SERDP

EXECUTIVE SUMMARY

Environmental Impact of DNAN and NTO on Plants

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SERDP Project ER-2723

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ACRONYMS AND ABBREVIATIONS

2ANAN	2-amino-4-nitroanisole
4ANAN	4-amino-2-nitroanisole
ADNT	amino dinitrotoulene
CDNB	1-chloro-2,4-dinitrobenzene
DAAN	2,4-diaminoanisole
DNAN	2,4-dinitroanisole
DoD	Department of Defense
GGT	gamma-glutamyl transpeptidase
GO	gene ontology
GSH	glutathione
GST	glutathione S-transferase
HADNT	hydroxylamino dinitrotoulene
HPLC	high-performance liquid chromatography
IMX-101	mixture of DNAN, NTO and 1-nitroguanidine
MATE	multidrug and toxin efflux
MDHAR6	monodehydroascorbate reductase 6
mRNA	Messenger RNA
MRP	multidrug resistance-associated protein
MS	Murashige and Skoog
NADH	nicotinamide adenine dinucleotide
NASC	Nottingham Arabidopsis Stock Centre
NTO	3-nitro-1,2,4-triazol-5-one
OPR	oxophytodienoate reductase
RDX	1,3,5-trinitrohexahydro-triazine
RNA	ribonucleic acid
SERDP	Strategic Environmental Research and Development Program
SPX	spexin
T-DNA	transfer DNA
TNT	2,4,6-trinitrotoluene
UGT	uridine diphosphate-glycosyltransferase

1.0 INTRODUCTION

Explosives are widespread chemicals of concern at Department of Defense (DoD) training ranges such that both land and groundwater are severely impacted. Current munition compositions such as Composition B mainly comprise 2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitrohexahydro-triazine (RDX); both compounds are toxic and a threat to environmental ecosystems. Whilst TNT and its transformation products bind strongly to clay and organic matter in soils, RDX is far more mobile, posing significant off-base groundwater impact risks. New composition mixtures containing 2,4-dinitroanisole (DNAN) and 3-nitro-1,2,4-triazol-5-one (NTO) are currently being phased in as replacements for Composition B to improve the safety of munitions. Relatively low aqueous solubility of DNAN means that, like TNT, it binds to organic matter in soil. However, each successive reduction of the nitro groups to amines reduces the hydrophobicity and increases migration potential through the soil. NTO and reported transformation products are far more water soluble and so also highly mobile in soils. Therefore, the new composition mixtures pose significant risks of land and groundwater impact.

Little is known about the effects of DNAN and NTO on plants, except soil studies that showed DNAN and IMX-101 (mixture of DNAN, NTO and 1-nitroguanidine) were toxic to grasses (Dodard et al., 2013; Richard and Weidhaas, 2014). However, microbial degradation pathways of DNAN have been identified and characterized from mixed consortia and pure cultures under aerobic and anaerobic conditions (Fida et al., 2014; Hawari et al., 2015; Liang et al., 2013; Perreault et al., 2012; Platten et al., 2010) as well as an endophytic bacterium isolated from willow (Schroer et al., 2015). The currently understood biodegradation pathway involves reduction of nitro groups to aromatic amines via a series of nitroso-intermediates (Figure ES-1). Reduction of the *ortho* nitro group is preferred in the reactions. Coupling can occur between nitroso-intermediates and aromatic amines to form azo dimers. Functional groups of azo dimers can then undergo further transformations such as demethylation, demethoxylation, and dehydroxylation (Liang et al., 2013; Olivares et al., 2013, 2016a; Perreault et al., 2012). Longer term fates of these azo dimers are not known.

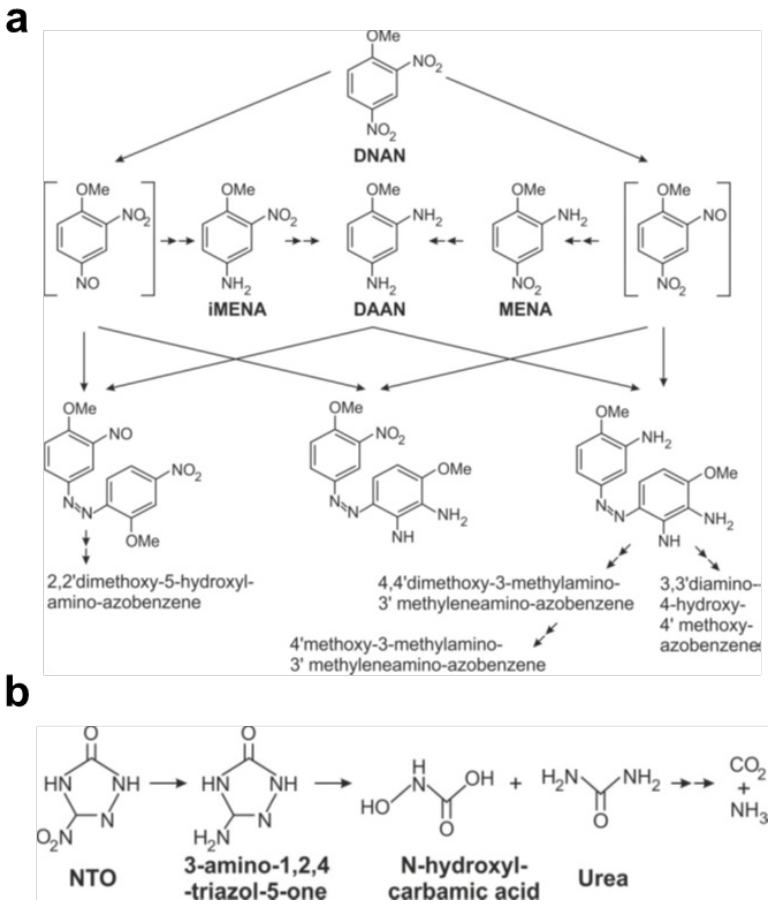

Figure ES-1. Summary of the Biotransformation Routes of (a) DNAN and (b) NTO.

Figure is based on Olivares et al., 2016a and Le Campion et al., 1999.

Less is known about the biodegradation of NTO. Microbial transformation studies in *Bacillus licheniformis* showed that NTO is transformed to 3-amino-1,2,4-triazol-5-one (ATO). Ring cleavage then occurs to generate N-hydroxyl-carbamic acid and urea (Le Campion et al., 1999) which can then be mineralized.

Toxicity studies on plants are limited and generally have been conducted using insensitive munition mixtures such as IMX-101. DNAN has been shown to inhibit seed germination and plant growth (Taylor et al., 2015) and is toxic to bacteria and earthworms (Dodard et al., 2013). Richard and Weidhass (2014) studied uptake of DNAN and NTO in the IMX-101 formulation but interestingly only found uptake of DNAN in the plant roots. However, the compounds were not in pure form and so the results may not be relevant to the study. Inhibitory concentrations of NTO in aquatic ecosystems have been determined on *Ceriodaphnia dubia* and *Selenastrum capricornutum* freshwater organisms (Haley et al., 2009). Toxicity thresholds are critical to understanding the ecological impacts of accidental release, better manage landscapes, and generate new plant DNAN- and NTO-resistant germplasms. Therefore, toxicity thresholds of DNAN and NTO require further research.

2.0 OBJECTIVES

In this project, the primary objective was to rigorously establish the fate and effects of DNAN and NTO on plants and the environment. This included understanding the physicochemical parameters of the compounds in the soil and assessing the toxicity of the compounds on plant species native to U.S. military training ranges. Further to this, the project team planned to elucidate mechanisms of NTO and DNAN toxicity in plants, identify *in planta* transformation intermediates of the compounds in Arabidopsis and, finally, resolve the biochemical pathways for the detoxification of these compounds in plants.

The findings from this project will facilitate the development of strategies to ensure the impact on DoD sites is minimal, thus mitigating costs associated with the deployment of future remedial and control technologies. The proposed work responds directly to the specific research objectives in Statement of Need number ERSON- 17-03 to determine effects of insensitive munitions compounds on vegetation in terms of toxicity and uptake.

3.0 TECHNICAL APPROACH

In Task 4, the project team first sought to establish the toxicity of DNAN on wildtype Arabidopsis plants by growing seedlings on ½ MS (Murashige and Skoog) agar plates containing a range of DNAN concentrations. A toxicity curve was then generated for DNAN and TNT as a comparison. After establishing the toxicity of DNAN, a high-throughput screen of Arabidopsis mutants was set up in order to try to identify genes that contribute to DNAN toxicity in the plant. Transfer DNA (T-DNA) insertional mutants were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Seeds were then germinated and grown up on ½ MS agar plates, in a 6-well plate format, dosed with 50 μM DNAN. After 14 days, seedlings were given a fitness score for ability to grow on 50 μM DNAN. Mutants with the highest fitness score were then grown on ½ MS agar plates and root lengths measured. Those with root lengths significantly greater than wildtype were identified and checked if the mutant gene was known to be linked to xenobiotic degradation pathways.

Previous studies that were funded by the Strategic Environmental Research and Development Program (SERDP) ER-1498 identified that monodehydroascorbate reductase 6 (MDHAR6) was a key driver of TNT toxicity in Arabidopsis plants. TNT and DNAN have high chemical similarity, thus the project team hypothesized that MDHAR6 may also play role in DNAN toxicity in plants. Wildtype and MDHAR6-mutant Arabidopsis plants were grown on media containing 50 μM DNAN and root lengths measured. Further to this, wildtype and MDHAR6-mutant plants were also grown on soil with DNAN at concentrations of either 0 or 100 mg/kg. MDHAR6 was recombinantly expressed, purified and assayed *in vitro* with DNAN substrate to confirm activity.

In Task 5, the project team sought to characterize the breakdown and transformation products of DNAN. First, liquid cultures were set up to compare phytotoxicity of DNAN and TNT across a range of concentrations. Fresh weights of plants were measured. Uptake and breakdown of the compounds were monitored using high-performance liquid chromatography (HPLC) by determining the concentration of each compound remaining in the media. Next, tissue of seedlings in shake flasks at day 4 and 7 were methanol extracted and analyzed for the presence of amino derivatives.

Toxicity of the amino derivatives on Arabidopsis plants was then determined by measuring root length from seedlings grown on ½ MS agar plates and measuring plant biomass from seedlings grown up in shake flasks. Finally, the project team set up hydroponic experiments in order to determine the concentrations of DNAN and transformation products in plant biomass. Plants were also grown in media containing TNT as a comparison. Plants were germinated and transferred to the hydroponic system with either 125 μM or 250 μM compound. Roots and shoots were separately methanol extracted and analyzed by HPLC.

In Task 6, the project team carried out transcriptomic analysis of Arabidopsis plants grown on DNAN- and NTO-containing media. Arabidopsis plants were grown up in shake flasks containing from 0-480 μM DNAN and the ribonucleic acid (RNA) extracted from plant tissue. RNA sample quality was assessed by TapeStation (Agilent) gel capillary electrophoresis. Messenger RNA (mRNA) was enriched for by poly(A) selection. Sequencing of mRNA was performed using the Illumina HiSeq 3000 platform. Gene ontology (GO) analysis was then performed on the differentially expressed RNA transcripts in order to identify genes associated with xenobiotic detoxification pathways. Interesting enzyme families were taken for recombinant expression and purification. The enzymes were then biochemically characterized to inform on their role in toxicity and biodegradation of DNAN and NTO in plants.

4.0 RESULTS AND DISCUSSION

4.1 TASK 4: ESTABLISHING THE MECHANISM OF DNAN AND NTO TOXICITY

Initial toxicity studies of DNAN and TNT on wildtype Arabidopsis plants established that both compounds significantly reduced root length and plant biomass. Toxicity was more acute at higher concentrations of TNT. Greater than 75% plant biomass was lost when grown on 50 μM DNAN, compared to approximately 98% plant biomass lost in the presence of 50 μM TNT. In the presence of 600 μM NTO, root lengths were greater than 30% smaller, seemingly less phytotoxic than TNT and DNAN. Next, T-DNA insertional Arabidopsis mutants obtained from NASC were grown up on 50 μM DNAN and given a fitness score based on the seedlings ability to grow on the media. Highest fitness lines (155) were then grown up on 50 μM DNAN and root lengths measured. Sixteen mutants had significantly greater root length than wildtype. However, none of these had mutations in genes known to be associated with xenobiotic degradation (Ramel et al., 2012). This report later detailed that some genes represented in this screen could degrade DNAN, such as the oxophytodienoate reductases (OPR) (Task 6). No observable change in root length was observed for these plant mutants, indicating redundancy in xenobiotic degradation pathways.

The previous studies (funded by SERDP ER-1498) identified that MDHAR6 was strongly associated with toxicity in plants exposed to TNT. *mdhar6* mutants grew significantly better than wildtype plants exposed to TNT (Johnston et al., 2015). DNAN and TNT share significant chemical similarity so *mdhar6* mutant and wildtype plants were exposed to DNAN. Mutants had greater root lengths and plant biomass. Soil studies nicotinamide adenine dinucleotide (NADH) replicated this data confirming that MDHAR6 had a role in DNAN toxicity in plants. Recombinantly produced MDHAR6 was also confirmed to have activity on DNAN, albeit minimal compared to TNT. In the presence of an NADH-recycling system, DNAN was not depleted by MDHAR6, also suggesting that, like TNT, toxicity in the plant was due to a futile cycle being established.

4.2 TASK 5: DETERMINING DEGRADATION INTERMEDIATES

Liquid cultures were set up in order to determine relative phytotoxicities of DNAN and TNT. Interestingly, whilst at 250 μM, the greatest decrease in biomass was observed for TNT, at 50 μM, DNAN caused the greater decrease in biomass. HPLC analysis of the media identified that TNT was rapidly depleted whereas DNAN persisted for much longer. This might explain the more chronic toxicity observed at 50 μM DNAN. Methanol extracted tissue from seedlings dosed with 250 μM DNAN indicated that plants could reduce DNAN to 2-amino-4-nitroanisole (2ANAN) (and a small amount of 4-amino-2-nitroanisole (4ANAN)). This conversion was likely a key step in detoxification of DNAN.

Nitro-reduced products have been reported to be less toxic in zebrafish and microbes (Liang et al., 2013; Olivares et al., 2016b), but little is known in regard to plants. The project team sought to establish the toxicity of 2ANAN, 4ANAN and 2,4-diaminoanisole (DAAN) to Arabidopsis plants. Interestingly, phytotoxicity experiments showed that DNAN significantly reduced root length and plant biomass, whereas 2ANAN only significantly affected plant biomass. This was likely due to the different developmental stages of plants used to measure root length and biomass. 4ANAN and DAAN had little effect at concentrations of less than 100 μM.

Locations of the xenobiotics in plants was investigated by a hydroponic system exposing plants to DNAN or TNT. Analysis of media indicated that DNAN was less readily taken up by the plants than TNT. Methanol-extracted tissue indicated that only nitro-reduced products 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene of TNT were present in the roots. However, DNAN and 2ANAN were detected in both roots and shoots of plants. These data suggest that TNT was more readily transformed by plants.

As for TNT and DNAN, liquid cultures were set up for NTO containing up to 1000 μM xenobiotic. Cultures had to be buffered with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to prevent acidification by NTO. Interestingly, very little difference in biomass was observed after seven days. This seemed at odds to the root lengths toxicity data but could have been due to the different developmental stages of the plants tested (as suggested for 2ANAN).

4.3 TASK 6: BIOCHEMICAL PATHWAY CHARACTERIZATION

The project team next sought to identify and characterize biochemical pathways involved in detoxification of DNAN. Seedlings were grown up in shake flasks dosed with concentrations ranging 0-480 μM DNAN. RNA was extracted from the plants and sequenced. Sequence analysis identified 2330 transcripts upregulated in 480 μM DNAN. GO analysis indicated that some of the most upregulated transcripts were associated with nitroreduction, conjugation, and transport. This was in agreement with previous studies on other xenobiotic compounds (Ramel et al., 2012). Of particular interest, a number of enzyme families were upregulated, including glutathione S-transferases (GST), uridine diphosphate-glycosyltransferases (UGT), and OPRs.

The project team previously demonstrated that GSTs could conjugate TNT to glutathione GSH) and thus prevent MDHAR6-driven phytotoxicity of TNT (Gunning et al., 2014; Johnston et al., 2015). Moreover, DNAN-GSH conjugates have been described in Arabidopsis (Schroer et al., 2017). Therefore, it was not surprising that 17 GSTs were upregulated in the presence of DNAN.

GST TAU subfamily 24 and 25 were most upregulated; these enzymes are known to conjugate TNT to GSH (Gunning et al., 2014). Notably, GST TAU 19 had by far the greatest abundance of transcripts. Nine of these GSTs were recombinantly expressed and purified from *E. coli*. Assays containing 1-chloro-2,4-dinitrobenzene (CDNB) and reduced GSH confirmed activity of each of these enzymes. Under similar conditions that conjugation of TNT was observed, the GSTs only had minimal activity on DNAN with no significant depletion of the compound observed.

UGTs are known to be upregulated in response to TNT, however conjugation to an activate nucleotide sugar group can only occur to the hydroxylamino dinitrotoulene (HADNT) or amino dinitrotoulene (ADNT) derivatives (Gandia-Herrero et al., 2008; Ross et al., 2001). In the presence of DNAN, 21 of the 107 Arabidopsis UGTs considered in the transcriptomic analysis were upregulated. Six of these are known to conjugate HADNT and ADNT. Future investigation into these enzymes is warranted to understand their possible role in conjugation of DNAN derivatives.

OPRs can catalyze the activation of TNT for conjugation to glucose. Through oxidation of NAD(P)H, OPRs concurrently reduce nitro groups to ADNT and HADNT products (Beynon et al., 2009). The Arabidopsis OPR family consists of six genes, whereby OPR4 and OPR5 are duplications of one another (Beynon et al., 2009). OPR1, OPR2, and OPR4/5 were upregulated in the presence of DNAN. OPR1, OPR2, and OPR4/5 were each recombinantly expressed and purified from *E. coli*. OPR1, OPR2, and OPR4/5 had activity on DNAN substrate, albeit minimal compared to TNT substrate. OPR1 and OPR2 were only expressed in plant roots, whereas OPR4/5 was expressed in plant shoots (Beynon et al., 2009). This suggested that OPR1 and OPR2 could contribute to accumulation of 2ANAN in the roots, whereas OPR4/5 contributed to accumulation in the shoots. Next, 35S-OPR overexpressor Arabidopsis plant lines were generated. 35S-OPR overexpressor lines were grown in media dosed with DNAN or TNT. After seven days, there was no significant difference in plant biomass from wildtype. However, analysis of the growth media hinted at some subtle differences. 35S-OPR1 lines contained significantly less DNAN in the media after seven days. Conversely, a significant increase in 2ANAN was observed in 35S-OPR2 line media. These data suggested that OPR1 and OPR2 may contribute to the breakdown of DNAN in plants.

The transcriptomic analysis also identified a few other notable genes upregulated, including five multidrug resistance-associated proteins (MRP). These proteins have been identified to have a role in transport of GSH conjugates (Klein et al., 2006; Lu et al., 1997). However, the most upregulated AtMRP3 was part of a different subfamily which did not transport GSH conjugates. GSH conjugates may undergo further processing inside the vacuole (Coleman et al., 1997). Gamma-glutamyl transpeptidases (GGT) catalyzed cleavage of Glu-Cys peptide bonds, of which Arabidopsis expresses four GGTs. Only GGT3 was localized to vacuoles but has been demonstrated to have activity on other conjugated xenobiotics (Ohkama-Ohtsu et al., 2007). However, this enzyme was not upregulated in the presence of DNAN in the analysis. Finally, an o-malonyltransferase and sulfotransferase were also upregulated. Indeed, malonylation of xenobiotics has been reported to increase their accumulation inside the cell (Taguchi et al., 2010). These enzymes warrant further investigation in their involvement of toxicity and detoxification of DNAN.

Transcriptomic analysis was also carried out on plants grown in the presence of NTO. Concentrations of 0-600 μM NTO were used, RNA extracted and sequenced. Significantly fewer transcripts were deferentially expressed in the analysis, indicative of the less phytotoxic effects of NTO on plants. Transcripts were upregulated in the presence of 600 μM NTO.

A number of gene families were upregulated including multidrug and toxin efflux (MATE) efflux transporters known to export xenobiotics (Gandia-Herrero et al., 2008), plant defensins that were implicated in heavy metal tolerance in metal hyperaccumulators (Mirouze et al., 2006), cytochrome P450s, SPX (spexin)-domain containing proteins that were involved in nutrient signalling (Duan et al., 2008; Secco et al., 2012; Wang et al., 2008), and GSTs already outlined.

GSTU5, 16 and 24 were upregulated in response to NTO and so were recombinantly expressed and purified for characterisation. Though GSTU5 and 24 were confirmed to have conjugating activity on CDNB to GSH, HPLC analysis indicated the enzymes could not conjugate NTO to GSH. These data suggested that the enzymes were likely unable to produce NTO-conjugates in plants.

Some of the upregulated proteins in the transcriptomic analysis were further examined by generating homozygous lines of single or multiple alleles. Seeds to this effect were obtained from NASC and included the MATE efflux transporters *ALMT1* and *DTX18*, *Allene oxide cyclase 2*, *SPX1* and *BBE/FOX1* (downregulated in the presence of NTO). 1/2 MS agar plate root lengths assays were performed; however no difference was observed to wildtype when dosed with 0, 300 or 600 μM NTO. These data suggested that these genes may not contribute to phytotoxicity of NTO.

5.0 IMPLICATIONS FOR FUTURE RESEARCH AND BENEFITS

In this project, the project team showed that DNAN exerted a more chronic toxicity on plants, evidenced by the slower breakdown of DNAN. NTO appeared significantly less toxic, though this may have depended on the plant development stage. Like TNT, DNAN toxicity was partially driven by MDHAR6. DNAN was reduced in the plant to the less toxic 2ANAN compound; however, the rate of conversion was markedly slower than that observed for TNT into its amino derivatives. DNAN also drove upregulation of a number of enzymes involved in xenobiotic degradation, including GSTs, UGTs and OPRs. In particular, the project team showed *in vitro* that recombinant OPRs could reduce DNAN and may also have increased breakdown of DNAN when expressed in plants. NTO drove upregulation of fewer enzymes, though a number of transporters and proteins involved in nutrient signalling were identified. A number of GSTs were also upregulated; however, biochemical characterisation of these enzymes could not conjugate NTO to GSH.

The combination of significantly slower transformation and conjugation steps of DNAN meant that plants were more exposed to the compound much longer than TNT. As a result, MDHAR6 was exposed to DNAN for longer, the key determinant of toxicity in plants. Moreover, lack of conjugation meant that DNAN remained more mobile in both root and aerial plant tissue. This posed a greater risk of toxicity to herbivores and the wider food chain. A number of studies have investigated microbial detoxification of DNAN, including identification of a *Nocardioides* species JS1661 strain that was able to breakdown the compound (Fida et al., 2014; Karthikeyan and Spain, 2016; Rylott and Bruce, 2019). However, without long-term effective, low-cost remediation strategies, DNAN poses a significant danger of leaving a poor environmental legacy similar to its predecessor, TNT. Further research is urgently required to address this.

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