BIOREMEDIATION OF TNT WASTES BY HIGHER PLANTS

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ABSTRACT

The uptake and biotransformation of TNT was studied in cell suspension cultures and in whole plants of Datura innoxia and Lycopersicon peruvianum. In cell culture, TNT was rapidly removed from the growth medium and recovered from the cell extract in the form of a variety of biotransformation products resulting from nitroreduction, deamination, N-acetylation and side chain oxidation to aldehyde and carboxylic acid metabolites. Whole plants of the same species grew well in soils contaminated with TNT up to 750 ppm; at 1000 ppm TNT the Datura plants showed some signs of phytotoxicity, while the Lycopersicon plants were severely affected. Both species removed TNT from soil and stored its metabolites at levels up to thirty times higher than the TNT soil concentrations. After a two week growth period, only 4 to 9.2% of the applied TNT was found in the soils.

KEY WORDS

bioremediation, 2,4,6-trinitrotoluene (TNT), soil, explosives, plants

INTRODUCTION

Soil contamination with 2,4,6-trinitrotoluene (TNT) and related explosives is a problem at many military installations and at some sites operated by the Department of Energy.

Explosives residues in soil can be mobilized by surface runoff and by leaching into ground water, resulting in contamination of streams and aquifers. TNT is classified as a possible human carcinogen, with a Drinking Water Equivalent Level (DWEL) of 20 μg/L and a lifetime health advisory of 2 μg/L [1]. In view of the high toxicity, cleanup of contaminated sites is mandatory to prevent surface water and aquifer contamination.

Incineration of TNT-contaminated soil is a radical cleanup method that produces a rather undesirable sterile soil residue. Bioremediation approaches have been studied by several research groups [2, 3] using adapted cultures of bacteria or fungi. Composting of TNT-contaminated soils is in the demonstration phase and reportedly works well to reduce TNT levels from several thousand ppm to 20-30 ppm.

Plants can absorb xenobiotics from soil and ground water, biotransform toxic contaminants to less toxic or non-toxic metabolites, and immobilize them by sequestration into vacuoles or incorporation into cell walls. Other benefits of plant growth in contaminated soils are upward movement of water by root uptake and evapotranspiration, counteracting of the downward movement of contaminants towards the water table, and increased microbial activity in the soil, as roots provide oxygen and organic carbon in form of exudates. Rhizobial and mycorrhizal communities are established in which the metabolic activities of bacteria, fungi, microfauna and plants form complementary systems capable of detoxifying and mineralizing xenobiotic contaminants.

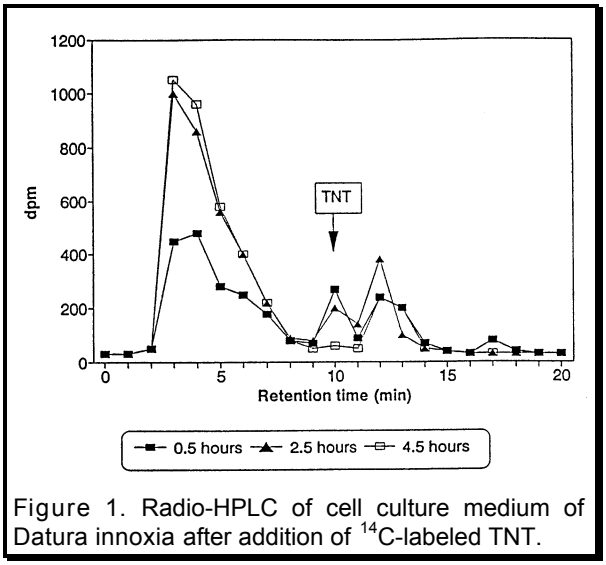

Figure 1. Radio-HPLC of cell culture medium of Datura innoxia after addition of ^{14}C-labeled TNT.

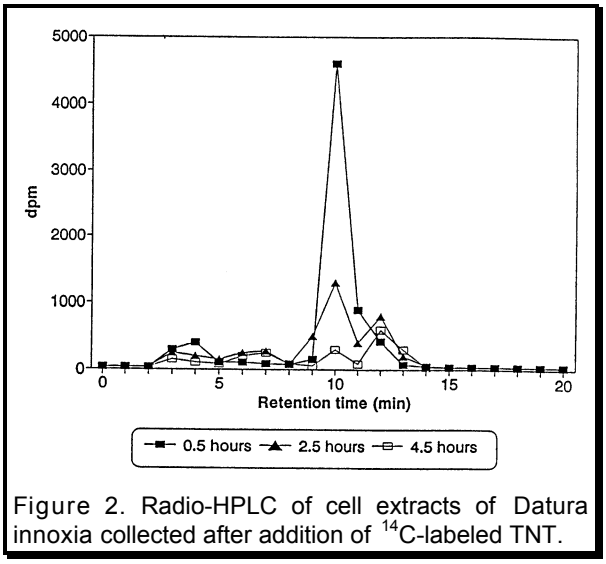

Figure 2. Radio-HPLC of cell extracts of Datura innoxia collected after addition of ^{14}C-labeled TNT.

UPTAKE AND BIOTRANSFORMATION OF TNT IN PLANT CELL CULTURES

The observation that growing cell cultures of Datura innoxia (Jimson weed) were able to decolorize "pink water" over night, removing TNT from >100 ppm to undetectable levels prompted us to investigate the mechanism of TNT removal in the cell cultures. In addition to Datura, a wild tomato species, Lycopersicon peruvianum, was included in the cell culture studies.

Procedures

Cells of Datura innoxia and Lycopersicon peruvianum were maintained as 150 ml batch suspension cultures in the dark at 30ºC. They were grown in a modified MS basal medium supplemented with vitamins, 2 mg/L 2,4-dichlorophenylacetic acid, and 250 µg/L kinetin. After ^{14}C-labeled TNT was added to the cell cultures, aliquots were removed at 30 minutes, 2.5, 4.5 and 24 hours and analyzed for TNT and metabolites. The cells were collected from the medium by centrifugation for 2 minutes at 2000 x g at 4ºC.

The cells were then washed twice in ice cold buffer containing 152 mg Tris-HCl, pH 7.4, 75 mg KCl, and 45 mg $MgCl_2$ in 100 ml distilled water. Pellets were resuspended in ½ the original volume of the same buffer containing 1.4 mg/L 2-mercaptoethanol, and homogenized in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged for 15 minutes at 12,000 x g. Levels of ^{14}C were measured by liquid scintillation counting in the original suspension, the 2000 X g supernatant (medium), the wash buffer, the extraction supernatant and the cell debris.

Aliquots of growth medium, wash buffer and cell extracts were further analyzed by high-performance liquid chromatography with a flow-through scintillation counter (radio-HPLC) in order to determine the ratio of TNT and metabolites. 65% methanol in 0.5% acetic acid was used as mobile phase on an RP-C18 column.

For isolation of the biotransformation products, volumes of 0.5 to 1 Ml of the cell extracts were chromatographed over a semi-preparative RP-C18 column (10 x 250 mm). The column effluent was collected in a fraction collector and, after liquid scintillation counting, fractions containing radioactivity were pooled in two groups which were further separated and purified by HPLC on the analytical column.

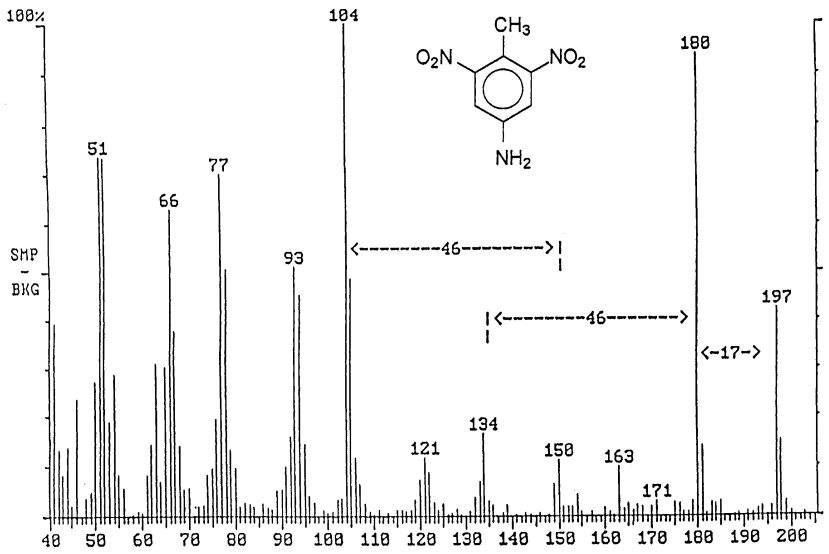

Figure 3. Mass spectrum of 4-amino-2,6-dinitrotoluene.

Isolated metabolite fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) either underivatized or after methylation with diazomethane. A Varian Saturn ion trap GC-MS system was used with a 30 m x 0.25 mm DB-5 column temperature-programmed from 40 to 280ºC.

Results

TNT and total radioactivity disappeared rapidly from the cell culture medium. Figure 1 shows the radio-HPLC chromatograms of medium samples taken at 0.5, 2.5 and 4.5 hours after addition of TNT-^{14}C to the culture. The TNT peak at retention time 10 has almost disappeared by 4.5 hours, and little other radiolabeled material was found in the medium.

The wash buffer, which was collected after washing the intact cells separated from the medium, contained very little radioactivity, indicating that little TNT remained adsorbed to the outside of the cells.

The radio-HPLC chromatograms of the cell extracts at 0.5, 2.5 and 4.5 hours are shown in Figure 2. Little TNT was found in the extracts even as early as 0.5 hours after TNT addition to the culture; the majority of the total radioactivity consisted of more polar metabolites eluting at shorter retention times than TNT. The chromatograms show the presence of a transient metabolite which is less polar than TNT (Rt 12 min.) and has its highest concentration at 2.5 hours.

GC-MS analysis of the isolated metabolite fractions yielded a number of mass spectra indicative of TNT biotransformation products. The primary products of nitroreduction, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene, had been described earlier as TNT metabolites in other organisms [4, 5]; the published mass spectra were matched well by the spectra of two components of the more non-polar group of metabolites shown in Figures 3 and 4. The spectra show the molecular ion at m/z 197, a loss of 17, which is characteristic for aromatic nitro-compounds, resulting in an intense peak at m/z 180 and consecutive losses of 46 or 47 as the two nitro-groups are lost as NO_2 or HNO_2, respectively.

After methylation, one of the more polar fractions yielded the mass spectrum shown in Figure 5, with a molecular weight of 211,

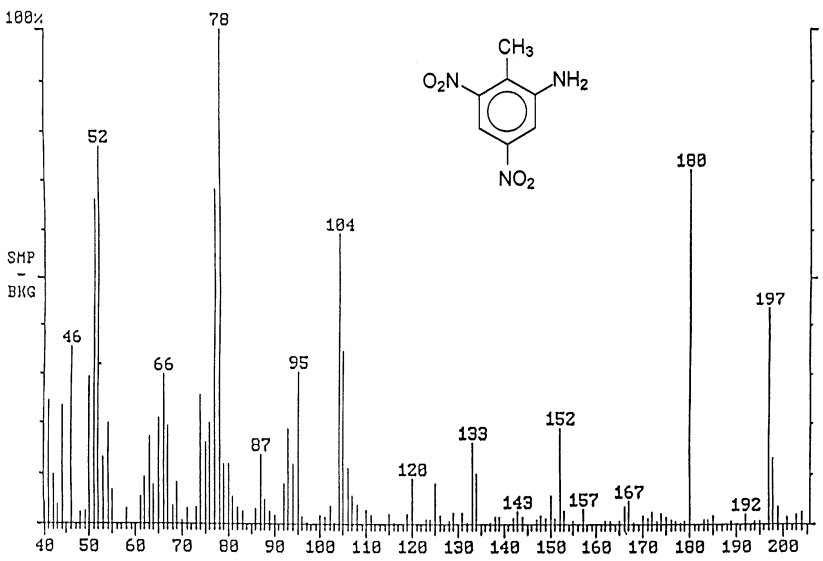

Figure 4. Mass spectrum of 2-amino-4,6-dinitrotoluene.

two consecutive losses of 17 producing the fragment ions at m/z 194 and 177, losses of 46 from either the molecular ion or the fragment ion at m/z 194, and a base peak at 118, which can be explained by a loss of 59 (-$COOCH_3$) from m/z 177. The proposed structure of this metabolite is a diamino-nitrobenzoic acid which was converted into the methyl ester by diazomethane treatment. Figure 6 shows the fragmentation scheme of this compound.

Another fraction gave a mass spectrum with a molecular ion at m/z 206, a base peak at m/z 149, and a strong tropylium ion at m/z 91 (Figure 7). The structure assigned to this metabolite is one of the two possible (2,4- or 2,6-) di-N-acetylaminotoluenes. The two major fragments arise from the consecutive losses of 57 (-$NCOCH_3$) and 58 (-$NHCOCH_3$).

The gas chromatogram of another metabolite fraction contained two well-resolved peaks with practically identical mass spectra, one of which is shown in Figure 7. The molecular ion is 163, and the two major fragments dominating the spectra are at m/z 105 and 77, the benzoyl and phenyl ions common to benzoyl compounds with the general formula C_6H_5CO-R. A search of the spectra against the 49K NIST mass spectral library showed a good match with N-acetylamino benzamide. This structure was excluded by the fact that it can not have any structural isomers, but the fraction contained two isomeric substances resolved by gas chromatography. The structures assigned to the two metabolites are 2-acetylamino benzaldehyde and 4-acetylamino benzaldehyde; their molecular weight is 163, and loss of 58 (-$NHCOCH_3$) yields the benzoyl ion at m/z 105, which can expel CO to form the phenyl ion at m/z 77.

Discussion

The initial uptake studies show that both Datura and Lycopersicon cell cultures absorb TNT readily from the nutrient medium and internalize and biotransform it rapidly. Within the 24 hour time period of these experiments, only small quantities of biotransformation products were released back into the medium, and TNT was removed completely. Biotransformation appears to be initiated by reduction of one nitro-group, resulting in the two isomeric amino- dinitrotoluenes found by other researchers in

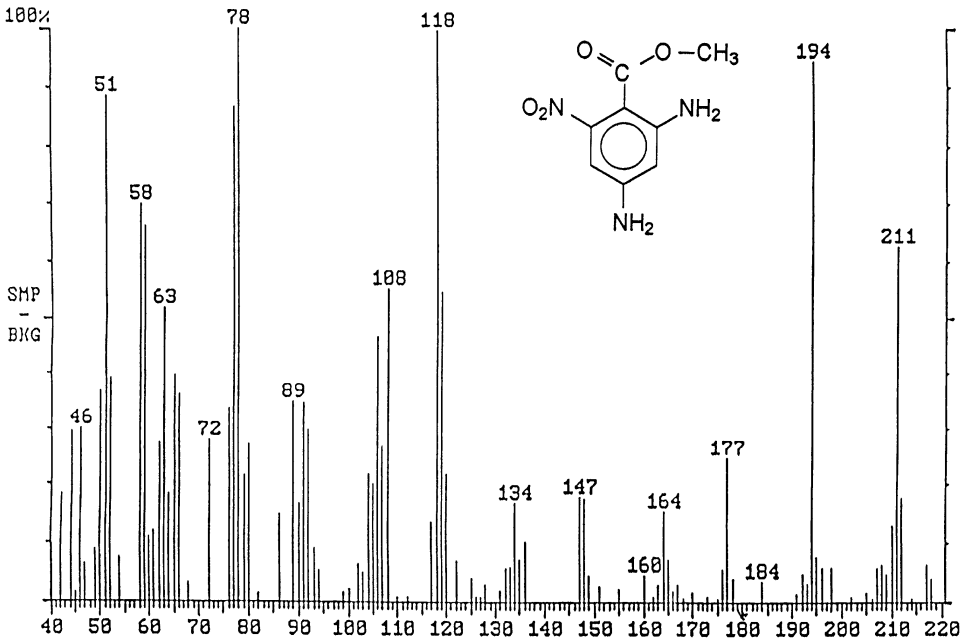

Figure 5. Mass spectrum of diamino-nitrobenzoic acid methyl ester.

bacteria, fungi and animals. This initial step is followed by further nitroreductions, cleavage of C-N bond, most likely by oxidative deamination, and acetylation of the amino groups remaining on the ring. The methyl group is subject to oxidation resulting in the benzaldehyde and benzoic acid derivatives that were identified. The combination of these biotransformation reactions produces a variety of polar metabolites that can be seen as broad unresolved peaks in the radio-HPLC chromatogram of the cell extract (Figure 2).

UPTAKE, BIOTRANSFORMATION AND DISTRIBUTION OF TNT FROM SOIL BY WHOLE PLANTS

After the studies with cell suspension cultures had shown that growing cells of Datura and Lycopersicon can rapidly absorb TNT from the nutrient solution and metabolize it to a variety of biotransformation products, we decided to investigate to which extent whole plants of the same species can take up TNT from contaminated soil and how the plants handle TNT after absorption. Two greenhouse studies were performed: in the first, TNT soil levels of 100, 150 and 250 ppm were used. As all plants grew very well and no signs of phytotoxicity were seen, a second set of experiments was added, in which concentrations of 500, 750 and 1000 ppm TNT in soil were used.

Procedures

Seeds of Datura innoxia were kept in running water for 15 days before planting into unpasteurized peat potting soil to improve germination. For Lycopersicon peruvianum, shoot cuttings of adult plants were dipped in Ferti-Lome rooting powder (0.1% indole-3-butyric acid) and planted into peat potting soil. The young plants were allowed to grow an average height of 15 cm before transplantation into the TNT-spiked soils.

To simulate southwestern desert soil, a mixture of sand and fine gravel was used for the TNT uptake studies. Batches of soil were treated with solutions of TNT in dichloromethane to produce TNT soil con-

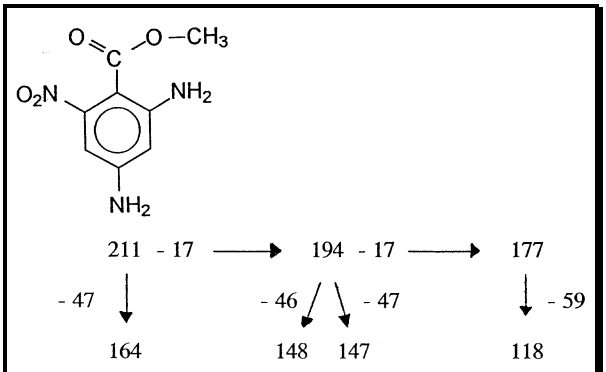

Figure 6. Fragmentation of diamino-nitrobenzoic acid methyl ester.

centrations of 100, 150, 200, 250, 500, 750 and 1000 ppm. After the spiking, the solvent was evaporated under a stream of nitrogen, then the batches were tumbled in the porcelain container of a ball mill for 30 minutes to ensure even distribution of the TNT. HPLC analysis of triplicate soil samples taken from the prepared batches showed a distribution of ±5% around the target levels. At each level, 1 kg of soil was spiked with 9 μCi of uniformly ring-labeled ^{14}C-TNT to allow the quantitative determination of uptake, distribution and biotransformation of TNT by the plants. Additional plants were grown in soils with the same TNT concentrations, but without radiolabeled TNT; their purpose was to increase the number of plants for phytotoxicity observation and to provide an additional source of TNT metabolites.

The plants were transplanted individually from the potting soil into containers with 100 g of TNT-spiked soil. They were kept for 14 days in the greenhouse at temperatures ranging from 25 to 35ºC and were watered daily to replace the evapotranspiration loss by approximate weight. After transplantation, the plants appeared healthy and continued to grow to an average of 20 cm.

On the 15th day the plants were removed from the soils and separated into roots, stems and leaves. Plant sections from replications were composited (e.g., the roots of all plants grown in soil with 100 ppm radiolabeled TNT were combined, etc.), and triplicate subsamples from each composite were analyzed for total radioactivity by combustion in a sample oxidizer (TRI-CARB 306) and liquid scintillation counting. The remaining plant tissue was homogenized in methanol to extract TNT and its metabolites. The soil in each growth container was sampled in triplicate and analyzed for total radioactivity by liquid scintillation counting and for residual TNT by HPLC with UV detection.

The methanol extracts were analyzed by HPLC with UV detection for residual TNT and by radio-HPLC for TNT and metabolites.

Results

Phytotoxicity

Compared with control plants grown in unspiked sandy soil, all plants grew well in soils with TNT levels up to 500 ppm. There was no reduction in growth or discoloration of leaves. At a TNT concentration of 750 ppm, Datura and Lycopersicon appeared to be slightly affected, showing some yellow spots on the leaves, but no decrease in flowering or significant loss of leaves. At 1000 ppm TNT in soil, the Lycopersicon plants showed moderate stress, with reduced flowering and drying and loss of some leaves and flowers. The Datura plants still grew quite well and looked healthy, with the exception of some yellow spots on the leaves.

TNT uptake and biotransformation

Tables 1 and 2 list the concentrations of TNT and/or metabolites in roots, stems and leaves of Datura innoxia and Lycopersicon peruvianum plants grown in soils containing 100 to 1000 ppm ^{14}C-labeled TNT. The ppm values were calculated from counts of total radioactivity, using the specific activity (mCi/mmol)of the TNT used for the experi-

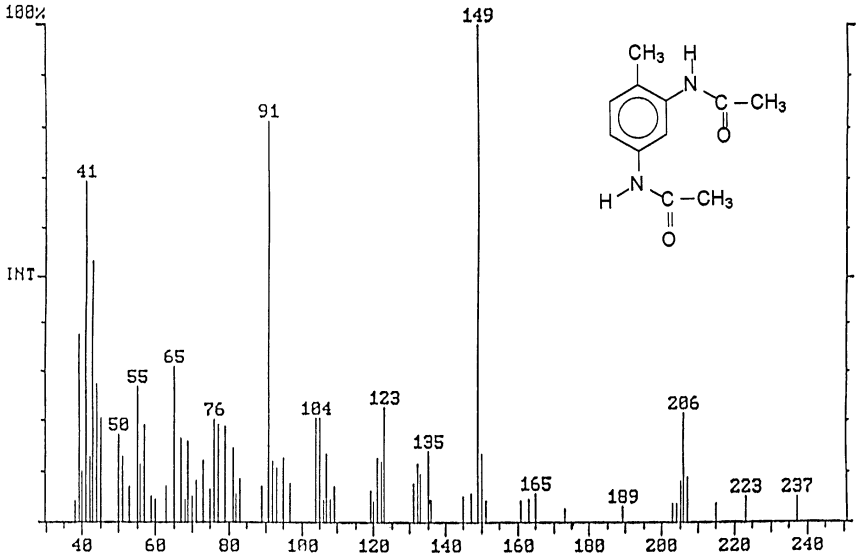

Figure 7. Mass spectrum of di-N-acetylaminotoluene.

ment and the molecular weight of TNT (227 g/mol).

At the lower concentrations of TNT in soil, more of the radiolabeled material was translocated from the roots into stems and leaves than at TNT soil levels of 500 ppm or more. Analysis of methanol extracts of the plant sections showed that no TNT was translocated into the above-ground parts of either species; all radioactivity was present in the form of more polar metabolites. Even in the roots, most of the radioactivity was present as metabolites, and only 0.3 to 1% of the residual radioactivity was found as TNT. Initial HPLC and GC-MS studies indicate that the same metabolites found in the cell culture studies are also present in the whole plant extracts.

At the end of the studies, only 4 to 9.2% of the TNT added to the soils was recovered, based on analysis of soil extracts by UV-HPLC and on direct liquid scintillation counting of soil samples.

Discussion

The studies with whole plants showed that both Datura and Lycopersicon plants can grow in soils contaminated with TNT up to at least 750 ppm. The plants absorb TNT through the roots and metabolize it readily into more polar products, which then are partially translocated into stems and leaves. The extent of translocation appears

TNT in soil (ppm)	Roots (ppm)	Stems (ppm)	Leaves (ppm)
100	2280	1785	3166
150	936	815	522
250	1324	1381	1392
500	6873	318	117
750	3883	229	116
1000	3326	166	72

Table 1. Concentration of TNT and metabolites in Datura innoxia grown in soils containing TNT.

TNT in soil (ppm)	Roots (ppm)	Stems (ppm)	Leaves (ppm)
100	1180	750	90
150	1054	2315	397
250	1130	3706	136
500	3234	226	24
750	2074	765	116
1000	4097	148	133

Table 2. Concentration of TNT and metabolites in Lycopersicon peruvianum grown in soils containing TNT.

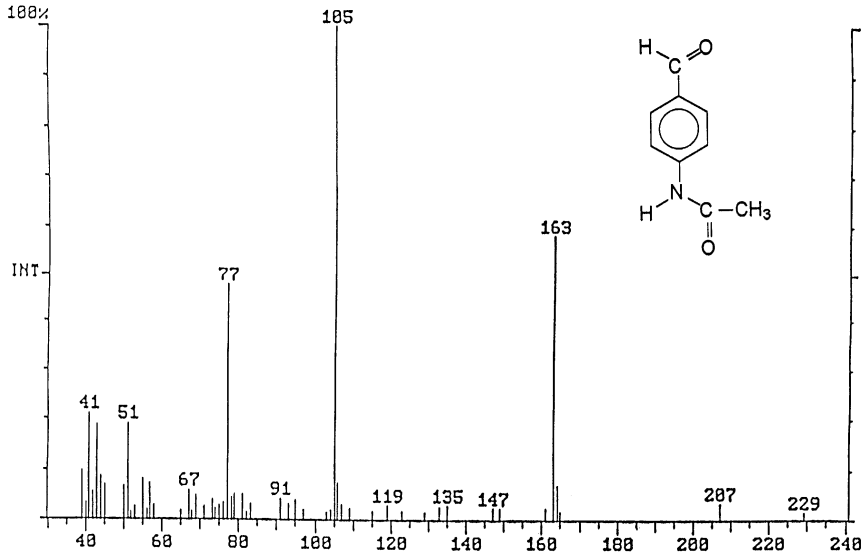

Figure 8. Mass spectrum of 4-N-acetylamino benzaldehyde.

to be dependent on the soil concentration of TNT; considering the short duration of the greenhouse studies, it is possible that the plants just did not have enough time to move the larger amount of metabolite material from the higher TNT levels from the roots into the upper plant parts. After the 14-day growth periods, the TNT levels in the soils were depleted to less than 10% of the starting concentrations; however, the total of the radiolabeled material recovered from soils and plant parts was only 10 to 25%. Since leaching as a source of label loss could be excluded by the fact that the planter cups had no drain holes, breakdown or volatilization are the only possible explanation for the loss of TNT from the soils.

CONCLUSIONS

The studies conducted so far with Datura and Lycopersicon peruvianum indicate that both plant species would be well suited for removal of TNT from contaminated soils at levels below 1000 ppm. The mass spectrometry data from the cell culture studies have shown that in the plant cells TNT undergoes nitroreduction, removal of nitrogen from the ring, probably by oxidative deamination, oxidation of the methyl group to the corresponding aldehydes and carboxylic acids, and N-acetylation of amino groups remaining on the ring. The results show clearly that in plants the biotransformation does not stop at the amino-dinitrotoluene level, but goes on to form a variety of products with greatly reduced toxicity.

The studies with whole plants in TNT-contaminated soils show that the two plant species can tolerate TNT soil levels in excess of 750 ppm, absorb the TNT through the roots, metabolize it and translocate the biotransformation products to the stems and leaves. Under the greenhouse conditions of the experiments, TNT levels in the experimental soils were reduced to less than 10% in two weeks.

Bioremediation of TNT-contaminated soils therefore appears to be a cleanup option at sites with low to intermediate levels of contamination as they exist at military sites such as shooting or bombing ranges, or at experimental blast sites. Plant bioremediation also may be an attractive approach to further reduce or completely remove residual TNT after composting of highly con-

taminated soils, which are typically cleaned up to target levels of 20-30 ppm.

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