

# Pathogenicity, Symptom Development, and Colonization of *Metrosideros polymorpha* by *Ceratocystis lukuohia*

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## Abstract

Extensive mortality of *Metrosideros polymorpha* (ʻōhiʻa) trees has been associated with *Ceratocystis* spp. on Hawaiʻi Island and was named rapid ʻōhiʻa death (ROD). Both *C. lukuohia* and *C. huliohia* have been associated with ROD, although *C. lukuohia* appears to be the more important pathogen. Crown observations and dissections of forest trees either wound-inoculated with, or naturally infected by, *C. lukuohia* were conducted to confirm pathogenicity and document patterns of host colonization. In pathogenicity trials, one of three and two of three trees inoculated with the fungus in February and August, respectively, exhibited crown wilt symptoms at 92 and 69 days after inoculation. Extensive, radial, black staining of the sapwood was found in main stems, while no crown wilt or xylem staining was found in control trees. Xylem staining, necrotic phloem, and fungus presence was noted in six trees inoculated in

May to June and harvested 37 to 42 days later, and these observations were compared with those in two naturally infected trees felled in early August. Contiguous xylem staining was found in the main stems and into crowns of all diseased trees, while discontinuous streaks of xylem staining extended into the main forks and side branches. Necrotic phloem associated with xylem staining occurred on the lower stems of inoculated trees. Aside from the necrotic phloem and radial staining of the sapwood, symptom development in ʻōhiʻa infected with *C. lukuohia* is similar to other systemic wilt diseases on hardwood trees. We propose *Ceratocystis* wilt of ʻōhiʻa as the official name of the disease.

**Keywords:** ʻōhiʻa, rapid ʻōhiʻa death, vascular wilt disease, Hawaiʻi, fungi, trees

ʻŌhiʻa (*Metrosideros polymorpha* Gaudich.) is a keystone species to Hawaiian native forests and a critical component to the islands' watersheds. On Hawaiʻi Island, ʻōhiʻa occupies nearly 250,000 ha of forest land and is the most abundant native tree. Highly variable in form, ʻōhiʻa is common across all islands of the state and can live in a variety of habitats from wet lowlands at sea level to upper elevation dry forests (Dawson and Stemmerman 1990). As an early colonizer of new lava flows, ʻōhiʻa is integral to the succession of Hawaiʻi's landscape, turning once barren lands into young monotypic stands that can later become diverse forests (Fig. 1A and B). Due to its land-transforming characteristics, the critical habitat it provides for endangered flora and fauna, and the extensive use of its wood, flowers, and other parts for cultural purposes, ʻōhiʻa holds tremendous utility and value to the people of Hawaiʻi (Mueller-Dombois et al. 2013).

Beginning in 2010, reports of an uncommon and rapid mortality of ʻōhiʻa in the Puna District of southeastern Hawaiʻi Island spurred investigations into its cause. Unlike earlier stand diebacks observed

decades ago, where the synchronized and gradual decline of canopy trees were primarily attributed to unfavorable environmental and abiotic conditions (Hodges et al. 1986; Mueller-Dombois 1985; Petteys et al. 1975), the recent mortality is patchy in distribution and progresses rapidly in trees and within stands. In general, symptoms include black staining of sapwood, crown wilt, and tree death (Fig. 1C and D) (Barnes et al. 2018; Keith et al. 2015). This mortality is commonly referred to as rapid ʻōhiʻa death (ROD) and has spread across the island to become the most damaging epiphytotic on ʻōhiʻa.

Using aerial imagery, Mortenson et al. (2016) described an increase in forest areas with suspected ROD mortality (stands with greater than 10% mortality) from 1,600 ha in 2012 to 6,400 ha in 2014 in the affected region. Currently (late 2019), suspected ROD mortality has been confirmed in all districts of Hawaiʻi Island. Remote sensing during mid-June 2017 flights detected nearly 43,000 ʻōhiʻa trees with brown leaves and over 500,000 leafless crowns on Hawaiʻi Island (Asner et al. 2018; Vaughn et al. 2018). Aerial digital sketch mapping has estimated over 76,083 ha of forest affected by ROD on Hawaiʻi Island (B. Tucker, *personal communication*); though ground-truthing is needed to verify the causal agent(s).

Keith et al. (2015) first reported the ROD pathogen as *Ceratocystis fimbriata* Ellis & Halst. based on pathogenicity, morphology, and ITS rDNA sequences, and they referred to the disease as *Ceratocystis* wilt. However, a second *Ceratocystis* sp. was associated with ROD-like mortality, and further DNA sequencing, molecular marker analysis, host range testing, and fungal mating assays concluded that two novel and distinct species, *C. lukuohia* I. Barnes, T.C. Harrin. & L.M. Keith and *C. huliohia* I. Barnes, T.C. Harrin. & L.M. Keith, were capable of causing mortality of ʻōhiʻa seedlings (Barnes et al. 2018). The new species are closely related to but distinct from earlier reported *Ceratocystis* spp. on Araceae in Hawaii, a *Syngonium* strain of *C. fimbriata* that is closely related to *C. platani* (J.M. Walter) Engelbr. & T.C. Harrin. and *C. uchidae* Q. Li, D. McNew & T.C. Harrin. on taro (*Colocasia esculenta*) (Li et al. 2017; Thorpe et al. 2005). Pathogenicity tests with *C. lukuohia* and *C. huliohia* on ʻōhiʻa seedlings resulted in similar wilt and xylem stain symptoms, yet these species failed to induce symptoms in taro, *Syngonium*, or London plane

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(*Plantanus × acerifolia*) (Barnes et al. 2018). Currently, *C. lukuohia* and *C. huliiohia* have been reported on Hawai'i Island and Kaua'i (Brill et al. 2019; Heller et al. 2019), and only *C. huliiohia* has been found on Mau'i and O'ahu (Dennison 2019).

Although both fungi caused xylem staining, wilt, and mortality in inoculated seedlings, observations of naturally infected trees in the field suggested substantial differences in external and internal symptomatology (Barnes et al. 2018). Trees infected by *C. lukuohia* generally show rapid crown wilt with extensive radial vascular streaking in the sapwood. Conversely, the crown symptoms associated with *C. huliiohia* tend to develop more slowly and are sometimes associated with wilt of individual limbs, which may eventually lead to tree death. With *C. huliiohia*, internal vascular discoloration may be patchy in distribution and appear diffuse in cross sections. Although both species have been recovered from dead and dying trees, *C. lukuohia* appears to be the more aggressive of the two pathogens and is associated with the vast majority of the `ohi'a mortality across the island (Barnes et al. 2018).

The goal of this research was to characterize the disease caused by *C. lukuohia* in a series of field inoculation studies and in naturally infected trees. The symptomatology and internal host colonization by the fungus were assessed to 1) fulfill Koch's postulates on wild-grown trees, and 2) document the pattern of internal colonization of the trees by the fungus. Based on our findings we propose the name *Ceratocystis wilt of `ohi'a* for the disease caused by *C. lukuohia*. Furthermore, we discuss the role of *Ceratocystis* wilt in the greater context of ROD-associated mortality in Hawaiian forests.

## Materials and Methods

**Site and tree selection for inoculations.** Studies were conducted at the Keaukaha Military Reservation (KMR) (19°42'54.71"N, 155°3'3.23"W) and Waiākea Forest Reserve (WFR) (19°36'6.62"N, 155°8'28.45"W) in Hilo, Hawai'i. Both sites had healthy and diseased `ohi'a trees in the immediate vicinity. Eighteen asymptomatic, single-stem trees (mean DBH = 15.0 ± 1.1 cm) were selected, tagged with metal labels, and GPS coordinates recorded before inoculation.

**Fungal isolates and inoculum production.** *C. lukuohia* was isolated from two diseased trees at WFR by baiting discolored sapwood between two carrot slices (Moller and DeVay 1968). One fungal isolate per diseased tree was made by transferring a single ascospore mass from a single perithecium on the colonized carrot slice to 10% V8 media amended with streptomycin (100 mg). Isolates P15-02 and P17-70 were obtained in 2015 and 2017, respectively.

Two inoculum types were used. For the agar slurry inoculum, 3 ml of a fungal suspension was uniformly spread onto a Petri plate (25 mm deep, 100 mm diam.) of 10% V8 or 2% malt yeast extract agar (MYEA) media and allowed to air dry under a laminar flow hood for 10 min, followed by the addition of 6.0 ml of vegetable oil (Crisco) to completely cover the agar surface and encourage the production of aleurioconidia on the medium. Cultures were incubated as above for 10 to 20 days at 25°C and 38% RH under continuous light. To prepare the slurry, five plates with the most abundant aleurioconidia (visible as melanized spores under 40× magnification) were selected. The vegetable oil was poured off the five plates and the colonized agar was added to an ethanol-sterilized blender with 50 ml of sterile water and blended in 30 s increments until a smooth consistency was obtained. Inoculum was placed into sterile 5 ml syringes (without the needle) and used within 24 h.

To produce filter paper inoculum (Keith et al. 2015), the isolates were grown on MYEA or 10% V8 agar media for 7 days. Culture plates were then flooded with 3 ml of sterile water, agitated with a sterile glass rod with rubber policeman scraper (Walter Stern Inc. Port Washington, NY), and adjusted to  $1.0 \times 10^6$  spores/ml with a hemocytometer. Sterile filter paper discs (32 mm diameter) (Whatman, GE Healthcare UK Limited, Buckinghamshire, UK) were soaked in the fungal suspension for 1 min, laid onto fresh media plates (one disc per plate), and incubated for 3 to 5 days at 25°C and 38% RH under continuous light. Inoculum for negative controls (sterile filter paper or agar slurry) were made in an equivalent manner.

**Host inoculations.** For the pathogenicity trials, 12 trees were labeled PT 1 to 12, and their main stem was wounded at 1.4 m height using a circular hole-saw drill bit (35 mm dia.) (Hole Dozer, Milwaukee Tool, Brookfield, WI) attached to a cordless drill. Wounds were made to a depth just beneath the vascular cambium, exposing fresh outer sapwood. The bark disc was carefully removed and 1 ml of *C. lukuohia* agar slurry inoculum was applied uniformly over each circular wound. The bark-plugs were reinserted into the wound and sealed over with plumber's putty (Oatey, Cleveland, OH).

Three trees were inoculated with *C. lukuohia* and three with sterile MYEA agar slurry (negative controls) in each of two experiments. The first inoculations were conducted in February 2017 at the WFR site with agar slurry of isolate P15-02. Trees received three to four wounds in each of two staggered rows, one row at 1.4 m height and one 10 cm higher, for a total of six to eight wounds per tree depending on the tree diameter. A second inoculation was conducted on 25 August 2017 at the KMR site with isolate P17-70. Trees received two wounds at breast height on opposite sides of the trunk. Trees were felled 92 and 69 days after inoculation (DAI), respectively.

To more closely examine the pattern of internal colonization, a third inoculation study of six trees (labeled IC 1 to 6) were inoculated with isolate P17-70 on 13 or 20 June 2017 at KMR. Three `ohi'a trees received *C. lukuohia* colonized filter paper (IC 1 to 3) and three were treated with agar slurry inoculum (IC 4 to 6). Negative controls were not used in this experiment. Trees were inoculated as above with two wounds per tree on opposite sides of the stem and monitored for the development of crown wilt symptoms. Trees were felled between 37 and 42 DAI.

**Tree harvest and dissection.** For the February to May pathogenicity trial, fungus-inoculated trees had the outer bark shaved with a draw knife to expose and measure the extent of necrotic phloem in the field. Each fungus-inoculated tree was then felled and dissected into stem sections using a chainsaw, first in the area between the two rows of inoculation points and then longitudinally in 10 to 30 cm increments until stained sapwood was no longer discernable on the top surface of the cut stem section. The presence of vascular staining in the sapwood was recorded and three to seven wood disks (2 to 3 cm thick) per tree were collected for pathogen assays and transported to the laboratory. Negative control trees were left standing and the extent of phloem necrosis was measured as above. The sapwood below each control inoculation point was exposed with a hatchet, the extent of discoloration measured, and a single sapwood chip per wound was collected for pathogen baiting.

For the August to November pathogenicity trial, trees were felled by chainsaw, cut into 1.22 m long main stem sections (bolts), and labeled according to their height along the bole. The stem sections with stained sapwood were transported to USDA Agricultural Research Service, Pacific Basic Agricultural Research Center (USDA ARS PBARC) in Hilo for processing. Outer bark was removed with an ethanol sterilized drawknife and the extent of phloem necrosis and surface xylem discoloration assessed. Two wood discs (8 cm thick) were cut from each 1.22 m bolt at approximately 15 and 85 cm from the bottom of each bolt section. In general, seven to eight disks per tree were obtained to assay for *C. lukuohia* presence. The circumference of each disk and the circumference and depth of xylem staining (at four equidistant points) were measured and recorded. If phloem necrosis and/or sapwood stain was not visible in the control trees, disks were removed at the inoculation points.

For the pattern of internal colonization study, each artificially inoculated tree was felled and the main stem sequentially sectioned (1.22 m lengths) using a chainsaw. Specifically, stem sections (bolts) with staining visible on either end as well as portions of the upper crown that exhibited xylem stain were transported to USDA ARS PBARC. Each tree was reconstructed on the ground with the appropriate sections and drawings made of tree architecture, including location of each section. For trees IC 1 to 3, between 4 and 10 stem disks were removed at a stem height of 20 to 560 cm, and for trees IC 4 to 6, 7 to 12 sapwood disks were sampled at heights between 20 and 530 cm. The circumference of each disk and the circumference

and depth of xylem staining (at four equidistant points) were measured and recorded.

**Naturally colonized trees.** To examine the spread of *C. lukuohia* within naturally infected `ōhi`a, two naturally established trees in a residential neighborhood were donated for dissection by a landowner in Volcano, HI, in August 2017. The first tree (VT1) was 15.0 m tall, 26 cm DBH, with a 75% wilted crown (approximately 75% of the crown area had wilted, browning, or dead leaves). The second tree (VT2) was 13.7 m tall, 24 cm DBH, with a 25% wilted crown. Prior to felling, presence of *C. lukuohia* was confirmed by qPCR assay of sapwood shavings (Heller and Keith 2018).

These two trees were felled and the main stems sectioned into 1.22 m long sections. Two cross sectional disks (8 cm thick) were cut out by chainsaw on site at approximately 15 and 85 cm along the length of each bolt and their position along the height of the bole labeled. Portions of the treetops (main branches and attached branches that exhibited sapwood stain) were also cut in the field. All disks and canopy sections were transported to USDA ARS PBARC and each tree reconstructed on the ground. Sapwood disks (11 and 16 per tree, respectively) were removed at a height ranging from 20 to 980 and 20 to 940 cm tall from VT1 and VT2. Additional wood disks were removed from branches. The circumference of each disk and the circumference and depth of xylem staining (at four equidistant points) was measured and recorded.

**Fungus bioassay.** To assess the distribution of viable *C. lukuohia* within the tree, carrot baiting was attempted on sapwood chips taken from along the main bole. When possible, pieces of phloem from the upper crown along the interface between healthy and necrotic tissue was also baited. Thin xylem or phloem sections (2 to 3 mm thick) were removed from the wood disks with an ethanol-sterilized chisel. For naturally infected trees (VT1 and VT2), similar baiting also was attempted from subsamples of small branches. To assess radial (inward) fungal colonization of wood disks obtained from lower stem sections, thin sections were sampled at 1 cm increments from the cambium.

Fungus isolation was attempted by using a modified carrot baiting technique (Moller and DeVay 1968). When possible, three replicate carrot baits were made per wood disc. Briefly, fresh carrot roots were sliced into discs (5 mm thick). In most cases, the roots were surface disinfested with 80% ethanol and peeled before slicing. Thin sections of symptomatic phloem or xylem were placed between two carrot discs, wrapped in laboratory film (Parafilm, Bemis Co. Inc., Oshkosh, WI), and individually bagged and incubated at room temperature. The symptomatic sections were usually misted with sterile water before placing between the carrot slices. Sample bags were periodically misted with sterile water to retain moisture and rated positive for *C. lukuohia* when grayish mycelia with perithecia and ascospore masses were visible. Small pieces of fungus-colonized carrot were excised by scalpel and assayed by qPCR (Heller and Keith 2018) if the mycelia appeared to be *Ceratocystis* but did not produce perithecia (e.g., due to bacterial contamination of the carrot).

To confirm that the perithecia on the carrot slices were *C. lukuohia*, ascospore masses were aseptically transferred from perithecia on the carrot baits to 10% V8 media, incubated for 10 to 12 days, and visually assessed for morphological characteristics, completing Koch's postulates (for pathogenicity trials). Additionally, the identity of *C. lukuohia* from at least three perithecia-positive carrot baits per each inoculated tree in the internal colonization study ( $n = 36$  baits) and the two naturally infected trees ( $n = 6$  baits) was confirmed by qPCR (Heller and Keith 2018).

## Results

**Pathogenicity of *C. lukuohia* on field grown trees.** For the February to May trial, one of three fungus-inoculated trees (PT 3) had a crown that was approximately 65% brown and wilted at 92 days. The other two *C. lukuohia*-inoculated showed no crown symptom at 92 DAI (Table 1). Total length of necrotic phloem in fungus-inoculated trees ranged from 44 cm to greater than 132 cm (Fig. 2A). The tree with wilt symptoms had the most necrotic phloem. Total length of xylem staining ranged from 221 to 1,146 cm for the three

trees. In two of three trees, the extent of phloem necrosis and xylem staining was much greater above the inoculation points than below (Fig. 2A).

During dissection, it became evident that the lower bole of PT 2 was composed of three small fused stems and the wood grain was highly contorted, likely limiting the upward extent of xylem staining (Fig. 2A). Tree PT 3 had extensive xylem staining that extended from the inoculation points to nearly the top of the tree crown's main fork (Fig. 2A). It was evident that staining had split at a branch fork 280 cm above the inoculation points. The staining was primarily seen in the larger diameter fork that supported nearly two-thirds of the tree's crown, which was completely wilted, while the smaller fork was much less extensively stained and had no wilt.

*C. lukuohia* was isolated from the stained sapwood of all wood discs taken from inoculated trees (Table 1). For the trees with no crown symptoms (PT 1 and 2), *C. lukuohia* was recovered from the sapwood at a maximum distance of 300 and 55 cm above inoculation points, respectively (Fig. 2A). For the partially wilted tree (PT 3), *C. lukuohia* was detected in sapwood discs 1,030 cm above the inoculation sites (Fig. 2A). Mock-inoculated trees (PT 4 to 6) were externally asymptomatic and showed very limited phloem necrosis around the wounds and only faint sapwood discoloration in the immediate vicinity (<1 cm) of the wounds (Fig. 2A). All samples from the control trees were negative in the carrot baiting assay.

For the August to November pathogenicity trial, fungus-inoculated trees PT 7 and PT 9 showed complete crown wilt (100%) with reddish-brown foliage throughout the crown, while PT 8 and all



**Fig. 1.** `ōhi`a (*Metrosideros polymorpha*) trees and crown symptoms associated with *Ceratocystis lukuohia* infection. **A**, Healthy, flowering pioneer seedling growing from crack in lava field; **B**, healthy, old-growth tree at the Hakalau Forest National Refuge, HI; **C**, partial and **D**, full crown wilt caused by *C. lukuohia* colonization. Photos (B, D): J. B. Friday, University of Hawai'i at Mānoa.

mock inoculated trees (PT 10 to 12) were externally asymptomatic 69 DAI (Table 1). Total length of phloem necrosis and xylem staining on wilted trees ranged from 670 to 805 cm (Fig. 2B). Although PT 8 had a green and asymptomatic crown, internal phloem necrosis extended 122 cm, and xylem staining was more extensive (390 cm) (Fig. 2B). For *C. lukuohia*-inoculated trees, phloem necrosis and sapwood stain extended further above than below the inoculation sites. Control trees displayed limited phloem necrosis (<9 cm long); xylem discoloration was diffuse brown, less than 21 cm long, and centered on the wound site (Fig. 2B). *C. lukuohia* was recovered from the sapwood of carrot baits for all wood disks sampled from fungus-inoculated trees, but it was not recovered from control trees (Table 1). The maximum height for pathogen detection ranged from 240 to 690 cm above the inoculation site (Fig. 2B). For both pathogenicity trials, all qPCR assays confirmed *C. lukuohia* presence on representative carrot baits ( $n = 12$ ) and in subcultures from carrot baits transferred to agar media ( $n = 6$ ).

**Colonization of artificially inoculated trees.** Trees IC 1 to 6 exhibited crown wilt symptoms by 6 weeks after inoculation, regardless of inoculum type (Table 2). Each of the three trees with filter paper inoculum completely wilted by 42 DAI, compared with agar-slurry inoculated trees, whose crown wilt ranged from 30 to 90% (mean = 53.3%). For the filter paper inoculum trees, the mean disk circumference with xylem staining ranged from 80 to 96% and mean maximum radial depth of stain was 0.46 to 1.34 cm into the sapwood (Table 2; Fig. 3A). The agar slurry inoculum tree with the lowest crown rating (IC 5) had a 65% mean disk circumference with staining and 0.55 cm mean maximum radial depth of stain (Table 2). Sapwood disks from trees IC 4 and 6 had >98% of their mean circumference showing xylem stain and a mean maximum stain depth of 1.7 cm.

For trees inoculated with filter paper inoculum (IC 1 to 3), the crowns were completely wilted (reddish-brown leaves) by 37 to 42 DAI. The main stem was forked at either 7.5 m or 13 m for two of the three trees. Continuous, dark xylem staining was observed to  $\geq 93\%$  of total tree height on either the main stem or the highest fork of each of the three trees, as well as in the second fork on two trees. Healthy phloem was seen adjacent to the highest sampled sections that had xylem staining on both the right and left forks of IC 2, but partial phloem discoloration (partly pink and partly brown) was associated with the highest sampled section on both forks of IC 1 and the main stem of IC 3. Continuous dark staining of the xylem was observed below the inoculation points down to the cutting height (0.2 to 0.3 m above ground) of all three trees. Xylem staining also extended into an exposed stilt root of IC 1.

The agar slurry inoculated trees (IC 4 to 6) were single-stemmed and exhibited crown wilt symptom ratings from 30 to 90% at 42 DAI (Table 2). Continuous dark xylem staining was observed up to 11.6 m, or 95% of total tree height, in IC 4, which had the most extensive crown wilt. In contrast, discontinuous dark staining

(streaked or spotty) (Fig. 3B and C) was observed up to 8.5 m (64.4% of tree height) and 13.5 m (93% of tree height) in trees IC 5 and 6, which had 30 and 40% wilted crowns, respectively (Table 2). Phloem associated with highest stem occurrence of xylem staining was generally “healthy” (pink) in IC 5, while partly pink and partly brown phloem was associated with the highest xylem staining in IC 4 and IC 6. Complete cinnamon to brown phloem necrosis was observed on lower stem sections adjacent to extensively stained xylem (Fig. 3D). Continuous dark xylem stain was observed from the inoculation points downward to the tree cutting height (0.1 to 0.3 m).

For trees IC 1 to 3, 18 to 29 stem disks were removed for carrot-baiting of xylem tissue, and 5 to 16 disks were used for baiting of phloem tissue (Table 3). The pathogen was recovered from 94 to 100% of the xylem samples and from 56 to 100% of the phloem samples. The maximum height of the trees from which *C. lukuohia* was isolated was the same for xylem and phloem tissues taken from two of the trees, but recovery was greater from xylem tissues than phloem for tree IC 2 (Table 3). Over 83% of the xylem sampled from 22 to 27 stained stem disks from agar slurry trees (IC 4 to 6) yielded the fungus, compared with 55% of the phloem tissues assayed from the same disks (Table 3; IC 6 depicted in Fig. 4A). The maximum height of pathogen recovery was between 11 and 13 m for both xylem and phloem tissue for these trees (Table 3).

**Naturally infected trees.** The two trees sampled in the community of Volcano, HI, had crowns that were 75% (VT1) and 25% (VT2) wilted. Although the trees differed in outward appearance, nearly the entire length of main stem and branches of both trees had sapwood heavily stained brown to black, often with radial streaking. Mean disk circumference with sapwood stain was greater than 50%, while mean maximum depth of staining was under 2.0 cm (Table 2). For VT1, phloem necrosis and xylem stain were visible into the small branches (<2 cm diam) of the upper crown to a height of 13 m.

*C. lukuohia* was isolated from 94% of the stained xylem samples taken from 34 wood disks of VT1 (Table 3). The pathogen was isolated from stained xylem of six branches greater than 10 m above ground and from samples taken 6 m above ground in two other branches. Although the phloem was sparsely sampled, *C. lukuohia* was isolated from 60% of the assayed necrotic phloem samples from tree VT1 (Table 3). For VT2, stained xylem and necrotic phloem were observed at the top of the main stem, with discontinuous streaking in otherwise healthy xylem in small branches at over 13 m above ground. Of the 31 wood disks sampled from VT2, *C. lukuohia* was detected in 92% of the stained sapwood samples, at a maximum radial depth of 4 cm (Table 3). The pathogen was isolated from stained xylem and necrotic phloem from all eight main limbs of VT2, with four limbs positive at 6 to 9 m height and four limbs positive above 10 m (Fig. 4B). The pathogen was isolated from nearly all stained xylem and necrotic phloem samples taken from near the apex of the VT2 (Table 3, Fig. 4B).

**Table 1.** Wilting and pathogen recovery from pole-sized *Metrosideros polymorpha* trees after inoculation of *Ceratocystis lukuohia* using the agar slurry method

Tree ID	DBH (cm)	Treatment	Fungal isolate	Field location <sup>a</sup>	Month of inoculation	Inoculation points (no.)	Days to final rating	Wilt at final rating (%)	Positive isolations/number of discs taken at different heights in the tree
PT 1	11.0	<i>C. lukuohia</i>	P15-02	WFR	February	4	92	0	7/7
PT 2	26.5	<i>C. lukuohia</i>	P15-02	WFR	February	8	92	0	3/3
PT 3	13.0	<i>C. lukuohia</i>	P15-02	WFR	February	7	92	65	5/5
PT 4, 5, and 6	18.0–26.0	Controls	Sterile agar <sup>b</sup>	WFR	February	6	92	0	0/23
PT 7	12.3	<i>C. lukuohia</i>	P17-70	KMR	August	2	69	100	8/8
PT 8	12.3	<i>C. lukuohia</i>	P17-70	KMR	August	2	69	0	7/7
PT 9	12.5	<i>C. lukuohia</i>	P17-70	KMR	August	2	69	100	7/7
PT 10, 11, and 12	11.0–11.9	Controls	Sterile agar <sup>b</sup>	KMR	August	2	69	0	0/10

<sup>a</sup> WFR = Waiākea Forest Reserve, KMR = Keaukaha Military Reserve.

<sup>b</sup> Control trees were inoculated with sterile malt yeast extract agar (MYEA) slurry.

## Discussion

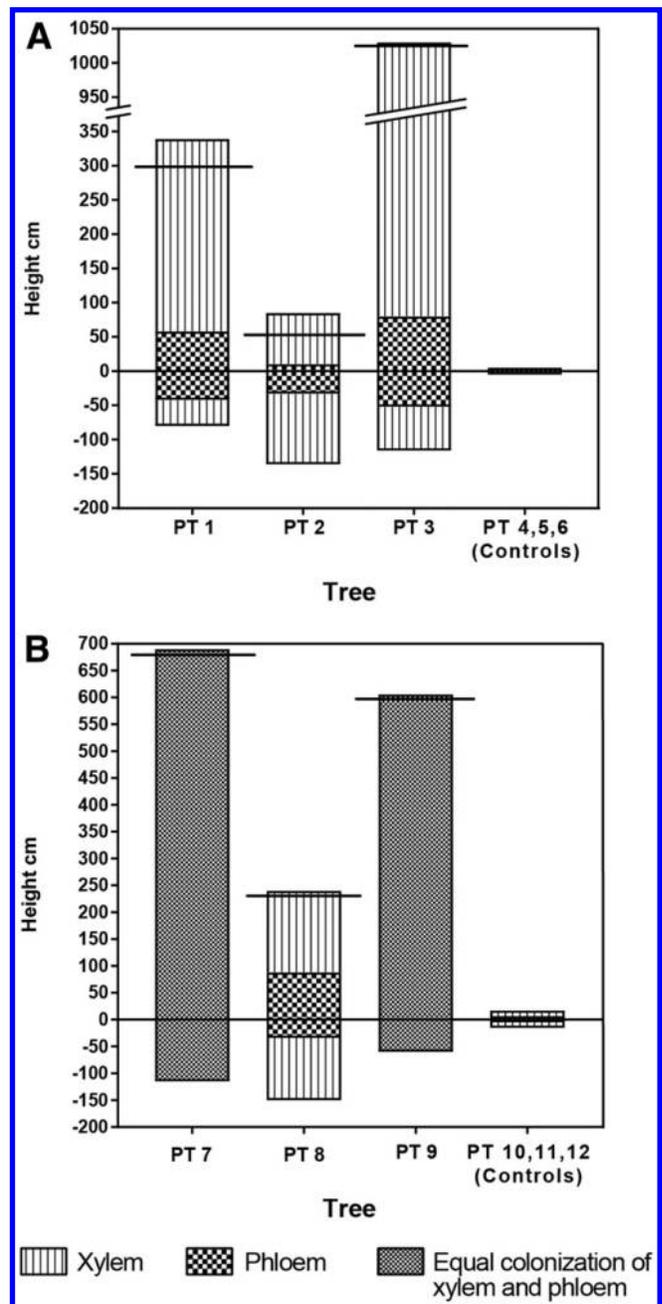
Artificial inoculation of pole-sized *M. polymorpha* trees with *C. lukuohia* caused external wilt and internal stain symptoms typical of those described for rapid ‘ōhi‘a death (Barnes et al. 2018) and those observed in the naturally infected trees we evaluated. The general pattern of pathogen spread and internal symptom development based on our results is as follows. Relatively rapid longitudinal (axial) spread follows initial *C. lukuohia* infection as evidenced by vascular staining and isolation of the pathogen, and the pathogen moves upward much faster than downward. The upper and lower margins (or advancing front) of the longitudinal spread is characterized by discontinuous xylem staining. Necrosis of the inner phloem (mixed coral and brown colors to total necrosis) generally occurs behind the advancing front of xylem staining. In a similar fashion, lateral spread in the outer xylem lags behind the axial spread, but within several weeks to several months it can lead to staining around the circumference of the outer xylem. Inward radial spread in the xylem occurs, but penetration is generally shallow (<2 cm) in depth in artificially inoculated trees at 6 weeks to 3 months. The combined lateral and radial progression of staining can result in a characteristic “starburst” pattern (Supplementary Fig. S1). The fungus can survive in necrotic phloem and xylem tissues based on baiting assays.

Green (1981) and Gibbs (2001) outlined the typical progression of vascular wilt diseases in woody plants: 1) for new infections to occur, a fresh wound into or near the xylem is needed; 2) after penetration, the pathogen enters and is strictly limited to the xylem tissue, where it moves passively in the transpiration stream; and 3) upon complete colonization, the pathogen is no longer confined to xylem elements and can grow outward into the living inner bark of moribund hosts. Other general characteristics of vascular wilt diseases include crown symptoms progressing from green to brown foliage, accompanied by leaf wilt, leaf death, and defoliation, along with xylem discoloration associated with the host’s resistance response. Wounds into, or near, the xylem are probably necessary for *C. lukuohia* infection to occur. Based on the results of our inoculation studies, the pathogen enters the xylem and rapidly moves upward and to a lesser extent downward, presumably as conidia in the flow of water during the diurnal transpiration cycle. Unlike classic vascular wilt fungi, the fungus appears to colonize living ray parenchyma and moves outward into the inner phloem of inoculated trees long before the host becomes moribund. In this respect, *C. lukuohia* resembles canker stain of *Platanus* species caused by *C. platani* and similar diseases caused by Latin American strains of *Ceratocystis* (Harrington 2013; Kile 1993; Tsopelas et al. 2017). However, all other characteristics we have documented lead us to classify *C. lukuohia* as a vascular wilt pathogen of *M. polymorpha*. Furthermore, we propose *Ceratocystis* wilt of ‘ōhi‘a as the official name of the disease, consistent with the first report of Keith et al. (2015).

Originally, the rapid ‘ōhi‘a death pathogen was reported as *C. fimbriata*, yet further work demonstrated instead that two novel species, *C. lukuohia* and *C. huliohia*, were associated with ‘ōhi‘a mortality (Barnes et al. 2018; Keith et al. 2015). Seedling inoculations were inadequate to distinguish the host colonization patterns by *C. lukuohia* versus *C. huliohia* (Barnes et al. 2018), but inoculation of larger trees demonstrate that *C. lukuohia* causes a systemic vascular wilt that is typical of the rapid mortality and extensive sapwood staining observed in *M. polymorpha* affected by ROD in natural landscapes. Thus, *Ceratocystis* wilt of ‘ōhi‘a represents the disease caused by *C. lukuohia*, and *Ceratocystis* wilt falls under the more broadly recognized condition of ROD. Parallel field experiments were conducted with *C. huliohia* on similar-sized, forest *M. polymorpha*. Results will be published separately, but preliminary findings indicate *C. huliohia* is not a systemic wilt pathogen (J. Juzwik, unpublished data).

At the time of harvest, tangential colonization around the circumference of the outer sapwood was nearly complete in our inoculated trees but was less so in the naturally infected trees (Supplementary Fig. S2). This may be an artifact of the inoculation procedure, in

which trees received a high dosage of inoculum into two (or more) wounds on opposite sides of the stem. In laurel wilt and oak wilt, caused by *Raffaella lauricola* T.C. Harr., Fraedrich & Aghayeva and *Bretziella fagacearum* (Bretz) Z.W.deBeer, Marinc., T.A. Duong & M.J. Wingf., respectively, a complete ring of brown-black discoloration that encircles the outer sapwood is seen only in the most recent annual rings, with little or no radial streaking along the ray parenchyma (Bretz 1953; Hughes et al. 2015). The internal vascular discoloration caused by *C. lukuohia* resembles that of *C. fimbriata* and *C. platani* in earlier stages of colonization, where distinct and separate longitudinal streaks are visible in the outer sapwood, along with radial streaking in cross-sections (Harrington 2013; Tsopelas et al. 2017). In heavily colonized or recently killed



**Fig. 2.** Extent of internal staining and *Ceratocystis lukuohia* colonization in the main stem of artificially inoculated ‘ōhi‘a trees in field pathogenicity trials. Bars denote extent of internal xylem staining and phloem necrosis. The inoculation point is indicated at height “0” and horizontal solid black lines indicate maximum longitudinal extent of pathogen recovery in sapwood. **A**, Feb. to May 2017 trial; **B**, late Aug. to early Nov. 2017 trial. Data from three control trees are shown as mean of three replicates.

ʻōhiʻa trees, *C. lukuohia* staining continues to intensify and penetrate radially and tangentially, eventually resulting in a wide band of stained sapwood across the entire cross-section.

In general, *C. lukuohia* penetrated deeper radially in the sapwood of naturally infected versus artificially inoculated trees. For the two naturally infected and symptomatic trees, *C. lukuohia* was isolated from stained xylem at a depth of 2 and 4 cm. Based on observations in dozens of dissected ʻōhiʻa trees killed by *C. lukuohia*, it appears that the fungus continues to penetrate radially toward the center of the tree after death, even into the red pathological heartwood tissues. Such sapwood colonization after death would be advantageous for the fungus as aleurioconidia in such tissue can serve as windborne inoculum if liberated as sawdust or ambrosia beetle frass (Harrington 2013; Roy et al. 2019).

*C. lukuohia* is an aggressive vascular pathogen that spreads throughout host trees, causing rapid wilt and mortality. Artificial inoculations resulted in whole-tree mortality and isolation of the pathogen at a height of over 10 meters above the inoculation points in under 100 days. Based on observed staining and results of carrot-baiting assays, the pathogen could move up from the inoculation point more than 10 cm per day, much faster than linear growth in a Petri dish on malt agar media. This pattern of movement is typical among vascular wilts where the spores of the pathogen are passively moved upward within the sap stream during transpiration (Gibbs 2001; Green 1981). With laurel wilt, Mayfield et al. (2008) were able to induce disease symptoms with

*R. lauricola* in field-inoculated *Persea borbonia* trees within 3.5 months using a single inoculation point, and in susceptible red oaks (*Quercus* section Lobatae) infected with oak wilt, symptoms can develop within weeks of infection (Juzwik et al. 2011). Like our results, Henry and Riker (1947) isolated the oak wilt pathogen (*B. fagacearum*) from the apex of most limbs of a stem-inoculated *Quercus velutina* soon after observable symptoms. These results indicated that extensive xylem spread of pathogen propagules can occur prior to the development of wilt. In artificially inoculated *M. polymorpha*, the pathogen also moved downward in the sapwood to a limited extent, perhaps in the sap stream during the nocturnal portion of diurnal sap flow, when the xylem tension relaxes. Similar to other vascular wilt pathogens, it is likely that *C. lukuohia* triggers the formation of tyloses, gums, and polysaccharides in vessel elements, which can result in occlusion and reduction of water conductance (Inch and Ploetz 2012; Inch et al. 2012; Ouellette 1962; Pomerleau 1970).

Within this study, both forms of inoculum used (filter paper and agar slurry) were able to induce disease in *M. polymorpha*. In the June inoculation trials, filter paper inoculum resulted in a higher percentage of crown wilt than the agar slurry, although the agar slurry inoculum had more fungal mass. Both inoculum types resulted in a similar amount of stained disc circumference, and radial staining penetrated slightly deeper with the agar slurry inoculum. Agar slurry inoculations for pathogenicity (February) and colonization studies (June and August) were conducted when monthly average

**Table 2.** Tree characteristics, crown wilt information, and internal symptom development for six trees artificially inoculated with *Ceratocystis lukuohia* and two naturally infected *Metrosideros polymorpha* trees<sup>a</sup>

ID	Tree characteristics			Crown wilt		Sample distribution		Vascular stain characteristics	
	Ht. (m)	DBH (cm)	Inoculum type <sup>b</sup>	Days to final rating	Wilt at felling (%)	Main or forked stem ht. sampled (m)	Main or forked stem disks taken (no.) <sup>c</sup>	Mean disk circumference with stain (% ± SE)	Mean disk max. depth of stain (cm ± SE)
IC 1	13.9	15	FP	37	100	0.4 to 3.1	7	95.0 ± 3.3	1.34 ± 0.10
IC 2	16.7	16.3	FP	42	100	1.1 to 5.6	10	95.5 ± 1.0	0.85 ± 0.05
IC 3	10.1	12.5	FP	42	100	0.2 to 1.0	4	79.7 ± 6.8	0.46 ± 0.07
IC 4	12.1	12.7	AS	42	90	0.4 to 2.8	7	99.3 ± 0.1	1.66 ± 0.08
IC 5	13.2	14	AS	42	30	0.3 to 5.3	12	65.2 ± 5.9	0.55 ± 0.05
IC 6	15.1	14.5	AS	42	40	0.2 to 3.8	9	98.1 ± 1.4	1.68 ± 0.07
VT 1	15	25.8	Nat	n/a	75	0.2 to 9.8	16	63.5 ± 5.4	1.80 ± 0.12
VT 2	13.7	23.6	Nat	n/a	25	0.2 to 9.4	11	71.0 ± 3.9	1.47 ± 0.12

<sup>a</sup> Trees were inoculated in June and felled in late July 2017. Naturally infected trees were felled early August 2017.

<sup>b</sup> *Ceratocystis lukuohia* inoculum type. FP = filter paper inoculum, AS = agar slurry inoculum, Nat = natural infection.

<sup>c</sup> Wood discs (8.0 cm thick) removed from main stem or stem fork at 50 to 70 cm intervals between sampled endpoints.



**Fig. 3.** Internal symptom development in *Metrosideros polymorpha* inoculated with *Ceratocystis lukuohia*. **A**, transverse section of cut log depicting the radial sapwood staining pattern. **B and C**, discontinuous brownish-black staining in sapwood under bark of main stem and in a small branch, respectively. **D**, cinnamon to brown phloem necrosis and internal sapwood staining in a partially de-barked stem.

temperature highs ranged from ~26 to 29°C, and disease severity increased with rising temperatures, suggesting seasonality may affect severity. However, other factors (fungal isolate, host genotype, etc.) may also contribute to variability in disease progression. For canker stain of plane trees (caused by *C. platani*), inoculations in spring and summer resulted in more rapid disease progression than in late autumn (Pilotti et al. 2016; Tsopelas et al. 2017). American elm and red oaks are most susceptible to Dutch elm disease (caused by *Ophiostoma ulmi* and *O. novo-ulmi* Brasier) and oak wilt, respectively, in spring and early summer, when large springwood vessels are produced (Juzwik et al. 2011; MacDonald and Hindal 1981).

*M. polymorpha* growing in Hawaii does not produce traditional growth rings of summer and springwood vessels; however, vessels are generally larger in the outer sapwood than those closer to the center of the stem (Fisher et al. 2007). The larger vessels in the outer sapwood, where wounding is more likely to occur, may contribute to the susceptibility of *M. polymorpha*, because wider and longer vessels are correlated with more susceptible phenotypes in elm species (Elgersma 1970; Solla and Gil 2002). Fisher et al. (2007) found that higher elevation *M. polymorpha* populations have narrower vessels when compared with lower elevation populations. Further research is needed to assess if, and to what extent, the roles of seasonality,

**Table 3.** Detection of *Ceratocystis lukuohia* by carrot-baiting of symptomatic sapwood or phloem tissue from stem disk samples taken from six artificially inoculated and two naturally infected *Metrosideros polymorpha* trees<sup>a</sup>

Tree ID <sup>b</sup>	Main or forked stem sample heights (range in m)	Wood disks taken (no.) <sup>c</sup>	Sapwood			Phloem		
			Samples assayed (no.) <sup>d</sup>	Fungus-positive samples (no.) <sup>e</sup>	Max. stem height of positive samples (m)	Samples assayed (no.)	Fungus-positive samples (no.) <sup>e</sup>	Max. stem height of positive samples (m)
IC 1	0.4–13.2	26	28	28	13.2	5	5	13.2
IC 2	1.1–15.4	29	35	33	15.4	16	9	12.7
IC 3	1.3–9.6	18	18	18	9.2	5	5	9.2
IC 4	0.4–11.5	22	25	23	11.5	5	3	11.5
IC 5	0.3–12.1	23	30	25	11.4	11	6	11.4
IC 6	0.2–14.2	27	32	28	13.5	12	9	13.5
VT 1	0.2–13.1	34	49	46	13.1	5	3	9.8
VT 2	0.2–13.3	31	49	45	13.3	15	14	13.3

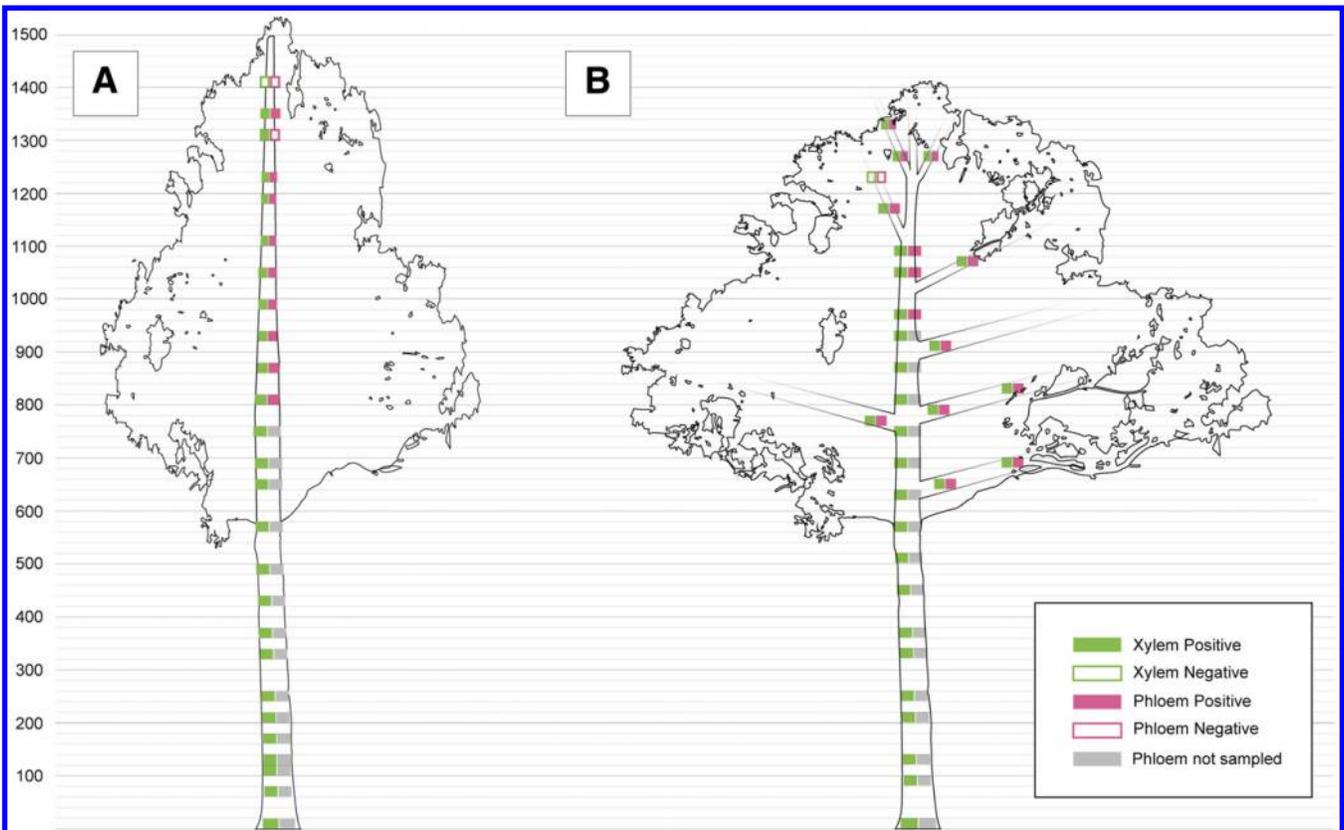
<sup>a</sup> Trees were inoculated in June and felled in late July 2017. Naturally infected trees were felled early August 2017.

<sup>b</sup> IC 1 through IC 3 = artificially inoculated using filter paper inoculum; IC 4 through IC 6 = artificially inoculated using agar slurry inoculum; VT 1 and VT 2 were naturally infected trees.

<sup>c</sup> Wood discs (8.0 cm thick) removed from main stem or stem fork at 50 to 70 cm intervals between sampled endpoints.

<sup>d</sup> Some wood discs were radially subsampled at intervals of 1 cm depth.

<sup>e</sup> Positive = Perithecia of *C. lukuohia* detected on carrot baits or, occasionally, mycelium on colonized baits identified using qPCR method of Heller and Keith (2018).



**Fig. 4.** Sample locations for viable *Ceratocystis lukuohia* in **A**, artificially inoculated (IC6) and **B**, naturally infected (VT2) *Metrosideros polymorpha* trees. The pathogen was detected using a carrot baiting assay. Tree IC6 was inoculated mid-June and felled late July 2017. Tree VT2 was felled in early August 2017.

elevation, and xylem anatomy play in `ōhi`a susceptibility and *Ceratocystis* wilt.

The epidemiology of *Ceratocystis* wilt of `ōhi`a is currently under investigation. Like other vascular wilts and *Ceratocystis*-caused diseases, exposed xylem wounds are likely necessary for pathogen ingress and colonization (Harrington 2013). Bark wounding and stripping by feral ungulates (sheep, goats, cattle, pigs) and branch breakage by strong winds may provide suitable infection courts for the pathogen. Insects may disperse conidia or ascospores to wounds, or wounds may be infected by contaminated tools or machinery. However, contaminated ambrosia beetle frass or sawdust are typically important sources of airborne or waterborne inoculum for *Ceratocystis* spp. in the Latin American Clade, such as *C. lukuohia* (Barnes et al. 2018; Harrington 2013; Souza et al. 2013). Exotic ambrosia beetles in the genus *Xyleborus* are commonly found infesting dead `ōhi`a trees at lower elevation sites. Roy et al. (2019) found a high percentage (62%) of ambrosia beetle galleries in trees killed by ROD released frass that contained *C. lukuohia* DNA, and the fungus was isolated from 17% of the frass samples. The potential of ambrosia beetles to act as direct vectors is under investigation. Unlike Dutch elm disease, oak wilt, and canker stain of plane tree (Harrington 2013; Juzwik et al. 2011), there is lack of evidence of root-grafting between *M. polymorpha*, and expanding centers of mortality associated with root graft transmission have not been evident in `ōhi`a forests.

`Ōhi`a, Hawai`i's most prominent native tree, is ecologically, economically, and culturally important. Collectively, `ōhi`a dominated forests support a variety of native plant and animal species and provide critical habitat for rare native birds and insects. On Hawai`i Island, Fortini et al. (2019) found 38 threatened and endangered plant species in 13 endemic genera whose geographical distributions overlap that modeled for *C. lukuohia*, and the ranges of six of those species overlap the pathogen by more than 95%. This suggests serious potential downstream effects on other species in `ōhi`a forests. In addition, these forests overwhelmingly constitute the upper watersheds that provide Hawai`i's citizens with clean drinking and agricultural water. Native forests on Hawai`i Island are experiencing an ecological crisis of widespread mortality referred to as ROD. We have shown that `ōhi`a is highly susceptible to *Ceratocystis* wilt caused by *C. lukuohia*, which is believed responsible for the epidemic levels of mortality first noted a decade ago. The disease caused by *Ceratocystis huliohia*, the second ROD pathogen, appears to be less important and is distinct in its pattern of host colonization (Barnes et al. 2018), and this is being further investigated. Further research is being conducted to determine the host range of *C. lukuohia*, and its potential effects on agricultural crops in Hawai`i. Research by a consortium of collaborators on disease management strategies is ongoing to protect this vital Hawaiian keystone species. Other Pacific regions and areas with native *Metrosideros* species, including New Caledonia, Tahiti, and New Zealand, should be aware of this pathogen and potential effects on their forests. Since there is currently no cure for *Ceratocystis* wilt of `ōhi`a, enhanced biosecurity with measures for prevention and early detection are critical.

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