How can rhizobacteria support crop cultivation and how do they function?

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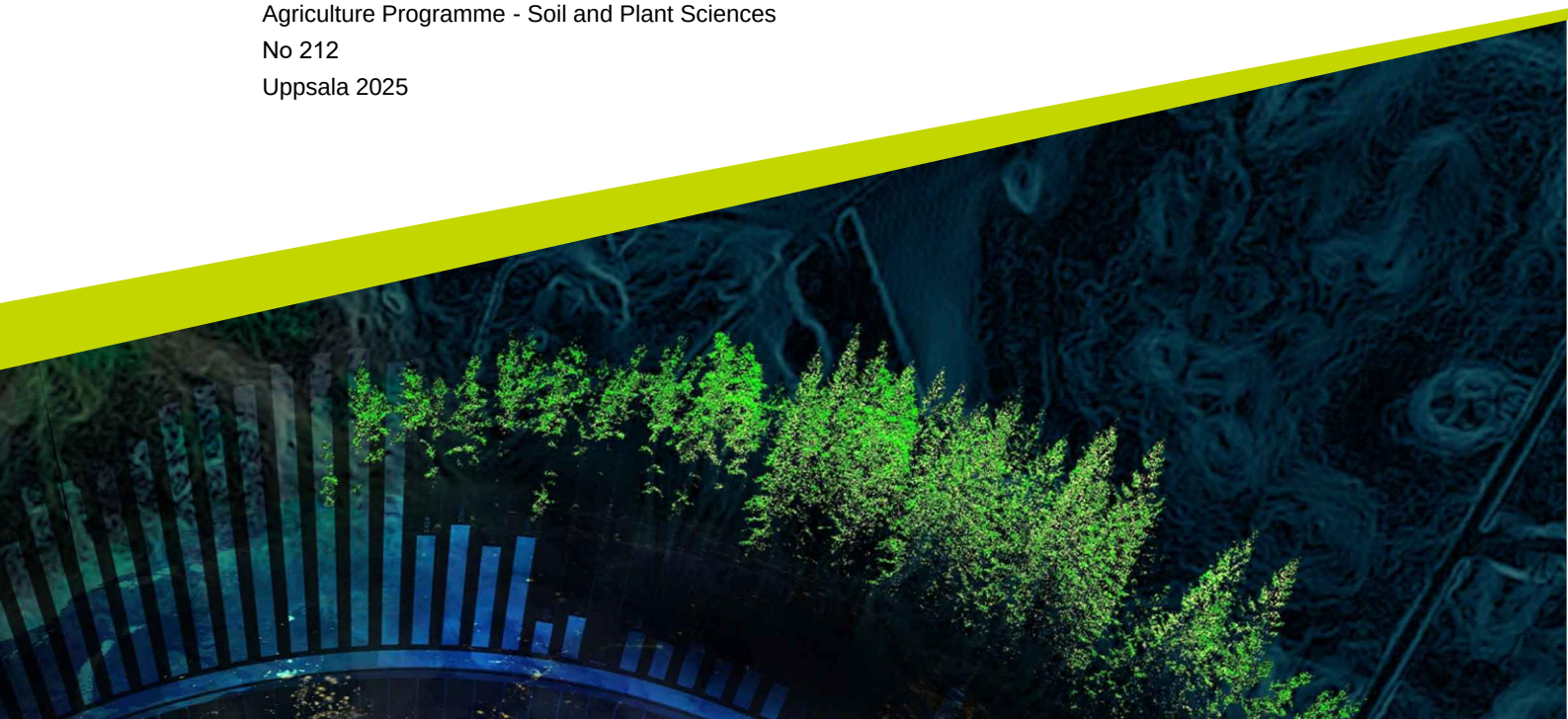
Independent project • 15 credits
Swedish University of Agricultural Sciences, SLU
Department of Plant Biology
Agriculture Programme - Soil and Plant Sciences
No 212
Uppsala 2025



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Credits:	15 credits
Level:	First cycle, G2E
Course title:	Independent project in Biology
Course code:	EX0894
Programme/education:	Agriculture Programme - Soil and Plant Sciences
Course coordinating dept:	Department of Aquatic Sciences and Assessment
Place of publication:	Uppsala
Year of publication:	2025

Keywords:	PGPR, rhizobacteria, food security, agricultural pollution, biofertilization, rhizoremediation, phytostimulation, stress control, biological control, ISR, rhizosphere competence, root colonisation, *Bacillus velezensis* UCMB5113.

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Abstract

As the global population is growing rapidly, and the amount of arable land is shrinking, food security stands out as one of humanity's primary challenges. Furthermore, the agricultural sector is a source of both harmful pollutants in the form of chemical pesticides, and nutrient leaching causing eutrophication. In this study, the utility of plant growth promoting rhizobacteria (PGPR) are considered in relation to crop growth and protection, highlighting their potential as contributors to food security. Moreover, ways in which PGPR might replace some conventional agricultural inputs, such as chemical pesticides and fertilisers, are explored. In relation to this, current obstacles to large scale implementation of PGPR are also discussed.

In a second part of the study, the PGPR *Bacillus velezensis* UCMB5113 was examined as a root growth promoter and plant root coloniser. Specifically, roots of a wild type, two mutants and two transformants of *Arabidopsis thaliana*, as well as a cultivar of *Brassica napus* ssp. *napus*, were inoculated *in vitro*, and some root growth parameters, gene expressions and root surface components were assessed in relation to colonisation. Results indicated a root growth promoting effect in *A. thaliana* wild type and transformants and a stimulation of tertiary root formation in *B. napus* ssp. *napus* roots following inoculation with UCMB5113. However, due to uneven colonisation, results regarding the extent and effect of colonisation are inconclusive. Several reasons for the colonisation results are discussed. Nevertheless, the results remain largely unexplained, why some suggestions for further study are given.

Keywords: PGPR, rhizobacteria, food security, agricultural pollution, biofertilization, rhizoremediation, phytostimulation, stress control, biological control, ISR, rhizosphere competence, root colonisation, *Bacillus velezensis* UCMB5113.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
Al	Aluminium
cfu	Colony forming units
Col-0	Columbia-0
Cu	Copper
Cyc1At	Cyclin 1 *Arabidopsis thaliana*
DNA	Deoxyribonucleic Acid
EU	European Union
Fe	Iron
g	Gram
GUS	*β*-glucuronidase
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
K	Potassium
l	Litre
LBA	Luria Bertani broth agar
M	Molar
m	Metre
MSA	Murashige-Skoog agar
MUT	Mutant
N	Nitrogen
NGO	Non-governmental organisation
N_2 (g)	Nitrogen gas
P	Phosphorus
PBS	Phosphate buffer solution
PGPR	Plant growth promoting rhizobacteria
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SAR	Systemic acquired resistance
TGG	*β*-thioglucoside glucohydrolase
TgN/year	Teragram nitrogen per year
UCMB5113	Ukrainian Collection of Microorganisms, section Bacteria 5113
USD	United States dollar
U/ml	Units per millilitre

vnd	Vascular-related NAC-domain
VOC	Volatile organic compound
Zn	Zinc
°C	Degrees Celsius

1. Introduction

Sustainable development goal number 2 of the United Nations aims at eliminating world hunger by 2030 (United Nations 2023), despite a rapidly growing world population estimated to reach about 8.5 billion by then (United Nations 2024), and the number of chronically hungry people in the world increasing by well over a hundred million since 2019 (United Nations 2023). Adding the fact that agricultural land is somewhat decreasing worldwide (Gomiero 2016; FAO 2022), increased productivity on this land becomes even more crucial. Complicating the matter further, agriculture is largely dependent on pesticides (Tostado 2022), which are often harmful for humans and the environment (Bernardes et al. 2015), and thus counteract the sustainable development goals numbers 6.3, 14.1 and 15 of the United Nations, that among other things aim to improve drinking water quality by reducing pollution, reduce marine pollution and conserve life on land by reducing pollution, respectively (United Nations 2023). There is, in other words, evident need for tools to both increase crop growth and decrease the need for pesticide use in agriculture worldwide.

When tackling these global problems, one should not disregard solutions so small as to be visible only through the eye of a microscope, namely among the microorganisms living within about a millimetre of the soil closest to the plant root surface. This region of soil, called the rhizosphere, is particularly abundant in bacteria (Hiltner 1904), among which plant growth promoting rhizobacteria (PGPR) are of special interest to the problems mentioned.

PGPR can be defined as bacteria living in the rhizosphere that are beneficial to the growth of the plant (Bhattacharyya & Jha 2012). This growth promotion can be provided in various ways, which will be considered further on in this text (Figure 1). Some PGPR promote plant growth directly, while others do so indirectly, for example by competing with pathogens (Bhattacharyya & Jha 2012; Backer et al. 2018), potentially reducing the need for a number of chemical pesticides. Thus, if PGPR can survive under field conditions, exert a substantial beneficial effect on the yield of crops grown there and be practically manageable as products used by farmers, it is possible that they can aid in achieving the sustainable development goals of reducing hunger and pollution mentioned above.

Many such products, often encompassed by the terms biostimulants or probiotics, already exist on the market. Tabassum et al. (2017) offers a summary of 49 selected PGPR products, and a summary of 60 PGPR strains which were reported to be effective for plant growth promotion. Half of these 60 strains were tested under field conditions (Tabassum et al. 2017). In Sweden, the biological seed

coating products Cedomon, Cerall and Cedress are available for farmers. These contain the PGPR strain *Pseudomonas chlororaphis* MA342, which is said to compete for resources and space with other microbes, enhance root growth and increase yield (Lantmännen 2021). Indeed, several field trials have shown the efficacy of the strain against a number of soilborne diseases (Johnsson et al. 1998), and a study of colonisation patterns on seeds indicated colocalisation with the pathogen *Pyrenophora teres,* suggesting competition with the pathogen (Tombolini et al. 1999).

PGPR seems to constitute a large and growing market worldwide, with estimates of the 2023 global bacterial agricultural market value ranging from 3.59–3.78 to 7.02 billion USD, with an expected increase to between 5.77–5.8 and 10.46 billion USD in 2030 (DataBridge 2023; Grand Research Store 2024; The Business Research Company 2024) (note that these numbers include non-PGPR use of bacteria). However, there are far more chemical pesticide products registered than biocontrol products (Tabassum et al. 2017). The global chemical pesticide market value has by comparison been estimated at about 61.42 billion USD for 2023 (Fortune Business Insights 2024).

Companies and interest groups propagating the use of PGPR exist in several countries, for instance the German Association for AgroBioTechnological Development and Production (ABiTEP), which promotes PGPR products among other biotechnological products for agricultural use (ABiTEP 2024). Although PGPR are thus sometimes referred to as a form of technology (Backer et al. 2018), perhaps bringing a human engineer to mind, their function is not the result of human design, but in many cases of a long evolutionary history that may have started earlier than when plants first grew on land (Partida-Martinez & Heil 2011). The results of an evolutionary study of plant microbiomes reveal an apparent gradual accumulation of biochemical functions of the host-microbiome over geological time, with mutualistic nitrogen (N)-fixing associations arising around 700 – 800 million years ago in chlorophytes, and plant associations with phosphorus (P)-mobilising fungi dating back to around 385 million years ago (Graham et al. 2018). Conversely, due to the inherently short generation times, high mutational rates and large population numbers of bacteria, some established plant-bacterial associations might evolve into mutualism in a relatively short period of time (Li et al. 2021). While some PGPR are consequently the result of millions of years co-evolution with plants, leading to complex mutual adaptations such as with root nodules of symbiotic N-fixing bacteria, many PGPR may represent completely new associations and host-bacterial combinations, discovered or devised by the researcher (especially when host specificity is low as with many but not all PGPR) (Kloepper 1996; Marasco et al. 2013; Rubin et al. 2017). One out of many possible evolutionary mechanisms for developing

mutualistic associations such as those between PGPR and plants involves host control, where microbes starting out as strong plant parasites are being rewarded by the plant when exhibiting weaker parasitic traits, thus gaining the upper hand over other, stronger plant parasites. In this way, the microbe gains energy and/or nutrients, while the plant gains a protector, moving the parasite along the parasitic-mutualistic continuum (Drew et al. 2021).

Even though the understanding of PGPR is constantly growing, and many potent plant growth promoting strains have been found, there is still a lot of unanswered questions regarding for example mechanisms of action, screening strategies for finding vigorous PGPR, or selecting the right strains or combinations of strains for specific purposes such as soil quality improvement. More controlled field trials to bridge the gap between research and practical use are also important (Bhattacharyya & Jha 2012). Furthermore, all steps from isolation of a promising PGPR strain to commercialised product will need to be carefully considered in the future (Backer et al. 2018). In other words, there is still much to be investigated regarding both the use of PGPR in agricultural crop cultivation and the ways in which they function. To this end, this text has been devised with the following purposes in mind.

1.1 Purpose and research questions

The purpose of this study is twofold:

1) To investigate how certain microorganisms, specifically plant growth promoting rhizobacteria (PGPR), can be used to support cultivation of agricultural crops, primarily by replacing conventional inputs (i.e. a literature survey).

2) To gain insight into how plant-microbe interactions can be studied with focus on some factors that can affect root colonisation through some basic experiments, primarily on *Bacillus velezensis* UCMB5113 and *Arabidopsis thaliana* (i.e. an experimental part).

This text will therefore hereafter be split into two parts; the review and the practical part, each of which will deal with one of the above purposes, respectively. Each part will try to answer the corresponding one of the following questions:

1) How can rhizobacteria support crop cultivation?
2) How well might a PGPR colonise plant roots under different conditions, and what is the effect on root growth? Specifically:

2.1) How well does *B. velezensis* UCMB5113 colonise the roots of a few different *A. thaliana* mutants (*tgg1,2* and *vnd1237* knockouts), and does the colonisation affect primary and lateral root growths in length and formation of lateral roots (Figure 2)?

2.2) Can *B. velezensis* UCMB5113 colonise the roots of a few different *A. thaliana* *β*-glucuronidase (GUS) transformants (*tgg1::GUS* and *Cyc1At::GUS*), does the colonisation affect primary and lateral root growths in length and formation of lateral roots, and is there a clearly visible difference in expression of the selected transformed genes following inoculation?

2.3) How well does *B. velezensis* UCMB5113 colonise differently pretreated roots of *Brassica napus* ssp. *napus* (cellulase and pectinase pretreatment), and does the colonisation affect primary and lateral root growths in length and formation of lateral roots?

2. Review: Materials and Methods

2.1 Materials

Scientific articles, course literature, websites concerning PGPR and plant interaction.

2.2 Methods

Relevant literature was found using databases including Google Scholar, Primo and PubMed, using search terms such as “PGPR induced systemic resistance” or “stress control by PGPR”. Here, only first page results were used in most cases. Some articles were also found through references in other articles. Internet information from various actors (authorities, companies, NGOs) was also studied. To find sources outside the scope of scientific articles, Google searches were used, with search terms such as “PGPR products available on the market” or “United nations sustainable developmental goals”. Again, only first page results were viewed.

This method of course limits the searches in several ways. Only viewing first page results hides all but the topmost articles from view, and it is likely that many relevant sources are missed this way. In addition, even though some kind of cutoff like this is necessary for practical reasons, and search engines are generally designed to favour results that are relevant to the search query, it is worth noting that both search engine owners and to some extent website owners can affect which results are included through indexing and which results are presented first, potentially producing a biased search result that leaves out many of the most relevant sources from the first page(s). On the other hand, finding papers through other papers, if practiced extensively, may entail a risk of giving a narrow view of a subject, as only papers who were read by the first author are found, potentially missing more recent articles outside of this network of article references. A possible remedy to this problem would be to find more recent related articles by searching for newer citations of an article on various databases.

Wherever possible, the original source of a certain referenced piece of information was determined. In many cases, several articles referring to another needed to be perused only to find that the original source did not contain the information referred to, the information was slightly misinterpreted to suit the writer of another article, or the information was simply outdated. Not referring to the original source makes it hard to confirm the reliability of a certain piece of

information, and the source of information can appear much more recent than it really is. This obscuring of information arguably makes a text less scientific, as it impedes scientific progress by generating confusion and misinformation.

More recent papers were generally preferred over older ones, in order to find the most relevant and up-to-date information. As far as possible, peer reviewed papers published in scientific journals were used, as this (although no hundred percent guarantee exists) provides some sort of affirmation as to the scientific quality of the text. When one scientist has read and approved the work of another, this reduces the risk of the author getting away with unscientific practices, such as drawing sweeping conclusions from insufficient results or tinkering with results to be able to draw remarkable conclusions. This peer review is, of course, not present when it comes to e.g. reports made by research companies. Although such a company might not have any interest to mislead the reader, and their reports might therefore provide the reader with useful information, the lack of peer review removes an assessment of scientific rigour, which makes it a less reliable source.

3. Review: Results and discussion

Listed below are the different ways in which PGPR can aid in crop production by promoting plant growth (see a brief overview in Figure 1).

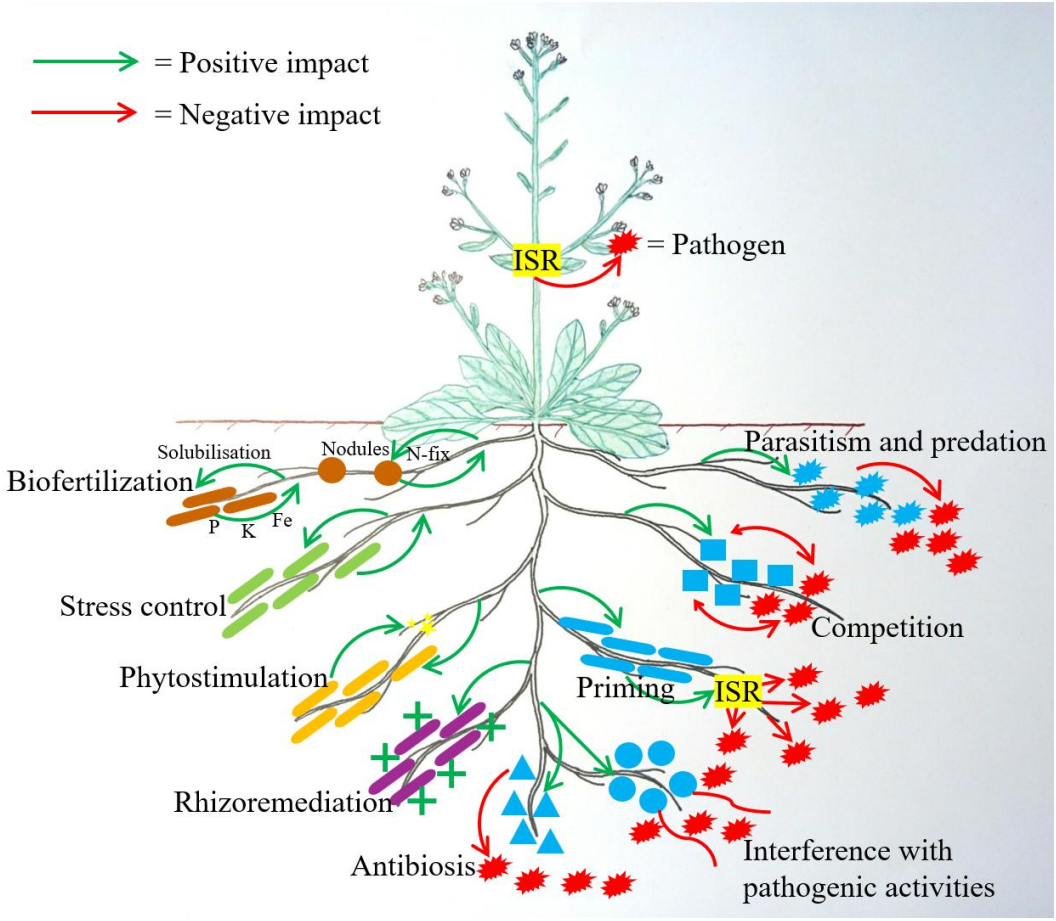

Figure 1. Overview of various ways in which PGPR (plant growth promoting rhizobacteria) can promote the growth of a plant. ISR = induced systemic resistance. Green arrows flowing from the plant towards PGPR indicate possible rewards such as root exudates. Brown colour signifies biofertilizers, light green colour signifies stress controllers, orange colour signifies phytostimulators, purple colour signifies rhizoremediators, light blue colour signifies various biocontrollers. Note that in reality, one PGPR might exhibit several of the above functions, and that communities are not as separated as in the picture.

3.1 Biofertilization

Biofertilization by rhizobacteria is the process by which PGPR aid plants in acquiring nutrients (Lugtenberg & Kamilova 2009). The most well-known example of this is probably bacterial fixation of atmospheric N_2 (g) in exchange

for energy rich carbon compounds from the plant. Examples of this include both symbiotic nitrogen fixation such as by root-nodule forming bacteria in the genera *Rhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Frankia* (Agrios 2005), and the so-called associative nitrogen fixation by bacteria living on the root surface or between root cells, including the genera *Azospirillum*, *Herbaspirillum* and *Azoarcus* (Xu & Wang 2023). Biological nitrogen fixation is already contributing about 17–31 TgN/year or around 17% of N to global agricultural crop production (Zhang et al. 2021), thereby reducing the need for synthetic nitrogen fertilisers. Furthermore, the significance and demand of legumes (and therefore symbiotic nitrogen fixation) has been projected to increase in the future (Nigam et al. 2021). The market of associative nitrogen fixating bacteria like *Azospirillum* and *Azotobacter* has also been projected to increase (DataBridge 2022; Global Market Insights 2024). Nevertheless, biological nitrogen fixation is limited by a number of factors, including socioeconomic constraints (Fouad Abobatta et al. 2021) and ecological factors such as pests and diseases like root rots and rust of legumes (Graham & Vance 2003). This puts a limit on the extent to which mineral fertiliser nitrogen can be replaced by biologically fixated nitrogen. Nitrogen fixing bacterial products are the most common biofertilizers, occupying as much as 80% of the global biofertilizer market (Basu et al. 2021).

Second-most common are the P-solubilising bacterial products, which occupy about 14% of the biofertilizer market (Basu et al. 2021). These bacteria solubilise P through the release of organic acids, which dissolve phosphatic minerals or release adsorbed phosphate through ligand exchanges from Fe (iron) and Al (aluminium) oxides (He et al. 2002), increasing P availability to the plant (Satyaprakash et al. 2017). For instance, an inoculation of chickpea with the P-solubilising bacterium *Pseudomonas striata* resulted in no significant yield loss when reducing P fertilisation with one-third (Dutta & Bandyopadhyay 2009).

PGPR biofertilization of other nutrients, such as K (potassium) solubilisation (Zhang et al. 2022) and Zn (zinc) solubilisation (Sirohi et al. 2015), have also been reported. Notably, siderophore production by certain PGPR can aid both in plant acquisition of Fe and in plant protection by competition, as the iron binding to the siderophores becomes unavailable to phytopathogens (Sayyed et al. 2013). Apart from N-fixing bacteria, biofertilizing PGPR function by making existing soil nutrients more available to the plant, thereby decreasing the need for fertilisation and thus probably also the risk of overfertilisation and eutrophication. However, the fact that they do not add any nutrients to the soil means that they can of course never fully replace fertilisers in the long run. The time it would take for the concentration of a soil nutrient to drop below a level acceptable for plant production in the absence of fertilisation would of course vary according to soil, plant type, nutrient pool and availability, but the fact that such a level of nutrient

depletion could be reached within a foreseeable time can be illustrated by a Swedish fertility experiment, where a drop in Fe and Cu (copper) levels in wheat kernels to below the critical level for nutrient deficiency was observed from a soil receiving conventional mineral fertiliser (no Fe or Cu) over a 40 year period (Kirchmann et al. 2010).

The commercial use of biofertilizing rhizobacteria can be exemplified by 11 PGPR products containing N-fixating or N uptake enhancing bacteria reportedly being available on the market in 2017, as well as 5 P-solubilising and 2 K-solubilising PGPR products (Tabassum et al. 2017).

3.2 Rhizoremediation

By rhizoremediation, plants or seeds of plants are protected from harmful soil pollutants through degradation by rhizobacteria. These bacteria mainly feed on root exudates, while using pollutants as an alternative nutrient source (Lugtenberg & Kamilova 2009). For example, phytotoxic effects of petroleum hydrocarbons adversely affecting growth and physiology of chickpea plants were reduced by up to 24% for agronomic traits (e.g. shoot or root length) and up to 35% for physiological traits (e.g. chlorophyll content) by a consortium of several PGPR, removing 74–80% of the petroleum hydrocarbons (Ali et al. 2023). In another study, a number of PGPR were found to be able to degrade the phytotoxic insecticide pentachlorophenol (Jagadeesh et al. 2011).

Although rhizoremediation does not directly substitute any conventional agricultural inputs, they could indirectly reduce the amount of pesticide needed by contributing to crop health and thereby resilience to pathogens and pests. Moreover, they could mitigate agricultural pollution by degrading e.g. herbicides that could otherwise be harmful to the plant, environment or humans. Rhizoremediation may well be combined with other PGPR (Backer et al. 2018). An important aspect of effective rhizoremediators is that they should not be dependent on the pollutant for energy to sustain themselves, but be able to use other sources of energy when pollutants are scarce (Lugtenberg & Kamilova 2009). The use of rhizoremediation in agriculture can be exemplified by the South African product AzoBac, using a PGPR strain of *Azospirillum* intended for bioremediation in several crops (Tabassum et al. 2017).

3.3 Phytostimulation

Phytostimulation by rhizobacteria is the production of plant growth promoting substances by PGPR. Certain plant hormones, notably auxin, the cofactor pyrrolquinoline quinone and a number of volatiles are examples of such substances (Lugtenberg & Kamilova 2009). For instance, cytokinins, which are plant hormones known to stimulate cell division and branching among other things (Fogelfors 2015), were indicated as contributing to the plant growth promotion of *Arabidopsis thaliana* by *Bacillus megaterium* through comparing inoculations of Arabidopsis wild types with mutants lacking certain cytokinin receptors (Ortíz-Castro et al. 2008). Another example involves the promotion of shoot biomass and length in tomato by the gibberellin-producing bacterial strain *Promicromonospora* sp. SE188 (Kang et al. 2012). Not only did the bacteria provide gibberellins, which are known to stimulate cell elongation and division in the stem (Fogelfors 2015), but biosynthesis of gibberellins by the host plant were also induced (Kang et al. 2012).

Although phytostimulation does not directly substitute any conventional agricultural inputs, their direct promotion of plant growth might serve as a way to increase yields and thus contribute to food security. They could also indirectly reduce the amount of pesticide needed by contributing to crop health and thereby resilience to pathogens and pests. The use of phytostimulation in agriculture can be exemplified by the Pakistani product Biozote-MAX, containing IAA (indole-3-acetic acid, an auxin) and gibberellin producing PGPR for use in several crops (Tabassum et al. 2017).

3.4 Stress control

PGPR that exhibit stress control improve plant physiology under abiotic stresses such as drought, heavy metals or salt, or biotic stresses such as pathogenic bacteria or fungi, thereby aiding plant growth and development (Mumtaz et al. 2022). One common way that certain rhizobacteria can achieve stress control is by decreasing plant ethylene levels caused by abiotic or biotic stresses. This can be accomplished through the production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase by the bacterium, blocking ethylene production by degrading an ethylene precursor (Lugtenberg & Kamilova 2009). For example, *Enterobacter* sp. UPMR18 was shown to produce ACC deaminase and improve gemination and early growth of okra under salt stress, by reducing ethylene levels among other things (Habib et al. 2016). Another example involves guinea grass (*Panicum maximum*), considered to be a valuable forage crop in tropical regions (Aganga & Tshwenyane 2004). Guinea grass plants germinated from seeds

inoculated with various PGPR (mostly strains of *B. licheniformis* and *B. subtilis*) and grown under conditions of salt and drought stress displayed significantly improved growth parameters compared to uninoculated plants, such as increased root and shoot lengths and dry weight, comparable to plants grown under normal conditions (Tiwari et al. 2018).

Other ways of attaining stress control besides production of ACC deaminase include the following examples: i) stimulation of antioxidant production (e.g. superoxide dismutase) counteracting harmful effects from excessive ROS (reactive oxygen species) buildup during various abiotic stresses (Nivetha et al. 2021), ii) exopolysaccharide production improving soil structure and consequent root growth and water uptake during drought stress (Ilyas et al. 2020), and iii) production of VOC (volatile organic compounds) aiding in the alleviation of salt stress through various mechanisms (Talaat & Shawky 2017). Biotic stresses can also be relieved by various forms of biological control (see below).

Stress control can reduce the amount of pesticide needed by contributing to crop health in environmentally stressful conditions, and thereby resilience to pathogens and pests. Additionally, they could increase usable arable land by providing adequate yields from crops grown on relatively saline or dry soils, thereby helping to increase food security in a warming climate. Moreover, yield reductions due to climate change induced stresses such as increased pathogen pressure (Singh et al. 2023) or extreme weather (Kompas et al. 2024) could be offset by the use of stress control PGPR. The use of stress control in agriculture can be exemplified by the Faisalabad-Pakistani product Rhizogold plus, containing *Rhizobium* and ACC deaminase producing PGPR for use in cereal crops (Tabassum et al. 2017).

3.5 Biological control

Common to all biocontrol PGPR is that they entail the possibility of reducing chemical pesticide need in agriculture by suppressing disease and increasing plant health. Tabassum et al. (2017) note the potential of biological control to surpass chemical pesticides in treatment success and cost-effectiveness. The wide variety of potential applications of PGPR intended for biological control can be illustrated by 25 such products reportedly available on the market (Tabassum et al. 2017). Listed below are different ways in which PGPR can protect plants from disease.

3.5.1 Antibiosis

PGPR aiding plant growth and development through antibiosis do so by harming pathogens through the production of secondary metabolites, e.g. antibiotics or biosurfactants (Doornbos et al. 2012), thus alleviating the plant from pathogenic pressure. For instance, surfactin produced by *B. subtilis* 6051 was shown to contribute to biocontrol of *Pseudomonas syringae* pv *tomato* DC3000 on Arabidopsis roots (Bais et al. 2004). A problem identified with antibiotic PGPR is that pathogens attacked by the bacteria can develop resistance against the mode of action of the PGPR (Lugtenberg & Kamilova 2009), creating a similar problem as with chemical pesticides.

3.5.2 Competition

PGPR aiding plant growth and development through competition do so by successfully competing with pathogens for space and nutrients in the rhizosphere, thus alleviating the plant from pathogenic pressure (Wang et al. 2021). For example, competitive exclusion including biofilm formation by *B. velezensis* T-5 was implicated as a successful control mechanism of *Ralstonia solanacearum* on tomato roots (Tan et al. 2016). In another study, spatial competition by biofilm formation of *B. velezensis* QST713 has been implicated as a mechanism in the successful control of *Trichoderma aggressivum* on cultivated mushroom, as genes related to biofilm formation were strongly expressed in the presence of the pathogen on mushroom (Pandin et al. 2019). Arguably, as all non-pathogenic rhizosphere inhabitants use resources and space, they are always competitively excluding pathogens to some extent (although less beneficial non-pathogenic microorganisms may of course also outcompete more beneficial ones). However, a pathogen seems to be effectively controlled by competition only if the PGPR inhabits the very same niche on the root (Pliego et al. 2008). The ability to successfully compete for root secretions and space with other microbes inhabiting the rhizosphere, known as rhizosphere competence (Lugtenberg & Kamilova 2009), is a desirable trait in all PGPR, as it encompasses traits (such as colonising ability) that are needed for a PGPR to be able to sustain its presence and exert beneficial effects in the rhizosphere (Compant et al. 2005).

3.5.3 Induced systemic resistance (ISR)

PGPR aiding plant growth and development through ISR do so by priming or triggering plant defence responses through plant ethylene and jasmonic acid signalling pathways stemming from a recognition of PGPR features such as flagellin, siderophores or lipopolysaccharides, thus alleviating the plant from

pathogenic pressure (Durrant & Dong 2004; van Loon 2007; Meena et al. 2020). Key to ISR is that the plant can benefit from having the preparation of systemic resistance responses stimulated by the PGPR prior to pathogen attack, a so-called priming of ISR, putting the plant one step ahead of the pathogen in the event of an attack (Meena et al. 2020). Similarly to ISR, systemic acquired resistance (SAR) also induces host resistance, but is triggered through salicylic acid signalling pathways following detection of a pathogen (Durrant & Dong 2004). Importantly, SAR induction is quite costly to the plant (Mosher et al. 2006), in contrast to the lower cost associated with priming of ISR (van Hulten et al. 2006). Examples of plant responses induced by ISR include production of ROS in tobacco after recognition of *Pseudomonas* lipopolysaccharides (van Loon et al. 2008) and production of ROS and autofluorescent phenolic compounds in rice contributing to resistance against *Magnaporthe oryzae* after root inoculation with *Serratia plymuthica* IC1270 (De Vleesschauwer et al. 2009).

3.5.4 Interference with pathogenic activities

PGPR can also help control diseases through interfering with pathogenic activities, such as sporulation or quorum sensing signalling (Lugtenberg & Kamilova 2009). For instance, *P. segetis* P6 was shown to improve symptoms of soft rot on tomato caused by several pathogens (e.g. *Dickeya solani*), using quorum quenching (disruption of intercellular bacterial communication, in this case related to pathogenesis) among other mechanisms (Rodríguez et al. 2020). Another example involves hyphal colonisation by *P. fluorescens* WCS365 that has been shown to impede spore germination of the tomato pathogen *Fusarium oxysporum* f.sp.*radicis-licopersici* (Kamilova et al. 2008).

3.5.5 Parasitism and predation

PGPR aiding plant growth and development through parasitism or predation do so by parasitising or preying on pathogens in the rhizosphere, thus alleviating the plant from pathogenic pressure (Murphey Coy 2014). For example, the endospore-forming bacterium *Pasteuria penetrans* has been shown to parasitise the plant pathogenic root-knot nematode *Meloidogyne javanica* on sugarcane roots, reducing nematode egg concentrations by up to 96% at a spore concentration of 50,000 endospores/g soil in a pot experiment (Bhuiyan et al. 2018). This form of biological control by PGPR does not seem to be widely reported (Lugtenberg & Kamilova 2009).

3.6 Obstacles to large-scale implementation of PGPR in agriculture

Several authors note the great potential of PGPR to increase in use in agriculture (Kaymak 2011; Bhattacharyya & Jha 2012; Kang et al. 2014; Tabassum et al. 2017; Backer et al. 2018). As noted, many PGPR products are indeed already available on the market, and many reportedly effective PGPR strains have been found (Tabassum et al. 2017; Kumari et al. 2019). However, when it comes to replacing conventional agricultural inputs, PGPR products seem to only have made a small dent (disregarding the substantial contribution to agricultural nitrogen supply provided by N-fixing bacteria). Compare, for instance, the size of the bacterial agricultural market, ranging from 3.59–3.78 to 7.02 billion USD (DataBridge 2023; Grand Research Store 2024; The Business Research Company 2024), with that of the chemical crop related pesticide market of about 61.42 billion USD (Fortune Business Insights 2024), as mentioned in the introduction.

This begs the question of why the apparently great potential of PGPR has not been realised, or in other words what stands in the way of PGPR replacing substantial parts of some of the conventional agricultural inputs, such as pesticides and fertilisers. The answer to this question might be approached by investigating the obstacles to large-scale implementation of PGPR in agriculture, of which there are many examples. To begin with, the many necessary steps from the discovery of a PGPR to its large-scale implementation in agriculture might be considered. Backer et al. (2018) outlines the steps that commonly need to be taken to develop a bacterial isolate into a commercially available product:

> (1) Isolation of the bacteria from roots or other plant tissues.
> (2) Laboratory and controlled growth environment screening.
> (3) Field screening for a range of crops, geographic locations, planting dates and soil types.
> (4) Evaluation of the possible combinations of strains and/or signals.
> (5) Consideration of the management practices (e.g., agrochemical use and rotation)
> (6) Refinement of the product.
> (7) Experiments confirming absence eco-toxicological effects.
> (8) Product delivery formulation – e.g., peat, granular, liquid or wettable powder.
> (9) Registration and regulatory approval of the product.
> (10) Product available on the market. (Backer et al. 2018)

Obstacles to successful implementation of PGPR in agriculture can conceivably arise at any of these steps, as ineffective or inadequately thorough PGPR product development will mean that fewer PGPR products are produced, or that they will lack some feature necessary for large scale commercial success. For instance, Lugtenberg & Kamilova (2009) note that potential strains of PGPR often work in a laboratory setting (2 on the list above), but lose their plant growth promoting effects when brought to the field (3 on the list). As the nature of the isolated strain will govern the success of all subsequent steps, finding efficient methods of isolating the most relevant strains for potential PGPR (1 above) is imperative to the long-term goal of developing strains capable of large-scale implementation in agriculture.

Several authors emphasise the importance of rhizosphere competence in a successful PGPR (Nakkeeran et al. 2006; Lugtenberg & Kamilova 2009; Bhattacharyya & Jha 2012). Inadequate rhizosphere competence and colonising abilities compared with indigenous microorganisms has been proposed as a probable cause of the observed loss of beneficial effects when moving a PGPR from a laboratory setting to the field (Benizri et al. 2001; Tabassum et al. 2017).

Furthermore, even if a PGPR is rhizosphere competent and exerts plant growth promoting effects in a certain environment, when applied to a different plant cultivar, or in a different environment, the bacterium or its interaction with the plant might be affected in a way that diminishes or removes the beneficial effects of the bacterium (related to 3 on the list above). In addition, different crops, cultivars of crops and different environments demand different types of PGPR based on the need of the specific plant and the needs that arise specifically in a certain environment (Tabassum et al. 2017). For example, a polluted soil might require addition of rhizoremediators to the PGPR formula, or a dry environment might require addition of stress control rhizobacteria. This presents another obstacle to large scale implementation, as there is no single PGPR formulation for all possible situations, which complicates the development and use of PGPR as agricultural inputs, as opposed to e.g. chemical pesticides or fertilisers. Of course, the use of chemical pesticides also requires adaptation to a certain plant type and environment, yet to a smaller degree (for example, a herbicide affecting many dicotyledons might be used in a grass crop, though not in many dicotyledonous crops, but soil conditions such as pH, moisture or microbial community composition does not affect the use of the herbicide much). The lack of knowledge of the effects on PGPR function of indigenous microorganisms entails a particularly difficult obstacle to overcome (Tabassum et al. 2017), as this cannot simply be sampled like pH, moisture or soil P content. However, recent advances in tools for DNA analysis allow for metagenomic or amplicon mapping of a particular soil that, if coupled to relevant traits of the microbial population, may

provide the information needed to be able to predict an appropriate PGPR consortia composition for a specific biotic environment (Liu et al. 2021; Jensen et al. 2024).

A further complicating factor in developing a PGPR product is that not only may a PGPR lose its beneficial effects when moving from the laboratory to the field, but the opposite may also be true. Thus, a strain might be uncultivable or not promote plant growth in the lab, but exert beneficial effects in its natural field environment. Alternatively, the strain might exert its beneficial effects in a long-term manner, promoting plant growth only at a later developmental stage (Backer et al. 2018), which requires much longer experiments to detect. This may lead to many PGPR strains with the potential of large-scale implementation in agriculture being abandoned at the laboratory screening stage (2) due to unsuccessful cultivation or lack of short-term effects.

When successively moving from research related steps towards commercial aspects of a PGPR (steps 4 to 5 to 6 etc.), it becomes clear that, as noted by Bhattacharyya & Jha (2012), the development of a commercially successful PGPR product relies on favourable collaborative relations between scientific institutions and agricultural companies. Moreover, even if a product works in principle, practical and economic aspects of the field application of the bacteria need to be considered (8 on the list), and might stop an otherwise effective PGPR from gaining widespread use (Ravensberg 2011; Glare et al. 2012). Next, the regulatory process and product registration can be slow and costly (9 on the list), especially for PGPR products aimed at biological control (Berg 2009). However, in recent years, the EU has changed the rules for registration of biological pesticides, aiming to facilitate their approval (Directorate-General for Health and Food Safety 2022). Nevertheless, EU legislation for biological control registration processes has still been criticised, including criticisms on ambiguous use of terminology causing confusion and slowing down registration processes (Stenberg et al. 2023). In Sweden, the registration processes are managed by the Swedish Chemicals Agency (KEMI) (Castensson 2017; Swedish Chemicals Agency 2024).

Having overcome all possible difficulties related to the above list, a fully functional, effective PGPR product (10) will still meet obstacles to large scale implementation in agriculture. For instance, PGPR products might become stuck in a sort of catch-22, where a certain degree of success is necessary for a major breakthrough on the market. There would, for example, likely be a cost reduction fuelling greater use of biopesticides only after the use of these products is widespread, and until a product is widespread, the absence of consequent practical evidence of their effectiveness can make it hard to gain the trust necessary from growers to start using them extensively (Tabassum et al. 2017).

In the end, the fate of PGPR products released to the market will be determined by factors like their general applicability (broad action), long-term efficacy, safety, storage properties and price, as well as the usual market forces of supply and demand (Bhattacharyya & Jha 2012).

3.7 Potential harms with using PGPR

Having outlined several ways in which PGPR are beneficial, the fact that PGPR can also have adverse effects should not be overlooked. Several PGPR strains have been identified as opportunistic human pathogens, raising concerns about the safety of handling them. In other cases, even though a PGPR may itself not be harmful to humans, they belong to the same genus as human pathogens. Moreover, as the traditional approach of 16S rRNA sequencing for identification cannot adequately be used to discern species level differences, other tools such as whole genome sequencing may be necessary to differentiate harmful strains from safe ones belonging to the same genus (Keswani et al. 2019).

In addition, PGPR could, at sufficient concentrations, disrupt indigenous microbial communities together with their related ecological functions, prompting the need for assessing unwanted environmental side effects from PGPR apart from human health concerns (Berg & Zachow 2011). An interesting side note here is that the much-desired rhizosphere competence might be precisely the thing making a PGPR able to outcompete beneficial indigenous parts of a rhizosphere community, conceivably leading to a net loss in some ecosystem services like degradation or even plant growth promotion itself.

Hence, it is also worth noting that not all PGPR are beneficial to crops in all situations. For example, a study involving inoculation of lettuce showed that *Pseudomonas mendocina*, previously shown to increase aggregate stability under non-saline conditions (Kohler et al. 2006), decreased aggregate stability under saline conditions (Kohler et al. 2010), contributing to a deterioration of growth conditions. This highlights the importance of testing PGPR in multiple environments and in specific host-bacterial combinations before introducing a certain inoculum to the broader agricultural market.

3.8 Suggestions for further study

Considering the time and effort required to complete all steps from isolation (1) to available PGPR product (10), the quality of the starting material is a crucial determinant in the amount of work needed to find a successful PGPR. Since many PGPR go through isolation, laboratory screening and field screening only to be found unviable under field conditions (Lugtenberg & Kamilova 2009), a suggestion for further study could be to conduct field screenings directly from isolated materials, postponing step (2) in the above list until an evidently rhizosphere competent strain has been identified through field screening.

In addition, since the success of a particular PGPR-plant interaction in aiding plant growth is dependent on factors that vary considerably between regions (Tabassum et al. 2017), studies of PGPR in different abiotic and biotic conditions (e.g. dry, moist, cold, warm, in the presence of indigenous microbial communities associated with different conditions) to understand how these affect the PGPR mechanisms seems a good way to increase the knowledge needed to develop site-specific PGPR formulations, aiding in passing stage (3) on the list above.

Finally, more formulations based on consortia of PGPR working together could be investigated, as the added diversity would likely increase the chances of the PGPR consortia as a whole surviving varying conditions through time, and bring the possibility of providing both direct and indirect benefits to the plant at the same time, e.g. growth stimulation and protection against pathogens (Tabassum et al. 2017). This sort of study would preferably combine stages (3) and (4) on the list above, aiding in the identification of broadly applicable PGPR formulations.

3.9 Conclusion

With the development of new screening strategies, the composition of various versatile and resilient PGPR consortia effective in multiple growing conditions, and successful collaboration across all related parties in PGPR product development and distribution (scientific institutions, companies and agricultural extension services), PGPR can hopefully make a substantial contribution to achieving world food security, and help achieving it in a way that minimises the risk of agricultural pollution from overfertilisation and harmful pesticides. Undoubtedly, both further investigations on how PGPR can support crop cultivation and conducting more experiments on their function is essential to reaching this goal, as without the necessary understanding, the full potential of PGPR in these matters can never be realised.

4. Practical part: Introduction

4.1 Introduction of the bacterial test strain *Bacillus velezensis* UCMB5113

One of the most common genera of PGPR is *Bacillus* (Liu et al. 2022; Azeem et al. 2023). This diverse genus of gram-positive, endospore-forming, rod-shaped bacteria (Turnbull 1996) contains, for instance, the extensively studied *B. subtilis* (Tabassum et al. 2017). *B. subtilis* strains have, for example, been shown to effectively colonise plant roots (Gao et al. 2013) and promote plant growth through the production of indole-3-acetic acid (IAA) and gibberellins (Reva et al. 2004). *B. subtilis* is also used in several commercial PGPR products (Tabassum et al. 2017).

Another widely studied member of the genus is *B. velezensis* (syn. *B. amyloliquefaciens, B. methylotrophicus*) (Tabassum et al. 2017), which has shown a very high versatility with regards to habitat, living in places as disparate as soils, intestines, plants and marine habitats (Reva et al. 2019). Among these bacteria there is the model strain UCMB5113 (Ukrainian Collection of Microorganisms, section Bacteria 5113) isolated from soil in Zakarpatye (Ukraine), a plant colonising endophyte requiring nutrient rich media, which has been described as having outstanding colonising and protective abilities on plants, outperforming other *B. velezensis* strains with regards to colonisation (Reva et al. 2019). Possessing high rhizosphere competence (Niazi et al. 2014; Asari et al. 2016), *B. velezensis* UCMB5113 has been shown to promote the growth of several different plants, for instance by increased root branching and surface area (Niazi et al. 2014).

For example, UCMB5113 can colonise the roots of oilseed rape (Reva et al. 2004), where it has been shown to increase root biomass (Sarosh et al. 2009). Interestingly, the strain does not seem to be able to grow on a medium of 10% oilseed rape root exudates alone (Reva et al. 2019). Increased plant growth has been observed after UCMB5113 root inoculation in both oilseed rape and *A. thaliana* (Col-0), with increased root branching in the Arabidopsis plants (Niazi et al. 2014). An increased outgrowth and elongation of lateral roots (Figure 2), as well as inhibition of primary root growth by UCMB5113 exudates, was found by Asari et al. (2017b) when inoculating *A. thaliana* (Col-0) roots at a concentration of 10^7 cfu×ml^{-1}. Bacterial production of plant hormones IAA and cytokinins, stimulated by root exudates, were implied in the observed changes in root growth pattern (Asari et al. 2017b).

In addition, significant plant growth promoting effects have been observed on Arabidopsis even at low concentrations of bacteria (Asari et al. 2016). The root growth stimulating effects of UCMB5113 have been strongly suspected to be linked to its ability to produce IAA growth regulators (Niazi et al. 2014). Conversely, increased shoot growth in Arabidopsis has been linked to the production of VOC by the strain (Asari et al. 2016). The strain has also been investigated in relation to other plants, showing for instance growth promotion and biocontrol potential in peas (Lagerlöf et al. 2020) and growth promotion and stress management support in wheat (Abd El-Daim et al. 2019).

A selection of indirect plant growth promoting effects observed by the strain include suppression of fungal pathogen growth on Brassica plants (Danielsson et al. 2007; Sarosh et al. 2009; Asari et al. 2017a), an apparent priming of plant induced systemic resistance (Niazi et al. 2014), and antagonism against *R. solanacearum*, lowering wilt symptoms in tomato (Reva et al. 2019).

In spite of all these promising abilities displayed by *B. velezensis* UCMB5113, it has not been thoroughly tested in field trials, currently making a prescription for the use of the strain in biological control or plant protection unreasonable (Reva et al. 2019). However, other strains of *B. velezensis* are already commercially available for plant growth promotion (Novobac 2024), as exemplified by the American PGPR product BioYield (*B. subtilis* GB03 and *B. amyloliquefaciens* IN937a), intended for broad spectrum control of pathogens in greenhouse cultivation of cucumber, pepper, tomato and tobacco (Tabassum et al. 2017). Additionally, ABiTEP (2024) markets biostimulants and plant protection products based on *B. velezensis* FZB42 and *B. atrophaeus* ABi05.

4.2 A note on *Bacillus* root colonisation

Different *Bacillus* strains have different colonisation preferences with regards to root part and root cell layer. For example, *B. subtilis* NCIB 3610 has shown a preference for aggregating at the root elongation zone of *A. thaliana* (Col-0) (Massalha et al. 2017), while *B. velezensis* FZB42 has shown a preference for colonising primary root tips, emerging lateral root tips and root hairs of the same plant (Fan et al. 2011). Furthermore, FZB42 has shown a preference for colonising the surface rather than the epidermis cell layer in maize roots (Fan et al. 2012), while preferring creases between epidermal cells in Arabidopsis roots (Fan et al. 2011). In addition, the rhizoplane surface of a root is initially very small. It is therefore conceivable that root colonisation of very young roots will start slowly, only increasing substantially when the preferred root part or cell layer becomes abundant, e.g. with the formation of lateral roots or root hairs. A

study on the colonisation preferences of *B. velezensis* UCMB5113 on two *A. thaliana* accessions (Ta-0 and Ms-0) showed inconclusive results, although indicative of a preference for root hairs in the case of Ta-0 and root tips in the case of Ms-0 (Matzén 2018).

Apart from root part and cell layer preferences affecting early colonisation, a host-bacterial association must be established between UCMB5113 and the plant before colonisation, requiring a considerable amount of intricate molecular communication including a suppression of the plant immune system or evasion of host pattern recognition receptors to avert the triggering of host defence against the bacterium (Niazi et al. 2014).

4.3 Introduction of experimental plant material

As noted by Tabassum et al. (2017); although many beneficial effects of PGPR on above-ground growth parameters have been demonstrated, the effect of PGPR on roots has remained hard to quantify. The authors further suggest the approach of *in vitro* inoculation of plant roots and looking for changes in primary root growth and lateral root formations as a remedy to this problem (Tabassum et al. 2017). In accordance with this approach, the following experiments were conducted using *in vitro* inoculated roots of Arabidopsis and oilseed rape, which were analysed for changes in primary and lateral root growths as well as lateral root formations and root colonisation. Additionally, comparisons regarding these parameters were made between wildtype and different mutants and transformants of Arabidopsis, as well as between different root pretreatments of oilseed rape.

The wildtype Arabidopsis cultivar used in these experiments is called Columbia-0 (Col-0), named ‘Columbia wild type’ by George Rédei who began working with the cultivar in Columbia in 1957 (Rédei 1992). Columbia-0 was selected for sequencing of the entire genome of Arabidopsis in 1996 (The Arabidopsis Genome Initiative 2000), and has since then become the most common wildtype used in Arabidopsis research (Woodward & Bartel 2018).

In the Arabidopsis transformants used in the experiment, GUS (*β*-glucuronidase) reporter genes had been fused to promoters of genes of interest, meaning that whenever and wherever the gene of interest was expressed in the plant, so was the GUS reporter gene, producing an easily detectible blue colour. One of the genes chosen was the Cyc1At gene, involved in cyclin production in the plant meristems, indicative of its coordinating function in cell division (Ferreira et al. 1994). In other words, where there is cell division, the Cyc1At gene would be expressed, more so where growth is stronger. This gene was included in the

experiment to investigate whether or not a considerably greater expression of Cyc1At (stronger growth) could be detected in the meristems of the inoculated plants compared to the non-inoculated plants, thus indicating a plant growth promoting effect of the inoculant. The other gene chosen for this experiment was the TGG1 gene, involved in the myrosinase-glucosinase defence system of the plant (Ahuja et al. 2021), as well as non-defence related regulatory functions in stomata (Zhao et al. 2008). Moreover, the gene has both a constitutive expression and an increased, induced expression, for instance following pathogen infection (Zeier et al. 2004). This gene is expressed in the guard cells of the stomata (Ahuja et al. 2021), where it has been linked to stomatal closure following wounding (Zhao et al. 2008). TGG1 is also especially abundant in myrosin cells of the phloem (Shirakawa & Hara-Nishimura 2018). These cells accumulate the the product of TGG genes, namely myrosinases, which degrade glucosinolates to produce toxic compounds used in plant defence (Andréasson et al. 2001; Ueda et al. 2006). The TGG1 gene was included to investigate whether or not the inoculation would lead to an activation of the plant defence system, thus indicating a possible induced systemic resistance by the bacteria. A study by Gao et al. (2022) found that the gene TGG1 in *A. thaliana* (Col-0) was expressed both when inoculated with a virulent *Pseudomonas syringae* pv. *maculicola* (Psm) ES4326 strain and when exposed to salt stress, which might indicate that this gene is part of a general response to stress, both biotic and abiotic.

According to Barth & Jander (2006), the breakdown of glucosinolates to toxins as part of the plant defence system is governed by both the TGG1 and TGG2 genes, making these genes functionally redundant in this regard, which means that both genes would need to be knocked out in order to determine the role of this part of the plant defence system in a PGPR-plant interaction. To avoid any such or other potential effects of redundance, a mutant with both TGG1 and TGG2 genes knocked out were used in this experiment (called *tgg1,2*), to investigate any effects of these genes on the colonisation of the bacteria or selected root growth parameters. The other Arabidopsis mutant used in this experiment was a *vnd1237* mutant. This is a quadruple mutant (Leoo 2021), altering the functions of vnd (vascular-related NAC-domain) genes 1, 2, 3 and 7, which are all involved in the biosynthesis of secondary cell walls in the xylem of Arabidopsis plants (Zhou et al. 2014). This means that the mutant would likely be dysfunctional with regards to xylem secondary cell wall synthesis, affecting transport of water, nutrients and other substances within the plant, allowing for an investigation of the importance of these functions for root colonisation and selected root growth parameters.

The other plant type used in this experiment is oilseed rape (*Brassica napus* ssp. *napus*) cv. Kumily (Lantmännen, Sweden). Rapeseed is the most widely cultivated oilseed crop in Europe, and the second-most cultivated oilseed crop in

the world (Snowdon et al. 2007; USDA 2024). It is also the most important oilseed crop in Sweden, where it is grown on 122,700 ha or about 4% of the agricultural land area (Karlsson 2024; Olsson 2024). Belonging to the same family, Arabidopsis and rapeseed might be expected to share some traits regarding plant-microbial interaction. For instance, sinapic acid and goitrin (a glucosinolate derived compound) are known to be produced by both species, and have been shown to disrupt microbial community composition and select for some PGPR (Wittstock & Burow 2010; Hussain et al. 2018; Siebers et al. 2018). This could make rapeseed an interesting object of further study after having observed PGPR colonisation and plant-microbial interactions on Arabidopsis. In this experiment, the importance of certain plant cell wall components for the colonisation of *B. velezensis* UCMB5113 was assessed, namely cellulose and pectin. To this end, the following cellulase and pectinase solutions were used, respectively: Cellulase Onozuka R-10 (C8001, Duchefa, Biochemie, Harlem, Netherlands) used at 100 U/ml. Pectinase Macerase® Rhizopus sp. (Calbiochem, La Jolla, CA), used at 50 U/ml. The lytic enzymes were dissolved in 0.45 M sucrose and sterile filtrated (0.2 µm) before use.

5. Practical part: Materials and Methods

5.1 Overview

To answer the research questions for the practical part, three basic *in vitro* experiments with root colonisation of oilseed rape/Arabidopsis with the strain UCMB5113 of *B. velezensis* were carried out: One comparing some root growth responses and root colonisation between two mutants (*tgg1,2* and *vnd1237*) and a wildtype (Columbia-0) of *A. thaliana*, one comparing some root growth responses, root colonisation and gene responses between two transformants (*tgg1::GUS* and *Cyc1At::GUS*) and a wildtype (Columbia-0) of *A. thaliana*, and lastly one experiment comparing some root growth responses and root colonisation between two destructive root pretreatments (cellulases and pectinases, respectively) and a control treatment of oilseed rape (*Brassica napus* ssp. *napus*). The first of these will hereafter be referred to as the mutant (MUT) experiment, the second, using GUS (*β*-glucuronidase) reporter genes, will be referred to as the GUS experiment, and the third will be referred to as the root (ROOT) experiment. All three experiments demanded a supply of test bacteria and agar plates, which were prepared beforehand as required throughout the course of the experiments.

5.1.1 Root colonisation experiment using mutants

For the MUT experiment, seeds of the three selected lines of *A. thaliana* were sterilised and grown densely on MSA (Murashige-Skoog agar) plates for 5 days. Plants of roughly equivalent root lengths were then selected and transferred to new MSA plates: three plants per plate according to the following experimental setup. Three plants from each line were selected as controls, receiving no bacterial root inoculations. Six plant roots from each line were inoculated with the selected bacterial strain: three of them were left to grow for 24 hours (t1) and three were left to grow for 7 days (t2) together with the controls. All plants were photographed at the time of inoculation (t0) and after 24 hours. At 24 hours, the roots of the three t1 plants were soaked in PBS (phosphate buffer solution), and the resulting solution was used in the spread plate method described below, to ascertain cfu as a measure of root colonisation. The remaining plants were photographed after 7 days and then received the same treatment as the t1 plants. Some root growth parameters (primary/secondary/tertiary root lengths and numbers) were assessed for the plants for each of the time points t0, t1 and t2 (Figure 2).

5.1.2 Root colonisation experiment – use of reporter genes for studies of gene expression

For the GUS experiment, seeds of the three selected lines of *A. thaliana* were sterilised and grown densely on MSA plates for 6 days. Plants of roughly equivalent root lengths were then selected and transferred to new MSA plates: three plants per plate according to the following experimental setup. Three plants from each line were selected as controls, receiving no bacterial root inoculations. Six plant roots from each line were inoculated with the selected bacterial strain: three of them were left to grow for 23 hours (t1), and three were left to grow for 72 hours (t2) together with the controls. All plants were photographed at the time of inoculation (t0) and after 23 hours. At 23 hours, the three t1 plants were each laid on agar plates for about 5 minutes to ascertain whether any root colonisation had taken place. The plants were then GUS stained, photographed and gene expression observed under a low power microscope. The remaining plants were photographed after 72 hours and then received the same treatment as the t1 plants. Some root growth parameters (primary/secondary/tertiary root lengths and numbers) were assessed for the plants for each of the time points t0, t1 and t2 (Figure 2).

5.1.3 Root colonisation experiment – root pretreatment

For the ROOT experiment, seeds of *B. napus* ssp. *napus* cv. Kumily were sterilised and grown densely on MSA plates for 3 days. Plants of roughly equivalent root lengths were then selected and transferred to new MSA plates: three plants per plate according to the following experimental setup. Six plants were selected as controls, receiving no bacterial root inoculations or root pretreatments. Eighteen plant roots were inoculated with the selected bacterial strain: nine of them were left to grow for 24 hours (t1), and nine were left to grow for 7 days (t2) together with the controls. Out of the nine t1 plants, three plants received an extra root pretreatment soaking in a cellulase solution for an hour, and three plants received a similar treatment but with pectinase solution instead of cellulase solution. Three t1 plants received no such root pretreatment, soaking in PBS (phosphate buffer solution) instead. The same subdivision by root pretreatment was made for the t2 plants. All plants were photographed at the time of inoculation (t0) and after 24 hours. At 24 hours, the roots of the nine t1 plants were soaked in PBS, and the resulting solution was used in the spread plate method described below, to ascertain cfu as a measure of root colonisation. The remaining plants were photographed after 7 days and received the same treatment

as the t1 plants. Some root growth parameters (primary/secondary/tertiary root lengths and numbers) were assessed for the plants for each of the time points t0, t1 and t2 (Figure 2).

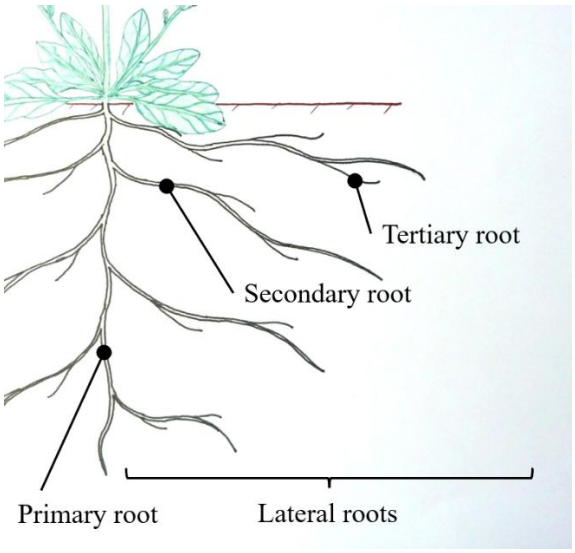

Figure 2. Principal outline of the root system structure of a typical dicotyledonous plant, showing primary root and lateral roots. Lateral roots are subdivided into secondary lateral roots (emerging from the primary root) and tertiary lateral roots (emerging from the secondary roots). Lateral roots emerging from the tertiary roots would be called quaternary roots and so on. With time, all these parts of the root system will develop root hairs.

5.2 Preparation of test bacteria

Cultivation of the bacterial strain UCMB5113 of the species *B. velezensis* was initiated in a laminar flow cabinet through transferring bacteria from a single bacterial colony to liquid LB broth (Luria Bertani broth) using a sterile pipette tip. These solutions were incubated at 28 ℃ overnight. The next day spores were selected for by a heat treatment at 70 ℃. The bacterial solution was then centrifuged and washed in PBS to remove LB broth. The centrifuged bacterial pellet was resuspended in PBS and serial dilutions were performed to determine cfu (colony forming units) on LBA (Luria Bertani broth agar) plates. This was carried out according to the spread plate method described below, and the cfu was found to be approximately $1.39*10^{12}$ cfu/ml. This solution was then diluted to a concentration of 10^7 cfu/ml, re-checking the cfu using the spread plate method again. The diluted solution was kept sterile and refrigerated in two Falcon tubes, and was used as stock solution for all three experiments.

5.3 LB Agar plate preparations

5.3.1 Materials

Two 1 l Duran bottles, 2 paper baking cups, autoclave indicator tape, cold storage room, distilled water (”MilliQ”), hand sanitiser, laminar flow cabinet, LBA powder mixture, oven kept at 55 ℃, paper towels, plastic storage box, scales, slightly more than 60 petri dishes (10 cm) with associated plastic packaging, spatula, spoon, surface disinfectant (70% ethanol) in a spray bottle.

5.3.2 Method

First, 800 ml of distilled water was measured out in each Duran bottle. Next, 28 g of LBA powder mixture was measured out per bottle using scales, spoon, spatula, paper baking cups and paper towels. The bottles were then screwed tight, shaken, labelled with autoclave indicator tape and autoclaved. After autoclaving, the bottles were put in the oven at 55 ℃. The laminar flow cabinet was disinfected, and 11 petri dishes were lined up at the back and filled with about 20 ml of warm LBA solution each from one of the heated bottles, which was immediately put back into the oven. A new row of 11 petri dishes was lined up in front of the first and the procedure repeated. After a couple of minutes, lids were put on the petri dishes containing the now solidified agar, and these were wrapped in sterile plastic packaging. New petri dishes were lined up and the procedure repeated until the contents of the bottles were emptied. The newly cast LBA plates were then put in the plastic storage box in the cold storage room for later use in the spread plate method described below.

5.4 Seed sterilisation and germination

5.4.1 Materials

PBS, 10% bleach (Klorin technical quality, 45 ml sterile distilled water (”MilliQ”), 5 ml bleach), 3 1.5 ml Eppendorf tubes with about 100–200 seeds for each mutant/transformant or wildtype, 3 MSA plates, gas burner, hand sanitiser, laminar flow cabinet, micropipette 0.1–1 ml with sterile tips, semipermeable sealing tape, sterile 1.5 ml Eppendorf tubes, sterile 15 ml Falcon tube, surface disinfectant (70% ethanol) in a spray bottle, tweezers, vortex mixer.

5.4.2 Method

The following steps were carried out at the start of all three experiments. Seeds were put in a sterile Eppendorf tube (MUT and GUS experiments) or 15 ml Falcon tube (ROOT experiment), and 0.5 ml (MUT and GUS experiments) or 5 ml (ROOT experiment) of bleach was added. The seeds were then incubated for about 5 minutes and shaken using the vortex mixer. After the incubation, the tubes were centrifuged briefly to sediment the seeds (in the ROOT experiment the seeds were simply left to sink to the bottom). Excess liquid was then removed with pipette, changing tips between different samples. The seeds were then rinsed with 0.5 ml (MUT and GUS experiments) or 5 ml (ROOT experiment) PBS for about five minutes, shaking the tubes now and then with the vortex mixer, centrifuging them at the end (MUT and GUS experiments) and removing excess liquid by pipette. This procedure was repeated four times. A small volume of liquid was then added to the seeds, and seeds were put out on designated dried MSA plates according to mutant/transformant type or rape seed. Arabidopsis seeds (MUT and GUS experiments) were put out densely using a sterile pipette tip, ideally one seed per drop, starting by placing seeds close to the top of the plate in a line. The rape seeds (ROOT experiment) were put out using burned off tweezers with about 1 cm distance between seeds in the row and about 1.5 cm between rows, transposing every other row to create a uniform dispersal pattern. Finally, the MSA plates were sealed with the semipermeable tape except at the bottom (root end) and put horizontally in a cultivation chamber with artificial sunlight. After about one hour, the plates were tilted vertically and left to grow for 5 (MUT), 6 (GUS) or 3 (ROOT) days, respectively.

5.5 Arabidopsis mutant and transformant root inoculations

5.5.1 Materials

PBS, 1 sterile well plate, 27 *A. thaliana* plants per experiment (9 Col-0, 9 *tgg1,2*, 9 *vnd1237* for the MUT experiment and 9 Col-0, 9 *tgg1::GUS*, 9 *Cyc1At::GUS* for the GUS experiment) on MSA plates, 9 sterile MSA plates, bacterial solution (10^7 cfu/ml of the bacterial strain *B. velezensis* UCMB5113), camera, disinfected cut out plastic template with holes for marking crosses where the plant hypocotyl should be placed for growth on the MSA plates (three crosses according to Figure 3 below), gas burner, hand sanitiser, laminar flow cabinet, micropipettes 0.1–1 ml with sterile tips, semipermeable sealing tape, surface disinfectant (70% ethanol) in a spray bottle, timer, two tweezers.

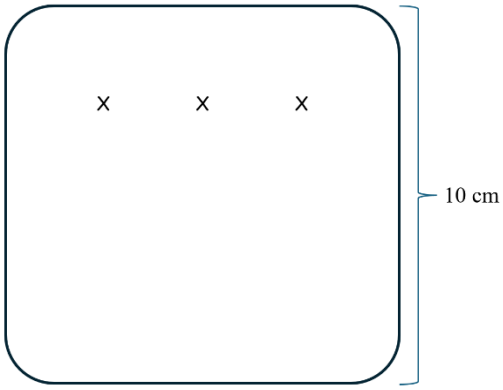

Figure 3. Illustration of plastic template used to mark spots for placing the hypocotyls of plants on MSA (Murashige-Skoog agar) plates according to the three crosses, with roots facing down. Plastic template dimensions are equal to those of an MSA plate.

5.5.2 Method

First, the MSA plates were dried and labelled according to the experimental setup. The plates were also marked with three crosses according to the plastic template (Figure 3). Bacterial solution was transferred to 18 wells: six wells for each line of *A. thaliana* (300 µl per well). PBS was then transferred in the same amount to nine wells: three per line of *A. thaliana*: for use as controls. Plants with a root length of about 1 cm were chosen for the MUT experiment, except for the larger *vnd1237* plants, where a root length of about 1.5 cm had to be chosen. For the GUS experiments, plants with a root length of about 2 cm were chosen, except for the smaller *Cyc1At::GUS*-plants, where a root length of 1 cm had to be chosen. Plants were picked up one by one, and each of them dipped for about 10 seconds in their designated well according to the experimental setup. Here tweezers were used, which were burned off and left to cool down between each plant. Each plant was then immediately transferred to their corresponding labelled MSA plate by carefully dragging the plant upwards across the agar surface, so that the hypocotyl ended up at the marked cross and the root was straightened (three plants per plate). The controls were dipped in PBS instead of bacterial solution. The MSA plates were sealed with the semipermeable tape except at the bottom of the plate (the root end), photographed and placed vertically after about one hour into a cultivation chamber with artificial sunlight.

5.6 Rapeseed root pretreatments and inoculations

5.6.1 Materials

Two sterile 24-well plates (at least 3 ml per well), 24 *Brassica napus* ssp. *napus* plants on MSA plates, 4 ml 0.2 M cellulase solution (sterile filtered), 4 ml 0.2 M pectinase solution (sterile filtered), 50 ml Falcon tubes with disinfected rack, 8 sterile MSA plates, at least 12 ml of bacterial solution (10^7 cfu/ml of the bacterial strain *B. velezensis* UCMB5113), at least 76 ml sterile PBS, camera, disinfected cut out plastic template with holes for marking crosses where the plant hypocotyl should be placed for growth on MSA plates (Figure 3), gas burner, hand sanitiser, laminar flow cabinet, micropipette 0.1–1 ml + sterile tips, semipermeable sealing tape, surface disinfectant (70% ethanol) in a spray bottle, timer, two tweezers.

5.6.2 Method

PBS was measured out in a Falcon tube. Solutions were transferred to wells according to the experimental setup shown in Figure 4 (2 ml per well):

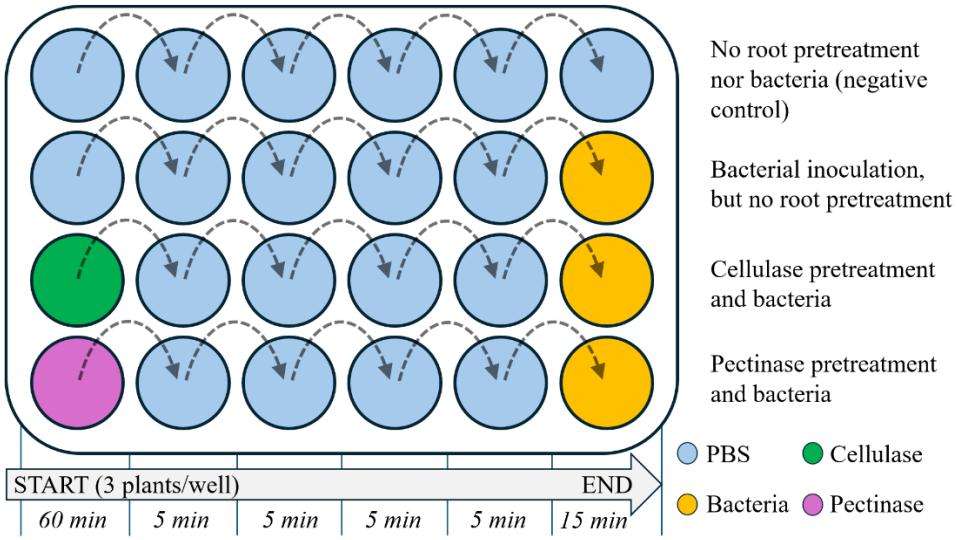

Figure 4. Experimental setup for root pretreatments and inoculations of rapeseed roots, starting by placing three plants in each of the four leftmost wells. Each well contained 2 ml of the corresponding solution. The same setup was duplicated in another set of wells, amounting to 24 plants in total. PBS = phosphate buffer solution.

Three rapeseed plants were transferred with burned off tweezers (any superfluous agar was wiped off as well as possible using the other tweezers) to each of the four leftmost wells according to the above setup (Figure 4). They were then left to soak for about an hour. The MSA plates were then dried and labelled according to the experimental design. The plates were also marked with three crosses

according to the plastic template (Figure 3). All plants were transferred to their corresponding well to the right of the one before (using burned off tweezers) according to the experimental setup above (Figure 4). They were then left to soak for 5 minutes, after which they were transferred to the next well, repeating the same procedure until reaching the last well, where they were left to soak for about 15 minutes. Each plant was then immediately transferred to their corresponding labelled MSA plate by carefully dragging the plant upwards across the agar surface, so that the hypocotyl ended up at the marked cross and the root was straightened (three plants per plate). The MSA plates were sealed with the semipermeable tape except at the bottom of the plate (the root end), photographed and placed vertically after about one hour into a cultivation chamber with artificial sunlight.

5.7 Use of reporter genes for studies of gene expression

5.7.1 Materials

0.45 ml 0.1 M Ferri-cyanide, 0.45 ml 0.1 M Ferro-cyanide, 180 µl 0.1 M X-gluc (0.1545 g Xgluc Apollo Mw 521.79; 2.96 ml dimethylformamide), 2.2 ml PBS, 27 LBA plates, 3 microscope slides, 5.94 ml distilled water (”MilliQ”), 70% ethanol, 9 MSA plates with 27 *A. thaliana* plants in equal proportion of the transformant lines (*tgg1::GUS* and *Cyc1At::GUS*) and wildtype (Columbia-0), aluminium foil, camera, Eppendorf tubes with rack, gas burner, low power microscope, micropipette 0.1–1 ml with sterile tips, Parafilm, tweezers.

5.7.2 Method

The following steps were carried out twice: once for the 23 hours incubated t1 plants, and once for the 72 hours incubated t2 and control plants, using one third of non-returnable materials for t1 and the rest for t2.

First, all stock solutions and chemicals mentioned in materials except ethanol were mixed according to the relative volumetric proportions listed there, to produce 3 ml (for the t1 plants) and 6 ml (t2 and control plants) of GUS staining solution, respectively. Next, plants were photographed and laid on one dried LBA plate each for about 5 minutes using burned off tweezers, for later assessment on whether any root colonisation had taken place. Plants were then similarly transferred back to their MSA plate. The plates were sealed with parafilm and incubated overnight in a dark cultivation chamber at 28 ℃. Each plant was then

transferred with tweezers to designated labelled Eppendorf tubes, where they were covered with 1 ml GUS staining solution. The tubes were wrapped in aluminium foil and incubated in darkness overnight until blue colour could be observed in some plants. After that, samples were rinsed in distilled water and incubated in 70% ethanol for several hours until chlorophyll was removed, and GUS stain easily observed. Plants were then rinsed in distilled water and put on a microscope slide, inspecting and photographing the plants through a low power microscope.

5.8 Spread plate method and counting colony forming units

5.8.1 Materials

Two tweezers, 9 or 18 *A. thaliana* plants (3 Col-0, 3 *tgg1,2*, 3 *vnd1237*) or 9 or 15 *B. napus* ssp. *napus* plants on MSA plates, 50 ml Falcon tube, autoclaved plate spreaders in beaker with aluminium foil (or disposable plate spreaders), beaker with about 200 ml 70% ethanol (for temporary disposal of reusable plate spreaders), disinfected Eppendorf tube rack, fine felt-tip pen, gas burner, hand sanitiser, inoculating turntable, laminar flow cabinet, micropipettes with sterile tips (0.1–1 ml and 20–200 µl), Parafilm, semipermeable sealing tape, sterile Eppendorf tubes, sterile LBA plates, surface disinfectant (70% ethanol) in a spray bottle.

5.8.2 Method

The following steps were carried out at time points t1 and t2 in the MUT and ROOT experiments, including the t1 plants at t1 and the t2 plants together with the control plants at t2. First, all plants were photographed. Next, PBS was measured out in the Falcon tube and then transferred to nine labelled Eppendorf tubes (1 ml/tube). The nine plants were transferred using tweezers (which were burned off between plants) to corresponding Eppendorf tubes and left to soak for about 10 minutes. The plants were then taken out (with tweezers) and thrown away in sealed MSA plates. Due to very small or non-existent cfus on the earliest completed spread plates, only the undiluted solutions in which the roots were left to soak were used in a first round of spread plates. Here, 0.1 ml of solution resulting from each plant was transferred to dried LBA plates, which was spread with a plate spreader on a turntable until relatively dry or resistance from the agar surface was sensed against the plate spreader. Plates were sealed with Parafilm and incubated overnight at 28 °C in a dark cultivation chamber, checking cfu the

next day. Based on the cfu result, appropriate dilution factors were estimated to produce representative and countable cfus (around 30 to a few hundred) for the plant roots in a second round of spread plates. Empty plates were simply replicated using undiluted root wash solution as before. Next, serial dilutions were performed in labelled Eppendorf tubes to the estimated dilution factor appropriate for each plant, using tenfold dilutions in each step up to maximally 10^6. Each dilution was then spread on corresponding LBA plates using the spread plate method as just described (two plates per plant and dilution factor, i.e. about six per plant not producing empty spread plates in the first round). Lastly, the LBA plates were sealed and incubated overnight at 28 ℃ in a dark cultivation chamber. They were then put in a cold-storage room at about 6 ℃ until counting cfu. Finally, plates were photographed and cfu counted using a fine felt-tip pen. For large cfu numbers, only a representative quarter (or eighth) of the plate surface was counted, multiplied by four (or eight).

5.9 Investigating mixing methods and stock solutions of test bacteria

As several of the root colonisations apparently failed, an investigation was conducted to eliminate or confirm the mixing method used prior to inoculation or spread plating, and/or bacterial stock solution viability as sources of error. The two Falcon tubes (A and B) containing stock solution at presumably 10^7 cfu/ml were both used in the test, to detect any differences between them. The mixing method used in the experiments before pipetting (M1) consisted of shaking the bacterial stock solution by hand back and forth about 4 times, turning it by hand about 20 times, shaking it using a vortex mixer for about 40 seconds, and an extra 7 turns by hand just before pipetting. This method was compared to a much longer mixing method (M2) thought to make an inadequate mixing of bacteria unlikely or at least produce a substantial difference in mixing success compared to the original method used, if the original method was inadequate. The longer mixing method consisted of the same procedure as the original method, adding 55 minutes in a shaking apparatus (turning the samples every 3 seconds), an extra 80 seconds of vortex mixing, an extra shaking 4 times back and forth by hand and an extra 20 times turning the solution by hand. Solution from Falcon tubes A and B were mixed according to the two different methods, after which dried and labelled LBA plates were inoculated according to the spread plate method described above: three plates per combination of bacterial solution from tube A or B and mixing method M1 or M2. The plates were incubated overnight at 28 ℃ in a dark cultivation chamber and cfus were later counted as described above.

5.10 Calculations and data analyses

The cfu of a plant root was attained by multiplying the plate cfu by the dilution factor, factoring in the last tenfold dilution when adding 0.1 ml of bacterial solution to the LBA plate. Plate cfus below 30 were excluded from calculations. The mean cfu of a plant was calculated using plate cfus (2–4 technical replicates per plant mean cfu; in three cases only one replicate was produced due to poor colonisation and time constraints). The cfu of a treatment (inoculated or not, with or without cellulase or pectinase root pretreatment) was attained by calculating the mean cfu from the plant cfus in that treatment. Means were calculated by adding the measurements in question and dividing by the number of added measurements.

Both two-tailed Mann-Whitney U-tests and two-tailed type two t-tests were used to calculate p-values for differences between treatments or lines of *A. thaliana*. As the Mann-Whitney U-test is considered more robust than the t-test, not assuming a normal distribution of the data, only the p-values from the U-tests will be displayed for every result. However, where the t-tests and U-tests differ in assessment of significance, p-values from both tests will be displayed; t-test p-values in cursive, and U-test p-values in non-cursive. Another viable alternative for statistical analyses would be the combined use of the ANOVA test and Tukeys HSD, which would regard the variance between treatments apart from variances within two treatments, further reducing the risk of false positives with regards to significance. Microsoft Office Excel was used for most calculations, including calculations of standard deviation. The Mann-Whitney U-tests were carried out with an online tool (Statistics Kingdom 2017), double-checking the calculations with another tool (Vasavada 2016).

6. Practical part: Results

6.1 Mutant experiment

Table 1 shows the amount of bacterial colonisation on inoculated wildtype and mutant Arabidopsis roots 1 and 7 days after inoculation. Although no quantifiable bacterial growth could be shown on wildtype roots in contrast to cfu counts pointing towards a larger bacterial growth potential on mutant roots, especially *vnd1237*, no statistically significant differences in bacterial root colonisation were found between any of the Arabidopsis lines. Control plants showed no bacterial growth.

*Table 1. Bacterial colonisation on roots of wildtype and mutant lines of A. thaliana 1 (t1) and 7 (t2) days after inoculation, respectively. Plant mean cfus were based on 2–3 technical replicates (*in two cases only one replicate was produced due to poor colonisation and time constraints). Arabidopsis line mean cfus were based on the three plant mean cfus (A,B,C) displayed closest to the left in the table, corresponding to a time point t1 or t2 for cfu count. Control plants showed no bacterial growth and are not displayed.*

Arabidopsis line	**Time point for cfu count**	**Plant**	**Plant mean cfu**	**Arabidopsis line mean cfu (from three plants A,B,C; one mean for each Arabidopsis line and time point t1 or t2)**
Col-0	t1	A	0	0
		B	0	
		C	0	
	t2	A	0	0
		B	0	
		C	0	
tgg1,2	t1	A	0	0
		B	0	
		C	0	
	t2	A	0	197444
		B	592333	
		C	0	
vnd1237	t1	A	0	0
		B	0	
		C	0	

t2	A	0	540200
	B	600*	
	C	1620000*	

Table 2 shows the increase in numbers of lateral roots and increases in root length for wildtype and mutant Arabidopsis plants 1 (t1) and 7 (t2) days after inoculation. Root growth in length is also divided into primary- and lateral root growth, and total root growth is displayed both as absolute growth (mm) and relative growth (percentage of initial root length). A relative root growth of 0% would then mean that no growth has occurred, while 100% would mean that the root has doubled in length. For example, looking at Table 2 or Figure 5, the 58% total relative root growth of uninoculated *tgg1,2* after 24 hours means that by 24 hours, the root had grown by 58% of the initial root length, or in other words it had grown to slightly more than 1.5 times the initial root length. Please see the endnote for details on the relevance of including relative root growth as a measurement[1].

Comparing inoculated to uninoculated plants within the same line of Arabidopsis, the only statistically significant ($p = 0.04$) difference found was a larger total relative root growth after 24 hours for uninoculated *tgg1,2* plants (58%), in contrast to inoculated plants of the same line (42%; Figure 5). However, the difference was no longer statistically significant after 7 days.

Looking exclusively at uninoculated plants, the only statistically significant (*$p = 0.03$* according to t-test, but $p = 0.1$ according to Mann-Whitney U-test[2]) difference between Arabidopsis lines found, was a larger total relative root growth after 7 days for *tgg1,2* (1540%) when compared to *vnd1237* (822%; Figure 6b). Please see endnote for details on how p-values are displayed[2].

When instead comparing all plants (inoculated or not, thus including potential synergistic effects between inoculation and mutant effects) of one Arabidopsis line with those of another, a statistically significantly (*$p = 0.02$*, $p = 0.06$)[2] larger total relative root growth was shown after 7 days for Col-0 (1417%) when compared to that of *vnd1237* (952%; Figure 6a). No similar difference was found when comparing only the inoculated or uninoculated plants of these two lines. However, a comparison of only inoculated plants showed a statistically significantly larger primary (*$p = 0.04$*, $p = 0.06$)[2] and total ($p = 0.04$) absolute root growth after 24 hours for *vnd1237* (5.8 mm and 6.2 mm, respectively), in contrast to Col-0 (4.8 mm in both cases; Figure 7). After 7 days, the difference was no longer statistically significant.

Making the same comparison between inoculated *vnd1237* and *tgg1,2* plants, the former mutant showed a statistically significantly larger primary ($p = 0.04$) and total ($p = 0.03$) root growth after 24 hours (5.8 mm and 6.2 mm, respectively) in contrast to the latter (4.7 mm in both cases; Figure 7). Again, however, the difference was no longer statistically significant after 7 days.

Table 2. Some growth parameters of roots of wildtype and mutant lines of A. thaliana after 1 (t1) and 7 (t2) days of inoculation, respectively. Control plants were not inoculated. Mean values from three plants are displayed.

Arabidopsis line	**Col-0**			***tgg1,2***			***vnd1237***		
Treatment	Control	t1	t2	Control	t1	t2	Control	t1	t2
Number of secondary root formations t2	14.3	-	9.3	15.7	-	10.3	9.0	-	13.7
Number of tertiary root formations t2	0.3	-	0.0	0.0	-	0.0	0.0	-	0.0
Total number of lateral root formations t2	14.7	-	9.3	15.7	-	10.3	9.0	-	13.7
Primary root growth t1 (mm)	5.7	5.3	4.3	6.0	4.3	5.0	5.3	5.7	6.0
Primary root growth t2 (mm)	67.0	-	59.7	72.7	-	64.0	62.0	-	64.0
Secondary and tertiary root growth t1 (mm)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7
Secondary and tertiary root growth t2 (mm)	91.0	-	66.0	86.0	-	48.0	41.3	-	88.0
Total root growth t1 (mm)	5.7	5.3	4.3	6.0	4.3	5.0	5.3	5.7	6.7
Total root growth t2 (mm)	158.0	-	125.7	158.7	-	112.0	103.3	-	152.0
Total root growth speed (mm/day)	22.6	-	18.0	22.7	-	16.0	14.8	-	21.7
Total root growth as percentage of initial root length t1 (%)	55	49	45	58	42	43	43	43	48
Total root growth as percentage of initial root length t2 (%)	1526	-	1309	1540	-	965	822	-	1082

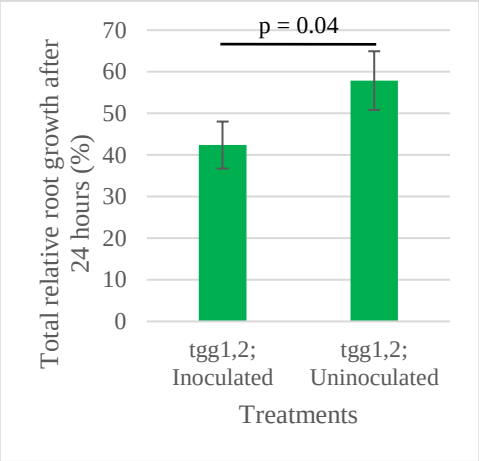

Figure 5. Total relative root growth (%) after 24 hours for inoculated and uninoculated tgg1,2 mutant plants of Arabidopsis.

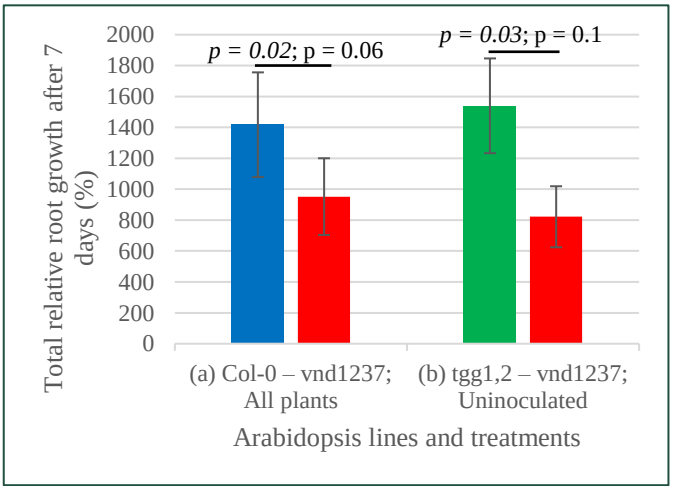

Figure 6. Total relative root growth (%) after 7 days for selected Arabidopsis lines and treatments (all plants meaning both inoculated and uninoculated plants from a line). Blue colour signifies Col-0, red colour signifies vnd1237 and green colour signifies tgg1,2. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

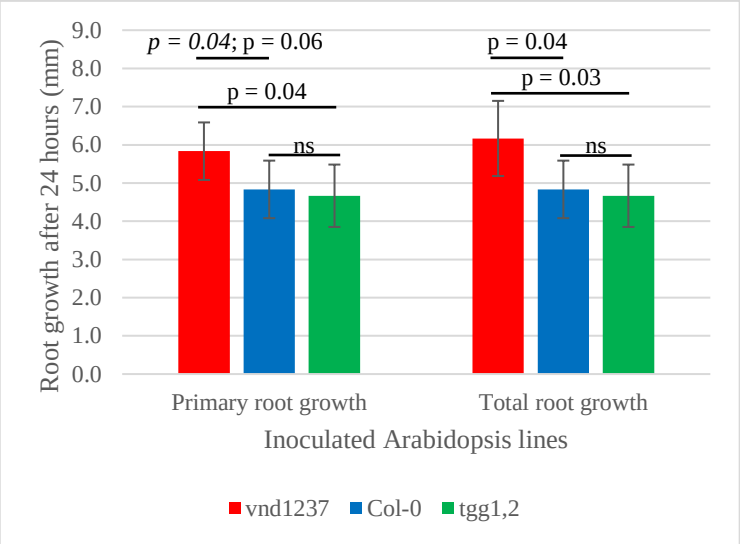

Figure 7. Primary and total absolute root growth (mm) after 24 hours for inoculated lines of Arabidopsis, where ns = no significant difference ($p > 0.05$). The cursive p-value is from a t-test; non-cursive p-values are from Mann-Whitney U-tests.

6.2 GUS experiment

Agar plates indicated bacterial colonisation on all roots of wildtype and transformant lines of *A. thaliana* 1 and 3 days after inoculation, respectively, and no bacterial colonisation on any control plants.

Table 3 shows increase in numbers of lateral roots and increases in root length for wildtype and transformant Arabidopsis plants 1 (t1) and 3 (t2) days after inoculation. Root growth in length is also divided into primary- and lateral root growth, and total root growth is displayed both as absolute growth (mm) and relative growth (percentage of initial root length). A relative root growth of 0% would then mean that no growth has occurred, while 100% would mean that the root has doubled in length. Please see the endnote for details on the relevance of including relative root growth as a measurement[1].

Comparing all inoculated plants from all Arabidopsis lines to all uninoculated plants (thus including any synergistic effects between inoculation and line effects), the uninoculated plants showed a statistically significantly larger root growth after 23 hours than the inoculated plants, both in terms of primary root growth ($p < 0.001$; 5.2 and 1.7 mm, respectively; Figure 8a) and total relative root growth ($p = 0.002$; 40% and 15%, respectively; Figure 9a). However, the difference was no longer statistically significant after 72 hours.

This higher growth rate of the uninoculated plants in the first 23 hours of incubation holds true at a statistically significant level also when comparing uninoculated to inoculated plants within the Arabidopsis lines, except for *Cyc1At::GUS*: In the case of *tgg1::GUS*, there were primary root growths of 5.3 mm and 1.0 mm (Figure 8b), respectively ($p = 0.01$, $p = 0.07$)[2], together with total relative root growths of 34% and 7% (Figure 9b), respectively ($p = 0.02$, $p = 0.07$)[2]. Regarding the Col-0 line, there were primary root growths of 6.0 mm and 2.0 mm (Figure 8c), respectively ($p = 0.03$, $p = 0.06$)[2], and total relative root growths of 40% and 13% (Figure 9c), respectively ($p = 0.02$, $p = 0.06$)[2]. The Col-0 line also showed statistically highly significant ($p < 0.001$, $p = 0.06$)[2] differences after 72 hours, but with a larger total absolute and relative root growth of the inoculated plants (33.3 mm; 218%; Figures 10a and 11, respectively) compared to the uninoculated plants (27.0 mm; 180%; Figures 10a and 11, respectively), contrary to the results from 23 hours (Figures 8 and 9).

Looking instead at inoculated plants, a statistically significant ($p = 0.03$, $p = 0.08$)[2] difference was found after 72 hours of incubation, with a larger total relative root growth of inoculated Col-0 plants (218%) compared to inoculated *tgg1::GUS* plants (132%; Figure 11).

Comparing other inoculated Arabidopsis lines with one another, Col-0 plants had statistically significantly ($p = 0.006$, $p = 0.07$)[2] more secondary root formations after 72 hours (4.7) compared to *Cyc1At::GUS* plants (0.7; Figure 12). The total root growth of Col-0 plants after 72 hours (33.3 mm) was also statistically significantly ($p = 0.009$, $p = 0.08$)[2] larger than that of *Cyc1At::GUS* plants (12.0 mm; Figure 10b).

Regarding the two transformants, inoculated *tgg1::GUS* plants had statistically significantly ($p = 0.005$, $p = 0.07$) more secondary root formations after 72 hours (3.3) than the *Cyc1At::GUS* plants (0.7; Figure 12). Furthermore, the secondary root growth after 72 hours was statistically significantly ($p = 0.02$, $p = 0.08$)[2] larger for the *tgg1::GUS* plants (6.3 mm) than the *Cyc1At::GUS* plants (1.0 mm; Figure 13).

Comparing uninoculated plants of the transformants, *tgg1::GUS* plants had a statistically significantly ($p = 0.048$, $p = 0.1$)[2] larger primary root growth after 72 hours (24.7 mm) than the *Cyc1At::GUS* plants (17.0 mm; Figure 10e). The very same statistically significant ($p = 0.03$, $p = 0.08$)[2] difference was found between the larger primary root growth after 72 hours of uninoculated Col-0 plants (24.7 mm) compared to uninoculated *Cyc1At::GUS* plants (17.0 mm; Figure 10d). Additionally, the uninoculated Col-0 plants showed a statistically significantly ($p = 0.04$, $p = 0.06$)[2] larger total root growth after 72 hours (27.0 mm) than the

uninoculated *Cyc1At::GUS* plants (18.3 mm; Figure 10c). Lastly, the uninoculated Col-0 plants showed a statistically significantly (*p = 0.002*, p = 0.06)[2] larger total relative root growth after 23 hours (40%) than the uninoculated *tgg1::GUS* plants (34%; Figure 9d), but the difference was no longer statistically significant after 72 hours.

Table 3. Some growth parameters of roots of wildtype and transformant lines of A. thaliana 1 (t1) and 3 (t2) days after inoculation, respectively. Control plants were not inoculated. Mean values from three plants are displayed.

Arabidopsis line	**Col-0**			***tgg1::GUS***			***Cyc1At::GUS***		
Treatment	Control	t1	t2	Control	t1	t2	Control	t1	t2
Number of secondary root formations t2	3.7	-	4.7	4.3	-	3.3	1.3	-	0.7
Primary root growth t1 (mm)	6.0	2.0	-	5.3	1.0	-	4.3	2.0	-
Primary root growth t2 (mm)	24.7	-	20.7	24.7	-	14.7	17.0	-	11.0
Secondary root growth t1 (mm)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Secondary root growth t2 (mm)	2.3	-	12.7	5.7	-	6.3	1.3	-	1.0
Total root growth t1 (mm)	6.0	2.0	-	5.3	1.0	-	4.3	2.0	-
Total root growth t2 (mm)	27.0	-	33.3	30.3	-	21.0	18.3	-	12.0
Total root growth speed (mm/day)	3.9	-	4.8	4.3	-	3.0	2.6	-	1.7
Total root growth as percentage of initial root length t1 (%)	40	13	-	34	7	-	45	24	-
Total root growth as percentage of initial root length t2 (%)	180	-	218	192	-	132	189	-	135

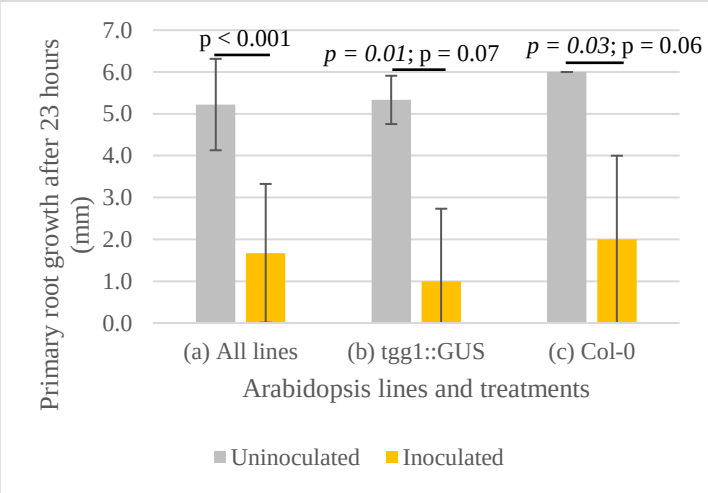

Figure 8. Primary absolute root growth (mm) after 23 hours for uninoculated and inoculated lines of Arabidopsis (all lines meaning both inoculated and uninoculated plants from both transformants and wild type). Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

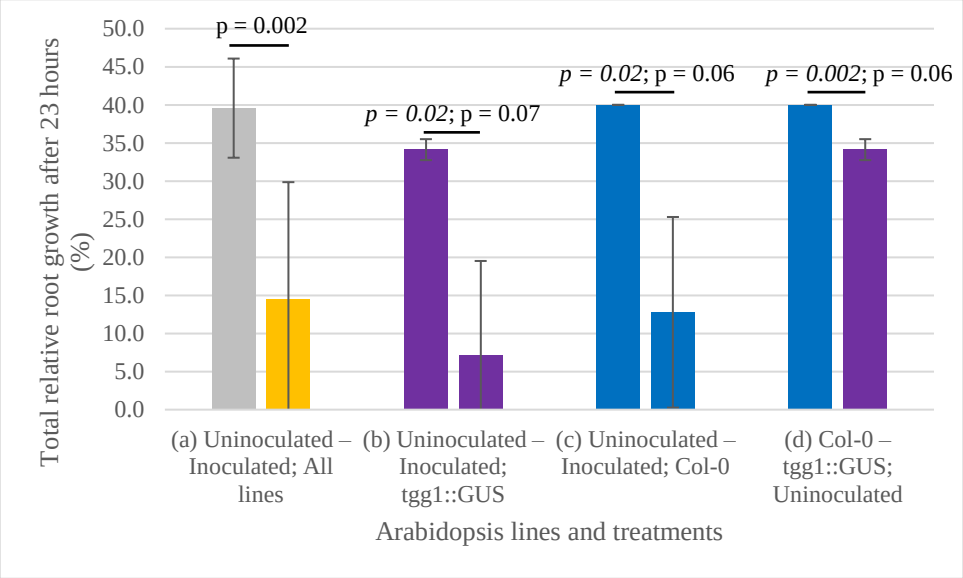

Figure 9. Total relative root growth (%) after 23 hours for selected Arabidopsis lines and treatments (all lines meaning both inoculated and uninoculated plants from both transformants and wild type). Grey colour signifies uninoculated, orange colour signifies inoculated, blue colour signifies Col-0 and purple colour signifies tgg1::GUS. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

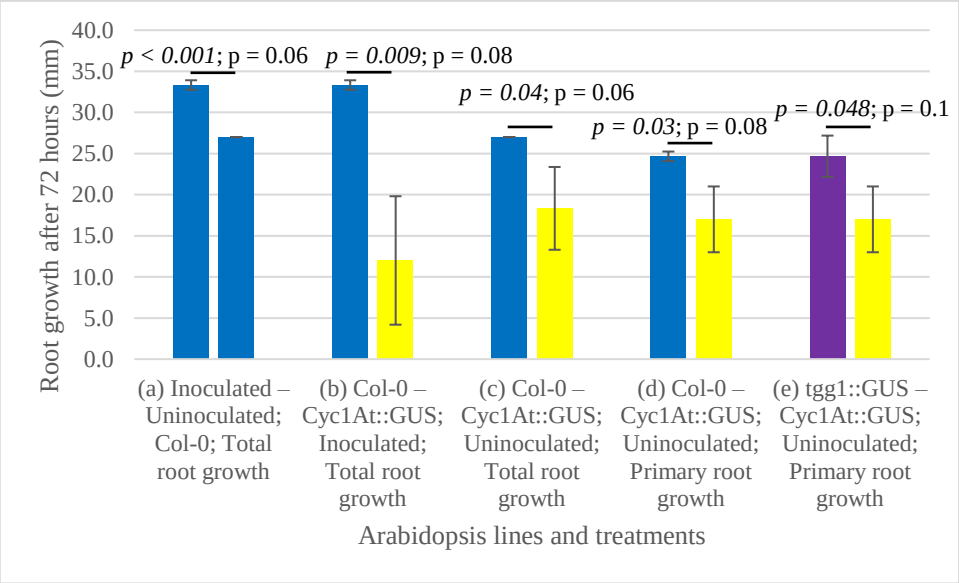

Figure 10. Total or primary absolute root growth (mm) after 72 hours for selected lines and treatments of Arabidopsis. Blue colour signifies Col-0, yellow colour signifies Cyc1At::GUS and purple colour signifies tgg1::GUS. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

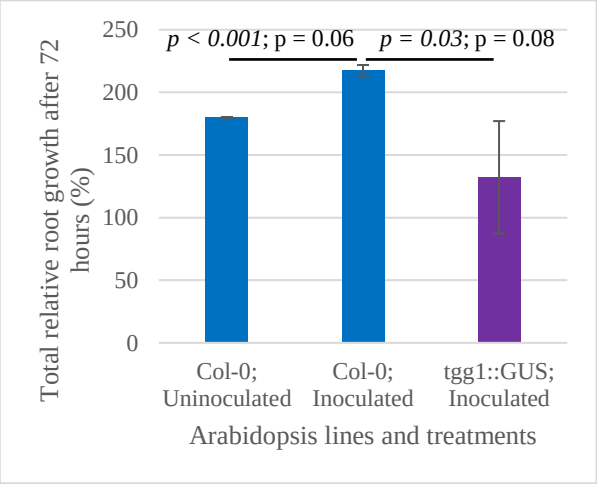

Figure 11. Total relative root growth (%) after 72 hours for selected Arabidopsis lines and treatments. Blue colour signifies Col-0 and purple colour signifies tgg1::GUS. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

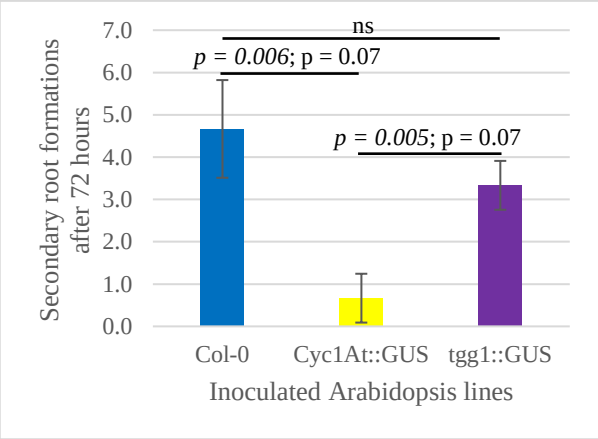

Figure 12. Number of secondary root formations after 72 hours for inoculated lines of Arabidopsis, where ns = no significant difference (p > 0.05). Blue colour signifies Col-0, yellow colour signifies Cyc1At::GUS and purple colour signifies tgg1::GUS. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

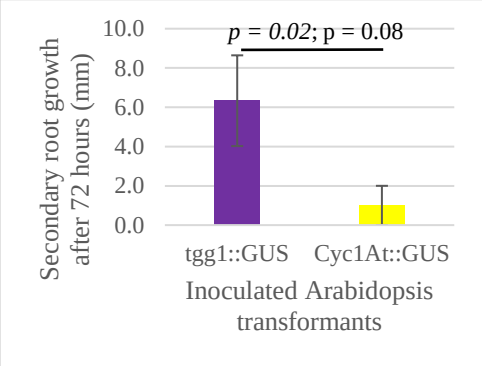

Figure 13. Secondary absolute root growth (mm) after 72 hours for inoculated transformants of Arabidopsis. Purple colour signifies tgg1::GUS and yellow colour signifies Cyc1At::GUS. The cursive p-value is from a t-test; the non-cursive p-value is from a Mann-Whitney U-test.

Table 4 shows where on the Arabidopsis plants blue colour could be observed after staining inoculated and uninoculated transformant and wildtype plants with *β*-glucuronidase after 23 hours (t1) or 72 hours (t2) of incubation. Great differences were observed between plants of different Arabidopsis lines, as there was no blue colour in the wildtype Arabidopsis line (Col-0), but stomata were blue in the *tgg1::GUS* plants and growing points were blue in the *Cyc1At::GUS* plants. Conversely, essentially no differences were noted within the same Arabidopsis line, regardless of time point or whether the plants were inoculated or not.

Table 4. GUS-stain observations on occurrences of blue colour indicative of gene expression in transformant and wildtype plants of A. thaliana.

Time point and plant	**Col-0**	***tgg1::GUS***	***Cyc1At::GUS***
t1 A	-	Stomata: leaves and stem	Growing points: shoot, new leaves, primary root tip and possibly at incipient secondary root
t1 B	-	Stomata: leaves and stem	Growing points: shoot, new leaves, primary root tip and possibly at incipient secondary root
t1 C	-	Stomata: leaves and stem	Growing points: shoot, new leaves, primary root tip and possibly at 3 incipient secondary roots. Primary root tip is missing.
t2 A	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips
t2 B	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips
t2 C	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips
t2 Control A	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips
t2 Control B	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips
t2 Control C	-	Stomata: leaves and stem	Growing points: shoot, true leaves and root tips

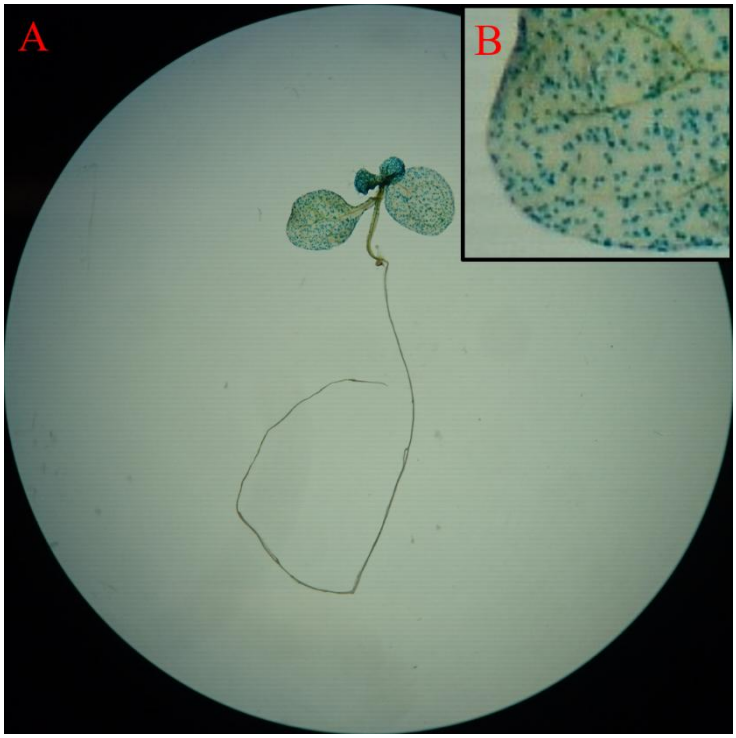

Figure 14. A: A 9 days old β-glucuronidase stained tgg1::GUS transformant of A. thaliana after 72 hours of inoculation with the strain UCMB5113 of B. velezensis. Note blue coloured stomata on leaves and stem. B: Closeup of blue-coloured stomata on leaves.

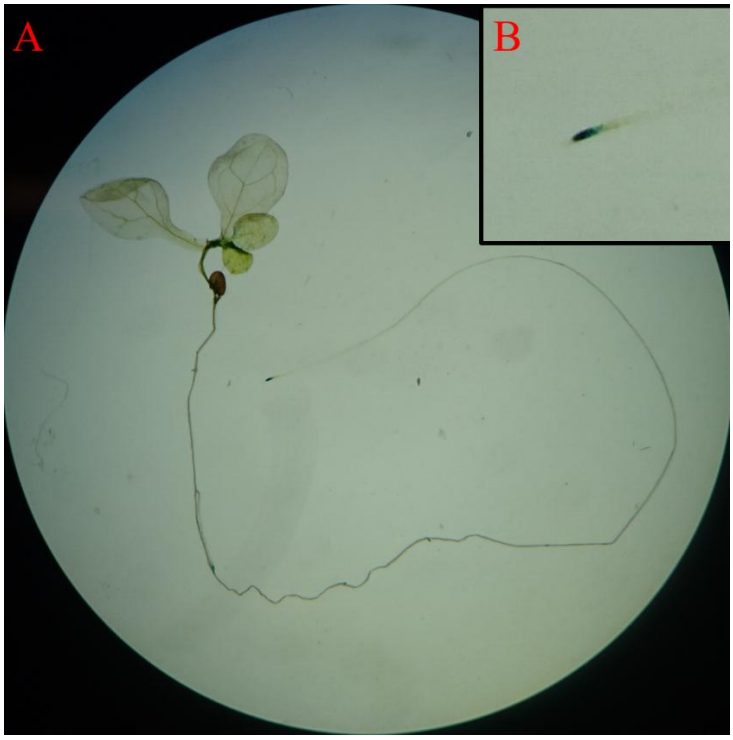

Figure 15. A: A 9 days old β-glucuronidase stained Cyc1At::GUS transformant of A. thaliana. Uninoculated control plant. Note blue coloured growing points. B: Closeup of blue-coloured root meristem.

Figures 14 and 15 show β-glucuronidase stained transformants with blue colour according to the observations listed in Table 4.

6.3 Root experiment

Table 5 shows the amount of bacterial colonisation on the differently pretreated rapeseed roots 1 (t1) and 7 (t2) days after inoculation. Even though the cellulase and pectinase treatments seem to average about ten times more cfu than the purely bacterial treatment at t2, no statistically significant differences in bacterial root colonisation were found between any of the treatments.

*Table 5. Bacterial colonisation on differently pretreated roots of B. napus ssp. napus 1 (t1) and 7 (t2) days after inoculation, respectively. Missing plants (A, B or C) were omitted due to possessing cfus larger than 0, but too low to be quantified (below 30). Plant mean cfus were based on 2–4 technical replicates (*in one case only one replicate was produced due to poor colonisation and time constraints). Control plants showed no bacterial growth.*

Root pretreatment	Time point for cfu count	Plant	Plant mean cfu	Treatment mean cfu
Bacteria	t1	A	500*	3805
		C	7110	
	t2	A	142400	255717
		B	334750	
		C	290000	
Cellulase (and bacteria)	t1	A	3970	2293
		B	615	
	t2	A	985000	2831250
		B	4677500	
Pectinase (and bacteria)	t2	A	5302500	2502500
		B	1420000	
		C	785000	

Table 6 shows increase in numbers of lateral roots and increases in root length for differently pretreated roots of rapeseed 1 (t1) and 7 (t2) days after inoculation. Root growth in length is also divided into primary- and lateral root growth, and total root growth is displayed both as absolute growth (mm) and relative growth (percentage of initial root length). A relative root growth of 0% would then mean

that no growth has occurred, while 100% would mean that the root has doubled in length. Please see the endnote for details on the relevance of including relative root growth as a measurement[1].

Comparing all inoculated to uninoculated plants (regardless of root pretreatment, thus including potential synergistic effects between inoculation and pretreatment effects), the only statistically significant ($p = 0.02$) difference found was more tertiary root formations after 7 days in the inoculated plants (9.4) than in the uninoculated plants (3.0; Figure 16a). Likewise, the only statistically significant ($p = 0.03$) difference between the plants that were only pretreated by inoculation (no cellulase or pectinase) and the uninoculated (control) plants was more tertiary root formations after 7 days in the former (13.3) compared to the latter (3.0; Figure 16b).

Similarly, there were statistically significantly ($p = 0.03$, $p = 0.07$)[2] more tertiary root formations after 7 days in the merely inoculated plants (13.3) than in the inoculated plants also pretreated with cellulase (5.7; Figure 16c). Conversely, there were statistically significantly ($p = 0.01$, $p = 0.1$)[2] more secondary root formations after 7 days in the inoculated cellulase pretreated plants (22.0) than in the merely inoculated plants (12.0; Figure 16d). Lastly, there were statistically significantly ($p = 0.001$, $p = 0.1$)[2] more secondary root formations after 7 days in the inoculated and cellulase pretreated plants (22.0) than in the inoculated and pectinase pretreated plants (12.0; Figure 16e). However, there were no statistically significant differences in the total number of lateral root formations between any of the treatments.

Table 6. Some growth parameters of differently pretreated B. napus ssp. napus roots after 1 (t1) and 7 (t2) days of inoculation, respectively. Control plants were not inoculated. Mean values from three plants are displayed.

Root pretreatment	**Control (no bacteria)**		**Bacteria**		**Cellulase (and bacteria)**		**Pectinase (and bacteria)**	
Time point for cfu count	t1	t2	t1	t2	t1	t2	t1	t2
Number of secondary root formations t2	11.0	19.7	-	12.0	-	22.0	-	12.0
Number of tertiary root formations t2	3.0	3.0	-	13.3	-	5.7	-	9.3
Total number of lateral root formations t2	14.0	22.7	-	25.3	-	27.7	-	21.3
Primary root growth t1 (mm)	0.0	0.0	0.0	0.0	0.0	-1.3	0.0	0.0

Primary root growth t2 (mm)	0.0	0.0	-	0.0	-	23.0	-	0.0
Secondary and tertiary root growth t1 (mm)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Secondary and tertiary root growth t2 (mm)	287.7	320.0	-	261.0	-	404.0	-	247.7
Total root growth t1 (mm)	0.0	0.0	0.0	0.0	0.0	-1.3	0.0	0.0
Total root growth t2 (mm)	287.7	320.0	-	261.0	-	427.0	-	247.7
Total root growth speed (mm/day)	41.1	45.7	-	37.3	-	61.0	-	35.4
Total root growth as percentage of initial root length t1 (%)	0	0	0	0	0	-5	0	0
Total root growth as percentage of initial root length t2 (%)	860	909	-	1038	-	1511	-	921

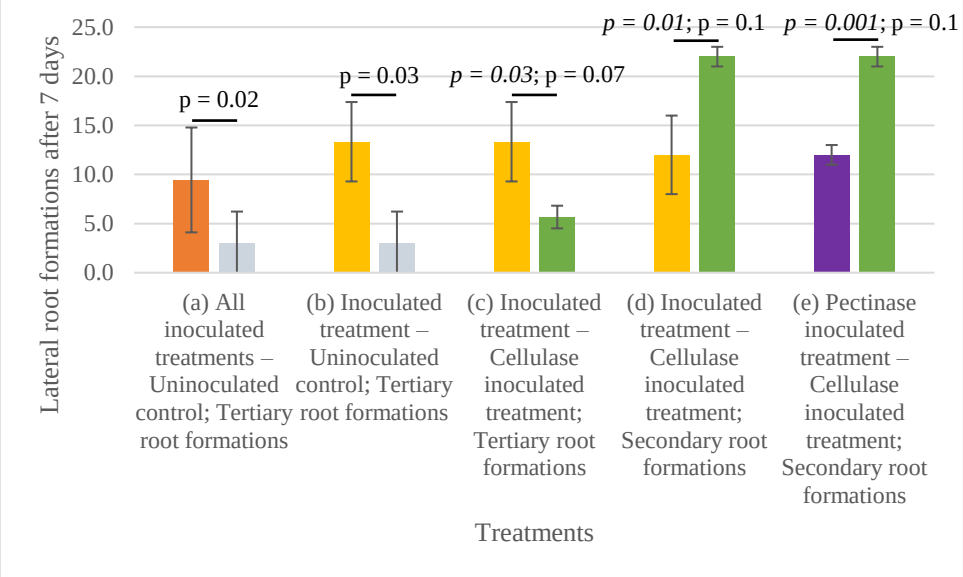

Figure 16. Number of tertiary or secondary roots formations after 7 days for different root pretreatments of oilseed rape plants. All inoculated treatments includes the (merely) inoculated treatment, cellulase inoculated treatment and pectinase inoculated treatment. Brown colour signifies all inoculated treatments, grey colour signifies uninoculated control, orange colour signifies inoculated treatment, green colour signifies cellulase inoculated treatment and purple colour signifies pectinase inoculated treatment. Cursive p-values are from t-tests; non-cursive p-values are from Mann-Whitney U-tests.

6.4 Mixing methods and stock solutions of test bacteria

Table 7 shows cfus of the two stock solutions (A and B) of the bacterial strain UCMB5113 of *B. velezensis* after use in the above experiments, mixed according to a shorter (M1) or longer (M2) mixing method. Means are displayed for stock solutions from tube A and B and for mixing methods M1 or M2 (using results from both tubes). Tube cfus acquired prior to the experiments are also displayed. No statistically significant differences were found between tubes ($p = 0.8$) or mixing methods ($p = 0.8$). Cfu seems to have dropped by about 99% between the start and end of the experiments (Table 7).

Table 7. Colony forming units (cfu) in stock solutions of the bacterial strain UCMB5113 of B. velezensis mixed according to a shorter mixing method (M1) or longer mixing method (M2).

Tube	**Mixing method**	**Solution cfu/ml**	**Mean solution cfu/ml**	**Mean tube cfu/ml**	**Mean mixing method cfu/ml**	**Previously acquired tube cfu/ml**
A	M1	49760	58107	54760	56173	10^7
		61840				
		62720				
	M2	40880	51413		56133	
		54240				
		59120				
B	M1	49040	54240	57547		10^7
		56320				
		57360				
	M2	64320	60853			
		58000				
		60240				

7. Practical part: Discussion

7.1 Mutant experiment

As seen in Figure 5, the only statistically significant effect of the bacterial inoculation was a short-term (24 hours) relative root growth inhibition in the *tgg1,2* line. As this effect was not observed in the wildtype, it could be assumed that the plant defence related TGG1 and/or TGG2 genes are somehow involved. However, although no statistically significant difference could be established between the bacterial root colonisation of the lines, the fact that no reliably quantifiable (cfu > 30) number of colonies could be produced from the Col-0 roots should make one hesitate to draw any conclusions from this result.

The absence of bacterial induced differences is perhaps not surprising, considering the largely unsuccessful root colonisations in this experiment (Table 1), even when accounting for the fact that UCMB5113 has been shown to promote the growth of *A. thaliana* (Col-0) even at low concentrations of bacteria (Asari et al. 2016).

Apart from a short-term (24 hours) larger primary and total root growth of the *vnd1237* plants (Figure 7), perhaps owing to the fact that the *vnd1237* roots were larger (c. 1.5 cm) than the roots of the two other lines (c. 1 cm) at inoculation, potentially giving them a growth speed advantage, the Col-0 and *tgg1,2* plants seemed to outgrow the *vnd1237* plants in the end, with larger total relative root growths after 7 days (Figure 6). A plausible explanation for this might be a reduced water and nutrient transport ability in the *vnd1237* plants, resulting from the mutations of the four vnd genes involved in xylem associated secondary wall biosynthesis (Zhou et al. 2014).

Considering the results in relation to the question of how well *B. velezensis* UCMB5113 colonises the roots of a few different *A. thaliana* mutants (*tgg1,2* and *vnd1237*), the answer would seem to be ‘not well’. Moreover, this makes it hard to give a reliable answer to the question of whether or not the colonisation affects primary and lateral root growths in length and formation of lateral roots. However, in view of the unexplained and uneven nature of the colonisation, the results of this experiment should by no means be regarded as a good basis for answering these questions, prompting the need for further studies on the matter. See a further analysis of the colonisation results in the general discussion below.

7.2 GUS experiment

Results indicate a short-term (23 hours) growth inhibition of inoculated roots (except for *Cyc1At::GUS* plants; Figures 8 and 9), that did not seem to have a long-term (72 hours) effect. For the inoculated plants to be able to catch up with the uninoculated plants in root growth, they would have to have averaged a faster growth than the uninoculated plants during hours 25–72. Perhaps the establishment of a host-bacterial relationship was initially taxing for the plant, but eventually offset by plant growth promoting effects by UCMB5113, such as IAA (growth stimulating hormone) production (Niazi et al. 2014; Asari et al. 2017b). The initial growth inhibiting effect could also be due to the primary root growth inhibition observed by the strain in another study (Asari et al. 2017b), seeing as there are no lateral roots present to compensate for any lack of primary root growth immediately after inoculation.

Further evidence that inoculated plants caught up with their uninoculated counterparts in root growth after 72 hours is provided by the fact that the total absolute and relative root growths of inoculated Col-0 plants exceeded that of uninoculated Col-0 plants after 72 hours (Table 3; Figures 10a and 11). This was, however, not observed in the transformants, which is surprising seeing as the GUS reporter gene should not have a significant negative impact on plant growth (Jefferson et al. 1987; Xiong et al. 2011). Nonetheless, a pattern of larger root growth in wild type plants compared to transformants emerges, as Col-0 surpassed *tgg1::GUS* and *Cyc1At::GUS* regarding several root growth parameters (Figures 9d, 10bcd, 11 and 12). Additionally, *tgg1::GUS* surpassed *Cyc1At::GUS* regarding several root growth parameters (Figures 10e, 12 and 13). A probable explanation for the greater root growth of Col-0 and *tgg1::GUS* compared with the *Cyc1At::GUS* plants in particular, could be the half initial root length of the latter (c. 1 cm) compared with the former (c. 2 cm), giving Col-0 and *tgg1::GUS* a head start in growth as well as larger initial rhizoplane surfaces (i.e. more space for UCMB5113 to grow and potentially exert plant growth promoting effects).

To summarise, the results indicate some significant effects of the bacterial inoculation, although differences between Arabidopsis lines cannot be fully explained. However, as root colonisation was merely qualitatively assessed due to time constraints, the relative impact of root colonisation between transformants cannot be quantified. Especially when looking at the poor colonisation results of the similarly executed MUT experiment, where a quantification of colonisation was attempted (Table 1), the actual extent and therefore effect of colonisation on root growth appears unclear.

Regarding observations on GUS reporter genes, these were expressed in all respective transformants in anticipated plant tissues regardless of whether they were inoculated or not (Table 4; Figures 14 and 15). In the case of *Cyc1At::GUS* plants, this was perhaps not surprising, as the function of the Cyc1At gene in cell division would be expressed in any growing Arabidopsis plant (Ferreira et al. 1994), and any difference in Cyc1At gene expression due to growth effects following UCMB5113 inoculation would maybe not be immediately visible through a stereo microscope. As the TGG1 gene can be expressed both constitutively and be induced as part of the plant defence system (Zeier et al. 2004), an assessment regarding the probable cause of the observed stomatal gene expression would seem difficult. However, no blue colour was observed in the plant phloem, indicating an absence of TGG1 expression in myrosin cells. This might indicate that the observed expression was merely constitutive and not part of an induced plant defence response, as TGG1 expression would then have been observed in both stomata and phloem. Another plausible contributing factor to the observed result could be provided by the fact that activity of the TGG1 gene has been implied as a response to both biotic and abiotic stresses; wounding specifically (Zhao et al. 2008; Gao et al. 2022). Indeed, the small Arabidopsis plants were difficult to handle during inoculation (for instance due to electrostatic effects between root and plastic when placing plants in Eppendorf tubes), and plants were consequently exposed to some measure of mechanical stress, perhaps resulting in the observed TGG1 expression even in the uninoculated plants. As no quantification of GUS expression was performed due to time constraints, an assessment on gene expression beyond immediately visible differences could not be made. Furthermore, as no such differences were observed, it is impossible to tell from these results whether UCMB5113 was involved in triggering the expression of the selected genes in any of the transformants.

In conclusion, the answer to the question of whether *B. velezensis* UCMB5113 can colonise the roots of a few different *A. thaliana* β-glucuronidase transformants (*tgg1::GUS* and *Cyc1At::GUS*) is yes, and the colonisation seems to affect primary and lateral root growths in length, although the actual extent of the colonisation and therefore effects thereof remain unclear due to colonisation being merely qualitatively assessed. Finally, there was no clearly visible difference in expression of the selected transformed genes following inoculation, prompting the need for detailed studies to quantify GUS-expressions as well as updated methods to minimise the risk of mechanical stress eliciting TGG1 gene responses during handling of plants (e.g. using larger tubes to reduce the risk of roots sticking to the rim or tube surface at transfer, or wetting plant roots prior to transfer to relieve electrostatic build-up that makes roots stick to the rim or surfaces of the new tubes).

7.3 Root experiment

Results clearly indicate a higher tertiary root formation in inoculated plants compared to control plants (Figure 16a and b). This result aligns with what could be expected based on increased root biomass (Sarosh et al. 2009) and growth (Niazi et al. 2014) observed in UCMB5113 root inoculated oilseed rape plants in other experiments. In addition, the strain has been shown to increase lateral root formations in *A. thaliana* (Niazi et al. 2014), also when using the same spore concentration of 10^7 cfu/ml at inoculation as in these experiments (Asari et al. 2017b).

Nevertheless, although all inoculated roots were clearly harbouring bacteria (however unevenly distributed between plants; Table 5), bacterial colonisation cannot be established as the sole contributing factor of this result, as all and only control plant roots showed definite or possible signs of mould growth 7 days after inoculation. As the results of this error would be systematic rather than random, and likely result in a growth inhibition of the control plants, it can not be excluded as a contributor of the comparatively lower tertiary root formation in this treatment.

Interestingly, the root pretreatment with cellulase seems to have altered lateral root formation patterns, as the cellulase pretreated plants formed more secondary roots compared with both pectinase pretreated and merely inoculated plants (Figure 16d and e), while the merely inoculated plants formed more tertiary roots than the cellulase pretreated plants (Figure 16c), and no statistically significant difference was found between treatments in lateral root formations as a whole.

In conclusion, the question of how well *B. velezensis* UCMB5113 colonises differently pretreated roots of *B. napus* ssp. *napus* (cellulase and pectinase pretreatment) cannot be adequately answered due to inconclusive results regarding colonisation, although results pointed towards cellulase and pectinase treatments facilitating root colonisation (Table 5). Furthermore, even though results suggest an increase in tertiary root formation following inoculation, potential effects of mould growth in control plants cannot be excluded as contributing to this result. In future studies, extra care should be taken to minimise the risk of introducing mould spores into the MSA plates in order to avoid subsequent systematic errors, for example by minimising the time that plate lids are open or by working further into the laminar flow cabinet to minimise exposure to unfiltered air.

7.4 General discussion

Looking at Table 7 on its own, the apparent 100-fold reduction of *Bacillus* spores in the stock solution between the start and end of the experiments could seem like an obvious explanation for the generally poor rhizobacterial yield produced in the experiments (Tables 1 and 5). However, due to time constraints, the cfu counts forming the basis of this result (Table 7) were unfortunately performed on plates with colonies far exceeding the reliably quantifiable number (amounting to over 4000 cfu per plate), rendering these numbers scarcely better than guesswork. The only thing certain from these results is that the stock solution contained at the very least about 5.6×10^4 cfu/ml, and that no immediately visible difference could be discerned between the mixing methods.

A more probable explanation for the generally poor root colonisation (Tables 1 and 5), at least pertaining to short-term results (24 hours), could be that, as noted in the introduction, initial colonisation speed might be low due to colonisation preferences for root tips and root hairs that are scarce or nonextant at first, coupled with the facts that host-bacterial partnership must be established for effective colonisation and that the rhizoplane surfaces of young plants (5–6 days old Arabidopsis plants and 3 days old oilseed rape plants in this case) initially are very small. As indicated by (admittedly inconclusive) results, UCMB5113 seems to prefer root tips or hairs of *A. thaliana* (Matzén 2018), and another PGPR *B. velezensis* strain; FZB42, also shows preference for root tips and hairs (Fan et al. 2011), pointing towards more extensive colonisation only after increased development of these structures. The roots used in these experiments were generally 1–1.5 cm (Arabidopsis) or about 3 cm (oilseed rape) at inoculation, with no lateral roots or visible root hairs. In other words, maybe the time frame of the experiments (7 days) was too short to observe a substantial colonisation of the roots. For instance, a similar experiment showing growth promoting effects by UCMB5113 used 12 days old Col-0 seedlings, letting the plants grow for 2 weeks after inoculation (Niazi et al. 2014).

On the other hand, another similar experiment showed significant growth promoting effects by the strain on Arabidopsis roots using 5 days old seedlings after only 6 days of inoculation, but here roots were not merely dipped in a 10^7 cfu/ml spore solution but were allowed to grow into a more concentrated spore solution ($2–5\times10^7$ cfu/ml) on the plate (Asari et al. 2017b). Furthermore, even though the time frame and slow initial colonisation might explain some of the lack of bacterial growth, this does not explain why in many cases there was absolutely no sign even after 7 days that the plants were colonised at all (Table 1), or the great and seemingly random unevenness in colonisation even within treatments (Tables 1 and 5). Considering the supposedly high rhizosphere competence (Niazi

et al. 2014; Asari et al. 2016) and excellent colonising abilities of the UCMB5113 strain (Reva et al. 2019), even if one were to grant a spore concentration as low as 5.6×10^4 cfu/ml, an inoculation should realistically result in more than 0 cfu after 7 days (Table 1).

Rejecting the abovementioned sources of error as clear and decisive contributors to the poor colonisation results, various errors in method or execution thereof stand out as all the more probable. For instance, Arabidopsis plants in these experiments were only dipped in UCBM5113 spore solution for 10 seconds (15 minutes for rapeseed plants, which indeed harboured more bacteria than the Arabidopsis plants in the MUT experiment after 7 days; Tables 1 and 5), and then left to grow for 7 days (72 hours in the GUS experiment), as opposed to letting them grow into the spore solution on a plate (Asari et al. 2017b), or, if only dipped, plants were left to grow for two weeks instead of one, or dipped seeds were left to grow for 18–21 days (Niazi et al. 2014). Perhaps the combination of the short dipping time and short growth period after inoculation contributed to the uneven colonisation, as the two similar experiments mentioned either had a longer spore solution exposure time (together with a higher spore concentration) or longer growth period. Additionally, the mixing method, although rather thorough, was possibly not effective enough in dislodging sedimented spores at the bottom of the stock solution tube (even though an investigation into the matter was attempted with no apparent differences resulting from a much longer mixing procedure). Besides a general inexperience in performing experiments of the present kind, possibly leading to unnoticed errors, no procedural failures were noted of a kind that were deemed likely to have affected colonisation results in any substantial way.

7.5 Conclusion and suggestions for further study

To summarise, successful root colonisation can give rise to a wide variety of effects in the plant, as discussed in the theoretical and introductory parts of this text (e.g. on root growth, root formation patterns and gene expression). Conversely, root colonisation success depends on a wide variety of factors, as displayed both by the controlled parameters applied (mutated genes, plant type, altered root surface components) and the unintentional and largely unexplained factors giving rise to the varying colonisation results of these experiments. This illustrates that experiments such as these need both careful planning and execution, and are thus both time consuming and require a fair amount of expertise and practical experience, e.g. in order to minimise contamination risks and exposure of the plants to (unequal amounts of) mechanical stress during

handling. In particular, a fruitful experiment seems to require allowing time to try out and evaluate methods before the start of the experiment by doing a test experiment, as well as allowing time to evaluate different possible sources of error, such as replicating unsuccessful cfu counts at other dilutions or properly examining stock solutions and mixing methods. The importance of conducting a test experiment becomes all the more evident as, even if flexibility is incorporated into the experimental schedule, unexpected circumstances like illness, unviable stock solutions and unsuccessful cfu counts at the estimated dilution factors can bring about a continuous revision of the schedule that can be hard to reconcile with logistical and biological aspects of the experimental procedure, like providing the right material in time for a particular part of the experiment or growing bacteria or plants at suitable intervals to create a functioning workflow.

A further study following the MUT experiment could be to simply redo the experiment, but adjust the method to try and minimise both random and systematic errors. Ways of alleviating random errors include trying to minimise mechanical stress from handling the plants, double-checking stock solution viability prior to inoculation if a certain time (e.g. 1 week) has passed since cfu for the solution was last checked, and increasing the number of biological and technical replicates to offset the effect of random errors on the data. One potential systematic error would be any growth effect resulting from differing root lengths at inoculation, which could be alleviated by adapting sowing dates of different Arabidopsis lines according to the growth speeds of each line to attain fairly equal root lengths between plants of different lines at inoculation. However, it is not entirely obvious that this procedure would create the most even starting position with regards to root growth for all plants at inoculation, as the differing plant physiological ages resulting from this approach could perhaps produce other systematic errors.

For the GUS experiment, apart from the suggestions given for the MUT experiment, a helpful way to clarify the results would be to quantify both colonisation (counting cfu) and gene expression (more precise measurements of the amount of blue colour through the microscope, e.g. by taking pictures of corresponding parts of different plants and analysing the amount of blue colour in the image with image analysis software). In this way, correlations between degrees of colonisation and gene expression could be established.

Regarding the ROOT experiment, apart from the suggestions given for the MUT experiment, extra treatments of uninoculated cellulase/pectinase pretreated roots could be added in order to better separate the effects of root pretreatment from that of inoculation (excluding any potential synergistic effects from simultaneous inoculation and root pretreatment). The rapeseed plants also appear intrinsically

more variable in growth compared with Arabidopsis plants, increasing the need for more biological replicates.

Other interesting prospects for further study would be to properly investigate colonisation preferences of *B. velezensi*s UCBM5113 on the roots of Arabidopsis or other plants, and to conduct field experiments with the strain to evaluate the endurance and potential long-term beneficial effects of the strain on e.g. rapeseed under field conditions.

Notes

[1] The relative root growth measurement (percentage of initial root length) was included in order to better compare root growths from plants with different initial root lengths, offsetting potential advantages in absolute root growth (mm) for plants with larger initial root lengths. Thus, a root with an initial length of 10 mm grown to 20 mm would have the same relative root growth as one initially 20 mm grown to 40 mm. Even though the latter root grew twice as fast in absolute terms (mm), perhaps this was mostly due to unequal initial growth conditions stemming from unequal root lengths, in which case the relative root growth better reflects the growth speed differences between the roots, showing that the plants grew equally fast in relation to their own size (and likely would have grown equally fast in absolute terms as well, if given equal initial root lengths). Possible growth speed advantages from a larger initial root length include a potentially larger initial shoot, providing more energy to root growth, a larger nutrient and water uptake, and larger rhizoplane surfaces where PGPR can colonise and exert beneficial effects on root growth. It should be noted that because the size of the effect on root growth stemming from unequal initial root lengths is unknown, it is likely that neither absolute nor relative root growth will capture the true difference in root growth speed in this scenario. Therefore, both measurements may well be included to highlight this fact. With equal initial root lengths, this issue, together with the use of measuring relative root growth, disappears, why this scenario is preferred. However, this was not the case in the present experiment, with initial root lengths varying between about 1–1.5 cm in the MUT experiment and about 1–2 cm in the GUS experiment.

[2] When only the t-test gave a statistically significant ($p < 0.05$) result, p-values from both the t-test and the Mann-Whitney U-test are displayed; first the p-value from the t-test in cursive, and then the p-value from the Mann-Whitney U-test in non-cursive. For example: $p = 0.04$, $p = 0.06$. All non-cursive p-values are from the Mann-Whitney U-test.

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Acknowledgements

I would like to extend my grateful appreciation for the invaluable support provided by my supervisor, Professor Johan Meijer, both in laboratory and theoretical matters.

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