

Root Turnover: An Important Source of Microbial Substrates in Rhizosphere Remediation of Recalcitrant Contaminants

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The growth dynamics and phenolic content of mulberry (*Morus* sp.) fine roots (<1 mm diameter) were determined and examined in relationship to rhizosphere remediation of recalcitrant soil contaminants. Root turnover measurements of rhizotron-grown plants showed that 58% of the fine roots produced during a 6-month growing season (June–November) died at the end of the season. The concentration of phenolic compounds in fine roots increased approximately 2-fold during the later stages of the season, and the total phenolic content of dead fine roots reached a maximum value of 38 mg/g dry weight. The late-season increase in total phenolics was primarily due to accumulation of three different flavones (morusin, morusinol, and kuwanon C). These three flavones were shown to support the growth of the bacterium *Burkholderia* sp. LB400, a degrader of polychlorinated biphenyls (PCBs). Thus, it has been established that, upon death, the fine roots of mulberry can serve as a source of substrate for PCB-degrading bacteria. These results establish for the first time that the chemical content and turnover rate of fine roots should be considered an important aspect of rhizosphere remediation.

Introduction

Provision of compounds that serve as growth substrates and/or enzyme inducers has been demonstrated as an effective means of stimulating the degradation of recalcitrant, slightly water-soluble contaminants such as polychlorinated biphenyls (PCBs) both in laboratory (1) and in field investigations (2). Large-scale implementation of this approach in remediation of contaminated soil has not occurred because of several obstacles: expense of the compounds, inability to evenly disseminate slightly water-soluble compounds into soil, and regulatory restrictions on some proven substrates/inducers (i.e., biphenyl). Rhizosphere remediation provides an opportunity to overcome these obstacles.

Under the supposition that naturally produced plant secondary compounds could replace commonly used biphenyl as a growth promoter and enzyme inducer for PCB-degrading bacteria, we showed that several flavonoids

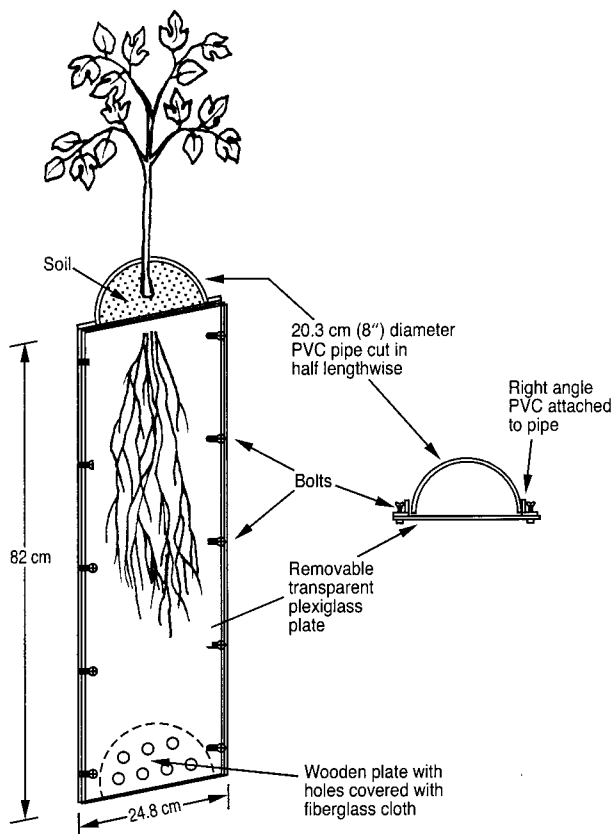


FIGURE 1. Design of the portable rhizotron pot.

equaled the performance of biphenyl for promoting both growth and PCB degradation by three strains of PCB-degrading bacteria (3). Screening studies conducted on 17 different plant species showed that root leachates collected in late fall from three plant species (mulberry, Osage orange, and apple) promoted the growth of *Alcaligenes eutrophus* H850, a PCB-degrading bacterium (4–6). Observation that plants harvested in late fall had a high density of dead fine roots and the knowledge that phenolic compounds accumulate in the vacuole (7, 8) suggested that the effective compounds were released as a result of cell death during root turnover, a phenomenon typical of tree roots during the fall (9).

It is our hypothesis that upon death, the fine roots of some plant species release compounds that promote the growth and activity of bacteria capable of degrading polycyclic organic pollutants. The present research conducted with mulberry addressed this hypothesis by quantifying root turnover, determining phenolic and flavonoid content as a function of root aging/death, identifying the most prominent fine root flavonoids, and demonstrating the growth-promoting properties of these flavones to *Burkholderia* sp. LB400, a PCB-degrading bacterium.

Experimental Section

Portable Rhizotron Pots. The growth of plants in specially designed portable rhizotron pots permitted both root imaging and selective root harvesting over the course of a growing season (Figure 1). Rhizotron pots were constructed from half-cylinders of 63 psi polyvinyl chloride (PVC) pipe (20.3 cm i.d.) that were 82 cm long, with removable 82 × 25 cm acrylic windows attached to the open sides. Wooden plates with

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drainage holes were covered with fiberglass cloth and bolted into the bottom to the pots in order to contain the soil while permitting drainage. Individual rhizotrons containing plants were placed into larger diameter PVC pipes positioned at a 55° angle in the ground to encourage root growth against the window. The openings between the rhizotrons and the outer pipe were sealed with foam rubber inserts at ground level in order to maintain near-ambient soil temperature and to reduce the exposure of roots to light.

Plant Materials and Growth. One-year-old, dormant, bare-rooted mulberry saplings were obtained from the Oklahoma State Forestry Division Tree Nursery (Goldsby, OK) where they had been grown from the seed of wild populations. On the basis of morphological examination and chemicals recovered in this investigation, the plants used in this study are thought to be a hybrid of the native species, *Morus rubra*, with *Morus alba*, a species native to Europe that has been planted extensively in the United States as windbreaks (10). It is common for mulberry species to hybridize in the wild and to display mixed characters (11) as we observed in the plants used in this study.

Saplings with approximately 1-cm basal stem diameter were planted in portable rhizotron pots in early April. Rhizotron pots were filled with 7.6 L of potting mix comprised of crushed black volcanic rock and dark compost (1:1) that had been passed through a 1.5-mm mesh screen. The homogeneous dark color and fine texture of this soil improved image analysis because it enhanced the contrast of root images and reduced background clutter. Plants were lightly fertilized with 500 mL of 20–20–20 (N–P–K) fertilizer at a concentration of 2.8 g/L in mid-June, July, and August, and water was provided daily with a drip system to maintain moist soil.

Plants were grown outdoors in full sunlight from the planting date in early April through late November. Average monthly high and low temperatures (°C) during each month of the study are as follows: April (22/9), May (26/14), June (31/19), July (34/21), August (34/21), September (29/17), October (23/10), and November (16/4). Plant roots were imaged and sampled on June 26, August 25, and November 28, as detailed below.

Root Imaging. Root images were prepared of five mulberry saplings on June 26, August 25, and November 28 of the same growing season. Photographs of roots were taken indoors on a copy-stand constructed to hold a rhizotron in a horizontal position with a camera mounted 60 cm from the soil surface. A Canon AE-1 camera equipped with a 50-mm lens and an 80A deep blue filter was used with Kodak Royal Gold 25 film. The observation window was removed from the rhizotron during photography to improve image quality. Roots, whenever temporarily exposed to the open air, were periodically sprayed with water. Photographic negatives were digitized onto CD-ROM (Kodak Photo CD by Kodalux Processing Services).

Root Image Analysis. Computerized analysis of root images was conducted with "Root Analysis Utility Program" (RAP), a software system recently developed specifically for root analyses by Tardis, Inc. (Los Alamos, NM) for the U.S. Environmental Protection Agency (Environmental Research Lab, Western Ecology Division, Corvallis, OR). The RAP program scans root images and tabulates the number of instances that scanlines intersect roots, providing a measure of root density. The RAP software is also capable of compiling the number of intersections according to root diameter classes. In this study, root density values were grouped into two root diameter classes: fine roots (<1 mm diameter) and mid-sized roots (1–2 mm diameter). Roots larger than 2 mm diameter occurred very infrequently along the rhizotron window and thus were excluded from analyses.

Calculation of Root Mortality. Root production and mortality were calculated by determining the difference between seasonal maximum and minimum root density measurements (12, 13). In this study, the seasonal maximum occurred in August. Temporal increases in density (from June to August) were used as indicators of production while subsequent decreases (August to November) represented mortality. Percent mortality represents the percent of root density produced during the season that subsequently died and was calculated using the following equation:

$$\% \text{ mortality} = 100 \times (\text{mortality}/\text{production})$$

Root Sampling and Phenolic Analyses. Root samples were collected from six plants on June 26, August 25, and November 28 immediately after root images were prepared. With the rhizotron window removed, samples of approximately 0.2 g FW of each root type were excised, using forceps and scissors, from the roots growing on the soil surface and then were frozen until extracted for phenolic analyses. The roots investigated were separated into two size categories: fine (<1 mm) and mid-sized (1–2 mm). Fine roots were further divided into three groups based on appearance and pigmentation: yellow living roots, brown living roots, and dead roots, which were black and withered. Repeated observation of specific roots from their origin until death had established that young, mature, and dead roots were colored yellow, brown, and black, respectively. On any single sampling date, <0.5% of the total fine root system was removed. This value was determined through measurements of total fine root biomass of 6 replicate plants grown under identical conditions that were destructively harvested.

Root samples were rinsed in distilled deionized water to remove soil particles and then blotted dry, and aliquots of 0.1 g fresh weight were oven-dried for 24 h at 60 °C and then reweighed. Dried roots were submerged in liquid nitrogen and then were ground to a fine powder in a mortar and pestle. The ground root material was placed in thick glass centrifuge tubes with 10 mL of 80% ethanol and boiled for 10 min in an 85 °C water bath (14). Extracts were clarified by centrifugation at 12000g for 15 min, pellets were rinsed, and extract volumes were adjusted to 25 mL with 80% ethanol and then were stored in capped vials at –10 °C until analyzed for phenolic content.

The total phenolic concentration of the ethanolic root extracts was determined colorimetrically using the method of Amorim et al. (15). The flavonoid morin was used as a standard at a range of concentrations between 0.025 and 0.25 mg/mL. All samples and standards were assayed in duplicate, and readings were averaged.

Root phenolics were characterized by subjecting extracts to high-performance liquid chromatography (HPLC). Extracts prepared as described above were dried, redissolved in 66:34 methanol:aqueous 0.3% H₃PO₄ and then filtered through 0.2-μm pore size Acrodisc CR PTFE syringe filters. A Macherey-Nagel C18 reversed-phase column, 250 mm × 4 mm (i.d.) with Nucleosil 100-5 packing was used with a flow rate of 1 mL/min. A linear gradient was run with solvent A consisting of aqueous 0.3% H₃PO₄ and solvent B consisting of methanol, changing from 60% B to 90% B over 75 min. Ultraviolet absorbance was measured at 275 nm, which was optimized based on diode-array detection data.

Identification of Root Flavones. Ten grams of dried roots (<1 mm diameter) taken from mature mulberry trees was boiled in 80% ethanol, and the extract was coarse-filtered, dried in a rotary evaporator, redissolved in methanol, and filtered through 0.2-μm pore size Acrodisc CR PTFE filters. The extract was first subjected to analytical HPLC as previously described to confirm similarity of phenolic components between the mature tree and the pot-grown

saplings. Semipreparative chromatography was performed using a Macherey-Nagel C18 reversed-phase column, 250 mm × 10 mm (i.d.) with Nucleosil 100-7 packing and the same gradient as described previously but with the omission of H₃PO₄ from the water and a flow rate of 6 mL/min. Fractions from each of the three major peaks were collected, dried completely in a rotary evaporator, and redissolved in methanol. When necessary, fractions were purified further with HPLC prior to nuclear magnetic resonance (NMR) analysis.

All NMR experiments were performed on a Varian VXR-500 spectrometer equipped with a 3-mm ¹H/¹³C switchable gradient microprobe (MDG-500-3) and a pulsed field gradient driver. Signals were reported in parts per million (δ), referenced to the solvent used. Electron impact mass spectra (EIMS) were measured on a Hewlett-Packard 5985 B mass spectrometer. The known compounds morusin (16), morusinol (17, 18), and kuwanon C (18) were identified by comparison of their mass spectral (MS) and proton NMR data, including correlation spectroscopy (COSY) experiment results, with literature values. For morusin and morusinol, ¹³C NMR data were also compared.

Microbial Substrate Experiment. The ability of a PCB-degrading bacterium to use three mulberry root flavones (morusin, morusinol, and kuwanon C) as sole carbon sources was tested by comparing growth with each flavone versus biphenyl. Petri plates were prepared with solid basal mineral medium (19) including 15 g/L of Noble agar (Difco, Inc., Detroit, MI). Substrates were applied directly to the agar surface in a sterile methanol solution to a final concentration of 100 μg/mL of agar medium. Methanol was evaporated from the plates for 2 h at room temperature in a laminar flow hood. The plates were streaked with the PCB-degrading bacterium *Burkholderia* sp. LB400, and colony growth rates were compared visually among the different substrates. Inoculated control plates lacking substrate but subjected to methanol treatment never exhibited growth of colonies. The bacterium *Burkholderia* sp. LB400 was selected for this experiment because it has been extensively studied for its PCB degradation properties (20) and has also been demonstrated to grow on other plant aromatic compounds (3). PCB-degrading bacteria isolated from the rhizosphere of mulberry were not available for this study, but this is a topic of ongoing research efforts.

Results and Discussion

Root Turnover. In all plants studied, net production of fine roots (<1 mm diameter) occurred in the spring and summer followed by a period of fine root mortality in the autumn (Figure 2). The percent mortality of fine roots ranged from 43 to 93% in the individual plants, and averaged 58% (Table 1), which is comparable to the 40–86% annual fine root death previously reported in several forest studies (21, 22) for this highly variable phenomenon (23, 24). Visual observations confirmed that the loss of mulberry fine root density in the autumn was due to an extensive senescence of the fine root system. In November, numerous dead fine roots, which appeared withered and black, were observed at locations in the rhizotron where young (yellow) and aging (brown) roots had previously been observed. Many dead fine roots had already decomposed and disappeared by November, an event that was established by careful examination of computerized images prepared on sequential dates.

The average mortality rate of 58%, although sizable, should be considered conservative. The “maximum minus minimum” method for calculating root mortality (12, 13) is generally regarded as a conservative estimate of true root dynamics (22, 25, 26) because it assumes that periods of root production and mortality are asynchronous, which is not necessarily true. In our study, the presence of dead roots in

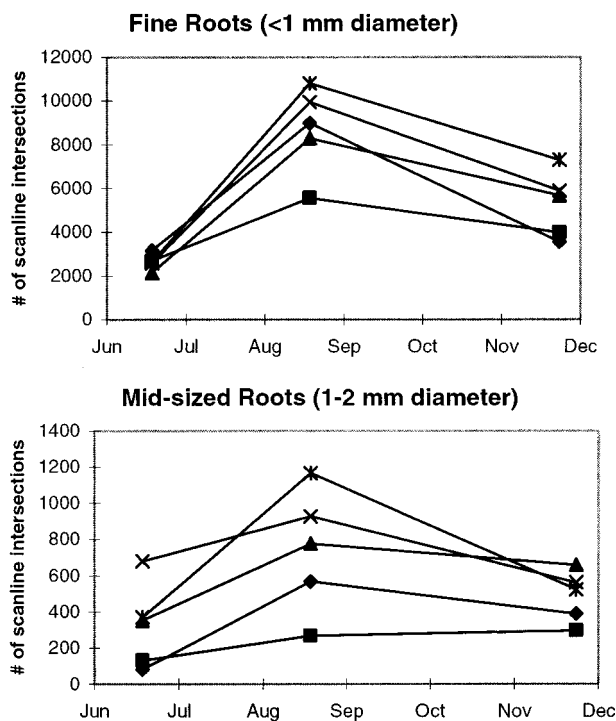


FIGURE 2. Seasonal changes in the root density of five mulberry saplings grown in rhizotron pots. Root density is expressed as the number of scanline intersections and is presented separately for fine roots (<1 mm diameter) and mid-sized roots (1–2 mm diameter).

TABLE 1. Mulberry Fine Root Production and Mortality during One Growing Season

plant	production ^a	mortality ^b	% mortality ^c
1	5826	5421	93
2	2877	1579	55
3	6156	2627	43
4	7366	4045	55
5	8175	3521	43
mean	6080	3439	58
% CV	33	42	36

^a Production represents root density increase between June and August (Number of scanline intersections). ^b Mortality represents loss in root density from August to November (Number of scanline intersections). ^c % mortality = 100 × (mortality/production).

August (the time of maximum root density) provides evidence that some root mortality occurred during the period of net production. Additionally, previous long-term studies on established forests have reported bimodal episodes of root mortality occurring in both the spring and the fall (9). Because our study began in the spring with bare-rooted saplings, it could not detect a spring mortality episode, which might further increase the annual root mortality estimate.

Mid-sized roots (1–2 mm), which were lignified and exhibited bright yellow to orange pigmentation in the outer root-bark regardless of root age, comprised a small portion of the overall root density (Figure 2). Although mid-sized root density also peaked in summer and declined in the autumn, the infrequent observation of dead mid-sized roots suggests that apparent losses in mid-sized root density were not due to root death. Rather, this trend may be explained by the observation that some mid-sized roots underwent further secondary growth over the summer and thereby progressed to the next higher root size class of > 2 mm. The growth dynamics of larger diameter roots (> 2 mm) were not measured in this study because they are not considered to have a high turnover rate (9).

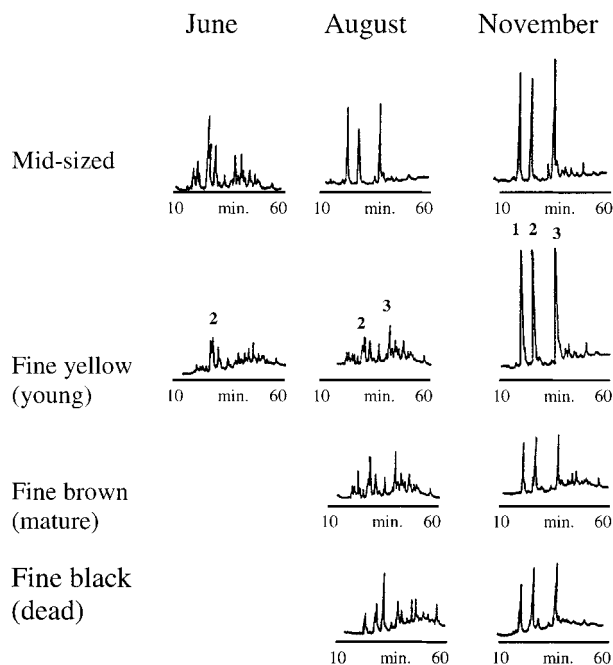


FIGURE 3. HPLC separations of mulberry root extracts at three times during the growing season.

TABLE 2. Total Phenolic Concentration in Different Root Types over One Growing Season^a

root diameter	root type	total phenolic concn (mg of morin equiv/g dry wt)		
		June 26	August 25	November 28
<1 mm	yellow (young)	22 (5)	25 (9)	47 ^b (10)
	brown (mature)	na ^c	37 (15)	40 (11)
	black (dead)	na	33 (11)	38 (7)
1–2 mm	yellow	27 (7)	31 (8)	30 (6)

^a Mean values are reported with standard deviations in parentheses.

^b Value significantly different from previous value ($p < 0.005$). ^c na, root type not present on this date.

Root Phenolics. The total phenolic concentration in fine yellow roots collected in June and August were 22 and 25 mg/g dry weight (DW), respectively, and then increased significantly ($p < 0.005$) by late November to 47 mg/g DW (Table 2). Older fine roots that had matured (brown) or died (black and shriveled) contained total phenolics in concentrations ranging from 33 to 40 mg/g DW, values that were not significantly different from those of fine yellow roots on either date. This indicates that the phenolic compounds remained in fine roots during their aging and death and thus were available to soil microorganisms as the dead roots decayed. Mid-sized roots (1–2 mm) averaged phenolic concentrations of approximately 30 mg/g DW and showed no seasonal change. Comparable total phenolic concentrations of 34–38 and 54–65 mg/g DW have been reported in the fine roots of *Vaccinium myrtillus* and *Picea abies*, respectively (27).

Qualitative comparisons of HPLC chromatograms showed that all root extracts contained a variety of compounds that absorbed ultraviolet light at 275 nm (Figure 3). In the young, mature, and dead fine roots, three peaks with retention times of 25, 30, and 45 min (peaks 1–3, respectively) significantly increased in size relative to the other components as the season progressed. Chromatograms prepared from mid-sized roots also showed a seasonal increase in the size of these three peaks; however, this occurred earlier in the season for mid-sized roots than for fine roots.

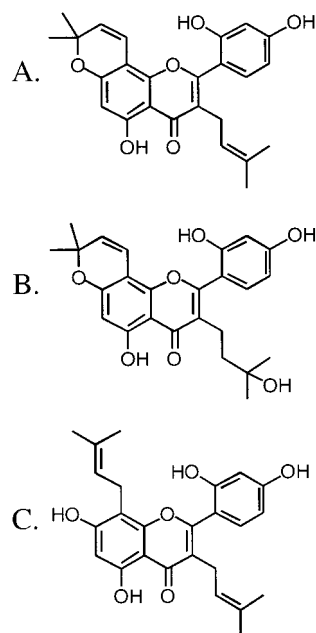


FIGURE 4. Chemical structures of (A) morusin, (B) morusinol, and (C) kuwanon C.

The three prominent peaks observed on HPLC chromatograms were found to consist of five major components, three of which were structurally determined as flavones with isoprenoid substituents (Figure 4). Peak 3 consisted entirely of morusin (Figure 4A) (16). Peak 2 consisted of three major components, two of which were identified as morusinol (Figure 4B) (17, 18) and kuwanon C (Figure 4C) (18). The third component of peak 2 as well as the major component of peak 1 could not be structurally determined conclusively using NMR due to apparent instability; however, both appeared to be roughly similar in structure to kuwanon C. Morusin, morusinol, and kuwanon C were first identified in the stem and root-barks of *M. alba* (16–18).

The component in peak 3 was identified as morusin on the basis of its low-resolution mass analysis, m/z 420 (M^+) and NMR data. The ¹H and ¹³C NMR data for peak 3 were initially acquired on methanol-*d*₄ (CD₃OD) and COSY. Hetero multiple quantum correlation (HMQC) and hetero multiple bond correlation (HMBC) experiments (28) were carried out in this solvent to assign the chemical shifts and establish the structure. When the structure that emerged from these experiments proved to be identical to that of morusin, the ¹H NMR of the sample was remeasured in dimethyl sulfoxide-*d*₆ (DMSO-*d*₆) whereupon it was observed that the proton chemical shifts matched within ± 0.05 ppm those reported for morusin (29). Only ¹H NMR and mass spectra were obtained for the components of peak 2. One of these components was identified as kuwanon C on the basis of the close correspondence of its chemical shifts in acetone-*d*₆ to literature values (± 0.09) in that solvent (29) and low-resolution mass analysis (m/z 422). Peak multiplicities and coupling as determined from a COSY experiment were also in full agreement with the structure. Likewise morusinol (m/z 438) was identified by a match (± 0.06) of its ¹H NMR data in DMSO-*d*₆ with literature data in that solvent (17). A COSY experiment confirmed the substitution pattern in the dihydroxyphenyl ring and the spin systems in the isoprene units. Although the total phenolic content of fine roots of a few species have been previously reported (27), we believe that this is the first identification of flavonoids in fine roots (<1.0 mm diameter) and evidence of their seasonal change and persistence in dead roots.

Microbial Growth on Root Flavones. When each of the three flavones isolated were provided as a sole carbon source to a PCB-degrading bacterium, *Burkholderia* sp. LB400, colonies grew at the same rate as when they were provided with biphenyl, whereas no growth occurred on substrate-free control plates. Thus, the naturally occurring mulberry flavones identified in this study appear equivalent to the synthetic, often-used biphenyl (1, 2) as a substrate for PCB-degrading bacteria.

Root Turnover in Rhizosphere Bioremediation. The mechanism of fine root turnover may be considered an injector system that facilitates rhizodegradation by delivering microbial substrates to the soil that promote the growth and activity of aromatic-degrading microorganisms. This investigation showed that 58% of mulberry fine roots die annually, and these dead roots contain flavones that support the growth of a PCB-degrading bacterium. The mechanism of root turnover releases phenolic compounds into the soil at a rate of 38 mg/gDW of dead root, a rate that equals or exceeds that released via exudation from living soybean roots (30). The phenolic content of plants and their fine root mortality rates vary among plant species and are also influenced by other factors including climate and environmental stresses (9, 31). Thus, the actual phenolic deposition through fine root turnover of mulberry may be expected to vary under different growth conditions.

In addition to providing substrates to degradative bacteria, root turnover also provides oxygen essential for the activity of dioxygenase and monooxygenase enzymes that catalyze the first step in aerobic degradation of aromatic contaminants (32, 33). Root turnover is considered a major contributor to soil aeration through formation of air channels created when roots die and decay (34, 35). Despite these observations, root turnover has received little attention in phytoremediation.

Results from this study support the position that slow, sustained rhizoremediation of soil contaminants including immobile, water-insoluble compounds (i.e., PCBs) is possible. It is not necessary for recalcitrant contaminants to move to the root because fine roots grow to the contaminants and, upon root death, serve as injectors of bacterial substrates and facilitators of soil aeration, both conducive to contaminant degradation. As the fine roots of a perennial plant continue to grow and die over time, they randomly probe the entire root zone and provide slow but sustained ecologically sound treatment of soil contaminants. Further study of the microflora associated with dead roots, which has not previously been a focus of agricultural or forestry rhizosphere research (36), holds great promise for the development of effective rhizoremediation technologies.

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