Vacuum steam treatment of *Metrosideros polymorpha* logs for eradication of *Ceratocystis huliohia* and *C. lukuohia*

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Abstract:

A new and devastating disease, rapid ohia death (ROD), in Hawaii led to a state quarantine that regulates inter-island transport of ohia wood and plant material to prevent spread of the causal pathogens. Heat treatments of ohia logs in commercial trade were considered for phytosanitary treatment. Vacuum steam (VS) was evaluated for its ability to eradicate the pathogens, *Ceratocystis lukuohia* and/or *C. huliohia*, in main stem logs from ROD-affected forest trees. Replicate loads of three de-barked logs (24 to 43 cm diameter; 1.7 to 2.0 m long) were VS-treated at 56° C for 30 min (5 loads) or 60° C for 60 min (4 loads) at a sapwood depth equal to 70% of log radius. Percent isolation of *Ceratocystis* from VS and ambient temperature logs prior to treatment and summarized by source tree ranged from 12 to 66% and 6 to 31% based on carrot baiting assays of tissue taken from outer and inner sapwood, respectively. No viable *Ceratocystis* was detected in either sapwood locations for the 60° C/ 60 min schedule or for the inner locations for the 56° C/ 30 min schedule following treatment. Only one subsample (0.48%, n=208) of the latter schedule treatment yielded *Ceratocystis*. Time required for treatment ranged from 7.4 to 15 h for the 56° C/ 30 min schedule and from 8.6 to 19.2 h for the 60° C/ 60 min schedule. These results demonstrate VS is an effective and efficient method for treating large diameter ohia logs that mill owners and regulatory plant pathologists may consider for use in Hawaii.

An emerging disease, Rapid Ohia Death (ROD), threatens the health and survival of the keystone forest tree species *Metrosideros polymorpha* (ohia) in the Hawaiian archipelago (Mortenson et al. 2016). Following the first visual detections between 2009 and 2012 of what is now known as ROD, the putative causal agent was determined to be *Ceratocystis fimbriata* in 2014 (Keith et al. 2015). However, further morphological observations and fungal interfertility and phylogenetic analyses has led to the differentiation of two distinct pathogens: *Ceratocystis lukuohia* I. Barnes, T.C. Harr. & L.M. Keith and *C. huliohia* I. Barnes, T.C. Harr. & L.M. Keith (Barnes et al. 2018). The first causes a systemic vascular disease and is associated with a rapid wilt of the entire crown of an infected ohia (Hughes et al. 2020) while the second causes distinct, but often extensive, cankers (Juzwik et al. 2019a). Multiple cankers caused by the latter are also capable of causing tree death, albeit over a longer timeframe than the wilt pathogen. Currently, *C. lukuohia* is known to occur on Hawaii Island and Kauai, while *C. huliohia* has been confirmed on Maui and Oahu in addition to Hawaii Island and Kauai (College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, 2021). In an early attempt to prevent spread of the pathogens from Hawaii Island to the rest of the state, the Hawaii Department of Agriculture enacted quarantine regulations restricting inter-island movement of any ohia plant parts and soil associated with the plant species (Hawaii Department of Agriculture Amendment to chapter 4-72, amendment §4-72-13). Off-island shipment of ohia logs has been permitted on a log-by-log basis since 2016 if a log was found to be free of ROD pathogen DNA based on qPCR assay (Heller and Keith 2018) of multiple samples taken along the length of the log. Besides its inability to distinguish between viable and non-viable pathogen propagules, this testing process is time-consuming and delays commercial log sales for the forest industry in Hawaii.

Ohia logs (locally referred to as poles and posts) of diameters from 8 to 50 cm and of various lengths (usually ranging from 3 to 6 m) are purchased for such uses as decorative poles and beams, fences, rustic bridges, and building structural supports. Price per log is based on base diameter and log length, with costs increasing with size. Although prices vary, the larger logs command prices of thousands of dollars(USD) each. Logs are de-barked early in the production process and often sold “green” (i.e., with a high moisture content).

Kiln-heating treatment at 56° C of prohibited wood materials was suggested as an eradicative treatment in the original quarantine; however, no certified heat treatment schedules were

available at the time. In response to the need, research efforts were initiated in 2017 to evaluate three different heat treatments and one chemical treatment potentially suitable for de-barked ohia logs. Because both ROD-associated *Ceratocystis* spp. colonize the xylem (Hughes et al. 2020; Juzwik et al. 2019a), depth of effective treatment was an important consideration. For mill owners in the state, cost of treatment and availability of equipment required for effective treatment are of concern. Chemical dip diffusion treatments were appealing because they are simple to apply, require minimal capital investment for mill owners, and are low in cost. However, incomplete eradication of *C. lukuohia* in the inner sapwood was found in log sections (9 to 17 dm diameter; 1.3 m long) immersed in a borate/quaternary ammonia solution with a 10-week diffusion period (Hughes et al. 2021).

Vacuum steam (VS) was recently evaluated for use with large diameter (41 to 61 cm), 1.9 m long logs cut from *Quercus rubra* trees that had recently wilted due to infection by *Bretziella fagacearum* (syn. *Ceratocystis fagacearum*), the oak wilt fungus. Two treatment schedules (60° C for 60 min. and 56° C for 30 min) were both found to eradicate living propagules of *B. fagacearum* from the sapwood; furthermore, DNA of the fungus was also degraded by the treatments (Juzwik et al. 2019b). Loads of three logs were tested in each trial. Between 6 and 10 hours were required to achieve the temperature / time specifications of the administered regime. A previously published study (Chen et al. 2017) found that the VS process had minimal deleterious effect on product (i.e., veneer) quality of five hardwood species tested. Because of similarities of *C. lukuohia*-caused wilt of ohia to *B. fagacearum*-caused wilt of oaks, VS holds promise for effective eradicative treatment of *Ceratocystis* species of ohia.

Outdoor experiments using a portable VS system were undertaken to evaluate the ability of the VS process to kill the ROD pathogens in colonized, debarked logs obtained from naturally infected *M. polymorpha* of large diameters (24 to 43 cm). The specific objectives were to: 1) evaluate the ability of VS heat treatments to eradicate viable propagules of *C. lukuohia* and/or *C. huliohia* at two sapwood depths, 2) document the temperature profile of the treated logs over the time-course of the experiments, 3) document the energy and time required for treating each load, 4) monitor for any changes in log quality (before versus after treatment), and 5) evaluate potential for pathogen re-colonization of treated wood.

Materials and Methods

Study trees. Naturally infected, ROD-symptomatic ohia trees located on woodlands used for cattle-grazing in Waipunalei, North Hilo District at elevation 530 m, and a private ranch in the District of Kau (location withheld to protect identity of land owner) on Hawaii Island were selected as sources of study logs between March and September 2018. During the initial scouting for trees at Waipunalea in March 2018, the identified candidate trees were in the early stages of crown wilt (≥ 25% severity) (data not shown) and had progressed to a range of 30 to 100% by the time of their last rating date (Supplemental Table 1). Scouting for study trees at the Kau site was conducted in July and November 2018. The numbers of days from their last crown rating (80 to 100% severity) to time of log treatment ranged from 73 to 215. Presence of *C. lukuohia* and/or *C. huliohia* in the main stems were verified using drill-shaving samples from the xylem and subjected to qPCR assay per published protocols (Heller and Keith 2018). The Waipunalei site is on the island's north lower flank of Mauna Kea and receives 4311 mm of rain annually (Giambelluca, et al. 2013). In contrast, the Kau ranch site is on the island's south side and receives 1616 mm annual rainfall. Diameters of the selected ohia trees ranged from 33 to 64 cm at 1.4 m stem height (dbh) and 32 to 58 cm dbh at the Waipunalei and Kau ranch sites, respectively. Permission to harvest trees on each site were obtained from current landowner or land lease holder.

Fifteen trees on the Waipunalei site that were determined to be positive for ROD *Ceratocystis* DNA using qPCR were felled in late December 2018. Twenty-nine candidate logs (2.1 m long) were cut from the main stems on site and the logs were transported to a University of Hawaii Manoa property in Hilo for temporary, outdoor storage. Five trees that tested positive for *Ceratocystis* spp. DNA on the Kau ranch site were felled in mid-January 2019, similar sized logs were removed from main stems, and 12 candidate logs transported to the same site in Hilo. Overall, 41 logs were obtained as candidates for treatment although not all were used in the trials.

Log preparation, measurements, and experimental design. In late January 2019, the logs were moved to a treatment site at the USDA ARS PBARC facility in Hilo. There they were sorted into three treatments (two levels of VS and the ambient temperature control) loads with attempts made to select three logs of similar diameters that had originated from different trees for VS treatment. In addition, logs with obvious injury from the harvesting process or with pre-

existing defects were excluded from use. Because commercial ohia logs are debarked prior to processing, the final selected logs were debarked using high pressure water and drawknives. A 28 cm long section was cut from the ends of each log, cut surfaces painted with liquid paraffin (Thompson's Water Seal, The Thompson's Company, Cleveland, OH), labeled, and stored at 5° C until further processed to obtain disks for pretreatment assay for viable *Ceratocystis*. In addition, for each log one cross-sectional disk (7.6 cm thick) was cut from the larger diameter end of the log, placed in a polybag, and used for pretreatment sapwood moisture content determination. The moisture content disks were processed as follows within one hour of collection. A pie-shaped section was cut from each disk, weighed (top-loading balance), placed in a labeled paper bag, and left in a drying oven (103° C) until constant weight was obtained.

End diameters and final length of each prepared log were then measured and recorded. Liquid paraffin was painted onto ends of each log and allowed to dry. Weight of each log was obtained immediately prior to treatment. Two heat treatment schedules (60° C / 60 min. and 56° C / 30 min.) were evaluated with four or five replicate chamber loads (tests) used per schedule. The unequal numbers of replicates were due to unexpected failure of the diesel generator during the last 60° C / 60 min test. Four logs were left untreated to determine whether ambient outdoor conditions reduced fungal viability over the time-course of a trial. For a control trial (2 logs per ambient temperature trial), pre-treatment samples were taken as previously described, logs stored under shade, and post-treatment samples removed 24 hours later.

Obtaining sample disks for fungal assay. Disks (7 to 8 cm thick) for pretreatment fungal assays were obtained using a bandsaw. The end sections obtained from each log were placed in a jig and cross-sectional cuts made using a bi-metal blade on a large bandsaw (Model JWBS-18-3 18" Bandsaw, 3 HP, JET Tools, Inc., La Vergne, TN). All sawdust was removed from the equipment and floor between processing of each log section. In addition, the blade, jig and cutting surface was sterilized with 70% ethanol in between sections to minimize fungal contamination between sampled disks. The disks were labeled, placed in polybags, and stored at 5° C until further processed.

Similar methods were used to obtain sample disks from post-treatment VS and control logs. End sections (28 cm long) were obtained from treated logs after they had cooled to a safe handling temperature following their removal from the VS chamber. Sample disks for fungal assay were obtained and stored until further processed as previously described. In addition, one

disk (7.6 cm thick) was obtained from each posttreatment log for moisture content determination using the previously described methods.

Fungal assays. To further minimize the possibility of contamination between pre-VS treatment (or control disks) and the post-VS-treatment disks, all posttreatment disk subsamples were prepared and assayed first. Using a circular template, lines delineating sixteen pie-shaped sections were drawn on each disk. Using the bandsaw, cuts were made along each line from the perimeter to approximately 5 or to 8 cm inward toward the geometric center of a disk. The disk was then stored in a polybag at 5° C and taken to the pathology laboratory for assay. Carrot-baiting (Moller and DeVay1968) was used to estimate frequencies of viable *Ceratocystis* presence in alternating outer (1 to 3 cm deep) and inner (4 to 6 cm deep) sapwood locations of each disk. A 2 cm wide sterile chisel was used to cut thin wafers of wood at each depth/location. Two or three wafers were misted with sterile water if they appeared dry, placed between two carrot disks (0.5 to 1.0 cm thick), bound together with a strip of laboratory film, placed in a polybag, and incubated at ambient temperature (~ 24° C) and lighting conditions for up to 30 days. Baits were considered positive for either *Ceratocystis* species if sexual fruiting bodies (perithecia) were visible on the wood tissue or surface of a carrot disk. If only greyish mycelia and/or immature perithecia were observed, a portion of the colonized carrot piece was placed in a sterile microcentrifuge tube for DNA extraction and subjected to qPCR assay (Heller and Keith 2018). In addition, a small subsample of carrot baits (based on stratified random sampling) also was subjected to qPCR to identify *Ceratocystis* species present.

Log temperature and energy use monitoring. After individuals of a three-log-load were weighed and placed on a wood pallet for insertion into the vacuum chamber, Omega K type thermocouple wires (TT-K-24; 260° C max) were inserted into predrilled holes located at ¼, ½ and ¾ along the length of each log (Supplemental Figure 1). The holes were drilled to a depth of 70% of the log radius at each point. In addition, one hole was drilled to the geometric center of the log at its mid-point and one thermocouple was affixed to the log surface at the mid-point. A final thermocouple was inserted into one end of the log to a depth equal to 70% of the log radius for the end probed. After a thermocouple wire was inserted to the bottom of a hole, the hole was plugged with pliable epoxy resin (plumber's putty) to block steam from entering it. The temperature probe wires were then connected to the data acquisition system to allow for the continuous recording of data. Voltage and current of each phase were measured every minute

during each trial using an energy meter (ELITEpro XC, Dent Instrument, Bend, OR) and data retrieved using Elog software (Dent Instrument). The kilowatt hours (kWh) were calculated by multiplying voltage x current. The measured wattages were recorded and converted to kWh per kg of wood treated (kWh/kg).

Vacuum steam treatment. The portable VS unit includes an electric steam generator with a 100kW boiler (Reimers Electra Steam Inc., Model RX100C3F, Clear Brook, VA), a 5 hp dry screw vacuum pump (Busch LLC, Virginia Beach, VA), and a custom-built vacuum chamber (1.5 x 1.5 x 3.0 m capacity) (Vacutherm Inc., Warren, VT). The unit was secured in position in the back portion of a 6.5 m long enclosed trailer (see White et al. 2017). The thermocouple wires exited a side wall of the chamber in a narrow port that was sealed and the wires connected to a computer equipped with data acquisition software (LabVIEW, National Instruments Corp., Austin, TX) that allowed for real-time monitoring of temperatures. Once a test load was inserted into the chamber and the door sealed, the vacuum treatment was initiated. After a vacuum of 100 mm Hg was reached, saturated steam (85° C) was introduced to the chamber until the chamber reached 85° C. This chamber temperature was maintained throughout the treatment cycle. Vacuum level varied between 400- and 600-mm Hg during treatment, depending on the duration of the treatment cycles. Temperatures of the targeted log locations and of the ambient chamber were monitored throughout the course of each trial until the target temperature was reached for all the probes inserted at 70% of each log's radius depth. The trials were then stopped when the prescribed temperature (either 56° C or 60° C) was achieved at the target depth. The steam was then shutoff and the target temperature held for the prescribed time (30 min for 56° C or 60 min for 60° C). At the end of the specified time, the remaining vacuum was released, and the chamber doors opened to allow for evacuation of the steam and condensate.

Log quality assessment. Visual observations for each log were recorded in writing and with photographs to document the effects of treatment on log color and structural degradation, such as end-checking and splitting.

Fungal inoculation and colonization of post-treatment logs. To assess if VS-treated wood was vulnerable to re-colonization by *C. lukuohia* and *C. huliohia*, logs were exposed to the fungi in a post-VS treatment artificial assay. Fungal isolates of *C. lukuohia* (14-1-1) and *C. huliohia* (16-8) were grown on 10% V8 media for seven days, plates flooded with sterile water and propagules collected into beakers. Sterile filter paper disks (Whatman no.1, 42.5 mm diameter)

were soaked in the fungal suspension for 1 minute, laid onto agar media and incubated for six days at 25° C until fully colonized (Keith et al. 2015).

Four VS-treated logs from each treatment schedule (30 min for 56° C; 60 min for 60° C) were randomly selected two weeks after VS treatment. Each filter paper inoculation site (8 x 8 cm square) was prepared by having the outermost layer of wood (1-2 mm) removed by a sterile drawknife blade and cut surface sprayed with sterile water to re-hydrate the site. Per each log, a single *C. lukuohia*, *C. huliohia* and water-soaked (negative control) filter paper disk was stapled onto the freshly-prepared wood surface, re-sprayed with water and allowed to incubate outdoors with sun and rain exposure (mean daily highs of 26.4° C) for 14 days. Inoculation sites were separated by 20 cm along the log length and were watered every 24 hrs. with a watering can unless rain occurred. To validate inoculum viability, two paper disks per treatment (*C. lukuohia*, *C. huliohia* and negative control) were placed between two fresh carrot slices and held in a plastic sandwich bag next to the logs. After two weeks, filter paper disks were removed from the logs and the wood surface underneath visually inspected for *Ceratocystis* fungal growth. An 8 x 8 cm square and approximately 1 cm thick section was then removed from each inoculation site. Thin wood strips were removed with a flame-sterilized chisel and carrot baited as above.

Data summarization and analyses. *Ceratocystis* spp. isolation data were summarized by sapwood depth and tree harvest site. A generalized linear mixed effects model (Agresti 2002) was used to investigate effects of these variables on isolation frequencies, particularly site and wood depth. The generalized linear mixed effect model has the form:

$$Y_{ijk} \sim Bernoulli(P_{ijkl})$$

$$\text{Logit}\,[P(Y_{ijk}) = 1] = \mu + S_i + D_j + SD_{ij}\,\alpha_k + \gamma_{l(k)}$$

$$\alpha_k \sim N(0, \sigma_{tree}) \; and \; \gamma_{l(k)} \sim N(0, \sigma^2_{log})$$

where P is the probability of detecting the fungus, μ is the overall mean, S is the site where trees were obtained (Kau or Waipunalei), D is the sapwood depth (inner or outer sapwood), α is the error associated with tree, and γ is the error associated with log. All calculations were performed using R (version 1.0.143; R Foundation for Statistical Computing, Vienna). The model was run as a generalized mixed effects model with lme4 (Bates et al. 2015). ANOVA (Type II Wald chi-square tests) for mixed effects model of pretreatment isolation data for all logs was conducted to investigate variable effects on the likelihood of fungus detection. Odds ratios and estimated probabilities of detecting *Ceratocystis* spp. from the inner and outer sapwood of

diseased trees were calculated using the lsmeans package (Lenth 2016). Temperature profiles for the monitored logs in each treatment trial were summarized graphically. Means and standard errors of wood moisture contents for all study logs were calculated for the pre- and the post-treatment assessment dates.

Results

Characteristics of test logs. The dimensions of the test logs after their ends were sampled for fungal assay and wood content, but before being subjected to VS or control treatments, ranged from 1.7 to 2.1 m in length (Supplementary Table 2). The diameters of the small end of each log ranged from 22 to 44 cm. Weights of the three logs in each load were determined immediately before the load was placed in the vacuum chamber. Logs of similar diameters were included in a load. The pre-VS-treatment log weights ranged from 90 to 329 kg.

Pathogen presence in logs. *Ceratocystis* spp. were detected using carrot-baits in sapwood of 27 of the 31 logs assayed before treatment. Sub-samples from the four *Ceratocystis*-negative logs also failed to yield the fungus in post-treatment assays. These particular logs were from four different trees in Waipunalei. When the *Ceratocystis*-positive, pre-treatment logs (n = 27) were grouped by source tree, the mean rates of pathogen detection ranged from 12.5 to 65.6% of the outer sapwood assayed locations and from 6.2 to 31.2% of inner sapwood locations (Table 1). The highest grouped mean was for tree EB02 whose two logs also had the highest outer and inner sapwood detection levels of all the study logs (Table 1). When all of the pre-treatment logs (n = 31) were summarized by frequency of detection level according to sapwood depth, more logs had higher percentages of *Ceratocystis* positive outer sapwood assay locations (n=16) compared to inner ones (n = 16) (Figure 1).

There was no difference in detection of *Ceratocystis* spp. from logs obtained from the two sites (P = 0.5997) based on results of the generalized linear effects model (Table 2). However, differences were found for probability of detection by depth of sapwood assayed. The estimated probability of viable pathogen detection for all pre-treatment logs (combined sites) was higher for sub-samples obtained from outer sapwood than those from inner sapwood (Table 3). Sub-samples taken from the inner sapwood were half as likely to yield *Ceratocystis* spp. compared to samples from the outer sapwood based on results of odds ratio contrasts analysis (odds ratio = 0.49; P = 0.0001).

When pre-treatment detection data were summarized by treatment (VS treatment schedules; ambient temperature control), 25.6 to 26.6% of outer sapwood and 13.9 to 18.8% of inner sapwood locations on disks from all logs yielded *Ceratocystis* using carrot-baits (Table 4). In comparison, no viable *Ceratocystis* was detected in outer and inner sapwood of logs after the 60° C / 60 min VS treatment and inner sapwood of the 56° C / 30 min treatment. Furthermore, only a single subsample (0.48%) (N = 208) from the outer sapwood yielded *Ceratocystis* on carrot baits after 56° C / 30 min treatment. In contrast, viable pathogen levels were similar before and after 24 hrs. of ambient temperature for control logs held outdoors under shade (Table 4). Only *C. lukuohia* was detected in the 28 carrot baits sub-sampled (from 432 total baits) and subjected to qPCR analysis.

Temperatures achieved, time and energy required for vacuum steam treatment. Three temperature probes were placed at a depth of 70% of calculated log radius in three locations along the length of the log. Temperatures in these locations were continuously monitored during treatment to determine when to begin the prescribed treatment time. The monitored placement depths ranged from 8.4 to 13.3 cm for logs in the five 56° C / 30 min schedule trials and 8.9 to 14.4 cm in the four 60° C / 60 min trials. The range of times for the probes to reach 56° C and hold for 30 min in five replicate trials was 442 to 924 min (7.4 to 15.4 h) (Table 5). In comparison, the range of times for the probes to reach 60° C and hold for 60 min in four replicate trials was 515 to 1151 min (8.6 to 19.2 h). Representative temperature profiles based on temperatures recorded during a 56° C / 30 min and a 60° C / 60 min treatment are shown in Figure 2. Energy required for the shorter treatment schedule ranged from 58 to 112 kWh or 0.15 to 0.26 kWh/kg on a weight basis (Table 5). The energy requirements for the longer schedule ranged from 73.8 to 121.4 kWh or 0.06 to 0.18 kWh/kg on a weight basis.

Sapwood moisture content. Log weights were recorded after VS treatment once logs were sufficiently cooled to handle safely or after 24 h for control logs to obtain an indirect measure of any changes in whole log moisture content. No or negligible changes were found for VS and control logs (Supplemental Table 2). Moisture content of sapwood in sub-sampled portions of pre-treatment VS log disks ranged from 52.9 to 97.4% with a mean of 71.3%, as calculated on a dry weight basis (data not shown). Similar moisture content measurements of sapwood for disks taken from 27 logs following treatment (when cooled sufficient to handle) ranged from 51.0 to

94.2 %, with a mean of 69.8%. On average, moisture content decreased by 1.5% following VS treatment.

Post treatment log quality. Both ends of each study log were visually evaluated and photographed before and within 2 hours following VS treatment to document physical conditions. Closer visual evaluations of logs were conducted two days after treatment ended for each load. Small end-splits were observed on ends of two pre-treatment logs. Small to medium length (half to three quarters of a log's radius measurement) end split lines were observed on most of the logs. However, the splits did not extend more than 15 cm deep into the logs. No change of log color was observed in post-treatment logs.

***Ceratocystis* inoculations of treated logs.** Carrot baits with *Ceratocystis*-infused filter paper disks applied had visible fungal mycelia and perithecia within five days, indicating viable inoculum was used in the assay. No fungal growth was visible on the wood surface of any VS-treated logs, similar to the sterile water filter-papers (negative control). Carrot baits from excised wood strips were all negative for *Ceratocystis* growth, regardless of fungal species.

Discussion

The VS process using a 56°C / 30 min schedule was effective in eradicating *Ceratocystis* in > 99.5% of sapwood samples assayed, while the 60° C / 60 min schedule resulted in total eradication of *Ceratocystis* in sapwood of ohia logs colonized by the ROD pathogens. Specifically, 22 to 44 cm dia (small end) logs from naturally infected ROD trees that were heat-treated to targeted depths of approximately 70% of log radius were evaluated. The targeted depths are greater than depths previously reported to yield *C. lukuohia* and *C. huliohia* in fungus colonization studies of naturally infected ROD trees (Hughes et al 2020; Juzwik et al. 2019a). However, the sapwood depths (between 4 and 6 cm) from which inner wood samples were obtained for carrot-baiting assays in the current study were at least one-half the maximum depth of the targeted VS treatment depth; thus, fungus detection was not attempted at the predetermined threshold temperature depth. In comparison, the maximum temperatures reached at 6 cm would be greater than either the 56° C or 60 C reached at a 70% of log radius depth (e.g., 12 cm for a 17 cm diameter log). Further refinement of the optimal depth for achieving the targeted temperature could be of value, particularly for purposes of reducing energy consumption

for treatment. The targeted depth (5 cm) for threshold temperature used for treating *B. fagacearum* colonized oak logs was based on the depth to the generally obvious sapwood - heartwood boundary (Juzwik et al. 2019b). The sapwood-heartwood boundary in ohia is indistinct. Furthermore, dark red staining (reaction wood) in cut ends of main stem sections from ROD infected ohia trees often extends past 70% of the log radius and is not distinct from heartwood even though the *Ceratocystis* may not be present.

The overall mean rates of *Ceratocystis* spp. detection were higher for the outer sapwood than the inner locations when logs were groups by source tree. The *Ceratocystis* associated with each symptomatic tree was determined when trees were being selected as sources for study logs. Eleven of the thirteen trees from which the study logs came were infected with *C. lukuohia*. Thus, if one assumes most of the logs were colonized by *C. lukuohia* the trend of higher pathogen isolation rates in the outer sapwood versus the inner is consistent with isolation depth of *C. lukuohia* from stained xylem found between 2 and 4 cm depth in earlier colonization studies of naturally infected *C. lukuohia* trees (Hughes et al. 2020). In the current study, pathogen detection levels were < 32% of outer sapwood and < 19% of inner sapwood for 85% of the *Ceratocystis*-positive logs. For isolation rates of *B. fagacearum* in pre-VS treated *Q. rubra* logs from naturally infected trees, values ranged from 9 to 36% for outer and 5 to 16% for inner sapwood locations (Juzwik et al. 2019b).

Negligible loss of sapwood moisture (avg. 1.5%) was found for VS treated ohia logs based on log disk samples. An even smaller loss (avg. < 0.1%) in sapwood moisture content was found for VS treated northern red oak logs colonized by *B. fagacearum* (Juzwik et al. 2019b) and an increase (2 to 4%) in sapwood moisture content for VS treated *Juglans nigra* logs colonized by the bark canker pathogen *Geosmithia morbida* (Juzwik et al. 2021). Treatment of oak and walnut logs with bark attached is likely responsible for lack of sapwood moisture loss in the oak and the slight gain in the walnut logs subjected to VS compared to the slightly greater loss found in the VS treated, de-barked ohia logs.

The differences in time required for treatment of three similar diameter logs per treatment load for either 56° C / 30 min or 60° C / 60 min schedule were attributed to differences in schedule temperature and holding time, although differences in log diameters used for a trial may have contributed to longer times. Although treatment times would logically be longer for treating more logs and longer logs in an operational run, VS is still a relatively fast treatment compared

to heat treatment with a drying kiln. Although phytosanitary treatment of dimension lumber and pallets using kiln-heating is recognized and accepted for global trade, its use for phytosanitary treatment of round wood has not been well studied. In comparison, VS has been shown to be an effective and efficient means of heating both rectangular sections of wood and round wood (Simpson 2001). Energy consumption for ohia log treatments would be an important consideration because energy costs in Hawaii are among the highest for states in the USA. For the current study, the authors calculated the cost per kg of log weight for the relatively small diameter logs (24 to 33 cm) in loads for tests 8 and 9 of the 56°C / 30 min treatment to be $0.016 and $0.012, respectively.

Ohia logs in Hawaii are sold "skinned" (de-barked) and "green" (not dried to a low moisture content). Up to the current time, the processed logs are shipped at varying moisture contents that are correlated with the length of time logs air dry while stored in the mill yard prior to sale and shipping. Thus, logs treated by VS process would be acceptable.

Superficial cracks or splits along the length and end checking of superficial depth of ohia logs are acceptable for traditional uses (e.g., decorative beams, porch railing systems). Large logs with deep cracks and splits, however, would not be suitable for structural supports of houses and load-bearing beams. In the current study, the effect of VS on ohia log quality was only evaluated two days after treatment and minimal damage was documented. Future tests could include quality evaluation of VS treated logs after several weeks or months of natural air drying in mill yards, i.e. the range of time logs are normally stored prior to shipping. Minimal end checking and splits of large logs of five different hardwood species were found on the same day treatment ended in earlier VS trials (Chen et al. 2017). We hypothesize that VS process would be an effective phytosanitary treatment that is energy and time efficient and would have no deleterious effects on quality of all sizes of ohia logs.

Recolonization of treated ohia logs was considered in this study because such logs could possibly need to be held for several weeks to months depending on the terms of a log sale and other factors. Such logs would likely be held in the mill yard where inoculum could be present in freshly cut logs coming directly from harvest sites in ohia forests. If phytosanitary treatment is conducted at the mill yard, the mill owner needs to know whether special precautions are needed to reduce potential for reinfestation by the ROD *Ceratocystis* species. Reinfestation experiments were conducted on *Juglans nigra* logs and natural wane lumber following different

412 phytosanitation treatments in Tennessee (Audley et al. 2016). *Pityophthorus juglandis* (walnut
413 twig beetle), the insect pest and primary vector of the thousand cankers disease pathogen
414 (*Geosmithia morbida*) was found to recolonize the tested products. Thus, efforts to exclude
415 insects from phytosanitatized bark on walnut wood products would be needed for commercial
416 trade. *Ceratocystis huliohia* and *C. lukuohia* were unable to colonize VS treated ohia logs in this
417 study. It is possible that treatment and subsequent air-drying reduced the surface xylem wood
418 moisture content below a threshold that could not support superficial Ceratocystis growth
419 (Tainter et al., 1984), or that the pathogens have limited saprophytic capacity to colonize non-
420 living tissues. Based on our findings of failure to re-colonize, we hypothesize that the exclusion
421 efforts needed for heat-treated walnut logs would not be needed for VS treated, de-barked ohia
422 logs.

423 Many practical matters should be considered before the VS process would be adopted and
424 used for ohia log treatments in Hawaii. Regulatory officials with the Hawaii Department of
425 Agriculture evaluate proposed, new phytosanitary treatments for various commodities, including
426 wood products. If approved, mill owners may consider among other things the capital
427 investment required for the needed equipment and extent of use for the equipment (i.e., conduct a
428 cost-benefit analysis). Currently, only one mill on Hawaii Island has a vacuum chamber suitable
429 for use in the VS process. Besides using VS for logs destined for off-island sales, the treatment
430 could be considered for safe utilization of wood products from dying and recently killed ohia
431 (i.e. salvage harvesting). Hundreds of thousands of ohia have been killed by ROD on Hawaii
432 Island (College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa
433 2021) and not utilized due to concerns over spreading the pathogen(s) to other areas of the island
434 and potential for increasing intensity of the disease in other areas. Salvage harvest of ROD-killed
435 ohia and phytosanitary treatment of cut logs could allow for safe utilization of ohia wood from
436 such trees. The forest industry in Hawaii is currently working toward increasing processing
437 capacity and overcoming barriers for utilization of invasive tree species in an effort to manage
438 them.

439 In summary, VS process (either 56 ° C for 30 min or 60° C for 60 min) was found to be an
440 effective and efficient method for killing viable *Ceratocystis* species associated with rapid ohia
441 death in initial tests with short length (~ 2 m), large diameter logs. Since a wide size-range of

ohia logs are produced by mills in Hawaii, further tests are needed to determine the range of treatment costs and total times required for different dimension products.

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TABLES

Table 1. Frequencies (numbers and percentages) of assayed subsamples yielding *Ceratocystis lukuohia* or *C. huliohia* on carrot baits for the outer and inner sapwood of logs taken from naturally infected *Metrosideros polymorpha* trees at Waipunalei and Kau ranch sites, Hawaii Island, prior to vacuum steam or ambient temperature (control) treatment.

Tree ID[a]	Number of logs[b]	Number of assayed locations	*Ceratocystis* – positive carrot baits by sapwood depth			
			Outer		Inner	
			number	percent	number	percent
W - T2	3	48	14	29.2	8	16.7
W- EB02	2	32	21	65.6	9	28.1
W – 202	2	32	6	18.8	4	12.5
W - 203	2	32	7	21.9	6	18.8
W - 204	1	16	3	18.8	2	12.5
W - 205	2	32	7	21.9	3	9.4
W - 206	2	32	8	25.0	4	12.5
W- 209	1	16	4	25.0	3	18.8
W - 210	1	16	2	12.5	1	6.2
K - 269	2	32	4	12.5	3	9.4
K - 270	1	16	7	43.8	5	31.2
K - 271	3	48	11	22.9	4	8.3
K - 272	5	80	18	22.5	12	15.0

[a] Letter preceding number indicates site where tree was located: W = Waipunalei; K = Kau.

[b] Sapwood sub-samples from four pre-treatment logs (one from each of Waipunalei trees 202, 203, 204 and 210) did not yield *Ceratocystis* in carrot-baiting assays. These *Ceratocystis*-negative logs were not used in calculating frequencies for this table.

518

Table 2. Coefficients of the generalized linear effects model fit to the *Ceratocystis* spp. carrot-baiting assay data for inner and outer sapwood depths of disks sampled prior to vacuum steam or ambient temperature treatments. Disks were from logs cut from main stems of thirteen naturally infected *Metrosideros polymorpha* trees at two sites on Hawaii Island.

Variable	Coefficient estimate	SE	P value
Intercept	-1.8739	0.3946	<0.0001
Site: Waipunalei	-0.2529	0.4818	0.5997
Depth: outer sapwood	0.6461	0.2865	0.0241
Site x depth	0.1244	0.3629	0.7318

519

520 [a/] Kau ranch site and inner sapwood are the reference levels.

521

Table 3. Estimated probabilities of *Ceratocystis* spp. detection using carrot-baiting assay of sapwood sub-samples from pre-treatment logs cut from diseased *Metrosideros polymorpha* trees. Probabilities are based on logistic regression of the interaction of tree source location (Kau or Waipunalei) and sapwood depth (outer or inner sapwood).

Tree source location	Sapwood depth	Estimated probability[b]	Standard error	95% Confidence interval
Kau	Outer	0.225	0.064	(0.066, 0.246)
Kau	Inner	0.389	0.132	(0.124, 0.374)
Waipunalei	Outer	0.198	0.039	(0.132, 0.287)
Waipunalei	Inner	0.103	0.025	(0.063, 0.164)
Combined[a]	Outer	0.216	0.025	(0.077, 0.179)
Combined[a]	Inner	0.119	0.039	(0.149, 0.301)

[a/] Because tree source site had no significant effect on likelihood of fungus detection, it is more appropriate to use the estimated probabilities from the pooled data for predictive purposes.

[b/] Probabilities and confidence intervals are back transformed from the logit scale.

Table 4. Numbers of assayed locations with subsamples yielding *Ceratocystis spp.* on carrot baits from the outer and inner sapwood of logs from naturally infected *Metrosideros polymorpha* trees at Waipunalei and Kau ranch sites, Hawaii Island, before and after vacuum steam treatment or exposure to ambient conditions for 24 hours.

Treatment schedule[a]	Number of tests	Number of logs[b]	Number of assayed sapwood locations	Pretreatment: Fungus – positive baits by depth		Posttreatment: Fungus – positive baits by depth	
				Outer	Inner	Outer	Inner
				(number)	(number)	(number)	(number)
56 / 30	5	13	208	54	29	1	0
60 / 60	4	10	160	41	23	0	0
Ambient / 24	2	4	64	17	12	18	8

[a] Treatments include: 56 /30 = 56° C held for 30 min; 60 / 60 = 60° C held for 60 min; and Ambient / 24 = Ambient temperature over 24 hrs. Threshold temperatures for treatments were at 70% of log radial depth. Initial vacuum 100 mm Hg and saturated steam 85°C.

[b] Three logs were used per treatment load for each test. However, sub-samples from four logs collected before and after one 56 / 30 and two 60 / 60 vacuum steam tests did not yield *Ceratocystis* spp. in carrot-baiting are excluded from this table.

Table 5. Vacuum steam treatment cycle time, heating rate and energy usage for the two treatment schedules with threshold temperature at 70 percent of log radius depth in sapwood of M*etrosideros polymorpha* logs.

Treatment schedule[a]	Test number[b]	Average initial log temp (° C)	Total cycle time (min.)	Heating rate (min / (° C)	Log load weight (kg)	Energy use KWh	Energy use kWh/kg
56 /30	1	24	442	13.8	312.4	58.00	0.1857
56 / 30	2	23	924	28	--[c]	112.05	--
56 / 30	6	27	474	16,3	332,9	73,67	0.2213
56 / 30	8	21	608	17.4	438.6	66.11	0.1507
56 / 30	9	20	499	13.9	296.6	75.76	0.2554
60 / 60	3	22	515	13.6	305.4	54.74	0.1792
60 / 60	4	25	1151	32.9	865.5	121.43	0.06364
60 / 60	5	24	853	23.7	564.3	83.73	0.1484
60 / 60	7	25	767	21.9	517.1	73.81	0.1427

[a/] Treatments: 56 /30 = 56° C held for 30 min; 60 / 60 = 60° C held for 60 min; and Ambient / 24 = Ambient temperature over 24 hrs. Threshold temperatures for treatments were at 70% of log radial depth. Initial vacuum 100 mg Hg and saturated steam 85° C.

[b/] The three logs per test were obtained from trees that were naturally colonized by *Ceratocystis* spp. that cause rapid ohia death.

[c/] Log weights for three logs in load were not taken.

Supplemental Table 1. Characteristics of rapid ohia death affected *Metrosideros polymorpha* trees used as sources for logs treated in vacuum steam and ambient temperature (control) tests.

Tree ID [a]	Tree DBH (cm)	Log IDs [b]	*Ceratocystis* species detected [c]	Crown wilt rating		Days from rating date to testing date (no.)
				Percent	Date	
W-T2	52	289, 291, 294	*C. lukuohia*	90	Oct. 2018	116 - 125
W-EB02	31	37, 39	*C. lukuohia*	95	Dec. 2018	51 - 56
W-202	34	277*, 279, 281	*C. lukuohia*	50	June 2018	224 - 233
W-203	43	283, 285, 287*	*C. hulioiha*	98	June 2018	225 - 232
W-204	--[d]	35, 275*	*C. lukuohia*	100	June 2018	231 - 233
W-205	33	31, 299	*C. lukuohia* + *C. huliohia*	100	June 2018	226 - 227
W-206	50	49, 51	*C. lukuohia*	100	Aug. 2018	162 - 164
W-209	39	53	*C. lukuohia*	30	June 2018	227
W-210	46	41*, 43	*C. lukuohia*	100	Aug. 2018	164 - 168
K-269	40	76, 80	*C. lukuohia*	80	Jul. 2018	214 - 215
K-270	32	61	*C. lukuohia*	100	Jul. 2018	206
K-271	50	66, 68, 70	*C. lukuohia*	95	Nov. 2018	73 - 80
K-272	52	82, 84, 86, 88, 90	*C. lukuohia*	85	Nov. 2018	73 - 76

[a/] Letter preceding number indicates site where tree was located: W = Waipunalei; K = Kau.

[b/] This table includes four logs denoted by asterisk for which no viable *Ceratocystis* was isolated using carrot-baiting assay (Moller and DeVay 1968) of sapwood from pre- and post-treatment logs.

[c/] Species detection result based on molecular assay. Specifically, sapwood shavings were collected from main stem of each tree using a battery powered drill. DNA was extracted from the shavings and a qPCR assay performed using protocol of Heller and Keith (2018).

[d/] Tree diameter was not obtained.

564 **Supplemental Table 2.** Characteristics of logs from rapid ohia death affected ohia
565 (*Metrosideros polymorpha)* trees that were prepared and treated in vacuum steam and ambient
566 temperature (control) tests.

Treatment schedule[a]	Test number	Log number[b]	Log length (m)	Log diameter by end (cm)		Log weight (kg) Relative to treatment	
				small	large	pre-	post-
56 /30	1	291	1.89	25.6	28.0	110.5	108.9
		281	1.89	24.3	26.5	92.1	89.2
		279	2.00	26.4	28.1	110.0	108.4
56 / 30	2	283	1.90	35.9	40.8	--[c]	--
		51	1.90	40.1	41.6	--	--
		82	1.90	38.1	40.8	--	--
56 / 30	6	37	2.00	26.4	29.2	110.7	108.4
		68	1.70	24.6	28.3	90.7	87.5
		66	1.98	28.0	29.1	131.5	129.7
56 / 30	8	285	1.90	32.6	32.9	169.6	168.3
		287*	1.90	29.5	32.3	140.2	138.8
		277*	1.70	28.5	33.3	128.8	128.4
56 / 30	9	80	1.71	24.3	27.5	71.7	72.6
		35	1.89	23.6	28.3	93.0	92.1
		294	1.92	26.6	29.5	132.0	134.7
60 / 60	3	31	1.90	25.0	27.3	110.4	108.2
		39	2.00	26.0	29.1	90.4	88.0
		88	1.80	26.7	27.9	104.6	104.4
60 / 60	4	41*	2.00	36.9	42.0	235.0	238.6
		289	2.00	44.2	45.7	329.3	324.8
		49	2.00	41.3	45.8	301.2	297.6
60 / 60	5	53	1.90	31.5	36.6	184.2	184.6
		299	1.90	34.2	35.7	184.2	180.5
		86	1.90	34.5	39.5	196.0	197.8
60 / 60	7	275*	1.90	31.0	32.5	157.8	157.8
		43	1.90	32.8	35.6	181.0	180.5
		76	1.90	33.8	33.9	178.2	178.7

Control 1	1	61	2.13	26.5	29.2	115.2	118.8
Control 2	3	70	1.93	36.9	38.7	210.0	207.7
Control 3	6	90	1.96	21.6	22.1	75.3	75.3
Control 4	9	84	1.85	43.2	43.8	270.3	269.4

[a/] Treatments: 56 /30 = 56° C held for 30 min; 60 / 60 = 60° C held for 60 min; and Ambient / 24 = Ambient temperature over 24 hrs. Threshold temperatures for treatments were at 70% log radial depth. Initial vacuum 100 mm Hg and saturated steam 85°C.

[b/] Log number followed by an asterisk denotes that the log did not yield viable Ceratocystis in subsequent pre-treatment and post-treatment assays using carrot-baiting technique (Moller and DeVay, 1969).

[c] Weights were not obtained.

575
576
577

FIGURE CAPTIONS

578
579

580 **Figure 1.** Numbers of pre-treatment logs (n = 31) yielding rapid ohia death *Ceratocystis* spp.by
581 sapwood assay depth (A = outer sapwood; B = inner sapwood) of logs cut from naturally
582 infected *Metrosideros polymorpha* trees. Detection level is displayed as percent of assayed disk
583 sampling location (16 per log) yielding the pathogen(s). Numbers based on isolation results of
584 two disks for each log. Wood tissues were assayed using the carrot-baiting technique described
585 by Moller and DeVay, 1968. Data for the two log-source sites are combined because no
586 difference (P = 0.5997) was found in fungus detection between them.

587

588 **Figure 2.** Representative temperature profiles of *Metrosideros polymorpha* logs treated in the
589 vacuum steam trials. (A) 56º C for 30 min treatment of log 285 (Test 8), and (B) 60º C for 60
590 min treatment of log 49 (Test 4).

591

592 **Supplemental Figure 1.** Locations of temperature probes used for monitoring thermal profile in
593 *Ceratocystis* colonized *Metrosideros polymorpha* logs during vacuum steam treatment trials.

594

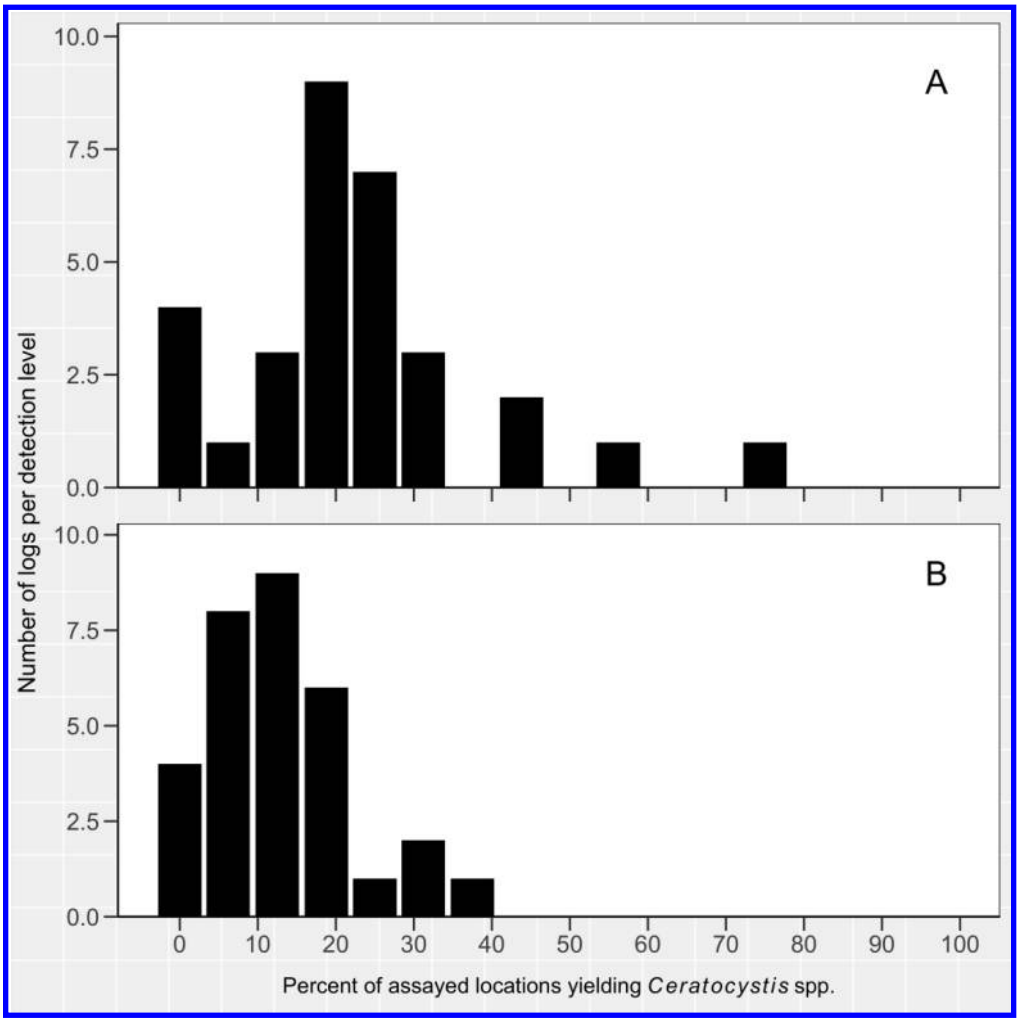
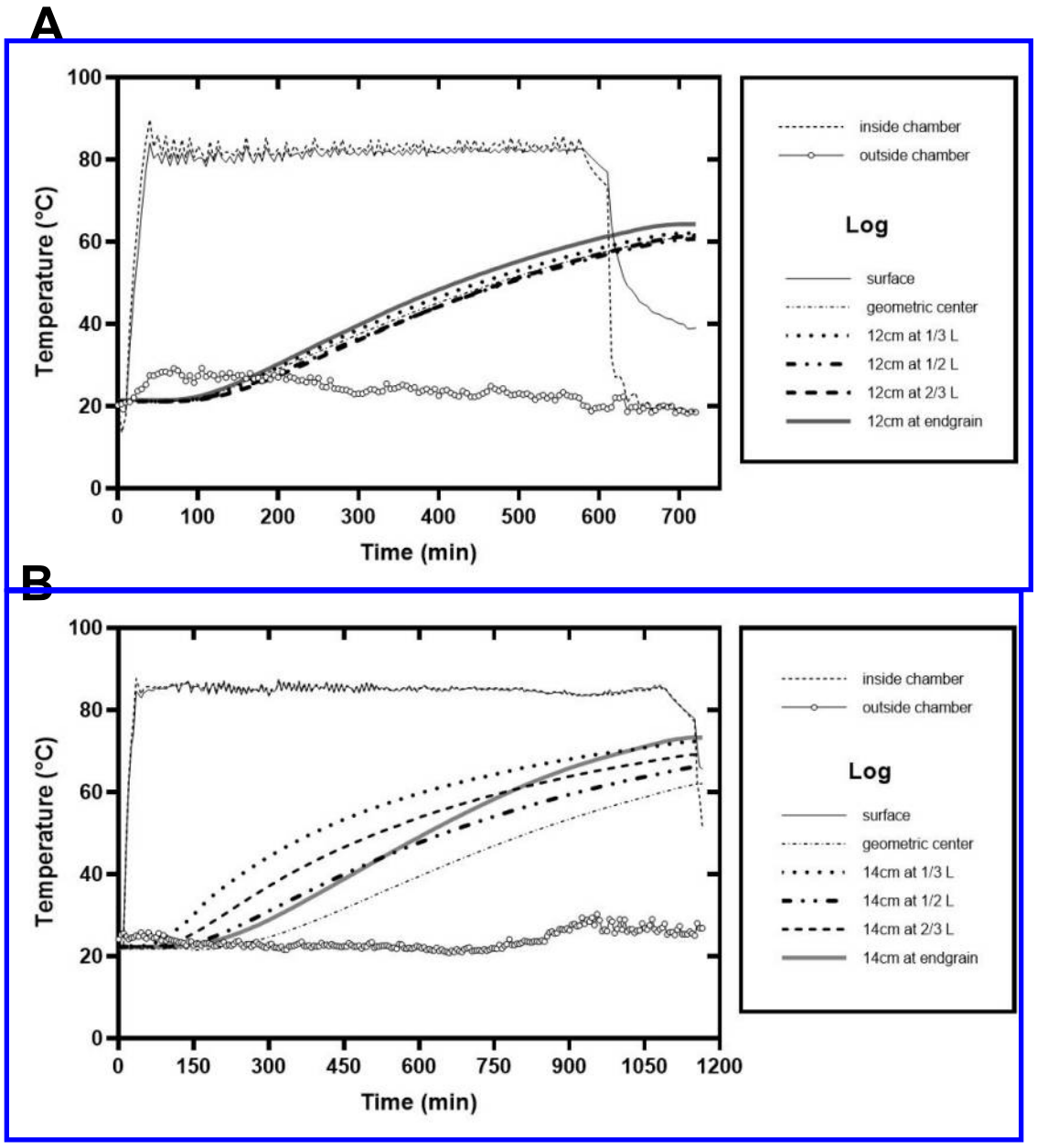
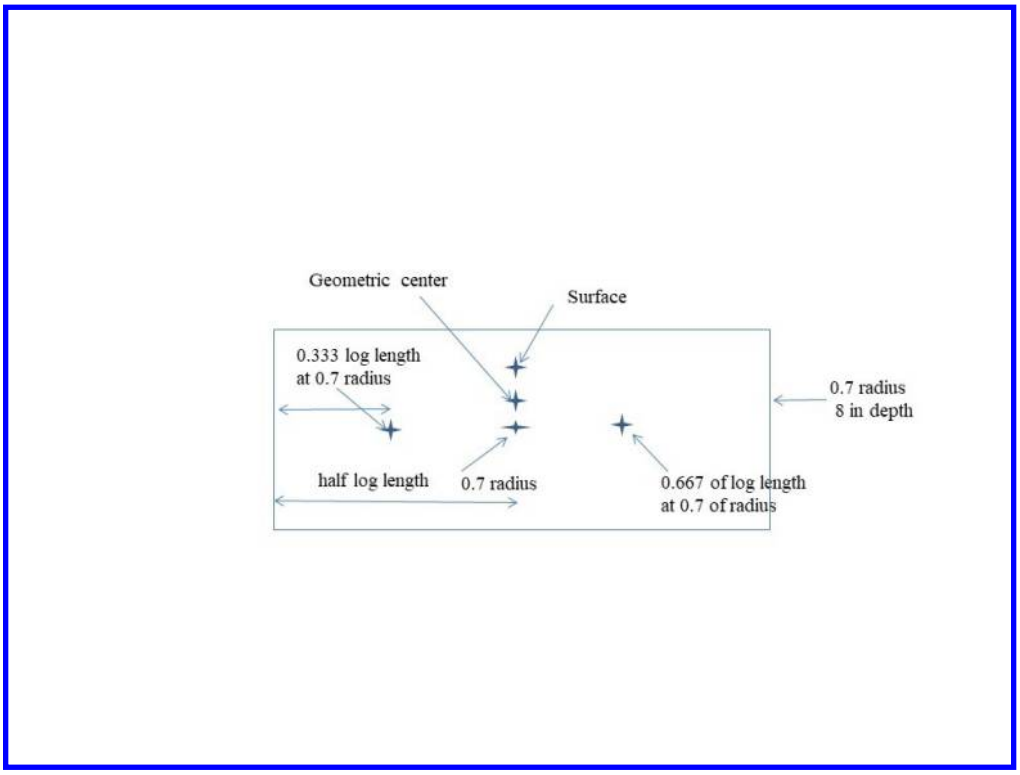


Figure 1. Numbers of pre-treatment logs (n = 31) yielding rapid ohia death Ceratocystis spp.by sapwood assay depth (A = outer sapwood; B = inner sapwood) of logs cut from naturally infected Metrosideros polymorpha trees. Detection level is displayed as percent of assayed disk sampling location (16 per log) yielding the pathogen(s). Numbers based on isolation results of two disks for each log. Wood tissues were assayed using the carrot-baiting technique described by Moller and DeVay, 1968. Data for the two log-source sites are combined because no difference (P = 0.5997) was found in fungus detection between them.

1234x1234mm (72 x 72 DPI)

Figure 2


A
Temperature (°C)
Time (min)
inside chamber
outside chamber
Log
surface
geometric center
12cm at 1/3 L
12cm at 1/2 L
12cm at 2/3 L
12cm at endgrain
B
Temperature (°C)
Time (min)
inside chamber
outside chamber
Log
surface
geometric center
14cm at 1/3 L
14cm at 1/2 L
14cm at 2/3 L
14cm at endgrain



Supplemental Figure 1. Locations of temperature probes used for monitoring thermal profile in *Ceratocystis* colonized *Metrosideros polymorpha* logs during vacuum steam treatment trials.

254x190mm (96 x 96 DPI)