Bacillus species applied in agriculture to increase crop yield and germination induction of *Bacillus* spores to promote plant growth

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Abstract

The increasing global population causes an increase in agricultural production pressure. At the same time infectious plant diseases, climate change and competition for available land cause a threat to food production. Therefore, methods need to be researched to increase crop yield. In this thesis the application of *Bacillus* species is proposed as a likely candidate for agricultural improvement. *Bacillus* is introduced here as a biocontrol agent that may partially replace the use of chemical pesticides. On top of the biocontrol properties of *Bacillus* species, additional positive characteristics for agriculture are mentioned. A general overview of the mechanism of plant growth promotion by bacteria is given in which the concepts of plant-microbe interactions are explained and the antimicrobial compounds produced by *Bacillus* species are outlined. The thesis sought to give an overview as to why the production needs to be increased and how this can be accomplished. A deeper insight in the process of germination, which is a requirement to implement *Bacillus* species in agriculture, is given in the final part of this thesis. In addition, this part will make it clear that more research is still required on the topic. This is followed by a discussion and conclusion that gives an overview on the positive and negative sides of *Bacillus* application in agriculture.

Contents

Introduction

Future challenges regarding food production

Estimations based on data gathered by the United Nations suggest that the global population will increase to a number exceeding far over ten billion people in the next few decades. This same dataset showed that the continent of Africa alone is expected to experience a population growth from one billion people currently to a population between 3.1 and 5.7 billion in the year 2100[1]. This massive increase of population in a world subjected to global warming and climate change poses major challenges regarding the production of a sufficient amount of food[2]. On top of climate change and global warming, which may result in the loss of fertile land by salination or desertification, competition of available land for energy production and urban development additionally poses a threat to food production[3]. Therefore, food production and efficiency of production on the available land will need to be increased dramatically in order to meet the growing demand[4]. However, achieving a higher efficiency of agricultural yield is a major challenge. For example, a big threat to the efficiency of production and food production in general is the decrease of yield caused by plant diseases[5,6]. On top of that, the ever-increasing production pressure results in an overexploitation of the fertile land, which in turn becomes exhausted in its nutrients[7]. The goal of this thesis, therefore, is to explore a way to enhance crop yield by reviewing the concepts of growth promotion by plant-microbe interactions. Specifically, the application of *Bacillus* species in agriculture.

Research in the past few decades has shown that microorganisms in the rhizosphere, which is the part of the soil in which plant growth takes place, plays an important role in plant growth[8,9,10]. A great deal of research has been conducted on the application of microorganisms in crop production. It has been proven that microorganisms have the ability to act as bio-fertilizers, bio-pesticides, bio-fungicides and bio-remediators[11,12]. These principles benefit the production of crops in agriculture and positively increase the crop yield. Therefore, microorganisms are a possible solution for the future improvement of agriculture. *Bacillus* species show a wide range of these plant growth promoting properties and can be used as biocontrol agents against fungi and bacteria[13,14]. The bio-pesticide and -fungicide properties are a result of the ability of *Bacillus* species to excrete compounds that display antimicrobial and antifungal properties[14]. These compounds are used to eliminate pathogens that infect plants and decrease the crop yield[15].

For the application and implementation of *Bacillus* species showing these plant biocontrol properties spore formation is utilized. Spores are small multi-layered spherical structures that can withstand extreme conditions and contain all the required information and contents of bacteria to form vegetative cells again[16]. A lot of spore forming bacteria are used currently as plant biocontrol agents and, therefore, efficient germination of the spores into living bacterial cells is an important factor for the application of these biocontrol agents in agriculture[17,18]. The application of microorganisms is promising but still a lot of research is required. Mechanisms of spreading microorganisms on crops, and negative treats need to be researched on top of screening for the effectiveness of *Bacillus* species against relevant pathogens.

Microbes in the rhizosphere and plant-microbe interactions can stimulate plant growth

The discovery of plant-microbe interactions

In 1904 a paper was published by Lorenz Hiltner who was the first to describe the rhizosphere with its microorganisms, marking the birth of the studies on rhizosphere interactions[19]. The years following this publication, a paper was published in 1915 by Percy Brown who investigated the influence of microorganisms on crops. This paper hypothesized that bacterial activity on nitrogen conversion was correlated to crop yield. The conversion of nitrogenous organic compounds by bacteria increases the nitrogen availability for crops and subsequently results in a larger yield. Therefore, the paper implied the possible importance of bacteria on crop growth and the possible applications of bacteria in agriculture to improve fertility of the soil resulting in a higher crop yield[20].

Another paper published in 1929 described and emphasizes the significance of plant-microbe interactions[21]. This paper, published by Robert L. Starkley, states that 'neither groups of organisms' referring to plants and microbes 'can develop long in the absence of the other in nature without showing abnormalities due to the absence of the other associate.' Starkly indicates that both organisms rely on each other. The bacteria requiring nutrients from the plant and the plant relying on the degradation and conversion of the remains of dead plants[21]. By then, it was already known that direct plant-microbe interactions play an important role in their growth and development by providing the required nutrients and habitats.

Advances in the field of microbiology on experimental techniques were made in the years following the early research on plant-microbe interactions. Examples are improvements on microscopy techniques in which higher resolution images could be taken and the fluorescence microscopes emerged. More recent developments occurred in genomics and metagenomics[22]. Discovery of plant-microbe interactions in the past were primarily based on gnotobiotic research combined with microscopy. Bacterial strains containing selection markers such as GFP-fused proteins were introduced in the rhizosphere and their root colonizing abilities were monitored. This was done by introducing the bacteria into the known system (rhizosphere) and follow their growth and division along with their distribution in the system on for example the roots of plants over time with microscopic techniques[23]. This technique was used after the initial studies on plant microbe interaction and is still used today to study the possible interactions that the bacteria have with the plant.

The recent application of metagenomics in the discovery of plant-microbe interactions has caused a tremendous increase of discovered possible interactions[24]. Modern research on plant microbe interactions often uses metagenomic techniques. With these metagenomic techniques the complete biological system (rhizosphere) is identified by sequencing all the DNA in the environment and correlate this to data available in databases. This metagenomic data is additionally connected to research on individual components of the system for example to research on single microorganisms to get an overview of the functions and relations of the plants and different microbes. This method is combined with mathematical and computing techniques to generate a model of the system. As a result of this systemic approach with metagenomics, a good understanding of the plant-microbe interaction have been obtained in recent years[25]. It is via metagenomics that *Bacillus* species are increasingly related to growth promoting plant-microbe interactions[26]

Strategies and mechanisms of plant microbe interactions

In the following section, a general overview will be presented to give an idea of how plant-microbe interactions function. To positively stimulate plant growth the bacteria need to be able to successfully interact with plants and other organisms in their proximity. Therefore, the ability to colonize plants is extensively researched[27]. Bacteria have developed specialized mechanisms for root colonization by excreting molecules such as amino acids, vitamin B1 and fimbriae or expressing proteins such as agglutinin, pili and proteins on the cell surface of the bacteria. Besides the excretion of these molecules which play a role in colonization, other mechanisms such as chemotaxis and the excretion of antimicrobial compounds play an important role during colonization. The result of all these molecules and mechanisms is the ability of the plant growth promoting bacteria to outcompete pathogens during the colonization of the plant[27]. Plant growth promoting rhizobacteria and pathogens do not only colonize the roots of the plants, they can also enter the internal environment of the plants. To enter the internal environment, no specialized mechanisms or proteins are required. However, some mechanisms may enhance the colonization of the internal structures of the plant. These mechanisms include lipopolysaccharide production, pili formation and flagella formation[27]. It is generally recognized that rhizobacteria that can enter the plant are more likely to display direct growth promoting properties than the bacteria that only colonize the roots. Cells use different mechanisms to colonize the plant which can act either alone or together to successfully inhabit the surfaces and insides of the plants.

Within the rhizosphere a large competition takes place between the different microorganisms. Therefore, bacteria have developed mechanisms to compete with these other inhabitants of the rhizosphere. Some of these mechanisms consist of the ability to excrete antimicrobial compounds, the ability to grow faster or having a higher motility[27]. Competition of pathogens with plant growth promoting rhizobacteria takes place for space on the root surface and for nutrients. The competition for space induces the production of antimicrobials of the plant growth promoting rhizobacteria to kill the competing pathogens[28]. The competition for nutrients results in a decrease in the amount of nutrients available for pathogens[28]. These competition mechanisms are biocontrol properties of bacteria displayed in nature that result in the death of the pathogens and subsequently in a decrease of pathogen related plant diseases[9].

Bacteria that may have entered the plant can induce a direct plant growth stimulus by excreting molecules that act as growth stimulating hormones or providing additional nutrients[29]. On top of that, the bacteria that entered the internal system can induce an immune response from the plant that makes them more resistant against pathogens, the so-called induce systemic response (ISR)[29]. This immune response is different than the immune response upon infections with pathogens and provides additional protection against pathogens. Although this makes the plant not fully resistant against pathogens it provides some additional level of protection. The ISR alongside the biocontrol properties of bacteria that colonize and compete for the roots result in a good defence system against pathogens. The next subsections will provide a deeper insight into direct and indirect plant growth promotion by different rhizobacteria.

Root exudates and volatile organic compounds

One of the possible ways by which plant-microbe interactions may be stimulated is via root exudates, which are (organic) compounds excreted by the roots of plants and crops. These exudates may stimulate certain microbes via chemotaxis to form interactions with the plant[30]. Other exudates can have the opposite effect by inhibiting microbes to interact with the plant, resembling an immune response of the plant. A bacterium living in the rhizosphere that undergoes an interaction with the plant stimulated by these root exudates can emit volatile organic compounds (VOC). These VOC may have varying effects on plants, some of which may be stimulating growth. The mechanisms to which VOC can stimulate crop growth differs in nature. Some VOC that exists mimic certain growth stimulating phytohormones such as indole-3-acetic acid, cytokinin and gibberellin and induce plant growth upon contact with the plant[31]. A different mechanism of how VOC may contribute to crop growth is by increasing the mineral and nitrogen availability in the soil[31]. Meaning that bacteria may excrete VOC that serve as biofertilisation. These VOC will allow the crops to gather the required resources for growth and proliferation more efficiently, resulting in an increasing growth rate[32].

Direct plant growth promotion via plant-microbe interactions

The rhizosphere contains a tremendously large amount of microorganisms[28,26]. It is a complex system in which a lot of plant-microbe and microbe-microbe interactions take place. The plant-microbe interactions function in a way that uses either biocontrol or biofertilisation properties as described above. Some of these plant-microbe interactions are beneficial for crop growth. The interactions can be positive for both the plants as well as the bacteria. The bacteria may provide protection or nutrients for the plants and the plants in turn may provide nutrients for the bacteria or places to grow and colonize[28]. The opposite is an interaction in which the bacteria have detrimental effects on crops by colonizing the crops and gaining nutrients from the plants but not providing any benefits in return[8]. These bacteria, or fungi are regarded as pathogens and will be described later. Neutral interactions in which neither the bacteria nor plants do not benefit or take advantage from the interactions are left out of consideration.

An example of plant-microbe interactions that uses biofertilisation, as described here, to stimulate plant growth is the ability of certain bacteria to fixate nitrogen. For example, the *Leguminosae* plant family is able to undergo symbiotic interactions with *Rhizobia*. These *Rhizobia* colonise parts of the plant that are known as nodules. These nodules are a bulge on the root of the plant that provide an ideal site for the bacteria to live[33]. The *Rhizobia* that live in these nodules are capable of fixating atmospheric nitrogen gas and convert it into ammonia which the plant can use to produce amino acids, nucleotides and other cell content from such as vitamins and hormones[34]. The interaction of the *Leguminosae* with the *Rhizobia* is so successful, that it makes *Leguminosae* the largest plant family to exist[35]. This interaction shows the potential of plant growth stimulation by bacteria via biofertilization. These kinds of interactions act in a direct way. This means that the bacteria provide nutrient to the plant in the form of VOC which the plant can use directly to grow. The product of the interaction stimulates plant growth. The next section will describe interactions that act in an indirect way. In these types of interactions, the product of the interaction results in better conditions for the plants to live, but do not provide any nutrients or phytohormones to plants.

Indirect plant growth promotion via plant-microbe interactions

In this thesis, emphasis lies most heavily on the indirect stimulation of plant growth by protecting the plant against pathogens. *B. subtilis,* for example, is known to be able to produce antimicrobials that can fight plant pathogens[36]. On top of that, *B. subtilis* can form a protective layer of cells, a biofilm, on the roots of plants that provides an additional protection for the plant against pathogens[9.] *Bacillus* species are able to kill or inhibit the growth of pathogenic bacteria and fungi. For instance, *Bacillus subtilis* has been shown to be effective against the bean pathogen *Uromyces appendiculatus*, a fungus that causes bean rust. Farm fields containing bean plants that were showing bean rust were treated with *B. subtilis.* Treatment with *B. subtilis* resulted in a decrease of bean rust of 86%. Additionally rust infected beans treated with supernatant of *B. subtilis* cultures also resulted in a decrease of rust indicating that excreted compounds by *B. subtilis* were responsible for the decrease of the rust disease causing pathogen[37].

An alternative mechanism for indirect plant growth stimulation is use of bacteria to clean land contaminated with heavy metals or organic compounds emitted by the industry. This will allow the contaminated land to be used again for agriculture[12]. In this final example of bioremediation, bacteria are used to clean contaminated land. As a result, the land becomes viable again for plants to grow[38]. A research conducted by Johnson et al. 2005 showed the principal of plant-microbe interaction during bioremediation. In this research they took samples of contaminated soil with poly aromatic hydrocarbons (PAH). Different conditions where used. In one sample they inoculated *Lolium perenne* and *Trifolium repens* with *Rhizobium leguminosarum* bv. *Trifolii.* These are two types of plants and a rhizobacterium all incapable of degrading PAH. In another setup they only planted the two plants. For 180 days they monitored the concentration of PAH as well as the amount of PAH degrading bacteria in the soil. They found that after 180 days, the setup with both plants and inoculated rhizobacteria resulted in a significant decrease of PAH (824 mg kg^{-1}) compared to a setup with no plants but only rhizobacteria inoculated (1017mg kg^{-1}). On top of that, the setup with plants but without inoculated rhizobacteria showed no significant decrease in PAH. This result indicates that the presence of both plants and inoculated rhizobacteria stimulates other bacteria to grow and degrade PAH.

Johnson et al. 2005 showed in their research that the soil samples already contained bacteria that where capable of degrading PAH. However, with plants that either received or did not receive an inoculum of rhizobacteria resulted in a significant increase in the number PAH degrading bacteria. This suggests the stimulation of bacterial growth by the plants. Additionally, the setup with plants and inoculum resulted in the largest increase in the number of PAH degrading bacteria, suggesting the positive effect of the rhizobacteria on other bacteria. As their final result, both the shoot and roots showed a significant increase in length and biomass respectively in the setup with plant and rhizobacteria compared to the setup with only plants. Indicating an additional positive effect of the rhizobacterium on the plant growth. The principle of bioremediation as presented here resulted in the soil becoming suitable again for agriculture that requires land without any industrial contaminated compounds[39]. This presented principle is one example of bioremediation and was explained to give an idea of the possibilities of using microorganisms for agricultural improvements.

Plant pathogens pose a threat to agricultural yield

The following sections will focus more on indirect plant growth promotion via the biocontrol properties of *Bacillus* species. As mentioned earlier, not all plant-microbe interactions are positive for plant growth. Some microbes may negatively influence plant growth and development. These microbes are known as plant pathogens and they can be either fungi or bacteria. Viruses can also infect plants and are therefore regarded as pathogens, but this is beyond the scope of this thesis[11].

An example of an agricultural relevant plant pathogens is *Puccinia graminis tritici*, which colonize wheat crops. This pathogen is believed to be a major threat to wheat production, possibly causing huge epidemics in the future[40]. Another example is the fungus *Fusarium oxysporum f. sp. cubense* that poses a major threat to banana production worldwide, also known as the Panama dissease[41]. Examples related to *Bacillus* research include *Uromyces appendiculatu* described in the previous section, *Exserohilum turcicum* that causes leaf blight of corn, *Pseudomonas savastanoi pv. Savastanoi* that causes olive knot disease and *Pseudomonas syringae* causing disease in *Arabidobsis*[37,42,43,9]. These are merely a few examples of the substantial amounts of plant pathogens out there that are threatening agricultural production, much more exist.

In the past, severe epidemics have been caused by infections of microorganisms in plants leading to a great loss of crops and posing a threat to a sufficient production of agriculture. Ultimately, this resulted in an increasing food price or even food shortage. Examples include an epidemic of potato blight caused by a fungus in Europe in 1845 that resulted in one third of the potato being lost[44]. And the fungus *Helmintosporium oryzae* infecting rice crops in India in 1943 that resulted in the death of over two million people due to starvation[45]. Most of these epidemics took place years ago, when transport and communication were not as developed as they are today. However, the introduction of large monocultures in which vast amounts of land is used for one crop only, poses a major risk for infectious disease in modern days[46]. Currently, still 20–30 percent of the crop yield is lost due to infectious disease on a yearly base[15]. Thus, with the increasing global population, climate change, and the resulting agricultural production pressure, more effort should be put in preventing these epidemics from occurring and pathogens being able to infect plants. One of these efforts lies in the field of *Bacillus* application during crop growth.

Bacillus species applied in agriculture

Why *Bacillus* species may be used to stimulate crop production

This thesis seeks to explore the ability of *B. subtilis* and other *Bacillus* species to protect crops from pathogens resulting in the promotion of plant growth. Chemical pesticides that are often used in agriculture are effective against most pathogens. The problem with these chemical pesticides, is that they can have severe negative side effects. Some of these effects include damaging human health, both mental and physiological as a result of binding to receptors found on the cell surface of cell in the nervous system and other parts of the body[47,48]. Additionally these compounds can damage the environment by poisoning animals and polluting the rhizosphere with dangerous chemical compounds. This environmental pollution results not only in the death of the intended pathogen but the bacteria that positively influence plant growth also get killed[49]. A complete narration on the positive and negative sides of chemical pesticides is not the goal of this section. Therefore, it will be assumed that the use of alternative biocontrol methods such as the application of *Bacillus* species are better and safer to use.

Bacillus species are commonly found in the topmost parts of the soil and are capable of adapting to the ever-changing environments. *Bacillus* species are frequently found to reside next to plants and capable of biofertilisation which decreases the need for additional fertilizing land. The mechanisms by which *Bacillus* stimulates plant growth are depicted in Figure 1. This figure shows the ability of *Bacillus* species to promote plant growth directly by providing nutrients to the plant via biofertilisation. The figure also depicts the indirect methods of plant growth promotion by acting as biocontrol agents such as bio-pesticides against pathogenic bacteria and bio-fungicides against pathogenic fungi[11].

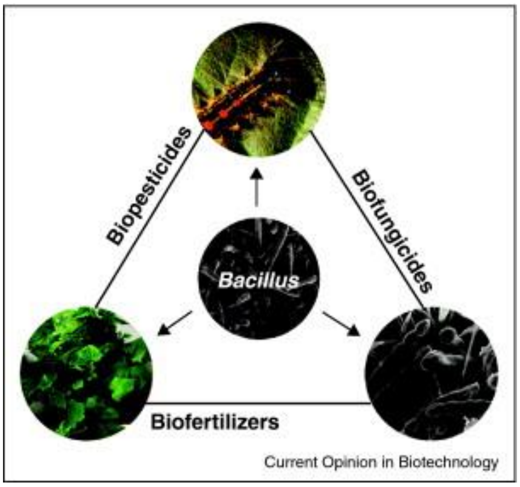

Figure 1: ways of plant growth promotion by Bacillus species. picture from Pérez-Garcia et al. 2011

Many bacterial species exist that are capable of protecting plants against pathogens or stimulating plant growth using either the same or other mechanisms mentioned above[29]. The reason why *B. subtilis* is considered here as a possible plant growth promoting bacteria is a result of several facts. The first reason being that *Bacillus* species are abundant throughout nature. They are found all over the globe and can live under a wide range of conditions, in different climates[50]. In addition to the abundancy of *Bacillus* species, they are also capable of withstanding harsh conditions in the soil. These conditions may be a result of changing weather conditions and even climate change. *Bacillus* species have multiple adaptation mechanisms to survive changing environments, the ultimate mechanism being the ability to from spores[51]. When a *Bacillus* has formed spores, it can withstand extreme conditions and survive for years. However, when the conditions are favourable again, the spores germinate to form vegetative cells[52]. This makes them capable again of growth and division, resulting in the possible promotion of plant growth. The fact that *Bacillus* species are capable of forming spores also make them easier to handle. As will become clear later these spores provide a way to produce commercial *Bacillus* strains for the implementation in agricultural systems.

How *Bacillus subtilis* promotes plant growth

Different *Bacillus* species may promote plant growth through varying mechanisms, the focus of this thesis, however, will be on *B. subtilis*. The first mechanism of biocontrol by *B. subtilis* to be considered is the formation of biofilms over the roots of the crops. A research conducted by Bais et al. 2003 showed that *B. subtills* is able to cover the entire root of *Arabidopsis* with a biofilm. With this research, they showed that this biofilm formation depends on surfactin production, a lipoprotein produced by *Bacillus* species. A strain lacking the genes responsible for surfactin production was shown to be deficient in biofilm formation. Within this same research they showed that no other bacteria were required for the formation of the biofilm, by inoculating *B. subtilis* on *Arabidopsis* in sterile soil. Arabidopsis infected with *Pseudomonas syringae* was inoculated with *B. subtilis.* As a result, most *P. syringae* cells died, leaving *B. subtilis* able to grow. They showed that the genes required for biofilm formation and subsequently production of surfactin was required for this biocontrol property of *B. subtilis*. Cells lacking the genes for surfactin were unable to protect the *Arabidopsis* from the *P. syringae.* The results they obtained upon inoculation of wild type *B. subtilis* with *P. syringae* infected *Arabidopsis* showed a mortality decrease from 85% to 10%[9]. The main point of criticism that can be given on the research conducted by Bais et al. is that this biocontrol property was tested on *Arabidopsis* which is not relevant for agriculture. To verify the biofilm mediated biocontrol properties of *B. subtilis* relevant for agriculture, crops should be used that are produced in agriculture. The next point of criticism is that only one pathogen was used in their research. This could have been a strain sensitive to competition for nutrients with other bacteria. To verify this as a relevant biocontrol property in agriculture, different crop-related pathogens should be screened.

There are, however, thousands of plant pathogens in nature that are also relevant for agriculture[53]. This shows the complexity of research on antimicrobial strategies against plant pathogens. The biofilm formation as shown on *Arabidopsis* with *B. subtilis* might not be as successful or even take place at all in other plant species. Nevertheless, the results showed a possible mechanism on how a biocontrol property of *B. subtilis* may function

Antimicrobial compounds produced by *Bacillus* species

The surfactin described by Bais et al. in the previous section is an example of a lipoprotein produced by *Bacillus* species that has antimicrobial properties against bacteria or fungi. Besides surfactins, *Bacillus* species can also produce iturins and fengycins. These lipoproteins are amphiphilic, meaning they have a lipid tail with a circular oligopeptide attached to it. The lipoproteins are produced in a non-ribosomal manner using a non-ribosomal peptide synthases[54]. Three representatives of these lipopeptides produced by *Bacillus* species that display antimicrobial activity are depicted in figure 2-4. The function of the three lipoprotein families lies not exclusively in the fact that they can kill plant pathogens but some of these molecules also facilitate the spread of the *Bacillus* species on the crops and some may stimulate the plants own immune system (ISR)[13]. The mode of action of these three lipoprotein families comes from their ability to interact with the lipid bilayer of the pathogen facilitated by their amphiphilic nature. Each of these lipoprotein families will be described in the sections that follow.

Lipoproteins

Surfactin

The surfactin lipoprotein family consists of seven amino acids that are linked to a β- hydroxy fatty acid with a length varying between 13-16 carbon atoms[55]. Surfactin acts by producing pores in the membrane of the pathogen. The lipid tail allows for the surfactin to insert into the membrane and when it reaches a critical concentration, pores start to form that destabilize the lipid bilayer. Surfactin acts like a detergent, but destabilized membranes stronger than typical detergents used in laboratory settings[56]. This membrane destabilization results in killing the pathogen. The efficiency of causing membrane leakage or cell lysis increases with an increasing number of surfactin molecules[57].

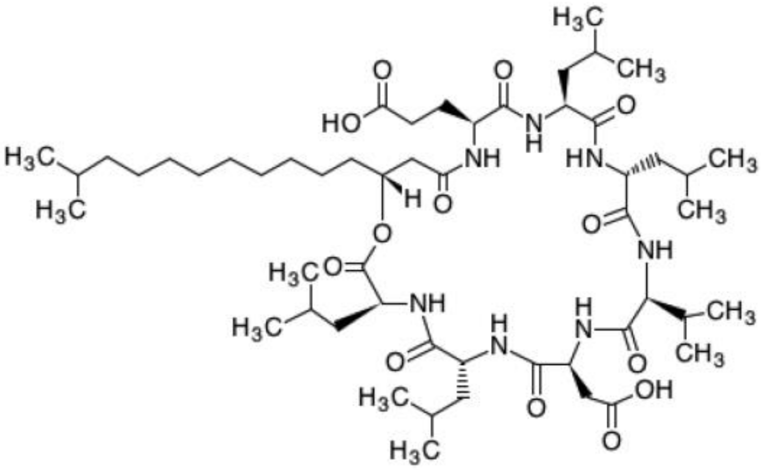

Figure 2: A representative of a Surfactin structure. derived from: W. Mongkolthanaruk.

Beside the ability of killing pathogens, surfactins are also known to interact with plant cells resulting in an immune-response (ISR) of the plant which in turn causes a resistance increase against pathogens[58]. Research conducted by Ongena, M. et al. 2007 showed that upon treatment with surfactin, ISR took place which resulted in a disease reduction of 33%[55]. In this research it was shown that this was the result of the ISR and not a result of the antimicrobial properties of surfactin. Thus, concluding that surfactin not only acts as an antimicrobial but also results in an induced systemic response.

Surfactin additionally plays an important role in the biofilm formation of *Bacillus* on plant roots, which already became clear from the section above[9].

Iturin

The iturin lipoprotein family consists of seven α-amino acids that are linked to a β-amino fatty acid with a length varying between 14-17 carbon atoms[55]. Iturin acts in a similar fashion as surfactin. Iturin is mainly effective against fungi[59]. But where pores made of surfactin destabilize the membrane of pathogens, the pores formed by iturin molecules allows for the flux of charged ions, such as K^+, that generates an osmotic shift resulting in the death of these pathogens[60]. The ability of Iturin to form ion conducting pores depends on the lipid length as well as the cyclic peptide attached to it[61].

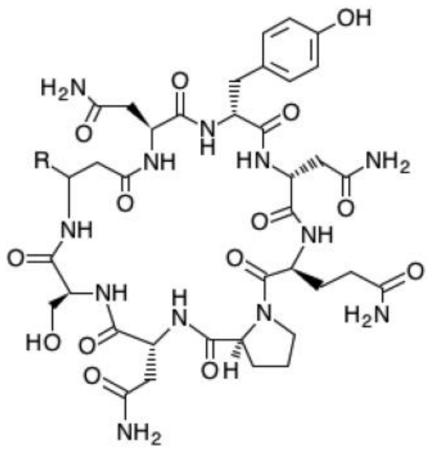

Figure 3: A representative of an Iturin structure. derived from: W. Mongkolthanaruk.

Fengycin

The fengycin family is made of 10 amino acids that are linked to a β-hydroxy fatty acid varying in length between 14-18 carbon atoms[55]. Fengycin acts different than the surfactin or iturin family. However, fengycin also interacts with the membranes of the pathogens. Fengycin acts as a destabiliser of membranes and cell walls and is capable of destroying mitochondrial membranes, which results in the release of reactive oxygen species inducing cell death of the pathogen[62].

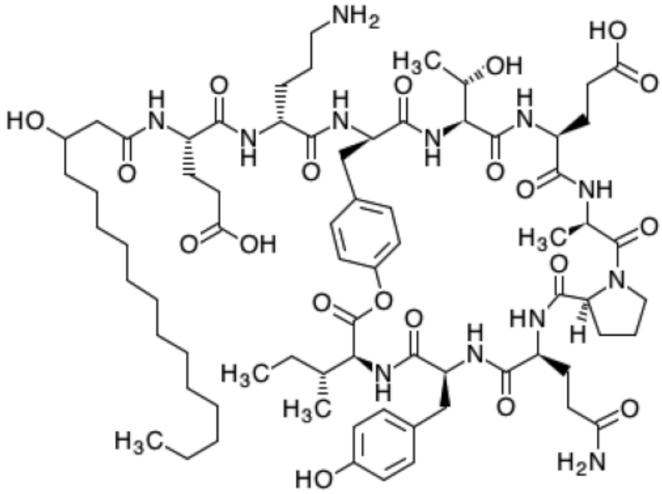

Figure 4: A representative of a Fengycin structure. derived from: W. Mongkolthanaruk.

Fengycin shows mainly antifungal properties and showed promising results in the protection of canola and wheat against pathogenic fungi, which is relevant for agriculture[63].

Besides the antimicrobial properties of fengycin, these molecules are also able to induce an immune response (ISR) from the plants enhancing the resistance of crops against patogens[58]. In the same research that proved surfactin to be an ISR inducing agent, fencing was shown to also induce ISR although to a lesser extent. The ISR induce by fengycin resulted in a disease decline of 14%.

Research has shown that these lipoproteins can also act synergistically with other lipoproteins and with other rhizosphere inhabitants to combat pathogens. This results in an increase of efficiency of these lipoproteins against pathogens[54]. Besides the three lipoproteins described in this section, *B. subtilis* is able to produce dozens of other different antibiotics and antimicrobials. Examples include: bacteriocins and polyketides some of which will be described below[36]. These compounds are not merely a product of *B. subtilis* but other *Bacillus* species are also capable of producing similar antimicrobial compounds[11]. A relevant example being the *B. subtilis* related bacteria, *Bacillus amyloliquefaciens* that can also produce a wide variety of antimicrobial compounds and is known to colonize plant roots[64]. The antimicrobial compounds described in these sections are produced throughout different *Bacillus* species and make the *Bacillus* species therefore promising in the field of agriculture as a biocontrol agent[13].

Bacteriocins

Bacteriocins produced by *Bacillus* species are peptides that can be classified in three classes. Class one are peptides that are posttranslational modified and contain unusual amino acids. This class of peptides are also known as lantibiotics. Some lantibiotics can form pores in the membranes. Others interfere with the enzyme responsible for the cell wall synthesis. All lantibiotics inevitably result in bacterial growth inhibition. The second class are peptides that are not posttranslational modified and the third class consists of large proteins[54]. These last two classes also show antimicrobial properties that function in a similar way as lantibiotics by targeting protein synthesis and disrupting the cell wall[54]. Three examples of bacteriocins are depicted in figure 5.

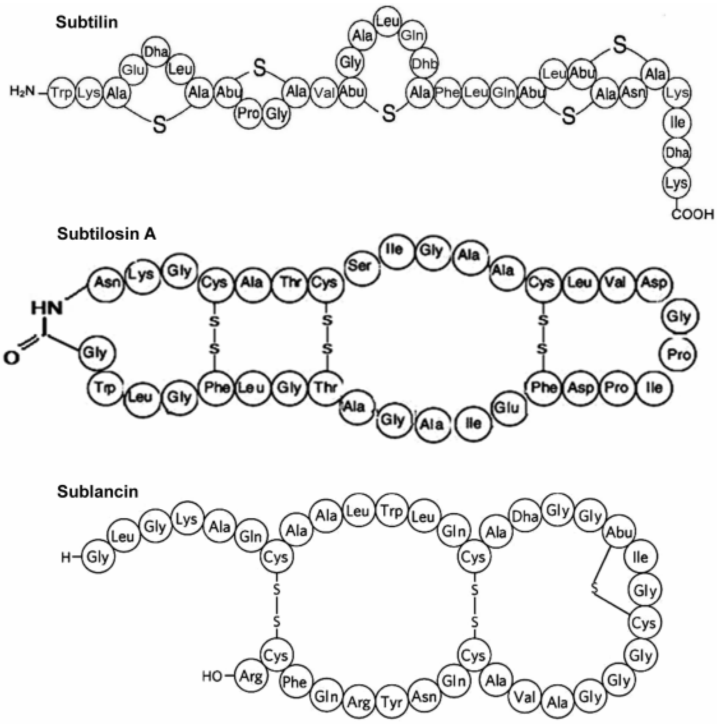

Figure 5: Representatives of the Bacteriocins. Derived from: W. Mongkolthanaruk

Polyketides

Polyketides are a class of antimicrobial compounds that are produced non-ribosomally via large multimodular synthethases (polyketide synthethases). Different polyketides exist and only the polyketides produced by *Bacillus* species are described below.

Macrolactin

Macrolactins are 24-membered ring lactones that are modified with for example glucose-β-pyranoside at the R1 position[54]. They are a polyketide family, that shows antibacterial properties[65]. Macrolactin targets the fatty acid synthesis machinery, namely the fatty acid synthase, and it can target the bacterial peptide deformase that removes formyl groups from the N-terminus of bacterial proteins[65,66].

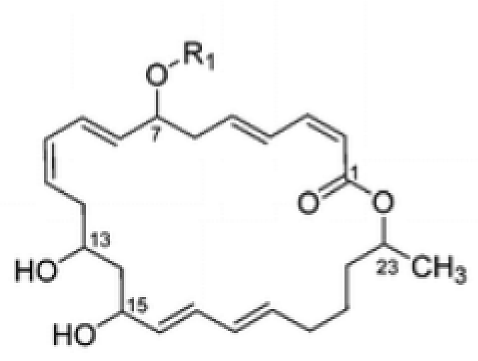

Figure 6: a Macrolactin structure. derived from: W. Mongkolthanaruk.

Bacillaene

Bacillaene is a linear open-chain enamine acid structure, attached to a hexaene which is an alkane with six double bonds[54,64]. Bacillaene is an antimicrobial compound produced by *Bacillus* species that targets protein synthesis[67]. This compound is very unstable and, therefore, hard to research[64]. Bacillaene shows primarily antifungal properties[68].

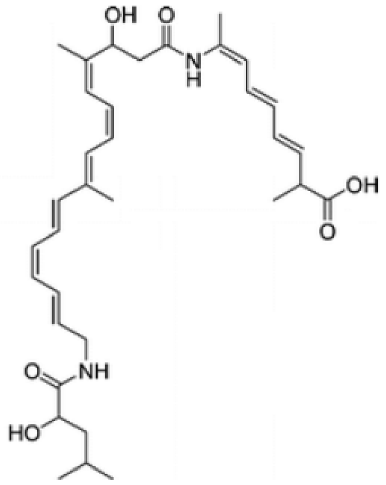

Figure 7: a Bacillaene structure. derived from: W. Mongkolthanaruk,.

Difficidin

Difficidin is a 22-membered macrolide (macrocyclic lactone) to which a phosphate group is attached[54]. Difficidin targets the cell wall and the membrane[69]. Cells treated with difficidin result in the destruction of bacterial cell walls and eventually causes severe membrane degradation such that the cell components leak to the extracellular environment leading to the death of the pathogen. The use of difficidin was proven to be successful against the agricultural relevant pathogen *Xanthomonas spp* that infects rice crops. After treatment of infected rice crops with difficidin a mortality decrease of 59% was achieved[67].

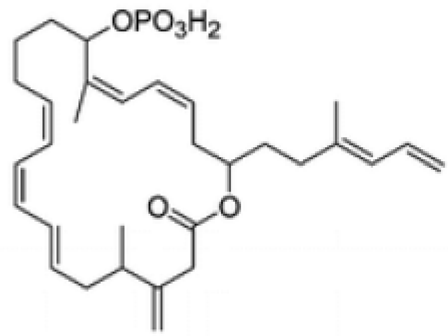

Figure 8: a Difficidin structure. derived from: W. Mongkolthanaruk.

The antimicrobial compounds described throughout these sections are a few representatives that are relevant for *Bacillus* species. These compounds consists of several structural variants but act in a similar fashion[54]. *Bacillus* species use these compounds to perform their biocontrol properties in agriculture and compete with other bacteria to obtain space on roots of crops or to obtain nutrients from the surroundings[27].

Germination, a mechanism that needs to be well understood for *Bacillus* application in agriculture

Sporulation of *Bacillus* species

All *Bacillus* species occurring in nature are known to be capable of forming spores. Sporulation of *Bacillus* species is an important survival strategy against the depletion of nutrients in their surroundings[70]. The process of sporulation in *B. subtilis* takes place via an asymmetric cell division. As a result of this asymmetric division a small dense daughter cell (the spore) appears besides a larger less dense daughter cell[18]. The less dense cell contributes a lot of its resources and energy to the spore formation and then lyses[70]. The resulting spore is a small encapsulated structure with hardly any metabolic activity[51]. This spore consists of an inner membrane, surrounded by a peptidoglycan cortex, that is followed by an outer membrane. This all is surrounded by a coat resulting in many layers that protect the spore[71]. The major constituent of the spore is located in the core of the spore, which are divalent calcium ions with dipicolinic acid, alongside the entire genome of the bacterium, additional enzymes and al the proteins required for germination (Figure 9)[71]. This spore is able to survive for a long time and can withstand harsh conditions, such as high temperatures and radiation levels[72,71]. The exact mechanism of sporulation has been subjected to a lot of research and is therefore reviewed extensively in other papers[70,73].

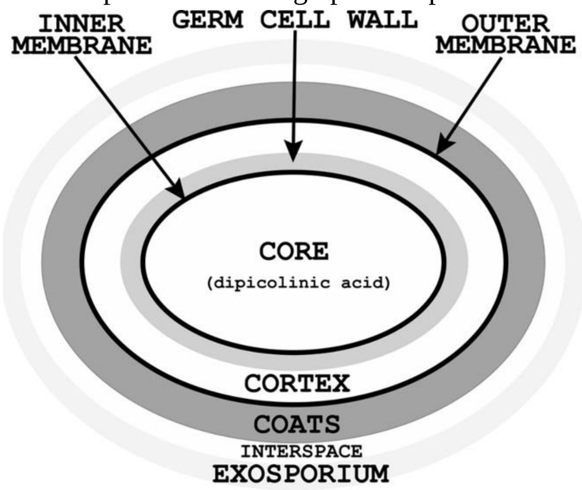

Figure 9: spores formed from Bacillus cells, a layered structure that can survive for years and cope with harsh conditions. Picture derived from the work of Peter Setlow

Germination of *Bacillus* spores

Spores of *B. subtilis* and other plant growth promoting bacteria are abundant in soil[74]. The reverse process of sporulation is germination. As a result of germination, spores become metabolically active again and able to grow and divide[75]. Therefore, it may be beneficial to know what factors influence the germination process in nature and how these factors may be used and applied to induce germination and subsequently promote plant growth by the mechanisms explained[11]. The process of germination is also important to be able to possibly implement *Bacillus* spores in agriculture as an additive.

The process of germination comprises of a few succeeding steps. The first step begins with the spore sensing nutrients and related molecules in the environment termed germinants. These germinants indicate the possibility of spores to survive after germination[52]. These molecules therefore are, for example, amino acids which may be required for the cell's ability of survival or sugars such as glucose and fructose. The germinants are sensed by receptors on the inner membrane of the spore and will induce the germination process[76]. After the spore has committed to germination, step two is initiated. In this step the spore's membrane becomes more fluid and allows the flux of ions to move out the spore. First, single charged ions are released to the extracellular environment such as Na^+ , K^+ and H^+ . This is followed by the release of Ca^{2+} and dipicolinic acid (Figure 9)[52]. The excreted K^+ is picked up again later and the cell will replace the dipicolinic acid and calcium with water during a rehydration process[76]. The rehydration step is followed by the degradation of the spore's cortex by lytic enzymes. The degradation of the cortex will allow the cell to take up additional water and swell to a normal sized bacterium. This all is followed by the final stage in which the spore becomes metabolically active again[77,52]. The spore becoming metabolically active in the final stage of germination indicates that for the entire process of germination no metabolic activity is required and the entire machinery is already present in the spore prior to germination[16].

Germinants and other factors that induce germination

As mentioned earlier, nutrient molecules are required to induce germination. Germinants have been researched extensively in laboratory settings[77]. One of the reasons why germination inducing agents have been researched so extensively is because it is relevant for the food processing industry. Some *Bacillus* species are known to cause food spoilage, and because spores are heat resistant it is hard to get rid of the contaminated *Bacillus* spores on crops using mild treatment conditions suitable for the food processing industry[77]. Therefore, germinants were researched that might efficiently induce germination of the resistant spores prior to food processing, resulting in the decrease of food spoil. However, for the application of *Bacillus* in crop growth promotion it is important to know which germinants induce germination in nature or what factors can be used to induce spores to germinate prior to introduction of *Bacillus* species to the soil. Unfortunately, no research has been done on this specific topic and no hard facts on the germinants used in nature to induce *Bacillus* spores are known.

Throughout *Bacillus* species, three receptors exist that monitor the nutrients in the surroundings. These receptors are located on the inner membrane of the spore (figure 9). The receptors GerA, GerB and GerK are clustered together on the inner membrane of the spore in a cluster termed the germinosome. The assembly of this structure is mediated by the protein GerD[78]. These receptors again consist of three substituents, in the case of GerA this is GerAA, GerAB and GerAC and each again have a different function. This already shows the complicated mechanisms of these receptors. The signal transduction pathways of these receptors are still not fully understood. The three germinant receptors (GerA/B/K) respond to different nutrients. It is generally recognized that in *B. subtilis,* the GerA receptor responds to L-alanine and L-valine in the environment[79]. The GerB and GerK receptor are believed to act synergistically, and correspond in a depending fashion to combinations of the nutrients: L-asparagin, D-glucose, D fructose and potassium (AGFK)[80]. The receptors are stereospecific and only respond to these isomers, other isomers might even inhibit the germination process[75]. However, these observations were done in a laboratory setting and do not represent the conditions and availability of these nutrients in natural soil. These germinants are however likely to be found in soil and therefore regarded as germination inducing factors in nature[8]. Also, different germinants have been found that artificially induce spores to germinate. However, these are not expected to exist in soil. These artificial germinants include lysozyme, dodecyl amine or physical germinants such a high pressure and temperature[77,81]. A second type of germinant that induces spore germination and may be present in soil in which the crops grow, and *Bacillus* species may live, is Ca^{2+}-dipicolonic acid. The Ca^{2+}-dipicolinic acid that is released from the spore during one of the early stages of germination, can induce the germination of other spores in close proximity. This can be explained by the fact that the germination of a spore indicates that there are enough nutrients available in the soil for *Bacillus* species to grow and proliferate. Because of this, the germination of one bacterium is a signal for other spores that the conditions are suitable for growth and survival[75]. Research has shown that the germination induced by nutrients takes place via different receptors than the germination induced by Ca^{2+}-dipicolinic acid[82]. Others however, doubt the contribution of Ca^{2+}-dipicolinic as a germinant in nature and believe that only nutrients are involved in natural induced germination[75]. Related to the idea that bacteria monitor the germination of other bacteria by sensing dipicolinic acid, is the idea that peptidoglycan components also serve as germinants[83]. Shah et al. 2008 showed that *B. subtilis* was able to germinate upon addition of peptidoglycan compounds to their environment. Growing Gram-positive cells excrete during their growth peptidoglycan components, Shah et al showed that *B. subtilis* is capable of monitoring these components and use them to sense the ability to grow in the environment[83].

Because germination contains a step in which the spore is rehydrated indicates that water in the environment is also an important requirement for germination. Without water, germination will not occur[84]. Besides induced germination by the presence of certain nutrients, a naturally slow rate of spontaneous germination exist, which results in the random germination of spores in nature, provided that there is water in the environment[85]. Also, when spores are prompted to germinate, not all the spores will actually germinate at the same time and rate[86]. Some spores are resistant to germinations and stay in their dormant state, which ultimately is an additional survival strategy of the *Bacillus* species. This so-called super dormancy is a result of a low number of germinant receptors on the inner membrane of the spore[78]. The process of germination is still not completely understood and heavily subjected to research. However, for the implementation of *Bacillus* species in agriculture it is important to know the process and how this can be stimulated. What can be concluded from laboratory research, however, is that germination occurs in soil that contains enough moisture and nutrients. Besides, soil in which already bacteria are able to grow and divide may be a sufficient condition for spore germination. In short, the soil should not be contaminated with heavy metals or industrial organic compounds and it should contain water and nutrients.

Discussion and conclusion

This thesis sought to explain and outline the possibilities of the application of plant growth promoting bacteria in agriculture. The production pressure induced by global population increase, demands us to investigate ways to improve our current production capacity and efficiency. The decreasing soil quality due to overexploitation in addition to yield loss by pathogens and to a lesser extent climate change, jeopardizes the current production capacity. The application of *Bacillus* species for agricultural improvement is suggested in this thesis as one of the potential solutions to obtain higher yields in agriculture.

Bacillus species show a promising potency in plant growth promotion, both in a direct and indirect way. Because of this, a lot of research has been done on the applications of *Bacillus* species. Some of these results are proven, for example by Johnson et al., to be successful. Some *Bacillu*s species are currently commercialized and distributed all over the world to improve crop production[17]. These *Bacillus* species are distributed in the form of spores[70]. These spores, however, still need to be germinated. Therefore, conditions need to be found which are both efficient and environmentally friendly in stimulating germination to turn the spores into vegetative cells again. An important factor for the implementation in agriculture is that the spores or vegetative cells need to be distributed on the relevant crops to exert their function. Current research should therefore not only be focussed on the production of additional strains with relevant antimicrobial properties, but also on the spread of the spores on crops. The spread of *Bacillus* spores on crops has already been researched using irrigation systems or by using insects[87,88,89,90]. This researched showed that the principle of distribution via insects is somewhat successful. However, more research is required to ensure a safe and efficient way for *Bacillus* application in agriculture. Other methods may be developed for the spread of *Bacillus* species for example using sprinkler installations. Techniques should be researched to utilize mechanism already used in agriculture to facilitate the spread of Bacillus species so that additional investments of expensive equipment are not required.

A negative side effect of using *Bacillus* species that stimulate plant growth in agriculture is that some *Bacillus* species can also spoil harvested crops. So, the *Bacillus* species applied in agriculture should also be checked on the ability to cause food spoilage. If this is the case, efficient ways of decontaminating the crops post harvesting need to be found. Altogether it is not as easy to implement *Bacillus* species in agriculture as it may seem.

The research on germination of *Bacillus* spores has been primarily focused on the application in the food processing industry. This was done to be able to kill germinating spores and subsequently decrease food spoil. Research should be focused more on germination processes in soil and other natural conditions to promote crop growth. Therefore, a shift should be made into investigating factors in nature such as weather conditions, soil components and soil inhabitants that can influence germination of spores. Research may be conducted using settings in which spores are added to soil that is completely sterile and in soil that contains bacteria that are known to inhabit the rhizosphere. The rate and efficiency of germination can be monitored subsequently to see if other bacterial species enhance the germination process. Research could be conducted in which root exudates are added to the soil to check if these exudates might stimulate germination. Different temperatures or humidity levels in a controlled environment in greenhouses may be screened, resembling the effects of different weather conditions on germination. In short: there are many possible ways to investigate the process of germination in nature. Still a lot of research is required, but nevertheless, undoubtedly some of the future principle of agriculture will rely on the implementation and application of *Bacillus* species.

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