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PLANT FUNCTIONAL TRAIT AND HYPERSPECTRAL REFLECTANCE RESPONSES TO
COMP B EXPOSURE: EFFICACY OF PLANTS AS LANDMINE DETECTORS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University

by

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Abstract

PLANT FUNCTIONAL TRAIT AND HYPERSPECTRAL REFLECTANCE RESPONSES TO COMP B EXPOSURE: EFFICACY OF PLANTS AS LANDMINE DETECTORS

By Paul V. Manley, II, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

Virginia Commonwealth University, 2015

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At least 110 million landmines have been planted since the 1970s in about 70 nations, many of which remain in place today. Some risk of detection may be mitigated using currently available remote sensing techniques when vegetation is present. My study focused on using plants as phytosensors to detect buried explosives. I exposed three species representing different functional types (*Cyperus esculentus* (sedge), *Ulmus alata* (tree), *Vitis labrusca* (vine)) to 500 mg kg⁻¹ of Composition B (Comp B; 60/40 mixture of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT)), a commonly used explosive mixture, and measured functional traits and reflectance over a nine-week period. *Cyperus esculentus* was not a good indicator for the presence of explosive compounds. Comp B treatment woody species, *U. alata* and *V. labrusca*, exhibited changes in pigment content, leaf area, specific leaf area, dry leaf biomass, and canopy reflectance. The efficacy of plants as landmine detectors is species and/or functional group dependent.

Introduction

Each year, approximately 15,000 to 25,000 people worldwide are killed or injured by landmines left behind from wars and conflicts (Walsh and Walsh, 2003). Prevalence of landmines stems from relative cheap cost of manufacturing (\$3 – \$30 USD per mine) compounded by the high cost of removal (\$300 – \$1000 USD per mine; Walsh and Walsh, 2003). Current detection methods require handheld or remote controlled mine detectors (De Leeneer and Pastijn, 2002) or the use of dogs, African giant pouch rats, and pigs (Habib, 2007). Animals are led on leashes sniffing for various chemical compounds used in unexploded ordnance (UXO; Habib, 2007). These demining efforts are expensive in terms of time and money and involve placing people and animals at risk. Some risk may be mitigated with remote sensing techniques which are currently available. The term remote sensing implies no physical contact with the study object and can be achieved by scanning an area with hyperspectral sensors mounted on a plane, unmanned aerial vehicle (UAV), or satellite. Hyperspectral sensors focus and measure reflected light, typically between 350 – 2500 nm, from over 200 spectral bands for different materials, each having their own “signature,” or typical response curve (Govender et al., 2008). The amount of land area that can be covered by the aforementioned crafts is significantly greater than that of ground demining teams over a given period of time. As many areas afflicted by landmine contamination are vegetated, hyperspectral imagery may be an effective tool for remote detection of landmines via plant reflectance.

Plants are rooted in soil and absorb nutrients and any contaminants within that soil (Pilon-Smits, 2005). Contaminant uptake by plants can cause physiological and morphological

responses (i.e. functional traits: changes in plant pigments; photosynthesis; specific leaf area). The extent to which functional traits are altered varies not only by contaminant and concentration, but by plant functional type (PFT) as well (Lavorel and Garnier, 2002; Dwyer et al., 2014). As an alternative method of classification, PFTs may be advantageous in better understanding how contaminants affect plants on a larger scale, rather than by specific species because this method groups plants by how they react to their environment. Using plants as bio-indicators of landmines is done by detecting changes in functional traits, as is done with a multitude of other anthropogenic and natural sources of stress, remotely via hyperspectral imagery (Ustin and Gamon, 2010). As the field of remote sensing expands to include vegetation of all functional types coupled with various sources of stress, accurate grouping of PFTs based on plant responses will be possible. This may expedite efforts to remediate landmine contamination as we better understand how different PFTs react to explosives uptake.

Two widely used energetic materials globally are TNT (2,4,6-trinitrotoluene, nitroaromatic compound) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine, nitramine; Khatisashvili et al., 2009; Pichtel, 2012; Panz and Miksch, 2012). Most landmines contain a mixture of these compounds known as Composition B (Comp B), which consists of 60% RDX, 39% TNT, and 1% paraffin wax (binding agent; Via et al., 2014; Ali et al., 2014; Taylor et al., 2015). The popularity of TNT and RDX for landmine production, as well as Comp B, is justification to gather information on how these energetic compounds affect vegetation both physiologically and spectrally. Landmines are designed to explode, but will leach compounds into the surrounding soil over time due to poor structural integrity of casings (Taylor et al., 2015). Soil moisture and rainwater may degrade the casing. Once this takes place the compounds are introduced into the soil and considered environmental contaminants (Rao et al., 2009; Taylor

et al., 2015) and are potentially available for plant uptake. Plant uptake of Comp B causes a multitude of stress responses including biomass reduction and leaf necrosis (Via et al., 2014), which are remotely detectable via measurable differences in plant leaf pigments, physiology, and other structural changes (Zinnert et al., 2013).

While many studies have focused on plant uptake of explosives and efficacy of hyperspectral imagery to detect plant stress, few have attempted to merge the two (Zinnert, 2012). By combining leaf-level information with data collected at the canopy- or landscape-level, it may be possible to locate forgotten landmines and UXOs. This may be plausible because of where TNT, RDX, and their metabolites localize within plants. TNT mainly concentrates in plant roots, as it is less mobile than RDX (Thompson et al., 1998; Best et al., 2008; Brentner et al., 2010), however, a number of metabolites can be produced and translocated to aerial portions of plant material (Sens et al., 1999; Robidoux et al., 2003). RDX alone can move quickly from soil to shoots and leaves within two days (Burken, 2003) where it can form conjugates via photolysis and enzymatic reactions (Just and Schnoor, 2004; Best et al., 2004; Khatisashvili et al., 2009). Both energetic compounds negatively affect plant physiology and morphology (i.e. plant functional traits) which may be remotely detectable using currently existing remote sensing technologies (Ali et al., 2014; Via et al., 2014). Some research has suggested Comp B has reduced effects on plant physiology compared to similar concentrations of TNT or RDX alone (Via et al., 2014). These effects, however, are likely to be species-specific. The goal of my study was to use remote sensing to determine the effectiveness of plants as bio-indicators to detect landmines.

Reflectance indices, or equations that use reflectance values from specific wavelengths (Peñuelas et al., 1995; Zarco-Tejada et al., 2003), calculated from hyperspectral data have been

devised to detect stressed vegetation; these use some variation of wavelengths from the visible (VIS), near-infrared (NIR), and short-wave infrared (SWIR) portions of reflectance spectra. For example, the Photochemical Reflectance Index (PRI) uses reflectance values at 531 and 570 nm where the former is a reference wavelength and the latter is sensitive to rapid pigment changes caused by stress (Peñuelas et al., 1995) that surround the green “hump” in a leaf’s reflectance signature (Sims and Gamon, 2002; Garbulsky et al., 2011). Reflectance-derived fluorescence techniques have allowed for stress detection before changes in pigments are visible (Zarco-Tejada et al., 2003; Moya et al., 2004; Naumann et al., 2010), though there are cases in which stress detected via reflectance is only possible after visible signs of stress have occurred (Naumann et al., 2010).

Presence of explosive compounds, as with other xenobiotics (foreign compounds), can inhibit photosynthesis, which may cause shifts in leaf fluorescence (Ali et al., 2006; Via et al., 2014). These shifts are detectable using plant reflectance indices for fluorescence that use wavelengths between 680 and 690 track changes in chlorophyll fluorescence (Zarco-Tejada et al., 2000). The challenge thus far has been isolating exact causes of stress via remote sensing techniques (Naumann et al., 2010), as the field of remote sensing is relatively novel.

My study used multiple plant species representing different plant functional types (PFTs), specifically *Cyperus esculentus* (nutsedge), *Ulmus alata* (winged elm), and *Vitis labrusca* (fox grape), to determine if changes in plant functional traits caused by the presence of Comp B in soil can be remotely detected using hyperspectral reflectance. Multiple PFTs were used to expand the research base on how effects of explosives translate to hyperspectral data. These species were chosen because they have been identified at one of our field sites that was contaminated with TNT, RDX, and Comp B “dummy devices.” Future studies will link

laboratory and field findings. I used fluorescence measurements and other functional traits, including chlorophyll content, leaf area, specific leaf area (SLA), and dry leaf biomass, concurrent with reflectance data to identify wavelengths associated with the presence of Comp B in soils. I hypothesized that plant stress from the presence of Comp B in soil will be detectable and discernable through analysis of functional traits, canopy reflectance, and reflectance indices.

Materials and Methods

Plants

My study compared differences in functional traits and reflectance spectra in response to Comp B exposure among three plant species that represent multiple PFTs. The species used for this study were *C. esculentus* (n = 5; Seed World USA), *U. alata* (n = 10; Hillis Nursery Company, Inc.), and *V. labrusca* (n = 10; Hillis Nursery Company, Inc.). These species were chosen for their varying growth forms and global distribution to serve as representative functional groups and species of areas of the world where landmines are present. *Cyperus esculentus* was started from seed because it grows and matures more rapidly than the two woody species. *Ulmus alata* and *V. labrusca* were purchased as 30-46 cm juveniles. All plants were kept in a glasshouse under relatively constant conditions (30 °C day/25 °C night), and watered twice a week. A 3:1 potting soil-to-sand ratio was used as potting media in this experiment (Naumann et al., 2010). Each individual pot contained 680-840 g of media, depending on initial plant and root size. The media for the experimental plants was treated with 200 mL of acetone containing 500 mg kg⁻¹ of Comp B following methods by Ali et al. (2006), while the control plants received 200 mL of acetone to control for any effects acetone may have on soil chemistry, as well as plant functional traits and canopy reflectance.

Functional Traits

At weeks 4 and 9, chlorophyll and carotenoid concentrations were analyzed by thoroughly grinding whole leaf samples with a mortar and pestle, forming a powder, following

Šesták (1971). Powder was placed in a 100% acetone solution to extract the chlorophyll, filtered, and then analyzed using a Spectronic 21 spectrophotometer. Chlorophyll and carotenoid concentrations were determined by standard equations (Holm, 1954). Leaf area and specific leaf area (SLA, $\text{cm}^2 \text{g}^{-1}$), or the ratio of leaf area to dry mass, was measured at weeks 4 and 9. Leaf fluorescence measurements, the fraction of absorbed photons that are used for photochemistry in a light-adapted leaf ($\Delta F/F'_m$; Genty et al., 1989), were taken using a Walz MINI-PAM (Heinz Walz GmbH). Dry leaf biomass was determined at the end of nine weeks after harvesting all leaves and drying them for 72 h at 70°C.

Canopy Reflectance

Within an hour of leaf fluorescence measurements, canopy reflectance spectra were quantified with a FieldSpec Pro Spectroradiometer (Analytical Spectral Devices, Inc.) for both experimental and control plants for nine weeks. A 3,200 °K, full-spectrum tungsten-halogen bulb was used as the illumination source to mimic full sunlight. An 8° field-of-view fore-optic was used for all species, held at nadir from a distance of 0.25 m. Instrument calibration was performed using a National Institute of Standards and Technology (NIST) reflectance standard that provides a white reference; dark reference calibration was done within the instrument by taking a reading with a closed shutter. This spectroradiometer provided a full-range waveform (350 – 2500 nm) that was collected by RS³ Spectral Acquisition Software (ASD Inc.) and analyzed by ViewSpecPro (ASD Inc.). Reflectance spectra were averaged by group for each species and used in further analysis. Only wavelengths between 400 and 2400 nm were included in analyses due to signal noise. Reflectance indices analyzed in this study represent specific

wavelengths associated with: carotenoid, chlorophyll, and anthocyanin content; biomass; reflectance-derived fluorescence; stress; and other functional traits.

Statistical Analysis

Independent two-sample t-tests were used to determine changes in week 4 and week 9 functional traits, canopy reflectance spectra, and reflectance indices between control and Comp B treatment plants. T-tests of chlorophyll concentrations and reflectance indices were adjusted with the Bonferroni correction. An alpha of 0.05/3 was used for chlorophyll concentrations and an alpha of 0.05/29 was used for reflectance indices. Reflectance spectra were averaged together by treatment group for each species. All analyses were performed with JMP statistical analysis software.

Results

Functional Traits

Pigment concentrations were significantly affected only at week 4 and limited to *V. labrusca* treatment plants (Table 1), but were not statistically different for any species at week 9 (Table 2). Leaf area, SLA, and $\Delta F/F'_m$ did not show differences at week 4 for any species relative to controls (Figures 1A, 1B and 1C). At week 9, leaf area was reduced in the *V. labrusca* treatment group ($t = 4.87$, $p = 0.0001$; Figure 2A). SLA was significantly greater in Comp B treatment groups of *U. alata* ($t = -2.49$, $p = 0.0285$) and *V. labrusca* ($t = -4.30$, $p = 0.0009$; Figure 2B). $\Delta F/F'_m$ was significantly higher only in treatment *U. alata* plants ($t = 4.57$, $p = 0.0003$; Figure 2C). *Vitis labrusca* was the only species to exhibit a significant reduction ($t = 4.48$, $p = 0.0003$) in dry leaf biomass due to Comp B exposure by the end of the experiment (Figure 2D). Though *U. alata* showed reduced dry leaf biomass, variability did not show significance (Figure 2D).

Canopy Reflectance

Reflectance of *C. esculentus* did not differ between treatment and control spectra at week 4 (Figure 3A). *Ulmus alata* treatment plants at week 4 showed significant differences from 617 – 696 nm, which represented changes in chlorophyll absorption (Figure 3B). Week 4 treatment *V. labrusca* plants exhibited less absorption by chlorophyll and carotenoid pigments from 489 – 514 nm (Figure 3C). In the same group, there were also significantly higher levels of reflectance from 570 – 709 nm attributed to less chlorophyll absorption (Figure 3C). Statistical differences

were calculated in chlorophyll absorption and the lower portion of the red edge (570 – 709 nm) for week 4 *U. alata* and *V. labrusca* treatment groups, relative to controls.

Cyperus esculentus again showed no changes in reflectance at week 9 (Figure 3D). *Ulmus alata* treatment plants, however, showed a reduction in reflectance in the chemical composition portion of the spectra between 497 – 606 nm (Figure 3E). *Ulmus alata* treatment reflectance was also significantly lower from 699 nm to 765 nm, the second half of the red-edge and into the cellular material region (Figure 3E). Decreased levels of absorption by chlorophyll and carotenoids were found in the spectra of *V. labrusca* at 425 – 504 nm and chlorophyll at 641 – 688 nm (Figure 3F). Differences in week 4 reflectance spectra were caused by leaf chlorosis and necrosis (Figure 4). Changes in reflectance were evident despite visual recovery of leaves at week 9 as compared to week 4 (Figure 5).

Reflectance Indices

Cyperus esculentus had no significant reflectance indices at either week 4 or 9 (Table 3 and Table 4). Of the 29 indices calculated, *U. alata* and *V. labrusca* had 8 and 18 significant indices at week 4, respectively (Table 3). Week 4 treatment *U. alata* and *V. labrusca* plants differed significantly from controls in terms of chlorophyll content based on multiple chlorophyll-related indices including the Photochemical Reflectance Index (PRI) and the Greenness Index (Table 3). Other notable differences involved changes in the Normalized Difference Vegetation Index (NDVI), which relates to biomass, as well as general plant stress indices (Table 3). At week 4 *V. labrusca* showed significance in almost all of the same indices as *U. alata* plus other modified indices that represent functional traits and plant health status: CI for chlorophyll content; Modified Chlorophyll Absorption in Reflectance Index 2 (MCARI2) for

leaf area index; and Lichtenthaler Indices 1 – 3 (LIC1 – 3) for stress (Table 3). By week 9, only the Orange/Red Chlorophyll Absorption Index (OCAR) was statistically different for *U. alata* (Table 4). The number of significant indices for *V. labrusca* dropped to zero by end of experiment (Table 4). Week 9 index results exhibited drastically less cross-species similarities compared to week 4 for *U. alata* and *V. labrusca*.

Discussion

Millions of landmines remain buried from past conflicts which pose threats to the lives of many across the globe (Walsh and Walsh, 2003). My study focused on observing the effects of Comp B, a commonly used energetic compound, on various plant functional traits and reflectance to make this research more applicable to a range of plant communities and ecosystems where landmines are present. Using multiple species representing a range of PFTs (e.g. sedges, vines, trees) will strengthen efforts to scale up remote detection of forgotten landmines and UXOs. Evidence of explosives contamination using hyperspectral remote sensing was revealed in the vine and tree species used. Though the sedge used in my study was not an effective candidate for remote detection of explosives, it may be a viable option for phytoremediation applications needed at military testing sites as *C. esculentus* is capable of uptake of TNT and RDX (Best et al., 2007).

Functional Traits

Vegetation remote sensing is still a developing discipline that requires comparisons of hyperspectral data to leaf-level measurements. This was attempted in my study by relating functional traits (pigments, SLA, $\Delta F/F'_m$, dry leaf biomass) to canopy-level foliar reflectance and reflectance-derived indices. Both natural and anthropogenic stress may affect leaf pigments in some manner (Gitelson and Merzlyak, 1997). Decreases in pigment concentrations are expected in the presence of xenobiotics when resulting stress is caused by photobleaching, peroxidation of chlorophyll molecules, or photoinhibition (Cañal Villanueva et al., 1985). In low doses, however,

xenobiotics may actually increase chlorophyll concentrations (Ketel, 1996; Miteva et al., 2005). Neither at week 4 nor at week 9 did *C. esculentus* respond significantly to Comp B exposure via pigment concentrations. Lack of significance may be attributed to variability around the mean from a small sample size ($n = 5$) because there was a non-significant mean reduction in pigments at week 4 and increase at week 9. At week 4, *U. alata* and *V. labrusca* displayed leaf interveinal chlorosis, which eventually led to laminar necrosis (personal observation). These occurrences due to chlorophyll degradation were attributed to RDX accumulation in aerial portions of plants (Vila et al., 2007b; Chen et al., 2011; Ali et al., 2014). Despite such high levels of visible damage, only pigments of *V. labrusca* treatment plants were significantly affected. By week 9, some *U. alata* and many *V. labrusca* leaves senesced which was followed by new growth. These newer leaves were probably exposed to less than 500 mg kg^{-1} Comp B and had healthier coloration than controls by end of experiment (personal observation). Changes in pigment concentrations were not evident at week 9 via spectrophotometer for any species. Visible damage remained prevalent for the few leaves that did not senesce from Comp B exposure. New growth of *U. alata* and *V. labrusca* at week 9 exhibited small areas of necrosis and morphological alterations by way of smaller leaves.

Typically, reductions in SLA due to stress are expected (Chaugool et al., 2013), though results may vary depending on the species and cause of stress (Wuytack et al., 2011). SLA may decrease in an attempt to minimize pollutant uptake, or increase in order to compensate for photosynthesis inhibition (Wuytack et al., 2011). The latter is a plausible explanation as TNT, RDX, and Comp B cause reductions in photosynthesis (Naumann et al., 2010; Ali et al., 2014; Via et al., 2014). Naumann et al. (2010) reported significant inhibition of photosynthesis (A_{net} , $\mu\text{mol m}^{-2} \text{ s}^{-1}$) in a coastal shrub, *Morella cerifera*, exposed to 30, 100, 250, and 500 mg kg^{-1}

TNT, as did Via et al. (2014) with the same species from exposure to various concentrations of RDX, TNT, and Comp B. In my study at end of the experiment, *U. alata* and *V. labrusca* both revealed significant increases in SLA from reduced leaf weight of new leaves, possibly as a result of quickly formed new growth.

Stressed plants without visible leaf damage (i.e., chlorosis, necrosis) will release excess energy from chlorophyll through fluorescence and heat (McFarlane et al., 1980; Porcar-Castell et al., 2014). Thus, before stress becomes visible, increased fluorescence measurements can indicate natural and anthropogenic causes of stress. Light-adapted fluorescence ($\Delta F/F'_m$) represents the effective quantum yield, or the operating efficiency of Photosystem II (PSII) to capture light (Genty et al., 1989). For Via et al. (2014), most concentrations of RDX used (up to 1,500 mg kg⁻¹) significantly reduced *M. cerifera* $\Delta F/F'_m$, whereas the authors only measured reductions from TNT exposure at the highest level used (500 mg kg⁻¹). Interestingly, Comp B had no effect on *M. cerifera* at any concentration reinforcing the notion that as a mixture Comp B may have reduced effects on vegetation compared to TNT or RDX alone (Via et al., 2014). By contrast, Ali et al. (2014) found reduced fluorescence in *Baccharis halimifolia* after exposure to 100, 300, and 750 mg kg⁻¹ Comp B. I hypothesized initially there would be declines in $\Delta F/F'_m$ attributed to Comp B exposure for all species since nitramines such as RDX are similar in structure to triazine herbicides that inhibit PSII (Peterson et al., 2001; Kuperman et al., 2009; Chen et al., 2011). *Ulmus alata* and *V. labrusca* displayed interveinal chlorosis and laminar necrosis at week 4 without increases in $\Delta F/F'_m$. By end of experiment, $\Delta F/F'_m$ of Comp B *U. alata* increased significantly indicating an increase in light capture efficiency, possibly due to regrowth of new leaves.

Dry leaf biomass of control and test plants was quantified after nine weeks for all species and was only reduced significantly in *V. labrusca* after leaf drop and regrowth. Variations in these findings are also found in other studies. In low concentrations, TNT and its metabolites cause leaf biomass reductions in some agricultural species, but not in some woody species (Khatisashvili et al., 2009; Chen et al., 2011). RDX at low concentrations can cause increases in biomass suggesting it may act as a nitrogen source (Low et al., 2008; Khatisashvili et al., 2009), but hinder root growth in higher concentrations (Winfield et al., 2004).

Throughout this nine-week study, *C. esculentus* showed no significant differences for any functional trait metric tested. While this may be attributed to a small sample size, it speaks to differences in stress responses between monocots and dicots. Winfield et al. (2004) compared germination and growth responses to RDX contamination between nine dicots and six monocots and found dicots to be more sensitive to RDX exposure than monocots. Gong et al. (1999) came to a similar conclusion from high levels of TNT. *Cyperus esculentus* proved to be much more resistant to Comp B exposure than the tree and vine species used and has been considered for phytoremediation of explosives on military testing ranges in the United States (Best et al., 2007).

Canopy Reflectance

While plant stress is important to detect early, in the case of explosive compounds it may not be the only factor in determining specific locations of landmines. Many affected areas that are also vegetated were contaminated decades ago. If the structural integrity of casings is compromised allowing explosive compounds to leach out into the soil, nearby plants may take up these compounds and display signs of initial stress in leaves, recover via leaf drop, and regrow indicating a pulse disturbance. Contrarily, this process may be more akin to a press

disturbance with a steady release of explosives compounds over a longer period of time (Taylor et al., 2015). *Cyperus esculentus* did not become chlorotic or necrotic; however, leaf senescence, drop, and subsequent regrowth occurred within the nine-week study in *U. alata* and *V. labrusca* treatment plants. These new leaves matured in the remaining weeks, but were stunted in size (personal observation) exhibiting morphological effects from Comp B exposure.

Canopy damage at week 4 was evident via hyperspectral reflectance for *U. alata* and *V. labrusca* in the visible spectrum (400 – 700 nm) from increased proportional reflectance past 550 nm. This increase in reflectance signified changes in light absorption, most notably via PRI, an index associated with nonphotochemical energy dissipation and chlorophyll and carotenoid levels (Barton, 2011; Zinnert, 2012). PRI was reduced significantly for both *U. alata* and *V. labrusca* Comp B plants suggesting changes in carotenoid levels (Zinnert, 2012), despite no significant differences in pigment measurements. Most other indices associated with carotenoid and chlorophyll levels also revealed significant differences for *V. labrusca* and to a lesser extent for *U. alata*. Increases in $\Delta F/F'_m$ were not measured for either woody species, but increases were detected in CUR index for *V. labrusca* which represented an change in canopy fluorescence (Zarco-Tejada et al., 2000). Though RDX is not toxic to some plants (Best et al., 2007; Chen et al. 2011), it is unlikely that *U. alata* leaves could have been as visibly damaged and not resulted in decreased pigment concentrations and increased $\Delta F/F'_m$. This may be a rare example of species-specific responses or an indication of measurement error and fluorescence sampling bias as the spectroradiometer and fluorometer may have been focused on healthier portions of leaves.

Ulmus alata control plants had higher reflectance in the 500 – 600 nm range than treatment plants by end of experiment. This is possibly because no nutrients were added over the course of the nine week experiment which caused leaves of control plants to become lighter in

color (personal observation). Week 9 *V. labrusca* control plants appeared to tolerate the low nutrient conditions better than *U. alata* controls. Most treatment *V. labrusca* plants by week 9 senesced, dropped, and regrew smaller leaves that showed some visible signs of stress such as small areas of necrosis and leaf curling, though not to the extent of pre-drop.

New leaves of *U. alata* were visibly smaller, possibly indicating a morphological response to Comp B uptake (Winfield et al., 2004; Via et al., 2014). At week 9, *U. alata* had a measured increase in SLA which can be interpreted in reflectance spectra with decreased reflectance in green, red, and SWIR regions as measured in my study (Vogelmann, 1993; Gitelson et al., 1996; Lymburner et al., 2000). Though an increase in SLA was measured for *V. labrusca* at week 9, decreased reflectance in the green and red portions of reflectance were not. Non-significant increase in NIR reflectance was, which is an indication of changes in SLA (Lymburner et al., 2000).

Conclusions

Unlike other experiments reported in literature, my study was carried out over a nine-week period. Many explosives studies focus on seedling germination and growth within a much shorter amount of time. In reality, landmines that were planted years ago in vegetated areas would have been done so around established vegetation, or have since revegetated. My study used matured vegetation to mimic real-world scenarios. Another distinguishing factor is that during those nine weeks, *U. alata* and *V. labrusca* leaves senesced and subsequently regrew while control leaves did not, which highlights distinctions between press versus pulse disturbances. It is important to note plants in contaminated areas may not be exposed to such

high concentrations of explosives at once, but smaller amounts over a longer period of time which may not cause such dramatic expressions of stress seen in this study.

Functional traits and canopy reflectance differed significantly, even following leaf drop and regrowth. Contrary to my hypothesis, pigment concentrations did not change for *U. alata*, though portions of reflectance spectra associated with chlorophyll content did show significant changes. It is unclear whether or not this is an indication that hyperspectral reflectance is more sensitive than traditional methods. Other significant differences exhibited by woody species only occurred after treatment plants regrew leaves. These responses may have been due to treatment woody plants translocating RDX in leaves and dropping them, thus reducing the amount of available RDX in soil (Reynolds et al., 2013). With TNT still located in roots, subsequent leaf growth may have been stunted, along with changes in other physiological metrics (Ali et al., 2006). Identifying quantities of parent compounds and conjugates in plants and their individual spectral responses may be crucial to understanding how explosives uptake can modify canopy reflectance.

Cyperus esculentus was not a useful phytosensor of explosives contamination as functional traits and reflectance spectra were unaffected. *Ulmus alata* and *V. labrusca* were efficacious at indicating presence of explosives. Increased SLA was attributed to reduced leaf weight, possibly from growth inhibition caused by TNT, and the only functional trait to differ across woody species. Increased SLA was also discernible via reflectance for *U. alata* but not *V. labrusca*. Change in fluorescence was limited to *U. alata* and not in the manner expected because of how RDX attacks PSII (Bhalla, et al., 2011). *Vitis labrusca* was the only species to exhibit significant reductions in dry leaf biomass though a reduction was measured with *U. alata* reflectance. Reflectance spectra of *U. alata* and *V. labrusca*, despite converse responses, gave a

more comprehensive indication of stress from explosives contamination than did functional metrics. Overall, reflectance of woody species used was more conclusive of explosives contamination than were functional traits. The drastic reduction of significant reflectance indices from week 4 to week 9 substantiates the need for an Explosives-Specific Index (ESI). If specific wavelengths are affected by plant uptake of explosives, this revelation will make remote detection of landmines both faster and safer.

As the field of remote sensing advances, allowing for better connections between leaf-scale and canopy-scale data (Serbin et al., 2015), more in-depth analyses will be necessary in order to determine exactly how uptake and compartmentalization of xenobiotics by vegetation transforms vegetation reflectance signatures. My results and similar studies emphasize the need to strengthen an understanding of how explosives affect plants of multiple functional types so that physiological and morphological data can be related to that obtained from remote sensing techniques. Some of these findings are either an indication of species-specific responses, or of the difficulties in disentangling a complex process. I did not examine natural or other anthropogenic stressors, so I cannot definitively state that observed differences were solely due to explosives contamination. In the future therefore, it is imperative to conduct analogous experiments that focus on both natural and anthropogenic stress in more controlled environments. With landmines contaminating so much of the world, the future of this research presents a hopeful step in landmine detection and remediation.

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Appendix

Table 1. T-tests of week 4 pigment concentrations adjusted with Bonferroni correction ($\alpha = 0.05/3$). Bold numbers denote significance relative to controls ($p < 0.01667$). Positive t -values indicate control means were greater than Comp B treatment means.

	<i>C. esculentus</i>			<i>U. alata</i>			<i>V. labrusca</i>		
	Chl <i>a</i>	Chl <i>b</i>	Carotenoids	Chl <i>a</i>	Chl <i>b</i>	Carotenoids	Chl <i>a</i>	Chl <i>b</i>	Carotenoids
Control Mean	1541.47	1310.29	401.64	80.51	72.60	25.53	108.79	94.98	41.21
Control S.E.	454.04	442.24	97.28	7.58	6.86	2.36	6.49	6.66	2.91
Treatment Mean	1018.84	985.82	229.32	107.88	100.34	26.15	62.71	55.36	21.70
Treatment S.E.	471.69	451.29	128.49	39.25	30.56	6.34	8.54	6.37	4.37
t -value	0.80	0.51	1.07	-0.68	-0.89	-0.09	4.30	4.30	3.71
p -value	0.4478	0.6215	0.3162	0.5034	0.3889	0.9276	0.0004	0.0004	0.0016

Table 2. T-tests of week 9 pigment concentrations adjusted with Bonferroni correction ($\alpha = 0.05/3$). There were no statistically significant differences ($p < 0.01667$) between treatment and control plants. Positive t -values indicate control means were greater than Comp B treatment means.

	<i>C. esculentus</i>			<i>U. alata</i>			<i>V. labrusca</i>		
	Chl <i>a</i>	Chl <i>b</i>	Carotenoids	Chl <i>a</i>	Chl <i>b</i>	Carotenoids	Chl <i>a</i>	Chl <i>b</i>	Carotenoids
Control Mean	228.24	150.87	102.98	46.75	53.09	18.15	73.28	63.58	34.55
Control S.E.	36.03	20.74	29.50	3.14	4.51	1.52	11.30	9.50	5.18
Treatment Mean	279.58	174.13	110.29	109.36	46.88	31.17	94.86	68.71	28.99
Treatment S.E.	81.46	49.85	35.42	50.89	10.81	9.16	9.27	6.58	4.01
t -value	-0.58	-0.43	-0.17	-1.23	0.53	-1.40	-1.48	-0.44	-0.68
p -value	0.5802	0.6779	0.8713	0.2372	0.6038	0.1802	0.1570	0.6621	0.5065

Table 3. Week 4 canopy reflectance indices t-tests adjusted with Bonferroni correction ($\alpha = 0.05/29$). Bold numbers denote significance relative to controls ($p < 0.0017$). Positive t -values indicate control means were greater than Comp B treatment means.

Index	Related to	<i>C. esculentus</i>		<i>U. alata</i>		<i>V. labrusca</i>	
		t -value	p -value	t -value	p -value	t -value	p -value
Photochemical Reflectance Index (PRI)	<u>Carotenoids</u> Chlorophyll	-0.30	0.7682	6.03	<0.0001	7.66	<0.0001
Chlorophyll Index (CI)	Chlorophyll Content	0.61	0.5565	3.76	0.0033	11.81	<0.0001
Gitelson and Merzlyak (GM1)	Chlorophyll Content	0.47	0.6518	0.87	0.3988	3.97	0.0019
GM2	Chlorophyll Content	0.53	0.6130	3.66	0.0048	13.14	<0.0001
Vogelmann Red Edge Index (VOG1)	Chlorophyll Content	0.52	0.6212	3.24	0.0089	13.47	<0.0001
VOG2	Chlorophyll Content	-0.10	0.9251	-1.84	0.0888	-7.01	<0.0001
VOG3	Chlorophyll Content	-0.10	0.9192	-1.84	0.0891	-7.15	<0.0001
Greenness Index (GI)	Chlorophyll Content	0.39	0.7038	4.30	0.0009	6.93	<0.0001
Orange/Red Chlorophyll Absorption Index (OCAR)	Chlorophyll Content	0.68	0.5178	1.90	0.0789	2.80	0.0174
Yellow/Red Chlorophyll Absorption Index (YCAR)	Chlorophyll Content	0.33	0.7528	4.75	0.0003	6.54	<0.0001
Normalized Difference Vegetation Index (NDVI)	Biomass	0.64	0.5440	4.08	0.0010	5.26	0.0005
NDVI ₂	Biomass	0.70	0.5057	3.93	0.0013	4.97	0.0007
NDVI _{green}	Biomass	0.78	0.4633	0.06	0.9503	1.82	0.0971
Structure Insensitive Pigment Index (SIPI)	<u>Carotenoids</u> Chlorophyll	-0.17	0.8739	-4.61	0.0012	-2.44	0.0371

Curvature Index (CUR)	Chlorophyll Fluorescence ($\Delta F/F'_m$)	-0.22	0.8301	-3.24	0.0060	-5.23	<0.0001
Water Index (WI)	Water Content	1.17	0.2777	-2.41	0.0391	-4.33	0.0013
Water Band Index (WBI)	Water Status	-1.16	0.2786	2.41	0.0388	4.36	0.0012
WBI/NDVI2	<u>Water Status</u> Biomass	-0.81	0.4448	-3.18	0.0079	-2.63	0.0273
Modified Chlorophyll Absorption in Reflectance Index (MCARI)	Leaf Area Index (LAI), Chlorophyll	0.06	0.9564	0.94	0.3638	1.94	0.0743
MCARI1	LAI	0.47	0.6508	1.07	0.2999	2.22	0.0459
MCARI2	LAI	0.53	0.6127	1.94	0.0705	4.23	0.0016
Plant Senescence Reflectance Index (PSRI)	<u>Carotenoids</u> Chlorophyll	-0.08	0.9356	-3.91	0.0034	-3.26	0.0098
Anthocyanin Index (AI)	Anthocyanin Content	-0.30	0.7691	-5.08	0.0004	-4.14	0.0025
Normalized Difference Nitrogen Index (NDNI)	Nitrogen Content	0.49	0.6351	-0.91	0.3775	-0.24	0.8137
Normalized Difference Lignin Index (NDLI)	Lignin Content	0.41	0.6896	-1.23	0.2408	-1.14	0.2782
Lichtenthaler Index (LIC1)	Stress	0.66	0.5277	4.21	0.0008	5.14	0.0006
LIC2	Stress	-1.85	0.1057	3.25	0.0051	9.55	<0.0001
LIC3	Stress	-1.22	0.2588	-1.90	0.0788	-6.35	<0.0001
R ₇₅₀ /R ₇₁₀	Photosynthesis	0.57	0.5853	3.37	0.0071	13.22	<0.0001

Table 4. Week 9 canopy reflectance indices t-tests adjusted with Bonferroni correction ($\alpha = 0.05/29$). Bold numbers denote significance relative to controls ($p < 0.0017$). Positive t -values indicate control means were greater than Comp B treatment means.

Index	Related to	<i>C. esculentus</i>		<i>U. alata</i>		<i>V. labrusca</i>	
		t -value	p -value	t -value	p -value	t -value	p -value
Photochemical Reflectance Index (PRI)	<u>Carotenoids</u> Chlorophyll	-0.48	0.6471	0.19	0.8538	-0.05	0.9590
Chlorophyll Index (CI)	Chlorophyll Content	-0.21	0.8402	-1.27	0.2362	1.27	0.2236
Gitelson and Merzlyak (GM1)	Chlorophyll Content	-0.05	0.9629	-2.57	0.0231	1.26	0.1227
GM2	Chlorophyll Content	-0.15	0.8888	-1.31	0.2245	1.38	0.1872
Vogelmann Red Edge Index (VOG1)	Chlorophyll Content	-0.28	0.7866	-1.67	0.1305	1.13	0.2728
VOG2	Chlorophyll Content	-0.47	0.6584	2.55	0.0320	-1.39	0.1810
VOG3	Chlorophyll Content	-0.45	0.6691	2.52	0.0335	-1.38	0.1837
Greenness Index (GI)	Chlorophyll Content	0.40	0.7015	2.45	0.0305	2.31	0.0407
Orange/Red Chlorophyll Absorption Index (OCAR)	Chlorophyll Content	0.43	0.6827	4.70	0.0002	2.96	0.0128
Yellow/Red Chlorophyll Absorption Index (YCAR)	Chlorophyll Content	0.34	0.7433	1.25	0.2423	1.45	0.1704
Normalized Difference Vegetation Index (NDVI)	Biomass	0.02	0.9827	0.75	0.4666	2.51	0.0307
NDVI2	Biomass	-0.06	0.9573	0.48	0.6390	2.35	0.0409
NDVI _{green}	Biomass	-0.25	0.8121	-3.24	0.0056	1.46	0.1622
Structure Insensitive Pigment Index (SIPI)	<u>Carotenoids</u> Chlorophyll	0.61	0.5677	-1.52	0.1636	-1.62	0.1390

Curvature Index (CUR)	Chlorophyll Fluorescence ($\Delta F/F'_m$)	-0.65	0.5370	-2.04	0.0588	-3.57	0.0023
Water Index (WI)	Water Content	-1.40	0.2044	2.12	0.0619	-0.51	0.6170
Water Band Index (WBI)	Water Status	1.40	0.2060	-2.13	0.0615	0.50	0.6264
WBI/NDVI2	<u>Water Status</u> Biomass	0.30	0.7776	-1.02	0.3323	-2.01	0.0735
Modified Chlorophyll Absorption in Reflectance Index (MCARI)	Leaf Area Index (LAI), Chlorophyll	0.58	0.5863	3.35	0.0061	1.33	0.2043
MCARI1	LAI	0.12	0.9064	2.69	0.0185	0.25	0.8091
MCARI2	LAI	0.13	0.9043	2.26	0.0385	1.35	0.1988
Plant Senescence Reflectance Index (PSRI)	<u>Carotenoids</u> Chlorophyll	0.07	0.9490	-2.50	0.0354	-1.46	0.1763
Anthocyanin Index (AI)	Anthocyanin Content	-0.03	0.9737	-1.64	0.1369	-1.73	0.1131
Normalized Difference Nitrogen Index (NDNI)	Nitrogen Content	-0.05	0.9606	1.04	0.3192	-1.79	0.0922
Normalized Difference Lignin Index (NDLI)	Lignin Content	-0.13	0.9045	1.73	0.1067	-0.53	0.6040
Lichtenthaler Index (LIC1)	Stress	-0.03	0.9761	0.78	0.4504	2.35	0.0411
LIC2	Stress	-1.41	0.2002	-1.01	0.3373	0.42	0.6802
LIC3	Stress	-0.67	0.5328	-0.54	0.5988	-3.04	0.0093
R ₇₅₀ /R ₇₁₀	Photosynthesis	-0.08	0.9424	-1.51	0.1661	1.07	0.2977

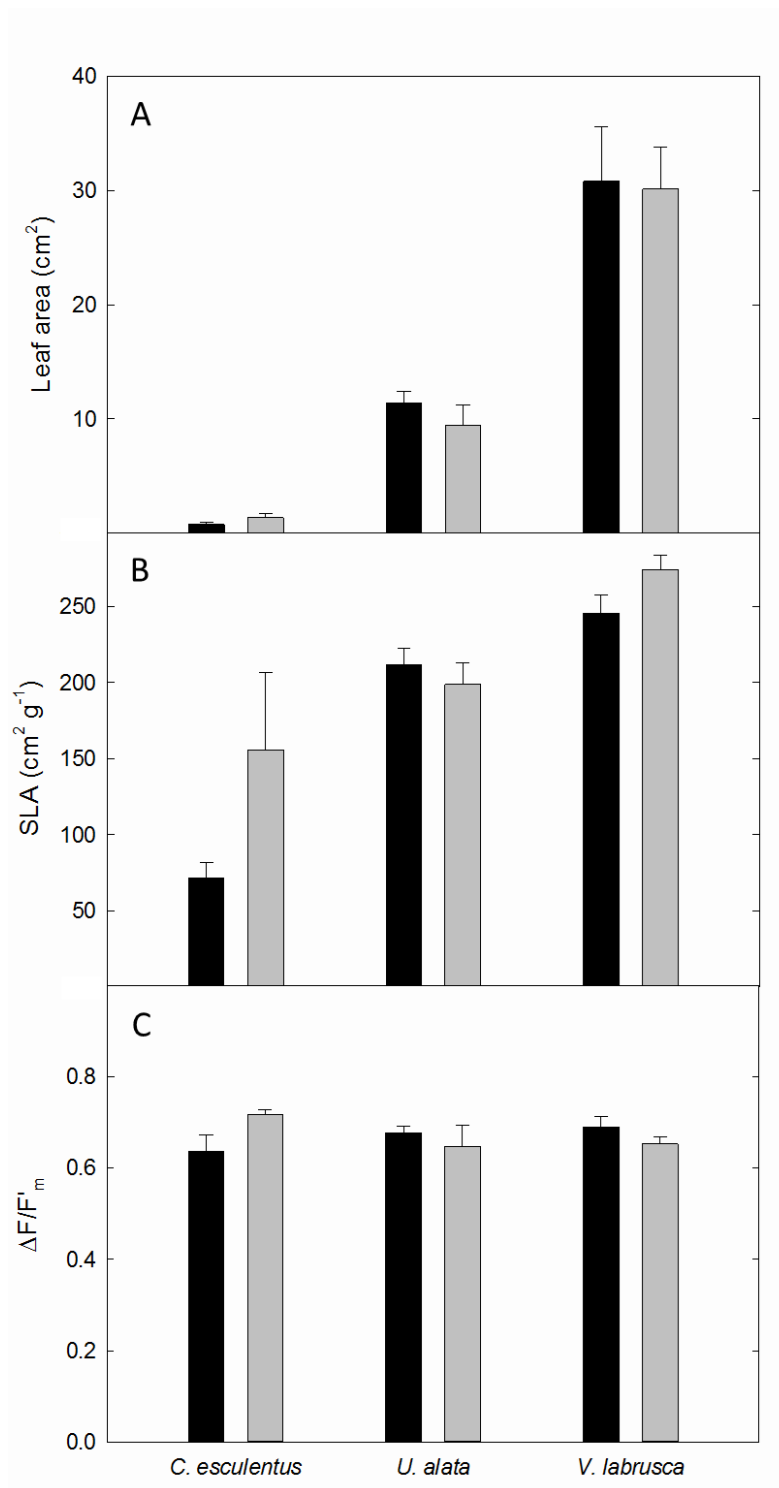


Figure 1. Week 4 leaf area (A), SLA (B) and leaf $\Delta F/F'_m$ (C) of *Cyperus esculentus*, *Ulmus alata*, and *Vitis labrusca* treated with 500 mg kg⁻¹ of Comp B. Black bars represent control plants, grey bars represent treatment plants. Means \pm 1 SE are presented. There were no statistically significant ($\alpha = 0.05$) differences between treated and control plants.

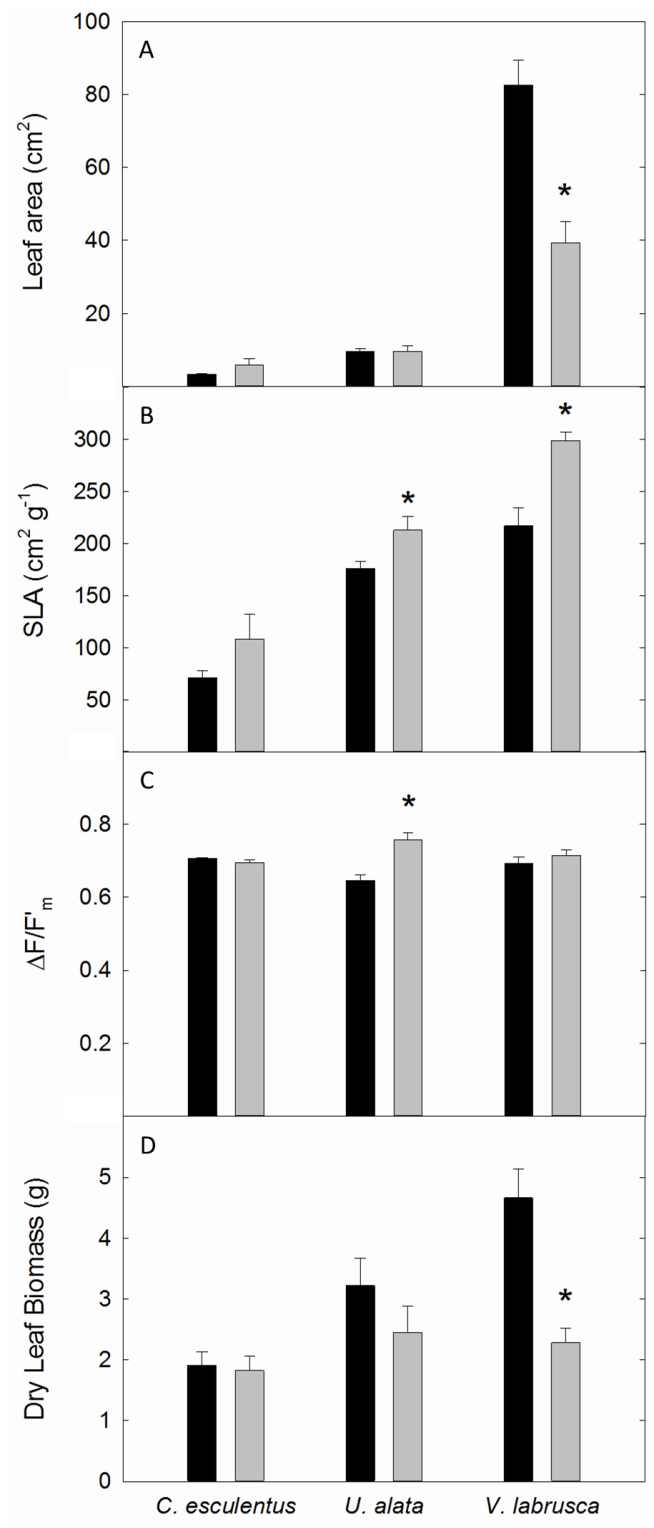


Figure 2. Week 9 leaf area (A), SLA (B), leaf $\Delta F/F'_m$ (C), and dry leaf biomass (D) of *Cyperus esculentus*, *Ulmus alata*, and *Vitis labrusca* treated with 500 mg kg⁻¹ of Comp B. Black bars represent control plants, grey bars represent treatment plants. Means \pm 1 SE are presented. * denotes significance ($\alpha = 0.05$) relative to control plants.

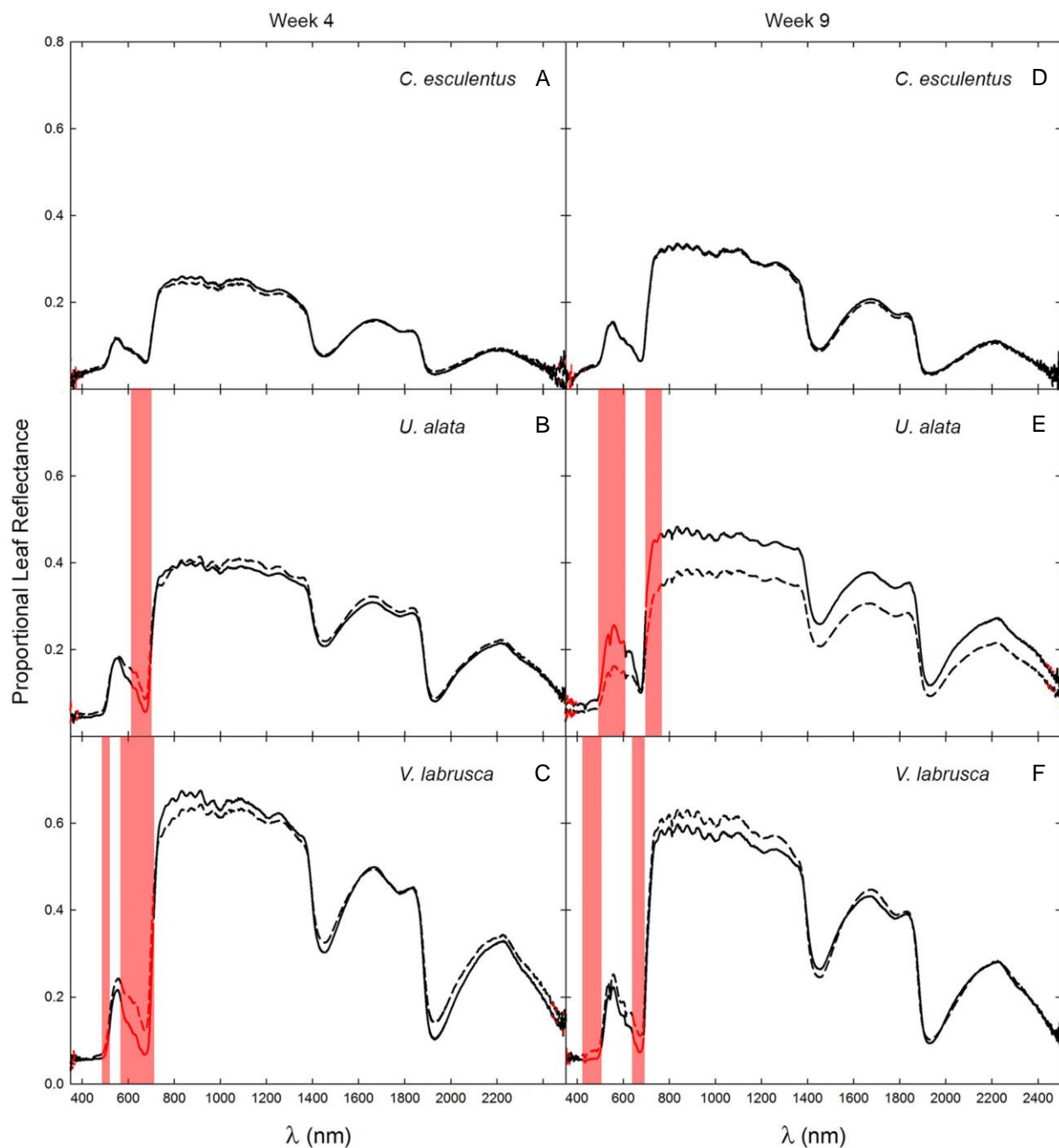


Figure 3. Average proportional canopy reflectance at week 4 and week 9 for *Cyperus esculentus* (A and D), *Ulmus alata* (B and E), and *Vitis labrusca* (C and F). Solid lines represent control plants; dashed lines represent plants treated with 500 mg kg⁻¹ of Comp B. Highlighted areas indicate statistical significance ($p < 0.05$).

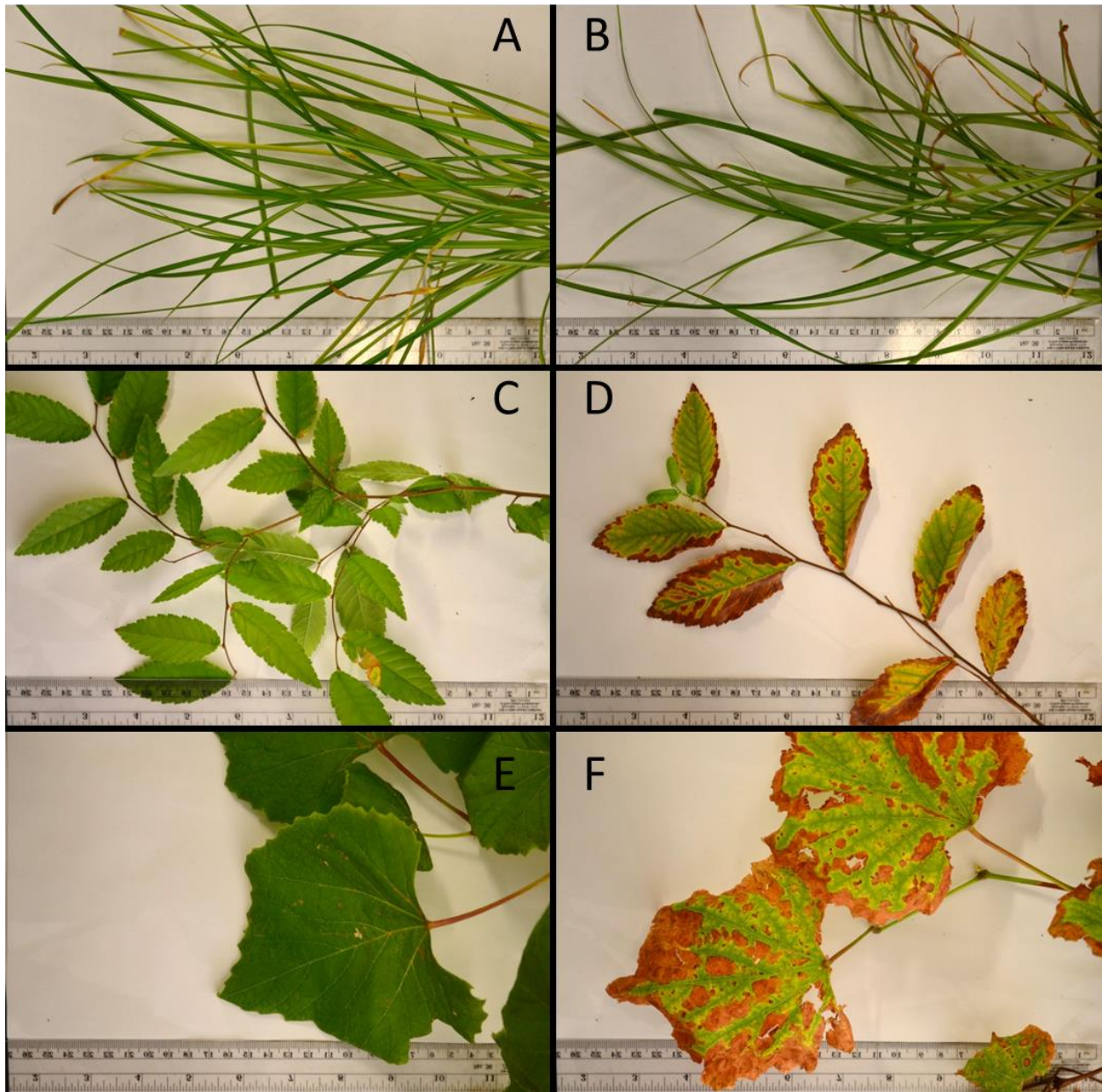


Figure 4. Examples of *Cyperus esculentus* (A and B), *Ulmus alata* (C and D), and *Vitis labrusca* (E and F) control and treatment leaves exposed to 500 mg kg⁻¹ Comp B in soil at week 4. Control leaves are on the left, treatment leaves are on the right.

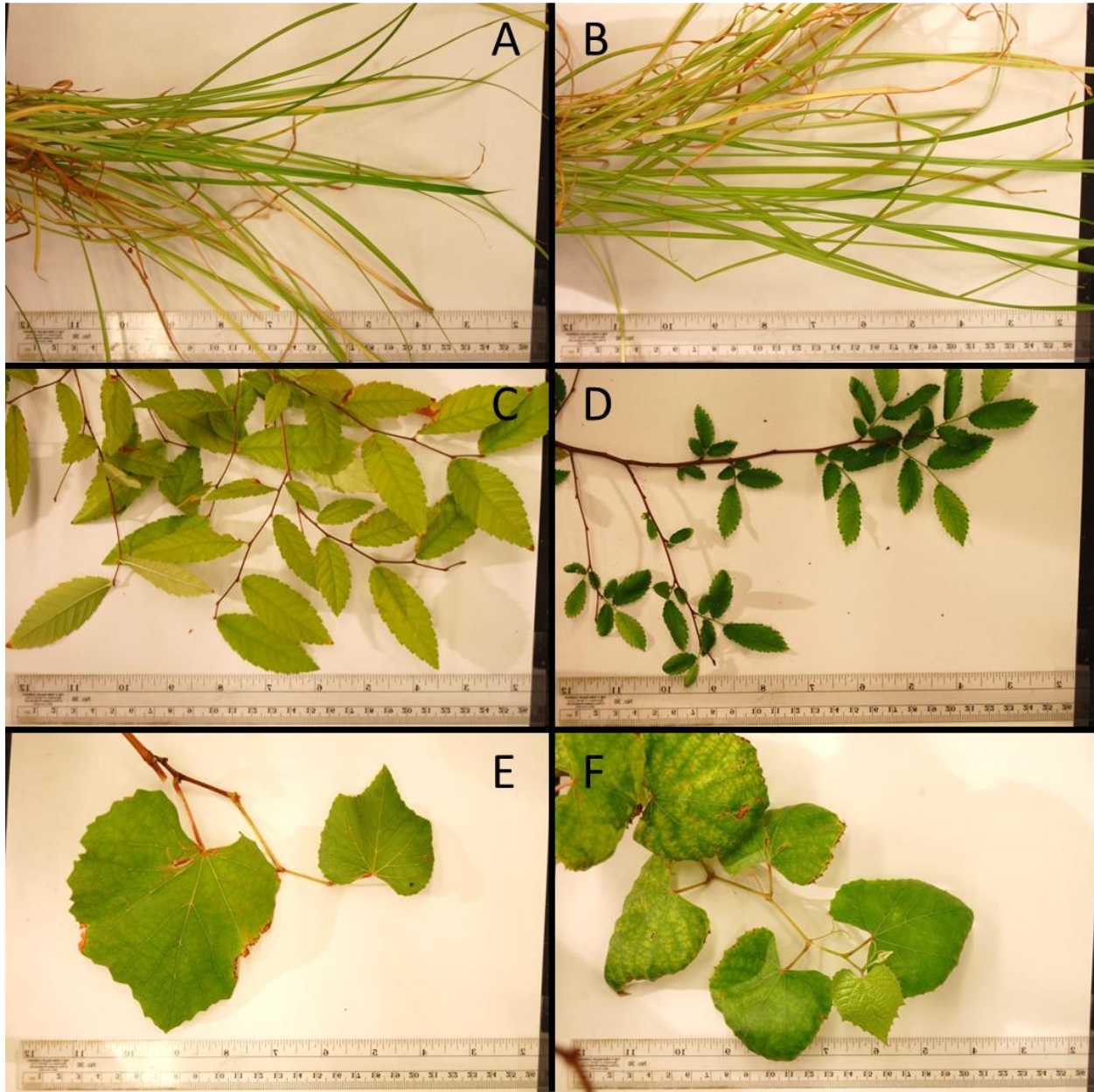


Figure 5. Examples of *Cyperus esculentus* (A and B), *Ulmus alata* (C and D), and *Vitis labrusca* (E and F) control and treatment leaves exposed to 500 mg kg⁻¹ Comp B in soil at week 9. Control leaves are on the left, treatment leaves are on the right.

Vita

Paul Vaughan Manley, II was born in Lynchburg, Virginia on 30 August, 1987. He attended high school at E.C. Glass High School in Lynchburg. He received a Bachelor of Science degree in Environmental Studies in 2010 from Virginia Commonwealth University. In 2012, he started his Master's program in biology. Initially he was going to work with another form of remote sensing called LiDAR (Light Detection and Ranging), but an opportunity arose to study remote detection of explosives using vegetation. This allowed him to connect two of his life goals: research and humanitarianism. During his Master's, he has been fortunate to travel to conferences, both national and international, to present his work and to learn of others'. He and his colleagues have also been published in various news outlets about their research on plants and landmines. Paul has recently started a Ph.D. program in Civil Engineering at the Missouri University of Science and Technology in Rolla, MO where he will continue to focus on remote detection of explosives.