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Strategies to design a new generation of biofertilisers for a more sustainable agriculture

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Strategies to design a new generation of biofertilisers for a more sustainable agriculture

by

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Abstract of the dissertation

Plant biostimulants (PBs) are an attractive and environmental friendly strategy to mitigate the continuous application of chemical fertilisers which disrupt the environment by degrading soil fertility and contaminating ground water. The research described in this PhD thesis explored strategies to select, characterise and design a new generation of PBs formulations. Numerous parameters are involved in the multistep process of formulating inoculants and add up to an infinite amount of possible approaches. This requires an elaborate and high-throughput screening to narrow down all the possible criteria. Several authors suggest that this is best achieved by using a stepwise screening approach. However, the scientific literature lack of a suitable screening strategy. We addressed this facet by designing a stepwise screening procedure to select the best microbial candidates as promising new active ingredients of PBs products. Furthermore, this screening was validated by using a case study: plant growth-promoting rhizobacteria (PGPR) combined with humic acids (HA) to be applied on tomato plants. This validation led us to select two PGPR, *Pantoea agglomerans* MVC 21 and *Pseudomonas (Ps.) putida* MVC 17 as potential candidates for PBs formulations.

Recently, co-inoculation of two or multiple PGPR is used to achieve prominent multifactorial effects on crop productivity. To develop a PGPR

consortium, we evaluated how *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 interact together and the effect of this interaction on tomato seedlings. Compatibility studies revealed that both strains may be combined in a biostimulant product. Experiments testing the effect of the PGPR interaction indicated that both PGPR interact together and with tomato seedlings mainly by volatile organic compounds (VOCs) produced by *P. agglomerans* MVC 21. Metabolomic studies pinpointed VOCs belonging to the family of alcohols, ketones and sulfide as the main VOCs released by *P. agglomerans* MVC 21. Moreover, the identification of the main VOC responsible of the effects of *P. agglomerans* MVC 21 VOCs deciphered new ecological roles of VOCs as chemical signals able to modulate behaviour of PGPR as well as the interaction between plants and PGPR.

Another promising area of PBs formulations is the use of HA combined with PGPR which have shown to better benefit plant growth. Our study, conducted under *in vitro* conditions, showed that HA synergistically modulate plant growth-promoting activities of *P. agglomerans* MVC 21. Whole genome sequencing analysis of *P. agglomerans* MVC 21 will be subject of future studies. Moreover, transcriptomic analysis will be carried out to better understand the effect of HA on *P. agglomerans* MVC 21 genes related to plant growth-promoting activities.

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Chapter 1

General introduction and thesis outline

General introduction

According to the United Nations, the global population will reach more than 9.47 billion people by 2050 (Rouphael and Colla, 2018). Feeding the growing global population adopting more effective and sustainable methods are some of the most important challenges that agriculture will face in the 21st century (Duca et al., 2014). In order to address those challenges, growers may find strategies to increase crop yield and productivity. Indeed, farmers have become dependent on chemical sources of fertilizers to provide essential nutrients for plant growth. However, these practices have negative impact on the environment (Glick, 2012). A promising and eco-friendly approach to chemical fertilizers would be the use of plant biostimulants (PBs). PBs were recently defined in the Regulations of the European Parliament and Council (Regulation EU 2019/1009) as "EU fertilising product (s) able to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (1) nutrient use efficiency, (2) tolerance to abiotic stress, (3) quality traits, or (4) availability of confined nutrients in the soil or rhizosphere". Within this concept, eight categories are included i) humic and

fulvic acids, ii) protein hydrolysates and other N-containing compounds, iii) seaweed extracts and botanicals, iv) chitosan and other biopolymers, v) inorganic compounds, vi) beneficial fungi and vii) beneficial bacteria (du Jardin 2015). The Marketsandmarkets.com (2017) database reveals that Europe is the largest PBs market representing 34% of the worldwide market share, followed by North-American and Asian-Pacific biostimulants representing 23 and 22% of the global market respectively (Rouphael and Colla, 2018). Particularly, among all categories of PBs, beneficial bacteria and humic substances (HS) have been already combined as plant growth promoter showing positive effects on crop yields (de Azevedo et al., 2019; Olivares et al., 2015, 2017).

1. Beneficial bacteria and humic substances as plant biostimulants

1.1. Beneficial bacteria

The rhizosphere concept was first coined by Hiltner to describe the narrow zone of soil surrounding the roots where microbes' populations are stimulated by root activities. This region is much richer in microorganisms than the surrounding bulk soil (Hiltner at al., 1904)**.** In this environment, rhizosphere microbes can benefit from the metabolites produced by plants which can be used as nutrients. This is due to the fact that high amount of carbon fixed by the plant, between 5 and 21%, is released as root exudate (Lugtenberg and Kamilova, 2009). Some

of these organic compounds include carbon-based compounds, such as amino acids, organic acids, sugars, secondary metabolites and high molecular weight compounds such as mucilage and proteins (Brescia et al., 2020). In this environment, rhizosphere microorganisms can be classified according to their effect on plants some being pathogens whereas others trigger neutral or beneficial effects (Kloepper et al., 1980). Particularly, the group bacteria inhabiting the roots and beneficial to plants are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1989). PGPR have the potential to contribute in the development of sustainable agricultural systems as they offer benefits to plants providing nutritional inputs and protecting them from pathogens. A wide range of bacterial groups are considered PGPR such as *Acinetobacter, Agrobacterium, Arthobacter, Azotobacter, Azospirillum, Burkholderia, Bradyrhizobium, Rhizobium, Serratia, Thiobacillus, Pseudomonads, and Bacillus* (Glick, 1995; Vessey, 2003). Moreover, PGPR are characterized by the following characteristics: i) they must colonise the root surface, ii) they must survive, multiply and compete with other microbiota at least for the time needed to express their plant growth promotion/protection activities, and iii) they must promote plant growth (Kloepper et al., 1994).

1.1.1. Classification of plant growth-promoting rhizobacteria

Somers et al. (2004) classified PGPR based on their activities as i) biofertilizers

(increasing the availability of nutrients to plant, ii) phytostimulators (producers of phytohormones), iii) rhizoremediators (degraders of soil pollutants), and iv) biopesticides (producers of antibiotics or antifungal metabolites).

On the other hand, PGPR can also be classified based on bacterial proximity to the root and intimacy of association. In this sense, PGPR can be grouped as i) extracellular (ePGPR) which live in the rhizosphere, on the rhizoplane, or in the spaces between cell of the root cortex and ii) intracellular (iPGPR) which exist inside root cells (Figueiredo et al., 2010).

1.1.2. Mode of action of plant growth-promoting rhizobacteria

Generally, PGPR promote plant growth directly by synthesizing phytohormones and/or increasing the accessibility and/or concentration of nutrients and indirectly by preventing plant diseases (Glick 1995) (Figure 1).

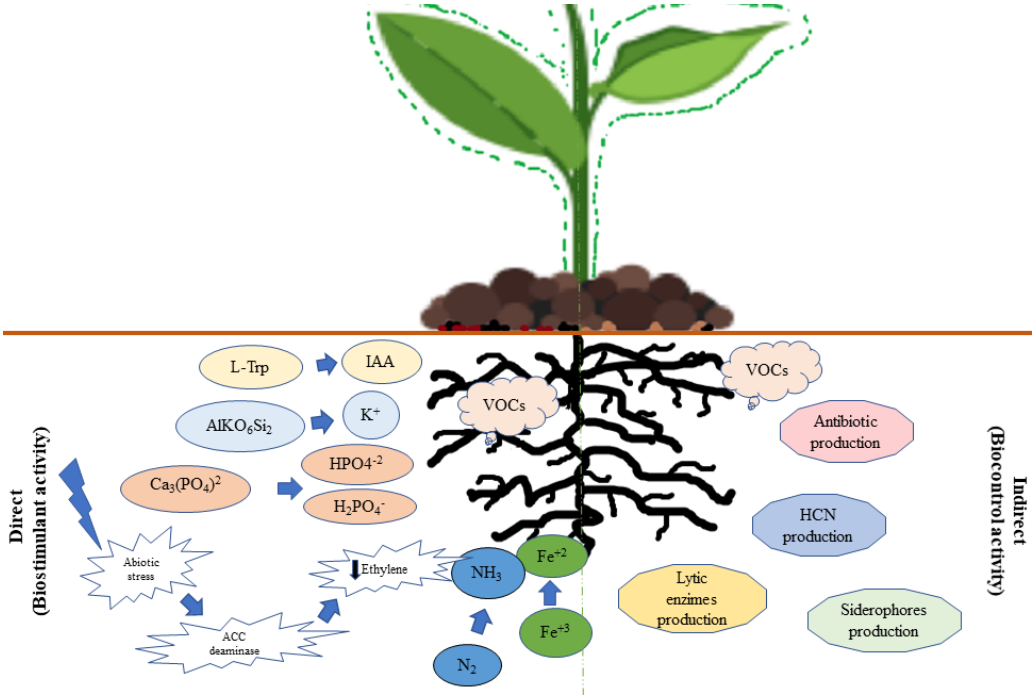

Figure 1: Schematic overview of the modes of action of PGPR. Adapted from Verma et al. (2019). L-Trp: L-Tryptophan; IAA: indole-3-acetic acid; VOCs: volatiles organic compounds; HCN: hydrogen cyanide.

1.1.2.1. Direct mechanisms

1.1.2.1.1. Phytohormone production

1.1.2.1.1.1. Indole-3-acetic acid

Some chemicals which are produced within the plant tissues can affect growth and development of plants. These compounds are known as plant growth regulators or phytohormones (Daman et al., 2016; Gouda et al., 2018). There are several classes of plant growth regulators such as auxin, cytokinin, gibberellin,

ethylene and abscisic acid (Sureshbabu et al., 2016). Particularly, the phytohormone auxin can be commonly produced by PGPR. It is recognised that more than 80 % of PGPR can synthesize indole-3-acetic acid (IAA) (Patten and Glick 1996) which is considered to be the most important native auxin (Spaepen et al., 2007). IAA functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses and cellular responses such as cell expansion, division, differentiation and cell regulation (Ryu and Patten, 2008). The scientific literature reveals that bacteria use IAA to interact with plants as a part of their colonisation strategy. Moreover, several authors indicate that IAA can be used as a signal molecule in bacteria leading to an important effect on bacterial physiology (Spaepen et al., 2007). The main precursor of IAA biosynthesis pathways is L-tryptophan which can be commonly found in root exudates (Etesami et al., 2009). Starting from tryptophan at least five different pathways have been described for the synthesis of IAA (for a detailed review see Spaepen et al., 2007; Spaepen and Vanderleyden, 2011) i) Indole-3-acetamide (IAM) pathway, ii) Indole-3-pyruvate (IPA) pathway, iii) tryptamine pathway, iv) Indole-3-acetonitrile (IAN) pathway, v) Tryptophan side chain oxidase (TSO) (Figure 2). The IAM pathway is present in several pathogens and can be used to increase the virulence of pathogens through the production of high amounts of IAA. This pathway is the best characterized pathway in bacteria and has been described in *Agrobacterium*

tumefaciens, *Pseudomonas savastanoi*, *P. syringae*, *Pantoea agglomerans*, but also in symbiotic nitrogen fixing bacteria belonging to *Rhizobium* and *Bradyrhizobium* species (Sekine et al., 1989; Morris, 1995; Theunis et al., 2004). In the IAM pathway, tryptophan is converted to IAM by the enzyme tryptophan-2-monooxygenase and then IAM is converted to IAA by an IAM hydrolase. The IPA pathway is the major auxin pathway in plants. This pathway is also present in some bacteria such as phytopathogens (*P. agglomerans*), plant beneficial bacteria (*Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Enterobacter cloacae*, *Paenibacillus*, *Pseudomonas* and *Rhizobium*), and even cyanobacteria (Baca et al., 1994; Brandl and Lindow, 1996; Costacurta et al., 1998; Koga et al., 1991; Patten and Glick, 2002; Ruckdäschel et al., 1988). In this pathway tryptophan is converted to IPA by an aminotransferase. IPA is then decarboxylated to indole-3-acetaldehide (IAAd) by indole-3-pyruvate decarboxylase. In the last step, IAAd is oxidized to IAA. In the tryptamine pathway, tryptophan is decarboxylated to tryptamine by a tryptophan decarboxylase and then tryptamine is converted to IAAd by an amine oxidase. This pathway was identified in *Bacillus cereus* and *Azospirillum* (Hartmann et al., 1983; Perley and Stowe, 1966). The IAN pathway is present in several *Agrobacterium* and *Rhizobium* spp. (Kobayashi et al., 1995). In this pathway nitrilases with affinity for IAN have been characterized. Via IAM, IAN is converted to IAA. Finally, the TSO pathway has been characterised in *P.*

fluorescens CHA0. In this pathway, tryptophan is converted to IAAld. In the last step, IAAld is oxidized to IAA (Oberhänsli et al. 1991).

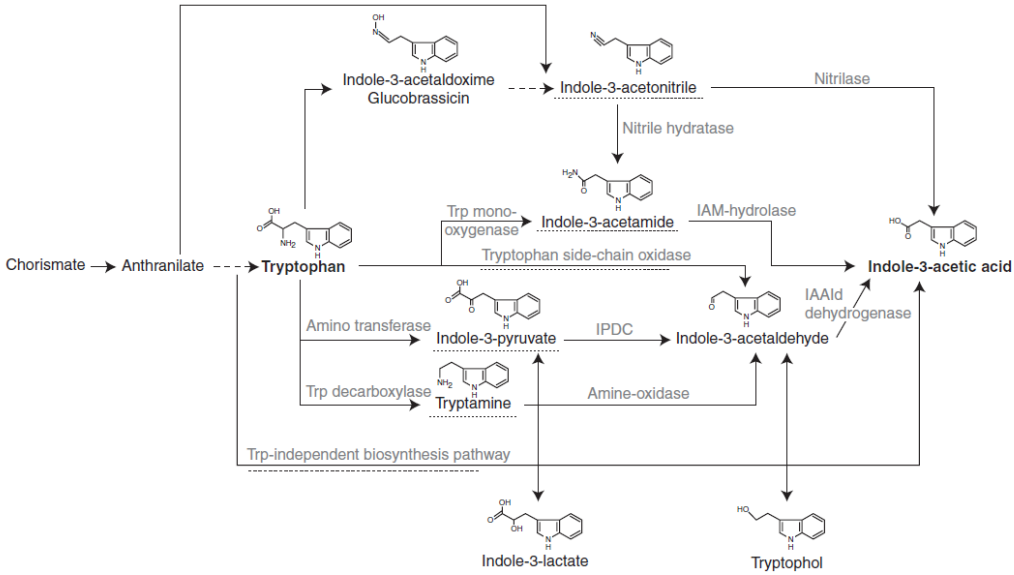

Figure 2: Summary of IAA biosynthesis pathways in bacteria. Dashes lines refer to the name of the pathway. IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IPDC, indole-3-pyruvate decarboxylase; Trp, tryptophan. Reproduced from Spaepen and Vanderleylen (2011).

On the other hand, it has been shown that bacteria and plants can produce IAA without tryptophan as precursor (Last et al., 1991; Normanly et al., 1993). In plants, the starting point of this pathway is indole-3-glycerol phosphate or indole. This pathway was suggested to occur in *A. brasilense* (Prinsen, 1993). However, no specific enzymes for plants and bacteria in this pathway have been characterized (Li et al., 2018; Spaepen and Vanderleyden, 2011).

1.1.2.1.1.2. 1-aminocyclopropane-1-carboxylate deaminase

The main mechanism employed by PGPR to reduce the stress leads to a decrease in the level of ethylene via hydrolysing 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC deaminase (Gupta and Pandey, 2019). The presence of ACC deaminase is quite common amongst soil microorganisms (Glick et al., 2007). ACC deaminase/activity genes were found in a wide range of bacterial isolates including *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas* and *Enterobacter* (Blaha et al., 2006). Moreover, ACC is the intermediate precursor of the hormone ethylene in plants. Ethylene is an essential metabolite for the normal growth and development of plants. Its production regulates important plant growth functions such as seed germination, seedlings establishment, root initiation, elongation and branching, Rhizobia nodule formation, flower and leaf senescence and leaf and fruit abscission, response to stress (Vacheron et al., 2016). Nevertheless, at high concentration, ethylene induces the defoliation and cellular processes that lead to the inhibition of root and stem growth together with premature senescence (Abeles and Wydosky 1987). High concentration of ethylene produced by its intermediate precursor ACC, had been found in plant growing under stress conditions (Wang et al., 2013; Liu et al., 2015; Abiri et al., 2017). Indeed, stress conditions such as drought, high/low temperature, salinity induce ethylene production. The production of ethylene is regulated by different factors including

temperature, light, nutrition, gravity and other plant hormones (Glick, 2005). A model proposed by Glick et al. (1998) describes how PGPR decrease ethylene levels leading to plant growth (Figure 3). In fact, several authors demonstrated that plants inoculated with PGPR containing ACC deaminase make the plant more resistant to various environmental stresses such as drought, salinity, and flood (Egamberdieva and Lugtenberg, 2014; Shameer and Prasad, 2018). In this model the PGPR binds to the surface of the plants (seeds or roots). In response to tryptophan and other molecules secreted as plant root exudates, PGPR synthesize and secrete IAA, some of which can be taken up by plants. This IAA together with endogenous IAA can stimulate plant cell proliferation and plant cell elongation or induce the transcription of ACC synthase which is the enzyme that catalyses the formation of ACC. ACC can be exuded from roots, seeds and leaves (Grichko and Glick, 2001; Penrose et al., 2001) and may be taken up by PGPR and subsequently cleaved by the enzyme ACC deaminase to ammonia and α-ketobutyrate lowering the ethylene levels in plants. As a direct consequence, PGPR can reduce the ethylene levels in plants which may cause a reduction in the stress levels in plants facilitating plant growth.

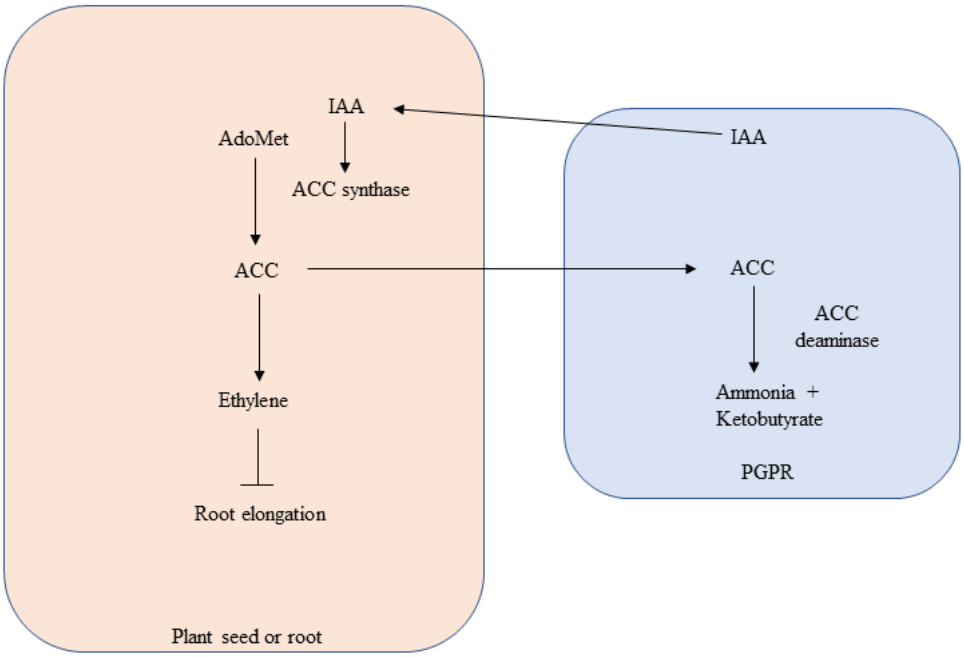

Figure 3: Overview of how PGPR prevents ethylene inhibition of root formation. The symbol ⊥ indicates inhibition. IAA, indole-3-acetic acid; AdoMet, S-adenosyl-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid. Reproduced from Glick et al. (1998).

Genetic studies in *Arabidopsis* and tomato have revealed that ethylene can inhibit lateral root formation (Ivanchenko et al., 2008; Negi et al., 2008). Negi et al., (2008) reported an enhanced in ethylene synthesis and signalling through the *eto 1* (ethylene overproducing) and *ctr-1* mutations (which confers constitutive ethylene signalling), or through the application of ACC, both negatively affect root branching. Ethylene affects root branching at the earliest stages of lateral root initiation (Ivanchenko et al., 2008) and alters auxin

transport, suggesting that cross talk with auxin might regulate ACC repression of root branching (Negi et al.,2008). The effects of the interactions of cross talk ethylene-auxin on elongation of primary root are different from those of lateral root formation, as they act synergistically to reduce primary root elongation, but antagonistically in lateral root formation (Stepanova et al., 2007; Lewis et al., 2011).

1.1.2.1.2. Nutrient uptake

1.1.2.1.2.1. Nitrogen

Nitrogen (N) is the first most important nutrient for plant growth. It is a key element required for cellular synthesis of enzymes, chlorophyll, DNA, RNA, amino acids, and others essential elements for all living organisms (Hayat et al., 2010). In the atmosphere nitrogen appears in large amount (78 %) but unavailable for a direct uptake by plants (Verma et al., 2019). Bacteria can fix dinitrogen (N_2) by a complex enzyme system known as nitrogenase (Postgate 1988; Leigh 2020). These microorganisms change dinitrogen to ammonia (NH_3) by a process called biological nitrogen fixation. There are two types of nitrogen-fixing bacteria: symbiotic (rhizobia and *Frankia*) (Ahemad and Khan 2012) and nonsymbiotic (*Cyanobacteria*, *Azospirillum*, *Azotobacter*, *Gluconobacter*, *Azoarcus*) (Bhattacharyya and Jha 2012; Roper and Gupta 2016).

Among all nitrogen fixers, root nodule-associated rhizobia are the most

studied and exploited (Postgate 1998). It is reported that in agricultural systems *Rhizobium phaseoli* increases growth and yield of *Phaseolus vulgaris* due to high nitrogen fixation (Yadegari et al., 2010). Symbiotic nitrogen fixation is a mutualistic relationship between the plant and a microbe. Firstly, the plant compounds induce the expression of nodulation (nod) genes in rhizobia which results in the production of lipo-chitooligosaccharide signals that trigger mitotic cell division in roots leading to nodule formation. In these nodules, nitrogen is fixed to ammonia and make it available for plants (Ahmed and Kibret 2014; Dakora 1995, 2003; Lhuissier et al., 2001; Matiru and Dakora 2004).

Rhizobium, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium* are the PGPR which belong to the group presented as symbionts with leguminous plants and *Frankia* with non-leguminous trees and shrubs (Zahran 2001).

1.1.2.1.2.2. Phosphorus

Phosphorus (P) is the second most-important plant growth limiting macronutrient after nitrogen (Azziz et al., 2012). On average, the P content in the soil is about 0.5 % (w/w); however only 0.1 % (w/w) of this P is available for plant use (Zhu et al., 2011). It is a component of many soil constituents and it plays a central role in several processes including photosynthesis, respiration, storage and transfer of energy, cell division and cell enlargement. In plants, adequate amount of phosphorus is needed for the promotion of early root

formation and growth (Khan et al., 2010). The low availability of P to plants is because the majority of P is found in insoluble forms whereas plants absorb P only in two soluble forms monobasic ($HPO4^{-2}$) and dibasic ($H_2PO_4^-$) ions (Glass 1989). Microorganisms able to solubilise P, often termed phosphate solubilizing microorganisms (PSM) are effective at releasing P from total soil P through solubilisation and mineralisation (Bhattacharyya and Jha, 2012). PSM increase the bioavailability of soil insoluble P for plant use (Zhu et al., 2011). In bacteria, P is essential to biological information storage and transfer, energy metabolism and membrane integrity (Yao et al., 2016). The major P pool in bacteria are RNA, DNA and lipids (Mitchell and Moyle, 1954). Bacteria genera like *Pseudomonas* spp., *Agrobacterium* spp., and *Bacillus circulans* (Babalola and Glick, 2012) have been reported to mobilise P by solubilisation and mineralisation. Other genera of PSM include *Azotobacter* (Kumar et al., 2014), *Bacillus* (Jahan et al., 2013), *Burkholderia* (Istina et al., 2015; Mamta et al., 2010; Zhao et al., 2014), *Enterobacter*, *Erwinia* (Chakraborty et al., 2009), *Kushneria* (Zhu et al., 2011), *Paenibacillus* (Bidondo et al., 2011), *Ralstonia* , *Rhizobium* (Tajini et al., 2012), *Rhodococcus*, *Serratia*, *Bradyrhizobium*, *Salmonella*, *Sinomonas*, and *Thiobacillus* (Postma et al., 2010). Moreover, PSM can also facilitate the growth and development of plants by releasing essential nutrients (Thomas et al., 2005), modifying the concentration of phytohormones such as IAA (Khan et al., 2009), showing biocontrol activity (Pandey et al.,

2006) through producing siderophores (Vassilev et al., 2006), antibiotics and cyanide (Yang et al., 2008), producing ACC deaminase leading to a modulation of ethylene levels (Anandham et al., 2008), and solubilising or reducing toxic metals (Khan et al., 2009).

There are several mechanisms of inorganic phosphate solubilisation (for detailed review see Alori et al., 2017, Zaidi et al., 2009). The main one is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxylic ions and CO_2 (Rodríguez and Fraga, 1999; Sharma et al., 2013). Among all the organic acids, gluconic acid is the most common agent of phosphate solubilisation (Alori et al., 2017). Organic acids are produced in the periplasmic space (Zhao et al., 2014). Their excretion is followed by a drop in the pH that produce an acidification of the microbial cell and its surroundings and P ions are released by a substitution of H^+ for Ca^{2+} (Goldstein, 1994). An alternative mechanism to organic acid production for phosphate solubilisation is the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase (Rodríguez and Fraga 1999). Moreover, it is known that the assimilation of NH_4^+ within microbial cells is followed by the release of protons which produces the solubilisation of phosphorus without the production of any organic acids (Sharma et al., 2013). Microorganisms can also solubilise phosphate by releasing inorganic acids (such as sulfuric, nitric and carbonic acids) and producing chelating substances (Alori

et al., 2017). In addition, another mechanism reported in the literature is the release of enzymes or enzymolysis in a medium containing lecithin which results in a decrease of the pH leading to the uptake of P (Zhu et al., 2011).

On the other hand, PSM can mineralise organic P. The major source of organic P in the soil is the organic matter. Its values in the soil can be around 30-50% of the total P (Alori et al., 2017). The soil organic P is commonly found in the form of inositol phosphate. Other organic P are phosphomonoesters, phosphodiesters, phospholipids, nucleic acids and phosphotriesters (Rodríguez and Fraga 1999). The mechanism of phosphate mineralisation is based on the removal of P that results in the dissolution of Ca-P compounds (Halvorson et al., 1990). Different kinds of enzymes are involved in the organic phosphate mineralisation such as phosphatases (Nannipieri et al., 2011) and phytases (Richardson and Simpson, 2011).

1.1.2.1.2.3. Potassium

Potassium (K) is considered the third most important macronutrient (Sedaghathoor et al., 2009). It plays a pivotal role in several physiological and metabolic processes in the plant (Zhao et al., 2001) including photosynthesis (Wang et al., 2012), plant growth, metabolism, rate of assimilation and accumulation of sugars (Sindhu et al., 2014). K improves crop quality since it is involved in the metabolism of carbohydrates, organic acids, fats, nitrogenous

compounds, protein synthesis, photosynthesis, improving resistance to drought, cold tolerance, and water use efficiency (Sindhu et al., 2014). Additionally, K mediates resistance against biotic stresses (Amtmann et al., 2008; Armengaud et al., 2010). K represents 2.6 % of the weight of the Earth's crust (Sardans and Peñuelas, 2015). Despite being an abundant element in the soil, only 0.1-0.2 % of the K is available to plants (Britzke et al., 2012; Wang et al., 2010). Several groups of PGPR such as *Bacillus*, *Acidothiobacillus*, *Paenibacillus*, *Pseudomonas*, *Burkholderia*, *Agrobacterium* and *Rhizobium* have been reported to solubilise K. Microbes solubilise K by direct, indirect, and other mechanisms (Sattar et al., 2018). In the direct mechanism, microbes solubilise K through i) organic acid production (Han et al., 2006) which is the main mechanisms of K solubilisation, ii) acidolysis of the rhizosphere (Gerke, 1992) and iii) carbonic acid based chemical weathering (Gadd, 2007; Park et al., 2009). Soil microorganisms excrete organic acids such as oxalic, tartaric and citric acid (Song and Huang, 1988) and H^+ ions which lowers the pH of the surrounding soil (Bennett, 1998) which lead to an increase in the releasing capacity of certain cations such as Fe, K and Mg. Moreover, microbial respiration, degradation of particulates and dissolved organic acid can elevate the concentration of organic acid at mineral surfaces which react with minerals increasing the rates of mineral weathering (Basak et al., 2017). Tartaric acid is the most common used agent for solubilisation of K minerals (Keshavarz Zarjani et al., 2013). Other organic acids

involved in the solubilisation of K-minerals are acetic, gluconic, lactic, propionic, malonic and fumaric acids (Wu et al., 2005). In the third mechanism, microbes accelerate the dissolution rate by creating a gradient between anion and cation concentrations in the solution (Welch et al., 2002).

On the other hand, microbes solubilise K by indirect methods of solubilisation such as i) chelation of cations bound to K silicate (Bennett et al., 2001; Song and Huang, 1988), ii) exchange reactions by forming metal-organic complexes with Al and Si ions associated to K-minerals and, as result, K ions is released in solutions (Gerke, 1992), iii) direct attachment of potassium solubilising microorganisms on mineral surfaces (Uroz et al., 2009), iv) metal complexing ligands (Basak et al., 2017) and v) release of phytohormones through microbes (Sattar et al., 2018).

Other mechanisms employed by microbes to solubilise K minerals is through releasing exopolysaccharides (EPS) and forming biofilm. Microbes able to produce EPS can excrete high amounts of organic acids on or around the mineral lowing the pH enhancing the solubilization of K (Liu et al., 2012). On the other hand, biofilm formation is the least studied mechanism of K reserve mobilisation. Some microbes form a biofilm on the rhizospheric mineral surfaces and release organic acids, metabolites which lowers the pH helping K mineral solubilization and uptake by plants (Balogh-Brunstad et al., 2008).

1.1.2.1.2.4. Iron

Iron (Fe) is an element relatively abundant in many cultivated soils. It is required for all living organisms in many cellular processes such as respiration, photosynthesis and nitrogen fixation (Litwin and Calderwood, 1993). Iron solubility and reactivity strongly depend on the pH. In the aerobic environment (at physiological pH), iron occurs mainly as Fe^{3+} and form insoluble hydroxides and oxyhydroxides and thus making it inaccessible for both plants and microorganisms (Albelda-Berenguer et al., 2019). PGPR able to release siderophores promote plant heath by improving iron nutrition, inhibiting the growth of other microorganism and hiding the growth of pathogens (Shen et al., 2013). Indeed, several authors reported the stimulation of plant growth through inoculation of PGPR able to release siderophores and thus increasing iron uptake by plants (Barzanti et al., 2007; Burd et al., 2000; Carrillo-Castañeda et al., 2002). In general, bacteria acquire iron by the secretion of low molecular mass chelators called siderophores, which are low molecular weight iron scavengers with strong affinity to complex iron (Ahmed and Holmström, 2014). Microorganisms produce a wide range of siderophores. Most of the bacterial siderophores are catecholates (i.e. enterobactin), carboxylates (i.e. rhizobactin) and hydroxamates (i.e. ferrioxamine B). Additionally, microorganisms can contain a mix of the main groups (pyoverdine) (Ahmed and Holmström, 2014).

Some PGPR release siderophores chelating iron, thus rendering it soluble (from Fe^{3+} to Fe^{2+}). Then iron diffuses toward the plant where it is reduced and absorbed through an enzymatic system present in the cell membrane (Neilands, 1995; Rajkumar et al., 2010). Bacteria belong to *Pseudomonas*, *Azotobacte*r, *Bacillus*, *Enterobacter*, *Serratia*, *Azospirillum* and *Rhizobium* are reported in the literature to release siderophores (Glick et al., 1999, Loper et al., 1999).

1.1.2.1.2.3. Volatile organic compounds

Bacterial volatilome encompasses many different type of molecules which can be divided into two main categories: organic and inorganic (Schulz and Dickschat, 2007). Volatile organic compounds (VOCs) are composed of terpenes, alkanes, nitrogen-containing compounds, ketones, pyrazines, sulfur-containing compounds, and acids (Kanchiswamy et al., 2015; Lemfack et al., 2014; Piechulla et al., 2017; Schmidt et al., 2015; Schulz and Dickschat, 2007). They are considered as either primary or secondary metabolism (Lemfack et al., 2020). Inorganic volatile compounds are represented by nitric oxide (NO), hydrogen sulfide (H_2S), hydrogen cyanide (HCN), ammonia (NH_3), and carbon dioxide (CO_2) (Bruisson et al., 2020). Microbial volatiles compounds are small molecules (<300Da) belonging to different chemical classes that can evaporate and diffuse easily through air-and water-filled pores. These physiochemical properties make volatiles ideal candidates for cooperation and competition

between soil microorganisms that do not live directly adjacent to each other (Schmidt et al., 2015). To date more than over 1000 microbial volatiles are described from a wide range of bacteria and fungi in a database called mVOC (Lemfack et al., 2018; 2014). Several studies have reported the positive effects of bioactive VOCs on plant growth. The first report was performed by (Ryu et al., 2003) who showed that volatiles emitted by *Bacillus subtilis* GB03 produced a ~5-fold increase of total leaf area of *Arabidopsis thaliana* after 10 days of exposition. In general, studies have shown that bacterial volatiles compounds (BVCs) produce drastic growth changes in their host plants (Blom et al., 2011; Ryu et al., 2003; Vespermann et al., 2007) by providing essential nutrients, changing the hormonal balance, sugar sensing or triggering induced systemic resistance. BVCs can modulate plant photosynthesis by affecting photosynthesis and carbohydrate accumulation. Iron is necessary for chlorophyll biosynthesis, electron transport chain activity and photosystem activity in plants (Briat et al., 2007). BVCs increase proton release, acidifying the rhizosphere and thus facilitating iron uptake (Zang et al., 2009). Moreover, BVCs can alleviate the negative feedback of sugar accumulation on photosynthesis (Zhang et al., 2008b). HEXOSE SENSOR KINASE 1 (HXK1) in *Arabidopsis* sense hexose sugar concentration after its accumulation during photosynthesis. HXK1 negatively regulates photosynthesis reactions by sensing high concentrations of sugar (Cho et al., 2010). Gene expression studies showed that BVCs emitted by

B. subtilis GB03 repress HXK1 signaling in *Arabidopsis*.

On the other hand, BVCs can facilitate the uptake of macro-microelements such as iron, copper, selenium, and sulfur. *Arthrobacter agilis* UMCV2 volatiles improve iron acquisition in both monocot and dicot plants (Castulo-Rubio et al., 2015). Treatment with *Bacillus subtilis* GB03 increased iron uptake up to 2-fold in *Arabidopsis* (Wang et al., 2017; Zhang et al., 2009). *B. amyloliquefaciens* BF06 volatiles increase selenium uptake by inducing the expression of sulfate transporter genes (Wang et al., 2017). Moreover, BVCs can be consumed as a source of nutrients. For instance, dimethyl disulfide, as a source of sulfur, increases *Arabidopsis* growth in sulfur deficient medium (Meldau et al., 2013). BVCs indirectly improve plant growth by alleviating biotic and abiotic stress. In this sense, dimethyl disulfide and 2-methylpentanoate are highly toxic to plant pathogens (Groenhagen et al.; 2013) and some such as acetoin, 2,3- butanediol and tridecane induce plant systemic resistance against pathogens (Lee et al., 2012). Moreover, some bacterial volatiles can also induce systemic tolerance to soil salinization and drought stress (Liu and Zhang 2015).

As regulators of plant hormones, some BVCs modulate the biosynthesis, perception and homeostasis of plant hormones such as ethylene, auxin, cytokinin, abscisic acid and gibberellin. For instance, plants treated with *B. subtilis* GB03 volatiles can enhance root proliferation by increasing lateral root formation through the auxin-dependent pathway (Zhang et al., 2007). Moreover,

the volatile indole also regulates auxin signaling in *Arabidopsis* (Bailly et al., 2014).

Over the past years, diverse microbial VOCs have been identified using gas chromatography coupled to mass spectrometry techniques (Korpi et al., 2009). The most reported way to assess the effects of bacterial VOCs is through split Petri dishes. This mechanism is based on a barrier which separates the dish into two compartments to guarantee the physical separation between bacteria-bacteria or bacteria-plants but sharing the same atmosphere. To prevent the escape of volatiles, dishes are sealed with double layer of semi-permeable Parafilm tape (Farag et al., 2017; Kai et al., 2016).

The main bioactive VOCs identified as growth inducers belong to alcohols, ketones, sulfur compounds, furans and terpenes which act at low concentrations (Fincheira and Quiroz, 2018). Particularly, 1-hexanol, 2-phenylethanol, 2,3-butanediol, acetoin, dimethyl disulfide, and indole are reported as the most frequent tested bacterial VOCs with an effect on plant growth (Piechulla et al., 2020). Without any physical contact, these volatiles are able to alter root system development, plant physiology, hormonal pathways, and biomass production (Bailly et al., 2014; Delaplace et al., 2015; Piechulla et al., 2017; Ryu et al., 2004).

On the other hand, it becomes clear that microbial VOCs play important roles in communication and competition between soil microorganisms. Indeed,

bacterial VOCs can have direct antagonistic effect against other bacteria. For instance, the volatile dimethyl disulfide emitted by *Pseudomonas (Ps). fluorescens* and *Serratia plymuthica* revealed bacteriostatic effects against two plant pathogens *Agrobacterium tumefaciens* and *A. vitis*. (Dandurishvili et al., 2011). In contrast, bacterial VOCs can also have positive effects on the growth of neighboring bacteria in the rhizosphere. For instance, VOCs emitted by *Collimonas pratensis* and *S. plymuthica* showed an increase in the growth of *Ps. fluorescens* Pf0-1 (Garbeva et al., 2014b). In addition to these effects, bacterial VOCs such as ammonia, trimethylamine, hydrogen sulfide, nitric oxide and 2-amino-acetophenone can alter biofilm formation and motility of bacteria (Audrain et al., 2015; Raza et al., 2016).

1.1.2.2. Indirect mechanisms

Indirect mechanisms involve all the process by which PGPR prevent or neutralize the deleterious effects of pathogens on plants by producing substances that increase the natural resistance of the host (Singh and Jha, 2015). In this mechanism, PGPR produce hydrolytic enzymes (chitinases, cellulases, proteases etc.) and antibiotics, induce systemic resistance against pathogens and pests, produce siderophores, VOCs and/or EPS (Gupta et al., 2015).

1.2. Humic substances

HS are natural components of the soil organic matter produced by decomposition of plant, animals, and microbial residues as well as from metabolic activities of soil microbes (du Jardin, 2014). The origin of HS can be diverse. They are extracted from organic matter (e.g. from peat or volcanic soils), from composts and vermicomposts, or from mineral deposits (leonardite) (du Jardin, 2012).

HS have been widely recognised as principal contributors to soil fertility due to the beneficial effects of HS on the physical, chemical, and biological properties of the soil (Cannellas and Olivares 2014). Indeed, HS can act by maintaining plant growth and terrestrial live in general, regulating soil carbon and nitrogen cycling, the growth of plants and microbes and the general stabilisation of the soil structure (Piccolo 1996). According to Hayes et al. (2006) HS can be divided into three categories based on their solubility in acid/alkaline solutions: humic acids (HA), humins and fulvic acids (FA).

The promotion of plant growth by HS is well documented in the literature (Piccolo et al., 1992; Nardi et al., 2000; Nardi et al., 2007; Canellas and Olivares, 2014). Generally, plants responses differ depending on the origin of HS, concentration, method of application and stage of plant development (Jindo et al., 2020). HS can be used directly on plants at low concentration (Canellas and Olivares 2014). The biostimulant effects of HS refer to i) root growth promotion,

ii) changes in primary and secondary metabolism and stress alleviation and iii) interaction with soil microbes (for a detailed review see Canellas and Olivares 2014; Canellas et al., 2015).

1.2.1. Mode of action of humic substances

1.2.1.1. Root growth promotion

The most reported biological effect of HS on plants is lateral root induction (Canellas and Olivares, 2014). Upon HS treatments, plans showed an increased in the emergence of lateral roots and an hyperinduction of sites of lateral root emergence (Canellas et al., 2020; Zandonadi et al., 2007). Canellas et al. (2002) provided evidences of small bioactive molecules such as auxin entrapped into the HS supramolecular which could be linked to the induction of lateral root emergences and H^+ATPases reported in his work. In addition, activation of plasma membrane H^+ATPases also promote plant nutrition by increasing the electrochemical proton gradient that regulates ion transport mechanisms (Morsomme and Boutry, 2000). Moreover, HS revealed nutrient accumulation such as N, K, Ca and Mg in leaves of pineapples (Baldotto et al., 2009). Additionally, HS can induce accumulation of nitric oxide on roots which results in an increase in the number of secondary roots, root thickness and root fresh weight (Mora et al., 2012).

On the other hand, Puglisi et al. (2008) showed that plants treated with HS

change of the exudation profile of organic acids in maize roots. Several factors can be involved in this increase including theresponse to apoplast acidification following an enhanced activity of plasma membrane H+-ATPase.

1.2.1.2. Changes on primary, secondary metabolism and stress alleviation

Glycolysis is the preferential way of energy for plant respiration (Jindo et al., 2020). It has been reported that HS may affect the enzymes activities related to glycolysis and tricarboxylic acid cycle (Nardi et al., 2007). Moreover, it was found an increase in sucrose (the starting point for the respiratory pathway) leading to an improvement of the activity of the enzyme rubisco in plants treatment with leonardite HS (Ertani et al., 2011). Additionally, it has been well documented the enhancement of N uptake/assimilation and N metabolism in plants treated with HS. For instance, (Nardi et al., 2000) reported that HS increased N uptake by decreasing the pH at root surface facilitating the H^+/NO^{3-} symport. Besides NO^{3-} uptake, HS can change N cell metabolism. In particular, Ertani et al. (2011) showed an increase in 65% in glutamine synthase root activities and 176 % in glutamate synthase in root and leaves which are key enzymes associated with N assimilation.

On the other hand, HS can affect the secondary plant metabolism. Particularly, HS treatment enhanced the expression of phenylalanine ammonia-

lyase involved in the biosynthesis of phenolics (Schiavon et al., 2010).

Other potential application of HS in agriculture resulted from its effect on drought stress alleviation. Under drought stress, plants revealed the capacity to regulate osmotic potential by maintaining water absorption and cell turgor in response to HS treatment (Azevedo and Lea, 2011). In this environment, plants generate reactive oxygen species (ROS) which can result in an induction of enzyme inhibition, chlorophyll degradation, damage of DNA and lipid peroxidation (Apel and Hirt, 2004). Plants use different ways to detoxify ROS. These mechanisms are described as enzymatic and non-enzymatic antioxidant defence system (Kellos et al., 2008). In the non-enzymatic system, plants produce compounds such as ascorbate, glutathione, alkaloids, phenols, tocopherols and carotenoids (Gratão et al., 2005). Particularly, alkaloids, phenols, tocopherols are stimulated by HA (Schiavon et al., 2010). Moreover, the enzymatic mechanism activated after HS treatment increase the peroxidase activity (a plant scavenging involved in regulating oxidative stress) (Pizzeghello et al., 2001).

High amount of heavy metals in soils represent an abiotic stress in plants which can also be influenced by HS (Dumat et al., 2006). FA was shown to alleviate Pb phytotoxicity by complexing highly toxic free Pb^{2+} in solution and thus reducing Pb uptake (Shahid et al., 2012).

1.2.1.3. Humic substances and soil microorganisms

Association of HS with beneficial microorganisms has been well documented (Naidu et al., 2013; Olivares et al., 2015; Siddiqui et al., 2008). As it is reported above, HS affect lateral root formation and root hair initiation. These changes may favor the mutualistic interactions by increasing the rhizosphere population and chemotaxis, bacterial attachment, and survival on plant surface as well as endophytic colonisation. Due to all these features and since HS are considered recalcitrant to bacterial degradation, HS have potential as enhancers of PGPR inoculation effects (Olivares et al., 2017). A combined effect of HS and bacteria was first demonstrated in surgane plantlets (Marques et al., 2008). The authors revealed an increase in the root growth of surgane seeds after HS treatment isolated from vermicompost. Moreover, plant growth-promoting effects of HS were described by Canellas et al. (2013) under field conditions. It was found that at early stages *Herbaspirillum seropedicae* treated with HS from vermicompost, activated plant metabolic processes such as enhancement of plasma membrane H+-ATPase activity, alteration of both sugar and N metabolism and increase of net photosynthesis.

2. Roadmap to commercialisation of plant growth-promoting rhizobacteria

The success and commercialisation of PGPR depend on the linkage between the scientific organisation and industries (Gupta et al., 2015). The different stages that take place during the development of microbial products for horticulture and agriculture have been described in many publications (Backer et al., 2018; Basu et al., 2021; Lobo et al., 2019; Kamilova et al., 2015; Parnell et al., 2016; Tabassum et al., 2017). In general, this sequence includes isolation and screening of potential strains in laboratory, greenhouse test and /or field trials, evaluation of strain safety, development of production protocols and suitable formulations and finally marketing and registration.

2.1. Isolation, characterization, and efficacy of plant growth-promoting rhizobacteria

The isolation and screening of PGPR mainly occur in the laboratory. The screening of region-specific local PGPR strains is needed because no microbial inoculant can be universal for all ecosystem (Adesemoye et al., 2009). Moreover, the PGPR strain should not be selective or highly targeted (to specific crops) in nature, and it should exhibit a broad host range (Basu et al., 2021).

2.1.1. Preference for plant growth-promoting rhizobacteria formulation

Commercial PGPR formulations prefer Gram-positive bacteria such as *Bacillus* and *Paenibacillus* sp. since they confer higher population stability during formulation and storage of inoculant products (Kokalis-Burelle et al., 2005). However, several Gram-negative bacteria such as *Azospirillum* and *Azotobacter* are difficult to formulate because they do not produce spores. Thus, formulation based on Gram-negative PGPR has a short shelf life, and bacteria are easily killed after desiccation of the formulation (Pérez-Garcia et al., 2011; Kamilova et al., 2015).

2.1.2. Screening for plant growth-promoting activities

Candidates are screened for different plant growth-promoting activities such as phosphate solubilisation, hormone production, nitrogen fixation, etc. After screening, the preference is given to strains with multiple properties. Commercial companies would therefore prefer microbial consortia. In this case, the compatibility of strains must also be analysed (Tabassum et al., 2017). However, this approach has some limitations. Some of the plant growth-promoting activities are inducible which means that they are expressed in certain conditions but not in others (Barriuso et al., 2008).

2.1.3. Evaluation in controlled environment and field conditions

Screening under growth chambers and/or greenhouses are considered robust methods to evaluate the agronomical validity of PBs (Vernieri et al., 2006). Plants are grown directly on soil, pots, or liquid media (hydroponic solution) and treated with prototypes (foliar and/or root application). This approach leads to define the best application methods, timing, and rates (Povero et al., 2016).

Once the agronomical performance of a prototype is determined, it is crucial to validate them by a number of trials under field conditions. Thus, prototypes should be tested in different climatic conditions and according to local agronomic practices (Povero et al., 2016).

2.2. Safety aspects

Risk-Groups and Biosafety-Levels (BSL) are used to classify and safely guide the use of microorganisms from the various risk categories (Keswani et al., 2019). The Word Health Organisation (WHO, 2015) has classified infectious agents (including microorganisms) in four different risk group with respect to their pathogenicity to adult human health, mode of transmission and the availability of preventing measures and treatments. Only strains from BSL-1 are considered as safe and can be considered for product development. Microorganisms belonging to BSL-2 are considered to have a moderate individual risk and low community risk. They have the potential to cause

infection in healthy human adults. However, treatment and vaccines are available to control the pathogen with a full recovery of the host. Indigenous and exogenous microorganisms are included in the BSL-3 which cause high individual risk and low community risk. Finally, BSL-4 include microorganisms with high individual and community risk. It includes organisms which cause fatal infection for which no treatments and vaccines are available.

Secretions of nutrients and root exudates convert the rhizosphere region into one of the reservoirs of opportunistic pathogens (Berg et al., 2015). Several opportunistic pathogens belonging to the genera *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus* and *Stenotrophomonas*, etc. have been reported as part of the plant rhizosphere microbiome with substantial plant-growth promotion and biocontrol activities (Halverson et al., 1993; Binks et al., 1995; Holmes et al., 1998; Tan et al., 1999; LiPuma et al., 2002; Chiarini et al., 2006; Mahenthiralingam et al., 2008; Selvakumar et al., 2014). Nowadays, strain identification by molecular biological methods, mainly at the DNA level, provides the possibility to classify strains in risk groups and identify opportunistic pathogens (Kamilova and De Bruyne 2013). Furthermore, it is highly recommended the analysis of the whole genome sequencing of candidates as an efficient, cheap and fast way to get maximum of phylogenomic information about novel isolates including their taxonomic relationship. This information

will allow a suitable selection of microbes as next generation of bacterial inoculants (Keswani et al., 2019).

Regarding the environmental safety issue associated to PGPR, they must have non-targeted effect on the native microbial community such as the replacement of the native non-target species through the release of toxins/allergens (Gullino et al., 2015).

2.3. Large-scale production

The goals of industrial or mass production are to minimize the fermentation cost and to produce the highest quantities of the microbe in the best physiological and metabolic state (Kamilova et al., 2015).

In general, the choice of an appropriate culture medium for the development of high amount of microbial biomass is an important issue (Liu et al., 2016). Different culture media have been optimized for the growth of PGPR in submerged and solid-stage fermentation processes. Standard culture media such as ammonium mineral salt broth, nitrogen-free broth and nutrient broth are suitable for laboratory assays (Chanratana et al., 2017). However, they are expensive for biomass production at larger scales (Trujillo-Roldán et al., 2013). New culture media have been optimised but the addition of some components such as peptones, yeast extract and tryptone continues to generate high production cost. In this sense, the decrease in nutrient concentration or

incubation time needed to reach the highest PGPR biomass can reduce production cost (Lobo et al., 2019).

2.3. Formulation

The formulation and the application method are probably among the most critical parameters determining the efficiency of PBs products (Le Mire et al., 2016). Formulation of microorganisms presents a very serious challenge specially for Gram-negative bacteria (non-spore formers) (Berg, 2009). Formulations play a role in stabilizing the microorganism, protecting the microbe at the target zone, aiding its delivery, and increasing the efficacy of the microorganisms against the target. Moreover, formulations should not be toxic to plants, must release the bacteria and should be easily dissolvable in water. Its cost effectiveness and compatibility with other agrochemicals have also to be considered (Nakkeeran et al., 2006).

The choice of the type of formulation is determined by i) the biology of the active microorganism, ii) the way of application of the product, iii) the existing irrigation system (Kamilova and De Bruyne 2013). Basically, the two most common types of formulations used are liquids and solids (Burges 1998). Solid formulations commonly include granules, micro granules, wettable powers, water dispersible granules and dust (Abadias et al., 2005; Guijarro et al., 2007, Larena et al., 2003). Liquid formulations are very popular for use in horticulture

and for seed treatments. However, shelf life of most liquid formulations for Gram-negative bacteria and fungi does not exceed 1-3 months.

Formulations may contain various additives such as carriers, nutrients, stickers, protectants, and emulsifiers (Kamilova et al., 2015). The selection of carrier is a key step for the survival and efficiency of PGPR. Carriers protect bacterial from desiccation, leading to an increase in the survival rate and shelf life but also provide a favourable microenvironment for the fast growth of bacteria after their release (Tabassum et al., 2017). The most common used carriers are sterilized peat, conditioned cereal grains, talc, agricultural clays and diatomaceous earth. Nutrients provide a primary boost for the microorganism immediately after application of the product. Stickers allow microbes to attach better to plant surfaces. Protectants defend microbes from desiccation, UV light and temperature changes (Kamilova et al., 2015).

Drying is part of many procedures for development of formulations of microbial inoculants. Freeze drying in particular, is the preferred method for transporting and storing vast culture collections of micro-organism strain types (Morgan et al., 2006). Designing of formulations that allow inoculant survival during drying procedure is a key step in the formulation (Arora et al., 2012).

2.4. Marketing and regulations

Market evaluation is extremely important for making a decision about which

strain would have a better chance to become a successful product (Kamilova and De Bruyne 2013).

Regulatory frameworks and product registration are used worldwide to guide the commercial development of microbial products. Thus, they are needed to ensure their practical, safe, and legitimate application (Yakhin et al., 2017). Today, the regulatory situation of biostimulants is very complex. One of the main reasons is the lack of a formal definition and acceptance of PBs by regulatory bodies so many EU and non-EU countries lack a specific legal framework (Caradonia et al., 2018; Rouphael et al., 2018; Yakhin et al., 2017). Within the EU, these compounds are regulated by national laws that vary from one member to another member. These discrepancies are a barrier to free internal market movement, limit the development of PBs and create doubts for controlling authorities and agencies (Caradonia et al., 2017).

Thesis outline

The development of a new generation of biostimulants in agriculture emerges as a key sustainable technology with the aim of making cropping systems more productive without questioning future negative impacts on the environment. However, unlike chemical fertilisers, this new technology depends on several factors for its success in promoting plant growth. My thesis attempts to address and shed new light on this topic of increasing concern. **Chapter 2** proposes and validates a stepwise screening procedure to select the best plant growth-promoting rhizobacteria (PGPR) candidates, which can be effectively combined with HA, as the main ingredient for novel plant biostimulants (PBs) formulations. The outcome of this chapter allows us to select two candidates, *Pantoea agglomerans* MVC 21 and *Pseudomonas* (*Ps.) putida* MVC 21 able to fulfil all the requirements included in our screening.

Many evidences indicate that microbial volatile organic compounds (VOCs) are responsible for inter- and intra-organismic communication playing a role in interactions among microbial communities and between microbes and their eukaryotic hosts. **Chapter 3** expands this knowledge highlighting how VOCs produced by *P. agglomerans* MVC 21 modulate microbe-microbe and microbe-plant interactions. Our attention was focused on VOCs affecting plant growth-promoting activities of *Ps. putida* MVC 17 and how VOCs modulate the interaction of *Ps. putida* MVC 17 and tomato plant.

Another emerging area in agriculture includes the use of microorganisms combined with active natural compounds such as Humic acids (HA) to exert a synergistic effect on plant growth. To address this aim, **chapter 4** investigates the effect of HA on plant growth-promoting activities of *P. agglomerans* MVC 21.

Finally, a general conclusion and perspectives are included in **chapter 5** and **6**. **Chapter 5** integrates the key findings of this thesis and **chapter 6** describes future outlook of this work.

Chapter 2

Selection of plant growth-promoting rhizobacteria sharing suitable features to be commercially developed as biostimulant products

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Abstract

Plant biostimulants (PBs) are an eco-friendly alternative to chemical fertilisers because of their minimal or null impact on human health and environment, while ensuring optimal nutrient uptake and increase of crop yield, quality and tolerance to abiotic stress. Although there is an increasing interest on microbial biostimulants, the optimal procedure to select and develop them as commercial products is still not well defined. This work proposes and validates a procedure to select the best plant growth-promoting rhizobacteria (PGPR) as potential PBs. The stepwise screening strategy was designed based on literature analysis and consists of six steps: (i) determination of the target crop and commercial strategy, (ii) selection of growth media for the isolation of microbial candidates, (iii) screening for traits giving major agronomical advantages, (iv) screening for traits

related to product development, (v) characterisation of the mode of action of PGPR and (vi) assessment of plant growth efficacy. The strategy was validated using a case study: PGPR combined with humic acids to be applied on tomato plants. Among 200 bacterial strains isolated from tomato rhizosphere, 39 % were able to grow in presence of humic acids and shared the ability to solubilise phosphate. After the screening for traits related to product development, only 6 % of initial bacterial strains were sharing traits suitable for the further development as potential PBs. In fact, the selected bacterial strains were able to produce high cell mass and tolerated drought, aspects important for the mass production and formulation. These bacterial strains were not able to produce antibiotics, establish pathogenic interaction with plants and did not belong to bacterial species associated to human, animal and plant diseases. Most importantly, five of the selected bacterial strains were able to promote tomato seedling vigour in experiments carried out *in vitro*. These bacterial strains were furtherly characterised for their mode of action assessing their ability to produce phytohormones, solubilise soil minerals and colonize effectively tomato plant roots. This characterisation led to the selection of the best plant growth promoter strains, which belong to *Pantoea* and *Pseudomonas* genera, that showed the ability to promote tomato plant growth in experiments carried out in greenhouse conditions. Overall, this work provides a flow diagram for the selection of PGPR candidates to be successfully developed and commercialized as PBs. The validation of the flow diagram led to the selection

of two bacterial strains belonging to *Pantoea* and *Pseudomonas* genera, potential active ingredients of new PBs.

Keywords

Plant biostimulants, plant growth-promoting rhizobacteria, stepwise screening strategy, *Pantoea*, *Pseudomonas*.

1. Introduction

Feeding the growing global population is one of the major challenges for agriculture (Rouphael and Colla 2020). To sustain and guarantee an adequate yield, crop production is getting more and more dependent on chemical fertilisers (Berg 2009), which has, unfortunately, a very negative impact on the environment (Vejan et al., 2016). Thus, the development of eco-friendly alternatives to chemical fertilisers greatly increased importance in recent time. One of them is the use of plant biostimulants (PBs), which is gaining interest globally (De Pascale et al., 2017). Indeed, the PB market is constantly increasing with an expected compound annual rate of 10.9 % until 2022 (Sessitsch et al., 2018). The main reasons of this growth are: (i) increasing importance of the organic farming, (ii) more PB use in developed countries and (iii) good acceptance of PBs among consumers (Biostimulant Market, 2014). Plant biostimulants include substances or microorganisms that enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits when applied to

plants (du Jardin 2015). Among PBs active ingredients, microorganisms residing in the plant rhizosphere received relevant attention, in particular plant growth-promoting rhizobacteria (PGPR). In fact, PGPR promote plant growth through several mechanisms as modulation of the hormone balance in plants through the release of indole-3-acetic acid (IAA) and synthesis of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase (Glick 2014; Spaepen and Vanderleyden 2011). Moreover, PGPR make soil elements, such as iron, phosphorus and potassium, more available to plants thanks to the release of siderophores, organic acids and enzymes (Ahmed and Holmström 2014; Parmar and Sindhu 2013; Rodriguez and Fraga 1999).

To develop commercial PBs based on PGPR, potential candidates are selected by following a step by step screening strategy based on testing different criteria, from laboratory to field-like conditions (du Jardin, 2015). However, without collaboration between stakeholders, farmers, researchers, and regulatory bodies to bring affordable and effective new bioproducts to the market is impossible (du Jardin 2015). For instance, considering both the mode of action of the candidates and the market demand, can be an effective combined criterion to decide which strain would be the most competitive and successful product (Kamilova and De Bruyne 2013). Many publications already described the general steps for the development of microbial bioproducts for agriculture (Backer et al., 2018; Nakkeeran et al., 2006; Pliego et al., 2011). Briefly, these include isolation of

microorganisms, screening in laboratory and under greenhouse conditions, assessment of ecological safety, development of suitable formulations, marketing and registration. The characterization of the mode of action of selected PGPR is an important aspect taken under consideration when new bacterial isolates are selected as potential PBs (Rouphael and Colla, 2020). Particular attention is given to the ability of bacterial isolates to protect plants against abiotic stresses as drought, salinity and chilling (Albdaiwi et al., 2019; Subramanian et al., 2015; Tiwari et al., 2016). Similarly, PGPR are commonly evaluated for their impact on plant uptake of soil nutrients (De Pascale et al. 2017). It is undeniable that the enhancement of nitrogen (N) assimilation received most of the attention so far and N-fixing bacteria as *Rhizobium* spp. are already developed as commercial PBs (Remigi et al., 2016). At the same time, scarce attention has been given to the ability of PGPR to solubilise other soil nutrients as phosphorous (P), that will less available in the next future (Granada et al., 2018).

Many other factors need to be taken into account during the development of a commercial PB (Backer et al., 2018). Some of these parameters are high competitive saprophytic activity, high rhizosphere competence, affordable mass multiplication, broad spectrum of action and enhanced plant growth. In addition, tolerance to heat, desiccation, UV radiation and oxidizing agents are also criteria to be considered for a successful practical application (Nakkeeran et al., 2006).

Moreover, it is also useful to take into consideration that the performance of commercial PBs may be region specifics due to the origin of the developed PGPR (Kristin and Miranda, 2013).

To support researchers and companies in the selection of PGPR to be developed as commercial PBs, we designed a stepwise screening strategy. It consists of several steps where various criteria are used, starting from the isolation of candidates to the effect of these candidates under greenhouse conditions. We validated this screening method by using a case study and efficiently selected PGPR able to fulfil all the criteria included in the program, ready to be scaled-up by companies.

2. Material and methods

2.1. Screening strategy

The screening strategy is made of six steps and the first consists of the selection of the target crop and the PB impact on the market (Fig. 1). The decision depends mainly on the market size, presence of competing products/solutions and advantages for the growers, which are all conditions that can create a sufficient market size to justify the investment for the development of the PB. The decision requires knowledge of the specific market and sufficient commercial experience to estimate possible economic constraints of the future PB and must also consider national/international regulations for PBs.

The second step is a crucial aspect and it is related to the decision of the most appropriate growth media to be used for the isolation of microbial candidates. In fact, the use of different growth media, i.e. synthetic, selective, poor or nutrient-rich media will end up in the isolation of different microbial groups.

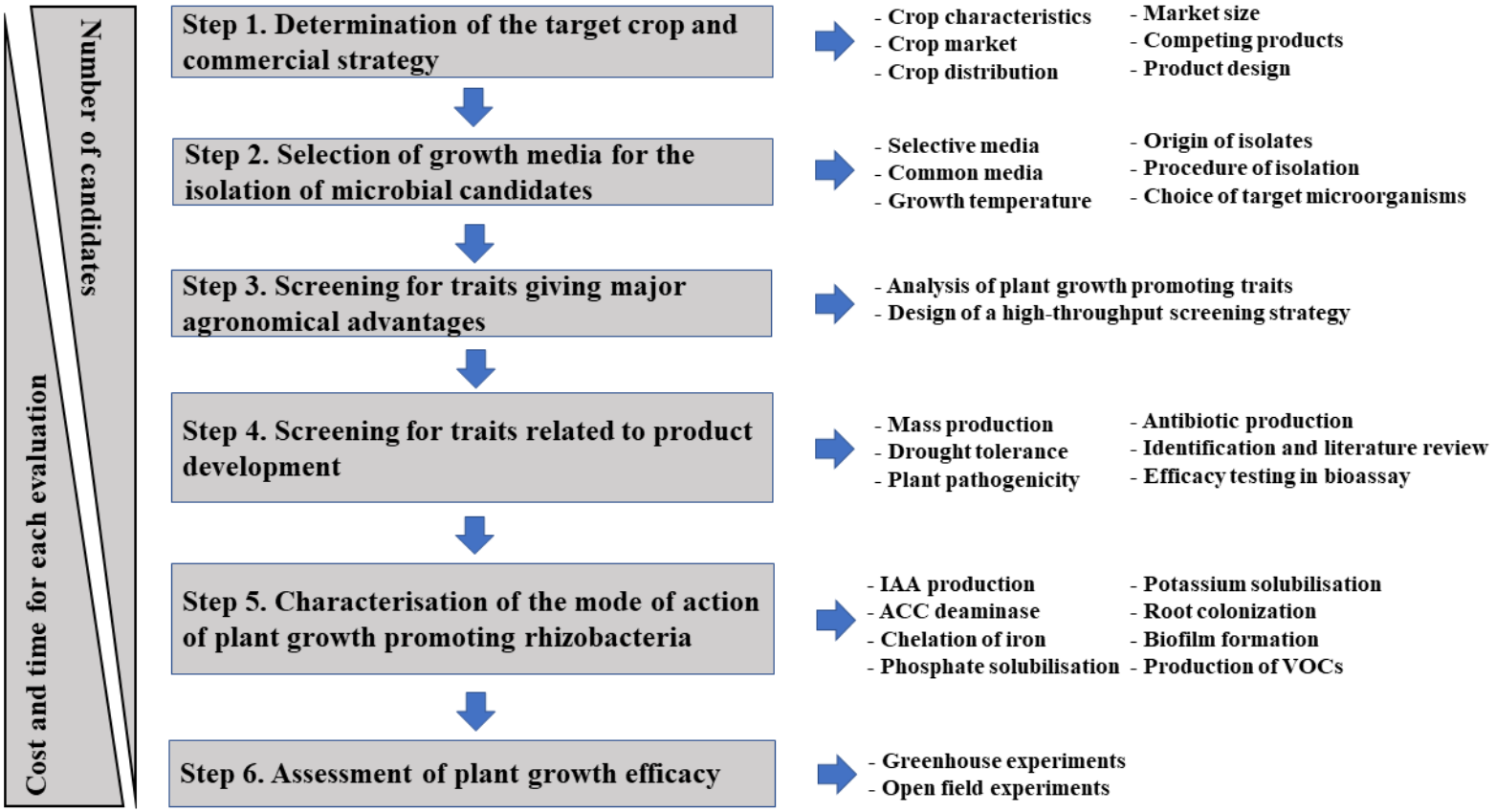

Figure 1. Stepwise screening strategy of microorganisms for commercial use as biostimulants

The third step consists of the preliminary screening based on the major agronomical advantages that the PB is expected to have. The availability/development of a high-throughput approach is crucial at this step, because screening of hundreds of candidates will increase the chances to select candidates with the desired features. The fourth step is the screening according to traits suitable for the development a successful PB product. At this stage, the industrial production and formulation approaches and the specific national/international standards and regulations laws must be carefully considered in the screening tests, because the economic and technical feasibility is highly depending on the specific microorganism's characteristics. For example, PBs candidates must reach a sufficient mass production on cheap growth medium in the industrial scale up, in order to achieve sustainable production costs. Another example is the tolerance to desiccation, if the final formulation is expected to be dry. This step must also include the safety issues, therefore taxonomic identification and detailed toxicological and ecotoxicological studies are needed to exclude human, plant or animal pathogens as well as microorganisms that can produce toxic metabolites. In this stage, a rapid throughput analysis consisting of *in vitro* bioassays on plants in controlled conditions is recommended allowing us to pre-screen candidates able to guarantee plant growth promotion efficacy without time-consuming approaches or expensive resources.

The fifth step is dedicated to the characterisation of the mode actions of the candidates to obtain knowledge to optimize the application and maximize their efficacy. In fact, candidates will be evaluated for their ability to make soil elements more available to the plants and to promote plant growth through the production of phytohormones and/or the modulation of plant hormone balance. Finally, in the sixth step, plant growth-promoting efficacy of candidates is evaluated under small-scale experiments mimicking the real environmental conditions, in order to select the most efficient one(s). In general, greenhouse experiments are proposed at this stage in order to control environmental conditions, while maintaining them as close as possible as the expected ones under future practical application.

2.2. Assessment of target crops and design of a commercial strategy

Target crop was chosen by consulting statistics reported by Food and Agriculture Organization (http://www.fao.org/faostat/) and Eurostat (https://ec.europa.eu/eurostat/data/database). The choice was oriented on a crop whose cultivation, production and organic production has grown constantly during the recent years. The commercial strategy was designed starting from a review process of the scientific works and patents related to PBs and PGPR published in the last five years (2015-2019) using the databases Espacenet (https://worldwide.espacenet.com) and Web of Science (https://apps.webofknowledge.com/). In Web of Science, an Advanced Search

was carried out using the forumulae: TI=plant biostimulants AND SU=agriculture and TI=plant growth-promoting rhizobacteria AND SU=agriculture. Similarly, the formulae TI = "Plant" OR TI = "Biostimulants" and TI = "Plant growth-promoting" OR TI = "rhizobacteria" were used to carry out an Advanced Search in Espacenet. The results were analysed to have a picture of: **i)** the progresses reached in the analysis of PGPR to be developed as PBs; **ii)** the procedures adopted to screen PGPR in the majority of the published literature; **iii)** new strategies that might be implemented to design next generation PBs. After the analysis, brainstorming meetings were programmed to formulate new commercial strategies. The ideas were subsequently discussed for their feasibility and impact on the market.

2.3. Isolation of bacteria from tomato rhizosphere soil

Soil adhering to tomato (*Solanum lycopersicum* cv. Tondo rosso) plant roots was collected, sieved at 2 mm mesh, placed in sterile 50 ml tubes and stored at 4 ºC until time of processing (approximately 24 h). Isolation of culturable bacteria was carried out by dilution plating method using the Rhizosphere Mimicking Agar (RMA, Brescia et al., 2020). Soil suspensions were prepared mixing 5 g of root-adhering soil in 45 ml of sterile saline solution (NaCl 0.85% w/v) contained in sterile 50 ml tubes. Subsequently, tubes were shaken at 200 rpm for 1 h at room temperature. At the end of the shaking, soil mixtures were serially diluted

(from 10^{-1} to 10^{-7}) in triplicate. A volume of 100 µl of diluted suspension (from 10^{-3} to 10^{-7}) was spread onto RMA medium amended with cycloheximide (100 mg/l). Petri dishes were incubated at 27 ºC and bacterial colonies selected after 72 h based on their morphology. Selected bacterial isolates were stored at length in glycerol 40 % at -80 ºC and routinely grown on Nutrient Agar (NA, Oxoid, United Kingdom) in Petri dishes (90 mm diameter). In all the experiments enlisted in the flow diagram (Fig. 1), bacteria were discarded if they did not share traits selected in each step except when otherwise indicated.

2.4. Screening of bacterial isolates for compatibility with humic acids and phosphate solubilization

The National Botanical Research Institute's phosphate growth medium (NBRIP) and R2A medium amended with 0.003 % of HA (Sigma Aldrich, United Kingdom) were respectively used to select bacterial isolates having the ability to solubilize phosphate and to be combined with humic acids. Briefly, bacterial isolates were grown in 5 ml of Nutrient Broth (NB, Oxoid) at 27 ºC on an orbital shaker (200 rpm). After 24 h, a volume of one ml of bacterial cell suspensions was centrifuged (13,000 rpm, 2 min) and pellets were suspended in NaCl (0.85% w/v) to a final optical density at 600 nm ($A_{OD600nm}$) of 0.1 corresponding to ≃ 1 × 10^8 colony forming units (CFU)/ml and used in all the experiments, except when otherwise indicated. A volume of five µl of bacterial cell suspension was spot inoculated into the above-mentioned growth media and incubated for 48 h

at 28 ºC. Bacterial isolates having both the ability to grow onto R2A amended with HA and develop a halo around the macrocolony on the NBRIP medium (Campisano et al., 2015) were selected for further characterisation.

2.5. Selection of bacterial isolates sharing traits useful for their development as a bioproduct

2.5.1. Evaluation of cell mass production and tolerance to desiccation

To assess cell mass production, bacterial isolates were grown in NB (5 ml) at 27 ºC on an orbital shaker (200 rpm). After 16 h, bacterial cell suspensions were serially diluted (from 10^{-1} to 10^{-8}) and dilutions (from 10^{-5} to 10^{-8}) were plated onto NA. Once inoculated, Petri dishes were incubated at 27 ºC and the developed CFU were counted after 48 h of incubation. Results were expressed as $\log_{10}$ CFU/ml.

Tolerance to desiccation was assessed by growing bacterial isolates in NB (5 ml) at 27 ºC on an orbital shaker (200 rpm) for 16 h. Subsequently, a volume of five µl of bacterial cell suspensions was spot inoculated (in triplicate) onto NA amended with sorbitol (0.53 M) to mimick drought condition [-2.5 MPa water potential (w.p.)]. Development of bacterial macrocolonies was assessed after 24 h incubation at 28 ºC.

Bacterial isolates able to reach a concentration ≥ 10^9 CFU/ml and to develop macrocolonies on NA amended with sorbitol were selected and further

characterised.

2.5.2. Assessment of plant pathogenicity and production of toxic secondary metabolites

The ability to establish a pathogenic interaction with plants was assessed according to Klement et al. (1964). Briefly, bacterial cell suspensions (1×10^8 CFU/ml) in sterile distilled water (SDW) were injected (in triplicate) into the intercellular spaces of healthy tobacco (*Nicotiana tabacum*) leaves. Tobacco plant leaves injected with SDW only were used as untreated control. Tobacco plants were kept in the greenhouse (25 ± 1 °C; 70 ± 10 % RH; 16 h photoperiod) and occurrence of a hypersensitivity reaction was visually assessed after 24 h.

Production of antibiotics was assessed by dual plate assay according to the procedure described by Puopolo et al. (2014a) with some modifications. Briefly, five µl of bacterial cell suspension (1×10^8 CFU/ml) was spot inoculated at 30 mm of the border of Petri dishes (90 mm diameter) containing Potato Dextrose Agar (PDA, Oxoid). After 24 h at 27 °C, plugs of mycelium of the phytopathogenic oomycete *Pythium* (*Py.*) *ultimum* (5 mm of diameter) were cut from the youngest region of the mycelium and placed at 25 mm far from the bacterial macrocolonies. *Pythium ultimum* was used as test microorganism based on its high sensitivity to microbial toxic secondary metabolites (Santos and Melo, 2016). PDA dishes seeded with mycelium plugs only were used as untreated controls. Inhibition of the mycelial growth was evaluated by measuring

the *Py. ultimum* colony diameter after 72 h incubation at 25 ºC.

Bacterial isolates not able to cause hypersensitivity reaction in tobacco plants and/or release toxic secondary metabolites against *Py. ultimum* were selected and further characterised.

2.5.3. Identification of bacterial isolates by 16S rDNA and literature review

Bacterial genomic DNA was extracted using the kit Power Soil™ DNA Isolation Kit (Mo Bio Laboratories, Inc., Carslband CA, USA) according to the manufacturer's instructions. Five µl of genomic DNA were used as template in PCR for the amplification of 16S rDNA region. Reaction mixtures (25 µl) containing 12.5 µl Go Taq Green Master Mix (Promega GmbH, Mannheim, Germany), 11.5 µl sterile deionized water and 0.5 µl of universal primers 16S-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1492R (5′GGTTACCTTGTTACGACTT-3′). Reaction was carried out in an automated thermal cycler (Biometra GmbH, Göttingen, Germany) with the following protocol: initial denaturation at 95 ºC for 2 min, 36 cycles of denaturation (95 ºC for 1 min), annealing (55 ºC for 1 min), extension (72 ºC for 1 min) and final extension at 72 ºC for 4 min.

PCR products were purified using Illustra ExoProstar Kit (Euroclone S.p.A., Italy) according to the manufacturer's instructions. To identify at the species level preferably, resulting nucleotide sequences were compared to known

sequences deposited in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm. nih.gov/nucleotide/) using BLASTN (Basic Local Alignment Tool).

Bacterial isolates belonging to human and/or animal harmful bacterial species were discarded.

2.5.4. Effect of bacterial seed treatment on the in vitro *tomato seedling vigour*

The ability of bacterial isolates to promote plant growth was carried out by a seed germination assay according to the procedure described by Smyth et al. (2011) with some modifications. Tomato seeds (*S. lycopersicum* var. Moneymaker, Justseed, Wrexham, UK) were surface sterilized by submerging them in the following solutions: 70 % ethanol for 2 min, 1 % sodium hypochlorite (NaOCl) for 5 min. After that, surface-sterilized seeds were washed five times with SDW. Inoculation of bacterial strains was carried out by soaking seeds in either 1 ml of bacterial cell suspension (1×10^8 CFU/ml; treated) or SDW (untreated) for 1 h and drying for 30 min under the laminar flow cabinet. Five seeds per bacterial isolate were placed on the top of four sterile filter papers soaked with 5 ml of SDW contained in sterile Petri dishes. Subsequently, Petri dishes were incubated in darkness for five days at 20 ºC. Germination was considered to occur if at least 1 cm of radicles appeared. To determine the vigour index, the following formula was applied:

Vigour index = (*Average root length* + *Average shoot length*) / *Germination rate* (%)

After this calculation, the change in the vigour index was evaluated as follows:

Change Vigour Index (%) = (*Average vigour index treated- Average vigour index untreated*) / *Average vigour index untreated*

Three replicates (Petri dishes) were used for each treatment and the experiment was carried out five times. Bacterial strains able to increase the vigour index of tomato seedlings at least in three out of five experiments were selected and further characterised.

2.6. Characterisation of bacterial strains for plant growth-promoting activities

2.6.1. Chelation of iron and solubilisation of phosphate and potassium

To determine the ability of bacterial strains to chelate iron through the release of siderophores, King´s B Agar dishes were overlaid with Chrome Azurol S (CAS) Agar medium (Schwyn and Neilands, 1987). Solubilisation of phosphate and potassium were evaluated using the NBRIP medium and Aleksandrow Agar (HiMedia GmbH, Germany) respectively. In all the tests, a volume of five µl of bacterial cell suspension (1×10^8 CFU/ml) was spot inoculated onto these media and incubated for 72 h at 28 °C. At the end of incubation, the areas of orange haloes (release of siderophores) and the clarification haloes (solubilisation of phosphate and potassium) formed around bacterial macrocolonies were

determined by capturing digital images with Bio-Rad Quantity One software implemented in a Bio-Rad Geldoc system (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.). The halo areas were subsequently measured using Fiji software (ImageJ1.50i; Schneider et al., 2012). For all the tests, three replicates (Petri dishes) were used and the experiment was repeated.

2.6.2. Production of Indole-3-acetic acid (IAA) and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase

IAA production was evaluated by a colorimetric detection test in liquid culture according to the procedure described by Campisano et al. (2015) with some modifications. Briefly, a volume of 500 µl of bacterial cell suspension (1×10^8 CFU/ml) was grown in five ml of DF salt minimal broth amended with 500 µg/ml of L-Tryptophan (Sigma-Aldrich) contained in sterile 15 ml tubes at 28 ºC on an orbital shaker (200 rpm). After 120 h, final cell densities were determined by measuring the absorbance at 600 nm ($A_{OD600nm}$). A volume of one ml of bacterial cell suspensions was centrifuged (13,000 rpm, 10 min) and 250 µl of supernatant were mixed with one ml of Salkowski's reagent. After 30 min incubation at room temperature, 150 µl of the mixture were transferred to 96-well polystyrene dishes (Thermo Fisher Scientific, Waltham, MA, USA). The intensity of pink red color was quantified by measuring the absorbance at 530 nm ($A_{OD530nm}$) by Synergy 2 Multiplate Reader (Biotek, Winooski, VT, USA) and concentration of IAA was determined by a standard curve prepared from

pure IAA solutions (Sigma-Aldrich) in a range from 0.5 to 100 µg/ml (Fig. S1). Quantity of IAA produced by bacterial cells was expressed as the ratio between $A_{OD530nm}$ and $A_{OD600nm}$.

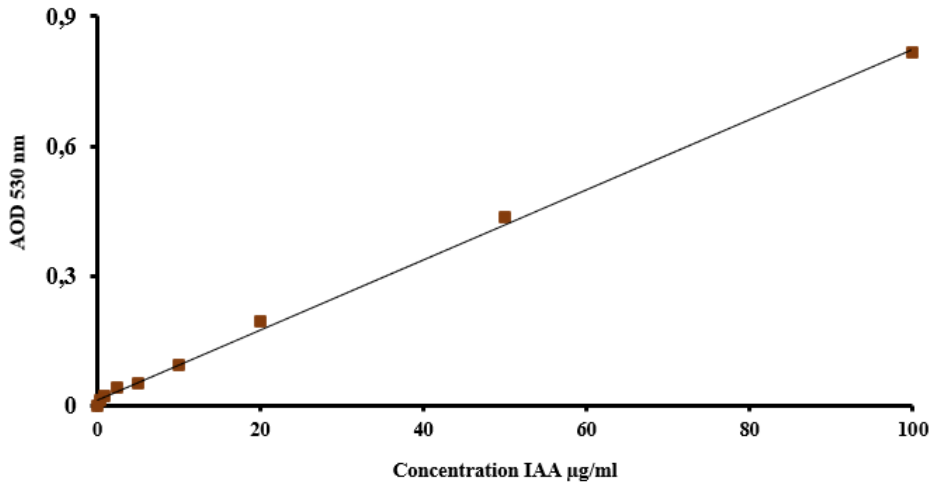

Figure S1. Standard curve of indole 3-acetic acid (IAA). Values of optical density at 530 nm of IAA concentration ranging from 0.5 to 100 µg/ml calculated by colorimetric detection assay (o, y = 0.0081x + 0.0144). Each data point represents the mean of three replicates and the column represents the standard error values.

ACC deaminase activity was assessed by a colorimetric ninhydrin assay according to the procedure described by Li et al., (2011). Briefly, bacterial cell suspensions (1×10^8 CFU/ml) were grown in sterile 15 ml tubes containing 5 ml of DF salt minimal medium supplemented with 3 mM of ACC as the only nitrogen source. The resulted Ruhemann's Purple depth was measured as absorbance at 570 nm ($A_{OD570\ nm}$) and associated to presence of ACC deaminase. ACC deaminase activity was calculated by dividing $A_{OD570\ nm}$ by the bacterial

cell density ($A_{OD600nm}$).

In both experiments, three replicates (15 ml tubes) were used for each bacterial strain and experiments were repeated.

2.6.3. Biofilm formation

Bacterial strains were evaluated for their ability to form biofilm using the procedure described by Puopolo et al., (2014b). Briefly, 1.5 µl of bacterial cell suspension (1×10^8 CFU/ml) was inoculated in 150 µl of NB distributed in sterile 96-well polystyrene dishes and incubated at 27 °C without shaking. NB not inoculated with bacterial strains was used as the untreated control. After 72 h, final cell density ($A_{OD600nm}$) was determined. Unattached cells were removed by inverting the plate and the remaining bacterial cells were attached to the well surfaces by an incubation period for 20 min at 50 °C. Bacterial cells were then stained and washed with 200 µl of crystal violet solution and SDW respectively. Adherent cells were decolorized with 200 µl of acetone/ethanol (20%/80%). A volume of 100 µl of each suspension was transferred to a new 96-well polystyrene plate. The density of adherent cells (biofilm formation) was determined by measuring the absorbance at 540 nm ($A_{OD540nm}$). To determine the Specific Biofilm Formation (SBF) the following formula was applied:

$SBF = (A_{OD540nm}X - A_{OD540nm}C) / (A_{OD600nm}X - A_{OD600nm}C)$

where X indicated the treated samples whereas C indicated the untreated control.

For each bacterial strain three replicates (wells) were used and the experiment was repeated.

2.6.4. Root colonisation

Seeds of tomato plants (*S. lycopersicum* var. Moneymaker, Justseed, Wrexham, UK) were surface-sterilized and bacterial strains were inoculated as mentioned above. Treated and untreated seeds (in triplicate) were dipped into perlite (3 g) contained in glass tubes (25 mm x 150 mm, Sigma Aldrich) and moistened with 10 ml of Hoagland nutrient solution. Subsequently, tubes were kept in a growth chamber (25 ± 1 °C; 70 ± 10 % RH; 16 h photoperiod). After 144 h, roots were weighted, cut and mixed in 5 ml of $MgSO_4$ 10 mM contained in sterile 15 ml tubes. Subsequently, tubes were shaken at 200 rpm for 30 min at room temperature. After shaking, suspensions were serially diluted (from 10^{-1} to 10^{-7}). A volume of 10 µl of diluted suspension (from 10^{-4} to 10^{-7}) was spot inoculated onto NA (Oxoid). Once inoculated, Petri dishes were incubated at 27 °C and the developed CFU were counted after 48 h. Results were expressed as $\log_{10}$ CFU/mg of tomato roots. Three replicates (tomato seedlings) were processed for each bacterial strain and the experiment was repeated.

2.7. Greenhouse experiment

The effect of the application of two selected bacterial strains on tomato plant growth was assessed under greenhouse conditions. Tomato seeds (*S.*

lycopersicum var. Moneymaker, Justseed, Wrexham, UK) were seeded in 100 ml pots containing DCM Ecoterra ® Zaaien & Stekken potting mix (DCM; Grobbendonk; Belgium) and grown in the greenhouse with an average temperature 22 °C ± 2 at night and 24 °C ± 2 during the day until the plants had produced one shoot with at least two true leaves. At this stage, a volume of 10 ml of three days old bacterial cell suspension (1×10^7 CFU/ml) was applied into the pots to reach a final cell density of 1×10^7 CFU/pot and plants were then kept in the greenhouse. After two days, tomato plants were transplanted to 4 l pots containing potting mix without fertilizer (DCM; Grobbendonk; Belgium) mixed with an organic fertilizer: DCM ECO-PLANT 2 (DCM; Grobbendonk; Belgium), and an organic amendment enriched in trace element: MICRO MIX DCM (DCM; Grobbendonk; Belgium) at 6 g/l and 0.3 g/l respectively. One week after soil transplantation, a volume of 100 ml of three days old bacterial cell suspension (1×10^9 CFU/ml) was applied into the pots to reach a final cell density of 2.5×10^7 CFU/pot. For both inoculations, the untreated control was treated with only water. Six weeks after the second application of bacterial strains, shoot length and shoot dry biomass (48 h incubation at 65 °C) was evaluated. Twenty replicates (tomato plants) were used for each treatment.

2.8. Statistical analysis

All experiments were carried out twice except seed germination test which was

repeated five times. Normality (Shapiro-Wilk test, $p>0.05$) and variance homogeneity (Levene´s test, $p>0.05$) were checked and parametric tests were used. Data from experiments were pooled when two-way ANOVA demonstrated non-significant differences between two experiments ($p> 0.05$). When significant differences between experiments were found, data presented were analysed from one representative experiment with similar results. Data were subsequently analysed using one-way ANOVA and mean comparisons between treatments were assessed by Tukey's test ($\alpha= 0.05$). Data were analysed with IBM SPSS software (Version 21).

3. Results

3.1. Determination of target crop and product design

Consultation of statistics from Eurostat and FAO (FAO, 2018) led us to choose tomato as the target crop for a future PB. In particular, the constant increase of world production and the increase of hectares deputed to organic production of fresh vegetables in EU played a major role in this choice (Fig. S2).

The advanced research on Web of Science revealed an increase in published articles having the terms PGPR and PBs in the title in the period of 2015-2019. In details, a total of 352 published articles contained the term PGPR in the title, whereas the term PBs appeared in a total of 48 published articles (Fig. S3).

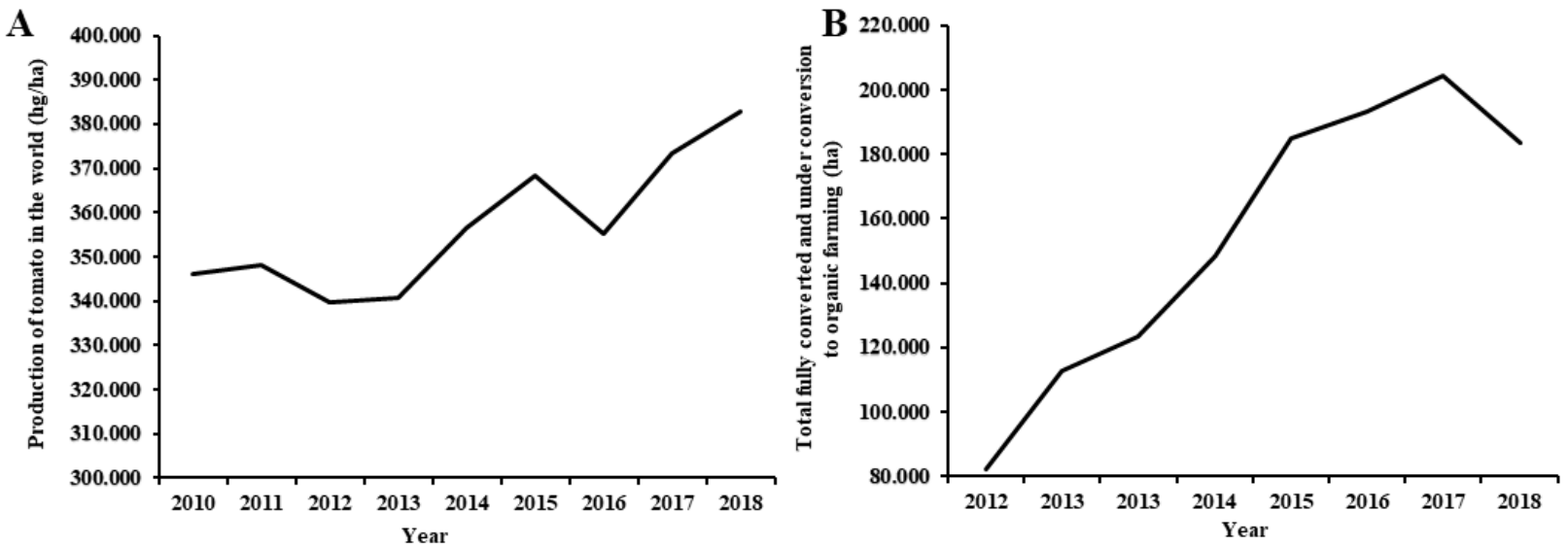

Figure S2. Analysis of tomato and organic farming. A) Trend of tomato production in the world according to statistics from Food and Agriculture Organization (FAO, 2018). B) Trend of organic farming in Europe according to statistics available on Eurostat (https://ec.europa.eu/eurostat/data/database).

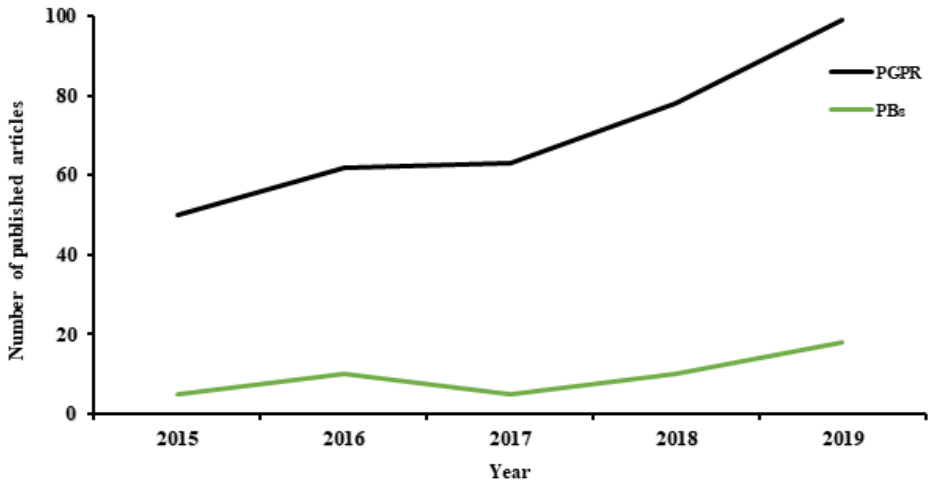

Figure S3. Number of documents published in the period 2015-2019. Web of Science - Advanced Search was used to carry out the literature search. The formulae: TI=plant biostimulants AND SU=agriculture and TI=plant growth-promoting rhizobacteria AND SU=agriculture was used to select documents having the terms "plant biostimulants" and "plant growth-promoting rhizobacteria" in the title, respectively.

Regarding patents, the advanced search in Espacenet showed the presence of 15 patents having the term "plant biostimulants" in the title, but only few of them were related to PGPR. A higher number of patents (27) contained the term "plant growth-promoting rhizobacteria" in the title and most of them referred to the application of rhizobacteria to stimulate plant growth.

Overall, the advanced searches carried out gave us a better idea in the steps that might be included in the screening procedure to make it more suitable for the selection of PGPR having traits useful for their future development as

commercial PBs. Moreover, results from these activities were discussed and the idea of a new commercial PB was conceived. Firstly, ability to solubilise phosphate was chosen as a plant growth-promoting activity that might characterize the future PB. Although the input of phosphorous in agriculture is lower than nitrogen input (Fig. S4), a relevant number of commercial PBs including PGPR are already available and this might represent a strong competition for a new commercial PB. Based on an internal research, the margin of a success of a future PB including PGPR able to solubilise phosphate is higher due to the small number of PBs with this plant growth-promoting activity. Based on the most recent published articles focused on PBs, we discussed and agreed on the fact that the combination of PGPR and humic acids a competitive advantage of the future commercial PB.

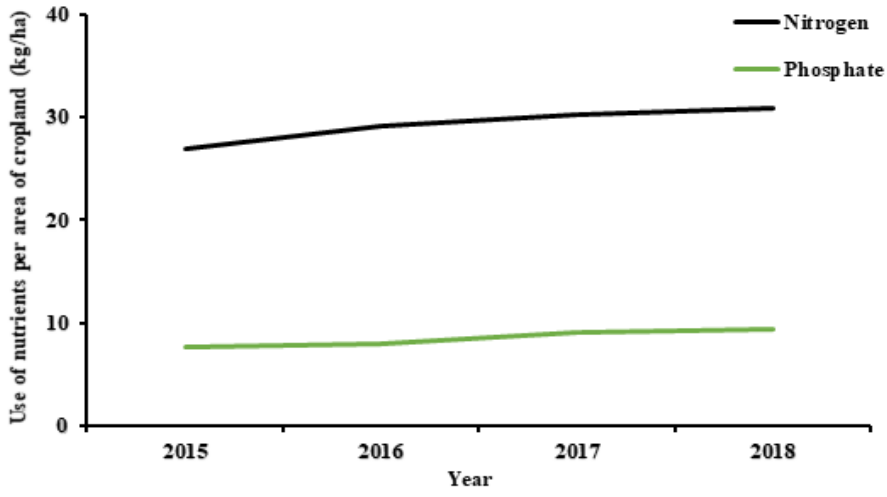

Figure S4. Use of nitrogen and phosphate in agriculture in the period 2015-2018. Trend

of the use of nitrogen and phosphate in the world according to statistics from Food and Agriculture Organization (FAO, 2018).

3.2. Isolation of bacteria from tomato plant rhizosphere soil and first screening

Once established the target crop and the potential of a new PB competitive product, a total of 200 culturable bacteria were isolated from the rhizosphere soil of tomato plants and rapidly screened for their ability to solubilize phosphate and grow in presence of humic acids. From this screening, 78 bacterial isolates having both the traits were selected and further evaluated.

3.3. Selection of bacterial isolates sharing traits useful for their development as a bioproduct

Out of 78 bacterial isolates selected from prior steps, 25 were able to reach 10^9 CFU/ml on NB after 24 h and tolerated desiccation condition. Seven of this group were discarded since they showed antifungal activity against *Py. ultimum* while the remaining 18 bacterial isolates were tested for their phytopathogenicity. After injection of bacterial isolates on tobacco leaves, only one showed hypersensitive reaction and discarded from the selection since it might represent a plant pathogenic bacterium.

Taxonomic identification of selected bacterial isolates showed different percentage of identity based on the similarity between nucleotides (Table 1). After a literature review, the bacterial isolates showing high sequence identity

level with *Bacillus cereus*, *Kluyveria intermedia*, *Pseudomonas* (*Ps.*) *plecoglossicida* and *Pseudomonas lini* were discarded for being human, animal or plant pathogen (Bottone, 2010; Nishimori et al., 2000; Sarria et al., 2001).

Table 1. Taxonomic identification of candidates by 16S rDNA.

Strain code	Bacterial species	Identity level (%)	Accession number
MVC 1	*Kluyvera intermedia*	99.7	MT374833
MVC 3	*Arthrobacter enclensis*	99.2	MT374834
MVC 6	*Paenarthrobacter nitroguajacolicus*	99.8	MT374835
MVC 11	*Bacillus aryabhattai*	99.3	MT374836
MVC 13	*Arthrobacter oxydans*	99.4	MT374837
MVC 15	*Pseudomonas plecoglossicida*	99.0	MT374838
MVC 16	*Arthrobacter pascens*	99.9	MT374839
MVC 17	*Pseudomonas putida*	99.8	MT374840
MVC 18	*Pseudomonas lini*	98.9	MT374841
MVC 21	*Pantoea agglomerans*	99.6	MT374842
MVC 22	*Paenarthrobacter nitroguajacolicus*	97.7	MT374843
MVC 23	*Paenarthrobacter nitroguajacolicus*	99.7	MT374844
MVC 31	*Bacillus pumilus*	99.1	MT374845
MVC 33	*Arthrobacter oxydans*	99.6	MT374846
MVC 41	*Pantoea cedensis*	99.3	MT374847
MVC 107	*Bacillus cereus*	99.9	MT374848
MVC 109	*Bacillus cereus*	99.7	MT374849

The effect on the vigour index of tomato plants compared to the untreated seeds varied among the different bacterial strains (Fig. 2). Among 12 bacterial strains previously selected, seven of them showed either slightly positive results, negative results or no differences in the vigour index compared to the untreated plants in most of the experiments carried out (Fig. 2). In contrast, tomato seeds

treated with *Paenarthrobacter* (*Pae*.) *nitroguajacolicus* MVC 6 showed a positive modulation of vigour index in all the experiments carried out. Similarly, *Arthrobacter enclensis* MVC 3, *Pseudomonas putida* MVC 17, *Pantoea agglomerans* MVC 21, and *A. oxydans* MVC 13 determined an enhancement in vigour index in four or three experiments carried out (Fig. 2). Based on these results, five bacterial strains that showed positive results in at least three independent experiments were selected to be further characterised.

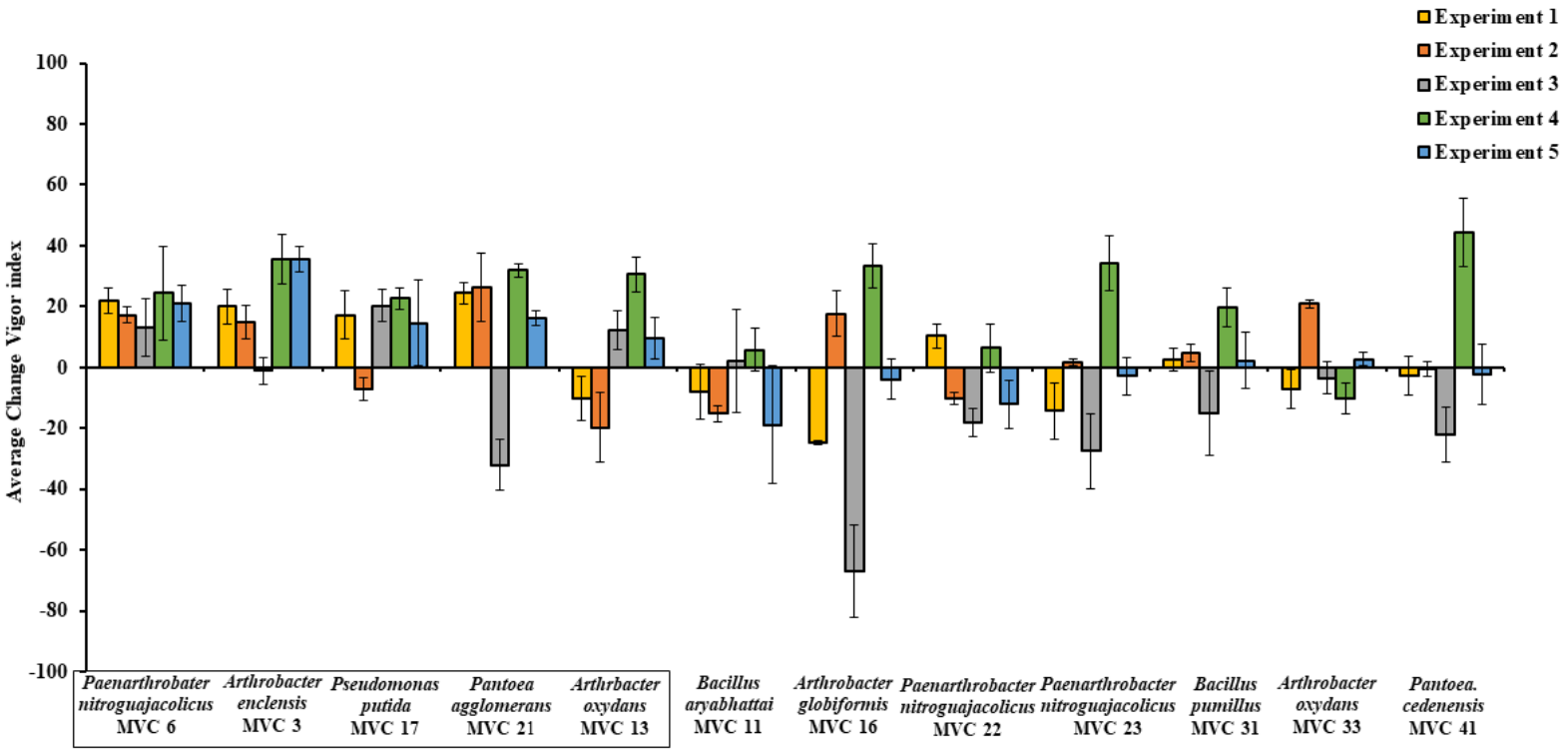

Figure 2. Efficacy of bacterial isolates on the in vitro seedling germination of tomato seeds. The vigour index was assessed from each treatment by the germination rate and the root and shoot elongation. Values were compared with the untreated (change vigour index) for

five experiments (columns). Mean and standard error values (columns) from fifteen replicates are represented for each treatment.

3.4. Screening for plant growth-promoting activities

Pantoea agglomerans MVC 21 was the best phosphate solubilising strain showing the largest clarification halo areas (Fig. 3A). Three bacterial strains were able to solubilise potassium and, in particular, *P. agglomerans* MVC 21, showed the largest clarification halo area (74.28 ± 6.30 mm^2, Fig. 3B). Similarly, three bacterial strains released siderophores and the most active bacterial strain was *Ps. putida* MVC 17 (1042 ± 24.055 mm^2; Fig. 3C). All the tested bacterial strains produced IAA and *P. agglomerans* MVC 21, with 281.84 ± 3.79 µg/CFU, showed the highest production (Fig. 3D). ACC deaminase activity was not detected in any tested bacterial strain.

All the tested bacterial strains were able to form biofilm and colonize tomato roots. *A. enclensis* MVC 3 was the best biofilm producer under the conditions tested (Fig. 3E) whereas *P. agglomerans* MVC 21 showed the highest values of root colonisation (6.47 ± 0.23 $\log_{10}$ CFU/mg of tomato roots; Fig. 3F).

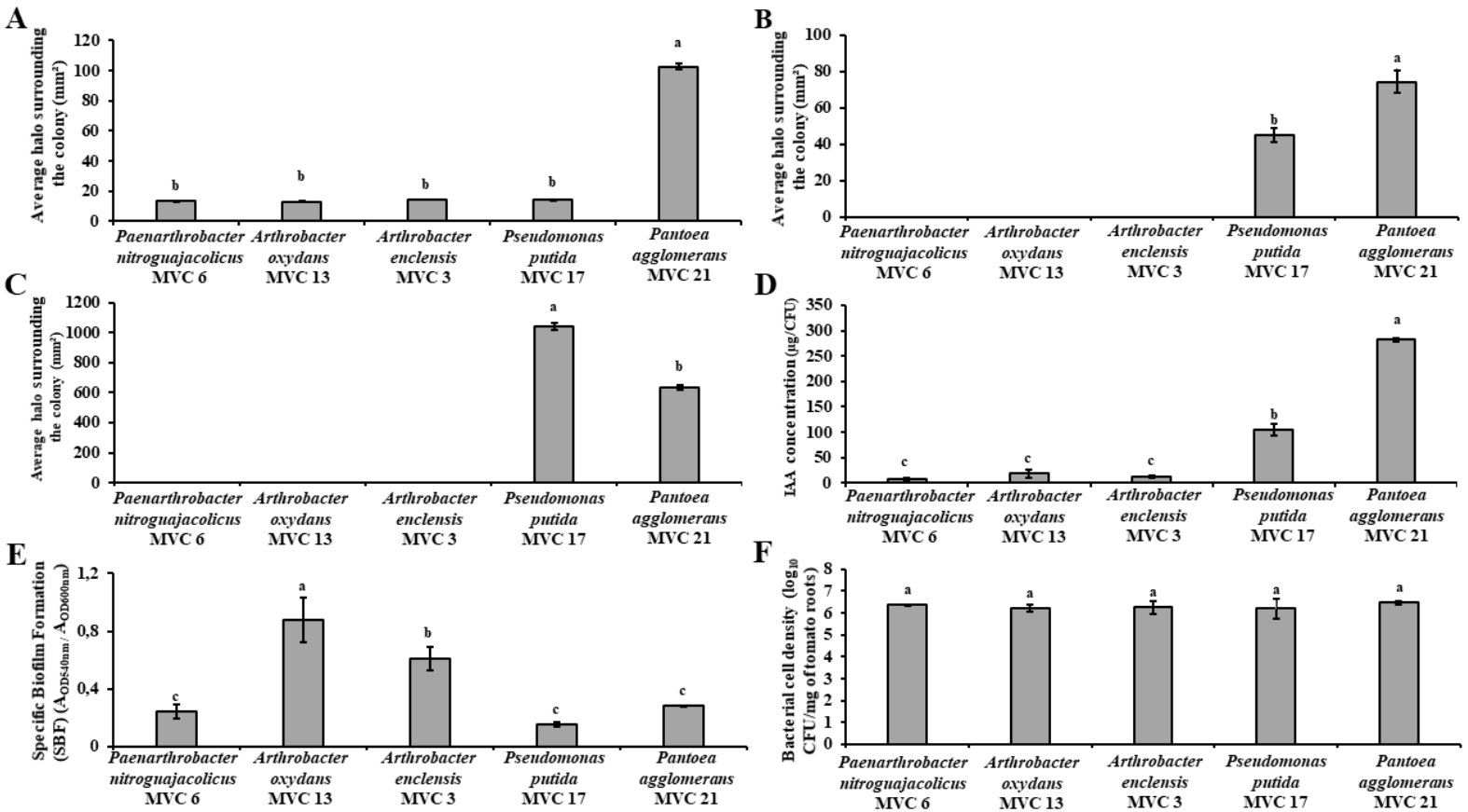

Figure 3. Quantitative assessment of plant growth-promoting traits. Phosphate (A) and potassium (B) solubilization, chelation of iron

(C), IAA production (D), biofilm formation (E) and root colonisation (F) were assessed for *Paenarthrobacter nitroguajacolicus* MVC 6, *Arthrobacter oxydans* MVC 13, *Arthrobacter enclensis* MVC 3, *Pseudomonas putida* MVC 17, and *Pantoea agglomerans* MVC 21. Mean and standard error values (columns) of six replicates from the two experiments are presented for each treatment (bacteria). Different letters indicate significant differences among treatments according to Tukey´s test (α = 0.05).

3.5. Effect of selected bacterial strains under greenhouse conditions

Under greenhouse conditions, tomato plants inoculated with either *P. agglomerans* MVC 21 or *Ps. putida* MVC 17 showed a significant increase in the shoot length and biomass compared to the untreated plants. Application of *P. agglomerans* MVC 21 determined the most significant increase in terms of shoot length (444.50 ± 16.06 mm; Fig. 4A). Regarding the shoot dry biomass, the application of *P. agglomerans* MVC 21 (12.45 ± 0.93 mg) and *Ps. putida* MVC 17 (11.25 ± 0.75 mg) determined a significant increase compared to the untreated tomato plants (8.76 ± 0.66 mg; Fig. 4B).

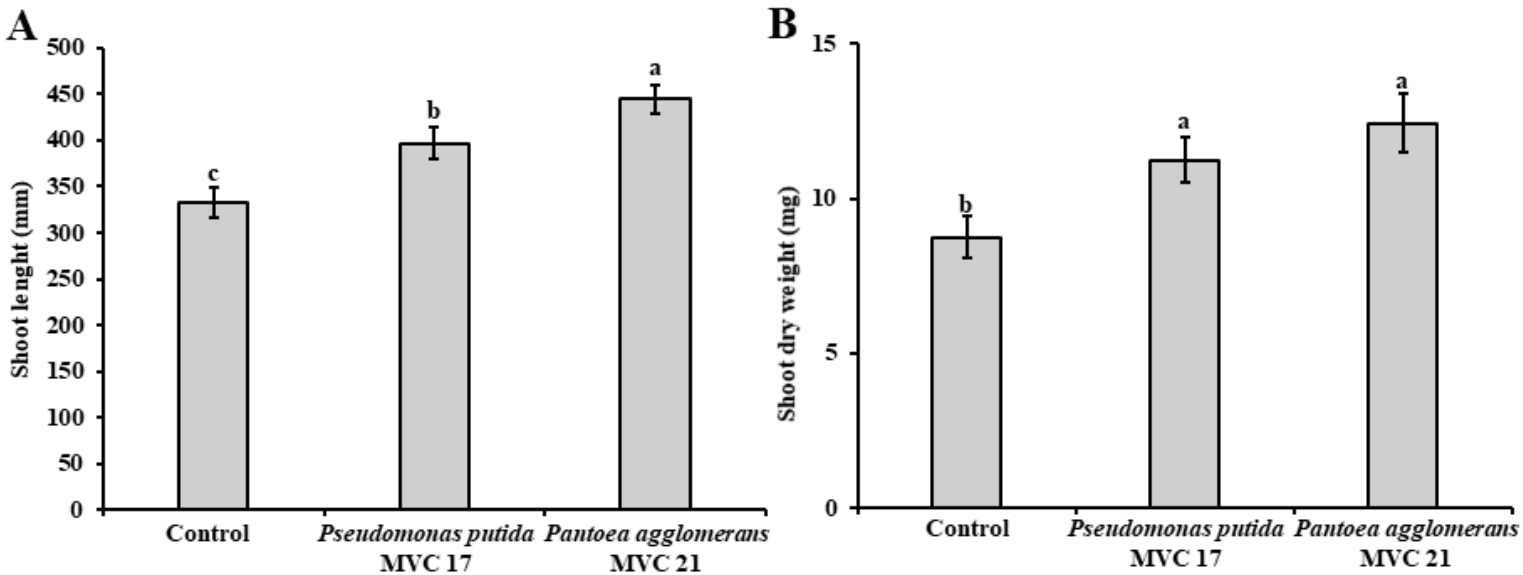

Figure 4. Plant growth-promoting effects of *Pantoea agglomerans* MVC 21 and *Pseudomonas putida* MVC 17 under greenhouse conditions. Shoot length (A) and shoot biomass (B) were evaluated after 6 weeks from the second inoculation. Mean and standard error values from 20 replicates are represented for each treatment. Different letters indicate significant differences according to Tukey´s test (α = 0.05).

4. Discussion

The design of flow diagrams including all the steps needed for the selection of microorganisms and their development as novel commercial bioproducts was addressed in the case of biocontrol agents (Köhl et al., 2011; Segarra et al., 2015). In contrast, this topic received scarce consideration in the case of the selection and characterization of PBs (Povero et al., 2016). In this work, we designed a straightforward stepwise screening program and we also validated it through the selection of two PGPR candidates able to fulfil all the requirements needed to be successfully commercialized as PBs.

The first step of our stepwise screening program was dedicated to the choice of the target crop and the strategy to realize a novel PB that might compete with PBs already available on the market. To decide the crop, the statistics published by Eurostat and FAO were examined (De Cicco, 2019; FAO, 2018). It is widely accepted that tomato is one of the most consumed vegetable worldwide (Nicola et al., 2008) and this is corroborated by the constant increase of world production in the last 20 years (FAO, 2018). The EU tomato production accounted for the 21.1% of the total value of total fresh vegetable production in 2017 corresponding to 7.3 billion € with the greatest planted area, corresponding to the 10.8% of the total EU area planted with fresh vegetables (De Cicco, 2019). Moreover, the increase of the organic farming in EU in the last years might contribute to a stimulus for the demand of PBs to be applied in organic

production. Based on these evidences, we decided to focus our attention on the selection of PGPR having a positive effect on the growth of tomato plants as this choice will guarantee a broad market to a future commercial PB.

Based on the analysis of patents and recently published articles, we considered that the combination of a PGPR and humic acids might have a competitive advantage on the market. Indeed, the combination of non-microbial and microbial biostimulants was recently suggested for the design and development of the second generation of PB products (Rouphael and Colla, 2018). Among non-microbial biostimulants, humic acids play an important role in the soil fertility and the PBs containing humic acids have been already evaluated for their efficacy in improving crop production (Canellas et al., 2015; Nardi et al., 2002 Olivares at al., 2017). A synergistic effect was already observed in the case of the combined application of humic acids with endophytic bacteria on tomato plants and the mycorrhizal fungus *Rhizophagus irregulare* on onion seedlings and perennial ryegrass (Bettoni et al., 2014; Galambos et al., 2020; Nikbakht et al., 2014). Furthermore, humic acids can be used as a vehicle to introduce beneficial microorganisms to the soil as they are considered recalcitrant to microbiological attack (Canellas and Olivares, 2014).

Based on these ideas, the work started from the isolation of bacteria from the rhizosphere of tomato plants. In this context, different growth media may be used to collect a sound number of bacterial isolates. Frequently, growth media

commonly used in microbiology laboratories are used to isolate bacteria from environmental samples. In our case, we opted for a semi-selective growth medium mimicking the nutrient conditions that the bacterial cells may find in the tomato rhizosphere (Brescia et al., 2020). Thus, all the collected bacterial isolates that were collected from rhizosphere soil shared the ability to grow in the presence of these nutrients, representing a first indication of their rhizosphere competence. In future, growth media mimicking the nutrient conditions of rhizosphere might be a step that might be implemented in the isolation of microorganisms inhabiting the rhizosphere soils of various crop plants.

To proceed with a first screening step, we decided to take into consideration the compatibility with humic acids and a plant growth-promoting activity. As plant growth-promoting activity, we focused our attention on the phosphate solubilisation. Indeed, phosphorous is the second of the most important inorganic nutrients necessary for plant growth (Alori et al., 2017). However, plants absorb and assimilate phosphorus as inorganic orthophosphate, a form with low availability in most soils (Herrera-Estrella and López-Arredondo, 2016). Microorganisms able to convert the inorganic orthophosphate to soluble forms will increase the uptake of this nutrient by crop plants (Khan et al., 2008). Moreover, we thought that the choice of phosphate solubilisation might present an advantage from a commercial point of view. Indeed, a relevant number of microbial PBs includes PGPR able to fix nitrogen whereas the number of PBs

with the ability to solubilise phosphate is increasing slowly (Parnell et al., 2016). As a consequence, the designed PBs having PGPR able to solubilize phosphate might face a less strong competition when it will be commercialized.

Based on these aspects, the collection of bacterial isolates was screened for the ability to grow in presence of humic acids and solubilize phosphate. A sensitive reduction of the bacterial isolate collection was registered with only the 39% of bacterial isolates able to grow in presence of humic acids and solubilise phosphate.

To narrow the list of bacterial isolates that might be developed as PB, we decided to introduce steps that are not frequently used in the published articles dedicated to the characterization of potential PGPR. For instance, we took into consideration parameters related to the mass production, as they might impact the costs related to the production of bacterial cells. Firstly, we selected the bacterial isolates able to reach a cell concentration $\geq 10^9$ CFU/ml in 16 h. This threshold was chosen since minimum incubation time to reach highest cell density may allow a reduction in production cost (Posada-Uribe et al., 2015).

Moreover, this value represents an optimal concentration to develop a successful commercial strain since a threshold of approximately from 10^8 to 10^9 CFU/g or CFU/ml of formulation is required for inoculant quality standards (Malusá and Vassilev, 2014). We included also another selecting step concerning the resistance to desiccation since drying procedures may be included in the

formulation of microbial inoculants (Validov et al., 2007). To simulate desiccation, we used sorbitol since it is the most commonly used stress-inducing agent in solid medium which acts by lowering the w.p. of the medium (Claeys et al., 2014). We considered -2.5 MPa w.p. an acceptable value to select bacterial isolates able to survive desiccation conditions since it is reported that rhizobacteria are able to survive to up to -3.5 MPa w.p. (Abolhasani et al., 2010). As PBs may not act as biocontrol agents (Validov et al., 2007), we introduced an additional step that is not frequently reported in the published articles dedicated to the screening of bacterial isolate collection for the identification of new PGPR. We decided to discard bacterial isolates able to produce antibiotics active against *Py. ultimum*, a soil-borne plant pathogen commonly found in soils (Rai et al., 2020) that shows high sensitivity to secondary metabolites with antibiotic activity (Santos and Melo, 2016). Noteworthy, bacterial isolates able to produce antibiotics might represent potential active ingredients for new commercial biopesticides. Thus, research groups and companies might discard these bacterial isolates or considering their transfer in the pipeline for the characterisation of candidates for the development of microbial biopesticides.

Looking at the final commercial PB product, bacterial isolates that may be a threat to animals, crop plants and humans were also discarded (Tabassum et al., 2017; Köhl et al., 2011). We decided to use the classical hypersensitive reaction on tobacco leaves to assess the potential to establish a pathogenic interaction

with plants. This additional step is not found frequently in published screening strategies even if it is a cheap and fast method and might guarantee the discard of unmarketable bacterial isolates before proceeding with more expensive steps. Hypersensitive reaction on tobacco leaves was followed by the identification at species level based on 16S rDNA sequencing to determine if the bacterial isolates belonged to bacterial species potentially pathogenic to animal, human and plants. Notably, the 16S rDNA sequencing allowed to discard *Ps. lini* MVC 18 even if this bacterial isolate did not trigger the hypersensitive reaction in tobacco leaves. This result proves the importance of the identification of the microorganisms based on molecular tools to correctly discard microorganisms that may cause problems during their registration. Moreover, the sequencing results also highlighted how human opportunistic pathogens find in the rhizosphere their ideal niche (Berg at al., 2005) as the two bacterial strains belonging to *B. cereus* and one bacterial strain belonging to *K. intermedia* that were discarded. At the end of this screening procedure, the 6 % of the initial 200 bacterial isolates showed all the traits suitable for the development as a PB product.

As efficacy testing in bioassays and field conditions is time consuming and expensive, candidates might be screened first under *in vitro* or *in planta* conditions selecting the most promising candidates (Köhl et al., 2011). We evaluated the effect of bacterial inoculation on the seed germination, root and

shoot elongation to quickly select plant growth promoter candidates since seed vigour and viability determine seedling establishment, crop growth and productivity (Nehra et al., 2016). Only five bacterial strains (*A. enclensis* MVC 3, *A. oxydans* MVC 13, *Pae. nitroguajacolicus* MVC 6, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17) guaranteed a reproducibility of the results registering a positive effect at least in three out of the five experiments carried out.

Using the growth medium reproducing the rhizosphere conditions contributed to select bacterial strains able to grow using nutrients released by plants in the rhizosphere (Brescia et al. 2020). Indeed, all the selected bacterial strains were able to colonize actively roots of tomato seedlings and this ability was corroborated by their ability to form biofilm, structures that help bacteria to survive in hostile environments leading to increase the chance of survival (Seneviratne et al., 2010).

Although plate assays are not indicative of the mechanisms of action implemented by PGPR during their interaction with crop plants (Cardinale et al., 2015), understanding plant growth-promoting activities endowed by PGPR may represent a benefit for a future PB product from a commercial point of view (Backer et al., 2018). Thus, the selected bacterial strains were evaluated for their ability to produce plant growth regulators and increase the nutrient availability in the rhizosphere favouring the nutrient uptake (Vejan et al., 2016). *Pantoea agglomerans* MVC 21 and *Ps. putida* MVC 17 were the most effective strains

for the release of siderophores and solubilisation of phosphate and potassium in agreement with previous results (Ahmad et al., 2008; Mukherjee et al., 2020). Similarly, these bacterial strains were the best producer of IAA, a phytohormone involved in plant cell enlargement, division, tissue differentiation and responses to light and gravity (Sureshbabu et al., 2016). *Pantoea agglomerans* MVC 21 and *Ps. putida* MVC 17 were endowed by all these plant growth-promoting activities and were able to positively stimulate tomato plant growth in greenhouse conditions. However, further investigation will be carried out to better elucidate the mechanisms of actions that are implemented by these two PGPR during their interaction with tomato plants.

Overall, most of the methods used in this screening procedure are cheap and fast, two important factors that need to be taken into consideration before arriving to the industrialization of bacterial strains. Moreover, this screening procedure is provided with steps dedicated to the evaluation of traits related to the product development that are not frequently used in published articles dedicated to the identification and characterization of PGPR. This complete screening strategy might contribute to the selection of new candidates endowed with traits that might encounter the interest of private companies involved in the development of PBs. Indeed, this strategy led to the selection of *P. agglomerans* MVC 21 and *Ps. putida* MVC 17, which represent good candidates for future steps aimed at designing a novel formulation with these bacterial strains.

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Chapter 3

Ecological role of volatile organic compounds emitted by *Pantoea agglomerans* as interspecies and interkingdom signals

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Abstract: Volatile organic compounds (VOCs) play an essential role in microbe-microbe and plant-microbe interactions. We investigated the interaction between two plant growth-promoting rhizobacteria, and their interaction with tomato plants. VOCs produced by *Pantoea agglomerans* MVC 21 modulates the release of siderophores, the solubilization of phosphate and potassium by *Pseudomonas (Ps.) putida* MVC 17. Moreover, VOCs produced by *P. agglomerans* MVC 21 increased lateral root density (LRD), root and shoot dry weight of tomato seedlings. Among the VOCs released by *P. agglomerans* MVC 21 only dimethyl disulfide (DMDS) showed effects similar to *P. agglomerans* MVC 21 VOCs. Because of the effects on plants and bacterial cells, we investigated how *P. agglomerans* MVC 21 VOCs might influence bacteria-plant interaction. Noteworthy, VOCs produced by *P. agglomerans* MVC 21 boosted

the ability of *Ps. putida* MVC 17 to increase LRD and root dry weight of tomato seedlings. These results could be explained by the positive effect of DMDS and *P. agglomerans* MVC 21 VOCs on acid 3-indoleacetic production in *Ps. putida* MVC 17. Overall, our results clearly indicated that *P. agglomerans* MVC 21 is able to establish a beneficial interaction with *Ps. putida* MVC 17 and tomato plants through the emission of DMDS.

Keywords: VOC; plant growth-promoting rhizobacteria; *Pantoea agglomerans*; *Pseudomonas putida*; tomato seedlings; dimethyl disulfide.

1. Introduction

Plant growth is affected significantly by the intimate interactions established with microorganisms residing in the rhizosphere and, indirectly, on the interactions among these microorganisms. The rhizosphere is indeed a hot spot where microbe-microbe and plant-microbe interactions occur (Bakker et al. 2013; Mendes et al. 2013). This ecological niche is occupied by a plethora of microorganisms that establish complex interactions with neighbouring microflora, microfauna and plant roots via production of secondary metabolites enabling them to respond and adapt to environmental changes (Raaijmakers et al. 2009).

Ranging from 10^6 to 10^{12} gene copy numbers/g of sample, bacteria represent the

most abundant class of microorganisms residing in soil, plant roots and rhizosphere (Leach et al., 2017). In these regions, bacteria cells regulate their activities through quorum sensing (QS) systems, based on the emission and perception of communication signals (Venturi and Keel, 2016; Waters and Bassler, 2005). These communication signals play an important role in the intraspecies interactions regulating, for example, biofilm formation, biosynthesis of antibiotics and plasmid conjugal transfer (Fuqua and Winans, 1994; Maddula et al., 2006; Pierson et al., 1994). They are also involved in the interspecies interactions by mediating competition and/or cooperativity among bacteria (Evans et al., 2018; Lloyd and Whitworth, 2017), but they can be sensed also by (micro)organisms belonging to other kingdoms (Venturi and Fuqua, 2013). Among chemical communication signals, N-acyl homoserine lactones (AHLs) received most of the attention so far (Fuqua et al., 2001; Abisado et al., 2018). As AHLs are produced by numerous bacterial species residing in the rhizosphere, they may act as interspecies signals modulating the behaviour of bacterial co-existing populations (Pierson et al., 1998). At the same time, AHLs can also constitute interkingdom signals and their perception by plants may trigger systemic plant resistance and/or affect plant growth and development (Nawaz et al., 2020; Schenk et al., 2012; Schikora et al., 2011).

Volatile organic compounds (VOCs) are a new class of interspecies and interkingdom signal compounds (Schulz-Bohm et al., 2017). They are small

molecules (<400 Da) belonging to different chemical classes that can evaporate and diffuse easily through air- and water-filled pores (Schulz and Dickschat, 2007). These properties make VOCs ideal signal candidates in the interactions with plant roots. Bacteria were found to produce more than 1,000 VOCs and non-organic volatile compounds (Audrain et al., 2015) and some of them increase yield and quality of crop plants. In fact, bacterial VOCs may modulate several physiological processes such as photosynthesis, plant hormone balance (Zhang et al., 2007), uptake of nutrients from soil (Aziz et al., 2016; Zhang et al. 2009), systemic plant resistance mechanisms (Lee et al., 2012) and tolerance to soil salinization and drought stress (Zhang et al., 2008a,b). At the same time, bacterial VOCs may act as interspecies communication signals playing a relevant role in the cooperation and competition among soilborne bacteria that are not in contact to each other (Tyc et al., 2017). Indeed, bacterial VOCs may can act as signal molecules between bacteria (Lee et al., 2015; Wang et al., 2001) and inhibit or promote the growth of other bacteria (Garbeva et al., 2014a; Raio et al., 2020; Tyc et al., 2014). While the involvement of these chemical compounds in the complex interaction webs of the rhizosphere is largely acknowledged their specific ecological roles are still far from being fully understood.

As bacterial VOCs are thought to be interspecies and interkingdom communication signals, the aim of our study was to clarify the interaction

between two plant growth-promoting rhizobacteria (PGPR) and tomato plants, by analysing the role of the bacterial VOCs in microbe-microbe interaction and, in parallel, their plant growth-promoting effect.

2. Material and methods

2.1. Microorganisms and plants

The bacterial strains used in this work, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17, previously isolated from tomato rhizosphere (Vasseur-Coronado et al., 2021), were stored at length in glycerol 40% at -80°C and routinely grown on Nutrient Agar (NA, Neogen, Miami, USA) in common Petri dishes (90 mm diameter). To prepare bacterial cell suspensions, bacterial strains were grown in 5 ml of Nutrient Broth (NB, Oxoid, Basingstoke, UK) at 27ºC on an orbital shaker (200 rpm). After 24 h, a volume of one ml of bacterial cell suspensions was centrifuged (13,000 rpm, 2 min) and pellets were suspended in NaCl solution (0.85% w/v) to a final optical density at 600 nm ($A_{OD600nm}$) of 0.1 corresponding to $\simeq 1 \times 10^7$ colony forming units (CFU)/ml and used in all the experiments, except when otherwise indicated.

Tomato seeds (*Solanum lycopersicum* var. Moneymaker; Justseed, Wrexham, UK), surface sterilised according to Vasseur-Coronado et al., (2021), were sown on common Petri dishes containing 25 ml of Murashige and Skoog Agar medium (MS, Murashige and Skoog, 1962) supplemented with 1.5% (w/v)

of sucrose (Sigma-Aldrich, St. Louis, MO, USA). Dishes were sealed with double layer of Parafilm tape and kept in a growth chamber [25 ± 1°C; 70 ± 10% relative humidity (RH); 16 h photoperiod] for 96 h. Four-days-old tomato seedlings were used in all the experiments, except when otherwise indicated.

2.2. Evaluation of compatibility between Pantoea agglomerans *MVC 21 and* Pseudomonas putida *MVC17*

A volume of 10 µl of *P. agglomerans* MVC 21 cell suspension was spot-inoculated onto NA in Petri dishes. Subsequently, 10 µl of *Ps. putida* MVC 17 cell suspension was spot-inoculated at 1 cm distance from *P. agglomerans* MVC 21. Similarly, the compatibility between the two strains was tested in split Petri dishes (92 mm) with two compartment and ventilation cams (Sarstedt, Nümbrecht, Germany) following the same procedure. The difference consisted in the presence of the separation border of the split Petri dish located in the middle of the 1 cm distance occurring between the two bacterial strains. Once bacterial strains were spot-inoculated, split Petri dishes were sealed with double layer of Parafilm tape (Beims, Neenah, WI, USA). Monocultures consisting of NA contained in common and split Petri dishes inoculated solely with *P. agglomerans* MVC 21 or *Ps. putida* MVC 17 were used as untreated controls. After 48 h incubation at 25°C, dishes were photographed with Bio-Rad Quantity One software implemented in a Bio-Rad Geldoc system (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.). Digital images were subsequently used to

measure the area of bacterial strain macrocolonies using the software ImageJ1.50i (Schneider et al., 2012).

To determine the quantity of *P. agglomerans* MVC 21 and/or *Ps. putida* MVC 17 viable cells residing in the macrocolony, plugs (5 mm diameter) were sampled from each macrocolony and transferred into sterile 1.5 ml microcentrifuge tubes containing 1 ml of sterile NaCl solution (0.85%). After 1 h incubation at 25°C on an orbital shaker (200 rpm), bacterial cell suspensions were serially diluted (from 10^{-1} to 10^{-7}) and those dilutions were plated onto 1/10 Tryptic Soy Agar [TS broth (Oxoid), Agar Technical No.2 16 g/l (Oxoid)]. Once inoculated, Petri dishes were maintained at 25°C and the developed CFUs were counted after 48 h incubation.

To assess the quantity of cell residing in the macrocolony, the following formula was applied:

$$Viable\ cells\ residing\ in\ macrocolony\ \ (log_{10}) =$$

$$\frac{Counted\ CFU\ \times Surface\ area\ of\ the\ macrocolony}{Surface\ area\ of\ the\ 5\ mm\ plug}$$

Six replicates (Petri dishes) were used for each treatment, and the experiment was repeated.

2.3. Assessment of the interaction effect on plant growth-promoting activities of Pantoea agglomerans *MVC 21 and* Pseudomonas putida *MVC 17*

The ability of the tested bacterial strains to chelate iron and to solubilise phosphate and potassium was assessed in monoculture and in pairwise combination both on common and split Petri dishes. Aleksandrow Agar (AA, HiMedia GmbH, Germany), Chrome Azurol (CAS) agar and National Botanical Research Institute's phosphate (NBRIP) medium were used respectively to assess iron chelation and solubilisation of phosphate and potassium according to Vasseur-Coronado et al. (2021). In the case of common Petri dishes, bacterial cell suspensions were spot-inoculated onto AA, CAS agar and NBRIP according to the procedure reported above. In the case of split Petri dishes, one compartment was filled with NA (25 ml) and the second one was filled either with five ml of AA, or CAS agar or NBRIP, respectively. A volume of 10 µl of *P. agglomerans* MVC 21 and/or *Ps. putida* MVC 17 cell suspension was spot-inoculated onto the compartment containing NA. Split Petri dishes were sealed with a double layer of Parafilm tape and incubated at 25°C. After 48 h, a volume of five µl of *Ps. putida* MVC 17 and/or *Ps. putida* MVC 17 cell suspension was spot-inoculated onto AA, CAS agar and/or NBRIP contained in the second compartment. Bacterial strains grown as monocultures on AA, CAS agar and NBRIP were used as untreated controls both common Petri dishes. Split Petri dishes having the compartment containing NA not inoculated with any bacterial

strain were used as untreated controls.

Once inoculated, dishes containing CAS agar were incubated at 25°C for 24 h whereas dishes containing AA and NBRIP were incubated at 25°C for 96 h. After each incubation period, common and split Petri dishes were photographed with Bio-Rad Quantity One software implemented in a Bio-Rad Geldoc system (Bio-Rad Laboratories, U.S.A.). In the case of split Petri dishes, ImageJ1.50i was used to measure the area of orange haloes (release of siderophores) and clarification haloes (solubilisation of phosphate and potassium) formed around bacterial macrocolonies. In the case of common Petri dishes, the haloes were measured on the macrocolony side without contact with the interacting bacterial strain (pairwise combination) or on the corresponding side in the untreated control. For each treatment, six replicates (Petri dishes) were used and the experiment was repeated.

2.4. Evaluation of the effect of VOCs emitted by Pantoea agglomerans *MVC 21 and* Pseudomonas putida *MVC 17 on tomato plant growth*

A volume of 20 µl of *P. agglomerans* MVC 21 and/or *Ps. putida* MVC 17 cell suspension was spot- inoculated into NA contained in one compartment of split Petri dishes. Subsequently, split Petri dishes were sealed with a double layer of Parafilm tape and incubated at 25°C. After 48 h, three tomato seedlings were placed in the second compartment containing MS medium (25 ml). Tomato

seedlings exposed to NA only were used as untreated control. Dishes were sealed with a double layer of Parafilm tape and incubated in a growth chamber (25 ± 1°C; 70 ± 10% RH; 16 h photoperiod). After 10 days, plant fresh weight (mg) and lateral root density [LRD; number of lateral roots/length main root (cm)] were determined according to Cordovez et al. (2018). Plant dry weight (mg) was determined after overnight incubation at 65°C in an incubator. Four replicates (split Petri dishes) were used for each treatment and the experiment was repeated.

2.5. Metabolite profiling of VOCs emitted by Pantoea agglomerans *MVC 21*

2.5.1. Preparation of samples for headspace analysis

For headspace volatile analysis, *P. agglomerans* MVC 21 was inoculated into headspace (HS) vials according to Lazazzara et al. (2017) with some modifications. Briefly, 5 ml of sterilised NA was poured into sterile HS vials (20 mL, La-Pha-Pack, Langerwehe, Germany) and placed horizontally under the laminar flow cabinet. The HS vials were left open overnight at room temperature under the laminar flow cabinet to avoid water condensation. A volume of 20 µl of *P. agglomerans* MVC 21 cell suspension was spot-inoculated onto NA and the HS vials were left to dry under the laminar flow cabinet for at least 2 h. HS vials containing only NA were used as untreated controls in order to distinguish the VOCs emitted by the culture medium. Each HS vial was then tightly sealed

with sterile metal caps containing 1.3 mm-silicone/PTFE septa (La-Pha-Pack) and incubated at 25°C for 144 h before GC-MS measurement. The 144 h time point was chosen as it showed the richest VOC profile of *P. agglomerans* MVC 21 growing in HS vials.

2.5.2. Headspace GC-MS analysis

The analysis of VOCs emitted by *P. agglomerans* MVC 21 was performed according to the procedure described by Vlassi et al. (2020). Briefly, a gas chromatograph (GC) was coupled to a mass selective detector (MSD). The GC-MSD was equipped with a multi-purpose autosampler (MPS), a dynamic headspace system (DHS, Gerstel), a thermal desorption unit (TDU) and a cooled injection system (CIS) unit. Following a 15 min incubation in the DHS at 27°C, VOCs were dynamically collected from the samples and trapped on a 2 cm TENAX trap at 30°C . The tenax tube was subsequently dried to remove potentially trapped water. VOCs were then thermally desorbed by heating the TDU unit from 30°C to 230°C at a rate of 60°C/min, followed by a hold time of 5 min and transferred to the GC-MS column by cooled injection in the splitless mode (from -150°C to 250°C at a rate of 2°C/s, hold time 6 min). The chromatographic separation was performed on a HP-MS (5% phenyl methylsiloxane) column (30 m × 0.25 mm × 0.25 μm). The GC oven temperature program was the following: 35°C for 2 min, raised to 200°C at 5°C/ min (hold

time 1 min) increased from 200°C to 250°C at 20°C/min (hold time 5 min). Helium was used as carrier gas at a flow rate of 1 ml/min.

The open-source software MetaboliteDetector (Hiller et al. 2009) version-3.1. (http://metabolite_detector.tu-bs.de/) was used to process the data. The initial parameters used were peak threshold 5.00, peak height 5.00, bins/scan 10 and deconvolution width (scan) 5.00. For compound annotation and identification a similarity score was calculated with the MetaboliteDetector program by combining the retention index (RI) fit and the similarity of the compound mass spectra. A similarity score ≥0.8 with ΔRI< 5.00 was required. For identification of compounds the spectra and RI values were compared to authentic standards measured under the same GC-MS conditions. Compound annotation was based on comparisons with entries from NIST 14 library (National Institute of Standards and Technology, USA, http://www.nist.gov). The RI was calculated by the software by comparing the experimental retention time to those of a series of n-alkanes (C8-C25) measured under the same chromatographic conditions. Identification levels were assigned according to the criteria described by Blaženović et al. (2018).

2.5.3. Preparation of volatile organic compound solutions for functional assays

Pure 2-phenylethyl alcohol (2PEA), 3-methyl-1-butanol (3M1B) and dimethyl disulfide (DMDS), were purchased (Sigma Aldrich) and tested for their

individual effects on tomato plant growth and on the ability of *Ps. putida* MVC 21 to chelate iron, produce IAA and solubilise phosphate and potassium.

To determine the dosage to be used for the VOC-mediated functional assays, the amount of each VOC emitted by *P. agglomerans* MVC 21 in the HS vials was estimated as follows:

$$Absolute\ amount\ of\ each\ standard\ (\mu g) =$$

$$\begin{pmatrix} Concentration\ of\ each\ standard\ (\mu g/\mu L)\ \times \\ Volume\ (\mu L) of\ standard\ used\ in\ HS\ vial \end{pmatrix}$$

$$Calculation\ factor\ of\ each\ standard\ (\mu g/Area) =$$

$$\frac{Absolute\ amount\ of\ each\ standard\ (\mu g)}{Average\ peak\ area\ measured\ for\ each\ standard}$$

$$Estimated\ amount\ of\ compound\ (\mu g) =$$

$$\begin{pmatrix} Average\ peak\ area\ measured\ for\ VOC\ in\ the\ GC-MS\ chromatogram \\ \times\ Calculation\ factor\ of\ each\ compound \end{pmatrix}$$

Stock solutions with a concentration of 0.02 ng, 0.2 ng, 2 ng and 20 ng per split Petri dish of DMDS; 0.09 ng, 0.9 ng and 9 ng, 90 ng per split Petri dish of 2PEA and 9 ng, 90 ng and 900 ng, 9000 ng per split Petri dish of 3M1B were prepared by serial dilution using ethanol (Merck KGaA, Darmstadt, Germany) to test the effects of pure VOC on PGP traits or methanol (Merck) to test the effects of pure VOC on tomato plant growth. Ethanol and methanol were used as solvents since they are reported to be nontoxic to bacteria and plants respectively (Schmidt et

al. 2016; Meldau et al. 2013).

2.6. Evaluation of the effect of pure VOCs on tomato plant growth and plant growth-promoting traits of Pseudomonas putida *MVC 17*

In all experiments, a filter paper (90 mm, VWR) was placed into one compartment of split Petri dishes and inoculated with 20 µl of the stock solutions of 2PEA, 3M1B and DMDS at different concentrations. In the case of the effect on tomato plant growth, three sterilised and pre-germinated tomato seeds were placed into the second compartment of the split Petri dish containing MS medium following the procedure reported above. To assess the effects of pure VOCs on the ability of *Ps. putida* MVC 17 to release siderophores and solubilise phosphate and potassium, CAS agar, NBRIP or AA medium were poured into the second compartment of the split Petri dish and inoculated with 10 µl of *Ps. putida* MVC 17 as mentioned above.

Pseudomonas putida MVC 17 or pre-germinated tomato seeds exposed to filter papers wetted with ethanol and methanol respectively were used as untreated controls. For each test, split Petri dishes were sealed with a double layer of parafilm tape and incubated following the timing mentioned above. After each respective incubation period, LRD, tomato root, shoot dry weight and the ability of *Ps. putida* MVC 17 to release siderophores, solubilise of phosphate and potassium were evaluated as mentioned above. To determine whether exposure

to pure VOC affected the viability of *Ps. putida* MVC 17 cells, the number of bacterial cells residing in the macrocolony area upon exposure to pure VOC was assessed as mentioned above. Four replicates (split Petri dishes) were used for each treatment and the experiment was repeated.

2.7. Effect of VOCs released by Pantoea agglomerans *MVC 21 on the interaction between* Pseudomonas putida *MVC 17 and tomato seedlings*

A volume of 20 µl of *P. agglomerans* MVC 21 cell suspension was spot-inoculated onto NA (25 ml) contained in one compartment of split Petri dish. Subsequently, dishes were sealed as reported above and incubated at 25°C. After 48 h, germinated tomato seeds were inoculated with one ml of *Ps. putida* MVC 17 cell suspension according to the procedure described by Vasseur-Coronado et al. (2021). Once inoculated, three tomato seeds were placed into the compartment of split Petri dishes containing MS (25 ml). Split Petri dishes having the compartment containing NA not inoculated with *P. agglomerans* MVC 21 and the compartment with MS containing tomato seeds inoculated with *Ps. putida* MVC 17 were used as untreated control. Dishes were sealed with a double layer of Parafilm tape and incubated in the growth chamber as reported above. After 10 days, plant fresh weight, LRD and plant dry weight were determined. Four replicates (split Petri dishes) were used for each treatment and the experiment was repeated.

2.8. Evaluation of the effect of dimethyl disulfide and volatile organic compounds emitted by Pantoea agglomerans *MVC 21 on the production of indole-3-acetic acid in* Pseudomonas putida *MVC 17*

IAA production was evaluated according to the procedure described by Vasseur-Coronado et al. (2021) with modifications. A volume of 20 µl of *P. agglomerans* MVC 21 cell suspension was added into one well of 25-well polystyrene plates (Thermo Fisher Scientific, Waltham, MA, USA) containing 3 ml of NA. The rest of wells were filled with 3 ml of DF salt minimal broth amended with 500 µg/ml of L-Tryptophan (Sigma-Aldrich). In the case of DMDS, a sterilised filter paper was placed into one well of 25-well polystyrene plates and was inoculated with 20 µl of a DMDS stock solution (8 mg/mL) to have a final concentration equivalent to 0.02 mg/Petri dish, assuming the complete evaporation of the VOC from the filter paper. Subsequently, the 25-well polystyrene plates were introduced in Petri dishes (150 mm diameter) and sealed with doubled layer of Parafilm tape and kept in the incubator at 25°C. After 2 days incubation, a volume of 300 µl of *Ps. putida* MVC 17 cell suspension was added to wells containing DF salt minimal broth (3 ml) amended with 500 µg/ml of L-Tryptophan. Petri dishes were sealed with a double layer of Parafilm tape and incubated at 28 °C on an orbital shaker (200 rpm). As untreated controls, 25-well polystyrene plates with wells containing non-inoculated NA and/or sterile filter papers were used. After 120 h, the quantity of IAA produced by bacterial

cells was monitored according to Vasseur et al. (2021) and expressed as the ratio between $A_{OD530nm}$ and AOD_{600nm}. For each treatment, six replicates (25-well polystyrene plates) were used and the experiment was repeated.

2.9. Statistical Analysis

All experiments were carried out twice. Normality (Shapiro-Wilk test, $p>0.05$) and variance homogeneity (Levene´s test, $p>0.05$) were checked and parametric tests were used. Non-significant differences were found between two experiments ($p>0.05$) after two-way ANOVA and thus, data from experiments were pooled. Data were subsequently analysed using one-way ANOVA and mean comparisons between treatments were assessed by Tukey's test ($\alpha= 0.05$). The statistical analysis was performed using IBM SPSS software (Version 21).

3. Results

3.1. Compatibility between Pantoea agglomerans *MVC 21 and* Pseudomonas putida *MVC 17*

No toxic effect was detected in the compatibility test carried out in Petri dishes. Instead, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 cell density increased significantly when they interacted, compared to when they grew alone (Table S1A). Similarly, no negative effect of VOCs emitted by *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 was observed on either bacteria, when they interacted in split Petri dishes (Table S1B).

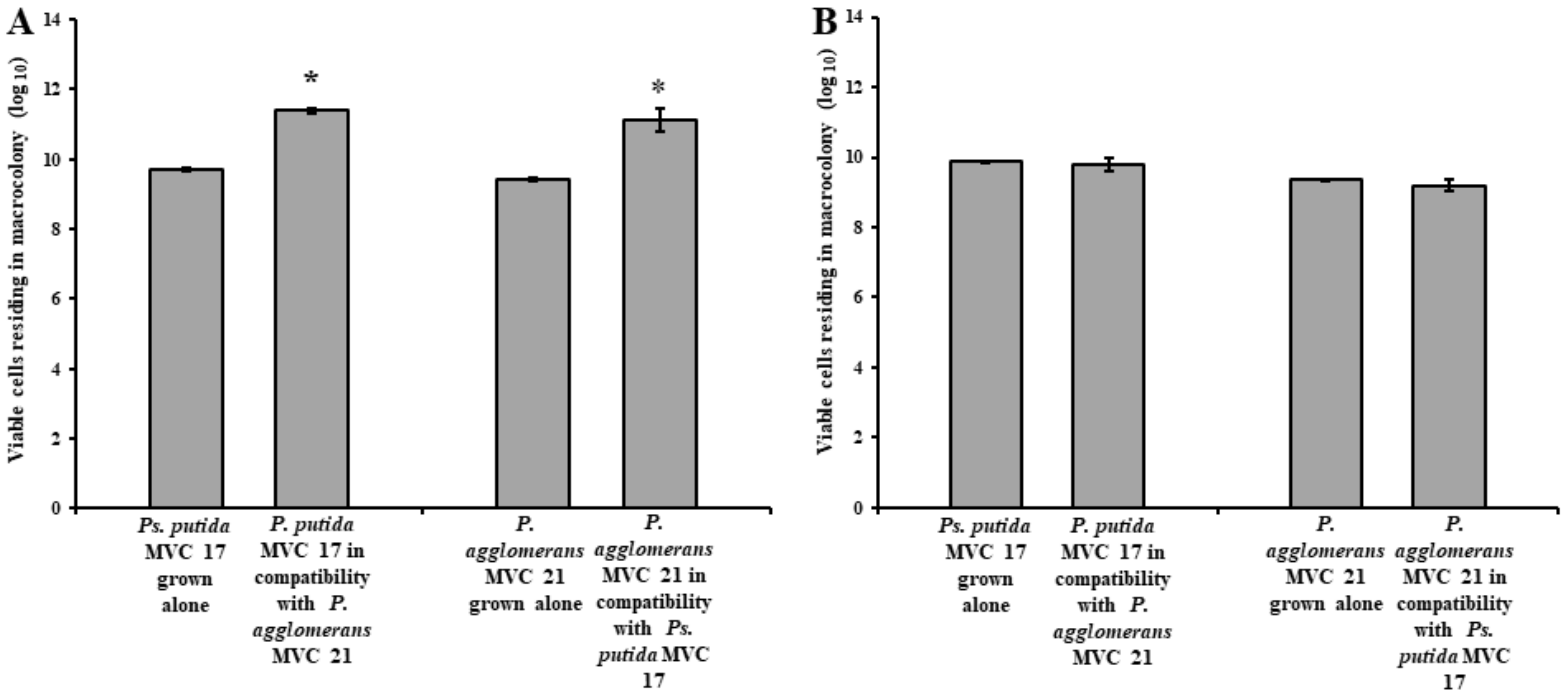

Figure S1. Compatibility between *Pantoea agglomerans* MVC 21 and *Pseudomonas* (*Ps.*) *putida* MVC 17. The quantity of viable cells residing in the macrocolonies of *P. agglomerans* MVC 21 and *P. putida* MVC 17 when grown in common Petri dishes (A) and in split Petri dishes (B) was assessed after 48 h incubation at 25 °C. Columns represent mean ± standard error values of six replicates (Petri dishes) for each treatment. Data from two independent experiments were pooled. Asterisks indicate significant differences between

treatments according to Student´s t-Test ($\alpha = 0.05$).

3.2. The interaction with Pantoea agglomerans *MVC 21 modulates the plant growth-promoting activities of* Pseudomonas putida *MVC 17*

No negative effect of VOCs on *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 cell viability was observed in these experiments (Fig. S2).

In common Petri dishes, *Ps. putida* MVC 17 significantly increased (19 %) the release of siderophores when interacting with *P. agglomerans* MVC 21 compared to the untreated control the (Fig. 1A). However, the co-inoculum significantly decreased the ability of *Ps. putida* MVC 17 to solubilise phosphate (17 %) and potassium (43 %) compared to the untreated control (Fig. 1B,C). A similar effect was observed with VOCs emitted by *P. agglomerans* MVC 21 that significantly increased the ability of *Ps. putida* MVC 17 to release siderophores (6 %) compared to the untreated control (Fig. 1D). Conversely, *P. agglomerans* MVC 21 VOCs caused a significant decrease in the ability of *Ps. putida* MVC 17 to solubilise phosphate (47 %) and potassium (35 %) compared to the untreated control (Fig. 1E,F). Differently, *Ps. putida* MVC 17 did not affect the plant growth-promoting activity of *P. agglomerans* MVC 21 compared to the untreated control in both experiments carried out in common and split Petri dishes (Fig. 1A-F).

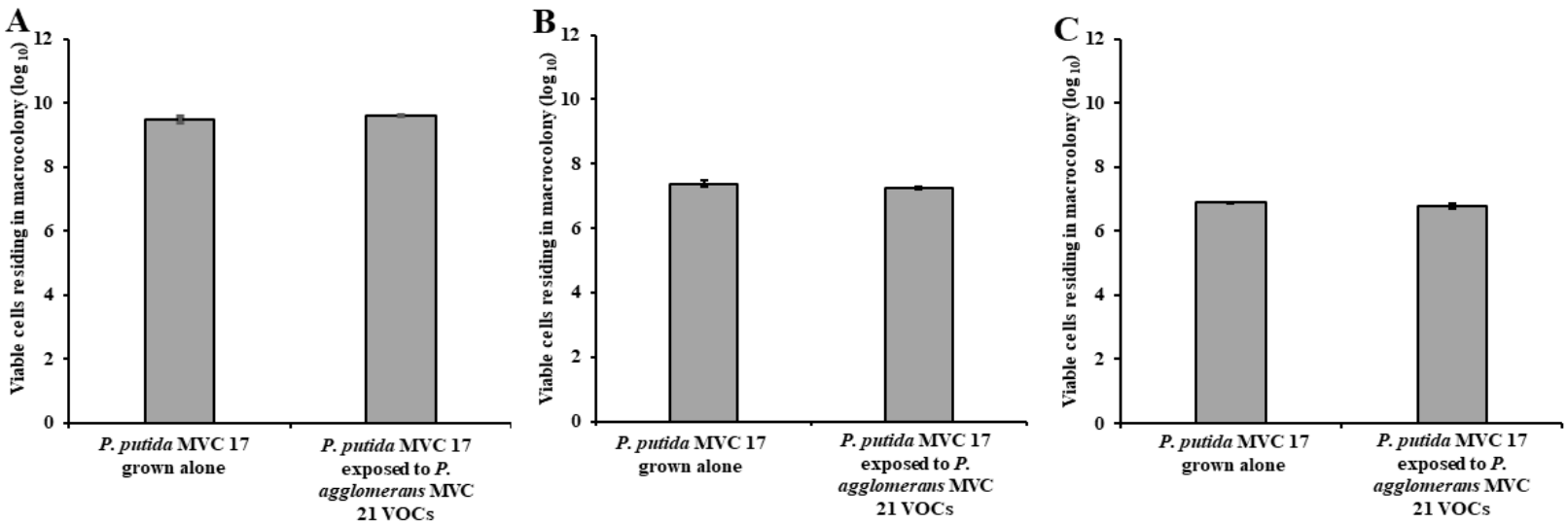

Figure S2. Viability of *Pseudomonas* (*Ps*). *putida* MVC 17 cells after exposure to *Pantoea agglomerans* MVC 21 volatile organic compounds (VOCs). The number of *Ps. putida* MVC 17 viable cells grown on different media for detecting the release of siderophores (A), solubilisation of phosphate (B) and potassium (C) was evaluated upon exposure to *P. agglomerans* MVC 21 VOCs. Columns represent mean ± standard error values of six replicates (Petri dishes) for each treatment. Data from two independent experiments were pooled. Asterisks indicate significant differences between treatments according to Student´s t-Test ($\alpha = 0.05$).

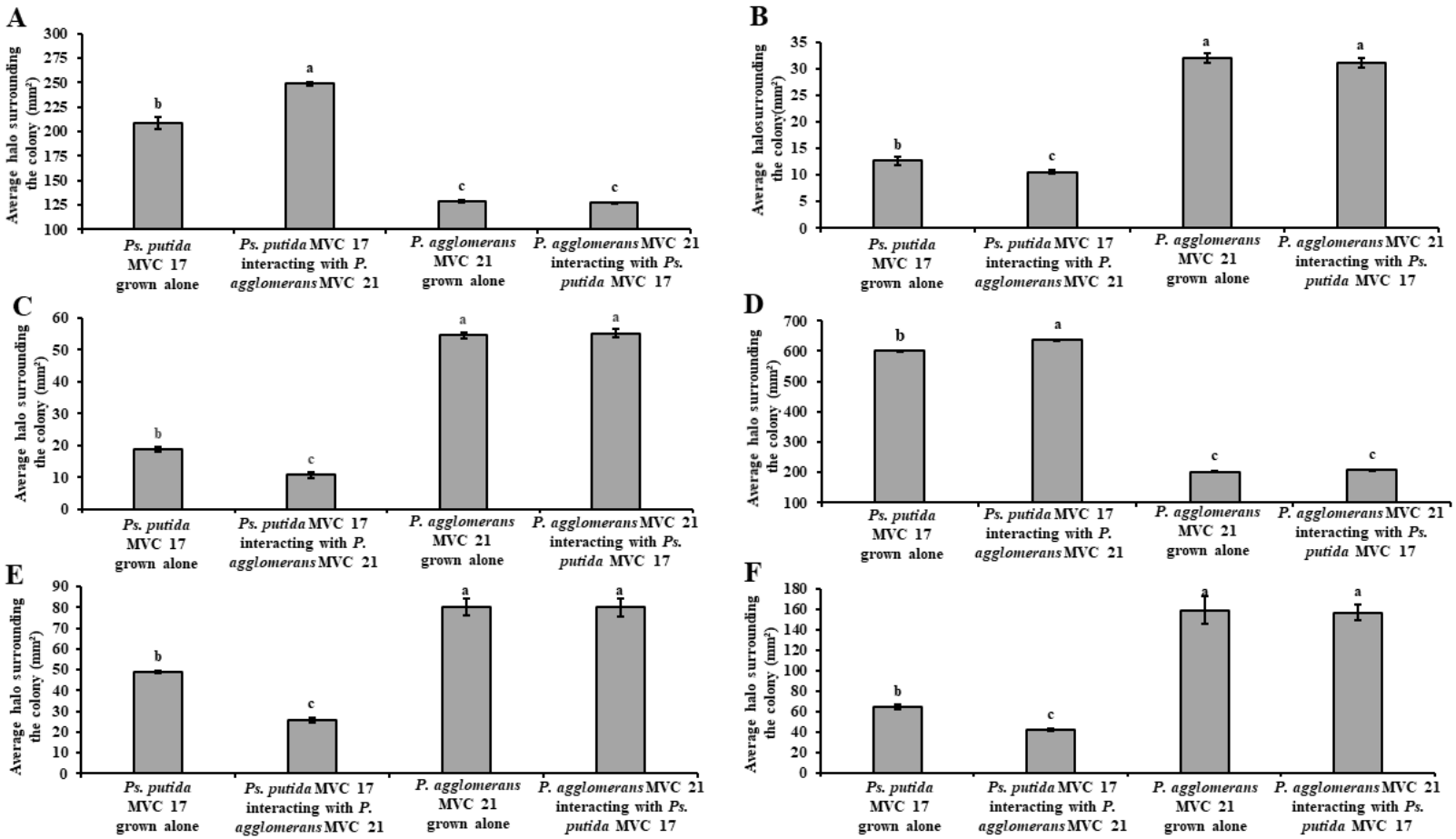
A
Average halo surrounding the colony (mm²)
300
275
250
225
200
175
150
125
100
b
a
c
c
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17
B
Average halo surrounding the colony(mm²)
35
30
25
20
15
10
5
0
b
c
a
a
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17
C
Average halo surrounding the colony (mm²)
60
50
40
30
20
10
0
b
c
a
a
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17
D
Average halo surrounding the colony (mm²)
700
600
500
400
300
200
100
b
a
c
c
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17
E
Average halo surrounding the colony (mm²)
90
80
70
60
50
40
30
20
10
0
b
c
a
a
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17
F
Average halo surrounding the colony (mm²)
180
160
140
120
100
80
60
40
20
0
b
c
a
a
Ps. putida MVC 17 grown alone
Ps. putida MVC 17 interacting with P. agglomerans MVC 21
P. agglomerans MVC 21 grown alone
P. agglomerans MVC 21 interacting with Ps. putida MVC 17

Figure 1. Effect of bacterial interaction on plant growth-promoting activities. Assessment of the ability of *Pantoea agglomerans* MVC 21 and *Pseudomonas* (*Ps*.) *putida* MVC 17 to release siderophores (A,D) and to solubilise phosphate (B,E) and potassium (C,F) during the interaction in common (A-C) and split Petri (D-F) dishes. Columns represent mean ± standard error of six replicates (Petri dishes) are reported for each treatment. Data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

3.3. Pantoea agglomerans *MVC 21 releases VOCs with a positive impact on tomato seedling growth*

The ability of *Ps. putida* MVC 17 and *P. agglomerans* MVC 21 to stimulate plant growth through the release of VOCs was evaluated in split Petri dishes. Upon exposure to *Ps. putida* MVC 17, no significant changes in tomato Lateral Root Density (LRD), root or shoot biomass were observed (Fig. 2A).

Conversely, when exposed to *P. agglomerans* MVC 21 VOCs, tomato seedlings significantly increased the LRD (125 %), the shoot (71 %) and root dry weight (81 %) compared to the seedings exposed to agar medium only (Fig. 2B,C).

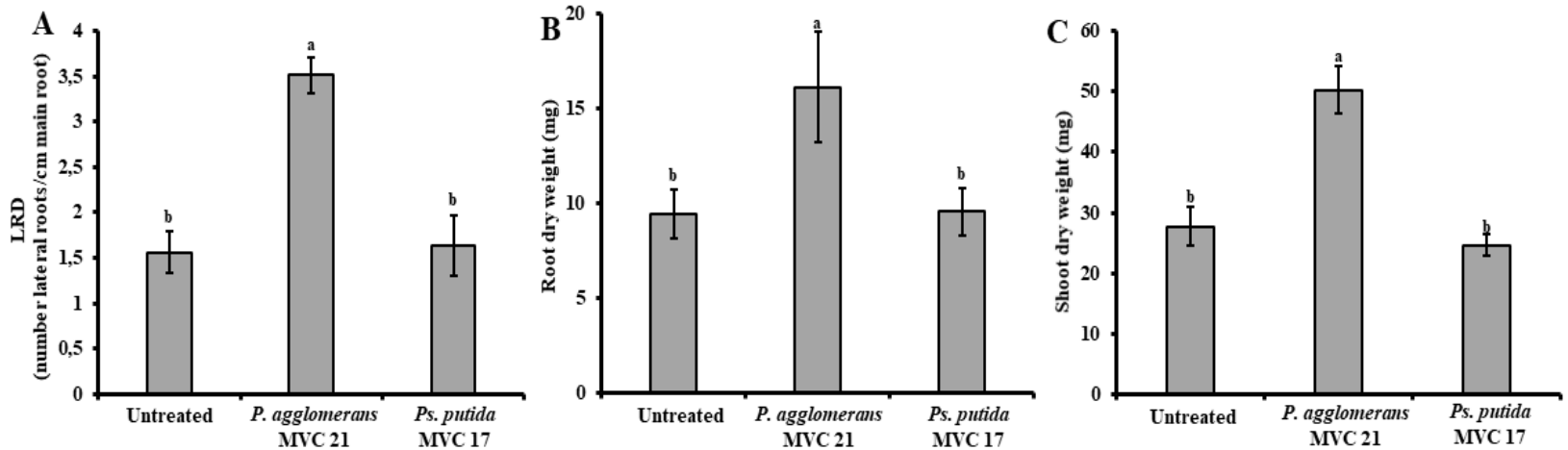

Figure 2. Plant growth-promoting effect of volatile organic compounds (VOCs) emitted by *Pantoea agglomerans* MVC 21 and *Pseudomonas (Ps.) putida* MVC 17. Lateral Root Density (LRD, A), root (B) and shoot (C) dry weight of tomato seedlings were assessed after 10 days of exposure to *P. agglomerans* MVC 21 and *Ps. putida* MVC17 VOCs. Untreated tomato seedlings were exposed to agar medium only. Columns represent mean ± standard error values of twelve replicates (tomato seedlings) are reported for each treatment. Data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

3.4. Headspace analysis of VOCs using GC-MS

Only a few VOCs were produced at levels above the limit of detection of the applied GC-MS method.

In total, seven VOCs were identified in the volatile profile of *P. agglomerans* MVC 21 (Table 1). One of them, namely 2-undecanone, was only found in one out of 10 replicate headspace (HS) cultivations. Additionally, some VOCs, namely 2-tridecanone, 2-nonanone, 1-tetradecanol, were found in two and five cultures out of 10, respectively. Three VOCs, namely 2-phenylethyl alcohol (2PEA), 3-methyl-1-butanol (3M1B) and dimethyl disulfide (DMDS), were most consistently (10 out of 10 HS vials) detected. After 96 h of incubation in the HS vial, the estimated amount of each VOC produced by *P. agglomerans* MVC 21 was 0.0021 µg of DMDS, 9.600 µg of 3M1B and 0.0912 µg of 2PEA.

Table 1. Volatile organic compounds (VOCs) detected by gas chromatography-mass spectrometry (GC-MS) in the headspace of vials inoculated with *Pantoea agglomerans* MVC 21 and incubated for six days.

Metabolite[1]	**RI[2]**	**Sim score[3]**	**Level of identification[4]**	**HS vials[5]**
Dimethyl disulfide	735	0.98	1	10/10
3-Methyl-1-butanol	752	0.99	1	10/10
2-Phenylethyl-alcohol	1115	0.97	1	10/10
2-Tridecanone	1498	0.89	1	5/10
2-Nonanone	1093	0.87	1	2/10
1-Tetradecanol	1671	0.91	1	2/10
2-Undecanone	1295	0.87	1	1/10

[1]VOCs not related to the untreated control. [2]Average retention index (RI). [3]Average similarity score ≥0.80. [4]Levels of identification were assigned according to Blaženović et al. (2018) where 1= confident identification by comparison of GC-MS spectrum and RI to standard compounds analysed under the same chromatographic and MS conditions. [5]Number of sample replicates in which the VOC was detected to the total number of replicates analysed.

3.5. Dimethyl disulfide shows a positive effect on Ps. putida *MVC 17 plant growth-promoting activities and* on *tomato plant growth*

Upon exposure to 2PEA, 3M1B and DMDS, the number of viable cells in *Ps. putida* MVC 17 macrocolony did not change compared to the untreated control (Fig. S3). 2PEA and 3M1B did not affect *Ps. putida* MVC 17 plant growth-promoting activities (Table S1-S2) whereas DMDS showed modulating activity in a concentration-dependent manner. In particular, DMDS increased the ability of *Ps. putida* MVC 17 to release siderophores at the highest concentration tested (Table 2). In contrast to this, DMDS decreased the ability of *Ps. putida* MVC 17 to solubilise phosphate and potassium (17 % and 40 %; Table 2).

At the tested concentrations, neither 2PEA nor 3M1B had any effect on tomato seedlings (Table S1-S2), whereas DMDS showed a significant increase in the LRD (64 %), root (367 %) and shoot (239 %) dry weight compared to the untreated control (Table 2).

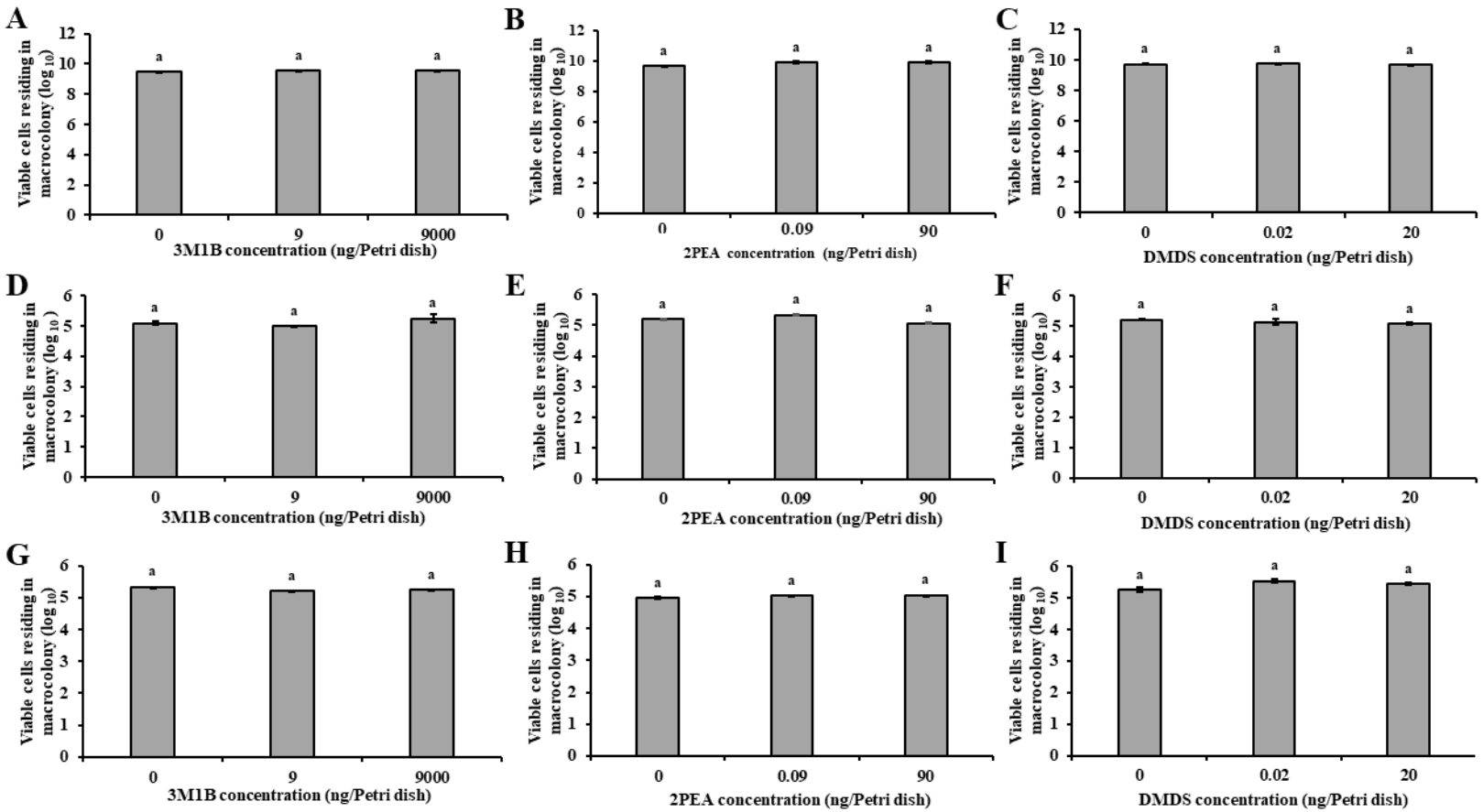

Figure S3. Effect of pure 3-metyl-1-butanol (3M1B), 2-phenylethyl alcohol (2PEA) and dimethyl disulfide (DMDS) on the viability of

Pseudomonas (Ps.) *putida* MVC 17 cells. The number of *Ps. putida* MVC 17 viable cells grown on media to detect release of siderophores (A-C) as well as to solubilise phosphate (D-F) and potassium (G-I) was assessed after exposure to different concentrations of 3M1B (A,D,G), 2PEA (B,E,H) and DMDS (C,F,I). Columns represent mean ± standard error values of six replicates (Petri dishes) are reported for each treatment. Data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

Table S1. Effect of pure 3-methyl-1-butanol (3M1B) on the plant growth-promoting activities of *Pseudomonas* (*Ps.*) *putida* MVC 17 and tomato plant growth.

3M1B concentration*	Siderophore release (mm^2)**	Phosphate solubilization (mm^2)	Potassium solubilization (mm^2)	Lateral root density ***	Root dry weight (mg)	Shoot dry weight (mg)
0	557.26 ± 1.45 a	28.79 ± 0.56 a	70.98 ± 1.45 a	1.49 ± 0.20 a	17.65 ± 1.26 a	40.99 ± 1.25 a
9	560.33 ± 2.13 a	29.30 ± 0.15 a	71.79 ± 1.33 a	1.52 ± 0.12 a	18.15 ± 1.23 a	39.13 ± 1.26 a
90	559.12 ± 3.02 a	28.95 ± 0.32 a	71.65 ± 2.25 a	1.59 ± 0.19 a	18.03 ± 2.26 a	40.45 ± 1.09 a
900	563.22 ± 1.26 a	28.77 ± 0.55 a	72.70 ± 1.26 a	1.58 ± 0.14 a	17.99 ± 1.09 a	41.45 ± 0.79 a
9000	562.36 ± 1.33 a	29.23 ± 0.12 a	72.15 ± 1.45 a	1.57 ± 0.19 a	18.63 ± 0.99 a	41.99 ± 1.33 a

The ability of *Ps. putida* MVC 17 to release siderophores, solubilise phosphate and potassium was assessed after exposure to different concentrations of 3M1B. Lateral root density, root and shoot dry weight were evaluated after 10 days of exposure to different concentrations of pure 3M1B. Mean ± standard error values of six replicates are reported for each treatment in the case of plant growth-promoting activities of *Ps. putida* MVC17 whereas mean ± standard error values of twelve replicates (tomato seedlings) are reported for each treatment in the case of tomato plant growth. In both cases, data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

*ng/split Petri dish, **average halo area surrounding the colony, ***number lateral roots/cm main roo**t**

Table S2. Effect of pure 2-phenylethyl alcohol (2PEA) on the plant growth-promoting activities of *Pseudomonas* (*Ps.*) *putida* MVC 17 and tomato plant growth.

2PEA concentration*	Siderophore release (mm^2)**	Phosphate solubilization (mm^2)	Potassium solubilization (mm^2)	Lateral root density ***	Root dry weight (mg)	Shoot dry weight (mg)
0	558.02 ± 2.56 a	27.99 ± 0.89 a	74.02 ± 2.02 a	1.47 ± 1.01 a	18.99 ± 1.03 a	34.01 ± 1.39 a
0.09	563.23 ± 2.31 a	28.10 ± 1.21 a	74.50 ± 1.23 a	1.45 ± 0.02 a	20.20 ± 1.26 a	33.98 ± 1.26 a
0.9	569.33 ± 1.26 a	28.65 ± 0.98 a	74.74 ± 0.69 a	1.49 ± 0.06 a	20.79 ± 1.03 a	34.02 ± 1.01 a
9	559.12 ± 1.75 a	28.90 ± 2.31 a	74.37 ± 1.06 a	1.50 ± 0.09 a	19.99 ± 1.49 a	33.99 ± 0.77 a
90	561.03 ± 2.02 a	28.75 ± 0.97 a	73.89 ± 1.31 a	1.49 ± 0.12 a	18.52 ± 1.33 a	33.08 ± 0.89 a

The ability of *Ps. putida* MVC 17 to release siderophores, solubilise phosphate and potassium was assessed after exposure to different concentrations of 2PEA. Lateral root density, root and shoot dry weight were evaluated after 10 days of exposure to different concentrations of pure 2PEA. Mean ± standard error values of six replicates are reported for each treatment in the case of plant growth-promoting activities of *Ps. putida* MVC17 whereas mean ± standard error values of twelve replicates (tomato seedlings) are reported for each treatment in the case of tomato plant growth. In both cases, data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

*ng/split Petri dish, *ng/split Petri dish, **average halo area surrounding the colony, ***number lateral roots/cm main roo**t**

3.6. Dimethyl disulfide and VOCs emitted by Pantoea agglomerans *MVC 21 affect the interaction between* Pseudomonas putida *MVC17 and tomato seedlings*

Based on the positive effect of the VOCs released by *P. agglomerans* MVC 21 on plant growth of tomato seedlings and plant growth-promoting activities of *Ps. putida* MVC 17, we investigated their effect on the interaction between tomato seedlings and *Ps. putida* MVC 17. Tomato seedlings inoculated with *Ps. putida* MVC 17 significantly increased LRD compared to the untreated control and tomato seedling exposed to *P. agglomerans* MVC 21 VOCs. In contrast, *Ps. putida* MVC 17 did not increase the root dry weight (Fig. 3). As reported above, *P. agglomerans* MVC 21 significantly increased tomato LRD and root dry weight compared to the untreated control (Fig 2).

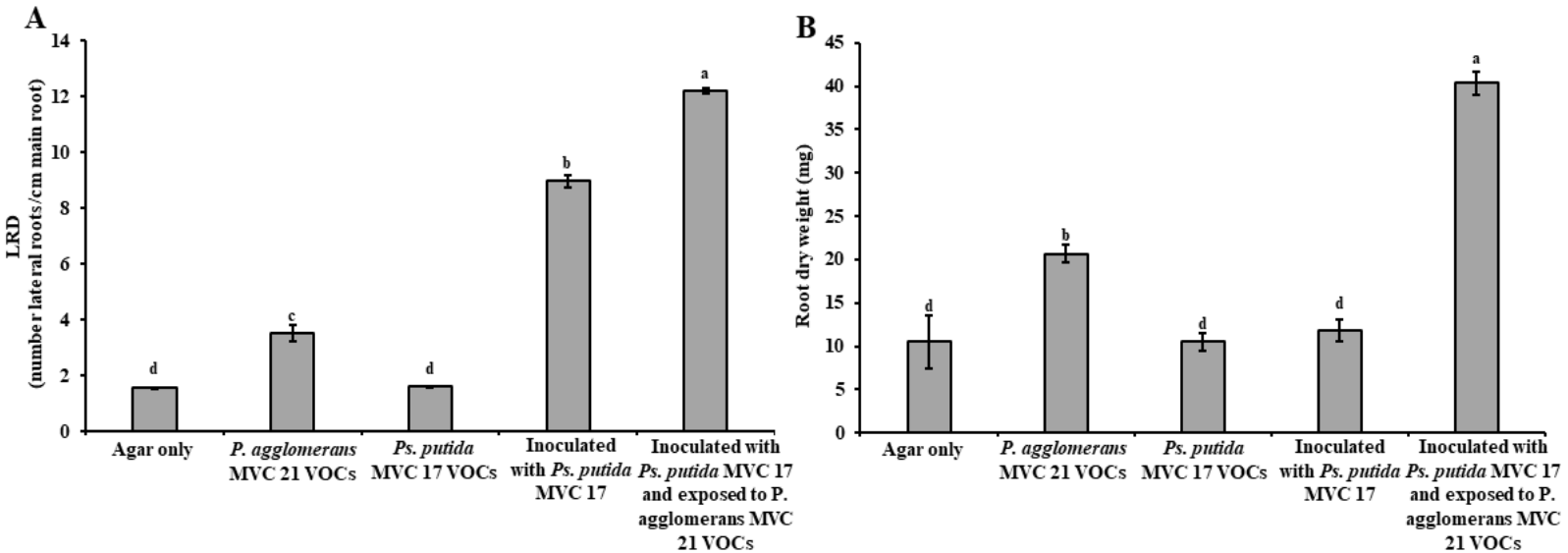

Figure 3. Effect of *Pantoea agglomerans* MVC 21 volatile organic compounds (VOCs) on the plant growth-promoting efficacy of *Pseudomonas* (*Ps.*) *putida* MVC 17. Lateral root density (LRD, A) and root dry weight (B) of tomato seedlings inoculated with *Ps. putida* MVC 17 were assessed after 10 days of exposure to *P. agglomerans* MVC 21 VOCs. Columns represent mean ± standard error values of twelve replicates (tomato seedlings) are reported for each treatment. Data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test (α =0.05).

Tomato seedlings inoculated with *Ps. putida* MVC 17 and exposed to *P. agglomerans* MVC 21 VOCs significantly increased the LRD and the root dry biomass compared to the untreated control. Similar effect was detected on tomato seedlings exposed to *P. agglomerans* MVC 21 VOCs and inoculated only with *Ps. putida* MVC 17 (Fig. 3A,B).

3.7. Dimethyl disulfide and VOCs emitted by Pantoea agglomerans *MVC 21 show a positive effect on the production of indole-3-acetic acid by* Pseudomonas putida *MVC 21*

VOCs emitted by *P. agglomerans* MVC 21 positively affected the production of indol-3-acetic acid (IAA, 29 %) by *Ps. putida* MVC 17 compared to the untreated control (Fig. 4). Similarly, DMDS positively modulated the production of IAA by *Ps. putida* MVC 17 . Particularly, the application of DMDS at 0.02 mg/Petri dish increased the production of IAA by 180 % (Fig. 4).

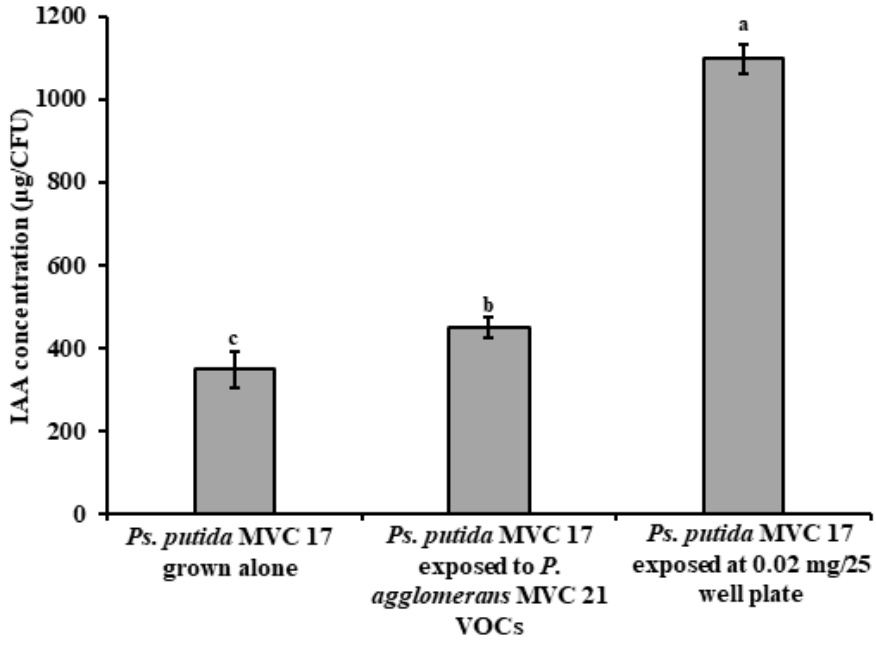

Figure 4. Effect of interaction mediated by volatile organic compounds (VOCs) on indol-3-acetic acid production by *Pseudomonas* (*Ps.*) *putida* MVC 17. The effect of VOCs emitted by *P. agglomerans* MVC 21 and of dimethyl disulfide (DMDS) on the ability of *Ps. putida* MVC 17 to produce indole-3-acetic acid (IAA) was assessed after 120 h of incubation in DF salt amended with L-Tryptophan. Columns represent mean ± standard error values of six replicates for each treatment. Data from two independent experiments were pooled. Different letters indicate significant differences among treatments according to Tukey´s test ($\alpha = 0.05$).

4. Discussion

Soil bacteria coexist in complex multispecies communities where VOCs play an important role in the interactions among the various microorganisms. In fact,

VOCs not only act as antimicrobials by suppressing other microorganisms competing for the same ecological niche, but also as chemical signals, affecting behaviour and gene expression of responding microorganisms (Tyc et al. 2017).

Under greenhouse conditions, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 promote tomato plant growth (Vasseur-Coronado et al. 2021), therefore in this experiment we aimed at understanding how these PGPR interact among themselves and with tomato seedlings, and how the interaction between the two PGPR may affect the development of tomato seedlings. Firstly, we proved that neither *P. agglomerans* MVC 21 nor *Ps. putida* MVC 17 released any diffusible toxic metabolite or VOC against the other. In fact, the number of *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 viable cells present in interacting macrocolonies grown in common Petri dishes were even higher compared to monocultures, whereas cell numbers were not significantly differing in the case of split Petri dishes. These results suggest that these two PGPR might coexist under natural conditions and share the same ecological niches when applied in the field. From a practical point of view, this information indicates that these two strains may be combined in a biostimulant product, as already reported for a combination of other PGPR belonging to *P. agglomerans* and *Ps. putida* (Malboobi et al., 2009a). From an ecological point of view, our results confirm previous evidences of how members of the genus *Pantoea* and *Pseudomonas* may coexist and interact in the phytobiome. In fact, it was already shown that *P.*

agglomerans strains were associated with *Ps. savastanoi* pv. *savastanoi*, the causal agent of olive (*Olea europaea* L.) knot disease, and their interaction determined the increase of olive knot size (Marchi et al., 2006).

These bacterial species were found to interact through a QS mechanism based on the production of AHLs (Hosni et al., 2011). In our case, interaction experiments carried out in common and split Petri dishes indicated that the interaction between *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 mainly relied on the ability of *P. agglomerans* to release VOCs. Particularly, the VOCs emitted by *P. agglomerans* MVC 21 were able to affect the ability of *Ps. putida* MVC 17 to release siderophores and solubilise phosphate and potassium. VOCs may affect different bacterial traits as cell motility, biofilm formation (Kim et al. 2013) cell metabolism, cell wall biosynthesis and response to stresses (Molina-Santiago et al., 2014). At the best of our knowledge, this is the first time that VOCs, as a communication signalling system, were shown to modulate plant growth-promoting activities in PGPR.

Notably, other communication signalling systems are controlling plant growth-promoting activities in bacteria. For instance, the AHL system in *Paracoccus denitrificans* (Zhang et al. 2018), Autoinducer-2 (AI-2) system in *Actinobacillus actinomycetemcomitans* (Fong et al., 2003) and *Porphyromonas gingivalis* (James et al,. 2006), and the *Pseudomonas* quinolone signal (PQS) system in *Ps. aeruginosa* (Bredenbruch et al., 2006) were found to mediate the ability of these

bacteria to chelate iron from the environment. Similarly, AHL and AI-2 systems were shown to be involved in phosphorous acquisition in the cyanobacterium *Trichodesmium consortia* (Van Mooy et al. 2012). Given the importance of iron and phosphorous in the physiology of bacterial cells, it is conceivable that different communication signalling systems evolved in bacteria to regulate the uptake of these elements from the environment.

Interestingly, we found that the VOCs emitted by *P. agglomerans* MVC 21 were also involved in the establishment of a positive interaction with tomato plants. Indeed, tomato seedlings exposed to *P. agglomerans* MVC 21 VOCs showed a significant increase in LRD, root and shoot dry weight compared to the untreated control and the ones exposed to *Ps. putida* MVC 17 VOCs. The ability of *P. agglomerans* strains to stimulate plant growth through production of siderophores, phytohormones and solubilisation of phosphate is widely accepted (Luziatelli et al., 2019; Malboobi et al., 2009b; Omer et al., 2004; Quecine et al., 2012; Viruel et al., 2011). In contrast, investigation on the involvement of VOCs produced by *P. agglomerans* in plant growth promotion is still in its infancy. However, the genome sequencing of plant beneficial *P. agglomerans* strains highlighted the presence of genes being putatively involved in the biosynthesis of VOCs that may stimulate plant growth (Luziatelli et al. 2019; Shariati et al., 2017). Moreover, Tyc et al. (2020) found that the endophyte *P. agglomerans* E44 was able to stimulate seed germination and increase primary root length as

well as fresh biomass of wild cabbage seedlings.

VOCs released by *P. agglomerans* E44 belong to the family of alkenes (1-undecene, cyclohexane), sulfides (DMDS and dimethyl trisulfide), and terpenes such as alpha-pinene (Tyc et al., 2020). In our case, *P. agglomerans* MVC 21 released VOCs belonging to the family of alcohols, ketones and sulfide. More specifically, 2PEA, 3M1B and DMDS were the VOCs detected in the headspace of all of the tested culture replicates of *P. agglomerans* MVC 21. Furthermore, bioassays testing the effect of pure VOCs indicates that DMDS is the main active VOC released by *P. agglomerans* MVC 21, able to modulate the plant growth-promoting activities of *Ps. putida* MVC 17 and to stimulate the growth of tomato seedlings in a dose-dependent manner.

Volatile sulfide compounds, such as DMDS, dimethyl sulfide and dimethyl trisulfide play an important role in plant-microbe and interspecific microbe-microbe interactions (Garbeva et al., 2014a; Tyc et al.,, 2015). DMDS is produced by several bacteria as *Bacillus* sp., *Pantoea* sp., *Pseudomonas* sp., *Serratia* sp., and *Stenotrophomonas* sp. (Meldau et al., 2013; Popova et al., 2014; Tyc et al., 2020). It is also the main volatile produced by *Serratia plymuthica* IC1270 which suppressed the growth of *Agrobacterium* strains (Dandurishvili et al. 2011). However, little is known on the impact of DMDS on the production of siderophores and solubilisation of phosphate and potassium by PGPR. Our results showed that DMDS, similarly to *P. agglomerans* MVC 21 VOCs,

significantly affected plant growth-promoting activities in *Ps. putida* MVC 17. As DMDS did not show any effect on the viability of *Ps. putida* MVC 17 cells, it is probable that this VOC may modulate expression of genes associated to plant growth-promoting activities, acting as an interspecies signal that allows bacteria to coordinate their behaviour according to neighbouring communities. In the future, transcriptome analysis of rhizosphere associated bacteria might be carried out to understand how DMDS affects their behaviour.

DMDS was the main active compound released by *P. agglomerans* MVC 21 responsible for the promotion of tomato seedlings in a concentration-dependent manner. Interestingly, after exposure to VOCs emitted by *P. agglomerans* MVC 21 and/or pure DMDS, tomato seedlings increased their LRD, as well as root and shoot biomass. DMDS may inhibit plant growth when applied at high concentrations, whereas it may inhibit plant pathogens and induce plant systemic resistance when used at low concentrations (Asari et al., 2016; Huang et al., 2012; Rojas-Solis et al., 2018). DMDS produced by *Burkholderia ambifaria* increased *Arabidopsis thaliana* biomass, resistance to gentamicin and kanamycin in *Escherichia coli* and inhibited *Alternaria alternata* and *Rhizoctonia solani* growth (Groenhagen et al. 2013). Similar to other VOCs, DMDS may modulate root system architecture by affecting plant hormone biosynthesis. Indeed, indole and DMDS promoted root development by modulating auxin signalling pathways in *A. thaliana*. In particular, DMDS

strongly enhanced auxin signalling near the root apical meristem (Bailly et al., 2014; Tyagi et al., 2019). Thus, we can hypothesize that *P. agglomerans* MVC 21 VOCs and DMDS had a similar effect by promoting auxin biosynthesis in tomato seedlings in our system.

As both DMDS and VOCs released by *P. agglomerans* MVC 21 influenced the activities of *Ps. putida* MVC 17 and the growth of tomato seedlings, we hypothesized that they might also influence the interaction between the plant and bacterium. Strikingly, our results showed that VOCs released by *P. agglomerans* MVC 21 positively influenced this interaction by boosting the plant growth-promoting efficacy of *Ps. putida* MVC 17. Indeed, tomato seedlings interacting with *Ps. putida* MVC 17 and exposed to VOCs released by *P. agglomerans* MVC 21 showed a significant increase of LRD and root dry weight. These results lead us to hypothesize that, similarly to what is reported in plants (Tyagi et al. 2019), DMDS and *P. agglomerans* VOCs might also have a promoting effect on indole-3-acetic acid production in bacteria. Our results clearly showed that DMDS and *P. agglomerans* VOCs enhanced indole-3-acetic acid production in *Ps. putida* MVC 17, providing thus the first evidence of DMDS to be a VOC signal that is able to affect the same biosynthetic pathway in two kingdoms.

In conclusion, this work suggests that VOCs and in particular, DMDS emitted by *P. agglomerans* species have an ecological role in the rhizosphere. In fact,

VOCs released by *P. agglomerans* may act as chemical signals by modulating the behaviour of tomato plants as well as PGPR belonging to the species *Ps. putida*, even when far from each other. From a practical point of view, our results show that DMDS, used as a commercial soil fumigant for the control of plant pathogens (Schulz-Bohm et al., 2017), may stimulate also plant growth and improve the interaction between plants and PGPR residing in agricultural soils.

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Chapter 4

Humic acids positively influence plant growth-promoting activities of *Pantoea agglomerans*

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Abstract

Associations of plant biostimulants (PBs) such as humic substances (HS) and plant growth-promoting rhizobacteria (PGPR) have already been demonstrated to benefit crop growth. The main objective of our study was to assess the effects of humic acids (HA) on the plant growth-promoting activities of *Pantoea agglomerans* MVC 21. Our study, conducted under *in vitro* conditions, has shown that HA increased plant growth-promoting activities of *P. agglomerans* MVC 21. Particularly, HA increased the ability of *P. agglomerans* MVC 21 to solubilise phosphate and potassium, release siderophores and indol-3-acetic acid and form biofilm. Moreover, no effect on *P. agglomerans* MVC 21 cell growth was observed in presence of HA suggesting that HA could modulate genes related to plant growth-promoting activities of *P. agglomerans* MVC 21.

Keywords

Plant biostimulants, humic acids, *Pantoea agglomerans* MVC 21, plant growth-promoting activities.

1. Introduction

One of the biggest challenges in the twenty-first century will be to develop a more sustainable crop production in order to avoid the most negative impacts of the current agricultural practices on soils and the environment (de Souza et al., 2015). This will need to be achieved while production should increase to to feed a growing global population. Altogether, there is a growing demand to find sustainable strategies in agriculture (Berg 2009). In particular, the use of plant biostimulants (PBs) appears an interesting option to reduce the utilization of chemical fertilizers. PBs were recently defined in the Regulations of the European Parliament and Council (Regulation EU 2019/1009) as "...EU fertilising product(s) able to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere:(1) nutrient use efficiency, (2) tolerance to abiotic stress, (3)quality traits, or (4) availability of confined nutrients in the soil or rhizosphere". Application of PBs to seeds, roots and/or soils have thus been demonstrated to improve fertilizer use, plant performance and soil fertility (Yakhin et al., 2017).

Among beneficial microorganisms included in the group of PBs, plant growth-promoting rhizobacteria (PGPR) are defined as bacteria living in the soil in association with plant roots, which can promote plant growth (Naik et al., 2019) through several mechanisms such as fixation of atmospheric nitrogen, solubilisation of minerals (i.e. phosphorus and potassium), production of siderophores, presence of ACC deaminase activity, synthesis of plant hormones such as indole-3-acetic acid (IAA), synthesis of hydrolytic enzymes (chitinases, cellulases, proteases etc.) and antibiotics, induce systemic resistance against pathogens (Bhattacharjee and Dey 2014; Singh and Jha, 2015).

Besides PGPR, several substances can also promote plant growth. Among them, humic substances (HS) have been shown to have both direct and indirect effects on plants. Indirect effects involve changes in soil physico-chemical properties, availability of nutrients, trace elements and growth regulators (Nardi et al., 1996). Direct effects are produced by an induction of H+-ATPase synthesis and transport proteins, hormone-like effects and effects on glycolysis and enzymes involved in the Krebs cycle (Nardi et al., 2002; Pinton et al., 1997; Pinton et al., 1992).

Among the common inhabitants of plant rhizosphere, members of the *Pantoea* genus is a cosmopolitan genus that lives in diverse environments, including soil (Omar et al., 1989), water (Mosso et al., 1994), insects (Dillon et al., 2001) and humans (de Champs et al., 2000). These species have been able to control plant

pathogens (Pusey et al., 2011; Sammer et al., 2012) and to promote plant growth (Malboobi et al., 2009a). For instance, *P. agglomerans* MVC 21 can fix atmospheric nitrogen, release phytohormones and siderophores and solubilise inorganic phosphates and can thus be considered as an interesting plant growth promoter (Vasseur-Coronado et al., 2021).

In addition to their positive effects on plants, HS have also been proposed as carrier to deliver beneficial microbials in the field (Canellas and Olivares 2015; Canellas et al., 2020). Furthermore, association of microbials with HS have been shown to increase their population and activity which results in plant growth promotion (Canellas and Olivares 2014). However, little attention has been focused on the effects of HS on the plant growth-promoting activities of PGPR. In this study, we investigated the effects of HA on the *in vitro* plant growth-promoting activities of *P. agglomerans* MVC 21

2. Materials and Methods

2.1. Maintenance and preparation of Pantoea agglomerans *MVC 21 cell suspension*

P. agglomerans MVC 21, previously isolated from tomato rhizosphere (Vasseur-Coronado et al., 2021), was stored at length in glycerol 40 % at -80 °C and routinely growth at 25 °C on Nutrient Agar (NA, Neogen, Miami, USA) in Petri dishes (90 mm diameter). To prepare bacterial cell suspension, *P.*

agglomerans MVC 21 was grown in 5 ml of Nutrient Broth (NB, Oxoid, Basingstoke, UK) at 27 ºC on an orbital shaker (200 rpm). After 24 h, a volume of one ml of *P. agglomerans* MVC 21 cell suspension was centrifuged (13,000 rpm, 2 min) and pellets were suspended in NaCl (0.85 % w/v) to a final optical density at 600 nm ($A_{OD600nm}$) of 0.1 corresponding to $\simeq 1 \times 10^8$ colony forming units (CFU)/ml and used in all the experiments.

2.2. Evaluation of the effect of humic acids on plant growth-promoting activities of Pantoea agglomerans *MVC 21*

The effects of HA on the plant growth-promoting activities were assessed using pure HA (Sigma Aldrich, St. Louis, MO, USA) at 0.003% (w/v).

2.2.1. *Chelation of iron and solubilisation of phosphate and potassium*

Tests were carried out according to Vasseur-Coronado et al. (2021) expecting that HA was added in the different media before autoclaving. King´s B Agar (Sigma-Aldrich) amended with HA were overlaid with Chromo Azurol S (CAS) agar medium. National Botanical Research Institute´s phosphate (NBRIP) medium and Aleksandrow Agar (AA, HiMedia GmbH, Germany) amended with HA were prepared. In all experiments, five µl of *P. agglomerans* MVC 21 cell suspension (1×10^8 CFU/ml) was spot inoculated onto these media and incubated for 72 h at 28 °C. As untreated control, King´s B Agar, NBRIP and AA not amended with HA were used. At the end of the incubation, orange haloes (siderophore production) or clarification haloes formed around bacterial

macrocolonies (solubilisation of phosphate and potassium) were determined by capturing digital images with Bio-Rad Quantity One software implemented in a Bio-Rad Geldoc system (Bio-Rad Laboratories, Inc., Hercules, California, U.S.A.). The halo areas were subsequently measured using Fiji software (ImageJ1.50i; Schneider et al., 2012). For all the tests, three replicates (Petri dishes) were used and the experiment was repeated.

2.2.2. Indole-3-acetic acid production

A volume of 500 µl of *P. agglomerans* MVC 21 cell suspension (1×10^8 CFU/ml) were grown in sterile 15 ml tubes containing five ml of DF salt minimal broth amended with HA and 500 µg/ml of L-Tryptophan (Sigma-Aldrich) at 28 °C on an orbital shaker (200 rpm). As untreated control, DF salt minimal broth not amended with HA was used. After 120 h, the quantity of IAA produced by bacterial cells was monitored according to Vasseur-Coronado et al. (2021) and expressed as the ratio between $A_{OD530nm}$ and $A_{OD600nm}$. Three replicates (15 ml tubes) were used for each bacterial strain and the experiment was repeated.

2.2.4. Biofilm formation

A volume of 1.5 µL of *P. agglomerans* MVC 21 cell suspension (1×10^8 CFU/ml) was grown in Nutrient Broth (NB, Oxoid) amended with HA distributed in 96-well polystyrene dishes and incubated at 27 °C without shaking. As untreated control, NB not amended with HA was used. After the incubation

period, the Specific Biofilm Formation (SBF) was determined according to Vasseur-Coronado et al. (2021). Three replicates (wells) were used and the experiment was repeated.

2.2.5. Statistical analysis

IBM SPSS software (Version 21) was used for statistics. All experiments were carried out twice. Normality (Shapiro-Wilk test, $p>0.05$) and variance homogeneity (Levene´s test, $p>0.05$) were checked. As both assumptions were met, data were analysed using statistical parametric test. Data from experiments were pooled when two-way ANOVA demonstrated non-significant differences between two experiments ($p> 0.05$). Mean comparisons between treatments were assessed using Student´s t-test ($p\leq 0.05$).

3. Results

3.1. Humic acids stimulated positively the plant growth-promoting activities of Pantoea agglomerans *MVC 21*

The presence of HA significantly increased the ability of *P. agglomerans* MVC 21 to solubilize phosphate (19.06 %) and potassium (13.62%), chelate iron through the release of siderophores (65.63 %), release IAA (24.66 %) and form biofilm (88.88 %) compared to the untreated control (Table 1).

Table 1: Effect of humic acids on plant-growth-promoting activities of *Pantoea agglomerans* MVC 21

Plant growth-promoting activities	Control	HA
Solubilisation of phosphate (mm² halo surrounding the colony)	80.17 ± 0.13	96.45 ± 0.15*
Solubilisation of potassium (mm² halo surrounding the colony)	161.23 ± 0.26	183.36 ± 0.31*
Chelation of iron (mm² halo surrounding the colony)	634.67 ± 18.13	1051.23 ± 10.65*
Production of indole-3-acetic acid (µg/CFU)	37.47 ± 0.22	46.71 ± 2.63*
Specific biofilm formation	0.27 ± 0.01	0.51 ± 0.04*

Plant growth-promoting activities in absence and presence of 0.003% w/v of HA. Data presented as the mean ± standard error of six replicates pooled from two independent experiments; asterisks indicate significant differences between treatments according to Student´s t-Test ($\alpha = 0.05$).

3.2. Humic acids did not affected cell concentration of Pantoea agglomerans *MVC 21*

P. agglomerans MVC cell growth did not change in presence of HA in none of the plant growth-promoting activities tested (phosphate and potassium solubilisation, chelation of iron, IAA production and biofilm formation) compared to the untreated control (Table 2).

Table 2: Assessment of the effects of humic on *Pantoea agglomerans* MVC 21 cell growth.

Plant growth promoting activities	Control	HA
Solubilisation of phosphate (mm² colony size)	21.12 ± 0.03	19.26 ± 0.01
Solubilisation of potassium (mm² colony size)	19.56 ± 0.21	18.98 ± 0.16
Chelation of iron (mm² colony size)	25.39 ± 0.15	24.37 ± 0.02
Production of indole-3-acetic acid (O.D 600 nm)	0.218 ± 0.06	0.214 ± 0.01
Specific biofilm formation (O.D 600 nm)	0.378 ± 0.04	0.383 ± 0.07

P. agglomerans cell growth in absence and presence of 0.003% w/v of HA. Data presented as the mean ± standard error of six replicates pooled from two independent experiments; asterisks indicate significant differences between treatments according to Student´s t-Test ($\alpha = 0.05$).

4. Discussion

Combinations of HS and PGPR have already been shown to be effective in ensuring high yield and quality of crops (Ekin 2019). Besides, HS induce lateral root formation and root hair initiation which may favor the fitness of mutualistic interactions by increasing rhizosphere population, chemotaxis, bacteria attachment, survival on plant surface and endophytic colonization (Olivares et al., 2017).

Increasing plant growth-promoting processes such as phosphate solubilization are particularly important in the transition from conventional agriculture to

organic production (Olivares et al., 2015). Several authors reported that HA and PGPR alone or in combination positively influence plant physiology. Additionally, molecular mechanisms underlying this event have been well studied (Canellas et al., 2020; Galambos et al., 2020; Schoebitz et al., 2016). Despite that, few information is available about the effects of HA on the mode of action of PGPR which could lead to an additional effect resulting in a better enhancement of plant growth. Recently, *P. agglomerans* MVC 21 was shown to share suitable characteristics to be developed as a PB product as well as multiple mode of actions to promote plant growth such as solubilization of phosphate and potassium, production of IAA and formation of biofilm (Vasseur-Coronado et al., 2021). In this work, we evaluated the effects of HA on the plant growth-promoting activities of *P. agglomerans* MVC 21. Interestingly, HA significantly improved the *in vitro* ability of *P. agglomerans* MVC 21 to solubilize phosphate and potassium, release siderophores and IAA and form biofilm. In plants, the main evidence of the biostimulant activity of HS is the lateral root induction (Canellas and Olivares, 2014). Trevisan et al. (2010) demonstrated that this phenomenon could be explained by the enhancement of the transcription of early auxin responsive gene *IAA19* in plants. Moreover, HS were found to increase nutrient uptake due to the activation of plasma membrane H^+ATPase increasing the ion transport across cell membranes (Morsomme and Boutry 2000). It is common to find accumulation of nitrogen, phosphorus, potassium, calcium, and

magnesium in plants treated with HS (Baldotto et al., 2009). At molecular level, tomato genes related magnesium, nitrogen, phosphate, sulphate, and zing transport were reported to be upregulated in presence of HS (Galambos et al., 2020). Moreover, application of HS to plants have shown to induce expression of Fe (III) -chelate reductase and Fe transporter genes (Tomasi et al., 2013).

As plant-growth promoting activities are increased in presence of HA, we may hypothesize that HA could affect bacterial cell growth resulting in an increased of *P. agglomerans* MVC 21 plant growth-promoting abilities. Overall, our work revealed that HA increased plant growth-promoting abilities of *P. agglomerans* MVC 21 without influencing *P. agglomerans* MVC 21 cell growth. Further studies will aim at evaluating the modulation of genes related to plant growth-promoting activities in *P. agglomerans* MVC 21 by quantitative PCR analysis. For that, the whole genome of *P. agglomerans* MVC 21 will be sequenced and genes will be annotated. Moreover, genes associated to plant growth-promoting activities will be selected and their modulation will be investigated in presence of HA. On the other hand, the sequencing analysis will also allow us to gain deeper knowledge about the main biological characteristics of *P. agglomerans* MVC 21 needed to reinforce its selection as a novel PBs product.

Chapter 5

Conclusions

The selection of suitable parameters to screen microbial candidates is a significant step in biostimulant development for commercial purpose. Due to the absence of an optimised screening strategy, we proposed and validated a procedure to select and characterise the best PGPR candidates, which can be successfully combined with HA, allowing them to be commercialised as PBs. This flow diagram commenced with cheap and straightforward screening of a large range of variables and concluding with more sophisticated and labour-intensive tests Particularly, the screening strategy involved methods dedicated to i) selection of a target crop and commercial methodology to develop PBs with a strong competitiveness in the market, ii) decision of an appropriate growth media, iii) characterisation of candidates able to fulfil a desired agronomical constraint, iv) screening for suitable characteristics for commercialisation such as industrial scale-up, formulation needs and regulation laws, v) *in vivo* plant growth-promoting efficacy, vi) screening for multiple mode of actions of candidates, and vii) plant growth-promoting efficacy in greenhouse conditions. Moreover, this stepwise screening strategy allowed us to select two candidates, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 as potential active ingredients to be developed as PBs.

After this selection, we focused our attention on designing a PGPR

consortium that may stimulate tomato plant growth. To achieve this goal, we evaluated how *P. agglomerans* MVC 21 and *Ps. putida* MVC 17 interact together and the effect of this interaction on tomato plant growth. Firstly, we showed that the cell viability of *P. agglomerans* MVC 21 and *P. putida* MVC 17 in interacting macrocolonies were higher compared to monocultures suggesting that these two PGPR can be used for designing a multi-species-inoculum based PB product.

The interaction between the PGPR, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17, and the effect of the PGPR-interaction on tomato plants were mediated mainly by VOCs produced by *P. agglomerans* MVC 21. It is known that the composition and functional properties of bacterial bouquet are influenced by the nutrient source where bacteria are grown. In our studies, we analysed the effects of VOCs and the volatiles profiles emitted by *P. agglomerans* MVC 21 grown on NA, a rich media with high protein content. VOCs produced by *P. agglomerans* MVC 21 modulated plant growth-promoting activities of *Ps. putida* MVC 17 such as release of siderophores, solubilisation of phosphate and potassium. In addition to that, the VOC bouquet emitted by *P. agglomerans* MVC 21 led to a positive interaction with tomato plants by increasing LRD, root and shoot biomass of tomato seedlings.

VOCs profiles of *P. agglomerans* MVC 21 obtained by GC-MS analysis revealed 2PEA, 3M1B and DMDS as the most dominated VOCs released.

Among these compounds, DMDS showed to promote tomato plant growth and *Ps. putida* plant growth-promoting activities similarly to *P. agglomerans* MVC 21 VOC bouquet. Based on these evidences, we hypothesized that DMDS modulates genes related to plant growth-promoting activities by acting as a signalling molecule able to change bacterial behaviour depending on their neighbouring communities.

On the other hand, to determine the effect of PGPR interaction on tomato seedlings, we evaluated how VOCs emitted by *P. agglomerans* MVC 21 affect plant growth-promoting efficacy of *Ps. putida* MVC 17. We found that VOCs emitted by *P. agglomerans* MVC 21 and/or DMDS increased the ability of *P. putida* MVC 17 to promote LRD and root biomass of tomato seedlings. As the VOC bouquet emitted by *P. agglomerans* MVC 21 and/or DMDS positively affected the IAA production of *Ps. putida* MVC 17, we may suggest that DMDS also affect the IAA pathways in PGPR.

Another interesting technique is the combination of HA and PGPR which have already shown to better benefit plant growth. To better understand a possible synergetic effect of HA on PGPR, we evaluated the effects of HA on the plant growth-promoting activities of *P. agglomerans* MVC 21. Noteworthy, HA increased the ability of *P. agglomerans* MVC 21 to chelate iron, solubilise phosphate and potassium, release IAA and form biofilm without affecting *P. agglomerans* MVC 21 cell growth.

Chapter 6

Perspectives

The work presented in this thesis selected two PGPR, *P. agglomerans* MVC 21 and *Ps. putida* MVC 17, as promising active ingredients to be developed as PBs formulations. Further characterisation of these candidates will be needed in terms of commercialisation such as determination of shelf life and proper type of formulation as well as the effect of PGPR candidates on fruit yield and quality of crops.

On the other hand, our results suggested that bacterial VOCs modulate the behaviour of rhizosphere bacteria and plants. In this sense, omics analysis might be implemented to have a deeper understanding of the mechanisms underlying VOCs mediated microbe-microbe-plant interactions.

The effects of HA on the plant growth-promoting activities reported on this thesis revealed that HA have a positive impact on PGPR. Further transcriptomic approaches will evaluate the modulation of genes related to plant growth-promoting activities of *P. agglomerans* MVC 21 in presence of HA. In addition, the genome sequencing of *P. agglomerans* MVC 21 will be investigated to get insights into the biological characteristics of *P. agglomerans* MVC 21 to support its selection as PBs.

Chapter 7

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A

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Plant biostimulants (PBs) are an environmental friendly strategy to mitigate the continuous application of chemical fertilisers. The research described in this PhD thesis explored strategies to select, characterise and design a new generation of PBs formulations. We designed a screening procedure to select the best microbial candidates as new active ingredients of PBs products. This screening was validated by using a case study: PGPR combined with humic acids (HA) to be applied on tomato plants. This validation led us to select *Pantoea agglomerans* MVC 21 and *Pseudomonas (Ps.) putida* MVC 17 as potential candidates for PBs formulations. Recently, co-inoculation of PGPR is used to achieve multifactorial effects on crop productivity. To develop a PGPR consortium, we evaluated how these PGPR interact together and the effects of this interaction on tomato seedlings. Compatibility studies revealed that these two strains can be combined in a biostimulant product. Experiments testing the effect of the PGPR interaction indicated that both interact together and with tomato seedlings mainly by volatile organic compounds (VOCs) produced by *P. agglomerans* MVC 21. Metabolomic studies pinpointed VOCs belonging to the family of alcohols, ketones and sulfide as the main VOCs released by *P. agglomerans* MVC 21. The identification of the main VOC responsible of the observed effects of *P. agglomerans* MVC 21 on *Ps. putida* MVC 17 and tomato seedlings deciphered new ecological roles of VOCs as chemical signals able to modulate behaviour of PGPR as well as the interaction between plants and PGPR. Another technology is the combination of HA and PGPR. Our study, conducted under *in vitro* conditions, showed that HA synergistically modulate plant growth-promoting activities of *P. agglomerans* MVC 21. Futher studies will aim at analysing the whole genome sequencing of *P. agglomerans* MVC 21. Transcriptomic analysis will be carried out to evaluate the effect of HA on *P. agglomerans* MVC 21 genes related to plant growth-promoting activities.

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