CHARACTERIZING THE PATHOGENICITY PROFILES OF *PHYTOPHTHORA COLOCASIAE*

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Abstract

The most significant plant disease affecting taro is taro leaf blight (TLB), caused by *Phytophthora colocasiae*. A taro breeding program was established at the University of Hawai‘i to develop taro varieties with improved characteristics including resistance to TLB. The program was initiated by crossing taro varieties with resistance to TLB from Palau, Indonesia, Guam, with a Hawaiian variety, ‘Maui Lehua’ taro, known to be susceptible to TLB. A previous study used detached leaf disc assays to challenge new hybrid cultivars with *P. colocasiae* isolate HPA 1, which was originally isolated from Pana‘ewa, Hawai‘i. Many of the hybrids were resistant to this isolate and were classified as resistant to TLB. However, when these cultivars were subsequently challenged with *P. colocasiae* isolate HPE 3 from Pepeekeo, Hawai‘i (approximately 10 miles from Pana‘ewa), some cultivars that were resistant to HPA 1 were susceptible to HPE 3. Further, a number of cultivars that were susceptible to the HPA 1 isolates were resistant to HPE 3. This current study aimed to determine whether the *P. colocasiae* populations in Pana‘ewa and Pepeekeo are homogenous with regard to pathogenicity. This was done using a panel of seven taro cultivars selected in the previous study in addition to cultivar ‘Bun-Long’ which was used as a susceptible control collected from Waiakea Research Station and a taro field in Pepeekeo. The cultivars were separated into four categories: cultivars that were resistant to both HPA 1 and HPE 3, cultivars that were susceptible to both HPA 1 and HPE 3, cultivars that were resistant to the HPA 1, but susceptible to HPE 1, and lastly, cultivars that were resistant to HPE 3, but susceptible to HPA 1. The cultivars were challenged against 20 isolates of TLB, 8 from Pana‘ewa and 12 from Pepeekeo. These isolates were designated HPA 2 – 9 and HPE 4 – 15, respectively. The original solates HPA 1 and HPE 3 were also analyzed in this study to evaluate whether changes occured in these cultures over

time. We used the pathogenicity patterns of these isolates to group them into pathotypes, which were determined using two trials of detached leaf disc assays. In addition, the influence of some environmental factors on the leaf disc assay were analyzed by comparing disc assays from leaves of the same variety from nursery plantings in Pana‘ewa, with field plantings from Pepeekeo. The influence of leaf size on disc assays was also evaluated by comparing the results of disc assays on large (older) and small (younger) leaves from a single plant. The first trial showed that the Pana‘ewa and Pepeekeo *P. colocasiae* populations consisted of multiple pathotypes resolved using the panel of eight selected cultivars. The second trial, performed ~ 2 weeks after the start of trial 1, revealed a change in pathogenicity patterns in a few *P. colocasiae* isolates. While some *P. colocasiae* isolates displayed a surprising degree of plasticity over time with regard to pathogenicity, the plants used in this study were unaffected by varying cultivation practices, location, and leaf site. Based on the results of this study, I conclude that *P. colocasiae* field populations are heterogenous, and that *P. colocasiae* cultures can exhibit changes in pathogenicity profiles within 2 weeks.

Table of Contents

List of Tables

List of Figures

Introduction

Colocasia esculenta

Colocasia esculenta (taro or kalo in Hawaiian language) is a plant of great cultural value to Native Hawaiian people. It is said that kalo came from the body of Hāloanakalaukapalili, the older brother of all Hawaiians. Hawaiians are taught to care for this older sibling, and in return, Hāloanakalaukapalili cares for his younger siblings by providing food for Hawaiians. For this reason, kalo is a symbol of family. By consuming kalo, Hawaiians can also create a connection with their ancestors (Wilson 1988). In addition, taro is a major staple food in the traditional Hawaiian diet. Shintani et al. (1991) found that maintaining this diet can improve cholesterol levels, blood pressure, and glucose levels in comparison with a "Western" diet. Taro has a high nutritional value, and is the fifth most commonly produced root vegetable worldwide (FAOSTAT 2013). In 2014, the production value of taro in Hawai‘i was $1,944,000 (United States Department of Agriculture 2014). Currently, taro is cultivated by propagating vegetative suckers (huli) from a mother plant (Nelson et al. 2011), which are cut ~ 1/8 – 1/4 of an inch below the top of the mother corm. Typically, a taro plant is ready for harvest after 7 – 11 months (CTAHR 1997).

Taro leaf blight (TLB) is the most important taro disease and causes significant declines in taro yields. Reduced taro yield results in economic hardship for those who cultivate taro, eventually resulting in a reduction in the number of taro farmers (Bourke 2012). Researchers are attempting to find effective disease management strategies that respect taro's significance in Hawaiian culture, while also being environmentally friendly. TLB resistance breeding is thus a suitable process towards that goal.

Phytophthora colocasiae

TLB is caused by *Phytophthora colocasiae*, a hemibiotrophic oomycete plant pathogen (Raciborski 1900). The word *Phytophthora* is derived from the Greek language that is literally translated into "plant destroyer". *Phytophthora infestans* is a well-known species of *Phytophthora*, which causes late blight (potato blight). It led to the notable Irish potato famine between the years 1845–1847 (Ristaino 2002). The life and disease cycles of *P. colocasiae* are well understood. The vegetative structure of *P. colocasiae* is called the mycelium, which is in turn composed of many branching strands of filamentous hyphae. Chlamydospores are sometimes produced and have thick walls allowing them to survive long periods of time without a host (Brookes 2005). *Phytophthora* species also produce asexual spores called sporangia (Figure 1), which can infect other plants when carried through wind or water. Sporangia can germinate directly via germ tubes which differentiate into hyphae, or *Phytophthora* can release zoospores (Figure 2) that have flagella allowing them motility in water. Zoospores eventually lose their flagella and encyst. The cyst can then germinate via a germ tube, becoming a hypha and eventually mycelium (Nelson et al. 2011). *P. colocasiae* can reproduce sexually if both mating types (A^1 and A^2) are present to produce an oospore (Ko 1979). Compatible hyphae develop oogonia (female portion) and antheridium (male portion) and undergo meiosis followed by plasmogamy, resulting in fertilization of the oogonium, which then develops into an oospore (Misra et al. 2008b). Oospores, as with chlamydospores, have thick walls that allow them to survive without a host for long periods of time (Nelson et al. 2011). However, a survey in 2012, revealed that only two isolates out of a total of 217 isolates from Hawai‘i were the A^1 mating type the rest being A^2 (Shrestha 2012). Despite the lack of both mating types, *P. colocasiae* populations can have high genetic diversity within regions. Nath et al. (2014) found this using

amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) analyses. These techniques can be powerful tools for determining the diversity of *P. colocasiae* populations. They also found that pathogenicity has no relationship with colony morphology, mating type distribution, and geographic origin (Nath et al. 2014).

According to Packard (1975), *P. colocasiae* is transmitted in a number of ways, most commonly through infected planting material and splashing rain, with the pathogen moving in the water droplets. After taro is harvested, *P. colocasiae* can survive on planting tops for three weeks. *P. colocasiae* can also infect adjacent plants when its spores are carried in the rain and by the wind. Because of this, the pathogen thrives in conditions with high humidity levels and frequent rainfall (Packard 1975). Weather conditions influence infection rates in all plants, including those with a degree of genetic resistance. A two-year study found that the infection of all crops was positively correlated with temperature up to 29°C, rainfall with a maximum of 198.20 mm, and relative humidity with a maximum of 84.8% (Shakywar et al. 2013). Spore formation is inhibited when temperature conditions were outside of the ideal range (20 – 28°C). However, the continuous favorable temperature in Hawai‘i results in a persistent favorable disease environment (Packard 1975). Giambelluca et al. and Frazier et al. reports that in 2014 Pepeekeo had a maximum air temperature of to 20.18°C and a minimum air temperature of 24.38°C. By understanding the pathogenic triggers of *P. colocasiae*, TLB can be better understood and managed.

Taro Leaf Blight

TLB is the most significant taro disease worldwide. It was first seen in Hawai‘i in 1920, and probably led to the extinction of more than 270 traditional Hawaiian cultivars (CTAHR 2009). According to Nelson et al. (2011), there have been reports of the disease in Asia, Africa,

the Caribbean, the Pacific, and the Americas. The disease was first introduced to American and Western Samoa in 1993, also causing great declines in taro production (Trujillo and Menezes 1995). TLB was also first seen in Nigeria in 2009, which lead to a rapid decline in taro yield (Bandyopadhyay et al. 2011).

An early pathogen diagnostic sign is a leaf symptom is the appearance of brown rapidly expanding necrotic lesions on leaves. Lesions can also occur anywhere on the leaf stalk. The lesions vary in length and are gray to brownish-black in color. Due to the reduced strength of the stalk due to necrotic tissue decay, the stalk may not be able to support the leaf causing it to collapse (Nelson et al. 2011). After becoming symptomatic, the entire leaf can be affected within three to five days (Packard 1975). Plants that are affected by TLB can experience a 95% decrease in leaf area, resulting in a loss of photosynthetic tissue, and reducing the corm yield (Nelson et al. 2011). In addition to leaf and stalk destruction and reduced corm yield, *P. colocasiae* can cause the corms to rot (pocket rot). In the early stages rotting tissue is usually subtle, but over time infected corm tissues develop a noticeable division between rotted and healthy flesh (Nelson et al. 2011).

TLB Management

The development of disease-resistant crops is the best management practice both environmentally and economically when managing pathogens (Nath et al. 2014). Breeding programs are beneficial because they can increase the genetic diversity in the population, thus increasing the likelihood of producing plants that carry genes for disease resistance (dela Peña 1984). There is low genetic diversity of taro in the Pacific islands. This is a concern, because a successful breeding program requires high genetic diversity (Mace and Godwin 2002; Kreike et al. 2004). This makes it challenging to produce taro hybrids with resistance to disease. While

taro can have low genetic diversity within geographic populations, taro between geographic populations can have high genetic diversity. Wild taro in particular has higher genetic diversity than cultivated taro (Kreike et al. 2004). Caillon et al. (2006) found that analyzing taro population's genetic diversity is an important step in conservation studies because it can determine the need for expanding the gene pool. AFLP PCR can be an effective way to analyze genes, but can only be used with the input from farmers who are familiar with the crops to aid in classification of cultivars based on morphology (Caillon et al. 2006). Genetic analysis and cultivar identification is important in crop breeding programs because these factors are important in selecting traits that improve crops.

When breeding taro, breeders focus phenotypes that would result in high yield and resistance to pests and disease. In addition, taste tests are conducted because palatability is important as a food crop (dela Peña 1984). To aid in the selection of these traits, genetic maps which use molecular markers are frequently used (Baird et al. 2008). Molecular markers can be a powerful tool in selecting traits for a breeding program because they can help describe relationships between gene pools, and allow for more rapid selection of desirable genes (Mace and Godwin 2002). Single nucleotide polymorphisms (SNPs) are a common type of molecular marker that is useful in genetic variety mapping because of their high density in genomes (Baird et al. 2008).

A genetic map using SNPs can be made using a genotyping-by-sequencing (GBS) approach. GBS is ideal for studying organisms with complex genomes because it uses restriction enzymes, used to cleave DNA at specific bases, to simplify genetic research by allowing researchers to analyze specific and smaller sequences. (Poland et al. 2012). GBS is used to discover novel SNPs based on high-throughput, next generation sequencing, which allows users

to sequence large amounts of DNA in a single run, which would have taken many more sequential or simultaneous DNA sequence readings in more traditional DNA sequence equipment. The advantage of GBS over sequencing approaches that analyze bases in fixed positions such as microarrays is that it allows genomic analysis of organisms with highly diverse nucleotides (Elshire et al. 2011). If enough SNPs are identified in a genome, they can be used in identification of genotypes, to identify different varieties of taro and as genetic markers linked to important traits (Cabezas et al. 2011). While SNPs are a powerful genetic tool, discovering and genotyping SNPs require a considerable amount of genetic sequencing research effort (Baird et al. 2008). To increase SNP discovery, restriction-site-associated DNA (RAD) can be used (Chutimanitsakun et al. 2011). RAD marker genotyping is used to identify DNA sequences around restriction sites of particular enzymes in a genome. This helps with SNP identification because the restriction enzymes associated with sites are partiality based on SNP frequency (Miller et al. 2007). In addition, RAD markers and SNPs can be used to create maps for the identification of quantitative trait loci (QTL) (Chutimanitsakun et al. 2011). QTL identification is helpful in breeding programs because it can specify the location on chromosomes that contain complex traits such as disease-resistance in which requires considering multiple factors.

To initiate a taro variety breeding program in Hawai‘i, Trujillo et al. (2002) used plants from Hawai‘i, Palau, Guam, Indonesia and Rota were evaluated for TLB resistance in 1995 - 1996. Initial studies showed that Palauan taro was the most resistant. There are approximately 70 varieties of Hawaiian taro; ‘Maui Lehua’ is desirable variety for making poi, but is susceptible to TLB. ‘Maui Lehua’ along with the highly resistant Palauan taro, Ngeruuch, were used as parental lines in breeding programs. Researchers were successful in producing a resistant line of taro named, ‘Pa‘lehua’ (pa to indicate the Palauan parental line and lehua to indicate the color).

Many individuals were selected from the Ngeruuch and ‘Maui Lehua’ cross before ‘Pa‘lehua’ was selected. Hybrid plants also need further horticulture research to confirm their soil and nutrient requirements, disease resistance levels, harvest time, and total yields. In addition to resistance ‘Pa‘lehua’ also produces a greater yield and has a shorter maturation time compared to ‘Maui Lehua’. ‘Pa‘lehua’ also has a comparable taste to ‘Maui Lehua’ and has ideal characteristics for poi (Trujillo et al. 2002). In addition, taro breeders also have produced taro hybrids that also have had high disease resistance and high yields (Miyasaka, Personal Communication, 2016).

TLB-resistant taro has been identified, and in at least one case, resistance was found to be linked to the presence of protease inhibitors (Ho and Ramsden 1998). Protease inhibitors have different inhibition mechanisms, and they are grouped into four groups (serine, cysteine, aspartic or metallo-proteases) based on the active amino acid in their reaction center (Koiwa et al. 1997). It was discovered that tarocystatin, a type of cysteine protease inhibitor, has strong antifungal activities and has the potential to be used as a fungicidal agent. Tarocystatins block proteinase activity in the fungal mycelium, which decreases nutrient digestion in fungal cells (Yang and Yeh 2005). Cysteine proteases have also shown to have antifungal effects in other plants such as in tomatoes which are highly involved in defending against *Phytophthora infestans* infections (Girard et al. 2007).

TLB-resistant taro varieties have been incorporated into breeding programs which are aimed at improving taro production in pathogen limited production environments. Genetic traits determine whether a plant is susceptible or resistant to a pathogen. Flor (1971) found a successful infection can only occur when there is a compatible interaction between the pathogen and the host. This is accepted as the gene-for-gene theory, which includes the proposition that

pathogens carry avirulence genes corresponding to plant disease resistance genes. Plant disease resistance is thus manifested when a plant carrying a resistance gene is inoculated with a pathogen carrying a corresponding avirulence gene (Flor 1971). Elicitors are released by plant pathogens, and in an incompatible interaction, the elicitors are recognized by the plant host and stimulate the plants immune response. During an incompatible interaction resistance is triggered; the pathogen's elicitor binds to the host receptors and induces cell death via apoptosis to limit the spread of the pathogen. Molecular plant-pathogen investigation revealed that a compatible interaction is one in which the pathogens produce elicitors that do not bind to the hosts receptor molecules found within the cell. This suppresses the host's ability to detect the infection and allows the pathogen to spread throughout the host's tissue (Dangl and Jones 2001). The gene-for-gene theory can also aid in co-evolutionary studies between pathogens and hosts, helping to understand molecular events that lead to the variability of resistance in populations. This theory can also be used to predict which host genes would be most effective in producing resistant crops (Dodds and Thrall 2009). Plant breeders are attempting to identify resistant genes to incorporate them into plants of interest to increase disease resistance (Dangl and Jones 2001). In the case of late blight, the gene Rpi-blb 1 isolated from wild potato species has shown to encode for complete resistance to *P. infestans* isolates with multiple virulence factors (Van Der Vossen et al. 2003). However, single resistance gene has not yet been identified in taro.

Breeding Program Issues

While the Hawaiian taro breeding program has been successful in producing hybrids with high yields and high resistant levels, relatively few hybrids have been adopted for commercial production (personal communication Miyasaka, 2016). Palauan taro varieties carry a few undesirable traits and score lower in taste tests than Hawaiian varieties. The undesirable traits

include producing long stolons with suckers resulting in a weedy disposition, thus complicating cultivation (CTAHR 2009). In contrast, Hawaiian varieties develop suckers close to the mother plant. In addition, fermentation varies depending on the taro variety (Allen and Allen 1933). The fermented Palauan poi is less palatable than ‘Maui Lehua’ making it less desirable in the Hawaiian market (Miyasaka, Personal Communication, 2016). In blind taste tests conducted in Hawai‘i, participants scored poi made from ‘Maui Lehua’ taro higher than poi made from Palauan taro in flavor, texture, and color (Hamasaki et al. 1998). Producing a variety with desirable traits can be challenging because many genes are often involved. Crossing Palauan with Hawaiian varieties can produce offspring with TLB resistance, but compromises other important traits mentioned previously (Trujillo et al. 2002). Maintaining a successful taro breeding program in Hawai‘i is challenging. Hawai‘i does not support the natural flowering conditions of taro; therefore, taro needs to be sprayed with gibberellic acid (GA) to induce flowering. However, even with GA, flowering is sporadic (dela Peña 1984). Another issue is the rare development of seeds. It is believed that taro can only reproduce sexually via hand pollination. Typically, female flowers become receptive before the pollen is released (Ivancic and Lebot 2000). Wild taro varieties in Papua New Guinea and Australia are pollinated by Drosophilidae species of flies. This pollination relationship is unknown in Hawai‘i and most other taro-growing regions (Carson and Okada 1980). In addition, it is thought that the limited amount of food in the small seeds causes a short viability duration. This is a concern because the ability to produce high numbers of viable seeds influences the ease and time of development of hybrid plants (Kikuta et al. 2014). The breeding program also requires a large amount of manpower, as breeders need to perform manual pollination, plant and maintain crops, as well as evaluate offspring.

Disc Assays

TLB management strategies are of critical importance. Screening large numbers of plants for resistance to field pathogenic organisms requires a large amount of time, space, and labor. Further, results can be affected by variables such as humidity and temperature (Brooks 2008). Fungal spore inoculation techniques can help further research in TLB as well as other plant diseases, because they can be used to rapidly evaluate germplasm for disease resistance (Xu and Ko 1998). Brooks (2008) found that laboratory detached bioassays are useful when studying TLB resistance because they are fast, space-saving, and reduce environmental variables effects on assays. However, detached-leaf bioassays are more sensitive to infection and symptom observation than attached leaf assays. When the results of detached-lead bioassays were compared to the results of attached leaf assays, attached leaves had smaller lesions than the detached leaves. This could lead to researchers rejecting germplasm with reasonably good disease resistance (Brooks 2008). Still detached-leaf bioassays are commonly used for evaluating disease resistance for the reasons above, and to avoid introducing inoculums in the breeding nursery. Overall, detached leaf assays are the first step of comprehensive disease resistance research in taro.

Pathogenicity of P. colocasiae

The infection mechanism of *P. colocasiae* is still not fully understood. However, Misra et al. (2008) found that TLB causes biochemical changes in taro. During the first stages of infection taro uses non-specific mechanisms to eliminate pathogens by increasing phenolic levels (Mishra et al. 2008), which may contribute to protecting cells from oxidation and accelerate recovery during inflammation (Gonçalves et al. 2013). To further confine the *P. colocasiae* infection to small tissue areas, taro plant tissue increases peroxidase, PR-proteins, and decreases sugar

production to induce tissue death, reducing the spread of disease (Misra et al. 2008a). Defense-related genes are expressed at the highest levels when TLB symptoms are most obvious as discolorations on leaves (Mishra et al. 2009). Molecular investigation of *P. colocasiae* found that the pathogen produce an elicitor which is recognized by its host, taro (Mishra et al. 2010). When an elicitor is detected, plants can limit the spread of pathogens via a hypersensitive response that induces apoptosis (Lam et al. 2001). The unaffected tissue then develops a systemic acquired resistance which renders the entire plant more resistant to pathogen attacks (Wang et al. 2003). The classes of elicitor protein produced vary among the species of *Phytophthora*. Mishra et al. (2009) found that *P. colocasiae* does not express its elicitor protein genes until after plant cell penetration, thus avoiding detection by the plant's defense responses. TLB systemic acquired resistance can be induced adding the elicitor glycoprotein into leaf tissues. When TLB-susceptible plants were infiltrated with the elicitor, and later inoculated with *P. colocasiae*, the plants were resistant for a week, and the disease was less severe than untreated crops. Understanding the biochemical response of taro to elicitors can be beneficial for mitigating TLB through genetic techniques (Mishra et al. 2010).

Previous Studies

In previous studies a *P. colocasiae* isolate from Pana'ewa (HPA 1) was inoculated on a number of Hawaiian taro hybrids using disc assays, and the results showed that many of the plant hybrids were resistant to it. Some of resistant hybrids were sent to the Hawaiian Agricultural Research Center on O'ahu and challenged with a *P. colocasiae* pathotype obtained from Pepeekeo. Some of the hybrids with resistance to *P. colocasiae* from Pana'ewa were susceptible to *P. colocasiae* from Pepeekeo. To test whether or not Hawai'i island's *P. colocasiae* population was homogenous, another pathotype of *P. colocasiae* was then isolated from

Pepeekeo, Hawai‘i (HPE 3). The taro cross using the cultivars ‘230’ x ‘255’ yielded a relatively large number of resistant plants. The cultivars of this cross were named ‘1025’, and the pedigree of ‘230’ x ‘255’ is presented in Figure 3. A number of progeny from that cross were challenged by HPA 1 and HPE 3, and revealed that some plants were resistant to both, some were susceptible to both, some were resistant to HPA 1 and susceptible to HPE 3, and some were susceptible to HPA 1 and resistant to HPE 3 (Table 1).

In this current study, I investigated whether a larger collection of *P. colocasiae* field isolates from each site would be pathogenically identical or similar to the initial isolates from each site or would consist of a mixture of different pathotypes. In other words, are the pathogenicity profiles of the isolates from Pepeekeo all comparable to the initial Pepeekeo isolate HPE 3, and are pathogenicity profiles from Pana‘ewa all comparable to the initial Pana‘ewa isolate HPA 1.

Methods

Preliminary Studies

In an on-going research project conducted by CTAHR (UH Mānoa, College of Tropical Agriculture and Human Resources), taro hybrid cultivars of the ‘1025’ cross were produced by crossing parental cultivars ‘230’ and ‘255’ (Figure 3). Unpublished research conducted within this research project found that many of the ‘1025’ cultivars showed promising TLB resistant results, with many of the hybrids producing little to no lesion in detached leaf disc assays when using the isolate HPA 1. Using the isolate HPE 3, these hybrids were be put into four different categories: cultivars that are resistant to both HPA 1 and HPE 3, cultivars that are susceptible to both HPA 1 and HPE 3, cultivars that are resistant to the HPA 1, but susceptible to HPE 1, and lastly, cultivars that are resistant to HPE 3, but susceptible to HPA 1 (Table 1). A subset of the

‘1025’ cultivars was use in this study to characterize of pathogenic profiles of the *P. colocasiae* populations in Pepeekeo and Pana‘ewa.

P. colocasiae Isolation

A total of 20 taro leaf blight lesions (Figure 4) were collected from symptomatic taro leaves found in Pepeekeo (HPE 4 - 15) and at the University of Hawai‘i at Hilo Farm in Pana‘ewa (HPA 2 - 9). *P. colocasiae* was isolated from by excising a small piece of tissue from the edge of a necrotic lesion. These excised pieces were surface sterilized with 10% bleach, blotted with sterile filter paper, and rinsed with sterile distilled water. They were further sterilized with 70% ethanol, blotted with sterile filter papers and rinsed with sterile distilled water. The tissues were then blotted with sterile filter paper and plated onto 10% V8 agar (150 mL V8 juice, 3 g $CaCO_3$, 15 g agar per liter) Petri dishes supplemented with 1 ml ampicillin (Ap) (50 mg/ml), 1 ml nystatin (Ny) (10 g/ml), and 50 µl Pentachloronitrobenzene (PCNB) (modified from Jeffers and Martin 1986). The plates were incubated at 27°C for five days (Figure 5).

Pure *P. colocasiae* cultures were established using a sandwich column (Figure 6a and 6b) using 10% V8 plates, 1.5% water agar plugs cut into a circle ~ 0.5 cm in diameter, and an agar plug from plates previously mentioned (*P. colocasiae* isolation). The samples were then incubated at 27°C. Sandwich columns were removed at T_1 (8 hours after inoculation), T_2 (24 hours after inoculation) T_3 (48 hours after inoculation), and T_4 (72 hours after inoculation). An agar plug was taken from the sandwich column with the earliest growth and plated onto V8 agar and incubated at 27°C for ~1 week. All samples were then examined using 0.01% chlorazol black e stain under a compound microscope at 400x magnification. *P. colocasiae* was indicated with aseptate hyphae and the presence of sporangia. In addition to isolates HPE 4 – 15 and

isolates HPA 2 – 9, isolates HPA 1 and HPE 3 were included in this study.

Taro Varieties

Taro cultivars were selected based on their responses to inoculation by HPE 3 and HPA 1. All of the cultivators in this study were of the '1025' cross. The cultivars were separated into four categories mentioned previously (Preliminary Studies). At least one cultivar from every category was selected using a random number generator to select the cultivars from each category (Table 1).

Zoospore Induction

The protocol for zoospore induction and leaf disc assays was previously described by Brooks (2008). Briefly, *P. colocasiae* was allowed to grow for 1 – 2 weeks on Petri dishes containing 10% V8 agar. The plates were then flooded with 10 ml of sterile distilled room temperature (~ 22 °C) water and refrigerated at 4 °C for 30 minutes. The plates were then taken out and left at room temperature (~ 22 °C) for 20 minutes. Zoospore concentration was determined by pipetting 10 µl of water from the plate onto a KOVA Glasstic Slide 10^{TM} grid slide and counting the number of zoospores following the supplied protocol. The concentration of zoospores was then diluted with distilled water to a concentration of 50 – 100 zoospores/10 µl.

Leaf Disc Assays

Leaf discs (~ 3.4 cm in diameter) were cut from leaves using a sharpened steel tube. The discs were then placed onto Petri dishes containing 1% water agar with the adaxial surface exposed. The leaf discs were then inoculated with a 10 µl aliquot of water containing 50 – 100 zoospores. The plates were covered and left at room temperature and photographed on day 3 and 4 post-inoculation (Figure 7). Four discs were challenged from each plant with each of the *P. colocasiae* isolates, and the lesion sizes were averaged. Two trials were conducted, but cultivar

‘1025-187’ was only tested once with isolates HPE 6 and 7, and isolates HPA 3 and 4 due the lack of leaves available at the field at time.

Data Analysis

The program ImageJ was used as a computer-based measuring tool (Rasband, 1997). Lesions of the ‘1025’ Crosses were measured in comparison to ‘Bun-Long’, a susceptible cultivar (CTAHR 2009). Plants with lesions ≤ 10% the sizes of ‘Bun-Long’ lesion were considered resistant. In addition to analyzing the data categorically, a resistance map (a heat map) was also made to analyze the relative lesion sized made by the isolates using a Euclidean formula. The map is a graphical way of representing the relative lesion sizes, as the colors intensify, the relative lesion sizes increase. It was made using the packages RColorBrewer (Erich, 2014), pheatmap (Raivo, 2015), vegan (Jari et al., 2016), and magrittr (Stefan and Hadley, 2014) in the statistical program R (R Core Team, 2016).

Analyzing the influence environmental factors on disc assay results

Leaves of the cultivars ‘Bun-Long’, ‘1025-81’, ‘1025-82’, and ‘1025-175’ were harvested from potted plants maintained in a nursery and some from in-ground and outdoor field plantings. These cultivars were chosen because they were used to characterize the different pathogenicity profiles mentioned previously, and they were also available at both the nursery and the in-ground planting site. To determine whether these environmental factors affected the disc assay results, leaf samples from the same cultivars from nursery and fields plots were challenged with the most virulent pathotypes from Pepeekeo and the University of Hawai‘i at Hilo Farm (HPE 8 and HPA 7). Isolates HPE 8 and HPA 7 were used to challenge the cultivars because they caused the largest lesion sizes from each population (Pepeekeo and Pana‘ewa), therefore were considered to be the most pathogenic. In these trials ‘Bun-Long’ was used as a

test cultivar to reduce confounding factors. Thus, the actual lesion size was used for analysis as opposed to the relative lesion size. A linear model was used to analyze the influence of location of leave harvest and the size (large and small) of leaves from Pepeekeo. Linear models describe the continuous variables (such as lesion sizes) using multiple factors (location and size) (R Core Team, 2016).

DNA Extraction

DNA sequences from these isolates obtained were genetically sequenced to confirm that they were *P. colocasiae*. Due to contamination, only isolates HPE 6 – 9, 11, 13, and 14 and isolates HPA 2, 4 – 7, and 9 were sequenced. Solution X DNA extraction solution was made according to a protocol kindly provided by Devin Leopold. To a clean 100 ml tube, 10 ml of a1 M Tris pH 8 stock was added, in addition to 1.86 g KCl (249.5µM), 0.37 g (10µM) ethylenediaminetetraacetic (EDTA), and 80 ml distilled water. The tube was then shaken until the solutes dissolved. The solution was then titrated with 1 M NaOH to a pH of between 9.5 – 10.0 and distilled deionized water was added to bring the total volume to 100 ml.

A 3% bovine serum albumin (BSA) stock was prepared by adding 3 g of BSA into a clean tube, and bringing the total volume of the tube to 100 ml using distilled water. The tube was then shaken until the BSA was dissolved.

P. colocasiae DNA was extracted by scraping *P. colocasiae* off of 2 week old cultures grown on 10% V8 agar plates using a sterile 20 µl tip. The tips were placed into a 0.2 ml tube containing 40 µl of DNA extraction solution (Solution X). The tubes were then incubated in a BioRad T100^{TM} thermal cycler set at 25˚C for 10 minutes; then shifted to 95˚C for 10 minutes. The tubes were removed from the thermal cycler and 120µl of 3% BSA was added to each tube. Due to some difficulty obtaining amplifiable DNA from isolate HPA 6 and HPA 7, DNA from

those isolates was extracted using the Macherey-Nagel NucleoSpin ® Plant II extraction kit, following the supplied protocol.

Polymerase Chain Reaction (PCR)

A PCR was performed using 4 µl of the extracted DNA mentioned above diluted with TE (10 mM Tris and 0.5mM EDTA) in a 1:20 ratio (4 µl of extracted DNA: 76 µl of TE buffer). The following were then added to a clean PCR tube: 3 µl of diluted DNA, 2 µl of a mixture ITS 3 (5' – GCATCGATGAAGAACGCAGC-3') and 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) DNA primers at a concentration of 50 ng/µl, 15 µl of Immomix, and 10 µl of water. The samples were placed in the thermal cycler and heated to 95˚C for 5 minutes. The following cycles were repeated 33 times: 95˚C for 20 seconds, 54˚C for 20 seconds, and 72˚C for 1 minute and 30 seconds. After the 33 cycles were completed, the tubes were left at 72˚C for 5 minutes.

Electrophoresis

Gel electrophoresis DNA migration was performed on the samples using 1% agarose in tris-acetate EDTA buffer (pH 8.3) to evaluate the quantity and quality of amplified DNA. The 1 Kb ladder from invitrogen ™ was used as the molecular weight markers using 18 µl of 1 Kb ladder (40ng/ µl) and 2 µl Molecular Probes invitrogen™ detection technologies SYBR® Gold. To the remaining wells 3 µl of the products were added, in addition to 1 µl of SYBR® Gold, 1 µl invitrogen™ 10X loading buffer, and 5 µl water. The electrophoresis was run for ~45 minutes at 60 volts. All samples were the expected size of ~ 800 bp (Figure 8).

Primer Removal and DNA Sequencing

The primers were removed from the PCR products using according to the ExoSAP-IT PCR Product Cleanup kit, following the manufactures protocol. Briefly, to a clean PCR tube, 13 µl of PCR product and 4 µl of ExoSAP-IT reagent were added. The PCR tube was placed in a

Thermal Cycler at incubated at 37˚C for 17 minutes, followed by inactivation at 80˚C for 15 minutes. The PCR products were then prepped for sequencing by adding 1.5 µl IT3 primer (25ng/µl) to 6 µl of each reaction. A second set of PCR products were prepared in the same manner using 1.5 µl ITS 4 primer (25ng/µl). The prepared PCR products were then sent for sequencing to the University of Hawai‘i at Hilo EPSCoR Hilo Core Genetics Facility using Big Dye Terminator v3.1 reagents. Sequences were cleaned after sequencing using the BigDye XTerminator Purification Kit, and subsequent sample-loading into the Applied Biosystems (AB) 3500 Genetic Analyzer instrument. The sequences were displayed using the program FinchTV 1.4.0 (Geospiza, Inc) (Figure 9). The NCBI (National Center for Biotechnology Information) BLAST (Zheng, 2000) tool was used identify identical sequences in GenBank, and Clustal Omega at EMBL-EBI (McWilliam et al., 2013) was used to align the sequences.

Results

Disc Assays

Results of the first trial showed that the HPE population from the Pepeekeo field could be separated into 5 pathotypes using 8 plant lines (Table 2). Isolate HPE 4 was unique in that it could successfully infect all plants except for the cultivar ‘1025-82’. HPE 5 was also unique in that it could infect all cultivars except for ‘1025-82’, ‘1025-225’, ‘1025-175’. Isolate HPE 6 was also a unique pathotype and would infect all cultivar except ‘1025-225’ and ‘1025-175’. Lastly, HPE 8 – 15 successfully infected all cultivars tested. HPE 3, obtained from a previous study, could successfully infect all cultivars except ‘1025-187’ and ‘1025-82’. Interestingly, HPE 3 was able to infect ‘1025-187’ and ‘1025-82’, which it previously was unable to infect. The results also showed that the HPA population from the University of Hawai‘i at Hilo Farm in Pana‘ewa had a total of 4 pathotypes. Isolates HPA 2 – 4 could infect all cultivars except ‘1025-225’.

Isolates HPA 5 – 9, could infect all cultivars. HPA 1 was collected prior to this study from this site and was also able to infect all of the cultivars. When all of the newly acquired isolates were compared to each other, there were total of 5 phenotypically unique pathotypes. When isolates HPA 1 and HPE 3 were included in this comparison there were a total of 6 phenotypically unique pathotypes.

A second trial was performed ~ 2 weeks after the first trial. In this trial the HPE isolates were all able to infect the tested cultivars (Table 3). In addition, there were also changes in the HPA population, and there were a total of 3 unique pathotypes. This time isolate HPA 4 which could infect all cultivars except '1025-225'. Isolate HPA 8 previously could infect all cultivars, but was unable to infect '1025 – 175' in this trial. Isolates HPA 2, 3, 5, 6, 7, and 9 could infect all cultivars. Both HPA 1 and HPE 3 were unique pathotypes in comparison to the newly acquired isolates. HPA 1 could infect all plants except for cultivars '1025-187', '1025-225', and '1025-175'. HPE 3 could infect all cultivars except '1025-82'. When all newly acquired isolates were compared to each other in the second trial there was a total of 3 phenotypically unique isolates. When isolates HPA 1 and HPE 3 are included in this comparison there were a total of 5 different phenotypically different pathotypes.

When the data was also analyzed using the Euclidean formula, results from the first trial showed that the Pepeekeo field had 2 unique groups of pathotypes. Euclidean formulas are a mathematical way of determining the greatest common divisor between positive numbers, which is useful in this study because it can help determine how similar the isolates are based on the lesion size they cause. However, this analysis does not consider specific plant-pathogen interactions to group the isolates, but emphasizes the number of resistant and susceptible interactions. One group included isolates HPE 5 and 6. The second group included isolates HPE

4 and HPE 7 – 15. The HPA 1 and HPE 3 isolates were significantly different in comparison to these recently obtained isolates and were more similar to each other than any other pathotype from Pepeekeo (Figure 10). My isolates from Pana‘ewa comprised 2 unique pathotypes. One group of pathotypes included isolates HPA 2 and 3 also included in this group were isolates HPA 1 and HPE 3. The second pathotype included isolates HPA 4 – 9 (Figure 11). When all of the isolates were compared to each other, they could be separated into 3 groups. Isolates HPA 1 and HPE 3 were significantly different from all of the pathotypes and were more closely related to each other than other pathotypes (Figure 12). A MANOVA test comparing the HPA population with the HPE population had a p-value of 0.87.

When the data from second trial was analyzed using the Euclidean formula, the field in Pepeekeo had a total of 2 unique pathotypes which included HPE 4 – 9 as one unique group, this group also included pathotypes HPA 1 and HPE 3, and pathotypes HPE 10 – 15 as another unique group (Figure 13). The field in Pana‘ewa was homogenous and included HPA 1 and HPE 3 (Figure 14). After the second trial, when all the isolates were compared to each other, there were a total of 2 unique isolate groups and the HPA 1 and HPE 3 pathotypes were included in these groups (Figure 15). A MANOVA test comparing the HPA population with the HPE population had a p-value of 0.20.

Comparing current disc assay results to previous data

The current study compared the HPA 1 and HPE 3 isolate results with the results from a previous study. The results showed that the disc assays had different results from what was previously recorded. The results from the first trial showed that the isolates were able to infect cultivars that were previously recorded as resistant to the isolates. The results from the second trial also differed from the first trial. In the second trial, some isolates could infect cultivars that

they could not in trial 1. There were also differences between these reactions and those observed three years ago. In 2012, HPA 1 and HPE 3 could not cause disease on many of the '1025' cultivars. However, in this current study, HPA 1 and HPE 3 could not cause disease on few of the '1025' cultivars (Table 2 and 3).

Testing for the influence environmental factors on disc assay results

Leaves from in-ground field planting in Pepeekeo were compared to leaves from potted plants at the CTAHR Waiakea Research Station nursery in leaf disc assays. When an ANOVA was used to analyze the influence of leave harvest location there was a p-value of 0.47 (df = 58, f-value = 0.53) which indicates no significant influence (Figure 16). When the influence of the use of large (older) leaves and small (younger) leaves from Pepeekeo were analyzed using an ANOVA there was a p-value of 0.019 (df = 26, f-value = 6.26), indicating significance (Figure 17).

Identification via DNA sequences

All isolates were identified as *P. colocasiae* according to the NCBI BLAST database (Table 4). All isolates had a 99% alignment with *P. colocasiae* with the exception HPA 1, which had a 100% alignment with *P. colocasiae*. All of the isolates were fully aligned with each other, with the exception of one base in the isolates HPA 2 and HPA 7 in which there is a base-call error (Figure 18).

Discussion

When classifying *P. colocasiae* pathotypes based on infection pathotype and using the Euclidean formula, results showed that populations in Pepeekeo and Pana'ewa are not homogeneous when the isolates were newly isolated. Results revealed a total of 6 pathotypes.

According to the results based on phenotype, the second trial showed that the isolates from Pepeekeo became homogenous over time and the isolates from Pana‘ewa had one more pathotype in comparison to the first trial. Based on the Euclidean formula, results from the second trial showed that the isolates from Pana‘ewa became homogenous over time and the isolates from Pepeekeo had the same number of unique pathotypes as the first trial. The MANOVA test showed no significant differences between study sites in both trials, meaning some isolates found at Pepeekeo were also found in Pana‘ewa.

The results showed that the isolates became more pathogenic over time. Isolates HPA 2 – 9 and HPE 4 – 15 were able to infect more cultivars in the second trial. Additionally, isolates HPA 1 and HPE 3 were also able to infect more cultivars after ~ 3 years. These results are inconsistent with a previous study which found that isolates that were collected and isolated more recently could cause a significant lesion on disc assays, and that isolates that were isolated earlier were weakly pathogenic or not pathogenic. The differences in results from the two trials may be due to mutations in the isolate cultures (Nath et al. 2014). Based on the results of the linear model, it can be concluded that the location in which leaves are harvested from is not significant. However, the size of the leave is significant, with larger leaves yielding smaller lesions. Changes in leaf assay results are may be due to changes within isolate culture as well as inconsistent with the selection of different sized leaves.

DNA sequencing results showed that all isolates that could be sequenced were *P. colocasiae* isolates. However, the isolates were too genetically similar to differentiate pathotypes using the ITS region.

Conclusions

I conclude that the *P. colocasiae* populations within taro fields are pathogenically heterogeneous. In addition, the differences in environmental conditions between field planting in Pepeekeo and nursery plants at Waiakea Research Station in Pana‘ewa do not affect leaf disc assay results. However, the size of leaves does affect leaf disc assay results. Lastly, *P. colocasiae* cultures can change with regard to pathotype within a time period of ~ 2 weeks. These changes in leaf disc assays results were a major confounding factor in this study. Future research should examine the genetics of *P. colocasiae.* Genetic analysis would increase the understanding of the degree and cause of heterogeneity of populations and may better define the mutation rate within culture and in field populations. In addition, the goal of the is breeding program is to develop taro varieties with good field resistance to TLB, so further work is needed to improve the ability of the detached leaf disc assays to evaluate field resistance. Some factors that could improve detached leaf disc assays include: being conscious of leaf sizes on the plant because leaf size has a significant influence on leaf assays and examining different resistant thresholds. For example, future research should use large leaves and classify leaves with lesion sizes that are 50% the size of ‘Bun-Long’ lesion.

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Tables

Table 1: Table showing the categories of the '1025' hybrids based on isolates HPE 3 and HPA 1. *Cultivars used in this study.

Resistant to HPE 3 and HPA 1	Susceptible to HPE 3 and HPA 1		Resistant to HPE 3	Resistant to HPA 1	
13	118	399	239	100	240
96	125	44	9	492	37
111	180*	287	188*	355	113
129	234	131	181*	480	81*
224	119	40	250	473	82*
281	237	418		332	168
387	122	64		19	87
507	187*	72		109	377
91	502	124		79	225*
174	130	114		248	317
99	120	186		35	312
175*	220	299		256	511
229	398	269		207	327
482	255	291		350	83
58	509	320		80	215
242	204	302		89	
51	74	283			
	60	288			
	510	278			
	71	274			
	56	297			

Table 2: Summary of the relative lesion sizes on the first trial of disc assays. Isolates beginning with HPA were from Pana'ewa, Hawai'i and isolates beginning with HPE were from Pepeekeo, Hawai'i. All cultivars are of the '1025-X' cross (for example '1025-187'). Hightlighed boxes indicate isolate resistance to the respective cultivar. Isolates HPA 1 and HPE 3 showed different results in this study in comparison to the results from a previous study. The HPE population showed a total of 4 unique pathotypes. The HPA population also showed a total of 4 unique pathotypes. *Previously recorded data.

Isolate	187	188	181	81	82	225	175
Previously Obtained Isolates							
HPA1*	0.78	0.79	0.83	0.00	0.00	0.00	0.00
HPA3*	0.86	0.04	0.03	0.45	0.55	0.66	0.05
HPA1	0.46	0.98	0.82	0.16	0.14	0.53	0.54
HPE3	0.06	0.89	1.30	1.23	0.04	0.51	0.51
Newly Obtained Isolates from Pepeekeo							
HPE4	0.98	1.03	1.57	0.46	0.00	0.68	0.93
HPE5	0.41	0.17	0.40	0.27	0.00	0.00	0.04
HPE6	0.45	0.65	0.41	0.16	0.59	0.03	0.06
HPE7	0.88	0.63	1.07	0.52	0.78	0.14	0.62
HPE8	1.10	0.87	1.14	1.07	0.83	0.35	0.71
HPE9	0.70	0.55	0.98	0.98	0.56	0.31	0.60
HPE10	0.81	0.69	1.25	0.76	0.53	0.61	0.78
HPE11	0.79	0.70	0.88	0.61	0.31	0.70	0.85
HPE12	0.88	0.64	0.91	0.73	0.45	0.68	0.74
HPE13	1.18	0.71	0.70	0.67	0.74	0.43	0.61
HPE14	0.94	0.62	0.88	0.74	0.41	0.66	0.70
HPE15	1.70	0.87	0.89	0.81	0.94	0.68	0.69
Newly Obtained Isolates from Pane'awa							
HPA2	0.69	0.45	0.64	0.20	0.38	0.09	0.30
HPA3	0.12	0.20	0.32	0.14	0.36	0.10	0.39
HPA4	1.50	0.90	1.10	0.46	0.38	0.06	0.86
HPA5	1.15	0.58	1.13	0.92	0.76	0.51	0.58
HPA6	0.76	0.69	1.14	0.77	0.78	0.60	0.84
HPA7	0.75	0.57	1.09	1.08	0.71	0.58	0.90
HPA8	0.96	0.82	1.13	0.92	0.70	0.86	0.84
HPA9	0.88	0.68	0.79	0.89	0.42	0.65	0.68

Table 3: Summary of the relative lesion sizes on the second trial of disc assays. Isolates HPA 1 and HPE 3 showed different results in this study in comparison to the results from a previous study and different results in comparison to the second trial. The HPE population also differed from the first trial showed that the field became homogeneous. The HPA population also differed from the first trial and showed a total of 3 unique pathotypes. *Previously recorded data from 2012.

Isolate	187	188	181	81	82	225	175
Previously Obtained Isolates							
HPA1*	0.78	0.79	0.83	0.00	0.00	0.00	0.00
HPA3*	0.86	0.04	0.03	0.45	0.55	0.66	0.05
HPA1	0.06	1.21	1.46	1.89	0.20	0.00	0.00
HPE3	0.41	1.35	0.88	0.67	0.10	0.29	0.00
Newly Obtained Isolates from Pepeekeo							
HPE4	1.22	0.82	1.00	0.60	0.78	0.12	0.62
HPE5	0.46	1.37	1.19	0.57	0.24	0.30	0.14
HPE6	-	0.58	0.78	0.63	0.13	0.43	0.52
HPE7	-	0.50	0.91	0.59	0.55	0.22	0.59
HPE8	0.82	0.64	0.94	0.72	0.85	0.21	0.60
HPE9	0.86	0.88	0.95	0.87	0.96	0.20	0.80
HPE10	0.93	1.15	1.03	0.93	0.77	2.18	0.58
HPE11	1.43	0.96	1.12	0.76	0.73	2.07	0.72
HPE12	1.12	0.94	1.52	0.88	0.33	1.63	1.15
HPE13	1.51	0.99	0.87	0.91	0.18	1.84	0.58
HPE14	1.45	0.88	1.31	0.85	1.26	0.87	1.77
HPE15	1.34	0.85	1.01	0.85	0.65	0.89	1.88
Newly Obtained Isolates from Pane‘awa							
HPA2	0.34	0.88	0.99	0.87	0.45	0.62	0.63
HPA3	-	0.54	0.59	0.50	0.39	0.34	0.40
HPA4	-	0.30	0.54	0.37	0.49	0.09	0.44
HPA5	0.74	0.71	0.81	0.74	0.81	0.34	0.61
HPA6	0.59	0.73	0.58	0.40	0.58	0.29	0.55
HPA7	1.06	0.83	1.07	0.74	0.55	0.59	0.73
HPA8	0.70	0.63	0.50	0.58	0.41	1.32	0.09
HPA9	0.73	0.65	0.94	0.69	0.35	0.17	0.69

Table 4: Summary of NCBI BLAST results. All isolates had 99% *P. colocasiae* sequence alignment, with the exception of HPA 1which had a 100% *P. colocasiae* sequence alignment.

Isolate	BLAST description	Percent Alignment
HPE 6	*P. colocasiae*	99%
HPE 7	*P. colocasiae*	99%
HPE 8	*P. colocasiae*	99%
HPE 9	*P. colocasiae*	99%
HPE 11	*P. colocasiae*	99%
HPE 13	*P. colocasiae*	99%
HPE 14	*P. colocasiae*	99%
HPA 2	*P. colocasiae*	99%
HPA 4	*P. colocasiae*	99%
HPA 5	*P. colocasiae*	99%
HPA 6	*P. colocasiae*	99%
HPA 7	*P. colocasiae*	99%
HPA 9	*P. colocasiae*	99%
HPA 1	*P. colocasiae*	100%
HPE 3	*P. colocasiae*	99%

Figures

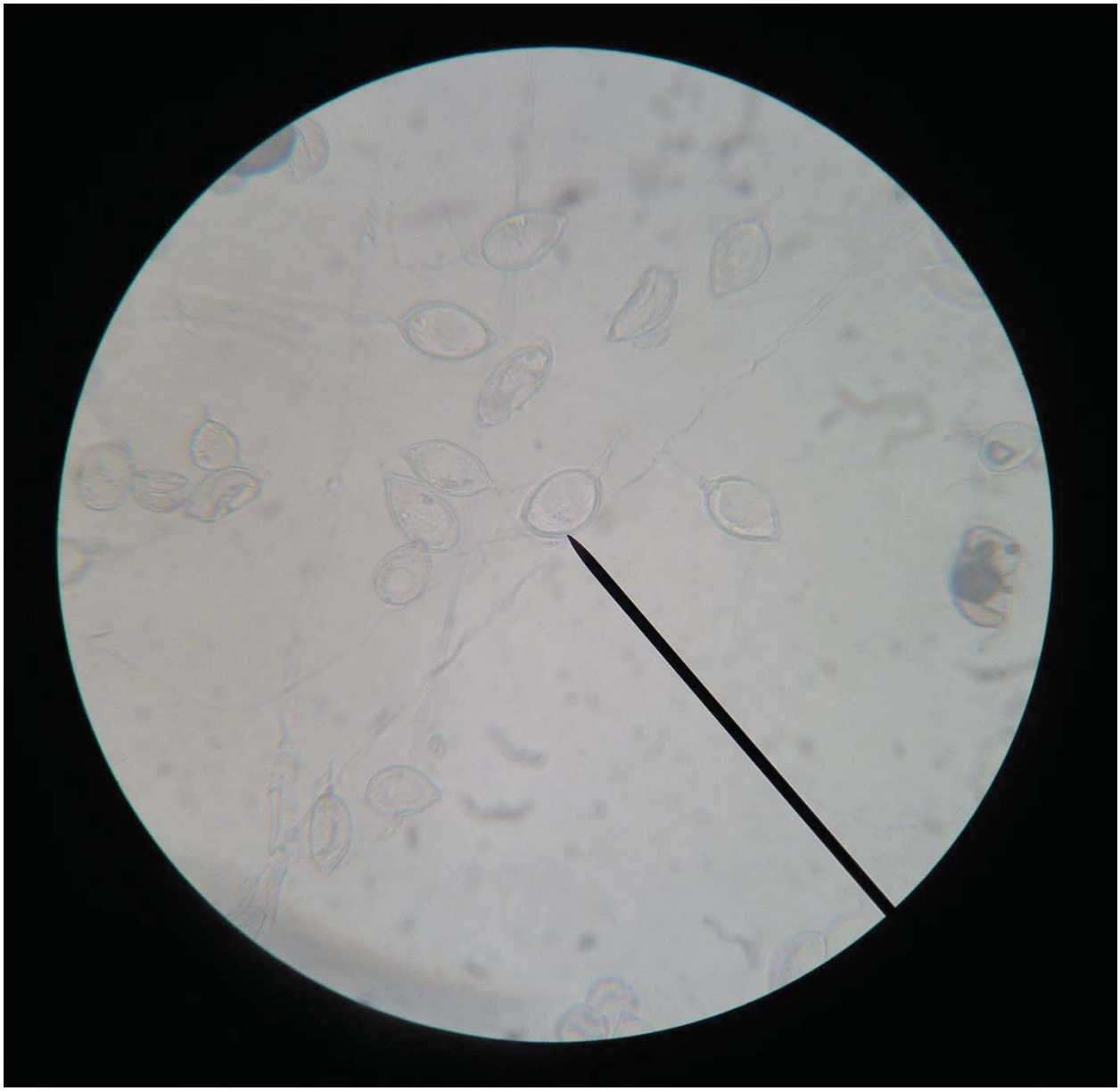

Figure 1: Sporaniga of *P. colocasiae* from isolate HPA 2 (400x).

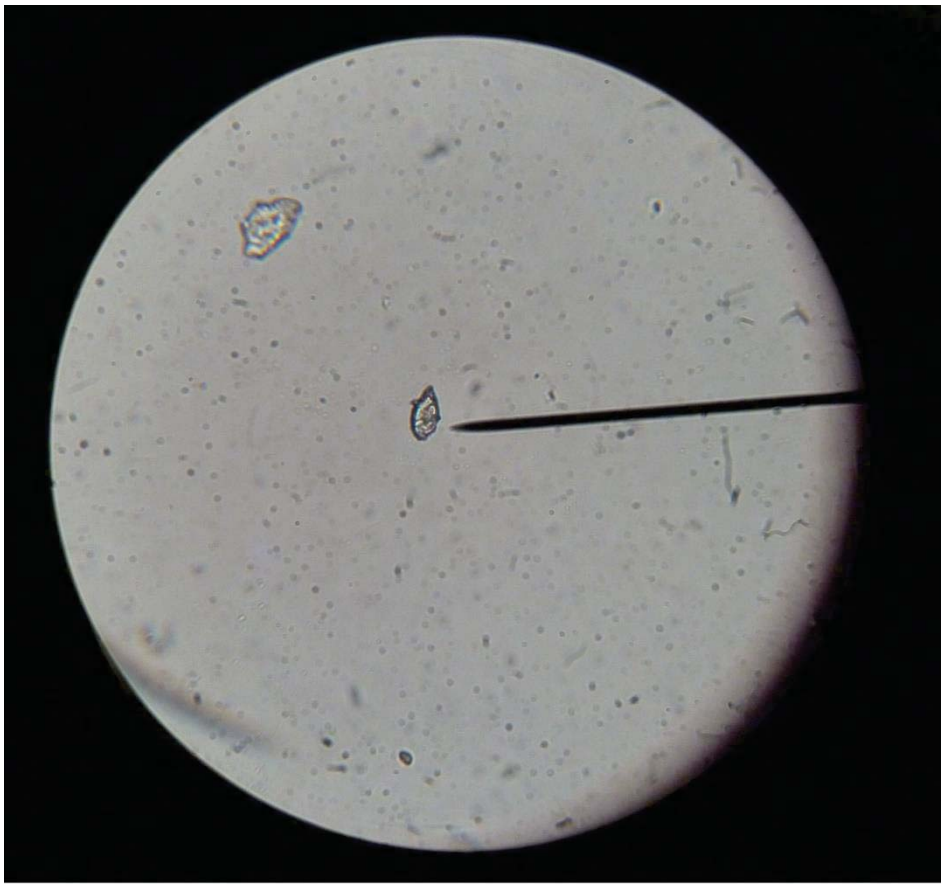

Figure 2: Zoospore (1000x)

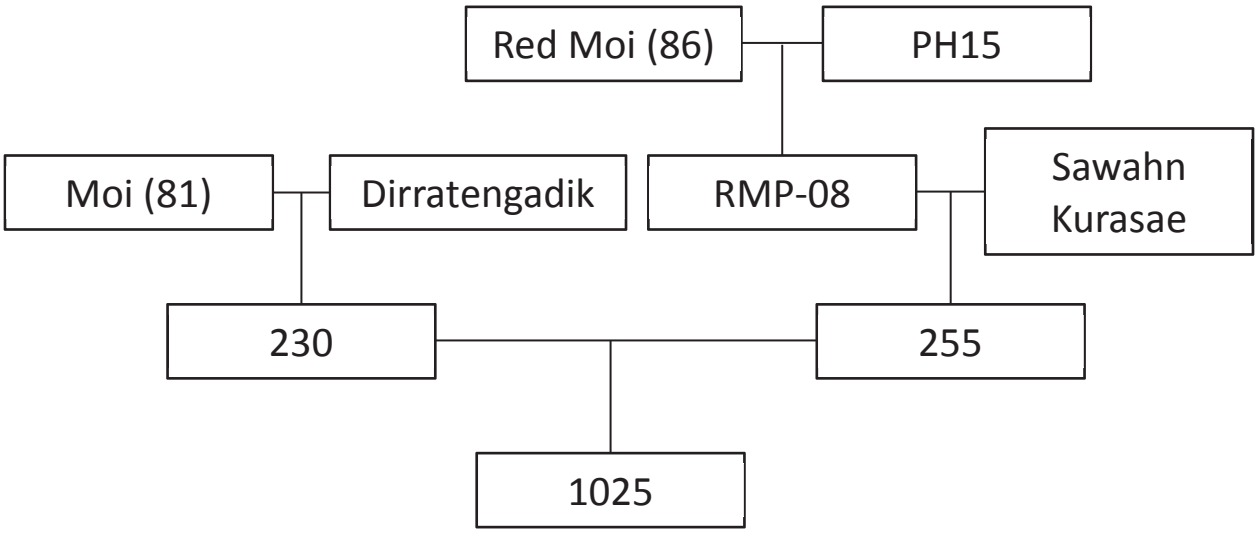

Figure 3: Pedigree of '230' and '255' cross. The progeny of the '1025' hybrid were using in this study (Miyasaka, Personal Communication, 2016).

Figure 4: Lesion on taro leaf (~2.5 cm).

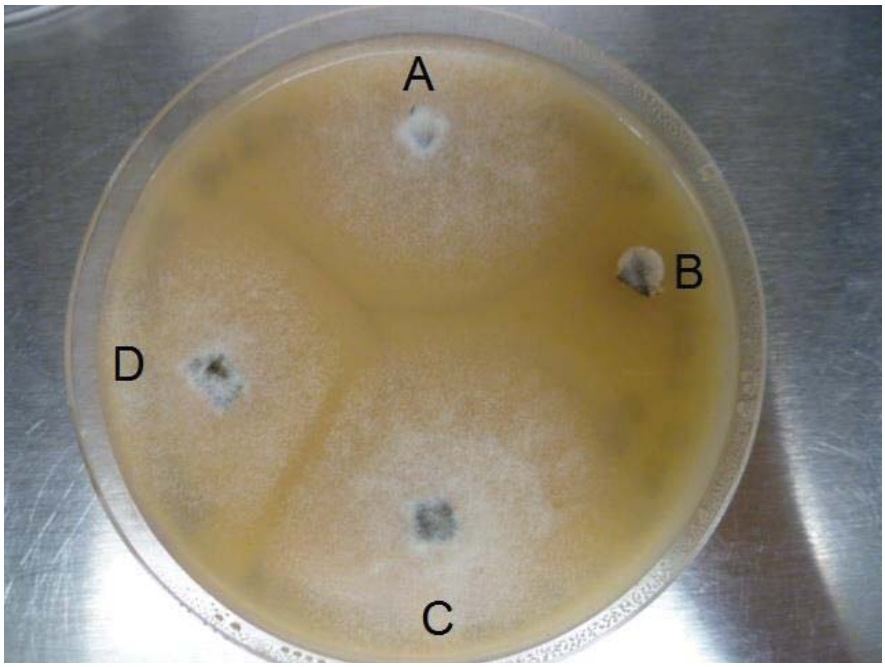

Figure 5: Taro lesions isolated from Pepeekeo after five days of incubation on 10% V8 supplemented with PCNB, NY, and amp. Isolates at this stage were selected based on the morphology. Isolates A, C, and D were selected for further isolation.

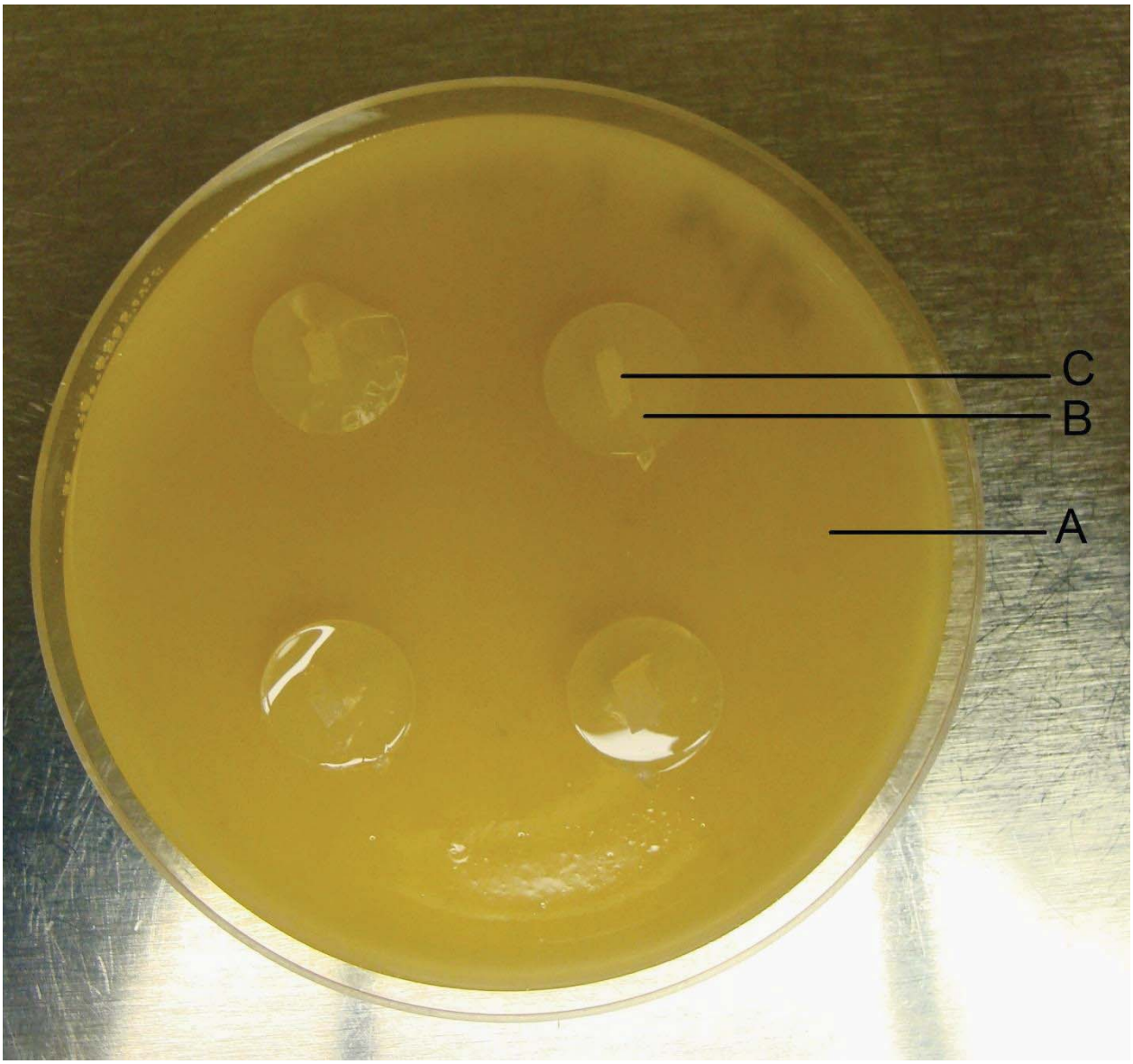

Figure 6a: Aerial view of sanwich column used to obtain a pure culture of *P. colocasiae* at T_0. A) 10% V8 agar B) 1.4% water agar C) Agar plug from lesion growth obtained from plates in Figure 5.

Figure 6b: Eye-level view of sanwich column used to obtain a pure culture of *P. colocasiae* at T_0. A) 10% V8 agar B) 1.4% water agar C) Agar plug from lesion growth obtained from plates in Figure 5.

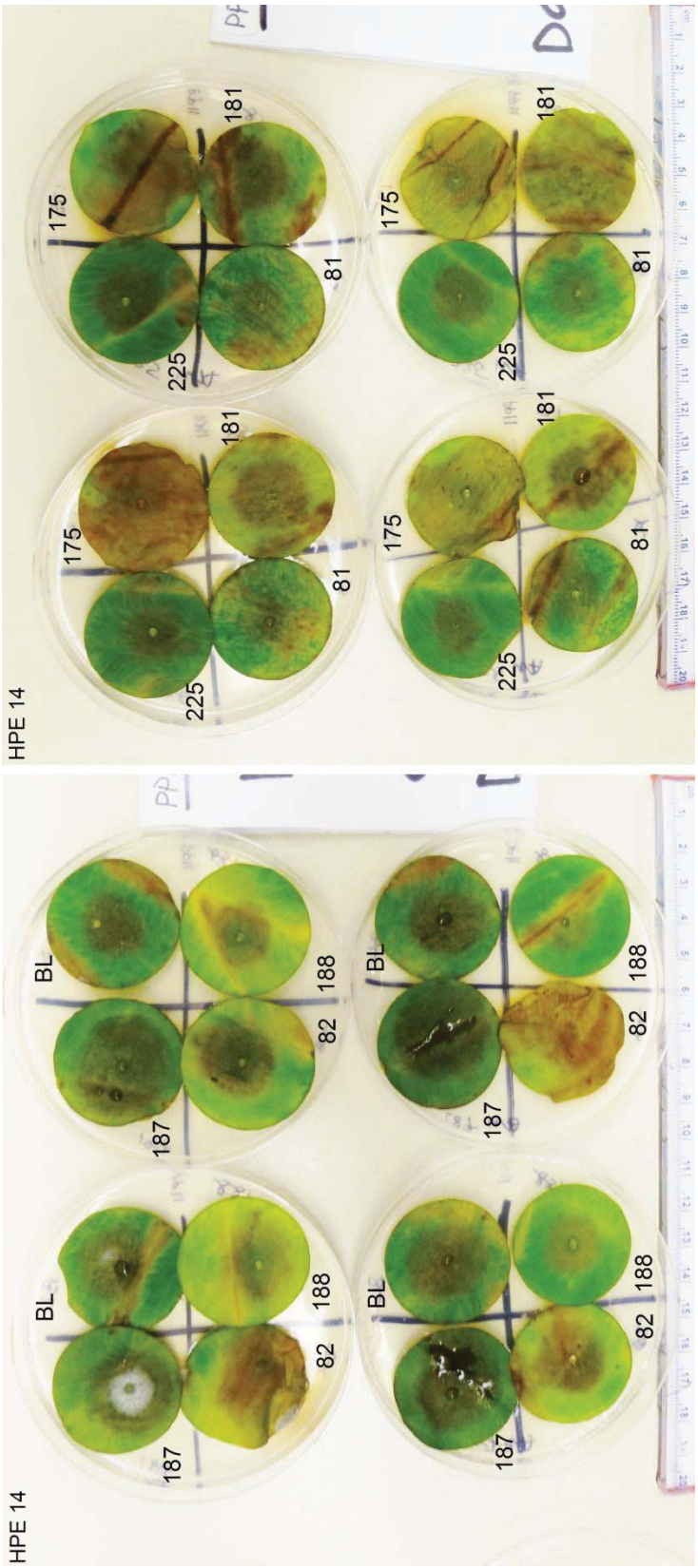

Figure 7: Disc assays for isolate HPE 14 after 4 days. All cultivars are of the '1025-X' cross for example '1025-187' with the exception of 'Bun-Long' (BL)

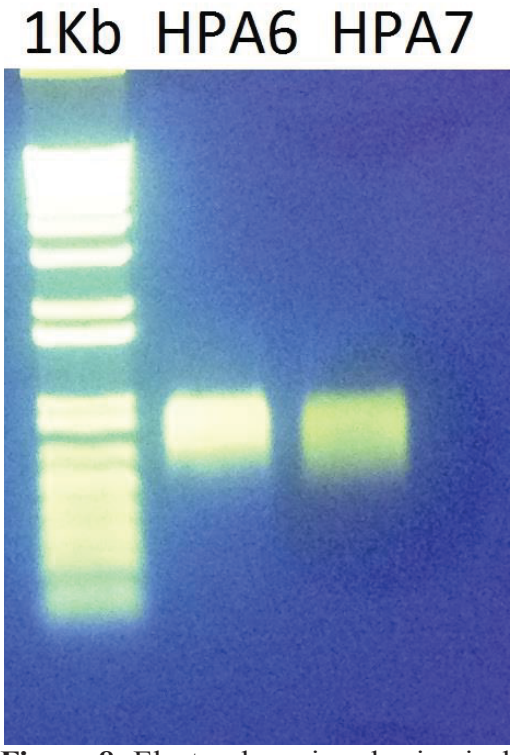

Figure 8: Electrophoresis gel using isolates HPA 6, HPA 7 and HPE 11. All of the samples are ~ 800 bp long.

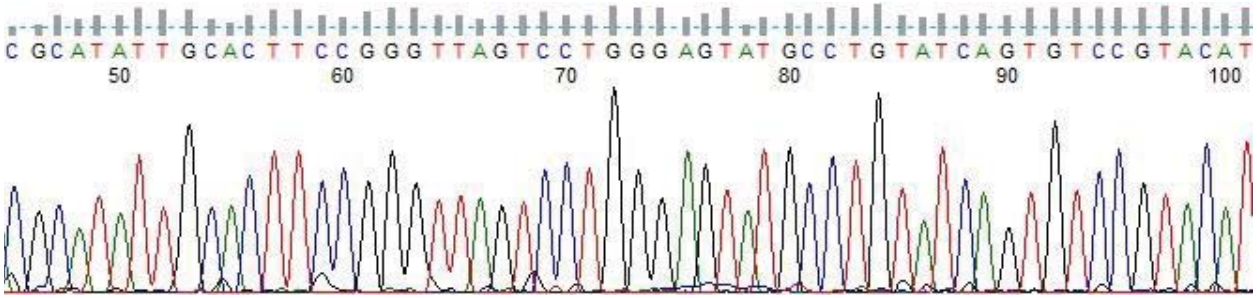

Figure 9: DNA sequences from FinchTV showing bases 45 – 101 from isolate HPE 3.

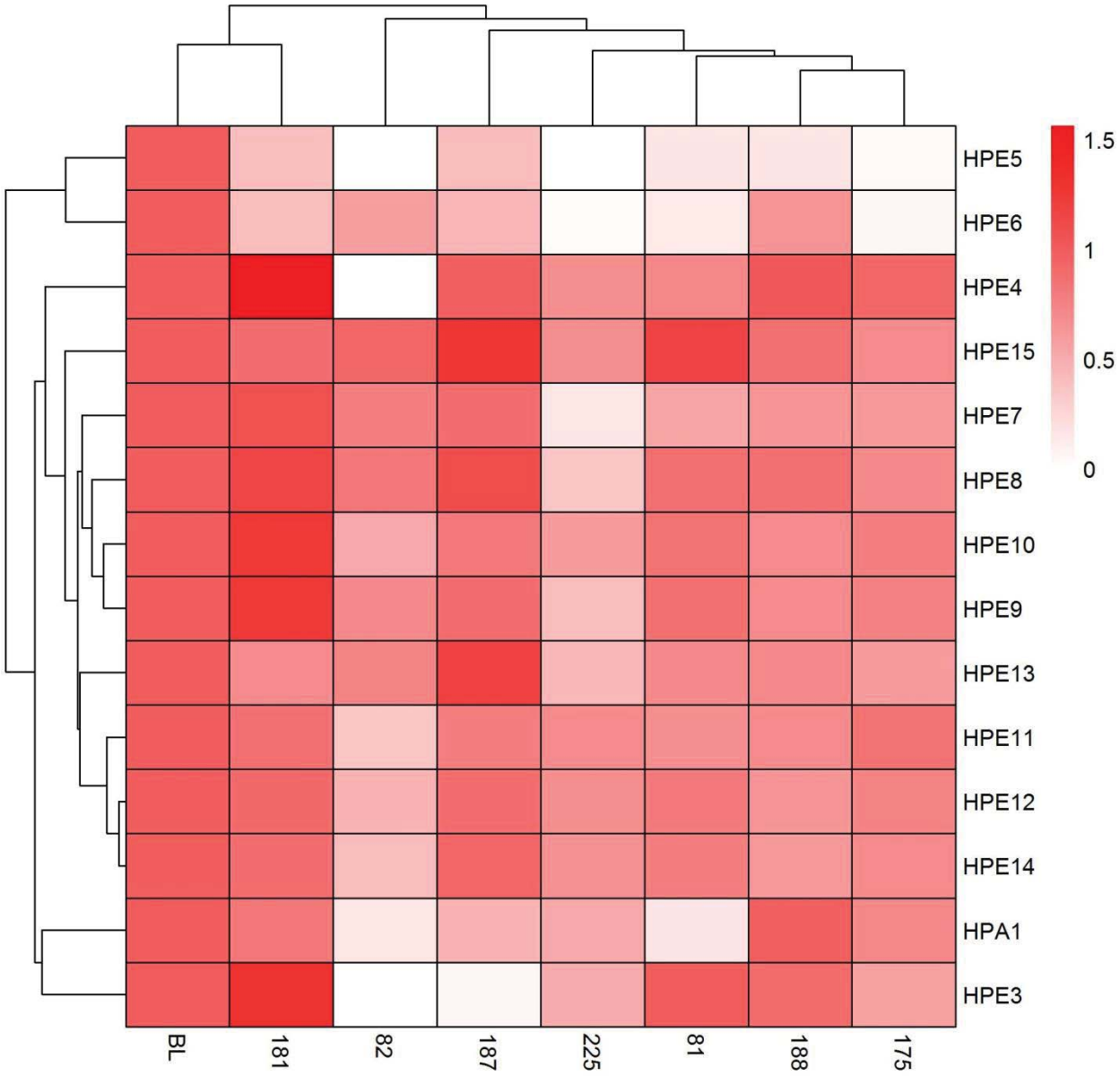

Figure 10: Heat map based on a Euclidean formula for the first trial of disc assays which used isolates HPA 1 (isolated from Pana'ewa, Hawai'i) and HPE 3 – 15 (isolated from Pepeekeo, Hawaii). The heat map shows a total of 3 mathematically unique pathotypes of *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the '1025-X' cross for example '1025-187' with the exception of 'Bun-Long' (BL) which was used as a positive control).

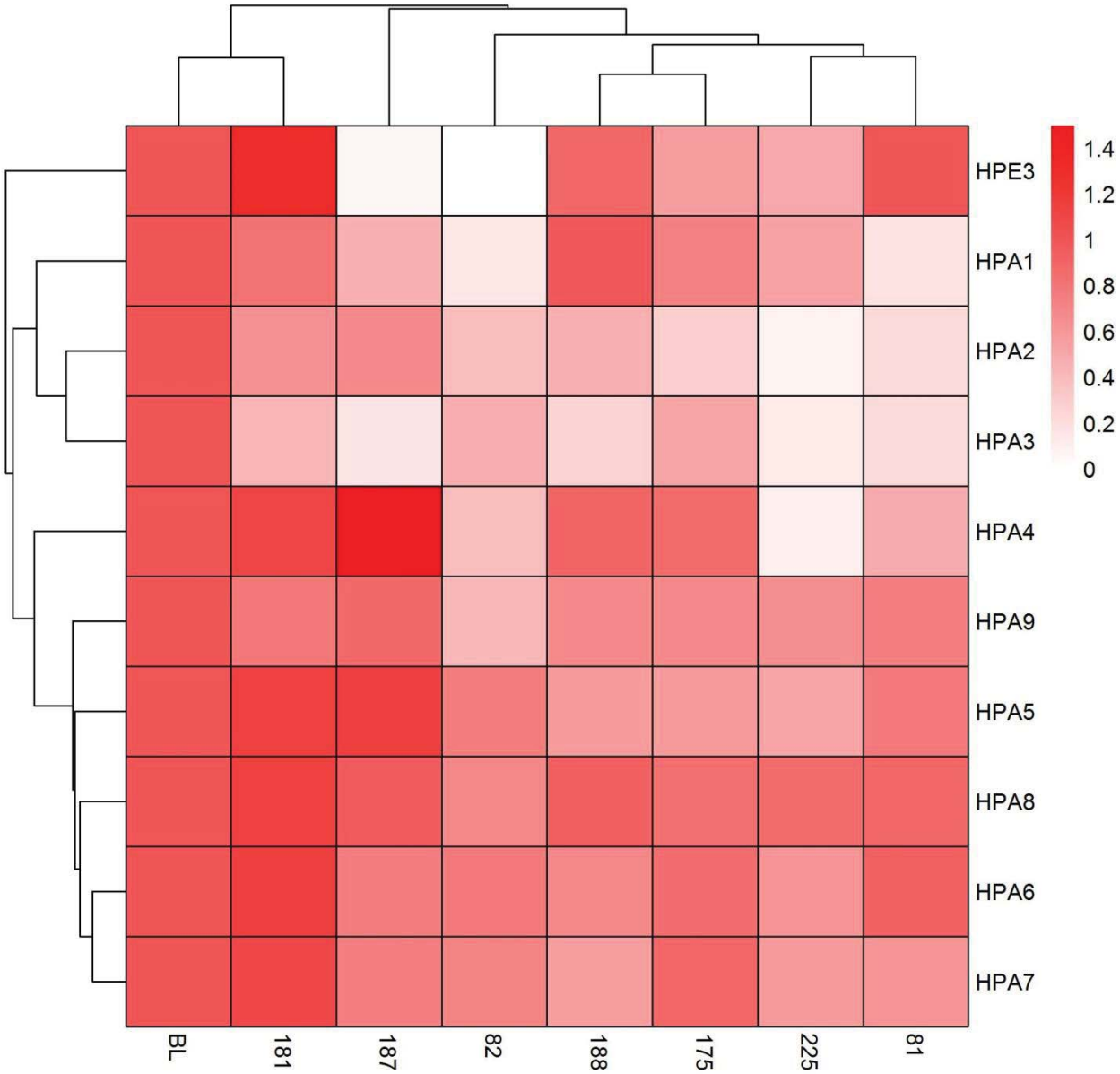

Figure 11: Heat map based on a Euclidean formula for the first trial of disc assays which use isolates HPE 3 (isolated from Pepeekeo, Hawai‘i) and HPA 1 – 9 (isolated from Pane‘awa, Hawai‘i). The heat map shows a total of 3 mathematically unique pathotypes of *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the 102 – X cross, for example ‘1025-187’ with the exception of ‘Bun-Long’ (BL) which was used as a positive control).

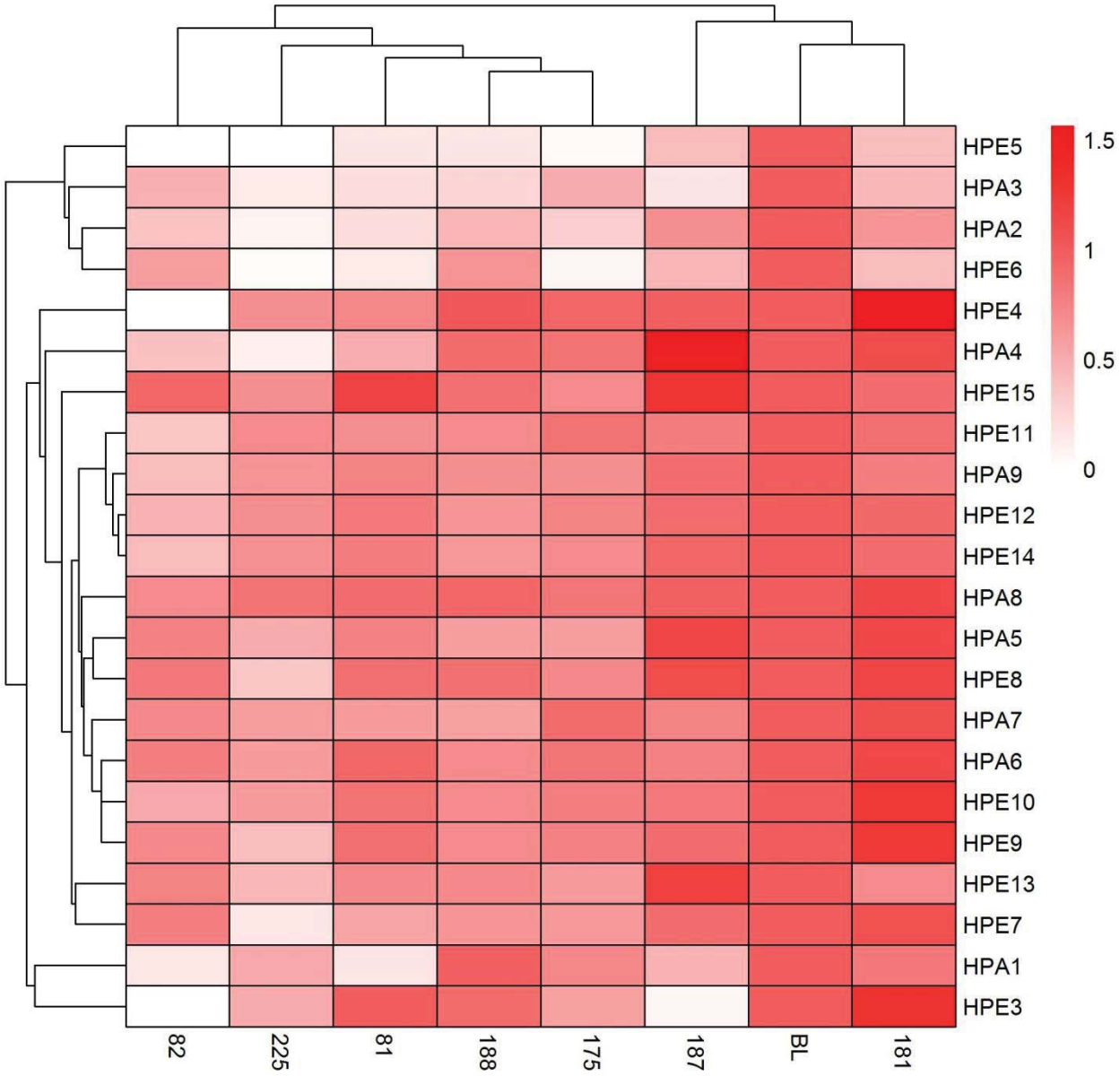

Figure 12: Heat map based on a Euclidean formula for the first trial of disc assays comparing all of the isolates used in this study HPA 1 – 9 (isolated from Pana'ewa, Hawai'i) and HPE 3 – 15 (Pepeekeo, Hawai'i). The heat map shows a total of 3 mathematically unique pathotypes of *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the '1025-X' cross for example '1025-187' with the exception of 'Bun-Long' (BL) which was used as a positive control).

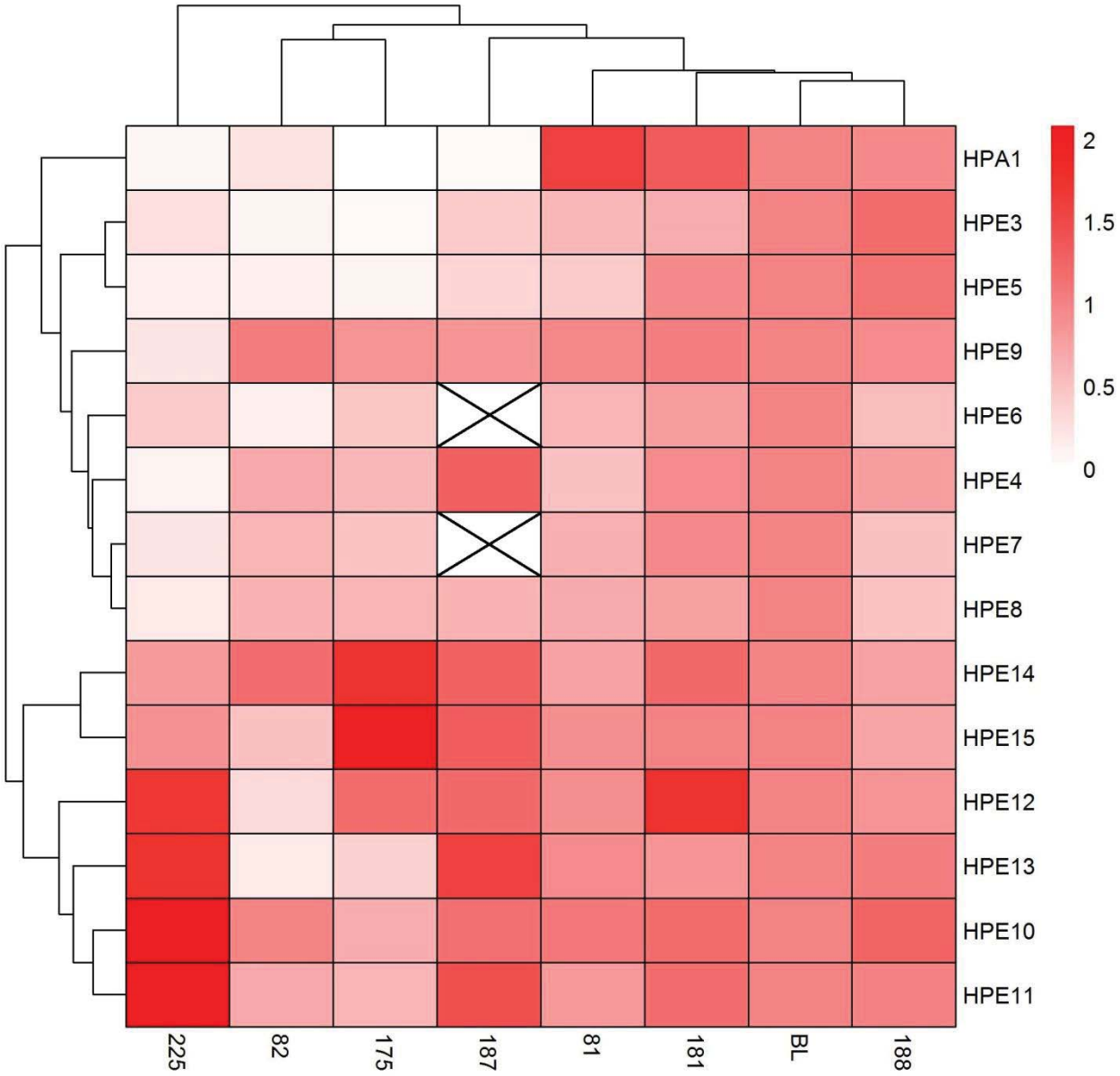

Figure 13: Heat map based on a Euclidean formula for the second trial of disc assays which use isolates HPA 1 (isolated from Pana'ewa, Hawai'i) and HPE 3 – 15 (isolated from Pepeekeo, Hawaii). The heat map shows a total of 2 mathematically unique pathotypes of *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the '1025-X' cross for example '1025-187' with the exception of 'Bun-Long' (BL) which was used as a positive control). Data is missing for HPE 6 and 7 on '1025-187' due to lack of avaliable leaves.

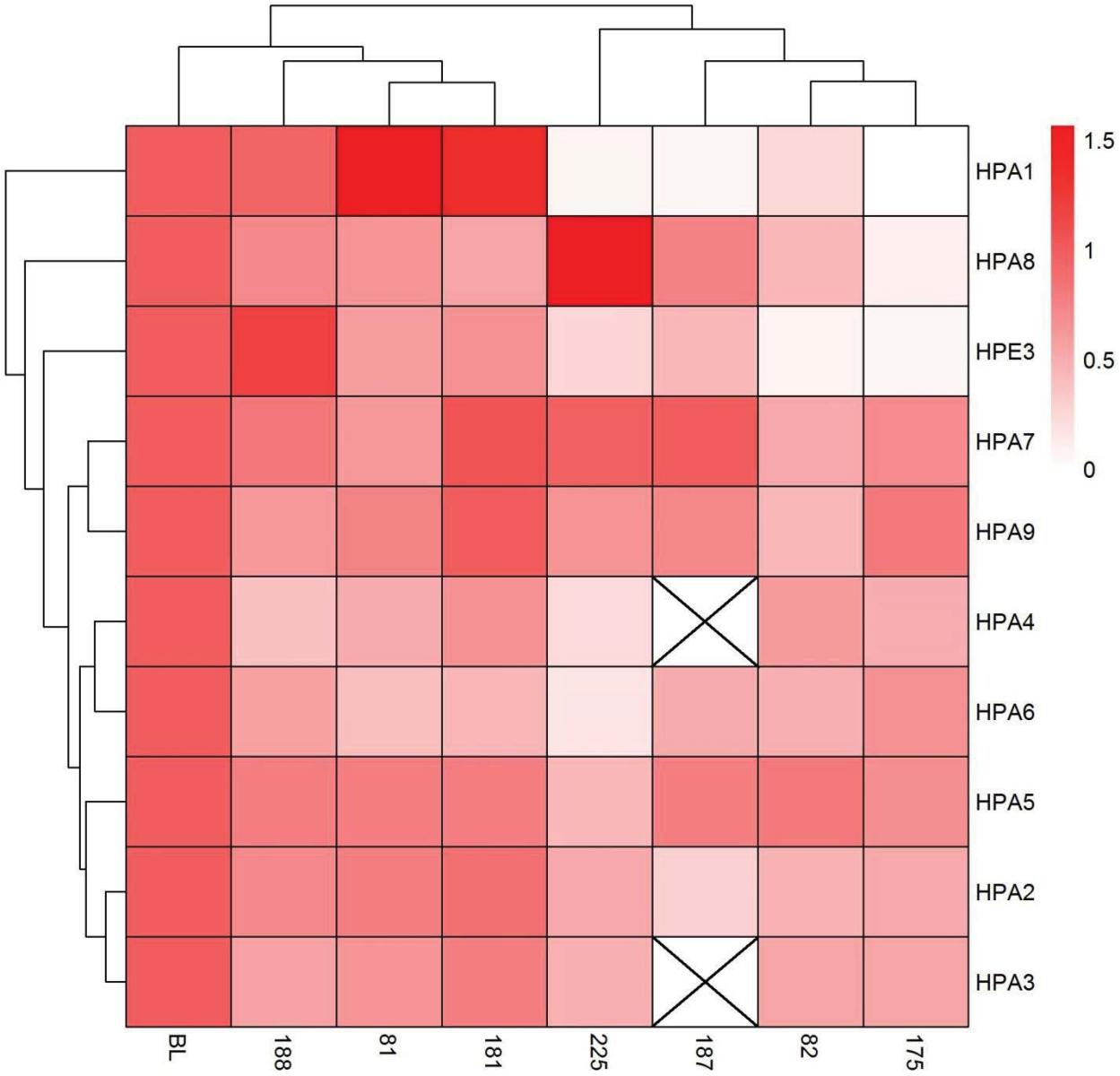

Figure 14: Heat map based on a Euclidean formula for the second trial of disc assays which use isolates HPE 3 (isolated from Pepeekeo, Hawai‘i) and HPA 1 – 9 (isolated from Pane‘awa, Hawai‘i). The heat map shows that the population is mathematically homogeneous *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the ‘1025-X’ cross, for example ‘1025-187’ with the exception of ‘Bun-Long’ (BL) which was used as a positive control). Data is missing for HPA 3 and 4 on ‘1025-187’ due to lack of avaliable leaves.

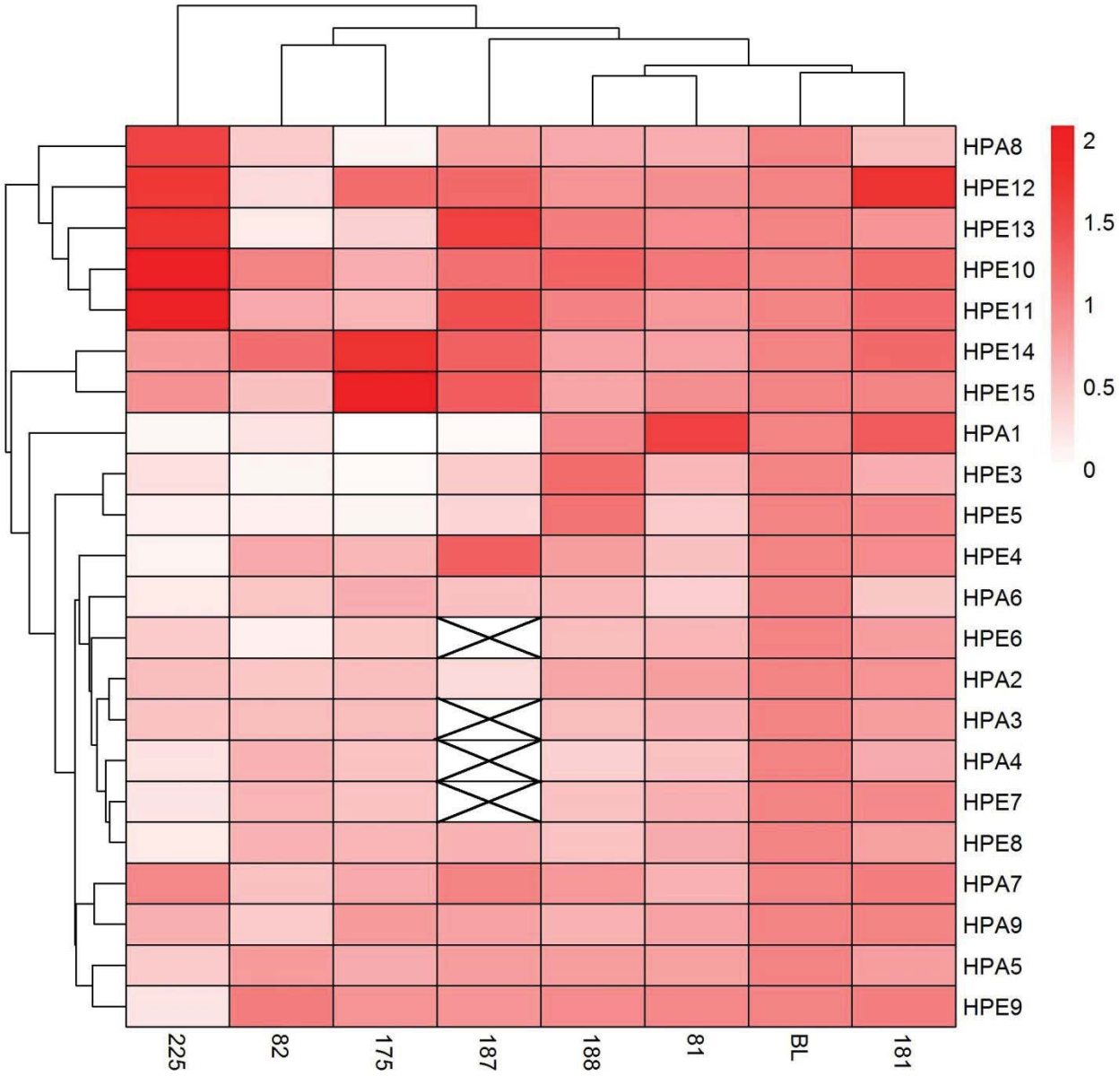

Figure 15: Heat map based on a Euclidean formula for the second trial of disc assays comparing all of the isolates used in this study HPA 1 – 9 (isolated from Panaʻewa, Hawaiʻi) and HPE 3 – 15 (Pepeekeo, Hawaiʻi). The heat map shows a total of 2 mathematically unique pathotypes of *P. colocasiae*. X-axis shows the isolate names. Y-axis shows cultivar names (all cultivars are of the '1025-X' cross which was used as a positive control). Data is missing for HPE 6 and 7 and HPA 3 and 4 on '1025-187' due to lack of avaliable leaves.

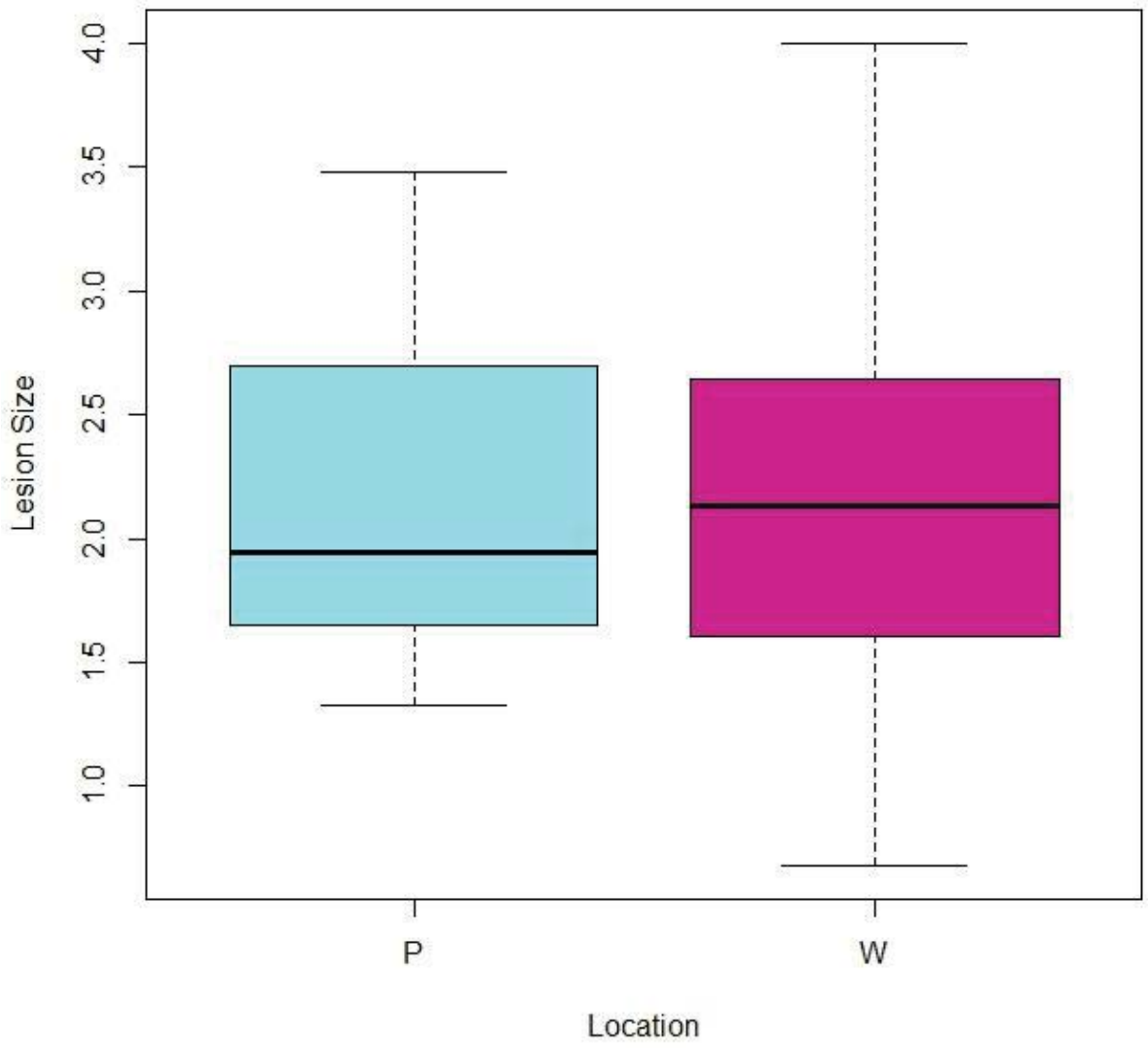

Figure 16: A) Box-plots comparing lesion sizes on leaves from Pepeepeko (P) (min = 1.33, Q1 = 1.66, median = 1.95, Q3 = 2.69, max =3.48) and Waiakea (W) (min = 0.68, Q1 = 1.62, median = 2.13, Q3 = 2.63, max =4.0) caused by the isolates HPE 11 and HPA 7 (df = 58, p-value = 0.47, f-value = 0.53).

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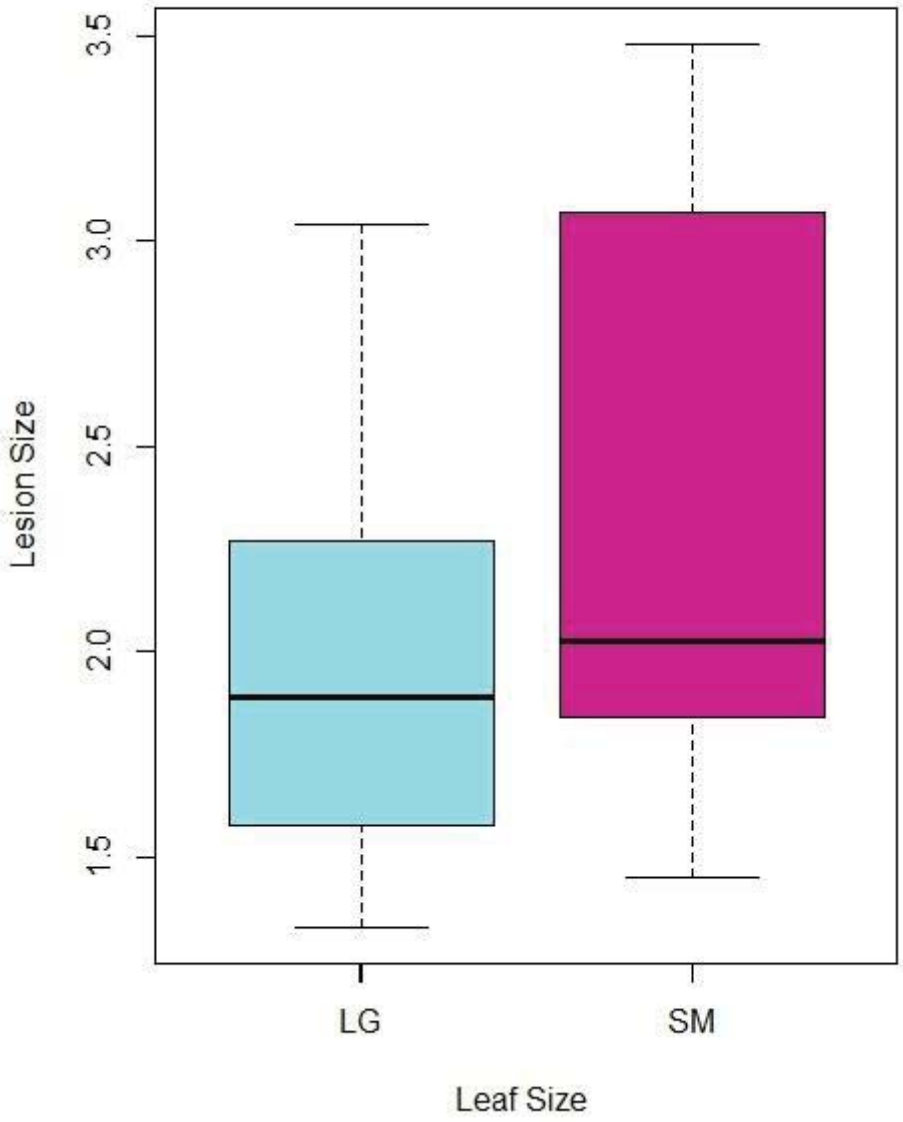

Figure 17: Box-plot comparing large (LG) (min = 1.33, Q1 = 1.59, median = 1.89, Q3 = 2.26, max =3.04) and small (SM) (min = 1.45, 1.84, median = 2.03, Q3 = 2.98, max = 3.48) leaves from Pepeekeo (df = 26, p-value = 0.019, f-value = 6.26).

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HPE14       GTCCTGGGAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGGCTTTCTTCCTTCCGTG     117
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HPA4        GTCCTGGGAGTATGCCTGTATCAGTGTCCGTACATCAAACTTGGCTTTCTTCCTTCCGTG     109
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HPE14       GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     477
HPE9        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     477
HPA4        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     469
HPE13       GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     469
HPE8        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     470
HPA7        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     477
HPE6        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     476
HPA5        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     478
HPA9        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     476
HPA6        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     475
HPE11       GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     480
HPA1        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     476
HPE3        GAATCGGCTTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGTGTCGATCCATT     479
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Figure 18: Isolate sequence alignment. Highlighted bases (W) indicate a base-call in which the sequencing could not differentiate between an A and T, and * indicate no difference in all of the isolates' sequences.