Received: 19 April 2022 | Accepted: 19 September 2022

DOI: 10.1111/ppa.13653

Plant Pathology WILEY

ORIGINAL ARTICLE

Ceratocystis lukuohia-infested ambrosia beetle frass as inoculum for Ceratocystis wilt of ʻōhiʻa (*Metrosideros polymorpha*)

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Funding information
Pacific Cooperative Studies Unit at University of Hawaiʻi at Mānoa; USDA-Forest Service, Institute of Pacific Islands Forestry in Hilo, Grant/Award Number: 19JV11272136-037; Hawaiʻi Division of Forestry and Wildlife

Abstract

Metrosideros polymorpha (ʻōhiʻa) trees in Hawaiʻi are dying from two distinct diseases, collectively referred to as rapid ʻōhiʻa death (ROD), caused by *Ceratocystis lukuohia* and *Ceratocystis huliohia*. Boring dust (frass) released when ambrosia beetles attack and colonize infected trees has been suspected as a transmission source. We sampled ambrosia beetle frass from six locations on Hawaiʻi Island and screened samples for *Ceratocystis* DNA and fungal viability. *Ceratocystis* DNA was detected in 79% of frass samples and 61% were viable. To assess the infectivity of *C. lukuohia*-colonized frass, *M. polymorpha* seedlings were wound-inoculated with frass in growth chamber trials. Wilt incidence was 40% in the first trial and 15% in the second. Frass particles naturally infested with *C. lukuohia* were treated with a cytoplasmic stain and microscopically examined; thick-walled chlamydospores were found in all samples. The chlamydospores appeared to be derived from aleurioconidia. *Ceratocystis* survival in frass was assessed in a baiting experiment conducted under varying environmental regimes; viability decreased with increasing temperature, frass age and decreasing humidity. After 6 months, 90% of the samples exhibited viable *C. lukuohia* at the lowest temperatures. Results confirmed that *C. lukuohia* chlamydospores are the fungal inoculum within ambrosia beetle frass and can induce wilt when introduced to *M. polymorpha* wounds. Although ambrosia beetles or other insects may serve as vectors, the present work supports the hypothesis that *Ceratocystis* species may be dispersed in frass, which can inform ongoing efforts to manage ROD across Hawaiʻi's native forests.

KEYWORDS
Ceratocystis huliohia, Hawaiʻi, rapid ʻōhiʻa death, tree disease, vascular wilt disease

1 | INTRODUCTION

Native forests of Hawaiʻi face a multitude of challenges to their health and persistence. Ecologically diverse and exhibiting multiple climatic zones across a small footprint, Hawaiʻi's forests support a variety of endemic and rare species. *Metrosideros polymorpha* (ʻōhiʻa) is the dominant, principal tree species within a variety of Hawaiian ecotypes. This native hardwood exhibits a highly variable morphology and, depending on habitat, may manifest as a dwarfish shrub up to a massive canopy tree that supports a diversity of native flora and fauna (Mueller-Dombois et al., 2013). *M. polymorpha* also forms the foundation of Hawaiian watersheds that provide the islands' abundant, clean drinking water. *M.*

polymorpha-dominated forests of Hawai'i Island and Kaua'i have experienced large-scale mortality due to two distinct diseases, collectively referred to as rapid 'ōhi'a death (ROD). The causal fungal pathogens are *Ceratocystis lukuohia* and *C. huliohia* (Barnes et al., 2018). *C. lukuohia* is the more aggressive of the two fungi and is considered the main pathogen associated with the epidemic expansion of *M. polymorpha* mortality across Hawai'i Island (Cannon et al., 2022). This pathogen causes Ceratocystis wilt of 'ōhi'a, a systemic vascular wilt disease capable of killing trees within months after initial infection (Hughes et al., 2020). Although *C. huliohia* is seldom associated with documented ROD outbreaks on Hawai'i Island, it causes cankers that may result in branch dieback, tree decline and mortality (Juzwik et al., 2019).

To date, the dispersal and infection processes of *C. lukuohia* and *C. huliohia* are not fully understood. However, like *Ceratocystis* spp. on other woody hosts, stem and trunk wounds represent the most important infection courts for ingress into trees (Harrington, 2013). Humans can cause wounds via mechanical damage through the use of heavy machinery, pruning and even rubbing with boots (Castro, 1991; Tarigan et al., 2011). Additionally, high wind events can damage branches and stems, simultaneously affecting large numbers of trees across the landscape. In Hawai'i, feral ungulates can rub, chew or strip patches of bark off *M. polymorpha*, and their presence in forests is strongly associated with elevated ROD mortality (Perroy et al., 2021). Prior studies with other hosts have demonstrated that *Ceratocystis platani* can travel between trees through functional root grafts (Tsopelas et al., 2017). However, patterns of disease spread in *M. polymorpha* stands do not appear similar to root graft transmitted tree diseases, such as oak wilt and Dutch elm disease (Haugen, 1999; Koch et al., 2010).

C. lukuohia belongs to the Latin American clade of *Ceratocystis*, along with tree pathogens *C. fimbriata*, *C. platani* and *C. cacaofunesta* (Barnes et al., 2018). Members of this clade may be highly aggressive to their tree hosts and are often associated with bark and ambrosia beetle attack (Harrington, 2013). As with sudden mango decline and canker stain disease of *Platanus orientalis*, woodboring beetles have been demonstrated to act as vectors of *Ceratocystis* (Al Adawi et al., 2013; Soulioti et al., 2015). Ambrosia beetles and wood borers may prove to be direct vectors of ROD fungi, and at least five species of ambrosia beetle (Coleoptera: Curculionidae: Xyleborini) colonize *Ceratocystis*-infected trees on Hawai'i Island (Roy et al., 2020). Frass (i.e., wood boring dust and faeces) of ambrosia beetles created when adults tunnel into diseased trees is hypothesized to serve as inoculum for Ceratocystis wilt diseases of cacao, eucalyptus, mango, planetree and others (Harrington, 2013; Iton, 1960; Ocasio-Morales et al., 2007; Souza et al., 2013). *C. lukuohia* and *C. huliohia* have been isolated from ambrosia beetle frass of at least five beetle species collected from *M. polymorpha* trees killed by ROD (Roy et al., 2019, 2020). Large volumes of frass can be released from trees colonized by ambrosia beetles in Hawai'i for more than a year after tree mortality; therefore, we hypothesize that frass powder serves as a dispersal agent of *Ceratocystis*. We also hypothesize that this frass is dispersed by wind and may lead to soil and water infestation. To increase understanding regarding the potential for ambrosia beetle frass to function as a dispersal agent of *Ceratocystis* inoculum, we focused on four research objectives: (1) to survey ROD-killed trees in various sites across Hawai'i Island, assessing the frequency of *Ceratocystis* liberation from trees in the form of frass; (2) to determine infectivity of frass collected from *C. lukuohia*-killed trees in growth chamber inoculation trials; (3) to determine the type and abundance of *Ceratocystis* propagules present in frass via light microscopy; and (4) to estimate the survival of *C. lukuohia* in frass over time.

2 | MATERIALS AND METHODS

2.1 | Island-wide survey of ambrosia beetle frass

Ambrosia beetle frass was collected from *M. polymorpha* trees (Figure 1a–c) at six different forest locations on Hawai'i Island between October 2017 and June 2020 (Figure 2). Sites on the windward (east) side of the island and their respective districts included Waiākea Forest Reserve (WFR) (UTM Z5 277082E, 2170131N) and Keaukaha Military Reservation (KMR) (UTM Z5 285122E, 2181192N) in the South Hilo district, Keau'ohana Forest Reserve (KFR) (UTM Z5 295055E, 2148037N) in the Puna district, and Waipunalei (WAI) (UTM Z5 262481E, 2211178N) and Laupāhoehoe Forest Reserve (LFR) (UTM Z5 261647E, 2207189N) in the North Hilo district. Frass was also collected from the leeward (west) side of the island at a private ranch in Kailua-Kona (KK) (UTM Z5 186028E, 2175108N) in the North Kona district. Metal spoons or scoopulas were sprayed with 70% ethanol, flame-sterilized and allowed to cool before frass was collected from the lower 2 m of the dead, ROD-symptomatic trees and placed in sterile 15-ml conical centrifuge tubes (Falcon) or sterile plastic sample bags. Tools were resterilized between each collection to avoid sample cross-contamination. Freshly excavated frass "tubes" extruded from gallery entrances or piles of loose powdery dust were collected from crevices between or under bark layers. Frass samples collected from entrances or below multiple galleries on the surface of a single source tree were aggregated to provide a composite or "bulk" frass collection for that tree (Figure 1b,c). Frass material appearing excessively dry, discoloured or old (i.e., hardened clumps) was avoided. Following collection, frass was stored at 4°C. Apart from frass obtained from boring dust traps (see below), frass used in all studies was collected in this manner.

2.2 | Molecular detection of *Ceratocystis* species

Ceratocystis DNA was detected in the survey and experimental frass samples following the method of Heller and Keith (2018). Approximately 50 mg (dry weight) of frass was transferred to a 2 ml screw-top grinding tube containing six 3 mm diameter zirconium beads (OPS Diagnostics). Samples were homogenized at 4.5 m/s

FIGURE 1 Expelled ambrosia beetle frass in the field and symptoms of inoculated *Metrosideros polymorpha* seedlings in the growth chamber. (a, b) Copious frass accumulating in the bark cracks and lower bole of a mechanically wounded tree killed by rapid ʻōhiʻa death (ROD) in Hilo; (c) bulk frass collected from a non-wounded tree in Waipunalei; and (d) seedlings in a growth chamber experiment that were (i) completely or (ii) partially wilted after inoculation with *Ceratocystis lukuohia*-infested ambrosia beetle frass and (iii) a healthy negative control.

for 60 s using a FastPrep-24 homogenizer (MP Biomedicals) prior to analysis. DNA extraction was performed using a commercial DNA spin column extraction kit (NucleoSpin Plant-II DNA kit; Machery-Nagel) using the manufacturer's protocol. Extracted DNA was amplified using two species-specific quantitative PCR (qPCR) assays to detect either *C. lukuohia* (primers: CP_For_A/CP_Rev_A) or *C. huliohia* (CP_For_B/CP_Rev_B) within the cerato-platanin (*CP*) gene (Heller & Keith, 2018). Both assays were multiplexed with a Myrtaceae-specific primer pair (MeNu47_+20For/MeNu47_+186R) and probe, which served as an internal amplification control for the detection of the host (ʻōhiʻa) DNA (Heller & Keith, 2018; Pillon et al., 2014).

2.3 | Viability testing of ambrosia beetle frass

To assess *Ceratocystis* viability, frass was spread and sandwiched between two freshly cut carrot root discs, which were subsequently wrapped together in Parafilm, placed in individual plastic bags and incubated at room temperature for up to 4 weeks (Moller & DeVay, 1968). Carrot discs were determined to harbour viable *Ceratocystis* material when greyish mycelia containing perithecia and ascospores were visible under a dissection microscope. In cases where mycelia developed on baits that appeared like *Ceratocystis* but did not produce perithecia (usually due to bacterial contamination), a frass-free segment of colonized carrot was excised and the presence of *Ceratocystis* was determined by qPCR (Heller & Keith, 2018).

2.4 | Pathogenicity trials in a growth chamber

Two-year-old potted seedlings of *M. polymorpha* (mean stem height and diameter at base 63.5 ± 1.9 cm and 6.3 ± 0.3 mm, respectively), exhibiting a single main stem, were acclimatized for 14 to 21 days before *Ceratocystis* inoculation in a growth chamber set to 25°C, the optimum temperature for *C. lukuohia* growth in culture (Barnes et al., 2018; Luiz & Keith, 2021). Based on qPCR and viability assays from a prior island-wide survey (Brian Tucker, Pacific Cooperative Studies Unit, oral communication, 2022), 16 *C. lukuohia*-positive trees were selected as sources for frass inoculum. Fresh bulk frass samples were collected from these trees, as previously described, and screened by qPCR to confirm the presence of *C. lukuohia* and absence of *C. huliohia* prior to use in the inoculation trials. A portion of each newly collected frass sample was also screened by carrot baiting to confirm *C. lukuohia* presence and viability. All collections of frass came from unique trees, and trees were not resampled. Prior to inoculation, frass (approx. 100 mg dry weight) was sifted through a coarse screen to remove bark pieces and debris, placed in glass Petri dishes, hydrated with 2–3 ml of sterile water, stirred into a thick paste, covered and held in an incubator at 25°C for up to 24 h before inoculation.

Plants were wounded at a sharp, downward angle with a flame-sterilized scalpel on the main stem at 4–8 cm above soil level, resulting in a 1.5 × 0.5 cm stem-flap. The exposed xylem underneath each flap was cross-hatched with a scalpel to provide a rough surface for frass adherence, and the frass paste (0.3 g wet weight) was spread over the wound. Stem flaps were subsequently closed, and wounds

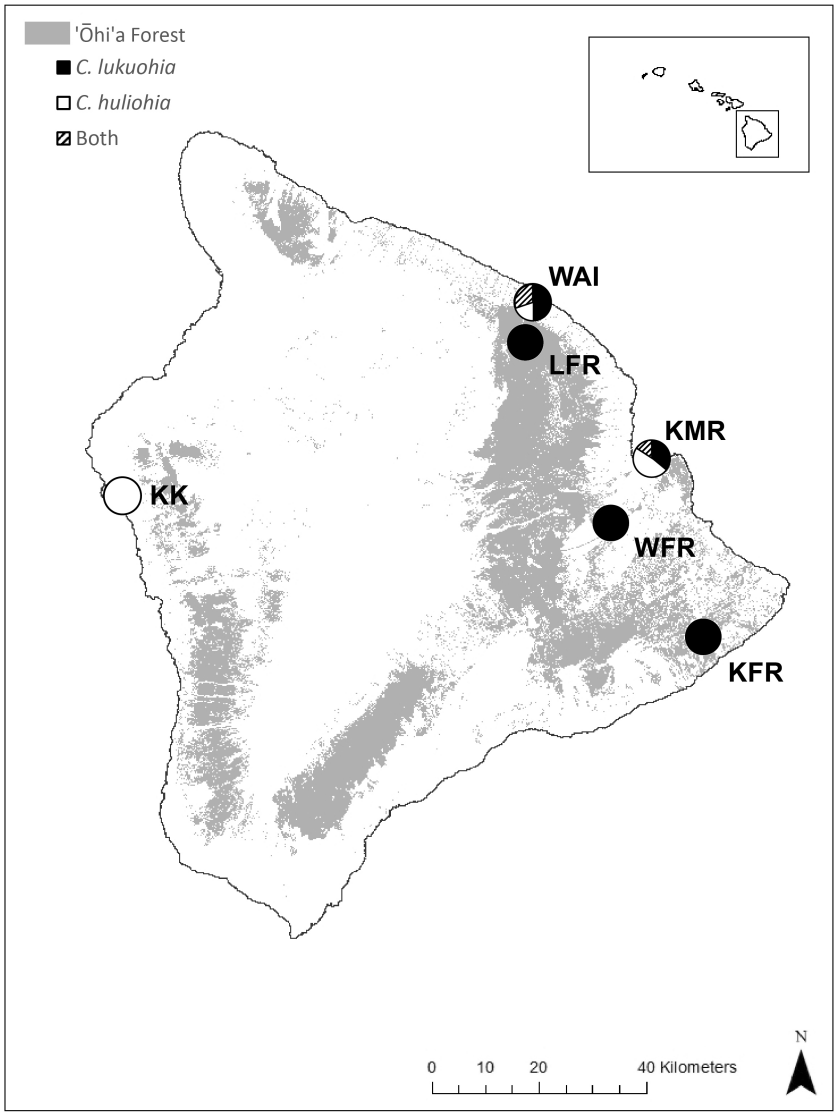

FIGURE 2 Locations on Hawai'i island where ambrosia beetle frass was sampled from *Metrosideros polymorpha* trees killed by rapid 'ōhi'a death: Keau'ohana Forest Reserve (KFR), Keaukaha Military Reservation (KMR), Waipunalei (WAI), Waiākea Forest Reserve (WFR), Laupāhoehoe Forest Reserve (LFR) and a private ranch in Kailua-Kona (KK). Pie charts indicate the proportion of each *Ceratocystis* species detected in the ambrosia beetle frass samples collected at each location.

were wrapped in sterile, water-soaked cotton balls, which were tied with garden wire around the stem to prevent desiccation. Instead of frass, positive control plants were inoculated with a *C. lukuohia*-colonized filter paper disc (fungal isolate P16-7) (Luiz et al., 2021) in an identical fashion. Negative control plants were inoculated in the same fashion, but with frass sterilized by autoclaving.

In the first inoculation experiment of December 2017, frass from four trees was collected from WFR and KMR in Hilo (eight trees in total). Frass from five trees from WFR and three trees from KFR in Puna (eight trees in total) were collected for the second experiment in 2018. In each of the two experiments, frass from each of the eight source trees was used to inoculate five replicate seedlings, with an additional five positive and five negative control seedlings, resulting in a total of 50 seedlings per experiment. Plants were randomly arranged in the growth chamber (PGR15; Conviron) and irrigated three times a week; cotton balls at the wound site were hydrated daily for the first 20 days to maintain inoculum moisture. Disease symptoms on seedlings were rated every other day on a 0 to 5 scale, where 0 = no symptoms, 1 = 1% to 20% of leaves wilted, 2 = 21% to 40%, 3 = 41% to 60%, 4 = 61% to 80%, 5 = 81% to 100%. After 60 days, the side branches of the seedlings were trimmed with flame-sterilized clippers, leaving only their central stem and the stems were wiped twice in 70% ethanol to remove superficial contaminants. Plants were debarked with a flame-sterilized grafting knife and checked for presence/absence of vascular staining, starting 3 cm above the inoculation point (to avoid typical wound-response discolouration). To avoid contamination from the frass inoculum, thin wood sections were removed from the main stem at least 3 cm above the inoculation point for qPCR and carrot baiting.

2.5 | Visualization of *Ceratocystis* propagules in ambrosia beetle frass

Ambrosia beetle frass was collected from dead *M. polymorpha* trees in October 2020 and January 2021 from trees at the WFR site that had been inoculated with *C. lukuohia* in June 2019. Frass was also

collected from trees naturally killed by *C. lukuohia* at KFR in Puna in June 2021. In addition, wood chips were collected with a hatchet from stained sapwood of three trees killed by *C. lukuohia* at KFR. All sampled trees had been shown to have died from *C. lukuohia* infection by diagnostic qPCR analysis of wood samples collected during previous survey operations (Brian Tucker, Pacific Cooperative Studies Unit, oral communication, 2022).

The frass samples were either collected in bulk or from individual beetle galleries. Bulk samples were collected from small piles accumulated in crevices of the outer bark below active ambrosia beetle galleries (Figure 1b,c). Such piles appeared fresh and had not noticeably dried, though some samples were wet from the rain. Bulk frass samples were scraped into sample vials or plastic bags using a flame-sterilized scoopula or spoon as previously described. To obtain the entire fraction of wood particles ejected from an individual ambrosia beetle gallery, additional samples were collected by boring dust traps (BDTs) constructed from 25 ml screw-cap tubes (54 × 27 mm, Sarstedt). The caps of the tubes had a small hole, which was placed over an individual gallery and fastened to the bark with screws (Roy et al., 2019; Figure S1). Tubes contained an opening at the bottom that was covered with a fine mesh screen to allow for airflow.

Frass and wood chip samples were air-dried on glass Petri dishes for several hours in a biosafety cabinet, and a small aliquot was screened by qPCR to reconfirm the presence of *C. lukuohia* and absence of *C. huliohia* DNA; all *C. huliohia* positive samples were excluded from the study. Small portions of frass from the two initial bulk frass samples collected from WFR were spread over the surface of three fresh carrot discs on moistened pieces of paper towel in a 90 mm Petri dish.

Frass particles were stained with the cytoplasmic stain lactophenol cotton blue (LCB; 0.1 g of cotton blue, 67 ml of anhydrous lactophenol, and 20 ml of water) in microcentrifuge tubes and spun to fully immerse the frass particles and eliminate air bubbles. Because of the limited availability of frass material in the second WFR samples, approximately 100 µl by volume of frass was mixed with 100 µl of sterile water, spun at 7000 × *g* for 2 min and half of the material was spread over three carrot discs. One or two drops of LCB were added to the remainder of the material and spun for an additional 2 min. In KFR samples, 200 µl of sterile distilled water was added to approximately 200 µl by volume of frass and spun at 10,000 × *g* for 10 min; approximately 25% of the wet frass was added to each of three fresh carrot discs, 50 µl of LCB was added to the remaining frass and the tube was spun for 5 min at 10,000 × *g*.

Stained frass was mounted in lactic acid on a glass microscope slide with a cover slip and examined at 500× or 1250× (40× or 100× objective lens, respectively) using light microscopy (BH2; Olympus). *Ceratocystis* propagules were counted from 25 or 26 frass particles per sample. Thin slices of stained *M. polymorpha* wood were cut with a razor blade and were similarly mounted in LCB on microscope slides. Photographs and measurements were taken with the Leica Application Suite v. 3.6.

2.6 | Estimation of *C. lukuohia* survival period in ambrosia beetle frass

To assess viability of *C. lukuohia* in frass over time, samples were collected from *M. polymorpha* trees killed by *C. lukuohia*, either from natural infection or artificial inoculation, at the KMR and WFR sites. Starting 10 August 2019, frass samples were collected over a 4-week period from trees showing visible wilt symptoms less than 6 months prior to sampling. The material was collected from multiple trees to obtain a sufficient volume of bulk frass, and a subsample of frass collected from each tree was screened by qPCR to confirm the presence of *C. lukuohia* DNA and the absence of *C. huliohia* DNA. Frass from trees positive only for *C. lukuohia* was pooled into a single, homogeneous mixture to obtain approximately 40 g of frass material.

Frass samples were stored at two locations on Hawai'i Island: a high elevation site at Volcano (UTM Z5 267835E, 2151476N, 1143 m a.s.l., mean annual temp. 18.2°C) and a low elevation site in Hilo (UTM Z5 281882E, 2179572N, 105 m a.s.l., mean annual temp. 20.7°C). A subset of the samples was also placed in an incubator set to 25°C as a control. Samples at both Volcano and Hilo sites were placed in covered, open-air locations to avoid excessive ultraviolet radiation and loss of samples due to rainfall. Frass was subdivided into 15 samples (approx. 2.6 g each) and placed in individually labelled white organza mesh bags (7.1 × 8.9 cm) with drawstrings. Each organza bag was placed in an open plastic pouch to minimize frass leakage. Five replicate organza bags per treatment (environment) were placed into an open-topped plastic bin (15.1 L) containing two temperature and relative humidity (RH) data loggers (DS1923-F5# Hygrochron; iButtonLink LLC). The average hourly temperature and RH recorded during the incubation period at the Hilo treatment site were 22.4°C ± 0.1 and 82.3% ± 0.1, 17.2°C ± 0.0 and 93.6% ± 0.1 at the Volcano treatment site, and 23.3°C ± 0.0 and 21.0% ± 0.3 in the control incubator. The data loggers for the Volcano site malfunctioned after the first 3 months, leaving an 85-day gap of data until the logger was replaced. Starting 13 September 2019, two subsamples (approx. 0.05 g) were removed at 14-day intervals from each of the five frass bags for carrot baiting, resulting in 10 baits per sampling date for each of the three treatments. Fourteen-day baiting cycles were repeated from September 2019 until the frass samples were exhausted 26 weeks later in March 2020, resulting in 13 baiting cycles and 390 carrot baits. Baits were held at room temperature and rated for fungal growth and development of perithecia, as described above. To confirm visual ratings, one visually positive carrot bait was screened for *C. lukuohia* DNA by qPCR for each baiting cycle.

2.7 | Statistical analysis

Mean disease progress was plotted over time for the inoculation study using mean wilt severity ratings that were converted to proportions based on the mid-points of the scale ranges (e.g., 21% to 40% wilt = 0.3). The area under the disease progress curve

(AUDPC) was calculated according to Simko and Piepho (2012) using SAS v. 9.4 software (SAS Institute Inc.). Due to the large number of severity ratings exhibiting a zero value and subsequent lack of homogeneity of pooled error variance, analyses of variance and multiple comparisons were not statistically appropriate. Consequently, we elected to report only mean and standard error values. For the data of *C. lukuohia* survival in frass samples, the percentage of *Ceratocystis*-viable carrot baits per sample period in each treatment environment was calculated, and data were analysed in R v. 4.0.2 using the RStudio interface (www.R-project.org). To test relationships amongst treatments (environments), time (frass age in weeks) and percentage of the carrot baits positive for *Ceratocystis*, two-way and one-way repeated measures analysis of variance (ANOVA) on generalized linear mixed effects (GLME) models were followed by post hoc comparisons using the Bonferroni correction with the lme4 and emmeans packages for R, respectively (Bates et al., 2015; Length, 2021). All three models included sample replicates as a random effect and *Ceratocystis*-viable carrot baits as the response variable. The first model used the shared prediction of both treatment (environment) and time, and temperature and RH predictors were tested in separate models due to their inherent relatedness. Statistical significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Island-wide survey of ambrosia beetle frass

C. lukuohia and/or *C. huliohia* DNA was detected in the frass of 93 of 118 sampled trees (79%), and viable spores were detected in frass of 72 trees (61%) using carrot baiting techniques (Table 1). *Ceratocystis* qPCR detection rates in frass ranged from 50% to 100% across the sites, and viable *Ceratocystis* was found in 34% to 100% of frass samples. In general, more positive *Ceratocystis* detections occurred with qPCR compared to carrot baiting, except at the KFR site, where the fungus occurred on carrot baits of two samples that yielded inconclusive results by qPCR. However, an inconclusive qPCR result indicates the lack of amplification of the *M. polymorpha* gene target (MeNu47) due to some sort of reaction inhibition.

For composite (bulk) frass samples, *C. lukuohia* was detected by qPCR in about 48% of samples, *C. huliohia* was detected in about 25% of samples, both *C. lukuohia* and *C. huliohia* were detected in about 6% of samples, and neither fungal pathogen was detected in about 21% of samples (Table 1). In KFR, LFR and WFR locations on the windward (eastern) side of the island, only *C. lukuohia* was detected by either qPCR or carrot baiting. *C. lukuohia* occurred on carrot baits from all frass samples from the KFR site, and it was detected by qPCR in the frass of all the sampled trees at the WFR site. In contrast, *C. lukuohia* DNA was detected in frass from only half of the trees at the LFR site. *C. lukuohia* and *C. huliohia* were detected by qPCR in similar percentages in frass samples from KMR and WAI sites, and these were the only locations where samples

TABLE 1 Detection of *Ceratocystis* species in ambrosia beetle frass collected from 118 individual *Metrosideros polymorpha* trees on Hawai'i Island by quantitative PCR (qPCR) and carrot baiting.

Study site, district	Collection date(s)	Total no. trees sampled	No. of tree samples positive for *Ceratocystis* by qPCR (%)				No. of tree samples positive for *Ceratocystis* by carrot baiting (%)
			C. lukuohia	*C. huliohia*	*C. lukuohia* and *C. huliohia*	Not detected or inconclusive	
Keau'ohana Forest Reserve (KFR), Puna	Oct 2018	9	7 (78)	0	0	2 (22)	9 (100)
Keaukaha Military Reservation (KMR), South Hilo	Oct 2017–Feb 2020	26	9 (34.6)	12 (46.2)	4 (15.4)	1 (3.8)	16 (61.5)
Waipunalei (WAI), North Hilo	Aug 2018–Apr 2019	12	5 (41.7)	2 (16.7)	3 (25.0)	2 (16.7)	8 (66.7)
Waiākea Forest Reserve (WFR), South Hilo	Dec 2017–Nov 2018	17	17 (100)	0	0	0	14 (82.3)
Private Ranch (KK), North Kona	Oct 2019	16	0	15 (93.8)	0	1 (6.3)	12 (75)
Laupāhoehoe Forest Reserve (LFR), North Hilo	Oct 2019–Jun 2020	38	19 (50.0)	0	0	19 (50.0)	13 (34.2)
Total		118	57 (48.3)	29 (24.6)	7 (5.9)	25 (21.2)	72 (61.0)

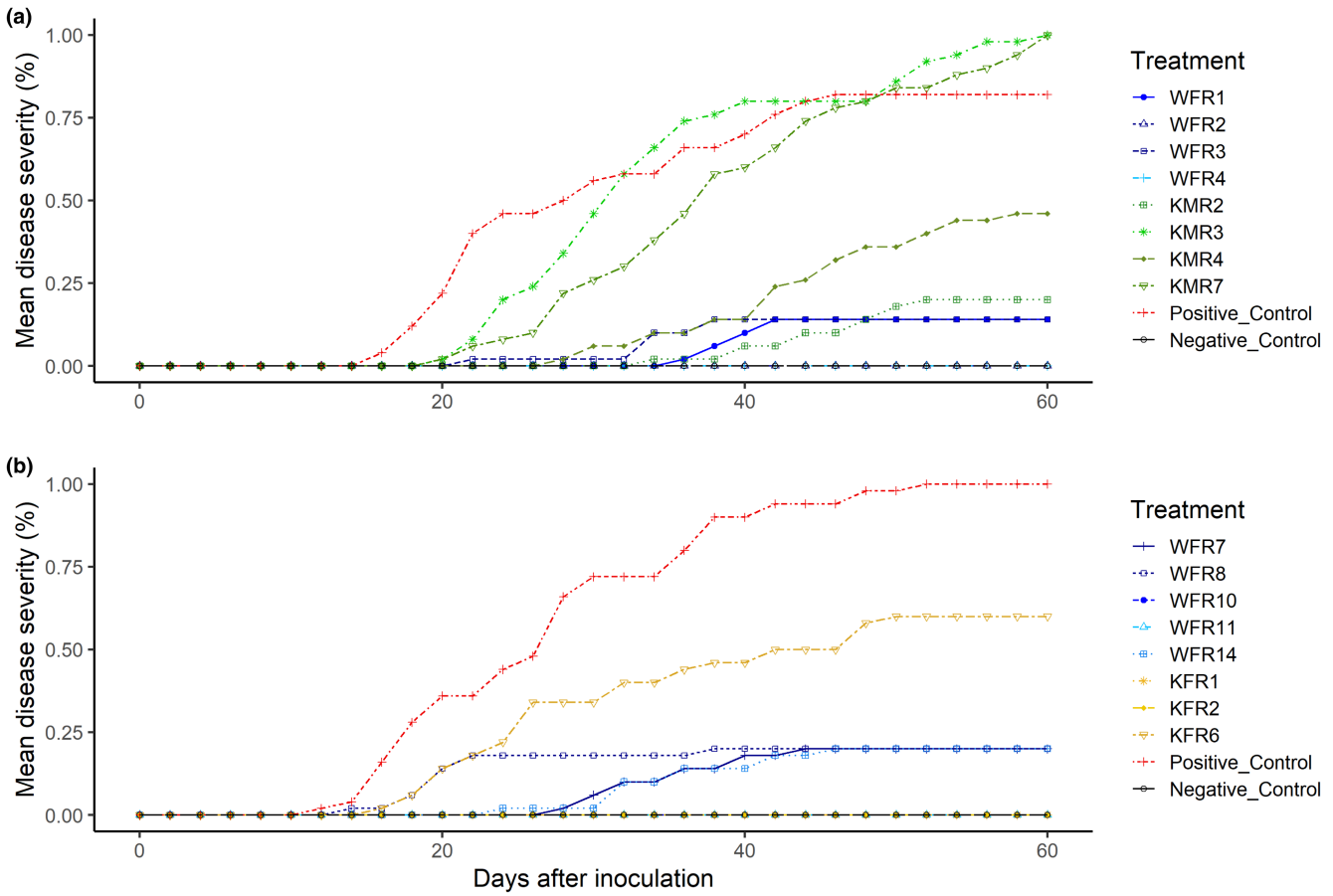

FIGURE 3 Disease progress curves in *Metrosideros polymorpha* seedlings after inoculation with ambrosia beetle frass in the (a) December 2017 and (b) November 2018 growth chamber experiments. The curves show the mean severity of foliar wilt (proportion of the canopy with symptoms) over time. Each line represents the mean of five replicate seedlings inoculated with the frass from one diseased tree. Frass had been collected from trees in Waiākea Forest Reserve (WFR), Keaukaha Military Reservation (KMR) and Keau'ohana Forest Reserve (KFR). Sterile frass and *Ceratocystis lukuohia*-colonized filter paper disks served as inoculum for negative and positive controls, respectively.

contained DNA of both *Ceratocystis* species (i.e., co-infested samples) (Figure 2; Table 1). Both species were detected in 25% of frass samples from the WAI site, which was greater than the number of detections of *C. huliohia* alone. At the KK site on the drier, leeward side of the island (Figure 2), only *C. huliohia* was detected in frass samples (Table 1).

3.2 | Pathogenicity trials in a growth chamber

Frass collected from six of eight *C. lukuohia*-infested *M. polymorpha* trees in the first growth chamber pathogenicity experiment (December 2017 trial) induced wilt in at least one of the five replicate seedlings (Figures 1d and 3a, Table 2). Internal xylem staining and detection of *C. lukuohia* by qPCR were congruent with external wilt symptoms. Xylem staining characteristic of Ceratocystis wilt of ʻōhiʻa occurred in 40% of frass-inoculated seedlings. Incubation periods for wilt symptoms of frass-inoculated plants ranged from 18 to 36 days, compared to a 16-day incubation period for seedlings that received *C. lukuohia*-colonized filter paper discs (positive controls) (Figure 3a). Mean AUDPC ranged from 0 to 27 for frass-inoculated treatments. Mean wilt, xylem staining incidence and seedling mortality were highly variable between, and sometimes within, frass treatment batches (Table 2). Approximately 33% of seedlings died after inoculation with frass, but this value was lower than that of positive control values (80%). Frass from the KMR site exhibited a 70% pathogen transmission rate, a value much higher than that of frass from the WFR site (10%). In addition, frass collected from two trees at the KMR site killed 100% of inoculated seedlings, a value comparable to that of positive controls (Table 2).

Wilt symptoms occurred in 15% of frass samples inoculated in the second experiment conducted in November 2018 (Table 3). Xylem staining manifested in 20% and *C. lukuohia* was recovered from about 23% of inoculated seedlings. Mortality occurred in 15% of frass-inoculated seedlings; this value was much lower than the 100% mortality values of the positive control. Seedlings with symptoms exhibited wilt 14–28 days after inoculation with frass; seedlings in the positive control exhibited wilting 12 days after inoculation (Figure 3b). Mean AUDPC values of frass treatments varied from 0 to 18 and were much lower than that of seedlings receiving the positive control treatment (Table 3). Out of all the frass samples tested,

TABLE 2 Disease symptoms, area under the disease progress curve and detection of *Ceratocystis lukuohia* in *Metrosideros polymorpha* seedlings inoculated with ambrosia beetle frass in the December 2017 growth chamber experiment.

Frass source tree	Symptom				*C. lukuohia* detection	
	Wilt	Xylem stain	Mortality	AUDPC[a]	Stem qPCR	Carrot baiting
WFR1	1/5[b]	1/5	0/5	3.0±3.0	1/5	1/5
WFR2	0/5	0/5	0/5	0.0±0.0	0/5	0/5
WFR3	1/5	1/5	0/5	3.9±3.9	1/5	1/5
WFR4	0/5	0/5	0/5	0.0±0.0	0/5	0/5
KMR2	1/5	1/5	1/5	3.2±3.2	1/5	1/5
KMR3	5/5	5/5	5/5	26.5±2.3	5/5	4/5
KMR4	3/5	3/5	2/5	8.3±4.7	3/5	3/5
KMR7	5/5	5/5	5/5	21.9±3.2	5/5	5/5
Negative control[c]	0/5	0/5	0/5	0.0±0.0	0/5	0/5
Positive control[d]	5/5	5/5	4/5	27.3±6.9	5/5	5/5

Abbreviations: WFR, Waiākea Forest Reserve; KMR, Keaukaha Military Reservation.

[a]Area under the disease progress curve based on the disease severity rating, calculated as in Simko and Piepho (2012).

[b]Number of replicates = 5.

[c]Negative control plants were inoculated with autoclave-sterilized frass.

[d]Positive control plants were inoculated with a *C. lukuohia*-colonized filter paper disk as in Luiz et al. (2021).

TABLE 3 Disease symptoms, area under the disease progress curve and detection of *Ceratocystis lukuohia* in *Metrosideros polymorpha* seedlings inoculated with ambrosia beetle frass in the November 2018 growth chamber experiment.

Frass source tree	Symptom				*C. lukuohia* detection	
	Wilt	Xylem stain	Mortality	AUDPC[a]	Stem qPCR	Carrot baiting
WFR7	1/5[b]	1/5	1/5	5.2±5.2	1/5	1/5
WFR8	1/5	1/5	1/5	8.0±8.0	1/5	1/5
WFR10	0/5	0/5	0/5	0.0±0.0	0/5	0/5
WFR11	0/5	1/5	0/5	0.0±0.0	1/5	1/5
WFR14	1/5	1/5	1/5	5.1±5.1	1/5	1/5
KFR1	0/5	0/5	0/5	0.0±0.0	0/5	0/5
KFR2	0/5	0/5	0/5	0.0±0.0	0/5	0/5
KFR6	3/5	4/5	3/5	18.4±8.4	5/5	5/5
Negative control[c]	0/5	0/5	0/5	0.0±0.0	0/5	0/5
Positive control[d]	5/5	5/5	5/5	33.7±4.4	5/5	5/5

Note: KFR indicates Keauʻohana Forest Reserve; and WFR, Waiākea Forest Reserve.

[a]Area under the disease progress curve based on the disease severity rating, calculated as in Simko and Piepho (2012).

[b]Number of replicates = 5.

[c]Negative control plants were inoculated with autoclave-sterilized frass.

[d]Positive control plants were inoculated with a *C. lukuohia* -colonized filter paper disk as in Luiz et al. (2021).

those from tree KFR6 proved the most infectious; all seedlings inoculated with frass from this tree were infected with *C. lukuohia*, and three of the five seedlings died. In contrast, frass from two trees from the KFR site and one from the WFR site failed to transmit *C. lukuohia*. Frass from trees at the WFR site infected 16% of seedlings with *C. lukuohia* based on evidence of internal xylem staining, qPCR

TABLE 4 Detection and viability of *Ceratocystis lukuohia* chlamydospores in ambrosia beetle frass particles by microscopic examination and carrot baiting.

Collection date	Site[a]	Sample ID	Sample type[b]	No. of particles with chlamydospores/no. of particles examined (%)	Total no. of chlamydospores (mean no. per particle)	No. of carrot discs with *C. lukuohia*/number of discs sampled
Oct 2020	WFR	USGS1	Bulk	22/25 (88)	72 (2.88)	3/3
		USGS2	Bulk	11/26 (42)	21 (0.81)	0/3
Feb 2021	WFR	BDT-2	BDT	12/26 (46)	17 (0.65)	2/3
		BDT-3	BDT	9/26 (35)	17 (0.65)	0/3
Jun 2021	KFR	Tree 3838	BDT	8/26 (31)	11 (0.42)	0/3
		Tree 3876	BDT	8/26 (31)	15 (0.58)	0/3
		Tree 3922	BDT	7/26 (27)	9 (0.35)	0/3
		Tree 3838	Bulk	4/26 (15)	5 (0.14)	0/3
		Tree 3876	Bulk	12/26 (46)	19 (0.73)	0/0
		Tree 3901	Bulk	7/26 (27)	8 (0.31)	3/3
		Tree 3922	Bulk	3/26 (12)	4 (0.15)	1/3

[a]Waiākea Forest Reserve (WFR) is in the South Hilo District and Keauʻohana Forest Reserve (KFR) is in the Puna district.

[b]Frass was collected from either individual boring dust traps (BDT) placed over a single ambrosia beetle gallery entrance or from the bark surface of the tree bole below ambrosia beetle galleries (Bulk).

results and carrot baiting results, and 12% of the seedings exposed to the frass wilted and died.

Seedlings inoculated with autoclaved frass did not show any wilt or xylem staining in either trial, and *C. lukuohia* was not detected in stem tissues in either case. All subsamples from carrot baits visually rated positive for *Ceratocystis* were confirmed to be infected with *C. lukuohia* by qPCR ($n = 16$).

3.3 | Visualization of *Ceratocystis* propagules in ambrosia beetle frass

C. lukuohi a grew profusely on each of the three carrot discs inoculated with bulk frass from sample USGS1 (Table 4), but *C. huliohia* was not isolated. Frass particles from this sample did not exhibit aleurioconidia when examined at 500× magnification, but numerous chlamydospores were observed. Chlamydospores were globose to subglobose, 5–10 μm in diameter, exhibited what appeared to be internally thickened and light brown pigmented cell walls of variable thickness, and contained deeply stained cytoplasm (Figure 4). Chlamydospores appeared attached to wood tissue, primarily the inner walls of the vessels and unattached chlamydospores were not observed in the mounting medium. The majority of the 25 examined frass particles from site USGS1 exhibited one or more chlamydospores (Table 4). No *Ceratocystis* was isolated from sample USGS2 using carrot baiting, but a total of 21 chlamydospores were found in 11 of 26 of the sampled frass particles. Thus, over three times as many chlamydospores were observed in fragments of frass from site USGS1, from which *C. lukuohia* was isolated on carrot baits, compared to frass samples from site USGS2, from which no *C. lukuohia* was isolated (Table 4).

Two frass samples were collected on February 2021 from boring dust traps (BDTs) over individual galleries of *Xyleborus ferrugineus* at WFR. Traps were placed on trees previously inoculated with *C. lukuohia*, and *X. ferrugineus* adults that had emerged from galleries were collected in traps along with frass. *C. lukuohia* was isolated on two of three carrot discs from sample BDT-2, but it was not isolated from the BDT-3 sample (Table 4). *C. huliohia* was not isolated from either sample. Forty-six percent of frass particles from the BDT-2 sample exhibited chlamydospores, which were 5–10 μm in diameter and had deeply stained cytoplasm, whilst 35% of the frass particles from the BDT-3 site sample exhibited chlamydospores. Aside from chlamydospores, no aleurioconidia or other spores attributable to *Ceratocystis* were observed in the frass particles.

At the KFR site, frass particles from BDTs on three trees killed by *C. lukuohia* were baited on carrots, but no *Ceratocystis* was isolated. Between 27% and 31% of frass particles from these samples exhibited chlamydospores; densities ranged from 0.35 to 0.58 chlamydospores per particle (Table 4). Bulk frass samples were also collected from the exterior of the same KFR trees as well as an additional tree, and *C. lukuohia* was isolated on three carrot discs from tree 3901 and one of three discs from tree 3922, but not from the other trees (Table 4). *C. huliohia* was not detected by qPCR or isolated on carrot baits from any of the samples used for microscopic examination.

Wood chips from trees killed by *C. lukuohia* were examined microscopically by slicing thin sections with a razor blade and mounting them in LCB. Most sections did not reveal spore structures or growth of *Ceratocystis*, even where streaks of vascular staining were evident; dark staining appeared to be due to gums and other host materials emanating from parenchyma cells, rather than from growth of *Ceratocystis* (data not shown). Some wood sections did reveal what appeared to be aleurioconidia produced from conidiophores in dense clusters within vessels. The developing aleurioconidia and

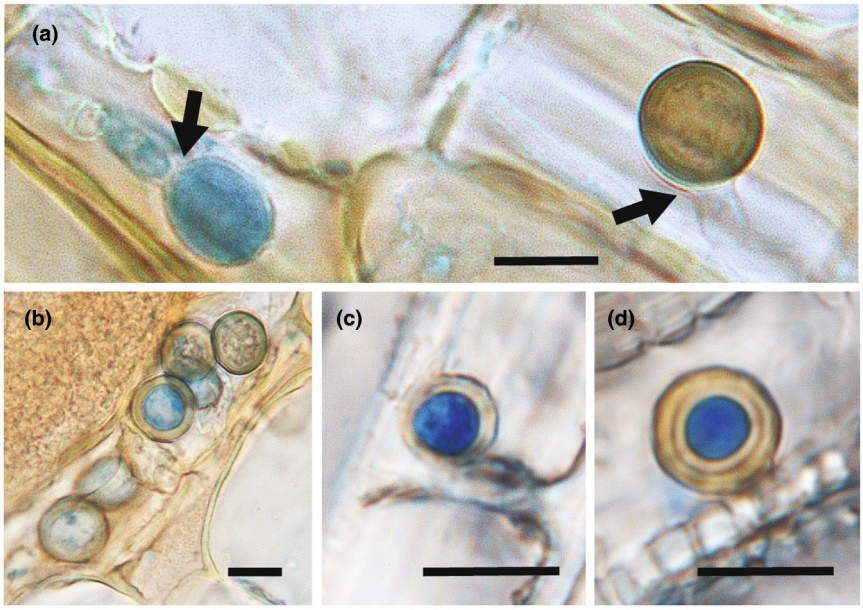

FIGURE 4 Aleurioconidia and chlamydospores of *Ceratocystis lukuohia* in wood and ambrosia beetle frass expelled from *Metrosideros polymorpha* trees killed by Ceratocystis wilt of ʻōhiʻa. (a) Developing, stained aleurioconidium (left) and mature, non-stained aleurioconidium (right) in wood, with arrows showing the point of attachment to conidiophores. (b) Aleurioconidia transitioning to chlamydospores in wood. (c, d) Chlamydospores on vessel walls in particles of ambrosia beetle frass. All specimens were stained with lactophenol cotton blue. Bars in all figures are 10 μm.

conidiophores were thin-walled and had cytoplasm stained by LCB, but the mature aleurioconidia had heavily pigmented cell walls and their cytoplasm failed to stain (Figure 4a). The mature aleurioconidia were obovoid, generally over 12 μm in width, and some exhibited a truncate base. However, subglobose spores with thicker, lightly pigmented walls were also observed in some wood chips (Figure 4b). These spores appeared to be intermediate or transitioning between aleurioconidia and chlamydospores, as the thickness of their spore walls varied, their cytoplasm sometimes stained lightly with LCB and their diameters ranged from 9.5 to 12.5 μm. Such diameters are comparable to the width of typical aleurioconidia formed in culture, which are pyriform to ovoid and 13–15 × 9.5–10.5 μm (Barnes et al., 2018).

3.4 | Estimation of *C. lukuohia* survival period in ambrosia beetle frass

Our GLME models on *C. lukuohia* viability in frass at different locations through time indicated a significant effect of environment (treatment site) (F [2363.31] = 175.802, $p < 0.001$, $\eta_p^2 = 0.49$) and time (F [12363.19] = 13.052, $p < 0.001$, $\eta_p^2 = 0.30$). Bonferroni-corrected post hoc comparisons revealed that viable *C. lukuohia* propagules were more likely to survive in frass at the Volcano treatment site compared to the Hilo site ($p < 0.001$) or control conditions ($p < 0.001$). Also, the Hilo site and control treatment (incubator) did not differ from one another ($p = 0.341$). Significant differences in *C. lukuohia* recovery occurred 4–8 weeks into the study period (Figure 5c; $p = 0.008$). Both of our one-way repeated measures ANOVAs on separate GLMEs on *C. lukuohia* viability in frass revealed a significant effect of temperature (F [32,285.25] = 21.965, $p < 0.001$, $\eta_p^2 = 0.71$) and the RH (F [31,286.26] = 21.985, $p < 0.001$, $\eta_p^2 = 0.70$). Temperatures at the Volcano site were consistently lower than at the Hilo and control treatment sites ($p < 0.001$) (Figure 5a), and the RH of the control treatment was consistently lower than that of both the Hilo and Volcano sites ($p < 0.001$) (Figure 5b). We did not observe a decline in viability of *C. lukuohia* inoculum in frass stored in the control treatment (incubator) during the first 4 weeks of incubation, but viability dropped precipitously up to the eighth week, after which time *C. lukuohia* was not recovered (Figure 5c). Viability of *C. lukuohia* in frass exposed to Hilo ambient temperature and RH conditions was intermediate of the three treatments; 70% of baits in Hilo yielded *C. lukuohia* for the first 6 weeks, after which time recovery steadily declined until the 20th week, the date of the last *C. lukuohia* recovery. Frass exposed to Volcano environmental conditions yielded the highest levels of *C. lukuohia* on carrot baits; recovery levels ranged from 50% to 100% throughout the study. At the end of the 26-week study period, 90% of frass baits at Volcano yielded viable *C. lukuohia*. The maximum length of viability could not be determined because the sample material was expended by 26 weeks.

4 | DISCUSSION

Results of the island-wide sampling indicated that ambrosia beetle frass from ROD-killed trees is frequently infested with *C. lukuohia* and *C. huliohia*. As expected, the *Ceratocystis* species recovered in frass was usually the predominant pathogen known to be killing trees at those sites. For example, at the WFR and KFR sites, *C. lukuohia* was the main species associated with ROD mortality (Brian Tucker, Pacific Cooperative Studies Unit, personal communication, 2022), and it was the only pathogen detected in frass samples. We collected samples at the KK site because the tree mortality there was attributed to *C. huliohia* in earlier ROD surveys, and indeed, *C. huliohia* was the only species detected in frass by qPCR. Continuing monitoring and field studies indicate that *C. lukuohia* is responsible for the vast majority of the

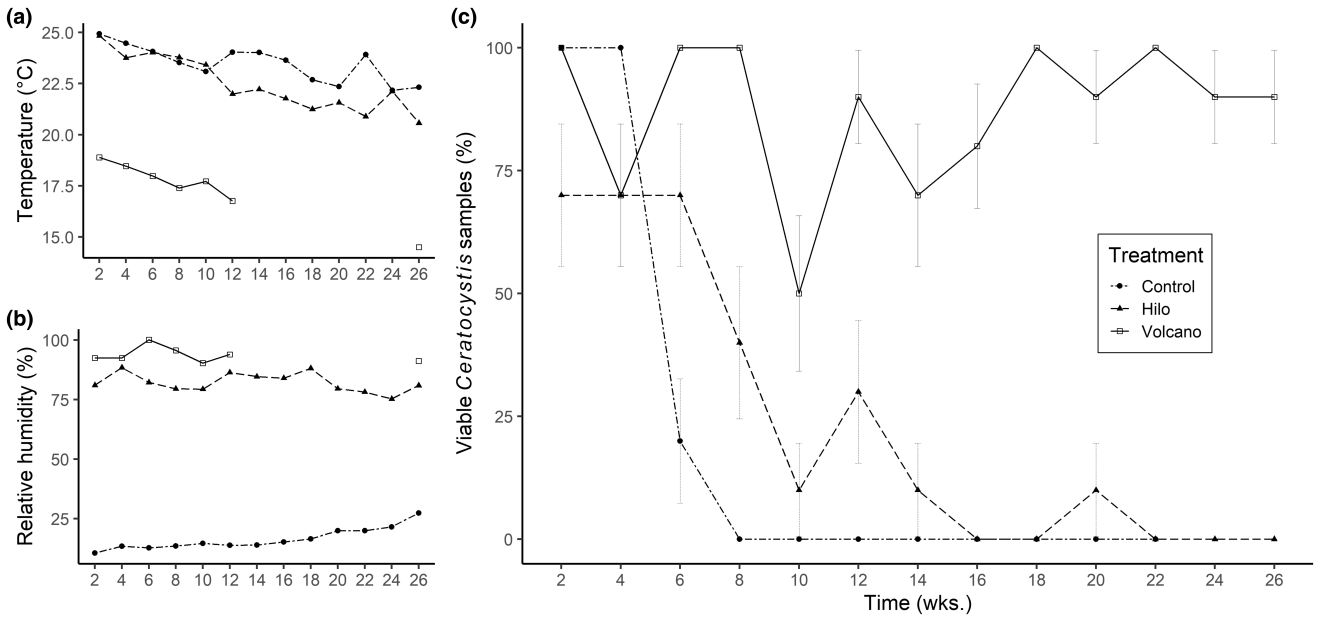

FIGURE 5 Duration of the viability of *Ceratocystis lukuohia* in ambrosia beetle frass in nylon mesh bags under field conditions in Hilo and Volcano sites, and in an environmental growth chamber set to 25°C (control); viability was determined by carrot baiting assays. (a) Temperature and (b) relative humidity for the three treatments (environments); data loggers at the Volcano site malfunctioned during week 14 and were reset for the final collection period. (c) The percentage recovery of viable *C. lukuohia* on carrot baits over time. Data points are the mean of 10 carrot baits, and vertical bars represent standard errors.

mortality across Hawai'i Island, especially in new outbreak areas of ROD (Cannon et al., 2022). *C. huliohia* appears to be less associated with the ROD epidemic across the landscape and, in surveys, tree mortality levels associated with this pathogen may be similar to natural background levels of mortality (F. Hughes, written communication). The presence of both *Ceratocystis* species in frass from KMR and WAI could be due to a background level of *C. huliohia* in declining trees followed by a subsequent wave of *C. lukuohia*-caused mortality. However, further research would be needed to disentangle the phenology of these events, and our limited sampling gives little indication as to the relative incidence of the two species across Hawai'i Island.

Of note, two of the three sites with *C. huliohia*-infested frass (WAI and KK) were in active pastures for cattle grazing, and diseased trees often exhibited extensive bark damage and gashes on the lower trunk, probably caused by the cattle. Ungulate damage and bark stripping negatively affect natural and plantation forests globally (Reimoser & Putman, 2011). In Hawai'i, these wounds may provide infection courts for *Ceratocystis*-laced frass dispersed by wind, rain and soil movement. Perroy et al. (2021) reported that the presence of ungulates (i.e., pigs and cattle) in previously animal-free areas was associated with rapid increases in ROD mortality. Direct transmission of pathogen-infested frass by animal bark-stripping, rooting and rubbing, or by insect vectors carrying the pathogens, have not been verified, but these alternative vector pathways are currently under investigation.

C. lukuohia causes a systemic vascular wilt disease that may completely colonize the stems of trees within months of infection (Hughes et al., 2020). Following mortality, the pathogen continues to colonize the sapwood of dead trees. *Ceratocystis* species are thought to produce aleurioconidia that can be liberated by chainsaws (i.e., in sawdust) or boring insects, such as bark and ambrosia beetles (Harrington, 2013; Iton, 1960; Luchi et al., 2013; Souza et al., 2013). Other members of the Latin American clade of *Ceratocystis*, including *C. fimbriata*, *C. cacaofunesta*, *C. platani* and the related *Davidsoniella australis*, are also believed to be spread through ambrosia beetle frass (Harrington, 2013; Iton, 1960; Kile & Hall, 1988; Ocasio-Morales et al., 2007; Souza et al., 2013). Attack and gallery development by predominantly non-native ambrosia beetles in trees killed by ROD ejects large volumes of *C. lukuohia*-infested frass into the environment that may then be dispersed by wind and rain-splash (Roy et al., 2019, 2020). Contaminated frass may also be deposited onto surrounding soils and can be spread elsewhere by animals, including ungulates and humans (Yelenik et al., 2020).

We believe this study to be the first demonstration that natural ambrosia beetle frass alone, without enrichment by nutrient media, functions as a dispersal agent of infectious *Ceratocystis* inoculum. Iton (1960) was able to induce wilt by artificial inoculation of cacao seedlings with frass enriched with liquid potato dextrose medium. In our trials, the majority of *C. lukuohia*-infected trees sampled in our first inoculation experiment produced frass capable of inducing wilt in at least one inoculated seedling, whilst other frass samples caused infections in all of the inoculated seedlings. The trees from the second trial yielded less infectious frass according to our subsequent seedling inoculations. The lack of infection produced by some frass samples could be due to the loss of viability associated with time since the death of the host tree, time since frass production, ultraviolet light and heat exposure, or density of fungal propagules within

the frass particles. Ambrosia beetles probably tunnel in and out of sapwood tissue colonized by *Ceratocystis*, and frass collected from a given gallery on different days could vary greatly in *Ceratocystis* propagule abundance. Additionally, competing fungi and bacteria also had an opportunity to grow on the frass samples under field conditions before collection. Although our approach avoided excessively dried material, and we confirmed fungal viability by carrot baiting prior to inoculation, we had no control over these biotic and abiotic factors before collection. Despite these constraints, we successfully infected healthy *M. polymorpha* seedlings with *C. lukuohia* using ambrosia beetle frass. However, we were not able to test the pathogenicity of *C. huliohia*-infested frass in this study because we were unable to identify enough trees recently killed by the pathogen to support a seedling inoculation trial.

Previous observations of *C. lukuohia*, *C. huliohia* and other Ceratocystidaceae in woody hosts led researchers to hypothesize that aleurioconidia seen in culture, infected wood samples and inoculated seedlings were the viable propagules in frass (Barnes et al., 2018; Harrington, 2013; Heller et al., 2019; Iton, 1960; Luiz et al., 2021). Aleurioconidia are "pigmented, thick-walled, chlamydospore-like spores borne on specialized conidiophores" (Harrington, 2013). *Ceratocystis* aleurioconidia are produced abundantly in chains, are dormant, heavily melanized and do not stain with LCB. They are thought to be the form of inoculum that allows some *Ceratocystis* species to survive for years in soil (Harrington, 2013; Sy, 1956). However, no aleurioconidia were seen in the hundreds of frass particles we examined. Instead, only chlamydospores with unpigmented to lightly pigmented cell walls and deeply stained cytoplasm were seen. Chlamydospores are secondarily formed from pre-existing hyphal cells or spores via internal cell wall thickening. True chlamydospores have been noted in a few *Ceratocystis* species (Johnson et al., 2005), but some researchers have erroneously referred to aleurioconidia as chlamydospores. The *C. lukuohia* chlamydospores we found in frass exhibited internally thickened walls, at times consisting of multiple layers and their cytoplasm stained deeply, suggesting that the thickened cell walls were more permeable than the walls of aleurioconidia and perhaps would more readily germinate if they landed on a suitable substrate. No conidiophores were associated with these chlamydospores, which were globose to subglobose and generally smaller than the obovoid aleurioconidia formed in culture (Barnes et al., 2018). Some particularly moist wood chip samples from trees killed by *C. lukuohia* exhibited darkly pigmented, obovoid aleurioconidia produced from conidiophores in vessels and some groups of aleurioconidia had individual spores that appeared to be transitioning to chlamydospores, in that they were smaller, globose, had thicker walls and had lightly stained cytoplasm. It is also possible that chlamydospores were formed from hyphae, but no such hyphae were observed. The transformation from aleurioconidia to chlamydospores could be initiated by lower moisture content or mechanical damage as the wood is masticated by ambrosia beetles. Iton (1960), studying Ceratocystis wilt of cacao, noted an abundance of "chlamydospores" produced from conidiophores surrounding *Xyleborus* boring tunnels and in frass particles. He was probably referring to aleurioconidia produced in the galleries, but his figure 6 illustrates what appears to be aleurioconidia transitioning to chlamydospores in a wood sample, much like the developing chlamydospores we observed in some ʻōhiʻa wood samples. Regardless of the confusion between these two spore types, we did not observe any other *Ceratocystis* propagule types in ambrosia beetle frass. The chlamydospores in frass were not clustered, and they appeared to have adhered to the inner cell walls of ʻōhiʻa vessels and thus, could be dispersed with the frass particles.

Based on the staining of cytoplasm with LCB, chlamydospores may not survive as long as aleurioconidia under harsh environmental conditions and microbial activity. *C. lukuohia* remained viable in some frass samples for the entire duration of the 26-week experiment. In comparison, *C. platani* can survive more than 15 weeks in soil (Accordi, 1989), and the sweet potato strain of *C. fimbriata* can survive more than 2 years in soil (Sy, 1956). According to our statistical models, the viability of *C. lukuohia* in frass was negatively affected by increased temperature, time and decreased RH. Over 75% of the samples at the Volcano site remained viable throughout the study period, probably due to the cool temperatures there compared to the warmer Hilo site and the control incubator. Similarly, *C. platani* survived the longest in cool winter soils compared to warmer soils in the spring and summer (Accordi, 1989). Although the Hilo site and the incubator had similar temperatures, the RH at Hilo was on average 82%, much higher than in the incubator (21%); this higher RH may be associated with the extended viability of *C. lukuohia* in the frass kept at Hilo (22 weeks compared to 8 weeks in the incubator). Other examples within the Ceratocystidaceae include the germination of *Berkeleyomyces basicola* aleurioconidia, which was highest in cool rather than warm soils (Papavizas & Lewis, 1971) and required a minimum RH of 81% to survive and germinate in laboratory assays (Brown & Harrower, 1997). Both temperature and RH affect wood moisture content and so are likely to affect *C. lukuohia* viability in frass. In *M. polymorpha* wood bolts, air-drying over a 10-week period reduced wood moisture content throughout the wood profile and decreased *C. lukuohia* viability compared to non-dried samples (Hughes et al., 2021). Due to our limited amount of *Ceratocystis*-colonized frass, we were not able to measure wood moisture content or expose frass to the natural wet/dry cycles that would occur on tree bark or in the soil to determine its effect on viability. However, in Volcano, *C. lukuohia* remained viable 6 months after collection and dispersal of *C. lukuohia* in frass would most probably take place within days or weeks of production.

In conclusion, we have found that ambrosia beetle frass liberated from diseased ʻōhiʻa trees can serve as inoculum in the ROD pathosystem, particularly frass from trees killed by *C. lukuohia*, which causes the devastating Ceratocystis wilt of ʻōhiʻa (Hughes et al., 2020). Ambrosia beetle frass contains thick-walled chlamydospores of *C. lukuohia*, and chlamydospores within the frass particles are probably the propagules that have been detected in soil (Heller

& Keith, 2018; Yelenik et al., 2020) and wind (Atkinson et al., 2019) in affected stands.

ACKNOWLEDGEMENTS

Funding for M.A.H. was provided by the Pacific Cooperative Studies Unit of the University of Hawai'i at Mānoa and the USDA-Forest Service, Institute of Pacific Islands Forestry in Hilo, HI (agreement number 19JV11272136-037). We would like to thank Ellen J. Dunkle and Dan Mikros (Hawai'i Cooperative Studies Unit [HCSU], the University of Hawai'i at Hilo) for field and laboratory support, Allison Lampe and Jenna Vickery (Iowa State University) for laboratory support, Bruce Mackey (USDA Agricultural Research Service), Kevin Brinck (HSCU), Robert W. Peck, (HCSU) and Helen Sofaer (U.S. Geological Survey) for their study design and/or statistical analysis support. We thank Craig Blaisdell (Keaukaha Military Reserve) and Steve Bergfeld (Hawai'i Division of Forestry and Wildlife) for field site access, and Dustin Swan and the ROD field team from the Big Island Invasive Species Committee for assistance in sample collection. We kindly acknowledge R. Flint Hughes (USDA Forest Service—Institute of Pacific Islands Forestry) for his presubmission review of this manuscript. Financial support for this work was provided by the U.S. Forest Service and Hawai'i Division of Forestry and Wildlife. Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Dept. of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable. Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data for the frass survival experiment is available in Roy and Dunkle (2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hughes, M.A., Roy, K., Harrington, T.C., Brill, E. & Keith, L.M. (2022) *Ceratocystis lukuohia*-infested ambrosia beetle frass as inoculum for Ceratocystis wilt of ʻōhiʻa (*Metrosideros polymorpha*). *Plant Pathology*, 00, 1–14. Available from: https://doi.org/10.1111/ppa.13653