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SURETY HOOD CULTURE OF PLANTS FOR AGENT–PLANT INVESTIGATIONS

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14. ABSTRACT:
We developed methods for investigating chemical warfare agent–plant interactions within a surety hood using live plants to maintain phytophysiological responses and obtain results applicable to contaminated battlefields. We selected VX [*O*-ethyl-*S*-(2-diisoproylaminoethyl) methyl phosphonothioate] and the grass plant *Echinochloa crus-galli* for the initial research. Grass is the most-prevalent higher plant worldwide, and natural distribution of *E. crus-galli* is one of the largest. Plant culture under controlled environmental conditions typically involves balancing heat loads with large chilling units too cumbersome for most surety hoods. We installed and tested a system of high-output, low-heat light-emitting diodes to supply high-quality photosynthetically active radiation (PAR). Environmental conditions within the surety hood were 300–350 µmol s^{-1} m^{-2} PAR, 16 h light/8 h dark, 21 °C, and relative humidity 50% ± 10. We successfully established critical parameters for assessing VX threat on natural environment battlefields, including visual characterization of VX effects on grass foliage, coefficient of VX wash-off from rainfall, contact transfer of VX from foliage onto army combat, and persistence and effective half-life of VX on grass foliage. These results provide critical parameter input for predictive models, direct experimental determinations for comparison of model outcomes, and information for decision-making affecting soldiers.

15. SUBJECT TERMS
Surety hood; Agent–Plant; Plant culture; Chemical warfare agent (CWA); *Echinochloa crus-galli*; Grass; Terrestrial plant; *O*-ethyl-*S*-(2-diisoproylaminoethyl) methyl phosphonothioate (VX)

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PREFACE

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SURETY HOOD CULTURE OF PLANTS FOR AGENT–PLANT INVESTIGATIONS

1. INTRODUCTION

Developing advanced defensive capabilities against chemical warfare agents (CWAs) that are disseminated in natural environments requires a greater understanding of the underlying principles involving CWA interaction with natural systems. Few experimental data existed that described interactions between CWA and terrestrial plants. Investigations from CWA field studies have provided some useful information involving agent–plant interaction; however, testing in the field has inherently uncontrolled environmental conditions (Reich, 1959a, 1959b). Variance in environmental parameters can make the data resulting from outdoor tests less useful as input for models, which predict outcomes using established scientific and empirical relationships. Laboratory investigations permit the assemblage of select controlled conditions to reduce interferences arising from the complexity of uncontrolled, open-ended natural environments, thereby allowing investigations to better focus on relationships of interest.

Methods for culturing intact living plants within a surety hood environment for extended periods of time did not exist. Such methods are necessary to maintain phytophysiological responses of plants within a surety hood to investigate and obtain results applicable to CWA-contaminated battlefields. Without a more complete understanding of agent–plant interactions, it is difficult to predict the presence and persistence of the potential exposure hazard posed by CWA-contaminated foliage. Therefore, capabilities for growing and sustaining healthy plants under controlled conditions within a chemical surety hood for extended periods of time are necessary to successfully investigate agent–plant interactions.

This report details newly developed methods and protocols for growing and maintaining healthy plants in a chemical surety hood for the conduct of controlled agent–plant interaction investigations. These plant culture methods were used to successfully establish critical parameters for assessing threats from *O*-ethyl-*S*-(2-diisoproylaminoethyl) methyl phosphonothioate (VX) on battlefields in natural environments, including the visual characterization of the effects of VX on grass foliage (Simini et al., 2016), coefficient of VX wash-off from rainfall (Haley et al., 2016), contact transfer (exposure) of VX from contaminated foliar surfaces onto an army combat uniform (Haley et al., 2017), and the persistence and effective half-life of VX on contaminated grass foliage (Checkai et al., 2017). The results of these investigations provided critical parameter input for predictive models, direct experimental determinations for comparison to model outcomes, and information for decision-making that can affect soldiers on VX-contaminated battlefields.

2. LIGHTING FOR PLANT CULTURE IN CONFINED SPACES

Light is one of the most-important environmental stimuli impacting plant growth and development (Kopsell et al., 2015). Terrestrial photoautotrophic plants require photosynthetically active radiation (PAR) within the range of 400–700 nm (McCree, 1972, 1981) for normal growth and physiology. Traditional high-intensity lighting systems, such as

high-pressure sodium, metal halide, fluorescent, and incandescent light combinations, require balancing of their heat loads to avoid scorching or damaging plants (Bula et al., 1991; Massa et al., 2008). However, heat-load balance for such plant growth systems is commonly accomplished using large chilling units, which are too cumbersome for use with most surety hoods (Bula et al., 1991).

2.1 Semiconductor Diodes as a Source of Radiation

The first commercially functional light-emitting diode (LED) was developed in the 1960s by combining three primary elements (Ga, As, and P) into a GaAsP diode to obtain a low luminous intensity red light source, and a comparable low luminous intensity green LED was commercially available by the late 1960s (Ehrenstein, 2014). These early LEDs were very low power and were usable only as low-intensity indicator lamps (Bourget, 2008). However, with their low heat output, they were especially useful in heat-sensitive electronic equipment.

In the United States, testing of LEDs as a source of radiation for the culture of higher terrestrial plants began primarily because of interest by the National Aeronautics and Space Administration in space-based plant-growth systems (Morrow, 2008). Early work with LEDs that focused on plant production as a source of food was undertaken to support research involving regenerative life-support systems for a space station and future Moon and Mars bases. As the performance of commercially available red LEDs improved, the intensity of red LED output became adequate for growing plants, especially if blue light was also provided using traditional sources of lighting (Morrow, 2008; Massa et al., 2008). However, the addition or supplementation by traditional means of supplying blue light sufficient for growing and maintaining healthy plants brought with it associated heat load (Bula et al., 1991). Even the U.S. Environmental Protection Agency (USEPA) plant test guidelines warned that care should be taken to ensure that plants are not affected by heat generated from supplemental lighting during ecological effects plant tests (USEPA, 2012).

2.2 Importance of Blue Light and Development of High-Intensity Blue LEDs

Light provided by improved-output red LEDs was found to be sufficient to initiate the growth of plants because a primary role of red light absorption is regulation of plant development (Massa et al., 2008). However, absorption of sufficient quality and quantity of blue light was needed to control primary and secondary metabolism, stomatal regulation, plant morphology, gene expression, and transition to flowering, which is the fundamental basis for fruiting and reproduction (Kopsell et al., 2015; Folta and Childers, 2008; Morrow, 2008). For photoautotrophic plants, incident PAR is a major environmental factor for controlling plant growth (Bula et al., 1991). In green plants, light is the energy source for conversion of carbon dioxide and water into complex organic compounds that are used in essential cellular functions, such as biosynthesis and respiration. Substantial flux of incident blue light is required to sustain normal, healthy photoautotrophic terrestrial plants (Hoenecke et al., 1992).

Before the 1990s, the intensity of light output from commercially available blue LEDs was barely visible (Ehrenstein, 2014). High-intensity output from commercially available blue LEDs was highly sought after but difficult to produce (Diep, 2014). Finally in the 1990s,

three researchers (Isamu Akasaki, Hiroshi Amano, and Shuji Nakamura), working in two different and independent research groups, produced bright blue light from LEDs that they had created in their respective laboratories. The first practical blue LED was developed in 1993 (Nakamura et al., 1996). Further development of LED technology led to the modern, commercially available, high-intensity blue LEDs in 1999 (Bourget, 2008). For their combined research breakthroughs that led to the initial creation of a functional high-intensity blue light LED using high-quality gallium nitride, the Nobel Prize in Physics (2014) was awarded to all three researchers.

Modern high-intensity LEDs now last up to 100,000 h compared with 10,000 h for fluorescent lights and 1,000 h for incandescent bulbs. Modern LEDs convert more than 50% of input electricity into light, compared with approximately 4% for incandescent lighting (Nobel Prize in Physics, 2014). Within the decade that followed the creation of bright blue LED, improved modern high-intensity LEDs have been incorporated into numerous products (Diep, 2014). However, by 2008, most commercially available LED lighting systems still did not provide light intensities that were sufficient for the successful growth and extended sustainment of healthy plants (Massa, 2008). Many plant lighting systems that are insufficient for extended sustainment of healthy plants remain on the commercial marketplace. It is notable that even white light LED-based plant growth systems are inefficient with regard to heat output, as compared with LED arrays that permit adjustment of the output-intensity for each type of LED within the array.

We recognized that some LED plant lighting systems began incorporating improved, modern, high-intensity LEDs into adjustable lighting arrays for the growth of plants (Morrow, 2008) and that this innovation was a key component for growing and sustaining healthy plants within a laboratory hood under surety constraints. Therefore, we began investigating commercially available LED array lighting systems.

3. METHODS AND MATERIALS

3.1 Lighting System Selected for the Culture of Plants within a Surety Hood

Because physiologically healthy living plants are necessary to investigate and record critical parameters for the effects of agent–plant interaction, we selected, installed, and tested a LumiBar (LumiGrow; Novato, CA) strip-lighting, high-intensity LED array system within a surety hood. The LED array consisted of the improved, modern, high-intensity output red, blue, and green LEDs to supply high-quality PAR. The PAR spectral outputs of modern high-intensity red and blue LEDs are required to supply radiation for absorption by plants that is sufficient for culturing normal, healthy, photoautotrophic plants. Green light is predominantly reflected by photoautotrophic plants, causing their green appearance. However, inclusion of green LEDs within an array allows inspection of plants under white light (red + blue + green; Figure 1), so that the effects of plant treatments may be recognized and characterized (Kim et al., 2004; Simini et al., 2016).

The LumiBar strip-lighting system includes a power-control module that permits external adjustment of the output-intensity for each type of LED within the array, thereby permitting exclusion of green LED output during plant culture periods (Figure 2).

Figure 1. Modern, high-intensity blue, red, and green LEDs (close-up photo).

Figure 2. Surety hood illuminated by an array of high-intensity output blue and red LEDs during the culture of grass plants (*Echinochloa crus-galli*).

3.2 Chemicals

The VX selected for method development and initial agent–plant interaction research was Chemical Agent Standard Analytical Reference Material (CASARM)-grade (U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD), Chemical Abstracts Service (CAS) no. 50782-69-9. VX purity was determined and verified by nuclear magnetic resonance spectroscopy. The VX was stabilized with 5% by weight diisopropylcarbodiimide (CAS no. 693-13-0; Sigma-Aldrich Company; St. Louis, MO). The water used in these investigations was ASTM Type I (ASTM, 2004) that was subsequently allowed to naturally equilibrate with air at ambient conditions. Miracle-Gro Water Soluble All Purpose Plant Food (Scotts Miracle-Gro Company; Marysville, OH) fertilizer (24% total nitrogen [calculated as N], 8% available phosphate [calculated as P_2O_5], 16% soluble potash [calculated as K_2O], 0.02% boron, 0.07% copper [water soluble], 0.15% iron [chelated], 0.05% manganese [chelated], 0.0005% molybdenum, 0.06% zinc [water soluble], and 1.14% ethylenediaminetetraacetic acid chelating agent) was used to prepare the dilute aqueous phytonutrient solution (530 mg/L). All other solvents and reagents were analytical grade or purer, and these were obtained from Sigma-Aldrich Company.

3.3 Plant Species

The plant species selected for method development and initial research was the grass plant *E. crus-galli* [L.] P. Beauv. (Figure 3). Grass is the most-prevalent type of higher plant worldwide and *E. crus-galli* is one of the most-ubiquitous grasses globally (CABI, 2017). *E. crus-galli* is tolerant of dry and wet natural habitats and is used as forage for grazing animals as well as for wildlife food and habitat (USDA–NRCS, 2015). This species is also one of the most-important weed species in crop systems globally; however, *E. crus-galli* is cultivated as a crop for human consumption in some parts of the world (CABI, 2017).

3.4 Plant Culture before and within Surety Hood Constraints

Within an environment-controlled plant-growth chamber (model PGC-9/2; Percival Scientific; Perry, IA), 20 *E. crus-galli* grass seeds (lot no. PM11452Q, 2014; Prairie View Nursery; Winona, MN) per 100 mm (4 in.) diameter pot were germinated by hydration with ASTM Type I water in potting mix (Miracle Gro Moisture Control potting mix; Scotts). Before use, each pot was prepared by lining the bottom of each with two pieces of absorbent paper and then filling with 170 g (77.1 g dry mass) of potting mix.

After 7 days post-germination, each individual grass plant seedling was transplanted into a new prepared pot and grown to mature leaf stage within a plant-growth chamber (Simini et al., 2016). To avoid the effects of nutrient deficiencies that could have existed in the soil, dilute phytonutrient solution was administered to the plants every 2 to 3 days to maintain the respective initial mass for moisture of each replicate plant system and to sustain healthy plants.

Figure 3. *E. crus-galli* grass plants in native habitat.

The following environmental conditions were maintained within the environment-controlled plant-growth chamber:

- 300–350 µmol s^{-1} m^{-2} PAR (at plant canopy),
- 22 ± 2 ºC (daytime),
- 18 ± 2 ºC (nighttime),
- 60 ± 5% relative humidity (RH), and
- 0.56 km/h (0.35 mph) airflow.

Temperature and RH were measured using an OM-DVTH data logger (Omega Engineering, Inc.; Stamford, CT). PAR, delivered by fluorescent and incandescent lighting, was measured periodically using an MQ-200 quantum meter equipped with an AM-310 sensor wand (Apogee Instruments; Logan, UT). Airflow was calculated by the manufacturer's technical personnel in accordance with the plant-growth chamber specifications (Percival Sci., 2017).

When the individual grass plants had developed two to three fully mature leaves (Figure 4), 18–21 days after transplanting, the individual seedlings within their pots were transferred into the plant culture conditions maintained within a surety hood. The selection of an individual plant and the pot position within the surety hood were randomized for agent–plant interaction experimental determinations involving VX on grass foliage. A sampling of *E. crus-galli* mature grass foliage was taken from additional plants that did not receive VX to determine the moisture status of the grass (water content) by mass (weight).

The following environmental conditions were maintained within the surety hood:

- 300–350 µmol s^{-1} m^{-2} PAR (at plant canopy),
- 21 ± 2 ºC (daytime),
- 21 ± 2 ºC (nighttime),
- 50 ± 10% RH, and
- 2.41 ± 0.14 km/h (1.5 ± 0.09 mph) airflow.

Temperature and RH were measured using an OM-DVTH data logger. PAR, delivered by a LumiBar strip lighting system, was measured periodically using an MQ-200 quantum meter equipped with an AM-310 sensor wand (Apogee Instruments, Inc.; Logan, UT). Airflow was measured at the face of the surety hood using an Airdata Multimeter ADM-870C (Shortridge Instruments, Inc.; Scottsdale, AZ).

Figure 4. *E. crus-galli* grass plants with two to three fully mature leaves within the environment-controlled plant-growth chamber before transfer into surety hood.

3.5 Dissemination of Agent Droplets onto Leaves

Plant stands were constructed to hold the pots and plants in fixed positions within the surety hood. A hole was cut in each Petri dish cover, and each pot was placed through the hole and onto the dish (Figure 5). Each pot was secured to a ring stand with an adjustable clamp. Grass leaves near the top of the plant canopy were randomly selected and laid horizontally across a ring. The grass leaves were secured to the rings by lengths of clear cellulose acetate tape that were folded in half lengthwise and placed across the leaf surface. This arrangement prevented sticky contact of acrylate adhesive with leaf surface. The ends of the folded tape were then secured to the ring with additional tape, which helped maintain a slight pressure on the leaf surface (Figure 6).

A droplet of agent was disseminated onto the surface of individual horizontally stabilized leaves, while other random and horizontally stabilized leaves served as controls (Figure 6). The stabilized leaves remained secured in this horizontal position during and after dissemination of VX. Single droplets of VX, a droplet size expected from CWA dissemination under field conditions (DPG, 2011), were individually dispensed using a calibrated 10 µL Hamilton (Reno, NV) gas-tight syringe onto single living leaves attached to a healthy grass plant.

Figure 5. *E. crus-galli* grass plants in surety hood with plant stands.

Figure 6. Close-up view of *E. crus-galli* leaves secured in horizontal position using tape folded in half lengthwise, which prevented leaf contact with adhesive. (VX was then disseminated onto the portion of the grass leaf held within the ring.)

4. RESULTS

Environmental conditions within the surety hood were sustained at levels similar to those maintained within the plant-growth chamber, and both sets of conditions were suitable for the culture of healthy plants (Table).

Table. Comparison of Environmental Conditions and Respective Durations within the Environment-Controlled Plant-Growth Chamber with Conditions Sustained within the Surety Hood Environment.

Environmental Factor	Daily Duration (h)	Plant-Growth Chamber	Surety Hood
Daytime PAR at canopy	16	300–350 μmol s^{-1} m^{-2}	300–350 μmol s^{-1} m^{-2}
Daytime Temperature	16	22 ± 2 ºC	21 ± 2 ºC
Nighttime Temperature	8	18 ± 2 ºC	21 ± 2 ºC
RH	24	60 ± 5%	50 ± 10%
Airflow Rate and Direction	24	0.56 km/h (Percival Sci., 2017) upward	2.41 ± 0.14 km/h horizontal

The horizontal stabilization of individual leaves provided the following technical advantages:

- no acrylate adhesive from clear tape came into contact with leaf surfaces,
- no leaf surface damage was caused by tape removal,
- the disseminated agent droplets came into contact with the leaf surfaces at the intended points, and
- the agent–leaf contact locations were easily identified for further investigation.

The methods of leaf stabilization and VX dissemination prevented droplets from merging on the foliage, which enabled analytical determination and characterization of the effects of VX.

All of the grass plants that did not receive agent droplets remained healthy and thrived, including the horizontally stabilized control leaves. The effects of the agent–plant interactions of disseminated VX with grass foliage were readily observable (Simini et al., 2016; Checkai et al., 2017). The percent moisture content of the grass leaves before VX dissemination was determined to be 88% water (88.1 ± 1.1%), based on the sampling of mature grass foliage from plants that did not receive VX. This moisture content comports with the available moisture commonly found in leaves of healthy *E. crus-galli* plants (Hamim et al., 2016).

The array of modern blue and red LEDs emitted high-intensity PAR within the range 400–700 nm, which supported the culture of healthy plants. The minimal heat load of the array of LEDs was readily handled by surety hood airflow. The LED array was adjustable by height above the plant canopy, and the luminous intensities for blue, red, and green wavelengths were individually adjustable. This not only permitted exclusion of green LED output during plant culture periods but allowed inclusion of green LED output for inspection of plants under normal

white light conditions. Plant illumination and intensity met USEPA (2012) quantity and quality specifications for ecological effects plant tests, including 350 ± 50 μmol s^{-1} m^{-2} PAR (measured at the top of the canopy in the wavelength range 400–700 nm), with a photoperiod of 16 h of light and 8 h of darkness.

The successful culture of plants within the surety hood environment permitted the investigation and determination of the following critical parameters for assessing threat from VX hazard on battlefields in natural environments:

- visual characterization of the effects of VX on grass foliage (Simini et al., 2016),
- coefficient of VX wash-off from rainfall (Haley et al., 2016),
- contact transfer (exposure) of VX from contaminated foliar surfaces onto an army combat uniform (Haley et al., 2017), and
- persistent and effective half-life of VX on grass foliage (Checkai et al., 2017).

The results of these investigations for VX on grass foliage were based on the successful culture of healthy plants within a surety hood environment, and coincide with the general trends that were observed historically (Reich, 1959a; 1959b), when effects on those studies from uncontrolled outdoor rainfalls (Haley, 2016; 2017) and temperature (Tevault et al., 2012) are taken into account. This outcome illustrates the usefulness of the present methods for investigating agent–plant interactions with additional classes of CWAs.

Extending the culture of healthy mature plants within a laboratory surety hood, rather than using costly walk-in exposure chambers, allows expenses to be minimized for investigations of agent–plant interactions and provides the necessary safety protection and precautions that are delivered by a surety hood environment.

Using the conditions established in these investigations, the culture of healthy plants within a surety hood for investigation of agent–plant interactions proved to be extremely successful. The only limiting factor may be the ultimate height of the plant species selected for investigation because of the height limit imposed by the dimensions of the surety hood.

Investigations of agent–plant interactions within a laboratory surety hood provide critical parameter input for predictive models, direct experimental determinations for comparison of model outcomes, and information for decision-making that affects soldiers on VX-contaminated battlefields.

5. CONCLUSIONS

Based on the results of these investigations of agent–plant interactions, we concluded the following:

- The environmental conditions maintained in the surety hood were suitable for the extended culture of healthy plants.

- The methods for horizontal stabilization of individual leaves and VX dissemination allowed the investigation to focus on agent–plant interactions on healthy living plants.

- The array of modern blue and red LEDs emitted high-intensity PAR that supported the culture of healthy plants. Plant illumination and intensity met USEPA (2012) quantity and quality specifications for ecological effects plant testing.

- The successful culture of plants within the surety hood permitted the investigation and determination of critical parameters for assessing the threat from VX hazards on battlefields in natural environments.

- The methods that we developed illustrated their usefulness for investigating agent–plant interactions for additional classes of CWAs.

- Extending the culture of healthy mature plants within a laboratory surety hood, rather than using costly walk-in exposure chambers, allowed expenses to be minimized for investigating agent–plant interactions.

- Investigations of agent–plant interactions within a surety hood provide critical parameter input for predictive models, direct experimental determinations for comparison of model outcomes, and information for decision-making that affects soldiers on VX-contaminated battlefields.

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ACRONYMS AND ABBREVIATIONS

CAS	Chemical Abstracts Service
CASARM	Chemical Agent Standard Analytical Reference Material
CWA	chemical warfare agent
LED	light emitting diode
PAR	photosynthetically active radiation
RH	relative humidity
USEPA	United States Environmental Protection Agency
VX	*O*-ethyl-S-(2-diisoproylaminoethyl) methyl phosphonothiolate

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