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Removal of Carbaryl, Linuron, and Permethrin by *Lupinus angustifolius* under Hydroponic Conditions

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The metabolism of organic pollutants by plants normally requires contaminant direct uptake by cells. Factors affecting this uptake and the later distribution of chemicals within the plant include the physicochemical properties of the compounds (concentration, structure, solubility, log k_{ow}, diffusion rate) and the biochemical characteristics of the plant. This paper reports the tolerance, uptake, and effects of the pesticides carbaryl, linuron, and permethrin on *Lupinus angustifolius* germination and growth as well as contaminant intraplant distribution and possible degradation. Lupine plants were grown in hydroponic culture containing either 1 or 5 mg of the individual pesticides, or combinations of these (1, 5, or 10 mg of each), in 100 mL nutrient and water solutions. Analysis of the remaining solutions 8 days post-germination showed the water solutions to have higher remaining pesticide concentrations than nutrient solutions. Furthermore, in the presence of pesticides, germination was more frequent in the water solutions. After 16 days of growth, the plants were harvested, and their tissues were microwaved digested and analyzed by reversed-phase liquid chromatography. Although only minor quantities of each pesticide were detected in plant tissues, their amount in the roots was higher than in the stems. No accumulation was noted in the cotyledons, and only 2% of linuron was detected in the leaves. Mass recovery at the end of the experiment showed that 57, 53, and 55% of carbaryl, linuron, and permethrin, respectively, were degraded and/or bound in an irreversible manner to plant material. The results suggest that *L. angustifolius* could be useful for the cleaning/remediation of pesticide-contaminated water.

KEYWORDS: ***Lupinus angustifolius*; pesticides; remediation**

INTRODUCTION

Over the past two decades, the increasing use of pesticides has led to concern about their presence in the environment and the threat they may pose to wildlife and humans. Although pesticides are useful in agriculture, exposure to them and their ingestion have been associated with the death of fish, reproductive failure in birds, and acute illnesses in humans. Additionally, adverse environmental consequences such as contaminated soils and water can be caused by misapplication, careless storage, or disposal of pesticides or their containers (*1*). Efficient methods to remediate contaminated areas are therefore needed. Plants offer the possibility of in situ remediation of contaminated sites. Phytoremediation uses plants and their associated rhizosphere microorganisms to remove, degrade, or contain contaminants in soils, sediments, groundwater, surface water, and even the atmosphere (*2*). Plants can be used to deal with most classes of contaminants, including petroleum hydrocarbons, chlorinated solvents, pesticides, metals, explosives, and excess nutrients (*3*).

Organic chemicals in soil/water−plant systems may undergo root adsorption, uptake, translocation, metabolic transformation, and/or volatilzation. When chemical contaminants in soil, water, or groundwater come into contact with roots, they may be adsorbed or bound to the root surface and cell walls (*2*, *3*). The adsorption process may be reversible or become irreversible when the contaminant undergoes a chemical or biological reaction at the root surface. Following uptake, organic chemical contaminants may be metabolically transformed. Plants have evolved compound-specific detoxification (metabolic) pathways. Generally, they enzymatically oxidize, conjugate, reduce, or hydrolyze organic contaminants (*4*). The products of these transformation reactions may be sequestered in vacuoles, diffused into the plant, or bound to insoluble cellular structures, such as lignin (*5*).

The biotransformation and sequestration of herbicides and pesticides have been extensively investigated in plants (*6*). Recently, Xia and Ma have studied the potential of water hyacinth (*Eichhornia crassipes*) to remove the organophosphorus pesticide ethion from water. They observed that the plant uptake and phytoremediation might be the dominant process for pesticide removal by the plant (*7*). The removal of

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organophosphorus pesticides {azinphos methyl assisted by alfalfa plants employing hydroponic cultures (*8*) and methyl parathion using common cattail *Typha latifolia* in water and artificial sediments (*9*)} has been also evaluated. Miglioranza et al. have studied the ability of the aquatic macrophyte *Schoenoplectus californicus* to bioconcentrate some organochlorine pesticides from a shallow lake (*10*). Additionally, plants can remediate organic pollutants by the release of exudates and enzymes that enhance the biochemical transformation and/or mineralization occasioned by mycorrhizal fungi and microbial activity in the rhizosphere (*3*). Suresh et al. have studied the ability of hairy root cultures of *Cichorium intybus* and *Brassica juncea* to uptake and degrade DDT, analyzing the role of the endogenous root enzymes in the breakdown of this pesticide (*11*).

One difficulty in using plants to remediate polluted sites is finding species that are adapted to the contaminated environment but which are also capable of removing the pollutant (*12*). Physiological factors of plant roots that control uptake of organic contaminants have been summarized by McFarlane (*13*). Ideally, the plant should be fast-growing and deep-rooted in order to reach the pollutant. *Lupinus* species can be grown and cultivated easily in sandy and acidic soils, and their root system are well-developed with numerous secondary roots (*14*). The potential use of these species in phytoremediation has been investigated by several authors. *Lupinus angustifolius* has been cultivated in nitroaromatic-contaminated soil under controlled conditions under glass (*15*), and Ximénez-Embún et al. have demonstrated the capability of *Lupinus* species to accumulate metals from wastewaters (*16*) and artificially contaminated sand (*17*). The ability of *L. angustifolius* seedlings to take up herbicides (simazine, atrazine, isoproturon, linuron) and insecticides (carbaryl, fenamiphos, permethrin) from aqueous solutions is also known, and its seeds have shown promise in the remediation of carbaryl-, linuron-, and permethrin-contaminated water (*18*).

The present paper aims to determine the removal of carbaryl, linuron, and permethrin by *L. angustifolius* seeds and plants under hydrophonic conditions, which allows both seeds and roots to be exposed to the chemicals in a highly homogeneous manner. The specific objectives of this research were focused on the following points: (i) determining the effect of carbaryl, linuron, and permethrin on *Lupinus angustifolius* germination and growing; (ii) quantifying pesticides uptake into seed and plant from spiked solutions; (iii) determining the intraplant distribution of the pesticides; and (iv) elucidating pesticides degradation.

MATERIALS AND METHODS

Apparatus. All analytical measurements were made with a HPLC system equipped with a CostaMetric 4100 series high-pressure pump (Thermo Separation Products, Waltham, MA) coupled to a SpectroMonitor 5000 photodiode-array detector (LDC Analytical, Thermo Separation Products). The analytical column (25 cm long, 4.6 mm i.d.) was packed with Hypersil 5 μm ODS (Symta, Madrid, Spain). Samples were injected manually through a sample injection valve (model 7725; Rheodine Inc.) fitted with a 200-μL loop.

An MPS 1000 CEM microwave oven (CEM Corp., Matthews, NC) equipped with a solvent detector was used to digest vegetation. This apparatus can simultaneously extract 12 solid samples in a Teflon-lined extraction vessel under identical conditions of temperature and pressure. Sample extracts were evaporated using a Univapo concentrator centrifuge coupled to a Unijet II refrigerated aspirator (Biogen Científica, SL., Madrid, Spain). A P Selecta (Barcelona, Spain) centrifuge was used to separate solids from pesticides solutions.

Reagents. Commercial samples of carbaryl—"Sevin 85" (85% w/w active ingredient carbaryl; Phytosanitary Products and Material Official Register Number (RN) 13807/02)—were supplied by Rhône-Poulenc Agro, S.A (Madrid, Spain). Linuron—"Linuron Zeltia" (50% w/w active ingredient linuron; RN 13807/02)—was supplied by Zeneca Agro, S.A (Madrid, Spain). Permethrin—"Permethrine" (25% w/v active ingredient permethrin; RN 13622/00)—was also supplied by Zeneca Agro S.A. Pesticide stock solutions of 1 mg mL^{-1} were prepared separately by dissolving 1.17 g of "Sevin 85", 2.0 g of "Linuron Zeltia", and 4 mL of "Permethrine" in 1000 mL of ultrapure Milli-Q water (Millipore Ibérica, Madrid, Spain). Solutions were stored at 5 °C in the dark. Working solutions were prepared in ultrapure Milli-Q water by appropriate dilution.

Hewitt's nutrient solution was made to contain the following: KNO_3, 19.4 mg mL^{-1}; $Ca(NO_3)_2 \cdot 4H_2O$, 69.4 mg mL^{-1}; $MgSO_4 \cdot 7H_2O$, 24.6 mg mL^{-1}; $Na_2H_2PO_4 \cdot H_2O$, 18.4 mg mL^{-1}; $MnSO_4 \cdot H_2O$, 2.49 mg mL^{-1}; $CuSO_4 \cdot 5H_2O$, 0.24 mg mL^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.296 mg mL^{-1}; H_3BO_3, 1.86 mg mL^{-1}; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.035 mg mL^{-1}; $CoSO_4 \cdot 7H_2O$, 0.028 mg mL^{-1}; NaCl, 5.85 g L^{-1}; EDTA-Fe, 0.026 mg mL^{-1}; KOH, 1 N; and $FeSO_4 \cdot 7H_2O$, 0.024 mg mL^{-1}. All reagents were supplied by Sigma Aldrich (Madrid, Spain). HPLC grade solvents, acetonitrile, methanol, *n*-hexane, and acetone were purchased from Scharlau (Barcelona, Spain).

Plant Material. *L. angustifolius* seeds were supplied by the Seed Centre of Badajoz (Spain), which were chosen since they are a bitter variety and not used as an animal feed. They are therefore appropriate for phytoremediation processes.

Procedures. *Hydroponic Culture.* Three *L. angustifolius* seeds were placed in 18 different series of three glass jars containing either 100 mL of nutrient or water solution (9 series each), and either 1 or 5 mg of each pesticide (carbaryl, linuron, and permethrin), or a combinations of these pesticides (at 10, 50, or 100 mg L^{-1} of each). Three grains of seeds (400−480 mg) were placed on a plastic mesh suspended at the surface of the solutions (to allow gaseous exchange). These seeds were germinated in the dark for 8 days (a black plastic cover was placed around each jar). Prior to germination, the seeds were soaked for 30 min in a solution containing sodium hypochlorite and rinsed copiously with distilled water in order to avoid fungal proliferation. Evapotranspiration losses from the jars were replaced with distilled water to maintain the nominal contaminant concentrations. After germination, the seedlings were moved outdoors to expose them to environmental conditions for 8 days. After this time, they were harvested and prepared for the analysis of pesticide uptake.

Two extra series of three jars without pesticides were also prepared with both nutrient and water solutions as controls. In addition, two further series of three jars for each contaminant or combination, and at all the described concentrations—but without the seeds—were prepared as reference solutions for determining the remaining pesticide concentrations at 8 and 16 days.

Determination of Pesticides in Test Solutions. Pesticides remaining in both the nutrient and water solutions at 8 and 16 days were determined using 1 mL samples from each jar. After centrifuging at 2500*g* and filtering through Millipore 0.45-μm nylon filters, these were analyzed by liquid chromatography (LC) and quantified by UV absorption (220−250 nm). The concentration of each pesticide in the blank test solutions was quantified in parallel to check for any pesticide degradation in the solutions.

Plant Tissue Extraction. After a growth period of 8 days (i.e., at day 16), the plants were removed from hydroponic culture, and their roots were washed with distilled water. The length of the main root and the stem were measured. Plants were then dissected into their individual leaves, cotyledons, stems, and roots. The fresh weight of each part was recorded. These tissues were then frozen and stored at −20 °C until their analysis. The tissues were homogenized in an agate mortar and transferred to PTFE-lined extraction vessels. A total of 10 mL of hexane:acetone (1:1, v/v) mixture was then added, and microwave extraction at 50% power was performed for 8 min at 90 °C (*19*). The extraction vessels were then cooled to room temperature before being opened. The supernatant from each vessel was filtered through a vacuum filtration system. The residue was rinsed with 2 mL of extraction solvent (hexane/acetone) and combined with the supernatant. Finally, the extracts were evaporated to dryness using a

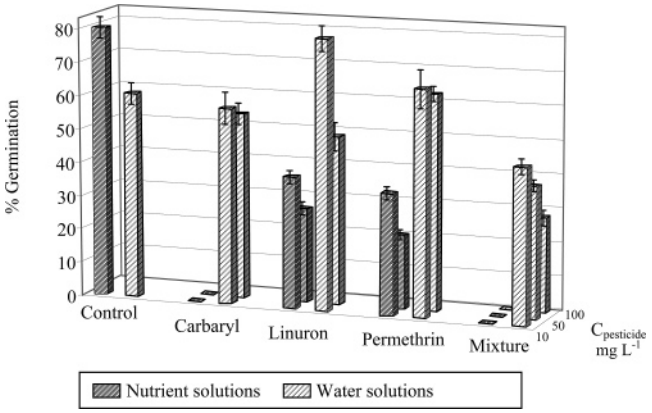

Figure 1. Germination of *L. angustifolius* seeds exposed to different concentrations of carbaryl, linuron, and permethrin, both individually and as mixtures, as compared to control seed germination.

concentrator centrifuge, and the residues were subsequently re-dissolved in 1 mL of the chromatographic mobile phase, water:acetonitrile 1:1 (v/v).

Chromatographic Conditions. Pesticide separation was performed using gradient elution at a flow rate of 1 mL min^{-1}. The chromatographic program was as follows: initially, a 5 min isocratic program with water:acetonitrile (60:40, v/v), followed by a 4 min linear gradient to water:acetonitrile (25:75, v/v). An additional 5 min of the gradient program reached the initial conditions for subsequent analysis. Absorbance was measured continuously in the range 200−356 nm by diode-array detection. Quantitative measurements of peak areas were made by LC−UV detection at 220 nm (carbaryl) and 250 nm (linuron and permethrin) in order to achieve maximum sensitivity. Analyte identification was accomplished on the basis of the retention times of the analytes and by comparison between the UV spectrum of the reference compound in the library and the UV spectrum of the detected peak in the sample.

Statistical Analyses. One way of analysis of variance (ANOVA) was used to test for differences between means when there was a possible variety due to a fixed-effect factor. One way ANOVA was performed to test statistically significant differences relative to controls at 5% level considering hydroponic medium as unique variation factor. Experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Seed Germination. The germination of *L. angustifolius* seeds was evaluated in the presence of 10 or 50 mg L^{-1} of carbaryl, linuron, or permethrin individually and in combination of these (10, 50 or 100 mg L^{-1} of each). Thus, nominal exposure to pesticide concentrations of 2, 10, and 20 times greater than the recommended agricultural application were selected. This range was chosen to achieve a balance between real environmental exposures and experimentally detectable quantities.

Figure 1 shows the percentage germination of *L. angustifolius* at the different concentrations of pesticides (in both solutions). As expected, the absence of pesticides improved the germination process in the control plants. Also, germination was more frequent in the nutrient than in the water solutions. The opposite occurred in the presence of contaminants: germination was less frequent in the nutrient than in the water solutions. Seed germination decreased with increasing concentration of the pesticides. In general, seed and plant litter and root exudates, as a defense mechanism, provide substances such as nitrate and phosphate that would reduce or eliminate the need for fertilizer additives (*1*). In our particular case, an excess of them in the nutrient solutions could explain the inhibition of the germination process. The presence of linuron and permethrin in nutrient solutions inhibited approximately 45 and 50% of the germination process, respectively, in comparison to control seeds. Carbaryl in nutrient solutions completely prevented germination, both alone and in combination with the other pesticides. Although carbaryl is an insecticide, it is sometimes used as a herbicide because of its inhibitory effect on cell division. Murthy and Raghu (*20*) reported an undesirable thinning of fruit after carbaryl application to apple trees.

The germination index (GI), defined as the measure of germination time in relation to germinative capacity, was calculated according to González-Zertuche and Orozco-Segovia (*21*), using the following equation (**Table 1**):

$$\mathrm{GI} = \sum(n_i t_i)/N$$

where GI = germination index; n_i = number of seeds germinated in the day i; t_i = number of days after planting; and N = total number of seeds planted.

In the experiments containing contaminated water solutions, germination was not significantly affected by exposure to any of the three contaminants on their own (compared to controls; $p > 0.05$). However, as a general behavior, the germination process was negatively affected by mixtures of the pesticides at the three concentration levels in both nutrient and water solutions (ANOVA, $p \leq 0.05$). This was more noticeable at higher pesticide concentrations and when germination took place in the presence of nutrients. This might be attributed to the carbaryl in the mixture.

Analysis of Remaining Pesticide at 8 Days. After germination, the amount of each pesticide remaining in the solutions was analyzed by RPLC. **Figure 2** shows the uptake/degraded quantity of each pesticide, calculated by the difference between the initial amount of pesticides spiked and the amount remaining in test solutions referred to initial weight of seeds. In nutrient solutions, the highest uptake was seen with permethrin, the lowest was seen with linuron (both individually and in mixtures), but no great competition between the pesticides was seen. Accumulation was greater when the pesticides were in combination, and in general, greater in nutrient than in water solutions. This was clearer in the case of permethrin, the uptake of which

Table 1. Germination Index of *Lupinus* Seeds under the Studied Conditions[a]

	Pesticide							
	Carbaryl		Linuron		Permethrin		Mixture	
Concn (mgL^{-1})	Nutrient Solutions	Water Solutions	Nutrient Solutions	Water Solutions	Nutrient Solutions	Water Solutions	Nutrient Solutions	Water Solutions
0 (control)	6.4	4.9	6.4	4.9	6.4	4.9	6.4	4.9
10	*	4.6	3.1	6.4	2.9	5.4	*	3.7
50	*	4.4	2.2	4.0	1.8	5.1	*	3.1
100	–	–	–	–	–	–	*	2.2

[a] Key: *, no germination. –, not tested.

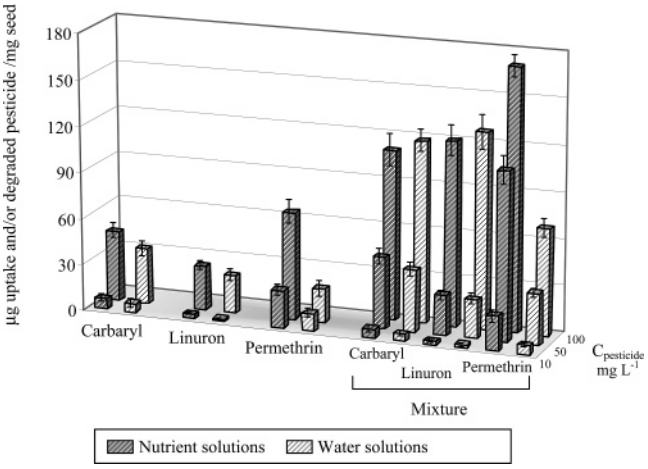

Figure 2. Total amount of uptaken and/or degraded pesticide by seeds.

was 70 μg g^{-1} seedling in nutrient solutions when tested individually and up to 170 μg g^{-1} in combination. Therefore, lupine seeds accumulated up to 400 μg of total pesticides when they were in combination. As has been commented on above, seeds release exudates and enzymes rather than enhance biochemical transformation of contaminants. These enzymes promote hydrolysis and oxidation processes, which make the pesticides more amenable to further degradation by increasing its water solubility, thereby increasing its bioavailability (*22*). In this case, the presence of nutrients seems to enhance the above-mentioned process, increasing the uptake/degradation of analytes. The amount of pesticide in distilled water in the absence of seeds was not reduced, and it can be deduced from **Table 2** that non-abiotic reactions could occur in this period. These facts indicate that the decrease in pesticide concentrations observed in the jars was due to uptake/degradation by the seedlings during germination. Adsorption can take place by non-metabolic processes when seeds are imbibing water or through diffusion. The factors that influence uptake by seeds are related to the physicochemical properties of the pesticide (concentration, structure, solubility, log k_{ow}, diffusion rate), the medium (temperature and pH), and the seed (size, characteristics, and permeability of the seed coat) (*2*). Polarity is the most important property of a compound with respect to its movement into and then within a plant. This property therefore determines the ease of movement across seed membranes (*23*). The fact that permethrin has a high log k_{ow} (**Table 2**) could explain why this pesticide was taken up most, due to its major ease of movement through seed membranes with respect to the other pesticides.

Pesticide Tolerance. To determine the effect of the pesticides on plant growth, root and shoot length and leaf appearance were monitored.

L. angustifolius was highly tolerant to permethrin at 10 and 50 mg L^{-1} in water solutions. Root elongation and shoot and leaf appearance in contaminated plants did not change significantly with respect to controls. In the presence of nutrients and permethrin, approximately 40 and 20% of the seeds germinated (when 10 and 50 mgL^{-1} of pesticide was added, respectively) and showed a poorly developed main root and no secondary roots.

The plants exposed to carbaryl without nutrients at 10 mg L^{-1} showed approximately a 50% reduction in the length of their main root and developed a strong secondary root system. At 50 mg L^{-1}, the root system was considerably reduced as compared to controls (approximately 85%), which led to lower water uptake and poor plant development. In the presence of nutrients, the seeds did not germinate; they only imbibed water from the solution and showed seed thickening.

Linuron at 10 mg L^{-1}, both with and without nutrients, caused a reduction of the main root length (approximately 80%) and an important development of the secondary root system. At 50 mg L^{-1}, plants showed serious symptoms of chlorosis (yellowing of leaf tissues due to a lack of chlorophyll), scorching, and significantly restricted growth. Linuron is a herbicide whose action is based on the inhibition of photosynthesis.

When the plants grew in the mixture of pesticides, the effect was similar but much more pronounced. In the absence of nutrients and at 100 mg L^{-1} concentrations, only 28% of the seeds germinated. No seedling development was observed at any of the concentrations tested when they were exposed to the mixture of pesticides in the nutrient solutions.

Uptake and Distribution of Pesticides in Plant Tissues. The above experiments showed that *L. angustifolius* was able to remove the pesticides from hydroponic culture during germination. To study the uptake/degradation of contaminants by *L. angustifolius*, the pesticides remaining in the solutions were analyzed and quantified when plants were harvested at 16 days. The pesticides were extracted from plant tissues and analyzed to determine their distribution within the plant. The yield of the extraction method was confirmed using spiked control tissues (with a mixture of standard pesticides), being the recoveries within the range 90−105%. Analysis of the dissected plant organs showed minimal quantities of pesticide in the plant tissues analyzed (**Figure 3**). Contaminant accumulation by *L. angustifolius* was detected primarily in the roots and to a lesser extent in the shoots. Only 2% of linuron was detected in the leaves, where a huge number of biochemical processes occurs and probably where the analytes degradation is the greatest. Finally, no pesticides were detected in the cotyledons. This is important in phytoremediation since it avoids the plants becoming a new contamination source after the cotyledons' function is over. **Figure 3** demonstrates that 57, 53, and 55% of carbaryl, linuron, and permethrin, respectively, were degraded and/or bound in an irreversible manner with plant material.

Analysis of the reference solutions indicated no losses of pesticides due to abiotic reactions such as hydrolysis, photolysis, or volatilization. These results were in agreement with those of numerous studies on pesticide abiotic reactions (**Table 2**). Therefore, the decrease of carbaryl, linuron, and permethrin in *L. angustifolius* cultures plants should be mainly caused by the metabolism of the plant.

Table 2. Physicochemical Properties of the Pesticides[a]

Pesticide	log k_{ow}	Henry's law constant (Pa m^3 mol^{-1})	Hydrolysis half-life	Photolysis half-life
Carbaryl (insecticide)	2.8	2.77×10^{-4}	pH 7; 12–17 days pH 5; >1500 days	pH 5; 21 days (distilled water)
Linuron (herbicide)	3.0	2.00×10^{-4}	pH 5, 7, 9; 945 days	
Permethrin (insecticide)	6.1	1.62×10^{-2}	pH 6, 8; 200 days	pH 5; 80, days

[a] Data from Noble (*30*), Sue (*31*), British Crop Protection Council (*32*), and U.S. Environmental Protection Agency (*33*).

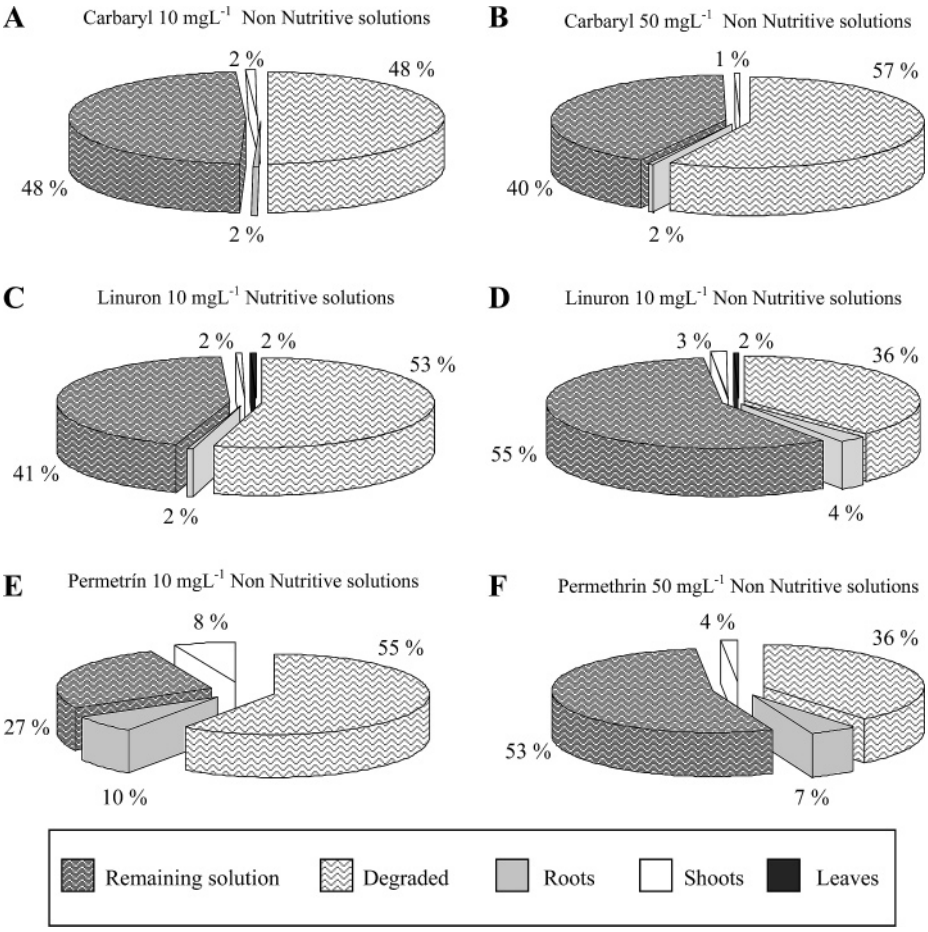

Figure 3. Distribution of analytes in the different parts of the plants.

Pesticide Degradation. Once accumulated, contaminants can be readily taken up by roots, where they are either stored unchanged, are bound to plant structural constituents, metabolized, or transported through the plant via the transpiration stream and volatilized through the leaves (*2*). Residues deposited in the lignin fraction of the plant seem to be unavailable even to animals that might feed on root tissue. Contaminants bound in such a manner are no longer available to most biological processes and ordinary chemical extraction protocols. As a consequence, they are often considered "degraded" (*24*). In addition, the compounds could be degraded in the solutions by exuded plant enzymes from the roots of the lupines. Many plant molecules released by roots and exudations resemble common contaminants chemically and can be used as cosubstrates.

Many authors claim that it is not clear whether the transformation of pesticides occurs at the cell surface or during transport into plant tissues (*3*). Although the transformation of pesticides has been demonstrated, the metabolic pathways and transformation mechanisms are not well-known. The main route of loss of carbaryl is probably through root exudation. The present results are in good agreement with those of other authors (*25*). Occasionally, degradation of carbaryl in crops occurs by hydrolysis inside the plants. Its metabolites are less toxic for humans than carbaryl itself (*26*). The main linuron degradation pathway is thought to be the hydroxylation of alkyl groups bound to nitrogen atoms (N-alkyl). This is a characteristic urea herbicide transformations reaction in plants. After this primary detoxification reaction, linuron is subject to oxidation and conjugation with endogenous compounds (peptides, sugars, amino acids, and organic acids) (*27*). The major degradation pathway for permethrin is again thought to be hydrolysis, followed by rapid conjugation with sugars (*28*). Enzymatic and nonenzymatic factors, such as light, heat, and humidity may also contribute to the degradation of all pesticides in plants (*29*).

In conclusion, obtained results showed that lupine seeds were found to accumulate up to 400 μg of pesticides/g of seedling 8 days after germination. At the end of the 16-day growth period, 57, 53, and 55% of carbaryl, linuron, and permethrin were degraded and/or were bound in an irreversible manner with plant material. Pesticides were therefore lost from both the nutrient and water solutions, but no significant quantities of contaminants were detected in the plant tissues. The high efficiency of pesticide recovery in sample extraction (90−105%) from the control plants and the results obtained from the reference solutions suggest that neither abiotic reactions (such as hydrolysis, photolysis, or volatilization) nor losses in sample treatment can explain the loss of pesticides observed with the test plants. Plant-mediated degradation and irreversible binding are therefore likely to be the main mechanisms involved. The ability of *L. angustifolius* to remove a high proportion of carbaryl, linuron, and permethrin from hydroponic culture suggests that this species may be of use to decrease the content of the tested pesticides in contaminated water.

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