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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*.

68 **Materials and methods**

69 ***Inoculation of seeds***

70 The selected bacterial strains were cultivated by transferring 5 µL of the bacterial stock into 30 mL
71 of 869 medium (Mergeay et al., 1985). A consortium was created as well by combining the selected
72 strains into one 50 mL tube. The bacterial suspensions were incubated at 30°C for 3 days.
73 Seeds of *Cucurbita pepo ssp. pepo* cultivar Raven (Johnny's Selected Seeds, Winslow, ME, USA)
74 were surface sterilized for 1 minute in 1% NaOCl and put in the bacterial suspension (10^9 cells mL^{-1})
75 for overnight inoculation.

76

77 ***Field characteristics***

78 The experimental field that was utilized for this trial is located on Lockwood Farm, owned by the
79 Connecticut Agricultural Experiment Station (N41,406786°; W72,906043°; Hamden, CT, USA).
80 The soil in the field was identified as being a Cheshire fine sandy loam soil containing 56% sand,
81 36% silt, and 8% clay. The percentage of organic carbon was 1.4 and an average pH of 6.7 was
82 measured. The cation exchange capacity of the soil was 18.6 cmol kg^{-1}.

83

84 ***Growth and harvest of plants***

85 The seeds were germinated between wet paper towels for 3 days at 30°C. The seedlings were
86 transferred to 750 mL plastic pots containing vermiculite. The bacterial suspension was again added
87 and plants watered daily with ¼ Hoagland nutrient solution [21]. After 7 days, the plants were of
88 sufficient size to be transferred to the field. Plants were set out in 5 rows covered with black plastic
89 to avoid weed growth. Each row containing 8 plants approximately 1 m apart (Figure 1). Holes were
90 made in the plastic foil to insert the plants. Soil samples were taken from each mound/planting site to
91 determine local DDE concentrations. The plants were watered continuously by a drip irrigation
92 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.

118 The concentration of DDx in plant tissues and soil was determined on an Agilent (Avondale, PA,
119 USA) 7890B gas chromatograph (GC) with a 5977A mass selective detector (MSD). Two microliters
120 of sample were injected into a multi-mode inlet (MMI) in pulsed splitless mode at 250°C with He as
121 the carrier gas and then onto an Agilent HP-5MS 30-m column with 0.25mm ID and guard column.
122 The GC oven initial temperature was 150°C for 1 min, then ramped to 250°C at a rate of 5°C min^{-1},
123 and then ramped at a rate of 50°C min^{-1} to a final temperature of 300°C which was held for 8 min.
124 After a 5 min solvent delay, the MSD detected analytes using scan mode at a mass to charge ratio
125 (m/z) 100-430. The instrument was calibrated with the standards described above, covering a range
126 of 25-1,000 ng mL^{-1}.
127

128 ***Cultivation-dependent isolation***

129 *Isolation of cultivable endophytes*

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

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

143 were calculated of the 3 replicates. The colonies were purified and in total 585 isolated strains were
144 stored in 15 $\%_w$ glycerol at -80°C.
145
146 *Genotypic characterization of cultivable bacterial strains*
147 The DNA of the 585 isolated strains was recovered with the Qiagen DNeasy blood and tissue kit
148 (Qiagen, Venlo, the Netherlands). A Nanodrop ND-1000 Spectrophotometer (Isogen Life Science,
149 De Meern, the Netherlands) was used to analyze the quality and quantity of the extracted DNA. The
150 DNA was used directly for 16S rDNA amplification with a universal 1392R primer (5-
151 ACGGGCGGTGTGTRC-3) and a bacteria-specific 26F primer (5-
152 AGAGTTTGATCCTGGCTCAG-3) [24]. The 16S products were digested and separated by gel
153 electrophoresis (1.5% agarose, 90V, 2h). The banding patterns were analyzed and 50 different DNA
154 fingerprints were distinguished. At least one representative of each pattern was selected and sent for
155 16S rDNA sequencing at Macrogen (Amsterdam, the Netherlands). Consensus sequences were
156 obtained with the Staden package and identification was acquired from the Ribosome Database
157 Project based on the most closely related species (http://rdp.cme.msu.edu/classifier/classifier.jsp) [25].
158 The Shannon-Wiener indices of all different communities were calculated to estimate the diversity
159 obtained after the different growth conditions [26, 27].
160
161 *Phenotypic characterization of cultivable bacterial strains*
162 The bacterial strains were tested *in vitro* for their plant growth-promoting traits. The bacteria were
163 grown in liquid 869 medium [23] at 30°C for 2 days, washed and resuspended in 1 mL sterile $MgSO_4$.
164 Twenty µl of this suspension was used for inoculation of 96 well microplate assays to investigate the
165 production of IAA using Salkowski reagent [28], siderophores using the Chrome Azurol S (CAS) assay
166 [29], 1-aminocyclopropane-1-carboxylate (ACC) deaminase using 2,4-dinitrophenylhydrazine and
167 NaOH [30], organic acids using Alizarin red S [31] and phosphate solubilization capacity using NBRIP

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

170 *Screening bacteria for DDE-degrading capacities*

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

174 One hundred µl of the bacterial suspension was plated on selective 284 medium. Immediately
175 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
176 added to the surface and smeared out on ¾ of the outer circle of the plate (Figure 4). The plates were
177 incubated at 30° for 6 days. Plates with bacteria growing on the total surface were considered neutral,
178 and were considered to be tolerant to the used concentrations of DDE and CuNPs. Plates on which
179 strains did not show growth on the outer circle containing DDE and CuNPs were considered negative,
180 since DDE was toxic to these bacterial strains. If the bacterial strains showed enhanced growth in the
181 area covered with DDE, the plates were considered positive and these strains are presumed to use
182 DDE as a carbon source while being tolerant to CuNPs.

183 *Statistical analysis*

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

186

187 ***Cultivation-independent molecular analysis***

188 *Extraction and sequencing*

189 The plant tissue samples were surface sterilized (3 min in sterile MilliQ water, 1.5 min ethanol 70%,
190 3 min NaOCl 1%, 1.5 min ethanol 70%, rinse 5 times in sterile MilliQ water) before being crushed
191 in liquid nitrogen. All genomic DNA was extracted using an Invisorb spin plant mini kit (Stratec
192 Biomedical, Germany). A template specific 967F primer (5- CAACGCGAAGAACCTTACC-3) was
193 combined with 1391R primer (5- GACGGGCGGTGWGTRCA-3) to target a 424 bp fragment in the

194 V3-V4 region. In a two-step PCR amplification, first the original primers were used, and then the
195 same primers with a 10 bp multiplex identifier (MID) were employed. PCR reactions contained 1x
196 Roche FastStart High Fidelity Reaction Buffer with 1.8 mM $MgCl_2$, 1.5U of Roche FastStart High
197 Fidelity DNA-polymerase, 0.2 mM Roche dNTP, 300 nM 341F, 100 nM 783Ra, 100 nM 783Rb, 100
198 nM 783Rc and 1 µl of 1/20 diluted template DNA (1 ng μl^{-1}) in a volume of 50 µl. The PCR program
199 consisted of an initial denaturation step of 95°C for 2 min, followed by 25 cycles (1st PCR) or 10
200 cycles (2nd PCR) of denaturation at 95°C for 30 s, annealing at 53°C for 40 s, extension at 72°C for 1
201 min, with a final extension of 5 min at 72°C. The amplicons were purified from 1.5% agarose gels
202 using a QIAquick gel extraction kit (Qiagen, Venlo, the Netherlands) and 1 µl was used for the second
203 PCR using MID-elongated primers. The PCR products were purified with a QIAquick PCR
204 purification kit (Qiagen, Venlo, the Netherlands). The DNA was quantitated using a Quant-IT
205 PicoGreen dsDNA assay kit (Life Technologies Europe, Gent, Belgium), after which equimolar
206 concentrations of the barcoded amplicons were collected per library and diluted to 100 µl using TE
207 buffer. The library was unidirectionally sequenced using a Roche 454 GS-FLX Plus Life Sciences
208 Genome Sequencer at Macrogen (Seoul, South Korea).
209
210 *Analysis of obtained pyrosequencing data*
211 The FASTA files containing the raw pyrosequencing data were accessed using Mothur bioinformatics
212 software [33] for processing and analysis according to Schloss et al. [34]. The obtained sequences were
213 denoised before barcodes and primers were removed. The remaining sequences were aligned and
214 classified along known sequences in the SILVA rRNA database [35]. Chimeric sequences,
215 mitochondrial and chloroplast sequences were deleted and the remaining sequences were grouped
216 into operational taxonomic units (OTUs) based on a 97% similarity criterion. Rarefaction curves were
217 starting to level off (Figure 5), but sequencing at a greater depth could have revealed more OTUs.
218 The similarity between samples and their resemblance to the cultivated communities were visualized
219 using Primer7 (Version 7.0.5, Primer-E Ltd.). Clustering of samples was based on S17 Bray-Curtis

220 similarity of the group average of the species after square root transformation of the samples. The
221 nMDS was based on S17 Bray-Curtis similarity as well, with square root transformation, Kruskall
222 stress formula 1 and minimum stress 0.01. ANOSIM (analysis of similarities) with 999 permutations
223 was used to test the spatial separation of the samples in nMDS.
224

225 **Results and discussion**

226 ***Plant weights***

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

235 ***Soil DDE concentrations***

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

240 ***DDE concentrations in plant tissues***

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

245 As expected, the BCFs of leaves are very low, ranging from 0.025 to 0.075; by comparison, BCFs of
246 roots and stems range from 19 to 25. This difference can be explained by the difficult translocation
247 of DDE in plant tissues due to the hydrophobic character of the molecule. Therefore, larger effects of
248 DDE on the endophytic communities can be expected in roots, in comparison to shoots.
249 With both the DDE concentrations of each plant compartment and weight of the respective
250 compartments known, the total amount of DDE that was extracted from the field per plant during the
251 entire growth period could be calculated as follows (Figure 4): (DDE concentration in plant tissue) x
252 (plant weight).
253 Although no significant differences were observed between inoculated plants and control plants for
254 the DDE concentrations, the total amount of DDE that was removed from the soil in one growth cycle
255 was significantly higher in plants that were inoculated with the consortium in comparison to non-
256 inoculated control plants.
257 By estimating the mound volume in which a *C. pepo* plant can grow, the total amount of DDE a plant
258 can reach was assessed. The mound had a surface area of 1m by 0.5m and a depth of 0.25m. This
259 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
260 2 presents the amounts of DDE that were present per mound, the amounts of DDE that were taken up
261 by the plants and the percentage this amount represents in comparison to the soil DDE content.
262 ***Endophytic communities***
263 *Genotypic identification*
264 The endophytic communities of the roots and shoots were investigated using both cultivation-
265 dependent and cultivation-independent techniques. The cultivation-dependent isolation of the
266 endophytic communities produced 530 cultivable strains, belonging to 4 phyla and 34 genera (Table
267 3 and Figure 6).
268 The cultivation-independent molecular analysis was conducted using 454 pyrosequencing. Macrogen
269 (Seoul, South Korea) delivered the raw data in 4 FASTA files. These files contained in total 573,227
270 strains with a mean length of 388.01 base pairs. The files were analyzed using Mothur [33] and the

271 sequences were filtered using following criteria: length >200 bp, <8 homopolymers, <2 differences
272 with the primers, <1 difference with the barcodes, pre-clustered to reduce sequencing errors, removal
273 of chimeras, and more than 95% in the same range of genes. After this filtering, 207,128 sequences
274 remained, containing a total of 26,862 unique sequences. Subsequently, all sequences belonging to
275 mitochondria, chloroplasts, archaea, and eukaryotes were deleted. After this step, 199,500 sequences
276 remained, implying that 3.66% of all sequences belonged to non-bacterial DNA. In the total of all
277 samples, 288 different genera were detected belonging to 20 different phyla, or subphyla in case of
278 the Proteobacteria (Figure 6). With a mean OTU length of 223.19 bp, identification to the species
279 level was not possible.
280 Although 288 different genera were detected during the pyrosequencing process, many of them were
281 only detected once across the different samples. Therefore, we focus on the 15 most abundant genera,
282 which account for a large portion of the total bacterial communities (ranging from 64.83% to 79.66%
283 of the total community).
284 Inoculating the plants with *Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1,*
285 *Enterobacter aerogenes UH1,* or a consortium combining the three strains increased their abundancy
286 in the community in the case of *S. taxi UH1* (Shoot)*, M. radiotolerans UH1, E. aerogenes UH1* (Root)
287 and the consortium but had no apparent effect on the presence of *S. taxi UH1* (Root) and *E. aerogenes*
288 *UH1* (Shoot) since even a slightly lower presence was detected in the inoculated plants (Figure 7).
289 It is important to evaluate the inoculation efficiency since an inoculation can only be considered
290 successful in case the presence of a bacterial strain is enhanced in the communities. However, in field
291 conditions, an effective establishment of the inoculated strain is not evident because of the large
292 number of bacterial genera competing for a position in the community.
293 The observed changes in abundance are larger for root communities of the plants inoculated with *M.*
294 *radiotolerans UH1, E. aerogenes UH1,* and the consortium. This is not surprising since all three
295 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

348 inoculated strains *S. taxi UH1* and *E. aerogenes UH1* were observed in slightly lower abundancies in
349 the shoot tissues of inoculated plants in comparison to the non-inoculated control plants. These
350 observations might be due to the fact that the endophytic strains were all originally isolated from roots
351 and thus predominantly or even exclusively colonized the roots of the inoculated plants.
352 When combining all these results, we can conclude that inoculation of *Cucurbita pepo* plants with a
353 consortium of *Sphingomonas taxi UH1, Methylobacterium radiotolerans UH1*, and *Enterobacter*
354 *aerogenes UH1* can significantly (46%) increase the phytoremediation potential of the plants in DDE-
355 contaminated soils.
356

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360

361 **Conflict of interest**

362 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

381 # positive = number of bacterial strains that scored positive on the auxanography, Control = not

382 inoculated, St = *Sphingomonas taxi UH1,* Mr = *Methylobacterium radiotolerans UH1,* Ea =

383 *Enterobacter aerogenes UH1,* Cons = consortium.

384

385

386 **FIGURE LEGENDS**

387

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

390

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

396

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

401

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

406

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

410

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

413 communities show the top 15 of detected species. Top: isolations from root material, bottom:
414 isolations from shoot material. T= Total community, C= Cultivable community, Control= non-
415 inoculated, St= inoculation with *Sphingomonas taxi UH1*, Mr= inoculation with *Methylobacterium*
416 *radiotolerans UH1,* Ea= inoculation with *Enterobacter aerogenes UH1*, Consortium= inoculation
417 with the consortium of *S. taxi UH1, M. radiotolerans UH1,* and *E. aerogenes UH1.*
418
419 **Figure 7** % increase in the relative abundancy of the inoculated bacterial strains in comparison to the
420 endophytic communities of the control plants. St S: *S. taxi UH1* Shoot, St R: *S. taxi UH1* Root, Mr
421 S: *M. radiotolerans UH1* Shoot, Mr R: *M. radiotolerans UH1* Root, Ea S: *E. aerogenes UH1* Shoot,
422 Ea R: *E. aerogenes UH1* Root, Co S: Consortium Shoot, Co R: Consortium Root.
423
424 **Figure 8** Cluster based on S17 Bray-Curtis similarity; cluster mode is the group average of the species
425 present in the samples; square root transformation; cophenetic correlation 0.94306. Hollow circles=
426 cultivable community root tissue, hollow squares = cultivable community shoot tissue, full circles=
427 total community root tissue, full square= total community shoot tissue. Blue= control, green=
428 inoculated with *S. taxi UH1*, red= inoculated with *M. radiotolerans UH1,* purple= inoculated with *E.*
429 *aerogenes UH1,* and orange= inoculated with a consortium of *S. taxi UH1, M. radiotolerans UH1,*
430 and *E. aerogenes UH1.*

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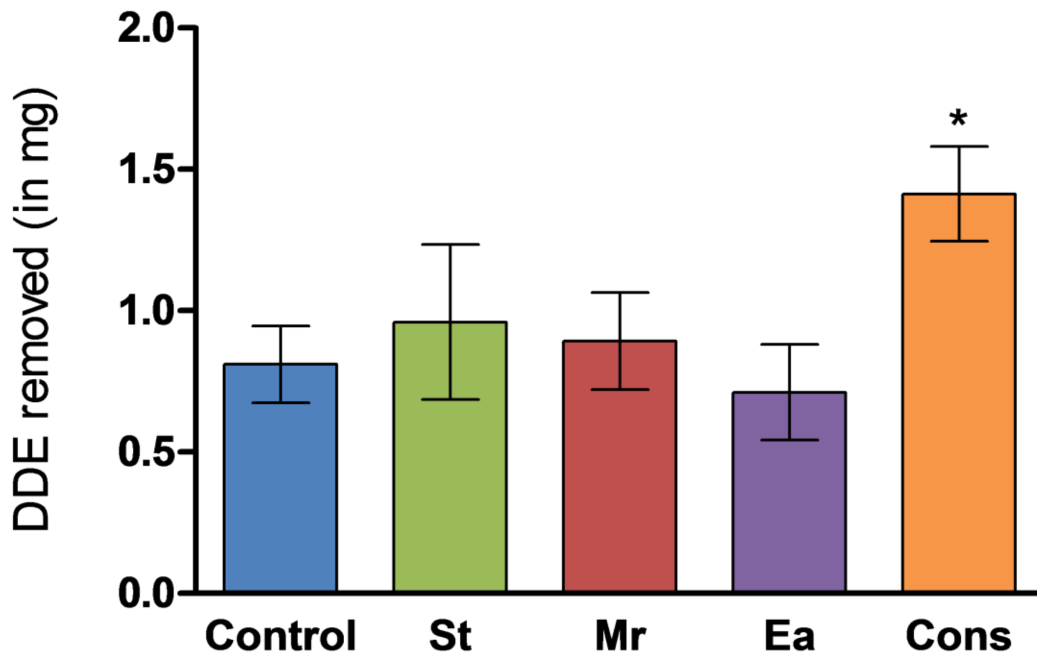
524
525

526 **Figures**

527 **Figure 4**



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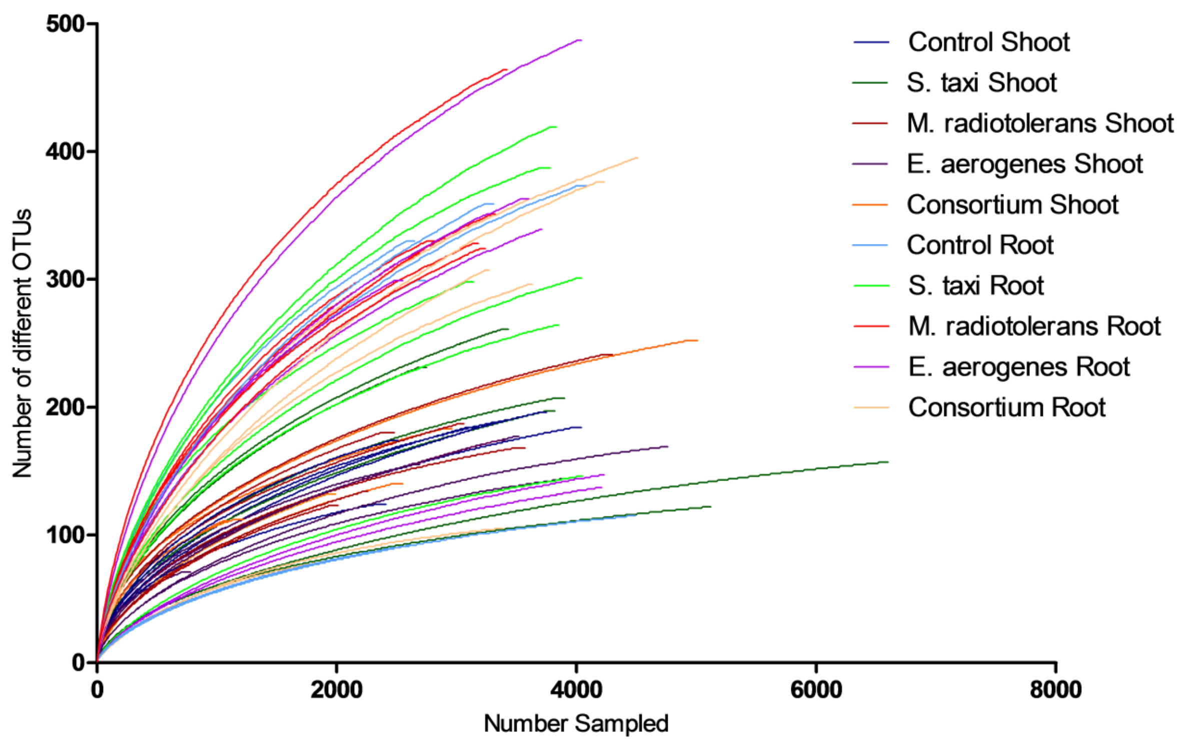

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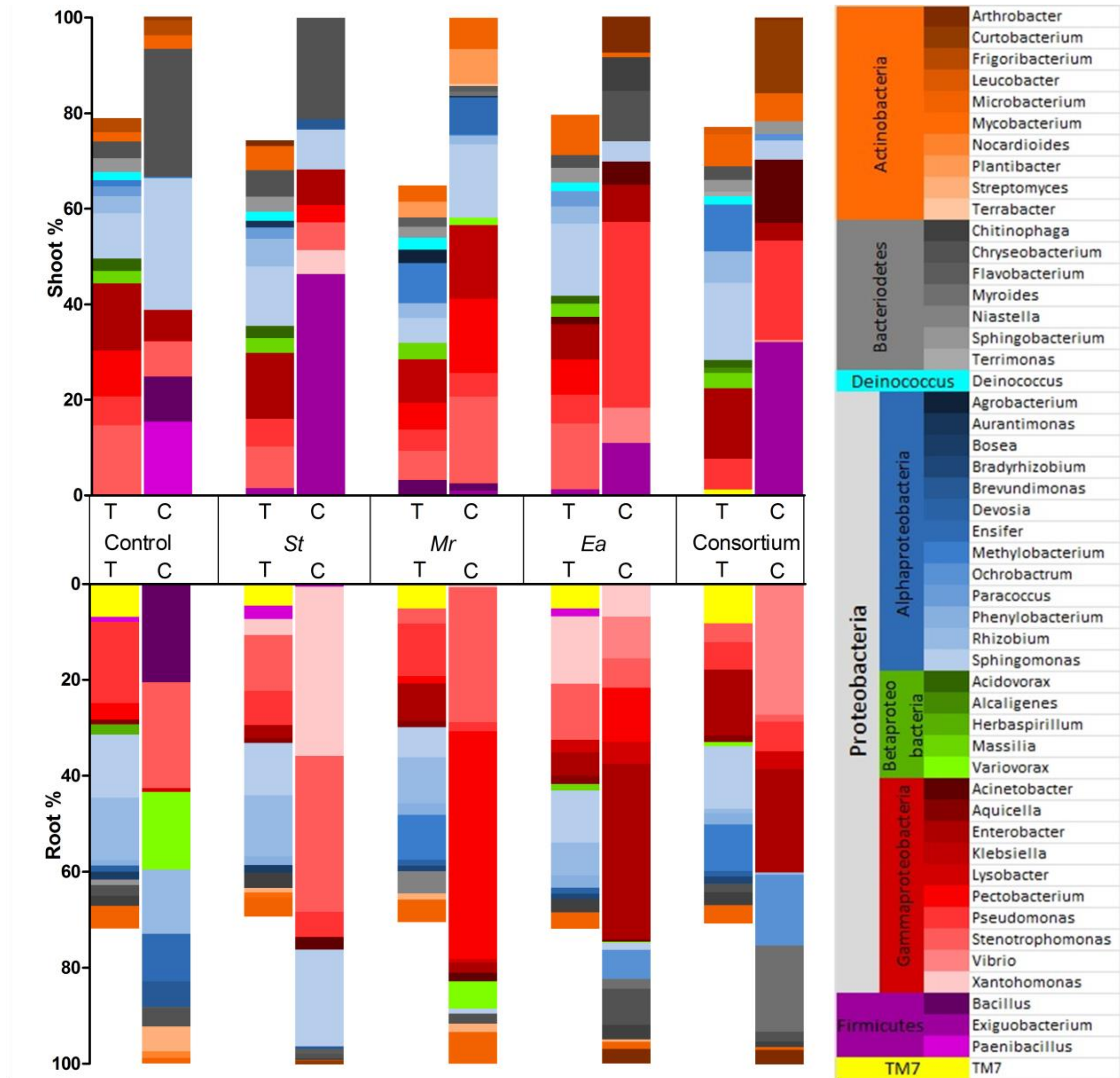

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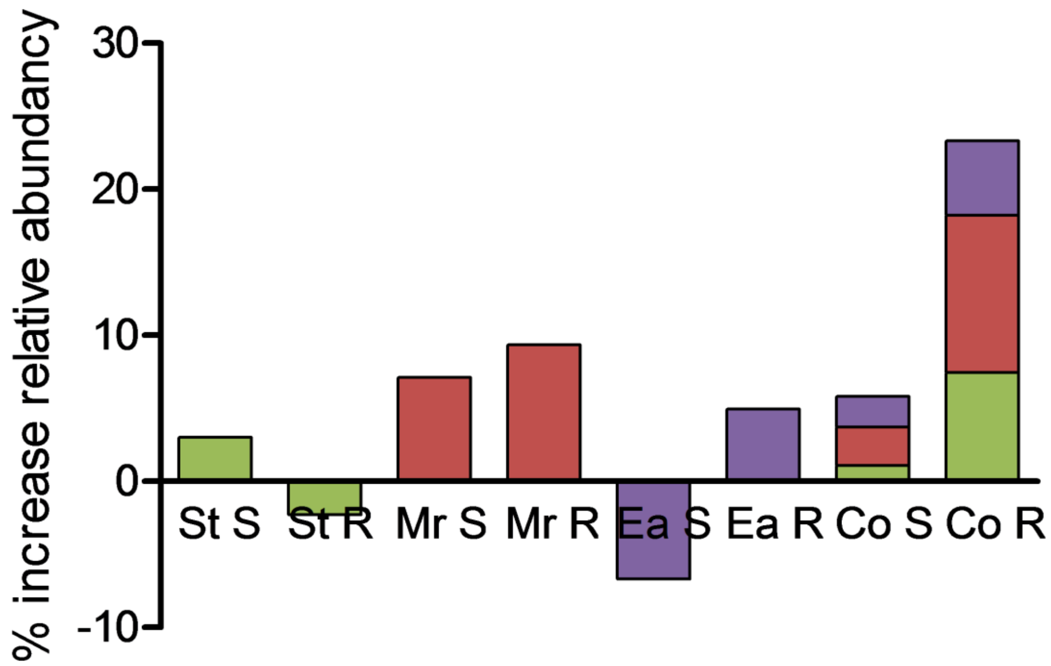

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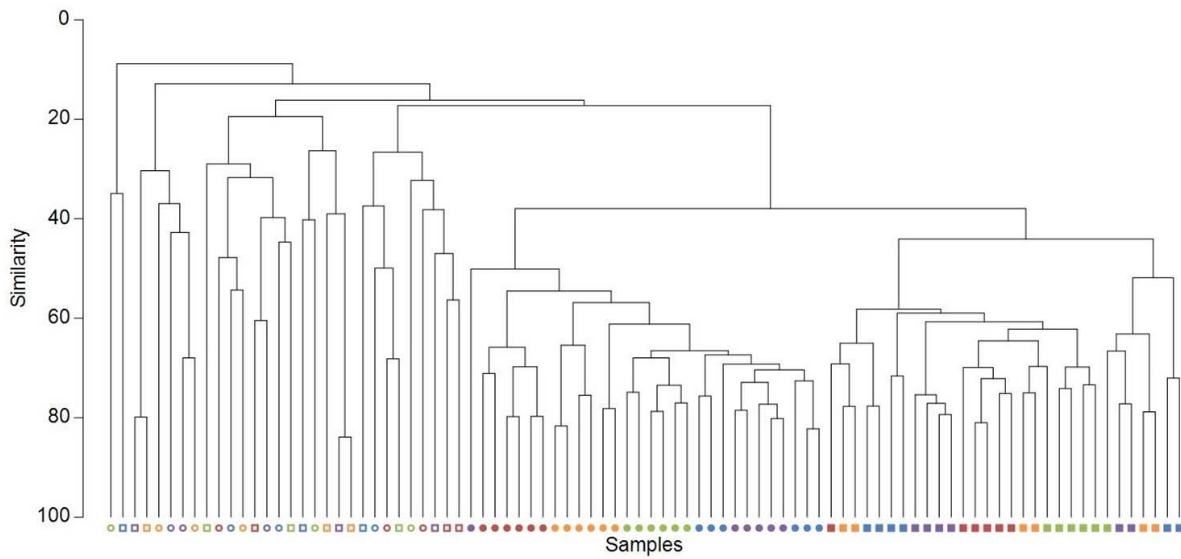
536 **Figure 8**



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