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Treatment of endosulfan contaminated water with *in vitro* plant cell cultures

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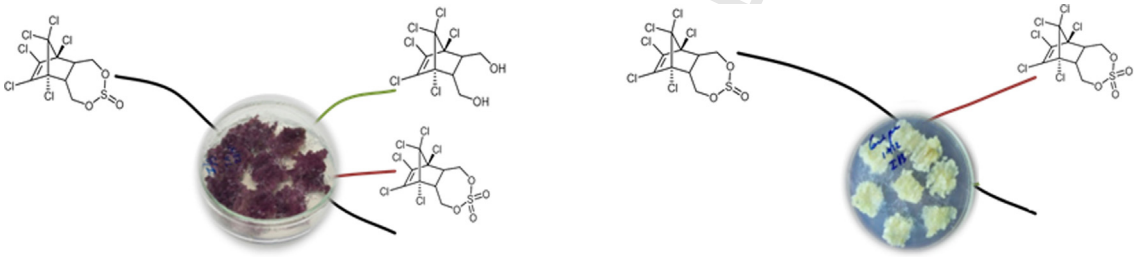
GRAPHICAL ABSTRACT



HIGHLIGHTS

- α- and β-endosulfan removal by plant cell cultures were studied for the first time.
- We designed a protocol to test axenic plant cell abilities to metabolize endosulfan.
- Time courses of technical grade endosulfan phytotransformations were evaluated.
- The tested plant species showed different behavior toward endosulfan metabolization.

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ABSTRACT

Endosulfan is a Persistent Organic Pollutant insecticide still used in many countries. It is commercially available as mixtures of two diastereomers, α- and β-endosulfan, known as technical grade endosulfan (TGE). A laboratory model based on the use of axenic plant cell cultures to study the removal and metabolization of both isomers from contaminated water matrixes was established. No differences were recorded in the removal of the two individual isomers with the two tested endemic plants, *Grindelia pulchella* and *Tessaria absinthioides*. Undifferentiated cultures of both plant species were very efficient to lower endosulfan concentration in spiked solutions. Metabolic fate of TGE was evaluated by analyzing the time course of endosulfan metabolites accumulation in both plant biomass and bioremediation media. While in *G. pulchella* we only detected endosulfan sulfate, in *T. absinthioides* the non-toxic endosulfan alcohol was the main metabolite at 48 h, giving the possibility of designing phytoremediation approaches.

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1. Introduction

Endosulfan is a synthetic organochlorine compound (OC), widely used as endocrine disrupting insecticide in agriculture, included in the list of Persistent Organic Pollutants (POP) by the Stockholm Convention in its fifth meeting (UNEP/POPS/COP.5-36, 2011) [1].

Although endosulfan is forbidden in over 60 countries, in large regions of the world, particularly in the southern hemisphere, it is still used. Technical grade endosulfan (TGE) is commercially available as a mixture of two diastereomers, known as α-endosulfan (or I) and β-endosulfan (or II) in ratios from 2:1 to 7:3. In most of the brands, the formulation is a mixture of the active diastereomers (95%) and inert ingredients (5%) [2]. Although there are not systematic data, the occurrence of endosulfan has been reported for punctual areas in Argentina before its prohibition in 2013. For example, in 2012, Gonzalez et al. reported levels of endosulfan between 0.10 to 1.15 ng L^{-1} in ground waters of La Pampa [3]. Concentrations of 0.2–13.5 μg L^{-1} were detected in Esteros del Ibera surface waters [4], while other authors reported quantities between 0.2 to 35.2 ng of endosulfan per gram of soil in the Patagonia [5].

The relatively low hydrophobicity of these OCs gives them a high potential to reach water sources, helped by irrigation practices and rainfall regime [6]. Additionally, they are particularly toxic to fish and aquatic invertebrates [7].

The ability of bacteria and fungi to degrade endosulfan from water and soil *in vitro* has been studied by several authors [7–14]. However, there are very few approaches using plants for such purpose. A study about the behavior of two Mexican species of basil (*Ocimum basilicum* and *Ocimum minimum*) in soils polluted with endosulfan, showed that both species can grow under endosulfan concentrations up to 1 g kg^{-1} soil and that the pesticide concentration decreased where *O. basilicum* was cultivated after 30 days [15]. Other authors reported that mustard (*Brassica campestris*) and corn (*Zea mays*) were capable of removing a mixture of α- and β-endosulfan from spiked soils at high levels of contamination, suggesting that the up-take and phytoextraction might be the dominant processes [16].

It is interesting to note that many of the plant xenobiotic metabolizing enzymes are constitutive, which make plants interesting remediation tools [17].

Different strategies can be assessed in order to use plants for remediation of contaminated waters and soils, such as plants cultured in fields, grown in hydroponic media and *in vitro* cultures. Although many features are shared by these systems, each of them offers unique characteristics that should be taken into account when designing experimental studies. In particular, the aseptic conditions of plant tissue cultures allow testing intrinsic genetic and metabolic capacity of plant species. Also, the use of these cultures offers the possibility of indefinite propagation, incubation in the dark, under non-photosynthetic conditions and they mostly release the metabolites into the medium, thus facilitating the chemical analysis [18,19]. Cell suspension cultures of *Blumea malcolmii* were successfully used for the phytodegradation of the triphenylmethane dye Malachite Green at high concentration with the formation of metabolites with reduced toxicity [20]. Also, the potential of tissue cultures and wild plants of *Portulaca grandiflora* to degrade a sulfonated diazo dye, at the same extent, was demonstrated [21].

In the present work we present an experimental model based on axenic cell cultures of *Tessaria absinthioides* and *Grindelia pulchella* to study the removal and phytometabolization of endosulfan. Both are endemic species widely distributed in several phytogeographical regions of Argentina and are involved in a research program devoted to the sustainable exploitation of native plants.

2. Experimental

2.1. Pesticide and metabolite standards

Certified purity standards were used: α-endosulfan (Sigma–Aldrich, 99.6%), β-endosulfan (Sigma–Aldrich, 99.8%), endosulfan sulfate (AccuStandard, 97.0%), endosulfan ether (Sigma–Aldrich, 99.9%), endosulfan alcohol (Sigma–Aldrich, 99.9%) and endosulfan lactone (Sigma–Aldrich, 99.9%). Stock solutions were prepared in toluene, isooctane or methanol pesticide grade and stored at −20 °C.

2.2. Instrumentation and operating conditions

2.2.1. General chromatographic procedures

Detection and quantification of endosulfan and its metabolites were performed by gas chromatography with electron capture detector (GC-ECD-Ni^{63}) following *Method A*. Two different alternative *Methods*, *B* and *C*, were used to confirm the identity of endosulfan metabolites. Peaks were identified by comparing their retention times with standards. Oven temperature program: (star temperature [°C]/holding time [min]/heating rate [°C/min]/plateau temperature [°C]/holding time [min]/heating rate [°C/min]/final temperature [°C]/holding time [min]). *Method A*: Chromatograph: PerkinElmer Autosystem XL; column: Restek® 1; oven temperature program: (50/3/20/210/0/5/260/10); injector temperature: 240 °C; carrier gas/make up: halogen-free ultrapure nitrogen; carrier gas flow: press: 49 psi—Aux1: 29; split flow: 40 mL/min; injection volume: 1 μL; detector temperature: 400 °C. *Method B*: Chromatograph: Varian CP3800; column: Restek® 5; oven temperature program: (50/3/20/210/0/5/260/9); injector temperature: 240 °C; halogen-free ultrapure nitrogen was used as carrier gas at a flow-rate of 2 mL/min; injection volume: 1 μL; detector temperature: 300 °C. *Method C*: Chromatograph: Varian CP3800; column: SPB608; oven temperature program: (150/4/8/250/30); injector temperature: 240 °C; halogen-free ultrapure nitrogen was used as carrier gas at a flow-rate of 2 mL/min; split flow: 5 mL/min; injection volume: 1 μL; detector temperature: 300 °C.

2.2.2. Analytical performance

Quantification of endosulfan isomers and their metabolites was performed with a standard curve prepared by injection of known concentrations of standards in isooctane.

The detection and quantification limits were determined as the lowest concentration of each compound that gave a response with a signal/noise in ratio of 3 and 10, respectively, both of them calculated from spiked samples.

The detection limits in culture media were 0.005 mg L^{-1}, except for endosulfan alcohol whose detection limit was 0.050 mg L^{-1}. Meanwhile, the detection limits in plant biomass were 0.010 μg g^{-1} except for endosulfan alcohol whose detection limit was 0.100 μg g^{-1}. The accuracy of the recovery method was evaluated by using samples spiked at different fortification levels (0.15, 1 and 10 mg L^{-1} in culture media; 1, 10 μg g^{-1} in plant biomass) obtaining recoveries ranging from 75% and 110%. Linearity was assessed by making serial dilutions of the standards in isooctane at seven concentrations (0.02–0.0005 μg mL^{-1}). The response of the detector was linear in the studied range with correlation coefficient of 0.98–0.99. Reproducibility was verified by analyzing different spiked samples in parallel. Relative standard deviation was ranged between 9 and 16% for each analite.

2.3. Plant cell cultures

T. absinthioides and *G. pulchella* callus cultures were initiated from leaves and maintained on Murashige Skoog (MS) agar [22]

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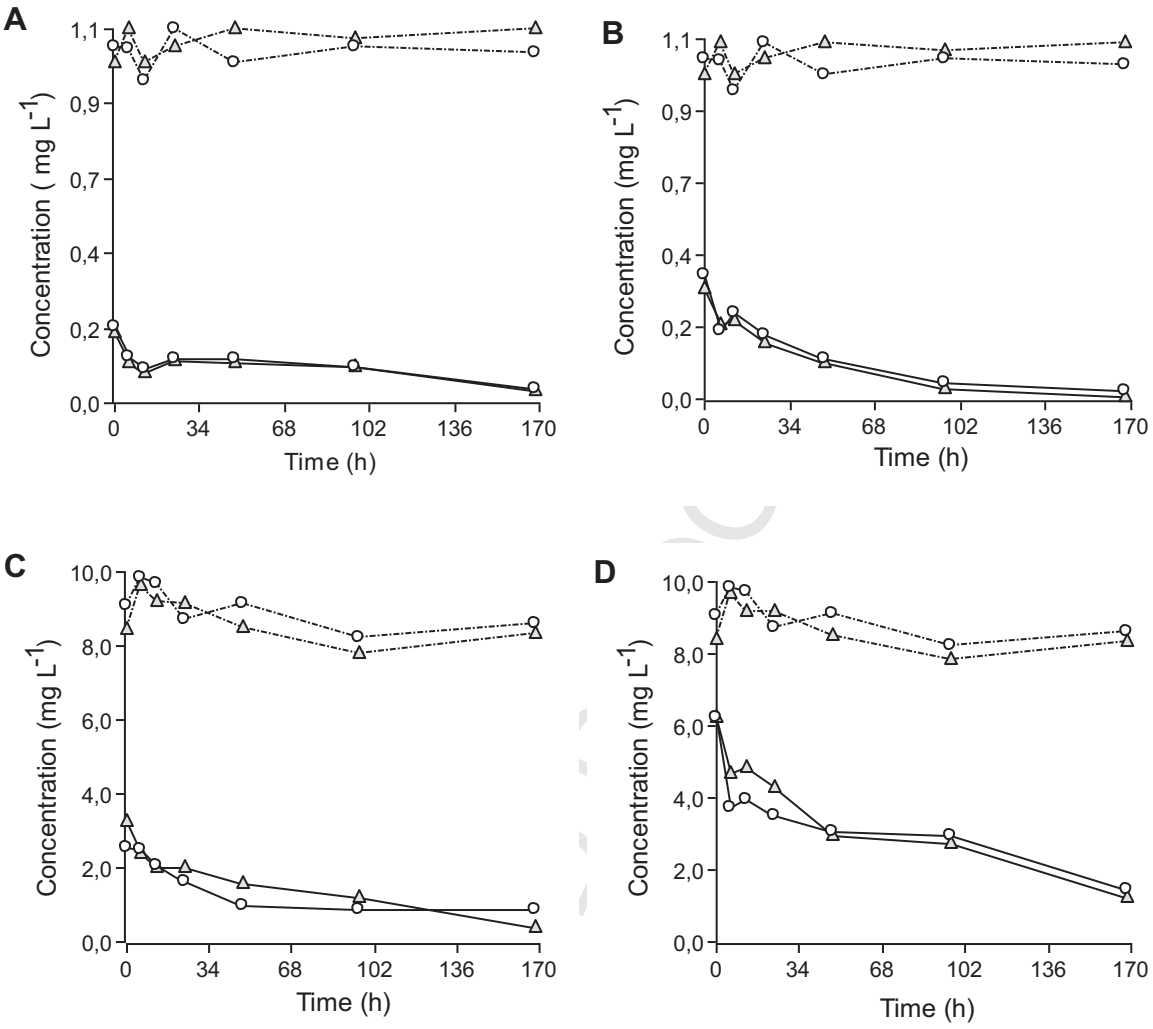

Fig. 1. α- and β-endosulfan removal by resting cell systems. (A) *G. pulchella* cells, contamination level 1.0 mg L^{-1}. (B) T. *absinthioides* cells, contamination level 1.0 mg L^{-1}. (C) *G. pulchella* cells, contamination level 10.0 mg L^{-1}. (D) *T. absinthioides* cells, contamination level 10.0 mg L^{-1}. Triangle: α-endosulfan, circle: β-endosulfan. Solid lines: experiments, dashed lines: controls.

supplemented with sucrose (30 g L^{-1}) and 20.0 μM of indol-3-butyric acid (IBA) and 18.0 μM of 6-bencylaminopurine (BAP), under a 16 h light/8 h dark cycle by fluorescent lamps at an irradiance of approximately 1.8 Wm^2 and 22 °C, as previously described [23].

Genomic DNA of *T. absinthioides* and *G. pulchella* undifferentiated cells were isolated as described by Dellaporta [24]. The presence of endophytic and/or epiphytic bacterial community in these cultures was analyzed by PCR amplification of 16S rDNA using a pair of bacterial primers 799F 5′-AACMGGATTAGATACCCKG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ [25]. These primers are able to differentiate between bacterial product approximately as 735 bp and a plant mitochondrial product as 841 bp. A bacterial suspension of *Roseomonas mucosa* CM15 was added on undifferentiated cells to detect both products as positive control. H_2O miliQ was used as negative control. The reaction mixture contained 100 ng of DNA extract, 0.4 μM of each primer, 200 μM of each dNTPís (Promega), *Taq* reaction buffer 1X and 1.25 U of *Taq* DNA polymerase (Sigma–Aldrich). The amplification program used was: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 52 °C for 45 s, 72 °C for 1 min, 72 °C for 8 min, 4 °C for an infinite period. The amplified bands were observed under UV illumination after electrophoresis on 1.5% (w/v) agarose gels and staining with ethidium bromide.

2.4. In vitro α- and β-endosulfan removal assays

Solutions of certified purity standards of each isomer: α-endosulfan (*ChemService*, 99.5%) and β-endosulfan (*ChemService*, 99.3%) were prepared in acetone. These were used to spike the flasks containing sterile phosphate buffer (0.1 M, pH 5.0) at two levels (1 mg L^{-1} and 10 mg L^{-1}). These amounts are close to the limits of the ranges generally used in pesticide microbial degradation and aquatic toxicology studies [26].

20-day-old *T. absinthioides* and *G. pulchella* calli were mechanically disrupted by gently pressing them with a spatula under sterile conditions and 2.5 g of fresh weight (FW) per flask were placed in the media. Incubation was carried out in an orbital shaker at 120 rpm, at 22 °C, under a 16 h light/8 h dark cycle. Samples (2 mL) were withdrawn at 0, 6, 12, 24, 48, 96 and 168 h under aseptic conditions, centrifuged in Eppendorf tubes for 5 min at 13,400 g and supernatants (1 mL) were extracted with ethyl acetate (0.5 mL × 2). Organic layers were pooled and preserved at −20 °C until GC-ECD analysis. Simultaneously, substrate blanks (spiked medium without cells) and culture blanks (cells suspended in medium with acetone (0.1%) and without endosulfan) were incubated. All the assays were performed in quadruplicate.

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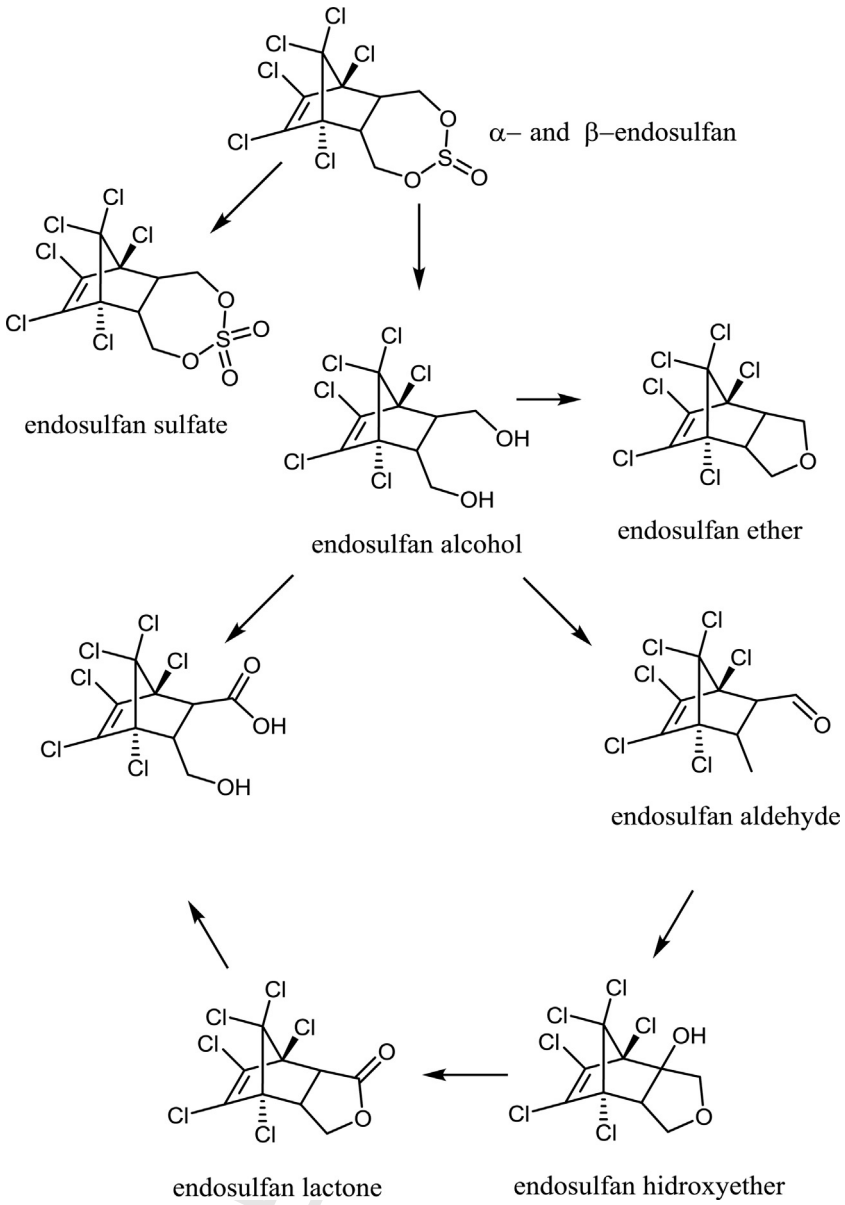

Fig. 2. Plausible endosulfan phytotransformation pathways.

2.5. Characterization of technical grade endosulfan (TGE) mixtures

Samples were prepared by weighing 100 mg of endosulfan technical grade and dissolved in isooctane until a final volume of 100 mL. Dilutions 1/10 in the same solvent were done and quantification was performed by GC-FID in a Varian CP3800 equipped with an Agilent J&W DB5 column, under the following experimental conditions: injector temperature 280 °C; oven program temperature: (80/4/15/150/5/270/6); carrier: halogen-free nitrogen, flow 2 mL/min; detector temperature 280 °C, split ratio: 5.

2.6. In vitro *TGE phytometabolization assays*

20-day-old *T. absinthioides* or *G. pulchella* disrupted calli (5 g of FW) were transferred to Erlenmeyer flasks containing 20 mL of 0.1 M phosphate buffer, pH 5.0 spiked with 10 mg L^{-1} of TGE.

Incubation conditions were the same as described in Section 2.4. Substrate and culture blank assays were performed in parallel. Samples were taken at 0, 6, 12, 24, 48, 96 and 168 h by a sacrificial vessel approach meaning that the whole volume of three replicates reactors was withdrawn at each incubation time. 20 mL of each single sample were transferred to Falcon tubes and centrifuged at 4100 g for 5 min to separate cell biomass from liquid media. Supernatant aliquots (2 mL) were centrifuged again in Eppendorf tubes for 5 min at 13,400 g and processed as described in Section 2.4. The cells were washed twice with 5 mL of phosphate buffer and samples of 1 g of FW were extracted with ethyl acetate (0.5 mL × 2), organic layers were pooled and preserved at −20 °C until GC-ECD analysis.

2.7. Statistical analysis

Statistical analysis was performed by using InfoStat, version 2013 [27]. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey test of comparison of means. *P* values less than 0.05 were considered significant.

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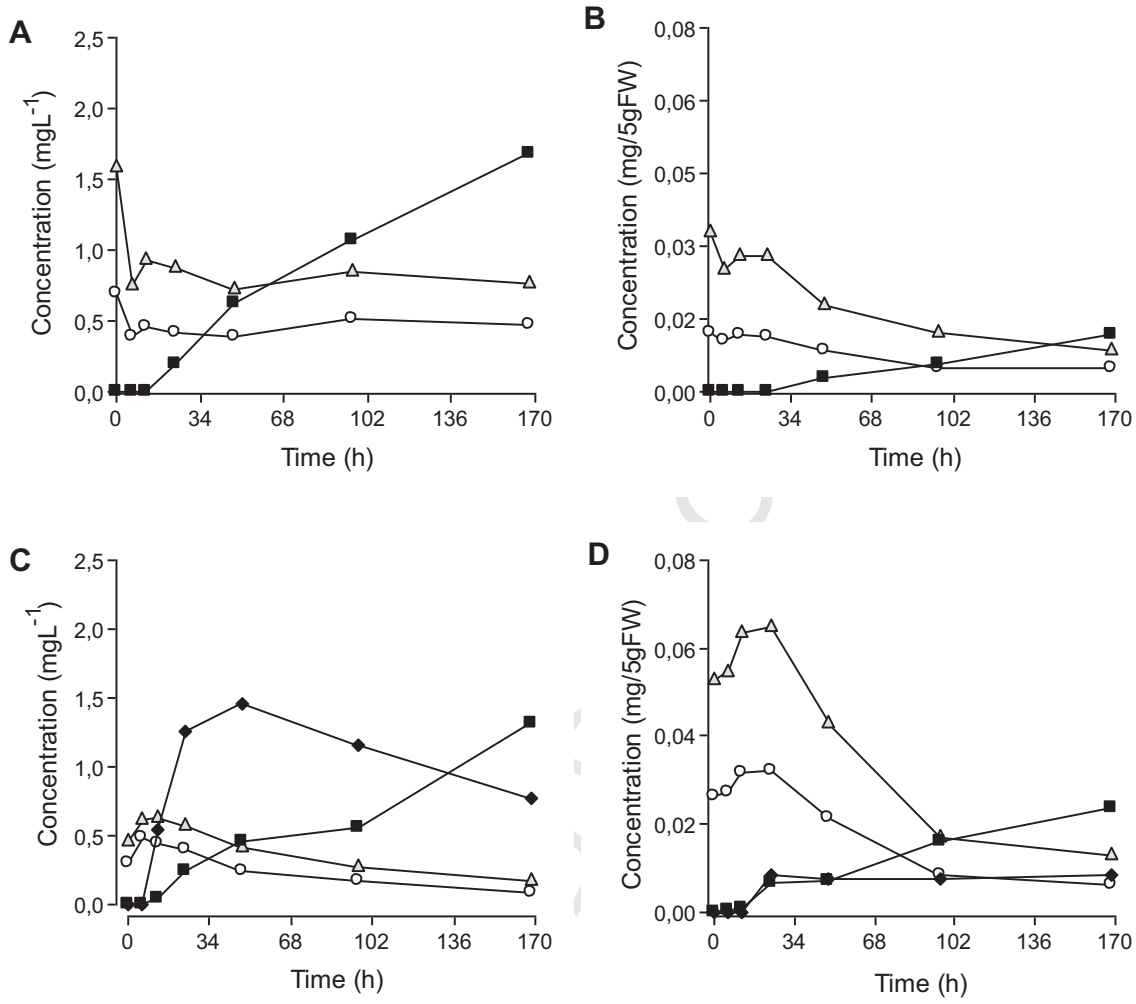

Fig. 3. Time course of TGE phytotransformation by resting cells. (A) *G. pulchella* media. (B) G. pulchella biomass. (C) *T. absinthioides* media. D) *T. absinthioides* biomass. Triangle: α-endosulfan, circle: β-endosulfan, diamond: endosulfan alcohol, square: endosulfan sulfate.

3. Results and discussion

3.1. Removal of α- and β-endosulfan by plant cell cultures

The experimental models were designed based on two endemic plant species, *T. absinthioides* and *G. pulchella*. Attempts to isolate endophytic and/or epiphytic microorganisms from both undifferentiated cultures were ineffective. Also the amplifications of bacterial gene 16S rDNA from plant tissues with a pair of bacterial specific primers 799F-1494R [25] were negative. Therefore, we assumed that the cultures were axenic.

Unlike other authors who used plant cell cultures as "growing cells" to phytodegrade the dye Malachite Green [20] we chose a resting cell procedure not only to facilitate work up and analytical assays, but also to mimic the conditions of a possible polluted aquifer in the bioremediation media. In addition, this model allowed us to study the ability of the cell lines to degrade endosulfan as sole hydrocarbon compound in the media. Furthermore, resting cells offer some other advantages such as recycled cell application, the lowered risk of undesirable microbial contamination and reduced bioconversion time [28,29].

Cells were exposed to endosulfan in the stationary phase (20 days old). Consequently, *T. absinthioides* and *G. pulchella* friable calli were transferred from solid MS media to batches containing 0.1 M phosphate buffer pH 5.0 that were previously contaminated with two different concentrations of α- and β-endosulfan (1 and 10 mg L^{-1}).

The removal of both isomers of endosulfan from the media in the course of 168 h was registered for the two contamination levels (Fig. 1). The biomass was 25 g (FW) in all experiments. The ANOVA analysis showed that, unlike the blank assays where no significant differences were observed for α and β endosulfan along the incubation time, removal was significant ($P < 0.05$) when the two plant cell cultures were used toward both contamination levels. The remaining quantities of endosulfan in the media at time zero indicated that an important fraction of the xenobiotic was bound to the biomass. Less than 30% of both isomers remained in the media when using *G. pulchella* cells at the two concentration levels, as well as with *T. absinthioides* cultures when contamination level was 1 mg L^{-1}. On the contrary, when solutions were spiked with 10 mg L^{-1} more than 60% of endosulfan was present in the media suggesting a differential performance of each cell species toward different quantities of the xenobiotic.

With both plant species, no differences were recorded in the removal of each individual isomer during the entire incubation period. In contrast, other authors have observed a preference toward one of the stereoisomers when using mixed bacterial cultures as well as bacterial isolates from endosulfan contaminated soil samples [30] or by using the fungi *Mortierella* sp. [31].

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A slight transient increase of endosulfan concentration in the media occurred at the beginning of the experiments (24–48 h) for both species, although it is more evident for *T. absinthioides* than for *G. pulchella*. This effect could be explained as desorption of the xenobiotic from the biomass as already reported by another author using microorganisms to degrade endosulfan [32]. From 24 h on, a steady decay in endosulfan concentration was observed.

Some authors have reported a notable pH decrease in more than two units during incubation of endosulfan with growing cells of *Aspergillus niger* due to metabolic activities, suggesting a chemical degradation of this compound [33]. However, despite a slight increase in pH at the very beginning (from 5.0 to 5.5), no significant changes in pH were observed in our systems from 6 h onward. This fact rules out the discussion of the existence of a degradative process driven by the increase in the acidity of the medium. Moreover, known endosulfan metabolites were not detected in the culture nor in the substrate blanks and the presence of signals that interfere with the identification and/or quantification of endosulfan or its metabolites were not recognized.

3.2. Phytotransformation of technical grade endosulfan

Since TGE is a mixture of two diastereomers (α- and β-endosulfan) in variable proportions, we characterized the commercial formulation prior to use it in phytotransformation assays, giving 68% α- and 32% β-isomer.

There are numerous endosulfan degradation studies performed with microorganisms [34,35] and, to a lesser extent, with plants and algae [15,16]. Based on them, we thought that the plausible metabolic route of our *in vitro* plant cell models should be contained in the general pathway depicted in Fig. 2.

Therefore, we optimized the chromatographic conditions for the quantification of the possible endosulfan metabolites. In this regard, we studied the linearity in the range $1 \times 10^{-4} - 1 \times 10^{-2}$ μg mL^{-1} at six different concentrations for each endosulfan isomer and the possible metabolites of which the analytical standards were available to us: endosulfan sulfate, alcohol, ether and lactone. Then phytotransformation of the spiked TGE media was carried out with both cell lines and the quantity of α- and β-endosulfan and their metabolites were analyzed in both cell biomass and bioremediation media over time. These experiments allowed finding out not only the metabolic fate of endosulfan but also the accumulation site of both untransformed isomers and the final metabolites (Fig. 3).

For *G. pulchella*, the only detected metabolite was endosulfan sulfate, which was accumulated in the biomass as well as in the bioremediation media following the same tendency in both curves. Meanwhile, for *T. absinthioides*, the metabolites accumulated in media and cells were endosulfan sulfate and endosulfan alcohol, showing that oxidative and hydrolytic reactions occurred. It is particularly interesting the accumulation profiles observed in the bioremediation media. From 6 h on, both metabolites showed a concentration increase, being more noticeable for the alcohol. Nevertheless, after 48 h, the quantity of the alcohol started to decrease as the sulfate kept its increasing rate. Chopra and Mahfouz observed in tobacco leaf that endosulfan isomers as well as endosulfan sulfate can directly hydrolyze into endosulfan alcohol [36]. Apparently, in our experiments both metabolic routes were independent one each other. Both referred pathways, the direct hydrolysis and the oxidation of the substrates, have been detected by other authors with fungi as degradation agents, but they have also reported endosulfan ether and endosulfan lactone as main metabolites [34]. In *T. absinthioides*, only endosulfan alcohol was detected as product of the possible action of a hydrolytic sulfatase.

Although the formation of endosulfan sulfate has been reported to occur through biological transformation, some authors have proposed that the hydrolysis yielding endosulfan alcohol occurs readily at alkaline pH values [37]. This was clearly not happening in our experiments since the pH value remained constant throughout the entire biodegradation process. The biotransformation into endosulfan alcohol was relevant since it is a non-toxic metabolite to fish and other organisms and could be further degraded to other non-toxic compounds such as endosulfan ether, hydroxyether and lactone [38]. For bioremediation purposes and based on the time-course experiments, it would be convenient to stop the process at around 48 h. At this time, the alcohol concentration in the media was the highest and, even if some sulfate was already detected, its concentration was low.

In a report dealing with the degradation of α-endosulfan to endosulfan sulfate and endosulfan ether by green algae *Chlorococcum* sp. and *Scenedesmus* sp. the authors stated that the metabolites appeared in non-stoichiometric amounts [39], which was in agreement with our own observations.

In summary, phytotransformation assays showed interesting differences between the two plant species. Hydrolytic and oxidative processes were observed with *T. absinthioides*. The corresponding metabolites, endosulfan alcohol and endosulfan sulfate, were detected in both the biotransformation media and in the biomass. On the other hand, endosulfan sulfate was the only metabolite detected with *G. pulchella,* and its accumulation was mainly extracellular. Besides, these *in vitro* axenic cell cultures offered a range of experimental advantages in studies aimed at examining the intrinsic metabolic capabilities of plant cells without the interference of epiphyte and endophyte microorganisms.

4. Conclusions

At the best of our knowledge this is the first study of the removal and phytotransformation of endosulfan by plant cell cultures. The recovery and chromatographic conditions to quantify endosulfan isomers and several possible metabolites were optimized, along with the designing of a reproducible protocol to test axenic plant cell abilities to metabolize TGE spiked solutions. This method was appropriate to give insights into the intrinsic metabolic capabilities of different plant species, thus useful to provide data for planning phytoremediation processes.

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