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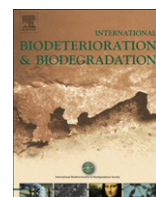
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Maize plants (*Zea mays*) root exudates enhance lindane removal by native *Streptomyces* strains

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ABSTRACT

The organochlorine pesticide (OP) lindane was removed from minimal medium (MM) by two *Streptomyces* native strains, while growing on maize root exudates (REs) as a primary carbon and energy source. REs supported 55 and 35% of lindane removal by *Streptomyces* sp. strains A5 and M7, respectively, corroborating the hypothesis that co-metabolism may be a plant/microbe interaction important to bioremediation. In addition, residual lindane concentration was more than half of the amount in MM supplemented with glucose compared to MM supplemented with REs, suggesting that exudates could be more appropriate carbon source to support aerobic dehalogenation of the pesticide. Lindane-degrading activity was detected in REs, which could explain 42% of lindane removal in REs-lindane assay, without microorganisms. Because *Streptomyces* sp. A5 showed maximum biomass and the highest pesticide removal in REs-lindane assay, it was found to be the most promising strain regarding their future application. These results showed that phytostimulation of OP-degrading actinobacteria by maize REs are therefore likely to be a successful strategy for the remediation of lindane-contaminated environments.

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1. Introduction

Lindane (γ -hexachlorocyclohexane) is a persistent organochlorine pesticide (OP) that has been used worldwide for crop protection and the control of vector-borne diseases (Manickam et al., 2008). Lindane is a potential carcinogen and is listed as priority pollutant by the US EPA (Walker et al., 1999). Although nowadays its use is restricted or completely banned in most countries, residues of lindane are found all over the world in soil, water, air, plants, agricultural products, animals, food, and humans (Piñero González et al., 2007; Kidd et al., 2008; Herrero-Mercado et al., 2010; Fuentes et al., 2011). Since lindane toxicity is well-known, it is imperative to develop methods to remove it from the environment. Bioremediation technologies, which use plants and/or microorganisms to degrade toxic contaminants, have become the focus of interest.

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Actinobacteria – the main group of bacteria presents in soils and sediments – have a great potential for bioremediating toxic compounds, since these Gram-positive microorganisms are already adapted to this habitat. In addition to their potential metabolic diversity, strains of *Streptomyces* may be well suited for soil inoculation as a consequence of their mycelial growth habit, relatively rapid rates of growth, colonization of semi-selective substrates, and their ability to be genetically manipulated (Shelton et al., 1996). However, little information is available on the ability of biotransformation of OPs by Gram-positive microorganisms and particularly by actinobacteria (Lal et al., 2010).

Recent studies demonstrate enhanced dissipation and/or mineralization of OPs at the root–soil interface (Kidd et al., 2008). This rhizosphere effect is generally attributed to an increase in microbial density and/or metabolic activity due to the release of plant root exudates (REs). REs contain water soluble, insoluble, and volatile compounds including sugars, amino acids, organic acids, nucleotides, flavonones, phenolic compounds and certain enzymes (Chen et al., 2002; Kuiper et al., 2004; Chaudhry et al., 2005). Since REs are complex mixtures of substrates, they not only provide a nutrient-rich habitat for pollutant degraders but can potentially enhance biodegradation in different ways: they may facilitate the co-metabolic transformation of pollutants with similar structures, induce

catabolic enzymes involved in the degradation process and/or enhance the contaminant bioavailability; some components of REs such as citric acid may increase the availability of xenobiotics in soils (Kidd et al., 2008; Gao et al., 2010). In addition, REs may directly induce contaminant degradation by root-driven extracellular enzymes (Barriada-Pereira et al., 2005; Gao et al., 2010). In this context, the phytostimulation of OP-degrading microorganisms by means of REs is therefore likely to be a successful strategy for the remediation of lindane-contaminated environments. However, limited research has evaluated this issue, still less employing natural REs. Successful application of maize to the remediation of xenobiotics was previously reported (Luo et al., 2006; Gao et al., 2010) on the basis of which an active role of this plant in lindane degradation was assumed.

In previous works, wild type *Streptomyces* strains were isolated and selected, which were able to remove lindane from different samples (Benimeli et al., 2003; Fuentes et al., 2010). Three of these strains showed promising results regarding their application for remediating polluted environments contaminated with OPs. The main objective of this work was to evaluate the effect of maize REs on lindane removal by *Streptomyces* sp. strains already isolated at the lab.

2. Materials and methods

2.1. Bacteria strains

Streptomyces sp. A5, M7 and A11 were previously isolated from sediments and soil samples contaminated with several OPs (Benimeli et al., 2003; Fuentes et al., 2010). Taxonomic identification of these strains have been confirmed by amplification and partial sequencing of their 16S rDNA genes [GenBank IDs: AY45953 (M7) (Benimeli et al., 2007), GQ867055 (A11) and GQ867050 (A5) (Fuentes et al., 2010)].

2.2. Collection of root exudates (REs)

Maize seeds (not treated with fungicide) were surface sterilized using 2% mercuric chloride (Benimeli et al., 2008). Then, seeds were placed onto sterile Petri dishes with filter paper Whatman No 1 moistened with sterile water, until germination. Groups of twenty seedlings were transferred aseptically to flasks, where they were grown in nutritive solution under sterile conditions, in a climate controlled room [25 °C, 16:8 (light/dark), 65% relative humidity]. The composition of the nutrient solution was: KNO₃ 1.5 mM; Ca (NO₃)₂ 1 mM; MgSO₄ 0.5 mM; NH₄H₂PO₄ 0.25 mM; EDTA–Fe 11.9 µM; H₃BO₃ 11.5 µM; MnSO₄ 1.25 µM; ZnSO₄ 0.2 µM; CuSO₄ 0.075 µM and (NH₄)₆Mo₇O₂₄ 0.025 µM. The solution was buffered at pH 5.8 with 2 mM Mes-Tris (Luo et al., 2006). The solution in the culture flasks was replaced twice daily with distilled and sterilized water in the morning and fresh nutrient solution in the evening, during two weeks. The nutrient solution collected in the evening from each flask was used as the source of REs. Exudates were lyophilized and stored at 4 °C until use.

2.3. Protein and carbohydrate determinations

The protein concentration was determined according to the Bradford method (Bradford, 1976). To 100 µL of sample (REs diluted in sterile water, see below) 1000 µL Coomassie Blue G-250 reagent was added. Sample was held for 10 min at room temperature and the protein concentration was then estimated at 590 nm using BSA as standard.

Carbohydrates were determined by the dinitrosalicylic acid (DNS) method described by Miller (1959) and modified as follows: 500 µL of sample and 750 µL of 1% DNS (dissolved in 6% NaOH) were mixed and incubated for 10 min in a boiling water bath and

subsequently absorbance was recorded at 590 nm. Glucose was used as standard.

2.4. Enzyme activity

Specific dechlorinase activity (SDA) of the REs was indirectly determined using a colorimetric assay, a modification of Phillips et al. (2001), in which phenol red sodium salt was added to the supernatant at a ratio of 1/10 as pH indicator. The change in color from red through orange to yellow in the presence of chlorides in the supernatant was indicative of lindane dechlorination and, therefore, a positive result. Chloride concentrations were determined colorimetrically at 540 nm with reference to standard HCl solutions using a Beckman spectrophotometer. One enzymatic unit was defined as the amount of chloride ions released (micromoles) in 1 h (EU = µmol Cl⁻ h⁻¹) and the SDA was defined as EU per milligram of protein.

2.5. Experimental conditions

Streptomyces sp. M7, A5 and A11 were plated on starch-casein medium (SC agar) at 30 °C for 7 days. SC agar contain (in grams per liter): starch, 10; casein, 1; K₂HPO₄, 0.5; agar 12. The pH was adjusted to 7 prior to sterilization. Spore suspensions were inoculated in flasks containing 30 mL of minimal medium (MM), whose composition (in grams per liter) is: (NH₄)₂SO₄ 4; K₂HPO₄ 0.5; MgSO₄ 7H₂O 0.2; FeSO₄ 7H₂O, 0.01 (Hopwood et al., 1985). The pH was adjusted to 7. Flasks were then amended with lindane, REs or/ glucose as sole carbon source. Lindane (99% pure, Sigma–Aldrich) was dissolved in methanol (pesticide grade, Merck), filter-sterilized (0.22 µm pore size) and then added to the MM at a final concentration of 1.66 mg L⁻¹ (Benimeli et al., 2008). Lyophilized REs were diluted in water, filter-sterilized and then added to the MM at the concentration of 1 g L⁻¹. Filter-sterilized glucose was added at the same concentration. Similar experiments were carried out without lindane, REs and/or glucose as controls. All cultures were incubated on a rotator shaker (100 rpm) at 30 °C, for 7 days. Centrifuged culture supernatants (9000×g, 10 min, 4 °C) were used to determine residual lindane using GC/µECD (see below). Biomass was estimated after centrifugation by washing the pellets with 25 mM Tris–EDTA buffer (pH 8) and drying to constant weight at 105 °C.

2.6. Gas chromatography

The residual lindane in cell-free supernatants was extracted by solid-phase extraction using a C18 column (Varian, Lake Forest, USA). Lindane concentration in extracts were quantified in a Gas Chromatograph Agilent 7890A equipped with a HP5 capillary column (30 m × 0.53 mm × 0.35 m) and ⁶³Ni µECD detector, a split/splitless Agilent 7693B injector and Agilent ChemStation software. Quantitative sample analysis was performed using appropriate calibration standards (AccuStandard).

2.7. Statistical analyses

Each experiment was carried out in triplicate and the results were arithmetic means. One-way ANOVA was used for variance analysis (Tukey post-test, *P* < 0.05). All statistical analyses were performed using a professional version of Infostat software.

3. Results

3.1. Maize REs composition and dechlorinase activity

Concentrate REs of five hundred plants of maize were analyzed. pH of REs soluble in water was 7.28. Carbohydrate and total protein

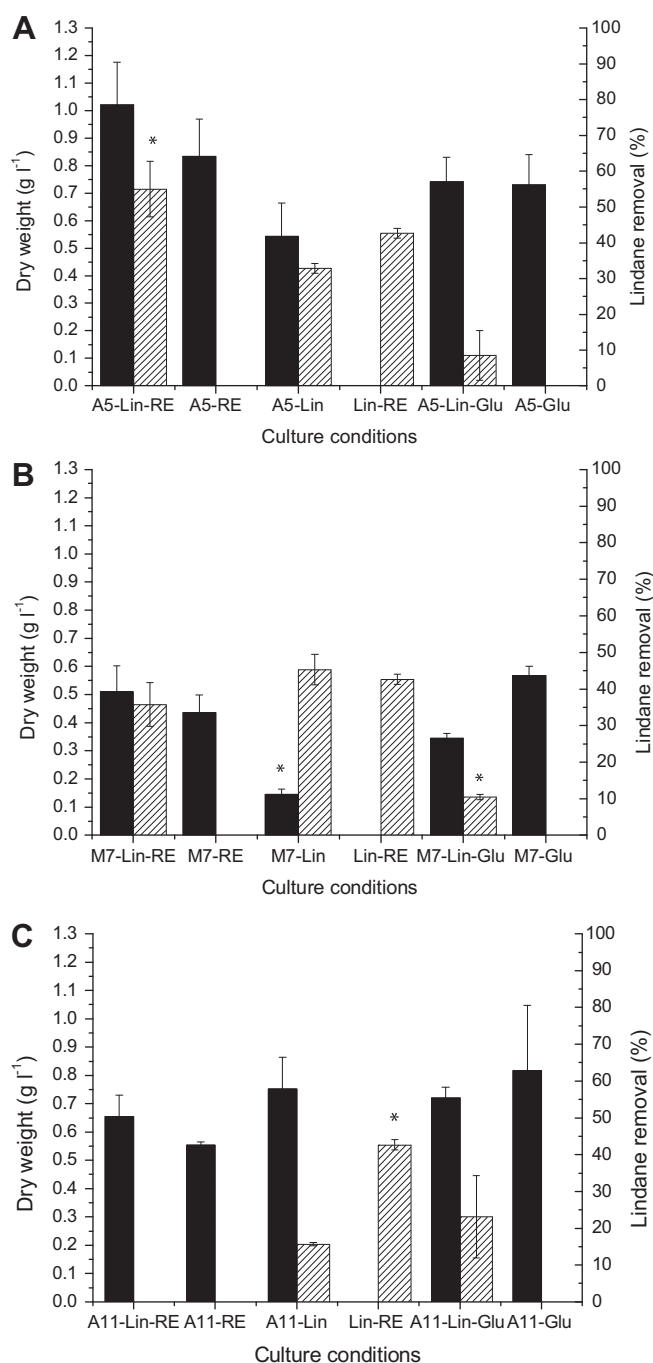


Fig. 1. Comparison of growth on different carbon sources (■) and lindane removal (▨) by *Streptomyces* sp. strains cultivated with maize root exudate (RE), glucose (Glu) or lindane (Lin). (A) *Streptomyces* sp. A5. (B) *Streptomyces* sp. M7. (C) *Streptomyces* sp. A11. Bars showing asterisk indicate that they were significantly different ($P < 0.05$, Tukey post-test).

content of exudates were 0.88 ± 0.02 (g L⁻¹) and 193.5 ± 16.0 (μg mL⁻¹), respectively. SDA was 10.8 ± 0.4 μmol Cl⁻ h⁻¹.

3.2. Growth of *Streptomyces* sp. on REs and lindane removal

All assayed strains were able to growth on MM supplemented with REs as sole carbon source (Fig. 1A–C). Maximum biomass was reached by *Streptomyces* sp. A5 when it was cultured in the

presence of REs-lindane (Fig. 1A). There was no evidence of microbial growth in MM without added carbon sources (control cultures, data not shown).

Lindane removal, calculated as percentage of lindane initial minus percentage of residual lindane, varied among 8.5–55% (Fig. 1A–C). The higher pesticide removal (55%) was reached by *Streptomyces* sp. A5, growing on REs-lindane (Fig. 1A), whereas lindane removal by *Streptomyces* sp. M7 was approximately a half at the same culture condition (Fig. 1B). No decrease in pesticide concentration was observed when *Streptomyces* sp. A11 was grown on REs-lindane, although biomass registered was elevated (0.7 ± 0.08 g L⁻¹) (Fig. 1C).

When MM amended with REs-lindane was incubated without microorganisms, pesticide removal reached 42% (Fig. 1A–C). Additionally, a micellar behavior was detected in this condition (data not shown).

Because no linear relationship was found between the residual lindane and microbial growth, the relationship between both parameters was calculated (Benimeli et al., 2007) (Table 1). *Streptomyces* sp. A5 showed a minimal ratio, since this strain showed the lowest concentrations of residual lindane (0.45 ± 0.08 mg L⁻¹) and maximum biomass (1.02 ± 0.3 g L⁻¹) in REs-lindane assay (Fig. 1A).

4. Discussion

During plant growth, roots release a range of organic compounds which potentially enhance biodegradation of xenobiotics in different ways. Stimulation of bacterial growth, provided they have the corresponding metabolic abilities, is one of this ways. In the present work, three native *Streptomyces* sp. strains previously isolated from highly contaminated sediments and soils (Benimeli et al., 2003; Fuentes et al., 2010) were able to grow on MM supplemented with maize REs as sole carbon source (Fig. 1A–C). This result indicates that these indigenous *Streptomyces* sp. strains are competitive at the maize rhizosphere level and that REs represent a convenient carbon and energy source and possible nitrogen, since there was no evidence of microbial growth in MM without added neither REs nor other carbon source (data not shown). Regarding this issue, Personeni et al. (2007) found that glucose is a very common sugar in root exudates of maize grown in hydroponics. In fact, we determine proteins and total carbohydrates in concentrate maize REs. It is known that hydroponics statics cultures underestimate amounts of secretions by plants because these are re-taken up by roots (Personeni et al., 2007). However, in our experiments, the nutritive growing solution was renewed daily, minimizing re-uptake. Hence, amounts of sugars and proteins detected could be considerate to reflect the amounts presents in the rhizosphere of maize.

As we expected, all *Streptomyces* sp. strains were able to remove lindane from the culture medium and/or degrade it, because the residual pesticide values detected were less than the initial concentration. Furthermore, the obtained residual lindane

Table 1
Relation between residual lindane concentration and microbial growth on different culture conditions.

Culture conditions	RL/B ^a		
	A5 ^b	M7 ^b	A11 ^b
Lindane + REs ^c	0.47	1.34	1.97
Lindane	1.31	4.02	1.19
Lindane + glucose	1.33	2.53	1.13

^a RL: residual lindane (mg L⁻¹); B: biomass (dry weight, g L⁻¹).

^b *Streptomyces* sp. strains.

^c REs: root exudates.

concentration was more than half of the amount in MM supplemented with glucose-lindane, compared to MM supplemented with REs-lindane, suggesting that REs could be more appropriate carbon source as electron donors to support aerobic dehalogenation of the pesticide (Cuozzo et al., 2009). In this connection, Benimeli et al. (2007) found that removal of different OPs by *Streptomyces* sp. M7 was more efficient when other carbon sources were present in the medium. In addition, REs may enhance pesticide biodegradation by increasing its bioavailability, as reported by Calvelo Pereira et al. (2006). These authors showed an increase in the aqueous solubility of different HCH (hexachlorocyclohexane) isomers (included lindane) and a reduction in their concentration in the rhizosphere of *Avena sativa* and *Cytisus striatus* in relation to the bulk soil. On the other hand, Gao et al. (2010) observed that artificial REs promote the release of polycyclic aromatic hydrocarbons (PAHs) from soil, making this more available for microbial degradation.

Streptomyces sp. A5 showed a minimal ratio between the residual lindane concentration and microbial growth (Table 1). Hence this strain was found to be the most efficient, considering their growth capacity and pesticide removal ability in the presence of REs-lindane. The strong biological effect of *Streptomyces* sp. A5 is very relevant taking into account the feasibility of its application: *Streptomyces* strains are already adapted to the habitat, and maize plants are well adapted to acidic conditions as generated during lindane degradation (Benimeli et al., 2008). Additionally, maize may create particularly good environmental conditions for soil microorganisms (Lin et al., 2008) and the high hydrophobicity (log K_{OW} 3.7–4.1, Willett et al., 1998) of HCH isomers make their uptake and translocation within the plant unlikely.

In contrast, no decrease in pesticide concentration was observed by *Streptomyces* sp. A11 after incubation with REs-lindane, although it grew considerably (Fig. 1C). This is not surprising considering that REs are a complex mixture of substrates and some of them could be repressing lindane-degrading activity of the microorganism. Similar results were obtained by Rentz et al. (2004) and Louvel et al. (2011), who studied the repression of phenanthrene-degrading activity of *Pseudomonas putida* in the presence of REs of different plants species.

Plants secrete enzymes that may also contribute to degradation of xenobiotics (phytodegradation) (Gao et al., 2010; Van Aken et al., 2010). For instance, Magee et al. (2008) reported recently dechlorination of polychlorinated biphenyls (PCBs) by crude extract of nitrate reductase from *Medicago sativa* and a pure commercial nitrate reductase from maize. In this study, we detect SDA in maize REs, which could explain the elevated lindane removal (42%) in REs-lindane assay, without microorganisms (Fig. 1A–C). Similar results were obtained by Barriada-Pereira et al. (2005), who analyzed data obtained from the bulk and rhizosphere soils from *A. sativa* and *C. striatus* and concluded that both plant species tend to reduce the levels of all HCH isomers in the rhizosphere due to secreted enzymes able to dechlorinate. Additionally to enzyme activity, we observed a micellar behavior in REs-lindane assay (without inoculated microorganisms) which could also contribute to pesticide reduction. As described by Nardi et al. (2002), organic acids occurring in maize REs are responsible for micellar behavior of carbonaceous humic substances in solution. On the other hand, due to its physicochemical characteristics, lindane tends to sorbs to organic material (Kidd et al., 2008). In this context, we hypothesize that lindane binds to organic acids and other carbonaceous components of REs to form a complex matrix in which pesticide being held and consequently it reduce in culture supernatant.

In conclusion, the results presented here show that maize REs markedly influenced the lindane removal by *Streptomyces* sp. strains, which corroborated the hypothesis that co-metabolism

may be a plant/microbe interaction important for bioremediation. In addition, the presence of lindane-degrading activity in REs may directly induce contaminant degradation. To our knowledge, this is the first report of phytostimulation of lindane-degrading actinobacteria by maize REs. Further studies evaluating the soil–plant–microbe system and its influence on lindane biodegradation are necessary so as to better explore and exploit an undoubtedly huge potential.

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