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ORIGINAL ARTICLE

Pathogenicity and colonization of *Metrosideros polymorpha* by *Ceratocystis huliohia*

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Abstract

Both *Ceratocystis lukuohia* and *C. huliohia* have been associated with Rapid ʻŌhiʻa Death (ROD), an emerging threat to ʻōhiʻa (*Metrosideros polymorpha*), a keystone forest tree species. The vascular wilt disease caused by *C. lukuohia* has been recently described and is responsible for the widespread ROD epidemic on Hawai'i Island. However, the role of *C. huliohia* in ROD development and tree death is not clear. Artificial inoculation of field-grown ʻōhiʻa with *C. huliohia* and dissections of naturally infected, early symptomatic forest trees were conducted to confirm pathogenicity on field grown trees and the pattern of internal colonization. In two trials, crowns of trees with main stems inoculated with *C. huliohia* were visually healthy at the time of tree harvest after 43–55 days in the first trial, and after 91 days in the second trial. However, elliptical inner bark cankers underlain by reddish-brown xylem were associated with the inoculation points. Similar canker and stain symptoms were found on stems and branches of ʻōhiʻa (24–26 cm trunk diameter) naturally infected by *C. huliohia*. This xylem stain manifested as multiple distinct elliptical cankers or the coalescing of multiple cankers. The pathogen was commonly isolated from the perimeter of the stained outer sapwood and to a depth of 4 cm. The coalescence of multiple cankers was associated with the crown symptoms observed on the naturally infected forest trees that were dissected. Multiple *C. huliohia* infections that lead to coalescing cankers which subsequently girdle stems likely occurs over one or more years compared to the shorter time (e.g., months) required for *C. lukuohia*-caused death to occur.

KEYWORDS
canker disease, *Ceratocystis* canker of ʻōhiʻa, rapid ʻŌhiʻa death

1 | INTRODUCTION

ʻŌhiʻa (*Metrosideros polymorpha* Gaudich.) is one of five recognized, endemic species of the genus in Hawaiʻi. This member of the myrtle family has eight named varieties that occur in different habitats and climate zones across the Hawaiian Islands (Dawson & Stemmermann, 1999). ʻŌhiʻa is the most abundant native forest tree on Hawaiʻi Island, HI, where it occurs on approximately 250,000 ha. The species is also the most ecologically and culturally important tree species in the state (Mueller-Dombois et al., 2013). Besides providing habitat for endangered flora and fauna, ʻōhiʻa forest ecosystems protect the watersheds that provide freshwater for the Hawaiian Islands (Burnett et al., 2017; Cannon et al., 2022). Besides its ecological role, ʻōhiʻa serves as a foundation for native Hawaiian biocultural practices and is a source of high-value local wood products.

An emerging disease, Rapid ʻŌhiʻa Death (ROD), has caused widespread and extensive mortality of ʻōhiʻa on Hawai'i Island since it was observed in 2010 (Mortenson et al., 2016) and has since been confirmed in all districts of Hawai'i Island and certain regions of Kaua'i and O'ahu. A single instance of ROD was discovered on Maui, yet the disease is now considered eradicated on this island. No active cases of ROD were known to exist on Maui in 2023 (L. Keith, personal communication). Symptom progression can occur quickly, starting with reddish-brown leaves on individual branches and ending with a completely dead crown with leaves attached within weeks to several months of initial symptom onset. A *Ceratocystis* sp. was first isolated in 2014 from stained wood samples obtained from a recently killed ʻōhiʻa. Pathogenicity was confirmed on 1- to 2-year-old ʻōhiʻa seedlings grown in a growth chamber and the fungus identified using ITS rDNA sequences as *Ceratocystis fimbriata* the following year (Keith et al., 2015). Shortly thereafter, a second *Ceratocystis* sp. was obtained from other recently killed trees with ROD-like symptoms. Two new *Ceratocystis* species (*C. lukuohia* and *C. huliohia*) were subsequently characterized and described based on morphological characteristics, results of phylogenetic analyses, pathogenicity and host range testing, and the biological species concept (Barnes et al., 2018). *Ceratocystis lukuohia* is now known to cause a systemic vascular wilt disease in ʻōhiʻa that may result in tree mortality in as little as 42 days after artificial inoculation (Hughes et al., 2020). The rapid spread of stain along the outer xylem of the stem as well as extensive wilting of the crown is consistent with passive transport of pathogen conidia in the sap stream of ʻōhiʻa. Radial streaks of stain occur as a ring around the outer sapwood in transverse stem sections of recently killed trees. In contrast, *Ceratocystis huliohia* is associated with an amorphous staining pattern in cross sections of trees infected with this species (Barnes et al., 2018). Slower, but similar wilting to that caused by *C. lukuohia* in young seedlings was found in early greenhouse pathogenicity tests with *C. huliohia*. Observations of ROD symptoms in naturally infected ʻōhiʻa in forested areas supported the assertion that disease progression is more variable throughout infected crowns and death in trees infected by *C. huliohia* (Figure 1) compared to those infected by *C. lukuohia*. Details, however, are lacking on how *C. huliohia* colonizes trees and causes ʻōhiʻa mortality.

Based on multigene phylogenetic analyses, Barnes et al. (2018) found the two ROD *Ceratocystis* species reside in two distinct lineages, the Latin American and Asian-Australian clades. Among other species, the Latin American clade includes *C. lukuohia* and *C. platani*, the causal agent of canker stain disease (CSD) of plane trees (*Platanus*). The latter is a serious threat to Oriental plane and London plane trees in Europe where the non-native pathogen has caused widespread mortality (Tsopelas et al., 2017). Like *C. lukuohia*, *C. platani* causes extensive wood staining in infected trees although it is found underlying sunken, cankered areas of the bark. Other important members of the Latin American clade include *C. cacoafunesta* that attacks cacao trees (*Theobroma cacao*) and the type species for the genus, *C. fimbriata* (Halsted, 1890). *Ceratocystis huliohia* belongs to the Asian-Australian clade, which includes species such as *C. uchidae*, *C. changhui*, and *C. cercfabiensis*. Pathogenic species within this clade are considered less aggressive than pathogens in the Latin American clade (Harrington, 2013; Li et al., 2016).

Since both *C. lukuohia* and *C. huliohia* are associated with ROD in Hawai'i, studies were initiated in 2017 to clarify the pathology and host colonization patterns of these species to ʻōhiʻa. Field-based inoculation studies with *C. lukuohia* led the authors to classify the disease it causes as a systemic vascular wilt and confirmed its original name as *Ceratocystis* wilt of ʻōhiʻa (Hughes et al., 2020; Keith et al., 2015). This pathogen is only known to exist on the islands of Hawai'i and Kaua'i, while *C. huliohia* has been found on O'ahu and Maui as well as Hawai'i and Kaua'i.

Here we report results of a companion investigation designed to characterize the disease caused by *C. huliohia* on *M. polymorpha*. Field studies involving both artificially inoculated and naturally infected trees were designed to (1) determine pathogenicity of *C. huliohia* on field-grown trees, and (2) to characterize the symptomology and internal colonization of both inoculated and naturally infected ʻōhiʻa by *C. huliohia*. A comparison of the diseases caused by *C. huliohia* and *C. lukuohia* is then provided based on these results. A preliminary report has been published (Juzwik et al., 2019).

2 | MATERIALS AND METHODS

2.1 | Sites and tree selection for inoculations

Forest stands with naturally regenerated *M. polymorpha* var. *incana* in Hilo, Hawai'i, were utilized for the studies. Both sites had healthy and ROD-diseased trees in the immediate vicinity. Sixteen single-stem trees (10.2–18.0 cm diameter at 1.4 m or DBH) with visually healthy crowns were selected in the lowland forests of the Keaukaha Military Reservation (KMR) (19°42′54.71″ N, 155°3′3.23″ W) for the pathogenicity trials involving artificial inoculations. Three diseased ʻōhiʻa (*M. polymorpha* var. *incana*) exhibiting early crown symptoms of ROD (branch dieback with yellow, wilting foliage) were selected from KMR and one other *M. polymorpha* var. *glaberrima* near Stainback Highway in a forest in Waiākea Forest Reserve (19°37′21.70″ N, 155°6′17.84″ W) for investigation of *C. huliohia* colonization in naturally infected trees.

2.2 | Fungus isolates and inoculum production

Two fresh isolates of *C. huliohia* were obtained in April 2017 from stained sapwood of two symptomatic ʻōhiʻa at KMR using the carrot-baiting protocol of Moller and DeVay (1968). Once in pure culture, a multi-spore isolate derived from an ascospore mass taken from a single perithecium was obtained from each tree sample and fungal species identity was verified using a qPCR protocol (Heller & Keith, 2018). The isolates were stored on 10% V8 media in petri dishes at 25°C in an incubator. A mixture of the two isolates was used to produce inoculum for the field experiments. Two types of

FIGURE 1 Slow progression of dieback in branches of *Metrosideros polymorpha* tree crowns results from infection by *Ceratocystis huliohia*. Symptomatic crowns of three multi-stemmed trees (in foreground) are shown here (Volcano, HI, 2017). *Photo source*: Wade Heller, USDA-ARS.

inoculum, a *C. huliohia*-colonized agar slurry and filter paper discs, were prepared according to protocols described in previously published *C. lukuohia* pathogenicity studies (Hughes et al., 2020) with one exception. Specifically, the two isolates were mixed in preparing each inoculum type. The inoculum for control treatments was either a sterile agar slurry or a sterile, moist filter paper disc.

2.3 | Host inoculation

For the May/June 2017 field pathogenicity trial, a battery-operated drill with a 35-mm-diameter circular hole-saw bit (Hole Dozer, Milwaukee Tool) was used to create one (for sterile control) or two (for inoculum involving mixed fungal isolates) wounds (approx. 10 cm^2 surface area) through the vascular cambium into the outermost sapwood at approximately 1.4 m stem height. The bark plug was carefully removed and subsequently used to cover the treated wound surface. For two trees, *C. huliohia* colonized filter paper circles were carefully placed with the fungus-colonized side against the exposed xylem of two wounds (on opposite sides of the stem). For two control trees, one sterile filter paper circle was similarly placed on the wounded xylem surface on the main stems. For the agar slurry treatments, *C. huliohia* colonized slurry was applied uniformly over the two exposed sapwood wounds on opposite sides of the stem of two trees, while sterile agar slurry was applied to one exposed wound on each of two trees. In all cases, the reserved bark plug was placed over the treated wound immediately after inoculum was applied and the plug edges sealed with plumber's putty (Oatey). Based on results of a preliminary pilot study that suggested slower xylem colonization rates associated with filter paper inoculum compared to agar slurry, the filter paper treatments were applied 12 days earlier than the agar slurry ones. In total, eight trees were inoculated for this (May/June 2017) trial (Table 1).

The same type and numbers of wounds were applied to another eight healthy ʻōhiʻa in the August 2017 field pathogenicity trial (Table 2). Both fungus and control inoculum types were used, but all treatments were applied on the same day (August 8). Bark plugs were placed over each treated wound surface and then sealed as described above. All study trees were regularly monitored for visible crown symptoms.

TABLE 1 Characteristics of inner bark cankers and underlying xylem stain within 43–55 days following artificial inoculation of pole-timber size (12.7–17.5 cm dia at 1.4 m stem height = DBH) *Metrosideros polymorpha* var. *incana* with *Ceratocystis huliohia*, in late May and early June 2017, Hilo, Hawai'i.

Inoculation month[a]	Inoculum type[b]	Inoculation points/tree[c] (number)	Treatment duration (days)	Canker dimensions (avg.)			Depth of stain (avg. max.)[e] (cm)	Recovery of fungus by depth (cm)[f] (number positive/number assayed)			
				Length (cm)		Area (cm^2)[b]		0–1	1–2	2–3	3–4
				Above IP[d]	Below IP						
May	FP	2	55	17.4	37.1	249.6	2	13/13	13/13	1/1	nt[g]
May	Con FP	1	55	1	1.6	18.1	0	0/2	0/2	nt	nt
June	AS	2	43	20	32.6	243.5	1.5	14/19	11/19	4/8	nt
June	Con AS	1	43	0.8	0.8	12.6	0.4	0/5	0/5	nt	nt

[a]Filter paper (FP and Con FP) inoculum was applied 26 May and agar slurry (AS and Con AS) inoculum applied 7 June.
[b]AS, slurry of *C. huliohia* – colonized agar medium; Con AS, slurry of sterile agar medium; Con FP, sterile filter paper; FP, *C. huliohia* – colonized filter paper.
[c]Two trees were used per inoculum type, but number of inoculation points used per tree differed by fungus versus control inoculum. A bark disc was removed with hole saw to expose outer sapwood ($10\,cm^2$) to which inoculum was applied.
[d]IP, inoculation point.
[e]Multiple stem discs were taken in a systematic manner along log length between each end of a visual canker. Number of discs taken depended on canker length. Radial depth of stain into the xylem was measured from the cambium for each disc.
[f]Thin wood wafers were removed with a sterilized chisel at specified depths on each disc and subjected to carrot-baiting assay (Moller & DeVay, 1968).
[g]Canker stain was not visible at these radial depths and thus samples were not tested (n.t.).

2.4 | Naturally infected trees

To compare with artificially inoculated trees, four naturally infected 'ōhi'a exhibiting early crown symptoms of individual branch dieback with wilted foliage (between 30% and 45% affected crown) were examined and *C. huliohia* presence confirmed by qPCR assay (Heller & Keith, 2018). However, extensive examination and dissection were conducted on only a single individual tree from each of the two study sites.

2.5 | Tree harvest and dissection

The artificially inoculated trees from the May/June trial were felled in late July 2017 (55- or 43-days post-inoculation for filter disc and agar slurry inoculated trees, respectively) (Table 1), while those in the August pathogenicity trial were felled in early November 2017 (91 days post-inoculation) (Table 2). Immediately after felling, close inspection was made of the stem portions above and below each inoculated point and the bark carefully removed in selected places to help delineate extent of any existing canker or otherwise necrotic tissue. Stems were marked for cutting at approximately 30 cm above or below the upper or lower margin of any observed canker/necrosis. The logs from these marked stem sections were labelled and transported to the USDA Agricultural Research Service, Pacific Basin Agricultural Research Center (ARS PBARC) in Hilo for processing. There the outer bark of each log was removed using an ethanol-sterilized drawknife to sequentially expose the phloem and the outer xylem. Cankers associated with each fungus-inoculated wound or necrotic tissue around sterile control wounds were delineated by red dots using a paint pen (Markal, LaCO Industries), photographed, and measured. The area of cankered/necrotic tissue on the outer xylem was calculated using the formula for a standard ellipse ($A = \pi \times a \times b$, where A is the area, a is length of the semi-major axis, and b is length of the semi-minor axis).

Four naturally infected trees were felled between late April and late July 2017. In April, the stems of one KMR tree (KMR Nat1) were carefully inspected on the ground immediately after felling with emphasis placed on portions associated with the lower stem or with a major side branch exhibiting wilting foliage. Following the felling of the KMR Nat1 tree, multiple logs (approx. 1.4 m long) were cut from the base of the tree to a 10-m height. The logs were labelled and the stem height at which each had been cut was recorded. All logs were then transported to PBARC for extensive examination. In July, two additional naturally infected trees with crown symptoms from KMR (KMR Nat 2 and KMR Nat 3) were felled and logs were transported to our field lab. Also in July (2017), a naturally infected tree (SB Nat 3) was selected from the Waiākea Forest Reserve near Stainback Highway and felled at 0.5 m above the ground for dissection and close examination. This was done to capture as much of the lower stem as possible due to the existence of a major side branch (at approximately 2 m stem height) that was exhibiting yellow, wilting foliage. Preliminary examination along the stem and the side branch

TABLE 2 Characteristics of inner bark cankers and underlying xylem stain 91 days following artificial inoculation of pole-timber size (10.2–18.0 cm dia at 1.4 m stem height = DBH) *Metrosideros polymorpha* var. *incana* with *Ceratocystis huliohia*, on 8 August 2017, Hilo, Hawai'i.

Inoculum type[a]	Inoculation points/tree[b] (number)	Canker dimensions (avg.) Length (cm)		Area (cm[b])	Depth of stain (avg. max.)[d] (cm)	Recovery of fungus by depth (cm)[e] (number positive/number assayed)			
		Above IP[c]	Below IP			0–1	1–2	2–3	3–4
FP	2	14.4	22.6	219.7	3.2	14/16	14/16	13/16	3/6
Con FP	1	2.6	2.8	16.8	0.4	0/5	0/5	nt[f]	nt
AS	2	32.9	68	458.2	3.8	15/17	11/17	10/17	2/2
Con AS	1	2.8	2.8	15.8	0.6	0/5	0/5	nt	nt

[a]AS, slurry of *C. huliohia*-colonized agar medium; Con AS, slurry of sterile agar medium; Con FP, sterile filter paper; FP, *C. huliohia* – colonized filter paper.

[b]Two trees were used per inoculum type, but number of inoculation points used per tree differed by fungus versus control inoculum. A bark disc was removed with hole saw to expose outer sapwood (10 cm^2) to which inoculum was applied.

[c]IP, inoculation point.

[d]Multiple stem discs were taken in a systematic manner along log length between each end of a visual canker. Number of discs taken depended on canker length. Radial depth of stain into the xylem was measured from the cambium for each disc.

[e]Thin wood wafers were removed with a sterilized chisel at specified depths on each disc and subjected to carrot-baiting assay (Moller & DeVay, 1968).

[f]Canker stain was not visible at these radial depths and thus samples were not tested (n.t.).

was made and three logs were cut and transported to PBARC for processing. The initial dissection involved careful removal of the bark with a drawknife to expose the extent of each canker present. Length and width were measured for each canker found and data were recorded by log section.

Logs from both the artificially inoculated and the naturally infected trees that exhibited one or more cankers upon bark removal were then taken to a local sawmill and, depending on diameter, were either half or quarter-sawn along the length of the log. The sawn log portions were taken to the wood dissection laboratory at the Komohana Research and Extension Center, University of Hawai'i at Mānoa, in Hilo, for examination and measurement of internal dimensions of canker stain in the xylem. Half or quarter discs were cut with a sliding compound mitre saw (Makita) from the log portions along the length of most cankers to provide samples to detect viable *C. huliohia* along the length of the canker and depth of the canker stain.

2.6 | Fungus bioassay

The visible canker stain on each obtained wood disc portion was delineated and the radial depths from the cambium inward were marked at 1 cm increments. Ethanol and flame-sterilized chisels and mallets were then used to tangentially open the outer disc to the middle of the marked depth zones and remove thin wood wafers for fungus bioassay. A modified version of the carrot-baiting assay developed by Moller and DeVay (1968) was used to detect presence of viable *Ceratocystis*. The modified protocol used is as described by Hughes et al. (2020). If the carrot baits of the 'ōhi'a wood wafers did not yield perithecia of *Ceratocystis* within 30 days but had greyish mycelia typical of ROD fungi, small pieces of the colonized carrot were excised with a scalpel and subjected to qPCR (Heller & Keith, 2018). In addition, qPCR assay was also performed on a representative subsample of the carrot baits with *Ceratocystis* perithecia to determine presence of *C. huliohia* and absence of *C. lukuohia*.

3 | RESULTS

3.1 | Pathogenicity of *C. huliohia* on field grown trees

No crown symptoms (yellowing, wilt, or dieback) were observed on the trees inoculated with *C. huliohia* in either May/June or August trials. However, long elliptical phloem (inner bark) cankers (220–458 cm^2 in area) (Figure 2a) were associated with fungus-inoculated stems compared to very limited bark necrosis surrounding the sterile control inoculations (Tables 1 and 2). Canker areas appeared similar between filter paper and agar slurry inoculum types in the May/June trial (Table 1); however, the filter paper inoculations were performed 12 days before the agar slurry ones. In contrast with the August trial (Table 2), canker areas for agar slurry inoculations were over twice as large as for filter paper inoculations when all treatments were applied on the same day. Upon further dissection, the xylem to the inside of bark cankers contained reddish-brown stain (Figure 2b). The canker stain extended above and below the inoculation point, radially inward, and laterally (to the side) in relation to the vertical (axial) stain (Figure 2a,b). Average lengths of fungus-caused cankers were greater below the inoculation points (23–68 cm) than above the points (14–33 cm) for both May/early June and August field trials (Tables 1 and 2, respectively). In cross-sections, the corresponding

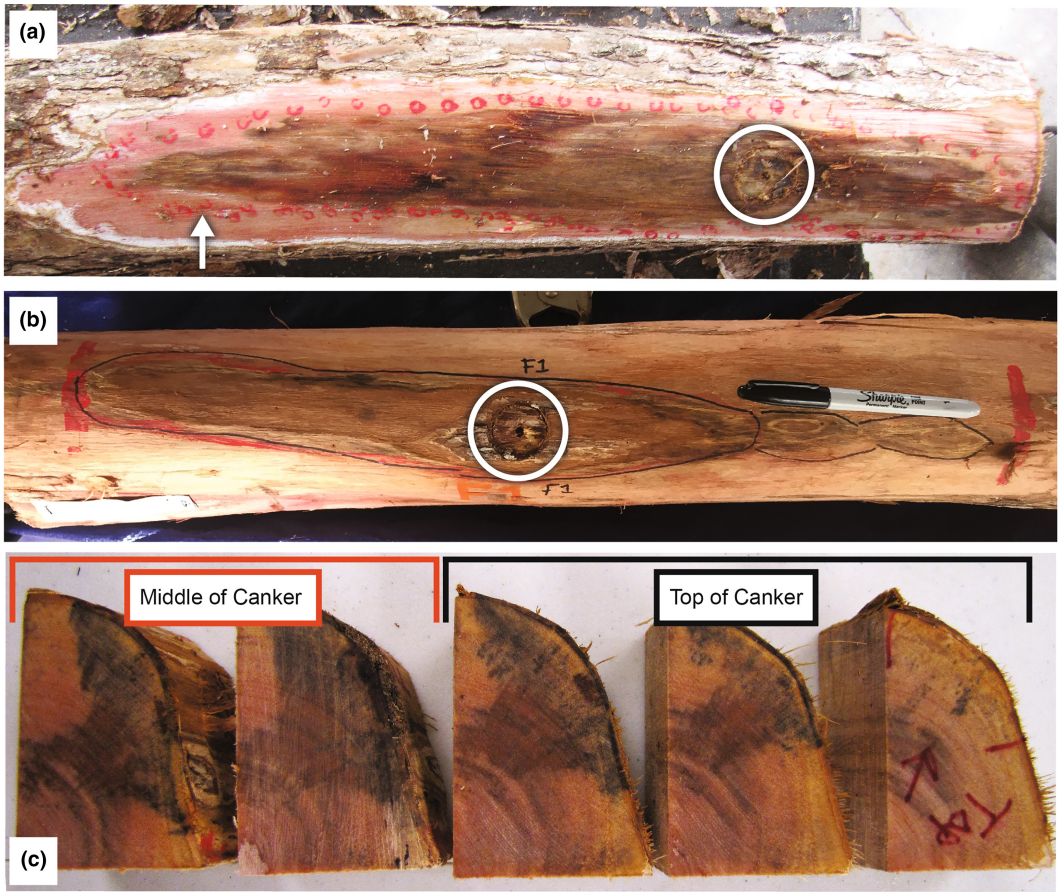


FIGURE 2 *Ceratocystis huliohia* associated canker stain in dissected stems of artificially inoculated *Metrosideros polymorpha*. (a) Delineated (red dots) canker of phloem (inner bark) and outer xylem underlying removed bark. (b) Exposed canker stain in outer xylem following bark removal. White circular lines indicate the inoculated area (3.5 cm diameter circular area). (c) Transverse sections from quarter-sawn log showing sequential depth and width of stain in xylem from middle to top of a canker.

but greyish-tinged stain had a "smudge-like" appearance (Figure 2c). The maximum depth of stain for fungus-inoculated stems ranged from 1.5 to 3.8 cm from the cambium (Tables 1 and 2) compared to the 0–0.6 cm depth for the dark brown stain associated with the control inoculations. Between 13 and 19 half or quarter discs were systematically cut along the length of the stem section taken from the inoculated trees with numbers depending on size of canker observed. Between two and five similar disc portions were cut from the necrotic areas on the control trees. Using our carrot-baiting assay, *C. huliohia* was commonly detected in thin wood wafers taken from the outer 0–2 cm of xylem of disc portions from May/June and August fungus-inoculated trees and for 2–3 cm radial depth of May/June fungus agar slurry trees and for all August fungus-inoculated trees (Tables 1 and 2). Because stain was infrequently or not observed at 3–4 cm depths, fewer carrot-baiting assays were conducted for that zone. However, *C. huliohia* was found at this greater depth for both fungus inoculum types 91 days after the August inoculations were made (Table 2). The fungus was not detected in assayed xylem from dark brown stained areas associated with control inoculations in either trial. Representative subsamples of carrot-baits yielding perithecia were found to yield only *C. huliohia*. Specifically, the pathogen species was verified using qPCR testing of a 21.8% subsample of the 55 perithecia-positive baits for May/June inoculated trees; however, the data for the subsamples from the August inoculated trees is missing.

3.2 | Colonization of naturally infected trees

Naturally infected 'ōhi'a trees with early crown symptoms observed between late April and late July were selected for comparative study. The lowland (22 m above sea level) forest tree, KMR Nat 1 (24.0 cm DBH, felled 26 April 2017), had 40% of its upper crown with symptoms of ROD (yellow foliage, dieback with thinning crown). Two other KMR trees (KMR Nat 2 & 3) with 30%–40% crown wilt located in the same forest were felled between mid- and late July but were not dissected as extensively as the first KMR tree. For the upland site, tree SB Nat 3 (26.4 cm DBH) was selected and felled on July 24 for extensive dissection. This tree had symptomatic foliage only on a large, lower fork and a side branch which were estimated to be 35% of the total tree crown (Figure 3a).

Closer inspection of the lower stem of SB Nat 3 and one of the KMR trees (KMR Nat 2) found copious amounts of exuded frass (ambrosia beetle boring powder) around the lower 1.4 m of the tree

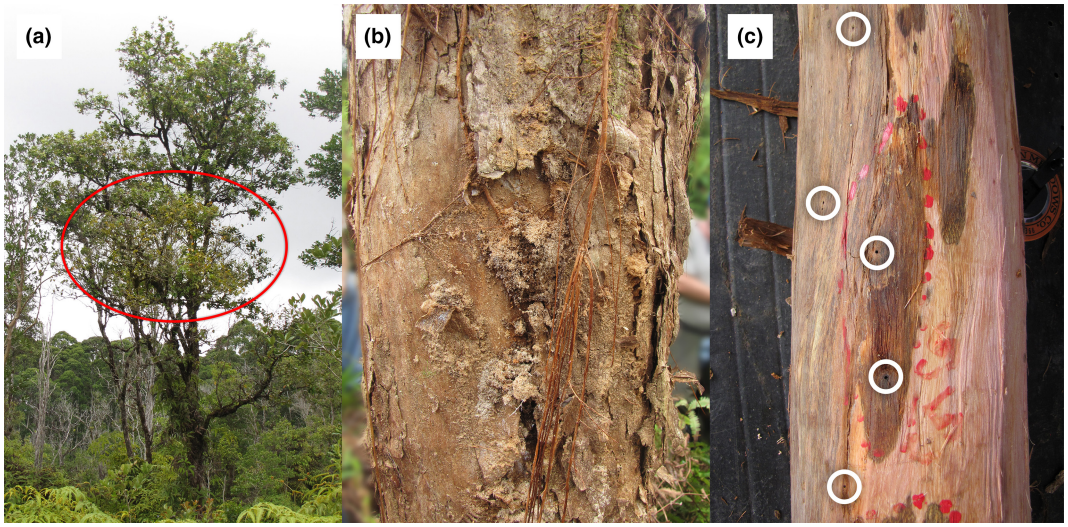
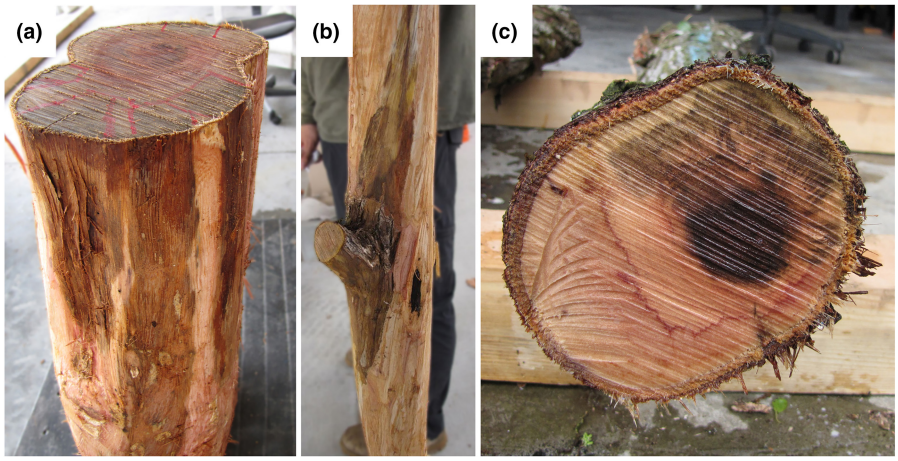


FIGURE 3 Visual symptoms associated with *Metrosideros polymorpha* colonization by *Ceratocystis huliohia* in naturally infected forest trees, Hilo, Hawai'i. (a) Lower portion of SB Nat 3 tree crown exhibiting yellowing symptoms (inside red line) in late July 2017. (b) Copious amounts of frass produced by ambrosia beetles on the lower stem of SB Nat 3 and (c) small pin-hole sized entry or exit holes characteristic of ambrosia beetles (white circular lines) on or near exposed xylem of canker on stem section of a KMR naturally infected tree.

FIGURE 4 Internal sapwood stain associated with *Metrosideros polymorpha* colonization by *Ceratocystis huliohia* in naturally infected forest trees, Hilo, Hawai'i. (a) Multiple and coalescing cankers associated with *C. huliohia* in the lower, de-barked stem of KMR Nat 2, (b) Main stem canker surrounding a dead branch higher on main stem of KMR Nat2, and (c) Cross-section of the main stem of a KMR naturally infected *M. polymorpha* tree showing amorphous, "smudge-like" stain in xylem (dark stain is natural heartwood).



stems (Figure 3b) and boreholes in the sapwood showed evidence of extensive attack and colonization by ambrosia beetles (Figure 3c). For the lower portion of the KMR Nat 2 tree colonized by ambrosia beetles, bark removal revealed multiple, coalescing cankers. Eight cankers were observed with four that extended the whole length of the lower main stem (0.4–1.2 m) (Figure 4a). Evidence suggesting pathogen spread from a side branch to the main stem was found after de-barking a stem section from 7.7 to 9.2 main stem height of KMR Nat 2 (Figure 4b). The "smudge" stain characteristic of natural *C huliohia* colonization was also observed in cross-section of the main stems of the felled trees (Figure 4c).

Six stem sections (each 121 cm long) cut from KMR Nat 1 were carefully debarked and revealed multiple, elongate cankers present on the lower 11 m of the tree's main stem (Table 3). The observed spiral orientation of the cankers reflected the spiral axial growth habit of xylem commonly found in field grown 'ōhi'a (evident in Figure 4b). In addition, two forks of an upper main stem section and sections of four side branches were cut and bark removed to expose cankers. Twelve cankers from this tree were more closely examined. The cankers ranged from 33.0 to 96.5 cm in length and 2.5–15.4 cm in width, as measured on the outer xylem (Table 3). Maximum radial depth of canker stain ranged from 2.0 to 7.0 cm for the cankers. Between three and seven half or quarter discs were cut along the length of each canker. Based on carrot-baiting assay results, viable *C. huliohia* was commonly detected in the outer 2 cm of xylem on the discs (Table 3). The frequency of fungus detection was higher for the outer 2 cm of the sapwood than the deeper 2–4 cm zones. The pathogen species was verified based on qPCR testing of a 19.6% subsample of the 133 perithecia-positive carrot baits assayed.

Three lower stem sections (between 1.5 and 4.0 m above ground) cut from SB Nat 3 tree were also carefully de-barked and revealed an encircling canker around the lower side branch of a stem fork (left fork canker 1 & 2 = LF1 & 2) that was exhibiting foliar wilt symptoms. This large canker (194 cm long × 31 cm wide) may have resulted from coalescing of two or more cankers (Table 3). A small canker (RF1) was found on the right fork (i.e., the lower branch) distal to the

TABLE 3 Dimensions of canker stain and characteristics of *Ceratocystis huliohia* isolations in secondary xylem of two *Metrosideros polymorpha* trees naturally infected with *Ceratocystis huliohia* and felled in late April (KMR Nat 1) and late July 2017 (SB Nat 3), Hilo, Hawai'i.

Tree ID[a]	Dimensions of canker stain					Characteristics of *Ceratocystis huliohia* isolations				
	Height of sampled stem section (cm)	Canker ID[b]	Canker stain dimensions (cm)[c]			Locations assayed along canker length (cm)[e]	Pathogen isolation by depth (cm)[f] (number. Positive/number assayed)			
			Length	Width (max.)	Depth (max.)		0–1	1–2	2–3	3–4
KMR Nat 1	242–363	3	74.8	7.0	5.2	2–72	5/5	5/5	5/5	5/5
	363–484	4	51.5	3.7	3.4	2–51	4/4	3/4	4/4	0/4
	484–605	5	96.5	>12.0	7.0	2–95	6/7	5/7	7/7	4/7
	726–847	7A	51.8	3.2	2.0	0–48	1/4	1/4	1/4	1/4
	726–847	7B	96.0	2.5	3.2	0–93	5/6	5/6	2/6	2/6
	726–847	7C	41.5	7.0	4.5	0–40	4/4	4/4	2/4	1/4
	847–968	8A	>36.0	8.6	4.0	0–35	4/4	3/4	2/4	3/4
	847–968	8B	43.7	6.4	4.1	0–40	3/4	1/4	1/4	2/4
	847–968	8C	n.d.[d]	n.d.	n.d.	n.d	n.d	n.d	n.d	n.d.
	968–1089	9A	110.0	15.4	3.0	0–108	5/7	5/7	3/7	2/7
	968–1089	9B	35.0	Coalesced	2.7	0–30	3/3	3/3	0/3	0/3
	968–1089	9C	33.0	3.0	2.0	0–33	3/3	2/3	1/3	0/3
SB Nat 3	146–206	LM 0	4.0	3.5	2.0	2	1/1	1/1	1/1	n.t.[g]
	206–448	LF1& 2	194.0	31.0	4.8	0–200	6/7	5/7	4/7	1/3
	206–326	RF1	14.0	32.0	3.5	7	1/1	0/1	1/1	n.t.

[a]KMR Nat 1 = 24.0 cm dia at 1.4 m stem height (DBH) tree exhibiting 40% crown symptoms; SB Nat 3 = 26.4 cm DBH tree exhibiting localized (lower side branch) crown symptoms (see Figure 3a).

[b]Bark was removed with a drawknife to expose canker(s) on each stem section.

[c]Length measurement based on exposed canker stain observed on outer xylem surface. Stem section was then half or quarter-sawn to expose internal wood stain so maximum stain width and stain depth could be obtained.

[d]n.d., not determined.

[e]Multiple stem discs were taken in a systematic manner along stem height between each end of a visual canker. Number of discs depended on canker length.

[f]Thin wood wafers were removed with a sterilized chisel at specified depths on each disc and subjected to carrot-baiting assay (Moller & DeVay, 1968).

[g]Canker stain was not visible at these radial depths and thus samples were not tested (n.t.).

large canker and another small canker below it on the main stem. Based on carrot-baiting assays, *C. huliohia* was commonly detected in the outer 3 cm of stained xylem for disc portions cut from the quarter-sawn stem sections (Table 3). The pathogen species was verified based on qPCR testing of a 26.0% subsample of 19 perithecia-positive carrot baits.

4 | DISCUSSION

Artificial inoculation of 10–18 cm DBH, single-stem *Metrosideros polymorpha* in two forest stands with *C. huliohia* applied as either colonized filter paper or colonized agar slurry resulted in elliptical inner bark cankers with the reddish-brown stain that extended into the sapwood xylem underlying the canker. Only small areas of inner bark necrosis with dark brown, shallow wood stain were associated with control inoculations (sterile filter paper or sterile agar slurry). The cankers resulting from artificial fungus inoculations were like those found on naturally infected ʻōhiʻa of similar size that were exhibiting early crown symptoms typical of ROD. The crown symptoms of the naturally infected trees were like those depicted in Barnes et al. (2018) for a *C. huliohia* infected forest tree. Furthermore, the "blotchy, diffuse black to grey staining" of sapwood evident in photos of the same publication is like the dark grey "smudge-like" discoloration found in our study. However, the characteristic elliptical inner bark cankers with underlying reddish-brown xylem staining were not reported by Barnes et al. (2018).

Although crown symptoms of ROD trees have been described as "rapid, synchronized death of leaves on individual branches" or "on a major trunk fork" (Barnes et al., 2018), the rate and spatial progression of crown symptom development differ for the disease caused by *C. huliohia* versus that caused by *C. lukuohia*. In our study, single cankers and multiple coalescing cankers were observed on ʻōhiʻa naturally infected by *C. huliohia* and expressing 30%–40% symptomatic crowns. Large coalescing cankers at the base of one tree was correlated with widespread yellowing and thinning of the upper crown of a KMR tree, whereas a lower side branch with yellow and wilting leaves on a tree whose remaining crown was asymptomatic was correlated with a large encircling canker of a Stainback Highway tree. The authors postulate that multiple infections may lead to coalescing cankers that girdle stems resulting in canopy death. However, one or more years may be required for this to occur. In contrast, artificial inoculations of ʻōhiʻa with either *C. lukuohia* colonized filter paper or agar slurry may lead to complete crown wilt within 2–3 months (Hughes et al., 2020). The rapid crown wilt is attributed to translocation of *C. lukuohia* conidia in the xylem vessels and extensive colonization, a characteristic of a systemic vascular wilt disease. Last, we have observed internal symptoms of co-infection of ʻōhiʻa by both ROD *Ceratocystis* pathogens. Specifically, a canker characteristic of *C. huliohia* and vertical dark vascular staining of *C. lukuohia* were observed on opposite aspects of the stem on a ROD symptomatic tree in Kaʻū District, Hawaiʻi Island (Figure 5). Molecular testing using qPCR protocol of Heller and Keith (2018) confirmed presence of *C. huliohia* in the elliptical brown cankers and *C. lukuohia* in the intermittent black streaked tissue. Although rare, co-infection of trees by both pathogens has been reported previously in diagnostic testing results of suspect ʻōhiʻa tissues (Heller & Keith, 2018).

FIGURE 5 Symptoms of internal colonization of *Metrosideros polymorpha* stem co-infected with *Ceratocystis* species associated with Rapid ʻŌhiʻa Death, Kaʻū District, Hawaiʻi Island. Left outlined box areas (solid white lines): Intermittent dark streaking associated with early vertical colonization of the xylem by *C. lukuohia*. Right box area (dotted white lines): Reddish brown xylem discoloration of outer sapwood associated with *C. huliohia* infection.

Hughes et al. (2020) proposed *Ceratocystis* wilt of ʻōhiʻa as the official common name for the *C. lukuohia*-caused disease based on their observations of external and internal symptom progression. The well-defined inner bark cankers with accompanying xylem stain resulting from artificial inoculation of ʻōhiʻa trees with *C. huliohia* and their presence in naturally infected forest trees reported herein support our proposal here of *Ceratocystis* canker of ʻōhiʻa as the common name for this disease. Both of these pathogens and their particular diseases fall under the already publicly and widely used over-arching term, Rapid ʻŌhiʻa Death, to denote ʻōhiʻa mortality caused by multiple species of *Ceratocystis*. The discovery, taxonomic description, and phylogenetic speciation of two or more *Ceratocystis* species

from diseased hardwoods or fruit trees without full exploration of pathogenicity on field-grown plants has led to confusion in common names for other *Ceratocystis* species (Nasution et al., 2019). In *Acacia* species, vascular stain, stem cankers, and eventual wilting of crowns have been attributed to multiple *Ceratocystis* species and referred to as *Ceratocystis* canker wilt and canker disease. The proposed common names for the *Ceratocystis* species associated with ROD in Hawai'i offer clarity for the 'ōhi'a situation.

Based on morphology, inner bark cankers and the underlying xylem stain caused by *C. huliohia* on naturally regenerated 'ōhi'a in forested areas resemble those of *Ceratocystis* canker of bitternut hickory (*Carya cordiformis*) caused by *C. smalleyi* in USA temperature forests (Park et al., 2010). The latter fungus was first described in 2005 and resides within the North American clade of *Ceratocystis* (Holland et al., 2019; Johnson et al., 2005). Detection of a bark canker is difficult based on visual inspection of infected stems for both *Ceratocystis* diseases. On hickory, dark-reddish liquid exudate ("bleeding") may be observed on the outer bark during the growing season and a slightly sunken canker revealed upon bark removal, although no outer bark discoloration is evident. However, no sunken appearance or "bleeding" symptoms have been observed on stems with developing *C. huliohia*-caused cankers on 'ōhi'a, although elliptical cankers are revealed upon bark removal. Coleopteran insect colonization is associated with *Ceratocystis* canker of 'ōhi'a and hickory, but the insect taxa involved are different. Removal of bark around entrance or exit holes of the hickory bark beetle (*Scolytus quadrispinnosus*) may reveal a characteristic *C. smalleyi*-caused canker and xylem stain around galleries of the beetle (Juzwik & Banik, 2011). The nature of the interactions between the two organisms in hickory requires further investigation. The presence of *C. huliohia* and/or *C. lukuohia* in the frass of five ambrosia beetle species (Subtribe Xyleborina) has been documented (Roy et al., 2020) and the pathogens have been detected in airborne frass particles (Atkinson et al., 2019; Heller et al., 2023). Frass collected from the outer bark of *C. lukuohia*-killed trees was found to be pathogenic to 'ōhi'a seedlings when introduced to stem wounds in growth chamber studies (Hughes et al., 2023). However, similar trials with *C. huliohia*-infested frass have not been conducted. These findings support the hypothesis that ambrosia beetles are involved in the indirect transmission of *C. lukuohia*. The possibility of direct transmission of both *Ceratocystis* species, i.e., dispersal of infectious propagules on exoskeletons of ambrosia beetles and transmission during insect colonization of healthy 'ōhi'a, has been demonstrated and recently published (Roy et al., 2023). Reddish-brown stain in the sapwood underlying *Ceratocystis* bark cankers is found in both hickory (Park et al., 2010) and in 'ōhi'a. Last, no evidence of *C. huliohia* or *C. lukuohia* transmission between diseased and healthy 'ōhi'a via root contact has been reported (Cannon et al., 2022). The possibility of *C. smalleyi* spread through connected roots of hickory has not been considered.

Pathogen colonization of hickory sapwood was correlated with impaired sap flow (xylem dysfunction) following multiple stem inoculations with *C. smalleyi* (Park et al., 2013). Infection by *C. smalleyi* results in vessel occlusion with gels and/or tyloses that lead to reduced sap flow (Park & Juzwik, 2014). Further investigation is needed to elucidate the relationship between sapwood colonization of 'ōhi'a by *C. huliohia* and observed foliage wilt.

In summary, *C. huliohia* causes inner bark cankers and xylem stains of infected *M. polymorpha* that is distinct from the axial vascular staining characteristic of *C. lukuohia* infection. Thus, two distinct *Ceratocystis* diseases are associated with ROD in Hawai'i. Although qPCR assay is needed to confirm presence of the causal species present in a tree, visual internal symptoms are also useful in distinguishing between the two diseases.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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