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TOLERANCE TO NITROGENOUS EXPLOSIVES AND METABOLISM OF TNT BY CELL SUSPENSIONS OF *DATURA INNOXIA*

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SUMMARY

Cell suspension cultures of *Datura innoxia* were incubated in the presence of the nitro-substituted explosives 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazine (RDX), and 1,3,5,7-tetranitro-1,3,5,7-tetraazocyclooctane (HMX). Cellular tolerance levels and TNT biotransformation kinetics were examined. Tolerance to TNT varied as cell suspensions aged. Concentrations of RDX or HMX in excess of reported solubility limits produced no observable changes in cell viability. GC/MS analysis of TNT-treated cell media and cell lysates revealed rapid removal of TNT. Within 12 h, less than 1% of the initial TNT remained in the growth medium. Aminodinitrotoluenes (ADNTs), known metabolites of TNT, accumulated transiently in cell lysates, and to a lesser extent in cell media. ADNT concentrations started to decrease after 3 h. After 12 h, less than 5% of the initial TNT could be detected as ADNT. Total ADNTs never exceeded 26% of initial TNT, suggesting that additional biotransformation steps also occurred. No other nitroaromatics were detected. A pseudo-first order rate constant for TNT clearance was calculated, $k = 0.40\ h^{-1}$. *D. innoxia* cell suspension cultures demonstrated virtually complete clearance of TNT and of subsequent ADNT metabolites in less than 12 h. This rapid metabolism of nitroaromatics by the *Datura* cell suspension system indicates the utility of this system for further molecular and biochemical studies.

Key words: phytoremediation; nitroaromatics; biotransformation; bioremediation.

INTRODUCTION

Since the early part of this century, 2,4,6-trinitrotoluene (TNT) has been used as an explosive in munitions manufacturing (Palazzo and Leggett, 1986; Fernando et al., 1990). In past years, wastes from TNT manufacturing, loading, and packing operations were routinely released into artificial holding lagoons ("pink water" lagoons), natural ponds, or freshwater streams (Boopathy et al., 1994). Significant contamination by TNT, other explosives, and their breakdown products now persist at these sites. Current munition disposal practices such as excavation and incineration or filtration of wastewater through granular activated carbon are expensive. For this reason, low-cost technologies such as composting, bioremediation, and phytoremediation have attracted a great deal of interest (Bradley and Chapelle, 1995; Cunningham and Ow, 1996; Montpas et al., 1997; Salt et al., 1998).

Bioremediation of TNT by fungi and other soil microbes has been investigated for at least 20 yr (reviewed by Fernando et al., 1990). In some cases, a consortium of microorganisms is required to transform TNT; in other cases, individual microbes can transform TNT into a variety of metabolites including toluene; and finally, selected microbes appear to be able to mineralize TNT (Boopathy et al., 1994; Alvarez et al., 1995; Shelley et al., 1996; Kalafut et al., 1998). While mineralization of TNT results in its complete decomposition, this can be a very slow process, taking months (Fernando et al., 1990).

Within the last 10 yr, a number of groups around the world have started to examine the phytoremediation of TNT. Initially this work developed out of a concern about the movement of TNT-derived toxins and mutagens into food chains following absorption of environmental TNT by plants (Palazzo and Leggett, 1986). Currently the focus of TNT phytoremediation studies is to determine the potential for plants as clean-up agents, either simply to concentrate TNT from lagoons or to clear contaminated soils (Schnoor et al., 1995). Since some of the observed and predicted metabolites of TNT are mutagens, it is crucial to understand completely the biotransformation process associated with remediation of environmental sources of TNT.

Uptake and nitroreduction of TNT has been demonstrated in several different types of plants or plant organ cultures. Several groups have demonstrated the accumulation of ADNTs in plant tissues following exposure of plants to ^{14}C-TNT (Palazzo and Leggett, 1986; Harvey et al., 1990; Thompson et al., 1998). In all of these cases, the presence of microflora in the plant cultures precluded the authors from unambiguously claiming that plant enzymes were responsible for the TNT biotransformation. Hughes et al. (1997) have demonstrated the elimination of TNT and the accumulation of ADNTs in aquatic *Myriophyllum* species and in hairy root cultures of *Catharanthus roseus*. This study used axenic cultures and demonstrated that plant enzymes performed the nitroreduction of TNT. However, the clearance of TNT was relatively slow, with a reported half-life of 60

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h for axenic *Myriophyllum* cultures and 12 h for *Catharanthus* root cultures.

Standard detoxification pathways in plants involve conjugation of xenobiotic substrates to more water-soluble molecules such as sugars and/or storage via transport to vacuoles or binding to cell wall components (Sandermann, 1992). ^{14}C-TNT tracer studies examining TNT metabolism by plants have consistently shown formation of ADNTs and partitioning of significant percentages of radiolabel with cell wall fractions (Palazzo and Leggett, 1986; Harvey et al., 1990; Hughes et al., 1997; Thompson et al., 1998).

In this report we describe the time course of nitroreduction of TNT together with the resulting accumulation and subsequent elimination of ADNTs in axenic cell suspensions of *D. innoxia*. The use of *D. innoxia* cell cultures provides an opportunity to characterize TNT biotransformation in detail in a plant suited to arid climates. Further, a dose response for TNT removal is determined over the range of 15 to 60 μg/mL (66 to 263 μ*M*) allowing an analysis of the kinetics of TNT biotransformation. The capacity of *D. innoxia* cell suspensions to tolerate exposure to the explosives HMX (1,3,5,7-tetranitro-1,3,5,7-tetraazocyclooctane) and RDX (1,3,5-trinitro-1,3,5-triazine) is also examined, since these explosives are frequently seen in field sites contaminated with TNT.

Materials and Methods

Cell suspension cultures. Cell suspension cultures were initiated from *D. innoxia* callus tissue, which had been maintained with 52 monthly subcultures on UM1A semisolid media (Uchimaya and Murashige, 1974). Approximately 5 g (fresh weight) of callus tissue was transferred to 25 mL liquid UM1A medium for each cell suspension. Cell suspensions were maintained on orbital shakers and subcultured into fresh UM1A medium at weekly intervals. Visual inspection and weekly plateouts onto tryptic soy agar, potato dextrose agar (Sigma Chemical Co., St. Louis, MO), and semisolid UM1A medium were used to check for the presence of common bacterial and fungal contaminants. Cell suspensions used for tolerance testing and biotransformation studies had undergone 6 to 12 subcultures.

Determination of cell line tolerance to TNT, HMX, and RDX. TNT (99%) was purchased from Chem Services Inc. (West Chester, PA) HMX and DRX (military specification) were donated by Doug Olson, Energetic Materials Research and Testing Center, NM Mining and Technology, Socorro, NM. Measured volumes of TNT (0.88 to 4.4 μmol), HMX (0.67 to 10.13 μmol), or RDX (0.90 to 13.51 μmol) in acetonitrile were placed in 125-mL Erlenmeyer flasks. The acetonitrile was allowed to evaporate before 20 mL sterile UM1A medium and 5 g of viable suspension cells were added. Flasks were sealed with air-permeable polyfoam stoppers covered with aluminum foil and placed on orbital shakers at 100 rpm for 7 d at 24° C under constant diffuse illumination at 4.78 μmol $s^{-1}m^{-2}$ photosynthetically active radiation. To determine viability, cells were stained with fluorescein diacetate (Duncan and Widholm, 1984), and samples were examined under a Zeiss light microscope with 450–490-nm filters.

TNT biotransformation. Aliquots containing 3 g (fresh wt) of harvested *D. innoxia* cell suspensions were transferred to Erlenmeyer flasks containing 15 mL of UM1A medium. These were placed on orbital shakers at 100 rpm and incubated overnight as described above. After 16 h, we prepared dead cell controls by transferring overnight cultures to a 100° C water bath for 8 min, after which no fluorescent cells could be seen in aliquots treated with fluorescein diacetate. Cell-free controls consisted of 23 mL of UM1A medium without added cells.

TNT from a 657 m*M* stock solution in dimethylsulfoxide (DMSO) was added to flasks at final concentrations of 66, 132, 197, or 263 μ*M*. No change in cell doubling time or percent viability was seen in cell suspensions exposed to DMSO alone (data not shown). Flasks were incubated as before, and duplicate samples were harvested at time points ranging from 30 min to 96 h by centrifugation through 0.45-μm nylon mesh filters at 3000 rpm for 5 min.

Harvested cells were rinsed two times for 5 min each on an orbital shaker in 12.5 mL of ice-cold wash buffer (50 m*M* Tris HCl, pH 7.4, 10 m*M* KCl, 10 m*M* $MgCl_2$). Wash buffer was removed by filter centrifugation. Filtrates from cell rinses were combined with cell media and stored as the "media" fraction, frozen in liquid nitrogen, and stored at −70° C.

We recovered TNT and ADNT residues adhering to the sides of culture flasks by rinsing each flask three times with 2 mL of methylene chloride, which was then collected in 20-mL glass vials. Methylene chloride was evaporated under nitrogen. Substances remaining in the vials were resuspended in 1 mL of internal standard solution (200 μg 2,4-dinitrotoluene per mL methanol) and analyzed by GC/MS as described below.

Extraction of TNT and ADNTs from cell media and lysates. Stored samples of cell media–wash buffer mixtures were thawed at 37° C and transferred to glass centrifuge tubes. Nitroaromatics were extracted three times with methylene chloride. The organic phase from each extraction was transferred to a 10-mL glass test tube and the solvent was evaporated under nitrogen. Samples were resuspended in 1 mL of internal standard solution, transferred to 1.5-mL autosampler vials with polytetra fluoroethylene (PTFE) septa, and stored at −20° C.

We prepared cell lysates by resuspending frozen cells in 20 mL of lysis buffer containing 50 m*M* Tris HCl, pH 7.4, 10 m*M* KCl, 10 m*M* $MgCl_2$, and 18 μ*M* β-mercaptoethanol. Samples were thawed at 37° C and homogenized in an Elvehjem tissue grinder. The lysate was extracted three times with methylene chloride as described above. After evaporation of methylene chloride, samples were resuspended in 1 mL of internal standard solution and transferred to autosampler vials.

GC/MS analysis of TNT, 2-ADNT, and 4-ADNT. TNT and the metabolites 2-ADNT and 4-ADNT were analyzed with a Varian Saturn GC/MS system. Gas chromatography utilized an HP-5MS capillary column which contained a cross-linked 5% phenyl-methylsiloxane coating, film thickness 0.25 μm, length 30 m, and an inner diameter of 0.25 mm. Temperature programming began at 70° C for 3 min followed by increases of 20° C/min to 200° C and then 5° C/min to 250° C. TNT and all observed metabolite peaks observed eluted at less than 200° C, which is well below the 240° C combustion point of TNT. Data were collected from a mass range of 50 to 450 atomic mass units.

We prepared calibration standards of TNT by drying 500 mg of TNT overnight at 40° C to remove H_2O, then preparing a 44 m*M* stock solution of TNT in methanol. Standards of 2-ADNT and 4-ADNT were purchased from Supelco, Inc. (Bellefonte, PA) as 1 mg/mL solutions in acetonitrile. The mass spectra seen for TNT and 2,4-dinitrotoluene were identical to reference mass spectra (NIST, 1998). 2-ADNT and 4-ADNT mass spectra were not present in the NIST library; however, the retention times and mass spectra measured in samples corresponded well with standards. Quantification of TNT was based on the ratio of the 210 mass peak of TNT, found at retention time 11:07, to the 165 mass peak from the internal standard, 2,4-DNT, found at retention time 9:51. Injections of 0.1 to 100 ng (representing final TNT values of 0.439 to 439.0 nmoles) produced a linear calibration curve with a correlation coefficient of 97.6%. Quantification of ADNT peaks relied on the ratio of the sum of three mass peaks characteristic of ADNTs (197 + 180 + 104) to the 165 mass peak of 2,4-DNT. The 2-ADNT was retained for 13:59 min. 4-ADNT was retained for 13:21 min. With ADNTs, injections of 5 to 100 ng (representing final ADNT values of 0.025–0.508 μmoles) provided a reproducible linear response with a correlation coefficient of 98%. However, injections of 0.1 to 5 ng produced a correlation coefficient of only 72%.

Each sample was analyzed twice by GC/MS. Since duplicate samples were prepared, this resulted in four measurements for each time point and initial TNT concentration tested. The mean and standard error of these four values were calculated.

Results

Cell line tolerance to TNT, HMX, and RDX. Cell suspensions of *D. innoxia* were incubated in the presence of different concentrations of TNT, HMX, or RDX to determine the effects of these compounds on cell viability. As demonstrated in Fig. 1, percent viability remained unchanged at TNT exposure levels as high as 88 μ*M*. Initial toxic responses were seen at 131 μ*M*, and at 219 μ*M*, TNT was lethal to slightly more than 60% of the exposed cells. This concentration is below the solubility limit of TNT in water (130 μg/mL [570 μ*M*], Anonymous, 1995a).

Tolerance to TNT was significantly dependent on the age of the cell suspension. In a preliminary study, cells that had been in sus-

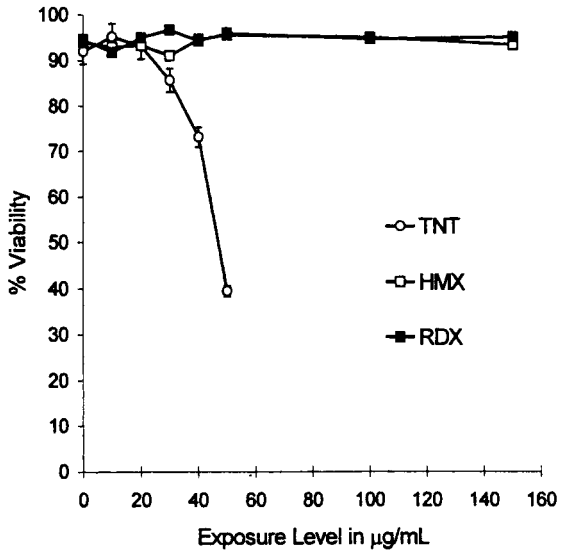

FIG. 1. Effect of TNT, HMX, and RDX on *D. innoxia* cell viability. Cell suspensions which had been initiated from callus 6 wk before the study were cultured for 1 wk at the indicated concentrations of TNT, HMX, or RDX (10 µg/mL = 44 µ*M* TNT, 34 µ*M* HMX, and 45 µ*M* RDX). Cell viability was determined by fluorescein diacetate staining. Values represent the mean percentage of viable cells in 15 samples. *Vertical bars* represent standard error. Aqueous solubility limits for HMX, RDX and TNT are 5, 60, and 130 µg/mL respectively.

pension for 18 mo. tolerated TNT-saturated media regardless of prior exposure to TNT (data not shown). In biotransformation studies reported below, which included doses of TNT as high as 263 µ*M*, neither reduced rates of TNT biotransformation nor decreased viability were observed. These cells had been in suspension only 2 wk longer than the cells tested here for TNT tolerance.

Cells treated with either HMX or RDX showed no decrease in cell viability regardless of treatment level (Fig. 1). The aqueous solubility limits are 5 mg/L (17 µ*M*) at 25° C for HMX (Anonymous, 1994) and 38–60 mg/L (173–270 µ*M*) at 20° C for RDX (Anonymous, 1995b). Higher treatments of HMX or RDX were not investigated. Such treatments would not have resulted in higher effective concentrations in the culture medium.

TNT biotransformation. The biotransformation of TNT by *D. innoxia* suspension cultures was characterized by quantification of the TNT, 2-ADNT, and 4-ADNT levels in cell lysates, cell media, and flask residues following inoculation of the cultures with a range of TNT concentrations. GC/MS analyses were performed on samples collected at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, and 96 h after addition of 66 to 263 µ*M* TNT. Analyses were also performed on heat-killed cells and cell-free media as controls. The cells were presumed to be viable throughout this time course at all doses because the packed cell volumes were visibly increased in the 48- and 96-h samples. No visible increases in packed cell volumes were observed in the heat-killed samples. The doubling time of the cell line used was approximately 48 h.

The time course for the biotransformation of TNT in cultures inoculated with 197 µ*M* TNT demonstrates the partitioning of TNT and ADNT between medium and cell lysate (Fig. 2). The cell media sample included washes of the pelleted cells, which would include

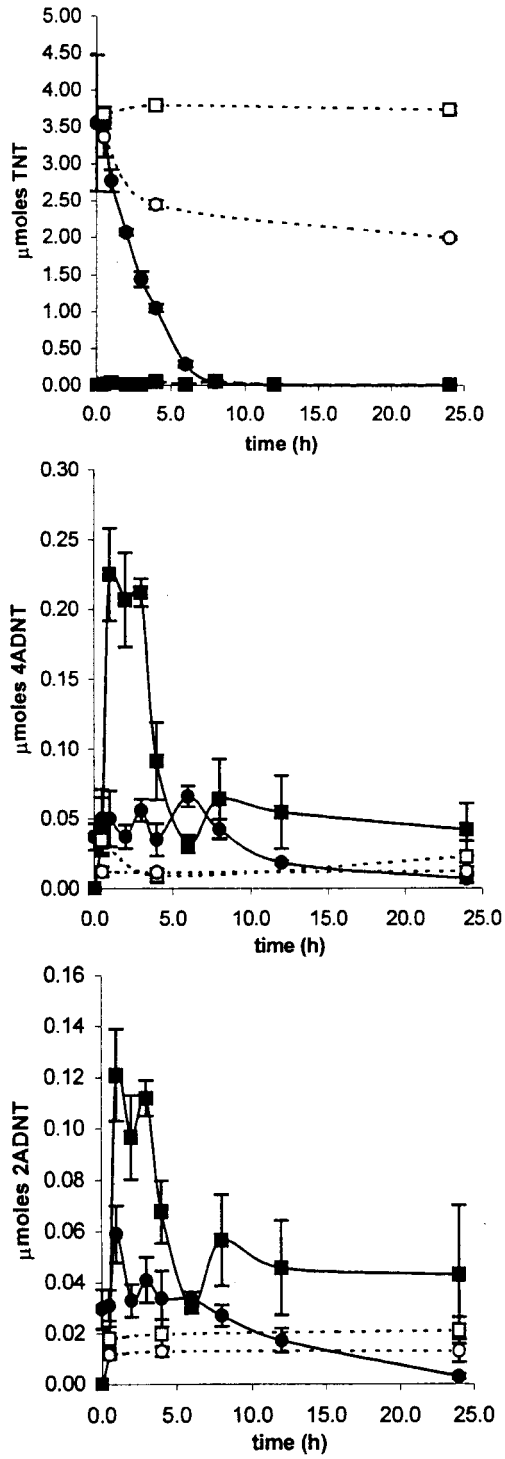

FIG. 2. Time course of TNT, 2-ADNT and 4-ADNT accumulation in media vs. cell lysates. Values represent the micromoles of the indicated nitroaromatic following inoculation at time zero of *D. innoxia* suspension cell culture with 197 µ*M* TNT. Nitroaromatics were quantified with GC/MS analysis of medium (*solid line, filled circle*), cell lysates (*solid line, filled square*), cell-free medium (*dashed line, open square*), or heat-killed cells plus medium (*dashed line, open circle*). *Vertical bars* represent standard error. Cells used in this study had been in suspension for 8 wk. Significant changes were not seen between 24 and 96 h; therefore data are not shown.

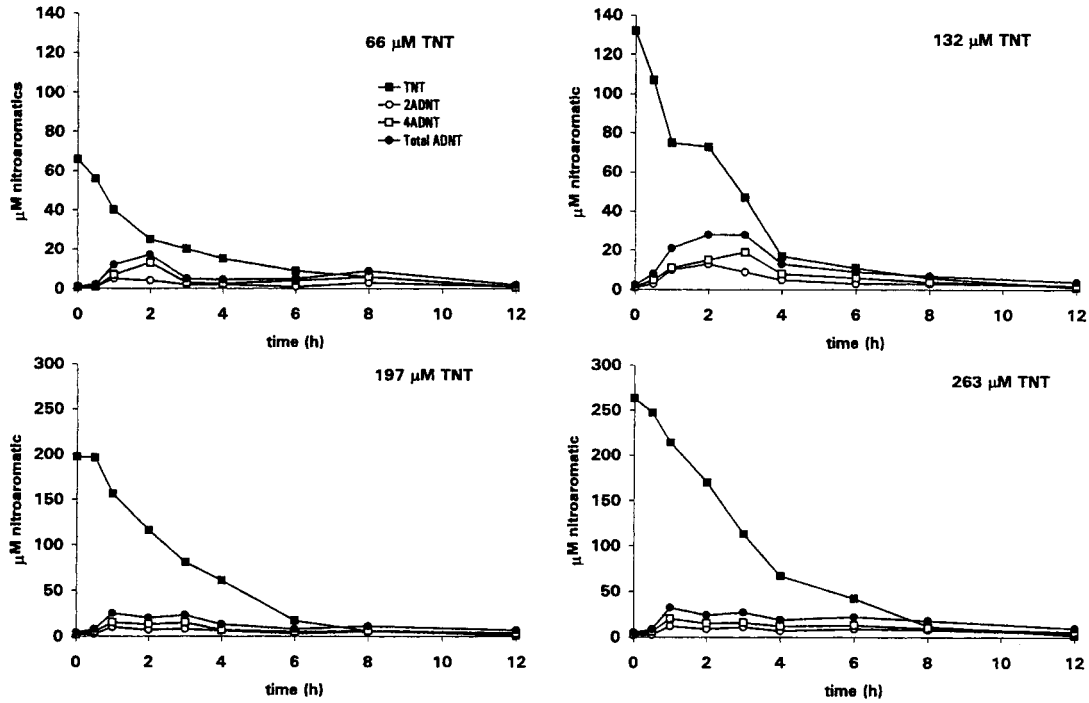

FIG. 3. Time course of total nitroaromatics in *D. innoxia* cell suspension cultures. Values represent the μ*M* concentrations of the indicated nitroaromatic detected in medium and cell lysate combined over the first 12 h of a 96-h time course. Initial TNT concentrations are indicated.

weakly adsorbed TNT and metabolites. The cell lysate sample included all of the intracellular volumes of the cell as well as strongly adsorbed TNT and metabolites. Neither TNT or ADNTs were detected in any of the flask residue samples.

TNT associated with cell lysates reached maximum levels within 3 to 8 h depending on the initial TNT concentration. Intracellular concentration of TNT can be approximated by an estimated cell density of 1 g/mL if one assumes that cell fresh weight during the first 12 h (when TNT concentrations are highest) remains static (3 g). Total TNT detected in the cell lysates can then be divided by 3 mL to give an approximate maximum intracellular TNT concentration of 19.7 μ*M* at the highest treatment level.

The time course for the biotransformation of TNT at all four treatment levels shows that TNT levels in the media dropped to virtually zero in less than 10 h (Fig. 3); transient and low levels of TNT were detected in the cell lysates (Fig. 2). The removal of TNT progressed rapidly at all four treatment levels with live cells, with less than 1% of the initial TNT remaining in either the medium or cells after 12 h (Fig. 3).

During the 96-h study, minor losses of TNT were also observed in the 264-μ*M* treatment level of cell-free medium and in all four treatment levels of heat-killed cell suspensions. These losses were much smaller than the loss of TNT from viable cell suspensions.

Aminodinitrotoluene accumulation. ADNTs have been reported to be primary metabolites of TNT in a variety of plant species (Palazzo and Leggett, 1986; Harvey et al., 1990; Hughes et al., 1997; Thompson et al., 1998). We were interested in the extent to which these molecules accumulate in plant cells during and following exposure to TNT. In this study, the trace levels of both ADNT isomers, present at the first sampling, increased during the initial hours following exposure to TNT in both media and cell lysates, with most of the ADNT accumulating inside the cell (Fig. 2). Peak levels were attained within 3 h regardless of TNT dose (Fig. 3). In cell lysates, 4-ADNT levels were nearly double the levels of the 2-ADNT isomer (Fig. 2).

For all four treatment levels, ADNT concentrations increased during the first 3 to 4 h, then declined subsequently (Fig. 3). Total ADNTs (2-ADNT + 4-ADNT) recovered in media and cell lysates were calculated as a percentage of initial TNT. Although nearly 100% of TNT was removed within 12 h, total ADNT concentrations never exceeded 26% of initial TNT at any one time point (data not shown). This was presumably due to the rapid subsequent transformation of ADNTs to unknown metabolites. Most of the ADNTs recovered were from the cell lysate fraction. At the 2-h time point, the approximate peak of ADNT accumulation, 15 to 19% of the total ADNT was present in the media fraction for all four TNT treatment levels. The majority (80 to 85%) of the ADNT was present in the cell lysate fraction (data not shown). The ADNT concentration in the cell media continued to decrease with time of incubation.

Kinetic analysis of TNT biotransformation. Precise calculation of TNT transformation kinetics in cell suspensions is confounded by the complexity of the cell culture itself. An approximation of the TNT transformation rate can be determined by assuming pseudo-first order kinetics. Using the integrated first order rate law $\ln([TNTi]/[TNTt]) = kt$, where [TNT*i*] represents the initial TNT concentration, [TNT*t*] represents the TNT concentration at time *t*, *k* equals the rate constant, and *t* = the time since the reaction began, one can plot $\ln([TNTi]/[TNTt])$ vs. time to determine the pseudo-first order rate constant of

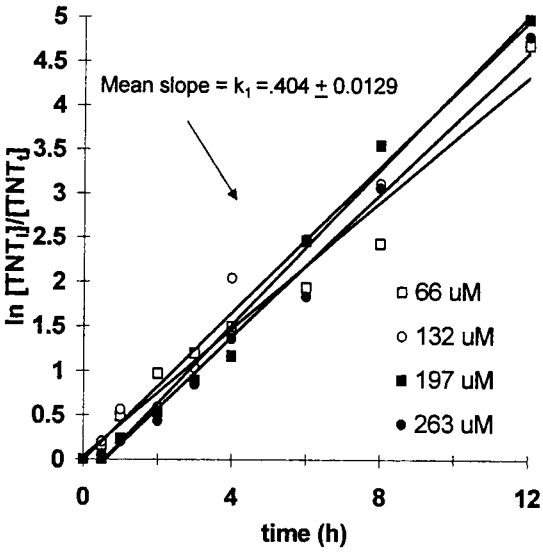

FIG. 4. Kinetics of TNT clearance by *D. innoxia* cell suspension cultures. A pseudo-first order rate plot ln ([TNT*i*]/[TNT*t*]) vs. time (0–12 h) is shown.

TNT biotransformation (Fig. 4). At TNT concentrations in excess of 1.25 μ*M*, this plot produces parallel lines for each initial TNT concentration, with slopes equal to 0.40 + 0.13. Therefore, the pseudo-first order rate constant k = 0.40/h and the $t_{1/2}$ = 1.7 h for TNT clearance by *D. innoxia* cell suspensions. The assumption of pseudo-first order kinetics for the TNT transformation was reasonable, given that the data points fit the semi-log plot very well. The regression coefficients for the fit of each of the four lines ranged between r^2 of 0.97 and 0.99.

DISCUSSION

This report demonstrates the intrinsic capability of *D. innoxia* suspension cell cultures to tolerate exposure to a variety of explosives commonly found at munitions waste sites, to rapidly clear TNT from growth media and cells, and to transform TNT via nitroreduction. *D. innoxia* grows wild in desert regions of the southwestern USA and differs from other plants proposed for use as remediators of explosives in that it thrives in arid climates.

Plant cells grown as suspension cultures do not develop extensive secondary cell walls or protective layers. Each cell or cell aggregate is in direct contact with the growth medium. In a field setting where soil adsorption reduces the effective concentration of a given xenobiotic and plant suberized and cutinized layers reduce contact with individual cells, lethal doses should exceed those reported here. *D. innoxia* cell cultures tolerated supersaturating concentrations of RDX and HMX in excess of reported aqueous solubility limits, suggesting that it is unlikely that soils contaminated with HMX or RDX will limit plant growth. Similarly, *D. innoxia* cell cultures readily tolerated up to 175 μ*M* TNT (Fig. 1), with a potential for selection for higher levels of tolerance. The variable tolerance observed may be due to a physiological adaptation, or to the genetic variability present in heterozygous, wild type *D. innoxia* cells. Preliminary studies with whole plants and soil mixes suggest that *D. innoxia* can survive on 900 μg TNT/g soil (Shojee, 1994). Other plants have been described to be more sensitive. In hydroponic systems, 5 mg TNT per L (22 μ*M*) reduced plant root growth for *Allium schoenoprasum* (Görge et al., 1994) and *Cyperus esculentus* (Palazzo and Leggett, 1986). Alfalfa is more resistant; root growth is inhibited at 10 mg/L (44 μ*M*), and plant growth is completely restricted at 50 mg/L (219 μ*M*) (Görge et al., 1994). In contrast, the water plant *Myriophyllum* appears to tolerate 100 mg TNT per L (438 μ*M*) (Vanderford et al., 1997).

The use of axenic cell suspension cultures has demonstrated that nitroreduction occurs without the assistance of plant-associated microorganisms. Because cells and cell aggregates in suspension come in direct contact with the media solution, the results of this study have afforded the unique opportunity to examine the kinetics of TNT uptake at the cellular level. The rapid disappearance of TNT, coupled with the appearance of ADNTs, indicates that nitroreductase activity is present in cells before TNT exposure.

Although the major portion of TNT was isolated from cell media, ADNTs accumulated in both media and cell lysates following exposure to TNT (Fig. 2). Accumulation was only temporary and was predominant in cell lysates. As TNT was consumed, ADNT concentrations also declined. It is significant that at all treatment levels, less than 1.7% of the initial TNT was present as either TNT or ADNT after 96 h. No adverse effects on the cell population were observed. It cannot be determined from these observations whether nitroreduction is occurring extracellularly by secreted enzymes; at the cell surface, followed by preferential transport of ADNTs into the cell; or within the cell, followed by the release of ADNTs into the cell media.

In cell-free medium, TNT loss may be due to photodegradation (Bazyl et al., 1996) and partitioning with polypeptides present in UM1A medium in the form of casein hydrolysate. Heat-treated cells lysed during time in culture, making it impossible to separate media from cell lysates. Therefore, values reported for heat-killed cells represent the total TNT or ADNT isomer found in media and lysates combined. In heat-killed cells, loss may be attributed to photodegradation, residual enzymatic activity, chemical reduction of TNT by reducing equivalents present in cell lysates, and/or partitioning interactions.

The temporal disappearance of ADNTs indicates that these molecules are catabolized or conjugated. We sought potential ADNT metabolite peaks by examining total ion chromatograms for peaks which appeared in a temporal fashion following addition of TNT. No such metabolites were observed. Very polar molecules, cell wall conjugates, and oxygen-sensitive metabolites (such as triaminotoluene) would not have been detected by these techniques. However, the less polar tetranitroazoxytoluene and diaminotoluene isomers should have been readily detected, and none were observed. Sugar beet suspension cell cultures have the capacity to degrade nitroglycerin (glycerol trinitrate) by denitrification of the nitrate ester and generate dinitrate and mononitrate glycerol as products (Goel et al., 1997). Tobacco seedlings can also denitrify nitroglycerin (French et al., 1999). *Pseudomonas* strains can remove nitro groups from TNT (Shelley et al., 1996). Denitrification of TNT by the *Datura* cultures is not likely, as these metabolites were not observed and should have been detectable by the GC/MS system.

The TNT clearance rate by *Datura innoxia* appears to be fast when compared with other published rates. Hydroponic cultures of *Populus* had TNT half-life of 34.6 h (Thompson et al., 1998). This finding was based on a single dose of TNT. These were not demonstrated to

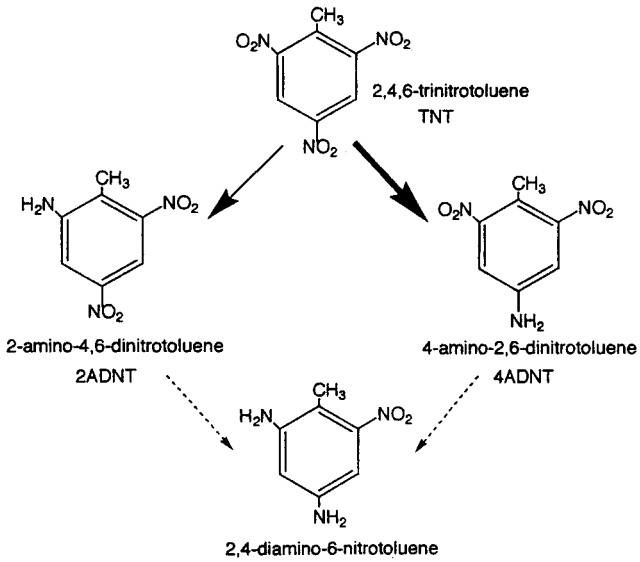

FIG. 5. Biotransformation of TNT by *D. innoxia* cell suspension cultures. Nitroreduction reactions predicted to take place, as indicated by *solid-line arrows*, are based on detection of intermediates; the width of the line indicates the relative abundance of products observed. *Dashed-line arrows* indicate nitroreduction reactions not predicted to take place in *Datura* cell cultures.

be axenic cultures, so some clearance of TNT could have been due to microflora. Hughes et al. (1997) reported the time course for TNT clearance from axenic *Myriophyllum* cultures and *Catharanthus* root cultures. Estimates from their published data indicate TNT half-lives of 60 h for *Myriophyllum* and 12 h for *Catharanthus*. Their published data again were for a single TNT dose for each culture. *Datura innoxia* cell cultures have demonstrated at least a 10-fold increase in TNT clearance rates over these axenic plant systems. These comparisons are based on aqueous systems. Studies on the clearance of TNT from soil by plants immediately become confounded by TNT bioavailability. In most cases, TNT clearance under these circumstances becomes slower and incomplete. Only 25% of the TNT in soil was cleared after 20 d by *Populus*, a plant proposed to be well suited to environmental clean-up (Thompson et al., 1998). Importantly, there are species-specific differences in TNT uptake capacity. Scheidemann et al. (1998) compared nitroaromatic accumulations in 11 different plants grown on TNT-supplemented soils. *Phaseolus vulgaris* was able to grow on soil contaminated at up to 500 mg/kg. *Phaseolus vulgaris*, along with several cultivars of wheat, accumulated the most aminodinitrotoluenes.

In addition to differences in rates of TNT uptake, differences exist in the proportions of ADNTs found. These *D. innoxia* cultures accumulated higher levels of 4-ADNT than 2-ADNT. This contrasts with the findings of Hughes et al. (1997) who reported higher concentrations of 2-ADNT than 4-ADNT in axenic *Myriophyllum* cultures. A schema for the biotransformations of TNT by *D. innoxia* is shown in Fig. 5.

D. innoxia cell suspension cultures have an excellent capacity for TNT uptake and biotransformation. They are able to rapidly absorb TNT from the surrounding aqueous phase and metabolize TNT into reduced intermediates. These metabolites are further processed into unknown compounds. Most of the ADNT metabolites accumulate in the plant cells; any metabolites that are secreted or present in the media appear to be cleared as well. There are two directions for future research: (1) use these cell cultures to isolate the genes encoding the enzymes responsible for nitroaromatic remediation, and (2) determine if the rapid clearance and metabolism of TNT demonstrated by *Datura* cell cultures will be demonstrated by the whole plant in a field setting.

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