2016•2017
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Can drought-resistant plant-growth promoting bacteria help plants to survive in arid environments?

Promotor :
Prof. dr. Jaak VANGRONSVELD

Elke Paredis

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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universiteit hasselt | Maastricht University

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LIST OF ABBREVIATONS

ACC	1-aminocyclopropane-1-carbocyclic acid
ANOSIM	Analysis of similarities
APOD	Ascorbate peroxidase
ARISA	Automated rDNA intergenic spacer analysis
BLAST	Basic Local Alignment Tool
BS	Bulk soil
BTB	Bromothymolblue
Car	Carotenes
CAS	Chrome-Azurol S
CAT	Catalase
Chla	Chlorophyll a
Chlb	Chlorophyll b
DNA	Deoxyribonucleic acid
DT	Drought tolerance
DTT	1,4-Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharides
FW	Fresh weight
GPOD	Guajacol peroxidase
IAA	Indole-3-acetid acid
IC	Indolic compounds
ISR	Induced systemic response
NBRIP	National Botanial Research Institute
NCBI	National Center for Biotechnology Information
nMDS	non-Metric Multidimentsional Scaling
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PGP	Plant growth promotion
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
Pro	Proline
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
rpm	Rounds per minute
RS	Rhizosphere soil
SID	Siderophores
SL	Shoot length
SMN	Sterile salt minimal medium
SOD	Superoxide dismutase
SP	Sampling places
SPOD	Sringaldazine peroxidase
U	Unit
VAM	Vesicular arbuscular mycorrhizal fungi

FOREWORD

This thesis is written as a final part of my five years of studying Biomedical Sciences at Hasselt University, which was not possible without the support and guidance of the following people.

First of all, I would like to thank Prof. dr. Jaco Vangronsveld for giving me the opportunity to perform this internship within the bacterial group. The past eight months would never been so interesting, educational nor pleasant, therefore I would like to extend my gratitude towards my supervisor Iva Cholakova for the huge amount of guidance during my internship. Thank you for improving my lab skills, for your confidence in my abilities and always making time to answer my questions. Thank you dr. Sofie Thijs for sharing your advice and experience with me. In addition, I would also like to thank Wouter Sillen, for helping me with the statistics. Furthermore I would like to thank everyone from the bacteria group who helped me out when a harvest was planned, for always answering loads of questions, and for the nice chats during the past eight months.

Last but not least, I would like to thank my parents and friends for their encouragement and big amount of support during the last five years. Phedra and Anneleen, I 'm truly happy that I had the chance to get to know you both better. We were together every day, had so much fun the past eight months, had endless discussions about our results, but when needed; we were there for each other! I can't complain that after this internship, I'm not only enriched with a lot of knowledge but also with two friends for the rest of my life!

SUMMARY

INTRODUCTION Drought is an insidious hazard of nature expected to cause serious plant growth problems for more than 50% of the arable lands by 2050. It is a worldwide, natural occurring phenomenon defined as sustained large-scale occurrence of a below average water availability. Together with the continuous rise in population, major challenges for the agricultural sector are implied to sustain food availability. Therefore, investigation towards an alternative method is necessary. Plant growth promoting bacteria (PGPB) may be beneficial to plants by providing nutrients, modulate hormone levels to protect plants from abiotic stresses. The aim of this research is to investigate if PGPB isolated from the drought resistant *Pistacia terebinthus* (in spring and autumn), a tree existing in the arid Bulgarian Rhodope Mountains, and PGPB isolated from the grass around the *Pistacia* (autumn) can transfer their drought resistance to wheat (*Triticum turgidum* var. *durum* cv. Vitron).

MATERIALS AND METHODS *In vitro* bioassays for plant growth promotion (PGP) and drought tolerance (DT) tests were performed for strains isolated from *pistacia* and grass, followed by *in planta* drought exposure experiments where wheat was inoculated with the most promising strains. Plant parameters such as biometric measurements, anti-oxidative capacity and photosynthetic parameters were measured. A genotypic characterization was performed for a subset of the community obtained from *Pistacia*. Automated rDNA intergenic spacer analysis (ARISA) fingerprinting was performed for DNA extracted from rhizosphere and bulk soil originating from *Pistacia* to compare total bacterial communities between the seasons. Furthermore, quantification of vesicular arbuscular mycorrhizal (VAM) fungi colonization was performed for *Pistacia* roots obtained from the different seasons of sampling (spring and autumn).

RESULTS When plants were not exposed to drought, no difference could be observed between the non-inoculated and inoculated conditions. Inoculation with strains derived from *Pistacia* caused an increase in the biometrical and photosynthetic parameters. With the exception for strain *157a Raoultella* sp., where a decrease in antioxidant capacity was observed. Overall, inoculation with strains derived from grass yield negative effects when considering the plant parameters. Genera were diverse between spring and autumn for the cultivable bacteria. A trend towards separation of total bacterial communities in spring and autumn was visible. *Pistacia* roots showed VAM colonization over the two different seasons.

DISCUSSION AND CONCLUSION Based on this research, our findings suggest that PGP activity is a stress-dependent and not a *per se* feature of the strains. Moreover, strains isolated from the *Pistacia* are better in alleviating drought stress in wheat than strains derived from grass. Thereby stating that drought-resistance PGPB obtained from *Pistacia* are cross compatible with different plant models. The abundance of VAM in the roots of *Pistacia* can lead towards promising results in further research. The use of selected microorganisms, alone or in a consortium may represent an important approach to decrease the deleterious effects of stress in crops.

SAMENVATTING

INLEIDING Naar verwachtingen zou in het jaar 2050 nog maar slechts 50% van de landbouwgronden beschikbaar zijn voor gewassenteelt te wijten aan droogte. Het is een natuurlijk veel voorkomend fenomeen dat wordt gedefinieerd als een langdurige ondermaatse water beschikbaarheid. Samen met de voortdurende stijging van de bevolking, oefent dit een enorme druk uit een op de agrarische sector om de voedselbeschikbaarheid te waarborgen. Onderzoek naar een alternatieve methode is nodig. Plantgroei-promoverende bacteriën (PGPB) kunnen gunstig zijn voor planten door voedingsstoffen te verschaffen, hormoonniveaus te moduleren, en zo bescherming te bieden tegen abiotische stress. Het doel van dit onderzoek is om te onderzoeken of PGPB geïsoleerd uit de droogtebestendige *Pistacia terebinthus* (in het voorjaar en in de herfst), een boom die zijn ontstaan kent in het droge Bulgaarse Rhodope-gebergte, en PGPB geïsoleerd uit het gras rond de *Pistacia* (herfst), hun droogteresistentie kunnen overdragen naar tarwe (*Triticum turgidum* var. *durum* cv. Vitron)

MATERIAAL EN METHODEN *In vitro* bioassays voor plantengroeipromoverende (PGP) - en droogte tolerantie (DT) -testen werden uitgevoerd voor stammen geïsoleerd uit *Pistacia* en gras, gevolgd door *in planta* droogte- blootstelling experimenten waarbij tarwe geïnoculeerd werd met de meest veelbelovende stammen. Plantparameters zoals biometrische metingen, anti-oxidatieve capaciteit en fotosynthetische parameters werden gekwantificeerd. Een genotypische karakterisering werd uitgevoerd voor een subset van de cultiveerbare bacteriële gemeenschap geïsoleerd uit *Pistacia*. DNA geëxtraheerd uit bulk en rhizosfeer bodem van *Pistacia* werd gebruikt om totale bacteriële populaties te vergelijken tussen de verschillende seizoenen. Vesiculaire arbusculaire mycorrhizale (VAM) schimmel kolonisatie werd gekwantificeerd op wortels verkregen van *Pistacia* (voorjaar en herfst).

RESULTATEN Geen verschil kon worden waargenomen tussen de niet-geïnoculeerde en geïnoculeerde condities, onder normale omstandigheden. Inoculatie met stammen afkomstig van *Pistacia* veroorzaakte een toename van de biometrische en fotosynthetische parameters. Met uitzondering van stam *157a Raoultella sp.*, werd er een afname van de antioxidant capaciteit waargenomen. Inoculatie met stammen afkomstig van gras veroorzaakte een afname in plant parameters. Genera van de cultiveerbare stammen waren divers tussen het voorjaar en herfst. Een trend in seizoensgebonden scheiding van de totale bacteriële populatie was zichtbaar. VAM kolonisatie was kwantificeerbaar in de wortels van *Pistacia* over de twee seizoenen.

DISCUSSIE EN CONCLUSIE Baserend op dit onderzoek kan er geconcludeerd worden dat PGP-activiteit een stressafhankelijke en niet *per se* kenmerk van de stammen is. Bovendien zijn stammen geïsoleerd uit *Pistacia* beter om droogtestress te overkomen in tarwe dan stammen geïsoleerd uit gras. Dit verduidelijkt dat deze droogte resistente PGPB niet enkel gerelateerd zijn aan hun gastheer plant. De aanwezigheid van VAM in de wortels kan leiden tot veelbelovende resultaten, al dan niet in een consortium met PGPB, in het verder onderzoek ter verbetering van de droogte tolerantie van gastheerplanten.

1. INTRODUCTION

1.1 DROUGHT STRESS

Plants can be exposed to different abiotic stresses such as salinity, extreme temperatures and drought. The latter is the most destructive abiotic stress that has an increased intensity over the past decades. Drought is expected to cause serious plant growth problems for more than 50% of the arable lands by 2050 (1). It is a worldwide, natural occurring phenomenon defined as sustained large-scale occurrence of a below average water availability. This insidious hazard is caused by a prolonged period of low precipitation and a rise in annual temperature. In Europe, large geographical and seasonal differences according to these two parameters are apparent (2). For this time period, an increase in winter precipitation for most of western and northern Europe is noticeable, while a decrease in southern Europe and parts of central Europe is apparent for the summer.

Figure 1 describes the projected changes in annual and summer precipitation for the period 2071-2100 compared to the baseline period 1971-2000. In parts of central Europe and northern Europe there will be a significant increase of 30% in precipitation and a decrease of 40% in southern Europe (left panel). During the summer, the zone where there is lower precipitation shifts northwards (right panel). This climate model is analogue with several others where there is a prediction for a general future increase in precipitation in northern Europe and a decrease in southern Europe, combined with a rise in annual temperature. Therefore, the areas in Europe suffering the most from drought are more specifically the southern-eastern parts (3).

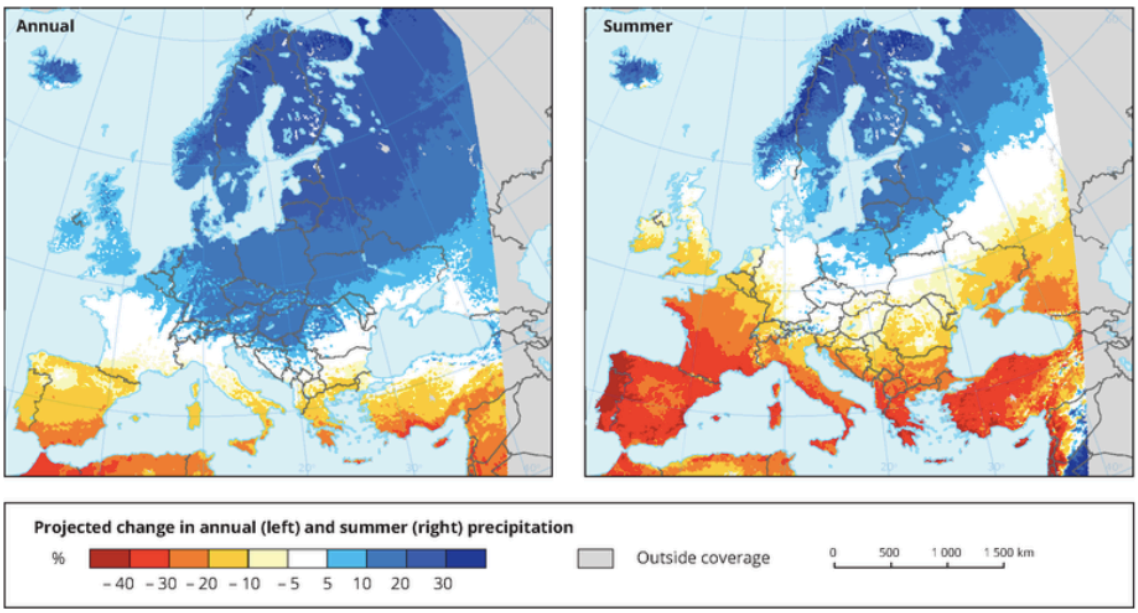

Figure 1. Projected changes in annual (left) and summer (right) precipitation (%) in the period 2071-2100 compared to the baseline period 1971-2000. Data provided by EURO-CORDEX initiative (3).

During the last few decades, major drought events have been recorded worldwide and thereby making farming exceedingly challenging in some countries. This resulted ultimately in lower crop yields and by that affecting world food security. For example Europe experienced an extreme drought event in 2003, which led to a dramatic reduction in primary production (4). Drought has not only a huge impact on the economy but the social effects are deleterious as well. Health is directly linked to water supply in any settlement. Clean drinking water, water for nutrition and sanitation helps to prevent illnesses and deaths. Fresh water levels during droughts are lower, resulting in less dilution in ecosystem waters. Leading towards a possible higher concentration of chemicals and a decline in the amount of dissolved oxygen, which affects organisms on each trophic level.

1.2 THE ROLE OF WATER IN PLANTS

Being by definition a living organism, such as a plant, exists out of two backbone functions – a cellular organization and a requirement for liquid water. Water is the central molecule of all physiological processes and it is used for various functions in plants e.g. transporting nutrients and organic compounds taken up by the roots or synthesized within the plant. It maintains the turgidity, which is caused by the osmotic flow of water from outside the cell into the cell's vacuole, which pushes the plasma membrane against the cell wall of the plant, hereby increasing the plants' rigidness. Water can buffer the plant against wide temperature fluctuations by the help of transpiration where the absorption, transport and release of water to the atmosphere by plants is involved. It is an obvious element in the living protoplasm of cells where it serves as a raw material for several chemical processes such as photosynthesis where carbohydrates are generated from carbon dioxide and water. Water is also required for several other processes in the plant such as germination, cell division and promotion of plant growth in height and width (5).

1.3 RESPONSES OF PLANT TO DROUGHT STRESS

The response of a plant facing a water deficit can be dependent on plant species, growth stage, duration and intensity of water deficit. Under rehydration after a mild stress, plants can turn to the normal physiological status. But when the water deficit is severe, recovery will be impossible. Under conditions of low water availability, stomatal closure, regulated by specific plant hormones, is known as one of the first strategies used by plants to diminish the transpiration rate and maintain sufficient cell turgor to continue plant metabolism (6).

1.3.1 REACTIVE OXYGEN SPECIES

Stomatal closure causes a progressive limitation of photosynthetic carbon assimilation leading towards a photosynthetic apparatus collapse, where the over-reduced photosynthetic apparatus may experience oxidative stress. This means limitation of CO_2 uptake and therefore photo respiratory production of H_20_2 in the peroxisome and production of superoxide, H_20_2 or singlet oxygen by the over reduced photosynthetic electron transport chain (6). The excessive production of reactive oxygen species (ROS) is known as oxidative stress and can cause membrane injuries, protein denaturation, enzyme inactivation, reduced synthesis and degradation of proteins, and defaults in membrane integrity (7). Under normal plant growth conditions, ROS are continuously produced by different organelles such as chloroplasts, mitochondria and peroxisomes. In order to

cope with the continuous ROS production, plants have developed several enzymatic and non-enzymatic antioxidant systems. The most important enzymes are catalase (CAT) and superoxide dismutase (SOD). With the exception from CAT, exclusively located in the peroxisomes, these enzymes are located throughout different compartments of the cell. SOD will catalyse the conversion of superoxide to oxygen or H_20_2, thereby generating another ROS species. The most prominent enzymes scavenging of H_20_2 are CAT and peroxidase e.g. ascorbate (APOD)-syringaldazine (SPOD)-, guajacol (GPOD)- peroxidase (7).

1.3.2 PHOTOSYNTHETIC PARAMETERS

Chlorophylls are plant pigments specifically arranged in photosystems embedded in chloroplasts or thylakoid membranes. They contain a stable porphyrin ring with free moving electrons. Therefore they have the possibility to capture energy out of the sunlight. In plants, there are two types of chlorophyll, chlorophyll a (Chl_a) the principal central photosynthetic pigment and chlorophyll b (Chl_b) the accessory peripheral photosynthetic pigment. Higher leave chlorophyll content is related with photosynthetic efficiency. However under drought stress, the low amount of CO_2 fixation leads to a reduction in the photosynthetic capacity followed by a decrement in the chlorophyll content. In addition, severe water deficit can inhibit photosynthesis by damaging chlorophyll components, and thereby changing the photosynthetic machinery (8). Besides the chlorophyll pigments, carotenoids can serve as accessory light-harvesting pigments by transmitting the light energy that they absorb to chlorophyll. In addition, carotenes serve as photo protective compounds by quenching triplet chlorophyll and singlet oxygen. Carotenoids present in the plant membrane play a key role in protecting plant cells subjected to drought stress (9).

1.3.3 MORPHOLOGICAL CHANGE

In a great majority of plants, drought stress results in morphological changes. These changes include wilting, shrinkage and reduction in the number of leaves, which leads to a lower photosynthetic rate and a lower biomass yield (5). Results from a study performed on corn (*Zea mays*, L) by JG Benjamin *et al.* revealed that the shoot growth was proportionately more affected than root growth, leading to an increased root/shoot ratio when exposed to drought to maintain osmotic pressure and facilitate water absorption (10).

1.4 MANAGING DROUGHT STRESS

With the changing climate and the continuous rise in population, which is predicted to reach 8 billion people by 2030, the sustainability of food production has become of more importance than in the past (11). Global maize and wheat production were projected to decline by 3.8% and 5.5% respectively with increasing temperature and decreasing precipitation, despite improved agricultural technologies. Nowadays chemical fertilizers are widely used in modern agricultural production to provide plants with several nutrients. However, the large-scale use of the fertilizers can cause harm such as waterway pollution, increased air pollution, acidification and mineral depletion of the soil (12). Further expansion of irrigation is also questionable due to lowering of the water table and potential salinization (13). Therefore other sustainable agricultural practices are needed while minimizing the threat to water resources. Despite several strategies, which have been suggested for controlling the negative impacts of drought stress, better alternatives are

necessary. A possible alternative strategy is to induce stress tolerance by priming the host plant with plant growth promoting bacteria (1).

1.5 PLANT GROWTH PROMOTING BACTERIA

The soil is well provided with millions of microbes that can inhabit plant tissues and root systems. Plants offer a wide range of habitats that support microbiological growth. These include areas rich in nutrients, but also exposed to environmental stresses. There they can form a possible complex community that influences plant growth and productivity through its metabolic activities and plant interactions. The interaction between the bacteria and plants can be beneficial, harmful or neutral. If the interaction between the associated bacteria with the roots or plant tissue alleviates plant stress, the microbes are known as plant growth promoting bacteria (PGPB). The most widely studied group of PGPB are plant growth promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere. Another group of PGPB are endophytes, found in the intracellular spaces where they establish endophytic populations visible in **Figure 2**. These beneficial microorganisms can promote the growth of plants through various direct and indirect mechanisms (**Figure 3**).

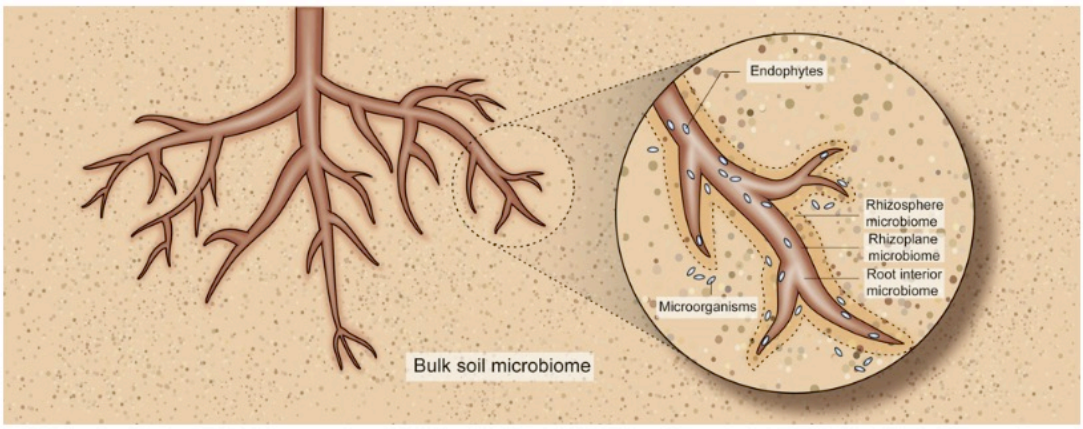

Figure 2. Model of the root microbiome: The root microbiome consists of microorganisms in the rhizosphere microbiome, the area surrounding the plant root; the rhizoplane microbiome, which is the root-soil interface and the internal root microbiome which consist out of endophytes. The bulk soil microbiome is the area outside of the influence of the plant roots. Figure adapted from *Gaiero et al.* (14).

1.5.1 DIRECT MECHANISMS

Direct mechanisms include the increasing of plant growth by supplying the plant with hormones. Plant growth promoting bacteria are able to produce indolic compounds (IC) such as the auxin phytohormone indole-3-acetic acid (IAA), which plays key roles in plant growth and development, and to escape or survive stressful conditions such as drought. The ability to produce these ICs is widely distributed among plant-associated bacteria. Indole-3-acetic acid is the best-characterized plant hormone produced by many plant-associated bacteria. Beneficial bacteria synthesize IAA through the indole-3-pyruvic acid pathway, a pathway dependent on L-tryptophan. Plant species inoculated with IAA producing bacteria show increased root elongation and increased root hair formation, followed by an increase in water and nutrient uptake (15).

Ethylene is another key phytohormone, participating in all processes of plants growth and development at low concentrations. But when detected in higher concentrations, ethylene can be

identified as a stress hormone. Under different biotic and abiotic stresses such as high temperature and drought, the amount of ethylene can be elevated and cause a reduction in root elongation, lateral root growth, root hair formation, and auxin transport. The production of this stress hormone can be lowered by PGPB due to the deamination of 1-aminocyclopropane-1-carboxyclic acid (ACC), the immediate precursor of ethylene. This will cause a degradation of ACC into ammonia and α-ketobutyrate. Plants' ACC can be excreted from the plant roots and taken up by the soil microorganisms where they hydrolyse this compound by the enzyme ACC deaminase, to lower the concentration of ACC in the environment (15).

Besides the production of hormones as a direct mechanism of microorganisms, they are also able to supply the plant with nutrients, mostly scarce available in agricultural soils with the consequence that plant/crop–growth is suboptimal. Nowadays, farmers are increasingly dependent on chemical sources of nitrogen and phosphorus. Moreover the fact that this is a costly affaire, the production of chemical fertilizers causes depletion of non-renewable resources and poses human and environmental hazards as stated before. Plant growth promoting bacteria can provide plants with the nutrients that they lack such as fixed nitrogen, iron and phosphorus.

Nitrogen is one of the most limiting nutrients for plant growth since atmospheric nitrogen occurs for 79% in the form of N_2 and is unavailable for use by most organisms, including eukaryotes. The molecule is almost inert because there is a triple bond between the two nitrogen atoms. Plants can only take up nitrogen in the form of ammonia (NH_3) or nitrate (NO_3). All organisms require nitrogen to synthesize biomolecules such as proteins and nucleic acids. It is even a major component of chlorophyll, the compound by which plants use sunlight energy to produce sugars from water and carbon dioxide (photosynthesis). Nitrogen fixation is the process responsible for the reduction of N_2 to ammonia or nitrate and is performed in diazotrophic microorganisms. Microorganisms able to fix nitrogen possess the reducing enzyme nitrogenase, an enzyme sensitive to oxygen that exists out of two metalloproteins, FeMo-protein and Fe-protein. Even though there are many physiological and genetic differences between the diazotrophics, they all contain the enzyme nitrogenase (16).

Phosphorus (P) is an essential macro-element, required for plant nutrition. It participates in metabolic processes such as photosynthesis, energy transfer and synthesis and breakdown of carbohydrates. Plants can absorb P in two soluble forms: the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}). The amount of phosphorus in the soil is generally quite high, but most of this compound is insoluble and therefore not available for the plants nutrition to support plant growth. Phosphate is highly reactive with other soluble components such as aluminum in acid soils (pH<7), and calcium in alkaline soils (pH>7). The insoluble form is mostly present in the form of an inorganic mineral, such as insoluble mineral complexes, or as an organic compound, incorporated into the biomass. Therefore the availability of P depends on the solubility of this element. Bacteria can enhance the uptake of phosphate by plants by solubilizing the phosphate in the soil. They can bring phosphorus back into the solution by producing organic acids, which chelate mineral ions (16).

Despite the fact that iron is the fourth most abundant element on earth, it is the third most limiting nutrient for plant growth and metabolism in many crop plants resulting in poor yields and reduced nutritional quality. In plants, iron is involved in chlorophyll synthesis, essential for the maintenance of chloroplast structure and function. It is found in proteins such as nitrogenase, ferredoxins and cytochromes. In aerobic soils, bacteria or plants do not easily assimilate iron because ferric ion (Fe3+), which is the predominant form, is only sparingly soluble so that the amount available for assimilation by living organisms is extremely low. This is the case because under aerobic conditions and at a physiologic pH, the reduced ferrous (Fe^2+) form is unstable and is easily oxidized to the oxidized ferric form. Plant growth promoting bacteria could perform uptake of iron from soil and satisfy nutritional plant requirements of this element in a specific way. Siderophores are low-molecular-mass microbial compounds with a high affinity for iron. They are secreted to solubilize iron from their surrounding environments to form a ferric-siderophore complex that can move by diffusion. Siderophores chelate ferric ion with high affinity, allowing its solubilization and extraction from most mineral and organic complexes (17).

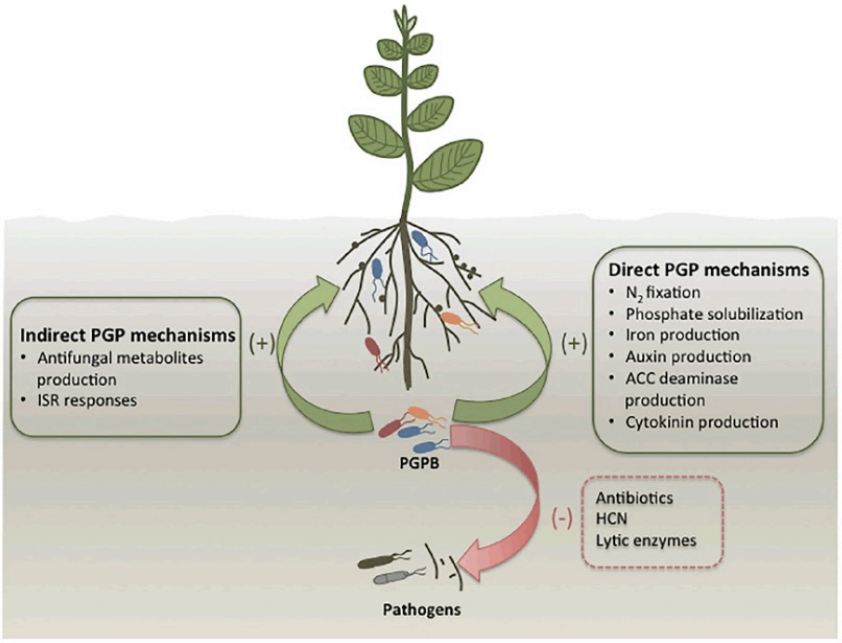

Figure 3: PGPB can promote growth through various direct and indirect mechanisms. Direct mode of actions includes nitrogen fixation, phosphate solubilisation, siderphore production, ACC-deaminase production and plant hormones production. The indirect mode of actions are largely based on excluding pathogens. Figure adapted from Premachandra et al (18).

1.5.2 INDIRECT MECHANISMS

On the other hand, indirect mechanisms to promote plant growth are the ability of PGPB to reduce the deleterious effects of plant pathogens on the plant growth. This involves the production of antibiotics in response to proliferation of plant pathogens as a biocontrol agent or the production of lytic enzymes such as chitinases, cellulases, 1,3-glucanases, proteases and lipases to lyse the cell walls of pathogenic fungi produced in response to proliferation of plant pathogens. Induced systemic response (ISR) is a mechanism of increased resistance at particular sites of the plants, activated only by induction of a pathogen. A jasmonate and ethylene signaling precede a range of defense responses to the pathogen. Another mechanism preventing plants from some pathogens is the production of siderophores to prevent pathogens acquiring a sufficient amount of iron and in

this way and suppress their ability to grow. Many bacteria have been reported to produce antifungal metabolites like cyanide and it is known that PGPR compete with detrimental microbes for nutrients and in this way limit the disease-causing agent (15, 17).

1.5.3 DROUGHT TOLERANCE

Except improving the growth of the plant via direct and indirect mechanisms, microorganisms can also improve the drought tolerance of plants by increasing the proline-content. Proline (Pro) is an amino acid, which is often synthesized by plants in response to various abiotic stresses. Pro functions as a free radical scavenger and subcellular structure stabilizator. More importantly, Pro is involved in osmotic adjustment during dehydration. Stressed plants can protect themselves against drought stress by accumulating these osmolytes. Different types of stress, such as drought, salinity and freezing, cause Pro to accumulate to high levels in many plants species (19). In several abiotically stressed plants, the level of Pro synthesis is even increased by the inoculation of beneficial bacteria such as *Burkholderia* (20), *Arthrobacter* and *Bacillus* (21). In this way, bacterial inoculation can be associated with the improvement of drought tolerance.

Some bacteria, like *Pseudomonas*, can survive under stress conditions due to the production of exopolysaccharides (EPS) (22). Exopolysaccharides, composed out of sugar residues, are believed to protect bacterial cells from water stress. Polysaccharides are hygroscopic and are therefore able to contain higher water content in the colony microenvironment then in the bulk soil as the external water potential declines in drying soil. Besides the increase in water content, there could also be an increase of nutrient availability within the bacterial colony (23). EPS are also known to produce biofilms, which is a complex association of bacterial cells established on various surfaces like roots and soil particles. Hereby enhancing the chances of microorganisms to attach and colonize and protect the plant roots from various pathogens (24). In previous studies, inoculation of EPS producing strains, such as *Pseudomonas* or *Bacillus* (25) resulted in better soil aggregation and increased resistance to water stress under drought conditions.

1.6 ARBUSCULAR MYCORRHIZA FUNGI

Besides the beneficial effects that bacterial inoculation can have on host plants, vesicular arbuscular mycorrhiza fungi (VAM) are reported to form associations with 80% of all terrestrial plants. The association creates a close link between plant roots and the soil, and thereby plays a pivotal role in the acquisition of nutrients. By enhancing the mobilization and uptake of several essential nutrients from the soil, they increase plant growth and development and give protection against pathogens. In addition, colonization may also enhance the plant's resistance to abiotic stresses such as drought (26). By the formation of an extensive hyphal network with plant roots, these VAM cause an improvement in soil texture and water relation and are therefore important in sustainable agricultural systems (27).

1.7 OBJECTIVES/ EXPERIMENTAL AIMS.

In this project the effect of inoculation on plant parameters, photosynthetic parameters and antioxidative capacity with bacterial strains derived from harsh environments will be analysed *in planta* under drought exposure. Durum wheat (*Triticum turgidum* var. *durum* cv. Vitron) will be used as the host plant. It is a monocotyledonous plant, like grass and widely used for human consumption as a source of calories used for e.g. the production of pasta. Next to the common wheat (*Triticum aestivum L.)* it is the most important *Triticum* species (28).

The bacteria were isolated from samples derived from the *Pistacia terebinthus* (September and May) and from the adjacent grassland (*Bromus sterilis, Hordeum murinum*) in September. The *Pistacia* is a deciduous, drought resistant tree growing to 10m tall, able to grow on a rocky substrate, native to the Rhodope Mountains in Bulgaria.

Studies have already been published that explain the effect of PGPB in relieving abiotic stress in different crop plants. In a study performed by Timmusk *et al.*, results demonstrate that the drought tolerance of wheat is significantly improved after inoculation with bacteria from harsh environments by a higher rate of survival, greater photosynthesis and biomass production in drought stressed plants (27). However to our knowledge, no studies focused on season of isolation (September- May) as a parameter influencing the capacity of PGPB in alleviating stress. Furthermore, since grass and wheat belong to the same *Poaceae* family, in which all cereal grasses, bamboos, and grasses of the cultivated lawns are included, we want to analyse the cross-compatibility between PGPB, derived from *Pistacia* and *grass* and the inoculated plant (29).

Therefore we hypothesize that PGPB isolated from the drought resistant *Pistacia* and PGPB isolated from the grass around the *Pistacia* can transfer their drought resistance to *Triticum.* Firstly, *in vitro* bioassays for plant growth promotion (PGP) and drought tolerance (DT) were performed. Additionally, the possible capacity of bacterial isolates derived from the *Pistacia* and grass to alleviate drought stress in wheat will be tested. A genotypic characterization will be performed for the cultivable strains and the total bacterial community will be compared between the different seasons (spring- autumn). Furthermore, the amount of VAM in *Pistacia* roots will be analysed in samples derived from the different seasons.

Since drought is a worldwide problem, affecting the agricultural yields and thereby having serious consequences for the society in terms of food security and health, the use of selected microorganisms may represent an important biotechnological approach to decrease the deleterious effects of stress in crops. Results from this project could therefore serve as a major improvement for the agricultural sector in the future.

2 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND ISOLATION OF BACTERIA

Samples (shoot, stem, roots, bulk (BS) and rhizosphere soil (RS)) from the *P. terebinthus* (spring and autumn 2014) were collected from the study site (Rhodope mountains, Bulgaria), followed by bacterial isolation at the Centre for Environmental Sciences (CMK). The isolates used for the present study consisted out of 13 strains obtained from the *Pistacia*, based on previous phenotypic data, such as plant growth promotion (PGP)-, drought tolerance (DT)-, and germination tests. By means of genotypic characterization following strains were characterized: 28a, 36c, 45e, 132a *Pseudomonas* sp., 36a, 45b, 54a *Arthrobacter* sp., 43d, 56b *Bacillus* sp., 50b *Rhodococcus* sp., 65 *Pantoea* sp., 99h *Stenotrophomonas* sp., and 157a Raoutella sp.. Samples (shoot, roots, BS and RS) derived from the grass around the *Pistacia* (September 2014) were also collected for isolation, and consisted of 242 isolates in total (not sequenced).

2.2 BIOASSAYS FOR PLANT GROWTH PROMOTION TESTS

Phenotypic characterization of 242 isolates derived from grass (shoot, root, RS and BS) around the *Pistacia* was done according to the following PGP-tests. 5µL of each bacterial strain was cultivated in 869 medium for three days in sterile 96-well masterblocks at 30°C by shaking at 120 rpm (30). After centrifugation at 2000 rpm for 15 minutes the supernatant was discarded and the pellet was washed with 10mM $MgSO_4$ or 0.01M PBS for the phosphate solubilisation. After a vortex and centrifugation step, the pellet was again dissolved in 10mM $MgSO_4$, or 0.01M PBS respectively.

2.2.1 NITROGEN FIXATION

25µL bacterial suspension was added into BTB (bromothymolblue) media, one with and one without NH_4Cl source, adapted from Xie G (31). The sterile 96-well masterblocks were incubated for two weeks at 30°C. The media containing the NH4Cl source was used as a positive control to observe whether bacteria can grow in the BTB medium. If the medium without the NH_4Cl source turned yellow, bacteria were able to fix nitrogen and grow. If the medium stayed blue, bacteria were not able to fix nitrogen and to grow.

2.2.2 IAA

25µL of bacterial suspension was inserted into a sterile 96-well masterblock containing 1ml IAA medium (1/10 869 containing L-tryptophan) and incubated for 4 days at 30°C and 125 rpm, wrapped in aluminum foil to protect from light. After the incubation period, the masterblocks were centrifuged for 15 minutes at 2000 rpm. 1mL Salkowski reagent, described by Patten C (31) was added to 0,5mL supernatant. After a vortex step of 5 min at 800 rpm, and 20 min of incubation, the masterblocks were evaluated. The wells containing bacterial suspension that scored positive for IAA- production, turned pink. On the other hand, wells containing bacterial suspension negative for IAA-production remained yellow.

2.2.3 BACTERIAL PHOSPHATE SOLUBILISATION

National Botanical Research Institute Phosphate (NBRIP) medium was poured into Petri dishes (32). When solid, three holes were made with sterile 1000µl tips to inoculate 50µl of bacterial

suspension into each hole. The halozone of the colony was measured after an incubation period of 12 days at 30°C. Bacteria with a halozone were found positive for phosphate solubilisation.

2.2.4 ACC-DEAMINASE

250µL bacterial suspension was inoculated into sterile 96-well masterblocks with 1,2 mL sterile salt minimal (SMN) medium with 5mM ACC as N-source. The masterblocks incubated for 3 days at 30°C and by shaking 125 rpm. After a centrifugation step for 15 minutes at 2000 rpm, the supernatant was discarded and the pellets were re-suspended in 100µL 0.1M Tris- HCl buffer (pH 8.5) and 3µL toluene. To this solution 10µL 0.5 ACC and 100µL 0,1M Tris- HCl buffer (pH 8.5) was added, followed by an incubation step for 30 minutes at 30°C (150rpm). 690µL 0,56N HCl and 0,2% 2,4- dinitrophenylhydrazine in 2N HCl was added. A color change from yellow to brown after adding 1mL 2N NaOH, was considered positive for ACC-deaminase activity. The bacterial strains without ACC-deaminase activity stayed yellow (33).

2.2.5 SIDEROPHORES

20µL of each bacterial suspension was added into the sterile 96-well masterblocks with 800µL 284 media containing different Fe (III)-citrate concentrations (0 µM, 0,25 µM, 3 µM) (34). The masterblocks were incubated five days at 30°C and 125 rpm. After the incubation period, 100µL Chrome-Azurol S (CAS) solution was added to all the wells and after four hours results could be obtained. Bacteria, which turned orange, were considered positive. Bacteria, which turned blue, were considered negative (34, 35)

2.3 DROUGHT TOLERANCE TESTS

Bacteria were cultivated in 869 medium in sterile 96-masterblocks for three days (36). The blocks were centrifuged at 2000 rpm for 10 minutes and the supernatants were discarded. The pellets were suspended in 1mL 10 mM $MgSO_4$. The suspensions were placed on the vortex for 5 minutes and centrifuged again for 10 minutes at 2000 rpm. This step was repeated once. Afterwards, 100µL of each bacterial strain was added into sterile 96-well masterblocks, one containing sterile 284 medium and one containing sterile 284 + 10% polyethylene glycol (PEG6000) with a molecular weight of 6000, further explained.

2.3.1PROLINE

After four days, proline (Pro) content was determined by the ninhydrine method as described by *Bates et al,* absorption was measured at 520nm (37).

2.3.2 EXOPOLYSSACHARIDE

After four days, the supernatant was collected and three volumes of ice-cold absolute ethanol were added. After 48h, the precipitated exopolyssacharides (EPS) were separated by centrifugation at 3000 rpm for 20 min at 4°C. The amount of EPS was visualized in terms of total carbohydrate content by phenol-sulfuric acid method, according to *Dubois et al.* (38).

2.4 GENOTYPIC CHARACTERIZATION OF THE BACTERIAL CULTIVABLE COMMUNITY

2.4.1 DNA EXTRACTION

DNA extraction was performed using the Ambion® MagMAX™ DNA isolation kit (Thermo Fisher Scientific, Waltham, USA) in order to obtain a clear view of the cultivable strains isolated from *Pistacia* (stem, root, rhizosphere and bulk soil). The masterblocks with bacteria were centrifuged for 20 minutes at 3000 rpm. The supernatant was decanted from the masterblocks and 50μL of lysisbuffer (180mg lysozyme; 108 mg Trition X-100; 180μL TE-buffer and 8820μL Rrnase free H_20) was added to the pellets. After 30 minutes of incubation at 37°C, the content of each well was transferred to a new MagMAX plate containing 50μL proteinase K solution. After 1 minute at 1000rpm and an incubation period of 30 minutes at 60°C, 200μL multi-sample DNA-lysis buffer was added. After 3 minutes at 1000 rpm, 18μL DNA-binding buffer was added to each sample followed by 3 minutes shaking at 800 rpm. Finally 280μL of 100% isopropanol was added, followed by 3 minutes at 800 rpm. The wash buffers and elution plate were prepared according to the manufacturer's protocol followed by loading the MagMAX (program = 44213021 DW blood).

2.4.2 PCR AMPLIFICATION AND SEQUENCING OF 16S RDNA

PCR amplification was done in 50μL reaction volume per sample using 1μL 16S rDNA Forward primer 27F (5' AGAGTTTGATCMTGGCTCAG 3'), 1μL 16S rDNA Reverse primer 1492R (5' TACGGYTACCTTGTTACGACTT 3') (both $1/10^{th}$ diluted from stock, 0,2μL Platinum *Taq* high fidelity, 5μL 10x hifi buffer, 1μL dNTP mix, 2μL 50nM $MgS0_4$, 1μL dNTP mix, and 38,8μL RNase free H_20 and was performed in the T100™ Thermal Cycler (Biorad). The thermocycling condition was initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation 94°C for 1 minute, annealing at 52°C for 30 seconds, extension at 72°C for 3 minutes and a final extension step at 72° for 10 minutes. PCR products were stored in the -20°C freezer upon sending for sequencing (Macrogen, Amsterdam, NL). Sequence analysis was carried out using the National Center for Biotechnology Information (NCBI) BLAST-tool for nucleotides. Results with the higher alignment score, query cover and an E value > 1E-04 were selected to match the sequence input.

2.5 GERMINATION EXPERIMENTS

Germination experiments were performed for the thirteen selected strains derived from *Pistacia*. Strains were cultivated in 869 medium for 1 day at 30°C by shaking at 120 rpm. The bacterial suspension was centrifuged for 10 minutes at 4000 rpm. The supernatant was removed and the pellet was dissolved in 14mL 15% polyethylene glycol (PEG 6000) with a molecular weight (MW) of 6000 to simulate water stress effects. PEG is a compound widely used for screening drought tolerance at early stages. At higher MW it is considered to block upstream water movement, thereby reducing water absorption followed by desiccation of the plant.

Germination tests were conducted with five and three replicates respectively for *Agrostis stolonifera* (bent grass), and *Triticum* per treatment with 10 seeds each. *A* filterpaper was folded containing A*grostis* seeds, purchased from Global Green Seeds. The filter paper was soaked in the solution and placed on a Petridish. *Triticum turgidum* var. *durum* cv. Vitron (obtained from Semillas Battle, SA) were soaked for 2 hours in the bacterial suspension and placed on a Petridish

containing 3 filters. The Petridishes were sealed with parafilm to prevent evaporation, but three small holes were made in the parafilm to allow gas exchange. They were stored in the dark (5 days - *Agrostis* 15% PEG) and in the growth chamber (5 days - *Agrostis* 10% PEG, 11 days -*Triticum*) accordingly to a completely randomized design with conditions set to maintain a 12h photosynthetic photon flux density, a day and night temperature of 23/20 °C, and a relative humidity of 65%. Germination was considered when the radicle was extended for at least 1mm.

2.6 PLANT INOCULATION

Six strains isolated from *Pistacia* (36a, 45e, 56b, 99h, 132a, 157a) and 6 strains isolated from the grass around the *Pistacia* (250a, 274a, 277e, 314a, 315d, 317c) were selected for inoculation on *Triticum* in the climate chamber with the aforementioned conditions. The strains were cultivated in 869 medium for 2 days at 30°C and 120 rpm. After a centrifugation step at 4000 rpm for 10 minutes, the supernatant was removed and the pellet was resuspended in 10mM $MgSO_4$. The *Triticum* seeds stayed overnight on the shaker in the bacterial suspension. For the control condition and for each strain, respectively 96 and 64 pots were filled with sand. Five seeds were placed per pot and watered with 1/10 Hoagland nutrient solution (39). After 8 days the seeds germinated and reinoculation was performed. The bacterial suspension was centrifuged for 10 minutes at 4000 rpm and dissolved in 10mM $MgSO_4$, subsequently 5mL was added to each pot. This procedure was repeated once again 27 days after sowing. The plants were irrigated with 1/10 Hoagland nutrient solution at day 9, 13, 16, 20 and 24 after sowing. At day 27, dH_20 was added to both conditions. 28 days after sowing, drought stress was induced. Thereby meaning that at day 28 and at day 30, only half of the control and inoculated pots received dH_20.

2.6. 1 BIOMETRIC MEASUREMENTS

33 days after sowing, the plants were harvested. Their height (cm), fresh and dry weight (g) were measured. Shoot length was measured directly and harvested shoots were dried for one week at 37°C to obtain the dry weight (DW).

2.6.2 MEASUREMENT OF PHOTOSYNTHETIC PARAMETERS

Each shoot sample was homogenized with mortar and pestle. The grinded chlorophyll-acetone mixture was centrifuged for 5 minutes at 6000 rpm. The volume of the supernatant was measured and a dilution (1/10) was made by adding 100µl to 900µl technical acetone. The blank and samples were measured at an absorption value of 663 nm, 646nm and 470 nm, respectively for chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total carotenes (Car). The formulas used to calculate the Chl_a, Chl_b and Car, while taking the fresh weight (~200mg), volume of the supernatant and dilution into account, are as following: Chl_a(µg/ml) = 12,21 A663 – 2,81 A646, Chl_b (µg/ml) = 20,31 A646 – 5,03 A663, Total car (µg/ml) = (1000 A470 – 3,27 Chl_a – 104 Chl_b) / 229.

2.6.3 MEASUREMENT OF ENZYMATIC CAPACITY

For each strain and condition, five times 200mg of the shoot was obtained for measuring the enzymatic activity of catalase (CAT), guajacol peroxidase (GPOD), seringaldazine peroxidase (SPOD), superoxide dismutase (SOD) and five times 200mg for ascorbate peroxidase (APOD). The frozen samples were homogenized with the addition of sand, polyvinylpyrrolidone (PVP) and 2mL

extraction buffer (0,1M Tris-HCl, 1mM DTT, 1mM EDTA). The samples were centrifuged at 4°C for 10 minutes at 12000 rpm. The pellet was discarded and the supernatant was used for the enzymatic activity measurements. The enzymatic activity of CAT, GPOD, SPOD, SOD, and APOD were determined by a spectrophotometric method. This was used to determine the quantity of the enzymes in the shoot by bringing the substrate of the enzyme in contact with the enzyme. The wavelength peak was set to a range of the molecule, which was formed by the enzyme (40). Results provided by the spectrophotometer were converted to enzymatic activity by using the Lambert-Beer equation and the correct extinction coefficient for each enzyme.

2.7 VESICULAR ARBUSCULAR MYCORRHIZA STAINING AND QUANTIFICATION

Root samples obtained from *Pistacia* in spring and autumn were used for Vesicular Arbuscular Mycorrhiza (VAM) staining and quantification. Firstly, the roots were immersed in 10% KOH, to remove the cytoplasm from the cortical cells, followed by acidification of the roots with 5% HCl for 1 min to improve colorization of the VAM-structures for 10 min with 0,05% Tryptan blue (10mL glacial acetic acid, 200mL glycerol, 0,2g tryptan blue, 190ml dH_20). Consequently a version of the gridline intersect method was used to determine the percentage of *Pistacia* roots that was colonized by VAM.

2.8 ANALYZING TOTAL BACTERIAL COMMUNITY: ARISA

Automated ribosomal intergenic spacer analysis (ARISA) was used for analyzing bacterial diversity and community structures in rhizosphere (RS) and bulk soil samples (BS) from *Pistacia*, obtained from three different sampling places in spring and autumn. ARISA is a PCR-based method that relies on the length heterogenity of the internal transcribed spacer (ITS) regions to fingerprint microbial communities.

2.8.1 DNA EXTRACTION AND PCR AMPLIFICATION

Total DNA from RS and BS was extracted in triplicate using the PowerSoil® DNA isolation kit (MOBIO Laboratories Inc., California, USA) following the manufacturer's protocol. The 16S-23S ribosomal intergenic spacer regions (IGS) were amplified by PCR. Amplification was performed in 25μL reaction volume including 1μL sample (30-110 ng μl^{-1}) using 0,5μL iTSF forward primer (5'-GTCGTAACAAGGTAGCCGTA-3'), 0,5μL ITSReub reverse primer (5'-GCCAAGGCATCCACC-3') (both 1/10 diluted), 0,5μL dNTP mix, 0,2μL Platinum ® *Taq* high fidelity, (Invitrogen, California, USA), 2,5μL 10xhifi PCR buffer, 1μL 50mM $MgSO_4$ and 19,4μL RNase free water. The amplification was performed in the T100™ Thermal Cycler (Biorad). The thermo cycling condition was denaturation at 94°C for 3 minutes; 30 cycles of 94°C for 1 minute, 55°C for 30s, 72°C for 1 minute and a final extension step at 72°C for 5 minutes.

2.8.2 PROCESSING FINGERPRINT DATA

The resulting PCR products were loaded onto the DNA-1000-chips (Agilent Technologies, USA) following the manufacturer's recommendations. Based on capillary electrophoresis, performed by the 2100 Agilent Bioanalyzer (Agilent Technologies, USA), the resulting DNA fragments were separated. By the means of 2100 Expert Software, digitalized ARISA fingerprints resulted in electropherograms. These acquired electropherograms in ASCII format were processed by using

the StatsFingerprints package 2.13.0 version of the R project (The R Project for Statistical Computing, Vienna, Austria). A baseline and the range of markers were defined, the area under the profiles was normalized and the background was deleted. By using RStudio, a non-Metric Multidimentsional Scaling (nMDS) plot was generated.

2.9 STATISTICAL ANALYSIS

Statistical analyses were performed by using the 3.3.2 version of R (The R Foundation for Statistical Computing, Vienna, Austria). For normalized data, the parametric ANOVA test was used with Dunnet's test of multiple comparisons as a post hoc analysis. For non-normalized data, the non-parametric Kruskall Wallis test was used with the Dunn test of multiple comparisons as post hoc analysis.

3. Results and discussion

3.1 In Vitro experiments

3.1.1 Bioassays for plant growth promotion

To interpret the potential of bacteria derived from grass around the *Pistacia* in plant growth promotion, the selected strains were examined for an array of PGP abilities *in vitro* focusing both on conventional and drought related PGP traits. Five bioassays for PGP were performed for 242 isolates derived from grass. Positive results were considered as having two positive scores out of the analysed triplicate. *In vitro* screening for the characteristics associated with PGP revealed that 52.48% out of the 242 strains were positive for the production of indole-3- acetic acid (IAA), 87.19% of the strains were positive for production of ACC-deaminase, 95.45% was positive for solubilisation of phosphates (PO4), all the strains were able to produce siderophores (SID), and 74.79% of all the strains were able to fix nitrogen (N-fix) (**Figure 5A**). Next, the amounts of PGP-capacities were analysed for each strain. There were no isolates positive for none or only one PGP-test. 2% of the strains were positive for 2 PGP tests. 15% were positive for 3 PGP-tests, 51% were positive for 4 PGP-test and 31% were positive for all the PGP-tests. The strains positive for all the PGP-tests were selected for drought tolerance tests (**Figure 5B**).

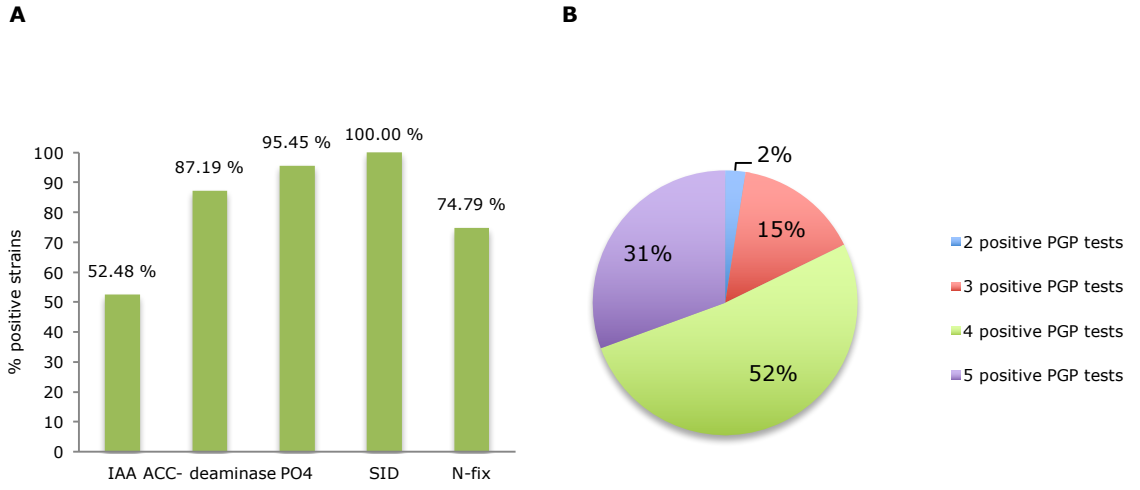

Figure 5. Bioassays for plant growth promotion for 242 strains derived from grass around Pistacia. *Panel A: percentage of strains derived from grass showing positive results for the respective trait. Indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, phosphates solubilisation (PO4), siderophores production (SID), and nitrogen fixation (N-Fix). Panel B: percentage of strains derived from grass showing the respective number of positive PGP-tests.*

More than half of the strains exhibit the capacity to metabolize L-tryptophan, the precursor of IAA, and thereby cause an elevation in the hormone IAA levels. IAA is the most active growth hormone from the group of the auxins inducing root formation and root hair growth (15). Our results are in concordance with other studies where endophytic and rhizosphere bacteria caused a higher production in IAA levels. However, a too high concentration can have inhibitory effects on the root elongation and root volume, as proven with endophytes derived from *Bacillus* strains (41).

However, in our study, the positive result of IAA was based on a change in colour and not on a spectrophotometric quantification.

Despite the essential role of the stress hormone ethylene in normal plant development, at higher concentrations it can induce damage to plants. In our study, almost all of the tested strains were able to metabolize ACC by ACC-deaminase, thereby lowering ethylene levels, stress susceptibility and favouring plant growth. This is in line with other findings where PGPB are found to produce ACC-deaminase and thereby lower the plant ethylene levels (42).

Phosphorus (P) is the second important plant growth-limiting nutrient after. The amounts available for plants are scarce since the majority is found in insoluble forms as an inorganic mineral, such as insoluble mineral complexes, or as an organic compound, incorporated into the biomass. Therefore the availability of P depends on the solubility of this element. From our results it can be seen that almost all the strains were able to form a clear halo on the media containing phosphate in an insoluble form, thus were able to solubilize phosphate. By reason of acidification or secretion of organic acids, bacteria are able to bring phosphorus back into the solution and make it an available nutrient for the plant (43).

Iron is the third limiting nutrient source for plant growth and metabolism resulting in poor yields and reduced crop quality whereas plants and bacteria do not easily assimilate the most prominent form Fe^{3+}. Siderophores produced by all the strains derived from the grass can promote plant growth by providing iron chelators. These help in the absorption of the generally low available iron. Furthermore, siderophores are beneficial for the plant, since its production inhibits iron in phytopathogens by binding the available form of iron Fe^{3+} in the rhizosphere. Production of siderophores has been reported for rhizosphere bacteria and endophytes in other studies (41, 44).

Nitrogen fixation is the process responsible for the reduction of N_2 to ammonia or nitrate and can be performed in diazotrophic microorganisms. In this study, ¾ of all the strains were able to fix nitrogen, a principal plant nutrient. They retrieved this capacity, by possessing the enzyme nitrogenase. Our findings that PGPR and endophytes carry this ability were in accordance with *Majeed et al.* (45).

It is well known that exposure to drought stress induced limitations in plant growth and productivity. Most of the strains, selected from an arid environment, were able to produce one or more PGP-traits related with improving nutrients availability and a changing the hormonal balance leading towards a higher stress tolerance in plants. Furthermore, strains having multi-functional traits are better in relieving stress compared with strains possessing single traits (45). For this reason, the strains positive for the five PGP-tests were selected for DT-tests.

3.1.2 DROUGHT TOLERANCE TESTS

DT-tests were performed under stressed and non-stressed conditions. Stressed conditions were generated by the addition of 10% PEG to the 284 culture media. All 72 strains positive for the five PGP-tests were screened for their production of EPS and Pro. For EPS, colours darker than the negative control were considered positive. The absorption of Pro was measured at 520nm. Under non-stressed conditions 22.22% of the strains were able to produce EPS, when exposed to drought 19.44% were able to produce EPS. In non-stressed conditions, 58.33% of the strains were able to produce Pro. When exposed to drought, 75% of the strains were able to produce Pro (**Figure 6**).

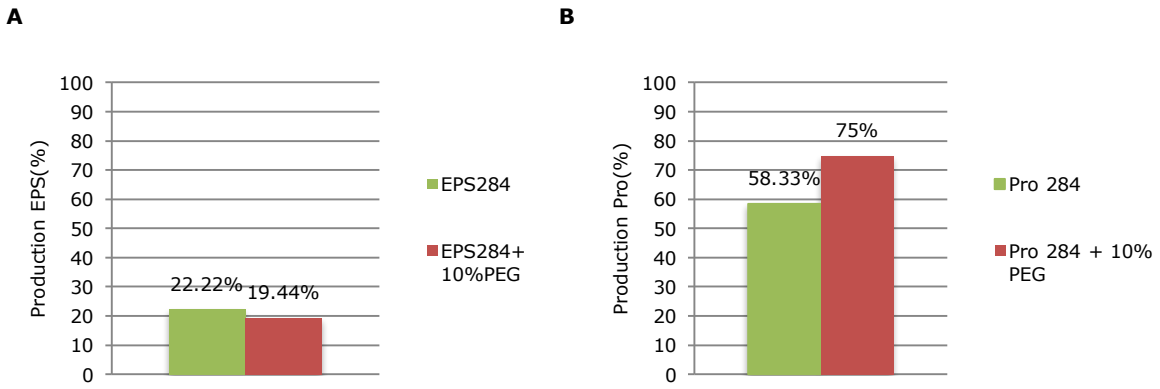

Figure 6. Production exopolysaccharides (EPS) and proline (Pro) under normal and exposed conditions (%). *Panel A: the percentage of strains positive for EPS production in normal conditions (284-medium) and under drought stress (284+10%PEG). Panel B: the percentage of strains positive for PRO production in normal conditions and under drought stress (284+10%PEG), measured by a spectrophotometer.*

With a difference of 2.78%, the number of strains producing EPS is slightly lower under exposed conditions. This is in contrast with the normal capacity of PGPB rendering drought tolerance by producing a higher amount of EPS upon exposure (46). In the study of *Vardharajula et al.,* inoculation of wheat with *Bacillus spp.* showed a higher accumulation of EPS under drought stress (47). However, in our study, strains that were not able to produce EPS under non-stressed conditions were able to produce EPS under stressed conditions. This indicates that EPS production by bacterial cells could occur as a response to stress. EPS production provides a biofilm and thereby creates a microenvironment that holds water and protects bacteria and roots against desiccation and fluctuations in water potential. Bacterial EPS production is known to improve nutrient uptake and thereby increase plant growth and protection, caused by better soil aggregation and maintaining of the water potentials around the roots.

Exposure of bacteria to drought caused an increase (16,67%) in Pro production. This result leads forward that the bacteria produced Pro as a response to drought stress. Many studies proved that the production of Pro by microorganisms under drought stress could be attributed to the up regulation of Pro biosynthesis pathway to maintain the water status, and thereby protecting membranes and proteins from stress (48). Besides the osmotic adjustment, Pro accumulation is also involved in the protection against oxidative damage, which is elevated under drought stress as stated previously (49). **Table 1** provides the strains selected for further plant experiments. These strains were positive for the array of 5 PGP-tests, EPS and Pro-production.

Table 1. Strains obtained from grass selected for plant inoculation experiment with Triticum. *Tested for indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, phosphates solubulisation (PO4), siderophores production (SID), nitrogen fixation (N-Fix), exopolyssacharides (EPS)- and proline (Pro) –production*

Strain	Sample origin	IAA	ACC-deaminase	PO4	SID	N-Fix	EPS	Pro
250a	Shoot	+	+	+	+	+	+	+
274a	Root	+	+	+	+	+	+	+
277e	Root	+	+	+	+	+	+	+
314a	Rhizosphere	+	+	+	+	+	+	+
315d	Rhizosphere	+	+	+	+	+	+	+
317c	Bulk	+	+	+	+	+	+	+

3.2 GENOTYPIC CHARACTERIZATION OF THE BACTERIAL CULTIVABLE COMMUNITY

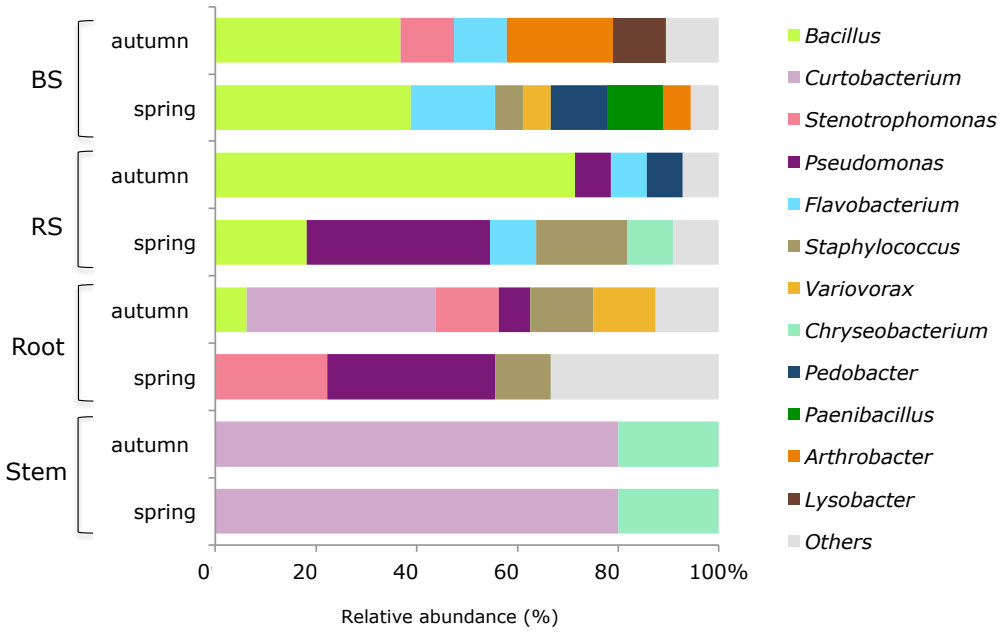

Figure 7. Genera obtained by 16S rDNA –sequencing from cultivable bacteria isolated from Pistacia. *Isolates obtained from BS, RS, stem, and roots in autumn and spring.*

To obtain more insight into the cultivable bacterial community of *Pistacia* and their abundance, DNA extraction was performed on a selection of strains isolated from stem, root, RS and BS, followed by 16S rDNA sequencing. Among the different isolation places, the genera *Bacillus, Pseudomonas, Stenotrophomonas, Chryseobacterium, Curtobacterium, Flavobacterium* and *Staphylococcus* were the most prominent genera. The abundance of *Bacillus* was mostly related to the BS and RS. While the highest abundance of the genera *Curtocobacterium* and

Chryseobacterium could be related to the stem. The population of *Flavobacterium* was almost equally divided over the RS and BS. Other less prominent sequences (≤1%) were grouped in the category others (**Figure 7**). Next, the seasonal separation of the genera for each sample place was observed. Seasonal differences were observed between spring and autumn communities in their relative abundance, except for isolates derived from the root. Besides characterization of a subset of the bacterial community of *Pistacia,* the strain 132a *Pseudomonas* sp., which was used for further inoculation experiments, could also be identified (**Table 2**).

Firstly, these results put forward a clear separation between genera in their different compartments. The most prominent sequenced genera *Bacillus, Pseudomonas, Stenotrophomonas, Chryseobacterium, Curtobacterium, Flavobacterium and Staphylococcus* were are already acknowledged as PGPB in previous research (46). Secondly, seasonal differences appear to have an effect on the abundance of genera in the roots, BS, and RS of *Pistacia*. However, the cultivable bacterial community compromises only a minor part of the total bacterial community (approx. 1%). To obtain more knowledge about the total microbial community of *Pistacia,* analysis of the non-cultivable bacteria is required.

3.3 *IN PLANTA* EXPERIMENTS

3.3.1 GERMINATION TESTS

The germination rate (GR) of different inoculants was assessed under exposure to drought, induced by the aforementioned compound PEG. Out of a subset of strains isolated from the *Pistacia*, 13 strains were selected based on preliminary data such as PGP-, DT-, and germination tests. Genotypic characterization of strains 28a, 36c, 45e *Pseudomonas* sp*.,* 36a, 45b, 54a *Arthrobacter* sp*.,* 43d, 56b *Bacillus* sp*.,* 50b *Rhodococcus* sp*.,* 65 *Pantoea* sp*.,* 99h *Stenotrophomonas* sp. and 157a Raoutella sp., were performed by 16S rDNA in previous research. The strain 132a was sequenced by 16S rDNA in this study and recognized as *Pseudomonas sp.* (**Figure 7**). *Agrostis* and *Triticum* seeds were exposed to drought by inoculation with a suspension of the strains containing a concentration of PEG. The strains were divergent in their sample origin and season of isolation (**Table 2**).

Table 2. Strains used for germination experiments, **their sample origin and season**.

Strains	**Sample origin**	**Season**
Control	/	/
28a *Pseudomonas* sp.	Rhizosphere	Spring
36a *Arthrobacter* sp.	Rhizosphere	Spring
36c *Pseudomonas* sp.	Rhizosphere	Spring
43d *Bacillus* sp.	Bulk	Spring
45b *Arthrobacter* sp.	Bulk	Spring
45e *Pseudomonas* sp.	Bulk	Spring
50b *Rhodococcus* sp.	Bulk	Spring
54a *Arthrobacter* sp.	Bulk	Spring
56b *Bacillus* sp.	Root	Spring
65 *Pontoea* sp.	Leave	Autumn
99h *Stenotropomonas* sp.	Root	Autumn
132a *Pseudomonas* sp.	Rhizosphere	Autumn
157a *Raoutella* sp.	Bulk	Autumn

Bacterial inoculation of *Agrostis* seeds exposed to 15% PEG showed no overall higher germination rate in comparison with the control condition, except a non-significant increase in GR for strain 45e. A reduction in the PEG solution to 10% caused a significant higher GR for the strains 45e and 50b, while achieving a significant lower germination rate for the strains 56b and 43d. The results between *Agrostis* exposed to 15% PEG and 10 PEG% were highly variable. For the inoculated *Triticum* seeds exposed to 10% PEG, no significant higher germination rate differences could be observed (**Figure 8**).

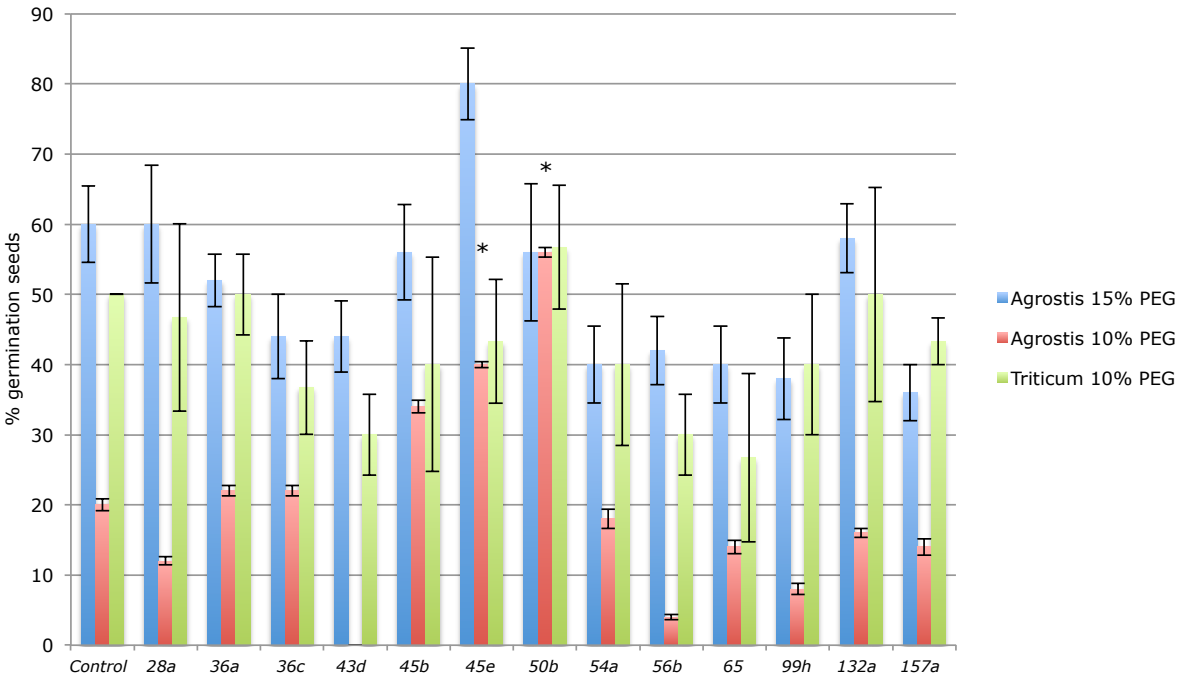

Figure 8. Germination rate (%) of Agrostis and Triticum under drought exposure, induced by PEG. *Values are means of 5 replicates (Agrostis), 3 replicates (Triticum) ± standard error of means (SEM). Only positive significant values are displayed (0.05<p-value<0.1).*

As discussed in a lot of studies, water stress can affect the seed germination. This germination experiment was performed to distinguish the effect of different bacterial inoculations under drought stress induced by PEG. The stress level (%PEG) plays also a major role in the capacity of the strains in alleviating the deleterious effects of drought to improve seed germination. From these results it is not visible whether a PEG solution of elevated strength caused less seed germination (**Figure 8**). However, this can be attributed to the fact that the indirect environment, the growth chamber, where germination of *Agrostis* 10% PEG was observed caused desiccation of the seeds resulting in an overall lower germination rate. Nevertheless, 10% PEG was used in the further *Triticum* experiment because the concentration was still a high enough to induce drought stress. Since the strains demonstrate positive *in vitro* results for the PGP-tests and DT-tests in preliminary data, they are expected to be drought resistant. While for the germination experiments *in vivo*, they do not provide the seeds with these capacities that clearly. Still, depending on the germination results of the three experiments six strains were selected for further experiments based on the highest germination rate per season of isolation and place of origin. For spring,

strains 36a, 45e and 56b were selected. For autumn, strains 99h, 132a and 157a were selected. Depending on this selection the selected sample origins (rhizosphere- bulk- root) can be compared between the seasons of isolation in the further experiments.

3.3.2 BIOMETRICAL MEASUREMENTS

The development of an optimal leaf area is important for photosynthesis and the dry matter yield, which refers to the biomass of a plant. The length of the shoot (SL) was measured at harvesting and after one week the dry weight (DW) was obtained. These two parameters were compared between the non-exposed and exposed conditions, as well as the control and inoculated conditions. The effect of inoculation on *Triticum* with strains derived from *Pistacia* and from grass exposed to drought stress was evaluated. In the first experimental set-up *Triticum* was inoculated with strains derived from *Pistacia*, in the second experimental set-up *Triticum* was inoculated with strains derived from grass.

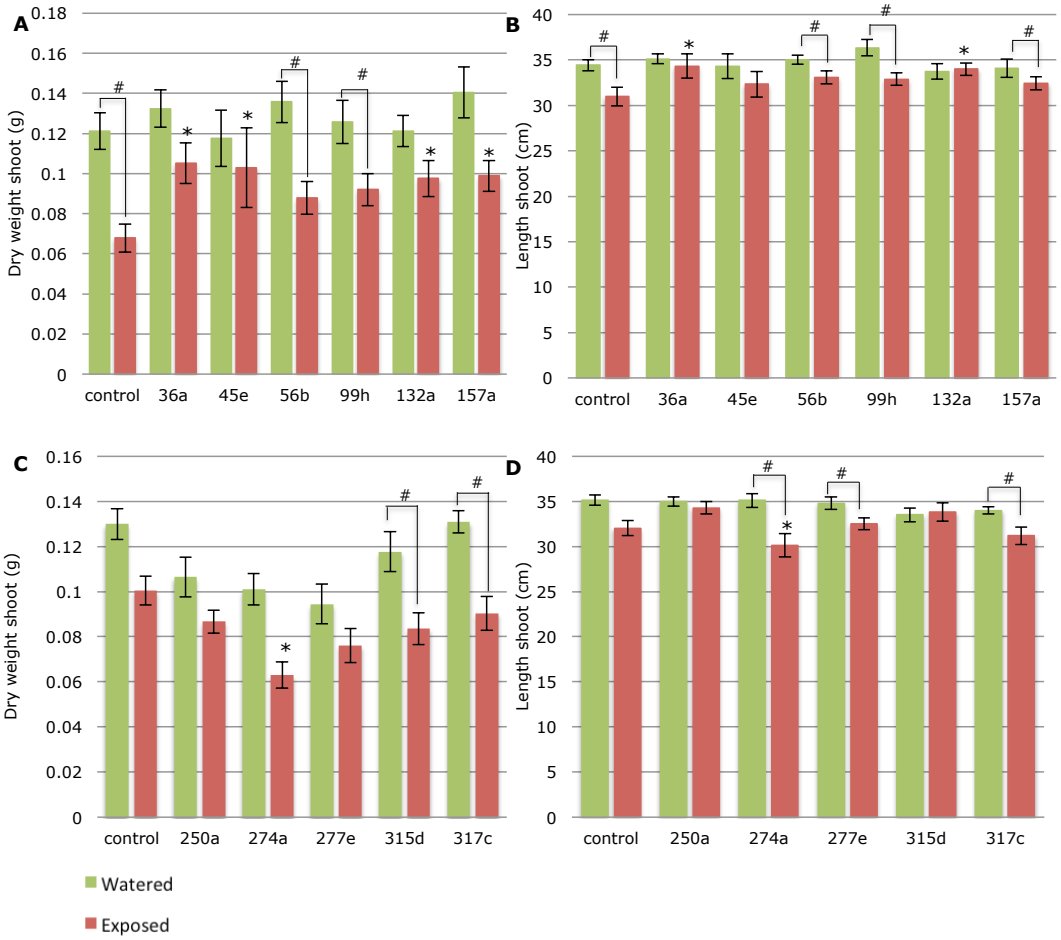

***Figure 9**. **Plant parameters measured 33 days after sowing of wheat inoculated with bacterial strains.** Panel A and B: respectively dry weight (DW) and shoot length (SL) for plants inoculated with strains derived from Pistacia. Panel C and D: dry weight (DW) and shoot length (SL) for plants inoculated with strains derived form grass. Exposure obtained by withholding irrigation for six days. Control received no bacterial inoculation. The height of the shoot was immediately measured, dry weight obtained after one week at 37°C. Values are mean of ten replicates ± standard error of means (SEM). With * (p-value <0.5) representing statistical difference between the control and # (p-value<0.05) representing statistical difference between the non-exposed and exposed conditions.*

The mean DW was decreased with 43.9% for the exposed non-inoculated plants when compared with the non-exposed plants in the first experimental set-up. For the plants inoculated with strains derived from *Pistacia* the mean DW was decreased with 20.5%; 12.3%; 35.3%; 26.9%; 19.6%; 29.6% respectively for the strains 36a, 45e, 56b, 99h, 132a and 157a. For the strains 36a, 45e, 132a and 157a there was a significant higher DW compared to the exposed non-inoculated control. A significant difference in DW could be observed between the non-exposed and the exposed conditions of the control, strain 56b and 99h (**Figure 9A**). The mean SL of the exposed non-inoculated plants was decreased by 10%. For the inoculated plants, there was a decrease of 2.2%; 5.7%; 5.6%; 9.5% and 4.8% respectively for the strains 36a, 45e, 56b 99h and 157a. For strain 132a there was an increase of 0.7% in the mean SL. Plants inoculated with the strains 36a and 132a showed a significant higher SL in comparison with the exposed control. The SL of the exposed plants was significantly lower than the non-exposed plants for the control, strain 56b and 99h.. Overall, no difference could be observed between the DW and SL of the isolates from the different seasons. (**Figure 9B**).

The mean DW was decreased with 22.69% for the exposed non-inoculated plants in the second experimental setup. For the plants inoculated with strains derived from grass the mean DW was decreased with 18.61%; 37,60%%; 19.56%; 29%; 30.95%; respectively for the strains 250a, 274a, 277e, 315d, and 317c. For the strain 274a there was a significant decrease in DW compared to the exposed non-inoculated control. The DW of the exposed strains 315d and 317c were significantly lower as their non-exposed inoculated strain, while for the control there was no significant difference between the exposed and non-exposed condition (**Figure 9C**).The mean SL was decreased with 8.8% for the exposed non-inoculated plants. For the plants inoculated with the strains derived from grass mean shoot length was decreased with 2.03%, 14.16%, 6.52%, 8.23% respectively for the strains 250a, 274a, 277e, and 317c. For the strain 315d there was an increase of 1.01%. The plants inoculated with the strain 274a had a significant lower SL in comparison with the non-inoculated exposed control. A significant lowering of the shoot length could be observed between the length of the non-exposed and exposed of strains 274a, 277e and 317c (**Figure 9D**).

As addressed in many other studies, drought has a huge impact on plants by limiting their growth and survival (50). Drought stress has serious effects on the non-inoculated plants visible by wilting of the leaves, less growth, and reducing the strength of the plant (SI, figure 1). Under non-exposed conditions, inoculation, regardless whether the strains' origin was from *Pistacia* or grass, had no significant effect on the plant parameters. This can be explained by the fact that exposure to drought, leading to higher stress levels, evoked specific PGP-capacities *in planta*. Showing that PGP activity is a stress-dependent and not a *per se* feature of the strains (45, 47, 51).

Under exposed conditions the strains derived from *Pistacia* caused an increase in the DW compared to the control. Scoring for a strain was considered positive when the percentage for decrease in DW between the non-exposed and the exposed inoculated plants is lower than the percentage for the control plants. However, not all the strains elicit the same results. Plants inoculated with the strains 36a, 45e and 132a and 157a showed a significant higher DW than the control plants upon exposure. While the DW of the control, strain 56b and 99h were significantly lower in the exposed conditions. Thereby meaning that the control and these strains showed the highest decrease in DW

under exposure (**Figure 9A).** Contingent on these results 36a *Arthrobacter* sp., isolated in spring, 45e Pseudomonas sp. isolated in spring and *132a Pseudomonas* sp*.* isolated in autumn from the *Pistacia* are considered as the strains improving the biomass of plants in drought stress. Apart from the significance of strain 45e, the same results were obtained for the SL of *Triticum* inoculated with the strains obtained from *Pistacia.* These PGPB were also found as alleviating drought stress in host plants in other studies (52). These results suggest that the season of isolation has no effect on improving the drought tolerance.

Inoculation of the plants with strains derived from grass had almost no, or even a significant negative effect on the DW when exposed to drought (strain 274a). Since there was no significant difference between the non-exposed and exposed control, no inoculation showed a smaller decrease in DW under exposed conditions than inoculation with the strains derived from grass (**Figure 9C**). The same was valid for the SL where the decrease between the non-exposed and the exposed control was smaller than for three of the strains. Thereby stating that inoculation with several strains derived from grass could not improve the drought tolerance.

Furthermore, the strains derived from grass showed promising *in vitro* results, showed negative results (274a) or affected plant growth in a bad way by e.g. limit their germination rate *in vivo* (SI, figure 1). This suggests that different mechanisms of growth promotion occur *in vitro* and *in vivo*. For *in vivo* experiments, growth promotion may be regulated by direct and indirect mechanisms, in contrary with *in vitro* tests; only substances secreted by PGPB can have an effect on the plant growth. In addition, environmental parameters such as watering, temperature, day/night light of the growth chamber were taken into account *in vivo*, while this is not the case for *in vitro* experiments (53).

Although the results of the SL were in line with the DW, the differences in SL between the non-exposed and the exposed conditions were less clearly visible as the differences for the DW. This could be attributed to the fact that drought was induced six days before harvesting. Plants are the most vulnerable to drought stress in the tillering stage, not in later flowering stage (54). Conclusively, it can be stated that strains associated with Pistacia are better in alleviating drought stress in wheat plants than strains associated with grass, stating that these drought-resistance PGPB are cross compatible with different plant models.

3.3.3 ANTIOXIDATIVE CAPACITY

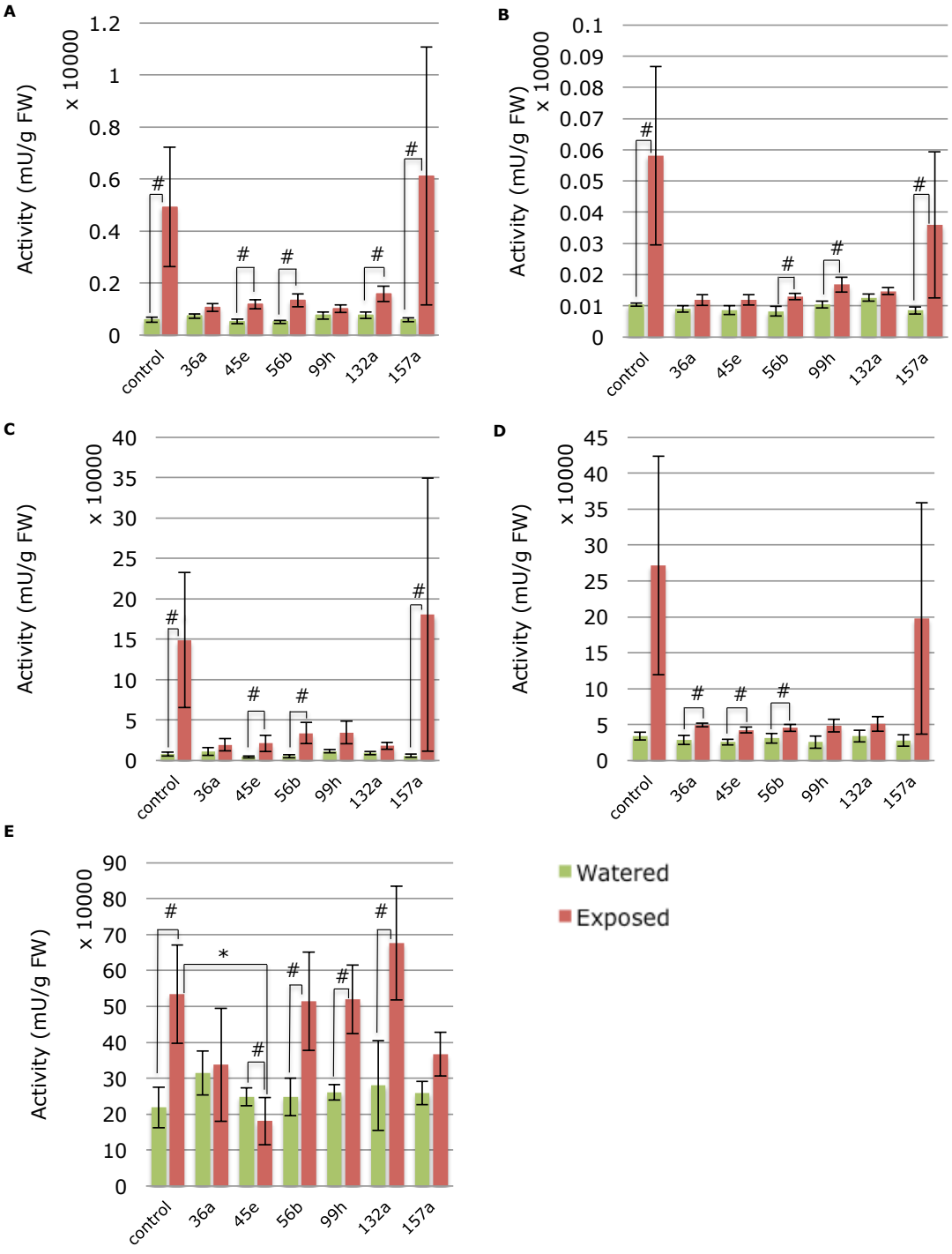

Figure 10. Antioxidative enzymes in Triticum shoots inoculated with strains derived from Pistacia measured in watered and exposed conditions. *For panel ABCDE respectively: Catalase (CAT), superoxide dismutase (SOD), guajacol peroxidase (GPOD), seringaldazine peroxidase (SPOD) and ascorbate peroxidase (APOD). Activity is represented as mU/g FW. Exposure obtained by withholding irrigation for six days. Control received no bacterial inoculation. The values given for each treatment are means of five replicates ± SEM. With * (p-value <0.05) representing statistical difference with the control and # representing (p-value<0.05) statistical difference between the non-exposed and exposed conditions.*

To assess the involvement of antioxidants against drought stress, wheat plants were inoculated with strains derived from *Pistacia* followed by measurement of the level of antioxidant in samples derived from the shoot. The results are presented in **Figure 10**. Under non-exposed conditions, no significant difference could be observed in the activity of the measured enzymes (CAT, SOD, GPOD, SPOD, and APOD) between the inoculated and non-inoculated treatments. For the control exposed to drought stress, an increase was measured for all the enzymatic activities in comparison with the non-exposed control. For the inoculated plants, an overall lower trend in the enzymatic activities could be observed compared with the exposed control under drought stress. However, an increase in the activity of CAT, SOD, GPOD and SPOD for all the strains and a multifold (significant for CAT, SOD and GPOD) increase for the activity of strain 157a were noticeable under exposure. In comparison with their non-exposed plants, CAT activity was significantly higher for the exposed strains 45e, 56b, and 132a (**Figure 10A**), the SOD activities of strain 56b and strain 99h were significant higher (**Figure 10B**), the GPOD activity of the exposed strain 45e and strain 56b were significantly higher (**Figure 10C**) and the enzymatic activity of SPOD was significantly higher for strain 36a, 45e and 99h (**Figure 10D**). The APOD activity was significantly higher for the strains 56b, 99h and 132a in comparison with their non-exposed plants. A decrease in APOD activity was observed between the exposed and non-exposed inoculated strain 45e (**Figure 10E**).

Under normal conditions, ROS are constantly being generated at basal levels. However, they are unable to cause damage as they are being scavenged by different antioxidant mechanisms, visible by the presence of the low activity of the scavenging enzymes under non-exposed conditions (55). Under stressed conditions ROS are known to increase and the plants' anti-oxidative system is responsible for protecting against extensive cellular damage by up-regulating the antioxidant capacities (56). Upon exposure, plants inoculated with bacterial strains showed an increase in antioxidant enzymes. However except for strain 157a, the increase was lower in comparison with the exposed non-inoculated treatment. This experiment leads to the conclusion that treatment with PGPB tends to reduce the activity of the enzymatic capacity upon drought exposure. Indicating that PGPB are able to elevate the plants' tolerance to drought stress measured by the lower activity the antioxidant system. *Sandhya et al.* reported similar results for maize where the PGPBs *Pseudomonas* spp. decreased the enzymatic activity of APOD, CAT and GPOD under drought stress compared with the non-inoculated treatment (57). In another study performed by Naseem *et al.,* inoculation with bacterial strains *Proteus peneri*, *Pseudomonas aeruginosa*, and *Alcaginenes faecalis* decreased the enzymatic activity of SOD, POD and CAT in drought exposed maize, thereby stating that PGPR lessen the adverse effect of drought stress measured by antioxidant enzyme activity in combination with an increase in biomass an chlorophyll content (58). However, there are some contradictory results presenting higher enzymatic activities upon drought exposure. These differences might be related to the plant age and tolerance/strategy towards water stress.

APOD has a higher activity even under non-exposed conditions in comparison with the other enzymes. This can be attributed to the fact that APOD is an enzyme located in every cellular ROS producing compartment and might function as a fine regulator of the intracellular ROS steady-state levels possibly for signalling purposes. In contrast with CAT, located exclusively in the peroxisomes, an enzyme which functions as a bulk remover of excess ROS production especially

under stress conditions (56). Peroxidases from class III (SPOD and GPOD), mostly found in cell walls and vacuoles, play a role in lignin polymerization. It has been well documented that severe drought stress can be charged for the increase in cell wall lignification. Lignification restricts water uptake and cell expansion. By having a lower production of class III peroxides in the inoculated plants in comparison with the control condition, we suggest that the inoculated plants are more tolerant against drought stress.(59) High SEM values can be explained due to biological variation between the samples. Therefore, this experiment could be repeated by using more repetitions to verify the observed effect of PGPB on the antioxidative capacity.

3.3.4 PHOTOSYNTHETIC PARAMETERS

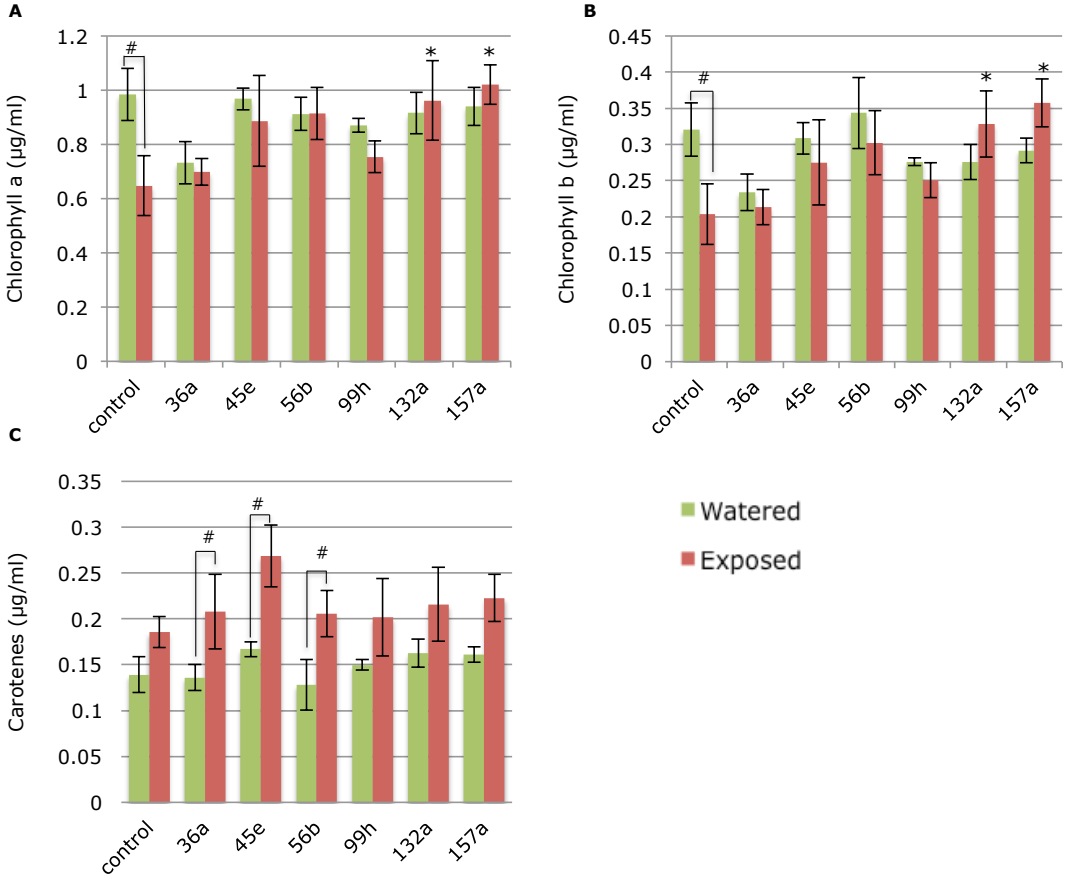

Figure 11. Pigment contents for the non-inoculated control and different PGPB inoculations in wheat shoots under watered and exposed conditions. *Panel A: Chl_a, panel B: Chl_b and panel C: Car content, all expressed in µg/ml. Parameters were measured 33 days after sowing of Triticum. Exposure obtained by withholding irrigation for six days. Control received no bacterial inoculation. The values given for each treatment are means of five replicates ± SEM. With * (p-value <0.05) representing statistical difference with the control and # (p-value<0.05) representing statistical difference between the non-exposed and exposed conditions.*

To see whether exposure to drought and inoculation with PGPB had an effect on the photosynthetic activity of the plant, the pigments chlorophyll a (Chl_a, the principal central photosynthetic pigment), chlorophyll b (Chl_b, accessory peripheral photosynthetic pigment) and carotenes were determined in fresh shoot samples 33 days after sowing. There were no significant differences between the non-exposed non-inoculated plants for the Chl_a content, except a significant lower

Chl_a for the non-exposed strain 36a. The Chl_a content of the exposed inoculated plant with strain 132a and 157a were significantly higher as the exposed non-inoculated control. A significant difference was observed between the non-exposed and the exposed control. A decrease of 34.17% in the mean Chl_a content between the non-exposed and the exposed control was visible. For the plants inoculated with the strains derived from the *Pistacia* the mean Chl_a content was decreased with 4.51%; 8.43% and 13.44% respectively for the strains 36a, 45e and 99h in comparison with the inoculated unexposed plants. For the strains 56b, 132a and 157a there was an increase of 0.08%, 5.04% and 8.53% (**Figure 11A**).

For Chl_b, no significant differences could be observed between the watered non-inoculated and the inoculated plants, except a significant lower Chl_b for the unexposed strain 36a. The Chl_b content of the exposed condition inoculated with strain 132a and strain 157a were significantly higher as the exposed non-inoculated control. A significant difference was observed between the non-exposed and the exposed control. Between the mean value for the non-inoculated and the exposed control there was a decrease of 36.45% in Chl_b content. For the plants inoculated with the strains derived from the *Pistacia* the mean Chl_b content was decreased with 8.70%; 10.83%, 12.04%; 9.23% respectively for the strains 36a, 45e, 56b and 99h in comparison with the inoculated unexposed plants. For the strain 132a and 157a there was an increase of 18.94% and 22.52%. The ratio Chl_a/Chl_b was 3.07 under watered circumstances, and contained 3.18 under exposed conditions for the non-inoculated conditions (**Figure 11B**).

For the content of the carotenes, there were no significant differences between the non-inoculated watered plants and the inoculated watered plants, except a significant lower Chl_b for the unexposed strain 36a. Between the mean value for the non-inoculated and the exposed control there was an increase of 33,35% in carotenes content. For the plants inoculated with the strains derived from the *Pistacia* the mean carotenes content was increased with 52.84%; 60.87%; 60.61%; 34.54%; 32.62% and 38.36% respectively for the strains 36a, 45e, 56b, 99h, 132a and 157a. There was a significant (p-value<0.05) higher carotenes content for the plants inoculated with the strains 36a, 45e, and 56b compared with their non-exposed inoculated plants, while there was no significant difference between the non-exposed and exposed control (**Figure 11C**).

The chlorophyll content of the leaves is known as a parameter for determining the photosynthetic efficiency of plants. Since there were no significant differences in the chlorophyll content under non-exposed conditions between the non-inoculated and the inoculated plants, we suggest that inoculation with PGPB has no effect on the photosynthetic efficiency of plants in normal conditions. The chlorophyll content was noticeably lower for exposed non-inoculated plants. These findings are in accordance with several other studies that reporting lower chlorophyll content under exposed conditions (50, 60). A possible explanation for the lower chlorophyll content can be associated with a decrease in the photosynthetic activity, followed by a decrease in stomatal conductance leading towards a lower chloroplast volume. It is well known that exposure to drought can cause oxidative stress due to the inhibition of the photosynthetic activity and imbalance between the light capture and its utilization, another possible explanation for the lower level of chlorophyll content under drought stress could be therefore chlorophyll degradation, change in chlorophyll synthesis or adaptations in the thylakoid membrane structure caused by oxidative stress. As reported in

another study performed by *Liu et al*., the Ch_a/Ch_b ratio was higher under drought stress. This can be explained by a decrease in peripheral light-harvesting complexes caused by wilting of the leaves as an effect of drought (50). However, for the exposed inoculated plants, there was an overall increase in Chl_a and Chl_b content in comparison with the exposed non-inoculated plants, indicating that inoculation caused a higher rate of photosynthesis and thereby generating a higher stress tolerance. This is in line with a study performed by *Gurani et al.* were *Solanum tuberosum* inoculated with *Bacillus* strains exhibited an increase in the photosynthetic efficiency. In another study performed by *Gusain et al*, the authors found increased chlorophyll content in susceptible rice cultivars (*Oryza sativa L.)* inoculated with different PGPR. PGPB are therefore known as important factors in enlighten the effects caused by drought on the photosynthetic apparatus (52).

Carotenes are important in photosynthetic organisms because they serve as accessory light-harvesting pigments by transmitting the light energy that they absorb to chlorophyll. Exposure to drought led to higher levels of carotenes in comparison with the non-exposed conditions. The rise in carotene content upon exposure is in line with the study performed by *Mohammadkhani et al.*, where water stress led to higher carotenoids contents, the main class organic pigments containing carotenes as a subclass, in two maize cultivars. (61). In this study, inoculation with strains derived from the *Pistacia* induced a rise in carotene content under exposure. These results are in accordance with the findings of *Dawwam et al.,* where *Ipomoea batatas* inoculated with *Bacillus cereus* and *Achromobacter xylosoxidans* showed a higher carotenoids content (62). Since the levels of carotenes content were not different under non-exposure, inoculation with PGPB only had an effect when exposed to stress. Carotenoids, and therefore also carotenes form a key part in the antioxidant defence system. Their protection against reactive oxygen species is essential for chloroplast functioning. Carotenes are also involved in the protection of the photosynthetic apparatus by scavenging singlet oxygen, and quench the triplet state of chlorophyll molecules. Therefore, a higher production can give rise to a higher tolerance of abiotic stress.

3.4 Quantification of Vesicular Arbuscular Mycorrhizal fungi

The colonization of Vesicular Arbuscular Mycorrhizal fungi (VAM) was quantified in roots from *Pistacia,* obtained from three different sampling places (SP). Between the different SP, there was no significant difference in the abundance of mycorrhizal fungi or vesicles. When considering the different sampling seasons, a significant higher mycorrhizal colonization could be observed for SP1 and SP3 in spring. The vesicles were significantly lower in spring than in the autumn for SP2.

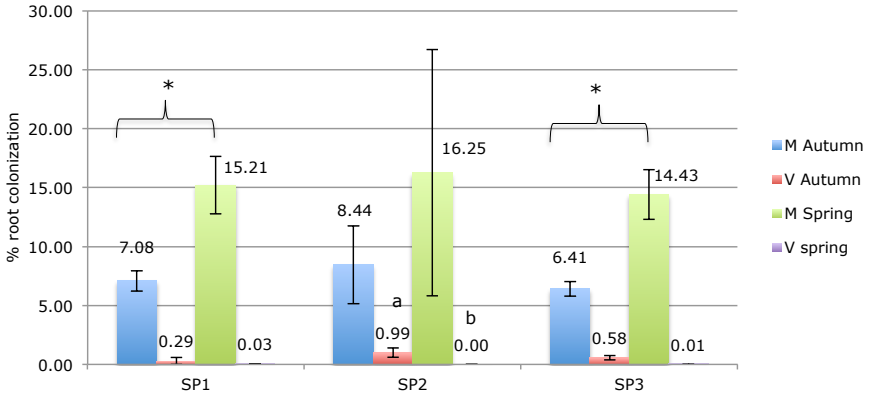

Figure 12. Visualization mycorrhizal fungi (M) and vesicles (V) in root samples obtained from Pistacia. *Quantification was performed for three different sampling places (SP) in spring (S) and autumn (A). The values given for each treatment are means of thee replicates ± SEM. Different letters indicate statistically significant differences for V and * indicate statistically significant differences for M (p-value<0.5).*

Vesicular Arbuscular mycorrhizal fungi (VAM), forming beneficial associations with the majority of terrestrial plant species, are known to increase plants' nutrient supply, plants' growth, restore the hormonal balance and increase the resistance to pathogens as stated before. The vesicles are known as a primary source of regrowth for AM fungal species and as storage structures in which VAM store energy as lipids for use during periods when resources are limited (63). According to our experiments, mycorrhizal fungi were found during both seasons in the *Pistacia* roots. Previous research showed that plants with mycorrhizal associations have a higher survival rate than non-mycorrhizal plants in arid environments (64). Although no differences concerning the amount of mycorrhizal and vesicular abundance could be detected between the different sampling places, a significant higher colonization could be observed during spring for two sample places. However, the roots sampled in spring were not that suitable for quantification followed by a realistic representation of mycorrhizal presence due to wood like structures and thickness of the roots. In literature, there is no overall consensus about the effect of season on VAM root colonization (64, 65).

Priming host plants with VAM originating from stressed soils appeared to improve plant development and health in terms of increasing shoot biomass, improvement plant yield, and higher nutrients availability. Numerous studies prove the positive synergistic interactions between VAM and PGPR under normal and stressed conditions. However, it has to be taken into account that a wide variation in synergistic reactions between different fungal and bacterial species is available (66).

3.5 TOTAL BACTERIAL COMMUNITY: ARISA

Bacterial ARISA fingerprints were created with the DNA extracted from rhizosphere and bulk soil samples from *Pistacia* among three different sampling places in spring and autumn. non- metric Multidimentsional Scaling (nMDS)-analysis with Bray-Curtis distance matrix was used to analyse the processed fingerprints in RStudio. An overall stress value of 0.16 could be obtained. In general, season provoked a separation of microbial communities (R-value= 0.3541, p-value= 0.001) obtained by analysis of similarities (ANOSIM). In addition, the sample place caused as well separation in microbial communities (R-value= 0.378, p-value= 0.001). However, no separation could be observed between BS and RS (R = 0.04783, p-value= 0.952).

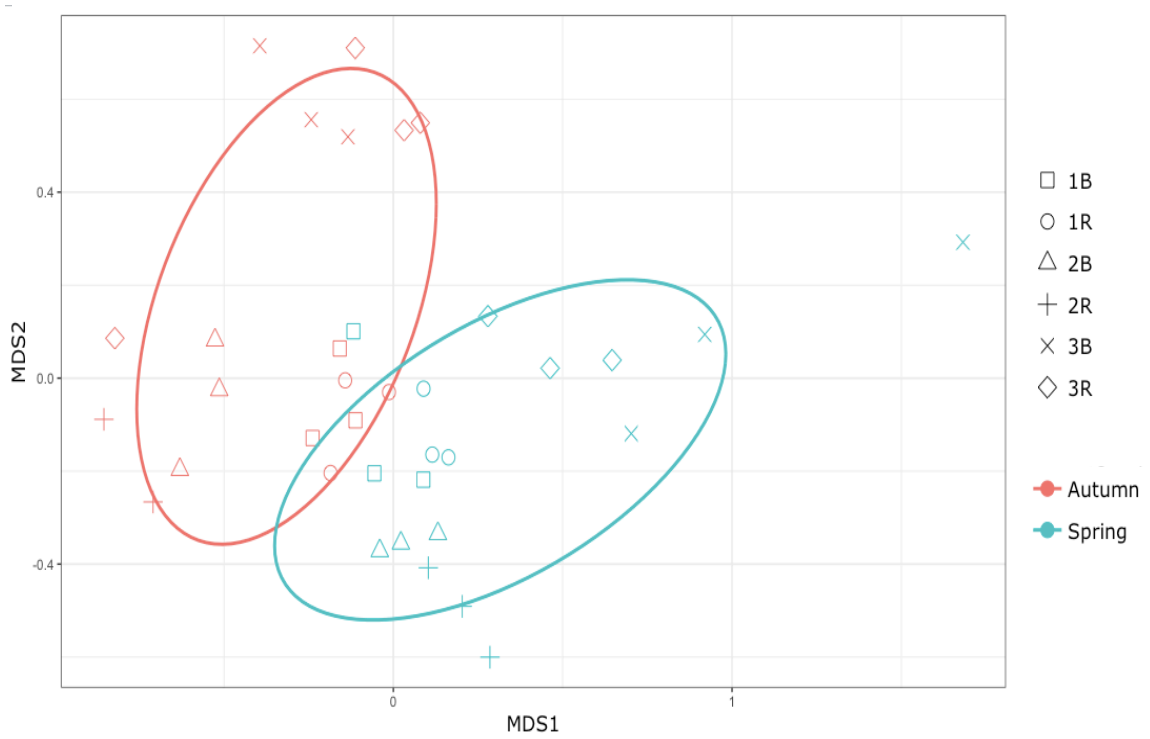

Figure 13. NMDS-analysis with the Bray-Curtis distance metric of the bacterial ARISA fingerprints (stress = 0.16) of communities originating from bulk soil (B) and rhizosphere soil (R) from three different sample places in autumn and spring*. Probability ellipses (standard deviation, p = 0.68) are shown for each sample type.*

By analyzing ARISA fingerprints, the data represents a trend in seasonal distinction between bacterial communities. A further step to form proper conclusions concerning the effect of season on the bacterial community would be to evaluate the bacterial diversity and abundance of microbes in various environments by the means of shotgun metagonomics. The communities where also different in each sample place, while they all originate from the harsh environment around the *Pistacia*. Even though the rhizosphere is the more tightly root-adhering soil containing the most microorganisms related to increasing PGP-capacities in comparison with bulk soil, no separation in bacterial communities was visible for RS and BS.

4 CONCLUSION

The main goal of this research was to reveal if PGPB derived from *Pistacia* and from grass are able to alleviate drought stress in crops relevant for humanity. To examine their possible capacity in relieving drought stress plant parameters such as biometric measurements antioxidative capacity and photosynthetic parameters were accomplished. When not exposed to drought, there was no difference between the non-inoculated and inoculated conditions showing that PGP activity is a stress-dependent and not a *per se* feature of the strains. Inoculation with strains derived from *Pistacia* caused increase in the DW, SL, Chl_a, Chl_b, and Car favouring towards a higher biomass rate and photosynthesis. Except for strain *157a Raoultella sp.*, inoculation caused a decrease in the antioxidant enzymes upon exposure showing in general that inoculation with bacteria derived from *Pistacia* can alleviate the stress caused by exposure to drought. Overall, inoculation of the plants with strains derived from grass yield no positive or even negative effects when considering the plant parameters. Therefore, we can conclude that strains isolated from the *Pistacia* are better in alleviating drought stress in *Triticum* than strains derived from grass, stating that these drought-resistance PGPB are cross compatible with different plant models. Analysis of the cultivable community and total bacterial community derived from BS and RS obtained from the *Pistacia,* showed a seasonal separation of the microbial community. The abundance of VAM in the roots of *Pistacia* can lead towards promising results in further research for improving the drought tolerance of host plants.

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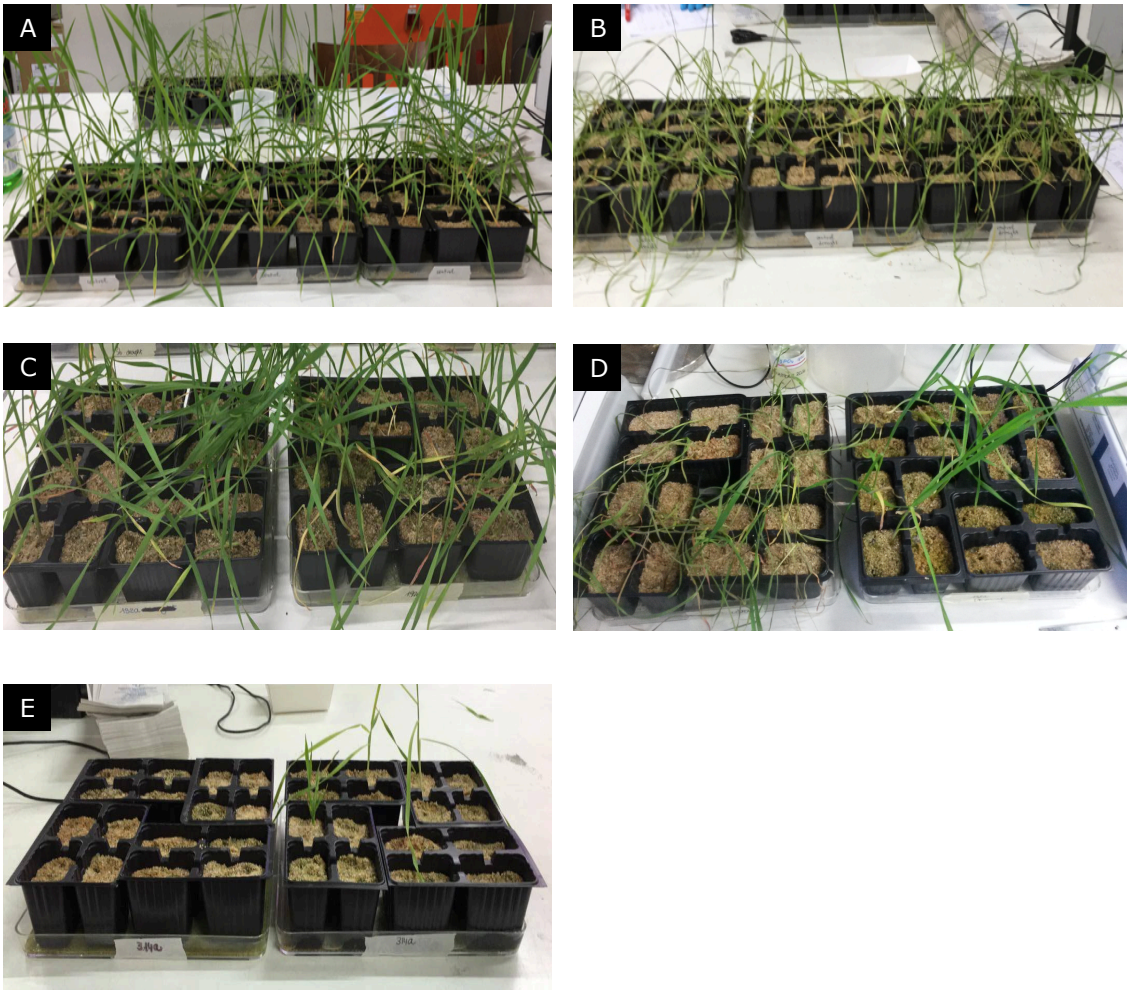
SUPPLEMENTARY INFORMATION



Supplementary figure 1: Comparison between different conditions of Triticum plants 33 days after sowing. A) Non-exposed non-inoculated plants. B) Non-inoculated plants exposed to drought by withholding irrigation for 6 days. C) Non-exposed plants inoculated with strain 132a *Pseudomonas sp*. obtained from *Pistacia*. D) Plants exposed to drought by withholding irrigation for six days inoculated with strain 132a *Pseudomonas* obtained from *Pistacia*. E) Non-exposed plants inoculated with strain 274a obtained from grass (not sequenced), showing a GR of 1,875%.

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2017**

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