Influence of Culture Media and Temperature on Growth and Sporulation of Ceratocystis lukuohia

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Influence of Culture Media and Temperature on Growth and Sporulation of *Ceratocystis lukuohia*[1]

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Abstract: *Ceratocystis lukuohia* is one of two newly described pathogens of *Metrosideros polymorpha* (ʻōhiʻa) causing Rapid ʻŌhiʻa Death, a phenomenon that is devastating sections of native forest across the state of Hawaiʻi, USA. *Ceratocystis lukuohia* research has increased as the threat of the pathogen has become more apparent, resulting in a need for efficient production of fungal cultures to meet research demands. Therefore, the temperature and medium-dependent mycelium growth and spore production of three isolates, one from the outbreak area of Leilani Estates and two from beyond the area, were assessed in culture. Fungal growth, measured as mycelium diameter, did not differ for the three isolates after 7 days of incubation. Optimal growth temperatures were 25–30 °C on 10% V8 agar and 25 °C on malt yeast extract agar (MYEA). Spore production, as assessed in one isolate, was most abundant at 25 °C and on MYEA, followed by 10% V8, and MEA. Mycelium growth and spore production on 10% V8 were less variable compared to the other media and, while mean spore production was highest on MYEA, the test isolate produced at least 1×10^6 spores/mL on 10% V8 agar, a commonly used spore concentration for inoculation experiments. Therefore, we recommend growing *C. lukuohia* at 25 °C on 10% V8 for inoculum production. These results contribute to the growing knowledge base of this newly discovered fungal pathogen and suggest a standardized methodology for propagating *C. lukuohia* for use in host-pathogen interaction studies.

Keywords: ROD, ʻōhiʻa, *Metrosideros polymorpha*, agar, *Ceratocystidaceae*

Ceratocystis lukuohia I. Barnes, T. C. Harr, & L. M. Keith (Microascales: Ceratocystidaceae) is the more aggressive of two newly described *Ceratocystis* species that are responsible for causing rapid wilting and mortality of the endemic Hawaiian tree *Metrosideros polymorpha* Gaud. (Myrtaceae), a phenomenon known as Rapid ʻŌhiʻa Death (ROD; Keith et al. 2015, Barnes et al. 2018). While currently found nowhere else in the world, *C. lukuohia* is believed to have been recently introduced to Hawaiʻi due to genetic homogeneity of isolates (Barnes et al. 2018). *Ceratocystis lukuohia* causes the disease known as *Ceratocystis* wilt of ʻōhiʻa, and typical symptoms include black-colored staining of the sapwood and rapid crown wilting resulting in death of the tree (Hughes et al. 2020). Although *C. lukuohia* was first discovered in 2014, trees showing ROD symptoms had been reported as early as 2010 (Keith et al. 2015), *C. lukuohia* is now associated with the widespread mortality on Hawaiʻi Island (Barnes et al. 2018). *Ceratocystis lukuohia* has the potential to devastate native Hawaiian forests, which are composed largely of *M. polymorpha*. Approximately 350,000 hectares (ha) of *M. polymorpha*

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forest exist in the state, most of which are found on Hawai'i Island (Mortenson et al. 2016). Using remote sensing techniques and aerial imagery, it is estimated that there are more than 72,000 ha of ROD-affected *M. polymorpha* (ROD SRP 2020).

Resistance of *M. polymorpha* to *C. lukuohia* infection is currently being investigated but the research is in its infancy. As ROD research increases, there must be an adequate and consistent amount of inoculum to support these initiatives. Two commonly used culture media in *Ceratocystis* research are 2% malt extract agar (MEA) and 2% malt yeast extract agar (MYEA) (Wingfield et al. 1996, Oliveira et al. 2016, Li et al. 2017), and previous experiments have been examined how isolates of *C. lukuohia* vary in spore morphology and mycelium growth using MEA (Barnes et al. 2018). However, 10% V8 agar is routinely used for *C. lukuohia* culturing (Keith et al. 2015, Heller and Keith 2018, Brill et al. 2019, Hughes et al. 2020). Oliveira et al. (2016) demonstrated that various culture media had different effects on mycelial growth and sporulation of *Ceratocystis fimbriata* Ellis and Halst. from mango (*Mangifera indica* L.). To date, there has not been a direct comparison of the effects of culture media on fungal growth and sporulation of *C. lukuohia*. Thus, the main objective of this study was to evaluate fungal growth and sporulation among three different, commonly used culture media in *Ceratocystis* research. The objective of this research is to optimize and standardize *C. lukuohia* culture methodology, thereby facilitating future research on *Ceratocystis* wilt of 'ōhi'a.

MATERIALS AND METHODS

Isolates

Isolates were selected from a collection stored at the USDA Agricultural Research Service in Hilo, HI which consisted of isolates from the main ROD outbreak areas in the Ka'ū, Puna, and Hilo Districts on Hawai'i Island. Isolates in the collection were preserved by storing chunks of colonized agar in sterile, water-filled test tubes. Three isolates of *C. lukuohia* were selected to represent distinct clusters of disease in 2016: P14-1-1 (Leilani Estates, Puna District, 312 m elevation; an area of high mortality), P15-80 (Fern Forest, Puna District, 693 m elevation; an area of low mortality), and, P16-7 (the Kahuku Unit of Hawai'i Volcanoes National Park, Ka'ū District, 704 m elevation; a new outbreak location). Isolates were transferred from storage, plated on 10% V8 agar, and incubated at 25 °C.

Mycelium Growth and Spore Production

To compare the effects of media and temperature on mycelium growth, 6 mm agar plugs were taken from the outer edge of the mycelium of 7-day-old cultures grown on 10% V8 agar and inserted into 6 mm gaps in the center of 15 × 100 mm Petri dishes containing 2% MEA (10 g malt extract, 10 g agar, 500 mL deionized water), 2% MYEA (10 g malt extract, 0.1 g yeast extract, 10 g agar, 500 mL deionized water), or 10% V8 agar (50 mL V8 juice, 0.01 g calcium carbonate, 10 g agar, 450 mL deionized water). Plates were incubated for 7 days at one of seven temperatures ranging from 10 °C and increasing by 5 °C increments up to 40 °C. Two perpendicular measurements of colony diameter were taken for each culture 7 days post-inoculation using a digital caliper and the average colony diameter was calculated. The experiment was conducted twice, resulting in six replicates of each isolate per temperature and medium combination.

Spore production was determined for the P14-1-1 isolate in a separate experiment by inoculating three replicate plates each of MEA, MYEA, and 10% V8 agar in 15 × 100 mm Petri dishes as above. Cultures were incubated for 7 days at temperatures ranging from 10 to 35 °C increasing by 5 °C increments, to observe whether temperature also played a role in spore production. Spore suspensions were obtained by flooding the culture plate with 4 mL of sterile distilled water and scraping the surface with a rubber policeman. Aleurioconidia, endoconidia, and ascospore concentrations were quantified on a hemocytometer and observed under 400× magnification using a compound microscope

(Nikon Eclipse E600, Japan). Spore concentration for each plate was measured twice and averaged. The experiment was conducted twice, resulting in six replicates.

Statistics

A two-way ANOVA was used to determine whether there were any differences in mean mycelium diameter among isolates and between trials. A two-way ANOVA and Tukey's HSD post-hoc tests were conducted to compare the mean mycelium diameter of all the isolates, collectively, across temperatures, and media types.

A Kruskal–Wallis test was conducted to compare the mean spore concentration between experimental replicates. Mean spore concentration was compared among temperatures for each media type, separately, due to unequal variances. One-way ANOVAs and Tukey's HSD post-hoc tests were used to compare mean mycelium diameter among the incubation temperatures tested for cultures grown on MEA and 10% V8, while a Welch's ANOVA with a Games-Howell post-hoc test was used to analyze the results for cultures grown on MYEA. Because media were analyzed separately, a Bonferroni correction was employed for these three tests ($\alpha = 0.016$). All statistical tests were conducted using R statistical software version 3.6.3 (R Core Team 2020). The Welch's ANOVA and Games-Howell post-hoc test were conducted using the "userfriendlyscience" package version 0.6-1 (Peters 2017). Graphs were created using the "ggplot2" package version 3.3.0 (Hadley 2020) in R statistical software.

RESULTS AND DISCUSSION

Mean mycelium diameter did not differ among isolates ($F = 1.32$; df = 2,372; $p = 0.27$), experimental replicate ($F = 1.98$; df = 1,372; $p = 0.16$), or their interaction ($F = 0.01$; df = 2,372; $p = 0.99$). Therefore, the mycelium diameter results from the three isolates and two experimental replicates were combined to assess the effects of temperature and agar medium on mycelium diameter. Mean mycelium diameter differed among media ($F = 121.71$; df = 2,357; $p < 0.01$), incubation temperature ($F = 741.31$; df = 6,357; $p < 0.01$), and the interaction of media and incubation temperature ($F = 23.65$; df = 12,357; $p < 0.01$).

All three isolates grew on 10% V8 agar, MEA, and MYEA at temperatures as low as 10 °C and as high as 35 °C (Figure 1). Mycelium growth decreased gradually from 25 °C to 10 °C, while growth decreased sharply from 30 °C to 35 °C. Mycelium growth at 40 °C was only observed on one plate of P15-80 and two plates of P16-7, all of which were less than 2 mm. Results indicate that temperatures above 30 °C are inhibitory to growth of *C. lukuohia*. MYEA at 25 °C and 10% V8 at 25 °C and 30 °C were found to be the combinations of incubation temperature and medium that produced the largest mycelium diameter. On average (mean ± standard deviation), the cultures grew to 46.6 ± 4.9 mm on 10% V8 at 25 °C, 47.7 ± 4.2 mm on 10% V8 at 30 °C, and 44.2 ± 9.1 mm on MYEA at 25 °C. The largest growth observed for MEA was at 25 °C (27.5 ± 4.8 mm), but it was significantly less than 10% V8 and MYEA at the same temperature.

The similarities in growth and morphology among the three isolates, as well as the clonal nature of the species (Barnes et al. 2018), suggested that a single isolate would be representative of *C. lukuohia* as a species. Therefore, isolate P14-1-1 was selected as the single isolate for evaluation of spore production. Spore concentration did not differ between experimental replicates (Kruskal–Wallis $\chi^2 = 0.16$, df = 1, $p = 0.69$), thus data from both experimental replicates were combined for further analysis. *Ceratocystis lukuohia* isolate P14-1-1 produced conidia at all temperatures and on all media types. Perithecia were not observed at temperatures ≤15 °C. Mean spore concentrations of cultures grown on MEA were similar across the six temperatures tested ($F = 2.48$; df = 5,30; $p = 0.054$). However, mean spore concentrations were significantly different among temperatures for cultures grown on MYEA ($F = 16.61$; df = 5,30; $p < 0.001$) and 10% V8 ($F = 15.84$; df = 5,30; $p < 0.001$). Overall spore concentration was the lowest on MEA and highest on MYEA (Figure 2). Spore

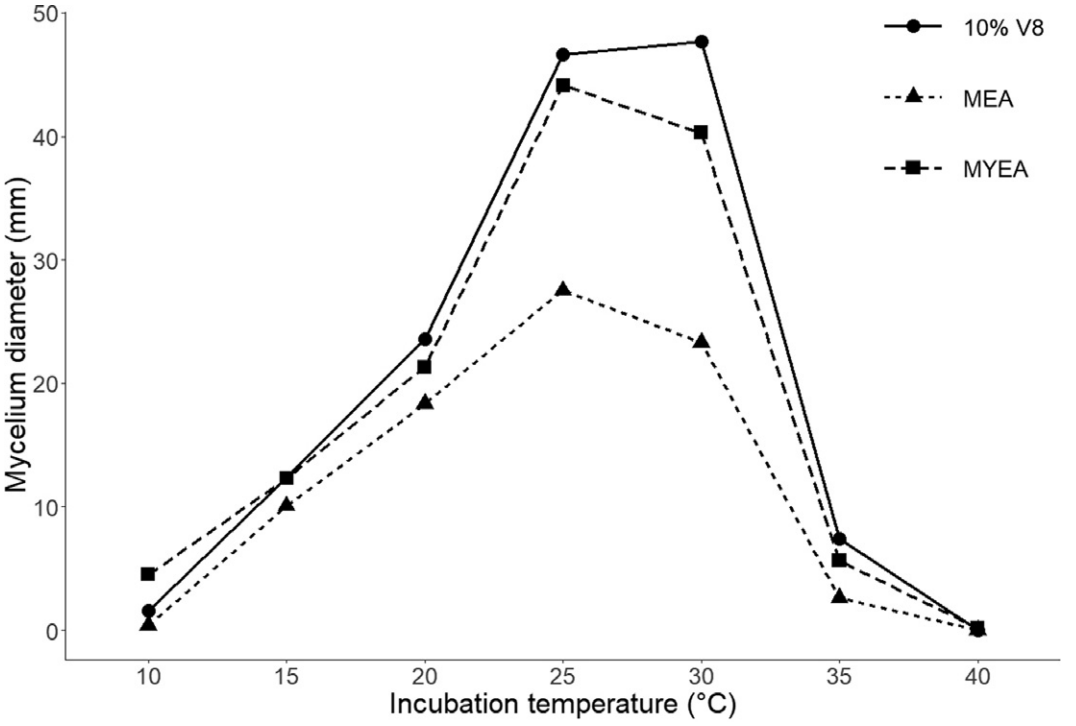

FIGURE 1. Diameter of colonies (in mm) of *Ceratocystis lukuohia* isolates grown on three different culture media at seven different temperatures. Measurements were taken 7 days post-inoculation.

concentration was highest at 25 °C for 10% V8 ($1.91 \times 10^6 \pm 0.73 \times 10^6$ spores/mL), MYEA ($7.28 \times 10^6 \pm 1.87 \times 10^6$ spores/mL), and MEA ($0.23 \times 10^6 \pm 0.08 \times 10^6$ spores/mL).

Results from this study showed that both MYEA and 10% V8 agar are suitable for growing *C. lukuohia* for inoculations, resulting in sufficient mycelium and spore production ($>1 \times 10^6$ spores/mL). However, while spore concentration was highest on MYEA, mycelial growth and spore concentration were more variable on MYEA than on 10% V8. Also, spore concentrations of up to 5×10^6 spores/mL after 7 days of growth have been obtained when centrally inoculating 10% V8 agar with ascospore masses (Luiz unpublished data), which is the method currently used for transferring cultures (Keith et al. 2015, Barnes et al. 2018, Hughes et al. 2020). This difference in spore production could be attributed to the inoculation method of the plates. The agar plugs were taken from the edges of mycelia which mainly contain hyphae and conidia, but few, if any, perithecia. The different propagules involved in each transfer might affect how the resulting culture grows. Nonetheless, inoculations of ʻōhiʻa seedlings have utilized concentrations of 1×10^6 spores/mL (Keith et al. 2015, Barnes et al. 2018, Hughes et al. 2020), which is attainable using both 10% V8 agar and MYEA. Therefore, due to the consistency of sporulation and mycelium production, we recommend growing *C. lukuohia* at 25 °C on 10% V8 agar.

Ceratocystis species cause disease and mortality of woody plants through a combination of xylem vessel occlusion and secretion of phytotoxins (Nasution et al. 2019, Silva et al. 2020). A large quantity of infective propagules is needed to cause such a major disruption to a vital plant system, so optimization of inoculum production is necessary to achieve reproducible results (Oliveira et al. 2016). Our study identified reliable methods for

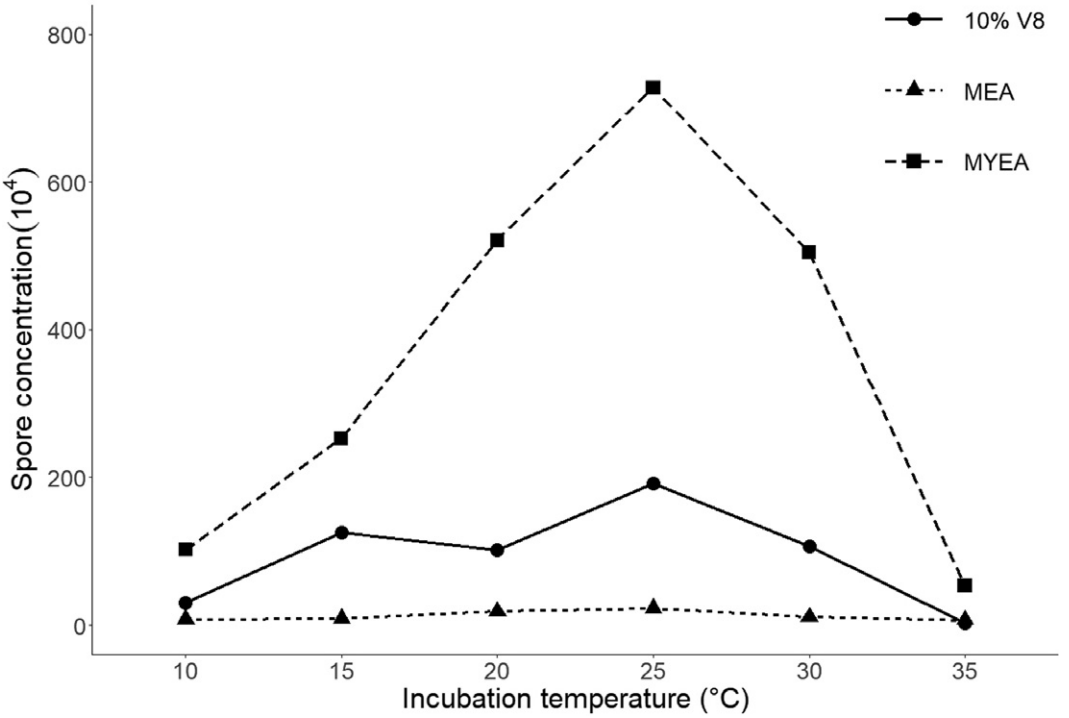

FIGURE 2. Spore concentration of *Ceratocystis lukuohia* isolates grown on three different culture media at six different temperatures. Measurements were taken 7 days post-inoculation.

growth and spore production of *C. lukuohia* for inoculation experiments. Our results highlight the importance of identifying the optimal growth medium and temperature for growth and sporulation of fungi and how they can positively affect production of cultures for pathological studies.

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