Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase

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There is major international concern over the wide-scale contamination of soil and associated ground water by persistent explosives residues. 2,4,6-Trinitrotoluene (TNT) is one of the most recalcitrant and toxic of all the military explosives. The lack of affordable and effective cleanup technologies for explosives contamination requires the development of better processes. Significant effort has recently been directed toward the use of plants to extract and detoxify TNT. To explore the possibility of overcoming the high phytotoxic effects of TNT, we expressed bacterial nitroreductase in tobacco plants. Nitroreductase catalyzes the reduction of TNT to hydroxyaminodinitrotoluene (HADNT), which is subsequently reduced to aminodinitrotoluene derivatives (ADNTs). Transgenic plants expressing nitroreductase show a striking increase in ability to tolerate, take up, and detoxify TNT. Our work suggests that expression of nitroreductase (NR) in plants suitable for phytoremediation could facilitate the effective cleanup of sites contaminated with high levels of explosives.

The manufacture, use, and disposal of explosives over the last hundred years has resulted in serious widespread contamination of the environment. Apart from the possible hazard of detonation, the progressive accumulation of explosive residues results in the environment becoming hostile to biological systems. TNT is one of the most highly toxic and recalcitrant explosives[1]. Currently incineration is the only effective treatment for TNT-contaminated soil, but this process produces unusable ash and has raised concerns about air pollution due to incomplete combustion. Large areas of land remain contaminated and continue to be polluted worldwide, and such demanding and expensive remediation procedures are clearly not an option for developing countries. The lack of affordable and effective cleanup approaches therefore demands the development of novel remediation processes. Recent attention has focused on phytoremediation, which is the use of plants to remediate environmental toxicity.

Plants have potentially impressive economic benefits as a robust and renewable resource. They have a remarkable ability to extract compounds from the surrounding environment, and their root systems are generally extensive and promote dramatically increased microbial numbers and activity in their rhizosphere[2,3]. Phytoremediation is encumbered by certain limitations that prevent wide-scale benefit from its significant economies. For example, plant species that accumulate metals to high levels are hampered by low biomass, and studies using trees have shown the chlorinated hydrocarbons, such as trichloroethylene (TCE), are taken up and degraded by the trees, but the degradation rates are low. Genetic engineering offers a way to overcome such restrictions; for example, the expression of microbial metal resistance genes and a mammalian cytochrome P450 has enabled plants to transform methylmercury[4] and TCE, respectively[5].

The potential for phytoremediation of explosives by both wild-type and transgenic plants has recently come under investigation. Explosives are phytotoxic compounds, and plants have been shown to possess detoxification systems resulting in their transformation[6–10], but the precise nature of these activities and the enzymes responsible have yet to be identified. Despite these innate detoxification activities, on exposure to low concentrations of TNT, plants have been shown to exhibit a range of adverse effects including stunted root and shoot development and bleaching[6,11]. Importantly, French et al.[11] demonstrated that the introduction and expression of a bacterial enzyme PETN reductase resulted in the enhancement of tobacco plants' ability to detoxify the explosive nitroglycerin.

The enzyme nitroreductase (NR) from *Enterobacter cloacae* has been found to be active against TNT (ref. 12). The enzyme utilizes NAD(P)H as a source of reducing equivalents to catalyze a two-electron reduction of TNT to HADNT, which is subsequently reduced to the ADNT derivative (Fig. 1). In this study we demonstrate how transgenic tobacco plants expressing NR are able to tolerate and detoxify TNT at levels that are commonly found in contaminated sites. Considering the availability of many microbial genes mediating detoxification systems and degradative pathways, this approach of engineering plants to remove toxic pollutants has the potential of providing an efficacious means of cleaning up land areas that have been polluted through military and industrial activities.

Results

Construction and selection of NR plant lines. The gene encoding the nitroreductase (*nfsI*) from *E. cloacae* NCIMB10101 was modified by PCR to contain a consensus start sequence AACAATGG to facilitate translation in plants[13]. The modified gene was transformed into tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks by *Agrobacterium*-

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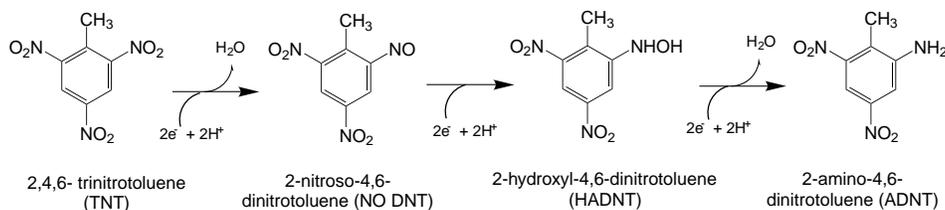
Figure 1. Proposed pathway for the reduction of TNT by nitroreductase.

mediated transfer. The transformation resulted in 25 independent T0 generation kanamycin-resistant plants that were selected for further investigation. PCR was used to determine lines containing the *nfsI* transgene, using the same primers originally used to clone the bacterial gene[14]. Of the 25 lines tested, 22 were shown to possess the transgene, with DNA from wild-type tobacco showing no product (data not shown).

The T1 generation plants were allowed to self-fertilize, and the seeds were collected. Studies were undertaken on T2 plants to establish whether the presence of the *nfsI* transgene in plants conferred increased tolerance to TNT. Seeds were germinated on plates containing 0.25 mM TNT (the solubility limit of TNT in aqueous solutions is ~0.5 mM). Phytotoxicity was determined by observation of stunting of roots and/or shoots and yellowing of shoots (bleaching). In the absence of TNT, all transgenic lines were indistinguishable from wild type, indicating that the insertion of the bacterial gene had no obvious deleterious effect. The growth of all but one of the 22 transgenic lines showed enhanced tolerance to TNT when compared to wild-type growth. The most TNT-tolerant transgenic line (designated NR 3-2) displayed only moderate signs of phytotoxicity at 0.25 mM TNT, relative to the extreme phytotoxicity displayed by wild-type plants, and was therefore chosen for further characterization. Northern blot analysis of RNA extracted from all transgenic lines revealed expression of the *nfsI* transgene only in tissue from the transgenic plants. The transgenic line NR 3-2 had both the highest TNT tolerance of the seedlings and the highest *nfsI* messenger RNA (mRNA) concentration of all the transgenic lines tested (data not shown).

TNT toxicity and transformation studies. A toxicity study was conducted to determine the effect of TNT on wild-type and transgenic seedlings. Duplicate batches of 50 seeds from both the wild-type and NR 3-2 lines were surface-sterilized, germinated, and grown in liquid medium for 14 days. Seedlings were then weighed and equal amounts of biomass (11 g) were aseptically transferred to sterile flasks containing 0, 0.1, 0.25, and 0.5 mM TNT. Growth after seven days in the presence of TNT was then determined as plant wet weight.

Control plants grown without TNT in the medium appeared healthy, an indication that the toxic effects observed were solely due to the presence of TNT and not the result of submersion in medium. The wild-type plants gained 48% in wet weight and the NR 3-2 line gained 52%. At a concentration of 0.1 mM TNT, wild-type plant biomass increased by 1% wet weight (see Table 1). The transgenic NR 3-2 plants showed no phytotoxic effects and gained 28% wet weight. After seven days at 0.25 mM TNT, the wild-type plants showed considerable phytotoxic effects and a substantial loss of biomass (34% wet weight). In contrast, the NR 3-2 plants appeared healthy and gained 18% in wet weight (Fig. 2). A toxicity study (without weight measurements) was also conducted at 0.5 mM TNT over seven days, during which the wild-type seedlings died and NR 3-2 plants remained healthy (data not shown).

The concentration of TNT and its metabolites in the medium were then measured by HPLC. Both wild-type and transgenic plants exhibited the ability to remove TNT from the growth medium. The wild-type plants removed 78% of TNT at 0.1 mM TNT by the end of the study (168 h) (Fig. 3A). At 0.1 mM TNT, the transgenic NR 3-2 plant line removed 71% of the TNT within the first 6 h (Fig. 3B). All of the TNT was removed by NR 3-2 plants within 20 h. In incubations with 0.25 mM TNT, wild-type plants removed only negligible amounts of TNT from the growth medium (Fig. 3C). The transgenic NR 3-2 line, in contrast, removed 50% of the TNT within the first 6 h and all of the TNT within 72 h (Fig. 3D). Negligible amounts of ADNT metabolite(s) were produced by both plant lines at both concentrations of TNT. Flasks of TNT without plant biomass revealed no significant loss of TNT, but addition of 0.1 mM or 0.25 mM TNT to tobacco seedlings in liquid medium resulted in a recovery of ~70% and 80%, respectively, indicating adsorption of some of the TNT to the tobacco biomass (Fig. 3).

Root morphology. Root formation in the presence of TNT was investigated, as normal root formation is essential in any potential phytoremediation application. Wild-type and NR 3-2 seeds were aligned in a horizontal array and grown vertically in Mirashige–Skoog medium containing a range of TNT concentrations to observe root development. The root length and tolerance indices[15] are shown in Table 2. There was no notable delay in the transgenic seed germination compared with wild-type on the control plate, and both plant lines developed similar root lengths with extensive secondary root branching and root hairs (Fig. 4A). At 0.05 mM TNT, the wild-type seedlings showed phytotoxicity in the form of stunted roots (Fig. 4B). The NR 3-2 seedlings showed negligible phytotoxic effects and produced extensive root branching, root length, and shoot length compared with the untreated seedlings (Fig. 4B). However, both wild-type and transgenic lines produced roots with extensive root hair growth. At 0.1 mM TNT, wild-type shoot development was severely retarded at the four-leaf stage. Radicles were produced at a length of ≤2 mm and only two on average per seedling. The radicles appeared swollen, with no secondary roots and no visible root hairs. In contrast, the NR 3-2 line displayed minimal signs of phytotoxicity. Shoot and root length and root hair production were similar to those at 0.05 mM

Table 1. TNT toxicity studies with wild-type and transgenic plants[a]

TNT (mM)	Wild-type			NR 3-2		
	Weight before TNT (g)	Weight after TNT (g)	Weight gain/loss (g)	Weight before TNT (g)	Weight after TNT (g)	Weight gain/loss (g)
0	11.37 ± 0.17[b]	16.9 ± 0.28[b]	+5.52 ± 0.45	11.08 ± 0.05[b]	16.88 ± 0.18[b]	+5.79 ± 0.23
0.1	11.2 ± 0.14	11.3 ± 0.02	+0.13 ± 0.15	11.4 ± 0.95	14.65 ± 0.07	+3.24 ± 0.88
0.25	11.59 ± 0.01	7.59 ± 0.19	−4.00 ± 0.21	11.93 ± 0.18	13.95 ± 1.20	+2.02 ± 1.01

[a]Wild-type and transgenic seeds (50 per flask) were surface-sterilized, germinated, and grown for 14 days. Identical plant weights were established for each flask (in duplicate), and seedlings were then aseptically transferred to TNT-containing medium (or continued to grow without TNT). Seedlings remained in the presence of TNT for seven days, after which wet weights were measured to establish gain (+) or loss (−) of plant biomass. This was used as an indicator of phytotoxicity.
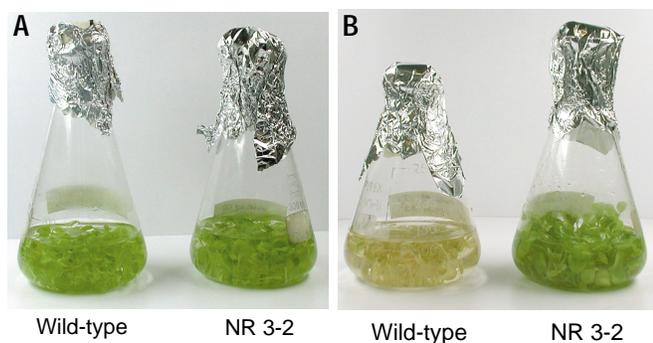[b]As TNT was not added to these flasks, weight measurements were taken at the same time as the addition of TNT to the other flasks.
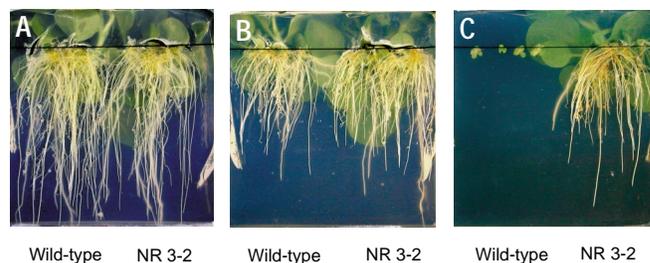
Figure 2. Growth of wild-type tobacco and transgenic NR 3-2 in liquid medium. (A) Fifty seeds per flask were surface-sterilized, germinated, and grown for 14 days. (B) Wild-type and transgenic seedlings were both incubated with 0.25 mM TNT in sterile water and remained in TNT solution for an additional seven days.

Figure 4. Growth of wild-type tobacco and NR 3-2 tobacco seedlings on explosives-containing medium 20 days after germination. Four of each seed type were planted on each plate. (A) Medium without TNT; (B) medium with 0.05 mM TNT; (C) medium with 0.1 mM TNT.

TNT. There was slightly less branching of the roots at this concentration compared with plants grown at 0.05 mM (Fig. 4C).

Analysis of TNT and its metabolites in seedlings. The TNT transformation studies with the NR 3-2 plant line indicated that TNT was completely removed from the medium and that minimal concentrations of TNT transformation product(s) were secreted by the seedlings into the media. To determine the concentration of TNT and possible metabolites in the seedlings, the plant tissue was extracted and analyzed by HPLC. The seedlings were separated into roots and shoots to evaluate TNT concentration in the different plant sections.

The results of HPLC analysis of the plant extractions revealed that TNT was found in both the wild-type roots (67 ± 28 nmol/g wet weight) and shoots (13 ± 6 nmol/g wet weight) (Fig. 5). ADNTs were also found in the roots and shoots again, with a higher concentration

extracted from the roots (~13 ± 3.2 nmol/g wet weight in contrast to 3 ± 0.33 nmol/g wet weight in shoots). No TNT was extracted from tissues of the NR 3-2 line. ADNTs (10 ± 3 nmol/g wet weight) were found in the roots with negligible concentrations in the shoots (0.47 ± 0.2 nmol/g wet weight).

Discussion

Earlier studies have shown that TNT and its metabolites have an inhibitory effect on plant growth resulting in the stunting of both root and shoot development[6,11]. To make any progress in the development of phytoremediation systems for TNT, this inhibition must be overcome. In this study, we demonstrated that transgenic tobacco seedlings expressing the bacterial nitroreductase (*nsfI* gene from *E. cloacae*) were able to tolerate TNT at concentrations up to the aqueous solubility limit of TNT. The levels of TNT tolerated by seedlings were significantly higher than the levels tolerated by germinating seeds. This implies that either the inhibitory effect of TNT on wild-type tobacco seedlings is more significant at germination, or the increased biomass or possibly elevated enzyme expression in older seedlings allowed more effective detoxification of TNT.

The weight studies (Table 1) revealed that the transgenic plants did show a reduced growth rate in the presence of TNT; however, the normal formation of the seedlings in the presence of TNT (Fig. 4) indicates that the transgenic plants are much more tolerant to TNT than the wild-type line.

Previous studies using whole plants and plant cell cultures have shown the transformation of TNT to ADNTs, which are found within the plant material and the surrounding growth medium[6–10]. The ADNTs are thought to be toxic and potentially carcinogenic; thus the lower the concentration released back into the plant's environment the more desirable the remediation system[1]. The wild-type plants were shown to extract a very limited amount of TNT from the growth medium, with the corresponding appearance of small amounts of ADNTs. The phytotoxic effects were clearly visible; the seedlings soon exhibited symptoms of extreme bleaching (Fig. 2), and transformation of TNT ceased (Fig. 3). In contrast, tobacco seedlings expressing NR were shown to be very efficient and removed all the TNT during the course of the experiment. These rates of TNT removal were considerably faster than were observed in similar studies with poplar cuttings[8] and produced negligible amounts of ADNTs.

TNT and metabolite extractions carried out on the wild-type and transgenic tobacco seedlings exposed to TNT showed an interesting distribution of TNT and ADNTs within the plant tissues. The concentration of TNT and the ADNTs in the roots of the wild-type seedlings corresponds well to the findings of other researchers working on a variety of plant species[9,10]. However, the complete submersion of plants in TNT solution will require that hydroponic experiments be conducted to determine transloca-
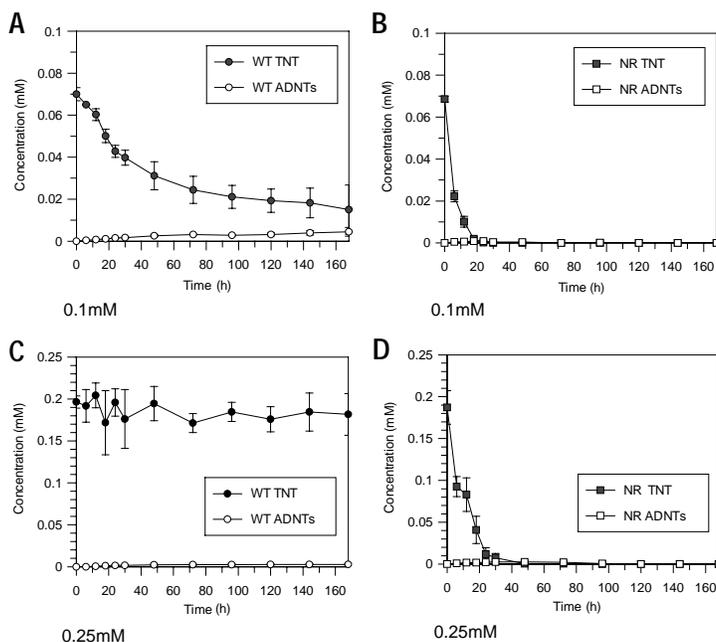
Figure 3. Transformation of TNT by wild-type tobacco and NR 3-2 seedlings. Wild-type and transgenic seedlings (50 per flask) were surface-sterilized, germinated, and grown for 14 days. Wild-type and transgenic seedlings were both incubated with 0.1 mM TNT in sterile water (A and B) or 0.25 mM TNT (C and D). Concentration of transformation products (ADNTs) were determined by HPLC. Results shown are the mean and standard deviation of three replicate flasks.

Table 2. Root growth for wild-type and transgenic seedlings after 21 days exposure to TNT[a]

TNT (mM)	Wild-type		NR 3-2	
	Root length (cm ± s.e.)	Root tolerance index (%)	Root length (cm ± s.e.)	Root tolerance index (%)
0	8.1 ± 0.1	100	8.1 ± 0.1	100
0.05	5.5 ± 0.6	68	6.2 ± 0.9	77
0.1	0.2	3	5.5 ± 0.9	68

[a]The results quoted are an average of the measurements made on 10 individual seedlings, and the tolerance index (root length of TNT-treated seedlings/root length of untreated control × 100)[15] was calculated for wild-type and transgenic seedlings at each TNT concentration tested.

tion. No TNT was extracted from the transgenic seedlings, indicating that it was either completely transformed or sequestered within the plant in a form that may be unextractable. Exhaustive attempts to extract and identify the conjugated transformation products proved unsuccessful. Plants are known to conjugate xenobiotic compounds with sugars, glutathione, amino acids, and malonic acid as part of their detoxification strategy[16]. The conjugates are then compartmentalized in the vacuole as cell wall material or lignin. A number of reports have suggested that this also occurs with TNT (refs 7,8) and its metabolites, explaining the difficulty most researchers have in extracting TNT and its metabolites from plant material[6,17]. The uptake and conjugation of TNT and its metabolites by the transgenic seedlings has great potential as a very effective phytoremediation process in which TNT is removed from the environment and bound up in the plant material before composting or harvesting and removal. Mass balance studies with ^{14}C-labeled TNT should elucidate the fate of TNT and its metabolites after compartmentalization in the transgenic and wild-type plants.

In conclusion, the data presented in this study clearly demonstrate that transgenic plants expressing a bacterial nitroreductase enzyme show enhanced ability to tolerate and detoxify the effects of TNT phytotoxicity. Further investigations are underway to determine the ability of these transgenic plants to remove TNT from soil. The enhanced metabolism demonstrated in transgenic tobacco indicates that the introduction of NR into fast-growing deep-rooted trees such as poplars, which are more suitable for phytoremediation purposes, could significantly increase TNT removal in the field. Such technology may provide the affordable, effective remediation systems that are urgently required.
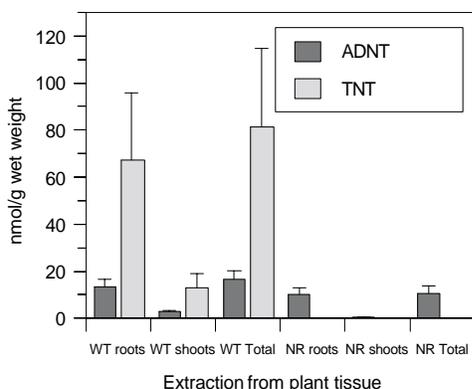
Figure 5. Extraction of TNT and transformation products from plant tissue. Wild-type and transgenic seeds (50 per flask) were surface-sterilized, germinated, and grown for 14 days. Both seedling types were incubated with 0.25 mM TNT in sterile water for an additional seven days before extraction was performed. Concentrations of ADNTs and TNT were determined by HPLC. Results shown are the mean and standard deviation of two independent studies.

Experimental protocol

DNA manipulation. DNA procedures were done according to standard protocols[18]. PCR was carried out using *Taq* polymerase (Gibco, Paisley, UK). For cloning of *nfsI* into plants, primers used were as follows:

Forward: G G AAT TC A ACA ATG GAT ATC ATT TCT GTC
*Eco*RI plant consensus sequence
Reverse: CG GGA TCC TCA GCA CTC GGT CAC AAT CG
*Bam*HI stop

Introduced restriction sites and base changes are underlined. The forward primer incorporates the modified start containing the plant consensus sequence[13] and *Eco*RI restriction site; the reverse primer incorporates a *Bam*HI restriction site. PCR conditions comprised 30 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C, 60 s extension at 72°C. The reaction volume used was 50 µl containing 50 pmol of each primer and 0.2 mM of each dNTP. Template was the *nfsI* clone, pNITRED1 (ref. 14). The PCR product was purified using the US–Bioclean kit of US Biochemical Corporation (Cleveland, OH), digested with *Eco*RI and *Bam*HI, and ligated into pART7 (ref. 19) and designated pNITRED2. Once constructed, the expression cassette, containing cauliflower mosaic virus (CaMV) 35S promoter, modified *nfsI*, and *nos* termination sequence, was excised using *Not*I and inserted in pART27 (ref. 19). This final construct was designated pNITRED3.

Transformation of tobacco. Construct pNITRED3 was introduced into *Agrobacterium* C58 using electroporation. Leaf disks of *N. tabacum* cv. xanthi were transformed as in Horsch *et al*[20].

Characterization of NR plants. Plant genomic DNA was prepared from leaves using the Phytopure kit (Scotlab, Coatbridge, UK).

Germination of seeds on TNT. Wild-type and NR seeds were planted on plates containing 0 mM, 0.025 mM, 0.05 mM, and 0.25 mM TNT, which was solubilized in *N,N*-dimethylformamide (DMF) before being added to warm Mirashige–Skoog (GM) agar (ICN Biochemicals, Basingstoke, UK). TNT was provided by the Defence Science and Technology Laboratory (Fort Halstead, UK). Approximately 100 seeds from each plant line were placed on the TNT plates under aseptic conditions. The plants were then allowed to germinate.

Toxicity study and transformation of TNT by plants. Wild-type and transgenic seeds (50 per flask) were surface-sterilized, germinated in 100 ml Mirashige–Skoog complete medium, and grown for 14 days in constant light at 25°C with rotary shaking at 121 r.p.m. TNT (solubilized in DMF) was added to the flasks under aseptic conditions. Samples were taken every 24 h for seven days and analyzed using a Waters 2690 HPLC using a Phenomenex C18 Ultracarb 250 × 4.6 mm column. Mobile phase consisted of 50:50 acetonitrile:water with 20 mM tetrabutylammonium dihydrogen phosphate (TBAP), pH 7. TBAP was added as the anion pair in the event that Hydride Meissenheimer complexes were formed. A 20 µl aliquot of each sample was injected onto the column. Retention times of known standards and their individual spectra were use to identify TNT and its transformation products. Standards of 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene were used for quantitation (Promochem, Herts, UK).

Extraction of TNT from plants. Plants used were those grown in flasks for the above study. Extraction of TNT and its transformation products was conducted according to Pavlostathis *et al*[5]. Extracts were concentrated by freeze-drying and resuspended in 500 µl pure acetonitrile. Samples of 100 µl of this concentrate were then analyzed by HPLC using the conditions described above to determine the concentration of TNT and its transformation products within the plant tissue.

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