

FINAL REPORT

Phytoremediation of Explosives from Contaminated Soil by Transgenic Grass

ESTCP Project ER-201436

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ACRONYMS AND ABBREVIATIONS

ADNT	Aminodinitrotoluene
ALA	α -aminolevulinic acid
AMC	Army Material Command
APHIS	Animal Plant Health Inspection Service
BRS	Biotechnology Regulatory Service
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CRREL	Cold Regions Research and Engineering Laboratory
DoD	Department of Defense
EPA	Environmental Protection Agency
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FMN	Flavin mononucleotide
GST	Glutathione-S transferase
GTase	UDP-glycosyltransferase
GTN	Glycerol trinitrate
HADNT	Hydroxylaminodinitrotoluene
HMX	High melting explosive (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine)
HPLC	High performance liquid chromatography
INRMP	Integrated Natural Resource Management Plan
IPTG	Isopropyl β -D-thiogalactopyranoside
ITAM	Integrated Training Area Management
LRAM	Land Rehabilitation and Maintenance
MEDINA	Methylendinitramine
MG	Mono glucoside
MMRP	Military Munitions Response Program
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NDAB	4-nitro-2,4-diazabutanal
NEPA	National Environmental Policy Act
<i>nfsI</i>	Gene encoding TNT-detoxifying nitroreductase from <i>Enterobacter cloacae</i>
NR	TNT-detoxifying nitroreductase enzyme from <i>Enterobacter cloacae</i>

OPR	Oxophytodienoate reductase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PETN	Pentaerythritol tetranitrate
PMSF	phenylmethanesulphonylfluoride
RDX	hexahydro-1, 3, 5-trinitro-1,3,5-triazine hexahydro- 1,3,5-trinitro-1,3,5-triazine (Royal demolition explosive)
TNT	2,4,6-Trinitrotoluene
UDP	Uridine diphosphate
USDA	United States Department of Agriculture
UW	University of Washington
UY	University of York
XplA	RDX-degrading enzyme from <i>Rhodococcus rhodochrous</i> 11Y
<i>xplA</i>	Gene encoding RDX-degrading enzyme from <i>Rhodococcus rhodochrous</i> 11Y
XplB	Reductase encoded adjacently to <i>xplA</i> in <i>Rhodococcus rhodochrous</i> 11Y genome
<i>xplB</i>	Gene encoding reductase next to <i>xplA</i> in <i>Rhodococcus rhodochrous</i> 11Y genome

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ABSTRACT

INTRODUCTION AND OBJECTIVES

Decades of military activity on live-fire training ranges have resulted in the contamination of land and groundwater by recalcitrant high explosives, in particular, TNT and RDX. While the transformation products of TNT, and TNT itself, are highly toxic, they tend to bind strongly to organic matter and clay in soil and so remain contained at the site of contamination. RDX however, is a major concern due to its high soil mobility. Contamination of RDX on training ranges, and subsequent contamination of groundwater poses a significant threat to drinking water sources. There are currently no cost-effective processes to remediate large areas of contaminated vegetated land on training ranges, or methods to contain the RDX. In previous SERDP-funded studies (ER-1318 & ER-1498), plants were genetically modified through the insertion of two genes, *xplA* and *xplB*, to degrade RDX from soils. The objective for this project is to demonstrate and evaluate, through the use of field-scale testing, the ability of XplAB-expressing grasses to contain and degrade RDX from explosives contaminated soil *in situ*.

TECHNOLOGY DESCRIPTION

We have shown that the unique cytochrome P450 enzyme XplA in combination with its partnering reductase XplB, from the soil bacterium *Rhodococcus rhodochrous* 11Y, metabolize RDX to produce nitrite, formaldehyde and the ring degradation products, 2-nitro-2,4-diazabutanal (NDAB; aerobic conditions) or methylenedinitramine (MEDINA; anaerobic conditions) (Jackson et al. 2007). In this report, plants genetically modified to express XplAB were assessed for their ability to remediate RDX-polluted soil.

TNT is often a co-contaminant alongside RDX, and is highly toxic, meaning that for the XplAB-expressing plants to remediate RDX, they also need demonstrative resistance to TNT. The bacterial gene *nfsI* encodes a nitroreductase (NR) which transforms TNT to the relatively unstable and non-toxic hydroxylamino dinitrotoluene (HADNT). This HADNT, and subsequent intermediate amino dinitrotoluene (ADNT) are both then conjugated to sugars (Gandia-Herrero et al. 2008) and ¹⁴C-labelling studies reveal subsequent incorporation into plant biomass (Brentner et al. 2010; Sens et al. 1999; Sens et al. 1998).

Initial work was conducted with *Arabidopsis*, understood genetically, but unsuitable for military land conditions. Thus, switchgrass (*Panicum virgatum*), western wheatgrass (*Pascopyrum smithii*) and creeping bentgrass (*Agrostis stolonifera*) were all transformed with the genes required to express XplA, XplB and NR.

PERFORMANCE AND COST ASSESSMENT

The performance of the transgenic materials was tested through four quantitative and three qualitative performance objectives. While we were not able to produce viable transgenic seeds nor significantly reduce soil levels of RDX, we did meet all the other performance objectives for the project.

Currently, the cost to implement the technology is heavily weighted to the requirements of the APHIS permit and monitoring process and the production of transgenic transplants. However, these costs will drop significantly if the materials can be deregulated and/or if viable transgenic seeds can be produced.

IMPLEMENTATION ISSUES

The main issue preventing the future implementation of XplAB-expressing switchgrass is regulatory, and specifically due to the transgenic nature of the plant. The switchgrass is currently limited to small, monitored testing under APHIS regulation 7 CFR part 340.

APHIS may be petitioned to show that a regulated article, such as XplAB-expressing switchgrass, is unlikely to pose a plant pest risk, and therefore should no longer be regulated under the plant pest provisions of the Plant Protection Act or the regulations at 7 CFR part 340. Deregulating the XplAB-expressing switchgrass will allow for both larger scale testing and widespread implementation.

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EXECUTIVE SUMMARY

INTRODUCTION

Decades of military activity on live fire training ranges have resulted in severe contamination of land and groundwater by recalcitrant high explosives, in particular, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). Both TNT and its transformation products are highly toxic, but as they bind strongly to clay and organic matter in soil, are largely contained at the site of contamination. However, RDX is a major concern because of its high mobility in soils and subsequent contamination of groundwater, which may move off base. RDX contamination of firing ranges is now proving to be a significant threat to drinking water sources, such as those close to the Massachusetts Military Reservation. These factors mean that RDX presents an immediate and future health problem to society. Additionally, once contamination is discovered, military operations at ranges can be suspended, interrupting soldier training.

Currently there are no cost effective, sustainable processes to contain or degrade RDX or to remediate large areas of contaminated vegetated land on training ranges. According to the EPA Technical Fact Sheet (Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), November 2017) there are a variety of methods to clean up RDX from military sites, however the processes are not environmentally friendly, they require space to perform these operations and often they require that the land be removed from military operations. Furthermore, given the scale of contamination, these methods would be prohibitively expensive and logistically challenging. Processes recommended include bioreactors, bioslurry treatments, composting, and passive subsurface biobarriers that have proven successful in reducing RDX concentrations. With the exception of very high rates of soil contamination, the technology proposed in this report will be more cost effective and a viable clean up method on ranges and manufacturing sites. Jenkins et al. (2006) has diagrammed a typical artillery training range showing that contamination can originate from firing points and within the impact area. Manufacturing areas can include those located within the Army Materiel Command (AMC) and other areas where RDX is produced.

While plants readily take up and accumulate RDX at low concentrations in the biomass, they can not significantly transform or degrade this compound. The technology demonstrated in this report uses commercially-available cultivars of species native to military ranges. These grasses have been modified to rapidly breakdown RDX into inert, naturally occurring plant metabolites that are reincorporated into the plant; plant harvesting is not necessary to remove the contaminated material from the site. An inadvertent benefit is that nitrogen-rich metabolites promote growth and establishment of the modified plants.

OBJECTIVES

The primary objective of this proposal was to evaluate, under field conditions, removal of RDX from contaminated soil by transgenic grasses that were developed under SERDP projects ER1318 and ER-1498. To achieve this objective, we assessed the ability of XplAB-expressing switchgrass to intercept and degrade RDX in field plots located at a military facility.

The secondary objective was to determine the lower bounds or reactivity of the transgenic plants. The lowest concentration of RDX in soil that the modified plants can remove and degrade is not known.

The EPA has established a lifetime health advisory guidance level of 2 µg/L for RDX in drinking water (2017 Fact Sheet). In our previous studies (Rylott et al. 2011), using soil-grown *Arabidopsis thaliana* we established a lower limit of detection of 21 µg/L. Conducting studies at 1mg/kg soil concentration would provide additional information on the capability of the plants to degrade RDX at lower levels. If successful, this information will be beneficial in predicting plant biomass requirements to meet drinking water standards, and will increase the use of the plants in additional areas containing less than 1 mg/kg of RDX, such as at firing points, wetlands, manufacturing, storage, and other sites.

The third objective was to conduct soil based, glasshouse studies to determine if plants can remove RDX in soil when grown in the presence of TNT. RDX is commonly found in soils with the co-pollutant TNT and the latter is highly toxic to plants.

TECHNOLOGY DESCRIPTION

The EPA Technical Factsheet details a number of methods for RDX clean-up at military bases, however, many of these are expensive or incompatible with the environment. The demonstration put forward in this report was the first of its kind to use vascular plants phytoremediate RDX-polluted soil, demonstrating that a biological system could provide help to maintain training activities occurring at the ranges. It has been demonstrated that the unique cytochrome P450 enzyme, XplA, in combination with its partnering reductase, XplB, from the soil bacterium *Rhodococcus rhodochrous* 11Y, metabolize RDX to produce nitrite, formaldehyde and the ring degradation products, 2-nitro-2,4-diazabutanal (NDAB; aerobic conditions) or methylenedinitramine (MEDINA; anaerobic conditions; Figure 1; Jackson et al. 2007). *Arabidopsis* plants expressing XplA and XplB have been shown to be able to remove saturating levels of RDX from soil leachate, whereas untransformed plants had no more impact upon RDX levels than control pots of soil without plants (Jackson et al. 2007; Rylott et al. 2011a). The technology is, therefore, innovative as it applies new methodology for the genetic modification of grasses to utilize novel explosive-detoxifying gene products.

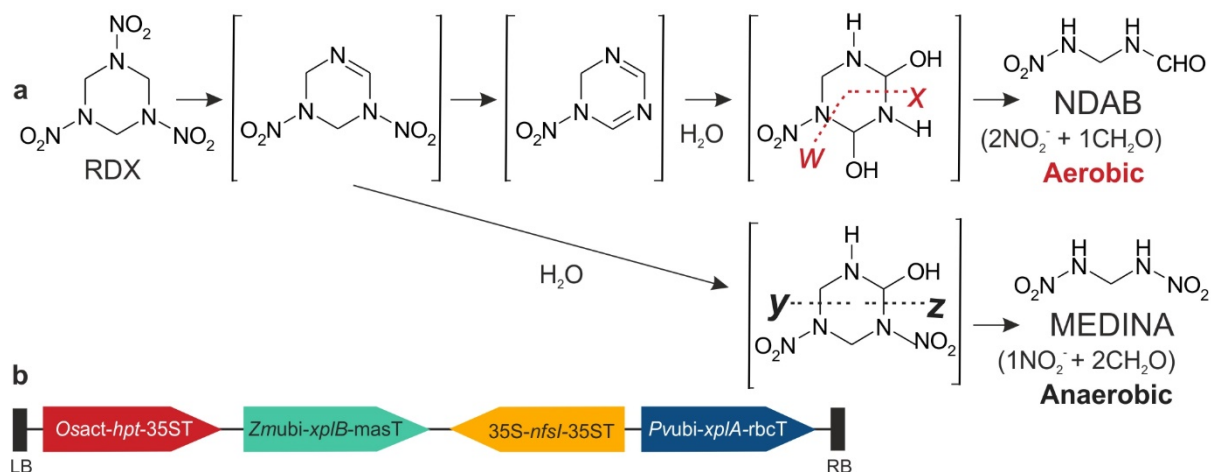


Figure 1. Pathway of RDX Catabolism by XplAB, and Transgene Cassette

(a) Ring cleavage occurs at wx and yz. Compounds in brackets are hypothetical, adapted from Jackson et al (2007). (b) The cassette transformed into the switchgrass lines described in Zhang et al (2017). The transgenes *xplA*, *xplB*, *nfsI*, and the selectable marker gene *hyg*, which encodes resistance to hygromycin, were controlled by the rice actin (*Osact*), maize ubiquitin (*Zmubi*), Cauliflower mosaic virus 35S, and switchgrass ubiquitin (*Pvubi*) promoters, respectively.

RDX is often found alongside its co-pollutant TNT, also highly toxic. For the transgenic plants to be able to thrive in soils polluted with both contaminants, they must also demonstrate resistance to TNT. Studies show that the *nfsI* encoded nitroreductase (NR) transforms TNT to the relatively unstable and non-toxic hydroxylamino dinitrotoluene (HADNT), which is then converted to amino dinitrotoluene (ADNT). Both HADNT and ADNTs are conjugated to sugars (Gandia-Herrero et al. 2008) and ¹⁴C-labelling studies reveal subsequent incorporation into plant biomass (Brentner et al. 2010; Sens et al. 1999; Sens et al. 1998).

The initial work was conducted with the model plant, *Arabidopsis*, which is understood genetically, but not well adapted to military lands. After demonstration of successful *XplA* and *XplB* expression, tobacco (*Nicotiana tabacum*), switchgrass (*Panicum virgatum*), and creeping bentgrass (*Agrostis stolonifera*) and western wheatgrass (*Pascopyrm smithii*) were all transformed with the genes required to express *XplA*, *XplB* and NR, and characterized switchgrass transformants for transgene expression levels and RDX degradation capability.

PERFORMANCE ASSESSMENT

Quantitative Performance Objectives

APHIS permit

Criteria required for the limited release of the transgenic grasses to the environment were met. A permit from the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) was required to enable controlled release of the transgenic plants into the environment. The APHIS permit was successfully awarded in 2016.

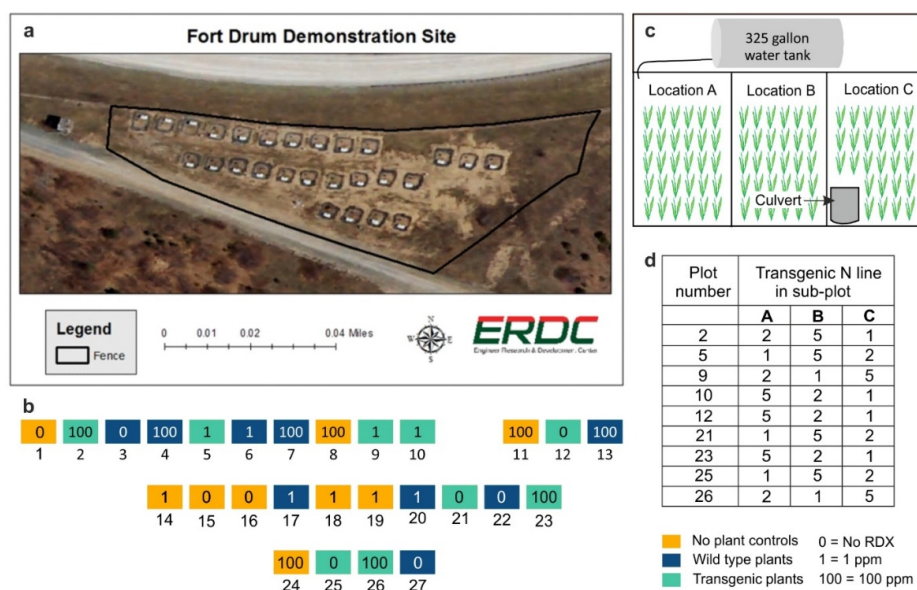


Figure 2. Plot Design and Lay-out

(a) Aerial photograph of the installed plots taken September 2015, (b) Randomized block lay-out of the plots, (c). Location of the plant lines on each plot, (d). Location of each of the three, independently-transformed XplAB-expressing lines N1, N2 and N5.

Field trial design

The lay-out of the plots are shown in Figure 2 and comprised a randomized complete block design with three soil treatments (1 mg/kg, 100 mg/kg and no RDX), three vegetation treatments (untransformed grass species, transformed grass species, and a no plant control) replicated three times for a total of 27 plots. Each vegetation plot was planted with approximately 250 clones, modified or unmodified, spaced on a 20 x 20 cm grid.

The cross-sectional design of each plot is shown in Figure 3a, and the appearance of a representative plot at the end of each growing season is shown in Figure 3b. Canopy closure was not fully obtained until the final growing season (2018). At the end of the trial, in the 100mg/kg RDX plots, the total aerial biomass of the transgenic plants was not significantly different to that of the wild type plants.

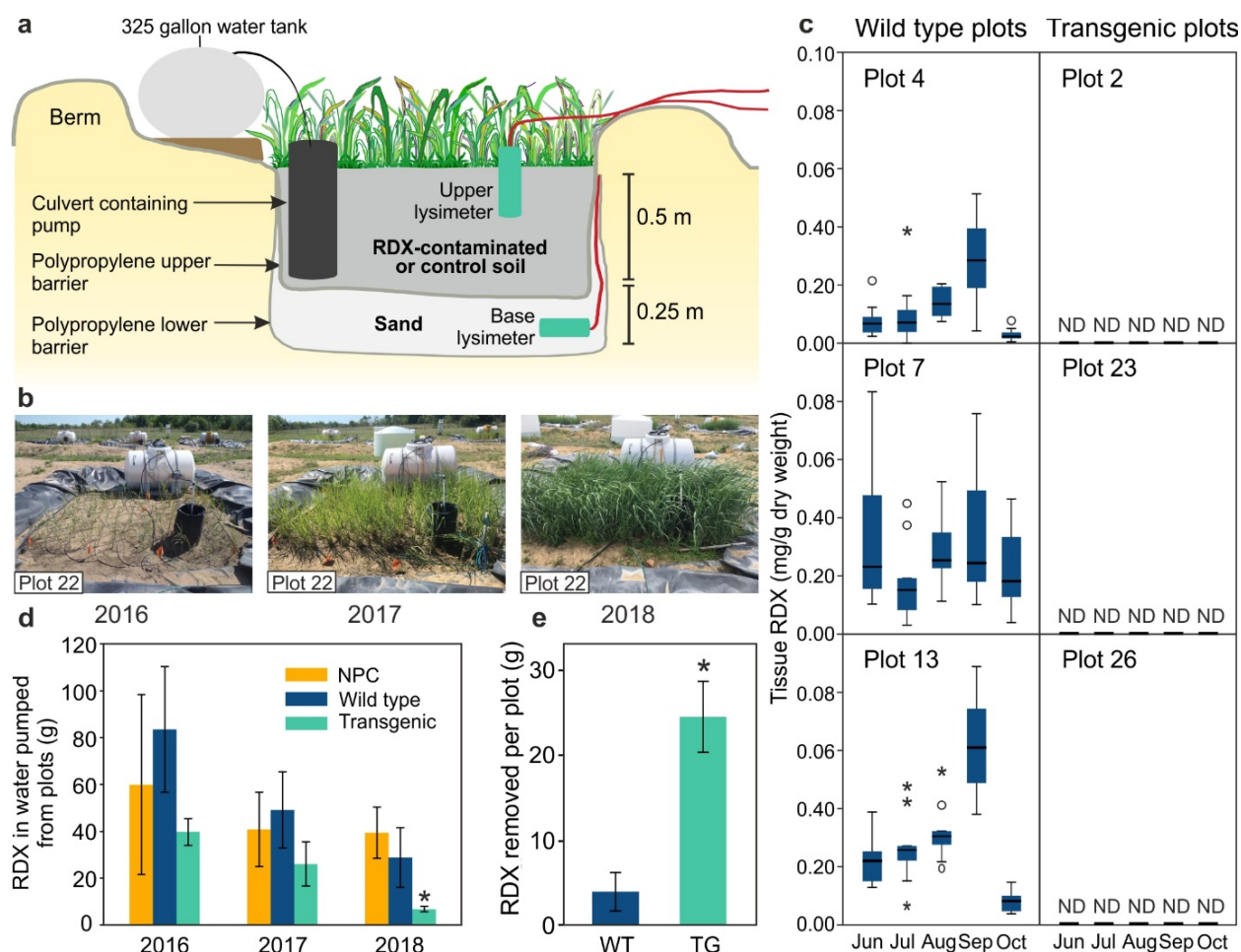


Figure 3. Plot Design and Performance

(a) Schematic showing a cross-section through a vegetated plot. (b) Appearance of the switchgrass on representative plots at the end of each growing season. (c) Levels of RDX in aerial tissues harvested across the 2018 growing season (ND = Not Detected, n = tissue from 9 plants). (d) Levels of RDX in the excess water pumped from the plots over the three-year duration of the trial (NPC; No Plant Control, n = 3 plots $\pm$ SD, * represents significantly different, $P = 0.015$, to NPC 2018). (e) Calculated level of RDX degraded in the transpiration stream from plants in the 100 mg/kg RDX plots over the 2018 growing season (WT; wild type, TG; transgenic, n = 3 plots $\pm$ SD, * represents significantly, $P = 0.015$). The statistical significance was performed by one-way analysis of variance followed by Tukey's *post hoc* test. $P < 0.05$ was considered as significant

For the 1mg/kg plots, RDX was not detected in any of the soil, water, or tissue samples, and data is presented for the 100mg/kg plots-only.

Removal of RDX by XplAB-expressing plants

For the soil samples, the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was not met. Our findings showed that the RDX was distributed heterogeneously across the plot, and unexpectedly, given its documented mobility in soils, RDX had not significantly moved below the 0-5 cm depth in the plots. In the field plots, the switchgrass roots were mainly in the lower (< 5 cm) depths of the plots and thus the majority of the RDX was not in contact with the plant roots.

For the soil-water samples, there was no significant difference in the levels of soil-water RDX in the NPC, WT, and TG plots across the 2018 growing season. However, the mean RDX level in the TG plot tanks was significantly less ($p = 0.021$) than in the tanks on the NPC plots, and at each month sampled, the RDX level was always lower than that in the NPC and WT plot tanks Figure 3d. Furthermore, across all three years, the concentration of RDX in the excess water pumped from the TG plots was always less than from either the NPC or WT plots. In the 2018 growing season, excess water pumped from the plots planted with the XplAB-expressing switchgrass contained significantly less ($p = 0.015$) RDX than excess water pumped from the NPC plots. Thus, we conclude from these data, that the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was partially achieved for soil water on the plots.

For the plant tissue samples, the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was met across all three growing seasons. During the 2018 growing season, levels of RDX in the aerial tissues of unmodified plants grown on the 100 mg/kg RDX plots varied within plots, between plots, and between monthly samplings. RDX was not detected in any of the aerial tissues sampled from the transgenic lines over the entire 2018 growing season, and across all three 100 mg/kg RDX plots Figure 3c. At the end of the 2018 growing season, the mean RDX concentration in the wild-type switchgrass from each of the three plots was 0.025 ± 0.02 mg/g dry weight. Previous measurements of tissue RDX levels using RDX-saturated soil column studies measured a maximum of 0.23 mg/g for wild-type switchgrass. Under these column conditions, RDX was detected in the XplAB-expressing switchgrass line N1, but at just 4 % of that in the unmodified switchgrass, suggesting that 0.23 mg/g is indicative of the likely upper limit for the RDX-degrading capacity of the transgenic lines in-the-field (Zhang, L. et al 2017), and that the XplAB-expressing plants in the 2018 season were at approximately 11 % of their RDX uptake capacity.

The levels of transpiration from each plot were estimated from the excess water pumped from the plots minus the rainfall measurements for the 2018 season. While it was not possible to determine the level of evaporation from the plots, given the near canopy closure for 2018 shown in Figure 3b, this factor was considered to be relatively low compared to that lost through transpiration. Multiplying the mean RDX concentrations in the soil water (from the lysimeter data) by the calculated volume of water transpired during the 2018 growing season, we calculated that the XplAB-expressing plants took up, and metabolized, significantly ($p = 0.002$) more RDX from the plots than the WT plants (Figure 3e), and equivalent to an RDX removal rate by the XplAB-expressing plants of 27 kg RDX per hectare. Based on our data in *Arabidopsis* (Rylott et al 2006; Rylott et al 2011), and grass species (Zhang et al 2017; Zhang et al 2018), it is likely that the wild-type plants did not significantly degrade any RDX.

Removal of RDX in the presence of TNT

RDX is commonly found in soils with the co-pollutant TNT and the latter is highly toxic to plants, and known to inhibit XplA activity (Jackson et al. 2007b). Alongside *xplA* and *xplB*, the switchgrass lines were also engineered to contain *nfsI* a nitroreductase that detoxifies TNT when expressed in tobacco (*Nicotiana tabacum*) (Hannink et al. 2007; Hannink et al. 2001). Gene expression studies on the XplAB-expressing switchgrass demonstrated that while the lines contained *nfsI*, transcript, or nitroreductase protein were not detectable. However, in the presence of TNT, the XplAB-expressing switchgrass lines, were able to remove significantly more RDX from the media than wild type, unmodified lines, indicating that the *nfsI* gene was expressed at albeit low levels. A possible reason was due to the design of the construct and gene promoters used. In our subsequent research, the viral 35S promoter and terminator for *nfsI* were replaced with a promoter from the monocot species rice (*Oryza sativa*) and the orientation of this expression cassette reversed. When transformed into western wheatgrass, the *nfsI* gene was expressed, and the plant lines removed significantly more TNT from media than unmodified, wild-type plants.

Qualitative Performance Objectives

At the end of the trial, the average aerial tissue biomass was 0.53 kg m⁻². Typical biomass yields for switchgrass are 1.08 kg m⁻² (Schneider & McCarl, 2003), thus, at the end of the three-year trial, the switchgrass had reached 50 % of its potential biomass. Despite the relatively poor biomass production, there was no significant differences in growth between the wild type and transgenic plants. The transgenic plants achieved the criterion that they were able to survive under field conditions as well as, or better than, wild type.

A number of techniques were employed at both UW and CRREL to gain seed from the transgenic lines, including the use of high-light growth chambers, a range of photoperiods, and outcrossing with wild type Alamo and Kanlow switchgrass. Under the conditions tested, viable seed was not obtained. Testing of vegetatively propagated materials demonstrated that the RDX-degrading properties were transferred to propagated material. For both the XplAB-expressing switchgrass, and western wheatgrass lines, viable seeds were not produced.

Throughout the process, the Regulatory Drivers outlined in section 1.3 of the final report were followed. APHIS personnel were involved from the start of the project, granting approval for testing of the XplAB-expressing lines under APHIS regulation 7 CFR part 340. Towards regulatory acceptance of the XplAB-expressing switchgrass, meetings with regulatory and DOD stakeholders, including APHIS, and the FDA have been held.

COST ASSESSMENT

Cost is an important part of the decision-making process when looking at remediation technologies. Currently there are no cost-effective processes to contain RDX or remediate large areas of contaminated vegetated land on training ranges. According to an EPA fact sheet (EPA 2017) there are a variety of methods to clean up RDX from military sites, however the processes are not environmentally friendly, they require space to perform these operations, and they require that the land be removed from military operations. Furthermore, given the scale of contamination, these methods would be prohibitively expensive and logistically challenging.

Cost model

The use of transgenic plant materials will reduce the costs associated with RDX remediation by reducing overall remediation costs. The main cost drivers associated with implementing this technology is given in Table 1. The basic cost model includes both fixed and variable costs. The fixed costs include the APHIS permit application, including the collection of site-specific data and the baseline characterization data. At most facilities the baseline characterization will have already been completed as part of the Military Munitions Response Program (MMRP). The required site-specific data may already be available through the facilities Environmental Office or, in the case of Army facilities, the Integrated Training Area Management (ITAM) program.

Table 1. Cost Model for Use of Transgenic Plants

Cost Element	Data Tracked During the Demonstration	Costs	
APHIS Permit	• Permit Application	To obtain permit 80 – 120 hours	\$4,000 - \$6,000
	• Site specific data (soil type, other related plant populations, T&E Species)	To review permit and provide necessary site data 40 hours	\$2,000
Baseline characterization	• Sample collection and data analyses	Field technician, 120 h Project engineer, 40 h	\$3,000 \$2,000
	• Detailed contaminant assessment required, costs associated with labor analyses	Chemical Analyses 6 – 10 composite samples per m ²	\$15,000
Plant production cost	Unit: \$ per transplant • Plants required per m ² • Area to be treated (m ²)	\$0.50 17 transplants per m ² \$8.50 per m ²	
Installation	Unit: \$ per m ² or ha	Planting Labor \$8.62 per m ²	
	• Mobilization cost	This is a variable cost that will depend on the area to be treated.	
	• Time required	0.34 hours per m ²	
	• Irrigation system with storage tank • Data logger, soil moisture sensors, and batteries	\$1,500 \$6,000	
Waste disposal	Standard soil disposal, no cost tracking	NA	
Operation and maintenance costs	• Monitoring time required for APHIS permit	4 hours per 100m ² every 2 weeks during growing season	
Long-term monitoring	• Standard soil and water analysis	Depends on site requirements	
	• Monitoring plant escape (monthly)	0.5 hours per 100m ²	

Average person can plant 50 plants per hour by hand. A typical person can thus plant 2.9 m² per person per hour, with a total of 23.2 m² per person per 8 hour day.

If we look at a simplified example for the use of our technology on a small (100m²) contaminated site, the typical costs would be:

Fixed costs:

Permit Application	\$ 6,000
Site specific data summary for permit	\$ 2,000
Baseline characterization of contamination	\$20,000

Variable costs:

Plant production (17 plants m ² * 100m ² = 1700 plants * \$0.50) =	\$ 850
Planting labor (\$8.62 per m ² * 100 m ²) =	\$ 862
Irrigation system to help establish transplants	\$ 1,500
Datalogger, sensors, and batteries – one of these is needed per site	\$ 6,000
Long term site monitoring escape (1 hour per month per 100m ²)	\$ 25
Long term soil and water monitoring per year	\$20,000

This would give a total for the fixed costs of \$28,000 per permit and a total of variable costs of \$29,237 per 100m² per year. It is important to note that permit fixed costs would be reduced after the first permit for a site is issued, as that data could be duplicated for each subsequent permit application. Also, if the plants can be deregulated, the cost associated with the APHIS permit application, site monitoring and maintenance activities and long-term monitoring would be reduced to zero. Thus requiring only the cost of plant production, planting and long term soil and water monitoring.

IMPLEMENTATION ISSUES

The main issue presented to the future implementation of XplAB-expressing switchgrass is regulatory, specifically due to the transgenic nature of the plant's development. The switchgrass is currently limited to small, monitored testing under APHIS regulation 7 CFR part 340. There have been discussions with both APHIS and the FDA on the topic of deregulating the XplAB-expressing switchgrass, and a white paper (Appendix D) on the need and likelihood of deregulation has been submitted to ESTCP.

The petition for deregulation must provide information under § 340.6(c)(4) related to plant pest risk which APHIS may use to determine whether the plant will pose more of a plant pest risk than the unmodified plant. If APHIS decides that there is no greater plant pest risk, the GE plants would no longer be subject to these regulatory requirements.

1.0 INTRODUCTION

1.1 BACKGROUND

RDX is increasingly understood to be a serious environmental pollutant. The US Environmental Protection Agency (EPA) consider it to be a possible human carcinogen and have placed it on the Contaminant Candidate List 4, also issuing a lifetime health advisory guidance level of 2 µg/L for RDX in drinking water. Of further concern is that RDX is recalcitrant to degradation in the environment, and extremely mobile, readily moving into the groundwater. These properties have resulted in the buildup of RDX in soil and groundwater at military sites that have then threatened, or affected, human health, such as contamination of a sole source aquifer at Cape Cod, and the exposure of individuals to RDX in the Bickford Case in Utah.

The remediation of this pollutant is an intractable challenge: the scale is enormous, in the US alone, 10 million hectares of military land is contaminated with munitions components. Contamination is heterogeneously spread, often unmapped, with unexploded ordnance restricting ground-access. Furthermore, there is the need to contain and perpetually remediate continuing contamination on active ranges. Traditional remediation methods such as land fill, incineration or advanced oxidation, are more suited to small-scale, highly polluted areas, where clean-up time is short (< 3 years), and overall costs are relatively low. The US Department of Defense (DoD) estimated that the remediation of its active ranges alone using currently-available methodologies would cost between \$16 billion and \$165 billion. To address this remediation challenge, the testing of novel cost-effective technologies is necessary.

There are many potential benefits to using plants to remediate environmental pollutants: they are minimally disruptive, promote restoration ecology, and are aesthetically-pleasing, with high levels of public acceptance. Furthermore, for longer-term (>3 years) remediation projects, phytoremediation has the potential to be low maintenance and cost effective. In plants, following uptake, RDX is located almost exclusively in the aerial tissues. Unlike bacteria, plants have little inherent ability to degrade RDX, with stored RDX becoming biologically available through the food chain via herbivory or returned as RDX back to the soil when the plant dies. It has been previously demonstrated, in soil-based laboratory studies, that *Arabidopsis thaliana* (Arabidopsis) plants transformed with *xplAB* are able to remove saturating levels of RDX from soil leachate. In these XplAB-expressing plants, RDX is broken down into inert, naturally occurring plant metabolites that are then incorporated into the plant, with no plant harvesting necessary to remove the contaminated material from the site. An inadvertent benefit is that these nitrogen-rich metabolites also promote growth and establishment of the modified plants.

1.2 OBJECTIVE OF THE DEMONSTRATION

The primary objective of this demonstration was to evaluate, under field conditions, removal of RDX from contaminated soil by transgenic grasses that were developed under SERDP projects ER-1318, and ER-1498. These projects identified RDX-degrading bacteria and the genes responsible, *xplA* and *xplB*. The insertion of *xplA* and *xplB*, first into model and then military relevant terrestrial plants, significantly enhanced the ability of the plants to degrade RDX into inert components. It was proposed to use this technology to intercept and degrade RDX by growing perennial grasses in field plots located at Ft Drum, NY.

The secondary objective was to determine the lower bounds or reactivity of the transgenic plants. The lowest concentration of RDX in soil that the modified plants can remove and degrade is not known. The EPA's standard for RDX in drinking water is 2 µg/L. In previous greenhouse studies, soil-grown *Arabidopsis* (*Arabidopsis thaliana*) were treated with aqueous solutions of 40 mg/l RDX and levels of RDX in soil leachate were measured using HPLC. After seven days, there was no RDX detectable in the soil leachate from the modified plants (Rylott et al. 2011) Under these experimental conditions, the lower limit of detection by the HPLC was 0.021 mg/l (21 µg/L). Conducting studies at various soil concentrations will provide additional information on the capability of the plants to degrade RDX at lower levels. If successful, this information will be beneficial in predicting plant biomass requirements to meet drinking water standards and will increase the use of the plants in additional areas containing less than 1 mg/kg of RDX, such as firing points, wetlands, manufacturing, storage, and other sites.

A third objective was to conduct soil based, glasshouse studies to determine if plants can remove RDX in soil when grown in the presence of TNT. RDX is commonly found in soils with the co-pollutant TNT and the latter is highly toxic to plants.

1.3 REGULATORY DRIVERS

Due to the unique nature of the transgenic materials, proposed study location and materials to be remediated, additional time was required to receive a movement and release permit than for a typical agronomic crop (120 days). A close working relationship with the APHIS regulators was established to meet their requests for additional information, accessibility to the study site, and concurrence of positive control of the regulated materials at all time.

Military lands must be maintained in settings that provide the opportunity to practice individual and battle-focused tasks and missions in realistic and challenging conditions. As noted in the Army Environmental Quality Technology Program A (2.5.e) Sustainable Army Live-Fire Range Design and Maintenance guidance from the Office of the Assistant Secretary of the Army, there are three dynamics adversely affecting the Army's ability to continue to maintain its training mission: the continued growth of environmental regulations, encroachment, and the Army's transformation. Some of the current environmental regulations include the Endangered Species Act, the Clean Water Act, Clean Air Act, and the Comprehensive Environmental Response, Compensation, and Liability Act.

Throughout the DoD, land stewardship and management of its natural resources fall under the Sikes Act of 1960, which promotes "effective planning, development, maintenance, and coordination of wildlife, fish, and game conservation and rehabilitation in military reservations on military lands". Military land stewardship integrates natural resources management objectives with land warfare training requirements. Environmental Compliance requirements that address these issues include: 2.1.b "...Range and Road Maintenance" and 2.5.e "Sustainable Army Live-Fire Range Design and Maintenance". The management of lands is funded and prioritized through the Integrated Natural Resources Management Plans (INRMP) process used at every military facility.

The research objectives for this project needed to respond to these regulations and or processes by helping installations be good stewards of land resources and supporting the Sikes Act and its provisions for no net loss of training land.

Furthermore, transgenic plant materials are regulated by the United States Department of Agriculture Animal Plant Health Inspection Service Biotechnology Regulatory Service (USDA-APHIS-BRS). In order to test regulated materials in the environment, a Movement and Release Permit is required for all release sites. The release permit is in effect for three years, and each movement permit is in effect for 1 year.

Additionally, USDA-APHIS required the creation of an approved training program for all staff or contractors who would be working with the materials at the research area. This training also included the creation of Standard Operating Procedures (SOPs) for all activities occurring at the research site.

The USDA APHIS permitting process provided the project with requirements for the release of transgenic plants to the environment. This required outlining which transgenes would be tested for each plant species, comparing the transgenic lines to the untransformed line and documenting plant physiological and establishment data to determine statistical differences in the transformed versus untransformed plants in the laboratory. The approved release permit package is included in Appendix B.

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2.0 TECHNOLOGY

2.1 TECHNOLOGY DESCRIPTION

There are, presently, no cost-effective methods to clean up RDX at military bases. The EPA Technical Fact Sheet lists a variety of methods to do so; most of these are expensive physical or chemical treatments that are not compatible with the environment. This demonstration was the first of its kind and focused on the use of vascular plants to phytoremediate RDX in the soil, thus demonstrating a biological system that would help to maintain training activities being carried out at the ranges. Phytoremediation studies have shown that plants readily take up RDX, accumulating it in the leaves and shoots, but do not have sufficient metabolic capability to degrade it (Best et al. 1999; Just and Schnoor 2004; Yoon et al. 2006; Vila et al. 2007; Zhang et al. 2009; Brentner et al. 2010). It has been demonstrated that the unique cytochrome P450 enzyme, XplA, in combination with its partnering reductase, XplB, from the soil bacterium *Rhodococcus rhodochrous* 11Y, metabolize RDX to produce nitrite, formaldehyde and the ring degradation products, 2-nitro-2,4-diazabutanal (NDAB; aerobic conditions) or methylenedinitramine (MEDINA; anaerobic conditions) (Jackson et al. 2007). While these degradation products are detectable *in vitro*, they have not been found *in planta* and it is likely that they are metabolized by endogenous plant enzymes. It has been shown that Arabidopsis plants expressing XplA and XplB were able to remove saturating levels of RDX from soil leachate (Figure 4) whereas untransformed plants had no more impact upon RDX levels than control pots of soil without plants (Jackson et al. 2007; Rylott et al. 2011a). This technology is innovative because it applies new methodology for genetic modification of grasses to utilize novel explosive-detoxifying gene products. This is an original approach to the problem of containing and remediating RDX and TNT waste on military sites. Additionally, the technology involves grasses which are not only cost effective to use, but are known to military personnel, who are experienced with these plants.

RDX is commonly found with the co-pollutant TNT, which is also highly toxic. Thus, in order for transgenic plants to thrive in soils contaminated with both RDX and TNT they may require resistance to TNT at contaminated sites. Previous studies show that the *nfsI* encoded nitroreductase (NR) transforms TNT to the relatively unstable and non-toxic hydroxylamino dinitrotoluene (HADNT), which is then converted to amino dinitrotoluene (ADNT) (Hannink et al. 2007; Hannink et al. 2001). Both HADNT and ADNTs are conjugated to sugars (Gandia-Herrero et al. 2008) and ¹⁴C-labelling studies reveal subsequent incorporation into plant biomass (Brentner et al. 2010; Sens et al. 1999; Sens et al. 1998). Although the toxicity of the TNT transformed intermediates has been studied (Rylott et al. 2011b), there is extremely little information on the toxicity or bioavailability of these intermediates once they are conjugated and incorporated into plant biomass.

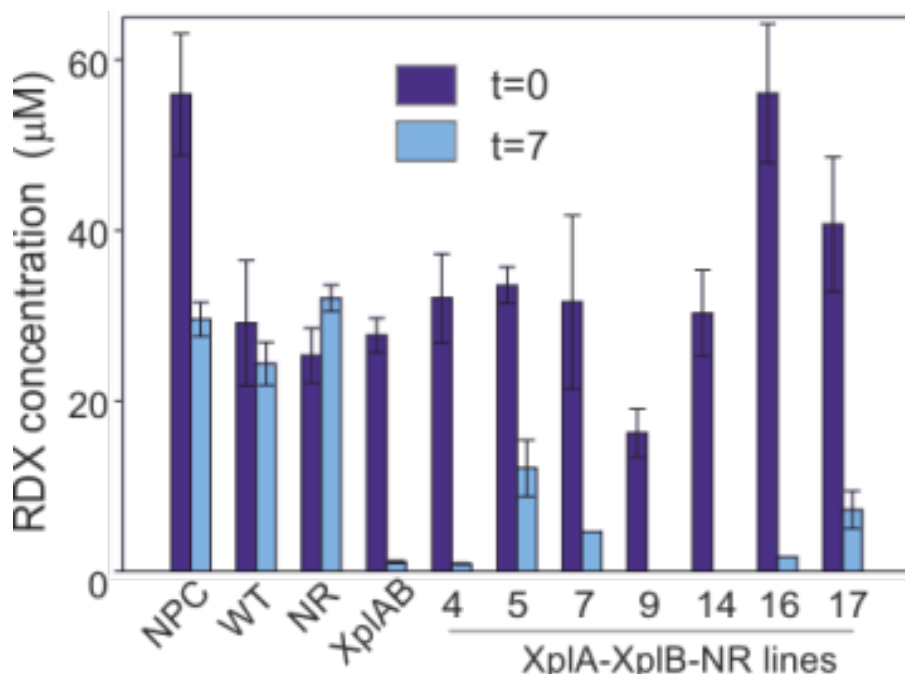


Figure 4. Levels of RDX in Soil Leachate from XplAB-expressing Arabidopsis

Plants were grown for six-weeks in uncontaminated soil then watered with 180 μ M RDX and flushed with an equal volume of water after 7 days. Results are the mean $\pm$ SE of eight replicate pots. NPC, no plant control; WT, wild type; NR, nitroreductase (adapted from Rylott et al. 2011).

2.2 TECHNOLOGY DEVELOPMENT

2.2.1 Vector construction

Four transgenes, *xplA*, *xplB*, *nfsI* and *hyg* were engineered into the switchgrass cultivar Alamo. The *hyg* gene, which encodes resistance to hygromycin, was used as a selectable marker to aid the identification of putative transformants. Following preliminary testing of prototype constructs (including pRCS2-HRM), the transgenes were near-constitutively expressed under the control of monocot promoters as follows: The *xplA* gene was under the control of the native switchgrass actin promoter *Pvubi-xplA-rbcT*; *xplB* under the control of the maize (*Zea mays*) ubiquitin promoter (*Zmubi-xplB-masT*); and *hpt* under the control of the rice (*Oryza sativa*) actin promoter (*Osact-hpt-35ST*). The rice actin promoter (*Osact*) and maize ubiquitin promoter (*Zmubi*) are widely used in monocot crops due to their ability to direct high levels of near constitutive gene expression (Cornejo et al., 1993; McElroy et al., 1990). The switchgrass ubiquitin promoter (*Pvubi*) has strong constitutive expression in switchgrass and rice (Mann et al., 2011). The TNT detoxifying bacterial nitroreductase was controlled by the cauliflower mosaic virus promoter, 35S (*35S-nfsI-35ST*). The pSAT vector series (Chung et al. 2005) was employed to clone the four genes into pRCS2-NABNR, the vector used to generate the switchgrass lines used in the field trial (Figure 5).

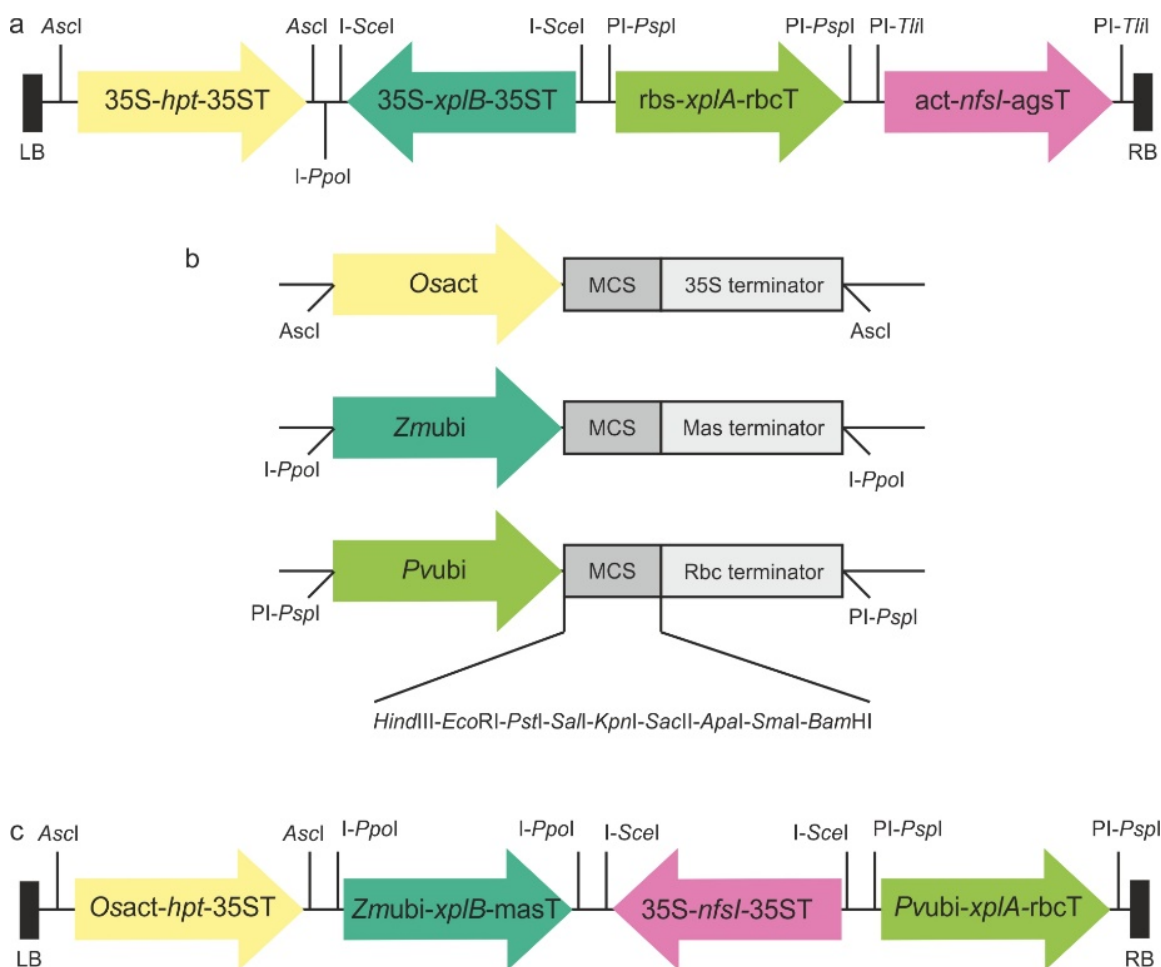


Figure 5. Construction of Vectors for Transformation of the Grasses

(a) T-DNA region of the binary vector plasmid pRCS2-ABNR-HR. The RDX degradation gene *xplA*, flavodoxin reductase gene *xplB*, and TNT detoxifying nitroreductase gene *nfsI* were constructed into versatile cloning vector pSATs (Chung et al 2005). Arrows show the direction of transcription. (b) The *Osact*, *Zmubi*, and *Pvubi* promoters were used to replace the promoters in the pSAT vectors resulting in pNSAT1a, pNSAT3a, and pNSAT6a respectively. (c) T-DNA region of the binary vector plasmid pRCS2-NABNR. The *hpt*, *xplA*, *xplB*, and *nfsI* genes were constructed into pNSAT1a, pNSAT6a, pNSAT3a, and pSAT4a respectively. The expression cassettes of these genes were integrated into the binary vector pPZP-RCS2 to produce pRCS2-NABNR. Abbreviations: 35s, CaMV 35s; *rbc*, rubisco small subunit; *act*, actin; *ags*, agropine synthase; *Osact*, *Oryza sativa* actin promoter; *Zmubi*, *Zea mays* ubiquitin promoter; *Pvubi*, *Panicum virgatum* (switchgrass) ubiquitin promoter; RB left border; RB right border.

Transformation of switchgrass, using a variety of promoters based on the pSAT vector system, has resulted in the development of scores of transgenic lines expressing *xplA/B* and *nfsI*. Preliminary constructs relied on strong promoters from dicots (i.e., not grasses), while newer constructs (pRCS2-HRM; HR lines and pRCS2-NABNR; N lines) used promoters from monocots, especially grasses. About 20 transformed lines have been verified by PCR and RT-PCR to contain and transcribe *xplA*, *xplB*, and *nfsI*. The presence of XplA protein in several of these lines has been confirmed by western blot analysis using antibody to XplA. RDX uptake studies showed the pRCS2-NABNR-transformed, N-lines to give the highest rates for RDX removal and degradation, so these plants were used for the subsequent characterization and field trials.

Switchgrass transformed using the pRCS2-NABNR vector removed RDX from media at 0.7 μg RDX/g plant tissue/h, twice that of the initial uptake by the wild type grass (Figure 6a). Importantly, RDX was recovered in the wild type plant tissues while transformed plants had no detectable RDX (Figure 6b), showing that RDX was degraded only in the transformed plants.

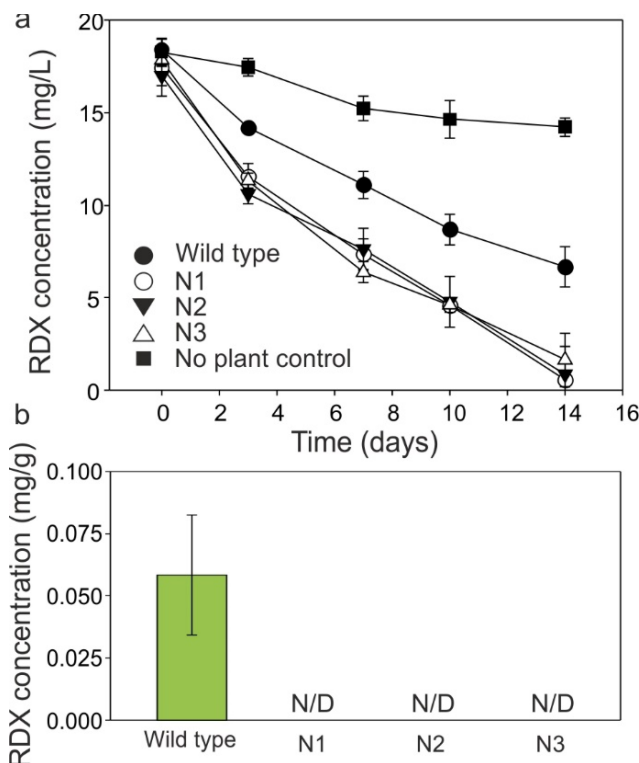


Figure 6. Uptake of RDX by XplAB-expressing Switchgrass Grown in Liquid Culture

(a) Concentration of RDX in culture medium over the course of the experiment. All three transgenic lines removed RDX from the medium at significantly faster rates than the wild type plants ($p = 0.051$, 0.0014 and 0.0016 for lines N1, 2 and 3 respectively at day 3; $p=0.0043$ for line N1 at day 7). (b) concentration of RDX in switchgrass tissue after 14 days ($n = 3 \pm SE$, N/D = none detected).

The function of RDX degrading genes in the transformed grasses was tested by measuring the rate of RDX removal from hydroponic solutions and the level of RDX in plant tissues at the end of the uptake test. Wild type plants have significant RDX in their tissue, though the total amount that the unmodified plants can take up is limited. Transformed plants have little or no RDX in their tissue, evidence that the RDX is degraded by the transgene product XplA. Several lines of transformed switchgrass have shown both improved RDX removal and less tissue RDX than the untransformed lines. Thus, there is confidence that the transformed switchgrass produced under SERDP ER-1318 and ER-1498 will be able to remove and degrade RDX from soil.

2.2.2 Transgenic plant development

The initial work conducted was with the model plant, *Arabidopsis*, which is well understood genetically, but not adapted to military lands. The successful expression of XplA and XplB was documented.

Tobacco (*Nicotiana tabacum*), switchgrass (*Panicum virgatum*), and creeping bentgrass (*Agrostis stolonifera*) were all transformed with the genes to express XplA, XplB and NR and characterized switchgrass transformants for transgene expression levels and RDX degradation capability (Zhang et al. 2017a; Zhang et al. 2017b). Several vectors that use a variety of promoters were constructed and transformed into switchgrass, with plant lines in various stages of regeneration and testing for RDX degradative potential.

RDX contamination occurs in a variety of climates and geographies, therefore for transgenic plants to be widely applicable to training ranges, additional species will need to be transformed, including additional switchgrass varieties. Towards this goal, a transformation protocol for western wheatgrass has been developed (Zhang et al 2018) and plastid-based transformation methods that will avoid the expression of the transgenes in pollen (Zhang et al. 2017b) have been investigated.

2.2.3 Herbivory risk assessment

An assay was designed to measure if herbivores exhibited any feeding preferences between the transgenic lines and unmodified wild type plants grown in the presence or absence of RDX. Pairwise comparisons were made between switchgrass lines N1, N2 and N5, and an unmodified, wild type.

To obtain plant material containing RDX, 25 cm sections were cut from fully expanded leaf blades from glasshouse-grown Switchgrass plants. The leaves were placed 12 per 50ml tube, in 40 ml of an aqueous solution of 150 μ M RDX, with 0.15 % DMSO. Treatment without RDX contained 0.15 % DMSO only. The tubes were wrapped in aluminum foil, to exclude as much light as possible from the RDX solution and were placed in polystyrene racks in a plant growth room with 16 h day-length. The leaf segments were allowed to take up the respective solutions for four days, after which the level of liquid in each tube had fallen to approximately 27 ml.

Leaf pieces were removed from the tubes, blotted dry then 3 cm cut off the basal end of each leaf piece, and the leaf piece trimmed to exactly 10 cm.

Leaf pieces were then fastened perpendicularly onto strips of cellophane tape, with the adaxial surface to the sticky side of the tape. Eight leaf pieces were arranged side-by-side on each strip of tape, with four pieces for each genotype/treatment, arranged pairwise. Leaf pieces overlapped the edge of the tape, at their basal end, by approximately 0.5 cm. The experimental setup is shown in Figure 7.



Figure 7. Experimental Design for Herbivory Experiments

Using the tape, the leaf pieces were then transferred to the inner surface of the wall of a 10cm square Petri plate and stuck down firmly so that they stood upright. The leaves were then photographed, by tipping the plate onto its side and placing a 20 cm square of 3 mm glass over the leaves, to flatten them. Once righted again, 80 ml water was poured into the Petri dish, making sure that the basal ends of the leaf segments were immersed in the water.

The lid was then placed on the plate and taped down firmly, so that it trapped and supported the leaves in their upright position. Each plate was placed inside a separate propagator tray (38 cm x 24 cm), with a well-fitting plastic lid, creating a ‘choice arena.’ A desk lamp was used to heat and illuminate each arena.

One 5th instar locust (*Schistocerca gregaria*) was then placed in each arena. Locusts had been starved of fresh food for a minimum of six hours prior to the experiment. Locusts were left with the leaf pieces until they had eaten approximately 50% of the leaf tissue of either sort. This process took between two and 24 hours, with each locust used only once. Following herbivory, the locusts were then removed, and the leaf segments photographed, as before. Surface area measurements were made using Image J software, developed by Wayne Rasband at National Institutes of Health and the Laboratory for Optical and Computational Instrumentation., and the results were compared before and after herbivory. Ten replicate experiments were set up, each with ten arenas, as described.

2.2.4 Results

Analysis of seven biological replicates showed that the locusts had no clear dietary preference for switchgrass leaves that had been treated with RDX solution over leaves that had been treated with 0.15 % DMSO only, or vice versa. This result was the same for the wild type as well as the transgenic lines (Figure 8).

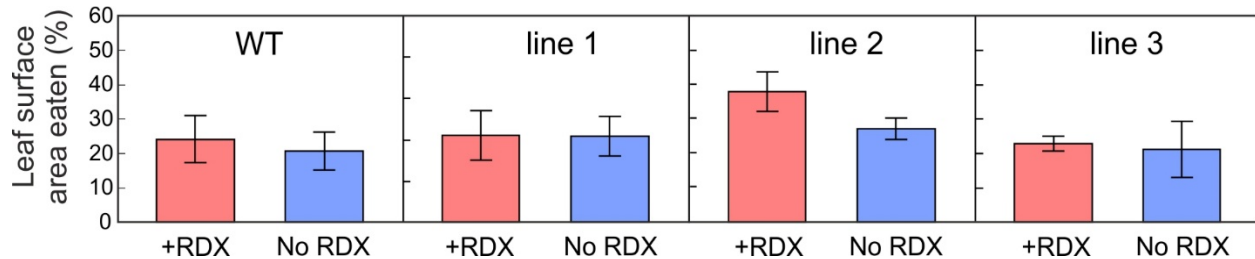


Figure 8. Herbivory Feeding Preference Testing in the Presence or Absence of RDX

Wild type (WT) and transgenic switchgrass lines.

The data in Figure 8 shows that the locusts exhibited no significant preference between the tissue grown in the presence, or absence, of RDX. Figure 9 shows that there was no significant preference between the wild type and transgenic switchgrass lines. However, there was a trend for the locusts to prefer wild type plant material over the transgenic lines, independent of RDX. On closer examination, it was noticed that the wild-type leaves had slightly different growth characteristics from those of the transgenic lines. The wild type leaves tended to be narrower, softer and suppler than those of the transgenic lines, features which may make them more palatable to locusts. The wild type line from CRREL-derived seed used in the herbivory experiments, has now been replaced with a wild type line from UW, which shares phenotypic characteristics indistinguishable from the transgenic lines. The results presented here indicate that during the field trial, biased herbivory, i.e., where foliar herbivores favor one plant line over another, would not occur.

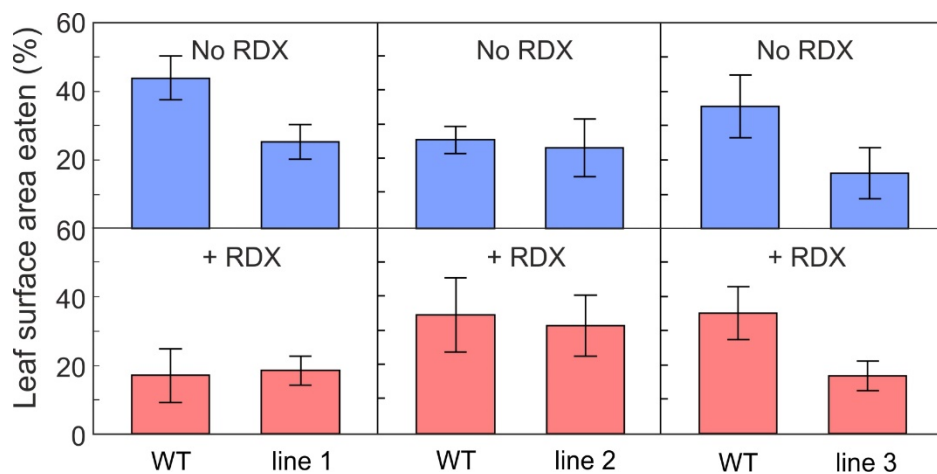


Figure 9. Herbivory Feeding Preference Comparisons between Plant Lines

Wild type (WT) and transgenic switchgrass lines.

2.2.5 Technology Maturity

For the demonstration to be successful, a method had to be developed to transfer the genes to a plant species that is adaptable to military sites. Published methods were used (Li et al. 2011; Luo et al. 2004; Yu et al. 2000) to successfully transfer the *xplA* and *xplB* genes into the model plants, *Arabidopsis*, and tobacco and later into creeping bentgrass and switchgrass. Switchgrass was selected as the primary demonstration plant as it is readily transformable, has a deep and robust root system, produces high biomass, and is a native species with a wide range of adaptability. It has also been shown, under SERDP and ESTCP project SI-0401, that switchgrass is compatible with the military mission and able to survive typical military training operations (Palazzo et al. 2012).

Transformation of switchgrass, using a variety of promoters based on the pSAT vector system, has resulted in the development of scores of transgenic lines expressing *xplA/B* and *nfsI*. Initial constructs relied on strong promoters from dicots (i.e., not grasses) (e.g., transformed lines designated HR), while recently constructed vectors (HRM and NABNR) have used promoters from monocots, especially grasses. About 20 NABNR and HRM transformed lines have been verified by PCR and RT-PCR to contain and transcribe *xplA*, *xplB*, and *nfsI*. The presence of XplA protein in several of these lines has been confirmed by western blot using antibody to XplA.

Switchgrass transformed using the NABNR vector removed RDX from media at 0.7 μg RDX/g plant tissue/h, twice that of the initial uptake by the wild type grass (Figure 10). Importantly, RDX was recovered in the wild type plant tissues while transformed plants had no detectable RDX (Figure 11), showing that RDX was degraded only in the transformed plants.

Using the RDX removal rate by transgenic grasses in vitro, the RDX removal by transformed switchgrass growing in training range soils was estimated, as follows. Switchgrass can grow to a biomass density of 10.8 tonnes/hectare (Schneider and McCarl 2003), which works out to about 3 kg plant wet weight/ m^2 . Thus, the potential removal rate of RDX per area of planting is about 2 mg RDX/ m^2 /h. If it is assumed an effective root depth is 1 m and an infiltration rate during a heavy rainfall event about 0.05 m/h, then the RDX contaminated water will be in contact with the root zone for approximately 20 hours. To calculate the rate of uptake by a switchgrass planting 0.8 μg RDX/g plant wet weight/h was multiplied by the 20 h root zone contact time. Thus approximately 50 mg RDX could be removed per m^2 of transformed switchgrass. Typical levels of RDX contamination around a target point are 0.05 mg RDX/kg soil (Walsh et al. 2003). If it is assumed that the RDX is distributed over a depth of 0.2 m and a soil density of 1200 kg/m^3 , then the areal concentration of RDX near the target would be approximately 12 mg RDX/ m^2 . This shows that by planting switchgrass there is a potential threefold increase in RDX removal rate than from unvegetated target areas.

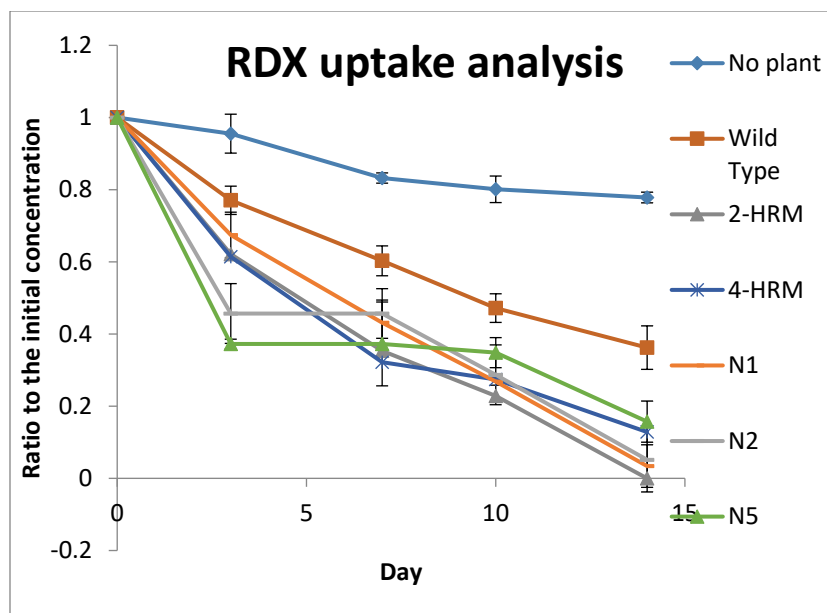


Figure 10. Removal of RDX from Hydroponic Culture by Wild Type and XplAB-expressing Switchgrass

Lines N1, N2, and N5 were NABNR transformed lines. Both HRM and NABNR lines used monocot promoters. Plants were incubated in open glass tubes each containing 5 mL of 18 mg/L RDX. Results are means of three replicates $\pm$ SEM.

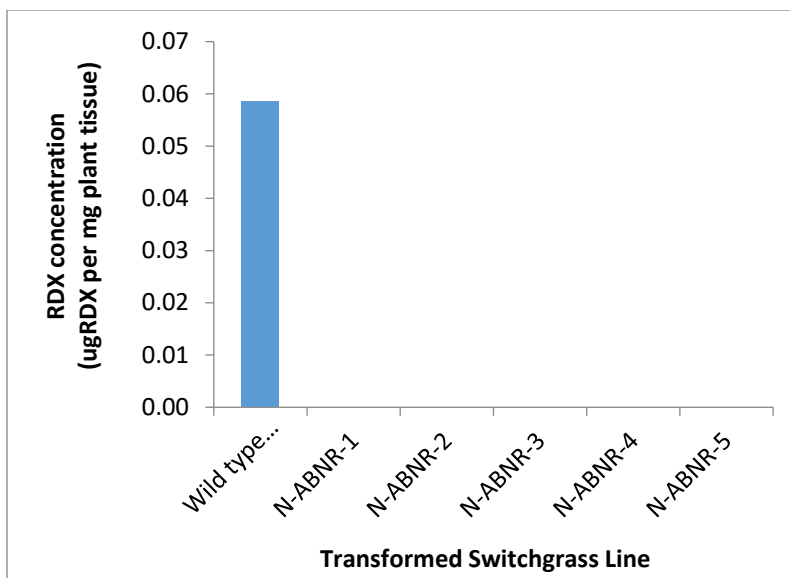


Figure 11. RDX detected in switchgrass tissue 14 days post dosing with 20mg/kg RDX.

The plant tissues were extracted in acetonitrile and the extracts analyzed by HPLC. Wild type plants were in duplicate because one of the replicate plants died.

2.2.6 Finalization of transgenic switchgrass characterization

To assess the level of transgene expression in the switchgrass N lines, quantitative PCR (qPCR) was used. For transcript analysis, mRNA was extracted from mature switchgrass leaf blades and Arabidopsis six-week-old rosette leaves using the Isolate II RNA Plant Kit (Bioline). Five micrograms of total RNA were used to synthesize cDNA using oligo (dT) 12-18 primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed using a StepOne Plus real-time PCR detection system with SYBR green (Applied Biosystems).

The following near-constitutively-expressed candidate genes, were tested for suitability as reference controls: a partial sequence of the 5.8S rRNA gene (GenBank: AM404348.1), eukaryotic Elongation Factor 1- α (eEF-1 α ; GenBank: GR876801) and eukaryotic Initiation Factor 4a (eIF-4a; GenBank GR877213) (Gimeno et al. 2014): Primers to these genes were designed using PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) with the following parameters: product size 50-200bp, T_m 58-60°C. Primer efficiency tests used serial dilutions of cDNA (5, 0.5, 0.05 and 0.005 ng/ μ l) in reaction mixtures containing 12.5 μ l SYBR green (Applied Biosystems Ltd), 5 μ L cDNA, 2 μ l 10 mM primers, 5.5 μ l sdH₂O. The reactions were run on an ABI machine. The amplification data (as Ct values) were plotted against cDNA concentration and the following R² values obtained: 0.191, 0.672 and 0.997 for 5.8S, eEF-1 α and eIF-4a respectively (for accurate quantification analyses, primer pairs are considered to require primer efficiency values of 100 % $\pm$ 5 %). Using StepOne software, version 2.3 (Life Technologies), primer efficiency values of -100, 5.63 and 100.97%, respectively, were obtained. Control reactions using previously designed primers for *xplB* gave an R² value of 0.99 and primer efficiency score of 97.11%. Based on these results, the eIF-4a primers PveIF-4aFq and PveIF-4aRq were selected (Table 2).

Table 2. Primers Used for qPCR

Primer name	Primer sequence (5' to 3')
PveIF-4aFq	TGATGTCATTTCAGCAAGCACAA
PveIF-4aRq	GGCATTCAACCAGGCCATAG
XPLA-Fq	CGACGAGGAGGACATGAGATG
XPLA-Rq	GCAGTCGCCTATACCAGGGATA
XPLB-Fq	CACCGCAATCGGTTTCG
XPLB-Rq	GTACAGGCCCGGAGCAAGA
NFSI-Fq	ACACGCCGGAAGCCAAA
NFSI-Rq	GGTGCATGTCTCGCGAAGTA

To provide RNA for primer efficiency tests, the top 20 cm of blades from mature leaves were ground in liquid nitrogen and RNA extracted using an RNAsy kit (Invitrogen). The qPCR was performed, using the primers shown in selected Table 2; results are shown in Figure 12a.

Expression levels for *xplA* and *xplB*, were broadly similar, showing 2 to 3-fold expression levels across all the lines (N1 to N8) tested, with plant line N4 exhibiting the highest levels of expression for these transgenes.

As a guide, qPCR was also conducted on the *xplA-xplB-nfsI* expressing Arabidopsis line 14 (Rylott et al. 2011a); transgene expression in this line conferred significant ability to remove RDX and TNT from contaminated media. However, while the expression levels of *xplA* and *xplB* were all significantly higher in the transformed switchgrass lines than in the Arabidopsis line 14, due to promoter and species differences, direct comparisons cannot be made. The levels of *nfsI* transcripts were significantly lower than for *xplA* and *xplB* in all the switchgrass lines (with the exception of line 7).

To determine protein levels from the transgenes, protein extracts were harvested from the top 20cm of mature leaf blades. Leaves were ground in liquid nitrogen, then 0.5 g fresh weight mixed, on ice, with 1000 µl protein extraction buffer (100 mM phosphate buffer pH 7 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol). Samples were centrifuged for 10 minutes, 10,000 rpm at 4 °C, then the supernatant transferred to fresh tubes. The protein concentration in the supernatant was determined using Bradford's Reagent (BioRad) with comparison to a standard curve generated using bovine serum albumin standards and eight micrograms of crude protein extract from leaf tissues was loaded per lane. Samples were mixed with 4 x sample loading buffer (250 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS and 0.05% (w/v) bromophenol blue), then heated at 95 °C for 5 mins, briefly centrifuged and loaded onto 10% SDS-PAGE gels, 5 µg protein per lane. Pre-stained protein markers were also loaded. Gels were electrophoresed in Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 200 v for 1 h. As part of the SERDP-1498 funding, antibodies specific to XplA, XplB and the nitroreductase NR, were produced in rabbit. These antibodies have been shown to specifically recognize their respective antigens, and we have published western blot analysis on XplA-XplB-NR-expressing Arabidopsis lines (Rylott et al. 2011a).

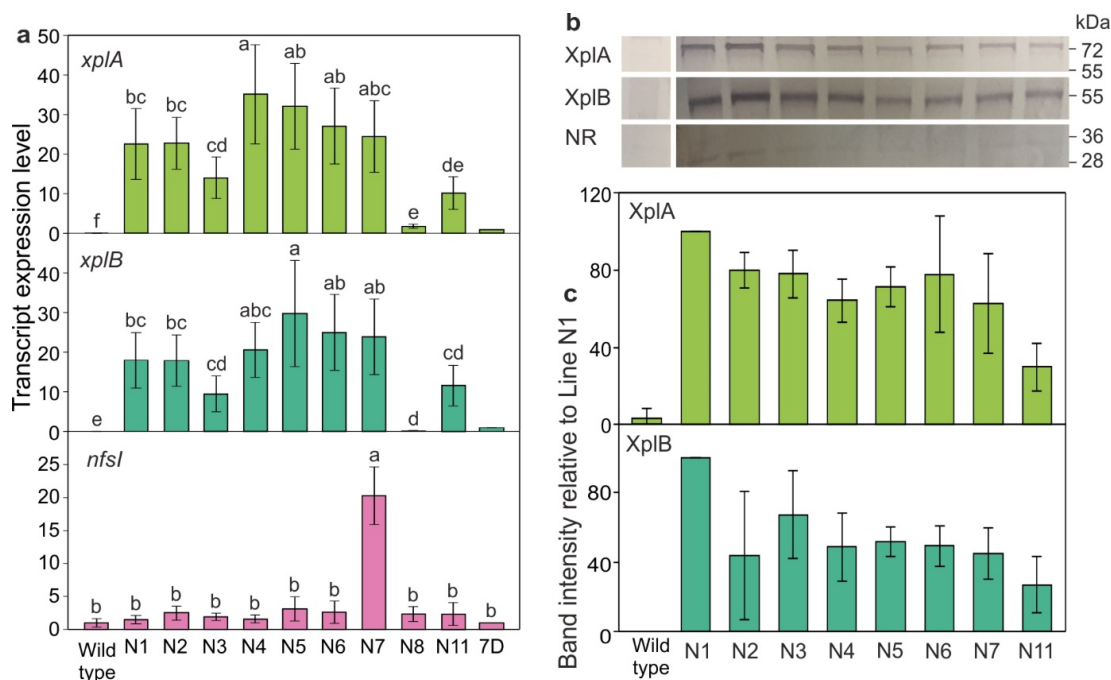


Figure 12. Molecular Characterization of XplAB-transformed Switchgrass

(a) Transcript abundance measured using quantitative RT-PCR on plant lines transformed with pRCS2-NABNR. Values were normalized to the switchgrass reference gene eIF-4a (Gimeno et al., 2014). Arabidopsis values were normalized to the reference gene ACT2. All values are relative to the expression levels of the xplA-xplB-nfsI expressing Arabidopsis line 7D (Rylott et al., 2011; $n = 4 \pm \text{SE}$). (b) Western blot analysis on leaf blades of switchgrass lines expressing XplA, XplB and nitroreductase (NR) protein. (c) Band intensities were quantified for XplA and XplB expression. Levels were normalized to the Coomassie-stained RUBISCO large subunit; results are from three replicate blots $\pm \text{SE}$.

To monitor integrity and loading uniformity of the proteins, gels were stained using InstantBlue (Expedeon). Unstained gels were blotted onto nitrocellulose using iBlot2 dry blotting system (ThermoFisher). Western blots were performed on the blotted membranes as follows: The blots were washed briefly in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), then blocked in PBS, 3% (w/v) BSA and 2% (w/v) milk powder for 2 hours. The blots were incubated in PBS, 3% (w/v) BSA containing a 1/1000 dilution (v/v) of the primary antibody, for 1 hour. The membranes were then washed twice with PBS, 0.1% Tween20 for 5 min, twice with PBS, 0.5% Tween20, 1M NaCl for 5 mins and rinsed briefly in PBS. The membranes were then incubated in PBS, 3% BSA containing the secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase) at a 1/20,000 dilution, for 1 h. The membranes were then washed twice with PBS, 0.1% Tween20 for 5 min, twice PBS, 0.5% Tween20, 1M NaCl for 5 min, and then rinsed briefly in PBS. The blots were developed using freshly made solutions from SigmaFAST tablets, then rinsed in water.

Photographs of the gels and blots were taken and the intensity of the protein bands quantified using ImageJ software. Band intensities were recorded from three replicate blots for each antibody and the mean and standard deviation recorded. The values were normalized to the corresponding Coomassie-stained, large subunit of RUBISCO, then expressed relative to switchgrass line N1.

The results shown in Figure 12b and c indicate that all eight lines tested were expressing significant, and broadly similar, levels of XplA and XplB with lines N1 and N3 exhibiting the highest combined levels of XplA and XplB expression. For NR expression, although bands were visible on blots probed with NR antibody, the band intensities were too low to quantify levels of NR using ImageJ.

2.2.7 Testing the RDX degrading activity in transgenic switchgrass

A ‘chopped leaf assay’ was developed as a robust, and relatively simple technique for measuring the RDX-degrading ability of switchgrass lines. The assay uses small (100 mg) quantities of fresh tissue, in non-sterile conditions, in combination with relatively simple, isocratic HPLC analysis, to measure RDX uptake over 0- 10 days. The setup was as follows: 100 mg of leaf blade material was chopped into 5 mm pieces and added to 5 ml of 200 μM TNT, or 200 μM TNT and 150 μM RDX, in 40 ml glass bottles. The bottles were loosely capped then incubated in the light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) on a shaker set to 100 rpm. Samples were taken at regular intervals and TNT and RDX levels measured using HPLC. Five replicate bottles were set up for each line.

RDX concentrations in culture media were analyzed with a modular Waters HPLC system consisting of a Waters 717 autosampler, two Waters 515 HPLC pumps, and a Waters 2996 photodiode array detector. A 4.6- by 250-mm Waters C18 column was used for separation, with concentration determined based on absorbance at 240 nm. Peak integrations and analyses were conducted using Millennium32 software (Waters, Milford, MA). The limit of detection of RDX by this method is 0.01 mg/L.

RDX uptake rates in the N lines, are shown in Figure 13. Leaves from all the N lines were able to remove RDX faster than wild type, unmodified leaves.

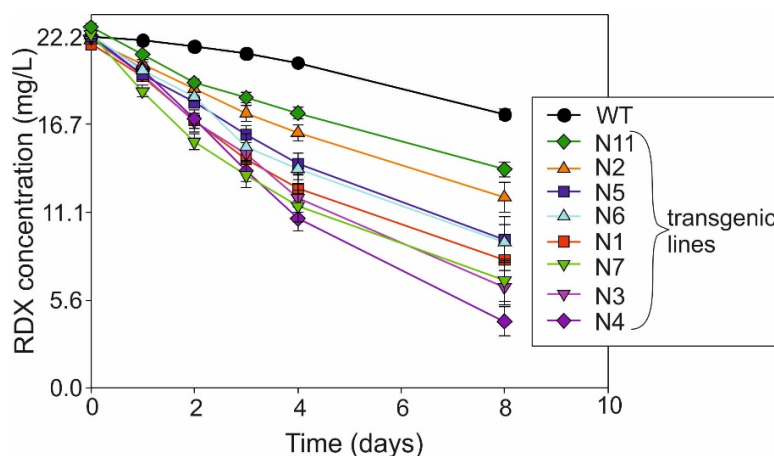


Figure 13. RDX in Media of XplAB-expressing Switchgrass Plant Lines Using Chopped Leaf Assay.

$N = 5 \pm SE$.

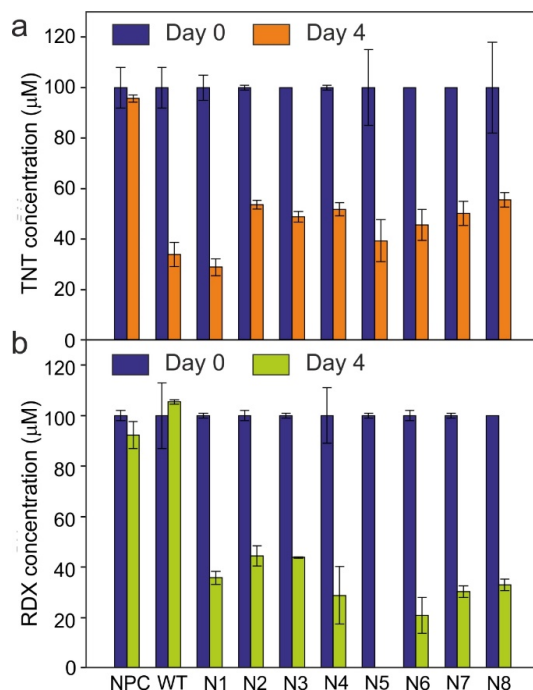


Figure 14. RDX and TNT Concentrations in Liquid Culture Grown XplAB-expressing Switchgrass

The ability of the N line switchgrass plants to take up both TNT and RDX was investigated using the chopped leaf assay. The results in Figure 14, show that all 8 lines removed less TNT from liquid media than wild type, unmodified plants. It is possible that the presence of RDX hinders the detoxification of TNT. However, even in the presence of TNT, which is known to inhibit XplA activity, the N lines were able to remove significantly more RDX from the media than wild type, unmodified lines. We have previously demonstrated that activity of XplA towards RDX substrate is inhibited by TNT (Jackson et al. 2007b). But, as we reported for *Arabidopsis*, when expressed *in planta*, XplA is able to metabolize RDX in the presence of TNT (Rylott et al. 2011a).

2.3 ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

The basis of the demonstration was to utilize transformed grasses to degrade RDX. The procedures to install this technology are well known. The application of this technology may also be used in remote sites.

Current remediation methods, such as landfill, incineration or advanced oxidation, are better suited to smaller-scale, highly polluted areas; where clean-up time is short (<3 years), and costs can be kept relatively low. The use of these new grasses is most likely to excel as an in-situ method on larger sites and/or when a longer-term clean-up time (>3 years) is permitted. With these new grasses, land managers will be able to design more resilient training ranges while decreasing cleanup costs associated with energetic compounds. Training ranges will be less prone to erosion, have better control over entry of RDX into the environment, and be more available to training requirements.

The only limitations of this technology will be the availability of the transgenic plant materials (seeds or transplants), and site-specific permitting required through APHIS.

The primary alternative technologies are both drastic and expensive and involve closing lands and physically removing the materials.

3.0 PERFORMANCE OBJECTIVES

The performance objectives are listed in Table 3. Primary performance objective of this demonstration project was to evaluate the ability of transgenic grasses (developed under SERDP projects ER-1318 and ER-1498) to remove RDX from the soil. Four quantitative performance objectives were established to directly measure the ability of the technology to meet the success criteria. Three additional qualitative performance objectives relate to the ability of the plants to survive in the field, the ability to produce seed – a more cost effective solution for large scale use, and the acceptance of the technology by regulators, DoD stakeholders and technology end users.

Table 3. Performance Objectives

Performance Objective	Data Requirements	Success Criteria	Response
Quantitative Performance Objectives			
Satisfy criteria for limited release of transgenic grasses to environment	- Location background - Transformation methods - NEPA documentation	Award of APHIS permit	Permit successfully awarded
Significant reduction of RDX levels in transgenic grass plots versus wild type	- Soil samples - Soil water collection - Plant tissue samples	Statistically significant ($p < 0.05$) decrease in RDX levels	- Soil samples – criterion not met - Soil water – criterion partially achieved - Plant tissue samples – criterion achieved
Qualitative Performance Objectives			
Reliability	Observations and data evaluation during demonstration	Ability to survive under field conditions as well as or better than wild type	Criterion achieved
Availability	Generation of robust plant lines	Viable seed production of switch grass and wheatgrass line	Viable seed not produced
Regulatory Acceptance	Feedback from other agencies (EPA, FWS, and APHIS) on project success.	Meeting of regulatory and DOD stakeholders to review project and produce a CRREL report of the meeting	Meeting and Report both achieved

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4.0 LABORATORY TESTING

4.1 COLUMN STUDIES WITH SWITCHGRASS

To gain an understanding of the ability of the transgenic grass lines to remove RDX from soils on military ranges, column studies containing a sand and gravel mix were conducted. The experimental setup is shown in Figure 15. Twelve polyvinyl chloride (PVC) columns were constructed with PVC tubing (90mm diameter, 0.5m long). Media for the columns was a mix of 75% gravel and 25% sand.

Eight matching columns were planted, four each, with wild type and transgenic grasses and the grasses were grown to over 0.5 m and pruned back to about 0.5 m uniform height. Then the columns were dosed with equal amounts of RDX on the first, third and fifth day of the first week, and, as needed, again on the following week on the same schedule. The RDX was dosed with aliquots of 125 mL of RDX solution containing approximately 7.5 mg RDX. The void volume of the planted columns was approximately 1.5 L. Following each dosing, the planted columns were incubated for one week with 125 mL 1X Hoagland's medium. Two days after the final RDX dosing, the planted columns were flushed with 5 L DI water and the effluent was collected in 500 mL aliquots, which were sampled for analysis of RDX. A total of 5 L DI water was used to flush the columns clean of RDX, until RDX was undetectable by HPLC, usually 3.5 to 4.5 L.

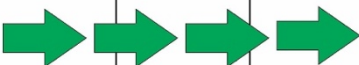
To determine RDX levels in plant tissues, 100 mg of fresh tissue per sample was freeze dried using a Labconco Freezone 4.5 Liter Freeze Dry System (Labconco, Kansas, USA) and ground to powder using a Fast Prep 24 (MP Biomedicals, LLC., Solon, USA). The plant tissue powders were immersed in 1 mL methanol and incubated for 12 hours at room temperature with shaking. The tubes were then centrifuged twice at 13000 rpm for 10 min. The supernatant (800 uL) was collected for HPLC analysis.



Figure 15. Soil Column Studies with Wild Type or N1 Transgenic Switchgrass

The RDX was applied to the columns containing wild type or line N1 switchgrass plants and flushed out three times over the course of two months, as outlined in Table 4.

Table 4. RDX Dosing Schedule for Column Studies

	Day 1 RDX added (one-third)	Day 2	Day 3 RDX added (one-third)	Day 4	Day 5 RDX added (one-third)	Day 6
Day 7	Day 8 water & nutrients tissue collected	Day 9	Day 10 water & nutrients tissue collected	Day 11	Day 12 water & nutrients tissue collected	Day 13
Day 14	Day 15 column flush tissue collected					Day 30 tissue collected

Following the first two applications, RDX was undetectable in the leachates of columns containing wild type and transgenic switchgrass, but wild type leaf tissue contained significantly more RDX than transgenic leaf tissue (data not shown). The results of the third application of RDX are shown in Figure 16a. About one fourth of the applied RDX was recovered in the leachate from the wild type columns, whereas RDX was not detected in the leachate from the transgenic columns. In the leaf tissues, RDX level was significantly less ($p = 0.0044$) in the transgenic leaf tissue compared to the wild type tissue (Figure 16b).

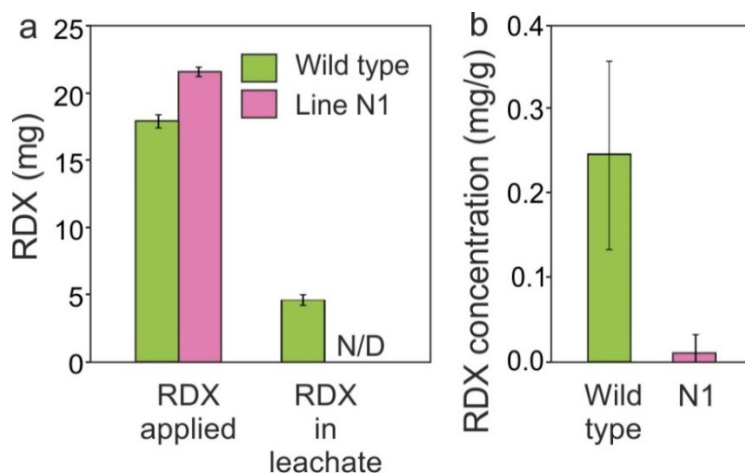


Figure 16. Recovery of RDX Applied to Switchgrass in Column Experiments

a) Mass of RDX applied as solutions containing 30 mg/L, and mass recovered in the leachate by flushing each column with 5 L water. b) RDX levels in leaf after 14 days ($n = 4 \pm \text{SE}$, N/D = none detected).

4.2 TNT RESISTANCE TESTING

The chopped leaf assay (described above) was used to assess the ability of the switchgrass lines to detoxify TNT, in the absence of RDX. The results, shown in Figure 17, demonstrate that the transgenic lines can remove TNT from the media, however, during this time, leaf material from unmodified, wild type lines removed comparable amounts of TNT, suggesting that removal rates are not linked to the activity of the *nfsI*-encoded nitroreductase. These findings are in-line with results for *nfsI* expression and western blots in Figure 12.

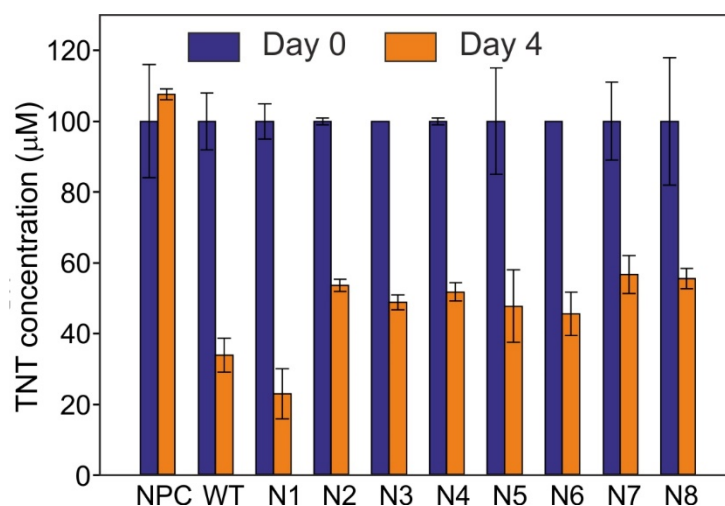


Figure 17. Levels of TNT in Media from Chopped Leaf Assays

4.3 USING MDHAR6 TO ENHANCE TNT RESISTANCE IN SWITCHGRASS

Research has demonstrated that monodehydroascorbate reductase (MDHAR6) activity is the primary cause of TNT toxicity in *Arabidopsis* (Johnston et al. 2015). Part of this research revealed that mutant *mdhar6* plants are resistant to TNT. This finding presents an opportunity whereby inactivating MDHAR6 in RDX-degrading switchgrass lines could enhance the ability of these lines to remediate both TNT and RDX. Towards this, there have been investigations into the distribution and conservation of the MDHAR6 protein sequence. As reported, MDHAR6 is specific to plants, but has homologs with high sequence similarity in *Amborella trichopoda* which is at the base of the Angiosperm lineage (Johnston et al. 2015). MDHAR6 is one of five homologs in *Arabidopsis*, but a key part of the toxicity caused by MDHAR6 is that unlike the remaining homologs (MDHAR1, 2, 3 and 4) it is localized to the mitochondria and plastids. This localization is achieved by organelle-specific targeting sequences on the protein, and it is this difference that enables MDHAR6 orthologs to be distinguished from other members of MDHAR families when searching protein sequence databases. Furthermore, MDHAR1 the *Arabidopsis* MDHAR family member most closely related to MDHAR6 shares only 41% identity.

Protein sequence alignments were conducted using the National Center for Biotechnology Information (NCBI) database. Switchgrass is in the Poaceae which comprises 780 genera including the major cereal crops. Accordingly, sequences from cereal species are well-represented in the NCBI database, and alignment against members of the Poaceae identified a mitochondrially targeted MDHAR6 ortholog in rice (*Oryza sativa*) with 78% identity (Sequence ID: XP_015649950.1).

Eleven species of *Pancium* are represented in the NCBI database, although the complete genome is only present for *P. hallii* (553.845 Mb). Searching the *Pancium* genera identified two *P. hallii* hypothetical proteins (Sequence ID: PAN33604.1 and PAN33605.1), both with 73% identity to MDHAR6. These two sequences are 100% identical to each other, with the exception of an additional –TSALRRKAAEM– sequence after the first amino acid in PAN33605.1. This is likely to be an artifact from a splice variant; there are four in MDHAR6 that together are thought to control organelle-specific targeting in Arabidopsis.

Using subcellular localization prediction programs (PSORT.org, www.cbs.dtu.dk/services/TargetP-1.1/) these putative MDHAR6 orthologs are predicted to have both mitochondrial and plastidial cleavage sites. The next closest match to MDHAR6 was a hypothetical protein (Sequence ID: PAN36681.1) which shares only 43% identity with PAN33604/5. Together, these factors indicate that ID: PAN33604/5 is the sole ortholog of *MDHAR6* in *P. hallii*. The PAN33604/5 gene sequence would enable identification of the corresponding ortholog in the *P. virgatum* line used to make the RDX-degrading switchgrass. As *P. virgatum* is now readily transformable, gene editing techniques could be used to disable the *MDHAR6* ortholog, and investigate the effects of this deletion on TNT resistance.

4.4 TNT RESISTANCE TESTING: ROLE OF GSTS IN TNT DETOXIFICATION

As part of the studies at UY, there have been investigations into alternative ways to engineer TNT resistance into plants. Towards this, there have been studies of bacterial nitroreductases (the *nfsI* encoded NR) and plant TNT-detoxifying enzymes including oxophytodienoic acid reductases (OPRs) and UDP-glucuronosyltransferase (UGTs). Now the focus is on glutathione transferases (GSTs). Earlier studies at UY, and others, had previously shown that GST transcripts were highly up regulated in response to TNT treatment (Ekman et al. 2003, Gandia-Herrero et al. (2008). Subsequently, (as part of SERDP-1498) it was demonstrated that the Tau class GST, AtGSTU25, catalyzes the formation of 2-glutathionyl-4,6-dinitrotoluene via substitution of a nitro group at the 2 or 6 position of TNT_{3,4}. The resulting conjugate is of interest in the phytoremediation of TNT as it is potentially more amenable to subsequent biodegradation than TNT; enzymatic degradation of TNT has not yet been reported. It can now be reported that the X-ray crystal structure for purified AtGSTU25, at a resolution of 1.99 Å has been obtained. Although the crystals were incubated with the TNT and GSH, occupancy of the active site with TNT was not found. Instead, multiple rounds of structure refinement cycles using the REFMAC5 program revealed two GSH molecules covalently linked by a disulphide bond, showing the structure of AtGSTU25 was in complex with the oxidized form of GSH, known as glutathione disulphide (GSSG). The locations of the active residues in the binding site were found to be similar to interacting sites in enzymes such as glutaredoxin, which is known to catalyze the oxidation of glutathione. There are now investigations into how the TNT molecule might sit in the active site to understand more about the formation of the denitrated TNT conjugate.

5.0 SITE DESCRIPTION

5.1 SITE LOCATION AND HISTORY

The US Army Garrison, Fort Drum, is located just east of Lake Ontario in upstate New York and is under the command of the U.S. Army Commands (ACOM), formerly Forces Command (FORSCOM). The primary mission of Fort Drum is to provide facilities and services to U.S. Armed Forces that require land and airspace to practice combat skills and operations year-round. Fort Drum is also home to the 10th Mountain Division (Light Infantry) whose mission is to deploy rapidly anywhere in the world. The 10th Mountain Division Light Infantry consists of light infantry brigades, an aviation brigade, a division artillery brigade, a division support command brigade, an engineer battalion, a signal battalion, an intelligence battalion, an air defense battalion, a military police battalion (provisional), a division band, and a headquarters company.

5.2 SITE GEOLOGY/HYDROGEOLOGY

Fort Drum encompasses two major physiographic provinces, the Lake Erie-Ontario Lowlands and the Adirondack Uplands. The southwestern two-thirds of the installation, where the proposed site is located, is part of the Lake Erie-Ontario Lowlands division. In this area, surface geological features include recessional moraines, small sand plains, drumlins, swamps, and drainage patterns resulting from Pleistocene glaciations. Fort Drum is underlain by a variety of metamorphic, igneous, and sedimentary bedrock ranging from Precambrian to Middle Ordovician. The oldest metamorphic rocks belong to the Grenville Complex and consist mainly of metamorphosed Precambrian quartzite, gneiss, schist, and marble. These rocks stretch in a wide northeast–southwest band across Fort Drum and border the igneous Adirondack massif and associated foothills to the east.

Fort Drum soils are generally developed from deltaic/lacustrine or glacial deposits. The soils vary from sandy gravels to loams to clays to mucks. Soils in the region are generally shallow and poorly drained; soil permeability is slow to moderate. The demonstration site is located on a Plainfield sandy soil and contains a mean of 92% sand, with small amounts of silt and clay. The area is relatively level and open with less than 25% tree canopy. Grasslands and meadows on sandy soils at Fort Drum are dominated by switchgrass, common hairgrass, stiff-leaved aster, poverty oat grass, and the sedge *Carex lucorum*. Grasslands on sandy soils are visually distinct from corresponding communities on less sandy soils, showing a relatively species-poor vegetative diversity with a predominance of native species. Fort Drum has a primarily humid, continental climate with relatively long, cold winters and short, warm and often humid summers. The mean annual temperature, averaged over the past 10 years, is 8.9 °C (48 °F). January is the coldest month, closely followed by February and December. Temperatures fall below –18 °C (0 °F) on about 20 days during these 3 months; below-freezing temperatures occur on about 104 days from December to March.

5.3 CONTAMINANT DISTRIBUTION

In order to better test the ability of the transgenic plants to remove RDX from the soils, all plots were constructed with uncontaminated field soil. This was to ensure that there was no other presence of contaminants and that levels of RDX could be controlled. Further details on contaminant distribution in the plots are described in Section 6.4.

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6.0 TEST DESIGN

6.1 CONCEPTUAL EXPERIMENTAL DESIGN

The transgenic plants expressing *xplA* and *xplB* were used in field trials to monitor the removal of RDX from contaminated soil within the root zone (upper 15 cm of soil). The experiment comprised a randomized complete block design with three soil treatments, three vegetation treatments (untransformed grass species, transformed grass species, and a no plant control) replicated three times for a total of 27 plots (Figure 18a). Three vegetation treatments were statistically necessary to prove that the transformed plants were reducing concentrations of RDX in the substrate. Each plot was 3.0 m in length x 3.0 m wide, and comprised a double lined containment system consisting of a reinforced polyethylene barrier placed 0.75 m below the existing soil surface. A layer of clean silica sand 0.25 m thick was placed above the barrier and a suction lysimeter placed in the sand layer for monitoring of RDX leachate. A second barrier was installed above the sand layer and filled with 0.5 m of clean local soil, Figure 18b.

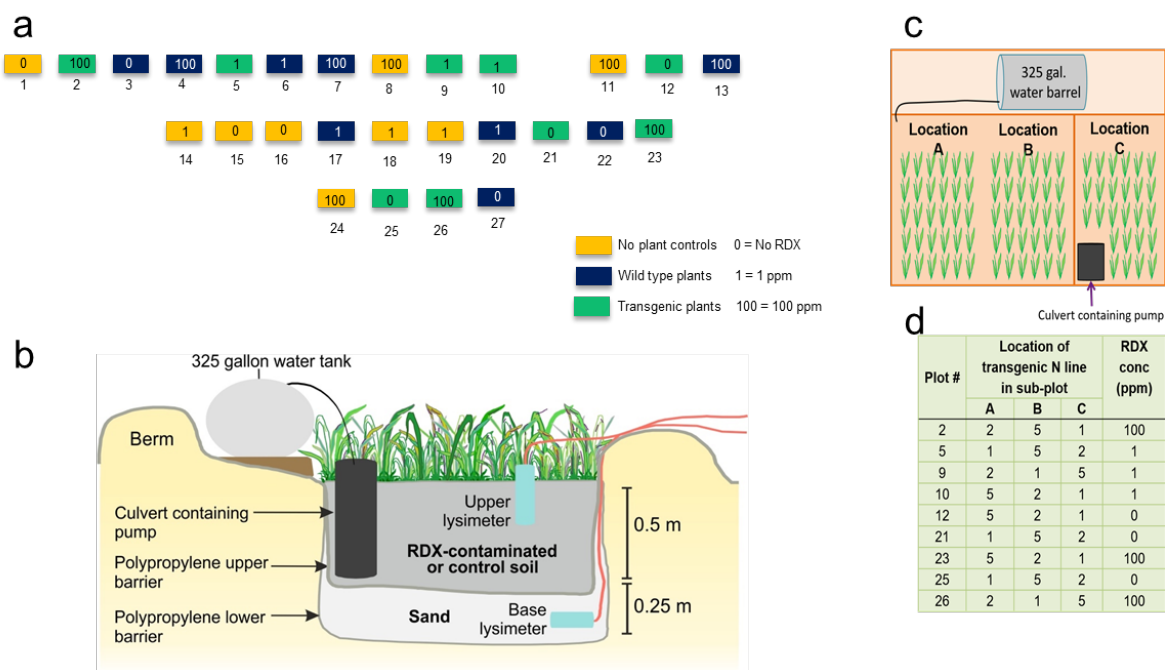


Figure 18. Plot Design and Lay-out

(a) Randomized block lay-out of the plots, (b) Schematic showing a cross-section through a vegetated plot, (c). Location of the plant lines on each plot, (d). Location of each of the three, independently-transformed *XplAB*-expressing lines N1, N2 and N5.

As per conversation with APHIS personnel, plant development was monitored weekly and all developing flower heads were removed from the plants to eliminate the potential for pollen production. Plots were monitored weekly for vegetation status (plant health, nutritional status, and soil moisture levels). Soils, plants, and leachate were sampled monthly to determine changes in RDX concentrations over time.

6.2 BASELINE CHARACTERIZATION

Baseline characterization activities consisted of soil sampling, below potential containment plots, to a depth of 3m. Soils were analyzed for munitions constituents, nutrients, and an array of heavy metals (Cd, Cr, Pb, Ni, and Zn) using EPA method 8830A and EPA method 3050, Table 5.

Table 5. Metals and Munitions Constituents Commercial Lab.

All results are in mg/kg

Metal	Sample 1	Sample 2	Sample 3	Munition	Sample 1	Sample 2	Sample 3
As	14	12	16	HMX	<0.04	<0.04	<0.04
Ba	16	13	15	1,3,5-TNB	<0.04	<0.04	<0.04
Ca	491	497	703	RDX	<0.04	<0.04	<0.04
Cu	7	6	7	1,3-DNB	<0.04	<0.04	<0.04
Fe	4789	3975	5612	TNT	<0.04	<0.04	<0.04
Mg	901	764	1014	NG	<0.04	<0.04	<0.04
Mn	68	55	66	2,4-DNT	<0.04	<0.04	<0.04
Na	69	64	77	2,6-DNT	<0.04	<0.04	<0.04
Ni	3	3	4	2-Am-DNT	<0.04	<0.04	<0.04
Pb	4	4	5	4-Am-DNT	<0.04	<0.04	<0.04
Sb	3	2	3				
Sr	2	2	2				
W	4	4	4				
Zn	14	12	15				

6.3 DESIGN AND LAYOUT OF TECHNOLOGY COMPONENTS

6.3.1 Production and testing of transgenic plants

As seed is not available for the current transgenic lines, CRREL propagated all the primary transformant lines in the greenhouse using standard horticultural practices, prior to release to the field site. UW supplemented these methods with additional micropropagation techniques developed specifically for switchgrass (Alexandrova et al. 1996). Micropropagation involved collecting nodal segments from the grasses, surface sterilization, splitting longitudinally and placed with the cut surface down on solid medium containing maltose and 6-benzylaminopurine. After 1.5 months in a growth chamber, the young shoots were transferred to medium without growth regulator for rooting. Hygromycin was added to the medium as a selection agent to maintain the transgenes. Over 1000 plantlets were created from this procedure.

Prior to planting, randomly-selected XplAB-expressing plants, from both UW and CRREL produced clones, were tested using PCR to confirm the presence of the transgenes, as described Zhang, L. et al. (2017a), and tested in soil-column studies, by UW, to verify RDX-degrading activity.

6.3.2 Field demonstration Setup

Twenty-seven test plots were constructed at the Wheeler Sack Army Air Field at Fort Drum, NY, in May 2014. The design of each plot is shown in Figure 18, each plot was constructed with a double layer of 60 mil reinforced polypropylene. The lower level contained a lysimeter to enable soil water measurement below the primary containment layer. This ensured that no RDX breached the upper containment layer. The upper layer was filled with the local soil and contained a lysimeter for monitoring of RDX concentrations in the active soil layer. Additionally, a water recycling system was installed to maintain soil water levels appropriate for optimum growth of the Switchgrass. The system contained a plastic culvert that has been drilled with holes every 5cm and wrapped in landscape fabric for water to permeate through. At the bottom of each culvert was a float valve-activated pump that automatically pumped excess water back into the 325-gallon storage tank included in each test plot.

6.4 FIELD TESTING

6.4.1 Plot planting and dosing

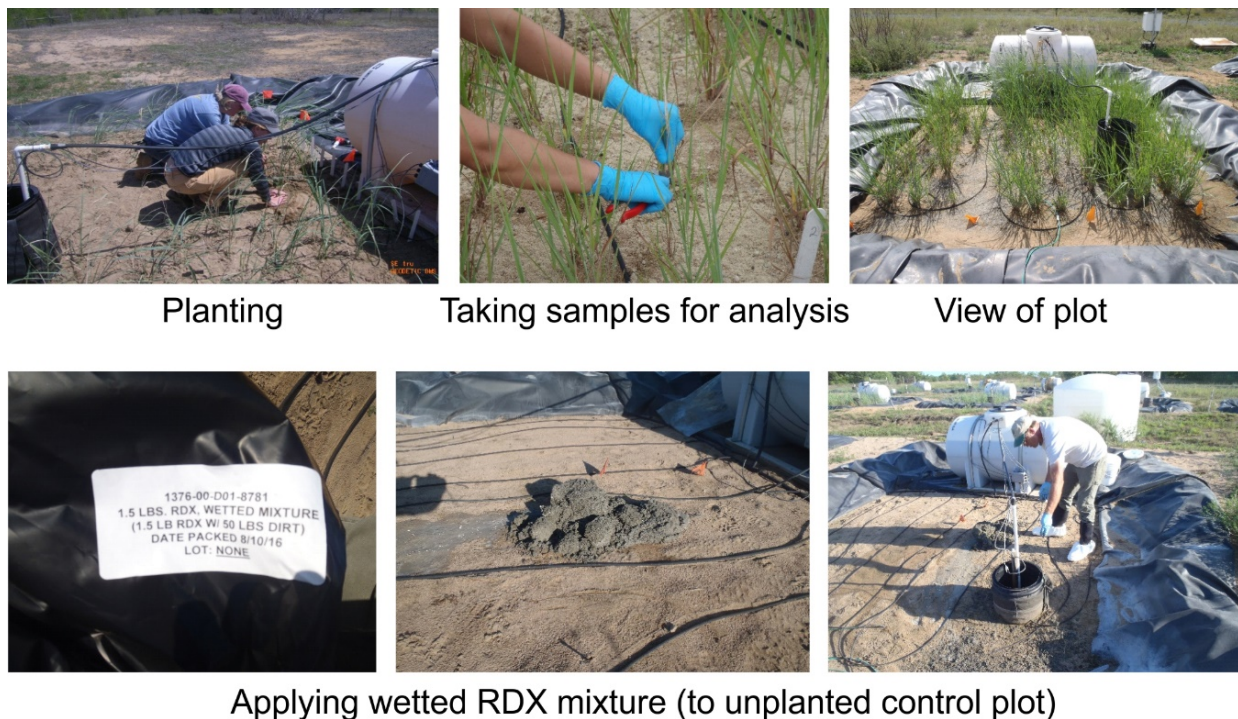


Figure 19. Photographs Setting Up of Irrigation, and Application of RDX-sand Mixture to the Plots

To simulate particulate munitions contamination around targets on training ranges, the plots were surface dosed with fine particulate RDX mixed with sand and irrigated with water. The selected rates simulate particulate munitions contamination around targets on training ranges (Walsh et al. 2003).

RDX was obtained from the ARDEC, Picatinny Arsenal, Picatinny, NJ. It consisted of a mixture of class 5 RDX mixed with Fort Drum sand and brought up to 40% moisture. Three concentrations of RDX (0, 1, and 100 mg/kg) were applied to the plots. The no RDX plots had clean sand applied to the surface and subsequently incorporated into the top 10 cm of the soil, the 1mg/kg treatment was applied through the irrigation system, and the 100 mg/kg treatment was surface applied and incorporated into the top 10 cm of the soil (Figure 19).

Each vegetation plot was planted with approximately 250 clones, modified or unmodified, spaced on a 20 x 20 cm grid (Figure 18c and d) and allowed to establish for approximately one month prior to RDX application to obtain a sufficient root system.

6.4.2 Plot maintenance, sampling and decommissioning

All plots were maintained at a constant soil moisture level (40%) using a Campbell Scientific CR1000 data logger system coupled to a CS650 soil moisture and temperature sensor to control both the sump pump and irrigation systems. In the event that the 1230L storage tank on a plot became full, and excess water remained on the plots, a sample of the storage tank water would be taken for analysis. The 1230L tank would then be pumped into a 5,678 L (1500 gallon) backup storage tank to maintain RDX containment and plot soil moisture content.

During the growing seasons, all plots were checked weekly and seedling weeds removed to reduce competition. In compliance with the APHIS-released permit, the switchgrass plants were checked for developing flower heads which were detached and destroyed. From April to Oct 2018, a complete 15-15-15 all-purpose fertilizer (Miracle Grow) was applied every week at a rate of 16g/m².

Over the winter season, plots were covered with polythene-covered cloches, to reduce plant losses to cold weather, reduce excess water/snow accumulation in the plots, and to maintain RDX containment.

6.4.3 Plot sampling and decommissioning

At the start and end of the trial, 5cm diameter soil cores were taken from 5 equidistant locations, and at three depths, across each plot. At monthly intervals across the 2018 growing season, plants were sampled from rows 2, 3, 4, 7, 8, 9, 12, 13, and 14 and from plants 2 through 9 as to remove bias from edge effects. One plant per row was sampled, a minimum of monthly to a maximum of every two weeks, from June through September. The selected plant was cut 15cm above the soil surface and allowed to regrow.

For each plot three soil cores were collected, chosen from random points in the plot. Soil water samples from the lysimeters in the plot and from the water holding tank were collected at each sampling period.

6.4.4 Extraction and quantification of RDX

All samples were analyzed using a method adapted from EPA method 8330A.

Briefly, soil samples were air dried at room temperature in the dark, ground, then a 5 g subsample, extracted with 10 mL acetonitrile, and incubated overnight on a rotary shaker. The supernatant was filtered through 0.45 µm PTFE filters, then analyzed using HPLC.

For plant samples, RDX was extracted from 100 mg ground plant tissue in 1.4 mL acetonitrile on a rotary shaker overnight, centrifuged 10 min at 12,000 g. The supernatant was applied to a 1 g florisil, 1 g alumina sample preparation column, and the concentrated, filtered samples analyzed using HPLC. The HPLC analysis was conducted using a modular Waters system consisting of a Waters 717+autosampler, two Water 515 HPLC pumps and a Waters 9926 photodiode array detector. A 250 x 4.6mm Hypersil Gold reverse phase column (ThermoFisher) was used for separation with a 60:40 mobile phase (HPLC-grade water: 83% methanol/17% acetonitrile) at 1 mL/min and monitored at 254 nm with frequent confirmation of RDX identification by spectral analysis using the photo diode array. Peak integration and data analysis were conducted using the Millennium software (Waters).

6.4.5 Determination of leaf surface area and transpiration rates

Polyvinyl chloride (PVC) columns were constructed with PVC tubing (90mm diameter, 0.5m long), and filled with a mix of 75% gravel and 25% sand. Plant were grown in the columns at 25 °C, 16-hour light and 8-hour dark photoperiod, 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity to a height of 50 cm. Transpiration efficiency was measured on second youngest, fully-expanded leaves using a SC-1 leaf porometer (Decagon Devices, Inc.). Ten leaves were sampled for each line.

6.4.6 Field demonstration decommissioning

In the spring of 2019, the field plot area was completely remediated and returned to the conditions prior to the construction of the containment cells. All vegetated plots (18 plots total) were treated with a non-selective herbicide (Pramitol 25E) to ensure complete removal of vegetation prior to plot deconstruction. Fourteen days following herbicide application, all RDX plots were treated with treated with 5kg/m² Ca(OH)₂, to increase the soil pH to 12.5. The increased soil pH was maintained until no RDX was detected in soil or soil water samples, by HPLC. The soil pH on the plots was restored by addition of 4kg/m² Al₂(SO₄). The containment liners were then removed and disposed of through the appropriate waste stream and the soils returned to the field location. The area was seeded with the standard Fort Drum seed m, consisting of 30 % fine fescue (*Festuca arundinacea*), 10 % sheep fescue (*Festuca ovina*), 30 % Kentucky bluegrass (*Poa pratensis*) and 30% perennial ryegrass (*Lolium perenne*).

The RDX in the storage tanks was degraded by addition of 10g/L Ca(OH)₂ per L storage water, Water samples were analyzed using HPLC to verify all RDX had been destroyed. Storage tank water was then treated with 8g/L Al₂(SO₄) to reduce the pH to previously measured levels, prior to release at the site.

6.5 SAMPLING RESULTS

Following the award of the APHIS permit (22 April 2016), the plots were planted (19 May 2016), and RDX applied (27 August 2016).

Lysimeters were applied to the plots, and soil, tissue and water samples taken at regular intervals. In total, during the course of the trial, over 3,500 samples were taken for analysis.

6.5.1 Data from plots

As shown in Figure 20, the plants were still establishing in 2016, with canopy closure not fully obtained until 2018. Thus, biomass recordings were only recorded for 2017 (Figure 21) and 2018 (Figure 22). At the end of the trial, in the 100mg/kg RDX plots, the total aerial biomass of the transgenic plants was not significantly different to that of the wild type plants.

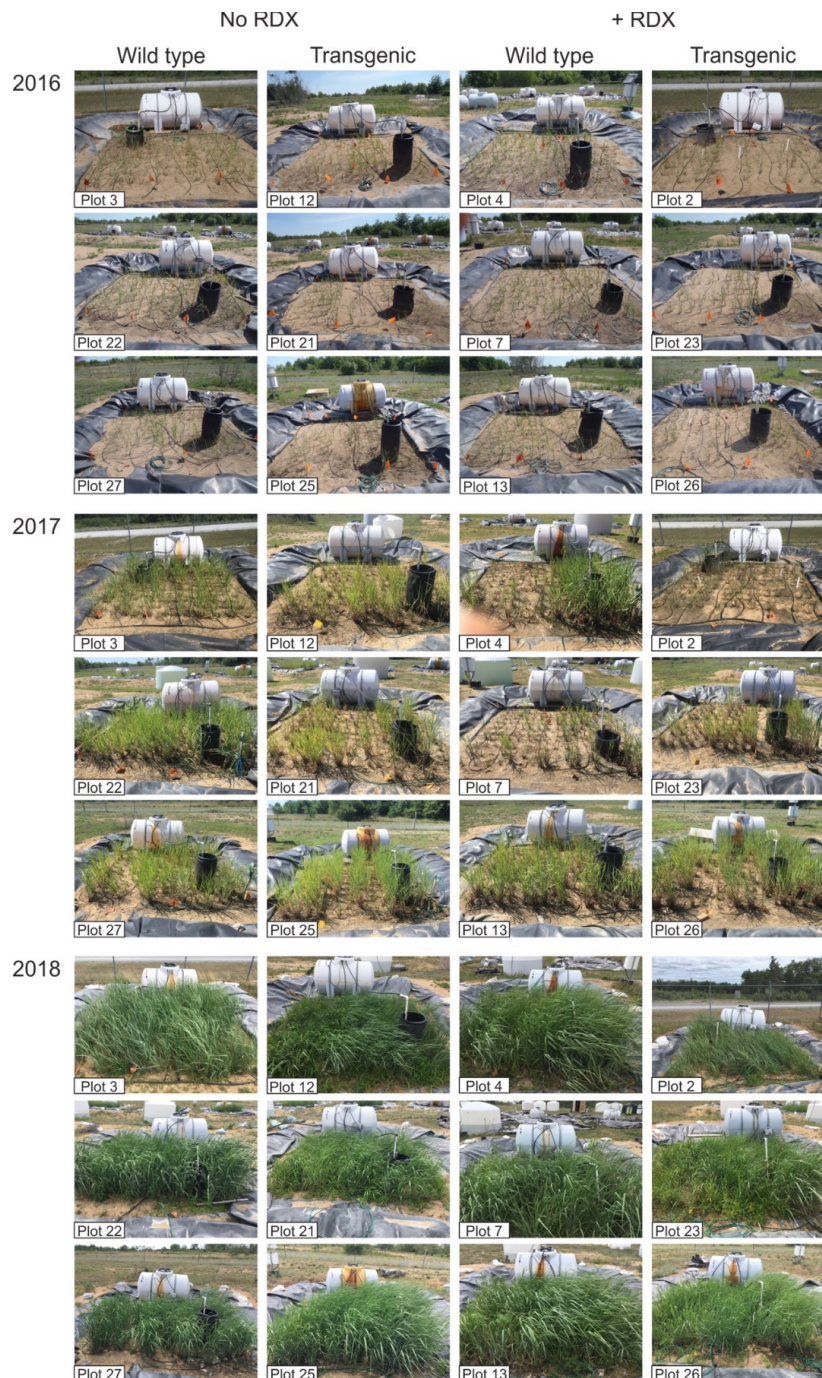


Figure 20. Appearance of the Uncontaminated, and 100mg/kg Plots
Photos taken near the end (July/August) of each growing season (2016, 2017, and 2018).

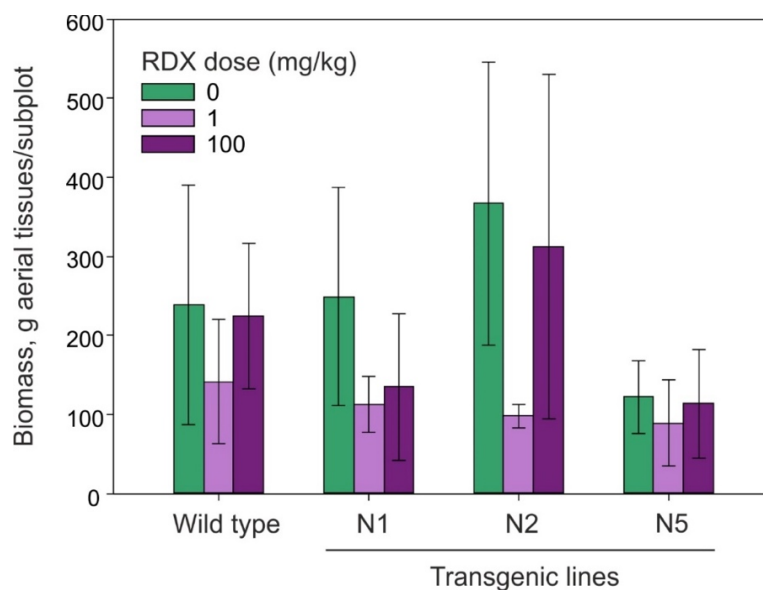


Figure 21. Aerial Plant Biomass Levels Per Subplot at the End of the Growing Season for 2017

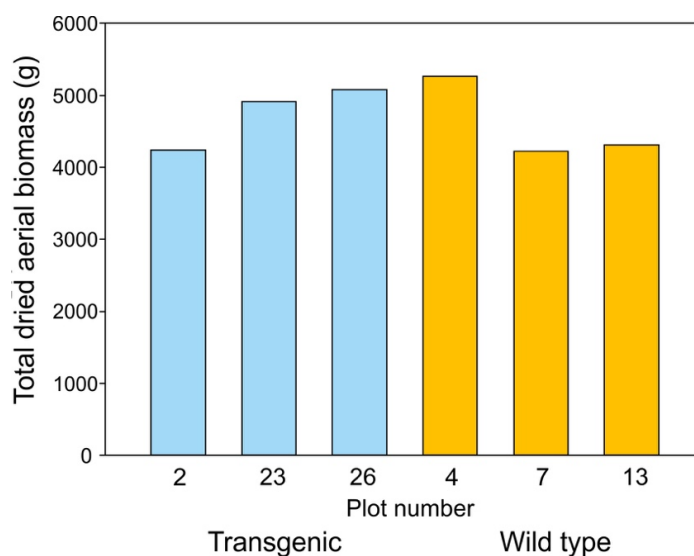


Figure 22. Aerial Plant Biomass Levels in the 100mg/kg RDX Plots at the End of the 2018 Growing Season

6.5.2 The 1 mg/kg plots

The 1 mg/kg plots were created to establish the secondary objective: was to determine the lower bounds or reactivity of the transgenic plants. The lowest concentration of RDX in soil that the modified plants can remove and degrade is not known. Across 2016 and 2017 growing season, RDX was not detected in soil, soils-water, or tissue from these plots. The 1mg/kg plots were kept covered for the 2018 season, and dismantled at the end of the trial.

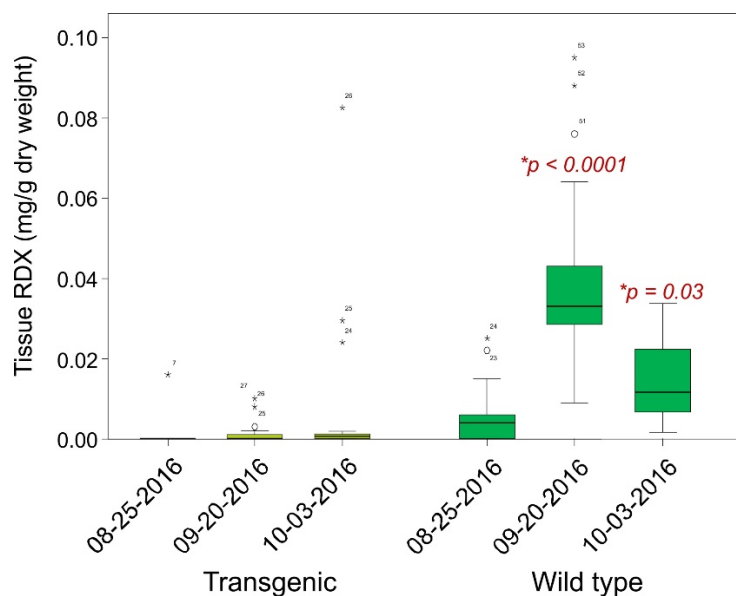


Figure 23. Tissue RDX Levels from 100 mg/kg Plots in 2016

Figure 23, Figure 24 and Figure 25 show the RDX tissue levels in the 2016, 2017 and 2018 plots respectively. In 2016, all time points from 9-20-2016 onwards had significantly less RDX in the leaf tissues of the transgenic plants compared to the unmodified wild type plants. In the 2017 and 2018 data, RDX was not detected in the transgenic leaves, with a mean of 0.026 mg/g and 0.0253 +/- 0.024 mg/g dry weight RDX respectively detected in the leaves of the unmodified plants.

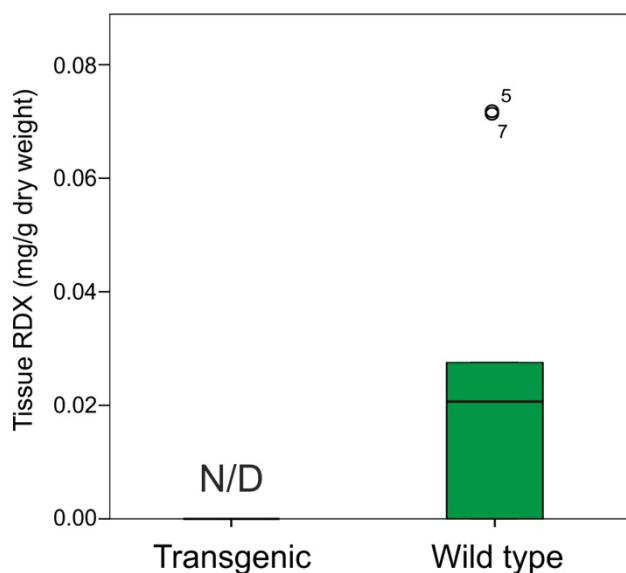


Figure 24. Tissue RDX Levels from 100 mg/kg Plots in July 2017

ND = not detected.

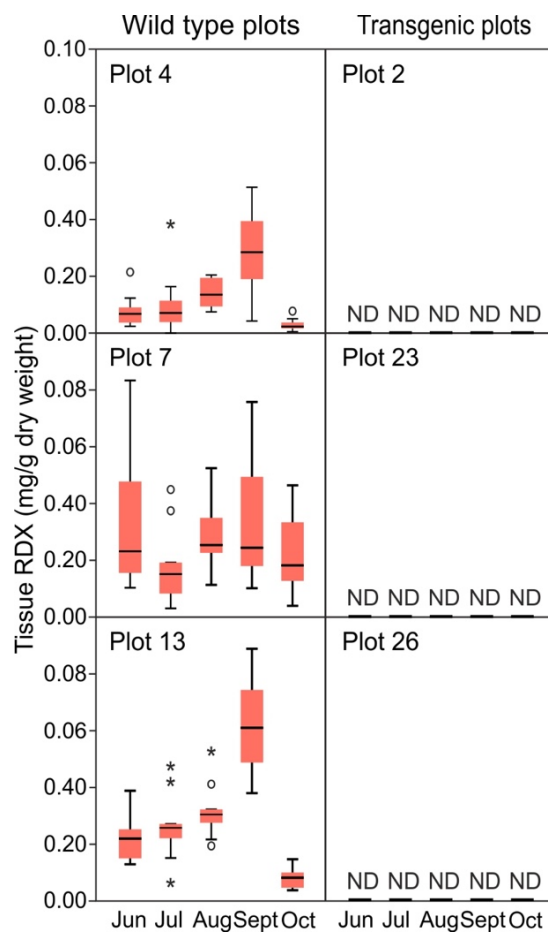


Figure 25. Levels of RDX in Aerial Tissues Harvested Across the 2018 Growing Season

(ND = Not detected, $n = 9$).

RDX concentrations in lysimeter water from samples (leachate water) from 100 mg/kg RDX plots in 2017 and 2018 are shown in Figure 26. No significant differences reported between RDX levels in lysimeters from the NPC, wild type or transgenic plots.

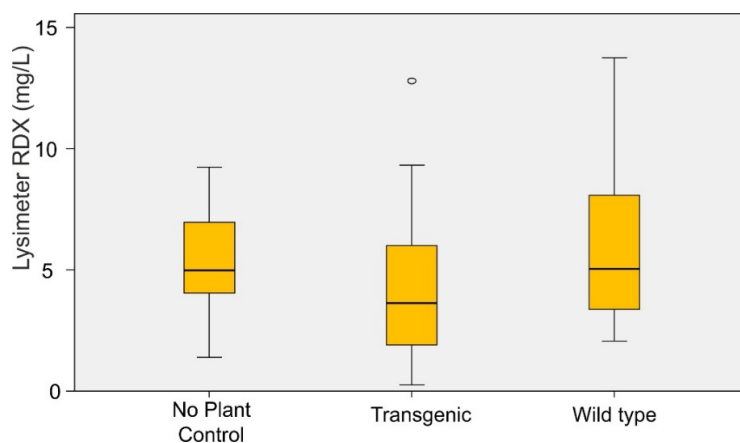


Figure 26. RDX Concentrations in Lysimeter Water Samples from 100 mg/kg Plots 2017

RDX concentrations in soil samples from 100 mg/kg RDX plots in and 2017 (Figure 27). No significant differences reported between RDX levels in soil RDX concentration from the NPC, wild type or transgenic plots.

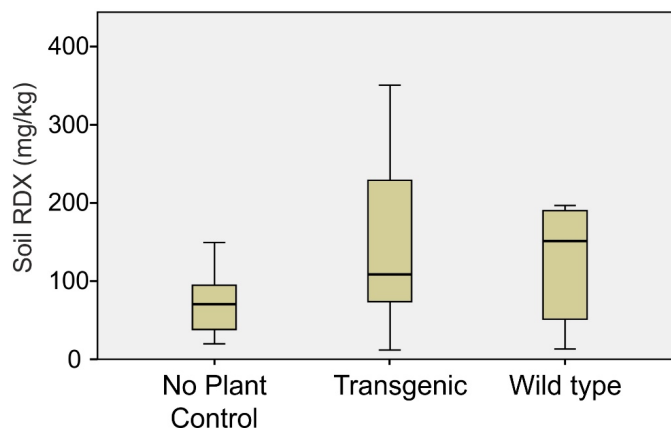


Figure 27. RDX Concentrations in Soil Samples from 100 mg/kg Plots 2017

Analysis of soil core samples from across the plots (Figure 28a) showed that the RDX was distributed heterogeneously across the plot. Unexpectedly, given its documented mobility in soils, RDX had not significantly moved below the 0-5 cm depth in the plots. However, higher concentrations tended to be clustered furthest away from the culvert areas, suggesting localized gradients of RDX occurred, as indicated by arrows on Figure 28b.

Levels of RDX in soil lysimeters (Figure 28c) indicated that there was no significant difference in the levels of soil water RDX in the no plant control (NPC), unmodified wild type (WT), and transgenic (TG) plots across the 2018 growing season. Similarly, there was no significant difference in the levels of RDX in the tanks on the NPC, WT and TG plots, although at each sampling point, the mean RDX level in the TG plot tanks was always lower than that in the WT plot tanks (Figure 28c). Each plot contained, embedded in sand between the polypropylene layers, a lysimeter to enable soil water measurement below the primary containment layer. Tests from these lysimeters confirmed that RDX had not breached the upper containment layer.

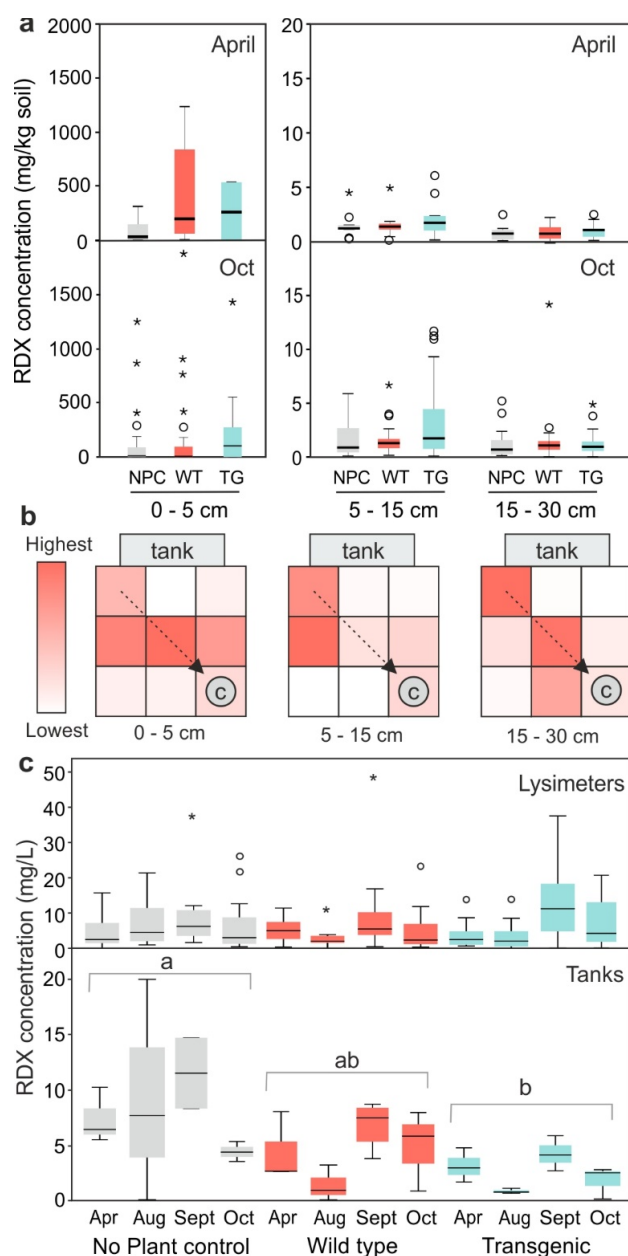


Figure 28. RDX Soil, Soil Water and Tank Concentrations in 2018 Growing Season.

(a) Soil RDX concentration in the 100 ppm plots measured at three depths at the start (April $n = 9$ soil samples) and end (Oct $n = 27$ soil samples) of the growing season (NPC; No plant control, WT; wild type, TG, transgenic). (b) Heat plot showing mean distribution of RDX across the 100 ppm plots at the three depths, c = position of culvert, tank = position of water tank. (c) Concentration of RDX in lysimeters and tanks across the 2018 growing season. Lysimeter data: $n = 12$ (NPC), 15 (WT), 15 (TG). Tank data: $n = 11$ (NPC), 12 (WT), 12 (TG). For the tank data, a Kruskal-Wallis H test showed the distributions of NPC, WT and TG values were statistically significantly different ($P = 0.026$) from each other. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons, and presenting adjusted P values. This post hoc analysis revealed that the RDX concentrations in the TG tanks were statistically significantly different from those in the NPC tanks ($P = 0.021$).

During the 2018 growing season, levels of RDX in the aerial tissues of unmodified plants grown on the 100 mg/kg RDX plots varied within plots, between plots, and between monthly samplings (Figure 29).

Although previous studies have shown that XplA- (Rylott, et al. 2006) and XplAB- (Zhang et al. 2017a) expressing plants have increased plant biomass when compared to unmodified plants, on the plots, the presence of RDX did not affect plant biomass levels in either XplAB-expressing or unmodified plants (Figure 29).

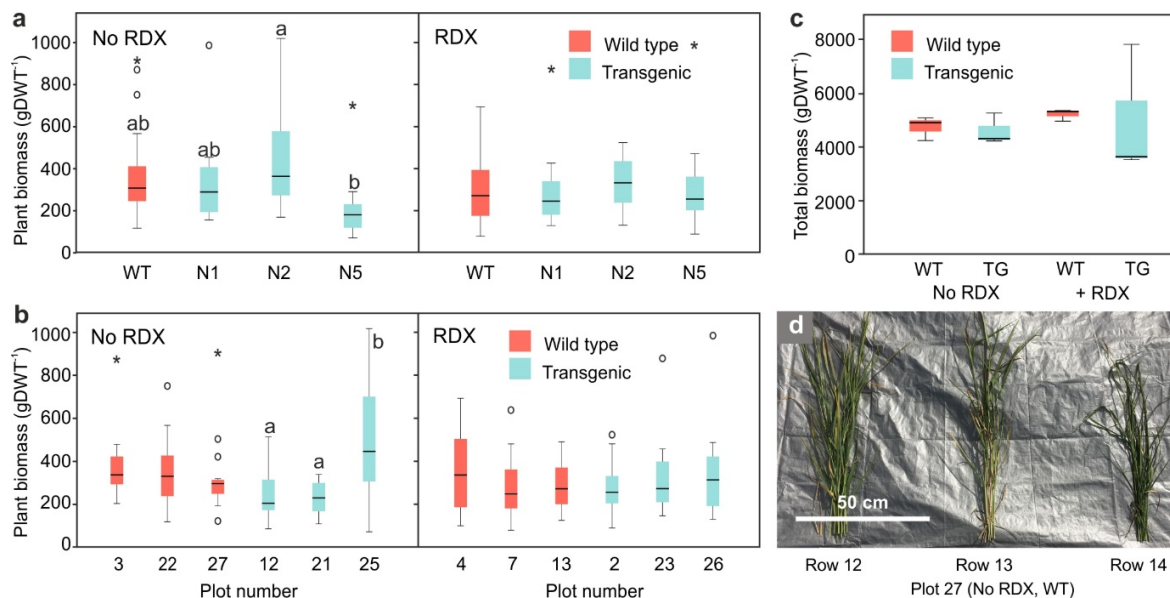


Figure 29. Aerial Tissue Weights Following Harvest at the End of the Field Trial

(a) Biomass of the WT (wild type) and individual transgenic N lines on uncontaminated plots (No RDX; $n = 45$ (WT), $n = 15$ (TG)). (b) Comparison of biomass from individual, uncontaminated plots (No RDX; $n = 15$). (c) Total biomass for each of the treatment types WT and transgenic (TG) plants ($n = 3$). (d) Appearance of representative tissue samples during harvest

At the end of the 2018 growing season, the mean RDX concentration in the wild type switchgrass from each of the three plots (Figure 30a) was 0.0253 ± 0.024 mg/g dry weight. Previous measurements of tissue RDX levels using RDX-saturated soil column studies measured a maximum of 0.23 mg/g for wild type switchgrass. Under the column conditions, RDX was detected in the XplAB-expressing switchgrass line N1, but at just 4% of that in the unmodified switchgrass, suggesting that 0.23 mg/g is indicative of the likely upper limit for the field RDX-degrading capacity of the transgenic lines (Zhang et al. 2017a), and that the XplAB-expressing plants in the 2018 season were at approximately 11% of their RDX uptake capacity.

Over the three-year demonstration, above average rainfall necessitated the removal of excess water from the plots. Significantly less water was pumped from the plots containing the XplAB-expressing plants than from the control plots containing wild type plants in 2017 and 2018 growing seasons. This difference in water pumped from the plots, was irrespective of the presence of RDX (Figure 30b).

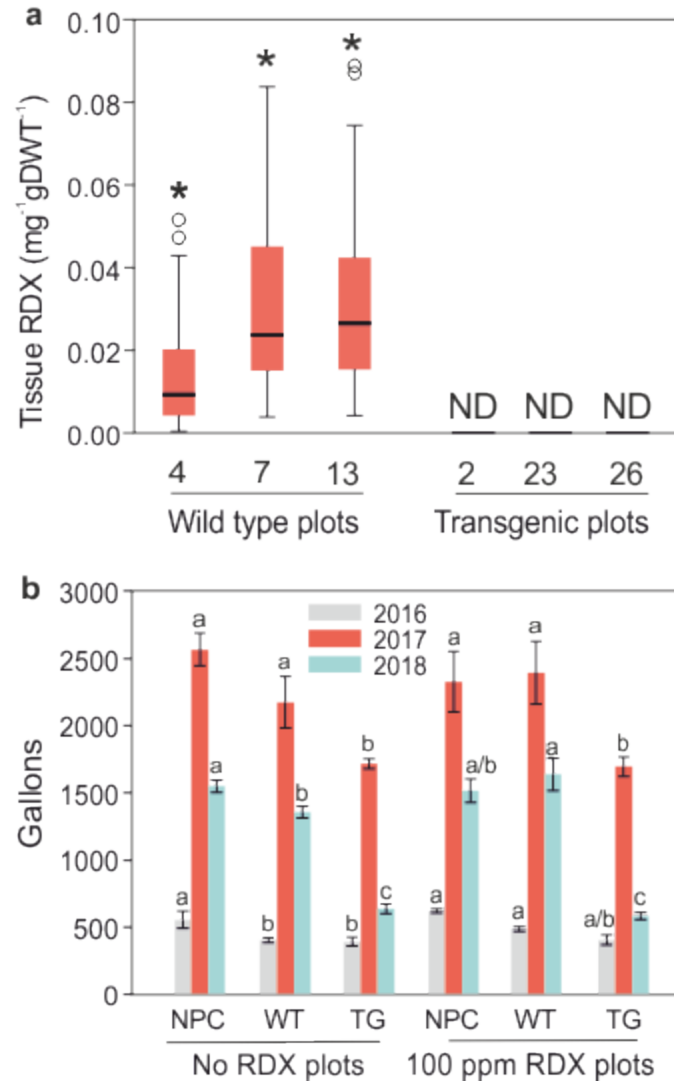


Figure 30. Total Tissue RDX, and Excess Water Pumped, from Plots over the 2018 Growing Season

(a) Mean aerial tissue RDX concentration in plants from the 100 mg/kg (ND, Not detected, $n = 45$). (b) Volume of excess water pumped from the plots over each year of the field trial (NPC; No plant control, WT; wild type, TG, transgenic, $n = 3$. 2016 data: Levene's test $F(5,12) = 3.34$, $p = 0.04$; $H(5) = 14.42$, $p = 0.013$; 2017 data: Levene's test $F(5,12) = 6.36$, $p = 0.004$; $H(5) = 13.43$, $p = 0.020$).

To investigate the discrepancy in water pumped from the plots further, leaf surface areas and transpiration rates were measured. Leaf surface areas were determined using WinRhizo (2011 Regent Instruments). Figure 31 shows the wild type and transgenic lines were not significantly different from each other, irrespective of the presence or absence of RDX.

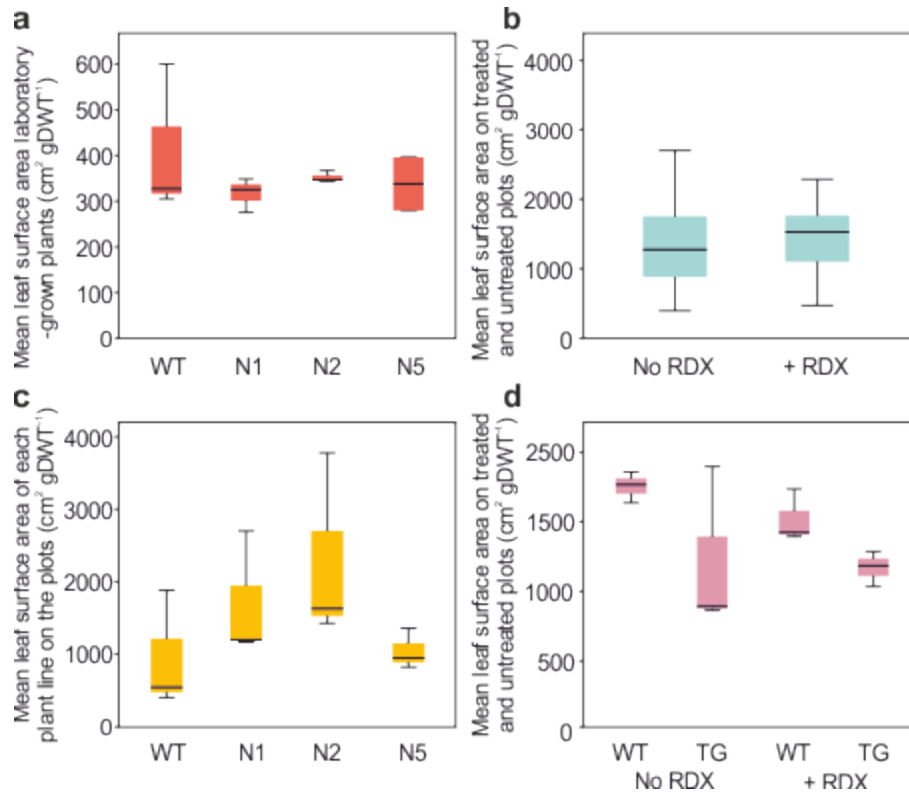


Figure 31. Leaf Surface Area of the Plants at the End of the 2018 Growing Season

(a) Mean leaf surface areas in the wild type (WT) and individual transgenic (TG) lines grown in the laboratory ($n = 3$, Homogeneity of variance was tested using Levene's test ($F(3,7) = 8.104$, $p = 0.011$; and significance using Kruskal-Wallis $H(3) = 1.273$, $p = 0.736$). (b) Comparison of mean leaf surface area of all WT and TG plants grown on the uncontaminated (No RDX) versus RDX-treated (+ RDX) plots ($n = 6$, Independent t -test values: $t(22) = 0.192$, $p = 0.20$). (c) Mean leaf surface areas in the WT and individual TG N lines grown in the field, independent of the presence of RDX ($n = 6$, Levene's test ($F(3,20) = 0.354$, $p = 0.79$; one-way ANOVA $F = 2.40$, $p = 0.10$). (d) Mean leaf surface areas in the WT and TG lines grown in uncontaminated versus RDX-treated plots ($n = 3$, Levene's test ($F(3,20) = 1.51$, $p = 0.24$; one-way ANOVA $F = 1.29$, $p = 0.31$).

To measure transpiration rates, polyvinyl chloride (PVC) columns were constructed with PVC tubing (90mm diameter, 0.5m long), and filled with a mix of 75% gravel and 25% sand. Plant were grown in the columns at 25 °C, 16-hour light and 8-hour dark photoperiod, 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity to a height of 50 cm. Transpiration efficiency was measured on second youngest, fully-expanded leaves using a SC-1 leaf porometer (Decagon Devices, Inc.). Ten leaves were sampled for each line. Figure 32 shows that transpiration rates measured for line N1 were not significantly different from wild-type plants. These differences were not observed in laboratory experiments, and we conclude they occurred by chance due to plot aspect, or other unknown factors.

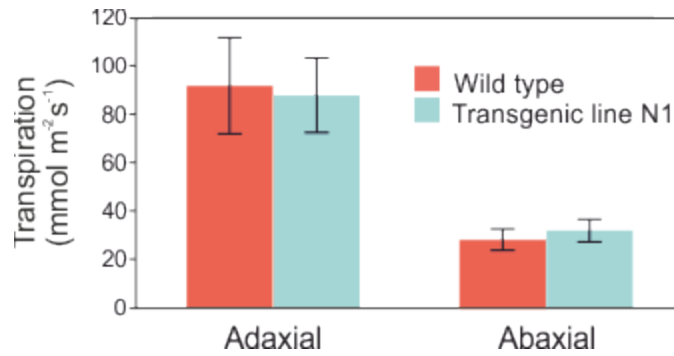


Figure 32. Leaf Transpiration Rates

Leaf surface transpiration rates for wild type and transgenic line N1 from adaxial ($n = 10$, $t(18) = -1.21$, $p = 0.95$) and abaxial ($n = 10$, $t(18) = -2.57$, $p = 0.13$) surfaces.

Figure 33 shows that the level of RDX in the excess water pumped from the plots containing the wild type control plants was not significantly different from the NPC plots, whereas water pumped from the plots planted with the XplAB-expressing switchgrass contained significantly less ($p = 0.015$) RDX than water pumped from the NPC plots.

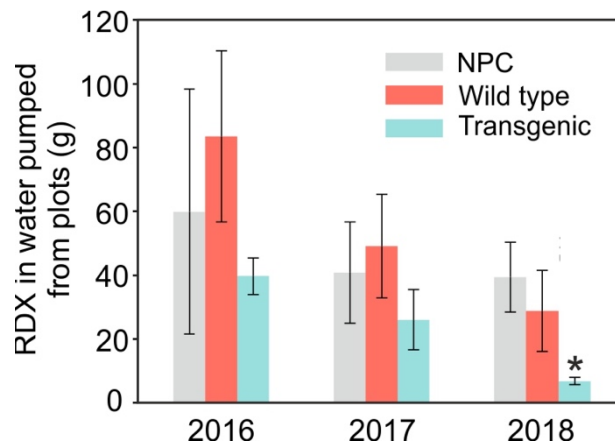


Figure 33. Levels of RDX in the Excess Water Pumped from the Plots

*Levels of RDX in the excess water pumped from the plots over the three-year duration of the trial (NPC; No Plant Control, $n = 3 \pm SD$, * represents significantly different, $p = 0.015$, to 2018).*

The levels of transpiration from each plot were estimated from the excess water pumped minus the rainfall measurements for 2018. While it was not possible to determine the level of evaporation from the plots, given the near canopy closure shown in Figure 20, this factor was considered to be relatively low compared to that lost through transpiration. Using the mean RDX concentrations in the soil water during the 2018 growing season (Figure 28c), we calculated that the transgenic plants took up and metabolized significantly ($p = 0.002$) more RDX from the plots than the wild type plants (transgenic: 25g, wild type: 4g; Figure 34), and equivalent to an RDX removal rate by the XplAB-expressing plants of 27 kg RDX per hectare. Based on our earlier data in *Arabidopsis* (Rylott et al. 2006; Rylott et al. 2011), and grass species, it is likely that the wild type plants did not significantly degrade any RDX. (Zhang et al. 2017a; Zhang et al 2018).

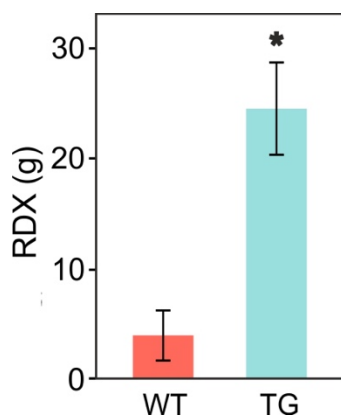


Figure 34. Calculated Level of RDX Degraded in the Transpiration Stream

From plants in the 100 mg/kg RDX plots over the 2018 growing season. Results are per 9m² plots, WT; wild type, TG; transgenic, $n = 3 \pm SD$, * represents significantly, $p = 0.015$

6.6 MOVING THE TECHNOLOGY INTO WESTERN WHEATGRASS

6.6.1 Creation of transgenic western wheatgrass

Following results from the expression of *xplA*, *xplB*, and *nfsI* in switchgrass and creeping bentgrass, the vector used in our former report (Zhang et al. 2017a), was modified to improve transgene expression. The 35S promoter driving expression of *nfsI* was replaced with the rice (*Oryza sativa*) actin promoter (*Osact*); and the switchgrass ubiquitin promoter (*Pvubi*) driving *xplA* expression was replaced with the maize (*Zea mays*) ubiquitin promoter (*ZmUbi*). The structure of the resulting vector, pEDLZ2014, is shown in Figure 35.

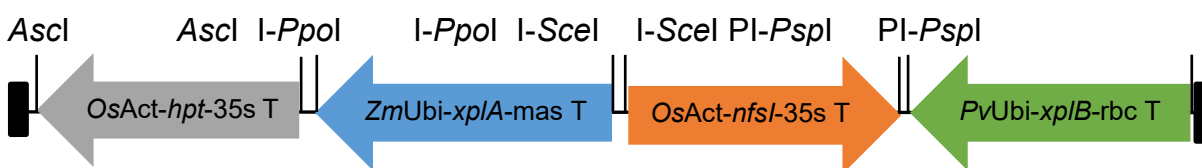


Figure 35. Construction of Vector pEDLZ2014 for Transformation of Western Wheatgrass

T-DNA region of the binary vector plasmid pEDLZ2014 used to transform western wheatgrass. The RDX degradation gene *xplA*, flavodoxin reductase gene *xplB*, and TNT detoxifying nitroreductase gene *nfsI* were constructed into versatile cloning vector pNSATs. Arrows show the direction of transcription. Abbreviations: *Osact*, *Oryza sativa* actin promoter; *hpt*, selection marker gene, *hygromycin B phosphotransferase* (*hpt*); 35s T, terminator of CaMV 35s gene; *Zmubi*, *Zea mays* ubiquitin promoter; *mas T*, terminator of *manopine synthase* gene of *Agrobacterium*; *Pvubi*, *Panicum virgatum* (switchgrass) ubiquitin promoter; *rbc T*, terminator of rubisco small subunit of tobacco; RB left border; RB right border.

To successfully transform western wheatgrass, vigorously-growing embryogenic calli were needed. Figure 36a shows the white or light-yellow, friable embryogenic calli obtained after 1-2 months of growth. Following additional culturing, calli suitable for transformation were obtained (Figure 36b) and bombarded with pEDLZ2014 vector DNA. Figure 36c shows the appearance of the surviving calli two months after bombardment; after three to four months, green and healthy plantlets were obtained (Figure 36d). In total, 10 plates of embryogenic calli were bombarded, with 98 calli showing hygromycin resistance and of these, 30 produced green and healthy plantlets (Figure 36e). To produce biomass for subsequent analysis, the plantlets were propagated on cluster shoot induction medium which initiated the formation of robust shoots and rhizomes as shown in Figure 36f. The rhizomes were cut into nodal sections, as shown in Figure 36g, and cultured to regenerate new plantlets. Figure 36h shows the appearance of the fully regenerated plants in soil.

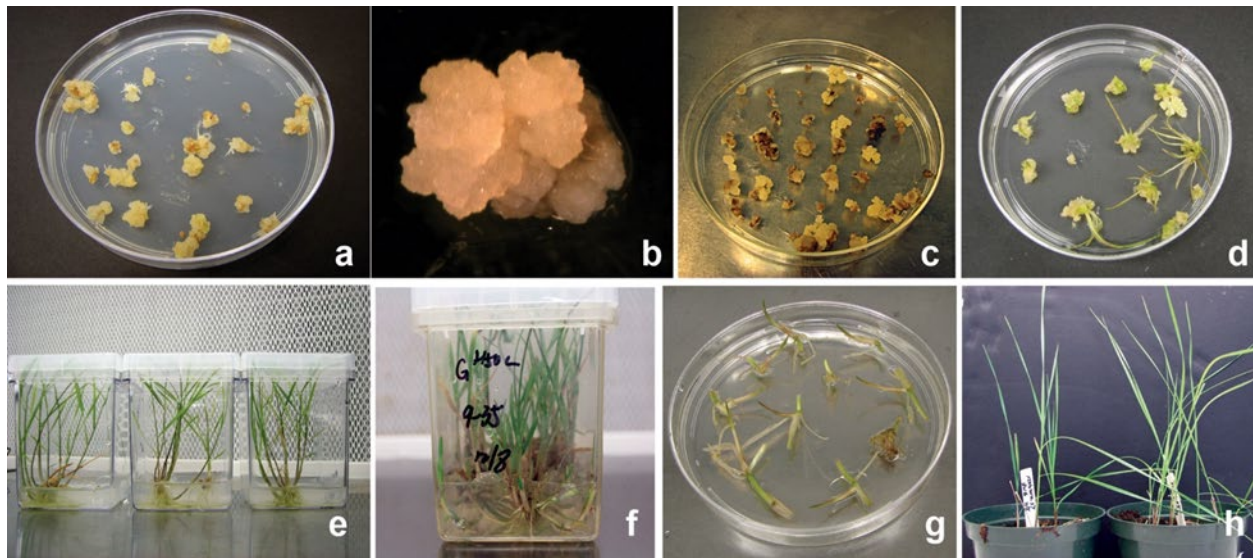


Figure 36. Tissue Culture, Transformation and Propagation of Western Wheatgrass

(a) Two month old calli induced from mature seeds. (b) Embryogenic calli that were used for bombardment with vector DNA. (c) Calli screened on Callus Induction medium with 75 mg/L hygromycin for two months after bombardment. (d) Hygromycin resistant plantlets from calli that were transferred to regeneration medium for 1-2 month. (e) Regenerated plantlets growing on MS medium with 75 mg/L hygromycin. (f) PCR and qRT-PCR positive plants were transferred to cluster shoot induction medium to induce cluster shoots and rhizomes. (g) Shoot induced on rhizomes sections with nodules on MS medium. (h) Transgenic plants growing in soil.

6.6.2 Confirming integration and expression of *xplA* and *xplB* in western wheatgrass

PCR and qRT-PCR analysis were conducted on thirteen, independently-transformed hygromycin-resistant plantlets. The results, presented in Figure 37a, show expression of *xplA*, *xplB* and *nfsI* in the transgenic lines, while the transgenes were not detectable in the wild-type, untransformed plants. Expression of the transgenes was highest in lines S35, N1, N9 and D22 and subsequent western blot analysis (Figure 37b) of these lines revealed the presence of 60, 45 and 24 kDa bands, which corresponded in size to the XplA, XplB and NR proteins, respectively.

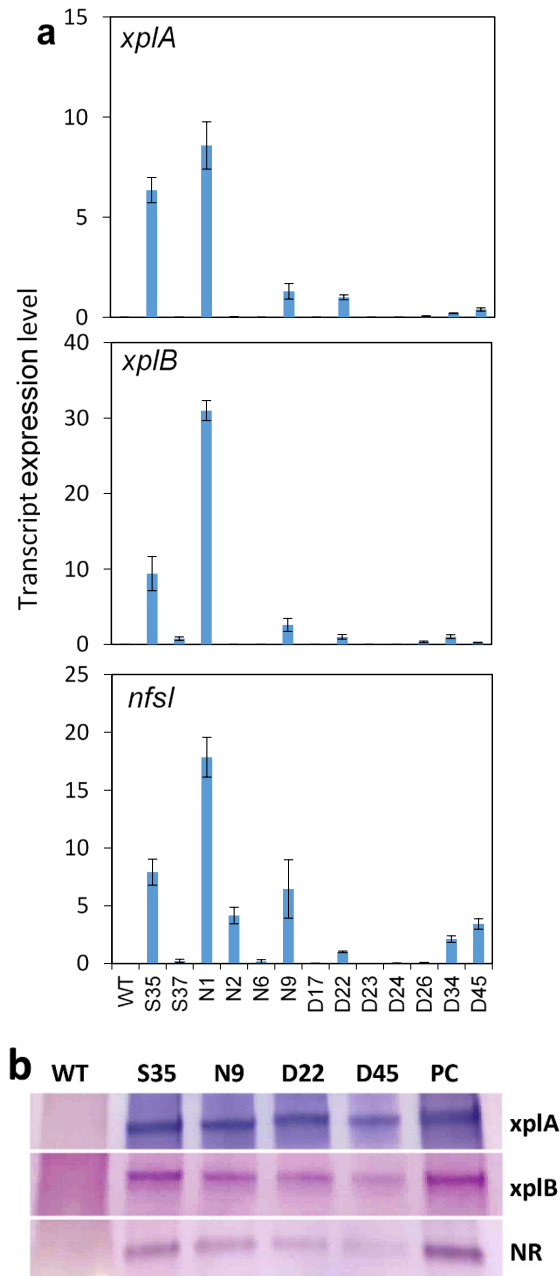


Figure 37. Molecular Characterization of *xplA-xplB-nfsI* Transformed Western Wheatgrass

a) Transcript abundance measured using quantitative RT-PCR on plant lines transformed with *xplA*, *xplB* and *nfsI*. Values were normalized to the 5.8s rRNA gene and relative to expression level of the transformed line D22. Data are the means $\pm$ SE, $n = 3$. **b)** Western blot analysis on leaf blades of western wheatgrass lines expressing XplA, XplB and nitroreductase (NR) protein. WT, wild type; PC, positive control, *xplA-xplB-nfsI* transformed *Arabidopsis* (Rylott et al. 2011a).

6.6.3 RDX uptake studies by XplAB-expressing Western wheatgrass

To determine the rate of RDX uptake, the plants were grown in liquid MS medium in open test tubes. Evaporation of RDX from the medium was minimal due to its low volatility (Xiong et al. 2009). Over the course of the experiment, all the transformed lines removed RDX from the medium faster than the wild type plants (Figure 38a). After 3 days, the RDX concentration in the medium containing transformed plants was significantly lower than that of wild type plants ($p = 2 \times 10^{-6}$). After 6 days, the difference between wild type and transformed lines further increased with lines N9 and D22 removing significantly more RDX than lines S35 and D45 ($p = 0.0003$). After 12 days of culture, 65 % of the RDX had been removed by wild type plants while 86 % and 87 % by line N9 and D22 respectively. After 12 days, wild type RDX leaf tissue levels were found to be 0.12 ± 0.03 mg/g RDX, in comparison, RDX was not detected in tissue from transformed lines tested, N9, D22, and S35, and RDX was at 0.033 ± 0.001 mg/g in transformed line D45 (Figure 38b). To confirm that the lack of accumulation of RDX in transformed tissue was caused by degradation, rather than by dilution in growing plant tissues, leaf blades of wild-type and lines, N9 and D22 were cultured in water with 20 mg/L or 40 mg/L RDX.

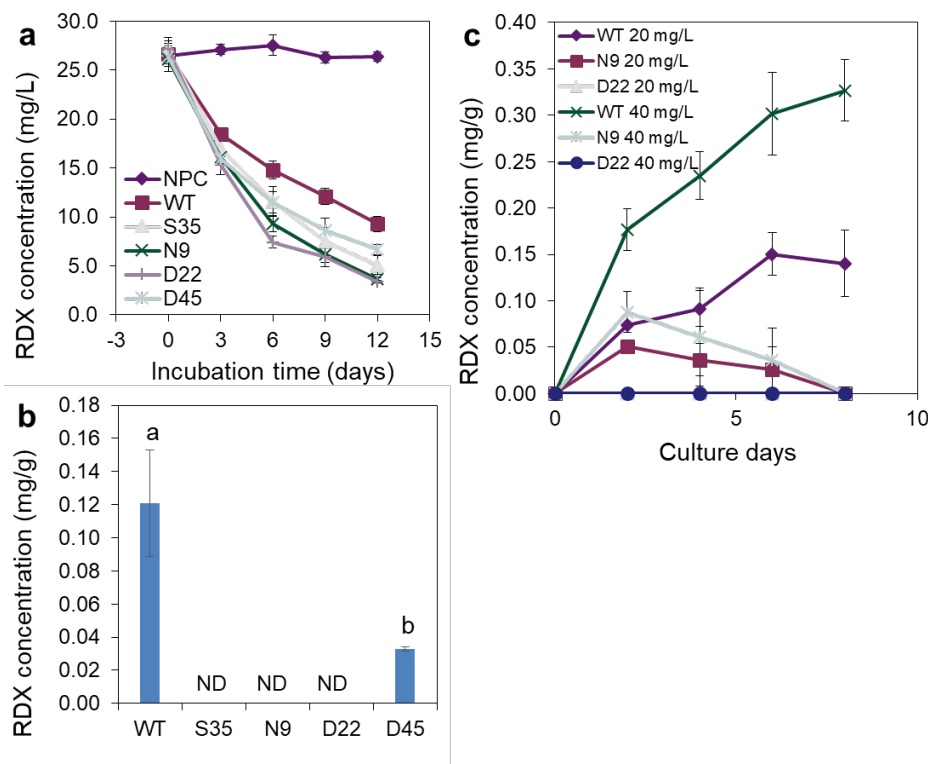


Figure 38. Uptake and Degradation of RDX by *xplA-xplB-nfsI* Transformed Western Wheatgrass

(a) Concentration of RDX in culture medium over the course of the experiment. (b) Concentration of RDX in plant tissue after 12 days culture. (c) Accumulation of RDX in leaf tissue during 8 days culture in 20 mg/L and 40 mg/L RDX solution. Letters indicate that RDX concentration in tissue were significantly different ($p < 0.05$) from other lines. Data are the means $\pm$ SE, $n = 3$; ND = None Detected; NPC, No Plant Control.

Figure 38c shows that while the concentration of RDX in the wild-type leaves increased from day 2 to day 8, no RDX was detected in the transformed line D22. Although RDX was detected in leaf tissue of transformed line N9, it was 28 % lower than that in wild type ($p = 0.004$) at day 2 when cultured in RDX solution at 20 mg/L, and 53% lower ($p = 0.033$) at day 2, with 40 RDX mg/L. In contrast to wild type, the RDX concentration in line N9 decreased after 2 days of culture and could not be detected on day 8. This result confirmed that both N9 and D22 are able to degrade RDX.

6.6.4 TNT detoxification studies on XplAB-expressing western wheatgrass

Though the main target of this research was to engineer grasses for RDX degradation, the grasses also need to be able to resist the phytotoxicity of TNT since training ranges are contaminated by both RDX and TNT, and TNT can also inhibit XplA activity (Jackson et al. 2007). Thus the *xplA-xplB-nfsI* transformed lines N9, and D22, which had the highest RDX degradation abilities, were tested for their resistance to TNT conferred by NR activity. When leaf sections were cultured in water containing 15 mg/L TNT, the transformed lines removed TNT more quickly than wild type plants ($p = 0.0019$ at 18 hr incubation). Line D22 removed the TNT most rapidly, with TNT undetectable in the solution after 48 hr. After 66 h, nearly all TNT had disappeared from solution from wild type and transformed plants (Figure 39a). Though wild type western wheatgrass removed TNT from the solution, when compared to the transgenic lines after 66 h, the wild type leaf sections exhibited symptoms of toxicity, with overall yellowing of the leaf surfaces and darkening of the cut edges. After 66 hours of culture in water containing 32 mg/L TNT, the wild type plants were unable to remove any TNT, while the transformed lines removed nearly all the TNT (0.38 ± 0.16 mg/L remaining for N9 and 0.51 ± 0.35 for D22; Figure 39b). Furthermore, the wild type plants showed signs of toxicity, as indicated by a reduction in growth rate such that the biomass gain of wild-type was 24 % and 30 % of that of N9 and D22, respectively after 3 days.

While in whole plants, TNT and transformation products are localized predominantly in the roots, when grown in liquid culture, leaf tissues are known to also contain these products (Hannink et al. 2007; Hannink et al. 2001). To verify that TNT was transformed in the tissues of the modified plants, TNT and ADNT levels, in leaf tissue were determined after 42 hours of culture in TNT solution (Figure 39c). When cultured in 12.5 mg/L TNT solution, TNT was not detected in the tissues of either wild type or transformed plants, while ADNT was detected in wild type leaf tissue at a level lower than for N9 and D22 ($p = 0.0188$). When cultured in 25 mg/L TNT solution for 42 hours, the wild type leaf tissue accumulated 11.91 ± 1.64 $\mu\text{g/g}$ TNT, about 3 times of that in lines N9 and D22 ($p = 0.0004$). At the same time, the ADNT concentration in the wild type plants was 6.01 ± 0.92 $\mu\text{g/g}$, about 60 % of that in leaf tissue of transformed plants ($p = 0.0012$). These results demonstrated that though wild type plants have the ability to uptake and detoxify limited amounts of TNT, the *nfsI*-transformed western wheatgrass are able to detoxify significantly more TNT, resulting in increased tolerance, and subsequent ability to take up overall more TNT.

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7.0 PERFORMANCE ASSESSMENT

The Performance Objectives of the project are listed in Section 3 Table 3.

7.1 QUANTITATIVE PERFORMANCE OBJECTIVES

7.1.1 Satisfy criteria for limited release of transgenic grasses to environment

A permit from the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) was required to enable controlled release of the transgenic plants into the environment. For the permit, National Environmental Policy Act (NEPA) documentation data were collected, in collaboration with the US Army Garrison Fort Drum, Environmental and Integrated Training Area Management, and Land Rehabilitation and Maintenance groups: descriptions of the proposed site (rainfall amounts, GPS coordinates, and orientation), demonstration of controlled site access, soil, flora and fauna surveys, and data on the cultivar phenotype. Comparisons between the wild type lines with the transgenic lines in greenhouse studies were also provided. A specific condition of the permit was the removal of all developing flower heads from the plots. The APHIS permit was successfully awarded.

7.1.2 Significant reduction of RDX levels in transgenic grass plots versus wild type

As shown in Figure 20, the plants did not establish vigorous growth until the third growing season (2018). Thus, to compare the performance of the transgenic grass plots against the wild type plots, we have focused data analysis on the results from the 2018 season.

For the soil samples, the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was not met. As can be seen in Figure 28a, analysis of soil core samples from across the plots showed that the RDX was distributed heterogeneously across the plot. Unexpectedly, given its documented mobility in soils, RDX had not significantly moved below the 0-5 cm depth in the plots. Higher RDX concentrations tended to be clustered furthest away from the culvert areas, suggesting localized gradients occurred, as indicated by arrows on Figure 28b. Our previous studies demonstrated that the XplAB-expressing switchgrass can remove all the RDX from saturated solutions in soil columns. In the field plots, the switchgrass roots were mainly in the lower depths of the plots and thus the majority of the RDX was not in contact with the plant roots.

For the soil water samples, as can be seen in Figure 28c, there was no significant difference in the levels of soil water RDX in the NPC, WT, and TG plots across the 2018 growing season. However, the mean RDX level in the TG plot tanks was significantly less ($p = 0.021$) than in the tanks on the NPC plots, and at each month sampled, the RDX level was always lower than that in the NPC and WT plot tanks. Figure 33 shows that across all three years, the concentration of RDX in the excess water pumped from the TG plots was always less than from either the NPC or WT plots. In the 2018 growing season, excess water pumped from the plots planted with the XplAB-expressing switchgrass contained significantly less ($p = 0.015$) RDX than excess water pumped from the NPC plots. Thus, we conclude from these data, that the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was partially achieved for soil water on the plots.

For the plant tissue samples, the criterion of a statistically significant ($p < 0.05$) decrease in RDX levels was met across all three growing seasons. During the 2018 growing season, levels of RDX in the aerial tissues of unmodified plants grown on the 100 mg/kg RDX plots varied within plots, between plots, and between monthly samplings. RDX was not detected in any of the aerial tissues sampled from the transgenic lines over the entire 2018 growing season, and across all three 100 mg/kg RDX plots, (Figure 25). At the end of the 2018 growing season, the mean RDX concentration in the wild type switchgrass from each of the three plots (Figure 30a) was 0.025 ± 0.02 mg/g dry weight. Previous measurements of tissue RDX levels using RDX-saturated soil column studies measured a maximum of 0.23 mg/g for wild type switchgrass. Under these column conditions, RDX was detected in the XplAB-expressing switchgrass line N1, but at just 4 % of that in the unmodified switchgrass, suggesting that 0.23 mg/g is indicative of the likely upper limit for the RDX-degrading capacity of the transgenic lines in-the-field (Zhang, L. et al 2017) , and that the XplAB-expressing plants in the 2018 season were at approximately 11 % of their RDX uptake capacity.

RDX is commonly found in soils with the co-pollutant TNT and the latter is highly toxic to plants, and known to inhibit XplA activity (Jackson et al. 2007b). Alongside *xplA* and *xplB*, the switchgrass lines were also engineered to contain *nfsI* a nitroreductase that detoxifies TNT when expressed in tobacco (*Nicotiana tabacum*) (Hannink et al. 2007; Hannink et al. 2001. Our gene expression studies on the XplAB-expressing switchgrass demonstrated that while the lines contained *nfsI*, transcript (Figure 12a), or nitroreductase protein (Figure 12b) were not detectable. However, in the presence of TNT, the XplAB-expressing switchgrass lines were able to remove significantly more RDX from the media than wild type, unmodified lines (Figure 14), indicating that the *nfsI* gene was expressed at albeit low levels. A possible reason was due to the design of the construct and gene promoters used. In the construct used to transform the XplAB-expressing switchgrass Figure 5, *nfsI* expression was controlled by the viral 35S promoter and terminator. Subsequently, these elements were replaced with a promoter and terminator from the monocot species rice (*Oryza sativa*), and the orientation of this expression cassette reversed. When this new cassette (Figure 35) was transformed into western wheatgrass, the *nfsI* gene was expressed, and the plant lines removed significantly more TNT from media than unmodified, wild type plants.

7.2 QUALITATIVE PERFORMANCE OBJECTIVES

7.2.1 Reliability

As shown in Figure 20, over the first two growing seasons, the plant biomass for both transgenic and wild type was low. A number of factors contributed towards this: the below seasonal averages of temperature, and above average levels of rainfall. The low temperatures over the winter caused some of the young plants to die, these were replaced in the spring with similar-sized reserve plants from the glasshouse. The low temperatures also led to a delay in spring growth. Alamo performs best in areas of 25 inches of annual precipitation, but in the 2017 and 2018 growing seasons there was unexpectedly high rainfall (2016: 13.3", 2017: 48.0", 2018: 31.25"). This excess rainfall resulted in the plots becoming water-logged, until additional reservoir tanks could be installed. This water-logging occurred because the plots were double-lined with waterproof polypropylene; a condition of the APHIS permit, and would not be an issue on the open range. Each plot contained, embedded in sand between the polypropylene layers, a lysimeter to enable soil water measurement below the primary containment layer. Tests from these lysimeters confirmed that no RDX breached the upper containment layer.

An additional restriction to plant vigor, was that the cultivar of wheatgrass selected for the trial, Alamo, is primarily adapted to regions south of I-70 which intersects Kansas east to west (38-39°N latitude); the field site was located at 44°, 4'47.03"N. This choice of mismatched cultivar, was a deliberate component for the APHIS permit, designed to reduce the likelihood of cross-pollination with closely-related, native species, and was in addition to the removal of flower heads, during the field trial.

At the end of the trial, the average aerial tissue biomass was 0.53 kg m⁻². Typical biomass yields for switchgrass are 1.08 kg m⁻² (Schneider & McCarl, 2003), thus, at the end of the three-year trial, the switchgrass had reached 50 % of its potential biomass. Despite the relatively poor biomass production, there was no significant differences in growth between the wild type and transgenic plants, as shown in Figure 29. The transgenic plants achieved the criterion that they were able to survive under field conditions as well as or better than wild type.

7.2.2 Availability

A number of techniques were employed at both UoW and CRREL to gain seed from the transgenic lines, including the use of high-light growth chambers, a range of photoperiods, and outcrossing with wild type Alamo and Kanlow switchgrass cultivars. Under the conditions tested, viable seed was not obtained. Testing of vegetatively-propagated materials demonstrated that the RDX-degrading properties were transferred to propagated material. For both the XplAB-expressing switchgrass, and western wheatgrass lines, viable seeds were not produced.

We are currently investigating options for seed production, outcrossing rates and potential gene flow to other switchgrass cultivars as part of our deregulation activities.

7.2.3 Regulatory Acceptance

Throughout the process, the Regulatory Drivers outlined in section 1.3 above were followed. APHIS were involved from the start of the project, granting approval for testing of the XplAB-expressing lines under APHIS regulation 7 CFR part 340. On 1-Aug-2017, a meeting of regulatory and DOD stakeholders was held to review project. The meeting included a site visit (Figure 40), and a CRREL report of the meeting is attached (Appendix B).



Figure 40. Site Visit by Regulatory and DOD Stakeholders 1-Aug-2017

As described in section 8.0 Implementation Issues, there has been discussions, and progress, with both APHIS and the FDA on the topic of deregulating the XplAB-expressing switchgrass.

8.0 COST ASSESSMENT

Cost is an important part of the decision-making process when looking at remediation technologies. Currently there are no cost-effective processes to contain RDX or remediate large areas of contaminated vegetated land on training ranges. According to an EPA fact sheet (EPA 2017) there are a variety of methods to clean up RDX from military sites, however the processes are not environmentally friendly, they require space to perform these operations, and they require that the land be removed from military operations. Furthermore, given the scale of contamination, these methods would be prohibitively expensive and logistically challenging.

8.1 COST MODEL

The use of transgenic plant materials will reduce the costs associated with RDX remediation by reducing overall remediation costs. The main cost drivers associated with implementing this technology is given in Table 1. The basic cost model includes both fixed and variable costs. The fixed costs include the APHIS permit application, including the collection of site-specific data and the baseline characterization data. At most facilities the baseline characterization will have already been completed as part of the Military Munitions Response Program (MMRP). The required site-specific data may already be available through the facilities Environmental Office or, in the case of Army facilities, the Integrated Training Area Management (ITAM) program.

USDA-APHIS permit: Since transgenic grass infected with a bacterial gene is being used, a permit from the USDA-APHIS is required. The permit application requires explanation of site and plant descriptions. Site descriptions require descriptions of the flora and fauna at the site. This information is easily accessible through the INRMPS documentation that is required to be developed at each military location. Information on the transgenic plant description will have been previously documented before this demonstration is completed. Therefore, the cost associated with acquiring the permit should be limited to reviewing information already known. There is no permit application fee.

Cost: \$5,000

Transgenic grass propagation: Propagating new plant material once the parent material is in hand is laborious but not difficult. Switchgrass profusely produces tillers that can be separated from the parent plant to be used to propagate new plants. It was estimated that plants would be planted on a one-foot grid and 1800 plants were required. It is assumed that these plants will be propagated from 25 parental plants. This process took approximately six months. Labor, potting material, and greenhouse space were needed, at an estimated a cost of \$25,000.

Site preparation: Only conventional planting equipment was required. This equipment is already available at military facilities and it is managed under Land Rehabilitation and Maintenance (LRAM) programs. The personnel are also available to perform this function and the cost should be covered under LRAM.

Monitoring: Monitoring costs for this demonstration will include instruments for analyzing RDX and irrigation.

8.2 COST DRIVERS

The primary factor in demonstrating the cost effectiveness of this demonstration is that this project does not use the 'dig and haul' methods recommended by the EPA; using instead a biological technology of which implementation is partially well known by military land managers. These land managers have expertise in the agronomy and so this will be used to full advantage; making the most of existing infrastructure and lines of plants. The primary costs for implementation are acquiring a USDA-APHIS permit, transgenic grass propagation, site preparation, and monitoring.

8.3 COST ANALYSIS

If we look at a simplified example for the use of our technology on a small (100m²) contaminated site, the typical costs would be:

Fixed costs:

Permit Application	\$ 6,000
Site specific data summary for permit	\$ 2,000
Baseline characterization of contamination	\$20,000

Variable costs:

Plant production ($17 \text{ plants m}^2 * 100\text{m}^2 = 1700 \text{ plants} * \$0.50$) =	\$ 850
Planting labor ($\$8.62 \text{ per m}^2 * 100 \text{ m}^2$) =	\$ 862
Irrigation system to help establish transplants	\$ 1,500
Datalogger, sensors, and batteries – one of these is needed per site	\$ 6,000
Long term site monitoring escape (1 hour per month per 100m ²)	\$ 25
Long term soil and water monitoring per year	\$20,000

This would give a total for the fixed costs of \$28,000 per permit and a total of variable costs of \$29,237 per 100m² per year. It is important to note that permit fixed costs would be reduced after the first permit for a site is issued, as that data could be duplicated for each subsequent permit application. Also, if the plants can be deregulated, the cost associated with the APHIS permit application, site monitoring and maintenance activities and long term monitoring would be reduced to zero. Thus requiring only the cost of plant production, planting and long term soil and water monitoring.

9.0 IMPLEMENTATION ISSUES

The main issue presented to the future implementation of XplAB-expressing switchgrass is regulatory, specifically due to the transgenic nature of the plant's development. The switchgrass is currently limited to small, monitored testing under APHIS regulation 7 CFR part 340. Currently, the GM switchgrass is regulated due to concerns over gene transfer and increased weediness. However, there have been discussions with both APHIS and the FDA on the topic of deregulating the XplAB-expressing switchgrass, and a white paper (Appendix D) on the need and likelihood of deregulation has been submitted to ESTCP.

APHIS may be petitioned to show that a regulated article, such as XplAB-expressing switchgrass, is unlikely to pose a plant pest risk, and therefore should no longer be regulated under the plant pest provisions of the Plant Protection Act or the regulations at 7 CFR part 340. The petition must provide information under § 340.6(c)(4) related to plant pest risk which APHIS may use to determine whether the plant will pose more of a plant pest risk than the unmodified plant. If APHIS decides that there is no greater plant pest risk, the GE plants no longer subject to these regulatory requirements. Deregulating the XplAB-expressing switchgrass will allow for both larger scale testing and also implementation. On 2-Dec 2019, Prof Neil Bruce and Mr Tim Cary attended a meeting with APHIS in Washington D.C. to discuss requirements for a petition to deregulate the XplAB-expressing switchgrass.

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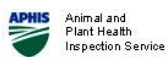
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APPENDIX A POINTS OF CONTACT

Point of Contact Name	Organization Name Address	Phone Fax Email	Role in Project
Prof. Neil C. Bruce	University of York Centre for Novel Agricultural Products Department of Biology Wentworth Way York	N/A	Lead Principal Investigator (University of York)
Mr Tim Cary	ERDC-CRREL CEERD-RR-N 72 Lyme Road Hanover New Hampshire 03755	N/As	Principal Investigator (ERDC- CRREL)

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APPENDIX B APHIS PERMIT SUBMISSION



Biotechnology
Regulatory
Services

4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

U.S. DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE BIOTECHNOLOGY REGULATORY SERVICE PERMIT UNDER 7 CFR 340 <i>(Genetically Engineered Organisms or Products)</i>	
This permit was generated electronically via the ePermits system	
Enclosed is the BRS Permit Application	
PERMITTEE NAME: Mr. Timothy Cary ORGANIZATION: ERDC-CRREL ADDRESS: 72 Lyme Road CEERD-RR-W Hanover, NH 03755 PHONE: 603-646-4358 FAX: 603-646-4845 DESTINATION: NH, NY, WA RELEASE: NY	PERMIT NUMBER: 16-025-103rm DATE ISSUED: April 22, 2016 EFFECTIVE: May 1, 2016 EXPIRES (Movement): May 1, 2017 EXPIRES (Release): May 1, 2019
INTRODUCTION TYPE: Interstate Movement and Release PERMIT TYPE: Standard PURPOSE OF PERMIT: Phytoremediation	

Regulated Article: Panicum virgatum	Under the conditions specified, this permit authorizes the following:
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Permit Number: 16-025-103rm	
THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS	
SIGNATURE OF BRS OFFICIAL Subray Hegde	DATE April 22, 2016

The donor organism was *Rhodococcus rhodochrous* extracted from RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) contaminated soils from the US. The donor organism for the TNT nitroreductase is *Enterobacter cloacae* obtained in the US from military impact areas. The recipient organism is *Panicum virgatum*, cultivar 'Alamo' purchased from Hancock Seed Company, Dade City, FL 33523. All vectors were constructed at the University of Washington.

Processes, Procedures, and Safeguards Description:

All plants have been grown in growth chambers and greenhouse facilities located at the University of Washington and ERDC-CRREL in Hanover, NH. Plants have been maintained in a non-sexually reproductive state through vegetative propagation and panicle removal through clipping. All waste material (whole plants, clippings and waste soil) were autoclaved to ensure enzymatic denaturing prior to disposal from either the laboratory or greenhouse facilities.

Plants were shipped to the ERDC-CRREL Greenhouse in April and May 2014 under APHIS transportation permit number 13-206-104m-al following 7 CFR340.4 and 340.8.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

<u>Name</u>	<u>Location</u>	<u>Contact Information</u>
Dr. Stuart Strand	University of Washington 490 Ben Hall IDR Bldg 616 NE Northlake Place Box 355014 University of Washington Seattle, WA 98105	Day Telephone: 206-543-5350 FAX: 206-685-9996 Email: sstrand@uw.edu
Mr. Timothy Cary	ERDC-CRREL 72 Lyme Road CEERD-RR-N Hanover, NH 03755 County: Sullivan	Day Telephone: 603-646-4358 FAX: 603-646-4845 Email: timothy.j.cary@usace.army.mil

11. PHENOTYPES/GENOTYPE

1) **Phenotypic Designation Name:** RDX degradation

Identifying Line(s):

Construct(s): Lines 20-1 HR, 8 HR, 5 HR

Mode of Transformation: *Agrobacterium tumefaciens*, disarmed

Phenotype Description: Hygromycin resistance, degradation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) pollutant, resistance to phytotoxicity of TNT (2,4,6 Trinitrotoluene)
A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.

Phenotype(s)

OO - RDX degradation

OO - Resist TNT phytotoxicity

MG - Hygromycin resistant

Genotype(s)

Selectable Marker

Promoter: CaMV35S **from** Cauliflower mosaic caulimovirus - Promoter from cauliflower mosaic virus, abbrev. 35S

Gene: hygromycin phosphotransferase (hpt) gene **from** *Escherichia coli* - The accession number for Hygromycin phosphotransferase (hpt) gene is V01499 from <http://www.ncbi.nlm.nih.gov/nuccore/V01499.1>

Hpt, the selective marker gene, detoxifies hygromycin within the plant. The hpt gene has been widely used in genetically modified plants without toxic effects in the environment. None of the gene products undergoes additional processing or cleavage to other products.

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Terminator: 35S **from** Cauliflower mosaic caulimovirus - Terminator from Cauliflower mosaic virus

Gene(s) of Interest

Promoter: 35S Promoter **from** Cauliflower mosaic caulimovirus - Promoter from Cauliflower mosaic virus

Gene: xplB **from** Rhodococcus rhodochrous 11Y - Accession number for XplB is AF449421.1, from <http://www.ncbi.nlm.nih.gov/nuccore/AF449421.1>

XplB is the reductase partner for xplA. It is a unique protein that has recently evolved in bacterial to degrade RDX (the true xenobiotic) and has no significant homology to other proteins. Codon usage optimized for plants. None of the products of RDX degradation by xplA or xplB lead to a product for industrial or any other use, food or otherwise. RDX is the only known substrate for xplA or xplB. Final products of xplA/B degradation are utilized with in the plant to produce additional biomass and are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or final disposition.

Engineering has had no affect on biological properties related to confinement measures.

Final products are expected to have no affects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: 35S Terminator **from** Cauliflower mosaic caulimovirus - 35S Terminator from Cauliflower mosaic virus.

Promoter: rbc **from** Chrysanthemum x morifolium - RuBisCO small subunit promoter from Chrysanthemum x morifolium.

Gene: xplA **from** Rhodococcus rhodochrous 11Y - Accession number for XplA is AF449421.1 from <http://www.ncbi.nlm.nih.gov/nuccore/AF449421.1>

Primary RDX degradation gene, encodes for RDX degrading enzyme xplA. It is a unique protein that has recently evolved in bacterial to degrade RDX (the true xenobiotic) and has no significant homology to other proteins. Codon usage optimized for plants. None of the products of RDX degradation by xplA or xplB lead to a product for industrial or any other use, food or otherwise. RDX is the only known substrate for xplA or xplB. Final products of xplA/B degradation are utilized with in the plant to produce additional biomass and are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or final disposition.

Engineering has had no affect on biological properties related to confinement measures.

Final products are expected to have no affects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: rbc **from** Chrysanthemum x morifolium - RuBisCO small subunit terminator from Chrysanthemum x morifolium.

Promoter: act **from** Arabidopsis thaliana - Promoter for actin gene in Arabidopsis thaliana

Gene: nfsI **from** Enterobacter cloacae - Accession number for nfsI is M63808.1 from <http://www.ncbi.nlm.nih.gov/nuccore/M63808.1>

Gene encoding for TNT detoxifying nitroreductase enzyme from Enterobacter cloacae. nfsI attacks TNT, reducing one of the nitroso moieties on the TNT molecule, reducing its toxicity. The gene nfsI acts on TNT, producing no product for industrial or other use; however, there will be no TNT in the proposed field trials. Additionally, final products are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or final disposition.

Engineering has had no affect on biological properties related to confinement measures.

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Final products are expected to have no effects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: **ags** from *Agrobacterium tumefaciens* - Terminator for Agropine synthase gene from *Agrobacterium tumefaciens*.

2) Phenotypic Designation Name: RDX degradation

Identifying Line(s):

Construct(s): Lines NABNR N1, NABNR N2, NABNR N5

Mode of Transformation: *Agrobacterium tumefaciens*, disarmed

Phenotype Description: Hygromycin resistance, degradation of RDX pollutant, resistance to phytotoxicity of TNT

A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.

Phenotype(s)

OO - RDX degradation

OO - Resist TNT phytotoxicity

MG - Hygromycin resistant

Genotype(s)

Gene(s) of Interest

Promoter: ubiquitin **from** *Zea mays* - ubiquitin promoter from corn (*Zea mays*)

Gene: xplB **from** *Rhodococcus rhodochrous* 11Y - The accession number for xplB is AF449421.1 from <http://www.ncbi.nlm.nih.gov/nuccore/AF449421.1>

XplB is the reductase partner for XplA. It is a unique protein that has recently evolved in bacteria to degrade the true xenobiotic RDX and has no significant homology to other proteins. Codon usage optimized for plants. None of the products of RDX degradation by xplA or xplB lead to a product for industrial or any other use, food or otherwise. RDX is the only known substrate for xplA or xplB. Final products of xplA/B degradation are utilized within the plant to produce biomass and are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or final disposition.

Engineering has had no effect on biological properties related to confinement measures.

Final products are expected to have no effects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: **mas** from *Agrobacterium tumefaciens* - Mannopine synthase terminator from *Agrobacterium tumefaciens*, abbreviated mas

Promoter: ubiquitin **from** *Panicum virgatum* - ubiquitin promoter from switchgrass (*Panicum virgatum*)

Gene: xplA **from** *Rhodococcus rhodochrous* 11Y - The accession number for XplA is AF449421.1 from <http://www.ncbi.nlm.nih.gov/nuccore/AF449421.1>

XplA gene encoding RDX degrading enzyme xpa, a unique protein that has evolved in bacteria to degrade RDX (the true xenobiotic compound) and has no significant homology to other proteins. Since it only degrades the pollutant and toxicant RDX, XplA reduces toxicity in the environment and reduces risk of harm to non-target organisms. RDX is the only known substrate for xplA or xplB. Final products of xplA/B degradation are utilized within the plant to produce biomass and are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or

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final disposition.

Engineering has had no affect on biological properties related to confinement measures.

Final products are expected to have no affects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: rbc **from** Chrysanthemum x morifolium - Rubisco small subunit terminator from chrysanthemum

Promoter: 35S **from** Cauliflower mosaic caulimovirus - 35S promoter from Cauliflower mosaic virus

Gene: nfsI **from** Enterobacter cloacae - The accession number for nfsI is M63808.1 from <http://www.ncbi.nlm.nih.gov/nuccore/M63808.1>

NfsI, TNT nitroreductase, attacks TNT, reducing one of the nitro moieties on the TNT molecule, reducing its toxicity. The gene nfsI acts on TNT, producing no product for industrial or other use; however, the transformed plants will not come into contact with TNT in this field trial. Additionally, all final products are not expected to have toxic effects on non-target organisms or effect any of the stages of release including removal of panicles, devitalization or final disposition.

Engineering has had no affect on biological properties related to confinement measures.

Final products are expected to have no affects on worker safety. Proper protective equipment while handling transgenic lines would be equivalent to EPA chemical protection Level D

Terminator: 35S **from** Cauliflower mosaic caulimovirus - 35S terminator from Cauliflower mosaic virus

Selectable Marker

Promoter: act **from** rice **from** Oryza sativa - actin promoter from rice.

Gene: hygromycin phosphotransferase (hpt) gene **from** Escherichia coli - The accession number for Hygromycin phosphotransferase (hpt) gene is V01499 from <http://www.ncbi.nlm.nih.gov/nuccore/V01499.1>

Hpt, the selective marker gene, detoxifies hygromycin within the plant. The hpt gene has been widely used in genetically modified plants without toxic effects in the environment. None of the gene products undergoes additional processing or cleavage to other products.

Terminator: 35S terminator **from** Cauliflower mosaic caulimovirus - terminator from cauliflower mosaic virus

12. INTRODUCTION

Point of Origin

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) ERDC-CRREL Greenhouse	72 Lyme Road CEERD-RR-N Hanover, NH 03755 County: Sullivan	1) Mr. Timothy Cary 72 Lyme Road CEERD-RR-N Hanover, NH 03755 USA County: Sullivan Day Telephone: 603-646-4358 Email 1: timothy.j.cary@usace.army.mil
2) [] - []	NY County: Jefferson	

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Destination

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) ERDC-CRREL Greenhouse/Laboratory - CRREL Agronomy Research Area CB-69	ERDC-CRREL Building CB-69 Rooms 101 - 108 72 Lyme Road Hanover, NH 03755 County: Sullivan Proposed Start Date: 5/1/2016 Proposed End Date: 5/1/2017 Quantity: 4000 Individual Leaves/Stems/ Soil/ Soil Water Samples Inspected by BRS or PPQ? Unknown Previous Permit No.:	1) Mr. Timothy Cary ERDC-CRREL CEERD-RR-N 72 Lyme Road Hanover, NH 03755 United States County: Sullivan Day Telephone: 603-646-4358 Alt. Telephone: 603-667-7824 Email 1: timothy.j.cary@usac e.army.mil Comments: This is to allow movement of plant tissue, soil, and soil water to the laboratory for analyses.
2) [] - [] []	NY [] County: Jefferson Proposed Start Date: 5/1/2016 Proposed End Date: 5/1/2017 Quantity: 6225 Individual Whole Plants Inspected by BRS or PPQ? No Previous Permit No.:	1) Mr. Timothy Cary 72 Lyme Road CEERD-RR-N Hanover, NH 03755 USA County: Sullivan Day Telephone: 603-646-4358 Email 1: timothy.j.cary@usac e.army.mil Comments: Mr. Cary serves as the overall contact for the experiment. This includes arranging contact with the appropriate facility staff. This would include contacting security to allow access to the site. 2) Mr. [] [] [] [] [] [], NY [] USA County: Jefferson Day Telephone: [] Email 1: [] Comments: []
3) University of Washington - Strand Lab	490 Ben Hall IDR Building 616 NE North Lake Place Seattle, WA 98105 County: King Proposed Start Date: 5/1/2016 Proposed End Date: 5/1/2017 Quantity: 4000 Individual Leaves/Stems/Soil and Soil Water Samples Inspected by BRS or PPQ? Unknown Previous Permit No.:	1) Dr. Stuart Strand 490 Ben Hall IDR Building 616 NE Northlake Place University of Washington Seattle, WA 98105 United States County: King Day Telephone: 206-543-5350

Release Site

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<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) []	[], NY County: Jefferson Proposed Release Start Date: 5/1/2016 Proposed Release End Date: 5/1/2019 No. of Releases: 3 Quantity: 6 acres Comments: One release per year from 2016 through 2018. 4600 plants (2300 wild type plants, and 2300 transgenic plants) per release (growing season).	1) Mr. Timothy Cary 72 Lyme Road CEERD-RR-N Hanover, NH 03755 USA County: Sullivan Day Telephone: 603-646-4358 Email 1: timothy.j.cary@usac.e.army.mil
Location Unique ID:	[]	
Location GPS Coordinates:	[], []	
Release Site History:	[]	
Critical Habitat Involved?:	☐ Yes ☒ No	

13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

Background:

Decades of military activity on live fire training ranges have resulted in severe contamination of land and groundwater by recalcitrant high explosives, in particular, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). TNT and its transformation products are highly toxic, but as they bind strongly to clay and organic matter in soil, are largely contained at the site of contamination. However, RDX is a major concern because of its high mobility in soils and subsequent contamination of groundwater, which may move off base. These factors mean that RDX presents an immediate and future health problem to society. Also, once contamination is discovered, military operations at ranges are suspended interrupting soldier training (Claussen et al. 2006).

Objectives:

The primary objective of this release is to evaluate, under field conditions, removal of RDX from contaminated soil by transgenic grasses that were developed under Strategic Environmental Research and Development Program (SERDP) projects ER-1318, and ER-1498 (attachments 1 and 2). These projects identified RDX-degrading bacteria and the genes responsible, xplA and xplB. The insertion of xplA and xplB, first into model and then military relevant terrestrial plants, significantly enhanced the ability of the plants to degrade RDX into inert components (Jackson et al 2007, Rylott et al. 2006, Rylott et al. 2011a, Rylott et al. 2011b). We propose to use this technology to intercept and degrade RDX by growing perennial grasses in field plots contaminated with RDX located at a military facility.

The secondary objective is to determine the lower bounds or reactivity of the transgenic plants. The lowest concentration of RDX in soil that the modified plants can remove and degrade is not known. The EPA's standard for RDX in drinking water is 2 ppb. In our previous greenhouse studies, soil-grown Arabidopsis (Arabidopsis thaliana) were dosed with aqueous solutions of 40 mg/l RDX and levels of RDX in soil leachate measured using HPLC. After seven days, there was no RDX detectable in the soil leachate from the modified plants (Rylott et al. 2011a). Under these experimental conditions, the lower limit of detection by the HPLC was 0.021 mg/l (21 ppb). Conducting studies at various soil concentrations will provide additional information on the capability of the plants to degrade RDX at lower levels. If successful, this information will be beneficial in predicting plant biomass requirements to meet drinking water standards, and will increase the use of the plants in additional areas containing less than 1 ppm of RDX, such as at firing points, wetlands, manufacturing, storage, and other sites.

Technology Description:

The EPA (2014) lists a variety of methods to cleanup RDX at military bases. Most of these are physical or chemical treatments that are expensive and not compatible with the environment. Phytoremediation studies have shown that plants readily take up RDX, but do not have sufficient metabolic capability to degrade it (Best et al. 1997, Best et al. 1999, Bhadra et al. 2001), with

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RDX subsequently accumulating in the leaves and shoots to a point of plant saturation and limiting uptake (Just and Schnoor2004, Van Aken et al. 2004). We have demonstrated that the unique cytochrome P450, XplA, in combination with its partnering reductase, XplB, from the soil bacterium *Rhodococcus rhodochrous* 11Y, metabolize RDX to produce nitrite, formaldehyde and the ring degradation products, 2-nitro-2,4-diazabutanal (NDAB; aerobic conditions) or methylenedinitramine (MEDINA; anaerobic conditions) (Jackson et al. 2007). While these products are detectable in vitro, they have not been found in planta and it is likely that they are metabolized by endogenous plant enzymes. We have shown that *Arabidopsis* plants expressing XplA and XplB were able to remove saturating levels of RDX from soil leachate (Figure 1) whereas untransformed plants (WT) had no more impact upon RDX levels than control pots of soil without plants (NPC) (Jackson et al. 2007, Rylott et al. 2011a).

RDX is commonly found with the co-pollutant TNT, which is also highly toxic. Thus, in order for transgenic plants to thrive in soils contaminated with both RDX and TNT they may require resistance to TNT at contaminated sites. Our studies show that the *nfsI* encoded nitroreductase (NR) transforms TNT to the relatively unstable and non-toxic hydroxylamino dinitrotoluene (HADNT), which is then converted to amino dinitrotoluene (ADNT). Both HADNT and ADNTs are conjugated to sugars (Gandia-Herrero et al. 2008) and ¹⁴C-labelling studies reveal subsequent incorporation into plant biomass (Brentner et al. 2010, Sens et al. 1998, Sens et al. (1999). Although the toxicity of the TNT transformed intermediates has been studied (Luo et al. 2004), there is extremely little information on the toxicity or bioavailability of these intermediates once they are conjugated and incorporated into plant biomass.

Transformation of Alamo switchgrass, using a variety of promoters based on the pSAT vector system, has resulted in the development of scores of transgenic lines expressing *xplA/B* and *nfsI*. Initial constructs relied on strong promoters from dicots (e.g., transformed lines designated HR), while recently constructed vectors (HRM and NABNR) have used promoters from monocots, especially grasses. About 20 NABNR and HRM transformed lines have been verified by PCR and RT-PCR to contain and transcribe *xplA*, *xplB*, and *nfsI*. The presence of XplA protein in several of these lines has been confirmed by western blot using an antibody to *xplA*.

Switchgrass transformed using the NABNR vector removed RDX from media at 0.7 µg RDX/g plant tissue/h, twice that of the initial uptake by the wild type grass (Figure 1). Importantly, RDX was recovered in the wild type plant tissues while transformed plants had no detectable RDX (Figure 2), showing that RDX was degraded only in the transformed plants.

We tested the function of RDX degrading genes in the transformed grasses by measuring the rate of RDX removal from hydroponic solutions and the level of RDX in plant tissues at the end of the uptake test. Wild type plants have significant RDX in their tissue, though the total amount that the unmodified plants can take up is limited. Transformed plants have little or no RDX in their tissue, evidence that the RDX is degraded by the transgene product XplA. Several lines of transformed switchgrass have shown both improved RDX removal and less tissue RDX than wild types. Our most recent transformants, NABNR lines using monocot promoters, removed RDX from medium at 0.7 µg RDX/g plant tissue/h, twice that of the initial uptake by the wild type grass (Figure 2). Importantly, Wild type plants have significant levels of RDX in their tissue, whereas transformed plants had little or no RDX in their tissue, evidence that the RDX is degraded by the transgene product XplA. Thus we have confidence that the transformed switchgrasses will be able to remove and degrade RDX from soil under field conditions.

CRREL has propagated the primary transformant lines in the greenhouse using standard horticultural practices during FY14 and FY15. The University of Washington supplemented these methods with additional micropropagated plantlets developed through previously published methods (Bhadra et al. 2001). Nodal segments from the parent grasses were surface sterilized, split longitudinally and placed with the cut surface down on solid medium containing maltose and 6-benzylaminopurine. After 1.5 months in a growth chamber, the young shoots developed from the parental nodal segment and were transferred to medium without growth regulator for rooting. Hygromycin was added to the medium as a selection agent to maintain the transgenes. The University of Washington produced 500 plantlets of each line.

Silencing, or loss of function, of an introduced transgene was a concern in the propagation of the transformed switchgrass. A subset of the clones (about 100 plants) produced by each round of propagation was tested for loss of *xplA* and *xplB* gene activity using RT-PCR to quantify levels of mRNA and protein for *xplA* and *xplB* and western blot and dot-ELISA (Yu et al. 2000) to monitor XplA and XplB protein in the plants using the polyclonal antibodies to the proteins developed previously. We used the housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase, and/or ascorbate peroxidase 3, as internal loading controls to normalize expression levels of XplA and XplB proteins. Additionally a smaller subset of plants (10-20) was tested for RDX uptake and degradation from hydroponic media.

Plot Design and Construction:

The proposed field experiment is a randomized complete block design with three soil treatments (1 ppm, 100 ppm and no RDX), three vegetation treatments (untransformed grass species, transformed grass species, and a no plant control) replicated three times for a total of 27 plots (Figure 3). In the transgenic vegetation treatments three blocks of five rows of each of the three best performing lines will be tested to further characterize RDX removal by the transgenic plant lines (Figure 4).

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Plots were constructed to be 3.0 m x 3.0 m meters with a 0.5 m depth. The plots are comprised of a double-lined containment system consisting of a reinforced polyethylene barrier placed 0.75 m below the existing soil surface. A layer of clean sand 0.25 m thick was placed above the barrier and suction lysimeters installed for monitoring of RDX leachate. A second barrier was placed above the sand layer and filled with clean local soil. Lysimeters were then placed within the plot to monitor the soil water concentration of RDX (Figure 5).

To ensure adequate soil moisture levels, an automated irrigation and storage system was incorporated into the plots (Figure 5). Each plot contains a 325 gallon tank that is connected to a drip irrigation system. When the soil moisture sensor indicates below optimum soil water content, an electronic valve will irrigate the plots. If excess water is noted in the system, a pump located in the drainage area will refill the storage tank. If additional water is needed, water can be added from municipal sources via a water trailer. No municipal sources of water are directly connected to the plot areas. Additional water for the plots is obtained either through natural rain fall or manually added to each plot. All additions of water will be monitored and recorded electronically.

Each vegetation plot will be planted with a maximum of 250 clones, modified or unmodified, spaced on a 20 x 20 cm grid and allowed to establish for approximately one month to obtain a sufficient root system. Plots receiving 1 ppm RDX will be dosed through the irrigation system. Plots receiving 100 ppm (i.e., 100 g/kg soil) will be dosed with fine particulate RDX mixed thoroughly with the top 10 cm soil and irrigated with water, in order to simulate particulate munitions contamination around targets on training ranges.

Plots will also be monitored bi-weekly for vegetation status (plant health, nutritional status, and soil moisture levels). Soils, plants, and leachate will be sampled monthly to determine levels of RDX remaining.

Sample analysis:

Soil samples will be extracted and analyzed by EPA methods 8330A and 8330B (EPA 2007, EPA 2006). Soil cores from surface to about 15 cm, 15-30 cm and 30-45 cm will be taken at three points in each plot. Samples will be dried at room temperature to a constant weight. All procedures will be done in a manner that avoids exposure to direct sunlight. The dried samples will ground to pass through a 30-mesh sieve. Subsamples (2 g) will be extracted with 10 mL of acetonitrile, and placed in a cooled ultrasonic bath for 18 h. Then 5mL of supernatant will be removed and combined with 5 mL of calcium chloride solution (5 g/L) and shaken. Then the supernatant will be filtered through a 0.45 μ m PTFE filter. The first 3 mL will be discarded and the remainder retained in a vial for HPLC analysis.

Aqueous samples of leachate and soil water (5 mL) will be extracted with 5 mL acetonitrile, shaken thoroughly and filtered through a 0.45 μ m PTFE filter. The first 3 mL will be discarded and the remainder retained in a vial for HPLC analysis. For aqueous samples suspected to have RDX levels below the detection limits of the simple extraction procedure, we will use the salting out method (EPA 2007). Sodium chloride (251.3 g) will be added to 770 mL of aqueous sample and stirred until the salt is dissolved. Acetonitrile (164 mL) will be added to the stirred solution and mixed for 15 minutes. After the phases separate, the acetonitrile layer will be transferred using a pasture pipette to a 100 mL flask. The extraction will be repeated with 10 mL of fresh acetonitrile and the extracts combined. Additional salt water will be added to the extracts and the mixture stirred for 15 minutes, followed by phase separation. The acetonitrile supernatant phase will be transferred, the extraction repeated, both extracts combined, and diluted 1:1 with reagent water. If the diluted extract is turbid it will be filtered as above. If we still have trouble reaching the desired detection limit of method, followed by 100 fold concentration as described in Chow et al. 2004.

Levels of RDX in plant extracts will be determined using EPA Method 8330B (EPA 2006). RDX will be extracted from ground, freeze-dried plant tissue (maximum 2.6 g fresh weight) using 2 x 10 mL volumes of methanol. Following solvent evaporation in a rotary vacuum, samples will be resuspended in 4 mL water: methanol (50: 50) and analyzed by HPLC. A minimum of five concentration levels will be used as external calibration standards for each run. Each run will also include selected samples that will be analyzed in quadruplicate as precision controls. Additionally we will spike selected samples in each run with a known concentration of RDX similar to that expected in the sample, in order to determine recovery efficiencies.

All the data collected from the field trial experiments will be collated, analyzed statistically and prepared into graphical formats. Key outputs will include a full mass-balance of RDX partitioning, which will be determined by measuring RDX levels in soil, groundwater, and plant biomass during the course of the experiment. The RDX that is not recovered will be assumed to be metabolized by the plants. Plant performance will be determined by measurement of plant biometric indicators, including, plant height, number of leaves and leaf surface area.

Sample handling and disposal.

Materials collected during the field trial for laboratory analysis will be double bagged in 5 mil plastic bags and stored in shipping containers as described in Section 7. Following laboratory analysis samples will be autoclaved, to devitalize all transgenic material, and then disposed of in accordance with existing laboratory Standard Operating Procedures.

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Entry Protocol:

All personnel entering the test site will need to be a U.S. citizen. Contact with program lead, Mr. Timothy Cary, must be made a minimum of five business days prior to visitation. The time requirement is to allow proper notification to security the office and/or vetting of visitors. Further information is in the appendix. Mr. Cary can be reached via telephone at 603-646-4358, cell phone at 603-667-7824 or email: timothy.j.cary@usace.army.mil. If Mr. Cary is not available, then contact should be made with [] through his contact information previously listed in the permit. The names, office affiliation, dates of access requested, time of arrival and departure will be required for the security office. If utilizing a non-government vehicle additional information will be required to obtain a vehicle pass including but not limited to vehicle registration, proof of insurance, and a valid state driver's license.

Additional Regulatory Agency Involvement:

The regulated article is not intended for use as food or feed so the Food and Drug Administration has no authority over its release.

The regulated material does not produce or contain pesticide active material, so the U.S. Environmental Protection Agency does not have authority over its release. The U.S. EPA is interested in this release as a potential phytoremediation technology to reduce levels of military munitions on training ranges and subsequent entry into the environment (soils, plants, surface water and ground water).

The U.S. Fish and Wildlife Service has been notified of this plant material, but has no regulatory authority for its release.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

The intended destination of the regulated product will be the field trial located at [] Jefferson County, NY. If the field trial is successful further research may be conducted at additional sites in the Northeastern US.

Plant materials, from all transformed lines, will be maintained at the ERDC-CRREL research greenhouse for continued development and research. ERDC-CRREL will continue to propagate and test transformed plants to determine other potential uses and future field trial locations.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations:

Our proposed containment system consists of three parts: 1. each research plot contains a double lined containment structure that is a physical barrier, 2. the cultivar of Switchgrass selected is a physiological barrier, and 3. the manual removal of the panicle while in the boot stage of development is a physical barrier to reproduction.

Each plot is double lined with 60 mil reinforced polypropylene (RPP). The RPP barrier provides a physical level of containment for the transgenic plants by eliminating the possibility of rhizomes rooting outside of the plots. All plants and contaminated soil are physically contained within the containment plots allowing for easy monitoring and control throughout the duration of the experiment. This is a closed system and irrigation water will be recirculated.

We specifically selected Switchgrass cultivar of Alamo for our project because it is a lowland ecotype from the extreme southern adapted range. Alamo's range is typically within USDA hardiness zone 9. The experiment site for this trial is in USDA hardiness zone 4a. As shown by Castler (2012) movement of a switchgrass cultivar more than 2 hardiness zones reduces the ability of this plant to cross with others of its species. Additionally since Alamo is a lowland ecotype it will flower later in the season than the Northern cultivars, has a different ploidy level (4x compared to 8x) of most Northern upland species, and when crossed with an 8x cultivar, contains a post fertilization incompatibility system between the ploidy levels that minimizes interploidy crosses and gene flow (Castler 2012, Vogel 2004).

While it is possible that the greenhouse grown switchgrasses may flower, we propose to eliminate this possibility by manually clipping the panicles while in the boot stage on a bi-weekly basis during the normal growing season (15 April to 31 October). We believe that bi-weekly monitoring will be appropriate since the cultivar is a southern lowland ecotype that requires a longer photoperiod in order to flower. The Northern climate does not produce enough growing degree days for Alamo Switchgrass to produce pollen and or seeds. Growth experiments at our greenhouse, in Northern NH, required the use of additional lighting to produce seeds from the Alamo switchgrass.

The buffer areas around the plots will be monitored bi-weekly for switchgrass introduction/escape during the normal growing season (15 April through 31 October) each year of the study. A 50' buffer around the outside of the plot area has been established and will be monitored bi-weekly for introduction/escape. As shown in Figure 6, the 50' buffer on the Northern edge of the plot

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area will be partially within the mowed embankment, and outside of the fenced area. While not contained within the fenced area, this buffer area will be marked with fiberglass posts and monitored for potential introductions during the bi-weekly monitoring. This area consists primarily of hard fescues and bluegrasses that are mowed several times during the growing season.

Similarly, on the southern edge of the plot area the 50' buffer extends past the fenced area, over a small dirt roadway. The roadway and the vegetated edge out to 50' will be monitored, during the normal growing season during the bi-weekly monitoring.

Within the fenced research area, all buffer areas between the plots will be treated with a nonselective herbicide, as needed, to keep the areas relatively plant free. These areas will be monitored bi-weekly to insure no introduction/escape of switchgrass occurs. All herbicide applications will follow standard agricultural practices.

Following the conclusion of the experiment and decommissioning of the site, all areas within the 50' buffer area and the research area will be monitored monthly for introduction/escape of switchgrass plants during the normal growing season for three years.

Cleaning of equipment:

All planting, sampling and removal of regulated materials will be done by hand. Separate and isolated sampling tools will be designated as either Wild Type or Transgenic for the duration of the study. All cleaning and decontamination activities will be conducted as described in the attached SOPs.

All equipment used on authorized sites, including hand equipment, will be dedicated to use on the authorized sites, posted as restricted to that use only, and secured or locked to prevent use by unauthorized personnel. All equipment will be cleaned and decontaminated prior to leaving the buffer areas, as outlined in the attached SOPs.

No permanent storage areas are available at the research location. Collected samples will be maintained in the transportation containers previously described and a Chain of Custody procedures implemented. All containers will be brought into the buffer area, the fenced area around the containment plots, to be filled, inventoried and locked prior to transport offsite.

Planting Area Restrictions: Following the termination of the experiment and subsequent harvest and devitalization of the regulated article, no switchgrass plants are allowed to be grown in the area for three years.

APHIS approved training: The proposed training program is attached to the appendix. It covers who must be trained, how often, applicable regulations (APHIS and DOD), proposed permit activities, requirements for specific activities (inspection, monitoring, recording), site specific information (entry protocol, security, contact information for all parties involved, sampling procedures to ensure containment, data to be collected, devitalization procedure, reporting and remediation requirements for accidental release, compliance requirements (inspections, records and audits, and report formatting requirements).

Final Disposition Method: ☒ Destruction/Devitalization ☐ Other ☐ Storage in Contained Facility

Final Disposition Description:

At the end of the experiment all transgenic grasses will be trimmed to 6 inches above ground level, the biomass collected and incinerated either onsite or at a certified facility. The remaining stubble will be treated with an application of non-selective herbicide to devitalize the remaining stems and roots.

The contaminated soils removed from the test cells containing transgenic grasses will be incinerated at an approved facility.

14. ATTACHMENTS

<u>Attachments</u>
APHIS Approved Training Program CBI Deleted (4/7/2016 @ 09:20 AM)
Approved SOPs for Field Activities CBI Deleted (4/7/2016 @ 09:14 AM)
Army Pamphlet 385-40 Army Accident Investigations and Reporting (4/5/2016 @ 09:51 AM)
Army Pamphlet 385-69 Safety Standards for Microbiological Laboratories (4/5/2016 @ 09:52 AM)
Army Regulation 385-10 Army Safety Program (4/5/2016 @ 09:49 AM)
Bhadra 2001 Cited Reference (4/5/2016 @ 09:27 AM)
Castler 2012 Cited Reference (4/5/2016 @ 09:27 AM)
Chow 2004 Cited Reference (4/5/2016 @ 09:28 AM)

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DoD Environmental Field Sampling Handbook Revision 1 April 2013 (4/5/2016 @ 09:55 AM)
EPA 2006 Cited Reference (4/5/2016 @ 09:29 AM)
EPA 2007 Cited Reference (4/5/2016 @ 09:30 AM)
EPA 2014 Cited Reference (4/5/2016 @ 09:31 AM)
Jackson 2007 Cited Reference (4/5/2016 @ 09:32 AM)
Lu Cited Reference (4/5/2016 @ 09:33 AM)
References and Figures CBI deleted (4/13/2016 @ 11:48 AM)
Rylott 2006 Cited Reference (4/5/2016 @ 09:33 AM)
Rylott 2011a Cited Reference (4/5/2016 @ 09:34 AM)
Rylott 2011b Cited Reference (4/5/2016 @ 09:35 AM)
SERDP ER-1318 Final Report (4/5/2016 @ 09:43 AM)
SERDP ER-1498 Interim Report (4/5/2016 @ 09:48 AM)
Vogel 2004 Cited Reference (4/5/2016 @ 09:40 AM)
Yu 2000 Cited Reference (4/5/2016 @ 09:41 AM)

15. ADDITIONAL INFORMATION

16. COURTESY JUSTIFICATION

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I, *Timothy Cary*, hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON*Timothy Cary***18. DATE***January 25, 2016*

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

SUPPLEMENTAL PERMIT CONDITIONS For Movement of *Panicum virgatum*

- (1) This authorization is strictly for movement and storage of the regulated article, as described in the permit. This authorization for movement under permit is valid for execution for a period of 1 year. This authorization is NOT valid for the release of this regulated article into the environment.
- (2) BRS should be notified in writing of any proposed changes to the permit application (or approved permit) including for example changes in movement protocols, additional transgenic lines or constructs, new destinations, or amount introduced. Changes usually require amendments to the permit and must be pre-approved by BRS. Requests should be directed to Regulatory Permit Specialist, USDA APHIS BRS, Biotechnology Permit Services, 4700 River Road, Unit 147, Riverdale, Maryland 20737.
- (3) The regulated article is to be shipped in containers as specified in the Title 7 Code of Federal Regulation Section 340.8 (7 CFR 340.8) unless a variance request has been reviewed and approved by APHIS/BRS.

The applicant has requested for a variance to the container requirements for movement of the regulated article as specified in the Title 7 Code of Federal Regulation Section 340.8 (7 CFR 340.8), and the variance request number 16-006 was approved.
- (4) While in storage all regulated articles covered under this permit are to be kept in a locked storage facility with limited access to only authorized personnel. Storage containers must be identified as containing a genetically engineered regulated article. At least one sign stating "Authorized Personnel Only" must be posted in each area where the regulated article is stored.
- (5) A careful control over the inventory and disposition of the regulated article must be maintained at all times. Records of the amount of regulated article used, disposition of the regulated article, chain of custody, and inventory are subject to APHIS auditing.
- (6) Upon completion of research, all regulated articles (except those retained for future studies) should be rendered non-viable by an appropriate method (e.g., heat or steam sterilization, bleach treatment, etc.).
- (7) There is to be no further distribution of this regulated article under this permit without prior approval from State (intrastate movement) and Federal regulatory officials (interstate movement).
- (8) APHIS/BRS and/or APHIS/PPQ personnel may conduct inspections of facilities and/or records at any time.
- (9) All necessary precautions must be taken to prevent escape of these regulated articles. In the event of an escape, notify this office by telephone.
- (10) Reporting an Unauthorized or Accidental Release

1. According to the regulation in 7 CFR § 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

- For immediate verbal notification, contact APHIS BRS Compliance Staff at (301) 851-3935 and ask to speak to a Compliance and Inspection staff member. Leave a verbal report on voicemail if the phone is not answered by a Compliance Officer.
- In addition, in the event of an emergency in which you need to speak immediately to APHIS personnel regarding the situation, you may call:

The APHIS/BRS Regional Biotechnologist assigned in the region where the field test occurs:
For Western Region, contact the Western Region Biotechnologist at (970) 494-7513
or e-mail: BRSWRBT@aphis.usda.gov
For Eastern Region, contact the Eastern Region Biotechnologist at (919) 855-7622 or e-mail: BRSERBT@aphis.usda.gov

Or

The APHIS State Plant Health Director for the state where the unauthorized release occurred. The list of APHIS State Plant Health Directors is available at:
http://www.aphis.usda.gov/services/report_pest_disease/report_pest_disease.shtml,
or
<http://pest.ceris.purdue.edu/stateselect.html>

2. Written notification should be sent by one of the following means:

By e-mail:
BRSCcompliance@aphis.usda.gov

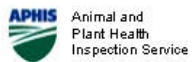
By mail:
Biotechnology Regulatory Services (BRS)
Regulatory Operations Program
USDA/APHIS
4700 River Rd. Unit 91
Riverdale, MD 20737

3. Additional instructions for reporting compliance incidents may be found at http://www.aphis.usda.gov/biotechnology/compliance_incidents.html
- (11) No person shall move a regulated article interstate unless the number of the limited permit appears on the outside of the shipping container.
- (12) ***Important***

Interstate movement, release/movement, and release permits may also be subject to PPQ domestic permit and/or quarantine requirements. Please call PPQ @ (877) 770-5990 for additional assistance in regards to their requirements.

Permit Number: 16-025-103m

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS	
SIGNATURE OF BRS OFFICIAL Subray Hegde	DATE April 22, 2016

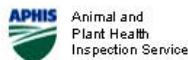


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4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

Permit Number: 16-025-103m

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SIGNATURE OF BRS OFFICIAL Subray Hegde	DATE April 22, 2016



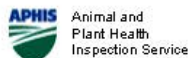
Biotechnology
Regulatory
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4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

SUPPLEMENTAL PERMIT CONDITIONS For Release of *Panicum virgatum*

- (1) Switchgrass plants that are part of the trial (genetically engineered or not) will not be allowed to flower. Plants will be monitored for the presence of panicles bi-weekly during the entire growing season, and all panicles will be removed at the boot stage.
- Plant materials removed at panicle removal will be destroyed by autoclaving or incineration, or composting in a closed container on the field trial site.
- Switchgrass plants or sexually compatible plants present in the field release area are to be treated as regulated material and will not be allowed to flower. The area outside the field plots are to be maintained free of switchgrass and any sexually compatible relatives.
- (2) BRS should be notified in writing of any proposed changes to the permit application (or approved permit) including for example confinement protocols, transgenic lines or constructs, release sites, acreage, etc. Changes usually require amendments to the permit and must be pre-approved by BRS. Requests should be directed to Regulatory Permit Specialist, USDA/APHIS/BRS, Biotechnology Permit Services, 4700 River Road, Unit 91, Riverdale, Maryland 20737.
- (3) Any regulated article introduced not in compliance with the requirements of 7 CFR Part 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).
- (4) This Permit does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including for the use of: (A) any non-genetically engineered plant pest or pathogens as challenge inoculum; (B) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (C) experimental use of unregistered chemicals; and (D) food, feed, pharmacological, biologic, or industrial use of regulated articles or their products and co-mingled plant material. In the latter case, depending on the use, reviews by APHIS, the U.S. Food and Drug Administration, or the U.S. Environmental Protection Agency may be necessary.
- (5) The procedures, processes, and safeguards used to prevent escape, dissemination, and persistence of the regulated article as described in the permit application, in APHIS-approved Standard Operating Procedures (SOPs) and, in the supplemental permit conditions must be strictly followed. The permittee must maintain records sufficient to verify compliance with these procedures, including information regarding who performed the activity. Persons performing such activities shall have received training as described in a training program submitted to and approved by APHIS. These records are subject to examination by APHIS. APHIS/BRS must be notified of any proposed changes to the protocol referenced in the permit application.
- (6) Inspections:
- APHIS/BRS and/or an APHIS/PPQ personnel may conduct inspections of the test site, facilities, and/or records at any time.
- APHIS may invite the FDA or State Regulatory Officials to participate in these inspections.
- Inspections will likely correspond to the beginning of the field test, mid-season or during flowering, at and/or following harvest, and during the post-harvest monitoring period.
- Inspections will include examination of records that verify compliance with regulations and SOPs.
- (7) Reporting an Unauthorized or Accidental Release
1. According to the regulation in 7 CFR § 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.
- For immediate verbal notification, contact APHIS/BRS Compliance Staff at (301) 851-3935 and ask to speak to a Compliance and Inspection staff member. Leave a verbal report on voicemail if the phone is not answered by a Compliance Officer.
 - In addition, in the event of an emergency in which you need to speak immediately to APHIS personnel regarding the situation, you may call:
- The APHIS/BRS Regional Biotechnologist assigned in the region where the field test occurs:
For Western Region, contact the Western Region Biotechnologist at (970) 494-7513
or e-mail: BRSWRBT@aphis.usda.gov
For Eastern Region, contact the Eastern Region Biotechnologist at (919) 855-7622 or e-mail: BRSERBT@aphis.usda.gov
- Or
- The APHIS State Plant Health Director for the state where the unauthorized release occurred. The list of APHIS State Plant Health Directors is available at:
http://www.aphis.usda.gov/services/report_pest_disease/report_pest_disease.shtml
or
<http://pest.oeris.purdue.edu/stateselect.html>
2. Written notification should be sent by one of the following means:
- By e-mail:
BRSCCompliance@aphis.usda.gov
- By mail:
Biotechnology Regulatory Services (BRS)
Regulatory Operations Program
USDA/APHIS
4700 River Rd, Unit 91
Riverdale, MD 20737
3. Additional instructions for reporting compliance incidents may be found at http://www.aphis.usda.gov/biotechnology/compliance_incident.shtml

Permit Number: 16-025-103m

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SIGNATURE OF BRS OFFICIAL	DATE
Subray Hegde	April 22, 2016



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4700 River Road, Unit 147
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20737-1236

(8) Reporting Unintended Effects:

According to the regulation in 7 CFR § 340.4(f)(10)(ii), APHIS shall be notified in writing as soon as possible but within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the permit application or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).

Written notification should be sent by one of the following means:

By e-mail:
BRSCompliance@aphis.usda.gov

By mail:
Biotechnology Regulatory Services (BRS)
Regulatory Operations Program
USDA/APHIS
4700 River Rd. Unit 91
Riverdale, MD 20737

(9) Perimeter Fallow Zone:

To ensure that transgenic plants are not inadvertently commingled with plants to be used for food or feed, a perimeter fallow zone of at least 50 ft. (unless a variance is granted by APHIS) must be maintained around the transgenic test site in which no crops are grown to be harvested or used for food or feed.

The permitted border rows of non-transgenic plants that are the same as, or sexually compatible with, the regulated article are considered part of the field test. The perimeter fallow zone shall start outside the border rows. No border rows are allowed for this trial.

The perimeter fallow zone shall be managed in a way that allows for detection and destruction of volunteer plants that are the same as, or sexually compatible with, the transgenic plants.

The perimeter fallow zone isolation distance area will be mowed biweekly. The area will be monitored biweekly and all plants sexually compatible with switchgrass will be removed and/or destroyed upon discovery by application of non-selective herbicide, incineration, or autoclaving.

(10) Dedicated Planting and Harvesting:

To ensure that the regulated article is not inadvertently removed from the site, planting and harvesting equipment must be dedicated for use in the permitted test site(s) from the time of planting through the end of harvesting.

After harvest, you will not be required to obtain APHIS authorization to use this equipment on APHIS-permitted sites (same sites or different sites) planted with same transgenic crop, with the target protein(s) authorized under this permit, in subsequent growing seasons under an extension of this permit or a different permit.

Authorization is required from APHIS before this planting and harvesting equipment can be used on sites planted to crops not included under this permit. The permittee must notify APHIS/BRS and the State Regulatory Official at least 21 calendar days in advance of cleaning this equipment for this purpose so that APHIS may schedule an inspection to ensure that the equipment has been cleaned appropriately.

(11) Cleaning of Equipment:

To minimize the risk of seed movement and commingling, equipment used for planting and harvesting, as well as other field equipment (e.g. tractors and tillage attachments, such as disks, plows, harrows, and subsoilers) used at any time from the time of planting through the post harvest monitoring period must be cleaned in accordance with procedures submitted to and approved by APHIS before they are moved off of the test site.

Equipment used to transport seeds or harvested material must be cleaned prior to loading and after transportation to the authorized site in accordance with procedures submitted to and approved by APHIS.

Seed cleaning and drying must be performed in accordance with the procedures submitted to and approved by APHIS to confine the plant material and minimize the risk of seed loss, spillage, or commingling.

(12) Use of Dedicated Storage Facilities:

Dedicated facilities (locked or secured buildings, bins, or areas, posted as restricted to authorized personnel only) must be used for storage of equipment and regulated articles for the duration of the field test.

Before returning these facilities to general use, they must be cleaned in accordance with procedures submitted to and approved by APHIS. The permittee must notify APHIS/BRS and the State Regulatory Official at least 21 calendar days in advance to allow for APHIS to schedule an inspection to ensure that the facilities have been cleaned appropriately. APHIS authorization should be received before facilities are returned to general use.

(13) Post Harvest Monitoring:

Following termination of the field trial the applicant will monitor the regulated trial area (the field trial site and the 50 ft isolation distance) for the presence of volunteer switchgrass plants monthly for three years, during the growing season. All volunteers will be destroyed upon discovery by application of non-selective herbicide. Viable plant material should not remain at the test site following termination.

(14) Post Harvest Land Use Restrictions:

Production of food and feed crops at the field test site and the perimeter fallow zone is restricted during the growing season that follows harvest or termination of the field test.

After the three year period of the field trial the area will be decommissioned and not used for planting switchgrass.

Permission must be obtained from APHIS/BRS prior to planting any food or feed crop at the field test site and perimeter fallow zone during the post harvest monitoring period. Requests for such permission are not encouraged and will not be granted in cases where there is a reasonable potential for plant material derived from, or

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Subray Hegde	April 22, 2016



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20737-1236

originating from, the regulated articles to become mixed with the proposed food or feed crop during harvesting.

(15) Reports and Notices:

Submit all reports and notices via ePermits using the link under "My Reports and Notices."

A link to instructions for submitting via ePermits is located here:

https://epermits.aphis.usda.gov/epermits/xml_schema/BRS_Reports_and_Notices_User_Guide.pdf

Other options are to submit reports and notices via email or paper, however, we strongly encourage submission via ePermits. If submitting using any other method, both CBI and CBI-deleted or non-CBI copies should be submitted via:

BRS E-mail:

BRSCompliance@aphis.usda.gov

BRS Mail:

Animal and Plant Health Inspection Service (APHIS)

Biotechnology Regulatory Services (BRS)

Regulatory Operations Program

4700 River Rd. Unit 91

Riverdale, MD 20737

a. Pre-Planting Notice

At least 7 calendar days before planting, submit a Pre-Planting notice that includes the following information for each field test site:

- Provide APHIS with the contact information for each field test site.
- Indicate if planting and harvesting equipment will be moved between authorized field test sites.
- A map that clearly identifies the site location to facilitate any inspections by USDA personnel.
- The planned number of acres for each gene construct.
- The planned planting date.

NOTE: THESE NOTICES SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

b. Planting or Environmental Release Report

Planting reports must be submitted to BRS by the 15th of the month following the month in which the environmental release was started and must include the following data:

- Permit number
- Regulated article
- State
- County
- Location Name (Unique ID)
- GPS coordinates of the planting
- Planting Unique ID
- Planting Start Date
- Total acreage of the regulated article planted or otherwise released
- List of all constructs planted

(This list is optional in the planting report but must be included in the Field Test Report)

NOTE: THESE REPORTS SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

c. Pre-Flowering Notice

At least 7 calendar days prior to the anticipated flowering of plants at the field site, submit a Notice indicating the anticipated flowering date and the contact information for each field test site. For multiple sites that may have different flowering times, submit the notice prior to the first anticipated flowering.

NOTE: THESE NOTICES SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

d. Pre-Harvest/Termination Notice

At least 21 calendar days prior to the anticipated harvest or termination, submit a Notice indicating the planned date of harvest or termination and the contact information for each field test site. For multiple harvests, submit the notice prior to the initial harvest.

NOTE: THESE NOTICES SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

e. Field Test Report

Within six months after the expiration date of the permit, the permittee is required to submit a Field Test Report. Field Test Reports provide the final status and observations at each location and must include:

- Permit number
- State
- County
- Location Name(s)
- Location Unique ID(s)
- Any plantings that occurred at each location
- GPS coordinates for each planting
- Size of the plantings (in acres) at each location

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- Phenotypic designations (all constructs that were planted)
- Indicate if any of the planted material was destroyed before harvest
- If so provide the Pre-Harvest destruction completion date and describe how the pre-harvested material was destroyed
- Indicate if any of the planted material was harvested
- If so provide the harvest completion date
- Describe how the harvested material was terminated
- If the material was terminated in the field and not removed from the field, provide the date the field test was completely terminated and describe the method of termination
- If material was removed from the field and terminated off site describe how it was disposed and provide the date of off site destruction.
- If material was removed from the field and placed in storage, provide the amount of material that was stored and provide a description of the storage location
- Describe any other disposition methods that may be applicable
- Describe any deleterious effects on plants, non target organisms, or the environment
- Describe methods of observations and resulting data and analyses
- Indicate if you have submitted any of the following:
 1. A report on the accidental or unauthorized release of the regulated article;
 2. A report that characteristics of the permitted species are substantially different from those listed in the application; or
 3. A report of any unusual occurrence

We encourage the inclusion of other types of data if the applicant anticipates submission of a petition for determination of non-regulated status for their regulated article. APHIS considers these data reports as critical to our assessment of plant pest risk and development of regulatory policies based on the best scientific evidence. Failure by an applicant to provide data reports in a timely manner for a field trial may result in the withholding of permission by APHIS for future field trials.

NOTE: THESE REPORTS SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

f. Monitoring Report

The report must include:

- Permit number
- State
- County
- Location Name(s)
- Location Unique ID(s)
- Dates when the field site and perimeter fallow zone were inspected for volunteers
- Number of volunteers observed
- Any actions taken to remove or destroy volunteers

The final monitoring report is due no later than three months from the end of the volunteer monitoring period.

NOTE: THESE REPORTS SHOULD BE SUBMITTED ON-LINE THROUGH ePermits

g. Storage Report

In cases where a regulated article is stored on a production site, a report must be filed with APHIS each year the regulated article remains in storage at the production site. The report, which must include the permit number, storage location, crop, event, and quantity of each regulated article, must be filed prior to the anniversary of the expiration date of the permit under which the regulated article was produced.

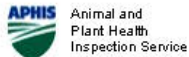
NOTE: THIS REPORT SHOULD BE SUBMITTED BY EMAIL TO COMPLIANCE (BRSCCompliance@aphis.usda.gov). There is no option for submitting this report via ePermits.

(16) ***Important***

Interstate movement, release/movement, and release permits may also be subject to PPQ domestic permit and/or quarantine requirements. Please call PPQ @ (877) 770-5990 for additional assistance in regards to their requirements

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Standard Permit Conditions for the Introduction of a Regulated Article

(7 CFR 340.4 (f))

Permit Conditions: A person who is issued a permit and his/her employees or agents shall comply with the following conditions, and any supplemental conditions which shall be listed on the permit, as deemed by the Deputy Administrator to be necessary to prevent the dissemination and establishment of plant pests:

- (1) The regulated article shall be maintained and disposed of (when necessary) in a manner so as to prevent the dissemination and establishment of plant pests.
- (2) All packaging material, shipping containers, and any other material accompanying the regulated article shall be treated or disposed of in such a manner as to prevent the dissemination and establishment of plant pests.
- (3) The regulated article shall be kept separate from other organisms, except as specifically allowed in the permit.
- (4) The regulated article shall be maintained only in areas and premises specified in the permit.
- (5) An inspector shall be allowed access, during regular business hours, to the place where the regulated article is located and to any records relating to the introduction of a regulated article.
- (6) The regulated article shall, when possible, be kept identified with a label showing the name of the regulated article, and the date of importation.
- (7) The regulated article shall be subject to the application of measures determined by the Administrator to be necessary to prevent the accidental or unauthorized release of the regulated article.
- (8) The regulated article shall be subject to the application of remedial measures (including disposal) determined by the administrator to be necessary to prevent the spread of plant pests.
- (9) A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment.
- (10) APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:
 - (i) Orally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;
 - (ii) In writing as soon as possible but not later than within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the application for a permit or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).
- (11) A permittee or his/her agent and any person who seeks to import a regulated article into the United States shall:
 - (i) Import or offer the regulated article for entry only through any USDA plant inspection station listed in 7 CFR 319.37-14;
 - (ii) Notify APHIS promptly upon arrival of any regulated article at a port of entry, of its arrival by such means as a manifest, customs entry document, commercial invoice, waybill, a broker's document, or a notice form provided for such purpose; and
 - (iii) Mark and identify the regulated article in accordance with 7 CFR 340.7.

Rev. January 1, 2010

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Any regulated article introduced not in compliance with the requirements of 7 CFR 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. Any regulated article introduced not in compliance with the requirements of 7 Code of Federal Regulation Part 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).

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APPENDIX C CRREL REPORT ON THE MEETING OF REGULATORY AND DOD STAKEHOLDERS

Transgenic Grass Review Meeting Report

11 August 2017

Timothy J. Cary, Neil Bruce, Stuart Strand, and Elizabeth Rylott

ABSTRACT

A field meeting was held on 1 August 2017 at Fort Drum, New York to demonstrate the effectiveness of transgenic grasses to degrade the Royal Demolition explosive (RDX) which is being funded under the Strategic Environmental Security Technology Certification Program (ESTCP) Project ER 201436 entitled Phytoremediation of Explosives from Contaminated Soil by Transgenic Grass. Participants included stakeholders, funding agencies, and researchers. Descriptions were made of the various researchers' involvement in the demonstration and an additional presentation was made by a representative from APHIS. Data was presented to show that the plants were degrading the RDX. Early results show that the mean of the three transgenic plant lines (N1, N2, and N5) have a 47-fold less RDX in their tissue and are, therefore, degrading the RDX that is taken up by these plants. A field tour was held in the afternoon to describe the demonstration plots. Participants asked questions and appeared to verbally approve the demonstration and felt that continued monitoring of the demonstration would be valuable and beneficial as a cost-effective method to remediate RDX in soils.

INTRODUCTION

This document is a report on the 1 August 2017 meeting of stakeholders, funding agencies, and researchers to demonstrate the effectiveness of transgenic grasses to degrade the Royal Demolition explosive (RDX) which is being funded under the Strategic Environmental Security Technology Certification Program (ESTCP) Project ER 201436 entitled Phytoremediation of Explosives from Contaminated Soil by Transgenic Grass.

The demonstration was developed to help remediate decades of military activity on live-fire training ranges that have resulted in the contamination of land and groundwater by recalcitrant high explosives, in particular, 2,4,6-trinitrotoluene (TNT) and RDX. TNT and its transformation products are highly toxic, but these tend to bind strongly to clay and organic matter in soil and are largely contained at the site of contamination; however, RDX is a major concern, because of its high mobility through soils and subsequent contamination of groundwater. There are approximately 10 million hectares of contamination on US military lands (USGAO, 2004). RDX contamination on training ranges is now proving to be a significant threat to drinking water sources. Currently, there are no cost-effective processes to contain RDX or remediate large areas of contaminated vegetated land on training ranges. The primary objective of this project is to demonstrate and evaluate the ability of XplA and XplB expressing grasses to contain and degrade RDX from explosives-contaminated soil *in situ*. Through field scale testing at a military facility in Fort Drum, NY. A secondary objective is to determine the lower bounds or reactivity

of the transgenic plants. We also want to show that the use of these plants is environmentally safe to flora through the permits we received to use them and to fauna through our herbivory studies. This meeting was held at the request of the ESTCP Program.

In our previous studies, we have genetically modified military relevant plants by expressing two bacterial genes enabling the plants to degrade RDX in soils. Under SERDP project ER-1318, Co-PIs at University of York demonstrated that expression of the rhodococcal genes, *xplA* and *xplB*, in the model plant system *Arabidopsis* (*Arabidopsis thaliana*) confers the ability to tolerate and degrade high concentrations of RDX (Jackson et al, 2007; Rylott et al 2006). They also demonstrated that expression of the bacterial nitroreductase *nfsI* gene in tobacco (*Nicotiana tabacum*) and *Arabidopsis* confers resistance to levels of TNT toxic to unmodified plants (Rylott et al, 2011). Under SERDP project ER-1498, Co-PIs at University of Washington developed switchgrass (*Panicum virgatum*) and bentgrass (*Agrostis stolonifera*) lines that expressed *xplA*, *xplB* and *nfsI*, respectively that were found to degrade RDX into inert components in the laboratory.

The EPA's standard for RDX in drinking water is 2 ppb. In our previous greenhouse studies, soil-grown *Arabidopsis* (*Arabidopsis thaliana*) plants were dosed with aqueous solutions of 40 mg/l RDX and levels of RDX in soil leachate measured using HPLC. After seven days, there was no RDX detectable in the soil leachate from the modified plants (Rylott et al, 2011). Under these experimental conditions, the lower limit of detection by the HPLC was 0.021 mg/l (21 ppb). Conducting studies at various soil concentrations will provide additional information on the capability of the plants to degrade RDX at lower levels. If successful, this information will be beneficial in predicting plant biomass requirements to meet drinking water standards, and will increase the use of the plants in additional areas containing less than 1 mg/kg (1 ppm) of RDX, such as at firing points, wetlands, manufacturing, storage, and other sites.

A release and movement permit application (Permit Number 16-025-103RM) for the use of the plants and a second movement permit (17-150-109M) was approved by the United States Department of Agriculture - Animal and Plant Health Inspection Service (APHIS). The release and movement permit is located in Appendix A. Two of the major concerns in using transgenic plants is cross pollination with similar plants and the effects on fauna.

Cross Pollination

We specifically selected Alamo switchgrass for our project because it is a lowland ecotype from the extreme southern adapted range and not adapted to this region. This means that it will flower later in the season than the Northern cultivars (upland types), has a ploidy level of 4x compared to 8x of most Northern species, and when crossed with an 8x cultivar, contains a post fertilization incompatibility system between the ploidy levels that minimizes interploidy crosses and gene flow (Castler 2012, Vogel 2004).

Herbivory Risk Assessment

We have conducted herbivory studies using the generalist herbivore locust (*Schistocerca gregaria*) to determine the toxicity of RDX using both switchgrass and Arabidopsis plants expressing XplA-XplB. Our results indicate that locusts show no feeding preferences between unmodified (wild type) and transgenic lines grown in the presence or absence of RDX. However, when grown on RDX-contaminated soil, the XplA-XplB expressing plant lines have between 40-100-fold less RDX in the leaf tissues than unmodified plants. Longer-term ingestion of RDX-rich tissues may have deleterious effects.

PARTICIPANTS

Performers

Neil Bruce
University of York

Liz Rylott
University of York

Timothy Cary
ERDC-CRREL

Stuart Strand
University of Washington

People involved with the demonstration

Cora Farnsworth
ERDC-CRREL

Michael Morgan
ERDC-CRREL

Heather Kase
ORISE

Nadia Podpora
ORISE

Mr. Ian Warden
DPTMS Fort Drum

Mr. Jimmy Horne
ERDC-CRREL

Nancy Parker
ERDC-CRREL

Antonio Palazzo
ORISE

Mr. Tom Lent
DPTMS Fort Drum

Invitees	
Dr. Afrachanna Butler ERDC-EL	Dr. Catharine Cook USDA-APHIS-BRS
Chuck Coyle USACE	Mr. Malcolm Garg OACSIM
Rajat Ghosh ACRONIC	Ms. Heidi Howard ERDC-CERL
Logan Hunt Wright State University	Stacey Jarvis ERDC-CRREL
Dr. Rick Lance ERDC-EL	Dr. Jerry Miller ERDC-EL
Ms. Robin Paul AFCEC	Mr. George Robitaille OACSIM
Evan Schultz Villanova University	Christopher Sutton Montana State University
Macrina Xavier Noblis	

AGENDA:

The meeting was lead by CRREL, and presentations were made by the three primary institutions responsible for different phases of this project that were involved in the development and demonstration of this technology. They include characterization of the bacterial genes responsible for RDX degradation and TNT detoxification, insertion of the genes into the plants, and designing and implementing the field demonstration.

Welcome and Introduction

Timothy Cary, ERDC-CRREL

History of project, Background on genes

Neil Bruce, University of York

Looking forward to deployment on training ranges

Stuart Strand, University of Washington

Field Demonstration overview

Timothy Cary ERDC-CRREL

Permits and Regulatory

Catharine Cook, APHIS

Site tour of phytoremediation plots

FIELD PROJECT DESCRIPTION

Plant selection for field study

Under SERDP project ER-1498, the author's transformed western wheatgrass and Alamo lowland switchgrass as described in the Background Data Section of this report. For the field study, we selected lowland switchgrass, which is a native warm season (NWS) perennial grass that is often grown as a forage crop, in natural wildlife habitat, or, increasingly, as a bioenergy crop. Although upland switchgrass habitats Fort Drum, we know of no stands of the lowland type. The two types are not generally sexually compatible so the chances for cross pollination is slim. Switchgrass can grow more than 10 feet tall, and well-managed stands may last for decades. Once switchgrass is established, its bunch-type growth habit makes it very competitive with weeds; however, it is not considered invasive. Another beneficial aspect in its use here, is that it produces a large root system that is capable for foraging soil contaminants.

Site construction and design

The field experiment is a randomized complete block design with three soil treatments (1 ppm, 100 ppm and no RDX), three vegetation treatments (untransformed grass species, transformed grass species, and a no plant control) replicated three times for a total of 27 plots (Figure 1). In each of the transgenic vegetation treatments there were three blocks of five rows of three different transgenic plant lines. The lines were N1, N2, and N5.

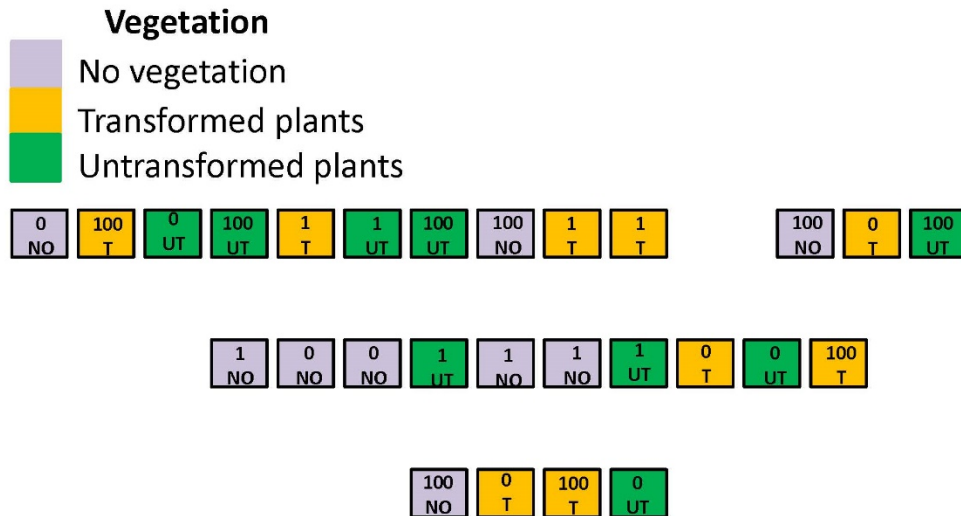


Figure 1. Field experiment design. The RDX treatments are: 0, 1, and 100 ppm. The plant treatments are: No = no plants, UT = non-transgenic plant, and T = transgenic plant.

Plots were constructed to be 3.0 m x 3.0 meters with a 0.5 m depth. The plots are comprised of a double-lined containment system consisting of a 60 mil reinforced polyethylene barrier placed 0.75 m below the existing soil surface. The reinforced polyethylene barrier provides a physical level of containment for the transgenic plants by eliminating the possibility of rhizomes rooting outside of the plots. A layer of clean silica sand, 0.25 m thick, placed above the barrier and suction lysimeters installed in the sand layer for monitoring of RDX leachate. A second barrier was placed above the sand layer and filled with clean local soil. Lysimeters were then placed within the plot to monitor the soil water concentration of RDX (Figure 2).

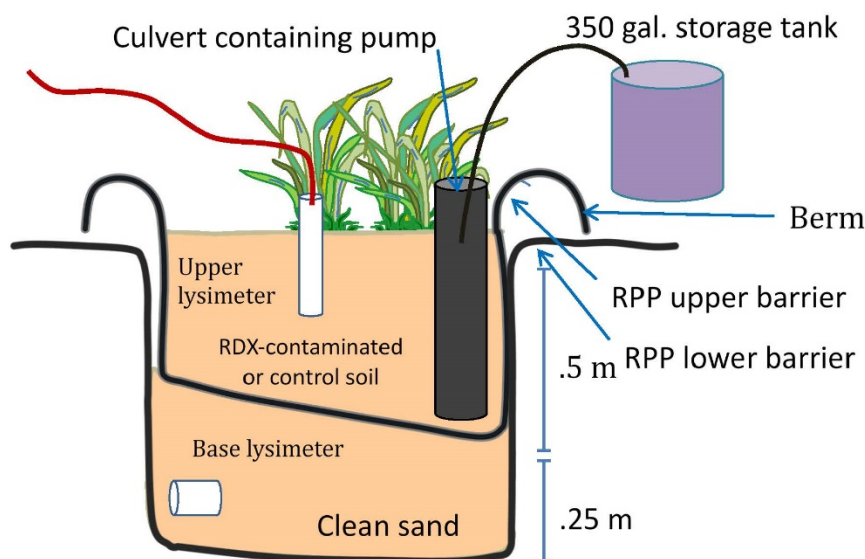


Figure 2. Double-lined containment system with lysimeters, soil moisture and temperature sensors and sump/irrigation system.

To better control soil moisture levels, an automated irrigation and storage system was incorporated into the plots (Figure 2). Each plot contains a 325 gallon tank that is connected to a drip irrigation system. When the soil moisture sensor indicates below optimum soil water content, an electronic valve will irrigate the plots. If excess water is noted in the system, a pump located in the drainage area will refill the storage tank. If additional water is needed, water can be added from municipal sources.

Each vegetation plot was planted with approximately 250 clones, modified or unmodified, spaced on a 20 x 20 cm grid and allowed to establish for approximately one month to obtain a sufficient root system. Plots receiving 1 ppm RDX were dosed through the irrigation system. Plots receiving 100 ppm (i.e., 100 g/kg soil) were dosed with fine particulate RDX mixed thoroughly with the top 10 cm soil and irrigated with water, in order to simulate particulate munitions contamination around targets on training ranges.

We have been monitoring plant development weekly and removing developing flower heads from the plants to control pollen production by preventing flowering. Plots have also been monitored weekly for vegetation status (plant health, nutritional status, and soil moisture levels). Soils, plants, and leachate are being sampled monthly to determine levels of RDX remaining (EPA 2006, EPA 2007).

All the data collected from the field trial experiments will be collated, analyzed statistically and prepared into graphical formats. Key outputs will include a full mass-balance of RDX partitioning, which will be determined by measuring RDX levels in soil, groundwater, and different plant parts during the course of the experiment. The RDX that is not recovered will be assumed to be metabolized by the plants. Plant performance will be determined by measurement of plant biometric indicators, including, plant height, number of leaves and leaf surface area.

A full site dismantling plan, including soil disposal route, will be developed. It is expected that dismantling will include the collection and incineration or composting of all plant material produced during the course of the field trial and remaining at the completion of the trial. Material collected during the field trial for laboratory testing will be, following analysis, disposed of by autoclaving and/or incineration at a certified facility, in accordance with existing laboratory Standard Operating Procedures.

DATA

Preliminary data from early sampling shows consistent results among the two different plants and three RDX treatment levels. RDX uptake by the wild, non-transgenic plants increased with increasing application rates of RDX (Figure 3). In contrast, the mean of the three transgenic plant lines (N1,N2, and N5) have a 47-fold less RDX in their tissue and are, therefore, degrading the RDX that is taken up by these plants.

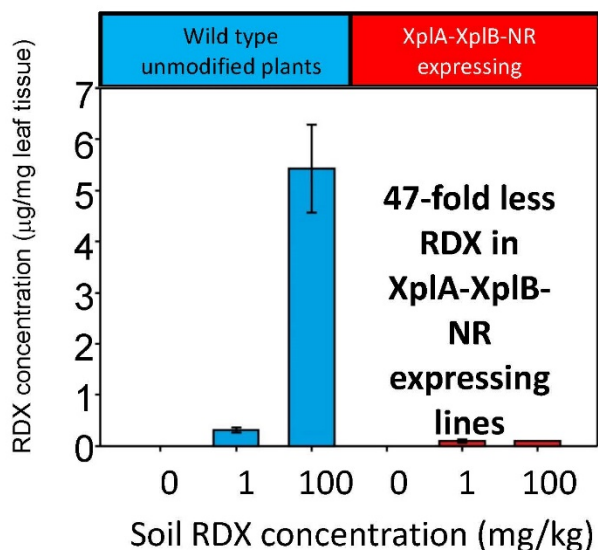


Figure 3. RDX levels in leaf tissues from plants three weeks after dosing the demonstration plots with RDX.

TRANSITIONING RESULTS TO OTHER CONTAMINANTS AND CLIMATES

The authors have transformed three grass species: Alamo upland switchgrass (*Panicum virgatum*), bentgrass (*Agrostis stolonifera*), and western wheatgrass (*Pascopyrum smithii*) lines that have expressed the gene products of *xplA*, *xplB* and *nfsI*: XplA, XplB and NfsI, respectively. The habitat of these three species covers most of the continental United States, however, now that this technology has been accomplished, other species may now be transformed to cover multiple climate, soil, and moisture conditions.

ADVANTAGES AND LIMITATIONS OF THE TECHNOLOGY

Potential Effectiveness of Plants in Field

The basis of the demonstration is to utilize transformed grasses to degrade RDX. The procedures to install this technology are well known. The application of this technology may also be used in remote sites.

Using the RDX removal rate by transgenic grasses in vitro, we estimated the RDX removal by transformed switchgrass growing in training range soils, as follows. Switchgrass can grow to a biomass density of 10.8 tonnes/hectare (Casler 2012), which works out to about 3 kg plant wet weight/ m². Thus the potential removal rate of RDX per area of planting is about 2 mg RDX/m²/h. If we assume an effective root depth of 1 m and an infiltration rate during a heavy rainfall event about 0.05 m/h, then the RDX contaminated water will be in contact with the root zone for approximately 20 hours. To calculate the rate of uptake by a switchgrass planting we multiplied 0.8 µg RDX/g plant wet weight/h by the 20 h root zone contact time. Thus approximately 50 mg RDX could be removed per m² of transformed switchgrass. Typical levels of RDX contamination around a target point are 0.05 mg RDX/kg soil [34]. If we assume that the RDX is distributed over a depth of 0.2 m and a soil density of 1200 kg/m³, then the areal concentration of RDX near the target would be approximately 12 mg RDX/m². This shows that by planting switchgrass there is a potential threefold increase in RDX removal rate than from unvegetated target areas.

With these new grasses, land managers will be able to design more resilient training ranges while decreasing cleanup costs associated with energetic compounds. Training ranges will be less prone to erosion, have better control over entry of RDX into the environment, and be more available to training requirements.

Limitations

The only limitations of this technology will be the availability of the transgenic plant materials (seeds or transplants), and site specific permitting required through APHIS. With the successful completion of the demonstration we will have the data required to reduce future permitting

requirements while increasing the demand for the transgenic materials. As demand increases, the cost of producing seeds and/or transplants should be reduced.

The primary alternative technologies are both drastic and expensive and involve closing lands and physically removing the materials.

ACKNOWLEDGEMENTS

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APPENDIX D WHITE PAPER: DEREGULATING XPLA/B SWITCHGRASS LINES FOR USE ON RDX CONTAMINATED SITES

Background

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) is responsible for the introduction of genetically engineered (GE) organisms into the environment. These organisms are regulated under various permits.

Permits are issued by APHIS for GE organisms being introduced that have the potential to pose a plant pest risk, such as insects, plants or microbes. Under ESTCP project ER-201436 switchgrass lines have been engineered to express the bacterial genes *xplA/B* enabling the explosive pollutant RDX to be broken down into inert naturally occurring plant metabolites. The XplA/B expressing switchgrass lines are currently under a standard permit for interstate movement and have previously been permitted for interstate movement and release. An alternative to permits are notifications which are, according to APHIS, more ‘administratively streamlined.’ⁱ The plant must meet eligibility criteria, which has been previously specified and the plant’s introduction has performance standards which are pre-defined.

Those who believe that a GE organism does not pose a plant pest risk and should no longer be regulated by APHIS under the plant pest provisions of the Plant Protection Act or the regulations at 7 CFR §340 are able to petition the Agency. A petition requires the submission of information under §340.6 (c) (4) related plant pest risk. The Agency may then decide if the regulated GE plant is more or less likely to pose a greater plant pest risk than the unmodified plant. Following a successful petition, the GE organism would no longer be subject to the plant pest provisions in the Plant Protection Act or the requirements of 7 CFR §340, as APHIS has determined that it is unlikely to pose a plant pest risk.ⁱⁱ

Current Regulations

Under the current regulations, a GE organism is regulated if the donor organism, recipient organism, vector agent or vector is a plant pest or if APHIS has reason to believe that the GE organism itself is a plant pest. Plant pests are currently defined at any living stage (active or dormant) of ‘insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof; viruses; or any organisms similar or allied with the foregoing’ which could directly and indirectly injure, or cause disease or damage to any plants or parts of plants.ⁱⁱⁱ A permit may allow for the introduction of regulated organisms if developers follow the conditions of the permit specified by the administrator to be ‘necessary’ to prevent the dissemination and establishment of the regulated GE organism. Under the petition procedure (outlined in §340.6) any person may submit a petition. Paragraphs b and c describe the form of the petition for the determination of nonregulated status and the information which must be provided within. As of December 2018, APHIS had granted 130 determinations of nonregulated status of the 162 petitions submitted, while 32 had been withdrawn. All of the aforementioned determinations have been for GE plants.^{iv}

The current APHIS regulations state that if a developer has collected enough evidence to demonstrate that a GE organism poses no more of a plant pest risk than its unmodified equivalent, they are permitted to petition APHIS to give the GE organism non-regulated status. Providing approval of the petition, the organism may be introduced into the environment within the US without further regulatory oversight from APHIS.^v

Within the petition-making process, any person may submit to APHIS a petition to see a ‘determination’ on an organism that should not be regulated under this section of the Plant Protection Act. A full statement must be presented explaining, on factual grounds, why the organism should be deregulated. The petition should include scientific literature, copies of unpublished studies (when available), and data recorded from tests upon which the Agency can base a decision on.^{vi}

Determinations of non-regulated status can also be extended, as the administrator may find that the regulated organism does not pose a potential for plant pest risk based on similarity of that organism to an antecedent organism, and therefore should not be regulated. A request can be made to APHIS to extend a determination of non-regulated status to other plants and organisms. The request should include information which establishes the similarity between the GE organism in question and the antecedent organism.^{vii}

Proposed Changes to Regulations

In June 2019, APHIS announced its plans to change the regulations surrounding GE organisms. These new enhancements would be, it claimed, more streamlined with an efficient internal process, as steps now deemed unnecessary would be eliminated. Timeframes for the completion of individual steps would be introduced. APHIS also intends to introduce ‘innovative tools’ which would aid managers in effectively tracking the progress of petitions in addition to assigning staff. The changes would also ‘enhance’ the opportunities for petitions to be made available for public input.^{viii}

The reason for these changes comes as APHIS argues that it has 3 decades of experience in evaluating GE organisms for plant pest risk. Evaluations to date have demonstrated that genetically engineering a plant with a plant pest vector, vector agent, or donor, ‘does not in and of itself result in a GE plant that presents a plant pest risk.’^{ix} The Agency intends to change its regulations in order to prepare for advances in the genetic engineering of plants. Once products are reviewed by APHIS and found unlikely to pose a plant pest risk, similar products would also become exempt from review.^x

Under the current system, decisions regarding regulatory oversight by APHIS of GE plants are made and assessed separately. This is done in spite of the fact that the new genetic material inserted in the plant may be ‘identical’ or ‘very similar’ to previously assessed GE organisms which had been granted nonregulated status. The proposed rule would change this, granting developers the option to request a permit or regulatory status review of a GE organism that had not been previously reviewed and given nonregulated status. The decisions on the regulatory status of that organism would be based on APHIS’s assessment of plant pest risk.^{xi}

The newly proposed §340.1 (c) would provide an exemption from plants with plant trait mechanism of action (MOA) combinations that had previously been evaluated by APHIS during a regulatory status review and deemed unlikely to pose a plant pest risk. The developer would be given the option to make a ‘self-determination’ as to whether the new GE plant belonged to a category listed under 340.1 (b) or (c), thus making it exempt from regulations. If the developer did decide that this was the case, they would have the opportunity to request ‘written confirmation’ that the determination they had made was valid by APHIS. APHIS argues that there are three ‘fundamental’ elements that they would consider when evaluating a GE plant:

1. The basic biology of the plant prior to the modification
2. The trait that had resulted from the genetic modification
3. The MOA^{xii}

APHIS also maintains that, based on the risk assessments performed over the past 3 decades, many cases would have been possible to evaluate without the need for field test data. The introduced trait of the organism, the Agency argues, provides the most ‘reliable indicator’ of the potential of the GE organism’s ‘deleterious’ effects on other plant products and plants. APHIS’s proposed changes would focus primarily on the evaluation of genetics and characteristics of the plant trait MOA combination and the potential for risk that, based on these characteristics, the plant would pose a plant pest risk should it be released into the environment as intended by the developer.^{xiii}

The proposed paragraph 340.4 (b) sets out the process for regulatory review. Upon receiving a request for a regulatory status review, APHIS would conduct a review of the plant pest risk that the GE plant could potentially provide, as well as any sexually compatible relatives that could inherit the GE trait. To do this, APHIS would use existing knowledge on the biology of the comparator to the GE plant, as well as sexually compatible relatives as the foundation of their consideration of potential plant pest risk. This would include information on the spatial and temporal distribution of the comparator plant, particularly in the absence of ‘intentional human assistance’ and the interaction and impact on other organisms in the environment. Evaluations would be made on whether introduction of the GE plant could result in indirect or direct harm to other non-target organisms, particularly those which are beneficial to agriculture, such as predators of plant pests and pollinators.^{xiv}

It is also recognized by APHIS that GE may be taken advantage of to introduce traits which increase the weedy impacts of a plant, such as distribution, density, or development. To negate this, APHIS would still continue what is the current practice of considering the weediness of the unmodified comparator plant, assessing whether the new GE plant would in any way affect the weediness. The weediness, and impact of the new trait on weediness, of other sexually compatible organisms would also be assessed due to its relevance to evaluating the GE organism’s plant pest risk.^{xv}

These changes come from APHIS’s desire, as outlined in its 2014-18 Strategic Plan, to ensure the best science is used in its decision making. It aims to use a science-based risk assessment framework, which would further enable a ‘sound decision-making process.’ This is in aid of the aim to respond to a rapidly changing scientific field and the need to adapt the regulatory system where it is deemed appropriate.^{xvi}

Successful Petitions for Nonregulated Status

The following section will consist of examples of petitions which were successful in their request for deregulation. As previously mentioned, as of December 2018, APHIS granted 130 determinations of nonregulated status of the 162 petitions submitted.^{xvii} When trying to assess the likelihood of a successful petition for deregulation for the XplA/B expressing switchgrass lines, these examples prove helpful as they demonstrate the level of information required into the weediness and likelihood of a GE plant having a high plant pest risk, as well as the main factors that APHIS are looking for when they grant nonregulated status.

When considering a plant for deregulation, APHIS appears to be looking for 7 main factors which determine a plant becoming a plant pest risk or having increased weediness. This consists of:

1. Whether a plant pest risk can be identified from the transformation process, the ‘insertion and expression of new genetic material,’ or changes that may occur in the metabolism in the new GE organism
2. Whether pest incidence or disease, and/or damage is expected to be increased by the introduction of the new GE plant
3. Based on the evidence, whether the GE plant is likely to ‘adversely impact’ non-target organisms which may be beneficial to the environment or to agriculture
4. The likelihood of the GE plant becoming weedier or ‘more difficult to control’ as a weed than its antecedent
5. How likely gene introgression from the GE plant into wild relatives in the US is, and, how likely this is to increase the weediness potential of any progeny
6. Whether the introduction of the GE plant would cause any significant changes to cultivation or agricultural practices
7. The likelihood of horizontal gene transfer of the new genetic material to other organisms, and whether it is expected to lead ‘directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens or parasitic plants.’^{xviii}

The increased weediness of the GE plant and the possibility of horizontal gene transfer are both factors which APHIS take seriously, however there does not necessarily seem to be a hard and fast rule as to how developers should deal with this. Indeed, this can be seen when comparing the petitions of Monsanto’s GE creeping bentgrass (petition number 15-300-01p) and Pioneer’s maize (petition number 11-244-01p). There has been one determination of nonregulated status for GE creeping bentgrass, with one other GE creeping bentgrass currently under assessment. The creeping bentgrass species itself has some weedy characteristics, however, Monsanto argued that it was not considered a major weed in US agriculture, citing scientific literature to support this. Furthermore, Monsanto announced that it was not its intention to commercialise this plant, and that all commercial seed stock had been destroyed.^{xix} The intention not to further propagate the GE plant was one of the major factors which encouraged APHIS to grant deregulation, informing its decisions in the plant pest risk assessment.^{xx}

In contrast to this, 4114 maize developed by Pioneer had been engineered to contain all genes at one single breeding locus, thus creating the expectation for the 4114 maize to be more efficiently bred into a wider variety of maize.^{xxi} Unlike previous determinations, the issue of horizontal gene transfer did not appear to raise an issue for APHIS's assessment of 4114 maize. Indeed, it was not included in the list of reasons in favor of deregulation.^{xxii} This proves promising for the chances of deregulation for XplA/B expressing switchgrass as the potential for horizontal gene transfer to others of its species does not prevent deregulation. Indeed, APHIS has ruled in other cases that it is the likelihood of this transfer to cause disease, damage, or injury to plants, as well as the creation of new plant pests, that is the real issue.^{xxiii}

Furthermore, the use of the 35S promoter and terminator, taken from the Cauliflower Mosaic Virus, in the XplA/B expressing switchgrass also does not appear to present an obstacle in a determination of nonregulated status. Indeed, previous APHIS determinations have issued nonregulated status to GE crops containing the 35S promoter, such as Bayer's LLRICE601 (petition number 06-234-01p) and Monsanto's reduced lignin alfalfa (petition number 12-321-01p). In its petition, Bayer argued that the use of the 35S promoter did not cause the rice to become a plant pest, as had been seen in other GE rice plants that had previously been deregulated by APHIS.^{xxiv}

Petitioning for Nonregulated Status for XplA/B Expressing Switchgrass

Based on the evidence presented above for previous successful petitions, there is probably a high likelihood for a successful petition for giving XplA/B expressing switchgrass lines a nonregulated status. Indeed, though the switchgrass uses promoters and terminators from the Cauliflower Mosaic Virus and has a vector derived from *Agrobacterium tumefaciens*, APHIS has acknowledged that these methods do not leave viable bacteria behind or confer plant pest traits, though they do facilitate the expression of other genes into the organism. Moreover, APHIS agrees that use of plant pests in this fashion (either as vectors for genetic material or as donors of 'regulatory sequences') has a 'long history' and does not result in the injury or disease to the organism receiving them or other organisms.^{xxv} The Agency argues that the impact of the GE plant, and its potential as a plant pest, must also be considered outside of its primary function or target, as these organisms are 'almost always intended for eventual release into the environment.' Consideration must be given to the indirect plant pest risks, particularly if the GE plant may impact non-target organisms that are beneficial to agriculture.^{xxvi} Attention must also be given to the potential allergenicity and toxicity of the GE plant. When determining a petition for deregulation, APHIS have also been known to use 'alternative characterizations' such as 'poses no more of a plant pest risk than its non- GE counterpart' or 'will not pose a plant pest risk.' The criteria used to assess this include conclusions made on the potential of the GE plant pest risks which involve the interactions of the organism with others or the environment.^{xxvii} Provided that the petition for XplA/B expressing switchgrass could demonstrate the lack of plant pest risk with factual information and evidence, chances of deregulation are favorable.

In order for a petition to be successful, APHIS argues, it needs to include a description of the biology of the recipient plant (unmodified), containing enough information necessary to identify it in the 'narrowest taxonomic grouping applicable.' This should be accompanied by a detailed description of the 'differences in genotype' between the GE plant and the non-modified comparator. APHIS requests that all scientific, common, and trade names are included, as well as the information needed to identify:

- The donor organism
- The nature of the transformation system (vector or vector agents)
- The inserted genetic material and its product(s)
- The regulated article^{xxviii}

The description of the phenotype of the regulated article needs to be detailed, describing the known (and possible) differences from the original recipient that would authenticate the premise that the GE plant is unlikely to pose a plant pest risk that is greater than the unmodified plant. This is including (but is not limited to):

- ‘Plant pest characteristics
- Disease and pest susceptibilities
- Expression of the gene product
- New enzymes or changes to plant metabolism
- Weediness of the regulated article
- Impact on the weediness of any other plant with which it can interbreed
- Agricultural or cultural practices
- Effects of the regulated article on non-target organisms
- Indirect plant pest effects on other agricultural products
- Transfer of genetic information to organisms with which it cannot interbreed’^{xxix}

In addition to this, any information that is known to the person making the petition that indicates the GE plant could pose a greater plant pest risk should also be included. It is important to consider what reasonable hypotheses can be put forward that would suggest environmental harm from the release of the *xplA/B* transgenes in the transgenic switchgrass lines. The cytochrome P450 XplA enzyme is only known to degrade RDX, and is not known to be active against substrates involved in disease promotion or are essential for cell metabolism. RDX is an important environmental pollutant of significant concern. So the expected result of the release of *xplA* into the environment would be the degradation of RDX in that environment, resulting in the improvement of the health and quality of the environment. All of this needs to be accompanied by the relevant experimental data and publications. The petition should also include field test reports for trials which were conducted under permit or notification, including the ‘APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, non-target organisms, or the environment.’^{xxx} This is all required by APHIS for a petition for nonregulated status for XplA/B switchgrass to be considered.

ⁱ Permits, Notifications and Petitions, APHIS, USDA. (<https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions>) accessed on 19/08/19

ⁱⁱ *Ibid*

ⁱⁱⁱ ‘7 CFR parts 340 and 372’ *Federal Register*, Vol. 82 No.109

^{iv} 7 CFR parts 340 and 372, *Federal Register*

^v *Ibid*

^{vi} Petition for determination of nonregulated status, APHIS (<https://www.govinfo.gov/content/pkg/CFR-2008-title7-vol5/pdf/CFR-2008-title7-vol5-sec340-6.pdf>) accessed 19/08/19

^{vii} *Ibid*

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- viii *Permits, Notifications and Petitions*
- ix 7 CFR parts 340 and 372, *Federal Register*
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- xvi 'Strategic Plan FY2014-FY2018', *APHIS USDA*
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- xxiv 'Application for an Extension of the Determination of Nonregulated Status for Glufosinate-Tolerant Rice' *Bayer*, 2006,
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- xxv 7 CFR parts 340 and 372, *Federal Register*
- xxvi *Ibid*
- xxvii *Ibid*
- xxviii *Petition for Determination of Nonregulated Status*
- xxix *Ibid*
- xxx *Ibid*