Environmental and Experimental Botany 99 (2014) 67–74

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Comparative physiological responses of *Morella cerifera* to RDX, TNT, and composition B contaminated soils

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ARTICLE INFO

Article history:
Received 26 July 2013
Received in revised form 17 October 2013
Accepted 1 November 2013

Keywords:
Explosives
TNT
RDX
Composition B
Plant physiology
Soil contamination

ABSTRACT

Surrounding vegetation is exposed to a variety of potentially toxic compounds due to unexploded ordnances leaching explosive compounds into the soil. These compounds are absorbed by roots, transported through the vascular system, and distributed throughout plant tissues. Research Demolition Explosive (RDX) (hexahydro-1,3,5-trinitro-1,3,5-triazine) and trinitrotoluene (TNT) (2-methyl-1,3,5-trinitrobenzene) are the most studied; however, mixtures of explosives are widespread in conventional munitions. Composition B (Comp B), a mixture of RDX and TNT, is the most common mixture. Our study objective was to quantify the comparative effects of RDX, TNT and Comp B on the physiology of an evergreen shrub, *Morella cerifera*. Adult *M. cerifera* plants were exposed for 7 weeks to soil amended with RDX up to 1500 mg kg^{-1} dry soil, TNT up to 500 mg kg^{-1} dry soil, and Comp B up to 750 mg kg^{-1} dry soil. Stomatal conductance, photosynthesis, leaf water potential, leaf fluorescence, and contaminant uptake values were measured at the end of the experiment. As contaminant concentration increased, significant declines in photosynthesis and leaf fluorescence occurred for all compounds. Overall responses varied between contaminants and impacts of Comp B were largely reduced compared to either RDX or TNT. Of all physiological parameters, photosynthesis was most impacted, making it a sensitive indicator for the detection of explosives. Yet, the intricate relationships within normal physiological processes appear to be severed in the presence of explosives. These disparate responses in plant physiology may serve as a method for explosive contamination stress detection. Our results highlight the importance of studying real world munition mixtures.

1. Introduction

Vegetation may be exposed to a variety of potentially toxic compounds due to over 110 million landmines and an unexploded ordnances (UXOs) buried across 68 nations (UNICEF, 1995) which leach explosives into the soil (Hawari et al., 2000). Explosive compounds are absorbed via roots and leaves, transported through the vascular system, and distributed throughout plant tissues (Paterson et al., 1990; Pilon-Smits, 2005; Vila et al., 2007; Verkleij et al., 2009). Explosives have industrial (i.e., demolition) and recreational uses (i.e., fireworks), so contamination may occur in areas far removed from military operations (Hawari et al., 2000) making them a very widespread threat. The presence of contaminants in soil can drastically alter the surrounding biological environment. Plants can be particularly impacted due to an intimate relationship with the soil. Plant health is coupled closely to surrounding environmental conditions, especially the soil, but effects of soils contaminated with explosive compounds on plants are poorly understood.

Research Demolition Explosive (RDX) (hexahydro-1,3,5-trinitro-1,3,5-triazine) and trinitrotoluene (TNT) (2-methyl-1,3,5-trinitrobenzene) are the most widely distributed organic explosive contaminants (Hawari et al., 2000; Rylott and Bruce, 2009; Khatisashvili et al., 2009). RDX, a nitroamine (cyclic nitroaromatic compound), is not readily degradable and typically bioaccumulates in stems and leaves; in some cases >90% of the RDX can be recovered in the parent form (Ahmadi et al., 1980; Thompson et al., 1999; Ampleman et al., 2003; Best et al., 2006; Laurent et al., 2007). In contrast, TNT, a nitroaromatic compound that can be metabolized by plants as a nitrogen source, is primarily bound within roots where up to 75% of the degraded products remain (Ahmadi et al., 1980; Ampleman et al., 2003; Pilon-Smits, 2005). Levels of contamination that plants may tolerate vary widely by species (Gong et al., 1999; Krishnan et al., 2000; Ait Ali et al., 2006; Vila et al., 2007; Khatisashvili et al., 2009).

Explosive compounds and by-products induce a variety of morphological responses that differ based on compound and the

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http://dx.doi.org/10.1016/j.envexpbot.2013.11.002

affected species (Winfield et al., 2004; Laurent et al., 2007). RDX can cause deformation and reduction of both leaves and stems (Winfield et al., 2004; Laurent et al., 2007) while TNT inhibits production of secondary roots and root hairs (Comfort et al., 1996; Comfort et al., 1998; D'Surney et al., 2003). Plant age can also induce variability in plant response (Gong et al., 1999; Vila et al., 2007; Winfield et al., 2004; Khatisashvili et al., 2009). RDX inhibits seedling germination in some species (Winfield et al., 2004), negatively impacts seedling establishment, and overall plant growth at any age (Winfield et al., 2004; Khatisashvili et al., 2009). Similarly, TNT impacts seed germination in some species (Gong et al., 1999; Krishnan et al., 2000; Sens et al., 1999; Khatisashvili et al., 2009), and reduces seedling establishment and adult growth (Gong et al., 1999). Relatively low doses of TNT (<30 mg kg^{-1}; Best et al., 1999) may promote plant growth while higher concentrations (>30 mg kg^{-1}; Best et al., 1999) inhibit it as observed in other types of soil contamination (Sun et al., 2009). RDX does not have a positive impact on vegetation at any concentration (Winfield et al., 2004; Laurent et al., 2007; Khatisashvili et al., 2009; Zinnert, 2012). In general morphological effects are coupled with physiological impairment but impacts on physiological functions remain unclear (Winfield et al., 2004; Naumann et al., 2010; Zinnert et al., 2012).

While uptake and morphological effects have been well studied, few physiological responses to explosive soil contamination have been quantified. RDX significantly reduced stomatal conductance as well as photosynthesis in the deciduous shrub *Baccharis halimafolia* (Groundsel tree; Zinnert, 2012) which also exhibited reduced function of Photosystem II (PSII), although the reduction disappeared as light levels were increased (Zinnert, 2012). Ait Ali et al. (2006) showed that TNT exposure induced photosynthetic electron transport impairment in *Lactuca sativa* (lettuce). Reduction in electron transport rate (ETR) was accompanied by a strong fluorescence quenching and increased dark minimal fluorescence (F_o) dissipation; evidence TNT was impairing PSII function (Ait Ali et al., 2006). TNT exposure also caused decreased transpiration in hybrid poplar trees (Thompson et al., 1998). The aforementioned studies on physiological responses of plants to RDX and TNT give some insight into the mechanisms of stress induction by those contaminants; however, there are even less data on the impact of real world munition mixtures on plant physiology.

Conventional munitions contain not one but mixtures of several explosive compounds (Pichtel, 2012). One of the most common munition mixtures, Composition B (Comp B) is 60% RDX, 39% TNT, and ~1% wax. When exposed to water, Comp B leaches TNT at a much faster rate than RDX resulting in pulses of contaminants released into the soil (Lever et al., 2005). This adds another layer of complexity that requires ecologically relevant studies to properly understand the full ecological impacts of such contamination, yet no studies have quantified the direct physiological impacts of Comp B. Our objective was to quantify the comparative effects of RDX, TNT, and Comp B on physiology of the evergreen shrub *Morella cerifera*. Specifically, we quantified photosynthetic function using photosynthesis and chlorophyll fluorescence and water status via stomatal conductance and water potential. We hypothesized that, due to the mixture of explosive compounds and the inherent effects of each, Comp B would induce the greatest impact on *M. cerifera* physiology.

2. Materials and methods

2.1. Plant material

Morella cerifera L. (wax myrtle, Myricaceae), also known as *Myrica cerifera*, was used as the target species because physiology and stress responses have been quantified in detail (Naumann et al., 2010; Sande and Young, 1992). Additionally *M. cerifera* exhibits growth characteristics that involve high nutrient/water absorption ensuring the uptake of the contaminant (Pilon-Smits, 2005). This evergreen shrub naturally colonizes disturbed sites, and is present on bombing ranges and near munition factories along the Atlantic coast of the United States. Adult native-stock plants were purchased from a commercial source (Pinelands Nursery & Supply, Columbus, NJ) and pruned to the woody stem. They were allowed to grow for 3 months prior to experimentation (≥30 cm).

Table 1
Concentrations of RDX and TNT relative to the range of Composition B concentrations in mg kg^{-1}.

RDX	TNT	Composition B
60	39	100
180	117	300
300	195	500
450	292.5	750

Composition B is comprised of 60% RDX, 39% TNT, and 1% wax (binding compound). All concentrations are in mg kg^{-1}.

2.2. Soil contamination

All plants were grown in a 3:1 mixture of low nutrient (5% nitrogen) topsoil and sand. This mixture approximated natural organic and nutrient content of field soils. The soil was amended with serial dilutions of RDX, TNT, and Comp B in 200 mL of acetone to bring the relative concentration to the proper level (Ait Ali et al., 2006; Naumann et al., 2010; Zinnert et al., 2012). After treating with contaminants, the soil was kept at room temperature (~25 °C) in the dark for 72 h to allow for the acetone to evaporate and prevent photodegradation of compounds (Ait Ali et al., 2006). Four concentrations, each having five replicates, were used for each contaminant and exposure was similar to those used in the aforementioned studies. RDX treated plants were exposed to concentrations of 0, 100, 500, 750, or 1500 mg RDX kg^{-1} soil. TNT treated plants were exposed to concentrations of 0, 30, 100, 250, or 500 mg TNT kg^{-1} soil. Comp treated plants were exposed to concentrations of 0, 100, 300, 500, or 750 mg Comp B kg^{-1} soil. Relative concentrations of RDX and TNT found in Comp B concentrations are presented in Table 1. Reference plant soil was treated with 200 mL of acetone containing no contaminant. Concentrations for each compound were relatively conservative compared to the concentrations found near munition sites in the field (Talmage et al., 1999). Plants were watered on a daily basis, kept at an ambient temperature of 30 °C ± 7 °C (relative humidity ≈60%), and were monitored for seven weeks. Catch dishes were placed under each plant to retain water and reduce the impact of leaching.

2.3. Uptake analysis

Dried plant leaves were separated and finely chopped using scissors. RDX and TNT were extracted from leaves using modifications of methods specified in EPA SW-846 Method 8330 (USEPA, 1989; Larson et al., 1998). Briefly, weighed tissue samples were placed in 20 mL glass vials with Teflon-lined caps and extracted using 10 mL of high performance liquid chromatography (HPLC) grade acetonitrile. Samples were vortexed for 1 min and placed in a cool ultrasonic bath for 18 h (USEPA, 1989). After sonication, the plant extracts were allowed to settle for an hour. Then, the supernatant (2.5 mL) was filtered through a clean-up column containing 0.25 g of Florisil and 0.25 g of basic alumina (wetted with fresh acetonitrile first) to clean up or remove chlorophyll plant pigments that interfere with UV detection of RDX and TNT. HPLC grade acetonitrile (2.5 mL) was passed through the same column and mixed with the supernatant. Thereafter, a sample of the supernatant was diluted