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Exposure of *Cucurbita pepo* to DDE-contamination alters the endophytic community: A cultivation dependent vs a cultivation independent approach

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ABSTRACT

2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene (DDE) is the most abundant and persistent degradation product of the pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) and is encountered in contaminated soils worldwide. Both DDE and DDT are classified as Persistent Organic Pollutants (POPs) due to their high hydrophobicity and potential for bioaccumulation and biomagnification in the food chain. Zucchini (*Cucurbita pepo* ssp. *pepo*) has been shown to accumulate high concentrations of DDE and other POPs and has been proposed as a phytoremediation tool for contaminated soils. The endophytic bacteria associated with this plant may play an important role in the remedial process. Therefore, this research focuses on changes in endophytic bacterial communities caused by the exposure of *C. pepo* to DDE. The total bacterial community was investigated using cultivation-independent 454 pyrosequencing, while the cultivable community was identified using cultivation-dependent isolation procedures. For both procedures, increasing numbers of endophytic bacteria, as well as higher diversities of genera were observed when plants were exposed to DDE. Several bacterial genera such as *Stenotrophomonas* sp. and *Sphingomonas* sp. showed higher abundance when DDE was present, while, for example *Pseudomonas* sp. showed a significantly lower abundance in the presence of DDE. These findings suggest tolerance of different bacterial strains to DDE, which might be incorporated in further investigations to optimize phytoremediation with the possible use of DDE-degrading endophytes.

1. Introduction

2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was used extensively beginning in 1943 against mosquitos and other insect pests, but its use was restricted globally in the 1970s due to concerns over its toxicity and persistency in the environment (Ahuja and Kumar, 2003; Lunney et al., 2004). The exception to this ban on use includes 19 countries where no other practical measures for malaria control exist (Porta and Zumeta, 2002). When exposed to weathering in the environment, DDT (aerobically) transforms continuously to 2,2-bis(p-chlorophenyl)-1,1-dichloro-ethylene (DDE) (Aslabie et al., 1997), causing a steady increase of DDE-concentrations. DDE is the most persistent and most frequently encountered degradation product of DDT in soils worldwide (Megharaj et al., 1997). Both DDT and DDE, and other less common metabolites such as 1-chloro-4-(2,2-dichloro-1-(4-chlorophenyl) ethyl)benzene (DDD), are listed as being Persistent Organic Pollutants (POPs) in the Dirty Dozen List of the Stockholm Treaty (Mitton et al., 2012; Porta and Zumeta, 2002); all are of significant environmental concern due to their high recalcitrance and toxicity (White et al., 2005). DDE has a log K_{OW} (octanol–water partitioning coefficient) of 6.51, which is indicative of hydrophobicity and a significant potential to bioaccumulate in lipids of exposed organisms and biomagnify within food chains (Ahuja et al., 2001; Chhikara et al., 2010; White et al., 2005).

Phytoremediation has been proposed as a possible solution for soils contaminated with DDE and other POPs (Lunney et al., 2010). Phytoremediation is a technology that uses the plants innate ability to remove organic or inorganic contaminants from the soil (Gent et al., 2007). For organic compounds such as DDE, remediation depends on plant processes related to contaminant uptake, translocation and metabolism; all of which lead to a diminished

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pollutant concentration in the soil (Afzal et al., 2014; Arslan et al., 2015; Mitton et al., 2012). *Cucurbita pepo* ssp. *pepo* cultivar Raven (Zucchini Raven) has demonstrated a strong potential to extract and translocate highly weathered *p,p′*-DDE from the soil (White, 2010). The root-to-soil and stem-to-soil bioaccumulation factors (BCF; ratio of contaminant concentration in the tissue to that of the soil) were reported as 19.9 and 23.7, respectively. However, plants are not the sole factor of importance when attempting efficient phytoremediation of organic contaminants. Endophytic bacteria that reside inside plant tissues are known to play a crucial role in the remediation of organic contaminants (Weyens et al., 2009c) as well as in plant growth in general (Barac et al., 2004). Plant-associated bacteria have been shown to degrade contaminants such as trichloroethylene (TCE), hydrocarbons and explosives (Weyens et al., 2009b; Thijs et al., 2014). Furthermore, many of these endophytic bacteria show plant growth-promoting capacities that might enhance plant fitness in marginal or stressful environments such as contaminated soils (Weyens et al., 2015). To optimize the phytoremediation efficiency of *C. pepo* in DDE-contaminated soils, the role of endophytic communities must be determined. This research focuses on the effect of DDE exposure on the endophytic communities of *C. pepo* by investigating both the total communities using 454 pyrosequencing, and the cultivable communities using cultivation-dependent isolations.

2. Materials and methods

2.1. Growing plants

C. pepo ssp. Raven seeds were purchased from Johnny's Selected Seeds (Winslow, ME, USA). The seeds were germinated in wet paper towels for 3 days at 30 °C. The seedlings were transferred to 750 ml plastic pots containing vermiculite. Half of the plants (n = 10) were watered with 30 ml regular ¼ Hoagland nutrient solution (Hoagland and Arnon, 1950), and the other half (n = 10) was watered with 30 ml ¼ Hoagland nutrient solution amended with 100 μg l^{-1} p,p'-DDE (analytical grade standard) that was dissolved in methanol before being diluted to contain 1% methanol. For 21 days, the plants were kept in the greenhouse before being harvested (humidity 60%; day–night cycle: day 7.00–22.00; temperature: day 23 °C, night 18 °C; light intensity 300 W m^{-2}). The steps after harvesting are depicted in Fig. 1 and described below.

2.2. Measuring DDE-content in plant tissues

At 21 days, root and shoot samples (n = 5 per condition) were separated at harvest. Freeze dried plant 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 GCMS as previously described by White (2010).

2.3. Cultivation dependent isolation

2.3.1. Surface sterilization of plant tissues

The vermiculite was rinsed from the plant tissues with tap water. Before separating roots and shoots, plant mass was determined. Root and shoot tissues were incubated separately in 1% NaOCl for external sterilization. Afterwards, the samples were rinsed 3 times in sterile dH_2O and dried on sterilized filter paper. A portion (100 μl) of the third rinsing water was transferred to a plate containing undiluted 869 medium (Mergeay et al., 1985) (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) to check for sterility.

2.3.2. Direct isolation of the bacteria

The sterilized tissues of three plants were transferred to sterilized mortars containing 5 ml sterile 10 mM $MgSO_4$ and 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 (Mergeay et al., 1985). 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 per gram of fresh plant material was determined. For each treatment, averages and standard errors were calculated of the 3 replicates. The colonies were purified and 168 isolated strains were stored in 15%$_w$ glycerol at −80 °C.

2.3.3. Selective growth of bacteria

One ml of each 0-dilution of crushed plant tissues (previous step) was transferred to a flask containing 100 ml liquid 284 + C medium (Schlegel et al., 1961) spiked with 1 mg l^{-1} DDE (per liter: 6.06 g Tris, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.03 g $CaCl_2 \cdot 2H_2O$, 0.04 g $Na_2HPO_4 \cdot 2H_2O$, 10 ml of a 48 mg l^{-1} solution of Fe(III)NH_4Citrate, 1 ml of SI7 trace elements [per liter: 190 × 10^{-3} g $CoCl_2$, 17 × 10^{-3} g $CuCl_2$, 62 × 10^{-3} g H_3BO_3, 100 × 10^{-3} g $MnCl_2$, 36 × 10^{-3} g $NaMoO_4$, 24 × 10^{-3} g $NiCl_2$, 144 × 10^{-3} g $ZnSO_4 \cdot 7H_2O$]. The following filter-sterilized carbon sources were added after autoclavation: 0.7 ml lactate, 0.52 g glucose, 0.66 g gluconate, 0.54 g fructose, 0.81 g succinate. The flasks were incubated at 30 °C and the medium was refreshed each 10 days by transferring 5 ml of the mixture into a new flask containing 100 ml 284 + C medium. After 30 days, 100 μl of the growth medium was plated onto Petri dishes containing 1/10 diluted 869 medium. The plates were produced in triplicate and incubated at 30 °C for 4 days. The colonies were purified and 243 isolated strains

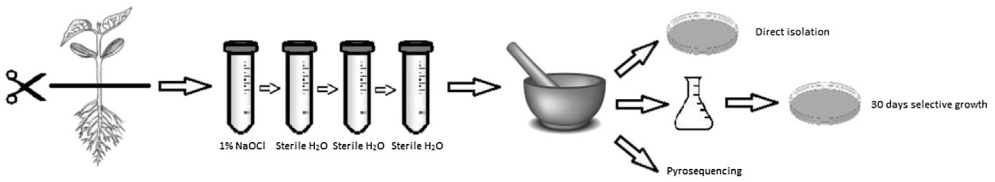

Fig. 1. Workflow for isolation of cultivable endophytic bacteria and sampling for pyrosequencing of total endophytic bacterial community. 10 pots per condition were harvested, each containing 2 plants.