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Short Communication

In Vitro Assays Suggest Rapid ʻŌhiʻa Death Fungi Can Colonize *Metrosideros polymorpha* Fine Roots

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Abstract

Rapid ʻōhiʻa death (ROD), caused by *Ceratocystis lukuohia* and *C. huliohia*, severely impacts ʻōhiʻa lehua (*Metrosideros polymorpha*), a keystone tree species on the Hawaiian Islands. This study examined the ability of these pathogens to colonize ʻōhiʻa fine roots in a series of root dip experiments under in vitro conditions, focusing on the potential roles of host genetics and root tissue condition in susceptibility. The results showed that both pathogens can colonize and form fruiting bodies on fine roots; however, *C. lukuohia* had greater colonization rates and produced more perithecia than *C. huliohia.* Fungal colonization rates were higher in fine roots damaged by excising the root cap, suggesting that wounds enhance susceptibility. These findings suggest that fine root damage may facilitate fungal ingress, raising concerns about root infection as a potential pathway for ROD spread.

Keywords: ʻōhiʻa, *Ceratocystis huliohia*, *Ceratocystis lukuohia*, forest conservation, Hawaiʻi, *Metrosideros polymorpha*, root damage, root infection

Plant pathogens are significant drivers of forest tree species distributions, structure, and successional patterns (Castello et al. 1995). Diseases such as chestnut blight and Dutch elm disease, caused by *Cryphonectria parasitica* and *Ophiostoma novo-ulmi*, respectively, have killed vast numbers of American chestnut and elm trees, irrevocably altering forests once dominated by these tree species (Brasier 1991; Rigling and Prospero 2018). The infection processes of tree diseases typically begin with successful establishment of a pathogen's propagules into or onto the susceptible host, with the extent and severity of the disease determined by a variety of biotic and abiotic factors. A clear understanding as to which plant tissues (e.g., roots, stems, or leaves) are initially colonized, as well as predisposing factors that allow infection to progress, is critically important in developing management strategies.

ʻŌhiʻa lehua (*Metrosideros polymorpha*) is Hawaiʻi's most abundant and biologically important native tree; it serves as a foundational keystone species instrumental to native forest composition, structure, and function and provides a primary habitat to native plant and animal species (Eldredge and Evenhuis 2003; Friday and Herbert 2006). ʻŌhiʻa-dominated forests account for 250,000 ha of the land cover on Hawaiʻi Island, and the tree is often dominant in both wet and dry forests across all islands

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of the state (Asner et al. 2018; Mueller-Dombois and Fosberg 1998). Beyond its ecological significance, ʻōhiʻa is intertwined with native Hawaiian culture (Hu et al. 2023; Mueller-Dombois et al. 2013).

In 2010, reports of rapid mortality of ʻōhiʻa in the Puna and Hilo districts of Hawaiʻi Island triggered a need for further investigation. This mortality phenomenon became commonly known as rapid ʻōhiʻa death (ROD) and is associated with black staining of sapwood, crown dieback, and wilt that leads to tree death (Barnes et al. 2018; Keith et al. 2015). Based on morphological and phylogenetic evidence, as well as the completion of Koch's postulates on ʻōhiʻa seedlings, the fungi *Ceratocystis lukuohia* and *C. huliohia* were identified as causal agents of ROD (Barnes et al. 2018, Keith et al. 2015). Although both fungi can induce similar general symptoms that include xylem staining, crown wilt, and mortality, further investigations into the pathology of each disease found that *C. lukuohia* causes a systemic vascular wilt disease (i.e., Ceratocystis wilt of ʻōhiʻa) that is more virulent compared with the canker disease of the inner bark and sapwood caused by *C. huliohia* (Ceratocystis canker of ʻōhiʻa) (Hughes et al. 2020; Juzwik et al. 2024). *Ceratocystis* is known to infect fresh wounds on trees (Harrington 2013; Roux et al. 2004). On Hawaiʻi Island, stem wounds created by human activity and storms create potential entry points for the disease (Cannon et al. 2022), and ROD mortality is strongly associated with feral ungulate (hooved animals) presence, which can lead to wounding of tree boles and woody surface roots during foraging and other behaviors (Perroy et al. 2021). Inoculation of boles of ʻōhiʻa trees with either pathogen can induce disease (Hughes et al. 2020; Juzwik et al. 2024). However, it remains unclear whether either can infect ʻōhiʻa through fine, non-woody roots and whether such infections lead to disease symptoms. The aim of this research was to investigate characteristics of *C. lukuohia* and *C. huliohia* colonization and growth on fine roots of ʻōhiʻa in vitro as a first step to understanding the potential of root infection and colonization.

Research was conducted at the USDA Forest Service Pacific Southwest Research Station, Institute of Pacific Islands Forestry, in Hilo on Hawaiʻi Island. To make the fungal inoculum, subcultures of *C. lukuohia* (isolate C21-29) and *C. huliohia* (isolate PL15-2) were transferred from our culture collection onto fresh circular Petri dishes (60-mm diameter × 15-mm height) of ½-strength potato dextrose agar (1.95% potato dextrose agar, 0.75% agar) amended with streptomycin sulfate (100 mg/liter). Cultures were incubated under continuous light for 7 days at 25°C. The inoculum was produced by flooding the cultures with 3 ml of autoclaved water, agitating the surface with a sterilized glass rod to release the conidia, then collected by pipette and adjusted to 1.0×10^6 CFU/ml by a hemacytometer. A separate inoculum batch was made for each *Ceratocystis* species and experimental replicate, which was used the same day. To collect ʻōhiʻa tissue for colonization assays, fine roots (4 to 6 cm long) and sapwood were harvested from 0.5- to 2.5-year-old, containerized, healthy ʻōhiʻa individuals, and these fresh tissues were used for inoculations on the same day.

In Experiment 1, we tested if *C. lukuohia* and *C. huliohia* were able to colonize the detached fine roots of different ʻōhiʻa genotypes. Fifteen individual fine roots (0.6 to 1.7 mm in diameter) were excised with sterilized shears from 10 ʻōhiʻa individual clonal genotypes to provide a total of 150 fine roots per trial replicate. To inoculate, five fine roots from each ʻōhiʻa genotype were soaked in 1.5 ml of one of three aqueous treatment solutions for 5 min. Treatment solutions included (i) *C. lukuohia* in sterile water (1.0×10^6 CFU/ml), (ii) *C. huliohia* in sterile water (1.0×10^6 CFU/ml), and (iii) sterile water without fungal propagules (0 CFU/ml) as a negative control. Immediately following inoculation, fine roots were trimmed to 2- to 3-cm lengths, leaving the root cap undamaged, and placed onto a damp filter paper circle (110-mm diameter, Whatman Grade 1001-110; Cytiva, Wilmington, DE, U.S.A.) within a Petri dish (100-mm diameter × 15-mm height). Fifteen fine roots from 10 ʻōhiʻa individual genotypes were inoculated to provide a total of 150 fine roots per trial replicate. As a positive control, ʻōhiʻa sapwood, a substrate suitable for the growth of *C. lukuohia* and *C. huliohia*, was used to confirm inoculum viability for each trial. Stem segments from healthy ʻōhiʻa seedlings were collected by hand pruners, and the outer bark of each segment was stripped off via a flame-sterilized grafting knife and cut into 2- to 2.5-cm lengths (2.5-mm diameter). Sapwood segments were inoculated with the *C. lukuohia*, *C. huliohia*, or no-fungus sterile water suspensions using the same procedure as above. All fine roots and stems were incubated in a growth chamber (PGR-15; Conviron, Manitoba, Canada) at 25°C, 92% relative humidity, and a 12-h daily light cycle. *Ceratocystis* spp. growth after 7 days was assessed using a dissection microscope (Stemi 508; Zeiss, Oberkochen, Germany) by counting the number of fruiting bodies (perithecia) that colonized fine roots and stem segments. The experiment was replicated twice. Due to the availability of plant material, the genotypes LEES 104, LEES 106, and LEES 122 were used in both trials, although fine roots from different individual plants within genotypes were selected between trials. The remaining 16 genotypes were tested in only one trial. In the second replicate trial, two separate collections of fine roots from the genotype LEES 121 were harvested from different individuals (Table 1).

In Experiment 2, we tested if fine root damage affected *Ceratocystis* colonization rates. Sixty individual fine roots (0.3 to 1.8 mm in diameter) were excised from 10 ʻōhiʻa individuals (i.e., genotypes). The 60 fine roots were randomized and sepa-

TABLE 1

Colonization of *Metrosideros polymorpha* detached fine roots after exposure to a *Ceratocystis* inoculum (trial replicates 1 and 2)

		C. lukuohia inoculum[a]		*C. huliohia* inoculum[a]	
M. polymorpha genotype	Trial no.	Colonized fine roots (%)[b]	Number of perithecia[c]	Colonized fine roots (%)[b]	Number of perithecia[c]
LEES 101	1	40	4	0	0
LEES 104		0	0	20	13
LEES 106		80	6	0	0
LEES 110		60	9	0	0
LEES 122		80	9	60	9
LEES 124		100	15	80	31
LEES 126		20	1	0	0
LEES 128		0	0	0	0
PUKA 056		60	8	0	0
KEMR 230		80	35	60	7
LEES 103	2	40	8	0	0
LEES 104		0	0	40	4
LEES 106		0	0	0	0
LEES 107		0	0	0	0
LEES 111		20	1	0	0
LEES 113		0	0	0	0
LEES 117		0	0	0	0
LEES 121a[d]		60	10	60	16
LEES 121b[d]		0	0	20	1
LEES 122		20	1	0	0

[a] Inoculation consisted of a 5-min soak in a conidial suspension (1.0×10^6 CFU/ml) and incubation at 25°C for 7 days.
[b] Percentage of colonized (out of five) fine roots for each plant genotype.
[c] Total number of *Ceratocystis* fruiting bodies (perithecia) per five fine roots treated.
[d] Roots belong to the same genotype collected from different individuals.

rated into two treatment groups: (i) undamaged fine roots and (ii) damaged fine roots, from which root caps had been removed with pruners. Undamaged fine roots exhibited no visible external wounds below the site where they were originally removed from the ʻōhiʻa individuals. In the damaged root treatment, fine roots were trimmed 0.2 to 1 mm above their tip to remove the root cap, leaving fresh wounds. Twenty damaged and 20 undamaged fine roots were individually soaked in an aqueous solution (150 µl) of *C. lukuohia* conidia (1.0×10^6 CFU/ml) for 5 min, exposing only the first 4 to 6 mm of the root tip to the solution to avoid exposure to the original harvesting wounds at the top of the fine root. Roots were individually plated into circular Petri dishes on top of damp filter paper circles (i.e., one fine root per Petri dish). The same procedure was duplicated using *C. huliohia* conidia (1.0×10^6 CFU/ml) and the no-fungus, sterile water (0 CFU/ml) negative control. To confirm the inoculum viability, 20 ʻōhiʻa stem segments were inoculated with *C. lukuohia*, *C. huliohia*, or the no-fungus treatment using the same procedure. Petri plates with fine roots were stored at a slight downward angle in the growth chamber to prevent runoff of the inoculum solution from migrating upward and entering the wound created from harvesting the fine root from the source plant. Growth of *Ceratocystis* spp. was assessed as previously described. The experiment was replicated twice.

A Bayesian approach was used for data analysis, allowing for interpretation of interval estimates as probability statements and to generate interval estimates even when there was no variability in the observed data. Given the exploratory nature of these experiments, this analysis focused on summarizing the posterior distribution rather than selecting models and testing hypotheses. In each experiment, the number of colonized versus non-colonized fine roots was modeled from a fixed number of roots using a binomial generalized linear model with a logistic probability function. Additionally, the total number of perithecia across fine roots was modeled using a Poisson generalized linear model with an exponential mean function. For Experiment 1, an interaction term between genotype of the ʻōhiʻa plant and species of the pathogen was used as the model input. For Experiment 2, the input was an interaction term between fine root condition (i.e., damaged or undamaged) and species of the pathogen.

Results were summarized using posterior means and 95% credible intervals. In models of colonized versus non-colonized fine roots, the posterior mean represented the log odds of a single fine root being colonized. In models of the number of perithecia, the posterior mean represented the log mean of the total number of perithecia across all fine roots in the experiment. All statistical analyses were done in version 4.4.1 R computer programming language (R Core Team 2024). Bayesian models were fit using version 2.21.0 of the brms package. Posterior means and credible intervals were found using version 1.10.3 of the emmeans package (Lenth 2024). Figures were made using version 3.5.1 of the ggplot2 package (Wickham 2016). For Bayesian inference, we used the same prior distribution on the log odds of probability of infection as we did on the log mean of the number of perithecia, namely, a normal distribution with a mean of 0 and standard deviation of 2.5.

The results of Experiment 1 indicate that both fungal species can colonize and produce perithecia on fine roots of ʻōhiʻa, but colonization was not uniform across all replicates (Fig. 1A to D). None of the fine roots treated with sterile water was colonized in either trial, but all positive control ʻōhiʻa stem segments (i.e., 40 of 40) yielded more than 200 perithecia per segment for *Ceratocystis* species (*data not shown*). In the first trial replicate (Table 1), *C. lukuohia* perithecia grew on at least one fine root (out of five fine roots per plate) in 8 of 10 incubated Petri plates, colonizing a total of 26 (i.e., 52%) individual fine roots ($n = 50$). *C. huliohia* perithecia grew on at least one fine root on 4 of 10 Petri plates, infecting 11 (i.e., 22%) individual fine roots in total ($n = 50$). In the second trial replicate (Table 1), 4 of 10 Petri dishes exhibited one or more fine roots with *C. lukuohia* perithecia, infecting seven (i.e., 14%) individual fine roots, and *C. huliohia* perithecia grew on 3 of 10 plates, infecting six (i.e., 12%) individual fine roots ($n = 50$ per trial). The number of perithecia fruiting bodies observed per 10 fine roots (i.e., both trials combined) per ʻōhiʻa genotype ranged from 0 to 35 and 0 to 31 for the *C. lukuohia* and *C. huliohia* inoculum, respectively (Table 1).

When comparing the fungal pathogens, *C. lukuohia* demonstrated higher log odds of fine root colonization and a greater mean number of perithecia compared with *C. huliohia* (Fig. 2A and B). A positive log odds per plant genotype indicated a probability greater than 50% for both fine root colonization and perithecia development, whereas a negative log odds indicated a colonization probability of less than 50%. Credible intervals that shifted left were associated with no colonization and zero variability among observations (Fig. 2A and B). Certain plant genotypes, such as LEES 124 and KEMR 230, exhibited a higher probability of growth for both *C. lukuohia* and *C. huliohia* on

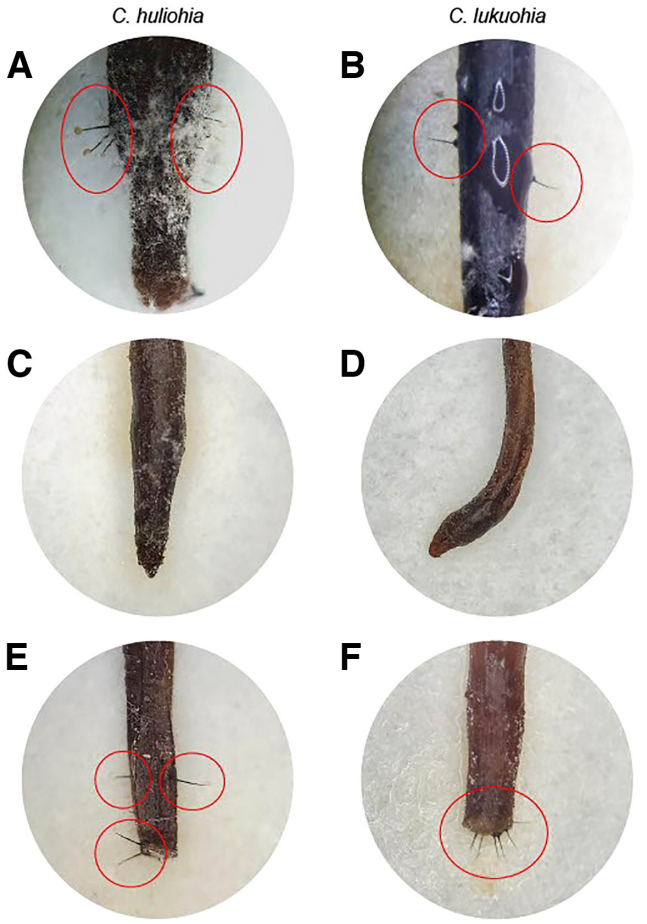

FIGURE 1
Growth of *Ceratocystis* fungal fruiting bodies (perithecia) on fine roots of *Metrosideros polymorpha* after artificial inoculation. Fine roots supporting the growth of mycelia and perithecia (red circles) of **A,** *C. huliohia* and **B,** *C. lukuohia.* Undamaged root tips exposed to **C,** *C. huliohia* and **D,** *C. lukuohia* conidia with no visual evidence of colonization and damaged (root caps removed) root tips exposed to **E,** *C. huliohia* and **F,** *C. lukuohia* conidia with perithecia fruiting body growth near the wound site.

their fine roots, whereas other genotypes, such as LEES 104 and LEES 128, did not show this pattern (Fig. 2A and B). The mechanism driving the variability in colonization rates between genotypes remains unknown, and further trials using live seedlings are needed to determine if this pattern is consistent with the results observed.

The results from Experiment 2 indicate that *C. lukuohia* colonized 6 of 20 (30%) undamaged fine roots and 11 of 20 (55%) damaged fine roots in the first trial, and *C. huliohia* colonized 2 of 20 (10%) undamaged fine roots and 12 of 20 (60%) damaged fine roots. In the second trial, *C. lukuohia* colonized 4 of 20 (20%) undamaged fine roots and 10 of 20 (50%) damaged fine roots, and *C. huliohia* colonized 0 of 20 (0%) undamaged and 5 of 20 (25%) damaged fine roots. The majority of perithecia development of the damaged fine root treatment was concentrated around the wound sites where the root cap was removed (Fig. 1E and F). Across both trials, damaged fine roots exhibited higher rates of colonization and greater perithecia counts for both pathogens compared with undamaged fine roots (Table 2). Damaged fine roots exposed to *C. huliohia* or *C. lukuohia* had greater log odds of infection than undamaged intact fine roots (Fig. 2C). Similarly, damaged fine roots also had greater log mean number of perithecia of *C. huliohia* and *C. lukuohia* than undamaged roots (Fig. 2D). The interpretation of credible intervals in this experiment was analogous to Experiment 1. Across both trial replicates, no damaged or undamaged fine roots treated with sterile water were colonized. In the first trial, all positive control wood stem segments were colonized by *C. lukuohia* (20/20) and *C. huliohia* (20/20). In the second trial, most stem segments were colonized by *C. lukuohia* (18/20) and *C. huliohia* (19/20) (*data not shown*). To confirm the identity of *Ceratocystis* species growing on fine roots tissues in these studies, DNA was extracted from colonized fine roots and stem (positive control) tissues and analyzed by qPCR according to the protocol of Heller and Keith (2018). In total, eight random samples were analyzed, and results from all samples were completely congruent to respective inoculation species treatment, indicat-

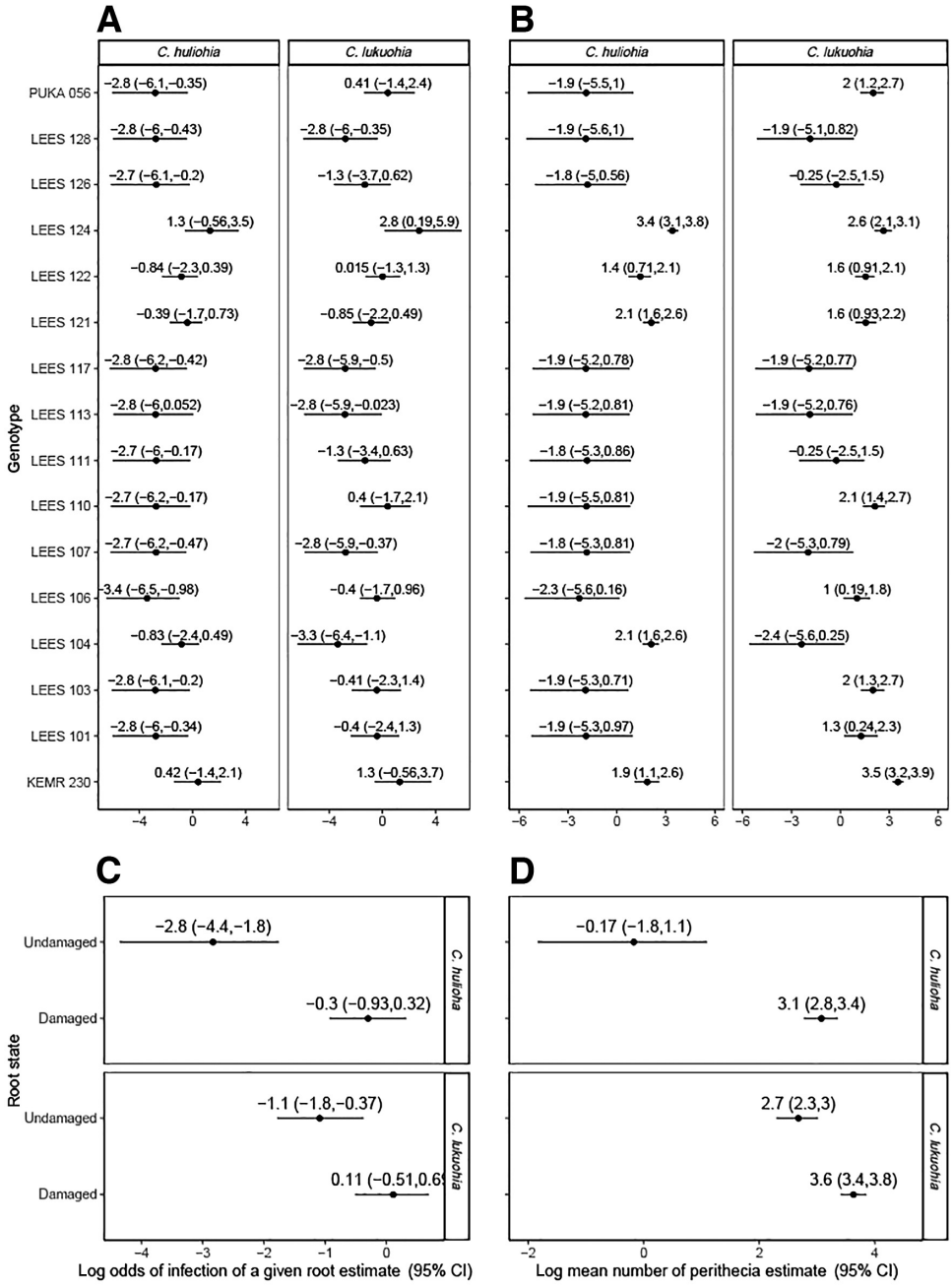

FIGURE 2
Probabilities of *Metrosideros polymorpha* fine root colonization by *Ceratocystis lukuohia* and *C. huliohia*. **A,** Log odds of root colonization per plant genotype and **B,** log mean number of perithecia produced per ʻōhiʻa genotype. **C,** Log odds of root colonization by *Ceratocystis* spp. based on ʻōhiʻa root damage treatment and **D,** log mean number of perithecia formed per *Ceratocystis* spp. based on ʻōhiʻa root damage treatment.

ing a lack of cross-contamination between treatments (*data not shown*).

Both *C. lukuohia* and *C. huliohia* exhibited the ability to grow and reproduce on ʻōhiʻa fine roots in these in vitro colonization assays. Damaged fine roots were generally more susceptible to colonization by both fungal species compared with undamaged fine roots. This trend is consistent with the current understanding of ROD pathogenicity, where stem or branch wounds of vascular tissues are required for *Ceratocystis* ingress and host colonization (Cannon et al. 2022; Hughes et al. 2020). Perithecia were typically observed around wound sites directly exposed to the fungal inoculum, whereas no perithecia were observed growing out of the opposite, untreated sides of excised fine roots. This may suggest that *Ceratocystis* growth is either slow or localized in ʻōhiʻa fine roots at the point of infection or growth is constrained to wound sites. It might also be possible that the pathogens require assistance from the negative pressure generated from living plants to move away from the wound site, which would not occur in a detached root assay. Future work investigating the growth of *C. lukuohia* and *C. huliohia* in fine roots of live ʻōhiʻa plants or trees could help clarify these movement patterns.

Generally, *C. lukuohia* exhibited a greater probability of colonizing ʻōhiʻa fine roots and developing perithecia than *C. huliohia* in both damaged and undamaged fine roots, suggesting potentially greater aggressiveness in this tissue. However, there was variability in this pattern and instances where neither fungus colonized roots for a particular genotype or, as in LEES 104, *C. huliohia* was a better colonizer. However, the overall general pattern aligns with our existing understanding of the relative virulence of *C. lukuohia*, where colonization within the host and disease symptom development are typically faster and more severe than *C. huliohia* (Hughes et al. 2020; Juzwik et al. 2024). Interestingly, variation in colonization across ʻōhiʻa genotypes suggested a potential role for host genetics in mediating susceptibility to *Ceratocystis* colonization.

Other *Ceratocystis* species are known to colonize and grow in root tissue. *Ceratocystis platani*, for example, can spread between infected plane trees via root grafts (Tsopelas et al. 2017), and spores surviving in river water may cause infections on damaged roots (Vigouroux and Stojadinovic 1990). Root infections by *C. fimbriata* isolates are documented to occur from a soilborne inoculum in mango as well as sweet potato (Halsted 1890; Oliveira et al. 2015). In ʻōhiʻa, we have observed woody surface or nurse log roots with vascular staining characteristic of *C. lukuohia* infection and isolated the pathogen in culture, suggesting that these tissues sustain fungal colonization (M. A. Hughes, *personal observation*). However, with these pathosystems, occurrences have only been reported in lignified, woody lateral roots or storage roots. To our knowledge, colonization by *Ceratocystis* in other disease systems has yet to be characterized for fine roots (<2.0-mm diameter) like those used in this study. These results suggest that fine roots could be a site for *Ceratocystis* infection in ʻōhiʻa or other hosts.

Although the findings of this study provide valuable insights into the colonization potential of *C. lukuohia* and *C. huliohia* on ʻōhiʻa fine roots, these experiments were conducted on detached fine roots in vitro. Additional research investigating the role of fine root colonization in living plants, as well as in forest trees, is needed to better assess this potential infection pathway. Indeed, if colonization of ʻōhiʻa fine roots can lead to increased infections and disease, then measures that prevent or minimize root damage, for example, practices that reduce soil compaction or damage to ʻōhiʻa roots from feral ungulates, may reduce ROD-related ʻōhiʻa mortality. By elucidating potential modes of *C. lukuohia* and *C. huliohia* colonization in ʻōhiʻa, this research contributes to ongoing efforts aimed at developing effective management strategies to mitigate the impact of ROD in Hawaiʻi.

TABLE 2

Comparison of *Ceratocystis* colonization on damaged versus undamaged individual *Metrosideros polymorpha* fine roots

	C. lukuohia inoculum[b]		*C. huliohia* inoculum[b]	
Fine root condition[a]	Colonized fine roots (%)[c]	Number of perithecia[d]	Colonized fine roots (%)[c]	Number of perithecia[d]
Undamaged	25	30	5	2
Damaged	52.5	76	42.5	44

[a] Fine roots were exposed to one of two treatments in which the apical tip (root cap) region was left intact (undamaged) or removed (damaged).
[b] Inoculation consisted of a 5-min soak in a *C. lukuohia* or *C. huliohia* conidial suspension (1.0×10^6 CFU/ml) and incubation at 25°C for 7 days. Only 4 to 6 mm of the apical tip (root cap) was exposed to the inoculum.
[c] Percentage of colonized fine roots for two trial replicates combined; 20 roots per treatment (40 fine roots for both replicates combined).
[d] Total number of *Ceratocystis* fruiting bodies (perithecia) for two trial replicates combined. Each *M. polymorpha* genotype had five fine roots inoculated per trial (10 roots for both replicates combined).

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