2011
2012

GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Optimization of DDE-phytoremediation by exploiting plant-associated bacteria and nanoparticles

Promotor :
dr. Nele WEYENS
Prof. dr. Jaak VANGRONSVELD

Nele Eevers

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

Maastricht University

Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek
Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt

2011
2012

GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Optimization of DDE-phytoremediation by exploiting plant-associated bacteria and nanoparticles

Promotor :
dr. Nele WEYENS
Prof. dr. Jaak VANGRONSVELD

Nele Eevers

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

Table of Contents

List of abbreviations

ACC Deaminase	1-aminocyclopropane-1-carboxulate deaminase
AgNPs	Silver Nanoparticles
ARDRA	Amplified rDNA restriction analysis
CAS	Chromium-Azurol S
DDD	1-chloro-4(2,2-dichloro-1-(4-chlorophenyl)ethyl)benzene
DDE	2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene
DDT	2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane
ENPs	Engineered Nanoparticles
GC/MS	Gas Chromatography-Mass Spectrometry
IAA	Indole-3-acetic acid
log(K_{ow})	Octanol-water partition coefficient
PCR	Polymerase Chain Reaction
PGPR	Plant-Growth Promoting Rhizobacteria
POPs	Persistent Organic Pollutants
PVP coated	Polyvinylpyrrolidone coated
SMN medium	Salts Minimal medium
ssp	Subspecies
TCE	Trichloroethylene
$\%_w$	Weight percentage

Acknowledgments - Dankwoord

Ik zou graag als eerste dr. Nele Weyens en prof. dr. Jaco Vangronsveld bedanken om mij de kans te geven om aan dit project te werken. Ik heb enorm genoten van de periode die ik in het labo heb doorgebracht. Bedankt Nele, om zo vaak tijd te maken om mijn eindeloze rij vragen te beantwoorden en om mij wegwijs in het labo te geven.

Ook aan alle doctoraatsstudenten van het CMK heb ik veel te danken. Ze stonden altijd klaar om mij te helpen wanneer ik hun nodig had. En ik mag natuurlijk Ann, Carine en Brigitte niet vergeten. Om producten voor mij bij te bestellen of mij de flow te laten gebruiken als ik weer iets dringend moest doen.

Then I would like to thank dr. Jason White and his team at the Connecticut Agricultural Experiment Station. They welcomed me into their lab for three months and trusted me with part of their research. I very much enjoyed working with all of you and of course having coffee at 10! Thank you all for helping me when I needed it most or if some package didn't arrive. I felt very much at home there. I also have to thank Pam and Mark to welcome me into their home and lives for 3 months. Jonezy, Makael and Kristen, you were the greatest friends to have while I was there! I will never see a Delirium again without thinking of you…

Ook al heeft het niets met labowerk of wetenschap te maken, ik ben mijn familie enorm dankbaar. Mama, Papa, Wouter en Pieter, bedankt om naar mijn verhalen over autoclaven, flows en groeipromotietesten te luisteren, zelfs als het enige wat blijft hangen het woord "courgetten" is.

En "Last but not least" wil ik Dorien en Eline hier zeker nog vermelden. Om naar mij te luisteren als ik alle frustraties eens moest luchten, voor de berichtjes "Half 12 eten?" en de geweldige middaguurtjes die volgden. Jullie maakten dat het hoogtepunt van de dag om half 12 viel! Ook toen ik aan de andere kant van de oceaan vertoefde, kon ik steeds op jullie rekenen voor een babbeltje of alle laatste nieuwtjes! Ook aan alle andere medestudenten, veel dank voor een geweldige periode.

Summary

Introduction Researchers at *The Connecticut Agricultural Experiment Station* observed a remarkable difference in DDE accumulation at the subspecies level of *Cucurbita pepo*. *C. pepo* ssp *pepo* accumulates DDE, while *C. pepo* ssp *ovifera* did not possess this capacity. In a first part, this study investigates whether the the differences in DDE accumulation can be related to differences in the plant-associated bacterial populations of the different subspecies . Bacteria were isolated from both seeds and plants of accumulating and non-accumulating *Cucurbita pepo* subspecies. The plants were exposed to 100 µg L^{-1} DDE.

The second part of this projects concentrates on silver nanoparticles (AgNPs) and their capability to increase the uptake of organic contaminants by plants. Here, the possibility to optimize DDE phytoremediation by exploiting both plant-associated bacteria as well as AgNPs was investigated. Plants were exposed to 100 µg L^{-1} DDE, 500 mg L^{-1} AgNPs or both. The effects of DDE and AgNPs on plant growth and on the associated bacterial populations were investigated. Plant biomasses were determined and endophytes from both subspecies in all conditions were isolated. All isolated bacteria were subjected to (1) genotypic identification, (2) characterization of their plant growth promoting capacity, (3) screening for AgNP toxicity and (4) screening for DDE degradation capacity.

Results DDE proved to have a toxic effect on plant growth, while AgNPs did not cause a decrease in biomass. The effect on the total number of plant-associated bacteria that could be isolated was the opposite. While DDE increased the number of endophytes in zucchini Raven, exposure to AgNPs caused a severe decrease. Endophytes associated with zucchini Raven demonstrated more plant growth promoting capacities than squash-associated bacteria. Bacteria that were isolated from plants that were exposed to DDE or AgNPs showed to experience less toxic effects when again exposed to these products.

Conclusion The DDE accumulator Zucchini Raven contains a higher number and diversity of associated bacteria and its bacteria show more plant growth promoting capacity and potential DDE degradation capacity than the bacteria associated with the non-DDE-accumulator squash Zephyr. These findings and the higher DDE-uptake of zucchini Raven makes it a suitable organism for DDE phytoremediation.

On one hand, AgNPs can be used to increase the DDE uptake of *Cucurbita pepo*, but on the other hand it has severe toxic effects on the plant-associated bacteria. Therefore, a good balance has to be discovered between the advantages and disadvantages of AgNPs for bacteria-enhanced phytoremediation of DDE using *Cucurbita pepo* ssp. *pepo*.

Samenvatting

Introductie Onderzoekers aan het *Connecticut Agricultural Experiment Station* stelden een opmerkelijk verschil vast in DDE-accumulatie tussen verschillende *Cucurbita pepo* subspecies. *C. pepo* ssp. *pepo* accumuleert DDE, terwijl *C. pepo* ssp. *Ovifera* niet beschikt over deze capaciteit. In een eerste deel van het onderzoek wordt onderzocht of dit verschil in accumulatiecapaciteit kan gerelateerd worden aan verschillen tussen de plant-geassocieerde bacteriële populaties. Bacteriën werden geïsoleerd van zowel zaden als planten van beide subspecies. De planten werden blootgesteld aan 100 µg L^{-1} DDE.

Het tweede deel van dit project concentreert zich op zilvernanopartikels (AgNPs) en hun capaciteit om de opname van contaminanten door planten te verhogen. Hierbij wordt de mogelijkheid onderzocht om fytoremediatie te optimaliseren door zowel endofyten als AgNPs te exploiteren. De effecten van DDE en AgNPs op de plantengroei en op de geassocieerde bacteriële populaties werden onderzocht. De biomassa's werden bepaald en de endofyten van beide subspecies onder alle condities werden geïsoleerd. Alle geïsoleerde bacteriën werden onderworpen aan (1) genotypische identificatie, (2) fenotypische karakterisatie, (3) een screening voor AgNP toxiciteit en (4) voor hun DDE-degraderende capaciteiten.

Resultaten DDE vertoonde een significant toxisch effect op de plantengroei, terwijl AgNPs geen afname in biomassa veroorzaakten. Het effect van beide producten was tegengesteld op vlak van het totaal aantal plant-geassocieerde bacteriën dat kon geïsoleerd worden. DDE veroorzaakte een toename van endofyten in Raven terwijl AgNPs een significante afname induceerde. In het algemeen vertoonden endofyten van Raven meer groeipromotie dan bacteriën geassocieerd met Zephyr. Bacteriën die geïsoleerd werden uit aan DDE of AgNPs blootgestelde planten ondervonden minder toxische effecten wanneer ze opnieuw aan deze producten werden blootgesteld.

Conclusie Zucchini Raven bevat een hoger aantal en een grotere diversiteit aan plantgeassocieerde bacteriën en deze endophyten vertonen een hoger percentage aan plantengroei promoverende capaciteiten dan de endophyten van Zephyr. Deze vaststellingen en de hogere DDE-opname door Raven maken dat het een geschikte plant is voor de fytoremediatie van DDE. Enerzijds kunnen AgNPs gebruikt worden om de DDE-opname van *Cucurbita pepo* te verhogen, anderzijds hebben ze een significant toxisch effect op de plant-geassocieerde bacteriën. Een goeie balans moet ontdekt worden tussen de voor- en nadelen die AgNPs kunnen uitoefenen op bacterie-gestimuleerde fytoremediatie van DDE met behulp van *Cucurbita pepo* ssp. *pepo*.

1. Introduction

1.1. DDE-contamination

2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) is a pesticide that has been used in agriculture and gardening since 1943 [1]. Since 1974, the use of DDT has been forbidden in Belgium because of its high toxicity for forest wildlife and its hormone disrupting properties [2].

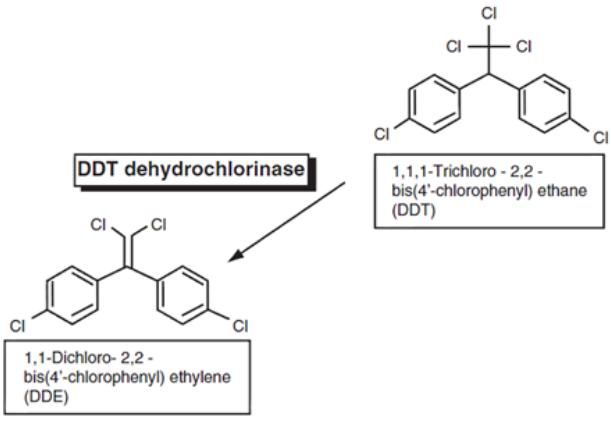

Figure 1: **Dechlorination of DDT forms DDE [3]**

When residing in soils, DDT degrades to 2,2-bis(*p*-chlorophenyl) -1,1-dichloro-ethylene (DDE) or 1-chloro-4- [2,2-dichloro-1-(4-chlorophenyl)ethyl] benzene. The degradation of DDT is caused by a loss of chlorine which can occur because of abiotic factors such as UV light and biotic factors like bacterial activity. DDT, DDE, and DDD are classified as Persistent Organic Pollutants (POPs) [4]. POPs are chlorinated hydrocarbons with high toxicity and recalcitrance. These products have half life values in soil frequently measured in years. Although DDT has not been used for over 3 decades, the product and its metabolites (DDE and 1-chloro-4(2,2-dichloro-1-(4-chlorophenyl)ethyl)benzene or DDD) are still often found in the soil. These chemicals are highly hydrophobic and they have a high log(K_{ow})-value (octanol-water partition coefficient) which causes a strong binding to organic matter in the soil. The hydrophobicity of the products induces the bioaccumulation of POPs in the lipids of exposed organisms, resulting in the potential biomagnification of DDT and its related metabolites in the food chain.

1.2. Phytoremediation of Persistent Organic Pollutants

Soil contamination by different contaminants is a growing problem worldwide. Because of the high recalcitrance of POPs, soil sanitation is needed to prevent long exposure times and biomagnification. Different engineered techniques for soil sanitation exist, but the technologies are often expensive and invasive to the environment and ecosystems.

Phytoremediation is a technique which is based on the *in situ* use of vegetation to remove or stabilize contaminants in soils [5,6]. This technique uses the natural ability of plants and plant-associated microorganisms to solubilize and extract nutrients and minerals from the soil. Notably, many plants accumulate toxicants that have a similar structure as their nutrients.

Despite the previously mentioned limitations, phytoremediation has been successfully applied to remediate several organic contaminants; e.g. TCE [7], aromatic hydrocarbons and explosives [8]. Research has also been performed on the possibility for phytoremediation of DDE-contaminated soils. Different plant species such as *Sinapis hirta* (mustard), *Brassica napus* (canola), *Arachis hypogaea* (peanut) and *Cucurbita pepo* (zucchini) have been found to be accumulators of DDE [9]. The phytoremediation potential not only varies between these different plant species, but also between different varieties of the same species [4]. *Cucurbita pepo* has been proven to phytoextract higher levels of DDE from the soil than other plant species. Researchers at *The Connecticut Agricultural Experiment Station* (New Haven, CT, USA) found significant differences in accumulation of DDE between the *Cucurbita pepo* ssp *ovifera* (squash) and the *Cucurbita pepo* ssp *pepo* (zucchini). Zucchini accumulates DDE, while Squash lacks the ability to accumulate DDE. The highest accumulation of DDE was found in *C. pepo* ssp *pepo* Costata (3.7%). For *C. pepo* ssp *ovifera* Yellow Crookneck, the accumulation was only 0.34%. The reason for this difference in DDE accumulation is not yet known.

There are, however, some limiting factors to this technique. First, not all plants are able to take up and accumulate or degrade the toxicants. Once the toxicants are taken up by plants, there is the risk that the contaminant will be transported to the leafs and transferred into the atmosphere. Another limiting factor to phytoremediation is the concentration of the toxicant in the soil. Depending on the sensitivity of the plant species, certain concentrations of contaminant can be phytotoxic and thus cause harm to the plant. However, these constraints can be overcome by exploiting the relationship between plants and their plant-associated bacteria. This will be discussed further in detail in topic 1.4. A third limiting factor for phytoremediation is the root depth of the plant. Phytoremediation is only successful in the area of soil that is under the influence of the plant roots. Therefore, it is beneficial to use plant species with a root system that grows into the contaminated area. A last limiting factor is the time that is needed to successfully apply phytoremediation to contaminated soils. For that reason, it is favorable to search for fast growing plants with high biomass.

1.3. Nanoparticles

Nanoparticles are particles with dimensions ranging from 1 nm to 100 nm [10]. This implies that a larger amount of atoms are situated on the outside of the nanoparticles in comparison to the bulk variant [11,12], resulting in a high surface to volume ratio. Because of their higher surface energy, these nanoparticles exhibit different electronic states, magnetic properties and catalytic reactions than their corresponding bulk materials [11,13,14,15]. Nanoparticles can occur in nature following volcanic activity, fire and erosion [15]. Engineered nanoparticles (ENPs) usually enter the environment by accidental release through atmospheric emissions, domestic wastewater and by their use in agriculture where they are added to pesticides to increase their uptake in plants [16]. Our interest relates to the use of silver nanoparticles (AgNPs). AgNPs are used in various applications for their antimicrobial activity, such as electronics, optics, textiles, medical bandages and food packaging [17]. They can also be used to remove contaminants such as chlorinated organics, toxic metals and other inorganic substances [18]. Because of the increased use of AgNPs in different applications, one may assume that there is also an increased release of nanoparticles into the environment. The research team of White (2009,2010) has investigated the possible toxic effects of AgNPs on *Cucurbita pepo* [10,14]. They concluded that for the germination and root elongation, there were no significant particle size dependent effects. However, when evaluating biomass, there was a significant decrease of 83% for squash and a 57% decrease for zucchini when the plants were exposed to 500 mg L^{-1} AgNPs for 14 days. This toxicity could only partly be explained by the higher amount of silver ions, the remainder of the phytotoxicity was linked to the nanoparticles themselves. Researchers assume that the toxicity of NPs might be due to several mechanisms. The NPs might block pores in roots [19], interact with plant cell transport pathways [14] and increase oxidative stress by transferring electrons to O_2 as acceptors to form O_2^- or H_2O_2 which are converted to the extremely reactive OH· radical[19]. Although some research has been conducted about nanoparticles toxicity to plants, little is known about the toxicity of NPs to plant-associated bacteria. Given that AgNPs are used as bactericides and that the uptake of AgNPs is 4.7 times higher than that of bulk silver in *Cucurbita pepo* [15], one may expect a negative influence of AgNPs on the plant-associated bacteria.

1.4. Plant growth promotion by plant-associated bacteria

Previous research has demonstrated that the efficiency of phytoremediation can be improved by using plant-associated bacteria [7,20]. Plant-associated bacteria include a wide range of bacterial species, which are associated with a high variety of plant species. In general, they can be divided in three major clusters: phyllospheric, rhizospheric and endophytic bacteria.

The phyllosphere is defined as all external regions of the above-ground plant parts: leaves, stems, blossoms and fruits. The dominant tissue is frequently the leaf because of its large surface area. In the phyllosphere, it is difficult to define which species are more common because of the great dependence on plant species, leaf physiology and environmental conditions [21,22].
The rhizosphere is defined as the narrow region of soil that is directly influenced by the root exudation of the plant and its associated bacteria. The diversity of bacteria in the rhizosphere is largely influenced by the exudates compositions of the roots. One group that is particularly interesting because of their beneficial effect on plant growth are known as Plant-Growth Promoting Rhizobacteria (PGPR).

Endophytic bacteria are defined as bacteria which colonize the internal tissues of plants without causing symptoms of infection or exerting a negative effect on their host [23]. Endophytic bacteria have been identified in an extensive range of host plants and a high diversity of bacterial taxa. In comparison with rhizospheric bacteria, endophytic bacteria have a sound advantage, as there is less competition for nutrients inside the plant than in the rhizosphere or in the soil.

Bacteria are often capable of promoting the growth and development of plants. These characteristics have been reported in numerous publications [5,20], and the effects have been linked to both direct and indirect plant growth promoting mechanisms.

1.4.1. Direct plant promotion

Nitrogen is one of the most abundant substances in the earth's atmosphere, but mainly in the form of dinitrogen (N_2) which is not accessible for plants. Diazotrophic bacteria however can produce nitrogenase: an enzyme that enhances the reduction of N_2 to ammonia. Ammonia is a form of nitrogen which is available for plant uptake. When plants engage in a symbiotic

relationship with these diazotrophic bacteria, they can benefit from this bacterial activity while the bacteria receive suitable location for colonization and can use nutrients and exudates released by the plants.

Iron is frequently present in the soil in an insoluble form, mainly ferric hydroxide (FeO_3). This form is not freely available for plants. Some bacterial species are able to produce organic compounds called siderophores, which bind Fe^{3+} (ferric form) and make it available for conversion to the form that is preferred by plants, Fe^{2+} (ferrous form). The bacterial siderophore-Fe complexes not only facilitate the uptake of iron for bacteria, but plants are also often able to recognize and absorb bacterial siderophore-Fe complexes [24]. Organic acids exert a similar effect on other nutrients. They assist plants in the solubilization of nutrients in surrounding soil, which enhances their uptake into the root system.

The bacterial production of phytohormones results in stimulation of the plant growth, but has no direct advantages for the bacteria itself. The most common phytohormone produced by symbiotic bacteria is an auxin, indole-3-acetic acid (IAA). IAA has multiple effects, mainly targeting the root system of plants. This phytohormone increases root length and has been associated with root hair proliferation [25,26]. In this case, there is however an indirect benefit for the bacteria. When the growth of a root system is stimulated by IAA, the colonization area available to the bacteria expands.

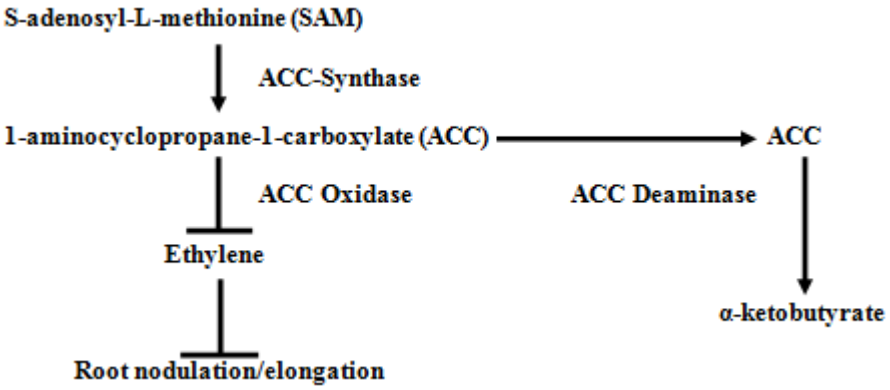

Figure 2: **Mechanism of ACC deaminase.** Disrupting ethylene biosynthesis in plants and thereby inducing increased root elongation and plant growth.

Ethylene is a phytohormone which is produced by plants when exposed to environmental stress; it's production causes an inhibition of root growth [27]. Consequently, the inhibition of ethylene production leads to an indirect promotion of root growth. Bacteria have the capacity to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that

counteracts the stress-induced ethylene by removing the amino groups of ACC. Since ACC is a precursor molecule of ethylene, the amount of ethylene itself is diminished.

1.4.2. Indirect plant growth promoting activity

Competition between pathogenic and beneficial plant-associated bacteria for nutrients and habitat is an important mechanism of biocontrol. Plant growth promoting bacteria have the advantage that they can produce siderophores and thus deprive their surroundings, and pathogenic bacteria, of iron. This mechanism allows them to limit the growth of competitive bacteria and fungi in their colonization area of the rhizosphere [8,12]. Furthermore, endophytic bacteria often produce enzymes and toxins that inhibit or eradicate pathogenic opponents. Because of the advantages that plant-associated bacteria have over pathogenic bacteria, the number of pathogenic bacteria will decrease. This implies that the pathogenic activity against the plant will be lowered as well.

1.5. Endophytic bacteria in phytoremediation

Many endophytic bacteria have, apart from their plant growth promoting effect, the potential to improve phytoremediation of organic contaminants. Bacteria frequently possess degradation genes for certain contaminants. Since these endophytic bacteria are mainly located in the xylem of the plant, organic contaminants can be degraded during their translocation from root to shoot. A consequence of this degradation is the reduced evapotranspiration of organic contaminants into the atmosphere [8].

1.6. Problem formulation and hypothesis

In this research, we will focus on the possible contribution of plant-associated bacteria to DDE-accumulation and degradation in a DDE-accumulating *Cucurbita* subspecies and a non-accumulating subspecies. We hypothesize that the endophytes associated with zucchini have the ability to facilitate the uptake and degradation of DDE, while squash-associated bacteria are missing these abilities. When proven, the zucchini-associated bacteria could be used to improve phytoremediation of DDE-contaminated soils. The study on the plant-associated bacteria has two main objectives concerning the phytoremediation of DDE. The first is to investigate the possible contribution of plant-associated bacteria to the difference in DDE-

accumulation between the two subspecies by making a genotypic and phenotypic comparison of the plant-associated bacterial communities of zucchini and squash upon exposure to DDE. The second objective is to test the DDE-degradation capacity of the isolated bacteria.

In a second facet of the research proposal, some of which has already been initiated at the Connecticut Agricultural Experiment Station (New Haven, CT, USA), the focus will be on the impact of engineered nanoparticles on phytoremediation efficacy. Previous research has shown that *Cucurbita pepo* demonstrates higher DDE-accumulation rates when carbon nanoparticles are added. We hypothesize that other nanoparticles such as AgNPs might be used to further optimize the phytoremediation of DDE-contaminated soils. The use of nanoparticles does pose a dilemma: on one hand it may improve the uptake of DDE by *Cucurbita pepo*, but on the other hand it might cause such toxic effects on the plant-associated bacteria that their positive effect on DDE-phytoremediation might be eliminated. The first objective of this part of the research is to determine the toxicity that AgNPs pose on the bacteria that were isolated from the plants. The second objective is to investigate the combined effects of AgNPs and plant-associated bacteria on the DDE-uptake of *Cucurbita pepo*.

2. Materials and methods

2.1. Cultivation of plants

Plants of 2 subspecies of *Cucurbita pepo* were grown: *Cucurbita pepo* ssp *ovifera* (Squash Zephyr) and *Cucurbita pepo* ssp *pepo* (Zucchini Raven). The seeds of each of the plants were rolled into humid paper towels and incubated at 28°C for 4 days to allow them to germinate. After germination, the plants were transferred to glass jars filled with 14 g of vermiculite. 4 different conditions were used: control, 100 ng L^{-1} DDE, 500 mg L^{-1} silver nanoparticles, and with both 100 ng L^{-1} DDE and 500 mg L^{-1} silver nanoparticles. DDE was added in ¼ Hoagland nutrient solution (Attachment 1: Media). AgNPs were added to the vermiculite before transferring the plants. The plants were grown for 8 days. After this period they were harvested. Part of the plants were used for measuring the DDE uptake, the other part for the isolation of plant-associated bacteria. The biomass of the plants for isolation of endophytes was determined before roots and shoots were separated.

Plants that were destined for DDE extraction were rinsed and chopped up before they were stored -4°C. The samples were thawed before they were mixed for 30 s with 25 mL of 2-propanol with 1 µg of o,p-DDE as an internal standard (in 100 µL of 2,2,4-trimethylpentane). 50 mL of petroleum ether was added and the sample was mixed for 5 min. The mixture was filtered through funnels lined with glass wool and the eluent was collected in a glass seperatory funnel. After resting for 15 min, draining occurred and the ether was rinsed 3 times at 20 min intervals with distilled water and a saturated sodium sulfate solution. The petroleum ether was drained into a vial containing 10 g of anhydrous sodium sulfate. A portion of the extract was used for cleanup on 4 mL florisil cartridges that had been preconditioned with 5 mL of petroleum ether. The extract was collected in a 8 mL vial and the volume of each extract was reduced to 1 mL under nitrogen at a heating block at 35°C. The vials were stored at 4°C until analysis.

2.2. Isolation of plant-associated bacteria

2.2.1. Isolation of seed endophytes

The seeds of 2 *Cucurbita pepo* subspecies were used: Squash Zephyr which is a non-accumulator of DDE and Zucchini Raven which is a DDE accumulator. The seeds were

weighed and sterilized externally with 1% chloride for 5 min. There were 3 replicates for each subspecies.

After external sterilization, the seeds (5) were washed 3 times in sterilized deionised water. From the third washing water, 50 µL was plated on rich (869) medium (Attachment 1: Media) to test the sterilization method. The seeds were crushed in a sterilized mortar with 5 mL of a 10 mM solution of $MgSO_4$ and following dilution series were made: 0, 10^{-1}, 10^{-2} and 10^{-3}. From each dilution, 100 µL were plated on 1/10 diluted rich (869) medium. The plates were incubated at 30°C for 4 days.

2.2.2. Isolation of plant endophytes

The bacteria were isolated from roots or shoots of the plants. The plant material was cut off and soaked in a 1% chloride solution for 5 min. For each species and condition there were 3 replicates. The rest of the isolation was identical to the isolation of bacteria from seeds.

2.2.3. Storage of isolated bacteria

After purification of all morphologically different isolated strains, bacteria were grown in falcon tubes with rich (869) medium for 5 days. The falcon tubes were centrifuged at 4000 rpm for 30 min. The supernatans was discarded and the pellet was resuspended in 2 mL of a sterilized 15%$_w$-glycerolsolution (75 g glycerol, 4.25 g NaCl, 420.75 g distilled water). 1.5 mL of the bacterial solution was stored in cryotubes at -80°C.

2.3. Genotypic characterization of plant-associated bacteria

2.3.1. DNA extraction

The bacteria were cultivated in 1 mL of rich (869) medium in a masterblock for 3 days at 30°C after which the bacterial suspension was transferred to 2 mL microcentrifuge tubes. The tubes were centrifuged for 10 min at 7500 rpm and the pellet was used for DNA extraction. This extraction was performed with a Qiagen DNeasy blood & tissue kit (Attachment 2: DNA extraction protocol). The DNA quality was tested spectrophotometrically by using a nanodrop (nanodrop spectrofotometer ND-1000 (Isogen Life Sciences, De Meern; The Netherlands).

2.3.2. PCR amplification of 16S rDNA (ARDRA)

To start up the PCR, a mastermix was made for the 16S rDNA amplification (for each reaction: 5 µL 10x high fidelity PCR buffer, 1 µL 10mM dNTP mix, 2 µL 50 mM $MgSO_4$, 1

µL bacteria specific 26F forward primer: 5'AGAGTTTGATCCTGCTCAG3', 1 µL universal 1392R reverse primer: 5'ACGGGCGGTGTGTRC3', 0,2 µL platinum Taq polymerase high fidelity, and 38,8 µL RNase free water). In each well of a microwellplate , 49 µL of the mastermix was mixed with 1 µL of the DNA sample. The samples were run in the PCR-machine (Cycle 1: 1x 5 min at 95°C; Cycle 2: 35x 1 min at 94°C, 30 sec at 72°C, 3 min at 72°C; Cycle 3: 1x 10 min at 72°C; store at 4°C).

2.3.3. DNA digestion

A mastermix was made for the digestion of the amplified DNA (for each reaction: 5 µL 1x New England Biolabs buffer 1, 0.5 µL HpyCH4IV 4-base specific restriction endonuclease with cutting place 5'…A▼CGT…3', 2 µL RNase (1%), 7.5 µL RNase free water). 8.6 µL of the mastermix was transferred in a well of a 96 microwell plate, together with 20 µL of the amplified 16S rDNA sample. The plate was incubated at 37°C for 2 hours. A gel electrophoresis was done (1.5 % agarose gel, constant V at 90V for 2.5 hours) to analyze which bacterial strains were different and a purified sample (PCR purification kit, Qiagen) of the amplified 16S rDNA of one representing strain for each different genotype was sent to Macrogen Inc. (Amsterdam, The Netherlands) for sequencing.

2.4. Phenotypic characterization

The bacteria were grown by introducing 5 µL of bacterial suspension from the glycerolstock to 1 mL of rich (869) medium in a sterile 96-well masterblock. The masterblock was incubated for 3 days at 28°C and 200 rpm.

2.4.1. Organic acid production

20 µL of the bacterial suspension was introduced in 48 well plates of which each well contained 800 µL of a sucrose tryptone medium that stimulates organic acid production. The microplates were incubated at 28°C and 200 rpm for 4 days. After this incubation period, 100 µL of an Alizarine red S reagent 0.1% was added as a pH indicator. After 15 min, acidic wells turned yellow and were considered positive, basic wells stayed pink and were considered negative.

2.4.2. IAA production

L-tryptophan was added at 0.5 g L^{-1} to a 1/10 rich (869) medium because bacteria can produce IAA from tryptophan. 20 µL of a bacterial suspension was introduced in 1 mL of the medium in a 96 well masterblock. The masterblock was incubated in the dark at 28°C and 200 rpm for 4 days. After 4 days the masterblock was centrifuged at 2000 rpm for 10 min. 0.25 mL of the supernatans was transferred in a new masterblock and 0.5 mL Salkowskireagent was added. Salkowskireagent turns pink if it is bound to IAA. Therefore, after 20 min, pink wells were considered positive. Control wells stayed yellow.

2.4.3. Siderophore production

A selective (284) medium was produced with different amounts of iron: no iron, 0.25 µM iron and 3 µM iron. 20 µL of the bacterial suspension was introduced in 48 well plates of which each well was filled with 800 µL of the different 284 media. 3 different plates were incubated at 28°C and 200 rpm for 4 days. When bacteria are grown in a poor medium without Fe, siderophore production is stimulated. After incubation, 100 µL of the blue Chromium-Azurol S (CAS) solution was added to each well. The CAS medium contains iron. When this iron is taken from the CAS medium by siderophores, the CAS solution turns yellow. After 4 hours the yellow wells were considered positive, blue wells were considered negative.

2.4.4. ACC deaminase production

Bacteria were cultivated in a masterblock with rich (869) medium for 2 days at 30°C and 150 rpm. The masterblock was centrifuged at 4000 rpm for 15 min. The pellets were washed 2 times with 10 mM $MgSO_4$ and afterwards resuspended in $MgSO_4$. 250 µL of this suspension was added to 1.2 mL liquid Salts Minimal (SMN) medium (Attachment 1: Media) with 5 mM ACC as sole nitrogen source. This mixture was incubated for 3 days at 30°C and centrifuged (4000 rpm, 15 min). The supernatans was discarded and the pellets were resuspended in 100 µL 0.1 M Tris-HCl buffer (pH 8.5) after which the cells were lysed by the addition of toluene. 10 µL 0.5 M ACC and 100 µL 0.1 M Tris-HCl buffer was added and the samples were incubated for 30 min at 30°C. 690 µL of 0.56 N HCl and 150 µL 0.2 % 2,4-dinitrophenylhydrazine were added and the masterblock was incubated for 30 min at 30°C. After the incubation, 1 mL 2 N NaOH was added. Yellow wells were considered positive, negative wells stayed brown.

2.4.5. Potential DDE degradation capacity: auxanography

The bacteria were grown in 5 mL of rich (896) medium for 4 days at 28°C and 200 rpm. The tubes were centrifuged at 4000 rpm for 20 min and the supernatans was discarded. The pellet was resuspended in 2 mL of sterile 10 mM $MgSO_4$. The optical density was measured at 660 nm and brought to 1. The bacterial suspension was diluted 10 000 times. 100 µL of the diluted suspension was plated at selective (284) medium and on a plate with selective (284) medium with a carbon mix (Attachment 1: Media). 2/3 of the outer circle of the plate was covered with a DDE solution with a concentration of 50 mg L^{-1} (3.835 mL from a 260 ng L^{-1} DDE in methanol stock in 10 mL of distilled water). If the bacteria grow on the entire plate, they are tolerant for DDE. If the bacteria grow on the entire plate, except for the area covered with DDE, DDE is toxic for the bacteria. If the bacteria grow better in the DDE covered area, they are expected use DDE as a carbon source, thus they are possibly able to degrade DDE.

2.4.6. Testing DDE degradation capacity

Bacteria that tested positive for the auxanography test were grown in different flasks containing (1) selective (284) medium with C-mix and 50 ng L^{-1} DDE or (2) selective (284) medium with C-mix, 50 ng L^{-1} DDE and autoclaved bacteria (dead) or (3) selective (284) medium with C-mix, 50 ng L^{-1} DDE and endophytic bacteria that were isolated from zucchini Raven. For each condition 3 replicates were made. 20 mL samples of each flask were taken at day 0, 3, 8 and 15. At the moment of sampling, 4 g NaCl was added to stop bacterial growth and activity. These samples were analyzed at *The Connecticut Agricultural Experiment Station* (New Haven, CT, USA) by measuring the DDE concentration using solid phase microextraction and GC/MS. A 30 µM film thickness PDMS fiber was used in a Combipal autosampler. 18 mL of the sample was spiked with a $13C_{12}$ p,p'-DDE internal standard to yield a 2 ng L^{-1} concentration in the solution. The DDE was extracted by immersing the SPME fiber in the solution for 40 min at 35°C. After this immersion, the DDE was desorbed from the fiber in the GC injection port for 75 min at 230°. For the actual GC/MS measurements a Varian 3800 GC/Varian 4000MS ion trap was used. The pressure settings for the GC injector port at 230°C were 0.1 min at 13 psi, increase to 25 psi at a ramp of 40 psi min^{-1} and hold for 3.4 min, decrease to 13 psi at a speed of 40 psi min^{-1} and immediately increase to 16.6 psi at a ramp of 1.2 psi min^{-1}, continue increasing at 0.03 psi min^{-1} until a pressure of 18.7 psi is reached, then increase the ramp to 0.06 psi min^{-1} until the final pressure of 20.6 psi is attained which will be held for 7.32 min. After this extraction cycle has been run in the injector port, the extracted solution will be moved to the GC oven. The solution arrives

at 100°C and will be hold there for 4 min. after which the temperature will increase to 160°C at a speed of 20°C min^{-1}, then it increases further at a ramp of 0.5°C min^{-1} to a temperature of 195°C and finally to 228°C with a ramp of 1°C min^{-1}. This final temperature will be maintained for 6 min after which the mass spectrometer can measure the DDE content in the extracted solution (emission current 60 µA, total ion current 5000 counts, maximum ionization count 25000 µs, multiplier offset ± 200 V, scan range 110 nm to 425 nm).

2.4.7. Testing toxicity of AgNPs to isolated bacteria: auxanography

A similar testing process as for testing the potential DDE degrading capacity was used (2.4.5. Potential DDE degradation capacity: auxanography). Instead of covering the 2/3 of the outer circle of the plate with DDE, it was covered with a AgNPs solution with a concentration of 500 mg L^{-1} of AgNPs in distilled water. If the bacteria grow on the entire plate, they are tolerant for AgNPs. If the bacteria grow on the entire plate, except for the area covered with AgNPs, AgNPs are toxic for the bacteria.

2.5. Statistical analysis

Normality and homogeneity were tested using R (R development core team, Vienna, Austria). None of the tested data sets was normally divided, if there would have been a normally divided dataset, a 2-way ANOVA would have been used. The datasets were log-transformed and the root of all data was taken, but still none of the data tested to be normally divided. For data that were not divided normally, a Kruskall-Wallis non-parametric test ($p < 0.05$) was used for analysis.

3. Results and discussion

Researchers from *The Connecticut Agricultural Experiment Station* (New Haven, CT, USA) have proven that *Cucurbita pepo* extracts higher levels of DDE from the soil than other plant species such as *Sinapis hirta* (mustard), *Brassica napus* (canola), *Arachis hypogaea* (peanut) [4,9]. Beside differences in phytoextraction between the different plant species, there are also differences in extraction potential between *Cucurbita pepo* subspecies. *Cucurbita pepo* ssp *pepo* (zucchini) shows a significantly higher capacity to accumulate DDE from a soil than does *Cucurbita pepo* ssp *ovifera* (squash). Up until now, no research has given any explanation for this difference in DDE accumulation. In this work, we investigate the possible contribution of plant-associated bacteria to the accumulation of DDE in *Cucurbita pepo*.
In a first part of the research, the bacterial populations associated with the accumulator (zucchini Raven) and the non-accumulator (squash Zephyr) were compared.

The second part of this project concentrates on the use of silver nanoparticles (AgNPs) to increase DDE uptake. AgNPs are used in agriculture to increase the uptake of herbicides and pesticides by plants [16]. On the other hand, AgNPs are also known for their bactericidal characteristics and therefore used in many applications such as electronics, optics, textiles, medical bandages and food packaging [16]. Because of their capacity to increase the uptake of organic contaminants [18], AgNPs might contribute to an improved DDE phytoremediation efficiency. However, because of their antimicrobial activity, they might harm the endophytic populations of plants and thus cause a decrease in the positive contributions of plant-associated bacteria. Therefore the toxicity of AgNPs towards plants and their associated bacteria will be investigated.

3.1. Comparison of squash Zephyr and zucchini Raven and their associated endophytes

In this part we tested the hypothesis that the difference in DDE uptake between the accumulating zucchini Raven and the non-accumulating squash Zephyr might be related to differences in the plant-associated bacterial population.

3.1.1. DDE accumulation of Cucurbita pepo subspecies

The DDE accumulation of *Cucurbita pepo* is depending on the concentration of DDE in the surrounding soil or nutrient media. Therefore, there is a need for an objective way to express the DDE accumulation in function of the concentration of DDE in the environment. The bioconcentration factor (BCF) describes the dry weight ratio of the DDE content in the plant tissues and the DDE concentration in the environment [4] and is therefore independent for different environmental DDE concentrations.

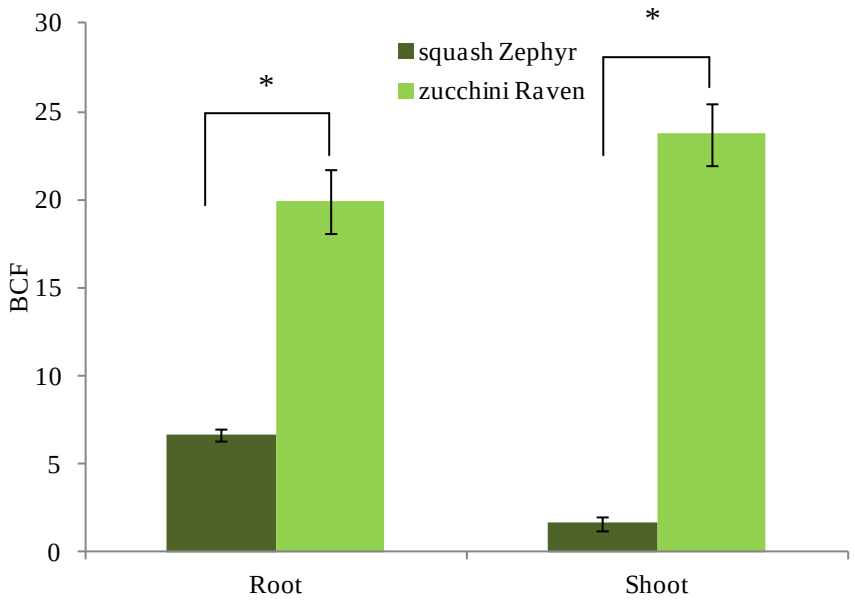

Figure 3: **DDE-uptake of *Cucurbita pepo* subspecies** Plants of *Cucurbita pepo* subspecies squash Zephyr and zucchini Raven were exposed to different concentrations of p,p'-DDE. The average bioconcentration factor (BCF) is given for roots and shoots for both subspecies. This is the dry weight ratio of DDE content in the tissue of the plant and the DDE concentration in the environment. Values are mean ± standard error of 3 biological independent replicates.

The bioconcentration factors of squash Zephyr and zucchini Raven were calculated for root and shoot *(Figure 3)*. In both plant compartments, the bioconcentration factor of zucchini Raven was 4 to 10 times higher than it was in squash Zephyr ($p < 0.001$). When comparing the bioconcentration factors of root and shoot, the BCF of the shoot is significantly lower than that of the root for squash Zephyr (6.65 and 1.63 respectively; $p < 0.001$). For zucchini Raven, the BCF of the shoot is 23.7, compared to a BCF of 19.9 in the root. These results indicate that (a) zucchini Raven is a better accumulator of DDE than is squash Zephyr and (b) the transport mechanisms for DDE from root to shoot are more efficient in zucchini Raven.

3.1.2. Plant biomass

When plants are exposed to organic contaminants such as DDE, these contaminants might be phytotoxic depending on their concentration. One possible marker for phytotoxicity is impaired plant growth.

32 plants of each subspecies were germinated and grown in ¼ Hoagland nutrient solution. Half of them were exposed to a concentration of 100 mg L^{-1} DDE, the other half was grown in regular media as control plants. After 8 days of exposure, the biomass of all plants was determined *(Figure 4)*.

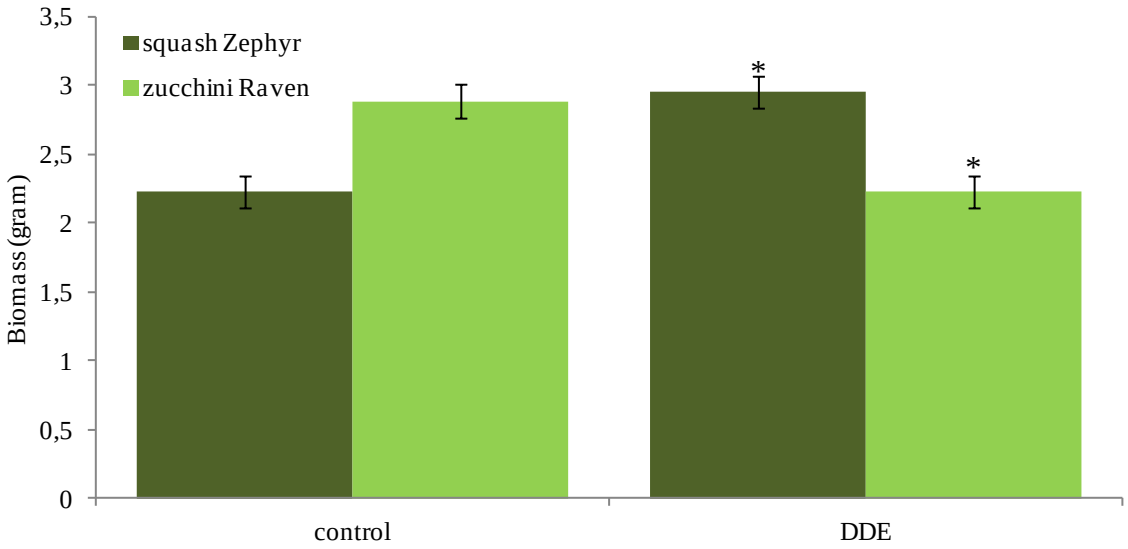

Figure 4: **Plant biomass.** Average plant biomass after 8 days of exposure. Control plants were grown in ¼ Hoagland nutrient solution, DDE plants were grown in ¼ Hoagland nutrient solution with a 100 mg L^{-1} concentration of DDE. Two subspecies of *Cucurbita pepo* were used. Values are mean ± standard error of 16 biological independent replicates. (*) are significantly lower than their control.

The average biomass of zucchini Raven decreased significantly when the plants were exposed to DDE in comparison to the control plants ($p < 0.05$). For squash Zephyr, this was the opposite. The higher uptake of DDE of zucchini Raven might cause higher phytotoxicity and thus decreased plant growth. DDE exerts a certain toxicity on plants depending on uptake.

3.1.3. Isolation of plant-associated bacteria

Endophytic bacteria can be passed on from generation to generation by incorporating them in the seeds. After isolation of the cultivable endophytes, a comparison can be made between the bacterial colonies in seeds and plants to investigate which species colonize plants after germination and which are passed on over generations.

The plants were exposed to 100 mg L^{-1} DDE for 8 days before isolation. Because of the higher uptake of DDE in the accumulator, a comparison between the bacterial species isolated from the accumulator and the non-accumulator might indicate the possible toxicity of DDE towards plant-associated bacteria.

Isolation of seed endophytes

The seed endophytes were isolated from both squash Zephyr and zucchini Raven after external sterilization of the seeds. The average number of seed endophytes was calculated in colony forming units per gram of seeds *(Figure 5)*. Three replicates were used. and the averages were tested for normality and homoscedasticity.

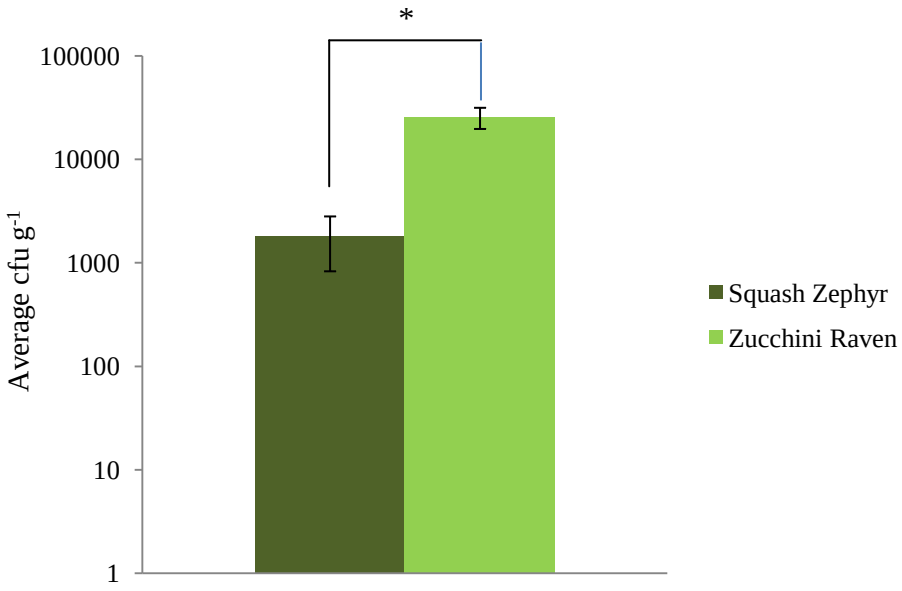

Figure 5: **Isolation of seed endophytes** Number of endophytes isolated from seeds. On the vertical axis the amount of bacteria is portrayed in colony forming units per gram of plant material**.** Values are mean ± standard error of 3 biological independent replicates. (*) are significantly different

The seeds of zucchini Raven contained on average 14 times more cultivable endophytic bacteria than did the seeds of squash Zephyr. This difference proved to be statistically significant ($p < 0.05$). It can be hypothesized that Zucchini Raven passes on a higher number of plant-associated bacteria to the next generation than squash Zephyr. Depending on the plant growth promoting capacities of these bacteria (3.1.5. Phenotypic characterization), this might cause an advantage during germination.

Isolation of plant endophytes

Endophytes were isolated separately from roots and shoots of both subspecies *(Figure 6)*. Three independent replicates were used.

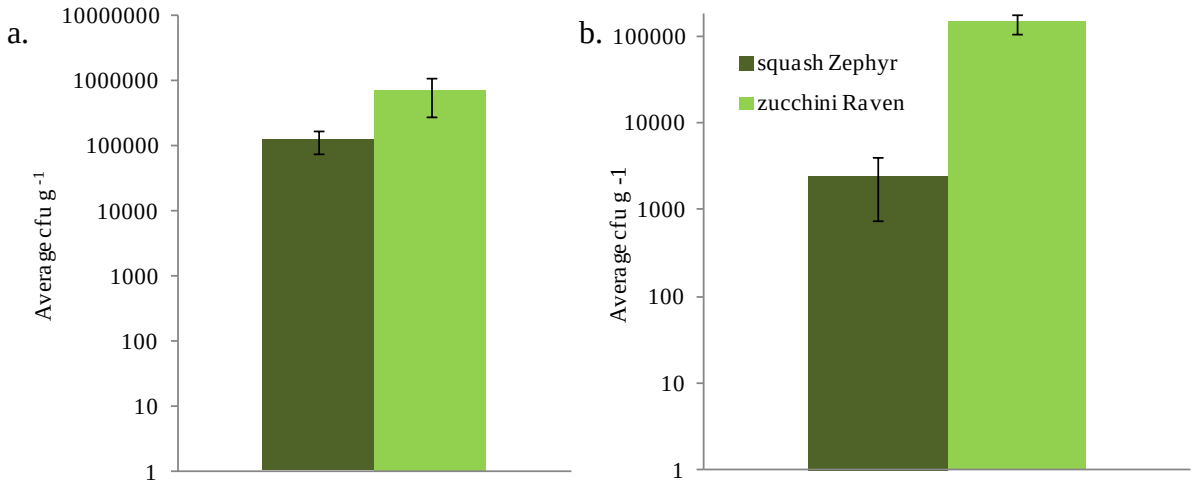

Figure 6 : **Isolation of plant endophytes** Number of endophytes isolated from plants. On the vertical axis, the number of isolated endophytes are portrayed in colony forming units per gram of plant material. . Two subspecies were tested: squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator. (a) Isolation from roots (b) Isolation from shoots. Values are mean ± standard error of 3 biological independent replicates.

The results of the isolation of plant endophytes show that, root tissues contain on average over 10 times more endophytic bacteria than shoot tissues from the same plant.

When the amount of endophytes is compared between the DDE accumulating zucchini Raven and the non-accumulating squash Zephyr, zucchini Raven proved to contain significantly more endophytes in both roots *(Figure 6a)* and shoots *(Figure 6b)*. This difference might be explained by the higher number of seed endophytes that is passed on from the previous generation of zucchini Raven *(Figure 5)* on the one hand and on the other hand, the higher concentration of DDE in the plant tissues of zucchini Raven (3.1.1. DDE accumulation of *Cucurbita pepo* subspecies) might provide an alternative carbon source for the bacteria to use in a possible co-metabolism mechanism. Thus, the higher uptake of DDE by zucchini Raven does not cause toxicity for the plant-associated bacteria when only considering the amount of bacteria.

3.1.4. Genotypic identification

All isolated endophytes were identified using DNA-extraction, ARDRA, gel electrophoresis and sequencing.

Genotypic identification of seed endophytes

Both subspecies, squash Zephyr and zucchini Raven, contained only one bacterial species: *Pseudomonas* sp. This means that there is no difference in the diversity of bacterial species that is transferred from one generation to the next between squash Zephyr and zucchini Raven, only a difference in the amount of bacteria is present (3.1.3. Isolation of plant-associated bacteria).

Genotypic identification of plant endophytes

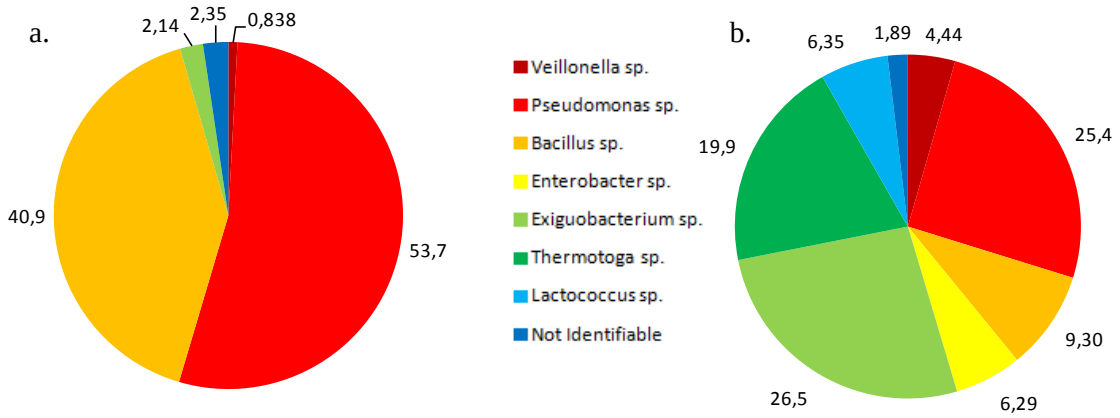

Figure 7: **Genotypic identification of plant endophytes** Representation of the distribution of bacterial species in plants. (a) squash Zephyr (b) zucchini Raven

The bacteria isolated from squash Zephyr proved to belong to 4 identifiable species: *Veionella* sp., *Pseudomonas* sp., *Bacillus* sp., and *Exiguobacterium* sp. *(Figure 7a).* One species, covering 2.35 % of the total amount of bacteria, was not identifiable. *Pseudomonas* sp. and *Bacillus* sp. make up most of the bacterial population (53.7 % and 40.9 % respectively). The high percentage of *Pseudomonas* sp. can be explained by the fact that *Pseudomonas* sp. were already present as seed endophytes.

Zucchini Raven contained 7 identifiable species: *Veionella* sp., *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Exiguobacterium* sp., *Thermotoga* sp., and *Lactococcus* sp. *(Figure 7b).* 1.89 % of the isolated bacteria were not identifiable. In this bacterial population, *Pseudomonas* sp. and *Bacillus* sp. include only 25.4 % and 9.30 % respectively.

The higher concentration of DDE that is present in zucchini Raven does not diminish the biodiversity present in the plant. On the contrary, it seems that the higher DDE content might even stimulate the diversity of the endophytic bacterial population.

3.1.5. Phenotypic characterization

Besides the genotypic identification, all isolated bacteria were also subjected to a phenotypic characterization. 5 different tests were performed evaluating the plant growth stimulating capacities and their potential to use DDE as a carbon source of the isolated bacterial strains.

Phenotypic characterization of seed endophytes

When the seed endophytes were screened for their capacity to use DDE as a carbon source using an auxanography (2.4.5. Potential DDE degradation capacity: auxanography), none of them proved to possess this ability *(Figure 8)*.

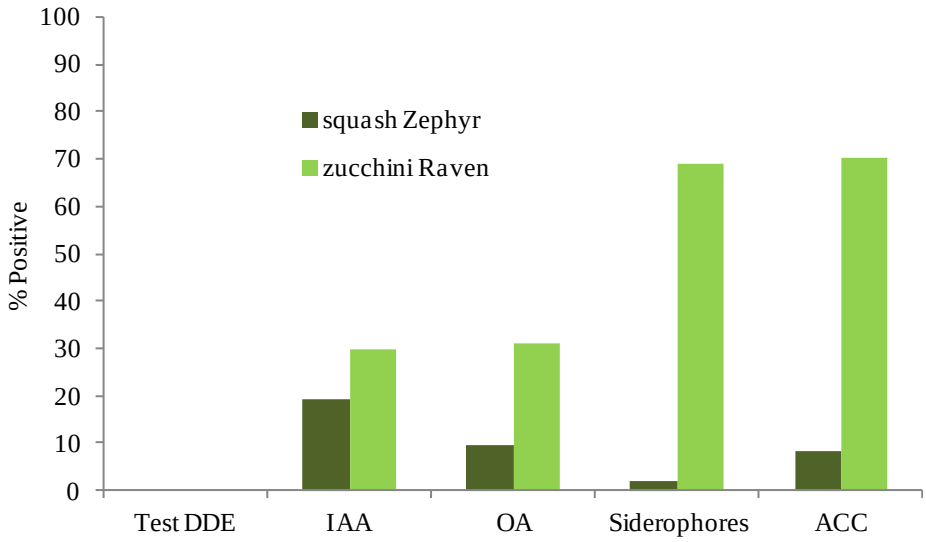

Figure 8: **Phenotypic characterization of isolated seed endophytes.** On the vertical axis, the percentage of isolated endophytes that scored positive are portrayed. Endophytes were isolated from two subspecies : squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator. Bacteria were analyzed in 5 tests: (1) test DDE: screening for possible degradation of DDE, (2) IAA: production of indole-3-acetic acid, (3) OA: production of organic acids, (4) Siderophores: production of siderophores, and (5) ACC: production of 1-aminocyclopropane-1-carboxylate deaminase.

When the 4 tests concerning the in vitro plant growth promoting capacity of endophytes were analyzed, the bacterial population isolated from zucchini Raven proved consistently to have a higher percentage of endophytes with plant growth promoting capacities.

Phenotypic characterization of plant endophytes

The plant endophytes were also subjected to an auxanography (2.4.5. Potential DDE degradation capacity: auxanography), to test their possible DDE degrading capacities *(Figure 9)*. In this case, 26 bacteria isolated from zucchini Raven (7.14 %) and 1 bacterium isolated

from squash Zephyr (2.14 %) displayed a possible DDE degradation capacity. If these data are further compared with the data obtained in the genotypic identification of the bacteria, 40.3 % of these bacteria belong to bacterial species that are not present in seed endophytes (full tables in Attachment 3: Genotypic and phenotypic identification). From the bacteria that show the potential to use DDE as a carbon source, the most promising strains are selected to confirm their DDE degrading capacity using GC/MS (3.1.6. DDE degradation experiment).

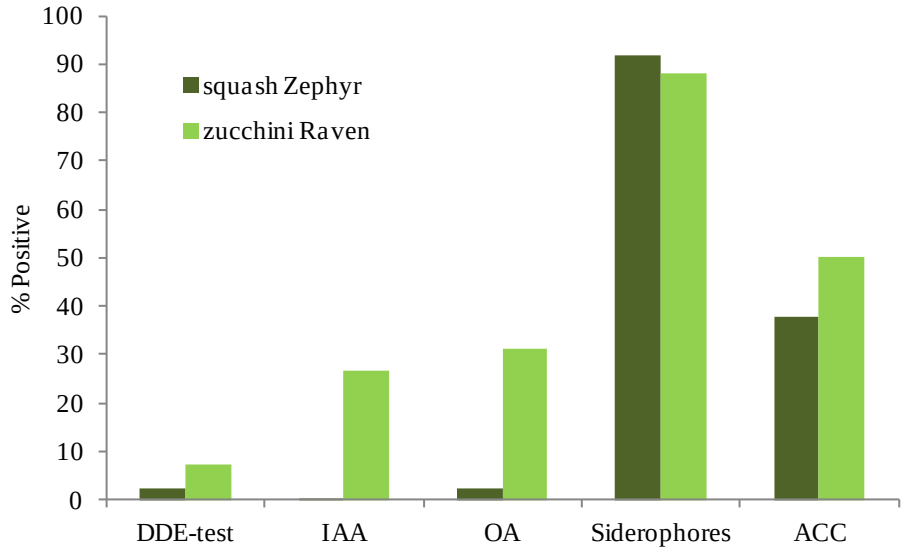

Figure 9: **Phenotypic characterization of isolated plant endophytes.** On the vertical axis, the percentage of isolated endophytes that scored positive are portrayed. Endophytes were isolated from two subspecies: squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator. Bacteria were analyzed in 5 tests: (1) test DDE: screening for possible degradation of DDE, (2) IAA: production of indole-3-acetic acid, (3) OA: production of organic acids, (4) Siderophores: production of siderophores, and (5) ACC: production of 1-aminocyclopropane-1-carboxylate deaminase.

When considering the plant growth promotion tests, endophytes from zucchini Raven show in general more plant growth promoting capacities than those of squash Zephyr, except for a slight difference in the amount of siderophore producing bacteria.

Both the results from the phenotypic characterization of the seed endophytes and the plant endophytes are very promising in the context of phytoremediation. If the bacteria that are associated with a contaminant accumulating plant species are capable of promoting plant growth, they can diminish the stress that is caused by the contaminant. By producing IAA, bacteria stimulate plant growth and thus counteract possible plant growth inhibiting effects of the contaminant. The production of organic acids and siderophores helps plants to obtain

scarce nutrients and ACC deaminase inhibits the production of ethylene, which is a stress hormone in plants.

Although the bacteria associated with zucchini Raven show a higher potential for plant growth promotion, the biomass of zucchini Raven plants is significantly lower than the biomass of squash Zephyr (3.1.2. Plant Biomass). This seems to be contradictory. However, when considering the higher DDE accumulation of zucchini Raven, it can be assumed that these plants experience more phytotoxic stress than can be counteracted by bacterial plant growth promotion.

3.1.6. DDE degradation experiment

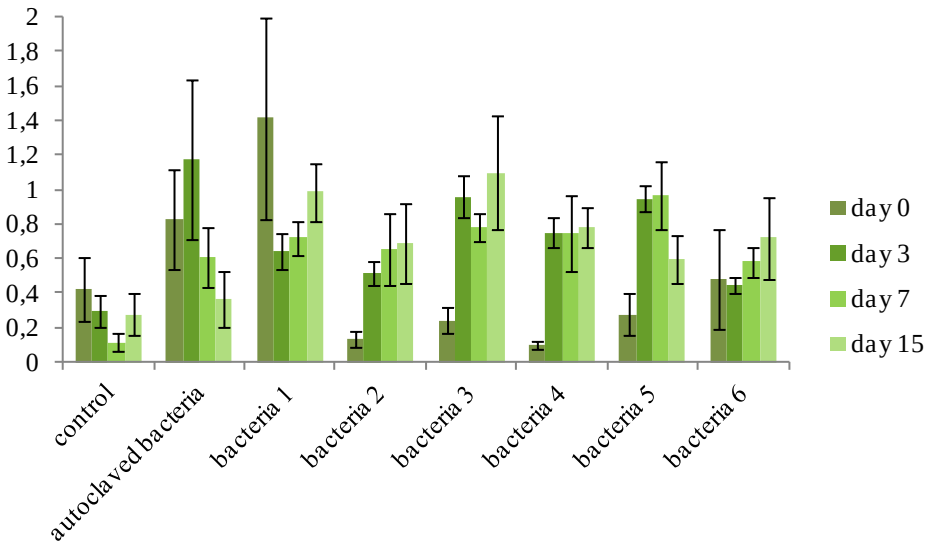

Figure 10: **DDE degradation experiment**. 6 different DDE degradation experiments were executed. (1) Control: Selective 284 medium with 50 ng L^{-1} p,p'-DDE. (2) autoclaved bacteria: Selective 284 medium with 50 ng L^{-1} p,p'-DDE and 2 mL of autoclaved bacterial suspension. (3-8) bacteria 1-6: Selective 284 medium with 50 ng L^{-1} p,p'-DDE and 100 µL of a bacterial suspension of endophytes that were isolated from *Cucurbita pepo* ssp *pepo* (zucchini Raven). Values are mean ± standard error of 3 biological independent replicates.

The 6 most promising bacteria that showed a potential DDE degrading capacity in the auxanography screening were selected for a DDE degradation experiment *(Figure 10)*. All the selected bacteria were originally isolated from zucchini Raven. The results of this experiment were not consistent with our expectations. Three different flaks were set up. The control flask only contained a selective (284) medium with 100 µg L^{-1} DDE. A second flask contained the same medium and DDE concentration, but 2 mL of autoclaved (dead) bacterial suspension was added. Furthermore, there were 6 flasks filled with selective (284) medium with 100 µg

L^{-1} DDE and living bacteria. The concentration of DDE that was present in the control flask should stay approximately constant over the course of the experiment, however, it varies over all 4 times of measurement. The same inconsistency occurs when observing the measurements of the suspensions containing bacteria. Several explanations for this phenomenon are possible. (1) DDE is highly hydrophobic, so although it was dissolved in methanol before water was added, it might re-crystallize when it is introduced in the medium and slowly resolve again over the period of the experiment. (2) The concentration of DDE might be too high to measure correctly using GC/MS. Further testing and experiments will be necessary to determine a correct and consistent testing method.

3.1.7. Overall evaluation of Cucurbita pepo ssp. pepo as DDE phytoremediation organism

When summarizing the results that were obtained in this first part of the research, following conclusions can be made. (1) Zucchini Raven has a high bioconcentration factor and therefore can be considered to be an accumulator of DDE. (2) DDE has a toxic effect on plant growth, however growth inhibition stays limited and is not expected to be detrimental in case of phytoremediation of DDE contaminated soils.

Since (1) zucchini Raven contains a higher number and diversity of associated bacteria, and (2) the Raven-associated bacteria show more plant growth promoting and potential DDE degradation capacity than the zephyr-associated bacteria, it can be hypothesized that the higher DDE concentrations present in the accumulator, zucchini Raven, are not toxic to the associated bacterial populations.

When all of the previous findings are considered, *Cucurbita pepo* ssp. *pepo* (zucchini Raven) shows promise to be a suitable organism for bacteria-enhanced phytoremediation of DDE.

3.2. Effects of DDE and/or AgNP exposure on *Cucurbita pepo* and its endophytes

Silver nanoparticles have been proven to increase the uptake of organic contaminants when they are added to the plant environment [17]. They are also know for their antimicrobial activity [16], which can restrict the possibility to use AgNPs in endophyte-enhanced phytoremediation. To use AgNPs for the optimization of phytoremediation of DDE contaminated soils, their toxicity towards both plants and their endophytes has to be investigated. Therefore, both zucchini Raven and squash Zephyr plants were exposed for 8 days to (1) 100 µg L^{-1} DDE, (2) 500 mg L^{-1} AgNPs, or (3) 100 µg L^{-1} DDE and 500 mg L^{-1} AgNPs, while unexposed plants were used as controls. After this exposure period (1) their

biomasses were determined and (2) their associated bacterial populations were isolated and characterized.

3.2.1. Plant biomass

A significant difference was observed between the biomasses of plants that were exposed to DDE (DDE and DDE + NPs) and control plants *(Figure 11)*. This confirms the findings in 3.1.2. that DDE has a phytotoxic effect on *Cucurbita pepo.*

When the biomass of control plants is compared to the biomass of plants exposed to NPs, no significant difference is found, the same applies to a comparison between plants exposed to just DDE and plants exposed to both DDE and NPs ($p < 0.05$). Therefore, the addition of AgNPs does not seem to have a deleterious effect on the biomass of plants.

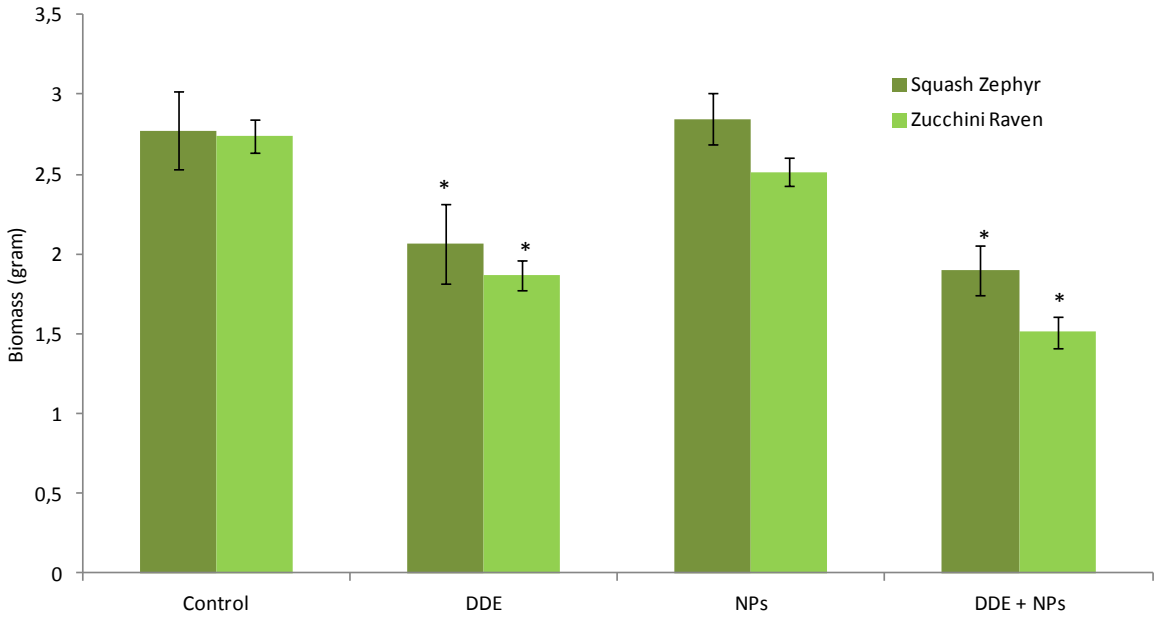

Figure 11: **Plant biomass.** Average plant biomass after 8 days of exposure. Two subspecies of *Cucurbita pepo* were cultivated in 4 different conditions: (1) Control (2) DDE: exposure to 100 ng L^{-1} of DDE (3) NPs: exposed to 500 mg L^{-1} of AgNPs (4) DDE + NPs: exposed to 100 ng L^{-1} of DDE and 500 mg L^{-1} of AgNPs. (*) are significantly different from their respective control . Values are mean ± standard error of 3 biological independent replicates.

3.2.2. Isolation of plant-associated bacteria

The plant-associated bacteria were isolated from plants grown under all 4 conditions and the average number of colony forming units per gram of plant material was calculated from 3 biologically independent replicates *(Figure 12)*.

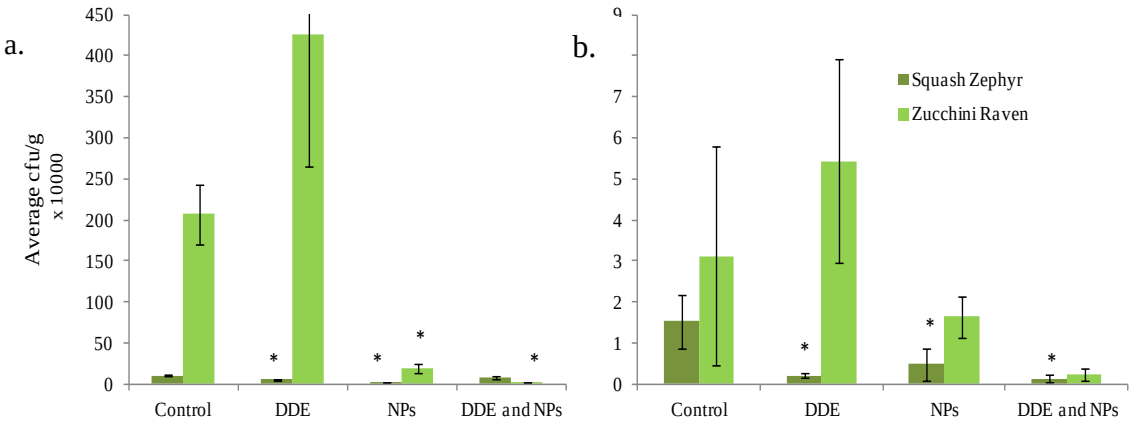

Figure 12: **Isolation of plant endophytes** Number of endophytes isolated from plants. On the vertical axis, the number of isolated endophytes are portrayed in colony forming units per gram of plant material. . Two subspecies of *Cucurbita pepo* were tested: squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator. 4 conditions were grown. (1) Control (2) DDE: exposure to 100 ng L^{-1} of DDE (3) NPs: exposed to 500 mg L^{-1} of AgNPs (4) DDE + NPs: exposed to 100 ng L^{-1} of DDE and 500 mg L^{-1} of AgNPs. (a) Isolation from roots (b) Isolation from shoots. (*) are significantly different from their respective control**.** Values are mean ± standard error of 3 biological independent replicates.

When comparing the number of isolated bacteria from the accumulator and the non-accumulator, the accumulator in general contains higher numbers of endophytes (except the shoots of plants that were exposed to DDE and NPs).

As expected, the number of bacteria that was isolated from roots *(Figure 12a)* was significantly higher than the number isolated from shoots ($p < 0.05$) *(Figure 12b).*

When plants were exposed to 100 ng L^{-1} DDE, a different effect can be observed on the endophytes of the accumulator and the non-accumulator. While the number of endophytes in the accumulator increases during DDE exposure, the amount of plant-associated bacteria in the non-accumulator decreases significantly ($p < 0.05$).

The number of bacteria in plants that were exposed to AgNPs decreased significantly when compared to control plants ($p < 0.05$). The effect of adding NPs to the growth medium of plants is smaller in shoots than it is in the roots. This might be caused by the direct contact between the roots and the NPs in the vermiculite, while the shoots never come in contact with the vermiculite and AgNPs. As expected, AgNPs have a toxic effect on the number of isolated endophytes of plants that were exposed.

The combined exposure to DDE an AgNPs causes a significant decrease ($p < 0.05$) in endophyte concentration in the roots of zucchini Raven and the shoots of squash Zephyr. The other tissues also showed a decrease, but this result was not statistically significant ($p < 0.05$).

3.2.3. Genotypic identification

The plant-associated bacteria of both squash Zephyr and zucchini Raven were isolated from plants grown under the following 4 conditions: (1) control, (2) exposed to 100 µg L^{-1} DDE, (3) exposed to 500 mg L^{-1} AgNPs, and (4) exposed to 100 µg L^{-1} DDE and 500 mg L^{-1} AgNPs. All isolated endophytes were identified using DNA-extraction, ARDRA, gel electrophoresis and sequencing.

Genotypic identification of the bacteria associated with squash Zephyr

The cultivable endophytic bacterial community associated with squash Zephyr plants grown under control conditions included 2 genotypically different bacterial strains: *Microbacterium* sp. (61.0 %) and *Klebsiella* sp. (39.0%) *(Figure 13a)*. Remarkable is that no *Pseudomonas* sp. were found, while they are the only species present in the seeds of squash Zephyr (3.1.4. Genotypic identification). A possible explanation for this observation is that these plants were grown from seeds from a different batch (since these experiments were performed during my stay in New Haven).

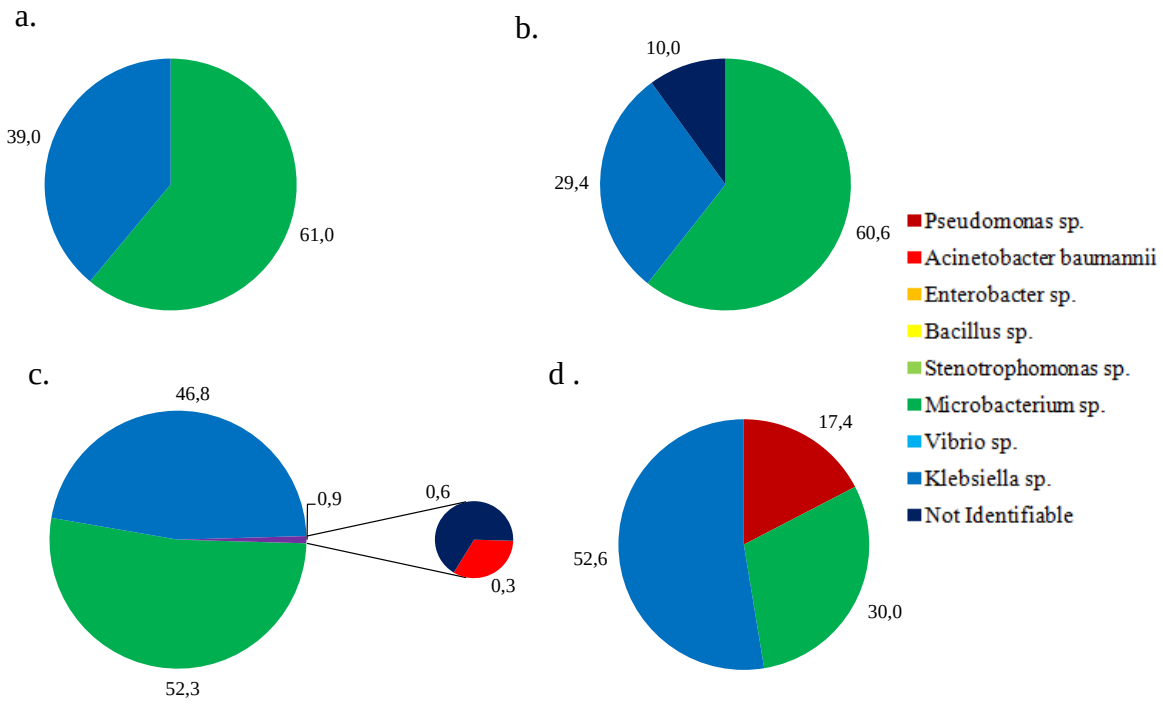

Figure 13: **Genotypic identification of plant endophytes of squash Zephyr** Representation of the distribution of bacterial species in squash Zephyr. (a) Diversity of cultivable endophytic bacteria in control plants; (b) Diversity of cultivable endophytic bacteria in plants exposed to 100 µg L^{-1} DDE; (c) Diversity of cultivable endophytic bacteria in plants exposed to 500 mg L^{-1} AgNPs; (d) Diversity of cultivable endophytic bacteria in plants exposed to 100 µg L^{-1} DDE and 500 mg L^{-1} AgNPs. The labels display the relative abundance of each genotypically different bacterial strain in the total population.

DDE exposure did not influence the endophytic diversity present in squash Zephyr *(Figure 13b)*. *Microbacterium* sp. (60.6 %) and *Klebsiella* sp. (29.4 %) were the only identified strains, while 10.0 % of the population was unidentifiable. Since squash Zephyr is a non-accumulator of DDE, the addition of DDE in the environment causes only minimal disturbance in the conditions inside the plant. Consequently, it would expected that changes in endophytic diversity are also minimal.

When squash Zephyr plants were exposed to NPs *(Figure 13c)*, the isolated endophytic community slightly changed. *Klebsiella* sp. (46.8 %) and *Microbacterium* sp. (52.3 %) remained prominently present in the population, while the remaining part of the population consisted of *Acinetobacter baumannii* (0.3 %) and 0.6 % of unidentifiable strains. The exposure of plants to AgNPs induced no shift in biodiversity. However, the effect of AgNPs is mainly perceptible in a significant decrease in the total number of isolated bacteria *(Figure 12)* (3.2.2. Isolation of plant-associated bacteria).

The community of cultivable bacteria associated with squash Zephyr when exposed to both DDE and AgNPs *(Figure 13d)* was again dominated by *Klebsiella* sp. (52.6 %) and *Microbacterium* sp. (30.0 %). However, the remaining 17.4 % of the endophytic population consisted of *Pseudomonas* sp. It was to be expected that *Pseudomonas* sp. were present in the plants since they were present as seed endophytes (3.1.4. Genotypic identification), but their absence in the 3 remaining conditions remains unexplained.

Klebsiella sp. and *Microbacterium* sp. are consistently dominant in all 4 conditions, the only different identifiable strains were *Acinetobacter baumannii* (0.3 %) when plants were exposed to NPs and *Pseudomonas* sp. (17.4 %) when squash Zephyr was exposed to DDE and AgNPs. In conclusion, DDE and/or AgNPs exposure only has a limited effect on the endophytic diversity of the non-accumulating squash Zephyr.

Genotypic identification of the bacteria associated with zucchini Raven

The community of cultivable endophytic bacteria associated with zucchini Raven in control conditions *(Figure 14a)* was dominated by *Klebsiella* sp. (79.9 %). *Pseudomonas* sp. and *Acinetobacter baumannii* both occupied 10 % of the population and the remaining 0.03 % contained *Enterobacter* sp. (0.01 %), *Bacillus* sp. (0.002 %), *Stenotrophomonas* sp. (0.02 %), and 0.002 % of unidentifiable strains.

If DDE was added to the plant environment of zucchini raven *(Figure 14b)*, the composition of the endophytic population changed drastically. *Enterobacter* sp. occupied a dominant position in the population (52.4 %), together with *Pseudomonas* sp. (38.1 %). The effect of

DDE on *Acinetobacter baumannii* is minimal, it still makes up 7.0 % of the bacterial community. The remaining part of the population exists of 0.1 % of *Vibrio* sp. and 2.3 % of a bacterial strain that was not identifiable. A remarkable absence is *Klebsiella* sp. which was dominantly present the zucchini Raven plants grown under control conditions. These drastic differences might be explained by the high uptake of DDE by zucchini Raven which causes a change inside the plant compared to control conditions.

When AgNPs are added to the environment of zucchini Raven, the relative abundance of *Vibrio* sp. increases drastically from 0 % to 54.1 % (*Figure 14c)*, while the percentage of *Pseudomonas* sp. decreases from 10.1 % to 0.2 %. *Acinetobacter baumannii* stays relatively constant at 11.3 %. 23.1 % of the population remained unidentified. This might explain the absence of other bacterial strains that were present in other conditions and makes it hard to draw solid conclusions concerning the effect of NPs on the endophytic diversity in zucchini Raven. More genotypic identifications in future experiments will have to bring clarification concerning the endophytic population of zucchini Raven.

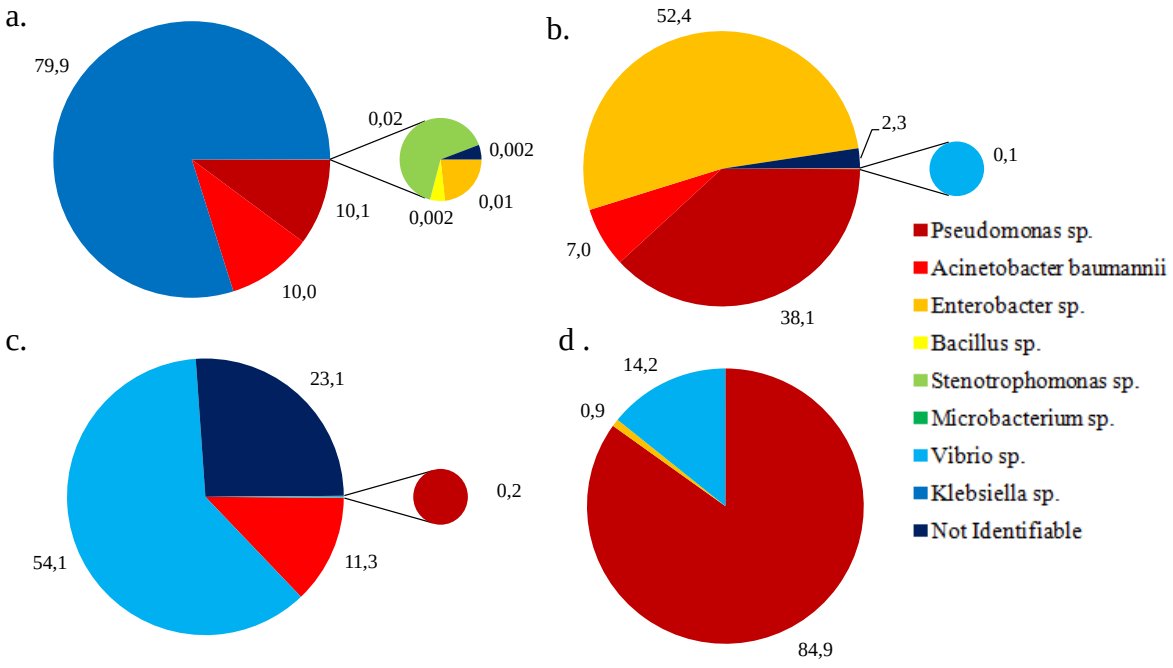

Figure 14: **Genotypic identification of plant endophytes of zucchini Raven** Representation of the distribution of bacterial species in zucchini Raven. (a) Diversity of cultivable endophytic bacteria in control plants; (b) Diversity of cultivable endophytic bacteria in plants exposed to 100 µg L^{-1} DDE; (c) Diversity of cultivable endophytic bacteria in plants exposed to 500 mg L^{-1} AgNPs; (d) Diversity of cultivable endophytic bacteria in plants exposed to 100 µg L^{-1} DDE and 500 mg L^{-1} AgNPs. The labels display the relative abundance of each genotypically different bacterial strain in the total population.

The amount of *Pseudomonas* sp. increases again (84.9 %) when zucchini Raven is exposed to both DDE and AgNPs *(Figure 14d)*. The remaining part of the population consisted of *Vibrio*

sp. (14.2 %) and *Enterobacter* sp. (0.9 %). *Acinetobacter baumannii* is absent in this community.

When comparing the endophytic population of zucchini Raven grown under the 4 different growth conditions, the highest diversity is observed in control conditions. The relative abundance of *Pseudomonas* sp. increases when the plants are exposed to DDE, while the same is true for *Vibrio* sp. after plants exposed to AgNPs. In general, the addition of AgNPs causes, next to a decrease in the total number of isolated endophytes *(Figure 12)*, also a decrease in the endophytic bacterial diversity.

If both the endophytic communities of squash Zephyr and of zucchini Raven are taken into consideration, several differences can be observed. While the populations of squash Zephyr mainly consist of *Microbacterium* sp. and *Klebsiella* sp., a higher diversity is detected in zucchini Raven with *Pseudomonas* sp. and *Vibrio* sp. as most dominant species. When squash Zephyr is exposed to DDE and/or AgNPs, no differences in bacterial diversity is noticeable, for zucchini Raven however, several changes are detected. Experiments already confirmed that zucchini Raven has a higher DDE uptake which might cause the observed changes after DDE exposure, but further research is needed to investigate whether the variation between squash Zephyr and zucchini Raven can be related to variations in the uptake of nanoparticles. This research is already in progress at the *Connecticut Agricultural Experiment Station*.

3.2.4. Phenotypic characterization

All bacteria that were isolated from both *Cucurbita pepo* subspecies grown under the 4 different conditions were subjected to (1) an auxanography to screen them for their possible DDE degrading capacity, (2) a second auxanography to test the toxicity of NPs, and (3) 4 tests evaluating their plant growth promoting capacity (production of IAA, organic acid, siderophore and ACC deaminase) *(Figure 15 and Table 1).*

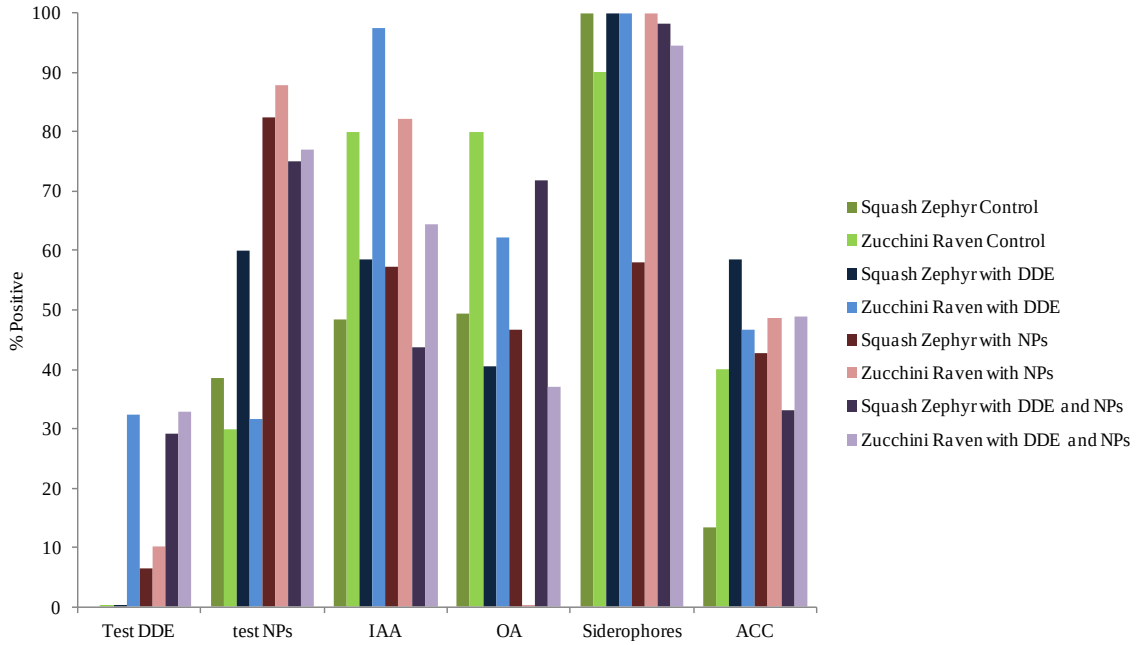

Figure 15: **Phenotypic characterization of isolated plant endophytes.** On the vertical axis, the percentage of isolated endophytes that scored positive are portrayed. Endophytes were isolated from two subspecies : squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator in 4 possible conditions: (1) Control (2) DDE: exposed to 100 ng L^{-1} of DDE (3) NPs: exposed to 500 mg L^{-1} of AgNPs (4) DDE + NPs: exposed to 100 ng L^{-1} of DDE and 500 mg L^{-1} of AgNPs. (a) Isolation from roots (b) Isolation from shoots. Bacteria were analyzed in six tests: (1) test DDE: screening for possible degradation of DDE, (2) test NPs : survival when exposed to 500 mg L^{-1} of AgNPs, (3) IAA: production of indole-3-acetic acid, (4) OA: production of organic acids, (5) Siderophores: production of siderophores, and (6) ACC: production of 1-aminocyclopropane-1-carboxylate deaminase.

Screening for potential DDE degrading strains

When comparing the endophytes that were isolated from plants exposed to DDE (DDE and DDE + NPs) with endophytes from plants that were not exposed (control and NPs), plants that were exposed to DDE have a higher percentage of bacteria with the potential to use DDE as a carbon source *(Figure 15).* For the DDE accumulating zucchini Raven, there is no difference between endophytes isolated from plants exposed to DDE and endophytes isolated from plants exposed to both DDE and AgNPs. Meanwhile, for the non-accumulator (squash Zephyr), a big difference is present between endophytes isolated from these conditions. We hypothesize that this difference is due to the increased uptake of DDE under the influence of AgNPs, which induces an increase in the number of potential DDE degrading endophytes.

Considering these results, it seems that once there is a certain amount of DDE present in the plant, plant-associated bacteria are subjected to natural selection for strains with the potential to degrade DDE.

Next to exposure to DDE, it seems that exposure to only NPs can also induce a natural selection for plant-associated bacterial strains with the potential to use DDE as a carbon source. This is hypothesized since bacteria from plants that were exposed to NPs show a

higher capacity for DDE degradation than bacteria from control plants. A possible explanation for this observation could be that bacteria that are exposed to stress conditions are more likely to develop alternative ways of feeding to create an advantage over other bacterial species that lack that ability.

Nanoparticle toxicity at the bacterial level

The same form of selective adaptation that was observed when bacteria came into contact with DDE in the plant, can be detected with AgNPs. Endophytes originating from plants that were previously exposed to AgNPs (NPs and DDE + NPs) experience less toxicity when they are exposed to 500 mg L^{-1} AgNPs in an auxanography. The percentage of bacteria that survives doubles when they have previously encountered NPs in the plant. This adaptation and decreasing toxicity of NPs to bacteria might prove useful when the DDE phytoremediation efficiency is enhanced by exploiting NPs as well as plant-associated bacteria.

Plant growth promoting capacity

The endophytes isolated from zucchini Raven show a higher percentage of IAA producing bacteria under all conditions of exposure than endophytes from squash Zephyr. When the endophytes isolated from DDE-exposed plants are compared to their respective controls, there is an increase in IAA production for both subspecies. The difference between the endophytes from controls and NP-exposed plants is smaller. All three of these findings can be considered positive in the context of DDE phytoremediation. However, when plants are exposed to both DDE and NPs, the IAA producing capacity of their associated endophytes decreases in comparison to the control.

The effects of growing plants under different conditions on organic acid production are not consistent. Therefore, no solid conclusions can be formed concerning organic acid production. The effect of NPs and/or DDE on siderophore and ACC deaminase production however, seems to be limited. The percentages of bacteria that can produce these molecules remain in the same range over different conditions. There are some remarkable outliers that will need further investigation. When endophytes are isolated from squash Zephyr plants that were exposed to NPs alone, an inexplicable decrease in siderophore production is observed. This is an unusual finding and will have to be re-investigated since NPs caused no effect when combined with DDE. In case of ACC deaminase production, the low percentage of bacteria that produce ACC deaminase in the controls of squash Zephyr seem odd.

Table 1: **Phenotypic characterization of isolated plant endophytes**

Condition	Species	total cfu/g	Test DDE	test NPs	IAA	OA	Siderophores	ACC
Control	squash Zephyr	4.58 x 10^4	0	55.3	47.8	70.9	100	19.3
	zucchini Raven	3.59 x 10^6	0.03	30.0	80.0	80.0	90	40.1
DDE	squash Zephyr	1.14 x 10^5	0.31	60.0	58.5	40.6	100	58.5
	zucchini Raven	2.87 x 10^7	32.3	31.5	97.4	62.1	100	46.8
NPs	squash Zephyr	1.55 x 10^4	6.45	82.5	57.3	46.7	57.9	42.8
	zucchini Raven	3.61 x 10^5	10.1	87.9	82.1	0.04	100	48.6
DDE and NPs	squash Zephyr	1.13 x 10^5	29.2	75.1	43.7	71.75	98.1	33.1
	zucchini Raven	2.63 x 10^4	32.9	77.0	64.5	37.0	94.4	48.8

Total number of isolated cultivable endophytes in colony forming units per gram of plant biomass and percentage of endophytes that scored positive. Endophytes were isolated from two subspecies : squash Zephyr, a DDE non-accumulator, and zucchini Raven, a DDE accumulator in 4 possible conditions : (1) Control (2) DDE: exposed to 100 ng L^{-1} of DDE (3) NPs: exposed to 500 mg L^{-1} of AgNPs (4) DDE + NPs: exposed to 100 ng L^{-1} of DDE and 500 mg L^{-1} of AgNPs. (a) Isolation from roots (b) Isolation from shoots. Bacteria were analyzed in six tests: (1) test DDE: screening for possible degradation of DDE, (2) test NPs : survival when exposed to 500 mg L^{-1} of AgNPs, (3) IAA: production of indole-3-acetic acid, (4) OA: production of organic acids, (5) Siderophores: production of siderophores, and (6) ACC: production of 1-aminocyclopropane-1-carboxylate deaminase.

3.2.5. Overall effects of AgNPs and/or DDE on Cucurbita pepo and its endophytes

When considering the overall effects of AgNPs and/or DDE at the plant level, it seems that the observed inhibition in plant biomass, that is observed after exposure to DDE or DDE + NPs, is mainly related to DDE phytotoxicity.

However, when the plant-associated bacteria are investigated, negative effects that are observed are mainly caused by the effects of AgNPs. A significant decrease in the amount of endophytic bacteria is demonstrated ($p < 0.05$) and, in case of the DDE accumulating zucchini Raven, the addition of NPs causes changes in the endophytic diversity. This change is not observed in squash Zephyr.

The combined exposure of DDE and NPs causes an increase in the DDE degradation capacity of the isolated plant-associated bacteria. When the NP toxicity is tested on separate endophytes that previously came into contact with NPs (isolated from plants exposed to NPs or DDE + NPs), we see a decrease in comparison to endophytes that never before were exposed (from control and DDE-exposed plants).

When considering the plant growth promoting capacities of endophytes, no general toxic trend can be observed after exposure to NPs and/or DDE. In some cases there is an increase in the amount of plant growth promoting strains, in other cases the opposite can be observed.

When considering the endophyte-enhanced phytoremediation of DDE, a balance will have to be maintained between the advantage of increasing DDE uptake by adding AgNPs and the disadvantage of eliminating possible positive effects of plant-associated bacteria.

4. Conclusion

Cucurbita pepo ssp. *pepo* (zucchini Raven) accumulates more DDE than *Cucurbita pepo* ssp. *ovifera* (squash Zephyr). This difference in accumulation has an effect on the plant growth, however the decrease in biomass is rather limited and will therefore pose no detrimental effects in the use of zucchini Raven for DDE phytoremediation.
Furthermore, zucchini Raven contains a higher amount and diversity of endophytes in both seeds and plants exposed to DDE and/or AgNPs. The relative abundance of endophytes with plant growth promoting capacities is also higher in zucchini Raven than in squash Zephyr. These findings are promising for the suitability of zucchini Raven as an organism for the endophyte-enhanced phytoremediation of DDE-contaminated soils.

The exposure of both *Cucurbita pepo* subspecies to silver nanoparticles has no significant effect on plant growth. When plants were exposed to both DDE and AgNPs, the relative number of bacterial strains that show DDE degrading capacity increases in both subspecies in comparison with the controls, although the amount of bacteria in general decreases under influence of AgNPs. Adding NPs to the environment of plants does not induce a change in diversity of the bacterial population of squash Zephyr, but is does have a negative effect on the endophytic community of zucchini Raven. This gives rise to the question whether there is, apart from a difference in DDE accumulation, also a difference in the accumulation of silver nanoparticles between both *Cucurbita pepo* subspecies. More clarity will come with the results of the research currently in progress at the *Connecticut Agricultural Experiment Station* where researchers are testing in identical conditions whether adding AgNPs to the growth medium of *Cucurbita pepo* increases the DDE uptake and how much of the AgNPs are entering the plant.
The effect of AgNPs on the plant growth promoting capacity of the isolated strains is inconsistent. AgNPs induce a decrease in IAA and organic acid producing bacteria, while an increase in siderophore and ACC deaminase production is observed.
Silver nanoparticles demonstrate both negative and positive effects on the potential endophyte-enhanced phytoremediation of DDE-contaminated soils. Therefore, a balance will have to be established between enhancing the DDE accumulation of zucchini Raven by adding AgNPs and harming the endophytic population of the plants and thus eliminating the positive effects that these bacteria might have on the phytoremediation efficiency.

Further research will be needed concerning the uptake of AgNPs by *Cucurbita pepo* and the effect of AgNPs on the DDE accumulation. Further, the DDE degradation capacity of the most promising bacterial strains needs to be confirmed. The testing protocol for this experiment needs further optimization. Once the DDE degradation capacity is confirmed, the best DDE degrading endophytes can be selected for inoculation experiments to optimize DDE phytoremediation.

References

1. Turusov, V., V. Rakitsky and L. Tomatis (2002). Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence and risks. *Environmental Health Perspectives* 110:125–128.
2. Colles, A. and G. Koppen (2007). DDE serumwaarden in landelijke regio's en de Albertkanaalzone boven het Vlaamse referentiegemiddelde: Enquete naar de oorzaak. *De Biomonitor, digitale nieuwsbrief van het medisch milieukundig netwerk (VITO).*
3. Thomas, J.E., L. Ou and A. Al-Agely (2008). DDE remediation and degradation. *Reviews of Environmental Contamination and Toxicology*: 55-70.
4. White, J.C. (2010). Inheritance of *p,p'*-DDE phytoextraction ability in hybridized *Cucurbita pepo* cultivars. *Environmental Science and Technology* 44: 5165-5169.
5. Pilon-Smits, E. (2005). Phytoremediation. *Annual review of Plant Biology* 56: 15-39.
6. Schwitzguébel, J.P., J. Kumpiene, E. Comino and T. Vanek (2009). From green to clean: a promising and sustainable approach towards environmental remediation and human health for the 21st century. *Agrochimica* 53: 209-237.
7. Vassilev, A., J.P. Schwitzguébel, T. Thewys, D. Van Der Lelie & J. Vangronsveld (2004). The use of plants for remediation of metal-contaminated soils. *The scientific world journal*, 4: 9-34.
8. Weyens, N., D. Van Der Lelie, T. Artois, K.Smeets, S. Taghavi, L. Newman, R. Carleer, J. Vangronsveld (2009). Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. *Environmental Science and Technology* 43: 9413-9418.
9. White , J.C., Z. Parrish, M. Isleyen, M. Gent, W. Ianmucci-Berger, B. Eitzer,M. Mattina (2005) Uptake of weathered p,p'-DDE by plant species effective at accumulating soil elements. *Microchemical journal* 81: 148–155.
10. Musante, C. and J.C. White (2010). Toxicity of silver and copper to Cucurbita pepo: differential effects of nano and bulk-size particles. *Environmental toxicology*, ahead of publication
11. Handy, R.D., F. Kamme, J.R. Lead, M. Hasselov, R. Owen and M. Crane (2008). The ecotoxicology and chemistry of manufactured nanoparticles. *Ecotoxicology* 17, 287-314.
12. Handy, R.D., R. Owen and E. Valsami-Jones (2008). The ecotoxicology of nanoparticles and Nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology* 17, 315-325.

13. Ma, X. and C. Wang, 2010. Fullerene nanoparticles affect the fate and uptake of trichloroethylene in phytoremediation systems. *Environmental Engineering science* 27, 989-992.
14. Stampoulis, D., S.K. Sinha and J.C. White (2009). Assay-dependent phytotoxicity of nanoparticles to plants. *Environmental Science and Technology,* 43: 9473-9479.
15. Christian, P., F. Von der Kammer, M. Baalousha and T. Hofmann (2008). Nanoparticles: structure, properties, preparation and behaviour in environmental media. *Ecotoxicology* 17, 326-343.
16. Zhang, W.X. and D.W. Elliott (2006). Applications of iron nanoparticles for ground water remediation. *Remediation* 16, 7-21.
17. Geriano, L., M. Heuberger and B. Nowack (2009). The behavior of silver nanotextiles during washing. *Environmental Science & Technology* 43, 8113-8118.
18. Wang, C. and W.X. Zhang. Nanoscale metal particles for dechlorination of PCE and PCBs. *Environmental Science & Technology* 31, 2154-2156.
19. Dietz, K.J. and S. Herth (2011). Plant nanotoxicology. *Trends in plant science* 16, 582-589.
20. Vangronsveld, J., R. Herzig, N. Weyens, J. Boulet, K. Adriaensen, A. Ruttens, T. Thewys, A. Vassilev, E. Meers, E. Nehnevajova, D. Van Der Lelie and M. Mench (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environmental science & pollution research*, 16: 765-794.
21. Weyens, N., S. Monchy, J. Vangronsveld, S. Taghavi and D. Van Der Lelie (2010). Plant-microbe partnerships. In: Timmis, K. N. (ed.), *Handbook of Hydrocarbon and Lipid Microbiology*, Springer-Verslag Berlin, Heidelberg: 2545-2574.
22. Weyens, N., D. Van Der Lelie, S. Taghavi, L. Newman and J. Vangronsveld (2009). Exploiting plant-microbe partnerships to improve biomass production and remediation. *Trends in Biotechnology,* 10: 591-598.
23. Weyens, N., D. Van Der Lelie, S. Taghavi and J. Vangronsveld (2009). Phytoremediation: plant-endophyte partnerships take the challenge. *Current opinion in Biotechnology,* 20: 1-7.
24. Saalfield, S.L. and B.C. Bostick (2009). Changes in iron, sulfur, and arsenic speciation associated with bacterial sulfate reduction in ferrihydrite-rich systems. *Environmental science and technology,* 43: 8787-8793.
25. Vessey, J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and soil,* 255: 571-586.

26. Taghavi, S., T. Barac, B. Greenberg, B. Borremans, J. Vangronsveld and D. Van Der Lelie (2005). Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. *Applied & environmental microbiology*, 71: 8500-8505.
27. Mayak, S., T. Tirosh and T.R. Glick (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant physiology & biochemistry*, 42: 565-572.

Attachments

Attachment 1: Media

¼ Hoagland medium

For 1 L:

- 250 mL macro-elements:
  For 1 L deionised water:
  - 10.2 g KNO_3
  - 7.08 g $Ca(NO_3)_2 \cdot 4H_2O$
  - 2.3 g $NH_4H_2PO_4$
  - 4.9 g $MgSO_4 \cdot 7H_2O$
- 2.5 mL micro-elements:
  For 1 L deionised water:
  - 2.86 g H_3BO_3
  - 1.81 g $MnCL_2 \cdot 4H_2O$
  - 0.08 g $CuSO_4 \cdot 5H_2O$
  - 0.09 g $H_2MoO_4 \cdot H_2O$
  - 0.22 g $ZnSO_4 \cdot 7H_2O$
- 1.5 mL Fe-EDTA:
  For 1L deionised water:
  - 7.6 g EDTA·Na
  - 5 g $FeSO_4 \cdot 7H_2O$
- 746 mL deionised water

Rich medium (869)

In 1 L deionised water:

- 10 g tryptone
- 5 g yeast extract
- 5 g NaCl
- 1 g glucose D+
- 0.345 g $CaCl_2 2H_2O$

Bring to pH 7 with NaOH

Add 15 g/L agar (for solid medium)

1/10 Rich medium

For 1 L: Take 100 mL of rich (869) medium and add 900 mL of deionised water.

Selective Medium (284)

in 1 L deionised water:

- 6.06 g Tris-HCl
- 4.68g NaCl
- 1.49g KCl
- 1.07 g NH_4Cl
- 0.43 g Na_2SO_4
- 0.2 g $MgCl_2 \cdot 6H_2O$
- 0.03 g $CaCl_2 \cdot 2H_2O$
- 40 mg $Na_2HPO_4 \cdot 2H_2O$ 1%
- 1 mL microelements solution:

  Per litre deionised water:

  - 1.3 mL 25% HCl
  - 144 mg $ZnSO_4 \cdot 7H_2O$
  - 100 mg $MnCl_2 \cdot 4H_2O$
  - 62 mg H_3BO_3
  - 190 mg $CoCl_2 \cdot 6H_2O$
  - 17 mg $CuCl_2 \cdot 2H_2O$
  - 24 mg $NiCl_2 \cdot 6H_2O$
  - 36 mg $NaMoO_4 \cdot 2H_2O$
- Carbon mix:
  - 0.52 g glucose
  - 0.66 g gluconate
  - 0.54 g fructose
  - 0.81 g succinate
  - 0.7 mL lactate

Bring to pH 7

20 g/L agar (for solid medium)

Glycerol stock medium

- 15 weight% glycerol
- 0.85 weight% NaCl

For 500 g:

- 75 g glycerol
- 4.25 g NaCl
- 420.75 g deionised water

Organic acid production

Sucrose tryptone medium

In 1 L deionised water:

- 20 g sucrose
- 5 g tryptone
- 10 mL of trace-element solution

Trace-element solution:

In 1 L of deionised water:

- 20 mg $NaMoO_4$
- 200 mg H_3BO_3
- 20 mg $CuSO_4.5H_2O$
- 100 mg $FeCl_3$
- 20 mg $MnCl_2.4H_2O$
- 280 $ZnCl_2$

Indicator

0.1 g of Alazarine red S reagent 0.1% in 100 mL of deionised water.

IAA production

1/10 869 medium

In 1 L deionised water:

- 1 g tryptone
- 0.5 g yeast extract
- 0.5 g NaCl
- 0.1 g Glucose D+
- 0.0345 g $CaCl_2 2H_2O$
- 0.5 g L-tryptophan

Salkowskireagent

1 mL of 0.5 M $FeCl_3$ in 49 mL of $HClO_4$ (35%).

Siderophore production

Selective medium (284)

in 1 L deionised water:

- 6.06 g Tris-HCl
- 4.68g NaCl
- 1.49g KCl
- 1.07 g NH_4Cl
- 0.43 g Na_2SO_4
- 0.2 g $MgCl_2 \cdot 6H_2O$
- 0.03 g $CaCl_2 \cdot 2H_2O$
- 40 mg $Na_2HPO_4 \cdot 2H_2O$ 1%
- 1 mL microelements solution:

    Per litre deionised water:

    - 1.3 mL 25% HCl
    - 144 mg $ZnSO_4 \cdot 7H_2O$
    - 100 mg $MnCl_2 \cdot 4H_2O$
    - 62 mg H_3BO_3
    - 190 mg $CoCl_2 \cdot 6H_2O$
    - 17 mg $CuCl_2 \cdot 2H_2O$
    - 24 mg $NiCl_2 \cdot 6H_2O$
    - 36 mg $NaMoO_4 \cdot 2H_2O$

0 µM Iron: do not add anything

0.25 µM Iron: add 0.0664952 mg L^{-1} Fe(III)citrate

3 µM Iron: add 0.07979424 mg L^{-1} Fe(III)citrate

CAS solution

For 25 mL:

- 1.5 mL HDTMA,
- 3.75 mL 10 mM HCl
- 0.375 mL $FeCl_3$
- 1.875 mL CAS
- 7.5 mL Piperazine
- 2.5 mL sulphosalicilic acid
- 7.5 mL deionised water

ACC-deaminase

SMN medium

for 1 L:

- 0.5 g ACC
- 1 g glucose
- 1 g sucrose
- 1 g sodiumacetate
- 1 g sodiumcitrate
- 1 g malic acid
- 1 g mannitol

0.56 N HCl

4.678 mL of 37% HCl in 100 mL of deionised water

0.2 % 2,4-dinitrophenylhydrazine in 2 N HCl

1.67 mL of 37% HCl in 10 mL of deionised water

20 mg of 2,4-dinitrophenylhydrazine

2 N NaOH

7.998 g of NaOH in 100 mL of deionised water.

Attachment 2: DNA extraction protocol

For DNeasy Blood & Tissue kit (Qiagen)

1. Cultivate bacteria in rich (869) medium for 2 days at 30°C and 150 rpm.
2. Prepare enzymatic lysis buffer:
   For 50 samples:
   - 180 µL 2* Tris-EDTA (TE) buffer pH 8
   - 108 mg 1.2% Triton X-100
   - 180 mg lysozyme
   - 8820 µL RNase free water
3. Harvest 1.5 mL bacterial solution in a microcentrifuge tube by centrifuging for 10 min at 7500 rpm. Discard supernatant.
4. Resuspend bacterial pellet in 180 µL enzymatic lysis buffer.
5. Incubate at 37°C for at least 30 min.
6. Add 25 µL proteinase K and 200 µL buffer AL and mix by vortexing.
7. Incubate at 56°C for 30 min.
8. Add 200 µL ethanol (96-100%) and mix by vortexing.
9. Pipet the mixture into the DNeasy mini spin column placed in a 2 mL collection tube. Centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.
10. Place the DNeasy mini spin column in a new 2 mL collection tube and add 500 µL buffer AW1. Centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.
11. Place the DNeasy mini spin column in a new 2 mL collection tube and add 500 µL buffer AW2. Centrifuge at 13200 rpm for 5 min to dry the DNeasy membrane. Discard flow-through and collection tube.
12. Place the DNeasy mini spin column in a 2 mL microcentrifuge tube and pipet 150 µL buffer AE onto the DNeasy membrane. Incubate at room temperature for 1 min. Centrifuge at 8000 rpm for 1 min.
13. Repeat step 12 for maximum DNA yield.

Attachment 3: Genotypic and phenotypic identification

Isolation seeds

squash Zephyr							
Species	**Subspecies**	**total cfu g^{-1}**	**Test DDE**	**IAA**	**OA**	**Siderophores**	**ACC**
***Pseudomonas* sp.**	2	4,67 x 10^2	0	+	+	0	+
	5	1,00 x 10^4	0	0	0	0	0
	6	4,67 x 10^2	0	0	0	+	0
	7	9,35 x 10^2	0	0	0	+	+
	8	9,35 x 10^2	0	0	+	+	+
	9	1,00 x 10^4	0	+	0	+	+
	10	7,08 x 10^1	0	0	+	0	+
% positive		**4,89 x 10^4**	**0**	**19,3**	**9,38**	**1,79**	**8,21**

zucchini Raven							
Species	**Subspecies**	**total cfu g^{-1}**	**test DDE**	**IAA**	**OA**	**Siderophores**	**ACC**
***Pseudomonas* sp.**	1	1,16 x103	0	+	0	0	0
	2	7,33 x 102	0	+	+	0	+
	3	1,22 x 102	0	+	+	+	+
	4	1,22 x 102	0	0	+	0	0
	5	7,30 x 103	0	0	0	0	0
	6	6,43 x 101	0	0	0	+	0
% positive		**9,50 x 103**	**0**	**29,7**	**31,1**	**68,9**	**70,3**

Isolation plants

zucchini Raven							
Species	**Subspecies**	**Total cfu g^{-1}**	**Test DDE**	**OA**	**IAA**	**Siderophores**	**ACC**
***Veillonella* sp.**	1	$3{,}71 \times 10^3$	0	+	0	+	+
	3	$3{,}81 \times 10^3$	0	0	0	+	0
	5	$3{,}81 \times 10^3$	0	+	0	0	0
	6	$1{,}42 \times 10^3$	+	0	0	0	0
***Pseudomonas* sp.**	1	$4{,}57 \times 10^4$	0	0	0	+	0
	2	$1{,}09 \times 10^3$	0	0	0	+	+
	3	$1{,}90 \times 10^4$	+	+	0	+	+
	6	$3{,}81 \times 10^3$	+	0	0	+	0
	7	$1{,}09 \times 10^3$	+	0	0	0	0
	8	$1{,}09 \times 10^3$	0	0	0	0	0
	9	$1{,}09 \times 10^3$	0	+	0	+	+
***Exiguobacterium* sp.**	1	$1{,}28 \times 10^4$	0	0	+	+	+
	2	$3{,}81 \times 10^3$	+	0	0	+	+
	3	$1{,}42 \times 10^3$	+	0	0	+	0
***Enterobacter* sp.**	1	$1{,}90 \times 10^4$	0	0	0	+	0
	2	$3{,}81 \times 10^4$	0	0	0	+	+
	4	$1{,}90 \times 10^4$	0	+	0	+	+
***Bacillus* sp.**	1	$1{,}50 \times 10^3$	0	0	+	+	+
	2	$3{,}28 \times 10^3$	0	0	0	+	0
	4	$3{,}28 \times 10^3$	0	+	0	+	+
	5	$3{,}28 \times 10^3$	0	0	0	0	0
	6	$1{,}86 \times 10^3$	0	0	0	+	+
***Thermotoga* sp.**	2	$1{,}90 \times 10^3$	0	+	+	0	0
	3	$1{,}90 \times 10^3$	0	0	0	+	0
	4	$1{,}90 \times 10^3$	0	+	0	+	+
***Lactococcus* sp.**	1	$5{,}66 \times 10^3$	0	0	0	+	0
	2	$3{,}28 \times 10^3$	+	0	0	+	0
	3	$1{,}90 \times 10^3$	+	0	0	0	0
	4	$1{,}90 \times 10^3$	0	0	+	0	0
	5	$1{,}90 \times 10^3$	0	0	0	0	0
	6	$3{,}71 \times 10^3$	0	+	+	+	+
Not Identifiable	1	$4{,}35 \times 10^3$	+	0	0	+	0
	2	$1{,}09 \times 10^3$	0	0	0	+	0
% positive		**$2{,}82 \times 10^5$**	**14,0**	**31,9**	**18,3**	**87,7**	**49,3**

squash Zephyr							
Species	**Subspecies**	**Total cfu g^{-1}**	**Test DDE**	**IAA**	**OA**	**Siderophores**	**ACC**
***Veillonella* sp.**	2	$8{,}16 \times 10^{1}$	0	0	0	0	0
	3	$1{,}09 \times 10^{2}$	0	0	0	+	0
	4	$2{,}72 \times 10^{1}$	0	+	0	+	+
***Pseudomonas* sp.**	1	$1{,}28 \times 10^{4}$	0	0	0	+	0
	2	$5{,}55 \times 10^{2}$	0	0	0	+	+
	3	$5{,}55 \times 10^{2}$	+	0	+	+	+
***Bacillus* sp.**	1	$5{,}55 \times 10^{2}$	0	0	0	+	0
	2	$6{,}04 \times 10^{3}$	0	0	0	+	+
	3	$2{,}01 \times 10^{3}$	0	0	0	0	0
	4	$2{,}01 \times 10^{3}$	0	0	+	+	+
***Exiguobacterium* sp.**	1	$5{,}54 \times 10^{2}$	0	0	0	+	0
Not Identifiable	1	$5{,}54 \times 10^{2}$	0	0	0	+	+
	2	$5{,}54 \times 10^{1}$	0	+	0	+	+
% positive		**$2{,}60 \times 10^{5}$**	**2,14**	**0,314**	**9,90**	**91,9**	**37,8**

Isolation plants from different conditions

Squash Zephyr Control								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Microbacterium* sp.**	11	$5{,}96 \times 10^{3}$	0	-	+	0	+	0
	12	$7{,}98 \times 10^{3}$	0	0	+	+	+	0
	13	$7{,}98 \times 10^{3}$	0	0	0	+	+	0
	14	$8{,}26 \times 10^{3}$	0	-	0	+	+	+
***Klebsiella* sp.**	1	$7{,}98 \times 10^{3}$	0	0	+	+	+	0
	2	$5{,}96 \times 10^{3}$	0	-	0	0	+	0
	3	$5{,}70 \times 10^{2}$	0	0	0	0	+	+
	4	$8{,}55 \times 10^{2}$	0	0	0	0	+	0
	5	$2{,}85 \times 10^{2}$	0	-	0	+	+	0
% positive		**$4{,}5827 \times 10^{4}$**	**0**	**55,3**	**47,8**	**70,9**	**100**	**19,3**

Squash Zephyr with DDE								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Microbacterium* sp.**	5	7,02 x 10^2	0	-	0	0	+	0
	6	3,51 x 10^2	0	0	0	0	+	0
	11	1,11 x 10^4	0	-	+	0	+	0
	15	2,25 x 10^4	0	0	+	0	+	+
	16	1,11 x 10^4	0	0	0	+	+	+
	17	1,11 x 10^4	0	-	+	0	+	+
	18	1,11 x 10^4	0	-	+	+	+	0
	19	3,51 x 10^2	0	-	0	+	+	0
***Klebsiella* sp.**	4	1,11 x 10^4	0	0	0	0	+	0
	5	3,51 x 10^2	0	-	0	+	+	0
	6	1,11 x 10^4	0	-	+	+	+	+
	7	1,11 x 10^4	0	0	0	+	+	+
Not Identifiable	1	1,11 x 10^4	0	0	0	0	+	0
	2	3,51 x 10^2	+	0	0	+	+	0
% positive		**1,14 x 10^5**	**0,3**	**60,0**	**58,5**	**40,6**	**100**	**58,5**

Squash Zephyr with NPs								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Microbacterium* sp.**	1	$4{,}80 \times 10^{1}$	0	0	0	0	0	+
	2	$9{,}73 \times 10^{2}$	0	0	0	0	+	+
	5	$1{,}16 \times 10^{3}$	0	-	0	0	+	0
	12	$1{,}41 \times 10^{3}$	0	0	+	+	+	0
	13	$2{,}69 \times 10^{2}$	0	0	0	+	+	0
	16	$9{,}73 \times 10^{2}$	0	0	0	+	+	+
	20	$1{,}06 \times 10^{3}$	0	0	+	0	0	+
	21	$6{,}21 \times 10^{2}$	0	0	0	+	0	0
	22	$7{,}03 \times 10^{2}$	0	0	0	0	0	0
	23	$7{,}03 \times 10^{2}$	+	0	+	0	0	+
	24	$3{,}40 \times 10^{1}$	+	0	+	0	0	0
	25	$3{,}40 \times 10^{1}$	0	0	+	0	0	0
	26	$1{,}36 \times 10^{2}$	0	-	+	0	0	0
***Klebsiella* sp.**	1	$1{,}32 \times 10^{3}$	0	0	+	+	+	0
	2	$9{,}60 \times 10^{1}$	0	-	0	0	+	0
	3	$6{,}69 \times 10^{2}$	0	0	0	0	+	+
	6	$5{,}70 \times 10^{1}$	0	-	+	+	+	+
	7	$2{,}69 \times 10^{2}$	0	0	0	+	+	+
	8	$4{,}13 \times 10^{2}$	0	-	+	+	+	0
	9	$2{,}07 \times 10^{2}$	+	0	0	+	+	+
	10	$2{,}07 \times 10^{2}$	0	0	0	+	+	0
	11	$7{,}03 \times 10^{2}$	0	0	+	0	+	0
	12	$7{,}03 \times 10^{2}$	0	0	+	0	0	0
	13	$7{,}03 \times 10^{2}$	0	-	+	+	0	0
	14	$7{,}03 \times 10^{2}$	0	0	+	+	0	+
	15	$7{,}03 \times 10^{2}$	0	0	+	0	0	+
	16	$1{,}14 \times 10^{2}$	0	0	+	0	+	+

	17	$5,70 \times 10^1$	0	0	+	+	+	+
	18	$1,14 \times 10^2$	0	0	0	0	0	+
	19	$5,70 \times 10^1$	+	0	0	0	0	0
	20	$5,70 \times 10^1$	0	-	0	0	0	0
	21	$1,14 \times 10^2$	0	0	0	0	0	0
Acetinobacter baumannii	1	$4,80 \times 10^1$	0	-	+	+	+	0
Not Identifiable	3	$4,80 \times 10^1$	0	-	0	0	+	0
	8	$4,80 \times 10^1$	0	0	0	0	0	0
% positive		**$1,55 \times 10^4$**	**6,4**	**82,5**	**57,3**	**46,7**	**57,9**	**42,8**

Squash Zephyr with DDE and NPs								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
Pseudomonas sp.	1	4,44 x 10^3	0	0	+	0	+	+
	2	2,31 x 10^3	0	0	0	+	+	0
	3	4,44 x 10^3	0	0	0	0	+	0
	4	4,25 x 10^3	0	0	+	+	+	+
	5	2,13 x 10^3	0	0	+	+	+	0
	6	2,13 x 10^3	0	-	+	0	+	+
***Microbacterium* sp.**	2	2,31 x 10^3	0	0	0	0	+	+
	3	4,62 x 10^3	+	0	+	0	+	0
	4	2,31 x 10^3	+	-	0	+	+	+
	5	4,69 x 10^3	0	0	0	0	+	0
	6	2,31 x 10^3	0	-	0	0	+	0
	7	6,30 x 10^2	+	0	0	0	+	+
	8	2,13 x 10^3	0	0	+	+	+	+
	9	2,13 x 10^3	0	-	0	0	0	+
	10	2,13 x 10^3	0	0	+	0	+	0
	11	2,13 x 10^3	0	-	+	0	+	0
	12	8,50 x 10^3	0	0	+	+	+	0
	14	6,20 x 10^1	0	-	0	+	+	+
	19	6,20 x 10^1	0	-	0	+	+	0
***Klebsiella* sp.**	1	8,50 x 10^3	0	0	+	+	+	0
	7	1,70 x 10^4	0	0	0	+	+	+
	8	8,50 x 10^3	0	-	+	+	+	0
	23	1,70 x 10^4	+	0	0	+	+	0
	24	8,50 x 10^3	+	-	0	+	+	0
	25	6,20 x 10^1	0	-	0	0	+	+
% positive		**1,13 x 10^5**	**29,2**	**75,1**	**43,7**	**71,7**	**98,1**	**33,1**

Zucchini Raven Control								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Pseudomonas* sp.**	3	$6,04 \times 10^2$	0	0	0	0	+	0
	4	$6,04 \times 10^2$	0	0	+	+	+	+
	5	$5,40 \times 10^1$	0	0	+	+	+	0
	7	$3,59 \times 10^5$	0	-	0	0	0	+
	8	$7,12 \times 10^2$	0	-	+	+	+	0
	9	$3,07 \times 10^3$	0	-	+	+	+	+
	10	$6,04 \times 10^2$	+	0	0	+	+	+
Acinetobacter baumannii	1	$3,59 \times 10^5$	0	-	+	+	+	0
***Klebsiella* sp.**	1	$7,17 \times 10^5$	0	0	+	+	+	0
	6	$7,17 \times 10^5$	0	-	+	+	+	+
	8	$7,17 \times 10^5$	0	-	+	+	+	0
	11	$3,59 \times 10^5$	0	0	+	0	+	0
	26	$3,59 \times 10^5$	0	-	0	+	+	+
***Enterobacter* sp.**	1	$1,62 \times 10^2$	0	0	+	+	+	+
	2	$5,40 \times 10^1$	0	-	+	+	+	+
***Bacillus* sp.**	1	$5,40 \times 10^1$	0	-	+	+	+	0
***Stenotrophomonas* sp.**	1	$6,04 \times 10^2$	+	0	+	0	+	+
Not Identifiable	4	$5,40 \times 10^1$	0	-	0	+	+	+
% positive		**$3,59 \times 10^6$**	**0,03**	**30,0**	**80,0**	**80,0**	**90,0**	**40,1**

Zucchini Raven with DDE								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Pseudomonas* sp.**	1	$2{,}73 \times 10^{6}$	0	0	+	0	+	+
	5	$8{,}86 \times 10^{5}$	0	0	+	+	+	0
	6	$4{,}16 \times 10^{2}$	0	-	+	0	+	+
	8	$1{,}07 \times 10^{6}$	0	-	+	+	+	0
	9	$2{,}26 \times 10^{6}$	0	-	+	+	+	+
	11	$6{,}72 \times 10^{5}$	+	-	+	0	+	+
	12	$6{,}72 \times 10^{5}$	0	-	0	+	+	+
	13	$2{,}26 \times 10^{6}$	0	-	+	0	+	0
	14	$1{,}99 \times 10^{5}$	+	-	+	0	+	0
	15	$1{,}99 \times 10^{5}$	+	0	+	0	+	0
Acinetobacter baumannii	2	$6{,}72 \times 10^{5}$	+	0	+	0	+	0
	3	$6{,}72 \times 10^{5}$	+	-	+	0	+	+
	4	$6{,}72 \times 10^{5}$	+	-	+	0	+	0
***Enterobacter* sp.**	1	$1{,}99 \times 10^{5}$	0	0	+	+	+	+
	2	$2{,}06 \times 10^{6}$	0	-	+	+	+	+
	3	$2{,}26 \times 10^{6}$	0	-	+	+	+	0
	4	$2{,}26 \times 10^{6}$	0	0	+	+	+	0
	5	$2{,}06 \times 10^{6}$	0	0	+	0	+	+
	6	$4{,}11 \times 10^{6}$	+	-	+	+	+	0
	7	$2{,}06 \times 10^{6}$	+	-	+	+	+	+
	8	$8{,}32 \times 10^{2}$	+	-	+	0	+	+
	9	$1{,}39 \times 10^{4}$	0	-	0	0	+	+
	10	$4{,}16 \times 10^{2}$	0	-	+	0	+	0
	11	$6{,}73 \times 10^{3}$	0	-	0	0	+	0
	12	$6{,}73 \times 10^{3}$	0	0	0	+	+	0
	13	$6{,}73 \times 10^{3}$	+	0	0	+	+	0
	14	$6{,}73 \times 10^{3}$	+	0	0	0	+	+
	15	$6{,}73 \times 10^{3}$	0	0	0	+	+	+
	16	$6{,}73 \times 10^{3}$	0	0	0	0	+	+
***Vibrio* sp.**	1	$2{,}16 \times 10^{3}$	0	0	+	0	+	+
	2	$2{,}16 \times 10^{3}$	0	0	0	+	+	+
	3	$2{,}16 \times 10^{3}$	+	0	0	+	+	+
	4	$2{,}16 \times 10^{3}$	0	0	0	0	+	0
	5	$2{,}16 \times 10^{3}$	+	0	+	0	+	0
	6	$2{,}16 \times 10^{3}$	0	-	+	0	+	0
	7	$2{,}16 \times 10^{3}$	0	0	+	0	+	0
	8	$2{,}16 \times 10^{3}$	0	-	0	0	+	+
	9	$2{,}16 \times 10^{3}$	+	0	+	+	+	0
	10	$2{,}16 \times 10^{3}$	0	0	0	0	+	+
Not Identifiable	5	$6{,}72 \times 10^{5}$	0	-	+	0	+	0
% positive		**$2{,}87 \times 10^{7}$**	**32,3**	**31,5**	**97,4**	**62,1**	**100**	**46,8**

Zucchini Raven with NPs								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
Acetinobacter baumannii	5	$3{,}22 \times 10^{4}$	0	0	+	0	+	0
	6	$1{,}28 \times 10^{2}$	0	0	+	0	+	+
***Enterobacter* sp.**	9	$3{,}22 \times 10^{4}$	0	-	0	0	+	+
***Pseudomonas* sp.**	1	$5{,}70 \times 10^{2}$	0	0	+	0	+	+
***Vibrio* sp.**	1	$7{,}15 \times 10^{4}$	0	0	+	0	+	+
	4	$3{,}22 \times 10^{4}$	0	0	0	0	+	0
	5	$3{,}63 \times 10^{4}$	+	0	+	0	+	0
	6	$7{,}06 \times 10^{3}$	0	-	+	0	+	0
	7	$7{,}70 \times 10^{4}$	0	0	+	0	+	0
	11	$3{,}53 \times 10^{3}$	0	-	+	0	+	+
	12	$1{,}28 \times 10^{2}$	+	-	0	0	0	0
	13	$1{,}28 \times 10^{2}$	0	0	+	+	+	+
Not Identifiable	6	$6{,}44 \times 10^{4}$	0	0	+	0	+	+
	7	$1{,}52 \times 10^{3}$	0	0	+	0	+	+
% positive		**$3{,}61 \times 10^{5}$**	**10,1**	**87,9**	**82,1**	**0,04**	**99,96**	**48,6**

Zucchini Raven with DDE and NPs								
Species	**Subspecies**	**cfu g^{-1}**	**Test DDE**	**test NPs**	**IAA**	**OA**	**Siderophores**	**ACC**
***Pseudomonas* sp.**	1	$3{,}72 \times 10^{3}$	0	0	+	0	+	+
	2	$1{,}46 \times 10^{3}$	0	0	0	+	+	0
	4	1514	0	0	+	+	+	+
	5	$1{,}46 \times 10^{3}$	0	0	+	+	+	0
	6	$1{,}13 \times 10^{3}$	0	-	+	0	+	+
	8	$1{,}06 \times 10^{2}$	0	-	+	+	+	0
	13	$1{,}18 \times 10^{3}$	0	-	+	0	+	0
	15	$5{,}30 \times 10^{1}$	+	0	+	0	+	0
	17	$2{,}26 \times 10^{3}$	0	0	+	0	+	0
	18	$2{,}26 \times 10^{3}$	+	0	0	0	+	0
	19	$1{,}13 \times 10^{3}$	0	0	0	0	+	+
	20	$1{,}46 \times 10^{3}$	+	0	0	0	+	+
	21	$1{,}46 \times 10^{3}$	0	0	0	0	0	0
	22	$1{,}46 \times 10^{3}$	+	-	0	+	+	0
	23	$1{,}46 \times 10^{3}$	+	0	+	+	+	0
	24	$1{,}06 \times 10^{2}$	+	0	+	+	+	+
	25	$5{,}30 \times 10^{1}$	+	-	0	0	+	+
***Enterobacter* sp.**	1	$4{,}60 \times 10^{1}$	0	0	+	+	+	+
	5	$4{,}60 \times 10^{1}$	0	0	+	0	+	+
	8	$4{,}60 \times 10^{1}$	+	-	+	0	+	+
	17	$4{,}60 \times 10^{1}$	0	-	+	0	+	+
	18	$4{,}60 \times 10^{1}$	0	0	+	0	+	0
***Vibrio* sp.**	1	$4{,}60 \times 10^{1}$	0	0	+	0	+	+
	5	$4{,}60 \times 10^{1}$	+	0	+	0	+	0
	7	$9{,}20 \times 10^{1}$	0	0	+	0	+	0
	13	$1{,}50 \times 10^{3}$	0	0	+	+	+	+
	14	$2{,}76 \times 10^{2}$	0	-	+	+	+	+
	15	$2{,}76 \times 10^{2}$	+	-	+	+	+	+
	16	$1{,}41 \times 10^{3}$	+	-	+	0	+	+
	17	$4{,}60 \times 10^{1}$	0	-	+	+	+	0
	18	$4{,}60 \times 10^{1}$	0	0	0	0	+	0
% positive		**$2{,}63 \times 10^{4}$**	**32,9**	**77,0**	**64,5**	**37,0**	**94,4**	**48,8**

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Optimization of DDE-phytoremediation by exploiting plant-associated bacteria and nanoparticles

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2012**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Eevers, Nele

Datum: **11/06/2012**