Research

Decontamination of *Ceratocystis* Pathogens Responsible for Rapid ʻŌhiʻa Death

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Abstract

Rapid ʻōhiʻa death (ROD) is caused by two recently described species of *Ceratocystis*, *C. lukuohia* and *C. huliohia*. These fungi are decimating ʻōhiʻa lehua (*Metrosideros polymorpha*), the keystone native tree species of Hawai'i. Viable *Ceratocystis* propagules can persist in ambrosia beetle frass (Coleoptera: Scolytinae), and movement of the frass may play a key role in the spread of the disease. In order to prevent the spread of ROD, we developed effective and practical surface (e.g., tools and shoes) decontamination methods to be used by researchers, managers, and the public alike. We first tested different household and laboratory disinfectants on the *Ceratocystis* fungi in culture, and then we applied the effective culture disinfectants to contaminated ambrosia beetle frass. Laboratory-grade ethanol (70, 80, and 95%), Clorox bleach (10%, 0.825% active ingredient [a.i.]), and isopropanol (70 and 91%), were all equally effective at decontaminating cultured *C. lukuohia* and *C. huliohia.* Although all concentrations of isopropanol (50, 70, and 90%) and ethanol (50, 70, and 90%) were effective disinfectants of *Ceratocystis*-contaminated frass, treatments of frass with up to 20% Clorox bleach (1.2% a.i.) were not completely adequate at killing the fungus. These data reveal that bleach is not a sufficient ROD disinfectant when frass is present, and isopropanol or ethanol are the more reliable options.

Keywords: decontamination, rapid ohia death, *Ceratocystis*, frass, Xyleborini

ʻŌhiʻa lehua (Myrtaceae: *Metrosideros polymorpha* Gaudich) is the keystone tree species of Hawai'i (Mueller-Dombois et al. 2013), accounting for more than 80% of native forest biomass (Loope et al. 2016). The tree is the dominant canopy species in the wet forest and is found across multiple ecological and altitudinal zones from sea level to 2,800 m (Mueller-Dombois and Fosberg 1998). ʻŌhiʻa are also important to the Hawaiian culture, which is evident in the many traditional uses and countless texts mentioning the trees and blossoms (Abbott 1992; Emerson 2005; Mueller-Dombois et al. 2013).

Rapid browning of ʻōhiʻa leaves followed by tree mortality was first noticed in the Puna District of Hawai'i Island in 2010. This phenomenon, referred to as rapid ʻōhiʻa death (ROD), has since spread to nearly all of the forested Hawaiian Islands, killing at least one million ʻōhiʻa trees (ROD Working Group, written communication 2020). Two novel *Ceratocystis* species are the causal agents of ROD, *C. lukuohia* I. Barnes, T.C. Harrin. & L.M. Keith and *C. huliohia* I. Barnes, T.C. Harrin. & L.M. Keith (Barnes et al. 2018; Keith et al. 2015); *C. lukuohia* is the more virulent and common of the two fungi (Fortini et al. 2019; Hughes et al. 2020). Due to both high mortality rates of infected trees and their important ecological and cultural value, ROD is now considered to be as potentially devastating to native Hawaiian ecosystems as chestnut blight and Dutch elm disease in the continental United States (Mortenson et al. 2016).

Ceratocystis fungi generally infect trees by entering through a wound, and the inoculum can be transported by insects, tools and equipment, and/or ambrosia beetle frass (fine particles of macerated wood or boring dust, beetle parts, and feces) (Harrington 2013). During gallery excavation, ambrosia beetles may come into contact with the *Ceratocystis* fungi and expel contaminated frass into the environment (Roy et al. 2019). *Ceratocystis*-contaminated frass can

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be transported on surfaces, in the soil, by wind, and/or by water (Harrington 2013). In Hawai'i, Xyleborini (Coleoptera: Scolytinae) frass containing viable fungal propagules is hypothesized to play a key role in the spread of ROD (Roy et al. 2019), a transmission pathway similar to that of *C. fimbriata* and *C. platani* (Harrington 2013). Decontamination of ROD-causing propagules embedded in frass may be difficult due to the thick-walled, environmentally resistant, long-lived aleurioconidia found in frass (Harrington 2013). Therefore, effective decontamination practices for *Ceratocystis*-contaminated ambrosia beetle frass may be crucial to prevent the further spread of ROD.

Our objective was to develop effective and practical methods for decontaminating surfaces (e.g., tools and shoes) that may harbor *C. lukuohia* and *C. huliohia* propagules. First, we aimed to develop a decontamination procedure for *Ceratocystis* conidia grown in culture using varying exposure times and concentrations of laboratory-grade ethanol and common household disinfectants including Simple Green, Lysol concentrate, hydrogen peroxide, Clorox bleach, and isopropanol. Based on results from the conidia trials, we then tested the effective products on *Ceratocystis*-contaminated frass, a condition that is likely to be encountered in the field, where removal of debris may be difficult.

Ceratocystis Culture Decontamination Trials

Suspensions of *C. lukuohia* isolate P14-1-1 and *C. huliohia* isolate P15-59 from the USDA-ARS Daniel K. Inouye U.S. Pacific Basin Agricultural Research Center collection in Hilo, Hawai'i, were made from 7-day-old cultures grown at 24°C under 24-h continuous fluorescent lighting. Three milliliters of sterile distilled water were added to each of three inoculum plates for both *Ceratocystis* spp., and the plates were gently scraped with a sterile rubber policeman to dislodge the spores. Using a hemocytometer, the concentration of the inoculum suspension was adjusted to 1×10^5 conidia/ml. Nine milliliters of each disinfectant (Simple Green [concentrate: heavy duty rate, 1:1; general purpose rate, 1:10; and light purpose rate, 1:30], Lysol concentrate [4 oz/gal], hydrogen peroxide [3%], Clorox germicidal bleach [8.25% sodium hypochlorite active ingredient diluted to 10%, therefore 0.825% sodium hypochlorite solution], isopropanol [70 and 91%], and laboratory-grade ethanol [70, 80, and 95%]) or sterile distilled water (control) was placed into a 15-ml Falcon tube. To each disinfectant, 1 ml of inoculum suspension was added for a final concentration of 1×10^4 conidia/ml. At each time interval (30, 60, 90, and 120 s), a 10-µl drop of inoculum and disinfectant suspension was plated onto three small plastic Petri dishes filled with 10% V8 agar per treatment. The droplet was spread evenly over the agar surface with a sterile cotton swab. All plates remained uncovered in a laminar flow hood until the surface was dry, at which time the plates were covered, sealed with Parafilm M, placed into a clean plastic bag, and incubated at 24°C for 7 days. After 7 days, each plate was checked for fungal growth. The experiment was repeated once.

Growth of *C. lukuohia* and *C. huliohia* occurred on all plates for 30, 60, 90, and 120 s exposure to sterile distilled water, Simple Green, and hydrogen peroxide. By contrast, no growth was observed on plates for 30, 60, 90, and 120 s exposure to Lysol (4 oz/gal), Clorox germicidal bleach (10%), isopropanol (70 and 91%), and laboratory-grade ethanol (70, 80, and 95%).

Frass decontamination trials. Fresh Xyleborini frass was collected from dying 'ōhi'a trees within 6 months of natural and artificial infection with *C. lukuohia*, the more virulent and common of the two ROD pathogens (Fig. 1A). Frass was collected from Waiākea Forest Reserve (UTM Z5 279259E, 2170951N), Keaukaha Military Reservation (UTM Z5 286601E, 2180379N), and 'Ōla'a Forest Reserve (UTM Z5 267835E, 2151476N) on Hawai'i Island. Frass was pooled, thoroughly mixed, divided into ~50 mg aliquots in sterile Petri dishes for treatment, and used within 1 week of collection.

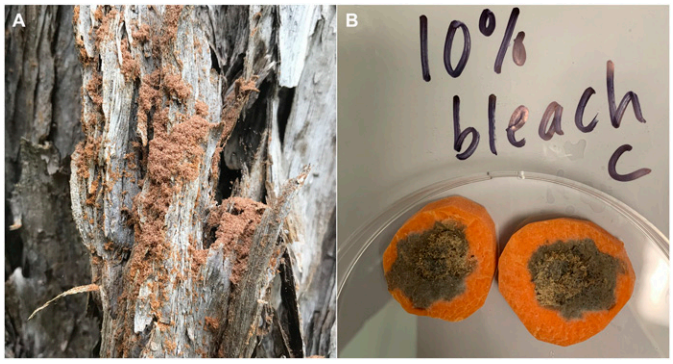

FIGURE 1

Ambrosia beetle frass **A,** collecting on the surface of 'ōhi'a bark and **B,** displaying *Ceratocystis lukuohia* growth using the carrot-baiting technique 7 days after treatment with 10% Clorox regular bleach.

Solutions of Clorox regular bleach (6% sodium hypochlorite active ingredient diluted to 5, 10, and 20%, therefore 0.3, 0.6, and 1.2% sodium hypochlorite solutions), isopropanol (99% diluted to 50, 70, and 90%), and laboratory-grade ethanol (100% diluted to 50, 70, and 90%) were freshly prepared with sterile water. Each disinfectant trial was paired with an equal number of sterile water controls. Bleach experiments were repeated six times with replicates of four to 12, and alcohol experiments were repeated four times with replicates of four. We decided against further tests using Lysol concentrate due to community feedback regarding public-user confusion caused by the term "Lysol" and lack of clear understanding of Lysol concentrate.

For each sample, 1 ml of the disinfectant solution was directly pipetted onto frass in Petri dishes, thoroughly mixed with a metal spatula, covered with the Petri dish lid, and incubated at room temperature for 1 h. After incubation, excess liquid was absorbed with Kimwipes, 1 ml of sterile water was pipetted onto the frass to rinse, and the liquid was absorbed again using a clean Kimwipe. Treated frass was tested for fungal viability using a carrot-baiting technique, in which frass was placed between two ~0.5-cm sterilized carrot discs and monitored for perithecial growth every 7 days up to 4 weeks (Moller and DeVay 1968). A subset of morphologically positive *Ceratocystis* cultures was confirmed by DNA analysis using methods described in Heller and Keith (2018).

To replicate in-field decontamination using household disinfectants, a final experiment applying isopropanol (99% diluted to 50, 70, and 90%) to frass was tested according to the above methods with a modified incubation period of 30 s. This test was repeated, and all data were summarized in R version 4.0 (R Core Team 2020).

Viable *C. lukuohia* was recovered from frass treated with all tested concentrations of Clorox regular bleach for 1 h, although the frequency at which viable *C. lukuohia* was recovered decreased with increasing bleach concentration (Figs. 1B and 2A). In contrast, all concentrations of ethanol and isopropanol were 100% effective at preventing *C. lukuohia* growth on carrots (Fig. 2B and C). After 30 s of disinfection with isopropanol, frass no longer contained viable *Ceratocystis* propagules (Fig. 2D). All tested cultures were positive by molecular diagnostics for *C. lukuohia* DNA.

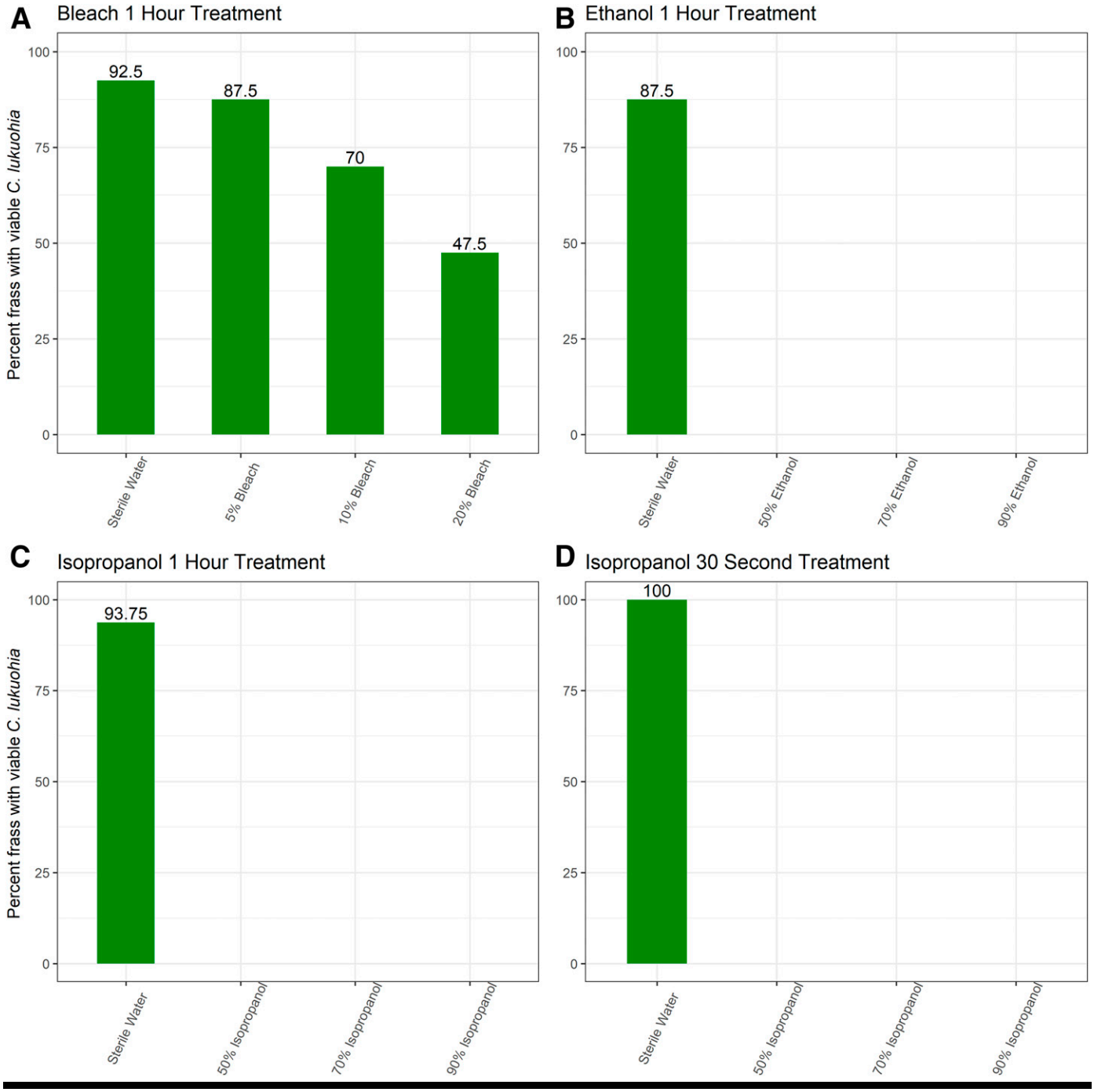

FIGURE 2

Percentage of viable carrot baits recovered from frass contaminated with *Ceratocystis lukuohia* after treatments with sterile water controls and **A,** Clorox regular bleach for 1 h; **B,** ethanol for 1 h; **C,** isopropanol for 1 h; and **D,** isopropanol for 30 s.

Discussion and Conclusions

Although Lysol concentrate, 10% Clorox germicidal bleach (0.825% sodium hypochlorite), isopropanol (70 and 91%), and laboratory-grade ethanol (70, 80, and 95%) killed pure conidia of *C. lukuohia* and *C. huliohia* in as little as 15 s, only isopropanol and ethanol (50, 70, and 90%) effectively decontaminated spores embedded in frass in all trials (Fig. 2B and C). The 20% Clorox regular bleach (1.2% sodium hypochlorite) was effective at preventing the growth of *C. lukuohia* in frass only 52.5% of the time (Fig. 2A).

Similar to our study, Andrews (1996) found that 70% ethanol is a more effective surface decontaminant than bleach solution, and Heninger et al. (2009) found that bleach requires extended exposure times for complete spore eradication. In our study, we found that even 1 h of contact time was not effective at killing *C. lukuohia* propagules embedded in frass, a scenario that is unlikely to occur in the field. Ryan et al. (2014) found that the presence of woody materials can influence the effectiveness of disinfectants, and Raugh and Taylor (1978) found that bleach can be rendered ineffective in the presence of organic materials, possibly explaining

why concentrated bleach solutions in our study were not effective disinfectants of contaminated frass.

Globally, decontamination recommendations for plant pathogens are similar to those tested in this study. For example, recommendations for *Phytophthora* spp. that cause sudden oak death in California emphasize the importance of removing all soil or plant debris from surfaces before using chemical decontamination methods (California Oak Mortality Task Force 2020). After thorough removal of debris, Lysol spray, 70% or greater alcohol, and 10% bleach are all recommended sudden oak death disinfectants (California Oak Mortality Task Force 2020). In efforts to contain the spread of *Ceratocystis platani* in Europe, 4% sodium hypochlorite and 50% denatured ethanol are recommended for disinfecting tools, and 2% sodium hypochlorite is recommended for disinfecting sawdust produced during tree felling (Panconesi 1999). Although 2 and 4% sodium hypochlorite may be effective disinfectant treatments for woody material containing *Ceratocystis*, these concentrations of sodium hypochlorite are respectively 3.3 and 6.6 times more concentrated than the recommended label use and are therefore hazardous to humans (Clorox Company 2019).

Household bleach solutions are unstable and highly corrosive (Clorox Company 2019). Concentrated Clorox bleach label strength is only guaranteed for 6 months, and diluting bleach concentrate, exposure to air, and exposure to light can all increase the degradation of sodium hypochlorite (Clarkson et al. 2001). In contrast, isopropanol and ethanol are considered to be stable solutions (Dubowski et al. 2002; Kucmanic 2009). Isopropanol at concentrations of 70% is often recommended for disinfection because higher concentrations of alcohol evaporate quickly, decreasing penetration time, whereas water concentrations higher than 30% may decrease sporicidal effectiveness (Block 2001). However, our study found that 50% diluted solutions of isopropanol and ethanol were still 100% effective at disinfecting viable *C. lukuohia* propagules embedded in frass. Lower concentrations of alcohol (i.e., 50 and 70% versus 90%) may be desirable because they offer reduced cost and flammability. Isopropanol prepared at 70% is a widely available commercial product and does not require additional dilution procedures.

Although we used germicidal bleach (8.25% sodium hypochlorite) in the culture experiment and regular bleach (6% sodium hypochlorite) in the frass experiment, the active ingredients were identical and tested at a higher concentration (1.2 versus 0.825%) for frass. Our study highlights that a variety of bleach products are available with different concentrations of active ingredients, and it highlights the importance of specifying specific disinfectant product names and dilutions to the general public. To reduce the spread of ROD, the use of bleach, isopropanol, and ethanol will effectively decontaminate properly washed tools and gear. However, during fieldwork or hiking when complete removal of debris is not practical, and contact with contaminated frass is likely, isopropanol and ethanol are the more reliable disinfectants for proper decontamination.

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Literature Cited

Abbott, I. A. 1992. Lā'au Hawai'i; Traditional Hawaiian Uses of Plants. Bishop Museum Press, Honolulu, HI.

Andrews, S. 1996. Evaluation of surface disinfection procedures for enumerating fungi in foods: A collaborative study. Int. J. Food Microbiol. 29: 177-184.

Barnes, I., Fourie, A., Wingfield, M. J., Harrington, T. C., McNew, D. L., Sugiyama, L. S., and Keith, L. M. 2018. New *Ceratocystis* species associated with rapid death of *Metrosideros polymorpha* in Hawai`i. Persoonia 40: 154-181.

Block, S. S. 2001. Page 238 in: Disinfection, Sterilization, and Preservation. Lippincott, Williams & Wilkins, Philadelphia, PA.

California Oak Mortality Task Force. 2020. Sanitation measures to minimize disease spread. http://www.suddenoakdeath.org/diagnosis-and-management/sanitation-reducing-spread/.

Clorox Company. 2019. Clorox Disinfecting Bleach Safety Data Sheet. https://www.thecloroxcompany.com/sds/clorox-disinfecting-bleach/

Clarkson, R. M., Moule, A. J., and Podlich, H. M. 2001. The shelf-life of sodium hypochlorite irrigating solutions. Aust. Dent. J. 46:269-276.

Dubowski, K. M., Goodson, E. E., and Sample, M. 2002. Storage stability of simulator ethanol solutions for vapor-alcohol control tests in breath-alcohol analysis. J. Anal. Toxicol. 26:406-410.

Emerson, N. B. 2005. Pele and Hi'iaka; A Myth from Hawai'i, Rev. Ed. Edith Kanakaole Foundation, Hilo, HI.

Fortini, L. B., Kaiser, L. R., Keith, L. M., Price, J., Hughes, R. F., Jacobi, J. D., and Friday, J. B. 2019. The evolving threat of rapid 'ōhi'a death (ROD) to Hawai'i's native ecosystems and rare plant species. For. Ecol. Manage. 448: 376-385.

Harrington, T. C. 2013. *Ceratocystis* diseases. Pages 230-255 in: Infectious Forest Diseases. P. Gonthier and G. Nicolotti, eds. CAB International, Wallingford, U.K.

Heller, W. P., and Keith, L. M. 2018. Real-time PCR assays to detect and distinguish the rapid 'ōhi'a death pathogens *Ceratocystis lukuohia* and *C. huliohia*. Phytopathology 108:1395-1401.

Heninger, S. J., Anderson, C. A., Beltz, G., and Onderdonk, A. B. 2009. Decontamination of *Bacillus anthracis* spores: Evaluation of various disinfectants. Appl. Biosaf. 14:7-10.

Hughes, M. A., Juzwik, J., Harrington, T. C., and Keith, L. M. 2020. Pathogenicity, symptom development and colonization of *Metrosideros polymorpha* by *Ceratocystis lukuohia*. Plant Dis. 104:2233-2241.

Keith, L. M., Hughes, R. F., Sugiyama, L. S., Heller, W. P., Bushe, B. C., and Friday, J. B. 2015. First report of Ceratocystis wilt on 'ōhi'a (*Metrosideros polymorpha*). Plant Dis. 99:1276.

Kucmanic, J. 2009. Long-term stability of ethanol solutions for breath-alcohols tests. J. Anal. Toxicol. 33:328-331.

Loope, L., Hughes, F., Keith, L., Harrington, T., Hauff, R., Friday, J. B., Ewing, C., Bennett, G., Cannon, P., Atkinson, C., Martin, C., and Melzer, M. 2016. Guidance document for rapid ohia death: Background for the 2017-2019 ROD Strategic Response Plan. University of Hawaii, College of Tropical Agriculture and Human Resources. https://www.fs.fed.us/psw/publications/hughes/psw_2016_hughes006_loope.pdf.

Moller, W. J., and DeVay, J. E. 1968. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. Phytopathology 58:123-126.

Mortenson, L. A., Hughes, R. F., Friday, J. B., Keith, L. M., Barbosa, J. M., Friday, N., Liu, Z., and Sowards, T. G. 2016. Assessing spatial distribution, stand impacts and rate of *Ceratocystis fimbriata* induced 'ōhi'a (*Metrosideros polymorpha*) mortality in a tropical wet forest, Hawai'i Island, USA. For. Ecol. Manage. 377:83-92.

Mueller-Dombois, D., and Fosberg, F. R. 1998. Vegetation of the Tropical Pacific Islands, Ecological Studies. Springer, New York, NY.

Mueller-Dombois, D., Jacobi, J. D., Boehmer, H. J., and Price, J. P. 2013. Ohia Lehua Rainforest; The Story of a Dynamic Ecosystem with Relevance to Forests Worldwide. Friends of the Joseph Rock Herbarium, Middletown, DE.

Panconesi, A. 1999. Canker stain of plane trees: A serious danger to urban plantings in Europe. J. Plant Pathol. 81:3-15.

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Raugh, J. S., and Taylor, T. 1978. Chlorine dioxide use in food processing applications. 10th Annual Symposium on Food Science. IFT-Intermountain Section, Sun Valley, ID.

Roy, K., Ewing, C. P., Hughes, M. A., Keith, L., and Bennett, G. M. 2019. Presence and viability of *Ceratocystis lukuohia* in ambrosia beetle frass from

rapid ʻōhiʻa death-affected *Metrosideros polymorpha* trees on Hawaiʻi Island. For. Pathol. 49:e12476.
Roy, K., Jaenecke, K. A., Bjontegard, N., Mikros, D., Dunkle, E. J., Yanger, C. and Peck, B. W. 2020. Hawaiʻi Island Rapid ʻŌhiʻa Death Decontamination 2019-2020. U.S. Geological Survey data release.
Ryan, S. P., Lee, S. D., Calfee, M. W., Wood, J. P., McDonald, S., Clayton, M., Griffin-Gachalian, N., Touati, A., Smith, L., and Nysewander, M. 2014. Effect of inoculation method on the determination of decontamination efficacy against *Bacillus* spores. J. Microbiol. Biotechnol. 30: 2609-2623.