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Microscopic analysis of barley plants during beneficial bacterial interactions under biotic stress

BAKALÁŘSKÁ PRÁCE

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Abstrakt

Předpokládá se, že lidská populace se bude stále rychleji rozšiřovat, a udržování stabilních globálních dodávek potravin pro tak obrovský počet lidí se stává každým dnem aktuálnějším problémem. Produkce potravin je značně ohrožena kvůli rostlinným patogenům, kteří jsou zodpovědní za pokles výnosu plodin. Jedním z nejčastějších patogenů obilovin je *Fusarium* spp. Nadměrné používání chemikálií pro ochranu obilovin před těmito patogenními houbami není šetrné k životnímu prostředí, a zapříčinilo na něj značné dopady, což vedlo ke zpřísnění předpisů o jejich používání v zemědělství. V nedávné době získaly pozornost půdní bakterie podporující růst rostlin, u kterých bylo zjištěno, že se přirozeně přizpůsobily růstu ve spojení s rostlinami, a u kterých bylo pozorováno, že podporují rostliny při adaptaci na stresové podmínky. Využití těchto bakterií může být ekologický a udržitelný přístup ke zvýšení produktivity plodin. V této studii byly použity dva bakteriální kmeny, *Enterobacter* sp. SA187 a *Pseudomonas argentinensis*, SA190. Oba bakteriální kmeny jeví prospěšné účinky při zvládání biotického stresu vyvolaného rostlinnými patogenními houbami. Cílem této studie bylo fenotypově a mikroskopicky potvrdit předpoklady o prospěšných vlastnostech těchto bakterií v přítomnosti patogenní houby *Fusarium graminearum* na rostlinách ječmene setého. V této bakalářské práci byla vyhodnocena měření délek kořenů a výhonků kontrolních a ošetřených rostlin ječmene, a proběhla také mikroskopická analýza kořenů ječmene.

Klíčová slova	biotický stres, ječmen setý (*Hordeum vulgare*) cv. Golden Promise, prospěšné bakterie, patogenní houby
Počet stran	46
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Abstract

World's human population is expected to expand rapidly, and the maintenance of a stable global food supply for such a huge number of people is becoming a more relevant issue each day. The food supply is under significant threat, as plant pathogens are responsible for the decline of crop yield. One of the most common pathogens of cereal crops is *Fusarium* spp. Excessive use of chemicals for *Fusarium* spp. control is not eco-friendly and had induced considerable effects on the environment which led to the strict regulations on their use in farming. Plant growth promoting rhizobacteria, which were found to have naturally adapted to growth in association with the plants, have gained attention since they have been observed to be supporting plants in adapting to stress conditions. This can be an eco-friendly and sustainable approach for enhanced crop productivity. Two different bacterial strains were used in this study, *Enterobacter sp.* SA187 and *Pseudomonas argentinensis,* SA190. Both bacterial strains seem to be prosperous for plants in handling stress induced by plant pathogenic fungi. The aim of this study was to phenotypically and microscopically confirm the presumptions about the beneficial properties of these bacteria in presence of *Fusarium graminearum* on *Hordeum vulgare* plants. Measurements of the lengths of the roots and shoots of control and treated barley plants were evaluated, and microscopical analysis of barley roots was done.

Key words	biotic stress, barley plants (*Hordeum vulgare*) cv. Golden Promise, beneficial bacteria, pathogenic fungi
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Content

Goals of the work

Theoretical part

- Elaboration of a literature search on the topic of the bachelor thesis

Experimental part

- Surface sterilisation and growth of plants under sterile conditions
- Analysis of the effect of pathogenic fungi (*Fusarium* sp.) on barley plants in the presence and absence of bacteria (*Pseudomonas* sp. and *Enterobacteria* sp.).

1 Introduction

World's human population is expected to reach over 9 billion by 2050 (FAO, 2009), and the maintenance of a stable global food supply for such a huge number of people is becoming a more relevant issue each day. The food supply is under significant threat, as plant pathogens are considerably responsible for the decline of crop yield. Moreover, due to reduction of the plant health caused by abiotic stress like salinity, crop plants are even more susceptible to the pathogens. One of the most common pathogens to the cereal crop is *Fusarium* spp. Plant health needs to be maintained to ensure a steady food, feed and fibre production. This is accomplished mainly by agrochemicals, which can be either inorganic chemicals like pesticides, gypsum, limestone, sulphuric acid and sulphur derivatives or organic chemicals which include farm manure and organic industrial wastes. Excessive use of such chemicals is not eco-friendly and had induced considerable effects on the environment which led to the strict regulations on their use in farming. Constant efforts are also made for the development of pathogen resistant crop varieties. Plant growth promoting rhizobacteria (PGPR), which were found to have naturally adapted to growth in association with the plants, have gained attention since they have been observed to be supporting plants in adapting to stress conditions (Kloepper *et al.,* 1989). This can be an eco-friendly and sustainable approach for enhanced crop productivity. Two different bacterial strains were used in this study which includes *Enterobacter sp.* SA187 and *Pseudomonas argentinensis,* SA190. *Enterobacter sp.* was previously reported to aid plants by diverse mechanisms, including the assimilation of nitrogen by fixation, lowering of ethylene levels by 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, production of siderophores and phytohormones, priming resistance against phytopathogens, solubilisation of nutrients, increase in nutrient availability, and by degradation of harmful xenobiotics (Andrés-Barrao *et al.*, 2017). Analysis of *Pseudomonas argentinensis* genome revealed, that they contain genes involved in an antifungal activity (Lafi *et al*., 2016). Both of these bacteria seem prosperous for plants in handling stress induced by plant pathogenic fungi.

2 Current state of the problem

2.1 Monocotyledons

Monocotyledonous plants (*Liliopsida*) belong to angiosperms. They have an embryo with one uterus. Their vascular bundles are scattered, meaning that their stem does not grow in width. Monocotyledonous plants do not have a main root, but the roots are bundled. In comparison to dicotyledons, monocotyledons have different root development. In dicotyledons the primary root continues to grow, in monocotyledons it disappears and is replaced by adventitious roots growing from stem nodes. The leaves have usually parallel veins, sessile or sometimes petiolate veins.

Among monocotyledonous plants are 11 orders, 77 families and about 60,100 species. One of the most numerous is the family of *Poaceae*, which includes cereals, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), millet (*Panicum sativum*), rice (*Oryza sativa*) and maize (*Zea mays*). The *Poaceae* family is widespread throughout the world, meaning that it is almost cosmopolitan. They are not only essential for food production, but they are also an ecologically important group of plants.

2.1.1 Barley

Barley (*Hordeum vulgare*) belongs to the class of monocotyledonous plants, the order *Poales* and the family *Poaceae*. It is an annual herb. The leaves are alternate, and the type of inflorescence is an ear. Barley is self-pollinating, diploid, and its genome consists of 7 chromosomes.

It is the fourth most important crop in the world after wheat, corn and rice. The largest barley production takes place in Russia, the United States, Germany, Ukraine, France, Australia, China, Spain and Canada. Barley is mostly used in malt production, which is necessary for production of beer and vinegar, but uses of barley are way broader. In addition to human food production, it is also crucial in the production of animal feed (Harwood, 2019). Estimations are that cereal production has to increase by at least 50% in the next 50 years to meet the growing demand (Miralles *et al.*, 2021). Nowadays, barley is also used as an experimental model in many fields of study, such as biotechnology, etc.

2.2 Beneficial plant- microbe interactions

Microbes are primitive organisms inhabiting the environment since the origin of life on earth. Due to their remarkable adaptability, they managed to survive millions of years of evolution, and as a result of that, microbes also became one of the most important organisms in the ecosystem, as they maintain ecological balance, and they play crucial roles in plants. Plants provide a habitat for microbes both in terrestrial and aquatic environments. Many microbes are essential for plant growth, but on the other hand, some microbes are able to cause diseases in plants, not having those beneficial interactions with plants. Plant-microbe interactions are studied by taxonomists, ecologists, agronomists, chemists, as well as evolutionary biologists.

2.2.1 Endophytes

Endophytes are usually non-pathogenic microorganisms, which spend at least some time of their life cycle inside of plants by colonizing the tissue of vascular plants. They have been isolated in most vascular plants (Brader *et al*., 2017, Fadiji and Babalola, 2020a). The term endophyte has been originally used for fungi inhabiting the plant, but it is known nowadays, that endophytes can be also bacteria. Endophytic microbial communities inhabit all plants, and they associate with various tissues and organs of plants, often without causing any negative effects, so the association is based on a symptomless nature.

These endophytic microbes have the ability to directly or indirectly enhance plant growth and yield, suppress plant pathogens, increase stress tolerance of plants, solubilise phosphate, contribute nitrogen to plants, which leads to proper growth of crops. Moreover, they have shown capability in production of compounds of biotechnological or pharmaceutical importance, such as antibiotics, antitumor and anti-infection agents. (Meena *et al.,* 2017). Endophytes can be divided into three groups: they are either microbes defending hosts from biotic stress; alleviating abiotic stress of the host; or supporting the host nutritionally (Bacon *et al*., 2015).

The endophytic interactions are found in various plant orders, families and genera, all around the world. Interestingly, all plants are considered to be symbiotic with endophytic microorganisms (Redman *et al*., 2011)

2.2.1.1 Prokaryotic endophytes

Bacterial endophytes include many genera and species. Their functions seem to depend on the host and environmental parameters, and they cannot be assigned to taxonomy. Most of the prokaryotic endophytes belong to the *Gammaproteobacteria*. Even though this group contains many phytopathogens, a few endophytic genera, such as *Pseudomonas, Enterobacter, Pantoea, Stenotrophomonas, Acinetobacter,* and *Serratia* can be found (Scortichini *et al*., 2010). An example of significant variations in behaviour can be found in the genera *Enterobacter,* as several species were stated as opportunistic pathogens, but others were described as beneficial to the host (Hardoim *et al.*, 2013).

2.2.1.2 Lifestyles of endophytes

Endophytes were classified as being facultative or obligate upon association with plants (Nair and Padmavathy, 2014). Facultative endophytes use nutrients provided by plants, they live freely outside plant tissues in the soil, but they can enter the plant endosphere under certain circumstances (Hardoim *et al.,* 2015). Obligate endophytic microorganisms need the plant tissues to live, as they complete a major part or even their entire life cycle inside plants (Hardoim *et al.,* 2015). Examples of obligate endophytes are found among mycorrhizal fungi. The behaviour of endophytes might be also opportunistic, meaning that their intention is not to colonize the plant, but might end up doing so, if wounds on plant surface appear. The microbes enhancing plant growth belong to facultative endophytes (Hardoim *et al.*, 2015).

2.2.1.3 Inoculation with endophytes

The method of inoculation of plants with endophytes has a noticeable influence on the endophytic colonization (Afzal *et al.*, 2013). Seed inoculation is one of the methods and comprises of a co-cultivation of liquid inocula with seed or seedling stage of the plant, usually in Petri dishes. Another frequently used method is soil inoculation. In this case, the liquid inoculum is introduced into root media or pots where the plants plants are grown. Maybe even more efficient method might be a so-called pruned-root dip, as wounds and subsequent leakage of plant exudates create great conditions for the endophytes (Bressan *et al.*, 2004). Spot-inoculation is an inoculation method forming a nodule at a desired location. Other method, a foliar spraying of endophytes showed the

enhancement tomato production, proving to be a useful method, too (Olivares *et al.,* 2015).

Inoculations with more varieties of endophytes are believed to be more efficient than individual inoculations (Knoth *et al.*, 2013; Knoth *et al*., 2014; Silva *et al.*, 2014). Security that the interrelation of endophytes consortium is beneficial is a key point in this case.

2.2.1.4 Colonization

The interactions and colonization by endophytes differ by microbial strains, and depend on type of the plant tissue, plant genotype, and biotic and abiotic environmental conditions. It was found out, that strains with relatively large genomes are mostly able to colonize a wider range of plant hosts, than strains with smaller genomes (Mitter *et al.,* 2013).

Regarding colonization of plant roots, many bacterial endophytes are attracted to the plants by root exudates and rhizodeposits (Philippot *et al.*, 2013). The movement of microorganisms is possible due to chemotaxis, a movement caused by different concentrations of a chemical substance (Begonia and Kremer, 1994). It has been suggested that the bacterial endophytes enter the roots via colonization of root hairs (Mercado-Blanco and Prieto, 2012). The penetration takes place where the lateral roots occur, and the bacteria use root cracks (Zakria *et al.*, 2007). Subsequently, some endophytes colonize intercellular spaces locally, and other, that are able to penetrate the endodermis, use the vascular tissues for movement to other parts of the plant (Fig. 1; James *et al.*, 2002; Johnston-Monje and Raizada, 2011). The endophytic vertical spread through the plant is very slow (Compant *et al*., 2008). It is not known why and if the endophytes try to reach a specific organ or tissue.

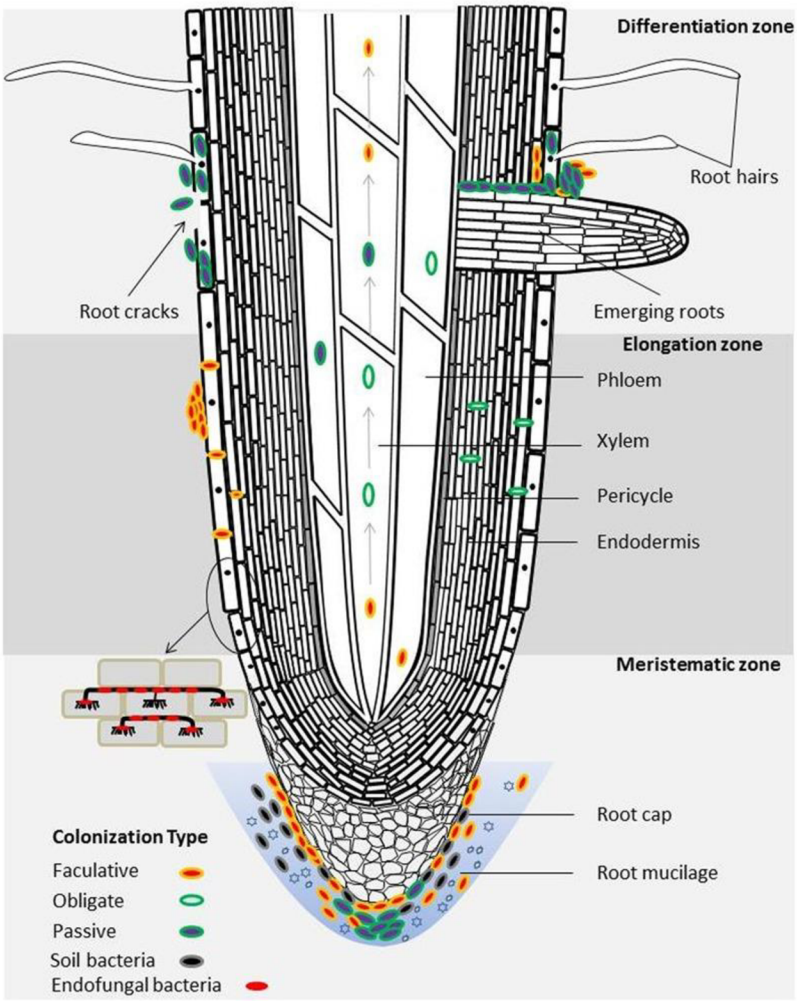

Figure 1 Bacterial colonization patterns of a plant root. The intercellular spaces and emerging sites of lateral roots are among the most common sites of colonization by bacteria. Vast amount of bacteria is also located in the root mucilage area, to where they are attracted by the root exudates. The bacteria are represented by ovals, each colonization type is distinguished by distinct colour. Arrows represent the movement of bacteria inside of the plant vascular system (Liu *et al*., 2017).

The place of beginning of colonization of the plant is not limited to the roots, some exudates attractive to microbes are also produced by leaf and stem, but only adapted microorganisms are able of this kind of colonization (Compant *et al.*, 2010). Moreover, penetration of plants through flowers and fruits is also possible in some cases. (Compant *et al.*, 2011). The advancement of endophytes is well visualized using green-fluorescent-protein (GFP) labelling.

2.2.2 Plant growth enhancing mechanisms of endophytes

2.2.2.1 Nitrogen fixation

Nitrogen fixation is a major mechanism for plant growth promotion, and it is especially essential for the plants growing in environments with low nitrogen contents. The nitrogenase gene, necessary for the fixation of atmospheric N_2, is abundant in higher number of endophytes (Hardoim *et al*., 2015). This suggests that the endophytes support plants under conditions of lack of nitrogen. Generally, the ability to fix nitrogen is well described in rhizobial and actinorhizal plant symbioses.

2.2.2.2 Alleviating biotic and abiotic stress

Upon interactions with the host, endophytes may induce induced systemic resistance (ISR), which are plant defence reactions resulting in an increased tolerance to pathogens (Robert-Seilaniantz *et al*., 2011; Zamioudis & Pieterse, 2012). Unlike pathogens, mutualists are able to avoid these host defence responses and colonize plants (Zamioudis & Pieterse, 2012). The most common bacterial strains inducing ISR belong to the genera *Pseudomonas* and *Bacillus* (Kloepper & Ryu, 2006). It was found out, that compounds like antibiotics, N-acylhomoserine, lactones, salicylic acid, jasmonic acid, siderophores, volatiles, and lipopolysaccharides are responsible for inducing ISR (van Loon *et al.,* 2008). Fungal endophytes have not been frequently reported to be involved in protection of their hosts via ISR, and their more significant properties are ability to produce compounds that have growth-inhibitory consequences towards plant pathogens and mainly insect herbivores. In addition, several reports have discussed the production of antiviral, antibacterial, antifungal, and insecticidal compounds by fungal endophytes (Gunatilaka, 2006).

Bacterial endophytes produce antimicrobial compounds, too. For example, it has been proved that the endophyte *Enterobacter sp*. strain 638 produces antibiotic substances, such as 2-phenylethanol and 4-hydroxybenzoate (Taghavi *et al.,* 2010). Generally, endophytic actinomycetes are the best-known examples of antimicrobial compound producers.

2.2.2.3 Siderophores

Siderophores are important compounds for iron acquisition by soil microorganisms. Some endophytes, both bacterial and fungal, produce these compounds. Siderophores might be essential for induction of ISR (van Loon *et al.*, 2008).

2.2.2.4 Phytohormone production

Phytohormone production may be one of the most understood mechanisms of plant growth promotion. Cytokinins are produced commonly by almost all endophytes, but production of auxins and gibberellins is the typical ability mainly of root-associated endophytes (Bastián *et al.*, 1998; Long *et al.*, 2008; Shi *et al.*, 2009; Merzaeva & Shirokikh, 2010; Khan *et al.*, 2012). A study suggested that an auxin indole-3-acetic acid (IAA) increases colonization efficiency (Suzuki *et al.*, 2003).

2.2.2.5 Other mechanisms

One study confirmed that adenine and adenine ribosides produced by endophytes function as growth-promoting compounds in Scots pine (Pirttilä *et al.,* 2004). Some bacterial volatile compounds seem to be beneficial for plant growth, too (Taghavi *et al.*, 2009; Johnston-Monje and Raizada, 2011).

Endophytic microorganisms are able to produce secondary metabolites, and to influence the secondary metabolism of the plant (Zhang *et al.,* 2006). Metabolites play important roles in signalling, defence, and genetic regulation of the establishment of symbiosis (Schulz & Boyle, 2005).

Root endophytes also increase phosphorus content in wheat, suggesting that these endophytes show promising potential for a phosphorus management (Taghinasab *et al.,* 2018).

1-aminocyclopropane-1-carboxylate (ACC) deaminase is an enzyme found in bacteria, which causes lowering of ethylene levels in the plant and thus preventing ethylene signalling by cleaving the ethylene precursor ACC to ammonia and 2-oxobutanoate. Apart from stress alleviation, the plant hormone ethylene is also an important regulator of colonization of plant tissue by bacteria, meaning, that ACC deaminase supports colonization of these endophytes (Iniguez *et al.*, 2005).

2.2.3 Rhizobacteria

Plant-microbe interactions might be located either in phyllosphere, endosphere or rhizosphere. Phyllosphere represents the aerial parts of the plant, and endosphere is the plant internal transport system. Rhizosphere is the region of soil influenced by the plant roots or directly in association with the roots of the plant, often extending a few mm from the root. This region of soil contains a lot of bacteria, and it is estimated that more than 4,000 microbial species are present per gram of such soil (Montesinos, 2003). The bacteria living in this area are generally termed as rhizobacteria.

Rhizobacterial interactions with plants can be divided into three categories: neutral, negative or positive. Most of the interaction are neutral, meaning that the rhizobacteria associated with plants are commensals with no visible effects on the host plant. During negative interactions, the phytopathogenic rhizobacteria produces harmful substances, and is negatively influencing the growth and physiology of the plant. The bacteria positively affecting the growth and physiology of the plants are called PGPRs.

2.2.3.1 PGPR

Bacteria were known to be useful in agriculture since ancient times, but the term "plant growth promoting rhizobacteria" was firstly introduced in 1978 by Kloepper and Schroth. PGPRs are defined as a group of soil bacteria, which can stimulate the growth of plants by colonizing their roots. PGPR seem to be especially useful, as they have the prospect of being used in modern sustainable crop production without the need of changing any genetic information. The concept of PGPR has now been confined to the bacterial strains that can fulfil at least two of the three criteria such as active colonization, plant growth stimulation and biocontrol (Weller *et al.,* 2002). Understanding how PGPR work is nowadays advancing at cellular, genomic and proteomic levels.

Plant growth promotion dominates with nitrogen fixation, phosphate solubilisation, production of phytohormones like auxin and cytokinin, and volatile growth stimulants such as ethylene and 2, 3-butanediol (Fig. 2; Ryu *et al.*, 2003; Vessey 2003), but the promotion can be also done indirectly by exclusion of pathogens or removal of phytotoxic substances. Moreover, iron-chelating siderophores, antibiotics and hydrogen cyanides might be produced by PGPR (Fig. 2), with the goal of reduction of phytopathogens (Ahl *et al.,* 2008). All this leads to increased harvest yield of crop plants. Biotization is a

metabolic response of plants to microbes resulting in developmental and physiological changes. It can be achieved both in *in vitro* and *ex vitro* conditions. The most commonly observed effects upon PGPR interactions with plants are a reduction of the growth rate of primary root, and an increase of the number and length of lateral roots and root hairs.

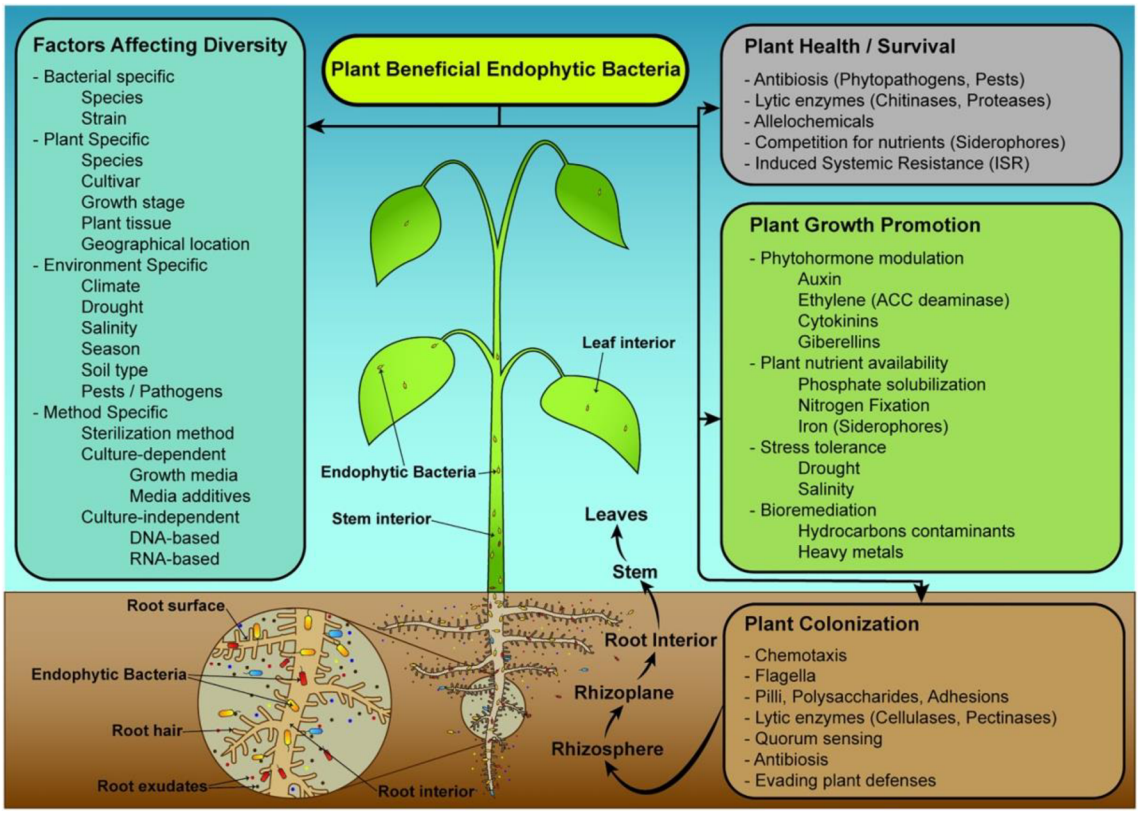

Figure 2 Mechanisms of promotion of plant growth and health, colonization, and factors affecting diversity of endophytic bacteria in host plant. Phytohormone modulation, providing of stress tolerance, bioremediation and improving of plant nutrient availability are ways of direct plant growth enhancement (green box). Indirect plant growth enhancements are phytopathogen and pest antagonization (grey box). Bacteria are attracted by the root exudates. The colonization starts in the rhizosphere and follows to the root surfaces. The bacteria then move into the interior parts of plants (brown box). Some endophytes can progress to the stem and leaves. The diversity of endophytic colonizers (blue box) is affected, among others, by plant and environment related factors (Afzal *et al.*, 2019).

PGPRs can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Martínez-Viveros *et al.,* 2010). The ePGPRs are present in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex. The iPGPRs are present in the specialized nodular structures of root cells. The bacterial genera such as *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* belongs to ePGPR (Gray & Smith 2005). The iPGPR includes mainly endophytes which can in symbiosis with higher plants fix atmospheric N_2. These endophytes are soil bacterial such as *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium*. Moreover, several actinomycetes strains are also prosperous because of their roles in soil nutrient cycling, among them *Micromonospora* sp., *Streptomyces* spp., *Streptosporangium* sp., and *Thermobifida* sp., and they have a prospect as a biocontrol agent against many root-pathogenic fungi (Franco-Correa *et al.,* 2010).

2.2.4 *Enterobacter* sp. SA187

SA187 are endophytic enterobacteria that have been isolated from the root nodes of native desert plants *Indigofera argentea* in Saudi Arabia, Jizan region. The genome of SA187 is 4 429 597 bp long and consists of only one chromosome. *Enterobacter* sp. SA187 lives in the rhizosphere and in association with various plants. This lifestyle of the bacterium allows it to adapt to the environment while supporting the plant growth. These bacteria have been found to contain a large number of genes for potential plant growth promotion (Andrés-Barrao *et al.*, 2017).

2.2.4.1 Effects of *Enterobacter* sp. SA187 on plants

It was found out that SA187 codes for a large amount of membrane transporters, which allow the exchange of bacterial metabolites and plant-produced nutrients. There are genes coding for ABC transporters, which among other things, are involved in the uptake of metals as iron or zinc, and uptake of phosphates, sulphates, nitrates or nitrites, and urea. 54 genes necessary for iron and manganese uptake were found in SA187. Apart from iron uptake transporters, the bacteria also produce siderophores (Andrés-Barrao *et al.*, 2017).

These bacteria also contain genes for carotenoid biosynthesis, as genes coding for phytoene synthase increase their expression upon association with plants (Andrés-Barrao *et al.*, 2017). Carotenoids are beneficial for association between plants and bacteria, and for UV protection of the plant (Mohammadi *et al.*, 2012; Bible *et al.*, 2016). Yellow pigment produced by SA187 could be a derivate of the carotenoid zeaxanthin. This leads to SA187 having two phenotypes, the white SA187W and yellow SA187Y. The yellow pigment could have some role in the root colonization. Moreover, zeaxanthin is a precursor in plant production of salicylic acid, an important plant hormone (Andrés-Barrao *et al.*, 2017).

Additionally, bacteria SA187 contain genes for biosynthesis of proline and trehalose, which act as osmolytes (Andrés-Barrao *et al.*, 2017). Proline aids plants during salt stress conditions (Hayat *et al.*, 2012).

2.2.5 *Pseudomonas argentinensis* strain SA190

SA190 are also endophytic bacteria that have been isolated from the root nodes of native *Indigofera argentea* desert plants. They are G-, and they belong to phosphate solubilising microbes, which are a group of beneficial microorganisms capable of hydrolyzing organic and inorganic insoluble phosphorus compounds to soluble form that can be easily assimilated by plants (Lafi *et al.,* 2016a).

2.2.5.1 Effects of *Pseudomonas argentinensis* strain SA190 on plants

Not much scientific research about SA190 has been done, and scarce information about this strain is available. Nevertheless, some genes supporting plant growth promotion activity were identified, such as gene-coding clusters for phosphate solubilisation, genes for pyrroloquinoline quinone synthesis, and a gene encoding ACC deaminase (Lafi *et al.,* 2016a). In addition, SA190 contains genes coding for glucan endo-1,3beta-D-glucosidase and chitinase, which have been proved to have the antifungal ability (Aktuganov *et al.,* 2008; Lafi *et al.,* 2016a).

2.2.6 Issues with endophytes and their prosperous properties

The definition of endophytes does not include pathogenic microorganisms, anyway, some problems and dilemmas with classifications exist. For example, if plant pathogens should be classified as endophytes, once they are no longer virulent. Moreover, it is becoming

more apparent, that the interactions of plants and endophytes might range and change from beneficial to pathogenic (Ryan *et al.*, 2009), meaning that not all plant-endophyte interactions are always mutualistic, which leads to problems with characterization of endophytes. In addition, it has been found out, that harmless bacteria can change their behaviour to pathogenic upon a change of host or host niche (Turrientes *et al.*, 2010), and that a bacterial group of fluorescent pseudomonads known to be beneficial to plants, turned out to be detrimental to a species of fern under specific conditions (Kloepper *et al.*, 2013). This proves that sometimes the interactions might change, and mutualistic interactions may become pathogenic for the host.

An important issue is that properties of endophytes are usually tested in a single plant species, but not that often over a taxonomically wide spectrum of plant species. Another problem is the low amount of the environmental conditions where these interactions are studied, as they are often based only on controlled conditions, which might not be realistic on the field. In addition, interactions between various endophytes of the community have rarely been studied. Bacterial and fungal endophytic communities are commonly investigated separately, and the interaction between both groups inside plants should be studied more.

2.2.7 Future prospects

The area of endophytic research is still in its beginning, and many endophytes may be still unknown to the world. As for the already discovered endophytic microorganisms, various mechanisms have been discovered and studied, but many of their properties also remain to be identified. The details of the expression of beneficial features are not well understood, and it is not certain what exact processes take place after inoculation. So far, many endophytes proved to be beneficial for plant growth enhancement, increase in tolerance towards pathogens and environmental changes. In the future, they might become crucial for adapting crops to climate change.

2.3 Plant pathogens

Pathogens are the causative agents of diseases with symptoms like abnormal cell division, decomposition or breakdown of cells, wilting, abscission, degeneration of some components as chlorophyll, etc. The most common plant pathogens are fungi, bacteria, viruses, viroids, mollicutes, parasitic green algae, nematodes, parasitic higher plants, and

protozoa. They are able to survive in the environment in which the host lives, and to penetrate the plant tissues like leaves, roots, fruits and other parts of plants for their profit.

Disease forms when pathogen, favourable environment and susceptible host are present. Plant pathogens are quite host specific, and while some have a wide range of hosts, others attack only one genus of plant, and some even target only one species of plant. They diverse by the location of infection, the plant type or the age of the organ or tissue they attack. Some grow on roots, others on stems or leaves. Some pathogens can only infect the young parts of plants, while some only infect the mature tissues (Doi *et al*., 1967).

The better understanding of plant-pathogen interactions might be achieved by identification and quantification of metabolites. Several techniques have been used in the past in metabolomics analysis, such as high-performance liquid chromatography, gas chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy.

2.3.1 Types of plant pathogens

Some plant pathogenic fungi are obligate pathogens, meaning that they are able to live only in the living host, and not elsewhere. Others are nonobligatory, as they can live on either living or dead hosts. Nonobligatory parasites can be either facultative saprophytes, which grow saprophytically on dead organic matter, or facultative parasites which are necrotrophs, however, they can cause damage to a plant under certain conditions. Overall, the harshness of the disease is not directly affected by the type of parasitism (Doehlemann *et al*., 2017).

As it was already outlined above, the plant pathogenic fungi can be also categorized as either biotrophs or necrotrophs. The fungi in the first group are dependent upon living cells of the host, and they do not kill the plant rapidly. Opposed to that, necrotrophs convert living tissues into dead matter and use the dead plant tissues as a resource, meaning that their pure intention is to kill their host (Doehlemann *et al*., 2017).

2.3.2 Development of disease

The disease development and pathogen propagation are a series of steps. The steps are inoculation, pre-penetration, penetration, infection, invasion, growth and reproduction of the pathogen (Dixon *et al.,* 1994). Some of the stages are described below.

2.3.2.1 Inoculation

Inoculation happens when the pathogen gets in contact with the plant and initiates the infection process. The contact can be carried through spores, sclerotia, mycelium, and by an intact cell in the case of inoculation by bacteria, protozoa, viruses, etc. There are two kinds of inoculum, primary and secondary. The primary inoculum is dormant for some time and causes the infection later on. The secondary inoculum is produced from the primary infections. Generally, the primary inoculum causes harsher diseases (Abdulkhair & Alghuthaymi, 2016).

2.3.2.2 Pre-penetration

While some pathogens start the penetration process of the plant directly by their vectors, others like fungi, bacteria, and parasitic higher plants get in contact with the external surface of the plant, and then penetrate the tissues. Mucilaginous substances found on the pathogen surface are responsible for the adhesion of the pathogen to the plant. The mucilaginous substances are various water insoluble polysaccharides, lipids and glycoproteins. These substances become sticky upon contact with liquids. Most nonobligatory parasites use lysozymes for degradation of the plant cell wall in order to invade and infect the host (Romantschuk, 1992).

2.3.2.3 Infection

An infection process is the contact of phytopathogen with its host. The host can be either susceptible or resistant. Infection of a susceptible host results in the appearance of symptoms which become apparent right after the incubation period. The symptoms might change in time.

2.3.2.4 Invasion and growth

The invasion process of plant pathogenic fungi and plant pathogenic bacteria is different. Fungi can directly grow their intracellular mycelia through the cells, or use intercellular mycelia, which grow between the cells. Some phytopathogens create mycelia which grow between the cuticle and epidermis, and other produce mycelia that form haustoria, which penetrate the epidermal cells. Plant pathogenic bacteria invade the plant tissues by using intercellular strategy (Perfect & Green, 2001). Fungi invade the plant in order to cause

vascular wilts by releasing spores in the vessels (Meredith, 1973). Either way, the phytopathogens continue their growth and spread within the plant tissues.

2.4 Plant pathogenic fungi

Over 20,000 species of fungi are parasites and can cause disease in plants. Fungi are ubiquitous in soils, but only some of them are pathogenic to plants. Nevertheless, they are the major originators of plant diseases, and cause considerable economic losses in crops production. Plant pathogens can be found across all fungal taxonomic groups, and they diverge in their host specificity.

The pathogenic fungi usually attack plant roots, even though some target the surface areas like the hypocotyls or stem bases. Taxes to roots is mediated via electrical charges or soluble or volatile exudates. Quite common is also a generation of mycelia on roots before the penetration via hyphae. To colonize plants and cause disease, pathogenic fungi use diverse strategies. Some fungi are biotrophs and only colonize the plant tissue, using effector molecules to avoid plant cell death. Necrotrophs secrete toxins to kill the plant tissue and get essential nutrients from dead matter. Fungal pathogens release virulence factors which have various functions. (Ritz, 2005; Doehlemann *et al.*, 2017).

2.4.1 *Fusarium graminearum*

Fusarium graminearum, also known by its sexual stage as *Gibberella zeae*, is a haploid ascomycete fungus from the family *Nectriaceae. F. graminearum*is a soil-borne pathogenic plant colonizer, which has been found attacking crops like barley, oats, rice, corn and wheat almost all around the world, but especially in temperate areas. It causes a head blight disease on barley and wheat, an ear rot or red rot disease on corn, and a root rot disease.

2.4.1.1 Life cycle of *Fusarium graminearum*

The fungal mycelium of *F. graminearum* forms perithecia, so called fruiting bodies, which grow on the surface of infected spikelets. They give rise to sexual spores (ascospores), which are discharged in order to land on the host plant (Beyer & Verreet, 2005). Upon that, germination starts within several hours. After that, the fungus produces macroconidia by asexual reproduction (Beyer *et al.*, 2004). Macroconidia remain dormant in the soil or plant residues during winter. Later on, they

give rise to the mycelium. Some members of the Fusarium genus do not have a sexual life cycle, such as *F. oxysporum,* but *F. graminearum* is able of completing the sexual stage, as was described above. Moreover, *F. graminearum* produces two types of spores, the first one spreads via wind and the second via water.

2.4.1.2 Fusarium head blight

FHB is also called a scab or a tombstone, and it is one of the most common fungal diseases affecting cereals worldwide, being prevalent mainly in humid areas. Warm and humid conditions during flowering and early stages of kernel development are especially prosperous for FHB development. More species of Fusarium are the causal agents of FHB, but *F. graminearum* seems to be the most common and the harshest one.

The main symptoms of FHB are premature bleaching and blighting of heads, which occur shortly after flowering. Shrinking of grains occurs, too. The damage induced by this pathogen is mainly of a qualitative nature, as it causes lowered seed quality, and the contamination of grain with poisonous mycotoxins. This leads to the fact that the grains are then unfit for human or animal consumption. Among the mycotoxins are trichothecene deoxynivalenol (DON) and zearalenone. DON is a type of vomitoxin, an antifeedant, and a strong biosynthesis inhibitor. Animals consuming high amounts of DON may suffer with reduced immune response and reproductive dysfunction. Zearalenone is a phytoestrogen, and the problematic issue is that it is very similar to mammals' estrogen. If it gets consumed by pregnant women, abortions might occur (Wang *et al.,* 2013). Generally, crops infected by *F. graminearum* negatively affect livestock feed, biofuel production, baking quality of wheat, and malting and brewing qualities of barley are also influenced.

2.4.1.3 Fusarium root rot

Fusarium root rot (FRR) is caused by *F. graminearum* and other *Fusarium* spp. Typical symptoms appear as dark brown to black, decaying or completely rotted roots, leading to disintegration of root structure and decrease of its functionality. It is often difficult to diagnose this disease, as is may get noticed only after the disease symptoms or necrosis occur on the plant organs above ground. The symptoms might also resemble effects of abiotic stress or other fungal pathogens (Looseley & Newton, 2014). The FRR disease patterns are not well-understood. Unlike in the case of FHB, soil-borne infestations by

F. graminearum have not been studied that much, due to the reason that for a quite lengthy period of time, *F. graminearum* was believed to cause only FHB.

2.4.1.4 Control of *F. graminearum*

The issue of inhibiting the activity of *F. graminearum* is of a great interest nowadays. Introducing less susceptible varieties in the field is one of the most important practises used for control of *Fusarium* induced diseases. Crop rotation, use of clean seeds, irrigation management, fungicide application, and post-harvest management are also essential steps for protection from this disease. Lookout for symptoms is necessary, too. Chemical compounds are not the best option for pathogen control due to safety problems and nutrition loss (Shi *et al.,* 2014). The most efficient and eco-friendly way is the use of plant cultivars resistant to pathogens. Control using antagonistic bacteria or fungi might be a great practice in the future, as they can protect the crops in times the fungicides no longer can. Root diseases are especially difficult to control, because the pathogens can stay in the soils for many years. Moreover, fungicides are not reliable and are not used frequently for *Fusarium* control (Raaijmakers *et al*., 2009).

3 Experimental part

3.1 Material

3.1.1 Tools

Autoclave indicator tape, beakers, microtubes, tubes, graduated cylinders, magnetic stirring bars, spoon spatulas, Pasteur pipettes, parafilm, tape, tweezers, razors, sterile toothpicks, sterile square Petri dishes, sterile round Petri dishes, wash bottles, miracloth, slides, coverslips.

3.1.2 Chemicals

Sigma-Aldrich:

Disodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$), potassium dihydrogen phosphate (KH_2PO_4), ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), potassium hydroxide (KOH), sodium hypochlorite (NaClO), trihydrogenboric acid (H_3BO_3), calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4\ H_2O$), manganese chloride tetrahydrate ($MnCl_2 \cdot 4\ H_2O$), Tween 20, EDTA iron salt (Fe-EDTA), calcium chloride ($CaCl_2$), propidium iodide ($C_{27}H_{34}I_2N_4$), LB broth

Duchefa Biochemistry:

Copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$), Microagar

Penta:

Ethanol (C_2H_5OH)

HiMedia:

Potato dextrose agar (PDA)

3.1.3 Apparatuses

Laboratory pre-scales S1502 - BEL (Italy), analytical scales XA110/2X - Radwag (Poland), electromagnetic stirrer MSH-420 - Boeco (Germany), laminar flow box Faster - Schoeller instruments (Czech Republic), cultivation chamber - Weiss Gallenkamp (Great Britain), refrigerator (4°C) Space plus - Electrolux (Sweden), pH meter

PC 2700 - Eutech Instruments (Singapore), Eppendorf pipettes Research plus - Eppendorf (Germany), centrifuge Scan Speed 1730 MR - Scala Scientific (The Netherlands), spectrophotometer - SmartSpec, BioRad (USA), bright-field microscope Carl Zeiss (Germany), spinning disk confocal microscope Observer Z1 Carl Zeiss (Germany), Plan-Apochromat 20×/0.8 NA Carl Zeiss (Germany)

3.1.4 Plant material

Seeds of wild type barley- *Hordeum vulgare* cv. Golden Promise

3.1.5 Bacterial cultures

1. *Enterobacter* sp. SA187 Y (GFP tagged)

2. *Pseudomonas* sp. 190 (GFP tagged)

3.1.6 Fungal culture

1. *Fusarium graminearum* (GFP tagged)

3.2 Methods

3.2.1 Sterilization of the plant material

Seeds of wild type barley (*H. vulgare* cv. Golden Promise) were surface sterilized in a laminar flow box. The seeds were placed in a 50 ml plastic tube. Subsequently, a solution of 70% ethanol (v/v) was added, and the seeds were washed for one minute. After that time, the ethanol was discarded, and the seeds were treated with 5% sodium hypochlorite (v/v) solution with 200 µl of 0.1% Tween (v/v) for 12 minutes (Tab. 1). After 12 min the hypochlorite solution was discarded carefully, and the seeds were washed again with ethanol for 1 min. After ethanol treatment the sterile seeds were washed thoroughly for 5 times with an interval of 1 - 2 min for each time with sterile MilliQ water. The tubes with the sterilized seeds were filled with distilled water and kept overnight at 4° C for imbibition.

Table 1 Composition of 5% sodium hypochlorite (v/v) sterilizing solution for barley seeds

Composition	Quantity
Sodium hypochlorite	25 ml
Tween 20	200 µl
MilliQ H_2O	Added to 50 ml

3.2.2 Germination of the seeds

After imbibition, the tubes containing seeds were transferred to laminar flow box, where it was possible to work under sterile conditions. The seeds were placed onto a plate (5 seeds per plate) containing solidified nitrogen free Fåhraeus medium (Tab. 2) and were then sealed with parafilm. For stratification, the seeds on the Petri dishes were placed at 4° C for 48 hrs. After sufficient stratification, the plates were put to a plant growth chamber for germination at 21° C, 70 % humidity, 16 hours light/8 hours darkness.

Table 2 Composition of Fåhraeus medium without nitrogen (pH 6,5)

Composition	Quantity
$MgSO_4 \cdot 7H_2O$	1 ml·l^{-1} (0,1232 g·ml^{-1})
KH_2PO_4	1 ml·l^{-1} (0,0953 g·ml^{-1})
$Na_2HPO_4 \cdot 2H_2O$	2 ml·l^{-1} (0,0712 g·ml^{-1})
Fe-EDTA	2,5 ml·l^{-1}
100 µl·l^{-1} $MnSO_4 \cdot H_2O$	100 µl·l^{-1} (0,001 g·ml^{-1})
100 µl·l^{-1} $CuSO_4 \cdot 5H_2O$	100 µl·l^{-1} (0,0015 g·ml^{-1})
100 µl·l^{-1} $ZnSO_4 \cdot H_2O$	100 µl·l^{-1} (0,0017 g·ml^{-1})
100 µl·l^{-1} H_3BO_3	100 µl·l^{-1} (0,001 g·ml^{-1})
100 µl·l^{-1} $Na_2MoO_4 \cdot 2H_2O$	100 µl·l^{-1} (0,0011 g·ml^{-1})
Microagar	13 g·l^{-1}
$CaCl_2$ (after autoclaving)	100 µl·l^{-1} (0,11098 g·ml^{-1})

3.2.3 Preparation of bacterial culture

Frozen glycerol stock cultures of *Enterobacter* sp. SA187 Y and *Pseudomonas* sp. SA190 were defrosted and transferred separately into 50 ml tubes containing 20 ml of LB broth (Tab. 3). The tubes were sealed and incubated at 28°C in a shaker for 24 to 48 hrs. After incubation, bacterial cells were harvested by centrifuging using 6000 ×g at 20° C for 8 min. After centrifugation, the supernatant was discarded, and pellet was resuspended in liquid Fåhraeus medium and incubated in a shaker at 28°C for another 4 hrs for the cells to get acclimatised to the new medium.

Table 3 Composition of LB broth (pH 7,2)

Composition	Quantity
LB broth	25 $g \cdot l^{-1}$

3.2.4 Inoculation of plants by bacteria

After 4 hrs, the optical densities (OD_{600}) of both bacterial cultures were measured in a spectrophotometer using sterile liquid Fåhraeus medium as a blank. Based on the OD obtained, the bacterial cultures were diluted using liquid Fåhraeus medium to obtain an OD 0.2. Subsequently, the bacterial cultures were poured on to a sterile Petri dish, and the roots of the seedlings were dipped in the culture for 1 minute. The seedlings were then placed back to the Petri dishes, sealed, and incubated for 24 hrs in the plant growth chamber before *Fusarium* treatment.

3.2.5 *Fusarium* treatment

For *Fusarium* treatment, control and treated plates were selected after inoculation by bacteria. Fusarium grown on potato dextrose agar (PDA) medium for 2 weeks at 28 °C was washed with sterile distilled water containing 0.01% Tween (v/v) and filtered through sterile miracloth membrane to obtain conidial spores (~1×10^5 spores.ml^{-1}) (Erayman *et al.,* 2015). Barley seedlings were infected by dipping in freshly prepared conidial suspension, sterile distilled water containing 0.01% Tween was used as control. The treated seedlings were then placed back to the Petri dishes, sealed, and incubated in the plant growth chamber. Microscopic and phenotypic analyses were performed on control and treated plants 24 h and 10 days post-infection.

3.2.6 Phenotypic analysis

The control and treated plates were scanned once per day from the day the seedlings were selected for initial bacterial treatment. The plants were carefully moved from the incubation chamber to the scanner to keep the plants in the least stress as possible. The length of the seedling roots and their number was then measured and determined in the ImageJ program. The measured data were processed using Microsoft Excel.

3.2.7 Microscopic analysis

For microscopic analysis, the roots of both control and treated samples 24 hrs after fungal inoculation were used. For this, the roots from the respective control and treatments were stained for 8 min with propidium iodide (1 mg.ml^{-1} final concentration) prepared using sterile liquid Fåhraeus medium. After staining, the 1.5 cm long root tips were cut and placed between slide and coverslip prepared using double sided tape. The cassettes

containing the stained roots were observed under spinning disk microscope (Carl Zeiss, Germany) equipped with Plan-Apochromat 20×/0.8 NA (Carl Zeiss, Germany) using a 2-channel excitation at 534 nm (for PI) and 488 nm (for GFP tagged fungi and bacteria). Image post-processing was done using ZEN 2010 software.

4 Results and discussion

F. graminearum causes a major loss of barley yield due to head blight and root rot diseases. Although *Fusarium* head blight was earlier believed to be a primary disease caused by *F. graminearum*, the root colonization by this pathogen is recognized as very important for immense economic losses. *Fusarium* root rot causes rapid necrosis, leading to a significant reduction in root growth and biomass, which is accompanied by the progression of the pathogen to the stem base (Smiley *et al*., 2005). The growth-inhibiting impact of the pathogen was assigned to the production of the mycotoxin deoxynivalenol (DON) (Masuda *et al.*, 2007). The hyphae colonize intra- and intercellular spaces in the root cortex in sensitive wheat cultivars, while the invasion in resistant cultivar is stopped at the epidermal cells (Wang *et al.*, 2015). The trend in the use of chemical pesticides to control fungal diseases is not very effective and economical because of the developing resistance among the fungi and high cost associated with it (Hu *et al.,* 2015). Recently biological control that uses microorganisms (like bacteria, actinomycetes and fungi) has gained high interest because of their eco-friendly nature (Chow *et al.,* 2018). Microbes with biocontrol properties possess both antagonistic and plant growth-promoting traits, which considered significant since they can provide both plant disease control as well as fruit yield (Sharma *et al.,* 2018).

4.1 Phenotypic differences in barley seedlings after *F.graminearum*

Changes in the observable traits of barley plants were significant in plantlets infected with *F. graminearum* (Fig. 3A & 3B). Severe brown coloration was observed in plants treated with *F. graminearum* alone. The coloration was formed because of the growth of mycelia and the production of mycotoxins like fusarin and deoxynivalenol (DON). Pigmentation is one of the most important parts of the fungal growth process which is usually progressing in a very consistent and predictable pattern. When the fungi germinate, they have a pale mycelium which then turns to a yellowish coloration on its third to fourth day of growth. By sixth day, it turns to orange tone and then to dark wine red/ brown (Cambaza, 2018). Bhandari *et al*. (2018) already described briefly the necrosis symptoms induced by the *F. graminearum* interactions at the root-shoot junction of wheat, but the processes involved in the infection progression on barley plants has not been studied in detailed yet. There is a significant connection between the production of major mycotoxins produced by *Fusarium* and pigments. Fungi with the altered aurofusarin

pigmentation was observed to produce an increased amount of toxins like zearalenone and epigenetic regulatory gene histone H3 lysine 4 methylation (H3K4me) responsible for the biosynthesis of both DON and aurofusarin (Liu et al, 2015). Plants with bacteria showed visible differences in the growth, especially in the root area, as the roots seemed to be longer when compared to the control plants. In the case of plants primed with bacteria treated with fungi, the plants mycelium growth was significantly low which was evident due to the less coloration from the mycelial growth (Fig. 3C & 3D). The plants treated with bacteria and *F. graminearum* were significantly bigger than both the mock control and bacteria alone treated plants.

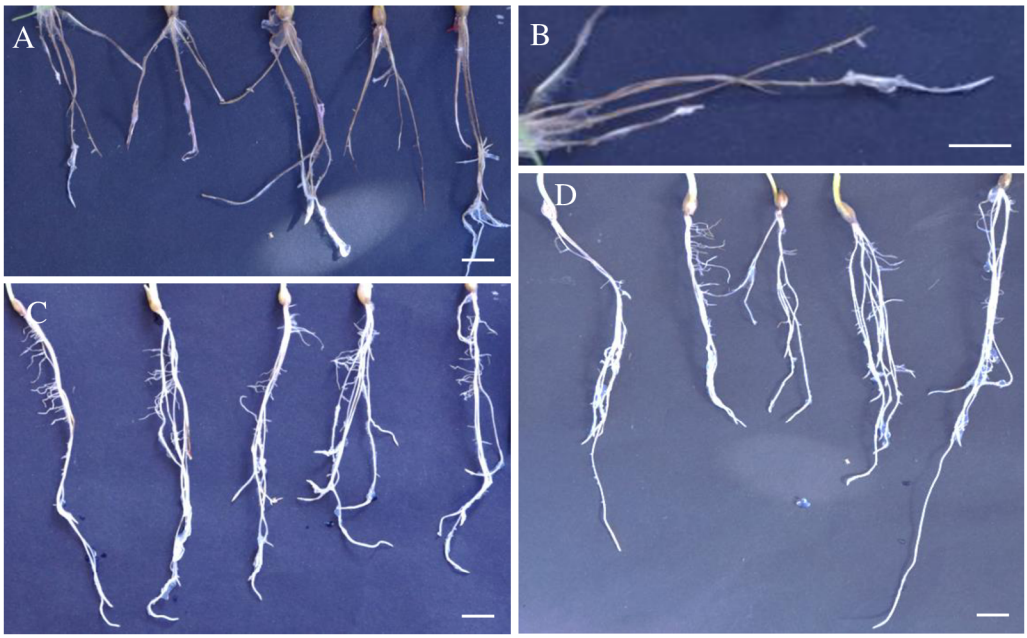

Figure 3 – Barley WT plants co-cultivated with bacteria (SA187 and SA190) and treated with *Fusarium graminearum* after 8 days post-infection. A – Barley WT plants treated with *F. graminearum*; B – WT plant with brown coloured roots after *F. graminarum* infection without bacterial priming; C – Barley plants co-cultivated with *Enterobacter* sp. SA187 treated with *F. graminearum* infection*;* D – Barley plants co-cultivated with *Pseudomonas* sp. SA190 treated with *F. graminearum* infection. Brown coloration of the WT plants treated with *F. graminearum* alone is a clear indication of mycelial growth on barley roots, whereas plantlets primed with bacteria showed less colouration. The plant roots treated with *F. graminearum* without primed bacteria were showing clear reduction in the overall length. Photo was taken on 10 DG (9 days after the beginning of co-cultivation with bacteria, and 8 days after the treatment with *Fusarium*). Scale bar = 1 cm.

The studies of van Peer *et al.* (1991) conducted on wheat observed the ability of *Pseudomonas* strain WCS417 to induced resistance against *Fusarium* wilt caused by *F. oxysporum* when the roots were inoculated with bacteria 1 week prior to stem inoculation with the pathogen. Wei (1991) demonstrated that *P. putida* 89B-27 and other non-pseudomonads induced resistance in cucumber leaves caused by *Colletotrichum orbiculare*. Strain 89B-27 also induced resistance in cucumber against angular leaf spot, caused by *P. syringae* and *Fusarium* wilt, caused by *F. oxysporum* (Liu *et al.,* 1995). For more specific results the roots and shoots of barley plants were measured after the treatment with bacteria and *Fusarium*, on the 10th day of growth.

4.1.1 Changes in root lengths

The average root length of control plants was 7,9 cm. Plants treated with *Fusarium* without the bacteria had the average root length of 5,3 cm, which is 2,6 cm less than in control plants (Fig. 4). Measurement of shorter roots in the presence of the fungus was expected, as infection-based root damage was clearly visible in these plants. The severe infection clearly decreased the growth of roots which are without bacterial priming. However, in the case of roots co-cultivated with *Enterobacter* sp. SA187 before *F. graminearum* infection, the roots had length of 8.8 cm which was 3,5 cm higher than the plants treated *F. graminearum* alone. Whereas for *Pseudomonas* sp. SA190 co-cultivated plants, the root length was 8.1 cm which was 2,8 cm higher than the *F. graminearum* treated. Interestingly, the plants treated with bacteria alone were showing no significant difference when compared to the untreated control. The average root length in plants co-cultivated with *Enterobacter* sp. SA187 and *F. graminearum* showed the highest root length when compared to all the other treatment indicating that the bacteria showed considerable beneficial effect on barley plants under *F. graminearum* induced biotic stress.

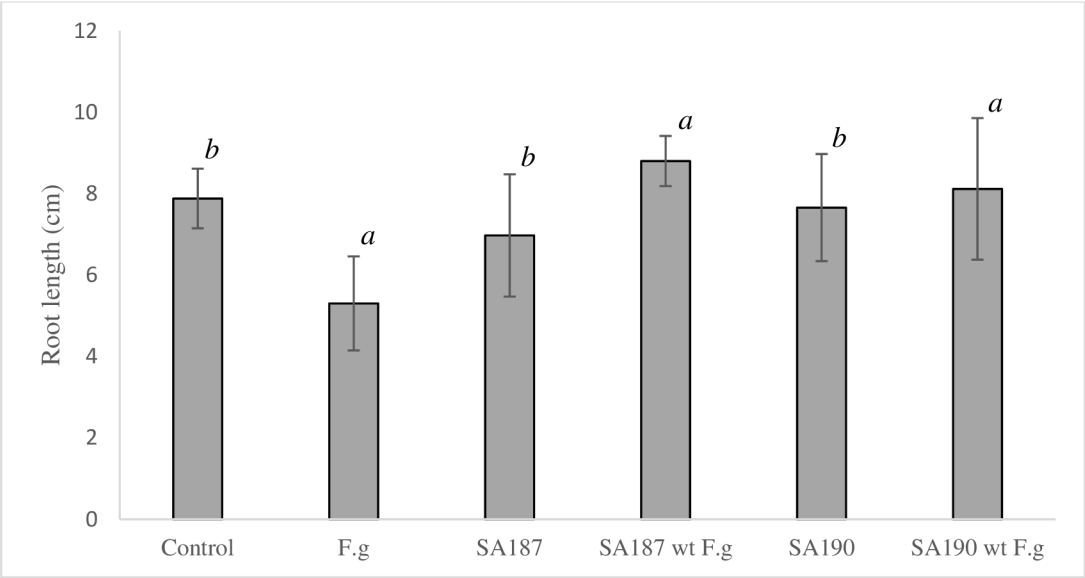

Figure 4 – Graphical representation of the average root lengths of barley plants after 10 days of growth. Plants with bacteria were measured 9 days after the beginning of co-cultivation. Plants with bacteria and *Fusarium* or *Fusarium* only were measured 8 days after the treatment with *Fusarium.* Data is represented as mean ± SD, n = 20; $p \leq 0.05$.

4.1.2 Changes in shoot lengths

The second parameter examined was shoot length of barley plants, also after 10 days of growth (Fig. 5). The average shoot length of control plants was 11,0 cm. Plants treated with *Fusarium* without the bacteria had the average shoot length of 8,5 cm. The length was higher by 3,9 cm when the plants treated with the pathogen were also co-cultivated with SA187, and by 2,8 cm when co-cultivated with *Pseudomonas* sp. SA190. The most significant increase in the length was also observed in plants co-cultivated with *Enterobacter* sp. SA187 and treated with *Fusarium.* The plants treated with bacteria alone were showing no significant difference when compared to the untreated control.

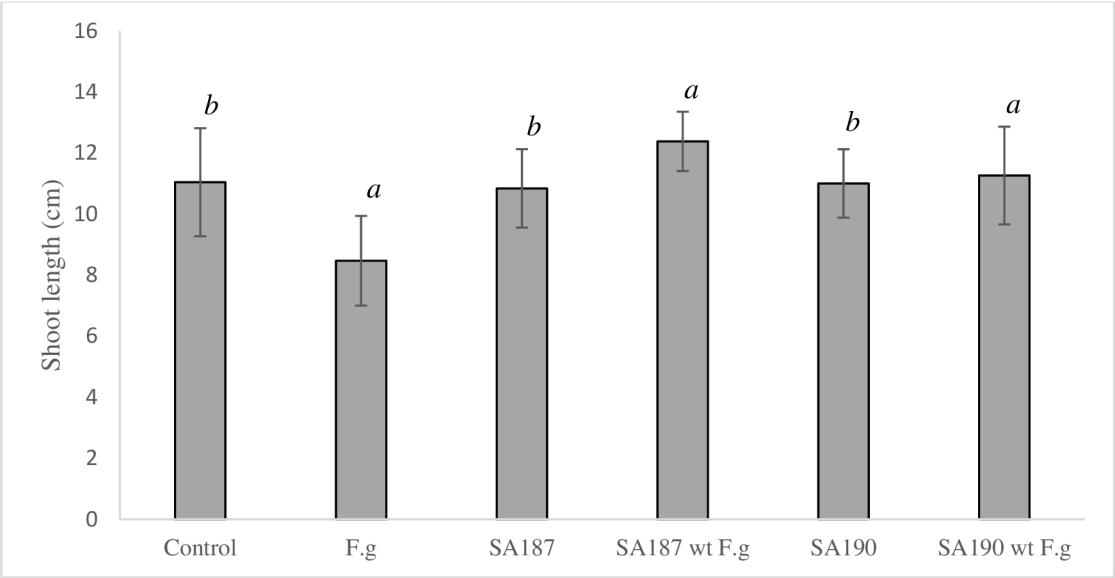

Figure 5 – Graphical representation of the average shoot lengths of barley plants after 10 days of growth. Plants with bacteria were measured 9 days after the beginning of co-cultivation. Plants with bacteria and *Fusarium* or *Fusarium* only were measured 8 days after the treatment with *Fusarium.* Data is represented as mean ± SD, n = 20; $p \leq 0.05$.

Based on their results on maize seedlings, Zhou *et al.* (2018) stated, that *F. graminearum* inoculation causes shoot elongation, which is dependent on dosage and *Fusarium* genotype. The shoot elongation occurrence upon *F. graminearum* infection is closely linked to seedling survival. Observing of shoot elongation may be used in determining of seedling resistance (Zhou *et al.,* 2018).

4.2 Microscopic analysis

4.2.1 Progression of *F. graminearum* in roots in presence and absence of bacteria

Observing of the viability of cells is a great index of a damage caused by *F. graminearum*. PI, which is a membrane non-permeable intercalating agent, allows the visualization of a non-viable cell by entering its disrupted plasma membrane. This compound subsequently intercalates with DNA, forming a bright red fluorescent complex in a nucleus. PI also stains the cell wall of both live and dead cells.

The vast progression of *F.graminearum* is indicated by large number of dead cells, which are well-visible on plants which were not co-cultivated with any bacteria. In addition, many fungal hyphae were observed in the intracellular spaces of the root epidermal cells. The number of dead cells was clearly higher in case of the pathogen alone treated roots (Fig. 6 D), whereas the number of dead cells was significantly lower in case of plants co-cultivated with *Enterobacter* sp. SA187. It is also visible from the images that the bacteria successfully colonized the intercellular spaces (Fig. 6 B). The *F. graminearum* mycelia were hardly found on the surface of the plants which were co-cultivated with *Pseudomonas* sp. SA190. Moreover, a major bacterial colonization with no fungal hyphae presence was visible on these roots (Fig. 6 C). It was quite interesting to find that the biofilm in the case ofSA190 was quite uniform and big whereas in the case of SA187 was plaque-like and small. It was found to have a significantly opposite effect in the growth since the growth induction was much higher in case of SA187 (Figure 4 & 5).

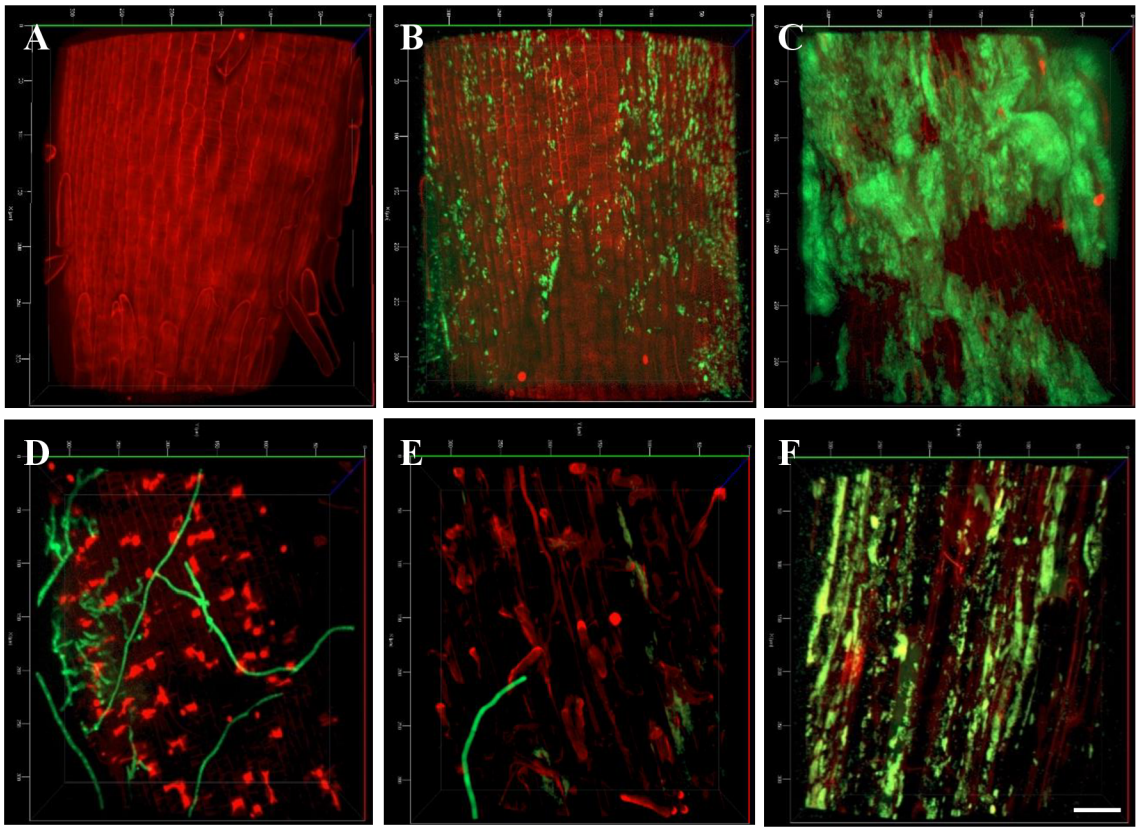

Figure 6 - Live dead staining of WT barley when treated with *Fusarium graminearum*. A- Untreated control, **B -** WT plants with SA187 (*Enterobacter* sp.); **C -** WT plants with SA190 (*Pseudomonas argentinensis*); **D -** WT plants with *Fusarium graminearum*; **E -** WT plants with SA187 (*Enterobacter* sp.) & *Fusarium graminearum*; **F -** WT plants with SA190 (*Pseudomonas argentinensis*) & *Fusarium graminearum.* Images were obtained by spinning disk microscope (Plan-Apochromat 20×/0.8 NA), scale bar = 50 µm

When the root tips were observed in bright-field microscope, the roots of WT barley were heavily colonized in the absence of endophytic bacteria, and the root tip was severely damaged and deformed (Fig. 7 A, B, C). The hyphae of the pathogenic fungi are visible in the vascular bundles and columella of plants co-cultivated with *Enterobacter* sp. SA187, but the damage caused by the fungi is significantly lower, and the root tip is in a better condition (Fig 7 D, E, F). Even lesser colonization of vascular bundles can be observed in the case of plants co-cultivated with *Pseudomonas* sp. SA190 (Fig. 7 G, H, I).

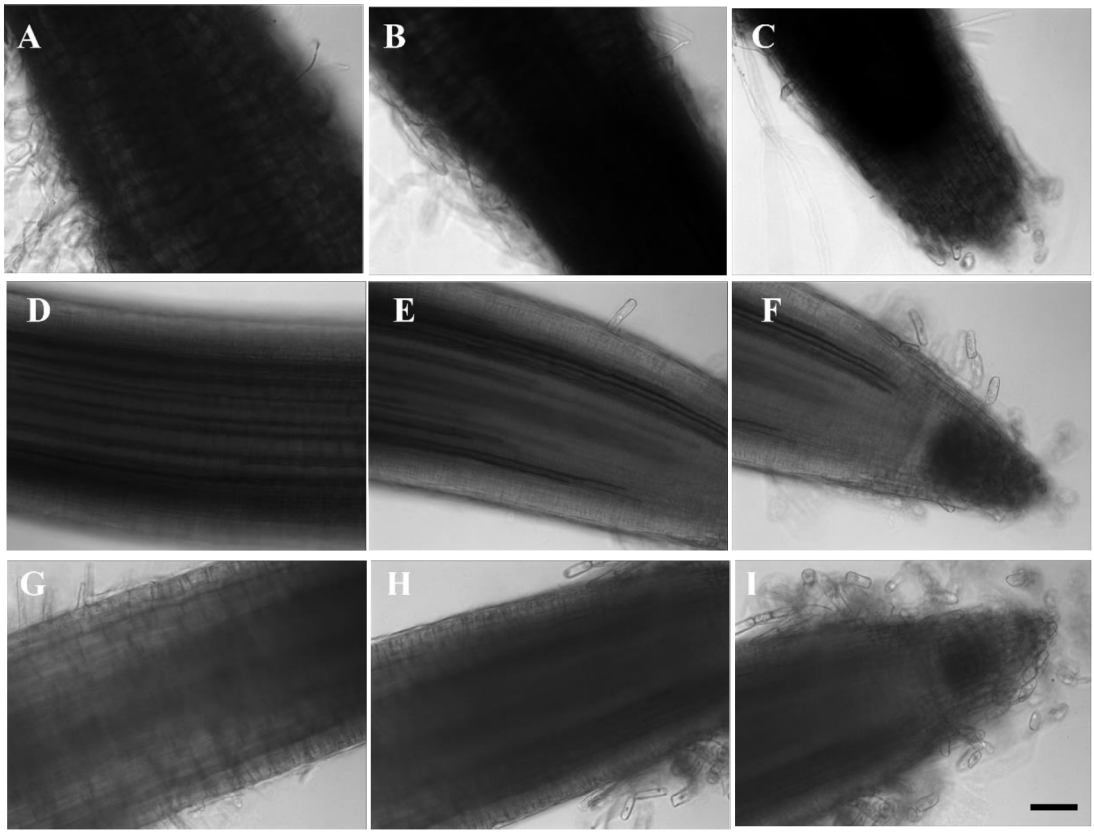

Figure 7 Difference in the root tip morphology of barley WT with and without bacteria (*Pseudomonas* and *Enterobacter* sp.) when treated with *Fusarium graminarum*. A-C – WT plants with *Fusarium*; D-F – WT plants with SA187 (*Enterobacter* sp.) & *Fusarium*; G-I – WT plants with SA190 (*Pseudomonas argentinensis*) & *Fusarium*. Images were obtained by bright-field microscope (Plan-Apochromat 20×/0.8 NA), scale bar = 50 μm.

Regarding the *Enterobacter* sp. SA187, its qualitative evaluation revealed that they solubilize zinc and produce siderophores (Andrés-Barrao *et al.,* 2017). Moreover, these bacteria possess mechanisms for iron acquisition, such as iron uptake transporters and synthesis of siderophore receptors, which are especially essential in exploitation of siderophores produced by other microbes. All these mechanisms contribute to protection of the plant by dispossessing iron from the pathogens (Taghavi *et al.,* 2010; Andrés-Barrao *et al.,* 2017). Following these findings, it is presumable that the microbial mechanisms mentioned above were at least partially responsible for reduced colonization of barley roots by *Fusarium* and thus lowered the damage induced upon these roots.

Pseudomonads, among which belongs *Pseudomonas argentinensis* SA190, might lower the impact of pathogens on plants by activating plant defence mechanisms (Henkes *et al.,* 2011). These bacteria activate ISR, leading to lowered pathogen impact on the plant (Pieterse *et al*., 2003). It was found out, that infected plant roots alert pseudomonads and stimulate their 2,4-diacetylphloroglucinol production, an antifungal bacterial toxin inducing ISR (Iavicoli *et al.,* 2003; Jousset *et al.,* 2011). In addition, they can inhibit the growth of wide variety of pathogens by synthesizing many antifungal compounds, and thus stop the evolvement of a disease in a plant (Compant *et al.*, 2005).

Currently, management of *F. graminearum* seedling blight in most countries is done with the help of standard seed fungicide treatment which is not always sufficient, and which also reported to promote the development of resistance in the pathogenic fungal population. Use of antagonistic microorganisms could be an effective alternative for the inhibition of *F. graminearum* infections if we could find a potential microbe. *Paenibacillus polymyxa* exhibited potent inhibition to *F. graminearum* growth and DON production under greenhouse conditions (He *et al*., 2009). Twenty-two bacterial strains, isolated from wheat anthers, of which nine strains significantly reduced both the disease severity and DON content in spikes, and five strains even decreased the mycotoxin to undetectable levels (Palazzini *et al.,* 2007). In the present study, an attempt was made to identify bacteria with biocontrol properties which could be used in the field for inducing resistance against *F. graminearum* induced blight and root rot in cereal crops.

5 Conclusion

The theoretical part of the thesis was mainly focused on describing and clarifying the properties of PGPB, specifically the rhizobacteria, which form a considerable part of the bacterial communities. Attention was paid principally to beneficial endophytic bacteria. Their various mechanisms of plant growth promotion were mentioned and explained briefly. Endophytic bacteria *Enterobacter* sp. SA187 and *Pseudomonas* sp. SA190 were introduced, and relevant up to date findings about these strains were cited. Plant pathogenic fungi were as well an integral part of the work, with *F. graminearum* representing the biotic part of the topic of the thesis.

The influence of *Enterobacter* sp. SA187 and *Pseudomonas* sp. SA190 on plants has been scarcely investigated so far, and no investigations have been done about their influence on barley plants under biotic stress. Due to this fact, the goal of this work was to bring some better understanding of the effects and behavior of *Enterobacter* sp. SA187 and *Pseudomonas* sp. SA190. The results support assumptions about beneficial properties of these bacteria upon co-cultivation with barley under biotic stress. They showed the ability to significantly enhance plant growth and to lower the damage on barley roots in the presence of *F. graminearum.* Nevertheless, the plant-growth promoting mechanisms apparently differ in each strain, and separate investigations of the bacteria should be done to understand their abilities properly.

6 References

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7 List of symbols and abbreviations

ABC transporters – ATP-binding cassette transporters

ACC – 1-aminocyclopropane-1-carboxylic acid

cv. – cultivar

DON – deoxynivalenol

EDTA – ethylenediaminetetraacetic acid

FHB – *Fusarium* head blight

FRR – *Fusarium* root rot

GFP– green fluorescent protein

IAA – indole-3-acetic acid

ISR – induced systemic resistance

JA – jasmonic acid

OD – optical density

PGPB – plant growth promoting bacteria

PGPR – plant growth promoting rhizobacteria

PI – propidium iodide

Sp. – species of bacteria

Spp. – subspecies of bacteria

v/v – volume to volume

WT – wild type

ZEA – zearalenone