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List of abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ARDRA	Amplified rDNA Restriction Analysis
CAS	Chromium-azurol S
Cfu	Colony forming units
CO_2	Carbon dioxide
DAPG	2,4-diacetyl phloroglucinol
FAM	Filtration acclimatization method
Fe	Iron
Fig	Figure
HCL	Hydrogen choride
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
$MgSO_4$	Magnesium sulfate
Min	Minutes
NH_4^+	Ammonium
N_2	Nitrogen gas
NaOH	Sodium hydroxide
NO_3^-	Nitrate
OA	Organic acids
OD	Optical density
P	Phosphorus
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
Rpm	Revolutions per minute
S	Seconds
SID	Siderophores
ST	Sucrose Tryptone
Tris-HCl	Tris hydrochloride
TSA	Tryptic soy agar

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Abstract (English)

INTRODUCTION The isolation of cultivable endophytic bacteria is very important regarding to the applications in which they can be used. Important applications of endophytic bacteria are for example the use of crop plants with higher biomass that are used for biofuel or for removing metals from contaminated soils. The cooperation between plants and endophytic bacteria is very important to increase the growth and thus the biomass of crops. In order to have a clear view of which endophytic bacteria can be used to increase the growth of plants, isolation and cultivation of plant bacteria is necessary. Isolation of a high diversity of cultivable endophytic bacteria is important to be able to use these bacteria to increase the plant growth. However, molecular-based approaches to study microbial ecology generally reveal a broader microbial diversity than what can be obtained by cultivation methods.

OBJECTIVES (1) This study aimed to improve the success and diversity of isolating endophytic bacteria from *Cucurbita pepo*. (2) A second objective of this study is to unravel which bacterial species might be obligate and facultative.

METHODS (1) In order to achieve a successful isolation with high diversity of endophytic bacteria, this study employed approaches such as different isolation methods (acclimatization, filtration acclimatization method), the use of different isolation media, the addition of plant extract and the use of different gelling agents (agar and gellan gum). The cultivable bacteria isolated from the roots, shoots, seeds and rhizosphere were counted, based on culture morphology. Next, the isolated strain species were phenotypically characterized by testing for different available agents such as organic acids, Indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores. Furthermore, the strains were genotypically characterized by 16S rRNA gen restriction analysis and sequencing. (2) The complete seed endophytic population, the root and shoot associated bacterial population and the rhizosphere colonizing bacteria were compared to unravel which bacterial species might be obligate and facultative.

RESULTS (1) The results show that the use of other isolation methods such as acclimatization, filtration acclimatization and the addition of plant extract or another gelling agent, in addition to the standard isolation procedure (plating directly on plates and incubate them) isolated more different strains in total in comparison when only one method is used. (2) A comparison of the complete isolated endophytic populations revealed that more obligate endophytes were isolated than facultative. However, it is thought that the total population is dominated by facultative endophytes or that obligate endophytes are more difficult to cultivate. This might be an indication that using different isolation methods, gelling agents and adding plant extract in addition to the standard isolation procedure can increase the cultivation and isolation of obligate endophytes.

CONCLUSION (1) The results pointed out that isolation of cultivable bacteria is based on specific parameters such as sufficient amount of nutrients, plant material, time to adjust to the environment and a habitat in this environment. Therefore, optimization of the number and diversity of isolated cultivable bacteria require a parallel application of different isolation methods. (2) A distinction between which endophytes were obligate and which facultative was made. However, characterization of total bacterial populations need to occur to find an indication of the cultivable fraction from the total population.

Abstract (Dutch)

INLEIDING De isolatie en cultiveerbaarheid van endofytische bacteriën zijn niet alleen belangrijk om de samenwerking tussen plant en bacteriën beter in beeld te kunnen brengen maar ook belangrijk aangaande hun toepassingen. Een voorbeeld van een belangrijke toepassing is het gebruik van endofytische bacteriën om plantengroei te stimuleren om uiteindelijk een hogere biomassa te verkrijgen. Deze biomassa wordt vervolgens gebruikt om bijvoorbeeld biobrandstof te produceren. De samenwerking tussen planten en endofytische bacteriën is het basisprincipe van de uiteindelijke plantengroei en biomassa stimulatie. Om een duidelijk beeld te krijgen welke endofytische bacteriën gebruikt kunnen worden voor de stimulatie van plantengroei, is de isolering en cultivering van endofytische bacteriën nodig. De isolatie en cultivatie hebben als doel een hoog aantal endofytische bacteriën met hoge diversiteit te karakteriseren zodat een aantal van deze endofytische bacteriën gebruikt kunnen worden voor de plantengroei stimulatie. Het probleem situeert zich in deze hoge aantallen en hoge diversiteit die door middel van één enkele methode redelijk beperkt blijven.

OBJECTIEVEN (1)Deze studie baseert zich op het verbeteren van het succesvol isoleren van hoge aantallen endofytische bacteriën met een hoge diversiteit van de *Cucurbita pepo* plant. (2) Een tweede objectief van deze studie is het achterhalen welke geïsoleerde bacteriële soorten obligaat zijn en welke facultatief.

METHODEN (1) Om een succesvolle isolatie van endofytische bacteriën met hoge diversiteit te bereiken, werden verschillende toepassingen in deze studie gebruikt zoals verschillende isolatie methoden (acclimatisatie, filtratie acclimatisatie methode), verschillende isolatie media met plantenextract en verschillende gel agens (Agar en Gellan gum). De cultiveerbare endofytische bacteriën geïsoleerd van de wortels, scheuten, zaden en rhizosfeer werden geteld op basis van hun cultuur morfologie. Vervolgens werden de geïsoleerde soorten fenotypisch gekarakteriseerd door deze endofytische bacteriën te testen voor verschillende aanwezige substanties zoals organische zuren, Indol-3-acetaat zuur (IAA), 1-aminocyclopropaan-1-carboxylaat (ACC) deaminase en sideroforen. Naast de fenotypische karakterisering werd er ook nog een genotypische karakterisering uitgevoerd door middel van een 16S rRNA gen restrictie analyse en sequencing. (2) De volledige endofytische populaties van de zaden, wortels, scheuten en rhizosfeer werden met elkaar vergeleken om bacteriële soorten obligaat of facultatief te benoemen.

RESULTATEN (1) Uit de resultaten blijkt dat het gebruik van andere isolatie methoden zoals acclimatisatie, filtratie acclimatisatie en het toevoegen van plantenextract of een ander gel agens naast de standaard isolatie procedure (direct uitplaten en incuberen) een hogere diversiteit oplevert dan wanneer één enkele methode gebruikt wordt. (2) Alhoewel, er gedacht wordt dat de totale populatie gedomineerd wordt door facultatieve endofyten of dat obligate endofyten moeilijker te cultiveren zijn, tonen de resultaten van de volledige geïsoleerde endofytische populaties aan dat meer obligate endofyten werden geïsoleerd dan facultatieve endofyten. Dit kan erop wijzen dat de verschillende isolatie methoden, gel agens en het toevoegen van planten extract, naast de standaard isolatie procedure, de isolatie en cultivatie van obligate endofyten kan verhogen.

CONCLUSIE (1) De resultaten tonen aan dat voor het optimaliseren van de isolatie en het hierbij verkrijgen van een hogere diversiteit afhangt van bepaalde parameters, die niet allemaal in één methode terug te vinden zijn. (2) Uiteindelijk kon een onderscheid worden gemaakt van welke endofyten obligaat zijn en welke facultatief. Verder zal er een karakterisering van de totale bacteriële populatie nodig zijn om een indicatie te hebben van de cultiveerbare fractie van de totale populatie.

Introduction

1. Plant microbe interactions.

Plants are known to be associated with a wide variety of microorganisms. They can be seen as habitats for microbial growth. Seeds, roots, leaves and fruits surfaces are mostly the sites that harbour large, diverse microbial communities. The most known and dominant microbial communities associated with plants are prokaryotes and primarily members of the Bacterial Domain. Beside the prokaryotes, eukaryotic microflora including fungi, yeasts, algae, protozoa, and nematodes, bacteriophages and viruses can also be members of these microbial communities.[1,2,3] In this project, the importance of plant-associated bacteria is studied more in detail.

A grouping of plant-associated bacteria can be made, based on the nature of the interaction with their host plant. If bacteria do not directly affect the plant, they are classified as commensals. In contrast to commensals, pathogens or parasites adversely affect their host. A class of pathogens, phytopathogenic bacteria, can harm the plant in such a way that it causes local areas of cell death such as leaf spots, cancers, scabby lesions, wilts, yellowing, tissue liquefaction, and tumor formation. In addition, bacteria can also have a beneficial effect on the plant and are called mutualists. Mutualisms between bacteria and plants is divided in 2 categories namely symbioses in which close associations between the bacteria and plant are involved. A second category known as **associative interactions** in which only loose associations are involved.[1,2,3] In this research we focus on the symbiotic bacteria that are also known as plant growth promoting bacteria.

2. Plant growth-promoting bacteria

A considerable number of bacterial species, are well known to be able to exert a beneficial effect upon plant growth. This group of bacteria has been termed 'plant growth promoting bacteria' (PGPB). The beneficial effect that PGPB are able to exert on plant growth is based on the use of different mechanisms. These mechanisms can be of two types: direct and indirect.[3,4,5,6]

2.1 Direct growth promotion

Direct growth promotion can occur through N_2 fixation, Phosphorus (organic acids), Fe (Siderophores) and regulation of plant hormones.

N_2 fixation

Nitrogen is primarily found in animal and plant proteins. In order to form these proteins with biological nitrogen atoms in plants, a nitrogen source is required. This nitrogen source needs to be traced, which can occur by nitrogen fixation. Beside, nutritional nitrogen from ammonia fixed industry fertilizers, biological nitrogen fixation occurs by bacteria. Most bacteria assimilate nitrogen in the form of NH_4^+, which they use in biosynthetic pathways. However, a limited set of the nitrogen fixing prokaryotes have the ability to convert atmospheric dinitrogen to NH_4^+. Catalyzation of biological nitrogen fixation occurs by the nitrogenase complex, which converts N_2 to NH_4^+. However, plants are able to use only very specific inorganic forms of nitrogen such as ammonium (NH_4^+) and nitrate (NO_3^-). Therefore, plants need the nitrogen fixation by bacteria to convert organic nitrogen and atmospheric dinitrogen into forms that can be taken up by the plant. Additionally, associative nitrogen-fixing organisms are different in the way they interact with their plant host. They give the plant benefits through nitrogen fixation but do not enter into host species-specific mutualisms, e.g. diazotrophic endophytes and diazothrophic plant surface colonists.[1]

P (organic acids)

Phosphorus (P) is one of the major essential macronutrients for biological growth and development. Microorganisms play a central role in the natural phosphorus cycle. This cycle occurs by means of the cyclic oxidation and reduction of phosphorus compounds, where electron transfer reactions between oxidation stages range from phosphine (-3) to phosphate (+5). Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of P fertilizers. A large portion of this soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants. Organic acids and phosphatase secreting bacteria solubilize phosphate by the so called 'mineral phosphate solubilization' process. In this process the insoluble phosphates are converted into soluble monobasic and dibasic ions, which increases the phosphorous availability to plants and to the plant uptake. PGPB require carbon and nitrogen sources, and metal ions as available nutrients to accomplish the phosphate solubilizing ability.[5,7,8]

Fe (siderophores)

Iron is one of the essential nutrients for almost all forms of life. With the exception of certain lactobacilli, all known microorganisms need iron. However, the problem with Fe is that it mainly exists as Fe^{3+} and other forms (insoluble hydroxides and oxyhydroxides) which are unavailable to microorganisms. Due to the requirement of iron, bacteria developed a strategy to acquire sufficient iron. A strategy, in which metal solubilization occurs for an efficient uptake by bacteria, is the production of siderophores. Siderophores are low-molecular mass iron chelators with high association constants for complexing iron. Therefore, the successful scavenging of iron by bacteria occurs by producing siderophores, which act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. This solubilization occurs by forming bacterial Fe^{3+}-siderophore complexes which can easily be taken up by bacteria. However, facilitating the uptake of iron into bacteria, due to these formed bacterial Fe^{3+}-siderophore complexes, is not the only benefit. Several plant species have the ability to recognize and take up these bacterial Fe^{3+}-siderophore complexes. Therefore, these siderophores-producing bacteria can assist plants with an improved, beneficial iron uptake.[9,10,11,12]

Regulation plant hormones

- **Production of plant growth hormones**

One of the most known beneficial plant-associated bacterial products are the **phytohormones.** An increase in root surface area is due to these phytohormones which stimulate the density and length of root hairs. Increasing the root surface area induces an improvement of water and mineral nutrients uptake from a large volume of soil.

Indole-3-acetic acid **(**IAA) is not only the most thoroughly plant growth regulator studied but also the most commonly occurring naturally produced auxin. It is actively produced by plant-associated bacteria by de novo synthesis and by conjugates releasing IAA.[13] This bacterial IAA is beneficial for root growth and length, root hair proliferation and root hair elongation. Therefore, IAA stands in for the directing of several aspects of plant growth and development in which it also induces and regulates in a variety of processes e.g., cell division, root extension, vascularization, apical dominance and tropisms. Research has demonstrated that the effects of IAA produced by plant-associated bacteria are concentration dependent and species specific.[14,15] In addition to IAA, bacteria such as *Azospirillum* and *Pseudomonas spp*. produce cytokinins and gibberellins. However the role of PGPB-produced cytokinins and gibberellins is to date poorly understood.[16]

- **Regulation of stress hormone ethylene**

In general, an increased ethylene production is present when plants are stressed. Root growth inhibition can occur due to the increased availability of the phytohormone ethylene. 1-aminocyclopropane-1-carboxylate (ACC) is an immediate precursor to ethylene. ACC deaminase is a cyclopropanoid amino acid that is most known and a common studied plant growth enzyme. ACC deaminase is interfering and causing a reduction mechanism in the biosynthesis of ethylene. By reducing the ethylene within this mechanism, root growth is not inhibited. Therefore, plant growth and development can be stimulated.[6,17 18]

2.2 Indirect growth promotion

The decrease or prevention of deleterious effects of pathogenic microorganisms on their host is defined as **indirect** growth promotion. Competition for habitat and nutrients between pathogens and PGPB is the most obvious indirect mechanism. Therefore, only one can be present and if PGPB are available, these can indirectly promote growth. Bacteria that synthesize volatile or non-volatile antibiotics, siderophores, enzymes and other secondary metabolites such as hydrogen cyanide (HCN) can also cause this decrease or prevention of deleterious effects.[1,5,19]

Antibiotics

PGPB antagonize the root pathogens by the production of antibiotics. Antibiotics, such as ammonia, 2,4-diacetyl phloroglucinol (DAPG), oligomycin A, oomycin A, xanthobaccin and zwittermycin A are mostly produced by different antagonistic bacteria. These are broad spectrum active antibiotics of which DAPG is the most potent and most extensively studied. In order to find the root pathogens' suppression role of individual antibiotic compounds, mutation analysis, molecular tools and purified antibiotic compounds were used. Isolation of the genes that encode the responsible enzymes for synthesis of few of the already mentioned antibiotics occurred. Furthermore, the transcriptional and post-transcriptional stages were studied. As a consequence of aforementioned studies, genetic improvement of antibiotic production was facilitated which led to a rapid selection of PGPB within a large number of antibiotics resistant bacteria. HCN is an example of a volatile antibiotic produced by PGPB. It inhibits cytochrome oxidase of many organisms. The bacterial strains that produce HCN are relatively insensitive to it, due to the alternate cyanide-resistant cytochrome oxidase they possess.[1]

Siderophores

PGPB produce siderophores which are inhibitors of root pathogens. This inhibition is based on the iron limiting conditions in the rhizosphere. These iron limiting conditions will lead to competition for Fe(III) between PGPB and pathogens. The successful scavenging of iron by PGPB occurs by producing siderophores, which act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. Bacterial Fe^{3+} siderephore complexes are formed, which facilitate iron uptake into bacteria and plants. However, pathogens do not form these complexes. Therefore, in iron limiting conditions, siderophores can sequester iron from pathogens and limit their growth. However the combination of various other traits are more likely the reason for the pathogen suppression siderophores-producing PGPB.[11,20]

The diversity of mechanisms by which bacteria can promote plant growth, the estimation difficulty of enhanced growth or improved health (with small increases) and the narrow range of abiotic and biotic conditions, under which growth promotion may occur, is challenging for the identification and characterization of these mechanisms.[1,9,10,11]

3. Applications of plant growth promoting bacteria

Plant growth promoting bacteria can play an important role when plants grow on nutrient-poor soils such as sandy soils, dry soils and contaminated soils. In ecosystems with low inputs and without any fertilization or soil amendment by humans, the nutrients availability to plants are derived from atmospheric inputs and weathering of soil's minerals. The association between microorganisms and plants contribute to plant nutrition. By using PGPB, the growth of plants on these types of soils can be promoted. Promotion of plant growth by PGPB makes it possible to use these soils for biomass production. Biomass production on these nutrient-poor soils can further be used to produce bio energy/biofuel.[4,21,22]

The use of biofuel as a renewable fuel and an alternative energy source can be a promising solution for the international growth of oil prices and the pressure to reduce atmospheric emissions of CO_2, ozone. Biomass, biodiesel and ethanol, are examples that can substitute petroleum, diesel and gasoline. Therefore a global increase in the use of biofuel and a rapid expansion of biofuel production is established. However, in comparison with fossil fuels, the dynamics of the production of biofuels are different. A high input of land is needed to produce the rather 'agricultural products' on which biofuels are based. Food crops are more and more used, to meet the rapidly increasing demand of biofuel, but this becomes a problem when the demand for biofuels keeps rapidly increasing. Because of the large amount of food crops that are used to produce biofuel, not only fertile land but also food supply is used to overcome the demand of biofuels. This induces other problems such as high, peaking prices and a declining in stocks. A conflict between food and bio energy crops is created in the form of a land use competition between food and biofuels. The use of nutrient-poor soils for biofuel crop production could be a solution for this conflict. Next to nutrient-poor soils, PGPB can also be exploited for a sustainable land management on contaminated soils.[1,3,9,21,22,23,24,25]

Environmental pollution, in particular soil and water pollution, is a well-known phenomenon. A lot of soil contaminants appear in the environment where they can be toxic to plants and microorganisms. **Phytoremediation** is defined as using plants and their associated microorganisms to remediate contaminated soils. However many plant species are sensitive to contaminants and show a reduced growth level. Therefore **plant growth-promoting bacteria** can be used to protect the plants from the toxic effects of the present pollutant.[3,6,9,23,24, 26,27,28,29]

4. Where to find plant-associated bacteria

The **rhizosphere** is defined as the direct environment around the root system of a plant. A large number of microorganisms can be found colonizing the rhizosphere. Root exudates are an ideal carbon source for microorganisms. Therefore a higher number of microorganisms, with a higher density, is found in the rhizosphere; this is in contrast with the bulk soil, which is the environment in the soil right next to the rhizosphere. This phenomenon is called 'the rhizosphere effect'. The most common genera of rhizospheric bacteria are *Aeromonas*, *Azoarcus*, *Azospirillum*, *Arobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Glucanobacter*, *Klebsiella*, *Pseudomonas* and *Serratia*.[23,30,44]

The **phyllosphere** can be defined as the external region of the above-ground plant parts, including: leaves, stems, blossoms and fruits. Research demonstrated that the highest amount of microorgansism colonizing the phyllosphere can be found at the leaves. In general, the taxa that can be found in the phyllosphere represent (1) a few taxa with a relatively large number of individuals and (2) many taxa with a small number of individuals. This commonly found composition is due to the plant's exposure to fluctuations in temperature, solar radiation and water availability, i.e. factors that can introduce large and rapid changes.[23]

Endophytes are the natural inhabitants of the internal tissues of plants in which they do not cause any visible external infectious signs or negative effects on their host. In contrast to the rhizosphere and phyllosphere

bacteria, endophytic bacteria are likely to interact more closely with their host. In these very close plant-endophytic interactions, plants and bacteria live in some kind of symbiosis, where plants provide nutrients and residency for bacteria, which can, in exchange directly or indirectly result in improvement of plant growth and health.[23] The endophytic bacteria have a large diversity, not only in plant hosts but also in bacterial taxa. The most common taxa of cultivable endophytic species are *Pseudomonaceae, Burkholdenaea* and *Enterobacteriaceae*.[9,23,24, 26,27,28]

A lot of plants are reported as endophytic harboring. All sorts of plants are used to do research on endophytes. Examples of mostly reported bacterial endophytes range between the classes of α, β, γ Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria and their orders. Examples of plant species in which these bacterial endophytes are harboring, are alfalfa, banana, carrot, citrus plants, coffee, dune grasses, grass *Miscanthus sinensis*, kallar grass, lettuce, maize, marigold, pineapple, radish, rice, scots pine, sugarcane, sorghum, soybean, sweet potato, tomato, wheat, yellow lupine,…. (Table1).[6,31] Within this research we will focus on the endophytic bacteria and their beneficial effects on plants.

5. Plant colonization by bacterial endophytes

Considering plant colonization by bacterial endophytes, the knowledge of all the mechanisms involved are not yet clarified. However a known fact is that the roots are the primary site where endophytic bacteria can gain entry into plants. This can be coupled to the lowering number of microorganisms that are found going from the rhizosphere, to the roots, stem and leaves. The entrance of microorganisms in plants can occur either passively or actively.[6]

Passive ways to colonize internal plant tissues are mainly through locations of epidermal damage, which can occur due to plant growth, or through root hairs and epidermal conjunctions. Moreover natural openings in the phyllosphere, such as leaf stromata, lenticels, nectarthodes and hydrathodes are a path to enter the plant. Vector organisms, such as insects, are another way for passively entering plants.[32,33]

Examples of **active** entry in plants are observed by an increasing bacterial production of cellulase and pectinase during endophytic colonization. Cellulase is an enzyme that catalyses cellulose, an important structural component of the primary cell wall of plants. Pectinase degrades pectin, a polysaccharide substrate that is also found in the cell wall of plants. By degrading pectin, polygalacturonic acid is split into monogalacturonic acid, which is a process that softens the cell wall.[6,34,35,36,37]

Within the endophytic bacteria there is a difference between ‘obligate’ and ‘facultative’ bacteria. These are classifications in accordance to the life strategies that endophytic bacteria can or will have.

Obligate endophytes are genetically transferred and are strictly dependent on the host plant for growth and survival. Moreover, these endophytes cannot survive without their host plant. Transmission to other plants occurs vertically or via vectors. The **seed endophytes** are an example of obligate endophytes which are available in the seeds themselves. These endophytes are transferred from one generation to the next through seeds.[9,38]

F**acultative endophytes** can be found in and around the hostplant. In a certain stage of their life cycle they exist and survive outside their host plant and afterwards they can colonize the plant actively or passively.[9,38,39]

Table 1: Examples of reported bacterial endophytes and plants harboring them. Rosenblueth, M. and E. Martinez-Romero, Bacterial endophytes and their interactions with hosts. Molecular Plant-Microbe Interactions, 2006. 19(8): p. 827-837.

Endophytes	Plant species	Reference
α Proteobacteria		
Azorhizobium caulinodans	Rice	Engelhard et al. 2000
Azospirillum brasilense	Banana	Weber et al. 1999
Azospirillum amazonense	Banana, pineapple	Weber et al. 1999
Bradyrhizobium japonicum	Rice	Chantreuil et al. 2000
Gluconacetobacter diazotrophicus	Sugarcane, coffee	Cavalcante and Döbereiner 1988; Jiménez-Salgado et al. 1997
Methylobacterium mesophilicum[a]	Citrus plants	Araujo et al. 2002
Methylobacterium extorquens	Scots pine, citrus plants	Araujo et al. 2002; Pirttilä et al. 2004
Rhizobium leguminosarum	Rice	Yanni et al. 1997
Rhizobium (*Agrobacterium*) *radiobacter*	Carrot, rice	Surette et al. 2003
Sinorhizobium meliloti	Sweet potato	Reiter et al. 2003
Sphingomonas paucimobilis[a]	Rice	Engelhard et al. 2000
β Proteobacteria		
Azoarcus sp.	Kallar grass, rice	Engelhard et al. 2000; Reinhold-Hurek et al. 1993
Burkholderia pickettii[a]	Maize	McInroy and Kloepper 1995
Burkholderia cepacia[b]	Yellow lupine, citrus plants	Araujo et al. 2001; Barac et al. 2004
Burkholderia sp.	Banana, pineapple, rice	Weber et al. 1999; Engelhard et al. 2000
Chromobacterium violaceum[a]	Rice	Phillips et al. 2000
Herbaspirillum seropedicae	Sugarcane, rice, maize, sorghum, banana	Olivares et al. 1996; Weber et al. 1999
Herbaspirillum rubrisulbalbicans	Sugarcane	Olivares et al. 1996
γ Proteobacteria		
Citrobacter sp.	Banana	Martínez et al. 2003
Enterobacter spp.	Maize	McInroy and Kloepper 1995
Enterobacter sakazakii[a]	Soybean	Kuklinsky-Sobral et al. 2004
Enterobacter cloacae[a]	Citrus plants, maize	Araujo et al. 2002; Hinton et al. 1995
Enterobacter agglomerans[a]	Soybean	Kuklinsky-Sobral et al. 2004
Enterobacter asburiae	Sweet potato	Asis and Adachi 2003
Erwinia sp.	Soybean	Kuklinsky-Sobral et al. 2004
Escherichia coli[b]	Lettuce	Ingham et al. 2005
Klebsiella sp.	Wheat, sweet potato, rice	Engelhard et al. 2000; Iniguez et al. 2004; Reiter et al. 2003
Klebsiella pneumoniae[b]	Soybean	Kuklinsky-Sobral et al. 2004
Klebsiella variicola[b]	Banana, rice, maize, sugarcane	Rosenblueth et al. 2004.
Klebsiella terrigena[a]	Carrot	Surette et al. 2003
Klebsiella oxytoca[b]	Soybean	Kuklinsky-Sobral et al. 2004
Pantoea sp.	Rice, soybean	Kuklinsky-Sobral et al. 2004; Verma et al. 2004
Pantoea agglomerans	Citrus plants, sweet potato	Araujo et al. 2001, 2002; Asis and Adachi 2003
Pseudomonas chlororaphis	Marigold (*Tagetes* spp.), carrot	Sturz and Kimpinski 2004; Surette et al. 2003
Pseudomonas putida[a]	Carrot	Surette et al. 2003
Pseudomonas fluorescens	Carrot	Surette et al. 2003
Pseudomonas citronellolis	Soybean	Kuklinsky-Sobral et al. 2004
Pseudomonas synxantha	Scots pine	Prittilä et al. 2004
Salmonella enterica[b]	Alfalfa, carrot, radish, tomato	Cooley et al. 2003; Guo et al. 2002; Islam et al. 2004
Serratia sp.	Rice	Sandhiya et al. 2005
Serratia marcescens[a]	Rice	Gyaneshwar et al. 2001
Stenotrophomonas[a]	Dune grasses (*Ammophila arenaria* and *Elymus mollis*)	Dalton et al. 2004
Firmicutes		
Bacillus spp.	Citrus plants	Araujo et al. 2001, 2002
Bacillus megaterium	Maize, carrot, citrus plants	Araujo et al. 2001; McInroy and Kloepper 1995; Surette et al. 2003
Clostridium	Grass *Miscanthus sinensis*	Miyamoto et al. 2004
Paenibacillus odorifer	Sweet potato	Reiter et al. 2003
Staphylococcus saprophyticus[b]	Carrot,	Surette et al. 2003
Bacteroidetes		
Sphingobacterium sp.[a]	Rice	Phillips et al. 2000
Actinobacteria		
Arthrobacter globiformis	Maize	Chelius and Triplett 2000a
Curtobacterium flaccumfaciens	Citrus plants	Araujo et al. 2002
Kocuria varians	Marigold	Sturz and Kimpinski 2004
Microbacterium esteraromaticum	Marigold	Sturz and Kimpinski 2004
Microbacterium testaceum	Maize	Zinniel et al. 2002
Mycobacterium sp.[b]	Wheat, Scots pine	Conn and Franco 2004; Prittilä et al. 2005
Nocardia sp.[b]	Citrus plants	Araujo et al. 2002
Streptomyces	Wheat	Coombs and Franco 2003a

6. Determining factors in the isolation and characterization of endophytes.

Defining endophytic bacteria as colonizers of internal plant tissue, they can only be isolated from surface sterilized plants. Most of the bacteria that are isolated are **non-cultivable** and not useful for inoculation experiments to enhance plant growth. Only a small range of endophytic bacterial strains can be further used for cultivation. Until now, research on plant-associated bacteria is mainly focussed on cultivable members. The isolation is a critical and essential step, especially when focussed on the endophytic bacteria. Looking at the commonly used protocols for isolation, a combination of plant tissue sterilization and plating onto nutrient agar is found.[40,41,42]

Sterilization and isolation

The **sterilization step** is a first determining factor. Sterilization exists of a few steps: plant tissue washing, surface sterilization, several aseptic rinses, sterility check and the product that will be used to isolate bacteria from. Duration of sterilization is linked to purpose of the isolation, more exactly, is the aim to isolate the total bacterial population, or only the cultivable bacterial population. Isolations of total populations mostly exist of longer sterilisation steps in contrast to sterilization that occurs for cultivable population measurements. In case of isolating the **total endophytic population**, the sterilization step not only does have to eliminate all living microorganisms on the outside of the plant, additionally it has to remove all the bacterial DNA that is present on the outside as well. After the sterilization step different protocols can be used.[12,43,44,45]

Surface-sterilized plant material can be used to isolate the cultivable endophytes. This can occur by plating on **different media**. The resulting isolation will not record the viable bacteria, but only the cultivable bacteria. It will be of importance to analyse the communities with molecular cultivable independent techniques to isolate a higher rate of the bacterial taxa that are present in the plants.[44,45]

The development of the ***in vitro* culturing technique**, based on microscopic counts in contrast to the total viable counts (total population), has still problems with the unculturability. The fact that certain bacterial species have never been identified, can be due to the fact that there are organisms with a low prevalence or slow-growing organisms that can be overlooked in cultural analyses. Also a lot of genetically distinct phylotypes are phenotypically indistinguishable and are lumped together if conventional biochemical methods for identification are used. However certain bacteria can have fastidious growth requirements including the need for specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere. Analyzing the effect of **different substrates** (other media) and culture conditions, such as **filtration acclimatization method**, on the growth of bacteria can be the first step to find crucial factors that are required for cultivation.[42] The filtration acclimatization method is a method that takes the low environmental substrate conditions, in which most bacteria survive, into account. Therefore, a slow transition from this low environmental substrate to a high concentration of standard microbial media will occur. Furthermore, the addition of a filtration step will give less dominant, smaller and slowly growing bacteria a chance to cultivate. Therefore, this method can give the opportunity to isolate and cultivate bacteria that are not cultivable with other standard methods. Nevertheless, they are an important fraction of the bacterial diversity.[41,42,46,47]

Characterization

After the endophytic bacteria are isolated, they can be **characterized**. Developmental techniques, that are molecular and culture-independent, have given the opportunity to expand the characterization of **total bacterial populations**. This characterization uses PCR amplification of housekeeping genes, particularly that encodes 16S rDNA gene, for cloning and purification. Sequencing is used for identification. New

phylotypes can be added to the already identified phylotypes among bacterial communities. Even though a variety of molecular methods are available to evaluate bacterial communities; analyzing cultures are far from unessential. Only if individual bacterial species are isolated in pure culture, comprehensive characterization of physiological properties and estimation of virulence potential can be done.[42,2748]

The characterization can be done **genotypically** and **phenotypically**. Genotypic characterization determines the different species isolated, the diversity, etc. Phenotypic characterization is used to determine the activity of metabolisms within the endophytes. Different techniques are used for both characterizations.[48]

Concluding from this information, cultivable bacteria can be characterized much better than non-cultivable bacteria. Characterization can occur in different forms e.g. genotypically, phenotypically and at different levels, total bacterial population or cultivable bacterial population. Additionally, the ability to cultivate the latter is an added value towards applications e.g. inoculation. Within these applications, knowledge and availability of cultivable bacteria is an enrichment due to the ability to isolate and characterize them for specific purposes. Therefore cultivable bacteria can be of important interest. However, optimization of cultivation is required to bring out all the advantages of cultivable bacteria and enabling the isolation and characterization of an important fraction of bacterial diversity.

Research question

In the lab there is often observed that after the original isolation, the bacteria do not grow on new plates when they are transferred to them. Researchers think this is due to the fact that with the first isolation still a small amount of plant extract is available for the endophytic bacteria. However with the next cultivations the plant extract will not be there anymore. If we take this together with the fact that a huge number of endophytic bacteria are non-cultivable, we can hypothesize that (1) medium enriched with plant roots and shoots will increase the efficiency for cultivation. Furthermore (2) the isolation of obligate endophytic bacteria will give evidence that production of phytohormones, enhancement of solubility of nutrients and phosphates, improvement of iron uptake and synthesis of ethylene and ammonia are specific properties that could be responsible for their beneficial character.

The purpose of this research is to look at the isolation and incubation techniques that are used for endophytic bacteria and see if changing the **medium** (agar, gellan gum, plant extracts,..), the acclimatization time can lead to an increase of cultivable bacteria isolated from the plant *Cucurbita pepo*. Moreover, changing the medium in time might also contribute to a higher amount of cultivable bacteria. Using e.g. the 'acclimatization method', the concentration of rich medium is increased in time. With this method, bacteria that are less dominant and require more time for growing, are given a chance to grow and cultivate, where they did not have the chance in other methods where they are supposed to grow fast and be dominant. Additionally, a distinction can hopefully be made between obligate and facultative endophytes, based on **genotypic** and **phenotypic identification**.

Materials and methods

Cultivation of plants

In this project, *Cucurbita pepo* seeds were selected to optimize the isolation of plant-associated bacteria. Seeds were rolled in a humid paper towel and placed in a plastic bag at 30°C for 4 days. After incubation, seedlings were planted in vermiculite and put in the greenhouse. The plants were watered with ¼ Hoagland nutrient solution. After one week, the plants were used for the isolation of bacteria.

Isolation of plant-associated bacteria

1. **Seed endophytes**

In order to isolate **seed endophytes**, 5 seeds were surface sterilized by rinsing them in a 1% chloride solution for 5 min. Then the seeds were rinsed 3 times in sterile water. After the rinsing steps, the seeds were left to dry on a filter and their weight was determined. Seeds were grinded with 5 mL 10 mM $MgSO_4$ and a dilution series (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) was prepared. One hundred microliters of the dilutions were plated on 1/10 rich (869) medium and 1/10 rich (869) medium with seed extract and plates were incubated for 4 days at 30°C (table 2). After incubation, colonies were counted and the amount of colony forming units (cfu) were calculated per gram seed material. All morphologically different bacteria were purified on rich (869) solid medium and rich (869) solid medium with seed extract by applying a sterile toothpick to pick up the bacteria from the plates, dilution in a droplet of $MgSO_4$ and dotting, striping this droplet dilution on rich (869) medium with agar, rich (869) medium with agar with plant extract and plates were incubated for 3 days at 30°C.

Application of a sterile toothpick to pick up the purified bacteria, occurred to **storage** the isolated bacteria. Next, 15 mL tubes filled with 5 mL liquid rich (869) medium were used to cultivate the bacteria in for 3-5 days at 30°C. Next, the 15 mL tubes were centrifuged at 4000rpm for 20 min at room temperature. After the supernatant was discarded, pellets were dissolved in 2 mL 15% glycerol of which 1.5 mL was stored in cryogenic tubes and frozen at -80°C.

2. **Root and shoot endophytes**

The **root** and **shoot endophytes** were isolated by using (a) different media and (b) an acclimatization and filtration acclimatization method.[46]

2.1 Different media

Plants were first rinsed with water to remove the surrounding vermiculite. Roots and shoots were separated and sterilized in a 1% chloride solution for 5 min. Furthermore, roots and shoots were rinsed 3 times in sterile water. After rinsing, the roots and shoots were dried on filters and weighed. The plant material was mixed with 5 mL 10 mM $MgSO_4$ and a dilution series (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) was prepared. One hundred microliters of the dilutions was plated on **different media** (Table 2). Plates were incubated for 4 days at 30°C. After incubation, colonies were counted and cfu was calculated per gram plant material. All morphologically different bacteria were purified on rich (869) solid medium. Storage occurred under the same conditions as the isolation of seed endophytes.

2.2 Acclimatization and filtration acclimatization method

The rinsing, sterilization and drying occurred under the same conditions as the isolation of plant material on different media. A dilution series (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) was prepared and 100 µL of the dilutions was plated on 1/10 rich (869) medium. Plates were incubated for 4 days at 30°C. After incubation, colonies were counted and cfu was counted per gram plant material. The **acclimatization method** (Figure 1) occurred by adding 100 µL of the lowest dilution (0) to each well of a 24 well plate and according to the method of Hahn et al. (2004), the acclimatization steps occurred (Figure 2). The eventually remaining obtained bacterial suspension was diluted up to 20 mL with $MgSO_4$. The diluted bacterial suspension was used to filter 10 mL with a Minisart filter (Sigma Aldrich) with a pore size of 0.2 µm (Sigma Aldrich, Europe). From the bacterial filtered suspension, 100 µL was plated on 1/10 rich (869) medium with 3 repetitions and plates were incubated for 7 days at 30°C. After incubation, colonies were counted and cfu was calculated per gram plant material. Next, 100 µL of the bacterial filtered suspension was added to each well of a 24 well plate for the **filtration acclimatization method** (Figure 1). The 24 well plates were used for the filtration-acclimatization method according to Hahn et al. (2004), given in the scheme in figure 2 with rich (869) medium and 284 medium.

Table 2. Media that were used to isolate bacteria from different compartments.

Compartment	Media	Gelling agent	Extra addition
Plant endophytes (Root and shoot)	1/10 rich (869) medium	agar	
	1/10 rich (869) medium	agar	+ autoclaved shoot extact
			+ autoclaved root extact
			+ filter sterilized shoot extract
			+ filter sterilized root extract
	1/10 rich (869) medium	gellan gum	
	1/10 rich (869) medium	gellan gum	+ autoclaved shoot extact
			+ autoclaved root extract
			+ filter sterilized shoot extract
			+ filter sterilized root extract
	1/10 284 medium	agar	
	1/10 284 medium	gellan gum	
	TSA medium	agar	
	TSA medium	gellan gum	
	Casein-Starch medium	agar	
	Casein-Starch medium	gellan gum	
	M3 medium	agar	
	M3 medium	gellan gum	
	M9 medium	agar	
	M9 medium	gellan gum	

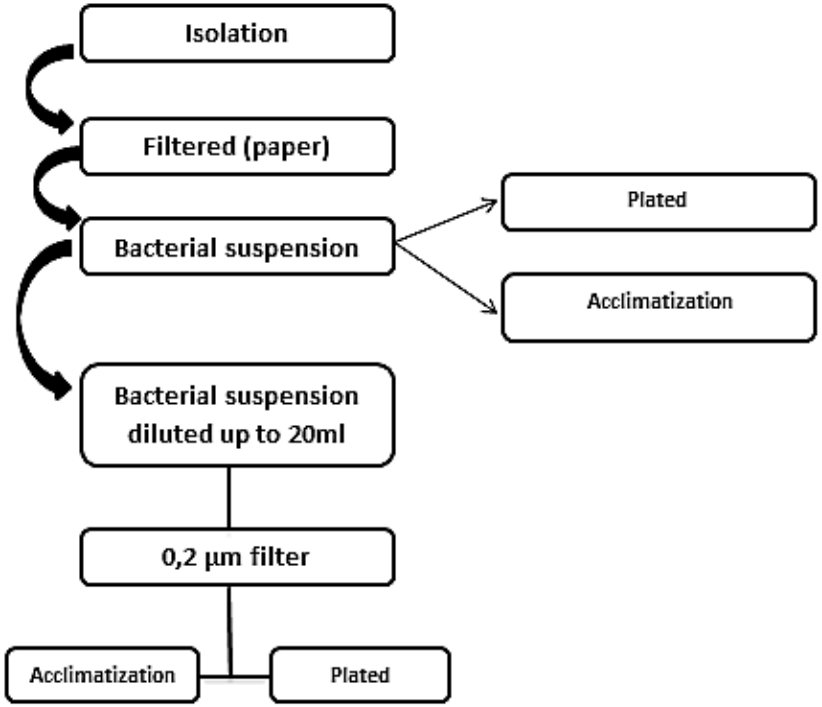

Figure 1. Set-up preparation acclimatization from isolation, equal to normal isolation, until plating and acclimatization with and without filtration method according to the method of Hahn et al. (2004).[46]

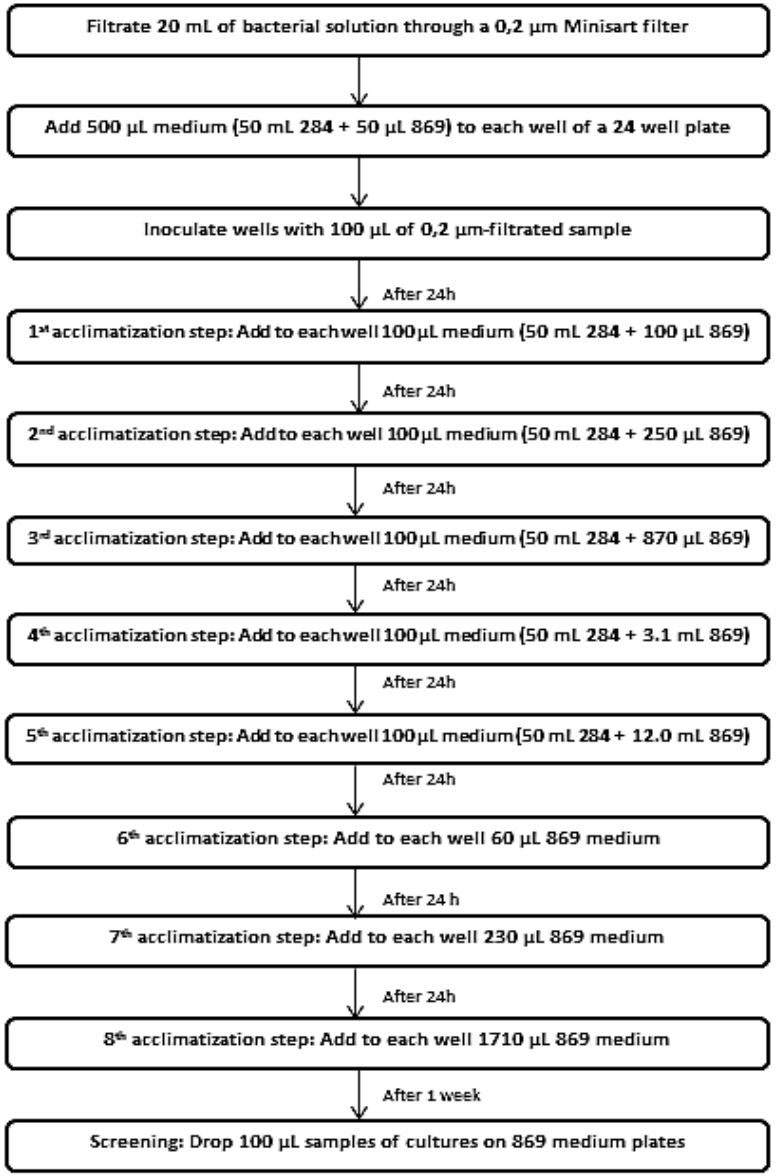

Figure 2. Standard FAM protocol (Hahn et al. 2004) of the stepwise addition of higher amounts of rich (869) liquid medium to give bacteria that are less dominant and require more time for growing the opportunity to grow and cultivate, which they do not have in other methods where they are supposed to grow fast and be dominant.[46]

3. Rhizosphere bacteria

R**hizosphere bacteria** were isolated using vermiculite from the plants' rhizosphere. The vermiculite was grinded in 5 mL 10 mM $MgSO_4$ and a dilution series (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) was prepared. Unused (clean) vermiculate was used as a control.

Plates of 1/10 rich (869) medium with agar, 1/10 rich (869) medium with gellan gum, 1/10 rich (869) medium with gellan gum and root extract were used to plate 100 µL of the dilutions and plates were incubated for 7 days at 30°C. After incubation, colonies were counted and cfu was calculated per gram vermiculite. All morphologically different bacteria were purified on rich (869) solid medium.

Next, 100 µL of the lowest dilution (0) was diluted in Erlenmeyer flasks with 50 mL of rich (869) liquid medium, rich (869) liquid medium with root extract and were incubated at 30°C. Optical density (OD) measurements of the liquid cultivations occurred daily under sterile conditions until maximum OD measurements (OD > 1) were obtained. After liquid cultivation, a dilution series (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) was prepared. One hundred microliters of the dilutions was plated on 1/10 rich (869) medium with agar, 1/10 rich (869) medium with agar with root extract, 1/10 rich (869) medium with gellan gum, 1/10 rich (869) medium with gellan gum and root extract. Next, the plates were incubated for 7 days at 30°C. After incubation, colonies were counted and cfu was calculated per gram vermiculite.

All **purifications** occurred by applying a sterile toothpick to pick up the bacteria from the plates, dilution in a droplet of $MgSO_4$ and dotting, striping this droplet dilution on rich (869) medium with agar, rich (869) medium with agar with plant extract and plates were incubated for 3 days at 30°C.

The **storage** of isolated bacteria occurred by applying a sterile toothpick to pick up the purified bacteria. The bacteria were cultivated in 15 mL tubes filled with 5 mL liquid rich (869) medium, at 30°C for 3-5 days. Next, the 15 mL tubes were centrifuged at 4000rpm for 20 min at room temperature. After the supernatant was discarded, pellets were dissolved in 2 mL 15% glycerol of which 1.5 mL was stored in cryogenic tubes and frozen at -80°C.

3.1 Phenotypic characterization of isolated bacteria

Some specific plant growth promoting features of all purified morphologically different bacteria were screened such as the capability to (a) produce siderophores (Sid), (b) organic acids (OA), (c) auxin indol-acetic-acid (IAA) and (d) 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Therefore, 10 µL of the bacterial glycerolstocks was cultivated in 96 well masterblocks containing 1mL liquid rich (869) medium. This bacterial suspension is further used to screen for phenotypic characteristics.

Siderophores

The bacterial suspension was used to detect which bacteria produced siderophores. Therefore, three 48-well plates containing 800 µL of 284 medium without iron, with 25 µM iron and 3 µM iron were used to add 20 µL of each bacterial suspension. The 48-well plates were incubated at 30°C, 200 rpm during 5 days. Siderophore production was detected with the method of Schwyn and Neilands (1987) by adding 100 µL of blue chromium-azurol S (CAS) reagent to all 48-well plates. After 4 hours, orange wells were considered as positive and blue wells were considered as negative.

Organic acids

The detection of organic acid production occurred by adding 20 µL of each bacterial suspension to 96 well masterblocks containing 800 µL of a Sucrose Tryptone (ST) medium. After 5 days of incubation at 30°C and 200 rpm, organic acids were detected according to the method of Cunningham and Kuiack (1992) by adding 100 µL of 0.1% alzarine red S pH indicator to all the 96 well masterblocks. After 15 min, yellow wells were considered as positive and pink indicated a negative result for organic acids.

IAA

The cultivated bacterial glycerolstock suspension was used to detect IAA production. Therefore, 10 µL bacteria were grown in the dark in 1 mL rich (869) medium with 0.5g/L tryptophan, a precursor of IAA, in 96 well masterblocks for 5 days at 30°C and 150 rpm. IAA production capacity was detected by using the Salkowski assay, adapted from Patten and Glick (2002) by centrifuging 96 well masterblocks at 4000g for 10 min, mixing 0.5mL supernatants with 1mL Salkowski's reagent. After 20 min, pink wells were considered as positive and yellow wells were considered as negative for IAA production capacity.

ACC deaminase

The cultivated bacterial suspension was centrifuged at 4000g for 15 min at room temperature. Next, 10 mM $MgSO_4$ was used to rinse the pellets 2 times. The pellets were resuspended in 500 µL $MgSO_4$ of which 205 µL was added to 1.2 mL salts minimal medium supplemented with 5 mM ACC. After incubating the bacterial suspension at 30°C during 3 days, the method of Belimov et al. (2005) was used to detect ACC deaminase production. The masterblocks were centrifuged at 4000 g for 15 min and the pellets were resuspended with 100 µL 0.1M Tris-HCl buffer. Cells were disrupted by adding 3 µL toluene. Then, 40 µL 0.5M ACC and 100 µL 0.1M Tris-HCl buffer for 30 min at 30°C, 690µL of 0.56N HCL and 150 µL 0.2% 2,4-dinitrophenylhydrazine in 2N HCl and 2N NaOH was added to the bacterial suspensions. A brown or yellow coloration of wells was considered respectively positive or negative for ACC deaminase production.

3.2 Genotypic characterization of isolated bacteria

DNA extraction

In order to extract genomic DNA, 10 µL bacteria of the glycerolstock were first grown in 2 mL rich (869) medium in 96 well masterblocks. **Extraction of total genomic DNA**, from all morphologically different purified bacteria, was performed using the **DNeasy® Blood and Tissue kit** (Qiagen). After 3 days of incubation at 30°C, total genomic DNA was extracted by centrifuging 1.5 mL bacterial solution during 10 min at 7500rpm. The supernatant was removed and the pellet was dissolved in 180 µL enzymatic lysis buffer. After disrupting the cells, DNA was attached to the DNeasy mini spin column membrane, which was used to do the washing steps. Finally, 300 µL DNA extract was obtained and stored at 4°C.

PCR

A polymerase chain reaction (PCR) of 16S rDNA was carried out per 96 reactions with 49 µL of PCR mastermix (Invitrogen, Carlsbad, CA, USA) and 1 µL sample. Amplification of the 16S rDNA occurred with the universal 1392R (5'-ACGGGCGGTGTGTGTRC-3') and the bacteria-specific 26F (5'-AGAGTTTGATCCTGGCTCAG-3') primers, according to the method described previously by Weyens et al. (2009). Cycling conditions consisted of: one denaturation cycle at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 72°C for 30s, and 72°C for 3 min and completed with an extension cycle of 10 min at 72°C and storage at 4°C.

Distinguishing the different 16S rDNA samples occurred by directly using the PCR products for the Amplified rDNA Restriction Analysis (ARDRA) which was performed with the *HpyCH4IV* restriction enzyme, a four-base-specific restriction endonuclease, which digested the PCR products at AC-bases. Therefore, DNA digestion occurred by adding 15 µL of digestion mastermix to 35 µL sample and incubating at 37°C for 2 hours. Finally, digestions products were examined by adding 3 µL loading dye to 5 µL of the amplified samples. Samples were loaded on a 1.5% agarose gel and separated with gelelectrophoresis, which ran at constant 90V for 2.5 hours. Furthermore, visualization occurred by using UV illumination. ARDRA patterns from bacterial strains that were the same, were grouped and one representative strain was selected for sequencing. In order to determine the sequences, PCR samples were send to Macrogen Europe (Amsterdam, The Netherlands) for sequencing. DNA sequence assembling, editing and sequence analysis occurred by using Staden Package (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Within Staden Package, pregap4 was used for base calling, end clipping and vector trimming. Gap4 was used for sequence assembly, contig editing and finishing. Sequence Match at the Ribosome Database Project was used for species identification. Furthermore, the number of identified species was calculated and visualized in pie charts and venn diagrams. The diversity index was calculated according to the Shannon-Wiener index and visualized with bar charts.

Results and discussion

In the first part of this work, we investigated if the proportion of cultivable endophytic bacteria can be increased by optimizing the isolation procedure. Different media (1.1) as well as different isolation procedures (1.2) were tested. Secondly, the optimized protocols are used to unravel the borderline between obligate and facultative endophytes by comparing cultivable bacterial populations of the roots, the shoots (characterized in part 1) with populations from the seeds (2.1) and the rhizosphere (2.2).

1. Optimization of isolation of cultivable endophytic bacteria

The optimization of the isolation of cultivable endophytes had the aim to isolate the highest number of cultivable bacteria and the highest diversity. In order to accomplish these objectives, the use of different media (1.1) and isolation procedures (1.2) was investigated.

1.1 Optimization of the medium that is used for isolation

In order to optimize the isolation medium, a broad variety of commonly used media were screened in combination with 2 different gelling agents (1.1.1). Next, a selection of the most optimal media was made and for these media the effect of adding plant extract for isolation of endophytes was investigated (1.1.2). Finally, a comparison of the different media was made, based on the phenotypic traits that were determined for the bacteria isolated on the most optimal media (1.1.3).

1.1.1 Screening of different commonly used media

Based on literature, common media used for the isolation of bacterial populations such as 284, 1/10 869, TSA, Casein-Starch, M3 and M9 were selected. These media have different characteristics: Casein-Starch, a medium that is mainly used to isolate *Saccharolytic marine* bacteria and *Actinomycetes*, M3 media were mainly applied for *Actinomycetes* growth, 284 medium is used as a selective nutrient-poor medium, M9 minimal salts medium is mostly used for the cultivation of *E. coli.* and 1/10 869 and TSA medium is utilized for a wide variety of fastidious and nonfastidious microorganisms. Next to the different media compositions, two gelling agents (agar and gellan gum) were screened for the optimization of isolation of cultivable endophytic bacteria.

When the total numbers (cfu/g) of cultivable bacteria that could be isolated on the different media are considered, it is clear that isolations on the rather rich media (such as 869 and TSA) resulted in higher numbers in comparison with the selective media (e.g. M3, M9 and Casein-Starch) (Figure 3). Cultivable bacteria showed the need for a medium that can be used by a wide variety of fastidious and nonfastidious cultivable bacteria such as TSA and rich (869) medium in order to isolate a high number of cultivable bacteria.

Furthermore, a comparison between the gelling agents (agar and gellan gum) total numbers (cfu/g) of cultivable bacteria showed no clear distinction between isolation results (Figure 3). There are media on which isolations resulted in the highest numbers with agar (such as 869, TSA) and there are media were the use of agar did not show this effect (e.g. 284, Casein-Starch). Some media showed the reverse where the isolations resulted in higher numbers when gellan gum was used (e.g. M9).

Together with the counting of the colonies of cultivable bacteria that could be isolated on the different media, an estimation of the diversity of the different colonies was made, based on phenotypic

distinction (such as color and form) (Figure 3). The diversity results showed that isolations on rich media (869) resulted in higher diversity in comparison with the selective media (e.g. 284, M3, M9 and Casein-Starch) and TSA medium. The latter showed mostly equal numbers of different bacteria with the selective media. In the shoots, the number of different bacteria resulted even higher than in the roots (M9). The results using different gelling agents (gellan gum and agar) showed again that for some media (e.g. 284) no differences in the number of different bacteria were made, for other media (Casein Starch) a higher diversity was shown in roots and shoots using gellan gum as gelling agent, for some media the opposite (e.g. TSA) and for some media it depended if the isolations occurred from the roots or the shoots (e.g. M9).

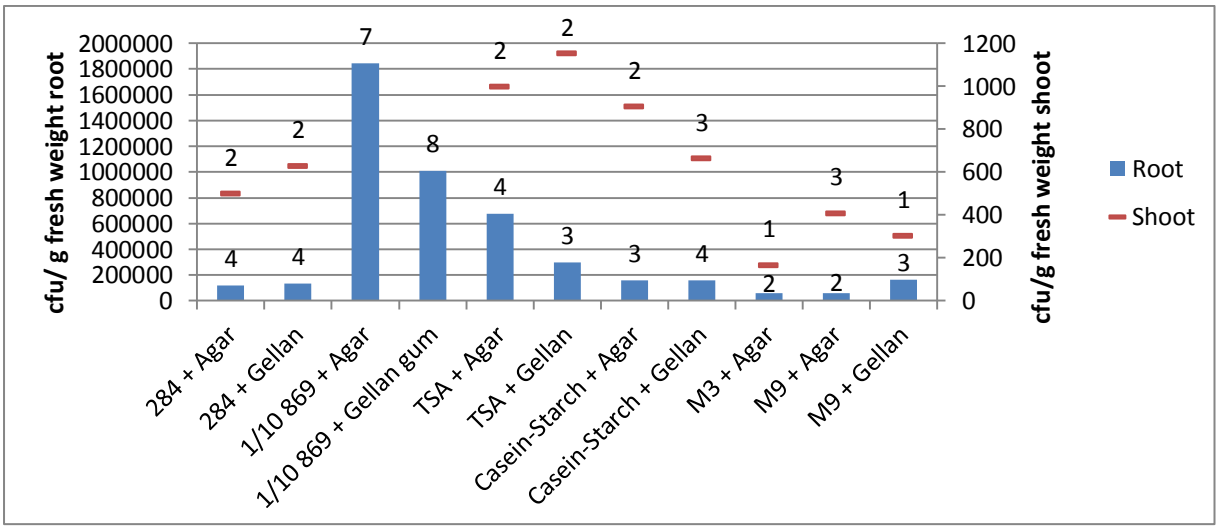

Figure 3. Number of cultivable bacteria isolated from roots and shoots on different media. The different media are 284, 1/10 rich (869), TSA, Casein-Starch, M3, M9 and two different gelling agents, agar and gellan gum. The left axis visualized the number (cfu/g plant material) for shoots and the right axis visualized the number (cfu/g plant material) for roots. Data labels refer to diversity estimations made of all the different colonies that were observed per media.

To conclude from these results, the 1/10 rich (869) medium with agar resulted in the highest number of cultivable bacteria and the highest diversity of cultivable bacteria that were isolated. Bacteria showed the need for a medium that can be used by a wide variety of fastidious and nonfastidious cultivable bacteria such as rich medium in order to isolate a high number and high diversity of cultivable bacteria. Therefore, the selective media (284, Casein-Starch, M3, M9) and the rich TSA medium were not further used to optimize the number and diversity of isolation cultivable bacteria.

The results showed that using a different gelling agent did not changed the number or the diversity of isolated cultivable bacteria on the selective media (284, Casein-Starch, M3, M9) and the rich TSA medium. However, together with the high number of cultivable bacteria isolated on 1/10 rich medium with agar, the use of gellan gum as gelling agents showed the second highest number of cultivable bacteria and the highest different cultivable bacteria isolated. Therefore, further analyses will occur using agar and gellan gum as gelling agents for 1/10 rich (869) medium.

1.1.2 The effect of plant extract addition

In the initial isolation step, there is still some plant extract available, because the surface-sterilized plant material was grinded and diluted in $MgSO_4$. However, this plant extract is no longer available in the further steps such as the purification step. Since some bacteria only survive the first isolation step and cannot be cultured anymore in the next steps, it seems that these bacteria need this extract to grow. For this reason, the effect of adding plant extract to 1/10 rich (869) medium was tested for the root and

shoot endophytic population. Addition of plant extract occurred by autoclaving the plant extract together with the culture medium.

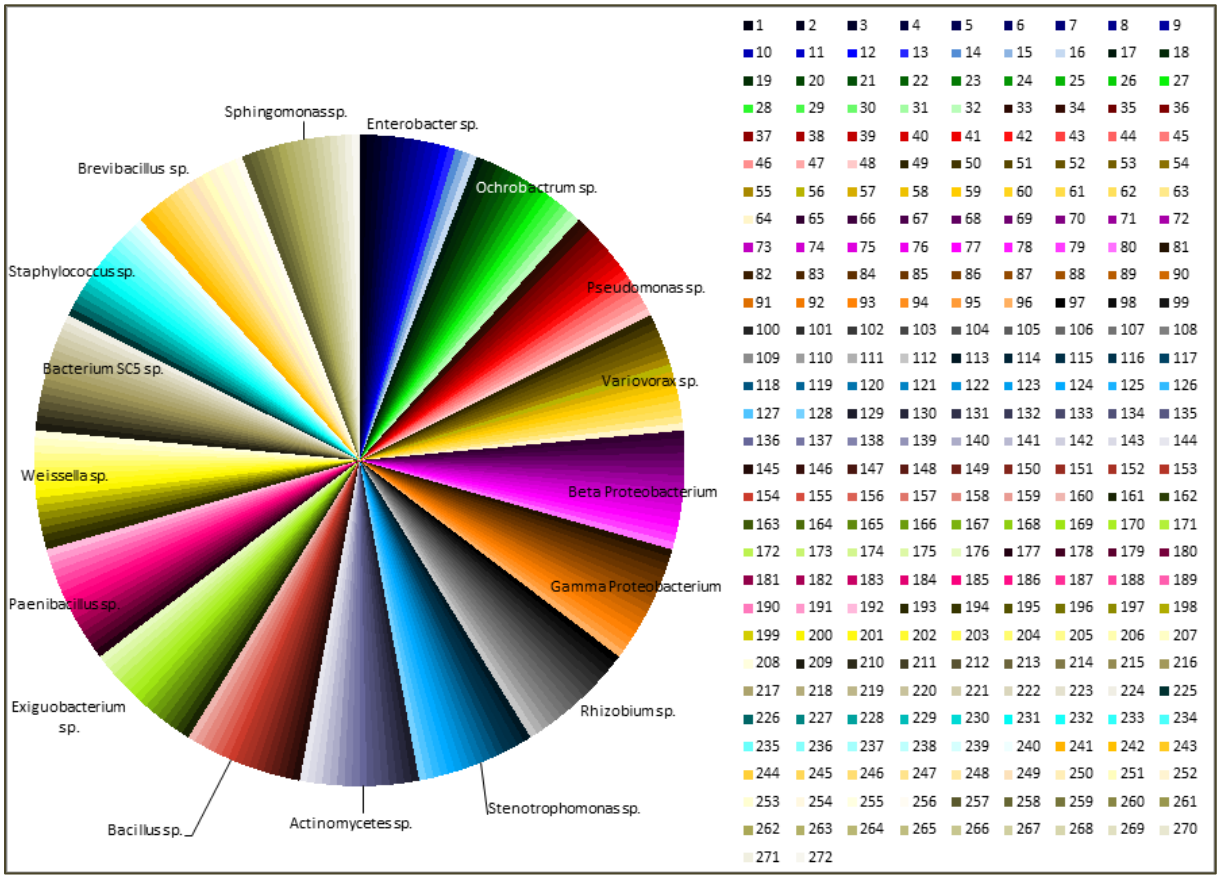

Figure 4: General legend for the different species that are used in all pie charts. For each genotypically identified species, a general different color is used. Moreover, subcolors (dark up to light) are indicating different phenotypic traits within one specific genotype. An overview of all the numbers with their genotypic as well as phenotypic information given in table 1 in attachement 5.

The total number (cfu/g) of cultivable bacteria from **the roots** (Figure 5), that could be isolated on the 1/10 rich (869) media with two different gelling agents (agar and gellan gum), with and without plant extract, indicated that isolations on 1/10 rich (869) media with agar without plant extract ($1.08\ 10^{+6}$) and gellan gum without ($2.90\ 10^{+6}$) and with plant extract ($1.86\ 10^{+6}$) resulted in more or less the same numbers for the medium, except for the isolations on 1/10 rich (869) medium with agar with plant extract ($8.20\ 10^{+5}$), which resulted in slightly lower numbers (Figure 5). The results regarding the total number (cfu/g) of cultivable bacteria from **the shoots** (Figure 6), that could be isolated on the 1/10 rich (869) media with agar without (3.70 10+5) and with plant extract (5.18 10+5) and gellan gum without (2.96 10+5) and with plant extract (6.54 10+5), were also more or less the same.

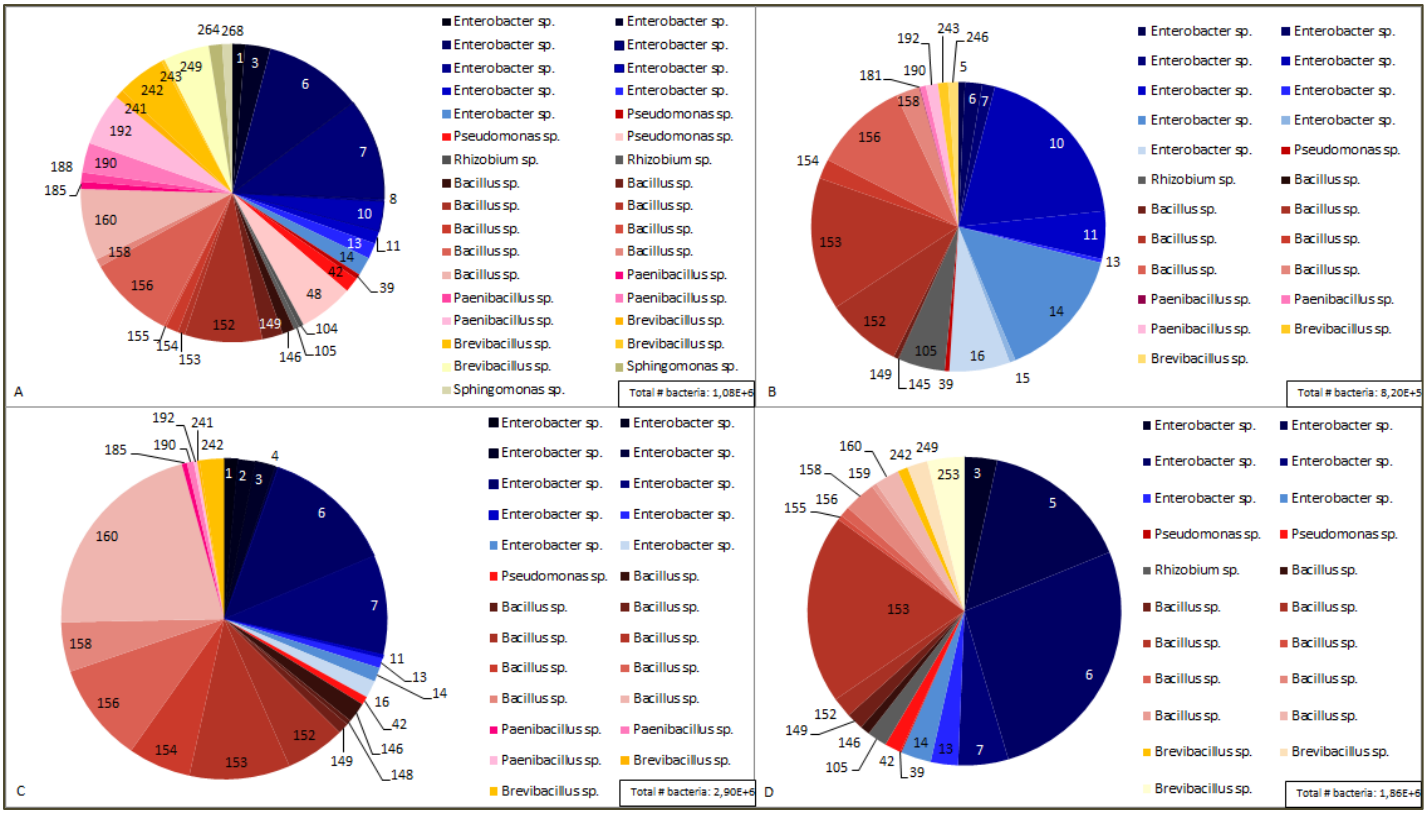

Figure 5: Different species of cultivable bacteria that could be isolated from the roots on 1/10 rich (869) medium with agar with (B) and without (A) root extract and on 1/10 rich (869) medium with gellan gum with (D) and without (C) root extract. The numbers refer to the unique numbers that are shown for each species in figure 4 and tables 2, 3, 4 and 5 of attachment 5.

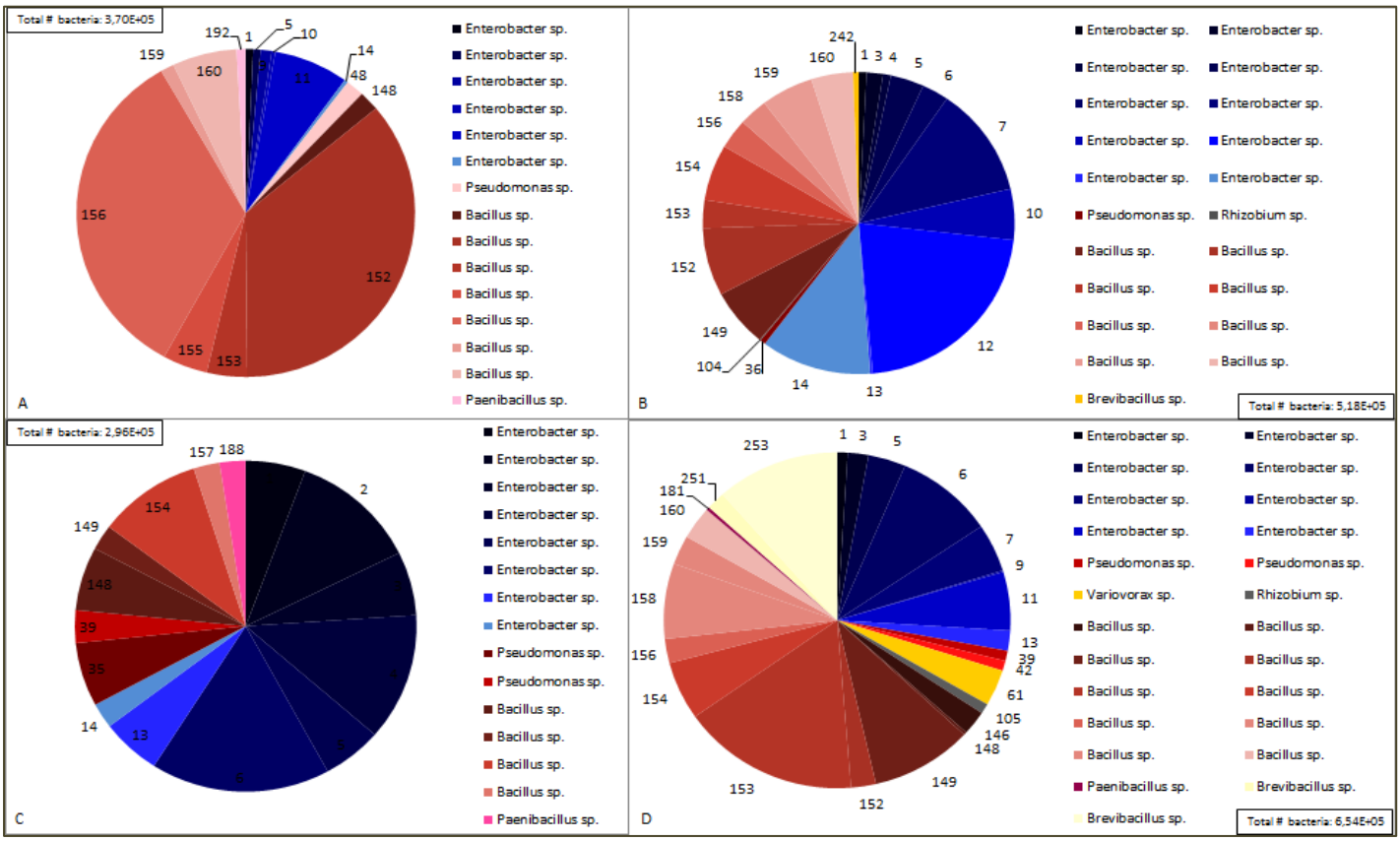

Figure 6: Different species of cultivable bacteria that could be isolated from the shoots on 1/10 rich (869) medium with agar with (B) and without (A) shoot extract and on 1/10 rich (869) medium with gellan gum with (D) and without (C) shoot extract. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 6, 7, 8 and 9 of attachment 5.

Besides, the total number (cfu/g) of cultivable bacteria, that could be isolated on the 1/10 rich (869) media with agar or gellan gum, with or without plant extract, a **diversity index** (Figure 7) was calculated, using the Shannon-Wiener diversity index. The higher the Shannon-Wiener index, the higher the diversity. In order to compare the effect of the plant extract addition to 1/10 rich (869) medium with agar or gellan gum, the diversity index was used. The results from **the roots** showed the highest diversity on the 1/10 rich (869) medium with agar without root extract (1.88) and slightly lower when root extract was added (1.63). When root extract was added to 1/10 rich (869) medium with gellan gum, this addition resulted in a higher diversity (1.55) in comparison with the medium with gellan gum, without root extract (1.34). However, the diversity of cultivable bacteria that could be isolated from the roots was the highest on 1/10 rich (869) medium with agar, different species were isolated with the use of gellan gum with and without root extract (Figure 5). The diversity index calculated for the cultivable bacteria isolated from **the shoots** (Figure 7) showed the highest diversity on 1/10 rich (869) medium with gellan gum and shoot extract (1.79). The cultivable bacteria that were isolated on 1/10 rich (869) medium with agar without (1.27) and with shoot extract (1.29) resulted in more or less the same diversity in comparison to cultivable bacteria that were isolated on the 1/10 rich (869) medium with gellan gum without shoot extract (1.36). The addition of shoot extract with the 1/10 rich (869) medium with gellan gum showed that different species could be isolated in comparison with the results isolated on 1/10 rich (869) medium with gellan gum without shoot extract. The results of the medium with agar with and without shoot extract showed in both cases the isolation of different species (Figure 6). When the results from the medium with agar with and without shoot extract were compared to the medium with gellan gum with and without shoot extract, differences in isolated species were shown (Figure 6).

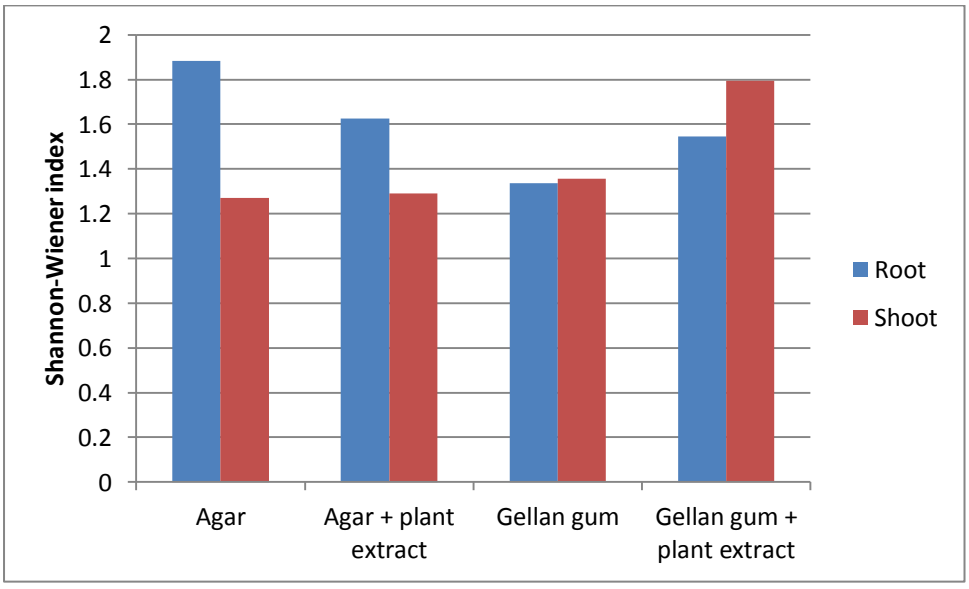

Figure 7: The Shannon-Wiener diversity index of the cultivable bacteria isolated from the roots and the shoots by using the standard isolation method on 1/10 rich (869) medium with agar with and without plant extract and 1/10 rich (869) medium with gellan gum with and without plant extract for the roots and the shoots.

Although the highest diversity did not result in the same way for the roots and the shoots, it is clear from the pie charts that the use of plant extract results in the isolation of different species. Species that were not isolated on 1/10 rich (869) medium with agar (with and without plant extract), were isolated on 1/10 rich (869) medium with gellan gum (with and without plant extract). For example, *Paenibacillus sp*. and *Rhizobium sp*. were not isolated on 1/10 rich (869) medium with agar with or without plant extract, but were isolated on 1/10 rich (869) medium with gellan gum with or without plant extract (Figure 5) (roots). And *Variovorax sp*. was isolated on 1/10 rich medium with gellan gum

and plant extract, which was not isolated on the other media with agar with/without plant extract or with gellan gum without plant extract (Figure 6) (shoot).

The use of gellan gum as gelling agent and the addition of plant extract showed bacteria from the roots and the shoots, that were previously uncultivable with the standard 1/10 rich (869) medium, to be cultivable. Therefore, the utilization of plant extract and a second gelling agent, in addition to the standard gelling agent and medium, pointed out to be useful according to the experimental objectives.

1.1.3 Effect of different media on phenotypic characteristics of cultivable bacteria

From previous results, it is clear that it seems very interesting to compare 1/10 rich (869) medium with agar or gellan gum, with and without addition of plant extract. Next to the total number of isolated strains and the bacterial diversity, the effect of using different media on the phenotypic characteristics of the isolated strains might also be of interest. These phenotypes can influence the potential of the isolated strains to assist their host plant and benefit plant growth and biomass production, which can be used in applications such as phytoremediation. According to the previous results, 1/10 rich (869) medium with agar or gellan gum and 1/10 rich medium with agar or gellan gum with plant extract were screened in order to visualize the effect of the used media on phenotypic characteristics such as siderophores, organic acids, IAA and ACC deaminase production of the bacteria isolated from the roots and the shoots.

The results of the phenotypic characterization in **the roots** (figure 8) showed that the amount of bacteria positive for siderophores production was the highest on 1/10 rich (869) medium with agar and root extract. The amount of isolated bacteria producing organic acid was the highest on 1/10 rich medium with gellan gum with root extract. The production of IAA and ACC deaminase was more or less the same with the highest production on 1/10 rich (869) medium with agar and gellan gum without root extract.

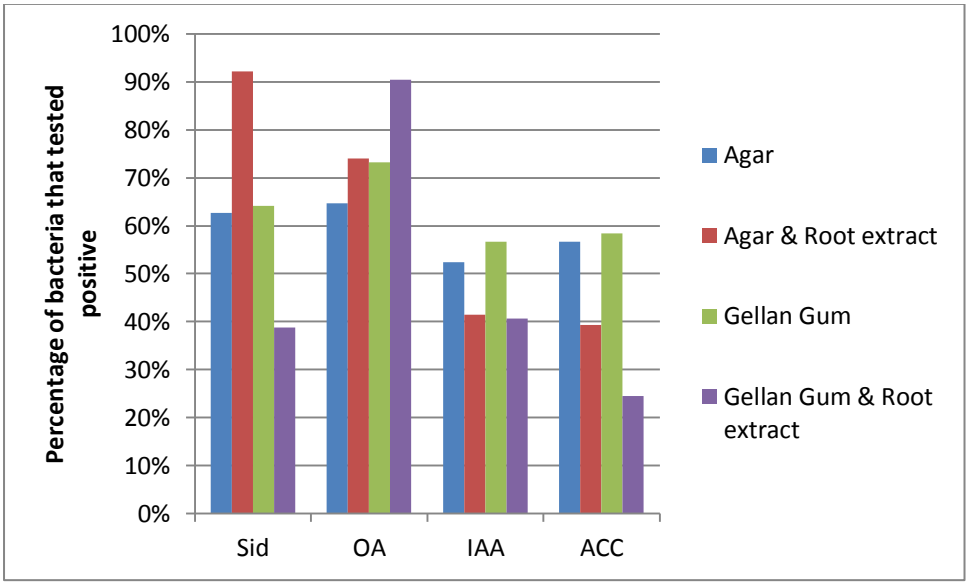

Figure 8: Phenotypic characteristics of bacteria isolated from the roots on 1/10 rich (869) medium with agar with and without addition of root extract and 1/10 rich (869) medium with gellan gum with and without addition of root extract. The production of siderophores (SID), organic acids (OA), IAA and ACC deaminase (ACC) from isolated cultivable bacteria from the roots were screened and visualized the percentage of bacteria that tested positive for the phenotypic tests.

The phenotypic characterization of isolated cultivable bacteria in **the shoots** (Figure 9) showed that the highest amount of siderophores producing strains was isolated on 1/10 rich (869) medium with agar without shoot extract. Organic acids production of the isolated cultivable bacteria on 1/10 rich

medium with gellan gum and shoot extract was the highest. The IAA producing strains were the highest on 1/10 rich (869) medium with gellan gum without shoot extract. The amount of ACC deaminase producing strains was high on 1/10 rich medium with agar and shoot extract.

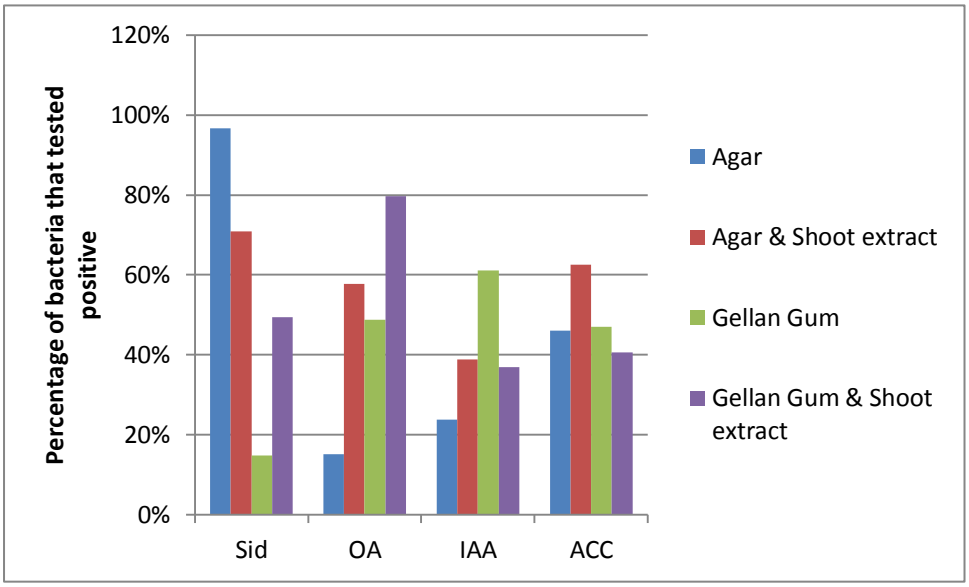

Figure 9: Phenotypic characteristics of bacteria isolated from the shoots on 1/10 rich (869) medium with agar with and without addition of shoot extract and 1/10 rich (869) medium with gellan gum with and without addition of shoot extract. The production of siderophores (SID), organic acids (OA), IAA and ACC deaminase (ACC) from isolated cultivable bacteria from the roots were screened.

The effect of different media on the phenotypic characterization showed that for some characteristics such as siderophores and organic acids production, the use of plant extract had an effect. The production of organic acids increased when the medium with gellan gum and plant extract was used for isolation from the roots and the shoots. Therefore, medium with gellan gum with plant extract could be assumed to be ideal to use when cultivable bacteria with high organic acids production are required. On the other hand, the siderophores production was also influenced by the used media. An increase of siderophores production by bacteria isolated from the roots was shown when the medium with agar and plant extract was used. However, the medium with agar with plant extract did not show an increase when bacterial strains were isolated from the shoots. The medium with agar without plant extract was assumed to be better for a high number of shoot bacteria that produced siderophores.

The other phenotypic characteristics, IAA and ACC deaminase showed differences for the different gelling agents used in the roots. However, in the shoots, the gelling agent affected the amount of positive strains for IAA production and the plant extract affected the amount of positive strains for ACC deaminase production.

To conclude, the amount of bacteria with some specific phenotypic characteristics was affected by the addition of gelling agent and/or plant extract to the medium. However, this effect was not consistent for all tested traits and could be positive or negative, depending on the tested characteristics.

1.2 Acclimatization/Filtration acclimatization method: an alternative isolation protocol

The **filtration acclimatization method** (Hahn et al. 2004) is based on (a) a filtration, which removes most of the readily cultivable bacteria that are able to overgrow slowly growing bacteria and (b) an acclimatization procedure that provides a slow transition from the low environmental substrate concentrations to the high concentration of standard media. Therefore, the filtration acclimatization method can be of value to increase the diversity of cultivable bacteria, due to the focus of the method on slowly growing bacteria and adaptation of the bacteria to the media. Within this experiment the

acclimatization (without previous filtration step) and the filtration acclimatization methods were used and compared with the standard isolation procedure on 1/10 rich (869) medium with agar or gellan gum, with and without plant extract.

In case the **acclimatization method** was applied, the dominated cultivable bacteria in **the roots** (Figure 10A) and **the shoots** (Figure 10B) were *Stenotrophomonas sp.* for respectively 77% and 91%. The remaining part consisted of *Ochrobactrum sp. (16%), Enterobacter sp*. (3%), *Paenibacillus sp*. (2%), *Pseudomonas sp.(1%), Gamma Proteobacterium sp.(1%), Beta Proteobacterium sp.(0,23%), Rhizobium sp. (0.08%) and Exiguobacterium sp.* (0,02%) in the **roots**. *Ochrobactrum sp*. (7%), *Actinomycetes sp*.(2%) and *Enterobacter sp*.(0.19%) were the other species found in the **shoots**.

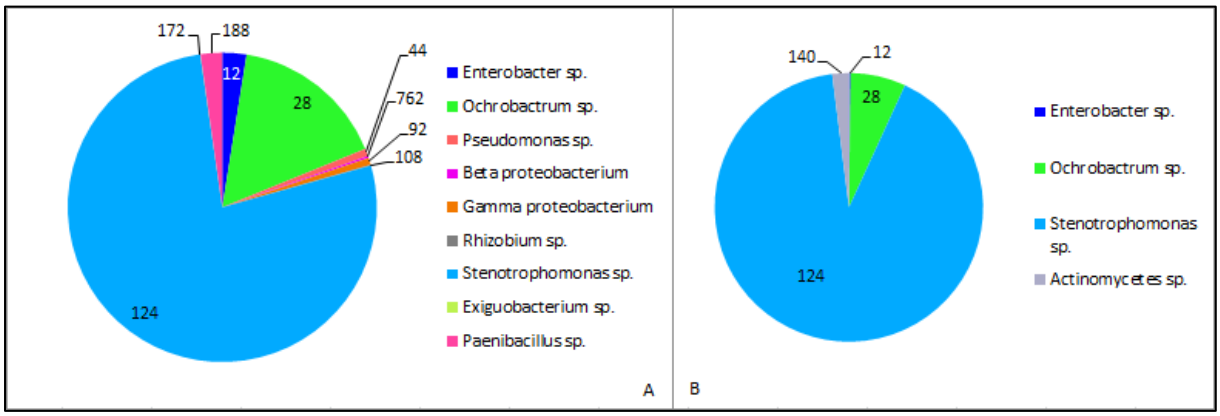

Figure 10: Different species of cultivable bacteria that could be isolated from the roots (A) and the shoots (B) by using the acclimatization method for isolation. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 15 and 16 of attachment 5.

In case **the filtration acclimatization method** (FAM) was applied in the **roots** (Figure 11A), the cultivable bacteria were dominated by the *Stenotrophomonas sp.* (82%). The remaining part was occupied by *Enterobacter sp. (7%), Staphylococcus sp. (7%), Ochrobactrum sp.* (4%) and *Paenibacillus sp*. (0.07%). In the **shoots** (Figure 11B), *Actinomycetes sp.* and *Bacterium SC5* dominated the cultivable bacterial population with respectively 54% and 35%. *Gamma Proteobacterium sp. (4%), Stenotrophomonas sp.*(3%), *Weissella sp.* (3%), *Ochrobactrum sp.(0.74%)* and *Paenibacillus sp*.(0.45%) represented the other cultivable bacterial species isolated from the shoots.

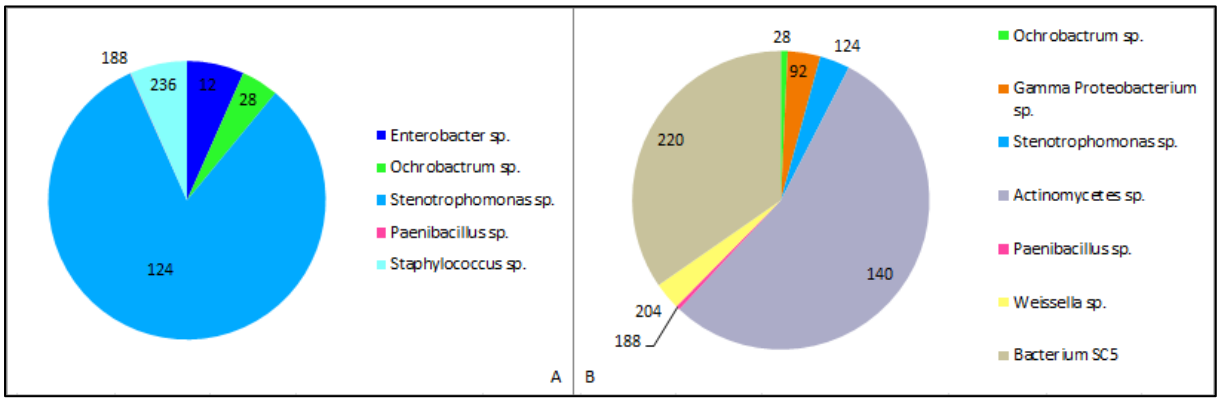

Figure 11: Different species of cultivable bacteria that could be isolated from the roots (A) and the shoots (B) by using the filtration acclimatization method for isolation. The numbers refer to the unique numbers that were shown for each species in figure 4 and table 17,18 of attachment 5.

When bacteria were isolated by the **standard protocol** the highest number of cultivable bacteria in the **roots** (Figure 5A) were *Enterobacter sp*. (34%) and *Bacillus sp*. (32%) and the remaining smaller parts consisted of *Brevibacillus sp*.(11%), *Paenibacillus sp*.(11%), *Pseudomonas sp*.(8%), *Sphingomonas sp*.(2%) and *Rhizobium sp*.(1%). Bacterial strains isolated from the **shoots** (Figure 6D) were also dominated by *Bacillus sp*. (52%) and *Enterobacter sp*. (28%), with a remaining part of *Brevibacillus sp*.(13%), *Variovorax sp*. (3%), *Pseudomonas sp*.(2%), *Rhizobium sp*.(1%) and *Paenibacillus sp*.(0.3%).

In order to easily compare the different methods of isolation, a **diversity index** (figure 12) was calculated using the Shannon-Wiener diversity index. The higher the Shannon-Wiener index, the higher the diversity. The diversity index was used to compare the different alternative methods with each other and with the isolation method from 1.1.2 (Figure 7) that resulted in the highest diversity on 1/10 rich (869) medium with agar for the roots and 1/10 rich (869) medium with gellan gum and plant extract for the shoots.

These results showed the highest diversity with the standard isolation protocol and a slightly lower diversity with the acclimatization method and FAM in the root and the shoot (Figure 12). The standard isolation protocol showed the highest diversity in the roots and slightly lower in the shoots. Isolation using FAM, showed a lower diversity compared with the standard isolation protocol. FAM showed a minor difference in diversity between the roots and the shoots. However, the acclimatization method showed a high diversity for the roots while this was only half the diversity in case of the shoots.

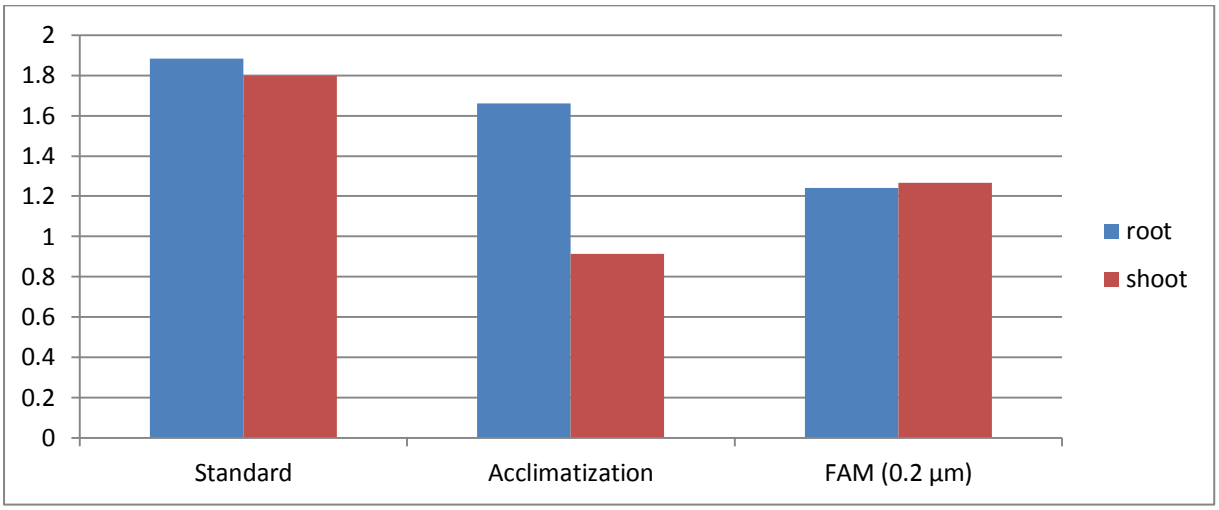

Figure 12: The Shannon-Wiener diversity index of the cultivable bacteria isolated from the roots and the shoots by using the standard isolation method on 1/10 rich (869) medium with agar for the roots and 1/10 rich (869) medium with gellan gum and plant extract for the shoots. The acclimatization method and the Filtration Acclimatization Method (FAM).

The difference in diversity between root and shoot increased by using the acclimatization method. This could indicate that especially bacteria that appeared in the roots and less in the shoots, required the acclimatization method to be cultivable. Furthermore, in the shoots the obtained diversity after applying FAM, was higher in comparison with the diversity obtained in the shoots after applying acclimatization. This increase could indicate that FAM showed to be a good method to increase the diversity of bacteria, that colonize the shoots. The additional filtration step might have eliminated dominant fast growing bacteria and enhance the isolation of less cultivable shoot endophytes.

Although the highest diversity was obtained after isolation with the standard isolation protocol (Figure 12), it is clear from the pie charts (Figures 5, 6, 10 and 11) that the different isolation methods result in

the isolation of different species. Species that were not cultivated with the standard isolation method (Figure 5 and 6), were cultivated with the acclimatization method (Figure 10) and FAM (Figure 11).

The acclimatization method showed bacteria from the roots and FAM showed bacteria from the shoots, that were previously uncultivable with the standard method, to be cultivable. Therefore, the utilization of a different method, in addition to the standard protocol, pointed out to be useful according to the experimental objectives. Especially in the roots, the standard method and the acclimatization method could both be used and in the shoots, the standard method and FAM can be used to increase the diversity of cultivable bacteria.

1.3 Conclusion optimization of isolation

Considering the total number of isolated cultivable bacteria and the number of morphologically different strains, the standard 1/10 rich medium with agar or gellan gum seems to be a very good medium to isolate endophytes. The additional use of plant extract resulted in isolations of different species in comparison to the use of medium without plant extract.

Next to the total number and the diversity, the results showed that the isolation medium also might affect the isolation of strains with specific phenotypic characteristics.

Besides changing the medium, changing the isolation procedure also has an effect on the diversity and composition of the isolated bacterial population.

Comparing the different isolation procedures (standard, acclimatization and filtration/acclimatization) learned us that the use of different methods (instead of applying only 1 method) can be useful in order to increase the variety of isolated cultivable bacteria in the roots and the shoots.

2. What is the borderline between facultative and obligate endophytes?

The aim of the second part of my thesis is to unravel the borderline between facultative and obligate endophytes. Facultative endophytes are living inside the plant, but are also able to survive and are expected to spend part of their life cycle, outside the plant. For that reason, it can be expected that facultative endophytes mainly originate from the rhizosphere and enter the plant through the roots. On the other hand, obligate endophytes only survive inside the plant and are hypothesized to be transferred from one generation to the next through the seeds.

Using the knowledge to optimize the isolation procedures of endophytes that I gathered in the first part of my thesis, I was able to isolate and genotypically characterize, as many as possible, cultivable seed endophytes (2.1) and the rhizosphere bacteria (2.2). After comparing these collections with the root and shoot associated bacteria that I characterized in the first part, it will become clear which species might be obligate and facultative (2.3)/

2.1 Seed endophytes

In order to isolate and characterize the complete cultivable seed endophytic bacterial population, seed endophytes were isolated using the standard isolation procedure followed by plating on 1/10 rich (869) medium with and without seed extract. We did not apply the acclimatization and/ or filtration acclimatization strategy because it is known that bacterial populations in the seeds are rather small with a low diversity and by consequence don't face much competition.

The number of isolated cultivable bacteria were equally on the media without ($2.76\ 10^{+4}$) and with ($2.09\ 10^{+4}$) seed extract (Figure 13). The results showed that seed endophytes were dominated by

Enterobacter sp. (33%) and *Pseudomonas sp*. (37%) for the isolates on the medium without extract. The remaining part of the isolated cultivable bacteria consisted of *Actinomycetes sp.* (13%), *Bacterium sc5* (11%), *Paenibacillus sp*. (11%) *and Bacillus sp*. (1%). The medium with seed extract showed that *Enterobacter sp*. (50%) dominated and the remaining part consisted of *Actinomycetes sp*. (15%), *Bacterium SC5* (12%), *Weissella sp*. (7%), *Exiguobacterium sp*. (6%), *Bacillus sp*. (4%), *Ochrobactrum sp.* (2%) and *Pseudomonas sp*. (2%).

These results confirm that using the seed extract increased the diversity of isolated cultivable bacteria from the seeds.

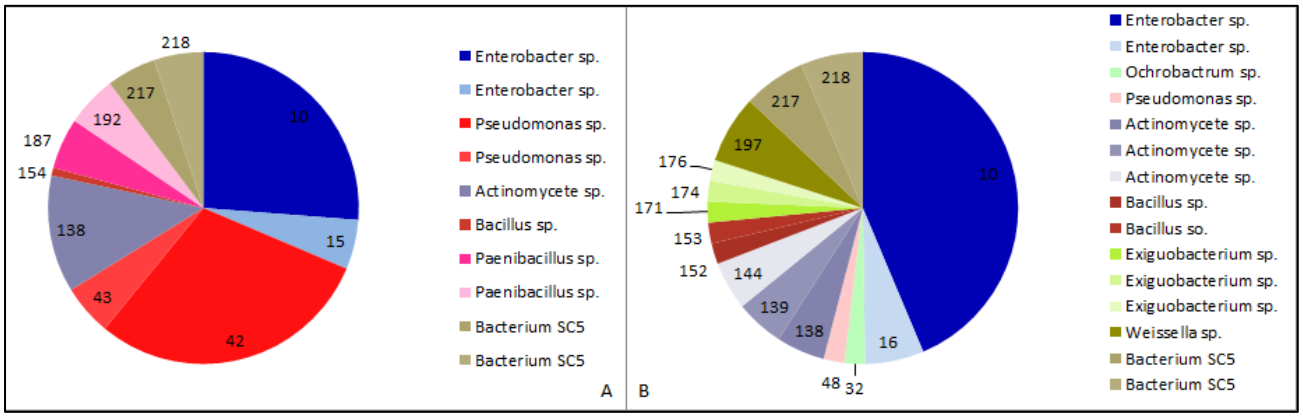

Figure 13: Different species of cultivable bacteria that could be isolated from the seeds by using the standard method for isolation followed by plating on 1/10 rich (869) medium with (B) and without (A) seed extract. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 10 and 11 of attachment 5.

In order to obtain a complete overview of all seed endophytic bacteria, both pie charts shown in figure 13 were combined in an overview pie chart of the complete cultivable seed endophytic population (Figure 14).

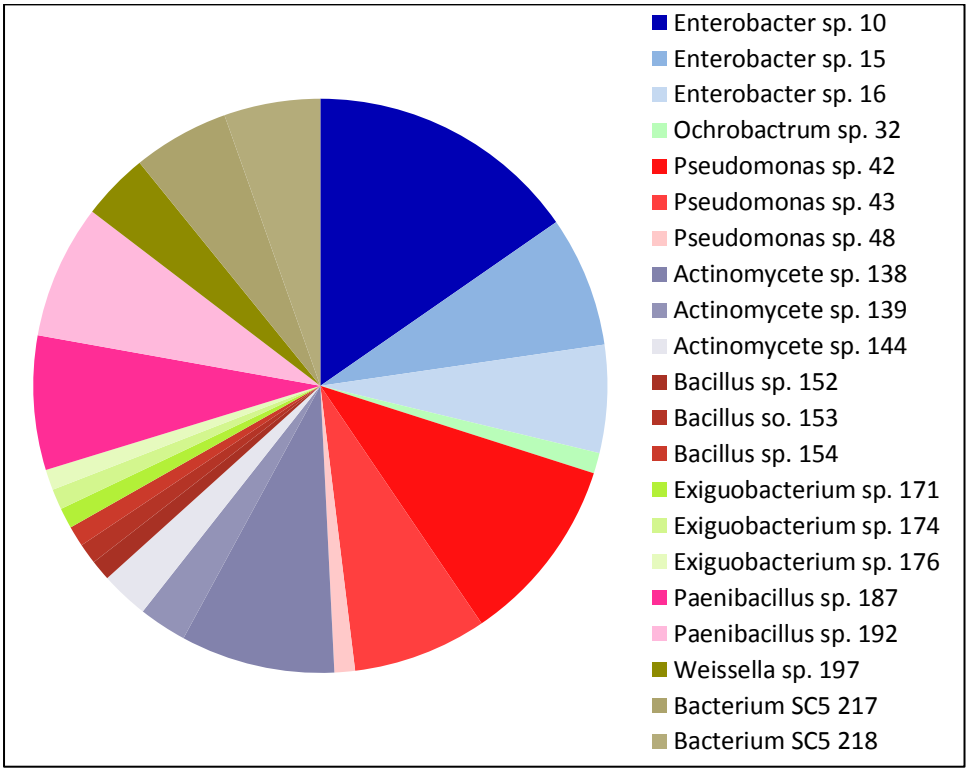

Figure 14: Overview of the complete cultivable seed endophytic population that could be isolated from *Cucurbita pepo*. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 10 and 11 of attachment 5.

2.2 Rhizosphere bacteria

In the next step, I attempted to isolate and genotypically characterize as many cultivable rhizosphere bacteria as possible. The rhizosphere is defined as the direct environment around the roots in which root exudates are released. As the rhizosphere is known to household the highest number as well as the highest diversity of bacteria, we decided to apply following different isolation strategies:

- Standard isolation procedure followed by plating on 1/10 rich (869) medium without plant extract
- Standard isolation procedure followed by plating on 1/10 rich (869) medium with root extract (to mimic root exudates)
- Type of acclimatization isolation procedure (first culturing in liquid medium before plating (see materials and methods for details) followed by plating on 1/10 rich (869) medium with root extract (to mimic root exudates)

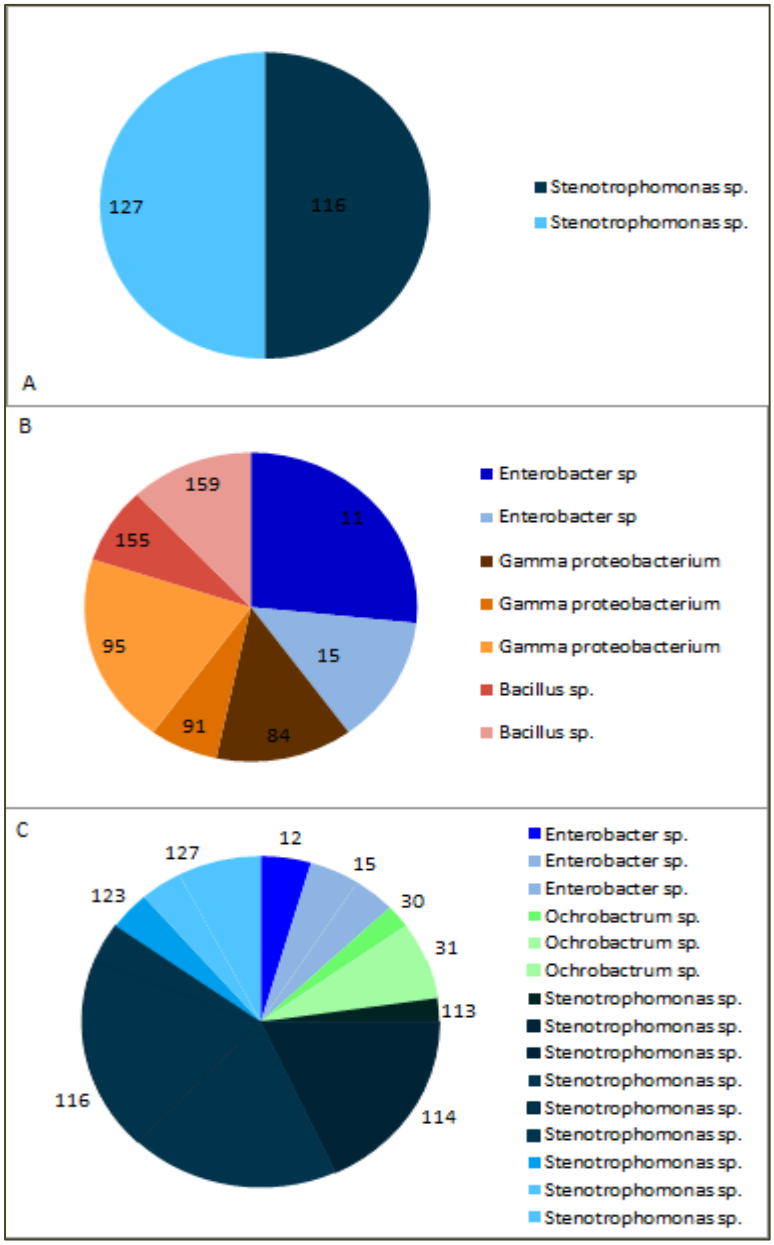

Figure 15: Different species of cultivable bacteria that could be isolated from the rhizosphere by using the standard method for isolation followed by plating on 1/10 rich (869) medium with (B) and without (A) root extract and by applying first a cultivation step in liquid medium with root extract before plating on 1/10 rich (869) medium with root extract (C). The numbers refer to the unique numbers that are shown for each species in figure 4 and table 12, 13 and 14 of attachment 5.

The rhizosphere bacteria that were isolated with the standard isolation procedure followed by plating on 1/10 rich (869) medium without plant extract showed that a number of 2.10 10^{+3} cultivable bacteria was fully dominated by *Stenotrophomonas sp*. (Figure 15A). When the standard isolation procedure was applied followed by plating on 1/10 rich (869) medium with root extract (Figure 15B), the diversity was clearly higher and the isolated population consisted of *Bacillus sp*.(20%), *Enterobacter sp*. (40%) and *Gamma proteobacterium sp*. (40%). Furthermore, the use of cultivation in liquid medium with root extract was used in hope to increase the diversity of cultivable bacteria from the rhizosphere (Figure 15C). The total number of cultivable bacteria was 4.07 10^{+3}, which was not higher than the normal isolation. The isolated rhizosphere population was dominated by *Stenotrophomonas sp*. (77%) and the remaining part consisted of *Enterobacter sp*. (13%) and *Ochrobactrum sp*. (10%). Furthermore, the diversity was higher when the addition of plant extract was used and when liquid medium was first used for cultivation before plating on standard medium agar.

Given that the 3 different used protocols resulted in the isolation of different (amounts of) species, it can again be confirmed that combining the results of these 3 protocols leads to a much more complete representation of the rhizosphere population.

Therefore, an overview pie chart in which the complete isolated rhizosphere population is presented, is shown in figure 16.

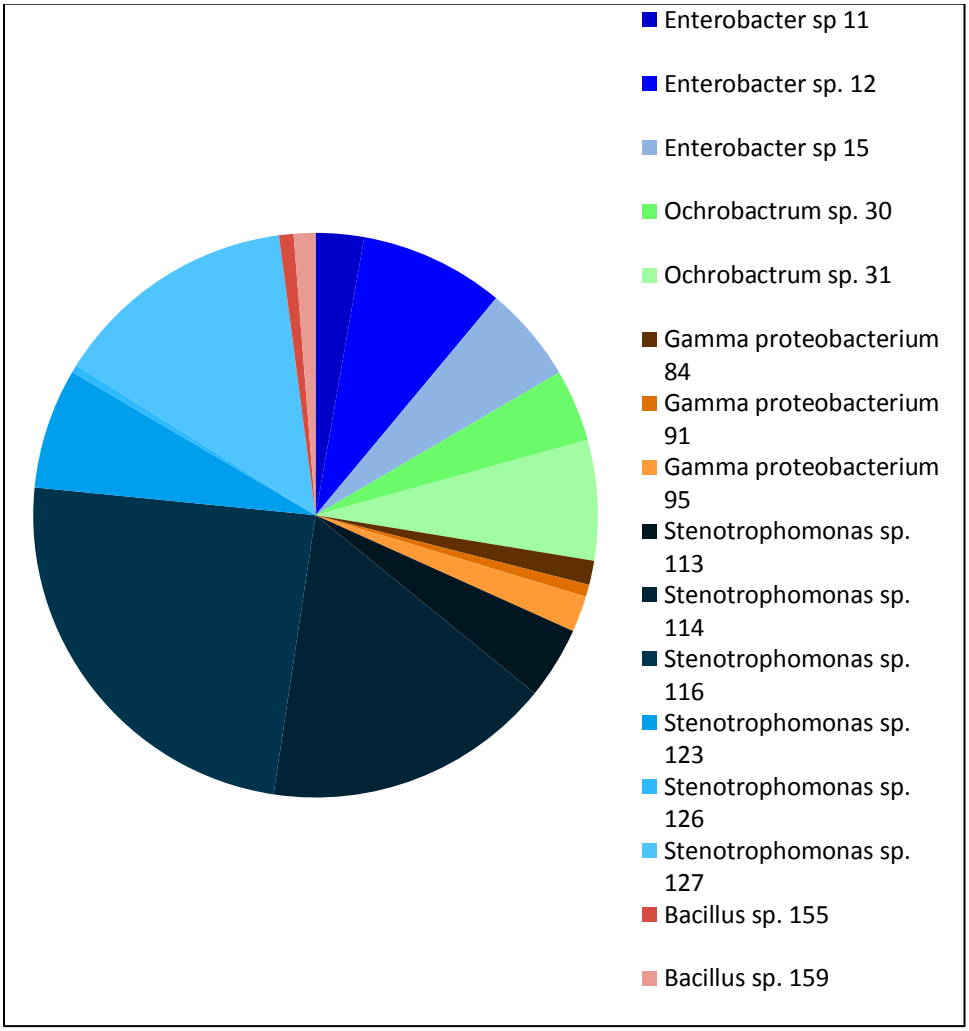

Figure 16:Overview of the complete cultivable rhizosphere population that could be isolated from Cucurbita pepo. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 12, 13 and 14 of attachment 5.

2.3 Obligate vs. facultative endophytes

In order to unravel which bacterial species might be obligate and facultative, the complete seed endophytic population, the root and the shoot associated bacterial population and the rhizosphere colonizing bacteria should be compared.

In case of the root and shoot associated bacterial population, an overview of all isolated bacteria, using the different protocols (Figure 17), was created in a similar way as for the seeds (Figure 14) and rhizosphere (Figure 16) population.

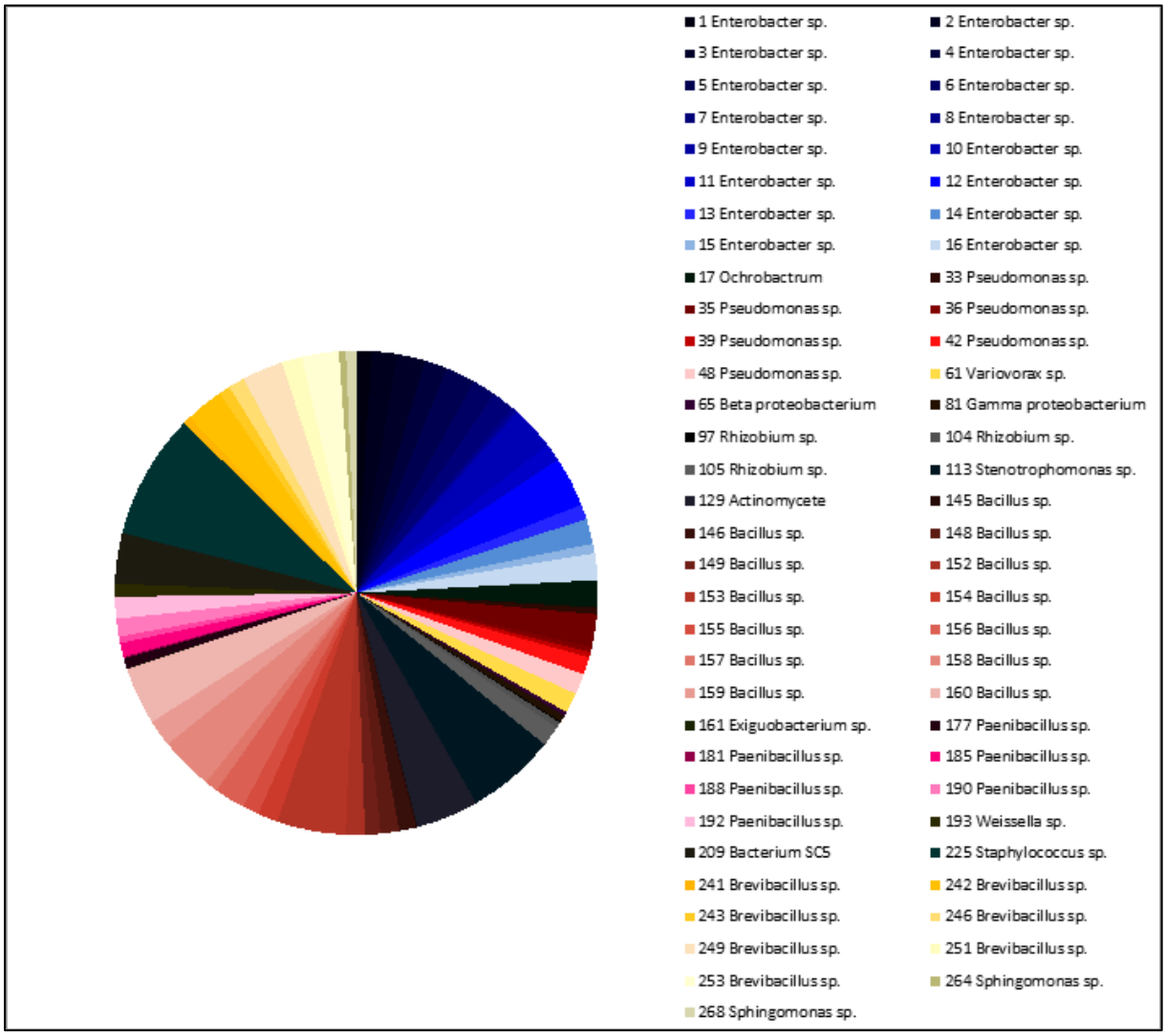

Figure 17: Overview of the complete cultivable root and shoot endophytes population that could be isolated from Cucurbita pepo. The numbers refer to the unique numbers that are shown for each species in figure 4 and table 2-9 of attachment 5.

In order to investigate if there are species that are typical obligate or facultative, a schematic overview of the compartmentalization of all isolated different (at the genotypic level) bacterial species was created (Figure 18).

These results showed that isolated cultivable bacteria from the seeds were also isolated from other compartments such as root and shoot and rhizosphere. Isolated cultivable bacterial species from the seeds and the roots and shoots are most likely to be obligate endophytes, because these endophytic bacteria are present inside the plant, which can indicate that these bacterial endophytes could also be important for the further growth and development of the plant. The presence of these endophytic

bacteria in the seeds, can indicate that these endophytes are transferred from one generation to the next generation, through the seeds. Therefore, bacterial species that were isolated from the seeds (Figure 14) and the roots/shoots (Figure 17) consisted of *Actinomycetes sp*., *Exiguobacterium sp*., *Pseudomonas sp*., *Paenibacillus sp*. and *Weissella sp*. and can be defined as most likely obligate endophytes (Figure 18).

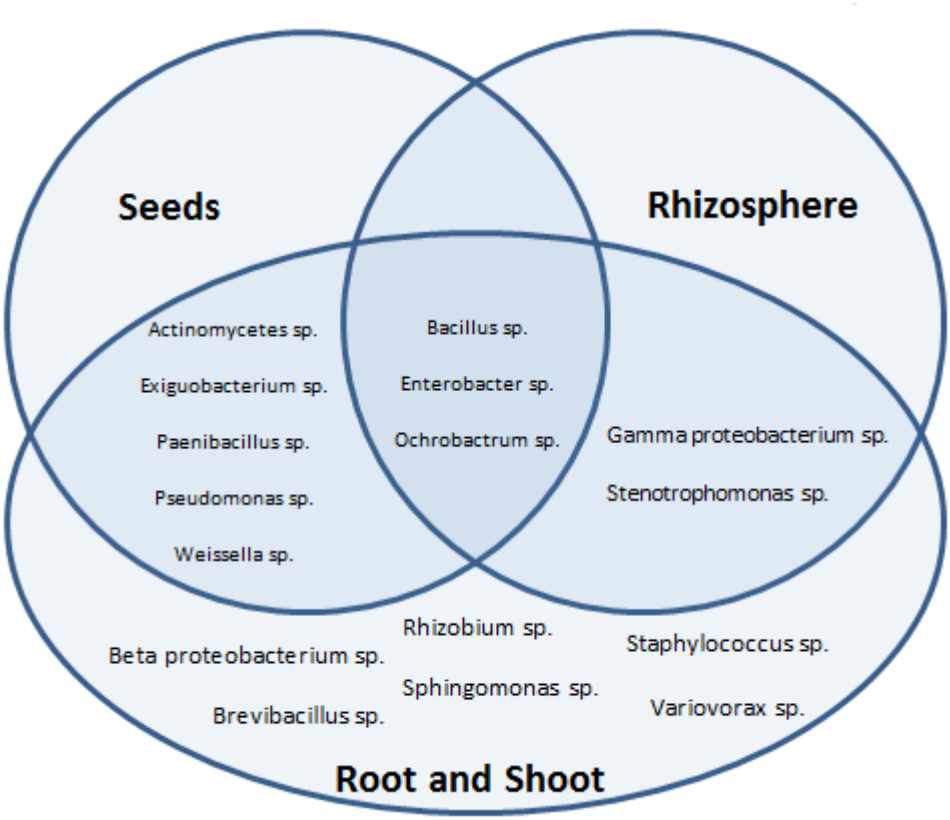

Figure 18: A schematic overview of the compartmentalization of all isolated different (at the genotypic level) bacterial species in the seeds, rhizosphere and the roots and the shoots.

The bacterial endophytes that are represented in the seeds, the roots and shoots and the rhizosphere are **facultative endophytes**, because these endophytes can also survive outside the host plant. The fact that these endophytic bacteria are present in most of the plant compartments and the seeds, can indicate that these bacterial endophytes are transferred from one generation to the next generation through the seeds. Isolated cultivable bacterial endophytic species from the seeds (Figure 14), the roots, the shoots (Figure 17) and the rhizosphere (Figure 16) consisted of *Bacillus sp*., *Enterobacter sp*. and *Ochrobactrum sp*., which are probably facultative endophytes (Figure 18).

Bacterial species that can be isolated from the roots and the shoots (Figure 17) are most likely **obligate endophytes**, because they do not survive outside a host plant. The fact that these bacterial endophytes are not present in the seeds, can indicate that these endophytic bacteria are less important to the plant development and that these bacterial endophytes are not transferred through the seeds from one generation to the next generation. In this study species such as *Beta proteobacterium sp*., *Brevibacillus sp*., *Rhizobium sp*., *Sphingomonas sp*., *Staphylococcus sp*. and *Variovorax sp* were isolated from the roots and the shoots and can be identified as obligate endophytes (Figure 18).

No cultivable bacterial species were represented by the rhizosphere alone. However, endophytic bacterial species from the roots, the shoots (Figure 17) and the rhizosphere (Figure 16) were isolated. These endophytic bacteria are typically facultative, because they can survive with (inside) and without

(outside) the host plant. Bacterial endophytic species such as *Gamma proteobacterium sp*. and *Stenotrophomonas sp*. were isolated from the roots and shoots and the rhizosphere, which make these species facultative species (Figure 18).

Normally it is thought that the total population is dominated by facultative endophytes or that obligate endophytes are more difficult to cultivate. However, the results from the isolated cultivable bacterial species were dominated by obligate endophytes. This might be an indication that by using the different isolation methods (standard, acclimatization, FAM), the addition of plantextract or a different gelling agent, the cultivation of obligate endophytes can also be increased.

Conclusion

The results of the first part of my thesis pointed out that there is not one ideal specific medium or isolation method that can be used to thoroughly optimize the isolation of cultivable bacteria. Therefore, in order to optimize the number and diversity of isolated cultivable bacteria, different media, with or without additional plant extract, as well as different isolation methods should be applied in parallel. This research revealed that important parameters during this isolation are based on the fact that bacteria require a sufficient amount of available nutrients and in some cases plant material in order to become cultivable. Additionally, bacteria might need time to adjust to their environment, (referring to the acclimatization steps) and a habitat to grow on (referring to the filtration step). In future work, even extra parameters could be screened for their importance during isolation, such as cultivation temperature, day and night cycles and humidity. In addition, total bacterial populations need to be characterized by using parallel sequencing in order to find an indication of the cultivable fraction from the total population. This information will reveal the proportion of bacteria that can be cultured by using the optimized protocols in parallel. In literature, it is often stated that only a small percentage of the total bacterial population can be cultured and grown in laboratory conditions.[40] However, taking into account the above results, this proportion is expected to increase significantly.

When a comparison was made between which endophytes could be defined as obligate and facultative, a distinction indeed could be made. However, the thought that the total population was dominated by facultative endophytes or that obligate endophytes were more difficult to cultivate, contradicted with the results where the isolated cultivable bacterial species were dominated by obligate endophytes. This might be another indication that by using the different isolation methods (standard, acclimatization, FAM), the addition of plantextract or a different gelling agent, in parallel with the standard isolation procedure, optimize the number and diversity of isolated cultivable bacteria and also increase the cultivation of obligate endophytes.

Finally, these experiments resulted in different opportunities to increase the number of cultivable bacteria and the diversity and with further research the fraction of cultivable bacteria will increase even more. This will contribute to an improved application of plant-associated bacteria (such as plant growth promotion, improved phytoremediation efficiency,…) because for these applications bacteria need to be enriched by means of inoculation which requires cultivation of the strain.

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Attachment

1. Specifications of the different components of the media

869 medium

In 1 L (pH 7)

- 10 g Tryptone
- 5 g Yeast extract
- 5 g NaCl
- 1 g Glucose D+
- 0,345 g $CaCl_2.2H_2O$

Tryptic soy agar (TSA) medium

In 1 L (pH 7)

- 15 g Tryptone
- 5 g Soytone
- 5 g NaCl

Casein-Starch

In 1 L (pH 7)

- 10 g Starch
- 2 g KH_2PO4
- 2 g KNO3
- 2 g NaCl
- 0,3 g Casein
- 0,05 g $MgSO_4.7H_2O$
- 0,02 g $CaCO_3$
- 0,01 g $FeSO_4$

M9 medium

In 1 L (pH 7)

- 200 mL M9 Salts solution:
  Per 1 L:
  - 64 g $Na_2HPO_4.7H_2O$
  - 15 g KH_2PO_4
  - 2,5 g NaCl
  - 5,0 g NH_4Cl
- 2 mL 1M $MgSO_4$
- 20 mL 20% glucose
- 100 µL 1M $CaCl_2$

M3 medium

In 1 L (pH 7)

- 0,466 g KH_2PO_4
- 0,732 g Na_2HPO_4
- 0,1 g KNO_3
- 0,29 g NaCL
- 0,1 g $MgSO_4.7H_2O$
- 0,02 g $CaCO_3$
- 0,2 g Sodium proprionate
- 200 µg $FeSO_4$
- 180 µg $ZnSO_4.4H_2O$
- 20 µg $MnSO_4.4H_2O$
- 50 mg Cycloheximide
- 4 mg Thiamine

284 medium

In 1 L (pH 7)

- 6,06 g Tris of HCl
- 4,68 g NaCl
- 1,49 g KCl
- 1,07 g NH_4Cl
- 0,43 g Na_2SO_4
- 0,2 g $MgCL_2.6H_2O$
- 0,03 g $CaCl_2H_20$
- 0,04 g $NaHPO_42H_2O$
- 10 mL of 0.048% Fe (III) NH4Citrate
- 1 mL S17 spore elements
  In 1 L
  - 1.3 mL 25% HCL
  - 144 mg $ZnSO_4{\cdot}7H_2O$

    - 100 mg $MnCL_2 \cdot 4H_2O$
    - 62 mg H_3BO_3
    - 190 mg $CoCL_2 \cdot 6H_2O$
    - 17 mg $CuCl_2 \cdot 2H_2O$
    - 24 mg $NiCl_2 \cdot 6H_2O$
    - 36 mg $NaMoO_4 \cdot 2H_2O$
- Cmix
    - 0,7 mL Lactate
    - 0,52 g Glucose
    - 0,66 g Gluconate
    - 0,54 g Fructose
    - 0,81 g Succinate

Gelling agents

Amount of gelling agents in 1 L

- 15 g Agar
- 30 g Gellan gum

2. Protocol of Seed extract/ plant extract addition to the medium

- Grinding:
    - 2 roots
    - 6 shoots
    - 5 seeds
- Dilution in 10 mL distilled water
- 2 times filtration with paper filter
- Filtration with Minisart 0.2 µm
- Addition of minimum 8 mL to 1 L of autoclaved medium

The last two steps could be replaced by:

- Addition of minimal 8 mL to 1 L medium
- Autoclaving extract with medium

3. Specifications of the different components of the used Solutions

10 % Chloride

In 1 L

- 100 mL 10% Chloride
- 900 mL Distilled water
- 10 drops of Tween 80

10 mM $MgSO_4$

In 1 L

- 2,4648 g $MgSO_4$

Glycerol stock (15%)

In 1 L

- 75 g Glycerol
- 4,25 g NaCl
- Add distilled water until 500 g
-

1/10 Hoagland nutrient solution

In 1 L

- 250 mL macro-elements:
  In 1 L:
  - 10,2 g KNO_3
  - 7,08 g $Ca(NO_3)_2 \cdot 4H_2O$
  - 2,3 g $NH_4H_2PO_4$
  - 4,9 g $MgSO_4 \cdot 7H_2O$
- 2.5 mL micro-elements:
  In 1 L:
  - 2,86 g H_3BO_3
  - 1,81 g $MnCL_2 \cdot 4H_2O$
  - 0,08 g $CuSO_4 \cdot 5H_2O$
  - 0,09 g $H_2MoO_4 \cdot H_2O$
  - 0,22 g $ZnSO_4 \cdot 7H_2O$
- 1.5 mL Fe-EDTA:
  In 1 L:
  - 7,6 g EDTA·Na
  - 5 g $FeSO_4 \cdot 7H_2O$
- 746 mL distilled water

4. Original protocol Filtration Acclimatization method

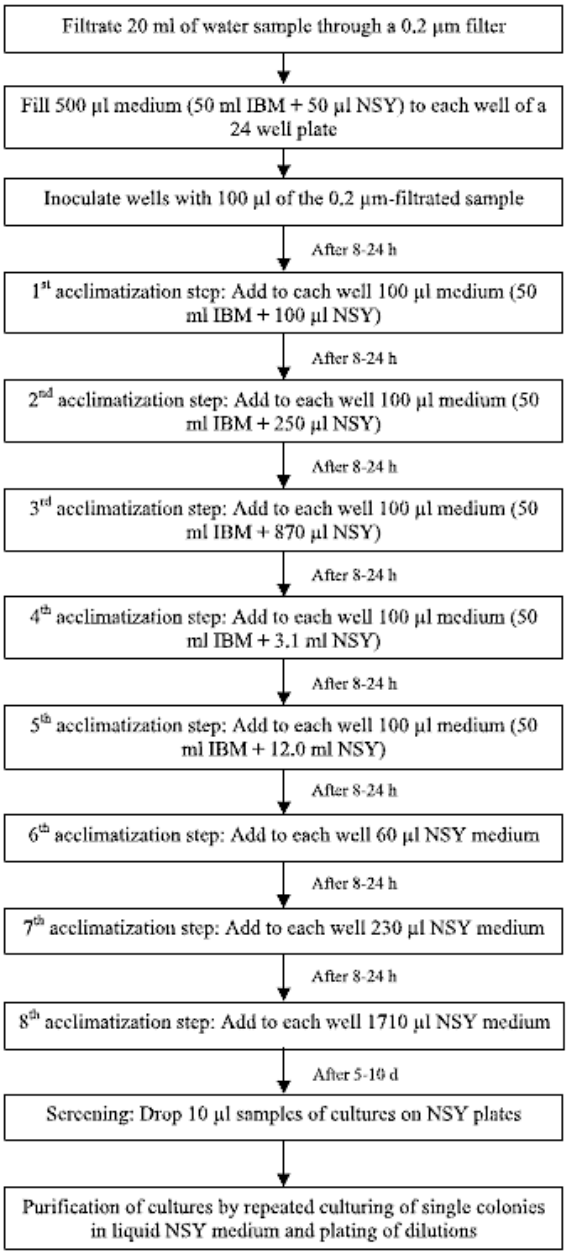

Figure 1. Standard FAM protocol (Hahn et al. 2004) of the stepwise addition of higher amounts of rich (869) liquid medium to give bacteria that are less dominant and require more time for growing the opportunity to grow and cultivate, which they do not have in other methods where they are supposed to grow fast and be dominant.

5. Specifications of the different components necessary for the Phenotypic characterization

Siderophores

- 284 medium (Schlegel et al., 1961) (Attachment 1: Specifications of the different components of the media)
- Stock 0.25 µM Fe(III) citrate (1L): 0.0664 mg Fe(III) citrate

pH:7

- Chroom-Azurol S (CAS)
  In 1 L
  - 60 mL HDTMA
  - 150 mL 10 mM HCl
  - 15 mL $FeCl_3$
  - 75 mL CAS
  - 300 mL Piperazine
  - 100 mL Sulfosalicylic acid

Organic acids

- Sucrose Tryptone (ST) medium
  In 1 L
  - 20 g Sucrose
  - 5 g Tryptone
  - 10 mL Trace elements solution (1L):
    - 20 mg $NaMoO_4.H_2O$
    - 200 mg H_3BO_3
    - 20 mg $CuSO_4.5H_2O$
    - 100 mg $FeCl_3$
    - 20 mg $MnCl_2.4H_2O$
    - 280 mg $ZnCl_2$
- Alizarin red S 0.1%
  In 1 L
  - 1 g Alizarin Red S

IAA

- 1/10 869 medium (Table 1.)

pH: 7

- Addition of 0.5g/L L-tryptophan: Tryptophan diluted in minimal volume of HCl (0,1M) with a pH 6-7 and sterilizing with Minisart 0.2 µm
- Salkowski reagent
  In 1 L
  - 980 mL $HClO_4$ (35%)

- 20 mL $FeCl_3$

ACC deaminase

- Salts Minimal (SMN) medium (Belimov et al., 2005)

  In 970 mL:

  - 0,4 g KH_2PO_4
  - 2 g K_2HPO_4

  pH 6,6

  After autoclaving:

  Addition with filter sterilization:

  - 10 mL $MgSO_4$ solution (2%)
  - 10 mL $CaCL_2$-solution (1%)
  - 10 mL micronutrient stock (100x):
    In 100 mL
    - 50 mg $FeSO_4$
    - 20 mg H_2BO_3
    - 50 mg $ZnSO_4$
    - 10 mg Na_2MoO_4
    - 30 mg $MnSO_4$
    - 10 mg $CoSO_4$
    - 10 mg $CuSO_4$
    - 10 mg $NISO_4$
  - 50 mL C-mix stock (20x)
    In 100 mL
    - 2 g glucose
    - 2 g sucrose
    - 2 g Na-acetate
    - 2 g Na-citrate
    - 2 g Malic acid
    - 2 g Mannitol
  - 10 mL ACC-stock (0.5 M)
    In 10 mL
    - 500 mg ACC
- 0.1 M Tris-HCl buffer (pH 8.5)

  In 1 L

  - 12,114 g Tris
  - Adjust pH to 7.0 with concentrated HCl
- 0.56 N HCl
  In 1 L
  - 46,78 mL HCl (37%)
- 0.2% 2,4-dinitrophenylhydrazine in 2N HCl

In 1 L

- 167 mL HCl (37%)
- 2 g 2,4-dinitrophenylhydrazine

- 2 N NaOH
  In 1 L
  - 79,98 g NaOH

6. Protocol DNeasy® Blood and Tissue kit

DNeasy® Blood and Tissue kit (Qiagen)

- Bacterial solutions from glycerol stock incubating for 3 days at 30°C
- Total genomic DNA extraction by centrifuging 1.5 mL bacterial solution during 10 min at 7500rpm in microcentrifuge tubes
- Remove the supernatant and dissolve the pellets in 180 µL enzymatic lysis buffer
- Incubation of at least 30 min at 37°C
- Add 25 µL proteinase K and 200 µL AL buffer
- Incubation for 30 min at 56°C
- Add 200 µL of ethanol (96-100%)
- Pipetting the solution in DNeasy mini spin colums
- Centrifuging 1 min at 8000rpm
- Add 500 µL buffer AW1
- Centrifuging for 1 min at 8000 rpm
- Remove elution microtubes
- Add 500 µL second buffer AW2 to the mini spin colums
- Centrifuging 5 min at 13200rpm
- Use 1.5 or 2mL microcentrifugetubes
- Add 150 µL buffer AE on the DNeasy membrane and incubate at room temperature for 1 min
- Centrifuging 1 min at 8000rpm
- Repeat addition 150 µL buffer AE on the DNeasy membrane and incubate at room temperature for 1 min
- Centrifuging 1 min at 8000rpm

7. Nanodrop ND-1000 Spectrophotometer (Isogen Life Science)

1 µL of the extracted genomic DNA, diluted in AE buffer was used for the nanodrop.

8. Specifications of the mastermix necessary for the Polymerase chain reaction

49 µL Mastermix (96 samples)

- 500 µL PCR buffer
- 200 µL $MgSO_4$
- 100 µL dNTP-mix
- 100 µL FW primer
- 100 µL RV primer
- 20 µL TAQ polymerase
- 3880 µL Rnase free water

1 µL sample

9. Specifications of the mastermix necessary for DNA-digestion

15 µL Mastermix

- 480 µL Buffer
- 48 µL HPYCH4IV
- 192 µL Rnase
- 720 µL Rnase free water

35 µL sample

10. Specifications of the different components necessary for Gelelectrophorese

50x TAE buffer (1L):

- 242 g Tris
- 57,1 mL acetic acid
- 37,2 g EDTA

Agarose gel (1,5%):

- 250 mL 1x TAE buffer
- 3.75 agarose
- 25 µL gelred

11. Phenotypic and genotypic results of isolated bacteria (Tables)

Table 1: Genotypic and phenotypic information such as production of Siderophores (Sid), Organic acids (OA),Indole-3-acetic acid (IAA) and ACC deaminase (ACC) of the different species isolated and cultivated.

Sid	OA	IAA	ACC	Enterobacter sp.	Ochrobactrum sp.	Pseudomonas sp.	Variovorax sp.	Betaproteobacterium sp.	Gammaproteobacterium sp.	Rhizobium sp.	Stenotrophomonas sp.	Actinomycetes sp;	Bacillus sp.	Exiguobacterium sp.	Paenibacillus sp.	Weissella sp.	Bacterium SC5 sp.	Staphylococcus sp.	Brevibacillus sp.	Sphingomonas sp.
-	-	-	-	1	17	33	49	65	81	97	113	129	145	161	177	193	209	225	241	257
-	-	-	+	2	18	34	50	66	82	98	114	130	146	162	178	194	210	226	242	258
-	-	+	-	3	19	35	51	67	83	99	115	131	147	163	179	195	211	227	243	259
-	-	+	+	4	20	36	52	68	84	100	116	132	148	164	180	196	212	228	244	260
-	+	-	-	5	21	37	53	69	85	101	117	133	149	165	181	197	213	229	245	261
-	+	+	-	6	22	38	54	70	86	102	118	134	150	166	182	198	214	230	246	262
-	+	+	+	7	23	39	55	71	87	103	119	135	151	167	183	199	215	231	247	263
+	-	-	-	8	24	40	56	72	88	104	120	136	152	168	184	200	216	232	248	264
+	+	-	-	9	25	41	57	73	89	105	121	137	153	169	185	201	217	233	249	265
+	+	+	-	10	26	42	58	74	90	106	122	138	154	170	186	202	218	234	250	266
+	-	+	-	11	27	43	59	75	91	107	123	139	155	171	187	203	219	235	251	267
+	-	-	+	12	28	44	60	76	92	108	124	140	156	172	188	204	220	236	252	268
-	+	-	+	13	29	45	61	77	93	109	125	141	157	173	189	205	221	237	253	269
+	+	-	+	14	30	46	62	78	94	110	126	142	158	174	190	206	222	238	254	270
+	-	+	+	15	31	47	63	79	95	111	127	143	159	175	191	207	223	239	255	271
+	+	+	+	16	32	48	64	80	96	112	128	144	160	176	192	208	224	240	256	272

Table 2. Cultivable root bacteria on Standard 1/10 rich medium with agar

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	3063	-	-	-	++	146
Bacillus sp.	3063	-	-	-	++	146
Bacillus sp.	3063	-	-	-	++	146
Bacillus sp.	2439	-	-	-	+	146
Bacillus sp.	2439	-	-	-	+	146
Bacillus sp.	5366	-	++	-	-	149
Bacillus sp.	5366	-	+	-	-	149
Bacillus sp.	5366	-	++	-	-	149
Bacillus sp.	5366	-	++	-	-	149
Bacillus sp.	2404	-	++	-	-	149
Bacillus sp.	3342	++	-	-	-	152
Bacillus sp.	3342	+	-	-	-	152
Bacillus sp.	3342	+	-	-	-	152
Bacillus sp.	5013	++	-	-	-	152
Bacillus sp.	5013	++	-	-	-	152
Bacillus sp.	5013	++	-	-	-	152
Bacillus sp.	5013	++	-	-	-	152
Bacillus sp.	5013	++	-	-	-	152
Bacillus sp.	2785	++	-	-	-	152
Bacillus sp.	2785	++	-	-	-	152
Bacillus sp.	2785	++	-	-	-	152
Bacillus sp.	2785	++	-	-	-	152
Bacillus sp.	2785	++	-	-	-	152
Bacillus sp.	4634	++	-	-	-	152
Bacillus sp.	4634	++	-	-	-	152
Bacillus sp.	4634	++	-	-	-	152
Bacillus sp.	26440	+	-	-	-	152
Bacillus sp.	4634	++	+	-	-	153
Bacillus sp.	3846	+	+	-	-	153
Bacillus sp.	5366	+	++	++	-	154
Bacillus sp.	4634	++	++	+	-	154
Bacillus sp.	4634	+	++	+	-	154
Bacillus sp.	2785	+	-	++	-	155
Bacillus sp.	3342	++	-	-	+	156
Bacillus sp.	3342	+	-	-	++	156
Bacillus sp.	3342	+	-	-	++	156
Bacillus sp.	5013	++	-	-	++	156
Bacillus sp.	5013	++	-	-	++	156
Bacillus sp.	5013	++	-	-	+	156
Bacillus sp.	5013	++	-	-	+	156
Bacillus sp.	5013	++	-	-	+	156
Bacillus sp.	2785	++	-	-	+	156
Bacillus sp.	2785	++	-	-	+	156

Bacillus sp.	2785	++	-	-	+	156
Bacillus sp.	4634	++	-	-	++	156
Bacillus sp.	26440	+	-	-	+	156
Bacillus sp.	26440	+	-	-	++	156
Bacillus sp.	4634	++	+	-	+	158
Bacillus sp.	4634	++	+	-	+	158
Bacillus sp.	4634	++	++	+	++	160
Bacillus sp.	26440	++	++	+	++	160
Bacillus sp.	26440	++	++	++	+	160
Bacillus sp.	26440	+	+	+	+	160
Brevibacillus sp.	3342	-	-	-	-	241
Brevibacillus sp.	3342	-	-	-	-	241
Brevibacillus sp.	3342	-	-	-	-	241
Brevibacillus sp.	3342	-	-	-	+	242
Brevibacillus sp.	26440	-	-	-	++	242
Brevibacillus sp.	26440	-	-	-	++	242
Brevibacillus sp.	3846	-	-	++	-	243
Brevibacillus sp.	26440	+	++	-	-	249
Brevibacillus sp.	26440	++	++	-	-	249
Enterobacter sp.	2439	-	-	-	-	1
Enterobacter sp.	2439	-	-	-	-	1
Enterobacter sp.	2439	-	-	-	-	1
Enterobacter sp.	2439	-	-	-	-	1
Enterobacter sp.	2404	-	-	-	-	1
Enterobacter sp.	2404	-	-	-	-	1
Enterobacter sp.	29267	-	-	++	-	3
Enterobacter sp.	5366	-	++	++	-	6
Enterobacter sp.	5366	-	++	+	-	6
Enterobacter sp.	5366	-	++	+	-	6
Enterobacter sp.	5366	-	++	+	-	6
Enterobacter sp.	29267	-	++	++	-	6
Enterobacter sp.	29267	-	++	++	-	6
Enterobacter sp.	29267	-	++	+	-	6
Enterobacter sp.	2439	-	++	+	-	6
Enterobacter sp.	2439	-	++	++	-	6
Enterobacter sp.	29267	-	++	+	++	7
Enterobacter sp.	29267	-	++	++	++	7
Enterobacter sp.	29267	-	++	++	+	7
Enterobacter sp.	15864	-	+	++	+	7
Enterobacter sp.	15864	-	+	++	+	7
Enterobacter sp.	2404	+	-	-	-	8
Enterobacter sp.	15864	+	+	++	-	10
Enterobacter sp.	15864	++	+	++	-	10
Enterobacter sp.	3846	++	++	++	-	10
Enterobacter sp.	3063	++	-	++	-	11
Enterobacter sp.	3846	++	-	++	-	11

Enterobacter sp.	3846	++	-	++	-	11
Enterobacter sp.	3846	++	-	++	-	11
Enterobacter sp.	2439	-	++	-	+	13
Enterobacter sp.	15864	-	++	-	++	13
Enterobacter sp.	5853	+	++	-	+	14
Enterobacter sp.	15864	+	++	-	++	14
Paenibacillus sp.	3063	++	++	-	-	185
Paenibacillus sp.	5366	++	++	-	-	185
Paenibacillus sp.	3063	+	-	-	++	188
Paenibacillus sp.	3063	++	-	-	++	188
Paenibacillus sp.	2404	+	-	-	+	188
Paenibacillus sp.	2404	+	-	-	++	188
Paenibacillus sp.	3063	++	+	-	+	190
Paenibacillus sp.	29267	+	++	-	++	190
Paenibacillus sp.	2439	+	+	-	+	190
Paenibacillus sp.	3063	++	++	++	+	192
Paenibacillus sp.	29267	+	++	+	++	192
Paenibacillus sp.	29267	+	++	+	+	192
Pseudomonas sp.	5853	-	++	++	++	39
Pseudomonas sp.	5853	+	++	+	-	42
Pseudomonas sp.	5853	+	+	+	-	42
Pseudomonas sp.	5853	+	++	++	-	42
Pseudomonas sp.	15864	+	++	+	+	48
Pseudomonas sp.	15864	+	++	+	++	48
Pseudomonas sp.	15864	++	++	+	++	48
Pseudomonas sp.	15864	++	++	++	+	48
Rhizobium sp.	5853	++	-	-	-	104
Rhizobium sp.	5853	++	++	-	-	105
Sphingomonas sp.	3846	++	-	-	-	264
Sphingomonas sp.	3846	++	-	-	-	264
Sphingomonas sp.	3846	++	-	-	-	264
Sphingomonas sp.	3846	++	-	-	-	264
Sphingomonas sp.	5853	++	-	-	+	268
Sphingomonas sp.	5853	++	-	-	+	268
Total # bacteria (%)	1.08E+06	62.67%	64.69%	52.39%	56.60%	

Table 3. Cultivable root bacteria on Standard 1/10 rich medium with agar with plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	676	-	-	-	-	145
Bacillus sp.	2164	-	+	-	-	149
Bacillus sp.	2164	-	++	-	-	149
Bacillus sp.	21409	++	-	-	-	152
Bacillus sp.	21409	++	-	-	-	152
Bacillus sp.	21409	+	-	-	-	152
Bacillus sp.	2164	++	-	-	-	152
Bacillus sp.	2164	++	-	-	-	152
Bacillus sp.	39419	+	++	-	-	153
Bacillus sp.	39419	+	++	-	-	153
Bacillus sp.	21409	+	++	-	-	153
Bacillus sp.	21409	+	++	-	-	153
Bacillus sp.	5410	+	++	+	-	154
Bacillus sp.	5410	+	++	+	-	154
Bacillus sp.	2164	+	++	+	-	154
Bacillus sp.	2164	+	++	+	-	154
Bacillus sp.	2164	++	++	+	-	154
Bacillus sp.	676	++	+	+	-	154
Bacillus sp.	21409	++	-	-	+	156
Bacillus sp.	21409	++	-	-	+	156
Bacillus sp.	21409	+	-	-	+	156
Bacillus sp.	21409	+	-	-	+	156
Bacillus sp.	21409	++	+	-	+	158
Brevibacillus sp.	9175	-	-	+	-	243
Brevibacillus sp.	9175	-	+	+	-	246
Enterobacter sp.	1758	-	++	-	-	5
Enterobacter sp.	1758	-	++	-	-	5
Enterobacter sp.	1758	-	++	-	-	5
Enterobacter sp.	676	-	++	-	-	5
Enterobacter sp.	1758	-	++	+	-	6
Enterobacter sp.	5410	-	++	+	-	6
Enterobacter sp.	5410	-	++	+	-	6
Enterobacter sp.	2164	-	++	+	-	6
Enterobacter sp.	676	-	++	+	-	6
Enterobacter sp.	5410	-	++	+	++	7
Enterobacter sp.	5410	-	++	+	++	7
Enterobacter sp.	676	-	++	+	++	7
Enterobacter sp.	39419	+	+	++	-	10
Enterobacter sp.	39419	+	++	++	-	10
Enterobacter sp.	39419	+	++	++	-	10
Enterobacter sp.	39419	+	++	++	-	10
Enterobacter sp.	39419	+	-	++	-	11
Enterobacter sp.	2164	+	-	+	-	11

Enterobacter sp.	2164	+	-	+	-	11
Enterobacter sp.	1758	-	++	-	++	13
Enterobacter sp.	1758	-	++	-	++	13
Enterobacter sp.	39419	+	+	-	++	14
Enterobacter sp.	13457	++	++	-	+	14
Enterobacter sp.	13457	+	++	-	+	14
Enterobacter sp.	13457	+	++	-	++	14
Enterobacter sp.	13457	+	++	-	++	14
Enterobacter sp.	13457	+	++	-	++	14
Enterobacter sp.	13457	+	++	-	+	14
Enterobacter sp.	5410	+	-	+	+	15
Enterobacter sp.	39419	+	+	++	++	16
Enterobacter sp.	13457	+	++	++	++	16
Enterobacter sp.	1758	+	++	++	++	16
Paenibacillus sp.	676	-	++	-	-	181
Paenibacillus sp.	5410	+	++	-	++	190
Paenibacillus sp.	5410	+	++	+	+	192
Paenibacillus sp.	5410	+	++	+	+	192
Pseudomonas sp.	1758	-	++	++	+	39
Pseudomonas sp.	1758	-	++	++	+	39
Pseudomonas sp.	676	-	++	++	++	39
Rhizobium sp.	13457	+	++	-	-	105
Rhizobium sp.	13457	+	++	-	-	105
Rhizobium sp.	13457	+	++	-	-	105
Rhizobium sp.	1758	+	++	-	-	105
Total # bacteria (%)	8.19E+05	58.77%	80.10%	41.17%	27.24%	

Table 4. Cultivable root bacteria on Standard 1/10 rich medium with gellan gum without plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	35396	-	-	-	++	146
Bacillus sp.	12981	-	-	-	++	146
Bacillus sp.	9985	-	-	-	++	146
Bacillus sp.	13979	-	-	++	++	148
Bacillus sp.	14978	-	++	-	-	149
Bacillus sp.	9693	-	++	-	-	149
Bacillus sp.	6710	-	++	-	-	149
Bacillus sp.	145122	+	-	-	-	152
Bacillus sp.	13979	++	-	-	-	152
Bacillus sp.	13979	++	-	-	-	152
Bacillus sp.	145122	++	++	-	-	153
Bacillus sp.	145122	++	++	-	-	153
Bacillus sp.	14978	+	++	+	-	154
Bacillus sp.	12981	+	++	+	-	154
Bacillus sp.	9985	+	++	+	-	154
Bacillus sp.	13979	+	++	+	-	154
Bacillus sp.	13979	+	++	+	-	154
Bacillus sp.	9693	+	++	++	-	154
Bacillus sp.	6710	+	++	++	-	154
Bacillus sp.	32881	+	++	++	-	154
Bacillus sp.	32881	+	++	++	-	154
Bacillus sp.	32881	+	++	++	-	154
Bacillus sp.	145122	+	-	-	+	156
Bacillus sp.	145122	++	-	-	++	156
Bacillus sp.	145122	+	+	-	+	158
Bacillus sp.	145122	++	+	+	++	160
Bacillus sp.	145122	++	+	+	++	160
Bacillus sp.	145122	+	++	+	+	160
Bacillus sp.	145122	++	+	+	+	160
Bacillus sp.	32881	+	++	++	+	160
Brevibacillus sp.	6710	-	-	-	-	241
Brevibacillus sp.	35396	-	-	-	++	242
Brevibacillus sp.	35396	-	-	-	++	242
Enterobacter sp.	14978	-	-	-	-	1
Enterobacter sp.	13979	-	-	-	-	1
Enterobacter sp.	9693	-	-	-	-	1
Enterobacter sp.	16975	-	-	-	+	2
Enterobacter sp.	32881	-	-	-	+	2
Enterobacter sp.	14978	-	-	+	-	3
Enterobacter sp.	9693	-	-	++	-	3
Enterobacter sp.	32881	-	-	++	-	3
Enterobacter sp.	9985	-	-	++	++	4
Enterobacter sp.	14978	-	++	+	-	6

Enterobacter sp.	14978	-	++	+	-	6
Enterobacter sp.	9985	-	++	+	-	6
Enterobacter sp.	12981	-	++	++	-	6
Enterobacter sp.	12981	-	++	+	-	6
Enterobacter sp.	12981	-	++	+	-	6
Enterobacter sp.	12981	-	++	+	-	6
Enterobacter sp.	12981	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	16975	-	++	+	-	6
Enterobacter sp.	13979	-	+	++	-	6
Enterobacter sp.	13979	-	++	+	-	6
Enterobacter sp.	13979	-	++	+	-	6
Enterobacter sp.	13979	-	++	+	-	6
Enterobacter sp.	9693	-	++	++	-	6
Enterobacter sp.	9693	-	++	++	-	6
Enterobacter sp.	6710	-	++	++	-	6
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	8052	-	+	++	-	6
Enterobacter sp.	32881	-	++	++	-	6
Enterobacter sp.	32881	-	++	++	-	6
Enterobacter sp.	35396	-	++	+	+	7
Enterobacter sp.	35396	-	++	+	++	7
Enterobacter sp.	14978	-	++	+	+	7
Enterobacter sp.	14978	-	++	+	+	7
Enterobacter sp.	9985	-	++	+	++	7
Enterobacter sp.	9985	-	++	+	++	7
Enterobacter sp.	12981	-	++	++	+	7
Enterobacter sp.	12981	-	+	++	+	7
Enterobacter sp.	9985	-	++	+	++	7
Enterobacter sp.	9985	-	++	+	++	7
Enterobacter sp.	9985	-	++	+	++	7
Enterobacter sp.	9985	-	++	+	+	7
Enterobacter sp.	9985	-	+	+	+	7
Enterobacter sp.	9985	-	++	+	+	7
Enterobacter sp.	9693	-	++	++	++	7
Enterobacter sp.	9693	-	+	+	++	7
Enterobacter sp.	32881	-	+	++	+	7
Enterobacter sp.	32881	-	++	++	+	7
Enterobacter sp.	9693	+	-	++	-	11
Enterobacter sp.	14978	-	++	-	++	13

Enterobacter sp.	6710	-	++	-	+	13
Enterobacter sp.	8052	-	+	-	++	13
Enterobacter sp.	16975	+	++	-	+	14
Enterobacter sp.	8052	+	++	-	++	14
Enterobacter sp.	8052	+	++	-	++	14
Enterobacter sp.	8052	+	++	-	++	14
Enterobacter sp.	16975	+	++	++	+	16
Enterobacter sp.	16975	+	++	+	+	16
Enterobacter sp.	8052	++	++	+	+	16
Enterobacter sp.	8052	+	++	++	+	16
Paenibacillus sp.	14978	++	++	-	-	185
Paenibacillus sp.	12981	+	++	-	+	190
Paenibacillus sp.	6710	+	+	-	+	190
Paenibacillus sp.	9985	+	++	+	+	192
Pseudomonas sp.	8052	++	++	+	-	42
Pseudomonas sp.	8052	+	++	+	-	42
Pseudomonas sp.	8052	+	++	++	-	42
Total # bacteria (%)	2.90E+06	64.14%	73.18%	56.61%	58.33%	

Table 5. Cultivable root bacteria on Standard 1/10 rich medium with gellan gum with plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	9394	-	-	-	++	146
Bacillus sp.	9394	-	-	-	+	146
Bacillus sp.	6530	-	++	-	-	149
Bacillus sp.	6530	-	+	-	-	149
Bacillus sp.	19591	-	++	-	-	149
Bacillus sp.	19591	++	-	-	-	152
Bacillus sp.	19591	++	-	-	-	152
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	61386	+	++	-	-	153
Bacillus sp.	9394	++	-	++	-	155
Bacillus sp.	19591	++	-	-	++	156
Bacillus sp.	61386	+	++	-	++	158
Bacillus sp.	9394	++	-	+	+	159
Bacillus sp.	9394	+	+	++	++	160
Bacillus sp.	9394	++	++	++	+	160
Bacillus sp.	9394	++	++	++	++	160
Bacillus sp.	9394	++	++	+	++	160
Bacillus sp.	9394	+	++	++	+	160
Brevibacillus sp.	19591	-	-	-	++	242
Brevibacillus sp.	19591	+	++	-	-	249
Brevibacillus sp.	19591	++	++	-	-	249
Brevibacillus sp.	9394	-	++	-	++	253
Brevibacillus sp.	61386	-	++	-	++	253
Enterobacter sp.	31346	-	-	+	-	3
Enterobacter sp.	31346	-	-	+	-	3
Enterobacter sp.	31346	-	++	-	-	5
Enterobacter sp.	31346	-	++	-	-	5
Enterobacter sp.	31346	-	++	-	-	5
Enterobacter sp.	31346	-	++	-	-	5
Enterobacter sp.	31346	-	++	-	-	5
Enterobacter sp.	61386	-	++	-	-	5
Enterobacter sp.	61386	-	++	-	-	5
Enterobacter sp.	6530	-	++	-	-	5
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	6710	-	+	++	-	6
Enterobacter sp.	1274	-	++	+	-	6
Enterobacter sp.	1274	-	++	++	-	6
Enterobacter sp.	1274	-	++	+	-	6

Enterobacter sp.	1274	-	++	+	-	6
Enterobacter sp.	743	-	++	++	-	6
Enterobacter sp.	743	-	++	+	-	6
Enterobacter sp.	743	-	++	+	-	6
Enterobacter sp.	743	-	++	+	-	6
Enterobacter sp.	743	-	++	+	-	6
Enterobacter sp.	10705	-	++	++	-	6
Enterobacter sp.	10705	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	++	-	6
Enterobacter sp.	44602	-	++	++	-	6
Enterobacter sp.	44602	-	++	++	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	44602	-	++	+	-	6
Enterobacter sp.	6710	-	+	++	+	7
Enterobacter sp.	1274	-	++	+	+	7
Enterobacter sp.	1274	-	++	+	+	7
Enterobacter sp.	1274	-	++	+	+	7
Enterobacter sp.	10705	-	++	+	+	7
Enterobacter sp.	10705	-	++	++	++	7
Enterobacter sp.	10705	-	++	+	++	7
Enterobacter sp.	10705	-	++	+	++	7
Enterobacter sp.	10705	-	++	++	+	7
Enterobacter sp.	10705	-	++	++	+	7
Enterobacter sp.	10705	-	++	++	+	7
Enterobacter sp.	10705	-	++	++	++	7
Enterobacter sp.	6530	-	++	-	+	13
Enterobacter sp.	6530	-	++	-	++	13
Enterobacter sp.	19591	-	++	-	++	13
Enterobacter sp.	19591	-	++	-	+	13
Enterobacter sp.	31346	++	++	-	++	14
Enterobacter sp.	6530	+	++	-	+	14
Enterobacter sp.	19591	++	++	-	+	14
Enterobacter sp.	743	+	++	-	+	14
Pseudomonas sp.	743	-	++	+	+	39
Pseudomonas sp.	743	-	++	+	++	39
Pseudomonas sp.	743	-	++	+	++	39
Pseudomonas sp.	31346	++	++	++	-	42
Rhizobium sp.	6710	++	++	-	-	105
Rhizobium sp.	31346	+	++	-	-	105
Total # bacteria (%)	1.86E+06	38.79%	90.39%	40.60%	24.50%	

Table 6. Cultivable shoot bacteria on Standard 1/10 rich medium with agar without plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	7128	-	-	++	++	148
Bacillus sp.	4791	++	-	-	-	152
Bacillus sp.	4791	+	-	-	-	152
Bacillus sp.	4791	++	-	-	-	152
Bacillus sp.	4791	++	-	-	-	152
Bacillus sp.	4791	++	-	-	-	152
Bacillus sp.	15811	++	-	-	-	152
Bacillus sp.	15811	++	-	-	-	152
Bacillus sp.	15811	++	-	-	-	152
Bacillus sp.	15811	++	-	-	-	152
Bacillus sp.	15811	++	-	-	-	152
Bacillus sp.	10801	++	-	-	-	152
Bacillus sp.	10801	++	-	-	-	152
Bacillus sp.	7128	++	-	-	-	152
Bacillus sp.	447	+	-	-	-	152
Bacillus sp.	447	++	-	-	-	152
Bacillus sp.	7128	++	+	-	-	153
Bacillus sp.	7128	++	+	-	-	153
Bacillus sp.	15811	++	-	+	-	155
Bacillus sp.	4791	++	-	-	+	156
Bacillus sp.	4791	++	-	-	+	156
Bacillus sp.	4791	++	-	-	++	156
Bacillus sp.	15811	++	-	-	++	156
Bacillus sp.	15811	++	-	-	+	156
Bacillus sp.	15811	++	-	-	+	156
Bacillus sp.	15811	++	-	-	+	156
Bacillus sp.	10801	++	-	-	+	156
Bacillus sp.	7128	++	-	-	++	156
Bacillus sp.	7128	++	-	-	++	156
Bacillus sp.	7128	++	-	-	+	156
Bacillus sp.	7128	++	-	-	++	156
Bacillus sp.	7128	+	-	-	++	156
Bacillus sp.	447	++	-	-	+	156
Bacillus sp.	4791	+	-	+	++	159
Bacillus sp.	4791	+	+	+	++	160
Bacillus sp.	10801	++	++	+	+	160
Bacillus sp.	7128	++	++	+	+	160
Enterobacter sp.	1297	-	-	-	-	1
Enterobacter sp.	1297	-	-	-	-	1
Enterobacter sp.	1297	-	++	-	-	5
Enterobacter sp.	1297	-	++	-	-	5
Enterobacter sp.	1297	+	++	-	-	9
Enterobacter sp.	1297	+	++	-	-	9

Enterobacter sp.	1297	+	++	-	-	9
Enterobacter sp.	1297	++	++	++	-	10
Enterobacter sp.	4791	+	-	+	-	11
Enterobacter sp.	10801	++	-	++	-	11
Enterobacter sp.	10801	++	-	++	-	11
Enterobacter sp.	1297	+	++	-	++	14
Paenibacillus sp.	3240	++	++	+	+	192
Pseudomonas sp.	3240	++	++	++	+	48
Pseudomonas sp.	3240	++	++	++	+	48
Total # bacteria (%)	3.70E+05	96.67%	15.09%	23.77%	46.04%	

Table 7. Cultivable shoot bacteria on Standard 1/10 rich medium with agar with plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	4192	-	++	-	-	149
Bacillus sp.	28147	-	+	-	-	149
Bacillus sp.	947	++	-	-	-	152
Bacillus sp.	947	++	-	-	-	152
Bacillus sp.	947	++	-	-	-	152
Bacillus sp.	947	+	-	-	-	152
Bacillus sp.	947	++	-	-	-	152
Bacillus sp.	947	+	-	-	-	152
Bacillus sp.	947	++	-	-	-	152
Bacillus sp.	15530	+	-	-	-	152
Bacillus sp.	15530	+	-	-	-	152
Bacillus sp.	15530	+	++	-	-	153
Bacillus sp.	15530	+	++	+	-	154
Bacillus sp.	15530	++	++	+	-	154
Bacillus sp.	947	++	-	-	++	156
Bacillus sp.	15530	++	-	-	+	156
Bacillus sp.	15530	++	+	-	+	158
Bacillus sp.	28147	+	-	+	++	159
Bacillus sp.	947	+	++	+	++	160
Bacillus sp.	2987	+	+	+	++	160
Bacillus sp.	2987	+	+	+	+	160
Bacillus sp.	15530	++	++	+	+	160
Brevibacillus sp.	2987	-	-	-	-	242
Enterobacter sp.	4192	-	-	-	-	1
Enterobacter sp.	447	-	-	+	-	3
Enterobacter sp.	4192	-	-	+	-	3
Enterobacter sp.	4192	-	-	+	-	3
Enterobacter sp.	4192	-	-	+	++	4
Enterobacter sp.	947	-	++	-	-	5
Enterobacter sp.	1493	-	+	-	-	5
Enterobacter sp.	15530	-	++	-	-	5
Enterobacter sp.	2987	-	+	+	-	6
Enterobacter sp.	2987	-	+	+	-	6
Enterobacter sp.	4192	-	++	+	-	6
Enterobacter sp.	4192	-	++	+	-	6
Enterobacter sp.	2987	-	++	+	+	7
Enterobacter sp.	2987	-	++	+	++	7
Enterobacter sp.	2987	-	++	+	+	7
Enterobacter sp.	15530	-	++	+	+	7
Enterobacter sp.	4192	-	+	+	+	7
Enterobacter sp.	4192	-	+	+	++	7
Enterobacter sp.	28147	-	++	+	+	7
Enterobacter sp.	28147	++	+	+	-	10

Enterobacter sp.	28147	++	-	-	+	12
Enterobacter sp.	28147	+	-	-	++	12
Enterobacter sp.	28147	+	-	-	++	12
Enterobacter sp.	28147	+	-	-	+	12
Enterobacter sp.	1493	-	++	-	+	13
Enterobacter sp.	2987	+	+	-	++	14
Enterobacter sp.	28147	+	+	-	+	14
Enterobacter sp.	28147	++	+	-	++	14
Pseudomonas sp.	2987	-	-	+	++	36
Rhizobium sp.	447	++	-	-	-	104
Total # bacteria (%)	5.18E+05	70.96%	57.79%	38.86%	62.61%	

Table 8. Cultivable shoot bacteria on Standard 1/10 rich medium with gellan gum without plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	18392	-	-	+	+	148
Bacillus sp.	7276	-	+	-	-	149
Bacillus sp.	7276	+	++	+	-	154
Bacillus sp.	7276	+	++	+	-	154
Bacillus sp.	7276	+	++	+	-	154
Bacillus sp.	7276	++	+	++	-	154
Bacillus sp.	7276	-	++	-	+	157
Enterobacter sp.	7276	-	-	-	-	1
Enterobacter sp.	9459	-	-	-	-	1
Enterobacter sp.	7276	-	-	-	++	2
Enterobacter sp.	9459	-	-	-	++	2
Enterobacter sp.	9459	-	-	-	++	2
Enterobacter sp.	9459	-	-	-	++	2
Enterobacter sp.	18392	-	-	+	-	3
Enterobacter sp.	18392	-	-	+	+	4
Enterobacter sp.	18392	-	-	+	+	4
Enterobacter sp.	7276	-	++	-	-	5
Enterobacter sp.	9459	-	++	-	-	5
Enterobacter sp.	7276	-	++	+	-	6
Enterobacter sp.	7276	-	++	+	-	6
Enterobacter sp.	7276	-	++	+	-	6
Enterobacter sp.	9459	-	+	+	-	6
Enterobacter sp.	9459	-	+	+	-	6
Enterobacter sp.	9459	-	+	+	-	6
Enterobacter sp.	7276	-	++	-	+	13
Enterobacter sp.	9459	-	+	-	+	13
Enterobacter sp.	7276	+	++	-	++	14
Paenibacillus sp.	7276	+	-	-	++	188
Pseudomonas sp.	18392	-	-	+	-	35
Pseudomonas sp.	9459	-	+	++	+	39
Total # bacteria (%)	2.96E+05	14.76%	48.72%	61.12%	46.96%	

Table 9. Cultivable shoot bacteria on Standard 1/10 rich medium with gellan gum with plant extract

Identification	cfu/g	SID	OA	IAA	ACC	Phen. char.
Bacillus sp.	15181	-	-	-	++	146
Bacillus sp.	1842	-	-	+	+	148
Bacillus sp.	18392	-	+	-	-	149
Bacillus sp.	6326	-	+	-	-	149
Bacillus sp.	6326	-	++	-	-	149
Bacillus sp.	6326	-	+	-	-	149
Bacillus sp.	6326	-	++	-	-	149
Bacillus sp.	18345	-	+	-	-	149
Bacillus sp.	15181	+	-	-	-	152
Bacillus sp.	11422	+	++	-	-	153
Bacillus sp.	11422	+	++	-	-	153
Bacillus sp.	15181	++	++	-	-	153
Bacillus sp.	15181	+	+	-	-	153
Bacillus sp.	18345	++	++	-	-	153
Bacillus sp.	18345	++	++	-	-	153
Bacillus sp.	18345	+	++	-	-	153
Bacillus sp.	18392	++	++	+	-	154
Bacillus sp.	18345	+	++	+	-	154
Bacillus sp.	15181	+	-	-	++	156
Bacillus sp.	1842	+	+	-	++	158
Bacillus sp.	15181	+	++	-	+	158
Bacillus sp.	15181	+	++	-	++	158
Bacillus sp.	15181	++	++	-	++	158
Bacillus sp.	18392	++	-	+	+	159
Bacillus sp.	1842	++	++	+	++	160
Bacillus sp.	1842	+	++	+	++	160
Bacillus sp.	1842	++	++	+	++	160
Bacillus sp.	15181	+	++	++	+	160
Brevibacillus sp.	11422	+	-	+	-	251
Brevibacillus sp.	1842	-	++	-	+	253
Brevibacillus sp.	18345	-	++	-	+	253
Brevibacillus sp.	18345	-	++	-	+	253
Brevibacillus sp.	18345	-	++	-	+	253
Brevibacillus sp.	18345	-	++	-	+	253
Enterobacter sp.	6326	-	-	-	-	1
Enterobacter sp.	6326	-	-	+	-	3
Enterobacter sp.	6326	-	-	+	-	3
Enterobacter sp.	11422	-	++	-	-	5
Enterobacter sp.	11422	-	++	-	-	5
Enterobacter sp.	1842	-	++	+	-	6
Enterobacter sp.	1842	-	++	+	-	6
Enterobacter sp.	1842	-	++	+	-	6
Enterobacter sp.	11422	-	++	+	-	6

Enterobacter sp.	11422	-	++	+	-	6
Enterobacter sp.	6326	-	++	+	-	6
Enterobacter sp.	6326	-	++	+	-	6
Enterobacter sp.	6326	-	++	+	-	6
Enterobacter sp.	6326	-	++	+	-	6
Enterobacter sp.	6326	-	+	+	-	6
Enterobacter sp.	1842	-	+	+	++	7
Enterobacter sp.	1842	-	+	+	+	7
Enterobacter sp.	11422	-	++	+	+	7
Enterobacter sp.	15181	-	+	+	+	7
Enterobacter sp.	995	+	++	-	-	9
Enterobacter sp.	18392	++	-	+	-	11
Enterobacter sp.	18392	++	-	+	-	11
Enterobacter sp.	6326	-	+	-	++	13
Enterobacter sp.	6326	-	++	-	+	13
Paenibacillus sp.	995	-	++	-	-	181
Paenibacillus sp.	995	-	++	-	-	181
Pseudomonas sp.	6326	-	++	+	++	39
Pseudomonas sp.	6326	+	++	+	-	42
Rhizobium sp.	6326	+	++	-	-	105
Variovorax sp.	11422	-	++	-	+	61
Variovorax sp.	11422	-	++	-	++	61
Total # bacteria (%)	6.54E+05	49.50%	79.67%	36.93%	40.68%	

Table 10. Cultivable bacteria on Standard 1/10 rich medium from seeds

Identification	cfu/g	SID	OA	IAA	ACC	Phenotypic characteristics
Actinomycete sp.	2.99E+03	+	+	+	-	138
Actinomycete sp.	5.51E+02	+	+	+	-	138
Bacillus sp.	2.36E+02	+	+	+	-	154
Bacterium SC5	1.50E+03	+	+	-	-	217
Bacterium SC5	1.50E+03	+	+	+	-	218
Enterobacter sp.	4.61E+03	+	+	+	-	10
Enterobacter sp.	1.50E+03	+	+	+	-	10
Enterobacter sp.	1.54E+03	+	+	+	-	10
Enterobacter sp.	1.50E+03	+	-	+	+	15
Paenibacillus sp.	1.54E+03	+	-	+	-	187
Paenibacillus sp.	1.54E+03	+	+	+	+	192
Pseudomonas sp.	3.07E+03	+	+	+	-	42
Pseudomonas sp.	4.61E+03	+	+	+	-	42
Pseudomonas sp.	2.36E+02	+	+	+	-	42
Pseudomonas sp.	7.09E+02	+	+	+	-	42
Pseudomonas sp.	1.54E+03	+	-	+	-	43
Total # bacteria (%)	2.77E+04	100.00%	77.92%	102.86%	6.27%	

Table 11. Cultivable bacteria on Standard 1/10 rich medium with seed extract from seeds

Identification	cfu/g	SID	OA	IAA	ACC	Phenotypic characteristics
Actinomycete sp.	5.51E+02	+	+	+	-	138
Actinomycete sp.	5.51E+02	+	-	+	-	139
Actinomycete sp.	5.51E+02	+	+	+	+	144
Bacillus so.	2.36E+02	+	+	-	-	153
Bacillus sp.	2.36E+02	+	-	-	-	152
Bacterium SC5	7.09E+02	+	+	-	-	217
Bacterium SC5	7.09E+02	+	+	+	-	218
Enterobacter sp.	1.42E+03	+	+	+	-	10
Enterobacter sp.	3.35E+03	+	+	+	-	10
Enterobacter sp.	6.69E+02	+	+	+	+	16
Exiguobacterium sp.	2.36E+02	+	-	+	-	171
Exiguobacterium sp.	2.36E+02	+	+	-	+	174
Exiguobacterium sp.	2.36E+02	+	+	+	+	176
Ochrobactrum sp.	2.36E+02	+	+	+	+	32
Pseudomonas sp.	2.36E+02	+	+	+	+	48
Weissella sp.	7.71E+02	-	+	-	-	197
Total # bacteria (%)	1.09E+04	92.95%	90.64%	79.99%	19.79%	

Table 12. Cultivable bacteria from Rhizosphere on Standard 1/10 rich medium						
Identification	cfu/g	SID	OA	IAA	ACC	Phenotypic characteristics
Stenotrophomonas sp.	9.94E+02	-	-	+	++	4
Stenotrophomonas sp.	9.94E+00	-	-	+	++	4
Stenotrophomonas sp.	9.94E+01	+	-	+	+	15
Stenotrophomonas sp.	9.94E+02	+	-	+	++	15
Total # bacteria (%)	2.10E+03	47.39%	0.00%	100.00%	100.00%	

Table 13. Cultivable bacteria from Rhizosphere on Standard 1/10 rich medium with plant extract						
Identification	cfu/g	SID	OA	IAA	ACC	Phenotypic characteristics
Bacillus sp.	1.85E+01	+	-	++	-	11
Bacillus sp.	2.78E+01	+	-	++	+	15
Enterobacter sp	6.17E+01	+	-	+	-	11
Enterobacter sp	3.09E+01	++	-	+	+	15
Gamma proteobacterium	3.09E+01	-	-	+	++	4
Gamma proteobacterium	1.54E+01	+	-	+	-	11
Gamma proteobacterium	4.63E+01	+	-	++	+	15
Total # bacteria (%)	2.31E+02	86.65%	0.00%	100.00%	58.70%	

Table 14. Cultivable bacteria from Rhizosphere cultivated in liquid medium with plant extract on Standard 1/10 rich medium with plant extract						
Identification	cfu/g	SID	OA	IAA	ACC	Phenotypic characteristics
Enterobacter sp.	1.85E+02	+	-	-	++	12
Enterobacter sp.	1.85E+02	+	-	+	+	15
Enterobacter sp.	1.54E+02	+	-	+	-	15
Ochrobactrum sp.	9.23E+01	+	+	-	++	14
Ochrobactrum sp.	1.54E+02	+	-	+	++	15
Ochrobactrum sp.	1.54E+02	+	-	+	-	15
Stenotrophomonas sp.	9.23E+01	-	-	-	-	1
Stenotrophomonas sp.	2.77E+02	-	-	-	++	2
Stenotrophomonas sp.	4.61E+02	-	-	-	+	2
Stenotrophomonas sp.	7.72E+02	-	-	+	++	4
Stenotrophomonas sp.	7.72E+02	-	-	+	++	4
Stenotrophomonas sp.	1.54E+02	-	-	+	++	4
Stenotrophomonas sp.	1.54E+02	+	-	+	-	11
Stenotrophomonas sp.	1.54E+02	+	-	+	++	15
Stenotrophomonas sp.	3.09E+02	+	-	+	++	15
Total # bacteria (%)	4.07E+03	37.87%	2.27%	72.78%	86.38%	

Table 15. Effect of Acclimatization on cultivable shoot bacteria isolated on 1/10 rich medium

Identification	cfu/g
Actinomycete sp.	7.29E+03
Enterobacter sp.	6.39E+01
Enterobacter sp.	6.39E+02
Ochrobactrum	2.43E+04
Stenotrophomonas sp.	2.56E+02
Stenotrophomonas sp.	6.39E+02
Stenotrophomonas sp.	6.39E+04
Stenotrophomonas sp.	7.29E+04
Stenotrophomonas sp.	7.29E+04
Stenotrophomonas sp.	7.29E+04
Stenotrophomonas sp.	4.86E+04
Total # bacteria	3.64E+05

Table 16. Effect of Acclimatization on cultivable root bacteria isolated on 1/10 rich medium with agar

Identification	cfu/g
Beta proteobacterium	2.18E+03
Enterobacter sp.	7.28E+03
Enterobacter sp.	2.91E+03
Enterobacter sp.	1.41E+04
Exiguobacterium sp.	2.12E+02
Gamma proteobacterium	7.07E+03
Ochrobactrum sp.	7.28E+03
Ochrobactrum sp.	7.28E+03
Ochrobactrum sp.	7.28E+02
Ochrobactrum sp.	7.28E+04
Ochrobactrum sp.	3.64E+03
Ochrobactrum sp.	3.64E+04
Ochrobactrum sp.	4.24E+02
Ochrobactrum sp.	2.83E+04
Paenibacillus sp.	1.46E+04
Paenibacillus sp.	7.07E+03
Pseudomonas sp.	7.28E+03
Pseudomonas sp.	1.41E+02
Rhizobium sp.	7.28E+02
Stenotrophomonas sp.	4.37E+04
Stenotrophomonas sp.	3.64E+04
Stenotrophomonas sp.	5.09E+03

Stenotrophomonas sp.	7.28E+04
Stenotrophomonas sp.	2.26E+04
Stenotrophomonas sp.	7.28E+03
Stenotrophomonas sp.	7.28E+04
Stenotrophomonas sp.	7.28E+04
Stenotrophomonas sp.	7.28E+04
Stenotrophomonas sp.	3.64E+04
Stenotrophomonas sp.	7.28E+04
Stenotrophomonas sp.	7.28E+03
Stenotrophomonas sp.	7.28E+03
Stenotrophomonas sp.	9.19E+02
Stenotrophomonas sp.	5.65E+03
Stenotrophomonas sp.	7.07E+03
Stenotrophomonas sp.	2.83E+04
Stenotrophomonas sp.	7.07E+04
Stenotrophomonas sp.	7.07E+04
Stenotrophomonas sp.	1.41E+04
Stenotrophomonas sp.	1.41E+04
Total # bacteria	9.62E+05

Table 17. Effect FAM 0.2 µm Minisart on cultivable shoot bacteria isolated on 1/10 rich medium with agar

Identification	cfu/g
Actinomycete	3.65E+04
Actinomycete	4.86E+04
Actinomycete	4.86E+04
Bacterium SC5	3.65E+04
Bacterium SC5	2.43E+04
Bacterium SC5	2.43E+04
Gamma proteobacterium	7.29E+03
Gamma proteobacterium	7.29E+02
Gamma proteobacterium	7.29E+02
Ochrobactrum spp.	1.09E+03
Ochrobactrum spp.	7.29E+02
Paenibacillus sp.	1.09E+03
Stenotrophomonas sp.	7.29E+03
Stenotrophomonas sp.	7.29E+02
Weissella sp.	7.29E+03
Total # bacteria	2.46E+05

Table 18. Effect of FAM 0.2 µm Minisart on cultivable root bacteria isolated on 1/10 rich medium with agar

Identification	cfu/g
Enterobacteriaceae	7.07E+04
Ochrobactrum	7.28E+03
Ochrobactrum	7.28E+03
Ochrobactrum	7.28E+03
Ochrobactrum	2.43E+04
Paenibacillus sp.	7.07E+02
Staphylococcus sp.	7.07E+04
Stenotrophomonas sp.	7.07E+02
Stenotrophomonas sp.	4.85E+04
Stenotrophomonas sp.	7.28E+05
Stenotrophomonas sp.	3.64E+03
Stenotrophomonas sp.	8.73E+03
Stenotrophomonas sp.	1.46E+03
Stenotrophomonas sp.	7.28E+03
Stenotrophomonas sp.	2.18E+03
Stenotrophomonas sp.	7.28E+04
Total # bacteria	1.06E+06

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Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2014**

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