricted Japanese region). Biovar 3 is the most virulent and destructive, being the dominant biovar in Europe, and the one that is present in the North region of Portugal (Moura *et al.*, 2015; Vaz *et al.*, 2018)

In Portugal, this disease is currently controlled with copper treatments, since the use of antibiotics in agriculture is forbidden in the European Union. Copper treatments are effective, but copper can be phytotoxic, induce bacterial resistance, and leave residual contamination in fruits (Wicaksono *et al.*, 2018). The use of endophytic antagonistic bacteria could be an alternative method of managing Psa and reducing the severity of this disease. Wicaksono *et al.* (2008) showed that three different endophytic *Pseudomonas* spp. were able to colonize kiwifruit plants and reduce bacterial canker development. Moreover, Biondi *et al.* (2015) showed that *B. amyloliquefaciens* strain D747 lead to a reduction of persistence of Psa on *A. chinensis* flowers by more than one order of magnitude just 48 h after pathogen application.

The main aim of this study was to evaluate the antagonistic capacity of the 21 PGPB isolates against Psa that, by reducing orchards yield, causes dramatic economic losses to producers each year. To screen the potential candidates to be used as

biocontrol agents against this disease, the isolates were tested against a Psa reference strain.

2. Material and Methods

2.1. Bacterial Isolates and Culture Conditions

In this work, all of the 21 isolates from the iB2 and UTAD collections were used. Psa CFPB 7286 was isolated from Kiwifruit plants in Italy in 2008 and was used in this work since it is a reference Psa strain. All of the isolates, including Psa, were grown in LB broth medium (Liofilchem, Italy) at 25 °C.

2.2. *In vitro* Screening for Antagonism Against Psa

Antagonistic activity of the bacterial isolates against Psa was tested using the double layer assay, according to Zenguerer *et al.* (2018). Psa culture grown overnight (16 h) was added to 0.7% LB top agar in a final OD₆₀₀ of 0.100 ± 0.005 . 3 mL of the LB top agar inoculated with Psa were poured onto LB plates. Bacterial cultures of the collection isolates grown overnight (16 h) were adjusted to an OD₆₀₀ of 0.100 ± 0.005 . 3 µL of each diluted test isolate were then applied in three equidistant spots, close to the margins of the plates. As a positive control, 3 µL of 11 mM chlorotetracyclin were applied to the centre of each LB plate. As a negative control, uninoculated LB broth was used. Inhibition halos were then measured after 24 h and 32 h of incubation, using ImageJ software (NIH, USA).

2.3. Statistical Analysis

Antagonism screening tests were performed using three individual replicates for each isolate, with three technical replicates per experiment. Presented values are the mean $\pm$ standard deviation of $n = 9$. Comparisons between treatments and the control were made using a one-way ANOVA test. When data were statistically different, the

Dunnett Comparison Test ($p < 0.05$) was applied. Graphpad™ Prism 7 software was used for the statistical analysis.

3. Results

3.1. *In vitro* Screening for Antagonism Against Psa

A total of 12 isolates produced clear inhibition halos on the LB top agar inoculated with Psa, indicating that they have antagonistic effects against Psa (Figure III.1). At the end of 24 h of incubation (Figure III.1.a), growth inhibition halos were observed in 1PB2, 2P2, 2PB1, 3, 5BB2, 5PB1, 7PB2, EA3 and PI34. After 32 h of incubation (Figure III.1.b), halos were visible for 3 more isolates, 9BB1, C9F and EA1.

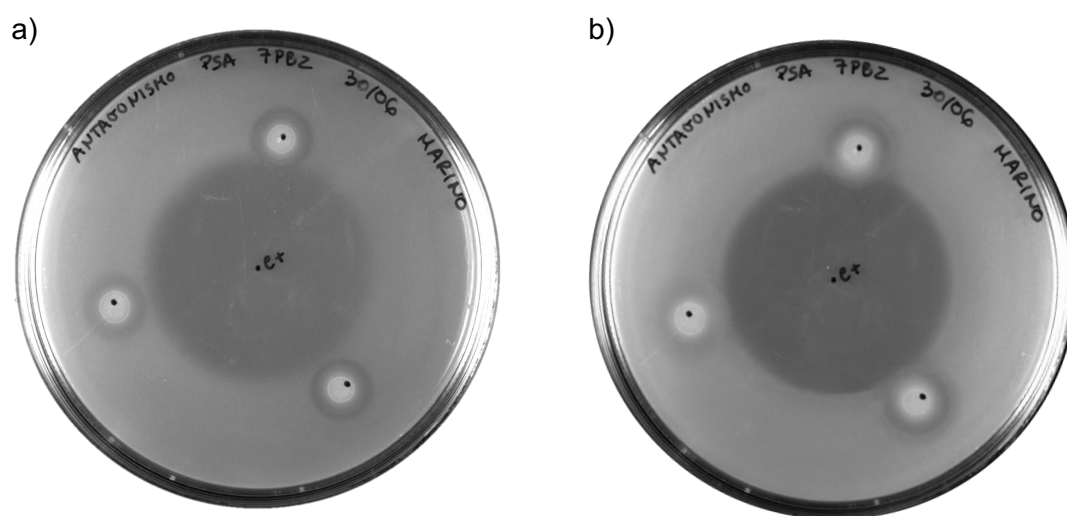


Figure III.1. Double layer antagonism assay. Inhibition halos formed around isolate 7PB2 (b) after 24 h of incubation (a) and 48 h of incubation (b).

Moreover, the diameter of inhibition halos varied among PGPB, indicating that some isolates were stronger antagonists than others. After 24 h of incubation, isolates 5BB2, 7PB2 and 3 presented significantly larger inhibition halos than the other isolates. Isolates 1PB2, 2P2, 2PB2, 5PB1, EA3 and PI34 all produced inhibition halos under 10 mm of diameter. 5PB1 and PI34 produced the smallest halos, significantly smaller than all of the isolates except for 2PB1 and EA3 (Table III.1).

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After 32 h of incubation, isolate 3 formed a significantly higher inhibition zone than all the other isolates. Although isolates 5BB2, 7PB2 and EA1 presented smaller inhibition halos than isolate 3, they still formed significantly bigger inhibition zones than isolates 1PB2, 2P2, 2PB1, 5PB1, 9BB1, C9F, EA3 and PI34. Isolates 5PB1 and PI34 were the weakest Psa antagonists, although their inhibitory effects were not significantly different from those of 1PB2, 2PB1, C9F and EA3.

After 6 days of incubation (data not presented here), the isolates that did not have any inhibitory effect after 24 h and 32 h of incubation (10PC, 11PC, 1PB1, 8BB2, EF1, EF2, EF3, UFG44 and FV46), still had not formed inhibition halos, suggesting that they are not antagonists of Psa. Moreover, inhibitory effects of the antagonistic isolates remained until the 6th day of incubation, since inhibition halos were still visible.

Table III.1. Inhibition halo diameter formed on the LB Top Agar by the isolates after 24 h of incubation (a), and 32 h of incubation (b). Presented values are means $\pm$ SD (n = 9) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$). n/i – no inhibition halo was observed.

Isolate	Diameter of inhibition zone (mm)	
	24 h incubation	32 h incubation
<i>B. amyloliquefaciens</i> 10PC	n/i	n/i
<i>B. simplex</i> 11PC	n/i	n/i
<i>B. megaterium</i> 1PB1	n/i	n/i
<i>Bacillus</i> sp. 1PB2	7.472 $\pm$ 0.241 c	8.149 $\pm$ 0.025 cd
<i>B. amyloliquefaciens</i> 2P2	8.442 $\pm$ 0.778 c	9.225 $\pm$ 1.137 c
<i>B. amyloliquefaciens</i> 2PB1	6.852 $\pm$ 0.346 cd	7.518 $\pm$ 0.192 cd
<i>Alcaligenes faecalis</i> 3	11.722 $\pm$ 0.984 b	17.018 $\pm$ 0.970 a
<i>A. faecalis</i> 5BB2	13.832 $\pm$ 1.039 a	12.899 $\pm$ 0.998 b
<i>B. zhangzhouensis</i> 5PB1	6.354 $\pm$ 0.607 d	7.102 $\pm$ 0.399 d
<i>B. amyloliquefaciens</i> 7PB2	12.087 $\pm$ 0.792 ab	14.072 $\pm$ 0.259 b
<i>Pseudomonas protegens</i> 8BB2	n/i	n/i
<i>Bacillus</i> sp. 9BB1	n/i	9.931 $\pm$ 0.672 c
<i>Brevibacillus laterosporus</i> C9F	n/i	7.801 $\pm$ 0.489 cd
<i>A. faecalis</i> EA1	n/i	13.156 $\pm$ 0.605 b
<i>Bacillus</i> sp. EA3	7.301 $\pm$ 0.371 cd	8.143 $\pm$ 0.601 cd
<i>B. amyloliquefaciens</i> EF1	n/i	n/i
<i>B. subtilis</i> EF2	n/i	n/i
<i>Bacillus</i> sp. EF3	n/i	n/i

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Isolate	Diameter of inhibition zone (mm)	
	24 h incubation	32 h incubation
<i>B. zhangzhouensis</i> UFG44	n/i	n/i
<i>B. pumilus</i> PI34	6.347 ± 0.396 d	7.098 ± 0.235 d
<i>B. safensis</i> FV46	n/i	n/i

4. Discussion

In this work, it was demonstrated that twelve of the tested isolates were able to inhibit the growth of a highly relevant kiwifruit plants' pathogen. *In vitro* tests (Figure III.1) showed that isolates 3, 5BB2 and EA1 (*Alcaligenes faecalis*) as well as 2P2, 2PB1, 7PB2 (*B. amyloliquefaciens*), 1PB2, EA3, 9BB1 (*Bacillus* spp.) 5PB1 (*B. zhangzhouensis*), PI34 (*B. pumilus*) and C9F (*Brevibacillus laterosporus*) were able to inhibit the growth of the Psa reference strain CFBP 7286.

Out of the twelve Psa antagonists, eight of them are *Bacillus* spp., with *B. amyloliquefaciens* 7PB2 being the strongest Psa antagonistic *Bacillus* isolate (Table III.1). These results are in line with the work of Biondi *et al.* (2012) who demonstrated that *B. amyloliquefaciens* strain D747 (commercially available in Portugal under the name Amylo-X) was able to inhibit the growth of two Psa strains, *in vitro*, using a similar method to the one used in this study. Furthermore, Biondi *et al.* also demonstrated that the D747 isolate was successful in colonizing leaves and roots of plants, offering continuous protection to plants. In contrast, Vaz *et al.* (2018) demonstrated that *B. subtilis* strain QST 713 (commercially available in Portugal under the name Serenade Max[®]) aggravated disease symptoms in Psa infected Kiwifruit orchards, under field conditions, but the increase of disease symptoms observed by the authors is most likely due to climate conditions, which strongly influence the growth and spread of Psa. Furthermore, Vaz *et al.* (2017) demonstrated that the application of this biological control product did not affect fruit quality and yield.

All of the three *A. faecalis* isolates were able to inhibit the growth of Psa. To date, there are no studies in literature regarding the antagonistic potential of *Alcaligenes* spp. isolates against Psa. However, species of this genus, namely *A. faecalis* are described as antagonists of other relevant plant bacterial and fungal

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pathogens, such as *Pantoea agglomerans* (Yokoyama *et al.*, 2013) and *Colletotrichum gloeosporioides* (Lahlali *et al.*, 2019).

However, besides being described in literature as a PGPB, *A. faecalis* is also known for being an opportunistic human (and animal) pathogen (Simmons *et al.*, 1981; Junejo *et al.*, 2018; Huang, 2020), mainly infecting immunocompromised individuals or people with underlying diseases (Tena *et al.*, 2015), so its application and release into the environment could pose public health risks and, therefore, should be done in a very cautious and controlled manner.

Similarly, *Brevibacillus laterosporus* isolate C9F also inhibited the growth of this pathogen. Even though there are no studies in literature regarding *Brevibacillus* spp. antagonistic activity towards Psa, *B. laterosporus* is described as a potential biocontrol agent, not only against bacteria such as *Ralstonia solanacearum* (the causal agent of tomato bacterial wilt) (Li and Jiang, 2007) but also against pathogenic fungi such as *R. solani* and *P. capsica* (Yue *et al.*, 2015).

Moreover, *Pseudomonas protegens* isolate 8BB2 had no inhibitory effect against Psa in this study, which is contradictory to the results obtained by Wickasono *et al.* (2018), who demonstrated that inoculation of Psa infected kiwifruit plants with endophytic *Pseudomonas* species significantly reduced disease severity.

Although this study did not focus on the action mechanisms behind the antagonistic effects of the PGPB isolates, the application of cultural extracts (data not presented here), obtained by filtration of the medium where isolates had grown, did not inhibit Psa growth, suggesting that probably these isolates are only able to antagonize this pathogen when in direct contact with it, possibly through the synthesis of antimicrobial compounds mediated by quorum sensing mechanisms (Altaf *et al.*, 2017). Tontou *et al.* (2015) demonstrated that an endophytic *Pseudomonas* strain had antagonistic activity against Psa, and this activity was mostly due to the production of non ribosomal peptides, although the hypothesis of quorum sensing mediated antimicrobial compounds synthesis and quorum quenching activity of an AHL-acylase were not excluded.

5. Conclusions

Plant growth promoting bacteria are known to inhibit the growth of many different plant pathogens and reduce the severity of such diseases in plants. In this study, 21 bacterial isolates, retrieved from the rhizosphere of chestnut trees, mushrooms, and kiwifruit and walnut phyllosphere were tested as possible biocontrol agents against a reference strain of the most relevant pathogen of kiwifruit orchards, Psa CFBP 7286. 13 of the isolates demonstrated the capacity of inhibiting this pathogen's growth, although some were stronger antagonists than others. Overall, isolates 7PB2 (*B. amyloliquefaciens*) and 5BB2, EA1 and 3 (*A. faecalis*) were the most efficient Psa antagonists.

These results suggest that these isolates are good candidates as biocontrol agents of Psa, as an alternative or additive to current antimicrobial chemicals and even biocontrol products already available. Future work should focus on testing combinations of different isolates, to improve the antagonistic effects. It would also be of relevance to study the antagonistic potential of these isolates against other important phytopathogens. Nonetheless, *in planta* studies must be made in order to infer further conclusions, as well as studies regarding the mechanisms of antagonism of these isolates.

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IV. Chapter 4: Effect of Plant Growth Promoting Bacteria on the Physiological Status of *Solanum lycopersicum*

Abstract

Plant growth promoting bacteria (PGPB) are gaining attention as a sustainable alternative to current agro-chemicals. Besides offering protection against pathogenic organisms, they are also able to improve plant health and growth. This study focused on evaluating the impact of three *Bacillus* spp. and one *Brevibacillus* sp., isolated from the rhizosphere of chestnut trees and kiwifruit plant leaves, on the photosynthetic and antioxidant performance of *Solanum lycopersicum* var. MicroTom plants. Physiological parameters such as root and shoot length and biomass were quantified in tomato seedlings under *in vitro* conditions. Photosynthetic parameters, such as pigments contents (chlorophyll (*a*, *b*), carotenoids and anthocyanins), chlorophyll *a* fluorescence, gas exchange, total soluble sugars (TSS), starch contents and, non-enzymatic defences were also assessed.

Results showed that PGPB treatments had different effects. *Brevibacillus laterosporus* C9F promoted a decrease in the chlorophyll amount and isolate *Bacillus safensis* FV46 stimulated the content of these pigments, compared to C9F. C9F also impaired the Photosynthetic quantum yield (Φ PSII) and decreased water usage efficiency (WUE). However, C9F induced positive effects, increasing TSS and decreasing superoxide (O_2^-) content. Also, *B. megaterium* 1PB1 led to a reduction of the O_2^- levels and an increase in TSS and starch. *B. zhangzhouensis* 5PB1 promoted an increase of stomatal conductance (*gs*) and starch content. FV46 increased the total phenol content levels, increasing the antioxidant power in tomato leaves. These isolates demonstrated different beneficial capacities for tomato plants, particularly 5PB1, that simulated photosynthesis, while 1PB1 and C9F showed as protectives against oxidative stress and FV46 stimulated the antioxidant defences.

Keywords

Biofertilizers, Chlorophyll *a* fluorescence, Gas exchange, Plant growth promoting bacteria (PGPB), *Solanum lycopersicum*, Sustainable agriculture

1. Introduction

Increasing global food demand has made agricultural practices extensively dependent on chemical fertilizers and pesticides. These agrochemicals, although of extreme importance, end up having huge negative impacts on environment, such as water pollution and soil degradation, which ultimately affect entire ecosystems and human health (Brown and Jameton, 2000; Bruhl and Zaller, 2019). However, sustainability in food production has been a priority in late years, so there is a growing interest for new environmentally friendly biotechnological tools that can potentially replace synthetic agro-chemicals or, at least, reduce their use and impact (Singh and Yadav, 2020). One of the proposed alternatives to traditional agrochemicals is the use of plant growth promoting bacteria (PGPB) (Basu *et al.*, 2017; Chandra *et al.*, 2018).

PGPB can improve plant growth and health in several ways. Direct benefits, the ones that affect plant metabolism, include modulation of phytohormone levels through the production of indole acetic acid (IAA) and other growth regulators (Khan *et al.*, 2014; Etesami *et al.*, 2015), improved nutrient availability, through the production of siderophores (Sharma and Johri 2003; Gull, 2012), nitrogen fixation (Yedagari *et al.*, 2010; Gopalakrishnan *et al.*, 2017) or phosphate solubilization (Kang *et al.*, 2008; Midekssa *et al.*, 2016). Indirect benefits include protection against pathogens, through the production of antibiotic compounds (Mnif and Ghribi, 2015; Mnif *et al.*, 2015), direct competition for nutrients (Miethke and Marahiel 2007; Gull, 2012) or by inducing systemic resistance in plants (Jain *et al.*, 2016). Besides, it is known that PGPB can also alleviate abiotic stresses, making plants more tolerant towards environmental adverse conditions (e.g. high salinity and drought), for example through the production of ACC deaminase (Glick *et al.*, 1998), which reduces stress ethylene production, or through phytohormone regulation (Egamberdieva, 2009).

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PGPB mediated changes in phytohormone levels and improved nutrition ultimately lead to metabolic changes. In fact, several studies suggest that PGPB inoculation can have beneficial effects on the photosynthetic metabolism of several plant species. Isolates of *B. amyloliquefaciens* and the mycorrhizal fungi *Rhizophagus irregularis* for instance improved the photosynthetic efficiency in *Cyrtostylis rotundifolia*, *Thymus serpyllum*, *Fragaria vesca* and *Trifolium repens*, either when inoculated alone or in combination (Xie *et al.*, 2018). Another study in sugar beet plants (*Beta vulgaris*), that used three PGPB isolates, *B. pumilus*, *Chryseobacterium indologene* and *Acinetobacter johnsonii*, showed that inoculation with those strains increased dry weight and plant height, and induced significant increases in the maximum efficiency of PSII (F_v/F_m), carbon assimilation as well as higher chlorophyll content, which lead to increased carbohydrates synthesis (Shi *et al.*, 2010). Stefan *et al.* (2013) showed that inoculation of runner bean plants (*Phaseolus coccineus*) with two siderophore and IAA-producing PGPB strains lead to higher chlorophyll content and photosynthetic rates, as well as a higher water-use efficiency compared to control plants.

Besides improving the fitness of healthy plants, studies show that PGPB inoculation also improves the photosynthetic metabolism in stressed plants. For example, inoculation of drought stressed cucumber plants with a combination of three PGPB strains, *B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21 lead to an increase of the leaf chlorophyll content compared to control plants, showing that these PGPB helped plants maintain their chlorophyll contents in adverse water conditions (Wang *et al.*, 2012). A halotolerant PGPB strain, *Curtobacterium albidum* SRV4, increased chlorophyll (Chl) *a*, *b* and carotenoid contents in *Oryza sativa* plants in saline stress conditions, comparatively to uninoculated control plants (Vimal *et al.*, 2018). Furthermore, *Pseudomonas aeruginosa* and *Burkholderia gladioli* inoculation of *Lycopersicon esculentum* cadmium stressed plants significantly improved gas exchange parameters such as net photosynthetic rate, stomatal conductance, intercellular CO₂ concentration and transpiration rate, comparatively to control plants. Besides that, an increase of the photosynthetic pigments contents as well as an upregulation of carbohydrates content were observed in PGPB treated plants (Khanna *et al.*, 2019). *B. subtilis* and *P. fluorescens* inoculation of radish plants stimulated plant growth, when alone or in combination with saline stress, leading to an increase of Chl *a*, Chl *b*, Chl *a/b* ratio and carotenoids contents in leaves. Similarly, inoculation with a *Bacillus* sp. in lettuce plants under saline stress conditions lead to an increase of Chl

content, as well as a higher photosynthetic rate and stomatal conductance (Han and Lee, 2005).

Tomato (*S. lycopersicum*) is one of the most important crops in the world, with more than 182 million tonnes of tomatoes being produced globally in 2018 (FAOSTAT, 2020, www.faostat.org). Its nutritional properties (e.g. great amounts of vitamin A and C) and versatility of processing into many different products make tomatoes extremely valuable vegetables. Its production has been increasing in late years, with a trend to future further increase. Hence, it is of great importance to develop sustainable ways of increasing yield and quality of tomatoes, without compromising the environment and at the same time ensuring food security.

In order to contribute to the better understanding of the interactions between PGPB and plants, the aims of this study were to evaluate the physiological effects of four different PGPB isolates on *S. lycopersicum* var. MicroTom fresh and dry weight, biomass, photosynthetic pigments, carbohydrate contents, chlorophyll *a* fluorescence and gas exchange parameters.

2. Material and Methods

2.1. Bacterial Isolates and Culture Conditions

For this work, four of the collection isolates (Table II.1) were used. Based on previous characterization and screening tests, the chosen isolates were 1PB1 (*B. megaterium*), 5PB1 (*B. zhangzhouensis*), C9F (*Brevibacillus laterosporus*) and FV46 (*B. safensis*). For both seedling inoculation and seed biopriming, isolates were cultured in LB broth medium (Liofilchem, Italy), at 25 °C.

2.2. Seedling Inoculation with PGPB

Seeds of *S. lycopersicum* var. MicroTom (Moles Seeds Ltd, UK) were disinfected in commercial bleach (5% sodium hypochloride) at final concentration of 50%, washed and soaked for 1 h in sterile water. After hydration, ten seeds were

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disposed in the middle line of each petri dish (11x11 cm) with Hoagland medium (pH= 5.7), prepared by diluting nutrient stock solutions, according to Table IV.1, at 0.7% agar (w/v). Bacterial cultures grown overnight (16 h) were adjusted to an optical density of $\lambda=600$ (OD₆₀₀) at 0.100 ± 0.005 . Using a sterile loop, bacteria were inoculated under the seeds, spaced by 0.5 cm. Plate dishes were disposed vertically on a phytotron under a 16 h light photoperiod, $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ of PAR radiation with a white fluorescent lamp (OSRAM, Germany) at $25 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$, during 12 days. After this period, seedlings shoot and root length as well as biomass were measured. Two temporal sampling points were established with one-week interval.

2.3. Seed Biopriming and Plant Growth Conditions

Seeds of *S. lycopersicum* var. MicroTom (Moles Seeds Ltd, UK) were germinated on sterile petri dishes with 5 mL of sterile water. Three days into the germination process, bacterial cultures grown overnight (16 h) were adjusted to OD₆₀₀ of 0.100 ± 0.005 , and 1 mL was inoculated for each experimental condition. After 6 days of germination, 10 seedlings of each condition were transferred to pots containing 190 g of soil. Plants were watered with $\frac{1}{2}$ strength Hoagland solution (pH = 5.7), prepared according to Table IV.1, two times a week, to 100% field capacity. Plant growth took place under greenhouse conditions, between March and July, with mean and maximum air temperatures ranging from 13 °C and 18 °C (March) to 25 °C and 33 °C (July), respectively (IPMA, 2020). Photoperiod ranged from 12 h of daylight (March) to 15 h of daylight (July). After 130 days in culture, when all plants were synchronized, in the flowering/fruit production stage, chlorophyll *a* fluorescence and gas exchange measurements took place, and additionally leaves were collected, immediately frozen in liquid nitrogen, and stored at -80 °C for further quantification of total soluble sugars (TSS), starch, pigments, total phenolic content, DPPH scavenging activity and superoxide semi-quantification. The experiment was temporarily repeated for another plant batch under the same culture conditions with 3 weeks shift.

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Table IV.1. Preparation of stock solutions and full strenght Hoagland solution.

Macro and Micro nutrients	Stock solution (g L ⁻¹)	Dilution of stock solution (mL L ⁻¹)
2M KNO ₃	202	2.5
2M Ca(NO ₃) ₂ ·4H ₂ O	472	2.5
Fe-EDTA	15	1.5
2M MgSO ₄ ·7H ₂ O	493	1
H ₃ BO ₃	2.86	1
MnCl ₂ ·4H ₂ O	1.81	1
ZnSO ₄ ·7H ₂ O	0.22	1
CuSO ₄ ·5H ₂ O	0.08	1
Na ₂ MoO ₄ ·H ₂ O	0.09	1
1M KH ₂ PO ₄	136	1

2.4. Pigments Quantification

Pigment quantification was performed following the method of Sims and Gamon (2002), using three replicates, each consisting of leaf pools from 8 plants. 100 mg of frozen leaves were used for each condition and the control. Leafs were macerated with 1 mL of extraction buffer [Acetone:Tris-HCl 50mM (80:20), pH = 7.8], using a mortar, and transferred to 2 mL microcentrifuge tubes. Another 0.5 mL of the extraction buffer were used to wash the mortar. Tubes were vortexed for 30 s and then centrifuged for 10 min at 10000 *xg* at 4 °C. Supernatants were transferred to 15 mL falcon tubes, and pellets were reserved. Tubes were placed in ice and kept in the dark. 1.5 mL of the extraction buffer were added to the reserved pellets. Tubes were once again vortexed and centrifuged for 10 min at 10000 *xg* at 4 °C. Supernatants were transferred to the corresponding falcon tubes. 150 µL of each sample were transferred to a 96-well microplate (in triplicates). For negative control/blank, 150 µL of the extraction buffer were used. Absorbances were read at 663 nm, 537 nm, 647 nm and 470 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA). Chlorophyll *a*, chlorophyll *b*, carotenoid and anthocyanin contents (mol g⁻¹) were estimated following the equations:

$$\text{Chlorophyll } a \text{ (}\mu\text{mol mL}^{-1}\text{)} = (0.01373 \times A_{663}) - (0.000897 \times A_{537}) - (0.003046 \times A_{647})$$

$$\text{Chlorophyll } b \text{ (}\mu\text{mol mL}^{-1}\text{)} = (0.02405 \times A_{647}) - (0.004305 \times A_{537}) - (0.005507 \times A_{663})$$

$$\text{Anthocyanins (}\mu\text{mol mL}^{-1}\text{)} = (0.08173 \times A_{537}) - (0.00697 \times A_{647}) - (0.00228 \times A_{663})$$

$$\text{Carotenoids (}\mu\text{mol mL}^{-1}\text{)} = \frac{(A_{470} - (17.1 \times (\text{Chl } a + \text{Chl } b) - 9.479 \times \text{Anthocyanins}))}{119.26}$$

2.5. Carbohydrates Content Quantification

TSS content was quantified by the anthrone method (Irigoyen *et al.* 1992), and the starch content according to Osaki *et al.* (1991) using three replicates, each consisting of leaf pools from 8 plants.

2.5.1 Total Soluble Sugars

For TSS quantification, 50 mg of frozen leaves (pool of 8 plants) preserved at -80 °C were used for each condition and for the control. Leaves were macerated with 1.5 mL of 80% (v/v) ethanol, using a mortar, and transferred to 15 mL falcon tubes. Another 1.5 mL of 80% (v/v) ethanol were used to wash the mortar. Additionally, 7 mL of 80% (v/v) ethanol were added to each tube, making the final volume of 10 mL. Tubes were incubated at 80 °C for 1 hour, and then were kept in ice for 10 min. Samples were vortexed for 30 s and then centrifuged for 10 min, at 10000 xg, at 4 °C. Supernatants were transferred to new tubes and the pellets were preserved. For the anthrone reaction, 30 µL of each supernatant were mixed with 750 µL of the anthrone solution [40 mg anthrone, 1 mL H₂O MilliQ, 20 mL 98% (v/v) H₂SO₄].

Reaction tubes were then placed in a dry bath at 100 °C for 10 min, and vortexed thoroughly. Tubes were kept in ice for 15 min and then 150 µL of each sample, white and standard were transferred to a 96-well microplate (in triplicates). Absorbances were read at 625 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA). TSS content was determined using a standard curve for glucose.

2.5.2. Starch

For starch content quantification, pellets reserved from the TSS quantification protocol were mixed with 5 mL of 30% (v/v) perchloric acid, and vortexed until they were resuspended. Tubes were incubated for 1 h at 60 °C. After that, tubes were kept in ice for 15 min and thoroughly vortexed for 30 s. Tubes were then centrifuged for 10 min, at 10000 $\times g$, at 4 °C. For the anthrone reaction, 30 μ L of each supernatant were mixed with 750 μ L of the anthrone solution [40 mg anthrone, 1 mL H₂O MilliQ, 20 mL 98% (v/v) H₂SO₄]. Reaction tubes were then placed in a dry bath at 100 °C for 10 min, and vortexed thoroughly. Tubes were kept in ice for 15 min and then 150 μ L of each sample, white and standard were transferred to a 96-well microplate (in triplicates). Absorbances were measured at 625 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA). Starch content was determined using a standard curve for glucose.

2.6. Photosynthetic Performance and Gas Exchange

Ten mature leaves of 8 plants of each treatment were used for gas exchange and chlorophyll *a* fluorescence determination. The portable photosynthesis system (LI-6400XT Photosynthesis System, LiCOR, Lincoln, Nebraska, USA) was used for the analysis of gas exchanges, which were performed under ambient growth conditions. Transpiration rate (E , mol m⁻² s⁻¹), stomatal conductance (g_s , mol m⁻² s⁻¹), net CO₂ photosynthetic rate (P_N , μ mol m⁻² s⁻¹) and intercellular CO₂ concentration (C_i , ppm) were determined according to Dias *et al.*, (2019). Chlorophyll *a* fluorescence parameters were determined by measuring the minimal fluorescence yield (F_0 , PSII centres open) in leaves adapted to dark for 40 min, using the LI-6400XT Photosynthesis System (LiCOR, USA) fluorometer. Following adaptation to dark, a 0.7 s saturating light pulse (>1500 μ mol m⁻² s⁻¹) was applied, and maximum fluorescence yield was determined (F_m , PSII centres closed). F'_0 and F'_m were also determined in leaves adapted to light for 30 min. Variable fluorescence values $F_v = F_m - F_0$ and $F'_v = F'_m - F'_0$ were estimated to assess the maximum efficiency of PSII and the effective photochemical efficiency of PSII (Φ_{PSII}). Additionally, photochemical quenching ($qP = (F'_m - F') / (F'_m - F'_0)$), non-photochemical quenching, ($NPQ = (F_m - F'_m) / F'_m$) and

effective efficiency of PSII ($\Phi\text{PSII} = (F'_m - F')/F'_m$) were determined (Klughammer and Schreiber, 2008; Murchie and Lawson, 2013).

2.7. Total Phenolic Content

Total phenolic content of plants treated with PGPB was assessed following the Folin-Ciocalteu method, according to Dewanto *et al.* (2002). Samples of 100 mg of frozen leaves (pool of 8 plants) were used for each condition and the control, in triplicates. Leaves were macerated with 2 mL of extraction buffer, containing dH₂O:methanol (9:1) using a mortar, and proceeding at -4 °C. A volume of 1 mL of extraction buffer was used to wash the mortar. Extracts were then filtered using a Buchner funnel and Whatman® paper filter, in a Kitasato flask, connected to a vacuum pump. Filtered extracts were collected in 15 mL falcon tubes. Extracts were centrifuged at 2500 rpm, for 10 min and -4 °C. 125 µL of each extract, as well as 125 µL of 7 galic acid standards (0, 25, 50, 100, 150, 200, 250 and 500 µg mL⁻¹) were transferred to new tubes. To each tube, 500 µL of dH₂O were added, as well as 125 µL of the Folin-Ciocalteu reagent. After 6 min of incubation in the dark, at room temperature, 1250 µL of 7% Na₂CO₃ were added, as well as 1 mL of water. Tubes were incubated for 90 min and then 150 µL of each sample, white and standards were transferred to a 96-well microplate (in triplicates). Absorbances were read at 760 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA).

2.8. DPPH Scavenging Activity

Scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was determined following the method of Harkat-Madouri *et al.* (2015) with some modifications. 100 mg of frozen leaves (pool of 8 plants) were used for each condition and the control, in triplicates. Leaves were macerated with 2 mL of extraction buffer (pure methanol) using a mortar and proceeding at -4 °C. 1 mL of extraction buffer was used to wash the mortar. Extracts were then filtered using a Buchner funnel and Whatman® paper filter, in a Kitasato flask, connected to a vacuum pump. Filtered extracts were collected in 15 mL falcon tubes. Extracts were centrifuged at 2500 rpm, for 10 min. 150 µL of each

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sample were diluted with 100 μL of methanol and 1250 μL of 0.1 mM DPPH was added to each sample. Simultaneously, 11 DPPH standards were prepared following the same method for samples. Tubes were incubated for 30 min in the dark and then 150 μL of each sample, white and standards were transferred to a 96-well microplate (in triplicates). Absorbances were measured at 517 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA).

2.9. Superoxide (O_2^-)

Superoxide (O_2^-) was measured by means of Nitroblue tetrazolium (NBT) reduction, according to Gajewska and Sklodowska (2007). 100 mg of frozen leaves (pool of 8 plants) were used for each condition and the control, in triplicates. 2 mL of extraction buffer, containing 0.01 M phosphate buffer (pH= 7.8), 0.05% (w/v) NBT diluted in 100 μL Dimethyl sulfoxide (DMSO) and 10 mM sodium azide were thoroughly mixed with the samples. After 1 h of incubation at room temperature, with regular shaking, tubes were centrifuged at 13000 xg for 2 min, at 4 °C. Samples were then incubated at 85 °C for 15 min and kept in ice for 10 min. Then, 150 μL of each sample were transferred to a 96-well microplate (in triplicates). Absorbances were read at 580 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific™, USA).

2.10. Statistical Analysis

Experiments regarding shoot and root length as well as biomass used 20 plantlets. The other experiments used 10 top mature leaves (with similar age) from 8 different plants, which were treated as biological replicates and/or as pools, with at least 3 independent technical replicates. Presented values are the mean $\pm$ standard deviation. Comparisons between all treatments and the control were made using One Way ANOVA test. When data was statistically different, the Dunnett Comparison Test ($p < 0.05$) was also applied. Graphpad™ Prism 7 was used. Multivariate analyses for data correlation used Principal Component Analysis and were performed with CANOCO V4.02 software for Windows.

3. Results

3.1. Shoot and Root Length and Biomass

No significant differences were observed regarding shoot length of PGPB treated seedlings. Treatment with 1PB1 and 5PB1 lead to a significant decrease of root length comparatively to the control. However, 5PB1 treatment was not different from treatments with C9F and FV46. No PGPB treatment led to significant differences in root and shoot fresh and dry weight of seedlings, comparatively to the control. However, C9F treatment lead to a slight increase of fresh and dry weight of seedlings' roots.

Table VI.2. Root and Shoot length (cm) and fresh and dry weight (g) of control seedlings and seedlings treated with 1PB1, 5PB1, C9F, FV46. Presented values are means $\pm$ SD ($n = 20$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$). FW – fresh weight; DW – dry weight.

	Control	1PB1	5PB1	C9F	FV46
Shoot length	3.237 ± 0.491 a	3.147 ± 0.527 a	3.082 ± 0.513 a	3.100 ± 0.489 a	3.124 ± 0.491 a
Shoot FW	0.0609 ± 0.0139 a	0.0558 ± 0.0007 a	0.0561 ± 0.0097 a	0.0572 ± 0.0054 a	0.0568 ± 0.0118 a
Shoot DW	0.00256 ± 0.00053 a	0.00252 ± 0.00011 a	0.00251 ± 0.00045 a	0.00253 ± 0.00047 a	0.00257 ± 0.00056 a
Root length	9.163 ± 1.761 a	6.211 ± 1.195 c	7.382 ± 1.261 bc	8.271 ± 2.074 ab	8.082 ± 1.694 ab
Root FW	0.012 ± 0.005 a	0.012 ± 0.006 a	0.010 ± 0.006 a	0.013 ± 0.003 a	0.010 ± 0.004 a
Root DW	0.00053 ± 0.00005 a	0.00054 ± 0.00011 a	0.00049 ± 0.00012 a	0.00063 ± 0.00018 a	0.00056 ± 0.00002 a

3.2. Pigments Content

No statistically significant differences between treatments were observed in the anthocyanins (Figure IV.1.a) and carotenoids (Figure IV.1.b) contents of PGPB treated plants comparatively to the control group, although there was a slight increase in plants treated with 5PB1 and FV46. Chl *a* (Figure IV.1.c) and Chl *b* (Figure IV.1.d) contents were also not significantly affected by inoculation with PGPB, even though 1PB1 and

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C9F seem to negatively impact these pigments, whilst 5PB1 and FV46 caused minor improvements. However, reductions in Chl *a* and Chl *b* in C9F were proportional, thus the Chl *a/b* ratio was maintained (Figure IV.1.e). Contrarily to FV46, which slightly increased Chl *b* levels and, consequently, reduced the Chl *a/b* ratio, although not significant. Nonetheless, inoculation with C9F caused a slight decrease in the contents of all the photosynthetic pigments, whilst inoculation with FV46 caused a slight improvement. Pigment contents in plants treated with 1PB1 and 5PB1 were almost identical to those of the control group, although 1PB1 seems to have a minor negative impact, contrarily to 5PB1.

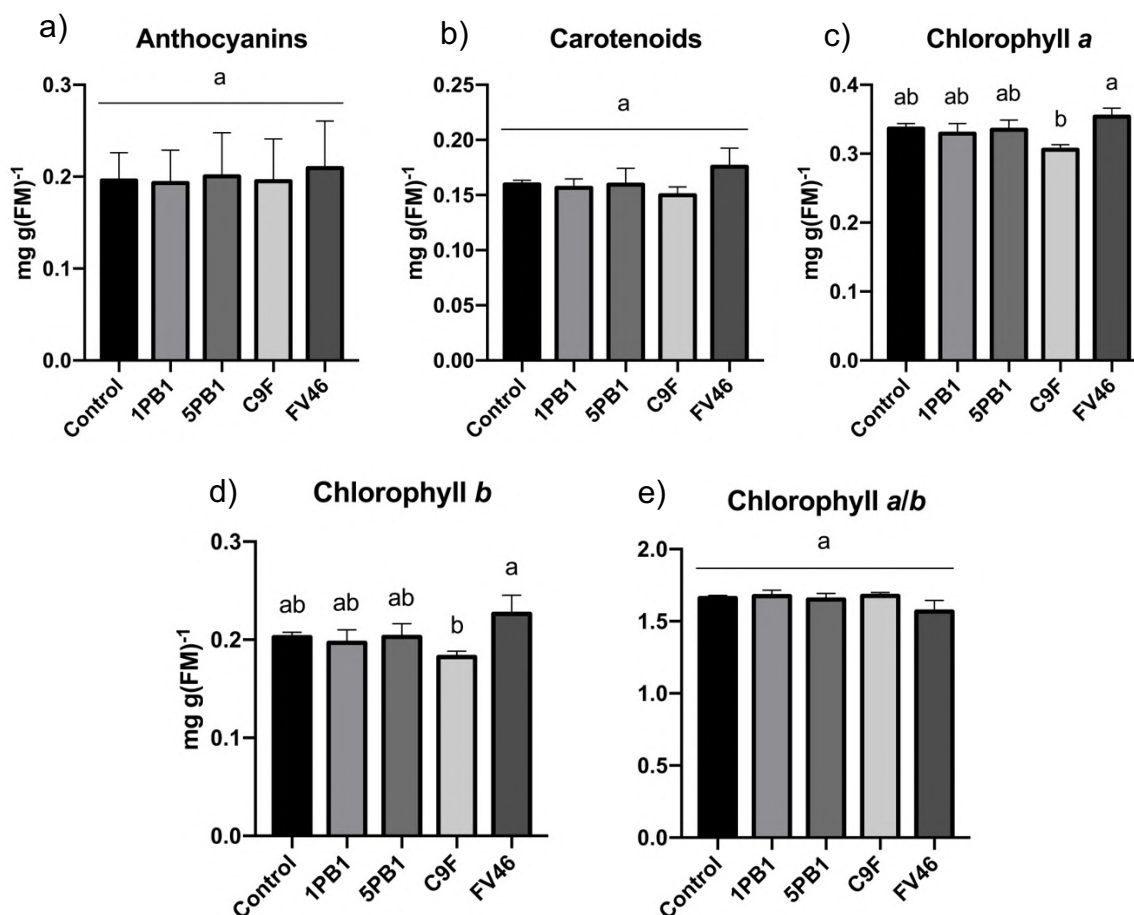


Figure IV.1. Effect of PGPB inoculants 1PB1, 5PB1, C9F and FV46 on Anthocyanins (a), Carotenoids (b) Chl *a* (c), Chl *b* (d), and Chl *a/b* (e) in leaves of *S. lycopersicum*. Presented values are means $\pm$ SD ($n = 3$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.3. Carbohydrates Content

Regarding TSS (Figure IV.2.a), plants inoculated with 1PB1 and C9F presented significantly higher TSS content comparatively to control, and slightly higher TSS content comparatively to the other two treatments, 5PB1 and FV46. Although there are no significant changes in the TSS content of plants treated with 5PB1 and FV46, there is a slight increase comparatively to the control group. Nonetheless, PGPB treatments resulted in higher TSS contents. Relatively to starch content (Figure IV.2.b), plants treated with 1PB1 and 5PB1 presented significantly higher starch content than control plants. In addition, 5PB1 inoculated plants had significantly higher starch content than plants inoculated with 1PB1. C9F and FV46 treatments did not significantly increase starch content of leaves comparatively to the control, although a small increment was observed.

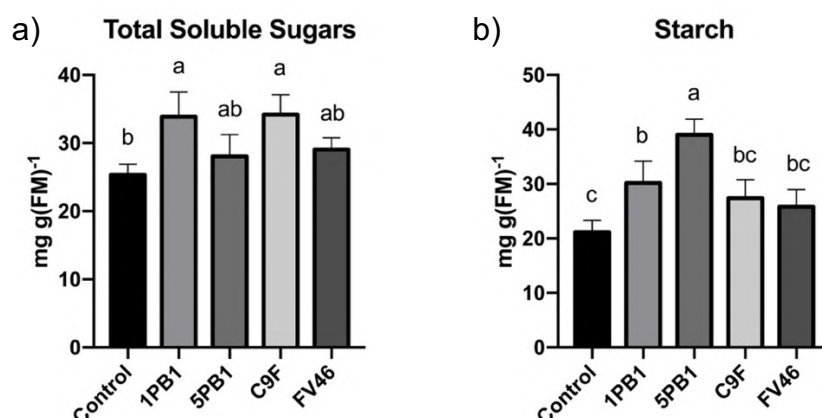


Figure IV.2. Effect of PGPB inoculants 1PB1, 5PB1, C9F and FV46 on Total Soluble Sugar (a) and Starch (b) contents, in leaves of *S. lycopersicum*. Presented values are means $\pm$ SD ($n = 3$) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.4. Photosynthetic Performance

Regarding maximum fluorescence (F_m) (Figure IV.3.a) plants inoculated with 5PB1 and FV46 did not significantly differ from those of the control group. However, inoculation of plants with C9F and 1PB1 led to a significant reduction in maximum fluorescence. Considering the F_v/F_m ratio (Figure IV.3.b), control plants showed a ratio ~ 0.82 , not significantly different from the ratio of plants treated with 1PB1 or 5PB1,

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although there was a minor increase in 5PB1 inoculated plants ~ 0.83 . C9F and FV46 treatments resulted in significantly reduced F_v/F_m ratios, of ~ 0.79 and ~ 0.81 . Considering the F'_v/F'_m ratio of leaves adapted to light (Figure IV.3.c), results were similar to those in dark-adapted leaves, with C9F significantly reducing this ratio, and no significant differences between plants inoculated with 1PB1, 5PB1 and FV46 and control plants. qP was significantly reduced in plants treated with C9F, with no differences for the other treatments (Figure IV.3.d). NPQ was not stimulated neither reduced upon inoculation with the bacterial isolates, although a slight increase was observed in plants inoculated with 5PB1, and a slight decrease in plants inoculated with 1PB1 and C9F (Figure IV.3.e). Regarding the efficiency of the PSII photosystem (Figure IV.3.f), inoculation with 1PB1, 5PB1 and FV46 resulted in no statistical differences, although 5PB1 lead to a slight increase. Inoculation with C9F significantly reduced the PSII efficiency.

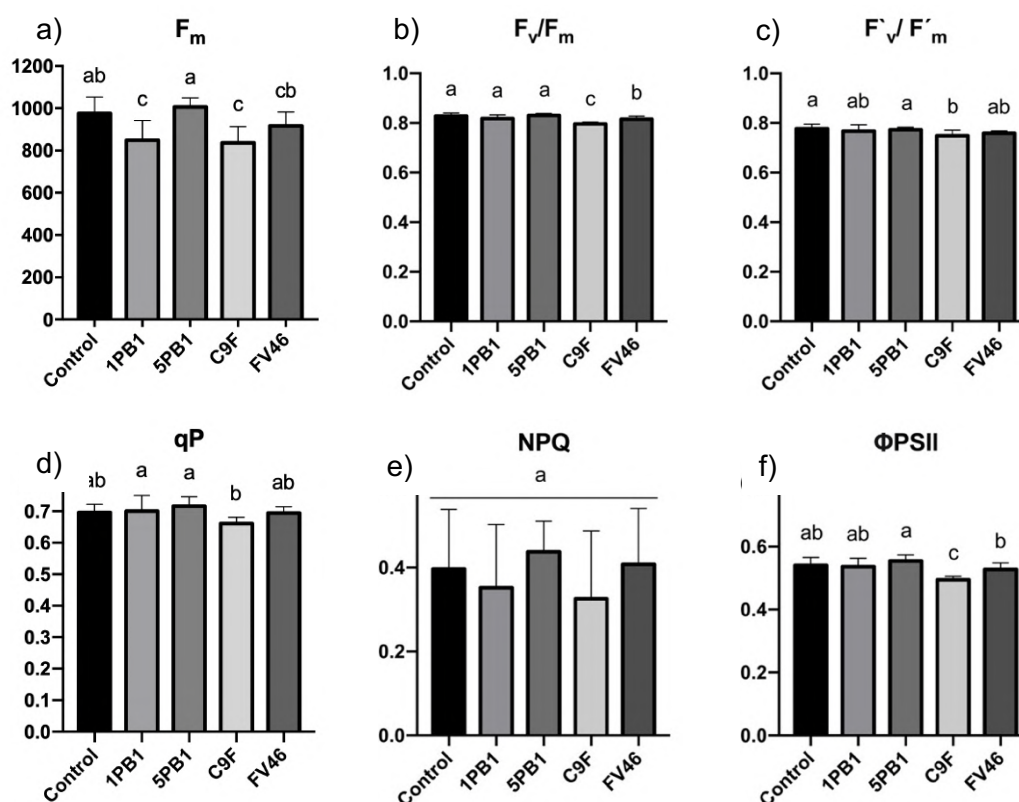


Figure IV.3. Effects of inoculation with isolates 1PB1, 5PB1, C9F and FV46 on PSII chlorophyll fluorescence. a) – Maximum Fluorescence; b) – Maximum quantum efficiency of photosystem II; c) – Maximum efficiency of photosystem II; d) – Fraction of photosystem II centers that are open; e) – Nonphotochemical quenching; f) – quantum yield of photosystem II. Presented values are means $\pm$ SD (n = 8) of measurements on each treatment group. Different letters mean significant values (p < 0.05).

3.5. Gas Exchange

Data for gas-exchange parameters shows that inoculation with C9F significantly decreased net CO₂ assimilation rate (P_N), while 1PB1, FV46 and 5PB1 did not significantly change P_N , although plants inoculated with 5PB1 had the highest P_N of all the conditions (Figure IV.4.a). Stomatal conductance (g_s) was significantly improved in 5PB1 comparatively to the control, while 1PB1, FV46 and C9F did not have significant effects (Figure IV.4.d), which is in line with the change of internal CO₂ concentration (C_i) (Figure IV.4.c). Transpiration rate (E) was not affected by inoculations with 5PB1 or C9F, but it was significantly reduced by 1PB1 and FV46 (Figure IV.4.b). C9F significantly reduced intrinsic water usage efficiency ($iWUE$) as well as water usage efficiency (WUE), while the other treatments were not statistically different from the control (Figure IV.4.e and Figure IV.4.f, respectively).

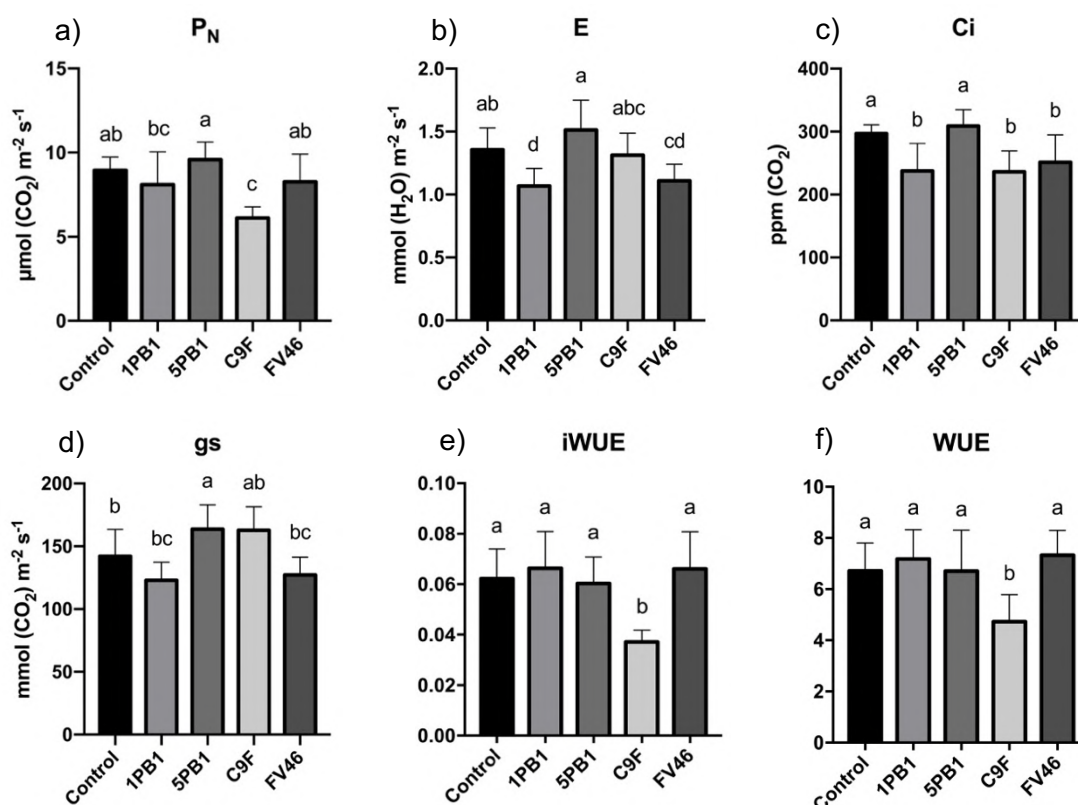


Figure IV.4. Effects of Inoculations with 1PB1, 5PB1, C9F and FV46 on gas exchange parameters; a) P_N – net photosynthetic rate; b) C_i – internal CO₂ concentration; c) g_s – stomatal conductance; d) E – transpiration rate e) intrinsic water use efficiency ($iWUE$) f) Water use efficiency (WUE). Different letters mean significant values ($p < 0.05$).

3.6. ROS Content and Non-enzymatic Antioxidant Activity

Inoculation of plants with 1PB1 and C9F resulted in lower superoxide concentration in leaves, while no significant differences were observed between control plants and plants inoculated with 5PB1 and FV46 (Figure IV.6.a). Inoculation of plants with FV46 resulted in significantly higher phenolic content, comparatively to the control. Inoculation with 1PB1, 5PB1 and C9F did not significantly change phenolic content of plants, although slight increases were observed on plants inoculated with 1PB1 and C9F (Figure IV.5.b). Results show that no significant differences between conditions regarding the DPPH free radical scavenging activity, although a slight increment was observed in plants inoculated with FV46 (Figure IV.5.c).

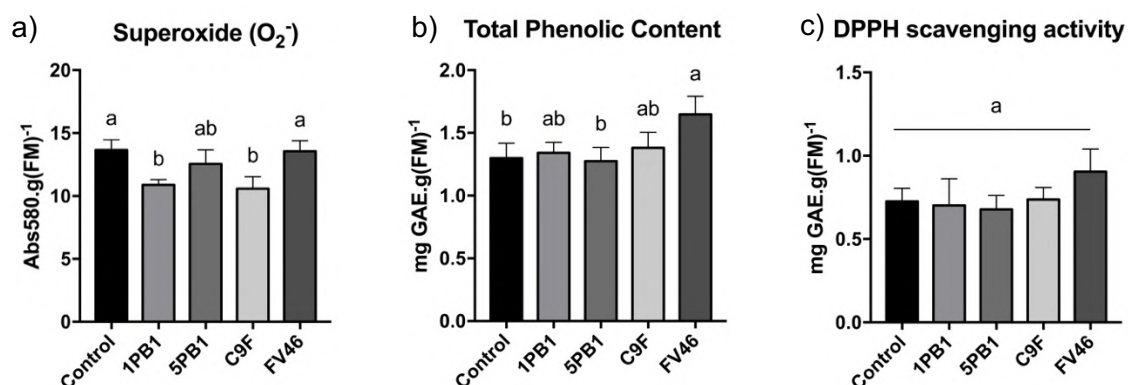


Figure IV.5. Effects of Inoculations with 1PB1, 5PB1, C9F and FV46 on superoxide (O_2^-) content (a), total phenolic content (b) and DPPH scavenging activity (c). Presented values are means $\pm$ SD (n = 3) of measurements on each treatment group. Different letters mean significant values ($p < 0.05$).

3.7. Principal Component Analysis

Principal component analysis (PCA) showed a separation between control and PGPB treatments, as well as separation between different PGPB treatments (Figure IV.6). This separation is more accentuated between treatments with FV46, C9F and 1PB1 and the control. 5PB1 was the treatment that scored the closest to the control. PC1 explained 48.2% of the variance and PC2 explained 29.5%. FV46 treatment is located on the left upper quadrant, being the treatment that scores the highest for photosynthetic pigments and non-enzymatic defense activity. 5PB1 is located on the

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lower left quadrant, along with the control, being the group that mostly scores for photosynthetic and gas-exchange parameters, such as F_v/F_m , $\Phi PSII$, C_i and P_N . C9F is located on the upper right quadrant, scoring the highest for TSS. 1PB1 is located on the lower right quadrant, scoring for g_s and Chl a/b .

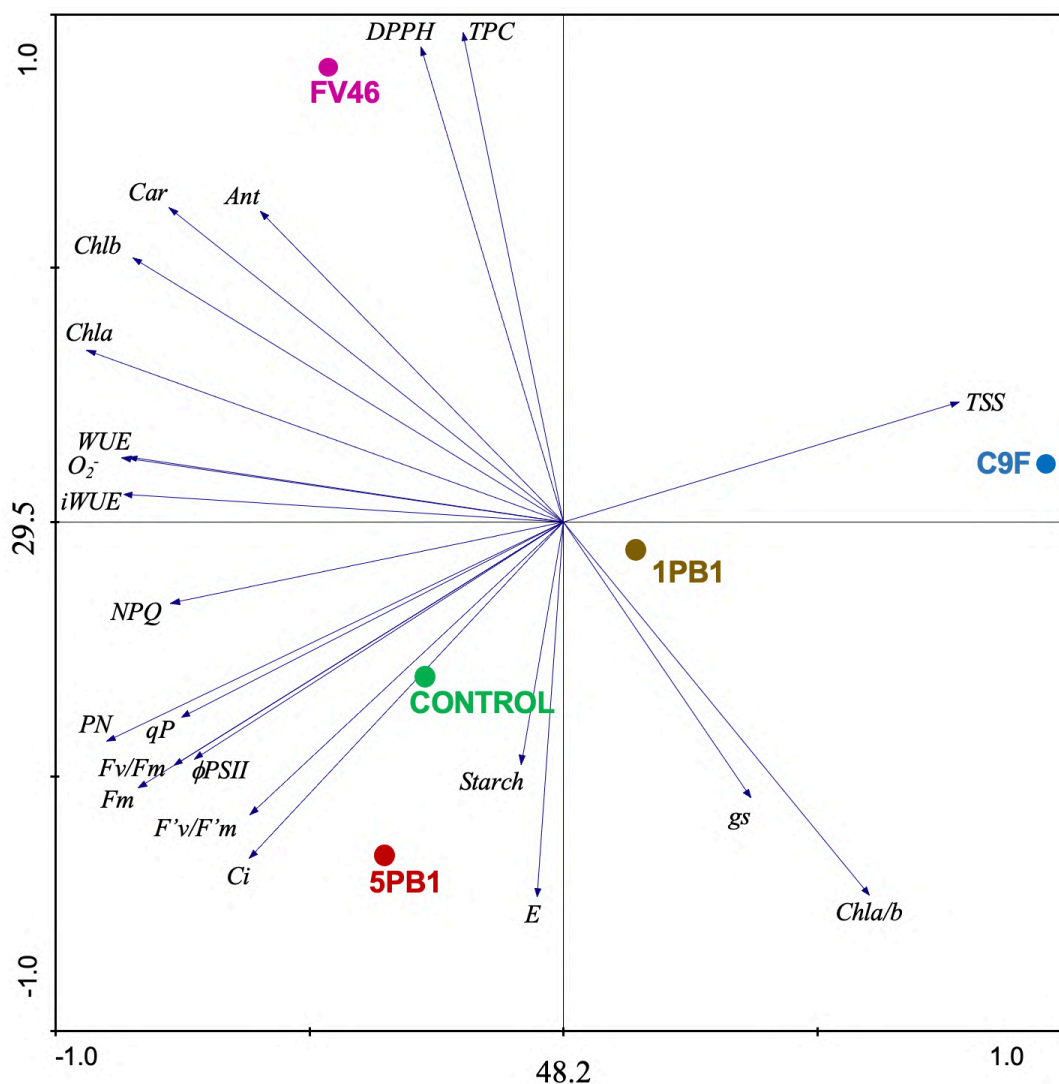


Figure IV.6. Principal component analysis biplot of the effects of inoculation of tomato plants with isolates 1PB1, 5PB1, C9F and FV46. Loading plot for the first axis PC1 explained 48.2% of the variance and second axis PC2 explained 29.5%. Ant – Anthocyanins; Car – Carotenoids; Chl a – Chlorophyll a ; Chl b – Chlorophyll b ; WUE – Water usage efficiency; O₂⁻ – Superoxide; iWUE – Intrinsic water usage efficiency; NPQ – Nonphotochemical quenching; P_N – Net carbon assimilation rate; qP – Fraction of PSII centres that are open; F_v/F_m – Maximum quantum efficiency of PSII. F_m – Maximum fluorescence; $\Phi PSII$ – Quantum yield/Efficiency of PSII; F'_v/F'_m – PSII maximum efficiency; C_i – internal CO₂; Starch – Starch content; E – Transpiration rate; g_s – stomatal conductance; TSS – Total soluble sugars; TPC – Total phenolic content; DPPH – DPPH scavenging activity.

4. Discussion

Plant growth promoting bacteria are known to improve plant growth and nutrition through production of hormones, such as IAA, and by solubilizing soil nutrients (Olanrewaju *et al.*, 2017). These mechanisms altogether end up influencing plants' physiological status. In this work it was demonstrated that inoculation of tomato plants with different PGPB isolates changed plants' physiological and antioxidant responses.

Growth related parameters, such as shoot and root length, as well as fresh and dry weight, were not sensitive to the PGPB treatments. However, treatments with 1PB1 and 5PB1 induced a reduction of root length, which could be due to an inhibitory effect of high IAA levels produced by these isolates (Chapter II), as reported by Tanimoto (2005). Nonetheless, results are in line with Cabra *et al.* (2017), who demonstrated that *B. subtilis* GIBI 200 had little effect on shoot and root length and biomass of treated tomato plants. Similarly, Gowtham *et al.* (2017) showed that inoculation of tomato plants with several PGPB strains (including *Bacillus* spp.) did not lead to changes in shoot and root length in plants growing under controlled conditions, but induced some increases in these parameters in plants growing under greenhouse conditions.

Considering the photosynthetic parameters, 5PB1 was the treatment that had the most positive influence on the photosynthetic performance of tomato plants. The F_v/F_m ratio, which estimates the maximum quantum efficiency of PSII photochemistry, varied between 0.79 and 0.83, which is in agreement with values described in literature for healthy unstressed plants (Zhuri *et al.*, 2015). These results are in line with the work of Yobo *et al.* (2009) who found that Inoculation of dry bean seedlings with *Bacillus* spp. isolates lead to a slight increase of maximum PSII efficiency (F_v/F_m), although over time that difference was not maintained comparatively to control plants. Similarly, Anusaraporn *et al.* (2020) demonstrated that inoculation of rice plants with *Bacillus* sp. N7 did not lead to changes in this ratio.

Regarding the F'_v/F'_m ratio, which estimates the maximum PSII efficiency when all the PSII centres are "open" (in light-adapted leaves), only the C9F treatment lowered this ratio. There is no data in literature regarding PGPB-mediated changes on the PSII effective efficiency (F'_v/F'_m) of tomato plants. However, in other species (*Eutropea oleracea*), De Castro *et al.* (2019), demonstrated that *B. subtilis* combined

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with *B. safensis* and other PGPB led to a significant increase of this parameter. Photochemical quenching (qP) was not affected by the PGPB treatments, although a small reduction was observed in plants inoculated with C9F, while 5PB1 led to a slight increase the proportion of PSII reaction centres that are open.

Furthermore, Φ PSII, which translates the effective quantum yield of the PSII electron transport, was not very susceptible to inoculation with 1PB1, while a slight improvement was observed in plants treated with 5PB1 and FV46. The PCA supports these findings, particularly for the 5PB1 treatment, which is positioned in the same quadrant as the control, near Φ PSII (Figure IV.6). Similar results were reported by Li *et al.* (2015), who found an increase of Φ PSII on *V. faba* plants inoculated with *B. subtilis*, but, in contrast, Jain and Jajoo (2019) verified a small decrease in Φ PSII on wheat plants treated with *B. subtilis* (NCIM 5594). In this work, only the treatment with C9F negatively influenced Φ PSII, and the PCA also confirms this finding (Figure IV.6). Moreover, a decrease of Φ PSII can result in a low availability of ATP and NADPH for the Calvin cycle.

The lack of beneficial effect in plants inoculated with 1PB1 and C9F could be due to the fact that these isolates were not obtained from the rhizosphere of tomato plants, so host-specificities might explain this fact, as already demonstrated by Vaikuntapu *et al.* (2014). These authors showed that PGPB isolates from tomato plants rhizosphere had a positive effect on tomato plants, but induced no effect, or negative effect, in other species. On the other hand, 5PB1 and FV46 seem to have less of a host preference, suggesting that they can benefit a broader range of plant species.

Nonphotochemical quenching (NPQ), which monitors the apparent rate constant for heat loss from PSII, was not changed by any treatment. Different results were reported by Samaniego-Gómez *et al.* (2016), who found that inoculation of pepper plants with *Bacillus* sp. M9 and *B. cereus* K46 led to a decrease of NPQ, but with an increase of qP. Similarly, Li *et al.* (2015) found that *B. subtilis* increased NPQ in *Vicia faba* plants.

In line with the observed for Φ PSII, gas exchange parameters also seem to be more positively influenced by 5PB1, and the PCA confirms this finding (Figure IV.6). The effect of 5PB1 on the P_N in tomato plants may be due to the lower restriction in E and g_s , and consequently high CO_2 availability (C_i). On the other hand, the restrictions observed on the Φ PSII of C9F treated plants may also explain the decline of P_N , as

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supported by the multivariate analysis (Figure IV.6). As a consequence, iWUE and WUE decreased. Contrarily, the other inoculants did not influence iWUE neither WUE. Different results were reported by Samaniego-Gómez *et al.* (2016), who demonstrated that inoculation of pepper plants with two *Bacillus* spp. strains lead to a significant increase of WUE.

Concerning pigments, inoculation with FV46 may stimulate the concentration of chlorophylls in tomato plants. The multivariate analysis supports this tendency, with the FV46 score positioned in the upper left quadrant, close to chlorophylls (Figure IV.6). Possibly, an improvement in the solubilization of micronutrients such as iron, zinc and manganese by PGPB can explain this increase (Roosta *et al.*, 2018). Our data are in agreement with the reported by Akram *et al.* (2019), who showed that a *B. megaterium* isolate lead to an increase in the levels of Chl *a*, Chl *b* and carotenoids in tomato plants. Also, Shah *et al.* (2020) obtained similar results when inoculating tomato plants with 4 different *Bacillus* isolates. This positive effect seems to be common for a wide range of species. Treatments with 5PB1 and 1PB1 had little influence on pigments, as confirmed by the PCA (Figure IV.6), while C9F negatively interfered with the chlorophylls, as these plants showed a tendency to have lower levels of these pigments. These results are in line with Morais *et al.* (2019) who showed that inoculation of strawberry plants with isolates of *Pedobacter* sp. CC1, *B. safensis* B106 and *B. subtilis* B167A exerted low or no impact on pigments content.

All isolates led, in general, to an increase of leaves' carbohydrates, with 1PB1 and 5PB1 particularly promoting a significant accumulation of starch, and 1PB1 and C9F an increase of TSS pool. PCA confirms that 5PB1 had a stronger positive influence on starch content, and 1PB1 and C9F on TSS (Figure IV.6). Gagné-Bourque *et al.* (2015) also demonstrated that inoculation of model grass *Brachypodium distachyon* with *B. subtilis* B26 lead to an accumulation of starch and TSS just after 8 days of growth. In the present work, the accumulation of TSS verified in plants inoculated with C9F might be linked to the observed P_N decrease. This finding is also supported by the PCA (Figure IV.6), indicating a reduction of TSS use. Moreover, isolates 1PB1 and C9F are able to produce more IAA than 5PB1 and FV46 (Chapter II) and exogenous IAA could justify the increase in carbohydrates in these plants (Li *et al.*, 2019). It is also known that plants usually provide sugars to beneficial bacteria, via root exudates, so higher sugar production may be linked to such interaction (Olanrewaju *et al.*, 2019). Moreover, accumulation of soluble sugars helps plants keep homeostasis,

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for instance under drought conditions, suggesting that these isolates could help plants withstand water deficit (Gagné-Bourque *et al.*, 2015).

Treatments with 1PB1 and C9F reduced the levels of superoxide content. This suggests that, despite not bringing benefits to photosynthesis, isolates 1PB1 and C9F might offer protection to plants by reducing the levels of superoxide, which could be advantageous in adverse environmental conditions. Moreover, it is known that bacteria produce defence enzymes such as superoxide dismutase (SOD) (Liu *et al.*, 2007), which could explain the lower superoxide contents in plants treated with these isolates. This negative correlation between superoxide and the 1PB1 and C9F treated plants was also confirmed by the PCA (Figure IV.6). Regarding total phenolic content (TPC), the inoculant FV46 favoured the accumulation of these compounds. These results are in line with Abd-Allah *et al.* (2017) who showed that a *B. subtilis* isolate led to an increase of the TPC in chickpea plants. Since phenolic contents have reducing properties and play a role in scavenging free radicals (Prakash *et al.*, 2007), results suggest that FV46 might improve non-enzymatic antiradical defences. In fact, the improvement of chlorophylls content by FV46 might be related with some protection provided by the TPC, which is also supported by the PCA (Figure IV.6).

5. Conclusion

In this work, four PGPB isolates obtained from chestnut tree roots and kiwifruit leaves were inoculated on tomato plants, in order to study their impact on plant health and performance. Overall, each isolate provided plants with different benefits. *B. zhangzhouensis* isolate 5PB1 improved the photosynthetic performance of plants, *B. safensis* isolate FV46 improved plant defences and promoted the accumulation of chlorophylls, while *B. megaterium* isolate 1PB1 and *Brevibacillus laterosporus* isolate C9F seem to protect plants against oxidative stress. Future studies should focus on using combinations of these isolates to test if they can act synergistically and provide plants with different benefits.

Although these isolates were not collected from tomato plants rhizosphere, they seem to have benefits in these plants, supporting the idea that these PGPB might have a broad-spectrum of action, which means they could improve plant growth and overall physiological status of a wide range of plant species, which is a desirable characteristic

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in terms of field/agricultural application and commercialization. However, additional studies with different plant species should be conducted to take further conclusions.

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V. Chapter 5: General Discussion and Conclusions

The main goal of this work was to characterize a collection of 21 bacterial isolates, that were isolated from the rhizosphere of chestnut trees, mushrooms, and the phyllosphere of kiwifruit plants and walnut trees, thus presumed to be plant growth promoting bacteria (PGPB), and test those isolates against a plant pathogen, as well as their biofortification potential, in tomato plants.

PGPB are described in literature as benefic microorganisms that live in association with plants or near plant roots, and present a variety of characteristics that stimulate plant growth and health and simultaneously inhibit the growth of several pathogens (Glick, 2020). These benefic bacteria gained attention in late years, emerging as an alternative to conventional agrochemicals such as fertilizers and pesticides, following the urge for sustainability in agricultural practices (Chandra *et al.*, 2018). There are several products that contain one or more PGPB strains available in the market, and being applied in field, therefore there is a big potential for exploring new PGPB strains that may possess better characteristics than the ones that are currently available.

In order to evaluate the potential of the collection isolates, they were characterized and screened for plant growth promoting (PGP) traits, such as Indole-3-acetic acid (IAA) production, siderophore production and inorganic phosphate solubilization. These traits were chosen because they are among the most important characteristics of these bacteria, and commonly studied when screening for new PGPB. IAA is the most common feature of PGPB, and there are numerous reports in literature regarding many different PGPB species that demonstrate IAA production, as shown by Bhutani *et al.* (2018), Marathe *et al.* (2017) and Hussain *et al.* (2020), among many others. Phosphate solubilization has also been reported in different PGPB species, as shown by the works of Prakash and Arora (2019) and Linu *et al.* (2018), for instance. Siderophore production is also a common and important PGP trait, as demonstrated by Yu *et al.* (2011) and Subramaniam and Sundaram (2020). Results of the characterization showed that all of the 21 isolates produce IAA, although some more than others. Furthermore, 13 were able to solubilize inorganic phosphate, and 5

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were able to produce siderophores. A general analysis shows that most of the isolates present at least two of the tested PGP traits, which is common among PGPB, and also desirable in biofertilizers/biopesticides. Molecular identification of the isolates confirmed that all of them are species described as PGPB, namely *Bacillus* spp. (1PB1, 1PB2, 2P2, 2PB1, 5PB1, 7PB2, 9BB1, 10PC, 11PC, EA3, EF1, EF2, EF3, UFG44, PI34 and FV46), *Alcaligenes faecalis* (3, 5BB2 and EA1), *Pseudomonas protegens* (8BB2) and *Brevibacillus laterosporus* (C9F).

While characterizing the isolates, and since plant pathogens are one of the main causes of losses in crop yield, the antagonistic potential of the isolates against a relevant plant pathogen was assessed *in vitro*. The chosen pathogen was a reference strain of *Pseudomonas syringae* pv. *actinidiae* (Psa), Biovar 3, the etiological agent of bacterial canker of kiwifruit, since it is currently the most severe disease of this culture (Moura, 2015). Some commercially available biocontrol products have been tested in field against Psa, with different results. Biondi *et al.* (2012) demonstrated that Amylo-X inhibited the growth of two Psa strains *in vitro*, while Vaz *et al.* (2018) found that Serenade Max was not effective in reducing disease symptoms in infected orchards. This shows the need for the discovery of new PGPB strains, in order to find more efficient biocontrol agents. In this study, the antagonistic potential was evaluated through the formation of a Psa growth inhibition halo around the zone where the PGPB isolates were inoculated, a common method for screening antagonistic PGPB strains. A total of 12 isolates were able to inhibit the growth of this pathogen, indicating that they might be used to control this disease, although field testing would be needed to further confirm this hypothesis.

The final aim was to assess the biofortification potential of the isolates 1PB1 (*B. megaterium*), 5PB1 (*B. zhangzhouensis*), C9F (*Brevibacillus laterosporus*) and FV46 (*B. safensis*), in tomato plants. There are studies that show that PGPB positively impact plant metabolism and photosynthetic processes of both healthy and stressed plants. For instance, Shi *et al.* (2010) showed that inoculation of sugar beet plants with PGPB including *B. pumilus* lead to increases in F_v/F_m ratio, carbon assimilation as well as chlorophyll content, which lead to increased carbon assimilation.

1PB1, 5PB1, C9F and FV46 were chosen based on the biochemical characterization and antagonistic potential presented in this work, and also based on screening tests (data not shown in this study). This work was done using S.

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lycopersicum var. MicroTom plants. An *in vitro* assay was performed to test the influence of PGPB on shoot and root size and plants' biomass. This study showed that the isolates do not seem to have influenced these parameters. Additionally, an *in planta* assay was done using plants grown under greenhouse conditions for 4 months, and parameters regarding carbon metabolism, photosynthesis and non-enzymatic plant defenses were used as a measure of plant health. Results showed that FV46 improved antioxidant plant defenses as well as pigment contents, 5PB1 improved photosynthesis and starch content, 1PB1 reduced the O_2^- levels and increased TSS and starch contents. C9F also reduced the O_2^- levels and increased TSS, but it impaired Φ PSII and decreased WUE.

5PB1 and FV46 had more beneficial effects than 1PB1 and C9F. These results suggest that 5PB1 and FV46 might have a broader spectrum of action than 1PB1 and C9F, since none of the isolates came from the rhizosphere of tomato plants. In this sense, 1PB1 and C9F seem to exhibit some sort of host preference, but additional studies would be needed to take further conclusions.

Overall, this study allowed the detection of PGPB strains that are able to produce IAA, improve plant nutrition by solubilizing nutrients, inhibit the growth of relevant plant pathogens, and improve the physiological status of tomato plants. These results suggest that the iB₂/UTAD collection isolates have the potential to be explored as more sustainable alternatives to chemical fertilizers and pesticides, and eventually be used in a biocontrol or biofertilizer formulation.

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