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Endophyte-enhanced phytoremediation of DDE-contaminated using *Cucurbita pepo*: a field trial

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Abstract

Although the use of the pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was banned from the mid-1970s, its most abundant and recalcitrant degradation product, 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene (DDE), is still present in terrestrial and aquatic ecosystems worldwide.

Zucchini (*Cucurbita pepo* ssp. *pepo*) has been shown to accumulate high concentrations of DDE and was proposed for phytoremediation of contaminated soils. We performed a field trial covering a full plant life cycle. *Cucurbita pepo* plants inoculated with the plant growth-promoting endophytic strains *Sphingomonas taxi UH1*, *Methylobacterium radiotolerans UH1*, *Enterobacter aerogenes UH1*, or a consortium combining these three strains were grown on a DDE-contaminated field for 100 days. The effects of these inoculations were examined at both the plant level, by evaluating plant weight and plant DDE-content, and at the level of the cultivable and total endophytic communities.

Inoculating plants with *S. taxi UH1*, *M. radiotolerans UH1*, and the consortium increased plant weight. No significant effects of the inoculations were observed on DDE-concentrations in plant tissues. However, the amount of DDE accumulated by *C. pepo* plants per growing season was significantly higher for plants that were inoculated with the consortium of the three strains. Therefore, inoculation of *C. pepo* with DDE-degrading endophytes might be promising for phytoremediation applications.

KEYWORDS: Pesticides, DDE, DDT, endophytes, field experiment

Introduction

The pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was used worldwide until the 1970s when its deleterious effects on human health and wildlife were recognized [1]. In soils, DDT rapidly degrades to the persistent 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene (DDE) and DDE-contaminated soils can still be found worldwide [2, 3]. A possible remediation strategy for these contaminated soils is phytoremediation. Phytoremediation is an *in situ* natural remediation technology that has been demonstrated to be successful in many cases [4-7]. The strategy relies on plants and their associated microorganisms to take up contaminants from the soil and metabolize or store them [8].

A primary requirement for the efficient phytoremediation of contaminated soils is an appropriate plant. *Cucurbita pepo* plants were shown to accumulate several organic contaminants under field conditions, including chlordane [9], Dieldrin, Endrin [10, 11], and hexachlorocyclohexanes (HCHs) [12, 13]. White et al. [14] also demonstrated the effective uptake of weathered DDE by *Cucurbita pepo ssp. pepo* cultivar Raven. This zucchini cultivar showed soil-to-plant bioconcentration factors up to 23.7 and is thus a suitable plant for phytoremediation of DDE-contaminated soils.

However, when envisaging efficient phytoremediation, plants are not the sole factor. Endophytic bacteria that reside inside plant tissues are known to play crucial roles in plant growth and development in general [15], as well as in the remediation of organic contaminants [16].

A collection of 585 bacterial endophytic strains that was established during earlier research [17] was investigated *in vitro* for plant growth-promoting traits and DDE-degradation potential. Suitable endophytes were selected and further identified through full genome sequencing[18-20]. After verifying the lack of pathogenic traits, the 3 full genome sequenced endophytic strains (*Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, Enterobacter aerogenes UH1)* were applied in the field experiment described in this manuscript. The main objectives of this experiment were to verify if the selected strains could promote both plant growth and DDE removal from a contaminated soil *in situ*.

Materials and methods

Inoculation of seeds

The selected bacterial strains were cultivated by transferring 5 µL of the bacterial stock into 30 mL of 869 medium (Mergeay et al., 1985). A consortium was created as well by combining the selected strains into one 50 mL tube. The bacterial suspensions were incubated at 30°C for 3 days.

Seeds of *Cucurbita pepo ssp. pepo* cultivar Raven (Johnny's Selected Seeds, Winslow, ME, USA) were surface sterilized for 1 minute in 1% NaOCl and put in the bacterial suspension (10^9 cells mL^{-1}) for overnight inoculation.

Field characteristics

The experimental field that was utilized for this trial is located on Lockwood Farm, owned by the Connecticut Agricultural Experiment Station (N41,406786°; W72,906043°; Hamden, CT, USA).

The soil in the field was identified as being a Cheshire fine sandy loam soil containing 56% sand, 36% silt, and 8% clay. The percentage of organic carbon was 1.4 and an average pH of 6.7 was measured. The cation exchange capacity of the soil was 18.6 cmol kg^{-1}.

Growth and harvest of plants

The seeds were germinated between wet paper towels for 3 days at 30°C. The seedlings were transferred to 750 mL plastic pots containing vermiculite. The bacterial suspension was again added and plants watered daily with ¼ Hoagland nutrient solution [21]. After 7 days, the plants were of sufficient size to be transferred to the field. Plants were set out in 5 rows covered with black plastic to avoid weed growth. Each row containing 8 plants approximately 1 m apart (Figure 1). Holes were made in the plastic foil to insert the plants. Soil samples were taken from each mound/planting site to determine local DDE concentrations. The plants were watered continuously by a drip irrigation system underneath the plastic.

93 Harvest ready fruits were collected 3 times per week. After a growing period of approximately 100
94 days, plants were harvested. Shoots were clipped and roots were excavated. All plant tissues were
95 transferred to the lab for further analysis.
96 Weights were determined separately for leaves, stems, roots, and fruits. All plant tissues were rinsed
97 thoroughly with tap water. Approximately 5 g of shoot and root tissue were separated for endophyte
98 isolation and 454 pyrosequencing, while the remainder of plant tissues were homogenized and stored
99 at 4°C for GCMS analysis to determine DDE concentrations.
100
101 ***DDE content in plant tissues***
102 DDE was extracted from plant tissues using the QuEChERS method [22]. Up to 15 g fresh tissue was
103 added to a 50 mL centrifuge tube with 15 mL acetonitrile and 30 µL o,p'-DDE from a 10 mg mL^{-1}
104 solution as an internal standard. The tubes were incubated on a wrist-action shaker for 10 min after
105 which 6 g $MgSO_4$ and 1.5 g $C_2H_3NaO_2$ were added. The tubes were shaken for 30 min and afterwards
106 centrifuged for 10 min at 3000 rpm. New 15 mL tubes were filled with 1.5 g $MgSO_4$ and 0.5 g of
107 primary secondary amine (PSA). Two mL of toluene was added to wet the powders before 10 mL of
108 the primary extract was transferred to each tube. The tubes were shaken for 30 s and centrifuged for
109 10 min at 3000 rpm. Six mL of each extract was concentrated to 1 mL under nitrogen pressure. These
110 extracts were amended with 100 ng mL^{-1} *o,p*'-DDE as an internal standard before the DDE
111 concentrations were determined using GCMS.
112 Standards of o,p'-DDE, p,p'-DDT, p,p'-DDD, and p,p'-DDE (Environmental Protection Agency
113 National Pesticide Standard Repository, Fort Meade, MD) were weighed in toluene to prepare
114 standards at 1,000 µg mL^{-1}, which were then diluted to obtain mixed calibration standards from 25-
115 1,000 ng mL^{-1}. Each calibration was furthermore amended with 100 ng mL-1 o,p'-DDE as an internal
116 standard since the breakdown of p,p'-DDT to p,p'-DDE/DDE in the GC inlet is unpredictable, total
117 DDx was calculated.

The concentration of DDx in plant tissues and soil was determined on an Agilent (Avondale, PA, USA) 7890B gas chromatograph (GC) with a 5977A mass selective detector (MSD). Two microliters of sample were injected into a multi-mode inlet (MMI) in pulsed splitless mode at 250°C with He as the carrier gas and then onto an Agilent HP-5MS 30-m column with 0.25mm ID and guard column. The GC oven initial temperature was 150°C for 1 min, then ramped to 250°C at a rate of 5°C min^{-1}, and then ramped at a rate of 50°C min^{-1} to a final temperature of 300°C which was held for 8 min. After a 5 min solvent delay, the MSD detected analytes using scan mode at a mass to charge ratio (m/z) 100-430. The instrument was calibrated with the standards described above, covering a range of 25-1,000 ng mL^{-1}.

Cultivation-dependent isolation

Isolation of cultivable endophytes

At harvest, plant tissues were thoroughly washed with tap water in order to remove soil particles and plant weight was determined. Root and shoot tissues were incubated separately in 1% NaOCl for surface sterilization. Subsequently, tissues were rinsed 3 times in sterile distilled water (dH_2O) and dried on sterilized filter paper. In order to verify surface sterility, an aliquot (100 µl) of the third rinsate was transferred to a Petri dish containing 869 medium [23] (per liter: 0.35 g $CaCl_2.2H_2O$, 1.00 g Glucose D+, 5.00 g NaCl, 10.0 g Tryptone, 5.00 g Yeast Extract, 15 g Agar; adjusted to pH 7 with HCl or NaOH).

The surface sterilized tissues of three individual plants were transferred to sterilized mortars containing 5 mL sterile 10 mM $MgSO_4$ and were crushed. The crushed root and shoot tissues were transferred to obtain serial dilutions (0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and each dilution (100 µl) was spread onto plates containing 1/10 diluted 869 medium [23]. All plates were prepared in triplicate and incubated at 30°C for 4 days. The colonies on the plates were counted and the number of colony forming units (cfu) per gram of fresh plant tissue were calculated. For each treatment, averages and standard errors

were calculated of the 3 replicates. The colonies were purified and in total 585 isolated strains were stored in 15 $\%_w$ glycerol at -80°C.

Genotypic characterization of cultivable bacterial strains

The DNA of the 585 isolated strains was recovered with the Qiagen DNeasy blood and tissue kit (Qiagen, Venlo, the Netherlands). A Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, De Meern, the Netherlands) was used to analyze the quality and quantity of the extracted DNA. The DNA was used directly for 16S rDNA amplification with a universal 1392R primer (5-ACGGGCGGTGTGTRC-3) and a bacteria-specific 26F primer (5-AGAGTTTGATCCTGGCTCAG-3) [24]. The 16S products were digested and separated by gel electrophoresis (1.5% agarose, 90V, 2h). The banding patterns were analyzed and 50 different DNA fingerprints were distinguished. At least one representative of each pattern was selected and sent for 16S rDNA sequencing at Macrogen (Amsterdam, the Netherlands). Consensus sequences were obtained with the Staden package and identification was acquired from the Ribosome Database Project based on the most closely related species (http://rdp.cme.msu.edu/classifier/classifier.jsp) [25]. The Shannon-Wiener indices of all different communities were calculated to estimate the diversity obtained after the different growth conditions [26, 27].

Phenotypic characterization of cultivable bacterial strains

The bacterial strains were tested *in vitro* for their plant growth-promoting traits. The bacteria were grown in liquid 869 medium [23] at 30°C for 2 days, washed and resuspended in 1 mL sterile $MgSO_4$. Twenty µl of this suspension was used for inoculation of 96 well microplate assays to investigate the production of IAA using Salkowski reagent [28], siderophores using the Chrome Azurol S (CAS) assay [29], 1-aminocyclopropane-1-carboxylate (ACC) deaminase using 2,4-dinitrophenylhydrazine and NaOH [30], organic acids using Alizarin red S [31] and phosphate solubilization capacity using NBRIP

medium [32]. For all plant growth promotion assays, the strains were assigned scores of + or – depending on the presence of a color in the colorimetric tests.

Screening bacteria for DDE-degrading capacities

An auxanography test was performed for all bacterial strains to screen them for potential DDE-degradation capability. Each bacterial strain was grown in liquid 869 medium [23]. After 4 days, 1 µL of each bacterial suspension was diluted in 999 µL sterile 10 mM MgSO4.

One hundred µl of the bacterial suspension was plated on selective 284 medium. Immediately thereafter, 50 µL of a sterile 50 mg L^{-1} DDE or a 50 mg L^{-1} DDE + 200 µg g-1 CuNPs solution were added to the surface and smeared out on ¾ of the outer circle of the plate (Figure 4). The plates were incubated at 30° for 6 days. Plates with bacteria growing on the total surface were considered neutral, and were considered to be tolerant to the used concentrations of DDE and CuNPs. Plates on which strains did not show growth on the outer circle containing DDE and CuNPs were considered negative, since DDE was toxic to these bacterial strains. If the bacterial strains showed enhanced growth in the area covered with DDE, the plates were considered positive and these strains are presumed to use DDE as a carbon source while being tolerant to CuNPs.

Statistical analysis

The averages and standard deviations were calculated from three replicates from a mixed sample of three plants. The samples were compared using a one way ANOVA with Dunn comparison test.

Cultivation-independent molecular analysis

Extraction and sequencing

The plant tissue samples were surface sterilized (3 min in sterile MilliQ water, 1.5 min ethanol 70%, 3 min NaOCl 1%, 1.5 min ethanol 70%, rinse 5 times in sterile MilliQ water) before being crushed in liquid nitrogen. All genomic DNA was extracted using an Invisorb spin plant mini kit (Stratec Biomedical, Germany). A template specific 967F primer (5- CAACGCGAAGAACCTTACC-3) was combined with 1391R primer (5- GACGGGCGGTGWGTRCA-3) to target a 424 bp fragment in the

V3-V4 region. In a two-step PCR amplification, first the original primers were used, and then the same primers with a 10 bp multiplex identifier (MID) were employed. PCR reactions contained 1x Roche FastStart High Fidelity Reaction Buffer with 1.8 mM $MgCl_2$, 1.5U of Roche FastStart High Fidelity DNA-polymerase, 0.2 mM Roche dNTP, 300 nM 341F, 100 nM 783Ra, 100 nM 783Rb, 100 nM 783Rc and 1 µl of 1/20 diluted template DNA (1 ng μl^{-1}) in a volume of 50 µl. The PCR program consisted of an initial denaturation step of 95°C for 2 min, followed by 25 cycles (1^{st} PCR) or 10 cycles (2^{nd} PCR) of denaturation at 95°C for 30 s, annealing at 53°C for 40 s, extension at 72°C for 1 min, with a final extension of 5 min at 72°C. The amplicons were purified from 1.5% agarose gels using a QIAquick gel extraction kit (Qiagen, Venlo, the Netherlands) and 1 µl was used for the second PCR using MID-elongated primers. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Venlo, the Netherlands). The DNA was quantitated using a Quant-IT PicoGreen dsDNA assay kit (Life Technologies Europe, Gent, Belgium), after which equimolar concentrations of the barcoded amplicons were collected per library and diluted to 100 µl using TE buffer. The library was unidirectionally sequenced using a Roche 454 GS-FLX Plus Life Sciences Genome Sequencer at Macrogen (Seoul, South Korea).

Analysis of obtained pyrosequencing data

The FASTA files containing the raw pyrosequencing data were accessed using Mothur bioinformatics software [33] for processing and analysis according to Schloss et al. [34]. The obtained sequences were denoised before barcodes and primers were removed. The remaining sequences were aligned and classified along known sequences in the SILVA rRNA database [35]. Chimeric sequences, mitochondrial and chloroplast sequences were deleted and the remaining sequences were grouped into operational taxonomic units (OTUs) based on a 97% similarity criterion. Rarefaction curves were starting to level off (Figure 5), but sequencing at a greater depth could have revealed more OTUs. The similarity between samples and their resemblance to the cultivated communities were visualized using Primer7 (Version 7.0.5, Primer-E Ltd.). Clustering of samples was based on S17 Bray-Curtis

similarity of the group average of the species after square root transformation of the samples. The nMDS was based on S17 Bray-Curtis similarity as well, with square root transformation, Kruskall stress formula 1 and minimum stress 0.01. ANOSIM (analysis of similarities) with 999 permutations was used to test the spatial separation of the samples in nMDS.

Results and discussion

Plant weights

Plant weight was determined to evaluate the effects of inoculation with endophytic bacterial strains on plant growth over the total life cycle (Figure 2); the weights were determined separately for leaves, stems, roots, and fruits. Significant increases in weight were observed for stems and roots of plants inoculated with the consortium of all three strains. Furthermore, plants inoculated with *S. taxi UH1* and *M. radiotolerans UH1 showed* tended to higher weights in comparison to non-inoculated control plants. However, plants inoculated with *E. aerogenes UH1* showed a slightly diminished growth in comparison to controls; weights for leaves, stem, and roots tended to be lower. This generally improved weight underlines the plant growth-promoting effects of the endophytic strains.

Soil DDE concentrations

At the moment of planting, soil samples were taken from each mound to determine the soil DDE concentrations (table 1). No significant differences in soil DDE concentrations were observed. The plants from different treatments were randomly planted (Figure 1) for an optimal evaluation and comparison between the different inoculations.

DDE concentrations in plant tissues

After determining the DDE concentrations in different plant tissues (leaf, stem, root, and fruit), the bioconcentration factors (BCFs) could be calculated (Figure 3). The BCFs are the ratio of the concentration of DDE accumulated in the plant tissues to the concentration of DDE present in the soil.

As expected, the BCFs of leaves are very low, ranging from 0.025 to 0.075; by comparison, BCFs of roots and stems range from 19 to 25. This difference can be explained by the difficult translocation of DDE in plant tissues due to the hydrophobic character of the molecule. Therefore, larger effects of DDE on the endophytic communities can be expected in roots, in comparison to shoots.

With both the DDE concentrations of each plant compartment and weight of the respective compartments known, the total amount of DDE that was extracted from the field per plant during the entire growth period could be calculated as follows (Figure 4): (DDE concentration in plant tissue) x (plant weight).

Although no significant differences were observed between inoculated plants and control plants for the DDE concentrations, the total amount of DDE that was removed from the soil in one growth cycle was significantly higher in plants that were inoculated with the consortium in comparison to non-inoculated control plants.

By estimating the mound volume in which a *C. pepo* plant can grow, the total amount of DDE a plant can reach was assessed. The mound had a surface area of 1m by 0.5m and a depth of 0.25m. This gives a volume of 125,000 cm^3 with a density of 1.14 g cm^{-3}, totaling 142.5 kg of soil per plant. Table 2 presents the amounts of DDE that were present per mound, the amounts of DDE that were taken up by the plants and the percentage this amount represents in comparison to the soil DDE content.

Endophytic communities

Genotypic identification

The endophytic communities of the roots and shoots were investigated using both cultivation-dependent and cultivation-independent techniques. The cultivation-dependent isolation of the endophytic communities produced 530 cultivable strains, belonging to 4 phyla and 34 genera (Table 3 and Figure 6).

The cultivation-independent molecular analysis was conducted using 454 pyrosequencing. Macrogen (Seoul, South Korea) delivered the raw data in 4 FASTA files. These files contained in total 573,227 strains with a mean length of 388.01 base pairs. The files were analyzed using Mothur [33] and the

sequences were filtered using following criteria: length >200 bp, <8 homopolymers, <2 differences with the primers, <1 difference with the barcodes, pre-clustered to reduce sequencing errors, removal of chimeras, and more than 95% in the same range of genes. After this filtering, 207,128 sequences remained, containing a total of 26,862 unique sequences. Subsequently, all sequences belonging to mitochondria, chloroplasts, archaea, and eukaryotes were deleted. After this step, 199,500 sequences remained, implying that 3.66% of all sequences belonged to non-bacterial DNA. In the total of all samples, 288 different genera were detected belonging to 20 different phyla, or subphyla in case of the Proteobacteria (Figure 6). With a mean OTU length of 223.19 bp, identification to the species level was not possible.

Although 288 different genera were detected during the pyrosequencing process, many of them were only detected once across the different samples. Therefore, we focus on the 15 most abundant genera, which account for a large portion of the total bacterial communities (ranging from 64.83% to 79.66% of the total community).

Inoculating the plants with *Sphingomonas taxi UH1*, *Methylobacterium radiotolerans UH1*, *Enterobacter aerogenes UH1*, or a consortium combining the three strains increased their abundancy in the community in the case of *S. taxi UH1* (Shoot), *M. radiotolerans UH1, E. aerogenes UH1* (Root) and the consortium but had no apparent effect on the presence of *S. taxi UH1* (Root) and *E. aerogenes UH1* (Shoot) since even a slightly lower presence was detected in the inoculated plants (Figure 7).

It is important to evaluate the inoculation efficiency since an inoculation can only be considered successful in case the presence of a bacterial strain is enhanced in the communities. However, in field conditions, an effective establishment of the inoculated strain is not evident because of the large number of bacterial genera competing for a position in the community.

The observed changes in abundance are larger for root communities of the plants inoculated with *M. radiotolerans UH1, E. aerogenes UH1,* and the consortium. This is not surprising since all three inoculated strains originated from roots of *C. pepo* plants exposed to DDE.

296 Several authors mentioned that, when isolating endophytes, many bacterial genera are unaccounted
297 for due to their inability to grow under laboratory conditions [36, 37]. A comparison of the number of
298 genera that are cultivable (n=34) and the number of genera present in the total bacterial communities
299 (n=288), seems to support this hypothesis. However, when comparing the percentages of the total
300 bacterial communities that were recovered by the cultivation-dependent techniques, percentages
301 ranging between 42.50% and 58.16% were observed at the genus level. This implies that although a
302 significant part of the total communities was indeed cultivable under laboratory conditions, many
303 endophytes that were present in the plant tissues were not cultivable.
304 When analyzing the similarity of all communities using the Analysis of Similarity (ANOSIM) and
305 Similarity Percentages (SIMPER) tools in Primer7, a clear clustering by the cultivation-dependent
306 and cultivation-independent techniques is observed (Figure 8). Due to the low cultivability rate of the
307 endophytic communities, the cultivable-dependent techniques show a lower similarity than the
308 cultivation-independent techniques that account for all endophytes present, 20.34% and 65.63%
309 average similarity, respectively. The samples inoculated in different conditions also cluster inside the
310 treatments, demonstrating the effect of inoculating the plants on the endophytic communities.
311 *Phenotypic identification*
312 Many endophytic bacterial strains were shown to possess plant growth-promoting capacities [15]. By
313 investigating the symbiotic relationships between plants and their associated microorganisms, they
314 might be exploited in phytoremediation processes [16]. Only cultivable endophytic strains can be tested
315 for their plant growth-promoting capacities *in vitro*. Table 4 describes the percentages of bacteria that
316 scored positive in the different plant growth promotion assays. Of the 530 strains that were examined,
317 2.83% (n=15) showed no *in vitro* plant growth promotion in the 5 assays that were performed, while
318 4.34% (n=23) of all strains scored positive on all 5 assays. No differences were observed between the
319 plant growth-promoting capacities of the endophytic communities isolated from non-inoculated
320 control plants or from inoculated plants; the numbers of strains displaying *in vitro* plant growth
321 promotion were very similar and apparently not influenced by inoculation.

Along with their plant growth-promoting capacities, all strains were also assessed for their *in vitro* DDE-degradation potential (Table 5). A total of 39 strains or 7.36% showed DDE-degradation potential. Of these strains, 38 were isolated from plants that were previously inoculated with DDE-degrading endophytic strains, while the remaining strain was isolated from a non-inoculated control plant.

Conclusion

The effects of inoculating plants with *Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1, Enterobacter aerogenes UH1*, or a consortium of all three strains were evaluated at different levels, plant growth, DDE-uptake, and endophytic communities.

Increasing tendencies were observed for the weight of plants inoculated with *S. taxi UH1, M. radiotolerans UH1*, and the consortium in comparison to the non-inoculated control plants. The effect of inoculation with the consortium was significant for roots and stems. In contrast, inoculation with *E. aerogenes UH1* led to a slight, yet statistically insignificant decrease of the plant weight.

The DDE concentrations in the soil were similar for the different treatments (non-inoculated control or inoculated plants). Inoculation with DDE-degrading endophytes did not affect the bioconcentration factors of DDE in the plant tissues. The ratio of the concentrations of DDE inside the plant tissues to the DDE concentrations in the soil did not change when plants were inoculated.

However, when the amounts were calculated of DDE that was removed from the soils per plant and per growth cycle, a significantly higher value was observed for plants inoculated with the consortium of the three endophytic strains. This increase is mainly due to the higher root and stem weights of the plants, these being the compartments containing the highest DDE concentrations.

When the total and cultivable endophytic communities were evaluated and compared over all treatments, no obvious differences were observed. However, when comparing the abundances of the endophytic strains that were used for inoculation were compared between the non-inoculated control and the inoculated plants, increases were observed for *S. taxi UH1* (shoot), *M. radiotolerans UH1* (root and shoot), *E. aerogenes UH1* (root), and the consortium (root and shoot). However, the

inoculated strains *S. taxi UH1* and *E. aerogenes UH1* were observed in slightly lower abundancies in the shoot tissues of inoculated plants in comparison to the non-inoculated control plants. These observations might be due to the fact that the endophytic strains were all originally isolated from roots and thus predominantly or even exclusively colonized the roots of the inoculated plants.

When combining all these results, we can conclude that inoculation of *Cucurbita pepo* plants with a consortium of *Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1,* and *Enterobacter aerogenes UH1* can significantly (46%) increase the phytoremediation potential of the plants in DDE-contaminated soils.

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Conflict of interest

None declared

363 **TABLES**

364 **Table 1** Average DDE concentrations in soils at moment of planting

Treatment	**Average DDE concentration (in ng g^{-1} dry weight) ± SD**
Non-inoculated control	149.7 ± 24.2
Sphingomonas taxi UH1	172.8 ± 26.0
Methylobacterium radiotolerans UH1	153.0 ± 34.8
Enterobacter aerogenes UH1	147.0 ± 24.0
Consortium	158.6 ± 50.1

365 Control plants are not inoculated, other plants were inoculated with the indicated strains, or a
366 combination of the three strains for the consortium.

367

368 **Table 2** Estimation of the amounts of DDE removed by *C. pepo* plants per mound during a full growth
369 cycle

	Control	***St***	***Mr***	***Ea***	**Consortium**
DDE concentration in soil (µg kg^{-1})	149.65	172.76	153.02	146.96	178.60
Amount of DDE per mound (mg)	21.667	25.012	22.154	21.277	25.858
Amount of DDE taken up by plant (mg)	0.81010	0.95981	0.83243	0.71163	1.4132
% of DDE removed by plant	3.74	3.83	4.03	3.34	5.47

370

371 **Table 3** Cultivable bacterial genera isolated from *Cucurbita pepo*

Phylum	**Class**	**Genus**
Actinobacteria	Actinobacteria	*Arthrobacter*
		Curtobacterium
		Frigoribacterium
		Microbacterium

		Nocardioides
		Plantibacter
		Streptomyces
		Terrabacter
Bacteriodetes	Flavobacteria	*Chryseobacterium*
		Flavobacterium
		Myroides
	Sphingobacteria	*Chitinophaga*
		Sphingobacterium
Proteobacteria	Alphaproteobacteria	*Agrobacterium*
		Brevundimonas
		Devosia
		Ensifer
		Methylobacterium
		Ochrobactrum
		Rhizobium
		Sphingomonas
	Betaproteobacteria	*Variovorax*
	Gammaproteobacteria	*Acinetobacter*
		Enterobacter
		Klebsiella
		Lysobacter
		Pectobacterium
		Pseudomonas
		Stenotrophomonas
		Vibrio
		Xanthomonas
Firmicutes	Bacilli	*Bacillus*
		Exiguobacterium
		Paenibacillus

372 Bacterial genera (phylum, class) that were isolated from *C. pepo* using cultivation-dependent
373 techniques.

374

375 **Table 4** Percentages of bacterial strains showing plant growth-promoting capacities

		OA	IAA	Sid	ACC	P-sol
Control	Root	40%	44%	43%	47%	58%
	Shoot	39%	57%	54%	47%	50%
Inoculated	Root	42%	60%	64%	58%	62%
	Shoot	41%	49%	67%	60%	68%

376 Plant growth-promoting capacities of tested bacterial strains (control n=104, inoculated n=426). OA
377 = organic acids, IAA = Indole-3-acetic acid, Sid = Siderophores, ACC = ACC-deaminase, P-sol =
378 phosphate solubilization.

379

380 **Table 5** Bacterial strains showing DDE-degrading potential

species	# positive	Isolated from
Arthrobacter sp.	2	Ea
Chitinophaga sp.	1	Ea
Chryseobacterium sp.	1	St
	2	Control
Enterobacter sp.	1	Ea
Exiguobacterium sp.	1	Cons
Microbacterium sp.	2	Ea
	2	Control
Pectobacterium sp.	1	Ea
Plantibacter sp.	3	Mr
Pseudomonas sp.	2	Mr
	3	Ea
Rhizobium sp.	1	Control
	1	Mr
Sphingomonas sp.	2	Mr
	1	Control
	1	Ea
Stenotrophomonas sp.	5	Control
	2	St
	2	Mr

Variovorax sp.	2	Control
Vibrio sp.	1	Cons

positive = number of bacterial strains that scored positive on the auxanography, Control = not inoculated, St = *Sphingomonas taxi UH1*, Mr = *Methylobacterium radiotolerans UH1*, Ea = *Enterobacter aerogenes UH1*, Cons = consortium.

FIGURE LEGENDS

Figure 1 Layout of the field experiment. One meter of space was between each plant inside rows as well as in between rows. Each row was covered in black plastic and a watering system was present.

Figure 2 Average fresh weights ± SD of *Cucurbita pepo* plants after harvesting in grams. St = *Sphingomonas taxi UH1,* Mr = *Methylobacterium radiotolerans UH1,* Ea = *Enterobacter aerogenes UH1,* Cons = consortium, Red line indicates weight of control plants. **a** Leaf fresh weight, **b** Stem fresh weight, **c** Root fresh weight, **d** Fresh weight of all fruits harvested during the total growth period. * are significantly different from control plants, $p<0.05$.

Figure 3 Bioconcentration Factors (BCFs) of different plant tissues (dry weight) from different treatments (average ± SD). BCF = DDE concentration in plant tissue/soil DDE concentration. St = *Sphingomonas taxi UH1,* Mr = *Methylobacterium radiotolerans UH1,* Ea = *Enterobacter aerogenes UH1,* Cons = consortium.

Figure 4 Average amount of DDE (in mg) removed ± SD per plant per growth season in milligram for the different treatments. St = *Sphingomonas taxi UH1,* Mr = *Methylobacterium radiotolerans UH1,* Ea = *Enterobacter aerogenes UH1,* Cons = consortium. * is significantly different from non-inoculated control plants ($p<0.05$).

Figure 5 Rarefaction curves of the different replicates and treatments of roots and shoots. The rarefaction curves were assembled showing the numbers of observed OTUs, defined at a 97% sequence similarity cut-off, relative to the total number of identified bacterial sequences.

Figure 6 Comparison of the compositions of the cultivable bacterial communities and the total bacterial communities for all treatments. Cultivable communities show all isolated species; total

communities show the top 15 of detected species. Top: isolations from root material, bottom: isolations from shoot material. T= Total community, C= Cultivable community, Control= non-inoculated, St= inoculation with *Sphingomonas taxi UH1*, Mr= inoculation with *Methylobacterium radiotolerans UH1,* Ea= inoculation with *Enterobacter aerogenes UH1*, Consortium= inoculation with the consortium of *S. taxi UH1*, *M. radiotolerans UH1,* and *E. aerogenes UH1*.

Figure 7 % increase in the relative abundancy of the inoculated bacterial strains in comparison to the endophytic communities of the control plants. St S: *S. taxi UH1* Shoot, St R: *S. taxi UH1* Root, Mr S: *M. radiotolerans UH1* Shoot, Mr R: *M. radiotolerans UH1* Root, Ea S: *E. aerogenes UH1* Shoot, Ea R: *E. aerogenes UH1* Root, Co S: Consortium Shoot, Co R: Consortium Root.

Figure 8 Cluster based on S17 Bray-Curtis similarity; cluster mode is the group average of the species present in the samples; square root transformation; cophenetic correlation 0.94306. Hollow circles= cultivable community root tissue, hollow squares = cultivable community shoot tissue, full circles= total community root tissue, full square= total community shoot tissue. Blue= control, green= inoculated with *S. taxi UH1*, red= inoculated with *M. radiotolerans UH1,* purple= inoculated with *E. aerogenes UH1,* and orange= inoculated with a consortium of *S. taxi UH1, M. radiotolerans UH1,* and *E. aerogenes UH1*.

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Figures

Figure 4

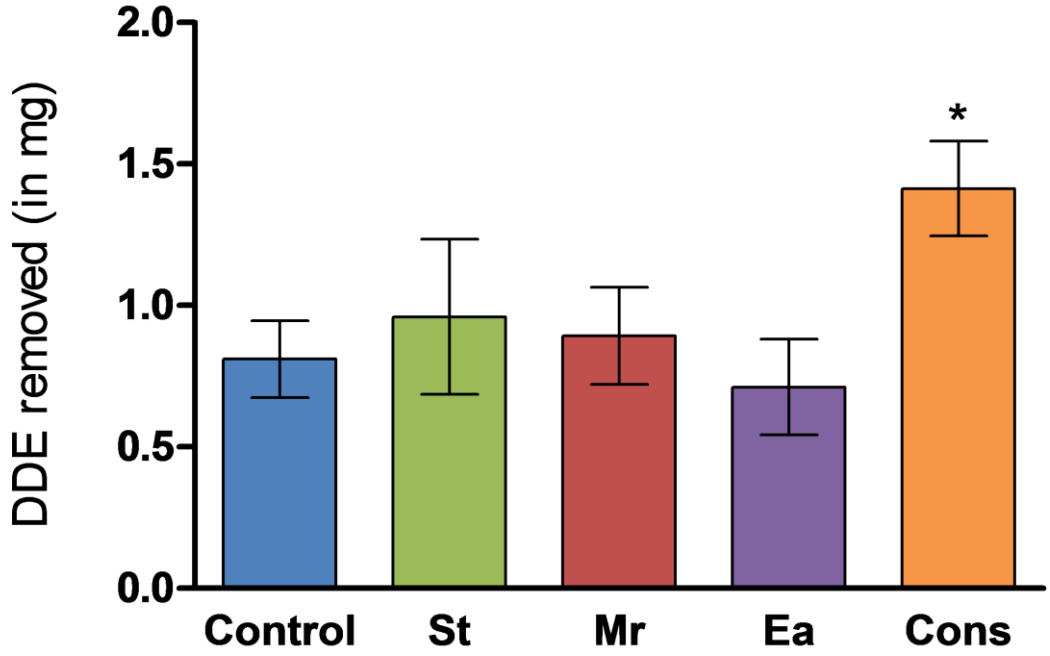


Figure 5

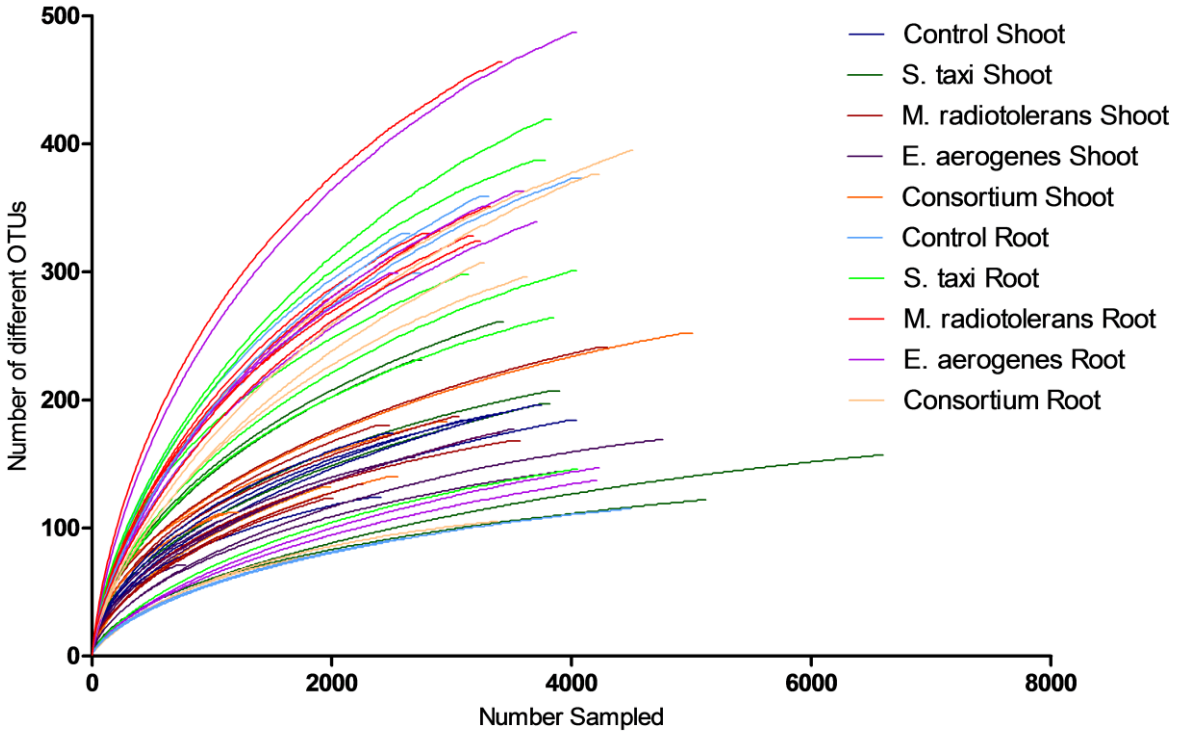


Figure 6

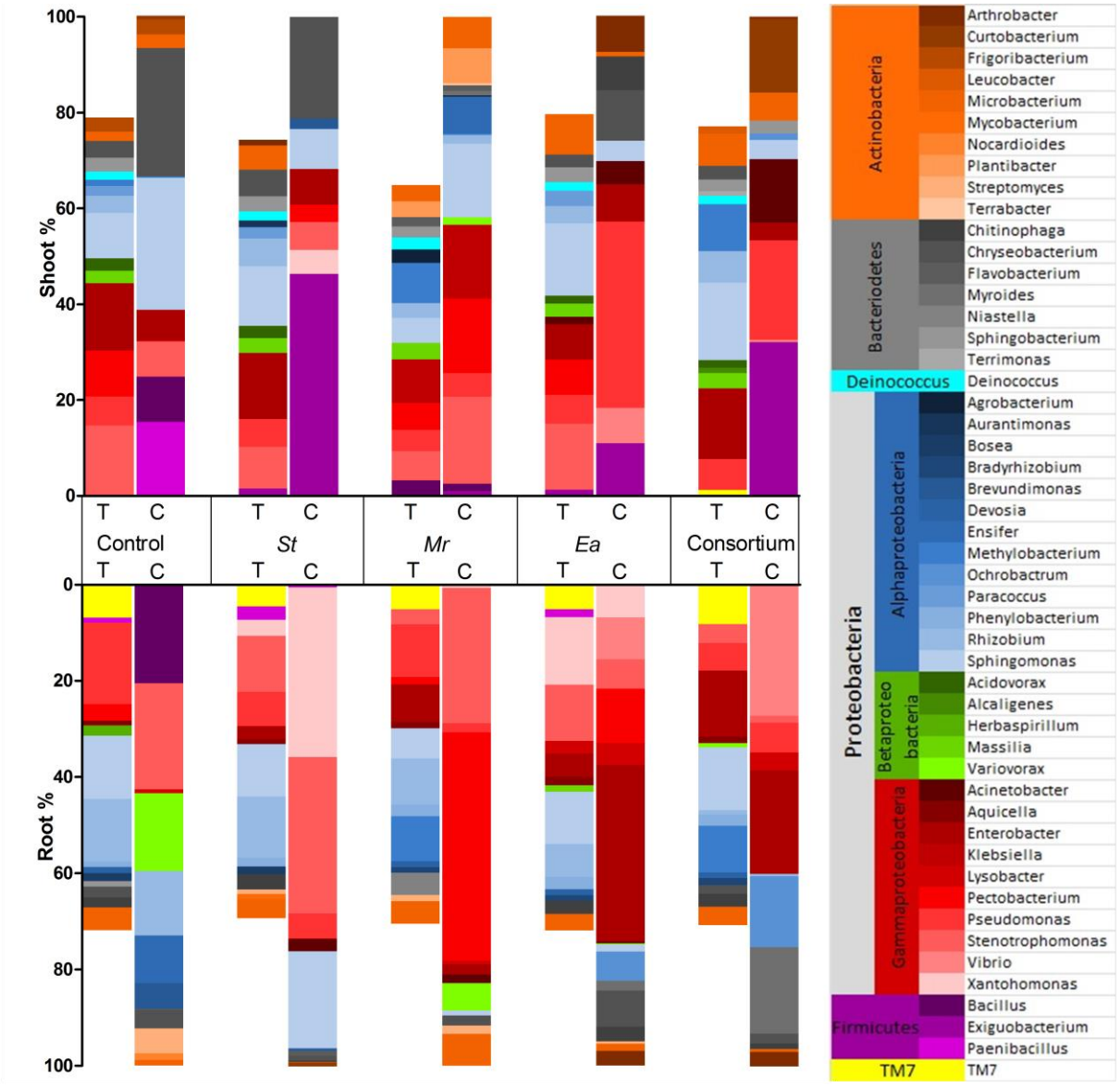
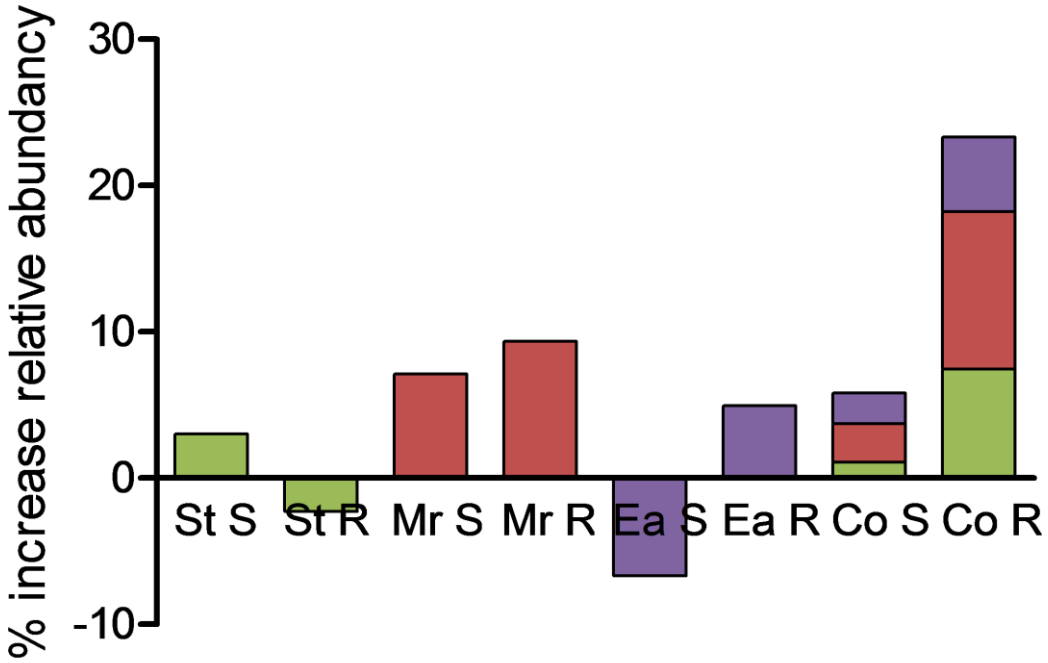


Figure 7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Mr R
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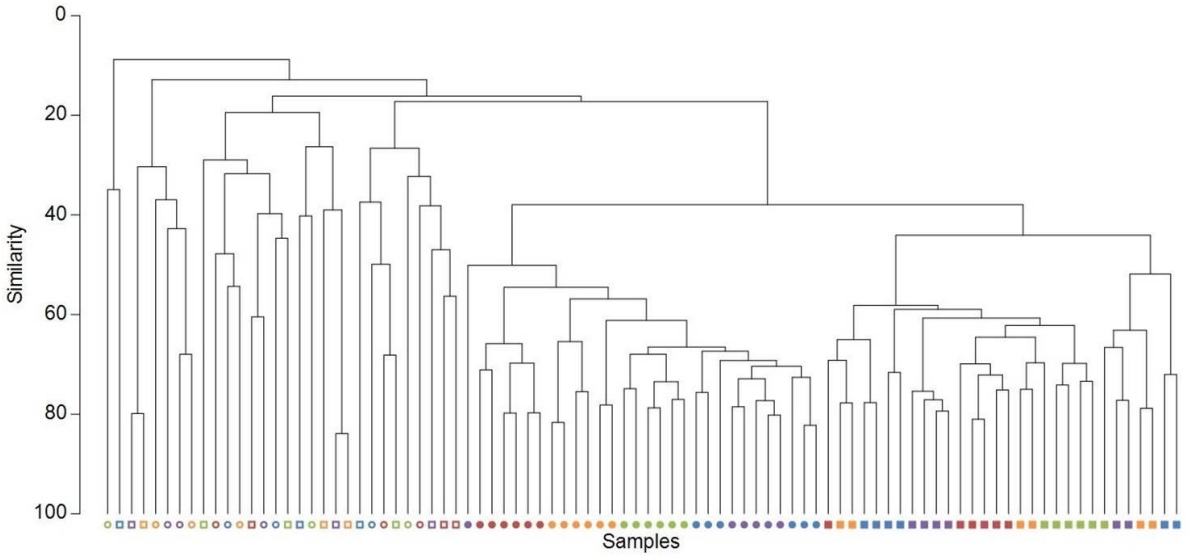
Ea R
Co S
Co R

Figure 8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