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Effect and disposition of TNT in a terrestrial plant and validation of analytical methods

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Effect and disposition of TNT in a terrestrial plant and validation of analytical methods

Antonio J. Palazzo and Daniel C. Leggett

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Little is known about the response of terrestrial plants to 2,4,6-trinitrotoluene (TNT). The objectives of this study were to develop and test a method for measuring the amounts of TNT and its metabolites in plant tissue and to assess their effects in yellow nutsedge (Cyperus esculentus L.). The method developed was tested for its precision and accuracy for measuring TNT and its metabolites. The minimum detection limits of the method were 0.4, 0.6 and 0.9 mg/kg for TNT, 4-ADNT and 2-ADNT, respectively. Homogenization of plant tissue prior to analysis did not improve precision or recovery of naturally incorporated residues. Spike recoveries ranged from 46% to 101%. Two plant growth and uptake studies were conducted by growing nutsedge in hydroponic cultures containing TNT concentrations ranging from 0 to 20 mg/L. The greatest changes in physiological activity occurred between solution concentrations of 0.5 and 5.0 mg/L of TNT. Within this range, new plant growth became increasingly inhibited. Physiological effects from TNT may occur at levels below 0.5 mg/L. Root growth was affected most, followed by rhizomes and leaves. TNT and metabolites were found throughout the plant. Since TNT was the only compound present in the cultures, the metabolites must have been formed within the plant. Increasing

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the TNT concentration in culture solutions increased the concentrations of this compound and the two metabolites in the plants. Concentrations of all three compounds were greatest in the roots, while the rhizomes contained the greatest quantities of TNT and metabolites.

PREFACE

This report was prepared by Antonio J. Palazzo, Research Agronomist, and Daniel C. Leggett, Research Chemist, of the Geochemical Sciences Branch, Research Division, U.S. Army Cold Regions Research and Engineering Laboratory. The authors gratefully acknowledge the funding provided by the U.S. Army Medical Research and Development Command, Ft. Detrick, Maryland, and the contracting officer's technical representatives, Capt. Hank Gardner and Dr. Howard Bausum.

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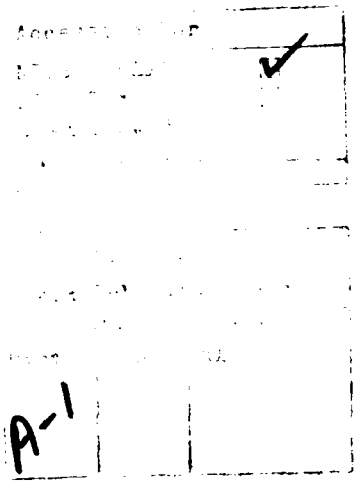

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Effect and Disposition of TNT in a Terrestrial Plant and Validation of Analytical Methods

ANTONIO J. PALAZZO AND DANIEL C. LEGGETT

INTRODUCTION

The explosive compound 2,4,6-trinitrotoluene (TNT) is used to manufacture munitions. It was first adopted by the Department of Defense in 1904 (Nay et al. 1974). After the manufacturing process, wash water from the cleaning and loading operations can contain 50–100 ppm of TNT. This water is usually directed to lagoons for primary treatment (Traxler 1974). In the lagoons the solid material is allowed to settle, and the effluent may later be discharged into streams and rivers (Klausmeier et al. 1973).

One area of concern in the waste handling process is that the TNT may be taken up by plants that grow near these storage lagoons and may eventually find its way into the environment. A literature review failed to locate information on the uptake of TNT by terrestrial plants (Palazzo and Leggett 1983).

Research on the effects of TNT on living organisms has been related primarily to aquatic systems and unicellular organisms. Schott and Worthley (1974) studied the toxicity of TNT to duckweed (*Lemna perpusilla*). They found the growth of this aquatic plant to be depressed at concentrations of 1 mg/L and above, and at 5 mg/L and above the plants died. The growth of freshwater algae is inhibited by TNT at concentrations of 2–15 mg/L (Liu et al. 1976, Smock et al. 1976, Won et al. 1976). Klausmeier et al. (1973) found that 50 mg/L of TNT severely inhibits the growth of gram-positive bacteria, actinomycetes, yeasts and fungi. Respiration of microorganisms in biological treatment systems is inhibited by TNT concentrations of less than 50 mg/L (Enzinger 1971, Nay et al. 1974).

A number of surveys of streams receiving ammunition wastes were conducted in the 1970s (Cairns and Dickson 1973, Fox et al. 1975a,b, Weitzel et al. 1975, Jerger et al. 1976, Stilwell et al. 1976, Sullivan et al. 1977a,b, Putnam et al. 1981). These studies generally showed that the discharge of ammunition waste changed or damaged downstream biological communities. However, it was not possible to conclude that TNT was responsible since other contaminants were also found.

TNT and related compounds are readily biotransformed or degraded; therefore, in many of these studies it is not clear to what extent toxicity was due to degradation products or metabolites of the compounds studied. For example, Smock et al. (1976) noted that TNT disappeared from solution during tests with algae, and Schott and Worthley (1974) found that 2,4-dinitrotoluene (DNT) was rapidly reduced to 4-amino-2-nitrotoluene in their culture medium during tests with duckweed.

To properly assess the fate of TNT in plant tissues, analytical methods were required for TNT and its metabolites. Our prior studies using grasses and legumes suggested that benzene was an adequate solvent for extracting TNT and its metabolites from plant tissue and soil (Palazzo and Leggett 1983). The extracts were analyzed by electron-capture gas chromatography, similar to a method reported by Hoffsommer et al. (1972). Neither of these reports gave an estimate of precision or accuracy.

Other researchers have used high-pressure liquid chromatography (HPLC) to measure TNT in plants. Lakings and Gan (1981) attempted to measure TNT, RDX and DNT in spiked leaves by reversed-phase HPLC. Interfering substances were coextracted when any one of several polar solvents were used. Thus they did not evaluate this method further for analysis of mixtures of RDX, TNT and DNT, although TNT may have been determinable in this matrix. A second method, using hexane-

isopropanol (98:2) as the extraction solvent, was developed and evaluated for measuring TNT and DNT in plant stems. This method recovered about 50% of the TNT and DNT from spiked tissue but was not tested on unspiked natural samples containing TNT or its metabolites. Preliminary work in our laboratory indicated that metabolites of TNT cannot be completely extracted unless the tissue is first hydrolyzed with acid. Therefore, recoveries of naturally incorporated TNT residues using this method may be considerably less than the 50% figure reported by Lakings and Gan (1981). The method of Lakings and Gan also required an evaporation step and solvent transfer prior to HPLC.

Electron-capture gas chromatography (ECGC) is inherently more sensitive for these compounds than HPLC with ultra-violet detection, so time-consuming extract preconcentration could be avoided. These considerations led us to decide to evaluate a benzene extraction and ECGC method incorporating an acid hydrolysis step for bound metabolites.

The purposes of this study were 1) to develop and test a method for measuring TNT and its metabolites in plant tissue and 2) to assess the toxicity, uptake, translocation and metabolism of TNT by yellow nutsedge (*Cyperus esculentus* L.) grown in solution cultures containing different concentrations of this compound. This species was selected because the Corps of Engineers is considering using it as an indicator species for evaluating contaminated materials (Folsom and Lee 1981).

Three separate studies were conducted. The first measured the precision and accuracy of the analytical method developed under this program. In FY83 we studied the growth of nutsedge and the uptake of TNT in plants grown in solutions containing 0, 5, 10 and 20 mg/L of TNT. During this study we discovered that the TNT concentrations were higher than that which causes initial toxicity to plants. To evaluate the physiological effects of TNT at low concentrations, we conducted a similar study in FY84 using TNT concentrations of 0, 0.5, 2.0 and 5.0 mg/L of TNT.

MATERIALS AND METHODS

In all three studies, nutlets or tubers of yellow nutsedge were germinated in a plant propagation mixture modified from that reported by Doty and Sweet (n.d.): 0.013 m^3 each of sphagnum peat moss and vermiculite, 340.2 g of $CaCO_3$, 1.5 g of $Na_2B_4O_7$, 3.7 g of sequestrine Fe, 28.8 g of NH_4NO_3, 78.15 g of NaH_2PO_4 (borax) and 18.77 g of KCl. Seedlings about 5-10 cm tall were selected for study on the basis of uniformity and size and were placed in hydroponic cultures.

The plants were grown in 35-L glass aquariums measuring 49 cm long, 24 cm wide and 29 cm deep (Fig. 1). The aquariums were painted black on the

Figure 1. Experimental setup of plant growth and uptake study.

Table 1. Nutrient solution composition. (After Epstein 1972.)

Compound	Element	Final concentration of element (ppm)
KNO_3	N	224
	K	235
$Ca(NO_3)_2 \cdot 4H_2O$	Ca	160
$NH_4H_2PO_4$	P	62
$MgSO_4 \cdot 7H_2O$	S	32
	Mg	24
KCl	KCl	1.77
H_3BO_3	B	0.27
$MnSO_4 \cdot H_2O$	Mn	0.11
$ZnSO_4 \cdot 7H_2O$	Zn	0.131
$CuSO_4 \cdot 5H_2O$	Cu	0.032
H_2MoO_4 (85% MoO_3)	Mo	0.05
Fe—sequestrene	Fe	1.12

outside to restrict light, and the tops were covered with a 1.25-cm-thick plywood board. Three equally spaced 1.25-cm-diameter holes were drilled through each board. A smaller hole was also added to each board for inserting the aeration tube.

The nutrient solution (Table 1) used in all aquariums was a modified version of that reported by Epstein (1972) and was used at half the recommended strength. The nutrient solution had an initial pH of 6.0 and was monitored twice weekly. Individual plants were placed in the holes in the plywood tops, one hole for each plant. The plants were supported with polyester fiber.

Stock solutions of TNT were prepared by adding 0.4 g of practical grade TNT (containing 10% water), obtained from Eastman Kodak, to 3.5 L of distilled water. The water was heated (< 80°C) and stirred for approximately 12 hours to dissolve the TNT. Each container of stock solution was sufficient to produce a final TNT concentration of 10 mg/L in a 35-L aquarium. The stock TNT solutions were added to the aquariums containing distilled water and nutrients to obtain the desired final TNT concentrations. Distilled water was then added to bring the volume to 35 L. The TNT concentrations selected for the initial study in FY83 were 0, 5, 10 and 20 mg/L and for the later study in FY84 were 0, 0.5, 2.0 and 5.0 mg/L. The four concentrations were randomly assigned to the aquariums, with four replications of each treatment. The aquarium solutions were analyzed twice weekly by the high-pressure liquid chromatography method developed by Jenkins et al. (1984) to assure that the desired TNT concentrations were being maintained and that metabolite concentrations were undetectable. Over time some TNT was lost and the pH lowered. Therefore, after three weeks the solutions were changed and fresh solutions added to restore the desired TNT concentration and pH.

The plants were periodically removed from the aquariums, and the roots and leaves were measured. Visual observations of the top and root color, vigor and other general features were also recorded.

After growing in these solutions for 42 days in FY83 and 58 days in FY84, the plants were harvested. At these times the control plants were at or near the stage of seedhead formation. The growing period for individual leaves of this species ranges from 24 to 40 days (Jansen 1971). Plants were separated into roots, leaves and crowns, rhizomes and tubers, and the parts were weighed. Separate portions of each sample were used for dry weight determination and plant analysis. Fresh tissue for analysis was chopped into 1-cm lengths and placed in a 20-mL scintillation vial filled with benzene. Another portion was dried at 70°C for 48 hours to determine weight loss.

Plant extracts were analyzed for TNT, 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). The latter compounds are metabolites of TNT that have been identified in other species (Palazzo and Leggett 1983). A double extraction procedure was used to separate the free forms from the bound forms of each compound. Free TNT and its metabolites were extracted three times by equilibrating the tissue overnight with benzene. After each benzene soaking, the extract was drawn off. The extracts were combined and diluted to volume for analysis. The resulting extracted residue was dried in a stream of air and weighed. To extract the bound forms, 5 N sulfuric acid was added to the dried residue and heated to 80°C for 90 minutes. This process completely digested any remaining plant residue. The acid remaining after this digestion process was extracted once with benzene. The free and bound extracts were dried with anhydrous sodium sulfate and the concentrations of compounds in each fraction determined by electron-capture gas chromatography (Leggett 1977). Total uptake was determined by multiplying the individual tissue concentrations by the dry weight for each plant part.

To assess the precision and accuracy of the analytical method, another group of plants was grown as before except that they were harvested after 21 days. Treatment levels were 0, 5 and 10 mg/L of TNT. Several aquariums were used at each level to

Table 2. Precision of TNT and metabolite analysis.

Treatment level (mg/L)	Plant part	Extraction type	No. of rep.	Concentration (mg/kg) TNT Mean	TNT Std. dev.*	TNT % Rel. std. dev.	4-ADNT Mean	4-ADNT Std. dev.	4-ADNT % Rel. std. dev.	2-ADNT Mean	2-ADNT Std. dev.	2-ADNT % Rel. std. dev.
10	Roots	Free	6	370	64.0	17	493	55.4	11	193	19.5	10
5	Roots	Free	7	195	17.1	9	390	92.4	24	105	11.7	11
0	Roots	Free	7	0.85	—	—	0.64	—	—	0.06	—	—
10	Leaves	Free	7	37.3	22.7	66	47.6	25.9	54	39.6	10.6	27
5	Leaves	Free	7	8.9	5.4	56	35.8	6.4	18	22.0	2.6	12
0	Leaves	Free	7	0.82	—	—	0.43	—	—	0.59	—	—
10	Roots	Bound	7	9.27	5.15	56	276	91.2	33	102	31.0	30
5	Roots	Bound	7	8.31	4.36	53	201	45.1	22	58.5	17.1	29
0	Roots	Bound	7	1.64	—	—	2.82	—	—	0.12	—	—
10	Leaves	Bound	7	2.36	0.94	40	96.8	35.9	37	76.0	17.2	22
5	Leaves	Bound	7	2.17	0.40	18	73.4	11.1	15	39.8	3.84	10
0	Leaves	Bound	7	0.11	—	—	0.32	—	—	0.15	—	—

* Controls not statistically anc';zed due to inhomogeneity of data.

Table 3. Precision of combined free and bound residue analysis.

Treatment level (mg/L)	Plant part	No. of rep.	Concentration (mg/kg) TNT Mean	TNT Std. dev.	TNT % Rel. std. dev.	4-ADNT Mean	4-ADNT Std. dev.	4-ADNT % Rel. std. dev.	2-ADNT Mean	2-ADNT Std. dev.	2-ADNT % Rel. std. dev.
10	Roots	6,7	376	78.1	21	781	88.1	11	300	38.7	13
5	Roots	7	2.03	14.4	7	590	27.4	22	163	22.2	14
0	Roots	7	1.77	1.73	98	3.5	3.2	91	0.178	0.184	103
10	Leaves	7	40	23.0	58	144	37.9	26	116	12.6	11
5	Leaves	7	11	5.54	50	109	15.5	14	61.8	3.99	6
0	Leaves	7	0.931	0.322	35	0.746	0.703	94	0.742	0.487	66

obtain sufficient tissue, and the tissues were pooled at harvest to obtain the sample. The tissues were prepared for analysis as before but in groups of seven replicates, and only the leaves and roots were analyzed. In addition, seven replicates of roots and leaves from the 10-mg/L treatment were first fresh-homogenized for 5–10 minutes in benzene using a Brinkman Polytron cell disruptor. Benzene was separated by centrifugation, and the tissue was extracted twice more by equilibrating overnight with benzene.

Control tissues were analyzed before and after spiking with three levels of TNT and the two metabolites in methanol to determine the accuracy of the method. The spike levels were chosen to simulate the low and high levels found in the treated tissues. Precision was estimated from the standard deviations calculated for each set of replicates of treated tissues. Data from the plant uptake study were subjected to an analysis of variance, and Duncan's Multiple Range Test was applied to the means (Little and Hills 1978). Since the variances of the mean plant TNT and metabolite data were not homogeneous according to Hartley's Test (Youden and Steiner 1975), all plant analysis data were transformed to square roots for the analysis of variance.

RESULTS AND DISCUSSION

Precision and accuracy

The results of the precision and accuracy tests are summarized in Tables 2–8. Analytical precision for individual analytes, expressed as percent relative standard deviation (% RSD), ranged from 9% to 66% for the 5- and 10-mg/L treatments (Table 2). In general the % RSDs remained relatively constant while the absolute values of the standard deviations increased with mean concentration (Fig. 2).

For the 5- and 10-mg/L treatments, the relative standard deviations of all three compounds combined averaged 28%—25% for the free forms and 30% for the bound forms (Table 2). When the data for free and bound residues were combined for each treatment and analyte, the precision improved with an average overall standard deviation of 22% of the means (Table 3). This is reasonable since free and bound residues are not independent of one another, and some conversion of free to bound forms during the sample preparation and extraction is possible. This would not be surprising since TNT metabolism is very dynamic. We have observed TNT being translocated and metabolized in this species within two hours after it was added to the media.* There could also be an interaction among TNT and its metabolites. This was tested, and the data are shown in Table 4 for total TNT and metabolites for each plant part and treatment level. This did not improve the precision of the metabolite analysis, however.

Table 5 shows the effect of homogenization on analytical precision and the recovery of naturally incorporated residues. The differences between treatment means using student's t-test (Hoel 1960) were in general not significant at a 95% confidence level. Only the difference between free 4-ADNT concentrations in homogenized and non-

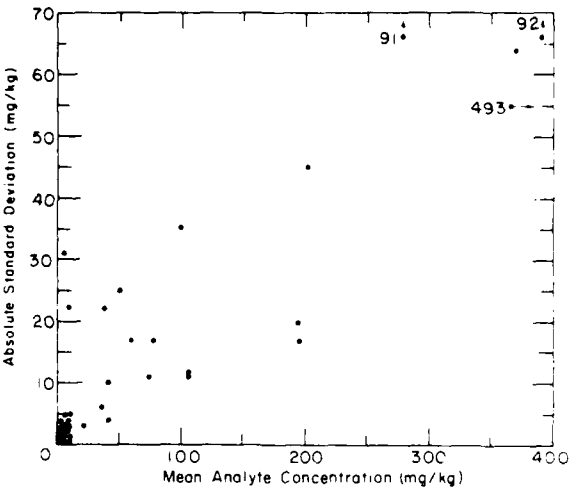

Figure 2. Relationship of precision (standard deviation) to mean analyte concentrations.

Table 4. Precision of total TNT and metabolite residue analysis.

Treatment level (mg/L)	Plant part	Homogenized	Concentration (mg/kg) Mean	Std. dev.	% Rel. std. dev.
10	Roots	No	1625*	182	11
10	Roots	Yes	1571	184	12
5	Roots	No	1031	202	20
10	Leaves	No	341	51.5	15
10	Leaves	Yes	297	91.9	31
5	Leaves	No	209	21.1	10

*As equivalent TNT $\left[\frac{\text{in moles(TNT + 4-ADNT + 2-ADNT)} \times 227}{\text{kg}}\right]$

* Unpublished results.

Table 5. Effect of homogenization on precision and recovery of naturally incorporated residues.

Plant part	Homog-enized	Extraction type	No. of rep.	Concentration (mg/kg) TNT Mean	TNT Std. dev.*	TNT % Rel. std. dev.	4-ADNT Mean	4-ADNT Std. dev.	4-ADNT % Rel. std. dev.	2-ADNT Mean	2-ADNT Std. dev.	2-ADNT % Rel. std. dev.
Roots	No	Free	6	370	64.0	17	493	55.4	11	193	19.5	10
Roots	Yes	Free	4	423	66.2	16	344	67.1	19	169	42.9	25
Leaves	No	Free	7	37.3	22.7	62	47.6	25.9	54	39.6	10.6	28
Leaves	Yes	Free	6	34.4	33.5	100	22.5	8.80	39	42.1	3.50	8
Roots	No	Bound	7	9.27	5.15	55	276	91.2	33	102	31.0	30
Roots	Yes	Bound	4	19.7	11.2	57	349	28.1	8	118	18.8	16
Leaves	No	Bound	7	2.36	0.94	40	96.8	35.9	37	76.0	17.2	22
Leaves	Yes	Bound	7	3.50	0.84	24	74.6	23.4	31	75.0	13.6	19

Table 6. Accuracy of TNT analysis at three spike levels.

Plant part	Extraction type	No. of rep.	TNT concentration (mg/kg) Added	Found Mean	Found Std. dev.	% recovery
Roots	Free	7	29.4	32.6	0.91	111
Roots	Free	7	21.0	21.1	0.58	100
Roots	Free	7	12.6	13.5	1.67	107
Leaves	Free	7	42.1	41.8	0.87	99
Leaves	Free	7	16.8	15.9	0.61	95
Leaves	Free	7	4.21	3.97	0.27	94
Roots	Bound	7	2.63	3.68	2.12	140
Roots	Bound	7	0.53	1.86	1.82	351
Leaves	Bound	7	5.26	4.90	0.75	93
Leaves	Bound	7	1.05	2.62	0.63	250

Table 7. Accuracy of 4-ADNT analysis at three spike levels.

Plant part	Extraction type	No. of rep.	4-ADNT concentration (mg/kg) Added	Found Mean	Found Std. dev.	% recovery
Roots	Free	7	82.3	92.0	1.55	112
Roots	Free	7	41.2	42.1	0.74	102
Roots	Free	7	25.7	25.2	1.56	98
Leaves	Free	7	82.3	83.1	1.58	101
Leaves	Free	7	41.2	40.6	1.70	99
Leaves	Free	7	15.4	15.5	0.67	101
Roots	Bound	7	51.5	42.5	7.74	83
Roots	Bound	7	25.7	19.1	0.40	74
Leaves	Bound	7	154	81.8	28.8	53
Leaves	Bound	7	51.5	23.4	13.3	45

Table 8. Accuracy of 2-ADNT analysis at three spike levels

Plant part	Extraction type	No. of rep.	2-ADNT concentration (mg/kg) Added	Found Mean	Found Std. dev.	% recovery
Roots	Free	7	15.1	19.7	2.84	130
Roots	Free	7	10.1	13.1	1.09	129
Roots	Free	7	5.04	5.94	0.71	117
Leaves	Free	7	45.4	44.5	1.33	97
Leaves	Free	7	25.2	27.9	2.02	108
Leaves	Free	7	10.1	10.7	0.95	100
Roots	Bound	7	15.1	15.2	2.87	100
Roots	Bound	7	7.56	7.56	1.30	98
Leaves	Bound	7	101	77.4	16.1	77
Leaves	Bound	7	50.4	34.9	10.2	69

homogenized roots was significant. Possibly more TNT was converted to 4-ADNT in the nonhomogenized roots during sample preparation. When residues are summed (Table 4), there is no difference between the results for homogenized and nonhomogenized tissues. Therefore, homogenization did not improve the accuracy or precision of the TNT and metabolite analyses.

The accuracy of spike recoveries is shown in Tables 6–8. The mean amounts of analytes recovered from spiked control tissue by extraction with benzene (recovering the free forms) ranged from 98% to 130%, with standard deviations generally less than 5% of the mean values.

When the control tissues were respiked after benzene extraction (simulating recovery of the bound forms) and then hydrolyzed with H_2SO_4, the accuracy of the recovery of the analytes was more difficult to analyze (Tables 6–8). High recoveries of TNT were probably due to the low spike levels (Table 6). Mean recoveries of 4-ADNT and 2-ADNT from roots receiving the intermediate and low-level spikes were 78% and 100%, respectively, while recoveries from the corresponding leaf samples were 50% and 73% (Tables 7 and 8). The recoveries from tissues spiked at a high level were very poor for both roots and leaves,* possibly because of a procedural error in which the extracts were not refrigerated for approximately two weeks prior to GC analysis. The long delay was apparently responsible since recoveries from roots were good for spiked tissues analyzed within a few days of extraction (Tables 6–8). We did not expect the benzene extracts of the hydrolyzates to be unstable, and we had not seen any instability in the benzene extracts of fresh tissue, some of which were held for three weeks with good recovery. This instability could also be partly responsible for the relatively low recoveries of analytes from leaves at the other two spike levels.

The detection limits of the chromatographic analysis were estimated from the precision of the measurements on the control tissues. This removed variability associated with differences between individual samples and looked at just the variability in the analysis for each set of duplicate samples, which should give the best estimate of instrument precision. The ranges associated with each pair of injections were expected to be normally distributed. A test for homogeneity (Youden and Steiner 1975) showed the ranges to be homogeneous at the 95% confidence level. The population standard deviation was then estimated from the mean range (Grant 1952). Only non-zero values were included in the test, and each analyte was evaluated separately. With the conservative criterion of three standard deviations, the detection limits were 0.37, 0.59 and 0.87 mg/kg for TNT, 4-ADNT and 2-ADNT, respectively.

FY83 plant growth and uptake study

The data in Table 9 show where significant differences were found in the measurements of plant growth and development for all TNT levels. Plant heights did not differ significantly until after 13 days of growth. Root lengths differed at the first measurement (after 15 days of growth), and the differences persisted throughout the study.

Table 10 shows the yield (on a dry weight basis) of individual plant parts and plant heights and root lengths after 42 days in the hydroponic cultures. Leaf and root growth was significantly low-

* Data not shown.

Table 9. Statistical analysis of differences in height and root length for all TNT levels.

Parameter	Days of growth	Significance*
Root length	15	0.01
	20	0.01
	34	0.01
	42	0.01
Plant height	1	NS
	6	NS
	13	0.01
	20	0.05
	33	0.01
	42	0.05

* Differences significant at the 0.05 and 0.01 levels of probability.
NS—not significantly different.

Table 10. Weights, heights and root lengths of plants grown in various TNT levels for 42 days in FY83.

	TNT level (mg/L)			
Parameter	0	5	10	20
Leaves (g)	3.94a*	1.93b	1.03b	1.41b
Roots (g)	1.18a	0.06b	0.03b	0.04b
Rhizomes (g)	2.10a	1.50ab	1.08b	0.93b
Tubers (g)	1.07a	0.32a	0.08a	0.19a
Root length (cm)	23a	6b	7b	6b
Plant height (cm)	28a	18b	17b	17b

* Means for each plant part across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978).

er than the control at all TNT concentrations. However, the yields of leaves, roots and rhizomes were not significantly different as TNT concentrations increased from 5 to 20 mg/L. Total plant yields were 54-74% lower than the control. Although yields were slightly lower at the higher concentrations, the TNT levels used in this study apparently are higher than those needed to inhibit growth. Figures 3-5 show leaves, roots and rhizomes grown in various TNT solutions.

Root growth (dry weight and length) was most affected by the TNT (Table 10). Roots grown in TNT weighed 95-97% less than those grown in the control solutions. Leaf growth was also seriously affected; leaf weights from the treated plants were 51-74% of the control. Rhizome growth was least affected; although there was a 29% reduction in rhizome weight between the 0- and 5-mg/L treatments, it was not statistically significant. Rhizome weights in the 10- and 20-mg/L treatments were

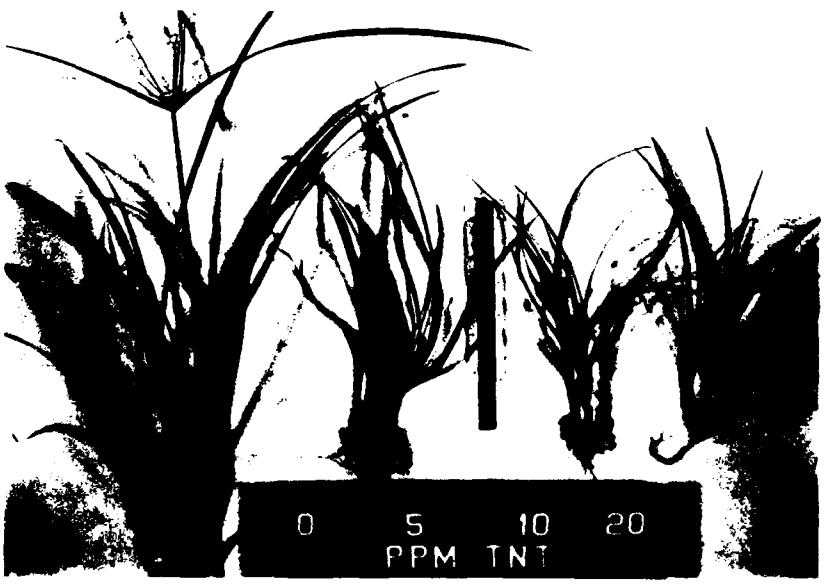

Figure 3. Leaf growth at various TNT concentrations.

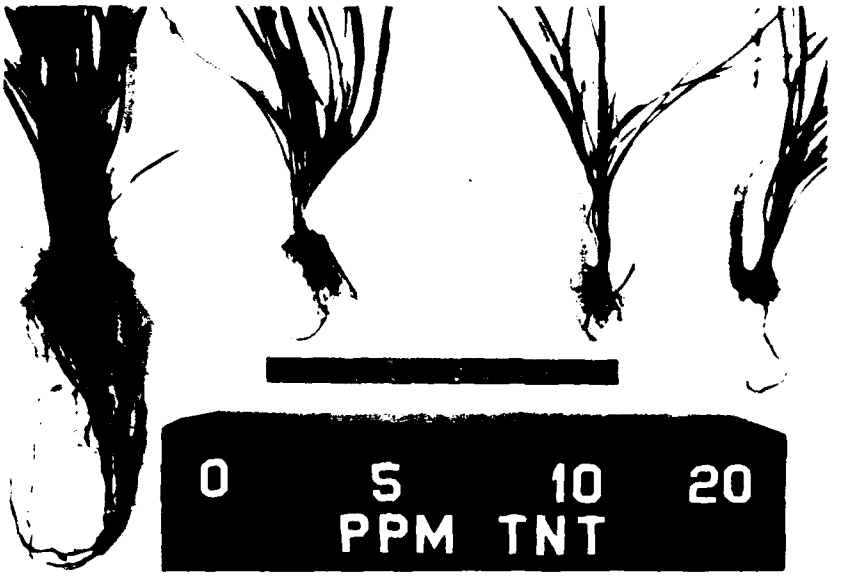

Figure 4. Root growth at various TNT concentrations.

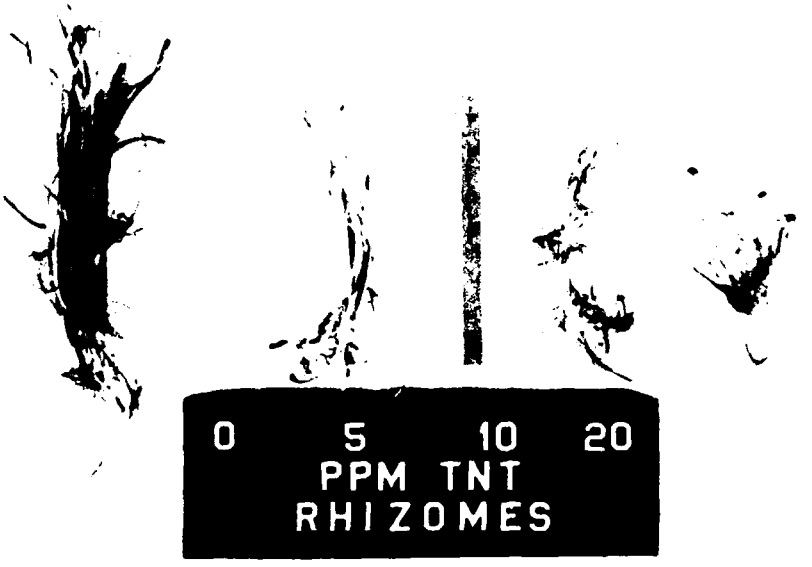

Figure 5. Rhizome growth at various TNT concentrations.

significantly lower than the control plants, amounting to a 50% reduction in weight. The dry weights of tubers did not differ significantly. We believe the similarity in tuber yields to be partially due to the tendency of these plants to produce tubers erratically under some environmental conditions. Visible symptoms of injury to roots consisted of discoloration and growth restriction. The leaves of treated plants were stunted, and new leaf growth appeared to be inhibited soon after initiation.

After 42 growing days, plant leaves, rhizomes, roots and tubers were analyzed for the bound and free forms of TNT, 2-ADNT and 4-ADNT. The

Table 11. Plant concentrations (mg/kg) of TNT and metabolites after 42 days in FY83.

Plant part	*Compound*	*Extraction type*	*TNT level (mg/L)* 0	5	10	20
Leaves	TNT	Free	1a*	3ab	3ab	13b
		Bound	<1a	<1a	<1a	<1a
	2-ADNT	Free	1a	14b	20bc	43c
		Bound	0a	24b	45c	62c
	4-ADNT	Free	1a	14a	17bc	48c
		Bound	0a	30ab	41b	77b
Roots	TNT	Free	9a	108b	290b	698c
		Bound	2a	3a	8ab	16b
	2-ADNT	Free	5a	190b	265b	274b
		Bound	2a	260b	336c	240c
	4-ADNT	Free	13a	615b	621b	872b
		Bound	5a	906b	1173bc	1307c
Rhizomes	TNT	Free	1a	19b	70c	93c
		Bound	0a	<1bc	1ab	2c
	2-ADNT	Free	0a	34b	51bc	74c
		Bound	1a	38b	42b	97c
	4-ADNT	Free	2a	85b	156bc	242c
		Bound	3a	95b	132b	287c
Tubers	TNT	Free	0a	10b	23b	66c
		Bound	0a	<1a	3b	4b
	2-ADNT	Free	0a	15b	30b	38b
		Bound	0a	11a	39b	74b
	4-ADNT	Free	0a	33b	101b	112b
		Bound	0a	28b	126c	204c

* Means for each plant part and compound across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978). Prior to statistical analysis, data were transformed to square roots of actual numbers since variances were not homogeneous.

concentrations of these compounds in plants grown at the different TNT levels were significantly different for all plant parts and compounds except bound TNT in leaves (Table 10). The smallest increases in concentrations as solution concentrations increased were for free TNT in leaves.

All three compounds were observed throughout the plant (Table 11 and Fig. 6). The roots, the probable point of entry into the plant, contained by far the greatest concentrations of the three compounds. The mean concentrations in the roots for all TNT levels were approximately 5.5–18.0 times those of the other plant parts. The TNT could also have been absorbed by the rhizomes, since they were grown directly in the solutions. Concentrations in the rhizomes were lower than those for roots, however, suggesting that root entry predominated.

Of the two metabolites, 4-ADNT was found in the greatest concentration in each of the plant parts (Table 11). Roots grown in the 20-mg/L solution of TNT contained almost 2200 mg/kg of 4-ADNT (both forms). Concentrations of TNT were usually lower than those of 2-ADNT.

Concentrations of TNT and metabolites in all plant parts generally increased as the concentrations of TNT in solution increased; the smallest increase was in the leaves (Table 11). This differs from the relationship in the plant weight data (Table 10), where yields were similar and lower than the control regardless of the TNT concentration in solution. The low concentrations of TNT in leaves could be a result of rapid metabolism (or other degradation) of this compound there, or metabolism in other parts of the plant followed by translocation, or both.

The distinction between bound and free forms of these compounds is based on their extractibility with benzene. We found most of the metabolites to be bound, in contrast to TNT, where the free

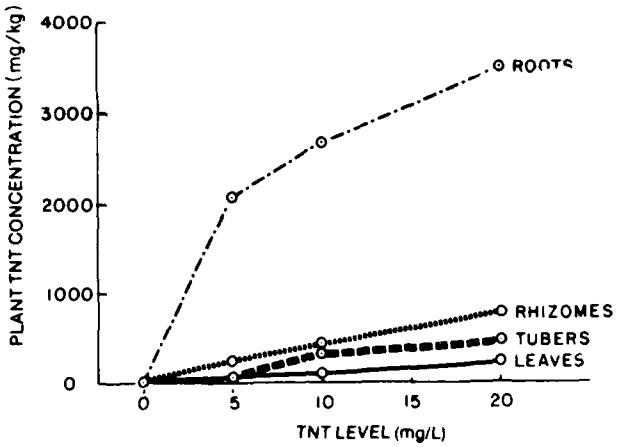

Figure 6. Concentrations of TNT and metabolites in various plant parts.

Table 12. Quantities (μg/aquarium) of TNT and metabolites in plants in FY83.

Plant part	*Compound*	*TNT concentration (mg/L)* 0	5	10	20
Leaves	TNT	3a*	6a	4a	21a
	2-ADNT	2a	71b	70b	147b
	4-ADNT	2a	76b	64b	176b
Roots	TNT	6a	7a	9a	25a
	2-ADNT	4a	28b	19b	24b
	4-ADNT	9a	97b	57b	86b
Rhizomes	TNT	2a	29b	64bc	85c
	2-ADNT	1a	109b	92b	148c
	4-ADNT	4a	271b	289b	475c
Tubers	TNT	0a	3a	2a	15b
	2-ADNT	0a	6a	9a	16a
	4-ADNT	0a	18a	28a	53a

* Means within each plant part across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978). Prior to statistical analysis data were transformed to square roots of actual numbers since variances were not homogeneous.

form predominates (Table 11). This observation is consistent with their chemical structures; the amine moiety of the metabolites is thought to react chemically with tissue components (Palazzo and Leggett 1983). The weaker extractant used in this study, benzene alone, was able to remove a greater percentage of TNT (free form) than of its metabolites. Therefore, TNT is less tightly bound within the plant and possibly more readily translocated and metabolized.

The total quantities of TNT and its metabolites in plants for the various treatments are shown in Table 12; the total quantity is calculated by multiplying the concentration by the dry weight of the plant. To evaluate the uptake of TNT and its metabolites, the bound and free forms of these com-

pounds were summed. The highest levels of statistical significance were for the rhizomes, where significant differences were found at the 1% level of probability for all three compounds.* The rhizomes contained about 58% of the TNT and metabolites taken up by the plants. The greater content in the rhizomes was due to their greater biomass and to their relatively high concentrations of these compounds (Tables 10 and 11). Leaves contained more than roots and tubers, especially at the higher levels of TNT; this also was related to the amount of biomass. The largest share of the total TNT and metabolites in the plants was 4-ADNT (63%). Only 10% of the total was TNT.

FY84 plant growth and uptake study

Table 13 shows the yield on a dry weight basis of leaves, roots and rhizomes after 58 days in hydroponic cultures containing 0, 0.5, 2.0 and 5.0 mg/L of TNT. The total yields of treated plants were 31–71% less than the control. In the FY83 study, where solution concentrations ranged up to 20 mg/L of TNT, yields were reduced by 54–74% (Table 10).

Table 13. Weights (g) of plants grown in various solutions of TNT for 58 days in FY84.

Plant part	*TNT level (mg/L)*			
	0	*0.5*	*2.0*	*5.0*
Leaves	4.00a*	3.48a	2.20b	1.38b
Roots	1.10a	0.60b	0.35bc	0.10c
Rhizomes	2.93a	1.50b	0.98b	0.85b

* Means for each plant part across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978).

The leaves were the least affected part of the plant, being reduced in growth by 13–66% (Table 13). There were no statistical differences in leaf yields for the 0- and 0.5-mg/L levels of TNT. As in FY83 the roots were the most sensitive to TNT, being reduced by 45–91%. The largest reduction in growth was at the lowest concentration of TNT, where growth was reduced by 45%. Higher levels of TNT caused even lower yields. The yield of rhizomes grown in TNT was significantly lower than the control, but the different TNT levels did not produce differences in growth. Rhizome growth was reduced by 49–71%. Visible differences in growth are shown in Figure 7.

Figure 7. Differences in plant growth for various levels of TNT.

Figure 8 shows plant heights. The leaves began to grow after nine days, but the control plants did not lengthen appreciably until after 23 days. This delay is related to the adjustment of the young plants to their new environment. The amount of leaf growth was directly correlated to the concentration of TNT in solution. As in FY83, plants in the 5-mg/L treatment did not grow much. At concentrations below 5 mg/L the growth rate increased as the TNT levels decreased.

The data in Figure 8 were used to correlate leaf elongation with time. R^2 values for all treatments, except the 5-mg/L level, were high. The 5-mg/L treatment had an R^2 of 0.18. The daily rate of leaf elongation from day 9 to day 58 was 40% and 60% lower for TNT concentration of 0.5 and 2.0 mg/L, respectively. Although there was a decrease in leaf growth, there were no obvious symptoms of TNT injury. In FY83 new leaves had died back.

Roots reacted similarly to leaves, with longer roots at the lower TNT concentrations (Fig. 9).

* Data not shown.

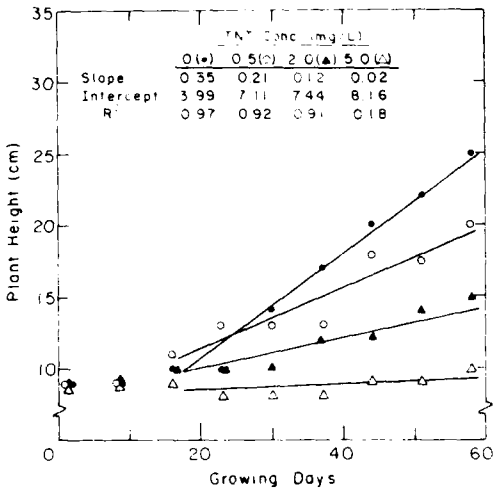

Figure 8. Plant height measurements in FY84.

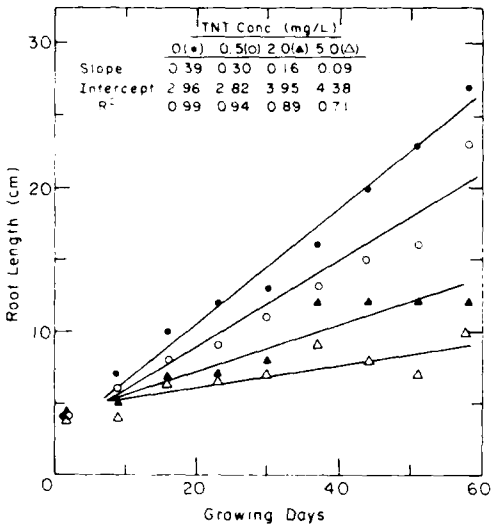

Figure 9. Root length measurements in FY84.

Unlike leaves, roots began to grow after the plants were in solution for only nine days.

The rate of root growth increased as the TNT concentration decreased (Fig. 9). The roots of the control plants grew 0.39 cm/day, which was similar to the rate of leaf growth. At 2.0 mg/L, root and leaf growth were also similar. At 0.5 mg/L, roots grew faster than leaves by 0.09 cm/day. There was a spurt in root length between day 51 and day 58, when the roots grew 7 cm. When the data point for day 58 is omitted, the rate of root growth was 0.25 cm/day, which is closer to that for leaves. Roots did grow at the 5-mg/L treatment but at the low rate of 0.09 cm/day. R^2 values for the root correlations declined as the TNT concentration increased. Symptoms of root injury were similar to those observed in FY83 and included a dark discoloration.

Both bound and free forms of TNT and metabolites were found throughout the plant (Table 14). As in FY83 the greatest concentration of TNT was in the free form, while most of the metabolites were in the bound form. Of the three compounds, 4-ADNT was found in the greatest concentration, making up 65% of the total at the 5-mg/L level. It was followed by 2-ADNT and TNT, which made up 21% and 9%, respectively.

All three compounds were found in their greatest concentration in the roots, which contained 1534 mg/kg, or 69% of the total in the plant at the 5-mg/L level (Table 14). Rhizomes contained 26% and leaves only 6%. Similar relationships among the three compounds were also found with the higher solution concentrations in FY83.

Concentrations of TNT and metabolites in the plants increased with increasing concentrations of TNT in the solutions (Table 14). The increases were smallest in leaves, followed by rhizomes. Relatively large amounts (7–17 mg/kg) of TNT and metabolites were found in several of the controls, probably because of sample contamination. Uptake was evident at the low TNT level of 0.5 mg/L and would probably have occurred at levels below this concentration.

Total quantities (concentration × plant dry weight) of TNT and the metabolites in plants are shown in Table 15. In general, as the concentration of TNT in solution increased, the increases were greatest for the metabolites. Increases were not as dramatic for TNT because TNT is unstable in the plant and because the combination of the small amount of contamination and the yields in the controls resulted in a high value for the control.

As in FY83, rhizomes contained the greatest quantities of TNT and metabolites, but differences between plant parts were smaller (Table 11). This was related to the greater amount of biomass produced at the lower TNT levels, especially for roots grown in the 0.5-mg/L treatment. Rhizomes contained about 45% of the compounds, and leaves and roots contained about 23% and 22%, respectively. The most common compound was 4-ADNT, followed by 2-ADNT and TNT.

Figure 10 shows the total quantities of the three compounds in the plant at each solution concentration for both FY83 and FY84. Although the studies were performed at different times, the re-

Table 14. Plant concentrations (mg/kg) of TNT and metabolites after 58 days in FY84.

Plant part	*Compound*	*Extraction type*	*TNT level (mg/L)* 0	0.5	2.0	5.0
Leaves	TNT	Free	10a*	13a	11a	16a
		Bound	<1a	<1a	<1b	1c
	2-ADNT	Free	<1a	2a	4b	15c
		Bound	<1a	5b	15c	32d
	4-ADNT	Free	<1a	6b	6b	15c
		Bound	<1a	8b	16c	44d
Roots	TNT	Free	17a	18a	45b	189c
		Bound	<1a	2b	10c	52d
	2-ADNT	Free	<1a	11b	28c	44d
		Bound	3a	43b	117c	246d
	4-ADNT	Free	2a	28b	53c	69d
		Bound	10a	216b	400c	934d
Rhizomes	TNT	Free	15a	15a	18a	56b
		Bound	<1a	<1a	2a	5b
	2-ADNT	Free	1a	6b	27c	46d
		Bound	<1a	12b	42c	89d
	4-ADNT	Free	7a	32b	90c	135d
		Bound	<1a	38b	107c	243d

* Means for each plant part and compound across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978). Prior to statistical analysis, data were transformed to square roots of actual numbers since variances were not homogeneous.

Table 15. Quantities (μg/aquarium) of TNT and metabolites in plants in FY84.

Plant part	*Compound*	*TNT concentration (mg/L)* 0	0.5	2.0	5.0
Leaves	TNT	40a*	47a	24b	24b
	2-ADNT	2a	21b	42c	64c
	4-ADNT	4a	42b	48bc	79c
Roots	TNT	18a	11a	18b	24b
	2-ADNT	4a	33b	51b	29b
	4-ADNT	14a	143b	154b	100b
Rhizomes	TNT	44a	24a	18a	52b
	2-ADNT	4a	29b	64c	89c
	4-ADNT	23a	106b	179c	323d

* Means for each plant part and compound across a row followed by the same letter were not significantly different at the 0.05 level of probability by Duncan's Multiple Range Test (Little and Hills 1978). Prior to statistical analysis, data were transformed to square roots of actual numbers since variances were not homogeneous.

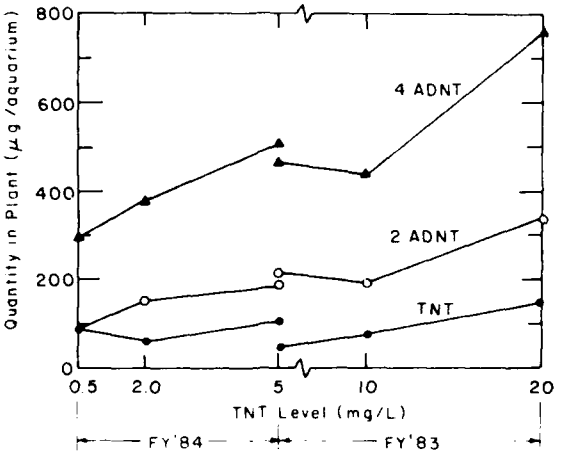

Figure 10. Quantities of TNT and metabolites in plants.

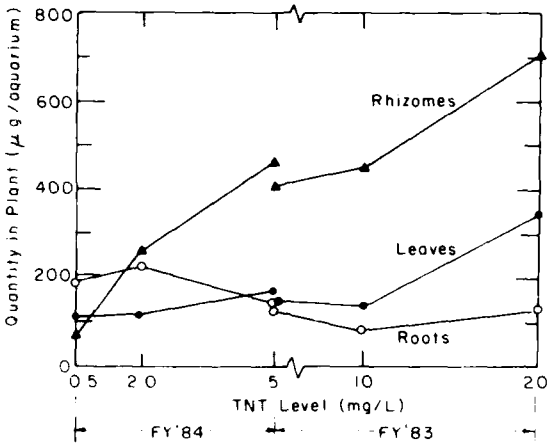

Figure 11. Location of TNT and metabolites in various plant parts.

sults generally agreed. Uptake increased as the solution concentration increased to 20 mg/L of TNT. The findings for the individual plant parts were similar (Fig 11).

CONCLUSIONS

A method was developed and validated for measuring TNT and two of its metabolites in plants grown in hydroponic cultures. The first step of the method is extraction of the fresh plant tissue with benzene. This removes the easily extracted (free) residues and the residual water. This is followed by dry weight determination and treatment with 5 N H_2SO_4 to free the chemically bound fraction, primarily the amino metabolites of TNT. The precision of the analysis seemed to depend primarily on the specific analyte levels, with precision generally improving with increasing analyte concentration. The average standard deviation for seven replicates was about 28% of the mean. Combining the free and bound fraction lowered the standard deviation slightly to 22%. This may not be significant, but it suggests that some free forms converted to bound forms during the sample preparation or extraction.

Summing all the residues for each plant part failed to improve precision over that for individual metabolite analyses. Homogenization failed to improve the precision or accuracy of the analyses. Spikes were recovered in the benzene extraction step (Tables 5-7). For the hydrolysis step some losses were evident, particularly for leaf samples, where recovery of 4-ADNT and 2-ADNT were about 50% and 73%, respectively. Some larger unexplained losses occurred during storage of the hydrolyzate extracts for several weeks. These results appear to be due in part to extraction inefficiencies and in part to chemical instability of the extracts. These problems should be addressed in future work.

It is apparent from the FY83 and FY84 studies that TNT affects the growth and development of *Cyperus esculentus* L. at solution concentrations as low as 0.5 mg/L. These effects include reduced yields and slower leaf and root growth. Plant growth became increasingly inhibited up to 5 mg/L of TNT; at higher concentrations no further significant reductions in yields were noted. Thus, the greatest changes in physiological activity apparently occur between concentrations of 0.5 and 5 mg/L of TNT. These changes would probably also occur at levels below 0.5 mg/L of TNT, but this very low range was not tested. In preliminary studies, plants died at concentrations of 60 mg/L of TNT.*

Visible symptoms of injury to roots included a very dark discoloration and apparent death at 5 mg/L or greater. New leaf growth is increasingly restricted up to 5 mg/L with no further reductions at higher levels. Older leaves did not reveal any toxic symptoms up to 20 mg/L.

Although the treatment concentrations in FY84 were lower than in FY83, the results of the two

* Unpublished results.

studies were similar in many ways. These included:

- The sensitivity of root growth in TNT solutions.
- The lower concentrations of TNT in leaves than in roots and rhizomes.
- The high concentration of compounds in roots.
- The high percentages of metabolites in the bound form and TNT in the free form.
- The increase in plant concentrations with increasing TNT in solution.

TNT and its metabolites were found throughout the plant. The metabolite 4-ADNT was present in the greatest amount. The roots were the probable point of entry into the plant and contained the highest concentration of these compounds; leaves contained the lowest. At the higher concentrations (> 2.0 mg/L) the rhizomes accumulated the greatest quantities of the three compounds. Although the yields for the different treatments did not differ significantly above concentrations of 5 mg/L, increasing the concentration of TNT in solution increased the concentration and accumulation of TNT and metabolites in plants.

The accumulation of these compounds by the rhizomes at all treatment levels is due to the greater amount of rhizomatous biomass produced, especially at the high concentrations of these compounds. The roots contained higher concentrations but produced only a small amount of biomass. Dry weights of leaves were not as adversely affected as those of roots.

The severe injury and high concentrations of compounds in roots may be related to a breakdown in root cell structure, allowing a greater rate of passive absorption. Although root injury was extensive, limited translocation to other plant parts does not seem to be the reason for high root concentrations. As shown in Table 12, the total content of TNT and metabolites in leaves is similar to or greater than that in roots.

Care was taken in this study to assure that TNT was the only compound taken up. Semiweekly analysis failed to detect metabolites in the culture solution. Solutions were replaced after three weeks to maintain the original levels of TNT and pH. Therefore, we believe that the metabolites of TNT are formed in the plant, although we cannot rule out a microbial origin since the plants themselves were not sterile. The metabolites 4-ADNT and 2-ADNT have been identified in other species (Palazzo and Leggett 1983).

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