FACULTY OF SCIENCES

Flavobacterium, a PGPR alleviating cold stress in *Lactuca sativa*

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I Abstract (EN)

Lettuce, an economically valuable crop that is eaten all year round, is sensitive to low temperatures. During the summer months, the leafy vegetable grows to a consumable size in four weeks, but in the cold season, this can take three times as long. Growth promotion of this plant during the cold winter months is therefore crucial for both food supply and to reduce the costs and negative environmental impact of heating greenhouses. A sustainable approach to increase the yield of crops can be found in the use of plant growth-promoting rhizobacteria. This project aims to gain insights into the microbiome of lettuce, and specifically into the growth-stimulating properties of three plant growth-promoting rhizobacteria.

A collection of bacteria isolated from the roots of lettuce, grown under cold temperatures was purified and characterized to serve as a basis for further research. A total of 145 bacteria were successfully purified and identified. These bacteria belonged to the phyla of the Proteobacteria, the Bacteroides, the Firmicutes, and the Actinobacteria. Next, the focus was on the genus *Flavobacterium*, because members of this genus are enriched in the rhizo- and endosphere of lettuce at cold temperatures. Additional bacteria of the genus were also purified from three different plants. In total 40 bacteria of this genus were identified. Additional research to isolate a greater diversity of *Flavobacterium* strains from lettuce could in the future contribute to a larger pool of possible plant growth-promoting bacteria for growth stimulation of lettuce.

Initially, 24 members of the genus *Flavobacterium* were tested for their growth-promoting properties on lettuce under cold temperatures. Three strains showed growth-promoting properties and were further investigated, showing that they could consistently promote lettuce growth under low temperatures. During the experiments, a lot of variation in growth-promoting results was observed, indicating a fragile balance between the presence and absence of growth-promoting properties in plant growth-promoting rhizobacteria. In the future, more molecular data could be collected to better characterize the observed growth promotion.

Lastly, the interaction between the three growth-promoting *Flavobacterium* strains towards each other and towards other *Flavobacterium* strains was examined. Antagonism assays were performed that revealed that several *Flavobacterium* strains reacted antagonistically. In the future, to better understand these interactions, it would be worthwhile to investigate which metabolites and enzymes these bacteria produce.

In conclusion, some members of the genus *Flavobacterium* have a growth-promoting effect on lettuce grown at low temperatures. In the future, after much additional research, these bacteria could perhaps be used in agriculture to improve the growth of lettuce in greenhouses in a sustainable way during the cold winter months.

II Abstract (NL)

Sla, een economisch zeer belangrijke plant die het gehele jaar door gegeten wordt, is gevoelig aan lage temperaturen. Gedurende de zomermaanden groeit deze in vier weken op tot consumeerbare grootte, maar in de winter kan dit drie keer zo lang duren. Groeibevordering van deze plant tijdens de koude wintermaanden is dus cruciaal zowel voor voedselvoorziening als om de kosten en negatieve impact op het milieu van de verwarming van serres te drukken. Een duurzame aanpak om de opbrengst van landbouwgewassen te verhogen kan gevonden worden in het gebruik van plantgroeibevorderende rhizobacteriën. Deze thesis beoogt inzichten te verwerven in het microbioom van sla en specifiek in de groeibevorderende eigenschappen van drie plantgroeibevorderende rhizobacteriën.

Een collectie van bacteriën, geïsoleerd uit de wortels van sla, gegroeid onder koude temperaturen, werd opgezuiverd en gekarakteriseerd om als basis voor verder onderzoek te dienen. In totaal werden er 145 bacteriën succesvol opgezuiverd en geïdentificeerd. Deze bacteriën behoorden tot de phyla van de Proteobacteria, de Bacteroïdetes, de Firmicutes en de Actinobacteria. Vervolgens was de focus op het genus *Flavobacterium*, omdat bacteriën van dit genus aangerijkt zijn in de rhizo- en endospheer van sla bij koude temperaturen. Er werden ook bijkomstige bacteriën van het genus opgezuiverd uit een aantal andere planten. In totaal werden er 40 bacteriën van dit genus geïdentificeerd. Bijkomend onderzoek om een grotere diversiteit aan bacteriën van het genus *Flavobacterium* te isoleren uit sla zou naar de toekomst toe kunnen bijdragen aan een grotere poel van mogelijke plantgroeibevorderende bacteriën voor de stimulatie van de groei van sla bij lage temperaturen.

Initieel werden verschillende 24 bacteriën van het genus *Flavobacterium* getest voor hun groeibevorderende eigenschappen op sla onder koude temperaturen. Drie strains toonden een positief effect op de groei van sla en werden verder onderzocht, waar uit bleek dat ze de groei van sla consistent konden bevorderen onder koude temperaturen. Tijdens de experimenten werd veel variatie in groeibevorderende resultaten vastgesteld, wat wijst op een delicate balans tussen de aan- en afwezigheid van groeistimulerende eigenschappen bij plantgroeibevorderende rhizobacterien. Naar de toekomst toe zou meer moleculaire data vergaard kunnen worden om de geobserveerde groeibevordering beter te karakteriseren.

In een derde luik werd specifiek gekeken hoe de drie groeibevorderende bacteriën van het genus *Flavobacterium* zich gedroegen ten opzichte van elkaar en van andere bacteriën van dit genus uit de collectie. Antagonistische testen werden uitgevoerd waaruit bleek dat een aantal *Flavobacterium* strains antagonistisch reageerden. Naar de toekomst toe kan er onderzocht worden welke metabolieten en enzymen deze bacteriën produceren om de interacties beter te begrijpen.

Er kan besloten worden dat een aantal leden van het genus Flavobacterium een groeibevorderend effect hebben op sla gegroeid bij lage temperaturen. In de toekomst zouden deze bacteriën misschien, na additioneel onderzoek gebruikt kunnen worden in de agricultuur om op een duurzame manier de groei van sla in serres te verbeteren tijdens de koude wintermaanden.

1 Introduction

1.1 Cold stress in plants

1.1.1 How global warming results in colder winters

Rising greenhouse gas emissions cause an increase in global temperature. However, paradoxically, this can also increase the occurrence of cold winters. Above the earth, there are strong air currents, and one of those is the Arctic polar vortex (Lindsey, 2021). It is a region of strong winds about 15 to 50 kilometers (km) in altitude above the Arctic. Variations in this stratospheric polar vortex impact the tropospheric polar jet stream below. This jet stream, which occurs at around 8 to 15 km altitude, forms the separation between warmer mid-latitude and colder polar air, and thus affects the weather conditions to a great extent (Lindsey, 2021). Studies have shown a link between Arctic sea-ice cover loss and the weakening of the Arctic polar vortex (Kim et al., 2014; J. Kim & Kim, 2020). Another factor disrupting the polar vortex is warm atmospheric air moving towards the north. This dwindling of the vortex causes the meandering of the jet stream towards the equator. Normally, the jet stream stays far north and contains a surface layer of cold air at the north pole, but when it moves towards the equator, the cold air will move southwards as well. This results in a higher chance of cold weather in winter for northern Europe, the United States of America, and parts of Northern Asia (**Figure 1**; Kidston, 2015; Lindsey, 2021). Hence, researching cold stress, and investigating how to make plants more resilient to cold will stay relevant in the future, since temperature stress majorly impacts crop yield (Asseng et al., 2010).

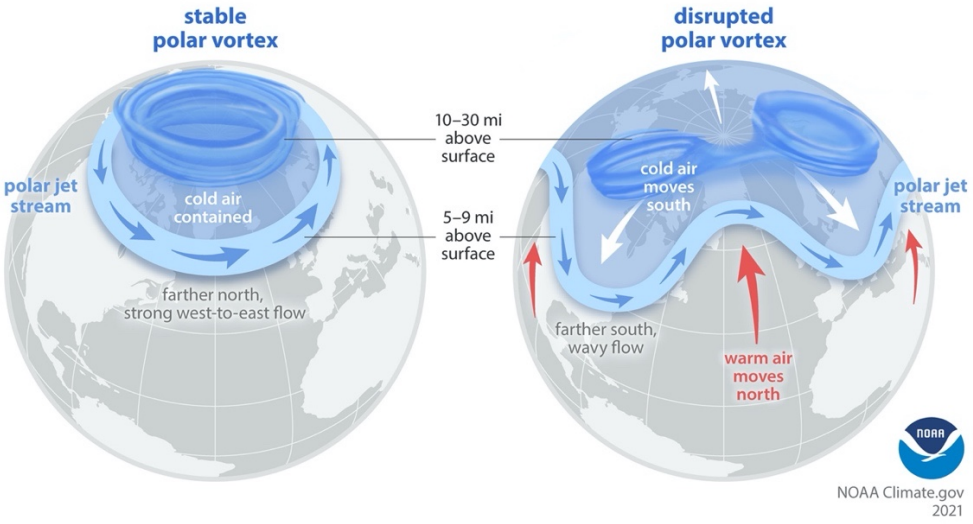


Figure 1. Contrast between the stable and disrupted Arctic polar vortex. (Left) A stable and strong polar vortex keeps the cold air contained at the North Pole. (Right) When the vortex is weakened or disrupted, it will cause the polar jet stream to shift towards the equator, allowing cold air to follow it, resulting in colder weather down south (Lindsey, 2021)

1.1.2 The effect of cold temperatures on plants

Cold stress is caused by chilling or freezing temperatures. Plants can encounter chilling stress in temperatures between 0 and 15 degrees Celsius (°C) and freezing stress when the temperature dips below 0°C. Upon previous exposure to low non-freezing temperatures, plants can improve their cold tolerance, through a process called cold acclimation. In temperate regions, plants are generally

tolerant to chilling stress in varying degrees, and some are cold-acclimated. On the other hand, tropical and subtropical species are often more sensitive to low temperatures (Miura & Furumoto, 2013). Many economically valuable are also to cold-sensitive, including lettuce, tomato, potato, rice, and soybean (Ritonga & Chen, 2020).

Depending on the level of tolerance to low temperatures, the time of exposure, and the plant species, the reaction to cold stress varies widely. General symptoms of cold stress include reduced germination, retardation of growth and reproductive development, chlorosis, wilting, and finally necrosis. Cold stress thus limits the growth and productivity of plants. The organelle most adversely impacted by cold stress is the chloroplast, thus highly affecting photosynthesis. For example, in rice, it has been shown that chlorophyll content will be strongly diminished after exposure to low temperatures. Additionally, stomatal closure often occurs in cold-tolerant plants, reducing CO_2 uptake. In total, this leads to a diminution of the metabolic rate of plants. Stomatal closure, however, also prevents dehydration. Other effects of low-temperature stress include a decrease in membrane fluidity, an accumulation of reactive oxygen species (ROS), and ultimately the impairment of membrane integrity, leading to the leakage of solutes from the cells (Yadav, 2010). Furthermore, chilling and freezing temperatures can also indirectly impact crop yield by reducing soil fertility and stimulating the growth of saprophytic fungi (Subramanian et al., 2016).

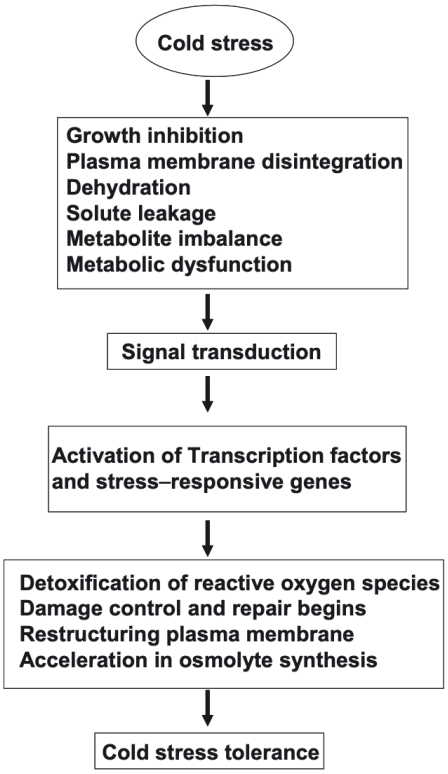


Figure 2. Overview of some of the main effects of cold stress on plants and the plant responses leading to cold stress tolerance. Cold stress severely impacts growth and productivity of plants. After perception of this stress, transcription factors and cold-responsive genes will be upregulated through signaling cascades. Hereby, different responses by the plant will be regulated, to minimize the damage (Yadav, 2010).

Upon membrane rigidification, calcium levels in the cell will increase. This causes calcium-binding proteins to undergo a conformation change, inducing interaction with other proteins, followed by the activation of phosphorylation cascades. Other molecules with a signaling function that are increased upon cold stress are phospholipase D, phosphatidic acid, and reactive oxygen species. Phytohormones, especially abscisic acid, influence the signaling cascades as well (Yadav, 2010; Venzhik et al., 2016). Eventually, these signal transduction cascades will lead to changes in the expression of transcription factors involved in the stress response, which in turn, regulate cold-regulated genes. The ICE-CBF-COR pathway is one of the most known pathways induced by the cold. It involves the inducer of CBF expression 1 (ICE1), the C-repeat binding factor (CBF), also known as the dehydration-responsive element-binding (DREB) proteins, and the cold-responsive (COR) proteins (Chinnusamy et al., 2003; Ritonga & Chen, 2020). This pathway, along with others, will then regulate various cold adaptation mechanisms, including damage repair, the detoxification of ROS, and the restructuring of the plasma membrane (**Figure 2**). Cryoprotection can also be caused by the

accumulation of osmolytes such as soluble sugars, proline, and cryoprotectant proteins to prevent the formation of ice crystals (Yadav, 2010).

One of the cold sensitive crops, as mentioned before that cannot adapt well to cold stress is lettuce.

1.2 Lettuce (*Lactuca sativa* L.)

The Asteraceae is a family containing many commercially valuable plants, of which lettuce (*Lactuca sativa* L.) is the most important from an economic standpoint. Belgium is one of the major producers and exporters of *L. sativa*, specifically butterhead lettuce (Subbarao, 1998). This leafy vegetable is consumed fresh and equally throughout the year (Zdravkovic et al., 2014), which means continuous cultivation is required. It is a beneficial component of many people's diet. *L. sativa* is a vegetable high in water and low in calorie content. It is known to be a healthy food due to it being a source of fiber, several minerals, vitamins, and bioactive compounds such as phenolics and folate. However, the composition of the nutrients does depend on the type of *L. sativa* and the leaf color (M. J. Kim et al., 2016).

Humans have used lettuce for millennia. First, hunter-gatherers collected wild lettuce, most likely *Lactuca serriola* L., until cultivation began in Ancient Egypt. The domestication of lettuce, and thus the origin of *L. sativa*, dates highly likely from around 5000 years ago in the Mediterranean region (Noumedem et al., 2017). At present, there are six main horticultural types of *L. sativa* based on morphology: butterhead, crisphead, looseleaf, oilseed, romaine, and stem (Zhang et al., 2017). However, there is also a lot of variation within each type, both in shape and color. Green is the prevalent color for *L. sativa* leaves, but red leaves also occur. The red color is due to an accumulation of anthocyanins, a pigment belonging to the flavonoids (Mulabagal et al., 2010).

The optimal growth temperature of *L. sativa* ranges from 20°C to 25°C. When cultivated in these conditions, it takes about six weeks for *L. sativa* to attain a consumable size. However, during the cold season, reaching this same size can take up to four months. In this project, microbes of the genus *Flavobacterium* will be investigated to identify their potential role as PGPR in aiding *L. sativa* growth in cold conditions. These bacteria, in the future, increase the turnover rate of the crop during wintertime.

There are multiple strategies to help cold-sensitive crops to cope with low temperatures, but these strategies should not just increase the yield, but also have a minimal impact on the environment and be economically sound. Heating greenhouses, for example, is very effective, but it is also expensive and requires a lot of energy (Ahamed et al., 2019). Another option is to use a gene-driven approach, making transgenic plants or breeding cold tolerance into crops. Both options also have their limitations, they are difficult to achieve and time-consuming, and regulations concerning gene editing in Europe are strict. Also, biostimulants could be used, hydrolyzed porcine blood derivatives, for instance, can reduce the impact of an intense cold period on *L. sativa* (Polo et al., 2006). This, however, raises ethical questions, as vegetarians and vegans do not agree with the use of animal-derived products. A more recent approach to enhance crop growth focuses on the use of micro-organisms.

1.3 Bacteria alleviating cold stress

Bacteria can also help to alleviate cold stress. For example, the bacterium *Burkholderia phytofirmans* will protect grapevine (*Vitis vinifera* L.) against the cold by causing an accumulation of soluble sugars, proline, and other cryoprotectant metabolites. These molecules will lower cell damage and improve photosynthesis (Theocharis et al., 2012). The bacterium also confers cold tolerance to *Arabidopsis thaliana* by causing an accumulation of chlorophyll a and b, pigment contents that were otherwise decreased in the cold. Another effect was the strengthening of the cell walls in the *Arabidopsis* leaves. Additionally, *Burkholderia phytofirmans* reduced the gene expression of *COR78* and *RbcL* (Su et al., 2015). The *Rbcl* gene is located in the chloroplast and encodes for the RuBisCO large subunit. The complete RuBisCo enzyme, consisting of a large and a small subunit, is responsible for the fixation of carbon in the process of photosynthesis.

For tomato (*Solanum lycopersicum* cv Mill), a cold-sensitive crop, four psychrotolerant bacterial strains were identified that consistently improved germination and growth of tomato plants at 15°C, namely *Pseudomonas frederiksbergensis* OS211, *Flavobacterium glaciei* OB146, *Pseudomonas vancouverensis* OB155, and *Pseudomonas frederiksbergensis* OS261. These strains reduced chilling damage by activating the expression of antioxidant enzymes and causing an accumulation of other molecules like proline (Subramanian et al., 2016).

Bacteria are not only able to stimulate plant growth in the cold but also in other stress conditions. These microbes are part of a large community of bacteria living in close contact with plants, on the surface of or inside leaves, stems, and roots. They can also live in the soil.

1.4 Plant Growth Promoting Rhizobacteria

Many micro-organisms live underground, with one gram of soil containing up to 10^{10} bacterial cells (Torsvik et al., 1990), with up to $5 \cdot 10^{4}$ different species (Roesch et al., 2007; Raynaud & Nunan, 2014). However, they are not evenly distributed within the soil. The soil type, the depth, and the environment, among others, all influence the number and diversity of cells present (Whitman et al., 1998). In relation to plants, the plant-soil interface can be divided into different microbial habitats. From outside to inwards there are the bulk soil, the rhizosphere, the rhizoplane, and the endosphere. The bulk soil is the soil not in contact with plant roots, while the rhizosphere is the layer of soil attached to the plant root. The rhizoplane consists of the root surface and the endosphere of the apoplastic spaces between the plant cells (**Figure 3**). The bulk soil has a typical density of approximately 10.8 bacterial cells, while the rhizosphere has an even higher number with 10^{10} bacterial cells (Raynaud & Nunan, 2014). This increased number of bacterial cells can be ascribed to plants influencing the rhizosphere composition by, for example, altering

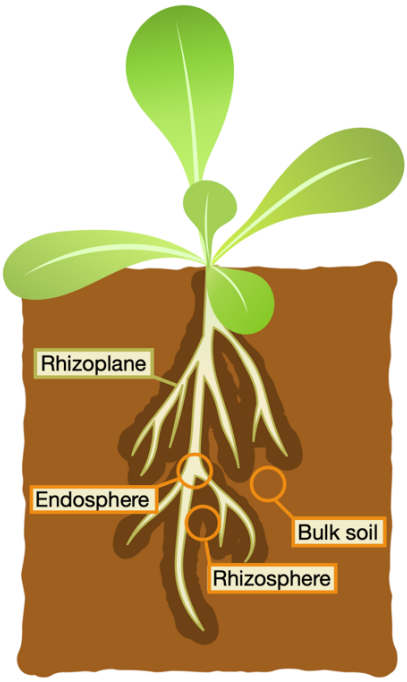


Figure 3. Schematic representation indicating the location of the bulk soil and the rhizosphere, the rhizoplane and the endosphere. The rhizosphere is the layer of soil in close contact with the plant roots (dark brown), while the endosphere is located inside the tissue of the plant roots. The rhizoplane, the root surface is indicated in light green.

the pH, the oxygen availability, and providing resources for microbes. These resources are called rhizodeposits (Baptist et al., 2015). They consist of exudates, nutrients, mucilage, and cells deposited by the plant roots. They are used as a food source by the micro-organisms living in the endo- and rhizosphere. They also contain signaling molecules that alter gene expression in favor of or against promoting interaction between the plant and beneficial or harmful bacteria, respectively (McNear Jr., 2013). Different plant species have different compositions of rhizodeposits, leading to variations in the microbial communities surrounding the plant's roots by the creation of specific niches. This community is also influenced by the type of soil the plant grows in (Santos-González et al., 2011) and by the climate (Zachow et al., 2014). Hence, certain bacterial taxa will be enriched in the rhizosphere and even more so in the endosphere, where there will be an even more rigorous selection by the plant and the specialized niche increasing the competition between bacteria. The main bacterial phyla in the endosphere are the Actinobacteria, the Proteobacteria, the Firmicutes, and the Bacteroidetes (**Figure 4;** Lagos et al., 2015).

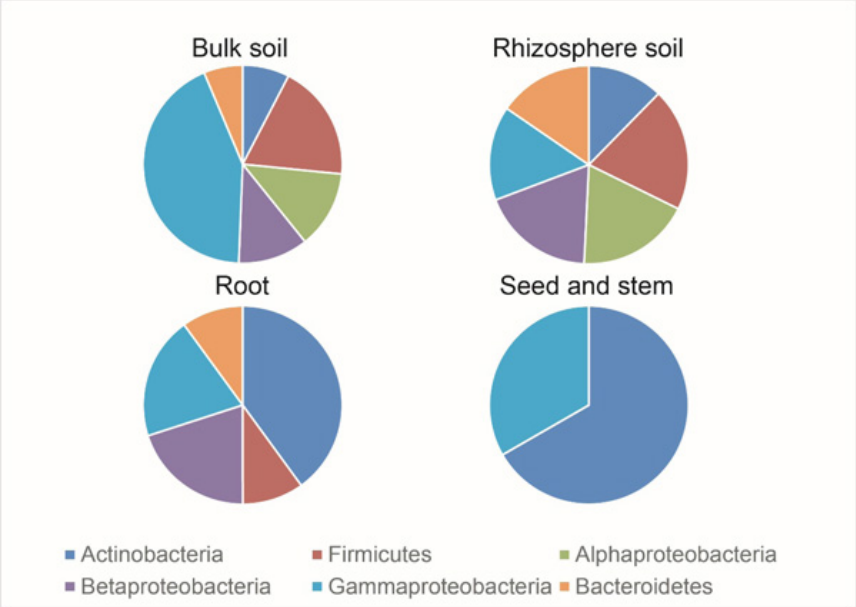


Figure 4. Overview of the composition of the dominant bacterial taxa in the bulk soil, rhizosphere, root or endosphere and the seed and stem. In the different compartments, different bacterial phyla are enriched, the main phyla are the Actinobacteria, the Proteobacteria, the Firmicutes, and the Bacteroidetes (Lagos et al., 2015).

All the bacteria interacting with the plant are collectively called the plant microbiome (Müller et al., 2016). The plant depends on this microbiome for growth stimulation and protection against pathogens. The bacteria within this microbiome are called plant growth-promoting rhizobacteria (PGPR). PGPR live mainly in the rhizosphere or the endosphere, and they can enhance plant growth through various mechanisms (**Figure 5**; (Bhattacharyya & Jha, 2012). They can act as biofertilizers (Vessey, 2003), reducing the dependence on synthetic fertilizers. They can increase the availability or the supply of nutrients, for instance, solubilizing phosphorus (Turan et al., 2012) or fixing nitrogen (Kuan et al., 2016), respectively. PGPR can also act as biocontrol agents (Sayeed Akhtar & Siddiqui, 2008) by improving plant growth in conditions of biotic stress. These bacteria can either stimulate the defense pathways in the plant or act directly antagonistic to pathogens. PGPR of this class are known to act as biocontrol agents against, for instance, *Fusarium oxysporum* (L. Liu et al., 1995; Loganathan et al., 2014). Furthermore, PGPR can promote plant growth by working as biostimulants, directly influencing regulatory pathways in the plant under numerous stress conditions such as, for example, cold stress (Zubair et al., 2019), drought (S. M. Gupta et al., 2020), or salt stress (Li et al., 2020).

1.5 Mechanisms of growth promotion

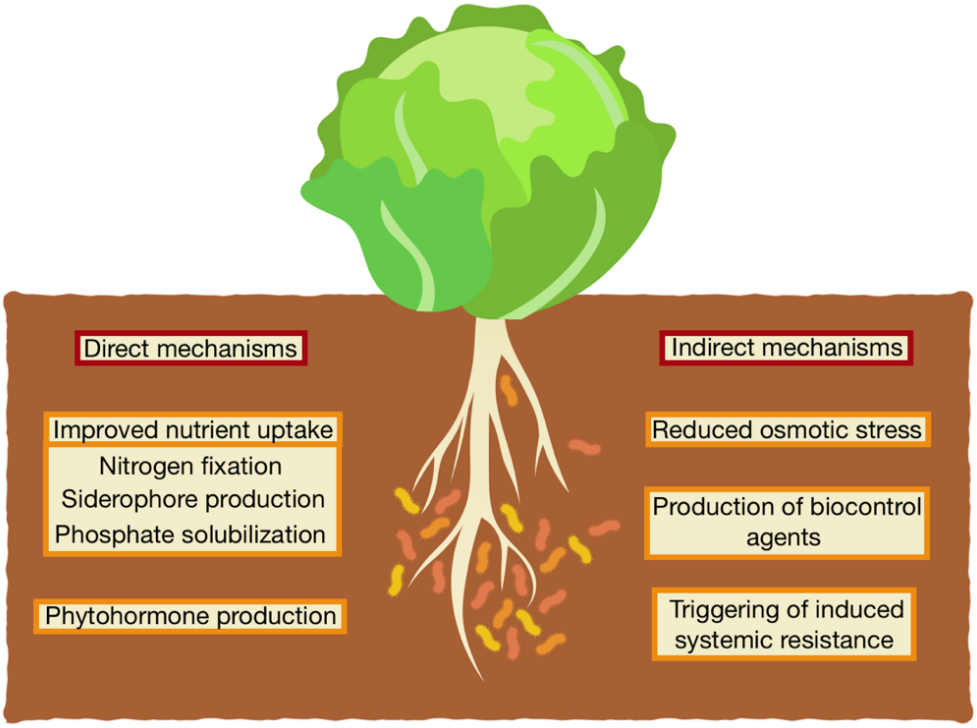


Figure 5. Overview of direct and indirect growth promoting properties of PGPR. Bacteria can promote plant growth directly by improving nutrient acquisition or interference in phytohormone pathways. Plant growth can also be facilitated indirectly through the reduction of abiotic or biotic stresses.

1.5.1 Biofertilizer

Major plant nutrients, such as nitrogen, phosphate, or potassium, are mainly present in sufficient amounts in the soil. However, they are often not readily accessible for uptake through the plant roots. PGPR can assist with nutrient acquisition by making certain nutrients available to the plant or by stimulating the transcription of transporters taking up nutrients.

Nitrogen fixation

About 78% of the atmosphere consists of nitrogen gas (N_2), but nitrogen in its gaseous form is not available for plants. Nitrogen is a vital element of DNA and RNA molecules, proteins, and chlorophyll, among others. The combination of the low supply and the high demand for this nutrient results in it often becoming limiting for plant growth. However, nitrogen-fixing bacteria can convert this atmospheric nitrogen (N_2) to ammonia (NH_3), through the use of a nitrogenase complex. This process is called Biological Nitrogen Fixation (BNF), it provides roughly two-thirds of the nitrogen used in agriculture today (Hayat et al., 2010). In contrast to N_2, plants can take up NH_3. In return, plants will provide carbon compounds, such as glucose, for the bacteria. Many different bacteria possess the ability to fix nitrogen, they are collectively called diazotrophs (Franche et al., 2009). Multiple of these prokaryotes act as PGPR, like *Bradyrhizobium spp.* (Hara et al., 2019) or *Burkholderia vietnamiensis* (Tang et al., 2010). The interaction with a plant host can range from rather loose, the bacteria staying free-living, to very close, with the formation of nodules on the plant roots. PGPR able to partake in this nodulation process include members of the genera *Frankia* and *Rhizobium*. Another example is

Paenibacillus mucilaginosus strain 3016, which has been found to significantly improve nodulation in soybean (Ma et al., 2018). Nitrogen-fixing bacteria that stay free-living and do not form nodules are often cyanobacteria, including species of the genera *Anabaena*, *Azotobacter*, and *Nostoc* (Postgate, 1982).

Phosphorus solubilization

Phosphorus is, next to nitrogen, the most plant growth-limiting nutrient. It is involved in multiple key processes, such as photosynthesis and energy transfer and it is an essential building block for nucleic acids, phospholipids, and adenosine triphosphate. Phosphorus is generally present in adequate quantities in the soil, however, only a fraction is available for plants. This fraction consists of two soluble forms: monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions. The remainder of the phosphorus is immobilized, insoluble, or in precipitated form (Bhattacharyya & Jha, 2012). PGPR, such as members of the genera *Bacillus*, *Pseudomonas* or *Rhizobium*, can assist with the release of this phosphorus from the soil to make it accessible for plants. They do so by secreting phosphatases, organic acids, or protons (Richardson et al., 2009), which lower the soil pH.

Siderophore production

Iron is the third most plant growth-limiting nutrient. It is invaluable to the plant as it plays a pivotal role in the synthesis of chlorophyll, among other processes. A deficiency causes yellowing of the leaves or chlorosis. Iron is mainly present as insoluble, oxidized Fe^{3+} in the soil. PGPR can facilitate the reduction of Fe^{3+} to Fe^{2+}, which plants can use as an iron source. These PGPR produce siderophores, small peptides, that bind Fe^{3+} with a relatively low affinity and reduce it. Chelators in the plant have a higher affinity for Fe^{2+}, and thus the plant will take it up. In addition, this will limit the iron available to pathogens in the soil (R. Sayyed et al., 2013).

1.5.2 Biocontrol

Many PGPR can suppress disease symptoms, especially members belonging to the phyla of the Actinobacteria, the Firmicutes, and the Proteobacteria have been found to protect plants against diseases (Mendes et al., 2011). This is called biocontrol or antagonism.

Bacteria can protect plants through different mechanisms. Firstly, certain micro-organisms can produce antibiotics stopping or slowing the growth of pathogens, mainly fungi. This process is known as antibiosis (Kenawy et al., 2019). Additionally, some PGPR produce lytic enzymes (Vivekananthan et al., 2004) that degrade cell walls, membranes, and virulence factors of infectious agents, or volatile organic compounds (VOCs) to control pathogens. *Bacillus amyloliquefaciens* FZB42 and *Bacillus artrophaeus* LSSC22 were found to work antagonistically against bacterial wilt disease in tobacco, which is caused by *Ralstonia solanacearum* (*Rsc*). They produce VOCs that inhibit the mobility and colony growth of *Rsc* and cause morphological abnormalities, among other things (Tahir et al., 2017).

Pathogenic micro-organisms communicate also with each other. This cell-cell communication between microbes is called quorum sensing. It works via the secretion of small chemical molecules (Miller & Bassler, 2001). Bacteria use it to relay information about the cell density of the colony and to synchronize their activity, for instance to synchronize an attack. Certain PGPR can interrupt this communication, preventing pathogens from becoming more virulent, through the process of quorum quenching (Dong et al., 2001).

Moreover, biocontrol can occur by the induction of systemic resistance. There are two forms: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Kamle et al., 2020). After the infection of a plant with a certain pathogen, other uninfected tissues of the plant will be primed, which makes them better prepared for an attack. This is SAR, the pathogenesis-related (PR) proteins will accumulate after infection with mainly biotrophic pathogens, that keep the host alive. Salicylate signaling plays an important role in this process. In contrast, in the case of ISR, non-pathogenic rhizobacteria, like *Pseudomonas fluorescens* (Vleesschauwer et al., 2008) or *Bacillus subtilis* (Cawoy et al., 2011), will prime the plants, making them react faster and stronger when a pathogenic attack takes place. Mainly ethylene and jasmonate signaling are enhanced within the plant during this process. This means ISR is mainly active against necrotrophs, pathogens that feed on dead tissue (Kamle et al., 2020).

1.5.3 Biostimulant: phytohormone modulation

Phytohormones play a crucial role in the life of plants. They control processes such as germination, cell division, elongation and differentiation, and apical dominance. Certain PGPR affect the architecture of the plant by modulating the phytohormone balance. These bacteria produce or degrade phytohormones or influence the plant's production of them and thus affect the development of plants as well as their defense (Tsukanova et al., 2017).

Auxin is a hormone mainly present in the form of indole-3-acetic acid (IAA) in plants. It is involved in many different processes including cell division, apical dominance, the differentiation of vascular tissue, gravitropism and phototropism (Tsukanova et al., 2017). Auxin also mediates cell elongation in a dose-responsive manner, if the concentration does not cross a certain threshold (Thimann, 1939). If it does, ethylene production will be induced, and plant growth will be attenuated. In the shoot, the optimum of IAA is much higher than in the root. Furthermore, auxin stimulates the initiation of lateral and adventitious roots (Tsukanova et al., 2017). The formation of these roots increases the root surface, this can help with the uptake of water and nutrients. Many rhizobacteria, including members of the genera *Enterobacter* (Ghosh et al., 2015) and *Pseudomonas* (Patten & Glick, 2002; Iqbal & Hasnain, 2013), can produce auxin or alter the plant's sensitivity to the hormone, which will accelerate germination and root growth.

Ethylene has many functions within the plant, especially in the developmental processes of leaves, flowers, and fruit. It also plays a role in senescence. Moreover, ethylene prevents elongation of the root, while promoting the formation of adventitious roots and root hairs. Various stresses will also result in the upregulation of the 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene and ethylene itself (Iqbal et al., 2017; Tsukanova et al., 2017). This will encourage chlorosis, senescence, and leaf abscission, and inhibit root growth (Iqbal et al., 2017). PGPR that produce ACC deaminase facilitate plant growth by lowering both the ACC and ethylene levels in the plant. For example, a consortium consisting of *Aneurinibacillus aneurinilyticus* and *Paenibacillus species (sp.)* was able to confer tolerance to French bean plants (*Phaseolus vulgaris*) coping with salt stress by lowering ethylene levels through ACC deaminase activity (S. Gupta & Pandey, 2019).

Cytokinin, another phytohormone, plays a crucial role as a signaling molecule regulating plant growth and development. It is known for its interplay with the phytohormone auxin. In contrast to auxin, however, cytokinin will slow down root growth and elongation (Werner et al., 2001; Stenlid, 2006), by promoting cell differentiation rather than cell division in the root apical meristem. In the shoot apical

meristem, a high concentration of cytokinin will stimulate cell division (Werner et al., 2001). Cytokinin also influences biotic interactions, the hormone, for instance, plays a role in the formation of root nodules (Suzaki et al., 2013). Other functions include the regulation of embryogenesis and vascular development (Werner et al., 2001;Tsukanova et al., 2017). An example of a PGPR producing cytokinin is *Bacillus subtilis,* which alleviates drought stress and stimulates mainly shoot growth in *Platycladus orientalis* seedlings by decreasing the cytokinin deficit present in shoots coping with drought stress (F. Liu et al., 2013).

Other hormones PGPR can affect include gibberellin, abscisic acid, and brassinosteroids (Tsukanova et al., 2017). Moreover, PGPR can possess many more functions to help plant growth. *Bacillus pumilis*, for example, has been shown to increase shoot fresh weight of tomato plants. They augment the antioxidant activity of mainly catalase and superoxide dismutase, subsequently alleviating osmotic stress (Sirajuddin et al., 2016), while other PGPR will assist in soil remediation (Zhuang et al., 2007). In fact, there are many more strategies to help plants cope with abiotic stresses. Many PGPR also do not have just one of these functions, they often affect plant growth in multiple ways. *Azobacter* spp., for instance, is not only known for nitrogen fixation, but also for the upregulation of auxin production, the breakdown of pesticides, the production of siderophores, etc. (Sumbul et al., 2020).

1.6 Commercialization of PGPR

PGPR have a lot of potential to be used in agriculture as they enhance crop growth and alleviate both abiotic and biotic stresses. However, the commercialization of these micro-organisms faces many challenges. Firstly, strains differ in their effectiveness depending on the crop, the climate, the soil type, and what stresses the crop is coping with. A second obstacle is the variability of field conditions compared to the environment in greenhouses. Hence, the colonization of the plant roots, the survival, and the proliferation of the selected strains should be considered. These factors can be positively impacted by inoculating the plants with the PGPR at the correct moment, using the right concentration of bacteria, and engineering the rhizosphere. Another challenge is the fact that not just the effectiveness of the strain decides if the strain can be brought onto the market. In fact, many other factors should also be taken into account, for instance, any health risks the strains pose by producing toxic components or allergens, how long the shelf life of these micro-organisms is, and how much it will cost to produce and apply (Tabassum et al., 2016).

Despite the many hurdles that need to be overcome before any bacteria can be brought to the market, there are examples of PGPR being successfully used in the field. Nodulator® Duo SCG, by the company BASF Canada Inc. contains the strains *Rhizobium leguminosarum* biovar *viceae* (strain 1435) and *Bacillus subtilis* (strain BU1814). These strains enhance nodulation, germination, nutrient uptake and the ability to cope with stress in peas and lentils (BASF Canada Inc., 2019). The product Cell-Tech® peat soybean by Novozymes is based on the bacterium *Bradyrhizobium japonicum*. It stimulates nodule formation in soybean formation to increase nitrogen levels available for the plant (Novozymes, 2019). There are many more products already commercially available and the demand for these products is only rising.

Various PGPR are thus already being used in the field or in greenhouses, helping plants cope with various stresses in a sustainable way. Some strains have even already been identified to promote crop

growth under low temperatures (**as described in 1.2**). However, not much is known about PGPR improving the growth of the cold-sensitive crop lettuce.

1.7 Microbiome analysis of the lettuce root microbiome

Previously to this project, a microbiome analysis was performed to visualize the rhizo- and endospheric bacterial communities of *L. sativa* plants and to look for bacterial groups enriched under the cold. This study was carried out by growing five different commercially available *L. sativa* cultivars in three different soil types (sand, loam and sandy loam) at cold (8-12°C) and control (18-22°C) temperatures. Bacterial samples were taken from the bulk soil, the rhizo- and the endosphere and were subsequently sequenced, after metabarcoding of the V4 region of the 16S rRNA gene. Principal Coordinates Analysis (PCoA) was performed to determine which samples clustered together according to the different compartments of soil, the soil type, and the temperature and relative abundances of the bacteria present were calculated.

The PCoA analysis shows a clear and gradual separation of the samples based on temperature (**Figure 6**). The bacterial composition of the bulk soil samples appears least influenced by a difference in temperature. Rhizosphere samples on the other hand start to separate out based on temperature, but the difference in most clear for the endosphere where samples from low and control temperature conditions clearly cluster away from each other. Concerning the relative abundances of bacterial groups, there are prominent differences in the most abundant groups in the rhizosphere versus the endosphere (**Figure 7A and 7B**, respectively). Three genera that are common in both soil compartments are *Flavobacterium*, *Pseudomonas*, and *Massilia*. These three groups all show significant enrichment under low temperatures, both in the rhizo- and endosphere. Other bacterial genera, for instance belonging to the *Streptomyces*, were depleted upon cold temperatures.

As members of the genus *Flavobacterium* are very abundant and enriched in both the endo- and rhizosphere of *L. sativa* plants in the cold, it is interesting to investigate their potential to promote *L. sativa* growth under low temperatures.

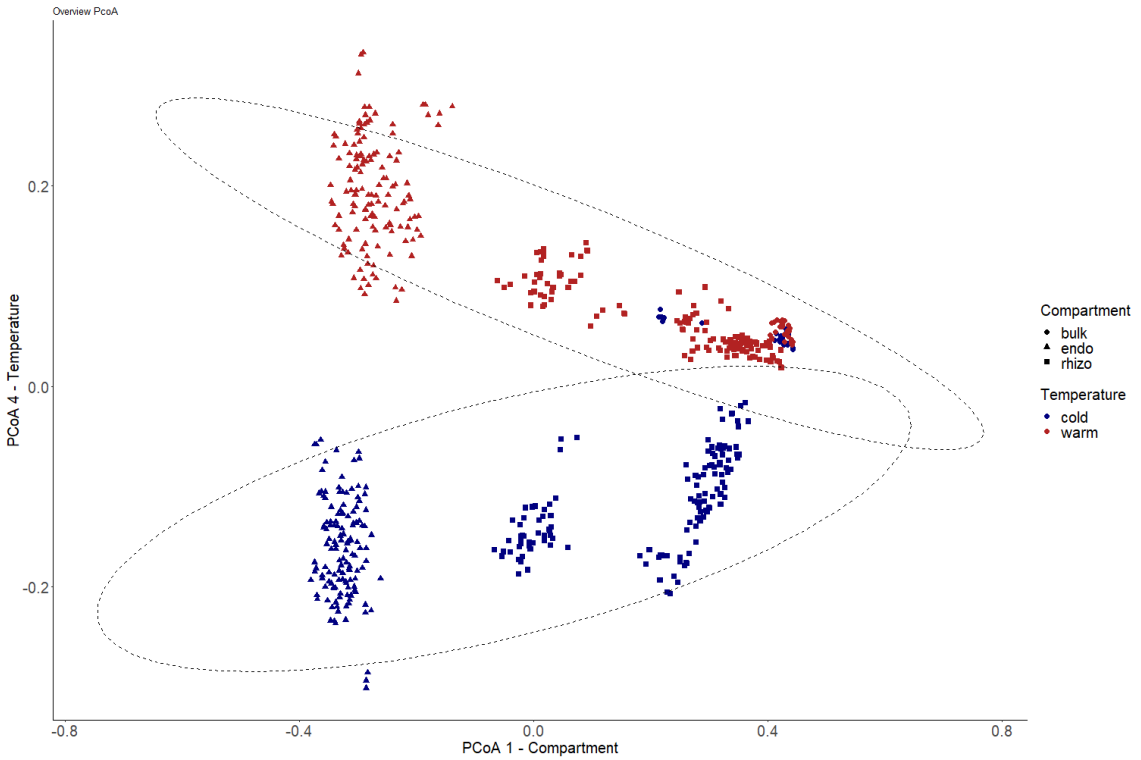
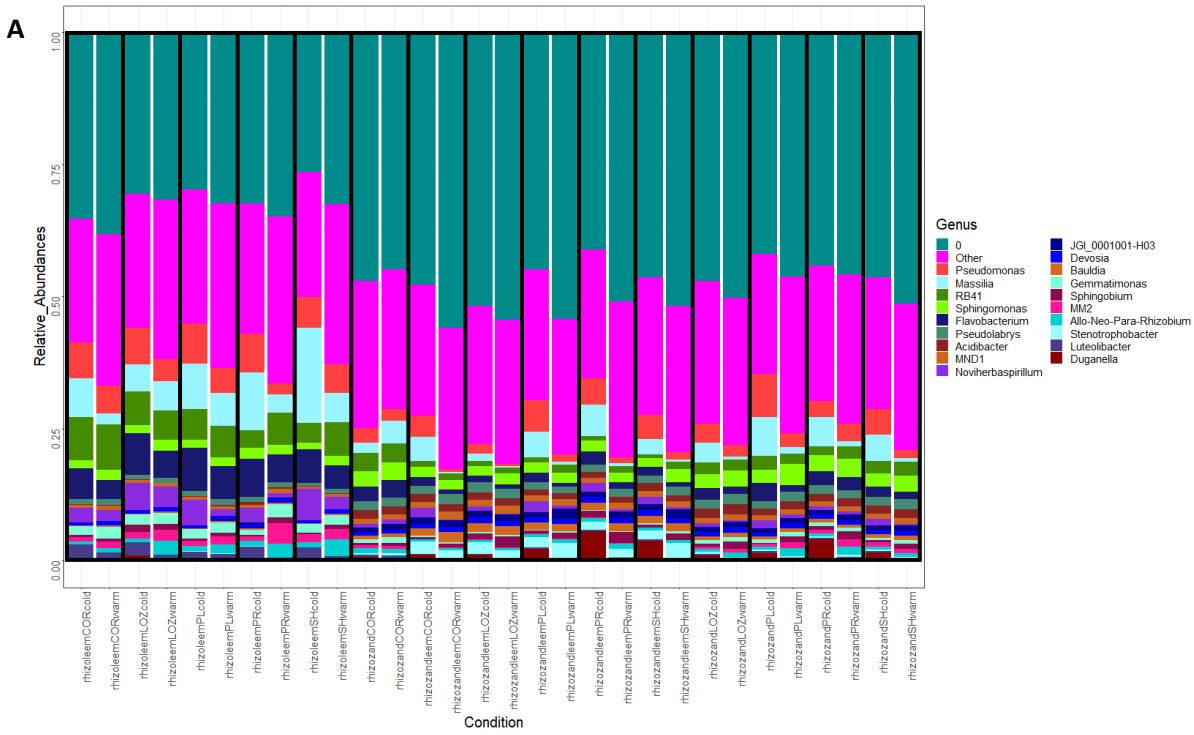


Figure 6. Principal Coordinates Analysis visualizing clustering of bacterial strains according to the soil compartment and the temperature. From the bulk soil to the endosphere there was a divergence of samples isolated in warm versus cold temperatures. The difference in temperature had the largest effect on the samples isolated from the endosphere.



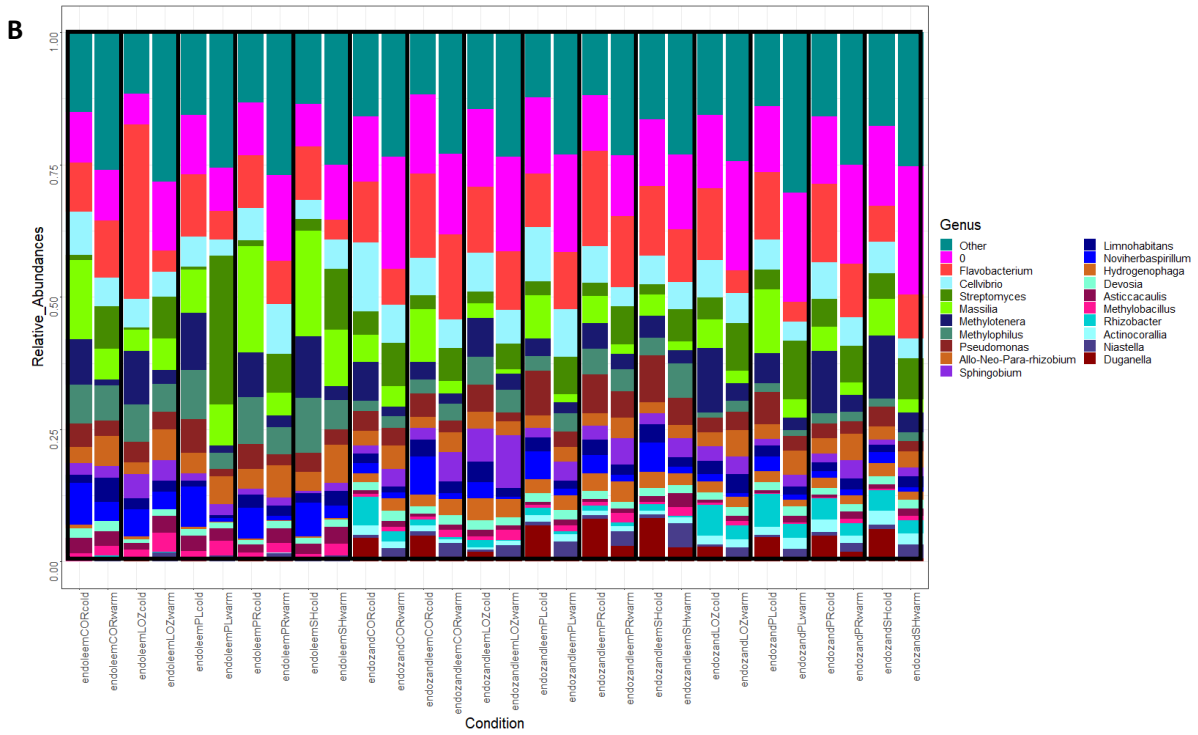


Figure 7. Relative abundances of bacteria isolated from the (A) rhizosphere and (B) endosphere of *L. sativa*. Differences in relative abundances according to temperature, cultivar and soil type are visualized. Multiple bacterial taxa are enriched or depleted in the cold.

1.8 *Flavobacterium* and its potential as a PGPR alleviating cold stress

The genus *Flavobacterium* belongs to the phylum Bacteroidetes and consists of Gram-negative, rod-shaped, and almost always strictly aerobic bacteria. It is the type genus within the family Flavobacteriaceae. It is a physiologically diverse genus, with a wide distribution, from the soil to saline and freshwater habitats. A large fraction of the species live in polar regions or cold environments and are psychrotolerant (Bernardet & Bowman, 2006), which means these bacteria have an optimal temperature of around 20 to 30°C, but they can also grow well around temperatures of 4°C. Furthermore, some *Flavobacterium* species are psychrophiles, which cannot grow in temperatures above 20°C. These psychrotolerant and psychrophile members have adaptations to make them cold-adapted: they may produce cold-active enzymes, cold shock proteins, and polyunsaturated branched-chain fatty acids.

Members belonging to the genus *Flavobacterium* can use malate as a carbon source, which is an important part of root exudates (Subramanian et al., 2016), making them excellent PGPR candidates. In fact, many members of this genus have been shown to possess growth-promoting characteristics. In *Brassica juncea*, *Flavobacterium sp*. stimulates root elongation, other members solubilize phosphorus or produce auxin. Other examples include *Flavobacterium sp.* improving drought tolerance in wheat (Gontia-Mishra et al., 2016), a consortium of *Flavobacterium sp.* and *Chitinophaga* suppressing fungal root disease (Carrión et al., 2019), *Flavobacterium sp.* TRM1 suppressing of disease development of *Ralstonia solanacearum* in tomato (Kwak et al., 2018), and *Flavobacterium sp.* playing an important role in the bioremediation of soils contaminated with hydrocarbon.

Many members of the genus *Flavobacterium* are known to be psychrotolerant, in addition the genus is enriched in the root microbiome of lettuce under cold conditions, moreover numerous members have been reported to possess growth-promoting characteristics. Ergo, members of the genus *Flavobacterium* are very promising candidates for increasing *L. sativa*'s crop productivity under low temperatures.

2 Objectives

The weakening of the polar vortex results in a greater chance of more frequent cold spells, which in turn severely impact crop productivity. Therefore, it is clear sustainable ways to improve crop growth are needed. One of these alternatives can be found in the form of PGPR. Many studies have been done to use these rhizobacteria as a way to promote plant growth and to help alleviate biotic and abiotic stresses. Yet, there is not that much known about PGPR alleviating cold stress and the mechanisms on how they do so. For this project, the growth-promoting effect of bacteria from the genus *Flavobacterium* will be investigated. More specifically, the effect these micro-organisms have on *L. sativa* when grown in cold conditions.

A first work package will focus on defining the root microbiome of *L. sativa*, using a previously isolated bacterial collection. These strains were collected from the endo- and rhizosphere of five *L. sativa* cultivars grown in low and control temperatures. The main focus will be on the identification of bacterial strains from the genus *Flavobacterium*, isolated from the endo- and rhizosphere from *L. sativa*, *Arabidopsis thaliana*, *Poa Annua*, and *Valerianella locusta*. Additionally, these strains will be compared to the top ten most abundant *Flavobacterium* ASV's from the endo- and rhizosphere enriched in the cold, identified in a preliminary study (**see 1.7**). The bacterial strains will be purified and identified via the sequencing of the 16S rRNA gene. Maximum Likelihood trees will be constructed for the collection of bacteria from the root microbiome of *L. sativa*, as well as for the bacteria from the genus *Flavobacterium*, to investigate the relatedness between species.

For the second work package, the focus will solely be on bacteria from the genus *Flavobacterium*. They will be tested in growth experiments to evaluate their potential to promote growth of juvenile *L. sativa* plants in the cold. The effect on growth will be assessed by measuring the plants' shoot fresh weight. Strains that promote growth could, in the future, be valuable in agriculture to increase the *L. sativa* turnover rate during the cold season.

In a third work package, the three most promising strains from the second work package will be screened against the other *Flavobacterium* strains to determine if they interact antagonistically, or synergistically. Ideally, a consortium will be built of strains that enhance each other's growth. To lower the number of possible combinations, antagonism assays are performed to discern which strains not to put together.

3 Materials and methods

3.1 WP 1 – Phylogenetic trees

3.1.1 Microbial strains

Bacterial strains were obtained from previously assembled in house collections, derived from the endosphere from *Lactuca sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*. Collections were stored at -70 degrees Celsius (°C).

3.1.2 Microbial growth conditions

All bacterial strains were grown on agar plates with Reasoner's 2A agar (R2A) medium (**see Appendix 8.2.1**), at room temperature (21°C), for purification and storage. Strains in liquid R2A were grown in a shaker at 28°C.

3.1.4 Purification

Contaminated bacterial strains were purified by picking and streaking single colonies on R2A agar plates for multiple rounds. When unsuccessful, colonies were grown overnight in liquid medium, followed by serial dilutions and plating of the bacterial suspension. After approximately six days of growth, colonies were picked.

3.1.5 Identification

After DNA extraction (**see Appendix 8.2.3**), the 16S rRNA gene was amplified via a Polymerase Chain Reaction (PCR) (**see Appendix 8.2.3**). After amplification, agarose gel electrophoresis was used to control the quality of the PCR product. Next, the PCR product was purified using HighPrep™ PCR (**see Appendix 8.2.3**). Finally, the DNA concentrations were checked using the Thermo Scientific Nanodrop and amplicons were sequenced using Sanger sequencing by Eurofins Genomics. Sequencing results were trimmed and merged in CLC main, and these sequences were BLASTed (Basic Local Alignment Search Tool) against a library from NCBI, for bacterial identification.

3.1.6 Phylogenetic trees

Maximum likelihood phylogenetic trees were assembled via Molecular Evolutionary Genetics Analysis X (MEGA X) and were further adjusted using Interactive Tree Of Life (iTOL). Any sequences too divergent to be aligned were removed. Phylogenetic trees with 16S rRNA gene sequences were made with 100 bootstraps, the phylogenetic tree with the V4 regions of the 16S rRNA gene sequences was made with 1000 bootstraps.

2. WP2 – Growth promotion experiments

3.2.1 Plant material

Butterhead lettuce seeds from the commercial *L. sativa* variety May Queen (Aveve) were used. Seeds were stored at 14°C.

3.2.2 Bacterial inoculum

For growth experiments, strains were grown in liquid R2A medium in a shaker at 28°C one day before inoculation. Subsequently, the bacterial suspension was centrifuged for 10 minutes at 3000 g, the pellet was resuspended in Phosphate Buffered Saline (PBS, 8 g/L NaCl, 0,2 g/L KCl, 1,44 g/L Na_2HPO_4, 0,24 g/L KH_2PO_4, pH 7,4). The optical density (OD) was measured, and the solutions were diluted, until an OD of 0,01 was reached.

3.2.3 Growth experiments

Seeds were placed in Erlenmeyer flasks with bacterial inoculum (**as described in 3.2.2**) or a control treatment treatment (30 mL of PSB). The Erlenmeyer flasks were shaken for two hours, after which the seeds were sown into pots loosely filled with soil and saturated with water. Trays, containing 15 pots, were covered with saran wrap and placed in a warm growth chamber (21°C, 16 hours daylight) for four days. Subsequently, the saran wrap was removed, plants from the corner were transferred to empty pots and the trays were moved to a cold growth chamber (14°C, 16 hours daylight).

3.2.4 Phenotypical analysis of *Lactuca sativa* shoots

At 32 days post inoculation an analytical balance was used to determine the fresh weight of shoots. Any plants with #L (all cotyledons and leaves visible with the naked eye) smaller than 5, were excluded from further analysis, due to being extremely small.

3.2.5 Statistical analysis

Outliers, values larger (or smaller) than the average plus (or minus) the interquartile range, were removed. Two-sided heteroscedastic student t-tests were performed in R to detect differences between plants treated with a bacterial suspension versus mock treated plants.

3.3 WP 3 – Antagonistic interactions

3.3.1 Antagonism assays

Bacterial strains were tested against each other for antagonistic interactions. The bacterial strains used in the experiment were grown in liquid medium (**as described in 3.1.2**) the night before use. Next, all bacterial suspensions were diluted to an OD of 0,5. Each time, 100 µL of a suspension was mixed with 6 mL of 75% heated R2A growth medium (11,25 g/L agar compared to 15 g/L). This mixture was poured on top of a 20 mL agar plate containing normal growth medium. On one plate six different bacteria were spotted by dripping 10 µL of each strain. Two repeats were done for each strain. After five days the results were analyzed by measuring the diameter of the original spot, the diameter of a spot if enlarged, and the diameter of the spot plus halo, an area around a spot without bacterial growth. The size of the halo was calculated as the diameter of the spot plus halo, minus the diameter of the spot, divided by two. The size of enlargement was calculated as the diameter of the enlarged spot, minus the diameter of the original spot, divided by two.

4 Results

4.1 WP1 - Phylogenetic trees

4.1.1 Bacterial collection: root microbiome *L. sativa*

The bacterial collection isolated from the endosphere of *L. sativa* contained approximately 300 strains, whether or not pure. After intensive purification, a total of 145 bacterial strains belonging to 36 unique genera were successfully identified via sequencing of the 16S rRNA gene. Most isolated bacteria, 71,03 percent (%), belonged to the phylum of the Proteobacteria. Other common phyla were the Bacteroidetes (15,17%), the Actinobacteria (10,34%), and finally, a few bacteria were a part of the Firmicutes phylum (3,45%). All purified bacteria belonged to one of these four phyla, which consisted out of seven classes in total (**Figure 8**). The five most common genera in this purified collection were *Variovorax*, *Pseudomonas*, *Agrobacterium*, *Enterobacter* and *Pedobacter* (**Figure 9**). In total, 36 unique genera were identified. When not taking into account the number of strains within a genus, the phylum of the Proteobacteria, remained the most represented, with 24 out 36 genera (66,67%), and the most common class within this phylum were the Alphaproteobacteria, with 11 genera (30,56%).

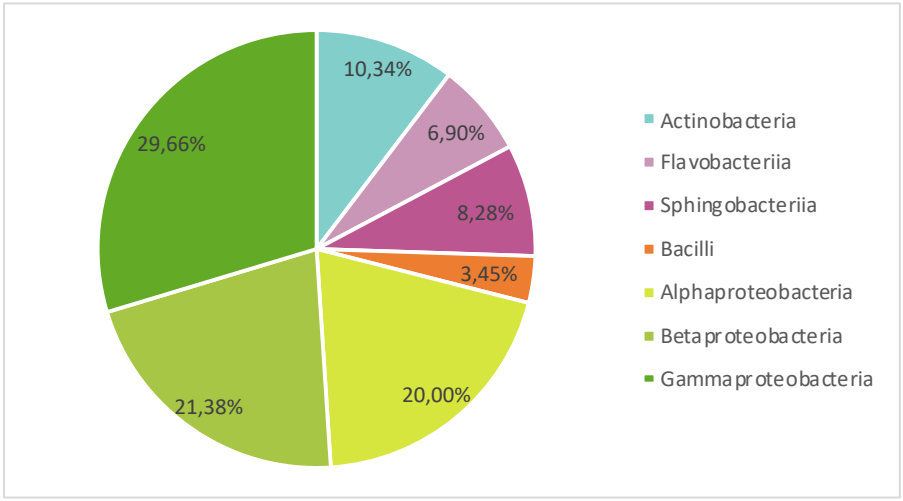


Figure 8. Overview of relative abundances of classes of purified bacterial strains. In total 145 bacterial strains were successfully purified. These strains belonged to 7 classes. The three most common classes all belong to the phylum of the Proteobacteria. The classes Flavobacterii and Sphingobacterii both are a part of the Bacteroidetes. The class Actinobacteria has the same name as its phylum and the Bacilli are a class within the Firmicutes.

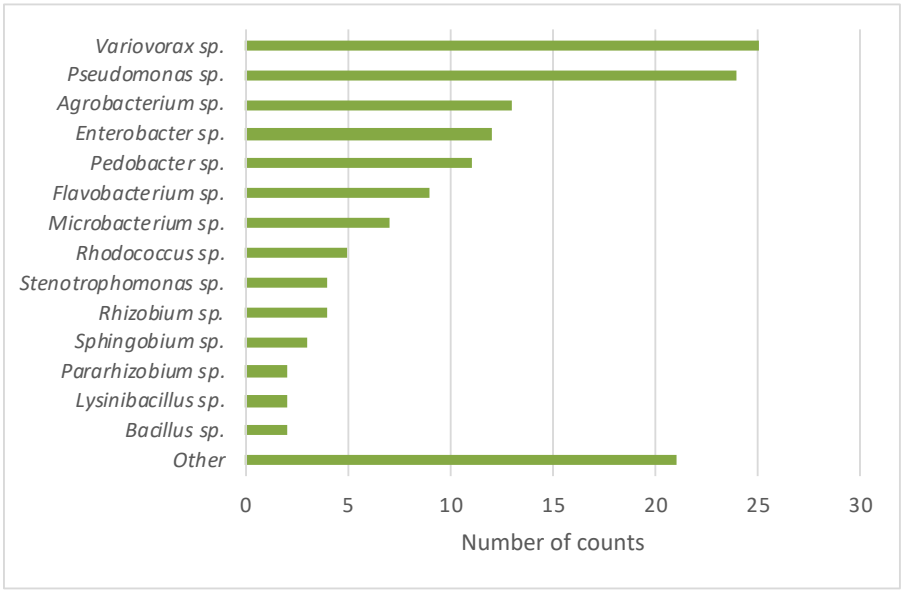


Figure 9. Overview of absolute abundances of purified bacterial strains. In total 145 bacterial strains were purified. These strains belonged to 36 different genera. The most prominent genera were *Variovorax*, *Pseudomonas* and *Agrobacterium*.

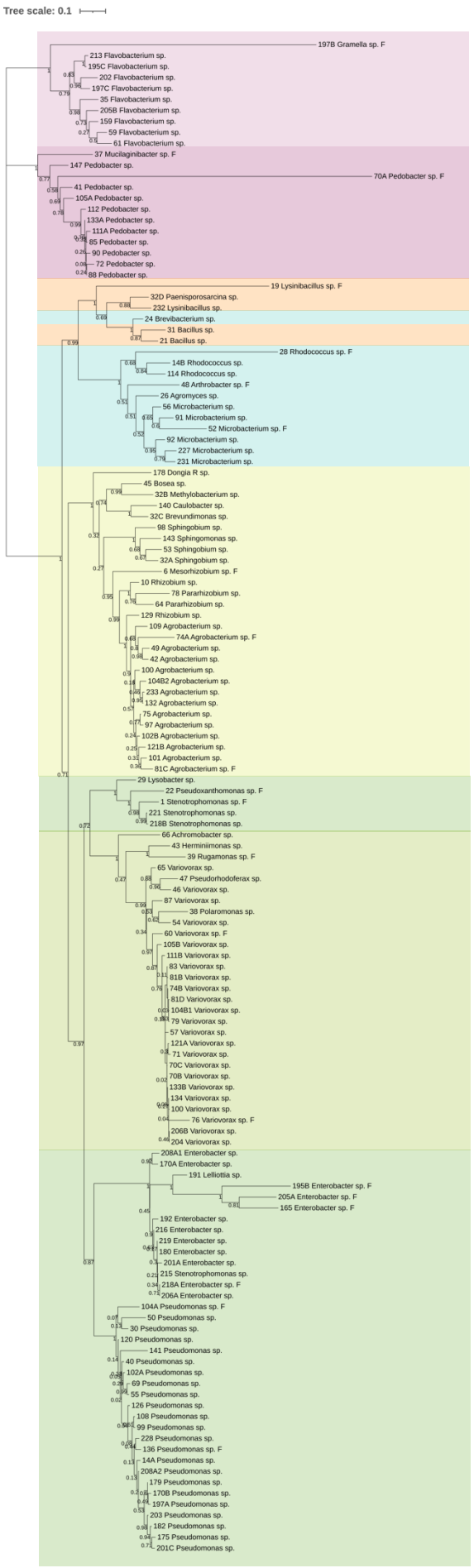


Figure 10. Maximum Likelihood tree of bacteria from the root endosphere of *L. sativa*. Bacteria were isolated from the endosphere of *L. sativa* plants, purified and identified via 16S rRNA gene. The values on each branch indicate the bootstrap values (100 bootstraps).

A Maximum Likelihood tree with the 16S rRNA gene sequences of all purified bacterial isolates was constructed via Mega X to investigate their phylogenetic relatedness to each other (**Figure 10**). The strains from the same classes all clustered together, with two exceptions. The genus *Brevibacterium* is a part of the Actinobacteria, but in this tree, it clustered together with strains from the class Bacilli. Also, one cluster of Gammaproteobacteria was more closely related to the Betaproteobacteria than to the other Gammaproteobacteria, both classes, however, do belong to the same phylum.

4.1.2 Bacterial collection: *Flavobacterium*

A Maximum Likelihood tree was built to evaluate whether the *Flavobacterium* strains isolated from the endosphere of *L. sativa* would all cluster together, or if they would be spread out amongst other *Flavobacterium* strains isolated from different plants (*Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta)*. Additionally, both some known species of the genus *Flavobacterium* and an outgroup of *Flexibacter* were added to the phylogenetic tree (**Figure 11**).

The *Flavobacterium* strains isolated from the *L. sativa* endosphere, did not all cluster together. Some strains, *Flavobacterium spp.* (61, 159, 59, and 205B), and *Flavobacterium spp.* (202B and 197C) did cluster together, because they were highly likely very similar, but *Flavobacterium* strains of other species also appeared closely related to them. As described in 4.2, three bacterial strains were found to have the most promising growth-promoting characteristics. These strains are *Flavobacterium spp.* (PA37, PA116, and PA403A). These three isolates did also not cluster together and are spread throughout the tree.

A second phylogenetic tree with the isolated *Flavobacterium* strains was made, where the top ten most abundant *Flavobacterium* isolates from the endo- and the rhizosphere of *L. sativa* plants grown in the cold were included (**Figure 12, see 1.7**). This was done to see if any of the purified *Flavobacterium* strains clustered together with the very abundant strains in the microbiome at low temperatures. When comparing both phylogenetic trees of *Flavobacterium* strains, it is very noticeable that the second tree, has much shorter branches, of practically length zero, towards the bottom of the tree, and many strains moved places, because only the V4 region of the 16S rRNA gene was used instead of the whole 16S rRNA gene.

The most abundant *Flavobacterium* strains did not cluster together according to the soil compartment they originated from, the endo- and rhizospheric bacteria were mixed in the phylogenetic tree. Many of these strains clustered together in a separate clade, apart from the purified *Flavobacterium* isolates. Some, however, did cluster together with the purified strains. For instance, *Flavobacterium sp.* (35A), was very closely related to the most abundant *Flavobacterium* strain from the endosphere and the fourth most abundant one from the rhizosphere. *Flavobacterium sp.* (2VL42), was in turn closely related to the fourth most prominent *Flavobacterium* strain from the endosphere.

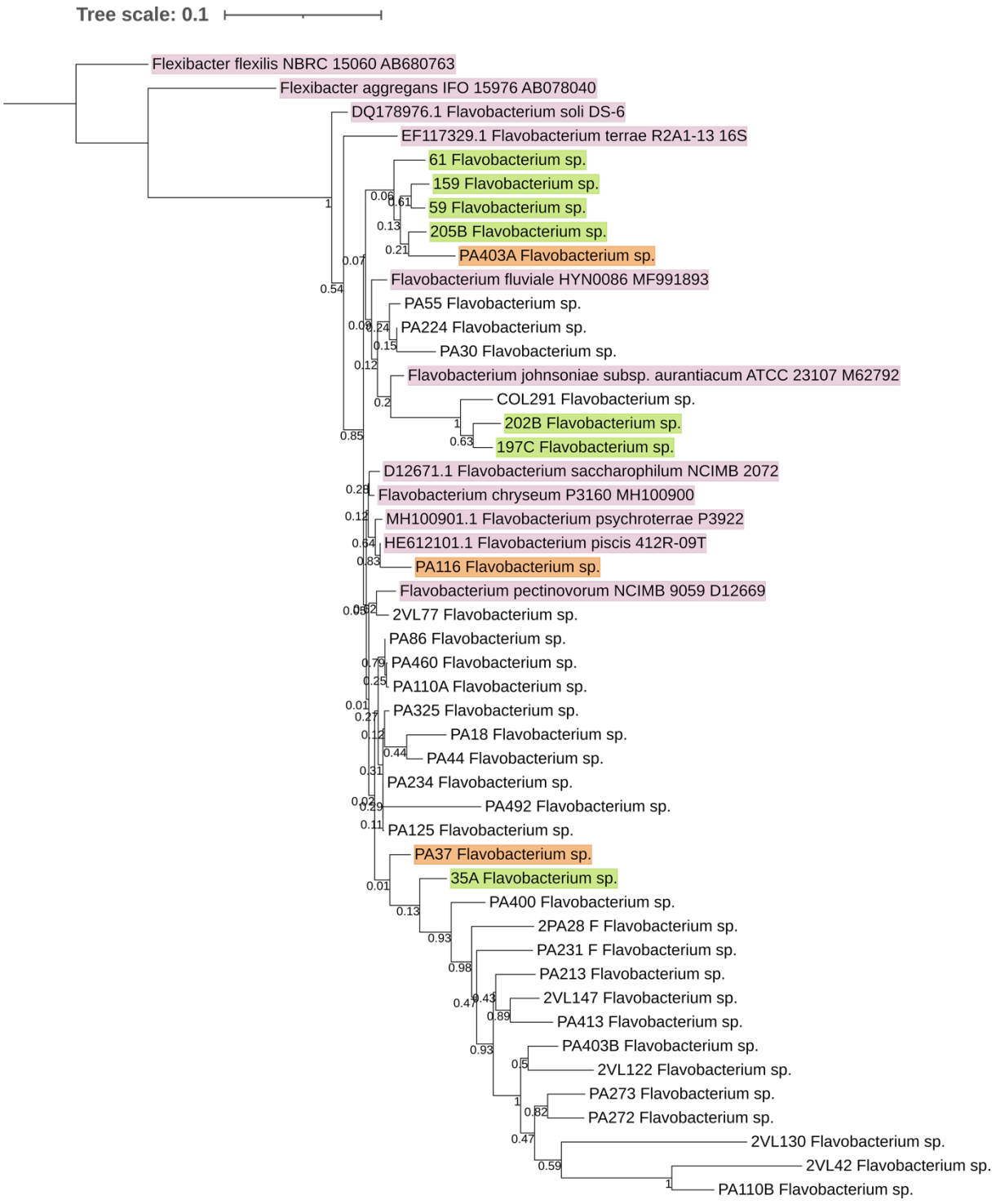


Figure 11. Maximum Likelihood tree of bacteria from the genus *Flavobacterium*. Bacteria were isolated from the endosphere of *L. sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*. The strains in green were isolated from the endosphere of *L. sativa*. In pink, some known different *Flavobacterium* species are shown, and also an outgroup of the related genus *Flexibacter* was added to the phylogenetic tree. In orange, the three most promising PGPR are shown (**as described in 4.2**). The numbers at each node indicate the bootstrap values (100 bootstraps).

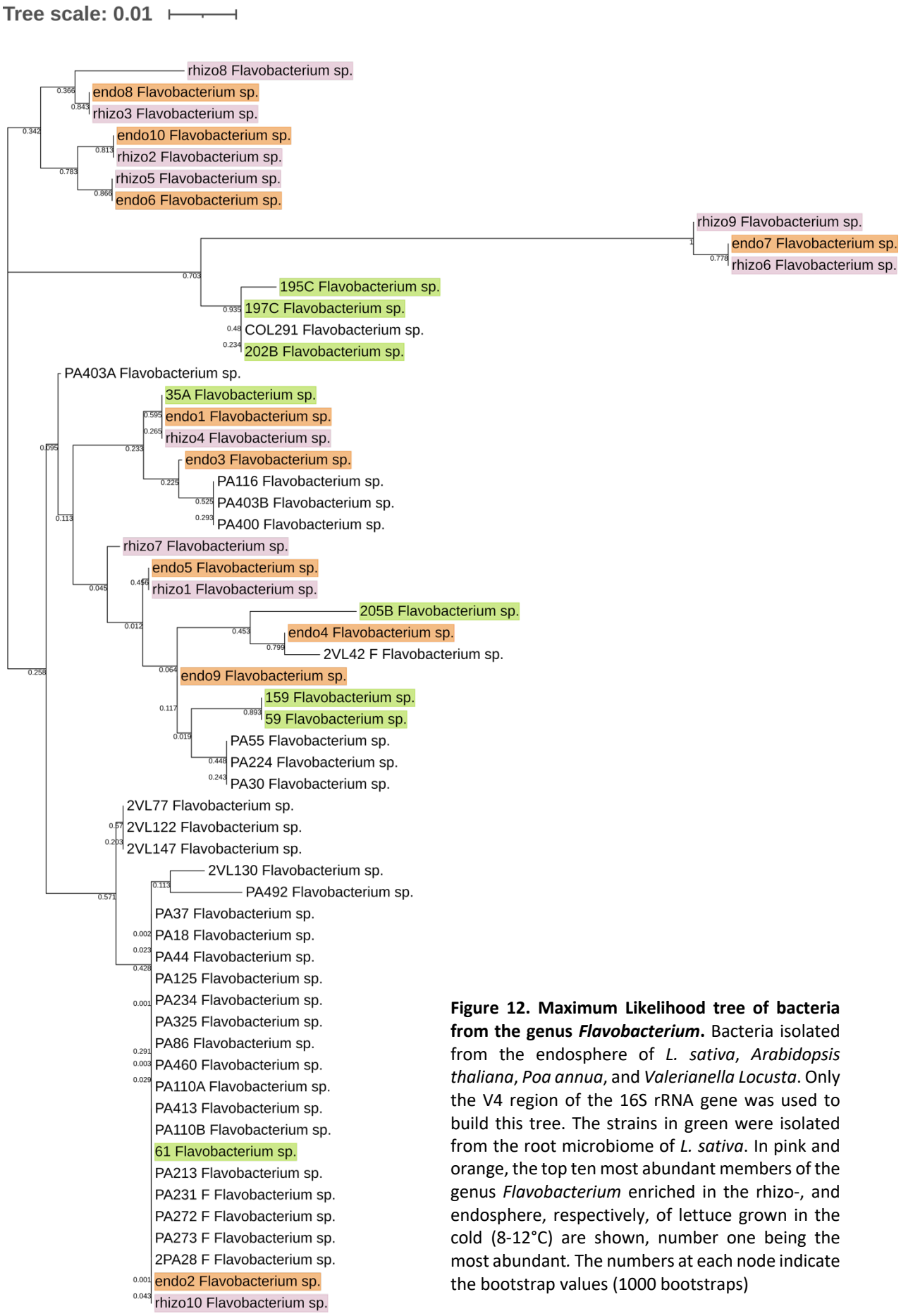


Figure 12. Maximum Likelihood tree of bacteria from the genus *Flavobacterium*. Bacteria isolated from the endosphere of *L. sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*. Only the V4 region of the 16S rRNA gene was used to build this tree. The strains in green were isolated from the root microbiome of *L. sativa*. In pink and orange, the top ten most abundant members of the genus *Flavobacterium* enriched in the rhizo-, and endosphere, respectively, of lettuce grown in the cold (8-12°C) are shown, number one being the most abundant. The numbers at each node indicate the bootstrap values (1000 bootstraps)

4.2 WP2 - Growth promotion experiments

To determine which substrate gave the best growth performance for *L. sativa* in the cold, three soil types were tested on mock treated seeds: sand, sand - potting soil (ratio 1:1) and potting soil (results not shown). Plants did not grow as well in sand as in the other two soil types, that did not differ significantly from each other. Since pure potting soil was the most straightforward substrate to use, all growth experiments were completed using it.

A selection of strains from the genus *Flavobacterium*, isolated from the root endosphere of *L. sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta,* were screened for their growth promoting effects on *L. sativa* grown at low temperatures (14°C). Seeds were either inoculated with a bacterial suspension of OD 0,01 abs or mock treated. Very often, more than three plants did not germinate which resulted in N < 15. To prevent N from becoming too low, two trays of each treatment were used in further experiments. Trays were placed in a warm growth chamber (21°C, 16 hours daylight) for four days, to allow seeds to germinate. Next, trays were moved to a cold growth chamber (14°C, 16 hours daylight) for 28 days. Thereafter, the effect of bacterial inoculation on the number of cotyledons and leaves present and forming, visible with the naked eye (#L), and shoot biomass was investigated.

After one biological repeat of each selected strain, two more biological repeats were performed for the three strains which showed the most promising growth promoting results. These three strains were *Flavobacterium sp.* (PA37), *Flavobacterium sp.* (PA116) and *Flavobacterium sp.* (PA403A). For each of these strains, in every biological repeat, the plants inoculated with a bacterial suspension of OD 0,01 abs differed significantly from the plants that were mock treated. Besides being visibly larger, and having a higher shoot weight, no obvious morphological differences, for instance a difference in color, could be observed.

L. sativa plants treated with *Flavobacterium sp.* (PA37) differed significantly from plants that received a mock treatment in all biological repeats (**Figure 13, Figure 14**). For the first biological repeat, there was an increase in shoot fresh weight of 82,32%, ($p < 0,001$), upon bacterial inoculation. For the second and the third biological repeat, this increase was lower, 27,24% and 24,08% respectively, but still significant ($p < 0,05$). #L also increased slightly upon treatment of plants with *Flavobacterium sp.* (PA37), but this increase was only significant in the first biological repeat with 13,17%, significant ($p < 0,001$). The other two repeats showed a non-significant increase in #L of 3,96% and 1,35%, respectively.

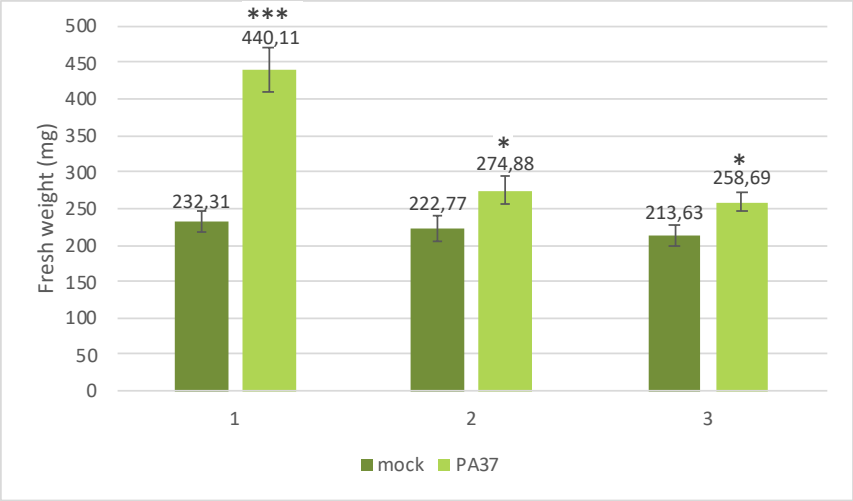


Figure 13. Shoot fresh weight (mg) of *L. sativa* plants grown in the cold. Comparison between mock treatment and seeds inoculated with *Flavobacterium sp.* (PA37). Plants were harvested 32 days after inoculation. Error bars indicate the standard error. '*', '**', and '***' represent the significance levels p<0,05, p<0,01 and p<0,001, respectively. (N >= 15)

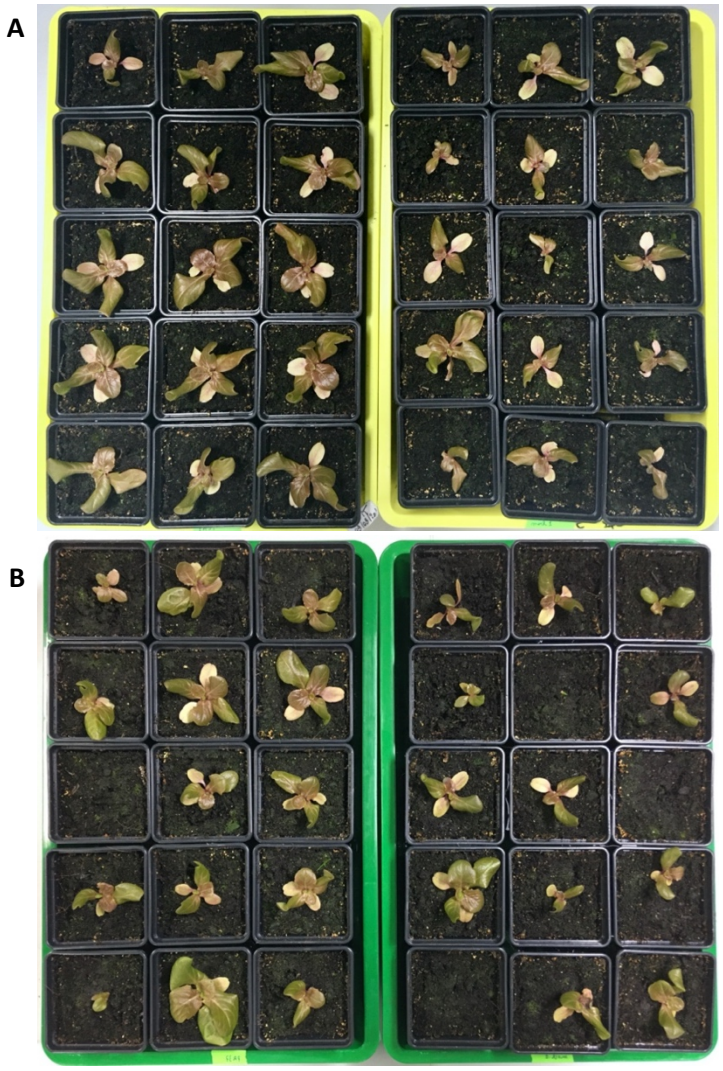


Figure 14. Growth promoting effect of *Flavobacterium sp.* (PA37) (left) versus control (right) *L. sativa* plants. Seeds were inoculated with a bacterial suspension with OD 0,01. Plants were harvested 32 days after inoculation, 4 days at 21°C and 28 days at 14°C. Not all plants included in statistical analysis are shown. (A) Part of first biological repeat (B) Part of second biological repeat.

Plants treated with a bacterial suspension of *Flavobacterium sp.* (PA116) differed significantly from control plants in each biological repeat (**Figure 15, Figure 16**). For the first biological repeat there was an increase in shoot fresh weight of 39,78% ($p < 0,05$), in the second biological repeat shoot weight increased 35,14% ($p < 0,01$), and for the third repeat this increase was 21,69% ($p < 0,05$). There was a trend of #L being larger for plants treated with *Flavobacterium sp.* (PA116), the increases for repeat

one to three were respectively 5,57%, 6,52%, and 1,75%. Only the increase in #L of the second biological repeat was significant ($p < 0,05$).

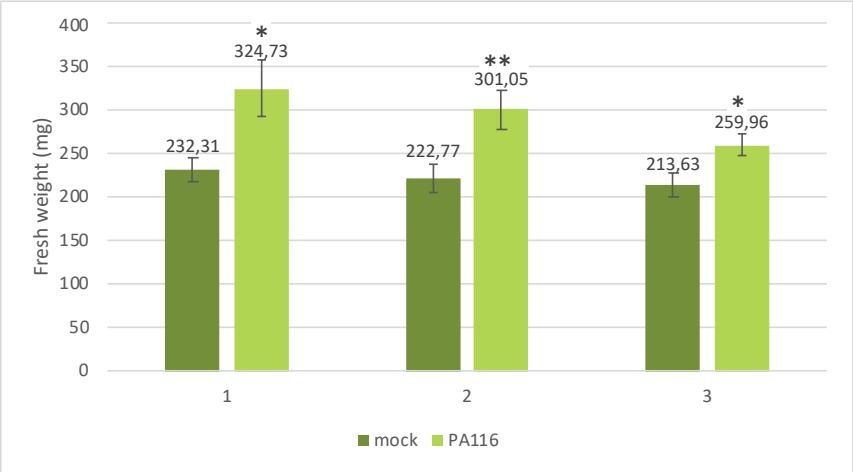


Figure 15. Shoot fresh weight (mg) of *L. sativa* plants grown in the cold. Comparison between mock treatment and seeds inoculated with *Flavobacterium sp.* (PA116). Plants were harvested 32 days after inoculation. Error bars indicate the standard error. '*', '**', and '***' represent significant levels $p<0,05$, $p<0,01$ and $p<0,001$, respectively.

Figure 16. Growth promoting effect of *Flavobacterium sp.* (PA116) (left) versus control (right) *L. sativa* plants. Seeds were inoculated with a bacterial suspension with OD 0,01. Plants were harvested 32 days after inoculation, 4 days at 21°C and 28 days at 14°C. Not all plants included in statistical analysis are show. (A) Part of first biological repeat (B) Part of second biological repeat.

L. sativa plants treated with a bacterial suspension of *Flavobacterium sp.* (PA403A) differed significantly from mock treated plants in each biological repeat (**Figure 17, Figure 18**). The increases in shoot fresh weight for biological repeat one to three were respectively 60,85% ($p < 0,01$), 38,47% ($p <$

0,05), and 21,29% ($p < 0,05$). Also, upon treatment *Flavobacterium sp.* (PA403A) there was a trend of a higher #L compared to the control plants, which was significant in the first and third repeat. #L increased with 10,39% ($p < 0,05$), 1,11%, and 11,47% ($p < 0,05$), for repeat one to three respectively.

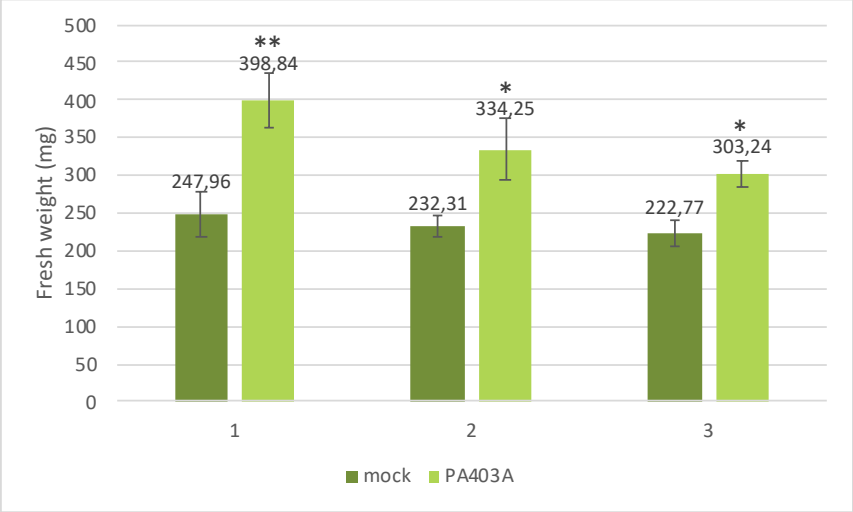


Figure 17. Shoot fresh weight (mg) of *L. sativa* plants grown in the cold. Comparison between mock treatment and seeds inoculated with *Flavobacterium sp.* (PA403A). Plants were harvested 32 days after inoculation. Error bars indicate the standard error. '*', '**', and '***' represent significant levels $p<0,05$, $p<0,01$ and $p<0,001$, respectively.
(N >= 15)

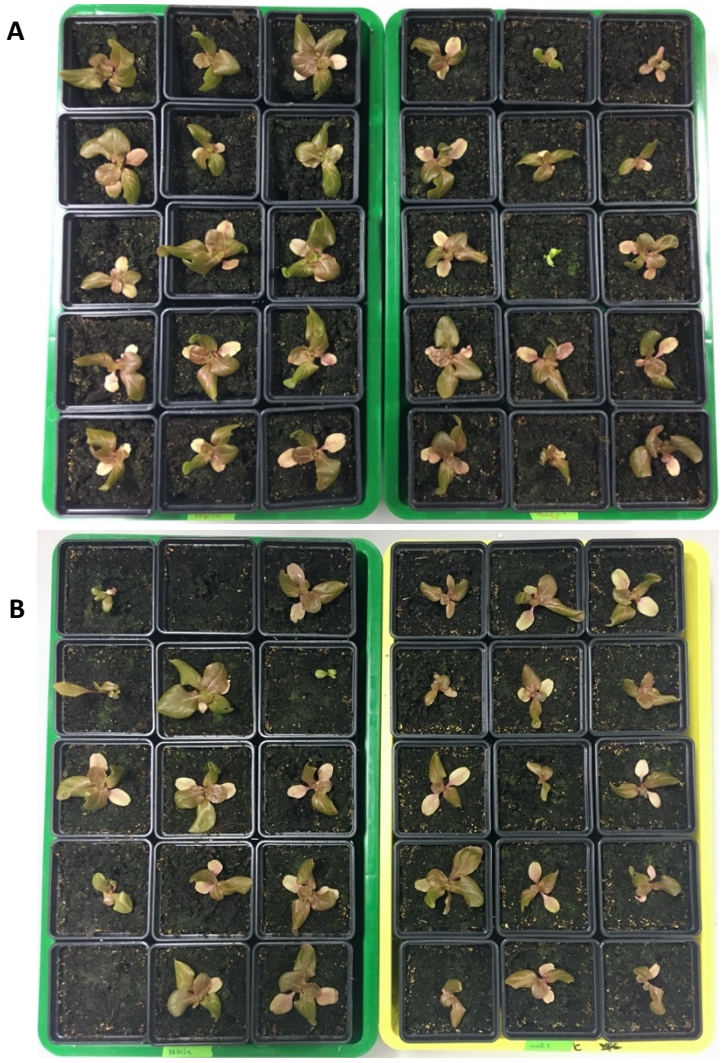


Figure 18. Growth promoting effect of *Flavobacterium sp.* (PA403A) (left) control (right) *L. sativa* plants. Seeds were inoculated with a bacterial suspension with OD 0,01. Plants were harvested 32 days after inoculation, 4 days at 21°C and 28 days at 14°C. Not all plants included in statistical analysis are shown. (A) Part of first biological repeat (B) Part of second biological repeat.

4.3 WP3 – Antagonistic interactions

For this project the focus was on finding strains of the genus *Flavobacterium* that could act as PGPR to promote *L. sativa* growth in the cold, as described in the previous paragraphs. A next step was to find out which do and do not act antagonistically with each other. Therefore, the three most promising *Flavobacterium* strains were tested against a selection of purified strains of this genus collected from the endosphere of various plants (**see Appendix 8.1.2**).

Antagonistic interactions were observed by looking for haloes, which are inhibition zones without bacterial growth around spots of different bacterial strains. Haloes were quantified by measuring the diameter of the spot plus the halo, minus the diameter of the spot, divided by two. However, these results were often hard to interpret, but some general trends could be observed. Firstly, haloes around spots, or enlargement of spots often appeared for the same bacterial strains on different underlayers. Secondly, some strains did not grow well on almost any underlayer. Thirdly, on some lawns all spots grew better or worse than on other lawns.

In total, haloes could be observed around 44,18% of spots on a lawn of *Flavobacterium sp.* (PA37), around 44,18% of spots on a lawn of *Flavobacterium sp.* (PA116), and around 26,47% of spots on a lawn of *Flavobacterium sp.* (PA403A). The haloes were on average 21 millimeters (mm), 18 mm and 24 mm for *Flavobacterium spp.* (PA37, PA116, PA403A), respectively. 32,35% of the strains spotted on the three different lawns resulted in at least halo-formation on two of those lawns. A halo could be observed around all spots of *Flavobacterium sp*. (2VL147) on the three different lawns.

Flavobacterium sp. (PA37) showed an intermediate lawn growth compared to the other two strains. In contrast, lawn growth of *Flavobacterium sp.* (PA116) was pronounced, while lawn growth of *Flavobacterium sp.* (PA403A) was less dense (**Figure 19**). Spots could not grow as well on very dense lawns. A spot was classified as 'not growing well', when it was less thick, and the color was whiter than the common yellow color. Therefore, a spot that expanded could still be classified as 'not growing well' (**Table 1**).

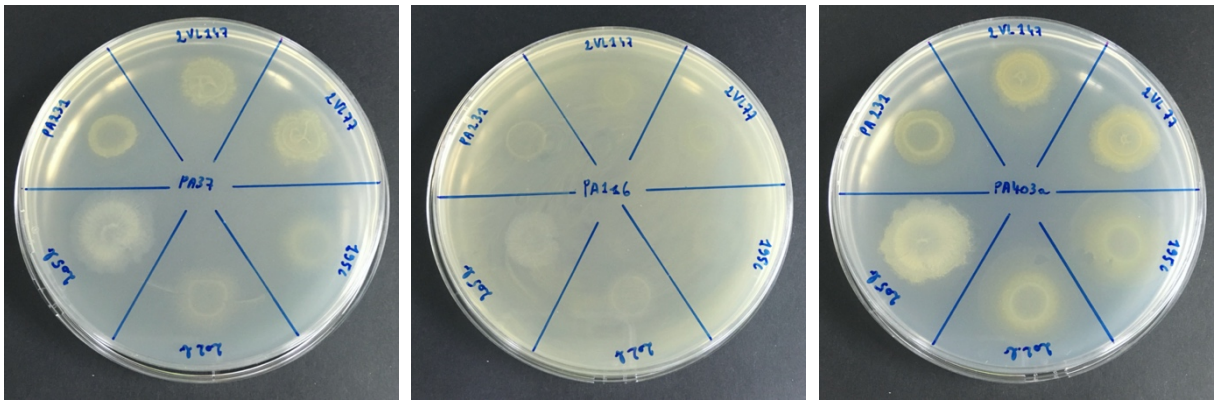


Figure 19. Comparison of the growth of lawns of *Flavobacterium spp.* (PA37, PA116 and PA403A). Strain *Flavobacterium sp.* (PA116) showed a distinctively denser lawn compared to the other two strains, while strain *Flavobacterium sp.* (PA403A) showed a distinctively less dense lawn. This also correlated with how well the spot could grow. The less dense the underlayer, the better a spot could grow.

A lawn of *Flavobacterium sp.* (PA116) resulted in most cases in one of two possibilities. Either the spot could not grow very well, as mentioned before, or the spot showed the formation of a halo (**Figure 20**).

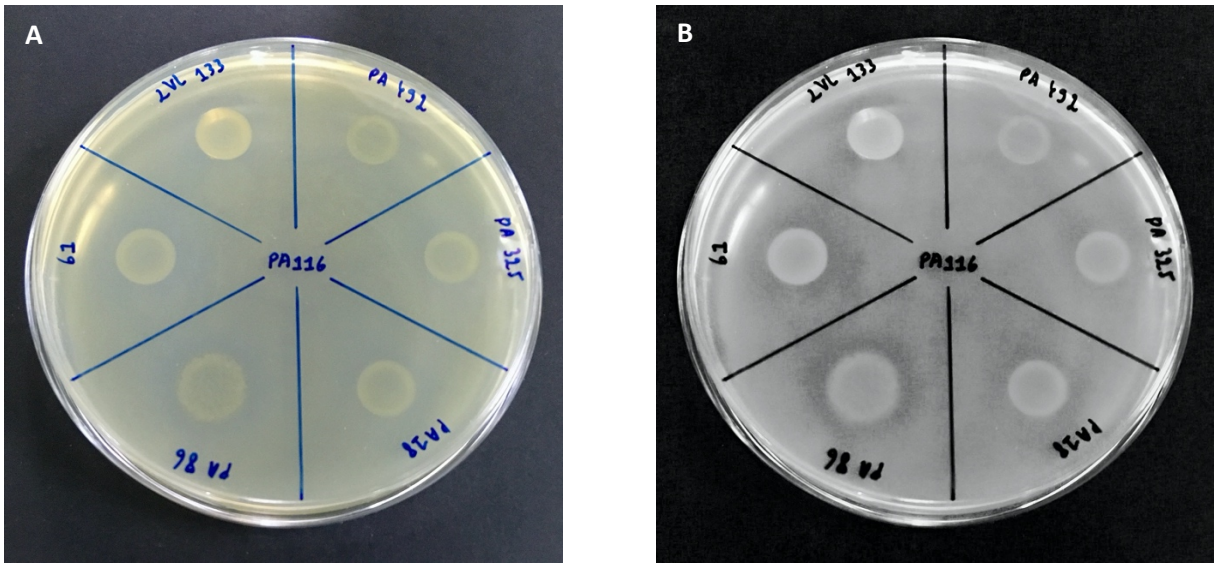


Figure 20. Antagonistic interactions between bacterial *Flavobacterium sp.* (PA116) and other *Flavobacterium sp.* strains. Around all spots haloes could be observed. Some spots showed clearer haloes than others. Often, instead of an area without any bacterial growth, the lawn was less dense around the spots. (A) and (B) show the same image, however in (B) the light intensity and saturation was changed to make the haloes appear clearer.

Almost all spots could grow well on a lawn of *Flavobacterium sp.* (403A) (**Figure 21**). This was especially clear for strain *Flavobacterium sp.* (159), which had a deep yellow color growing on top of the bacterial underlayer. When growing on top of other lawns, the color was white, and the spot was thin, almost see-through.

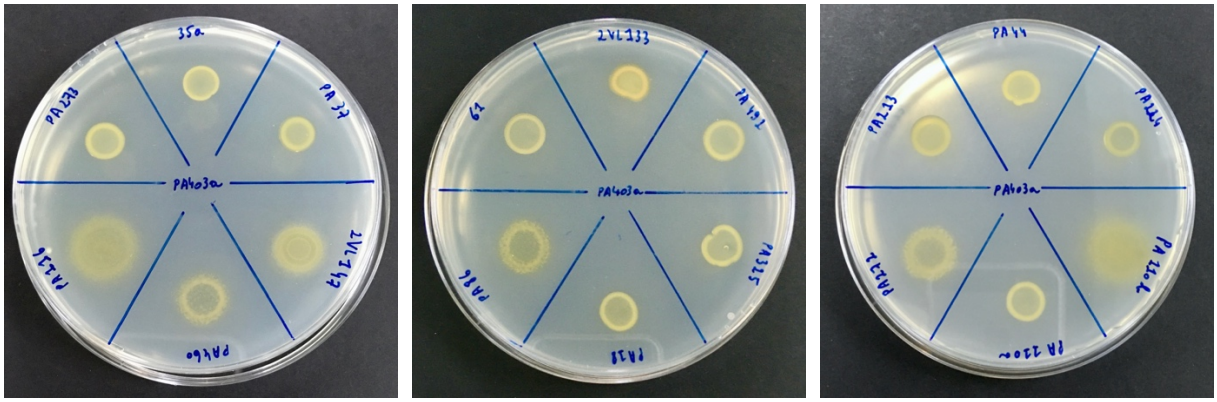


Figure 21. Antagonistic interactions between *Flavobacterium sp.* (PA403A) and other *Flavobacterium sp.* strains. No haloes could be observed. All spots had a distinctly yellow color compared to when grown on top of a different strain.

	Flavobacterium sp. (PA37)			*Flavobacterium sp.* (PA116)			*Flavobacterium sp.* (PA403A)	
Strain	Size halo	Size enlargement	Strain	Size halo	Size enlargement	Strain	Size halo	Size enlargement
PA460	++++		PA86	+++		PA231	++++	+++
PA272	++++		PA37	+++		2VL147	++++	+++++
PA86	+++		PA492	++		PA30	+++	++
PA55	+++	+	2VL133	++		PA234	+++	
195C	+++	+++	PA18	++		PA37	++	
PA231	+++	++	PA44	++		PA224	++	
2VL147	++	+++	PA325	++		2VL77	++	++++++
2VL130	++	++	PA272	++	++	2VL130	++	++
202B	++	+++	61	++		PA487	++	
PA413	++	++	PA224	++		195C		++++++
PA116	++	++++	PA213	++		202B		++++++
2VL42	++	+++++	COL291	++		205B		++++++
205B	+	++++	2VL147	+	++	PA116		++++++
2VL77	+	+++	195C	+++		PA403B		++++++
COL291	+	+	202B	++		2VL42		++++++
PA400		++++	PA110B		+++++	PA400		++++++
PA403B		++++	2VL42		+++	COL291		+++++
PA110B		+++	PA403B		+++	PA55		+++
61			205B		+++	PA110B		+++
159			PA460		+++	PA86		++
35A			PA400		+++	PA460		++
PA403A			2VL77		++	PA272		++
			PA55		++			
			2VL130		+			
			PA30		+			
			PA413		+			
			PA231		+		**Legend**	
			35A				+	<= 1 mm
			159				++	> 1 mm
			PA37				+++	> 2 mm
			PA234				++++	> 3 mm
			PA403A				+++++	> 4 mm
			PA487				++++++	> 5 mm

Table 1. Overview of antagonistic interactions and enlarged spots. The different bacterial strains are ranked from largest to smallest halo, then largest to smallest spot without a halo, and lastly spots that did not grow well are mentioned. The size of a halo was calculated by subtracting the diameter of the spot from the diameter of the halo and dividing it by two. If a halo occurred around a spot grown on two different lawns it is shown in darker green, a spot was present around *strain Flavobacterium sp.* (2VL147) on all three lawns and is indicated in very dark green. How much the spot expanded was calculated by subtracting the diameter of the original spot from the diameter of the enlarged spot and dividing it by two. The sizes of the haloes and expansions of spots were converted to symbols, as can be seen in the legend. Spots that did not grow well are indicated by an orange box.

5 Discussion

5.1. Phylogenetic trees

5.1.1 Bacterial collection: root microbiome *L. sativa*

A Maximum Likelihood tree was constructed using the 16S rRNA gene sequences of the purified bacterial strains from the endosphere of *L. sativa*. This phylogenetic tree was made to investigate which strains occur in the endosphere of *L. sativa* and to investigate their relatedness to each other. All bacteria were part of one of four phyla, namely the Proteobacteria, the Bacteroidetes, the Actinobacteria, and the Firmicutes. This was expected, as these phyla are often dominant in the rhizo- and endosphere of plants (Lagos et al., 2015).

Strains from the same genus clustered together, but sometimes, the branch lengths were almost zero. It is thus highly likely that some of the 145 successfully isolated bacteria are duplicates and that some strains might not be unique. This seems to be the case for the five most common genera, namely *Variovorax*, *Pseudomonas*, *Agrobacterium*, *Enterobacter*, and *Pedobacter*. Strains of these genera were highly likely the main sources of contamination. However, even without taking the number of species within a genus into account, the Proteobacteria remained the most common. This phylum is often slightly enriched in the rhizosphere compared to the bulk soil, and even more so in the endosphere, for many different plant species. Within this phylum, the class Alphaproteobacteria was often the most prominent (Hamonts et al., 2018; Poudel et al., 2019; Trivedi et al., 2020). The abundance of Proteobacteria in the rhizo- and endosphere can partly be explained by the fact they are good root colonizers, fast-growing species, reacting quickly to carbon sources (Lagos et al., 2015; Mitter et al., 2017).

Because the strain [*Brevibacterius*] *sp.* (24) was situated in an unexpected place in the phylogenetic tree (**Figure 10**), it was BLASTed again, to check for any mistakes. The strain was found to have the same percentage similarity to multiple *Bacillus sp.* as to [*Brevibacterius*] *sp.*, according to the NCBI database. Hence, another database, Ez BioCloud, was used to identify the sequence. Here, the highest similarity was 89,44% to a *Bacillus sp.*, thus it is more likely that this strain belongs to the class Bacilli in the phylum of the Firmicutes, and more specifically the genus *Bacillus*, which the phylogenetic tree also indicates. The second unexpected part of the phylogenetic tree is a cluster with strains from the phylum Gammaproteobacteria that was situated by the Betaproteobacteria. The sequences of this cluster were also re-identified via Ez Biocloud. These sequences all remained within the same genera, with high similarities varying between 93% and 100%. Also, a few branches were longer than expected, namely of the strains *Gramella sp.* (197B), *Pedobacter sp.* (70A), *Lysinibacillus sp.* (19), *Rhodococcus sp.* (28), and lastly *Enterobacter spp.* (195B, 205A, and 165). This is because the sequences could not be merged via CLC Main, only the forward sequences were used, and the sequences were shorter than the others, so they could not be trimmed to the same length. Therefore, their relatedness to other strains might be incorrect and cannot be trusted. Most likely, these branches in the phylogenetic tree are longer than what is the case in reality, due to Mega X interpreting the shorter sequences as having more variation.

5.1.2 Bacterial collection: *Flavobacterium*

A second phylogenetic tree was built to compare the relatedness of *Flavobacterium spp.* isolated from the root endosphere of *L. sativa*, to members of this genus isolated from the root microbiota of different plant species, namely *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*. Also, an outgroup of the genus *Flexibacter*, and some additionally known *Flavobacterium spp*. from the literature were added (**Figure 11**). *Flavobacterium* strains isolated from the root endosphere of *L. sativa* did not all cluster together, although some did. This means, that highly likely, the same *Flavobacterium spp.* occur in the microbiota of different plant species, and that the bacteria of the genus *Flavobacterium* isolated from the root endosphere of *L. sativa* are thus not unique to the plant species.

A third phylogenetic tree was constructed to compare the *Flavobacterium spp.* originating from the root endosphere of *L. sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*, to the top ten most abundant *Flavobacterium* strains from both the endosphere and the rhizosphere of *L. sativa* grown under low temperatures (8-12 °C) (**Figure 12**). This last phylogenetic tree is not as correct compared to the other two, because only a fragment (V4) of the 16S rRNA gene sequences was used, and the strains in the phylogenetic tree all belonged to the same genus and are thus very similar. This makes it more difficult to draw conclusions from this tree. The most abundant *Flavobacterium spp.* from the endosphere seemed to be different from the most abundant ones from the rhizosphere. Some of the top ten strains seemed to be quite randomly distributed in the phylogenetic tree, but many strains did cluster together apart from the isolated bacteria originating from the endosphere of *L. sativa*, *Arabidopsis thaliana*, *Poa annua*, and *Valerianella Locusta*. This indicates that the variation of the most abundant *Flavobacterium spp.* in the endo- and rhizosphere is higher than the variation in isolated bacteria from the endosphere. Therefore, it would be worthwhile to isolate more *Flavobacterium spp.*, this would increase the genetic variation between isolated strains, and thus increase the chances of finding growth-promoting strains. To isolate these additional *Flavobacterium spp.*, different isolation methods could be used, with different growth media. *Flavobacterium* strains are often sensitive to inhibitory compounds produced in growth media were phosphate and agar interact during autoclaving (Tanaka et al., 2014), this is the case for the R2A medium. An alternative for this growth medium could be phosphate separately autoclaved R2A supplemented with cycloheximide and tobramycin (Nishioka et al., 2016).

5.2 Growth promotion experiments

Cold stress majorly impacts agriculture by causing reduced germination, stunted seedlings, wilting, chlorosis, necrosis, and overall, it lowers crop productivity (Yadav, 2010). The inoculation of plants with plant growth promoting rhizobacteria (PGPR) can stimulate plant growth in such stressful conditions. This project focused on identifying single bacterial strains able to increase *L. sativa* seedling growth at low temperatures. Members of the genus *Flavobacterium* were found to be enriched in the root microbiome of *L. sativa* at low temperatures (**Figure 9**). Many have also been found to be successful PGPR in various stressful environments (Gontia-Mishra et al., 2016; Kwak et al., 2018; Rai et al., 2018; Carrión et al., 2019). This made them excellent candidates to investigate their effect on *L. sativa*, in cold stress conditions. Three different strains were found to have a significant positive effect on shoot fresh weight in three biological repeats. However, within each treatment, there was a lot of variation. This could partly be resolved by removing the outliers, but this in turn lowered the statistical power.

The germination of seeds occurred rather non-synchronously and there was a large variability in the growth of plantlets within and between biological repeats and treatments. There are many factors contributing to this variation. First of all, even though a growth room is a more controlled environment than a greenhouse or the field, it is not completely uniform (Potvin & Tardif, 1988). The position of trays in the growth chamber can explain some of the variation, because slight differences in humidity, light intensity, ambient CO_2 concentration, nutrients in the soil or temperature could all impact the growth (Measures et al., 1973; Potvin & Tardif, 1988). Secondly, the success of inoculation might also vary. In the future, it should be quantified how much bacteria colonize the plant roots, by counting the colony-forming units. Another possible method to quantify the bacteria is through the use of flow cytometry (Vandeputte et al., 2017). Hereby, the bacterial cells of specific strains would be fluorescently labeled before inoculating the seeds, these cells can then be sorted after the growth experiments. If the abundances of the bacteria are low, and the colonization is thus not very successful, it might be interesting to repeat the growth experiments using different colonization methods or growing the seeds in a different substrate. Thirdly, the effect of PGPR is also dependent on the genotype of the plant. For example, the growth stimulation of two Arabidopsis ecotypes differed, even though the same PGPR were used (Schwachtje et al., 2012). This indicates that highly likely, there will be a difference in the effect of the bacterial strains depending on different *L. sativa* cultivars. In addition, the presence of a lot of variation indicates that there is a fine line between PGPR being growth-promoting or not. One reason might be a trade-off between growth promotion and upregulation of defense pathways in the plant (Lara-Chavez et al., 2015). This makes the practical application of PGPR in agriculture more challenging.

Following this project, it would be interesting to investigate the mechanisms the successful strains use to promote *L. sativa* growth under low temperatures. Members of the genus *Flavobacterium* have been reported to be able, among other things, to solubilize phosphate, produce siderophores, and enzyme 1- aminocyclopropane-1-carboxylate (ACC) deaminase (Rai et al., 2018). Hence, the three most successful strains might use one or some of these mechanisms to stimulate *L. sativa* growth in the cold. Firstly, the ability of the *Flavobacterium* strains to solubilize phosphate can be screened through spot inoculation of the strains on Pikovaskaya's agar medium (Pikovskaya, 1948). A clear halo around a spot indicates the capability of the strain to solubilize calcium phosphate. Also, the production of siderophores can be screened by inoculating the strains on and Chrome Azurol S agar medium (Schwyn & Neilands, 1987). The formation of an orange halo around spots is the result of siderophore production (Pant et al., 2016). Additionally, various enzymatic activities, such as cellulolytic and proteolytic activities of the strains can be tested via the agar disk method (Rai et al., 2018). Furthermore, the ability of the bacteria to produce indole-3-acetic acid (IAA) can be tested via the use of a tryptophan-containing nutrient broth (Loper & Schroth, 1986). Production of IAA by bacteria can stimulate the root growth of plants (Patten & Glick, 2002), resulting in a larger root surface area through which more nutrients can be taken up. In addition, the production of ACC deaminase can be looked into. Ethylene signaling plays a negative role in the freezing tolerance of *Arabidopsis* (Shi et al., 2012). Hence, ACC deaminase production might help plants cope with cold stress. The production of ACC deaminase could be screened by growing bacteria on sterile minimal Dworkin and Foster (DF) salts medium (M Dworkin & Foster, 1958), with 3 mM ACC instead of $(NH_4)_2SO_4$ as the sole nitrogen source. If colonies can grow on this medium, they can highly likely produce ACC deaminase (S. Gupta & Pandey, 2019).

All these tests would give insight into the capabilities of the bacteria. However, the ability of bacteria to produce IAA, for instance, does not necessarily mean these bacteria can influence hormone levels *in planta* (Schwachtje et al., 2012). Therefore, it would be worthwhile to investigate if the most successful strains can modulate different phytohormonal pathways *in planta*, via the use of inoculated *Arabidopsis* GUS reporter lines. In a preliminary study, the growth promoting effect of *Flavobacterium spp.* (37 and 116) was tested on two *Arabidopsis* ecotypes grown under low temperatures, Col-0 and Cvi-0. Col-0 is a cold-tolerant ecotype, while Cvi-0 is cold-sensitive. The results were variable but similar for both ecotypes, sometimes there was a significant growth-promoting effect of the bacterial strains, but mostly the results were not significant. Inoculation of both ecotypes with *Flavobacterium sp.* (37) even resulted in a decrease of rosette weight compared to the control plants in one of the repeats. These results show that the effect of the strains on *L. sativa* might be different than on *Arabidopsis*. This indicates that the GUS assays might not be entirely correct. Lastly, it would be interesting to repeat the growth experiments, but keeping the inoculated plants in the warm growth chamber (22°C, 16 hours daylight) for a longer period, for instance, ten days, before switching to the cold growth chamber (14°C, 16 hours daylight). The reason for this being that in reality, the temperature in a greenhouse can fluctuate, and the bacteria should still be able to maintain their growth-promoting effects after a period of warmer conditions.

5.3 Antagonism assays

This project focused on finding single strains of the genus *Flavobacterium* that could promote *L. sativa* growth in the cold. However, the ultimate goal is to define a consortium consisting of multiple PGPR, because it is expected that a consortium might perform better than a single strain. Several recent studies support this hypothesis (Berg et al., 2018; Molina-Romero et al., 2021). A consortium might be more successful than single strains because it might encompass more modes of action as well as being able to adapt to a broader range of environments. However, this can only be the case if the bacteria put together are compatible (Thomloudi et al., 2019). To downsize the number of possible combinations, antagonism assays were performed to assess which bacteria not to combine. The three most promising *Flavobacterium spp.* were tested against other purified strains of this genus (**Table S2**). Ideally, these three strains would be tested against the entire collection. However, due to time constraints, the focus was solely on the genus *Flavobacterium*.

An antagonistic interaction was present when a halo formed around a spot. However, the results are not straightforward to interpret because the haloes varied not only in size but also in clarity and morphology. Furthermore, sometimes, the bacteria spotted on top of the lawn spread out, at times with a halo around it, and other times without one. It was not clear if this was the case due to the two strains growing synergistically, or rather the spotted strain inhibiting the growth of the lawn and therefore being able to expand. Occasionally, a very thin white line could be observed around the spot, it was not always clear if this circle meant that there was a halo present or forming, but only areas with less dense or without any bacterial growth were considered haloes. Because of these variations and unclarities in the results and only two replicas were done, the antagonistic screenings should be looked at as more qualitative than quantitative (**Table 1, Table S3**).

Different factors influenced the results of the antagonism assays. The most prominent one being the temperature of the layer of soft agar when mixing it with the bacteria. When the agar was too hot, it killed the bacteria, while it being too cold made it impossible to homogenously mix them with the agar.

The right balance had to be found, which was obtained at a temperature around 55°C (Hockett & Baltrus, 2017). Another major factor appeared to be how well the bacteria could grow in liquid or on top of solid R2A medium. It could be that antagonistic interactions could not be observed due to certain strains not growing as well in the growth medium. A few examples of strains often not growing as well, were *Flavobacterium sp*. (PA403A), *Flavobacterium sp.* (PA35A), and *Flavobacterium sp.* (159) (**Table 1**). The less dense lawn of strain *Flavobacterium sp*. (PA403A), gave other strains spotted on top the chance to grow very well or to even expand. The opposite also seemed to be true. Some strains, like *Flavobacterium sp*. (2VL174), *Flavobacterium sp*. (PA460), and *Flavobacterium sp*. (PA116) could grow quickly which made it more difficult for spots to grow or expand.

Even taking these limitations into account, haloes were observed rather often, on average around 35,15% of all spots, which could be explained by extracellular enzymes, such as amylase, cellulase, chitinase, and peptidases, that many members of the *Flavobacterium* genus produce. They can use these enzymes not only for the turnover of organic compounds in the soil but possibly also for the degradation of bacteria and other organisms (Kolton et al., 2016). For future research the metabolites the antagonistic bacteria produce could be examined using molecular techniques (Enisoglu-Atalay et al., 2018), to better understand why some bacteria are antagonistic.

How quickly strains can grow in the rhizo- or endosphere of *L. sativa* plants differs for low versus control temperatures. Firstly, the temperature will affect the speed of growth of various strains differently. Secondly, the growth medium R2A contains a lot of nutrients, which outside of these antagonism assays might not always be the case. Lastly, in field or greenhouse experiments, the bacteria interact not only with the added strains but also with the plant and with other naturally occurring microbes in the soil. This vastly increases the complexity, which can only partly be grasped in these antagonistic experiments. The antagonism assays are thus a useful tool to simplify which strains to put together in a consortium.

Some adjustments could be made to the antagonism assays. For example, it would be worthwhile to repeat the antagonistic screenings with different growth media to observe if the interactions between the bacteria stay consistent. As the goal is to form a bacterial consortium able to promote plant growth in the cold, it would also be useful to repeat the antagonistic screenings at lower temperatures to better mimic the actual growing conditions of *L. sativa* under low temperatures. Temperature, after all, is an important factor modulating bacterial growth and can cause a shift in community structure (**Figure 9**; (Habtewold et al., 2021).

6 General conclusions

The isolated bacteria of the genus *Flavobacterium* from the collection were found to be less diverse than the most abundant *Flavobacterium* strains from the rhizo- and endosphere of *L. sativa* grown under low temperatures. In the future, research to isolate additional *Flavobacterium* strains could increase the number of potential plant growth-promoting bacteria to improve *L. sativa* growth in the cold. In this project, three members of the 24 initially tested *Flavobacterium* strains were able to promote the growth of *L. sativa* under low temperatures, consistently over three biological repeats. To better understand the interaction between the *Flavobacterium* strains and *L. sativa*, further research could investigate the mechanisms these strains use to stimulate *L. sativa* growth. However, throughout the growth experiments, a lot of variation in the growth-promoting results was observed, indicating a fine balance between the presence and absence of growth-promoting properties in plant growth-promoting rhizobacteria. In addition, the interaction between the three growth-promoting *Flavobacterium* strains and other members of this genus was examined. Antagonistic screenings were performed that showed some bacterial strains inhibit others. In some instances, however, it was difficult to distinguish if the bacterial strains acted synergistically or antagonistically. To better comprehend these interactions, the metabolites and enzymes some of the antagonistic Flavobacterium strains produce could be examined. Overall, this project showed that some *Flavobacterium* strains are able to improve *L. sativa* growth in cold conditions. In the future, the application of some members of the genus *Flavobacterium* might serve as a way to sustainably improve *L. sativa* growth in greenhouses during the cold winter months.

7 References

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8 Appendix

8.1 Experimental data

8.1.1 Selection of *Flavobacterium spp.* screened in growth experiments

Flavobacterium sp. (35A)	*Flavobacterium sp.* (PA30)
Flavobacterium sp. (61)	*Flavobacterium sp.* (PA37)
Flavobacterium sp. (159)	*Flavobacterium sp.* (PA44)
Flavobacterium sp. (195C)	*Flavobacterium sp.* (PA55)
Flavobacterium sp. (202B)	*Flavobacterium sp.* (PA116)
Flavobacterium sp. (205B)	*Flavobacterium sp.* (PA125)
Flavobacterium sp. (2PA28)	*Flavobacterium sp.* (PA213)
Flavobacterium sp. (2VL42)	*Flavobacterium sp.* (PA234)
Flavobacterium sp. (2VL130)	*Flavobacterium sp.* (PA400)
Flavobacterium sp. (2VL133)	*Flavobacterium sp.* (PA403A)
Flavobacterium sp. (COL291)	*Flavobacterium sp.* (PA413)
Flavobacterium sp. (PA18)	*Flavobacterium sp.* (PA492)

Table S1. Flavobacterium strains screened in growth experiments. In total 24 bacteria belonging to the genus *Flavobacterium* were screened for their growth-promoting potential on lettuce grown under low temperatures (14°C)

8.1.2 Selection of *Flavobacterium spp.* screened in antagonism assays

Flavobacterium sp. (35A)	*Flavobacterium sp.* (PA86)
Flavobacterium sp. (61)	*Flavobacterium sp.* (PA110A)
Flavobacterium sp. (159)	*Flavobacterium sp.* (PA110B)
Flavobacterium sp. (195C)	*Flavobacterium sp.* (PA116)
Flavobacterium sp. (202B)	*Flavobacterium sp.* (PA213)
Flavobacterium sp. (205B)	*Flavobacterium sp.* (PA224)
Flavobacterium sp. (2VL42)	*Flavobacterium sp.* (PA231)
Flavobacterium sp. (2VL77)	*Flavobacterium sp.* (PA272)
Flavobacterium sp. (2VL130)	*Flavobacterium sp.* (PA273)
Flavobacterium sp. (2VL133)	*Flavobacterium sp.* (PA325)
Flavobacterium sp. (2VL147)	*Flavobacterium sp.* (PA400)
Flavobacterium sp. (COL291)	*Flavobacterium sp.* (PA403A)
Flavobacterium sp. (PA18)	*Flavobacterium sp.* (PA403B)
Flavobacterium sp. (PA30)	*Flavobacterium sp.* (PA413)
Flavobacterium sp. (PA37)	*Flavobacterium sp.* (PA460)
Flavobacterium sp. (PA44)	*Flavobacterium sp.* (PA492)
Flavobacterium sp. (PA55)	*Flavobacterium sp.* (PA487)

Table S2. Flavobacterium strains screened in antagonism assays. In total 34 bacteria were used in the antagonistic screenings.

8.1.3 Antagonistic interactions

	Flavobacterium sp. (PA37)				*Flavobacterium sp.* (PA116)				*Flavobacterium sp.* (PA403A)			
	Strain	Size halo (mm)	Size enlargement (mm)	Comments	Strain	Size halo (mm)	Size enlargement (mm)	Comments	Strain	Size halo (mm)	Size enlargement (mm)	Comments
	PA460	4,0		Thin line around halo	PA86	2,5		Thin line around halo	PA231	3,5	3,0	Very vague halo
	PA272	3,5		Thin line around halo	PA37	2,5		Halo very difficult to see	2VL147	3,3	4,3	Slight halo
	PA86	3,0		Thin line around halo	PA492	2,0		Very vague halo	PA30	3,0	1,8	Very vague halo
	PA55	2,8	0,5	Thin line around halo	2VL133	2,0		Slight halo	PA234	2,5		
	195C	2,3	3,0	Halo and enlargement seem to be combined	PA18	2,0		Very vague halo	PA37	2,0		Very vague halo
	PA231	2,3	1,5	Thin line around halo	PA44	2,0		Very vague halo	PA224	2,0		
	2VL147	2,0	2,5	Thin line around halo	PA325	2,0		Very vague halo	2VL77	2,0	5,5	Slight halo
	2VL130	2,0	1,3	Matte circle around spot	PA272	1,5	1,5	Very vague halo	2VL130	1,8	1,8	Very vague halo
	202B	2,0	3,0	Halo and enlargement seem to be combined	61	1,5		Slight halo	PA487	1,8		
	PA413	1,8	1,5	Matte circle of 1,5 mm thickness around enlarged spot	PA224	1,5		Not grown very well, very vague halo	195C		8,3	Thin line around enlarged spot
	PA116	1,5	4,0		PA213	1,5		Very vague halo	202B		8,0	Thin line around enlarged spot
	2VL42	1,5	4,8	Thin line around halo	COL291	1,3		Clear halo combined with a bit of enlargement	205B		7,0	Thin line around enlarged spot
	205B	1,0	4,0		2VL147	0,8	1,8	Not grown very well, thin line around halo	PA116		7,0	
	2VL77	1,0	2,8		195C		2,3	Not grown very well, halo and enlargement seem to be combined	PA403B		7,0	
	COL291	1,0	1,0	Halo and enlargement seem to be partly combined	202B	2,0		Not grown very well, halo and enlargement seem to be combined	2VL42		6,8	
	PA400		3,8	Matte circle of 1,5 mm thickness around enlarged spot	PA110B		4,5		PA400		6,8	
	PA403B		3,8		2VL42		3,0		COL291		4,5	
	PA110B		3,0	Matte circle of 2 mm thickness around spot	PA403B		2,8	Not grown very well	PA55		3,3	
	159		0,0	Not grown very well	205B		2,5	Not grown very well	PA110B		2,5	
	35A			Not grown very well	PA460		2,5	See through circle of 1 mm around original spot, thin line around enlarged spot	PA86		2,0	
	PA403A			Not grown very well	PA400		2,3	Not grown very well	PA460		2,0	
	PA30			Matte circle of 1,5 mm thickness around spot	2VL77		1,8	Not grown very well	PA272		1,5	
					PA55		1,3	Enlargement might be combined with halo				
					2VL130		1,0	Not grown very well				
					PA30		1,0					
					PA413		1,0	Not grown very well				
					PA231		0,5	Not grown very well				
					35A			Not grown very well				
					159			Not grown very well				
					PA37			Not grown very well				
					PA234			Not grown very well				
					PA403A			Not grown very well				
					PA487			Not grown very well				

Table S3. Overview of antagonistic interactions and enlarged spots. The different bacterial strains are ranked from largest to smallest halo, then largest to smallest spot without a halo. The size of a halo was calculated by subtracting the diameter of the spot from the diameter of the halo and dividing it by two. How much the spot expanded was calculated by subtracting the diameter of the original spot from the diameter of the enlarged spot and dividing it by two. Lastly, some comments are mentioned, for example if the spot did not grow well or there was a thin line present around the halo.

8.2 Protocols

8.2.1 R2A medium

- 0,5 g/L protease peptone
- 0,5 g/L casamino acids
- 0,5 g/L yeast extract
- 0,5 g/L dextrose
- 0,5 g/L soluble starch (potato starch)
- 0,3 g/L K_2HPO_4
- 0,05 g/L Mg-sulfate
- 0,3 g/L pyruvic acid sodium salt
- pH 7 +/-0,2
- 15 g agar/L

8.2.2 Alkaline lysis buffer

- 2.5 ml 10% SDS (10 g in 100 ml MQ)
- 5.0 ml 1M NaOH (Merck, 1.06498.1000; 4 g in 100 ml MQ)
- 92.5 ml sterile MQ

The solution has to be filter-sterilized, using a nylon membrane filter unit with pore size 0.2µm.

8.2.3 Protocol 16S sequencing

DNA extraction

- Pick, with a pipette tip, a bacterial colony of a plate with growth medium and suspended it in a well containing 20 µL alkaline lysis buffer.
- Add 40 µL of 50% SDS-page.
- Incubate for 15’ at 95 °C and cool on ice immediately.
- Add 120 µL sterile MQ H_20.
- Centrifuge for 5’ at 4000 g.
- If not used directly, pipet the supernatans over to new PCR-tubes or 96 well plates and store in the fridge until further use.

Polymerase Chain Reaction: primers

Primer forward: 5’-AGAGTTTGATCMTGGCTCAG

Primer reverse: 5’-GGTTACCTTGTTACGACTT 3’-AAGTCGTAACAAGGTAACC*

Polymerase Chain Reaction

- For one sample mix:
  - 12,4 µL MQ H_20
  - 4 µL buffer
  - 0,4 µL primer forward
  - 1 µL primer reverse

    - 1 µL dNTP
    - 0,2 µL taq polymerase
- Add 1 µL DNA to the mixture.
- Put in PCR machine at settings:
    - 98°C for 30 s
    - 98°C for 10 s
    - 60°C for 20 s
    - 72°C for 45 s
    - Repeat 30 times
    - 72°C for 5 min
    - 12°C for infinity

Magnetic bead purification

- Put HighPrep™ PCR at room temperature 30 min before use.
- Shake thoroughly to resuspend magnetic beads.
- Add 30 µl HighPrep™ PCR to each PCR sample, and pipet up and down 6-8 times to mix.
- Incubate for 5 min at room temperature.
- Place the sample plate on the 96 magnetic separation device for 3 min, until the beads are put to the side of the well and the solution clears.
- Remove and discard the supernatant by pipetting
- Add 200 µL of 80% ethanol to each well, incubate for 30 s at room temperature and remove and discard supernatant. Repeat once.
- Dry the magnetic beats at room temperature for 10 – 15 min, until all traces of alcohol are removed without over drying the beads.
- Remove sample plate from the 96 magnetic separation device.
- Add 40 µL of elution buffer to each well and pipet up and down 5 times to mix.
- Incubate for 2 min at room temperature.
- Place the sample plate on the 96 magnetic separation device for 1 min, until solution clears.
- Transfer the eluate, the clear supernatans, to a new PCR tube or 96 well plate for storage.