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Phytoremediatory effect and growth of two species of *Ocimum* in endosulfan polluted soil

M. Ramírez-Sandoval[a], G.N. Melchor-Partida[a], S. Muñiz-Hernández[b], M.I. Girón-Pérez[a], A.E. Rojas-García[a], I.M. Medina-Díaz[a], M.L. Robledo-Marenco[a], J.B. Velázquez-Fernández[a,*]

[a] *Laboratorio de Contaminación y Toxicología Ambiental. Universidad Autónoma de Nayarit, Tepic, Mexico*
[b] *Subdirección de Investigación Básica, Instituto Nacional de Cancerología, Secretaría de Salud México (SS), Mexico City, Mexico*

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ABSTRACT

Endosulfan is a hazardous organochlorine pesticide banned or restricted in several countries. However, it has been found in the environment and in animal samples. To study a potential way to bioremediate soils contaminated with this pesticide, two plant species of the genus *Ocimum* were studied: *Ocimum basilicum L.* and *Ocimum minimum L.*, since they are economically feasible and well adapted to the climatic conditions of the Nayarit zone (Mexican pacific coast). Young plants were transplanted into soil experimentally polluted with endosulfan. Growth of both species was not affected by endosulfan, the plants grew, flourished, and produced seeds; 30 days later, endosulfan concentration was lower in the soil with *O. basilicum* than in the soil without plants. On day 90, no differences in endosulfan concentrations were found between soil with or without *O. minimum*. At day 1, plants in the polluted soil showed lipoperoxidation, as measured by thiobarbituric acid-reactive species (TBARS). Interestingly, a higher TBARS value was observed at day 3 in transplanted plants as compared to non-transplanted plants. In conclusion, both species can endure endosulfan pollution (as high as 1 g kg^{-1}) in soils. *O. basilicum* seems to be an adequate candidate for bioremediation of soils polluted with endosulfan.

1. Introduction

Endosulfan is an organochlorine insecticide widely used in agriculture, although it has been banned or restricted in several countries. At the present time, it has not been included in the Stockholm Convention's list as banned, but it is under consideration [1]. Endosulfan can be naturally degraded, but when attached to soil particles, it could persist for years [2]. Thus, endosulfan has been found in human sera and in environmental samples from water bodies or agricultural areas in several countries during the last decade [3–8]. Despite these findings, few strategies for its removal from the environment have been studied or developed.

Bioremediation is a group of technologies that use organisms (or organism-derived molecules) such as bacteria, fungi, or plants to avoid the effects of pollutants, by means of their degradation, biotransformation, confinement, or elimination from the environment. Although, bioremediation of organochlorine pesticides has been the subject of intensive research [9–11]; the selection of an organism for bioremediation purposes must be "tailored" for each environment to be treated. Phytoremediation, i.e., the use of plants to bioremediate an environment, is based on the physiological and biochemical mechanisms of the plant itself or root-associated (rhizosphere) microorganisms. For instance, it has been shown that some plants can take up organochlorine pesticides (OCP's) [12] that could be transformed by the rhizosphere, thereby diminishing the OCP concentration in the environment [13,14]. A good candidate for bioremediation should be a plant able to grow in polluted soil, but also able to improve the natural decrease of the pollutant's concentration in the soil. Nevertheless, bioaugmentation should be avoided, i.e., the possible bioremediation candidate should not be a new species for the environment where it will be grown. Basil (*Ocimum*) meets these features because it adapts easily to warm environments, it does not reproduce aggressively, and does not spread out-of-control. In this sense, the aim of this work was to evaluate whether two species of *Ocimum*, *Ocimum minimum L.* and *Ocimum basilicum L.*, which are adapted to grow in the soil of the Nayarit zone, could: (a) grow in soil experimentally polluted with endosulfan, and (b) promote the decrease of endosulfan concentrations in the soil.

Abbreviations: OCP's, organochlorine pesticides; TBARS, thiobarbituric acid reactive species or substances; C, control plant (non-transplanted plant); CP, control plant (plant in non-polluted soil); CS, control soil (non-polluted soil); PP, "polluted" plant (plant in polluted soil).

* Corresponding author at: CEMIC03, Sria Inv y Posgrado, Univ. Auton. Nayarit; Av. De la Cultura s/n Cd. De la Cultura; CP63190 Tepic, Nayarit, Mexico.
Tel.: +52 311 2118800x8965; fax: +52 311 2118800x8951.
E-mail address: jesusbvf@gmail.com (J.B. Velázquez-Fernández).

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2. Experimental

2.1. Material

Seeds of *O. minimum L.* and *O. basilicum L.* were purchased from a local store. Germination and growth was achieved under greenhouse conditions. Technical grade endosulfan (Thiodan®) was used for all experiments. Chromatography OCP's standards were purchased from ChemService, Inc. (West Chester, PA, USA). Hexane and acetone were of pesticide grade.

2.2. Treatment with endosulfan

Young plants of 8 cm approximately were transplanted to 1 kg soil contained in plastic bags. Previously, the soil was spiked with endosulfan at a final concentration of 0.1 g kg^{-1} soil (unless otherwise indicated). Two controls were made: plant grown in non-polluted soil (CS), and polluted soil without plant (CP, see below). Three or four (n, as indicated in figure legends) plants were grown individually for the experiment. Height was measured from soil surface to the tips at the top part of the plant with an accuracy of 0.1 cm. Thirty days after transplanting, soil samples of approximately 100 g were collected and frozen until analysis. After 90 days, plants and soils were frozen until their analyses.

2.3. Determination of lipoperoxidation

Lipoperoxidation was determined using the thiobarbituric acid reactive species (TBARS) method, as follows: 2 g of plant leaves were homogenized with 50 mL of water and 2 mL of 40 mM butylated hydroxytoluene (BHT), adding 500 μL of trichloroacetic acid to 1 mL of the homogenate. After vortexing, the tubes were incubated in an ice bath during 2 h. Tubes were centrifuged for 10 min at 3500 rpm. To 1 mL supernatants, 250 μL of 1% thiobarbituric acid in 0.05 N NaOH and 75 μL of disodium EDTA were added. Tubes were placed in boiling water for 15 min and, after cooling, absorbance was measured at 532 and 600 nm. Blanks were prepared without boiling to eliminate possible interferences of other plant compounds. After subtracting the absorbance at 600 nm from that obtained at 532 nm ($A_{532\,nm} - A_{600\,nm}$), the molar extinction coefficient of adduct (0.156 mM^{-1} cm^{-1}) was used to calculate TBARS levels.

2.4. Endosulfan concentration measurement

Endosulfan concentration was determined according to the method described by UNEP/IAEA [15] modified for soils. Samples of polluted soil (100 g) were collected at 30 or 90 days and dried at <50 °C up to constant weight. Dried samples were reduced with mortar and pestle, and, then, sieved using a 0.25 mm mesh; 10 g of the powdered sample was extracted with 50 mL of hexane in a Soxhlet apparatus. Extracts were concentrated with a rotoevaporator (Büchi, Switzerland) and purified using column chromatography. The column was packed with Florisil and sodium sulfate. To elute the sample, mixtures of 9:1 and 8:2 hexane:ether were used. Eluted samples were concentrated by rotoevaporation and, subsequently, subjected to a nitrogen stream to dryness. After reconstitution in an amber vial with 2 mL of hexane, samples were frozen until analyzed. Samples (1 μL) were injected in an Agilent 6890 N gas chromatograph-ECD (radiation source of Ni_{63}) with an SPB-5 capillary column (fused 5% phenylmethyl silicone), 30 m length × 0.25 mm ID × 0.25 mm film thickness. Helium and nitrogen were used as carrier and auxiliary gas with a flow rate of 1 and 30 mL min^{-1}, respectively. Temperatures used were: injector, 260 °C; column, 2 min at 90 °C; 30 °C min^{-1} from 90 to 180 °C, 3 °C min^{-1} from 180 to 270 °C (total elution time: 35 min); detector, 320 °C. Endosulfan concentration was calculated by interpolation to an external standard curve made with five different concentrations in triplicate. Sample measurements were made in duplicate.

2.5. Statistical analysis

The results were expressed as means ± standard deviation (SD). One-way ANOVA-Bonferroni and Student's t-test were applied and a $P < 0.05$ was used for significance. The STATA program, version 8.0 (Stata Corp., College Station, TX), was used for all statistical calculations.

3. Results and discussion

3.1. Development and growth of plants after endosulfan exposure

Ocimum plants were able to grow in soil polluted with endosulfan. Two closely related plant species were used: *O. basilicum L* and *O. minimum L.* Plant growth was measured within a period of 90 days and no difference in height was found between exposed or control plants (Fig. 1); no differences in other characteristics (like leaf color or width) were observed (data not shown). Each species exhibited a particular growth rate; while *O. basilicum* reached 50 cm in 90 days, *O. minimum* did not exceed 25 cm. At this time, no other difference was observed between plants in soil with or without endosulfan: plants flowered and produced seeds normally and the growth rate of each species did not seem to be related to the presence of endosulfan in the soil.

3.2. Endosulfan concentration in the soil with plants

It is possible that both species are able to grow in polluted soil, but this does not imply the use of *Ocimum* as a phytoremediator. To know whether the presence of the plant could help bioremediate the endosulfan-polluted soil, we evaluated the concentration of endosulfan after transplantation.

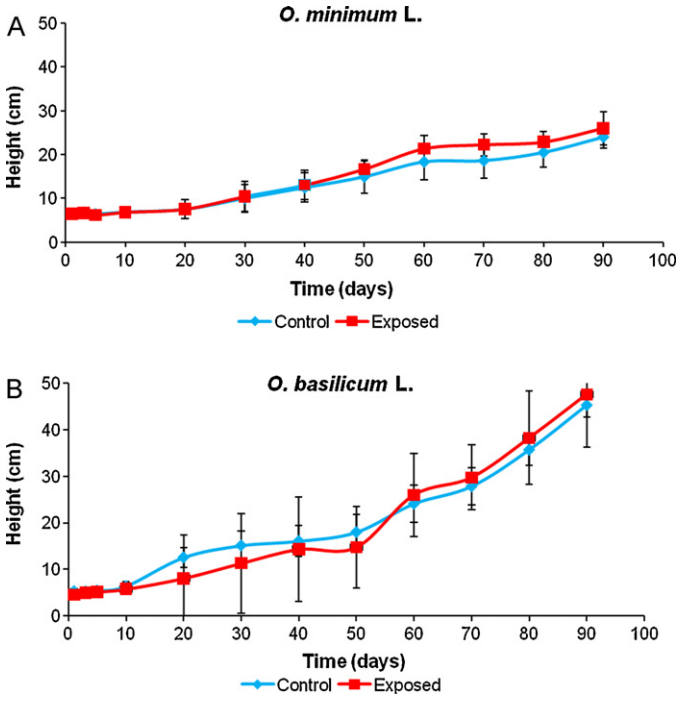

Fig. 1. Height of *O. minimum* (A) and *O. basilicum* (B) plants grown in control soil (◆ diamonds) or amended soil (■ squares) ($n = 4$). Soil was amended with 0.1 g endosulfan kg^{-1} of soil.

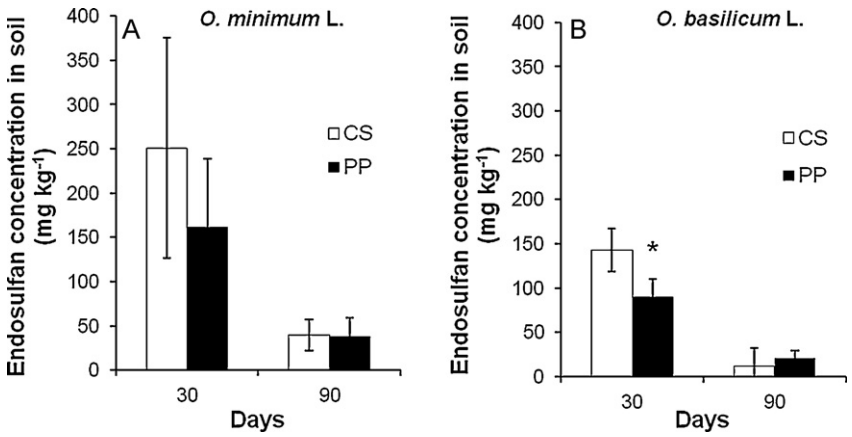

Fig. 2. Endosulfan concentrations in soil without (CS) or with plant (PP). *O. minimum* (A) or *O. basilicum* (B) was used. Mean ± SD values are shown. * Statistically significant difference with CS ($P < 0.05$, Student's *t*-test) ($n = 4$).

In *O. minimum*, no differences were observed in endosulfan concentrations at either 30 or 90 days (Fig. 2). After 30 days of transplanting *O. basilicum*, endosulfan concentration in the soil (PP) was lower than that in the soil without plant (CS). At 90 days, no difference was observed in the concentration of endosulfan in soil with or without *O. basilicum L.* (Fig. 2). The decrease observed at 30 days with *O. basilicum* could be explained by the metabolism of the plant itself, by the natural biostimulation of the rhizosphere or by a mixture of both. It has been demonstrated that plant-rhizosphere symbiosis can be useful for the bioremediation of other pollutants [16–18]. Recently, it has been shown that other organochlorine pesticides can be degraded by soil bacterial communities [14]. In a previous study, it was shown that *Pseudomonas spinosa*, *Pseudomonas aeuriginosa*, and *Burkholderia cepacia* are efficient in biotransforming endosulfan from soil spiked with endosulfan (0.1 g L^{-1}) to endosulfan diol and ether [19]. Similarly, *Anabaena*, *Fusarium ventricosum*, and *Pandoraea* species biotransformed endosulfan to different metabolites: endosulfan diol, endosulfan hydroxyether, endosulfan lactone, endosulfan sulfate [13,20]. The kind of metabolites depends mainly on the organism. In these studies, cultures with high density of microorganisms were used, and degradation occurred between 10 and 20 days, depending on the species, temperature, or pH. The decrease in endosulfan concentration due to the presence of *O. basilicum* was observed at 30 days, but the plant presence did not seem to be relevant at 90 days. This could reflect an active metabolism reaching a plateau at one or two months. Several characteristics, like plant population density, presence of worms, could affect or improve the bioremediation process. To this respect, further studies on the fate of endosulfan through plant or rhizosphere metabolism are being carried out.

3.3. Lipoperoxidation in plants exposed to endosulfan

Lipid peroxidation in plants has been used as a measurement of environmental stress, since it is generated by several environmental agents. Lipid peroxidation can be quantified by thiobarbituric-acid-reactive species (TBARS) levels. For instance, TBARS levels have been used to evaluate the damage caused by saline or water-logging stresses [21,22].

Endosulfan has been shown to produce oxidative stress in organisms from different taxa. In the yeast *Saccharomyces cerevesiae*, 250 μM (~102 mg L^{-1}) of endosulfan induces an increase in TBARS. Moreover, 50 μM (~20 mg L^{-1}) of endosulfan caused impaired growth in the yeast and a decrease in viability of human Hela or HepG2 cell lines [23]. Soaked seeds of sorghum in a solution with 0.2% of endosulfan showed oxidative damage and a decrease in germination rate and shoot length [24].

To evaluate a possible damage at a biochemical level on both species, young plants of both species, *O. basilicum* and *O. minimum*, were separately transplanted to 1 kg of soil with (or without) 0.1 g of endosulfan, and TBARS were measured at 90 days thereafter. In both cases, TBARS showed no differences relative to control. Although a small increase of lipoperoxidation was observed, it was not statistically significant (Fig. 3).

To study if endosulfan could induce oxidative damage in our species model at some other concentrations, we tested two more concentrations in the soil, and the time of analysis was reduced to 3 and 7 days. The response was analyzed only in *O. basilicum* because its effect on endosulfan concentration pointed it out as a possible remediator. (Fig. 2); we decided to perform this study in a shorter time period to determine the effect of endosulfan at a few days of plant growth. Higher concentrations of endosulfan would be expected to produce higher values of lipoperoxidation. Surprisingly, at any concentration used, no oxidative damage was observed in plants at 3 or 7 days after transplanting *O. basilicum* into the polluted soil (Fig. 4). This could be due to an adaptation of the plant within the first 3 days, but the possible effect of the transplant per se should be considered. In keeping this in mind, not transplanted (C) control plants were used. Oxidative damage was evaluated daily during the first 3 days after transplantation to the soil with (PP) or without (CP) endosulfan (Fig. 5). At day 1 after transplantation, plants grown in the polluted soil (PP) showed higher TBARS levels than plants grown in non-polluted soil (CP) or non-transplanted plants (C), this could indicate that pollution plus transplantation itself induces stress in the plant. Additionally, it was interesting to note that at day 3, both transplanted plants (CP and PP) showed significantly higher TBARS values respect to those plants that were not transplanted (C) (Fig. 5). These results suggest that both, transplant per se and endosulfan, may cause oxidative damage in the plant and it seems that *O. basilicum* can adapt to endosulfan concentrations as high as 1000 mg kg^{-1}. A plant suitable for bioremediation should endure or tolerate high concentrations of the pollutant, if that were not the case, the plant would die before decreasing the concentration of the pollutant. In the case of *Ocimum*, tolerance was developed within the first week after transplantation. The use of lipoperoxidation biomarker could reduce the time to assess whether a plant can tolerate a pollutant and is suitable for bioremediation. Nevertheless, more studies are required to establish if this is the case only for herbs or for plants in general.

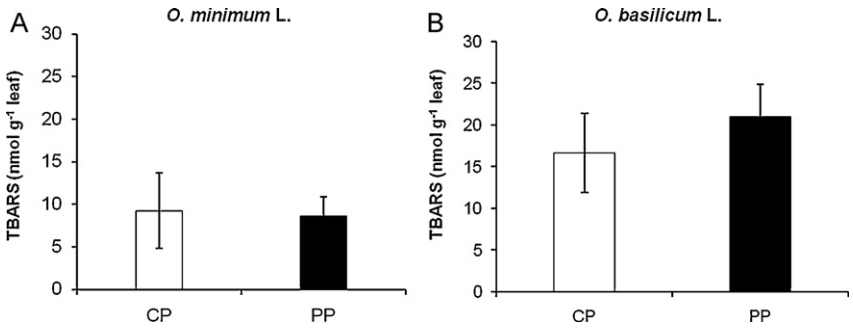

Fig. 3. Lipoperoxidation of plants grown in soil (CP) or polluted soil (PP). The plants grown were *O. minimum* (A) or *O. basilicum* (B). Mean ± SD values are shown ($n = 4$). No statistically significant difference was observed.

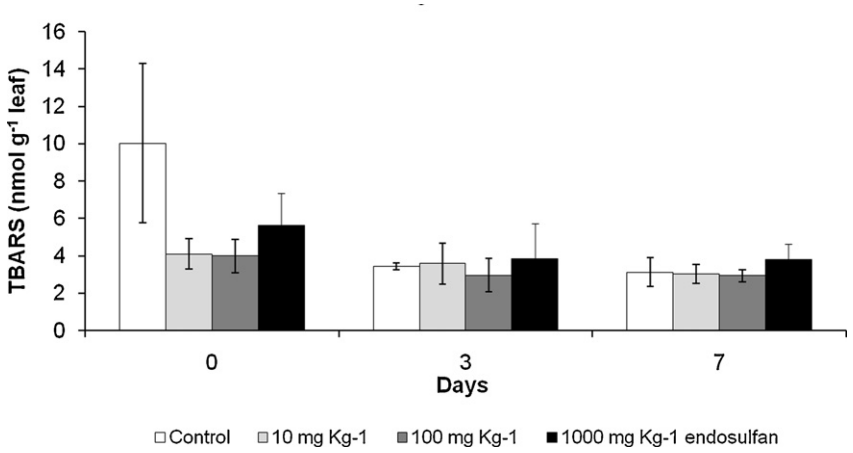

Fig. 4. Lipoperoxidation of *O. basilicum L.* plants grown at different endosulfan concentrations in soil at 0, 3, or 7 days after transplanting. Mean ± SD values are shown ($n = 2$ for control, $n = 3$ for exposed plants). No statistically significant difference was observed with respect to control at each time.

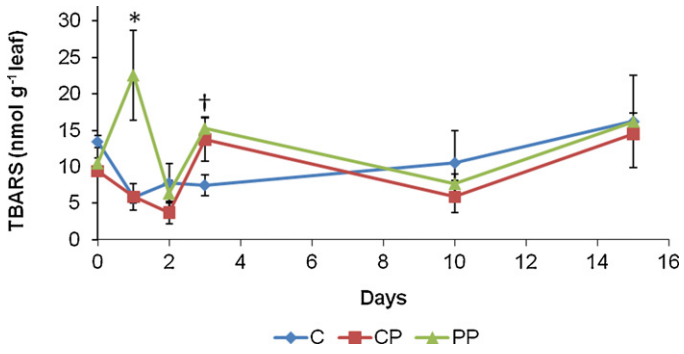

Fig. 5. Lipoperoxidation of *O. basilicum L.* plants grown in soil without (C and CP) or with 1000 mg endosulfan kg^{-1} (PP) at several times. "C", plants were not transplanted (control for transplantation) whereas CP means transplanted to non-polluted soil. Mean ± SD values are shown. *Symbol at day 1 data means that PP was statistically different from both controls ($P < 0.05$); † symbol at day 3 data means that both groups of transplanted plants (CP and PP) showed a statistically significant higher TBARS value than the C group ($P < 0.05$) ($n = 3$).

4. Conclusions

O. minimum L. and *O. basilicum L.* show no differences in growth rate in soil with or without endosulfan. It seems that plants could adapt to endosulfan-polluted soil within 10 days after transplantation; although the transplant per se could cause oxidative damage. The adaptation process could involve diminishing the endosulfan effect on the plant by means of plant exudates, antioxidants, or metabolism or through its symbiosis with the rhizosphere. Whichever the mechanism is, endosulfan concentration in soil was reduced 37% when *O. basilicum* was grown in it, but not with *O. minimum*. Therefore, *Ocimum basilicum* could be an adequate candidate for phytoremediation of endosulfan-polluted soils. More studies on the metabolism and oxidative stress in plants caused by pollutants or conditions like transplantation, humidity, among others, are needed to understand the complex mechanisms of plant adaptation.

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