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EFFECT OF THE INOCULATION OF PLANT GROWTH PROMOTING BACTERIA ON MAIZE PLANTS TO IMPROVE THEIR GROWTH UNDER DROUGHT STRESS

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa*
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ABSTRACT

Global warming and bad agricultural practices are affecting the environment, with an impact on food productivity. Applying sustainable practices in agriculture is essential to maintain soil fertility and to promote crop productivity. It has been demonstrated that plant growth promoting bacteria (PGPB) have positive effects on plants because of their ability to influence plant's growth through direct and indirect effects, such as production of phytohormones, increase of nutrient availability and/or synthesis of biocontrol agents. From Escola Superior de Biotecnologia collection of PGPB, thirteen strains were screened *in vitro* for abilities such as osmotic tolerance and indole acetic acid production under different osmotic potentials. Two bacterial strains were selected, *Pseudomonas fluorescens* (S3X) and *Ralstonia eutropha* (1C2) for a greenhouse assay using maize (*Zea mays*) as target plant to assess their effect on plant productivity under water stress. Different inoculation treatments were applied (control, commercial inoculum, S3X, 1C2 and co-inoculation of both strains) as well as different inocula size (V1 and V2, 3.3×10^3 and 2.5×10^6 cell. g^{-1} dry weight, respectively). Maize plants were subjected to different water regimes (80, 60 and 40% of water holding capacity (WHC)). The water regime at 40% of WHC affected to higher extent plant growth, such as elongation and biomass, as well as plant nutrient uptake and activity of soil enzymes. Application of the commercial inoculum proved to be the less effective treatment in enhancing plant growth under the water stress deficit. The highest improvement on plant growth was observed with the co-inoculation of strains S3X and 1C2 at 80 and 60% of WHC and no differences were observed between the two inocula size applied. The co-inoculation improved shoot biomass by 20 and 28% in V1 and by 16 and 47% in V2 at 80 and 60% of WHC, respectively. The consortia at 60% caused 11% increase in root biomass in V2. Nitrogen and phosphorous accumulation in shoot and root was also improved by the addition of both strains into the rhizosphere at 80 and 60%. Nutrient use efficiency was also calculated and the inoculation of both strains enhanced this parameter. Overall, the highest enhancement of plant performance occurred when the mixture of *P. fluorescens* S3X and *R. eutropha* 1C2 was used, with similar effects with the two inocula size. Combinations of PGPBs may prove to be of significant advantage for the design of bioinoculants for agriculture.

Keywords: drought, maize, PGPB.

RESUMO

O aquecimento global e práticas agrícolas desadequadas estão a afetar o meio ambiente, causando um grande impacto na produtividade alimentar. Deste modo, o uso de práticas sustentáveis na agricultura é essencial para manter a fertilidade do solo e para promover o aumento da produtividade de culturas economicamente importantes. Vários estudos demostraram que o uso de bactérias promotoras do crescimento vegetal (do inglês PGPB) tem efeitos positivos em diferentes espécies, o que está relacionado com a sua capacidade em produzir fitohormonas, com o aumento da absorção de nutrientes e/ou com a síntese de agentes de biocontrolo. Da coleção da Escola Superior de Biotecnologia de PGPB, foram testadas treze estirpes *in vitro* para avaliar a sua tolerância osmótica e a produção de ácido indolacético sob condições de stresse osmótico. Foram selecionadas duas estirpes, *Pseudomonas fluorescens* (S3X) e *Ralstonia eutropha* (1C2), para avaliar o seu efeito de promoção de crescimento de milho (*Zea mays*) quando sujeitas a diferentes regimes hídricos (80, 60 e 40% de capacidade de retenção água no solo). Os tratamentos incluíram diferentes regimes de inoculação (controlo, inóculo comercial, S3X, 1C2 e co-inoculação de ambas as estirpes) assim como diferentes volumes de inóculo (V1 e V2 -$3.3x10^3$ e $2.5x10^6$ células. g^{-1} peso seco, respetivamente). O regime hídrico severo foi o que mais afetou o crescimento das plantas, ao influenciar negativamente parâmetros de crescimento, tais como elongamento e biomassa, absorção de nutrientes e a atividade de algumas enzimas no solo. A aplicação do inóculo comercial mostrou-se menos eficaz na promoção de crescimento das plantas sob o stress hídrico. A co-inoculação das estirpes S3X e 1C2 resultou no tratamento mais vantajoso para o crescimento das plantas, no entanto os volumes de inóculo aplicados não afetaram a performance das plantas. A co-inoculação promoveu um aumento de 20 e 28% de biomassa na parte aérea das plantas no tratamento V1 e 16 e 47% no V2 a 80 e 60% de humidade, respetivamente. Também causou um aumento de 11% na biomassa das raízes sob 60% de humidade no tratamento V2. A acumulação de azoto e fósforo, tanto na parte aérea como nas raízes das plantas, também foi positivamente influenciada pela co-inoculação das estirpes e ainda a inoculação de ambas as estirpes incrementou a eficiência de uso de nutrientes. De forma geral, o melhor desempenho das plantas ocorreu quando a co-inoculação das estirpes *P. fluorescens* S3X e *R. eutropha* 1C2 foi usada, sendo que as combinações de PGPB podem constituir uma grande vantagem para o desenho de bioinoculantes para a agricultura.

Palavras-chave: seca, milho, PGPB.

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1. INTRODUCTION

Agriculture is the production of food by growing crops and livestock to provide the most basic of human needs and can be classified in two different categories: crop farming, in which plant photosynthesis is used to produce grain, fruit, and fiber, and livestock farming for the production of meat, milk, wool, and other animal products[1].

According to the United Nations, the current world population is 7 billion people and is estimated that by 2050 the population will reach 9.6 billion people[2], which implies a major challenge for the agricultural sector to improve crop production and to ensure food availability in the near future.

However, some agricultural practices, such as the application of artificial fertilizers, displacement of native plants, destruction of wildlife habitats, erosion, and others, have brought environmental problems and a decrease in the fertility of agricultural lands[1]. Moreover, global warming is another issue of concern because it represents a severe threat to agriculture. In addition, crops are constantly exposed to a variety of environmental stresses that affect their growth and development as a consequence of bad agricultural practices and global warming. This means that plants are subject to several abiotic stresses including salinity, drought and temperature fluctuation, which most likely occurs simultaneously[3].

Drought is one of the major limiting factors for plant growth because it is vital for them to constantly absorb and evaporate water. The mechanism used by plants to absorb water is through the roots, and the loss of water occurs by the stomata in the leaves through transpiration[4]. Thus, drought stress is the most devastating environmental problem that affects agricultural productivity and occurs when the available water in the soil is limited and the atmospheric conditions causes continuous loss of water by transpiration or evaporation[5]. In fact, drought could be chronic in areas where there is a low availability of water, or random, due to acute weather conditions, being these effects more notorious in arid and semi-arid areas.

Therefore, growth and development of plants have been compromised, leading to the reduction in crop yield and serious threats to food security. The reason why drought affects plant growth and development is related to water deficit. Plants suffers dehydration and osmotic stress, which leads to nutrient deficiency and promotes hormonal imbalance[6]. Water deficit stress can also reduce photosynthesis by decreasing the amount of chlorophyll synthesized by plants[7]. The reduction occurs due to the damage of chloroplasts caused by reactive oxygen species (ROS) produced during stressful conditions[8].

Consequently, there is a decrease in crop yield and in food security, affecting thousands of people globally and representing huge losses in the economics of agriculture and forestry[6]. In response to water deficit stress, plants can promote physiological responses to increase the water availability and survive until overcoming the stress:

1) **Abscisic acid (ABA) increases.** ABA is a phytohormone produced in response to stress, particularly osmotic stress. When plants are exposed to drought and to prevent lack of water, the levels of ABA reaches high concentrations, stimulating the stomata closure and avoiding water loss through transpiration[9]. High concentrations of ABA induce stomata closure by stimulating the production of ROS, alkalization and changes in Ca^{2+} and K^{+} fluxes within the cells[10].

2) **Accumulation of compatible solutes.** Production and accumulation of compatible solutes called osmolytes, which include aminoacids like glutamate, glutamine, proline, alanine, etc; quartenary amines like glycinebetaine and sugars like sucrose, trehalose and polyglucosyl granules[11]. The accumulation of these compatible solutes improves cell growth because they reduces water potential in the cytoplasm and maintains the cell turgor in order to prevent a degenerative process[11].

3) **Increase expression of aquaporins.** Aquaporins are pores that facilitate the passive diffusion of water across cells membranes. During water deficit, there are changes in the post-transcriptional regulation of aquaporin, increasing their expression since aquaporin activity is known to be controlled by phosphorylation. Thus, with abundant aquaporins, cells can maintain turgor during drought[12].

4) **Enzymatically and non-enzymatically changes in the cell wall**. Some alterations in the cell wall could cause a disruption of the membrane integrity of the cell[3], these changes could be:

4.1) **Enzymatic changes**, which can be related to alterations of the cell wall, for example by ROS activity. These species are produced as natural bioproducts of photosynthesis and respiration and have an important role in cell signaling and homeostasis[13]. However, when plants are exposed to environmental stresses, such as drought, ROS levels can rise and as a consequence a significant damage in cell structure can occur, leading up to lipid peroxidation, protein degradation, membrane injury and necrosis[14].

4.2) **Non-enzymatic changes,** involves a negative impact on the interaction between pectate and calcium in the cell wall. Pectate is a product obtained by the degradation of pectin in plants and cytosolic calcium (Ca^{2+}) is an essential element for the growth and development of plants because it determines the structural rigidity of the cell wall[15].

During the development of cell wall, the acidic pectin residues are secreted as pectin methylesterase, releasing carboxyl groups that will bind to the calcium. Therefore, calcium is important in the cross-linking acidic pectin residues. So, lower concentration of calcium will weaken the cell wall and will be easily ruptured, whereas higher concentration of calcium will make the cell wall thicker[15].

According to Instituto Português do Mar e da Atmosfera,(IPMA) the geographical situation of Portugal is favorable for the occurrence of drought, which has detrimental consequences in the agriculture, livestock, water resources and well-being of people. Drought in Portugal is specially accentuated in the central and southern regions, being these lands more vulnerable and more affected over the years[16].

During the years 2004 – 2006, it was recorded one of the most severe drought periods in Portugal, which affected 100% of national territory and being one of the most intense, taking into account the number of consecutive months presenting extreme and severe drought[17].

Actually, IPMA calculated the Palmer Drought Severity Index (PDSI) over the past decades to observe how drought has evolved among the years in Portugal. PDSI is a meteorological index that detect the length of time of drought period and classifies them according to their intensity. The study concluded that the last decades have presented more frequently drought situations, which indicates an increase risk and vulnerability to this phenomenon and consequently an impact in the agriculture[17/18].

Re-valorization of soils is an important issue nowadays to enhance crop productivity, especially in soils under stress conditions. Over time, genetic engineering has been developed in order to implement a sustainable agriculture and enhance drought stress tolerance in plants through the development of drought-tolerant varieties. Examples of such strategies include the introduction of transgenic crops, with introduced or altered expression of some genes. However, these strategies are expensive, time consuming and have some limitations related to ethical issues[19].

Application of bioinoculants, such as plant growth promoting bacteria (PGPB) which can be rhizospheric or endophytic and arbuscular mycorrhizal fungi (AMF) into the rhizosphere of plants with high economic value, has the potential to become a strategy for sustainable agriculture [20]. The application of bioinoculants into the soil has shown positive effects in plant growth and stress alleviation, constituting an attractive and interesting biotechnology tool that could be used to improve plant's survival and growth under limited water availability, overcoming water deficit stress, enhancing soil fertility and improving food productivity[21].

1.1 Plant Growth Promoting Bacteria

Plant growth promoting rhizobacteria constitutes a heterogeneous group of beneficial bacteria that can be found within the soil, specifically in the rhizosphere, at root surfaces or may interact with the roots of plants[22], while endophytic bacteria (EB) correspond to the population of bacteria that colonize the inner tissues of healthy plants[23].

The reason why there is a higher concentration of bacteria in the rhizosphere than in the rest of soil it is because presumably there exists a positive interaction between the microorganisms and the plants. The plants are beneficiaries of this association because bacteria will protect plants from abiotic and/or biotic stresses while microbial population will obtain nutrients like amino acids, sugars and organic acids that are exuded from the roots of plants and that will be used by the microorganisms to enhance their growth and metabolism[24].

Plant growth promoting bacteria can be applied in agriculture because of their functional activities as biofertilizers, by increasing the availability of nutrients to plants; phytostimulators, by producing phytohormones; rhizomediators, by degrading organic pollutants and biopesticides, by the production of antibiotics and antifungal metabolites[25].

The choice of strains that will be inoculated in plants is essential in order to guarantee their resistance and proliferation under the adverse environment caused by drought[26]. The selection of these strains is based on the indirect or direct effects that PGPB have on plants and the interaction established between the roots of host plants and the microorganisms[27].

1.1.1 Indirect plant growth promoting effect

In general, indirect effects occurs when the presence of PGPB in the rhizosphere decreases or prevents the negative effects possibly caused by a pathogen through the synthesis of biocontrol agents, explained in the bellow sections.

1.1.1.1 Antibiotics and antifungal metabolites

Antibiotics are a heterogeneous group of organic, low-molecular-weight compounds that are toxic to the growth or the metabolic activities of other microorganisms[28]. In fact, PGPB have the ability to secrete these extracellular metabolites in order to prevent the harmful effects or even the proliferation of plant pathogens at low concentrations. This type of ability is defined as antagonistic activity and in this case the antibiotics synthesized by PGPB have antiviral, antibacterial and anthelmintic activity and are insect and mammalian antifeedant[29].

Different types of antibiotics can be produced by PGPB, meaning that different mechanisms of action prevent pathogens proliferation, such as the inhibition of the synthesis of their cell walls, the influence on membrane structure of pathogen cells and inhibition of the formation of proteins or ribosomes[30]. In addition to this antipathogenic action, PGPB also synthesizes antibiotics that contribute to disease suppression[15] and this type of ability is referred as biocontrol agent. PGPB can have the ability to produce, as well, antifungal metabolites such as hydrogen cyanide (HCN). This is a toxic gas with the property to affect negatively roots metabolism and growth of weeds, nonetheless can also suppress the development of some phytophatogenic organisms and indirectly enhance plant growth[30].

1.1.1.2 Siderophores

Iron (Fe) is one of the most important elements for the survival of living organisms, nonetheless a deficiency of soluble Fe is present in nature. Therefore, organisms living in the rhizosphere enters in competition because of Fe uptake. The fact that PGPB has the ability to synthesize siderophores, which are molecules with high affinity for this nutrient and consequently act as a biocontrol agent, reduces the opportunity of other organisms, which have no ability to produce this agent, such as fungi, to absorb Fe, limiting their proliferation[30].

1.1.1.3 Extracellular enzymes

Plant growth promoting bacteria can synthesize some extracellular enzymes, such as lipases, cellulases, proteases and pectinases[31]. These enzymes can indirectly improve plant growth because they can lyse pathogenic fungal cells walls as a mechanism of fungal inhibition[30].

1.1.2 Direct plant growth promoting effect

It involves the solubilization of nutrients present in the soil and the production of phytohormones that promote plant growth.

1.1.2.1 Facilitating uptake of nutrients

Plant growth promoting bacteria can promote plant growth directly by facilitating the absorption of some nutrients, which are limited in soil, such as nitrogen (N), phosphorus (P) and Fe. These PGPB traits are important in agriculture to obtain a higher yield and to minimize the use of chemical fertilizers, which could represent a risk to human health.

1.1.2.1.1 Nitrogen fixation

Nitrogen is the most vital nutrient for plant growth and development. However, N is unavailable for direct use by most organisms because it has a triple bond between the two N atoms, making the molecule almost inert. In order to be absorbed by plants, it must be fixed to ammonium by some bacteria[32]. The ability of some bacteria to fix atmospheric N depends on the synthesis of two enzymes, dinitrogenase and dinitrogenase reductase[27]. These enzymes catalyze the reduction of atmospheric N into ammonia.

The N-fixation can occur either symbiotically or non-simbiotically[33]. Symbiotic N-fixation is referred to the mutualistic relationship between bacteria and plants. The process is based on the entry of the microorganism into the roots of plants and the formation of nodules in which the N fixation occurs. Non-symbiotic interaction is based on the fixation of N by microorganisms without symbiotic association with plants. These microbes are free living and the amount of N fixed is relatively small[34].

1.1.2.1.2 Siderophores

Iron is one of the most abundant elements in nature, being the ferric ion or Fe^{3+} the predominant form. Despite this fact, living organisms rarely assimilate ferric ion because it is slightly soluble, in consequence the amount of Fe available for assimilation by the living organisms is extremely low[30].

To provide Fe to plants, some PGPB have the ability to synthesize siderophores, which are molecules with membrane receptors that have high affinity for Fe. The formation of a complex of Fe - siderophores facilitate the uptake of this nutrient promoting plant's growth[30].

1.1.2.1.3 Phosphate solubilization

Phosphorus is an important mineral element essential for the promotion of plant growth and development. Plants absorb P in a soluble form ($H_2PO_4^-$ and HPO_4^{-2}), however the major proportion of soil P is in an insoluble form, turning their bioavailability limited in soil[35].

Some PGPB have the ability to solubilize soil insoluble P forms in order to make it available for plants. This solubilization can occur by the production of organic acids such as formic acid, propionic acid, latic acid, glycolic acid, fumaric acid, succinic acid, gluconic acid, 2-ketogluconic, oxalic acid, citric acid, acetic acid and malic acid[35]. However, some PGPB can also mineralize the insoluble P through the excretion of extracellular enzymes, namely phosphatases. In fact, these two mechanisms, solubilitazion and mineralization, can coexist in the same PGPB[35], improving the availability of P in soil and enhancing directly plants growth and development.

1.1.2.2 Modulating phytohormone levels

When plants are under stressful conditions, their growth could be compromised and even inhibited until the stress is removed. In such cases, plants can produce phytohormones, which will allow them to overcome the stress and survive in such stressful conditions.

Phytohormones are naturally occurring organic substances which influence the growth and development of plants at low concentrations and there are 5 classes of phytohormones: auxins, cytokinins, gibberellins, ethylene and abscisic acid[30]. PGPB can also synthesize phytohormones, and their presence in the rhizosphere can modify plants hormonal balance and its response to stress.

1.1.2.2.1 Ethylene

The ethylene is essential for normal growth and development in plants. In fact, under normal environmental conditions, plants produce the necessary concentration of ethylene, conferring positive effects on them and regulating many physiological responses[36]. It can promote root initiation, inhibit root elongation, promote fruit ripening and flower wilting, stimulate seed germination, promote leaf abscission and activate the synthesis of other phytohormones[30]. Therefore, many plant tissues are affected by ethylene, as well as all stages of plant development[24].

However, ethylene is also regarded as a “stress hormone” since under stressful conditions, ethylene levels increase[37] and consequently the symptoms of the stress are intensified, leading up to plant growth inhibition, by root shortening, or even death, reducing crop performance[30].

1-aminocyccloprpane-1-carboxylate (ACC) is the immediate precursor of ethylene produced by plants. Under stressful conditions, ACC levels rises, resulting in high levels of ethylene that consequently cause damage in plants[24]. The mechanism that plants have to reduce ethylene concentration is the synthesis of ACC deaminase[24]. Some PGPB strains can synthesize this enzyme, decreasing ethylene levels on plants and consequently its inhibitory effect on growth[31/36].

1.1.2.2.2 Auxins - Indole acetic acid (IAA)

Auxins are important phytohormones that coordinate some plant processes, such as cell elongation, lateral root initiation, vascular differentiation, ethylene production, floral meristem initiation, differentiation of pholoem and xylem, floral bud formation and fruit development[38].

IAA is the most abundant auxin produced by plants and the aminoacid tryptophan is the main precursor, being important to modulate the biosynthesis of this hormone[25]. The presence of IAA in the rhizosphere promotes plant growth because it affects plant cell proliferation, extension and differentiation, stimulates seed to germinate, increases the rate of xylem and root development; controls processes of vegetative growth; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of some metabolites and resistance to stressful conditions[30].

Many rhizobacteria have the potential to synthesize IAA, which means that they can affect the concentration of this phytohormone in the rhizosphere and in consequence, the physiological processes are also altered, promoting plant growth and a greater yield[38].

1.1.2.2.3 Cytokinins and Gibberellins

Cytokinins and gibberellins, are hormones produced by plants and bacteria. Cytokinins are important for plant growth because their production improves tissue expansion, cell division and cell enlargement in plants[34]. When there is a stressful situation, such as drought, cytokinins levels decrease and simultaneously the stomata closes to prevent water loss, contributing to drought tolerance[39].

Gibberellins also improves plant growth because of their metabolic functions such as seed germination, stem elongation, sex expression, flowering, formation of fruits and senescence[40].

1.2 Soil enzymes

A healthy soil is vital for agriculture, therefore there is a direct relationship between soil and food quality. A fertile soil provides the right amounts of essential macronutrients and micronutrients that plants need to survive, as well as oxygen, water and root support[41].

To maintain soil healthy, three components must be in balance, a chemical component, where soil pH, cations, organic matter and nutrients must be in equilibrium, a physical component, where soil texture, density, porosity, erosion and moisture should be in balance and a biological component characterized by the microorganisms living in the soil and their activities[42].

In fact, the activity of enzymes in soil are altered before other soil quality indicators can be easily detectable. The majority of enzymes present in the rhizosphere come from microbial synthesis and their production and activity indicates the state of health of soil. However, not all sources of soil enzymes have origin in living microorganisms. Dead bacteria, plants roots and soil animals can also produce enzymes which accumulate in the soil. This means that enzyme activity includes the cumulative effect of long term microbial activity and activity of viable population.

The importance of soil enzymes relies on: (1) the biochemical functions that they perform through organic matter decomposition, (2) the catalytic reactions that are important in the life process of microorganisms, (3) the organic waste decomposition and (4) nutrient cycling[43].

Soil is a living ecosystem and the synthesis and activity of enzymes is not stable, it can vary over time or it can be different between soils. Thus, enzyme activity can change according to the amount of organic matter present in the soil, the presence of different microorganisms and/or the presence of some abiotic stresses in the environment[44]. Soil enzymes may include dehydrogenase, glucosidases, urease, amidases, phosphatases, arylsulphatase, cellulase and phenol oxidases[43]. Some of them are presented in the sections below.

1.2.1 Catalase

Many environmental stresses, including drought, have as a consequence the accumulation of ROS, such as: superoxide ion, hydrogen peroxide and hydroxyl radicals. The presence of these compounds can cause cellular damage through oxidation of lipids and proteins, chlorophyll bleaching and damage to nucleic acids, leading up to cellular death[45].

To overcome this situation and protect themselves against oxidative stress, organisms can produce some antioxidative enzyme, such as catalase, which maintains ROS at low levels. Its function is to catalyze the decomposition of hydrogen peroxide into water and oxygen. Therefore, the activity of this enzyme is increased when a stress is affecting the environment, being frequently used as indicator of oxidative stress in plants.

1.2.2 Fluorescein diacetate hydrolysis (FDA)

As it was already discussed, the activity of microorganisms in the rhizosphere not only affects the development of plants but also, the quality of the soil. So, total microbial activity is a good indicator of how fertile the soil is.

Fluorescein diacetate hydrolysis is an analysis that indicates the soil microbial activity. In fact, fluorescein diacetate is a substance that can be hydrolyzed by different enzymes, such as proteases, lipases and estereases, synthesized by microorganisms which makes this assay nonspecific. At the end of the hydrolysis, the quantity of fluorescein produced is proportional to the microbial activity present in the rhizospheric soil[46].

1.3 Maize (*Zea mays*)

Cereals are the crops that provide most of human caloric food intake, as well as animals feed, and maize is one of the most important and consumed cereal around the world, after wheat and before rice[47].

In fact, according to the Food and Agriculture Organization of the United Nations (FAO), the forecast for world maize production in 2016/2017 was 1.027 million tones[48], which represent a large production, not only in developing countries, but also in developed ones. This is due to the high rate of photosynthetic activity of the crop, which leads to high grain and biomass yield[19].

In developing countries, maize continues to be used as a basic food crop, while in developed countries in recent years, the production of this crop has been used not only as staple food but the mainly percentage for poultry, pigs and ruminant feeding[49].

Nowadays, the use of non-petroleum energy sources has been increase gradually in order to reduce the dependence of petroleum and simultaneously slow global warming due to fossil fuel emission[50]. Being maize a versatile crop, it has started to be used as a bioenergy source because of the large quantity of biomass obtained during its production. Globally, most bioethanol production is from two crops, maize and sugar cane, and the production of bioethanol can be used for domestic and industrial purposes as well as for transport[50/51].

1.3.1 Maize inoculated with PGPB

Some studies have demonstrated that the inoculation of PGPB into the rhizosphere enhances plant growth of many crops, being one of them maize, under different stressful conditions. Ansary et al.[52] showed that the application PGPB can enhanced phytohormones content of *Z. mays* plants under water stress condition improving their growth. In fact, they demonstrated that bacterial inoculation increased proline, ABA, IAA, gibberelline and cytokinin content, which help plants to overcome the stress. Pereira et al.[53] also demonstrated that the inoculation of *Z. mays* plants with PGPR enhanced plant growth and nutrition under P-deficient conditions. They showed an improvement on shoot and root biomass, on P accumulation and on synthesis of pectinases, proteases and lipases in maize plants.

1.4 Aims of the research

The main aim of this thesis was to evaluate the effect of PGPB inoculation on maize growth and resilience under different water regimes (80, 60 and 40% of water holding capacity (WHC)), using PGPBs from the collection of Escola Superior de Biotecnologia (ESB) and a commercial inoculum. Two secondary aims were proposed: (1) to assess the influence of inocula size on plant growth under water deficit stress and (2) to evaluate if the co-inoculation of PGPB were more efficient than single strain inoculation on promoting plant growth under different water regimes.

2. MATERIALS AND METHODS

2.1 Bacterial strains selection and characterization of *in vitro* traits

2.1.1 Bacterial strains selection

Thirteen bacterial strains (Table 2.1) were isolated and identified in previous works [23/26/53/54]. These strains were chosen because of their ability to promote plant growth under stressful conditions, such as heavy-metal pollution and low concentrations of phosphate in soil [20/23/26/53/54].

Table 2.1 PGPB´s from Escola Superior de Biotecnologia (ESB) used in the present study.

	Bacterial strains	**Type of Bacteria**
EC35	*Rhodococcus sp* [53]	Rhizobacteria and Phosphate solubilizer bacteria
EAPAA	*Arthrobacter nicotinovorans* [53]	Rhizobacteria and Phosphate solubilizer bacteria
EAV	*Pseudomonas sp.*[53]	Rhizobacteria and Phosphate solubilizer bacteria
EDP28	*Pseudomonas reactans* [54]	Rhizobacteria
S3X	*Pseudomonas fluorescens* [54]	Rhizobacteria
1C2	*Ralstonia eutropha* [26]	Rhizobacteria
EC1B	*Rhizobium radiobacter* [26]	Rhizobacteria
ZR3-5	*Ochrobactrum haematophilum* [23]	Endophytic Bacteria
ZS1-5	*Sphingomonas paucimobilis* [23]	Endophytic Bacteria
ZS1-6	*Agrobacterium larrymoorei* [23]	Endophytic Bacteria
ZS1-11	*Sphingomonas paucimobilis* [23]	Endophytic Bacteria
ZS2-2	*Agrobacterium larrymoorei* [23]	Endophytic Bacteria
ZS3-6	*Pantoea allii* [23]	Endophytic Bacteria

According to Pereira et al.[31/53] and Moreira et al.[26] strains have *in vitro* plant growth promoting traits like IAA, siderophore, HCN and ammonia production, ACC deaminase activity and P solubilization ability. In addition, positive results were observed in enhancing plant growth under stressful conditions.

2.1.2 *In vitro* osmotic stress tolerance

Water deficit stress effects on bacterial growth can be studied using *in vitro* methods, such as the amendment of growth medium with an osmotic solution. Therefore, polyethylene glycol (PEG6000) was used in this study as a non-toxic osmotic solution for stimulating osmotic stress[5].

To characterize strains for drought tolerance, 7.5 g of trypticase soy broth (TSB) medium was amended with 0, 21.25, 32, 40 and 52.80 g of PEG 6000 in 250 mL of distilled water in order to simulate different osmotic potentials (0; -0.10; -0.20; -0.30; -0.70 Mpa)[55]. Three replicates of each strain were prepared. After incubation at 28 °C under shaking conditions (120 rpm) for 24 h, bacterial growth was estimated by measuring the optical density (OD) at 600 nm using a Helios Gamma (Thermo Spectronic Unicam) spectrophotometer. Percentage of growth inhibition was calculated for all bacterial strains for the different osmotic potentials.

2.1.3 IAA production

This assay was performed according to the method described by Gordon et al.[56]. The reagents and solutions used during this assay are described in Table 2.2.

To perform this assay, bacteria were grown in TSB for 24 h. Cells were collected by centrifugation at 7000 rpm, for 10 min at 4 °C and the supernatant discarded. Then, 3 mL of the buffer solution, with different osmotic potentials, namely 0, -0.30 and -0.70 Mpa, and 1 mL L-tryptophan (1%) were added to the pellet. After 48 h of incubation at 30 °C and 120 rpm, 2 mL of 5% TCA and 1 mL of 0.5 M $CaCl_2$ were added. The mixtures were then centrifuged (7000 rpm, 20°C, 8 min) and 500 µL of the supernantant was mixed with 350 µL of Salkowski solution and incubated for 30 min at 25 °C in the dark. IAA production was estimated by measuring OD at 535 nm. A calibration curve was prepared using IAA standard (0 to 100 µg/mL) solution.

Table 2.2 List of the reagents and solutions used during the IAA production and their function.

Reagents / Solutions	Preparation / Concentration	Function
Buffer Solution	· Phosphate buffer (pH 7.5) · Glucose 1% · PEG 6000	-
L-Tryptophan	1%	IAA precursor.
Trichloroacetic acid (TCA)	5%	Inactivate the enzymes involved during the assay[57].
$CaCl_2$	0.5 M	Inactivate the enzymes involved during the assay[57].
Salkowski Reagent	· Percloric acid 35% · Iron (III) chloride ($FeCl_3$)	Reacts with the IAA and produce tris (indole-3-acetato) iron III.

2.2 Greenhouse experimental design

At the beginning of the experimental design, the objective was to cultivate wheat seeds under different water regimes (80, 60 and 40% WHC) in order to observe the effect of the inoculation on wheat growth under water deficit stress. Wheat plants were chosen in this study based on the fact that this cereal is one of the most important crops cultivated around the world and it represents an important renewable resource for food, feed and industrial raw material. Another reason to use wheat during this study was the fact that it is a non-irrigated crop which it is highly affected by drought periods, thus wheat is watered only using rainfall water, being a serious problem especially in drylands.

Nonetheless, one month after the seeds were planted on the pots, it was observed that the plants did not growth properly (Figure 2.1). This result was related to the presence of a high concentration of metals, namely copper, in soil which led to a high toxicity that prevented plants to grow properly.

Figure 2.1 Photographs of the wheat experimental design, 15 weeks after the seeds were planted in the pots.

As such, the experimental design was redefined and a new experiment was assembled using maize seeds and an agricultural soil collected in the North of Portugal. Soil physico-chemical characteristics are shown in Table 2.3.

Table 2.3 Soil properties.

Parameters	**Results**
pH (H_2O)	6.06 ± 0.01
Organic matter	2.15 ± 0.01%
Electrical conductivity	116 ± 2 µS/cm
Organic carbon	1.24 ± 0.04%
Total N	0.12 ± 0.02%
P (P_2O_5)	13.9 ± 0.4 mg/kg
K (K_2O)	128.2 ± 1.3 mg/kg
Calcium (CaO)	989.2 ± 0.9 mg/kg
Magnesium (MgO)	98.7 ± 0.4 mg/kg
Sulfur	68.1 ± 0.1 mg/kg
Fe	139.4 ± 0.1 mg/kg
Boron	0.08 ± 0.0 mg/kg
Copper	0.5 ± 0.1 mg/kg
Zinc	1.3 ± 0.2 mg/kg
Sodium	16.8 ± 0.1 mg/kg

The use of maize instead of wheat allowed to prepare a bigger experimental design since maize plants require less amounts of soil and as such pots of 1 kg were used instead of 8 kg (used during the wheat experiment).

The study was carried out in the Escola Superior de Biotecnologia, Universidade Católica Portuguesa during the year 2016/2017. The experiment in the greenhouse consisted in a factorial design with 3 water regimes: 80% of WHC (normal conditions), which was used as control, 60% of WHC (moderate osmotic stress) and 40% of WHC (severe osmotic stress) and 8 types of inoculation (Table 2.4). Each treatment was replicated 5 times. According to the results obtained in the *in vitro* assays, two rhizobacterial strains were selected, S3X (*P. fluorescens)* and 1C2 (*R. eutropha),* for the pot trial.

Table 2.4 List of the treatments (and their nomenclature) used during the study and inoculum size (cells. g^{-1} dry weight (dw)).

Treatments		**Inoculum size**
Control	Non-inoculated soil.	-
CI	Soil inoculated with commercial inoculum.	3.3 x 10^3 cell. g^{-1} dw / 3.3 µL
B1 V1	Soil inoculated with *Pseudomonas fluorescens* (S3X)	3.3 x 10^3 cell. g^{-1} dw / 3.3 µL
B2 V1	Soil inoculated with *Ralstonia eutropha* (1C2)	3.3 x 10^3 cell. g^{-1} dw / 3.3 µL
MIX V1	Soil inoculated with *Pseudomonas. fluorescens* (S3X) and *Ralstonia eutropha* (1C2)	3.3 x 10^3 cell. g^{-1} dw / 3.3 µL
B1 V2	Soil inoculated with *Pseudomonas fluorescens* (S3X)	2.5 x 10^6 cell. g^{-1} dw / 25 mL
B2 V2	Soil inoculated with *Ralstonia eutropha* (1C2)	2.5 x 10^6 cell. g^{-1} dw / 25 mL
MIX V2	Soil inoculated with *Pseudomonas fluorescens* (S3X) and *Ralstonia eutropha* (1C2)	2.5 x 10^6 cell. g^{-1} dw / 25 mL

The soil was sieved (2 mm) and 1 kg of soil was placed in each plastic pot. The maize seeds var. DKC3014 (Dekalb, France) were hydrated overnight and then sterilized with a solution composed by 50% of bleach and 50% of water during 10 min under agitation. At the end, seeds were washed with sterilized water in order to remove all the sterilization agents used before. The sterilized seeds were placed on agar for 6 days to germinate and then 7 seedlings were transferred to the plastic pots. Pots were placed in a room with 12 h of photoperiod and a temperature which ranged from 15 to 20 °C. After a few weeks, the number of plants was reduced to 5 per pot.

Bacterial inoculation was performed by spraying the soil surface one week after the seedlings were transferred to the pots and a reinoculation was performed 9 weeks later. Bacterial strains were grown overnight at 120 rpm and 30 °C in TSB medium. Cells in the exponential phase were harvested by centrifugation at 7000 rpm for 10 min and ressuspended in saline solution (0.85% NaCl).

After 3 weeks, plants received 50 ppm of nitrogen solution (32 N Solution). In addition, 50 mL of 1.36×10^2 M phosphate solution was added four times before the osmotic stress was applied. The addition of these nutrients was performed in order to fulfill plant's requirements.

The water regimes were applied 8 weeks after sowing. In order to apply the water regimes, pots were watered until the field capacity and the weight of each pot was recorded. The difference between the weight of the soil during the field capacity and the dry weight of the soil (1 kg) represented 100% of soil moisture. The levels of 80, 60 and 40% of WHC were calculated according to that. Therefore, pots were weighted daily in order to determine if the soil presented the target moisture, and in case the moisture was lower, it was watered until the value previously calculated.

2.2.1 Chlorophyll measurements

For chlorophyll determination, a SPAD 502 plus was used. This is a non-invasive device that estimates the total chlorophyll amount in leaves by absorbance, where higher values indicates healthier plants. A first measurement was performed 5 weeks after the seeds were planted and before the application of the water regimes. The chlorophyll measurement was taken in each plant of each pot at three different locations of the 3rd leaf. A second measurement was made at the end of the experiment, 13 weeks later.

2.2.2 Plant analysis

After 13 weeks, plants were harvested and separated in roots and shoots and shoot elongation was registered. The dry biomass of the plants was determined after shoots and roots were oven dried at 65 °C for 4 weeks. Then, shoot and root samples were grinded until dust and digested. The digestion process consist in an acid digestion used to remove all organic components present in the samples. For this process, 4.5 mL of 0.7 M sulfuric acid (H_2SO_4) and 4.5 mL of 35.3 mM hydrogen peroxide (H_2O_2) were added. During this process, high temperatures and high pressure are reached in order to catalyze the reaction.

2.2.3 Nutritional parameters

2.2.3.1 Nitrogen determination

Determination of N was performed according to Wallinga et al.[58], using the "Berthelot reaction", which is a colorimetric method performed to determine the concentration of N in plants. Table 2.5 shows the solutions needed for this assay.

Table 2.5 List of the different solutions needed to prepare the required reagents to determine the N concentration in the digested samples and their function.

Solution	Components	Function
A	· 1 M Sodium salicylate solution.	Berthelot reagent component.
	· 1×10^{-3} M Sodium nitroprusside dehydrated solution.	Catalysts agent.
	· 3×10^{-3} M EDTA Solution.	Chelate Agent.
B	· 5×10^{-2} M Disodiumhydrogenphosphate solution.	Buffer Solution.
	· Hypochlorite Solution.	Berthelot reagent component.

To perform this assay, 20 µL of each digested sample were diluted in 180 µL of distilled water. Then, 3 mL of Solution A was added as well as 5 mL of Solution B. The tubes rested for 2 h and the absorbance was measured. Standard solutions (0 to 15 mg/L) were prepared using a stock solution of ammonium sulfate ($(NH_4)SO_4)$). The absorbance of N concentration was determined at 660 nm and the concentration was calculated through the formula:

$$[\,(a - b) * v\,] / w$$

a: N concentration in the digested samples (mg/L).
b: N concentration in the zero samples (mg/L).
v: Total volume of the digestion process (L).
w: Dry weight of samples (kg).

2.2.3.2 Phosphorus (P) determination

Determination of P was performed according to Wallinga et al.[58]. Two solutions were prepared for the assay (Table 2.6).

Table 2.6 List of the different solutions needed to prepare the required reagents to determine the P concentration in the digested samples and their function.

Solution	Components	Function
A	5×10^{-3} M Ammonium molibdate Solution.	Reagent.
	0.7 M Sulfuric acid.	Turn the medium acid.
	3×10^{-2} M Ascorbic acid.	Reducing agent.
	6×10^{-3} M Antimonyl potassium tartrate.	Reagent.
B	Solution A.	-
	H_2O.	-
	Aerosol 22.	Wetting agent.

To perform this assay, 100 µL of each digested sample were diluted in 900 µL of distilled water. Then, 3.8 mL of Solution B was added. The tubes rested for 1 h and the absorbance was measured at 880 nm. Standard solutions (0 to 5 mg/L) were prepared using a stock solution of monopotassium phosphate (KH_2PO_4). The P concentration was calculated through the formula:

$$[\,(a - b) * v\,] / w$$

a: P concentration in the digested samples (mg/L).
b: P concentration in the zero samples (mg/L).
v: Total volume of the digestion process (L).
w: Dry weight of samples (kg).

2.2.3.3 Nutrient Use Efficiency (NUE)

Nutrient Use Efficiency for N and P was calculated according to the formula explained by Nguyen et al.[59].

$$NUE = Total\ dry\ biomass\ /\ Total\ nutrient\ absorbed$$

$$where,\ Total\ nutrient\ absorbed = Nutrient\ concentration \times Total\ dry\ biomass$$

2.2.4 Soil enzymes

2.2.4.1 Catalase activity

Catalase activity determination was performed according to the method described by Johansson et al.[60]. The reagents used during the assay are described in Table 2.7.

Table 2.7 List of the different reagents needed to determine the activity of catalase in the soil and their function.

Reagents	**Function**
100 mM Potassium phosphate	Buffer solution.
Methanol	Hydrogen donor.
35.2 mM H_2O_2	Initiates the reaction.
10 M Potassium hydroxide (KOH)	Stops the reaction.
34.2 mM Purpald	Chromogen solution.
65.2 mM Potassium periodate	Oxidize the product formed by the formaldehyde and the purpald.

To perform this assay, 5 mL of buffer solution and 1.5 mL of methanol was added to 1 g of soil (composite sample for each treatment). The samples were incubated for 1 h at 30 °C and 120 rpm. The reaction was initiated with 700 µL of H_2O_2 and the sample were incubated again for 20 min at 30 °C and 120 rpm. The reaction was stopped by adding 1.5 mL of KOH and 1 mL of purpald was added. The sample were incubated again at the same conditions and 300 µL of potassium periodate was added. At the end, samples were centrifuged at 7000 rpm during 7 min in order to obtain the supernatant and the absorbance at 540 nm was read. Standard solutions (0 to 200 uM formaldehyde/mL) were prepared using a stock solution of formaldehyde (CH_2O). The catalase activity was calculated through the formula:

$$(a * v) / (w * h)$$

a: Catalase activity in soil (µmoles formaldehyde).
v: Total volume of the assay (L).
w: Dry weight of samples (g).
h: Number of hours during the assay (h).

2.2.4.2 Fluorescein diacetate (FDA) hydrolysis

The FDA assay was performed according to Adam et al. [46]. The reagents used during the assay are listed in Table 2.8.

Table 2.8 List of the different reagents needed to hydrolyzed fluorescein diacetate into fluorescein and their function.

Reagents	Function
60 mM Sodium phosphate	Buffer solution.
4.8 mM FDA	Initiates the reaction.
Acetone	Stops the reaction.

To perform this assay, 3 g of soil were weighted (composite sample for each treatment) and 5 mL of buffer solution was added. Then, 25 µL of FDA was added to each sample to initiate the reaction and they were incubated at 30 °C for 20 min at 110 rpm. After incubation, 2.5 mL of acetone was added to stop the reaction and samples were centrifuged at 5000 rpm during 10 min. At the end, the absorbance at 490 nm was recorded. Standard solutions (0 to 200 µM fluorescein/mL) were prepared using a stock solution of fluorescein. FDA activity was calculated through the formula:

$$(a * v) / (w * h)$$

a: FDA activity in soil (µmoles fluorescein).
v: Total volume of the assay (L).
w: Dry weight of samples (g).
h: Number of hours during the assay (h).

2.2.5 Statistical analysis

All statistical analyses were performed using SPSS 23.0 Software package. The data were analyzed through analysis of variance (ANOVA). Two way ANOVA was performed to observe the influence of the bacterial inoculation and the water regimes on each parameter tested. One way ANOVA was also performed to analyze the effects of microbial inoculation on each parameter for each water regime. The post hoc Duncan test ($P < 0.05$) was performed to determine the significant differences between group means.

3. RESULTS

3.1 Characterization of *in vitro* traits

3.1.1 *In vitro* osmotic stress tolerance

All PGPB were screened for drought tolerance *in vitro*, using PEG 6000, which is an agent that induces osmotic stress (-0.10; -0.20; - 0.30 and – 0.70 Mpa) (Figure 3.1).

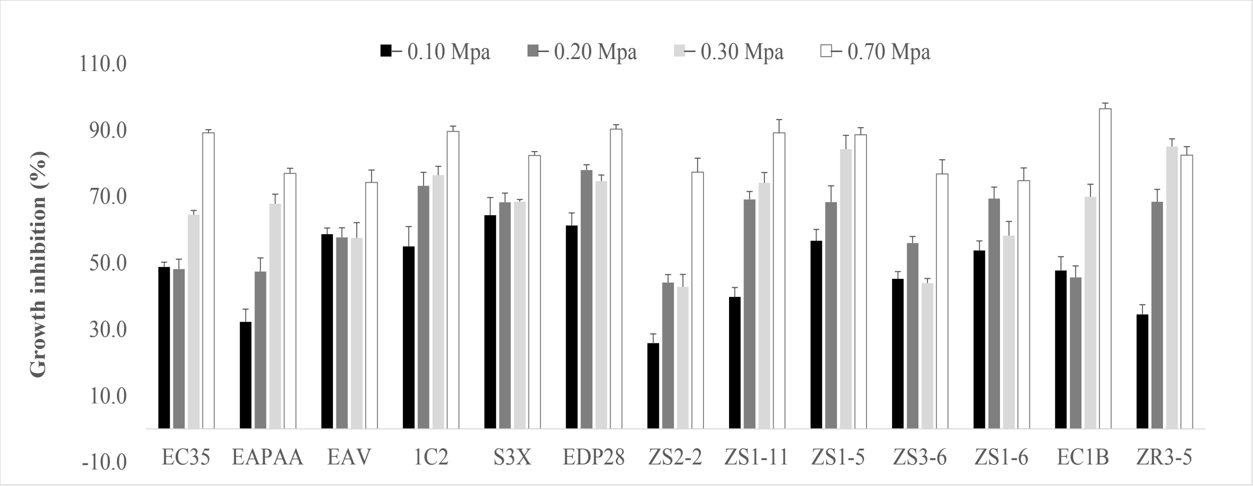

Figure 3.1 Percentage of growth inhibition of the different bacterial strains exposed to different osmotic potentials (-0.10; -0.20; -0.30 and -0.70 Mpa).

Values are means ± standard error (n = 4 to 9). One way ANOVA was performed to determine the influence of the different osmotic potentials on the growth inhibition for each bacterial strain. The *F* values of one-way ANOVA are $F = 8.100$ ($P < 0.001$), $F = 9.688$ ($P < 0.001$), $F = 17.582$ ($P < 0.001$) and $F = 5.106$ ($P < 0.001$) for the osmotic potentials – 0.10; -0.20; -0.30 and -0.70 (Mpa), respectively.

All strains showed significant ($P < 0.001$) differences in growth inhibition for all osmotic potentials tested. In fact, all bacterial strains were able to grow on TSB medium supplemented with different concentrations of PEG 6000 simulating different osmotic potentials. As the osmotic potentials increased, the growth inhibition also increased in all strains tested.

The lowest percentage of growth inhibition were recorded at -0.10 Mpa. Strains EAPAA (*A. nicotinovorans*) and ZS2-2 (*A. larrymoorei*) had the highest tolerance facing this osmotic potential. In general, the percentages of growth inhibition at -0.20 and at -0.30 Mpa were quite similar for all strains. However, at 0.20 Mpa, strains EC35 (*Rhodococcus* sp), EAPAA (*A. nicotinovorans*) and EC1B (*R. radiobacter*) showed the highest tolerance while at 0-30 Mpa, strains ZS2-2 (*A. larrymoorei*) and ZS3-6 (*P. allii)* presented the highest drought tolerance. The highest growth inhibition was registered at -0.70 Mpa with the strains ZS2-2 (*A. larrymoorei*), EAV (*Pseudomonas sp.*) and ZS3-6 (*P. allii*) presenting the highest tolerance.

Overall, strain ZS2-2 (*A. larrymoorei*) showed the best performance, since it presented the lowest percentage of growth inhibition at -0.10, -0.20 and -0.70 Mpa. The bacterial strain EAPAA (*A. nicotinovorans*) and strain ZS3-6 (*P. allii*) also showed high tolerance at -0.10 and -0.20 Mpa and at -0.30 and -0.70 Mpa respectively.

3.1.2 IAA Production

The IAA production by the bacterial strains exposed to different osmotic potentials is presented in Figure 2.3. All bacterial strains produced IAA in the presence of the amino acid tryptophan after 48 h, either in the control (0 Mpa) or under osmotic stress (-0.30 and -0.70 Mpa). As presented in the Figure 2.3, different trends on IAA production were observed with increasing osmotic potentials. The presence of PEG 6000 generally increased the production of IAA when compared to the control (0 Mpa), which means that a higher amount of IAA was synthetized when the strains where under osmotic stress.

Nonetheless, some strains decreased their production among the different osmotic stress, namely, the strains S3X and ZR3-5 (*P. fluorescens* and *O. haematophilum* respectively). Other strains (1C2, EDP28, ZS1-11 and ZS1-5) also showed a decrease in the production of IAA at -0.30 Mpa, however an increase was observed at -0.70 Mpa.

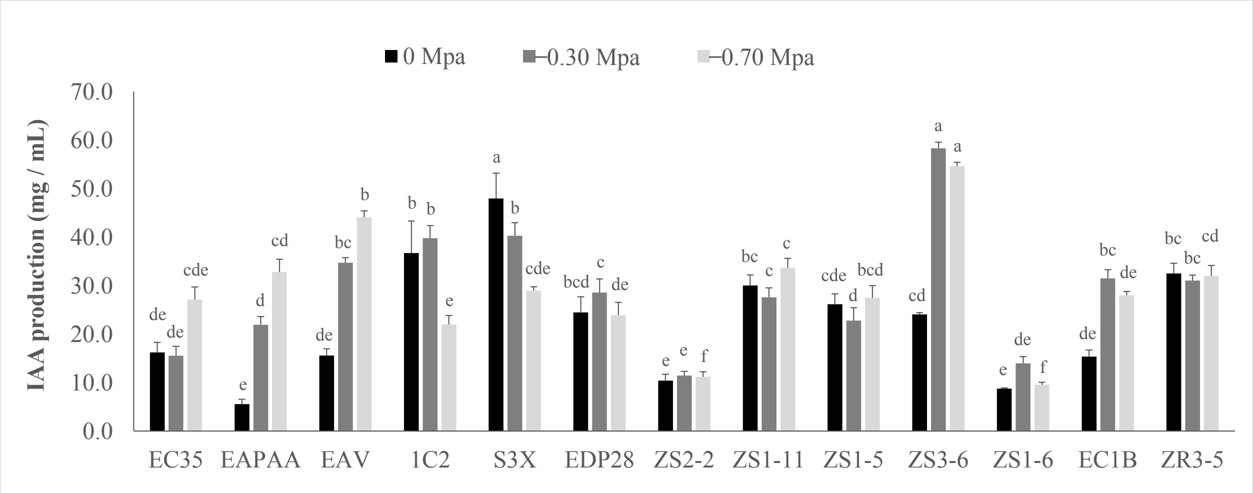

Figure 3.2 Indole acetic acid (IAA) production by bacterial strains exposed to different osmotic potentials (0; -0.30 and -0.70 Mpa).

Values are means ± standard error (n = 6 to 25). One way ANOVA was performed to determine the influence of the different osmotic potentials on IAA production for each bacterial strain. Means for the same osmotic potential showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. For IAA production, the F values of one way ANOVA are $F = 16.587$ ($P < 0.001$), $F = 16.547$ ($P < 0.001$) and $F = 11.931$ ($P < 0.001$), for 0, -0.30 and -0.70 Mpa, respectively.

3.2 Greenhouse experiment

From the initial thirteen bacterial strains selected, the strains *R. eutropha* (1C2) and *P. fluorescens* (S3X) were selected for the greenhouse pot assay. These strains were chosen because they demonstrated in the *in vitro* characterization good tolerance facing the osmotic potentials tested and they synthesized higher values of IAA under osmotic stress, which may indicate higher efficiency in improving plant growth under water deficit. Another reason to select these two rhizobacterial strains was their proven activity as plant growth promoting agents under seriously environmental stresses, such as salinity and heavy metals.

At the end of the experiment, some photographs were taken choosing randomly pots of each water regime and each bacterial inoculation treatment.

(a)

(b)

(c)

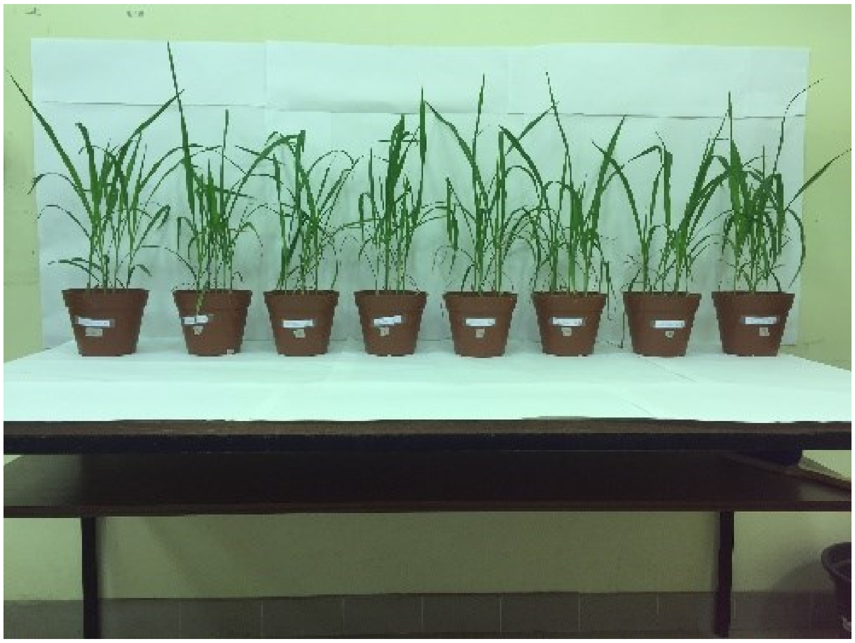

Figure 3.3 Photographs of pots selected randomly of 80% (a), 60% (b) and 40% (c) of WHC of the maize experimental design, 13 weeks after the seeds were planted.
From left to right, Control; CI; B1 V1; B2 V1, MIX V1, B1 V2, B2 V2 and MIX V2 treatments.

3.2.1 Plant Parameters

3.2.1.1 Chlorophyll

Chlorophyll content in leaves, before (a) and after (b) applying drought stress are shown in Figure 3.4. Values in the chlorophyll content of plants before the application of water regimes were quite similar. In fact, one way ANOVA showed no significant ($P > 0.05$) differences, so the bioinoculants applied to soil did not promote any difference in chlorophyll content before the osmotic stress was applied.

When results are compared before and after applying the water regimes on plants, it is observed that chlorophyll content in leaves was negatively affected by water deficit, with a remarkable reduction after the stress in all treatments. Nonetheless, according to the results of the two way ANOVA, the water regimes and the bacterial inoculation, as well as the interaction between both factors, had no significant ($P > 0.05$) effect on chlorophyll content in leaves after the stress was applied. Also, no significant differences were observed between bacterial treatments in each water regime.

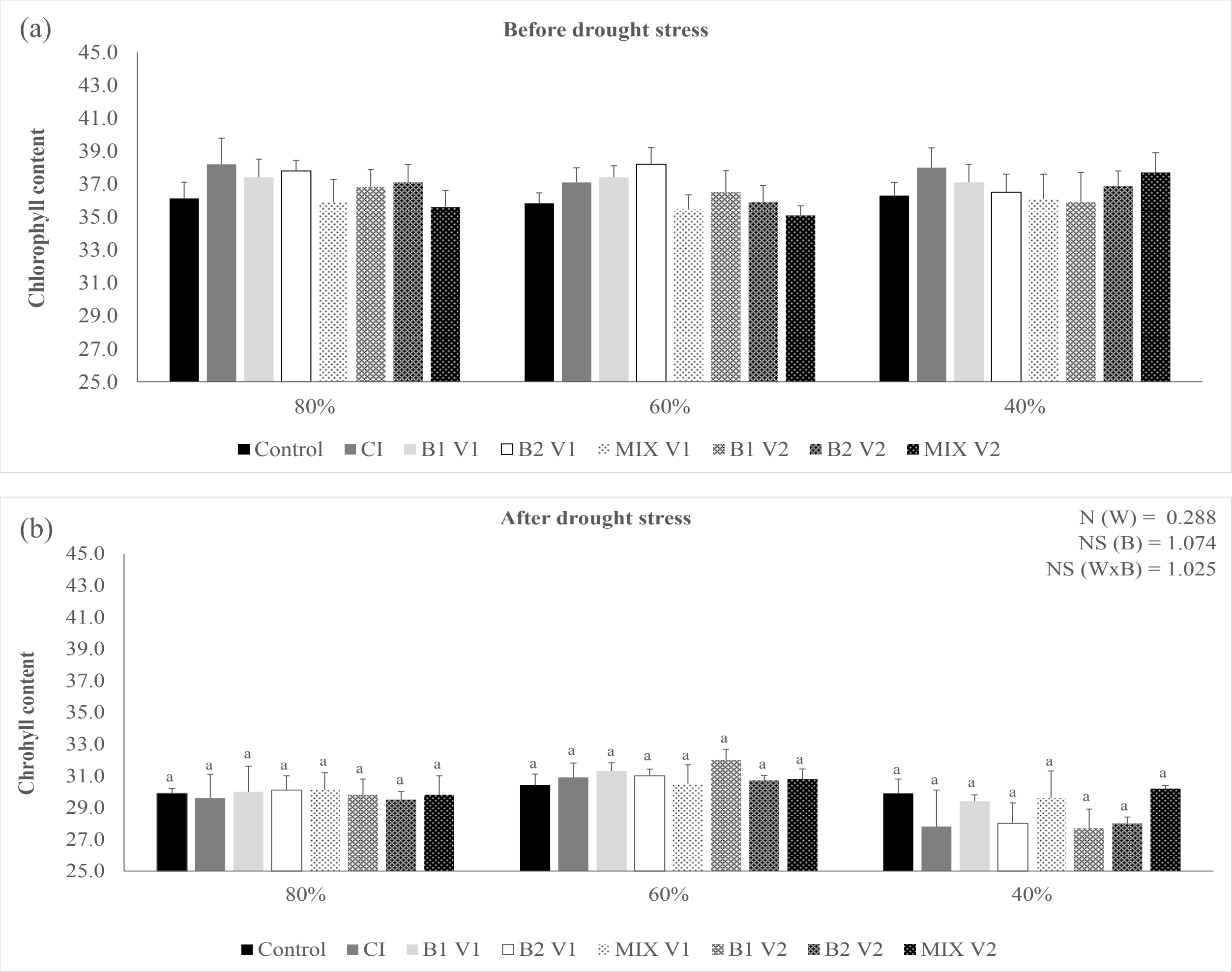

Figure 3.4 Chlorophyll measurements before (a) and after (b) applying drought stress to plants. Values are means ± standard deviation (n = 5). A two way ANOVA was only performed in (b) to determine the influence of bacterial inoculation and the water regimes on chlorophyll content. The results are shown with the test statistic for each case (W: Water regimes; B: Bacterial treatments; W x B: Water regimes x Bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of the bacterial inoculation on chlorophyll content before the stress was applied. The *F* value correspond to $F = 0.824$ (NS). One way ANOVA was also performed to determine the influence of the water regimes on chlorophyll content after the stress was applied. The *F* values of one way ANOVA are $F = 1.054$ (NS), $F = 1.063$ (NS) and $F = 0.693$ (NS) for 80, 60 and 40% of WHC, respectively.

3.2.1.2 Shoot elongation

Shoot elongation is presented in Figure 3.5. According to the results of two way ANOVA, the water regimes applied to the soil significantly ($P < 0.05$) influenced shoot elongation of maize plants. At 80 and 60% of WHC maize plants showed a quite similar shoot elongation, however this parameter was affected when inoculated plants were grown in the severe water regime, with a proportional relation between shoot elongation and the increasing water deficit stress, which demonstrated that lower moisture percentage in soils corresponded to a lower shoot elongation.

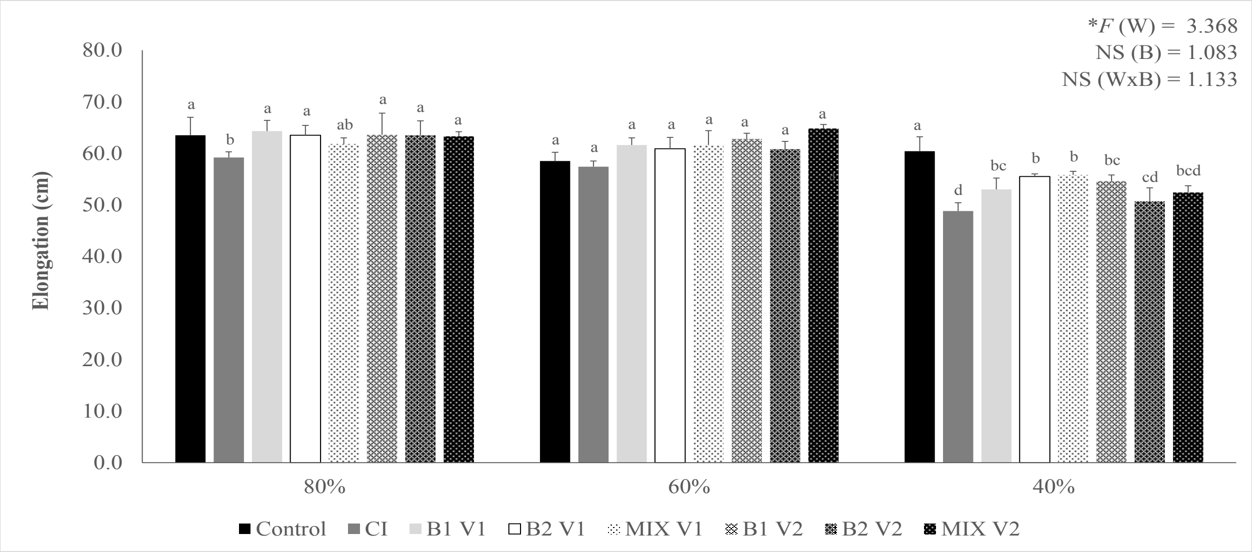

Figure 3.5 Shoot elongation of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± to standard deviation (n =4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on shoot elongation. (W - water regimes; B - bacterial treatments; W x B - water regimes x bacterial treatment) and as NS - Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of bacterial treatments on shoot elongation for each water regime. Means for the same water regime showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. The F values of one way ANOVA are $F = 2.162$ ($P < 0.05$), $F = 0.825$ (NS) and $F = 7.334$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively.

Maize shoot elongation at 80% of WHC ranged from 59.2 to 64.3 cm (CI and B1 V1, respectively), at 60% ranged from 57.4 to 64.8 cm (CI and MIX V2, respectively) and at 40% varied between 48.8 to 60.4 cm (CI and Control treatments, respectively). According to the results obtained, CI treatment significantly presented the lowest shoot elongation at 80 and 40% of WHC, when comparing to the others treatments.

At 80% of WHC, for all inoculation treatments, with the exception of CI, which influenced negatively shoot elongation, no significant ($P < 0.05$) differences were observed among treatments. A similar trend was observed at 60% where bioinoculants did not influence shoot elongation. However, at 40%, all bioinoculants significantly ($P < 0.05$) decrease shoot elongation.

3.2.1.3 Dry biomass

The results obtained for shoot (a) and root (b) biomass are shown in Figure 3.6. Shoot biomass was significantly influenced by water regimes and bacterial treatments ($P < 0.001$). Overall, a decrease in shoot biomass as the percentage of soil moisture decreased, especially at 40%, was observed (Figure 3.6a). Shoot biomass at 80% ranged from 1.460 to 2.419 g (CI and B2 V1, respectively), at 60% ranged from 1.670 to 3.150 g (Control and MIX V2, respectively) and at 40% varied between 0.960 and 1.704 g (CI and Control, respectively).

At 80% of WHC, all inoculation treatments showed significant ($P < 0.001$) effects on shoot biomass, with the exception of B1 V1 and B2 V2 treatments. The inoculum size applied seemed to not influence this parameter. Within this regime, CI treatment was the only one that presented negative effects on shoot biomass when compared to the control. At 60%, all bacterial treatments significantly ($P < 0.001$) promoted shoot biomass, in fact, MIX V2 was the treatment that better performed within this regime, enhancing by 47% the production of biomass. However, at 40% a different trend was observed, with the inoculation significantly ($P < 0.001$) decreasing shoot biomass.

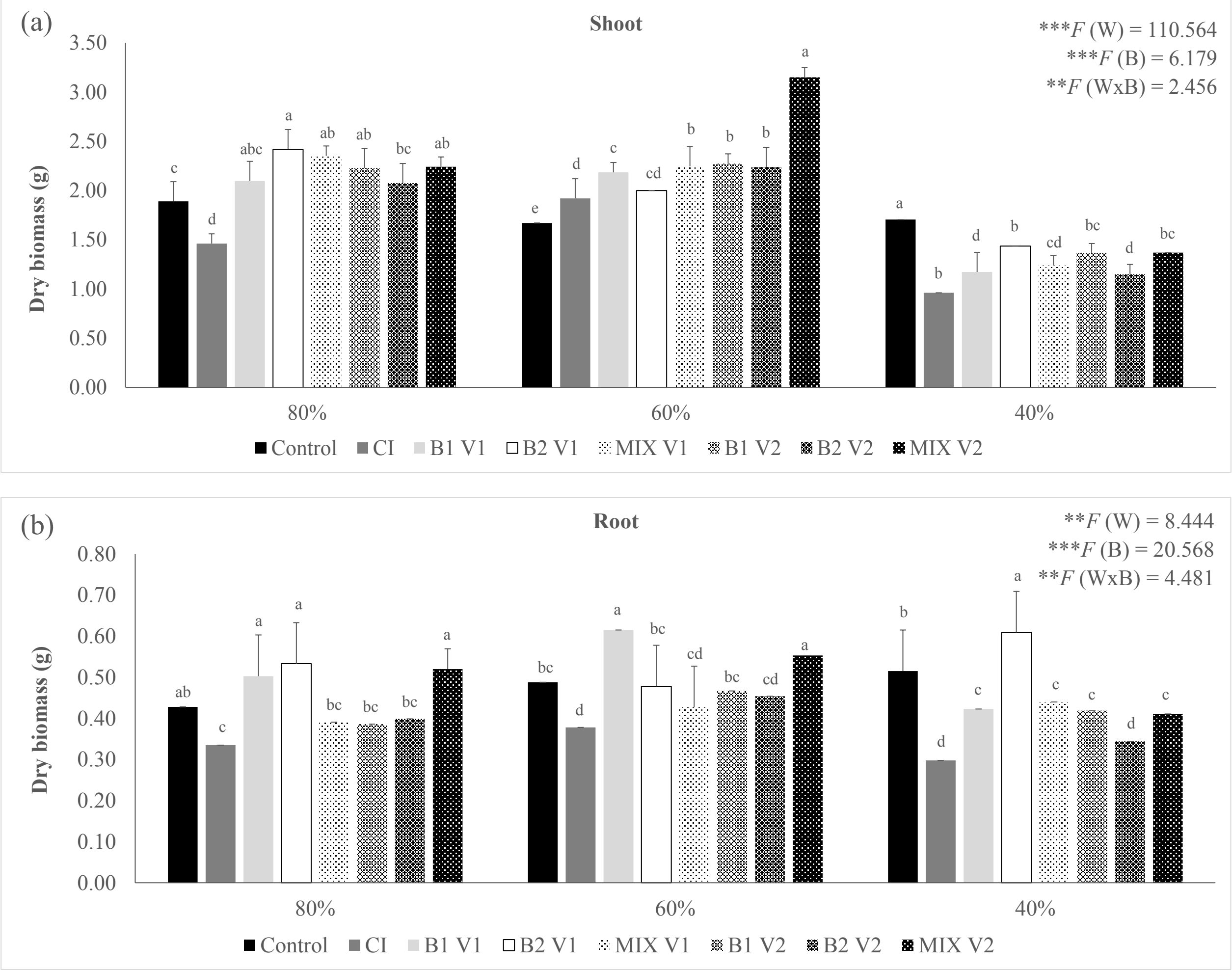

Figure 3.6 Shoot (a) and root (b) dry biomass of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± standard deviation (n = 4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on shoot and root biomass. (W - water regimes; B - bacterial treatments; W x B - water regimes x bacterial treatments) and as NS - Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of bacterial inoculation on shoot and root biomass for each water regime. Means for the same water regime showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. For shoots, the *F* values of one way ANOVA are $F = 9.207$ ($P < 0.001$), $F = 30.821$ ($P < 0.001$) and $F = 17.152$ ($P < 0.001$), respectively for 80, 60 and 40% of WHC. For roots, the *F* values of one way ANOVA are $F = 6.414$ ($P < 0.001$), $F = 6.776$ ($P < 0.001$) and $F = 23.665$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively.

Bacterial treatments and water regimes also significantly ($P < 0.01$) influenced root biomass (Figure 3.6b). However, the decrease of moisture in soil did not cause a decrease in root biomass of non-inoculated plants. Root biomass at 80% ranged from 0.319 to 0.540 g (CI and B1 V1, respectively), at 60% ranged from 0.379 to 0.652 g (CI and B1 V1, respectively) and at 40% varied between 0.345 to 0.566 g (CI and Control, respectively).

At 80% of WHC, none of the treatments influenced significantly ($P < 0.001$) this parameter, except for CI treatment, which negatively affected root biomass. At 60%, the treatments B1 V1 and MIX V2 significantly ($P < 0.001$) promoted root biomass. CI treatment was significantly different as well, however a negative influence was observed. At 40%, all treatments were significantly ($P < 0.001$) different, with B2 V1 outperforming the other treatments and influencing positively root biomass. According to Figure 3.6b, CI treatment presented the lowest root biomass values in all water regimes tested in the soils, when comparing to the others treatments within each regime.

3.2.2 Nutritional Parameters

3.2.2.1 Nitrogen accumulation

The results obtained for N accumulation in shoot (a) and root (b) of maize plants growing under drought stress are presented in Figure 3.7. N accumulation was higher in shoots than in roots for all bacterial treatments and water regimes. Overall, the water regimes and the bacterial inoculation significantly ($P < 0.001$) influenced N accumulation in shoots and roots.

Shoot N accumulation at 80% of WHC ranged from 1442.5 to 1843.0 mg N. kg^{-1} dw (MIX V1 and B1 V2, respectively), at 60% ranged from 1360.3 to 2232.4 mg N. kg^{-1} dw (MIX V2 and Control, respectively) and at 40% varied between 1481.0 and 1879.4 mg N. kg^{-1} dw (B2 V2 and Control, respectively). The higher accumulation was observed in non-inoculated plants growing at 60%. At 80%, the treatment B1 V2 was the only treatment that significantly ($P < 0.001$) increased N accumulation in shoots. At 60 and 40%, bioinoculants significantly ($P < 0.001$) decreased the amount of N in shoots.

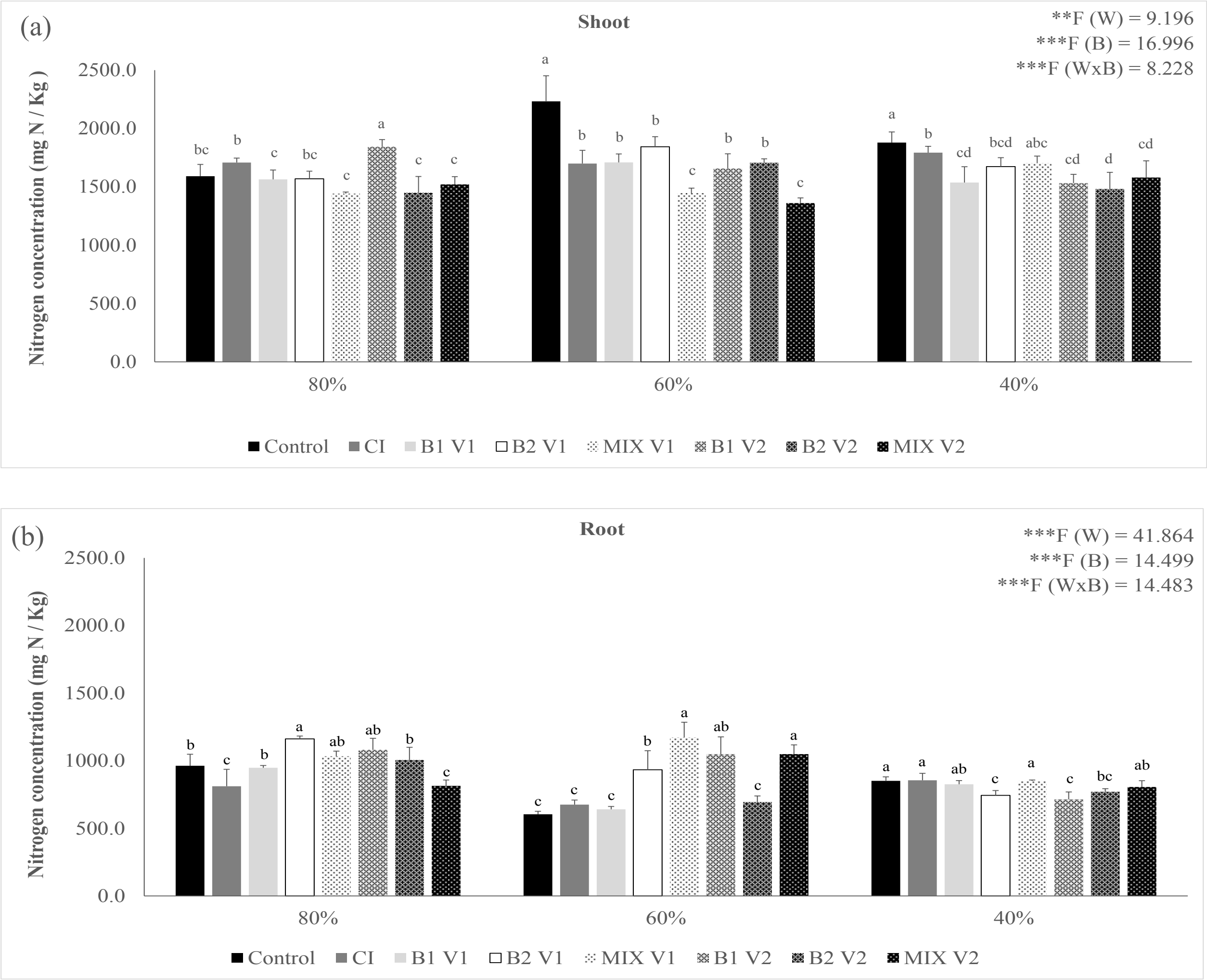

Figure 3.7 Nitrogen accumulation in shoot (a) and root (b) of plants growing under different water regimes (80, 60 and 40 % of WHC).

Values are means ± standard deviation (n = 4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on shoot and root N content. (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of bacterial inoculation on shoot and root N accumulation for each water regime. Means for the same water regime showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. For shoots, the F values of one way ANOVA are $F = 8.637$ ($P < 0.001$), $F = 17.643$ ($P < 0.001$) and $F = 5.460$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively. For roots, the F values of one way ANOVA are $F = 8.254$ ($P < 0.001$), $F = 20.756$ ($P < 0.001$) and $F = 6.195$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively.

Nitrogen accumulation in roots at 80% of WHC ranged from 811.9 to 1162.5 mg N. kg^{-1} dw (CI and B2 V1, respectively), at 60% ranged from 604.6 to 1172.2 mg N. kg^{-1} dw (Control and MIX V1, respectively) and a 40% varied between 715.4 to 855.3 mg N. kg^{-1} dw (B1 V2 and CI, respectively). One way ANOVA was performed within the water regimes, and significant ($P < 0.001$) differences were observed at 80% of WHC. CI and MIX V2 influenced negatively N accumulation in roots, however B2 V1 significantly raised the amount of N accumulated in roots. At 60%, only 4 treatments significantly ($P < 0.001$) increased the concentration of N in roots (B2 V1, MIX V1, B1 V2 and MIX V2). Meanwhile at 40%, three treatments, namely B2 V1, B1 V2 and B2 V2, were significantly different when compared to the other treatments, nonetheless, their presence in the rhizosphere decreased N accumulation in roots.

3.2.2.1.1 Nitrogen use efficiency

The results calculated for N use efficiency are presented in Figure 3.8. According to the results of the two way ANOVA, the water regimes and bacterial inoculation, as well as the interaction between both factors, had a significant ($P < 0.001$) effect on N use efficiency.

At 80% of WHC, treatment B1 V2 influenced negatively N use efficiency ($P < 0.001$) while other treatments had no effect. At 60%, all treatments enhanced N use efficiency ($P < 0.001$), especially MIX V1 and MIX V2, improving in 24 and 29% this parameter when compared to the control. At 40%, most bacterial treatments (B1 V1, B2 V1, B1 V2, B2 V2 and MIX V2) significantly ($P < 0.001$) increased N use efficiency on plants.

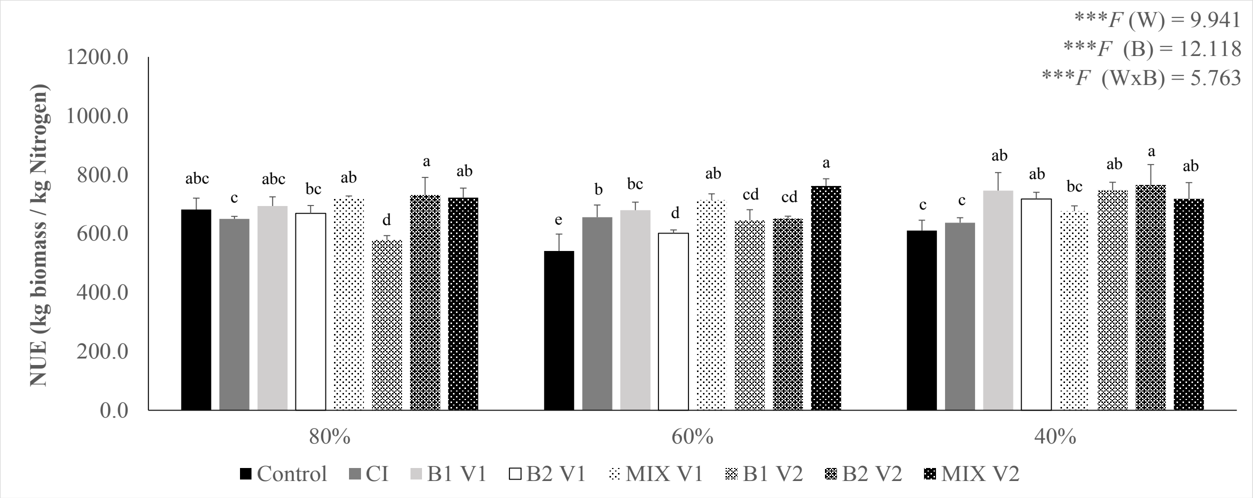

Figure 3.8 Nitrogen use efficiency of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± Standard Deviation (n = 4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on N use efficiency. (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of the water regimes on N use efficiency for each treatment. Means for the same concentration showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. The F values of one way ANOVA are $F = 7.471$ ($P < 0.001$), $F = 12.906$ ($P < 0.001$) and $F = 5.193$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively.

3.2.2.2 Phosphorus accumulation

Results obtained for P accumulation in shoot (a) and root (b) are presented in Figure 3.9. P in shoots and roots was significantly affected by water regimes and bacterial inoculation ($P < 0.001$ and $P < 0.05$, respectively). Roots accumulated lower concentration of P than shoots. In fact, an inverse relation was verified, P accumulation tended to decrease in shoots and increase in roots with the decrease of moisture in soil.

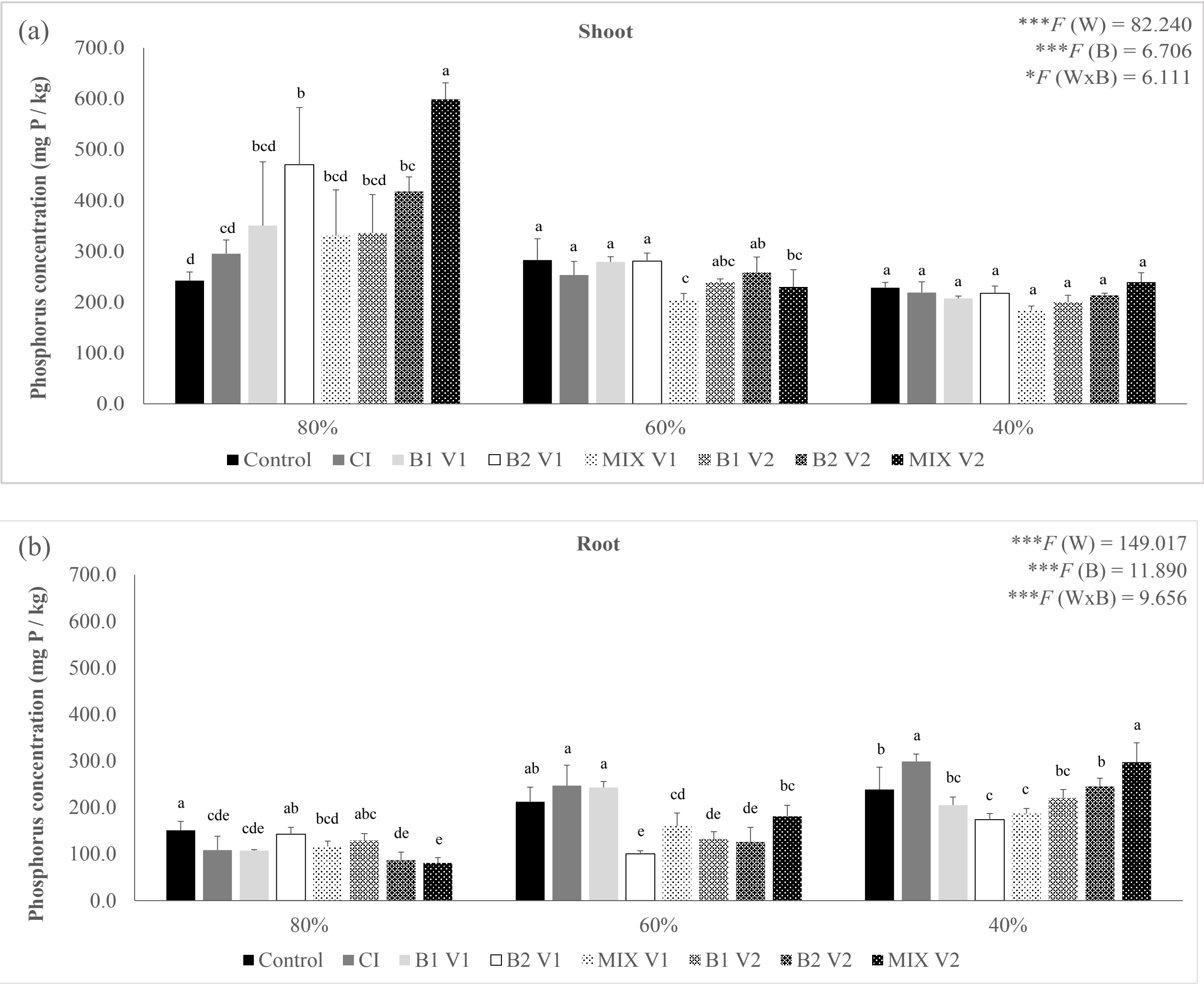

Figure 3.9 Phosphorus accumulation in shoot (a) and root (b) of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± standard deviation (n = 4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on shoot and root P accumulation (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of the bacterial treatments on shoot and root P accumulation for each water regime. Means for the same water regime showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. For shoots, the F values of one way ANOVA are $F = 6.754$ ($P < 0.001$), $F = 3.739$ ($P < 0.001$) and $F = 1.049$ (NS), respectively for 80, 60 and 40% of WHC. For roots, the F values of one way ANOVA are $F = 6.615$ ($P < 0.001$), $F = 13.857$ ($P < 0.001$) and $F = 9.416$ ($P < 0.001$), for 80, 60 and 40% of WHC, respectively.

Shoot P accumulation at 80% ranged from 242.3 to 598.8 mg P. kg^{-1} dw (Control and MIX V2, respectively), while at 60% and 40% ranged from 203.0 to 282.9 mg P. kg^{-1} dw (MIX V1 and Control, respectively) and from 183.7 to 239.3 mg P. kg^{-1} dw (MIX V1 and MIX V2, respectively), respectively. Plants growing at 80% presented higher P values in their tissues than plants growing under 60 and 40% of WHC. Within this water regime, B2 V1, B2 V2 and MIX V2 significantly ($P < 0.001$) increased P accumulation on shoots, however MIX V2 accumulated P in plants tissues to a concentration much higher than the other two treatments, by increasing 60% this parameter when compared to the control. At 60%, significant ($P < 0.001$) differences were observed between MIX V1 and MIX V2, nonetheless, both treatments presented negative effects on P accumulation. Statistical analysis for 40% of WHC revealed no significant differences among the means.

P accumulation in roots ranged from 80.8 to 151.1 mg P. kg^{-1} dw (MIX V2 and Control, respectively) at 80% and from 100.3 to 212.4 mg P. kg^{-1} dw (B2 V1 and Control, respectively) and from 173.9 to 298.8 mg P. kg^{-1} dw (B2 V1 and MIX V2, respectively) at 60 and 40%, respectively. At 80% of WHC, significant ($P < 0.001$) differences were observed in almost all treatments, with the exception of B2 V1 and B1 V2. At 60% of WHC, most bacterial treatments (B2 V1, MIX V1, B1 V2, B2 V2 and MIX V2) decreased P accumulation in roots, while at 40%, CI and MIX V2 showed a positive and significant influence on this parameter.

3.2.2.2.1 Phosphorus use efficiency

Phosphorus use efficiency results are presented in Figure 3.10. According to the results of the two way ANOVA, the water regimes and bacterial inoculation, as well as the interaction between both factors, had a significant ($P < 0.001$) effect on P use efficiency.

Figure 3.10 shows that plants growing at 80% presented a reduce P use efficiency when compared to the 60 and 40% of WHC. At 80%, only B2 V1, B2 V2 and MIX V2 were significantly ($P < 0.001$) different, however a negative influence was observed within these treatments. At 60%, MIX V1, B1 V2 and MIX V2 significantly ($P < 0.001$) improved P use efficiency, enhancing this parameter by 26, 16 and 17% respectively, when compared to the control. At 40%, the same trend was observed, where B1 V1, B2 V1, MIX V1 and B1 V2 significantly ($P < 0.001$) increased this parameter, especially MIX V1, which enhanced P use efficiency by 20% when compared to the control.

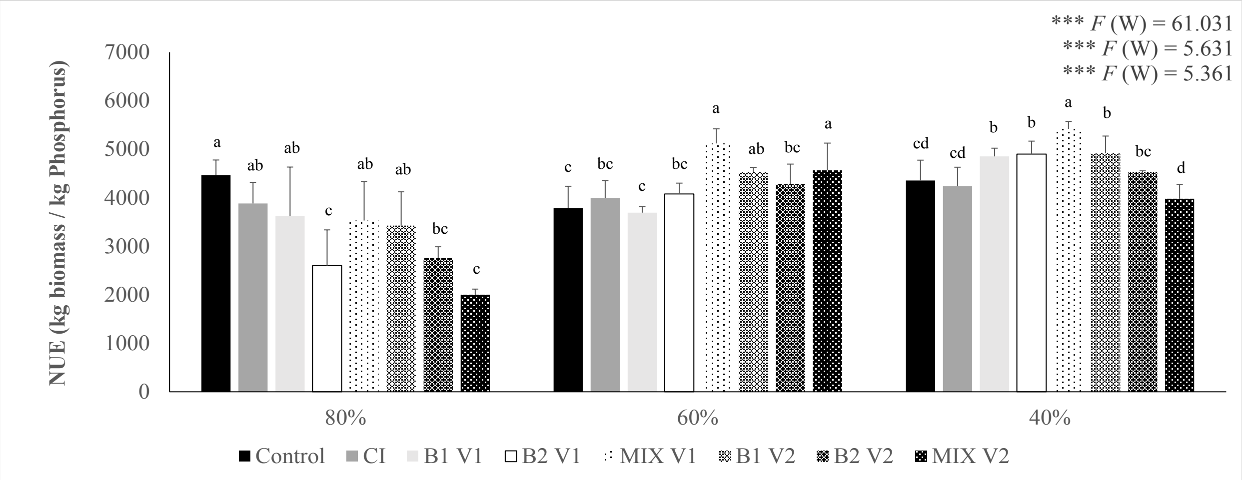

Figure 3.10 Phosphorus use efficiency of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± Standard Deviation (n = 4). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on P use efficiency. (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of the water regimes on P use efficiency for each treatment. Means for the same concentration showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. The F values of one way ANOVA are $F = 4.893$ ($P < 0.001$), $F = 5.431$ ($P < 0.001$) and $F = 8.230$ ($P < 0.001$) for 80, 60 and 40% of WHC, respectively.

3.2.3 Soil enzymatic activities

3.2.3.1 Catalase activity

Results obtained for the determination of the catalase activity in rhizospheric soils of plants growing under the different water regimes are shown in Figure 3.11. According to the result of the two way ANOVA, the water regimes and bacterial inoculation, as well as the interaction between both factors, had a significant ($P < 0.05$) effect on soil catalase activity.

At 80% of WHC, the treatments B1 V1 and MIX V2 negatively ($P < 0.01$) influenced catalase activity. At 60 and 40%, the inoculation of MIX V2 also significantly ($P < 0.05$) decreased catalase activity.

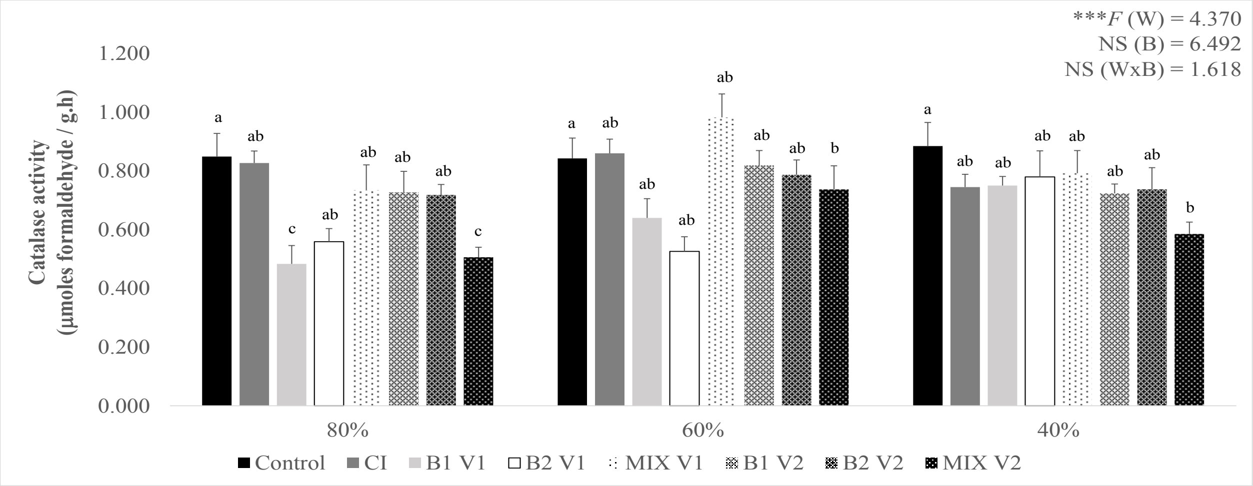

Figure 3.11 Catalase activity in rhizospheric soil of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± Standard Error (n = 5 to 6). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on catalase activity in soil. (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of the water regimes on catalase activity for each treatment. Means for the same concentration showing different letters are significantly different from each other ($P < 0.05$) according to Duncan test. The F values of one way ANOVA are $F = 4.267$ ($P < 0.01$), $F = 1.487$ ($P < 0.05$) and $F = 1.487$ ($P < 0.05$) for 80, 60 and 40% of WHC, respectively.

3.2.3.2 FDA hydrolysis activity

Results of the determination of FDA activity are described in Figure 3.12. According to the result of the two way ANOVA, only the water regimes applied to the soil had significant effect in the activity of FDA. In fact, FDA activity was higher in the rhizospheric soil of plants growing at 80% WHC, being significantly reduced at 40%.

At 80% of WHC, none of the treatments was significantly different between them, so bacterial inoculation did not influenced this parameter. The same results were observed at 60%. At 40%, significant ($P < 0.05$) differences were observed between B1 V1 and MIX V1, nonetheless, none of them influenced positively FDA activity.

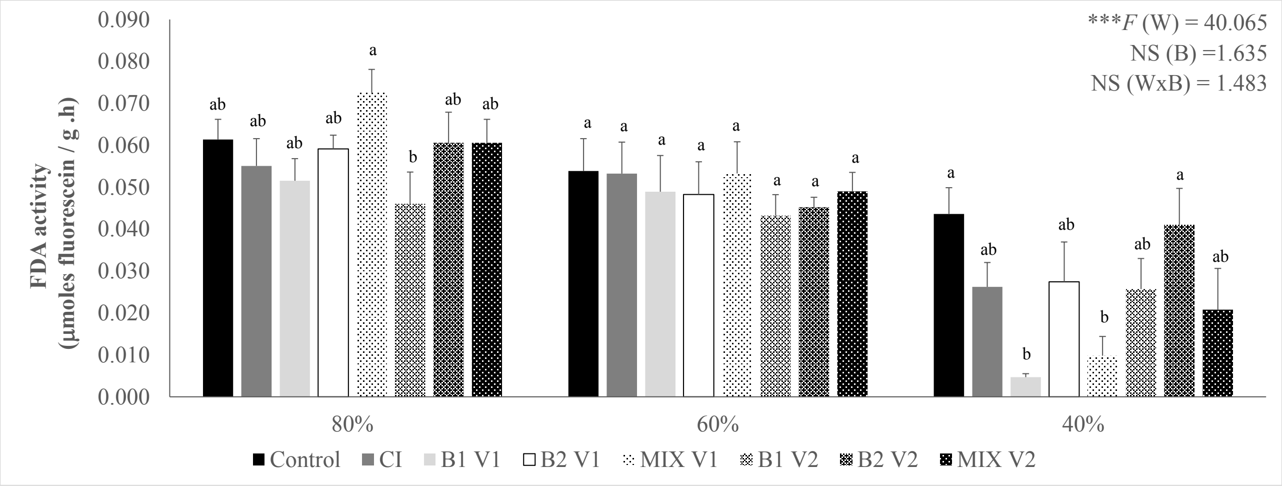

Figure 3.12 FDA activity in the rhizospheric soil of maize plants growing under different water regimes (80, 60 and 40% of WHC).

Values are means ± standard error (n = 3 to 8). A two way ANOVA was performed to determine the influence of bacterial inoculation and the water regimes on FDA activity in soil. (W: water regimes; B: bacterial treatments; W x B: water regimes x bacterial treatments) and as NS: Non significant at the level $P > 0.05$; * significant at the level $P < 0.05$; ** $P < 0.01$; *** significant at the level $P < 0.001$, respectively. One way ANOVA was performed to determine the influence of bacterial inoculation on FDA activity for each water regime. Means for the same water regime showing different letters are significantly different from each other ($P < 0.05$) according to Duncan Test. The *F* values of one way ANOVA are $F = 1.686$ ($P < 0.05$), $F = 0.302$ (NS) and $F = 2.653$ ($P < 0.05$) for 80, 60 and 40% of WHC, respectively.

DISCUSSION

In general, the water stress regimes applied to soil negatively affected maize growth and development. The severe water regime (40% of WHC) caused detrimental effects on plants and on soil microbiota; in fact, shoot elongation, shoot and root biomass, shoot N accumulation, root P accumulation and FDA activity were seriously affected by this water regime. From all bacterial treatments tested, at the different water regimes, the best results were observed on plants inoculated with the mixture of *P. fluorescens* and *R. eutropha,* enhancing some plant parameters, such as shoot and root biomass, root N accumulation, shoot and root P accumulation and N and P use efficiency, and no differences were observed between the two inocula size applied to plants.

Soil moisture plays an important role in plant growth because it can affect the germination of the seeds, plant growth and nutrition, microbial decomposition of the organic matter, altered nutrients transformation and others[61]. As such, an optimal water balance must be accomplish in the rhizospheric soil in order to enhance plant growth and allow the survival of the microorganisms in the soil. If excessive water is present in the soil, plants will continue to absorb water until they reach a turgor situation, where cells swell because of the pressure applied, until they burst leading to the plant death in extreme situations. On the other hand, lack of water causes nutrient deficiencies, reduces photosynthesis and promote hormonal imbalance, leading to a low yield of plants, wilting and death in the worst cases[6].

One of the methods to evaluate drought is using the meteorological index PSDI, which detects drought and classified them according to their intensity. This calculation is based on the quantity of precipitation, the air temperature and the water capacity available in soil[18]. With this index, different wet and drought conditions can be classified and the categories are shown in Table 4.1. For this study, according to PSDI index, the three water regimes applied, 80, 60 and 40% of WHC, correspond to normal conditions, moderate and severe drought, respectively.

Table 4.1 Classification of wet/drought conditions in mild, moderate, severe and extreme according to PSDI[18].

Category	PSDI classification
Extremely wet	4.00 or more
Very wet	3.00 to 3.99
Moderately wet	2.00 to 2.99
Slightly wet	0.50 to 1.99
Normal	0.49 to -0.49
Mild drought	-0.50 to -1.99
Moderate drought	-2.00 to -2.99
Severe drought	-3.00 to -3.99
Extreme drought	-4.00 or less

Many studies have demonstrated that the presence of environmental stresses, such as drought, impairs plant growth, which is reflected in the reduction of shoot elongation, dry biomass and other parameters[62]. Zahir et al. [37] studied the effectiveness of *P. fluorescens* and *P. putida* on growth promotion of peas (*Pisum sativum*) under drought stress. In fact, they tested four water regimes (25, 50, 75 and 100% of WHC) and found that root and shoot elongation as well as biomass decreased among the regimes. Boutraa et al. [63] found similar results on wheat. They cultivated wheat under different water regimes (30, 50 and 80% of WHC) and results indicated a decline in shoot and root dry biomass at moderate and severe water deficit.

In the present study, results demonstrated a reduction in shoot elongation at 40% of WHC. In fact, plants facing water deficit stress tend to reduce their leaves area, through the reduction of cell elongation in order to maintain water supply, increasing the chances of plants to survive[63]. Results showed that the inoculation of PGPB in the rhizosphere of maize plants did not improve shoot elongation among the different water regimes applied. Actually, at 40% bacterial inoculation promoted a negative influence on this parameter. Despite that, results showed higher plant biomass for shoots and roots of inoculated plants at 60% than at 80% when compared to the control. Some reports show promotion of shoot elongation with bacterial inoculation. Naveed et al.[19] observed an increase in the number of leaves per plant, leaf area, shoot and root biomass both under normal and reduced irrigation. In this study, four microbial treatments at 80% of WHC and all bioinoculants at

60% positively influenced shoot biomass. The presence of the bacterial strains *P. fluorescens* S3X and *R. eutropha* 1C2 and the co-inoculation of both strains in the rhizosphere increased shoot biomass. Similarly, Vardharajula et al.[64] demonstrated that the inoculation of sunflower with strains of the genera *Pseudomonas sp.,* significantly improved shoot elongation as well as total dry biomass under no stress and drought stress conditions because of their capacity to synthesize exopolysaccharides (EPS). In fact, *Pseudomonas sp.* are known to produced EPS, which are polymers composed by sugar residues known for their ability to protect microorganisms and plants from water deficit stress by enhancing water retention[43]. In addition, the results obtained in this study might be related to the ability of these bacterial strains to synthesize plant growth promoting substances, such as IAA, which can increase water and nutrient uptake, helping plants to survive during lack of water[65]. In fact, it was observed that these two rhizobacterial strains had the ability to produce *in vitro* high levels of IAA under the different osmotic potentials tested, being also able to withstand severe water deficit stress. Vardharajula et al.[66] showed similar results on maize under drought stress. They screened the ability of several rhizobacterial strains to grow at minimal water potential (-0.73 Mpa) and also tested the production of IAA, under stressed and non-stressed conditions. Some isolates showed drought tolerance and production of IAA under these conditions and were inoculated in maize plants to analyze their performance under water stress deficit. They found that all strains promoted plant growth.

MIX V2 was the only treatment that stood out from the others treatments at 60% of WHC in shoot biomass. Actually, this treatment also showed good performance at 80% of shoot, and at 60% of root, presenting positive effects on biomass. The co-inoculation of both strains improved maize growth, especially at 60% level of water regime. Actually, a normal situation in the rhizosphere is to find interactions between different bacteria which may have different or complementary modes of action or abilities which allow them to survive under stressful conditions and simultaneously enhance plant growth. The fact of having positive results using a co-inoculation of two PGPB means that both of them did not enter in competition and stimulated plant growth[66].

Some studies has demonstrated that a co-inoculation can positively affect plant growth. The results obtained in this study are in line with what Akram et al.[6] demonstrated. They co-inoculated wheat seedlings with drought tolerant bacteria under water deficit conditions (100, 75 and 50% of WHC). They observed that the combined application of bacteria as consortium showed better results than single inoculation, significantly increasing dry weights of roots and shoots of wheat.

These results can be explained because a co-inoculation of several bacteria can improve their adaptability to soil and environmental conditions, can have a better colonization of the rhizosphere and can have better capacities to overcome pathogens attacks. Kumar et al.[67] also proved that when a consortia of bacteria (*Pseudomonas putida* and *Bacillus amyloliquefaciens*) were applied to chickpea (*Cicer arietinum L.*) under drought stress, a better performance was observed, enhancing plant growth and ameliorating the negative effects of water deficit stress.

The selection of the adequate strains to be inoculated in soil is an important issue to guarantee the survival of the bioinoculants in the environment and simultaneously to help plants to develop under stressful conditions. Nonetheless, the amount of the inoculated bacteria is another issue of concern. Low quantity of bacteria in the rhizosphere could lead to a low colonization, preventing rhizobacteria to enhance plant growth[26]. In this study, two different inocula size, V1 ($3.3x10^3$ cells g^{-1} dw) and V2 ($2.5x10^6$ cells g^{-1} dw), of each microbial treatment were applied to plants to evaluate differences in plant growth. However, overall no significant differences were observed between both levels of the inocula applied. These results are in accordance to Moreira et al.[26], that applied two different inocula size ($0.25x10^7$ and $0.5x10^7$ cells. g^{-1} dw) of the rhizobacterial strains *R. eutropha, Chryseobacterium humi, P. fluorescens, R. radiobacter* and *P. reactans* in maize seedlings and no significant effects in improving plant biometric parameters were observed. These results suggested that the improvement of plant growth is not necessarily dependent of the quantity of bacteria inoculated on the rhizosphere, but depends mainly on the traits that the bacteria exhibit in order to adapt itself to stressful conditions that can be present in the environment and at the same time, also depends on the establishment of a symbiotic relation with root plants, so rhizobacteria can colonize this area and improve growth and development of plants.

Chlorophyll is a vital pigment produced by plants during photosynthesis. When an abiotic stress, such as drought, is present in the environment, plants do not synthesize adequate amounts of chlorophyll, and consequently occurs a reduction of the photosynthesis[68]. In this study, under non-stressed conditions (before application of water regimes), no significant differences were observed on chlorophyll content in all bacterial treatments. Nonetheless, when the water deficit stress was applied (after 8 weeks of sowing), a decrease on chlorophyll content was observed in all water regimes and inoculation patterns. It is known that chloroplasts are organelles very sensitive to different stressful situations, such as drought[68]. Probably, the decrease on the chlorophyll content happens due to chloroplast damage that it is caused by ROS during the stress, in fact these compounds initiate damage in the chloroplast and cause a cascade of damaging effects, leading to chlorophyll destruction and as consequence, reduction in photsynthesis[8]. However, the chlorophyll reduction observed does not seem to negatively influence plant photosynthesis as at the end of the experiment the leaves remained green and no symptoms of lack of chlorophyll were observed. This result could be due to the short experiment length, which might be not enough to cause prejudicial damage on photosynthesis. Heidari et al.[62] demonstrated that rhizobacteria inoculated on the rhizosphere of plants of basil (*Ocimum basilicum* L.) during water deficit stress, improved chlorophyll content on leaves plants. Nonetheless, during this experience, bacterial inoculation did not increase leaves chlorophyll content in the maize plants.

In this study, results showed a higher accumulation of N in shoots than in roots, which may be explain by the fact that N is needed for photosynthesis in leaves, tissue respiration, conversion and storage of photosynthesis[69]. Curiously, it was observed that when water deficit stress is present, plants tend to increase the accumulation of N in shoots, as it was observed in non-inoculated plants as well as in inoculated. However, an opposite trend was observed for roots. The different accumulation of this nutrient in different compartments during non-stressed and drought stressed conditions is explain because plants tend to increase N concentration in leaves to maintain growth under dry conditions[69].

Phosphorus is another important nutrient required by plants for their growth and development. Results demonstrated a higher accumulation of P in shoots than in roots. This can be explained because P it is used by plants for the production of proteins, so a usual situation for plants is to accumulate higher amounts of this nutrients in this tissue[69]. However, results suggested that while on shoot, the highest accumulation of P was recorded in plants growing at 80% of WHC and a reduction of this nutrient was observed as water deficit increased, in roots, P concentration increased with the reduction of soil moisture, presenting the highest value at 40%. Thus, an inverse relation was observed between shoots and roots. This might be related to the fact of, in the presence of stress, plants have the ability to balance the distribution of the nutrients among their compartments, as a strategy to response to the changes occurring in the environment[69].

Some studies have demonstrated the positive effects of inoculation of PGPB on plants in increasing nutrients uptake. Erdogan et al.[70] showed that strawberry plants, under drought conditions, inoculated with three PGPR strains (*Paenibacillus polymyxa*, *Rhodococcus* *erythropolis* and *P. fluorescens*) generally grew better and had higher N and P uptake than control plants. Pereira et al.[20] demonstrated higher accumulation of N on roots than on shoots, and they also showed that bacterial inoculation increased the accumulation of this nutrient in sunflower tissues under salinity stress. For roots, MIX V1 and MIX V2 were the two treatments that increased the most N accumulation at 60% of WHC. Curiously, MIX V2 treatment also presented the highest value of P concentration in shoot at 80% of WHC and moreover MIX V2 also increased accumulation of this nutrient at 40% in roots. The presence of both strains simultaneously in the rhizosphere might enhanced N and P accumulation in plant compartments, as it was already discussed, because of a non-competitive relation between them.

Nutrient use efficiency is a useful tool to indicate how well plants are using the nutrients present in the soil. In this study, this parameter was calculated for N and P and results suggested that MIX V1 and MIX V2 were the most efficient treatments in improving NUE under 60 and 40% of WHC. However, at normal conditions none of the treatments influenced positively this parameter for both nutrients. In fact, the ability of PGPB in enhancing NUE has been studied. Adesemoye et al.[71] tested the hypothesis that microbial inoculants could not only increase maize growth but also enhance nutrient uptake, turning the use of these nutrients more efficient for the plants. Their results demonstrated that the presence of PGPR and AMF into the rhizosphere promoted maize growth and yield. It also increased N and P uptake efficiency, improving NUE and reducing potential losses of these nutrients to the environment. Hui et al.[72] also showed that a mixture of PGPR (*B. amyloliquefaciens and B. pumilus*) improved plant growth and increased NUE in tomato plants growing in calcareous soils.

Soil moisture has an important role in the maintenance of soil microorganisms, so changes in the irrigation level can affect soil microbial properties, such as soil enzyme activities. Catalase is one of the intracellular enzymes usually found in all aerobic bacteria[73]. According to the results obtained in this study, under drought stress, specifically at 60 and 40% of WHC, in general, it was observed a significant increase in catalase activity in the rhizospheric soils of inoculated maize plants. This increase can be related to the higher amounts of H_2O_2 produced by bacteria as a consequence of oxidative stress caused by water deficit stress. Ali et al.[74] concluded that changes of water regimes and K^+ rates influenced soil microbial properties, such as enzyme activities, including catalase activity. Erdogan et al.[70] have also shown an increase of catalase activity with the increasing levels of water deficit in inoculated and non-inoculated strawberry plants. They suggest that the activities of the antioxidant enzymes increased under water-deficit stress because plant cells stimulate different antioxidant enzymes, such as catalase, to eliminate ROS or suppress their formation. However, in this study, bioinoculants did not had a positive influence on catalase activity when compared to the control in all water regimes tested.

FDA hydrolysis is used as a measure of the microbial activity in environmental samples because it comprises protease, lipase and esterase activities[75]. In fact, bacterial cells transport fluorescein diacetate inside the cells, where it is hydrolyzed into fluorescein. When the storage capacity is full, the cells release it into the extracellular environment. Thus, FDA hydrolysis is generally proportional to the viable microbial population present in the sample[75]. This study demonstrated that at 80 and 60% of WHC, a representative amount of FDA was hydrolyzed into fluorescein, indicating that a larger microbial population was present on these samples. Among the water regimes, the FDA hydrolysis decreased, showing an important reduction of FDA hydrolysis at 40% of WHC, suggesting that the microbial density present in the rhizosphere decreased remarkably under this water regime. Similar results were obtained by Rietz et al.[76] that showed a decline of FDA activity with increasing salinity and sodicity in soils. Bacterial treatments in all water regimes tested did not influence positively FDA activity. These results can explain why at 40% of WHC bacterial treatments did not improve plant's performance. Probably the bacteria inoculated in the rhizosphere during the experiment could not stand the severe osmotic stress applied to plants, and eventually the majority of the microorganisms disappear, not enhancing plant growth.

According to the results obtained in this study, the commercial inoculum treatment was the less effective treatment tested. In fact, it influenced negatively plant parameters, decreasing shoot elongation and shoot and root biomass, except for shoot biomass at 60% of WHC. CI did not improved the nutritional parameters tested, with the exception of P uptake in roots at 40%. For the soil enzymes, no significant effects were observed in the plants inoculated with this treatment.

CONCLUSION

This study concluded that applying different water regimes to maize plants reduced their growth with decreasing moisture in soil. Catalase activity was slightly increased at moderate and severe water stress, which suggested that in fact, microorganisms were in oxidative stress. It was observed that severe water regime was the most devastating stress for plants, showing a reduction in shoot elongation, biomass, nutrients uptake and FDA activity. Actually, an important reduction of FDA activity at 40% of WHC indicates that the soil microbial activity on this regime was minimal, when compared to the others water regimes. At 80 and 60% of WHC, bacterial inoculation showed the best performance, enhancing some parameters tested, especially when *P. fluorescens* S3X and *R. eutropha* 1C2 were co-inoculated in the rhizospheric soil. Treatments were applied in two different inocula size, and no particular effect in the improvement of plants growth was observed. A commercial inoculum was also tested and results suggested that it was the less effective treatment applied to the plants, enhancing almost none of the plant parameters tested.

It is conclude that the inoculation of maize plants with bacterial strains is an alternative to reduce the prejudicial effects of drought stress on plant's growth.

FUTURE WORK

The present study was based on an exploratory approach to evaluate the effect of PGPB to enhanced maize growth under drought stress. Considering this thesis as a base, it would be important to perform more *in vitro* assays, namely ACC deaminase, siderophores and exopolysaccharide production to have a better understanding of PGPB behavior under drought stress. Moreover, the production of ABA and the accumulation of compatible solutes, like proline, are typically increased during water deficit stress and their study *in vitro* would be important, as well.

Lipid peroxidation would be another assay that could be performed in the future. This is a mechanism of cellular damage that occurs because of diseases or because of environmental stresses. This assay would give information about the oxidative stress that the plants were subject under the drought stress conditions applied.

It would be interesting to analyze the activity of other soil enzymes, such as dehydrogenase activity (DHA). This enzyme is one of the most important in the soil environment because it is used as an indicator of overall soil microbial activity. Studying DHA along with FDA activity would give better information about microbial activity under drought stress.

An interesting analysis would be following the inoculants in the soil during the assay. With molecular biology techniques, such as denaturing gradient gel electrophoresis (DGGE), it would be possible to know if in fact the bacterial inoculants remained in the rhizosphere along time and despite the drought stress applied to the soil.

A new approach would be performing a new greenhouse experimental design this time using PGPB directly isolated from drylands. It would be also interesting to inoculate mycorrhizal fungi along with the PGPB to observe if the mixture would improve plant growth under water deficit stress.

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