Use of Rhizobacteria for the Alleviation of Plant Stress

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Cover: Scanning electron micrograph of biofilm on root hair of wheat
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Abstract

Plant growth promoting rhizobacteria are beneficial microbes able to induce plant stress tolerance and antagonise plant pathogens. The present study showed that wheat seedlings pre-treated with *Bacillus thuringiensis* AZP2 had better tolerance to severe drought stress and showed 78% greater plant biomass and five-fold higher survivorship compared to wheat seedlings not treated with the bacterium. The effect of *B. thuringiensis* AZP2 also resulted in improved net assimilation and reduced emission of stress volatiles.

The study investigated the effect of the inactivation of sfp-type phosphopantetheinyl transferase in plant growth promoting bacterium *Paenibacillus polymyxa* A26. The inactivation of the *sfp* gene resulted in loss of NRP/PK production such fusaricidins and polymyxins. In contrast to the former *Bacillus* spp. model the mutant strain compared to wild type showed greatly enhanced biofilm formation ability. Its biofilm promotion is directly mediated by NRP/PK, as exogenous addition of the wild type metabolite extracts restores its biofilm formation level. Further, increased biofilm formation was connected with enhanced ability of the *sfp* inactivated strain to remarkably protect wheat seedlings by improving its survival and biomass under severe drought stress conditions compared to wild type.

Fusarium graminearum and *F. culmorum* are the causing agents of a destructive disease known as Fusarium head blight (FHB). The disease is the leading cause of contamination of grain with *Fusarium* mycotoxins that are severe threat to humans and animals. Biological control has been suggested as one of the integrated management strategies to control FHB causing agents. The present study showed that *P. polymyxa* A26 is a potent antagonistic agent against *F. graminearum* and *F. culmorum*. In order to optimize strain A26 production, formulation and application strategies traits important for its compatibility need to be revealed. Hence, a toolbox comprising of dual culture plate assays and wheat kernel assays including simultaneous monitoring of the FHB causing pathogens, A26 and mycotoxins produced was developed in the present study. Using this system results showed that, besides the involvement of lipopeptide antibiotic production by *P. polymyxa* in the antagonism process, biofilm formation ability may play a crucial role in the case of A26 *F. culmorum* antagonism.

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Keywords: Plant drought tolerance, Biocontrol, NRPS/PKS, Rhizobacterial biofilm, sfp-type PPTase, Stress volatiles, DON, ZEA

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Dedication

To my beloved ones

“If you wish to make an apple pie from scratch, you must first invent the universe.”
Carl Sagan, Cosmos.

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Timmusk S, **Abd El-Daim IA**, Copolovici L, Tanilas T, Kannaste A, Behers L, Nevo E, Seisenbaeva G, Stenström E, Niinemets U (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. *PloS One* 9 (5):e96086. doi:10.1371/journal.pone.0096086

II Timmusk S, Kim S, Nevo E, **Abd El-Daim IA**, Ek B, Bergquist J, Behers L (2015) Sfp-type PPTase inactivation promotes bacterial biofilm formation and ability to enhance wheat drought tolerance. *Frontiers in Microbiology*; doi: 10.3389/fmicb.2015.00387

III **Abd El-Daim IA**, Häggblom P, Stenström E, Karlsson M, Timmusk S, (2015) *Paenibacillus polymyxa* A26 sfp-type phosphopantetheinyl transferase inactivation limits bacterial antagonism against *Fusarium graminearum* but not of *F. culmorum* (Manuscript submitted)

The contribution of Islam A. Abd El-Daim to the papers included in this thesis was as follows:

I Contributed to experiments design, conducted greenhouse experiments, conducted plant phenotypes analysis, performed photosynthesis and volatiles analysis and contributed to data analysis and paper writing

II Contributed to drought stress experiment design, conducted greenhouse experiment and plant phenotypes analysis and contributed to data analysis

III Contributed to experiments design, performed the antagonism assays and qPCR analysis and contributed to data analysis and paper writing

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
APX	Ascorbate Peroxidase
CAT	Catalase
CFU	Colony Forming Unit
DHAR	Dehydro Ascorbate Reductase
DOM	Deepoxyvomitoxin
DON	Deoxynivalenol
ESEM	Environmental Scanning Electron Microscopy
FHB	Fusarium Head Blight
GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSG	Glutathione Disulfide
IAA	Indole Acetic Acid
IPM	Integrated Pest Management
MDHAR	Mono-Dehydro Ascorbate Reductase
NFS	North Facing Slop
NRP	Non-Ribosomal Peptides
NRPS	Non-Ribosomal Peptide Synthetases
PGPR	Plant Growth Promoting Rhizobacteria
PK	Polyketides
PKS	Polyketide Synthases
PPTase	Phospho-Pantetheinyl- Transferase
RAS	Roots Adhering Soil
ROS	Reactive Oxygen Species
SFS	South Facing Slop
SOD	Superoxide Dismutase
TSB	Tryptone Soy Broth
VOCs	Volatile Compounds
WUE	Water Use Efficiency
ZEA	Zearalenone

1 Background

Foods demands have increased substantially during the last decade (FAO 2012). An increasing world population is the main factor for this steady rise. The United Nations estimates that the world population is predicted to increase from close to 7 billion in 2010 to about 9.15 billion by 2050. In addition, many people lack food security (Chrispeels 2000), the majority of them are living in developing countries. For instance, the US department of agriculture estimated the number of food-insecure people in the developing countries to be 833 million in 2009 (USAD 2009). Wheat (*Triticum aestivum*) is the most widely grown cereal grain, occupying about 17% of the total cultivated land in the world. Moreover, wheat constitutes the major staple food for nearly 35% of the world's population (Curtis et al. 2002; Farooq 2009). It is estimated that the world will require a 60% increase in wheat production by 2020. However, this is a major challenge due to the environmental constraints which cause major growth, yield and quality losses that limit the production of wheat (Tolmay 2001; Conway et al. 2012). Hence, a sustainable utilization of the environment and natural resources are critical to maintain and secure food supply for mankind (FAO 2012).

Abiotic and biotic stresses are limiting factors negatively affecting crop growth and productivity worldwide (Ji-Ping et al. 2007). Plants responses to such factors are very complex which manifest in a range of developmental, molecular and physiological modifications that lead to either stress sensitivity or tolerance/resistance (Harb et al. 2010). Several economically important plants such as wheat, maize and rice are known for their sensitivity to stresses which often results in substantial losses for crop production under unfavourable conditions (Bita and Gerats 2013). Hence, increasing crop plant productivity and enhancing resistance or tolerance against various stress factors has become major aims for modern agriculture (Farooq et al. 2009). In sustainable agriculture, Integrated Pest Management (IPM) is considered the most efficient strategy to manage stress causing agents, such strategy rely on combining several approaches including using resistant varieties, crop rotation,

monitoring pests, biocontrol and in sever situations employing pesticides in an attempt to keep stress agents under control (Wegulo 2012). Biological control form an integral part of the IPM strategy (Landa et al. 2004). Plant growth promoting rhizobacteria (PGPR) refer to a group of bacteria that can improve plant growth and productivity by several mechanisms (Bashan et al. 2006). Further, several PGPRs have been suggested as a potential biocontrol agents (Beneduzi et al. 2012). The main aim of the present study was to use rhizosphere bacteria for wheat stress tolerance alleviation.

2 Introduction

2.1 Plant and stresses

Plants are often challenged by several environmental stresses. Lichtenthaler (1998) defines the term stress as any un-favorable condition or substance that affects or blocks a plant's metabolism, growth or development, which can be induced by various natural factors. Stress factors are divided into biotic (living) and abiotic (non-living) stresses. Biotic stress includes a variety of pathogenic microorganisms, insects and higher animals including interference from humans. On the other hand, abiotic stress include factors such as water logging, drought, heat, cold, wind, intense light, soil salinity and inadequate or excess of mineral nutrients (Vinocur and Altman 2005; Wahid et al. 2007).

2.2 Drought stress

Growth rates of several plants are directly proportional to the availability of water in the soil (Song et al. 2009). Plant or cellular water deficit occur when the rate of transpiration exceeds water uptake resulting in the reduction of the relative water content, cell volume and cell turgor (Lawlor and Cornic 2002). Cellular water deficit is a common component of several different stresses including drought, salinity and low and high temperature (Bray 1997; Song et al. 2009). The effects of drought range from morphological to molecular levels and are evident at all growth stages of plant growth at whatever stage the water deficit occurs (Farooq et al. 2009). The first and foremost effect of drought is poor germination (Kaya et al. 2006). For instance, drought stress has been reported to severely reduce germination and seedling development in sunflower and wheat (Kaya et al. 2006; Nezhadahmadi et al. 2013). A variety of physiological responses are directly influenced by drought stress including relative water content, leaf water potential, stomatal conductance, rate of transpiration and leaf temperature

(Machado and Paulsen 2001). A major effect of water scarcity is compromised photosynthesis efficiency, which arises by a decrease in leaf expansion, impaired photosynthetic machinery and premature leaf senescence (Wahid and Rasul 2005). Very severe drought conditions limit photosynthesis due to a decline in Rubisco activity (Bota et al. 2004).

Drought stress is a leading cause for generation of reactive oxygen species (ROS) including superoxide anion radicals (O_2^{-}), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), alkoxy radicals ($RO^{\cdot}$) and singlet oxygen ($^1O_2$) in plant (Munné-Bosch and Penuelas 2003). ROS are very energetic and often react with proteins, lipids and DNA causing oxidative damage and impairing the normal functions of cells (Foyer and Fletcher 2001). To cope with ROS cellular damage plants have developed a very complex defence system relaying on both enzymatic and non-enzymatic components. Enzymatic components include superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase. On the other hand, non-enzymatic components include compounds such as cysteine, reduced glutathione and ascorbic acid (Gong et al. 2005). The ascorbate–glutathione pathway, also known as the Halliwell-Asada cycle is considered one of the most studied antioxidant defense mechanism in plants, which is catalyzed by a set of four enzymes (Fig. 1) (Fazeli et al. 2007). Hydrogen peroxide is scavenged via the oxidation of ascorbate by ascorbate peroxidase (APX). This enzyme is involved in the oxidation of ascorbate to mono-dehydroascorbate, which can be converted back to ascorbate via mono-dehydroascorbate reductase (MDHAR). Mono-dehydroascorbate that escapes this recycling is converted rapidly to dehydroascorbate which is converted back to ascorbate by the action of dehydroascorbate reductase (DHAR). DHAR utilizes reduced glutathione (GSH), which is regenerated by glutathione reductase (GR) from its oxidized form, glutathione disulfide (GSSG) (Murshed et al. 2008). Overall, the production of ROS positively correlates with the severity of drought stress in plants hence, measuring ROS and its associated defence components such as antioxidant enzymes are used to monitor stress severity in plants (Wahid et al. 2009).

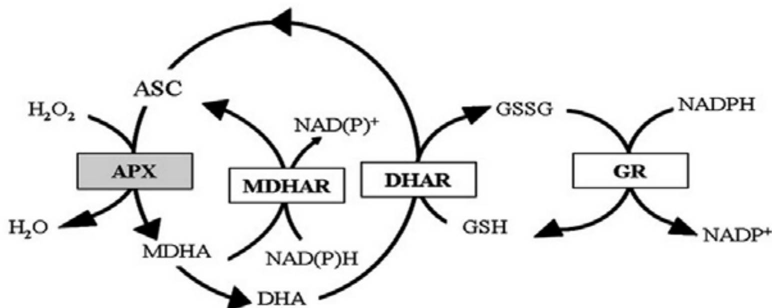

Figure 1. The Ascorbate–Glutathione Pathway adopted from Zechmann (2014)

2.3 Induced stress volatiles

Several volatile compounds (VOCs) are emitted from plants leaves. However, VOCs emission is known to substantially increase under several abiotic and biotic stress conditions in plants (Loreto and Schnitzler 2010; Copolovici et al. 2014). Numerous VOCs have been identified and most belong to a few broad classes such as volatile isoprenoids, volatile products of shikimic acid pathway (phenylpropanoids, benzenoids, and indole), carbohydrate and fatty acid cleavage products (Niinemets et al. 2013). VOCs may play several roles during stress conditions, for instance, as defense and priming signals within the individual as well as between closely located plants (Heil and Bueno 2007). On the other hand, VOCs biosynthesis consumes a considerable amount of carbon which requires reallocating of plant metabolic resources (Niinemets 2004; Loreto and Schnitzler 2010). A strong correlation between VOCs emission and stress severity has been well stablished for several plants (Holopainen and Gershenzon 2010; Niinemets et al. 2013). Hence, monitoring VOCs emission could be used as non-invasive strategy for stress severity monitoring.

2.4 Fusarium head blight (FHB)

Fusarium head blight (FHB) is a destructive disease on cereals that is caused by a group of *Fusarium* species including *Fusarium graminearum* and *F. culmorum* (Nazari et al, 2014). FHB is a serious threat to agricultural production due to yield losses, but also constitutes a major safety concern when humans and animals consume *Fusarium*-contaminated wheat products due to the accumulation of several mycotoxins (Champeil et al, 2004). Both *F. culmorum* and *F. graminearum* are soil borne and cause not only FHB, but also fusarium foot and root rot on cereals around the globe especially during wet seasons (Nicolaisen et al. 2009; Scherm et al. 2013). The infection can develop in several stages but the anthesis is the most susceptible stage for *Fusarium* infection, especially the opening of the florets which allows the fungal hyphae to establish infection more easily (Siou et al 2014).

2.5 *Fusarium* mycotoxins

Several toxicologically important mycotoxins have been connected to *Fusarium* spp. including deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA) and fumonisin B1 (FB1) (Fig. 2). *Fusarium* mycotoxins can cause both acute and chronic toxic effects for both animals and human. The severity of the toxins is dependent on the mycotoxin type, the level and duration of exposure. Intake of high doses of mycotoxins may lead to acute mycotoxicoses (Antonissen et al. 2014). DON and ZEA are by far the most studied *Fusarium* spp toxins (Peraica et al. 1999). Higher levels of both toxins in wheat grains are usually connected to infection with *F. culmorum* and *F. graminearum* (Sniders 1990; Scherm et al. 2013). Both toxins are known with their special

mode of actions. DON is known to inhibit protein synthesis while ZEA possesses estrogenic properties and belongs to the group of endocrine disruptors (Döll and Dänicke 2011).

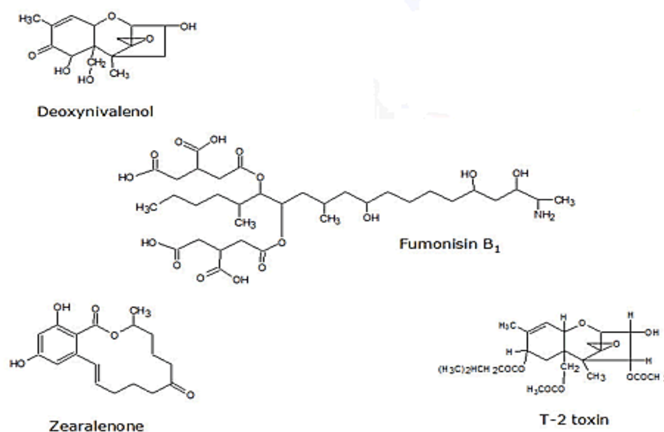

Figure 2. Chemical structures of *Fusarium* spp. mycotoxins adopted from Zain (2011)

2.6 Managing stress

Several strategies could be employed to manage the deleterious effects of both abiotic and biotic stress factors on plants. For decades the most adopted strategy relied heavily on conventional plant breeding for genetics improvement aiming for resistant/tolerant varieties (Cattivelli et al. 2008; Rudd et al. 2001). However, conventional plant breeding techniques have practical limitations. For instance, plant breeding is a relatively slow process often dependent on costly programs and highly influenced by seed companies (Conway 2012). On the other hand, genetic improvement could be also achieved by utilizing biotechnology aiming to engineer resistant/tolerant varieties carrying modified genes (Conway 2012). The potential of genetically modified crops have received a great attention from the scientific community, however, it is still not fully accepted by the general public due to possible environmental and health concerns (Key et al. 2008). Another well-known stress management strategy is to control the stressful agent, for instance, farmers have long relied on fungicides to control pathogens such as *F. culmorum* and *F. graminearum* (Dal Bello et al. 2002). However, the reliability of fungicides is limited and the strong dependence on chemical fungicides in modern agriculture may lead to unwanted, negative effects on the environment and on human health (Hasan et al. 2012). Stress causing agents could be

biologically controlled which is considered a much safer strategy (Dal Bello et al. 2002). Biological control could be utilized to manage both biotic and abiotic stress factors. For instance, several plant pathogens including *Fusarium* spp. could be controlled using antagonistic microbial agents (Dal Bello et al. 2002). Plant growth promoting rhizobacteria (PGPR) are known for their abilities to induce plant defence/tolerance, promote plant growth as well as antagonise several plant pathogens and have been considered as potential biocontrol agents (Planchamp et al. 2014; Barriuso et al. 2008).

2.7 Rhizosphere and plant growth-promoting rhizobacteria (PGPR)

The rhizosphere refers to a unique zone formed by soil under the influence of a plant root system (Berendsen et al 2012). Root's rhizosphere is characterized by a great microbial diversity as well as complex interactions between microorganisms and the roots (Bakker et al. 2013). Bacterial communities are well established in the rhizosphere, typically numbering 10^6 to 10^9 g^{-1} bacteria of rhizosphere soil. The concentration of bacteria in the rhizosphere is higher than in bulk soil due to the production of root exudates that can support bacterial growth and metabolism (Bais et al. 2006). Plant microbe interactions within the rhizosphere zone are very complex and might be beneficial, harmful, or neutral for the plant (Berendsen et al 2012). A schematic representation for plant rhizosphere interactions is shown in Fig. 3. Beneficial bacteria include both those that form a symbiotic relationship, which involves the formation of specialized structures as in the genus *Rhizobia*, and those that are free-living in the soil (Valdenegro et al. 2000). Beneficial free-living bacteria, referred to as PGPR are a characterized component of the plant rhizosphere and have been found in association with many different plant species including wheat (Majeed et al. 2015; Vacheron et al. 2013). Beneficial microbes could limit pathogen progress through production of biostatic compounds, consumption of (micro) nutrients or by stimulating the immune system of the plant (Berendsen et al 2012). Further, several PGPRs are known for the ability to colonize plant roots and often lead to direct plant growth promotion through producing phytohormones such as indole acetic acid (IAA) (Bruto et al 2014).

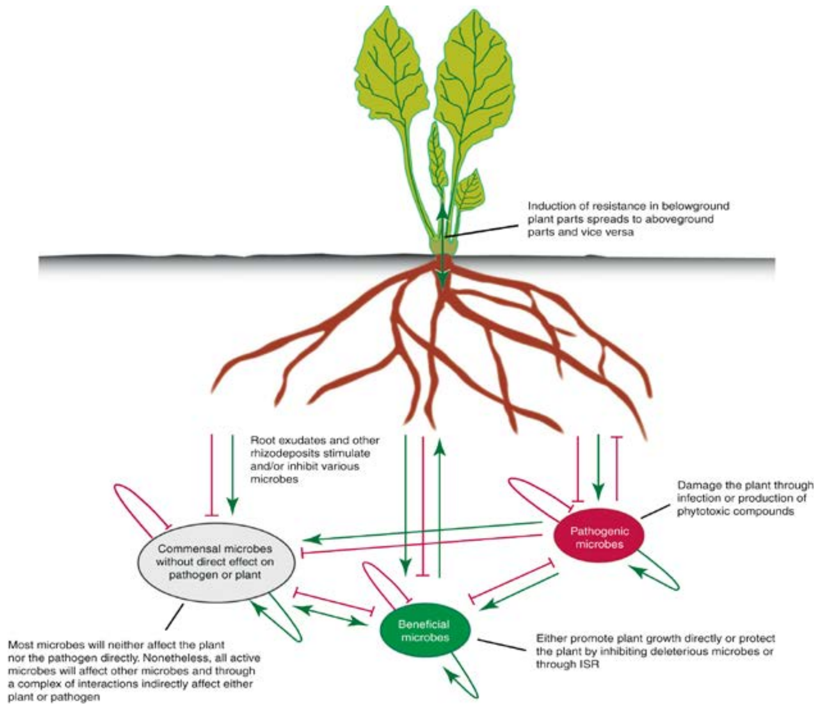

Figure 3. A schematic representation for plant rhizosphere; showing the complexity of the interactions between root and rhizosphere components; Adopted from Berendsen et al. (2012) (Reproduced by publisher permission)

2.8 PGPR improve abiotic stress tolerance

Application of PGPR to induce abiotic stress tolerance in plants is extensively investigated as an attractive strategy to control plant stress (Dimkpa et al. 2009; Kasim et al. 2013; Rejeb et al. 2014). The ability of *Paenibacillus polymyxa* to alleviate drought stress was first found in *Arabidopsis thaliana* by Timmusk and Wagner (1999). After that, various groups have reported the ability of PGPR to induce plant stress tolerance (Yang et al. 2009; Rejeb et al. 2014). Recently, it was reported that *Bacillus amyloliquefaciens* can improve heat and drought stress tolerance in wheat (Kasim et al 2013; Abd El Daim et al 2014). PGPR utilize several mechanisms to induce abiotic stress tolerance in plants (Fig. 4) (Dimkpa et al. 2009; Yang et al. 2009). PGPR can enhance plant growth directly by providing plants with nutrients such as nitrogen via nitrogen fixation or by supplying phosphorus from soilbound phosphate (Omar et al 2009; Berg 2009). PGPR are known for their ability to synthesize several plant growth hormones such as auxins and cytokinins (Berg 2009; Yang et al. 2009).

Besides direct phytohormone production, PGPR can modulate levels of the plant stress hormone ethylene via producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase. It degrades ACC the primary precursor of ethylene and diminishes its negative effects under stress condition (Glick 2014). For instance, it have been reported that *P. polymyxa* with ACC deaminase activity are potential drought stress tolerance enhancers (Timmusk et al. 2011). PGPR form biofilms composed by bacteria and extracellular matrix (Yang et al. 2009; Dimkpa et al. 2009; Conrath et al. 2006). Biofilms contain several classes of sugars that can play various roles in improving plant abiotic stress tolerance through maintaining significant water availability in the rhizosphere (Timmusk and Nevo 2011).

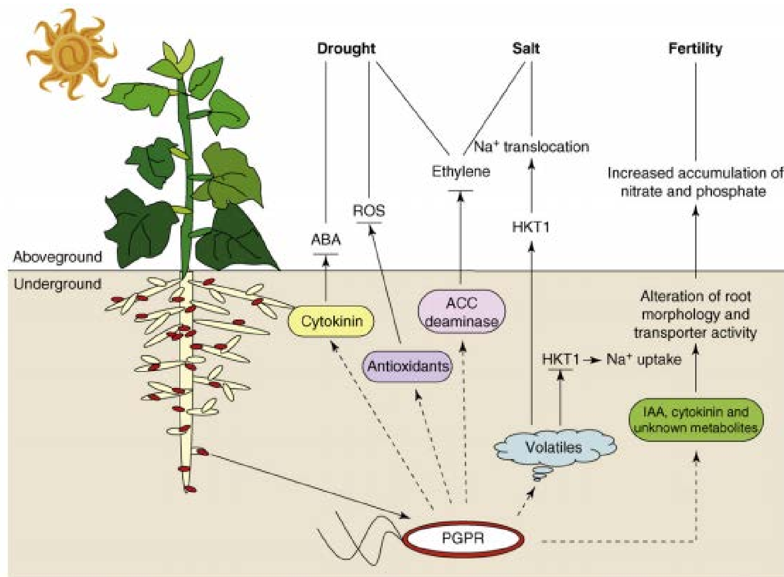

Figure 4. Induced systemic tolerance (IST) elicited by PGPR against drought, salt and fertility stresses underground (root) and aboveground (shoot); Adopted from Yang et al. (2009) (Reproduced by publisher permission)

2.9 PGPR as a biocontrol agents

PGPR have been employed to control several plant pathogens, including *Fusarium* spp. (Dal Bello et al. 2002). Biological control could be achieved either by using the ability of several PGPR strains to antagonise the disease causing agents or inducing plant resistance (Siddiqui 2006; Van Loon and Bakker 2006). For instance, *P. polymyxa* have been successfully used to control several plant diseases caused by *Botrytis* spp. and *Fusarium* spp. (Raza

et al. 2008). Further, Shi et al (2014) reported that *B. amyloliquefaciens* antagonised *F. graminearum* growth which in turn significantly inhibited DON production in wheat seeds. Several PGPR are also capable of mycotoxin detoxification as shown by Cheng et al (2010) that reported the ability of two *Bacillus* isolates to detoxify DON in wheat and maize. The detoxification was achieved by transforming DON to a less toxic product deepoxyvomitoxin (DOM).

2.10 Antagonism mechanisms

Productions of toxic and microbial growth inhibiting metabolites are widely considered the most powerful mechanism employed by rhizobacteria against plant pathogens (Cawoy et al 2014). It is estimated that some *Bacillus* and *Paenibacillus* species devote from 4% to 8% of their genomes for genes encoding proteins involved in synthesising bioactive compounds (Cawoy et al 2014). The biosynthesis of such compounds in rhizobacteria is complex and poorly understood, however, the majority of these compounds are predicted to be non-ribosomal peptides (NRP) synthesized by nonribosomal peptide synthetases (NRPS), or polyketides (PK) synthesised by polyketide synthases (PKS) (Pimental-Elardo et al. 2012; Mongkolthanaruk 2012; Raza et al. 2008). PKS are multi-domain enzymes containing numerous enzymatic domains organized into functional units. Correspondingly, NRPS are large multifunctional enzymes synthesizing NRP, which is a class of peptide secondary metabolites having an extremely broad range of biological activities (Hwang et al 2013). Despite the enormous chemical diversity both PKS and NRPS share a common point of regulation. Hence, all of these enzymes require activation by 4-phosphopantetheinyl transferase (PPTase) (Beld et al 2014; Owen et al 2012). Bacterial PPTases are classified into two groups based on their sequence conservation and substrate spectra. The members of the first group are associated with primary metabolism and catalyze the activation of the fatty acid acyl carrier domains (Bunet et al. 2014). The second group type is a PPTase sfp which activates peptidyl carrier protein domains (Bunet et al. 2014; Quadri et al. 1998).

3 Objectives

Employing rhizobacteria to control both abiotic and biotic stresses is a very attractive strategy for sustainable and environment friendly agriculture. However, several aspects need to be explored in order to efficiently utilize rhizobacteria for such purposes. The overall aim of the present work was to develop methods for wheat (*T. aestivum*) stress alleviation utilizing the abilities of some rhizobacterial isolates to induce plant abiotic stress tolerance and antagonise plant pathogens.

The specific objectives:

3.1 Determine the potential of rhizobacterial isolates from contrasting habitats to induce wheat drought stress tolerance (manuscript I).

3.2 Develop non-invasive strategies to gauge drought stress severity in wheat (manuscript I).

3.3 Assess the impact of *P. polymyxa* A26 sfp-type-PPTase inactivation on the rhizobacterial ability to induce drought stress tolerance in wheat (manuscript II).

3.4 Determine the potential of selected rhizobacterial isolates to antagonise the FHB causing agents (manuscript III).

3.5 Develop an *in vivo* experimental model to monitor *P. polymyxa* A26 antagonistic ability against FHB causing pathogens (manuscript III).

3.6 Determine the effect of sfp-type-PPTase inactivation on *P. polymyxa* A26 antagonistic ability against FHB causing pathogens (manuscript III).

4 Materials and Methods

4.1 Bacterial isolation

Rhizosphere bacteria were isolated from several locations including the North-Israeli ‘Evolution Canyon’ both from more stressed south facing slop (SFS) and less stressed north facing slop (NFS) sites (SFS and NFS strains), *B. thuringiensis* AZP2 was isolated from ponderosa pine (*Pinus ponderosa*) roots grown on gneiss rock at Mt. Lemmon, AZ, USA (32.38568° N, 110.69486° W elevation 2150 m). *P. polymyxa* B was isolated from salty rice (Oryza sativa) rhizosphere at Tina plain, North Sinai, Egypt (31.044° N, 32.6661° E, elevation 13 m).

4.2 Bacterial inoculation, plant growth and stress treatment (manuscript I, II)

Three wheat cultivars (spring wheat cv. Sids1, drought-sensitive winter wheat cv. Stava and drought-tolerant winter wheat cv. Olivin) were used in the present study. Seeds were surface sterilized using 5% chlorine solution. Bacteria were grown in tryptone soy broth (TSB) medium at 28°C overnight. Culture density was determined by colony forming unit analysis (CFU). Inoculation was performed by soaking grains in solutions containing 10^7 bacteria ml^{-1} for 4 hours at 28°C. For the control treatment, another set of grains was soaked in sterile TSB media. Seeds were sown in plastic pots filled with 450 g sand or sand mixed with 10% greenhouse soil. Both inoculated and non-inoculated treatments were replicated twelve times and each treatment had three plants per pot. The pots were incubated in controlled environment in a MLR-351H (Phanasonic, IL, USA) growth chamber with 24/16°C (day/night) temperature, and 16 h photoperiod at a quantum flux density of 250 µmol m^{-2} s^{-1}. Soil moisture content was kept constant during the first 10 days of seedling growth.

In 10 days after seed germination, drought stress was induced by stopping watering. Plants grown in sand were stressed for 10 days and plants grown in sand mixed with 10% greenhouse soil were stressed for 14 days. Soil volumetric water content was evaluated using 5TE soil moisture sensors (Decagon Devices, Inc, Pullman, WA, USA).

4.3 Plant survival and growth analysis (manuscript I, II)

Plant survival was calculated daily after stress application using 32 stressed plants that were randomly selected and divided into two groups with 16 plants each. After stress application, the plants were watered and allowed to recover for 4 days. The recovered plants were counted as survived plants. Eight days after application of drought stress, the survived and recovered plants were harvested, washed and blotted dry between filter paper. Plant roots were counted and their length was estimated with Root Reader 3D Imaging and Analysis system (Clark et al. 2011). Root-adhering soil was evaluated in twelve plants per treatment. Roots with adhering soil (RAS) were carefully separated from bulk sand and sand soil mix by shaking. Shoot, soil and root dry mass (RT) were recorded after drying the samples at 105°C to a constant mass, and RAS/RT ratio was calculated. Water use efficiency (WUE) was calculated as the ratio of total plant dry mass to total water use during the experiment.

Root hair length and density were evaluated using twelve plants. Plants were carefully separated from soil by shaking. After separation of loosely attached soil, plant roots were washed in distilled water and left to drain in Petri dishes containing 5 ml of water. The other set of plant was homogenised and used for AZP2 identification and quantification. The dried root system characteristics (root hair density and length) were evaluated using Zeiss LSM 710 microscope.

4.4 Foliage gas exchange and VOCs measurements (manuscript I)

Steady-state net assimilation and transpiration rates and stomatal conductance were recorded immediately after stress application (day 0) and in 2, 5, 8 and 10 days from start of stress application using a Walz GFS-3000 portable gas exchange system equipped with a LED-array/PAM-fluorimeter 3055-FL (H. Walz GmbH, Effeltrich, Germany). Volatiles were trapped by sampling 4 L of chamber air from the Walz GFS-3000 cuvette outlet onto a (Shimadzu Corporation, Kyoto, Japan).

4.5 Scanning Electron Microscopy (manuscript I, II)

Environmental scanning electron microscopy (ESEM) micrographs of the samples were obtained with a Hitachi TM-1000- µDex variable pressure scanning electron microscope. Samples were deposited on a carbon tape and coated by gold using Sputter Coater 108 auto (Cressington).

4.6 Protein extraction and antioxidant enzyme activity measurements (manuscript I, II)

Leaf samples for enzyme activity determination were harvested after 8 days from drought-treated and well-watered plants. Plant tissue was mixed with 10 ml extraction buffer as described by Knöerzer et al. (1996). The mixture was centrifuged at 14,000 rpm (Eppendorf, 5415C) for 10 min at 5°C, and the supernatant was used to determine protein content and activity of key antioxidant enzymes. Monodehydroascorbate reductase (MDAR) activity was determined following the decrease in light absorbance at 340 nm due to NADH oxidation as described by Hossain et al. (1984). Glutathione reductase (GR) activity was determined by increase in absorbance at 412 nm according to Smith et al. (1989). Superoxide dismutase (SOD) activity was determined by reduction in light absorbance at 490 nm using an Oxiselect SOD activity assay kit (Cell Biolabs, San Diego, CA, USA.) according to manufacturer's instructions. Catalase (CAT) activity was measured by reduction in light absorbance at 520 nm, using an OxiselectTM CAT activity assay kit (Cell Biolabs). For CAT and SOD, enzyme activities were determined per gram of fresh mass (FM).

4.7 Biofilm formation assay (manuscript II)

The assay was performed based on pellicle weights as described by Beauregard et al. (2013). Cells were cultured from 1 day old colonies re-suspended in 3 ml potato dextrose broth (PDB). After 2 hours the cells were diluted 1:100 in 3 ml PDB. The dilution was repeated two more times. After the last dilution, cells were harvested at OD 600 <0.5 and adjusted to a final OD600 of 0.3. The assays were performed in a 24 well plates. Pre-weighed PELCO prep-eze individual wells with a mesh bottom (opening size 420 µM) (Ted Pella) were put in the wells to which 1 ml medium and 14 µl of cells were added. Plates were incubated at 30^{0}C for 96 h to allow pellicles to develop. Individual wells were then removed, dried and weighed. Values are the means of four experiments.

4.8 Bioassay of in *vivo* antagonism between *P. polymyxa* and FHB causing agents (manuscript III)

4.8.1 Plate assay:

Inhibitory studies between *P. polymyxa* A26 and E1 and *F. culmorum* and *F. graminearum* were conducted on King's B plate. The bacterial strains were streaked onto the plates after inoculation with fungal plugs. Plates were incubated at 28°C for 5 days.

4.8.2 Assay on wheat grains:

Sterile 150 ml conical flasks containing 20 g sterile wheat grains were inoculated with 15 ml 1×10^{7} cells/ml *P. polymyxa* A26, A26Δ*sfp* and bacterial culture filtrate solutions. Controls were treated with 15 ml sterile water. Flasks were incubated at room temperature for 8 hours, and then inoculated with 1 cm^{2} agar plugs from 2 week old cultures of either *F. graminearum* or *F. culmorum*, and incubated at room temperature. Fungal growth was assessed visually and 1 g samples (≈15 grains) were taken from each flask at 4 time points; i.e. 0, 5, 10 and 15 days after fungal inoculation, and stored at -20°C. Samples were subjected to fungal and bacterial DNA quantification using quantitative PCR as well as fusarium mycotoxins DON and ZEA analysis.

5 Results and Discussion

5.1 Rhizosphere bacteria isolated from harsh environments improved the survival and biomass of drought stressed wheat (manuscript I)

Several rhizobacterial isolates were screened for their abilities to induce drought stress tolerance in wheat. Data presented in (Table 1: manuscript I) revealed that rhizosphere isolates originated from harsh environments were superior in drought tolerance enhancements. *B. thuringiensis* AZP2 topped the list of 12 screened rhizosphere bacterial isolates originated from harsh environments. Compared to the un-inoculated drought stressed seedlings, AZP2 treated seedlings showed delayed response to drought stress. For instance, more than 40% AZP2 treated seedlings have managed to survive for 8 days without water compared to 0% in their AZP2 untreated counterparts (Fig. 5). The survival of *B. thuringiensis* AZP2 drought stressed wheat was further improved to 80% in sand soil mixed with 10% greenhouse soil (Fig. 6A).The *B. thuringiensis* AZP2 treatment resulted in significantly higher dry weight in shoots (Fig. 6B) and recovered plant phenotypes (Fig. 6C). The effect of AZP2 was also evident in wheat roots. Hence, several root growth traits were found to be enhanced after AZP2 treatments including root dry weight, length and root hair counts (Table 2: manuscript I). Bacterial biofilm formation on plant root surface was estimated as the amount of soil attached to roots. Two to three times more soil was attached to AZP2-trated roots under drought stress and up to two times more under normal watering (Table 2: manuscript I). Electron microscopic imaging of the AZP2 treated wheat seedlings grown under drought stress confirmed the bacterial biofilm formation on root hairs (Fig. 7). An early study showing the potential of rhizobacteria to induce drought stress tolerance was reported by Timmusk et al. (1999) where the ability of *P. polymyxa* to improve drought stress tolerance in *A. thaliana* was showen. Further, the induction of drought stress tolerance was also reported in wheat treated with *B. amyloliquefaciens* (Kasim et al. 2013). The ability of PGPR to induce drought stress tolerance is often attributed to several mechanisms such as hormones production, ACC deaminase activity and biofilm formation (Yang

et al 2009). AZP2 genome sequencing revealed gene clusters for alginate, ACC deaminase, and auxin (IAA) production and regulation. Hence, the present study suggests that such traits alone, and in combination could have been responsible for the bacterial drought tolerance induction. On the other hand, the roots ability to extract moisture and nutrients from the soil is key traits determining plant survival under drought stress conditions (Werner et al. 2010). Improved nutrient and water extraction capacity can be achieved by various ways (Werner et al. 2010). Results obtained in the present study indicate that AZP2 inoculation resulted in major modifications of the wheat root system, for instance two to three times longer root hairs, and longer and denser lateral roots were detected in the present study. Root hair length and density are critical when it comes to water and nutrient acquisition from the surrounding environment. Although root hair formation can be massively enhanced, this increase should not necessarily show up as an increase in total root dry mass (Comas et al. 2013). Another important root trait in plant protection against drought stress is the creation of bacterial biofilm with attached soil mulch. AZP2 induced denser and longer root hair framework that forms an excellent matrix for the bacterially-excreted biofilm comprised of cells and extracellular matrix producing a thick sticky layer around root hair. Hence, induction of long and dense root hair should be considered as an important drought stress tolerance enhancement strategy. The dense biofilm matrix also limits diffusion of biologically active compounds secreted by bacteria and these are therefore concentrated on the root surface, facilitating plant uptake. In addition, biofilm formation on root hair substantially improves root-to-soil contact, enhancing plant nutrient acquisition from soil and suggesting that biofilm formation importantly contributes to improving plant nutrition as well (Fig. 7, manuscript I; Table 2).

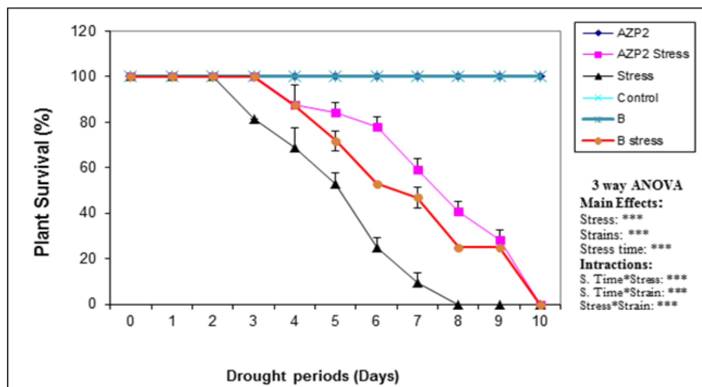

Figure 5. Effect of *B. thuringiensis* AZP2 and *P. polymyxa* B treatment on seedlings survival % during 10-day drought stress episode; the statistical analysis is based on a three-way ANOVA (stress, strains (i.e. AZP2 and B) and stress exposure time). ANOVA was conducted on two plant groups with 16 replicates in each group. *** indicate highly significant effects for the tested factor at $P \leq 0.01$

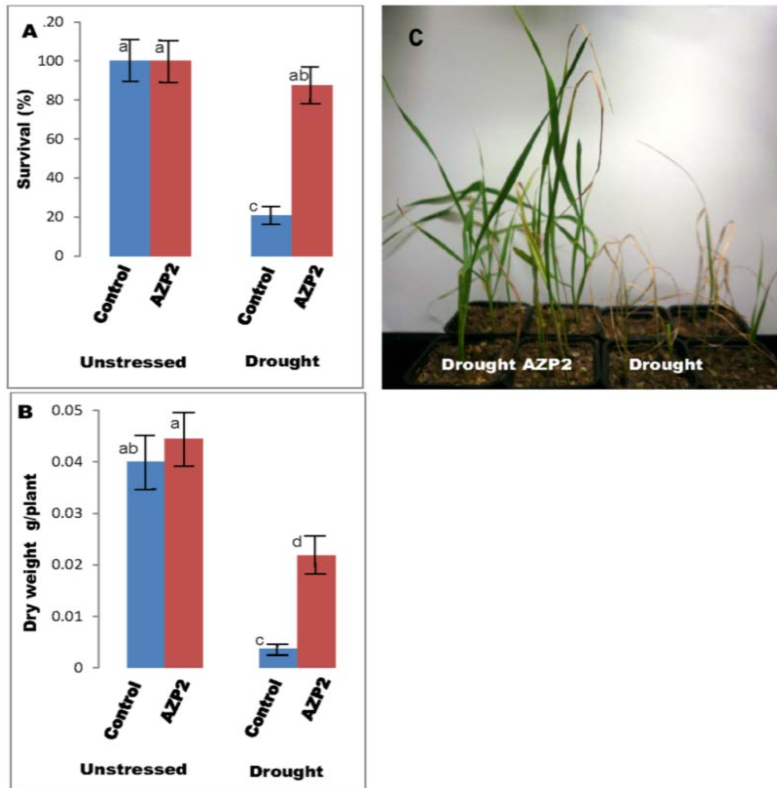

Figure 6. Increase of wheat drought stress tolerance by *B. thuringiensis*AZP2 in sand mixed with 10% greenhouse soil. Effect of AZP2 inoculation on wheat survival **(A, C)** and dry mass **(B)** after 14 days of drought stress. Eight independent experiments were performed, and treatments with the same letter are not significantly different at $P \leq 0.01$

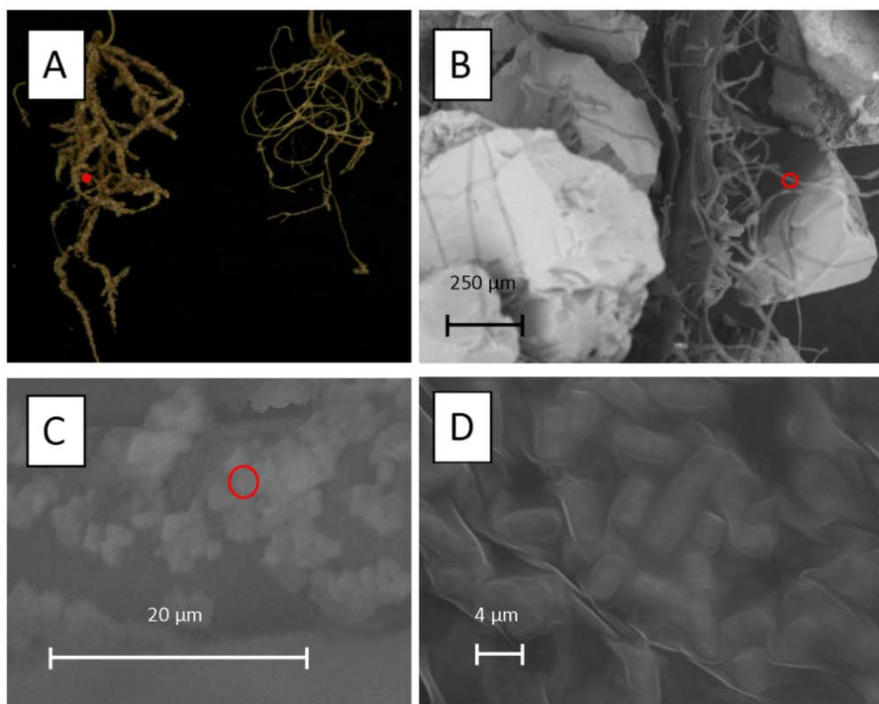

Figure 7. Formation of sand soil mulch and biofilm on root hairs of winter wheat by *B. thuringiensis* AZP2; Scanning electron micrographs were made of AZP2-treated wheat root systems after 10-day drought stress and show sand mulch **(A, B)** and bacterial biofilm formation on root hair **(C, D)**. Significantly more soil mulch is attached to the AZP2 treated plant (A, left) in comparison to control (A, right). Red circles indicate the areas magnified

5.2 Rhizobacterial treatment improved photosynthesis and antioxidant defense response (manuscript I)

Total net assimilation rate and stomatal conductance were monitored for both bacterial treated or not treated wheat seedlings every other day from stress initiation (zero time) till 10 days (Fig. 8). A steady decline in net assimilation rate and stomatal conductance was recorded in all stressed wheat plants. Both photosynthetic parameters were almost totally inhibited in non-bacterial treated wheat seedlings within 8 days since withholding water. *B. thuringiensis* AZP2-traeted plants exhibited significantly higher net assimilation rate compared to the non-inoculated controls. A regression analysis demonstrated a very strong positive correlation between net assimilation rate and plant survivorship through the drought-stress period ($r^2 = 0.95$, $P<0.001$). Due to the importance of antioxidant enzymes in ROS scavenging, the activities of MDHAR, GR, CAT and SOD were studied after 8 days in drought-stressed and well-watered plants. The relative activity of MDHAR was increased by drought stress and AZP2 colonization. About 2 fold increase in GR activity was recorded in AZP2

treated wheat seedlings under drought stress. Both SOD and CAT activities were significantly increased by AZP2 under drought stress (table 2: manuscript I). Reduction in plant's photosynthetic capacity is a major consequence of drought stress (Farooq 2009). The effect is either due to stomatal limitations in response to decreasing stomatal conductance and/or to non-stomatal limitations as a result of less optimal conditions for the photosynthesis process including chlorophyll oxidation and decline in Rubisco activity (Bota et al. 2004). In the present study, AZP2 treatments reversed the inhibitory effect of drought stress on wheat seedlings photosynthetic activity which was evident by higher total net assimilation rate and stomatal conductance. The effect of AZP2 treatment on photosynthesis was connected to upregulating in several antioxidant enzymes which suggest stronger plant response to oxidative stress (Loggini et al. 1999; Ali and Ashraf 2011).

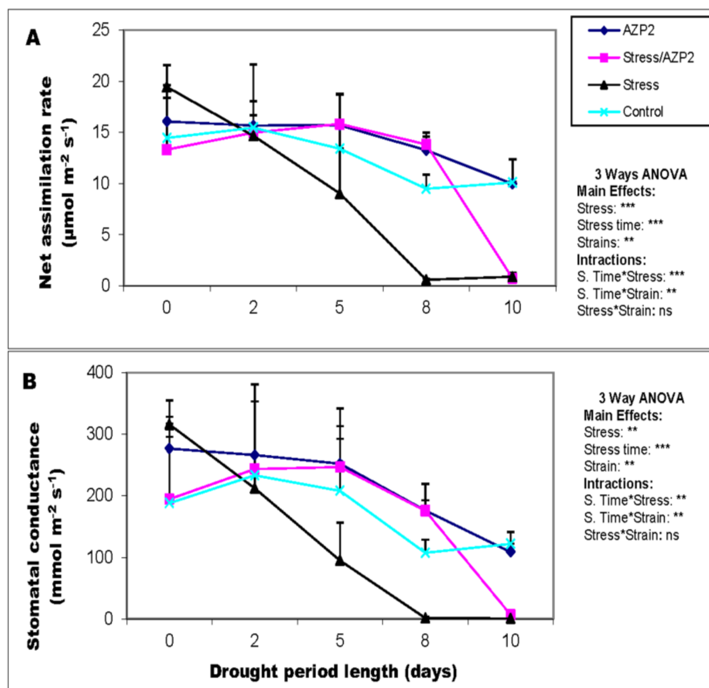

Figure 8. Net assimilation rate **(A)** and stomatal conductance **(B)** of *B. thuringiensis* AZP2-trated wheat seedlings under drought stress. The data are shown for plants grown for 0, 2, 5, 8 and 10 days without water. The error bars indicate standard deviation for three biological replicates. Statistical analysis is based on three-way ANOVA with stress, strains and stress exposure time as factors. ***, ** and ns, indicate highly significant, significant or non-significant effects for the tested factor at $P \leq 0.05$

5.3 Reduced VOCs emission in response to bacterially induced plant drought stress tolerance (manuscript I)

VOCs profiling using GC-MS analysis showed that seven terpenoid and benzenoid compounds were emitted from wheat leaves including α-pinene, limonene, para-cymene, α- phellandrene and camphene. Among the compounds, benzaldehyde, β-pinene and geranyl acetone were most responsive to drought stress and exhibited greatest differences among the treatments. Benzaldehyde emissions increased with increasing the drought stress period. The emission reached a maximum value when non-primed wheat plants were grown without water for 8 days. On the other hand, *B. thuringiensis* AZP2-trated stressed plants showed modest benzaldehyde emission compared to the non-treated stressed seedlings (Fig. 9A). The emission of β-pinene significantly increased within 2 days of drought stress initiation in un-treated AZP2 wheat seedlings. Levels of β-pinene emission remained stable by increasing drought stress exposure time in un-treated AZP2 wheat seedlings. Significantly lower β-pinene emission levels were detected in AZP2 treated drought stressed wheat seedlings at all-time points (Fig. 9B). Drought stress also resulted in higher emission levels of geranyl acetone where pronounced levels were detected within 5 days from water withholding and kept raising with increasing stress exposure time. As with the other VOCs geranyl acetone levels were significantly reduced in AZP2 treated drought stressed wheat seedlings (Fig. 9C). Increasing VOCs emission was always correlated with decreased survival and less efficient photosynthesis in drought stressed plants. It has been demonstrated that plants may lose up to 10% (exceptionally up to 50%) of the carbon fixed by photosynthesis as cost for VOCs emission under stressful conditions (Loreto and Schnitzler 2010; Sharkey and Loreto 1993). Hence, the present results suggest that the reduced VOCs emission in the AZP2 treated seedlings was connected with lower physiological cost under drought stress conditions which in turn was reflected in more efficient photosynthesis and potentially contributing to greater productivity under stress conditions. The present results provides evidences connecting stress severity with VOCs emission and suggest that monitoring the emission of β-pinene and geranyl acetone could be an attractive non-invasive strategy to detect drought stress at very early stage which offer a great opportunity to manage stress before the plant sustain any irreversible damages.

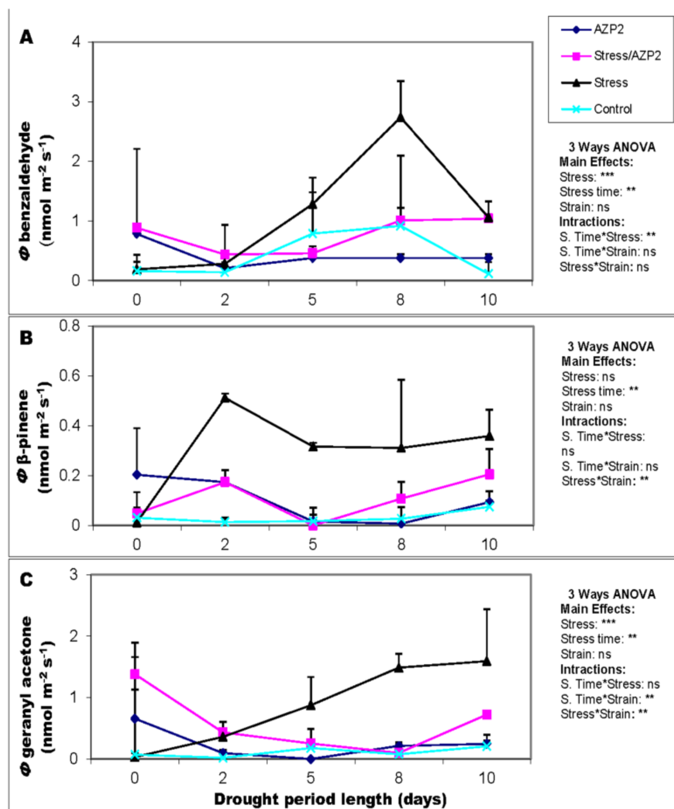

Figure 9. Temporal variations in the emission rates of some benzenoids and terpenoids emitted by wheat plants; Benzaldehyde **(A)**, β-pinene **(B)** and geranyl acetone **(C)** emission rates from leaves of drought-stressed (0, 2, 5, 8 and 10 days without water) wheat plants after inoculation with *B. thuringiensis* AZP2 are demonstrated. The error bars indicate +SE for three biological replicates. Statistical analysis is based on three-way ANOVA with stress, strains and stress exposure time as factors. ***, ** and ns, indicate highly significant, significant or non-significant effects for the tested factor at $P \leq 0.05$

5.4 Inactivation of *P. polymyxa* A26 sfp-type PPTase results in loss of non-ribosomal peptide production and enhanced biofilm formation (manuscript II)

The analysis of the *P. polymyxa* A26 genome shows that it contains a single sfp-type PPTase. The gene shares 97, 92 and 91% homology with *P. polymyxa* E681, SC2 and M1 PPTase genes respectively. The sfp-type PPTase gene was disrupted leading to the *P. polymyxa* A26 sfp-type PPTase mutant strain A26*Δsfp*. The mutant strain was also complemented with a fully functional *sfp* gene. All three strains were subjected to several phenotypic and chemical analyses (manuscript II, Fig. 2). LC-MS analysis suggests that A26 is able to

produce fusaricidins of molecular weights 883, 897, 911, 931, 947 and 961 Da and a polymyxin of molecular weight 1,094 Da. Further, neither fusaricidins nor polymyxin was detected in A26*Δsfp* by MALDI-TOF MS. In order to confirm that the inability of A26 to biosynthesis fusaricidins and polymyxin was due to the *sfp* inactivation, the complemented strain was subjected to MALDI-TOF MS analysis as well which revealed that production of both fusaricidins and polymyxin was restored in the complemented strain. Several microorganisms produce NRPs and PKs, which are biologically active products of the reactions catalysed by NRPSs and PKSs (Mongkolthanaruk 2012). Activity of sfp type PPTase is crucial for the activation of NRPS and PKS (Sunbul et al. 2009; Beld et al. 2014). Hence, the presents results indicate that, similar to NRPS/PKS synthesis in various beneficial and pathogenic bacteria, *P. polymyxa* A26 is dependent on the presence of a single functional sfp- type PPTase. The first report on *sfp* function in *B. subtilis* was published by Nakano et al. (1988). They reported that *B. subtilis* 168 was not able to produce iturin, fengycin and surfactin due to a frameshift mutation in *sfp* gene coding for 4-phosphopantetheinyl transferase which is responsible for conversion of NRPSs to their active holoforms.
Various assays were used to evaluate biofilm formation of the wild type, A26Δ*sfp* and complemented strain (Fig. 10). Generally, results revealed that A26Δ*sfp* had remarkably enhanced biofilm formation compared to the wild type and complemented strain. For instance, the deletion of the sfp-type PPTase gene resulted in about 40% higher biofilm formation based on pellicle weight assay (Fig. 10A). Further, the enhanced biofilm formation in A26Δ*sfp* was confirmed using electron scanning microscopy (SEM) which showed that significantly more porous extracellular matrix is formed by A26Δ*sfp* when colonizing wheat root tips (Fig. 10B). Another biofilm assay was performed with the A26 and A26Δ*sfp* inoculated plant roots grown in sand, washed and left in 5 ml water on Petri plates. Biofilm formation was observed to significantly enhance root hair growth of A26Δ*sfp* inoculated plants (Fig. 10C). Additional quantitative estimation of biofilm formation was performed based on amount of soil attached to roots (manuscript II, Table 1). Two times more soil was attached to the wheat seedling roots inoculated with A26Δ*sfp* (manuscript II, Table 1). Although *P. polymyxa* is one of the best rhizosphere biofilm formers, the mechanism of biofilm formation remains poorly explored (Raza et al. 2009). However, considerable information is available for biofilm formation mechanisms in *B. subtilis* which include a connection between sfp activity and biofilm formation (McLoon et al. 2011; Lopez et al. 2009; Vlamakis et al. 2013). It is generally known that the sfp inactivation impairs *B. subtilis* biofilm formation and for that reason root colonization is also impaired (Chen et al. 2009; Zeriouh et al 2014). Still, that was not the case in *P. polymyxa* A26 where the present study provides evidence suggesting that *sfp* inactivation enhance biofilm formation substantially in A26.

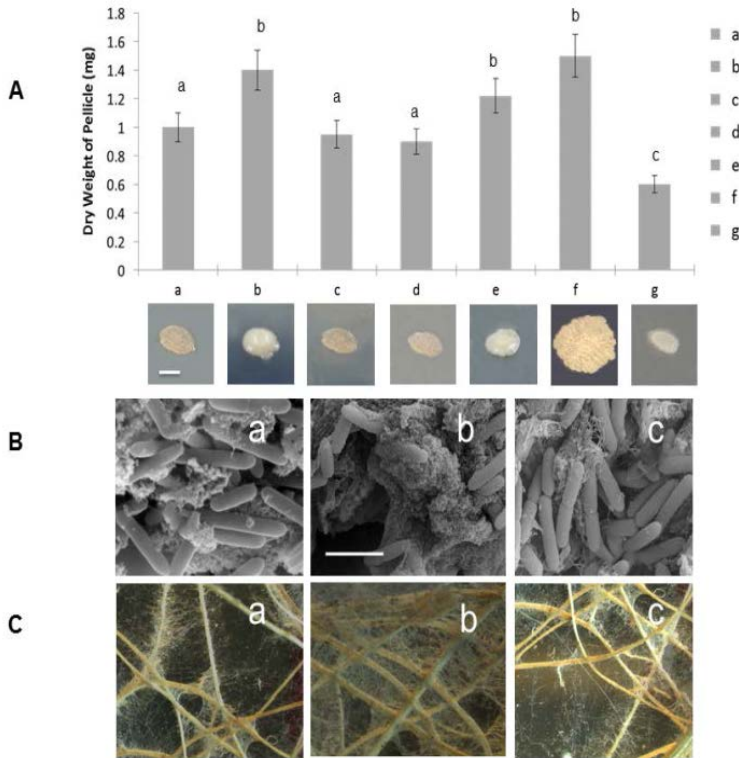

Figure 10. Biofilm and root hair formation analysis of *P. polymyxa* sfp- type PPTase mutants. **A.** *In vitro* biofilm formation of A26 (a), A26*Δsfp* (b) A26*Δsfp* pHPS9*sfp* (c), E681 (d), E681*Δsfp* (e), compared to *B. subtilis* 3610 (f), and *3610Δsfp* (g). Colony phenotypes of the strains are shown. Colonies were grown on PDA agar for 4 days at 30C. The scale bar represents 2 mm. **B.** Scanning electron microscopic images of A26 (a), A26*Δsfp* (b) A26*Δsfp* pHPS9*sfp* (c) inoculated wheat roots. Significantly more biofilm compared to A26 is formed on the roots inoculated with A26*Δsfp*; complementation of the strain restores the wild type biofilm formation level. The scale bar represents 3 um. **C.** Light microscopic images of biofilm and root hair formation on wheat roots inoculated A26 (a), A26*Δsfp* (b) and A26*Δsfp* pHPS9*sfp* (c). Note that compared to A26 significantly more root hair and biofilm are formed on wheat roots inoculated with A26*Δsfp*. Complementation of A26*Δsfp* restores the wild type of root hair and biofilm formation levels

5.5 Inactivation of *P. polymyxa A26* sfp-type PPTase improved A26 ability to induce wheat drought stress tolerance (manuscript II)

Comparative effects of the wild type A26 and A26Δ*sfp* on wheat water use efficiency and relative water content were studied. The mutant strain significantly increases seed germination, root hair length, density, amount of soil attached to roots and plant water use efficiency (manuscript II, Table 1). 100 % of the A26Δ*sfp* treated seeds germinated under normal and stress

conditions. A26Δ*sfp* inoculation resulted in 4.5 and 2.5 times improvements in root hair length and density, respectively. This is about twice the improvements obtained with the wild-type strain. Both the wild type and mutant strains improved the relative water contents in drought stressed wheat. However, about 2 fold higher relative water contents was recorded in the A26Δ*sfp* treated wheat after 6 days without water (Fig. 11). Moreover, A26Δ*sfp* inoculated seedlings showed significantly higher antioxidant responses compared to their A26 treated counterparts under drought stress (manuscript II, Table 1).

It is well known that bacterial capacity to form biofilms on the root is required for colonization and biocontrol effect (Timmusk and Nevo 2011; Timmusk et al. 2009; Zeriouh et al. 2014). However, in the present study wheat roots colonization by the wild type mutant and complemented mutant did not differ significantly, hence, a connection between drought stress tolerance enhancement and colonization due to enhanced biofilm formation in A26Δ*sfp* could not be supported. Still, biofilm can be involved in many different processes leading to better plant drought stress tolerance. For instance, bacterial biofilms are comprised of cells and extracellular matrix and form layers around a root hair (Fig. 10). The dense biofilm matrix limits diffusion of ACC deaminase and biologically active compounds secreted by bacteria, and these are therefore concentrated for plant uptake. Moreover, biofilms may act as soil adhesive which in turn helps to reserve soil moisture (Donlan 2002). Such findings are also well supported in manuscript I. On the other hand, the present study suggests that A26 sfp-type PPTase mediated NRPS/PKS driven compounds induce negative effects in wheat seedlings and affect plant drought tolerance (manuscript II, Fig. 6). It has been previously reported that plant growth promoting *P. polymyxa* strains may cause mild negative effects on plant root tips (Timmusk et al. 2005; Timmusk and Wagner 1999). It has been suggested previously that microbial hydrolytic enzymes and auxins may be responsible for the deleterious effects (Timmusk et al. 2005; Timmusk and Wagner 1999; Ludwig-Muller 2015). However the present study suggests that NRP/PK compounds produced by *P. polymyxa* may be the primary reason for its temporary mild deleterious influence on wheat roots (manuscript II, Fig. 5).

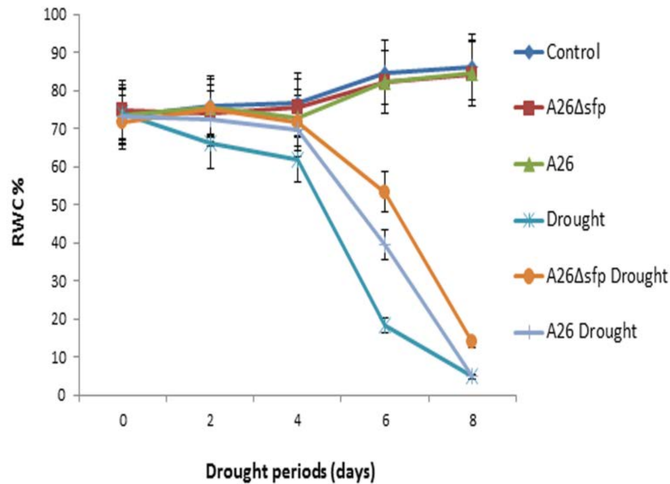

Figure 11. Relative water content (RWC) of *P. polymyxa* A26Δ*sfp*, A26 and untreated wheat under drought and well watered regime

5.6 *P. polymyxa* antagonized FHB causing agents *F. culmorum* and *F. graminearum* (manuscript III)

The antagonistic ability of two *P. polymyxa* strains (A26 and E1) against the FHB causing pathogens *F. culmorum* and *F. graminearum* was assayed on King's agar plates (manuscript III, Table 2). Results showed that both *P. polymyxa* A26 and E1 were very potent antagonistic agents against both *F. culmorum* and *F. graminearum*. However, *P. polymyxa* A26 showed superior ability to antagonise both pathogens with 17 mm inhibition zone for *F. graminearum* and 16 mm in case of *F. culmorum*. The ability of other *P. polymyxa* strains to antagonise *Fusarium* have been previously reported by He et al (2009) when they used several *P. polymyxa* strains to inhibit *F. graminearum* growth aiming to control the progress of FHB in wheat. They reported that *P. polymyxa* W1-14-3 and C1-8-B had higher antagonistic ability and they suggested that the strain characters may play a significant role in the antagonistic activity.

5.7 Inactivation of *P. polymyxa* A26 sfp-type PPTase impaired A26 ability to antagonise *F. culmorum* and *F. graminearum* (manuscript II and III)

Sfp-type-PPTase inactivation resulted in a total loss of the *P. polymyxa* A26 ability to antagonise either *F. culmorum* or *F. graminearum* on agar plates. The effect was very remarkable with no inhibition detected for either pathogen. Further, to confirm that the loss of the antagonism trait was triggered by *sfp*

inactivation the antagonistic ability of A26 strain complemented with *sfp* was also verified and found to be similar to the wild type strain (Fig. 12; Table 2, manuscript III). The compromised ability of A26Δ*sfp* to antagonise both pathogens was expected considering that no NRPS/ PKS lipopeptide antibiotics are produced by the *sfp* inactivated strain (manuscript II). Hence, the results confirm the role of sfp-type-PPTase mediated compounds in the antagonism process (Mootz et al. 2001).

Figure 12. Inhibitory effect of wild type A26 **(a)**, A26Δ*sfp* **(b)** and complemented strain A26Δ*sfp* pHPS9*sfp* **(c)** against *F. graminearum*; Note that the zone of antagonism observed with wild type has disappeared with mutant and is fully restored with complemented strain

5.8 *P. polymyxa* A26 antagonism against *F. culmorum* and *F. graminearum* in wheat grains (manuscript III)

Plate assays have been extensively used to study microbial antagonism (Pereira et al 2013). It's simple, rapid and offer good visualization for the antagonism effects (Nielsen and Sorensen 1996). However, plate assays are very artificial and the result is dependent on the growth medium (Whipps 2001; Yang et al. 2012). In the present study a gnotobiotic system on wheat kernels was developed in order to study *P. polymyxa* A26 antagonism against *F. culmorum* and *F. graminearum*. Compared to plate assays, the system provides a surface for colonization as well as nutrition source that might be used by both the pathogen and the biocontrol agent (BCA) under field conditions. It also allows qPCR monitoring of pathogen, BCA A26 and A26Δ*sfp* as well as mycotoxin production. Visual inspection of wheat grains over the experimental period revealed increased amounts of *F. culmorum* and *F. graminearum* mycelia in the pathogen control treatment (Fig. 13). Both *F. culmorum* and *F. graminearum* were completely antagonized by *P. polymyxa* A26 by day 5,

which didn't change during the course of the 15 day studies (Fig. 13A and B). The visual observations were confirmed by quantification of pathogen DNA. Only trace amounts of *F. culmorum* and *F. graminearum* DNA was detected in the *P. polymyxa* A26 treated wheat grains, while in the absence of the bacteria up to 260 and 382 ng pathogen DNA/ ng wheat DNA were detected after 15 days for *F. culmorum* and *F. graminearum*, respectively (manuscript III, Table 3). Further, the successful antagonism for both pathogens were confirmed by not detecting any significant levels of their associated mycotoxins DON and ZEA on wheat grains treated with *P. polymyxa* A26 (manuscript III, Table 4). Biological control of *Fusarium* has been achieved using a variety of antagonistic microbes before in several studies with a variable success. For instance, Franco et al (2011) reported the growth inhibition of *F. graminearum* using several lactic acid bacteria. Moreover, Dal Bello et al (2002) studied the antagonistic efficiency of 52 plant growth promoting bacteria strains isolated from wheat rhizosphere against *F. graminearum* and reported that several *Bacillus* isolates were the most promising candidates specially *B. cereus* in inhibiting *F. graminearum*. The ability of the bacteria to inhibit fungal growth could be due to antagonism between the pathogen and the BCA which could be attributed to the competition between both organisms on the available resources or the ability of the bacteria to produce active antifungal compounds (Franco et al 2011 and Dogi et al 2013).

5.9 *P. polymyxa* A26Δ*sfp* antagonism against *F. culmorum* and *F. graminearum* in wheat grains (manuscript III)

Considerable *F. graminearum* mycelia were clearly visible on wheat grains treated with A26Δ*sfp* at 15 days post infection (Fig. 13A). Further, significant levels of *F. graminearum* DNA (62.66 ng fungal DNA/ng wheat DNA after 15 days of fungal infection) in the grains treated with A26Δ*sfp* was also detected (manuscript III, table 3). Moreover, significant levels of both mycotoxins DON (0.3-1.5 mg/kg) and ZEA (0.24-0.41) were recorded in A26Δ*sfp* treated wheat grains after *F. graminearum* infection (manuscript III, Table 4). On the other hand, unlike what was seen on the plate assays, the sfp-type-PPTase inactivation seems to play only a very minor role in the antagonistic effect of *P. polymyxa* A26 in wheat grains against *F. culmorum*. This suggests, that the antagonistic effect was related to the pathogen targeted. Hence, no significant difference was observed in the effect of A26Δ*sfp* and the wild-type strain against *F. culmorum* (Fig. 13B). The inability to detect any significant amount of *F. culmorum* DNA in A26Δ*sfp* treated wheat grains was confirmed by qPCR (manuscript III, Table 3). Also, no detectable levels of neither, DON nor ZEA were found in A26Δ*sfp* treated seeds infected with *F. culmorum* (manuscript III, Table 4).

Production of bioactive compounds is commonly employed by bacteria to antagonise pathogens (Cawoy et al 2014). For instance, in *B. subtilis,* the most frequently reported antagonism mechanisms are connected to nonribosomally produced cyclic lipopeptides (Cawoy et al 2014). Lipopeptides which are amphiphilic molecules with an amino or hydroxy-fatty acid integrated into a peptide moiety, interact with the biological membranes of microbial pathogens, resulting in cell leakage and death (Zeriouh et al. 2011). An examination of the A26 genome indicates that production of polymyxins, fusaricidins as well as quite a number of potentially new nonribosomal lipopeptides/antibiotics are potentially mediated by its sfp-type PPTase. Moreover, the present study provides evidences confirming that A26Δ*sfp* was not able to synthesise polymyxins and fusaricidins (manuscript II). Such findings strongly suggest that NRPS/PKS bioactive compounds driven by sfp such as polymyxins and fusaricidins (manuscript II) are potentially mediating the A26 antagonism against *F. graminearum*. However, the fact that A26Δ*sfp* successfully antagonized *F. culmorum* in wheat grains suggest the involvement of other mechanisms. Hence, the present study attempted to explore such possibility by treating wheat grains with A26 and A26Δ*sfp* culture filtrates. A cell free culture supernatant assay showed that the culture filtrates of A26Δ*sfp* were unable to antagonise *F. culmorum* in wheat grains (Fig. 13C). This suggests that niche exclusion, i.e. antagonist biofilm occupation of the pathogen colonization sites, could also be responsible for the observed antagonism as previously reported by Timmusk et al. (2009); Haggag and Timmusk (2008). In this connection, A26Δ*sfp* has enhanced biofilm formation (40% higher compared to wild type) (manuscript II). Microbial biofilms are comprised of cells and extracellular matrix and can produce a protective layer around infection sites. The dense biofilm matrix limits diffusion of compounds secreted by bacteria and these are therefore concentrated at pathogen infection sites of action.

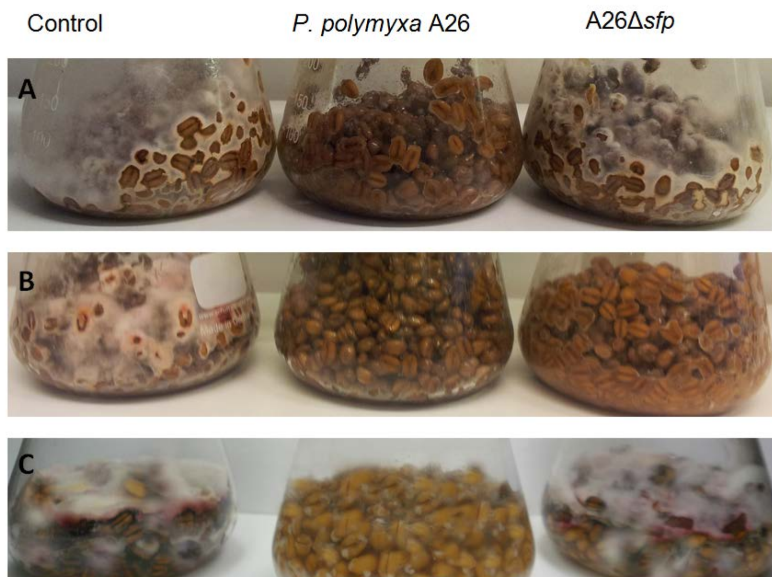

Figure 13. F. graminearum and *F. culmorum* antagonism in wheat kernel assay; *F. graminearum* growth in wheat grains inoculated with *P. polymyxa* A26 orA26Δ*sfp* **(A)**, *F. culmorum* inoculated with *P. polymyxa* A26, A26Δ*sfp* **(B)** and *F. culmorum* treated with *P. polymyxa* A26, A26Δ*sfp* culture filtrates **(C)** after 15 days incubation

5.10 Monitoring of the antagonistic agents (manuscript III)

The versatility of the gnotobiotic system on wheat kernels also allows simultaneous monitoring of the antagonistic agents as well. Hence, bacterial DNA was detected at all-time points after inoculation (Fig. 14). In most cases, increasing the incubation time did not lead to a significant effect on the detected DNA levels. The only exception was the detection of significantly higher *P. polymyxa* A26 DNA levels in *F. graminearum* infected wheat grains (12.12 pg bacterial DNA/ 100 ng plant DNA after 15 days from infection) compared to (4.53 pg bacterial DNA/ 100 ng plant DNA) in *F. culmorum* infected wheat grains (Fig. 14A and B). By using specific PCR primers (manuscript III, Table 1) it was always possible to differentiate between the wild type and the mutant strain at all-time points which confirmed the stability of *sfp* inactivation during the experiment (Fig. 14C).

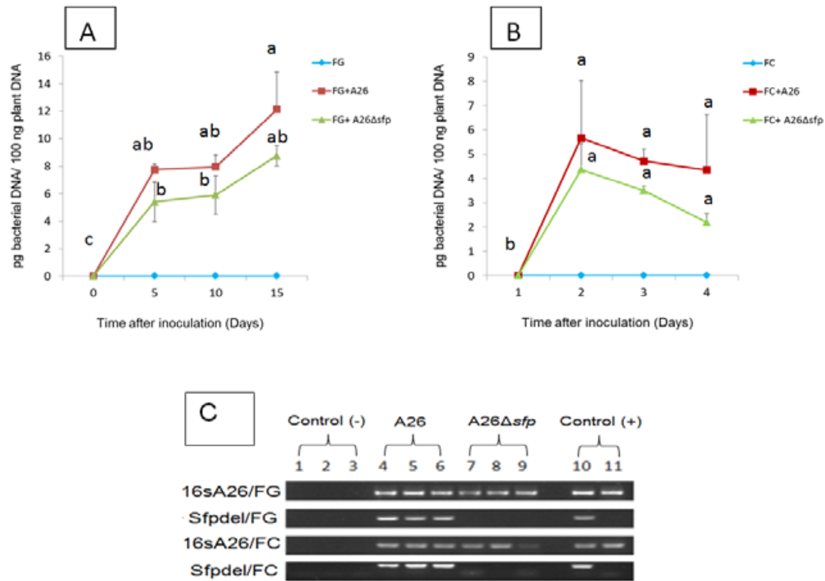

Figure 14. A26 and A26Δ*sfp* quantification in wheat kernel assay. qPCR quantification of bacterial DNA extracted from wheat grains inoculated with A26 and A26Δ*sfp* as well as un inoculated wheat grains after 5, 10 and 15 days **(A)** *F. graminearum* and **(B)** *F. culmorum*; (pg bacterial DNA/ 100 ng plant DNA). Data shown as a means of two experiments; Bars represents standard deviation; Different letters indicate statistically significant differences ($P \leq 0.01$) based on the LSD test. **C.** PCR analysis for bacterial DNA using 16S A26 primers (identifying both A26 and A26Δ*sfp*) and sfpdel primers (identifying only A26). DNA extract from pure cultures of A26 and A26Δ*sfp* was used as a positive control while DNA extracted from untreated wheat grains was used as a negative control

6 Conclusions

Rhizobacteria isolated from harsh environments are potent drought stress tolerance inducers. That was evident in wheat seedlings treated with AZP2 strain isolated from ponderosa pine roots grown on gneiss rock at Mt. Lemmon, AZ, USA. AZP2 treatment altered multiple physiological responses in drought stressed wheat including higher net assimilation and stomatal conductance, stronger antioxidant defense response as well as reduced emission of stress volatiles. That was correlated with improved wheat biomass and survival under drought stress conditions. The beneficial effects of AZP2 seem to be connected with its ability to protect drought stressed wheat roots through biofilm formation which resulted in better utilization of soil water contents and overall improved drought stress tolerance in wheat.

The emission of stress volatiles such as β-pinene, geranyl acetone and benzaldehyde was found to be correlated with drought stress severity in wheat which could be employed as a non-invasive approach to monitor stress responses at early stages before any visible un-reversible distractive stress phenotypes appearance.

An active sfp-type-PPTase is crucial for biosynthesis of NRP/PK metabolites such as fusaricidins and polymyxins in *P. polymyxa* A26. The activity of *sfp* is also negatively involved in A26 biofilm formation. The superior ability of A26Δ*sfp* to produce biofilm resulted in enhanced bacterial abilities to induce drought stress tolerance in wheat. However, other mechanisms involved in the improved A26Δ*sfp* potential to mediate drought tolerance in wheat may include minimizing plant exposure to *sfp* driven metabolites such as polymyxin B and E that results in deleterious effects on drought stressed wheat and even significantly impair wheat germination.

P. polymyxa A26 is very efficient in antagonising both *F. graminearum* and *F. culmorum in vitro*, and it has the potential to be used as a BCA against FHB and fusarium foot and root rot diseases in wheat. The reduction of *F. culmorum* and *F. graminearum* biomass by *P. polymyxa* A26 was accompanied by a reduction of DON and ZEA contamination in wheat grains. The present study, suggest that dual plate assays alone are not enough to characterise microbial biocontrol potential. The results suggest that synthesis of NRP/PK such as

fusaricidins and polymyxins could be a potential mechanism contributing to the antifungal ability of *P. polymyxa* A26 against *F. graminearum*. However, the involvement of biofilm formation in the antagonistic process is also possible, which was evident in the case of *F. culmorum.*

7 Future perspectives

The results provided in this thesis (manuscript I) suggests that rhizobacteria could be harnessed to manage abiotic and biotic stress consequences in wheat cultivation. The results further suggest that rhizobacteria isolated from harsh environments are likely superior for such purposes. More strains isolated from different habitats needs to be tested to confirm the findings.

We suggest plant stress volatiles as a potential strategy to monitor drought stress severity. However, the sensitivity of such technique needs to be adapted under field conditions. Hence, we would like to develop the method further to be able to discriminate between different stresses in natural conditions.

The successful employment of rhizobacterial isolates to improve plant stress tolerance and antagonise plant pathogens requires deep understanding of their mechanisms. The present study shows that a single gene deletion has a great impact on the bacterial activity (manuscript II, III). For instance, the significant enhancement in *P. polymyxa* A26Δ*sfp* to produce biofilm is interesting and calls for extensive study on the molecular mechanisms.

Another factor contributing to the success of rhizobacteria in the field will be its ability to colonize the host plant which in turn depends on its fate in the rhizosphere. The development of reliable and sensitive tracking approaches will be crucial if we want to know the fate of the introduced bacteria in the rhizosphere. The available methods have mostly relied on molecular and microscopic assays. Hence we believe that more robust but sensitive assays are needed in the future.

Introduction of beneficial microorganisms by plant and soil inoculation offers a convenient and promising solution for sustainable agriculture. We believe that using beneficial microbes should be combined with sustainable agriculture practices which require several studies to understand the optimal conditions needed for the microbes.

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