2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Optimizing the efficiency of endophytic bacteria-enhanced phytoremediation of DDE-contaminated soils with Cu-NPs

Promotor :
dr. ir. Nele WEYENS

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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List of abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ANOSIM	Analysis of Similarity
ARDRA	Amplified rDNA Restriction Analysis
BCF	Bioconcentration factor
bp	Base pairs
CAES	Connecticut Agricultural Experiment Station
CAS	Chromium-azurol S
Cfu g^{-1}	Colony-forming units per gram
Cu-NPs	Copper nanoparticles
DDD	1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
DDE	2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene
DDMU	1-chloro-4-[2-chloro-1-(4-chlorophenyl)ethenyl]benzene
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
GC-MS	Gas Chromatography-Mass Spectrometry
HiFi	High Fidelity
IAA	Indole-3-acetic acid
ICP	Inductively Coupled Plasma Analysis
MID	Multiplex Identifier
NBRIP	National Botanical Research Institute's phosphate
OA	Organic acids
OTUs	Operational Taxonomic Units
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
POPs	Persistent organic pollutants
Rpm	Revolutions per minute
SID	Siderophores
SIMPER	Similarity Percentages
SMN	Salts Minimal
ST	Sucrose Tryptone

Foreword

This thesis is written as a completion to my master in Clinical Molecular Sciences at Hasselt University. A lot of people made it possible for me to write this thesis. Therefore, I would like to thank everyone for their support, effort and confidence.

First of all, I would like to express my appreciation and gratitude towards my first supervisor dr. ir. Nele Weyens, for making it possible for a 'Clinical student' to do an internship at the Centre for Environmental Sciences. I would like to thank her for her confidence in my abilities and always making time to answer my questions. I would also like to express my deep gratitude towards my second supervisor Nele Eevers, to initiate me into the wonderful world of (bright pink) bacteria, for understanding me when I was super-enthusiastic about 'my big plants' and 'my bacteria' but most of all, for her enthusiastic encouragements and useful critiques of this thesis. Without her help, performing the experiments and writing of this thesis would have never been possible, nor would it have been pleasant. My grateful thanks are also extended to all the other (Phd-) students for their help and knowledge in and around the lab and Carine for our nice talks and her useful advices.

My gratitude also extends to the people at the Department of Analytical Chemistry of the Connecticut Agricultural Experiment Station (CAES) for their advice concerning my experiments, for sharing data and for performing experiments necessary for my thesis.

Last but not least, I would like to thank my family and friends for their enthusiasm when I was talking about my plants and bacteria and their genuine interest in what I did the last eight months. I would especially like to thank my friends Charlotte Cosemans and Sanne Verspeek for their support, knowledge and the attention they have given me, and Charlotte, thank you for trying to fix lunch dates during our busy schedules, luckily we managed to lunch together at least five times.

Summary

Background DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) is a pesticide developed in 1939 that had a broad range of agricultural and non-agricultural applications worldwide. For many years it was used to control insects on agricultural crops and insects that carry diseases like malaria and typhus. However, the use of it was banned in the seventies due to its deleterious effects on animal and human health. DDT and its main metabolite, DDE (2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene), are persistent organic pollutants (POPs), environmental contaminants that, to a varying degree, resist photolytic, biological and chemical degradation. This leads to widespread and persistent contamination of soils worldwide.

A possible strategy for the remediation of soils contaminated with DDE, is phytoremediation. This technology uses the natural ability of plants and their associated microorganisms to remove, inactivate, or degrade harmful contaminants in soils and sediments. *Cucurbita pepo* ssp. *pepo* (zucchini) has proven to accumulate high levels of DDE and other POPs and has been proposed as a phytoremediation tool for contaminated soils. Previous research indicates that phytoremediation efficiency of DDE by *C. pepo* can be enhanced by endophytic bacteria. Manufactured copper nanoparticles (Cu-NPs) are often added to improve the uptake of pesticides by plants. Therefore, it would also be plausible to apply Cu-NPs to enhance the phytoremediation efficiency. This research exploits the prospects of using endophytic bacteria combined with Cu-NPs, hypothesizing that their combined use can further optimize the endophyte-enhanced phytoremediation of DDE-contaminated soils with *C. pepo*. Therefore, the possible toxicity of Cu-NPs and subsequently, the combined effect of DDE and Cu-NPs on the endophytic community of *C. pepo* was examined. All isolated bacteria were subjected to (1) genotypic identification, (2) characterization of their plant growth-promoting capacity and (3) screening of their DDE-degrading capacity. The total endophytic community was identified using 454 pyrosequencing.

Results Cu-NPs did not have any deleterious effects on plant growth nor any significant effect on the endophytic community. Several bacterial strains, such as *Stenotrophomonas* and *Enterobacter*, showed plant growth-promoting as well as DDE-degrading capacity.

Conclusion Our findings suggest tolerance of different bacterial strains, especially *Stenotrophomonas* and *Enterobacter*, to Cu-NPs combined with DDE. This might suggest that both genera have a selective advantage when residing in DDE-contaminated environments and were therefore able to dominate the endophytic community. Further investigations with specific endophytes displaying plant-growth promoting properties and DDE-degrading capacity will give an indication whether these findings can be used to further optimize the endophyte-enhanced phytoremediation of DDE-contaminated soils with *C. pepo*.

Samenvatting

Introductie DDT (1,1,1-trichloor-2,2-di(4-chlorofenyl)ethaan) is een pesticide ontwikkeld in 1939 en wereldwijd gebruikt voor (non-)agrarische doeleinden. Het werd vele jaren ingezet om gewassen te beschermen tegen insecten die ziektes, zoals malaria en tyfus, overbrengen. Het gebruik van DDT werd in de jaren zeventig verboden omwille van zijn schadelijk effect op de gezondheid van mens en dier. DDT en zijn belangrijkste metaboliet, DDE (2,2-bis(p-chloorfenyl)-1,1-dichloor-ethyleen), beide geclassificeerd als persistente organische polluenten (POPs), zijn verontreinigende stoffen die persistent zijn in het milieu. Dit heeft er toe geleid dat wereldwijd DDT en DDE nog steeds in de bodem aanwezig zijn en dus een impact hebben op zowel mens als dier.

Een mogelijke strategie om DDE-verontreinigde bodems te saneren, is door gebruik te maken van fytoremediatie, een technologie die gebruik maakt van de natuurlijke capaciteit van planten en de daarmee geassocieerde micro-organismen om schadelijke stoffen uit de bodem te verwijderen, te inactiveren of af te breken. Onderzoek heeft aangetoond dat *Cucurbita pepo* ssp. *pepo* (de courgetteplant) in staat is om hoge concentraties DDE en andere POPs uit de bodem te accumuleren. Bijkomend onderzoek toonde aan dat de efficiëntie waarmee *C. pepo* DDE uit de bodem accumuleert, verbeterd kan worden door gebruik te maken van endofyten. Koper nanopartikels (Cu-NPs) worden vaak toegevoegd aan pesticiden om de opname ervan te verhogen. Het is dus een logische denkwijze dat Cu-NPs, net zoals endofyten, het fytoremediatie-proces kunnen verbeteren. De hypothese van deze studie is dat de combinatie van Cu-NPs en endofyten de fytoremediatie van DDE door *C. pepo* nog verder zal optimaliseren. Eerst werd de mogelijke toxiciteit van Cu-NPs op de endofytische gemeenschap van *C. pepo* onderzocht. Daarna werd het effect van de combinatie van DDE en Cu-NPs op de gemeenschap getest. Alle geïsoleerde bacteriële stammen werden onderworpen aan (1) een genotypische screening, (2) een screening van plantengroei-stimulerende activiteit en (3) een screening van het vermogen tot DDE-degradatie. De totale gemeenschap werd geïdentificeerd door 454 pyrosequencing.

Resultaten Cu-NPs hadden geen negatief effect op plantengroei of op de endofytische gemeenschap. Enkele stammen, zoals *Stenotrophomonas* en *Enterobacter*, vertoonden plantengroei stimulerende eigenschappen en het vermogen om DDE af te breken.

Conclusie Onze bevindingen suggereren tolerantie van bacteriële stammen, zoals *Stenotrophomonas* en *Enterobacter,* voor de combinatie van DDE met Cu-NPs. Beide genera hebben mogelijks een selectief voordeel wanneer ze in een DDE-gecontamineerde omgeving groeien waardoor ze de endofytische gemeenschap kunnen domineren. Verder onderzoek met endofyten die plantengroei stimuleren én DDE kunnen afbreken is noodzakelijk om een indicatie te geven of deze stammen al dan niet gebruikt kunnen worden om de efficiëntie van de fytoremediatie van DDE met *C. pepo* nog verder te verhogen.

1 Introduction

1.1 DDE-contamination

DDT (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane) is an organochlorine pesticide developed in 1939 that had a broad range of agricultural and non-agricultural applications worldwide (1). It has been very popular due to its effectiveness, long residual persistence and low cost. For many years it was used to control insects on agricultural crops and insects that carry diseases like malaria and typhus (1, 2). In 1962, Rachel Carson published 'Silent Spring', a book documenting the detrimental effects on the environment of the indiscriminate use of pesticides, with DDT as a prime example. Eventually, this led to a ban of the use of DDT since the seventies, because of its threat for animal and human health, its toxicity for forest wildlife and its hormone disrupting properties. The use of DDT was banned in the United States in 1972 and in Belgium in 1974. Despite the international treaty that banned most uses of DDT and other organochlorine pesticides, the 2001 Stockholm Convention on Persistent Organic Pollutants included an exemption for the use of DDT for vector-borne diseases, such as malaria, until effective and affordable alternatives are available (3).

Before 1972 when it was banned, DDT entered the air, water, and soil during its production and use as an insecticide. When DDT is exposed to weathering in the environment, it aerobically transforms to DDE (2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene), the most persistent and most frequently encountered degradation product of DDT in soils worldwide. Under anoxic conditions, DDT transforms to DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane), a less common metabolite (2, 4) (Figure 1).

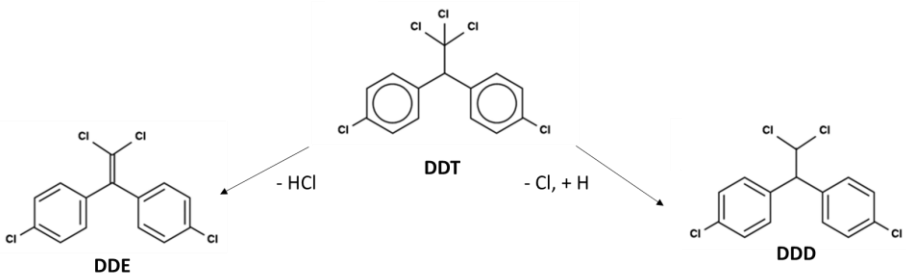

Figure 1: *Molecular structure of p,p'-DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane and its primary metabolites, p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene) and p,p'-DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane). Technical DDT is a mixture of various isomers of which p,p'-DDT is the most prevalent (65–80%).*

DDT, DDE and DDD are manufactured chemicals and are not known to occur naturally in the environment. DDT, DDE and DDD are all classified as persistent organic pollutants (POPs); environmental contaminants that, to a varying degree, resist photolytic, biological and chemical degradation (5, 6). POPs include many of the first generation insecticides such as dieldrin, DDT, toxaphene and chlordane, and several industrial chemical products or byproducts including polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (dioxins) and dibenzo-p-furans (furans). These

chemicals are highly hydrophobic. In addition, they are highly lipophilic, which is expressed as the octanol–water partition coefficient (K_{ow}). Their high log(K_{ow})-value causes them to strongly bind to organic matter in the soil. POPs are characterized by long half-lives in the environment and due to their hydrophobicity, they display the potential for bioaccumulation (increasing concentration of a chemical in an organism which exceeds that in its environment) in the lipids of exposed organisms resulting in the potential biomagnification (increasing concentration of a chemical in an organism as a function of trophic level) of DDT and its related metabolites in the environment.

Thus, although the use of DDT has been banned since the seventies, concentrations of DDT and its metabolites are still present in the soil worldwide having a potential impact on human health and the environment (1). Although little is known about its long-term effects on the human nervous system, at high doses, DDT is considered a potent neurotoxin. Numerous studies including laboratory animals experiments and *in vitro* assays, have established that at low doses it can act as an endocrine disruptor (7). The primary metabolite of DDT, DDE, is far more persistent than the parent compound and thus still found in the environment at low levels. For all these reasons, the removal of DDT and its residues from the soil is a priority.

1.2 Soil remediation

Several technologies and methods have been developed to remove contaminants from the soil. To remove a contaminant, either the soil is removed or the soil is remediated by using chlorine as a sanitation agent or by using antimicrobial washing solutions such as O_3 and UV–C radiation (8). Another method is to reduce the risk posed by the contaminant by reducing exposure, which can be achieved by decreasing contaminant bioavailability using a combination of plants and soil amendments. These techniques are rapid but are costly from both an economic and an environmental point of view. They are labor-intensive and have a deleterious impact on the soils physical, chemical, and biological properties (9).

Another possible strategy for the remediation of soils contaminated with DDE, is phytoremediation. This technology uses the natural ability of plants and their associated microorganisms to remove, inactivate, or degrade harmful contaminants in soils and sediments. When taking up nutrients from the soil, plants unintentionally take up contaminants as well, thereby reducing their presence in the soil. Phytoremediation offers a promising biological alternative for the more expensive sanitation techniques that put a high burden on the environment (8). Phytoremediation is a novel, cost-effective, eco-friendly, *in situ* applicable and solar powered remediation strategy that requires minimal site disturbance and maintenance, resulting in a low cost and a high public acceptance. It is a technology that can be used to clean-up and/or stabilize inorganic as well as organic contaminants (6, 9, 10).

Phytoremediation has already been successfully applied to remediate several organic contaminants, such as trichloroethylene (TCE) and aromatic hydrocarbons (10, 11). Research has been performed on the possible use of phytoremediation for DDE-contaminated soils. Several plants, such as *Sinapis hirta* (mustard), *Brassica napus* (canola), *Cucurbita pepo* ssp. *pepo* (zucchini) and *Arachis hypogaea* (peanut), show the capacity to accumulate DDE (12). *C. pepo* proved to phytoextract the highest levels of DDE from the soil (with a root bioconcentration factor (BCF) of 13) compared to the other accumulators mustard, canola and peanut (with root BCFs of 0.71, 0.70 and 1.3 respectively) (12).

However, the efficiency of phytoremediation as an environmental remediation technology depends on several factors, such as the extent of soil contamination, the availability and accessibility of contaminants for rhizosphere microorganisms and uptake into roots (bioavailability), and the ability of the plant and its associated microorganisms to intercept, adsorb, accumulate, or degrade the contaminants. Plants suitable for phytoremediation have to be able to grow in a polluted environment, but in general, the presence of organic pollutants in soil can reduce plant development and eventually phytoremediation efficiency (13, 14). A promising solution could be to exploit the plant-bacteria partnership, in which plants are used in combination with pollutant-degrading, plant growth-promoting microorganisms for the clean-up of polluted soil (14, 15).

1.3 Plant-associated bacteria

Plant-associated bacteria include endosphere, phyllosphere and rhizosphere bacteria (Figure 2). Endophytic bacteria are those that colonize the internal tissue of the plant, such as leaf, root and vascular tissue, without causing disease symptoms to their host. They are numerously present and reside in a latent state or actively colonize plant tissues (16). Endophytic bacteria can proliferate inside the plant tissue, thus are likely to interact closely with their host, face less competition for nutrients, and are more protected from adverse changes in the environment than bacteria in the rhizosphere, the below-ground microbial habitat, and the phyllosphere, the total above-ground portions of plants as habitat for microorganisms (14, 15, 17).

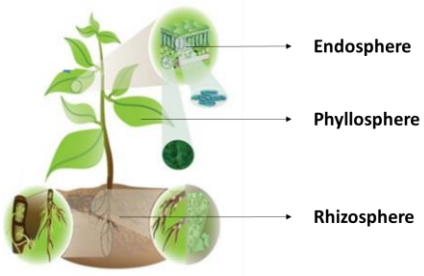

Figure 2: Plant-associated bacterial communities. *Schematic overview of the major sources for bacteria that compose the plant-associated community: the endosphere, the phyllosphere and the rhizosphere.*

1.4 Plant–endophyte partnerships for the remediation of contaminated soil

Endophytes can originate from the phyllosphere by entering through natural openings such as stomata, the rhizosphere and the soil environment. With the exception of seed-transmitted bacteria, which are already present in the embryo, the root is the primary site where endophytes gain entry into plants. Root hair cells, sites of epidermal damage and epidermal conjunctions are the main entry points on roots (14, 18). Moreover, cell wall-degrading enzymes facilitate the penetration of such bacteria into plants and plant exudates leaking through wounds are a nutrients source for the colonizing bacteria and hence create favorable conditions. Once inside the plant, endophytic bacteria either remain localized in a specific plant tissue or colonize the plant systematically by transport through the vascular system or the apoplast (14, 15, 18).

Endophytes have a variety of interactions with the plants, ranging from being active pathogens, opportunist pathogens, bacteria that live within the plant and gain some physical protection, to bacteria that actively interact with their host plant for the improvement of both. Moreover, many endophytic bacteria, particularly those inhabiting plants growing in a polluted environment, produce degradation enzymes and contribute to the degradation of several types of organic compounds present in the rhizosphere and endosphere (14, 16, 19).

Previous research has proven that the efficiency of phytoremediation can be improved by further exploiting endophytes (10). On the one hand, plants provide the habitat as well as nutrients to their associated endophytic bacteria. On the other hand, endophytic bacteria with appropriate degradation pathways and metabolic activities enhance degradation of organic pollutants. In addition, endophytic bacteria can possess plant growth-promoting activities which enhance the plant's adaptation and growth in soil contaminated with pollutants (14). At Hasselt University, the research group of Environmental Biology was able to isolate and identify three important bacterial strains, *Enterobacter aerogenes, Sphingomonas taxi,* and *Methylobacterium radiotolerans*, that live within *Cucurbita pepo* ssp. *pepo* (20-22). These bacteria display characteristics promising for DDE-phytoremediation, such as the presence of genes coding for plant growth–promoting capacities, which enables them to enhance the growth and development of the plants in contaminated areas. But, most importantly, these bacteria display the ability to degrade DDE. The combination of these traits makes them promising strains for the phytoremediation of soils contaminated with DDE.

1.4.1 DDE-degradation

DDT is reductively dechlorinated to DDD and dehydrochlorinated to DDE. Both metabolites may undergo further transformation but the extent and rate are dependent on soil conditions and microbial populations present in soil (1, 23). Endophytic bacteria can possess genes coding for enzymes that can

contribute to the degradation of organic compounds, such as DDE. During the phytoremediation of DDE, endophytic bacteria can produce dehalogenases, dioxygenases, and hydrolases, enzymes associated with DDE-degradation. At Hasselt University, they identified three bacterial strains of which the draft genome revealed the presence of these enzymes (20-22). Although little is known about the microbial metabolism of DDE, several aerobic degradation pathways of DDE have been proposed, but none has been fully verified (1, 4). Co-metabolism, a process in which the microbes derive nutrients from sources other than the compound of concern, of DDE by *Pseudomonas sp.*(24) and *Terrabacter sp.* (25) grown on biphenyl has been reported. However, not all bacterial species able to produce biphenyl dioxygenase were able to degrade DDE. Recent laboratory experiments in marine sediment showed that DDE is dechlorinated to DDMU (1-chloro-4-[2-chloro-1-(4-chlorophenyl)ethenyl]benzene) under methanogenic or sulfidogenic conditions (23). The only pure culture reported to degrade DDE under anaerobic conditions was the denitrifier *Alcaligens denitrificans* (1, 14).

1.4.2 Plant growth promotion

There is a close relationship between rhizosphere, endophytic and phyllosphere bacteria suggesting that they all use similar mechanisms to benefit their host plant. Mechanisms of plant growth-promotion by plant-associated bacteria vary greatly, and have been broadly categorized into two groups, direct and indirect plant growth promotion. Direct plant growth-promoting mechanisms may involve the supply of unavailable nutrients such as phosphorus and iron, the synthesis of siderophores which can sequester iron from the soil and provide it to plant cells, the production of organic acids (OA) which can solubilize inorganic phosphorus, the production of plant growth regulators such as the auxin indole-3-acetic acid (IAA), and the suppression of ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. A bacterial strain may directly affect plant growth and development using any of these mechanisms. Plant-associated bacteria can indirectly benefit the plant growth by preventing the growth or activity of plant pathogens through antibiotic production, competition for space and nutrients, production of fungal cell wall lysing enzymes and biosurfactants (15, 18, 26). Since many plant growth-promoting bacteria possess several of these traits, an endophyte may utilize different traits at various times during the life cycle of the plant.

A. Direct plant growth promotion

Solubilization of unavailable nutrients

Phosphorus (P) is an important plant macronutrient, forming a component of key molecules such as nucleic acids, phospholipids, and ATP. Therefore, plants cannot grow without a reliable supply of this nutrient. Although phosphorus is abundantly present in the soil, it is often present in unavailable forms or in forms that are only available outside the rhizosphere. Only a very small fraction (~0.1%) is available to plants. Plants can only take up phosphorus when it is in its monobasic ($H_2PO_4^-$) or dibasic

(HPO_4^{2-}) soluble form. Microbial solubilization of inorganic phosphorus has been attributed mainly to the production of OA. In certain plant species, root clusters are formed in response to a limited availability of phosphorus. These root clusters excrete OA, which acidify the soil and chelate metal ions around the roots, resulting in the mobilization of phosphorus and some micronutrients (27, 28). P-solubilizing and P-mineralizing bacteria that can enhance plant growth are common in the rhizosphere, where it is most important, but endophytic bacteria have also been reported to solubilize immobilized mineral phosphorus (15, 28, 29).

Iron (Fe) is, like phosphorous, one of the essential elements for a proper plant development and often present in the environment in its highly insoluble ferric hydroxide form. Since it is a cofactor of many metabolic pathways, iron deficiency may lead to the disruption of many processes including respiration and photosynthesis. Iron can exist in aqueous solution in two states: Fe^{2+} and Fe^{3+}. However, Fe^{3+}-forms are not readily utilizable by plants and microbes. Many bacteria produce organic compounds, called siderophores, capable of chelating iron with high affinity and in a reversible manner. Siderophores bind Fe^{3+} and make it available for conversion to the preferred form, Fe^{2+}. Bacterial Fe^{3+}-siderophore complexes may facilitate uptake of iron not only into bacteria, but into plants as well. (15, 30).

Phytohormones and Plant Growth-Promoting Compounds

The phytohormone IAA belongs to the group of auxins and is the most thoroughly studied plant growth regulator. IAA directs several aspects of plant growth and development, e.g. cell division, root extension, vascularization, apical dominance, and tropisms. Enhanced rooting leads to improved mineral and nutrient uptake and root exudation that subsequently stimulates bacterial proliferation on the roots (15, 31, 32).

The phytohormone ethylene, which is found in all higher plants, is an important modulator of normal plant growth and development as well as a key feature in the response of plants to a wide range of stresses. During periods of environmental stress, plants produce high levels of ethylene which affects many aspects of the growth of plant tissues such as roots, stems, leaves, flowers and fruits. The most commonly observed mechanism to reduce ethylene production levels is by bacterial ACC-deaminase activity. ACC-deaminase cleaves the ethylene precursor ACC, thereby lowering the level of ethylene which leads to some protection against the inhibitory effects of various stresses (15, 33).

B. Indirect plant growth promotion

Bacterial endophytes and plant pathogens colonize a similar ecological niche. By competing with pathogens for space and nutrients and thus suppressing the growth or activity of these pathogens, endophytes can potentially benefit plant growth indirectly. Many plant growth-promoting bacteria, particularly Pseudomonads, produce high-affinity Fe^{3+}-binding siderophores under iron-limiting

conditions. By binding available iron, these bacteria deprive pathogenic bacteria and fungi of iron, which could limit their growth (15).

In addition to competition, antibiosis, which is the production and release of molecules that either kill target pathogens or inhibit their growth, is the best-known mechanism by which microbes can control plant diseases. Furthermore, some plant-associated bacteria produce hydrolytic enzymes that cause cell wall lysis, which can be used to control fungal pathogens. Biosurfactants are also being investigated as antimicrobial compounds. As pathogens frequently form a biofilm on the root surface, it is interesting to note that some biosurfactants prevent biofilm formation and even degrade existing biofilms (15).

The combined use of plants and endophytic bacteria is an emerging approach for the clean-up of soil polluted with organic compounds. Plant-endophyte interactions are often considered mutualistic, the microorganisms gain nutrients and a protected niche to occupy, whereas the host benefits from bacterial activities. During the phytoremediation of organic pollutants, endophytic bacteria produce different enzymes to mineralize organic pollutants and decrease both the phytotoxicity and evapotranspiration of volatile organic pollutants. Furthermore, endophytic bacteria improve the plants adaptation and growth by the virtue of their plant growth-promoting activities, and consequently improve phytoremediation activity (10, 14, 15, 34, 35).

1.5 Nanoparticles

Nanomaterials, particles with dimensions less than 5 nm, are gaining interest for a wide range of engineering and technical agricultural applications due to their physicochemical properties. They exhibit unique magnetic and optical properties, electronic states, and catalytic reactivity that differ from corresponding bulk materials. Nanoparticles (NPs) have, for example, a larger surface-to-volume ratio, which provides them with a higher reactivity due to a greater proportion of atoms on the surface relative to the interior of the structure (36-39). Naturally occurring NPs can be found in volcanic ash, ocean spray, fine sand and dust, and biological matter (e.g. viruses). Manufactured NPs enter the environment through atmospheric emissions, wastewater and agriculture, where they are added to improve the uptake of pesticides and herbicides by plants (39). Manmade NPs are categorized as incidental or engineered. With the former being more variable in size, shape and composition of material and the latter being more well-defined, designed to have specific surface characteristics (40).

The interest of this study is directed towards copper nanoparticles (Cu-NPs). In 1761, copper was used for the first time in agriculture when it was discovered that seed grains soaked in a weak solution of copper sulfate inhibit seed-borne fungi. Since then, Cu-compounds have been used widely in agriculture practices as fungicide, pesticide, algaecide, and herbicide (41, 42). Nanotechnology

enhances the antimicrobial activity of Cu by manipulating it to NPs. The improved antimicrobial activity of Cu-NPs is due to the unique properties of NPs as mentioned before (41).

Although exposure to Cu-NPs can result in statistically significant ($p<0.001$) decreases in plant biomass and transpiration volume relative to untreated control plants (40), zucchini seed germination was unaffected after exposure to Cu-NP (37). Nonetheless, Cu-NPs can be used as a novel antifungal agent in agriculture to control the plant pathogenic fungi (41). Preliminary data indicates that when Cu-NPs are added to the soil, *C. pepo* shows higher growth rates and an increased resistance to fungal infections (41). In addition, many modern pesticides utilize Cu-NPs to optimize the plants uptake of the products; therefore it would be plausible to apply Cu-NPs to enhance the uptake of contaminants such as DDE (37, 43).

1.6 Problem statement & hypothesis

Previous research indicates that phytoremediation efficiency of DDE by *C. pepo* can be enhanced by specific endophytic bacteria as well as by administering Cu-NPs to the plant. However, the combination of Cu-NPs and endophytic bacteria to even further enhance this efficiency has yet to be investigated. Furthermore, little is known about the toxicity of Cu-NPs to endophytic bacteria. This research exploits the prospects of using endophytic bacteria in combination with Cu-NPs to increase organic pollutant degradation in the soil and thus the efficiency of phytoremediation.

The hypothesis of this research is that the use of Cu-NPs can further optimize the endophyte-enhanced phytoremediation of DDE-contaminated soils with *C. pepo*. Therefore, two objectives were formulated. First, the possible toxicity of Cu-NPs on *C. pepo* and its endophytic community was evaluated. Subsequently, the combined effect of DDE and Cu-NPs on the endophytic community of *C. pepo* was evaluated by examining its genotypic and phenotypic characteristics and DDE-degrading capacity. By performing this research, the aim is to define the optimal concentration of Cu-NPs for efficient endophyte-enhanced phytoremediation of DDE-contaminated soils.

Since DDE-accumulation in the soil is a worldwide problem, having significant consequences for human health as well as for the environment, results from this project could serve as an important development in environmental biology. Phytoremediation offers a promising biological alternative for the more expensive and invasive sanitation techniques that are currently used. Phytoremediation has a low impact on the environment, is *in situ* applicable, and is solar-driven. It is suitable for application at very large field sites where other remediation methods are not cost-effective or practically feasible. In addition, phytoremediation has low installation and maintenance costs compared to other remediation options. It could lead to durable land management where phytoextraction gradually improves soil quality for subsequent cultivation of crops with higher market value.

2 Materials & methods

2.1 Growth of plants

Cucurbita pepo ssp. *pepo* Raven seeds, purchased from Johnny's Selected Seeds (Winslow, ME, USA), were germinated on wet paper towels and incubated for four days at 30°C. After germination, the seedlings were transferred per two to plastic pots, each pot containing approximately 36g of vermiculite. Four different concentrations of Cu-NPs (Copper Oxide Nanopowder, CuO, 99%, 40nm, US Research Nanomaterials, Inc., Twig Leaf Ln, Houston, USA), i.e. 100, 200, 300 and 400 µg g^{-1}, were added to the vermiculite in each pot. Three replicates were set up per condition. Six control pots were prepared without Cu-NPs. The plants were kept in a greenhouse for 20 days (humidity 60%; day night cycle: day 7.00–22.00; temperature: day 23°C, night 18°C; light intensity 300 W m^{-2}) prior to harvest and were watered every other day with ¼ Hoagland solution (Supplementary table 1).

2.2 Measuring DDE-content in plant tissues

In order to determine whether Cu-NPs enhance the uptake of DDE, the Department of Analytical Chemistry of the Connecticut Agricultural Experiment Station (CAES, New Haven, US) performed an experiment in which the uptake of DDE was determined. Plants were grown under exact the same circumstances as described earlier (2.1 Growth of plants). Six plants were grown and were watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. To three of the six plants, 150 µg g^{-1} Cu-NPs was added to the vermiculite. At day 20, plants were harvested and root and shoot samples (n=3 per condition) were separated. Samples were blended for 30 s before 50 mL of petroleum ether was added, after which the plant samples were further blended for 5 min. The extracts were filtered through a glass-wool lined funnel, the eluent was collected in a glass funnel with Teflon stopcock. The eluent was drained for 15 min and rinsed 3 times with distilled water and a saturated sodium sulfate solution. The petroleum ether was drained into a vial containing 10 g anhydrous sodium sulfate. One ml was used for cleanup on 4-mL Florisil cartridges that were preconditioned with 5 mL petroleum ether. The 1-mL extract was loaded on the cartridge, which was then eluted with 6 mL of 6% diethyl ether in petroleum ether. The extract was collected and reduced to 1 mL under nitrogen on a heating block at 35°C. These extracts were amended with 100 ng ml^{-1} o,p'-DDE as an internal standard before the DDE-concentrations were determined using Gas Chromatography-Mass Spectrometry (GCMS) as previously described by White (44). Unpaired t-tests were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA) in order to determine significant differences.

2.3 Defining the uptake of Cu-NPs

A. Sample preparation

The concentration of Cu-NPs in the plants was determined by performing an Inductively Coupled Plasma (ICP) analysis (Figure 4) (45), executed by the Department of Analytical Chemistry of the Connecticut Agricultural Experiment Station (CAES, New Haven, US). Prior to ICP, the 42 samples (5 control samples, 6 samples of plants exposed to 200 µg g^{-1} Cu-NPs, and 10 samples of plants exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE, roots and shoots separately, per pot) were freeze-dried at Hasselt University by storing them first at -45°C in 50 mL tubes. The frozen samples were placed in a lyofilisator for five days until the vacuum reached 10 µbar.

B. Statistical analysis

Before analyzing the data, significant outliers (α=0.05) were detected by performing a Grubbs' test. Values that were significant outliers, were removed from the data set. Unpaired t-tests were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA) to determine whether the uptake of Cu-NPs differed between the groups, i.e. roots and shoots exposed to 200 µg g^{-1} Cu-NPs (control group) and roots and shoots exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE.

2.4 Isolation of endophytes from plants exposed to Cu-NPs

A. Surface sterilization of plant tissue

After 20 days, the plants were harvested and rinsed with tap water to remove the vermiculite. Roots and shoots were separated and plant mass of each individual plant was determined in order to investigate the possible effect of Cu-NPs on plant growth. Unpaired t-tests (GraphPad Prism version 5.00) were performed for the different groups in order to determine whether there were significant differences in plant mass and consequently in plant growth. Subsequently, mixed samples were prepared from two plants originating from the same pot. Plant tissues were surface-sterilized (Figure 3) by washing them in 1% NaOCl. Afterwards, the plant tissues were rinsed three times in sterile dH_2O and dried on sterile filter paper. In order to check the sterility, 50 µL of the third batch of rinsing water of roots and shoots was transferred to plates containing 869 medium (46) (Supplementary table 2). The biomass of roots and shoots, used for bacterial isolation, was determined to calculate the number of colony-forming units per gram fresh plant material (Cfu g^{-1}).

B. Isolation of endophytes

Sterile plant tissues were crushed in sterile mortars containing 5 mL sterile 10 mM $MgSO_4$. The crushed plant material was used to make a serial dilution (0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) with 10 mM $MgSO_4$. Of each dilution, 100 µL was transferred to plates containing 1/10 diluted 869 medium (Figure 3). The

plates were incubated at 30°C for four days. After four days, the different types of colonies and the number of colonies were counted to determine the cfu g^{-1} for each dilution of each condition.

Bacterial colonies were purified by picking up the colonies of interest with sterile toothpicks and dissolving them in a drop of sterile 10 mM $MgSO_4$. This solution was transferred to plates containing 869 medium which were incubated at 30°C for four days.

After four days, individual colonies were picked up with sterile toothpicks and transferred to 15 mL tubes containing 5 mL of liquid 869 medium and incubated for two days (30°C, 120 rpm). Afterwards, the tubes were centrifuged at 3000 rpm for 10 min and the pellets of the isolated strains were re-suspended in 2 mL of 15%$_w$ glycerol (Attachment 2). The solutions were transferred to cryotubes and stored at -45°C.

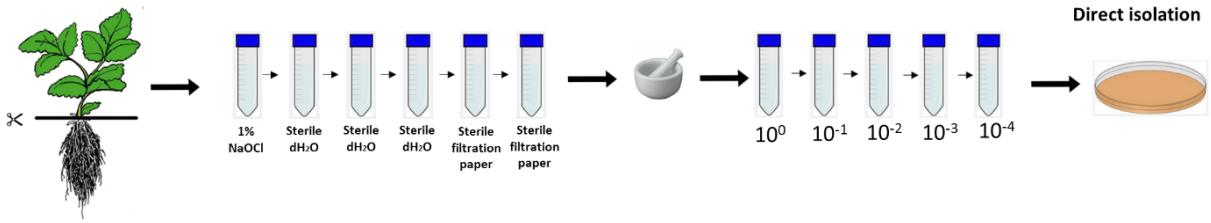

***Figure 3**: Workflow for the isolation of cultivable endophytes exposed to different concentrations of Cu-NPs, i.e. 100, 200, 300 and 400 µg g^{-1}. dH_2O:distilled water, Cu-NPs: Copper nanoparticles.*

2.5 Isolation of endophytes exposed to Cu-NPs and DDE

For the second part of the study, the same methods (Figure 4) as for the first part were used to grow the plants and to isolate and store the bacterial strains. Again seedlings were transferred per two to a pot and kept in a greenhouse for 20 days. This time, 20 plants (10 pots) were grown in the presence of 200 µg g^{-1} Cu-NPs and watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. The second group consisted out of 12 plants (6 pots) grown in the presence of 200 µg g^{-1} Cu-NPs and were watered every other day with ¼ Hoagland solution without DDE. Six control pots were prepared without Cu-NPs and were not exposed to DDE either.

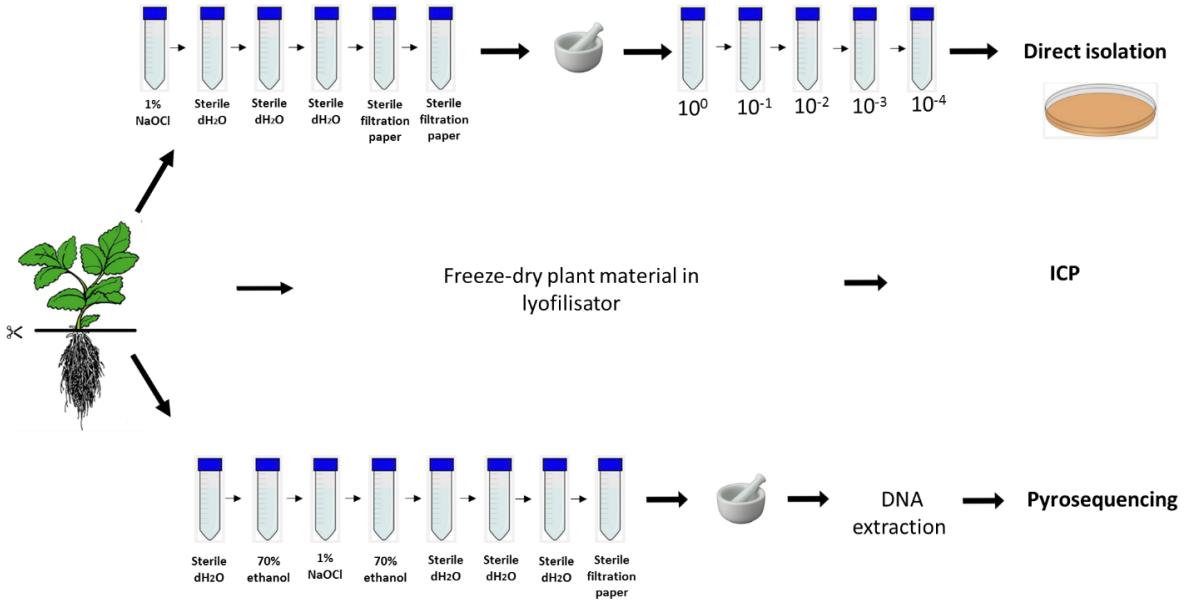

***Figure 4**: Workflow for (1) the isolation of cultivable endophytes, (2) the ICP-analysis of plants exposed to Cu-NPs (200 µg g^{-1}) whether or not in combination with DDE (100 µg L^{-1}) and for (3) the pyrosequencing process of the total endophytic community. dH_2O:distilled water, ICP: Inductively Coupled Plasma analysis.*

2.6 Genotypic characterization of cultivable bacterial community: ARDRA

A. Extraction, amplification and digestion of 16S rDNA

Bacteria (5 µL) were cultivated in rich (869) medium (1 mL) in sterile 96-well masterblocks for three days at 30°C. Genomic DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen Benelux N.V., Venlo, The Netherlands) following the protocol (Supplementary protocol 2) provided by the manufacturer. The quality and quantity of the DNA was checked using the Nanodrop ND-1000 Spectrophotometer (Isogen Life Sciences, Temse Belgium). The 16S DNA obtained from the bacterial cells was amplified in a PCR (Supplementary table 8, Supplementary table 9, Supplementary table 10).

PCR products of the 16S DNA were digested for 2h at 37°C in a digestion mastermix (Supplementary table 11). The digestion products obtained were separated on a 1.5% agarose gel (2h, 90V) and visualized with GelRed™ under UV light.

B. Data analysis

ARDRA-patterns were grouped, and strains with representative patterns were selected to be sent for 16S Sanger Sequencing to Macrogen (Seoul, South-Korea). These sequences were analyzed using Staden Package (©1998 Whitehead Institute for Biomedical Research) and then blasted against the NCBI Ribosomal Database Project to identify the bacterial colonies.

2.7 Screening of the total bacterial community: Pyrosequencing

A. Sampling and DNA extraction

Mixed samples of roots and shoots of each pot (ten pots exposed to 200 µg g^{-1} Cu-NPs in combination with 100 µg L^{-1} DDE and six pots exposed to 200 µg g^{-1} Cu-NPs) were used for the pyrosequencing process. Surface sterilization (Figure 4) was performed by sequentially washing them with sterile dH_2O (1 min), followed by immersion in 70 % ethanol (1 min), 1% NaOCl (1 min), 70% ethanol (1 min). Samples were rinsed three times in sterile dH_2O and dried on sterile filtration paper. To confirm sterility, 50 µL of the third batch of rinsing water of roots and shoots was transferred to plates containing 869 medium which were incubated for four days at 30°C. The samples were homogenized using a sterile pestle and mortar under liquid N_2. The DNA of the samples was extracted by using the Invisorb® Spin Plant Mini Kit following the protocol (Supplementary protocol 1) provided by the manufacturer (Stratec Biomedical AG, Birkenfeld, Germany). Quantity and quality of extracted DNA was determined using the Nanodrop ND-1000 Spectrophotometer (Isogen Life Sciences, Temse, Belgium).

B. Test PCR

A PCR (Supplementary table 3, Supplementary table 5) was conducted in order to test primer pair combinations (Table 1). The primer pairs were tested on six samples from roots and shoots, on one blanc and on one sample of bacterial DNA. The activity of the primer pairs was checked on a 1.5% agarose gel (2.5 h, 90 V) and visualized with GelRed™ under UV light.

Table 1: *Summary of primers used for screening of the total bacterial community.*

PCR	Primer Pair	Primer Sequence (5'-3')
Test PCR	341F	CCTACGGGNGGCWGCAG
	783abcR	CTACCAGGGTATCTAATCCTG
	783aR	*GTCCTAATCTATGGGACCATC*
	783bR	*GCCCTAATCTATGGGGCCATC*
	783cR	*GGCCTAATCTATGGGCCCATC*
	799F	AACMGGATTAGATACCCKG
	1391R	GACGGGCGGTGWGTRCA
	799F	AACMGGATTAGATACCCKG
	1193R	ACGTCATCCCCACCTTCC
Final PCR 1	799F	AACMGGATTAGATACCCKG
	1391R	GACGGGCGGTGWGTRCA
Final PCR 2	967F	CAACGCGAAGAACCTTACC
	1391F	GACGGGCGGTGWGTRCA

Primers are indicated as forward (F) or reverse (R). Primer 783abcR is a primer mix composed of a, b and c.

C. *Final PCR*

The 16s DNA obtained from the bacterial cells was amplified in a PCR (Supplementary table 4, Supplementary table 5). Samples were run on a 1.5% agarose gel (2.5h, 90V) to separate bacterial DNA from mitochondrial DNA, plasmids and plant DNA. The bands were visualized with GelRed™ under UV light and the bacterial amplicons were excised and extracted from the gel using the Qiaquick Gel extraction Kit (Qiagen Benelux N.V., Venlo, The Netherlands) following the protocol (Supplementary protocol 3) provided by the manufacturer. The obtained DNA was amplified in a second, nested PCR (Supplementary table 6, Supplementary table 7) with a second set of primers to add multiplex identifier tags (MIDs). The PCR products were purified using the Qiaquick PCR Purification kit (Qiagen Benelux N.V., Venlo, The Netherlands). Purified amplicon libraries were quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Both steps were performed following the protocol (Supplementary protocol 4 and 5) provided by the manufacturer. After performing the Quant-iT PicoGreen dsDNA reagent assay, DNA concentrations were determined using the FLUOstar Omega Microplate Reader (BMG LABTECH, Isogen Life Science, The Netherlands). All 32 samples were pooled in equimolar concentrations (5.06 10^{10} ng μL^{-1}) by diluting each sample in deionized water. The resulting 2 barcoded amplicon pools, each of them containing 16 samples, were each sequenced on 1/8 of a Pico Titer Plate by Macrogen (Seoul, South Korea).

D. *Data analysis*

The raw data from the 454 pyrosequencing were delivered by Macrogen (Seoul, South-Korea) in FASTA-files which were accessed using Mothur bioinformatics software (47). The dataset was simplified by working with only the unique sequences and removing barcodes and primers. The remaining sequences were aligned and classified along the SILVA ribosomal RNA database (48). Sequences classified as chimeric, mitochondrial, chloroplast and 'unknown', i.e. sequences that could not be classified at the Kingdom level, were removed as well as archaeal and eukaryotic 16S/18S rRNAs. The remaining sequences were grouped into operational taxonomic units (OTUs) based on a 97% similarity criterion. The similarity between samples and their resemblance to the cultivated communities was visualized using Primer7 (Version 7.0.5, Primer-E Ltd.). Analysis of similarity (ANOSIM) with 999 permutations was performed to compare the variation in species abundance and composition among sampling units in terms of the different conditions. In addition, a Hierarchical Cluster analysis was performed based on the distance matrix calculated by using the Bray-Curtis distance matrix. The Non-metric Multi-Dimensional Scaling (nMDS) was based on the distance matrix provided by the Bray Curtis similarity as well, using the Kruskall stress formula 1 and minimum stress 0.01. Similarity percentages (SIMPER) were calculated to identify the species that are most important in creating the observed pattern of similarity using the Bray-Curtis measure of similarity.

Further statistical analysis of the pyrosequencing data was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA).

2.8 Phenotypic characterization of cultivable bacterial community

For the phenotypic characterization, 5 µL of each bacterial strain was cultivated in 1 mL liquid 869 medium for four days at 30°C in sterile 96-well masterblocks, except when mentioned otherwise. After four days, 20 µL of each bacterial suspension was added to a test-specific medium.

A. IAA

Five microliter of each bacterial strain was incubated in 1 mL IAA medium (Supplementary table 2) for five days (30°C, 120 rpm) in sterile 96-well masterblocks. Each masterblock was wrapped in aluminum foil to protect from light. After incubation, 1 mL Salkowski reagens (Attachment 2) was added to 500 µL of the bacterial suspension. The solution was vortexed for 3 min (300 rpm). After 20 min of incubation, the wells containing bacterial suspension that scored positive for IAA-production, turned pink. The ones that scored negative remained yellow (31).

B. Bacterial phosphate solubilization

NBRIP medium (Supplementary table 2) was poured into petri dishes. When solid, a hole was made in the medium with a sterile 10 mL pipette tip, to inoculate 50 µL of each bacterial suspension. Plates were incubated for twelve days at 30°C. Bacteria that displayed a solubilization zone around the colonies were considered positive for the test (49).

C. ACC Deaminase

After four days of growth, 96-well masterblocks were centrifuged for 20 min at 2000 rpm. Supernatans was discarded and 600 µL SMN medium (Supplementary table 2) was added to each pellet. After three days at 30°C, masterblocks were again centrifuged for 20 min at 2000 rpm. The supernatans were discarded and the pellets were re-suspended in 100 µL 0.1M Tris-HCl buffer (pH 8.5). Cells were disrupted by adding 3 µL toluene. To this cell suspension, 10 µL 0.5M ACC and 100 µL 0.1M Tris-HCl buffer (pH 8.5) was added. The solution was incubated for 30 min at 30°C at 150 rpm. 690 µL 0.56M HCl and 150 µL 0.2% 2.4-dinitrophenylydrazine in 2M HCl was added. After adding 1 mL 2M NaOH, a color change from yellow to brown was considered positive for ACC-deaminase activity. The ones that scored negative remained yellow (50).

D. Organic Acids

After four days of growth, 20 µL of the bacterial suspension was added to sterile 96-well masterblocks containing 800 µL ST-medium (Supplementary table 2). Masterblocks were incubated for six days at 30°C at 120 rpm. Afterwards, 100 µL of alzarine red S 0.1% was added and incubated for 15 min. The

wells containing bacteria that were considered positive, turned yellow, the ones that were considered negative turned pink (51).

E. Siderophores

After an incubation time of four days, 20 µL of each bacterial suspension was added to sterile 96-well masterblocks containing 800 µL 284 medium (Supplementary table 2) with 0 µM, 0.25 µM and 3 µM Fe(III)-citrate. The masterblocks were incubated for six days at 30°C at 200 rpm. After incubation, 100 µL of CAS (Attachment 2) was added. Four hours after adding CAS, the bacteria that were considered positive turned orange, the ones that were considered negative, remained blue (52).

F. Screening bacterial strains for potential DDE-degrading capacity in the presence of Cu-NPs

An auxanography (Figure 5) was performed for all bacterial strains to test their potential DDE-degrading capacity in the presence of Cu-NPs. Of each bacterial strain, 2 µL was cultivated in sterile 96-well masterblocks containing 1 mL 869 medium in each well. After four days, 1 µL of each bacterial suspension was diluted in 999 µL $MgSO_4$. A sterile 50 mg L^{-1} DDE solution was made by autoclaving the bottle and filter sterilizing the DDE twice. A sterile 200 µg g^{-1} Cu-NPs solution was made by diluting 6 mg of Cu-NPs in 30 mL sterile dH_2O and then autoclaving the solution.

First, 100 µL of the bacterial solution was spread on selective 284 medium. After a couple of minutes, 50 µL of the 50 mg L^{-1} DDE solution and 50 µL of the 200 µg g^{-1} Cu-NPs were added to the surface and smeared out on ¾ of the outer circle of the plate (Figure 5). The plates were kept in an incubator for six days at 30°C. Bacteria that grow on the entire plate are considered neutral and are tolerant for DDE and Cu-NPs. Bacteria that grow on the entire plate, except for the area covered with DDE and Cu-NPs, are considered negative. For these bacteria DDE combined with Cu-NPs is toxic. If the bacteria grow better in the DDE and Cu-NPs covered area, they are considered positive and are expected to use DDE as a carbon source, thus are possibly able to degrade DDE in the presence of Cu-NPs.

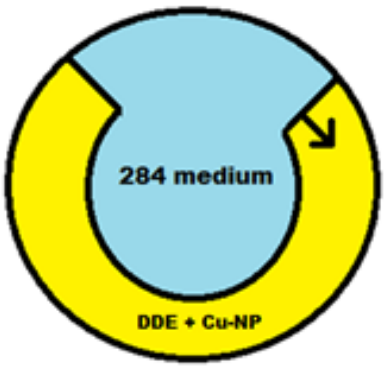

Figure 5: *Auxanography of bacteria grown on 284 medium in the presence of DDE and Cu-NPs. DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene, Cu-NPs: Copper nanoparticles.*

3 Results & Discussion

The combined use of plants and endophytic bacteria is an emerging approach for the clean-up of soil polluted with organic compounds such as DDE. The research group of Environmental Biology at Hasselt University has already proven that phytoremediation efficiency can be improved by exploiting the plant-endophyte relationship (10). They were able to isolate and identify three important bacterial strains that live within *Cucurbita pepo* ssp. *pepo* (20-22). These bacteria possess genes coding for plant growth–promoting capacities, which enable them to enhance the growth and development of the plants in contaminated areas. But, most importantly, these bacteria possess genes coding for enzymes reported to be involved in DDE-degradation. The combination of these traits makes them promising strains for the phytoremediation of soils contaminated with DDE and other contaminants. In addition to endophytes, the use of NPs to improve phytoremediation efficiency is an interesting field to be investigated. Manufactured NPs are often added to improve the uptake of pesticides and herbicides by plants. Therefore it might be interesting to apply NPs to enhance the uptake of DDE and thereby phytoremediation efficiency. The interest of this study is directed towards Cu-NPs. Cu-NPs can be used as a novel antifungal agent in agriculture to control infections with plant pathogenic fungi. In addition, Cu-NPs are able to enhance the growth of *Cucurbita pepo* ssp. *pepo* (41). Therefore, it would be plausible to apply Cu-NPs to contribute to the efficiency of the uptake of pesticides such as DDE.

Since little is known about the possible toxicity of Cu-NPs to endophytes, the first part of the research focused on examining the effect of Cu-NPs on both the endophytic community and *C*. *pepo*. The results of this part also provided an indication of the concentration of Cu-NPs that needs to be used in further experiments.

In the second part of the study (Figure 6), the combined effect of Cu-NPs and DDE on the endophytic community of *C. pepo* and the effect on the plant itself was investigated. The total bacterial community was investigated performing 454 pyrosequencing. The cultivable community was analyzed genotypically using ARDRA, and phenotypically by analyzing its plant growth promoting capacities. All isolated strains were also tested for their DDE-degrading capacity.

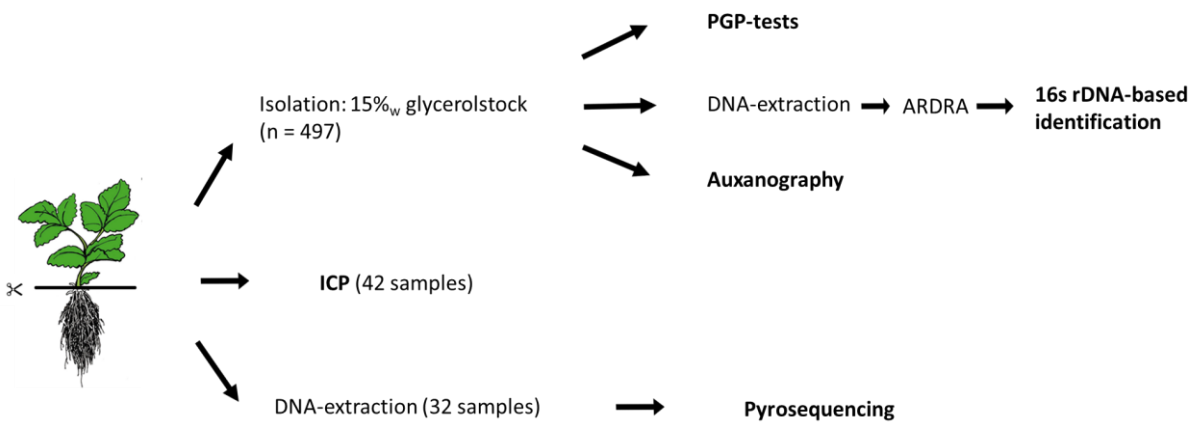

Figure 6: *Overview of the different tests performed on the control plants, plants exposed to different concentrations of Cu-NPs, i.e. 100, 200, 300 and 400 µg g^{-1}, and plants exposed to 200 µg g^{-1} Cu-NPs with or without 100 µg L^{-1} DDE. Pyrosequencing is only performed for plants exposed to 200 µg g^{-1} Cu-NPs and 200 µg g^{-1} Cu-NPs with 100 µg L^{-1} DDE. PGP-tests: Plant Growth Promotion tests, ARDRA: Amplified Ribosomal DNA Restriction Analysis, ICP: Inductively Coupled Plasma analysis*

3.1 DDE-content in plant tissues

The DDE-content in the plant tissues was determined for root and shoot samples of six plants, all exposed to 100 µg L^{-1} DDE and of which three were also exposed to 150 µg g^{-1} Cu-NPs. The average DDE-content ± standard error (Figure 7) in roots exposed to DDE was 5883.17 ng g^{-1} (±431.70 ng g^{-1}). A higher amount of DDE, an average of 7789.34 ng g^{-1} (±472.17 ng g^{-1}), was detected in plants exposed to DDE and Cu-NPs. The average DDE-content ± standard error (Figure 7) in shoots was 680.12 ng g^{-1} (±43.43 ng g^{-1}) for the three DDE-exposed plants, compared to an average of 873.72 ng g^{-1} (±63.94 ng g^{-1}) for the three plants exposed to Cu-NPs and DDE, being significantly more than when no Cu-NPs were present ($p<0.01$). Likewise as for roots, shoots do take up significantly more DDE when exposed to Cu-NPs ($p<0.01$). In addition, roots take up significantly more DDE than shoots ($p<0.01$) as roots come into direct contact with the DDE, whereas the shoots depend on translocation of DDE in the plant. These results confirm earlier findings that NPs can be used to enhance the uptake of pesticides, such as DDE, from the environment by plants (39).

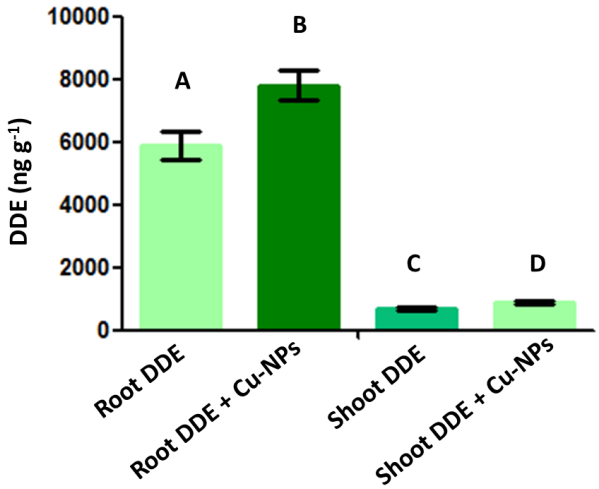

Figure 7: DDE-content in plant tissues. *DDE-content was determined using GS-MC for root and shoot samples grown with 100 µg L^{-1} DDE and with (n=3) or without (n=3) 150 µg g^{-1} Cu-NPs to examine the effect of Cu-NPs on the uptake of DDE. All samples differed significantly from each other. Significant differences have been marked using letters (p<0.01). Values are mean ± SEM. DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene, Cu-NPs: Copper nanoparticles, GC-MS: Gas Chromatography-Mass Spectrometry.*

3.2 Defining the uptake of Cu-NPs

As mentioned earlier, NPs can be used to enhance the uptake of pesticides such as DDE and for this research, we have proven that adding Cu-NPs to the plants environment significantly increases the uptake of DDE for both roots and shoots (3.1 DDE-content in plant tissues). A higher uptake of Cu-NPs could thus lead to a higher uptake of DDE. In order to determine whether there is a difference in the uptake of Cu-NPs, the concentration of Cu-NPs in roots and shoots of the different plants was determined by performing an ICP-analysis.

For the plants grown without Cu-NPs, nor DDE, the amount of Cu-NPs present, was below detection limits. A significant difference ($p<0.0001$) for the uptake of Cu-NPs was observed between the roots and shoots of the group exposed to 200 µg g^{-1} Cu-NPs as well as between the roots and shoots exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE ($p<0.0001$, Figure 8). The uptake of Cu-NPs proves to be higher in the roots than in the shoots which can be explained by the roots direct contact with the vermiculite containing the Cu-NPs, while the concentration in the shoots depends on transport. The uptake also differed significantly ($p<0.05$) between the roots exposed to Cu-NPs and roots exposed to Cu-NPs and DDE. Roots exposed to Cu-NPs solely were able to take up significantly more. Binding of Cu-NPs to DDE may enhance the uptake of DDE, but on the other hand, binding of Cu-NPs to DDE may impede the uptake of Cu-NPs as DDE is highly hydrophobic and thus hinders the transport of Cu-NPs to the roots. These findings support the hypothesis that binding of DDE to Cu-NPs decreases the uptake of Cu-NPs thereby impeding the possible negative effect of Cu-NPs on certain genera present in *C. pepo* (3.4 B Plants exposed to Cu-NPs and DDE). A significant difference, was also reported between roots exposed

to Cu-NPs and shoots exposed to Cu-NPs and DDE, were the latter takes up significantly ($p<0.0001$) less Cu-NPs than the former.

Cu-NPs are thus taken up by roots and transported to the shoots, but binding of Cu-NPs to DDE partially impedes its transport to the shoots of the plant. Due to DDE's high hydrophobicity and log(K_{ow}), there is sorption of Cu-NPs to the roots, albeit already lower than when exposed to Cu-NPs alone, and limited translocation to the stems and leaves.

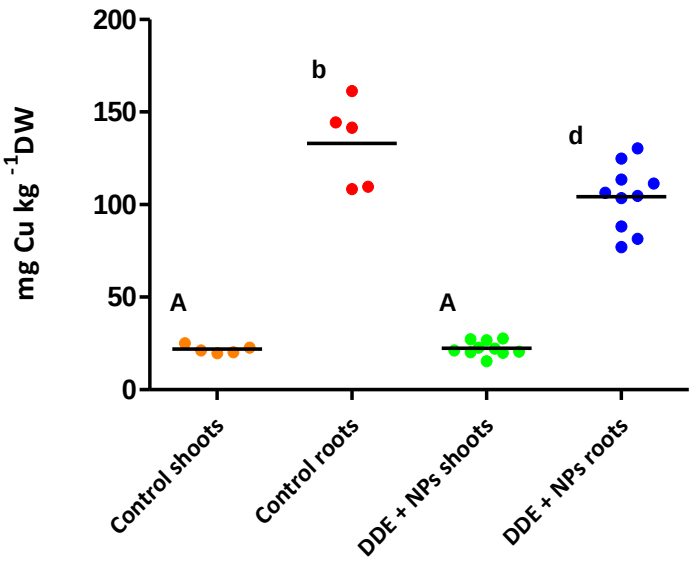

Figure 8: Uptake of Cu-NPs. *Uptake of Cu-NPs by plants exposed to 200 µg g^{-1} Cu-NPs (control, n=12) and plants exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE (n=20) was determined by performing an ICP analysis. After 20 days of exposure, plants were harvested, roots and shoots were separated and Cu-NPs content for roots and shoots was determined. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene, ICP: Inductively Coupled Plasma Analysis, DW: dry weight. Significant differences have been marked using letters: capital letter ($p<0.05$), lowercase letter ($p<0.0001$).*

3.3 Plants biomass

A. Plants exposed to Cu-NPs

Impaired plant growth is a possible marker for phytotoxicity and therefore, 32 plants were germinated and grown per two in a pot. They were watered every other day with ¼ Hoagland solution. To 12 pots, different concentrations of Cu-NPs were added, i.e. 100, 200, 300 and 400 µg g^{-1}, 3 pots per condition. To six pots no Cu-NPs were added, this was the control group. After 20 days, the plants were harvested, roots and shoots were separated and their weight was determined. No significant difference ($p=0.5202$) was observed between the weight of the roots of the control group and the weight of the roots of the group exposed to Cu-NPs (Figure 9A). Likewise, no significant difference ($p=0.9929$) could be observed for the weight of the shoots (Figure 9B). Although not significant, the biomass of roots seems to increase when exposed to 200, 300 and 400 µg g^{-1} Cu-NPs compared to the control group. For shoots, the biomass is only higher when exposed to 300 µg g^{-1} Cu-NPs. These results, although not

significant, were to be expected as the addition of Cu-NPs to plant environment has proven to increase the growth of *C. pepo* (41). Cu-NPs are taken up significantly less by shoots than by roots (Figure 8), which explains the larger effect of Cu-NPs on biomass observed for roots. It can be stated that in this experiment, the Cu-NPs did not have a negative effect on the growth of the *C. Pepo* plants.

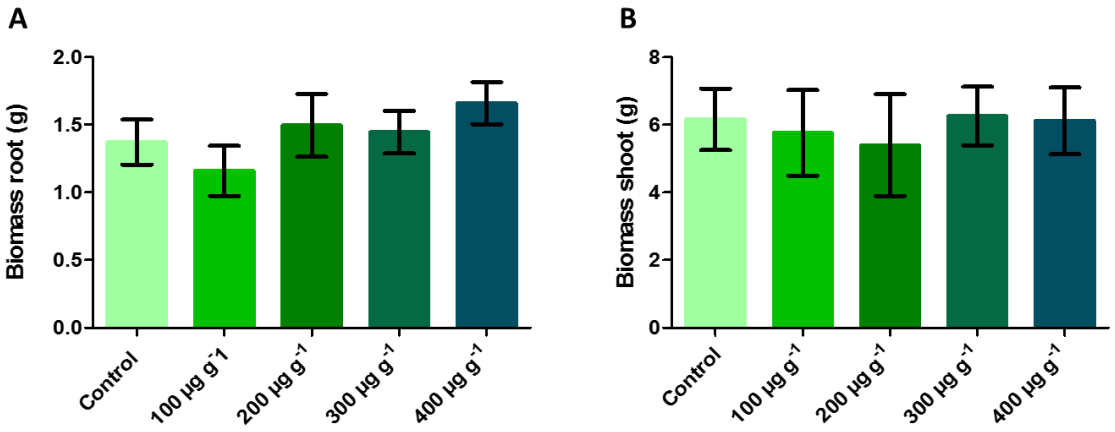

Figure 9: Biomass of roots and shoots*. Average biomass of roots (A) and shoots (B) of plants after 20 days of exposure. Control plants (n=10) were watered every other day with ¼ Hoagland solution. Cu-NPs plants (n=23) were watered every other day with ¼ Hoagland solution and grown in the presence of different concentrations of Cu-NPs, i.e. 100, 200, 300 and 400 µg g^{-1}. No significant differences were observed for root and shoot samples. Values are mean ± SEM. Cu-NPs: Copper nanoparticles.*

B. Plants exposed to Cu-NPs and DDE

In a second experiment, 42 plants were germinated and grown per 2 in a pot. Six pots served as the control group. To six other pots, 200 µg g^{-1} Cu-NPs were added. Both groups were watered every other day with ¼ Hoagland solution. To ten pots, 200 µg g^{-1} Cu-NPs were added, but were watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. After 20 days of growth, plants were harvested. Roots were separated from shoots and their weight was determined in order to evaluate the combined effect of Cu-NPs and DDE on plant growth. For root biomass (Figure 10A) as well as for shoot biomass (Figure 10B) no significant differences ($p>0.05$) could be observed between the control group, plants exposed to Cu-NPs and plants exposed to Cu-NPs and DDE. However, biomass of roots and shoots seems to be larger, albeit not significant, when exposed to 200 µg g^{-1} Cu-NPs and when exposed to 200 µg g^{-1} Cu-NPs and DDE. This again confirms earlier findings that Cu-NPs can enhance the growth of *C. pepo* (41). Cu-NPs as well as the combination of Cu-NPs with DDE did not show to have a negative effect on the growth of the *C. Pepo* plants.

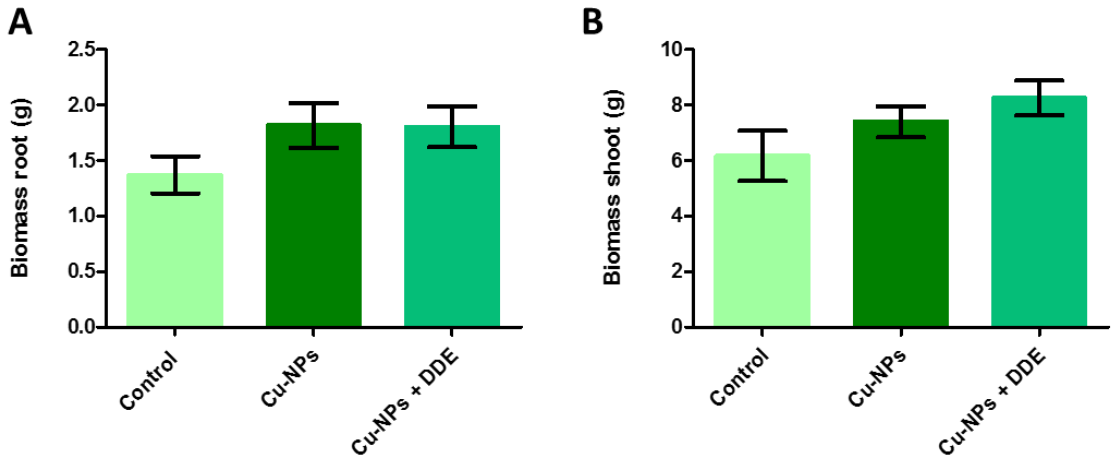

Figure 10: Biomass of roots and shoots*. Average biomass of roots (A) and shoots (B) of plants after 20 days of exposure. The control plants (n=10) and the Cu-NPs (200 µg g^{-1}) plants (n=12) were watered every other day with ¼ Hoagland solution. Twenty other plants exposed to 200 µg g^{-1} Cu-NPs were watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. No significant differences were observed between the control group, plants exposed to Cu-NPs and plants exposed to Cu-NPs and DDE. Values are mean ± SEM. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

3.4 Isolation of endophytes

After separating roots and shoots, mixed samples were prepared from two plants originating from the same pot. Plant mass was sterilized, crushed, diluted and different dilutions were plated out on 1/10 869 (Figure 3). After incubating the plates for four days, the cfu g^{-1} of fresh plant material was determined for the different conditions (Supplementary table 13).

A. Plants exposed to Cu-NPs

In order to determine the optimal concentration of Cu-NPs in which the endophytes can grow, the average cfu g^{-1} of fresh plant material was determined for the different conditions (Figure 11). As expected, the average number of cfu g^{-1} associated with shoot tissues, is lower than the average number of cfu g^{-1} associated with root tissues (53-55). No significant differences in cfu g^{-1} could be detected for the roots. Although the control group seems to have higher amounts of cfu g^{-1} than the groups exposed to Cu-NPs, all differences although large, are not significant due to the high biological variation. This in contrast to the shoots group, when exposed to 200 µg g^{-1} Cu-NPs, significantly more cfu g^{-1} were observed than for the control group and the group exposed to 300 µg g^{-1} Cu-NPs. *Enterobacter* and *Stenotrophomonas* are the genera responsible for the significantly higher numbers of colonies observed when plants are exposed to 200 µg g^{-1} Cu-NPs. Endophytes present in roots do not experience any significant effect of the Cu-NPs. For this reason and supported by the results indicating that Cu-NPs do not have a negative effect on the growth of the *C. Pepo* plants, it was decided to work with 200 µg g^{-1} Cu-NPs for the remaining experiments.

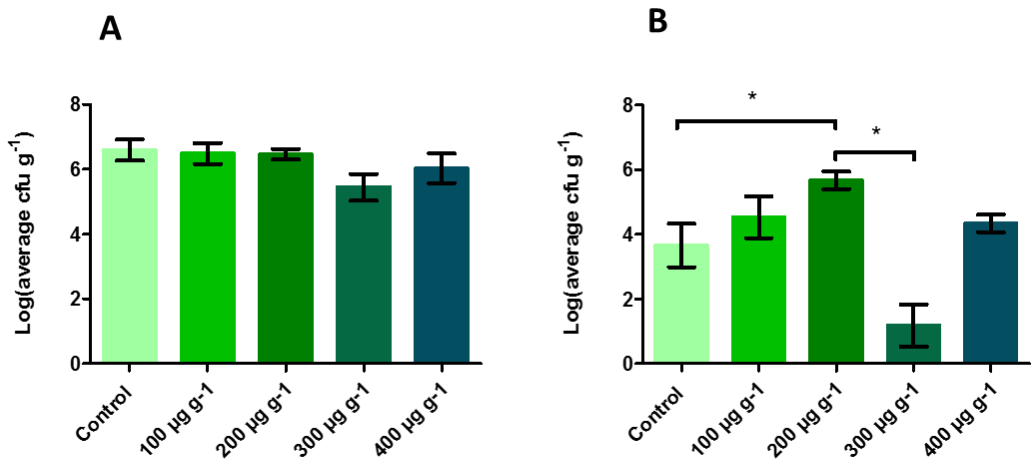

***Figure 11: Average cfu g^{-1} plant material**. Logarithmic value of the average number of cfu g^{-1} roots (A) and shoots (B) for the control plants (n=10) and plants exposed to different concentrations, i.e. 100, 200, 300 and 400 µg g^{-1}, of Cu-NPs (n=23). No significant differences were observed for the root samples. Shoots exposed to 200 µg g^{-1} Cu-NPs differed significantly from the group exposed to 300 µg g^{-1} Cu-NPs and the control group. Values are log(mean) ± SEM. Cu-NPs: Copper nanoparticles, Cfu g^{-1}: colony-forming units per gram of plant material. (*) p<0.05.*

B. Plants exposed to Cu-NPs and DDE

The main question of this research is whether combining endophytes and Cu-NPs enhances the phytoremediation efficiency of DDE-contaminated soils even further. An earlier experiment proved that Cu-NPs nor the combination of Cu-NPs with DDE had an effect on the growth of *C. pepo* (Figure 9, Figure 10). Endophytes present in roots did not experience any significant effect when exposed to Cu-NPs. Endophytes present in shoots, however, formed significantly more cfu g^{-1} when exposed to 200 µg g^{-1} Cu-NPs compared to other conditions (Figure 11). Therefore, plants were exposed to 200 µg g^{-1} Cu-NPs with or without 100 µg L^{-1} DDE. To investigate the effect of this combined use of DDE and Cu-NPs on the endophytes present in roots and shoots, the cfu g^{-1} of fresh plant material was determined.

In agreement with literature (53-55), for all conditions more colonies were formed by endophytes present in the roots (Figure 12A), than by endophytes isolated from the shoots (Figure 12B). However, this difference is not significant. The only significant difference was detected within the roots. Endophytes present in the roots of *C. pepo* of the control group (Figure 12A) were able to form significantly more colonies compared to the roots of the plants exposed to 200 µg g^{-1} Cu-NPs and DDE. A trend noticeable for all conditions is the decrease of the number of endophytic bacteria when plants are exposed to Cu-NPs. This is the opposite of what is seen for shoots when exposed to 100, 200 and 400 µg g^{-1} Cu-NPs in the previous experiment (Figure 11B). However, when exposed to Cu-NPs in combination with DDE, this number increases again. The effect of Cu-NPs on endophytes can be observed more clearly in root tissues than in shoot tissues which can again be explained by the direct contact of the roots with the surrounding vermiculite containing Cu-NPs. However, the increase of cfu g^{-1} that is observed when Cu-NPs and DDE are combined, is larger for shoot than for root tissues.

Although Cu-NPs or the combination of Cu-NPs and DDE did not have a negative effect on the growth of the plants, it is possible that some endophytes present in the roots and shoots might experience a negative effect from Cu-NPs. This was to be expected, as Cu-NPs are known for their anti-microbial activity (41). Observed changes are more apparent for the roots than for the shoots, likely due to the fact that the roots come into direct contact with the Cu-NPs and/or DDE present in the vermiculite, whereas the shoots depend on translocation of Cu-NPs and/or DDE in the plant. In addition, roots take up significantly more Cu-NPs than shoots (Figure 8). As a consequence, the same possible negative effect of Cu-NPs is lower for endophytes present in shoots. A trend that is observed, is that the number of cfu g^{-1} decreases when exposed to Cu-NPs alone, but increases again when exposed to Cu-NPs in combination with DDE. This can be explained by the fact that binding of the hydrophobic DDE to Cu-NPs possibly decreases the uptake of Cu-NPs thereby impeding the possible negative effect of Cu-NPs on certain genera present in *C. pepo*. In addition, adding DDE to the plants environment has already proven to increase the number of endophytic bacteria (20-22, 56). These results indicate that Cu-NPs could affect the endophytes present in roots and shoots, however, this effect is not fatal as certain genera are still able to grow in the presence of DDE and Cu-NPs.

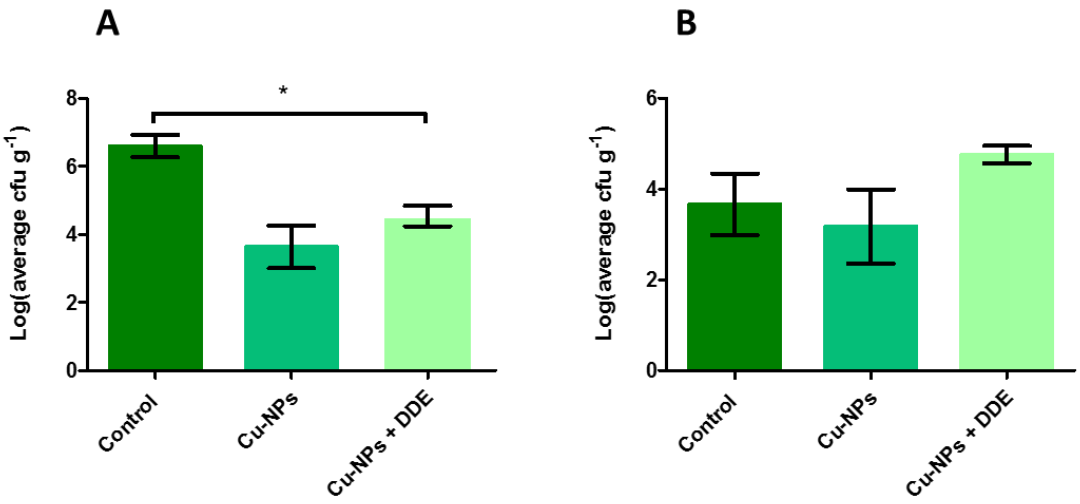

***Figure 12: Average cfu g^{-1} plant material**. The logarithmic value of the average amount of cfu g^{-1} roots (A) and shoots (B) for the control plants (n=10), the plants exposed to 200 µg g^{-1} Cu-NPs (n=12) and the plants exposed to 200 µg g^{-1} of Cu-NPs and 100 µg L^{-1} DDE (n=20). Roots exposed to Cu-NPs and DDE differed significantly from the control group. Values are log(mean) ± SEM. Cu-NPs: Copper nanoparticles, Cfu g^{-1}: colony-forming units per gram of plant material. (*) p<0.05.*

3.5 Genotypic characterization of the endophytic community

In order to identify the cultivable endophytic community of *C. pepo* when exposed to Cu-NPs and Cu-NPs with DDE, bacterial strains (n=497) were isolated from roots and shoots of these plants, DNA was extracted, amplified and digested and an ARDRA was conducted. For the identification of the total bacterial community when plants are exposed to Cu-NPs and Cu-NPs with DDE, DNA was extracted from root (n=16) and shoot (n=16) samples, DNA was amplified and barcodes were attached to each sample in a nested PCR. In order to identify the bacterial strains present in the samples, 454 pyrosequencing was performed on the resulting 2 barcoded amplicon pools (each containing 16 samples) by Macrogen (Seoul, South Korea).

A. Screening of the cultivable endophytic community

In total, 13 different genera, belonging to 4 different phyla could be detected performing ARDRA (Table 2). *Stenotrophomonas* (52.71%), *Enterobacter* (35.01%), and *Citrobacter* (4.63%) were the 3 most abundant genera observed, while 0.20% of the samples was not identifiable.

Table 2: *Cultivable bacterial genera isolated from C. pepo.*

Phylum	**Class**	**Genus**
Actinobacteria	Actinobacteria	*Streptomyces*
Deinococcus-Thermus	Deinococcus-Thermus	*Truepera*
Firmicutes	Bacilli	*Bacillus*
		Brevibacillus
		Paenibacillus
		Staphylococcus
	Clostridia	*Clostridiales*
Proteobacteria	Alphaproteobacteria	*Rhizobium*
	Gammaproteobacteria	*Citrobacter*
		Enterobacter
		Pseudomonas
		Rhodanobacter
		Stenotrophomonas
Unclassified	Unclassified	*Unclassified*

Bacterial genera (phylum, class) that were isolated from *C. pepo* control plants (n=10), *C. pepo* plants exposed to 200 µg g^{-1} Cu-NPs (n=12), and *C. pepo* plants exposed to 200 µg g^{-1} Cu-NPs combined with 100 µg L^{-1} DDE (n=20). Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

When investigating the effect of Cu-NPs and the combination of Cu-NPs and DDE on the cultivable endophytic community of the roots (Figure 13A), lower abundancies could be observed for *Stenotrophomonas, Enterobacter* and *Paenibacillus* when exposed to Cu-NPs and DDE. The presence of *Stenotrophomonas* made up 80.18% of the endophytes of the control group, 86.19% of the endophytes of plants exposed to Cu-NPs, while a lower abundancy (48.27%) was observed in plants exposed to Cu-NPs together with DDE. Likewise, *Enterobacter* accounted for 16.12% of the cultivable community in the control group, 9.13% of the endophytes in the plants exposed to Cu-NPs, and 4.26% of the endophytes in plants exposed to DDE and Cu-NPs. The decrease of *Paenibacillus* was less

pronounced: *Paenibacillus* accounted for 3.20% in the control group, a lower abundancy (0.60%) could be detected in plants exposed to Cu-NPs, and *Paenibacillus* could not be detected anymore in plants exposed to DDE and Cu-NPs. The opposite was true for *Brevibacillus,* which was not present in the control group or in plants exposed to Cu-NPs, but displayed an abundancy of 32.13% when exposed to Cu-NPs together with DDE. Likewise, *Clostridiales* could not be detected in the control and the Cu-NPs group, but formed 6.35% of the endophytic community of plants exposed to Cu-NPs and DDE. *Pseudomonas* was not present in the plants exposed to Cu-NPs, but accounted for 0.19% of the community present in the control group. Higher abundancies of *Pseudomonas* (9.00%) could be detected in plants exposed to Cu-NPs and DDE. These results might suggest that the presence of *Stenotrophomonas* is not strongly affected by the addition of Cu-NPs solely, but is so by the combination of DDE and Cu-NPs, although it still accounts for 48.27% of the colonies formed. The opposite is true for *Brevibacillus* which is able to grow well when exposed to DDE and Cu-NPs. The observed results indicate that some genera are affected by the presence of Cu-NPs, but are still able to form colonies, even when DDE is added. For some, the number of colonies formed even increases again when DDE is present. This again confirms our findings that binding of DDE to Cu-NPs impedes the uptake of Cu-NPs, thereby decreasing the effect of Cu-NPs and that adding DDE to the plants environment has a positive effect on the number of endophytic bacteria. These results suggest that certain genera might possess genes that enable them to grow in the presence of DDE and Cu-NPs and these genes are activated when exposed to these compounds. These genes might, for example, provide them with the capacity to degrade DDE and perhaps even give them the ability to use it as a carbon source.

When analyzing the cultivable endophytic community present in shoots, far smaller differences could be observed (Figure 13B). A decreasing presence could be observed for *Enterobacter* and *Brevibacillus.* The highest abundancy (50.00%) for *Enterobacter* could be detected in the control group. The abundancy was lower (3.01%) in plants exposed to Cu-NPs. When plants are exposed to Cu-NPs with DDE, *Enterobacter* accounts for 12.93% of the endophytic community. *Brevibacillus* accounted for 2.74% of the community in the control group and was not present in plants exposed to Cu-NPs and plants exposed to the combination of DDE and Cu-NPs. The only sharp increase that could be observed, was for the presence of *Stenotrophomonas*. *Stenotrophomonas* made up 46.68% of the endophytic community of the control group. A higher abundance (92.81%) was observed in plants exposed to Cu-NPs. In the plants exposed to Cu-NPs with DDE, *Stenotrophomonas* accounted for 86.44% of the community. These results suggest that *Enterobacter, Brevibacillus* and S*tenotrophomonas* experience an effect from Cu-NPs but for *Enterobacter* this effect reduces when exposed to Cu-NPs and DDE. This could again be explained by the fact that binding of DDE to Cu-NPs hinders the uptake of Cu-NPs,

impeding its possible effect on the endophytes, and by the positive effect on the number of endophytes after adding DDE. *Enterobacter* and *Stenotrophomonas* are still able to grow in the presence of DDE, together they account for 99.37% of the colonies observed, suggesting that they both possess the capacity to degrade DDE.

When analyzing the diversity of the cultivable endophytic community, no significant differences could be observed between roots and shoots. Likewise, no significant differences were observed within the roots and shoots group for the different conditions (Figure 17). Cu-NPs nor the combination of Cu-NPs with DDE had an effect on the diversity of the cultivable endophytic community of *C. pepo.*

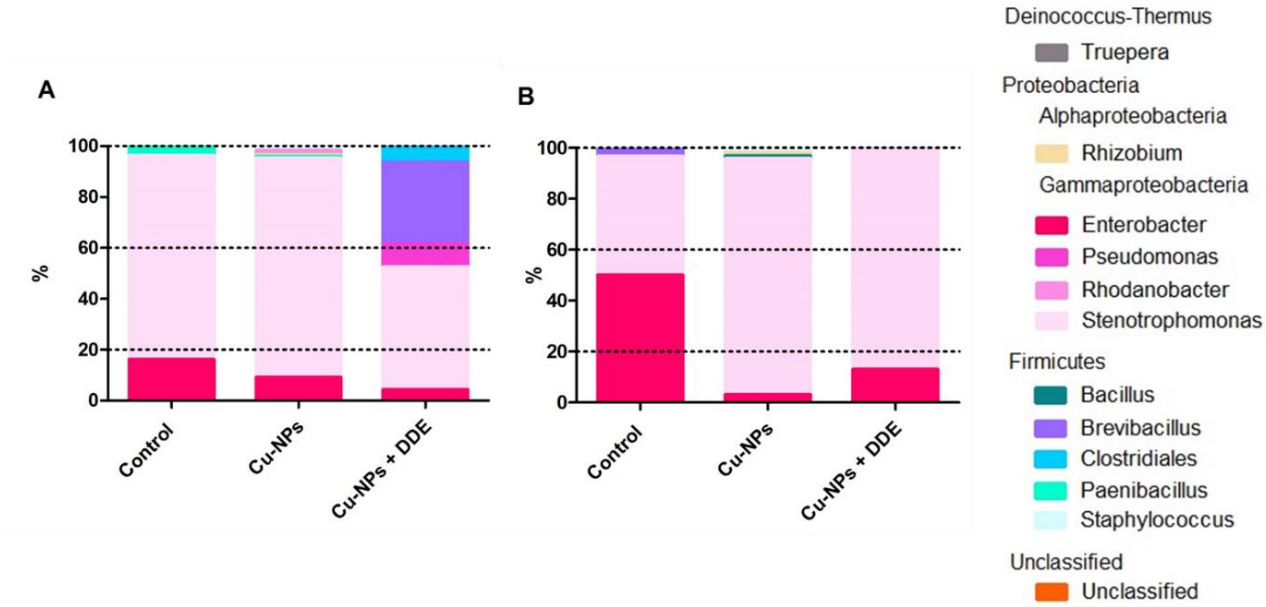

***Figure 13: Cultivable bacterial community of roots and shoots**. Composition of the cultivable bacterial community of roots (A) and shoots (B) expressed in genera and based on condition. All isolated species are shown. The control group (n=10) was not exposed to Cu-NPs nor DDE. Plants were exposed to 200 µg g^{-1} Cu-NPs (n=12) or 200 µg g^{-1} Cu-NPs in combination with 100 µg L^{-1} DDE (n=20). Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

B. Screening of the total endophytic community

Macrogen (Seoul, South Korea) performed the 454 pyrosequencing for two pools and delivered the raw data in FASTA files. The first FASTA-file contained 117105 sequences with a mean length of 379.605 base pairs (bp). The second FASTA-file contained 113891 sequences with a mean length of 362.176 bp. Both files were combined and further analyzed using Mothur (47). After removal of barcodes and primers sequences, the remaining sequences were filtered and analyzed according to the following criteria: maximum two different bp compared to the primer sequence, maximum one different bp compared to the barcode sequence, a minimum sequence length of 200 bp and a maximum length of homopolymers of eight bp. Subsequently, the remaining sequences were aligned to the SILVA rRNA database (48). Alignments were filtered and pre-clustered, and chimeras were removed, all in order to reduce sequencing errors. After this step, a total of 124908 sequences remained; 2298 of these were

unique and the average length was 256.95 bp. A final step to improve data quality was the removal of sequences classified as "chloroplast", "mitochondria", or "unknown" (those sequences that could not be classified at the Kingdom level) as well as archaeal and eukaryotic 16S/18S rRNAs. After this filtering, 27490 sequences remained, containing 1329 unique sequences with an average bp-length of 257.074 bp. Mothur also provided a file containing the number of reads per sample, giving rise to rarefaction curves, a measure of the diversity and quality of the measurement (Figure 14).

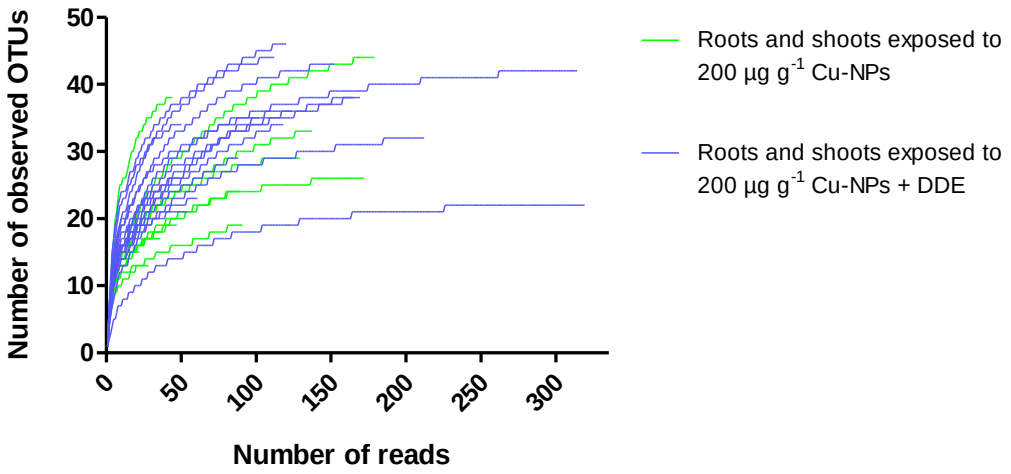

Figure 14: Rarefaction curves describing the dependence of discovering novel OTUs as a function of sampling effort for OTUs. *The number of OTUs per sample as a function of the number of reads per sample for roots and shoots exposed to 200 µg g^{-1} Cu-NPs (n=12) and for roots and shoots exposed to 200 µg g^{-1}Cu-NPs and 100 µg L^{-1} DDE (n=20). OTU: Operational Taxonomic Unit, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene, Cu-NPs: Copper nanoparticles.*

For all the samples analyzed, 909 different OTUs were detected, belonging to 4 different phyla and 86 genera. Of all the samples, 41.05% could not be identified to the genus nor the phylum level and are thus grouped as 'unclassified' (Figure 15). Although 86 different genera could be detected, many of these genera could only be detected once or twice across all samples. Therefore the discussion will further focus on the ten most abundant genera, which accounts for almost all bacteria present (Root with Cu-NPs: 97.33%, Root with Cu-NPs and DDE: 94.33%; Shoot with Cu-NPs: 98.21%; Shoot with Cu-NPs and DDE: 94.92%).

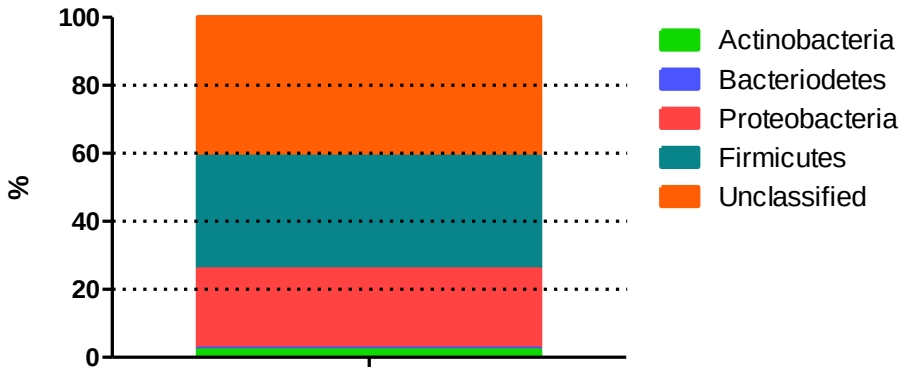

Figure 15: Total bacterial community. *Composition of the total bacterial community based on phyla.*

The unclassified endophytes form the most abundant genus for both roots and shoots (Figure 15, Figure 16). A possible explanation is that, for certain samples, DNA was of lower quality which makes it more difficult to process the samples. When not taking into account these unclassified endophytes, the most abundant genus for roots exposed to Cu-NPs solely is *Salmonella* (22.23%) (Figure 16A). The addition of DDE together with Cu-NPs to the plant environment causes a shift in this dominance. Upon exposure to DDE with Cu-NPs, *Staphylococcus* is dominantly presence (18.71%) and *Salmonella* could not be detected anymore. This effect was not observed in shoot tissues (Figure 16B), where *Staphylococcus* proved to be the most abundant genus in the total community when exposed to Cu-NPs and Cu-NPs together with DDE (24.22% and 58.86% respectively). A trend that is visible for roots exposed to Cu-NPs and Cu-NPs with DDE and for shoots exposed to Cu-NPs and Cu-NPs with DDE, is that *Enterobacteriaceae* forms the second most abundant genus present (4.14% and 14.17% for root, and 1.67% and 4.07% for shoots respectively). These results suggest that Firmicutes (*Staphylococcus*) and Gammaproteobacteria (*Enterobacteriaceae* and *Salmonella*) are phyla that seem to thrive quite well in the presence of DDE and Cu-NPs. As assumed earlier, this could be due to the fact that certain genera possess genes that, when necessary, provide them with the capacity to degrade DDE and perhaps even use it as a carbon source.

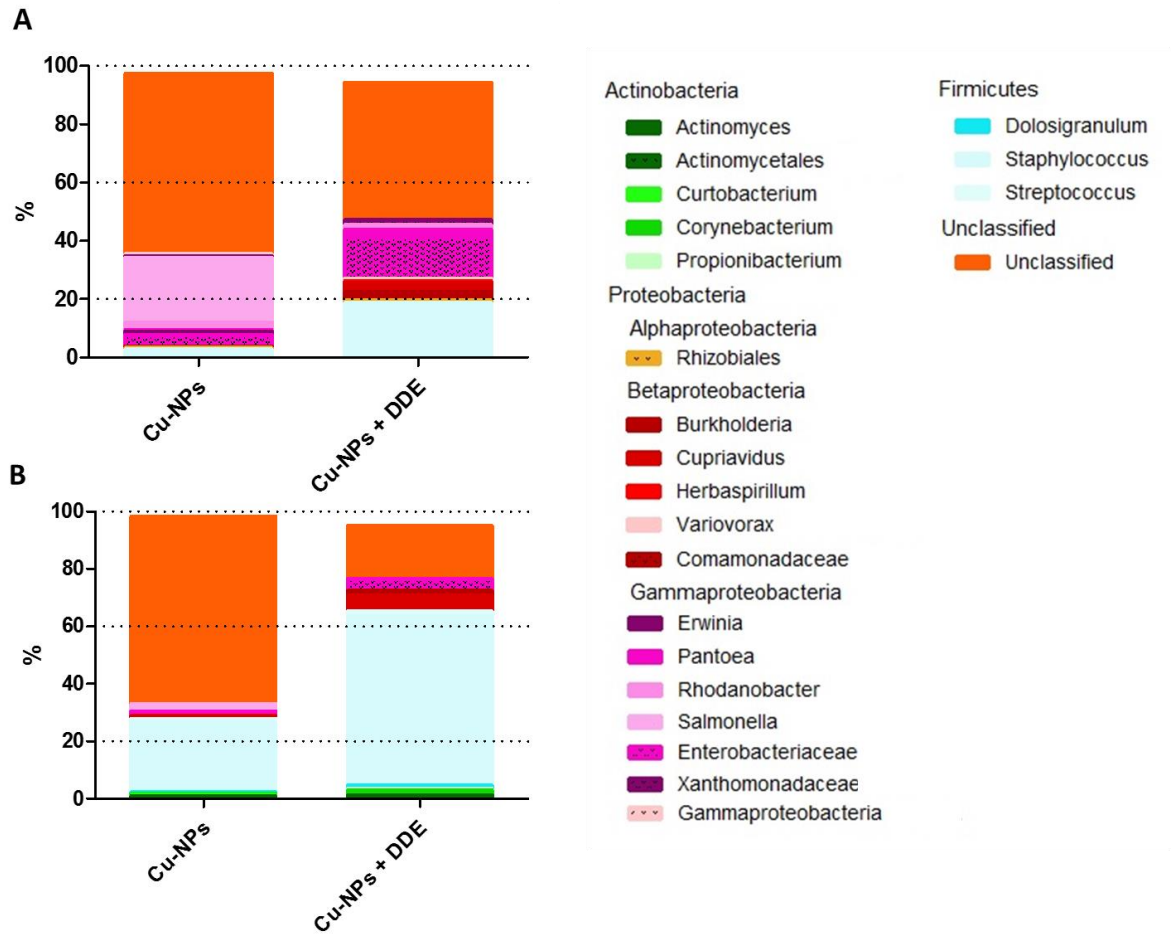

Figure 16: Total bacterial community of roots and shoots. *Composition of the total bacterial community of roots (A) and shoots (B) expressed in genera and based on condition. Only the top ten of detected species is shown. Plants were exposed to 200 µg g^{-1} Cu-NPs (n=12) or 200 µg g-1 Cu-NPs in combination with 100 µg L^{-1} DDE (n=20). Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

When analyzing the Shannon-Wiener diversity indices for the total endophytic community, no significant differences could be observed between roots and shoots (Figure 17). Likewise, no significant difference could be observed for roots and shoots when Cu-NPs were combined with DDE. However, several trends are visible. When exposed to Cu-NPs in combination with DDE, the observed diversity seems to be higher than when exposed to Cu-NPs alone (0.987 and 1.330 for roots, and 1.176 and 1.350 for shoots respectively). Shoots also display a slightly higher diversity than roots in both conditions.

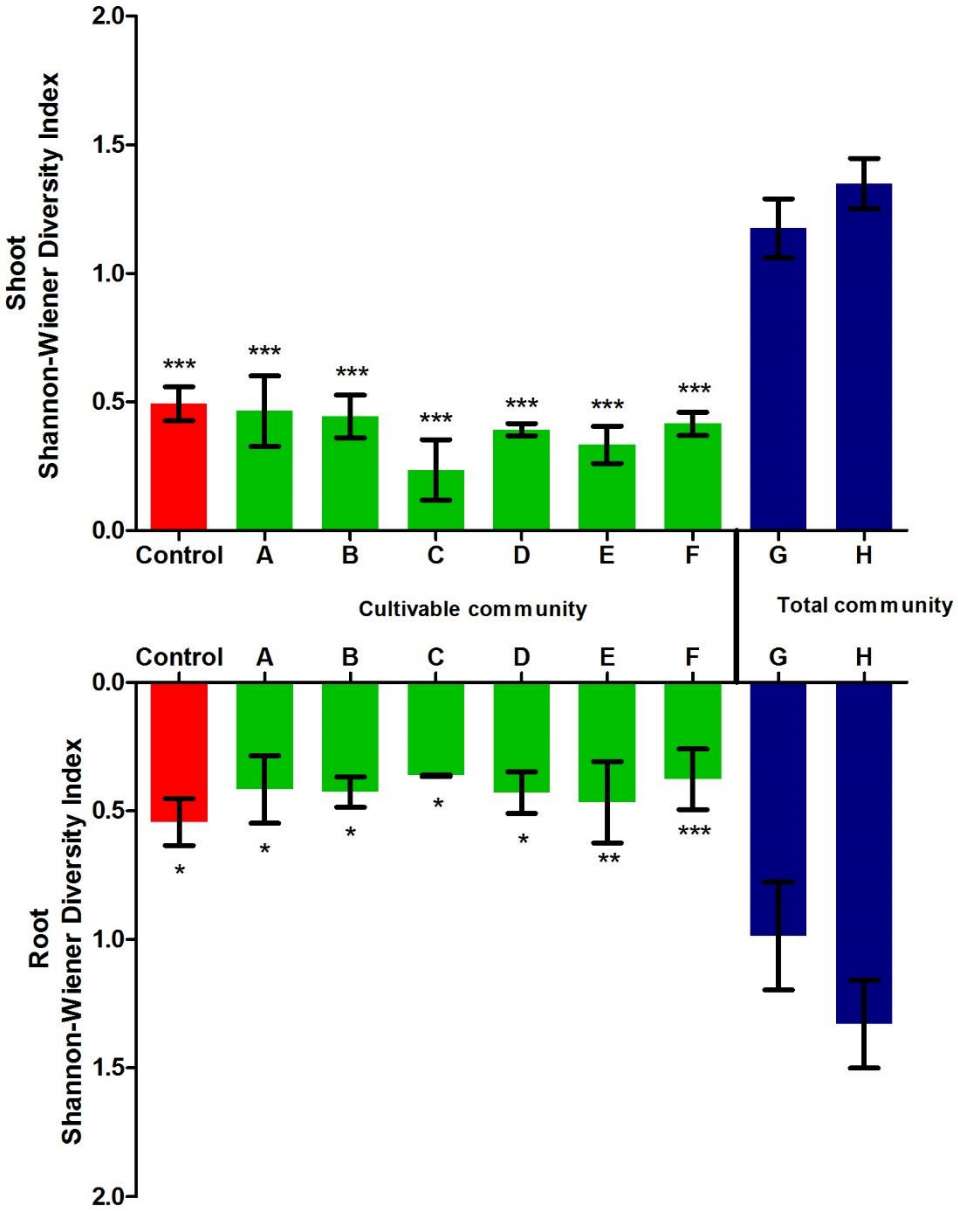

Figure 17: Comparison of the Shannon-Wiener Diversity Indices of the cultivable and the total bacterial community of roots and shoots. *The control group (n=10) was not exposed to Cu-NPs nor DDE. A-D: plants exposed to 100, 200, 300 and 400 µg g^{-1} Cu-NPs respectively, E and G: plants exposed to 200 µg g^{-1} Cu-NPs, F and H: plants exposed to 200 µg g^{-1} Cu-NPs + 100 µg L^{-1} DDE. For the shoots, from control group to group F, all differ significantly from H. For the roots, from control group to group F, all differ significantly from group G and H. Values are mean ± SEM.(*) p<0.05, (**) p<0.01, (***) p<0.001.Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

C. Comparing cultivable and total endophytic community

When analyzing the obtained results by using Analysis of Similarity (ANOSIM) and Similarity Percentages (SIMPER) tools in Primer7, a clear clustering could be observed for the different conditions (Figure 18). In addition, a large overlap between the total and the cultivable endophytic community is present (Figure 18, Figure 19), indicating a high cultivability efficiency. The overall p-value of ANOSIM is 0.001 indicating that there is a significant difference in communities between the different samples.

However, the overall R-value is 0.371, indicating a mediocre separation between levels of the investigated factor; it is likely that all the most similar samples are within the same groups.

The dendrogram based on S17 Bray–Curtis similarity (Figure 19) showed a clear overlap between the total and cultivated community. Within the different conditions, limited clustering could be observed for the endophytic community of roots and shoots of the control plants.

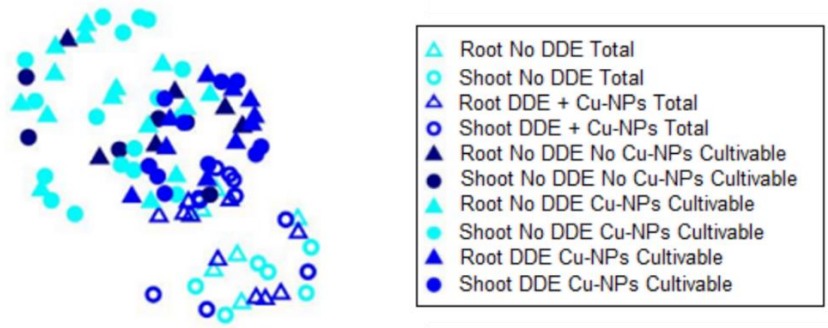

Figure 18: Similarity between the different conditions of the total and the cultivable endophytic community. *2D nMDS based on S17 Bray-Curtis similarity, Kruskall stress formula 1; Minimum stress 0.01; Stress value 0.01. 2D nMDS: 2D Non-Metric Multidimensional Scaling, Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

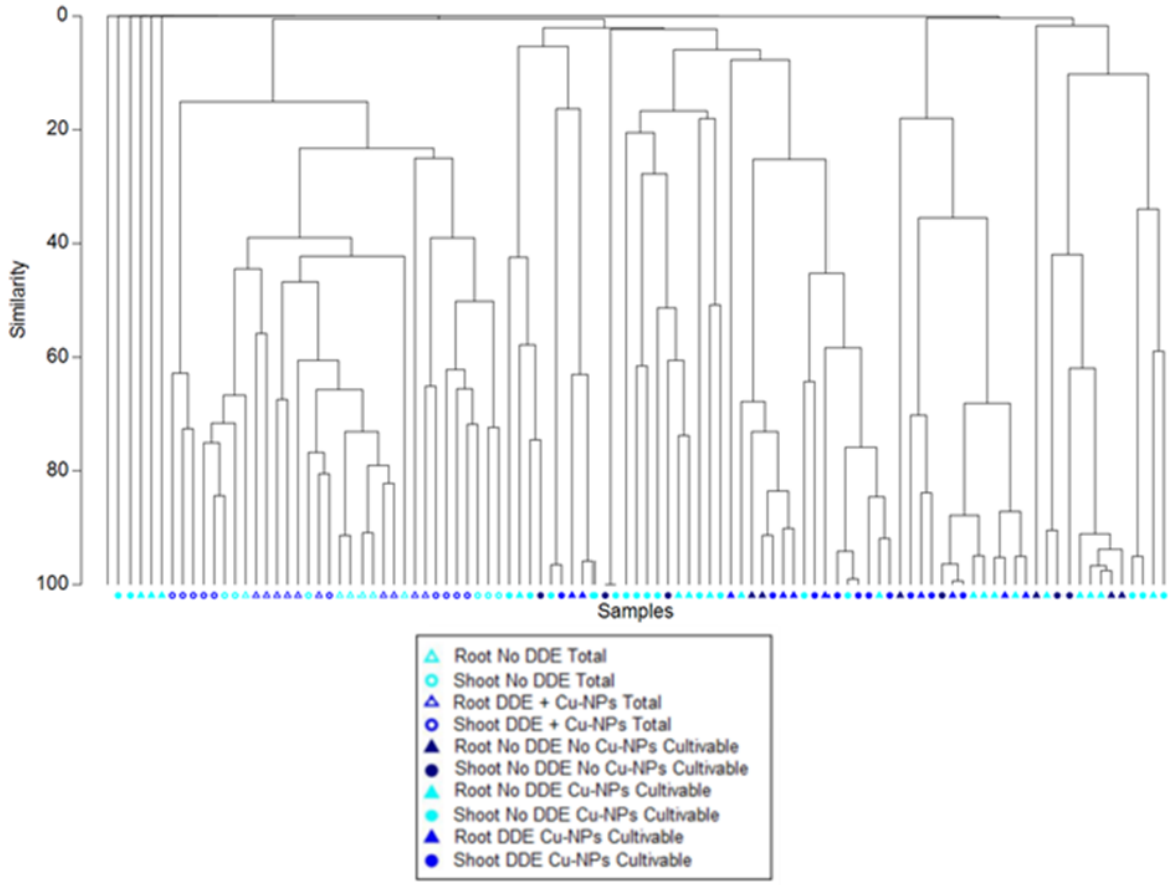

Figure 19: Dendrogram*. Cluster based on S17 Bray–Curtis similarity, cophenetic correlation 0.87367. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

Although there was a high cultivability efficiency, certain genera, such as *Staphylococcus* in root and shoot tissues, showed a dominant presence in the total community of plants exposed to DDE and Cu-NPs, but it has a very low representation in the cultivable community of plants exposed to DDE and Cu-NPs. This can be due to a reduced ability to grow under laboratory conditions. *Enterobacter* however, showed a dominant presence in the cultivable community of plants exposed to DDE and Cu-NPs and the family of *Enterobacteriaceae* was dominant as well in the total endophytic community. It has already been proven that *Enterobacter* species are rather easily cultivable under laboratory conditions (57). For *Stenotrophomonas,* the same observations as for *Enterobacter* could be made. *Stenotrophomonas* displayed a high abundancy in the cultivable endophytic community of plants exposed to DDE and Cu-NPs and, although *Stenotrophomonas* was not amongst the ten most abundant genera of the total community of plants exposed to DDE and Cu-NPs, it was still ranked quite high.

The lowest variability and thus highest similarity, was observed within the total community. The average similarity between members of the group exposed to Cu-NPs, based on Bray-Curtis similarity measure, is 58.44% for roots and 43.52% for shoots. A lower similarity was observed for the group exposed to Cu-NPs combined with DDE (41.45% and 27.33% for roots and shoots respectively). The results indicate that adding DDE to the plant environment increases the diversity of the endophytes present, however, this increase was not significant (Figure 17).

The highest variability was detected within the cultivable community. Roots and shoots of the group exposed to Cu-NPs showed a similarity of 17.26% an 11.50% respectively. When plants were exposed to Cu-NPs together with DDE, higher similarity values were detected for both roots and shoots (40.16% and 47.91% respectively). Plants of the control group displayed mediocre values, 28.74% and 21.07% for roots and shoots respectively. These results indicate that adding Cu-NPs to the plants environment increases the diversity, but this diversity decreases when Cu-NPs and DDE are combined. However, these results are not significant (Figure 17). This could be due to the fact that only a few genera, with *Staphylococcus* and *Enterobacteriaceae* being the most abundant ones, are able to grow in the presence of DDE and Cu-NPs and thereby lower the diversity.

When comparing the Shannon-Wiener diversity indices of the total communities and the cultivable communities, all the conditions tested for the cultivable community present in shoots, differed significantly from the group of the total community exposed to Cu-NPs in combination with DDE. For the roots, all the conditions tested for the cultivable community differed significantly from both groups of the total community (Figure 17). The total community has a significantly higher diversity than the cultivable community which is to be expected as all bacterial genera are detected.

When a pairwise comparison was made between the samples taken from plants grown with and without DDE, and with and without Cu-NPs, few significant differences could be observed. Root tissues of the cultivable community exposed to Cu-NPs with or without DDE (Table 3) displayed a high dissimilarity (82.34%), which was significant ($p<0.05$) although they do not differ strongly from each other (R=0.147). Likewise, shoot tissue exposed to the same conditions, display a high and significant dissimilarity (85.21%, $p<0.01$), however, their difference is not very strong (R=0.229). The total community displayed no significant average dissimilarities for root and shoot tissue exposed to Cu-NPs with or without DDE (Table 3).

Table 3: *Dissimilarity between the bacterial community from plants grown with and without DDE.*

Technique	Compartment	Condition	Average dissimilarity	R-value	P-value
Total	Root	Cu-NPs	57.68	0.093	0.171
	Shoot	Cu-NPs	66.53	-0.071	0.706
Cultivable	Root	Cu-NPs	82.34	0.147	0.041
	Shoot	Cu-NPs	85.21	0.229	0.002

Average dissimilarities calculated using ANOSIM and SIMPER in Primer7. They represent the dissimilarities between the different conditions for plants grown with 100 µg L^{-1} DDE and plants grown without DDE. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

When analyzing the effect of Cu-NPs on the dissimilarity of root and shoot tissues of the cultivable community, no significant differences could be detected (Table 4).

Table 4: *Dissimilarity between the cultivable bacterial community from plants grown with and without Cu-NPs.*

Technique	Compartment	Condition	Average dissimilarity	P-value
Cultivable	Root	No DDE	80.23	0.771
	Shoot	No DDE	81.43	0.996

Average dissimilarities calculated using ANOSIM and SIMPER in Primer7. They represent the dissimilarities between the different conditions for plants grown with 200 µg g^{-1} Cu-NPs and plants grown without Cu-NPs. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

Cu-NPs combined with DDE does have a significant effect on the average dissimilarity of root and shoot tissues of the cultivable community. Root tissues exposed to Cu-NPs combined with DDE display an average dissimilarity of 76.62% compared to root tissue of the control group, which is a significant ($p<0.01$) and mediocre (R=0.426) difference (Table 5). *Stenotrophomonas* (42.34%) and *Enterobacter* (39.36%), contributed the most to this dissimilarity. For the shoot tissues, the same observations could be made. An average dissimilarity of 79.16% was detected which was significant ($p<0.01$) and of mediocre strength (R=0.474). Again, *Stenotrophomonas* (47.28%) and *Enterobacter* (39.20%), contributed the most to this dissimilarity.

Table 5: *Dissimilarity between the cultivable bacterial community from plants grown with DDE and Cu-NPs vs. plants grown without DDE nor Cu-NPs.*

Technique	Compartment	Average dissimilarity	R-value	P-value
Cultivable	Root	76.62	0.426	0.001
	Shoot	79.16	0.474	0.006

Average dissimilarities calculated using ANOSIM and SIMPER in Primer7. They represent the dissimilarities between the different conditions for plants grown with 100 µg L^{-1} DDE and 200 µg g^{-1} Cu-NPs, and plants grown without DDE nor Cu-NPs. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

These results indicate that, for the cultivable community, Cu-NPs alone do not affect the dissimilarity of the endophytic communities from roots and shoots. Adding DDE to the plants environment led to a high dissimilarity that is significant, but this observed dissimilarity is not very strong. However, combining Cu-NPs and DDE causes a dissimilarity that is significant and strong.

3.6 Phenotypic characterization of cultivable bacterial community

Plant-associated bacteria are often capable of promoting growth and development of plants. These effects have been linked to both direct and indirect plant growth promoting mechanisms. All cultivable bacterial strains isolated from *C. pepo* (n=497) were subjected to a phenotypic characterization. They were subjected to five different tests evaluating their plant growth-stimulating capacities and an auxanography to screen them for their potential to use DDE as a carbon source.

A. Screening of plant growth promotion characteristics

Endophytes isolated from roots and shoots from *C. pepo* grown under six different conditions, i.e. exposure to (1) 100, 200, 300 and 400 µg g^{-1} Cu-NPs, (2) 200 µg g^{-1} Cu-NPs in combination with 100 µg L^{-1} DDE, and (3) none of both, were evaluated for their plant growth promotion. They were subjected to five different tests: (1) examining their ability to produce organic acids (OA), (2) examining their ability to produce siderophores, (3) measuring their ACC-deaminase activity, (4) examining their ability to produce the phytohormone IAA and (5) examining whether they have the capacity to solubilize phosphate.

When analyzing the results of the five different tests, high amounts of endophytes tested positive for all the tests (Figure 20). However, a trend that is immediately visible is that almost none of the endophytes isolated from roots and shoots were able to produce OA. Plants were grown in the lab under conditions that provided sufficient nutrients, making the presence of OA not essential for the growth of the plant. Therefore, the genes needed for the production of OA were not activated and no or few OA were produced. Another trend visible is that the endophytes that test positive for the different tests are, in the shoots, dominated by Gammaproteobacteria (72.17%) of which *Enterobacter* and *Stenotrophomonas* form the largest part. In the roots there is a dominance of Gammaproteobacteria (61.64%), especially *Enterobacter, Stenotrophomonas* and *Pseudomonas*, and

Firmicutes (35.92%) of which *Paenibacillus* forms the largest part. In roots, more endophytes scored positive for ACC-deaminase activity. Exposure to Cu-NPs and DDE induces stress and as a reaction, plants produce high levels of ethylene, which is counteracted by bacterial ACC-deaminase activity. The higher ACC-deaminase activity observed in roots can be explained by the fact that roots come into direct contact with Cu-NPs and DDE and thus suffer more from stress. Whereas the exposure of shoots to Cu-NPs and DDE depends on the translocation of these compounds which exposed them less to these stress-inducing compounds. No other major differences could be detected between roots and shoots, When examining the results for the different conditions, no major differences could be detected. Adding Cu-NPs or the combination of Cu-NPs with DDE does not seem to affect the capacity of the endophytic community to promote plant growth.

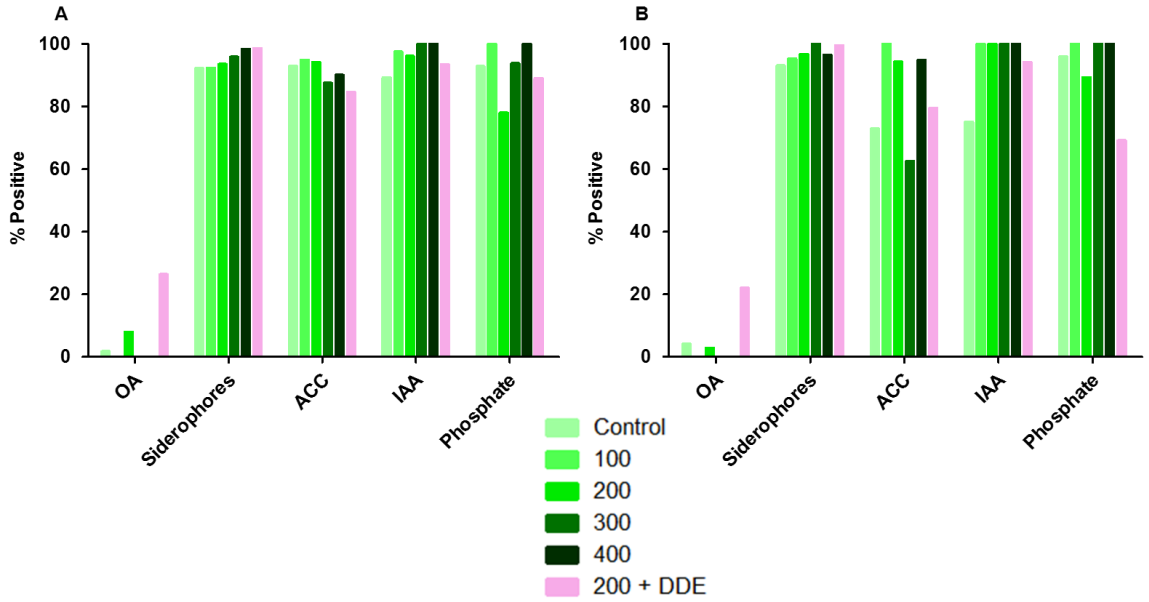

Figure 20: Plant growth promoting tests. *Endophytes isolated from roots (A) and shoots (B) were evaluated for their capacity to promote plant growth by performing five different tests: (1) examining their ability to produce OA, (2) their ability to produce siderophores, (3) measuring their ACC-deaminase activity, (4) examining their ability to produce the phytohormone IAA and (5) examining whether they have the capacity to solubilize phosphate. Each test was performed for eight different groups: control, 100, 200, 300, and 400 µg g^{-1} Cu-NPs, control, 200 µg g^{-1} Cu-NPs and 200 µg g^{-1} Cu-NPs + 100 µg L^{-1} DDE. For the control group and the plants exposed to 200 µg g^{-1} Cu-NPs the mean value was calculated and presented as one group in the figure. All plants were watered every other day with ¼ Hoagland solution. The control groups were nor exposed to Cu-NPs nor DDE, the other groups were exposed to 100, 200, 300, and 400 µg g^{-1} Cu-NPs. The 200 + DDE group was exposed to 200 µg g^{-1} Cu-NPs and watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. Results are presented as the percentage of endophytes that scored positive. OA: organic acids, ACC: 1-aminocyclopropane-1-carboxylate, IAA: indole-3-acetic acid, Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

B. Screening bacterial strains for potential DDE-degrading capacity in the presence of Cu-NPs

All isolated strains were screened for their potential to degrade DDE in the presence of Cu-NPs by performing an auxanography (Figure 5). Bacteria that were able to grow on the entire plate were considered neutral and are tolerant for DDE and Cu-NPs. Bacteria able to grow on the entire plate, except for the area covered with DDE and Cu-NPs, were considered negative. If the bacteria were able

to grow better in the DDE- and Cu-NPs-covered area, they are considered positive and are expected to use DDE as a carbon source, thus probably possessing the ability to degrade DDE, even in the presence of Cu-NPs (Figure 21).

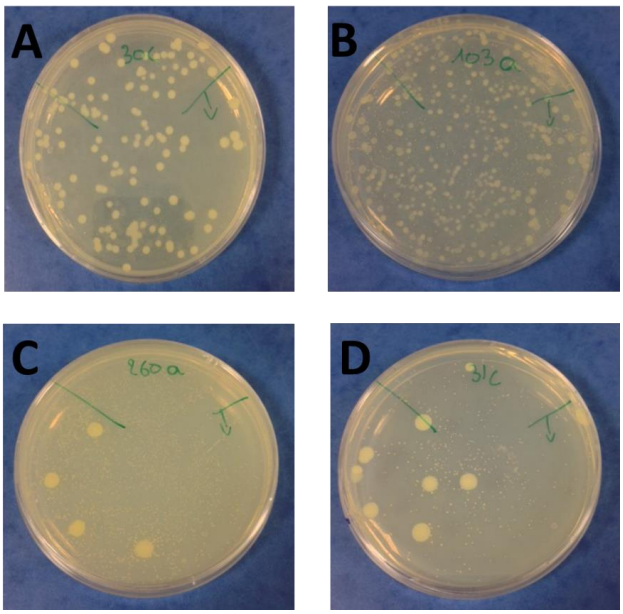

Figure 21: Auxanography to screen for the DDE-degrading capacity of the isolated strains in the presence of Cu-NPs. *Neutral auxanography response towards DDE and Cu-NPs by Enterobacter (A and B) and positive auxanography response towards DDE and Cu-NPs by Stenotrophomonas (C) and Enterobacter (D). Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

None of the tested strains were considered negative. Of all the strains tested (n=497), 98.59% of them scored neutral and 1.41% scored positive. Of those that tested positive, 43.00% were endophytic strains isolated from shoots and 57.00% were endophytes isolated from roots. *Enterobacter* made up 28.60% of the bacteria that tested positive, and *Stenotrophomonas* covered the remaining 71.40%. When looking at the different conditions to which the endophytic bacteria where exposed, 71.40% of those that scored positive and thus are able to grow in the presence of Cu-NPs and even prefer to grow when DDE is present, where originating from plants that were exposed to 200 µg g^{-1} Cu-NPs together with DDE. The remaining 28.60% of the endophytes that scored positive, were isolated from plants exposed to 100 µg g^{-1} Cu-NPs (14.30%) and isolated from the control group (14.30%). As 200 µg g^{-1} Cu-NPs was chosen to be the best concentration of Cu-NPs in this research, the results of the strains isolated from the control group, the group exposed to 200 µg g^{-1} Cu-NPs and the group exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE were compared (Figure 22). *Stenotrophomonas* and *Enterobacter* are two genera that score quite well. For shoots, only endophytes from the control group and the plants exposed to Cu-NPs and DDE were considered positive. For the control group, 100% of those that were considered positive, belonged to the genus *Enterobacter.* For the group exposed to Cu-NPs and DDE, 50% belonged to the genus *Enterobacter* and 50% belonged to the genus *Stenotrophomonas*. For roots,

only endophytes from the plants exposed to Cu-NPs and DDE scored positive, 100% of them belonged to the genus of *Stenotrophomonas.*

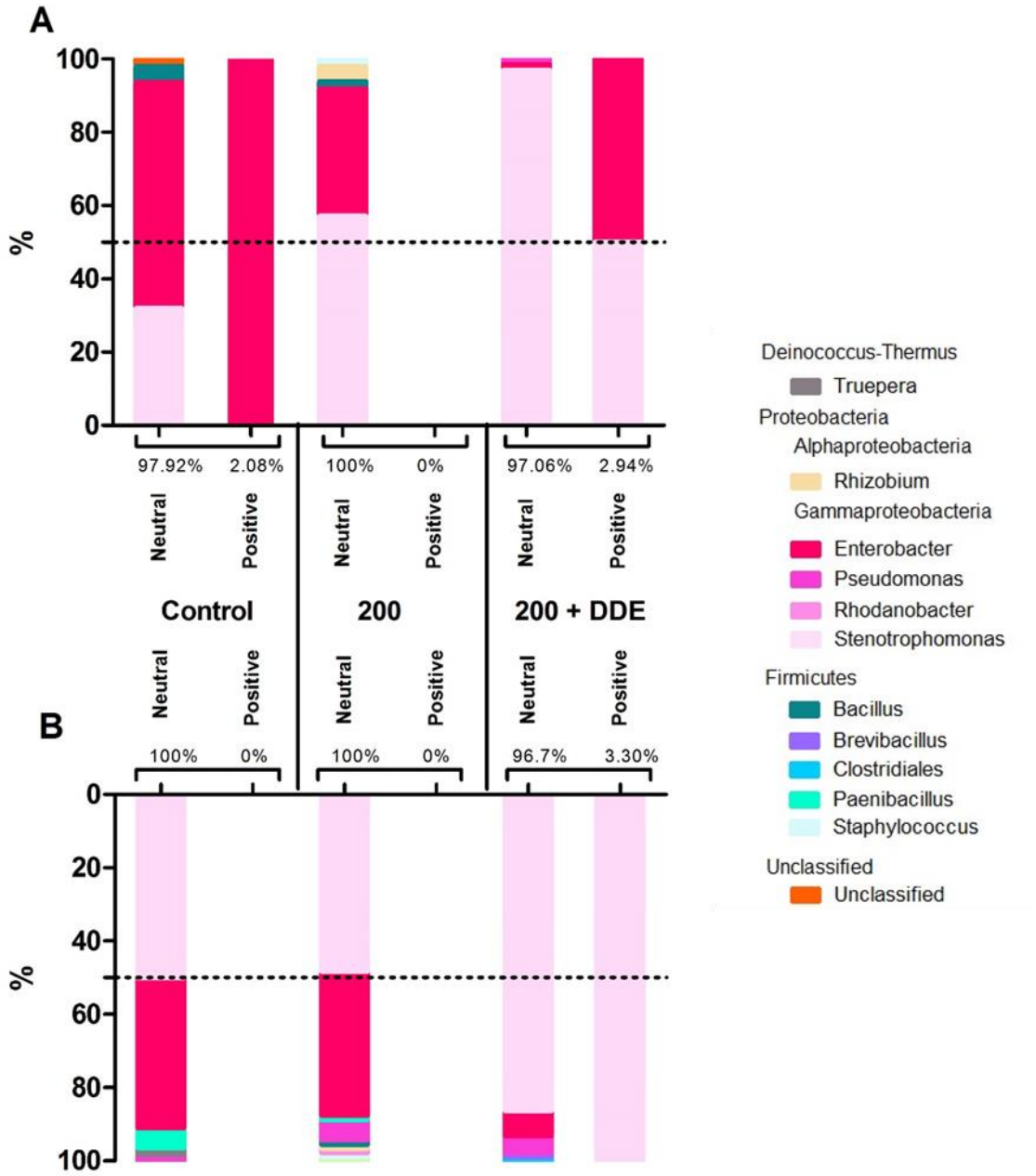

Figure 22: *Endophytes isolated from shoots (A) and roots (B) were screened for their potential to degrade DDE in the presence of Cu-NPs by performing an auxanography. The results of the genera isolated from the control groups (2 groups, for both n=10), the groups exposed to 200 µg g^{-1} Cu-NPs (2 groups, n=6 and n=12) and the group exposed to 200 µg g^{-1} Cu-NPs and 100 µg L^{-1} DDE (n=20) are shown. The x-axis represents the percentage of endophytes that were considered neutral or positive per condition. The y-axis represents the ratio of genera that were considered neutral or positive. For shoots, only endophytes from the control group and the plants exposed to Cu-NPs and DDE were considered positive. For the control group, 100% of those that were considered positive, belonged to the genus Enterobacter. For the group exposed to Cu-NPs and DDE, 50% belonged to the genus Enterobacter and 50% belonged to the genus Stenotrophomonas. For roots, only endophytes from the plants exposed to Cu-NPs and DDE scored positive, 100% of them belonged to the genus of Stenotrophomonas. Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.*

A high abundance of endophytes that scored neutral could be observed. These endophytes display a tolerance for DDE as well as for Cu-NPs. This suggest that they are able to grow in the presence of Cu-NPs and that they might possess the ability to degrade DDE when DDE is the only carbon source present. For the endophytes that tested positive, these results might suggest that endophytes present in plants grown in the presence of a certain amount of DDE were subjected to natural selection and probably have the potential to degrade DDE. However, 28.60% of the endophytes that tested positive, were isolated from plants exposed to Cu-NPs or plants from the control group. Bacteria often possess genes that are only activated when needed. A possible explanation for the observations could thus be that plants grown in the presence of Cu-NPs or plants from the control group do possess the genes needed for the degradation of DDE and these genes are activated when DDE is present. Additional tests for these specific endophytes that scored positive or neutral are needed to confirm their DDE-degrading capacity.

When considering the results of the phenotypic characterization of the isolated endophytes, no general toxic trend could be observed for the plant growth-promotion tests after exposure to Cu-NPs and/or DDE. For the DDE-degrading experiment, the combined exposure to DDE and Cu-NPs causes an increase in the number of endophytes that score positive for the test, which could be indicative for natural selection.

When looking at the different classes that are phenotypically characterized, the Gammaproteobacteria, from which *Stenotrophomonas* and *Enterobacter* stand out most, score best. This class accounts for more than 60% of the endophytes that scored positive for the plant growth-promotion tests and makes up 100% of the endophytes that scored positive in the DDE-degrading experiment. This might suggest that both genera had a selective advantage and were therefore able to dominate the community.

4 Conclusion

The main goal of this research was to examine whether combining Cu-NPs and specific endophytes could be an option to further enhance the phytoremediation efficiency of DDE by *C. pepo.* Therefore, in this work we tested (1) if Cu-NPs induce toxic effects at both the plant and bacterial community level and (2) what the combined effect is of DDE and Cu-NPs on the endophytic bacterial community of *C. pepo*. Primarily it was important to examine whether *C. pepo* was indeed capable of taking up the Cu-NPs and if so, whether or not they would increase the uptake of DDE. Both statements were confirmed and Cu-NPs even significantly enhanced the uptake of DDE. Further results of the study indicate that nor Cu-NPs nor the combination of Cu-NPs with DDE had a negative effect on plant growth. When looking at the effect on the endophytes present in *C. pepo,* results indicate that Cu-NPs alone significantly increased the colonies formed by Gammaproteobacteria in shoots, however this effect is not observed when plants are exposed to Cu-NPs combined with DDE. When plants are exposed to Cu-NPs in combination with DDE, significantly less endophytes were present in roots. Nevertheless, this effect is not fatal as certain genera were still able to grow and form high numbers of colonies in the presence of DDE and Cu-NPs. ,

When looking at the different classes that were genotypically and phenotypically characterized, the Gammaproteobacteria, from which *Stenotrophomonas* and *Enterobacter* stood out most, scored best. These are the genera that were able to grow best when exposed to Cu-NPs and DDE. In addition, the Gammaproteobacteria accounted for more than 60% of the endophytes that scored positive for the plant growth-promotion tests and made up 100% of the endophytes that scored positive in the DDE-degrading experiment. This might suggest that both genera have a selective advantage when residing in DDE-contaminated environments, such as possessing genes that enables them to produce the enzymes necessary for DDE-degradation and to produce compounds that stimulate plant growth, and were thus able to dominate the endophytic community.

When answering to the question whether the combination of Cu-NPs and specific endophytes provide the opportunity to enhance the phytoremediation efficiency of DDE, it can be stated that results are very promising. However, further research is needed. The findings of this study suggest tolerance of different bacterial strains to Cu-NPs combined with DDE. A future perspective is to perform inoculation experiments under greenhouse conditions with Cu-NPs and plant growth-promoting strains on DDE contaminated soils. This will give an indication whether findings of this study can be used to further optimize the endophyte-enhanced phytoremediation of DDE-contaminated soils with *C. pepo.*

5 Communication

Parts of this research will be published in A1 journals and presented at conferences:

Poster presentation at the 13th International Phytotechnologies Conference: Plant-Based Solutions for Environmental Problems from Lab to Field; September 26-28, 2016; Hangzhou City, Zhejiang Province, China.

> Using copper nanoparticles for phytoremediation of DDE-contamination: a screening for new possibilities. N. Eevers, K. Witters, J.C. White, N. Weyens, J. Vangronsveld.

Poster presentation at the 19th Mosa Conference; June 21-22, 2016; Maastricht, The Netherlands.

> Optimizing the efficiency of endophytic bacteria-enhanced phytoremediation of DDE-contaminated soils with Cu-NPs. K. Witters, N. Eevers, N. Weyens, J. Vangronsveld.

Chapter in the doctoral dissertation of Nele Eevers.

> Endophyte-enhanced phytoremediation of DDE-contaminated soils using *Cucurbita pepo.* N. Eevers, J.C. White, N. Weyens, J. Vangronsveld.

Article 'Optimizing the efficiency of endophytic bacteria-enhanced phytoremediation of DDE-contaminated soils with Cu-NPs'. N. Eevers, K. Witters, J.C. White, N. Weyens, J. Vangronsveld.

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Supplementary Information

Attachment 1: Specifications of the different components of used media

Supplementary table 1: ¼ Hoagland Solution. *Amount of the different components needed for the production of 1L ¼ Hoagland Solution.*

¼ Hoagland solution	
250 mL macro-elements	For 1L macro-elements:
	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 1L micro-elements :
	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 $H_2MoO_4 \cdot H_2O$
	0.22g $ZnSO_4 \cdot 7H_2O$
1.5 mL Fe-EDTA	For 1L Fe-EDTA:
	7.6g EDTA·Na
	5g $FeSO_4 \cdot 7H_2O$
746 mL deionized H_2O	

Supplementary table 2: *Concentrations of the different components needed for the production of bacterial growth media and the production of media for Plant Growth Promotion tests.*

	869 Medium	**284 + C**	**IAA Medium**	**NBRIP Medium**	**ST Medium**	**SMN Medium**
ACC						0.500
$CaCl_2.2H_20$	0.345	0.030	0.035			0.100
$Ca_3(PO_4)_2$				5.000		
$CoCl_2$		0.190				
$CoSO_4$						0.001
$CuCl_2$		0.017				
$CuSO_4.5H_2O$					0.0002	0.001
$FeCl_3$					0.001	
$Fe(III)NH_4$ Citrate		0.005				
$FeSO_4.7H_20$						0.005
Fructose		0.540				
Gluconate		0.660				
Glucose		0.520		10.000		1.000
Glucose D+	1.000		0.100			
H_3BO_3		0.062			0.002	0.002
KCl		1.490		0.200		
K_2HPO_4						2.000

KH_2PO_4						0.400
L-tryptophan			0.500			
Lactate		0.350				
Malic acid						1.000
Mannitol						1.000
$MgCl_2.6H_20$		0.200		5.000		
$MgSO_4.7H_20$				0.250		0.200
$MnCl_2$						
$MnCl_2.4H_2O$		0.100			0.0002	
$MnSO_4.4H_20$						0.003
Na-acetate						1.000
Na-citrate						1.000
$Na_2HP0_4.2H_20$		0.040				
Na_2MoO_4						0.001
$NaMoO_4.H_2O$		0.036			0.0002	
Na_2SO_4		0.430				
NaCl	5.000	4.680	0.500			
$(NH_4)2SO_4$				0.100		
NH_4Cl		1.070				
$NiCl_2$		0.024				
$NiSO_4$						0.001
Succinate		0.810				
Sucrose					20.000	1.000
Thiamine.HCl						
Tris-HCl		6.060				
Tryptone	10.000		1.000		5.000	
Yeast Extract	5.000		0.500			
$ZnCl_2$					0.003	
$ZnSO_4.7H_20$		0.144				0.005

Products are given in gram per liter deionized water. 869 is a rich growth medium. 284 is a selective growth medium with different carbon sources. IAA, NBRIP, ST and SMN are media for Plant Growth Promotion tests. In case of liquid media, gelling agents are not added. When needed, pH was adjusted with either HCl or KOH before autoclaving. Filter sterilization (Minisart single use filter unit 0.2 µm, Sartorius Stedim Biotech, Germany) was applied for the products marked in blue, before adding them to autoclaved media. IAA medium: Indole-3-acetic acid medium, NBRIP: National Botanical Research Institute's phosphate growth medium, ST medium: Sucrose Tryptone medium, SMN medium: Salt Minimal medium.

Attachment 2: Specifications of the different components of the used solutions

$MgSO_4$ 10 mM

2,4648 g in 1 L

15%w Glycerolstock

For 500 g:

- 75 g glycerol
- 4,25 g NaCl
- Dilute in distilled H_2O until 500 g is reached

DDE

100 µg L^{-1} DDE: 1000 µg in 1 mL methanol

- 100 µL in 900 µL water
- 1 mL in 1 L ¼ Hoagland

1.5% agarose gel

- 250 mL 1X TE
- 3.8 g agarose
- 25 µL GelRed

Salkowskireagent

- 49 ml 35% $HClO_4$ (70 % $HClO_4$, dilute to half)
- 1 ml 0.5 M FeCl3

Chroom-Azurol S (CAS)

For 25 mL CAS:

- 1.5 mL HDTMA
- 3.75 mL 10 mM HCl (10 mM: 0.00835 mL HCl Supra pur in 100 mL water)
- 0.375 mL FeCl3

Add slowly:

- 1.875 mL CAS
- 7.5 mL Piperazine
- 2.5 mL sulfosalicylic acid
- Add 100 mL deionized water to correct volume

DDE

For 100 mL of 50 mg L^{-1} DDE

- Dissolve 5 mg DDE in 1000 µL methanol
- Add 100 mL sterile dH_2O

Attachment 3: Specifications for the different Polymerase Chain Reactions

Supplementary table 3: *Products and their concentration needed for the mastermix of the test-PCR for pyrosequencing.*

Product	**Initial concentration**	**Volume (µL) for 8 reactions**
Molecular Grade Water		172.60
Fast Start HiFi buffer (10x) +MgCl	18 mM	22.00
dNTP mix	10 mM each nucleotide	4.40
Forward primer	10 µM	5.00
Reverse primer	10 µM	5.00
Platinum Taq Polymerase	5 U/µL	2.20
Template DNA		1.00 (1/50 dilution)
Mastermix volume		24.00
Total volume		25.00

HiFi: High Fidelity, dNTP: Deoxynucleotide, Forward primer 1: 341, Forward primer 2: 799, Forward primer 3: 799, Reverse primer 1: 783abc, Reverse primer 2: 1391, Reverse primer 3: 1193.

Supplementary table 4: *Concentration and volume of the products needed for the mastermix of PCR1 for pyrosequencing.*

Product	**Initial concentration**	**Volume (µL) for 40 reactions**
Molecular Grade Water		863.00
Fast Start HiFi buffer (10x) +MgCl	18 mM	110.00
dNTP mix	10 mM each nucleotide	22.00
Forward primer	10 µM	25.00
Reverse primer	10 µM	25.00
Platinum Taq Polymerase	5 U/µL	11.00
Template DNA		1.00 (1/50 dilution)
Mastermix volume		24.00
Total volume		25.00

HiFi: High Fidelity, dNTP: Deoxynucleotide, Forward primer: 799, Reverse primer: 1391.

Supplementary table 5: *Cycling conditions of the test-PCR and PCR1 for pyrosequencing.*

Phase	**Number of cycles**	**Duration**	**Temperature**
Initial denaturation	1	3 min	94°C
Denaturation	35	1 min	94°C
Annealing	1	1 min	53°C
Extension	1	1 min	72°C
Final extension	1	10 min	72°C
			Storage at 4°C

Supplementary table 6: *Concentration and volume of the products needed for the mastermix of PCR2 for pyrosequencing.*

Product	**Initial concentration**	**Volume (µL) for 33 reactions**
Molecular Grade Water		680.625
Fast Start HiFi buffer (10x) +MgCl	18 mM	90.75
dNTP mix	10 mM each nucleotide	18.15
Forward primer (967+MIDs)	10 µM	36.30
Reverse primer (1391+B-adaptor)	10 µM	36.30
Platinum Taq Polymerase	5 U/µL	9.075
Template DNA		1.00 (1/50 dilution)
Mastermix volume		24.00
Total volume		25.00

HiFi: High Fidelity, dNTP: Deoxynucleotide, MIDs: Multiplex identifiers

Supplementary table 7: *Cycling conditions of PCR2 reaction for pyrosequencing.*

Phase	**Number of cycles**	**Duration**	**Temperature**
Initial denaturation	1	3 min	94°C
Denaturation	25	1 min	94°C
Annealing	1	1 min	53°C
Extension	1	1 min	72°C
Final extension	1	10 min	72°C
			Storage at 4°C

Supplementary table 8: *Products and their concentration needed for the mastermix of the PCR for ARDRA.*

Product	**Initial concentration**	**Volume (µL) for 96 reactions**
HiFi PCR Buffer	10x	555.00
MgSO4	50 mM	222.00
dNTP-Mix	10 mM each	111.00
Bacteria specific 26F primer		111.00
Universal 1391R primer		111.00
HiFi Platinum TAQ DNA-polymerase		22.20
Rnase free water		4306.80
Template DNA		1.00
Mastermix volume		49.00
Total volume		50.00

HiFi: High Fidelity, dNTP: Deoxynucleotide, ARDRA: Amplified Ribosomal rDNA Restriction Analysis

Supplementary table 9: *Primers used to amplify bacterial 16S DNA.*

PCR	Primer	Primer Sequence (5'-3')	Target
ARDRA	26F	AGAGTTTGATCCTGGCTCAG	16S-23S internal transcribed spacer
	1391R	ACGGGCGGTGTGTRC	16S bp 1392-1406

ARDRA: Amplified Ribosomal DNA Restriction Analysis, bp: base pairs

Supplementary table 10: *Cycling conditions of the PCR for the amplification of the 16S rRNA gene.*

Phase	Number of cycles	Duration	Temperature
Initial denaturation	1	5 min	95°C
Denaturation	35	1 min	94°C
Annealing	35	30 sec	52°C
Extension	35	3 min	72°C
Final extension	1	10 min	72°C
			Storage at 4°C

Supplementary table 11: *Products and their concentration needed for the digestion mastermix for ARDRA.*

Product	Initial concentration	Volume (µL) for 96 reactions
NEB Buffer	10x	302.90
HPYCH4IV	10 000U/mL	30.30
Rnase	1%	121.10
Rnase free water		
Template DNA		20.00
Mastermix volume		8.60
Total volume		28.60

NEB: New England Biolabs, ARDRA: Amplified Ribosomal rDNA restriction Analysis

Attachment 4: Protocols

Protocol 1: Invisorb® Spin Plant Mini Kit - DNA extraction from fresh or dried plant material and from food of plant origin (fresh, frozen or dried material)

Important *Transfer the needed amount of* ***Elution Buffer*** *into 2.0 mL Receiver Tube (not included in the kit) and place the tube a 65°C.*

1. Homogenization of the starting material

Homogenize about 60 mg of starting material by a pestle under liquid N_2.

Commercially available equipment for homogenization also can be used.

Note: *Use 120-180 mg of starting material if extraction from material containing more water (fruits, algae).*

2. Lysis of the starting material

Transfer the "plant powder" into a 1.5 mL reaction tube. Add 400 µL **Lysis Buffer P** and 20 µL **Proteinase K** and vortex briefly. Incubation at 65°C for 30 min or longer (incubation in a thermo mixer under continuous shaking is recommended). During incubation place the Prefilter into a 2.0 mL Receiver Tube.

3. Filtration of Lysis Solution and realizing optimum binding conditions

Transfer of Lysis Solution onto the Prefilter. Centrifuge for 1 min at 11.100 x g (11.000 rpm). Discard the Prefilter. Add 200 µL of **Binding Buffer A** and vortex thoroughly.

Note: *To remove RNA (if it is necessary) from the sample add 40 µL of RNase A to the filtrate (10 mg/mL), vortex briefly and incubate for 5 min at room temperature.*

4. DNA Binding

Place a Spin Filter into a 2.0 mL Receiver Tube. Transfer the suspension onto the Spin Filter and incubate for 1 min. Centrifuge at 11.100 x g (11.000 rpm) for 2 min. Discard the filtrate and place the Spin Filter again into the 2.0 mL Receiver Tube.

5. Washing I

Add 550 µL **Wash Buffer I** and centrifuge at 11.100 x g (11.000 rpm) for 1min. Discard the filtrate, place the Spin Filter again into the 2.0 mL Receiver Tube.

6. Washing II

Add 550 µL **Wash Buffer II** and centrifuge at 11.100 x g (11.000 rpm) for 1min. Discard the filtrate, place the Spin Filter again into the Receiver Tube and repeat the washing step once again. Finally discard the filtrate and centrifuge for 4 min at 11.100 x g (11.000 rpm) to remove residual ethanol.

7. Elution of the DNA

Place the Spin Filter into a new 1.5 mL Receiver Tube and add 100 µL of the prewarmed Elution Buffer. Incubate for 3 min. Centrifuge for 1 min at 11.100 x g (11.000 rpm).

Note: *The DNA can also be eluted with a lower or a higher volume of Elution Buffer (depends on the expected yield of genomic DNA). But pay attention, that minimum volume for the elution is 50 µL. If quite large amount of DNA is expected, the volume of elution can be increased (100-200 µL).*

To maximize the final yield we recommend a second elution step with the equal volume of Elution Buffer.

Protocol 2: DNeasy blood & tissue kit, Qiagen – Rapid purification of total DNA

Preparation

1. Cultivate bacteria (5µL) in rich (869) medium (1mL) in masterblocks for 3 days at 30°C.
2. Prepare the enzymatic lysis buffer for 50 samples (25 samples)
    - 180 µL (90 µL) 2x Tris-EDTA (TE) buffer pH 8 (1/50 diluted from 100x)
    - 108 mg (54 mg) 1,2 % Triton X-100
    - 180 mg (90 mg) lysozyme (add right before use)
    - 8820 µL (4410 µL) RNase free water

Procedure

1. Centrifugate 1,5 mL bacterial solution 10 min at 7500 rpm in microcentrifuge tubes and discard the supernatans.
2. Resuspend the pellet in 180 µL enzymatic lysis buffer.
3. Incubate for at least 30 min at 37°C.
4. Add 25 µL proteïnase K and 200 µL AL buffer , vortex.
5. Incubate 30 min at 56°C.

6. Add 200 µL ethanol (96-100 %), vortex.
7. Apply the sample to the DNeasy mini spin columns, centrifuge 1 min at 8000 rpm, discard tube with the flow-through.
8. Place the DNeasy mini spin columns into a new tube, add 500 µL buffer AW1, centrifuge 1 min at 8000 rpm, discard tube with the flow-through.
9. Place the DNeasy mini spin columns again into a new tube, add 500 µL buffer AW2, centrifuge 5 min at 13200 rpm, discard tube with the flow-through.
10. Place DNeasy mini spin columns in 1,5 of 2 mL microcentrifugetubes, apply 150 µL buffer AE to the DNeasy membrane. Incubate at roomtemperature for 1 min, centrifuge 1 min op 8000 rpm.
11. Repeat step 10.
12. Store DNA at 4°C.

Protocol 3: Qiaquick Gel extraction Kit, Qiagen

Notes before starting

- This protocol is for the purification of up to 10 µg DNA (70 bp to 10 kb).
- The yellow color of Buffer QG indicates a pH ≤7.5. DNA adsorption to the membrane is only efficient at pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg gel ~ 100 µL). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of

the mixture is orange or violet, add 10 µL 3 M sodium acetate, pH 5.0, and mix. The mixture turns yellow.

4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 mL collection tube or into a vacuum manifold. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µL, load and spin/apply vacuum again.
6. If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 500 µL Buffer QG to the QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
7. To wash, add 750 µL Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube. Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, bluntended ligation), let the column stand 2–5 min after addition of Buffer PE. Centrifuge the QIAquick column in the provided 2 mL collection tube for 1 min to remove residual wash buffer.
8. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
9. To elute DNA, add 50 µL Buffer EB (10 mM Tris · Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µL Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

Protocol 4: Qiaquick PCR Purification kit, Qiagen

Notes before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µL 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in a provided 2 mL collection tube or into a vacuum manifold. For details on how to set up a vacuum manifold, refer to the QIAquick Spin Handbook.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 mL Buffer PE to the QIAquick column centrifuge for 30–60 s or apply vacuum. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 mL collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, add 50 µL Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0– 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

Protocol 5: Quant-iT PicoGreen dsDNA Assay Kit

Allow the **Quant-iT Picogreen** reagent to warm to room temperature before opening and protect from light.

Preparing assay buffer

20X TE buffer: used for dilution of Picogreen reagent, DNA samples and assay itself

Prepare **1X TE buffer**:

40ml: 2ml 20X TE + 38ml molecular grade H_2O

Preparing Picogreen reagent

In plastic as reagent may absorb to glass surface

Protect from light as reagent is susceptible to photodegradation

Use solution within few hours of preparation

200 assays: 100µl picogreen + 19.9ml 1X TE

50 assays: 25µl picogreen + 4.975ml 1x TE

40 assays: 20µl picogreen + 4.980ml 1x TE

DNA standard curve

1. Prepare a **stock solution of dsDNA** in 1x TE: use bacteriophage λ of calf thymus DNA

   2µg/ml: 30µl bacteriophage λ (100µg/ml) + 1.47ml 1X TE → *use for high range*

   50ng/ml: 37.5µl of 2µg/ml stock solution + 1.4625ml 1X TE → *use for low range*

2. **High range** standard curve (1ng/ml – 1µg/ml)

Volume (µl) of 1X TE	**Volume (µl) of 2µg/ml DNA stock**	**Final DNA concentration**
0	1000	1 µg/mL
900	100	100 ng/mL
990	10	10 ng/mL
999	1	1 ng/mL
1000	0	blank

1µg = 1000ng, 1ng = 1000pg

3. **Low range** standard curve (25pg/ml – 25ng/ml)

Volume (µl) of 1X TE	**Volume (µl) of 50ng/ml DNA stock**	**Final DNA concentration**
0	1000	25 ng/mL
900	100	2.5 ng/mL
990	10	250 pg/mL
999	1	25 pg/mL
1000	0	blank

1µg = 1000ng, 1ng = 1000pg

Sampling

Black 96-well plate

1. High range: 100µl of each dilution + 100µl Picogreen reagent
2. Low range: 100µl of each dilution + 100µl Picogreen reagent
3. Samples: 1µl sample + 99µl 1X TE reagent + 100µl Picogreen reagent

Sample analysis

Settings microplate reader:

- Filter: excitation 485nm, emission 520nm
- Number of flashes: 20-200
- Gain and focus: perform automated gain and focus adjustment

Subtract the fluorescence value of the **reagent blank** from that of each of the samples (noise of Picogreen reagent).

Protocol 6: Screening endophytic bacteria for *in vitro* plant-growth-promotion-traits

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A. Bacterial IAA production

Tryptophan doesn't dissolve in water, therefore solve it in a minimal volume (± 40 ml) of 0.1M HCl, bring it to pH 6-7 with KOH, filter sterilize it with a blue 0.20 µm syringe filter and add it to the sterile 1/10 869 medium. Do not use NaOH to bring the tryptophan-solution to a pH of 6-7, because it can make a false positive in the IAA test. Tryptophan is sensible to light, so always store in the dark.

Salkowski reagent:

49 ml 35% $HClO_4$ (70 % $HClO_4$, dilute to half)

1 ml 0.5 M $FeCl_3$

Under laminar air flow:

1. Insert 5µL of bacterial stock in a masterblock with 1 mL IAA medium. Incubate for four days at 30°C, 150 rpm in the dark (wrap in aluminium foil)
2. Centrifuge for 20 minutes at 2000 rpm.

Under fume hood:

3. Take 0.5 ml supernatans and add 1 ml Salkowskireagent.
4. Vortex, 3min, 300rpm
5. Wait for 20 minutes. Pink colour means positive for IAA production, yellow is negative.

B. Bacterial ACC-deaminase activity

Work under laminar air flow:

1. Cultivate bacteria (5µL) in a sterile masterblock with 1 mL rich medium (869) for four days at 30°C at 150 rpm.
2. Centrifuge at 2000 rpm during 20 min at room temperature.

3. Discard supernatans.
4. Add 1mL SMN medium with 5 mM ACC as N-source to the pellet.
5. Incubate for three days at 30°C.
6. Centrifuge at 2000 rpm for 20 min.

Work under the fume hood:

7. Discard supernatans and resuspend pellets in 100 µL 0,1M Tris-HCl buffer (pH 8,5).
8. Add 3µL toluene for cell lysis and vortex for 5 min.
9. Add 10µL 0,5 M ACC and 100 µL 0,1M Tris-HCl buffer (pH 8,5), vortex and incubate for 30 min at 30°C and 150 rpm.
10. Add 690 µL 0,56N HCl *[4,678 mL 37% HCl in 100 mL water]* and 150 µL 0,2 % 2,4-dinitrophenylhydrazine in 2N HCl *[1,67 mL 37 % HCl in 10 mL water and add 20 mg D028]*
11. Add 1 mL 2N NaOH *[7,998g N027 in 100 mL water]*.
12. Evaluate the results obtained. Negative: Yellow, Positive: Brown.

C. Siderophores

1. Grow bacteria (5µL) in rich (869) medium (1mL) in sterile masterblocks for four days at 30°C at 150rpm.
2. Fill new sterile masterblocks with 800 µL 284 medium (0 µM, 0,25 µM en 3 µM Fe).
3. Add 20 µL of the bacterial suspension.
4. Incubate for six days at 30°C and 120rpm.
5. Add 100 µL Chroom-Azurol S (CAS). (method of Schwyn and Nielands (1987))

   CAS 25 mL (10 mL)

   - 1,5 mL HDTMA (0,6 mL) *[10 mM: 0,182 g H016 in 50 mL water]**
     - ➔ Put at 60°C to solve
   - 3,75 mL 10 mM HCl (1,5 mL) *[10 mM: 0,0835 mL HCl supra pur in 100 mL water]**
   - 0,375 mL $FeCl_3$ (0,1 mL) *[1 mM: 0,0811 g I004 in 50 mL water, dilute 1/10, always make fresh]*

   Add slowly:

   - 1,875 mL CAS (0,75 mL) *[2 mM: 0,0605 g C043 in 50 mL water]**
   - 7,5 mL Piperazine (3 mL) *[3,589 g P017 in 25 mL water, op pH 5,6 met HCl pure]**
   - 2,5 mL sulfosalicilic acid (1 mL) *[40 mM: 0,508 g S010 in 50 mL water]**
   - Add water to correct volume

* Are in fridge

6. After 4 hours, orange = positive, blue = negative (chelator takes Fe from coloring, blue turns orange)

D. Organic acids

1. Grow bacteria (5µL) in rich (869) medium (1mL) in sterile masterblocks for four days at 30°C at 150rpm.
2. Fill 48-well plates with 800 µL ST-medium.
3. Add 20 µL of the bacterial suspension.
4. Incubate for six days at 30°C and 120 rpm.
5. Add 100 µL alizarine red S 0,1 % (0,1 g A037 in 100 mL)
6. After 15 min: yellow = positive, pink = negative

E. Phosphate solubilization

Work under the flow:

1. Grow the bacteria (5µL) in rich (869) medium (1mL) in sterile masterblocks for four days at 30°C at 150rpm.
2. Add the NBRIP medium to petri dishes, and make a hole with a sterile pipette (10mL) when the medium is solid, to inoculate later the bacterial suspension into that hole.
3. Inoculate bacterial suspension on NBRIP medium (50 µl per hole)
4. Incubate plates at 30°C for 12 days and evaluate halo (solubilisation zone) around the colonies.

Attachment 5: Genotypic and phenotypic characterization of isolated strains

Supplementary table 12*: Overview of the results of the plant growth-promotion tests of all the strains isolated from roots and shoots of plants exposed to different conditions.*

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
1	**CONTROL**	R	*Enterobacter*	63.16	-	+	+	+	+	+	+	0
2	**CONTROL**	R	*Enterobacter*	63.16	-	+	+	+	+	+	+	0
3	**CONTROL**	R	*Enterobacter*	2.63	-	+	+	+	+	-	+	0
4	**CONTROL**	R	*Enterobacter*	2.63	-	+	+	+	+	+	+	0
5	**CONTROL**	R	*Enterobacter*	10.53	-	+	+	+	-	+	+	0
6	**CONTROL**	R	*Stenotrophomonas*	10.53	-	+	+	+	+	+	+	0
7	**CONTROL**	R	*Stenotrophomonas*	50.00	-	+	+	+	+	+	+	0
8	**CONTROL**	R	*Enterobacter*	50.00	-	+	+	+	+	+	+	0
20	**CONTROL**	R	*Stenotrophomonas*	11.85	-	+	+	+	+	+	+	0
21	**CONTROL**	R	*Stenotrophomonas*	11.85	-	+	+	+	+	+	-	0
22	**CONTROL**	R	*Stenotrophomonas*	27.49	-	+	+	+	+	+	-	0
23	**CONTROL**	R	*Stenotrophomonas*	27.49	-	+	+	+	+	+	-	0
24	**CONTROL**	R	*Enterobacter*	39.34	-	+	+	+	+	+	+	0
25	**CONTROL**	R	*Enterobacter*	39.34	-	+	+	-	+	+	+	0
26	**CONTROL**	R	*Enterobacter*	2.37	-	+	+	+	+	+	+	0
27	**CONTROL**	R	*Stenotrophomonas*	2.37	-	+	+	+	+	-	+	0
28	**CONTROL**	R	*Truepera*	2.37	-	+	+	+	+	+	+	0
41	**CONTROL**	R	*Stenotrophomonas*	6.43	-	+	+	+	+	-	+	0
42	**CONTROL**	R	*Stenotrophomonas*	6.43	-	+	+	+	+	+	+	0
43	**CONTROL**	R	*Stenotrophomonas*	11.43	-	+	+	+	+	+	+	0
44	**CONTROL**	R	*Stenotrophomonas*	11.43	-	+	+	+	+	+	+	0
45	**CONTROL**	R	*Stenotrophomonas*	845.71	-	+	+	+	+	+	+	0
46	**CONTROL**	R	*Stenotrophomonas*	0.71	-	+	+	+	+	+	-	0
52	**CONTROL**	R	*Enterobacter*	25.18	-	+	+	+	+	+	+	0
53	**CONTROL**	R	*Enterobacter*	25.18	-	+	+	+	+	-	+	0
54	**CONTROL**	R	*Enterobacter*	845.71	-	+	+	-	+	+	+	0
55	**CONTROL**	R	*Enterobacter*	21.76	-	+	+	-	+	+	+	0
56	**CONTROL**	R	*Enterobacter*	21.76	-	+	+	+	+	+	+	0
57	**CONTROL**	R	*Enterobacter*	4.65	-	+	+	+	+	+	+	0
58	**CONTROL**	R	*Enterobacter*	4.65	-	+	+	+	+	+	+	0
69	**CONTROL**	R	*Enterobacter*	5.12	-	+	+	+	+	+	+	0
70	**CONTROL**	R	*Enterobacter*	5.12	-	+	+	+	+	-	+	0
71	**CONTROL**	R	*Paenibacillus*	17.06	-	+	+	-	+	+	+	0
72	**CONTROL**	R	*Enterobacter*	17.06	-	+	+	+	+	+	+	0
73	**CONTROL**	R	*Enterobacter*	1.37	-	+	+	+	+	+	+	0
74	**CONTROL**	R	*Enterobacter*	1.37	-	+	+	+	+	+	+	0
75	**CONTROL**	R	*Enterobacter*	1.37	-	+	+	-	+	+	+	0
76	**CONTROL**	R	*Pseudomonas*	1.37	+	+	+	+	+	-	+	0
77	**CONTROL**	R	*Stenotrophomonas*	29.69	-	+	+	-	+	+	+	0
78	**CONTROL**	R	*Enterobacter*	29.69	-	+	+	-	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
79	**CONTROL**	R	*Paenibacillus*	29.69	-	+	-	-	+	+	+	0
80	**CONTROL**	R	*Stenotrophomonas*	24.23	-	+	+	+	+	+	+	0
81	**CONTROL**	R	*Paenibacillus*	24.23	-	+	-	-	+	+	+	0
82	**CONTROL**	R	*Stenotrophomonas*	24.23	-	+	+	+	+	+	+	0
83	**CONTROL**	R	*Stenotrophomonas*	0.68	-	+	+	+	+	+	+	0
84	**CONTROL**	R	*Stenotrophomonas*	0.68	-	+	+	+	+	+	+	0
95	**CONTROL**	R	*Stenotrophomonas*	10.48	-	+	+	-	-	+	+	0
96	**CONTROL**	R	*Stenotrophomonas*	10.48	-	+	+	+	-	+	+	0
97	**CONTROL**	R	*Stenotrophomonas*	0.95	-	+	+	+	+	+	+	0
98	**CONTROL**	R	*Stenotrophomonas*	1904.76	-	+	+	+	+	+	+	0
99	**CONTROL**	R	*Stenotrophomonas*	1904.76	-	-	+	+	+	+	+	0
100	**CONTROL**	R	*Stenotrophomonas*	2.86	-	+	+	+	-	+	+	0
101	**CONTROL**	R	*Stenotrophomonas*	2.86	-	+	+	+	+	+	+	0
102	**CONTROL**	R	*Stenotrophomonas*	14.29	-	+	+	+	+	+	+	0
103	**CONTROL**	R	*Stenotrophomonas*	14.29	-	+	+	+	+	+	+	0
104	**CONTROL**	R	*Stenotrophomonas*	0.95	-	+	+	+	+	+	+	0
9	**CONTROL**	S	*Enterobacter*	0.59	-	+	+	+	-	+	+	0
10	**CONTROL**	S	*Enterobacter*	3.02	-	+	+	+	+	+	+	0
11	**CONTROL**	S	*Enterobacter*	3.02	-	+	+	+	-	+	+	0
12	**CONTROL**	S	*Enterobacter*	3.52	-	+	+	+	-	+	+	0
13	**CONTROL**	S	*Stenotrophomonas*	3.52	-	+	+	+	+	-	+	0
14	**CONTROL**	S	*Stenotrophomonas*	19.60	-	+	+	+	+	+	+	0
15	**CONTROL**	S	*Stenotrophomonas*	19.60	-	+	+	+	+	-	+	0
16	**CONTROL**	S	*Stenotrophomonas*	35.18	-	+	+	+	+	+	+	0
17	**CONTROL**	S	*Stenotrophomonas*	35.18	-	+	+	+	-	-	+	0
18	**CONTROL**	S	*Stenotrophomonas*	149.75	-	+	+	+	+	+	+	0
19	**CONTROL**	S	*Stenotrophomonas*	149.75	-	+	+	+	+	+	+	0
29	**CONTROL**	S	*Enterobacter*	1.79	-	+	+	+	+	-	+	0
30	**CONTROL**	S	*Enterobacter*	1.79	-	+	+	-	+	+	+	0
31	**CONTROL**	S	*Enterobacter*	2.15	-	+	+	+	+	+	+	0
32	**CONTROL**	S	*Enterobacter*	2.15	-	+	+	+	+	+	+	0
33	**CONTROL**	S	*Enterobacter*	10.39	-	+	+	+	+	+	+	0
34	**CONTROL**	S	*Enterobacter*	10.39	-	+	+	+	+	+	+	0
35	**CONTROL**	S	*Enterobacter*	1.08	-	+	+	+	+	+	+	0
36	**CONTROL**	S	*Enterobacter*	1.08	-	+	+	+	+	+	+	0
37	**CONTROL**	S	*Enterobacter*	0.72	-	+	+	+	+	+	+	0
38	**CONTROL**	S	*Enterobacter*	0.72	-	+	+	-	+	+	+	0
39	**CONTROL**	S	*Enterobacter*	4.66	-	+	+	+	+	-	+	0
40	**CONTROL**	S	*Enterobacter*	4.66	-	+	+	+	+	+	+	0
47	**CONTROL**	S	*Stenotrophomonas*	0.24	-	+	+	+	+	+	-	0
48	**CONTROL**	S	*Stenotrophomonas*	2.17	-	+	+	+	+	+	-	0
49	**CONTROL**	S	*Enterobacter*	2.17	-	+	+	+	+	-	+	0
50	**CONTROL**	S	*Enterobacter*	3.14	-	+	+	-	+	-	+	0
51	**CONTROL**	S	*Enterobacter*	3.14	-	+	+	-	+	-	+	0
59	**CONTROL**	S	*Enterobacter*	3.74	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
60	**CONTROL**	S	*Enterobacter*	3.74	-	+	+	+	+	+	+	+
61	**CONTROL**	S	*Enterobacter*	9.77	+	+	+	+	+	+	+	0
62	**CONTROL**	S	*Enterobacter*	9.77	-	+	+	-	+	-	+	0
63	**CONTROL**	S	*Enterobacter*	478.16	-	+	+	-	+	-	+	0
64	**CONTROL**	S	*Enterobacter*	478.16	-	+	+	+	+	-	+	0
65	**CONTROL**	S	*Enterobacter*	0.86	-	+	+	+	+	+	+	0
66	**CONTROL**	S	*Enterobacter*	0.86	+	+	+	-	+	+	+	0
67	**CONTROL**	S	*Enterobacter*	1.44	-	+	+	+	+	+	+	0
68	**CONTROL**	S	*Enterobacter*	1.44	-	+	+	+	+	+	+	0
85	**CONTROL**	S	*Brevibacillus*	10.70	-	+	+	-	-	+	+	0
86	**CONTROL**	S	*Brevibacillus*	10.70	-	+	+	-	-	-	+	0
87	**CONTROL**	S	*Stenotrophomonas*	44.19	-	-	+	+	-	+	+	0
88	**CONTROL**	S	*Stenotrophomonas*	44.19	-	+	+	+	-	+	+	0
89	**CONTROL**	S	*Stenotrophomonas*	104.19	-	+	+	+	-	+	+	0
90	**CONTROL**	S	*Stenotrophomonas*	104.19	-	+	+	+	-	+	+	0
91	**CONTROL**	S	*Enterobacter*	11.63	-	+	+	+	-	+	+	0
92	**CONTROL**	S	*Stenotrophomonas*	11.63	-	+	+	+	-	+	+	0
93	**CONTROL**	S	*Stenotrophomonas*	1.40	-	+	+	+	+	+	+	0
94	**CONTROL**	S	*Non identifiable*	1.40	-	+	+	+	-	+	+	0
105	**100**	R	*Stenotrophomonas*	14.35	-	+	+	+	-	+	+	+
106	**100**	R	*Stenotrophomonas*	14.35	-	+	+	+	+	+	+	0
107	**100**	R	*Stenotrophomonas*	1.35	-	+	+	+	+	+	+	0
108	**100**	R	*Stenotrophomonas*	1.35	-	+	+	+	-	+	+	0
109	**100**	R	*Stenotrophomonas*	3.14	-	+	+	+	+	+	+	0
110	**100**	R	*Enterobacter*	3.14	-	+	+	+	+	+	+	0
111	**100**	R	*Enterobacter*	3.14	-	+	+	+	+	+	+	0
112	**100**	R	*Enterobacter*	3.14	-	+	+	-	+	+	+	0
113	**100**	R	*Enterobacter*	49.33	-	+	+	+	+	+	+	0
114	**100**	R	*Enterobacter*	49.33	-	+	+	+	+	+	+	0
115	**100**	R	*Enterobacter*	87.44	-	+	+	+	+	+	+	0
116	**100**	R	*Enterobacter*	87.44	-	+	+	+	+	+	+	0
117	**100**	R	*Enterobacter*	1.79	-	+	+	+	+	+	+	0
118	**100**	R	*Enterobacter*	1.79	-	+	+	-	+	+	+	0
119	**100**	R	*Enterobacter*	0.45	-	+	+	-	+	+	+	0
125	**100**	R	*Enterobacter*	57.85	-	+	+	-	+	+	+	0
126	**100**	R	*Enterobacter*	57.85	-	+	+	+	+	+	+	0
127	**100**	R	*Enterobacter*	57.85	-	+	+	+	+	+	+	0
128	**100**	R	*Enterobacter*	22.31	-	+	+	+	+	+	+	0
129	**100**	R	*Enterobacter*	22.31	-	+	+	-	+	+	+	0
130	**100**	R	*Enterobacter*	1652.89	-	+	+	-	+	+	+	0
131	**100**	R	*Enterobacter*	1652.89	-	+	+	+	+	+	+	0
132	**100**	R	*Enterobacter*	3.31	-	+	+	+	+	+	+	0
133	**100**	R	*Enterobacter*	3.31	-	+	+	+	+	+	+	0
134	**100**	R	*Enterobacter*	3.31	-	+	+	+	+	+	+	0
135	**100**	R	*Enterobacter*	4.96	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0μM)	SID (0.25μM)	SID (3μM)	ACC	IAA	P	Aux.
136	**100**	R	*Enterobacter*	4.96	-	+	+	+	+	+	+	0
137	**100**	R	*Enterobacter*	47.11	-	+	+	+	+	+	+	0
138	**100**	R	*Enterobacter*	47.11	-	+	+	-	+	+	+	0
139	**100**	R	*Enterobacter*	47.11	-	+	+	+	+	+	+	0
140	**100**	R	*Pseudomonas*	1.09	-	+	+	+	+	+	+	0
141	**100**	R	*Pseudomonas*	1.09	-	+	+	-	+	+	+	0
142	**100**	R	*Enterobacter*	36.96	-	+	+	+	+	+	+	0
143	**100**	R	*Enterobacter*	36.96	-	+	+	+	+	+	+	0
144	**100**	R	*Pseudomonas*	1086.96	-	+	+	+	+	-	+	0
145	**100**	R	*Pseudomonas*	1086.96	-	+	+	+	+	+	+	0
146	**100**	R	*Pseudomonas*	0.54	-	+	+	+	+	+	+	0
147	**100**	R	*Pseudomonas*	1.09	-	+	+	-	+	+	+	0
148	**100**	R	*Pseudomonas*	1.09	-	+	+	+	+	+	+	0
120	**100**	S	*Enterobacter*	31.34	-	+	+	+	+	+	+	0
121	**100**	S	*Enterobacter*	60.07	-	+	+	+	+	+	+	0
122	**100**	S	*Enterobacter*	60.07	-	+	+	+	+	+	+	0
123	**100**	S	*Enterobacter*	60.07	-	+	+	-	+	+	+	0
124	**100**	S	*Enterobacter*	0.63	-	+	+	+	+	+	+	0
149	**100**	S	*Pseudomonas*	6.47	-	+	+	+	+	+	+	0
150	**100**	S	*Enterobacter*	6.47	-	+	+	+	+	+	+	0
151	**200**	R	*Enterobacter*	1.15	-	+	+	+	+	+	+	0
152	**200**	R	*Enterobacter*	1.15	-	+	+	-	+	+	+	0
153	**200**	R	*Pseudomonas*	0.57	-	+	+	-	+	+	+	0
154	**200**	R	*Pseudomonas*	0.57	-	+	+	+	+	+	+	0
155	**200**	R	*Pseudomonas*	45.69	-	+	+	+	+	+	+	0
156	**200**	R	*Pseudomonas*	45.69	-	+	+	+	+	+	+	0
157	**200**	R	*Pseudomonas*	66.67	-	+	+	+	+	+	+	0
158	**200**	R	*Enterobacter*	66.67	-	+	+	+	+	+	+	0
159	**200**	R	*Enterobacter*	0.29	-	+	+	+	+	+	+	0
166	**200**	R	*Enterobacter*	38610.04	-	+	+	-	+	+	+	0
167	**200**	R	*Enterobacter*	38610.04	-	+	+	+	+	+	+	0
168	**200**	R	*Enterobacter*	11.97	-	+	+	+	+	-	+	0
169	**200**	R	*Streptomyces*	11.97	-	+	+	+	+	+	+	0
170	**200**	R	*Enterobacter*	2.32	-	+	+	+	+	+	+	0
171	**200**	R	*Enterobacter*	2.32	-	+	+	+	+	+	+	0
172	**200**	R	*Enterobacter*	0.77	-	+	+	-	+	+	+	0
173	**200**	R	*Bacillus*	0.77	-	+	+	-	+	+	+	0
174	**200**	R	*Enterobacter*	6.18	-	+	+	+	+	+	+	0
175	**200**	R	*Enterobacter*	6.18	-	+	+	-	+	+	+	0
176	**200**	R	*Enterobacter*	6.18	-	+	+	+	+	+	+	0
180	**200**	R	*Enterobacter*	1.77	-	+	+	+	+	+	+	0
181	**200**	R	*Enterobacter*	1.77	-	+	+	+	+	+	+	0
182	**200**	R	*Enterobacter*	19.50	-	+	+	+	+	+	+	0
183	**200**	R	*Enterobacter*	19.50	-	+	+	+	+	+	+	0
184	**200**	R	*Enterobacter*	7.09	-	+	+	+	-	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
185	**200**	R	*Enterobacter*	7.09	-	+	+	+	+	+	+	0
160	**200**	S	*Enterobacter*	3.39	-	+	+	+	+	+	+	0
161	**200**	S	*Enterobacter*	3.39	-	+	+	+	+	+	+	0
162	**200**	S	*Enterobacter*	3.39	-	+	+	+	+	+	-	0
163	**200**	S	*Enterobacter*	35.31	-	+	+	+	+	+	+	0
164	**200**	S	*Enterobacter*	35.31	-	+	+	+	+	+	+	0
165	**200**	S	*Enterobacter*	0.28	-	+	+	-	+	+	+	0
177	**200**	S	*Enterobacter*	29.37	-	+	+	+	+	+	+	0
178	**200**	S	*Enterobacter*	15.03	-	+	+	+	+	+	+	0
179	**200**	S	*Stenotrophomonas*	15.03	-	+	+	-	+	+	+	0
186	**200**	S	*Enterobacter*	0.94	-	+	+	+	+	+	+	0
187	**200**	S	*Enterobacter*	0.94	-	+	+	+	+	+	+	0
188	**200**	S	*Enterobacter*	4.38	-	+	+	+	+	+	+	0
189	**200**	S	*Enterobacter*	4.38	-	+	+	+	+	+	+	0
190	**200**	S	*Enterobacter*	0.31	-	+	+	+	+	+	+	0
191	**300**	R	*Enterobacter*	15.65	-	+	+	+	+	+	+	0
192	**300**	R	*Enterobacter*	15.65	-	+	+	+	-	+	+	0
193	**300**	R	*Enterobacter*	4.79	-	+	+	+	+	+	+	0
194	**300**	R	*Enterobacter*	4.79	-	+	+	-	+	+	+	0
195	**300**	R	*Enterobacter*	126.52	-	+	+	+	+	+	+	0
196	**300**	R	*Enterobacter*	126.52	-	+	+	+	+	+	+	0
200	**300**	R	*Enterobacter*	5.13	-	+	+	-	+	+	+	0
201	**300**	R	*Enterobacter*	5.13	-	+	+	+	+	+	+	0
202	**300**	R	*Enterobacter*	33.70	-	+	+	+	+	+	+	0
203	**300**	R	*Enterobacter*	33.70	-	+	+	+	+	+	-	0
209	**300**	R	*Enterobacter*	2.60	-	+	+	+	-	+	+	0
210	**300**	R	*Enterobacter*	2.60	-	+	+	+	+	+	+	0
211	**300**	R	*Enterobacter*	10.98	-	+	+	+	+	+	+	0
212	**300**	R	*Enterobacter*	10.98	-	+	+	+	+	+	+	0
213	**300**	R	*Enterobacter*	13.29	-	+	+	+	+	+	+	0
214	**300**	R	*Enterobacter*	13.29	-	+	+	+	+	+	+	0
197	**300**	S	*Enterobacter*	1.68	-	+	+	+	-	+	+	0
198	**300**	S	*Enterobacter*	1.68	-	+	+	+	+	+	+	0
199	**300**	S	*Enterobacter*	0.34	-	+	+	+	-	+	+	0
204	**300**	S	*Enterobacter*	0.23	-	+	+	+	-	+	+	0
205	**300**	S	*Enterobacter*	3.52	-	+	+	+	+	+	+	0
206	**300**	S	*Enterobacter*	3.52	-	+	+	+	+	+	+	0
207	**300**	S	*Enterobacter*	2.11	-	+	+	+	+	+	+	0
208	**300**	S	*Enterobacter*	2.11	-	+	+	+	+	+	+	0
215	**400**	R	*Enterobacter*	0.52	-	+	+	+	+	+	+	0
216	**400**	R	*Enterobacter*	0.52	-	+	+	+	+	+	+	0
217	**400**	R	*Enterobacter*	59.84	-	+	+	+	+	+	+	0
218	**400**	R	*Enterobacter*	59.84	-	+	+	+	+	+	+	0
219	**400**	R	*Enterobacter*	12.86	-	+	+	+	+	+	+	0
220	**400**	R	*Enterobacter*	12.86	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
221	**400**	R	*Enterobacter*	0.26	-	+	+	+	-	+	+	0
228	**400**	R	*Citrobacter*	0.25	-	+	+	+	+	+	+	0
229	**400**	R	*Citrobacter*	8.25	-	+	+	+	+	+	+	0
230	**400**	R	*Citrobacter*	8.25	-	+	+	+	+	+	+	0
231	**400**	R	*Citrobacter*	8.25	-	+	+	+	+	+	+	0
232	**400**	R	*Citrobacter*	28.00	-	+	+	+	+	+	+	0
233	**400**	R	*Citrobacter*	56.00	-	+	+	+	-	+	+	0
234	**400**	R	*Citrobacter*	56.00	-	+	+	+	+	+	+	0
235	**400**	R	*Citrobacter*	1.00	-	+	+	+	+	+	+	0
236	**400**	R	*Citrobacter*	1.00	-	+	+	+	+	+	+	0
243	**400**	R	*Enterobacter*	7.00	-	+	+	+	+	+	+	0
244	**400**	R	*Enterobacter*	7.00	-	+	+	+	+	+	+	0
245	**400**	R	*Citrobacter*	10.00	-	+	-	+	+	+	+	0
246	**400**	R	*Citrobacter*	10.00	-	+	+	+	+	+	+	0
222	**400**	S	*Enterobacter*	0.25	-	+	+	+	+	+	+	0
223	**400**	S	*Enterobacter*	11.58	-	+	+	+	+	+	+	0
224	**400**	S	*Enterobacter*	11.58	-	+	+	+	+	+	+	0
225	**400**	S	*Enterobacter*	173.40	-	+	+	+	+	+	+	0
226	**400**	S	*Enterobacter*	173.40	-	+	+	+	+	+	+	0
227	**400**	S	*Citrobacter*	0.25	-	+	+	+	+	+	+	0
237	**400**	S	*Citrobacter*	11.76	-	+	+	+	+	+	+	0
238	**400**	S	*Citrobacter*	11.76	-	+	-	+	+	+	+	0
239	**400**	S	*Citrobacter*	2.85	-	+	+	+	+	+	+	0
240	**400**	S	*Citrobacter*	2.85	-	+	+	+	+	+	+	0
241	**400**	S	*Citrobacter*	6.95	-	+	+	+	+	+	+	0
242	**400**	S	*Enterobacter*	6.95	-	+	+	+	+	+	+	0
247	**400**	S	*Citrobacter*	49.61	-	+	+	+	+	+	+	0
248	**400**	S	*Citrobacter*	49.61	-	+	+	+	+	+	+	0
249	**400**	S	*Citrobacter*	1.17	-	+	+	+	+	+	+	0
250	**400**	S	*Citrobacter*	1.17	-	+	-	+	+	+	+	0
251	**400**	S	*Citrobacter*	31.25	-	+	+	+	+	+	+	0
252	**400**	S	*Citrobacter*	31.25	-	+	+	+	-	+	+	0
253	**400**	S	*Enterobacter*	0.39	-	+	+	+	+	+	+	0
254	**200**	R	*Rhizobium*	0.41	-	+	+	+	+	+	-	0
255	**200**	R	*Paenibacillus*	0.41	+	-	+	-	+	-	-	0
262	**200**	R	*Enterobacter*	4.76	+	+	-	+	+	+	+	0
263	**200**	R	*Stenotrophomonas*	4.76	+	+	+	+	+	+	+	0
264	**200**	R	*Enterobacter*	3.57	+	+	+	+	+	+	+	0
265	**200**	R	*Stenotrophomonas*	3.57	-	+	+	+	+	+	-	0
266	**200**	R	*Enterobacter*	8.33	-	+	+	+	+	+	+	0
267	**200**	R	*Enterobacter*	8.33	+	+	+	+	+	-	-	0
268	**200**	R	*Rhodanobacter*	1.19	-	+	+	+	+	+	-	0
269	**200**	R	*Staphylococcus*	1.19	-	+	-	+	+	+	+	0
270	**200**	R	*Stenotrophomonas*	1.19	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
281	**200**	R	*Stenotrophomonas*	9.24	-	+	+	+	+	+	+	0
282	**200**	R	*Stenotrophomonas*	9.24	-	+	+	+	+	+	+	0
283	**200**	R	*Stenotrophomonas*	0.84	-	+	+	+	-	+	+	0
284	**200**	R	*Stenotrophomonas*	3.36	-	+	-	+	+	+	+	0
285	**200**	R	*Stenotrophomonas*	3.36	-	+	+	+	+	+	+	0
286	**200**	R	*Stenotrophomonas*	6.72	+	+	+	+	+	+	+	0
287	**200**	R	*Stenotrophomonas*	6.72	-	+	+	+	+	+	-	0
288	**200**	R	*Stenotrophomonas*	51.26	-	+	+	+	-	+	+	0
289	**200**	R	*Stenotrophomonas*	51.26	-	+	+	+	+	+	-	0
290	**200**	R	*Stenotrophomonas*	7.56	-	+	+	+	+	+	+	0
291	**200**	R	*Stenotrophomonas*	7.56	-	+	+	+	+	+	-	0
292	**200**	R	*Stenotrophomonas*	2.52	-	+	+	+	+	+	-	0
293	**200**	R	*Stenotrophomonas*	2.52	-	+	+	+	+	+	-	0
294	**200**	R	*Stenotrophomonas*	0.84	-	+	+	+	+	+	+	0
295	**200**	R	*Stenotrophomonas*	28.95	-	+	+	+	+	+	+	0
296	**200**	R	*Stenotrophomonas*	28.95	-	+	+	+	+	+	+	0
297	**200**	R	*Stenotrophomonas*	1.05	-	+	+	+	+	+	+	0
298	**200**	R	*Stenotrophomonas*	1.05	-	+	+	-	+	+	+	0
299	**200**	R	*Stenotrophomonas*	23.68	-	+	+	+	+	+	+	0
300	**200**	R	*Stenotrophomonas*	23.68	-	+	+	+	+	+	+	0
309	**200**	R	*Stenotrophomonas*	58.33	-	+	+	+	+	+	+	0
310	**200**	R	*Stenotrophomonas*	58.33	-	+	-	-	-	+	-	0
311	**200**	R	*Stenotrophomonas*	7.50	-	+	+	+	+	+	-	0
312	**200**	R	*Stenotrophomonas*	7.50	-	+	+	+	-	+	-	0
313	**200**	R	*Stenotrophomonas*	6.67	-	+	+	+	+	+	-	0
314	**200**	R	*Stenotrophomonas*	6.67	-	+	+	+	+	+	-	0
315	**200**	R	*Stenotrophomonas*	6.67	-	+	+	+	+	+	-	0
316	**200**	R	*Stenotrophomonas*	6.67	-	+	+	+	+	+	-	0
317	**200**	R	*Stenotrophomonas*	25.00	-	+	+	+	+	+	-	0
318	**200**	R	*Stenotrophomonas*	25.00	-	+	+	+	+	+	-	0
319	**200**	R	*Stenotrophomonas*	18.33	-	+	+	+	+	+	-	0
320	**200**	R	*Stenotrophomonas*	18.33	-	+	+	+	+	+	+	0
321	**200**	R	*Stenotrophomonas*	0.83	-	+	+	+	+	+	+	0
327	**200**	R	*Stenotrophomonas*	0.96	-	+	+	+	+	+	+	0
328	**200**	R	*Stenotrophomonas*	0.96	-	+	+	+	+	+	-	0
329	**200**	R	*Stenotrophomonas*	13.46	+	+	+	+	+	+	-	0
330	**200**	R	*Stenotrophomonas*	13.46	+	+	+	+	+	+	+	0
331	**200**	R	*Stenotrophomonas*	7.69	-	+	+	+	+	+	+	0
332	**200**	R	*Stenotrophomonas*	7.69	-	+	+	+	+	+	+	0
256	**200**	S	*Enterobacter*	15.06	-	+	+	+	+	+	+	0
257	**200**	S	*Staphylococcus*	15.06	-	+	+	+	+	+	+	0
258	**200**	S	*Rhizobium*	10.51	+	+	+	+	+	+	-	0
259	**200**	S	*Rhizobium*	10.51	+	+	+	+	-	+	-	0
260	**200**	S	*Bacillus*	7.36	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0μM)	SID (0.25μM)	SID (3μM)	ACC	IAA	P	Aux.
261	**200**	S	*Enterobacter*	7.36	-	+	+	+	+	+	+	0
271	**200**	S	*Stenotrophomonas*	2793.30	-	+	+	+	-	+	+	0
272	**200**	S	*Stenotrophomonas*	2793.30	-	+	-	+	+	+	+	0
273	**200**	S	*Stenotrophomonas*	10.34	-	+	+	+	+	+	+	0
274	**200**	S	*Stenotrophomonas*	10.34	-	+	+	+	+	+	+	0
275	**200**	S	*Stenotrophomonas*	9.50	-	+	+	+	+	+	+	0
276	**200**	S	*Stenotrophomonas*	9.50	-	+	+	+	+	+	+	0
277	**200**	S	*Stenotrophomonas*	13.13	-	+	+	+	-	+	+	0
278	**200**	S	*Stenotrophomonas*	13.13	-	+	+	+	+	+	+	0
279	**200**	S	*Enterobacter*	12.57	-	+	+	+	+	+	-	0
280	**200**	S	*Enterobacter*	12.57	-	+	+	+	+	+	+	0
301	**200**	S	*Stenotrophomonas*	13.97	-	+	+	+	+	+	+	0
302	**200**	S	*Stenotrophomonas*	13.97	-	+	+	+	+	+	+	0
303	**200**	S	*Stenotrophomonas*	0.32	-	+	+	+	+	+	+	0
304	**200**	S	*Stenotrophomonas*	12.70	-	+	+	+	+	+	+	0
305	**200**	S	*Stenotrophomonas*	12.70	-	+	+	+	+	+	+	0
306	**200**	S	*Stenotrophomonas*	10.79	-	+	+	+	+	+	+	0
307	**200**	S	*Stenotrophomonas*	10.79	-	+	+	+	+	+	+	0
308	**200**	S	*Stenotrophomonas*	0.32	-	+	+	+	+	+	+	0
322	**200**	S	*Stenotrophomonas*	3.80	-	+	+	-	+	+	-	0
323	**200**	S	*Stenotrophomonas*	3.80	-	+	+	+	+	+	-	0
324	**200**	S	*Stenotrophomonas*	3.16	-	+	+	+	-	+	+	0
325	**200**	S	*Stenotrophomonas*	3.16	-	+	+	+	+	+	+	0
326	**200**	S	*Stenotrophomonas*	0.32	-	+	+	+	+	+	+	0
333	**200**	S	*Stenotrophomonas*	13.04	-	+	+	+	+	+	+	0
334	**200**	S	*Stenotrophomonas*	13.04	-	+	+	-	+	+	+	0
335	**200**	S	*Stenotrophomonas*	16.11	-	+	+	+	+	+	+	0
336	**200**	S	*Stenotrophomonas*	16.11	-	+	+	+	+	+	+	0
337	**200**	S	*Stenotrophomonas*	11.51	-	+	+	+	+	+	+	0
338	**200**	S	*Stenotrophomonas*	11.51	-	+	+	+	+	+	+	0
482	**200 + DDE**	R	*Stenotrophomonas*	32.14	-	+	+	+	+	+	-	0
483	**200 + DDE**	R	*Stenotrophomonas*	32.14	+	+	+	+	+	+	-	0
484	**200 + DDE**	R	*Stenotrophomonas*	1.79	-	+	+	+	+	+	+	0
485	**200 + DDE**	R	*Stenotrophomonas*	35.71	-	+	+	+	+	-	+	0
486	**200 + DDE**	R	*Stenotrophomonas*	35.71	+	+	+	+	-	+	+	0
487	**200 + DDE**	R	*Stenotrophomonas*	141.07	+	-	+	+	-	+	+	+
488	**200 + DDE**	R	*Brevibacillus*	141.07	+	+	+	+	-	-	+	0
489	**200 + DDE**	R	*Stenotrophomonas*	1.79	+	+	+	+	+	-	+	0
490	**200 + DDE**	R	*Stenotrophomonas*	25.00	+	+	+	+	+	-	-	0
339	**200 + DDE**	R	*Stenotrophomonas*	4.92	-	+	+	+	+	+	+	0
340	**200 + DDE**	R	*Enterobacter*	4.92	-	+	+	+	+	+	+	0
341	**200 + DDE**	R	*Enterobacter*	17.21	-	+	+	+	+	+	+	0
342	**200 + DDE**	R	*Enterobacter*	6.56	-	+	+	+	+	+	+	0
343	**200 + DDE**	R	*Enterobacter*	6.56	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0μM)	SID (0.25μM)	SID (3μM)	ACC	IAA	P	Aux.
344	**200 + DDE**	R	*Stenotrophomonas*	9.02	+	+	+	+	+	+	+	0
345	**200 + DDE**	R	*Stenotrophomonas*	9.02	-	+	+	+	+	+	+	0
346	**200 + DDE**	R	*Stenotrophomonas*	18.85	-	+	+	-	+	+	+	+
347	**200 + DDE**	R	*Stenotrophomonas*	18.85	-	+	+	+	+	+	+	0
348	**200 + DDE**	R	*Stenotrophomonas*	2.46	-	+	+	+	+	+	+	0
349	**200 + DDE**	R	*Stenotrophomonas*	2.46	-	+	+	+	+	+	+	0
358	**200 + DDE**	R	*Stenotrophomonas*	4.32	-	+	+	-	+	+	-	0
359	**200 + DDE**	R	*Stenotrophomonas*	4.32	-	+	+	+	+	+	-	0
360	**200 + DDE**	R	*Stenotrophomonas*	10.79	-	+	+	+	+	+	-	0
361	**200 + DDE**	R	*Stenotrophomonas*	10.79	-	+	+	+	+	-	-	0
370	**200 + DDE**	R	*Stenotrophomonas*	0.60	-	+	+	-	+	+	+	0
371	**200 + DDE**	R	*Stenotrophomonas*	11.31	-	+	+	+	+	+	+	0
372	**200 + DDE**	R	*Stenotrophomonas*	11.31	-	+	+	+	+	+	+	0
373	**200 + DDE**	R	*Stenotrophomonas*	12.50	-	+	+	+	+	+	+	0
374	**200 + DDE**	R	*Stenotrophomonas*	12.50	-	+	+	+	+	+	+	0
375	**200 + DDE**	R	*Stenotrophomonas*	56.55	-	+	+	+	+	+	+	0
376	**200 + DDE**	R	*Pseudomonas*	56.55	-	+	+	+	+	+	+	0
377	**200 + DDE**	R	*Stenotrophomonas*	20.24	-	+	+	+	+	+	+	0
378	**200 + DDE**	R	*Stenotrophomonas*	20.24	-	+	+	+	+	+	+	0
379	**200 + DDE**	R	*Stenotrophomonas*	4.76	+	+	+	+	+	+	+	0
380	**200 + DDE**	R	*Pseudomonas*	4.76	-	+	+	+	-	+	+	0
385	**200 + DDE**	R	*Stenotrophomonas*	0.50	-	+	+	+	+	+	+	0
386	**200 + DDE**	R	*Stenotrophomonas*	17.41	-	+	+	+	+	+	+	0
387	**200 + DDE**	R	*Stenotrophomonas*	17.41	-	+	+	+	+	+	+	0
388	**200 + DDE**	R	*Stenotrophomonas*	1.00	+	+	+	+	+	+	+	0
389	**200 + DDE**	R	*Stenotrophomonas*	1.00	-	+	+	+	+	+	+	0
390	**200 + DDE**	R	*Stenotrophomonas*	21.89	-	+	+	+	+	+	+	0
391	**200 + DDE**	R	*Stenotrophomonas*	21.89	-	+	+	+	+	+	+	0
392	**200 + DDE**	R	*Stenotrophomonas*	27.86	-	+	+	+	+	+	+	0
393	**200 + DDE**	R	*Clostridiales*	27.86	-	+	+	+	+	+	+	0
398	**200 + DDE**	R	*Stenotrophomonas*	8.33	-	+	+	+	+	+	+	0
399	**200 + DDE**	R	*Stenotrophomonas*	8.33	-	+	+	+	+	+	+	0
400	**200 + DDE**	R	*Stenotrophomonas*	17.59	+	+	+	+	+	+	+	0
401	**200 + DDE**	R	*Stenotrophomonas*	17.59	-	+	+	+	+	+	-	0
402	**200 + DDE**	R	*Stenotrophomonas*	55.56	-	+	+	+	+	+	-	0
403	**200 + DDE**	R	*Stenotrophomonas*	55.56	-	+	+	+	+	+	-	0
413	**200 + DDE**	R	*Stenotrophomonas*	8.82	-	+	+	+	+	+	+	0
414	**200 + DDE**	R	*Stenotrophomonas*	8.82	-	+	+	+	+	+	+	0
415	**200 + DDE**	R	*Stenotrophomonas*	1.47	-	+	+	+	+	+	+	0
416	**200 + DDE**	R	*Stenotrophomonas*	1.47	+	+	+	+	+	+	+	0
417	**200 + DDE**	R	*Stenotrophomonas*	5.88	-	+	+	+	+	+	+	0
418	**200 + DDE**	R	*Stenotrophomonas*	5.88	+	+	+	+	+	+	+	0
419	**200 + DDE**	R	*Pseudomonas*	5.15	-	+	+	+	+	+	+	0
420	**200 + DDE**	R	*Stenotrophomonas*	5.15	+	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0μM)	SID (0.25μM)	SID (3μM)	ACC	IAA	P	Aux.
421	**200 + DDE**	R	*Stenotrophomonas*	8.09	+	+	+	+	-	+	+	0
422	**200 + DDE**	R	*Stenotrophomonas*	8.09	+	+	+	+	-	+	+	0
430	**200 + DDE**	R	*Enterobacter*	9.90	+	+	+	+	+	+	+	0
431	**200 + DDE**	R	*Enterobacter*	9.90	+	+	+	+	+	+	+	0
432	**200 + DDE**	R	*Stenotrophomonas*	38.61	+	+	+	+	+	+	+	+
433	**200 + DDE**	R	*Stenotrophomonas*	38.61	-	+	+	+	-	+	+	0
434	**200 + DDE**	R	*Stenotrophomonas*	0.99	+	+	+	+	+	+	+	0
435	**200 + DDE**	R	*Stenotrophomonas*	0.99	+	+	+	+	+	+	+	0
436	**200 + DDE**	R	*Stenotrophomonas*	9.41	+	+	+	+	+	+	+	0
437	**200 + DDE**	R	*Stenotrophomonas*	9.41	-	+	+	+	+	+	+	0
438	**200 + DDE**	R	*Stenotrophomonas*	5.94	-	+	+	+	+	+	+	0
439	**200 + DDE**	R	*Stenotrophomonas*	5.94	-	+	+	+	+	+	+	0
440	**200 + DDE**	R	*Stenotrophomonas*	1.49	-	+	+	+	+	+	+	0
441	**200 + DDE**	R	*Stenotrophomonas*	1.49	-	+	+	+	+	+	+	0
447	**200 + DDE**	R	*Stenotrophomonas*	17.86	+	+	+	+	+	-	+	0
448	**200 + DDE**	R	*Stenotrophomonas*	17.86	+	+	+	+	+	+	+	0
449	**200 + DDE**	R	*Stenotrophomonas*	22.62	-	+	+	+	+	+	+	0
450	**200 + DDE**	R	*Stenotrophomonas*	22.62	-	+	+	+	+	+	+	0
451	**200 + DDE**	R	*Stenotrophomonas*	89.29	-	+	+	+	+	+	+	0
452	**200 + DDE**	R	*Stenotrophomonas*	89.29	-	+	+	+	+	+	+	0
453	**200 + DDE**	R	*Stenotrophomonas*	22.62	-	+	+	+	+	+	+	0
454	**200 + DDE**	R	*Stenotrophomonas*	22.62	-	+	+	+	+	+	+	0
455	**200 + DDE**	R	*Stenotrophomonas*	1.19	-	+	+	+	+	+	+	0
456	**200 + DDE**	R	*Stenotrophomonas*	1.19	+	+	+	+	-	+	+	0
466	**200 + DDE**	R	*Stenotrophomonas*	0.93	-	+	+	+	+	+	+	0
467	**200 + DDE**	R	*Stenotrophomonas*	3.70	-	+	+	+	+	+	+	0
468	**200 + DDE**	R	*Stenotrophomonas*	3.70	-	+	+	+	+	+	+	0
469	**200 + DDE**	R	*Stenotrophomonas*	0.93	-	+	+	+	-	+	+	0
470	**200 + DDE**	R	*Stenotrophomonas*	3.70	-	+	+	+	-	+	+	0
471	**200 + DDE**	R	*Pseudomonas*	3.70	-	+	+	+	-	+	+	0
472	**200 + DDE**	R	*Stenotrophomonas*	204.63	-	+	+	+	-	+	+	0
473	**200 + DDE**	R	*Stenotrophomonas*	204.63	-	+	+	+	-	+	+	0
474	**200 + DDE**	R	*Stenotrophomonas*	0.93	-	+	+	+	-	+	+	0
350	**200 + DDE**	S	*Stenotrophomonas*	6.13	-	+	+	+	+	+	+	0
351	**200 + DDE**	S	*Stenotrophomonas*	6.13	-	+	+	+	+	+	+	0
352	**200 + DDE**	S	*Stenotrophomonas*	3.07	-	+	+	+	+	+	+	0
353	**200 + DDE**	S	*Stenotrophomonas*	3.07	-	+	+	+	+	+	+	0
354	**200 + DDE**	S	*Stenotrophomonas*	13.79	+	+	+	+	+	+	+	0
355	**200 + DDE**	S	*Stenotrophomonas*	13.79	-	+	+	+	+	+	+	+
356	**200 + DDE**	S	*Stenotrophomonas*	38.31	-	+	+	+	+	+	-	0
357	**200 + DDE**	S	*Stenotrophomonas*	38.31	+	+	+	+	+	+	-	0
362	**200 + DDE**	S	*Stenotrophomonas*	7.02	-	+	+	+	-	+	-	0
363	**200 + DDE**	S	*Stenotrophomonas*	7.02	-	+	+	+	+	+	+	0
364	**200 + DDE**	S	*Stenotrophomonas*	0.44	-	+	+	+	+	+	-	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0μM)	SID (0.25μM)	SID (3μM)	ACC	IAA	P	Aux.
365	**200 + DDE**	S	*Stenotrophomonas*	3.51	-	+	+	+	+	+	-	0
366	**200 + DDE**	S	*Stenotrophomonas*	3.51	-	+	+	+	+	+	+	0
367	**200 + DDE**	S	*Stenotrophomonas*	0.44	-	+	+	+	+	+	-	0
368	**200 + DDE**	S	*Stenotrophomonas*	37.72	-	+	+	+	+	+	+	0
369	**200 + DDE**	S	*Stenotrophomonas*	37.72	-	+	+	+	+	+	+	0
381	**200 + DDE**	S	*Stenotrophomonas*	37.08	-	+	+	+	-	+	+	0
382	**200 + DDE**	S	*Stenotrophomonas*	37.08	-	+	+	-	+	+	+	0
383	**200 + DDE**	S	*Stenotrophomonas*	1.87	-	+	+	+	+	+	+	0
384	**200 + DDE**	S	*Stenotrophomonas*	1.87	-	+	+	+	+	+	+	0
394	**200 + DDE**	S	*Stenotrophomonas*	5.79	-	+	+	+	+	+	+	0
395	**200 + DDE**	S	*Stenotrophomonas*	5.79	-	+	+	+	+	+	+	0
396	**200 + DDE**	S	*Stenotrophomonas*	4.21	-	+	+	+	+	+	+	0
397	**200 + DDE**	S	*Stenotrophomonas*	4.21	-	+	+	+	+	+	+	0
404	**200 + DDE**	S	*Stenotrophomonas*	9.13	-	+	+	+	+	+	-	0
405	**200 + DDE**	S	*Stenotrophomonas*	9.13	-	+	+	+	+	+	-	0
406	**200 + DDE**	S	*Stenotrophomonas*	13.04	-	+	+	+	+	+	-	0
407	**200 + DDE**	S	*Stenotrophomonas*	13.04	-	+	+	+	+	+	-	0
408	**200 + DDE**	S	*Stenotrophomonas*	170.43	-	+	+	+	+	+	-	0
409	**200 + DDE**	S	*Stenotrophomonas*	170.43	-	+	+	+	+	+	-	0
410	**200 + DDE**	S	*Stenotrophomonas*	0.43	-	+	+	+	+	+	-	0
411	**200 + DDE**	S	*Stenotrophomonas*	57.39	+	+	+	+	+	+	-	0
412	**200 + DDE**	S	*Stenotrophomonas*	57.39	-	+	+	+	+	+	-	0
423	**200 + DDE**	S	*Stenotrophomonas*	0.74	+	+	+	+	+	+	+	0
424	**200 + DDE**	S	*Stenotrophomonas*	0.74	-	+	+	+	+	+	+	0
425	**200 + DDE**	S	*Stenotrophomonas*	3.68	-	+	+	+	+	+	+	0
426	**200 + DDE**	S	*Stenotrophomonas*	3.68	+	+	+	+	+	+	+	0
427	**200 + DDE**	S	*Stenotrophomonas*	0.25	+	+	+	+	+	+	+	0
428	**200 + DDE**	S	*Stenotrophomonas*	0.49	+	+	+	+	+	+	+	0
429	**200 + DDE**	S	*Stenotrophomonas*	0.49	+	+	+	+	+	+	+	0
442	**200 + DDE**	S	*Stenotrophomonas*	2.49	-	+	+	+	+	+	+	0
443	**200 + DDE**	S	*Stenotrophomonas*	0.23	+	+	+	+	+	+	+	0
444	**200 + DDE**	S	*Stenotrophomonas*	0.23	-	+	+	+	-	+	+	0
445	**200 + DDE**	S	*Enterobacter*	21.04	-	+	+	+	-	+	+	0
446	**200 + DDE**	S	*Stenotrophomonas*	21.04	-	+	+	+	-	+	+	0
457	**200 + DDE**	S	*Stenotrophomonas*	0.57	-	+	+	+	-	+	+	0
458	**200 + DDE**	S	*Enterobacter*	2.29	+	+	+	+	-	+	+	+
459	**200 + DDE**	S	*Stenotrophomonas*	2.29	+	+	+	+	+	+	+	0
460	**200 + DDE**	S	*Stenotrophomonas*	2.29	-	+	+	+	-	+	+	0
461	**200 + DDE**	S	*Stenotrophomonas*	2.29	+	+	+	+	+	+	+	0
462	**200 + DDE**	S	*Pseudomonas*	1.14	-	+	+	+	+	+	+	0
463	**200 + DDE**	S	*Stenotrophomonas*	1.14	-	+	+	+	+	+	+	0
464	**200 + DDE**	S	*Stenotrophomonas*	18.86	+	+	+	+	+	+	+	0
465	**200 + DDE**	S	*Stenotrophomonas*	18.86	-	+	+	+	+	+	+	0
475	**200 + DDE**	S	*Stenotrophomonas*	2.88	-	+	+	+	-	+	+	0
476	**200 + DDE**	S	*Stenotrophomonas*	2.88	-	+	+	+	+	+	+	0

Sample	Condition	Origin	Genus	Cfu g^{-1}	OA	SID (0µM)	SID (0.25µM)	SID (3µM)	ACC	IAA	P	Aux.
477	**200 + DDE**	S	*Stenotrophomonas*	8.64	-	+	+	+	+	+	+	0
478	**200 + DDE**	S	*Stenotrophomonas*	8.64	+	+	+	+	+	+	+	0
479	**200 + DDE**	S	*Stenotrophomonas*	27.16	-	+	+	+	+	+	+	0
480	**200 + DDE**	S	*Stenotrophomonas*	27.16	-	+	+	+	-	+	+	0
481	**200 + DDE**	S	*Stenotrophomonas*	0.41	-	+	+	+	+	+	-	0
491	**200 + DDE**	S	*Stenotrophomonas*	5.51	-	+	+	+	+	-	-	0
492	**200 + DDE**	S	*Stenotrophomonas*	1.57	-	+	+	+	+	-	+	0
493	**200 + DDE**	S	*Stenotrophomonas*	1.57	-	+	+	+	-	+	-	0
494	**200 + DDE**	S	*Stenotrophomonas*	64.57	-	+	+	+	-	+	-	0
495	**200 + DDE**	S	*Stenotrophomonas*	64.57	+	+	+	+	+	+	-	0
496	**200 + DDE**	S	*Stenotrophomonas*	3.54	-	+	+	+	-	-	-	0
497	**200 + DDE**	S	*Stenotrophomonas*	3.54	-	+	+	+	-	-	+	0

Plants were exposed to six different conditions: control (n=10), 100 µg g^{-1} Cu-NPs (100, n=5), 200 µg g^{-1} Cu-NPs (200, n=6), 300 µg g^{-1} Cu-NPs (300, n=6), 400 µg g^{-1} Cu-NPs (400, n=6), 200 µg g^{-1} Cu-NPs with 100 µg L^{-1} DDE (200 + DDE, n=20). Origin: R = roots, S = shoots, Cfu g^{-1}: colony-forming units per gram of fresh plant material. Plant growth promotion tests, samples are scored as positive (+), negative (-), or neutral (0): OA: organic acids, SID: siderophores, ACC: 1-aminocyclopropane-1-carboxylate, IAA: indole-3-acetic acid, P: phosphate solubilization. Aux.: Auxanography, Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

Attachment 6: Colony-forming units per gram of fresh plant material

Supplementary table 13: *Average Cfu g^{-1}isolated from C. pepo.*

Condition	Plant compartment	Average cfu g^{-1} (SEM)
Without Cu-NPs, without DDE	Root	1.02×10^7 (4.74×10^6, N = 5)A
	Shoot	3.2×10^4 (1.06×10^4, N=5)AB
With Cu-NPs, without DDE	Root	1.99×10^4 (7.86×10^3, N = 6)AB
	Shoot	1.15×10^4 (5.88×10^3, N=6)AB
With Cu-NPs, with DDE	Root	1.83×10^5 (1.06×10^5, N=10)B
	Shoot	1.05×10^5 (3.21×10^4,N=10)AB

The average number of cfu g^{-1} (standard errors) of root and shoot tissues were calculated from an average of five samples made from a mix of two plants that were either grown without or with DDE, and with or without Cu-NPs. Control plants (n=10) were watered every other day with ¼ Hoagland solution. Cu-NPs plants (n=23) were watered every other day with ¼ Hoagland solution and grown in the presence of different concentrations of Cu-NPs, i.e. 100, 200, 300 and 400 µg g^{-1}. Twenty other plants exposed to 200 µg g^{-1} Cu-NPs were watered every other day with ¼ Hoagland solution containing 100 µg L^{-1} DDE. All plants were grown for 20 days in greenhouse conditions. Significant differences have been marked using letters: B ($p<0.05$), Cfu g^{-1}: Colony-forming units per gram of fresh plant material, SEM: Standard Error of the Mean, Cu-NPs: Copper nanoparticles, DDE: 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene.

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Optimizing the efficiency of endophytic bacteria-enhanced phytoremediation of DDE-contaminated soils with Cu-NPs

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2016**

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Witters, Katrien

Datum: **5/06/2016**