Universidade de Aveiro Departamento de Biologia
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PAULO JORGE DA ROCHA CARDOSO

Promoção do crescimento de plantas por bactérias em condições de seca: a importância da comunicação volátil

Bacterial-induced plant growth promotion under drought: the importance of airborne communication

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Bacterial-induced plant growth promotion under drought: the importance of airborne communication

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Professora Doutora Etelvina Maria de Almeida Paula Figueira (Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro), da Professora Doutora Sílvia Maria da Rocha Simões Carriço (Professora Auxiliar do Departamento de Química da Universidade de Aveiro) e do Professor Doutor Artur Jorge da Costa Peixoto Alves (Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro).

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palavras-chave

Bactérias promotoras do crescimento das plantas, diversidade, compostos voláteis, alterações climáticas, seca

resumo

As plantas são habitadas por bactérias que lhes podem trazer benefícios, como por exemplo a promoção do seu crescimento, a melhoria da aquisição de nutrientes, defesa contra agentes patogénicos e predadores, e melhoria da tolerância a fatores abióticos como por exemplo a seca. O desenvolvimento de aplicações biotecnológicas utilizando estas bactérias desde há muito que interessa a comunidade científica e o setor da agricultura. Enquanto que as espécies de plantas cultivadas têm recebido bastante atenção, as espécies de plantas a crescer em ambientes naturais têm sido negligenciadas e também devem ser exploradas nesse sentido. Este interesse é redobrado num contexto de ameaça à produtividade agrícola por parte das alterações climáticas, e em particular a seca e associada desertificação. Esta tese teve como objetivos explorar a diversidade de bactérias existentes nos nódulos das raízes de plantas leguminosas a crescer em ambiente selvagem em Portugal continental, perceber se as condições edafoclimáticas do local de origem afetam a osmotolerância das bactérias, e avaliar as capacidades de promoção de crescimento de uma planta modelo, não leguminosa, a *Arabidopsis thaliana*. As estirpes bacterianas isoladas a partir de várias espécies de plantas leguminosas a crescer em Portugal continental foram caracterizadas por BOX-PCR fingerprinting e amplificação parcial e sequenciação do gene que codifica para o 16S rRNA. Estas estirpes, predominantemente dos géneros *Flavobacterium* e *Pseudomonas*, foram utilizadas para avaliar a sua capacidade de evidenciar capacidades promotoras do crescimento das plantas (produção de sideróforos, ácido indol acético, emissão de compostos voláteis) e a sua tolerância ao stress osmótico. Um conjunto representativo de estirpes de diferentes géneros foi utilizado para estudar os mecanismos de resposta ao stress osmótico (osmólitos e mecanismos antioxidantes), tendo sido possível apontar o alginato intracelular como um possível novo mecanismo de osmotolerância bacteriana. Nesta tese, um enfoque particular foi dado ao mecanismo de promoção de crescimento de plantas através da emissão de metabolitos voláteis pelas bactérias. Os metabolitos voláteis emitidos por duas estipes bacterianas (*Flavobacterium* sp. D9 e *Rhizobium* sp. E20-8), promotoras do crescimento de *A. thaliana*, foram captados por microextração em fase sólida e analisados por cromatográfica gasosa e cromatografia gasosa bidimensional e espectrometria de massa. Foram também avaliados os efeitos da emissão de voláteis em diversos parâmetros fisiológicos e bioquímicos das plantas, em condições controlo e condições de stress osmótico (seca). Globalmente, os resultados desta tese evidenciam o potencial das espécies de leguminosas selvagens como fontes de bactérias que promovem o crescimento e tolerância à seca de espécies de plantas, incluindo leguminosas e não leguminosas, e que devem ser usadas como prática agrícola para aumentar a produtividade, particularmente em condições de seca.

keywords Plant growh promoting bacteria, diversity, volatiles, climate change, drought

abstract

Plants harbor bacteria which can provide them with benefits such as growth promotion, enhancement of nutrient uptake, defense against pathogens and predators, and improvement of tolerance to abiotic factors such as drought. The development of biotechnological applications using these bacteria has been the focus of research and interest from the scientific community and agricultural sector. While crops have received significant attention, plant species growing in natural environments were neglected and should also be explored. This is particularly important in the paradigm of climate change and its threat to plant productivity, especially due to drought and desertification. This thesis aimed to study the diversity of bacteria living in legume root nodules from wild areas in continental Portugal., to understand if the site of origin and climate conditions influence bacterial osmotolerance, and to evaluate the plant growth promotion abilities of bacterial strains towards the non-legume plant model *Arabidopsis thaliana*. The bacterial strains isolated from different wild legume species were characterized by BOX-PCR and partial 16S rRNA gene sequencing. These strains, which belonged mainly to the genera *Flavobacterium* and *Pseudomonas*, were used to evaluate their plant growth promoting abilities (production of siderophores, indol acetic acid, emission of volatiles) and their tolerance to osmotic stress. A representative set of strains from the different genera was used to study the mechanisms behind the response to osmotic stress (osmolytes and antioxidant mechanisms). It was possible to suggest intracellular alginate as a new mechanism of bacterial osmotolerance. In this thesis, a particular focus has been given to the mechanism of plant growth promotion by bacterial volatiles. The volatile metabolites released by two bacterial strains (*Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8) were captured using solid-phase microextraction and analyzed by gas chromatography – mass spectrometry and two-dimensional gas chromatography – mass spectrometry. The effects of bacterial volatiles emission on several physiological and biochemical endpoints of A. thaliana were also evaluated, in control and osmotic stress (drought). Globally, the results of this thesis evidenced the potential of wild legume plant species as sources of bacteria promoting the growth and tolerance to drought of plant species, including legumes and non-legumes, which should be the used as an agricultural practice to increase crop production, particularly in drought conditions.

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Abbreviations

16S rRNA – 16S ribosomal ribonucleic acid

ACC - Aminocyclopropane-1-carboxylate

AICRPDA - All India Coordinated Research Projects on Agri-Meteorology

ANOVA - Analysis of variance

APX – Ascorbate peroxidase

ATP - Adenosine triphosphate

BLAST - Basic Local Alignment Search Tool

BOX-PCR - BOX-A1R-based repetitive extragenic palindromic-PCR

BSA - Bovine serum albumin

BVOCs - Bacterial volatile organic compounds

CAS - Chemical abstracts service

CAS - Chrome azurol S

CAT – Catalase

CDNB - 1-Chloro-2,4-dinitrobenzene

CR- Congo red

CRIDA - India's Central Research Institute for Dryland Agriculture

DMBB - Dimethyl methylene blue

DTT- Dithiothreitol

EDTA - ethylenediaminetetraacetic acid disodium salt dihydrate

ETS - Electron transfer system

EU - European Union

FAO – Food and Agriculture Organization of the United Nations

GC-MS - Gas chromatography-mass spectrometry

GCxGC-ToFMS - Two-dimensional gas-chromatography coupled to time-of-flight mass spectrometry

GSH - Glutathione

GSTs - Glutathione S-transferases

HDTMA - Hexadecyltrimethylammonium bromide

HS-SPME- Headspace solid phase microextraction

IAA - Indol acetic acid

IPCC - Intergovernmental Panel on Climate change

ISR - Induced systemic resistance

LPO - Lipid peroxidation

MDA - Malondialdehyde

MS - Murashige and Skoog medium

NAD+ - Nicotinamide adenine dinucleotide (oxidized)

NADH - Nicotinamide adenine dinucleotide (reduced)

NBT - Nitro blue tetrazolium

OD - Optical density

PCA – Principal component analysis

PCO - Principal coordinate analysis

PCR - Polymerase chain reaction

PEG- Polyethylenoglycol 6000

PGP - Plant growth promotion

PGPB - Plant growth promoting bacteria

PGPR - Plant growth promoting rhizobacteria

POD - Peroxidase

PVP - Polyvinylpyrrolidone

ROS - Reactive oxygen species

SOD - Superoxide dismutases

SPME - Solid-phase microextraction

TBARS - Thiobarbituric acid reactive substances

TCA - Trichloroacetic acid

Tris-HCl – Tris buffer

U - Unit of enzyme activity

UN - United Nations

UNDP- United Nations Development Programme

UPGMA - Unweighted pair group method with arithmetic mean

USDA - United States Department of Agriculture

VOCs- Volatile organic compounds

YMA- Yeast extract mannitol

YMB - Yeast mannitol broth

Chapter 1

General introduction

Abstract

Plant productivity in natural and agricultural ecosystems is under threat due to global changes. Among the global changes, climate change, with predicted frequency and severity of droughts in particular, can have a significant impact. Strategies that can alleviate the effect of drought on plants and that are also sustainable can complement or enhance agriculture. One of the proposed approaches is the development and application of plant growth promoting bacteria (PGPB), which span different bacterial genera and can improve plant productivity by several mechanisms, for instance by increasing nutrient availability, producing phytohormones or fighting phytopathogenic agents. One of these mechanisms is the plant growth promotion by volatile organic compounds produced by the bacteria, which has been reported to elicit growth promotion, induce tolerance to drought, resistance to diseases, and improve nutrient assimilation. In this chapter the current knowledge concerning the importance of PGPB to plant productivity is reviewed, with a focus on the importance of bacterial growth promotion via emission of volatiles, its role in a drought scenario and the effects of drought on the plant, on the bacteria and on the mechanism. This chapter also includes the objectives and outline of the thesis.

1. Plant productivity and sustainable development

The United Nations has identified 17 Sustainable Development Goals to pursue, and hopefully achieve until 2030, a response to the global challenges of poverty, inequality, climate, environmental degradation, and peace and justice (UN, 2018a). The increasing human population requires strategies that can improve agricultural productivity. This need for high yields is reinforced by global changes, which challenge current agricultural practices by altering the areas available for farming, changing abiotic factors such as temperature and water availability, or introducing toxic substances. Several Sustainable Development Goals of the UN can be allocated to these challenges, namely Zero Hunger, Responsible Consumption and Production, Climate Action and Life on Land (Figure 1). Zero Hunger deals with starvation, food security and the dependence of 40% of the total population whose income comes from agriculture. Among the targets of this goal are the doubling of agricultural productivity of small-scale producers and also the achievement of sustainable production systems that can maintain ecosystems and be resilient to climate change and drought (UN, 2018b). Responsible Consumption and Production deals, among other things, with the fact that agriculture is the biggest user of water worldwide, with irrigation claiming about 70 percent of all freshwater for human use (UN, 2018c). Climate Action is focused on improving resilience to climate-related

threats and on increasing awareness of climate change mitigation and adaptation (UN, 2018d). Finally, Life on Land is dedicated to combat desertification, land degradation and biodiversity loss, since arable land is being lost at more than 30 times the historical rate, and considers the fact that microorganisms are key to ecosystem services, yet scarcely studied and acknowledged (UN, 2018e).

Figure 1. United Nations Sustainable Development Goals related to this thesis: Zero Hunger, Responsible Consumption and Production, Climate Action and Life on Land (Source: United Nations).

Plant productivity is limited by many factors, for instance nutrient and water availability, temperature, soil contamination with biocides, elements such as metals and other toxicants. Moreover, biotic factors such as pathogenic agents, beneficial agents, herbivores and birds can also have great impact on crop yields. Changes in land use accompanied by the development of agricultural technologies have been happening for millennia but have increased in the last decades (Houghton, 1994), and nowadays intensive farming (as opposed to extensive farming) is common practice. Intensive farming delivers high yields and provides food security, but at the same time can have serious environmental consequences. These include desertification (Banin and Fish, 1995), eutrophication (Tilman et al., 2002), emission of greenhouse gases (Robertson, 2000) and soil pollution (Pain et al., 1991).

Several global change trends affect plant productivity and soil microorganisms. These include, but are not restricted to, climate change, wildfires, desertification, contaminants, urbanization, and invasive species. The pressure arising from increasing human population drives for increased agricultural productivity and pollution, impacting Earth in a variety of ways, some still unknown, while others are already identified, such as climate change. However, these impacts also embody an opportunity to complement current agricultural practices with more environmentally sustainable approaches. The application of soil bacteria as inoculants to boost plant productivity has gathered significant attention as a means to improve agriculture yield and contribute to global sustainability (e.g. Glick, 2012)). Ubiquitous in both natural and agricultural fields, many bacterial species and strains have been reported as capable of promoting plant growth (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009). The beneficial traits can

be obtained from free living soil bacteria, bacteria living in the rhizosphere or endophytic bacteria (bacteria living inside plant tissues) (Bashan and De-Bashan, 2005). Plant models such as *Arabidopsis thaliana* and several edible plants (e.g. lettuce, maize, tomato) have been reported to benefit from efficient strains (e.g. García-Fraile et al., 2012; Gholami et al., 2009; Ryu et al., 2005; Schuhegger et al., 2006). Higher plant yields can be achieved by bacterial production of phytohormones (e.g. indol-3-acetic acid) (Spaepen et al., 2007), volatiles that stimulate plant growth (Ryu et al., 2003), alleviation of abiotic stress (Vurukonda et al., 2016), antagonism against pathogenic organisms (Compant et al., 2005) or enhancement of nutrient acquisition (e.g. biological nitrogen fixation, phosphate solubilization) (van Rhijn and Vanderleyden, 1995; Vassilev et al., 2006). The benefits provided by plant growth promoting bacteria are particularly important for areas of the globe currently facing or predicted to suffer from climate events such as severe and prolonged droughts. However, while it is well documented that bacteria can alleviate global change effects on crops, such as drought (Vurukonda et al., 2016), knowledge on how these factors affect the bacteria themselves has comparatively received much less attention. Moreover, the striking mechanism of plant growth promotion by bacterial volatile organic compounds (VOCs) requires further investigation on the impacts and feedback of bacterial VOCs on plants productivity and ecological safety.

Here, we review the existing literature regarding the effects of drought on soil bacteria and highlight the importance of plant and bacterial VOCs under this scope. Instead of providing an overview on the benefits of application of these bacteria to alleviate drought stress in plants and other benefits, which have been extensively reviewed (e.g. Vurukonda et al. (2016)), we explore how drought stress can affect the bacterial side. The interest behind application of and mechanisms by which PGPB can promote plant productivity have been reviewed comprehensively (for instance in Ahemad and Kibret, 2014; Glick, 2012; Lugtenberg and Kamilova, 2009). Nonetheless, the literature concerned on the effects of these global changes on PGPB is comparatively scarce. In this review, we parallel the knowledge concerning PGPB as a strategy to fight global change adverse effects with the knowledge concerning how global change affects these beneficial bacteria themselves. We highlight their potential role in a sustainable development-minded paradigm, identify exciting recent advances and suggest topics that remain to be addressed. Moreover, the beneficial traits of some bacteria to promote growth via volatiles, which do appear not to be restricted to the specific host plant, but rather have broad biotechnological potentialities and ecological importance to vegetation in general is focused in this chapter.

2. Plant growth promoting bacteria

Soil can be populated by high numbers of bacteria. Some of these are deleterious to plant growth, for instance by inducing plant diseases or by co-existing with plants in a parasitic manner. However, other bacteria provide several benefits for plant growth. Due to the ecosystem services that they provide, these bacteria have a high environmental interest. At the same time, they also have high biotechnological potential, thus can also play an important role in agriculture. The increasing human population and the deterioration of agricultural fields due to pollution or desertification stresses for sustainable agricultural approaches that can stand as alternatives or complement modern agricultural technology, such as the use of biofertilizers. Plant growth promoting bacteria (PGPB) might be used as biofertilizers, as biopesticides, and as agents to minimize the impacts of stressful abiotic factors such as drought (Vurukonda et al., 2016).

PGPB are beneficial microorganisms that modulate plant growth in natural and agricultural sites in a positive manner. Plant growth promoting rhizobacteria (PGPR) are associated with plant roots and are some of the most well-known PGPB. Nevertheless, other bacteria, both endophytic and in free-living form can be regarded as PGPB, and can colonize other plant tissues besides roots and soil beyond the proximity of roots. Due to the promising potentialities of PGPB to be used as biofertilizers, extensive research exists concerning the diversity of these bacteria and the mechanisms by which plant growth promotion occurs, along with studies on the intricacies of the establishment of symbiosis and other relationships with plants, fungi and other organisms. Some of the most well-known genera are *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Bacillus* and *Azospirillum*, among others. Bacteria which are not pathogenic to humans are of most importance since these can be actually applied in the agroindustry systems. Thus, for genera which include some pathogenic species such as *Pseudomonas* and *Bacillus*, application is restricted to the non-pathogenic species.

The mechanisms by which PGPB promote plant growth encompass direct growth promotion through the action of phytohormones (e.g. indol acetic acid) and (VOCs), enhancement of nutrient acquisition (e.g. biological nitrogen fixation), antagonism against phytopathogenic and herbivore agents, activation of plant defenses against pathogens and abiotic stress (e.g. drought, contamination with metals).

3. Soil bacteria as mitigators of global change effects on plant productivity

PGPB can be useful allies to cope with the effects that global changes can have on plant productivity and food security. While already employed in bioremediation and in the

enhancement of phytoremediation approaches, their growth promotion effect on plants can be an alternative or complement to chemical fertilizers and biocides, conducting to a reduction in the production and application of agrochemicals. Research on the mitigation of drought stress in particular, using PGPB, is in its infancy (Ngumbi and Kloepper, 2016) and recent discoveries have been carefully reviewed by Vurukonda and collaborators (Vurukonda et al., 2016) and Ngumbi and Kloepper (Ngumbi and Kloepper, 2016). Due to the multiple effects that drought stress have on plants, which include physiological and morphological alterations related to water potential and turgor, oxidative stress inducement and nutrient uptake decrease (Vurukonda et al., 2016), a multitude of effects of PGPR can be of use to alleviate the impact of low water availability on plant productivity, and can be linked to production of phytohormones, ACC deaminase, induced systemic tolerance and production of exopolysaccharides by the bacteria (Vurukonda et al., 2016).

Research concerning the use of drought tolerant bacteria to increase productivity of plants in arid regions has been happening for quite some time (Zahran, 2001). Vast areas of the globe already suffer from drought and saline conditions and these areas are predicted to increase in the light of climate change estimates. Thus, strategies to counter drought impact on agriculture including bacteria application are under study. It is important, however, to include a screening of tolerance to abiotic stresses such as drought in studies that screen bacterial strains for plant growth promotion (PGP), since this will likely affect the persistence and efficacy of the bacteria.

4. Drought effects on beneficial soil bacteria

4.1. Drought and climate change

Drylands are 41 % of the landmass, 44 % of cultivated areas, and support one third of the world population (Johnson et al., 2006). Desertification affects 70 % of global arable land and is an important factor driving poverty, affecting directly 250 million people and threatening another billion (Johnson et al., 2006). A decrease in total rainfall can lead to desertification. While drylands are more susceptible to desertification, several parts of the globe are under drought stressful events that have been increasing in frequency, duration and severity. As examples, the IPCC 2014 report on Climate Change Impacts, Adaptation and Vulnerability (IPCC, 2014) reports that widespread drought in southeast Australia lead to economic losses, that extreme events such as droughts and floods can have high impact on natural and human systems in Africa, and that for Europe extreme weather events already have significant economic and health impacts (IPCC, 2014). Even areas of the globe that are not usually under severe impact of drought can be at

risk. For instance, the IPCC 2007 report on Climate Change Impacts, Adaptation and Vulnerability indicated that water stress will increase over central and southern Europe (IPCC, 2007). In fact, Europe has suffered recent drought events linked to heat waves, although they might be more common in the long term history that initially thought (Hanel et al., 2018). While animals can migrate easily, plants and their associated communities require longer time to disperse. Thus, they are at special risk, especially in areas where plants are not well adapted to tolerate water scarcity. This is further reinsured by the fact that even dryland suffers a decrease in microbial diversity and abundance due to increasing aridity (Maestre et al., 2015).

4.2. Drought effects on soil bacteria

Tolerance to water stress by bacteria is a subject that has received research attention for a long time now, with reviews on the subject ranging as back as 1976 (Brown, 1976). On a physiological and biochemical level, bacteria cope with drought stress by producing and accumulating osmolytes (glycine betaine, proline, trehalose), producing exopolysaccharides and heat shock proteins, and controlling oxidative stress, among other strategies (Ngumbi and Kloepper, 2016). Any possible role of VOCs is currently undisclosed, although since bacterial VOCs production can be altered under stress (Cardoso et al., 2017), it is a possibility that should not be discarded.

Soil is alive, thriving with different organisms and organic reactions catalyzed by extracellular enzymes and other molecules with biological origin. In the field, drought can have a profound effect on the community composition of soil bacteria and can put plant-microbe interactions, fundamental for ecosystem functioning, at risk (Ochoa-Hueso et al., 2018). The field of ecological metabolomics, which can be regarded as an integrated approach that benefits from recent analytical advances (Peñuelas and Sardans, 2009), may prove useful in highlighting the dynamics of the cell, organism, community and ecosystem response to drought events. Soil communities can be affected by global changes in a deterministic or stochastic manner, in which they are not only determined by the specific environmental conditions, but also to random effects such as birth, death and loss of biodiversity (Zhang et al., 2016). These effects of environmental changes on the soil communities highlight the complexity with which they might respond to a stressor or a set of stressors. Moreover, interactions between microorganisms and other organisms such as plants further increase the complexity of the system. Exposure of soil microbial communities to heat in combination with drought leads to a decrease in microbial diversity and a community shift that appears to result more from the plant-soil-microbial dynamics than from direct effects of drought and heat stress alone (Rein et al.,

2016). Thus, research on interactions, especially on interkingdom interactions, such as volatile communication between bacteria and plants, are fundamental under the scope of global changes. In fact, recent advances, reviewed extensively by Naylor and Coleman-Derr (Naylor and Coleman-Derr, 2018), show that the effects of drought on bacterial communities include not only direct effects, but also indirect effects due to alterations in the soil chemistry and plant phenotypes. Since the metabolome, including the volatilome, depends on the substrates available, on the species and strains of bacteria that could be inhabiting the soil, and also strongly from other environmental factors, one could argue that community shifts due to drought can favor the release of certain VOCs in detriment of others. These VOCs might affect the remaining microbial communities, a possibility that is virtually unexplored. Another interesting question that might be raised, is that due to lower water availability in the soil, diffusion of infochemicals should be harder, making communication between organisms, including plant-bacteria interactions, more difficult. Communication through volatiles in particular might be protected or even enhanced by this effect, due to the volatile nature of the compounds, since they might travel in the porous soil, and even be less retained due to the absence of water.

Pinpointing drought stress effects on microorganisms is easier under more controlled conditions, using more lab based, microcosm and mesocosm approaches, that might not be feasible to escalate to field conditions or provide conclusions that are not directly relevant and transferable to the field level, can still be useful in shedding light on some of the fundamental soil processes, for instance, plant-microbe and microbe-microbe interactions. Nevertheless, care should be taken when advancing conclusions, since stress induced in vitro assays is usually very severe using high concentrations of stress inducing-agents (Claeys et al., 2014), and might not represent most of the conditions found in the environment.

Studies on the emission of VOCs in conditions of drought induced stress are scarce. This is true for plant emitted volatiles (Ngumbi et al., 2014). Moreover, to the best of our knowledge, no studies concerned with the effects of drought stress on emission of bacterial VOCs exist, and it seems to be the case for any type of stress, with the exception a work from our own team, but which was dealing with Cd induced stress (Cardoso et al., 2017).

5. Airborne communication and global change

Bacteria can produce a wide diversity of VOCs (Schulz and Dickschat, 2007). Soil bacteria VOCs can travel further than heavier substances released by these bacteria,

thus they can be of interest even for surrounding plants and for the ecosystem level, and be important in microbial communication and interaction (Schmidt et al., 2015). Bacterial volatiles have been shown to promote plant growth. The first report published in 2003, by Ryu and colleagues, indicated 2,3-butanediol and acetoin behind growth inducement of *A. thaliana* (Ryu et al., 2003). Since this report, several compounds of different chemical families have been reported, arising from different bacterial species, and with different bioactivities of interest for plant productivity (Table 1). These have been reviewed comprehensively in Kai et al. (2009), in Bailly and Weisskopf (2012) and in Liu and Zhang (2015).

Table 1. Bacterial volatile metabolites with reported plant growth promotion effects.

Compound	Bioactivity	Reference
Dimethyl disulfide	Plant growth promoter (PGP), antifungal, plant growth inhibitor	(Groenhagen et al., 2013); (Vespermann et al., 2007); (Meldau et al., 2013)
Dimethyl trisulfide	Antifungal	(Kai et al., 2007); (Groenhagen et al., 2013)
2-Undecanone	Antifungal	(Groenhagen et al., 2013)
Decanal	Antifungal	(Fernando et al., 2005)
Nonanal	Antifungal	(Fernando et al., 2005)
2R,3R-Butanediol	PGP; induced systemic resistance (ISR); ISR against drought	(Ryu et al., 2003); (Ryu et al., 2004); (Cho et al., 2008); (Han et al., 2006)
Acetoin (3-hydroxibutanone)	PGP; ISR	(Ryu et al., 2003); (Ryu et al., 2004)
Indole	PGP	(Blom et al., 2011)
1-Hexanol	PGP	(Blom et al., 2011)
Pentadecane	PGP	(Blom et al., 2011)
3-methyl-1-butanol	Potential PGP*	(Farag et al., 2006)
2-methyl-1-butanol	Potential PGP*	(Farag et al., 2006)
Butane-1-methoxy-3-methyl	Potential PGP*	(Farag et al., 2006)
Dimethylhexadecylamine	PGP	(Velázquez-Becerra et al., 2011)
2-Pentylfuran	PGP	(Zou et al., 2010)

*Plant growth induction by synthetic compound not confirmed.

Plants emit a complex variety of VOCs, which fulfill functions related to growth, reproduction and defense, and which have impact on other organisms and on the atmosphere, and whose emission can in turn also be affected by drought, climate change

and other global change events (Peñuelas and Staudt, 2010). Research points that the rise in global temperatures may abruptly increase the quantity of VOCs with biological origin emitted to the atmosphere (Peñuelas and Staudt, 2010). If this is true for VOCs with plant origin, one could argue that there is the possibility that the same may be observed in microbial communities. A high-volume effect considering cyanobacteria and aquatic bacteria that exist in huge amounts might occur. The increase of emission of VOCs from soil microorganisms should be not as significant in volume as the cyanobacteria volatiles. However, due to the complex plant-microbe interactions and recent discoveries concerning bacterial VOCs effects on plants, an increase in soil VOCs emission may trigger a cascade of biochemical, physiological and biochemical alterations that at the moment are hard to predict.

Improvement of tolerance to drought stress in crops by beneficial soil bacteria has been reviewed extensively by Vurukonda et al. (2016), including the action of bacterial volatiles under this scope. Mitigation of abiotic stress in particular by VOCs has been review by Liu and Zhang (2015). Cho and colleagues reported that *Pseudomona*s *chlororaphis* strain O6 could improve *A*. *thaliana* tolerance to drought by 2R, 3R-Butanediol, which mediated stomatal closure and drought resistance (Cho et al., 2008). Zhang et al. (2008) reported that bacterial volatiles of *Bacillus subtilis* GB03 enhances salt tolerance of *A. thaliana*. The team used a divided plate setup to check for the mitigation of salt stress effects on *A. thaliana* growth, and both the plant and the bacteria were subjected to the stress. This is realistic since, for non-endophytic bacteria, in the environment if the plant is under salt or drought conditions, so is the bacteria. The endpoints measured in this study were on the plant side, since it was the purpose to the work. Checking how volatiles emitted by the bacteria change compared to a control condition could also be relevant, together with studying what biochemical changes bacteria underwent and which may explain the expressed volatilome. Zhang and colleagues showed that *Bacillus subtilis* GB03 can induce systemic tolerance to osmotic stress in *A*. *thaliana* (Zhang et al., 2010). The experiments were carried out using mannitol to simulate water stress in divided plates and in pots by withholding water (Zhang et al., 2010). Plant tolerance to drought was achieved in both cases. Yet, the changes on the biochemical, physiological and volatilome of the bacteria remain to be investigated and could provide interesting insights on the dynamics of the tolerance of the bacteria, on the volatile communication between bacteria and plant, while at the same time raising questions about the implications for surrounding organisms (plants, microorganisms) that may not be the target of the volatiles, and which can benefit or be negatively affected by volatiles.

Studying how global changes and drought stress in particular might affect growth promotion by bacterial volatiles is important, yet no studies exist to date, probably because this is a relatively recent discovery. Nevertheless, even when considering the most studied mechanisms of promotion, one could argue that most studies are directed to the isolation of microorganisms displaying PGP abilities, and how they help plants grow or cope with biotic and abiotic stress, but not much research has been devoted to the mechanisms of tolerance of the bacteria themselves.

To study the effect of bacterial VOCs on plants the approach used by Ryu el al. (2003), and Farag et al. (2006), described in detail in Farag et al. (2017) has been employed. Effects on *A. thaliana* are checked using a divided plate system or growing the bacteria in a separate compartment than plants, and growing the bacteria in vials and extracting the volatiles using solid-phase microextraction (SPME) and analyzing the volatiles by gas chromatography-mass spectrometry (GC-MS). While developments in this approach (e.g. using the more powerful two-dimensional gas-chromatography time-of-flight mass spectrometry (GCxGC-ToFMS) instead of GC-MS) might improve the detection of new compounds, it is also worth considering that headspace SPME (HS-SPME) opens a wide range of applications, and can be used not only in the lab, but also in the environment to study the dynamics of the volatilome under drought, in real time. Signatures or profiling of the organisms is necessary to know whose volatiles are (the plant's or the bacteria's, of each species, strains) and is made difficult by the fact that many of the microbial and plant volatiles overlap, and shift according to environmental factors apart from drought, but the possibility that one can detect shifts in the volatilome as a response to drought stress in a plate, microcosm, mesocosm or in the field is not far-fetched.

6. Way to go

Most studies expose plants to reduced water availability, which at the same time also reduces the availability of water to the bacteria which are inoculated in the soil, plants or seeds. However, while the beneficial effects on the plant are assessed, the effects on surrounding plants of other species are not mimicked, nor the effects that long-range communication mechanisms (such as VOC released by the bacteria) have in these conditions. These compounds can be bioactive; thus, they can have unsuspected outcomes in the biological network. Moreover, pot studies for instance will probably provide the same conditions for the bacteria and plant in terms of water. But since VOCs travel, and microsites with different conditions can exist in the soil in the same area, a case could exist in which only the bacteria or the plant are submitted to water stress, but

still their VOCs (both bacteria or plant's) can elicit responses on surrounding organisms including bacteria and plants. Thus, it is important not only to look how alleviation of drought stress can be achieved in plants using bacteria, but also how the bacteria are affected, and if there may be effects on non-target organisms and inter-specific relations.

7. Thesis outline

The work presented is this thesis aimed to explore the diversity of bacteria harbored in wild legume nodules growing in mainland Portugal and to obtain soil bacteria that can be used as biosimulants to improve agricultural productivity and enhance crops tolerance to drought. The thesis is particularly focused in the fascinating mechanism of inducement of plant growth by the emission of rhizobacterial volatiles. Moreover, the thesis evaluates physiological and biochemical responses of the bacteria and plants in response to drought.

To obtain bacteria experiencing different drought levels isolation from different legume species growing in four sites in Portugal, with different climatic conditions was performed. Isolates were identified to the genus level using PCR and partial 16S rRNA gene sequencing. The identified isolates were screened *in vitro* for plant growth promotion abilities and osmotolerance. Plant growth inducement and enhancement of tolerance to drought of *Arabidopsis thaliana* via airborne communication through volatiles was also studied. Finally, the volatiles emitted by the bacteria were analyzed using gas chromatography, two-dimensional gas chromatography and mass spectrometry to identify the molecules responsible for the effects observed on the plants.

This chapter **(Chapter 1)** is an introductory chapter that provides the scientific background and rationale for the thesis, and deals with the importance of bacterial volatile organic compounds (BVOCs) in a changing climate, with predictions of increase in duration, spread and severity of drought.

Chapter 2 explores the diversity of bacteria living inside nodules of legume plants in natural ecosystems in Portugal, checking for probable differences according to the different water availabilities of the sampling sites and for plant growth promoting abilities.

Chapter 3 examines the physiological and biochemical mechanisms behind the response to drought in bacteria from different genera isolated in Chapter 2.

In **Chapter 4**, the mechanism of airborne plant growth promotion through bacterial volatile metabolites is examined on two strains presenting high plant growth promotion levels (*Rhizobium* sp. E20-8 and *Flavobacterium* sp. D9) highlighting the mechanism as

non-specific to a host species and screening the volatilome of each strain for compounds with biotechnological and ecological interest.

In **Chapter 5** the mechanism of airborne plant growth promotion through bacterial volatile metabolites evaluated in Chapter 4 was is challenged by drought conditions, to check how drought might affect the dynamics of growth promotion by bacterial volatiles.

In **Chapter 6** findings are discussed considering future perspectives for research and applications.

In **Chapter 7** a list of references cited is this thesis is provided.

Chapter 2

Bacteria from nodules of wild legume species: Phylogenetic diversity, plant growth promotion abilities and osmotolerance

Abstract

The demand for food with high nutritional value that can sustain the growth of human population while safeguarding sustainability, deserves urgent attention. A possible strategy is the inoculation of crops with plant growth promoting (PGP) bacteria. Plants are naturally colonized by bacteria that can exert beneficial effects on growth and stress tolerance. N_2 fixation by rhizobia in the root nodules of legumes is a well-known PGP effect. These bacteria can be used as inoculants to boost legume productivity and can be especially interesting if they are able to survive to abiotic stresses, such as drought. Herein we report the phylogenetic diversity of bacteria colonizing the root nodules of several wild legume species, from four geographic locations in Portugal with different bioclimates. Interestingly, *Flavobacterium* and *Pseudomonas* and not rhizobia were the dominant genera. Plant growth promotion (PGP) abilities other than N_2 fixation (production of indol acetic acid, siderophores and volatile organic compounds) and osmotolerance were screened. Location and host plant species did not influence PGP abilities and osmotolerance. Taken together, results evidence that bacterial strains from wild legumes displaying PGP abilities and osmotolerance can be regarded as good candidates for inoculants of a broad range of hosts, including non-legumes.

Keywords

Wild legumes, endophytic bacteria, plant growth promotion, climate change, drought

1. Introduction

Legumes are an important protein source for human consumption (Ben Romdhane et al., 2009; Singh and Singh, 1992) and animal feed (Hanbury et al., 2000). This is particularly true for developing countries (Iqbal et al., 2006) and semi-arid and tropical regions (Singh and Singh, 1992). Plant productivity is affected by drought, which is a key constraint (Vurukonda et al., 2016), especially for grain legumes (Tuteja et al., 2012). For southern Europe, the area where this study was undertaken, the number of extreme drought events is predicted to double by the 2090s, whilst the duration of these events is expected to increase by a factor of six (IPCC, 2007). The increase of temperatures will also be observed (IPCC, 2007), along with a decrease in summer rainfall, which will both contribute to higher evaporation and thus lower summer soil moisture and more frequent and intense droughts (IPCC, 2007). However, an ever-growing global population and limited arable land demand an increase in crop yields, while safeguarding sustainability. Thus, environmentally friendly solutions that boost agricultural productivity with a low cost are required. One possible strategy to boost plant productivity, with lower impact on the environment than the use of inorganic fertilizers and pesticides, is the inoculation of

plants with plant growth promoting rhizobacteria (PGPR). These bacteria colonize plant roots and can have several beneficial effects for the plant, namely the synthesis of compounds that stimulate plant growth, increase nutrients bioavailability, relieve abiotic stress or work as biocontrol agents of plant pathogenic organisms (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009).

Among the capabilities of plant growth promoting bacteria are the production of phytohormones such as indol acetic acid, which stimulates lateral root development (Ahemad and Kibret, 2014), the production of siderophores, which increase iron availability for plants (Ahemad and Kibret, 2014), and the emission of volatile organic compounds which can promote growth (Ryu et al., 2003; Bailly and Weisskopf, 2012). Several beneficial effects of PGPR can be associated with enhancement of plant's tolerance to drought, such as the production of phytohormones, alteration in root morphology, induction of osmolytes accumulation and emission of volatile organic compounds (Vurukonda et al., 2016; Schulz and Dickschat, 2007; Garnica-Vergara et al., 2016; Cho et al., 2008; Gutiérrez-Luna et al., 2010; Fincheira et al., 2016). The effects of bacterial VOCs on plant growth are a recent discovery (Ryu et al., 2003), and can have important ecological and economic roles since VOCs can travel the soil easier than non-volatile molecules (Schmidt et al., 2015).

Extensive research has been undertaken concerning nitrogen fixing rhizobia, which are harbored in nodules of crop legume species. However, in wild areas plant growth promotion effects of nitrogen fixing and endophytic (rhizobia and non-rhizobia) bacteria in general have been overlooked, despite their influence in wild legumes growth. In fact, wild legumes are commonplace in arid areas, and might be more tolerant to stress than crop legumes (Zahran, 2001). Thus, knowledge on the bacteria living inside wild legumes nodules is important since these can be a source of interesting PGPR which can be used both in agricultural and environmental contexts as inoculants for legume and non-legume crops. Rhizobia are among the most well studied endophytic bacteria in legumes, however other bacteria can be found in legume root nodules (Martínez-Hidalgo and Hirsch, 2017). The diversity of plants that exist in an ecosystem is, among other factors, influenced by the prevailing climatic conditions. This diversity can in theory have also an effect on the microbiome associated to plants. Therefore, the more diverse the sites are the more likely it is to obtain bacteria with different plant promotion traits, which can be used as inoculants.

Improvement of legume productivity in soils affected by drought can be aimed by selecting tolerant legume varieties but also by rhizobial inocula that are also adapted to

these conditions (Coba de la Peña and Pueyo, 2012). Although little is known about the microbiome of wild legumes, it is expected that these plants harbor different bacteria in their root nodules when compared with crop legumes. This bacterial diversity can be a source of new inoculants to be used in agriculture. Along with PGP abilities, osmotolerance of the endophytic bacteria might depend on the site where the bacteria are collected, and is also a feature to be looked for when selecting inoculants.

Taking into consideration that climate change also influences legume species diversity and their symbionts due to altered rainfall profiles, warming, changes in soil properties and wildfires (Reverchon et al., 2012), the aim of this study was to explore the diversity of bacteria harbored in the root nodules of legume species growing in wild areas of Portugal, an approach that can provide new bacteria strains with particular PGP abilities, when compared to bacteria isolated from cultivated legume species. With this objective in mind, four sampling sites with different geographic and climatic conditions in Portugal were selected. We hypothesized that different sites would present different legume species, and consequently different bacteria being harbored in the root nodules. From each site the dominant legume species were collected and the bacteria colonizing the root nodules isolated. Isolates were identified by 16S rRNA gene sequencing and their tolerance to drought and plant growth promotion abilities (production of indol acetic acid, production of siderophores and emission of volatiles that induce plant growth) were screened.

2. Materials and methods

2.1. Plant sampling and identification

Specimens from different legume species were sampled from four sites in continental Portugal in spring 2015. The geographic location of each site is presented in Figure 1. Sites were chosen based on different bioclimatic conditions. Murtosa (MT) is located in inferior humid and inferior mesomediterranic (ambrotype/thermotype), Vale de Cambra (VC) in inferior hyperhumid and inferior mesotemperate, Alvito (AV) in superior dry and inferior mesomediterranean, and finally Aljustrel (AT) in inferior dry and superior termomediterranean (Mesquita, 2005). Fifteen wild legume plants at the flowering stage were collected randomly from each site. The aerial parts of plants were preserved in the herbarium of the University of Aveiro and identified according to *Flora Iberica* (Castroviejo, 2012).

2.2. Isolation of bacteria

Five nodules were randomly picked from each plant. Endophytic bacteria from root nodules were isolated following the method described in Somasegaran and Hoben (1994), with some modifications. Nodules were surface sterilized by soaking in 96 % ethanol for 5 s, and then immersed for 2 min in a 3 % hydrogen peroxide solution. Nodules were then rinsed twice in sterile deionised water and crushed. The macerate was streaked onto yeast extract mannitol (YMA) plates, containing 1 g mannitol, and supplemented with Congo red (CR). Morphologically distinct single colonies evidencing low absorption of CR were further re-streaked onto YMA+CR plates and allowed to grow at 26 °C. Following this methodology, 180 isolates were obtained. A loopful of each isolate was used to inoculate a tube of 5 mL of YMB. Tubes were incubated at 26°C and 500 µL of the resulting cultures were transferred to microbubes. These cultures were preserved by adding 500 µL of a sterile solution of 30% glycerol, vortexing and storing at -80°C.

Figure 1. Map of Continental Portugal with the four harvesting sites marked: Aljustrel (AT); Alvito (AV); Murtosa (MT); and Vale de Cambra (VC).

2.3. Bacteria identification

2.3.1. PCR-based fingerprinting

Since bacteria were isolated from multiples nodules of the same plant, isolates were typed using BOX-PCR to screen for isolates with unique fingerprints, before proceeding to 16S rRNA gene amplification. This way the identical genotypes can be identified and only a representative isolate of each fingerprint pattern proceeds to identification by 16S

RNA gene sequencing. Isolates were inoculated onto YMA+CR and single colonies were used to prepare a bacterial suspension in 100 µL of autoclaved deionised water. Each PCR reaction tube contained a mixture of 1 µL bacterial suspension, 1 µL BOXA1R primer (5′-CTACGGCAAGGCGACGCTGAC-3′; Versalovic et al., 1994), which was diluted in sterile Milli-Q water to 10 µmol/µL, 6.25 µL NZYTech 2X Taq Green Master Mix (NZYTech), and autoclaved Milli-Q water to fill up the mixture to 25 µL. Amplification was performed applying one cycle at 95 °C (7 min), 30 cycles at 94 °C (1 min), 53 °C (1 min), 65 °C (8 min), and a final cycle at 65 °C (16 min). GelCompar II (Applied Maths, Belgium) was used to calculate the Pearson correlation coefficient and analyze the clusters formed applying the unweighted pair group method with arithmetic mean (UPGMA). A representative isolate of each distinct fingerprint was selected randomly. This procedure yielded 100 representative isolates.

2.3.2. 16S rRNA gene amplification and phylogenetic analysis

The 16S rRNA gene from the representative isolates obtained in section 2.2 was amplified using primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′; Lane, 1991) and 1492r (5′-GGTTACCTTGTTACGACTT-3′; Lane, 1991) and the NZYTaq 2× Green Master Mix (NZYTech, Portugal) in 25-µL tubes by applying one cycle at 94 °C (5 min), 30 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (1.5 min), and a final cycle at 72 °C (10 min). PCR products were sent to GATC Biotech (Germany) for sequencing. Each sequencing reaction tube contained 5 µL PCR product, 2.5 µL 27f primer and 2.5 µL autoclaved MilliQ water. Sequences were edited with the software FinchTV V1.4.0 (Geospiza, USA). A BLAST search against the GenBank database was performed to identify the bacteria to genus level. The partial 16S rRNA gene sequences from the representative isolates were deposited in GenBank.

2.5. Plant growth promotion abilities

2.5.1. Production of siderophores

To assess the ability of bacteria to produce siderophores, the strains (100) that were identified by 16S rRNA gene sequencing were grown for six to ten days on YMA medium supplemented with a chrome azurol S (CAS) solution, which consisted of 1.21 mg mL^{-1} CAS, 0.1 mM $FeCl_3 \cdot 6H_2O$ and 1.82 mg mL^{-1} hexadecyltrimethylammonium bromide (HDTMA). The presence of an orange halo around the colonies was considered as positive for siderophore production (Alexander and Zuberer, 1991). Data are presented as a ratio between the diameter of the halo and the diameter of the colonies.

2.5.2. Production of indol acetic acid (IAA)

To quantify the production of indol acetic acid (IAA), the strains were grown in 5 mL tubes of yeast-mannitol broth (YMB) supplemented with 100 µg/mL of tryptophan, the precursor of IAA. Cells were grown at 26ºC, 150 rpm, until 1.0 optical density at 600 nm or maximum growth after five days was reached. After centrifugation at 10 000 g 4 ºC, the supernatant was collected and 500 µL of supernatant reacted with 200 µL Salkowsky reagent (0.5M $FeCl_3 \cdot 6H_2O$, 35 % $HClO_4$) for 10 min at room temperature. The color was then measured at 530 nm using a spectrophotometer. The concentration of IAA was determined using IAA (Sigma) as a standard (Asghar et al., 2002). Data are presented in concentration of IAA normalized by the optical density (OD) of each strain.

2.5.3. Growth promotion of a non-legume through the emission of volatiles

To screen for growth promotion of a non-legume the setup used by Ryu *et al*. (2003) was followed, with modifications. *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) seeds were surface sterilized by immersion in 70% ethanol, followed by 100% ethanol, and allowed to dry. Seeds were then transferred to plates containing half-strength Murashige and Skoog medium, pH 5.7, supplemented with 1.5% sucrose, which were vernalized at 4 ºC. After 2 days, plates were placed in a growth chamber set for 16 h light/ 8 hours dark, 25 ºC and 85 % humidity, and allowed to germinate for 3 days. For the trials, center divided plates containing YMA in side I and Murashige and Skoog (MS) in side II were used. A strain per plate was spotted in side I. In side II of the plate, ten seedlings were planted. Non-inoculated plates were used as controls. Plates were sealed with parafilm and placed randomly in the chamber during 14 days. At the end of the trials, *A. thaliana* total leaf area and total fresh weight were recorded. Total leaf area was measured using Easy Leaf Area (Easlon and Bloom, 2014). Data are presented as means of the ratios between the total fresh weight or leaf area of strains isolated from each host species and the controls.

2.6. Bacteria osmotolerance

The osmotolerance of the rhizobacteria strains was screened in YEM (Somasegaran and Hoben 1994) with different polyethylenoglycol 6000 (PEG) concentrations: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0 and 30 %. For each strain and PEG concentration at least four replicates were performed. Inoculated tubes were incubated at 26 °C in an orbital shaker (200 rpm) for 48 h. For growth measurement, optical density (620 nm) was determined (Figueira et al., 2005). These results were used to calculate IC50 values, i.e. the concentration that inhibited growth by 50 %.

2.7. *Data analysis*

Venn diagrams were built using the Venn tool from the Bioinformatics & Evolutionary Genomics, University of Ghent (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Data from the production of siderophores, production of IAA, growth promotion of a non-legume through the emission of volatiles and osmotolerance was submitted to univariate analysis, testing for differences displayed by bacteria isolated from the different plant species using Kruskal-Wallis One Way Analysis of Variance on Ranks and pairwise multiple comparison by Dunn's method (differences among plant species were considered significant when $p<0.05$). The analysis was performed using SigmaPlot 11 (Systat Software).

Dendrograms from BOX-PCR fingerprints were built using GelCompar II (Applied Maths, Belgium) to calculate the Pearson correlation coefficient and analyze the clusters formed applying the unweighted pair group method with arithmetic mean (UPGMA).

Principal component ordination (PCO) was performed using Primer+Permanova (Primer-E, Plymouth). Data from the production of IAA, production of siderophores, VOCs influence in plant growth (*A. thaliana* leaf area and fresh weight), and bacteria osmotolerance was normalized and similarity matrix using Euclidean distance was calculated and used to perform PCO using the host plant species or the site as factors.

3. Results

3.1. Legumes species diversity

The legume species present at each site differed. Of the 16 species harvested none was found in all four sites. Moreover, only one (*Ornithopus compressus* L.) was present at three sites. AT (1 species), VC (2 species) and MT (6 species) presented legumes that were exclusive to the site. On the other hand, legume species from AV were also present in AT (3), VC (2) and MT (1) (Figure 2A).

3.2. Bacterial diversity

Molecular typing of bacterial isolates (n =180) by BOX-PCR revealed 100 distinct profiles. One isolate representative of each profile was selected for 16S rRNA gene-based identification and further characterization. Most of the strains belonged to genera *Flavobacterium* (56%) or *Pseudomonas* (28%) and were isolated from different plant hosts at the four harvesting sites. The remaining strains (15%) belong to different genera and are sporadic. From *Lysobacter*, *Variovorax*, *Achromobacter*, *Acinetobacter*, *Rhizobium*, *Stenotrophomonas* and *Paenibacillus* only one strain was obtained and from *Erwinia* and *Herbaspirillum*, two and four strains, respectively (Supplementary Table 1).

From AT site, 34 strains were obtained, 11 from AV, 32 from MT and 23 from VC. The bacterial genera isolated from the root nodules of legumes at each site showed low variation (Figure 2B), with two genera (*Flavobacterium* and *Pseudomonas*) being isolated from the four sites and accounting for 86% of the strains. However, some variation is observed among sites. MT is the most diverse site with 6 genera, AT and AV have 5 and VC 3 (see Supplementary Table 1 and Figure 1B). Since the majority of strains belong to *Pseudomonas* or *Flavobacterium*, dendrograms were generated for these two genera to check if the site from which the bacteria were isolated influenced similarity (Supplementary Figure 1A and B). However, these dendrograms show that there is no clear grouping of strains according to sites.

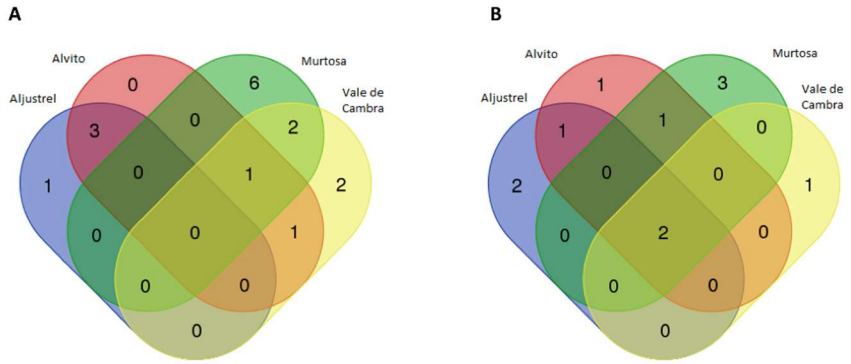


Figure 2. Venn diagrams showing the legume species (A) and bacteria genera (B) distribution in the four harvesting sites: Aljustrel (AT); Alvito (AV); Murtosa (MT); and Vale de Cambra (VC).

Comparing the two genera, most of *Flavobacterium* strains were isolated from *Vicia* (39%), *Medicago* (18%) and *Ornithopus* (16%) genera at the four sites. On the other hand, *Pseudomonas* strains were isolated from different plant genera (Supplementary Table 1).

3.3. Bacteria plant growth promotion (PGP) abilities

The ability of strains to synthesize two important traits promoting plant growth were also analyzed in this study. Half of the strains (49.4%) synthesize IAA, although with distinct performances (0.02-18.6 µg/mL IAA/ OD) (Table 1). No statistical significance ($p>0.05$) was found among the mean of IAA production of strains isolated from each legume species, albeit the host species from which bacteria were isolated influenced the ability of strains to produce IAA. In fact, the three strains isolated from *Trifolium repens* L. all synthesize IAA. The strains producing the higher concentrations of IAA (> 14.5 µg/mL IAA/OD) were isolated from *Vicia* and *Medicago* genera mostly at MT (80%) (Figure 3A).

In contrast, strains from *Scorpiurus vermiculatus* L., *O. compressus* L., *Ornithopus sativus* Brot. and *Vicia benghalensis* L. did not synthesize IAA (Figures 3A and 3B).

The ability to synthesize siderophores also varied among strains from different host species (Figures 3C and 3D), despite the absence of statistically significant differences ($p>0.05$). A high percentage (38-75%) of strains of *S. vermiculatus*, *Ornithopus pinnatus* (Mill.) Druce, *O. sativus* and *Vicia sativa* L. are able to synthesize siderophores (Figure 3D). It is also in these host species that strains displayed the highest ability to produce siderophores and are all from VC site (Supplementary Table 1 and Figure 3C).

The promotion of *A. thaliana* growth by the emission of bacterial volatiles was observed for many of the strains, with 47 of the strains yielding at least a 50% gain compared to the control (no inoculation) when fresh weight is considered and 55 strains for total leaf area (Supplementary Table 1). Some of the strains lead to increments of up to 4-fold of fresh weight and up to 5-fold of total leaf area (Figures 3E, 3G and 3I). Growth promotion through the emission of volatiles seems to be independent of the host legume species, since no statistically significant difference in *A. thaliana* leaf area and fresh weight was observed amongst strains isolated from the different legume species ($p>0.05$). However, the proportion of strains that promote, inhibit or do not have an influence on *A. thaliana* varies amongst host species (Figures 3F and 3H). For instance, considering leaf area (Figure 3F), some host legumes such as *O. pinnatus* present strains with no effect, but also with the ability to promote or inhibit growth, while for the majority of strains isolated from a host species, they either promote growth or do not have an effect (Figure 3F). Interestingly, when plant fresh weight is considered, only two host species present strains that inhibit *A. thaliana* growth (Figure 3H), while strains from four host species inhibited leaf area (Figure 3F).

3.4. Bacterial osmotolerance

Strain's osmotolerance was evaluated by growth in YEM supplemented with different PEG concentrations. Most strains (67%) presented IC_{50} between 8 and 15% PEG, and were considered moderately tolerant. Eighteen strains tolerated high PEG concentrations ($IC_{50} \geq 15\%$ PEG) and were considered tolerant, and 11 strains displayed IC_{50} lower than 8% PEG and were classified as sensitive to osmotic stress (Supplementary Table 1). The osmotolerance level of the bacteria was not significantly different amongst genera ($p>0.05$), with an IC_{50} between 11 and 14% PEG, except for

Herbaspirillum. The average IC_{50} for this genus was 21.3% PEG and it included two of the most osmotolerant strains (IC_{50} of 25.8 and 26.4% PEG) (Supplementary Table 1).

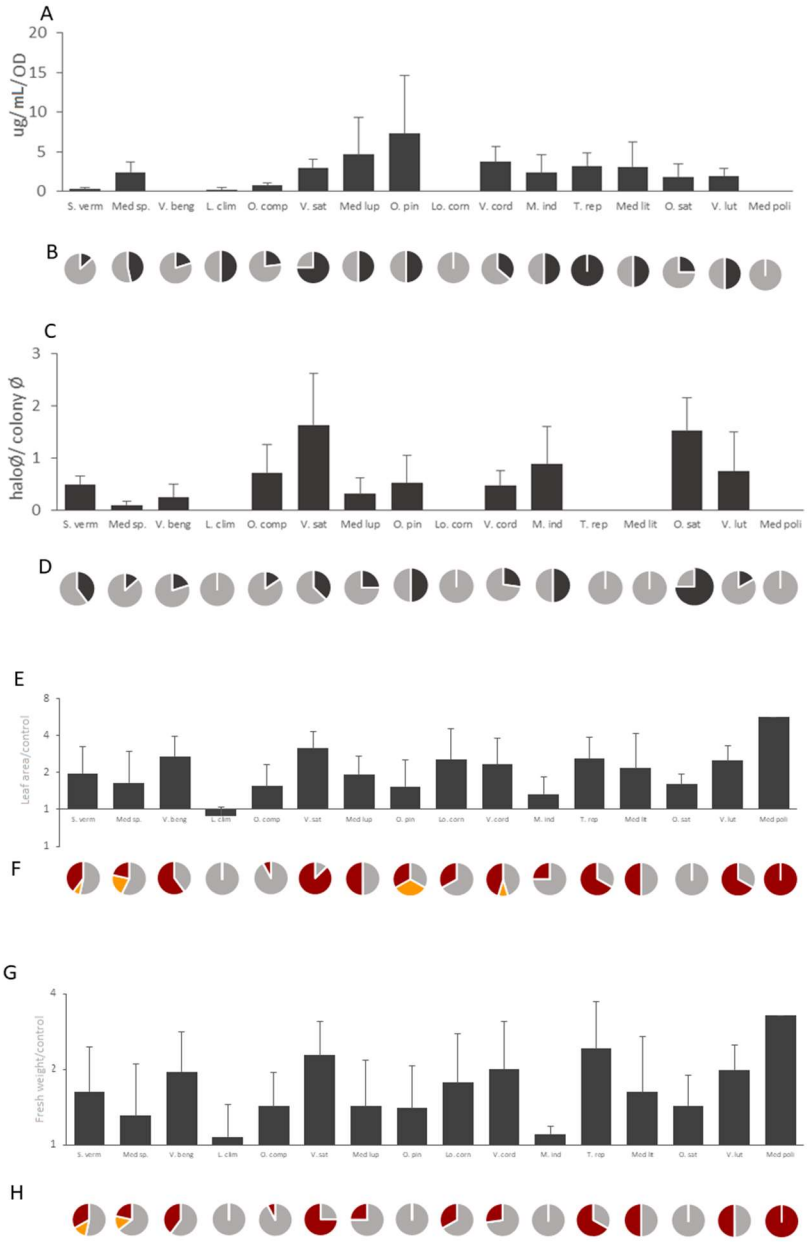


Figure 3. Plant growth promotion traits of bacteria isolated from different host legume species: *Scorpiurus vermiculatus* (S. verm); *Medicago* sp. (Med sp.); *Vicia benghalensis* (V. beng); *Ornithopus compressus* (O. comp); *Vicia sativa* (V. sat); *Medicago lupulina* (Med lup); *Ornithopus pinnatus* (O. pin); *Lotus corniculatus* (Lo. corn); *Vicia cordata* (V. cord); *Melilotus indicus* (M. ind); *Trifolium repens* (T. rep); *Medicago litoralis* (Med lit); *Ornithopus sativus* (O. sat); *Vicia lutea* (V. lut); *Medicago polymorpha* (Med poli). (A) Indol acetic acid (IAA) produced by bacteria ((µg/mL)/optical density). (B) Proportion of strains able (dark grey) or unable (light grey) to synthesize siderophores. (C) Siderophores (halo Ø/colony Ø). (D) Proportion of strains able (dark grey) or unable (light grey) to synthesize siderophores. (E) Leaf area of *Arabidopsis thaliana* exposed to bacterial strains, compared to the control. (F) Proportion of strains that promote (red), inhibit (orange) or do not influence (grey) *A. thaliana* leaf area. (G) Fresh weight of A. thaliana exposed to bacterial strains, compared to the control. (H). Proportion of strains that promote (red), inhibit (orange) or do not influence (grey) *A. thaliana* fresh weight. (I) Promotion of *A. thaliana* growth by bacterial volatile organic compounds. Example of a control plate (A) and inoculated plate (B).

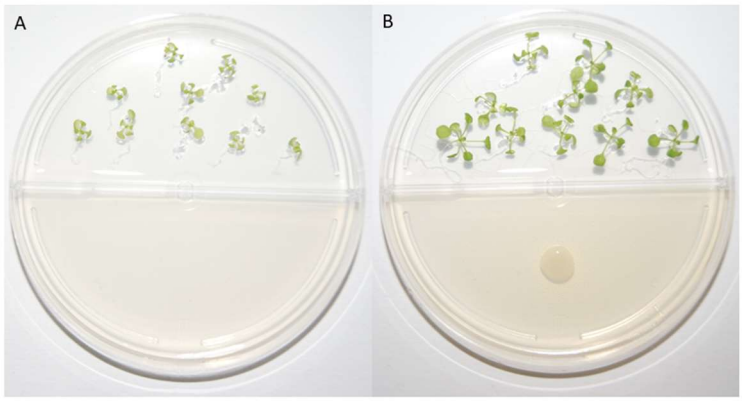


Figure 3 (continued).

The host species from which bacteria were isolated influenced their osmotolerance (Supplementary Table 1 and Figure 4). Strains isolated from *V. benghalensis* (AT), *O. compressus* (AV, MT and VC), *Medicago lupulina* L. (MT), *Medicago littoralis* Rohde ex Loisel. (MT) and especially *Medicago polymorpha* L. (VC) presented high tolerance to osmotic stress, since 40 to 100% of the strains were classified as tolerant and the remaining are moderately tolerant. Strains isolated from *Lotus corniculatus* L., *Vicia cordata* Hoppe. and *Vicia lutea* L. presented higher sensitivity, only including sensitive (9 to 33%) and moderately tolerant strains and all coming from MT and CV. Strains isolated from *Lathyrus clymenum* L. (AT and AV), *O. pinnatus* (MT), *Melilotus indicus* (L.) All. (MT), *T. repens* (MT), and *O. sativus* (MT and VC) are all moderately osmotolerant. Three plant species host strains with the three levels of tolerance and are from AT and AV (*S. vermiculatus* and *Medicago* sp.) or from AV and VC (*V. sativa*). The results evidence that strains osmotolerance is more associated with the host species than with location. In fact, the sites AT and AV have percentages of sensitive and tolerant strains similar to CV site. Most strains (88%) from MT are moderately tolerant and the number of sensitive strains is the lowest of the four sites. However, the strains displaying the highest osmotolerance (O3, S7 and P2) were isolated from the two southern sites (Supplementary Table 1).

3.5. *Principal component ordination (PCO)*

The PCOs of PGP abilities and osmotolerance using the host plant species as factor revealed that there is not an observable influence of host plant species in these bacterial properties (Supplementary Figure 2). The influence of the site is also not observed (Supplementary Figure 3).

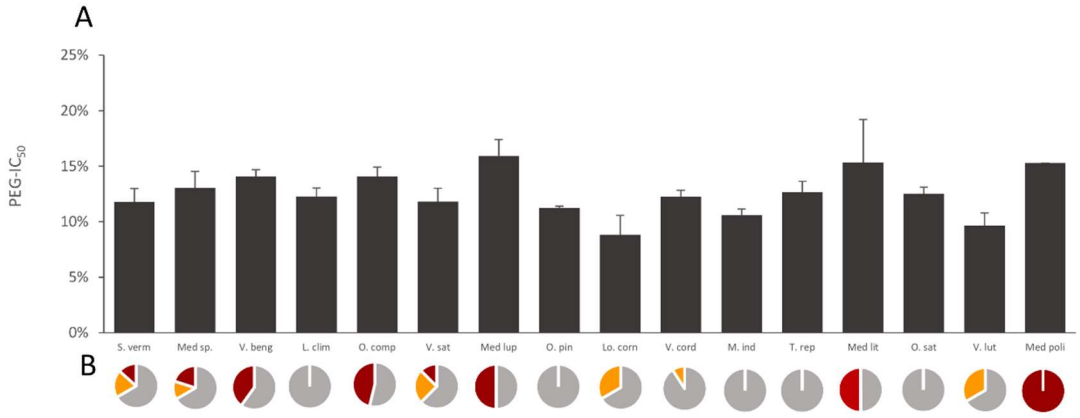


Figure 4. Osmotolerance of bacteria isolated fromdifferent host legume species: *Scorpiurus vermiculatus* (S. verm); *Medicago* sp. (Med sp.); *Vicia benghalensis* (V. beng); *Ornithopus compressus* (O. comp); *Vicia sativa* (V. sat); *Medicago lupulina* (Med lup); *Ornithopus pinnatus* (O. pin); *Lotus corniculatus* (Lo. corn); *Vicia cordata* (V. cord); *Melilotus indicus* (M. ind); *Trifolium repens* (T. rep); *Medicago litoralis* (Med lit); *Ornithopus sativus* (O. sat); *Vicia lutea* (V. lut); *Medicago polymorpha* (Med poli). (A) Concentration of PEG (%) inducing 50% growth inhibition (IC50). (B) Proportion of strains with different osmotolerance levels: Sensitive (IC50 b 8% PEG)-yellow; moderately tolerant (8% ≤ IC50 b 15% PEG)-grey; tolerant (IC50 ≥ 15% PEG)- red. Values are means of 3 replicates ± standard deviation.

4. Discussion

In the present study, we explored the diversity of bacteria harbored in the root nodules of legume species growing in wild areas of Portugal from distant geographical sites and distinct bioclimatic conditions. Since the different prevailing conditions between sites could have an impact on microbial diversity and performance, the isolation of bacteria from different sites and at the same time from different wild legume species was expected to yield different endosymbionts adapted to the conditions of the host when inside nodules and to the prevailing environmental conditions when in free living form. As expected, the different sites presented different legume species. However, a weak link of bacterial genera between the site of origin and the host species was observed. The genera isolated from the root nodules of the legumes collected in this study were all previously described as endophytic bacteria: *Flavobacterium* (Maheshwari, 2011), *Pseudomonas* (Maheshwari, 2011), *Erwinia* (Procópio et al., 2009), *Herbaspirillum* (Maheshwari, 2011), *Lysobacter* (Lòpez-Fernàndez et al., 2017), *Variovorax* (Maheshwari, 2011), *Achromobacter* (Dawwam et al., 2013), *Acinetobacter* (Maheshwari, 2011), *Agrobacterium* (Maheshwari, 2011), *Stenotrophomonas* (Maheshwari, 2011) and *Paenibacillus* (Costa et al., 2012). Several of these genera were already isolated from roots or other organs of legumes, including *Flavobacterium* (UmaMaheswari et al., 2013), *Pseudomonas* (UmaMaheswari et al., 2013) and *Paenibacillus* (Costa et al., 2012). Martínez-Hidalgo and Hirsch (2017) reported that amongst the most common non-nodulating endophytes found in legume nodules are *Azospirillum* (*Trifolium*, *Phaseolus*, *Vicia*, *Medicago*), *Variovarax* (*Crotalaria* and *Acacia*),

Pseudomonas (*Vigna*) and *Paenibaclillus* (*Medicago* and others). All these genera were also found in our study. Msaddak et al. (2017) found three genera in the root nodules of *Lupinus micranthus* Guss., *Bradyrhizobium* was an expected genus, but *Microvirga* and *Phyllobacterium* were unexpected. Moreover, of the fifty isolates obtained by Msaddak et al. (2017), almost half belonged to *Microvirga* and *Phyllobacterium*. These reports, along with this study, evidence the diversity of bacteria that colonize legume nodules, and challenge the perspective that rhizobia are the prevalent bacteria in legume nodules. Therefore, as more studies dealing with wild legumes species are published, the currently published known diversity of bacteria living inside legume nodules might increase. Bacteria that do not nodulate legumes can enter the nodule when rhizobia induce nodule formation (Ibáñez et al., 2009; Leite et al., 2017). Furthermore, a synergy between *Mesorhizobium* sp. and an endophytic *Pseudomonas chlororaphis* isolated from the root nodules of the legume *Sophora alopecuroides* L. has been reported by Zhao et al. (2013). These authors described an increase in siderophore production, phosphate solubilization, organic acid production, IAA production and antifungal activity *in vitro* when the two different bacteria were co-inoculated (Zhao et al., 2013). Thus, the lack of knowledge about the role of non-nodulating bacteria, alone or in consortia, understimates their potential benefits to the host plant.

The vast majority of the strains obtained in this study belong to the genera *Flavobacterium* and *Pseudomonas*. This is a result which is in accordance with the fact that these genera are dominant in plant microbiomes (Piechulla et al., 2017). Furthermore, these genera were found independently of the host legume species. It has been accepted for long that there is a molecular specificity between the two partners of the symbiotic relationship, that is the bacteria and the legume species (Somasegaran and Hoben, 1994), yet this knowledge is mainly based on the symbiosis between rhizobia and crop legumes. Unlike crop species, wild legumes seem to be more promiscuous in the interaction with rhizobia (Mutch and Young, 2004). Based on our results, this promiscuity seems to extend to non-rhizobial bacteria. A recent study by Hartman et al. (2017) reported abundant *Pseudomonas* isolates in the *Trifolium* root microbiome. In the same study, *Flavobacterium*, *Stenotrophomonas* and *Paenibacillus* were also observed (Hartman et al., 2017). *Flavobacterium*, *Pseudomonas* and *Paenibaclillus* were the dominant fraction of non-rhizobia isolates found inside root nodules of native legumes in a study in Flanders (Meyer, 2011). Both *Flavobacterium* and *Pseudomonas* have been reported as capable of inhibiting plant pathogens (Singh et al., 2016). Some *Pseudomonas* strains are plant growth promoting rhizobacteria used as inoculants to promote plant growth and alleviate stress in plants (Ahemad and Kibret, 2014).

Pseudomonas were also isolated from the rhizosphere of legumes, such as chickpea (*Cicer arietinum* L.) and green gram (*Vigna radiata* (L.) Wilczek) (Sindhu and Dadarwal, 2001). Ibáñez et al. (2009) reported that *Pseudomonas* entered the nodules of peanut (*Arachis hypogaea* L.) in an opportunistic manner. Nonetheless, these authors found that these *Pseudomonas* strains improved plant yield. Moreover, some *Pseudomonas* were described as diazotrophic (Santi et al., 2013). Regarding *Flavoabacterium,* negative effects on the growth of *Trifolium pratense* L. were reported, which could be counteracted when other bacteria were co-inoculated (Hartman et al., 2017). However, in our study some *Flavobacterium* strains displayed high ability to produce IAA and to promote *A. thaliana* growth through the emission of VOCs. Soltani et al. (2010) and Tsavkelova et al. (2005) also reported *Flavobacterium* as a IAA producer and a bacteria genus promoting plant growth (Ahemad and Kibret, 2014).

The fact that in this study non-rhizobial bacteria were found in root nodules of different wild legume species collected from four distant geographical sites, suggests that nodule colonization by non-rhizobial bacteria is not an anedoctal phenomenon but a common ocurrence, at least in legumes that are growing in land not used for agricultural purposes. It could be that in agricultural soils the isolation of these non-rhizobial bacteria is less frequent, due to the presence of different species usually cultivated in different conditions (fertilized and irrigated soils). The nutritional conditions provided to plants and from the plants to the microssymbionts can also differ. In agricultural areas, due to the growth of crop legumes in monocultures, large increases in rhizobial populations with higher affinity to the host occur (Hirsch, 1996; Kucey and Hynes, 1989). In wild areas, a high diversity of both legume and non-legume plants co-habit, and these non-rhizobial bacteria can play important roles as endophytic bacteria, providing benefits to plants, which can be crucial in the predominant conditions of these sites, where there is no input of fertilizers, irrigation and pesticides as in agricultural fields, and thus plants are more vulnerable to nutrient defficencies, water scarcity and infection.

Approximately half of the strains obtained in this study are IAA producers, and therefore have the potential to alleviate legumes suffering from drought-induced stress (Hussain et al., 2014). This can be put at risk if the bacteria cannot survive in an environment subjected to drought. Most of the strains in this work are moderately tolerant to PEG, although strains range from sensitive to tolerant. This information is important when selecting PGPR to be used as inoculants in areas frequently subjected to drought. Although inside the nodule some level of protection might be offered by the plant, the bacteria must be able to survive in the soil prior to infection.

The low specificity between the non-rhizobial endophytic bacteria and the host legume species observed in our study is an important feature both from an agronomical and an ecological point of view. If the strains selected as inoculants for legumes do not present a narrow specificity between the bacteria and the legume crop species, and instead bacteria are able to colonize a broad range of hosts, than strains with interesting PGP capabilites can effectively be used to enhance growth of different legume and non-legume species. In fact, the PGP abilites which were evaluated in this study (siderophores, IAA and volatiles capable of promoting the growth of *A. thaliana*) show that these bacteria can provide benefits to plant productivty which are not restriced to the host from which they were isolated. This becomes especially apparent with the airborne growth promotion of a non-legume, *A. thaliana*, by these strains (isolated from legumes). Moreover, bacterial VOCs that are able to promote plant growth might be beneficial in the field not only for their host, but also to surrounding plants. Bacteria from the genera *Bacillus*, *Pseudomonas*, *Burkholderia*, *Stenotrophomonas*, *Arthrobacter* and *Chromobacterium* have been reported previously as emitting VOCs with effects on *A. thaliana*, *Medicago sativa* L., *Nicotiana tabacum L.*, *Triticum aestivum* L. or *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Bailly and Weisskopf, 2012). Apart from effects observed from strains of the already reported bacterial genera (e.g. *Pseudomonas* and *Stenotrophomonas*), in this study *A. thaliana* growth promotion by several strains of *Flavobacterium*, *Herbaspirillum*, *Variovorax*, *Achromobacter*, *Erwinia*, *Acinetobacter*, *Agrobacterium/Rhizobium*, and *Paenibacillus* was shown (see Supplementary Table 1), thus extending the known range of bacterial genera capable of promoting plant growth through the emission of VOCs. Morevover, based on our results, these benefits are not restricted to a geographical area and its prevailing conditions, neither to a bacterial genus nor host legume species. This approach reduces the effort for the selection of effective strains which instead of being specific for a reduced set of plant species, can have a more broad use. From an ecological point of view, this issue is also important since legume species colonizing the same site can all benefit from the advantages provided by the most effective strains present in each site, be it growth improvement, resistance to infection or drought tolerance.

Concluding remarks

Our study shows that other bacteria besides rhizobia can be widely found in wild legumes nodules, particulary *Flavobactrium* and *Pseudomonas*, and might influence the growth of legumes and therefore should be studied so that they can be used in concert to improve legume and non-legumes productivity (Zahran, 2010). This becomes specially important as new mechanisms of PGP are found, such as the emission of

bacterial volatiles that can have multiple benefits for the plants (Piechulla et al., 2017), and which can strengthen the biotechnological potential of PGP bacteria to be used in the improvement of legume productivity while keeping environmental sustainability in mind.

Chapter 3

Alginate as a feature of osmotolerance differentiation among soil bacteria

Abstract

Agriculture in the 21st century has to produce more food to feed a growing population and agricultural production will have to increase at the same time that governments are trying to slash global greenhouse gas emissions and that less land area is suitable for agriculture. A possible strategy is the inoculation of crops with plant growth promoting (PGP) bacteria. Plants are naturally colonized by bacteria that can exert beneficial effects on growth and stress tolerance. These bacteria can be used as inoculants to boost crop productivity and natural plants resilience, and can be especially interesting if they are able to survive to abiotic stresses, such as drought. Herein we report the mechanisms that soil bacteria resort to tolerate drought and we also explore the influence of each mechanism to the level of drought tolerance exhibited. Of the determined osmolytes, betaine, trehalose and alginate increased in the majority of the strains exposed to polyethylene glycol 6000 (PEG). Betaine was the osmolyte with higher increases, evidencing the important role of this compound in the tolerance of bacteria to drought and helping to explain how bacteria tolerate conditions with high osmotic potential. However, betaine and trehalose levels were not significantly different among bacteria with different osmotolerance levels (sensitive, moderately tolerant and tolerant), thus not explaining why bacteria display different levels of osmotic tolerance. Moreover, several biochemical endpoints (protein content, superoxide dismutase, catalase, glutathione-S-transferases) related to oxidative stress were assessed, since although oxidative damage has been reported in drought conditions, little information exists. Nevertheless, in this study the oxidative stress parameters were not sufficient to explain the differences in osmotolerance observed for the tested strains. In contrast, alginate showed significant differences among the three levels of osmotolerance, enabling to relate for the first time the level of osmotolerance with the ability of soil bacteria to synthesize and accumulate alginate intracellularly. The use of alginate, applied directly to the soil or by inoculation with bacteria with a high ability to synthesize alginate, could be used to develop methodologies aiming at protecting communities of bacteria more susceptible to drought, thus preserving biodiversity and enhancing plant growth in drought affected soils.

Keywords

Plant growth promoting bacteria, drought, osmolytes, alginate, superoxide dismutase, catalase

1. Introduction

Drought is perhaps the most common environmental stress that soil microorganisms experience (Schimel et al., 2007). One third of the Earth's surface is arid, semi-arid, or

seasonally arid (Gurevitch et al., 2002). In Mediterranean ecosystems, surface soils frequently experience long dry periods followed by a relatively rapid wetting (Fierer and Schimel, 2002). Moreover, the number of extreme drought events is predicted to double by the 2090s, and the duration of these events is expected to increase by a factor of six (IPCC, 2007). Thus, more frequent and intense droughts are projected, with unknown impacts on agriculture. Several programs have been launched to increase crop production in drought affected areas (e.g. United Nations Development Programme (UNDP) and the European Union (EU) joint Programme to Combat Desertification in 2007, USDA Drought Resilience Partnership, India's Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad and the All India Coordinated Research Projects on Agri-Meteorology and Dryland Agriculture (AICRPAM and AICRPDA), FAO (Coping with Water Scarcity). However, little attention has been given to communities inhabiting these systems, although they may have a preponderant influence on systems productivity and resilience.

Soil microorganisms constitute less than 0.5% (w/w) of the soil mass, but they play a key role in soil properties (Yan et al., 2015). Microbial driven processes such as oxidation, nitrification, ammonification and nitrogen fixation lead to mineralization of organic matter into plant available nutrients (Yan et al., 2015). Therefore, microbes are essential to maintain a productive and valuable soil system (Yan et al., 2015). Disturbance of the soil environment can shift microbial communities and can have detrimental effects on soil nutrient cycling(French et al., 2009), being of utmost importance to maintain high microbial activity in soils (Yan et al., 2015).

The osmotic strength of environments is one of the physical parameters that determines the ability of organisms to proliferate in a given habitat (Patel et al., 2017). Exposure of cells to high external osmolarity results in water efflux (Csonka, 1989; Patel et al., 2017) and consequently to the increase of intracellular metabolites concentration (Csonka, 1989). Because an elevation in the concentrations of various intracellular ions may become toxic (Walderhaug et al., 1987), many cellular properties may change and perturbation of cell metabolism may overcome (Wood, 2015). Thus, passive alteration of the cell volume is not adequate for adaptation to changes in the osmolarity of the environment (Csonka, 1989). Evidence suggests that the regulation of cytoplasmic composition and hydration is a key feature of cellular homeostasis (Wood, 2011) and small differences in osmotolerance may reflect subtle differences in osmoadaptation mechanisms, with these differences influencing the relative fitness of individual species and strains (Freeman et al., 2013).

The increase of specific solutes, which are compatible with cellular processes even at high concentrations is an efficient mechanism of tolerance to drought (Brown and Simpson, 1972). However, uptake and synthesis of osmolytes require large amounts of energy, resulting in reduced growth and activity (Oren, 1999; Schimel et al., 2007; Wichern et al., 2006).

Compatible solutes are similar among phylogenetically distant organisms and comprise inorganic ions and few classes of organic osmolytes (Yancey et al., 1982). The prominent compatible solutes found in bacteria are K^+ ions, the amino acids glutamate and proline, the quaternary amine glycinebetaine, and the sugars sucrose and trehalose (Flowers et al., 1977; Imhoff, 1986; Reed, 1986; Yancey et al., 1982). Dinnbier et al. (Dinnbier et al., 1988) and Ohwada and Sagisaka (Ohwada and Sagisaka, 1988) reported that accumulation of K^+ was only transient and that 30 min after the exposure of *Escherichia coli* to hyperosmotic media, cells excreted K^+, returning to pre-stress value (Roller and Anagnostopoulos, 1982). Measures, (1975) found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. Yaakop et al. (Yaakop et al., 2016) described proline to be a critical osmoprotectant for *Jeotgalibacillus malaysiensis*. On the other hand, Perroud and LeRudulier (Perroud and Le Rudulier, 1985) found that the intracellular concentrations of glycinebetaine maintained by *E. coli* were proportional to the osmolarity of the medium. Trehalose has been found to be synthesized in a number of bacteria (Galinski and Truper, 1982; Larsen et al., 1987; Mackay et al., 1984; Reed, 1986), in response to osmotic stress and mutations which resulted in accumulation impairment of trehalose increased sensitivity to osmotic stress (Giaever et al., 1988). The osmoprotectants glycinebetaine and proline were able to suppress the osmotic accumulation of K^+ in enteric bacteria (Sutherland et al., 1986) and this ability is consistent with the notion that these compounds are less toxic to cellular processes than K^+ and are accumulated preferentially over K^+ by cells as a means of maintaining turgor (Csonka, 1989). Accumulation of compatible solutes such as proline, glycinebetaine and trehalose protect proteins from denaturation (Schobert, 1977), by enhancing proteins solubility (Schobert and Tschesche, 1978), increasing thermotolerance of enzymes, stabilizing proteins in moieties that would otherwise lead to their denaturation (Csonka, 1989), scavenging hydroxyl radicals (Marulanda et al., 2009), and regulating the NAD/NADH ratio (Marulanda et al., 2009).

Other mechanisms that help bacteria to cope with water stress include production of extracellular polymeric substances (Freeman et al., 2013; Ngumbi and Kloepper, 2016). The production of exopolymeric compounds, such as alginate, encapsulate

bacterial cells protecting them from dehydration (Chang et al., 2007; Freeman et al., 2013). Alginate is a linear anionic co-polymer of β-d-mannuronic acid (M) and α-l-guluronic acid (G) (1–4)-linked residues, arranged either in heteropolymeric (MG) and/or homopolymeric (M or G) blocks (Larsen et al., 2003; Yang et al., 2011). Alginate is hygroscopic, can hold several times its weight in water thereby keeping cells hydrated (Robyt, 1998; Sutherland, 2001). Moreover, alginate production was reported to clearly provide a competitive advantage for bacteria in water-limited environments, leading to increased ecological success (Chang et al., 2007), and helping maintain membrane integrity (Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007; Welsh, 2000).

Besides osmotic effects, drought stress also results in the accumulation of reactive oxygen species (ROS) (Benabdellah et al., 2011; Ngumbi and Kloepper, 2016). However, there is very limited information about the oxidative response of soil microorganisms in response to drought (Benabdellah et al., 2011). Oxidative damage is caused by increased concentrations of ROS, that can react with a large variety of biomolecules causing irreversible damage (Kim et al., 2008; Rivero et al., 2007; Wu and Ni, 2015) . Effects include changes in protein conformation, protein denaturation, restricted enzyme efficiency, changes in electron transport chains (Bérard et al., 2015; Vriezen et al., 2007) and lipid peroxidation (Potts, 1999). However, living organisms are equipped with antioxidant mechanisms to regulate intracellular ROS concentrations. ROS scavenging mechanisms include the enzymes superoxide dismutases (SOD), catalase (CAT) and glutathione-S-transferases (GSTs). SOD catalyzes superoxide radicals dismutation into hydrogen peroxide and is considered the first line of defense against ROS (Nunes et al., 2018). CAT, detoxifies H_2O_2 to H_2O and O_2 (Nunes et al., 2018). GSTs catalyze the conjugation of GSH with xenobiotics with exogenous or endogenous origin (Sharma et al., 2004)) and convert toxic aldehydes (resulting from peroxydized polyunsaturated fatty acids) to less toxic alcohols (Korpi et al., 2009; Schmidt et al., 2015).

Since drought is a current problem and it is projected to worsen, understanding the mechanisms behind bacteria tolerance to drought is a subject deserving attention and will contribute to mitigate drought effects in soil bacterial communities and to improve strategies for the use of beneficial bacteria in mediating drought tolerance in other inhabitants of drought impacted ecosystems, namely plants. Indigenous bacterial populations may have adapted to stress conditions and evolved the capacity to survive in stressed soils (Marulanda et al., 2009), selection of drought tolerant strains isolated

from stressed ecosystems can be a more effective strategy in reducing plants susceptibility to drought.

With this in mind, the central focus of this study was to untangle the intracellular osmolytes used by phylogenetically diverse soil bacteria isolated from different edaphoclimatic conditions, and to understand the determinants driving the level of osmotolerance. To achieve this goal, representatives of all genera of bacteria previously isolated from wild legumes growing at different edaphoclimatic environments were used (Cardoso et al., 2018). Furthermore, strains with different osmotolerance levels from the most represented genera (*Pseudomonas* and *Flavobacterium*) were also studied. Strains were grown in non-stressed (control) and drought stressed (PEG % inducting 50% growth inhibition) conditions and osmotic (proline, glycinebetaine, trehalose, alginate), antioxidant (SOD, CAT), biotransformation (GSTs) and damage (lipid peroxidation) endpoints were studied.

2. Material and methods

2.1. Bacterial strains

Bacteria were isolated from the nodules of plants from different wild legume species harvested from four sites in continental Portugal in spring 2015, as described by Cardoso et al. (2018). Molecular typing of bacterial isolates identified 100 distinct profiles, belonging to 11 genera, with 85% of strains belonging to *Pseudomonas* and *Flavobacterium*) and displaying different plant growth promotion (PGP) abilities and different tolerance levels to politethylene glycol (PEG) (Cardoso et al., 2018).

From this set twenty strains were selected using biodiversity and tolerance to PEG as selection criteria (Table 1). All bacterial genera are represented in this study. In genera with higher number of strains different levels of tolerance, sensitive (S -EC_{50} < 10%PEG), medium tolerant (MT -EC_{50} ≥ 10% and < 15% PEG) and tolerant (T -EC_{50} ≥ 15%PEG) strains were used.

2.2. Experimental conditions

Strains were grown in tubes containing 5 mL of yeast broth mannitol (YMB) medium (Somasegaran and Hoben, 1994) supplemented at control (no PEG addition) and PEG (at EC_{50} PEG %s -table1, rounded to unit%). Inoculated tubes were incubated at 26 °C in an orbital shaker (150 rpm) until late exponential phase (14 h). Growth was determined by measuring optical density at 620 nm. The relationship between optical density and cell concentration was obtained by direct cell counting in a Neubauer chamber. Cell concentration was expressed in million cells per milliliter (M cells mL^{-1}). Three

independent experiments were carried out with 3 replicates per condition. Cells from each tube were collected separately after centrifugation at 10,000 × g for 10 min at 4°C, washed twice with deionized water, and frozen at -80°C for further use. The growth media and cell wash water from one replicate of each experiment were pooled and also frozen at -80°C for extracellular alginate quantification.

Table 1. Selected endophytic bacteria differing in osmotolerance. Percentage of PEG that inhibits 50% growth (PEG%). and osmotolerance (OsmT) level (Sensitive - PEG% < 10, Moderately tolerant - 10 ≤ PEG% < 15 and Tolerante- PEG% >15%). Strains were isolated from the root nodules of wild legumes (Legume host species) growing in four sites (Aljustrel-At, Alvito-Av, Murtosa-M and Vale de Cambra-V) in Continental Portugal (Site). Bacteria genera identified by 16S rRNA gene sequencing.

Strain	PEG%	Legume host species	Site	Bacteria genera
Sensitive				
Q1	7.69	*Medicago* sp.	At	*Flavobacterium*
U6	7.41	*Ornithopus pinnatus*	M	*Agrobacterium/Rhizobium*
M1	7.25	*Vicia sativa* subsp. *sativa*	Av	*Flavobacterium*
D4	5.94	*Lotus corniculatus*	V	*Flavobacterium*
Moderately Tolerant				
N1	14.06	*Scorpiurus vermiculatus*	At	*Herbaspirillum*
K6	13.43	*Ornithopus compressus*	Av	*Achromobacter*
K1	13.29	*Vicia sativa* subsp. *sativa*	Av	*Erwinia*
U1	14.15	*Medicago lupulina*	M	*Acinetobacter*
H5	13.17	*Medicago lupulina*	M	*Pseudomonas*
I9	11.18	*Ornithopus sativus* subsp. *sativus*	M	*Pseudomonas*
V4	14.80	*Medicago lupulina*	M	*Stenotrophomonas*
E1	10.91	*Vicia sativa* subsp. *sativa*	V	*Flavobacterium*
A7	13.86	*Ornithopus sativus* subsp. *sativus*	V	*Pseudomonas*
Tolerant				
O4	18.71	*Medicago* sp.	At	*Herbaspirillum*
Q4	15.56	*Medicago* sp.	At	*Lysobacter*
N9	15.69	*Vicia benghalensis*	At	*Variovorax*
O3	25.8	*Scorpiurus vermiculatus*	Av	*Herbaspirillum*
B3	20.50	*Ornithopus compressus*	V	*Flavobacterium*
A10	18.19	*Ornithopus compressus*	V	*Paenibacillus*
C11	17.82	*Ornithopus compressus*	V	*Pseudomonas*

2.3. Cell damage

A replicate from each experiment was suspended 20% (v/v) trichloroacetic acid (TCA) and lysed in an ultrasonic probe for 20 s, keeping tubes in an ice bath, and extracts centrifuged at 12,000 × g for 10 min at 4°C. Lipid peroxidation (LPO) was measured by quantification of thiobarbituric acid reactive substances (TBARS), according to the protocol described by Buege and Aust (Buege and Aust, 1978), based on the reaction of lipid peroxidation products such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA), forming TBARS. The amount of TBARS was quantified spectrophotometrically at 532 nm and calculated using the molar extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}). Results were expressed in nmol of MDA equivalents per million cells (nmol MDA eq M $cells^{-1}$).

2.4. Osmotolerance

Alginate determination was adapted from the method described by Johnson et al. (2009), using dimethyl methylene blue (DMBB) A replicate from each experiment was suspended in sodium phosphate buffer (0.1M pH 7.25), lysed in an ultrasonic probe for 20 s and extracts centrifuged at 12,000 × g for 10 min at 15°C. DMBB was added both to the supernatant and to the growth media in order to quantify intra and extracellular alginate, respectively. Absorbance was immediately measured at 525 nm and alginate standards (1.25 – 25 μg mL^{-1}) were used. Results were expressed in ng alginate per million cells (ng M $cells^{-1}$).

Betaine was determined following the method described by Grieve and Grattan (1983). A replicate from each experiment was suspended in deionized water, lysed in an ultrasonic probe for 20 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, sulfuric acid (1M) and KI-I_2 were added and incubated overnight in ice. Tubes were centrifuged at 12,000 × g for 30 min at 4°C, the supernatant discarded, dichloroethane was added and tubes vortexed. After 2h the absorbance was measured at 365 nm and betaine standards (50 – 200 μg mL^{-1}) were used. Results were expressed in ng alginate per million cells (ng M $cells^{-1}$).

Proline was determined following the method described by Bates et al., (1973) with some modifications. A replicate from each experiment was suspended in 3% sulfosalicylic acid, lysed in an ultrasonic probe for 20 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, 2.5% acid ninhydrin and glacial acetic acid were added and incubated 1h at 100 °C and cooled in an ice bath. Toluene was added and vortexed vigorously for 15-20 s. The toluene phase was separated from the aqueous phase, and absorbance measured at 520 nm and proline standards (1 – 6.25 μg mL^{-1}) were used. Results were expressed in ng proline per million cells (ng M $cells^{-1}$).

Trehalose was determined following the method described by Dahlqvist (1968) with some modifications. A replicate from each experiment was suspended in citric acid buffer (0.135 M, pH 5.7), lysed in an ultrasonic probe for 20 s and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was collected, trehalase solution (0.2 unit/ml) was added and incubated 15 min at 37 °C. The glucose formed was determined using an assay kit (NZYTech, Portugal). Absorbance was measured at 510 nm and trehalose (15-240 μg mL^{-1}) and glucose (15-240 μg mL^{-1}) standards were used. Results were expressed in ng trehalose per million cells (ng M $cells^{-1}$).

2.5. Antioxidant and biotransformation responses

Cells were suspended in sodium phosphate buffer (50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dihydrate; 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT), pH 7.0) and lysed in an ultrasonic probe for 20 s, keeping tubes in an ice bath, and extracts centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was immediately used or frozen (-80°C) for Protein content, catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferases (GSTs).

Protein content was determined by the Biuret method (Robinson and Hogden, 1940). The amount of protein was determined spectrophotometrically at 540 nm, using bovine serum albumin (BSA) as standard (1.25 to 10 mg mL^{-1}). Results were expressed in µg protein per million cells (µg M $cells^{-1}$).

Catalase (CAT) activity was determined by the reaction of CAT with methanol in the presence of H_2O_2 (Johansson, 1988). A standard curve was built using formaldehyde standards (2.5 – 30 µM). One unit (U) was defined as the amount of enzyme that caused the formation of 1.0 µmol formaldehyde, per min. Results were expressed in milliunits (mU) per million cells (mU M $cells^{-1}$).

Superoxide dismutase (SOD) activity was determined by the reaction of nitro blue tetrazolium (NBT) with superoxide radicals to form NBT diformazan, based on the method described by (Beauchamp and Fridovich, 1971). Absorbance was measured at 560 nm and SOD standards (0.01 – 60 U) were used. One unit of enzyme activity (U) corresponds to a 50% reduction of NBT. Results were expressed in milliunits (mU) per million cells (mU M $cells^{-1}$).

Glutathione-S-transferases (GSTs) activity was determined using the method described by (Habig et al., 1974) and modified by (Corticeiro et al., 2013). GSTs catalyze the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH), forming a thioester product. The reaction can be followed by the absorbance increase at 340 nm and GSTs activity determined using CDNB extinction coefficient (9.6 mM cm−1). Results were expressed in nanounits (mU) per million cells (nU M $cells^{-1}$).

2.6. Statistical analysis

All parameters tested were submitted to hypothesis testing. One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA). Parameters were analyzed

following a one-way hierarchical design, with PEG conditions (0-control and EC_{50}) as fixed factor. The null hypothesis tested was: no significant differences exist between tested conditions. Significant differences were considered for $p \leq 0.05$, and were identified in figures with different lowercase (for control) and uppercase (PEG) letters.

A matrix gathering the descriptors (LPO, trehalose, alginate, betaine proline, Prot, SOD, CAT, GSTs) per strain and condition was used to calculate a Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on the strains and conditions, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biomarkers (correlation > 0.60) were provided as supplementary variables and superimposed on the PCO graph, allowing to identify the descriptors that contributed more to differences among strains and between conditions.

3. Results

3.1. Cell density

Since osmotolerance of strains was already published (Cardoso et al., 2018) and in this study the concentrations of PEG used were the EC_{50} of each strain, only cell density in control condition was presented (Figure 1).

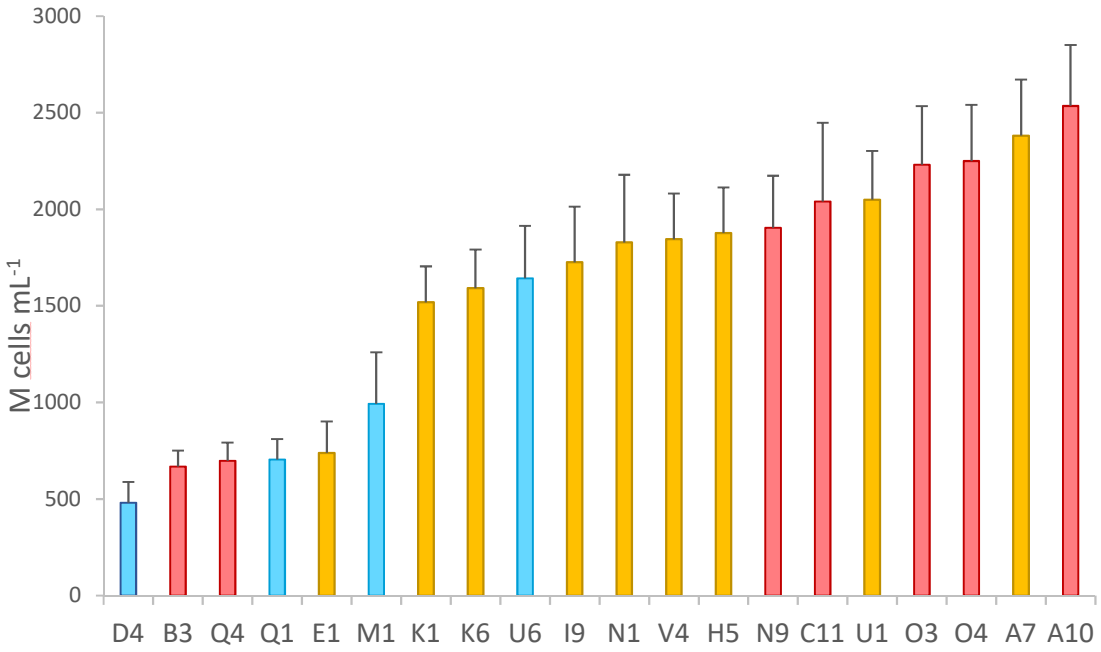


Figure 1. Cell density of selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) grown under control conditions. Values (+ standard error) are means of at least 3 replicates of 3 independent experiments.

Strains displayed disparate cell densities (the difference between the strain having the highest and the lowest cell density is greater than 5-fold). The influence of strains osmotolerance level on cell density is recognized, of the 6 strains with cell densities lower than 1000 M cells mL^{-1}, three (50%) are sensitive, two are tolerant and one is moderately tolerant. The strain with the lowest cell density (480 M cells mL^{-1}) is sensitive. Eight strains had cell densities between 1000 and 2000 M cells mL^{-1}, of which 6 (75%) are moderately tolerant, one is tolerant and one is sensitive. Seven strains had cell densities higher than 2000 M cells mL^{-1}, four (57%) tolerant, three (43%) moderately tolerant and no sensitive strains displayed such high cell densities (Figure 1).

3.2. Cell damage

LPO levels of strains in the absence (control) and presence of PEG were represented in a coordinate plan (control in the abscissa and PEG in the ordinate) (Figure 2A), in order to evince the relationship of LPO levels at both conditions. The dotted line marks identical LPO values between the two conditions, symbols (circles) above the line represent higher values in PEG than in the control condition, circles below the line represent higher values in control than in the PEG condition. Variability in LPO levels among strains is observed. For most strains LPO increased (between 1.5 and 9.1 times) in PEG relatively to control. In five strains LPO did not vary or even decreased in PEG relatively to control condition. Three strains clearly stood out above the line showing higher increase in LPO (between 6.7 to 9.1 fold) in the presence of PEG relatively to control.

LPO by strain's osmotolerance level of PEG and control conditions are shown in Figure 2B. In control, no significant differences were observed among osmotolerance levels. PEG increased LPO relatively to control but significant differences were only noticed in T and MT strains. Significant lower LPO in S compared to T and MT strains was observed (Figure 2B).

3.3. Osmotolerance

The osmolytes to which cells resorted to counteract the effects of exposure to increased osmolarity are presented in Figure 3. This figure presents strains separately (Figures 3A, 3C, 3E and 3G) and grouped by tolerance level (Figures 3B, 3D, 3F and 3H).

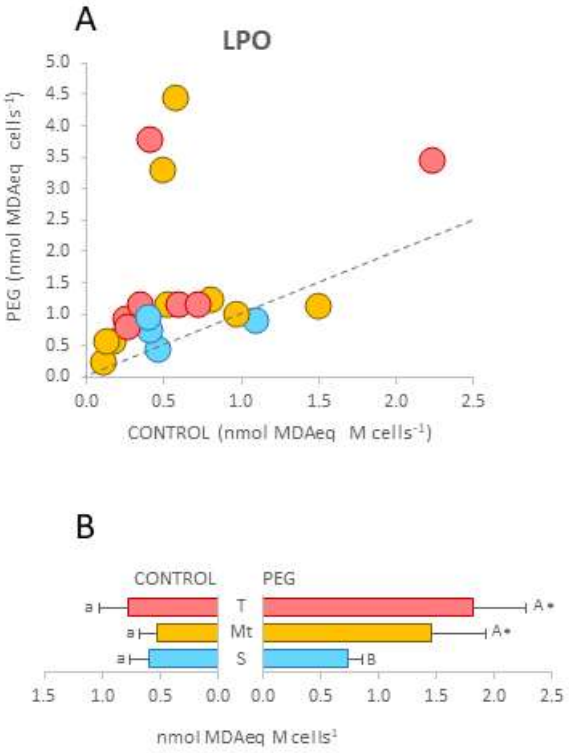


Figure 2. Lipid peroxidation (LPO) in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. A) LPO levels for each strain in control versus PEG condition; dashed line represents equal concentrations in both conditions for each strain; values are means of at least 3 replicates. B) LPO levels for strains grouped by tolerance level; values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

Proline concentrations of most strains (80%) were lower than 0.5 ng M cells^{-1} in control, but four strains displayed higher proline concentrations (between 0.75 and 3 ng M cells^{-1}). PEG induced (above dashed line) proline in 60% of strains and reduced (below dashed line) in 15% of strains (Figure 3A). Osmotolerance level influenced proline concentrations in control and PEG conditions (Figure 3B). MT strains had significantly lower proline concentrations than S and T strains at control. Proline levels were not changed by PEG in T strains but increased in the MT and S strains, although only significantly in MT ones (Figure 3B).

At control betaine levels of most strains (70%) were below 10 ng M cells^{-1}, the remaining 30% displayed values between 16.1 and 25.6 ng M cells^{-1} (Figure 3C). In one strain betaine was not increased by PEG, in the other 19 PEG induced increases of this osmolyte between 3 and 37-fold relatively to control (Figure 3C). Betaine concentration of strains grouped by osmotolerance level (Figure 3D) showed significantly higher betaine levels in S strains compared to T and MT strains in control. PEG quashed this difference and betaine concentration was not significantly different among

osmotolerance levels. Comparing conditions, the large betaine increase by PEG relatively to control was noticed for all levels of tolerance. These increases were more expressive for the T and MT strains (13 and 12-fold, respectively) than for S strains (8-fold) (Figure 3D).

Variability in trehalose content (between 8.3 and 78.6 ng M cells^{-1}) were observed among strains. Most (65%) of them were clearly above the dashed line, evidencing increased trehalose levels in presence of PEG. However, some strains (6) are over the line (no concentration change between control and PEG) and one strain is below the line (trehalose decrease by PEG) (Figure 3E). The average response of strains per tolerance level evidenced an inverse (though not statistically significant) relationship between trehalose content and osmotolerance in both control and PEG conditions. However, trehalose concentrations were higher in PEG than in control for all tolerance levels, but significant increases were only observed for strains with higher osmotolerance (T and MT) (Figure 3F).

Two strains were not able to synthesize alginate nor in control neither in PEG conditions. The amount of alginate present extracellularly was considered negligible (less than 5%) and therefore were not presented. In the other 18 strains a high variability was observed, with most strains (80%) synthesizing between 1.5 and 5.5 ng alginate M cells^{-1} in the control and two stains producing more than 10 ng M cells^{-1}. Among the strains able to synthesize alginate, PEG increased between 1.5 and 9.1-fold the amount of alginate in 70% of them (Figure 3G). Results presented by osmotolerance level (Figure 3H) showed the lower ability of S strains to synthesize alginate both in control and PEG conditions, evidencing the inability of S strains to induce alginate synthesis in presence of PEG. On the contrary, PEG increased by 1.7 and 2.4 fold trehalose levels in MT and T strains, respectively (Figure 3H).

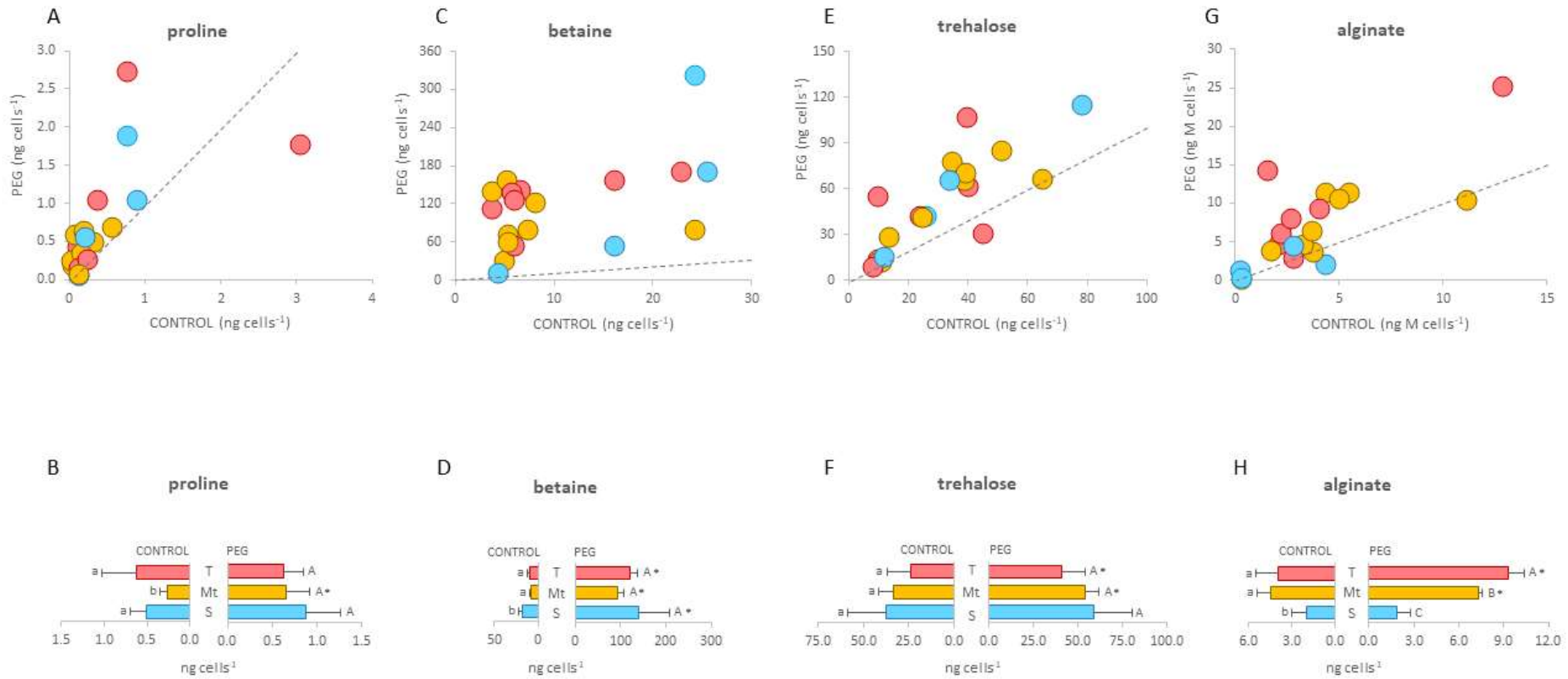


Figure 3. Intracellular concentration of osmolytes in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. Concentration of osmolytes for each strain in control versus PEG condition (A- proline, C- betaine, E- trehalose, G- alginate); dashed line represents equal concentrations in both conditions for each strain; values are means of at least 3 replicates. Osmolytes concentrations for strains grouped by tolerance level (B- proline, D- betaine, F- trehalose, H- alginate); values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

3.4. Antioxidant and biotransformation response

The metabolic effort to adapt to changes induced by high osmolarity is presented by protein levels (Figures 4A and 4B). The antioxidant (SOD, CAT) and biotransformation (GSTs) mechanisms strains resorted as a way to minimize oxidative damage and toxicity of endoxenobiotics by exposure to high osmolarity is also presented separately (Figures 4C, 4E and 4G) or grouped by tolerance level (Figures 4D, 4F and 4H)

Protein content of most strains (60%) was lower than 1 µg M cells^{-1}, the other strains had protein levels between 1 and 3 µg M cells^{-1} (Figure 4A). Most strains do not show variation in the amount of protein between conditions (PEG and control), but three strains had 50% lower and two 100% more protein in the presence of PEG relatively to control (Figure 4A). Protein content by osmotolerance level (Figure 4B) showed that in control condition S strains presented 2-fold ($p< 0.05$) more protein than MT and T strains, but PEG narrowed this difference and at PEG condition no significant difference was observed among tolerance levels (Figure 4B).

Although most strains had CAT activities between 0.2 and 0.5 mU M cells^{-1} in the control, four strains displayed activities above 1 mU M cells^{-1}. In most strains PEG duplicated CAT activity, but in five strains no increase was observed in CAT activity between conditions (Figure 4C). Overall performance by osmotolerance level (Figure 4D) showed that S strains exhibited significantly higher activity than MT strains both in presence and absence of PEG. However, PEG increased CAT activity relatively to the control at all tolerance levels (Figure 4D).

Most strains displayed SOD activities below 120 mU M cells^{-1} for both conditions, but three strains had higher activities. SOD activity was not changed by PEG in 55% of strains, but increases (between 3 and 19-fold) and decreases (between 2 and 6 fold) were recorded in 3 and 6 strains, respectively (Figure 4E). Overall performance by osmotolerance level (Figure 4F) evidenced significant differences among osmotolerance levels both presence and absence of PEG, but no differences were observed between conditions (Figure 4F).

Most (70%) strains had GSTs activities close to 1 mU M cells^{-1} in the control condition. In the other strains (30%) activities between 2.5 and 4.5 mU M cells^{-1} were observed. With the exception of four strains, PEG increased (1.5 to 2.7-fold) the activity of GSTs (Figure 4G). Analysis by osmotolerance level (Figure 4H) showed higher activity in S strains in both conditions and increased activity by PEG relative to the control at all osmotolerance levels (Figure 4H).

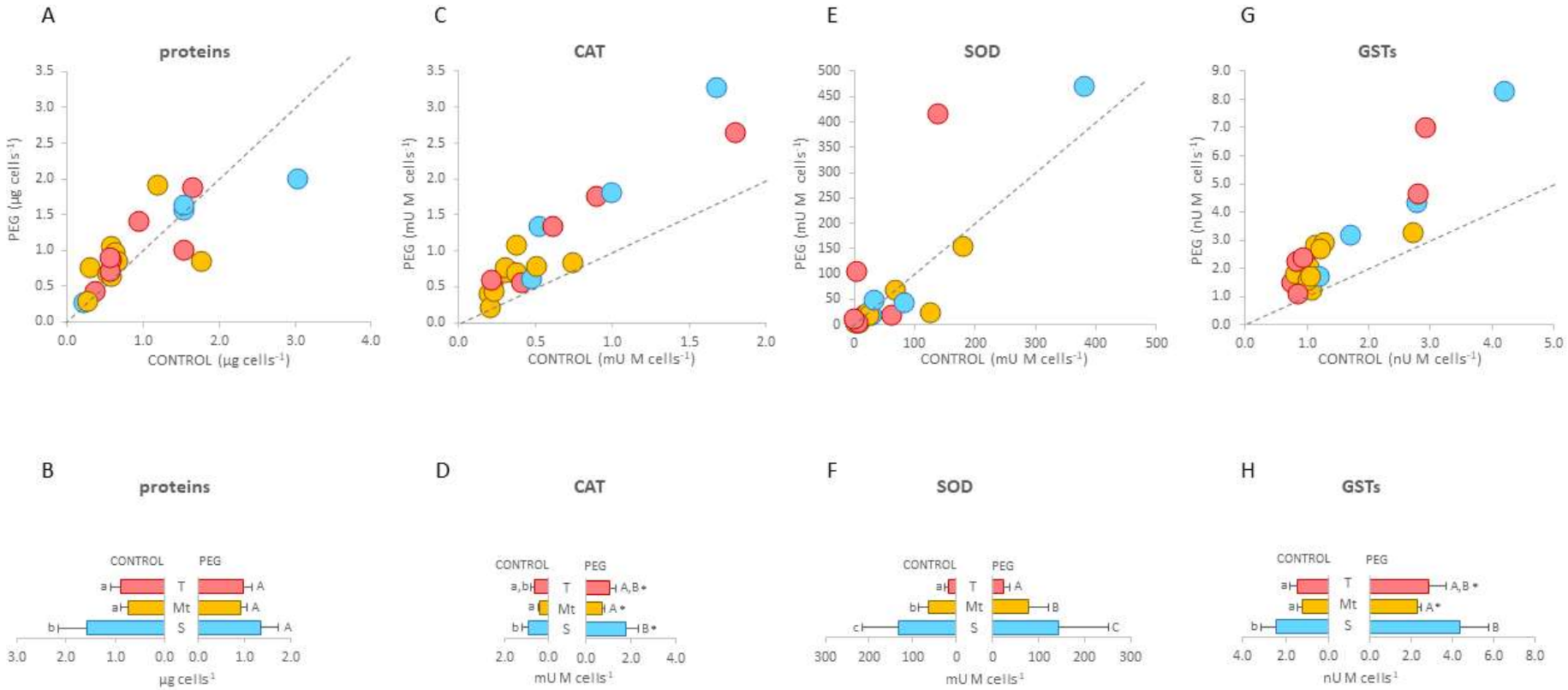


Figure 4. Soluble protein and activity of antioxidant (CAT, SOD) and Biotransformation (GSTs) enzymes in selected endophytic bacterial strains with different osmotolerance levels (blue - sensitive, yellow – moderately tolerant, pink – tolerant) exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. Protein concentration and enzyme activity for each strain in control versus PEG condition (A- protein, C- CAT, E- SOD, G- GSTs); dashed line represents equal levels or activity in both conditions for each strain; values are means of at least 3 replicates. Protein concentration and enzyme activity for strains grouped by tolerance level (B- protein, D- CAT, F- SOD, H- GSTs); values are means (+ standard error) of 12 to 27 values; different lowercase letters indicate significant differences among tolerance levels in control condition and uppercase letters indicate significant differences among tolerance levels in PEG condition.

3.5. Multivariate analysis

Principal Components Ordination (PCO) diagram evidenced that the two axes represented explained more than 90% of the total variation obtained among strains exposed to two conditions (Figure 5). Along PCO1 (explaining 51.9% of total variation) strains with different levels of tolerance were separated, 86% of the T strains are on the positive side, 88% of the S strains are on the axis or on the negative side, and MT strains are on both sides of the axis. PCO2, which explained 39.2% of total variation, separated the two conditions, all strains in control condition are on the axis or on the negative side, and 85% of strains grown with PEG are on the positive side. Strains osmotolerance level was highly correlated (r=0.96) with SOD activity, evidencing that SOD activity (both at control and PEG conditions) influenced the level of osmotolerance displayed by strains. Exposure to PEG was highly correlated with osmolytes, such as betaine (r=0.94) and trehalose (r=0.66), with CAT (0.79) and GSTs (0.74) activity, and with alginate (0.65), evidencing the effort of cells to regulate osmotically and to fight oxidative stress imposed by PEG exposure. Despite the activation of these mechanisms exposure to PEG was also highly correlated with LPO (0.70), evidencing the damage that PEG-induced oxidative stress caused in membranes.

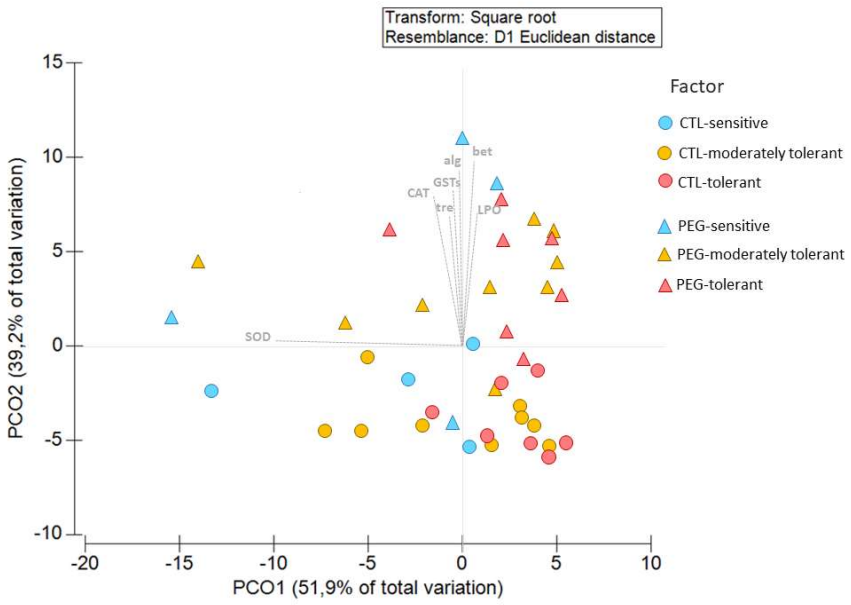


Figure 5. Principal Coordinates (PCO) with Centroids ordination of in selected endophytic bacterial strains with different osmotolerance levels exposed to control and PEG (% PEG inducing 50% growth inhibition) conditions. Pearson correlation vector imposed lipid peroxidation (LPO), betaine (bet), alginate (alg) and trehalose concentrations, and superoxide dismutase (SOD), glutathione S-transferases (GSTs) and catalase (CAT) activity (r ≥ 0.65).

4. Discussion

Several studies addressed the effect of osmotic stress on bacteria (Benabdellah et al., 2011; Chang et al., 2007; Csonka, 1989; Fierer and Schimel, 2002; Freeman et al., 2013; Marulanda et al., 2009; Ngumbi and Kloepper, 2016; Patel et al., 2017; Schimel et al., 2007; Wood, 2015; Yaakop et al., 2016; Yan et al., 2015; Yancey et al., 1982) and some assessed the mechanisms of tolerance (Benabdellah et al., 2011; Chang et al., 2007; Csonka, 1989; Freeman et al., 2013; Yaakop et al., 2016; Yancey et al., 1982), yet these studies focus on a strain or a set of strains of the same species and results are hardly extrapolable to other bacterial groups and accepted as a general bacterial response.

The present study aimed to investigate the mechanisms underlying the tolerance differences observed in soil bacteria isolated from wild legumes (Cardoso et al., 2018) growing at different edaphoclimatic environments and the relation between tolerance level and bacteria diversity (genus level). In this way, representatives of all genera isolated, and of strains with different osmotolerance levels from most represented genera (*Pseudomonas* and *Flavobacterium*) were studied.

Differences observed among strains of the same genus overlapped differences among genera. However similar response patterns among strains with the same level of osmotolerance were noticed, regardless of the genus and the edaphoclimatic provenance.

The osmotolerance level of strains was not limited by the level of oxidative stress, since when exposed to PEG sensitive strains presented lower LPO levels than more tolerant ones. Sensitive (S) strains already presented higher activity of antioxidant (CAT, SOD) and biotransformation (GSTs) enzymes in the control, which only increased significantly in the presence of PEG for CAT, but that were sufficient to control oxidative stress and limit LPO. Indeed, the presence of PEG did not increase LPO significantly relatively to control in S strains.

Since strains sensitivity was not associated with oxidative stress, it may be related to changes in other cellular functions, such as osmotic adaptation. General osmotolerance mechanisms are similar across diverse bacteria and include the accumulation of compatible solutes by *de novo* synthesis and uptake. The compatible solutes accumulated by bacteria differ among species and even strains but include the disaccharide trehalose (D'Souza-Ault et al., 1993; Freeman et al., 2010; Kurz et al., 2010), glycinebetaine (Chen and Beattie, 2008, 2007; Csonka, 1989; D'Souza-Ault et al., 1993) and proline (Csonka, 1989; Ngumbi and Kloepper, 2016; Yaakop et al., 2016). Accumulation of compatible solutes such as proline, glycinebetaine and trehalose

increase the thermotolerance of enzymes, inhibit proteins denaturation, and help maintain membrane integrity (Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007; Schobert and Tschesche, 1978; Welsh, 2000). The observed protection may have resulted from the interaction of these compounds with proteins resulting in the coating of proteins with a hydrophilic shell that would enhance their solubility (Csonka, 1989). Additional mechanisms of water stress tolerance include cellular aggregation (Monier and Lindow, 2003) and production of exopolymeric substances such as alginate (Chang et al., 2007). In our study, exposure to PEG increased trehalose and proline in 60% of strains with different levels of osmotolerance. Freeman and collaborators (2013)(Freeman et al., 2013) also observed that trehalose contributed to the osmotolerance of two *Pseudomonas* strains differing in osmotolerance and found that trehalose was a much larger contributor to osmotolerance than other osmolytes (Freeman et al., 2013). Measures (1975) found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. Csonka (1989) reported that some species respond equally to both proline and glycinebetaine, other are stimulated more dramatically by glycinebetaine than by proline. In soil bacteria, such as *Rhizobium meliloti* glycinebetaine also accumulated in cells grown in media with high osmolarity (Smith et al., 1988). In our study, glycinebetaine increased much more in PEG-exposed cells relatively to control than did other osmolytes (trehalose, proline), constituting a preferential mechanism of osmotolerance in strains of all levels of tolerance and providing a particularly strong fitness benefit to osmotically stressed cells. Yet, there were no significant differences in the osmolytes commonly referred in the literature (trehalose, proline and glycinebetaine) among strains form different osmotolerance levels.

Therefore, the present study searched for alternative mechanisms that could explain the differences in the osmotolerance observed. The production of exopolymeric substances, such as alginate, was proposed by several authors (Chang et al., 2007; Freeman et al., 2013) to increase bacteria osmotolerance (Chang et al., 2007), by encapsulating bacterial cells and thus protecting them from desiccation (Chang et al., 2007). In our study, quantification of extracellular alginate evidenced very low concentrations, falling to evidence differences among strains with different levels of osmotolerance. However, S strains accumulated low intracellular concentrations of alginate at control and were unable to increase alginate synthesis in presence of PEG. In contrast, T (tolerant) and MT (medium tolerant) strains had constitutively (control) higher intracellular concentrations of alginate, and were able to significantly increase alginate concentration under PEG, especially T strains, where the average response was

a 2.4-fold increase of alginate concentration and with one strain increasing 9-fold alginate concentration intracellularly. (Freeman et al., 2013) also observed osmoinduction of the alginate biosynthesis genes and alginate production in a *Pseudomonas* osmotolerant strain but not in the sensitive one and hypothesized that the lack of osmoinduction of the alginate biosynthesis in the sensitive strain could be associated with its lower osmotolerance. (Singh et al., 1992) and (Fialho et al., 1990) had already described the ability of *Pseudomonas* spp. to stimulate alginate production in presence of high osmolarity and Flores and collaborators (Flores et al., 2013) restricted the production of alginate in bacteria to the genera *Pseudomonas* and *Azotobacter*. We further extended this ability to other bacteria genera and related this capacity to the level of osmotolerance evidenced by strains.

The results of this study thus demonstrate the ability to synthesize alginate and to increase its concentration under high osmotolerance conditions, as an essential attribute for soil bacteria to tolerate and survive in environments with high osmolarity, extending to other bacteria the statement made by Chang et al. (Chang et al., 2007) for pseudomonads that alginate production could clearly provide a competitive advantage in water-limited environments, leading to increased ecological success.

Although it is assumed that alginate is produced by bacteria belonging to genus *Pseudomonas* (Chang et al., 2007; Fialho et al., 1990; Flores et al., 2013; Freeman et al., 2013), in our study of the five *Pseudomonas* strains used three presented high amounts of alginate (both at control and PEG conditions) and two not only produced low alginate levels at control condition, but in the presence of PEG the alginate biosynthesis was reduced. Moreover, other bacterial genera (*Flavobacterium*, *Erwinia*, *Herbaspirillium*, *Stenotrophomonas*, *Achromobacter*, *Lysobacter*) produced higher alginate levels than *Pseudomonas* at both conditions, leading to question the nearly universal ability of *Pseudomonas* species to produce alginate (Chang et al., 2007) and the prevalence of alginate biosynthesis capabilities among pseudomonads (Fialho et al., 1990).

Contrary to most literature that describes alginate as an exopolimeric compound (Chang et al., 2007; Flores et al., 2013), less than 5% of the alginate produced was excreted outside cells, being accumulated intracellularly and contributing preponderantly to the level of osmotolerance observed in strains. But how does alginate accumulation inside cells increase bacteria osmotolerance?

Alginate was described as having antioxidant properties. Kelishomi et al. (2016) reported the antioxidant and radical scavenging properties of alginate, which they

attributed to functional groups such as hydroxyl, carbonyl and carboxyl groups and double bonds between C-4 and C-5. (Sellimi et al., 2015) also reported alginate to have high free-radicals scavenging activity, including hydroxyl radicals and high reducing power of Fe^{3+}. Moreover, alginate hygroscopic properties (Robyt, 1998; Sutherland, 2001) contribute to keep cells hydrated (Chang et al., 2007) and its interaction with various proteins (Imeson et al., 1977; Schwenke et al., 1977) increase the stability of enzymes to heat (Wingender and Winkler, 1984) and possibly to other stresses. Thus, alginate may reduce cell dehydratation, stabilize enzymes and decrease oxidative stress originated by high osmolarity, increasing cell osmotolerance. In fact, S strains were unable or synthesized low amounts of alginate, whereas strains with higher levels of alginate presented higher osmotolerance with or without higher concentrations of other osmolytes (trehalose, betaine, proline), and many with mild increases in the activity of antioxidant enzymes (CAT and SOD).

Thus, results evidence alginate as an efficient mechanism to increase osmotolerance in soil bacteria, but its protective effect is not extensive to membranes and tolerant strains, which were exposed to high PEG concentrations evidenced high damage, as LPO levels prove. The metabolism of lipid peroxides originates endoxenobiotics, such as aldehydes, some of them presenting high toxicity towards cells, which in strains with higher osmotolerance (T and MT) should be abundant, since lipid peroxidation is high. However, GSTs can convert the products resulting from the metabolism of lipid peroxides in less toxic compounds, thus minimizing the interference with biomolecules with important functions, such as proteins and nucleic acids. Indeed, in our study GSTs activity of T and MT strains increased significantly in PEG exposed relatively to control cells, evidencing the cell effort to contain toxicity resulting from lipid peroxides metabolism and rendering cells more tolerant to osmotic effects.

Some of the strains displaying higher osmotolerance, and therefore more likely to survive in environments subjected to frequent droughts, have the capacity to promote plant growth, either by production of phytohormones (indole acetic acid), siderophores, or VOCs (Cardoso et al., 2018), being excellent candidates for and implementation in natural and agricultural environments subjected to drought after validation in controlled conditions, such as mesocosm studies. Moreover, some of the osmoprotectants (such as proline, choline and trehalose) synthesized by bacteria in drought stress can be excreted, and if absorbed by plants can directly increase drought stress, or induce osmoprotective mechanisms in plants. For example trehalose metabolism in plant growth promoting rhizobacteria (PGPR) is key for signaling plant growth, yield, and adaptation to abiotic stress, and its manipulation may have a major agronomical impact

on plants (Rodríguez-Salazar et al., 2009). PGPR also improves the stability of plant cell membranes by activating the antioxidant defense system (SOD, CAT, APX, POD), also enhancing drought tolerance in plants (Gusain et al., 2015).

5. Conclusions and application

This study relates for the first time the level of osmotolerance with the ability of soil bacteria to accumulate alginate intracellularly. Thus, alginate can be applied in order to minimize the effects of low water availability (drought or high salinity) in soil bacteria communities, protecting biodiversity in stress conditions. The ability to synthesize alginate should be an attribute present in bacteria to be used as inoculants at sites potentially subjected to conditions generating low water activities in soils both at natural and agronomic systems. Additionally, since alginate is able to form complexes with divalent cations (Grant et al., 1973) and (Davis et al., 2003), some of them being micronutrients (Cu, Mn, Mg, Fe, Ca) its application can thus further promote plant growth.

Chapter 4

Volatile-mediated growth promotion of *Arabidopsis thaliana* by *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8: plant biochemical alterations

Abstract

Rhizobium and *Flavobacterium* are important soil bacteria that display several plant growth promotion abilities. Their beneficial effects to plants include nitrogen fixation, production of phytohormones, enhancement of nutrient acquisition and production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, among others. While mechanisms such as the establishment of nodules for nitrogen fixation found in rhizobia require an intricate and specific molecular cross-talk between the host legume and the bacteria, other mechanisms such as production of indol acetic acid are somewhat unspecific and open the door for wider application beyond legume plants. Here we report a novel mechanism of growth promotion induced by *Rhizobium*, which is unspecific to legumes, as shown by growth promotion of the non-legume *Arabidopsis thaliana*, and which was shared with a *Flavobacterium* strain, also isolated from a legume. This mechanism consists on the release of volatile organic compounds that promote plant growth and although it has been reported before for other genera of bacteria, this ability integrates an extensive list of benefits of rhizobia, highlights the possibilities of its use with non-legumes and raises important questions regarding plant-bacteria communication. We also examined several biochemical endpoints of the plant in response to the bacterial volatile organic compounds (BVOCs) released, having found responses in chlorophyll content, protein, electron transfer system, lipid peroxidation and superoxide dismutase. This is also, to our knowledge, the first report applying GC×GC-ToFMS to plant growth promoting bacteria (PGPB), and also comparing the results obtained by GC×GC-ToFMS and by GC-MS. We were able to discriminate distinct VOCs profiles (with a wide range of compounds belonging to several different chemical families) between the two PGPB bacteria and to identify BVOCs with reported bioactivity.

Keywords

plant growth promotion*; Rhizobium*; *Flavobacterium;* bacterial volatiles; non-legume plants

1. Introduction

The global increase in human population is requiring an intensification in the production of food, both locally and globally. At the same time, due to pollution, climate change and ecosystems degradation there is an existing concern regarding the need for sustainable agriculture practices. Modern technology such as the application of chemical fertilizers and biocides in developed countries has provided high agricultural yields and food security. Nevertheless, there can be high environmental costs associated with the

intensive use of chemical fertilizers and other agrochemicals, such as nutrient run-off and eutrophication or the contamination of soils with toxic substances, such as pesticides and metals. On the other hand, due to economic constraints, many areas of the globe are not able to rely on chemical fertilization. The use of soil microorganisms, solely or in combination with chemical fertilizers, that promote plant growth has been proposed as a sustainable strategy to enhance plant productivity (Souza et al., 2015).

Some bacteria are capable of inducing plant growth, living inside plant tissues (endophytic) or in a free-living form, in the rhizosphere or in the near soil of plants. These bacteria are often referred to plant growth promoting bacteria (PGPB). Their diversity and mechanisms of plant growth promotion have been studied extensively (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009). These bacteria can benefit plant productivity by producing phytohormones such as indol acetic acid (IAA - which promotes root development), by enhancing nutrient acquisition (for example by fixing atmospheric nitrogen or releasing siderophores that chelates iron) by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase (lowering ethylene levels and promoting plant growth, among other effects), by acting antagonistically against phytopathogenic agents (producing compounds with antimicrobial effects), by inducing plant defenses such as systemic acquired resistance (SAR) against pathogens and herbivores, by inducing tolerance to drought and other abiotic factors and by promoting tolerance to contaminants such as metals (Ahemad and Kibret, 2014; Lugtenberg and Kamilova, 2009). These beneficial effects highlight the value of soil bacteria to be used as biofertilizers and biocide producers. *Rhizobium* is a well-known genus of rhizobia (denotation which encompasses *Rhizobium*, *Bradyrhizobium* and other related genera) which fixes atmospheric nitrogen in symbiosis with legumes. Apart from this important feature, these bacteria also display other plant growth promotion activities, such as production of indol acetic acid (IAA) (Datta and Basu, 2000), siderophores (Datta and Chakrabartty, 2014) and phosphate solubilization (Chabot et al., 1996), which help rhizobia to promote growth of legumes but also of non-legumes (Antoun et al., 1998; Chabot et al., 1996; Flores-Félix et al., 2013; Jiménez-Gómez et al., 2018; Mehboob et al., 2012; Noel et al., 1996). *Flavobacterium* is a bacterial genus that can be endophytic (Maheshwari, 2011) and is one of predominant bacteria in plant microbiomes (Piechulla et al., 2017). Some *Flavobacterium* strains can help fight plant pathogens (Vijayabharathi et al., 2016), promote plant growth and ameliorate plant stress (Ahemad and Kibret, 2014). A representative of this genus has also been reported to be a nitrogen fixer (Kämpfer et al., 2015).

A new mechanism of growth promotion was reported for the first time by Ryu et al. (2003). This mechanism consists on the emission of volatile organic compounds by bacteria promoting plant growth a mechanism that leads to fascinating questions regarding how communication through volatile molecules between organisms takes place, particularly between different kingdoms (bacteria-plant). Moreover, it is of great ecological and biotechnological relevance since these compounds easily diffuse through soils and atmosphere. Since the report by Ryu *et al*. (2003), several studies disclosed the potential of bacterial VOCs to activate induced systemic resistance (ISR) (Han et al., 2006; Ryu et al., 2004), induce systemic tolerance to drought (Cho et al., 2008), improve photosynthesis and produce antifungal compounds (Fernando et al., 2005; Groenhagen et al., 2013; Kai et al., 2007), among other benefits, and VOCs were recognized as a prevalent mechanism among rhizosphere bacteria (Blom et al., 2011). PGPB can also alter the activity of antioxidant enzymes, such as superoxide dismutase (Vurukonda et al., 2016).

In this work, we set out to test the hypothesis that *Rhizobium* can promote the growth of non-legumes besides fixing N_2 through the emission of volatiles and that this feature is also present in the genus *Flavobacterium*. Moreover, we tried to understand if the blend of volatiles released by the different strains were responsible for biochemical alterations in the plants, and if the mechanisms affected were the same between strains and with the same level of alteration. For this, we carried out experiments to check for *Arabidopsis thaliana* growth promotion and biochemical effects by *Rhizobium* sp. E20-8 and *Flavobacterium* sp. D9 volatiles, assessing leaf area and physiological and biochemical endpoints (chlorophyll content, carbohydrates content, protein content, superoxide dismutase activity, electron transfer system and lipid peroxidation). Then, we captured the volatiles emitted by *Rhizobium* sp. E20-8 and *Flavobacterium* sp. D9 using HS-SPME and analyzed them by gas chromatography–mass spectrometry (GC-MS) and two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-ToF-MS). A detailed inspection of the chromatograms of the two bacteria allowed us to identify candidate VOCs for inducing of growth and to pinpoint differences between the two strains.

2. Material and methods

2.1. Bacterial strains and plants

Rhizobium sp. strain E20-8, isolated from *Pisum sativum* L. (Figueira, 2000), GenBank accession number KY491644, and *Flavobacterium* sp. strain D9, GenBank accession number MH236732, isolated from *Ornithopus compressus* L. (Cardoso et al., 2018),

were used in the trials. These two strains were selected from our rhizobacterial collection due to their plant growth promoting abilities (Cardoso et al., 2018). Bacteria (stored in 15% glycerol at -80 °C) were grown in yeast mannitol agar (YMA) (Somasegaran and Hoben, 1994) plates for 4 days at 26 ºC for the experimental uses described below. *Arabidopsis thaliana* ecotype Columbia (Col-0) seeds were used as plant material.

2.2. Experiments with two compartment Petri plates

The trials were based in the protocol described in Farag et al. (2017) and Ryu et al. (2003), with modifications. Seeds were surface sterilized by immersion during 5 minutes in a microtube containing 500 µL of an aqueous solution of 70% ethanol and 0.05% Tween-20. Seeds were then centrifuged for 1 minute at 10 000 *g* and the supernatant was discarded. 500 µL of ethanol (100%) was added and discarded after 5 minutes.

After sterilization, seeds were transferred to Petri plates containing half-strenth Murashige and Skoog (MS) culture medium (Duchefa Biochemie), supplemented with 1.5% sucrose and 0.8% agar, adjusted to pH 5.7. Plates were sealed with parafilm, covered with aluminum foil and vernalized for 2 days at 4 ºC. To germinate the seeds, plates were placed in a growth chamber (Snidjers Scientific ECP01E) programmed for 22 ºC, 12h light/12 h dark cycle, and 85% relative humidity. After germination, 2-day-old seedlings were used for the trials using two-compartment Petri plates. These plates have a center partition that restricts interaction between the two compartments to a volatile nature. Side I of the plates contained full-strength (MS) medium. Side II of the plates contained yeast extract mannitol agar (YMA) medium, pH 6.8 (Somasegaran and Hoben, 1996). In each plate, 10 *A. thaliana* seedlings were placed on side I, while side II was spotted with *Rhizobium* sp. E20-8 and *Flavobacterium* sp. D9 previously grown in YMA plates and stored at 4 ºC. A total of 9 inoculated plates were used per strain. Non-inoculated plates (n=9) were used as controls. The divided plates were placed in the growth chamber in the same conditions used for germination. After 14 days, the total leaf area of plant fresh weight was recorded. Leaf area was estimated using the software Easy Leaf Area (Easlon and Bloom, 2014). Plants were collected, roots were detached the fresh weight of the aerial parts was recorded before storing at -80 ºC for the further analysis.

2.3. Bacterial VOCs effects on plant physiology and biochemical endpoints

2.3.1. Extraction of plant tissue

Two plants from each plate of each condition (Control – C; *Rhizobium* sp. E20-8 – E20-8; *Flavobacterium* sp. D9 – D9) were randomly combined to make composite replicate

samples for the following procedures. For chlorophyll content, carbohydrates, and lipid peroxidation, each sample was composed of 6 plants, which were collected from three plates (two from each plate). For the remaining parameters (electron transfer system, protein carbonylation, protein and superoxide dismutase) each sample comprised 12 plants (three from each plate). Fresh weights were recorded to normalized the results of the physiological and biochemical parameters. Plants were collected and roots were detached. The aerial parts were homogenized in using a mortar and pestle, kept in ice. Chlorophylls were measured in fresh, while for the remaining parameters the samples were frozen at -80 ºC. Extraction and maintenance of samples was carried out in ice for the following assays.

2.3.2. Chlorophyll content

Plants were homogenized with a mortar and pestle in 200 µL of 80% acetone, in the dark (Lichtenthaler, 1987). The homogenate was transferred to a microtube, covered in aluminum foil, and centrifuged for 5 minutes at 4 ºC. Absorbance at 663 and 645 nm was measured with a spectrophotometer. 80 % acetone was used as blank.

2.3.3. Protein content

Frozen samples were homogenized in 600 µL 0.1 M Tris-HCl pH 8.5, 15% (w/v) PVP, 153 uM magnesium sulfate (MgSO4) and 0.2% (v/v) Triton X-100. After centrifugation for 20 minutes at 10 000 *g*, protein content was determined in the supernant by the Biuret method (Robinson and Hogden, 1940). Samples were diluted 10X in the homogenization buffer. Biuret reagent (300 µL) was added to samples or standard (25 µL) and the reaction was incubated at 30 ºC during 10 minutes. The amount of protein was determined spectrophotometrically at 540 nm, using bovine serum albumin (Sigma) as standard.

2.3.4. Carbohydrate content

Samples obtained in section 2.3.3. were diluted 10X in the homogenization buffer. Carbohydrates were analyzed by the method of Masuko et al. (2005) using sucrose as a standard. Total carbohydrate was measured by adding 30 µL of 5 % phenol and 150 µL of 95 % sulfuric acid 50 µL of sample and reading the absorbance at 490 nm using a microplate reader.

2.3.5. Electron transport system

Samples were extracted in 300 µL homogenization buffer and centrifuged at 3000 *g* 4ºC for 20 minutes. In a microplate, 36 µL of a solution of 1.7 mM NADH and 250 µM NADPH

was added to 30 µL of the supernatant. Then, the reaction was started by adding 71 µL of 8 mM p-iodonitrotetrazolium. Absorbance at 490 nm was read at 25 s intervals during a total period of 10 minutes. The quantity of formazan formed in the linear range of each reaction (each sample) was calculated using the extinction coefficient (Ɛ=15900 M^{-1} cm^{-1}).

2.3.6. Lipid peroxidation

Samples were extracted in 200 µL of 20 % trichloroacetic acid (TCA) solution and vortexed. The homogenate was centrifuged at 10 000 g for 20 minutes at 4 ºC. 50 µL of the supernatant (sample) was added to 200 µL of thiobarbituric acid (TBA) solution containing 0.5% TBA dissolved in 20 % TCA and 150 µL TCA. A blank was prepared by mixing 200 µL TCA and 200 µL TBA. Microtubes were vortexed and incubated at 96 ºC during 25 minutes. To stop the reaction, tubes were placed in ice. 300 µL were transferred to a microplate and absorbances at 532 and 750 nm were measured using a microplate reader. The extinction coefficient of malondialdehyde (MDA) (1.56×10^5 M^{-1} cm^{-1}) was used to calculate lipid peroxidation.

2.3.7. *Superoxide dismutase activity*

Superoxide dismutase activity was assayed with a protocol based on Beauchamp and Fridovich (1971). 25 µL of the supernatant obtained in section 2.3.3. was mixed with 25 µL of xanthine oxidase (51.6 mU/ml) and 200 µL of nitro blue tetrazolium reaction buffer (68,4 uM NBT in 50 mM Tris-HCl (pH 8), 0.1 mM DTPA, 0.1 mM hypoxanthine). After 20 minutes of incubation at room temperature, absorbance at 560 nm was read in a microplate reader. One unit of enzyme activity (U) corresponds to 50% reduction of NBT.

2.4. HS-SPME

A current methodology to study bacterial VOCs consists in collecting the volatiles by headspace-solid phase microextraction (HS-SPME) and analyzing using a gas chromatography coupled to mass spectrometry (GC-MS). HS-SPME is a very versatile technique that requires little sample preparation while GC-MS is a powerful methodology to analyze compounds with a volatile nature. This was the strategy employed to collect volatiles emitted by *Rhizobium* sp. strain E20-8 and *Flavobacterium* sp. strain D9, following the method by Farag et al. (Farag et al., 2006), with modifications. The main modification was instead of growing the bacteria in medium inside a sealed vial, in this work the bacteria were grown in Petri dishes, and then collected into chromatography vials to be analyzed (Figure 1). Briefly, bacteria were grown in YMA plates sealed with parafilm for 26 ºC. After 4 days, the colonies were collected with a stainless-steel spatula

into 12×32 mm, 2 mL screw vials for chromatography with screw caps and polytetrafluoroethylene (PTFE)/silicon septa. Each vial contained 300 mg of bacterial colony. Care was taken to avoid collection of culture medium.10 µL of an aqueous solution containing 1 µg of (Z)-3-Hexenyl acetate (Sigma, CAS 3681-71-8) was spiked into each vial prior to extraction to be used as internal standard, using a Hamilton syringe. The internal standard was prepared fresh by diluting the compound in Milli-Q water. A stable flex divinylbenzene/carboxen/PDMS 50/30 µM 1 cm fiber (Supelco) was inserted into the vial. Extraction was performed by placing each vial in a water bath (50 ºC) for 60 minutes. Six vials of each strain were extracted (three were analyzed in GC-MS and the remaining three in GC×GC-ToFMS).

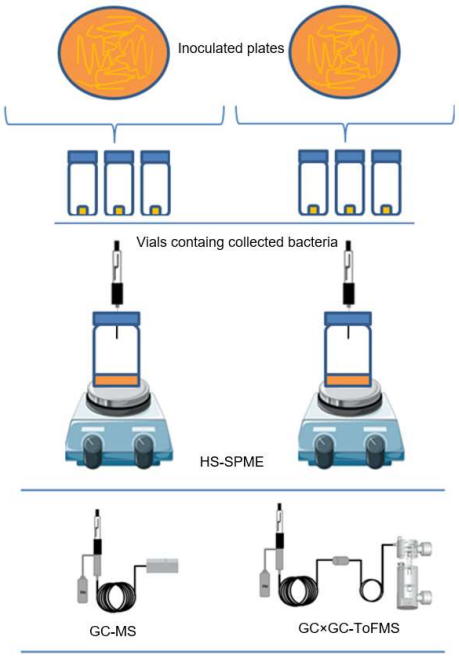


Figure 1. Workflow of the headspace solid phase microextraction (HS-SPME), gas chromatography–mass spectrometry (GC-MS) and two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC-ToF-MS) analysis of *Flavobacterium* sp. D9 and *Rhizobium* sp. strain E20-8 volatiles.

2.5. Gas chromatography coupled to mass spectrometry (GC-MS)

The GC-MS system consisted of an Agilent Technologies 7890B GC System coupled to an Agilent Technologies 5977A MS. The column used was a Tekno TRB-5MS (Teknokroma). The GC-MS method was based on the one reported by Farag *et al.*, (2006). The main difference was the MS source temperature, which in our case was 230 instead of 200 ºC.

2.6. Two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC-ToFMS)

The GC × GC-ToF-MS system was a LECO Pegasus 4D (LECO, St. Joseph, MI, USA) equipped with an Agilent GC 7890A gas chromatograph, a dual stage jet cryogenic modulator (Zoex), a secondary oven and a high-speed time-of-flight (ToF) mass spectrometer. The ^{1}D column was an Equity-5 (30 m×0.32 mm I.D., 0.25 µm film thickness (Supelco, Inc., Bellefonte, PA, USA) and the ^{2}D column was a DBFFAP (0.79 m × 0.25 mm I.D., 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA).

2.7. Data analysis

ANOVA and Kruskal-Wallis tests of leaf area and the physiological parameters was performed using R (R Core Team, 2016). GC-MS peak integration and deconvolution was done in Enhanced Chemstation (Agilent Technologies). GC×GC-ToF-MS peak integration and deconvolution was done in Chromatof (LECO). Compounds were tentatively identified using NIST14 database. An alkane standard solution was also analyzed, both in GC-MS and GCxGC-MS, and the calculated Kovat's index was also used to tentatively identify compounds. Principal component analysis and heatmaps were built using Metaboanalyst 3.0 (peak areas were autoscaled) (Xia et al., 2015).

3. Results

The experiments with the two-compartment Petri plates showed that inoculation with *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8 significantly promoted growth of *A. thaliana*, when compared with the non-inoculated control plates ($p<0.05$) (Figure 2). The mean leaf area (total leaf area per plate) of plates treated with the bacterial strains was approximately 3-fold (D9) and 2-fold (E20-8) the mean leaf area of untreated plates (C).

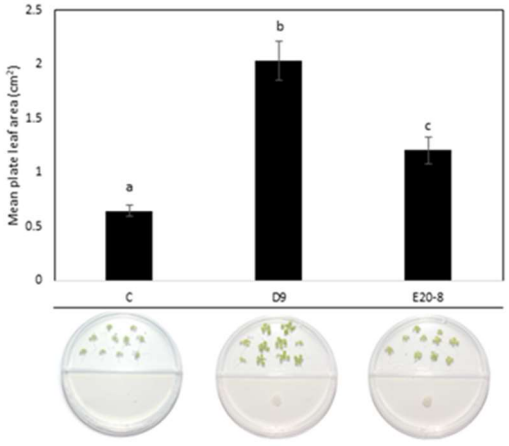


Figure 2. Mean leaf area of *Arabidopsis thaliana* planted in center divided plates inoculated with *Flavobacterium* sp. D9 (D9), *Rhizobium* sp. strain E20-8 (E20-8) or non-inoculated (Control). Bars represent the mean leaf area of 9 plates. In each plate contained 10 A. thaliana seedlings were planted. Error bars represent standard deviation. Statistically significant differences ($p<0.05$, Kruskal-Wallis) are represented by different letters.

Apart from the observed effect on *A. thaliana* growth, several physiological and biochemical endpoints were assessed to check for alterations due to exposure to the bacterial volatiles (Figure 3). Chlorophyll content (Figure 3A), carbohydrate content (Figure 3B), protein content (Figure 3C), electron transport system activity (Figure 3D), lipid peroxidation (Figure 3E) and superoxide dismutase activity (Figure 3F) were assessed. The amount of total chlorophyll increased significantly in plates inoculated with *Flavobacterium* sp. D9 compared to the control (Figure 3A), but not for plates inoculated with *Rhizobium* sp. E20-8. Contrarywise, total carbohydrate content (Figure 3B) appears to be lower for treated plates, although differences are not statistically different ($p<0.05$). Protein content (Figure 3C) was significantly lower ($p<0.05$) in bacterial exposed conditions than in control conditions, albeit with no significant differences between strains. The electron transfer system (ETS) activity was significantly increased in plates treated with *Flavobacterium* sp. D9 compared to control plates or with the plates treated with *Rhizobium* sp. E20-8 (Figure 3D). Lipid peroxidation was lower in plants exposed to both D9 and E20-8 released volatiles, compared to control plants (Figure 3E). A similar response was observed for superoxide dismutase activity, although not significantly different between E20-8 and control condition (Figure 3F).

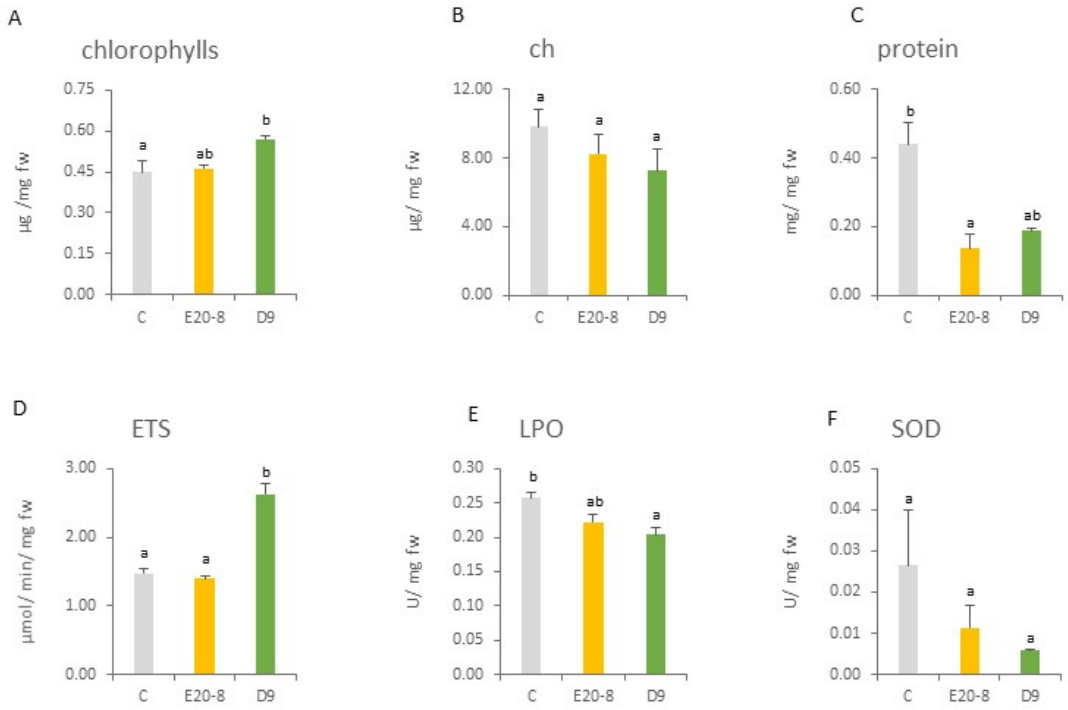


Figure 3. Physiological and biochemical endpoints of *Arabidopsis thaliana* exposed to bacterial volatiles. (A) Chlorophyll content; (B) Carbohydrate content; (C) Protein content; (D) Electron transport system; (E) Lipid peroxidation; (F) Superoxide dismutase activity. Statistically significant differences ($p<0.05$, ANOVA) are represented by different letters.

The HS-SPME and GC-MS methodology that was followed allowed the detection of 11 peaks (Table 1), from different chemical families. It was possible to identify a VOC, phenethyl alcohol, that was present in *Rhizobium* sp. E20-8 chromatograms but not in *Flavobacterium* sp. D9 chromatograms.

The HS-SPME and GC×GC-ToFMS provided the tentative identification of 39 compounds (Table 2), belonging to several different chemical families: alkanes, alkenes, alcohols, ketones, aldehydes, esters, aromatic compounds and volatile sulfur compounds. Most compounds were detected in both bacterial strains, yet 4 compounds were exclusive of *Rhizobium* sp. E20-8 and 8 were exclusive of *Flavobacterium* sp. D9. Exclusive compounds of *Rhizobium* sp. E20-8 were 2-Undecanone, methyl oleate, benzaldehyde and phenylethyl alcohol. Exclusive compounds of *Flavobacterium* sp. D9 were 3-Hexen-1-ol (isomer), 4-Methyl-2,3-pentanedione, 5-Methyl-3-hexanone, 3,4-Dimethyl-2-hexanone, methyl isovalerate, methyl 4-methylpentanoate, methyl 2-ethylhexanoate and methyl phenylacetate. The chemometric multivariate analysis (principal component analysis and heatmap) of GC×GC-ToFMS data (Figure 4A and Figure 4B) show that there is a distinct volatile profile rendered by the analysis of the VOCs released by the different bacterial strains.

4. Discussion

Several bacteria have been reported as being capable of promoting the growth of different plant species via emission of volatile metabolites, with increases in growth that can range up to fivefold (Ryu et al., 2003). This ability appears to be commonplace among rhizosphere bacteria, extending from *Bacillus*, *Pseudomonas* and *Serratia*, to other bacterial genera such as *Burkholderia*, *Stenotrophomonas* and *Cupriavidus* (Blom et al., 2011). In Camarena-Pozos et al. (2018) study, approximately 90% of the bacterial strains of different genera promoted plant growth. Cardoso et al. (2018) also reported that the VOCs released by strains belonging to other genera were able to induce growth of *A. thaliana*, but this induction was only observed in less than 50 % of the bacterial strains evaluated, which belonged to 11 genera. Plant growth promotion by VOCs has also been reported for different plant species, namely *A. thaliana* (Cardoso et al., 2018; Ryu et al., 2003), *Nicotiana attenuata* (Meldau et al., 2013), *Medicago sativa* (Velázquez-Becerra et al., 2011) and *Lactuca sativa* (Fincheira et al., 2016).

Table 1. Volatile organic compounds (VOCs) detected in the headspace of *Flavobacterium* sp. D9 and/or *Rhizobium* sp. E20-8 by solid phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS).

$^{1}T_R$[a]	Compound	CAS[b]	Formula	RI_{calc}	RI_{lit}
15.38	2-Ethyl-1-hexanol	104-76-7	C8H18O	998	1040
17.25	Phenylethyl alcohol	60-12-8	C8H10O	1138	1139
17.81	Unknown	-	-	1177	-
17.99	Unknown	-	-	1189	-
18.44	Dodecane	112-40-3	C12H26	1220	1201
18.5	Unknown	-	-	1224	-
21.23	3-hydroxy-2,2,4-trimethylpentyl isobutyrate	77-68-9	C12H24O3	1393	1380
21.35	Tetradecane	112-95-8	C20H42	1399	1400
22.17	Geranyl acetone	689-67-8	C13H22O	1446	1436
22.97	2,4-Di-tert-butylphenol	96-76-4	C14H22O	1489	1526
23.09	Butylated hydroxytoluene	128-37-0	C15H24O	1488	1519

[a] Retention times of first dimension.

[b] Chemical Abstracts Service (CAS) - short string that refers to a chemical substance.

RI_{calc}: retention index obtained through the modulated chromatogram. [e] RI_{lit}: retention index reported in the literature for one-dimensional GC with 5%- phenyl-methylpolysiloxane GC column or equivalent (Silva et al. (2010), Salvador et al. (2013), Santos et al. (2015), Costa et al. (2016), Li et al. (1998), Jarunrattanasri et al. (2007), Qiming et al. (2006).

Table 2 Volatile organic compounds (VOCs) detected in the headspace of *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8 by solid phase microextraction (HS-SPME) and comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC-ToFMS).

$^{1}T_{R}$[a]	$^{2}T_{R}$[b]	Compound	CAS	Formula	RI_{calc}	RI_{lit}
Alkanes						
594	0.500	Decane	124-18-5	C10H22	1000	1000
890	0.510	Dodecane	112-40-3	C12H26	1200	1200
1014	0.510	Tridecane	629-50-5	C13H28	1301	1300
1126	0.520	Tetradecane	629-59-4	C14H30	1401	1400
1230	0.530	Pentadecane	629-62-9	C15H32	1501	1500
1330	0.540	Hexadecane	544-76-3	C16H34	1601	1600
1422	0.550	Heptadecane	629-78-7	C17H36	1696	1693
1510	0.560	Octadecane	593-45-3	C18H38	1801	1800
Alkenes						
602	1.510	6-Methyl-5-hepten-2-one	110-93-0	C8H14O	1006	997
1194	1.230	6,10-Dimethyl-,5,9-undecadien-2-one	3796-70-1	C13H22O	1467	1451
Alcohols						
154	2.870	3-Methyl-1-Butanol	123-51-3	C5H12O	779	751
338	3.170	3-Hexen-1-ol (isomer)	928-96-1	C6H12O	875	861
670	2.310	2-Ethy-1-hexanol	104-76-7	C8H18O	1050	1040
Ketones						
194	1.570	4-Methyl-2,3-pentanedione	7493-58-5	C6H10O2	799	-
270	1.090	5-Methyl-3-hexanone	623-56-3	C7H14O	838	865
410	1.490	2-Heptanone	110-43-0	C7H14O	911	895
426	1.250	3,4-Dimethyl-2-hexanone	19550-10-8	C8H16O	918	906
762	1.070	2-Nonanone	821-55-6	C9H18O	1109	1097
1018	1.000	2-Undecanone	112-12-9	C11H22O	1305	1291
Aldehydes						
522	1.020	2-Ethylhexanal	123-05-7	C8H16O	965	958
774	1.050	Nonanal	124-19-6	C9H18O	1118	1111

1142	0.970	Dodecanal	112-54-9	C12H24O	1416	1416
1530	0.950	Hexadecanal	629-80-1	C16H32O	1825	1815
Esters						
62	1.040	S-Methyl thioacetate	1534-08-3	C3H6OS	730	699
186	0.880	Methyl isovalerate	556-24-1	C6H12O2	794	770
402	1.270	Methyl 4-methylpentanoate	2412-80-8	C7H14O2	907	861
686	0.840	Methyl 2-ethylhexanoate	816-19-3	C9H18O2	1060	1045
766	2.710	Methyl benzoate	93-58-3	C8H8O2	1113	1101
882	2.920	Methyl phenylacetate	101-41-7	C9H10O2	1196	1186
1086	1.930	1-Hydroxy-2,4,4-trimethylpentan-3-yl 2-methylpropanoate	74367-33-2	C12H24O3	1366	1364
1110	1.690	3-Hydroxy-2,4,4-trimethylpentyl 2-methypropanoate	74367-34-3	C12H24O3	1387	1387
1338	0.890	1-[2-(Isobutyryloxy)-1-methylethyl]-2,2-dimethylpropyl 2-methylpropanoate	74381-40-1	C16H30O4	1609	1607
1434	1.000	Methyl myristoleate	56219-06-8	C15H28O2	1711	1715
Aromatic compounds						
506	2.090	2,5-Dimethylpyrazine	123-32-0	C6H8N2	958	930
546	1.000	Benzaldehyde	100-52-7	C7H6O	977	965
666	1.590	2,3,5-Trimethylpyrazine	14667-55-1	C7H10N2	1047	1016
726	3.690	Acetophenone	98-86-2	C8H8O	1087	1093
810	0.650	Phenylethyl alcohol	60-12-8	C8H10O	1143	1139
Volatile sulfur compounds						
146	1.090	Dimethyl disulfide	624-92-0	C2H6S2	773	757

[a] Retention times of first dimension.

[b] Retention times of second dimension.

[c] Chemical Abstracts Service (CAS) - short string that refers to a chemical substance.

[d] RI_{calc}: retention index obtained through the modulated chromatogram.

[e] RI_{lit}: retention index reported in the literature for one-dimensional GC with 5%- phenyl-methylpolysiloxane GC column or equivalent (Aaslyng et al., 1998; Adams, 2000; Alves et al., 2015; Bicalho et al., 2000; Caldeira et al., 2012; Costa et al., 2016; Guichard and Souty, 1988; Jarunrattanasri et al., 2007; Loureiro et al., 2014; Owens et al., 1997; Rocha et al., 2013, 2012; Rout et al., 2007; Salvador et al., 2013; Santos et al., 2015; Scheidig et al., 2007; Silva et al., 2015, 2009; Verdier-Metz et al., 1998; Wu et al., 2007)

Our results show the potential that *Rhizobium* has to be used as inoculant of non-legumes, even without the formation of N_2-fixing root nodules. The opposite situation, i.e., the growth promotion of legumes by a non-rhizobial species (*Pseudomonas fluorescens*) has also been reported (Hernández-León et al., 2015). Thus, communication via volatiles does not seem to require a fixed pair of microorganism-plant species. Moreover, our results show that *Flavobacterium* sp. D9, isolated from the root nodules of the legume *Ornithopus compressus* L. (Chapter 2), can also promote growth of *A. thaliana*, highlighting the plasticity of the mechanisms of airborne growth promotion.

Data from the metabolomics approach revealed that methyl myristoleate was a peak present in E20-8 chromatograms. This volatile is not reported as plant growth promoter. Methyl myristoleate might be involved in fatty acid signaling and other signaling and regulation mechanisms. Methyl myristoleate is an ester, derived from fatty acids. This is the first fatty acid ester to be reported as a potential plant growth promoter and reveals the potential of this family of compounds. Plants are able to sense microorganisms by fatty acids in plant-pathogen interactions (Walley et al., 2013). Another VOC characteristic of *Rhizobium* sp. E20-8 volatile profile is 2-phenylethanol which is produced by diverse bacteria (Schulz and Dickschat, 2007). It is an antimicrobial produced by *Candida albicans* (Lingappa et al., 1969) and by *Saccharomyces cerevisiae* (Eshkol et al., 2009). In *C. albicans*, the compound has been reported as an autoantibiotic (Lingappa et al., 1969). It inhibits growth of some bacteria, gram-negative in particular, being able to collapse the permeability barrier of the cytoplasmic membrane at a concentration of only 0.5 % (Silva et al., 1976). While these VOCs (methyl myristoleate 2-Phenyl-ethyl-alcohol) can be regarded as candidates to explain plant growth promotion observed in plants exposed to VOCs released by E20-8, the comparison between VOCs released by E20-8 and D9, reveals six common compounds. Moreover, *Flavobacterium* promoted growth at a higher level, so other compounds might be responsible for the observed effects on growth, which could be emitted exclusively or at higher levels by *Flavobacterium* but not detected by the GC approach used. Thus, a higher resolving and detection power such as GC×GC-ToFMS was used and proved to be useful when studying PGPB metabolites. In fact, in this work the number of compounds that could be resolved from the two-dimensional gas chromatography time of flight mass spectrometry approach was much higher (39 compounds compared to 11 compounds) that from the one-dimensional gas chromatography, rendering GC×GC-ToFMS a more effective technology for the screening of bacterial VOCs.

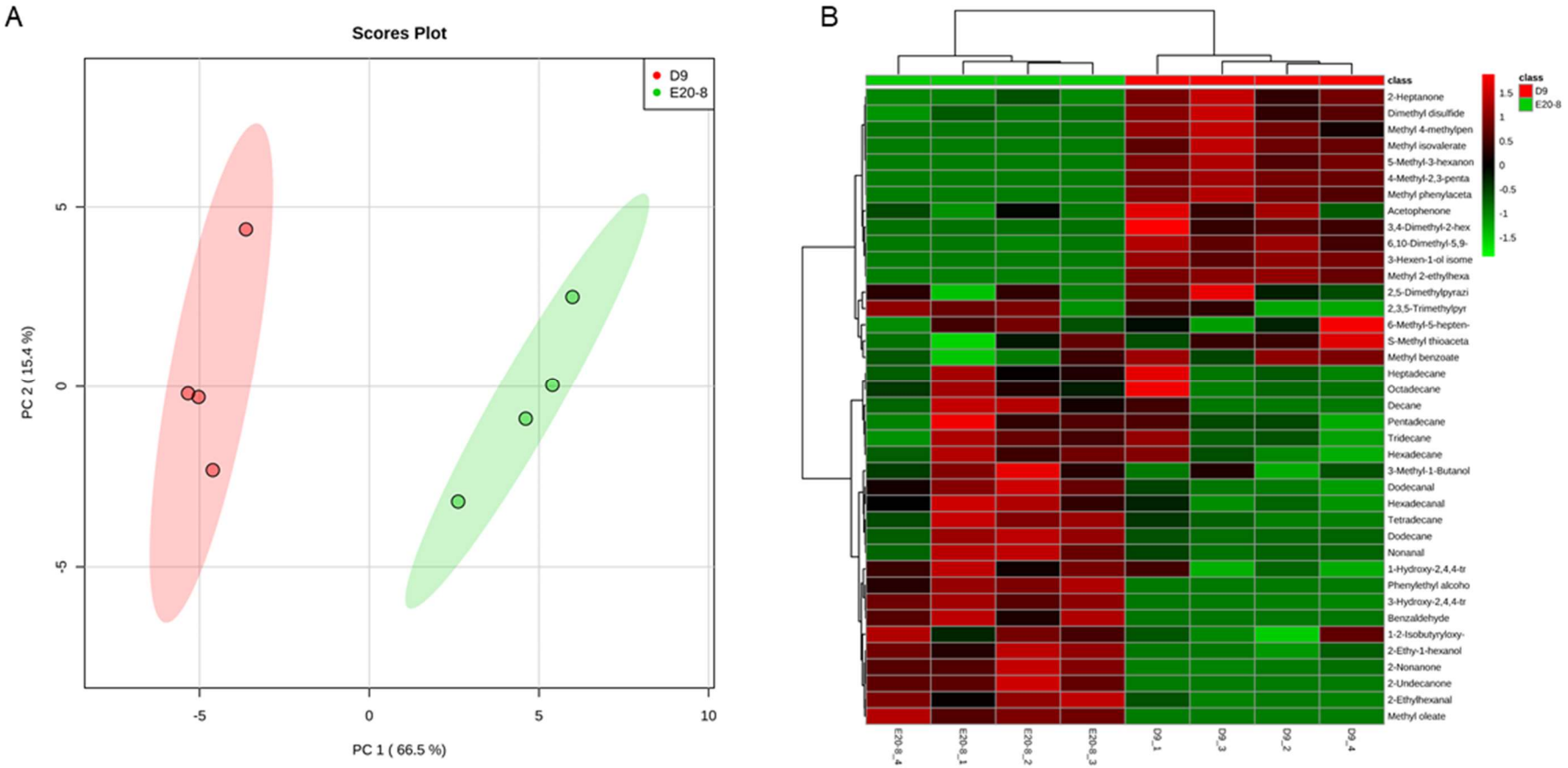


Figure 4 – Principal component analysis (A) and heatmap (B) of the relative peak area of volatile organic compounds (VOCs) emitted by *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8 analyzed by headspace solid phase microextraction (HS-SPME) and two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC×GC-ToF-MS). Red denotes higher relative abundance, green lower relative abundance.

Many VOCs identified in this work have been reported previously to be produced by microorganisms, for example butylated hydroxytoluene (GC-MS), which can be produced by green algae and cyanobacteria, or methyl thioacetate (ethanethioic acid, S-methyl ester), benzothiazole and carbon disulfide (GC×GC-ToFMS) (Schulz and Dickschat, 2007). The chemical families reported in this work, coming from either GC-MS or GC×GC-ToFMS have all been reported for bacteria before (Schulz and Dickschat, 2007). Many of the compounds can be originated from different pathways and their intermediate reactions (Schmidt et al., 2015), but in a general manner alkanes, alkenes, alcohols, ketones and esters can be originated from fatty acids (from their biosynthesis or degradation), nitrogen containing compounds (including pyrazines and azoles) from amino acids, volatile sulfur compounds from dimethylsulfoniopropionate, inorganic sulfide or L-methionine, aromatic compounds from the shikimate pathway (degradation of L-phenylalanine or L-tryptophan), and the volatile halogenated compounds, that do not have many different representatives, and that in our work we were able to detect one, methyl iodide, common in terrestrial and marine bacteria, including *Rhizobium*, and is the result of the methylation of iodide by an enzyme with *S*-adenosylmethionine as the methyl donor. (Schulz and Dickschat, 2007).

So far the bacterial VOCs reported as plant growth promoters are 2R,3R-Butanediol (Ryu et al., 2003), acetoin (3-hydroxibutanone) (Ryu et al., 2003), dimethyl disulfide (Groenhagen et al., 2013), indole, 1-Hexanol and pentadecane (Blom et al., 2011), dimethylhexadecylamine (N, N-dimethyl-hexadecanamine) (Velázquez-Becerra et al., 2011), 2-Pentylfuran (Zou et al., 2010). Moreover, 3-methyl-1-butanol, 2-methyl-1-butanol and butane-1-methoxy-3-methyl were reported as potential growth promoters (Farag et al., 2006). These compounds belong to several different chemical families, encompassing diols, ketones, volatile sulfur compounds, alcohols, ketones and amines. Given that bacteria can emit a wide diversity of VOCs (Schulz and Dickschat, 2007), the potential for the discovery of more bacterial plant growth promoters is high. We did not find these reported compounds using GC-MS, however that does not mean they were not released, since growth medium formulation (Schulz and Dickschat, 2007) and methodology of analysis greatly impact the outcome of analysis. Moreover, our procedure did not allow us to identify highly volatile compounds (low carbon number) of interest, since the two compounds here reported have a relatively high carbon number. In fact, our results suggest that a novel family of compounds might be behind growth promotion, the esters derived from membrane fatty acids. Methyl myristoleate was abundant in *Rhizobium* sp. strain E20-8, and was not detected in *Flavobacterium* sp. D9. Nevertheless, when the data obtained from GC×GC-ToFMS is considered, several

reported bioactive bacterial VOCs could be detected. Dimethyl disulfide, emitted by both strains, is a plant growth promoter (PGP) (Groenhagen et al., 2013), antifungal (Vespermann et al., 2007) and plant growth inhibitor (Meldau et al., 2013). 3-Methyl-1-butanol is a potential PGP (Farag et al., 2006) and was detected in both strains. 3-Nonanal and 2-Undecanone are antifungal compounds (Fernando et al., 2005; Groenhagen et al., 2013). Interestingly, 2-Undecanone was released by E20-8 but not by D9. Pentadecane is also a PGP (Blom et al., 2011) and was released by both strains, as so as while acetophenone was reported as toxic and inducer of oxidative stress in plants (Moreira, 2013). The compounds detected in *Flavobacterium* sp. D9 but not in *Rhizobium* sp. E20-8 may be regarded as candidates to explain the higher growth-inducement by D9 compared to E20-8. Therefore, the pure compounds should be tested to screen for PGP ability. These compounds were mainly esters (methyl isovalerate, methyl 4-methylpentanoate, methyl 2-ethylhexanoate and the methyl phenylacetate) and ketones (4-Methyl-2,3-pentanedione, 5-Methyl-3-hexanone and 3,4-Dimethyl-2-hexanone), but an alcohol, 3-Hexen-1-ol (isomer), was also exclusive of D9. Nevertheless, E20-8 also had an exclusive ester, methyl myristoleate, and a ketone, 2-Undecanone, an antifungal compound (Groenhagen et al., 2013). The remaining E20-8 exclusive compounds were 2-phenylethanol, and benzaldehyde, two aromatic compounds produced by different bacteria (Schulz and Dickschat, 2007).

The effects of bacterial VOCs on plant physiology, gene expression, and metabolome has received some attention, and was reviewed by Farag *et al.* (2013) and Bailly and Weisskopf (2012). Growth promotion effects seem to depend on the bacterial strain used, since Ryu and his team (Ryu et al., 2003) have reported that growth promotion did not appear to be linked with plant growth regulators such as cytokinins and gibberellic acid for one strain, but was linked to cytokinin signaling pathway when another was considered. Enhancement of plant nutrition by bacterial VOCs can also promote growth. Meldau et al. (2013) reported that dimethyl disulfide can improve sulfur nutrition. So far, the literature indicates that the effects of bacterial VOCs on plant growth due to the PGP VOCs acetoin, 2,3-butanediol, dimethylhexadecylamine and 2-pentyl furan is associated with the ethylene, cytokinins, auxin and abscisic acid pathways (Bailly and Weisskopf, 2012). Nevertheless, the amount of research done on the growth promotion by bacteria largely surpasses what is known about the effects on the plant metabolome, on an organism level. Despite noticeable differences in plant growth (Figure 2), in our work not all tested biochemical parameters were altered by plant exposure to the released bacterial VOCs. The content of carbohydrates was unaltered due to inoculation with the bacterial strains. Nevertheless, chlorophyll content increased

in response to *Flavobacterium* sp. D9 volatiles (Figure 3A) and a similar response was recorded for ETS activity (Figure 3D). These effects may be due to increased metabolic activity to sustain a higher growth rate, with the enhancement of photosynthesis and the consumption of energy reserves to feed growth. Stimulation of photosynthesis due to bacterial VOCs has been reported previously (Sharifi and Ryu, 2018). Yet, sugars, which in our work decreased in bacterial treated plates (more than 16% for *Rhizobium* sp. E20-8 and 26% for *Flavobacterium* sp. D9), although not statistically significant), have been reported to accumulate due bacterial VOCs (Sharifi and Ryu, 2018). Regarding ETS, we believe our report is the first to evaluate this parameter in plants exposed to bacterial VOCs and evidences the higher activity of electron transport chain to produce adenosine triphosphate (ATP) used in anabolic activities related to growth. Our results evidence LPO decrease in exposed plants due to bacterial VOCs, even when oxidative respiration is more active (higher ETS activity). The lower LPO may be linked to the interaction of VOCs with membrane lipids. Most of these compounds have a lipophilic nature thus making the interaction with membrane lipid bilayers possible. Some VOCs were already described as antioxidants (Cardoso et al., 2017), thus it is possible that they can protect membrane lipids from oxidation. Bacterial VOCs of both strains significantly reduced the protein levels of *A. thaliana* plants. In the literature BVOCs have already been reported to regulate gene expression in plants (Zhang et al., 2007) and thus the maximum number of copies and activity of many enzymes, altering markedly the plants metabolism. This seems to be the case with the two strains under study, since the results show that bacterial VOCs have reduced substantially the protein levels in *A. thaliana*. This generalized decrease in protein levels leads to the possibility that VOCs interfere with protein synthesis or generally repress gene expression. Hence, the total activity of proteins such as SOD, being down-expressed, is lowered although the specific activity does not suffer alterations. The activity of antioxidant enzymes, such as SOD, has already been reported to be altered by PGPB (Gusain et al., 2015).

GCxGC-ToFMS allowed the detection of many compounds which could not be detected or resolved in GC-MS. To the best of our knowledge this is the first work applying GC×GC-ToFMS to the analysis of PGPB volatiles. The results indicate that this a much powerful methodology than GC-MS. Moreover, our work shows that *A. thaliana* plants respond at a physiological and biochemical level to the blend of volatiles emitted by *Rhizobium* and *Flavobacterium*, two bacteria genera whose PGPB volatiles were previously unexplored. We were able to identify known PGP VOCs, but the wide array of compounds here reported opens the door for the discovery of new compounds displaying PGP abilities.

Chapter 5

Bacterial volatile-induced plant growth promotion under polyethylene glycol-induced drought stress

Abstract

Plant growth promoting bacteria have been reported as capable of alleviating drought stress in plants by several mechanisms. One of the mechanisms is the production of volatile organic compounds**.** In this work we tested the influence of two bacterial strains (*Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8) previously reported (Chapter 4) as inducers of *Arabidopsis thaliana* growth on the tolerance of the plants to osmotic stress. Two levels of osmotic stress (induced by polyethylene glycol-6000) were applied. Growth and several biochemical endpoints were assessed in *A. thaliana*. Our results show that inoculation with the bacteria promoted plant growth at all stress levels, although different responses were obtained for biochemical parameters. The most significant alterations due to the influence of bacterial volatiles where observed for chlorophyll (increased), carbohydrates and superoxide dismutase (decreased). Taken together, our results highlight the potential of bacteria to mitigate drought impacts on plant productivity via bacterial volatiles, especially if the bacteria themselves are capable to survive in osmotically stressed conditions, and reinforce the interkingdom bacteria-plant communication, which may osmotically influence plants with biochemical alterations in the plants exposed to bacterial volatiles.

Keywords

Plant growth promoting bacteria (PGBP); drought; bacterial volatiles; physiological endpoints; biochemical endpoints

1. Introduction

Drought is a major limiting factor for plant productivity (Osakabe et al., 2014) which can result in economic losses (IPCC, 2014). Moreover, due to climate change the duration and severity of drought events is expected to increase (IPCC, 2007). The impact of low water availability in agricultural systems extends further from direct effects on plants, since negative effects on other players, such as soil microorganisms that improve soil fertility, may also be negatively affected by drought, inducing indirect effects on agricultural yields. Effects of drought on soil microflora and soil functioning can also have a strong impact on natural areas, where no fertilizers are used and no irrigation is practiced and therefore the primary production of ecosystems is totally dependent of the on-site nutrient availability and cycling, which are heavily dependent on soil microorganism's activity.

A fertile soil thrives with microbial biomass, which includes bacteria and fungi that are beneficial to plant growth, and that can be found associated to plant roots, living

inside plant tissues or in free-living form (Santoyo et al., 2016). Bacteria in particular, depending on the genera and strain, can display several beneficial traits for plant growth. These bacteria can be collectively referred to as plant growth promoting bacteria (PGPB). Their beneficial traits might include direct plant growth promotion through hormones such as indol acetic acid or volatile organic compounds (VOCs) (Ryu et al., 2003), enhancement of nutrient assimilation (e.g. production of siderophores to increase iron availability) (Radzki et al., 2013), antagonistic effects against phytopathogens (Compant et al., 2005), or enhancement of the plant tolerance to biotic (e.g. herbivores) (Brock et al., 2018) and abiotic factors, namely drought (Zahir et al., 2008).

Bacterial volatiles are particularly interesting since several molecules, from different organic families, have been reported to promote plant growth (Ryu et al., 2003), have antifungal properties (Fernando et al., 2005), enhance nutrition (Meldau et al., 2013), induce systemic acquired resistance (Ryu et al., 2004) and induce systemic acquired tolerance to drought (Cho et al., 2008). Induction of systemic tolerance of *A. thaliana* to drought by 2,3-Butanediol was reported by Cho et al. (2008). Improvement of *A. thaliana* salt tolerance by *Bacillus subtilis* (GB03) volatiles was reported by Zhang et al. (2008). *B. subtilis* strain GB03 also improved osmotic stress tolerance of *A. thaliana* by increasing the levels of choline, a precursor of the osmoprotectant glycine betaine (Zhang et al., 2010).

Plants can respond or adapt to water deficit by triggering physiological and biochemical responses. These responses can be related to the closing of stomata, osmotic and metabolic adjustment, protection of photosynthesis and ROS scavenging (Osakabe et al., 2014) and root initiation and elongation (Jupp and Newman, 1987). Among other responses at the cellular level, cells can adapt by avoiding stress or protecting membrane and proteins (e.g. by accumulating osmolytes) or by repairing damage (e.g. inducing gene products responsible by protein degradation) (Bray, 1997). Some plant growth promoting bacteria are able to alleviate plant stress due to low water availability or other osmotic stress (Numan et al., 2018; Vurukonda et al., 2016). However, evaluation of bacterial VOCs (BVOCs) effects on osmotic stress of plants have not been addressed.

This study aims to test the hypothesis that plant tolerance to osmotic stress can be altered by the bacterial VOCs, and that this alteration is due to alterations in physiological and biochemical traits. Plants (*Arabidopsis thaliana* Col-0) and bacteria (*Rhizobium* sp. strain E20-8 and *Flavobacterium* sp. strain D9, used in **Chapter 4**) were grown in divided Petri plates, in control and two levels of osmotic stress induced by

polyethylene glycol 6000 (PEG-6000). Several endpoints were assessed (leaf area, chlorophyll content, protein content, carbohydrate content, activity of electron transfer system, lipid peroxidation and superoxide dismutase activity) to evaluate the effects of osmotic stress on *A. thaliana* plants, when they are challenged under the influence or not of bacterial VOCs by three levels of stress (0, 200 and 300 g/L PEG-6000).

2. Material and methods

2.1. Plant material and bacterial strains

Arabidopsis thaliana Columbia (Col-0) seeds were surface sterilized by immersion in a microtube containing a solution of 70% ethanol. After 5 minutes, the solution was replaced for 100% ethanol, which was discarded after 5 minutes. Seeds were then kept in the dark for 2 days at 4ºC. Sterilized seeds were transferred to half-strength Murashige and Skoog (MS) medium Petri plates, pH 5.7, containing 1.5% sucrose and 0.8% agar, and germinated in a growth chamber (Snidjers Scientific ECP01E) set at 22ºC, 12h light / 12h dark photoperiod. The bacterial strains used were *Rhizobium* sp. strain E20-8 and *Flavobacterium* sp. strain D9, previously used in **Chapter 4**.

2.2. Polyethylene glycol 6000 drought induced experiments

Since polyethylene glycol does not allow agar to solidify, the method of van der Weele et al., (2000) was used to prepare petri plates containing PEG-6000. Briefly, 10 mL of full-strength MS medium were poured side I of center partitioned Petri dish, and 10 mL of extract mannitol (YMA) (Somasegaran and Hoben, 1994) into side II. After solidification, 10 mL of autoclaved liquid (containing no agar) MS or YMA containing 0 (control), 400 or 600 g/L of PEG-6000 were poured onto the top of the solidified medium. The liquid medium was allowed to diffuse into the solid medium plates for 24 h, after which the solution was poured off. Three different conditions were obtained for each medium (MS-plant or YMA-bacteria): control, 20% PEG (400 g/L PEG) and 30% PEG (600 g/L) PEG, simulating no water stress, and two different levels of simulated water deficit.

Ten days-old *A. thaliana* seedlings were planted onto side I of center partitioned Petri plates containing the full-strength MS medium. On side II of the plates, which contained YMA, bacterial strains *Rhizobium* sp. E20-8 or *Flavobacterium* sp. D9 were inoculated. Uninoculated plates were used as controls. Plates were sealed with two layers of parafilm, and placed for 1-week in the growth chamber. At the end of the drought induced experiments, total *A. thaliana* leaf surface area of each plate was estimated using Easy Leaf (Easlon and Bloom, 2014). Fresh weight was also recorded. Roots were detached

and discarded, while the aerial parts were used to determine the chlorophyll content in fresh plants. The remaining plants were frozen at -80 ºC to be used for the assessment of other physiological and biochemical endpoints.

2.3. Physiological and biochemical endpoints

The physiological and biochemical endpoints were assessed using the methodologies described in **Chapter 3** (proline and glycinebetaine) and **Chapter 4** (remaining parameters).

3. Results

Growth of *A. thaliana* plants not exposed to bacterial VOCs was strongly inhibited by osmotic stress, in a dose dependent manner. Growth at 200 g PEG/L (20%) induced a growth inhibition slightly lower than 50 %. Growth at 300 g PEG/L (30%) induced a strong (89%) growth inhibition (Figure 1). VOCs produced by bacteria promoted *A. thaliana* growth (Figure 1). In non-osmotically stressed plants (0% PEG) E20-8 induced higher growth than D9 strain. In PEG (20% and 30%) exposed plants, bacteria volatiles (both D9 and E20-8) significantly induced growth (Figure 1).

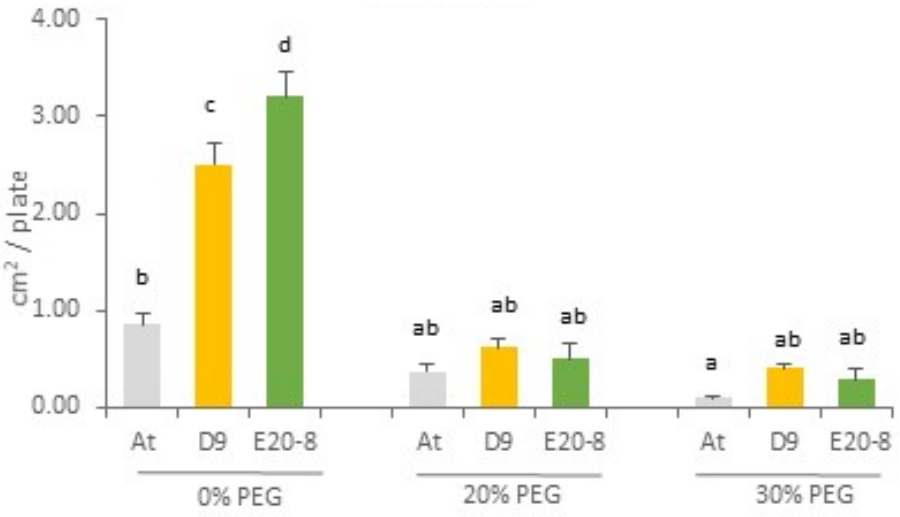


Figure 1. Influence of bacterial volatile organic compounds (VOCs) released by *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8 on *Arabidopsis thaliana* growth (leaf area) at different levels of osmotic stress (0, 20 and 30% polyethylene glycol-6000).

A. thaliana underwent physiological and biochemical changes when exposed to the VOCs released by *Flavobacterium* strain D9 and *Rhizobium* strain E20-8 VOCs and to PEG (Figure 2).

When plant and bacteria were not osmotically stressed (0% PEG), chlorophyll content was significantly increased by bacterial VOCs (Figure 2A). At 20% PEG, chlorophyll content of plants not influenced by BVOCs was not significantly different from plants at 0% PEG. BVOCs did not increase (E20-8) or increased, but not significantly (D9,) the chlorophyll content of plants compared to plants not influenced by BVOCs. When stress level was raised to 30% PEG, the chlorophyll content of plants not influenced by BVOCs decreased significantly compared to non-osmotically stressed plants. However, at the same level of stress (30% PEG) BVOCs of both strains increased almost 3-fold plant chlorophyll content (Figure 2A).

BVOCs (both D9 and E20-8) decreased protein content of non-osmotically stressed plants (Figure 2B). Exposure to PEG increased the amount of proteins both in plants influenced or not by BVOCs, and in some conditions (20%PEG-D9, 20%PEG-E20-8 and 20%PEG-D9) protein content was not significantly different between VOC influenced and not influenced plants (Figure 2B).

Carbohydrate content was lower in plants grown in the presence of D9 VOCs for the three PEG concentrations (0, 20 and 30%). E20-8 VOCs decreased plant carbohydrate levels compared to plants not influenced by BVOCs at 0% and 30% PEG (Figure 2C). At 20% PEG, plants not influenced by BVOCs and under the influence of E20-8 VOCs presented similar carbohydrate levels (Figure 2C).

The activity of the electron transport system (ETS) was not influenced by bacterial VOCs (Figure 2D). However, ETS activity gradually increased as plants grew at increasing PEG concentrations of PEG (Figure 2D).

Superoxide Dismutase (SOD) activity increased as the level of osmotic stress increased in plants not exposed to BVOCs (Figure 2E). BVOCs of both strains inhibited SOD activity over 90% independently of PEG concentration (Figure 2E).

Lipid peroxidation was more influenced by PEG concentration than by BVOCs (Figure 3F). However, at 30% PEG LPO levels were higher in plants under BVOCS influence, especially E20-8 strain (Figure 2F).

Proline was more influenced by PEG concentration than by BVOCs (Figure 2G). At 0% PEG proline levels of both BVOCs influenced and non-influenced plants were similar. In osmotically stressed plants BVOCs increased proline levels both at 20% and 30% PEG (Figure 2G).

Glycinebetaine was influenced both by BVOCs and by PEG concentration (Figure 2H). PEG increased and BVOCs decreased glycinebetaine in *A. thaliana* plants. VOCs

influence is lower in the presence of PEG and for the same bacterial influence (none, D9 or E20-8) glycinebetaine significantly increased, following PEG concentration (Figure 2H)

4. Discussion

When looking for suitable bacterial biofertilizers to be used in areas with water shortage, it is important to use bacteria that not only display PGP abilities and can work as PGPB to the target plant, but also that can increase plant tolerance to drought. Several studies have reported the potential of PGPB to alleviate drought effects on plants, and it should be highlighted that it is important to consider the osmotolerance of bacteria when screening for isolates with PGP and plant tolerance increase potential. Therefore, more studies such as Niu et al. (2018), which explored the application of drought tolerant bacteria to drought-stressed foxtail millet should be undertaken. In this study we challenged bacterial airborne growth promotion of *A. thaliana* plants by submitting both the bacteria and plants to drought stress, and evaluated the effects of this influence on the plant. Apart from an observed effect on growth of *A. thaliana* due to exposure to the bacterial volatiles, which in general promoted the growth of the plants in either control or stressed conditions, we also studied the effects on several plant biochemical endpoints.

Bacteria can have effects on the activity of drought stressed plant's antioxidant enzymes like superoxide dismutase (SOD) (Gusain et al., 2015). An increase in the activity of this enzyme appears to drive for tolerance of plants to drought or salinity, due to the scavenge of reactive oxygen species (Fikret et al., 2013). In our study, SOD activity was also evaluated and BVOCs strongly inhibited SOD activity independently of the osmotic stress level plants were exposed to. We also checked for oxidative damage (lipid peroxidation) and effects on the contents of chlorophylls, protein and carbohydrates. Although SOD activity was strongly (more than 90%) inhibited by BVOCs, interestingly lipid peroxidation at 0% PEG was lower in BVOCs influenced plants and at osmotic stress conditions lipid peroxidation increased less than 50% (16% to 48%) in BVOCs influenced compared to non-influenced plants for the same level of osmotic stress. Lipid peroxidation increases in plants stressed by drought or salt induced stress. Santos et al. (2018) described an increase of 31% in LPO of salt stressed compared to non-stressed plants. PGPB were reported to be capable of lowering plant stress levels under different water availabilities by lowering LPO (e.g. Sahin et al. (2015)). However, the study by Sahin et al. (2015) was not focused on bacteria volatile organic compounds.

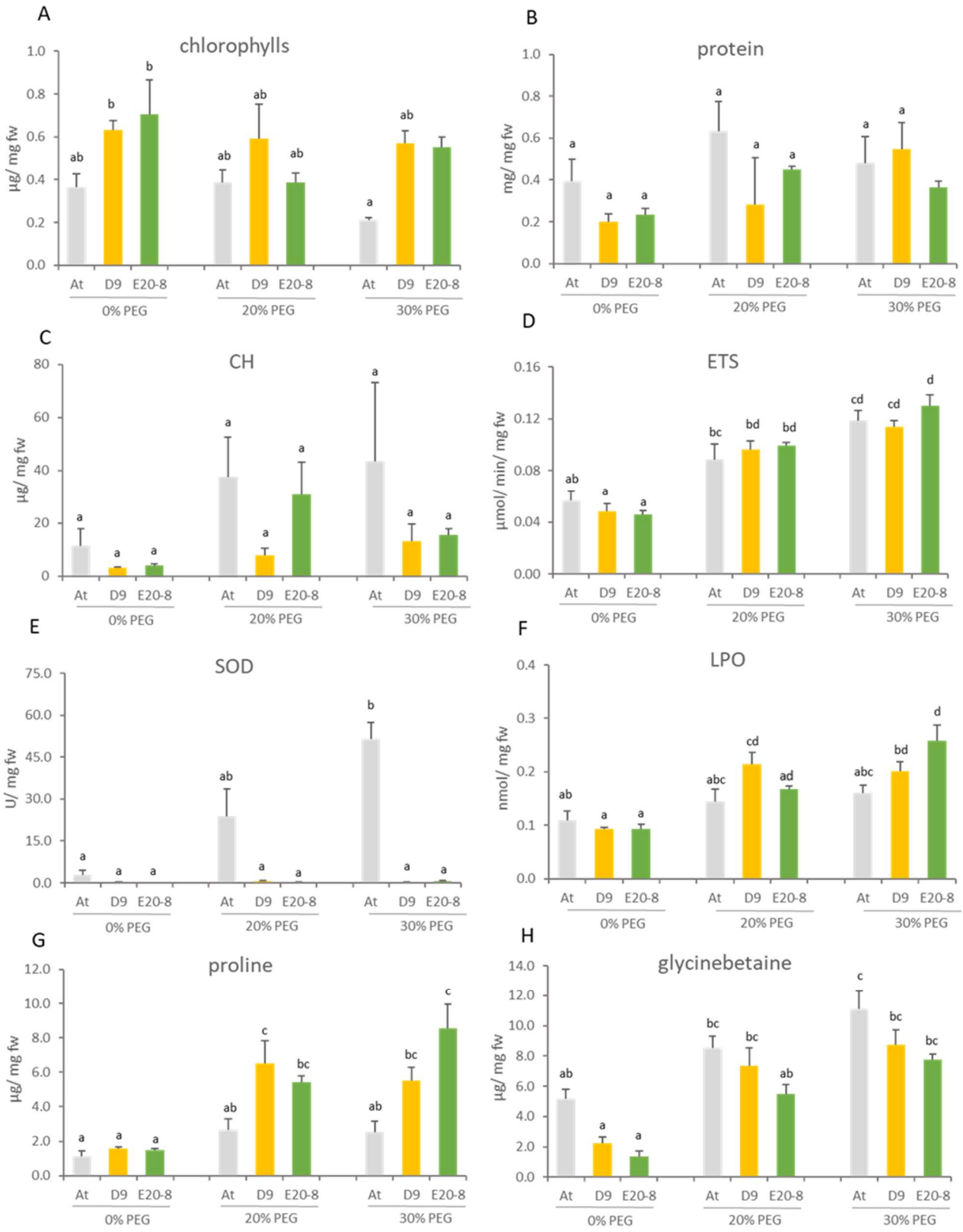


Figure 2. Influence of bacterial volatile organic compounds (VOCs) released by *Flavobacterium* sp. D9 and *Rhizobium* sp. E20-8 on *Arabidopsis thaliana* physiological and biochemical endpoints at different levels of osmotic stress (0. 20 and 30% polyethylene glycol-6000). (A) Chlorophyll content; (B) Protein content; (C) Carbohydrate content; (D) Electron transport system activity; (E) Superoxide dismutase; (F) Lipid peroxidation; (G) Proline; (H) Glycinebetaine. Statistically significant differences ($p<0.05$, ANOVA) are represented by different letters.

Stress can lead to alterations in plants apart from the antioxidant machinery. Water deficit can have a severe impact on photosynthesis due to the low CO_2 levels resulting from closure of stomata (Chaves et al., 2009) or due to alterations in metabolism (Lawlor, 2002). The chlorophyll content of pepper was reduced due to salt stress in the study of Lima et al. (2017). In our work, BVOCs tended to increase chlorophylls both at osmotically stressed and non-stressed plants. This increase in chlorophyll content should increase photosynthesis, thus providing plants with more resources (energy and compounds) to fight osmotic stress. Indeed, and in spite of the increased chlorophyll levels, plants under BVOCs influence have lower carbohydrates levels, which should be used for anabolic processes, such as the synthesis of osmolytes like proline and glycine betaine, since energy expenditure (ETS) is not different between plants under the influence or not of BVOCs.

PGPB have been suggested to be used to improve drought tolerance in plants (e.g. (Khan et al., 2018). While it has already been reported that bacterial VOCs can improve osmotic stress in plants (Zhang et al., 2008), our study assessed the influence of BVOCs on plants biochemical status under different osmotic conditions and, allowed a better understanding of the potential of bacterial VOCs in a drought scenario.

Chapter 6

Final remarks and future work

The results reported in this thesis revealed a high diversity of bacteria living in wild legume nodules growing in continental Portugal that extends beyond rhizobia. Most of the bacterial strains identified belonged to *Flavobacterium* and *Pseudomonas*, two genera that are also plant growth promoting (Chapter 2). The isolates with distinct BOX-PCR fingerprinting and 16S rRNA gene sequences (100) were screened for plant growth promoting (PGP) abilities. Osmotolerance was also screened, and results showed that the level of osmotolerance displayed by the strains was not related with the site of origin (and their inherent environmental conditions) nor with the host plant species.

Since osmotolerance of bacteria is important to develop effective inoculants in areas affected by drought, the biochemical mechanisms of tolerance to osmotic stress of strains from different genera were studied (Chapter 3). Strains were able to induce mechanisms of tolerance to drought stress such as improved synthesis of osmolytes trehalose, proline and betaine. Interestingly, a novel mechanism of tolerance, the intracellular accumulation of alginate, appears to be a major driver explaining differences of osmotolerance among the strains.

Some of the strains proved to be capable of promoting growth of *Arabidopsis thaliana* via emission of volatiles in *in vitro* trials, a mechanism that is not specific to host plants (Chapter 2). Two bacterial strains that promoted growth of *A. thaliana* through emission of volatiles, *Flavobacterium* sp. strain D9 (isolated in this work) and *Rhizobium* sp. strain E20-8 (from our rhizobacterial collection) were studied in detail, evaluating the effects of their released volatiles on the physiology and biochemistry of *A. thaliana* (Chapter 4). Several plant parameters were altered as a response to the bacterial volatiles, namely chlorophyll content, protein, electron transfer system, lipid peroxidation and specially superoxide dismutase activity. The strains volatilomes were also extracted by headspace solid phase microextraction (HS-SPME) and analyzed by gas-chromatography-mass spectrometry (GC-MS) and two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-ToFMS). GC×GC-ToFMS emerged as an efficient tool to screen plant growth promoting bacteria (PGPB) volatiles and revealed distinct volatile profiles for the two strains, being possible to identify compounds with reported bioactivity and potential novel compounds with plant growth promotion ability.

Moreover, *Flavobacterium* sp. strain D9 and the *Rhizobium* sp. strain E20-8 showed potential to alleviate the effects of drought stress on *A. thaliana* via emission of volatiles that exerted an effect on the plant's biochemistry, including production of the osmolyte proline (Chapter 5). This suggests that volatiles can have an important role in

drought conditions. Unlike other plant growth promoting molecules, these molecules do not need to be in solution in liquid water to diffuse, since they can diffuse through pores of the soil and through the air, making them excellent to function as infochemicals at a distance.

As future work, the following topics emerged from the present study:

- Study the dynamics of the volatile communication bacteria- plant: check for example how the bacterial volatiles alter the volatiles emitted by plants (in control and drought stress conditions)
- Explore the remaining isolates hunting for novel VOCs that promote plant growth
- Test the sole exposure to each of the VOCs detected that can be candidates to explain the promotion of growth in plate setup
- Volatile induced plant growth promotion in control and under drought stress: sole and combined effects of different bacteria (consortia) and pure compounds
- Evaluate VOCs profiles of bacteria growing in different media
- Investigate the ability of VOCs to travel dried soil compartments, an ability that water diffusible infochemicals do not have.
- Perform greenhouse and field trials with crops to test the efficacy of some of the strains reported in this work in plant growth promotion and alleviation of drought effects
- Develop and implement the commercial production of the bacteria
- Test the inoculation of single strain inoculants or consortia of inoculants - the mixture of strains improves the odds that at least some of the bacteria will survive in different abiotic conditions
- Study the genomic, transcriptomic, proteomic and metabolomic response of plants under exposure to bacterial volatiles
- Test the combined effect of other abiotic conditions, that are frequently associated with drought, such as high temperature and other conditions such as metals, nanomaterials, pesticides, microplastics and antibiotics
- Check if the bacteria themselves are influenced by plant volatiles
- Further explore the bacterial diversity of wild legume nodules using a metagenomics approach

Chapter 7

References

Aaslyng, M.D., Elmore, J.S., Mottram, D.S., 1998. Comparison of the Aroma Characteristics of Acid-Hydrolyzed and Enzyme-Hydrolyzed Vegetable Proteins Produced from Soy. J. Agric. Food Chem. 46, 5225–5231. https://doi.org/10.1021/jf9806816

Adams, R.P., 2000. Systematics of smooth leaf margin *Juniperus* of the western hemisphere based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 28, 149–162. https://doi.org/10.1016/S0305-1978(99)00047-2

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. J. King Saud Univ. - Sci. 26, 1–20. https://doi.org/10.1016/j.jksus.2013.05.001

Alexander, D.B., Zuberer, D.A., 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol. Fertil. Soils 12, 39–45.

Alves, Z., Melo, A., Figueiredo, A.R., Coimbra, M.A., Gomes, A.C., Rocha, S.M., 2015. Exploring the *Saccharomyces cerevisiae* Volatile Metabolome: Indigenous versus Commercial Strains. PLoS One 10, e0143641. https://doi.org/10.1371/journal.pone.0143641

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R., Laland, R., 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes : Effect on radishes (Raphanus sativus L .). Plant Soil 204, 57–67.

Asghar, H., Zahir, Z., Arshad, M., Khaliq, A., 2002. Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in Brassica juncea L. Biol. Fertil. Soils 35, 231–237. https://doi.org/10.1007/s00374-002-0462-8

Bailly, A., Weisskopf, L., 2012. The modulating effect of bacterial volatiles on plant growth: Current knowledge and future challenges. Plant Signal. Behav. 7, 1–7. https://doi.org/10.4161/psb.7.1.18418

Banin, A., Fish, A., 1995. Secondary Desertification Due to Salinization of Intensively Irrigated Lands: The Israeli Experience, in: Desertification in Developed Countries. Springer Netherlands, Dordrecht, pp. 17–37. https://doi.org/10.1007/978-94-009-1635-7_3

Bashan, Y., De-Bashan, L.E., 2005. Bacteria, in: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment. Oxford, pp. 103–115.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 287, 276–287.

Ben Romdhane, S., Trabelsi, M., Aouani, M.E., de Lajudie, P., Mhamdi, R., 2009. The diversity of rhizobia nodulating chickpea (*Cicer arietinum*) under water deficiency as a source of more efficient inoculants. Soil Biol. Biochem. 41, 2568–2572. https://doi.org/10.1016/j.soilbio.2009.09.020

Benabdellah, K., Abbas, Y., Abourouh, M., Aroca, R., Azcón, R., 2011. Influence of two bacterial isolates from degraded and non-degraded soils and arbuscular mycorrhizae fungi isolated from semi-arid zone on the growth of *Trifolium repens* under drought conditions: Mechanisms related to bacterial effectiveness. Eur. J. Soil Biol. 47, 303–309. https://doi.org/10.1016/j.ejsobi.2011.07.004

Bérard, A., Ben Sassi, M., Kaisermann, A., Renault, P., 2015. Soil microbial community

responses to heat wave components: drought and high temperature. Clim. Res. 66, 243–264. https://doi.org/10.3354/cr01343

Bicalho, B., Pereira, A.S., Aquino Neto, F.R., Pinto, A.C., Rezende, C.M., 2000. Application of High-Temperature Gas Chromatography−Mass Spectrometry to the Investigation of Glycosidically Bound Components Related to Cashew Apple (*Anacardium occidentale* L. Var. *nanum*) Volatiles. J. Agric. Food Chem. 48, 1167–1174. https://doi.org/10.1021/jf9909252

Blom, D., Fabbri, C., Connor, E.C., Schiestl, F.P., Klauser, D.R., Boller, T., Eberl, L., Weisskopf, L., 2011. Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environ. Microbiol. 13, 3047–3058. https://doi.org/10.1111/j.1462-2920.2011.02582.x

Bray, E., 1997. Plant responses to water deficit. Trends Plant Sci. 2, 48–54.

Brock, A.K., Berger, B., Schreiner, M., Ruppel, S., Mewis, I., 2018. Plant growth-promoting bacteria Kosakonia radicincitans mediate anti-herbivore defense in *Arabidopsis thaliana*. Planta 248, 1383–1392. https://doi.org/10.1007/s00425-018-2964-0

Brown, A.D., 1976. Microbial water stress. Bacteriol. Rev. 40, 803–46.

Brown, A.D., Simpson, J.R., 1972. Water Relations of Sugar-tolerant Yeasts: the Role of Intracellular Polyols. J. Gen. Microbiol. 72, 589–591. https://doi.org/10.1099/00221287-72-3-589

Buege, J.A., Aust, S.D., 1978. Microsomal Lipid Peroxidation. Methods Enzymol. 52, 302–10. https://doi.org/10.1016/S0076-6879(78)52032-6

Caldeira, M., Perestrelo, R., Barros, a. S., Bilelo, M.J., Morête, a., Câmara, J.S., Rocha, S.M., 2012. Allergic asthma exhaled breath metabolome: A challenge for comprehensive two-dimensional gas chromatography. J. Chromatogr. A 1254, 87–97. https://doi.org/10.1016/j.chroma.2012.07.023

Camarena-Pozos, D.A., Flores-Núñez, V.M., López, M.G., López-Bucio, J., Partida-Martínez, L.P., 2018. Smells from the desert: Microbial volatiles that affect plant growth and development of native and non-native plant species. Plant. Cell Environ. https://doi.org/10.1111/pce.13476

Cardoso, P., Alves, A., Silveira, P., Sá, C., Fidalgo, C., Freitas, R., Figueira, E., 2018. Bacteria from nodules of wild legume species: phylogenetic diversity, plant growth promotion abilities and osmotolerance. Sci. Total Environ. 645, 1094–1102. https://doi.org/10.1016/j.scitotenv.2018.06.399

Cardoso, P., Santos, M., Freitas, R., Rocha, S.M., Figueira, E., 2017. Response of *Rhizobium* to Cd exposure: A volatile perspective. Environ. Pollut. 231, 802–811. https://doi.org/10.1016/j.envpol.2017.08.067

Chabot, R., Antoun, H., Cescas, M.P., 1996. Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* biovar. *phaseoli*. Plant Soil 184, 311–321. https://doi.org/10.1007/BF00010460

Chang, W.-S., van de Mortel, M., Nielsen, L., Nino de Guzman, G., Li, X., Halverson, L.J., 2007. Alginate Production by *Pseudomonas putida* Creates a Hydrated Microenvironment and Contributes to Biofilm Architecture and Stress Tolerance under Water-Limiting Conditions. J. Bacteriol. 189, 8290–8299. https://doi.org/10.1128/JB.00727-07

Chaves, M.M., Flexas, J., Pinheiro, C., 2009. Photosynthesis under drought and salt

stress: regulation mechanisms from whole plant to cell. Ann. Bot. 103, 551–560. https://doi.org/10.1093/aob/mcn125

Chen, C., Beattie, G.A., 2008. *Pseudomonas syringae* BetT is a low-affinity choline transporter that is responsible for superior osmoprotection by choline over glycine betaine. J. Bacteriol. 190, 2717–25. https://doi.org/10.1128/JB.01585-07

Chen, C., Beattie, G.A., 2007. Characterization of the osmoprotectant transporter OpuC from *Pseudomonas syringae* and demonstration that cystathionine-beta-synthase domains are required for its osmoregulatory function. J. Bacteriol. 189, 6901–12. https://doi.org/10.1128/JB.00763-07

Cho, S.M., Kang, B.R., Han, S.H., Anderson, A.J., Park, J.-Y., Lee, Y.-H., Cho, B.H., Yang, K.-Y., Ryu, C.-M., Kim, Y.C., 2008. 2R,3R-Butanediol, a Bacterial Volatile Produced by *Pseudomonas chlororaphis* O6, Is Involved in Induction of Systemic Tolerance to Drought in Arabidopsis thaliana. Mol. Plant-Microbe Interact. 21, 1067–1075. https://doi.org/10.1094/MPMI-21-8-1067

Claeys, H., Van Landeghem, S., Dubois, M., Maleux, K., Inze, D., 2014. What Is Stress? Dose-Response Effects in Commonly Used in Vitro Stress Assays. PLANT Physiol. 165, 519–527. https://doi.org/10.1104/pp.113.234641

Coba de la Peña, T., Pueyo, J.J., 2012. Legumes in the reclamation of marginal soils, from cultivar and inoculant selection to transgenic approaches. Agron. Sustain. Dev. 32, 65–91. https://doi.org/10.1007/s13593-011-0024-2

Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E.A., 2005. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl. Environ. Microbiol. 71, 4951–4959. https://doi.org/10.1128/AEM.71.9.4951-4959.2005

Conlin, L.K., Nelson, H.C.M., 2007. The natural osmolyte trehalose is a positive regulator of the heat-induced activity of yeast heat shock transcription factor. Mol. Cell. Biol. 27, 1505–15. https://doi.org/10.1128/MCB.01158-06

Corticeiro, S., Freitas, R., Figueira, E., 2013. The role of GSTs in the tolerance of *Rhizobium leguminosarum* to cadmium. Biometals 26, 879–86. https://doi.org/10.1007/s10534-013-9664-9

Costa, C.P., Silva, D.G., Rudnitskaya, A., Almeida, A., Rocha, S.M., 2016. Shedding light on *Aspergillus niger* volatile exometabolome. Sci. Rep. 6, 27441. https://doi.org/10.1038/srep27441

Costa, L.E. de O., Queiroz, M.V. de, Borges, A.C., Moraes, C.A. de, Araújo, E.F. de, 2012. Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*). Brazilian J. Microbiol. 43, 1562–1575. https://doi.org/10.1590/S1517-83822012000400041

Csonka, L., 1989. Physiological and Genetic Responses of Bacteria to Osmotic Stress. Microbiol. Rev. 53, 121–147.

D'Souza-Ault, M.R., Smith, L.T., Smith, G.M., 1993. Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. Appl. Environ. Microbiol. 59, 473–8.

Dahlqvist, A., 1968. Assay of intestinal disaccharidases. Anal. Biochem. 22, 99–107. https://doi.org/10.1016/0003-2697(68)90263-7

Datta, B., Chakrabartty, P.K., 2014. Siderophore biosynthesis genes of Rhizobium sp. isolated from *Cicer arietinum* L. 3 Biotech 4, 391–401.

https://doi.org/10.1007/s13205-013-0164-y

Datta, C., Basu, P.S., 2000. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, Cajanus cajan. Microbiol. Res. 155, 123–127. https://doi.org/10.1016/S0944-5013(00)80047-6

Davis, T.A., Volesky, B., Mucci, A., 2003. A review of the biochemistry of heavy metal biosorption by brown algae. Water Res. 37, 4311–30. https://doi.org/10.1016/S0043-1354(03)00293-8

Dawwam, G.E., Elbeltagy, A., Emara, H.M., Abbas, I.H., Hassan, M.M., 2013. Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. Ann. Agric. Sci. 58, 195–201. https://doi.org/10.1016/j.aoas.2013.07.007

Dinnbier, U., Limpinsel, E., Schmid, R., Bakker, E.P., 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch. Microbiol. 150, 348–57.

Easlon, H.M., Bloom, A.J., 2014. Easy Leaf Area: Automated digital image analysis for rapid and accurate measurement of leaf area. Appl. Plant Sci. 2, 1400033. https://doi.org/10.3732/apps.1400033

Eshkol, N., Sendovski, M., Bahalul, M., Katz-Ezov, T., Kashi, Y., Fishman, A., 2009. Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain. J. Appl. Microbiol. 106, 534–542. https://doi.org/10.1111/j.1365-2672.2008.04023.x

Farag, M.A., Ryu, C.-M., Sumner, L.W., Paré, P.W., 2006. GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry 67, 2262–2268. https://doi.org/10.1016/j.phytochem.2006.07.021

Farag, M.A., Song, G.C., Park, Y.-S., Audrain, B., Lee, S., Ghigo, J.-M., Kloepper, J.W., Ryu, C.-M., 2017. Biological and chemical strategies for exploring inter- and intra-kingdom communication mediated via bacterial volatile signals. Nat. Protoc. 12, 1359–1377. https://doi.org/10.1038/nprot.2017.023

Farag, M.A., Zhang, H., Ryu, C.-M., 2013. Dynamic Chemical Communication between Plants and Bacteria through Airborne Signals: Induced Resistance by Bacterial Volatiles. J. Chem. Ecol. 39, 1007–1018. https://doi.org/10.1007/s10886-013-0317-9

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C., 2005. Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biol. Biochem. 37, 955–964. https://doi.org/10.1016/j.soilbio.2004.10.021

Fialho, A.M., Zielinski, N.A., Fett, W.F., Chakrabarty, A.M., Berry, A., 1990. Distribution of alginate gene sequences in the *Pseudomonas* rRNA homology group I-Azomonas-Azotobacter lineage of superfamily B procaryotes. Appl. Environ. Microbiol. 56, 436–43.

Fierer, N., Schimel, J.P., 2002. Effects of drying–rewetting frequency on soil carbon and nitrogen transformations. Soil Biol. Biochem. 34, 777–787. https://doi.org/10.1016/S0038-0717(02)00007-X

Figueira, E., 2000. Aspectos da tolerância salina em *Pisum sativum* L.: influência da nutrição azotada. Universidade de Aveiro.

Figueira, E.M. de A.P., Gusmão Lima, A.I., Pereira, S.I.A., 2005. Cadmium tolerance

plasticity in *Rhizobium leguminosarum* bv. viciae: glutathione as a detoxifying agent. Can. J. Microbiol. 51, 7–14. https://doi.org/10.1139/w04-101

Fikret, Y., Manar, T., Şebnem, E., Şebnem, K., Özlem, U., 2013. SOD, CAT, GR and APX Enzyme Activities in Callus Tissues of Susceptible and Tolerant Eggplant Varieties under Salt Stress. Res. J. Biotechnol. 8, 45–51.

Fincheira, P., Venthur, H., Mutis, A., Parada, M., Quiroz, A., 2016. Growth promotion of *Lactuca sativa* in response to volatile organic compounds emitted from diverse bacterial species. Microbiol. Res. 193, 39–47. https://doi.org/10.1016/j.micres.2016.09.008

Flores-Félix, J.D., Menéndez, E., Rivera, L.P., Marcos-García, M., Martínez-Hidalgo, P., Mateos, P.F., Martínez-Molina, E., Velázquez, M. de la E., García-Fraile, P., Rivas, R., 2013. Use of *Rhizobium leguminosarum* as a potential biofertilizer for *Lactuca sativa* and *Daucus carota* crops. J. Plant Nutr. Soil Sci. 176, 876–882. https://doi.org/10.1002/jpln.201300116

Flores, C., Moreno, S., Espín, G., Peña, C., Galindo, E., 2013. Expression of alginases and alginate polymerase genes in response to oxygen, and their relationship with the alginate molecular weight in *Azotobacter vinelandii*. Enzyme Microb. Technol. 53, 85–91. https://doi.org/10.1016/j.enzmictec.2013.04.010

Flowers, T.J., Troke, P.F., Yeo, A.R., 1977. The Mechanism of Salt Tolerance in Halophytes. Annu. Rev. Plant Physiol. 28, 89–121. https://doi.org/10.1146/annurev.pp.28.060177.000513

Freeman, B.C., Chen, C., Beattie, G.A., 2010. Identification of the trehalose biosynthetic loci of *Pseudomonas syringae* and their contribution to fitness in the phyllosphere. Environ. Microbiol. 12, 1486–97. https://doi.org/10.1111/j.1462-2920.2010.02171.x

Freeman, B.C., Chen, C., Yu, X., Nielsen, L., Peterson, K., Beattie, G.A., 2013. Physiological and Transcriptional Responses to Osmotic Stress of Two *Pseudomonas syringae* Strains That Differ in Epiphytic Fitness and Osmotolerance. J. Bacteriol. 195, 4742–4752. https://doi.org/10.1128/JB.00787-13

French, S., Levy-Booth, D., Samarajeewa, A., Shannon, K.E., Smith, J., Trevors, J.T., 2009. Elevated temperatures and carbon dioxide concentrations: effects on selected microbial activities in temperate agricultural soils. World J. Microbiol. Biotechnol. 25, 1887–1900. https://doi.org/10.1007/s11274-009-0107-2

Galinski, E.A., Truper, H.G., 1982. Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris*. FEMS Microbiol. Lett. 13, 357–360. https://doi.org/10.1111/j.1574-6968.1982.tb08287.x

García-Fraile, P., Carro, L., Robledo, M., Ramírez-Bahena, M.-H., Flores-Félix, J.-D., Fernández, M.T., Mateos, P.F., Rivas, R., Igual, J.M., Martínez-Molina, E., Peix, Á., Velázquez, E., 2012. *Rhizobium* Promotes Non-Legumes Growth and Quality in Several Production Steps: Towards a Biofertilization of Edible Raw Vegetables Healthy for Humans. PLoS One 7, e38122. https://doi.org/10.1371/journal.pone.0038122

Garnica-Vergara, A., Barrera-Ortiz, S., Muñoz-Parra, E., Raya-González, J., Méndez-Bravo, A., Macías-Rodríguez, L., Ruiz-Herrera, L.F., López-Bucio, J., 2016. The volatile 6-pentyl-2 H -pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and ETHYLENE INSENSITIVE 2 functioning. New Phytol. 209, 1496–1512. https://doi.org/10.1111/nph.13725

Gholami, A., Shahsavani, S., Nezarat, S., 2009. The Effect of Plant Growth Promoting Rhizobacteria (PGPR) on Germination, Seedling Growth and Yield of Maize. Int. J. Agric. Biosyst. Eng. 3, 9–14.

Giaever, H.M., Styrvold, O.B., Kaasen, I., Strøm, A.R., 1988. Biochemical and genetic characterization of osmoregulatory trehalose synthesis in Escherichia coli. J. Bacteriol. 170, 2841–9.

Glick, B.R., 2012. Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica (Cairo). 2012, 1–15. https://doi.org/10.6064/2012/963401

Grant, G.T., Morris, E.R., Rees, D.A., Smith, P.J.C., Thom, D., 1973. Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Lett. 32, 195–198. https://doi.org/10.1016/0014-5793(73)80770-7

Grieve, C.M., Grattan, S.R., 1983. Rapid assay for determination of water soluble quaternary ammonium compounds. Plant Soil 70, 303–307. https://doi.org/10.1007/BF02374789

Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S., Weisskopf, L., 2013. Production of Bioactive Volatiles by Different *Burkholderia ambifaria* Strains. J. Chem. Ecol. 39, 892–906. https://doi.org/10.1007/s10886-013-0315-y

Guichard, E., Souty, M., 1988. Comparison of the relative quantities of aroma compounds found in fresh apricot (*Prunus armeniaca*) from six different varieties. Zeitschrift f�r Leb. und -forsch. 186, 301–307. https://doi.org/10.1007/BF01027031

Gurevitch, J., Scheiner, S., Fox, G., 2002. The ecology of plants. Sinauer Associates, Sunderland.

Gusain, Y.S., Singh, U.S., Sharma, A.K., 2015. Bacterial mediated amelioration of drought stress in drought tolerant and susceptible cultivars of rice (*Oryza sativa* L.). African J. Biotechnol. 14, 764–773. https://doi.org/10.5897/AJB2015.14405

Gutiérrez-Luna, F.M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H.R., Macías-Rodríguez, L., 2010. Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis 51, 75–83. https://doi.org/10.1007/s13199-010-0066-2

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139. https://doi.org/10.1017/CBO9781107415324.004

Han, S.H., Lee, S.J., Moon, J.H., Park, K.H., Yang, K.Y., Cho, B.H., Kim, K.Y., Kim, Y.W., Lee, M.C., Anderson, A.J., Kim, Y.C., 2006. GacS-Dependent Production of 2R , 3R-Butanediol by *Pseudomonas chlororaphis* O6 Is a Major Determinant for Eliciting Systemic Resistance Against *Erwinia carotovora* but not Against *Pseudomonas syringae* pv . tabaci in Tobacco 19, 924–930.

Hanbury, C.., White, C.., Mullan, B.., Siddique, K.H.., 2000. A review of the potential of *Lathyrus sativus* L. and *L. cicera* L. grain for use as animal feed. Anim. Feed Sci. Technol. 87, 1–27. https://doi.org/10.1016/S0377-8401(00)00186-3

Hanel, M., Rakovec, O., Markonis, Y., Máca, P., Samaniego, L., Kyselý, J., Kumar, R., 2018. Revisiting the recent European droughts from a long-term perspective. Sci. Rep. 8, 9499. https://doi.org/10.1038/s41598-018-27464-4

Hartman, K., van der Heijden, M.G., Roussely-Provent, V., Walser, J.-C., Schlaeppi, K.,

2017. Deciphering composition and function of the root microbiome of a legume plant. Microbiome 5, 2. https://doi.org/10.1186/s40168-016-0220-z

Hernández-León, R., Rojas-Solís, D., Contreras-Pérez, M., Orozco-Mosqueda, M. del C., Macías-Rodríguez, L.I., Reyes-de la Cruz, H., Valencia-Cantero, E., Santoyo, G., 2015. Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by *Pseudomonas fluorescens* strains. Biol. Control 81, 83–92. https://doi.org/10.1016/j.biocontrol.2014.11.011

Hirsch, P.R., 1996. Population Dynamics of Indigenous and Genetically Modified Rhizobia in the Field. New Phytol. 133, 159–171.

Houghton, R.A., 1994. The Worldwide Extent of Land-Use Change. Bioscience 44, 305–313. https://doi.org/10.2307/1312380

Hussain, M.B., Zahir, Z.A., Asghar, H.N., Asgher, M., 2014. Can catalase and exopolysaccharides producing rhizobia ameliorate drought stress in wheat? Int. J. Agric. Biol. 16, 3–13.

Ibáñez, F., Angelini, J., Taurian, T., Tonelli, M.L., Fabra, A., 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Syst. Appl. Microbiol. 32, 49–55. https://doi.org/10.1016/j.syapm.2008.10.001

Imeson, A.P., Ledward, D.A., Mitchell, J.R., 1977. On the nature of the interaction between some anionic polysaccharides and proteins. J. Sci. Food Agric. 28, 661–668. https://doi.org/10.1002/jsfa.2740280802

Imhoff, J.F., 1986. Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Lett. 39, 57–66. https://doi.org/10.1111/j.1574-6968.1986.tb01843.x

IPCC, 2014. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.

IPCC, 2007. Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Iqbal, A., Khalil, I.A., Ateeq, N., Sayyar Khan, M., 2006. Nutritional quality of important food legumes. Food Chem. 97, 331–335. https://doi.org/10.1016/j.foodchem.2005.05.011

Jarunrattanasri, A., Theerakulkait, C., Cadwallader, K.R., 2007. Aroma Components of Acid-Hydrolyzed Vegetable Protein Made by Partial Hydrolysis of Rice Bran Protein. J. Agric. Food Chem. 55, 3044–3050. https://doi.org/10.1021/jf0631474

Jiménez-Gómez, A., Flores-Félix, J.D., García-Fraile, P., Mateos, P.F., Menéndez, E., Velázquez, E., Rivas, R., 2018. Probiotic activities of *Rhizobium laguerreae* on growth and quality of spinach. Sci. Rep. 8, 295. https://doi.org/10.1038/s41598-017-18632-z

Johansson, L.H., 1988. A Spectrophotometric Method for Determination Small Tissue Samples of Catalase Activity in 336, 0–5.

Johnson, P.M., Mayrand, K., Paquin, M. (Eds.), 2006. Governing Global Desertification: Linking Environmental Degradation, Poverty and Participation. Ashgate Publishing.

Jupp, A.P., Newman, E.I., 1987. Morphological and anatomical effects of severe drought on the roots of *Lolium perenne* L. New Phytol. 105, 393–402.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B., 2009. Bacterial

volatiles and their action potential. Appl. Microbiol. Biotechnol. 81, 1001–1012. https://doi.org/10.1007/s00253-008-1760-3

Kai, M., Uta, E. V, Berg, G., 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani* 351–360. https://doi.org/10.1007/s00203-006-0199-0

Kämpfer, P., Busse, H.-J., McInroy, J.A., Xu, J., Glaeser, S.P., 2015. *Flavobacterium nitrogenifigens* sp. nov., isolated from switchgrass (*Panicum virgatum*). Int. J. Syst. Evol. Microbiol. 65, 2803–9. https://doi.org/10.1099/ijs.0.000330

Kelishomi, Z.H., Goliaei, B., Mahdavi, H., Nikoofar, A., Rahimi, M., Moosavi-Movahedi, A.A., Mamashli, F., Bigdeli, B., 2016. Antioxidant activity of low molecular weight alginate produced by thermal treatment. Food Chem. 196, 897–902. https://doi.org/10.1016/j.foodchem.2015.09.091

Khan, N., Bano, A., Zandi, P., 2018. Effects of exogenously applied plant growth regulators in combination with PGPR on the physiology and root growth of chickpea (*Cicer arietinum*) and their role in drought tolerance. J. Plant Interact. 13, 239–247. https://doi.org/10.1080/17429145.2018.1471527

Kim, B.-E., Nevitt, T., Thiele, D.J., 2008. Mechanisms for copper acquisition, distribution and regulation. Nat. Chem. Biol. 4, 176–85. https://doi.org/10.1038/nchembio.72

Korpi, A., Järnberg, J., Pasanen, A.-L., 2009. Microbial volatile organic compounds. Crit. Rev. Toxicol. 39, 139–193. https://doi.org/10.1080/10408440802291497

Kucey, R.M.N., Hynes, M.F., 1989. Populations of *Rhizobium leguminosarum* biovars phaseoli and viceae in fields after bean or pea in rotation with nonlegumes. Can. J. Microbiol. 35, 661–667. https://doi.org/10.1139/m89-107

Kurz, M., Burch, A.Y., Seip, B., Lindow, S.E., Gross, H., 2010. Genome-Driven Investigation of Compatible Solute Biosynthesis Pathways of *Pseudomonas syringae* pv. *syringae* and Their Contribution to Water Stress Tolerance. Appl. Environ. Microbiol. 76, 5452–5462. https://doi.org/10.1128/AEM.00686-10

Lane, D.J., 1991. 16S/23S rRNA sequencing, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New York, pp. 115–175.

Larsen, B., Salem, D.M.S.A., Sallam, M.A.E., Mishrikey, M.M., Beltagy, A.I., 2003. Characterization of the alginates from algae harvested at the Egyptian Red Sea coast. Carbohydr. Res. 338, 2325–36.

Larsen, P.I., Sydnes, L.K., Landfald, B., Strøm, A.R., 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch. Microbiol. 147, 1–7.

Lawlor, D.W., 2002. Limitation to Photosynthesis in Water-stressed Leaves: Stomata vs. Metabolism and the Role of ATP. Ann. Bot. 89, 871–885. https://doi.org/10.1093/aob/mcf110

Leite, J., Fischer, D., Rouws, L.F.M., Fernandes-Júnior, P.I., Hofmann, A., Kublik, S., Schloter, M., Xavier, G.R., Radl, V., 2017. Cowpea Nodules Harbor Non-rhizobial Bacterial Communities that Are Shaped by Soil Type Rather than Plant Genotype. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.02064

Li, W., Wang, H., Sun, Y., Huang, A., Sun, Y., 1998. Capillary gas chromatographic analysis of volatile components in goat feces. Fenxi Huaxue 26, 935–939.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 148, 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Lima, N. da S., Morais, M.B. de, Silva, Ê.F. de F. e, Camara, T.R., Willadino, L., 2017. Production and antioxidative metabolism in bell pepper grown with saline water in hydroponic system. Rev. Bras. Eng. Agrícola e Ambient. 21, 675–680. https://doi.org/10.1590/1807-1929/agriambi.v21n10p675-680

Lingappa, B.T., Prasad, M., Lingappa, Y., Hunt, D.F., Biemann, K., 1969. Phenethyl alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans*. Science 163, 192–4. https://doi.org/10.1126/SCIENCE.163.3863.192

Liu, X., Zhang, H., 2015. The effects of bacterial volatile emissions on plant abiotic stress 6, 1–6. https://doi.org/10.3389/fpls.2015.00774

Lòpez-Fernàndez, S., Mazzoni, V., Pedrazzoli, F., Pertot, I., Campisano, A., 2017. A Phloem-Feeding Insect Transfers Bacterial Endophytic Communities between Grapevine Plants. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00834

Loureiro, C.C., Duarte, I.F., Gomes, J., Carrola, J., Barros, A.S., Gil, A.M., Bousquet, J., Bom, A.T., Rocha, S.M., 2014. Urinary metabolomic changes as a predictive biomarker of asthma exacerbation. J. Allergy Clin. Immunol. 133, 261-263.e1–5. https://doi.org/10.1016/j.jaci.2013.11.004

Lugtenberg, B., Kamilova, F., 2009. Plant-Growth-Promoting Rhizobacteria. Annu. Rev. Microbiol. 63, 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918

Mackay, M.A., Norton, R.S., Borowitzka, L.J., 1984. Organic Osmoregulatory Solutes in Cyanobacteria. J. Gen. Microbiol. 130, 2177–2191. https://doi.org/10.1099/00221287-130-9-2177

Maestre, F.T., Delgado-Baquerizo, M., Jeffries, T.C., Eldridge, D.J., Ochoa, V., Gozalo, B., Quero, J.L., García-Gómez, M., Gallardo, A., Ulrich, W., Bowker, M.A., Arredondo, T., Barraza-Zepeda, C., Bran, D., Florentino, A., Gaitán, J., Gutiérrez, J.R., Huber-Sannwald, E., Jankju, M., Mau, R.L., Miriti, M., Naseri, K., Ospina, A., Stavi, I., Wang, D., Woods, N.N., Yuan, X., Zaady, E., Singh, B.K., 2015. Increasing aridity reduces soil microbial diversity and abundance in global drylands. Proc. Natl. Acad. Sci. 201516684. https://doi.org/10.1073/pnas.1516684112

Maheshwari, D.K., 2011. Bacteria in Agrobiology: Crop Ecosystems. Springer Science & Business Media.

Martínez-Hidalgo, P., Hirsch, A.M., 2017. The Nodule Microbiome: N 2 -Fixing Rhizobia Do Not Live Alone. Phytobiomes 1, 70–82. https://doi.org/10.1094/PBIOMES-12-16-0019-RVW

Marulanda, A., Barea, J.-M., Azcón, R., 2009. Stimulation of Plant Growth and Drought Tolerance by Native Microorganisms (AM Fungi and Bacteria) from Dry Environments: Mechanisms Related to Bacterial Effectiveness. J. Plant Growth Regul. 28, 115–124. https://doi.org/10.1007/s00344-009-9079-6

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., Lee, Y.C., 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Anal. Biochem. 339, 69–72. https://doi.org/10.1016/j.ab.2004.12.001

Measures, J.C., 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature 257, 398–400.

Mehboob, I., Naveed, M., Zahir, Z.A., Ashraf, M., 2012. Potential of Rhizobia for

Sustainable Production of Non-legumes, in: Crop Production for Agricultural Improvement. Springer Netherlands, Dordrecht, pp. 659–704. https://doi.org/10.1007/978-94-007-4116-4_26

Meldau, D.G., Meldau, S., Hoang, L.H., Underberg, S., Wünsche, H., Baldwin, I.T., 2013. Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. Plant Cell 25, 2731–2747. https://doi.org/10.1105/tpc.113.114744

Mesquita, S., 2005. Modelação bioclimática de Portugal Continental. Univerisdade Técnica de Lisboa.

Meyer, S., 2011. Diversity of root nodule endophytes associated with native legumes in Flanders. Ghent University.

Monier, J.-M., Lindow, S.E., 2003. Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. Proc. Natl. Acad. Sci. 100, 15977–15982. https://doi.org/10.1073/pnas.2436560100

Moreira, J., 2013. *Solanum nigrum* L., a fine tool for phytoremediation of acetophenone. Universidade do Minho.

Msaddak, A., Durán, D., Rejili, M., Mars, M., Ruiz-Argüeso, T., Imperial, J., Palacios, J., Rey, L., 2017. Diverse Bacteria Affiliated with the Genera *Microvirga*, *Phyllobacterium*, and *Bradyrhizobium* Nodulate *Lupinus micranthus* Growing in Soils of Northern Tunisia. Appl. Environ. Microbiol. 83, e02820-16. https://doi.org/10.1128/AEM.02820-16

Naylor, D., Coleman-Derr, D., 2018. Drought Stress and Root-Associated Bacterial Communities. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.02223

Ngumbi, E., Fadamiro, H., Kloepper, J., 2014. Evaluating the effects of drought stress on cotton plant volatile emissions.

Ngumbi, E., Kloepper, J., 2016. Bacterial-mediated drought tolerance: Current and future prospects. Appl. Soil Ecol. 105, 109–125. https://doi.org/10.1016/j.apsoil.2016.04.009

Niu, X., Song, L., Xiao, Y., Ge, W., 2018. Drought-Tolerant Plant Growth-Promoting Rhizobacteria Associated with Foxtail Millet in a Semi-arid Agroecosystem and Their Potential in Alleviating Drought Stress. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02580

Noel, T.C., Sheng, C., Yost, C.K., Pharis, R.P., Hynes, M.F., 1996. *Rhizobium leguminosarum* as a plant growth-promoting rhizobacterium: direct growth promotion of canola and lettuce. Can. J. Microbiol. 42, 279–83.

Numan, M., Bashir, S., Khan, Y., Mumtaz, R., Shinwari, Z.K., Khan, A.L., Khan, A., AL-Harrasi, A., 2018. Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: A review. Microbiol. Res. 209, 21–32. https://doi.org/10.1016/j.micres.2018.02.003

Nunes, T., Cardoso, P., Freitas, R., Figueira, E., 2018. Protective effects of farnesol on a Rhizobium strain exposed to cadmium. Ecotoxicol. Environ. Saf. 165, 622–629. https://doi.org/10.1016/j.ecoenv.2018.07.125

Ochoa-Hueso, R., Collins, S.L., Delgado-Baquerizo, M., Hamonts, K., Pockman, W.T., Sinsabaugh, R.L., Smith, M.D., Knapp, A.K., Power, S.A., 2018. Drought consistently alters the composition of soil fungal and bacterial communities in grasslands from two continents. Glob. Chang. Biol. 24, 2818–2827.

https://doi.org/10.1111/gcb.14113

Ohwada, T., Sagisaka, S., 1988. The Differential Roles of K + , Proline and Betaine in Osmoregulation of *Escherichia coli* B. Agric. Biol. Chem. 52, 313–319. https://doi.org/10.1080/00021369.1988.10868659

Oren, A., 1999. Bioenergetic Aspects of Halophilism. Microbiol. Mol. Biol. Rev. 63, 334–348.

Osakabe, Y., Osakabe, K., Shinozaki, K., Tran, L.-S.P., 2014. Response of plants to water stress. Front. Plant Sci. 5. https://doi.org/10.3389/fpls.2014.00086

Owens, J.D., Allagheny, N., Kipping, G., Ames, J.M., 1997. Formation of Volatile Compounds During *Bacillus subtilis* Fermentation of Soya Beans. J. Sci. Food Agric. 74, 132–140. https://doi.org/10.1002/(SICI)1097-0010(199705)74:1<132::AID-JSFA779>3.0.CO;2-8

Pain, B., Jarvis, S., Clements, B., 1991. Impact of Agricultural Practices on Soil Pollution. Outlook Agric. 20, 153–160. https://doi.org/10.1177/003072709102000305

Patel, S., Jinal, H.N., Amaresan, N., 2017. Isolation and characterization of drought resistance bacteria for plant growth promoting properties and their effect on chilli (*Capsicum annuum*) seedling under salt stress. Biocatal. Agric. Biotechnol. 12, 85–89. https://doi.org/10.1016/j.bcab.2017.09.002

Peñuelas, J., Sardans, J., 2009. Ecological metabolomics. Chem. Ecol. 25, 305–309. https://doi.org/10.1080/02757540903062517

Peñuelas, J., Staudt, M., 2010. BVOCs and global change. Trends Plant Sci. 15, 133–144. https://doi.org/10.1016/j.tplants.2009.12.005

Perroud, B., Le Rudulier, D., 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. 161, 393–401.

Piechulla, B., Lemfack, M.C., Kai, M., 2017. Effects of discrete bioactive microbial volatiles on plants and fungi. Plant. Cell Environ. 40, 2042–2067. https://doi.org/10.1111/pce.13011

Potts, M., 1999. Mechanisms of desiccation tolerance in cyanobacteria. Eur. J. Phycol. 34, 319–328. https://doi.org/10.1080/09670269910001736382

Procópio, R.E.L., Araújo, W.L., Maccheroni Jr, W., Azevedo, J.L., 2009. Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. Genet. Mol. Res. 8, 1408–1422.

Qiming, X., Haidong, C., Huixian, Z., Daqiang, Y., 2006. Chemical composition of essential oils of two submerged macrophytes,Ceratophyllum demersum L. andVallisneria spiralis L. Flavour Fragr. J. 21, 524–526. https://doi.org/10.1002/ffj.1588

R Core Team, 2016. R: A language and environment for statistical computing.

Radzki, W., Gutierrez Mañero, F.J., Algar, E., Lucas García, J.A., García-Villaraco, A., Ramos Solano, B., 2013. Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek 104, 321–330. https://doi.org/10.1007/s10482-013-9954-9

Reed, R., 1986. Organic solute accumulation in osmotically stressed cyanobacteria. FEMS Microbiol. Lett. 39, 51–56. https://doi.org/10.1016/0378-1097(86)90060-1

Rein, I., Gessler, A., Premke, K., Keitel, C., Ulrich, A., Kayler, Z.E., 2016. Forest

understory plant and soil microbial response to an experimentally induced drought and heat-pulse event: the importance of maintaining the continuum. Glob. Chang. Biol. 22, 2861–2874. https://doi.org/10.1111/gcb.13270

Reverchon, F., Xu, Z., Blumfield, T.J., Chen, C., Abdullah, K.M., 2012. Impact of global climate change and fire on the occurrence and function of understorey legumes in forest ecosystems. J. Soils Sediments 12, 150–160. https://doi.org/10.1007/s11368-011-0445-1

Rivero, R.M., Kojima, M., Gepstein, A., Sakakibara, H., Mittler, R., Gepstein, S., Blumwald, E., 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc. Natl. Acad. Sci. U. S. A. 104, 19631–6. https://doi.org/10.1073/pnas.0709453104

Robertson, G.P., 2000. Greenhouse Gases in Intensive Agriculture: Contributions of Individual Gases to the Radiative Forcing of the Atmosphere. Science (80-.). 289, 1922–1925. https://doi.org/10.1126/science.289.5486.1922

Robinson, H.W., Hogden, C.G., 1940. The biuret reaction in the determination of serum proteins. J. Biol. Chem. 135, 707–725.

Robyt, J.F., 1998. Essentials of carbohydrate chemistry. Springer-Verlarg, New York.

Rocha, S.M., Caldeira, M., Carrola, J., Santos, M., Cruz, N., Duarte, I.F., 2012. Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry. J. Chromatogr. A 1252, 155–163. https://doi.org/10.1016/j.chroma.2012.06.067

Rocha, S.M., Freitas, R., Cardoso, P., Santos, M., Martins, R., Figueira, E., 2013. Exploring the potentialities of comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry to distinguish bivalve species: Comparison of two clam species (*Venerupis decussata* and *Venerupis philippinarum*). J. Chromatogr. A 1315. https://doi.org/10.1016/j.chroma.2013.09.049

Rodríguez-Salazar, J., Suárez, R., Caballero-Mellado, J., Iturriaga, G., 2009. Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants. FEMS Microbiol. Lett. 296, 52–9. https://doi.org/10.1111/j.1574-6968.2009.01614.x

Roller, S.D., Anagnostopoulos, G.D., 1982. Accumulation of carbohydrate by *Escherichia coli* B/r/1 during growth at low water activity. J. Appl. Bacteriol. 52, 425–434. https://doi.org/10.1111/j.1365-2672.1982.tb05073.x

Rout, P.K., Rao, Y.R., Sree, A., Naik, S.N., 2007. Composition of essential oil, concrete, absolute, wax and headspace volatiles of *Murrarya paniculata* (Linn.) Jack flowers. Flavour Fragr. J. 22, 352–357. https://doi.org/10.1002/ffj.1804

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Kloepper, J.W., Paré, P.W., 2004. Bacterial Volatiles Induce Systemic Resistance in *Arabidopsis*. Plant Physiol. 134, 1017–1026. https://doi.org/10.1104/pp.103.026583

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wei, H.-X., Paré, P.W., Kloepper, J.W., 2003. Bacterial volatiles promote growth in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 100, 4927–4932. https://doi.org/10.1073/pnas.0730845100

Ryu, C.-M., Hu, C.-H., Locy, R.D., Kloepper, J.W., 2005. Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. Plant Soil 268, 285–292. https://doi.org/10.1007/s11104-004-0301-9

Sahin, U., Ekinci, M., Kiziloglu, F.M., Yildirim, E., Turan, M., Kotan, R., Ors, S., 2015. Ameliorative Effects of Plant Growth Promoting Bacteria on Water-yield Relationships, Growth, and Nutrient Uptake of Lettuce Plants under Different Irrigation Levels. HortScience 50, 1379–1386.

Salvador, Â.C., Baptista, I., Barros, A.S., Gomes, N.C.M., Cunha, Â., Almeida, A., Rocha, S.M., 2013. Can Volatile Organic Metabolites Be Used to Simultaneously Assess Microbial and Mite Contamination Level in Cereal Grains and Coffee Beans? PLoS One 8, e59338. https://doi.org/10.1371/journal.pone.0059338

Santi, C., Bogusz, D., Franche, C., 2013. Biological nitrogen fixation in non-legume plants. Ann. Bot. 111, 743–767. https://doi.org/10.1093/aob/mct048

Santos, A. de A., Silveira, J.A.G. da, Bonifacio, A., Rodrigues, A.C., Figueiredo, M. do V.B., 2018. Antioxidant response of cowpea co-inoculated with plant growth-promoting bacteria under salt stress. Brazilian J. Microbiol. 49, 513–521. https://doi.org/10.1016/j.bjm.2017.12.003

Santos, M.C., Nunes, C., Rocha, M.A.M., Rodrigues, A., Rocha, S.M., Saraiva, J.A., Coimbra, M.A., 2015. High pressure treatments accelerate changes in volatile composition of sulphur dioxide-free wine during bottle storage. Food Chem. 188, 406–414. https://doi.org/10.1016/j.foodchem.2015.05.002

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., Glick, B.R., 2016. Plant growth-promoting bacterial endophytes. Microbiol. Res. 183, 92–99. https://doi.org/10.1016/j.micres.2015.11.008

Scheidig, C., Czerny, M., Schieberle, P., 2007. Changes in Key Odorants of Raw Coffee Beans during Storage under Defined Conditions. J. Agric. Food Chem. 55, 5768–5775. https://doi.org/10.1021/jf070488o

Schimel, J., Balser, T.C., Wallenstein, M., 2007. Microbial stress-response physiology and its implications for ecosystem function. Ecology 88, 1386–94.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., Garbeva, P., 2015. Volatile affairs in microbial interactions. ISME J. 9, 2329–2335. https://doi.org/10.1038/ismej.2015.42

Schobert, B., 1977. Is there an osmotic regulatory mechanism in algae and higher plants? J. Theor. Biol. 68, 17–26.

Schobert, B., Tschesche, H., 1978. Unusual solution properties of proline and its interaction with proteins. Biochim. Biophys. Acta 541, 270–7.

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., Hutzler, P., Schmid, M., Van Breusegem, F., Eberl, L., Hartmann, A., Langebartels, C., 2006. Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. Plant. Cell Environ. 29, 909–18.

Schulz, S., Dickschat, J.S., 2007. Bacterial volatiles: the smell of small organisms. Nat. Prod. Rep. 24, 814–842. https://doi.org/10.1039/b507392h

Schwenke, K.D., Kracht, E., Mieth, G., Freimuth, U., 1977. [Protein isolation by means of complexing agents. 2. Formation of insoluble complexes of sunflower albumins with alginate or pectin]. Nahrung 21, 395–403.

Sellimi, S., Younes, I., Ayed, H. Ben, Maalej, H., Montero, V., Rinaudo, M., Dahia, M., Mechichi, T., Hajji, M., Nasri, M., 2015. Structural, physicochemical and antioxidant properties of sodium alginate isolated from a Tunisian brown seaweed. Int. J. Biol. Macromol. 72, 1358–1367. https://doi.org/10.1016/j.ijbiomac.2014.10.016

Sharifi, R., Ryu, C.-M., 2018. Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future. Ann. Bot. 122, 349–358. https://doi.org/10.1093/aob/mcy108

Sharma, R., Yang, Y., Sharma, A., Awasthi, S., Awasthi, Y.C., 2004. Antioxidant Role of Glutathione S-Transferases: Protection Against Oxidant Toxicity and Regulation of Stress-Mediated Apoptosis. Antioxid. Redox Signal. 6, 289–300. https://doi.org/10.1089/152308604322899350

Silva, I., Coimbra, M.A., Barros, A.S., Marriott, P.J., Rocha, S.M., 2015. Can volatile organic compounds be markers of sea salt? Food Chem. 169, 102–113. https://doi.org/10.1016/j.foodchem.2014.07.120

Silva, I., Rocha, S.M., Coimbra, M.A., 2009. Headspace solid phase microextraction and gas chromatography–quadrupole mass spectrometry methodology for analysis of volatile compounds of marine salt as potential origin biomarkers. Anal. Chim. Acta 635, 167–174. https://doi.org/10.1016/j.aca.2009.01.011

Silva, M.T., Sousa, J.C., Macedo, M.A., Polónia, J., Parente, A.M., 1976. Effects of phenethyl alcohol on *Bacillus* and *Streptococcus*. J. Bacteriol. 127, 1359–69.

Sindhu, S.S., Dadarwal, K.R., 2001. Chitinolytic and cellulolytic *Pseudomonas* sp. antagonistic to fungal pathogens enhances nodulation by *Mesorhizobium* sp. Cicer in chickpea. Microbiol. Res. 156, 353–358. https://doi.org/10.1078/0944-5013-00120

Singh, S., Koehler, B., Fett, W., 1992. Effect of osmolarity and dehydration on alginate production by fluorescent pseudomonads. Curr. Microbiol. 25, 335–339.

Singh, U., Singh, B., 1992. Tropical grain legumes as important human foods. Econ. Bot. 46, 310–321. https://doi.org/10.1007/BF02866630

Smith, L.T., Pocard, J.A., Bernard, T., Le Rudulier, D., 1988. Osmotic control of glycine betaine biosynthesis and degradation in Rhizobium meliloti. J. Bacteriol. 170, 3142–9.

Somasegaran, P., Hoben, H.J., 1994. Handbook for Rhizobia. Springer-Verlag, New York, USA.

Souza, R. de, Ambrosini, A., Passaglia, L.M.P., 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. Genet. Mol. Biol. 38, 401–19. https://doi.org/10.1590/S1415-475738420150053

Spaepen, S., Vanderleyden, J., Remans, R., 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev. 31, 425–448. https://doi.org/10.1111/j.1574-6976.2007.00072.x

Sutherland, I., 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147, 3–9. https://doi.org/10.1099/00221287-147-1-3

Sutherland, L., Cairney, J., Elmore, M.J., Booth, I.R., Higgins, C.F., 1986. Osmotic regulation of transcription: induction of the proU betaine transport gene is dependent on accumulation of intracellular potassium. J. Bacteriol. 168, 805–14.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677. https://doi.org/10.1038/nature01014

Tuteja, N., Gill, S.S., Tuteja, R. (Eds.), 2012. Improving Crop Productivity in Sustainable Agriculture. John Wiley & Sons.

UmaMaheswari, T., Anbukkarasi, K., Hemalatha, T., Chendrayan, K., 2013. Studies on phytohormone producing ability of indigenous endophytic bacteria isolated from tropical legume crops. Int. J. Curr. Microbiol. Appl. Sci. 2, 127–136.

van der Weele, C.M., Spollen, W.G., Sharp, R.E., Baskin, T.I., 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J. Exp. Bot. 51, 1555–1562. https://doi.org/10.1093/jexbot/51.350.1555

van Rhijn, P., Vanderleyden, J., 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. 59, 124–42.

Vassilev, N., Vassileva, M., Nikolaeva, I., 2006. Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. Appl. Microbiol. Biotechnol. 71, 137–144. https://doi.org/10.1007/s00253-006-0380-z

Velázquez-Becerra, C., Macías-Rodríguez, L.I., López-Bucio, J., Altamirano-Hernández, J., Flores-Cortez, I., Valencia-Cantero, E., 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. Plant Soil 339, 329–340. https://doi.org/10.1007/s11104-010-0583-z

Verdier-Metz, I., Coulon, J., Pradel, P., Viallon, C., Berdagué, J., 1998. Effect of forage conservation (hay or silage) and cow breed on the coagulation properties of milks and on the characteristics of ripened cheeses. J. Dairy Res. 65, S0022029997002616. https://doi.org/10.1017/S0022029997002616

Versalovic, J., Schneider, M., de Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5, 25–40.

Vespermann, A., Kai, M., Piechulla, B., Vespermann, A., Kai, M., Piechulla, B., 2007. Rhizobacterial Volatiles Affect the Growth of Fungi and *Arabidopsis thaliana* Rhizobacterial Volatiles Affect the Growth of Fungi and *Arabidopsis thaliana* □ 73, 17–20. https://doi.org/10.1128/AEM.01078-07

Vijayabharathi, R., Sathya, A., Gopalakrishnan, S., 2016. A Renaissance in Plant Growth- Promoting and Biocontrol Agents by Endophytes, in: Singh, D., Singh, H., Prabha, R. (Eds.), Microbial Inoculants in Sustainable Agricultural Productivity Vol. 1: Research Perspectives. Springer India, New Delhi, pp. 37–60.

Vriezen, J.A.C., de Bruijn, F.J., Nusslein, K., 2007. Responses of Rhizobia to Desiccation in Relation to Osmotic Stress, Oxygen, and Temperature. Appl. Environ. Microbiol. 73, 3451–3459. https://doi.org/10.1128/AEM.02991-06

Vurukonda, S.S.K.P., Vardharajula, S., Shrivastava, M., SkZ, A., 2016. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. Microbiol. Res. 184, 13–24. https://doi.org/10.1016/j.micres.2015.12.003

Walderhaug, M., Dosch, D., Epstein, W., 1987. Potassium Transport in Bacteria, in: Ion Transport in Prokaryotes. Elsevier, pp. 85–130. https://doi.org/10.1016/B978-0-12-596935-2.50005-0

Walley, J.W., Kliebenstein, D.J., Bostock, R.M., Dehesh, K., 2013. Fatty acids and early detection of pathogens. Curr. Opin. Plant Biol. 16, 520–526. https://doi.org/10.1016/j.pbi.2013.06.011

Welsh, D.T., 2000. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol. Rev. 24, 263–90.

Wichern, J., Wichern, F., Joergensen, R.G., 2006. Impact of salinity on soil microbial communities and the decomposition of maize in acidic soils. Geoderma 137, 100–108. https://doi.org/10.1016/j.geoderma.2006.08.001

Wingender, J., Winkler, U.K., 1984. A novel biological function of alginate in Pseudomonas aeruginosa and its mucoid mutants: stimulation of exolipase. FEMS Microbiol. Lett. 21, 63–69.

Wood, J.M., 2015. Bacterial responses to osmotic challenges. J. Gen. Physiol. 145, 381–388. https://doi.org/10.1085/jgp.201411296

Wood, J.M., 2011. Bacterial Osmoregulation: A Paradigm for the Study of Cellular Homeostasis. Annu. Rev. Microbiol. 65, 215–238. https://doi.org/10.1146/annurev-micro-090110-102815

Wu, Q., Ni, X., 2015. ROS-mediated DNA methylation pattern alterations in carcinogenesis. Curr. Drug Targets 16, 13–19.

Wu, S., Zorn, H., Krings, U., Berger, R.G., 2007. Volatiles from submerged and surface-cultured beefsteak fungus, *Fistulina hepatica*. Flavour Fragr. J. 22, 53–60. https://doi.org/10.1002/ffj.1758

Xia, J., Sinelnikov, I. V., Han, B., Wishart, D.S., 2015. MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Res. 43, W251–W257. https://doi.org/10.1093/nar/gkv380

Yaakop, A.S., Chan, K.-G., Ee, R., Lim, Y.L., Lee, S.-K., Manan, F.A., Goh, K.M., 2016. Characterization of the mechanism of prolonged adaptation to osmotic stress of *Jeotgalibacillus malaysiensis* via genome and transcriptome sequencing analyses. Sci. Rep. 6, 33660. https://doi.org/10.1038/srep33660

Yan, N., Marschner, P., Cao, W., Zuo, C., Qin, W., 2015. Influence of salinity and water content on soil microorganisms. Int. Soil Water Conserv. Res. 3, 316–323. https://doi.org/10.1016/j.iswcr.2015.11.003

Yancey, P., Clark, M., Hand, S., Bowlus, R., Somero, G., 1982. Living with water stress: evolution of osmolyte systems. Science (80-.). 217, 1214–1222.

Yang, J.-S., Xie, Y.-J., He, W., 2011. Research progress on chemical modification of alginate: A review. Carbohydr. Polym. 84, 33–39. https://doi.org/10.1016/j.carbpol.2010.11.048

Zahir, Z.A., Munir, A., Asghar, H.N., Shaharoona, B., Arshad, M., 2008. Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. J. Microbiol. Biotechnol. 18, 958–63.

Zahran, H., 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. J. Biotechnol. 91, 143–153. https://doi.org/10.1016/S0168-1656(01)00342-X

Zhang, H., Kim, M.-S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., Farag, M.A., Ryu, C.-M., Allen, R., Melo, I.S., Paré, P.W., 2007. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta 226, 839–851. https://doi.org/10.1007/s00425-007-0530-2

Zhang, H., Kim, M., Sun, Y., Dowd, S.E., Shi, H., Paré, P.W., 2008. Soil Bacteria Confer Plant Salt Tolerance by Tissue-Specific Regulation of the Sodium Transporter HKT1 21, 737–744.

Zhang, H., Murzello, C., Sun, Y., Kim, M.-S., Xie, X., Jeter, R.M., Zak, J.C., Dowd, S.E.,

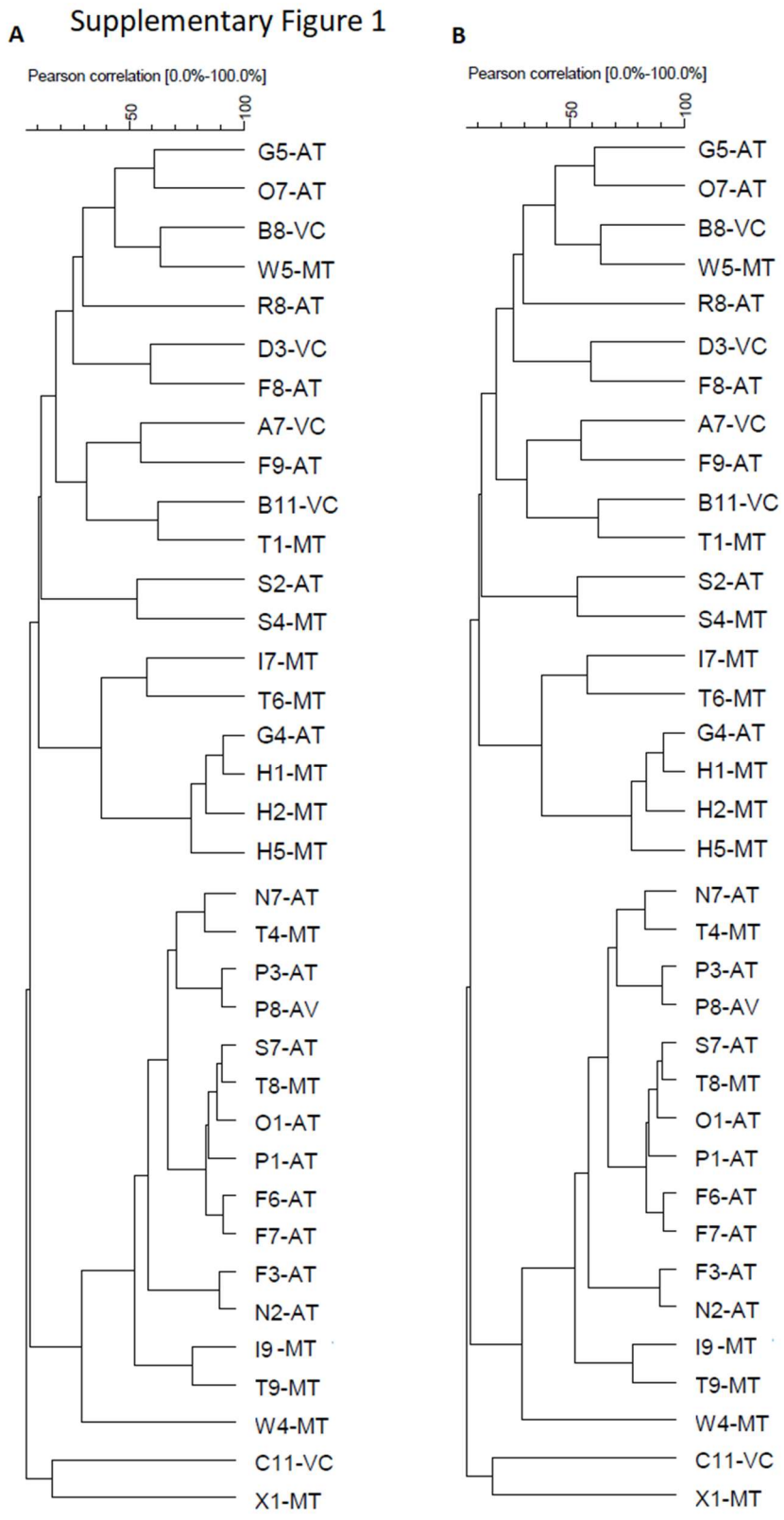
Paré, P.W., 2010. Choline and Osmotic-Stress Tolerance Induced in *Arabidopsis* by the Soil Microbe *Bacillus subtilis* (GB03). Mol. Plant-Microbe Interact. 23, 1097–1104. https://doi.org/10.1094/MPMI-23-8-1097

Zhang, X., Johnston, E.R., Liu, W., Li, L., Han, X., 2016. Environmental changes affect the assembly of soil bacterial community primarily by mediating stochastic processes. Glob. Chang. Biol. 22, 198–207. https://doi.org/10.1111/gcb.13080

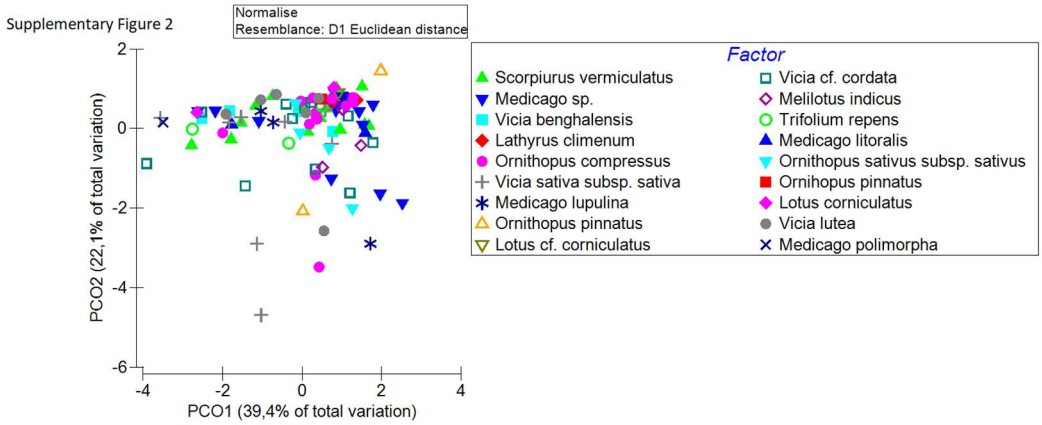
Zhao, L.F., Xu, Y.J., Ma, Z.Q., Deng, Z.S., Shan, C.J., Wei, G.H., 2013. Colonization and plant growth promoting characterization of endophytic *Pseudomonas chlororaphis* strain Zong1 isolated from *Sophora alopecuroides* root nodules. Brazilian J. Microbiol. 44, 629–637. https://doi.org/10.1590/S1517-83822013000200043

Zou, C., Li, Z., Yu, D., 2010. *Bacillus megaterium* Strain XTBG34 Promotes Plant Growth by Producing 2-Pentylfuran 48, 460–466. https://doi.org/10.1007/s12275-010-0068-z

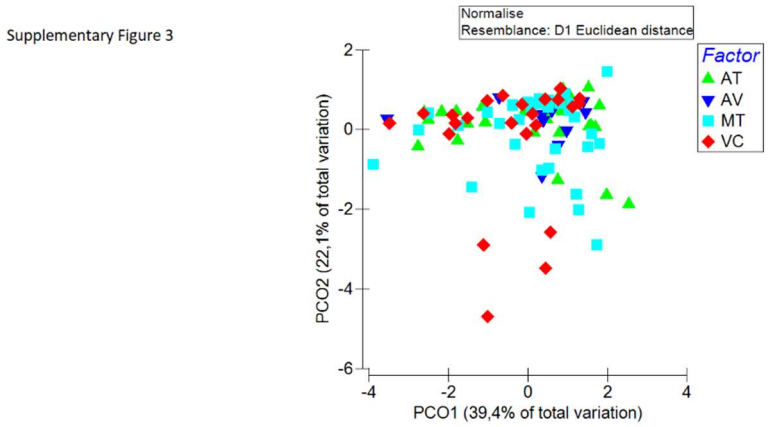
Supplementary Material



Supplementary Figure 1. (A) Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based BOX-PCR patterns of Pseudomonas, n=24, (A) and Flavobacterium, n=48, (B) strains isolated from the root nodules of wild legumes growing in four sites in Continental Portugal (n = 24).



Supplementary Figure 2. Principal component ordination (PCO) of production of IAA, production of siderophores, A. thaliana leaf area and fresh weight, and bacteria osmotolerance using the host plant species as factor.



Supplementary Figure 3. Principal component ordination (PCO) of production of indol acetic acid (IAA), production of siderophores, *A. thaliana* leaf area and fresh weight, and bacteria osmotolerance using the site as factor.

Supplementary Table 1. Endophytic bacteria isolated from the root nodules wild legumes growing in four sites in Continental Portugal. Identification by 16S rRNA gene sequencing, host species, osmotolerance (percentage of PEG that inhibits 50% growth) and the plant growth promotion abilities production of indol acetic acid-IAA (µg/mL normalized by optical density), production of siderophores (ratio between diameter of halo and diameter of colony) and *Arabidopsis thaliana* growth promotion through the emission of bacterial volatiles (ratios of leaf area in cm2 and fresh weight in mg between inoculated plates and non-inoculated controls).

Strain	Bacteria genera	Legume host species	PEG	IAA	Siderophores	Leaf area	Fresh weight
Aljustrel (AT)							
F4	*Flavobacterium*	*Scorpiurus vermiculatus* L.	11.52	-	-	3.25	2.22
L1	*Flavobacterium*	*Medicago sp.*	10.63	0.02	-	1.13	0.98
L8	*Flavobacterium*	*Medicago sp.*	10.27	-	-	1.04	1.13
Q1	*Flavobacterium*	*Medicago sp.*	7.69	14.22	1	0.97	0.89
F1	*Flavobacterium*	*Scorpiurus vermiculatus* L.	9	-	1.43	0.80	0.66
F2	*Flavobacterium*	*Scorpiurus vermiculatus* L.	11.2	-	1.33	1.76	1.65
G6	*Flavobacterium*	*Vicia benghalensis* L.	12.71	-	-	3.36	2.88
Q5	*Flavobacterium*	*Medicago sp.*	12.31	18.4	-	0.56	0.50
Q6	*Flavobacterium*	*Scorpiurus vermiculatus* L.	9.06	1.83	1.05	0.57	0.78
Q7	*Flavobacterium*	*Medicago sp.*	12.62	1.07	-	0.55	0.46
Q8	*Flavobacterium*	*Scorpiurus* vermiculatus L.	6.7	-	-	0.74	0.89
R7	*Flavobacterium*	*Medicago sp.*	8.93	1.88	-	1.60	1.07
Q3	*Flavobacterium*	*Medicago sp.*	8.07	3.16	-	1.21	1.47
N1	*Herbaspirillum*	*Scorpiurus vermiculatus* L.	14.06	2.13	-	1.65	1.31
O4	*Herbaspirillum*	*Medicago sp.*	18.71	-	-	2.82	2.08
P2	*Herbaspirillum*	*Medicago sp.*	26.41	8.09	-	1.31	0.95
Q4	*Lysobacter*	*Medicago sp.*	10.56	3.16	0.5	0.91	0.74
N9	*Variovorax*	*Vicia benghalensis* L.	15.69	-	-	1.91	1.62

F3	*Pseudomonas*	*Scorpiurus vermiculatus* L.	13.26	-	0.5	3.56	2.38
F6	*Pseudomonas*	*Scorpiurus vermiculatus* L.	9.18	-	1.56	3.65	2.82
F7	*Pseudomonas*	*Vicia benghalensis* L.	15.29	-	-	4.54	2.82
F8	*Pseudomonas*	*Scorpiurus vermiculatus* L.	12	-	-	1.22	1.31
F9	*Pseudomonas*	*Scorpiurus vermiculatus* L.	11.48	-	1.46	4.62	3.27
G4	*Pseudomonas*	*Medicago sp.*	11.48	-	-	4.87	2.91
G5	*Pseudomonas*	*Medicago sp.*	12.17	-	-	3.91	2.98
N2	*Pseudomonas*	*Scorpiurus vermiculatus* L.	15.36	-	-	1.20	1.26
N7	*Pseudomonas*	*Vicia benghalensis* L.	13.94	-	-	2.13	1.52
O1	*Pseudomonas*	*Vicia benghalensis* L.	12.75	0.06	1.25	1.60	0.92
O7	*Pseudomonas*	*Medicago sp.*	8.37	-	-	1.41	1.20
P1	*Pseudomonas*	*Scorpiurus vermiculatus* L.	6.52	-	-	1.15	1.40
P3	*Pseudomonas*	*Scorpiurus* vermiculatus L.	13.66	-	-	2.24	1.33
R8	*Pseudomonas*	*Lathyrus clymenum* L.	13.04	-	-	1.00	1.33
Alvito (AV)							
K6	*Achromobacter*	*Ornithopus compressus* L.	13.43	2.34	-	1.97	1.26
K1	*Erwinia*	*Vicia sativa subsp. sativa* L.	13.29	0.99	-	1.60	1.18
I3	*Pseudomonas*	*Vicia sativa subsp sativa* L.	13.38	-	-	5.12	3.78
J5	*Flavobacterium*	*Scorpiurus vermiculatus* L.	7.89	-	-	2.16	2.60
J6	*Flavobacterium*	*Medicago sp.*	7.26	-	1	0.68	0.99
M1	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	7.25	5.1	1	2.05	1.18
M2	*Flavobacterium*	*Ornithopus compressus* L.	17.39	-	-	1.34	1.40

M9	*Flavobacterium*	*Lathyrus clymenum* L.	11.51	0.47	-	0.78	0.82
P9	*Flavobacterium*	*Ornithopus compressus* L.	10.05	3.7	-	1.56	1.75
O3	*Herbaspirillum*	*Scorpiurus vermiculatus* L.	25.8	-	-	0.89	0.55
P8	*Pseudomonas*	*Ornithopus compressus* L.	15.95	-	2.79	1.42	1.42
Murtosa (MT)							
U1	*Acinetobacter*	*Medicago lupulina* L.	14.15	-	-	2.77	2.26
U6	*Agrobacterium/Rhizobium*	*Ornithopus pinnatus* (Mill.) Druce	11.41	14.54	1.05	2.62	1.99
K4	*Erwinia*	*Lotus cf. corniculatus* L.	8.55	-	-	1.25	1.18
D1	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	11.73	-	-	2.53	1.78
D8	*Flavobacterium*	*Melilotus indicus* (L.) All.	9.49	9.02	-	1.08	1.04
J1	*Flavobacterium*	*Trifolium repens* L.	13.88	6.02	-	2.15	2.16
J4	*Flavobacterium*	*Trifolium repens* L.	10.71	3.41	-	4.05	3.83
J8	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	9.99	3.16	-	3.04	1.49
U7	*Flavobacterium*	*Medicago littoralis* Rohde ex Loisel	11.47	6.2	-	0.78	0.87
V3	*Flavobacterium*	*Ornithopus pinnatus* (Mill.) Druce		-	-	0.64	0.69
G2	*Flavobacterium*	*Medicago lupulina* L.	19.93	-	-	2.40	1.86
H8	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	7.92	14.78	-	4.18	2.88
T2	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	12.34	7.69	-	0.64	0.76
T3	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	12.92	-	0.63	0.83	0.96
T7	*Flavobacterium*	*Vicia cf. cordata* Hoppe.	13.41	15.57	-	1.10	1.53
U3	*Flavobacterium*	*Melilotus indicus* (L.) All.	10.6	-	0.56	0.84	1.22

U9	*Flavobacterium*	*Trifolium repens* L.	13.37	0.26	-	1.58	1.25
H1	*Pseudomonas*	*Medicago littoralis* Rohde ex Loisel	19.2	-	-	3.57	2.38
H2	*Pseudomonas*	*Vicia cf. cordata* Hoppe.	12.39	-	-	3.76	3.49
H5	*Pseudomonas*	*Medicago lupulina* L.	13.17	0.02	-	1.10	0.86
I7	*Pseudomonas*	*Vicia cf. cordata* Hoppe.	14.76	-	1.8	4.91	4.28
I9	*Pseudomonas*	*Ornithopus sativus subsp. sativus* Brot.	11.18	6.99	3.02	1.47	0.86
S4	*Pseudomonas*	*Ornithopus sativus subsp. sativus* Brot.	13.02	-	1.94	1.18	1.30
T1	*Pseudomonas*	*Melilotus indicus* (L.) All.	12.14	0.31	-	1.37	1.10
T4	*Pseudomonas*	*Vicia cf. cordata* Hoppe.	12.12	-	-	1.73	1.52
T6	*Pseudomonas*	*Vicia cf. cordata* Hoppe.	13.11	-	2.79	1.45	1.53
T8	*Pseudomonas*	*Melilotus indicus* (L.) All.	10.1	-	3	2.05	1.06
T9	*Pseudomonas*	*Vicia cf. cordata* Hoppe.	14.16	-	-	1.47	1.79
W4	*Pseudomonas*	*Ornithopus compressus* L.	10.17	-	-	1.44	1.75
W5	*Pseudomonas*	*Ornihopus pinnatus* (Mill.) Druce	11.09	-	-	1.34	1.52
X1	*Pseudomonas*	*Ornithopus compressus* L.	11.2	-	-	1.61	1.95
V4	*Stenotrophomonas*	*Medicago lupulina* L.	15.8	18.63	1.25	1.46	0.72
Vale de Cambra (VC)							
B3	*Flavobacterium*	*Ornithopus compressus* L.	13.5	-	-	0.96	0.70
B4	*Flavobacterium*	*Ornithopus sativus subsp. sativus* Brot.	12.06	-	-	1.84	1.93
C1	*Flavobacterium*	*Lotus corniculatus* L.	12	-	-	4.86	2.92
D2	*Flavobacterium*	*Vicia lutea* L.	7.02	-	-	2.45	2.34

D4	*Flavobacterium*	*Lotus corniculatus* L.	5.94	-	-	1.60	1.21
D5	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	14.9	2.5	5	2.96	2.35
D6	*Flavobacterium*	*Vicia lutea* L.	10.79	4.74	4.5	1.68	1.48
D7	*Flavobacterium*	*Vicia lutea* L.	6.71	3.65	-	2.37	1.70
D9	*Flavobacterium*	*Ornithopus compressus* L.	11.4	-	-	0.86	0.85
E1	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	10.91	8.28	7	3.04	2.67
E2	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	6.66	4.91	-	2.84	2.10
E3	*Flavobacterium*	*Vicia lutea* L.	10.44	-	-	1.53	1.50
E4	*Flavobacterium*	*Vicia lutea* L.	8.76	-	-	3.32	2.15
H4	*Flavobacterium*	*Vicia lutea* L.	14.18	-	-	3.60	2.77
C4	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	16.15	-	-	3.11	2.55
E9	*Flavobacterium*	*Vicia sativa subsp. sativa* L.	12.02	2.13	-	4.38	2.37
R4	*Flavobacterium*	*Ornithopus compressus* L.	15.04	-	-	0.79	0.95
A10	*Paenibacillus*	*Ornithopus compressus* L.	18.19	-	6.55	1.40	1.22
A7	*Pseudomonas*	*Ornithopus sativus subsp. sativus* Brot.	13.86	-	1.1	1.93	1.65
B11	*Pseudomonas*	*Ornithopus compressus* L.	11.19	-	-	1.30	1.22
B8	*Pseudomonas*	*Ornithopus compressus* L.	17.59	1.91	-	3.77	2.70
C11	*Pseudomonas*	*Ornithopus compressus* L.	17.82	1.57	-	1.81	1.33
D3	*Pseudomonas*	*Medicago polymorpha* L.	15.3	-	-	5.63	3.28