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# Rhizobacterial promotion of maize growth and drought tolerance: perpectives from the laboratory, greenhouse and field

# Promoção do crescimento e tolerância à seca no milho por rizobactérias: evidências de laboratório, estufa e campo

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Paulo Jorge da Rocha Cardoso, Investigador Auxiliar do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro, e coorientação da Professora Doutora Etelvina Maria de Almeida Paula Figueira, Professora Auxiliar do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro.

Ao meu pai, que me ensinou a ser sempre a melhor (possível).

**o júri**

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**palavras-chave**

Alterações climáticas; bactérias promotoras do crescimento de plantas; milho; seca; BOX-PCR; parâmetros bioquímicos

**resumo**

Devido às alterações climáticas, prevê-se uma subida na temperatura mundial igual ou superior a 1.5 °C. Consequentemente, espera-se que a seca impacte mais de 50% da terra arável, afetando várias colheitas, incluindo o milho, que é a terceira principal colheita mundial. De facto, antecipa-se que a produtividade do milho a nível mundial diminua cerca de 15% devido ao stress hídrico. Por outro lado, a população mundial continua a aumentar, atingindo potencialmente 9 mil milhões até 2050. Logo, é fundamental garantir a disponibilidade de alimentos e segurança alimentar, de modo a responder às necessidades de uma população humana cada vez maior. Neste contexto, as rizobactérias surgem como uma alternativa mais sustentável ou um complemento ao uso de fertilizantes químicos. Estas bactérias existem naturalmente no solo e são usadas para promover o crescimento de plantas e para induzir tolerância a fatores abióticos, como a seca, e por isso são denominadas rizobactérias promotoras de crescimento de plantas. Várias espécies de bactérias têm sido aplicadas nas colheitas como biofertilizantes, aumentando a sua produtividade. Posto isto, esta tese tem como objetivo explorar o processo através do qual são desenvolvidos biofertilizantes compostos por rizobactérias promotoras de crescimento de plantas. Para tal, plantas de milho foram inoculadas com rizobactérias isoladas de raízes de leguminosas selvagens, bem como, rizobactérias isoladas de raízes de milho, e foram crescidas em estufa, sob condições normais, em que as plantas foram irrigadas e condições de seca, para encontrar potenciais candidatos para aplicar em testes de campo. Posteriormente, nos testes de campo, a produtividade do milho foi averiguada para determinar o crescimento do milho, e alguns parâmetros bioquímicos foram analisados de modo a entender-se se a inoculação com rizobactérias melhora o desenvolvimento desta colheita. Os resultados evidenciaram que a inoculação contribuiu para o aumento do crescimento do milho e a sua tolerância à seca em estufa e os parâmetros bioquímicos analisados revelam o efeito positivo da inoculação das bactérias nos estudos em campo. Adicionalmente, foram isoladas rizobactérias de raízes de milho que fora crescido em três níveis de défice hídrico para entender se haveria alguma diferença nas suas características. As capacidades de promoção de crescimento, bem como a osmotolerância foram avaliadas. De facto, a comunidade microbiana associada com as raízes do milho foi afetada pela seca. Ainda assim, várias estirpes foram capazes de produzir sideróforos e as bactérias isoladas de condições sujeitas a seca tiveram uma menor osmotolerância. No geral, os resultados desta tese evidenciam o potencial da aplicação de rizobactérias no milho para mitigar o stress causado pela seca e melhorar o seu crescimento, aumentando consequentemente a produtividade desta colheita.

**keywords**

Climate change; plant growth promoting bacteria; maize; drought; BOX-PCR; biochemical parameters

**abstract**

Due to climate changes, global temperature is projected to increase 1.5 °C or more. Consequentially, drought is expected to impact over 50% of the arable lands by 2050, affecting several crops, including maize, which is the third main food crop in the world. In fact, maize yield globally is expected to suffer a reduction of 15% because of drought stress. On the other hand, world population is predicted to reach 9 billion by 2050. Thus, there is the need to ensure food availability and security, to respond to an increased need to feed the growing population. In this context, rhizobacteria emerge as a more sustainable alternative or complement to chemical fertilizers. These bacteria exist naturally in the soil and are used to promote plant growth, and induce tolerance to abiotic factors, like drought, and are therefore called plant growth promoting rhizobacteria. Several strains have been applied in crops as biofertilizers, increasing productivity. Hence, this thesis aimed to explore the process of development of biofertilizers composed by plant growth promoting bacteria. To achieve that, maize plants were inoculated with rhizobacteria isolated from wild legumes, as well as with rhizobacteria isolated from maize plants and were grown in greenhouse under irrigated and drought conditions to screen for potential candidates to apply in the field. Posteriorly, in the field tests, maize yield was assessed to determine plant growth, and biochemical parameters were analyzed to understand how rhizobacteria inoculation improved maize development. Results evidenced maize growth and drought stress mitigation in the greenhouse, and biochemical parameters analyzed reveal the positive effect of bacterial inoculation, in the field. Additionally, rhizobacteria were isolated from maize plants growing under three levels of water deficit to understand if there would be any differences in their characteristics. Growth promotion abilities were evaluated as well as osmotolerance. In fact, microbial community associated with maize roots was affected by drought. Nevertheless, several strains were able to produce siderophores and bacteria isolated from conditions subject to water deficit had a lower osmotolerance. In general, the results of this thesis evidenced the potential of rhizobacteria to be applied in maize crops to mitigate drought stress and improve growth, ultimately increasing crop production.

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Three conditions were considered: A and D – w100 control condition (100% irrigated), D and E – w50 (50% irrigated), C and F – w0 (0% irrigated). Results represent the diameter of the colonies growing under control conditions (dark green) and PEG 20% (light green). Values are means of three replicates and error bars represent standard deviation.

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## Abbreviations

16S rRNA – 16s ribosomal ribonucleic acid

ABA – Abscisic acid

APX – Ascorbate peroxidase

BSA – Bovine serum albumin

BOX-PCR – BOX-A1R-based repetitive extragenic palindromic – polimerase chain reaction

CAS – Chrome azurol S

CAT – Catalase

CFU – Colony forming units

DTT – Dithiothreitol

DNA – Deoxyribonucleic acid

EDTA – Ethylenediamine tetraacetic acid

EPS – Extracellular polymeric substances

GR – Glutatione reductase

HDTMA – Hexadecyltrimethylammonium bromid

HI – Harvest index

HPSs – Heat shock proteins

IAA – Indole acetic acid

IPCC – Intergovernmental Panel on Climate Change

IST – Induced systemic tolerance

MDA - Malondialdehyde

NBT – Nitro blue tetrazolium

OD – Optical density

PAR – Photosynthetically active radiation

PEG – Polyethylene glycol

Permanova – Permutational multivariate analysis of variance

PGPB – Plant growth promoting bacteria

PVP – polyvinylpyrrolidone

RIDER - Rhizobacterial-induced drought endurance and resilience

ROS – Reactive oxygen species

RPM – Rotation per minute

RUE – Radiation use efficiency

SEM – Standard error of the mean

SOD – Superoxide dismutase

TAE – Tris-acetate-ethylenediamine tetraacetic acid

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TCA – Trichloroacetic acid

U – Unit of enzyme activity

UPGMA – Unweighted pair group method with arithmetic mean

VOCs – Volatile organic compounds

YMA – Yeast mannitol agar

YMB – Yeast mannitol broth

# Chapter I

# General Introduction

**Abstract**

Drought affects crop growth and productivity and is expected to impact over 50% of the arable lands by 2050. Furthermore, its effects will intensify because of global warming, decreasing food production globally. For instance, drought is predicted to reduce 15% of annual yield in maize (*Zea mays* L.), which is one of the most produced and consumed cereal globally. Consequently, there is pressure to improve crop production given that world population is expected to reach 9 billion by 2050. Therefore, it is crucial to find sustainable strategies to enhance drought tolerance in plants, maintaining high yields to assure food availability and security. In fact, plants have natural strategies to withstand drought stress, as well as microorganisms. Additionally, some soil microorganisms interact with plants, mitigating stress. Particularly, plant growth promoting rhizobacteria inhabit plants rhizosphere and have beneficial influence in plant growth and yield though several mechanisms, like alteration in phytohormonal content, antioxidant defense, accumulation of osmolytes, exopolysaccharides production, and volatile organic compounds. Thus, they can be applied as an alternative or a complement to chemical fertilizers and biocides, gradually decreasing the use of synthetic agrochemicals, promoting a sustainable agriculture. Accordingly, there is a need to find more microorganisms with these beneficial characteristics to apply on crops, increasing the global use of biofertilizers. As a result, the main goal of this thesis is to search for bacterial strains with potential to be applied on the field and improve crops productivity.

**Keywords**

Climate change, Drought, Maize, Plant growth promoting rhizobacteria, Biofertilizers

## 1. Drought effects in agriculture production

Crops are influenced by biotic and abiotic stresses. Drought is an abiotic stress and can be defined in various ways, according with the variable considered (Wilhite and Glantz, 1985). Drought associated with periods with lack of precipitation is meteorological drought; drought associated with insufficient water resources for the needs of water use uses of a given water resources management system is hydrological drought; inadequate system of water resources to satisfy water demand is socio-economic drought; and during lack of water in the soil occurs agricultural drought, that can lead to crop failure (Wilhite and Glantz, 1985). In fact, drought is a major threat to crop growth and productivity in the world, and is expected to affect over 50% of the arable lands by 2050 (Vinocur and Altman, 2005). Furthermore, drought effects, severity, and frequency, will be amplified because of global warming, resulting in less production of food globally and a reduction in arable area (IPCC, 2007). This is corroborated in the Intergovernmental Panel on Climate Change (IPCC) Climate Change

2021 report (IPCC, 2021), that states how global temperature is expected to reach or exceed 1.5 °C of warming. Additionally, with a 2 °C increase in global temperature, heat extremes would more often reach critical tolerance thresholds for agriculture and health (IPCC, 2021). In fact, climate change is expected to cause more severe periods of drought in crop lands, affecting cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.) and maize (*Zea mays* L.) crops (IPCC, 2007).

Since world population is increasing and it is expected to reach 9 billion by 2050, there is pressure to keep up with the need for food availability and security (IPCC, 2007). Concern is rising to find strategies to enhance drought tolerance in plants maintaining high yield, like agro-biotechnological approaches, including development of transgenic plants by introducing novel genes, or altering the expression levels of the existing genes (Lu et al., 2013). Another efficient tactic is resorting to genetic engineering and plant breeding in order to create drought tolerant varieties, simultaneously with natural resource management, improving both agriculture productivity and water use efficiency (Warren, 1998). However, this may not be the best solution, because it is hard and long drawn to establish new tolerant varieties due to the complexity of the mechanisms involved in abiotic stress tolerance. Also, there is some hesitation to accept the usefulness of genetically modified plants in some regions of the world (Wahid et al., 2007).

These approaches do not take into consideration the ecological context of the soil environment were the crops are grown (Morrissey et al., 2004). The crops are grown under sterilized conditions, so the results might not translate into practical applications (Ngumbi and Kloepper, 2016). Also, plants are not independent organisms, only regulated by their genetic code and cellular physiology, like considered by classical breeding and genetic engineering (Barrow et al., 2008; Coleman-Derr and Tringe, 2014).

## 2. Maize production and drought effects

Maize (*Zea mays* L.) is one of the most produced and consumed cereal globally, along with rice and wheat (Awika, 2011), and it is the third main food crop in the world in terms of human nutrition (Edmeades, 2008; Olesen et al., 2011). An area of almost 200 million hectares is cultivated each year and almost 800 million tons of grain are harvested, and United States of America are the biggest producers of maize, and Europe produces 11.2% of globally produced maize (FAOSTAT, 2021). In 2019, 70.1 million tons of grain maize were harvested in the Europe Union, 1.1 million tons more than in 2018 (Eurostat, 2021). This crop is especially productive in Southern Europe and Mediterranean (Edmeades, 2008; Olesen et al., 2011). This cereal is used in energy industry for fuel ethanol industry, and to feed livestock (Klopfenstein et al., 2013).

Due to change in the climate conditions, especially drought, it is expected 15% of annual yield losses in maize production globally (Edmeades, 2008). Southern Europe and Mediterranean regions face an increased risk of yield reduction in the production of maize (Edmeades, 2008). In these regions with a Mediterranean-type climate, thus precipitation is rare during maize growing season, irrigation strategies have been used to stabilize and maximize yield (Yang et al., 2017). Climate change amplifies the need for irrigation to mitigate yield reductions due to enhanced crop water stress that prejudices physiological process, for example canopy cover expansion and stomatal functions (Döll, 2002; Fischer et al., 2007; Wolf and Van Diepen, 1995).

Maize is a key crop for agri-food sector in Portugal, occupying the largest area amongst annual crops (Nóbrega, 2006). Since southern Iberia is expected to experience a rise in temperature and a decrease in precipitation due to climate change, this crop production will be affected (IPCC, 2013a). Furthermore, an increase in water demand by other socioeconomic sectors is reducing water availability for agriculture uses (Iglesias et al., 2007; Iglesias and Garrote, 2015).

**3. Drought adaptations**

Plants have natural strategies to grow and survive under drought stress conditions, evidencing drought resistance (Chaves et al., 2003; Levitt, 1972). These strategies can be considered: (1) drought escape when the plant undergoes dormancy during the drought period and the life cycle is completed before the drought period begins (Farooq et al., 2009; Levitt, 1972), (2) drought avoidance and phenotypic flexibility during drought periods when the plant can maintain its normal water status by increasing water assimilation from the soil or decreasing transpiration (Blum, 2005), (3) drought tolerance when the plant is able to maintain usual plant growth and metabolic activities by osmotic adjustment, maintenance of root viability and membrane stability, accumulation of proteins and other metabolites associated with structural stabilization (Huang et al., 2014; Nilsen and Orcutt, 1996). These mechanisms also include morphological adaptations, optimization of water resources, antioxidant systems against reactive oxygen species (ROS) associated to drought, and induction of different stress-responsive genes and proteins (Farooq et al., 2009).

Similarly, drought affects soil microorganisms causing osmotic stress and changes in protein conformation affecting the membrane characteristics through changes in the composition of phospholipid fatty acid, restriction in enzyme efficiency and changes in electron transportation chain leading to accumulation of free radicals (Bérard et al., 2015; Schimel et al., 2007; Vriezen et al., 2007), that in their turn induce protein denaturation and lipid peroxidation, resulting in cell lysis (Potts, 1999). Since soil

microorganisms are constantly in contact with soil water and their membranes are semipermeable, they have to accumulate compatible solutes to decrease their internal water potential when water potential in soil declines, otherwise they face dehydration and death (Schimel et al., 2007). Soil bacteria have several physiological mechanisms to protect cell structure and organelles, for example, accumulation of compatible solutes, production of exopolysaccharides and production of spores (Allison and Martiny, 2008; Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007). Some of the compatible solutes accumulated provide membrane integrity, an increase in thermotolerance of enzymes, and inhibition of thermal denaturation of proteins, and some of them are proline, glycine betaine, and trehalose (Bérard et al., 2015; Conlin and Nelson, 2007; Schimel et al., 2007; Welsh, 2000). There is also synthesis of heat shock proteins (HSPs) which can recognize and bind to other proteins when they are in non-native conformations (Feder and Hofmann, 1999; Hecker et al., 1996), or storage of high quantities of ribosomes to a faster protein synthesis when stress occurs (Placella et al., 2012), and production of extracellular polymeric substances (EPS) that protects, not only the cell, but also their surroundings (Rossi et al., 2012). Lastly, soil bacteria are able to increase resources efficiency and re-allocation within microbial cells (Tiemann and Billings, 2011). Some strategies used by soil bacteria to endure drought stress are similarly used by plants under the same conditions, for example, production of compatible solutes like proline and glycine betaine (Ngumbi and Kloepper, 2016).

**4. Plant Growth Promoting Rhizobacteria**

Some soil microorganisms interact with plants and can provide protection against different stresses, expanding the natural systems plants already have, and increasing growth and yield (Marulanda et al., 2007). This is magnified under stress conditions, as soil microorganisms boost plants metabolic activity to alleviate stress. Especially under stress conditions, microbial communities can keep a moist environment conducive to root development, supply of nutrients, hormones and promote plant growth, that influence biological balance and soil sustainability (Kavamura et al., 2013; Kennedy and Smith, 1995). Some of these soil microorganisms are called rhizobacteria, because they inhabit the rhizosphere or the plant roots, and a few of them are considered plant growth promoting rhizobacteria (PGPR), since they have beneficial influence in plant growth and yield (Kloepper et al., 1989). This effect has been documented in *Arabidopsis thaliana* and other edible plants like lettuce, maize and tomato (García-Fraile et al., 2012; Gholami, A., Shahsavani, S., Nezarat, 2009; Ryu et al., 2005; Schuhegger et al., 2006). PGPR can also protect plants against drought effects as they are adapted to adverse conditions, leading to an increasing in crop productivity in arid or semiarid areas (Kasim

et al., 2013; Kavamura et al., 2013; Marulanda et al., 2007). There are several reports of PGPR able to induce drought tolerance in plants, including wheat, maize, sunflower, sugarcane and green gram (Kasim et al., 2013; Moutia et al., 2010; Sandhya et al., 2009; Saravanakumar et al., 2011; Vardharajula et al., 2011). Also, growth and nutrient efficiency improvement have been reported in maize inoculated with PGPR under water deficit conditions (Pereira et al., 2020).

This beneficial characteristics are common in free living soil bacteria, bacteria living in the rhizosphere or endophytic bacteria, which live inside plant tissues (Bashan and De-Bashan, 2005). Furthermore, it is believed that rhizobacteria worth will be overcome by endophytic bacteria, since these ones are not affected by competition with other microorganisms present in the rhizosphere and can accomplish more profound interaction with plant tissue (Naveed et al., 2014).

These bacteria can be applied as an alternative or a complement to chemical fertilizers and biocides, resulting in a reduction in production and use of agrochemicals, leading to a sustainable crop production (Vurukonda et al., 2016). In fact, since 1990 some PGPR-based products containing mostly strains of *Bacillus* sp. are commercially available in the United States and new products are currently under development (Kloepper et al., 2004). Also, *Azotobacter*, *Azospirillum*, *Bacllius*, *Rhizobium*, *Bradyrhizobium* have been used as PGPR-based fertilizers available in Europe (Artyszak and Gozdowski, 2020; Dal Cortivo et al., 2020; García-Fraile et al., 2017; Mącik et al., 2020; Mustafa et al., 2019). Also, phosphate solubilizers *Bacillus megaterium*, *Frateuria aurantia* and *Rhizophagus irregularis* (Dal Cortivo et al., 2020; García-Fraile et al., 2017), potassium solubilizer *Frateuria aurantia* (García-Fraile et al., 2017), phytostimulator *Pseudomonas azotoformans* (Mustafa et al., 2019), and for biocontrol *Pseudomonas chlororaphis* (Mustafa et al., 2019) have been commercialized and applied in Europe.

**5. Mechanisms of PGPR mediated drought stress tolerance**

Several PGPR possess the ability to alleviate the stress plants undergo during drought, so they have the potential to be used in sustainable agriculture (Vurukonda et al., 2016). These mechanisms involve alteration in phytohormonal content (Khalid et al., 2006), antioxidant defense, accumulation of osmolytes, exopolysaccharides (EPS) production (Vanderlinde et al., 2010), production of heat-shock proteins (HSPs) (Berjak, 2006), dehydrins (Timmusk and Wagner, 1999) and volatile organic compounds (VOCs) (Ryu et al., 2004) (Figure 1).

Yang et al., 2009, has established that enhanced tolerance to abiotic stresses caused by physiological and biochemical changes in plants induced by microorganisms

is designated Induced Systemic Tolerance (IST). Another designation for the process of mitigation of the impact of drought stress on plants by PGPR is rhizobacterial-induced drought endurance and resilience (RIDER) and also includes physiological and biochemical changes (Kaushal and Wani, 2015).

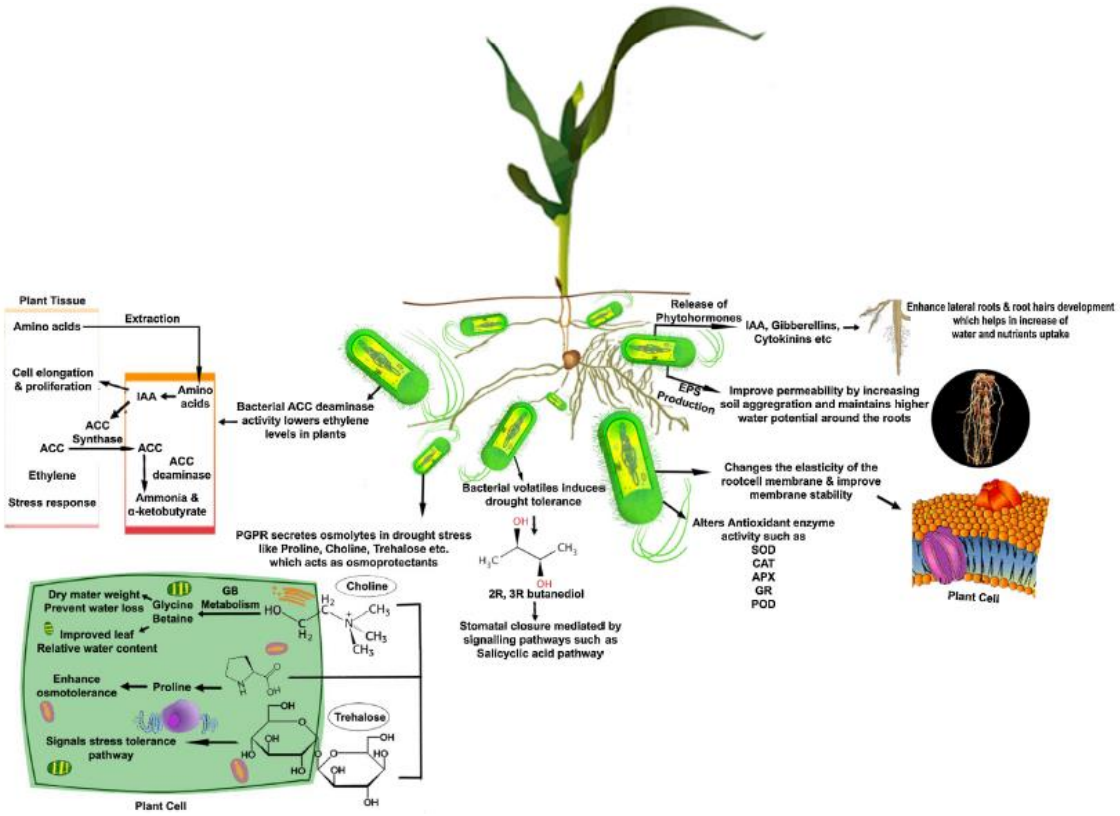


**Figure 1.** Schematic representation of mechanisms of PGPR mediated drought stress tolerance. In: (Vurukonda et al., 2016).

### 5.1. Modification of phytohormonal activity

Plants produce phytohormones, like indole acetic acid (IAA), gibberellins, ethylene, abscisic acid (ABA) and cytokinins with crucial role in their growth and development (Barea and Brown, 1974; Egamberdieva, 2013; Teale et al., 2006) (Barea and Brown, 1974; Teale et al., 2006). Some also have influence during periods of stress caused by the environmental conditions, helping plants survive (Fahad et al., 2015; Skirycz and Inzé, 2010). Similarly, PGPRs can synthetize phytohormones that stimulate plant growth and tolerance against abiotic stresses (Glick, 2012).

IAA is the most active auxin in plant growth and development and, when a plant is inoculated with bacteria that can produce IAA, it causes an increased root growth and/or enhanced formation of lateral roots hairs (Dimpka et al., 2009). This helps plants cope with water deficit, by increasing water and nutrient uptake (Egamberdieva and Kucharova, 2009; Mantelin, 2003). For instance, inoculation of *Azospirillum brasilense*, which produces nitric oxide, a signaling molecule in the IAA pathway, in maize seedlings

improved relative and absolute water contents when compared to non-inoculated plants. These bacteria increased the root growth, biomass, foliar area, and proline accumulation in leaves and roots, despite it dropped the water potential. These effects were more significant at 75% reduction in water supply, compared to 50% reduction (Casanovas et al., 2002).

ABA controls stomatal closure and stress signal transduction pathways, therefore, regulates water loss (Yamaguchi-Shinozaki and Shinozaki, 1994). The production of this stress hormone is induced by cell dehydration during water deficit condition (Kaushal and Wani, 2015). The inoculation of *Azospirillum lipoferum*, which produces ABA and gibberellins, alleviated drought stress in maize plants (Cohen et al., 2009).

### 5.2. Antioxidant defenses

Oxygen, hydrogen peroxide and hydroxyl radicals are reactive oxygen species (ROS) produced in organelles at low levels in normal situations of plant growth (Apel and Hirt, 2004). However, when cells are under stress conditions due to water deficit, photosynthetic machinery is disrupted and photorespiration increases, leading to an enhanced production of ROS. Subsequently, excess of ROS results in enhanced lipid peroxidation, damaging proteins, DNA, and lipids (Pompelli et al., 2010). Nevertheless, ROS act as a signal pathway for the activation of stress-response and defense pathways (Pitzschke et al., 2006). Thus, it is imperative to maintain balance between ROS production and ROS scavenging systems.

To deal with the oxidative damage that occurs during drought, plants are equipped with antioxidant defense mechanisms scavenging ROS (Miller et al. 2010). These mechanisms can be performed by enzymatic components, like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR); and non-enzymatic components including cysteine, glutathione, and ascorbic acid (Kaushal and Wani, 2015).

Oxidative stress tolerance in plants has been linked to high activities of antioxidant enzymes (Štajner et al., 1997). Furthermore, inoculation of plants with PGPR reduces the damage on antioxidant enzymes activity caused by drought stress (Han and Lee, 2005). For instance, a significant reduction of malondialdehyde (MDA) content was observed in PGPR-inoculated seedlings when comparing with control condition under water stress (Gontia-Mishra et al., 2016). Likewise, maize plants reduced the activity of APX and GPX, developing protection against drought stress, when inoculated with *Bacillus* species (Vardharajula et al., 2011). Furthermore, salt-treated maize seedlings inoculated with *Bacillus aquimaris* DY-3 had a significant increase SOD activity when compared to control condition (Li and Jiang, 2017). Also, SOD activity was higher in

maize inoculated with *Pseudomonas aeruginosa* under drought stress conditions (Naseem and Bano, 2014).

### 5.3. Accumulation of osmolytes

Drought leads to cell turgidity losses, so both plants and bacteria need to adjust osmotically, by accumulating osmolytes, including proline, glycine betaine and trehalose (Chen et al., 2007; Rodríguez-Salazar et al., 2009; Sakamoto & Murata, 2002; Vendruscolo et al., 2007). They protect membrane integrity, preventing protein denaturation (Farooq et al., 2009; Hoekstra et al., 2001).

During changes due to osmotic adjustments, hydrolysis of proteins, and subsequently, accumulation of amino acids occurs (Iqbal et al., 2011; Krasensky and Jonak, 2012). Therefore, elevated levels of amino acids are considered an indicator of drought stress (Zhu, 2002), and have been reported in sorghum, pepper and wheat (Yadav et al., 1995). Also, the synthesis of proline is associated not only with osmotic adjustment, but also free radical scavenging and stabilization of subcellular structures in plant cells to overcome drought impacts (Hare et al., 1998).

In fact, glycine betaine improved growth of maize plants under drought stress (Agboma et al., 1997). Similarly, an accumulation of glycine betaine has been reported in maize inoculated with *Pseudomonas* genera isolates in greenhouse conditions (Gou et al., 2015). Also, *Azospirillum brasilense* improved drought tolerance and biomass in maize through trehalose accumulation (Rodrí-guez-Salazar et al., 2009). Furthermore, maize inoculated with *Azospirillum* sp. has an increased proline content when subjected to drought (García et al., 2017). This was also observed in maize inoculated with *Bacillus* sp. (Vardharajula et al., 2011), and maize inoculated with *Pseudomonas* (Sandhya et al., 2010). *Pseudomonas* spp. degrades the starch, contributing to biosynthesis and consequential osmotic adjustment resulting is stress effects mitigation (Sandhya et al., 2010). This strain helps to hydrolyze starch, increasing sugar availability which contributes for osmotic adjustment, alleviating the effects of drought stress (Naseem and Bano, 2014). As previously mentioned, soluble sugars are important osmolytes for osmotic adjustment in plants under drought stress (Dekánková et al., 2004).

### 5.4. Production of EPS

EPS are composed of 97% water enclosed in a polymer matrix to prevent desiccation (Bhaskar and Bhosle, 2005). EPS enhance microaggregates that increase its stability and root-adhering soil / root tissue ratio, resulting in increased uptake of water and nutrients and enhancing plant growth and tolerance to drought stress (Vardharajula et al., 2011).

It has been reported that when inoculated with EPS-producing bacteria, maize plants tolerated drought stress, decreasing activity of APX, CAT and GPX enzymes (Naseem and Bano, 2014).

### 5.5. Volatile compounds production

When plants are under stress occurs synthesis of volatiles to signal the development of priming and systemic responses within the plant itself and others surrounding it (Choudhary et al., 2008; Heil and Silva Bueno, 2007; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Niinemets, 2010). Timmusk et al. (2014) considers the use of volatiles to assess crop drought stress and its mitigation as a promising rapid and non-invasive technique.

An improvement in plant stress tolerance associated with bacterial inoculation has been reported (Timmusk et al., 2014). For instance, wheat seedlings under drought stress were treated with *Bacillus thuringiensis* AZP2, causing increase in plant biomass, higher photosynthesis, and reduced emission of volatiles resulting in higher survival (Timmusk et al., 2014).

Similarly, the volatile 2R,3R-Butanediol, produced by *Pseudomonas chlororaphis* O6 alleviates drought stress in *Arabidopsis thaliana*, in comparison with inoculation with bacteria deficient in 2R,3R-Butanediol (Cho et al., 2008). Also, this volatile induced tolerance to drought stress through a mechanism dependent of salicylic acid (SA) (Cho et al., 2008).

Likewise, *Bacillus subtilis* induces systemic tolerance to plants against salinity, since it produces VOCs with the ability to induce tissue-specific gene regulation of high affinity $K^+$ transporter which restricts $Na^+$ uptake in roots and increases shoot to root translocation (Zhang et al., 2008)

## 6. Biofertilizers

Since the Green Revolution synthetic fertilizers, pesticides, and other agrochemicals were applied to enhance crop productivity (Kesavan and Swaminathan, 2018), but their overuse has led to deterioration of biological and physicochemical conditions of arable soil, which in turns has been affecting agriculture productivity over the past few decades (Pingali, 2012). In this context of shrinkage of land resources, it is important to promote sustainable agriculture and gradually decrease the use of synthetic agrochemicals. Biofertilizers or bioinoculants have been emerging as an eco-friendly alternative to mitigate the damaging effects of synthetic agrochemicals whilst promoting plant growth, tolerance to environmental stresses, and disease control, ensuring high agriculture productivity and improving soil health (Souza et al., 2015). The process of synthesis of biofertilizers is schematized in Figure 2, and begins with isolation of potential

candidates, followed by laboratory and greenhouse screening and field evaluation (Basu et al., 2021). In fact, some microorganisms have been used to make bacterial inoculants. *Azospirillum* bacteria have been used to coat maize seeds that were commercialized (Reis, 2007), and *Rhizobium* inoculation on different cereal crops, as rice, maize, and wheat, has had positive effects (Mia and Shamsuddin, 2010). Bacteria from genera *Bacillus, Pseudomonas*, and microorganisms like mycorrhiza, *Trichoderma* and yeast have been used as bioinoculants (Aremu et al., 2017; Tahir et al., 2017). Therefore, it is important find more microorganisms with characteristics considered as plant growth promoters to apply on crops and improve global use of biofertilizers, and rhizosphere is rich in microorganisms with potential to be used in developing bio-inoculants for enhancement of growth and yield of crop plants (Joshi and Bhatt, 2011). This approach leads to a sustainable agriculture, maintaining and improving human health, benefiting the environment, responding to a global increase of food production to feed the growing human population (Bishnoi, 2015).

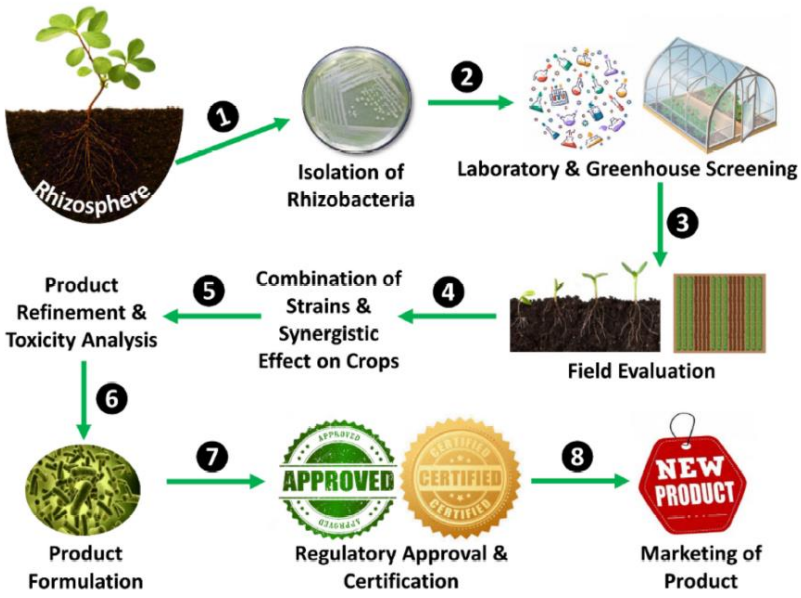


**Figure 2.** Steps in the development and commercialization of plant growth promoting rhizobacteria (PGPR)-based biofertilizers. In: (Basu et al., 2021).

## 7. Objectives and thesis outline

All United Nations Member States adopted in 2015 the 2030 Agenda for Sustainable Development, in which 17 Sustainable Development Goals (SDGs) resume how it is important to end poverty while simultaneously improve health and education, reducing inequality and increasing economic growth, meanwhile responding to the climate change crisis ahead, preserving oceans and forests (United Nations, 2015).

This thesis can be associated with four out of the seventeen goals: Zero Hunger, Responsible Consumption and Production, Climate Action, and Life on Land (Figure 3).

Briefly, Zero Hunger aims to end hunger, achieving food security and improved nutrition, promoting sustainable agriculture; Responsible Consumption and Production seeks to ensure sustainable consumption and production patterns; Climate Action wants to take urgent action to combat climate change and its impacts, Life on Land aims to protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss (United Nations, 2015).


**Figure 3.** United Nations Sustainable Development Goals related to this thesis: Zero Hunger, Responsible Consumption and Production, Climate Action and Life on Land In: (United Nations, 2015).

The main goal of this thesis is to characterize bacterial strains with potential to be applied on the field and improve crops productivity. Thus, several steps of the development of PGPR-based biofertilizers were employed.

**Chapter I** encapsulates a broad view on environmental changes affecting crop production, especially drought effects on maize production, and solutions to respond to this problem. Also, microorganisms considered plant growth promoting rhizobacteria are mentioned as they are important biofertilizers.

**Chapter II** is a study on both greenhouse and field, applying previously characterized rhizobacteria, isolated from wild-legumes plants in Portugal, to test if any of them could potentially be applied on maize crops to improve productivity.

In **Chapter III** bacteria isolated from plants from Angola are applied on growing maize under drought stress in greenhouse conditions to determine if these bacteria could alleviate drought stress. Then, the bacteria are also tested on maize growing on the field.

**Chapter IV** explores the diversity of bacteria isolated from maize roots growing in the field under drought stress conditions and characterize these bacteria on plant growth promoting capacity.

**Chapter V** provides general conclusions and suggests potential future work to further investigate these topics.

# Chapter II

# Growth promotion of maize by bacteria – a lab and field study

**Abstract**

Maize (*Zea mays L.*) is the third most consumed and produced cereal in the world. Since this crop is affected by temperature and water availability, its yield production is expected to be reduced by 15% due to drought intensification because of climate change and global warming. In response to this problem, conventional breeding and plant engineering have been used to develop new tolerant varieties. However, these strategies are time-consuming, cost and labor-intensive, and can potentially impact the environment. Therefore, sustainable, and more approachable alternatives are needed. In fact, inoculation of maize with plant growth promoting rhizobacteria have been reported as an effective solution, improving growth and mitigate stress caused by biotic and abiotic stresses. Hence, in this study, bacterial strains were screened to find potential candidates to apply in maize crops. Previously characterized bacteria were firstly tested in a greenhouse, then the best candidates T1 and T7, which lead to the highest increase in maize shoot and root dry weight, were inoculated in the field. Maize productivity was assessed, as well as biochemical parameters to better understand strains effect on maize growth. In the field trial, no significant difference in yield was observed when maize was inoculated with these strains. However biochemical parameters show the positive effect of bacterial inoculation in maize, with an increase in protein content and soluble sugars, and a decrease in lipid peroxidation when maize was inoculated with the bacterial strains.

**Keywords**

Climate change, maize, plant growth promoting rhizobacteria, biochemical analysis, greenhouse, field

## 1. Introduction

Maize (*Zea mays* L.) is one of the most consumed and produced cereals globally, along with rice and wheat (Awika, 2011). An area of almost 200 million hectares is cultivated each year and almost 800 million tons of grain are harvested (FAOSTAT, 2021). According to FAOSTAT (2021), United States of America are the biggest producers of maize, and Europe produces 11.2% of globally produced maize. In 2019, 70.1 million tons of grain maize where harvested in the Europe Union, 1.1 million tons more than in 2018 (Eurostat, 2021). This cereal is also used in energy industry for fuel ethanol industry, and to feed livestock (Klopfenstein et al., 2013).

The production of these crops is influenced by abiotic factors, like temperature and water availability (Awika, 2011). Due to climate changes, and especially the increase in frequency and intensity of droughts, 15% of annual yield losses in maize production globally are expected (Edmeades, 2008). To mitigate the effects of water scarcity on

maize production new tolerant varieties are being developed through conventional breeding and plant engineering (Ashraf, 2010; Atkinson and Urwin, 2012; Bakhsh and Hussain, 2015). Another strategy has been to apply inorganic and organic chemicals, including osmoprotectants and plant hormones (Travaglia et al., 2010). However, these approaches have some disadvantages, as they are time-consuming, cost and labor-intensive, and transgenic genes may unwantedly be transferred to the environment (Atkinson and Urwin, 2012).

Thus, there is a need for alternative solutions, which are more sustainable and easier to apply. The inoculation of beneficial microorganisms in plants to improve growth and mitigate stress caused by biotic and abiotic stresses has been considered (Dimpka et al., 2009). Some reports have shown that inoculation of maize with plant growth-promoting rhizobacteria (PGPR) increases plant height, plant dry weight, root length and weight, yield, leaf area, and nutrient uptake (Biari et al., 2008; Sachin, 2009; Yazdani et al., 2009). These results have been demonstrated both in greenhouse and filed conditions. Youseif (2018), reports shoot height and root length increase, shoot and root fresh and dry weigh increase, associated with inoculation of maize plants with rhizobacteria in greenhouse trials. Similarly, Zhao et al. (2014), reports increase in leaf area, length, and shoot and root dry weight, after inoculation of maize plants with rhizobacteria in greenhouse conditions. Likewise, Breedt et al. (2017), reports an increase in maize productivity in the field associated with application of rhizobacteria able to perform biological nitrogen fixation (BNF) and acid indole acetic (IAA) production. Similarly, Di Salvo et al. (2018), reports higher grain yield due to application of bacteria strains able to produce IAA and solubilize phosphate in a field study.

This study fits in the first stages in the development of commercial formulations of bioinoculants to increase agricultural productivity. It aims to screen bacterial strains isolated from nodules of wild legume species, testing their ability to enhance growth and yield in maize crop, both in growth chamber and under field conditions. To better comprehend maize response to bacterial strains inoculation on the field trial, several biochemical endpoints were assessed.

## 2. Materials and Methods

### 2.1. Bacterial Strains

In this study, bacterial strain isolated in Spring 2015 from nodules of wild legume species (*Ornithopus compressus* L., *Medicago lupulina* L., *Scorpiurus vermiculatus* L., *Vicia sativa* subsp. *sativa* L., *O. sativus* subsp. *sativus* Brot., *V. benghalensis* L., *O. pinnatus* (Miller) Druce, *Lotus corniculatus* L. *and Medicago* sp.) were used (Cardoso et al., 2018). The endophytic bacteria were isolated from the root nodules wild legumes growing in

four sites in Continental Portugal, Aljustrel (37 55 49.127 N 8 06 26.485 W), Alvito (38 16 20.447 N 008 00 08.377 W), Murtosa (40 46 28.907 N 008 38 51.865 W), and Vale de Cambra (0 51 09.113 N 008 18 32.222 W), in a previous work (Cardoso et al., 2018). Most of the strains belonged to genera *Flavobacterium* or *Pseudomonas*, and the remaining strains belong to different genera, *Erwinia*, *Herbaspirillum*, *Variovorax*, *Acinetobacter*, *Agrobacterium/Rhizobium*, and *Paenibacillus* (Cardoso et al., 2018). *Rhizobium* sp. strain E20-8 previously isolated from root nodules of *Pisum sativum* L. (Figueira, 2000) was also tested. Bacterial strains used are mentioned in the supplementary table 1.

### 2.2. Greenhouse trial procedure

A greenhouse trial was made to test if bacterial strains could promote growth of maize plants. Plastic pots of 200 mL were filled with a mixture (2:1) of autoclaved sand, previously washed, and peat. In each pot, three *Zea mays* (Dekalb DKC 6031) seeds were sown. Pots were regularly irrigated with tap water when substrate on top was dry. After 12 days, seeds which did not germinate properly were removed, leaving pots with two plants and pots with one plant.

After 12 days of seedling germination, to test for bacterial ability to promote growth, pots were inoculated with 2 mL of each bacterial strain per plant, previously grown in 8 mL yeast mannitol broth (YBM) for 24 h at 26 °C and 150 rotations per minute (rpm) on an orbital shaker. Optical density (OD) was measured at 620 nm to determine bacterial growth (Supplementary Table 1). The bacterial cultures were applied on the base of the stem. Control was inoculated with 2 mL of growth medium. For control condition, six replicates were considered, and for each bacterial strain, there were three replicates.

Plants were grown for a total of 28 days in greenhouse conditions at approximately 17 ± 2 °C during the day and 13 ± 2 °C during the night, at natural light with a 12 h light / 12 h dark cycle. However, plants grew in control condition and in the presence of bacteria for 16 days.

After the test, plants were washed and dried on a greenhouse at 60 °C until weight was constant. Then, shoot and root were weighted separately. Dry weight was used to assess the ability of each strain to promote growth compared to the control condition.

### 2.3. Field trial procedure

To assess the effects of the bacterial strains on a more realistic scenario, a field trial was established. The experiment was conducted from 05/23/2019 to 08/31/2019 at INOVMILHO - Centro Nacional de Competências das Culturas do Milho e Sorgo Estação Experimental António Teixeira (INIAV), 38º56’28.32’’N/8º30’36.66’’W.

The best strains T1 and T7, which lead to the highest increase in maize shoot and root weight in the greenhouse trial previously performed were inoculated in the field. Also, I9, a *Pseudomonas*, was mixed with T1 and added in the field test, since it produces alginate (Cardoso et al., 2018; Sá et al., 2019). Accordingly, selected bacteria during the greenhouse trial were grown in plates containing yeast extract mannitol agar (YMA) medium (Somasegaran and Hoben, 1994) during 2 to 3 days at 26 °C . Then, bacterial strains were grown in tubes containing 5 mL of yeast broth mannitol (YMB) medium (Somasegaran and Hoben, 1994). Inoculated tubes were incubated at 26 °C in an orbital shaker (150 rpm), for 4 days. Then, to prepare an additional pre-inoculum, tube's content was poured into 90 mL flasks, incubated for 3 days at 26 °C in an orbital shaker (150 rpm). Pre-inoculum growth was determined by measuring OD at 620 nm. The final inoculum for the field inoculation was prepared using containers of 20 L, only filled with 9 L of culture medium and 90 mL of the previously pre-inoculum. Compressed air was diffused into the containers using sterile diffusers, emerged in the medium. Containers were sealed with sterile cotton, and this material was also placed inside the tubes, which guaranteed sterile conditions. Bacteria were grown for 3 days. During all the procedure, inoculum was always grown in aseptic condition. Inoculum growth was determined by measuring OD at 620 nm (Supplementary Table 2).

Maize was previously sown in the field, with application of nitrogen, but no herbicide. There were three blocks, in a total area of 540 $m^2$. Each block had a length of 10 m and 4.5 m of width. Each block had a unit for control condition and the others were for the other conditions being tested. In each unit, 6 lines, each with 70 plants, were sown, with 13 to 16 cm in between plants. Of the six lines seeded in each block only the central two 16-day old plants were inoculated with 9 L of culture medium with grown bacteria by pulverization with a knapsack sprayer (Preininger et al., 2018).

For each condition there were three replicates in a randomized complete block design. Plants were irrigated by center pivot irrigation. Leaf samples were collected on four different stages of maize development, on 06/05/2019, 07/05/2019, 07/31/2019, and 08/31/2019, to perform biochemical tests.

At the end of the trial, maize plants were harvested per replicate from each condition, from only the two inner rows at approximately 14% grain moisture. After harvesting, the average grain moisture content was determined to calculate the grain yield mass according to a standard of 14% moisture content. Results include thousand grains weight and 1 $m^2$ grain weight considering 14% moisture content and not considering it.

### 2.4. Biochemical analysis

#### 2.4.1. Protein content

Frozen samples were homogenized using mortar and pestle in 600 µL of Tris extraction buffer, constituted by 0.1 M Tris-HCl pH 8.5, 15% (w/v) polyvinylpyrrolidone (PVP), 153 µM magnesium sulfate (MgSO4) and 0.2% (v/v) Triton X-100. After centrifugation, for 20 min at 10 000 *g*, protein content was determined in the supernatant by the Biuret method (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used as a standard (1.25 to 10 mg $mL^{-1}$). Sample or standard (25 µL) was mixed with 300 µL of Biuret reagent and incubated in the dark for 10 min at 30 ºC. The amount of protein was determined spectrophotometrically at 540 nm. Final concentration was calculated using standards curve and results were expressed in mg / g fresh weight.

#### 2.4.2. Starch content

Samples were extracted by homogenization with mortar and pestle in phosphate buffer, which consists of 50 mM potassium phosphate (pH = 7), 1mM ethylenediamine tetraacetic acid (EDTA), 1% (v/v) Triton X-100, 1mM dithiothreitol (DTT). After centrifugation, for 20 min at 10 000 *g*, starch content was determined in the supernatant according to the methodology described by DuBois et al. (1956). Sample or standard (10 µL) was mixed with 100 µL of phenol 5% (v/v) and 600 µL of 98% sulfuric acid ($H_2SO_4$) and incubated for 30 min at room temperature in the dark. Then, 300 µL of mix was pipetted to a microplate and absorbance was read at 492 nm. Final concentration was calculated using standards curve and results were expressed in mg / g fresh weight.

#### 2.4.3. Soluble sugars content

Soluble sugars content was determined in the supernatant according to the methodology described by DuBois et al. (1956). Samples were extracted in phosphate buffer, composed of 50 mM potassium phosphate (pH = 7), 1mM EDTA, 1% (v/v) Triton X-100, 1mM DTT. After centrifugation for 20 min at 10 000 *g*, 10 µL of sample or standard was mixed with 100 µL of phenol 5% (v/v) and 600 µL of 98% sulfuric acid ($H_2SO_4$) and incubated for 30 min at room temperature in the dark. Then, 300 µL of the mix were transferred to a microplate and absorbance was read at 492 nm. The standard curve was used to calculate the final concentration, expressed in mg / g fresh weight.

#### 2.4.4. Lipid peroxidation

Frozen samples were milled using liquid nitrogen. Samples were extracted mixing 0.5 g of maize leaf and 1 mL of 20% (v/v) trichloroacetic acid (TCA) solution (1:2 w/v). The homogenate was centrifuged at 10 000 *g* for 20 min at 4 ºC. Supernatant (sample) was collected and used for thiobarbituric acid reactive substances (TBARS) according with (Buege and Aust, 1978). Supernatant (50 µL) was mixed with 200 µL of thiobarbituric

acid (TBA) solution (0.5% TBA dissolved in 20% TCA), and 150 µL TCA. Blank consisted of 200 µL TCA and 200 µL TBA. Microtubes containing mixes were vortexed and incubated at 96 ºC for 25 minutes, then they were transferred to ice to stop the reaction. Then, 300 µL of the mix were transferred to a microplate and absorbances were measured at 532 and 750 nm using a microplate reader. Lipid peroxidation was calculated using the extinction coefficient of malondialdehyde (MDA) ($1.56 \times 105\ M^{-1}\ cm^{-1}$). Results were expressed in mili Mol of MDA / g of fresh weight (mM / g).

### 2.4.5. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by the reaction of nitro blue tetrazolium (NBT) with superoxide radicals to form NBT diformazan (Beauchamp and Fridovich, 1971). Samples were extracted in phosphate buffer, which consists of 50 mM potassium phosphate (pH = 7), 1mM EDTA, 1% (v/v) Triton X-100, 1mM DTT. Supernatant (25 µL) was mixed with 25 µL of xanthine oxidase (51.6 mU / ml) and 250 µL of NBT reaction buffer (68.4 µM NBT in 50 mM Tris-HCl (pH 8), 0.1 mM DTPA, 0.1 mM hypoxanthine) and incubated for 20 min at room temperature in an orbital incubator. Absorbance was measured at 560 nm. Results were expressed in unit per mg of fresh weight (U / mg). One unit of enzyme activity (U) corresponds to 50% reduction of NBT.

## 2.5. Statistical analysis

Data from maize shoot and root dry weight in the greenhouse, maize field yield, and biochemical performance was analyzed performing permutational multivariate analysis of variance (Permanova+) tests, using Primer (PRIMER-e, Plymouth), considering differences significant when p-value ≤ 0.05.

# 3. Results

## 3.1. Greenhouse maize dry weight

When maize plants were grown under watered conditions in the greenhouse test, there was no significant difference between shoot and root dry weight when compared to the control (Figure 1). A total of 25 bacterial strains were considered in pots with two maize plants, and 15 (60%) of them had a higher shoot dry weight relative to the control non inoculated maize plants (Figure 1A), and 13 (52%) of them had a higher shoot dry weight relative to the control non inoculated maize plants (Figure 1B). A total of 39 bacterial strains were considered in pots with two maize plants, and only 9 (23%) of them had a higher shoot dry weight relative to the control non inoculated maize plants (Figure 1C), and 18 (46%) of them had a higher shoot dry weight relative to the control non inoculated maize plants (Figure 1D).

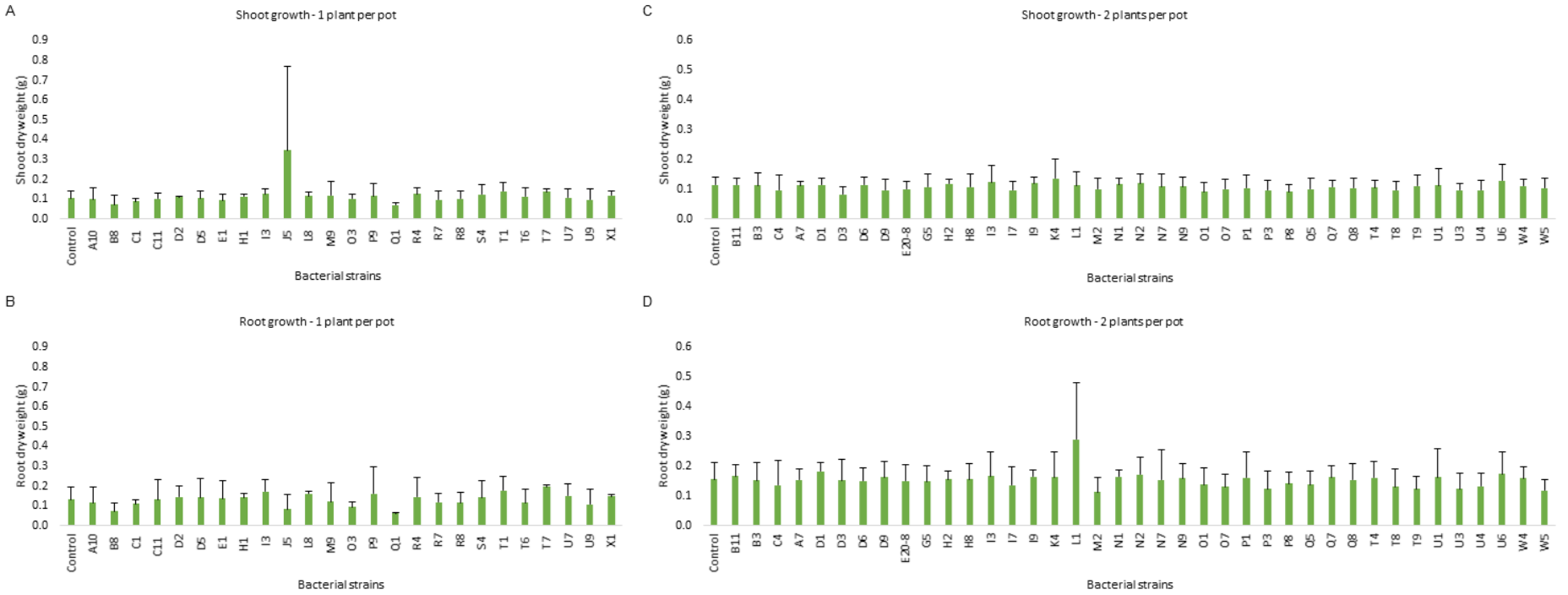


**Figure 1.** Maize growth represented as shoot and root dry weight under greenhouse conditions. A) Shoot growth of maize considering one plant per pot. B) Root growth of maize considering one plant per pot. C) Shoot growth of maize considering two plants per pot. D) Root growth of maize considering two plants per pot. Error bars represent standard deviation. Permanova+ tests to shoot and root weight were performed using Primer (PRIMER-e, Plymouth), no significant difference was observed.

T1 and T7 strains had the highest increase in shoot dry weight relative to the control, respectively 25.6 and 23.3% (Figure 1A). Similarly, both strains had the highest increase in root dry weight relative to the control, respectively 25.8 and 32.7% (Figure 1B). Thus, these strains were selected to further test in the field trial. I9 was also tested in the field, despite it only had a 5.4 and 6.3% increase in shoot and root weight, respectively (Figure 1C and Figure 1D), because it has been reported that this strain produces alginate (Cardoso et al., 2018; Sá et al., 2019). This strain was mixed with T1.

### 3.2. Field productivity

Maize productivity was determined considering thousand grains weight and 1 $m^2$ grain weight considering 14% moisture content and not contemplating it. In all the parameters considered, thousand grains weight (Figure 2A), grains weight per $m^2$ (Figure 2B), thousand grains weight considering 14% humidity (Figure 2C), and grains weight per $m^2$ considering 14% humidity (Figure 2D), there was no significant difference when comparing inoculated maize weight and control condition (Figure 2).

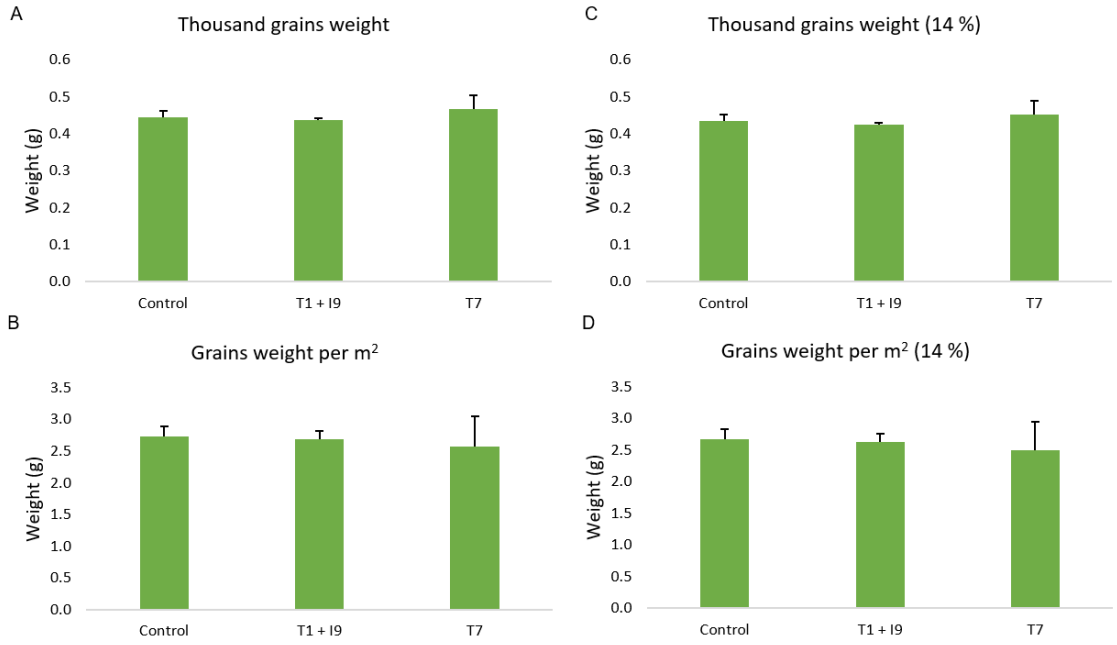


**Figure 2.** Field productivity. A) Thousand grains weight. B) Grains weight per $m^2$. C) Thousand grains weight considering 14% humidity. D) Grains weight per $m^2$ considering 14% humidity. Error bars represent the standard deviation. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth), no significant difference was observed.

### 3.3. Biochemical analysis

#### 3.3.1. Protein content

In the first sampling, protein content was higher on maize inoculated with T7, than maize inoculated with T1 + I9 and maize not inoculated (control), but the standard deviation of results is big. T1 + I9 also lead to a slight increase in protein content relative to control,

although control had a considerable standard deviation. In the second sampling the same tendency was observed, but standard deviation was smaller in control and T7, and larger in T1 + I9. In the third sampling, protein content was lower in every condition relative to previous sampling stages. T1 + I9 had the highest protein content, but all the standard deviation bars are large. In the fourth sampling, the values of protein content were like those observed in the first and second sampling for T1 + I9 and T7 with relatively small standard deviation bars, and protein content was the highest in control condition, however standard deviation bar was larger. None of these differences were significant (Figure 3A).

### 3.3.2. Starch content

In the first sampling, starch content was higher on maize not inoculated and inoculated with T7, despite values obtained in T7 are slightly lower than control, and maize inoculated with T1 + I9 had the lowest starch content. In the second sampling, all three conditions had similar values. A decrease was observed in starch content when comparing control and T7 values to the first sampling, and an increase was determined in starch content when maize was inoculated with T1 + I9 relative to the first sampling. In the third sampling starch content increased from previous samplings in every condition. Starch content was higher in the control, followed by T1 + I9 and lastly T7 condition. In the fourth sampling, starch content was similar in the three conditions, and control values were slightly lower and T7 were slightly higher relative to the third sampling. None of these differences were significant (Figure 3B).

### 3.3.3. Soluble sugars content

In the first sampling, soluble sugars content was higher in maize inoculated with T1 + I9 and T7 relative to the control. Contrarily, in the second sampling, a decrease was observed relative to the control with both inoculations. In the third sampling, soluble sugars content was slightly higher when maize was inoculated with T1+ I9 and slightly lower when maize was inoculated with T7. Also, an increase in soluble sugars is observed in all the three conditions relative to prior samplings. In the fourth sampling, soluble sugars content was lower when maize was inoculated with T1+ I9 and T7 (Figure 3C).

### 3.3.4. Lipid peroxidation

In the first sampling, lipid peroxidation was significantly lower when maize was inoculated with T7 compared to maize not inoculated (control), similarly lipid peroxidation was lower when maize was inoculated with T1 + I9 relative to the control, however this decrease was not significant. Contrarily, in the second sampling, lipid peroxidation was higher in T7 compared to control and T1 + I9 had similar lipid peroxidation values. Also, lipid

peroxidation was lower for control condition in the second sampling compared to the first sampling and the opposite was observed in maize inoculated with T7. Similarly, to the first sampling, in the third sampling, lipid peroxidation was lower when maize was inoculated with T7 compared to the control, and it was lower when maize was inoculated with T1 + I9 relative to the control, however these differences were not significant, and the values were lower than those obtained in the first sampling. In the fourth sampling, lipid peroxidation was lower in both T1 + I9 and T7 conditions relative to the control, yet control condition had a large standard deviation (Figure 3D).

### 3.3.5. Superoxide dismutase (SOD) activity

In the first sampling, superoxide dismutase (SOD) activity was similar in all the three conditions considered, but standard deviation was larger in T1 + I9 and T7 relative to the control. In the second sampling, all values were slightly lower than in the first sampling. SOD activity was significantly lower in T7 compared to control condition, and a decrease was also observed in the T1 + I9 relative to the control, but this difference was not significant. In the third sampling, all values were slightly higher than in the second sampling, and SOD activity was similar in all the three conditions. In the fourth sampling, SOD activity was lower in the control condition compared to values determined in the previous samplings, and it was slightly higher in both T1 + I9 and T7 compared to the control (Figure 3E).

## 4. Discussion

In the current study, previously characterized bacteria (Cardoso et al., 2018) were screened to select possible candidates which potential to promote maize growth. Despite the difference between shoot and root dry weight was not significant when comparing inoculated conditions to the control, T1 and T7 strains had the highest increase in weight relative to the uninoculated plants. These bacteria belong to the genera *Pseudomonas*, and *Flavobacterium*, respectively (Cardoso et al., 2018). A significant increase in shoot and root dry weight of maize seedlings has been previously reported *in vitro* on maize inoculated with *Pseudomonas* as well as *Bacillus* (Almaghrabi et al., 2014). Also, *Flavobacterium* sp. strain NGB-31 inoculation in maize lead to an increase in root length, and maize inoculated with this strain had an increase in shoot and root fresh and dry weights under greenhouse conditions (Youseif, 2018). Similarly, an increase in aerial biomass was observed in maize plants inoculated with *Pseudomonas fluorescens and P. putida* and the highest underground biomass was obtained when maize was inoculated with *Azospirillum lipoferum* in a greenhouse test (Noumavo et al., 2013).

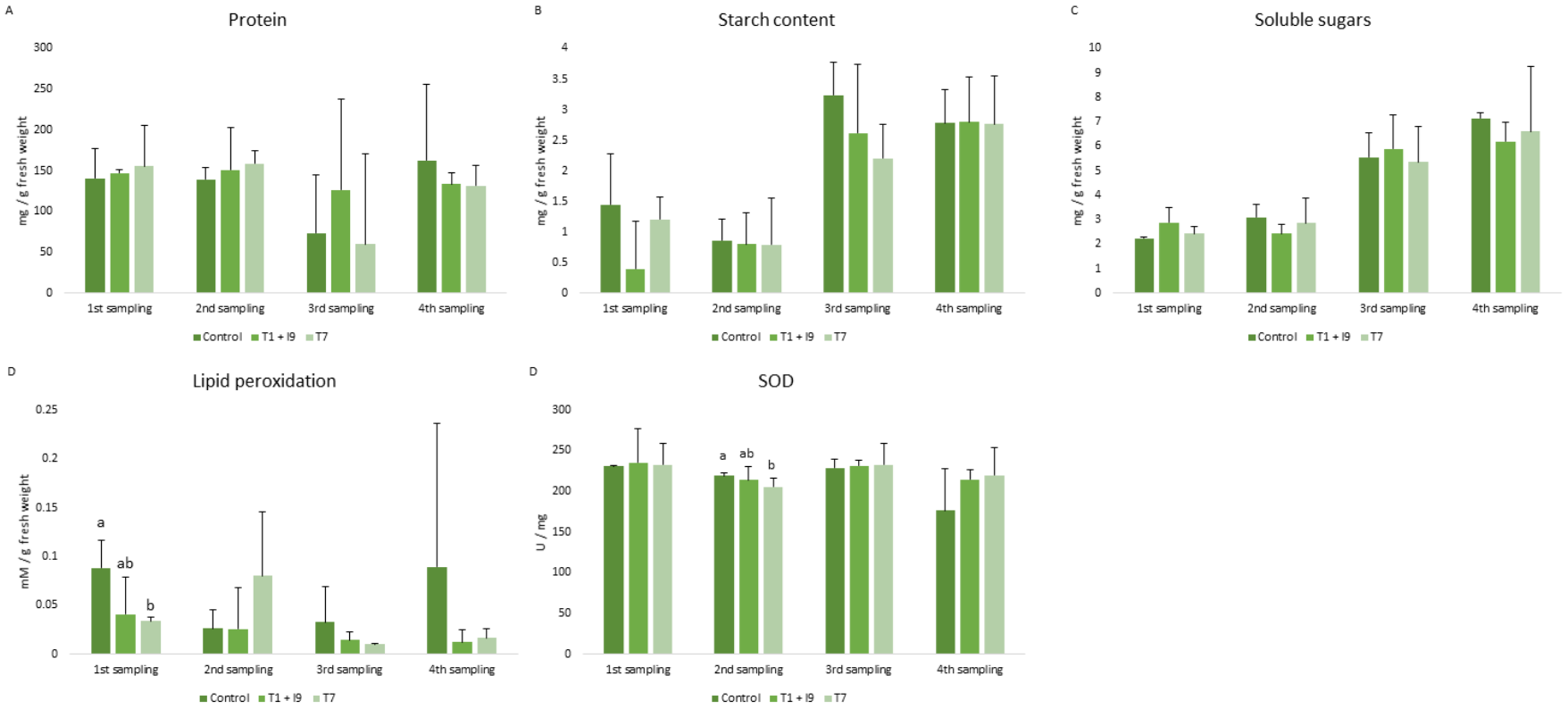


**Figure 3.** Biochemical analysis of maize plants grown in control (dark green) and bacterial consortium (light green) condition, in four different sampling stages. A) Protein content results were expressed in mg / g fresh weight. B) Starch content results were expressed in mg / g fresh weight. C) Soluble sugars results were expressed in mg / g fresh weight. D) Lipid peroxidation results were expressed in mili Mol of MDA / g of fresh weight (mM / g). E) Superoxide dismutase (SOD) activity results were expressed in unit per mg of fresh weight (U / mg). Error bars represent the standard deviation. Permanova+ tests to biochemical parameters were performed using Primer (PRIMER-e, Plymouth). Different lowercase letters indicate significant differences in each sampling stage among conditions tested (p-value < 0.05).

Additionally, it was observed an increase of 32.7% in root dry weight of maize inoculated with T7 relative to the control. This may be explained by the ability of this strain to produce 15.57 µg / mL of IAA (Cardoso et al., 2018). In fact, IAA is an auxin important for plant growth and development, thus when a plant is inoculated with a bacteria that can produce IAA, it causes an increased root growth and/or enhanced formation of lateral roots (Dimpka et al., 2009), consequently increasing water and nutrient uptake, mitigating water deficit stress (Egamberdieva and Kucharova, 2009; Mantelin, 2003). Both strains were chosen for further testing growth promotion of maize in field.

Moreover, I9, another *Pseudomonas*, was mixed with T1 and added in the field test, since it has been reported that this strain produces alginate (Cardoso et al., 2018; Sá et al., 2019). Alginate is an hygroscopic extracellular polymeric substance, with the ability to retain several times its weight in water thus maintaining cells hydrated, mitigating stress caused by water deficiency (Freeman et al., 2013; Ngumbi and Kloepper, 2016; Robyt and John, 1998; Sutherland, 2001).

In the field trial, no significant differences in yield were observed when maize was inoculated with the chosen strains. In fact, inconsistent results are usually reported concerning plant growth promotion under field conditions (Zahir et al., 2004). This may occur due to competition of inoculated strains and native microbiome (Smith et al., 1992). Environmental conditions such as soil type, soil texture, temperature, water availability may also influence PGPR success under field conditions (Babalola, 2010). Nevertheless, an increase in maize grain yield of up to 18.9%, and cob weight, cob length, thousand-grain and straw weight was significantly enhanced by 20.8, 11.6, 17.2, and 27.1%, respectively, when maize was inoculated with *Pseudomonas* isolates (Javed et al., 1998). Similarly, when *Pseudomonas* isolates were combined with *Azotobacter*, a significant increase in grain yield (19.8%), cob weight (21.3%), cob length (20.6%), thousand grain weight (9.6%), plant height (8.5%) was observed when compared to the non-inoculated control (Zahir et al., 1998). Likewise, maize yield increased from 24 to 34% when maize was inoculated with *Bacillus* under field conditions (Breedt et al., 2017).

Increase in protein content is part of the plant response to environmental stress and adaptation to changes in environmental conditions (Yancey et al., 1982). Protein accumulation protects against denaturation and decomposition of the cellular molecules and components (Campbell and Close, 1997). Our results show an increase in protein content in both T1 + I9 and T7 condition relative to the control in the first and second sampling, also an increase in protein content in T1 + I9 relative to control and similar values regarding T7 inoculation in the third. Similarly, protein content increased in maize leaves inoculated with PGPR relative to control plants (Ullah et al., 2013). Contrarily, a

reduction in both inoculated conditions relative to the control in the fourth sampling were observed.

Our results show a decrease in starch content when maize was inoculated with T1 + I9 and T7 in the first and third samplings, and similar values to the control were determined in the second and fourth samplings. This decrease may be associated with osmotic adjustment since both these strains T1 and T7 belong are *Pseudomonas*. Strains from these genera have the ability to facilitates starch hydrolysis, increasing sugar availability therefore contributing for osmotic adjustment to alleviate the effect of drought stress (Naseem and Bano, 2014; Sandhya et al., 2010).

Likewise, our results show an increase in soluble sugars when maize plants were inoculated with T1 + I9 and T7 relative to the control in the first sampling. In the field, maize plants were irrigated, nevertheless, soluble sugars have an important role in the cells even under normal conditions. Soluble sugars are a substrate in biosynthesis mechanisms, they are used to produce energy, and are involved in metabolic regulation as regulatory signal molecules (Gibson, 2005; Sheen et al., 1999; Smeekens, 2000).

Water stress boosts production of reactive oxygen species (ROS), like oxygen ($O_2$), hydrogen peroxide ($H_2O_2$), and hydrogen radical ($OH^{-1}$), due to a disruption in photosynthetic machinery and enhanced photorespiration. As a result of the excess of ROS lipid peroxidation is increased, damaging proteins, DNA, and lipids (Pompelli et al., 2010). Malondialdehyde (MDA) is often used as an indicator of cell membrane oxidative damaged caused by ROS and is accumulated in tissues (Parida and Das, 2005). In our study, lipid peroxidation was lower when maize was inoculated with T1 + I9 and significantly lower when inoculated with T7 in the first sampling. This decrease is also observed in the third and fourth samplings. Similarly, lipid peroxidation significantly reduced in chickpea (*Cicer arietinum*) inoculated with *Bacillus* under drought stress (Khan et al., 2018). However, in the third sampling, similar values were determined when maize was inoculated with T1 + I9 and an increase in lipid peroxidation was observed when maize was inoculated with T7 relative to the control.

Furthermore, antioxidant activity may be improved thought enzymatic and non-enzymatic mechanisms, responding to oxidative damage caused by ROS as a result of drought stress (Miller et al. 2010), increasing the plant tolerance to drought (Timmusk et al., 2014). SOD is an essential element of antioxidant defense system, because it protects cells against oxidative damage, converting superoxide radicals to $H_2O_2$ quickly (Verma and Dubey, 2003). Actually, a significant increase in SOD activity was observed in maize leaves when plants were inoculated with PGPR relative to the control (Ullah et al., 2013). Contrarily, our results show a reduction in SOD activity when maize was inoculated with T7 in the third sampling, and a slight decrease when maize was

inoculated with T1 + I9, however an increase in SOD activity was observed in the fourth sampling.

## 5. Conclusion

In this study, several bacterial strains, previously characterized, were screened to find potential candidates to apply in maize crops to enhance maize growth. Strains were firstly tested in a greenhouse, and despite the difference between shoot and root dry weight was not significant when comparing inoculated conditions to the control, T1 and T7 strains had the highest increase in weight relative to the uninoculated plants. These bacteria are a *Pseudomonas* and a *Flavobacterium*, respectively (Cardoso et al., 2018). Furthermore, T7 inoculation in maize in the greenhouse trial lead to an increase in root dry weight which may be associated with its ability to produce IAA (Cardoso et al., 2018), an auxin associated with increased root development (Dimpka et al., 2009). Even so, maize productivity in the field was not enhanced by bacterial inoculation, which is expected, because field conditions are more unpredictable and harder to control, affecting bacterial influence in plant growth. Nevertheless, a positive effect of bacterial inoculation in maize is evident through the biochemical parameters assessed, with an increase in protein content and soluble sugars, and a decrease in lipid peroxidation was observed when maize was inoculated with selected bacterial strains.

# Chapter III

# Maize growth promotion by bacteria under drought conditions – greenhouse and field approach

**Abstract**

Climate change and global warming is expected to reduce 15% of maize (*Zea mays L.*) yield, because this crop is affected by temperature and water availability. This is a problem because maize is the third most consumed and produced cereal in the world and it is crucial to find a sustainable approach to improve productivity. Plant growth promoting rhizobacteria emerges as an alternative or complement to the use of chemical fertilizers, improving growth and alleviating drought stress. Additionally, these bacteria can be applied individually or as a consortium, which is more advantageous because each strain can have a positive effect on plant development and in group this effect is enhanced. Therefore, in this study, a bacterial consortium was tested in greenhouse to understand if it could mitigate drought stress and could potentially be applied in the field. In fact, a positive effect resulted of bacterial consortium inoculation in maize in the greenhouse under drought stress. However, when applied in the field, the results were not as promising, as bacterial consortium inoculation did not lead to an increase in yield in maize in the field trial. Nonetheless, biochemical parameters show an increase in protein content, and soluble sugars, and a decrease in starch content and a lower lipid peroxidation which are associated with stress alleviation.

**Keywords**

Maize, drought stress, plant growth promoting rhizobacteria, bacterial consortium, greenhouse, field, biochemical response

## 1. Introduction

Drought is characterized by the lack of precipitation, resulting in water scarcity, and it can occur globally. It has an impact on health, agriculture, economics, energy, the environment, and affects 40% of world's population (World Health Organization, 2021). In fact, drought is a major threaten to crop growth and productivity in the world, and is expected to affect over 50% of the arable lands by 2050 (Ashraf and Wu, 1994; Kasim et al., 2013; Vinocur and Altman, 2005). This may lead a decrease in food availability and to malnutrition, and micronutrient deficiency, such as iron-deficiency anemia (World Health Organization, 2021). Recently, the Intergovernmental Panel on Climate Change (IPCC) Climate Change 2021 report (IPCC, 2021) predicts an increase of 1.5 °C or more in global temperature, whereas heat extremes would more often reach critical tolerance thresholds for agriculture and health if temperature increase reaches 2 °C (IPCC, 2021).

One of the most produced and consumed food crops around the world is maize (*Zea mays* L.), with almost 800 million tons of grain being harvested in a cultivated area of almost 200 million hectares (FAOSTAT, 2021). Abiotic factors, like temperature and water availability, have a great influence on crop growth (Awika, 2011). This crop is

susceptible to drought (Awika, 2011) and its yield has been affected by drought, resulting in a reduction of nearly 40% globally (Daryanto et al., 2016). Maize is a key crop for agri-food sector in Portugal, occupying the largest area amongst annual crops (Nóbrega, 2006). Since southern Iberia is expected to experience a rise in temperature and a decrease in precipitation due to climate change, this crop production will be affected (IPCC, 2013a).

Some soil microorganisms named rhizobacteria, inhabit the rhizosphere or the plant roots, and a few of them are considered plant growth promoting rhizobacteria (PGPR), since they have beneficial influence in plant growth and yield (Kloepper et al., 1989). Studies have reported the efficiency of these bacteria in promoting growth in edible plants such as maize (Adjanohoun et al., 2011), rice (Gopalakrishnan et al., 2013), wheat (Islam et al., 2014), and others. Furthermore, some PGPR potential possess the ability to alleviate the stress plants undergo during drought (Vurukonda et al., 2016). In fact, PGPR have been proved to mitigate drought stress in maize plants (Naseem and Bano, 2014; Sandhya et al., 2010; Shirinbayan et al., 2019).

These bacteria can be applied as an alternative or a complement to chemical fertilizers and biocides, resulting in a reduction in production and use of agrochemicals, leading to a sustainable crop production (Vurukonda et al., 2016). Likewise, PGPR can be applied individually or as a consortium. This last option has some advantages because some positive interaction can happen between rhizobacteria, as well as coupling of bacteria with different positive effects in one product. So, a combination of two or more PGPR can help in colonization, increase plant growth, diminish stress, and combat pathogens. This has been reported in various studies (Berendsen et al., 2018; Kumar et al., 2016; Shanmugam et al., 2013; Sharma et al., 2018; Wang et al., 2012).

Our study aims to find if a bacterial consortium composed by rhizobacteria previously isolated from roots of wild maize plants can be applied, both in greenhouse and under field conditions, to enhance growth and yield in maize crops under drought stress, increasing agricultural productivity, as a first stage in the development of commercial formulations of bioinoculants.

## 2. Materials and Methods

### 2.1. Bacterial strains

Bacteria tested were previously isolated from maize roots of plants sampled in Angola, and most of the strains belonged to genera *Pseudomonas* and *Enterobacter* and are mentioned in the supplementary table 3.

## 2.2. Greenhouse trial procedure

### 2.2.1. Consortium test

A greenhouse trial was put together to test if the bacterial consortium could promote growth under drought conditions. Plastic pots of 200 mL were filed with a mixture of 2:1 washed and autoclaved sand and peat. In each pot, three *Zea mays* (Dekalb DKC 6031) seeds were sown. Pots were irrigated with tap water when needed. After 12 days, plants that did not germinate correctly were removed, leaving two plants per pot.

After seedling germination, pots were inoculated with 4 mL of bacterial consortium, previously grown for 24 h at 26 °C in 5 mL yeast mannitol broth (YBM) on an orbital shaker (200 rpm), and control was inoculated with 4 mL of growth medium. For each condition there were 6 pots. W – Control – Watered and not inoculated; W + I – Watered and inoculated; D – Not watered and not inoculated; D + I – Not watered and inoculated (Figure 1).

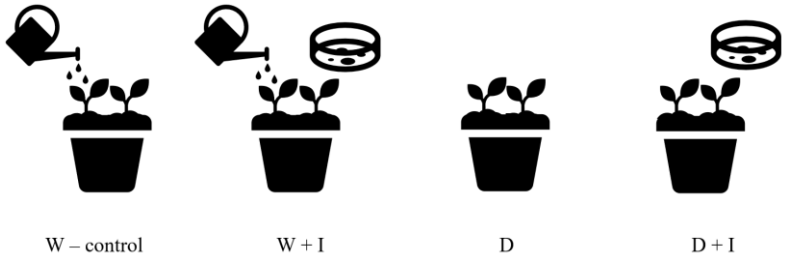


**Figure 1.** Schematic representation of the four conditions considered on the greenhouse test. Each condition had 6 replicates. W – Control – Watered and not inoculated; W + I – Watered and inoculated; D – Not watered and not inoculated; D + I – Not watered and inoculated.

Plants grew in bacteria presence for 16 days. Plants were grown for a total of 28 days in greenhouse conditions at approximately 17 ± 2 °C during the day and 13 ± 2 °C during the night, at natural light with a 12 h light / 12 h dark cycle.

### 2.2.2. Isolated strains test

To assess how each individual strain affects plant growth, a similar procedure was made applying each individual strain to maize plants grown in plastic pots, under water conditions. For each strain there was three replicates, thus there were three plastic pots, each with two maize plants. In this process, plants were watered when top substract was dry. OD was measured at 620 nm using a spectrophotometer to ensure bacterial growth of the inoculum used in the greenhouse trial (Supplementary Table 3).

### 2.2.3. Dry weight

Plants were used to determine dry weight. After the test, plants were washed and dried on a greenhouse at 60 °C until weight was constant. Then, shoot and root were weighted

separately. Dry weight was used to test the ability of the consortium to promote growth under drought conditions compared to the control watered condition.

### 2.3. Field trial procedure

To assess the effects of the same bacterial consortium on more realistic scenario, a field trial was established. The experiment was conducted from 05/23/2019 to 08/31/2019, at INOVMILHO - Centro Nacional de Competências das Culturas do Milho e Sorgo Estação Experimental António Teixeira (INIAV), 38º56'28.32''N/8º30'36.66''W.

Bacterial strains were grown in plates containing yeast extract mannitol agar (YMA) medium (Somasegaran and Hoben, 1994) during 2 to 3 days at 26 °C in an incubator. Then, the strains were inoculated and grown in tubes containing 5 mL of yeast broth mannitol (YMB) medium (Somasegaran and Hoben, 1994). Inoculated tubes were incubated at 26 °C in an orbital shaker (150 rpm), for 4 days. Tube's content was poured into 90 mL flasks to prepare another pre-inoculum. Inoculated flaks were incubated at 26 °C in an orbital shaker (150 rpm), for 3 days. Pre-inoculum growth used in the field trial was determined by measuring optical density at 620 nm (Supplementary Table 3). Flask's content was then used to prepare the final quantity of medium used for the field inoculation, which was prepared using containers of 20 L, but only filled with 9 L of culture medium. All the bacterial strains were grown individually in each pre-inoculum and added together for the inoculum preparation. Compressed air was diffused into the containers using sterile diffusers, emerged in the medium. Containers were sealed with sterile cotton, and this material was also placed inside the tubes, which guaranteed sterile conditions. Bacteria were grown for 3 days. During all the procedure, inoculum was always grown in aseptic condition. Inoculum growth was determined by measuring optical density at 620 nm (Supplementary Table 4).

Maize was previously sown in the field, with application of nitrogen, but no herbicide. There were three blocks, in a total area of 540 $m^2$. Each block had a length of 10 m and 4.5 m of width. Each block had a unit for control condition and another for the consortium being tested. In each unit, 6 lines, each with 70 plants, were sown, leaving 13 to 16 cm in between plants. Of the six lines seeded in each block only the central two 16-day old plants were inoculated with 9 L of culture medium with grown bacteria by pulverization with a knapsack sprayer (Preininger et al., 2018). For both conditions there were three replicates in a randomized complete block design. Plants were irrigated by center pivot irrigation. Leave samples were collected on 06/05/2019, 07/05/2019, 07/31/2019, and 08/31/2019 to assess biochemical conditions.

At the end of the experiment, plants were harvested per replicate from each condition, from only the two inner rows at approximately 14% grain moisture. After harvesting, the average grain moisture content was determined to calculate the grain

yield mass according to a standard of 14% moisture content. Results include both thousand grains weight and 1 $m^2$ grain weight. Also, results were determined taking into consideration 14% moisture content.

### 2.4. Biochemical analysis

Biochemical analysis followed the same methodologies described in Chapter 2

### 2.5. Statistical analysis

Statistical analysis followed the same methodologies described in Chapter 2.

## 3. Results

### 3.1. Greenhouse maize dry weight

Maize's shoot and root dry weight was analyzed. There was a non-significant decrease in both shoot (Figure 2A) and root (Figure 2B) growth when watered maize was inoculated with bacterial consortium (W – control and W + I). Contrariwise, there was a non-significant increase in both shoot (15%) (Figure 2A) and root (22%) (Figure 2B) growth when comparing not inoculated and not watered with inoculated and not watered condition (D and D + I). Moreover, a significant decrease in shoot growth was established when comparing watered conditions (W – control and W + I) and non-watered conditions (D and D + I) (Figure 2A). Similarly, a significant decrease in root growth was determined when comparing watered conditions (W – control and W + I) and not inoculated and not watered condition (D), however this decrease is not significant when comparing watered conditions (W – control and W + I) and inoculated and not watered condition (D + I) (Figure 2B).

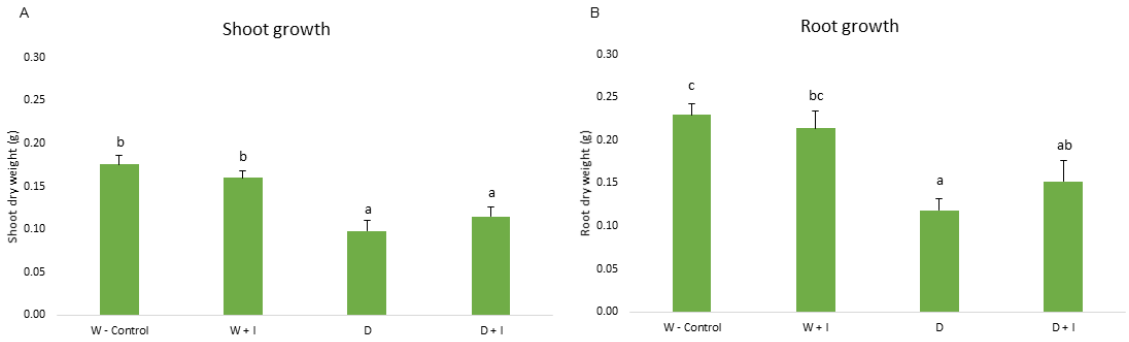


**Figure 2.** Shoot (A) and root (B) maize growth represented as shoot and root dry weight respectively. W – Control – Watered and not inoculated; W + I – Watered and inoculated; D – Not watered and not inoculated; D + I – Not watered and inoculated. Error bars represent the standard error of the mean (SEM). Permanova+ tests to shoot and root weight were performed using Primer (PRIMER-e, Plymouth). Different lowercase letters indicate significant differences among conditions tested (p-value < 0.05).

Yet, when maize plants, grown under watered conditions on greenhouse test, were inoculated with each bacterial strain of bacterial consortium, there was no significant difference between shoot (Figure 3A) and root (Figure 3B) dry weight when compared to the control. However, a slight decrease is observed in shoot and root growth when maize was inoculated with every individual strain, when comparing with control.

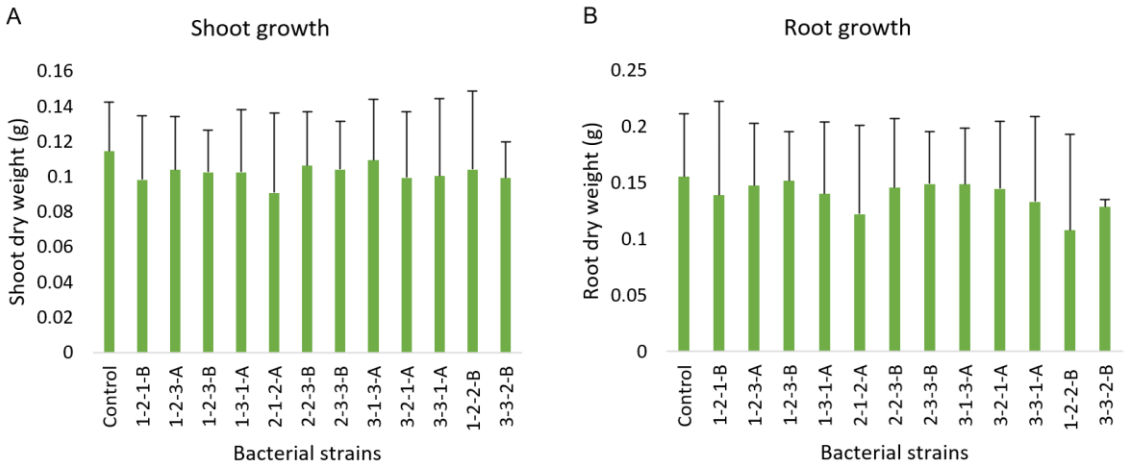


**Figure 3.** Shoot (A) and root (B) maize growth represented as shoot and root dry weight respectively. Error bars represent the standard deviation. Permanova+ tests to shoot and root weight were performed using Primer (PRIMER-e, Plymouth), no significant difference was observed.

### 3.2. Field productivity

Maize productivity in the field was determined considering thousand grains weight and 1 $m^2$ grain weight. Also, results were determined taking into consideration 14% moisture content. In all the parameters considered, thousand grains weight (Figure 4A), grains weight per $m^2$ (Figure 4B), thousand grains weight considering 14% humidity (Figure 4C), and grains weight per $m^2$ considering 14% humidity (Figure 4D), there was no significant difference when comparing inoculated maize weight and control condition. Still, a slight decrease of both grains weight per $m^2$ (14.26%) (Figure 4B) and grains weight per $m^2$ considering 14% humidity (14.55%) (Figure 4D), was observed when maize was inoculated with the bacterial consortium relative to the control condition.

### 3.3. Biochemical analysis

#### 3.3.1. Protein content

In the first sampling, protein content was slightly higher when field growing maize was inoculated with bacterial consortium, comparing with control. This was also verified in the maize samples collected in second sampling, despite results standard deviation was higher. Protein content in both samplings was similar in each condition. In third sampling, protein content was lower in control condition, but similar values were determined on

samples from maize inoculated with bacterial consortium. Consequently, protein content was higher when field growing maize was inoculated with bacterial consortium, comparing with control. In the fourth sampling, the opposite was observed. Protein content was lower when field growing maize was inoculated with bacterial consortium, comparing with control. None of these differences were statistically significant (Figure 5A).

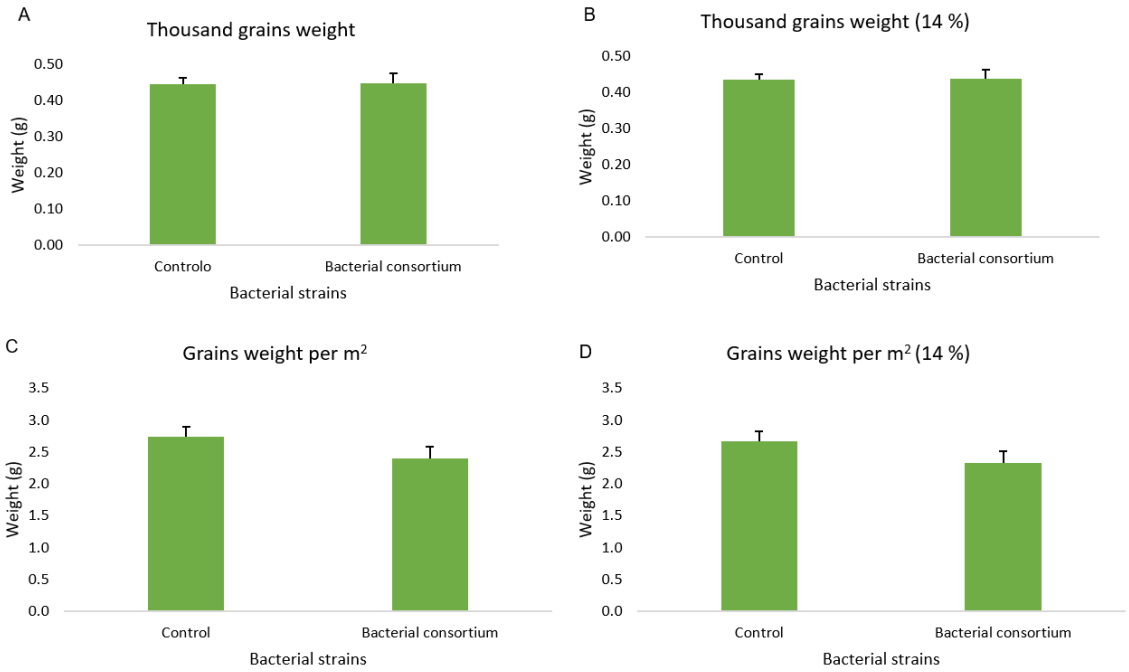


**Figure 4.** Field productivity. A) Thousand grains weight. B) Thousand grains weight considering 14% humidity. C) Grains weight per $m^2$. D) Grains weight per $m^2$ considering 14% humidity. Error bars represent the standard deviation. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth), no significant difference was observed.

### 3.3.2. Starch content

In the first sampling, starch content was slightly lower when field growing maize was inoculated with bacterial consortium, comparing with control. This was also verified in the maize samples collected in second sampling. And a reduction in values determined in both conditions also decreased in the second sampling. Contrarily, starch content increase in the last two samplings in both conditions. Both in the third sampling and the fourth sampling, starch content was slightly lower when field growing maize was inoculated with bacterial consortium, comparing with control. None of these differences were statistically significant (Figure 5B).

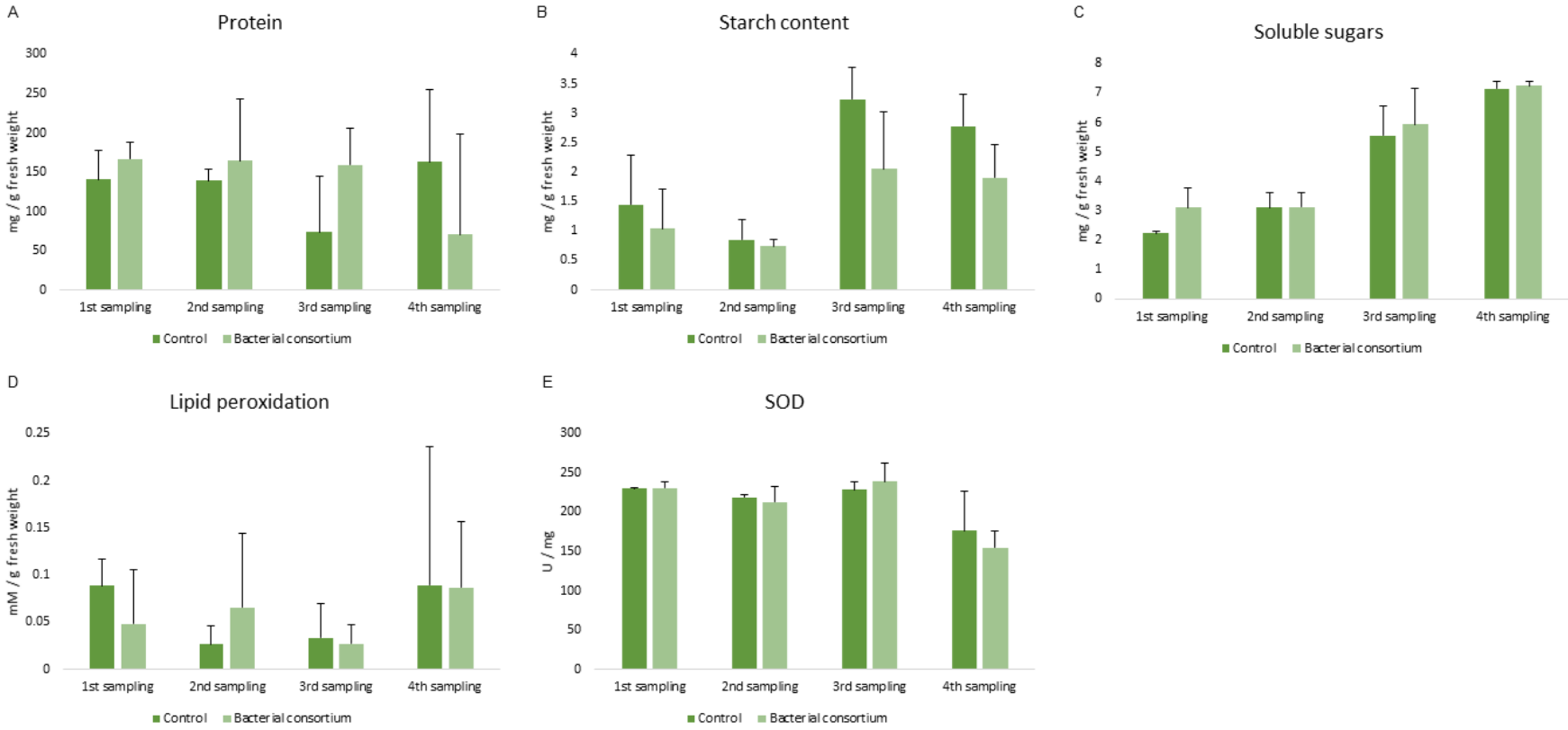


**Figure 5.** Biochemical analysis of maize plants grown in control (dark green) and bacterial consortium (light green) condition, in four different sampling stages. A) Protein content results were expressed in mg / g fresh weight. B) Starch content results were expressed in mg / g fresh weight. C) Soluble sugars – results were expressed in mg / g fresh weight. D) Lipid peroxidation – results were expressed in mili Mol of MDA / g of fresh weight (mM / g). E) Superoxide dismutase (SOD) activity results were expressed in unit per mg of fresh weight (U / mg). Error bars represent the standard deviation. Permanova+ tests to biochemical parameters were performed using Primer (PRIMER-e, Plymouth). No significant difference was observed.

### 3.3.3. Soluble sugars content

In the first sampling, soluble sugars content was higher in maize inoculated with bacterial consortium relative to the control. Contrarily, in the second sampling, similar values were obtained for both conditions. In the third sampling, there was a slight increase in soluble sugars content in maize inoculated with the bacterial consortium. Also, an increase in soluble sugars is observed in both conditions relative to prior samplings. In the fourth sampling, soluble sugars content was again similar in both conditions (Figure 5C).

### 3.3.4. Lipid peroxidation

Lipid peroxidation was lower when field growing maize was inoculated with bacterial consortium, comparing with control, in the first sampling. The contrary was verified in the second sampling, as lipid peroxidation was higher when field growing maize was inoculated with bacterial consortium, comparing with control. In the third sampling, lipid peroxidation was slightly lower when field growing maize was inoculated with bacterial consortium, comparing with control. In the fourth sampling, similar values were determined, but standard deviation was higher in the control condition. None of these differences were statistically significant (Figure 5D).

### 3.3.5. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was similar in both control and bacterial consortium condition, in the first, second and third sampling. A slight decrease is observed in SOD activity when field growing maize was inoculated with bacterial consortium, comparing with control, in the second sampling, and the contrary was verified in the third sampling. In the fourth sampling, SOD activity was lower in both condition when comparing to previous samplings, and a slight decrease is observed in SOD activity when field growing maize was inoculated with bacterial consortium, comparing with control. None of these differences were statistically significant (Figure 5E).

## 4. Discussion

In the current study, positive effects were observed in greenhouse trails, when maize was inoculated with bacterial consortium under drought stress conditions. Even though increase on weight when the bacterial consortium is present under drought conditions in the greenhouse trials is not significantly different, an increase of 15% on shoot weight and 22% on root weight could be a possible indicator of alleviation to drought stress. Contrarily, when irrigated maize was inoculated with bacterial consortium and with each individual strain in the greenhouse trial, a decrease in shoot and root weight was observed. This also occurred in the field trial when irrigated maize was inoculated with bacterial consortium. Thus, further testing should be done exposing inoculated maize to drought in the field to check if bacterial consortium has the same

effect observed in the greenhouse trial. Likewise, no significant difference was observed in the field maize productivity when inoculated with bacterial consortium composed by *Pseudomonas* and *Enterobacter* isolates. However, a significant increase in plant growth measured by shoot and root length, and dry biomass, has been reported in maize inoculated with *Pseudomonas* under drought stress (Sandhya et al., 2010). Also, an improvement in dry weight when maize was inoculated with *Pseudomonas* genera isolates in greenhouse conditions under drought stress has been reported (Gou et al., 2015). Furthermore, maize tolerance to drought stress increased when inoculated with *Enterobacter* sp. FD17 in a greenhouse test (Naveed et al., 2014). As a result, findings regarding positive effects of plant growth-promoting rhizobacteria (PGPR) inoculation have been reported, but when it comes to field trials, results are often inconsistent (Zahir et al., 2004). This could be explained by competition happening between introduced rhizobacterial strains and native flora (Smith et al., 1992).

Increase in protein content protects against denaturation and decomposition of the cellular molecules and components especially during abiotic stress conditions, like drought (Campbell and Close, 1997). Our results show an increase in protein content when maize was inoculated with bacterial consortium in the field trial in the first three samplings. This has been observed under stress condition, as inoculation with *Pseudomonas* strains significantly improved protein and sugar concentration in maize leaves (Naseem and Bano, 2014). Likewise, protein content was improved in maize plants grown under drought stress inoculated with *Pseudomonas* (Vardharajula et al., 2011).

Our results show a decrease in starch content when maize was inoculated with bacterial consortium relative to the control. Similarly, a decrease in starch content was observed in maize plants grown under drought stress inoculated with *Pseudomonas* (Sandhya et al., 2010). *Pseudomonas* spp. contributes to biosynthesis as it degrades the starch for osmotic adjustment resulting is stress effects mitigation (Sandhya et al., 2010). This was further corroborated, with inoculation of EPS-producing *Pseudomonas* sp. in maize under water stress condition, and a decrease in starch content was also determined as this strain facilitates starch hydrolysis, increasing sugar availability consequently contributing for osmotic adjustment to alleviate the effect of drought stress (Naseem and Bano, 2014). In fact, soluble sugars are important osmolytes for osmotic adjustment in plants under drought stress (Dekánková et al., 2004).

Furthermore, in our study, there was an increase in soluble sugars in maize inoculated with the bacterial consortium relative to the control in the first sampling. Likewise, an increase in soluble sugars was observed in maize inoculated with *Azospirillum* under drought stress (Qudsia et al., 2013). Similarly, it was observed an

increase in soluble sugars in salt-stressed maize inoculated with *Azospirillum chroococcum* and *Azotobacter chroococcum* (Abdel Latef et al., 2020). These osmolytes mitigate water deficit stress maintaining turgor pressure and resisting osmotic stress (Kordrostami et al., 2017; Silva-Ortega et al., 2008), stabilizing cellular membranes (Hoekstra et al., 2001).

Water stress causes a disruption in photosynthetic machinery and photorespiration increases, leading to an enhanced production of oxygen ($O_2$), hydrogen peroxide ($H_2O_2$), and hydrogen radical ($OH^{-1}$) which are reactive oxygen species (ROS). Consequently, excess of ROS results in enhanced lipid peroxidation, damaging proteins, DNA, and lipids (Pompelli et al., 2010). Malondialdehyde (MDA) is considered to be an indicator of cell membrane oxidative damaged caused by ROS and is accumulated in tissues (Parida and Das, 2005). Furthermore, a significant reduction of MDA content was observed in PGPR-inoculated seedlings when comparing with control condition under water stress (Gontia-Mishra et al., 2016). *Bacillus aquimaris* DY-3 alleviated the salt stress in maize, likely through the integration of the antioxidant enzymes and the non-antioxidant systems that improve the plant response, causing a 9.55% decrease in MDA content (Li and Jiang, 2017). Similar results were obtained with maize plants inoculated with mycorrhiza grown under temperature stress (Zhu et al., 2010). Our results show a decrease in LPO when maize was inoculated with bacterial consortium in the first and third sampling. However, LPO was higher in the second sampling relative to the control, and it had similar values in the fourth sampling, showing unconclusive results. To further investigate this tendency, more samplings could potentially be assessed to have a more frequent LPO determination.

Under stress conditions, rhizobacteria may improve antioxidant capacity thought enzymatic and non-enzymatic mechanisms, thus increasing the plant tolerance to drought (Timmusk et al., 2014), responding to oxidative damage caused by ROS (Miller et al. 2010). SOD converts superoxide radicals to $H_2O_2$ quickly, and it protects cells against oxidative damage, so it is an essential component of the antioxidative defense system (Verma and Dubey, 2003). Furthermore, inoculation of plants with PGPR reduces the damage on antioxidant enzymes activity caused by drought stress (Han and Lee, 2005). For instance, severe water stress increased 63% SOD activity in maize plants inoculated with *Pseudomonas* (Rezazadeh et al., 2019). Similarly, SOD activity in salt-treated with *Bacillus aquimaris* DY-3 maize seedlings increased significantly when compared to control condition (Li and Jiang, 2017). Also, dual inoculated flax (*Linum usitatissimum* L.) plants with arbuscular mycorrhizal fungi and *Pseudomonas* alleviated reactive oxygen species damage resulting in improve water stress tolerance (Rahimzadeh and Pirzad, 2017). SOD activity was higher in maize inoculated with

*Pseudomonas aeruginosa* under drought stress conditions (Naseem and Bano, 2014). However, our results show similar values of SOD activity in maize inoculated with bacterial consortium and the control through the exposure, and a slight decrease in the fourth sampling. This may happen since maize in under no stress, so there is balance between ROS production and ROS scavenging systems (Apel and Hirt, 2004).

## 5. Conclusion

In this study, a bacterial consortium was tested in greenhouse to understand if it was able to mitigate drought stress and could potentially be applied in the field. Bacterial consortium inoculation had a positive effect on maize growing under drought stress, in the greenhouse, which is a possible indicator of alleviation to drought stress. However, this effect was not observed in the field, probably because maize was irrigated. Nevertheless, inoculation with bacterial consortium lessened the adverse effect of drought stress on the antioxidant enzymes activity, reducing lipid peroxidation has it has been previously reported (Han and Lee, 2005). Likewise, an increase in protein content, and soluble sugars, and a decrease in starch content was observed, which also contributes to drought stress mitigation.

# Chapter IV

# From the field to the lab – isolation and characterization of maize associated bacteria in different water regimes

**Abstract**

Global temperature is predicted to increase, impacting agriculture and human health. Drought is expected to affect 50% of the arable lands by 2050, causing a decrease in global food production. Maize is one of the most consumed and produced cereal across the world, but its yield will be impacted by drought increase. In fact, a reduction of 15% yield is predicted in maize production globally. Mineral fertilizers, genetic engineering, and increased irrigation have been used to reduce drought impacts and obtain high yields. However, these solutions have disadvantages, which makes it a priority to find more sustainable options, taking into consideration the ecological environment of crop development, exploiting beneficial interactions between plants and plant growth promoting rhizobacteria associated with them, to mitigate drought effects and improve crop yields, to feed a growing population, expected to reach 9 billion by 2050. Hence, bacterial strains isolated from root of maize plants growing under three different water stress conditions were isolated and characterized, testing their ability to tolerate drought stress, produce siderophores, and solubilize phosphate. Our results demonstrate how drought reduced maize productivity and impacted the microbiome associated with root, reducing the number of strains isolated from drought conditions relative to the control. Nevertheless, several strains were able to produce siderophores which is a characteristic previously associated with plant growth promotion. Furthermore, bacteria isolated from conditions subject to water deficit had a lower osmotolerance.

**Keywords**

Drought, plant growth promoting rhizobacteria, plant growth promotion, diversity, microbiome, osmotolerance

## 1. Introduction

Global warming is happening at a faster pace than previously estimated. The Intergovernmental Panel on Climate Change (IPCC) Climate Change 2021 report (IPCC, 2021), states that global temperature is expected to reach or exceed 1.5 °C of warming. Also, this report shows how for a 2 °C increase in global temperature, heat extremes would more often reach critical tolerance thresholds for agriculture and health (IPCC, 2021). This has already been in discussion, as the IPCC Climate Change 2007 report (IPCC, 2007), also acknowledged how global warming is leading to an increase in the severity and frequency of drought, and consequentially the loss of arable lands may double by the end of the century, causing a decrease in global food production (IPCC, 2007). Likewise, The IPCC Climate Change 2013 report, also predicted a rise in global temperature and how it would increase soil dehydration on Mediterranean regions,

and reduction in soil humidity (IPCC, 2013b). In fact, water scarcity affects 40% of world's population, disturbing economies, agriculture, and health (World Health Organization, 2021), and it is expected to have an impact on over 50% of the arable lands by 2050 (Ashraf and Wu, 1994; Kasim et al., 2013; Vinocur and Altman, 2005).

One of the most consumed and produced cereals is maize (*Zea mays* L.), only behind rice and wheat (Awika, 2011). Due to climate changes, especially increase in drought, 15% of annual yield losses are expected in maize production globally (Edmeades, 2008). In fact, a meta-analysis shows a 39.3% yield reduction in maize production at approximately 40% water reduction (Daryanto et al., 2016). This could be explained since maize was originated from wetter regions (van Heerwaarden et al., 2011). Also, maize was more sensitive to drought during reproductive phase, however yield losses were small when drought stress only affected vegetative phase (Daryanto et al., 2016). Drought stress during reproductive phase could lead to ovule abortion and pollen sterility, which affects maize yield along the run (Araus et al., 2012). Water deficit during vegetative phase induces stomatal closures and inhibits photosynthesis, limiting carbohydrate synthesis, and consequently cell division and expansion (Barnabás et al., 2008).

To reduce the impacts of drought and obtain high yields, mineral fertilizers are often applied to the soil (Bijay-Singh et al., 1995), along with genetic engineering (Warren, 1998) and increased irrigation (Döll, 2002). All these strategies have problems associated with them. For instance, the application of fertilizers to natural ecosystems increases concentrations of nitrate in surface freshwater and groundwater, losses of nitrate by leaching, and others (Bijay-Singh et al., 1995). Likewise, resorting to genetic engineering is a complex solution, since it is hard to establish new tolerant varieties due to the complexity of the mechanisms involved in abiotic stress tolerance, and these methods are not well accepted in some regions of the world (Wahid et al., 2007). Furthermore, an increase in water demand by other socioeconomic sectors is reducing water availability for agriculture uses (Iglesias et al., 2007; Iglesias and Garrote, 2015). Which means, there is a need to find a more sustainable solution to increase crops yield to feed an ever growing population, expected to reach 9 billion by 2050 (IPCC, 2007).

The approaches mentioned before do not take into consideration the ecological context of the soil environment were the crops are grown (Morrissey et al., 2004). Also, the intensification of maize production, not inadequate nutritional management  Thus, a more sustainable, economic, and less time-consuming solution to mitigate drought stress and increase crop tolerance alternative is to apply beneficial bacteria, exploiting

beneficial interactions between plants and microbiome associated with them (Asghar et al., 2015).

These bacteria are referred to as plant growth promoting rhizobacteria (PGPR), since they have beneficial influence in plant growth and yield (Kloepper et al., 1989). PGPR can also protect plants against drought effects if they are adapted to adverse conditions, leading to an increase in crop productivity in arid or semiarid areas (Kasim et al., 2013; Kavamura et al., 2013; Marulanda et al., 2007). Some studies report an enhance in maize yield when inoculated with these bacteria (Mehnaz et al., 2010; Wu et al., 2005). Thus, there is a justified global interest in PGPR use for maize crop.

However, not many studies have covered the global area of maize cultivation, and results of PGPR application vary widely with factor, such as climate, natural microbiota, available nutrients, and crop characteristics (Ercole et al., 2021). Hence the importance of studying the PGPR associated with specific agroecosystems and optimize its use (Vassilev et al., 2015). Also, there is a lack of knowledge concerning bacteria associated with maize rhizosphere, particularly when maize is under water stress. This information could be important to further understand plant-bacteria interaction and it may lead to finding promising strains useful to improve tolerance to drought. As a result, the aim of these study was to isolate and characterize a collection of bacteria obtained from roots of maize growing under three different water stress conditions. Bacteria was isolated from outside and inside the roots, since it is believed that rhizobacteria worth will be overcome by endophytic bacteria, since these ones can achieve more intense interaction with plants and are not affected by competition with other microorganisms present in the rhizosphere (Naveed et al., 2014). These conditions were then compared in terms of maize productivity, diversity of bacteria isolated and their tolerance to drought stress, their ability to produce siderophores, and their capability to solubilize phosphate.

## 2. Materials and methods

### 2.1. Maize growth, productivity, and sampling

Maize (Dekalb DKC 6031) was sown at INOVMILHO - Centro Nacional de Competências das Culturas do Milho e Sorgo Estação Experimental António Teixeira (INIAV), 38º56’28.32’’N/8º30’36.66’’W. There were three blocks, one for control and two others for two different water stress conditions. Each block had a length of 10 m and 1.5 m of width. In each block, 2 lines, each with 70 plants, were sown, leaving 13 to 16 cm in between plants.

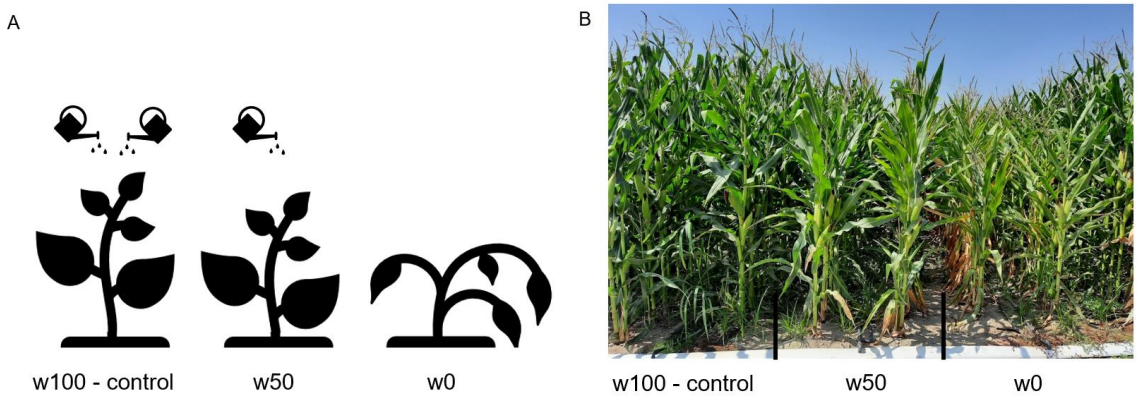


**Figure 1.** Maize growth. A) Irrigation scheme. Three conditions were considered: w100 – control (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). B) Maize growth on sampling day.

Maize plants were grown during 65 days before sampling (Figure 1B), under three different conditions: w100 control (100% irrigated) and two stress conditions, w50 (50% irrigated), and w0 (0% irrigated) (Figure 1A). Five plants were randomly sampled from these three different conditions to further analysis, which included, bacterial isolation (Figure 2A), bacterial typing (Figure 2B), plant growth promoting abilities screening and osmotolerance (Figure 2C), and all the data collected was then analyzed statistically (Figure 2D).

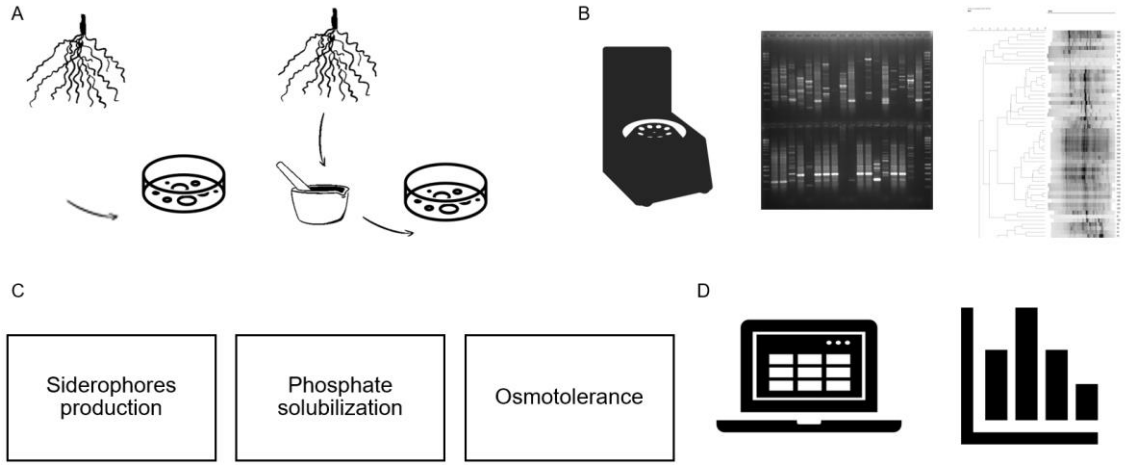


**Figure 2.** Bacteria isolation and characterization. A) Bacterial isolation from outside and inside (endophytic) the root. B) Bacterial typing. C) Plant growth promoting abilities screening and osmotolerance. D) Statistical analysis.

At the end of the field trial, maize productivity was assessed. After harvesting, the number of maize plants per $m^2$ was counted, as well as the number of maize cobs per $m^2$. Maize grain weight, and thousand maize grains weight was determined, along with humidity and specific weight.

## 2.2. Colony forming units (CFU)

Colony forming units (CFU) were determined on three samples of soil collected from each condition. A soil sample of 1 g was diluted on 9 mL of sterile distilled water for the $10^{-1}$ dilution. Then, serial dilutions ($10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$) were achieved, by successive dilutions of 1 mL of previous solution on 9 mL of sterile distilled water. Only 100 µL from $10^{-3}$, $10^{-4}$, and $10^{-5}$ in each one of the three replicates of each condition were plated onto yeast mannitol agar (YMA) medium (Somasegaran and Hoben, 1994). Plates were incubated and colonies were counted after ten days of growth at 26 °C. Results are expressed in CFU per gram of soil (CFU / g soil) and were obtained from $10^{-4}$ dilution (Figure 3).

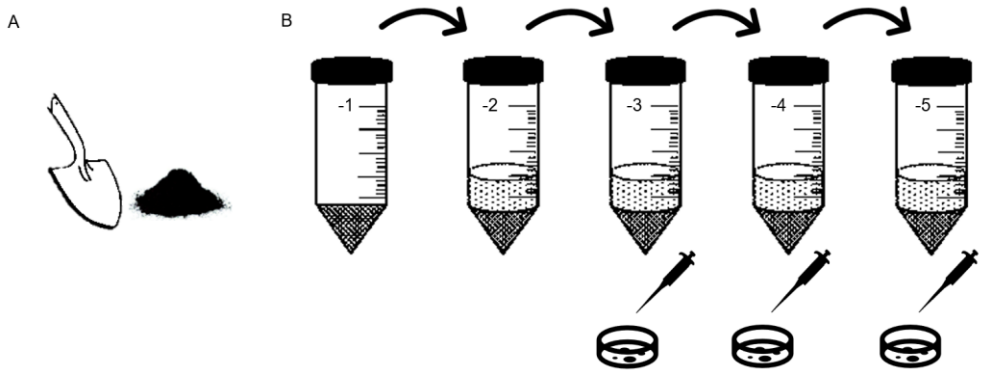


**Figure 3.** Colony forming units' assessment. A) Soil sampling. B) Serial dilution and plating of $10^{-3}$, $10^{-4}$, and $10^{-5}$ dilution.

## 2.3. Isolation of bacteria

Bacteria were isolated from maize roots (Figure 4) following the method described in Somasegaran and Hoben (1994). Three pieces of the tip of the root were randomly picked from each plant. These root pieces were firstly washed with sterile deionized water and streaked onto yeast extract mannitol (YMA) plates, containing 1 g mannitol, to isolate bacteria from outside the root (w100o, w50o, and w0o). Then, the same root pieces were surface sterilized by soaking in 96% ethanol for 5 s, and then immersed for 2 minutes in a 3% hydrogen peroxide solution. Root pieces were then rinsed three time in sterile deionized water and crushed. The macerate was streaked onto YMA plates, to isolate endophytic bacteria (w100i, w50i, and w0i). After growing at 26 °C, morphologically distinct single colonies were further re-streaked onto YMA plates and allowed to grow at 26 °C until obtaining 244 isolates. Each isolate was then preserved at -80 °C, by mixing 500 µL of the resulting culture medium containing the isolate and the same amount of a sterile solution of 30% glycerol.


**Figure 4.** Root samples from each condition. w100 – control, 100% irrigated; w50, 50% irrigated; w0, 0% irrigated.

### 2.4. Bacterial typing using PCR-based fingerprinting

For typing isolates with unique fingerprints and evaluate the genetic variability of the collection, BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) was performed. With this procedure it is possible to identify identical genotypes, comparing all the fingerprints. Isolates were previously grown on YMA medium and single colonies were used to prepare a bacterial suspension in 50 µL of sterile Milli-Q water. To perform the PCR reactions, 1 µL of bacterial suspension was mixed with 2 µL BOXA1R primer (5′-CTACGGCAAGGCGACGCTGAC-3′; (Versalovic et al., 1994), previously diluted 1:10 in sterile Milli-Q water to 10 µmol/µL, 6.25 µL NZYtaq II 2X Taq Green Master Mix (NZYTech, Portugal), and 15.75 µL Milli-Q water to obtain a final volume of 25 µL. PCR amplification was performed applying one 7 min cycle at 95 °C, a 30 cycles repetition of 1 min at 94 °C, 1 min at 53 °C, and 8 min at 65 °C, and a final 16 min cycle at 65 °C. PCR products were then run on a 1.5% agarose gel, prepared with Tris-Acetate-Ethylenediamine tetraacetic acid (TAE) buffer 1X, to obtain typing profiles. In each well, 10 µL of each PCR product was loaded, and the negative control (previously prepared by mixing 2 µL BOXA1R primer, 6.25 µL NZYtaq II 2X Taq Green Master Mix, and 15.75 µL Milli-Q water), and 3 µL of the molecular weight marker NZYDNA Ladder III (NZYTech, Portugal) was applied at the beginning and the end of each row of wells. Electrophoresis was run at 80 V for 70 min. Then, the gel was stained in ethidium bromide for 20 min and the excess staining was removed in distilled water for 20 min. Gels were scanned under UV light and profiles were obtained with Image Lab software (Bio-Rad, Portugal). Pearson correlation coefficient was calculated, and the clusters formed applying the unweighted pair group method with arithmetic mean (UPGMA) were analyzed using GelCompar II (Applied Maths, Belgium). For further tests, a representative isolate of each distinct fingerprint was selected randomly. This procedure yielded 168 different isolates.

### 2.5. Plant growth promotion abilities

#### 2.5.1. Phosphate solubilization

To determine the ability of bacteria to solubilize phosphate, three replicates of the strains isolated from maize roots were grown for ten days on YMA medium, composed by 5 g of calcium phosphate, instead of dipotassium phosphate. Colonies with an halo were considered as capable of solubilizing phosphate (Pikovskaya, 1948).

#### 2.5.2. Production of siderophores

To determine the ability of bacteria to produce siderophores, the isolated strains were grown for ten days on YMA medium supplemented with the indicator solution chrome azurol S (CAS), containing 1.21 mg $mL^{-1}$ CAS, 0.1 mM $FeCl_3 \cdot 6H_2O$ and 1.82 mg $mL^{-1}$ hexadecyltrimethylammonium bromide (HDTMA). Colonies surrounded by an orange halo were characterized as siderophore producing strains (Alexander and Zuberer, 1991). Three replicates were considered. Results represent the ratio between the diameter of the halo and the diameter of the colonies.

### 2.6. Bacteria osmotolerance

Rhizobacteria osmotolerance was tested using the method of van der Weele et al., (2020) adapted for bacteria, because polyethylene glycol (PEG) does not allow agar to solidify. Petri plates containing PEG-6000 were prepared by pouring 10 mL of extract mannitol (YMA) (Somasegaran and Hoben, 1994) and, after its solidification, 10 mL of autoclaved liquid (containing no agar) containing 0 (control) or 400 g of PEG-6000 were poured onto the top of the solidified medium. After 24 h of letting the liquid medium diffused into the solid medium, the solution was poured off. Two conditions were obtained: control and 20% PEG (400 g / L PEG), simulating no water stress and one level of simulated water deficit. Results are the means of three replicates of diameters of colonies measured on both conditions after growing for four days at 26 °C.

### 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/).

BOX-PCR fingerprints dendrograms 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).

Data from the production of siderophores, osmotolerance and CFU was analyzed performing permutational multivariate analysis of variance (Permanova+) tests, using Primer (PRIMER-e, Plymouth), considering differences significant when p-value ≤ 0.05.

## 3. Results

### 3.1. Field productivity

Maize productivity on the field was evaluated. The number of maize plants per $m^2$ (12) and number of maize cobs per $m^2$ (12) was the same in all three conditions considered. There is a reduction on maize grain weight, 100 maize grains weight, and specific weight when maize is grown under w0 condition (0% irrigated), in relation to control w100 and w50 condition. Maize grain weight was 14.8% lower when maize was grown under 50% irrigated condition, and 188.4% lower under 0% irrigated condition, when compared to maize grown under control condition (100% irrigated). Regarding thousand maize grain weight, this reduction is not as distinct, since weight was 5.9% lower on maize grown under 50% irrigated condition, and 38.6 % lower under 0% irrigated condition, when compared to maize grown under control condition (100% irrigated). Humidity also is lower when maize is grown under water stress conditions, 6.7%, and 14.4% when comparing maize grown under 50% irrigated and 0% irrigated condition, respectively, comparing with control. However, maize grown under 50% irrigated condition had a 0.3% increase in specific weight when compared to maize grown under no water deficit, and maize grown under 0% irrigation had a 3.0% decrease in specific weight when compared to maize grown under no water deficit (Table 1).

**Table 2.** Field productivity of maize growing in three conditions: w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated).

| Conditions | Number of maize plants per $m^2$ | Number of maize cobs per $m^2$ | Maize grain weight (g) | 1000 maize grains weight (g) | Humidity (%) | Specific weight (g) |
|---|---|---|---|---|---|---|
| w100 – Control | 12 | 12 | 2.636 | 359 | 17.5 | 74.9 |
| w50 | 12 | 12 | 2.296 | 339 | 16.4 | 75.1 |
| w0 | 12 | 12 | 0.914 | 259 | 15.3 | 72.7 |

### 3.2. Colony forming units (CFU)

Maize plants were grown in non-axenic field conditions. CFU were similar on samples from soil isolated from 100 and 50% irrigated condition. Nevertheless, the highest number of CFU was on samples from soil isolated from 50% irrigated condition, but its standard deviation is also the highest. There was a significant decrease when comparing CFU from 0% irrigated soil, comparing to soil collected from 100% (231.8% decrease) and 50% (234.8% decrease) irrigated. When comparing w100 and w50, no significant difference was observed (Figure 5).

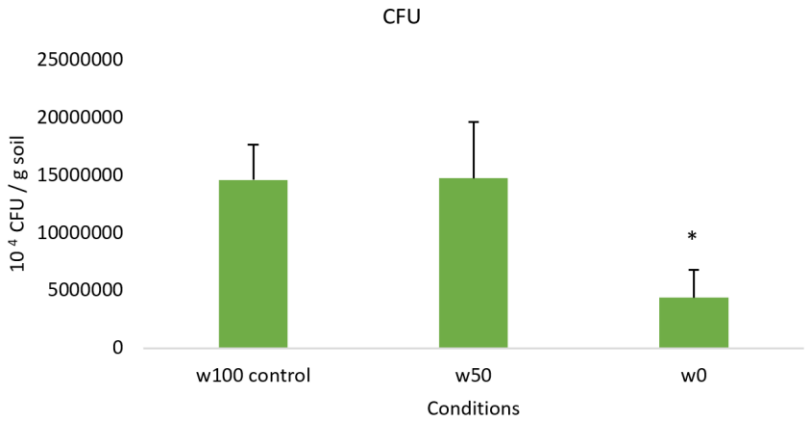


**Figure 5.** Colony forming units (CFU). Three conditions were considered, w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Values are means of three replicates. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth). Error bars represent standard deviation, and asterisks indicate significant differences between conditions (p-value < 0.05).

### 3.3. Bacteria diversity

BOX-PCR performed for molecular typing of bacterial isolated yielded 168 distinct typing profiles (Figure 6).

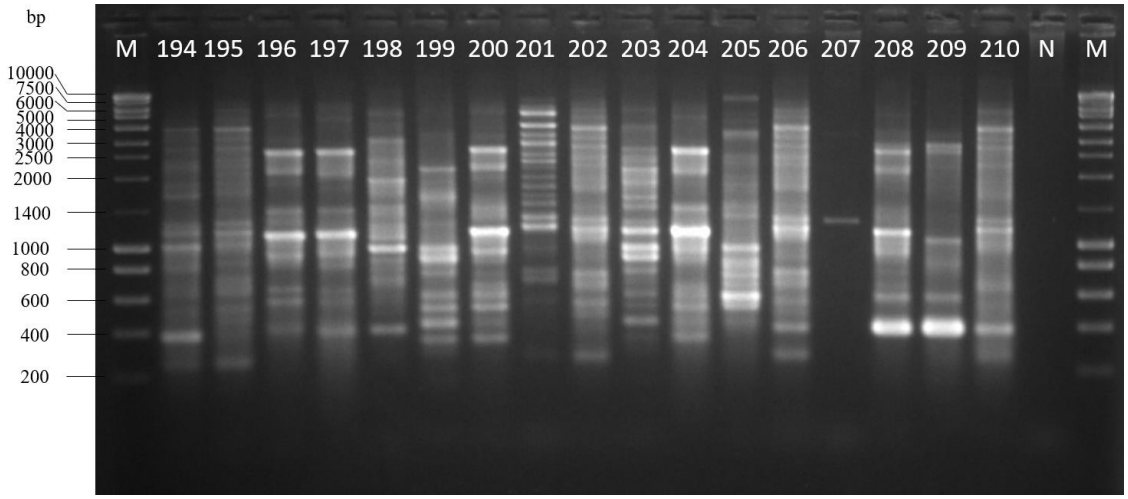


**Figure 6.** Molecular typing gel electrophoresis agarose results. M – molecular weight marker with respective band size (bp); N – negative control; 194 to 210 - DNA samples. Sample 196 and 197 are the same strains according to further analysis. Similarly, 200 and 204 are the same strain according to further analysis.

Bacterial strains were isolated from three different conditions. A total of 72 bacteria were isolated from w100 (control condition 100% irrigated), 57 from w50 (50% irrigated), and 47 from w0 (0% irrigated). Also, bacterial strains were isolated from inside and outside the roots of maize plants. From inside the root, 43 strains were isolated from

w100, 28 strains from w50, and 29 strains from w0. From outside the root, 29 strains were isolated from w100, 29 from w50, and 18 from w0 (Figure 7).

The condition with the highest bacteria counting was w100, from inside the root, and the lowest was w0, from outside the root. Similar number of strains were isolated from the other conditions (Figure 7).

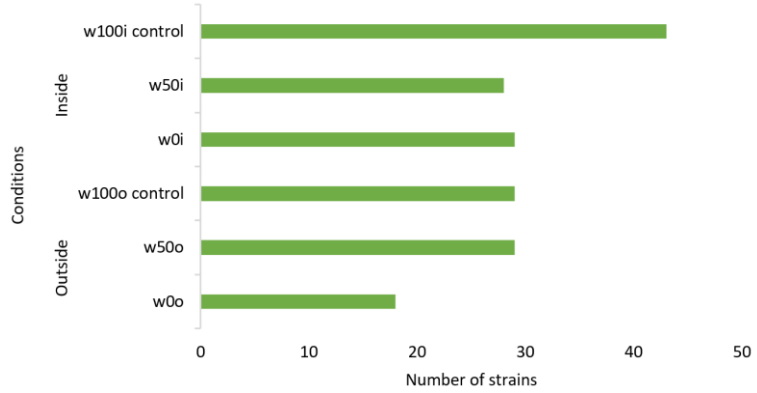


**Figure 7.** Number of strains isolated from each condition. Three conditions were considered, w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Bacterial strains were isolated from both inside and outside maize roots.

Bacterial distribution in the three conditions considered showed low repetitiveness since only one strain was common on all the three conditions inside the root, and none when considering outside the root isolated strains.

In fact, no common strain was isolated from outside the root on all the three conditions. Two strains were common between w100i and w50i, one strain was common between w50i and w0i, and no strains were common when comparing w100i and w0i. Comparing w100i and w100o, a strain is common between these two conditions, and the same happens when comparing w50i and w50o. Contrarily, no strain was common between w0i and w0o (Figure 8).

### 3.4. Bacteria plant growth promotion (PGP) abilities

Plant growth promoting traits were assessed. None of the bacteria tested was able to solubilize phosphate. Of all 168 the bacteria tested, 12 strains were not able to grow on the medium used for the test, only 2 strains were not able to produce siderophores, and a total of 154 were able to produce siderophores (91.7%). From plants grown under control condition (w100) 40 bacteria isolated from inside the roots were able to produce siderophores (Figure 9A), and 27 from outside the roots (Figure 9D). From plants 50% irrigated (w50), 24 bacteria isolated from inside the roots were able to produce siderophores (Figure 9B), and 26 from outside the roots (Figure 9E). From plants 0%

irrigated (w0), 28 bacteria isolated from inside the roots were able to produce siderophores (Figure 9C), and 17 from outside the roots (Figure 9F).

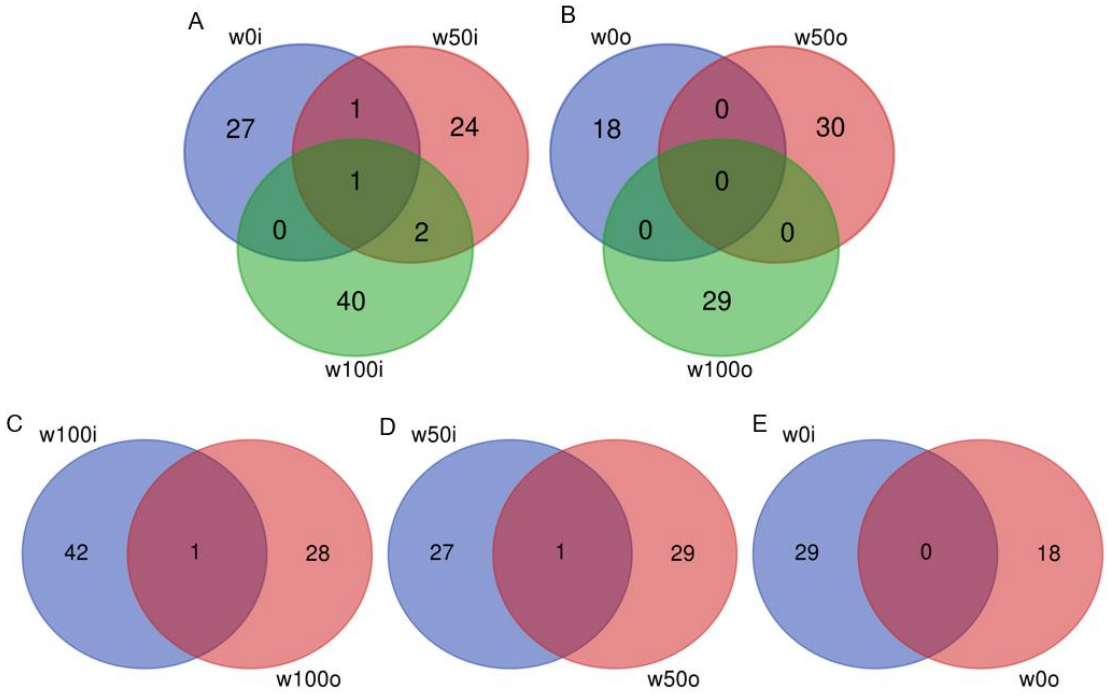


**Figure 8.** Venn diagrams showing bacterial strain distribution in the three conditions considered w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). A) Bacterial distribution of strains isolated from inside the root in the three conditions considered. B) Bacterial distribution of strains isolated from outside the root in the three conditions considered. C) Bacterial distribution of strains isolated from w100 – control condition from inside and outside the root. D) Bacterial distribution of strains isolated from w50 condition from inside and outside the root E) Bacterial distribution of strains isolated from w0 condition from inside and outside the root.

Moreover, 6 strains isolated from w100i had a ratio between the diameter of the halo and the diameter of the colony equal or superior to 2 (Figure 9A), 9 strains from w50i (Figure 9B), 3 strains from w0i (Figure 9C), 7 strains from w100o (Figure 9D), 8 strains from w50o (Figure 9E), and 5 strains from w0o (Figure 9F).

Additionally, 1 strain isolated from w100i had a ratio between the diameter of the halo and the diameter of the colonies equal or superior to 3 (Figure 9A), 2 strains from w50i (Figure 9B), 0 strains from w0i (Figure 9C), 3 strains from w100o (Figure 9D), 1 strain from w50o (Figure 9E), and 2 strains from w0o (Figure 9F).

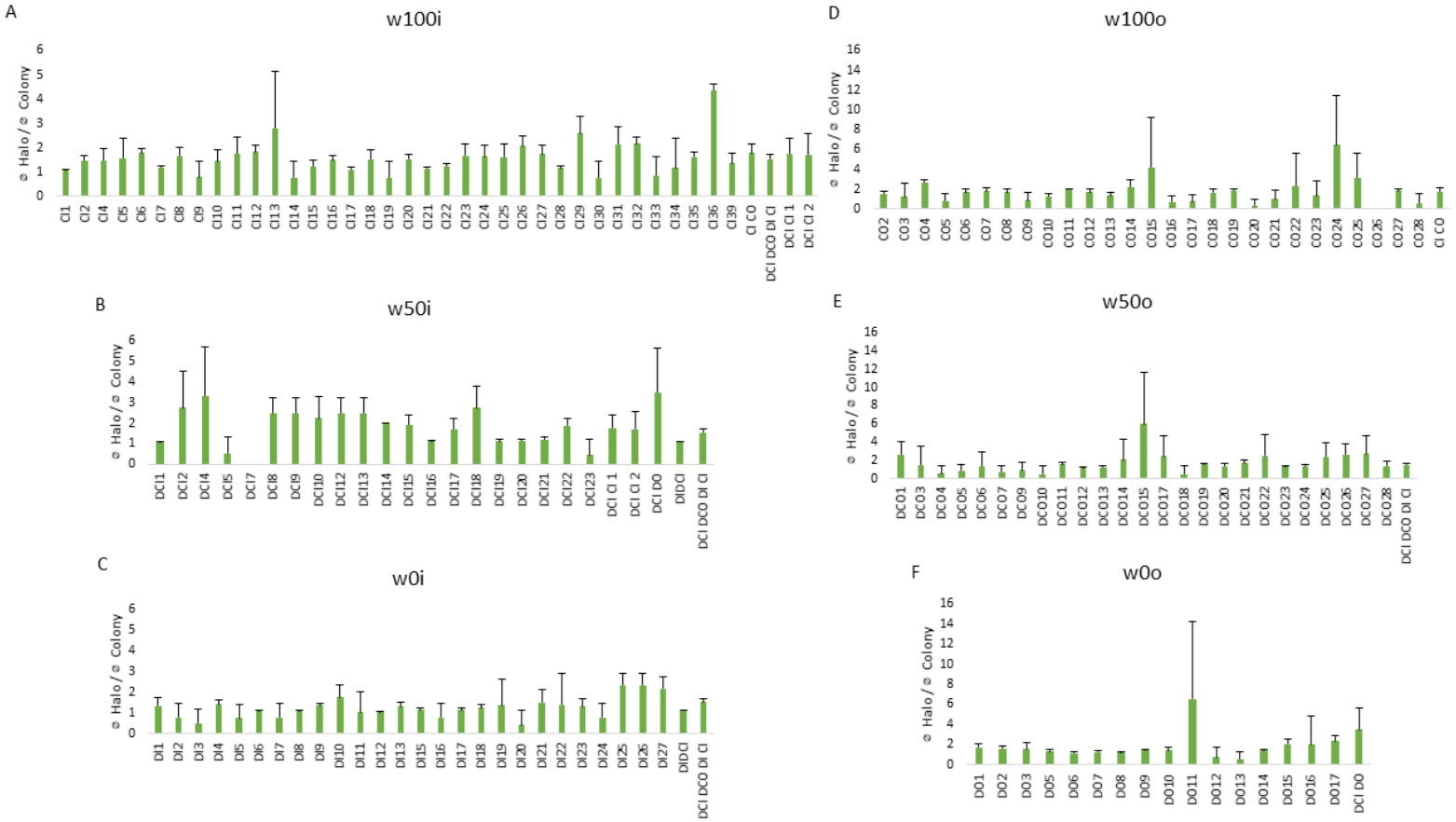


**Figure 9.** Siderophores production of bacterial strains isolated from inside (A, B, and C) and outside (D, E, and F) the root of maize plants grown under different water conditions. Three conditions were considered: A and D – w100 control condition (100% irrigated), D and E – w50 (50% irrigated), C and F – w0 (0% irrigated). Results represent the ratio between the diameter of the halo and the diameter of the colonies. Values are means of three replicates and error bars represent standard deviation.

Comparing siderophore production of bacterial strains isolated from different conditions, it is observable a significant lower ratio between the diameter of the halo and the diameter of the colonies between strains isolated from inside the root of plants grown on w0 condition (0% irrigation). This condition has a ratio of 1.25, 31% lower than the mean of ratios from all bacteria considered (1.65). This is the condition with the lowest ratio recorded. The highest ratio is from condition w0o (0% irrigation, outside the root), presenting a ratio of 1.87, 12% higher than the mean of all ratios (Figure 10).

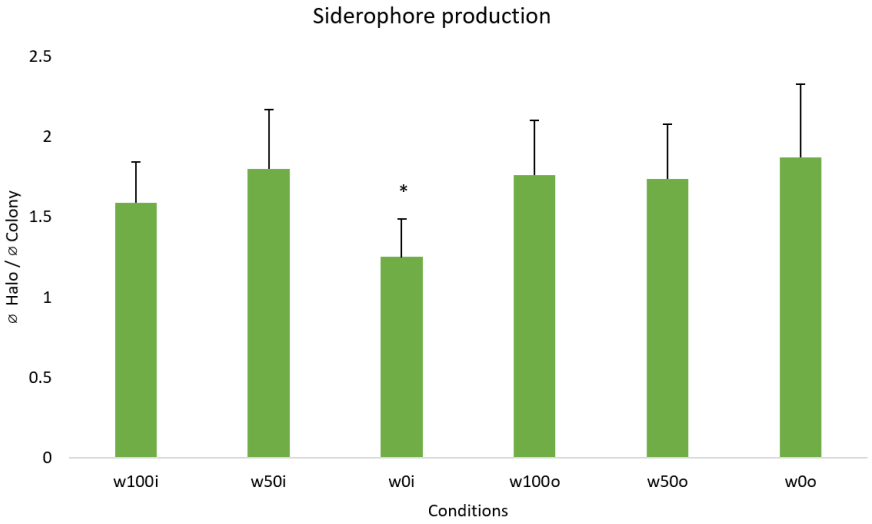


**Figure 10.** Siderophores production of bacterial strains isolated from inside (w100i, w50i, and w0i) and outside (w100o, w50o, and w0o) the root of maize plants grown under different water conditions. Three conditions were considered: w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Results represent means of all the ratios between the diameter of the halo and the diameter of the colonies of all bacteria strains isolated from each condition. n (w100i) = 40; n (w50i) = 25; n (w0i) = 28; n (w100o) = 28; n (w50o) = 26; n (w0o) = 17. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth). Error bars represent standard error of the mean, and asterisks indicate significant differences between conditions (p-value < 0.05).

Comparing siderophore production of bacterial strains isolated from different conditions, no significant difference was observed between ratios considered. The w0 condition had the lowest ratio mean recorded (1.48), 11% lower than the mean of ratios from all bacteria considered (1.65), and w50 had the highest ratio recorded (1.77), 6% higher than the mean of ratios. Lastly, w100, the control condition, had intermediate ratio values (1.66), similar with the mean of ratios (Figure 11).

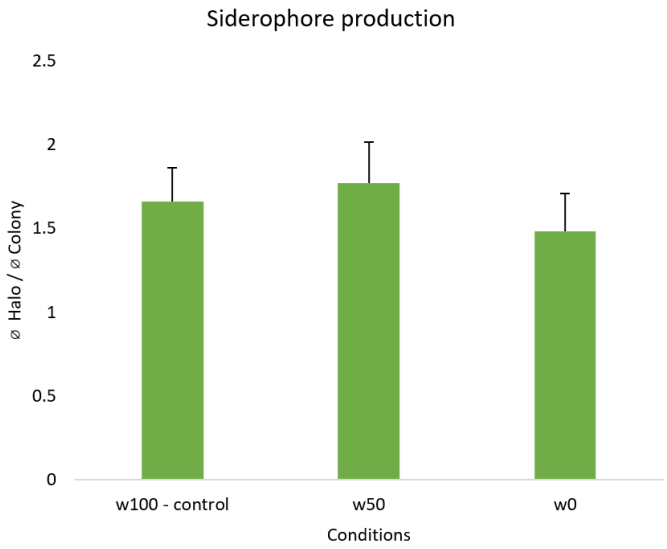


**Figure 11.** Siderophores production of bacterial strains isolated from the root of maize plants grown under different water conditions. Three conditions were considered: w100 control (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Results represent means of all the ratios between the diameter of the halo and the diameter of the colonies of all bacteria strains isolated from each condition. n (w100) = 68; n (w50) = 51; n (w0) = 45. Error bars represent standard error of the mean. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth), no significant difference was observed.

### 3.5. Bacteria osmotolerance

Bacterial strains' osmotolerance was evaluated, growing each strain in YMA medium supplemented with PEG 20% and comparing each diameter to bacteria grown only in YMA medium. Of all 168 the bacteria tested, a total of 8 were not able to grow in PEG medium, 3 from w100i, 1 from w50i, 2 from w100o, and 2 from w50o. Furthermore, from plants grown under control condition 11 bacteria isolated from inside the roots had a bigger colony diameter when growing on YMA medium supplemented with PEG (Figure 12A), and 7 from outside the roots (Figure 12D). From plants 50% irrigated, 2 bacteria isolated from inside the roots had a bigger colony diameter when growing on YMA medium supplemented with PEG (Figure 12B), and 1 from outside the roots (Figure 12E). From plants 0% irrigated, 7 bacteria isolated from inside the roots had a bigger colony diameter when growing on YMA medium supplemented with PEG (Figure 12C), and 1 from outside the roots (Figure 12F).

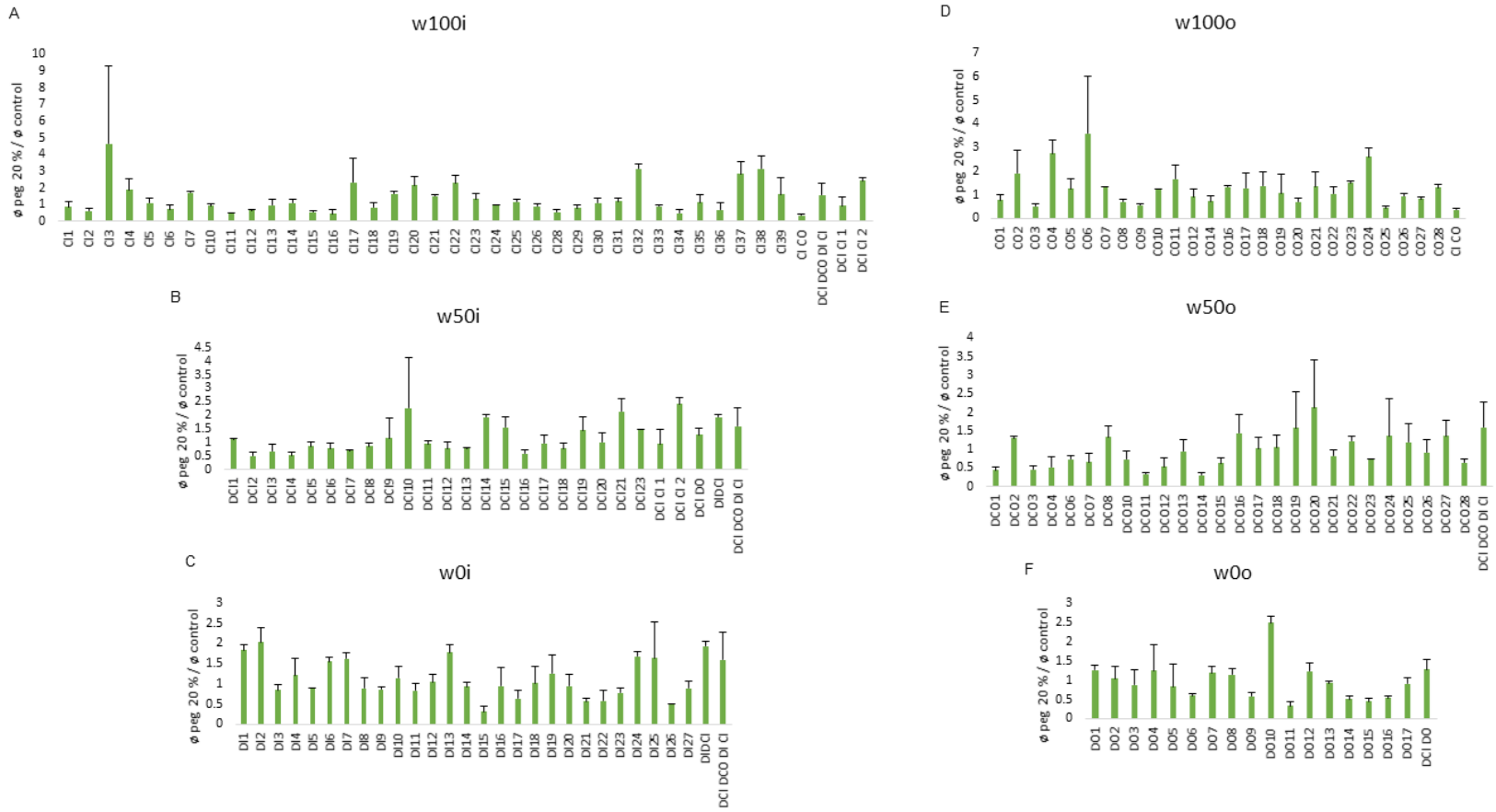


**Figure 12.** Bacteria osmotolerance of bacterial strains isolated from inside (A, B, and C) and outside (D, E, and F) the root of maize plants grown under different water conditions. Three conditions were considered: A and D – w100 control condition (100% irrigated), D and E – w50 (50% irrigated), C and F – w0 (0% irrigated). Results represent the diameter of the colonies growing under control conditions (dark green) and PEG 20% (light green). Values are means of three replicates and error bars represent standard deviation.

Comparing osmotolerance of bacterial strains isolated from different conditions, it is observable a significant lower ratio between the diameter of the colony grown on YMA medium supplemented with PEG 20% and the diameter of the colony grown solely on YMA medium in w50o relative to w100i, w50i, and w100o. This reduction is not significant when comparing w50o with w0i and w0o. All the other condition, w100i, w50i, w0i, w100o, and w0o were not significantly different. The highest ratio was obtained when comparing the diameters of strains isolated from w100i (1.33), and a similar ratio value was determined with w100o (1.27), as a result of only a 4.5% higher ration on w100i. Comparing w50i (1.19) and w50o (0.92), the ratios obtained were 22.4 % higher in the first condition mentioned, which was a significant increase. Likewise, the ratios obtained with bacteria from w0i (1.11) were 13.7% higher than bacteria from w0o (0.96), but this difference was not significant (Figure 13).

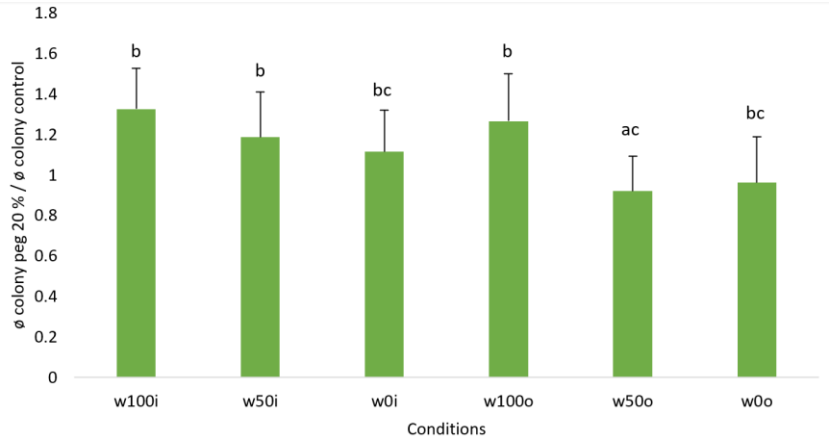


**Figure 13.** Osmotolerance of bacterial strains isolated from inside (w100i, w50i, and w0i) and outside (w100o, w50o, and w0o) the root of maize plants grown under different water conditions. Three conditions were considered: w100 control condition (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Results represent means of the ratios between the diameter of colonies grown under stress from PEG 20% and the diameter of colonies grown on YMA medium, from all bacteria strains isolated from each condition. n (w100i) = 40; n (w50i) = 27; n (w0i) = 29; n (w100o) = 27; n (w50o) = 27; n (w0o) = 18. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth). Error bars represent standard error of the mean (SEM), and lower-case letters indicate significant differences between conditions (p-value < 0.05).

Comparing osmotolerance of bacterial strains isolated from different water stress conditions, there was a significant higher ratio between the diameter of the colony grown on YMA medium supplemented with PEG 20% and the diameter of the colony grown solely on YMA medium on bacterial strains isolated from 100% irrigated condition than strains from w50, recording a 19.3% higher ratio. This increase is also noticeable when comparing

strains isolated from 100% irrigated condition with strains isolated from 0% irrigated condition, however this increase is not significant, being only 18.9% higher. Similar ratio values were determined when comparing strains osmotolerance of bacteria isolated from w50 and w0 (Figure 14).

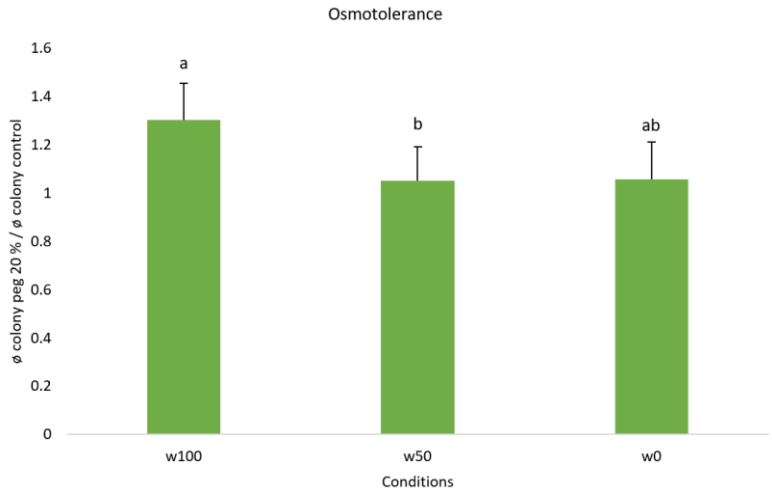


**Figure 14.** Osmotolerance of bacterial strains isolated from the root of maize plants grown under different water conditions. Three conditions were considered: w100 control (100% irrigated), w50 (50% irrigated), and w0 (0% irrigated). Results represent means of the ratios between the diameter of colonies grown under stress from PEG 20% and the diameter of colonies grown on YMA medium, from all bacteria strains isolated from each condition. n (w100) = 67; n (w50) = 54; n (w0) = 47. Permanova+ tests were performed using Primer (PRIMER-e, Plymouth). Error bars represent standard error of the mean, and lower-case letters indicate significant differences between conditions (p-value < 0.05).

## 4. Discussion

In this study, it was noticeable how maize productivity was affected by drought stress. Maize grain weight, thousand grains weight, and specific weight was lower when maize was 0% irrigated, relative to maize 50% and 100% irrigated. In fact, accordingly to a meta-analysis, maize yield may experience a 39.3 % reduction at approximately 40% water reduction shows a (Daryanto et al., 2016). Also, mild and severe water stress reduced by 63 and 85% the final grain yield in maize (Earl and Davis, 2003). This reduction in maize grain yield and could be due to three main processes. First, leaf area expansion and early leaf senescence induced by drought can reduce whole canopy absorption of incident photosynthetically active radiation (PAR) (Bennett et al., 1986; Wolfe et al., 1988; Xianshi et al., 1998). Secondly, radiation use efficiency (RUE) is reduced by drought because water deficit reduces the efficiency with which absorbed PAR is used by the crop to produce new dry

matter (Bennett et al., 1986; Stone et al., 2001). Thirdly, harvest index (HI) is reduced due to drought stress if the exposure occurs during a fundamental development stage around silking (Earl and Davis, 2003). Drought stress can reduce photosynthate availability needed for ovaries development (Bassetti and Westgate, 1993; Schussler and Westgate, 1991), it can additionally reduce silk receptivity consequentially preventing ovary fertilization (Bassetti and Westgate, 1993) and reduce kernel water potential therefore causing kernel to stop growing too soon (Grant et al., 1989; Schussler and Westgate, 1991). However, to understand how drought stress affected maize plants in our study, further investigation should be made, taking into consideration these three parameters mentioned.

Due to global changes and consequentially drought increase, crops productivity will be affected, thus there is a lot of interest in the study of PGPR in different plant species to understand how to use them to benefit agriculture. In the present study, the diversity of bacteria harbored in the roots of maize growing under three different conditions, control and two different drought conditions, was explored.

Rhizosphere bacteria play a critical role as plant growth promoters, induction of disease resistance (Kent and Triplett, 2002; Reuben et al., 2008), and improving tolerance to abiotic stress such as drought (Yang et al., 2009). Rhizosphere and, consequentially, rhizobacteria are influenced by exudation, because bacteria utilize carbon sources exuded by roots for their growth, and different type and amount of exudation is associated with different types of bacterial communities for various plants (Marschner et al., 2001; Smalla et al., 2001). Also, abiotic factors, such as drought, can impact bacterial community composition and abundance (Sanaullah et al., 2011), due to the direct effect of physical stress on the microorganisms, as well as indirectly because of alterations in plant root exudation (Yang et al., 2017). In fact, some bacterial communities can sense plant signal molecules under stress, triggering increase or decrease on microbial populations (Naylor et al., 2017; Ullah et al., 2019). It has been previously reported a decrease in rhizosphere microorganisms in 20 selected tropical garden plants when exposed to drought stress (Reuben et al., 2013). Additionally, drought stress caused alterations on microbial community and the enrichment of specific microbial species in peanut rhizosphere (Dai et al., 2019). Likewise, the total number of CFUs decreased when soil from grassland in Scotland was submitted to water stress (Griffiths et al., 2003). Similarly, soil from alfalfa rhizosphere had a reduced number of CFUs per g of soil under water stress conditions (Bogino et al., 2013). Similarly, in this study, the maize microbial community under normal conditions and drought stress was examined through counting of CFU. Although root

exudation was not determined and quantified, it is noticeable how drought affected rhizobacteria communities since CFU is significantly lower when maize was not irrigated relative to watered conditions. This may happen due to inhibition or killing of sensitive species and selection of tolerant species by drought stress interference (Bérard et al., 2011). Nevertheless, it is important to notice how values determined for 100% and 50% irrigated conditions were similar.

Furthermore, the response pattern of bacteria isolated from inside and outside the root is different. The number of strains isolated from inside the root of 100% irrigated maize is higher than both 50% and 0% irrigated maize which had similar values. However, the number of strains isolated from inside the root of 100% irrigated maize is similar to 50% irrigated maize, and the lowest value was obtained in 0% irrigated maize. The reduction in strains isolated from outside the root of maize 0% irrigated relative to inside the root may happen because endophytic bacteria are not affected by competition with other microorganisms present in the rhizosphere and can accomplish more profound interaction with plant tissue (Naveed et al., 2014), resulting in a higher protection from drought stress. Additionally, Santos-Medellín et al. (2017), reported that compartment was the main source of variation in rice root-associated bacterial communities, as a result of a potential differential response to water deficit in the three compartments considered: rhizosphere, endosphere, and bulk soil (Santos-Medellín et al., 2017). Moreover, drought effect was the highest in the endosphere and rhizosphere, because of the differences in water deficit effect on both communities (Santos-Medellín et al., 2017). Also, the plant itself could enhance the changes in bacterial communities as drought triggers a complex molecular and physiological response to which associated microbes can actively react (Sheibani-Tezerji et al., 2015). For example, to the enrichment of particular bacteria in the endosphere could be facilitated by root exudation (Henry et al., 2007; Song et al., 2012) and synthesis and accumulation of osmolytes in the root (Janiak et al., 2016). This may explain the similar values in strains isolated from 50% and 0 % irrigated maize inside the root, and 100% and 50% irrigated maize outside the root. Nevertheless, to our understanding, there is a lack of knowledge in how maize microbiome is affected under drought stress. Thus, further studies should uncover how maize is affected by changes in the composition and abundance of bacterial communities.

The BOX-PCR profiles were considered different when the similarity was equal to or lower than 93%. After dendrogram analysis, molecular typing of bacterial isolated yield 168 distinct typing profiles. BOX-PCR fingerprinting is an accurate technique to differentiate bacterial strains, determining genetic relatedness and diversity (Kim et al., 2002), and

identifying at the strain level bacteria otherwise impossible to distinguish only through their morphology (Borah et al., 2019). Thus, this procedure is particularly important in studies where many samples are considered (Gardan et al., 1999; Marques et al., 2008; Viana et al., 2020). However, BOX-PCR fingerprinting is not enough to know the bacteria diversity, so identification is needed and 16S ribosomal ribonucleic acid (16S rRNA) sequencing could be applied to identify isolates at the genus level. In fact, bacteria diversity associated with maize roots has been previously documented, and several genera has been considered to potentially promote plant growth, for instance bacteria of the genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Enterobacter* (Bomfim et al., 2020; Ikeda et al., 2020; Khan, 2019; Nascimento et al., 2021; Szilagyi-Zecchin et al., 2014). Furthermore, PGPR intended for use as inoculants must be identified to the species level to exclude strains that are pathogenic to plants, animals, and humans (Benami et al., 2013).

PGPR have mechanisms through which they are able to promote plant growth (Ahemad and Kibret, 2014). Hence, mechanisms of plant growth promotion were assessed, particularly phosphate solubilization and siderophore production.

Phosphorus is the second most important nutrient to plant growth, only behind nitrogen (Khan et al., 2009), but its availability for plants is usually low, since plants can absorb it only in the monobasic ($H_2PO_4^-$) and the dibasic ($HPO_4^{2-}$) ions, and most of the soil phosphorus is found in insoluble forms (Bhattacharyya and Jha, 2012). To increase availability of phosphorus in agriculture fields, there are frequent applications of phosphatic fertilizers, but plants absorb small amounts of it and the rest is rapidly converted into insoluble forms (McKenzie and Roberts, 1990). This regular application is costly and detrimental for the environment. Therefore, alternative options have been applied to improve crop production in an ecological and economical improved way (Ahemad and Kibret, 2014). In this context, several bacteria genera have been reported as significant phosphate solubilizing bacteria, as they are able to solubilize inorganic phosphorus through action of low molecular weight organic acids, thus providing the available forms of phosphorus to plants (Zaidi et al., 2009). As a result, bacterial genera like *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Pseudomonas, Rhizobium and Serratia* are promising biofertilizers, supplying plants with phosphorus otherwise insoluble and unavailable for plants (Bhattacharyya and Jha, 2012). In this study, no strain was able to solubilize phosphate, however there are reports of bacteria isolated from maize with this ability, for instance bacteria of the genus *Bacillus*, including *B. amyloliquefaciens*, *B. megaterium*, and *B.*

*subtilis*, isolated from maize (Bomfim et al., 2020; Nascimento et al., 2021; Babu et al., 2017).

Similarly, iron is another essential nutrient for plant development, as it acts as an enzyme cofactor in biochemical pathways involved in plant physiological processes, like respiration, photosynthesis, and biological nitrogen fixation (Sansinenea, 2019). Usually, iron is inaccessible to both plants and microorganisms as it occurs as $Fe^{3+}$ and is prone to form insoluble hydroxides and oxyhydroxides (Rajkumar et al., 2010). Consequentially, bacteria acquire iron through siderophore, low-molecular mass iron chelators, secretion which have high association constants for complexing iron (Ahemad and Kibret, 2014). Since siderophores act as solubilizing agents for iron under conditions of iron limitation (Indiragandhi et al., 2008), and are able to form stable complexes with other heavy metals such as Al, Cd, Cu, Ga, In, Pb and Zn, and radionuclides including U and Np (Kiss and Farkas, 1998; Neubauer et al., 2000), bacterial siderophores are able to mitigate stress caused by heavy metals on plants (Ahemad and Kibret, 2014). In fact, there are reports of plant growth promotion associated with siderophore-mediated Fe-uptake when plants are inoculated with siderophore producing rhizobacteria (Rajkumar et al., 2010). For instance, siderophores produced by rhizosphere microorganisms deliver iron to oat (Crowley and Kraemer, 2007), *Pseudomonas fluorescens* C7 contributed to increase of iron inside Arabidopsis thaliana plant tissues, improving plant growth (Vansuyt et al., 2007), and inoculation of *Pseudomonas* strain GRP3 led to a decline in chlorotic symptoms and iron chlorophyll a and chlorophyll b content increased on *Vigna radiate* when compared to non-inoculated plants (Sharma et al., 2003). In the present study, a total of 154 (91.7%) strains were able to produce siderophores, indicating their significance in the context of plant growth. However, further testing must be done to test the potential of these bacterial strains under greenhouse and field conditions, to explore their utility and elucidate the mechanisms of growth promotion under more realistic scenarios.

Understanding strains osmotolerance is important when looking for potentials candidates to improve crops tolerance to drought, to know how well they tolerate stress (Manjunatha et al., 2019). Bacteria isolated from conditions subject to water deficit had a lower osmotolerance. Comparing osmotolerance of bacterial strains isolated from different conditions, it is observable a significant lower ratio between the diameter of the colony grown on YMA medium supplemented with PEG 20% and the diameter of the colony grown solely on YMA medium in w50o relative to w100i, w50i, and w100o. Manjunatha et al. (2019) reported a higher osmotolerance in endophytic bacteria isolated from drought-tolerant pearl

millet (Manjunatha et al., 2019). In fact, endophytic bacteria are able to induce tolerance to stress, allowing plants survival (Kumar and Verma, 2018; Manjunatha et al., 2017). On the other hand, osmotolerance of bacteria isolated from wild legume species was not significantly different among different climatic conditions (Cardoso et al., 2018).

## 5. Conclusion

Our study shows that drought stress deceases maize grain yield and has an impact on the bacterial community associated with maize roots, leading to a decline in microorganisms. This can consequently affect plant development since rhizosphere bacteria play a critical role as plant growth promoters, induction of disease resistance (Kent and Triplett, 2002; Reuben et al., 2008), and improving tolerance to abiotic stress such as drought (Yang et al., 2009). Drought exposure also affected the number of strains isolated from each condition, but bacterial identification is needed to further understand how bacterial community diversity was affected. Nevertheless, several strains were able to produce siderophores which is an important characteristic associated with growth promotion because of siderophore-mediated Fe-uptake (Rajkumar et al., 2010). Also, bacteria isolated from conditions subject to water deficit had a lower osmotolerance.

# Chapter V

# Final remarks and future work

With the likelihood that changes in global climate and increase in global temperature will adversely affect crop yields, it is crucial to take to our advantage sustainable methods to enhance plant development, for instance potential microbial communities and plant growth promoting rhizobacteria. It is important to know which are the best rhizobacteria to apply in the field, promoting plant growth and tolerance to abiotic stress like drought, whilst understanding how water deficit affects microbials communities and characterize and isolate rhizobacteria from maize roots which is one of the most produced and consumed cereals globally, to improve maize production.

In this thesis, three strains, two *Pseudomonas* and a *Flavobacterium* successfully increased maize growth in greenhouse conditions. However, the same was not verified in the field. This is to expect, because field conditions are more unpredictable and harder to control, which could affect bacterial influence in plant growth.

Furthermore, inoculation with a bacterial consortium promoted maize growth under drought conditions in the greenhouse, which is a possible indicator of alleviation to drought stress. The same was not observed in field cultivated maize, although inoculation lessened the adverse effect of drought stress on the antioxidant enzymes.

Additionally, drought stress had an adverse impact in maize grain yield and the bacterial community associated with maize roots. Water deficit significantly reduced the number of microorganisms, nevertheless identifications is vital to better understand the impact on the bacterial community. Even so, several strains were isolated and were able to produce siderophores which are associated with plant growth promotion.

Some topics left unanswered and could potentially be studied in the future are:

- Test double application of PGPR in the greenhouse and field tests to know if maize growth is enhanced
- Test PGPR ability in the strains used in the bacterial consortium to better understand how they might be mitigation drought stress
- Study the bacterial strains of the consortium individually under drought to know which strains have more response or if the response is similar even when strains are inoculated individually
- Perform phylogenetic characterization of the strains isolated from maize roots to know how strains are distributed in the different conditions, similarities, and differences in the bacterial community
- Isolate rhizobacterial strains from different stages of maize grown under drought stress to assess changes in bacterial community in the different stages when maize is under drought stress

- Further explore the bacterial diversity in maize under different water regimes using a metagenomic approach
- Screen isolated strains from maize under different water regimes under greenhouse conditions to look for candidates to apply in the field
- Apply potential candidates in the field, testing their ability to mitigate drought stress
- Test how other abiotic conditions, frequently associated with drought, such as high temperatures, would impact PGPR abilities
- Adapt maize inoculation in the field to improve consortium ability to mitigate drought stress
- Develop and implement the commercial production of the bacteria

# Chapter VI

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## Supplementary material

**Supplementary Table 1.** Bacterial strains applied in the greenhouse and field trials. For each strain, the respective code is presented as well as the greenhouse inoculum's optical density (OD) and the pre-inoculum's OD for the field trial.

| **Strain 2 plants** | **Genera** | **OD greenhouse inoculum** | **OD field pre-inoculum** |
|---|---|---|---|
| A7 | *Pseudomonas* | 0.42 | - |
| B11 | *Pseudomonas* | 0.51 | - |
| B3 | *Flavobacterium* | 0.6 | - |
| C4 | *Flavobacterium* | 0.46 | - |
| D1 | *Flavobacterium* | 0.46 | - |
| D3 | *Pseudomonas* | 0.49 | - |
| D6 | *Flavobacterium* | 0.9 | - |
| D9 | *Flavobacterium* | 0.33 | - |
| E20-8 | *Rhizobium* | 0.49 | - |
| G5 | *Pseudomonas* | 0.49 | - |
| H2 | *Pseudomonas* | 0.44 | - |
| H8 | *Flavobacterium* | 0.47 | - |
| I3 | *Pseudomonas* | 0.42 | - |
| I7 | *Pseudomonas* | 0.44 | - |
| I9 | *Pseudomonas* | 0.47 | 1.19 |
| K4 | *Erwinia* | 0.44 | - |
| L1 | *Flavobacterium* | 0.54 | - |
| M2 | *Flavobacterium* | 0.52 | - |
| N1 | *Herbaspirillum* | 0.63 | - |
| N2 | *Pseudomonas* | 0.42 | - |
| N7 | *Pseudomonas* | 0.65 | - |
| N9 | *Variovorax* | 0.45 | - |
| O1 | *Pseudomonas* | 0.66 | - |
| O7 | *Pseudomonas* | 0.6 | - |
| P1 | *Pseudomonas* | 0.58 | - |
| P3 | *Pseudomonas* | 0.59 | - |
| P8 | *Pseudomonas* | 0.57 | - |
| Q5 | *Flavobacterium* | 0.45 | - |
| Q7 | *Flavobacterium* | 0.56 | - |
| Q8 | *Flavobacterium* | 0.67 | - |
| T4 | *Pseudomonas* | 0.48 | - |
| T8 | *Pseudomonas* | 0.53 | - |
| T9 | *Pseudomonas* | 0.58 | - |
| U1 | *Acinetobacter* | 0.55 | - |
| U3 | *Flavobacterium* | 0.42 | - |
| U6 | *Agrobacterium/Rhizobium* | 0.67 | - |
| W4 | *Pseudomonas* | 0.48 | - |
| W5 | *Pseudomonas* | 0.46 | - |

| **Strains 1 plant** | | | |
|---|---|---|---|
| A10 | *Paenibacillus* | 0.72 | - |
| B8 | *Pseudomonas* | 0.67 | - |
| C1 | *Flavobacterium* | 0.65 | - |
| C11 | *Pseudomonas* | 0.52 | - |
| D2 | *Flavobacterium* | 0.51 | - |
| D5 | *Flavobacterium* | 0.57 | - |
| E1 | *Flavobacterium* | 0.48 | - |
| H1 | *Pseudomonas* | 0.62 | - |
| I3 | *Pseudomonas* | 0.54 | - |
| J5 | *Flavobacterium* | 0.48 | - |
| L8 | *Flavobacterium* | 0.57 | - |
| M9 | *Flavobacterium* | 0.51 | - |
| O3 | *Herbaspirillum* | 0.75 | - |
| P9 | *Flavobacterium* | 0.56 | - |
| Q1 | *Flavobacterium* | 0.5 | - |
| R4 | *Flavobacterium* | 0.7 | - |
| R7 | *Flavobacterium* | 0.65 | - |
| R8 | *Pseudomonas* | 0.66 | - |
| S4 | *Pseudomonas* | 0.67 | - |
| T1 | *Pseudomonas* | 0.72 | 1.16 |
| T6 | *Pseudomonas* | 0.72 | - |
| T7 | *Flavobacterium* | 0.49 | 0.48 |
| U7 | *Flavobacterium* | 0.7 | - |
| U9 | *Flavobacterium* | 0.64 | - |
| X1 | *Pseudomonas* | 0.78 | - |

**Supplementary Table 2.** Optical density (OD) of the control and bacterial strains of the inoculums applied in the field.

| **Condition** | **OD** |
|---|---|
| Control | 0.000 |
| T1 + I9 | 0.157 |
| T7 | 0.363 |

**Supplementary Table 3.** Bacterial strains applied in the greenhouse and field trials. For each strain, the respective code is presented as well as the optical density (OD) of the greenhouse inoculum and the OD of the pre-inoculum for the field trial.

| **Code** | **Bacterial strain** | **OD inoculum greenhouse trial** | **OD pre-inoculum field trial** |
|---|---|---|---|
| 3-3-1-A | *Pseudomonas plecoglossicida* (Pse4) | 0.59 | 0.78 |
| 3-2-1-A | *Enterobacter cancerogenus* (E3) | 0.44 | 1.40 |
| 2-3-3-B | *Enterobacter cancerogenus* (E3) | 0.77 | 1.22 |
| 1-2-3-A | *Enterobacter CP034769_s* (E2) | 0.71 | 1.10 |
| 3-3-2-B | *Pseudomonas batumici* (Pse1) | 0.43 | 1.27 |
| 2-2-3-B | *Enterobacter CP034769_s* (E3) | 0.87 | 1.42 |

| | | | |
|---|---|---|---|
| 1-3-1-A | *Pseudomonas batumici* (Pse1) | 0.14 | 0.38 |
| 1-2-2-B | Not identified | 0.33 | 0.53 |
| 3-1-3-A | *Curtobacterium luteum* (C1) | 0.53 | 0.70 |
| 1-2-3-B | Not identified | 0.71 | 1.95 |
| 1-2-1-B | Not identified | 0.73 | 1.11 |
| 2-1-2-A | *Pseudomonas batumici* (Pse1) | 0.49 | 0.90 |

**Supplementary Table 4.** Optical density of the control and bacterial consortium of the inoculum applied in the field.

| **Condition** | **OD** |
|---|---|
| Control | 0.000 |
| Bacterial consortium | 1.087 |