Metabolism of the soil and groundwater contaminants, ethylene dibromide and trichloroethylene, by the tropical leguminous tree, *Leuceana leucocephala*

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Abstract

Ethylene dibromide (EDB; 1,2-dibromoethane) and trichloroethylene (TCE) are hazardous environmental pollutants. The use of plants to treat polluted sites and groundwater, termed phytoremediation, requires plants that can both effectively remove the pollutant as well as grow in the climatic region of the site. In this paper, we report that the tropical leguminous tree, *Leuceana leucocephala* var. K636, is able to take up and metabolize EDB and TCE. The plants were grown in sterile hydroponic solution without its symbiont, Rhizobium. EDB and TCE were both metabolized by the plant, as indicated by the formation of bromide ion from EDB and trichloroethanol from TCE. Each plant organ was independently capable of debromination of EDB. *L. leucocephala* is being used to treat perched groundwater as part of a remedial alternative to address an accidental EDB spill in Hawaii. Bromide levels of plant tissues from the trees grown in the phytoremediation treatment cells at the Hawaii Site were elevated, indicating uptake and degradation of brominated compounds in the trees. This report is the first evidence of a tropical tree effectively metabolizing these common organic pollutants. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Ethylene dibromide (EDB; 1,2-dibromoethane) was used as a gasoline additive and as a soil fumigant to kill nematodes before it was banned in 1983 following evidence of its acute toxicity and mutagenic properties (for a review of the toxic effects of EDB, see [1]). Of 40 chemicals evaluated by the University of Florida, EDB was ranked as having the greatest potential for groundwater intrusion [2]. In the United States Environmental Protection Agency (EPA) Report, Pesticides in Groundwater Database, the EPA reported that 2918 of 20,221 wells sampled contained detectable levels (greater than 0.01 μg/l) of EDB.

Two pathways for EDB degradation occur in mammals (Fig. 1). The conjugative, glutathione-S-transferase (GST) pathway produces a carcinogenic reactive episulfonium ion which forms mutagenic adducts to DNA through the action of the DNA repair enzyme, O(6)-alkylguanine-DNA alkyltransferase [3].

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Metabolism of EDB through the GST pathway also interferes with oxidative phosphorylation in mitochondria [4]. The cytochrome P450 pathway produces free bromide ion and bromoacetaldehyde [5]. Bromoacetaldehyde is further metabolized to bromoacetic acid and thiodiacetic acid which are excreted. The P450 responsible for the oxidation of EDB is P450 2E1 which has a high affinity and turnover of EDB [5].

Bacteria also metabolize EDB, releasing bromide ion as one of the products. Haloalkane dehalogenases from Xanthobacter autotrophicus [6], Mycobacterium [7], Rhodococcus rhodochrous, and Pseudomonas putida [7] have been cloned. Mycobacterium strain GP1 produces bromide ion and 2-bromoethanol in the initial step of EDB metabolism and can utilize EDB as a sole carbon source [7].

Trichloroethylene (TCE) was commonly used as a degreaser of engine parts, as a dry cleaning solvent, and as an anaesthetic. TCE is now one of the most common pollutants in the industrial world and is present in 40% of all Superfund Sites in the United States. TCE is hepatotoxic, mutagenic, and suspected to be carcinogenic (for review, see [9]). Like EDB, TCE is metabolized in mammals through the GST and P450 pathways. The oxidative pathway that involves P450 2E1 is the predominant one. The resulting TCE metabolites include chloral, trichloroethanol, trichloroacetate, and dichloroacetate. Trichloroethanol exists mostly as a conjugate to a glycoside both in mammals [9] and in plants [10].

Phytochemistry, the use of plants for remediation of polluted sites, has been demonstrated to be an effective means of removing toxic wastes from soil and water (recently reviewed in [11]). Plants are being studied for the remediation of heavy metals such as selenium and mercury, chlorinated hydrocarbons such as TCE and carbon tetrachloride, explosives such as trinitrotoluene, aromatics such as benzene and toluene, and hydrophilic compounds such as methyl tertbutyl ether and 1,4-dioxane. Plants can be used to take up and metabolize the pollutant, enhance rhizosphere microorganisms, stabilize metals in soil, or volatilize the pollutant through transpiration.

One difficulty in using plants to remediate polluted sites is identifying plant species that are both adapted to the environment of the polluted site as well as able to effectively remove the pollutant. Ideally, the plant should be fast-growing and deep-rooted to effectively reach the pollutant. Poplar trees have these characteristics and are effective at removing TCE [12]. However, poplar trees are limited to the temperate zone. There is a need to determine which plants can be used for remediation that are suitable to other geographical regions, such as the tropics.

In this study, we examined the fast-growing tropical tree, Leuecanea leucocephala var. K636, for its ability to metabolize EDB and TCE. L. leucocephala is used in the tropics for multiple purposes including as fodder for livestock, fuel wood, post wood, erosion control, soil improvement and simply for shade. It was used in India in a study on remediation of fly ash [13]. Due to its superior growth and soil stability properties, it has been studied extensively by the University of Hawaii (AgroForester web site). EDB was previously used in Hawaiian agriculture. As a result of an accidental release, EDB is one of the Chemicals of Concern for a Superfund site in Hawaii. TCE contamination is common to military sites due to its use as an engine degreaser, so remediation of this pollutant is necessary in the tropics as well. In this work, we demonstrate that L. leucocephala can effectively take up and metabolize EDB and TCE, thus making this tree a suitable candidate for phyto remediation in tropical climates.

2. Materials and methods

2.1. Growth of L. leucocephala

Leuecanea leucocephala K636 seeds were purchased from AgroForester™ Tropical Seeds (Hawaii). Seeds were surface-sterilized with 10% bleach for 15 min, rinsed, and soaked in sterile water for 30 min. After the seeds germinated on water agar, the seedlings were
transferred to sterile 125-ml flasks containing sterile (filtered through 0.2 μm filters) half-strength Hoagland’s Solution [14]. The flasks were covered with foil, and the area around the stem was plugged with sterile cotton. The solution was changed weekly and as the plants grew, they were transferred to 250- or 500-ml flasks. All transfers of fresh hydroponic solution were done in a sterile hood, the solution was not aerated, and the solution remained clear and free of visible contamination throughout the study. The plants were not nodulated.

2.2. Growth of tobacco and poplar for EDB metabolism comparison experiment

Cuttings from tobacco (Nicotiana tabacum var. xanthi, n.c.) and poplar (Populus trichocarpa x P. deltoides clones H11-11 and 184-402) were surface-sterilized and grown in sterile hydroponics as described [15].

2.3. Exposure to EDB or TCE

EDB (99+%) and TCE (99+%) were purchased from Sigma-Aldrich. Cuttings grown hydroponically for 6–8 weeks were transferred from sterile hydroponics into sterile 500-ml test flasks with a side-arm and flat top (see Fig. 2). To minimize escape of volatile EDB and TCE, two sets of glass plates with center holes were placed around the stem at the top of the flask and sealed with Plumber’s Putty (Home Depot) and Fluorolube (Fisher Scientific). The side arm was sealed with a septum-lined cap. Samples of a concentrated solution of equilibrated TCE or EDB were added through the side-arm to give a final concentration of either 50 μg/ml TCE or the specified amount of EDB. The hydroponic solution was allowed to equilibrate for approximately 1 h before samples of the solution were taken for quantification. During the exposure period, the plants were placed in a fume hood equipped with 400-W high pressure sodium lights at a 12 h light/dark cycle.

2.4. Isolated tissue experiments

L. leucocephala seeds were surface-sterilized and placed in sterile jars containing 1/2 X Murashige and Skoog media agar [16]. After 6 weeks, three seedlings were separated into roots, stems, and petioles with leaves, and transferred to 125-ml flasks containing a modified Hoagland’s solution with reduced nitrate and chloride. The solution was dosed to a level of 5 μg/ml EDB, and the flasks were incubated on a shaker for 5 days. The tissues were rinsed with sterile water and were extracted for bromide as described below.

2.5. EDB and TCE detection in hydroponic solutions

Samples of hydroponic solutions were taken 1 h after dosing and at the end of the 5-day exposure period. The samples were diluted into HPLC-grade water and analyzed using a Tekmar (Cincinnati) Purge and Trap concentrator in line with a Perkin-Elmer Autosystem XL GC with electron capture detector. Three concentrations of EDB and TCE were run to generate standard curves for quantification.

2.6. EDB uptake experiments

Twenty-three plants and 15 glass rod controls were exposed to 2, 5, 10, 13, or 20 μg/ml EDB for 5–14 days. The average loss of EDB from the hydroponic solution per day was calculated using the EDB concentration at the beginning and end of the experiment.

2.7. Bromide ion detection in hydroponic solutions

After the 5-day exposure to EDB, 1 ml of hydroponic solution was loaded onto a washed Superclean LC-18 column (Supelco) and eluted with 2 ml of HPLC-grade water. The solution was then filtered through a 0.2 μm filter (ISC Bioexpress), evaporated to dryness in a 105°C oven, and resuspended in 1 ml HPLC-grade water. Samples were run on a Dionex DX-120 ion chromatograph with a Dionex AS40 autosampler. Data were analyzed with Dionex PEAKNET 5.01 software. Dilutions of sodium bromide were used to generate a standard curve for quantification.

2.8. Bromide ion extractions from tissues

Leaves, stems, and roots were frozen in liquid nitrogen immediately and stored in a −80°C freezer. Tissues were ground with liquid nitrogen using a mortar
and pestle, and the powders were fully dried by baking. Samples weighing 0.5 g (or all available if less than 0.5 g) were placed in plastic 50-ml conical tubes (Sarstedt). Three milliliter of HPLC-grade water were added, and following vortexing, the tubes were placed on a shaker for 3 h. The samples were collected into 10-ml syringes containing a small amount of glass wool that had been thoroughly rinsed with HPLC-grade water and dried. The filtered extract was further cleaned with a phenol/chloroform extraction. After filtering through a 0.2 μm filter (ISC Bioexpress), 2 ml of the samples were loaded onto LC-18 columns and analyzed for bromide ion as described above.

The tissues for the data presented in Fig. 3 were extracted with 10 ml HPLC-grade water and treated as described above except without the phenol/chloroform or LC-18 columns. Since this less-clean extract was damaging to the IC column, all other extractions were done with the additional purification steps.

2.9. EDB in tissues

One-tenth gm (wet-weight) of tissue was added to 1 ml HPLC-grade water in a 40-ml vial sealed with a septa-lined cap, and analyzed for EDB using the Purge and Trap Concentrator in solids mode.

2.10. L. leucocephala nodule bacteria

Root nodules from L. leucocephala plants grown in Hawaiian soil were surface-sterilized with 20% bleach and rinsed in sterile water. The nodules were crushed and the extract was plated on a TYC plate (tryptone/yeast extract/CaCl₂). The bacteria were restreaked to obtain single colonies. A few colonies of what appeared to be a pure culture were used to inoculate liquid TYC media, and the grown culture was frozen in glycerol. All experiments with the nodule bacteria utilized bacteria grown from this stock.

Viability tests following EDB exposure were done by diluting the culture, plating on MG/L media [17], and counting colonies. Bromide extraction from the bacteria was done by drying the cells, extracting with 10 ml of HPLC-grade water, and filtering the supernatant through 0.2 μm filter disks.

2.11. Trichloroethanol detection

The tissues were extracted with MTBE and analyzed on a GC-ECD as described [15]. Detection of the glycoside-conjugate form of trichloroethanol was done as described [10].

3. Results

3.1. Growth of L. leucocephala in hydroponics

To assess the ability of the plants to take up and metabolize EDB and TCE apart from its usual microbial associations (Rhizobium and mycorrhizae), we chose to do all the experiments in sterile hydroponics. L. leucocephala cuttings root poorly, so we started plants from surface-sterilized seeds.

![Fig. 3. Bromide levels in L. leucocephala plants exposed to 10 μg/ml EDB for 10 days. The actual bromide levels per tissue type, without normalization to grams of tissue, are shown.](image-url)
3.2. **EDB toxicity**

To determine which levels of EDB were toxic to the plants, axenic *L. leucocephala* seedlings were grown in hydroponics for 4 weeks before being transferred to sealed flasks (Fig. 2) and exposed to various levels of EDB. Two plants each were exposed to 0, 4, 10, 20 or 30 μg/ml EDB for 5 days. The leaves of the plants at the highest dose wilted and died. As an indicator of plant health, we measured the amount of solution taken up by the plants. A significant decrease in water uptake occurred when EDB was present. The uptake of hydroponic solution after the 5-day exposure was 110 g/ml. The uptake of the solution was 80 and 70 g/ml for the two undosed plants, 80 and 50 ml for the plants at 10 μg/ml EDB, 80 and 70 g/ml for the plants at 20 μg/ml EDB, and 70 and 60 for the plants at 30 μg/ml EDB.

3.3. **EDB uptake from hydroponic solution**

A series of experiments was done to measure EDB uptake. Since EDB uptake cannot be measured directly, we calculated the EDB lost from the hydroponic solution during the experiment due to either uptake by the plant, volatilization into the headspace of the flask, or leakage through the seals. Glass rods were used in place of plants to determine the amount of the latter two possibilities. The average loss of EDB per day from the planted solutions was 6.4% whereas the average loss from the rod controls was 1.5%, indicating that the plants are capable of taking up EDB from solution. The variation in levels of EDB uptake and metabolism between plants was probably due to the genetic variability of the plants and due to variable amounts of leakage of volatile EDB.

3.4. **Metabolism of EDB releases bromide ion**

Plants dosed with EDB were analyzed for bromide ion, an EDB metabolite. Since there was no background level of bromide ion in the plant tissues, bromide ion made an ideal indicator of EDB metabolism. Eight plants were transferred to the 500-ml test flasks, and four of them were exposed to a level of 10 μg/ml EDB for 10 days while the other four were left undosed as controls. A sample of each tissue was reserved for measuring EDB, and the remaining tissue was extracted for total bromide ion. None of the undosed plants contained detectable levels of bromide ion. As shown in Fig. 3, each of the dosed plants had bromide ion in the tissues, with leaves and petioles containing the most bromide. Conversely, EDB levels were lowest in the leaves and petioles, with five times as much intact EDB in stems and 7 times as much in root. Since the EDB concentration was least in the leaves yet the organ having the most bromide ion was leaf, either the EDB is metabolized in the other tissues and then the resulting bromide ion was transported to leaves or the EDB was more quickly metabolized in the leaves.

3.5. **Localization of EDB metabolism activity**

While bromide ion was detected in the roots, stems, and leaves of EDB-dosed plants, it was not known if the EDB was metabolized in all three organs or the bromide ion was transported throughout the plant. To address this issue, three plants were grown in sterile vessels and the three tissue types were separated and placed in sealed flasks and dosed individually with EDB. All three tissues demonstrated EDB metabolism to bromide ion. To compare the metabolism by each tissue, the data were converted to μg bromide per gram of tissue. However, the main stems of the seedlings were very small compared to the amount of petioles and leaves. On a per gram basis, stems produced the most bromide ion (Fig. 4). The average bromide ion per gram of tissue was 72 μg in root, 133 μg in stem, and 64 μg in leaf. However, the amount of bromide ion per organ was still highest in leaves and petioles.

3.6. **Comparison of EDB metabolism in L. leucocephala, poplar, and tobacco**

Poplar and tobacco are currently used in phytoremediation studies, so we compared EDB metabolism of these model plants with that of *L. leucocephala*. The plants were exposed at a level of 5 μg/ml EDB for 5 days, and the tissues were extracted for bromide ion. Possibly due to the genetic variation in the *L. leucocephala* seedlings compared to the poplar and tobacco clones, there was variation in the ability to metabolize EDB to bromide ion. As shown in Fig. 5, the plants with the highest and lowest bromide ion levels were *L. leucocephala*. However, tobacco plants produced an average of 196 (std. dev. 12) μg bromide ion per gram of tissue, while poplar produced 138 (std. dev. 45) and *L. leucocephala* produced 99.3 (std. dev. 85).

3.7. **Nodule bacteria dosed with EDB**

To determine if the Rhizobium normally associated with *L. leucocephala* can also metabolize EDB, we isolated the bacteria from root nodules of soil-grown *L. leucocephala* plants and grew the bacteria in liquid media. Duplicate flasks of broth containing either 0, 1, 5, 10, 20, or 40 μg/ml EDB were inoculated with overnight culture of the nodule bacteria. There was no effect on viability at even the highest EDB dose. However, no bromide ion was detected in the media or in the cell extracts.
3.8. EDB metabolism in phytoremediation treatment cell-grown trees

During the laboratory phase of the phytoremediation study, two high density polyethylene-lined treatment cells were installed near the Superfund Site in Hawaii. The lined treatment cells were filled with 60% soil, 20% mature yard waste compost, and 20% manure. One of the treatment cells was planted with the *L. leucocephala* plants while the other treatment cell was used as an unplanted (land treatment) control.

Perched groundwater containing site chemicals including EDB was pumped from a series of perched extraction wells installed for the study. The perched groundwater was used to irrigate both the land treatment cell and the phytoremediation treatment cell. Leaf and root samples from the phytoremediation treatment cell were analyzed for bromide ion.

Fig. 4. Bromide levels in isolated tissues of three plants of *L. leucocephala*. Each of three plants were divided into roots, stems, and leaves and exposed individually to 2 μg/ml EDB in sealed flasks for 5 days. Bromide was normalized to grams of dry weight of each tissue type.

Fig. 5. Comparison of EDB metabolism to bromide ion in poplar, tobacco, and *L. leucocephala* after exposure to 5 μg/ml EDB for 5 days. Tissues were dried and weighed and extracted for bromide ion. Bromide levels were totalled for roots, stems, and leaves and normalized to μg bromide per gram of tissue. Undosed poplar, tobacco, and *L. leucocephala* had less than 1 μg bromide ion in the tissues.
In *L. leucocephala* trees grown in the phytoremediation treatment cell, leaf and root samples contained $16 \pm 0.1 \mu g \text{ bromide/gm}$ and $4.7 \pm 0.9 \mu g/gm$ of dried tissue, respectively. In untreated control trees, the leaf and root samples contained $12.8 \pm 1.4 \mu g/gm$ and $1.4 \pm 0.03 \mu g/gm$, respectively. Bromine is found in tropical soils and in groundwater, therefore, it is not unexpected to find bromide ions in the untreated controls. The trees were approximately 3 m tall, so a substantial amount of EDB was taken up and metabolized per tree.

Since there were higher levels of bromide ion in the treated plants compared to in the untreated plants, and since the plants grown from seed in the laboratory do not contain bromide ion, it provides qualitative evidence that the *L. leucocephala* plants have the ability to uptake and metabolize EDB to bromide ion.

### 3.9. Metabolism of TCE to trichloroethanol

To examine whether *L. leucocephala* can metabolize TCE, a more common pollutant than EDB, we exposed plants and glass rod controls to $50 \mu g/ml$ TCE for 5 days and compared them to undosed plants. The experiment was repeated, and the data compiled and shown in Fig. 6. The five planted flasks had an average loss of TCE from the hydroponic solution of 42% (standard deviation of 17%), and the rod flasks had an average loss of 19% (std. dev. of 6%). The TCE metabolite, trichloroethanol, was extracted from the tissues and both the free trichloroethanol and its glycosylated form were quantified. The total trichloroethanol per plant is shown in Fig. 6. Trichloroethanol is clearly present in the dosed plant tissues indicating TCE metabolism occurred. The presence of small amounts of trichloroethanol in two of the undosed plants may be from metabolism of volatile TCE from the dosed plants. In support of this hypothesis, the trichloroethanol of these undosed plants was present only in leaves, whereas trichloroethanol was present in leaves, stems, and roots of the dosed plants.

### 4. Discussion

We have provided evidence of metabolism of the environmental pollutants, EDB and TCE, by the tropical tree, *Leuceana leucocephala*. There was significant variation between individual plants and their ability to metabolize these compounds; however, all the plants were able to metabolize the compounds to some extent. In mammals, metabolism of EDB and TCE is initiated with the cytochrome P450 2E1. As with *L. leucocephala*, there is variation in the population in the extent of metabolism of xenobiotics. In mammals, the variation has been shown to correspond to differing levels of P450 expression in the population [18]. There is some precedence for homologues of mammalian P450 cytochromes being present in plants [19]. We attempted immunodetection of P450 2E1 in *L. leucocephala* protein extracts by western blotting using antibodies to rat 2E1 but there was no corresponding protein. Either P450 2E1 is not present in *L. leucocephala* or the enzyme is too dissimilar to the rat enzyme. Other enzymes, such as

![Fig. 6. Trichloroethanol production in TCE-dosed *L. leucocephala* plants and percent TCE loss from the hydroponic solutions. Plants were exposed to 50 μg/ml TCE for 5 days. The total trichloroethanol produced (in both the free form and glucosylated form) were quantified per plant. Loss of TCE from the solution was calculated by comparing the TCE concentrations at the beginning and end of the experiment.](image-url)
dehalogenases or lignin peroxidases, may be responsible for the observed activity. Since bromide ion was produced in roots, stems, and in leaves, the enzyme must be common to all three tissue types.

Although the average *L. leucocephala* plant did not metabolize EDB as effectively (produce as much bromide ion per gram of tissue) as did poplar and tobacco plants, some of the individual plants did outperform these two model phytoremediation species. We have examined a total of 27 *L. leucocephala* plants and all of them produced bromide ion when dosed with EDB. Despite the superior performance of the tobacco plants in degrading EDB to bromide ion in this study, these annual plants would not be likely candidates for phytoremediation due to their smaller size and the need to replant.

Naested and coworkers reported that plants, including tobacco, do not express detectable haloalkane dehalogenase activities [20]. In their studies, plant extracts were exposed to EDB or DCE, and halide production was monitored by a colorimetric assay. Our work demonstrates that tobacco, poplar, and *L. leucocephala* do have dehalogenase activities. The differences can perhaps be explained due to the fact that our experiments used living, whole plants whereas Naested et al. used plant extracts; and we used ion chromatography while they explained due to the fact that our experiments used living, whole plants whereas Naested et al. used plant extracts; and we used ion chromatography while they used a less sensitive colorimetric assay.

In nature, *L. leucocephala* trees are associated with *Rhizobium* and mycorrhizae. Although the nodules bacteria in culture did not demonstrate dehalogenation of EDB, the bacteria may metabolize EDB when it is associated with the plant in root nodules. Experiments are underway to determine if the plant with its two symbiotic partners is better able to metabolize EDB than when it is in isolation.

The results of the phytoremediation study in Hawaii utilizing *L. leucocephala* (“Kao Haole”) to remediate an EDB spill were recently published [21]. The report describes the successful use of the trees for remediation of contaminated ground water. Irrigation of the plantation with several hundred thousand gallons of pumped well water containing an average of 25 μg/l EDB over a three year period resulted in no visible phytotoxicity, and the leachate from the site contained no detectable EDB (detection limit of 0.02 μg/l). The report indicated that microbial degradation of EDB may have been the predominate mechanism, however, the trees allowed for a greatly increased treatment capacity of the soils. Since we detected EDB metabolites in the trees from both the field experiment and from sterile hydroponics, it is clear that the plants do have the capacity to degrade EDB.

5. Conclusions

For the phytoremediation technology to succeed, a wide variety of tree species of remediation potential must be available in order to serve in a range of climatic regions. In this paper, we demonstrated that the tropical tree *L. leucocephala* is able to take up and metabolize the common environmental pollutants, EDB and TCE, thus making it a good candidate for phytoremediation of these organic pollutants in tropical environments.

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