Bioinformatic and biotechnological study of biocontrol *Bacillus* suitable for crop protection and plant growth promotion

by

Liberata Mwita

Student number: 10661558

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School of Biological Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

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SUBMISSION DECLARATION

I, Liberata Mwita, declare that the thesis, which I hereby submit for the degree (Philosophiae Doctor) in the Department of Biochemistry, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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DEPARTMENT OF BIOCHEMISTRY

Full name: Liberata Mwita

Student number: 10661558

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SUMMARY

The main focus of this research was to study in detail the selected plant growth promoting bacteria (PGPR) from *Bacillus* group. PGPR have been described as possible solution to the negative effects of chemicals fertilizers experienced by human beings, other living organisms and the environment through reducing or replacing the use of chemical fertilizers and pesticides. The work aimed at finding plant promotion and plant protection activity of selected PGPR species. Different bioassays were performed in the laboratory and greenhouse to find if the selected bacteria promote plant growth and protect plants through inhibiting pathogens, or protect them during drought conditions. RNA sequencing technology was carried out to study gene expression regulation of a *Bacillus atrophaeus* UCMB-5137. The selected bacteria showed some plant growth promotion and protection abilities. *Bacillus atrophaeus* UCMB-5137 and *Bacillus amyloliquefaciens ssp. plantarum* UCMB-5007 actively promoted wheat growth. All strains showed some pathogen inhibition, while some strains induced drought tolerance abilities. Genes responsible for plant colonization of the *Bacillus atrophaeus* UCMB-5137 and transcription factors regulating gene expression were identified. This work will create a basis for the possible development of industrially potential biofertilizers and biopesticides for agricultural practices.

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LIST OF ABBREVIATIONS

PGPR	Plant Growth Promoting Bacteria
UCMB	Ukrainian Collection of Microorganisms (section Bacteria)
RNASeq	Ribonucleic acid sequencing technique
B.	*Bacillus*
MS	Murashige and Skoog broth
MSA	Murashige and Skoog Agar
A.	*Arabidopsis*
RE	Root exudates
B. napus	*Brassica napus*
LB	Luria broth

Chapter 1: LITERATURE REVIEW

Food is a basic human need required for the survival of each being. Plants are the main source of food to human beings and it is therefore important to produce sufficient food and protect plants from different factors that may threaten plant growth. Chemical fertilizers and pesticides are usually applied to promote plant growth and protect them from pests (Aktar et al., 2009, Sivasakthi et al., 2014). Application of chemical fertilizers and pesticides nevertheless brings about negative effects to human beings, other life forms and the environment (Aktar et al., 2009). Plant Growth Promoting Bacteria (PGPR) use in promoting and protecting plants offers several advantages over the application of chemicals (Adesemoye and Kloepper, 2009, Sivasakthi et al., 2013).. These and other factors sparked our interest in the study of PGPR. The work particularly focused on PGPR from the *Bacillus* group, which provides many advantages over other PGPR (Fan et al., 2013). *Bacillus atrophaeus* UCMB-5137's ability of promoting plant growth (Lapa and Reva, 2005) unlike other soil dwellers and availability of its complete genome sequence (Chan et al., 2013) raised our interest of studying this strain in detail, using a combination of biotechnological and bioinformatics techniques. Biotechnology is a technology that uses living systems and organisms to make or modify products while bioinformatics addresses biological problems usingcomputational techniques (En.wikipedia.org, 2017). In this study, further tests on the plant growth promoting ability of the *Bacillus atrophaeus* UCMB-5137 were performed; the results were compared to other strains of the *B. subtilis* group, after that, plant colonization behavior of the strain was studied.

1.1 Overview of the application of plant growth promoting bacteria in agriculture

Plant growth promoting rhizobacteria (PGPR) are soil bacteria inhabiting around or on the plant root surface, they are directly or indirectly involved in promoting plant growth (Ahemad and Kibret, 2013). They include free living, those that form symbiotic relationship with the plant, endophytes and cyanobacteria (Glick, 2012). PGPR is divided into extracellular PGPR and intracellular PGPR based on their location in the rhizosphere. Extracellular PGPR (ePGPR) are found in the rhizosphere, on the rhizoplane or in spaces between the cells of the root cortex, while intracellular PGPR (iPGPR) exist inside root cells particularly in nodules (Gray and Smith, 2005). Rhizoplane is

the root surface (Nihorimbere et al., 2011). As roots grow, they cast off dead cells and must navigate around soil particles, making the rhizoplane highly irregular (Knief et al., 2011). The rhizoplane is the site of water and nutrient uptake and the release of exudates into the soil (Soilhealth.com, 2017).Rhizosphere is the soil immediately surrounding plant roots, where the roots influence soil bacteria by producing multiple energy rich and bioactive compounds and in return, the plant cells are influenced by bacterial metabolites. Plants create preferable conditions for bacteria contributing most to their fitness by releasing organic compounds. At the same time, the plants synthesize several antimicrobial compounds to control plant pathogens and competitors of the beneficial microflora (THCFarmer Community, 2017). In the rhizosphere, large quantities of metabolites which act as chemical signals for motile bacteria to move to the root surface are released from living root hairs or the fibrous root systems (Nihorimbere et al., 2011). Plants coexist with a large number of microorganisms; nevertheless, the bacteria are most abundant hence influencing plant physiology (THCFarmer Community, 2017). Microorganisms compete for water, nutrients and space in the rhizosphere (Nihorimbere et al., 2011). Rhizosphere microorganisms act as intermediate linkers between the plant and the soil (Prashar et al., 2013). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as possible biofertilizers.

Mechanisms by which PGPR species exert their beneficial effects on plants can be very diverse. They are divided mainly into direct and indirect mechanisms. Direct mechanism is the one that acts by directly providing nutrients or growth regulators while indirect mechanism is the one that protects plants from infections or environmental stress (Sivasakthi et al., 2014, Goswami et al., 2016).

Direct mechanisms include: (i) Facilitating resource acquisition: providing plants with lacking nutrients, for example through sequestering iron, through the solubilization of inorganic phosphorous and the mineralization of organic phosphorous, through nitrogen fixation (Glick, 2012), and also through the production of volatile organic compounds (Ping and Boland, 2004). Plants require different resources for their growth and survival, some are major and some are minor. Nitrogen is one of the major resources; it is needed in the synthesis of basic building blocks of animals, plants and other organisms such as nucleotides and amino acids. Phosphorous is a major essential macronutrient for biological growth and development while iron is an essential growth element for all living organisms (Sivasakthi et al., 2014). Plant growth is suboptimal when

agricultural soils lack any of these compounds (Glick, 2012). (ii) Modulating phytohormone levels: plants need hormones for their growth and development; in addition, hormones help plants respond to stressful conditions and pollutants (Glick, 2012, Pérezet al., 2014).

Indirect mechanisms consist of: (i) production of antibiotics that prevent proliferation of plant pathogens, lytic enzymes which lyse cell wall portions of the pathogenic fungi, and siderophores which prevent phytopathogens from acquiring sufficient amount of iron and thus unable to proliferate. (ii) Competition: PGPR rapidly colonize soil surface and use available nutrients making pathogen growth difficult. (iii) Induced systemic resistance: help the plant react faster and strongly to pathogen attack by inducing defense mechanisms (Glick, 2012; Beneduzi et al., 2012), (iv) Interfere with the quorum sensing system (Pérezet al., 2014).

Regardless of the mechanism(s) used for plant growth promotion, PGPR must first colonize the rhizosphere (around the roots), the rhizoplane (root surface) or the root itself (within root tissue) (Singh et al., 2011) in order to bring the desired effect. The growth responses of plants to PGPR differ from strain, crop, variety and site specificity, also mode of application and type of soil (Zahir, 2004). Since each bacterial strain may affect different plants differently (Glick, 2012), it is important to study the benefit of each bacterial strain on a particular plant. An increase in the worldwide population and ongoing environmental damage present a great need of applying PGPR in agriculture, using PGPR is an environmental friendly method needed for sustainable agriculture (Glick, 2012).

1.1.1 The problem of food security around the world and in developing countries

Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food which meets their dietary needs and food preferences for an active and healthy life (Fao.org, 2017). Food security problem affects many countries, particularly developing countries, where hunger challenges 780 out of 795 million people worldwide, everyday (FAO, 2015; IFAD, 2015; WFP, 2015).

Causes of food insecurity in Africa and other third world countries include, drought and extreme weather events, pests, animal or plant diseases, climate change, military conflicts, lack of emergency plans, political instability and rapid population growth (Harvesthelp.org.uk, 2017). Hunger, poverty

and diseases are interlinked and they all result from food insecurity, food insecurity also causes malnutrition (Ilaboya et al., 2012). Other challenges contributing to food insecurity include overreliance on primary agriculture, low fertility soils, minimal use of external farm inputs, pre- and post- harvest crop losses and lack of adequate storage facilities (Ilaboya et al., 2012; FAO, 2015; IFAD, 2015; WFP, 2015).

Great effort has been made by FAO (Food and Agricultural Organization of the United Nations), IFAD (International Fund for Agricultural Development) and WFP (World Food Program) to alleviate and monitor the problem of food security using different strategies. They have almost met the commitment to half the percentage of hungry people at the global level from 1990 to 2015. This was assessed by measuring the undernourishment and prevalence of underweight children less than five years of age. The problem of food security faces many people in developing countries (FAO, 2015; IFAD, 2015; WFP, 2015), it is thus crucial that countries set means to eradicate the problem.

Agriculture provides a major share of national income and export earnings in many developing countries, while ensuring food security, income and employment to a huge proportion of the population (Singh et al., 2011). Application of PGPR in agriculture is important in reducing the problem of food security.

1.1.2 Plant growth promoting bacteria as biofertilizers and biopesticides

Plants are a major source of food to most living organisms, source of medicine, energy (charcoal, biofuels), shelter, income etc. It is therefore, important to preserve them. Each person needs safe and sufficient amount of food. To achieve high yields and better quality plants, agriculture heavily depends on the use of chemical fertilizers and pesticides (Aktar et al., 2009, Sivasakthi et al., 2014). This dependence is associated with problems such as environmental pollution, health hazards, interruption of the natural ecological nutrient cycling and destruction of biological communities (Sivasakthi et al., 2014). Also, the chemical fertilizers are costly (Glick, 2012). The major strategies to combat plant pathogens are chemical pesticides and resistant cultivars (Boriss, 2011), however "agrochemicals do not prevent all diseases, and toxic residues can accumulate in the soil and food chain (Boriss, 2011).

Agrochemicals have a negative effect to life forms and the environment. Production workers, formulators, sprayers, mixers, loaders and agricultural farm workers are highly exposed to pesticides; they may be affected with cardiovascular and respiratory diseases, diabetes and cancer diseases (Aktar et al., 2009). Consumers may be affected when pesticide residues exceed acceptable levels in foods (Aktar et al., 2009). Pesticides are also a threat to other life forms, heavy pesticides treatment can cause a decline of beneficial soil microorganisms (Aktar et al., 2009). Pesticides also affect the environment: can contaminate soil, surface water through run-offs from treated plants and soil as well as ground water contamination, also contamination of air and non-target vegetation (Aktar et al., 2009).

Biofertilizers and biopesticides are produced from microorganisms residing naturally in soil. Promoting sustainable agriculture through the use of microorganisms and reducing the use of pesticides and fertilizers is important in sustaining high production in agriculture (Zahir, 2004). If microorganisms are used adequately, they provide an added dimension in optimizing soil and crop management practices, maintenance of soil quality and biocontrol of plant diseases (Singh et al., 2011). "PGPR are novel and potential tools to provide substantial benefits to agriculture" (Sivasakthi et al., 2013). Some of the benefits derivable from plant–PGPR interactions include: improvements in seed germination rate, root development, shoot and root weights, yield, leaf area, chlorophyll content, hydraulic activity, protein content, and nutrient uptake (Adesemoye and Kloepper, 2009). Application of PGPR in agriculture is an essential alternative to chemicals. PGPR can be used to replace or supplement chemicals.

1.1.2.1 Application of PGPR as biofertilizers and biopesticides in different countries - problems and prospects

"Agriculture on its own can trigger growth in countries with a high share of agriculture in GDP,but even if other sectors of the economy, such as mining or services, were to grow, agriculture, through targeted investments, can become an avenue through which the poor participate in the growth process" (FAO, 2012; IFAD, 2012; WFP, 2012). Empirical evidences suggest that agricultural growth in low-income countries is three times more effective in reducing extreme poverty compared with growth in other sectors. "In the Sub-Saharan Africa, agricultural growth can be 11 times more

effective inreducing poverty than growth in other non-agricultural sectors" (FAO, 2012; IFAD, 2012;WFP, 2012).

In Europe, the use and effectiveness of biofertilizers depends on several factors including climatic conditions in the region, andnature and fertility of the soil. Biofertilizer is mainly applied in cereals and grains, fruits and vegetables, oilseeds and pulses, as well as in plantations (Micromarketmonitor.com, 2017). "The European Union's 'Common Agricultural Policy' promotes bio-based products and offers nearly 30% of the total budget as direct green payment to farmers that adhere to sustainable agricultural practices" (Hexaresearch.com, 2017).

There have been many attempts to use biofertilizers/microbial inoculant materials (various rhizobacteria, yeasts, free-living nitrogen fixers such as azospirillum) in North America, but there wereno consistent results. The exception is the use of humic acids, where growth in the use of these products is rapid. There are several biofertilizer products circulating throughout southeastern North America. These products aremarketed by small and large fertilizer dealers, although no one really has a good feel of how widespread their usage is, as products come and go so rapidly from the market that they are hard to keep up with. When they are used, they are used mainly as a spray adjuvant for liquid fertilizer solutions. The most widespread commercial use appears to be in the turf and organic markets in Florida (Ipni.net, 2017).

The use of biofertilizers has been duplicating yearly in Brazil since the company Embrafós started the production in 1995. Other companies working on biofertilizers in Brazil are Instituto de Fosfato Biológico (IFB) which is the current leader in the business; Biofosfatos do Brasil, and Liderfós Luís (Prochnow and Casarin, 2011). The use of biofertilizers in Southern America (Argentina, Paraguay, Bolivia and Uruguay), is mostly on the soybean crops which are inoculated with Bradyrhizobium. It is estimated that 70% of the area is being inoculated. Other inoculants or biofertilizers are growth promoters such as *Pseudomonas* sp. and *Azospirillum brasilense*. These products are recommended mainly for wheat and maize. Responses were quiet variable but, in responsive situations, yield increases are of 4-9% (Info.ipni.net, 2017).The use of biofertilizers in China has been there since the 1950s, and some products, mainly rhizobium inoculants, have been used in crop production and adopted by farmers. The biofertilizer application area in China has passed 167 million hectares at present (Cheng, 2006).

In India, the government has been promoting the use of biofertilizers in agriculture through the National Project on Development and Use of Biofertilizers (NPDB) and the state governments via subsidization and extension. Commonly explored biofertilizers in India are Rhizobium (RHZ), Azotobacter (AZT), Azospirillum (AZS), Blue Green Algae (BGA),Azolla and Phosphate solubilizing (PSB)/Mobilizing biofertilizer. However, while positive responses have been observed in a wide range of field trials, there is a remarkable inconsistency in responses across crops, regions and other conditions. Even for a given crop, the range of response is quite high (Ghosh, 2003; Nilay et al., 2014).

Adesemoye and Egamberdieva (2013) presented a good piece of work on the use of PGPR in developing economies, highlighting factors that can affect performance and effectiveness of PGPR in different regions, thus hindering wide acceptance and use of PGPR: "variability in colonization efficiency, rhizosphere competence, and field performance", also abiotic factors such as soil type, availability of macro and micro nutrients in the soil, and crop cultivar. Furthermore, the authors mentioned factors hindering agricultural development in many developing economies: socioeconomic, political, cultural, environmental factors, low technological development, bad agricultural methods and policies. They stated that the benefits of resident soil microbes are hardly explored, and when commercial inoculants are used, they are usually not derived from microbes isolated locally and so may not be effective. They also pointed out that environmental stresses such as salt and drought, areas with low levels of soil fertility, arid regions of low rainfall and high evaporative demand, as well as soil-borne pathogens cause inestimable crop losses in many developing regions with more noticeable consequences in Africa.

Several research have shown the necessity of using PGPR in Africa. Inoculation of some *Bacillus* strains isolated from rhizoplane of grasses in South Africa had significant growth promotions on wheat and tomato (Hassen and Labuschagne, 2010). Several studies that were conducted in different African countries showed that PGPR have a high potential in Africa; however commercial inoculants are not yet available (Adesemoye and Egamberdieva, 2013). "There is need for more studies on plant–microbeinteractions and their activities in different regions and ecologies, including stressed environments, for instance, in arid and tropical regions" (Adesemoye and Egamberdieva, 2013).

To benefit from PGPR, commercial products developed from microorganisms isolated and experimented in Africa are needed. "It will be very useful to match correctly the appropriate PGPR

with the right plant and environmental condition to achieve the best results on plant growth" (Pérezet al., 2014). Another recommended solution is of applying biofertilizers in combination with small amounts of chemical fertilizers (Adesemoye and Kloepper, 2009).

1.1.2.2 Species diversity of PGPR - the place of *Bacillus* among PGPR

Studying bacteria diversity in the rhizosphere is complex due to large numbers of bacteria present in such a little space, and their variation in different ecological niches (Barriuso et al., 2008). The rhizosphere bacteria composition varies depending on type and age of the plant, temperature, humidity, stress free or stressful conditions, etc (Barriuso et al., 2008), hence it is recommended to study the diversity in a specific plant or niche, at different stages of the plant growth etc. PGPR species are very diverse; they include Gram positive and Gram negative species. PGPR genera include *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Bacillus*, *Paenibacillus*, *Enterobacter*, *Rhizobium*, *Erwinia* and *Flavobacterium* (Rodriguez and Fraga, 1999; Raj et al., 2005). Molecular methods have eased the identification and characterization of PGPR species. However, in determining genetic diversity among *Bacillus* species, gene expression, biochemical and morphological aspects must be considered in addition to molecular techniques (Alina et al., 2015).

Among the diverse bacteria identified as PGPR, *Bacilli* and *Pseudomonas* are the predominant ones (Podile and Kishore, 2007) and the most studied. The genus *Bacillus* belongs to the phylum Firmicutes which consists of rod-shaped, endospore forming bacteria (Rooney et al., 2009). They are Gram positives and can use various nutritional substrates (Alina et al., 2015). They are found in virtually every environment: some species are pathogenic e.g. *Bacillus anthracis* and *Bacillus cereus* but most of them are not pathogenic thus suitable for biotechnological applications (Rooney et al., 2009).

1.2. *Bacillus* as a paradigm of PGPR

"*Bacillus* is a heterogeneous taxon with ubiquitous spread in nature; most of its members move using peritrich flagella" (Alina et al., 2015).Using *Bacillus* species as PGPR has several advantages

over others: "*Bacillus* species are one of the attractive candidates for developing improved bioinoculants, since they are endospore forming; their production can be cheaper because they can be produced without the addition of stabilizing carriers and they can be stored for nearly unlimited times" (Boriss, 2014). They are easily cultivated, form thermostable and chemically-resistant endospores (Fan et al., 2013) that allow them to survive for extended periods under unfavorable environmental conditions,thus have a long shelf life (Bouizigarne, 2013). They have been reported to promote a wide range of plants and they are effective in greenhouse and field trials (Bouizigarne, 2013).

Several products have been commercialized from *Bacillus* species. The first PGPR product, Alinit, was developed from *Bacillus subtilis* (Kilian et al., 2000). Other commercial products include Kodiak, RhizoPlus and Taegro from *Bacillus subtilis*, Yield Shield and SONATA from *Bacillus pumilus*, RhizoVital 42 liquid and BioYield from *Bacillus amyloliquefaciens* (Boriss, 2011). However, a successful use of *Bacillus* requires more knowledge about the basic mechanisms of interaction between bacteria and plants (Boriss, 2011).

1.2.1 Systematic and phylogeny of the *Bacillus subtilis* group

Bacillus subtilis species exist in vegetative form, and spores form when conditions are not favorable, they can be isolated in terrestrial and aquatic environments, and they can also grow on plant roots and animal gastrointestinal tracts (Earl et al., 2008), this shows that they are phenotypically diverse. Classification of *Bacillus* species was started by Cohn and Koch who used bacterial morphology to differentiate species (Cohn, 1962; Koch, 1962). Advances in technology made reclassification necessary: combinations of biochemical, physiological and morphological tests were used to classify bacteria (Maughan and Auwera, 2011). Biochemical tests were very useful, however, they could only be used "to characterize bacteria that grow under normal laboratory conditions and it was thus difficult to standardize between laboratories" (Maughan and Auwera, 2011). DNA hybridization methods emerged and they were helpful than biochemical tests even though they could not differentiate very closely related species, the 16s ribosomal DNA method is currently ideal in classifying species although still not perfect for closely related species such as *Bacillus* (Maughan and Auwera, 2011).

Bacillus subtilis group has three subspecies *Bacillus subtilis* subsp. *subtilis*, subsp. *spizizenii* and subsp. *inaquosorum*. The group consists of *Bacillus subtilis* and other closely related species with high genetic and or biochemical similarities. These are *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. licheniformis*, *B. malacitensis*, *B. mojavensis*, *B. pumilus*, *B. sonorensis*, *B. tequilensis*, *B. vallismortis* (Alina et al., 2015). In a recent publication by Dunlapet al. (2015), the subspecies *B. amyloliquefaciens plantarum*that had been considered as a paradigm of PGPR, was suggested to be renamed to the species *B. velezensis*. To avoid confusion, in this work, the taxonomic name *B. amyloliquefaciens* ssp. *plantarum* was used as a synonym of *B. velezensis* as the former name is common in the literature on plant growth promoting bacteria. It is difficult to differentiate these species using phenotypic and biochemical characteristics.The degree of similarity between *Bacillus subtilis* and other closely related species is ≥ 99% using the 16s RNA sequence (Rooney et al., 2009) which is highly conserved in bacteria and suitable for bacteria identification and classification (Alina et al., 2015). Using DNA-DNA hybridization, the complementarity at genome level between *Bacillus subtilis* and other closely related species is up to 70% (Wang et al., 2007). Despite the higher degree of similarity between them, the genomes are diverse. Molecular and biochemical techniques are usually combined to be able differentiate the species of *Bacillus subtilis* group (Alina et al., 2015). Species that demonstrated plant protection and plant promotion activities can be used in agriculture. Example: *B. subtilis* species eliciting induced systemic resistance (ISR) (Choudhary and Johri, 2009), quorum quenching, antibiotics producers, antifungal producers and chitinase activity (Alina et al., 2015).

1.2.2 Regulation of plant colonization, stress response and biofilm formation in bacteria of the *Bacillus subtilis* group

"For the effective establishment of PGPR beneficial effects, the ability to colonize plant roots by introduced bacteria is an important trait" (Bouizigarne, 2013). PGPR use flagella to move to the root surface (Nihorimbere et al., 2011). In the soil, PGPR overcome opposition from plants and other soil microorganisms to successfully colonize plants. The PGPR colonization process involves seed attachment, respond to seed exudates, root surface attachment and root colonization, "it includes the following steps: attraction, recognition, adherence, colonization and growth and other strategies to establish interaction" (Nihorimbere et al., 2011). Successful plant colonization involves several aspects, including but not limited to: mechanisms to withstand environmental stresses, efficient

chemotaxis, effective communication within bacteria and between bacteria and plant which is performed by quorum sensing auto inducers (Lengeler et al., 1999) as well as transcriptional adaptation. “To establish sufficient population on the host roots, compatibility with the host root exudates and other compounds released by the rhizosphere microorganisms is crucial” (THCFarmer Community, 2017). To understand the genetic mechanisms underlying interaction between *Bacillus* PGPR and the host plant is important for their effective use as biofertilizers and biopesticides; however there are only a few gene regulation studies during plant colonization from *Bacillus subtilis* group (Fan et al., 2012).

B. subtilis produces biofilm: an extracellular matrix that holds the cells together in multicellular communities (Beauregard et al., 2013). The biofilm is used to attach on root surface where the bacteria provide the plant with many benefits (Vlamakis et al., 2013). *B. subtilis* coordinates the expression of matrix genes in response to shifting environmental conditions using a complex regulatory network (Vlamakis et al., 2013).

When bacteria colonize plant roots, they encounter different stress conditions from plants and the environment. Bacteria are able to survive stressful environments by altering their gene expression which is controlled at transcription level (Borukhov and Nudler, 2003). *Bacillus subtilis* cells’ adaptation to stress and starvation is crucial for survival in nature because these unfavorable conditions are the rule in natural ecosystems (Petershon et al., 2001).

Plant colonization, stress response and biofilm formation in the bacteria are controlled at transcription level. Transcription factors involved in the processes are explained in section 1.2.3. The *B. subtilis* group has a σ/B-dependent general stress regulon with more than 200 genes which are expressed following bacterial exposure to heat, acid, ethanol, salt stress, entry into stationary phase, or starvation for glucose, oxygen or phosphate (Petershon et al., 2001, Price et al., 2001). The group has other sigma factors which respond to different stress conditions. SigI controls a class of heat shock genes. SigL controls the utilization of arginin, acetoin and fructose, required for cold adaptation. SigM controls adaptation to inhibitors of the peptidoglycan synthesis. SigV controls resistance to lytic enzymes. SigX controls resistance to cationic antimicrobial peptides (Subtiwiki.uni-goettingen.de, 2017).

1.2.3 Transcription regulation and important transcription factors in PGPR *Bacillus*

The gene regulation network in *B. subtilis* was well studied by many researches and it was summarized in the SubtiWiki (Michna et al., 2015) and the DBTBS databases (Sierro et al., 2008). We used these sources of information to create a local database of transcriptional regulation in bacteria of the *B. subtilis* group, which is visualized in Fig. 2.1 (Mwita et al., 2016).

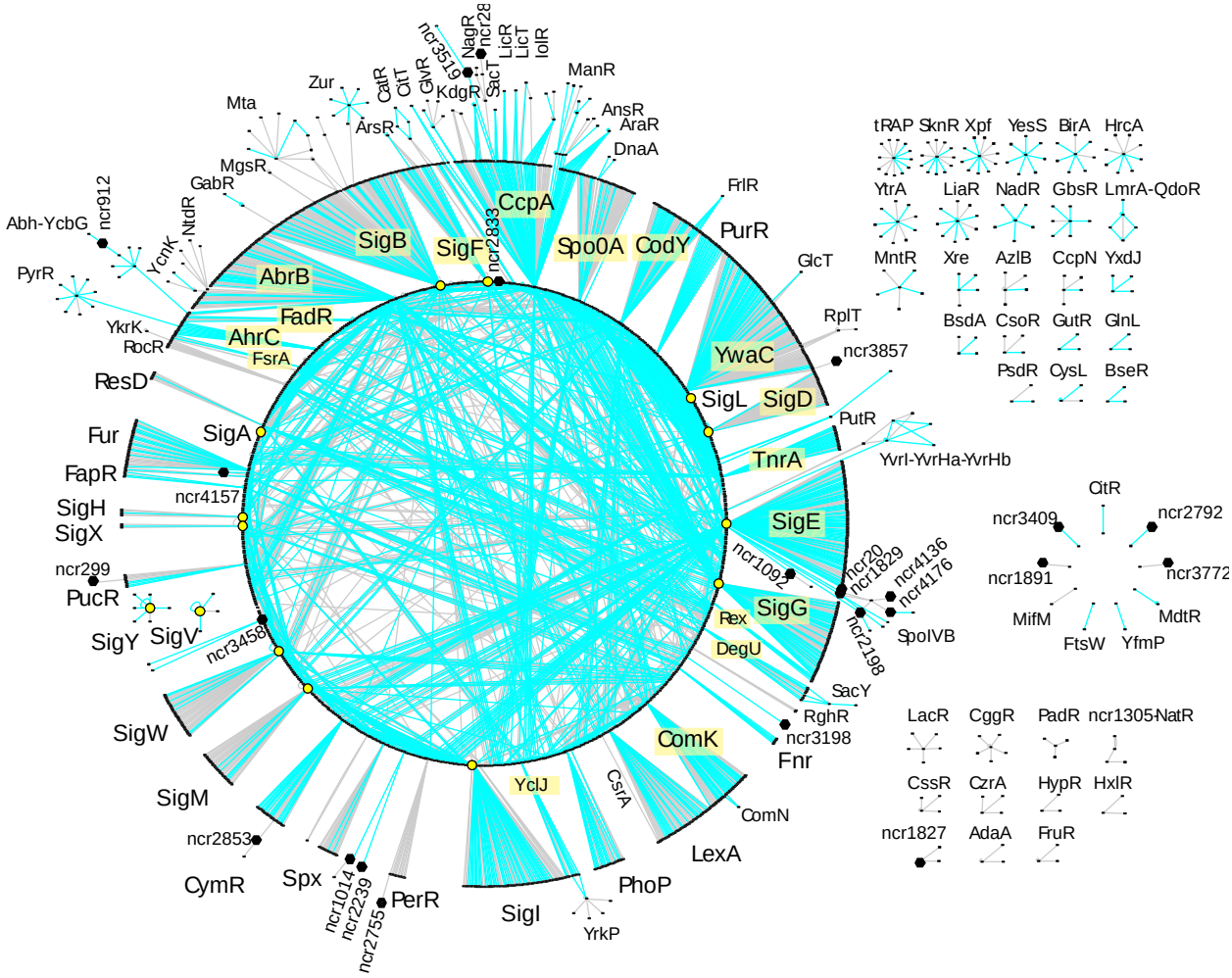

Figure 1.1: Genes of *B. subtilis* are depicted by black dots. They were grouped around sigma-factors and transcription regulators labeled on this figure. Graph color intensity represents the level of dependence of gene expression on the corresponding transcription factors.

It was demonstrated in numerous publications that the plant colonization capacity and ability to withstand biotic and abiotic stresses depends a huge extent, on an appropriate regulation of gene

expression by the corresponding transcription factors (TF) (Fan et al., 2013; Kohlstedt et al., 2014). The most important TFs in this respect are explained below.

"Sigma factors are multi-domain subunits of bacterial RNA polymerase (RNAP) that play critical roles in transcription initiation, including the recognition and opening of promoters as well as the initial steps in RNA synthesis" (Paget, 2015). Sigma is usually denoted by the symbol (σ). SigB (sigma factor B or σ^B) was the first alternative sigma factor to be found in bacteria (Haldenwang, 1995), it controls the general stress response in *Bacillus subtilis* (Paget, 2015). SigE, SigF, SigG and SigK are known as sporulation sigma factors (Haldenwang, 1995), they are involved in transcription of sporulation genes (Feucht et al., 2003; Clarkson et al., 2004; Wang et al., 2006). SigE, SigF, SigG and SigK also control endospore formation in *Bacillus subtilis* (Paget, 2015). *Bacillus subtilis* usesSigD for expression of motility and chemotaxis apparatus (Hamoen et al., 2003); it also regulates flagella and autolysis (Serizawa et al., 2004). SigW controls resistance and detoxification (Cao et al., 2002).

CcpA (carbon catabolite protein A) mediates carbon catabolite repression (Fujita, 2009) which is a "mechanism controlling carbon and energy sources metabolism to maximize metabolic efficiency" (Marciniak et al., 2012). Bacteria first utilize most preferred sugars: glucose, fructose or malate exclusively before other sugars in the environment (Marciniak et al., 2012). CcpA can act as a positive or negative regulator of genes responsible for carbon metabolism (Sonenshein, 2007). CcpA is usually produced regardless of the availability of most favored carbon sources (Miwa et al., 1994). Apart from carbon metabolism, it also regulates amino acid anabolism, overflow metabolism and nitrogen assimilation (Fujita, 2009).

AbrB controls regulation of gene expression during the transition from growth to stationary phase, including those for biofilm formation, antibiotic production, motility, development of competence for DNA uptake, synthesis of extracellular enzymes and sporulation (Chumsakul, 2011). It regulates other transcription factors; SinR and AbrB positively regulate ComK (Ogura et al., 1997). Development of competence, sporulation, biosynthesis of degradative enzymes and chemotaxis are some of the ways in which *Bacillus subtilis* adaptively responds to deficiency of nutrients and many stresses that naturally occur in their environment (Ogura et al., 1997). AbrB represses the expression of many genes during the exponential growth in *Bacillus subtilis*.

CodY controls expression of genes responding to nutrient availability of the environment (Stenz et al., 2011). "The ability to adapt to changes in nutrient availability is central to bacterial colonization of diverse habitats" (Handke et al., 2008). The adaptive responses controlled by CodY include chemotaxis and motility to migrate to an environment richer in nutrients, secretion of macromolecule degrading enzymes, induction of transport systems for the uptake of amino acids, peptides, and other available nutrients (Villapakkam et al., 2009). CodY also controls activation of intracellular catabolic systems, production and secretion of antibiotics that could aid in reducing the competition for limited resources, development of competence that enables the intake of exogenous DNA and lastly, sporulation if the nutritional status has not improved (Villapakkam et al., 2009).

Spx is a negative and positive regulator of many genes (Zuber, 2009). It is a transcription regulator of oxidative stress response in *Bacillus subtilis* (Choi et al., 2006). DegU is involved in the regulation of degradative enzymes, expression of late competence genes and other processes in *Bacillus subtilis* (Hamoen et al., 2000). ComK is a competence transcription factor in *Bacillus subtilis*, it regulates competence and DNA uptake (Ogura et al., 2002, Kearns, 2005). ComK is regulated by several other factors including MecA, SinR, AbrB and Rok (Hamoen et al., 2003). *Bacillus subtilis* consists of three Fur homologs (Fur, Zur and PerR) which control gene expression in the presence of iron ions (Fuangthong et al., 2002, Imlay, 2015). PerR (peroxide stress regulator) regulates hydrogen peroxide stress response in *Bacillus subtilis* (Fuangthong et al., 2002, Imlay, 2015). Fur is a main sensor of iron status in *Bacillus subtilis* cells and it regulates iron homoeostasis (Smaldone et al., 2012). GlnR regulates glutamine synthesis (Wray and Fisher, 2008). Spo0A regulates entry into sporulation in *Bacillus subtilis* (Molle et al., 2003). (p) ppGpp alarmone controls stringent response (adaptive cellular response to starvation) in *Bacillus subtilis* (Eymann et al., 2002).

1.2.4 Application of genomics and transcriptomics approaches to elucidate the mechanisms of positive action of the PGPR *Bacillus*

Next generation sequencing is a DNA sequencing technology which has revolutionized genomic research (Behjati and Tarpey, 2013). It generates a lot of data in a short time with low cost compared to the previous techniques (Goodwin et al., 2016). It also facilitates a good progress in microbiology research (Behjati and Tarpey, 2013). Knowledge on gene content, expression and

diversity of plant-associated organisms helps our understanding of the basis of their interactions with plants and in developing strategies to modify such interactions (Llaca, 2012). The availability of PGPR genome sequences has increased the knowledge of plant bacteria interactions. It is important to elucidate the molecular principles determining the beneficial effects on plant growth of each PGPR (Boriss, 2014).

Microarray techniques have been used for transcriptomic analyses in previous *Bacillus* studies (Fan et al., 2012, Xie et al., 2015) nevertheless, RNA-Seq offers several advantages over microarray limitations including large dynamic range of expression levels and the experiment requires less RNA sample (Wang et al., 2009). In the recent publications by Fan et al. (2012) and Xie et al. (2015) where the microarray technique was used, it was found that the maize root exudates and rice seedlings could cause significant changes in gene regulation profile of the PGPR bacteria *B. amyloliquefaciens* ssp. plantarum FZB42 and *Bacillus subtilis* OKB105 respectively. Interaction of rice seedlings with *Bacillus subtilis* OKB105 resulted in an up-regulation of genes involved with metabolism, transport and stress while down-regulated genes were involved with chemotaxis, motility and sporulation, the majority of genes were down-regulated (Xie et al., 2015). In Fan et al. (2012), majority of genes were up-regulated. Up-regulated genes were corresponding to nutrient utilization, chemotaxis and non-ribosomal synthesis of the antimicrobial peptides and polyketides. Microarray techniques have also been used to study gene regulation in Gram-negative bacteria (Mark et al., 2005).

1.2.5 Simulation of plant colonization by root exudates

Plants root exudates are the nutrient rich components secreted by the plant root (Lugtenberg and Kamilova, 2009). Their components belong to three classes: low-molecular weight, high-molecular weight and volatiles (Uren, 2007). Low-molecular-weight compounds represent the main portion and consist of sugars, amino acids, organic acids, phenolics, vitamins, and various secondary metabolites (Uren, 2007). They mediate interactions with neighboring plants and microbes (Bais et al., 2004; 2006, Weir et al., 2004, Broeckling et al., 2008) initiating a vast array of biological responses on the bacterium (Fan et al., 2012). They can be collected separately and applied on the bacteria to study bacteria gene expression in the absence of the plant host (Mark et al., 2005, Fan et al 2012). Only exudates collected from sterile plants growing under artificial conditions such as

sterile filter paper or sterile plant nutrient solution are sufficiently concentrated to be analyzed successfully (Lugtenberg and Kamilova, 2009). This technique enables a complete recovery of root exudates and prevents interference of other organism's genes (Fan et al., 2012), however, the results should be interpreted with enough prudence or further investigated in a soil system (Fan et al., 2012).

1.3 Problem statement

Chemical fertilizers and pesticides used by farmers to promote plant growth could be hazardous to human health and the environment (Pimentel, 1996). Using plant growth promoting bacteria is a safe alternative to agrochemicals; however their use is not common in Africa. Understanding molecular principles involved when PGPR interacts with the plant host is crucial in improving biofertilizers and biopesticides developed; nevertheless this is not fully explored in PGPR *Bacillus*. Although *Bacillus* are common in rhizosphere communities and frequently are used in biopesticides (Podile and Kishore 2007; Fan et al., 2013), only a few studies on gene regulation in PGPR *Bacillus* have been reported. In various studies it was demonstrated that the maize root exudates were useful to mimic in vitro interactions between different plants and PGPR by initiating a range of biological responses within the bacteria (Mark et al., 2005; Broeckling et al., 2008; Fan et al., 2012; Kierul et al., 2015). Differential gene expression regulations caused by the maize root exudates were reported previously on *Bacillus amyloliquefaciens* FZB42 that was proposed as a paradigm of PGPR *Bacillus* (Fan et al., 2012; Kierul et al., 2015). Gene regulation in *Bacillus subtilis* BSB1 under abiotic stresses was reported by Kohlstedt et al. (2014). It was not clear whether these reports could be extended to other PGPR *Bacillus*, as it had been found that the activities of biotechnological importance were strain specific (Safronova et al., 2012). There was little knowledge available regarding plant colonization behavior of other bacteria of the *B. subtilis* group, such as *B. atrophaeus*. A collection of 12 potential plant growth promoting *Bacillus* strains was used in this study. The rational to use this collection was that all these strains were well characterized in previous studies by their phenotype and bioactivities on different models. Initially all these strains were identified by phenotypic treats as representatives of *B. subtilis* (Reva et al., 2001), but further sequencing of taxonomic marker genes (Reva et al., 2004) followed by complete genome sequencing allowed distribution of these organisms to different taxa of the *B. subtilis*/

B. amyloliquefaciens taxonomic group. All these strains showed various PGPR activities, but they have never been compared to each other. A special interest was paid to the strain *B. atrophaeus* UCMB-5137. The strain *B. atrophaeus* UCMB-5137 was isolated from rhizosphere in Ukraine in 1989. In an array of bioassays, *B. atrophaeus* UCMB-5137 showed an ability to protect plants and crops from bacterial and fungal phytopathogens, and to promote the plant growth (Lapa and Reva, 2005). The complete genome sequence of *B. atrophaeus* UCMB-5137 has been achieved recently (Chan et al., 2013). *B. atrophaeus* is a common soil inhabitant. Spores of *B. atrophaeus* have been used in biotechnology to control sterilization processes because of the resistance of the spores to extreme temperatures and chemical detergents (Pinzón-Arango et al., 2009). Except for UCMB-5137, the strains of *B. atrophaeus* were not reported in the literature as active plant-associated growth promoters or protectors, in contrast to strains of the closely related species *B. amyloliquefaciens* and *B. subtilis*. This thus initiated our interest to study this bioactive strain, UCMB-5137, to contribute to the knowledge on gene regulation in PGPR *Bacillus*. Current knowledge on this issue is biased towards the paradigm examples of *B. amyloliquefaciens* ssp. *plantarum* (Reva et al., 2004; Chen et al., 2007; Fan et al., 2011; 2012; 2013; 2015). It was therefore interesting to study further the gene expression regulation in *B. atrophaeus* UCMB-5137 under the standard laboratory conditions through simulation with the root exudates, to compare the gene expression profile with the published results for other PGPR *Bacillus*. To identify other transcription factors and genes specifically involved in root colonization by *B. atrophaeus* UCMB-5137, the obtained gene regulation profiles were superimposed over known regulatory networks in *B. subtilis* (Kohlstedt et al., 2014; Michna et al., 2015).

Research aim and objectives

Aim:

To uncover the plant promotion and plant protection ability of selected *Bacillus* species.

Objectives

(i) To investigate the plant growth promoting activities of the selected*Bacillus* isolates on different bioassay models, which will include:

a. Growth characteristics of the strains on rich media, minimal mineral media and root exudates as a sole source of nutrients.
b. Inhibition of fungal plant pathogens by the *Bacillus* strains.
c. Promotion of seedling germination and growth promotion by the *Bacillus* strains in controlled laboratory conditions on the model plants.
d. Improvement of drought tolerance of the plants by treatment with the PGPR *Bacillus*.

(ii) To elucidate the specific gene regulation pattern of *Bacillus atrophaeus* UCMB-5137 under root exudate's stimuli to underline the specificity of plant colonization behavior of this strain.

(iii) To identify major transcription regulation factors including ncRNA involved in gene regulation by the root exudate's stimuli in the *B. atrophaeus* UCMB-5137.

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CHAPTER 2: EVALUATING THE PLANT PROMOTION AND PLANT PROTECTION ACTIVITIES OF THE *BACILLUS* STRAINS

Abstract

In this chapter, plant promotion and plant protection activities of *Bacillus* strains were determined, using different bioassay models. The strains UCMB-5137 and UCMB-5007 actively promoted wheat growth. All strains showed some degree of fungal pathogen inhibition. Some strains improved *Brassica napus* tolerance to drought conditions. Plant growth promoting bacteria were sensitive to root exudates stimulation.

2.1 Introduction

Plants are a major source of food to human beings. Pathogens affect plant health, threatening food production and ecosystem worldwide (Compant et al., 2005). Most African farmers usually apply fertilizers and pesticides to promote plant growth and health. Fertilizers and pesticides, improve productivity, food quality, prevent yield reduction and control diseases, however, they have negative impacts on the health of human beings and the environment (Aktar et al., 2009). In addition, the cost of buying the chemicals is high to local farmers. The mentioned challenges presented a need for finding an alternative: cheaper, effective and safe solution to farmers, consumers and the environment. Using PGPR to control plant pathogens is the environmentally friendly alternative to chemical pesticides (Bouizgarne, 2013) and hence a current practical solution to the mentioned challenges.

Plant Growth Promoting Rhizobacteria (PGPR) promote plant growth directly by providing, chemicals needed by the plant, including iron chelating siderophores, antibiotics, lytic and detoxification enzymes and indirectly, through induced systemic resistance (Compant et al., 2005). PGPR also help the plant to adapt to harsh environments, including drought stress (Hu et al., 2008; Lim and Kim, 2013; Timmusk et al., 2014). *Bacillus* is one of the PGPR group; they can be applied in plants as spores or inoculants (Reva et al., 2004). Biopreparations based on *Bacillus* are easy to produce, store and manipulate due to an ability of these bacteria to form thermo-stable and chemically resistant endospores (Fan et al., 2013). Some commercial products are available from the

Bacillus species (Kilian et al., 2000; Boriss, 2011). PGPR perform differently in different plants and perhaps in different weather conditions. Available products are not commonly used in African countries. It is therefore recommended to find biocontrols that are suitable for the African crops.

In this chapter, 12 biocontrol strains from the *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus mojavensis* and *Bacillus atrophaeus* were studied. The strains were isolated from plants and different types of soils in Europe and Asia: *B. subtilis* ssp. *subtilis* UCMB-5021 and UCMB-5121, and *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007 and UCMB-5044 were isolated from cotton plant tissues in the Tadjikistan; *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113 and UCMB-5140, *B. subtilis* ssp. *spizizeni* UCMB-5014 and *B. atrophaeus* UCMB-5137 were isolated from grass rhizosphere in Ukraine; *B. mojavensis* was isolated in Ukraine from animal furage; the strains *B. amyloliquefaciens* ssp. *plantarum* At1 and *B. subtilis* ssp. *spizizenii* At2 and At3 were isolated in Sweden from the germinated surface-sterilized seeds of *Arabidopsis thaliana* and thus they were considered as representatives of the endophytic microphlora. Taxonomic relations between these strains and several sequenced reference strains are shown in Fig. 2.1.

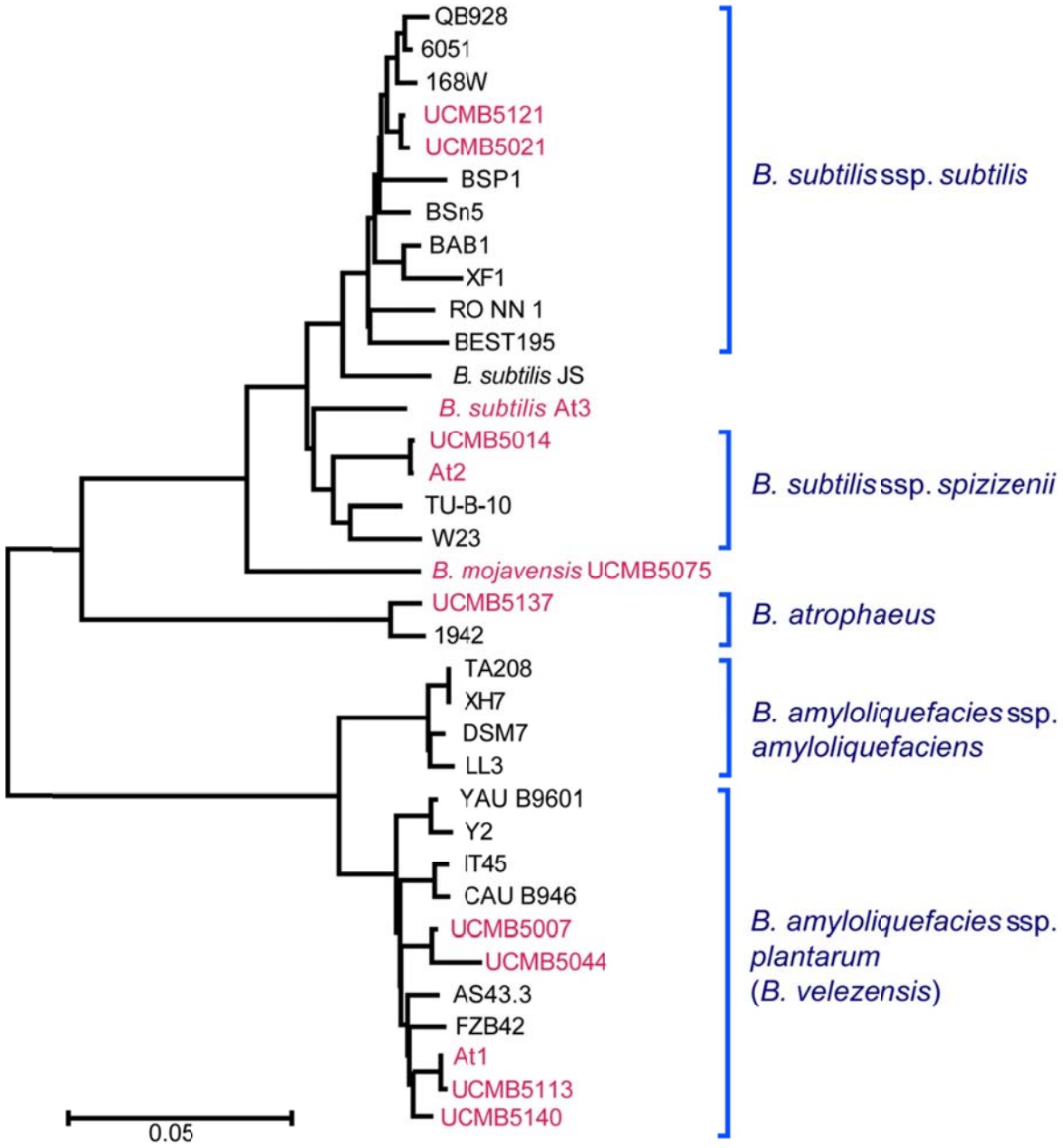


Figure 2.1: Neighbor-joining species tree constructed for selected organisms of the *Bacillus subtilis* group (the tree was constructed from orthologous genes of the twelve strains).

The selected strains showed significant antibacterial and antifungal activities and were demonstrated to be suitable for biotechnological application as biocontrol agents (Lapa and Reva, 2005). Genomes of all these strains were sequenced in previous unpublished studies.Chan et al., 2013 reported genome sequence information of the UCMB-5137.This chapter describes different bioassays that were performed to verifyif the strains could promote or protect plants. The PGPR from the *Bacillus*

group were tested on the following plants: wheat (*Triticum aestivum)*; an important food crop in many parts of the world (Wheat production Guideline, 2010), *Arabidopsis thaliana*; a model research plant and the oilseed rape; a cash crop. Some strains showed potential to promote plants, with some active in more than one bioassay while others were not active at all.

2.2 Materials and methods

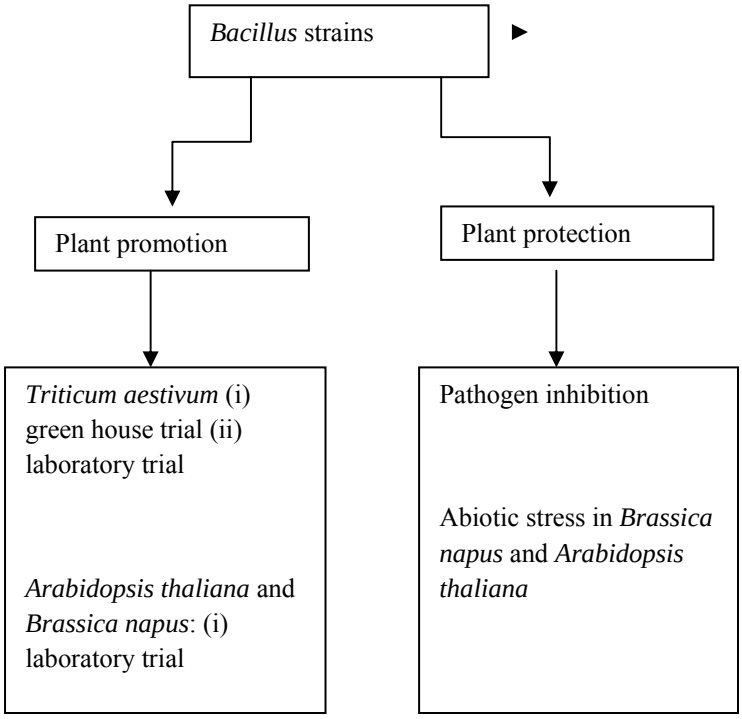


Figure 2.2: The diagram below is a summary of tests that were performed on model plants to evaluate biological activities of *Bacillus* strains.

2.2.1 Cultivation of the *Bacillus*

The bacteria were inoculated in 1 litre flasks containing 300 ml Luria Broth (DuchefaBiochemie) at 28°C with shaking at 200 rpm for two weeks. The cultures were dispensed in small centrifuge flasks and heat shock was applied at 65°C for 5 min to remove vegetative cells. This was followed by

centrifugation (Sorvall (model: Evolution RC), Wilmington, *Delaware, USA*) at 10,000g for 10 min. The supernatant was sterilized by filtering and kept in the cold room at 4°C. The supernatant fluid was used to investigate the possible antifungal activity of metabolites produced by the *Bacillus* into the culture medium. The pellets of spores were resuspended in 25 ml and centrifuged at 10,000g again to wash out the spores. After centrifugation, the supernatant was discarded and the pellets were resuspended in 25 ml of sterile distilled water. The stock suspensions were kept in the cold room at 4°C.

2.2.2 *Bacillus* growth on root exudates

Aim: To study the dynamics of growth of the *Bacillus* strains on *Brassica napus* root exudates.

Brassica napus exudates collection

Brassica napus seeds (Larissa, spring variety, line, Scandinavian Seed AB) were surface sterilized,by soaking in 70% ethanol for 5 min followed by soaking in 1% sodium hypochlorite and shaking for 5 min. Finally, the seeds were rinsed with sterile distilled water and shaken for 5 min 3 times. Surface sterile seeds were pre-germinated in Petri dishes containing 0.2 M MSA (Murashige and Skoog mixed with plant agar (DuchefaBiochemie)). Seedlings of the same size were selected and transferred to a 250 ml flask containing 100 ml of the 0.5 M MS (Murashige and Skoog, broth (DuchefaBiochemie B.V., Haarlem, The Netherlands)). Seedlings were grown for two weeks at 22°C under 16 h light/8 h dark photoperiod with shaking (INFORS AG, Basel, Switzerland) at 110 rpm. The exudates were collected after two weeks, and lyophilized. The dry residues of the root exudates were dissolved in 50 ml sodium phosphate (Na_3PO_4). The solutions were sterilized by filtering (0.22 µm pores) and kept in 50 ml aliquots in the cold room. 1% and 10% of the root exudates were working solutions prepared from the stock solution.

Growth curve experiment

Bacteria were cultured overnight in Luria broth (DuchefaBiochemie). The cultures were diluted to the optical density of 0.4. Aliquots of 10 µl of the suspensions were loaded into wells of the 96 well plates containing either 90 µl of the media (LB or M9), or 70 µl of the medium M9 mixed with 20 µl of the 1% or 10% *Brassica napus* root exudates calculated from stock in advance and as it was already described above. Therefore, the resulting concentrations of the root exudates in each well

were 0.2% and 2% respectively. Wells loaded with 100 µl of the M9, LB, or M9 with1% and 10%root exudates (RE) were used for negative growth controls. The M9 minimal salt medium (Sigma-Aldrich Co, St Louis, MO, USA) contains mineral salts (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl, $CaCl_2$and $MgSO_4$) and 0.4% glucose as a source of carbon and energy. The 96-well plates were incubated in a plate reader spectrophotometer (FLUOstar Omega, BMG LABTECH) at 28°C with shaking for 16 h and 22 min. Optical density measurements were recorded automatically at regular intervals of 8 min. The distribution of wells on the plates is shown below in Fig. 2.3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	2	2	2	2	2	2	2	2	2	2	2	2
C	3	3	3	3	3	3	3	3	3	3	3	3
D	4	4	4	4	4	4	4	4	4	4	4	4
E	w	w	w	w	w	w	w	w	w	w	w	w
F	1	1	1	1	4	4	4	4	M9			
G	2	2	2	2	w	w	w	w	1% root exudate			
H	3	3	3	3	LB				10% root exudate			

Figure 2.3: Distribution of wells with culture growth on the 96-well plate. Wells containing M9, M9+0.2%RE, M9+2%RE and LB media are depicted by different colors.

Wells with media names were used as media density controls. Wells marked by 'w' remained empty. Numbered cells were inoculated with bacteria: 1 – UCMB-5021 (plate 1) or UCMB-5007 (plate 2); 2 –UCMB-5075 (plate 1) or UCMB-5044 (plate 2), 3 – UCMB-5137 (plate 1) or UCMB-5113 (plate 2); 4 – At3 (plate 1) or At1 (plate 2). Each strain was represented on the plate by 4 wells. Average values of optical densities in 4 respective wells were calculated and the average values of the optical density of the wells containing the corresponding medium without bacteria (blank) were subtracted. The experiment was repeated twice.

2.2.3 Re-isolation of *Bacillus* strains from the wheat roots

Aim: To measure the rate of survival of *Bacillus* strains on wheat roots.

Wheat seeds from PANNAR (South Africa) were surface sterilized, by soaking in 70% ethanol and gently shaken for 5 min, followed by soaking in 1% sodium hypochlorite and gently shaken for 1 min. Finally, the seeds were rinsed with sterile distilled water 5 times for 5 min. Surface sterile

seeds were germinated in Petri dishes containing 7 ml of autoclaved water (1:1 sterile water, tap water). The Petri dishes were incubated in the dark at 25°C for 7 days (to allow the wheat seedlings to achieve a main root length of 5 cm or more).

Each *Bacillus* strain was separately inoculated in 10 ml nutrient broth (MERCK) and incubated at 37°C for 16 h. The resulting culture was centrifuged (Hettich Universal 2s Zentrifugen D-7200 Tuttlingen) at 3000×g for 10 min and the pellet was diluted in Ringer's solution to an optical density of 0.5 (Hettich Universal 2s Zentrifugen D-7200 Tuttlingen). Colony forming units (CFU) were calculated from this optical density at 10^6dilutions.

One wheat seedling per each strain was dipped for 1 min into the diluted solution obtained above. The seedling was then transferred into a new 10 ml Ringer's solution and incubated overnight at 25°C in the conviron (EF7H Controlled Environments Ltd Winnipeg Manitoba Canada) in the presence of light. The seedling was removed from the solution, and the CFU were then determined in the solution after 10^6 dilutions. The seedlings were left to dry on a paper towel, thereafter the root was excised and its weight measured to normalize the CFU values by the root weight. The experiment was replicated three times.

2.2.4 Plant growth promotion

Aim: To assess abilities of *Bacillus* strains to promote wheat growth.

Wheat (*Triticum aestivum*) seeds from PANNAR (South Africa) were surface sterilized by soaking in 70% ethanol and gently shaken for 5 min, followed by soaking in 1% sodium hypochlorite and gently shaken for 1 min.Finally, the seeds were rinsed with sterile water 5 times for 5 min. The surface-sterilized seeds were germinated in sterile vermiculite for one week and then transferred into plastic pots containing 500 g steam-pasteurized sandy loam soil. Five seedlings were planted in each pot. The pot trial was conducted at the University of Pretoria ProefPlaas greenhouse. The greenhouse temperature was maintained at 20-26°C; plants received municipal tap water after every other day.

Bacterial pure cultures were preserved in 20% glycerol; the cultures were revived by inoculating in nutrient agar (MERCK) before use. Bacteria inoculum was prepared by incubating a streak of

bacteria grown on nutrient agar into 10 ml nutrient broth (MERCK) overnight, at 25°C with shaking. An overnight culture (5 ml) was transferred into 250 ml nutrient broth. This new culture was incubated at 25°C and 150×g for 24 or 48 h to achieve a count of 10^7cfu/ml. In this experiment, twelve strains of *Bacillus* were tested for their ability to promote wheat plants. Bacterial cultures were cultivated at 25°Cfor 48 h and then transferred to 50 ml falcon tubes. The tubes were centrifuged at 3000×g (Hettich Universal 2s Zentrifugen D-7200 Tuttlingen) for 10 min. The pellets were resuspended in a sterile Ringer's solution to a concentration of 10^7cfu/ml. In biotrials, 50 ml Ringer's solution aliquots containing 10^7cfu/ml of the bacteria were applied into the soil around each plant in a pot when the plants were ten days old (at this time the plant had at least one true leaf). Each pot was planted with 5 plants. Treatments of plants by different bacterial strains were replicated 5 times. Negative control plants were treated by the same volume of water. A commercial preparation BP0103 from BECKER UNDERWOOD (South Africa) was used as a positive control.

Plants were harvested after one month by pulling them gently from the soil. The roots were rinsed with tap water, then excised, and shoot and root wet weight were measured separately. The roots and shoots were oven-dried at 65°C for three days and thereafter the dried weight was measured. The mean and standard error were calculated in Excel to determine the significance of plant growth promotion (Method modified from Idriset al., 2007).

2.2.5 Inhibition of phytopathogenic fungi

Aim: To study inhibition of pathogen growth by *Bacillus* strains.

The following fungal pathogens were used in this study: *Alternaria brassicicola* 20297, *Verticillium longisporum* D11, *Sclerotinia sclerotiorum* 13MM and *Fusarium oxysporum*. Pathogens were inoculated in the center of PDA (Applichem, Darmstadt, Germany) plates and then incubated at room temperature in the dark for five days, except for *Alternaria brassicicola* 20297 that was incubated at the same conditions under a 16/8 light/dark photoperiod.

Both, the spore suspensions and supernatant fluid samples were used to test the antifungal activity. Aliquots of 25 µl of the spore suspensions and supernatant samples were pipetted onto paper disks placed at the edge of the Petri dishes with the phytopathogen pre-incubated atthe center of the Petri

dishes for 3days. Then, the inhibition zones around the disk with the growth of *Bacillus* and supernatants were measured after 8 and 14 days of incubation at the conditions described above.

2.2.6 Inducing drought tolerance in plants by treatments with *Bacillus*

Aim: To study drought tolerance improvement in the plants inoculated with the *Bacillus* strains.

Arabidopsis thaliana seeds were surface sterilized by soaking in 1% sodium hypochlorite and shaking for 5 min, followed by rinsing with sterile distilled water with shaking for 5 min 3 times. *Brassica napus* followed the same procedure, except that 70% ethanol was added and seeds shaken for 5 min before addition of sodium hypochlorite.

Two trials were performed either by treating the plant seeds with the suspensions of spores of the *Bacillus* or by applying the spores of the *Bacillus* onto the soil where the seeds were planted. For the soil treatment experiment, the surface-sterilized seeds were first germinated in S-soil (Hasselfors Garden, Örebro, Sweden). Alternatively, the surface sterilized seeds were soaked in bacterial spore suspensions for two hours with shaking (LIC, Stockholm, Sweden) at 10 rpm. Seeds used as non-inoculated control were soaked in sterile water. Then, seeds were planted in the S-soil.

After 5 days, seedlings were transferred to the P-soil (plant-soil) (Hasselfors Garden, Örebro, Sweden). For soil treatment, 1 ml of spore suspensions (the same volume of water for control) was applied directly into the rhizosphere area of each plant after the plants were transferred to the P-soil. The seedlings grew until the plant had at least one true leaf. One group of seedlings was subjected to drought conditions (without supply of water) while another group was watered every second day. The experiment lasted for 10 days. Recovery allowed for drought-subjected plants by supplying water for one week following the standard regiment. To determine the photosynthesis efficiency of the plants, the concentration of chlorophyll in leaves was measured by chlorophyll meter (Konica Minolta, Tokyo, Japan). Shoots were harvested and the wet weight was then measured. The leaves were dried at 60°C for two days and the dry weight was then measured.

In this experiment, eight strains of *Bacillus* were tested for their ability to enhance drought tolerance in model plants. For every run, 18 seedlings of the model plant were used for the treatment by suspensions of spores of the different *Bacillus* strains, and another18 seedlings were inoculated into

sterile water and used as negative controls. The density of live cells in the suspensions was controlled by the serial dilution method and by suspension opacity. It was then adjusted to 1×10^7 cells per ml.

2.3 Results and Discussion

2.3.1 *Bacillus* growth on root exudates

Dynamics of growth of the *Bacillus* strains on *Brassica napus* root exudates was studied as described in the method (section 2.2.1). Growth of the bacteria in root exudates was compared to the growth in LB and M9 media. The strains were divided into two groups: the strains of *B. amyloliquefaciens* and *B. subtilis* clades.

Many bacteria were able to grow on M9; but At3, At1 and UCMB-5113showed very slow growth on this nutrient-poor medium (Figures 2.4 and 2.5). UCMB-5044 grew adequatelyon M9 (Figure 2.4), almost as on LB (Figure 2.6).

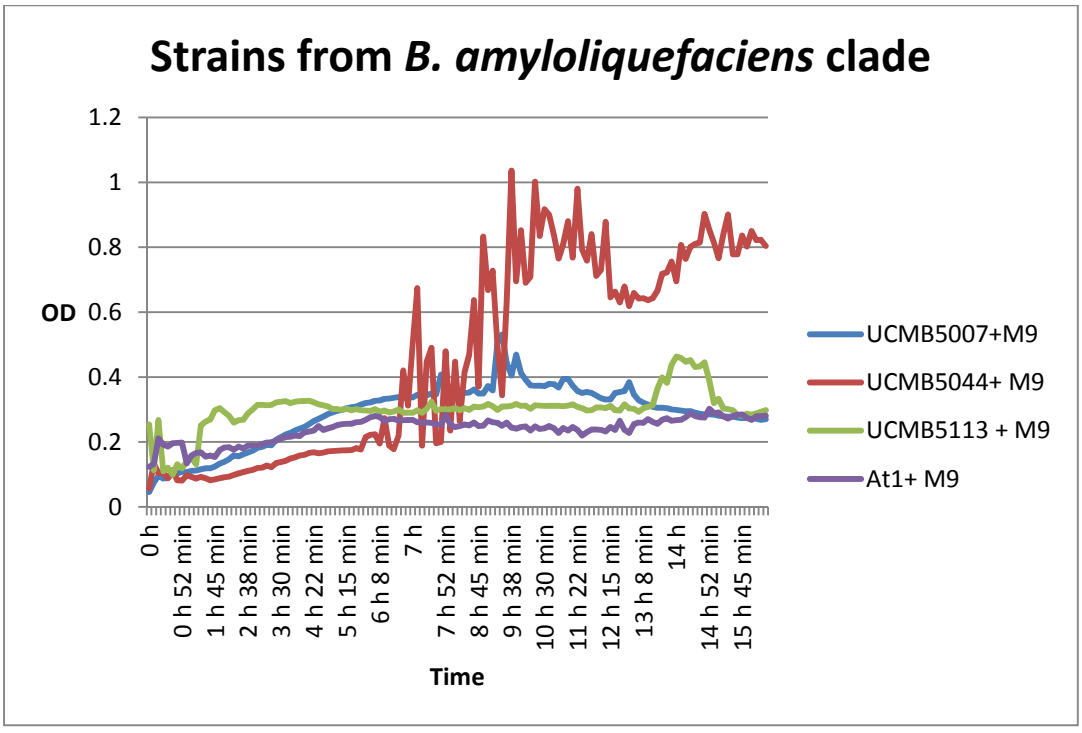


Figure 2.4: Growth curves of *B. amyloliquefaciens* strains in M9.

Growth curves of the *B. amyloliquefaciens* ssp. *plantarum* UCMB 5044, *B. subtilis ssp subtilis* UCMB-5021and *B. atrophaeus* UCMB-5137were of a zigzag fashion (Figure 2.4 and 2.5). This could be explained by phase changes of the bacterial growth in a series of autonomous alterations in every 25-30 min of the clamping of cells into micro-biofilm, followed by a scattering of the plankton cells that was previously described by Norman et al. (2013). These phase changes could have influenced the medium turbidity.

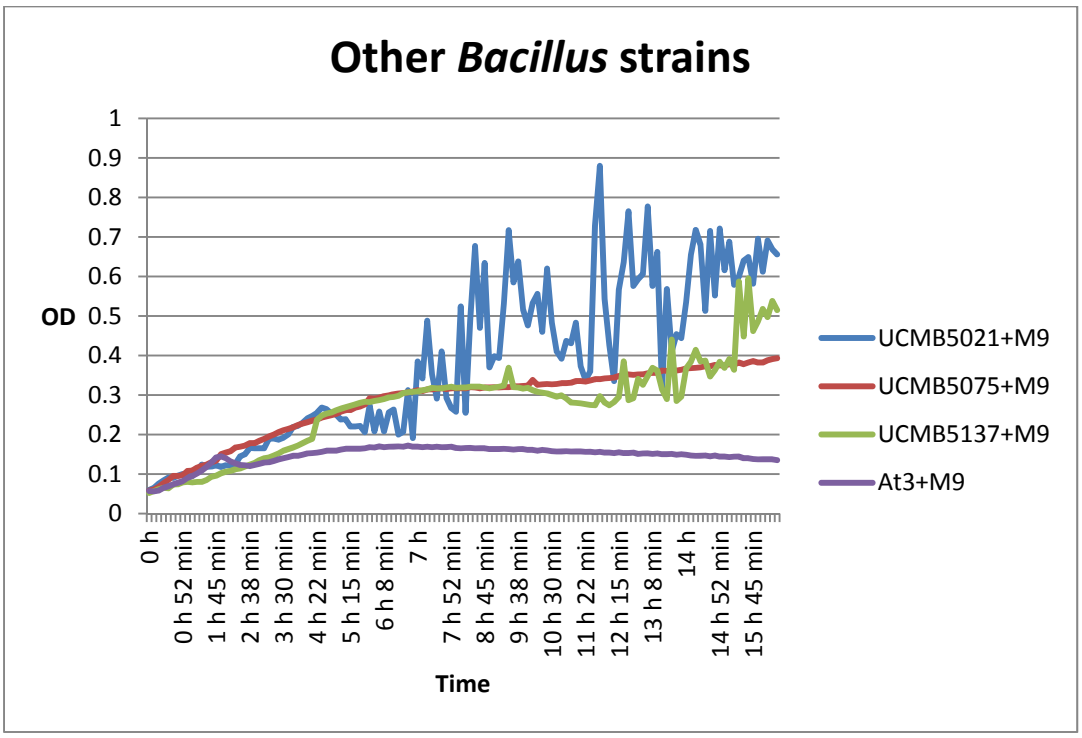


Figure 2.5: Growth curves of the strains of the *B.subtilis* group in M9.

All the strains of the *B. subtilis* and *B. amyloliquefaciens* groups grew very well on LB (Figures 2.6 and 2.7). After 7-9 h of incubation they all reached OD of 1, but then some continued to grow up to an OD of 1.4, but the strains *B. mojavensis* UCMB-5075 and *B. amyloliquefaciens* UCMB-5044 declined after 9 h of the logarithmic growth. It may indicate that the nutrient rich media are not appropriate for these strains because of either the fast acidification of the medium, or a higher sensitivity of these strains to the pH of the media, or perhaps these strains produce some self-toxic compounds, or possibly because of all these factors acting together.

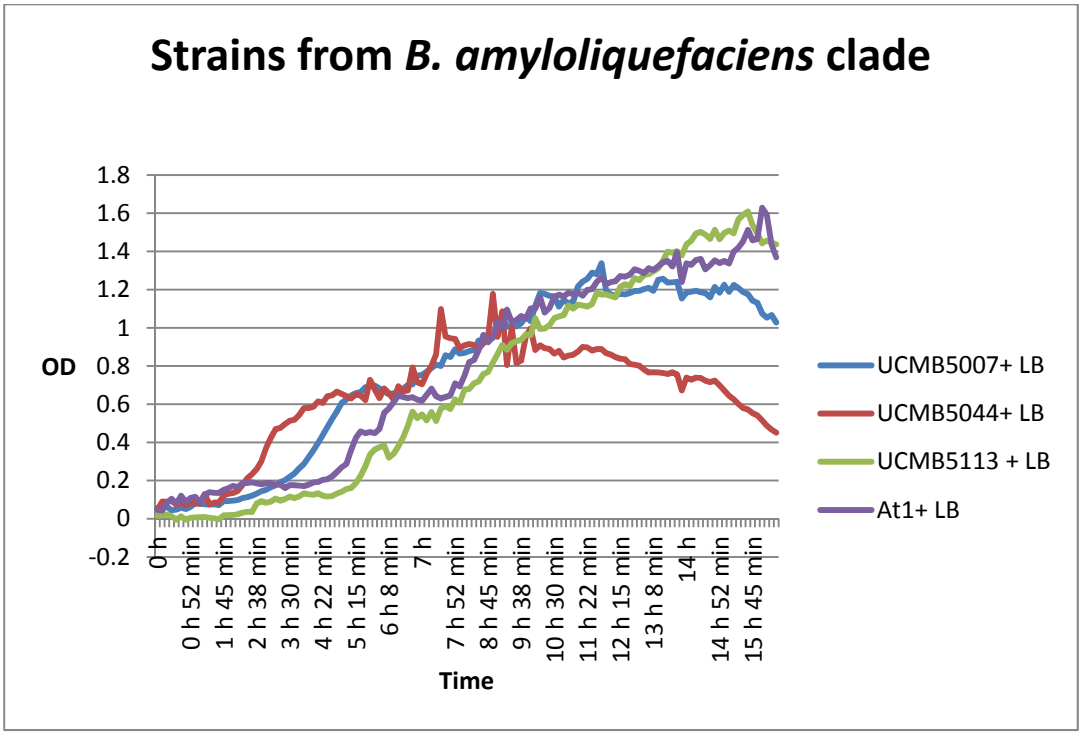


Figure 2.6: Growth curves of the *B.amyloliquefaciens* strains in LB.

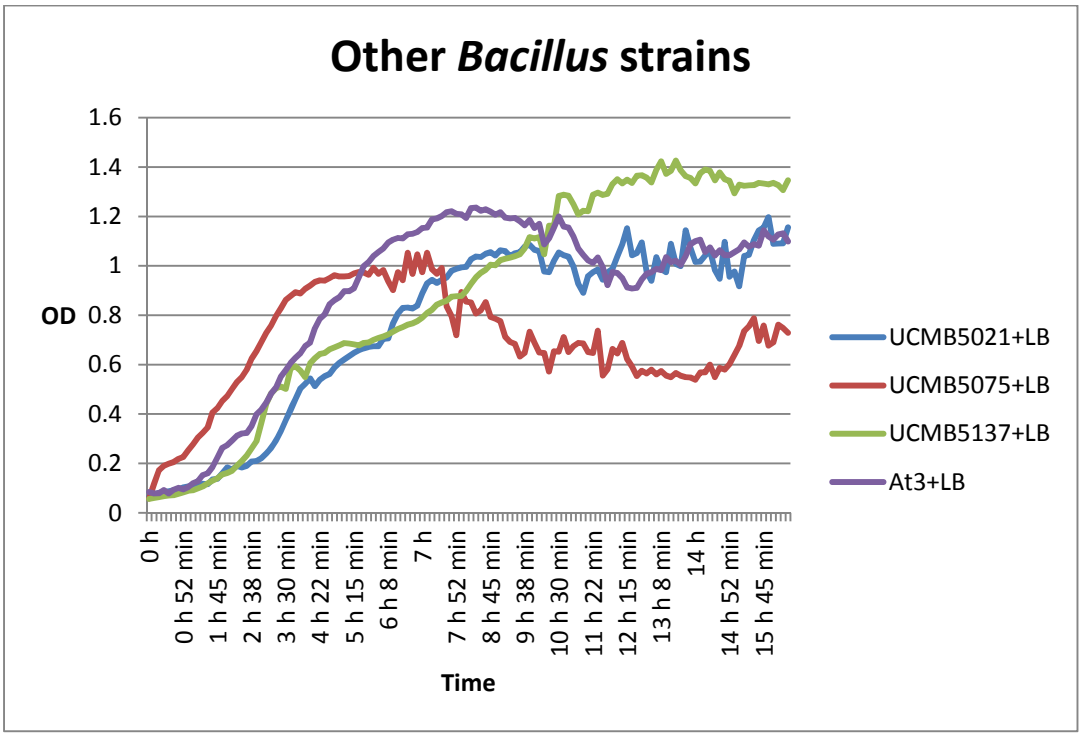


Figure 2.7: Growth curves of the strains of *B. subtilis* group in LB.

It was expected that the root exudates (RE) may influence the growth of PGPR *Bacillus*. The hypothesis was tested by mixing up 70 µl of the medium M9 with 20 µl of the 0.2% and 2% of root exudates collected from *Brassica napus* and 10 µl of the suspensions. The strains UCMB-5007 (Figures 2.8 and 2.9), UCMB-5021 and UCMB-5075 (Figures 2.10 and 2.11) responded positively even to the 0.2% RE, and they grew vigorously on 2% RE.

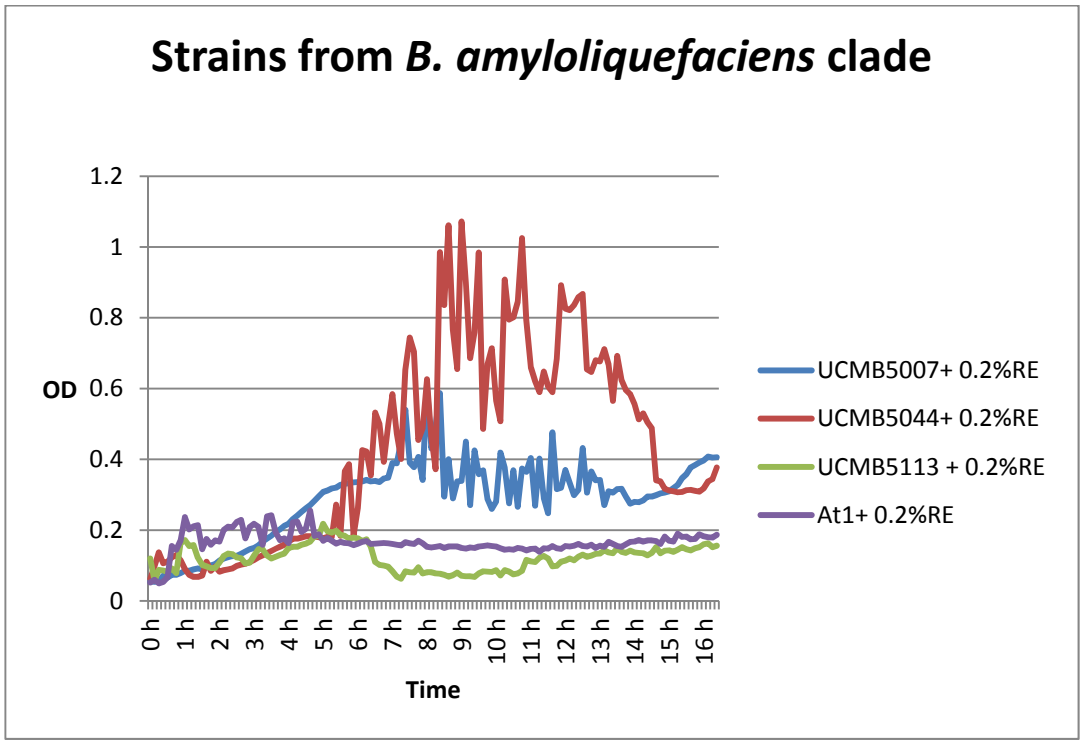


Figure 2.8: *B. amyloliquefaciens* strains growing in 0.2% RE.

The growth of UCMB-5007 on 2% RE outperformed the growth of UCMB-5044 (Figure 2.9). The growth curve of the strain UCMB-5007 was relatively smooth on the media M9 and LB, but addition of the RE caused a high amplitude opacity oscillations associated with phase transitions. The strain UCMB-5044 grew on RE as well as on M9 alone without showing any significant growth escalation (Figure 2.9 and Figure 2.4 respectively). Surprisingly, At1 and UCMB-5113 grew on RE even worse than on M9 (Figures 2.8, 2.9 and Figure 2.4 respectively).

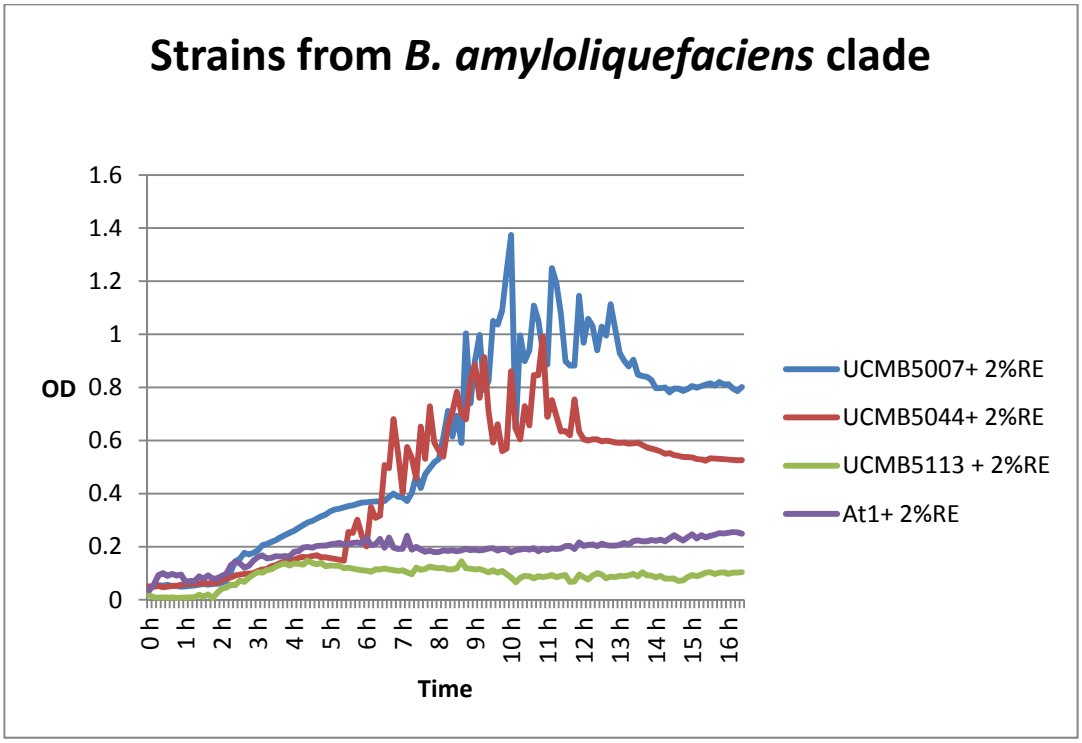


Figure 2.9: *B.amyloliquefaciens* strains growing in 2% RE.

The strains *B. subtilis* ssp. *subtilis* UCMB-5021and *B. mojavensis* UCMB-5075 responded positively to the addition of the root exudates. The growth curve of UCMB-5021was characterized by a significant lag phase at the beginning; on the medium with 2% RE, but eventually, it outgrew the strain UCMB-5075. An interesting observation was that the addition of RE stopped the growth curve oscillations, which were characteristic for the strain UCMB-5021 on M9 medium without RE. The response of the strains *B. atrophaeus* UCMB-5137 and *B. subtilis* ssp. *spizizenii At3* to the RE addition was rather moderate with a long lag phase that lasted for 5 h (Figures 2.9 and 2.11). A characteristic deflection of the growth curve of the strain UCMB-5137on the 9^{th}-10^{th} h of the growth may be associated with formation of the micro-biofilm; however, this hypothesis was not checked.

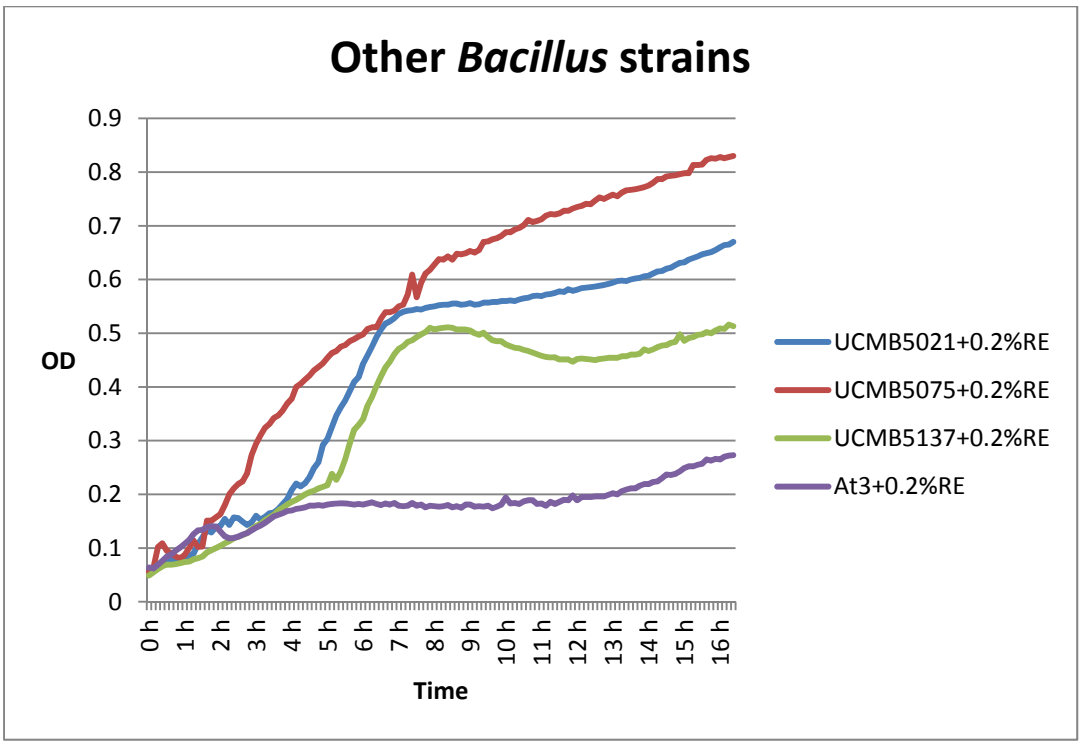


Figure 2.10: *Bacillus* strains growing in 0.2% RE.

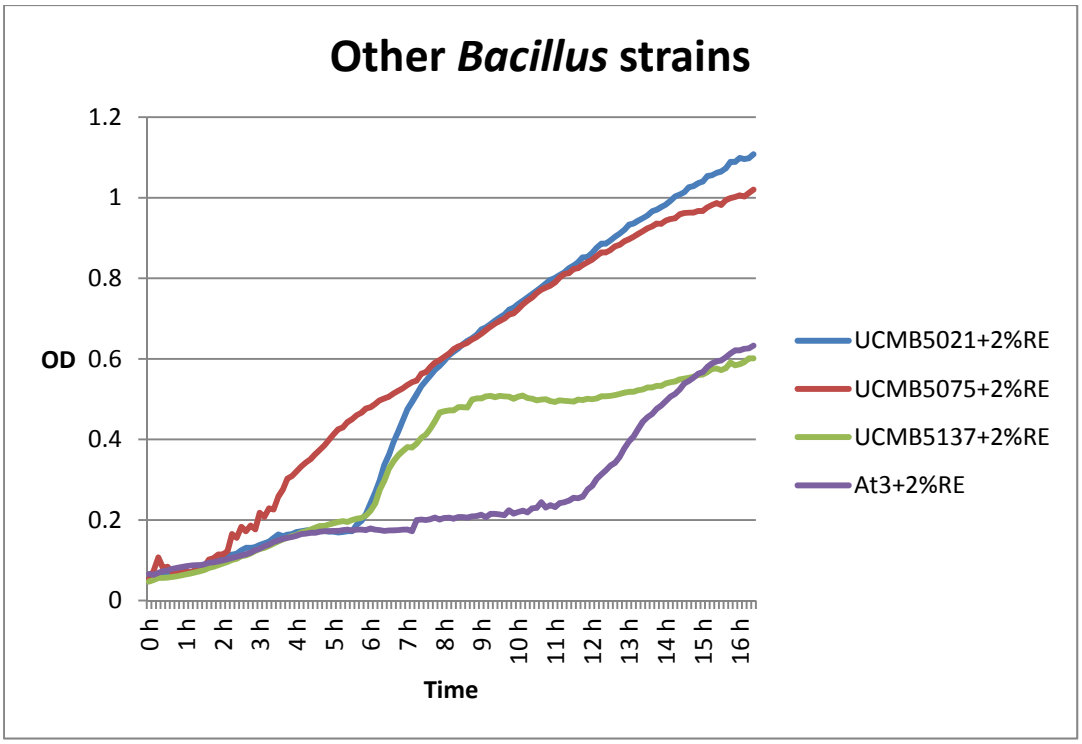


Figure 2.11: *Bacillus* strains growing in 2% RE.

This experiment showed that the closely-related strains of the group *B. subtilis/B. amyloliquefaciens* have quite different media preferences. The strains *B. mojavensis* UCMB-5075 and *B. amyloliquefaciens* ssp. *plantarum* UCMB-5044 grew better on nutrient-poor mineral media, but responded positively to the root exudates supplements. *B. mojavensis* was isolated for the first time from the soil of the Mojave Desert (Roberts et al., 1994). The strain UCMB-5044 was isolated from tissues of cotton plants cultivated in the arid areas of the Tadjikistan. An adaptation to nutrient-poor environments may be characteristic for the strains of *Bacillus* isolated from desert soils and plants. Also it may be assumed that these strains are better suitedfor plant promotion and biocontrol in arid areas. On the contrary, the red-pigmented strains *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113 (isolated from the nutrient rich Ukrainian black soil) and At1, and the black pigmented *B. subtilis* ssp. *spizizenii* At3 (the latter two strains were isolated in Sweden from the germinated surface sterilized seeds of *A. thaliana*) required nutrient-rich media, and a supplementation of the M9 medium with the RE was not sufficient to maintain their growth. In previous yet unpublished studies, the pigmented strains of *Bacillus* were supposed to be plant endophytes that may explain their requirement of a nutrient rich media. Other strains of the *B. subtilis* and *B. amyloliquefaciens* clades had intermediate requirements and responded to the RE addition in various ways. It may be concluded that these strain-specific growth requirements of the PGPR *Bacillus* should be taken into consideration when new biopesticides/biofertilizers are designed. Shapes of the growth curves were highly reproducible in two independent experiments including the numbers and amplitudes of the auto induced phase transitions characteristic for several strains. The standard deviation of the recorded values was 0.00047 OD.

2.3.2 Re-isolation of *Bacillus* strains from wheat roots

In the current experiment, a repeated isolation of bacteria from wheat roots was controlled. It was published before that many *Bacillus* cannot survive even 10 min on the plant roots, most likely because of the oxidative stress caused by plant tissues (Reva et al., 2004). In the current work, suspensions of 10^7 cells per ml of the *Bacillus* strains were applied on the wheat roots grown up in water in Petri dishes. After 1 min of incubation at room temperature, the suspensions were removed and the roots were dipped into 10 ml of water then incubated at 25°C overnight. Aliquots of 1 ml were then plated on LB medium to count Cfu. Cfu numbers were normalized by the root weights (Fig. 2.12).

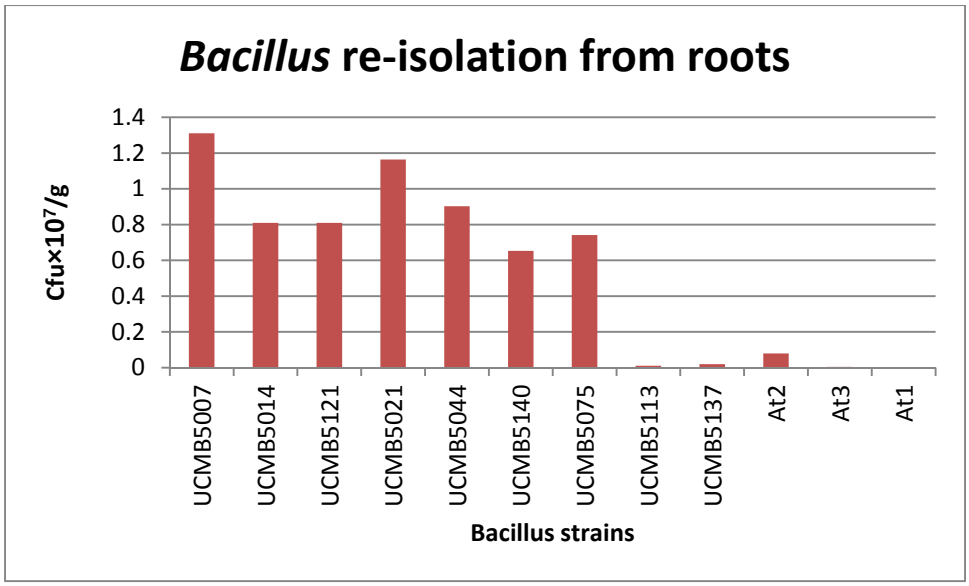


Figure 2.12: The normalized Cfu×10^7/g counts of bacterial cells washed out from wheat roots after an overnight incubation. The difference between Cfu of the first 7 and last 5 cultures was statistically reliable with $p < 0.05$.

It was found out that all the strains of the *B. amyloliquefaciens* ssp. *plantarum* except for two representative of this species with an unusual red pigmented phenotype (UCMB-5113 and At1), and also the strain *B. mojavensis* UCMB-5075 could be found in wheat roots in high titres, while the titres of cells of five other strains was remarkably lower. This experiment demonstrated a significant difference in biology of the PGPR *Bacillus*; however, it should not be concluded that the strains UCMB-5113, UCMB-5137, At1, At2 and A3 could not survive on plant roots. In previous studies, the fluorescent microscopy of roots of the model plant *Hordeum vulgare* var. Optic treated with cells of the *B. atrophaeus* UCMB-5137 and *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113 did prove that the bacteria were able to colonize plant roots by creating thick biofilm layers; however, individual cells outside the biofilm indeed died (Fig. 2.13). Biofilm formation may take longer than 30 min of the experiment and if it had happened, it could be difficult to wash these cells out from the root surface. It may be the reason for the differences in the Cfu numbers observed in this experiment. A more detailed analysis of gene regulation by root exudates stimuli in comparison between the *B. atrophaeus* and *B. amyloliquefaciens* ssp. *plantarum* will be presented in the next chapter.

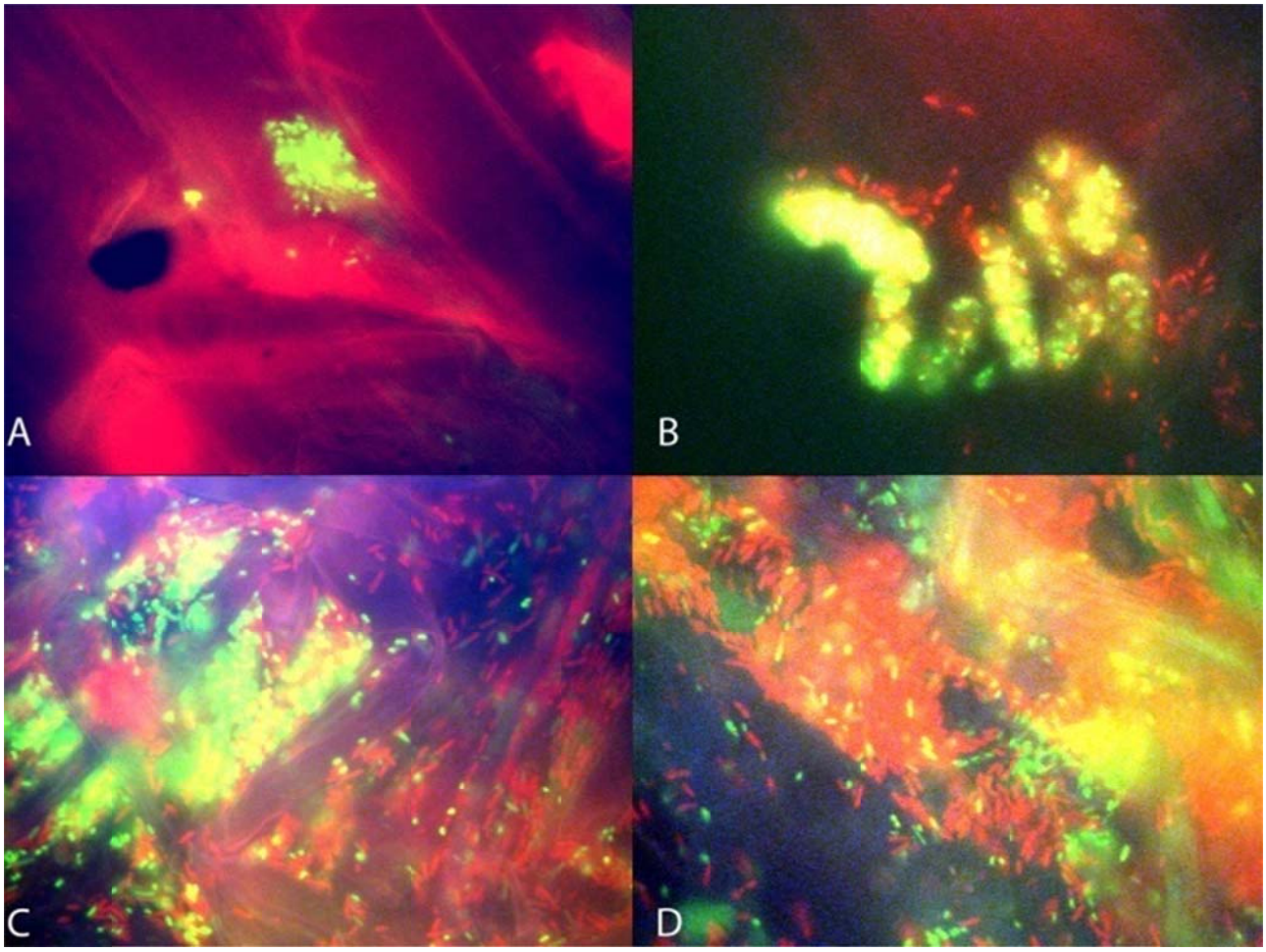


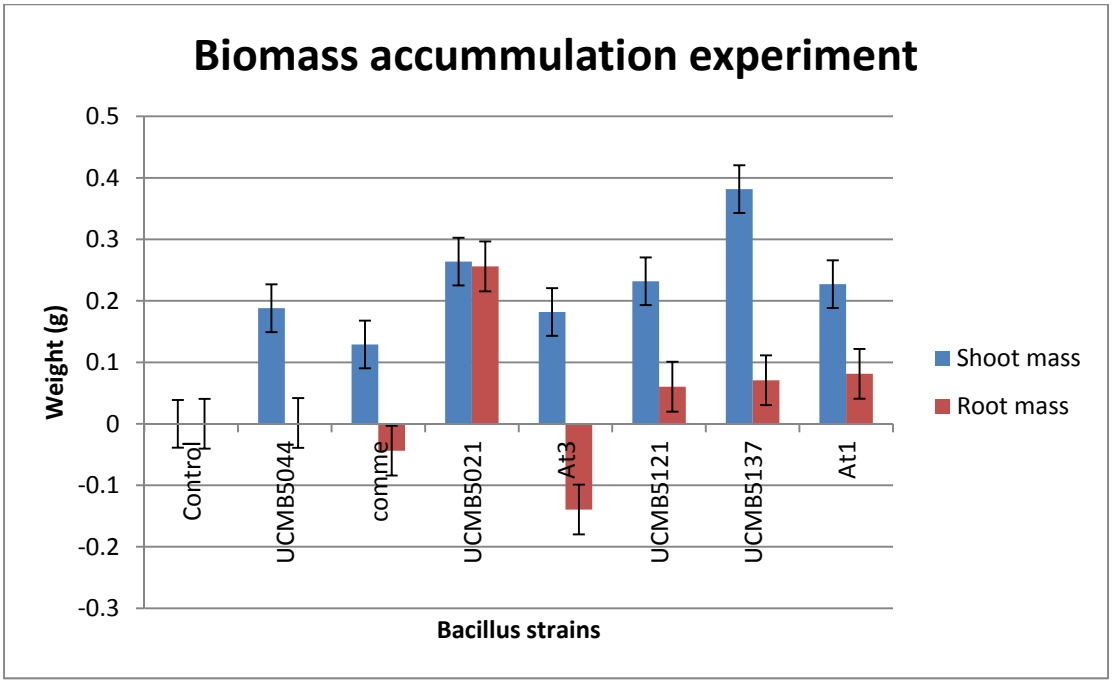
Figure 2.13: Fluorescent microscopy images of the *Bacillus* growth on barley roots. Green staining depicts live cells and the red staining indicated that the cells were dead. Root colonization by the *B. atrophaeus* UCMB-5137 and by the *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113. This previously unpublished photo was donated by Prof. O. Reva are shown in sections A and B for *B. atrophaeus* UCMB-5137 (sections C and D) for *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113

2.3.3 Plant growth promotion

Bacterial strains used in this study were cultivated until they had reached adensity of 1×10^7 cells per ml.Suspensions of bacterial cells were prepared and used for treatment of the soil with the germinated wheat seedlings as described above (section 2.2.1).

The effect of the treatment of seedlings by bacterial spores was controlled by weighing the harvested roots and shootings. The mass of shootings increased, compared to the control in all experiments. However, the mass of roots remained more or less the same or in several cases, even decreased (Fig.

2.14). Possible explanations could be that the bacteria either inhibited root development directly or that there were no stimuli for a vigorous root development due to a better nutrient supply caused by the bacterial metabolic activity. It was a promising observation that the bacteria selected for this experiment were even more active than the commercial preparation used as the positive control in this experiment. The strains *B. atrophaeus* UCMB-5137and UCMB-5007 showed the highest activity on shoot growth promotion. Another interesting strain was the *B. subtilis* ssp. *subtilis* UCMB-5021, which actively promoted both plant growth and root development. Treatment of the wheat seedlings with suspensions of the *B. amyloliquefaciens ssp. plantarum* UCMB-5007, UCMB-5113 and *B.* UCMB-5075 *mojavensis* stimulated shoot growth. Strain UCMB-5007 also promoted root growth; however, the strains UCMB-5075 and UCMB-5113 inhibited it. Root growth was also inhibited by the *B. subtilis ssp. spizizeni* At2 and by the commercial preparation used as a positive control (Fig 2.14).



A.

B.

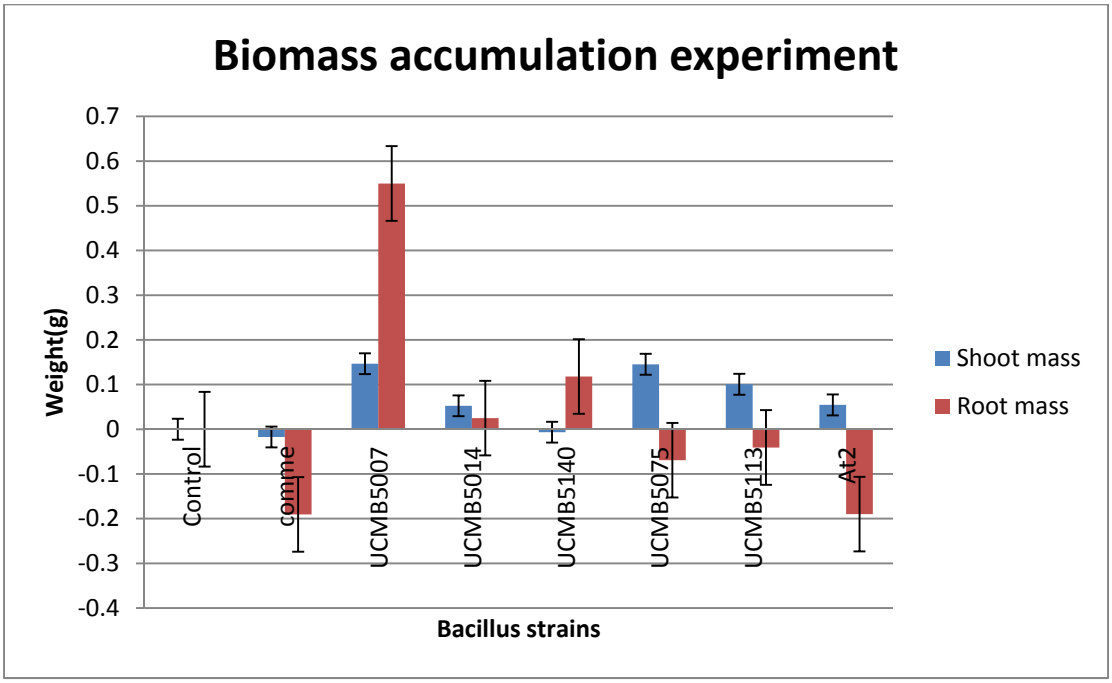


Figure 2.14: Average weights of the wheat plants biomass obtained after seed treatment with the *Bacillus* strains compared to the untreated plants (zero line);A) plants treated with the strains *B. amyloliquefaciens* ssp. *plantarum*UCMB 5044, *B. subtilis* ssp. *subtilis*UCMB-5021, UCMB5017, *B. atrophaeus* UCMB-5137, *B. amyloliquefaciens* ssp. *plantarum* At1, *B. subtilis* ssp. *spizizenii* At3 and the commercial preparation used as a positive control (comme); B) plants treated with the strains *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007, UCMB-5014, UCMB-5140, UCMB-5113, *B. mojavensis* UCMB-5075, *B. subtilis* ssp. *spizizenii* At2 and the commercial preparation used as a positive control (comme).

Our results are consistent with the previously published reports on plant growth promotion by *Bacillus* cell suspensions (Idris et al., 2007; Bai et al., 2002, Bashan et al., 2013), where it was proposed, that the PGPR brings about desirable effects after it comes into contact with the plant root, attaching to the root surface and colonizingthe plant root. PGPR promote root and shoot growth by improving plant nutrition and influencing the physiology of the whole plant (Vacheron et al., 2013).

2.3.4 Inhibition of phytopathogenic fungi

Inhibition of four fungal pathogens was studied for eight strains of the *Bacillus*. *Sclerotinia sclerotiorum* is a fungus that affects cultivated and wild plants, including oilseeds, pulses, forage legumes, vegetables and ornamentals (Purdy, 1979) with a wide host range of about 75 families and

225 genera (Bolland and Hall, 1994). It damages plant tissues, causing soft rot or white mould of the plant (Purdy, 1979). It causes stem rot in *Brassica napus*, its spores (sclerotia) remain viable in the soil for a long time and whenever the environment becomes favorable, it infects the plant. *Sclerotinia sclerotiorum* infection was managed by conventional methods, chemical fungicides and biological control (Fernando et al., 2004). Conventional methods such as crop rotation were not effective while "accumulation of pesticide residues in the edible parts threatened the scope for export of the commodities to other countries" (Fernando et al., 2004). Some *Bacillus* species and other bacteria showed potential to control *Sclerotinia sclerotiorum* infection (Fernando et al., 2004; Hu et al., 2011; Monteiro et al., 2013).

Alternaria brassicicola is a fungus causing a dark leaf spot of crucifers (vegetables of the family Brassicaceae) including broccoli, cabbage, canola, and mustard. It secretes numerous toxic secondary metabolites and proteins that cause plant cell death (Mamgain et al., 2013). It can affect the whole plant, including pods, seeds, and stems, and it is also a post-harvest disease, which may result in 20-50% yield loss (Genomeportal.jgi.doe.gov, 2017). *Alternaria brassicicola* infection is managed by conventional methods (water treatment of seeds at 50°C for 30 min), chemical fungicides, resistant plants, herbal extracts and natural products and bio-agents (Mamgain et al., 2013). Application of fungicides is a common method of controlling *Alternaria*, however, since this method is associated with several health hazards, emphasis is put on the use of other economical, safe and eco-friendly methods such as growing disease resistant varieties, the use of plant and natural products, bio-control agents and alterations in agronomic practices (Mamgain et al., 2013). The eight strains that were tested in this study inhibited the *Alternaria brassicicola* (Table 2.1, Table 2.2); they are therefore potential biocontrol agents for the dark leaf spot disease of crucifers.

Verticillium longisporum growth was inhibited by *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007, At1 and UCMB-5113. It was also inhibited by the *B. subtilis* ssp *subtilis* UCMB-5021, *B. atrophaeus* UCMB-5137, and *B. subtilis* ssp *spizizeni* At3 (Table 2.1, Table 2.2). *Brassica napus* is the primary host of *Verticillium longisporum* (Zeise and von Tiedemann, 2002), when it infects, it causes an early senescence and ripening of the host plant which reduces yield (Phytopathology.uni-goettingen.de, 2017). Verticillium wilt is managed by conventional methods, use of disease resistant varieties, elemental sulphur as an antifungal agent and potential bacteria strains (Lindbeck and Plant Health Australia, 2011).

The results of inhibition of the *Sclerotinia sclerotiorum*, *Alternaria brassicicola* and *Verticillium longisporum* around the wells with filtered supernatant fluids of the bacterial cultures and around the colonies of *Bacillus* grown from spore inoculates on the 8th day after inoculation, are shown in Table 2.1, and on the 14th day – in Table 2.2.

Table 2.1: Inhibition of fungal growth by the selected *Bacillus* strains on the 8th day after inoculation

***Bacillus* strains**	**Fungal pathogens**		
	Sclerotinia sclerotiorum	***Alternaria brassicicola***	***Verticillium longisporum***
5007	+/+	++/++	++/++
5021	-/-	++/++	-/++
5044	-/++	++/++	-/-
5075	-/-	+/++	-/-
5113	-/-	+/++	-/++
5137	+/++	+/++	-/++
At1	-/+	+/++	-/+++
At3	-/+	+/++	-/++

Supernatant samples/spores samples growth inhibition values are: '-'– no inhibition; '+' - zones of inhibition in average are between 3-5 mm; '++' - zones of inhibition in average are between 6-10 mm; '+++' - zones of inhibition in average are above 10 mm; results are given in a form: filtered supernatant inoculation/bacteria spore inoculation (section 2.2.1 in the methods).

The highest inhibition activity against these three fungal phytopathogens was shown by the strain *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007. The inhibition zones were observed around the supernatant samples and spore samples that indicated an extracellular extraction and accumulation of the antifungal compounds synthesized by this bacterium. Another active strain was the

B. atrophaeus UCMB-5137; however, the inhibition activity of its supernatant samples was weaker. All the tested strains inhibited the growth of *A. brassicicola*. Supernatant samples of these bacteria were less active than the bacterial cultures grown from the spores. All the tested *Bacillus* strains were inactive against the *Fusarium oxysporum*– a fungal pathogen which causes vascular wilt in a variety of plants.

Table 2.2: Inhibition of fungal growth by selected strains of *Bacillus* on the 14th day after inoculation

***Bacillus* strains**	**Fungal pathogens**		
	Sclerotinia sclerotiorum	***Alternaria brassicicola***	***Verticillium longisporum***
5007	+/+	+/++	++/++
5021	-/-	+/++	-/++
5044	-/++	++/++	-/-
5075	-/-	+/++	-/-
5113	-/-	+/++	-/++
5137	+/++	+/++	-/++
At1	-/+	+/++	-/+++
At3	-/+	+/++	/++

Supernatant samples/ spores samples growth inhibition values are: '-'– no inhibition; '+' - zones of inhibition in average are between 3-5 mm; '++' - zones of inhibition in average are between 6-10 mm; '+++' - zones of inhibition in average are above 10 mm.

2.3.5 Induction of drought tolerance in plants by treatments with *Bacillus*

Several papers have been published, which reported a possibility to inducedrought tolerance of agricultural plants by treating them with preparations based on PGPR (Yang et al., 2009;

Vardharajula et al., 2011). "Although the exact mechanisms of plant drought stress tolerance enhancement by rhizosphere bacteria remain largely speculative, possible explanations include: production of hormones like abscisic acid, gibberellic acid, cytokinins, and auxin, production of the essential enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants, induced systemic resistance by bacterially produced compounds and formation of the bacterial biofilm" (Timmusk et al., 2014). Other studies have shown that the application of the PGPR endophytes (Naveed et al., 2014) including *Bacillus* species (Hu, 2008) increased the chlorophyll content.

In the current project, the *Brassica napus* seeds were treated with a $1x10^7$ of the bacteria inoculum and then exposed to drought conditions as explained in section 2.2.6 of the methods. Phenotypes of plants from the different groups were documented by photography (Fig. 2.15). To determine the photosynthetic efficiency of the plants, the concentration of chlorophyll in leaves was measured withchlorophyll meter. Concentrations of chlorophyll in leaves were compared between treated and non-treated plants.The wet and dry masses of the harvested shoots were compared between treated and non treated plants. The comparison included the plants subjected to the drought conditions and plants under the normal regiment of watering. Significant differences were determined by calculating mean and standard deviation in Excel.

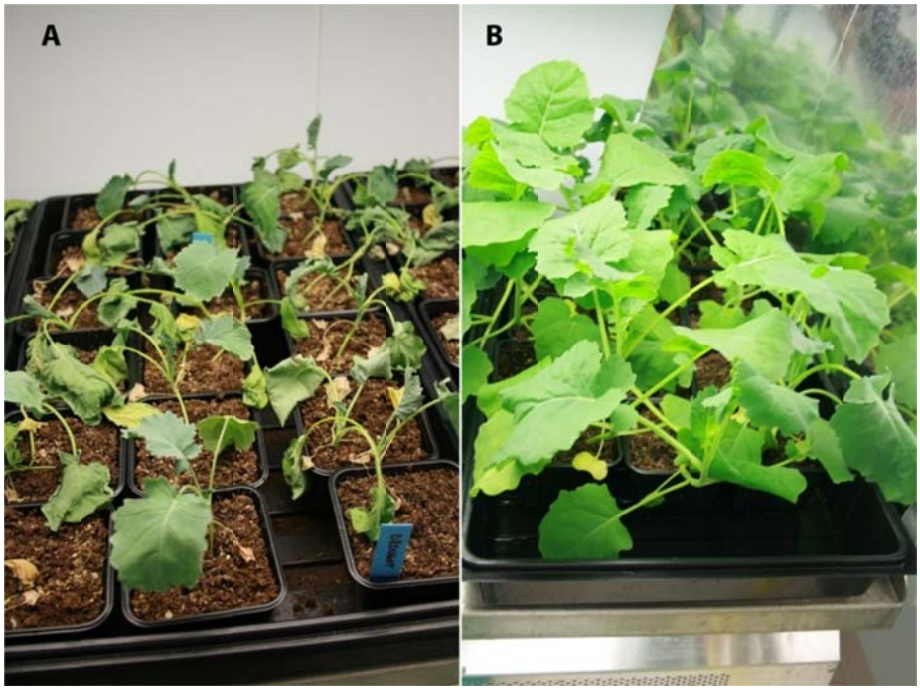


Figure 2.15: The drought tolerance experiment: A) plants of the group subjected to the drought conditions for 10 days; B) watered plants of the same age.

Figure 2.16 shows the concentrations of chlorophyll in plants of different groups. The first observation was that the treatment of seeds by spores of the PGPR *Bacillus* had usuallyincreased the chlorophyll concentration in seedlings except for plants treated with the *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113 and *B. atrophaeus* UCMB-5137, in which the chlorophyll concentration remained the same as in the control plants. However, these strains made the plants more resistant to the drought conditions in terms of their chlorophyll loss. The percentage of the chlorophyll loss under drought conditions in the treated plants and in the control group is shown in Fig. 2.17. The effect of the strains *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007 and *B. subtilis* ssp. *subtilis* UCMB-5021should be explained. Treatment of plant seeds by the spores of these strains resulted in the highest level of chlorophyll in the seedlings. However, this high level of chlorophyll in these plants was sensitive to abiotic stresses and under drought conditions, it dropped to the level recorded in the untreated plants in the case of UCMB-5007, or still remained on a relatively high level in the case with UCMB-5021. Promising results were obtained for the strain *B. amyloliquefaciens* ssp. *plantarum* UCMB 5044, which increased the level of chlorophyll in seedlings and improved the drought tolerance of the plants by keeping this high level of chlorophyll under stress conditions.

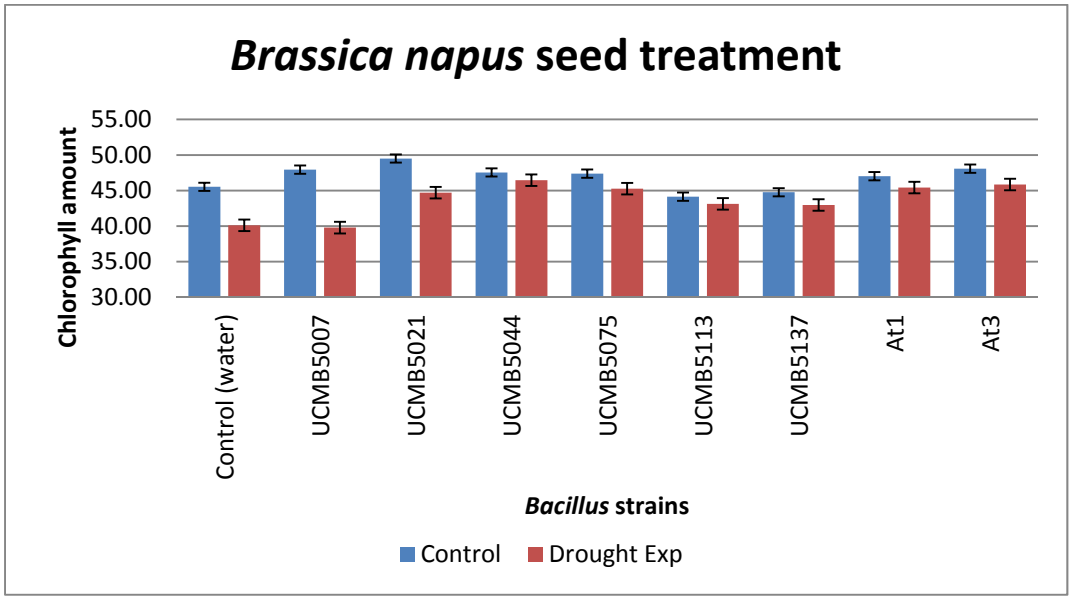


Figure 2.16: Concentration of chlorophyll in leaves of seedlings of *Brassica napus* plants grown from seeds treated with the *Bacillus* strains. Groups of plants watered regularly are depicted by the blue bars; groups of plants subjected to drought conditions are depicted by the red bars.

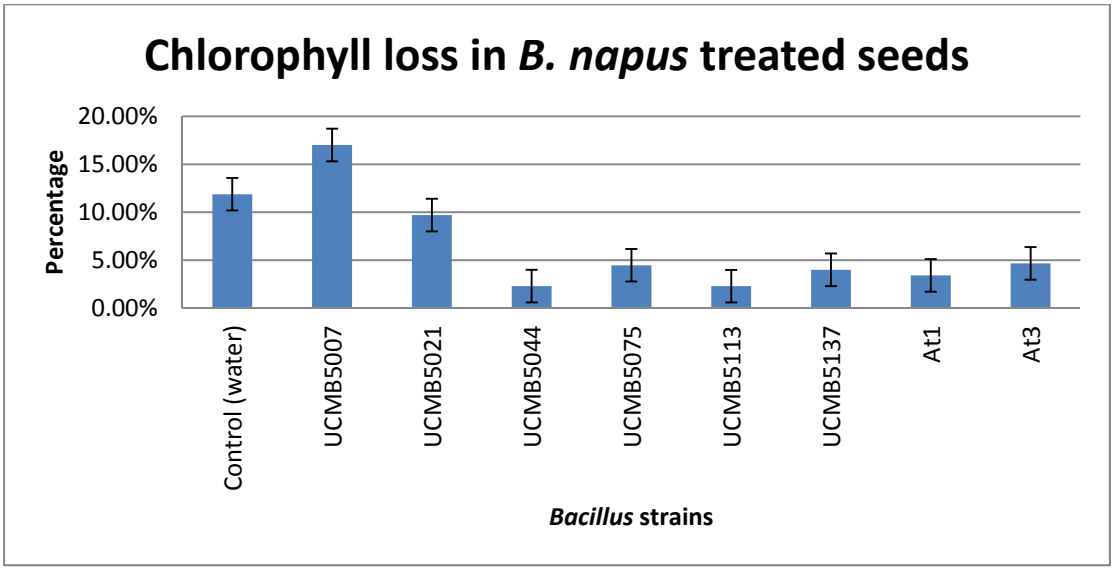


Figure 2.17: Percentage of chlorophyll loss in *Brassica napus* plants subjected to drought conditions.

It should be noted that the chlorophyll concentration in leaves is not a direct indication of plant tolerance and health. In the following experiments, the plants from the different groups were weighed to find out how the drought and plant treatment by the PGPR *Bacillus* may have affected biomass accumulation by the plants. The mass loss under the drought conditions was calculated as the percentage of the mass difference in comparison to the plants grown under the normal regiment of watering. Variations of plant mass in different pots with the plants of the same group were calculated (Figure 2.18). In general, the mass loss in treated plants was in the same level as in the control group. The highest mass loss was observed in the plants treated by the strain *B. amyloliquefaciens* ssp. *plantarum* UCMB-5044which indicated that a preservation of the high concentration of chlorophyll in leaves may not be sufficient enough to improve the drought tolerance of the plants. A positive effect was reported only for two strains; *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007 and *B. subtilis* ssp. *subtilis* UCMB-5021.

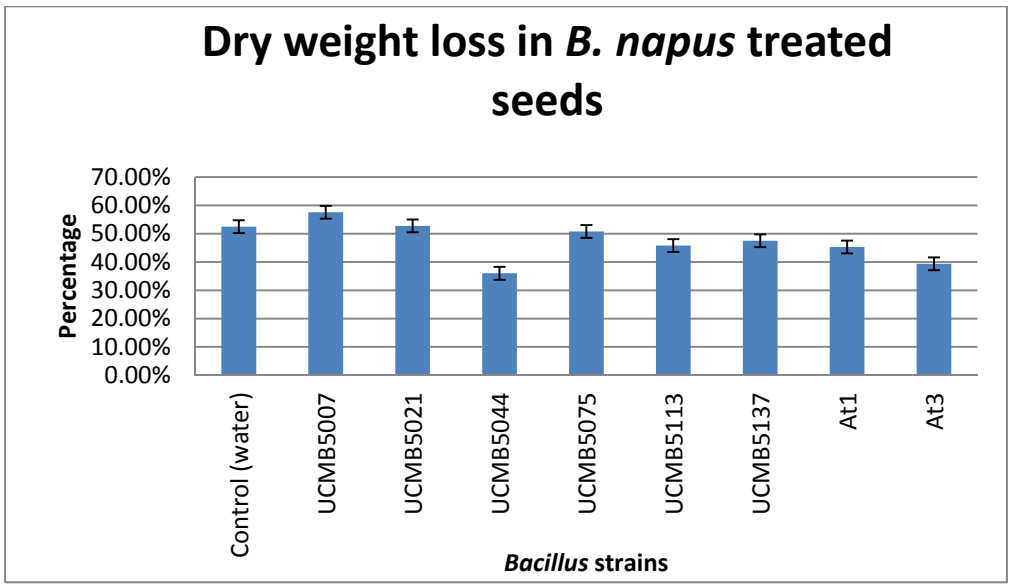


Figure 2.18: Percentage of dry weight loss when *Brassica napus* seedlings arising from seeds treated with *Bacillus* strains were subjected to drought conditions.

Different schemes of application of the PGPR in biopesticides and biofertilizers are used in agricultural practices: the preparations may be used for seed treatment before sowing; for treatment of leaves of seedlings or matured plants, or the preparations may be applied to the soil. In the following set of experiments, the bacterial cultures were inoculated into the rhizosphere of the germinated seedlings before they were exposed to the drought conditions. Then, the chlorophyll concentration and biomass loss were measured as in the experiment described above. Surprisingly, the results were quite different. Inoculation of soil of all strains of the *Bacillus* caused a significant loss of the chlorophyll in leaves of the seedlings even if they were watered regularly. In the case of the drought conditions, the chlorophyll loss was even higher (Fig. 2.19).

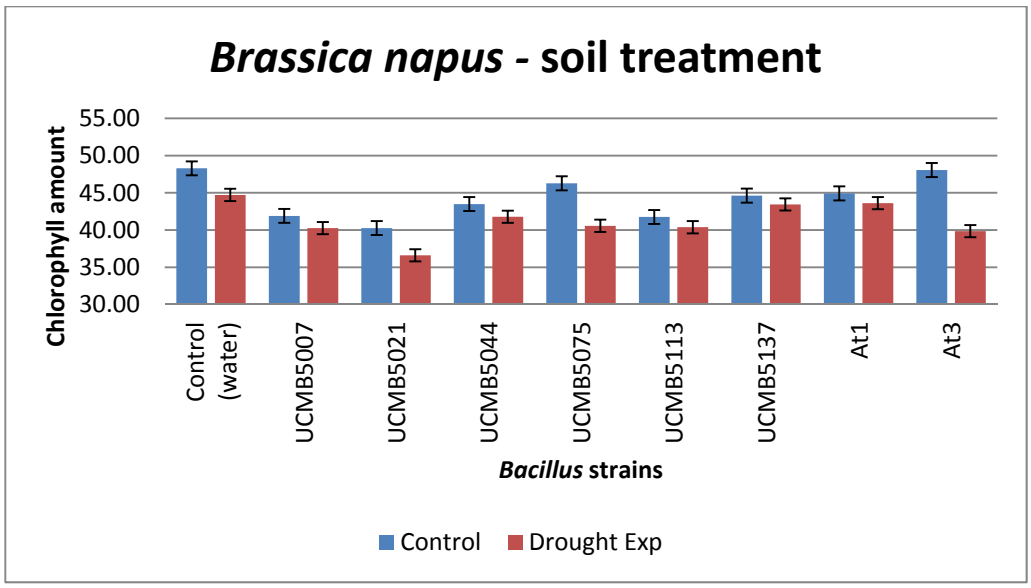


Figure 2.19: Concentration of chlorophyll in leaves of *Brassica napus*plants under the normal regiment of watering (blue bars) and under the drought conditions (red bars).

The percentage of chlorophyll loss under stress conditions is shown in Figure 2.20. It should be noted that even if the percentage was not high, the concentration of chlorophyll in leaves of the treated plants, was lower than in untreated plants. A conclusion may be made that the treatment of seeds would be a better practice to improve the drought tolerance of plants rather than the introduction of bacterial suspensions into the rhizosphere of seedlings.

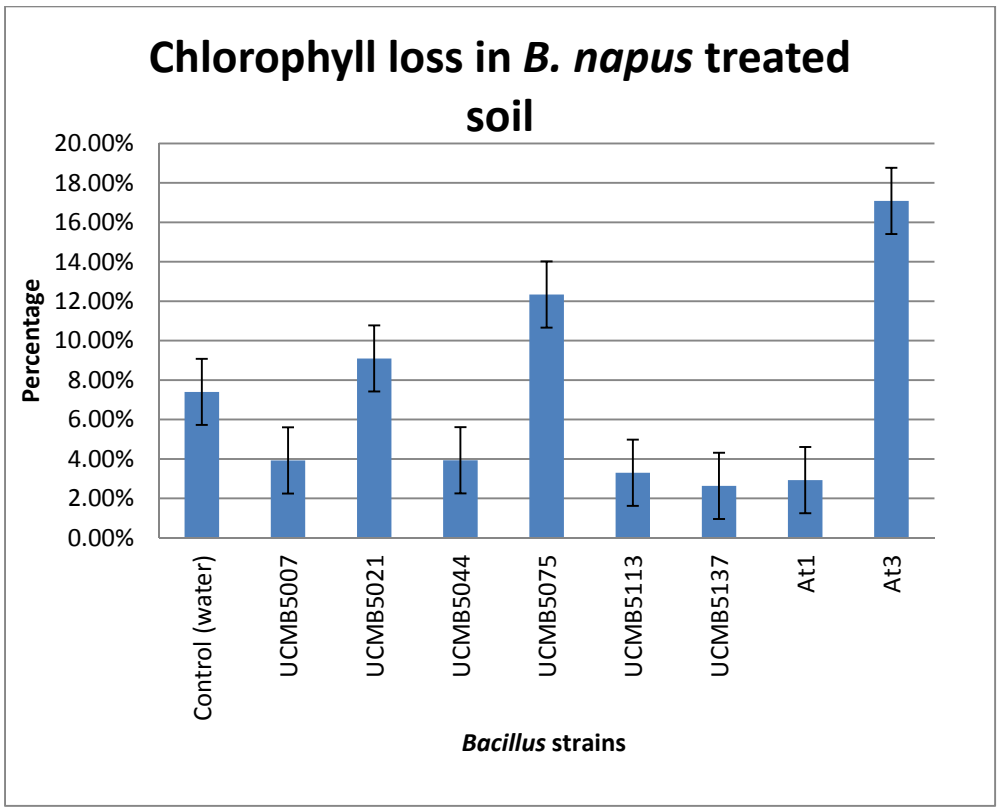


Figure 2.20: Percentage of chlorophyll lost after soil, where *Brassica napus* was growing, was treated with the bacteria then subjected to drought conditions.

This conclusion was confirmed by measuring of the mass loss in treated and untreated plants after the exposure to the drought conditions (Figure 2.21). Usually, the mass loss in the treated plants was the same or higher compared to the untreated plants. A positive protective effect was recorded only for the plants treated by the *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007.

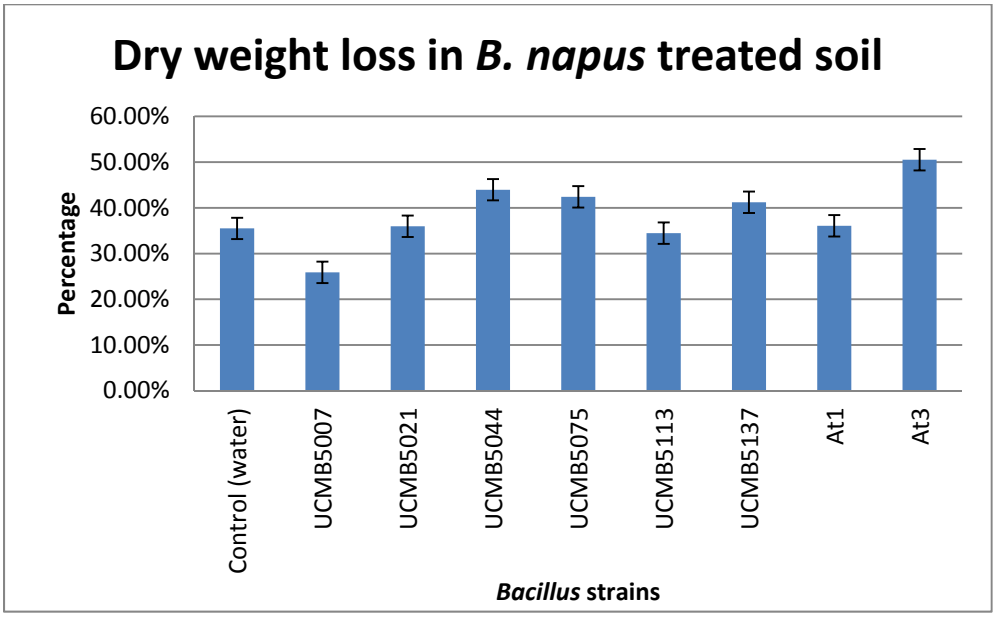


Figure 2.21: where *Brassica napus* was growing, was treated with the bacteria then subjected to drought conditions. Percentage of dry weight lost after soil, where *Brassica napus* was growing, was treated with the bacteria then subjected to drought conditions.

It is known that the results on plant growth promoting or protection received on one model plant may not be applicable to other plant species. For example, the paradigm PGPR strain *B. amyloliquefaciens* ssp. *plantarum* exploited the different strategies of colonization of the different plant hosts (Fan et al., 2012). To check this hypothesis, the described above experiment on seed treatment by spores of the *Bacillus* performed on *Brassica napus* seeds was repeated on *Arabidopsis thaliana*.

Arabidopsis thaliana seeds were treated by spore suspensions of $1x10^7$ cfu/ml as described above. Chlorophyll content was not measured in *A. thaliana* because the leaves were too small for SPAD measurement. The treatment of seed improved the drought tolerance of *A. thaliana* plants (Fig. 2.22). The highest positive effect was obtained for the strain *B. mojavensis* UCMB-5075 followed by the *B. amyloliquefaciens* ssp. *plantarum* UCMB-5113 and At3. The strains *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007 and *B. subtilis* ssp. *subtilis* UCMB-5021, which were the most effective in the experiment on *B. napus* seeds, showed a rather moderate positive effect on the *A. thaliana* plants.

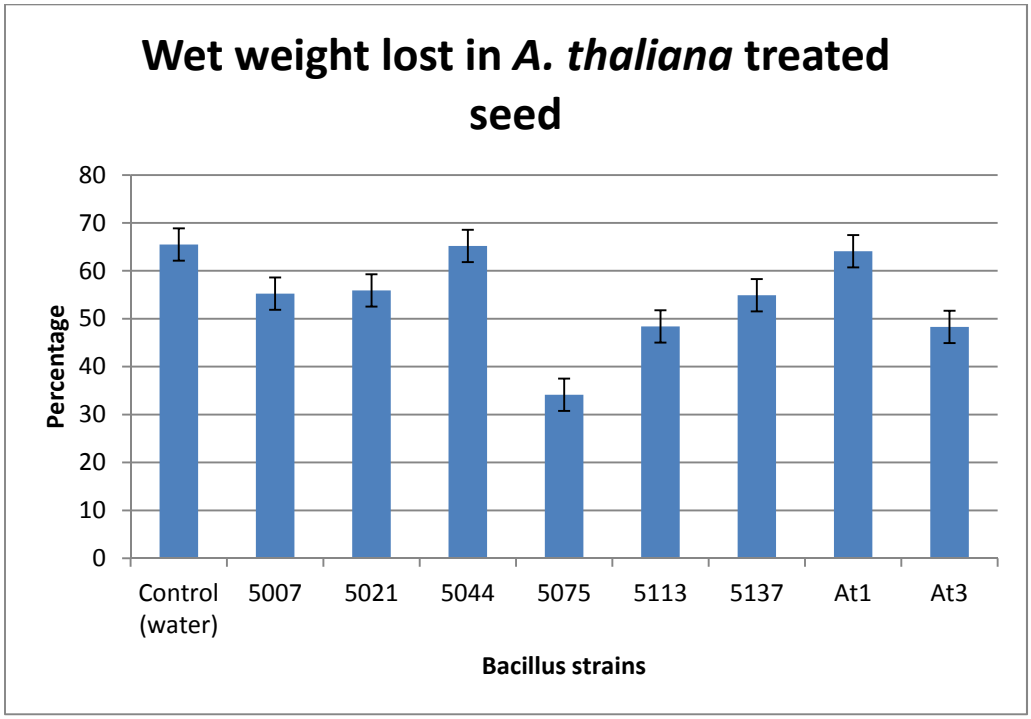


Figure 2.22: Percentage of wet weight loss in *A. thaliana* plants under drought conditions in controls and after seed treatment with the *Bacillus* spores.

Inoculation of the *Bacillus* spores into the rhizosphere of *A. thaliana* was not effective or even negative with all the strains of *Bacillus* except for the *B. amyloliquefaciens*ssp *plantarum* At1, which significantly reduced the weight loss of the *A. thaliana* plants under drought conditions (Fig. 2.23).

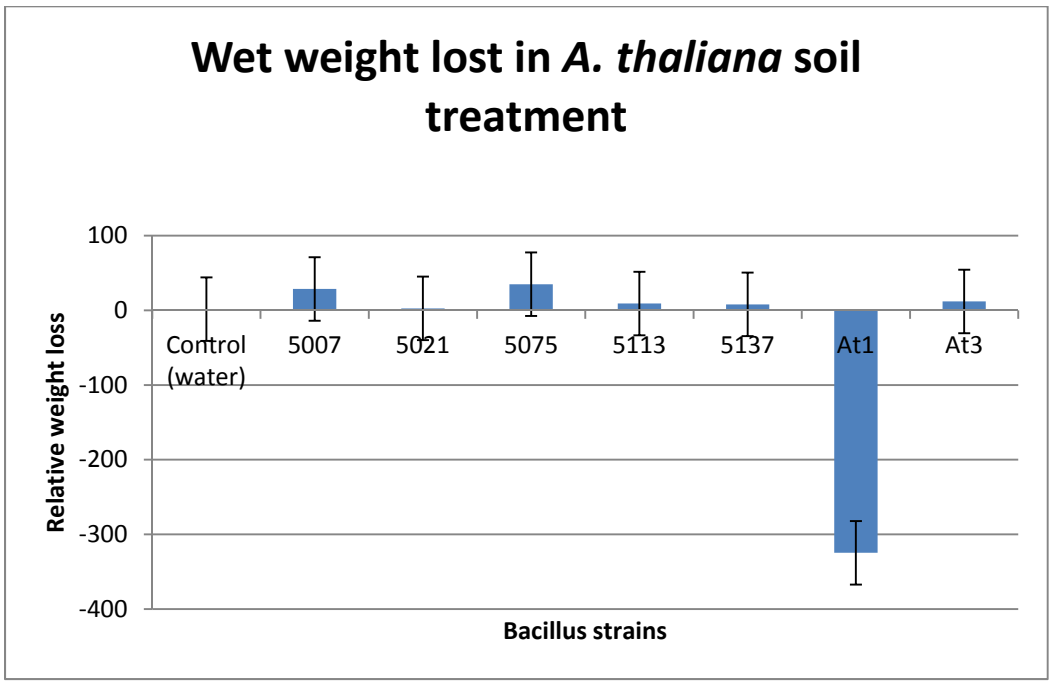


Figure 2.23: The relative weight loss in *A. thaliana* plants under drought conditions after soil treatment in relation to the weight loss in the control group of plants.

2.4 Conclusion

The selected *Bacillus* strains showed the PGPR activities in various biotrials as summarized in Table 2.3.

Table 2.3: A comparison of strains from the results obtained in section 2.3

Method	**Section**	**The strains showing the highest activity**
Growth in:	2.3.1	
M9		UCMB 5044
0.2% RE		UCMB-5021,UCMB-5075 and UCMB 5044
2% RE		UCMB-5021and UCMB-5007
LB		All
Plant growth promotion	2.3.3	UCMB-5137and UCMB-5007
Inhibition of phytopathogenic fungi	2.3.4	
Sclerotinia sclerotiorum		All except UCMB-5075, UCMB-5021and UCMB-5113
Verticillium longisporum		All except UCMB-5075 and UCMB 5044
Alternaria brassicicola		All
Inducing drought tolerance in plants	2.3.5	All

The results from this chapter presented a series of active plant promoters and protectors that may be suitable for biotechnological application in future. *B. atrophaeus* UCMB-5137and *B. amyloliquefaciens* ssp *plantarum* UCMB-5007 actively promoted wheat growth, while *B. mojavensis* UCMB-5075 and *B. atrophaeus* UCMB-5137 were active root promoters in *Arabidopsis*

thaliana. All *Bacillus* strains effectively inhibited the plant pathogen *Alternaria brassicicola*; however they were defeated by *Fusarium oxysporum*. In *Brassica napus*, all strains maintained plant chlorophyll amount during drought except for the UCMB-5021and UCMB-5007 (for seed treated), UCMB-5021, UCMB-5075 and At3 (for soil treated). When water was not available, shoot weight was maintained by UCMB-5044(for seed treated) and UCMB-5007 (for soil treated) in *Brassica napus*. In *Arabidopsis thaliana*, the best performing strain was UCMB-5075; it preserved plant weight when water was not available to plants. Some strains had poor activity in all tests, for example the UCMB-5121, while others were active in more than one tests, for example, the UCMB-5137and UCMB-5007. Further research was aimed at investigating gene regulation in active PGPR strains under the root exudates stimuli. Patterns of gene regulation are available from literature for the paradigm of PGPR – *B. amyloliquefaciens* ssp. *plantarum* FZB42. We decided to investigate gene regulation in the strain *B. atrophaeus* UCMB-5137 that showed remarkable PGPR activities. *Bacillus atrophaeus* isolates are abundant in soils but they have never been reported as active plant growth promoters. By comparison of the transcription profiles developed for this strain and for the strain FZB42, the extent of patterns overlap in different rhizobacteria will be identified.

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Chapter 3: REGULATION OF GENE EXPRESSION IN *BACILLUS ATROPHAEUS* UCMB-5137 STIMULATED BY MAIZE ROOT EXUDATES

Abstract

In the previous chapter, the strain *B. atrophaeus* UCMB-5137 demonstrated plant growth promotion and protection activities. In the current chapter, gene regulation pattern of the strain was studied under maize root exudates stimuli. Genes and metabolic pathways up- and down-regulated by maize root exudates were identified. Transcription factors and non coding RNAs controlling gene regulation were also identified.

3.1 Introduction

The strain *B. atrophaeus* UCMB-5137 is a gram-positive, aerobic, endospore-forming, rod-shaped bacterium (Nakamura, 1989). It was isolated from the rhizosphere in Ukraine in 1989 and at first, based solely on the phenotype; and was identified as *B. subtilis*. In an array of bioassays, *B. atrophaeus* UCMB-5137 showed the ability to protect plants and crops from bacterial and fungal phytopathogens, and to promote plant growth (Lapa and Reva, 2005). The complete genome sequence of *B. atrophaeus* UCMB-5137 has been achieved recently: it showed multiple horizontally-acquired unique genes, which were hypothesized as possible sources of an extraordinary activity in plant root colonization (Chan et al., 2013). This strain has also shown significant potential of promoting plant growth in greenhouse trials in this study (refer chapter two results).

To develop effective biofertilizers and biopesticides based on PGPR, it is important to better understand the genetic mechanisms underlying the interactions of these bacteria with the host plants at early stages of the rhizosphere colonization. *Bacillus* strains are common in the rhizosphere communities (Podile and Kishore, 2007; Fan et al., 2013). However, only a few studies on gene regulation in PGPR *Bacillus* have been reported. Differential gene expression regulations caused by the maize root exudates were reported previously on *Bacillus amyloliquefaciens* FZB42 that was proposed as a paradigm of PGPR *Bacillus* (Fan et al., 2012; Kierul et al., 2015). Gene regulation in the *Bacillus subtilis* BSB1 under abiotic stresses was reported by Kohlstedt et al. in 2014. It was not clear whether these reports could be extended to other PGPR *Bacillus*, as it had been found that the

activities of their biotechnological importance were strain-specific (Safronova et al., 2012). There was also very little knowledge regarding plant colonization behavior of the other bacteria of the *B. subtilis* group, such as *B. atrophaeus*.

B. atrophaeus is a common soil inhabitant. Spores of the *B. atrophaeus* have been used in biotechnology to control sterilization processes because of the resistance of these spores to extreme temperatures and chemical detergents (Pinzón-Arango et al., 2009). Except for UCMB-5137, the strains of *B. atrophaeus* were not reported in literature as active plant-associated growth promoters or protectors, in contrast to the strains of the closely related species *B. amyloliquefaciens* and *B. subtilis*. It was therefore interesting to study the bioactive strain UCMB-5137, to contribute to the body of knowledge on gene regulation in the PGPR *Bacillus*. Current knowledge on this issue is biased towards the paradigm examples of *B. amyloliquefaciens* ssp. *plantarum* (Reva et al., 2004; Chen et al., 2007; Fan et al., 2011, 2012, 2013, 2015). It was also interesting to compare gene regulation patterns between two closely related strains, both belonging to the *Bacillus subtilis* taxonomic group: *Bacillus atrophaeus* UCMB-5137 and *Bacillus amyloliquefaciens* FZB42.

Root exudates are the nutrient rich components secreted by the root (Lugtenberg and Kamilova, 2009). In various studies, it was demonstrated that the maize root exudates were useful to mimic *invitro* interactions between different plants and PGPR by initiating a range of biological responses within the bacteria (Mark et al., 2005, Broeckling et al., 2008, Fan et al., 2012, Kierul et al., 2015). Only exudates collected from sterile plants growing under artificial conditions are sufficiently concentrated to be analyzed successfully (Lugtenberg and Kamilova, 2009). Use of gnotobiotic system to study the effect of maize root exudates on bacteria has several advantages including enabling the complete recovery of root exudates and preventing interference of other organism's genes; however the results should be interpreted with enough prudence (Fan et al., 2012). It was therefore important to study the gene expression regulation in *B. atrophaeus* UCMB-5137 under the standard laboratory conditions by simulation with the root exudates.

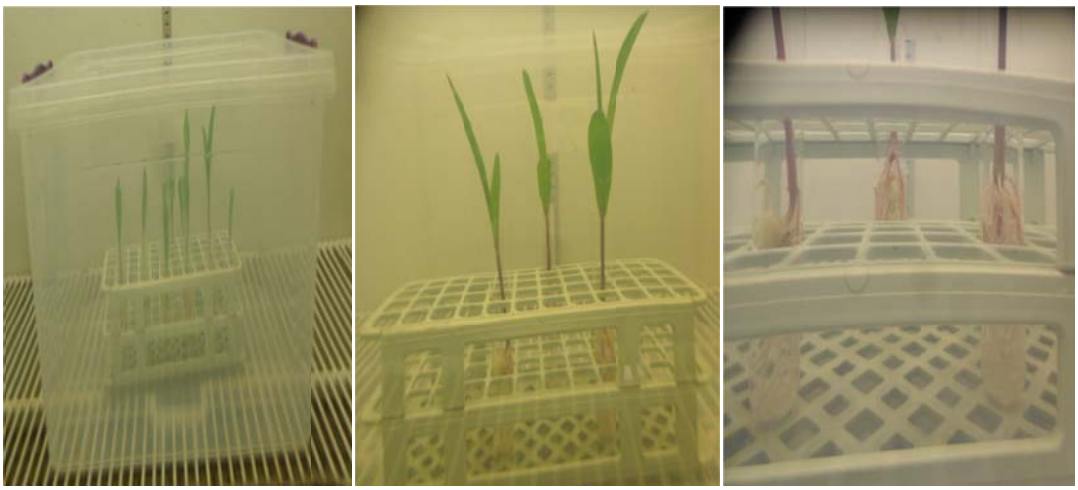
Differential transcription regulation stimulated by the root exudates in *B. atrophaeus* UCMB-5137 and *B. amyloliquefaciens* FZB42 revealed substantial differences between these bacteria. To identify other transcription factors and genes specifically involved in root colonization by the *B. atrophaeus* UCMB-5137, the obtained gene regulation profiles were superimposed over known regulatory

networks in *B. subtilis* (Kohlstedt et al., 2014; Michna et al., 2015). A possible involvement of the non-coding RNA (ncRNA) in gene regulations during plant colonization was studied.

3.2 Materials and methods

3.2.1. Root exudates preparation

Root exudates were extracted from maize roots as described by Fan et al. (2012). Seeds of the maize breed 5Q-751BR were surface-sterilized by treating them with 70% ethanol for 3 min and then with 5% (v/v) sodium hypochlorite for 3 min followed by rinsing 5 times with sterile distilled water. The seeds were germinated at 28°C until the main root was at least 2 cm long before transferring into the test tubes with sterilized water in a way that only the roots were submerged into water. The test tubes were kept in plant growth chamber (16 h light/8 h dark) at 24°C for 8 days. Water aliquots were collected daily from the third to eighth day and the tubes were refilled with the same amount of fresh sterilized water. Each collection was kept separate, and 100 µl from each sample was spread on the nutrient agar to check for contamination. Contaminated root exudates samples were discarded and the clean samples were pooled, freeze dried and stored at -20°C. The lyophilized exudates were weighted, dissolved in 100 µl of water and centrifuged. The supernatant was filter-sterilized. Concentrations of the root exudates were adjusted to 0.25 g/l and the solutions were kept as stocks at -80°C.



(a) (b) (c)

Figure 3.1: Maize plants growing (a) fourth day, (b): shoots: sixth day, (c): roots: sixth day, after their transfer tothe test tubes.

3.2.2. Bacterial growth conditions and RNA preparation

The *Bacillus atrophaeus* UCMB-5137 was obtained from the Ukrainian collection of microorganisms at the Danylo Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine, Kiev, Ukraine. Bacterial cultures were inoculated from a frozen stock culture and incubated at 37°C overnight on solid Luria agar medium (MERCK). Colonies from the overnight culture were inoculated into a 1C medium and cultivated at 24°C for 14 h with shaking at 180 rpm. Composition of the 1C medium was the same as suggested by Fan et al. (2012): 0.7% w/v pancreatic digest of casein, 0.3% w/v papain digest of soya flour, 0.5% w/v NaCl and 0.1% glucose (all the mentioned reagents were purchased from MERCK). One ml aliquots of the overnight cultures were inoculated into conical flasks with 20 ml of fresh 1C medium in control, and supplemented with 0.25 mg/ml of the maize root exudates in treatments. Control and treatment cultures were grown at 24°C for 14 h 30 min with shaking at 180 rpm to achieve the transition to stationary growth phase, which was controlled by the medium opacity of $OD_{600} \approx 1.0$ units. Growth curve analysis showed that the bacterial cultures reached the transition to stationary phase in average after 14 h 30 min of cultivation at 24°C with shaking ($OD_{600} \approx 1.0$ units) at both treatment and control conditions. Bacterial cells were harvested for the total RNA extraction by mixing with 2 volumes of the ice cold killing buffer (20 mM Tris–HCl from BDH Laboratory, 5 mM $MgCl_2$ from MERCK and 20 mM NaN_3 from SIGMA; pH was adjusted to 7.5) (Völker et al.,1994). The mixture was centrifuged at 5000 rpm for 3 min at room temperature. The final pellet was washed with 1 ml of the killing buffer and immediately frozen at -80°C until RNA extraction. Six RNA samples (3 controls and 3 treatments) were obtained from three independent experiments. However, one sample of the treated bacteria did not pass the RNA quality control and was discarded.

3.2.3. Total RNA extraction and sequencing

Total RNA was isolated using the ZR Fungal/Bacteria RNAMini Prep kit from the Zymo research Corp. and according to the manufacturer's instruction. Concentration and quality of the RNA samples were checked by a NanoDrop. Ribolock Ribonuclease inhibitor (Thermo Scientific) was added to prevent RNA degradation. Paired-end RNA sequencing was performed on the MiSeq Illumina platform at the Inqaba Biotech (Pretoria, South Africa, http://www.inqababiotec.co.za/). RNA-Seq datasets and the results of the statistical analysis by CLC Genomics Workbench 7 were

deposited to the NCBI GEO database under the accession number GSE68543. The RNA-Seq reads were trimmed from the adapter sequences and mapped against the predicted coding and non-coding loci of the reference genome sequence of the *B. atrophaeus* UCMB-5137(CP011802) using a CLC Genomics Workbench 7.0.3 (currently this program is distributed by QIAGEN — http://www.clcbio.com/products/clc-genomicsworkbench/). The Estimated Degree of Gene Expression statistics approach (Magoc et al., 2013) was used to identify the up- and down-regulated genes. Different cut-off values of the fold change and p-values were applied as explained below.

3.2.4. Complete genome sequence of *B. atrophaeus* UCMB-5137

A draft genome sequence of the *B. atrophaeus* UCMB-5137 (NZ_CM001847.1) comprising 21 contigs was obtained recently by Illumina HiSeq sequencing (Chan et al., 2013). DNA reads obtained by RNA-Seq for the current work were used for gap closing and resolving ambiguities in the previous assembly by mapping reads to contigs by the CLC Genomics Workbench 7.0.3. Prediction of the coding genes in the complete genome sequence was performed by using the RAST Genome annotation robot (Aziz et al., 2008) and then checked manually. Nomenclature of the gene names, where it was possible, was adopted from that of the *B. subtilis* 168 (NC_000964). Locations of prophages and other horizontally-transferred genomic islands were identified in the genome by using the SeqWord Genome Island Sniffer program (Besideet al., 2009).

3.2.5. Gene orthology and phylogenetic studies

Orthologous genes in the genomes of *B. atrophaeus* UCMB-5137, *B. amyloliquefaciens* ssp. *plantarum* FZB42 (NC_009725), *B. amyloliquefaciens* ssp. *plantarum* Y2 (NC_017912), *B. amyloliquefaciens* ssp. *plantarum* CAU B946 (NC_016784), *B. amyloliquefaciens* ssp. *plantarum* IT-45 (NC_020272), *B. amyloliquefaciens* ssp. *amyloliquefaciens* DSM7 (NC_014551), *B. subtilis* ssp. *subtilis* 168 (NC_000964), *B. subtilis* ssp. *subtilis* BSn5 (NC_014976), *B. subtilis* ssp. *spizizeni* TU-B-10 (NC_016047), *B. atrophaeus* 1942 (NC_014639), *B. atrophaeus* C89, *B. atrophaeus* 9372–1, *B. atrophaeus* 9372–2, *B. atrophaeus* Dugway, *B. atrophaeus* Detrick-1, *B. atrophaeus* Detrick-2, *B. atrophaeus* Detrick-3, *B. atrophaeus* 1013-1, *B. atrophaeus* 1013-2, *B. atrophaeus* 49822-1, *B. atrophaeus* 49822-2, *B. atrophaeus* BACI051-E and *B. atrophaeus* BACI051-N were

identified by the EDGAR software platform (Blom et al., 2009). The strains with the NC (RefSeq) accession numbers were downloaded from the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) and the genomes of other strains were obtained from the PATRIC (Wattam et al., 2014; http://patricbrc.vbi.vt.edu/portal/portal/patric/Home). The identified groups of orthologous genes were then used for the superimposition of the gene regulation profiles obtained in this work and those known from literature. A super-alignment of the 856,373 amino acid residues of concatenated alignments of the orthologous proteins was used to determine the phylogenetic position of the strain UCMB-5137, by the neighbor-joining clustering algorithm implemented in MEGA 6.0.6 (Tamura et al., 2013).

3.2.6. Gene co-expression analysis

Operons were predicted as syntenies of genes transcribed in the same direction with the spacer regions between genes smaller than 200 bp. It was assumed that the operon was regulated if it comprised at least one gene regulated by more than 2 folds in comparison to the control condition with a calculated p-value of ≤ 0.05 and without contradictions to the regulations of other genes in the operon. The 2 folds threshold was used in this case to make the results comparable with the stress response regulation patterns published by Sappa et al. in 2013 and Kohlstedt et al. in 2014. Operon predictions and their transcriptional regulations were checked by using the SubtiWiki web-based resource (Michna et al., 2015). Putative transcription factors binding sites were identified by using the software tools provided by the DBTBS server (Sierro et al., 2008; http://dbtbs.hgc.jp/). Loci of 200 bp upstream of the first gene in the operon were searched for transcriptional factor (TF) binding sites by the DBTBS tools. Co-regulation of genes at different conditions was estimated as 4-fold phi-correlation (Fleiss et al., 2013):

$$\varphi = \frac{n_{11}n_{22} - n_{12}n_{21}}{\sqrt{(n_{11}+n_{21})(n_{12}+n_{22})(n_{11}+n_{12})(n_{21}+n_{22})}}$$

where n11—number of genes activated at both conditions; n12—number of genes activated at the first condition, but repressed at the second condition; n21 — number of genes repressed at the first condition, but activated at the second condition; and n22 — number of genes repressed at both

conditions. The whole genome regulation network was constructed and visualized using the Cytoscape 3.2.1 for Windows (64 bit) (http://www.cytoscape.org/cy3.html). Metabolic pathways for regulated genes were identified using the Pathway Tools software version 13.0 for 64-bit Windows 7 (Karp et al., 2010).

3.2.7. Identification of the differentially expressed non-coding RNA

The non-coding RNA (ncRNA) loci were identified by differential density of the Illumina reads aligned against the intergenic spacer regions of the genome of *B. atrophaeus* UCMB-5137. They were considered as regulated by the root exudates if their level of differential transcription exceeded a 3-fold threshold with at least 50 reads mapped to the region in the sample with the highest expression. To avoid false-positive identification of the untranslated regions (5′-UTR) as ncRNA, only the loci flanked by oppositely regulated genes were considered in this work. Identified sequences were compared to the records of ncRNA in the SubtiWiki database (Michna et al., 2015) and those reported by Fan et al. (2015) in *B. amyloliquefaciens* FZB42. The level of sequence conservation of the predicted ncRNA was checked by BLASTN search through the complete genome DNA sequences of *B. subtilis* ssp. *subtilis* 168 (NC_000964), *B. amyloliquefaciens* ssp. *plantarum* FZB42 (NC_009725), *B. licheniformis* ATCC 14580 (NC_006270), *B. pumilus* SAFR-032 (NC_009848), *B. halodurans* C-12 (NC_002570) and *B. cereus* ATCC 14579 (NC_004722). If the conserved regions were found at least in 3 different genomes, possible mRNA targets for predicted ncRNA were then identified by using CopraRNA (Wright et al., 2014). Predictions of the target mRNA with a p-value of $\leq 1 \times 10^{-5}$ was considered reliable.

3.3 Results and Discussion

3.3.1. A complete genome sequence of the strain UCMB-5137 and its phylogeny

In total, 2,233,142 paired-end Illumina MiSeq reads were generated from the RNA samples obtained from the two bacterial cultures treated with the root exudates and 5,555,122 reads were obtained from the untreated cultures. The previously published complete genome sequence of the *B. atrophaeus* UCMB-5137 (Chan et al., 2013) was updated by closing the gaps with the newly generated reads. A new version of the genome was deposited in the GenBank database under the accession number CP011802 (RefSeq NZ_CP011802.1, NCBI ASM38596v2). A BLASTP search through sequences of the genomes of 22 other publicly available microorganisms belonging to the *B. subtilis* group revealed 3025 clusters of the orthologous protein coding genes. To clarify the phylogenetic position of the strain UCMB-5137 within the *B. subtilis* taxonomic group, a super-alignment of all orthologous genes was analyzed by the Neighbor-Joining algorithm implemented in MEGA 6.0. The phylogenetic position of the strain UCMB-5137 is shown in Figure 3.2. The strain UCMB-5137 was grouped together with the reference strain of *B. atrophaeus*. However, it was separated from the cluster of other multiple sequenced strains of this species. It was hypothesized that UCMB-5137 might represent a sub-species of the *B. atrophaeus* but this hypothesis was not further analyzed in this study.

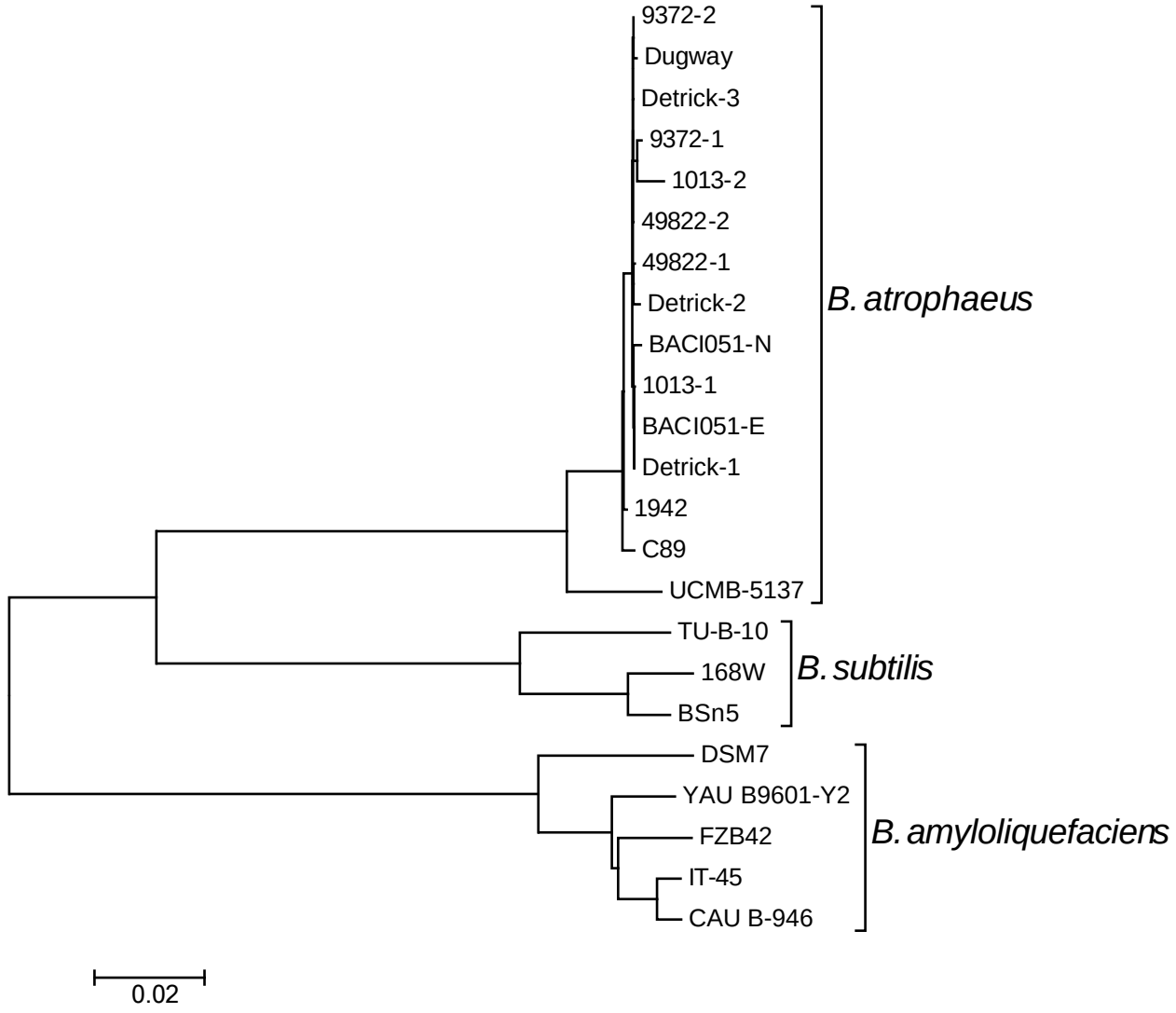


Figure 3.2: A Neighbor-joining species tree constructed for selected organisms of the *Bacillus subtilis* group based on concatenated amino acid sequences of orthologous proteins.

3.3.2. Gene expression profiling

Up- and down-regulated genes in samples treated by the root exudates compared to the control samples were determined with the fold difference exceeding the factor of 3 and p-value ≤ 0.05. The detected genes were grouped by their functional associations (Table 3.1). A ratio of 54 up-regulated versus 152 down-regulated genes was observed in the total set of samples. Particularly: amino acid, nucleotide, carbohydrate and fatty acid biosynthetic pathways, anaerobic respiration pathway and also the associated ABC-transport systems were mainly down-regulated by the root exudates. It was

also true for all genes associated with the identified prophages and horizontally transferred genomic islands. On the contrary, several genes for cell surface protein biosynthesis and post-translational processing, aerobic respiration genes and also multiple transcriptional regulators were stimulated by the root exudates. Genes involved in DNA replication and cell division processes were mostly up-regulated by the root exudates. However, the bacteria treated with the root exudates did not show any increase in the rate of growth. More details on metabolic pathway regulation may be found in Table S1.

Many of the genes activated by the root exudates were associated with stress response and detoxification. Activation of the GABA shunt and spermidine biosynthesis indicated the preparation to oxidative and/or acidic stresses (Feehily and Karatzas, 2013). Activation of the stress response genes in PGPR organisms by the root exudates was reported in a previous study (Matilla et al., 2007). Through the observed up-regulation of the SasA alarmone biosynthesis pathway, it was hypothesized that the level of (p) ppGpp might be increased in bacteria treated with the root exudates. The alarmone (p) ppGpp could trigger a stringent response in bacteria experiencing nutrient exhaustion. No signs of the stringent response were observed at the control conditions when the medium was the same but without the root exudates. We therefore concluded that there should be no real nutrient exhaustion in the medium. According to Braeken et al. (2006), (p) ppGpp alarmone is also involved in many physiological processes including adaptation to changed environmental conditions and interactions between bacteria and eukaryotic cells.

Aligning of the DNA reads against the non-coding intergenic loci of the complete genome sequence of *B. atrophaeus* UCMB-5137 showed some multiple differently-expressed regions, which could not be translated into any putative proteins. A search for differentially regulated intergenic spacer regions revealed at least 49 loci of the putative ncRNA. The genomic location of the identified ncRNA, their neighboring genes, possible mRNA targets and known ncRNA orthologs are listed in Table 3.1. Out of these 49 ncRNA loci, 40 were up-regulated by the root exudates. There were many other down-regulated intergenic spacer loci; however, since it was difficult to distinguish them from the putative 5′-UTR as they were surrounded by the down regulated neighbor genes, these loci were not considered in this study.

3.3.3 Superimposition of the gene regulation profile in *B. atrophaeus* over the regulatory network of *B. subtilis*

Bacillus atrophaeus is the closest relative of *B. subtilis*. The gene regulation network was studied in *B. subtilis* by many researches and summarized in the SubtiWiki (Michna et al., 2015) and the DBTBS databases (Sierro et al., 2008). Both species belonged to the same group of related organisms, which were not easily distinguished even by the 16S rRNA sequences (Safronova et al., 2012). We concluded that the transcription regulatory network known for *B. subtilis* might be fully applied to study the regulation of orthologous genes in the *B. atrophaeus*. Table 3.1 below presents the known activators and repressors associated with the regulated genes.

Table 3.1: Functional grouping of the genes of *Bacillus atrophaeus* UCMB-5137 regulated by the maize root exudates (fold change ≥ 3; p ≤ 0.05).

Functional groups and reactions		Up-regulated	Down-regulated	Activa-tor*	Repres-sor*
Aerobic respiration					
ctaA	cytochrome oxidase biogenesis, heme A synthase	D068_15390		ResD	SigE
ykuU	alkyl hydroperoxidereductase	D068_14700			AbrB
qcrB	menaquinol-cytochrome c reductase cytochrome b		D068_22850	ResD	AbrB
Anaerobic respiration					
nfrA	nitrate respiration	D068_40270		Spx	Spo0A, SigD
narG	respiratory nitrate reductase alpha chain		D068_39480	Fnr	
yyaE	selenocysteine-containing anaerobic dehydrogenases		D068_43360		
Amine and polyamine metabolism					
speD	spermidine biosynthesis I	D068_28960			CcpN
gubD	4-aminobutyrate degradation II and III; GABA shunt	D068_32090		GabR, SigB	

Amino acid biosynthesis, degradation and transportation					
cysE	cystein biosynthesis I		D068_00800		
hisA	histidine/imidazole biosynthesis		D068_36810		
hisD			D068_36840		
hisF			D068_36800		
hisH			D068_36820		
	homocystein biosynthesis		D068_36350		
proB	proline biosynthesis I		D068_13560		
ycgM	proline dehydrogenase/oxidase		D068_03140	PutR, Spo0A	CodY
ycgN	delta-1-pyrroline-5-carboxylate dehydrogenase		D068_03150	PutR, Spo0A	CodY
cysC	sulfate activation for sulfonation		D068_16200		CymR
hutU	histidine degradation I		D068_41790		CcpA, CodY
hutM	histidine transport permease		D068_41820		CcpA, CodY
yckJ	TcyB L-cystine ABC transporter		D068_32510		
ydgF	D-serine/D-alanine/glycine transporter		D068_42470		
yxeN	amino acid ABC transporter		D068_32320		CymR
ycsG	branched chain amino acids transporter		D068_31920	TnrA	KipR
ycgO	PutPproline/sodium symporter		D068_03160	PutR	
Antibiotic and bacteriocin biosynthesis					
yshB	colicin V biosynthesis	D068_28480			
pksE-R	bacillaene biosynthesis operon		D068_17790-17890	CodY	AbrB
ycbN	bacitracin transporters		D068_02530		

ycbO			D068_02540		
plpC	plipastatin/fengycinsynthetase		D068_20095		
srfAA	Surfactinsynthetase		D068_03520	ComA, PerR, PhoP	Abh, CodY , Spx
srfAB			D068_03530		
yuiI	trilactone hydrolase, bacillibactinsiderophore biosynthesis		D068_33600		AbrB, Fur
ybdB	SkfF cannibalism toxin		D068_01840	PhoP, Spo0A	AbrB
Aromatic compound biosynthesis and degradation					
yitW	aromatic ring hydroxylating enzyme	D068_11280			
Biofilm formation and regulation					
luxS	S-adenosyl-L-methionine cycle I; autoinducer AI-2 biosynthesis	D068_30750			
ymcA	biofilm formation regulation	D068_17660			
yugO	putative K+ channel protein		D068_32890		SinR
ywqE	protein dephosphorylation		D068_38300		AbrB
Carbohydrate biosynthesis and degradation					
sacA	sucrose degradation III (sucrose invertase)		D068_40170		CcpA
yfnG	galactose degradation I (Leloir pathway); galactose degradation III; UDP-D-galactose biosynthesis		D068_07090	SigK	
ycbC	D-glucarate/D-galactarate degradation II		D068_02410		YcbG
ycbD			D068_02420		YcbG
pelB	pectin synthesis/degradation		D068_41580		
licH	arbutin/salicin-6-phosphate hydrolysation		D068_27630	LicR	CcpA
glgC	glycogen biosynthesis I (from ADP-D-glucose)		D068_31080		SigE
xynD	cellulose and hemicellulose degradation		D068_19950		AbrB

	(cellulolosome)				
yveB	Levanase		D068_36400	DegU	
RhiN	rhamnogalacturonides degradation protein		D068_06760		
	chitin binding protein		D068_21820		
yyaE	RpiRsialic acid utilization regulator		D068_01620		
yisS	myo-inositol degradation II, myo-, chiro- and scillo-inositol degradation		D068_04260		
IolF	myo-inositol transporter		D068_42110		CcpA
iolI	Inososeisomerase		D068_42080		CcpA
rbsK	alpha-D-ribofuranosephosphorilation to D-ribose 5-phosphate		D068_37920	AbrB	CcpA
acoB	Transketolase		D068_07970		CcpA
yufO	unspecified monosaccharide ABC transporters		D068_33130		CodY
yufP			D068_33140		CodY
Carbon metabolism and glycolysis					
ywkA	gluconeogenesis I; glycolysis III; heterolactic fermentation; sucrose biosynthesis I, glycerol degradation to butanol	D068_39220		MalR	
gapA		D068_35950		CggR	
yqiK	glycerophosphoryldiester,phosphodiesterase	D068_24670			
glpD	aerobic glycerol-3-phosphate dehydrogenase	D068_09280		AbrB	CcpA
pdhA	pyruvate dehydrogenase E1 component alpha subunit		D068_15080		ppGpp
pyrC	Dihydroorotase		D068_16080		ppGpp
iolS	oxidoreductase, aldo/ketoreductase		D068_06830		IolR
Cell division					
divIVA	cell division initiation protein	D068_16000			Spo0A

yyaA	ParB chromosome partitioning protein	D068_43440		ComK	
ftsW	cell division protein	D068_40290		YofA	
gpsB	cell division protein	D068_22460			
Cell wall biosynthesis and membrane proteins					
yocA	antigen A homolog; putative transposon-related lytic enzyme	D068_20960			
tagD	teichoic acid biosynthesis	D068_37680		WalR	PhoP
tagO	peptidoglycan biosynthesis I and V (beta-lactam resistance)	D068_37460			
secG	preproteintranslocase subunit	D068_35560			
yocH	cell wall binding protein	D068_21050		WalR, Spo0A	AbrB
yfmQ	holin associated protein, membrane protein	D068_23710			
yneJ	integral inner membrane proteins	D068_19650			
yozB		D068_20970			
yqjG		D068_24390		MifM	
yuiD		D068_33670			
gtaB	sucrose degradation II, UDP-glucose biosynthesis	D068_37620		SigB	
tuaA	undecaprenyl-phosphate galactosephosphotransferase		D068_37540	PhoP	CcpA
yubE	N-acetylmuramoyl-L-alanine amidase (cell wall degradation/ turnover)		D068_05240		
ykfC	cell wall endopeptidase NLP/P60		D068_13420		CodY
	LysM domain containing peptidoglycan-binding protein (horizontally acquired)		D068_05530		
			D068_05540		
Chemotaxis and motility					
flaA	FlaAflagellin protein	D068_37280			SigD, CodY,

					CsrA, ScoC
flbD	Flagellinproteins		D068_16900		
flhB			D068_16990	SigD	
fliY			D068_16930	SigD	
flhA			D068_17000	SigD	
tlpA	methyl-accepting chemotaxis protein		D068_32780	SigD	AbrB
Co-factor biosynthesis and utilization					
ppnK	NAD kinase	D068_29420			
ywfI	HemQhemoprotein	D068_39940			
atpI	ATP synthase protein I2	D068_39040		ppGpp	
moaC	molybdenium cofactor biosynthesis	D068_04680			
ribT	vitamin B synthesis, acetyltransferase	D068_23580			
yqeY	GatBtransamidase	D068_25960			
ytaP	biotin biosynthesis; fatty acid biosynthesis		D068_30330		
bioD			D068_30270		BirA
bioB			D068_30260		BirA
yqjQ			D068_03660		
tenI	thiamin biosynthesis and salvage		D068_11830		
thiC			D068_08720		
thiF			D068_42760		
yaaD	PdxST pyridoxine biosynthesis glutamine amidotransferases		D068_18930		Spo0A
yaaE			D068_18940		
Fatty acid biosynthesis					
glpQ	glycerophosphoryldiester,phosphodiesterase		D068_02080		CcpA
pcrB	S-3-O-geranylgeranylglyceryl phosphate		D068_06290		

	synthase				
yxaA	glycerate kinase		D068_42360		AbrB
yusK	fatty acid beta-oxidation pathway		D068_34610	SdpR	CcpA, FadR
yqjQ	short-chain dehydrogenase/reductase		D068_03670		
pgsA	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase		D068_17520		
yqiD	ThiJ/PfpI family protein, biosynthesis of lipids		D068_36480		
Iron metabolism					
yvrA	adenosylcobalamin salvage from cobinamide II, iron metabolism		D068_34950		
dhbC	1,4-dihydroxyl-2-naphthoate biosymthesis I; 2,3-dihydrobenzoate biosynthesis		D068_33580		AbrB, Fur
dhbA			D068_33590,		AbrB, Fur
dhbE	vibriobactin biosynthesis		D068_33570		AbrB, Fur
Nitrogen utilization					
nasA	nitrate/nitrite transporter		D068_03270	tnrA	
ureA	urea degradation II		D068_38790	PucR, TnrA	CodY, GlnR, SigH
ureB			D068_38780		
Nucleotide biosynthesis and degradation					
guaC	nucleotide biosynthesis/acquisition		D068_33750	CodY	PurR
purA	adenosine ribonucleotides de novo biosynthesis		D068_42840		PurR
purN	5-aminoimidazole ribonucleotide biosynthesis; tetrahydropholate salvage		D068_06180		PurR
purS	5-aminoimidazole ribonucleotide		D068_06130		PurR

	biosynthesis				
	pyrimidine deoxyribonucleotides biosynthesis		D068_04970		
pyrB	UMP biosynthesis; pyruvate decarboxylation to acetyl CoA		D068_16070		PurR
	adenine/guanine phosphoribosyltransferases		D068_02880		
	ATP/GTP-binding protein		D068_02890		
Protein translation, maturation, activation and utilization					
yfhP	cysteinyl-tRNAsynthetase related protein	D068_08500			
ppiB	protein folding acceleration	D068_23750			
prfA	peptide chain release factor 2	D068_37200			
yflG	N-terminal amino acid release	D068_07470			
sipT	leader sequence cleavage, signal peptidase	D068_14890		DegU	
sipS		D068_23650			
ykuE	protein kinases	D068_14540			
yjbH		D068_11720			
yrzF		D068_27510			
prkC			D068_16370		
Ribosomal proteins					
rbfA	ribosome-binding factor A	D068_17260			ppGpp
rpsB	SSU ribosomal protein S2p	D068_17100			ppGpp
rpsL	SSU ribosomal protein S9p	D068_01340			ppGpp
rpsT	SSU ribosomal protein S20p	D068_26110			
rpsU	SSU ribosomal protein S21p	D068_25970			
rplT	LSU ribosomal protein L20p	D068_28780			ppGpp
ytiA	LSU ribosomal protein L31p	D068_30790		SigB	Zur

rpmF	LSU ribosomal protein L32p	D068_15640			ppGpp
ylbN	ribosomal protein clustered with L32p	D068_15630			ppGpp
yvyD	ribosomal subunit interface protein	D068_37220		SigB, PhoP, SigH	
rplB	LSU ribosomal protein L2p		D068_01060		ppGpp
rplP	LSU ribosomal protein L16p		D068_01080		ppGpp
rimM	16S rRNA processing protein		D068_16620		
ybxB	ribosomal RNA small subunit methyltransferas		D068_00930		ppGpp
Replication, repair, recombination					
dnaN	DNA polymerase III beta subunits	D068_00020			Spo0A
dinG		D068_26790			
Resistance to antibiotics and toxins					
yvaE	ethidium bromide-methyl viologen resistance protein EmrE		D068_04110		
pbpE	beta-lactamase		D068_05680		SigW
lmrB	drug resistance transporter EmrB/QacA		D068_02650		LmrA
yoaV	drug/metabolite transporter DMT		D068_41730		
Sporulation					
yraG	spore coat protein F	D068_08690			SigG
sspJ	spore protein	D068_35180			SigG
yndM	sporulation protein	D068_19520			
yqfU	sporulation protein	D068_25650			SigG
yraF	sporulation protein		D068_01970	SigG	
yisY	chlorination of organic molecules		D068_29590	SigG, SigE	GerE, SigK

spsG	CMP-N-acetylneuraminate biosynthesis II, spore coat protein		D068_38680	GerE	SigK
spsC	PglE 4-keto-6-deoxy-N-Acetyl-D-hexosaminyl-Lipid carrier aminotransferase		D068_35870	GerE	SigK
ytcA	UDP-alpha-D-glucoronate biosynthesis from UDP-glucose		D068_35880		SigK
cotJC	spore coat protein		D068_06660	SigE, SpoIIID	
yesJ	GNAT family acetyltransferase		D068_06670	SigE, SpoIIID	
ypjB	spore formation membrane associated protein		D068_22820	SigE	
gerKA	spore germination protein		D068_37880	SigG	SpoVT
ybbE	nylon-6 oligomer degradation		D068_01600		
Stress response, detoxication, antibiotic resistance					
nfrA	oxygen-insensitive NADPH nitroreductase (oxidative stress)	D068_40270		Spx	Spo0A, SigD
hxlB	thiaminase II, resistance against oxidative stress	D068_03470		HxlR	
ywbC	lactoylglutathionelyase, oxidative stress resistance	D068_40560			
ygaF	thiol peroxidaseantioxidant proteins, resistance against oxidative stress	D068_08610			
yjbC		D068_11650		SigB, SigX, SigW	PerR, SigM
yjbL	thiol management oxidoreductase component	D068_11730			
ykuV	thiol-disulfideisomerase	D068_14710			AbrB
sodA	superoxide radicals degradation	D068_25560		SigB	
trxA	thioredoxin pathway	D068_28370		SigB,	CtsR

trxA		D068_31340		Spx	
trxB		D068_36650		Spx	
yusE		D068_34550		Spo0A	
mrgA	metal ion oxidation for incorporation into corresponding proteins	D068_34740			PerR
yocK	general stress protein	D068_21090			
ytxJ	NAD kinase, general stress response	D068_29850		SigB, SigH	
cspC	cold-shock proteins	D068_03770			
		D068_23670			
ykrL	HtpXheat shock protein	D068_13940			YkrK, Rok
yflT	heat stress induced protein	D068_07310		SigB	
ykzA	OhrB organic hydroperoxide resistance protein	D068_13600		SigB	
ylmG	YggTintegral membrane protein involved in response to extracytoplasmic stress osmotic shock	D068_15980			Spo0A
nhaX	stress response protein	D068_09730		SigB	
yfkM	ThiJ/PfpI family protein	D068_07580		SigB	Fur
ytxH	general stress response proteins	D068_29860		SigB	SigH
ytxG		D068_29870		SigB	SigH
ydaG		D068_31760		SigB	
gsiB		D068_31500		SigB, SigI	
gspA		D068_40610		SigB	
yugI		D068_32960			ppGpp
yxiE		D068_41660			CcpA

yfhL	SdpC immunity factor	D068_08450		SigB, SigW	
ywsB		D068_37970		SigB	
clpP	proteolysis and hydrolysis, general stress response	D068_36470		SigB	CtsR
yfhM		D068_08460		SigB, SigW	
clpX		D068_28080			CtsR
ydiL	stress protection CAAX amino terminal protease	D068_04730			
yvgN	stress response protein	D068_01940		SigB	SinR
		D068_35260		SigB	SinR
ydaM	general stress protein		D068_31640	SigB	
dnaJ	chaperone protein DnaJ		D068_26020		HrcA
ydfO	MhqOglyoxalase/dioxygenase		D068_29690	SigB	MhqR
ispF	methylerythriol phosphate pathway, lipid biosynthesis, stress response		D068_00770	SigB	
Transcriptional regulation					
ywaC	SasA protein, stringent response,ppGpp biosynthesis	D068_40690		SigM	SigW
perR	peroxide stress regulator PerR	D068_08620			
spo0A	stage 0 sporulation two-component response regulator	D068_24730			SigH
lexA	LexA SOS-response repressor	D068_19550			
yozG	Cro/CI family transcriptional regulator	D068_21840			
ydeB	CarD-like transcriptional regulator	D068_03780			
ypdC	anti-sigW regulation	D068_23280			
ytvA	positive sigma-B regulator, blue light GTP-binding receptor	D068_30400		Spx	

paiB	sporulation negative regulatory protein PAI-2	D068_03850			
sinR	SinR regulator of post-exponential-phase responses genes competence and sporulation	D068_25150			AbrB, ScoC, Spo0A
bmrU	transcription regulator, stress response, resistance against toxins and antibiotics	D068_24490		SigB	
yqgZ	MgsR transcriptional regulator of stress response	D068_25310		SigB	
dps	non-specific DNA-binding protein Dps,iron-binding ferritin-like antioxidant protein	D068_30720		SigB	
ydhC	GntRtranscriptional regulator	D068_43070			
ykvE	MhqRtranscriptional regulator, resistence to methyl-hydroxyquinone	D068_14120			
ykoM	MarR family transcriptional regulator	D068_13800			
ypoP		D068_21940			
yvnA		D068_35190			AbrB, CcpA
mecA	MecA negative regulator	D068_11680			
ypbH		D068_23310			
glnR	GlnR transcriptional regulator of nitrogen metabolism	D068_18140			TnrA
fnr	Fnrtranscriptional regulator	D068_39520		ResD	NsrR
yvdT	TetRtranscriptional regulator	D068_36420			
yvaP	CatR/HxlRranscriptional regulator, viability in the presence of catechol	D068_35610			
cggR	CggR glycolytic genes regulator	D068_35960			
yhdE	NsrR nitrite-sensitive transcriptional repressor, resistance against nitric oxide, phenolic acids, flavonoids, oxalate	D068_09360			

ykmA	OhrR organic hydroperoxide resistance transcriptional regulator	D068_13590			
hrcA	HrcA heat-inducible transcription repressor	D068_26050			
ykoB	RsbRBpiezosome protein	D068_13690			
abh	Abh transcriptional regulator, transition from growth to stationary phase	D068_14960		SigM, SigX	
ylbF	ComK regulatory protein, antagonist of biofilm repression by SinR	D068_15530			
tnrA	TnrA transcriptional regulator, regulation of nitrogen assimilation	D068_13790			GlnR
fur	Fur regulation of iron homoeostasis By repression of transcription of ferri-siderophore uptake genes	D068_23940			PerR
zur	Zur transcriptional regulator, trace metal homeostasis	D068_25630			
yjbD	Spx transcriptional regulator	D068_11660		SigB, SigW, SigM, SigX	PerR
xpf	Xpf transcriptional regulator, prophage transcription activator		D068_12870		ppGpp
yvrL	YvrL regulator, controls acid stress proteins		D068_35050		
Transport and uptake (in addition to mentioned above amino acid, hydrocarbon and secondary metabolite transporters)					
ypaA	RibU riboflavin ECF transporter	D068_23390			
	siderophoreFe-uptake	D068_09870			
expZ	ABC-transporters	D068_34750			
yfiM			D068_08200		
yfiL			D068_08190		
yhcG			D068_09020		

yvrO			D068_35100	ComK	AbrB
yclI			D068_32270	YclJ	
fhuG	ABC-type Fe3+-siderophore transports		D068_35130		Fur
fhuB			D068_35140		Fur
ycdI	zinc ABC transporter		D068_02790		Zur
ssuA	aliphatic sulfonate ABC transporters		D068_08760		CymR
ssuC			D068_08770		
ybbF	PTS transporters		D068_01610		
mtlA			D068_32010	MtlR	CcpA
sacP			D068_40180	SacT	CcpA
appB	OppBoligopeptide transport system permease protein		D068_11530		CodY, ScoC
pstA	PstA phosphate ABC transporter		D068_25510	PhoP	
yqeW	sodium-dependent Na+/anion phosphate transporter		D068_25980		
gltP	proton/glutamate-aspartatesymport protein		D068_02290		
gltP	glycerol-3-phosphate transporter		D068_02100		
yclF	di-/tripeptide transporter		D068_32430		ScoC
dctP	aerobic C4-dicarboxylate transporter for fumarate, L-malate, D-malate andsuccunate		D068_31430		CcpA, FsrA
Other enzymes					
	acetylxylan esterase	D068_19930			
tatCY	R-CoA hydrolysis, twin-arginine translocation pathway	D068_13460			
yneT	succinyl-CoA synthetase	D068_19790			
yfmJ	putative oxidoreductase		D068_07240	Spo0A	
yxeL	putative acetyltransferase		D068_32340		CymR

yxeK	Nitrilotriacetatemonooxygenase		D068_32350		CymR
yurQ	endo/excinuclease amino terminal domain protein		D068_34420		

*Information about gene repressors and activators was taken from DBTBS (Sierro et al., 2008 and http://dbtbs.hgc.jp/) and checked with data in SubtiWiki (http://subtiwiki.uni-goettingen.de/wiki/index.php/Main_Page).

A visualization of the superimposition of gene regulations in UCMB-5137 under the root exudates stimuli over the regulatory network of *B. subtilis* are shown in Fig. 3.3. Clusters of co-regulated genes in Fig. 3.3 were titled by the corresponding TFs or sigma-factors. Inconsistence in regulation of the protein coding genes and the corresponding TFs was depicted by red edges linking the nodes of the genes and TFs. For example, if a gene encoding a transcriptional activator was up-or down-regulated, it was expected that all the genes dependent on this activator would be regulated accordingly. On the contrary, regulation of a repressor should cause an opposite regulation of all the related genes.

If a gene was regulated by several TFs, this regulation was accepted as consistent if there was consistency in the regulation with at least one TF. Contradictions depicted in Fig. 3.3 might indicate our limited knowledge on TFs involved in the gene regulation at the given condition. The biggest number of the conflicted gene repressions was observed among the genes regulated by the SigB (stressosome), SigF (sporulation regulon), AhrC (arginine metabolism regulon), FadR (fatty acid degradation regulon), FapR (fatty acid biosynthesis regulon), Fnr (anaerobiosis and overflow metabolism) and TnrA (nitrogen assimilation) regulons. Conflicted gene activations were observed in the YwaC (ppGpp associated regulon independent from stringent response) and SigW (resistance and detoxification) regulons.

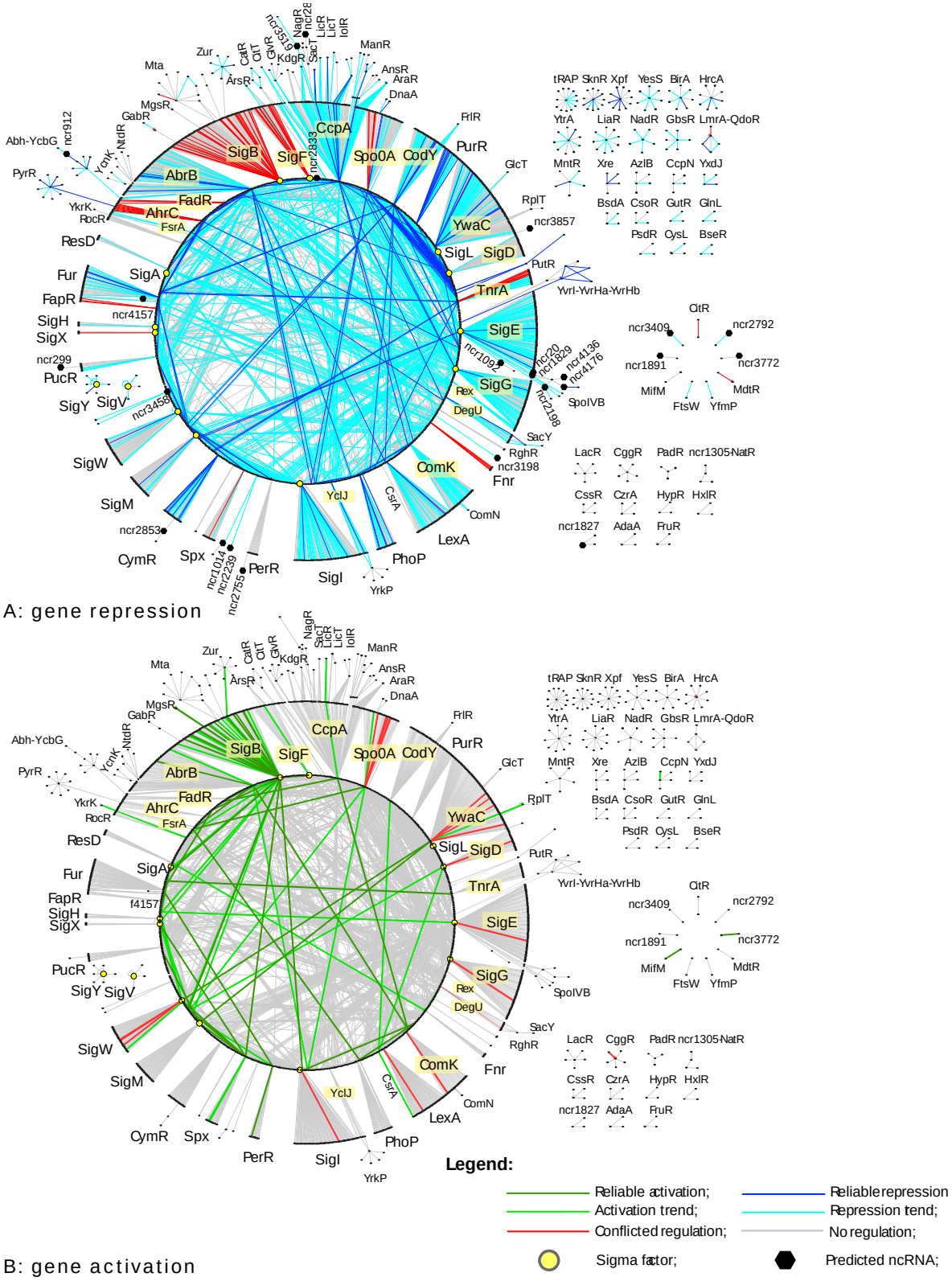


Figure 3.3: A regulatory network constructed based on the regulon predictions for *B. subtilis* 168 as presented in the SubtiWiki database. The repressions and activations of the genes in UCMB-5137were superimposed over the regulatory network in parts A and B, respectively.

The majority of the genes controlled by the up-regulated Spo0A (sporulation initiation) were activated or repressed oppositely to what might be expected. It was expected that all these genes were additionally controlled either by other TFs, or Spo0A was somehow inactivated by other regulatory factors triggered by treatment with the root exudates. An interference of the regulatory ncRNA molecules in this process was assumed.

Many general stress response genes were up-regulated by the root exudates, especially those controlled by the SigB stressosome (Fig. 3.3A). To identify specific regulations not associated with the general stress response, the pattern of gene regulation observed in the current study was compared to the stress response patterns reported earlier (Sappa, 2013; Kohlstedt et al., 2014). A significant correlation was observed between the gene regulation patterns triggered by the root exudates and those under nutrient stringency and high temperature stresses, while congruence with the low temperature growth and osmotic stress conditions was lower. It might be explained in a way that the nutrient stringency, higher temperature growth and plant root colonization were all associated with an increased oxidative stress resulted from an accumulation of free radicals (Lamb and Dixon, 1997). The majority of genes activated at the stress conditions and in the current experiment were controlled by the SigB and partly by the SigW regulons (Table 3.2).

Down-regulations of gene expression at all these conditions were controlled by the CodY and several other repressors. The genes regulated oppositely to the patterns of the general stress response were of interest for us as they were most likely associated with the specific responses of the *B. atrophaeus* UCMB-5137 to the root exudates stimuli. In total, 250 operons were identified comprising 426 protein coding genes. All these genes were controlled by multiple TFs and sigma factors (Table 3.2), particularly by the AbrB and Abh (transition to stationary phase), PerR (peroxide response), CggR (glycolysis activator) and WalR (synthesis of cell wall proteins). However, the regulation of many of these genes in *Bacillus* was obscured. A search for possible TF binding sites in 200 bp flanking regions up-stream to the operon start codons was performed. The identified TFs are listed in Appendix 1.

Table 3.2: Overlaps between gene expression patterns regulated by the root exudates and different stress conditions.

Stress conditions	**Up-regulated genes**		**Down-regulated**		**Pearson correlation coefficient**
Root exudates	**Up-regulated**	**Down-regulated**	**Up-regulated**	**Down-regulated**	
Stationary growth	25	23	5	64	0.505
Growth at 51°C	11	24	0	26	0.404
Growth at 16°C	20	20	8	27	0.280
Growth with 1.2M NaCl	3	11	4	20	0.059
Regulatory TFs	SigB, SigW	PutR, CodY, AbrB, SigE, SifF, SpoIIID, ScoC, TnrA, Fur, Spo0A	SigD, SinR, SigB, CggR, ResD, Fnr, NsrR, Spo0A	CodY, SigD, SigK, SigW, AbrB, CcpA, TnrA, KipR, FapR, MntR , Spo0A	

A combined overview of the known and predicted TFs involved in regulation of these operons is shown in Figure 3.4. The major repressors activated by the root exudates stimuli were CcpA (mediated carbon catabolite repressor), CodY (pleiotropic repressor), TnrA (nitrogen assimilation regulator) and Spo0A. However, the role of the latter regulator should be taken cautiously as at the treatment condition, the effect of Spo0A was most likelymodulated by other unknown transcriptional factors (see Figure 3.3 and the discussion above). The major activators were AbrB and SigH (both regulated the transition to stationary phase and the cell growth rate), SigB and PerR (general stress and oxidative stress response, respectively). Fig. 3.4 highlighted a possible crucial

role of the DegU in regulation of the plant root colonization by *Bacillus*. The DegU binding sites were identified in front of almost all the regulated operons; however, only some of them were reported in the literature as parts of the DegU regulon. It is known that the DegU in *B. subtilis* is an important regulator of many processes including chemotaxis, motility, extracellular secretion, quorum sensing and biofilm formation (Msadek et al., 1991; Murray et al., 2009; Gupta and Rao, 2014; Omer et al., 2015). It was reported that the inactivation of DegU in *B. amyloliquefaciens* FZB42 led to an impairing in the efficiency of root colonization (Budiharjo et al., 2014). Finding of the DegU binding sites in front of the operons specifically regulated by the root exudates confirmed the importance of this TF for plant colonization behavior.

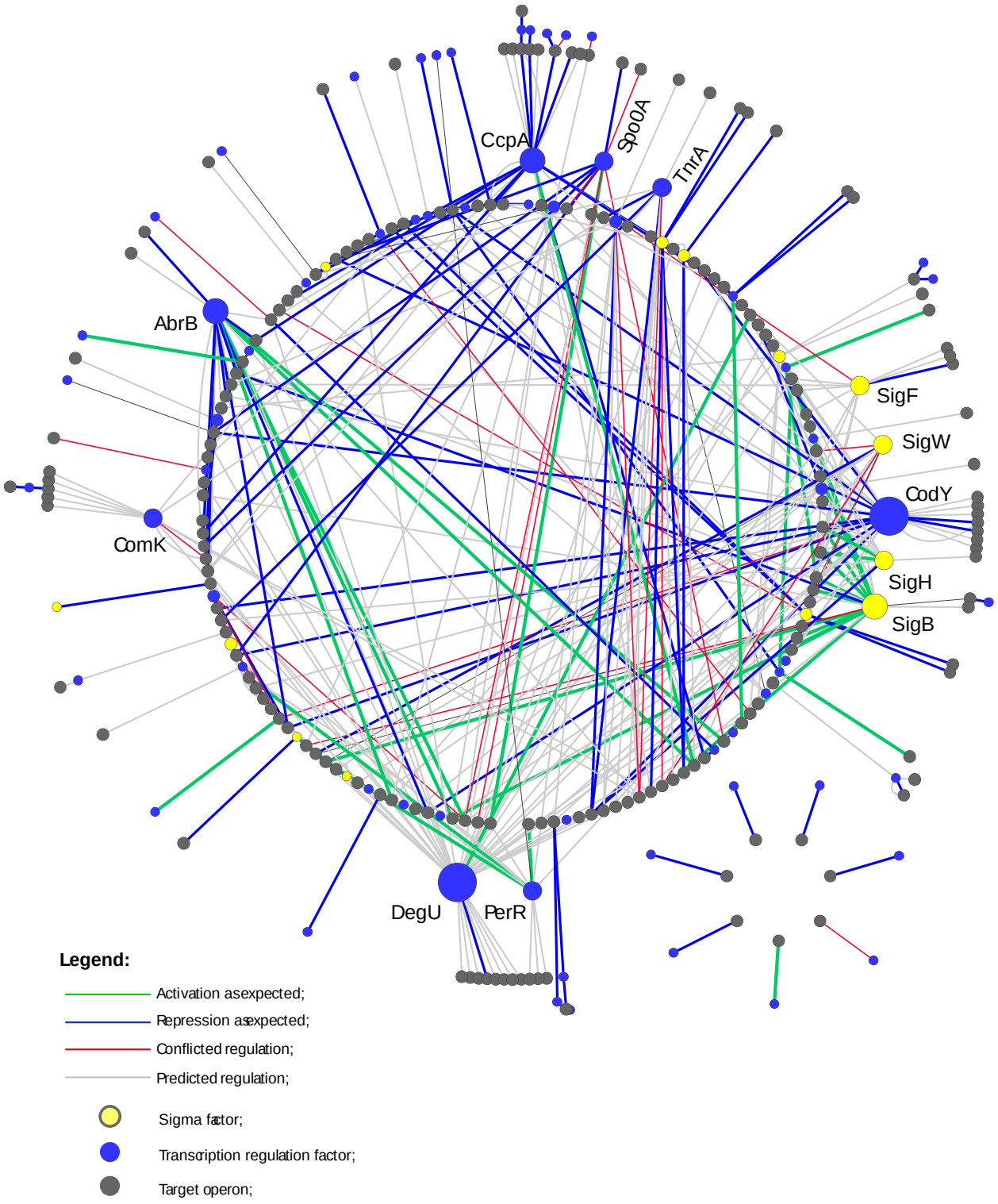


Figure 3.4: A regulatory network of the genes, whose regulation by the root exudates was incongruent to the general stress response regulation. The nodes representing transcriptional factors were sized according to the numbers of regulated genes. Labeled TFs were involved in regulation of at least 5 protein coding genes.

3.3.4. The role of ncRNA in gene regulation under root exudates stimuli

It was known that many TFs were controlled by ncRNA. For example, it was reported for AhrC regulator (Brantl and Brückner, 2014). A conflicted regulation of the genes controlled by the AhrC was observed in this study. Also, conflicts were observed in the regulation of genes controlled by SigB, SigW, SigF, YwaC, Spo0A, FadR and several other TFs (Fig. 3.4). The majority of ncRNA identified so far in *B. subtilis* were antisense translational inhibitors including the dual-function genes encoding both the regulatory RNA and small regulatory proteins. One example of the dual-function genes was SR1 (Brantl and Brückner, 2014). In *B. atrophaeus* UCMB-5137, the SR1 corresponded to the predicted protein coding gene D068_15140, which was not regulated by the root exudates. Two other genes, D068_34490 and D068_15550, predicted in UCMB-5137as small unknown proteins had shared sequence similarities with the bsrI and csfG regulatory RNA of *B. subtilis*, respectively. These genes were repressed by the root exudates.

Multiple cis-encoded phage related anti-toxins were also repressed by the root exudates together with all the phage related genes. Many ncRNA predicted in this study had showed significant sequence conservation in different species of the *Bacillus* despite being located in variable intergenic spacer regions. This conservation allowed prediction of possible target mRNA by using a CopraRNA Web-service (Table 3.3). Contributions of the found ncRNA to the gene regulatory network of *B. atrophaeus* UCMB-5137 were summarized in Fig. 3A. Six predicted ncRNA; ncr628, ncr818, ncr2198, ncr3198, ncr3519 and ncr3877, were located near the promoter regions of the genes, which were their most likely targets. Other six ncRNA; ncr20, ncr628, ncr1092, ncr1829, ncr2198 and ncr3198, aimed at different transcriptional regulators. Particularly, the genes of the SpoIVB regulon including mraZ, which was reported in several studies as a new TF inhibiting the cell division (Meile et al., 2006; Eraso et al., 2014), most likely, were under control of the predicted ncRNA (Fig. 3.3A). Among other potential targets of the predicted ncRNA, there were several genes, which have not been associated with any TF regulons in the SubtiWiki. In Table 3.3, these genes were depicted by the bold typeface.

Table 3.3: Non-coding loci regulated by the maize root exudates, locations of loci are given as is in the NCBI genome submission CP011802.

Name	Location	Upstream gene	Fold change	Predicted targets	Orthologs*
ncr20	22230..22278	D068_03770	19.94	**YllB MraZ protein**; SpoIVB peptidase;	
ncr23	25176..25569	D068_03810	3.96	No conservation;	
ncr28	28250..28322	D068_03850	3.1	NagP component of PTS system;	
ncr63	67120..67569	D068_04240	5.99	No conservation;	
ncr86	95107..95160	D068_04490	3.29	No conservation;	*bsrF*
ncr299	4000114..4000377	D068_19000	15.39	PucD xanthine dehydrogenase;	*scr* small cytoplasmic scRNA
ncr409	377769..378299	D068_07850	3.35	No conservation;	
ncr628	599457..599519	D068_09620	-inf	Downstream gene: CueR transcriptional regulator;	
ncr818	777740..777830	D068_11560	-2.44	Downstream gene: tryptophan-tRNA ligase TrpS;	*trpS-oppA*, bar094
ncr912	853200..853261	D068_12600	4.27	**YlbQ 2-dehydropantoate reductase**; YeaB transporter;	
ncr1014	939698..939779	D068_13670	5.6	**YkrV, MtnE transaminase;** CotE spore coat protein;	
ncr1068	997865..998060	D068_14210	-inf	No conservation;	
ncr1092	1020314..1020360	D068_14450	-47.94	SpoIVB peptidase; **YqjI 6-phosphogluconate dehydrogenase;**	*ykwD-pbpH*
ncr1258	1108507..1180808	D068_16150	3.03	No significant match found;	
ncr1305	1226294..1226547	D068_16630	12.04	NatB sodium efflux ABC transporter permease; RpoA DNA-directed RNA polymerase;	
ncr1509	1477643..1478293	D068_18730	4.1	No conservation;	

ncr1648	1625065..1625385	D068_20480	3.06	No conservation;	
ncr1827	1821647..1822003	D068_22450	14.59	**YfhS and YgzB hypothetical proteins;**	
ncr1829	1823159..1823313	D068_22470	7.92	**YllB MraZ protein**; YetA hypothetical protein; YesN transcriptional regulator; SpoIVB peptidase;	
ncr1891	1877568..1877676	D068_23110	3.44	**MiaA tRNA dimethylallyltransferase;**	
ncr1947	1925995..1926105	D068_23670	5.55	No conservation;	
ncr2198	2143072..2143195	D068_26260	3.4	Downstream gene: YqeG hypothetical protein; **YllB MraZ protein**; SpoIVB peptidase	
ncr2239	2178179..2178362	D068_01700	3.29	CotE spore coat protein;	*glmM-glmS*
ncr2499	2463009..2463121	D068_31760	22.34	No significant match found;	*bsrC*
ncr2755	2697733..2697777	D068_29190	-inf	AhpF NADH dehydrogenase;	bar067
ncr2792	2734908..2735126	D068_28810	4.2	**YqgL hypothetical protein;**	
ncr2833	2779026..2779093	D068_28380	12.28	RpsD 30S ribosomal protein S4; NhaX stress response protein;	*trxA-xsa*
ncr2852	2795834..2795898	D068_28200	-inf	No conservation;	
ncr2853	2796437..2796600	D068_28190	3.59	**YdiK lipoprotein**; TcyP L-cystine uptake protein;	
ncr2893	2839504..2839767	D068_27790	6.14	No significant match found;	
ncr3022	2955963..2956313	D068_26460	3.42	No conservation;	
ncr3198	3034438..3034536	D068_33310	3.61	Downstream gene: DegQ transcriptional regulator;	
ncr3267	3105503..3105823	D068_34020	3.85	No conservation;	
ncr3314	3150551..3150665	D068_34530	5.04	No significant match found;	

ncr3409	3244049..3244404	D068_35490	15.74	**YjlA possible transporter;**	*ssrA* tmRNA
ncr3458	3290801..3290900	D068_36020	3.56	RpsG 30S ribosomal protein S7; **YkoQ metallophosphoesterase;** **YqjP** and YtnM hypothetical proteins;	
ncr3519	3349197..3349330	D068_36640	2.37	Downstream gene: YvcI Nudix hydrolase; **TenA thiaminase;**	*yvcI-trxB*
ncr3522	3351953..3352333	D068_36670	7.75	No conservation;	
ncr3526	3356453...3356663	D068_36720	3.46	No conservation;	
ncr3628	3460797..3460941	D068_37800	-inf	No conservation;	
ncr3736	3555042..3555497	D068_38890	-8.92	No conservation;	bar073
ncr3772	3583861..3583995	D068_39260	2.03	**GpsB cell cycle protein;**	bar096
ncr3826	3637975..3638305	D068_39820	3.52	No conservation;	
ncr3877	3685045..3685132	D068_40350	4.2	Downstream gene: QoxA quinol oxidase subunit;	
ncr3952	3752185..3752625	D068_41140	3.03	No conservation;	
ncr4000	3793213..3793326	D068_41680	-inf	CysB cyclodextrin-binding protein;	bar084
ncr4136	3931507..3931653	D068_43080	3.42	**YllB MraZ protein;**	
ncr4157	3956921..3957068	D068_43320	3.57	RapC aspartate phosphatase C response regulator and 10 other paralog genes;	
ncr4176	3973567..3973915	D068_43510	6.56	**XhlA phage-like protein**; **YllB MraZ protein;**	

*Orthologous ncRNA were searched by sequence similarity among sequences of the ncRNA found in *B. amyloliquefaciens* FZB42 (Fan et al., 2015) and indicated as bar0##, and in the SubtiWiki database. In the later database the ncRNA found in *B. subtilis* 168 were presented either by names, or by names of the flanking genes: *trpS-oppA* for example.

In general, ncRNAs were considered in the literature as translational inhibitors. However, several ncRNAs were reported to be able to prolong the lifetime of mRNA (Wagner and Romby, 2015). It

may explain the observed differences in RNA abundance of several genes, which presumably, were controlled in *B. atrophaeus* UCMB-5137 exclusively by the ncRNA under the root exudates stimuli.

3.3.5. A comparison of the gene expression profiles of the *B. atrophaeus* UCMB-5137 and the *B. amyloliquefaciens* FZB42 stimulated by the root exudates

It was interesting to study to which extent the gene regulation by the root exudates stimuli in *B. atrophaeus* UCMB-5137 was congruent to that reported previously for the paradigm of PGPR *B. amyloliquefaciens* FZB42. The gene expression profile of *B. atrophaeus* UCMB-5137 from the current research was compared to the microarray and proteomics profiles of the *B. amyloliquefaciens* FZB42 published earlier (Fan et al., 2012; Kierul et al., 2015). The transcriptional profiles appeared to be substantially different (Table 3.4).

Table 3.4: A comparison of the gene expression profiles reported for the *B. atrophaeus* UCMB-5137 and the *B. amyloliquefaciens* FZB42

Genes, pathway and/or metabolic processes	**UCMB-5137**	**FZB42**	
	RNA-Seq (this work)	**Microarray hybridization data (Fan et al.,2012)**	**Proteomics data (Kierul et al.,2015)**
Similar response to root exudates in both organisms			
Biofilm and quorum sensing: *ymcA* and *luxS* genes.	Up-regulated	Up-regulated	Not reported
Proteins secreted in late exponential phase: SodA (superoxide dismutase), Tpx (thiol peroxidase), AhpC (alkyl hydroperoxide reductases C), YodJ (D-Alanine carboxypeptidase), CysK (cysteine synthase A) and FbaA (fructose 1,6-bisphosphate hydrolase)	Up-regulated	Not reported	Up-regulated
PenP (beta-lactamase precursor)	Down-regulated	Not reported	Down-regulated
All phage-associated genes	Down-regulated	Not reported	Down-regulated (associated with the late exponential phase)
Cold shock protein CspA	Up-regulated	Not reported	Up-regulated
Dissimilar response to root exudates			

Genes involved in carbon and nitrogen utilization	Down-regulated	Up-regulated	Not reported
Genes involved in mobility and chemotaxis	Down-regulated	Up-regulated	Not reported
Non-ribosomal synthesis of secondary metabolites with antimicrobial action such as surfactin synthetase	Down-regulated	Up-regulated	Up-regulated
Inositol metabolism IolFGEDBC	Down-regulated	Up-regulated	Not reported
Lipids metabolism: FabI enoyl-(acyl-carrier-protein) reductase and FabF acyl carrier protein synthase II	Down-regulated	Not reported	Up-regulated
ChbA chitin-binding protein	Down-regulated	Not reported	Up-regulated
Tricarboxylic acid (TCA) cycle	Differentially regulated but below statistical reliability	Up-regulated	Not reported
Oligopeptide ABC transportes OppADF	Down-regulated ($p > 0.05$)	Up-regulated	Not reported
Phosphotransferase system (PTS)	Down-regulated	Up-regulated	Not reported

For example, the chemotaxis and motility genes were down-regulated in *B. atrophaeus* (see also Table 3.1) in contrast to what was reported for *B. amyloliquefaciens*. All the mentioned genes were under control of the SigD, which was two folds down-regulation by the root exudates in the current experiment. While SigD was down-regulated, its repressor CodY was two fold up-regulated by the root exudates in *B. atrophaeus* UCMB-5137. From the literature, it is known that the sporulation

repressor CodY is activated in *Bacillus* by the catabolic repressor CcpA (Ratnayake-Lecamwasam et al., 2001). It implied a possible role of the CcpA in gene regulation by the root exudates. The similarity between *B. atrophaeus* UCMB-5137 and *B. amyloliquefaciens* FZB42 was in activation of the genes ymcA and luxS controlling biofilm formation (Nicolas et al., 2012). It is known that luxS is also an activator of synthesis of quorum sensing autoinducer AI-2 (Ruzheinikov et al., 2001). Activation of the ylbF, which is an antagonist of the biofilm repressor SinR (Kearns et al., 2005), was also observed in *B. atrophaeus* UCMB-5137 treated by the root exudates. FlaA, flagella protein synthesis, was activated by the root exudates in both organisms. It was reported in a study on *Bacillus cereus* that FlaA was important for biofilm formation and had nothing to do with other flagellin proteins (Houry et al., 2010), which were down-regulated by the root exudates in *B. atrophaeus* UCMB-5137. These observations confirmed the activation of the quorum sensing triggered biofilm formation in *B. atrophaeus* UCMB-5137 and *B. amyloliquefaciens* FZB42 in response to the root exudates stimuli. The most striking and unexpected effect on gene expression in *B. atrophaeus* UCMB-5137 treated by the root exudates was the strong stringent response presumably triggered by the alarmone (p)ppGpp. A summarized scheme of interactions between the key TFs and the regulated genes is shown in Figure 3.5.

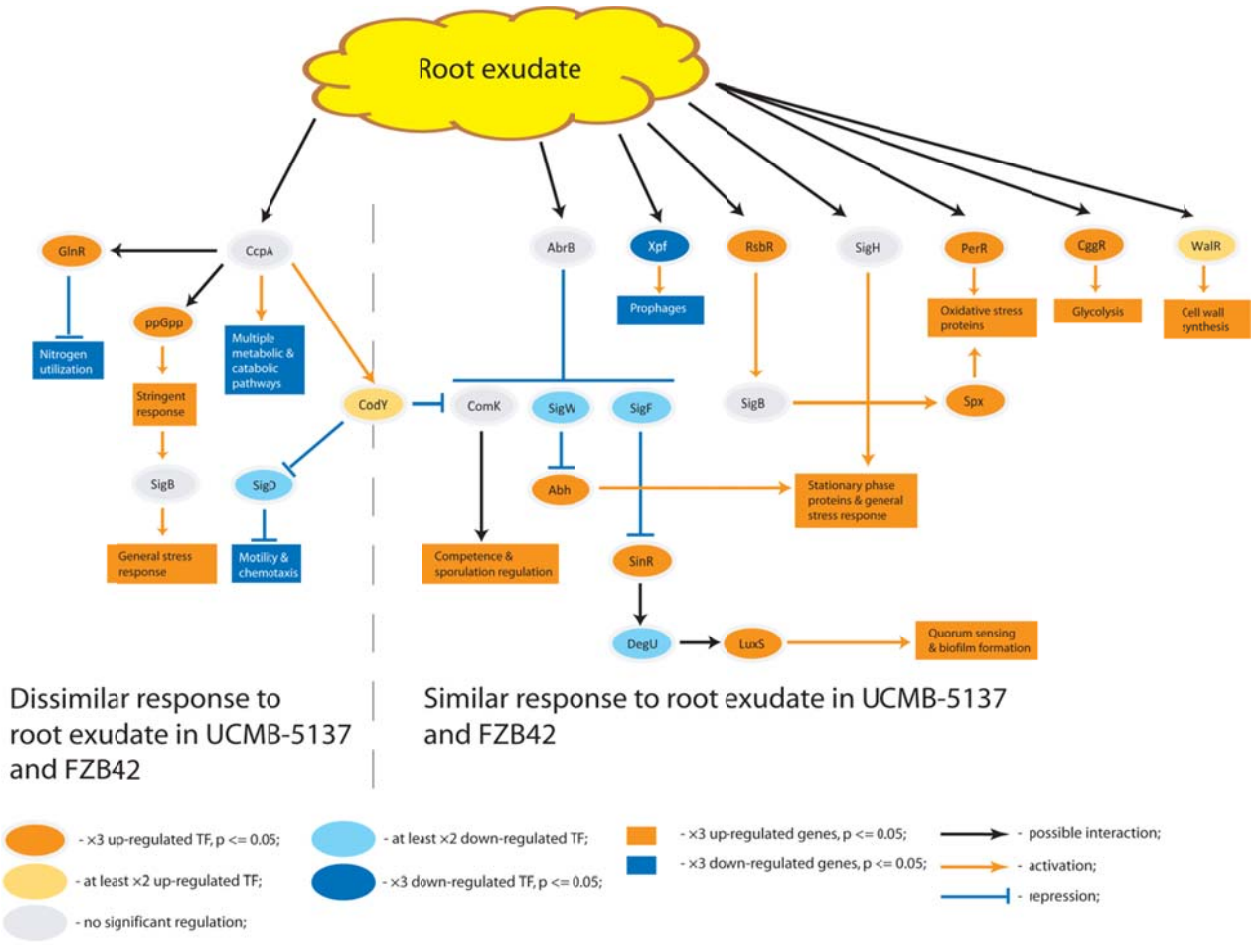


Figure 3.5: A schematic representation of the regulation of the top level transcriptional factors in UCMB-5137, which may explain the differences and similarities in gene expression profiles between the *B. atrophaeus* UCMB-5137 and *B. amyloliquefaciens* FZB42.

Similarities in responses to treatment with root exudates in the *B. atrophaeus* UCMB-5137 and the *B. amyloliquefaciens* FZB42 were represented on the right part of Fig. 3.5, and the dissimilar regulations were shown on the left part of the figure. The stringent response and general catabolic repression by the CcpA could explain all the differences between the expression profiles of these two microorganisms. The stringent response was known to be accompanied by the general stress response (Eymann et al., 2002) and by activation of the SigB stressosome that caused an up-regulation of many general stress response genes (Hecker et al., 2007).

The results of our study were in agreement with the previous publications that reported a significant down-regulation of the SigD activator of motility and chemotaxis by the different stress conditions (Sappa, 2013; Kohlstedt et al., 2014). This effect might be linked to the activation of the CodY

repressor, which also repressed the amino acid metabolism and many other metabolic pathways at the time of transition from the exponential to stationary growth phases in response to shortage of nutrients (Serror and Sonenshein, 1996, Bergara et al., 2003). It remains unclear which factors could trigger such strong stress on the *B. atrophaeus* UCMB-5137, which resulted from an addition to the medium of a tiny amount of the root exudates. It was hypothesized that the stress could be associated with the metabolic repression caused by the CcpA. The major function of the CcpA repressor is to optimize the carbon and nitrogen metabolism by repressing the pathways of utilization of the less convenient sources of nutrients, when there is an abundance of easily accessible sugars and ammonium (Wacker et al., 2003, Görke and Stülke, 2008). Normally CcpA is activated by glucose, which is a major component of the root exudates (Fan et al., 2012). However, other components, i.e. organic acids, melibiose and traces of amino acids, may intensify this response.

The amount of the root exudates added to the medium could not provide any sufficient increase in the amount of nutrients, but it could mislead the bacteria by signaling the presence of plant roots suitable for colonization in the close vicinity, which could be a source of a surplus of plant sugars (Dennis et al., 2010). The strong carbon metabolism repression without any supply of additional sugars could cause an abrupt shortage of energy and nutrients that led to an accumulation of the uncharged tRNA molecules, which in their turn, had triggered the stringent response through a ppGpp alarmone activation (Eymann et al., 2002) and a further repression of many metabolic pathways by ppGpp activation of the CodY repressor (Wünsche et al., 2012; Geiger and Wolz, 2014).

3.4 Conclusion

The current study showed limitations of our knowledge of the gene regulatory network in *B. subtilis* and related organisms. The whole dimension of the gene regulations by small non-coding RNA is still to be discovered. It was demonstrated that ncRNA interfered with gene expression regulation at the time of rhizosphere colonization. Significant alterations in the gene regulation profiles between the *B. atrophaeus* UCMB-5137 and the paradigm PGPR strain of the *B. amyloliquefaciens* FZB42 demonstrated that the PGPR could use different strategies for plant colonization. It may be important to count for these differences to achieve an optimal performance of the biopesticides and biofertilizers based on PGPR. It was concluded in this work that these differences could be explained by a hypersensitivity of the UCMB-5137 to the root exudates stimuli, impelling it to a sessile root colonization behavior through the CcpA-CodY-AbrB and probably, the DegU regulations. It was found that the general stress response genes were significantly up-regulated in UCMB-5137 by the root exudates that might result from a repression of catabolism by the CcpA activated by chemical components of the root exudates. Regulation of many genes by the root exudates was inconsistent with our knowledge on gene regulation in *B. subtilis*.

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GENERAL CONCLUSION AND RECOMMENDATIONS

In the current situation where agrochemicals present threats to people and environment, and food security problem is high in many developing countries, application of PGPR for plant growth promotion is essential. Despite many benefits that the application of PGPR offers to agriculture, there is little application of PGPR in most developing countries due to little research in this area, and the available researches have not reached to the stage of producing commercial inoculants. Using *Bacillus* species as PGPR has several advantages. The current study has added knowledge in the available limited information about gene regulation in the bacteria of the *Bacillus* group.

The first aim of this research study was to investigate the plant growth promoting and protecting abilities of selected strains of *Bacillus* isolated from the rhizosphere in different bioassays. A special attention was paid to an investigation of the strain *Bacillus atrophaeus* UCMB-5137 in comparison with the other potential PGPR *Bacillus*. To achieve the aim, we performed the experiments presented in Chapter two, from which we concluded that the selected strains despite being related to the genetically and morphologically homogenous *Bacillus subtilis/B. amyloliquefaciens* group, showed the different levels of bioactivities. There were several promising PGPR isolates, but none of them was the best in all aspects. It may be assumed that a combinatorial use of these different *Bacillus* strains in biopreparations can be the most practical solution for the agricultural application of biofertilizers and biopesticides. Differences in biological activities and specific gene regulations in PGPR bacteria by environmental stimuli should be taken into consideration when new combinatorial biopreparations are designed.

The strain *B. atrophaeus* UCMB-5137 showed some positive effects in many bioassays. As it was demonstrated on the wheat model, this strain outperformed other tested strains in promoting behavioral growth and development; however, it did not influence root development that was significantly promoted by the strain *B. amyloliquefaciens* ssp. *plantarum* UCMB-5007. We also learned that the *B. atrophaeus* UCMB-5137 produced antimicrobial compounds, which inhibited the growth of fungal pathogens: *Sclerotinia sclerotiorum*, *Alternaria brassicicola* and *Verticillium longisporum*. The strain also promoted the drought tolerance that was demonstrated on the *Brassica napus* and *A. thaliana* models by measuring the plant weights and chlorophyll amount in leaves. The *B. atrophaeus* UCMB-5137 could grow on the root exudates as a sole source of nutrients; however, not as good as the paradigm plant colonizers of the *B. subtilis* and *B. amyloliquefaciens* and with a

significant lag phase. Microscopy of plant roots treated with the spores of this strain demonstrated ability of the organism to survive and colonize the roots by forming thick biofilms on the root surface. The survival rate of the cells outside of the biofilm formations was apparently low and that was dissimilar to the tested *B. amyloliquefaciens* strains. It was hypothesized that the gene regulation in *B. atrophaeus* UCMB-5137 by root exudates stimuli may differ from the transcriptional pattern determined for the paradigm PGPR strains of the *B. amyloliquefaciens* ssp. *plantarum* FZB42.

Sequencing of the total mRNA extracted from the cells of *B. atrophaeus* UCMB-5137 under the influence of the stimuli of the maize root exudates compared to the mRNA pool of the cells grown on the same medium but without the root exudates, demonstrated a profound re-organization of the cellular metabolism. It may explain the observed extended lag phase on the growth curve. Many up-regulated genes were associated with stress response and detoxification. A down regulation of the biosynthetic pathways was observed, especially the amino acid, nucleotide, carbohydrate and fatty acid pathways. Transcription factors responsible for gene regulation during plant colonization were determined and a computational model of the gene regulation was constructed. Repression of numerous metabolic pathways was caused by the CcpA carbon catabolite repressor and a CodY pleiotropic repressor, which interplayed with the nitrogen assimilation regulators TnrA and AbrB transcriptional factors in preparing the cells to consume nutrients provided by the root exudates. Involvement of the DegU transcriptional factor in plant root colonization was confirmed. Adaptation to the oxidative stress caused by the plant produced oxygen radicals was controlled by the PerR regulator and SigB stressosome.

Analysis of the differential transcription from the non-coding chromosomal sequences allowed an identification of the multiple ncRNAs, some of which were predicted for the first time. Interfering of the ncRNAs could explain conflicted gene regulations observed in this experiment. Given similar conditions, gene regulation in the *Bacillus atrophaeus* UCMB-5137 was different to that of *Bacillus amyloliquefaciens* FZB42, which was considered a paradigm PGPR. More studies on gene regulation in *Bacillus* species are recommended. This aim contributed to the knowledge of gene regulation in *Bacillus* as well as highlight limitations available in gene regulation studies, which should be solved in some future studies.

Generally, the strains investigated showed different plant promotion and plant protection activities. There is a need to conduct field trials for those strains that promoted plant growth and find reasons

that led to growth reduction by some strains in future researches. Future research should also include other plants, apart from that, strains which showed different results can be combined to see whether they can achieve both effects in one plant. Although the strains originated from European continent, they performed well in South Africa despite the weather differences. It is hence possible to use commercial products from other countries; however research on Africa original strains should be highly encouraged.

This study is of its own kind and unique in Africa, where the need of commercial inoculants from PGPR is high, also there is little or no use of PGPR by farmers. Use of PGPR is easy, safe and cheap alternative thus can be used even by small-scale farmers. More studies of this kind are needed.

Research outputs

This research has been presented in local and international conferences and published in peer reviewed scientific journal.

Publication:

Mwita, L.N., Chan,W.Y., Pretorius,T., Lapa,S.V., Avdeeva, L.V., Lyantagaye S.L. and Reva, O. (2016).Gene expression regulation in the plant growth promoting *Bacillus atrophaeus* UCMB-5137 stimulated by maize root exudates.*Gene*, 590, pp.18–28.

Conferences:

1. ECCB'14, 7th to 10th September 2014, Strasbourg, France.

Poster: Comparison of gene expression profiles of two plant growth promoting strains *Bacillus atrophaeus* UCMB-5137 and *Bacillus amyloliquefaciens* FZB42 stimulated by maize root exudates.

2. Joint SASBi-SAGS Congress, 23-26 September 2014 Tshwane, South Africa.

Poster: Gene expression regulation of a plant growth promoting rhizobacterium *Bacillus atrophaeus* UCMB-5137 in response to maize rootexudates stimulation.

3. ISCB-Africa ASBCB Conference on Bioinformatics, 9-11 March 2015, Dar es salaam, Tanzania.

Poster: Operons used for plant colonization by a plant growth promoting rhizobacterium *Bacillus atrophaeus* UCMB-5137.

4. BIOTECHNO2015, 24th to 29th May 2015, Rome, Italy.

Talk: Gene Expression Profile of a Plant Growth Promoting Rhizobacterium *Bacillus atrophaeus* UCMB-5137in Response to Maize Root Exudates Stimulation.

5. ProkaGENOMICS 2015, 29 September- 2 October 2015 Gottingen, Germany.

Poster: Overlap between plant colonization behavior and stress response in plant growth promoting rhizobacterium *Bacillus atrophaeus* UCMB-5137.