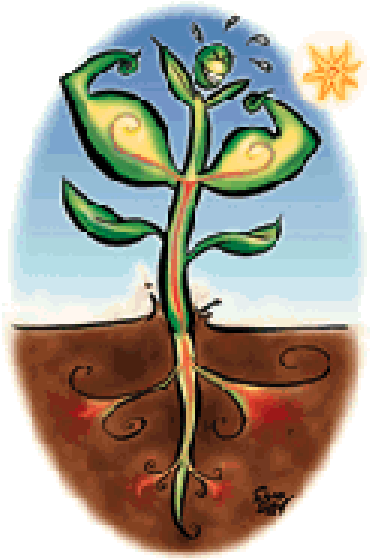
Professional Bachelor Agro- and Biotechnology

In planta screening for the positive growth effect of inoculated DDE degrading endophytic bacteria in Cucurbita pepo



Laurens Vanoppen

Promotors:

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Dr. ir. Nele Weyens	CMK-UHasselt
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Foreword

First of all, I would like to thank dr. ir. Nele Weyens for letting me fulfil my undergraduate internship at the Centrum for Environmental Science. Secondly I want to thank my promotors drs. Nele Eevers and dr. Ilse Smets for their guiding and evaluation of my work. Thanks to their advice I could bring my bachelor thesis to a good end. And thirdly, I would like to thank the colleagues in the lab for showing me all the stuff I could not find once in a while…

During my bachelor thesis it did not all went very well from the first time. For instance, I had to inoculate the seeds and let them grow in the greenhouse for three times, because I could not manage to keep my plants alive. That was a difficult time for me, but I kept carrying on. Eventually after the third time I succeeded in letting my plants grow enough for harvesting them. I felt pretty stupid, maybe even ashamed afterwards, because I had to put a lot of work to inoculate the seeds each time and eventually all my plants really extra needed was water with more minerals… Cultivating plants, it is something I totally did not expected to learn during my bachelor thesis. My mother surely would be ashamed if she knew about it.

I believe I may say that I have learned a lot more about phytoremediation in the past few months and that my interests for environmental sciences have increased ever since.

Abstract (EN)

DDT (Dichloro-Diphenyl-Tricholoroethane) is a pesticide that has been intensively used from 1945 to 1973 in Belgium. Because DDT and its primarily metabolites (DDE (Dichloro-Diphenyl-Ethylene) and DDD (Dichloro-Diphenyl-Ethane)) are highly persistent, traces are still being found today in the soil and the groundwater. DDE is the most abundant DDT-linked substance found in soil/groundwater, though still low in concentration. But thanks to its hydrophobic characteristic, DDE has the tendency to accumulate in fatty tissues up to a concentration where it can have negative effects on the health of animals and the ecosystem. This effect is called biomagnification. The focus of removing DDT-linked contaminants from soil/groundwater is put on DDE, because it is the most found DDT-linked substance in the soil/groundwater.

The use of mechanical methods for the removal of contaminants from soil/groundwater is rather expensive and has a destructive impact on the soil quality, causing it not to be desired by the public opinion. The use of phytoremediation, on the other hand, is much cheaper and is environmentally friendly. But phytoremediation is less efficient than mechanical methods and takes several years. For the phytoremediation of DDE-contaminated soils and groundwater, *Cucurbita pepo* will be used since it has the ability the extract (phytoextraction) large amounts of DDE without storing it in its fruits (zucchini). But the efficiency of phytoremediation of *Cucurbita pepo* can be upgraded by adding PGPBs (Plant Growth Promoting Bacteria). Normally, in healthy soils, there are sufficient amounts and different species of PGPBs present. But this is not the case in DDE-contaminated soils, since DDE is toxic to many bacterial species. Adding DDE-tolerant bacterial species to *Cucurbita pepo* can therefore benefit the plant growth and make the phytoremediation process of DDE-contaminated soils more efficient.

First of all, an appropriate endophytic bacterial strain had to be identified by performing a phenotypical characterization on a collection of 530 endophytic bacterial strains that were previously isolated from the endosphere of *Cucurbita pepo* in a field experiment on a DDE-contaminated soil. The major plant growth promoting capabilities were investigated, such as the production of IAA (Indole-3-Acetic Acid), ACC (1-aminocyclopropane-1-carboxyl)-deaminase, siderophores, organic acids, and the capability of solubilising phosphor in the soil. Afterwards, a selection was made based on the tolerance of the endophytic strains for DDE by performing an auxanography test with DDE. Ten DDE-tolerant endophytic bacteria were selected and individually inoculated to *Cucurbita pepo* seeds. After germination and a growth period of 15 days, the roots and shoots were weighed separately to determine the plant growth. Afterwards, the bacteria present in the endosphere were isolated, purified and genotypically identified by sequence alignment of the amplified 16s rDNA gene on NCBI (National Center for Biotechnology Information). Afterwards the inoculation efficiency of the inoculant was evaluated as well as the possible positive effect of the inoculant on the plant growth by comparing the biomass of the inoculated plants with the biomass of the control plants that were not inoculated.

From the results it appears that less than 50% of the inoculations were successful. The inoculation of *Ensifer sp.* can be seen as the only one that was successful. Unfortunately, there was no improved plant growth visible when the mass of the plant was compared with the control plants. Inoculating more plants at the same time with *Ensifer sp.* and letting the plants grow on a DDE-contaminated soil, might give a clearer difference in plant growth between the inoculated and the uninoculated plants.

Samenvatting (NL)

DDT (Dichloor-Difenyl-Trichloorethaan) is een pesticide dat intensief gebruikt werd sinds 1945 tot 1973 in België. Omdat DDT en zijn primaire afbraakproducten, DDE (Dichloor-Difenyl-Ethyleen) en DDD (Dichloor-Difenyl-Dichloorethaan) zeer persistente moleculen zijn, worden er nog steeds sporen gevonden in de bodem en het grondwater. DDE is de voornaamste DDT-gelinkte stof die voorkomt in de bodem en het grondwater, doch in lage concentraties, maar dankzij bio-accumulatie van deze hydrofobe stof in het vetweefsel van dieren, kan het geconcentreerd geraken tot een niveau waarop het schade toe kan brengen aan de gezondheid van dieren en het ecosysteem. Aangezien DDE het grootste deel uitmaakt van DDT gelinkte stoffen in de bodem en het grondwater, wordt er gefocust op het verwijderen van DDE.

Het gebruik van mechanische technieken voor het verwijderen van contaminanten uit de bodem en het grondwater zijn duur en destructief voor de bodemkwaliteit en valt daarom ook niet in de smaak bij het ruime publiek. Het gebruik van fytoremediatie daarentegen is veel natuurvriendelijker en vooral goedkoper. Alleen is deze techniek minder efficiënt dan mechanische technieken en heeft het meerdere jaren nodig. Voor fytoremediatie van DDE-vervuilde bodems, wordt *Cucurbita pepo* gebruikt omdat het grote hoeveelheden DDE kan extraheren (fytoextractie) en omdat het geen DDE opslaat in zijn vruchten (courgetten). Maar de efficiëntie van de fytoremediatie door *Cucurbita pepo* kan wel nog verbeterd worden door het toevoegen van PGPBs (PlantenGroei-Promoverende Bacteriesoorten). In een gezonde bodem zijn zulke PGPBs normaal vanzelf in voldoende hoeveelheid en diversiteit aanwezig. Maar in DDE-vervuilde bodems is dit niet het geval. DDE is namelijk toxisch voor een groot deel van de bacteriesoorten. Het toevoegen van plantengroei-promoverende bacteriesoorten die een tolerantie hebben voor DDE kan de groei van *Cucurbita pepo* verbeteren en dus ook de efficiëntie van het fytoremediatie-proces.

Allereerst werd er gezocht naar de geschikte endofyten (plantgeassocieerde bacteriën afkomstig van de endosfeer) d.m.v. een fenotypische karakterisatie op een collectie van 530 endofytische bacteriestalen, geïsoleerd uit de endosfeer van *Cucurbita pepo*, gekweekt op een DDE vervuilde bodem. De voornaamste plantengroei-promoverende eigenschappen werden onderzocht: de productie van IAA (Indoleen-3-ethaanzuur), ACC (1-aminocyclopropaan-1-carboxyl) -deaminase, sideroforen, organische zuren en de mogelijkheid om fosfor op te lossen in de bodem. Daarnaast werd er ook geselecteerd naar DDE-tolerante soorten door een auxanografietest met DDE uit te voeren. Er werden tien DDE-tolerante endofytische bacteriën geselecteerd en individueel geïnoculeerd aan *Cucurbita pepo* zaden. Na ontkieming en een groeiperiode van 15 dagen, werd de plantengroei bepaald door de massa van de wortelen en stengel met bladeren apart te bepalen. Hierna werden de bacteriën uit de endosfeer geïsoleerd, opgezuiverd en genotypisch gekaraktiseerd via DNA-sequentie alignering van het geamplificeerde 16s rDNA gen op NCBI (National Center for Biotechnology Information). Hierna kon er geconcludeerd worden of de inoculant aanwezig was in de endosfeer en of deze een positief effect kon hebben op de plantengroei door de massa van de plant te vergelijken met deze van de niet-geïnoculeerde planten.

Uit de resultaten blijkt dat minder dan 50 % van de inoculaties gelukt zijn. De inoculatie van *Ensifer sp.* kan als enige als succesvol beschouwd worden. Helaas is er geen verbeterde plantengroei zichtbaar door deze inoculant. Meerdere planten tegelijk met deze bacteriesoort inoculeren en de planten laten groeien in aanwezigheid van DDE, kan uitwijzen op een duidelijker groeiverschil met de controle planten.

Table of contents

Abbreviations

ACC	1-aminocyclopropan-1-carboxyl
CAS	Chroom-Azurol S
Cfu	Colony forming units
Cmix	Carbon source mix
DDD	2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane
DDE	2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene
DDT	2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane
DDTs	DDT and its metabolites: DDE, DDD, …
DNA	Deoxyribonucleic acid
IAA	Indole-3-acetic acid
LD50	Lethal Dose, 50 = the percentage of dead in a population after a specific dose
NCBI	National center for biotechnology information
PCB	Polychlorinated biphenyls
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

Introduction

DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane) is an organochlorine insecticide that has been intensively used from 1945 until 1973 in Belgium. Traces of DDTs are still being found in the soil and water worldwide. DDE (2,2-bis(p-chlorophenyl)-1,1-dichloroethylene) is the main metabolite of DDT. Due to its persistence and its tendency to accumulate in fatty tissues, DDE has adverse effects on the nervous system, is hormone disrupting and has bio accumulating capabilities due to its hydrophilic and lipophilic properties. An efficient method for cleaning up DDE contamination is necessary to reduce further health risks to wildlife and humans worldwide [1].

An intensely studied method to remove contaminants from soil or water is phytoremediation. This is the use of plants to remove, degrade or immobilise contaminants from the soil or water. This technique has many advantages over conventional mechanical methods. It is an ecologically friendly, solar-energy driven clean-up technology, based on the concept of using nature to cleanse nature [2]. But phytoremediation is a rather slow process and therefore it is in need of some help. Bacterial help to be precise. To enhance a phytoremediation process, plant-associated bacteria with plant growth-promoting or contaminant degrading or immobilising capabilities could be added [3].

Cucurbita pepo (zucchini) can be used for the phytoremediation of DDE-contaminated soils [4]. These are vascular plants that are able to accumulate high concentrations of DDE. In this project, a screening of a bacterial collection extracted from the endosphere from *Cucurbita pepo* grown on a DDE-contaminated field, was performed in order to investigate if there is an endophytic bacterial strain that can enhance the phytoremediation process of DDE-contaminated soils with *Cucurbita pepo* by their plant growth promoting capabilities.

A collection of genotypically identified endophytic bacterial strains, that were isolated from *Cucurbita pepo* plants cultivated in a DDE-contaminated field, was available. These bacterial strains were then phenotypically identified, by testing their ability to produce IAA (Indolene-3-Acetic Acid), ACC (1-aminocyclopropan-1-carboxyl), siderophores and organic acids and to solubilize $Ca_2(PO_4)_3$, as well as their tolerance for DDE. Afterwards, a selection of DDE-tolerant endophytic bacterial strains are inoculated in *Cucurbita pepo,* in order to find out if these bacteria also have an *in planta* plant growth promoting effect. The endophytic bacteria were again extracted, purified and genetically identified by amplifying the conserved 16s rDNA domain after DNA-extraction and were sequenced. By comparing the resulting DNA sequences to references at online databases, the corresponding bacterial species were discovered and the presence of the inoculant in the endosphere was evaluated. If the inoculant was present in the endosphere, the biomass of the inoculated plants can be compared with control plants that were not inoculated, to evaluate whether the inoculant had a positive effect on the plant growth.

1. Literature

1.1 DDE pollution and impact on the environment and to humans

1.1.1 DDT/DDE use and pollution

DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane) (figure 1) is an organochlorine insecticide that was first discovered in 1874. It is not a natural product, but is synthetically created. DDT was a commonly-used pesticide for insect control in Belgium until it was forbidden in 1973 together with most western countries. It was initially used by the military of the allies during and after WWII in Europe to control malaria, typhus, body lice [5]. Cases of malaria fell from 400,000 in 1946 to virtually none in 1950 [6]. DDT is still used today Africa and Asia [7, 8] for this purpose. This demonstrated the effectiveness of DDT and farmers got permission to use it as a pesticide on a variety of food crops worldwide. DDT was also used for pest control in buildings. Therefore, DDT was used on a large scale worldwide because of its effectiveness, relative low manufacture cost, and high persistency in the environment compared to modern-day pesticides.

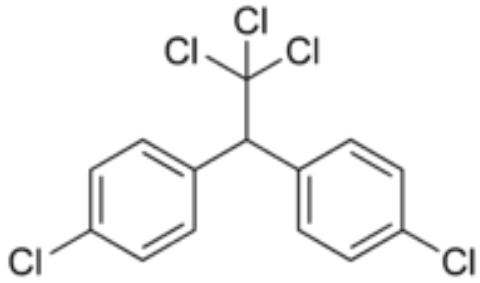


Figure 1: chemical structure of DDT [11]

By the 1960s, evidence indicated that DDTs, such as DDE, persist in the environment, accumulate in fatty tissues (biomagnification), and can cause adverse health effects on wildlife [9]. In addition, resistance occurs in some insects (like the house fly) who developed the ability to quickly metabolize DDT [5].

The mechanism of DDT is to affect the nervous system by interfering with normal nerve impulses. DDT opens sodium ion channels in neurons, causing the nerve cells to repeatedly generate an impulse which accounts for the repetitive body tremors in exposed animals [10].

1.1.2 DDE production

DDE (2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene) is not naturally found in the environment, it is only found as a recalcitrant degradation product of DDT. DDE is created by dehydrohalogenation of DDT. The loss of HCl results in a double bond on the central (previously) quaternary carbon atoms [11].

DDT can be degraded to several different metabolites through aerobic biotic degradation, abiotic dehydrochlorination (figure 2), and even through photochemical decomposition. DDE, however, has been reported to be more persistent than DDT and its other metabolites. DDE can therefore be found in soil decades after the last DDT treatment. DDE is also the most frequently encountered degradation product of DDT in soils worldwide [12]. DDE toxicity and persistence are regarded to be a serious environmental problem [11].

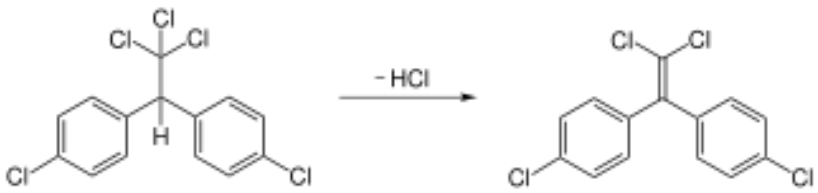


Figure 2: dehydrohalogenation of DDT to DDE [11]

1.1.2 DDTs toxicity on the environment and to humans

Because DDTs (DDTs = DDT and its metabolites: DDE, DDD, …) are highly persistent organic pollutants (POP), they are able to stay in the soil and water for decades and adversely affect the present organisms. Because of DDTs chemical properties (hydrophobic and lipophilic), they have the tendency to accumulate in animals. As animals lower on the food chain are eaten by other animals higher up, DDE becomes concentrated in the fatty tissue of predators. This continues until the primary predator of the food chain receives the highest dose, which may lead to negative health effects [1]. This phenomenon is called biomagnification or bioaccumulation.

1.1.2.1 Acute toxicity

DDT has a LD50 (Lethal Dose for 50% of subjects) for rats by oral intake of 113 mg/kg [9] for DDE this is 850 mg/kg [13].

1.1.2.2 Chronical toxicity

DDT and DDE are both endocrine disruptors that are similar to estrogens and can therefore trigger hormonal responses in animals. These can negatively affect the development of the reproductive system of both female and male animals [14].

The available data about neurological effects from DDT/DDE in wildlife species, suggest that these effects are similar to those observed in humans. The problem is that it is difficult to fully understand the neurological effects on humans, because it is not allowed to observe at lethal doses, while in animals this is possible. The nervous system seems to be the number one target for DDT toxicity in humans. During an experiment in the 1940s, volunteers were exposed with an acute dose of DDT and experienced disturbance of sensitivity of the lower part of the face, malaise, cold moist skin, hypersensitivity and severe vomiting. Another study was performed on workers who produced DDT in a factory. They seemed to have an overall poorer performance in verbal attention and the ability to synchronize visual information with physical movement [14].

1.1.2.3 Carcinogenicity

There is no clear evidence that DDTs can cause cancer to humans. Though in several experiments it did cause liver cancer to mice, rats, and hamsters after chronic dietary exposure to DDE. The EPA (Environmental Protection Agency) has estimated an oral cancer potency factor for DDT and DDE of $3.4x10^{-1}$ mg/kg/day by oral intake. This was based on the incidence of liver tumors in mice and rats. There have been speculations that DDTs are responsible for breast cancer. Yet many studies revealed there is no significance relation between breast cancer and DDTs [15, 16, 17, 18]. Though a study of elderly women with breast cancer who were at the of age 12 to 20 years during the 1950s did showed a significance result to DDT [19]. The use of DDT peaked around 1950, so it was more likely to get potentially-breast-cancer-inducing doses around that time [14].

1.1.2.4 Breast milk

DDTs have the tendency to accumulate in fatty tissues due to its hydrophobic and lipophilic chemical characteristics. Milk also contains fat and can therefore be a way for DDTs to be passed on to juvenile mammals. This can have adverse effects to the growth development, since this is controlled by many systems and functions that are depended on the timely activation of hormones, especially by sexual steroids. Interfering with such actions during development can lead to a wide array of effects that may include altered metabolic, sexual, immune, and neurobehavioral functions [14].

1.1.2.5 Eggshell thinning

Biomagnification in birds especially occurs in predatory birds that consume fish [20]. Due to the biomagnification process of DDE in the sea from plankton up to fish, these birds can accumulate a high dose DDE from the fish they consume. The reproductivity is adversely affected because of the eggshell thinning, so the eggs cannot be breaded out.

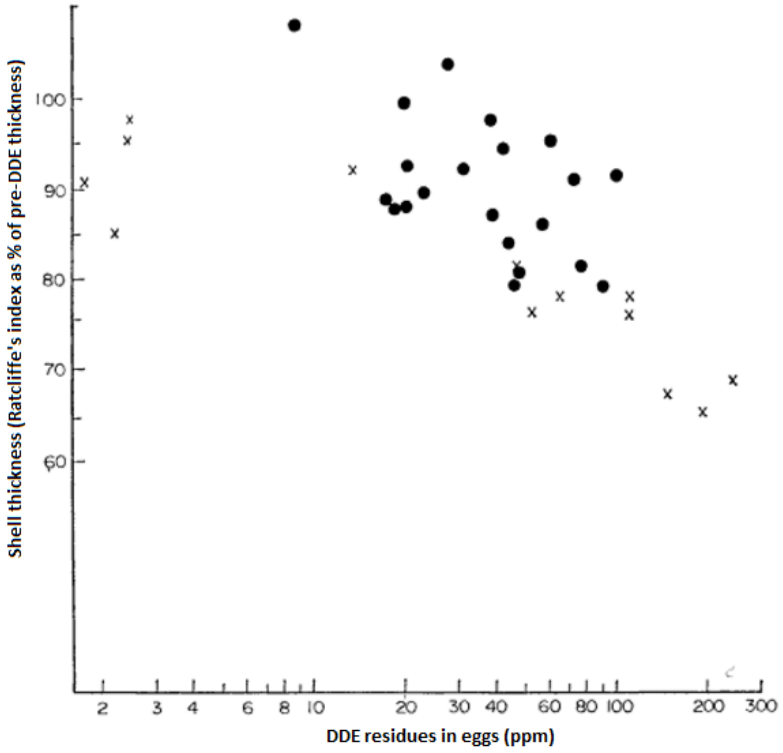


Figure 3: Relation between shell thickness and DDE residues in eggs. This figure shows the relationship between the shell thickness and the DDE residues found in the eggs of American kestral eggs. These values are represented by a dot. A cross represents the value of experimentally dietary induced eggshell thinning with DDE. [21]

It can be concluded from figure 3 that rising DDE values in birds cause egg shell thinning. As a result, when a bird wants to go sit on her or his eggs to incubate them, the eggs are crushed by the weight of the bird. DDE contamination has been proven to be de cause of eggshell thinning in many reports and seems to be more potent than DDT [14]. Especially the bird of prey, such as the bald eagle and brown pelican [20] suffer from declining populations due to DDE contamination.

1.2 A natural solution: Phytoremediation enhanced with plant-associated bacteria

The generic term “phytoremediation” consists of the Greek prefix *phyto* (means: plant), attached to the Latin root *remedium* (means: to correct or remove an evil).

With phytoremediation, plants are used to revitalize polluted soils. The definition for phytoremediation is as followed: The use of plants for the removal/degradation/stabilisation of harmful environmental contaminants from soil or water.

Phytoremediation is an extensively studied concept [22]. Reports of these studies indicate that certain plants are able to grow in polluted soil and water without being seriously. This means it is possible to use plants to remediate contaminated soils or water through agricultural and biotechnological approaches. Some plant

species grow better than other, depending on the contaminant. Plants are equipped with metabolic and absorption capabilities, as well as transport systems that can take up nutrients or contaminants selectively from the growth matrix. Higher plants, plants with vascular tissues, can possess abilities for the metabolism and degradation (phytodegradation) of many contaminants and their recalcitrants [23, 24]. These plants can be considered as “green livers”, acting as an important biological sink for environmentally damaging chemicals [25]. Phytoremediation involves growing, or encouraging the growth of, plants in a contaminated matrix, either artificially (constructed wetlands) or naturally for a required growth period to remove contaminants from the matrix or facilitate immobilization (binding / containment) or degradation (detoxification) of the pollutants.

Phytoremediation can be used to remove metals, pesticides, solvents, explosives, crude oil, and polyaromatic hydrocarbons residues from soil or water [2].

Phytoremediation can be considered an alternative or complimentary technology that can be used along with or, in some cases, instead of conventional mechanical clean-up technologies that often require high capital inputs and are labour and energy intensive. Phytoremediation is an *in situ* remediation technology that utilizes the inherent abilities of living plants. It is also an ecologically friendly, solar-energy driven clean-up technology, based on the concept of using nature to cleanse nature [26].

1.2.1 Importance of plant-associated bacteria

Previous research has revealed that there are possibilities in improving the phytoremediation efficiency by using plant-associated bacteria [2, 3]. Plant-associated bacteria consist of a wide range of bacterial species, which are associated with a wide range of plant species. These bacteria can be divided in 3 categories according to their habitat: phyllospheric, rhizospheric and endophytic bacteria [27].

The phyllosphere is a term used in microbiology to refer to the total above-ground portions of plants as habitat for micro-organisms [28, 29]. The phyllosphere can be further subdivided into the caulosphere (stems), phylloplane (leaves), anthosphere (flowers), and carposphere (fruits). Most work on phyllosphere microbiology has focused on leaves, a more dominant aerial plant structure. Bacteria are by far the most numerous micro-organisms on leaves [30].

The rhizosphere is a thin layer of soil that is being directly influenced by the root excretions of the plant. The majority of the soil is not a part of the rhizosphere and is called bulk soil. Plants secrete many compounds through their roots to serve symbiotic functions in the rhizosphere. The diversity of rhizospheric bacteria is dependent on the composition of the excretions of the roots. The rhizospheric microorganisms compete for water, nutrients and space and sometimes improve their competitiveness by developing an intimate association with plant. Rhizospheric bacteria with plant growth promoting capabilities are categorized as PGPR (Plant Growth Promoting Rhizobacteria). Symbiotic nitrogen-fixing bacteria, such as *Rhizobium* species belong to this group [31].

The endosphere is defined as all the internal tissues of a plant. Endophytes are a group of micro-organisms that are able to colonize the endosphere without causing negative effects to the plant. Endospheric bacteria have the advantage that they are protected from the weather elements and there is less competition in the endosphere than there is in the rhizosphere. This is due to the abundance of available nutrients in the endosphere compared to what is available in the rhizosphere. Also, the interaction between plants and bacteria is more strongly interwoven to each other in the endosphere than in the rhizosphere or phyllosphere. An interestingly fact about endophytic bacteria is that they can also colonise the endosperm of the seeds. This means that the endophytic bacterial community of a plant can be carried over to its descendants through the seeds [32].

Phyllospheric, rhizospheric or endophytic bacteria can all possess plant growth promoting capabilities. These can be divided into direct and indirect plant growth promoting capabilities.

1.2.1.1 Direct plant growth promoting capabilities

Direct plant growth promotion includes capabilities such as the production of plant growth stimulating hormones (auxins, cytokines and gibberellins), the suppression of the production of ethylene (stress hormone) and the solubilisation of bio-unavailable nutrients such as phosphorus, Fe^{3+} and other minerals by the secretion of organic acids and siderophores.

IAA (indole-3-acetic acid) is an important auxin which has a positive effect on root proliferation, growth and elongation. IAA can be produced by some bacterial species when its precursor tryptophan is present. When a bacterial strain is able to produce this auxin, the plant-bacteria symbiosis can be stronger.

Suppression of stress ethylene production could be exerted by bacteria that possess 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. There are bacterial strains that are able to consume ACC and thereby lower the stress response of the plant, if the bacteria possess the gene that codes for enzyme 1-aminocyclopropan-1-carboxyl deaminase. This enzyme is responsible for the hydrolysis of the plants ethylene precursor, ACC, into ammonia and α-ketobutyrate. Bacteria can use ammonia as N-source. ACC (1-aminocyclopropan-1-carboxyl) is the precursor of ethylene. Ethylene is a stress hormone that is produced when the plant is in a stressful situation. This stress hormone causes plant growth inhibition to plants [33].

In figure 5, [33] the production of ACC deaminase and synthesis of IAA by growth-promoting bacteria are. An interesting observation is the connection between the IAA and ACC production. So when a bacterial strain contains both these capabilities, it can influence the ACC production from the plant by metabolising tryptophan to IAA. These plant growth-promoting capabilities do not necessarily have to be simultaneously present in one bacterial species, but can also be combined from several bacterial species in a consortium.

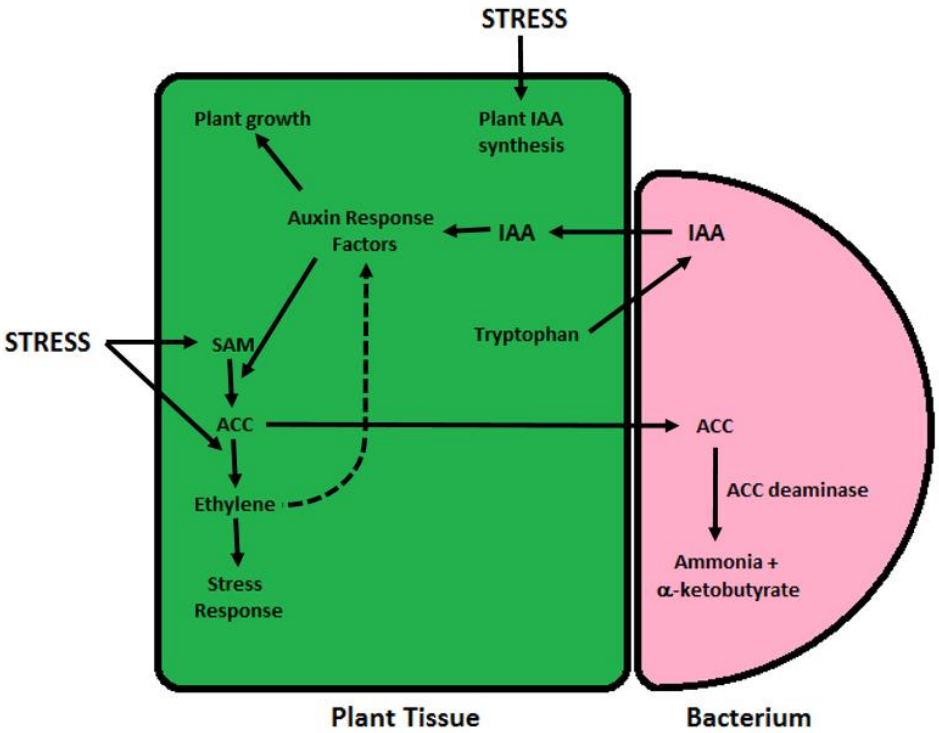


Figure 4: Model of the effect of ACC deaminase and IAA production by a bacterium to a plant [33]

The bioavailability of $Fe^{3+}$is limited because of its low solubility. Some bacterial species are able to produce siderophores to take up sufficient amounts of Fe^{3+}. Siderophores are very strong iron chelators which can keep Fe^{3+} soluble at high concentrations [34].

Clay humus complexes in the soil contain positive and negative charges that can attract cation and anion nutrients causing decreased solubility and diminished availability to the organisms. Unless organic acids are being produced and secreted by bacteria, which can increase the solubility of the nutrients

Phosphorus is one important mineral of which the bioavailability can be facilitated by the excretion of organic acids. Phosphorus is a component of the complex nucleic acid structure of plants, which regulates protein synthesis. Phosphorus is therefore important in cell division and development of new tissues. Phosphorus is also associated with complex energy transformations in the plant. Although it is present in abundance in the soil, the bioavailability of phosphorus is often limited [35]. This is because it is generally in its insoluble form, $Ca_3(PO_4)_2$. But phosphorus is one very important element that is mostly necessary in the first stages of a plants life. Certain bacterial strains are able to solubilize these phosphates by lowering the pH around the roots through the production and secretion of organic acids. As a result, the following reaction takes place:

$$Ca_3(PO_4)_2\ (s) + 6\ H_3O+\ (aq) \rightleftarrows 3Ca^{2+}\ (aq) + 6\ H_2O\ (l) + H_3PO_4\ (aq)$$

(S) = solid, (aq) = aqueous

1.2.1.2 Indirect plant growth promoting capabilities

PGPB can indirectly promote the plant growth and development by decreasing the inhibitory effects of various pathogens. The application of microorganisms to control diseases, which is a form of biological control, is an environment-friendly approach. This involves production of hydrolytic enzymes, antibiosis, induction of plant defence mechanisms and inhibition of pathogen-produced enzymes or toxins and competition for nutrients between pathogenic and PGPB. PGPB in the rhizosphere that are able to produce organic acids and/or siderophores have the advantage of making surrounding bio-unavailable nutrients bioavailable. Pathogenic bacteria lacking these capacities can only consume what is left behind by the PGPB [36].

1.2.2 Different forms of phytoremediation

Phytoremediation is a collective term of different methods that can be accommodated for soil/water remediation. Depending on the underlying processes, phytoremediation can be divided into 6 categories [37]:

Mostly used for organic contaminants:

- Phytodegradation
- Phytostimulation
- Phytovolatilisation

Mostly used for metal contaminants:

- Phytoextraction
- Rhizofiltration
- Phytostabilization

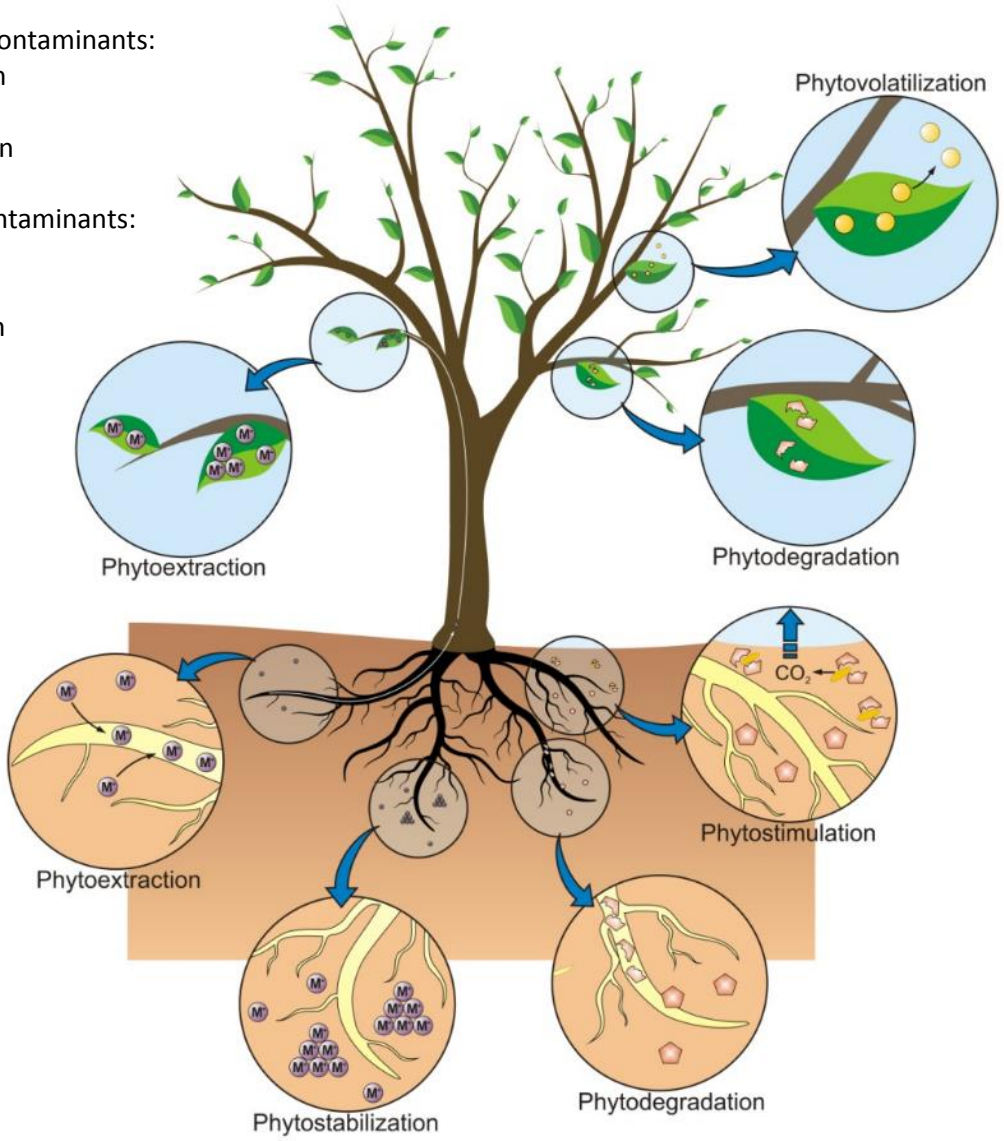


Figure 5: Scheme of the difference phytoremediation processes [37]

1.2.2.1 Phytodegradation

Phytodegradation or phytotransformation is the breakdown of complex organic molecules, that are surrounded by a plant or are taken up by a plant, through degradation effects of compounds (such as enzymes) produced by the plant itself or its associated microorganisms. By breaking the complex organic pollutants down to simple molecules, these molecules can be integrated into the plant tissues or used as nutrients. Phytodegradation has been observed to remediate some organic contaminants, such as chlorinated solvents [38], herbicides [11], and munitions [39], and it can address contaminants in soil, sediment, or groundwater [40].

1.2.2.2 Phytovolatilisation

Phytovolatilization is the uptake and transpiration of organic contaminants by a plant. Transpiration will lead to the release of the contaminant in its initial or a modified (phytodegraded) form into the atmosphere. The contaminant migrates from the roots up into the leaves where water can evaporate together with its dissolved substances into the atmosphere. The occurrence of this process depends on whether the contaminant has a hydrophilic characteristic. [40]

1.2.2.3 Phytostimulation

Phytostimulation or rhizodegradation, is the breakdown of organic pollutants in the soil through roots secretion or by microbial activity that is enhanced by the presence of the rhizosphere. Microorganisms consume and digest organic substances in the soil and can also digest fuels, solvents or other contaminants that are hazardous to humans and wildlife. Natural substances released by the plant roots (root exudates) such as sugars, alcohols and acids, contain energy for microorganisms, and the additional nutrients enhance their activity. Phytostimulation is also aided by the way plants loosens the soil and transports water into the area [41].

1.2.2.4 Phytoextraction

Phytoextraction, also known as phytoaccumulation, is the uptake of contaminants from soil or water by plant roots into plant tissues. Accumulation into above ground plant tissues permits accumulated contaminants to be harvested without completely destructing the plant. Certain plants, called hyperaccumulators, are able to absorb unusually large amounts of metals and organic contaminants [42]. After a growth period, they will be harvested and can be composted to recycle the metals or they are combusted for energy production. In case of combustion, the ashes must be disposed of in a hazardous landfill. The mass of ashes is only a fraction of the mass that would have to be discarded if the contaminated soil would have to be removed. This process can be repeated as many times as necessary to bring the contaminant concentration below an acceptable limit [40].

1.2.2.5 Rhizofiltration

Rhizofiltration (‘rhizo’ means ‘root’) is the adsorption or precipitation onto plant roots (or absorption into the roots) of contaminants that are in solution surrounding the root zone. This is very similar to phytoextraction, but the plants are more efficient in cleaning up contaminated groundwater than soil. The plants to be used for clean-up are raised in a greenhouse with their roots in the water. Contaminated water is collected and brought to the plants in the greenhouse. This makes it possible to remediate *in situ* or *ex situ*. Plants grown with their roots in water in a greenhouse can still be used for another phytoremediation process after a remediation process. The plants can of course also be planted in the contaminated area itself, where the roots take up the water and the contaminants dissolved in it [40]. For example, sunflowers were successfully used to remove radioactive cesium contaminants from pond water in a test at Chernobyl, Ukraine [43].

1.2.2.6 Phytostabilization

Phytostabilization is the use of certain plant species for the immobilisation of contaminants in the soil or groundwater through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants (rhizosphere). With this phytoremediation technique it is possible to reduce the mobility of contaminants and therefore prevent them from further migration into groundwater or air. This also means that the bioavailability is reduced and that it will be more difficult for a contaminant to occur in the food chain. This technique can re-establish a vegetative cover and is therefore applied at sites where natural vegetation and biodiversity has dropped due to high metal concentrations in surface soils. Metal-tolerant species can be used to restore vegetation to the sites, thereby decreasing the potential migration of contamination through wind erosion and transport of exposed surface soils and leaching of soil contamination to groundwater [40].

1.2.3 Advantages and disadvantages

It is important to consider the advantages and disadvantages of different remediating techniques before chosing which one to apply. Phytoremediation is a cost effective technique compared to the traditional mechanical removal of contaminated soil or water. A big disadvantage to this technique is that it can only be used to remediate sites with a limited contaminant concentration, because plants can only tolerate a certain concentration. Another problem is that smaller plants only work to a depth of approximately 50 cm. This is obviously not the case when fast growing trees are used, trees can grow deeper than 50 cm into the soil. Furthermore, it is not possible to remove pollutans to a level dating from before the pollution by using phytomremediation. Though this is of less importance, because polluted soils only have to be remediated to a certain degree [2].

In Table 1, the most common advantages and disadvantages/limitations have been listed:

Table 1 the most common advantages and disadvantages/limitations of phytoremediation [44, 45]

Advantages	Disadvantages/Limitations
Adaptable to a wide range of organic and inorganic contaminants.	Limited to sites with shallow contamination within the rooting zone of remediative plants.
In situ applications decrease the amount of soil disturbance compared to conventional mechanical methods.	A long remediation periode is often necessary, up to several years.
Harvested plant biomass from phytoextraction in case of heavy metal contaminant, can be used as bio-ore of heavy metals.	Limited to sites with low contaminant concentrations
In situ applications decrease the spreading of contaminants via air and water. Less secondary contamination through off-site migration via water than with traditional methods.	Harvested plant biomass from phytoextraction may be classified as hazardous waste. Disposal should be proper.
Expensive in research but very cheap in use. Does not require expensive equipment, higly specialized personnel nor high energy usage. It is cost-effective for large volumes of water and for large areas having low concentrations of contaminants.	Consumption/utilization of contaminated plant biomass is a cause of concern. Contaminants can still enter the food chain through animals/insect that eat plant material containing contaminants.
In large scale application the potential energy stored can be utilized to generate bio energy plant	Introduction of non-native species may affect biodiversity
	Climate is an important factor that affects the rate of growth of plants that can be utilized.

1.2.3 Phytoremediating promoting capabilities of PGPB

Plant growth promoting bacteria can enhance a phytoremediation process by simply increasing the plant growth and therefore helping immobilising the contaminant (phytostabilization). But, they can also ameliorate phytoremediation processes on a more specific way. For instance, phytoextraction can be enhanced by PGPB that produce natural chelators like siderophores and organic acids (citrate, oxalate, malate, …). Beside the fact that this capability promotes the plant growth (1.2.1.1), it can also promote the phytoremediation process by chelating metal contaminations to solubilise them and making them available for the plants [46]. Phytoremediation processes are overall being enhanced by adding PGPB that are tolerant to the contaminant or have a contaminant degrading capacity. Many endophytic and rhizospheric bacteria possess capabilities to degrade organic contaminants as they would use it as nutrients. Bacteria with such capabilities are able to promote a phytodegradation processes in soil contaminated with organic pollutants. Phytovolatilisation is sometimes not sufficient for remediating soils contaminated with organic contaminants that have volatile characteristics. Because plants and their rhizospheric and endospheric micro-organisms only partially degrade such contaminants since they do not get enough contact time with the contaminant as they migrate from the roots to the leaves. In result, plants emit the contaminants through evapotranspiration causing the contaminants to be freely dispersed into the environment and thereby undermining the purpose of phytoremediation. Though, succeeding in the inoculation of the endosphere with endospheric bacteria capable of degrading such contaminants, has already been proven in the field that such bacteria can improve the phytoremediation results. Endophytic bacteria can further degrade organic contaminants while these are migrating from roots to the shoots through the xylem [3].

1.2.4 *Cucurbita pepo* and its applicability in phytoremediation

Cucurbita pepo is a vascular plant that can be used for DDE remediation, because it has DDE accumulating capabilities [46]. It is also a fast growing plant, capable of extracting large amounts of water necessary for its fruits, the zucchinis. DDE does not appear to be able to migrate into the fruit [47]. This means that there is a low chance for DDE to get in the food chain by consumption of its fruits.

1.3 Principles of the phenotypic characterisation of plant-associated bacteria

These phenotypic characterisation tests are commonly used for plant-associated bacteria to determine if they possess one of the following plant growth-promoting characteristics: Production of IAA, siderophores, ACC-deaminase, organic acids. The ability to solubilise phosphorus and if they are tolerant for DDE.

1.3.1 Auxanography

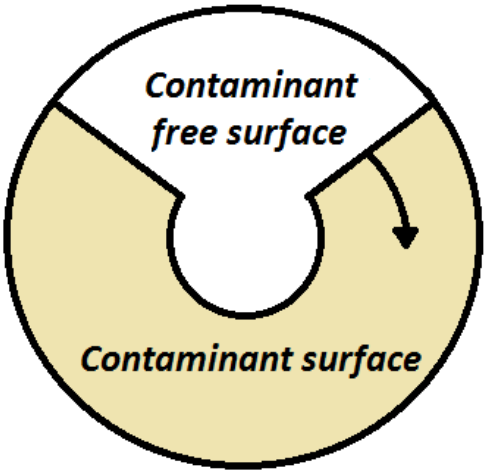


Figure 6: Scheme of auxanography

An auxanography is a biological method to determine the ability of the growth of microorganisms on various carbon or nitrogen sources or to test its ability to tolerate some compounds, e.g. a pollutant or contaminant. The cultivated bacteria are plated out on a culture medium allowing the growth of the bacteria. Directly afterwards the pollutant containing solution is applied on the culture plate as shown in figure 6. After an incubation period of 4 to 7 days, colonies should be visible. When colonies are only visible in the contaminant free surface, then the bacteria strain is not tolerant for the contaminant. If colonies are also visible in the polluted surface, then the bacterium has a tolerance for the pollutant. If the colonies are visible in a higher degree on the contaminated surface than on the contaminant free surface, then the bacteria strain has a degrading ability for the contaminant. This technique is a simple, cheap, low labour phenotypical identification [48]. This makes this technique suitable for a screening. In figure 7 and 8 are given an example of a negative and a positive test.

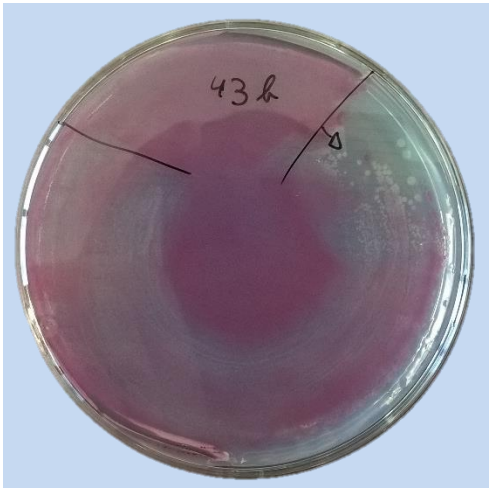


Figure 8: negative example of an auxanography test with DDE as pollutant. Strain 43 b: Sphingomonas sp.

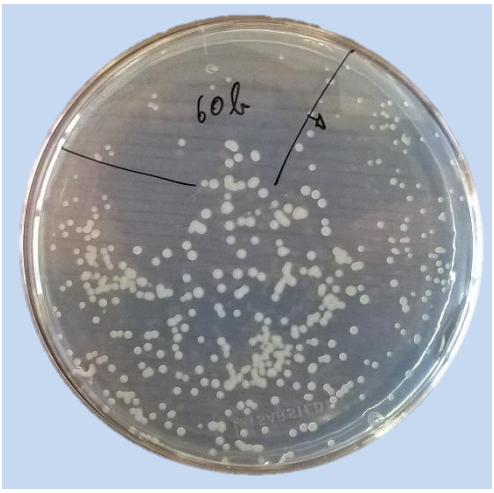


Figure 8: positive example of an auxanography test with DDE as pollutant. Strain 60b: Ensifer sp.

1.3.2 IAA test

To determine whether a bacterial strain is able to produce IAA (Indolene-3-Acetic Acid), it can be cultivated in a general cultivating medium with an excess of tryptophan. This will induce IAA production, because tryptophan is the precursor of IAA. IAA can then be detected after addition of the Salkowskireagent. When this reagent is mixed with IAA, tris-(indole-3-acetato) iron(III) complexes are formed causing pink coloration. If a bacterial strain is capable to metabolize tryptophan to IAA, the solution colours pink and the test result is positive (Pink = positive; yellow = negative) [49]. An example is given in figure 9. This examples shows that it is not always very clear if a bacterial strain is positive or not.

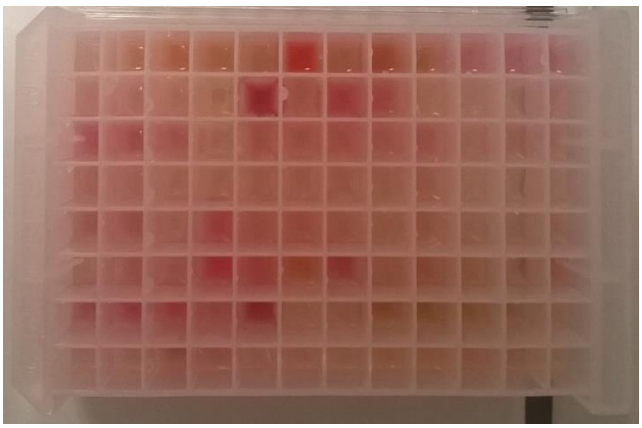


Figure 9: example of an IAA test

1.3.3 ACC deaminase test

To test whether a bacterial strain is able to produce ACC-deaminase, production is induced by cultivating the bacteria in a selective medium that contains ACC as a sole N-source (ammonia after cleavage). After an incubation period, a Brady 's test can be executed. This is a staining reaction that detects the presence of ketones/aldehydes. α-ketobutyrate contains a ketone on C3 (figure 8) and can thereby be detected by this test. So when a bacterial strain is able to produce ACC deaminase and cleave ACC, α-ketobutyrate will be produced and can be detected.

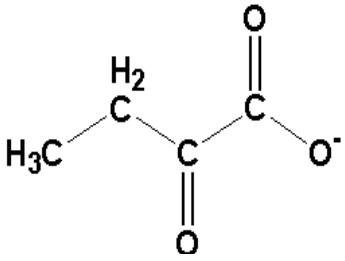


Figure 10: Chemical structure of α-ketobutyrate [64]

Brady 's test:

2,4-Dinitrophenylhydrazine can be used to qualitatively detect the carbonyl functionality of a ketone or aldehyde functional group. A positive test is signalled by a yellow or red precipitate (known as a dinitrophenylhydrazone).

$RR'C=O + C_6H_3(NO_2)_2NHNH_2 \rightarrow C_6H_3(NO_2)_2NHNCRR' + H_2O$

This reaction can be described as a condensation reaction, with two molecules joining together with loss of water. It is also considered an addition-elimination reaction: nucleophilic addition of the $-NH_2$ group to the C=O carbonyl group, followed by the removal of a H_2O molecule [50]. In figure 9 an example is given of this test.

Figure 11: Example of Brady's test to detect production of α-ketobutyrate

1.3.4 Siderophores test

By using the method of Schwyn & Neilands [51], it is possible to detect the production of siderophores. The detection method is based on the affinity of siderophores for Fe^{3+} and is therefore independent of the structure of the chelator. This means this test is able to detect other iron chelators as well. CAS (Chrome-Azurol S) is added to detect the presence of siderophores. This is a solution that holds a highly coloured iron dye complex. Siderophores can chelate the iron from this complex and this will cause a colour change from blue to orange (blue = negative, orange = positive; chelator takes Fe^{3+} from colouring, blue turns orange) [51].

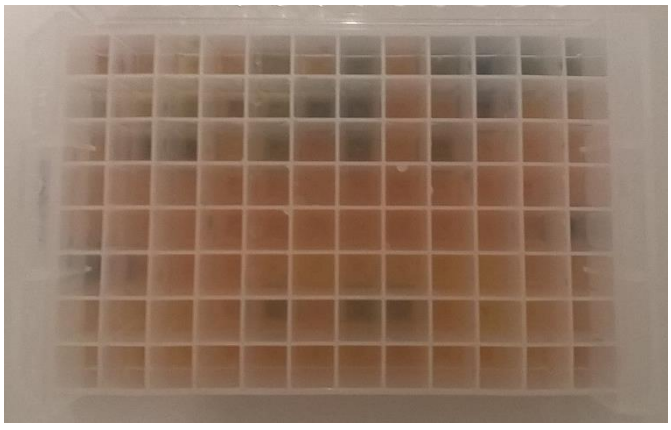


Figure 12: Example of an siderophores test

1.3.5 Phosphorous solubilisation test

The phosphorus solubilisation capability of a bacterial strain can be determined growing the bacteria on agar plates with insoluble $Ca_3(PO_4)_2$. The plates are inoculated in a single hole with a micropipette. from this spot only one large colony will grow. Around this colony, a clear halo zone will appear if the bacterium is able to produce and secrete amounts of organic acids [52].

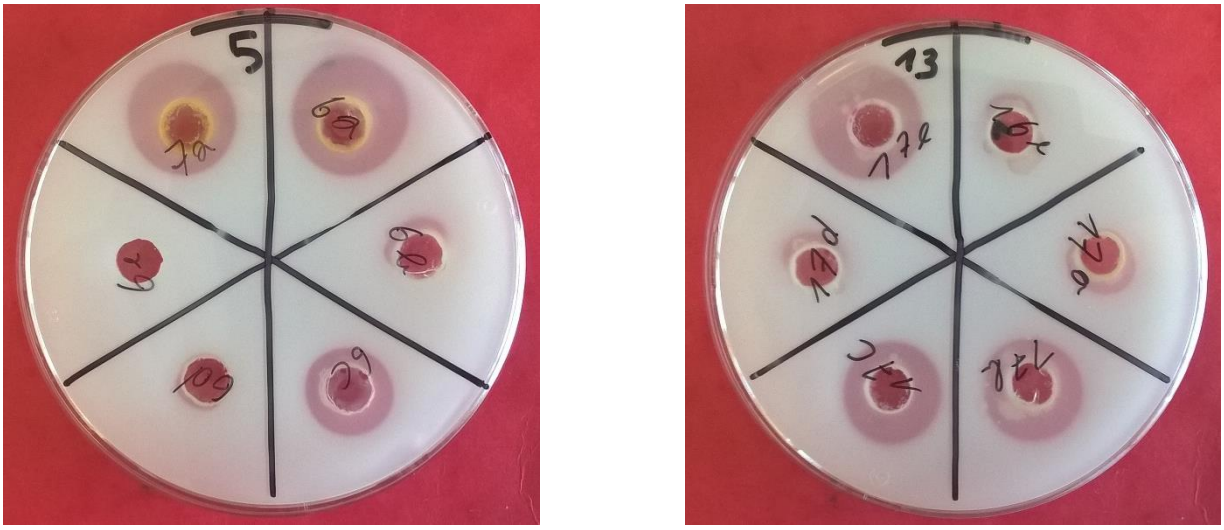


Figure 13: example of phosphorus solubilisation test, 2 plates with each 6 different bacterial strains.

1.3.6 Organic acids test

To test whether bacterial strains can produce and secrete organic acids, they are cultivated in a sucrose-tryptone medium. This medium induces the production of organic acids. After an incubation period, a pH-indicator can be added. For this test, alizarin red can be used as pH indicator (Yellow = positive, pink = negative) [52].

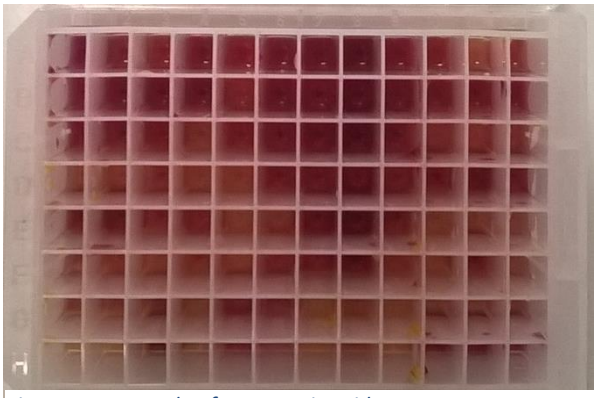


Figure 14: Example of an organic acids test

1.4 Inoculating the plants endosphere

When suitable bacteria for assisting a specific phytoremediation process are not present in the soil nor in the seeds, human interference in the form of inoculation is necessary to populate the endosphere of the plant. Inoculation can be described as effectively introducing new bacterial species to the endosphere by adding them as a bacterial suspension to the seeds and to let the seeds use this moisture for germination [32].

1.5 DNA-extraction from bacteria

Extracting DNA from bacteria is generally performed by the following steps: cell lysis, purification, concentrating and analysis via spectrophotometry. Cell lysis is necessary to set the DNA free in a pH-buffered suspension. A proteolysis step subsequently is performed on the lysate by adding Proteinase K. Purification of the lysate can be done by using a membrane that can bind the negatively charged DNA and lets other cell particles pass through when a centrifugal force is applied. After a wash step, the DNA can be eluted in an empty tube. The quality of a DNA-extract is determined by measuring the absorbance of DNA at A260, Proteins at A280 and organic solvents at A230. This qualification is necessary to conclude if a DNA-extract contains PCR (Polymerase Chain Reaction) inhibitors and so can or cannot be used for PCR amplification [53, 54].

1.6 Species determination through amplification and sequencing of the 16s rDNA gene

To determine the species of an isolated bacterial strain, the conserved characteristics of the 16s rDNA gene can be consulted. Highly conserved sequences are often genes required for basic cellular functions, like the function of the transcripted 16s rRNA which has a structural role in the 30S small subunit of prokaryotic ribosomes and is therefore an important factor for the synthesis of proteins. The sequence of the 16s rDNA gene varies in an orderly manner across phylogenetic lines and contains segments that are conserved at the species, genus, or kingdom level. A list of universal primers exists for the amplification of the 16s rDNA gene. These are oligonucleotide primers that are targeted at the 5’ and 3’ extreme ends of the 16s rDNA gene [55]. The sequence of the PCR-product can then be determined and afterwards aligned at the online nucleotide database of NCBI (National Center for Biotechnology Information). This database holds the largest collection of nucleotide sequences. When a nucleotide sequence is aligned on this database, the database gives a list of species in return that possess a highly similar sequence. This list is sorted on the identity percentage. The identity percentage represents the percentage that a search result is equal to the subject’s DNA sequence. The species with highest identity percentage can be presumed to be the same species as the subject. This applies only when the highest identity percentage is 90 % or higher.

2. Materials and methods

2.1 Experimental design:

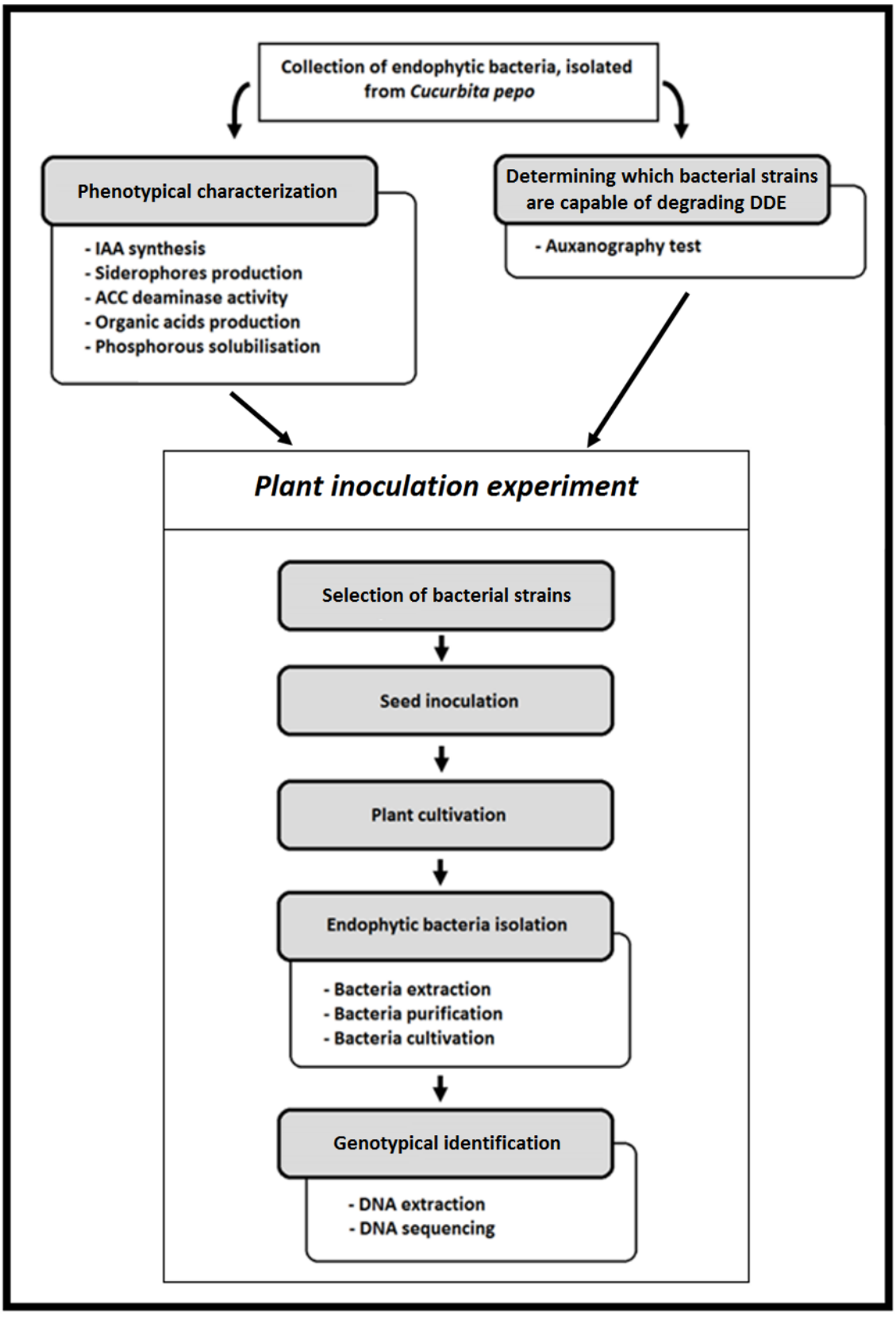


Figure 15: Scheme of the experimental design

A collection of 530 bacteria strains extracted from 40 *Cucurbita pepo* plants from an earlier field experiment was available. The field experiment dated from 10/06/2015 to 01/09/2015 and was performed on DDE-contaminated soil in the United States (Lockwood Farm, New Haven, Connecticut, USA). The endophytic bacteria were isolated and stored in 15 %w glycerol at -80 °C. These bacterial strains were already genetically identified by ARDRA (Amplified Ribosomal DNA Restriction Analysis) and DNA sequencing of the 16s rDNA gene.

This collection was cultivated and underwent a phenotypical characterization for IAA, siderophores and organic acids production. And for ACC deaminase activity and for the ability to solubilise phosphorus. At the same time, an auxanography test was performed to visualise any DDE tolerance or degrading capacity.

In the plant inoculation experiment (figure 15) the influence of the plant growth promoting capabilities of the DDE-tolerant bacterial endophytes were tested in *Cucurbita pepo*. This was done by introducing the DDE tolerant bacterial endophytes back into the endosphere by inoculating *Cucurbita pepo* seeds and evaluating the plant biomass.

After a growth period of 15 days in a greenhouse, the endophytic bacteria were isolated from the endosphere. First the plant mass, roots and shoots separately, were weighed to visualise any growth speed differences on the plant. To isolatethe bacteria from roots and shoots, the surfaces first were chemically sterilised to exclude any rhizo- or phyllospheric bacteria from the isolation. The dicot leafs were not included, since the endophytic bacterial community possibly differs from the rest of the plant. Plant tissues were crushed in sterile $MgSO_4$ using mortar and pestle.

The purification of an isolate of a mixture of different bacterial species was done by plating them out on agar plates. After an incubation period, the colonies were differentiated from each other by their colour and shape. Picking one individual colony gives the insurance that only one bacterial species is being transferred to a new medium or agar plate. To insure this even more, the selected colony was transferred to a new agar plate to dilute the bacteria from the colony and detect any other bacterial species that might be inside the colony in low numbers.

After all the colonies were differentiated, counted and given an ID, the next part of this experiment could start. All the selected colonies needed to be cultivated in liquid medium to have enough DNA for DNA extraction. The quality of the DNA was analysed by using spectrophotometry, thanks to the aromatic rings from nucleic acids, to decide whether a DNA extract was in need for an additional purification step or if the DNA should be diluted necessary for PCR.

For determining the species of the selected bacteria, the 16s rDNA gene was amplified. This gene is commonly used for the genotypically identification of bacteria thanks to its highly conserved characteristic. A list of universal primers exists for the amplification of the 16s rDNA gene. The amplified 16s rDNA from the PCR-product was sequenced. The DNA sequence was compared via “sequence alignment” with other DNA sequences on an online databases tool from NCBI.

After all the selected colonies from the bacteria purification were genetically identified, all the information was obtained to compare the endophytic bacterial community from the control plants with the plants that were inoculated. And link this result to the plant growth by comparing the biomass of the control plants with the inoculated plants.

2.3 Cultivating bacterial collection

Purpose:
Cultivating the bacterial strains so a large enough population of bacteria is produced to apply the following tests on them.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain)

Method:

- Add 1 ml rich (undiluted) medium 869 (See attachments) to each well to be inoculated
- Inoculate each well with the correct bacterial stock
- Incubate at 30 °C for at least 48 hours
- To extend the lifetime of a cultivation, remove medium and add 1 ml fresh medium 869 (undiluted).

2.3 Phenotypic characterization tests

2.3.1 Auxanography with DDE

Purpose:
Determining which bacterial strains are able to grow on DDE and which are not.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain)

Method:

Following actions must be performed under a laminar flow:

- Pipet 25 ml medium 284 + C-mix for each plate (See attachments to prepare medium)
- After solidifying, place plates at room temperature to dry
- Add 990 µl $MgSO_4$ (10 mM, final concentration 100 µM) to each masterblock well.
- Dilute 10 µl of the bacterial suspension in to the masterblocks.
- Add a drop of 100 µl 100x diluted bacteria solution on the agar plate.
- Spread with ent spatula until dry.
- Add a 30 µl drop of DDE solution (100 µg/l) on the arrow.
- Spread from the arrow to the second bar.
- Incubate at 30 °C for 5 days.

2.3.2 IAA test

Purpose:
Determining which bacterial species are able to produce a growth promoting auxin, IAA.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain)

Method:

- Prepare IAA medium:
  - Prepare a liquid medium 869 (1/10 diluted) (see attachments).
  - Add 0,5 g L-tryptophan in 1 l medium:
    - Solve the desired amount of tryptophan in a minimal volume HCl (0,1 M).
    - Bring to pH 6-7 with KOH (10 M).
    - Add to the medium via filter sterilisation (0,20 µm pore).
- Prepare Salkowskireagens (34,3 % $HClO_4$, 10 mM $FeCl_3$):
  - 539 ml $HClO_4$ (35%) (70 %, dilute to half).
  - 11 ml $FeCl_3$ (0,5 M, 81,1 g/l).

  Caution!!! Highly exothermic while dissolving, do not rinse!!!: Add water on top of the weighed amount $FeCl_3$ instead of the other way around.
- Grow bacteria in 1 ml IAA medium in masterblock (see masterblock inoculation tables).
  - Use 8-channel electronic pipet to inoculate.
- Incubate at 30 °C for 4 days.
- Centrifuge at 2000 rpm for 15 min.

Note: Don't centrifuge > 2000 rpm, masterblock will burst and lose liquid.

- Pipet 0,5 ml supernatants to empty masterblock.
- Add 1 ml Salkowskireagens.
- After 20 min: pink = positive, yellow = negative.

2.3.3 Siderophores test

Purpose:

Determining which bacterial species are able to produce siderophores in order to solubilise sufficient Fe^{3+}.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain)

Method:

- Prepare liquid 284 medium + C-mix (Attachments) with 0,00 µM; 0,25 µM; 3,00 µM Fe(III)citrate:
  - Add Fe(III)citrate before autoclave to autoclave bottle:
    - 0,25 µM → Add 0,061 g/l Fe(III)citrate.
    - 3,00 µM → Add 0,743 g/l Fe(III)citrate.
- Fill masterblocks with 800 µl 284 medium (0,00 µM; 0,25 µM; 3,00 µM Fe(III)citrate).
- Add 20 µl of the in liquid 869 medium (undiluted) cultivated bacteria.
- Incubate for 5 days at 30 °C.
- Prepare 200 ml Chroom-Azurol S (CAS).
  - 12 ml HDTMA (10 mM: 0,182 g in 50 ml H_2O).
  - 30 ml HCl (10 mM: 0,0835 ml HCl supra pure in 100 ml H_2O). Wear gloves + under hood
  - 3 ml $FeCl_3$ (1 mM: 0,0811 g in 50 ml H_2O, dilute 1/10, always make fresh).

  Add following substances slowly:
  - 15 ml CAS (2 mM: 0,0605 g in 50 ml H_2O).
  - 60 ml piperazine (3,589 g in 25 ml H_2O, set pH to 5,6 with 37 % HCl).

- 20 ml sulfosalicilic acid (40 mM: 0,508 g in 50 ml H_2O.
- Add distilled H_2O to correct volume to 200 ml (Should be 60 ml H_2O).

- Add 100 µl Chroom-Azurol S to each well.
- After 4 hours, orange = positive, blue = negative (chelator takes Fe^{3+} from colouring, blue turns orange).

2.3.4 ACC deaminase test

Purpose:
Determining which bacterial species are able to produce ACC deaminase, in order to lower a plant 's stress response.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain), Centrifuge (SL40R, ThermoScientific, Massachusetts, United States).

Method:

- Prepare a salts minimal (SMN) medium:

  - Prepare a ACC-stock solution (0,5 M)
  - Prepare a Cmix2 stock solution (20x):
    - D^+ glucose 20 g/l
    - Sucrose 20 g/l
    - Na-acetate 20 g/l
    - Na-citrate 20 g/l
    - Malic-acid 20 g/l
    - Mannitol 20 g/l

  - Prepare a salt solution:
    - KH_2PO_4 0,4 g/l
    - K_2HPO_4 0,5 g/l
    - Bring to pH to 6,6 with HCl.
    - Autoclave 15 min on 15 bar.

  - Add the following solutions with filter sterilization (20 µm pore) to the autoclaved salt solution:

	Final concentration
MgSO4 (10 mM)	0,1 µM
CaCl2 (90 mM)	0,1 µM
SI7 (Attachments) (1x)	0,01x
Cmix2-stock (20x)	1x
ACC-stock (0,5 M)	5 mM

- Centrifuge the in liquid 869 medium (10/10) cultivated bacteria at 2000 rpm during 15 min at room temperature.

Note: Don't centrifuge > 2000 rpm, masterblock will burst and lose liquid.

- Wash pellets to times with sterile $MgSO_4$ (10 mM).
- Resuspend pellets in 250 µl sterile $MgSO_4$ (10 mM).
- Add 1,2 ml SMN medium (5 mM ACC as N-source).
- Incubate for 72 hours at 30 °C.

- Centrifuge masterblocks at 2000 rpm during 15 min at room temperature.
- Discard supernatants and resuspend pellets in 100 µl Tris-HCl (0,1 M pH 8,5) (Stock in fridge).
- Add 3 µl toluene for cell lysis and vortex. Toluene is very toxic, work under hood with gloves!!!

Note: Do not pour toluene in a plastic reservoir. The plastic will dissolve. Use a glass petri dish instead.

- Add 10 µl ACC (0,5 M) and 100 µl Tris-HCl (0,1 M pH 8,5).
- Incubate for 30 min at 30 °C.
- Add 690 µl HCl (0,56 N) (4,678 ml 37 % HCl in 100 ml H_2O).
- Add 150 µl 2,4-ditrinophenylhydrazine with HCl (2 N) (1,67 ml 37 % HCl in 10 ml H_2O, add 20 mg 2,4-ditrinophenylhydrazine).
- Incubate for 30 min at 10 °C.
- Add 1 ml NaOH (2 N) (7,998 g in 100 ml water). Exothermic while dissolving, may become hot.
- Colours immediately: Yellow = positive, red = negative.

2.3.5 Phosphorous solubilisation test

Purpose:

Screening which bacterial strains are able to produce organic acids in order to solubilise sufficient PO_4^{3-}.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain),

Method:

- Use NBRIP medium to pour plates.
- After solidifying, make a hole in each plate with the upper side of a sterile 1000 µl pipet tip.
- Inoculate with 50 µl bacterial suspension in the correct hole.
- 6 to 12 days' incubation at 30 °C.
- Evaluate the size of the appearing clear halo (solubilisation zone) daily.

2.3.6 Organic acids test

Purpose:

Screening which bacterial strains are secreting organic acids. Organic acids can increase the solubility of nutrients in the soil.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain),

Method:

- Prepare sucrose tryptone medium:

	Final concentration
▪ Sucrose	20 g/l
▪ Tryptone	5 g/l
▪ Trace elements SET	10 ml/l
▪ $MgSO_4.7H_2O$	0,625 g/l
▪ KCl	0,5 g/l
▪ $(NH_4)_2SO_4$	0,25 g/l

- Agar no. 2 Microbiological 37,5 g/l

- Fill masterblocks with 800 µl sucrose tryptone medium.
- Inoculate by adding 20 of the in liquid 869 medium (10/10) cultivated bacteria.
- Incubate for 5 days at 30 °C.
- Add 100 µl alizarine red S (0,1 %) (0,1 g in 100 ml).
- After 15 min: Yellow = positive, pink = negative.

2.4 Plant inoculation experiment

In this experiment the influence of the plant growth promoting capabilities of the DDE tolerant bacterial endophytes to the plant growth of *Cucurbita pepo* was tested *in planta*. This was done by introducing the selected DDE tolerant/degrading bacterial endophytes back into the endosphere by inoculating *Cucurbita pepo* seeds and evaluating the plant growth by its biomass.

2.4.1 Plant-inoculation

Purpose:
Trying to bring DDE-tolerant endophytes into the endosphere of *Cucurbita pepo*.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain),

Method:

- Cultivation of DDE-tolerant endophytes:
  - Prepare an 869 medium (10 ml per endophyte).
  - Pipet 10 ml 869 medium in centrifuge tubes (15 ml).
  - Inoculate with 5 µl in liquid 869 medium (10/10) cultivated bacteria.
  - Incubate for 48 hours at 30 °C.

- Inoculation of *Cucurbita pepo* seeds:
  - Centrifuge the in centrifuge tubes cultivated bacteria.
  - Decant the 869 medium.
  - Resuspend bacterium pellet with 10 ml $MgSO_4$ (10 mM).
  - Fold up 1 or more seeds into 1200 cm^2 paper towel.
  - Moisten with 10 ml bacteria suspension and an additional 5 ml $MgSO_4$ (10 mM).
  - Control seeds (blank) may not get inoculated.
  - Put this in a plastic bag (small autoclave bag) and incubate at 30 °C in a dark room.
  - After 48 hours, approximately 95 % of seeds are germinated.

- Cultivating *Cucurbita pepo:*
  - Remove the paper towel from the seedlings and put them in plant pots filled with perlite.
  - Water the seedlings with ¼ Hoagland medium.
  - Make sure the water from a plant pot cannot get in contact with the water from other plant pots through beneath the holes.

    Note: place a bowl beneath every plant pot.
  - Place the plant pots in a greenhouse for maximum growth efficiency
  - Water the plants each day with ¼ Hoagland medium

2.4.2 Bacterial isolation

Purpose:

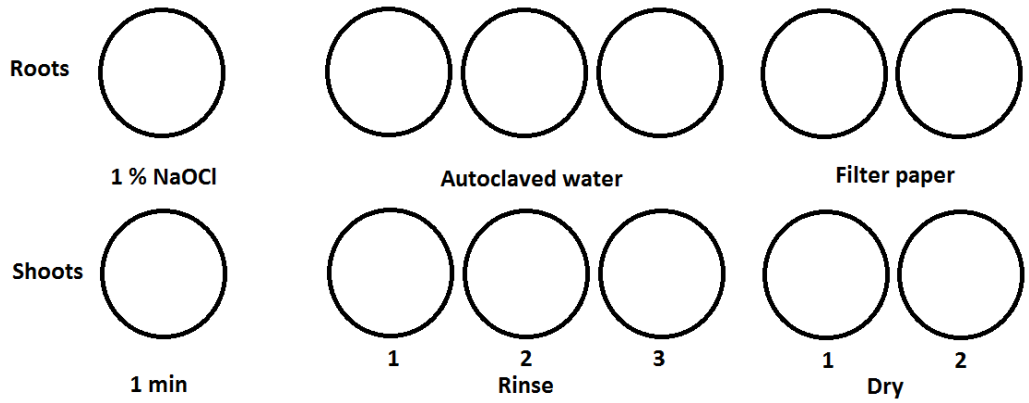
Extracting and purifying the endophytic bacteria from *Cucurbita pepo*. This to later identify the genetic identity of the present endophytic bacteria.

Materials:

Incubator (Analis, Memmert, Schwabach, Germany), Laminar flow cabinet (BH-100, Telstar, Terrassa, Spain),

Method:

- Bacteria extraction:
    - Remove the roots from the shoots.
    - Wash any perlite left over from the roots away.
    - Weigh the plant mass of the roots and shoots separately.
    - Prepare a plant tissue sterilisation set-up with petri dishes:



    - Weigh the second petri dish with filter paper before adding the roots/shoots.
    - 1 min sterilisation with 1 % NaClO solution.
    - Rinse 3 times with autoclaved water.
    - Dry 2 times on the filter paper.
    - Weigh the plant tissue isolation mass at the second dry step.
    - Bring the plant tissue over to a mortar together with 5 ml $MgSO_4$ (10 mM) and homogenise with pestle.
    - Make a dilution of the plant tissue suspension with $MgSO_4$(10 mM): 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}.
    - Plate 100 µl of each dilution on 869 medium (1/10) plates.
    - Incubate for 2 days at 30 °C.

- Bacteria purification:
    - Select a plate with individual colonies.
    - Count the colonies and differentiate them from other colonies by their colour and formation.
    - It is possible to calculate the cfu/ml, cfu/g and total plant cfu from the total numbers of colonies present at each dilution:

$$\frac{cfu}{ml} = \frac{Total\ number\ of\ colonies}{Total\ dillution\ factor}$$

$$\frac{cfu}{g} = \frac{cfu}{ml} x\ isolation\ weight$$

$$Total\ plant\ cfu = \frac{cfu}{g} x\ total\ plant\ weight$$

  - Select the colonies which are to be inoculated at new 869 medium (1/10) plates:
    - Select a colony with a sterile toothpick.
    - Dip the toothpick in a drop of $MgSO_4$ (10 mM).
    - Dip with an ent needle into that drop and inoculate the 869 medium (1/10) plate.
  - Incubate the plates for 2 days at 30 °C.

- Bacteria cultivation:
  - Fill a falcon tube (15 ml) with 10 ml 869 medium (10/10) for each colony.
  - Inoculate these by scraping a colony from the bacterial purification plates and dip it in the falcon tube.
  - Incubate for 2 days at 30 °C and at 150 rpm.

2.4.3 DNA-extraction

Purpose:

Extracting the DNA from the individual *bacterial species*.

Materials:

Microcentrifuge (Centrifuge 5415R, Eppendorf, Hamburg, Germany), DNA-extraction kit (Qiagen Blood &Tissue DNA extraction kit Qiagen Cat. no. 69504, Hilden, Germany) Spectrophotometer (Nanodrop-1000, Isogen Life Science, Utrecht, Nederland), Thermomixer (Compact, Eppendorf, Hamburg, Germany).

Method:

- Prepare the enzymatic lysis buffer:

  Note: careful with triton X-100, this is toxic

# samples	25	50
2x Tris-EDTA (TE) buffer pH 8	90 µl	180 µl
1,2 % Triton X-100	54 mg	108 mg
Lysozyme (Right before actual use)	90 mg	180 mg
RNase free water	4410 µl	8820 µl

- Harvest 1,5 ml bacterial solution in a microcentrifuge tube by centrifuging for 10 min at 7500 rpm. Discard supernatant.
- Resuspend bacterial pellet in 180 µl enzymatic lysis buffer.
- Incubate for at least 30 min at 37°C.
  After incubation heat the heating block or oven to 56°C for incubation in step 5.

- Add 25 µl proteïnase K and 200 µl buffer AL (without ethanol). Mix by vortexing.
  Do not add proteinase K directly to buffer AL.
- Incubate at 56°C for 30 min.
- Add 200 µl ethanol (96-100 %) to the sample and mix thoroughly by vortexing.
  It is important that the sample and the ethanol are mixed thoroughly to yield a homogenous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy mini spin column. The precipitate does not interfere with the DNeasy procedure.
- Pipet the mixture (including any precipitate) into the DNeasy mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.
- Place the DNeasy mini spin column in a new 2 ml collection tube (provided), add 500 µl buffer AW1 and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube.
- Place the DNeasy mini spin column in a new 2 ml collection tube (provided), add 500 µl buffer AW2 and centrifuge for 4 min at 13200 rpm to dry the DNeasy membrane. Discard flow-through and collection tube.
  It is important to dry the membrane since residual ethanol may interfere with subsequent reaction. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.
  Following centrifugation remove the DNeasy spin column carefully so that the column does not come into contact with the flow-through, since this will result in carrying over of ethanol. If carrying occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 13200 rpm.
- Place the DNeasy mini spin column in a 1,5 ml or 2 ml microcentrifuge tube (not provided) and pipet 200 µl buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min and centrifuge for 1 min at 8000 rpm to elute.
  Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield.
- Recommended: for maximum DNA yield, repeat the elution once as described in the previous step.
  This leads to increased overall DNA yield.
  A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine eluates, the microcentrifugation tube from step 10 can be reused. Do not eluate more than 200 µl into a 1,5 ml microcentrifuge tube because the DNeasy mini spin column will come into contact with the eluate.

Store the DNA extracts in the fridge (4°C). Log the samples (papers on the fridge).

2.4.4 DNA amplification

Purpose:

Copying the 16s rDNA from the different bacterial species in order to get a measurable amount that can be detected on gel electrophoresis. The 16s gene will be amplified by using universal 16s rDNA primers; forward pA (5'AGAGTTTGATCCTGGCTCAG); reverse pH (5'AAGGAGGTGATCCAGCCGCA).

Materials:

Microcentrifuge (Centrifuge 5415R, Eppendorf, Hamburg, Germany), Thermomixer (Compact, Eppendorf, Hamburg, Germany).

Methods:

Note: When entering the post-PCR room, put a post-PCR lab coat on top of your own or switch coats to avoid contamination.

Make the mastermix:

- Let the PCR products melt on ice (primers, 10x hifi buffer, $MgSO_4$, dNTP mix).
- Vortex and short spin all the products.
- Make the mastermix (volumes per sample):
    - 5 µl 10x hifi PCR buffer
    - 2 µl 50mM $MgSO_4$
    - 1 µl 10 mM dNTP mix
    - 1 µl 16S rDNA Forward primer ($1/10^{th}$ diluted from 100 µM stock)
    - 1 µl 16S rDNA Reverse primer ($1/10^{th}$ diluted from 100 µM stock)
    - 38,8 µl RNase free water
- Take the Platinum Taq high fidelity out of the freezer and short spin.
- Add to the mastermix (volumes per sample):
    - 0,2 µl Platinum Taq high fidelity
- Vortex the mastermix and shortspin.

Make the amount of samples plus 10 % extra mastermix.

Pipetting of the mastermix and samples:

- Place a 96 well PCR plate, 8 strip PCR tubes or PCR tubes (0.2 ml) in a Isofreeze.
- Distribute 49 µl per tube/well.
- Include at least 1 NTC.
- Ad 1 µl of the bacterial DNA extract to the tubes/wells and close them.
- Spin the plate or strips in the big centrifuge at 1000 rpm for 1 min.
  The PCR tubes can be centrifuged in a microcentrifuge (0.2ml adaptors) at 13200 rpm for 1 min.

PCR program

- Place the tubes/strips/plate into the PCR machine. Make sure all caps are tightly closed and the tubes make good contact with the machine.
- Close the lid in the right way.
- Set the program:
    - 1x 5 min 95 °C
    - 35x
        - 1 min 94 °C
        - 30 s 52 °C
        - 3 min 72 °C
    - 1x 10 min 72 °C
- Adjust the sample volume to 50 µl and start the program.

3. Results and discussion

3.1 Phenotypic characterization

A collection of 530 bacteria strains extracted from *Cucurbita pepo* from an earlier field experiment was available. These bacterial strains were already genetically identified using ARDRA (Amplified Ribosomal DNA Restriction Analysis) and DNA sequencing of the 16s rDNA gene. These bacteria were tested for their capability of producing organic acids, IAA, ACC deaminase, siderophores and the solubilization of phosphor. They were also tested for DDE degradation, because the bacteria are to be introduced in plants growing in DDE-contaminated soils. The results for these test are shown in Table 2-4. Table 2 represents the results from the bacteria extracted from the roots and shoots combined, table 3 from only the roots and Table 4 from only the shoots. The contents of the tables are sorted on the total number of bacterial strains that were tested of a bacterial species (column 2 in each table).

Results bacteria from roots and shoots

The percentages from this table give a total view of all the results from the phenotypical characterization tests performed on 530 bacterial strains. The total percentages are shown in the last row of the table. This value gives the total percentage of bacterial strains that were able to produce organic acids, IAA, siderophores or ACC deaminase or have the ability to solubilise phosphor. The last column shows the percentage of bacteria strains that have a tolerance for DDE, determined via auxanography (2.3.1). This is a rather crude method and therefore it was not always clear whether a bacterial species was able to grow better on the DDE part than on the free DDE part of the agar plate. But it was clear that there were a few (39 in total) bacterial strains that were able to grow better on the DDE contaminated surface. This gave a total percentage of 7 % bacterial strains that have a tolerance or degradation capacity for DDE. It is hard to say from these results which bacterial species has the greatest percentage of DDE tolerant bacteria, because some bacterial species had a very low number of strains and could give a high percentage by coincidence (e.g. *Plantibacter sp.*).

Table 2: Phenotypical characterization results from endophytic bacteria extracted from the endosphere from the roots and shoots combined

Bacterial species	Number	Frequence	Organic acids	IAA	Siderophores	ACC deaminase	P-solubilisation	DDE tolerance
Stenotrophomonas sp.	78	15%	45%	76%	60%	50%	37%	12%
Sphingomonas sp.	50	9%	36%	60%	60%	46%	30%	8%
Chryseobacterium sp.	49	9%	14%	67%	73%	37%	55%	6%
Pseudomonas sp.	48	9%	46%	42%	79%	50%	63%	10%
Microbacterium sp.	40	8%	43%	65%	40%	40%	78%	10%
Enterobacter sp.	30	6%	57%	53%	63%	60%	77%	3%
Exiguobacterium sp.	23	4%	61%	52%	74%	65%	74%	4%
Bacillus sp.	20	4%	25%	75%	75%	55%	20%	0%
Pectobacterium sp.	19	4%	79%	84%	79%	32%	53%	5%
Acinetobacter sp.	18	3%	56%	56%	94%	50%	89%	0%
Ochrobactrum sp.	13	2%	0%	62%	92%	38%	62%	0%
Variovorax sp.	13	2%	54%	85%	92%	31%	38%	15%
Curtobacterium sp.	12	2%	42%	92%	25%	8%	75%	0%
Vibrio sp.	12	2%	83%	67%	50%	92%	75%	8%
Myroides sp.	11	2%	45%	64%	82%	73%	64%	0%
Xanthomonas sp.	11	2%	36%	73%	73%	64%	73%	0%
Flavobacterium sp.	10	2%	0%	70%	70%	50%	60%	0%
Lysobacter sp.	9	2%	0%	78%	89%	56%	22%	0%
Chitinophaga sp.	8	2%	13%	100%	63%	38%	50%	13%
Rhizobium sp.	8	2%	13%	25%	88%	38%	63%	25%
Arthrobacter sp.	7	1%	14%	43%	71%	57%	57%	29%
Brevundimonas sp.	6	1%	67%	67%	67%	33%	50%	0%
Ensifer sp.	6	1%	0%	100%	100%	67%	17%	0%
Paenibacillus sp.	6	1%	83%	50%	50%	17%	50%	0%
Streptomyces sp.	5	1%	60%	40%	80%	80%	40%	0%
Klebsiella sp.	4	1%	25%	100%	75%	25%	25%	0%
Plantibacter sp.	3	1%	0%	100%	33%	0%	33%	100%
Devosia sp.	2	0%	0%	50%	100%	0%	100%	0%
Frigoribacterium sp.	2	0%	0%	0%	50%	0%	0%	0%
Sphingobacterium sp.	2	0%	0%	100%	100%	0%	100%	0%
Terrabacter sp.	2	0%	50%	100%	50%	50%	50%	0%
Agrobacterium sp.	1	0%	100%	0%	100%	100%	0%	0%
Methylobacterium sp.	1	0%	0%	0%	100%	0%	0%	0%
Nocardioides sp.	1	0%	0%	100%	100%	100%	100%	0%
Total Shoots + Roots	530	100%	39%	65%	68%	47%	54%	7%

Table 3: Phenotypical characterization results from endophytic bacteria extracted from the endosphere from the roots

Bacterial species	Number	Frequency	Organic acids	IAA	Siderophores	ACC deaminase	P-solubilisation	DDE tolerance
Sphingomonas sp.	36	14%	28%	61%	50%	47%	33%	11%
Stenotrophomonas sp	36	14%	61%	58%	61%	64%	36%	3%
Pseudomonas sp.	27	10%	30%	41%	74%	44%	67%	19%
Microbacterium sp.	26	10%	46%	50%	27%	42%	88%	8%
Chryseobacterium sp.	23	9%	17%	65%	74%	30%	74%	4%
Exiguobacterium sp.	23	9%	61%	52%	74%	65%	74%	4%
Bacillus sp.	15	6%	20%	80%	87%	53%	27%	0%
Enterobacter sp.	15	6%	60%	53%	67%	47%	73%	0%
Acinetobacter sp.	10	4%	40%	80%	90%	70%	90%	0%
Curtobacterium sp.	10	4%	50%	100%	30%	10%	70%	0%
Pectobacterium sp.	7	3%	71%	71%	71%	29%	29%	0%
Brevundimonas sp.	4	2%	100%	50%	75%	0%	75%	0%
Xanthomonas sp.	4	2%	0%	75%	100%	100%	75%	0%
Plantibacter sp.	3	1%	0%	100%	33%	0%	33%	100%
Arthrobacter sp.	2	1%	50%	0%	100%	100%	100%	0%
Chitinophaga sp.	2	1%	0%	100%	50%	50%	100%	50%
Ensifer sp.	2	1%	0%	100%	100%	50%	0%	0%
Frigoribacterium sp.	2	1%	0%	0%	50%	0%	0%	0%
Klebsiella sp.	2	1%	50%	100%	50%	50%	0%	0%
Rhizobium sp.	2	1%	0%	0%	50%	50%	100%	50%
Sphingobacterium sp.	2	1%	0%	100%	100%	0%	100%	0%
Terrabacter sp.	2	1%	50%	100%	50%	50%	50%	0%
Vibrio sp.	2	1%	50%	50%	50%	100%	50%	0%
Agrobacterium sp.	1	0%	100%	0%	100%	100%	0%	0%
Flavobacterium sp.	1	0%	0%	100%	0%	0%	100%	0%
Methylobacterium sp.	1	0%	0%	0%	100%	0%	0%	0%
Myroides sp.	1	0%	0%	100%	100%	0%	0%	0%
Ochrobactrum sp.	1	0%	0%	100%	100%	0%	0%	0%
Variovorax sp.	1	0%	0%	0%	0%	0%	100%	0%
Total Roots	263	100%	40%	60%	63%	47%	58%	7%

Table 4: Phenotypical characterization results from endophytic bacteria extracted from the endosphere from the shoots

Bacterial species	Number	Frequency	Organic acids	IAA	Siderophores	ACC deaminase	P-solubilisation	DDE tolerance
Stenotrophomonas sp	42	16%	31%	90%	60%	38%	38%	19%
Chryseobacterium sp.	26	10%	12%	69%	73%	42%	38%	8%
Pseudomonas sp.	21	8%	67%	43%	86%	57%	57%	0%
Enterobacter sp.	15	6%	53%	53%	60%	73%	80%	7%
Microbacterium sp.	14	5%	36%	93%	64%	36%	57%	14%
Sphingomonas sp.	14	5%	57%	57%	86%	43%	21%	0%
Ochrobactrum sp.	12	4%	0%	58%	92%	42%	67%	0%
Pectobacterium sp.	12	4%	83%	92%	83%	33%	67%	8%
Variovorax sp.	12	4%	58%	92%	100%	33%	33%	17%
Myroides sp.	10	4%	50%	60%	80%	80%	70%	0%
Vibrio sp.	10	4%	90%	70%	50%	90%	80%	10%
Flavobacterium sp.	9	3%	0%	67%	78%	56%	56%	0%
Lysobacter sp.	9	3%	0%	78%	89%	56%	22%	0%
Acinetobacter sp.	8	3%	75%	25%	100%	25%	88%	0%
Xanthomonas sp.	7	3%	57%	71%	57%	43%	71%	0%
Chitinophaga sp.	6	2%	17%	100%	67%	33%	33%	0%
Paenibacillus sp.	6	2%	83%	50%	50%	17%	50%	0%
Rhizobium sp.	6	2%	17%	33%	100%	33%	50%	17%
Arthrobacter sp.	5	2%	0%	60%	60%	40%	40%	40%
Bacillus sp.	5	2%	40%	60%	40%	60%	0%	0%
Streptomyces sp.	5	2%	60%	40%	80%	80%	40%	0%
Ensifer sp.	4	1%	0%	100%	100%	75%	25%	0%
Brevundimonas sp.	2	1%	0%	100%	50%	100%	0%	0%
Curtobacterium sp.	2	1%	0%	50%	0%	0%	100%	0%
Devosia sp.	2	1%	0%	50%	100%	0%	100%	0%
Klebsiella sp.	2	1%	0%	100%	100%	0%	50%	0%
Nocardioides sp.	1	0%	0%	100%	100%	100%	100%	0%
Total Shoots	267	100%	39%	70%	74%	47%	50%	7%

In table 2 and 3, the results from respectively the roots and the shoots are presented. 263 out of the 530 bacterial strains were isolated from the roots and 267 bacterial strains were isolated from the shoots. There is no noticeably difference to be seen when the total percentages are compared with these from the table of the shoots presented as a diagram in figure 9. The total percentages of ACC deaminase and DDE tolerance are exactly the same and the total percentages of the other tests from shoots and roots were similar to each other.

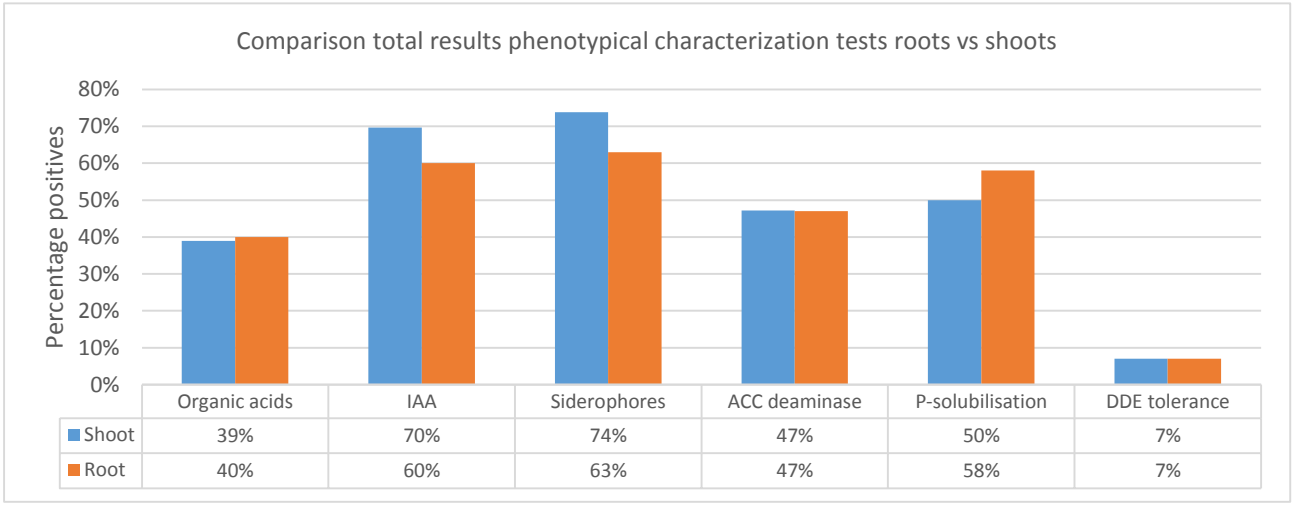


Figure 16: Comparison of the total percentages of the phenotypically characterization tests between roots and shoots

3.2 Plant inoculation experiment

3.2.1 Plant cultivation

There were 10 bacterial strains selected with as many different bacterial species and plant growth promoting capabilities as possible and at the same time gave the clearest sign of DDE degrading or tolerance. Table 5 gives an overview of the selected bacterial strains.

These bacteria strains were cultivated and afterwards inoculated to 3 *Cucurbita pepo* seeds per bacterial strain. After incubation 95% of the seeds germinated. The seedlings were transferred to pots with perlite in a greenhouse and were watered every day with ¼ Hoagland medium. After a growth period of 15 days, the plants were ready to be harvested. Though not all plants could be harvested. Only the plants with leaves other than its dicots leaves can be harvested. Also, not all seedlings had grown. This is why some inoculants did not even have one seedling out of three that had grown into a plant that could be harvested. This was the case for *Stenotrophomonas sp.* (131e) and *Sphingomonas sp.* (75b). The remaining bacterial strains that were selected did have one, two or three plants that had grown and were able to be harvested.

Table 6: Bacterial strains selected for plant-inoculation

Bacterial species	Strain	Organic acids	IAA	Siderophores	ACC deamiase	P-solubilisation	DDE Tolerance	Compartment
Chitinophaga sp.	134c	-	+	-	+	+	+	Shoot
Chryseobacterium sp.	45a	-	+	+	+	-	+	Shoot
Chryseobacterium sp.	125a	-	+	+	+	-	+	Root
Ensifer sp.	60b	-	+	-	+	-	+	Root
Exiguobacterium sp.	5b	-	+	+	+	-	+	Shoot
Microbacterium sp.	92d	+	-	-	-	-	+	Root
Rhizobium sp.	37b	-	+	+	+	+	+	Shoot
Sphingomonas sp.	75b	-	-	+	+	+	+	Shoot
Stenotrophomonas sp.	34a	-	-	+	+	-	+	Root
Stenotrophomonas sp.	131e	-	-	-	-	+	+	Root

Table 5: Biomass of roots and shoots used for bacteria isolation.

	Roots						Shoots					
	Biomass (g)					Total cfu/g	Biomass (g)					Total cfu/g
Plant ID	Plant 1	Plant 2	Plant 3	Average	Isolation		Plant 1	Plant 2	Plant 3	Average	Isolation	
Blank 1	0,61	2,09	-	1,35	1,56	26092	2,29	11,93	-	7,11	1,92	9675
Blank 2	5,60	2,40	-	4,00	1,92	10390	18,37	10,78	-	14,58	2,40	24827
Blank 3	3,31	-	-	3,31	1,89	31591	10,75	-	-	10,75	2,76	5251
5b	2,99	-	-	2,99	1,37	34523	6,91	-	-	6,91	1,96	8241
34a	5,37	1,31	-	3,34	2,23	8006	15,06	4,35	-	9,71	2,86	31042
37a	3,25	2,94	2,98	3,06	1,41	13277	10,98	9,80	12,50	11,09	1,56	35543
45a	3,59	5,78	-	4,69	2,79	3405	15,40	18,64	-	17,02	2,75	12830
60b	2,41	-	-	2,41	1,08	2801	7,03	-	-	7,03	1,47	5614
92d	0,72	-	-	0,72	0,59	91160	2,46	-	-	2,46	0,71	28967
125a	3,78	5,25	4,72	4,58	1,22	3707	13,52	14,44	11,79	13,25	2,08	35781
134c	9,40	-	-	9,40	1,37	60826	22,32	-	-	22,32	2,93	13049

The plant ID is the same ID of the bacterial strain it got inoculated with from table 5. The average is calculated from plant 1,2 and 3. "Isolation" is the weight of the biomass that was used for isolating the endospheric bacteria. The total cfu/g is given for roots and shoots separately.

The plants were removed from the perlite and the roots were separated from the shoots before being weighed (table 5). Table 4 shows the weight of the roots and shoots of every plant together with an average weight. The "isolation" column shows the weight of biomass that was used for the bacterial isolation.

The average weight of the roots and shoots are depicted in figure 10. There is a clear link between the mass of the roots and shoots. Because sometimes only one out of three plants had grown, the standard deviation equals 0 and therefore gives a false image of reliability. This is the case for Blank 3, 5b, 60b, 92d and 134c.

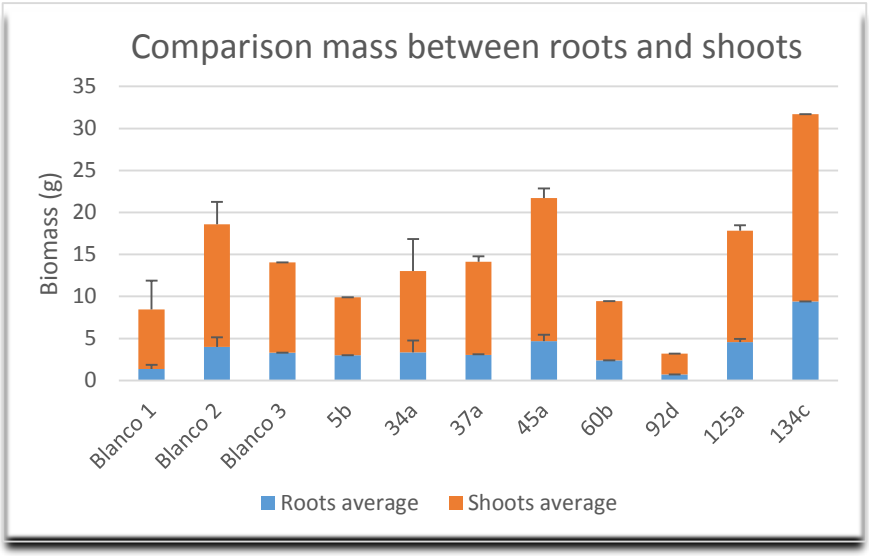


Figure 17: Comparison of the biomass between roots and shoots. The average values of the roots and shoots are calculated from 1, 2 or 3 plants according to table 5. The black error bars represent the standard deviation. Because sometimes only one out of three plants had grown, the standard deviation equals 0 and therefore gives a false image of reliability. This is the case for Blank 3, 5b, 60b, 92d and 134c.

3.2.2 Bacteria genotypically characterization

The identified bacterial species from blank 1, 2 and 3 are put together in one diagram (figure 18). So these results are derived from 5 plants in total (see table 6). These blank diagrams can be used as control for the bacterial identification for comparison with the inoculated plants.

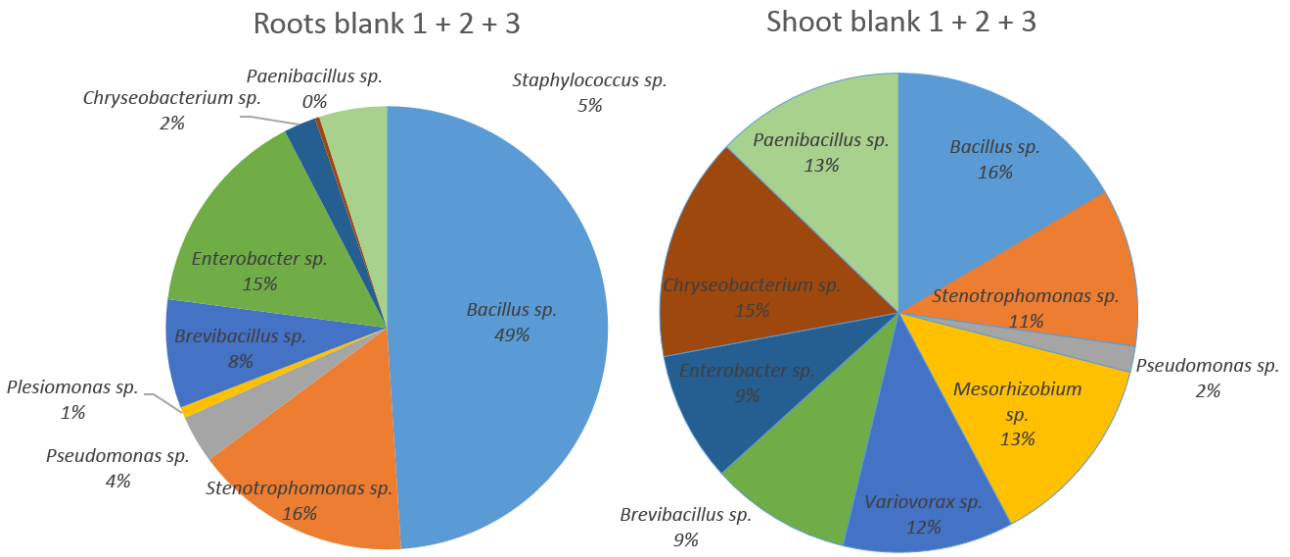


Figure 18: These diagrams represent the community of the endophytic bacteria from the 3 blanks combined, roots and shoots separately. The %cfu from each bacterial species of all 3 blanks were summed and divided by 3 to give the average percentage cfu of each bacterial species that was present in the blanks.

Eight bacterial strains were left from table 5, because not one out of three *Cucurbita pepo* seeds that were inoculated with *Stenotrophomonas sp.* (131e) and *Sphingomonas sp.* (75b) had germinated or had grown enough for harvesting. But the bacterial isolation from plant the that got inoculated with *Chryseobacterium sp.* (125a) failed. So below are 7 figures that show the genotypically identified bacterial species that were isolated from the inoculated plants together with their cfu/g biomass. This was calculated by dividing the total cfu by the isolation weight (table 6).

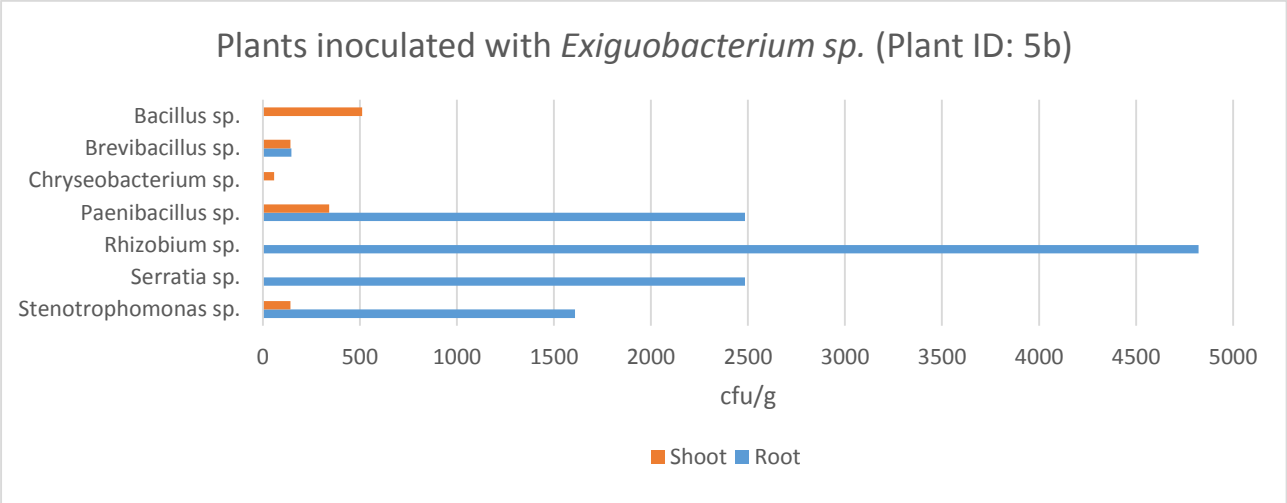


Figure 19: Cfu/g per bacterial species for the Exiguobacterium sp. inoculated plant

The inoculant, *Exiguobacterium sp.,* was not identified in the bacterial community of the endosphere. The inoculation of the bacterial species is therefore not successful.

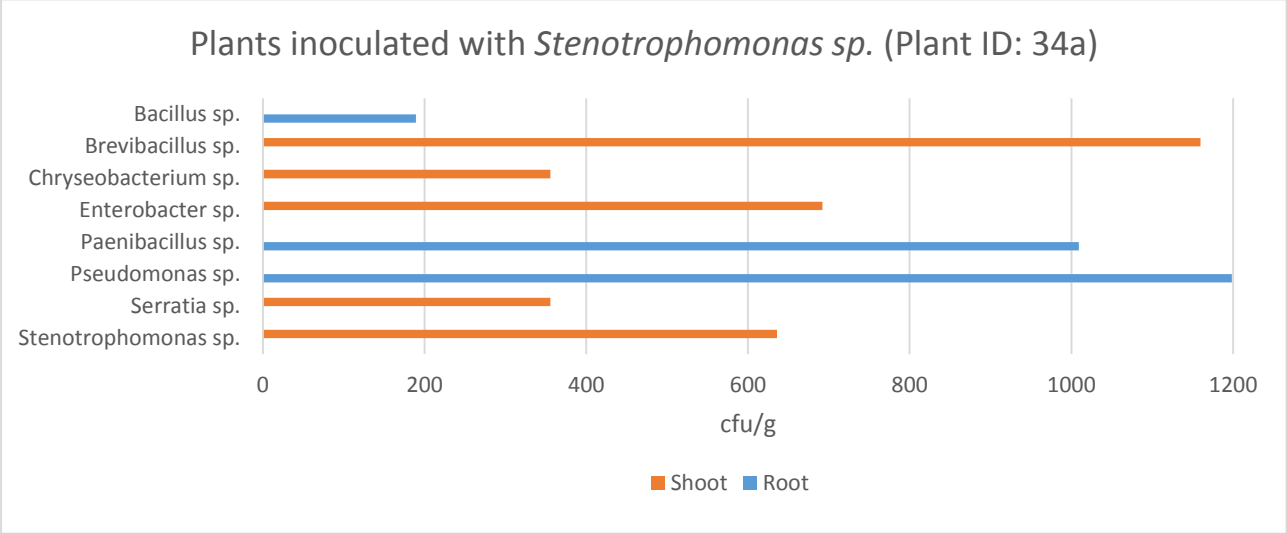


Figure 20: Cfu/g per bacterial species for the Stenotrophomonas sp. *inoculated plant*

The inoculant, *Stenotrophomonas sp.,* was identified in the bacterial community of the endosphere of the shoots. The inoculation of the bacterial species therefore seems to be successful. However, in the control, the bacterial community of the shoots consisted for 11% out of *Stenotrophomonas sp.* (Figure 11). That is why it is not possible to exclude that this result is coincidence, because this bacterial species could have originated from the seeds themselves (1.2.1.1) Plant growth seems to be better compared to that of the controls. But it is not possible to say whether it is thanks to the inoculant or because of any other endophytic bacteria that was identified in the endosphere of plant 34a.

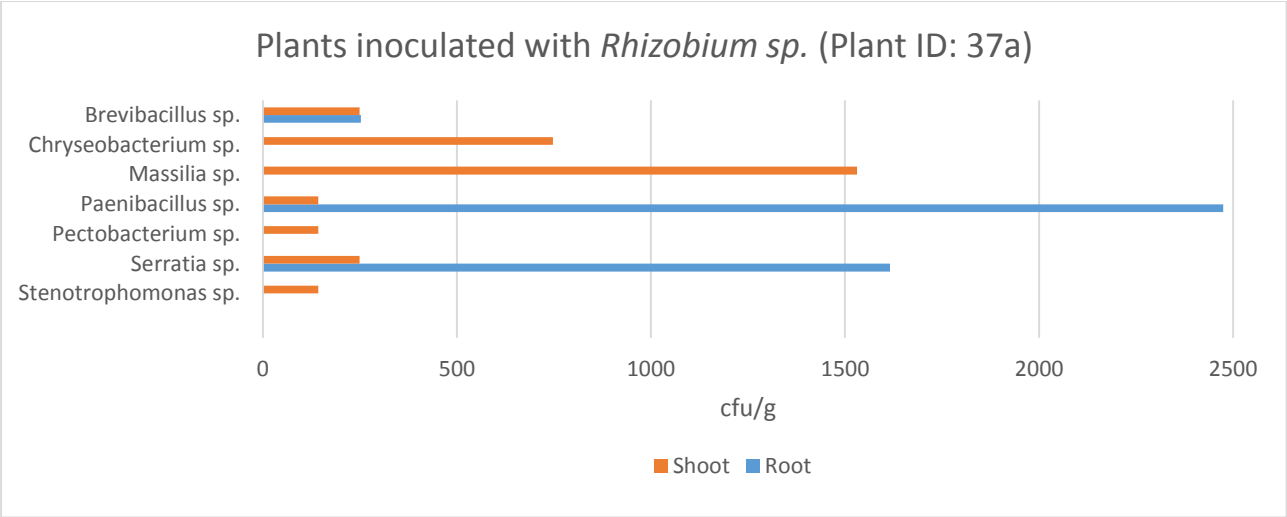


Figure 21: Cfu/g per bacterial species for the Rhizobium sp. *inoculated plant*

The inoculant, *Rhizobium sp.,* was not identified in the bacterial community of the endosphere. The inoculation of the bacterial species is therefore not successful.

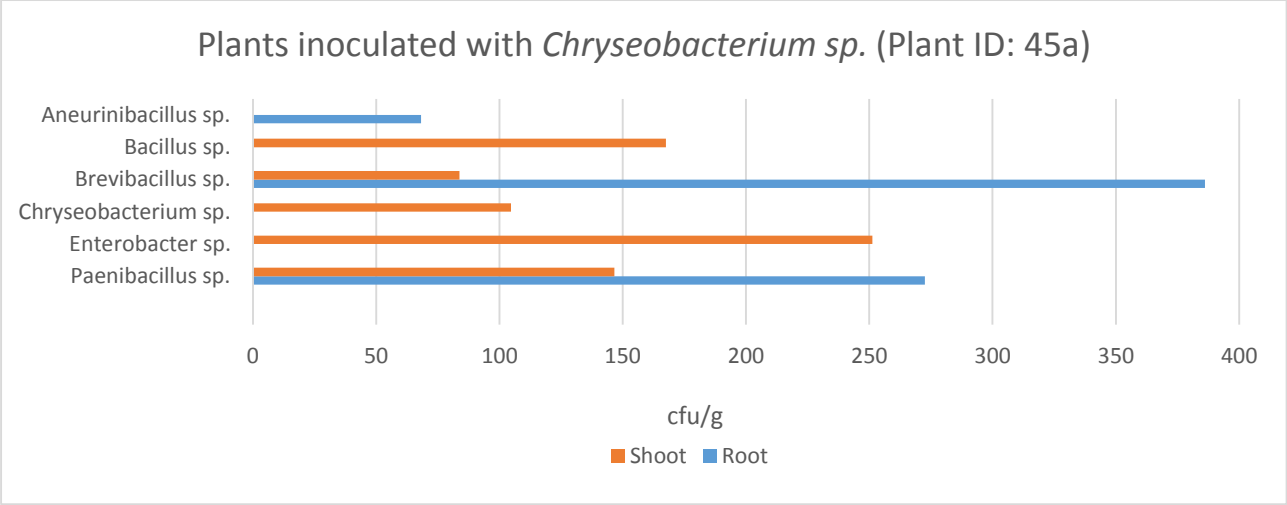


Figure 22: Cfu/g per bacterial species for the Chryseobacterium *sp. inoculated plant*

From this diagram we can conclude that *Chryseobacterium sp.*, which was inoculated to the endosphere, was also identified in the endosphere. The inoculation of the bacterial species therefore seems to be successful. Although, *Chryseobacterium sp.* was also present in the blanks for 15 % (figure 11), so this bacterium is also present in the seeds. Therefore, it is not possible to say if this *Chryseobacterium sp.* is the same *Chryseobacterium sp.* that was inoculated to the seeds.

The plant growth is similar to that of the blanks, so there was also no improved plant growth because of the inoculant.

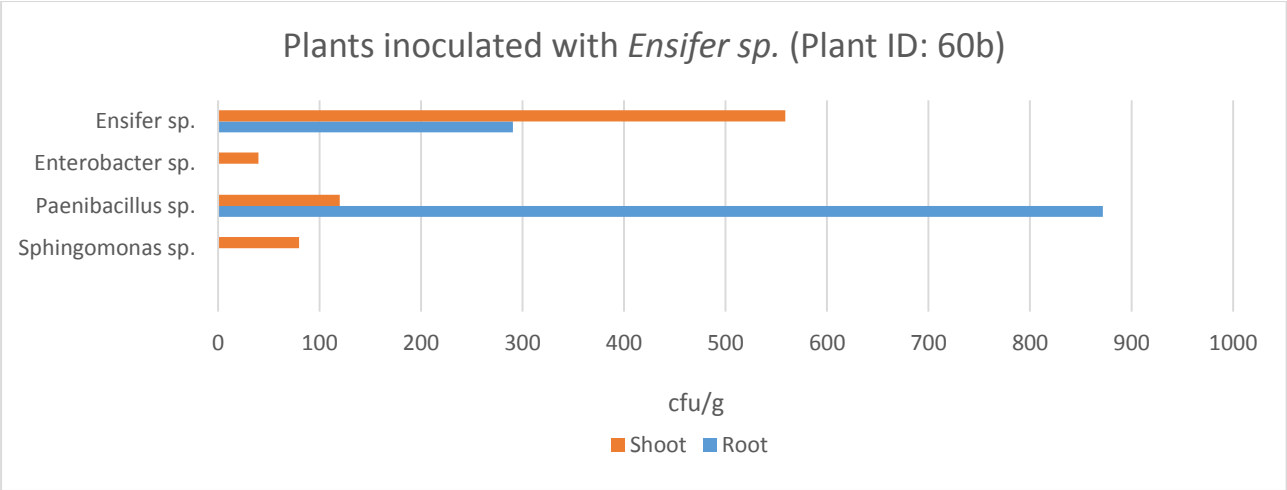


Figure 23: Cfu/g per bacterial species for the Ensifer *sp. inoculated plant*

This is a very promising result. The endosphere of plant 60b that was inoculated with *Ensifer sp.,* shows a bacterial community that consists for almost about 30 % out of *Ensifer sp.* This bacterial species is not present in the blanks, nor in any other inoculated plant. This strengthens the reliability of this outcome that the inoculation of *Ensifer sp.* seems to have been successful.

Ensifer sp., bacterial strain 60b, was positive for IAA, ACC deaminase production (table 4), yet it did not seem to have any positive effects on the growth of *Cucurbita pepo.* The weight of this plant was similar to the average weight of the blanks (figure 8). But this does not necessarily have to mean that *Ensifer sp.* did not have any positive effects on the plant growth. Like earlier said in the introduction, this is only a quick screening and therefore the experiment was performed in a small scale of 3 plants per inoculant. Increasing this number will give more accurate results, because this result might be just a coincidence.

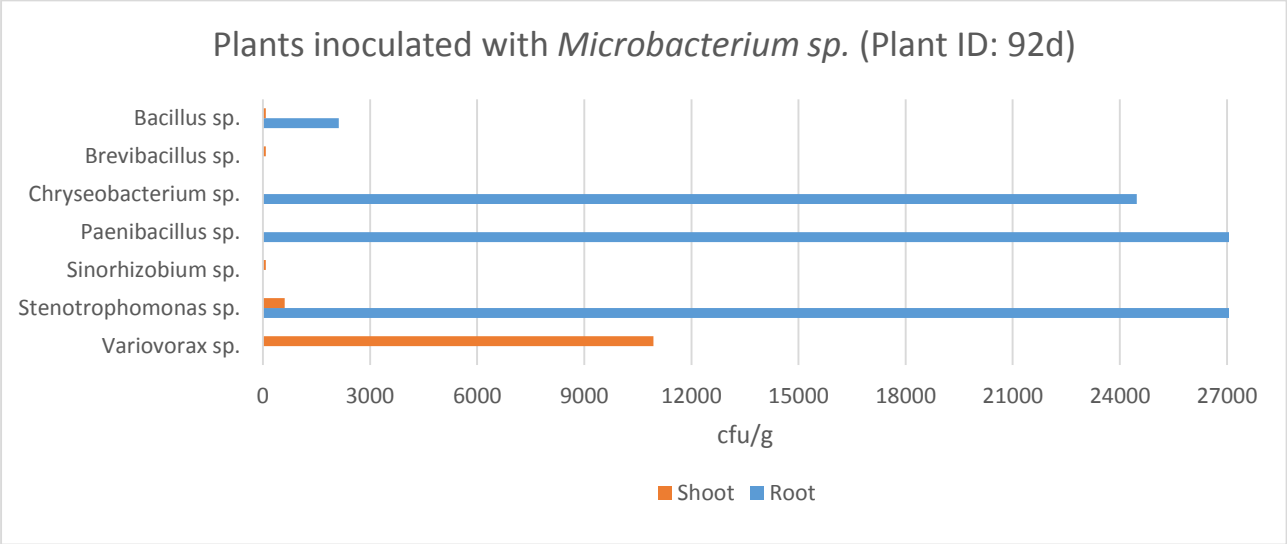


Figure 24: Cfu/g per bacterial species for the Microbacterium *sp. inoculated plant*

The inoculant, *Microbacterium sp.,* was not present in the bacterial community of the endosphere after identification. The inoculation of the bacterial species is therefore negative.

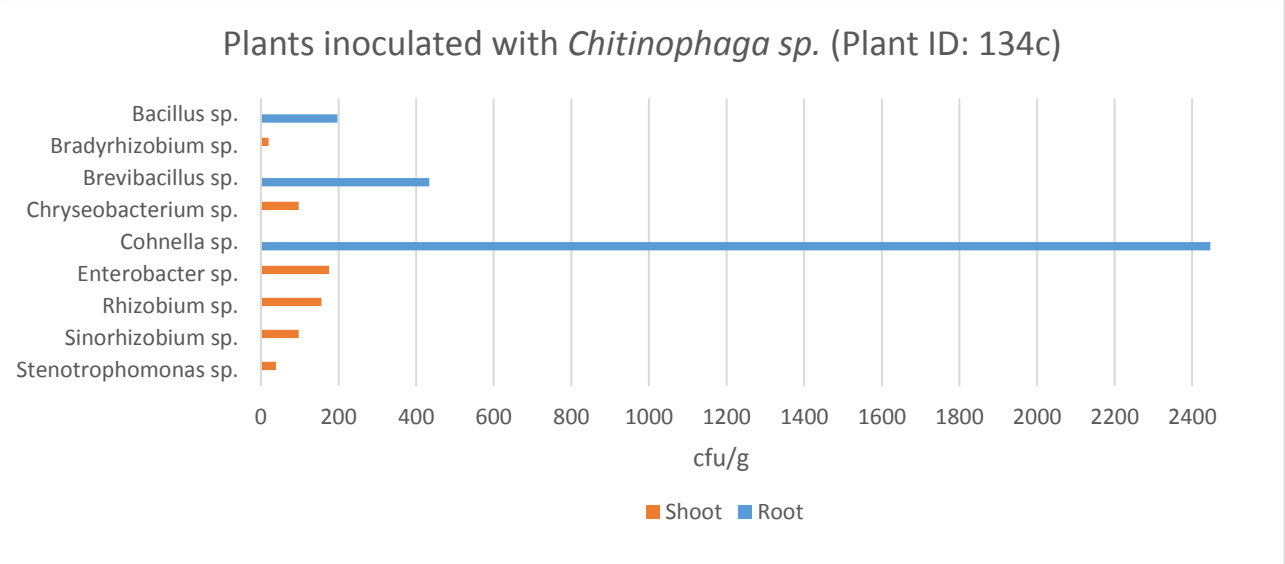


Figure 25: Cfu/g per bacterial species for the Chitinophaga *sp. inoculated plant*

The inoculant, *Chitinophaga sp.,* was not identified in the bacterial community of the endosphere. The inoculation of the bacterial species is therefore negative. This plant does have the greatest biomass, but there cannot be said which bacterial species is responsible for this growth, because 99 to 99,99 % of the microscopically countable bacterial cells are not cultivatable or very difficult to culture [56, 57].

Overall discussion

The number of different bacterial species that got extracted from the roots and shoots are rather low, compared to the bacterial species collection used for the phenotypically characterization, which also got extracted from *Cucurbita pepo*. This can be explained by the fact that the *Cucurbita pepo* plants from this experiment were cultivated in perlite and in laboratory conditions. Perlite is an amorphous volcanic glass that, after it is quarried, is being heated to 900 °C to make it porous and thereby also making it sterile. *Cucurbita pepo* from the field experiment was cultivated outside in the open environment where there is a lot more bacterial diversity in the soil than there is in the perlite [58] and were able to grow 85 days longer than the plants from this experiment. About the cfu/g, this varies between $2{,}8x10^3$ and $9{,}9\ 10^4$ cfu/g. These values are interpreted as normal as they were compared with values from other articles [59, 57].

The inoculation process has failed for 3 out of 7 plants. Although this cannot be said with certainty, because only a fraction (>0,1 %) [57, 56] of the total bacterial cells in the endosphere is cultivatable. Identifying the total endospheric bacterial community can be done by extracting all the 16s rRNA present in the endosphere and using a highly sensitive sequencing technique, such as pyrosequencing. The inoculation process could be enhanced by sterilising the exterior of the *Cucurbita pepo* seeds, so there is less competition for the inoculant to infiltrate into the endosphere. Growing the plants in the presence of DDE contamination might also enhance the inoculation efficiency since this would cause a selective advantage for DDE tolerant bacterial species, which was not the case in this experiment. This gives the inoculated bacterial strain a competitive advantage. Also extending the plant growth duration might give the inoculant more time to populate the endosphere. A phenotypical identification of all the extracted endophytic bacteria might be an option to improve the reliability of the results. Because with these data it is not possible to say whether a plant has improved in growth due to the inoculant. It might also be improved by one of the other endophytic bacterial species that were extracted from the endosphere.

Conclusion

The results from the plant inoculation experiment were negative. Only *Ensifer sp.* seems to be a promising bacterial species that is easy to inoculate to *Cucurbita pepo*. But it cannot be confirmed whether it had any positive effects on the plant growth, since the plants biomass was similar to that of the control plants. More research is necessary to conclude whether *Ensifer sp.* does have a positive effect on the plant growth of *Cucurbita pepo*. Though it can be stated that *Ensifer sp.* is easy to inoculate into the endosphere of *Cucurbita pepo*.

Like mentioned in the introduction, this was mere a screening and therefore the experiment was performed in a small scale of 3 plants per inoculant. Increasing this number will give more accurate results. Growing the plants in the presence of a DDE contamination, might also enhance the inoculation process. Because then there would be selection on DDE tolerant bacterial species, which was not the case in this experiment.

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List of figures

List of tables

Attachments

1. Media compositions used in this study

1.1 General rich (10/10) cultivation 869 medium

Table 7: Composition of 869 medium

	Concentration
Trypton (large cabinet)	10 g/l
Yeast extract (large cabinet)	5 g/l
NaCl (N013)	5 g/l
Glucose D^+ (G003)	1 g/l
$CaCl_2.2H_2O$ (C007)	0,3450 g/l

- Set to pH 7 with NaOH or HCl.
- For plates: add agar no. 2 (microbiological) 20 g/l.
- Autoclave 15 min at 15 bar.

1.2 SI7 Micro nutrients solution

Table 8: Composition of the micro nutrients solution

	Concentration
HCl	9,62 mM
$ZnSO_4.7H_2O$ (Z004)	144 mg/l
$MnCl_2.4H_2O$ (M002)	100 mg/l
H_3Bo_3 (B010)	62 mg/l
$CoCl_2.6H_2O$ (C027)	190 mg/l
$CuCl_2.2H_2O$ (K028)	17 mg/l
$NiCl_2.6H_2O$ (N059)	24 mg/l
$NaMoO_4.2H_2O$ (N029)	36 mg/l

- This solution does not have to be autoclaved.
- Storage in fridge.

1.3 SET Trace elements solution

Table 9: Composition of the trace elements solution

	Concentration

$NaMoO_4.H_2O$ (N029)	20 mg/l
H_3BO_3 (B010)	200 mg/l
$CuSO_4.5H_2O$ (K029)	20 mg/l
$FeCl_3$ (I004)	100 mg/l
$MnCl_2.4H_2O$ (M002)	20 mg/l
$ZnCl_2$ (Z005)	280 mg/l

- This solution does not have to be autoclaved.
- Storage in fridge.

1.4 Medium 284 + Cmix1

Table 10: Composition of medium 284+C-mix

		Concentration
284	Tris of HCl (T019)	6,06 g/l
	NaCl (N013)	4,68 g/l
	KCl (K005)	1,49 g/l
	NH_4Cl (A025)	1,07 g/l
	Na_2SO_4 (N035)	0,43 g/l
	$MgCl_2.6H2O$ (M002)	0,2 g/l
	$CaCl_2.2H_2O$ (C007)	0,03 g/l
	$Na_2HPO_4.2H_2O$ (N039)	0,04 g/l
	Fe(III)NH_4Citraat (A051)	4,80 mg/l
	SI7 Micro nutrients (see 2.1.2)	1 ml/l
Cmix1	Lactate (N056)	0,70 g/l
	D^+ glucose (G003)	0,52 g/l
	Gluconate (G019)	0,66 g/l
	Fructose (F014)	0,54 g/l
	Succinate (N057)	0,81 g/l

- Set to pH 7 with concentrated NaOH or HCl
- For plates: add agar no. 2 (microbiological) 20 g/l
- Leave stir bar in autoclave bottles during the autoclave process
- Autoclave 15 min at 15 bar

1.5 DDE solution (100 µg/l)

- Autoclave a bottle of the desired volume
- Dissolve 1000 µg in 1 ml methanol
- Dissolve 100 µl in 900 µl milliQ
- Dissolve this 1 ml in 1 l $^1/_4$ Hoagland
- Add desired volume to the autoclaved bottle via a sterilising filter (0,2 µm pore) in laminar flow

1.6 $MgSO_4$ solution (10 mM)

- Dissolve 2,648 g/l $MgSO_4$ (M005) in distilled H_2O
- For sterile: autoclave 15 min on 15 bar

1.7 NBRIP medium

Table 11: Composition of NBRIP medium

	Concentration
D^+ glucose (G003)	10 g/l
$Ca_3(PO_4)_2$ (C051)	5 g/l
$MgCl_2.6H_2O$ (M002)	5 g/l
$MgSO_4.7H_2O$ (M005)	0,25 g/l
KCl (K005)	0,2 g/l
$(NH_4)_2SO_4$ (N019)	0,1 g/l
Agar no. 2 Microbiological (large cabinet)	15 g/l

- Set to pH 7 with concentrated NaOH or HCl.
- Autoclave 15 min at 15 bar.

1.8 ¼ Hoagland medium

Table 12: Composition of the 1/4 Hoagland medium

	Concentration	1/4
Macro - elements	KNO_3	2,55 g/l
	$Ca(NO_3)_2.4H_2O$	1,775 g/l
	$NH_4.H_2PO_4$	0,575 g/l
	$MgSO_4.7H_2O$	1,225 g/l
Micro-elements	H_3BO_3	7 mg/l
	$MnCl_2.4H_2O$	4,6 mg/l
	$CuSO_4.5H_2O$	0,2 mg/l
	$ZnSO_4.7H_2O$	0,5 mg/l
	$H_2MoO_4.H_2O$	0,2 mg/l
Fe-EDTA	EDTA.Na	11,4 mg/l
	$FeSO_4.7H_2O$	7,5 mg/l