APPLYING MERISTEM TIP CULTURE AND THERMO THERAPY TO ELIMINATE VIRUSES FROM PINEAPPLE (*ANANAS COMOSUS*) AND TARO (*COLOCASIA ESCULENTA*)

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I AT HILO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

TROPICAL CONSERVATION BIOLOGY AND ENVIRONMENTAL SCIENCE

MAY 2020

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Keywords: Pineapple mealy bug wilt associated virus, Taro vein chlorosis virus, Meristem tip culture.

ACKNOWLEDGEMENTS

The establishment and completion of this project would not have been possible without the help and contribution from my supervisors, coworkers, peers, family, friends and loved ones. First and foremost, I would like to thank Dr. Tracie Matsumoto of the United States Department of Agriculture Agricultural Research Service Pacific Basin Agriculture Research Center, Tropical Plant Genetic Resource and Disease Research (USDA ARS DKI PBARC TPGRDR) for believing, challenging, and giving me the honor to take on this project, in addition to your leadership and guidance. Thank you to Dr. Mike Shintaku for serving as my primary advisor and for your guidance, teachings, support, and helping me grow as both a student and researcher. I would also like to thank Dr. Jonathan Awaya for serving on my committee, giving me advice, guidance, and especially for referring me to Daven Vicente when the USDA ARS DKI PBARC was looking for student applicants towards their program. Without your referral, I would not be in the position that I am in today. In addition, a big thank you to Claire Arakawa and Donna Ota for sharing your tissue culture knowledge, techniques, advice, and for all of your help with preparations, media, and sub culturing. Thank you to Blaine Luiz for your navigation throughout the TCBES program and help in data processing. Thank you to Eva Brill for helping me capture meristem culture photos and for your motivational backing. A big mahalo to the USDA ARS DKI PBARC, TCBES graduate program, and the UH Hilo Biology department for your financial support, teachings and foundational guidance. Finally, thank you to Alyssa E. Hatada for editing my photos used in this project and for your monumental emotional support and sacrifices in helping me accomplish my research and institutional goals.

ABSTRACT

Viruses in important crops such as pineapple (*Ananas comosus*) and taro (*Colocasia esculenta)* decrease plant growth, fruit or tuber yield, and overall crop production (Sether et al. 2001). Farmers are also negatively impacted by a decrease in profit due to subpar produce quality as a consequence of virus/viruses infecting crops. Lower taro yields affect the culture and diet of Pacific Island regions and countries because taro is a significant component of their diet, cultural events, and practices (Deo et al. 2009). Plant virus outbreaks also have environmental consequences; serious outbreaks can result in farmers abandoning fields and clearing land to establish new ones. This recently occurred on the island of Hawai‘i when an outbreak of banana bunchy top virus prompted the move of banana plantings (approximately 200 acres) from Kea‘au to Hāmākua. The elimination of viruses in such crops are important to minimize the negative economic, cultural and environmental impacts of plant viral infections. (Deo et al. 2009). Worldwide, pineapple is the most highly traded tropical fruit, ranked second in tropical fruit production in 2018 at approximately 28.3 million tons. Costa Rica is the largest producer and exporter of pineapple, followed by Brazil (Altendorf, 2019). In 2018 Hawai‘i produced 2.99 million pounds of taro representing a 1.97-million-dollar industry (Quickstats.nass.usda.gov, 2020). Currently only 70 (69 native to Hawai‘i) of the 150 landraces previously cultivated by Hawaiians remain in Hawai‘i (Helmkampf et al. 2017). The United States Department of Agriculture Agricultural Research Service Pacific Basin Agriculture Research Center, Tropical Plant Genetic Resource and Disease Research unit in Hilo Hawai‘i (USDA ARS DKI PBARC TPGRDR) houses and maintains 186 *Ananas* accessions ranging from local to wild type pineapples. The loss of unique culturally significant taro varieties and the need for maintaining and preserving the genetic diversity of both crops warrants a protocol for aiding germplasm maintenance, which is the aim of this study. Methods such as meristem tip culture, thermo

therapy, or a combination of the two on certain crops has been shown to eliminate viruses at varying rates of efficacy. This study will be the first in Hawai‘i to quantify the effectiveness of meristem tip culture followed by in vitro thermo therapy growth rates to achieve “virus free” pineapple and taro plants compared to other methods. Information derived from this study can play an integral part in establishing uninfected pineapple and taro plant material that will benefit researchers, farmers and the agricultural community as a whole leading to other studies applying similar techniques and measuring virus removal efficiencies on other important crops.

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LIST OF ABBREVIATIONS

µl ..... Microliter

µM ..... Micromolar

ARS ..... Agricultural Research Service

BA ..... Benzyl Adenine

BaMV ..... Bamboo Mosaic Virus

C ..... Celsius

CBDV ..... Colocasia Bobone Disease Virus

cDNA ..... complimentary Deoxy Nucleic Acid

cm ..... centimeter

CP ..... Coat Protein

CPd ..... Coat Protein duplicate

DI ..... Deionized

DKI ..... Daniel K. Inouye

DNA ..... Deoxy Nucleic Acid

dNTP ..... deoxynucleoside triphosphate

DsMV ..... Dasheen Mosaic Virus

FAO ..... Food and Agriculture Organization

HCl ..... Hydrochloric Acid

HP ..... Hapa

HSP70 ..... Heat Shock Protein 70

HTS ..... High Throughput Sequencing

M ..... meter

mL ..... milliliter

mm ..... millimeter

mM ..... millimolar

MMLV ..... Moloney Murine Leukemia Virus

MP ..... Mother Plant

MS ..... Murashige-Skoog

MWP ..... Mealybug Wilt of Pineapple

N ..... Population size

NF ..... Nuclease-Free

NGS ..... Next-Generation Sequencing

Nm ..... nanometer

ORF ..... Open Reading Frame

P ..... Probability-value

PBARC ..... Pacific Basin Agriculture Research Center

PCR ..... Polymerase Chain Reaction

PMWaV ..... Pineapple Mealybug Wilt associated Virus

qPCR ..... quantitative Polymerase Chain Reaction

RdRp ..... RNA dependent RNA polymerase

RNA ..... Ribonucleic Acid

RT ..... Reverse Transcription

RT ..... Room Temperature

SDW ..... Sterile Distilled Water

SL ..... Slip

TaBCHV ..... Taro Bacilliform CH Virus

TaBV ..... Taro Bacilliform Virus

TaRV ..... Taro Reovirus

TaVCV ..... Taro Vein Chlorosis Virus

TC ..... Tissue Culture

TCBES ..... Tropical Conservation Biology and Environmental Science

TPGRDR ..... Tropical Plant Genetic Resource and Disease Research

UH ..... University of Hawai‘i

USDA ..... United States Department of Agriculture

CHAPTER 1: LITERATURE REVIEW

1A. Pineapple:

1A.1. Taxonomy and History

Pineapple (*Ananas comosus*) is the most important commercial grown crop within the Bromeliaceae family. In 2018 the Food and Agriculture Organization (FAO) determined pineapple to be second overall throughout the world in mass fruit production at approximately 28.3 million tons per year (Altendorf, 2019). Amidst all tropical fruit, following bananas and citrus, pineapples are considered the most essential tropical fruit in the world (Bartholomew et al. 2003).

These perennial herbaceous monocots range from a height and width of 0.91-1.98m (Bartholomew et al. 2003) and were said to be domesticated in the Americas prior to the arrival of Christopher Columbus (Collins, 1960). It wasn't until the early 1800's that pineapples were first documented in Hawai‘i, which subsequently led to Hawai‘i's monumental contributions to production, research, and processing for the pineapple industry (Collins, 1960). Hawai‘i is home to the first pineapple processing machine to peel and core pineapples at a rate of 90-100 fruit per minute which eventually led to canning for exportation (Bartholomew et al. 2003). The "Smooth Cayenne" variety which tends to increase in acidity during winter months was the primary cultivar for canning processes (Gortner 1963) however a shift in preference for fresh pineapple fruit paralleled with the development of refrigerated shipping made it less desirable (Collins, 1960). In response to the shift in market preference, this led to the development of variety "MD-2/Del Monte Gold Extra Sweet". Bred by the Pineapple Research Institute of Hawai‘i, this cultivar is known for its sweetness, appealing fragrance, long storage life, large size, and low acidity during winter (Bartholomew, 2009).

1A.2. Importance of Pineapple and Mealybug Wilt of Pineapple Disease

Pineapple crops contaminated with viruses affect production and may lead to mealybug wilt of pineapple (MWP) disease which can be found in all pineapple crops in high density growing areas throughout the world (Sether et al. 2001). Mealybug wilt of pineapple symptoms are morphologically evident on plant leaves displaying leaf downward curling, reddening, wilting, or gradual dying of the tips (Fig. 2.1) (Sether et al 2001; Sether et al. 2005). The presence of MWP symptoms on plants within the first three months of growth was associated with fruit weighing half of fruit from uninfected plants (Sether et al. 1998). In the event pineapple plants displayed MWP symptoms at the later stages of growth, fruit mass was not affected (Sether & Hu 2002). However, fruit weight of plants symptomatic of MWP shortly after one year had slightly higher increased fruit mass compared to uninfected plants, indicative of a growth response to viral induced stress (Sether & Hu 2002; Sether et al. 1998). In most cases plant stress induced by MWP symptoms subsequently lead to plant death (Sether et al. 2001).

Fig. 2.1. Symptoms of mealy bug wilt of pineapple disease displayed on host pineapple plants. Reddening of the leaves (A, B, C); leaf downward curling (B, C); dieback of leaf tips (A, B, C); severe wilting of leaves (D). Adopted from: Dey et al. (2018).

1A.3. Species of PMWaV

Recent genetic analysis utilizing high-throughput sequencing comparing open reading frame proteins and amino acid similarity across the family *Closteroviridae* has led to the reclassification and identification of three confirmed and one presumed species of pineapple mealybug wilt associated viruses. Previous studies have indicated the presence of four PMWaVs. However, an 85-87% similarity in RNA-dependent RNA polymerase, heat shock protein 70, and coat protein between PMWaV-1 and PMWaV-4 indicates PMWaV-4 is not a distinct species but instead a strain of PMWaV-1, hence the renaming to PMWaV-1 (strain 4) (Fig. 2.2). (Green et al. 2019; Sether et al. 2009). Although the presence of a putative PMWaV-5 species has been detected in Australia (Gambley et al. 2008) further characterization of its genome sequence is

needed. Pineapple mealybug wilt associated viruses are positive sense flexuous rod-shaped RNA virions measuring approximately 1200nm in length and 10-12nm in diameter with genomes varying from 13-15.5 kilobases within the family *Closteroviridae* and genus *Ampelovirus* (Dey et al. 2018; Sether & Hu 2002; Sether et al. 2001; Sether et al. 2009 Martelli et al. 2002).

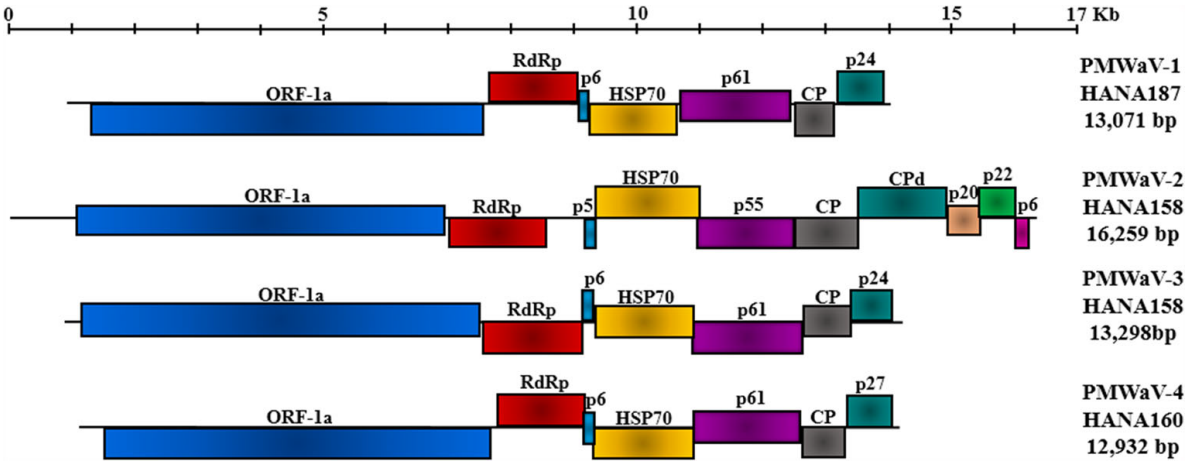


Fig. 2.2. Genome organization of PMWaV-1, PMWaV-2, PMWaV-3, PMWaV-1 strain 4 (PMWaV-4). Genome organization of PMWaV-1, PMWaV-2, PMWaV-3, PMWaV-1 strain 4 (PMWaV-4). Key abbreviations: ORF (open reading frame); RdRp (RNA dependent RNA polymerase); HSP70 (heat shock protein 70); CP (coat protein); CPd (coat protein duplicate). Adopted from: Green et al. (2019).

1A.4. Presence and effects of PMWaV

PMWaV-1 is present in most pineapple crops throughout the pineapple growing areas in the world, detected in 80% of MWP symptomatic plants and 78% in asymptomatic plants (Sether et al. 2001). The results of PMWaV-1 infection show a decline in crown abundance, slips and suckers used in plant regeneration, fruit size, and yield which negatively impacts the income of commercial pineapple growers (Sether et al. 2005). A survey conducted by Sether et al. (2001) showed one or multiple PMWaVs present in either symptomatic or asymptomatic crops (Sether et al. 2001). In Hawai‘i the presence of PMWaV-2 in infected plants ranges from 0-20% (Sether et al. 2005). Pineapple varieties grown in Hawai‘i and foreign countries such as Brazil, Costa

Rica, Malaysia, Guyana, and India with MWP symptoms all tested positive for the presence of PMWaV-2 indicating PMWaV-2 to be the primary factor of MWP disease. (Sether et al. 2001; Sether et al. 2005). Similar to PMWaV-3, the presence of PMWaV-1 alone in pineapple does not lead to MWP symptoms in Hawai‘i (Sether et al. 2005) however this was not the case in Australia as MWP symptoms were present with other PMWaVs (Gambley et al. 2008).

1A.5. PMWaV vectors

Whiteflies, aphids, and mealybugs are known vectors of viruses in the *Closteroviridae* family. Vectors known to transmit PMWaV in pineapples are the pink mealybug (*Dysmicoccus brevipes*) and grey mealybug (*Dysmicoccus neobrevipes*) (Fig. 2.3). Although not a vector of PMWaV, the presence of ants can be observed on mealybug infested plants and are associated with the spread of PMWaV through their mutualistic relationship with mealybugs (Sether et al. 1998; Sether & Hu 2002). Mealybugs feeding on pineapple plants have a strong correlation with the occurrence of MWP symptoms, however the mechanism of virus transmission is yet to be determined (Sether et al. 2001, Sether et al. 2009). While the presence of PMWaV-2 is associated with MWP symptoms, studies have shown that the presence of PMWaV-2 alone without mealybug feeding does not lead to MWP (Sether et al. 2001). Pineapples rarely produce seeds, consequently pineapple farmers utilize vegetative propagation to regenerate their crops. Therefore, it is common for ratoons from asymptomatic plants not tested for virus presence to be used for plant regeneration, promoting virus presence and replication (Sether et al. 2005).

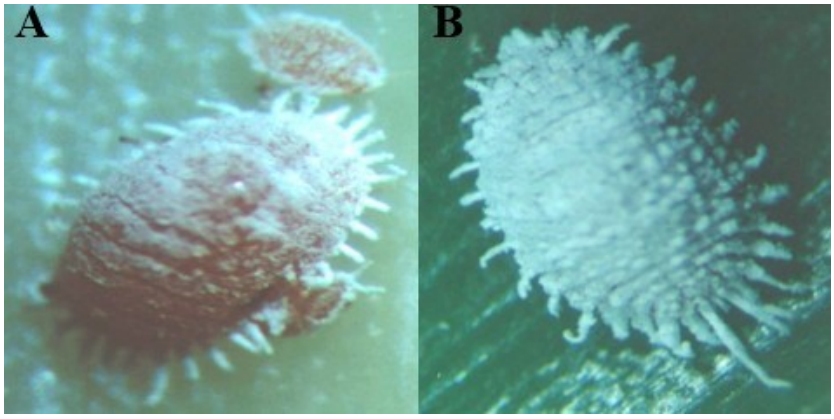


Fig. 2.3. PMWaV vectors: pink mealybug (Dysmicoccus brevipes) and grey mealybug (Dysmicoccus neobrevipes). Adopted from Dey et al. (2018).

1B. Taro

1B.1. Origin and Distribution

Taro *Colocasia esculenta* (L.) Schott is an herbaceous perennial corm-based crop in the Araceae family and genus *Colocasia* (Macharia et al. 2014). Grown primarily in tropical and subtropical regions, this edible aroid is a genetically diverse species (Caillon et al. 2006) consisting of nearly 100 different genera and more than 1500 species (Mandal et al. 2013). Although not a staple crop in Asia, the use of multiple genetic markers indicates taros derived from this region to display the greatest genetic diversity (Helmkampf et al. 2017). Its origin and domestication are unclear, but some studies point to the South-east Asia region (Rashmi et al. 2018). It was not until Polynesians migrated to Hawai`i that this edible aroid was introduced to the islands (Greenwell, 1947).

1B.2. Cultural and Economic Importance of Taro in the Tropics and Subtropics

Taro plays a significant role in diet composition throughout the Pacific Island regions and countries. Decrease in taro quality and yield affects the Pacific Islands both culturally and economically. Composed of mostly starch, taro tubers also provide protein while taro leaves are

high in vitamins A, B, C and abundant in minerals such as iron, potassium, calcium, riboflavin, and thiamine (Deo et al. 2009; Rashmi et al. 2018). Taro comprises 40% of the calorie intake in Tonga and 10% in the Solomon Islands (Deo et al. 2009). In some Pacific cultures, taro plays a role in socio-cultural importance. For example, certain taros are chosen for a select few of the highest stature and are present in cultural traditional feasts (Deo et al. 2009). In parts of Africa, taro plays a critical role as an alternative food supply when conditions are not favorable for traditional crops (Tewodros et al. 2013).

Diseases present in taro crops throughout the tropics has led to a reduction of tuber mass, quality, and a loss of up to 20% or more in production yield (Deo et al. 2009). Grown for numerous purposes, a reduction in taro production corresponds with a decrease in domestic and exported food supply, animal feed, and processed taro-based products such as alcohol, cosmetics, and infant formula (Deo et al. 2009). Overall taro ranks 9th among worldwide food crops (Rashmi et al. 2018) demonstrating its importance in economics and food security.

1B.3. Taro Viruses and Their Effects

Throughout the Pacific islands there are six viruses known to infect taro individually or simultaneously (Deo et al. 2009), including the recently-discovered taro bacilliform CH virus (TaBCHV) (Yusop et al. 2019). The most prevalent is dasheen mosaic virus (DsMV), followed by other viruses such as taro bacilliform virus (TaBV), taro reovirus (TaRV), Colocasia bobone disease virus (CBDV), and taro vein chlorosis virus (TaVCV). DsMV is a potyvirus of the family *Potyvirdae* consisting of rod-shaped virions that are vectored by aphids (Deo et al. 2009; Yongwei et al 2002). Taro plants infected with DsMV display chlorotic and plumy leaf patterns that are pale green, grey or light yellow in color in addition to undersized plant growth (Deo et

al. 2009). TaBV of the family *Caulimoviridae*, genus *Badnavirus* (Yusop et al. 2019) is the only known DNA virus to infect taro. Symptomatic plants show leaves with yellow mosaic vein chlorosis usually near leaf margin areas, backward bending of leaves, along with stunted plant growth (Deo et al. 2009; Carmichael et al. 2008). The spread of this disease can be via vegetative propagation, pollen or seeds and mealybugs. Studies have indicated mealybugs can vector TaBV (Revill et al. 2005a; Yang et al. 2003). Although in the same *Badnavirus* genus, a new virus TaBCHV was first discovered in China (2013), eventually in Africa (2014-15) and Hawai‘i (2016) (Kazmi et al. 2015; Kidanemariam et al. 2018; Wang et al. 2017). TaBCHV's two additional open reading frames, difference in TATA box location, and existence of a polyadenylation signal differentiates it from TaBV (Kazmi et al. 2015).

Another recently discovered taro virus is the Taro reovirus (TaRV). Known to be associated with other taro viruses, there is limited data on its effects on taro. The Colocasia bobone disease virus (CBDV) spread by planthopper (*Tarophagus proserpina*) has been demonstrated to cause bobone disease. Taro infected with bobone disease have leaves that exhibit severe stunted growth with veins that are thick and surrounded by brittle feathery mosaic patterns (Carmichael et al. 2008). Further symptoms show leaf lamina that may be twisted or exhibit backwards curling, dark green colored leaves with potential swelling of plant tissue on the petioles or large veins (Deo et al. 2009; Carmichael et al. 2008). Some plants show mild symptoms and contain leaves with patches that are light green and wrinkled but are still able to recover (Carmichael et al. 2008). When multiple viruses are present in a plant, the combination of TaVCV, CBDV, or TaBV are associated with alomae disease. Alomae disease causes symptoms similar to bobone disease but may subsequently lead to plant death due to systemic

necrotic rot (Deo et al. 2009; Carmichael et al. 2008). Taro variety and recovery of infected taro aid in determining alomae from CBDV infections (Carmichael et al. 2008).

1B.4. Taro Vein Chlorosis Virus (TaVCV)

The Taro Vein Chlorosis Virus was recently introduced to the Hawaiian Islands. Symptoms on infected plants are typically seen on mature taro plants (Carmichael et al. 2008). Mature leaves of symptomatic plants display vein chlorosis which spreads between leaf veins, eventually forming a network of yellow streaks in addition to leaf downwards bending (Fig. 2.4) (Carmichael et al. 2008). Other symptoms are leaves that may be torn and or show necrosis which are considered unmarketable in the taro industry (Carmichael et al. 2008). Although the virus is not mechanically transmitted, confirmed vectors of TaVCV have yet to be identified. Recent studies suggest leafhoppers and planthoppers are suspects since they are known vectors of nucleorhabdoviruses such as CBDV (Revill et al. 2005; Lamprecht et al. 2008).

Fig. 2.4. TaVCV symptoms present on young and mature leaves displayed on taro plants used in this experiment.

Taro vein chlorosis virus belongs to the family *Rhabdoviridae* and genus *Nucleorhabdovirus* (Revill et al. 2005; Deo et al. 2009). This rhabdovirus consists of bullet-shaped or bacilliform particles approximately 200 x 70 nm in size as determined by electron microscopy (Fig. 2.5) (Revill et al. 2005; Lamprecht et al. 2008). The genome of rhabdoviruses contains a lone strand of negative sense RNA encoding five proteins necessary for viral structure and replication (Revill et al. 2005; Lamprecht et al. 2008). Replication of nucleorhabdovirus occurs in the nucleus of a cell followed by budding in the nuclear membrane to accumulate in the perinuclear space (Revill et al. 2005). Further elucidation of the nuclear rhabdovirus replication processes in their insect and plant hosts may allow researchers to determine the TaVCV vector.

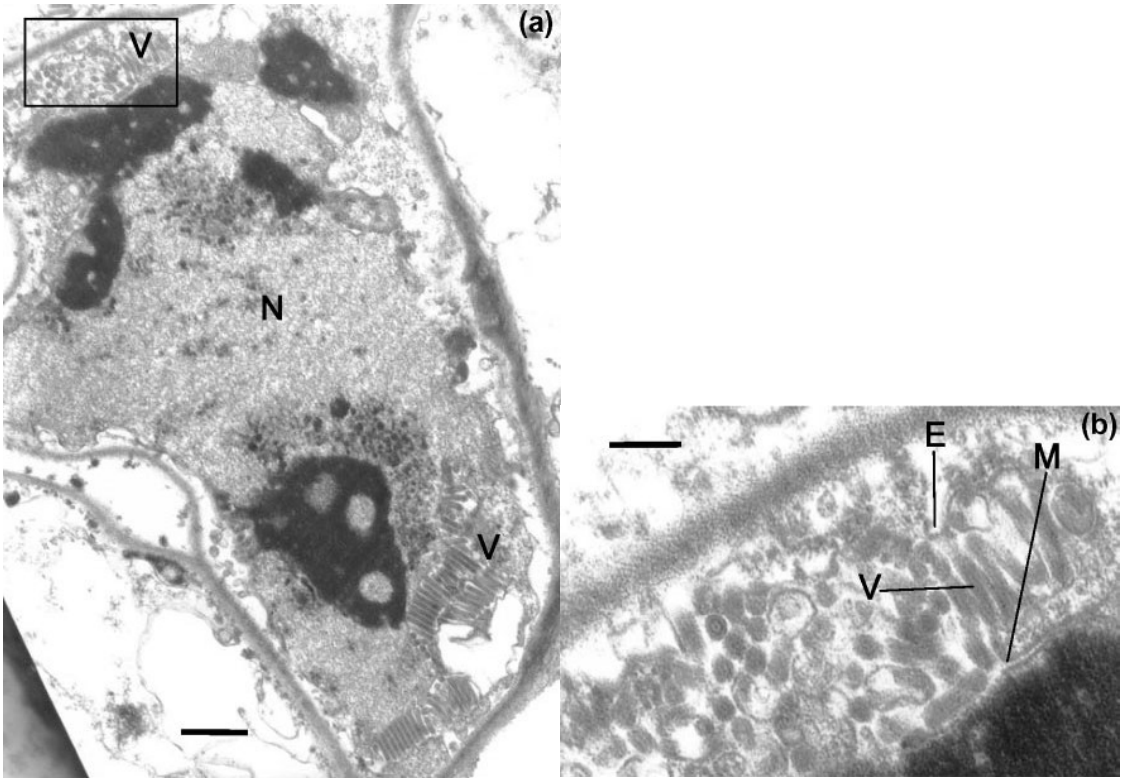


Fig. 2.5. Electron micrograph of a cross-section of a cell (a) Infected with TaVCV. Rod shaped virions (V) are depicted between the inner (M) and outer (E) membrane of the nucleus (N). (b) is enlarged to show detail. Bar (a): 500nm; Bar (b): 200nm. Adapted from Revil et al. (2005).

1C. Meristem Tip Culture

1C.1. History and Benefits of Meristem Tip Culture

Meristem tip culture is a form of in vitro propagation in which the meristem apex consisting of 2-3 of the youngest leaf primordia of a plant is excised. This technique is commonly used to obtain "virus free" plants (Hall 1999; Nehra and Kartha, 1994) by separating newly-formed cells at the meristem apex from virus-infected tissue (Rabinowitch & Currah 2002). Limmaset and Cornuet (1949) determined viruses to be unevenly distributed within host plants and observed low titers at the apical meristem region. This subsequently led the earliest studies of meristem tip culture done by Morel and Martin in 1952 resulting in "virus free" Dahlias (Nehra and Kartha, 1994). Meristem tip culture explants range in size (0.1-0.5mm)

however this is also dependent on the type of crop, as morphological structures may limit how small explants can be. Utilizing meristem tip culture also maintains genetic stability. As an apical meristem is already established, this reduces the growth of adventitious shoots and callus formation to limit somaclonal variation (Scowcroft et al. 1987; Grout B.1999).

A strong negative correlation exists between "virus free" plants recovered and the meristem propagative tissue size. In potatoes, meristem tips ranging from 0.2-0.5mm has successfully yielded "virus free" plants with approximately 25-44% efficiency across three potato varieties (Ali et al. 2014). Meristem tip culture of pineapple trimmed to 1mm has been shown to be effective in eliminating PMWaV-1 presence at a success rate up to 92% (Sether et al. 2001). Similar results of virus elimination through meristem tip culture across multiple species of plants indicates that smaller sized explants, although lower in growth rate, show a higher recovery of "virus free" plants.

1C.2. Plant Specific Meristem Tip Culture Media

Meristem tip culture media specific to plants, plant variety and temperature are contributing factors to plant growth and virus elimination efficiency. The proper balance and combination of plant hormones such as gibberellic acid and the growth regulator benzylaminopurine has shown to improve virus elimination in potatoes by approximately 15% and improves plant survival by 9-12% (Ali et al. 2014). Murashige-Skoog (MS) medium typically used to grow many varieties of taro needed to be modified by lowering levels of naphthaleneacetic acid to allow Solomon Island taro *Colocasia esculenta* var. esculenta to grow (Yam et al. 1990). Utilizing the optimal media for certain crops may allow the plant to outcompete the virus, which would increase the yield of virus free plants. Transplanted pineapple

grown from meristem tip culture remained PMWaV-free for over a year in field conditions (Sether et al. 2001).

1C.3. Plant Varieties Differ in Viral Infection Rates

Having the option to propagate "virus free" plants from infected mother plants through meristem tip culture allows farmers to maintain genetic stability of desired crop varieties. In Fiji, the virus-eliminated 'Samoa' taro variety derived from meristem tip culture propagated in separate plots have more than double the yield of tubers when compared to their asymptomatic huli (taro top planting material) field plot counterpart (Jackson et al. 2001). Results of a three-year trial comparing 'Samoa' meristem tip culture DsMV-free planted field plots to asymptomatic huli field plots showed large differences in infection rates. Huli grown plots had a DsMV infection rate of 73% compared to a 14% infection rate from meristem tip culture field plots based on symptoms. However similar results were not achieved when tested on the 'Toakula', 'Samoa hybrid' and 'Tausala ni Samoa' varieties with no difference in plant height and corm yields, indicating that certain varieties may be more resistant to virus infection than others. (Jackson et al. 2001).

1D. Thermo Therapy

1D.1. Minimal Viral Elimination Effectiveness Using Thermo Therapy Alone

Prior to planting, the application of heat treatment on vegetatively-propagated plants in an attempt to remove viruses and other diseases is one of the earliest practices dating as far back as 1869 (Varveri et al. 2015). Applying high temperatures decreases the rate of survivability by inhibiting viral RNA synthesis (Ali et al. 2014), while increasing the amount of virus free plants (Hall 1999). The trade-off between the abundance of surviving plants versus virus free plants

produced must be taken into consideration when determining an appropriate thermotherapy temperature, as plant varieties differ in survivability and virus elimination rates. However, applying thermotherapy alone as a virus removal treatment on pineapple propagules has shown to have minimal or no impact. Experiments using heat treatments of infected pineapple crowns have been conducted to eliminate PMWaV-1. Prior to isolating meristems for tissue culture, pineapple crowns were immersed in hot water baths at varying temperatures ranging from 35°C, 58°C, and 56°C for extended periods of time ranging from 24 hours to 40 and 60 minutes. These treatments failed to eliminate PMWaV-1from resulting explants (Sether et al. 2001). Similarly, thermotherapy alone failed to remove virus from garlic cloves grown in 37°C dry heat (Torres et al. 2000).

1D.2. Viral Elimination Effectiveness Utilizing Thermotherapy Pre Versus Post Meristem Tip Culture

The practice of thermotherapy pre versus post meristem tip culture significantly affects the abundance of virus free plant yield. Taro corms left out to dry for a month and treated at 38°C for 30 days until shoots grew to a height of 2-3cm prior to meristem tip culture did not show an increase in the abundance of virus free plants. (Yongwei et al. 2002). Applying thermo therapy treatment after meristem tip excision showed an overall virus elimination efficiency of 54% in garlic plants when meristems were grown in 37°C for 35 days (Torres et al. 2000). Meristems grown at 40°C showed a decrease in plant survival but had a 90% recovery of virus free plants for an overall efficiency rate of 18% indicating the tradeoffs between plant quantity versus quality (Torres et al. 2000). A similar pattern is seen when heat treating three varieties of potato post meristem tip culture. Control groups observed at 27° had the highest plant survival rate ranging from 21-28% with a low virus free rate of 14-15% (Ali et al. 2013). At 30°C plant

survival ranged from 18-26% with a virus free rate of 38-39%. The highest virus free rate of 43% occurred when the plants were treated at 35°C however plant survival rates were the lowest ranging from 17-23% (Ali et al. 2013).

1E. Virus Detection

Virus detection methods may be a limiting factor in determining the effectiveness of virus elimination techniques and the abundance of virus free crops. Previous studies have utilized immunological assays or visual inspection of virus presence via electron microscopy, and/or western blots. In a study by Hsu et al. (2000), immunological assays combined with electron microscopy had a 50-70% detection rate for Bamboo mosaic virus (BaMV) in bamboo, However, 100% detection of virus presence resulted when utilizing RNA hybridization probes on the same samples (Hsu et al. 2000). In addition to higher sensitivity, RNA hybridization can be used to detect other types of viruses (RNA or DNA) at low concentrations along with other virus forms such as single stranded, double-stranded, encapsidated, or non-encapsidated (Hsu et al. 2000).

Polymerase chain reaction (PCR) targeting viral nucleic acids is the most utilized method for virus detection in plants; having a higher detection sensitivity compared to immunological assays. The majority of plant viruses require the molecular machinery of their host for replication and typically have an RNA genome (Varveri et al. 2015). For RNA viruses, a reverse transcription (RT) step is necessary prior to PCR, hence the name reverse transcription PCR (RT-PCR). Primer design may be a limiting factor when using RT-PCR to detect plant viruses. This can be problematic since RNA viruses that infect vegetative propagated plants tend to have vast genetic diversity (Subr & Glasa et al. 2013). Therefore, it is important to utilize primers that encompass conserved regions of the genome to enable detection of a wide range of viruses. To

increase sensitivity and accuracy, a nested PCR utilizing multiple primer pairs targeting similar locations of the targeted genome can be utilized. This technique was applied to specifically detect PMWaV-2 with higher sensitivity compared to traditional PCR (Dey et al. 2012). Real-time quantitative PCR (qPCR) using fluorescent probe activation during DNA amplification may be 10-100 times more sensitive than PCR while having a lower risk of mis amplification due to nested-primer specificity. Recently qPCR probes and primers have been developed for PMWaV 1,2,3 with an increased sensitivity 10-1000 times greater compared to RT-PCR (Chang et al. 2015; Subere et al. 2011; Hu et al. 2015).

To best establish virus free plants, next-generation sequencing (NGS) or high-throughput sequencing (HTS) can be incorporated with other detection methods. By sequencing entire plant transcriptomes, small RNAs are assembled and scanned for virus/es. (Kreuze et al. 2009). This led to the discovery of novel cryptic viruses, isolates, the transcription of whole virus genomes such as PMWaV-1, 2, 3 and the recent reclassification PMWaV-4 to be a strain of PMWaV-1 (Kreuze et al. 2009; Green et al. 2019). The ability to sequence whole viral genomes allows further analysis to better understand viral etiology, classification, and identify conserved regions within viruses. Conserved regions are used to design primers that can detect multiple viruses or strains. Although HTS is a useful tool, further research is needed to reduce application costs.

CHAPTER 2: APPLYING IN VITRO METHODS MERISTEM TIP CULTURE FOLLOWED BY THERMO THERAPY TO ELIMINATE VIRUSES PRESENT IN PINEAPPLE (*ANANAS COMOSUS*)

Abstract

Pineapple mealybug wilt associated viruses (PMWaV) cause mealybug wilt of pineapple disease (MWP) in pineapple crops resulting in loss of plant vigor and eventual plant death. Viral transmission occurs during conventional vegetative propagation such as the use of crowns, suckers, slips or other propagative material. Plants are usually asymptomatic, thus the development of a protocol that will effectively and efficiently provide farmers, stakeholders, and conservationists access to clonal virus and disease-free material is needed. Meristem tip culture, thermo therapy, or a combination of the two have been shown to eliminate viruses at varying rates of efficiency. Here, we investigate the effects of explant sizes (1mm and 2mm) and location of buds on the mother plant (top, middle, and bottom thirds) for eliminating PMWaV. We also investigated these parameters on survival rates during the early stages of meristem tip culture. In addition, thermotherapy treatments were conducted to measure the growth rates of tissue cultured pineapple plant sections (top and middle) against temperature treatments (37°C, 35°C, 30°C, RT). This will provide conditions for future treatments of infected pineapples to establish virus free plant material. Differences in recovery of virus free plants from PMWaV (PMWaV-1,2,3,1-strain 4) infection was observed when comparing 1mm and 2mm explant sizes ($P < 0.048$). Initiating bud explants 1mm in height eliminated PMWaV from 97% of plants, while 83% were free of PMWaV when initiated at 2mm. Location of bud explants on the stem of the pineapple was also important in the exclusion of PMWaVs ($P < 0.035$). Higher incidences of PMWaV was observed when bud explants were located on the lower part of the mother plant. Buds harvested from the top third were 97% clean compared to 84% clean when taken from the

middle, and 75% clean if harvested from the bottom. Overall, 399 buds were initiated and 193 (48%) survived (were successfully disinfested and grew). Survival rates showed 2mm buds (N=229) had a 16% higher chance of surviving versus 1mm (N=170). This could be attributed to smaller buds being more prone to over sterilization by sodium hypochlorite disinfestation. The highest rate of bud survival (67%) was observed when 2mm buds were harvested from the top section of the mother plant suggesting that explants derived from the younger, actively growing tissue trimmed to 2 mm is optimal for survival.

Regardless of growth temperatures, comparing pineapple explants isolated from the top and middle section of tissue culture plantlets revealed that the top sections grew more quickly (0.51mm/day) compared to middle sections (0.085mm/day). Although at 37°C top sections grew the least, an initial growth spurt was observed for 30 days before plateauing. A similar trend was observed for 35°C indicating higher temperatures treatments may still be used to inhibit viral RNA synthesis.

Introduction

Worldwide pineapple is the most highly traded tropical fruit, ranked second in tropical fruit production in 2018 with a production volume of approximately 28.3 million tons. Costa Rica is the largest producer and exporter of pineapple, followed by Brazil (Altendorf, 2019). Mealybug wilt of pineapple (MWP) disease can be found throughout the world in pineapple crops grown in high density. In Hawai‘i, MWP is highly associated with the presence of PMWaV-2 (Sether et al. 2001) but has also been associated with the presence of PMWaV 1 and 3 in Australia (Gambley et al. 2008). Mealybug wilt symptoms on pineapple include downward curling of leaves, reddening, wilting, or gradual dying of the tips (Sether et al 2001; Sether et al. 2005). The presence of MWP symptoms on plants at earlier stages of plant growth is associated

with fruits weighing an average of 55% less compared to plants showing symptoms at later stages (Sether and Hu, 2002). In most cases, MWP symptoms lead to plant death (Sether et al. 2001). The heightened plant damage caused by PMWaV-2 can be attributed to increased virulence factors. PMWaV-2 produces two local suppressors and three systemic suppressors to overcome plant RNA silencing, which is a mechanism used for antiviral defense (Dey et al. 2015; Dey et al. 2018). PMWaV-1 is present in most pineapple crops throughout the pineapple growing areas in the world and is found positive in 80% of MWP symptomatic plants and 78% of asymptomatic plants. The presence of PMWaV-2 in infected plants range from 0-20% (Sether et al. 2005) but was more recently detected in 85% of the accessions at USDA ARS germplasm repository in Hilo, HI (Hu et al. 2009). PMWaV-1 produces one RNA silencing suppressing protein which allows this virus to be more prevalent among pineapples without having severe lethal effects upon plant hosts (Dey et al. 2015; Dey et al. 2018). PMWaV-1 infection in pineapples may lead to a decline in crown abundance, slips and suckers used in plant regeneration, fruit size, and yield which may be costly to the commercial pineapple growers (Sether et al. 2005). In several instances, multiple PMWaVs may be present within a single plant (Sether et al. 2001; Gambley et al. 2008). Previously thought to be a separate PMWaV species, newest studies utilizing high-throughput sequencing have confirmed PMWaV-4 to be a strain of PMWaV-1 named "PMWaV-1 strain 4" (Green et al. 2019). Although the presence of PMWaV-5 has been detected in Australia (Gambley et al. 2008), detection of this virus for this project was not included.

Pineapple mealybug wilt associated viruses are flexuous rod-shaped RNA virions measuring approximately 1200nm in length and 10-12nm in diameter varying from 13-15.5 kilobases (Dey et al. 2018) within in the *Closteroviridae* family (Sether & Hu 2002). Further

analysis has determined all PMWaVs to be of the genus *Ampelovirus* (Martelli et al. 2002). Whiteflies, aphids, and mealybugs are known vectors of the *Closteroviridae* family however the pink mealybug (*Dysmicoccus brevipes)* and grey mealybug (*Dysmicoccus neobrevipes)* are known to transmit PMWaV in pineapples (Sether et al. 1998). Although not a vector of PMWaV, ants can be observed on mealybug infested plants and are associated with the spread of PMWaV through their mutualistic relationship with mealybugs (Rohrbach 1988; Sether et al. 1998; Sether & Hu 2002).

The application of meristem tip culture, thermo therapy, or a combination of the two has been demonstrated to remove viruses and other diseases with varying degrees of effectiveness. Meristem tip culture is a form of in vitro propagation in which the meristem apex consisting of the youngest leaf primordia of a plant is excised (Hall 1999). In doing so, viral, fungal, and bacterial diseases can be removed by separating potentially infected outer layer tissue from newly formed cells at the apical meristem (Hall 1999; Rabinowitch & Currah 2002). Meristem propagative sizes may range from < 1-5mm or greater depending on crops as some may not need to be excised at very small sizes to eliminate targeted diseases. Excised buds at a size of 1mm or less increases the abundance of yielding "virus free" plants, however it has been demonstrated to promote a lower success rate in growth and survivability compared to larger excised meristem sizes (Hall 1999; Sastry & A. Zitter 2014; Verma et al. 2004). Some factors dictating viral elimination among plants can be attributed to the type of virus being eliminated, the physiological condition of the mother plant, and the locality of the meristem on the mother plant (Verma et al. 2004). The application of meristem tip culture has been effective in removing PMWaV-1 in 92% of pineapples plants containing the virus (Sether et al. 2001) but the effectiveness of the method has yet to be determined with pineapple viruses such as PMWaV-2,

PMWaV-3 PMWaV-1 strain 4, PMWaV-5. Attaining meristem tip culture sizes of ≤1 mm is a tedious process that requires proper skill and craft while incorporating aseptic technique. Thus, identifying the optimum site and size for meristem culture that eliminates virus infection at a reasonable survival rate would be ideal in protocols specific for pineapple.

At 30°C or higher, most viruses within plants have their replication abilities arrested or hindered (Sastry & A. Zitter 2014). Applying high thermo therapy temperatures decreases the rate of survivability while increasing the amount of "virus free" plants produced (Hall 1999) by inhibiting viral RNA synthesis (Ali et al. 2014). Constraining viral RNA synthesis by RNA degradation is associated with RNA silencing mechanisms naturally found within plants, and has a tendency to increase with temperature (Wang et al. 2008). Similar to meristem tip culture, tradeoffs between plant survival versus "virus free" plants produced must be taken into consideration when determining appropriate thermo therapy temperatures. Plants grown in higher temperatures are more likely to be "virus free" at the risk of dying due to high temperature stress. In previous studies, the application of thermo therapy alone as a virus elimination treatment has shown to have little effectiveness in pineapple. Heat treatment of infected pineapple crowns has been done in an attempt to eliminate PMWaV-1. Prior to meristem tissue culture, pineapple crowns were immersed in hot water baths at varying temperatures ranging from 35°C, 58°C, and 56°C for extended periods of time ranging from 40 minutes to 24 hours and showed minimal influence in eliminating PMWaV-1 (Sether et al. 2001). The percentage yield of obtaining clean plant material differs among plants but is typically less than 50%. This highlights the need to increase efficiency in obtaining "virus free" plants for further propagation.

A study using pineapple apical buds at 5-7mm in size incubated at 27°C resulted in buds still containing PMWaVs, compared to 1mm buds under the same conditions resulting in a 50% success rate in removing PMWaVs (Souza et al. 2012). This indicates that apical meristems placed in culture should be smaller than 5mm when working with pineapple. Utilizing meristem tip culture followed by thermo therapy in growing garlic at 37°C for 35 days showed an overall virus elimination efficiency of 54% (Torres et al. 2000). Meristems grown at 40°C showed a decrease in plant survival but had a 90% rate of "virus free" plant yield demonstrating the tradeoffs between plant quantity versus quality (Torres et al. 2000). A similar pattern is seen when heat treating three varieties of potato post meristem tip culture. Control groups observed at 27° had the highest plant growth rate 21-28% with a low 14-15% rate of "virus free" plants (Ali et al. 2013). At 30°C plant survival ranged from 18-26% with a "virus free" plant rate of 38-39%. The highest "virus free" success rate of 43% occurred when the plants were treated at 35°C however plant survival rates were the lowest ranging from 17-23% (Ali et al. 2013). The difference of "virus free" obtained plants between the two studies demonstrates how different plants vary in different levels of virus removal and crop survivability success.

The United States National Plant Germplasm pineapple collection is housed at the USDA ARS Daniel K. Inouye Pacific Basin Agriculture Research Center in Hilo, Hawai`i. The repository maintains 186 *Ananas* accessions including cultivated and wild pineapple relatives. (Pineapple Collection: USDA ARS. 2017). The mission of the repository is the conservation, maintenance, and distribution of the pineapple collection. Production of disease-free plant material furthers the mission of the repository to preserve and maintain the genetic diversity of the pineapple collection, as well as distribute disease-free material to the research community throughout the world. Methods such as meristem culture, thermotherapy, or a combination of the

two has been shown to effectively eliminate viruses. This study will evaluate the effectiveness of meristem culture to remove pineapple mealybug wilt associated viruses (PMWaV-1, PMWaV-2, PMWaV-3, and PMWaV-1 strain 4) by testing different explant sizes (1mm and 2mm) and location (top, middle, and bottom) of the explants from the donor pineapple plant. In addition, we investigate different growing temperatures to regenerate PMWaV-free pineapples from infected pineapples plants in tissue culture.

2B. Materials and Methods

2B.1. Initial Plant Virus Screening

Fifty-eight *Ananas comosus* variety *comosus* and one *Ananas macrodontes* were screened for PMWaV from the USDA ARS repository Hilo, HI. Samples were harvested from pineapple plants following removal of fruits taken for descriptor data and regeneration of the collection. In previous immunological screenings for pineapple closterovirus, leaves from crowns during fruit production were most dependable for detection (Hu et al.1997). Similarly, young leaf samples collected for virus testing ranged from the second to fourth leaf whorl from available slips, stem shoots, or suckers. Once harvested, leaf samples were kept cool by immediately placing them in a cooler containing ice packs.

2B.2. Reverse-Transcription PCR (RT-PCR)

Each leaf was either tested as an individual sample or pooled together with two replicates during the RNA extraction process. Each sample consisted of 100-150mg taken from the basal leaf tissue (white in color) collected from the youngest section of the leaf base to increase RNA yield (Hu et al. 1997). Young leaf tissue was sliced into 2-3mm segments and placed into 2ml Polypropylene Disruption Tubes (OPS Diagnostics, Lebanon, NJ). Each tube contained six pre-

loaded 3mm Acid Washed Zirconium Beads (OPS Diagnostics, Lebanon, NJ), 800µl of Lysis Binding Solution RNAqueous® Kit (Ambion, Austin, TX) and 100µl Plant RNA Isolation Aid (Thermo Fisher Scientific Waltham, MA). FastPrep-24 with QuickPrep™ Adapter (M.P. Biomedicals, Santa Ana, CA) was used to homogenize the tissue at speed 6.0 M/S for 1 minute. Wash and filtration followed the standard protocol of the RNAqueous® Kit and final RNA was eluted three times at volumes of 15µl, 10µl, 10µl for a total of 35µl. Following RNA extraction, samples were either stored at -80°C or quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA) for reverse transcription and PCR provided in Melzer et al. (2001.); Melzer et al. (2008.); Sether et al. (2005.); and Sether et al. (2009.) From each RNA sample, 3µl was aliquoted into a 0.2ml (PCR) thin walled tube (Thermo Fisher Scientific Waltham, MA) and incubated at 70°C for 8-10 minutes in a thermal cycler (MJ Research Inc. Waltham, MA) and immediately chilled on ice. An RT master mix was made including 6.5µl nuclease free (NF) water (Qiagen, Hilden, Germany), 5µl of dNTPs (2mM) (Invitrogen), 4µl M-MLV 5X RT Buffer (Promega Madison, WI), 1µl Random Hexamer (Invitrogen) 0.5 µl RNasin (40u/µl) (Promega), and 1µl MMLV RT (5u/µl) (Promega) added for each sample. The RT master mix was gently agitated and 17µl was added to each sample tube. All samples were again gently mixed and centrifuged for 30 seconds. Following the spin down, the samples were incubated in a thermal cycler at 42ºC for 45 minutes, then 95ºC for 5 minutes.

During RT incubation, the PCR master mix was made for each PMWaV virus. The following reagents was added for each sample; 10µl 2X GoTaq Green Master Mix (Promega), 8µl NF water, 0.5µl of (10µM) forward/reverse primers (Table 3.1) targeting PMWaV heat shock protein (Hsp70h). All PCR master mix reagents were gently mixed and 19µl was aliquoted. Lastly 1µl of cDNA was added to each sample tube. PCR cycling parameters applied were

(step1) 95 ºC for the first seven minutes, followed by (step 2) 95 ºC for one minute, (step 3) 54 ºC for one minute, (step 4) 72 ºC for one minute, back to (step 2) for 42 cycles, finishing off at 72 ºC for seven minutes, and 4 ºC indefinitely.

Table 3.1. Primer sequences used in this study targeting the HSP 70 homolog genes of PMWaV-1, PMWaV-2, PMWaV-3, PMWaV-1 (strain 4), and amplicon product size.

Target Virus	Primer	Sequence (5' → 3')	Amplicon Size (nt)
PMWaV-1	225	ACA GGA AGG ACA CTC AC	590
	226	CGC ACA AAC TTC AAG CAA TC	
PMWaV-2	223	CAT ACG AAC TAG ACT CAT ACG	610
	224	CCA TCC ACC AAT TTT ACT AC	
PMWaV-3	263	ATT GAT GGA TGT GTA TCG	499
	264	AGT TCA CTG TAG ATT TCG GA	
PMWaV-1 (strain 4)	267	GGT ACA GGC CCG ATA AA	475
	268	ACT TGG GCG TCG TA	

2B.3. Gel electrophoresis

Gel electrophoresis using 1.5% agarose (Invitrogen) in 1x Tris-acetate-EDTA (TAE) buffer (Omega Bio Tek, Norcross, GA) (mass/volume). A mixture of 10µl PCR-product with 2µl loading dye (VWR EZ-Vision One, Radnor, PA) was added to each gel-well and run at 92 volts (Thermo EC Mod: EC105-115) for 25-30 minutes. Band presence within gels lanes were observed using ultra violet imaging (UVP BioDoc-It Imaging System). Among the 61 mother plants tested, most plants that had both replicate samples test positive for the same or combination of PMWaVs was selected to move forward into the meristem tip culture initiation phase.

2C.4. Meristem Tip Culture-Leaf Harvest for PMWaV Testing

2C.4.1. Bud Explant

Prior to bud initiation, mother plants were separated and placed on a covered benchtop outside of the greenhouse and removed from any water or irrigation supply for a 5-6week period. This allowed plants to dry to reduce fungal contamination from excess moisture and optimize plant texture and firmness for easier removal of leaf sheaths from buds during the dissection phase of initiation. After the drying period, the peduncle was trimmed to approximately 20.32cm. The base of the mother plant was cut at soil level. The peduncle was utilized as a handle to hold the plant firmly as each leaf was carefully peeled off without damaging the underlying axillary bud. Removal started from the lowest leaf sequentially working up to the top youngest leaves. Following leaf removal, the stem was gently washed using a mild soap detergent; carefully scrubbing in the same direction of the axillary bud. Measuring from the lowest bud to the top youngest axillary bud (not including buds on peduncle), the mother plant stem was divided into thirds designated as the top, middle, and bottom sections. A steel razor blade was used to explant each bud into approximately 1.5-2cm cubes (Fig. 3.1). The exterior leaf sheath typically brown in color were carefully removed using a scalpel. The trimmed product was further cubed into 0.5-1cm blocks with a flat back (for easier handling during dissection phase). The pre-surface sterilization product was off-white at the bud base while white/brown on the actual bud itself. The buds were placed into a deionized (DI) water filled plastic beaker labeled with its appropriate bud location to allow them to keep moist while excising the rest of the buds from the pineapple stem. All explanted buds were then quadruple rinsed with DI water to remove excess debris and placed into autoclaved 5.08cm Pyrex petri dishes for bud disinfestation.

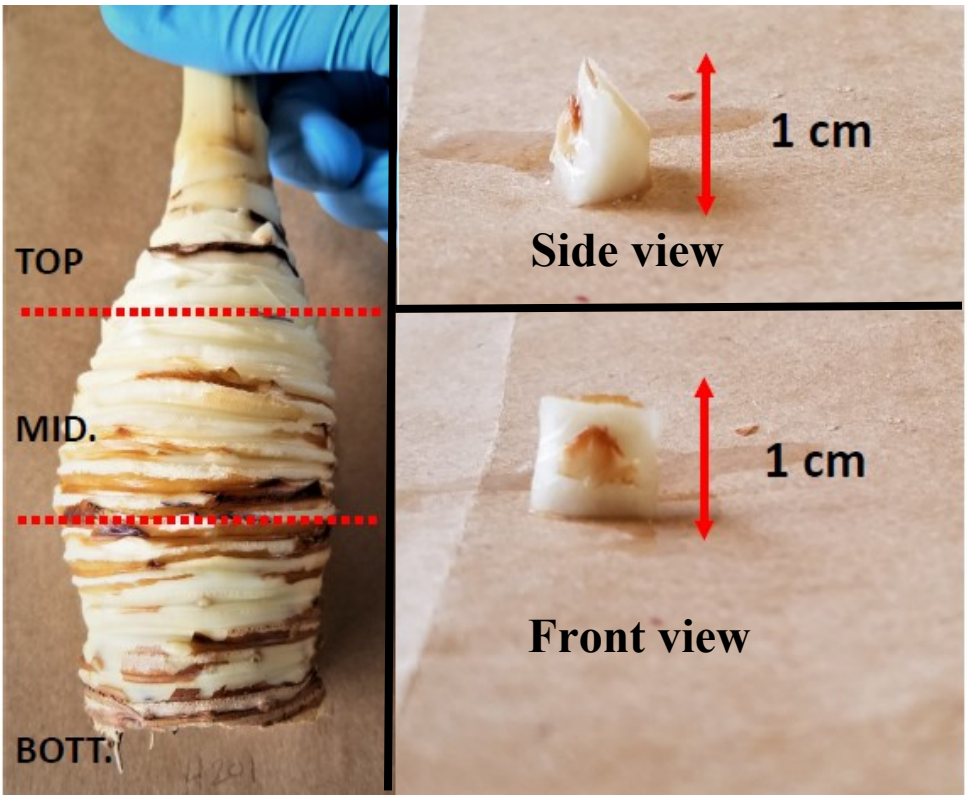


Fig. 3.1. Pineapple stem separated into top, middle, bottom (left) and bud explants dissected prior to surface sterilization (right).

2C.4.2. Bud Disinfestation

Surface disinfestation utilized a series of four concentrations of sodium hypochlorite (bleach)/sterile distilled water (SDW) diluted solutions containing 2 drops (100ml bleach solution) or 3 drops (200ml bleach solution) of Tween 20. All bleach solutions were freshly prepared before handling the mother plant. The first submersion consisted of 15% bleach (1.24% sodium hypochlorite) for 15 minutes and 10% (0.83% sodium hypochlorite) for 10 minutes. All bleach solutions were decanted immediately after each soak. Petri dishes with the disinfested buds were moved to the tissue culture room onto a benchtop sterilized with 80% ethanol. All buds were then triple rinsed with SDW and decanted to remove excess bleach residue, limiting over sterilization. Under a dissecting microscope, a 10.16cm sterile petri dish was placed over a

thin transparent plastic ruler (used for bud sizing) and was filled to a depth of approximately (3.175mm) with 5% bleach (0.41% sodium hypochlorite). Sterilizing the dissection tools by flame between each bud sample, forceps and scalpels was used to tediously dissect each bud while submerged in the 5% bleach solution. Each leaf sheath was removed one at a time until the 2nd or 3rd leaf primordia or apical meristem was reached. Upon leaf removal, the surrounding bud base was trimmed to form an explant size of 1mm or 2mm (Fig. 3.2) All buds 1mm or smaller were designated as 1mm while buds that were >1mm but ≤2mm were designated as 2mm. After sizing, buds were pooled together based on size and explant location into new 5.08cm sterile petri dishes slightly filled with SDW. Once all buds were processed, SDW from bud-pooled petri dishes was decanted and buds were immersed in 5% bleach for five minutes followed by a 1% bleach (0.08% sodium hypochlorite) soak for one hour and triple rinsed with SDW. If a shortage of time became a factor, buds were left to soak in SDW overnight and transferred to media the following day.

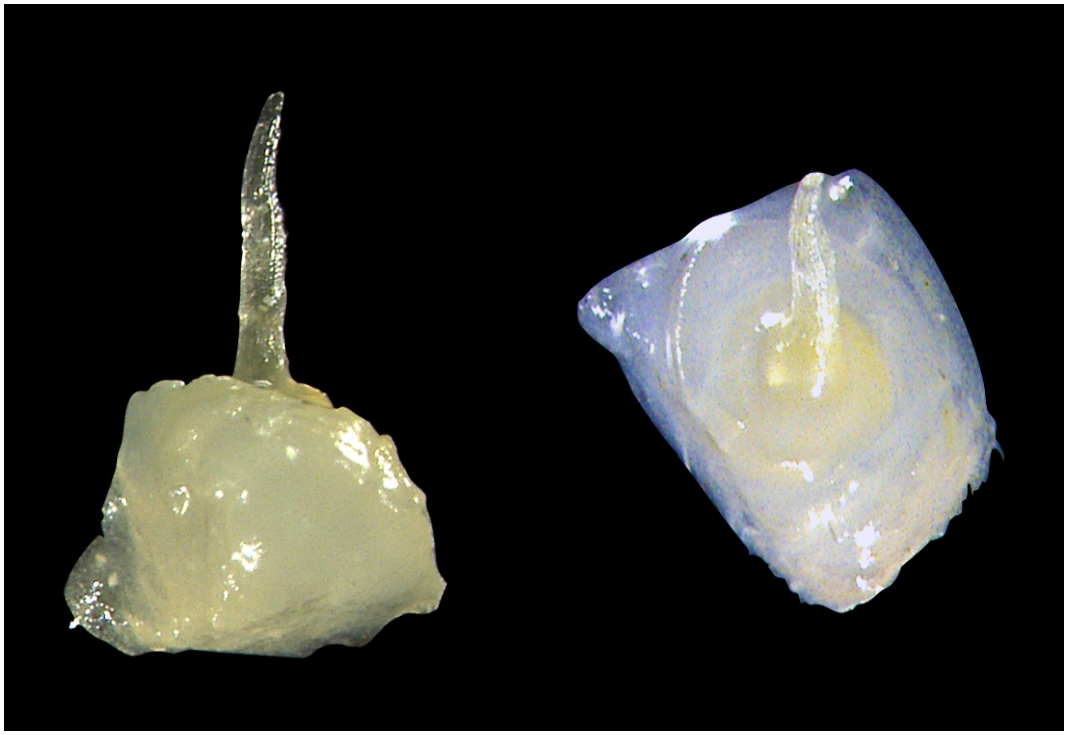


Fig. 3.2. 1mm pineapple meristem tip culture side (left) and top (right) view.

2C.4.3. Bud Media Initiation and Tissue Culture Plant Maintenance

Buds were transferred into sterile solid agar growth media containing Murashige and Skoog (MS) salts with benzyl adenine (BA) (Table 3.2) in a laminar flow hood. Each bud was planted at least 2/3 the depth of its height into individual test tubes (14mm) filled with approximately 15ml of growth media. Following planting, the top of the tubes and inner caps were briefly flame sterilized and sealed with parafilm. Each tube was labeled specifying the accession number, variety name, initiation date, transfer date, bud location, and bud initiation size. All buds were grown in a 16-hour light/8-hours dark photo period under Phillips F32T8 Daylight fluorescent bulbs at room temperature (~25°C). After one week all buds were inspected for bacteria or fungal contamination, or over sterilization. Dark colored buds due to oxidation of phenolic substances, or media with contamination were immediately removed. All contaminated tubes were autoclaved to prevent contaminants from entering other tubes. Remaining clean buds

were sub cultured into new media. Three weeks after the first subculture, buds were inspected for additional contamination, oxidation, and over sterilization. During this inspection phase, most healthy buds showed greening as buds began to swell and open while those that remained completely white, dark pigmented or translucent were deemed as over sterilized and/or dead.

Table 3.2. Pineapple tissue culture growth media with supplemental MS vitamin stock, and thiamine stock recipe.

Pineapple Growth Medium Recipe (500ml)	
Add 500mL of DI H20 to 1L glass beaker	
MS salts	2.15g
Sucrose	30g
MS vitamin stock	5ml
BA (stock 1mg/ml)	1ml
Thiamine Stock	4ml
Adjust to pH 5.7-5.8	
Agar per 500ml bottle	4.5g
MS vitamin stock (500ml)	
Add 500ml the following to DI water in 500ml Pyrex bottle:	
myo-inositol	0.10g
Glycine	0.020g
Nicotinic Acid	0.050g
Pyridoxine HCl	0.050g
Thiamine HCl	0.010g
Cover with foil and store at 4°C	
Thiamine Stock Solution (100ml)	
Add 0.010g of Thiamine HCl to 100ml of autoclaved DI water	

Once leaves emerged (Fig. 3.3), the plantlets were transferred into maintenance media (half strength of growth media except for MS vitamins, and excludes BA) to prevent or limit multiple shoots from forming.

Fig. 3.3. Growth stage a pineapple bud was sub cultured onto maintenance media.

2C.4.4. Leaf Sample Collection for PMWaV Retesting

Plantlets were continuously sub cultured until 100-150mg of leaf tissue (minimum five 2.54-3.81cm leaves) could be harvested for virus testing. Leaf harvests took place in a laminar flow hood during sub culturing. Half or more of plantlet leaves from each sample was harvested, diced into ≤0.5cm squares and pooled into a 1.5ml centrifuge tube containing RNAlater Solution (Ambion, Austin, TX). Leaf samples were stored at -80°C until RNA extraction. RT-PCR testing and gel electrophoresis procedures were repeated from sections *2B.2* and *2B.3.*

2C.4.5. Pineapple Thermotherapy Across Temperature Treatments

Growth rates at various temperatures (37°C, 35°C, 30°C, RT (25°C)) were used to treat pineapple tissue cultures to determine potential thermotherapy conditions where pineapples would be able to grow while potentially inhibiting viral RNA synthesis. Maui Gold pineapple

tissue culture plants were utilized for this study. To induce active growth, plantlets were sub cultured into fresh media and grown for 2 weeks before dissection. Whole mature plantlets were dissected into top and middle sections under aseptic conditions. Top sections were dissected to ~0.635cm in height consisting of an apical shoot and base. Middle sections comprised the remaining green section of the stem above the root system and were dissected to ~0.318cm in height (Fig. 3.4). Sections that were brown in color or containing roots were not used.

Fig. 3.4. Pineapple top (left)and middle (right) plant sections sized for in vitro thermo therapy treatment.

In addition to plantlet sections, young whole plants without roots were also treated for visual growth comparison. All plant samples were grown in individual test tubes. Top and young plantlets were planted in maintenance media while middle sections were planted in growth media containing BA to elicit plant growth. Throughout the entire treatment, none of the plant samples were sub cultured into fresh media. Each temperature treatment consisted of 10 top, middle, and whole young plantlets for a total of 30 plant samples per treatment totaling 120 samples for the overall experiment. All plant samples were sub cultured and placed into incubation chambers set at either 37°C, 35°C, 30°C, or RT on the same day and grown in the dark to enhance growth rates via etiolation. Plants were only exposed to light when documenting changes in height but were immediately placed back into their incubation chamber once completed. Using a ruler, plant heights were determined by measuring the explant base to their tallest point. Any roots that developed after initiation from all samples were not accounted for during measurements.

Measurements were taken every Monday and Thursday for a period of 57 days. In addition, photos were taken with a ruler beside the plantlets. To ensure measurements and pictures were taken from the same position, all tubes were snugly placed and grown in a modified foam tube rack with a mounted black background for enhanced visibility (Fig. 3.5). All measurements were documented to determine differences of average growth rates per day (mm/d) among the different plant sections across the four temperature treatments.

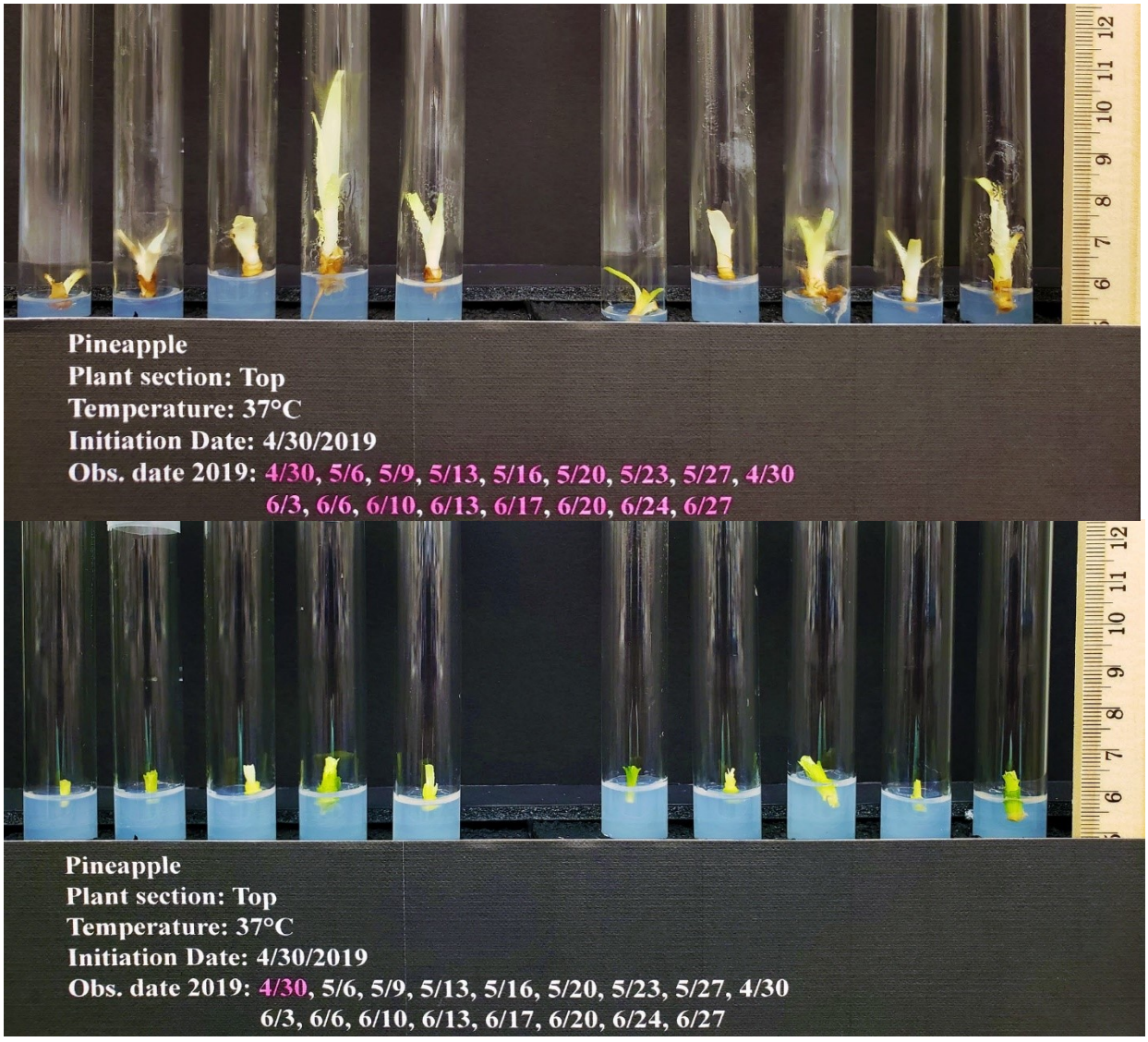


Fig. 3.5. Thermotherapy treated pineapple top sections comparing the first (bottom) and last (top) day of 37ºC treatment.

2D. Results

2D.1. Pineapple Sampling and Initial PMWaV Testing

The initial PMWaV screening consisted of testing 59 mother plants (50 different varieties) (Table 3.3). In this screening based on PMWaV being present individually or combined, PMWaV-1 was present in 41% of the plants followed by PMWaV-2 (64%), PMWaV-3 (42%), PMWaV-1 strain 4 (5%), while 7% tested negative. Comparing the number of viruses present, 49% contained single PMWaV infection while 51% had multiple. Eighteen mother plants with both replicates testing positive for the same or combination of PMWaVs were selected for meristem tip culture (Table 3.3). In addition, plants infected with three PMWaVs were selected to determine efficacy of meristem tip culture in removing PMWaVs. Among the 18 plants, 39% contained a single PMWaV while 61% contained multiple PMWaVs. The lone accession, H60 *Ananas comosus* var. comosus 'Spanish Guatemala' was positive for PMWaV-1,2,3, while H160 *Ananas comosus* var. comosus 53-116 was the only plant positive for PMWaV-1 strain 4. All plants used in this experiment are of the *Ananas comosus* genus species except for H181 N04-4 *Ananas macrodontes.* Throughout the duration of this project, none of the mother plants during the initial screening or tissue culture phase displayed MWP disease symptoms.

2D.2. Pineapple Bud Culture Survival Relative to Size and Location

Preliminary data on bud survival for the first two subcultures following initiation was documented to demonstrate some of the challenges faced when applying meristem tip culture. Bacterial and fungal contamination is a common problem during plant initiation and will always affect overall success rates for initiation. However, after the second subculture, or one month

after initiation, buds that turn green are more likely to survive to maturity. Factors such as explant size and location play a role in survival rates. Bud explants that were 1mm tend to be more susceptible to over sterilization while 2mm buds were more susceptible to contamination from internal or external sources. In the case of pineapples, buds that are closest to the top were more succulent in texture. This indicates top buds are more prone to absorbing bleach deep within their tissue during surface sterilizations resulting to buds having a soft mushy texture. Buds located on the bottom were smaller and dome shaped but not as active compared to higher buds. Middle explanted buds had a crisp firm texture making it easier to work with during the dissection phase. Overall, 399 buds were explanted and 193 (48%) survived. Comparing survival rates between sizes, 2mm buds (64%, N=124) had a 28% higher chance of surviving over 1mm (36%, N=69). The highest rate of bud survival 67%) was observed when 2mm buds were harvested from the top. Buds that were set aside to allow more time to green survived 3% of the time.

2D.3. Pineapple Bud Explant Initiation Size and PMWaV Presence

A total of 167 pineapple plantlets from 1mm and 2mm buds was retested post meristem tip culture treatment to determine an association between size and PMWaV presence. A total of 34 (1mm) and 133 (2mm) buds survived to the PMWaV retesting phase. Overall, 144 buds inclusive of both sizes were free of PMWaVs with 23 still infected for an 86% virus removal success rate. Pineapple plantlets initiated at 1mm had 33 that tested negative, with 1 positive for a 97% success rate. In contrast, 2mm buds had 111 that tested negative and 22 positives for an 83% success rate. The difference in efficiency between the sizes was 14%. More than 20% of expected frequencies within the contingency table was less than 5, a Fisher's exact test was

applied. Results revealed an association between bud explant initiation size and virus presence/absence ($P < 0.048$).

2D.4. Pineapple Bud Location and PMWaV Presence

The location of buds along the mother plant indicated as top, middle, and bottom was observed, and tested to determine an association between bud explant location and PMWaV presence. Based on bud location along the mother plant, 38 buds were harvested from the top, 61 from the middle, and 68 from the bottom. Plantlets that had buds explanted from the top resulted in 37 that tested negative versus 1 positive producing a 97% rate of buds being PMWaV-free. Middle buds had an 85% clean rate with 53 plantlets that tested negative and 8 positives. From the bottom location, 74% were free of PMWaV with 54 that tested negative and 14 positives. A chi-squared test was applied and concluded that an association exists between the location in which pineapple bud explants were harvested along the mother plant and PMWaV presence ($P < 0.035$).

2D.5. Pineapple Size, Bud Location, and PMWaV Presence

Results of this study have determined that the size and location of pineapple bud explants played a significant role in PMWaV presence or absence. By analyzing the two factors together and comparing it with PMWaV presence, the main contributing factor towards eliminating PMWaVs via meristem tip culture was the initiation explant size of 1mm. Buds 1mm in size harvested from the top and middle locations were 100% negative for PMWaV (N=19). Although buds harvested from the bottom location had a PMWaV virus free rate of 74%, when initiated at 1mm, 93% of the buds were clean (N=15). When initiating buds at 2mm, buds collected from the top were 97% clean (N=29). A slight drop-off was observed when 2mm buds were harvested

from the middle (84%, N=51). The lowest PMWaV-free rate (75%, N=53) was observed when buds were harvested from the bottom third of the mother plant and initiated at 2mm.

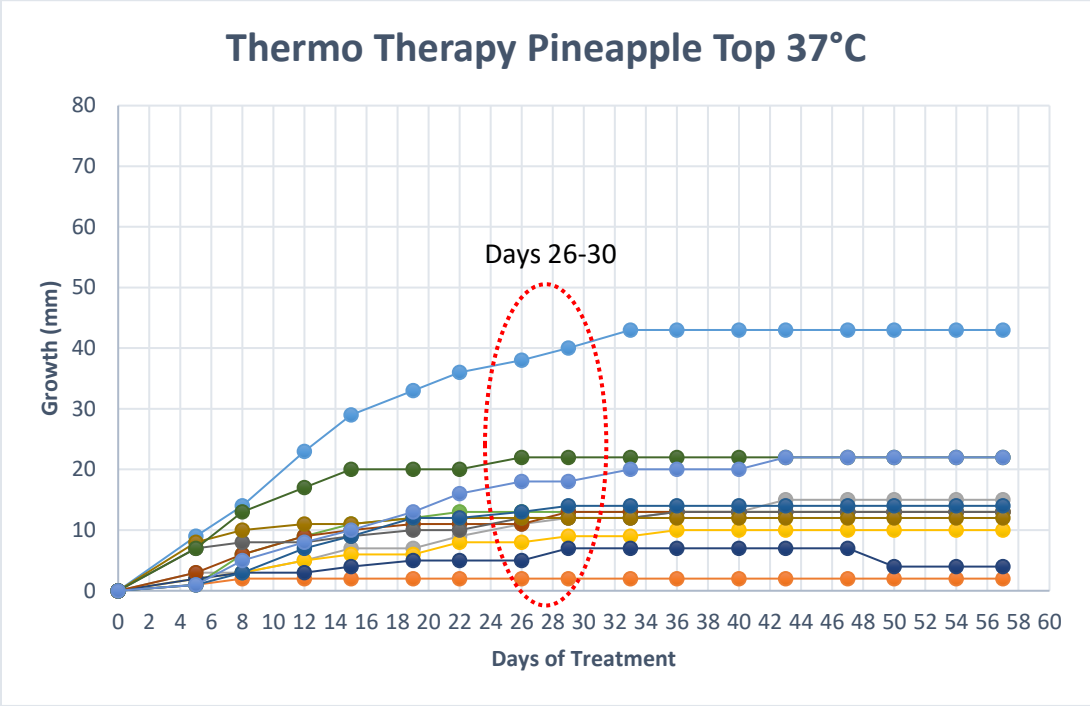
2D.6. Elimination Rates of Each PMWaV

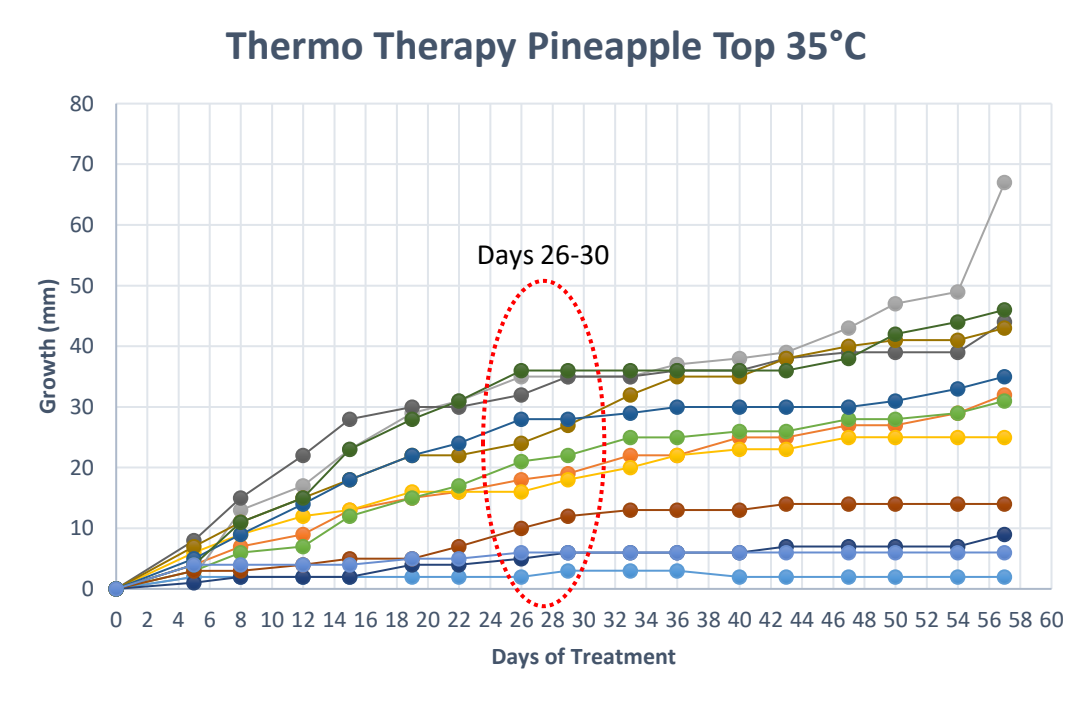
Results from meristem culture treatment and bud location varied when comparing efficacy rates of removal in relation to each species of PMWaV individually or combined. Among 167 meristem tip cultured plantlets, 96 were infected with PMWaV-1. A total of 14 ((7)-*2mm-Middle*, (7)-*2mm-Bottom*) remained positive after initiation into tissue culture resulting in an elimination rate of 85%. For PMWaV-2, a total of 81 plantlets were initially positive while 2 ((1)-*1mm-Bottom*, (1)-*2mm-Bottom*) were still infected for a removal rate of 98%. PMWaV-3 had 43 positive plantlets while 7 ((*1)-1mm-Top*, (3)-*2mm-Middle*, (3)-*2mm-Bottom*) remained positive for a success rate of 84%. Lastly 23 plantlets containing PMWaV-1 strain 4 had four ((4)-*2mm-Bottom*) remain positive resulting in an 83% elimination rate.

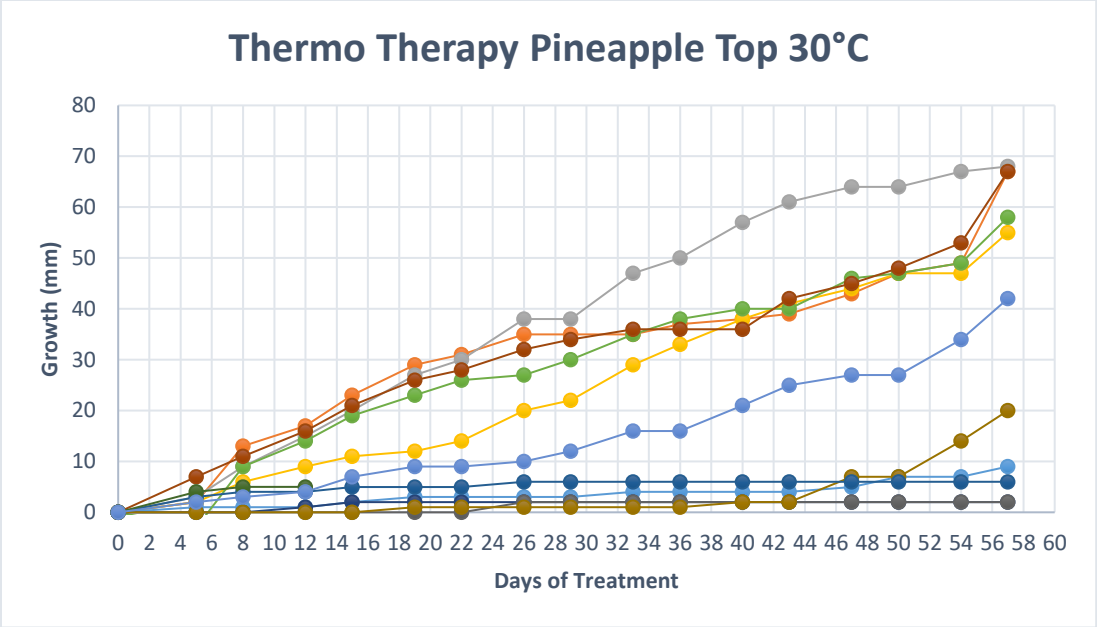
2D.7. Thermotherapy Growth Rates Across Temperature Treatment

The growth rates of pineapple explant sections from top and middle against temperature treatments (37°C, 35°C, 30°C, RT) were observed over a period of 57 days. Top sections grew fastest as an apical meristem was already established. A trend of faster growth rates was observed as temperature treatment decreased. At 37°C top sections grew slowly at a rate of (0.26mm/d) followed by (0.51mm/d) at 35°C, (0.62mm/d) at 30°C, and (0.65mm/d) at room temperature. When comparing middle section growth rates, almost no growth occurred at 37°C (0.01mm/d) followed by (0.04mm/d) at 35°C, (0.18mm/d), at 30°C and (0.11mm/d) at room temperature. Although top sections had the slowest growth rate at 37°C, a growth spurt was observed until the 30th-day of treatment before plateauing (Fig. 3.6). A similar trend was also

noted for top sections at 35°C however unlike 37°C, growth at 35°C, 30°C, RT were continuous throughout the experiment displaying long internodes resulting from etiolation.







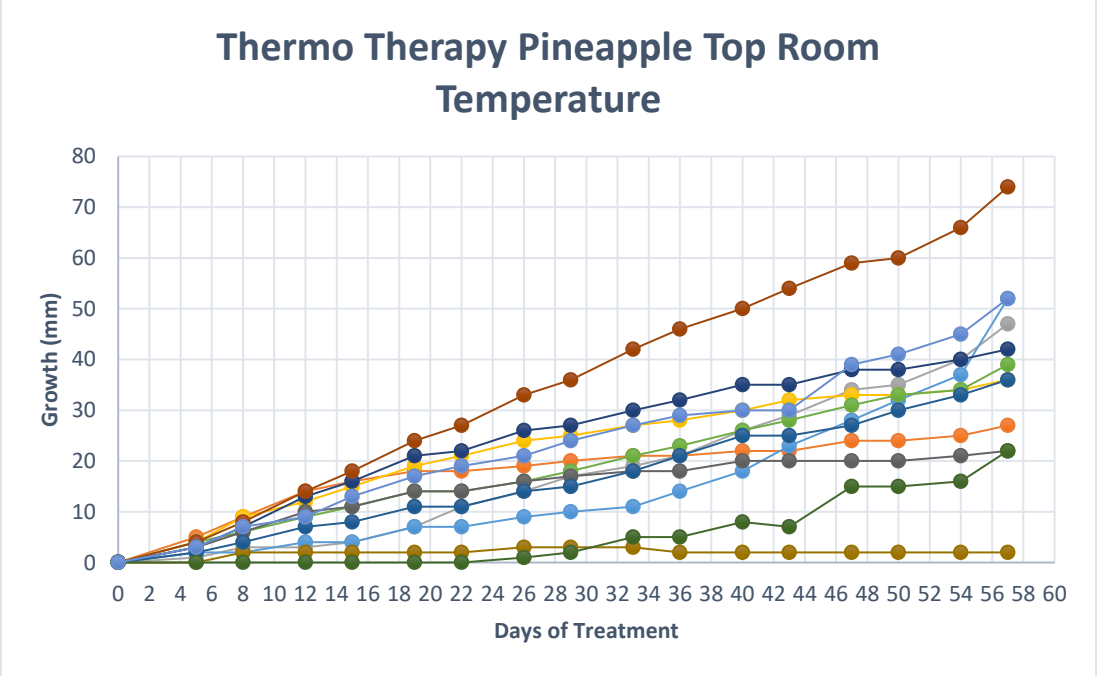


Fig. 3.6. Growth rates of thermo therapy treated (37°C, 35°C, 30°C, RT) pineapple top sections. Each line represents an individual plant. Red-dotted circles indicates the peak of initial growth spurts prior to plateauing.

2E. Discussion

This study was the first in Hawai`i to correlate bud explant size and elimination of single or multiple PMWaVs from pineapple plants. The initial screening for PMWaV among 58 asymptomatic *Ananas comosus* plants and one *Ananas macrodontes* showed the highest incidence (64%) for PMWaV-2 individually or combined with other PMWaV. This is less than previous results (85%) reported from (Hu et al. 2009). The second highest incidence (41%) was observed for PMWaV-1 compared to 78% from (Sether et al. 2001).

To determine meristem tip culture viral removal efficacy against each PMWaV (PMWaV-1, 2, 3, PMWaV-1 strain 4), infected plants were selected based on being infected with individual or mixed viruses. PMWaV-2 had the second highest incidence among all tissue cultured plants, but was most susceptible to elimination (98%). This is important as PMWaV-2 infection combined with mealybug presence causes MWP disease in Hawai`i (Sether et al. 2002) Although PMWaV-1 had the highest incidence it had similar elimination rates as PMWaV-3 and PMWaV-1 strain 4, ranging from 83-85%.

Trends in size and location were evident. As bud initiation size decreased, PMWaV elimination increased similar to trends described in Hall (1999). The highest PMWaV elimination rate (97%) was observed when buds were initiated at 1mm compared to 83% at 2mm. The 1mm rates were similar to those of Sether et al. (2001) when PMWaV-1 was eliminated from 92% of pineapple plants. However, results of this study contrast those of Souza et al. (2012) who observed a 50% success rate using 1mm apical meristem explants from in vitro pineapple plants.

Presence of PMWaV and bud explant location was also noted. Buds that were higher on the mother plant showed higher levels of PMWaV elimination. The highest PMWaV infection-free rate (97%) based on location and inclusive of both sizes came from buds explanted from the

top-third location. With a sample size of N=9, buds that were explanted from the top location and initiated at 1mm were 100% PMWaV-free. This result was also observed at 1mm middle buds (N=10) however, middle buds that were initiated at 2mm were 84% clean suggesting initiation size is more critical for eliminating PMWaVs. Based on visual observation, middle sections of both explant sizes tend to reach maturity faster. Therefore, sections and size must be taken into consideration when initiating buds from PMWaV infected plants.

Although initiating buds at 1mm was highly effective in eliminating PMWaVs, dissecting buds to achieve such size is a challenge that requires specialized skills. Besides being more prone to over sterilization, smaller bud sizes are highly susceptible to bud shearing or breaking in which the meristem is accidentally removed or damaged. In addition, top sections were smaller in diameter limiting the number of buds available. For these reasons, 1mm buds represented 20% of all buds used in this experiment. One month after initiation, 1mm buds excised from all locations on the pineapple stem had a 41% survival rate compared to 57% for 2mm buds. Nonetheless, surviving 1mm buds isolated from the top section of the pineapple plant had increased survival percentages. Buds located on the top section of the plant are younger, more actively growing, and are triangular shaped compared to round dome-capped shaped buds closer to the bottom.

Top sections in thermotherapy displayed a higher growth rate (0.51mm/day) compared to middle sections (0.085mm/day). This is attributed to the presence of actively growing apical meristems versus dormant axillary meristems in the middle section. As temperature treatments increased, decreases in growth rates were observed, similar to trends described by (Hall 1999). At 37°C top section growth rates were less at (0.26mm/d) followed by (0.51mm/d) at 35°C, (0.62mm/d) at 30°C, and (0.65mm/d) at room temperature (25°C). In the past, pineapple buds

from crowns survived treatment temperatures ranging from 35-58°C for periods ranging from 40 minutes to 24 hours with minimal success in eliminating PMWaV-1 (Sether et al. 2001). In this experiment, pineapple tissue cultured top sections were shown to be able to etiolate and grow at 37°C during their initial growth spurt up to 30 days. Ideally, higher temperature treatment will have a stronger impact in arresting plant viral RNA replication (Sastry & A. Zitter 2014). In the event that pineapples placed into tissue culture test positive for PMWaV, by dissecting the top sections to a height of approximately 6.35mm they could be initiated and treated at 37°C. After 26-30 days, the newest growth section would then be harvested and transplanted into fresh media in hopes of obtaining PMWaV-free plants.

The ideal method to obtain PMWaV-free plants based on the results of this experiment would be to initiate pineapple buds 1mm in size taken from the top and middle sections of the stem. Although achieving 1mm buds appear initially inefficient due to lower survival rates, most of the culling would take place in the earlier stages of post initiation. Having a higher percentage of clean plants on hand, other tissue culture multiplication procedures could be utilized to rapidly produce and provide clean plant material for farmers, researchers, and conservationists. Considering meristem tip cultured plants may take up to one year to mature (depending on variety), the time, effort, and materials needed to make media, sub culture every 21 days, in addition to the extra dishware would be considered inefficient only to discover potentially 25% of the plant population is still infected. In this event the positive population could be treated with thermo therapy at 37°C for 30 days and have their newest growth transferred to new media. Although the plant sections were able to grow at 37°C, the PMWaV elimination rate among the various treatment temperatures have yet to be determined. In the case that virus titers may be too low for RT-PCR detection, utilizing more sensitive detection practices such as real-time

quantitative PCR could be incorporated. Probes and primers have been developed for PMWaV 1, 2, 3 shown to have 10-1000 increased sensitivity compared to RT-PCR (Chang et al. 2015; Subere et al. 2011; Hu et al. 2015) which would enhance the credibility of detection results. Considering PMWaV-4 was recently confirmed to be a strain of PMWaV-1 named "PMWaV-1 strain 4" instead of a separate species (Green et al. 2019), the need for a PMWaV-4 probe and primer may not be needed.

Table 3.3. Pineapple accessions initially screened and used for meristem tip culture (green). Key abbreviations: SL (slip); MP (mother plant); HP (hapa)

Hana No.	Genus, Species	Leaf Sample location	PMWaV Results
45	*Ananas comomus var. comosus*	SL	1,2,3
158	*Ananas comomus var. comosus*	MP	2,3
160	*Ananas comomus var. comosus*	MP	4
187	*Ananas comomus var. comosus*	MP	1,2
209	*Ananas comomus var. comosus*	MP	2
235	*Ananas comomus var. comosus*	SL	3
160	*Ananas comomus var. comosus*	SL	4
233	*Ananas comomus var. comosus*	SL	1,2
40	*Ananas comomus var. comosus*	SL	1,2
213	*Ananas comomus var. comosus*	SL	1,2
130	*Ananas comomus var. comosus*	SL	1,2
25	*Ananas comomus var. comosus*	SL	1,2,3
27	*Ananas comomus var. comosus*	SL	3
56	*Ananas comomus var. comosus*	SL	1,3
140	*Ananas comomus var. comosus*	SL	1,2
161	*Ananas comomus var. comosus*	SL	2

211	*Ananas comomus var. comosus*	SL	2
12	*Ananas comomus var. comosus*	SL	2,3
42	*Ananas comomus var. comosus*	SL	2
54	*Ananas comomus var. comosus*	SL	1,3
71	*Ananas comomus var. comosus*	SL	3
110	*Ananas comomus var. comosus*	SL	1,2,3
140	*Ananas comomus var. comosus*	SL	1,2
212	*Ananas comomus var. comosus*	HP	1,2
208	*Ananas comomus var. comosus*	HP	2
216	*Ananas comomus var. comosus*	SL	1,2
25	*Ananas comomus var. comosus*	SL	1,2,3
42	*Ananas comomus var. comosus*	SL	2
43	*Ananas comomus var. comosus*	SL	1,3
57	*Ananas comomus var. comosus*	SL	1,3
126	*Ananas comomus var. comosus*	SL	Negative
177	*Ananas comomus var. comosus*	SL	1,3
226	*Ananas comomus var. comosus*	SL	1,2
230	*Ananas comomus var. comosus*	HP	2,3
1	*Ananas comomus var. comosus*	SL	2
2	*Ananas comomus var. comosus*	SL	3
3	*Ananas comomus var. comosus*	SL	2
27	*Ananas comomus var. comosus*	SK	2,3
56	*Ananas comomus var. comosus*	SL	1,3
129	*Ananas comomus var. comosus*	HP	3
189	*Ananas comomus var. comosus*	SL	1,2,3

63	*Ananas comomus var. comosus*	SL	Negative
181	*Ananas macrodontes*	HP	2,3
201	*Ananas comomus var. comosus*	SL	2
205	*Ananas comomus var. comosus*	SL	1
17	*Ananas comomus var. comosus*	SL	1,2
39	*Ananas comomus var. comosus*	SL	2,3
60	*Ananas comomus var. comosus*	SL	1,2,3
62	*Ananas comomus var. comosus*	SL	3
6	*Ananas comomus var. comosus*	SL	3
42	*Ananas comomus var. comosus*	SL	Negative
79	*Ananas comomus var. comosus*	SL	2,3
152	*Ananas comomus var. comosus*	SL	2
169	*Ananas comomus var. comosus*	SL	2
187	*Ananas comomus var. comosus*	SL	2
7	*Ananas comomus var. comosus*	SL	Negative
11	*Ananas comomus var. comosus*	SL	Negative
94	*Ananas comomus var. comosus*	SL	1
209	*Ananas comomus var. comosus*	SL	Negative

CHAPTER 3: APPLYING IN VITRO METHODS MERISTEM TIP CULTURE FOLLOWED BY THERMO THERAPY TO ELIMINATE VIRUSES PRESENT IN TARO (*COLOCASIA ESCULENTA)*

Abstract

Taro vein chlorosis virus (TaVCV) was recently found in Hawai‘i, and can cause severe symptoms and crop loss. Meristem tip culture was conducted using different bud explant sizes (1mm and 2mm) and different bud locations along the mother plant (top, and middle-bottom) to determine efficacy in eliminating TaVCV. In addition, overall bud survival rates during early stages of meristem tip culture was observed. Thermo therapy treatment was conducted, to measure the growth rates of tissue cultured taro plant sections (top and middle) against temperature treatments (37°C, 35°C, 30°C, RT (25°C)), to provide options for future treatment of infected taro towards establishing disease free plant material. For taro, initiating 2mm bud explant sizes was as effective as initiating 1mm explants in eliminating TaVCV ($P > 0.25$), exclusive of taro varieties (3) and bud location. Both explant sizes were able to rid TaVCV from 97% of all plants maintained in vitro after testing at >6months. No correlation between bud location and TaVCV presence was detected ($P > 1$). However, correlation between taro varieties (Mana Ulu, Purple, and Purple 2) and TaVCV presence was observed ($P < 0.046$). Purple-2 had all (100%, N=13) buds tested negative while the Purple variety had (98.5%, N=67) free of TaVCV. Variety Mana Ulu had the lowest rate of clean plants (77.7%, N=11). Bud survival rates among 1mm and 2mm buds for the first month were nearly identical at 89% and 90% however, buds harvested from the top location had an 8.5% higher survival rate. At 35°C, taro explants grew 0.58mm/day, while at 30°C plants grew 1.52mm/day.

Introduction

Viruses present in important crops such as taro (*Colocasia esculenta*) decrease plant growth, the abundance of corm yield, and overall crop production (Babu et al. 2001; Jackson et al. 2001). In addition, it also impedes the international movement of taro cultivars (Revill et al. 2005). Farmers are also impacted by decreases in profit due to subpar produce quality as a consequence of virus infection. Lower taro yields affect the culture and diet of Pacific Island regions and countries because taro is a significant component of their diet and cultural practices (Deo et al. 2009). In 2018, Hawai‘i produced 2.99 million pounds of taro representing a 1.97-million-dollar industry (Quickstats.nass.usda.gov, 2020). Currently only 70 (69 native to Hawai‘i) of 150 Hawaiian landraces remain in Hawai‘i (Helmkampf et al. 2017). The elimination of viruses in taro crops are important to minimize the negative economic and cultural loss of uniquely significant taro varieties.

Taro vein chlorosis virus (TaVCV) belongs to the family *Rhabdoviridae* and genus *Nucleorhabdovirus* (Revill et al. 2005; Deo et al. 2009). Common morphological structures of rhabdovirus consist of bullet-shaped or bacilliform particles approximately 200 x 70nm in size. (Revill et al. 2005; Lamprecht et al. 2008). TaVCV has been detected in Papua New Guinea, Palau, Fiji, Micronesia, New Caledonia, Vanuatu, Tuvalu (Carmichael et al. 2008), the Hawaiian Islands in 2013 (Long et al 2014), and most recently American Samoa (Atibalentja et al, 2018). Taro vein chlorosis symptoms are typically observed on mature infected taro plants (Carmichael et al. 2008). Mature leaves predominantly displayed foliar vein chlorosis that eventually spreads between leaf veins ultimately forming a network of yellow streaks. Other symptoms include leaves that are torn and or necrotic (Carmichael et al. 2008) which is an unmarketable trait for the taro industry. The virus is not mechanically transmitted between plants and confirmed vectors of TaVCV have not been identified. However, leafhoppers and planthoppers are

suspected as other studies demonstrate them to vector other *Nucleorhabdoviruses* such as Colocasia bobone disease virus (CBDV) (Revill et al. 2005; Lamprecht et al. 2008).

In this work, meristem culture is the primary method for removing taro vein chlorosis virus from taro plants. It is a form of in vitro propagation in which the meristem apex consisting of the youngest leaf primordia of a plant is excised (Hall 1999). In doing so, viral, fungal, and bacterial diseases can be removed by separating potentially infected outer layer tissue from newly formed cells at the apical meristem (Hall 1999; Rabinowitch & Currah 2002). Meristem propagative sizes may range from <1mm–5mm or greater depending on crops, as some crops may not need to be excised at very small sizes to eliminate targeted diseases. Excised buds at a size of 1mm or less increases the abundance of yielding "virus free" plants, however it also has a lower survival rate compared to larger excised meristem sizes (Hall 1999; Sastry & A. Zitter 2014; Verma et al. 2004). Some factors dictating viral elimination can be attributed to the type of virus being eliminated, the physiological condition of the mother plant, and the locality of the meristem along the mother plant (Verma et al. 2004). Attaining meristem sizes of ≤1 mm is a tedious process that requires skill and dexterity while incorporating aseptic technique. The application of meristem tip culture on taro has been effective in removing DsMV in some varieties of taro leading to more than double the yield of tuber production and decreased reinfection rates compared to vegetative propagated counter parts (Jackson et al. 2001). Thus, identifying the optimum size for meristem culture that eliminates virus infection at a reasonable survival rate would improve protocols specific for taro to provide clean plant material at large scale quantities for conservationists, farmers, researchers, and stakeholders.

At 30°C or higher, most plant viruses have their replication abilities arrested or hindered (Sastry & A. Zitter 2014). Applying high temperatures to plants decreases the rate of survival

while increasing the amount of “virus free” plants produced by inhibiting viral RNA synthesis (Ali et al. 2014). Constraining viral RNA synthesis by RNA degradation is associated with RNA silencing mechanisms naturally found within plants and has a tendency to increase with temperature (Wang et al. 2008). Tradeoffs between plant survival and “virus free” plants were taken into consideration when determining appropriate thermo therapy temperatures. Plants grown in higher temperatures are more likely to be “virus free” but at higher risk of dying due to high temperature stress. In previous studies, the application of thermo therapy alone as a virus elimination treatment has shown to have little effectiveness in taro. Taro corms left out to dry for a month treated at 38°C for 30 days until shoots grew to a height of 2-3cm prior to tissue culture propagation, resulted in the survival of 28% “virus free” plants (Yongwei et al. 2002). The percentage yield of obtaining clean plant material differs among plants but is typically less than 50%. This highlights the need to increase efficiency in obtaining “virus free” plants for further propagation.

Utilizing meristem culture followed by thermo therapy in garlic grown at 37°C for 35 days showed an overall virus elimination efficiency of 54% (Torres et al. 2000). Meristems grown at 40°C showed a decrease in plant survival but had a 90% rate of “virus free” plant yield demonstrating the tradeoffs between plant quantity versus quality (Torres et al. 2000). A similar pattern is seen when heat treating three varieties of potato post meristem culture. Control groups observed at 27 had the highest plant growth rate 21-28% with a low 14-15% rate of “virus free” plants (Ali et al. 2013). At 30°C plant survival ranged from 18-26% with a “virus free” plant rate of 38-39%. The highest “virus free” success rate of 43% occurred when the plants were treated at 35°C however plant survival rates were the lowest ranging from 17-23% (Ali et al. 2013). The

difference of “virus free” obtained plants among studies demonstrates how different plants vary in levels of virus removal and crop survival success.

Methods such as meristem culture, thermo therapy, or a combination of the two on certain crops has been shown to eliminate viruses at varying rates of efficiency. This study will be the first in Hawai`i to evaluate the effectiveness of applying quantified meristem tip culture and provide in vitro thermo therapy growth rates to improve production of “virus free” taro plants compared to other methods. Information derived from this study can play an integral part in establishing uninfected taro plant material that will benefit researchers, conservationists, farmers and the agricultural community as a whole.

3B. Materials and Methods

3B.1. Leaf Sample Collection and RNA Extraction

Ten TaVCV symptomatic taro mother plants were harvested from a botanical garden taro patch located in Hilo Hawai`i. Vein chlorosis symptoms were evident on the leaves of what appeared to be three different taro varieties. These samples were tested for the presence of TaVCV and DsMV. According to the taro patch groundskeeper, the green stem variety was Mana Ulu, while the other two purple/brown stem varieties were of the lehua group. For this experiment they will be referred to as Purple (dark purple petiole) and Purple-2 (light purple petiole). Collection of leaf samples were carried out utilizing resealable plastic bags prelabeled with the plant’s variety name, plant number, and location within the patch. In addition, the base of the plants that had leaf samples taken were also flagged by tying a ribbon around the base labeled with its plant number corresponding to the plastic bag. The collection bags were turned inside out serving as a glove and was used to remove and pool small samples from the first and second youngest leaves excess of 150mg to increase the chances of yielding quality RNA from

each plant. Immediately after harvest, each bag containing leaf samples were placed into a cooler with ice to maintain sample integrity before testing. Testing of the leaves was done on the same day as the harvest utilizing the RNAqueous® Kit, (Ambion, Austin, TX) with Plant RNA Isolation Aid (Thermo Fisher Scientific, Waltham, MA). Leaf tissue from both leaves were pooled together for a combined mass of 150mg and were homogenized using liquid nitrogen. Once ground to a fine powder while making sure the mortar was not too cold to freeze liquids, 800µl of Lysis Binding Solution and 100µl Plant RNA Isolation Aid was added to the mortar and further homogenized. The homogenate was then poured into a 2ml sterile centrifuge tube and placed in ice until all samples were processed. Following the standard RNAqueous extraction procedures, 64% ethanol was added to each sample tube followed by slightly mixing the solution by gently inverting the tubes several times. A fiberglass filter-clogging thick dark green tacky precipitate developed, so the sample solution was centrifuged for 2 minutes at a speed of 14,500g to separate the precipitate from the rest of the light green solution and transferred into a new sterile 1.5ml centrifuge tube. The rest of the RNA extraction procedure was done following the RNAqueous® Kit standard protocol and eluted three times at volumes of 15µl, 10µl, 10µl for a total of 35µl into a final collection tube. Following RNA extraction, samples were stored at -80°C or quantified using a Qubit Fluorometer (Invitrogen Carlsbad, CA) prior to the reverse transcription/cDNA synthesis phase.

3B.2. cDNA Synthesis

cDNA synthesis and PCR procedures followed (Gosai 2016). From each RNA sample 2µl was aliquoted into a 0.2ml (PCR) thin walled tube (Thermo Scientific) combined with 6.5µl (NF) water (Qiagen, Hilden, Germany), 1µl of random primer (10µM) (470 (5'-GCC GGA GCT CTG CAG AAT TCN NNN NN-3')) and incubated at 70°C for 8-10 minutes in a thermal

cycler (MJ Research Inc. Waltham, MA) and immediately chilled on ice. During incubation, the RT master mix was prepared including 5µl of dNTPs (2mM) (Invitrogen), 4µl M-MLV 5X RT Buffer (Promega, Madison, WI), 0.5µl RNasin (40u/µl) (Promega, Madison, WI), and 1µl MMLV RT (5u/µl) (Promega, Madison, WI). The RT master mix was gently mixed and briefly centrifuged. 10.5µl of RT master mix was added to each sample for a total volume of 20µl. The samples were once again gently mixed and briefly spun down prior to the second incubation of 25°C for five minutes, 42°C for 55 minutes, followed by 4°C indefinitely. Samples were retrieved within 18 hours.

3B.3. Polymerase Chain Reaction

During RT incubation, the PCR master mix was prepared for TaVCV detection. The following reagents were added to each sample; 10µl 2X GoTaq Green Master Mix (Promega, Madison, WI), 7µl NF water, 1µl (10µM) of each forward and reverse primers (Table 4.1) targeting TaVCV's RNA-dependent RNA polymerase protein gene. Into each tube, 19µl of PCR master mix was aliquoted. Lastly 1µl of cDNA was added to each tube. PCR cycling parameters applied were (step1) 95ºC for the first seven minutes, followed by (step 2) 95ºC for 30 seconds, (step 3) 60ºC for 30 seconds, (step 4) 72ºC for 1 minute, back to (step 2) for 35 cycles, finishing off with a final extension of 72 ºC for five minutes, and 4 ºC indefinitely.

Table 4.1. Primers targeting TaVCV-RdRp genes used for virus detection in this experiment.

Target Virus	Primer	Sequence (5' → 3')	Amplicon Size (nt)
TaVCV	DCGF4	ATC CAG GAT ATT CCC CTT GG	240
	DCGR4	CTC TTT CCC GCT CTT GTC AC	
TaVCV	DCGF5	AGG GGY TGA GRC AAA AGG GGT	442
	DCGR5	CGC TCY TTC ATA CAT GCS GCC TT	

3B.4. Gel Electrophoresis

Gel electrophoresis was conducted using 1.5% UltraPure™ Agarose (Invitrogen) in 1x TAE buffer (Omega Bio Tek, Norcross, GA) (mass/volume). From each sample, 10 µl of PCR-product mixed with 2µl loading dye (VWR EZ-Vision One, Radnor, PA) was loaded into a well and run at 92 volts (Thermo EC Mod: EC105-115) for 25-30 minutes. Band presence within gels were observed using ultra violet imaging (UVP BioDoc-It Imaging System). Mother plants that had samples positive for a gel band present at 240 or 442 base pairs were selected to move forward to the meristem culture initiation phase.

3C. Taro Meristem Tip Culture and TaVCV Retesting

3C.1. Taro Mother Plant Harvest and Meristem Tip Culture Preparation

A total of ten taro corms positive for TaVCV were processed for meristem tip culture. The plants were harvested and rinsed with water to remove loose debris and soil. All roots and dead petioles were removed, while fully but attached whorls were trimmed to a height of 7.62cm and left intact. Using a mild soap detergent and a soft bristled scrubbing brush, additional cleaning of the corms was done by applying brush strokes in the same direction that bud tips

were pointing to prevent bud breakage. This was followed by another rinse to remove debris and soap. A 10% bleach solution was made to surface sterilize all corms in a clean bucket to fully submerge the tallest corm (petiole included) for 5 minutes while swirling occasionally. Without rinsing off the bleach, all corms were laid out to dry. As a precaution to prevent over drying that could lead to a soft bud texture during the dissection phase, corms were dried in an indoor air-conditioned environment but only if the corms were to be initiated into the media on the second day of drying. Corms that were scheduled to be initiated later than two drying days were air dried under a shaded outdoor benchtop.

3C.2 Bud Explanting

Buds located under intact/attached petioles on the corm or cormels were grouped as top section buds while all other buds were grouped in the middle-bottom location (Fig. 4.1).

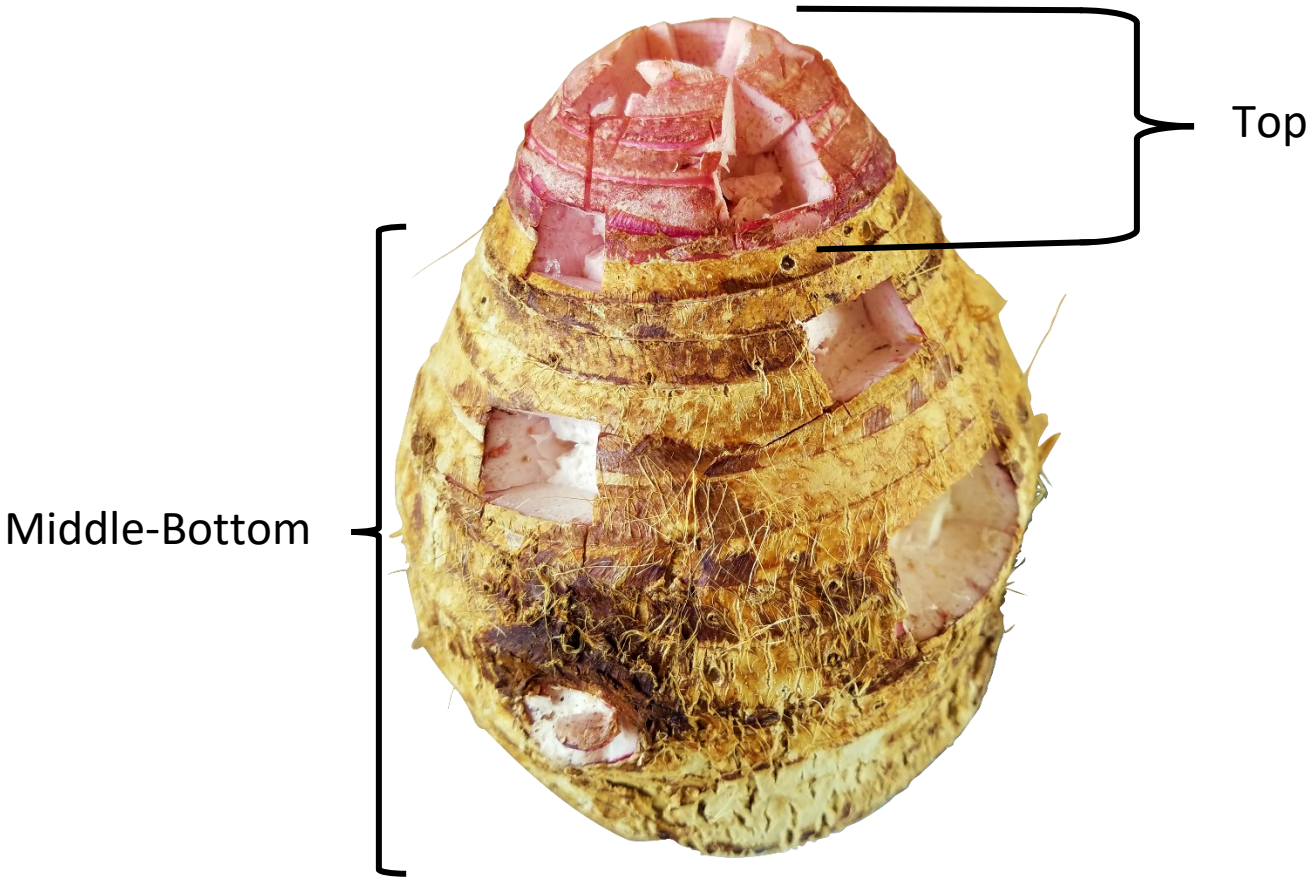


Fig. 4.1. Indication of bud harvest locations top and middle-bottom along a taro mother plant corm.

Each bud was initially trimmed as $2cm^3$ cubes using a steel razor blade. Switching to a scalpel, two to three bud leaf layers were removed in addition to trimming off tissue exposed to soil or brown in color. The cube was further trimmed to approximately 1cm in size with 5 flat sides (except bud face) for easier handling during the dissection phase. The buds were placed into a plastic beaker labeled with bud location and filled with deionized water to allow the buds to keep moist while dissecting the rest of the buds from the mother plant. All dissected buds were then quadruple rinsed with DI water to remove excess debris and placed into sterile 5.08cm Pyrex petri dishes for bud disinfestation (Fig. 4.2).

Fig. 4.2. Taro bud explant dissection prior to surface sterilization.

3C.3. Taro Bud Disinfestation

All buds were processed through a series of surface disinfestation by fully submerging them in 15%, 10%, 5%, and 1% bleach solutions diluted with SDW and two drops of tween 20 if preparing a 100ml solution or three drops for a 200ml solution. All bleach solutions were freshly prepared on the same day as initiation. The first bleach soak consisted of 15% bleach (1.24% sodium hypochlorite) for 15 minutes followed by 10% (0.83% sodium hypochlorite) for 10 minutes. Following each soak, bleach solutions were immediately decanted to prevent over sterilization. Petri dishes with the buds were transferred into a tissue culture room onto a benchtop surface sterilized with 80% ethanol. All buds were then triple rinsed with SDW and decanted to remove excess bleach. Under a dissecting microscope, a 10.16cm sterile petri dish was placed over a thin transparent plastic ruler (used for bud sizing) with 5% bleach (0.41% sodium hypochlorite) filled to a depth of ~3.175mm. Flame sterilizing between each bud sample,

sterile forceps and scalpels was used to remove bud sheaths while submerged in the 5% bleach solution. Sequentially each bud leaf sheath was removed one at a time until the 2nd or 3rd leaf primordia or apical meristem was reached. The surrounding bud base was trimmed to form an explant size of 1mm or 2mm (Fig. 4.3). All buds 1mm or smaller were designated as 1mm while buds that were >1mm but ≤2mm were designated as 2mm.

Fig. 4.3. Taro 2mm meristem tip culture.

After sizing, buds were pooled together based on size and explant location into new sterile petri dishes slightly filled with SDW. At this stage buds were kept moist until all buds were processed. Once completed, SDW from bud-pooled petri dishes were decanted and all buds underwent further sterilization utilizing the same petri dish. Pooled buds were immersed in 5% bleach for five minutes followed by a 1% bleach soak (0.08% sodium hypochlorite) for one hour

and triple rinsed with SDW. At this stage, buds were soaked in SDW overnight and transferred to media the following day.

3C.4. Bud Media Initiation and TC Plant Maintenance

Bud initiation into sterile solid agar growth media containing Murashige and Skoog salts with benzyl adenine (BA) (Table 4.2) was done in a laminar flow hood. Each bud was planted at least 2/3 the depth of its height into individual test tubes (22mm) filled with approximately 15ml of growth media. Following planting, the top of the tubes and inner caps were briefly flame sterilized before capping followed by a parafilm seal. Each tube was labeled specifying the bud's mother plant variety name, initiation date, transfer date, bud location, and bud initiation size. All buds were grown in a 16-hour light/8-hour dark photo period under a Phillips F32T8 Daylight fluorescent bulb at room temperature. After one week all buds were inspected for bacterial and fungal contamination, or over sterilization. Media with contamination were immediately removed and autoclaved to prevent contamination of other tubes. All clean buds were sub cultured into a different sterile tube. Three weeks after the first subculture, buds were inspected for additional contamination, phenolic oxidation (dark pigmented buds), in addition to over sterilization. During this inspection phase, most healthy buds showed greening as buds began to swell and open while those that remained completely white, dark pigmented or translucent were considered as over sterilized and/or dead.

Table 4.2. Taro growth media used for meristem tip culture bud initiation.

Taro Growth Medium Recipe (500ml)	
Add 500mL of DI H20 to 1L glass beaker	
MS salts	2.15g
Sucrose	15g
MS vitamin stock	5ml
BA (stock 1 mg/ml)	1.25ml
Adjust to pH 5.7-5.8	
Agar per 500ml bottle	4.5g
MS vitamin stock (500ml)	
Add 500ml the following to DI water in 500ml Pyrex bottle:	
myo-inositol	0.10g
Glycine	0.020g
Nicotinic Acid	0.050g
Pyridoxine HCl	0.050g
Thiamine HCl	0.010g
Cover with foil and store at 4°C	

Once leaves began to develop and take shape, the plantlets were transferred into maintenance media (same as growth media excluding BA) to prevent or limit multiple shoots from forming. Growth rates varied among buds based on size, bud location, plant variety or a combination of these factors.

3C.5. Taro In Vitro Leaf Sample Collection for TaVCV Testing

Plantlets were continuously sub cultured until 100-150mg of leaf tissue (minimum two 1.90cm -2.54cm leaves) could be harvested for virus testing. In most cases, 2-3 taro leaves were present on in vitro plants. Petioles or in some cases root tissue were also harvested when leaf tissue was limited. Plant tissue harvests took place in a laminar flow hood during sub culturing. Collected plant tissue was diced into ≤0.5cm squares and pooled into a 1.5ml centrifuge tube

containing RNAlater Solution (Ambion, Austin, TX). All samples were stored in -80°C until ready for testing. RT-PCR testing and gel electrophoresis procedures were as described in sections *3B.2–3B.4.*

3C.6. Taro Thermotherapy

Previous studies utilizing thermo therapy on taro have been done prior to tissue culture initiation with poor success in eliminating targeted viruses. This study was done to determine taro meristem tip culture growth rates at various temperatures (37°C, 35°C, 30°C, RT (25°C) to evaluate upon future heat treatments in which taro would be able to grow, while inhibiting viral RNA synthesis. Taro plants treated in this experiment consisted of the varieties Mana Ulu, Purple, and Purple-2 that tested negative for TaVCV and DsMV. All taro plants selected for thermotherapy treatment was mature, actively growing, and given two weeks after their last sub culture to acclimate prior to dissection. For this experiment, top sections were harvested under aseptic conditions taking place in a fume hood. To accurately quantify growth rates and establish a standard baseline, top sections were dissected approximately 5mm in total height consisting of its terminal shoot (~3mm) and base of ≤2mm (Fig. 4.4).

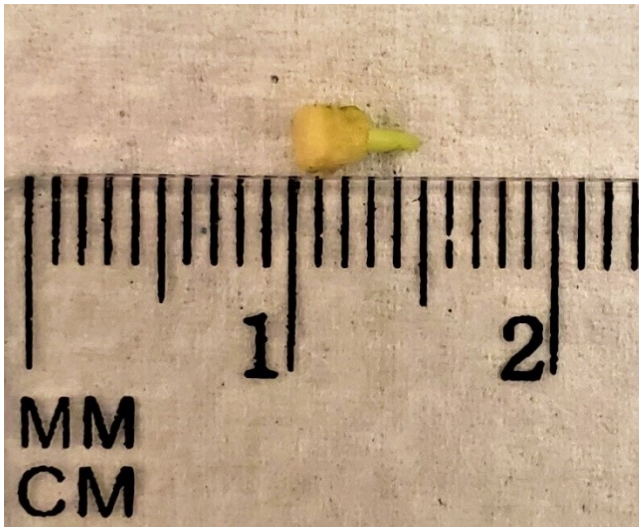


Fig. 4.4. Taro bud thermo therapy initiation size (0.5cm).

All plant samples were individually grown in test tubes (16mm) containing taro maintenance media. Throughout the entire treatment, none of the plant samples were sub cultured into fresh media. Each temperature treatment consisted of 10 top sections for a total of 40 plant samples overall. All plant samples were initiated and placed in their corresponding incubation chambers on the same day and grown without light to enhance growth rates via etiolation. Plants were only exposed to light when documenting changes in height. Using a ruler, plant heights were determined by measuring the explant base to their tallest point. Any roots that developed after initiation were not included in plant height measurements. Measurements were taken every Monday and Thursday for a period of 29 days and photos were taken with a ruler beside the plantlets for scale. To ensure measurements and pictures were being taken from the same position, all tubes were snugly placed and grown in a modified foam tube rack with a mounted black background for enhanced visibility (Fig. 4.5). All measurements were documented to determine differences of average growth rates per day (mm/d) among the four temperature treatments.

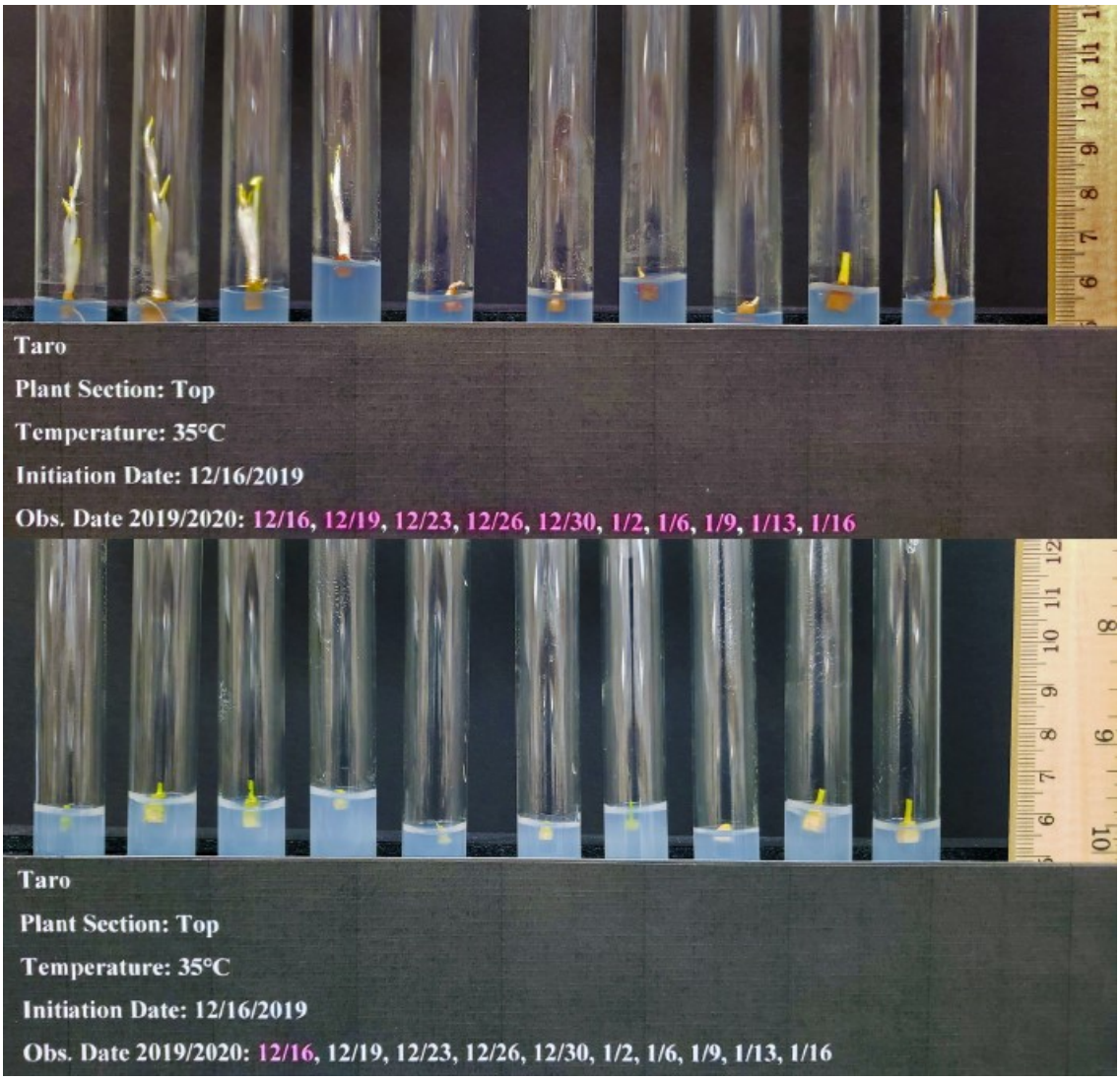


Fig. 4.5. Imaging of first (bottom) and last day (top) of thermo therapy treated (35ºC) taro top sections.

3D. Results

3D.1. Overall Sampling and Testing

Three taro "varieties" were 1) confirmed for TaVCV infection; 2) field harvested; 3) isolated via meristem tip culture; 4) in vitro-grown; and 5) retested observing the effectiveness of meristem tip culture initiation size, and bud location along the mother corm in eliminating TaVCV. A total of 91 plantlets survived the initiation process consisting of 67 Purple, 13 Purple-

2, and 11 Mana Ulu. Overall, 88 plants tested negative for TaVCV while 3 remained positive resulting in a 97% efficacy in eliminating TaVCV.

3D.2. Taro Bud Survivability Rates One Month Post Initiation

Preliminary data of bud survival after the first and second sub culturing was documented to demonstrate some of the challenges faced when applying meristem tip culture. Bacterial and fungal contamination is a common problem therefore overall survival rates varied. However, after the 2nd sub culture or 1-month post initiation, buds that show greening combined with sub culturing every three weeks increased the plants' chances to survive to maturity. In this experiment taro buds began to green one week after initiation. Factors such as explant size and location may play a role in survival rates. Bud explants that were 1mm tend to be more susceptible to over sterilization while 2mm buds may be more susceptible to contamination from internal or external sources. In the case of taro, all buds regardless of location had a crisp firm texture making it easier to work with during the dissection phases. This is most likely attributed to the buds being harvested from a corm-based mother plant. Overall, 142 buds were explanted and 127 (89%) survived. Comparing survival rates between sizes showed only a 1% difference. Contrasting survival rates amid location, top based buds had a 9% higher chance of surviving. Survival rates for buds set aside to allow more time to green were not collected due to overall initial high survival rates.

3D.3. Taro Bud Explant Initiation Size and TaVCV Elimination

Thirty plantlets were initiated at 1mm and 61 were initiated at 2mm. A total of 28 (1mm) buds tested negative and two positives for a TaVCV-virus free rate of 93%. At the 2mm range, 60 buds tested negative and one positive for a TaVCV elimination rate of 98%. Utilizing RStudio

for all statistical analysis, a Fisher's exact was applied and determined that taro buds initiated at 2mm are just as effective as 1mm in eliminating TaVCV ($P < 0.25$).

3D.4. Taro Bud Location and TaVCV Elimination

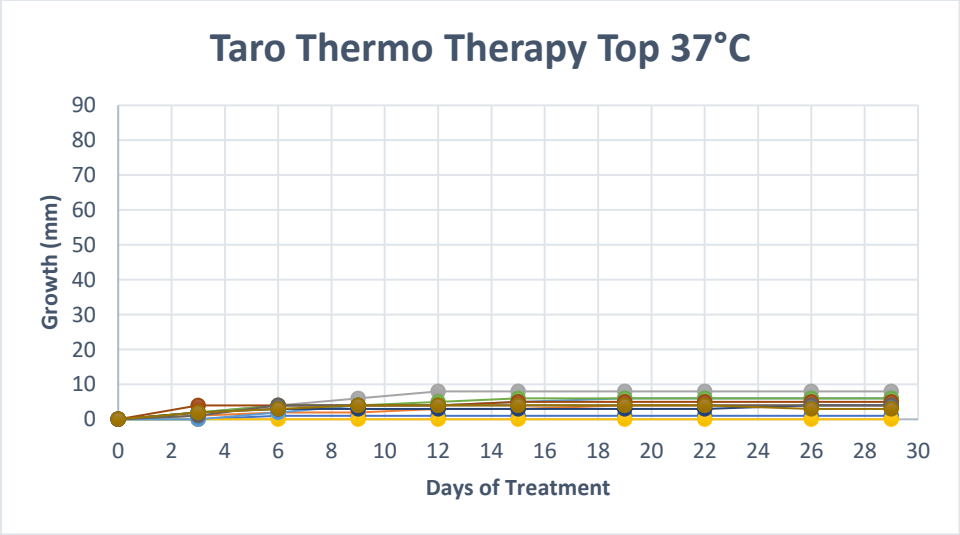
From the top portions of taro corms, 49 plantlets remained throughout the meristem tip culture process while 42 originated from the middle-bottom section. Top plantlets consisted of 47 that tested negative for TaVCV while two remained positive resulting in a 96% efficacy in eliminating TaVCV. Similarly, middle-bottom plantlets had 41 that tested negative while one remained positive resulting in a 97.6% efficacy in removing TaVCV. As a result, a Fisher's exact test was applied indicating no association between bud explant location and the presence of TaVCV ($P > 1$).

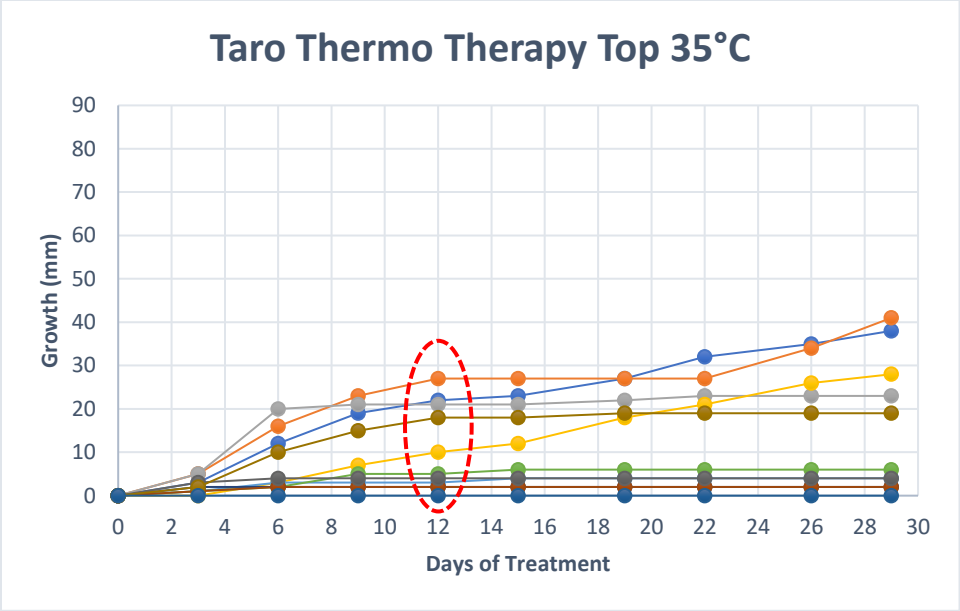
3D.5. Taro Variety and TaVCV Elimination

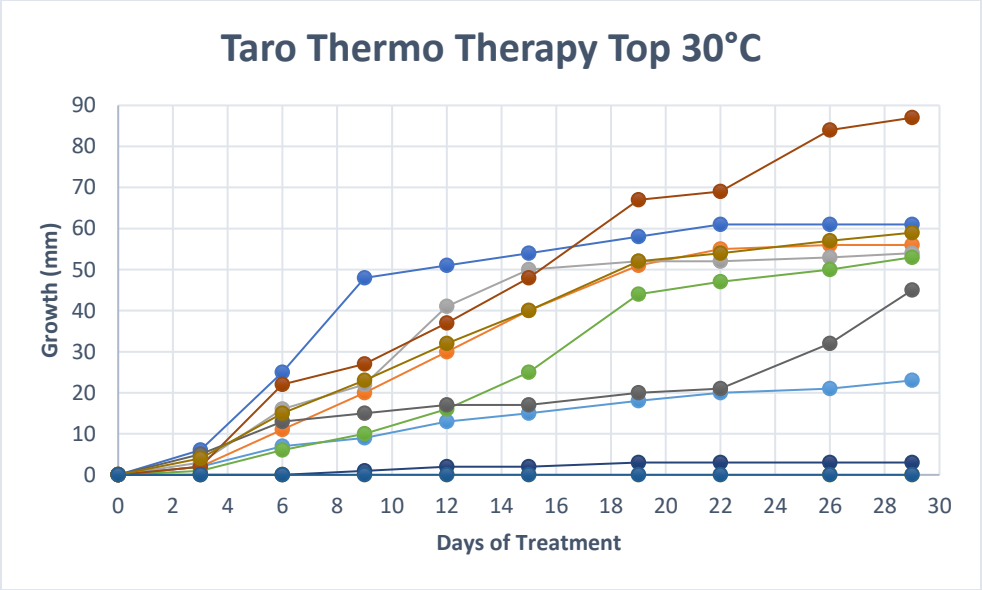
Among the three varieties tested within the experiment, 67 plantlets were of the Purple variety (seven mother corms), 13 of the Purple-2 (1 mother corm), and 11 of the Mana Ulu variety (two mother corms). The Purple variety included 66 plantlets that tested negative while one remained positive for a 98.5% susceptibility in TaVCV elimination. Purple-2 plantlets all tested negative for a 100% TaVCV eliminating susceptibility. Within the Mana Ulu variety, nine plantlets tested negative while two remained positive for a 78% TaVCV eliminating susceptibility rate. A Fisher's exact test was applied and demonstrated taro variety to be a factor in the susceptibility of TaVCV removal ($P < 0.047$) when applying meristem tip culture.

3D.6. Thermo Therapy Growth Rates Across Temperature Treatment

The growth rates of taro top section explants among temperature treatments (37°C, 35°C, 30°C, RT) were observed over a period of 29 days. At 37°C top sections grew the least at a rate of (0.14mm/d) followed by (0.58mm/d) at 35°C, (1.52mm/d) at 0.30°C, and (0.65mm/d) at room temperature. A trend of faster growth rates was evident as temperature treatment decreased with the exception of 30°C growing 61% faster than room temperature (Fig. 4.6). At 37°C, growth was miniscule. Any growth that occurred took place up to the 12th day before plateauing. A similar trend was observed for 35°C at the 12th day however some plants remained continuous in growth. At 30°C and room temperature, majority of the plants actively grew throughout the 29-day treatment period.




Taro Thermo Therapy Top 35°C
Growth (mm)
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days of Treatment


Taro Thermo Therapy Top 30°C
Growth (mm)
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days of Treatment

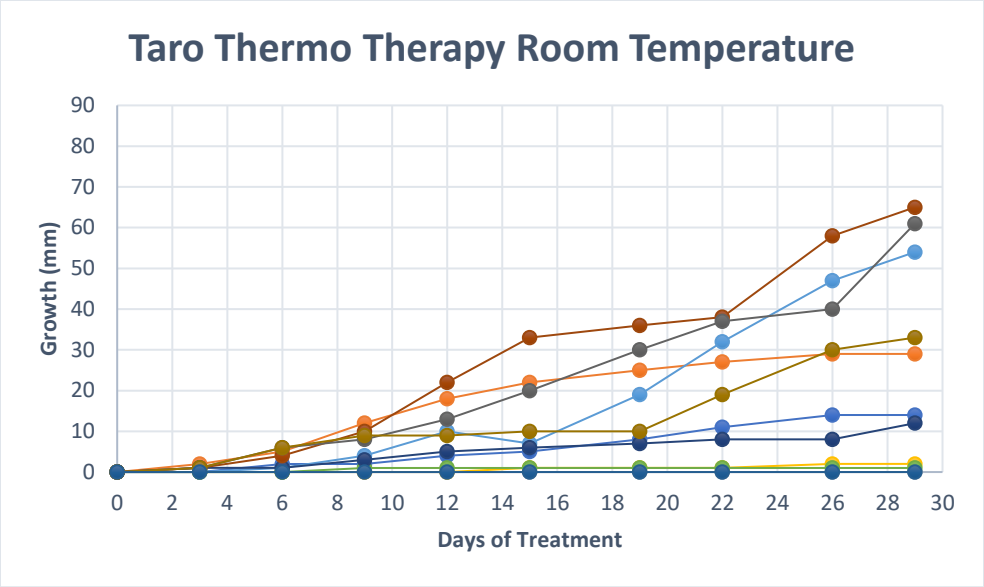


Fig. 4.6. Taro in vitro plant height measurements plotted throughout thermo therapy treatment among temperatures 37°C, 35°C, 30°C, RT. Each line represents the growth rate of an individual plant while the red-dotted circles indicates the peak of an initial growth spurt prior to plateauing.

3E. Discussion

This study was the first to correlate bud explant sizes and location on mother plant for eliminating TaVCV. Previous studies on other plants showed that smaller bud-initiated sizes resulted in fewer infected plants (Hall 1999; Sastry & A. Zitter 2014; Verma et al. 2004), however this was not the case in this experiment. Regardless of size (1mm or 2mm) or location, ≥93% of TaVCV-free plants can be achieved utilizing meristem tip culture alone. However, the results of these methods seem to vary dependent on taro variety. Based on the distinct dark purple petiole and leaf blade shape, the "Purple" cultivar resembles characteristics of Lehua Palaii (Ctahr.hawaii.edu, 2020) however the "Purple-2" cultivar is still unknown. The Mana Ulu cultivar showed a 78% TaVCV elimination rate however, 4 other plantlets displayed viral-like disease symptoms (Fig. 4.7) similar to the TaVCV infected plantlets (Fig. 4.8)

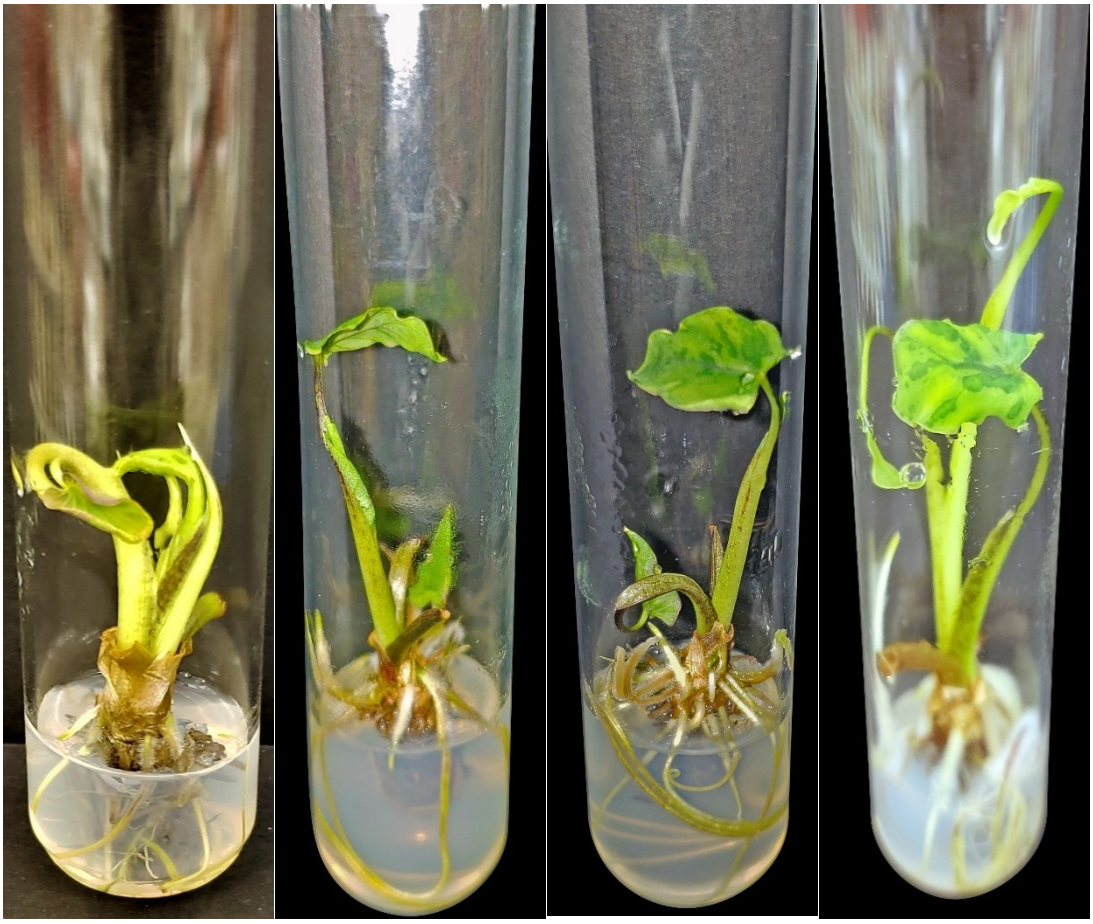


Fig. 4.7. Mana Ulu in vitro taro plantlets displaying viral symptoms. TaVCV and DsMV tests were negative.

Symptoms such as poor root development, downward bending and curling of petioles, zero to limited leaf development in addition to yellow mottling and crinkled leaf blade edges was evident in plants five months and older. Despite viral symptoms, this could indicate the presence of multiple viruses or different TaVCV strains known to be present in Hawai`i (Atibalentja et al. 2018; Long et al. 2014). Other viruses were not tested for, and this indicates a smaller initiation size may be necessary and/or the addition of other treatments such as antivirals or thermo therapy. In the event thermo therapy treatment is utilized, 35°C appears promising taking into consideration the combination of growth rate and higher temperature treatment. Although 30°C had the fastest overall growth rate, the TaVCV elimination rate among the various temperature

treatments have yet to be determined. Despite the infected Mana Ulu variety displaying disease symptoms, the lone Purple TaVCV infected plantlet appeared normal compared to its uninfected counterparts. One of the two TaVCV infected plants was limited in root and petiole development therefore both the roots and petiole tissue were harvested. It was later determined that TaVCV could be detected by testing root tissue alone and can be a source of testing material moving forward in the event leaf and petiole tissue is less than the 100mg recommended for RNA extractions. In addition, as variety plays a role in TaVCV elimination rates, future studies could be done increasing the sample size for both Purple-2 and Mana Ulu cultivars. In this experiment only a small amount of symptomatic plants per variety were available. Perhaps other cultivars that are commercially or culturally favored and susceptible to diseases could be tested to determine elimination rates for TaVCV and other taro-threatening diseases.

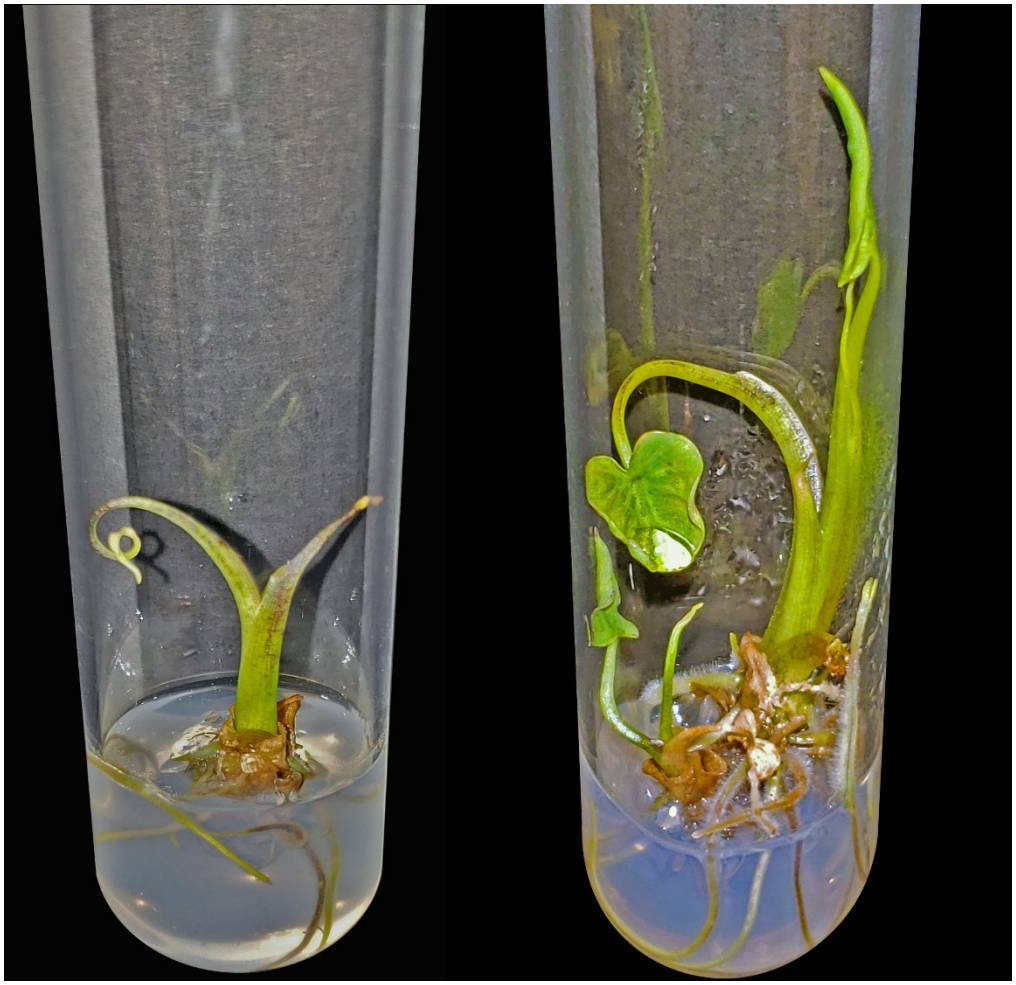
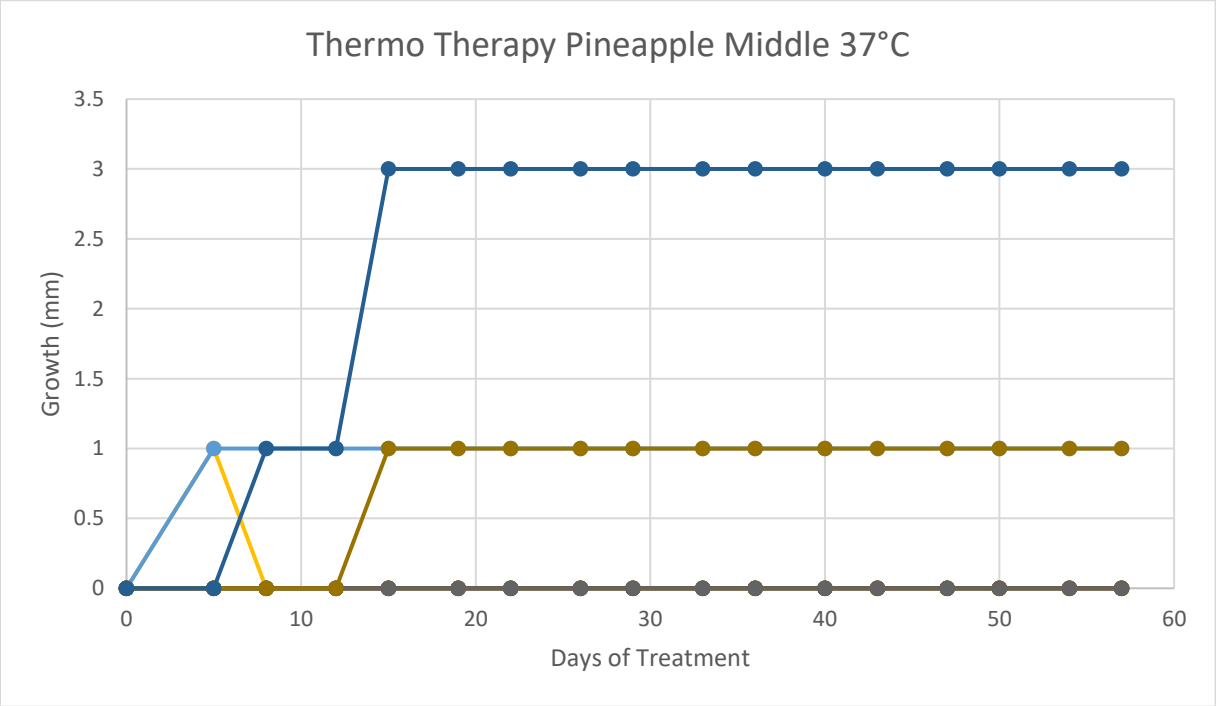
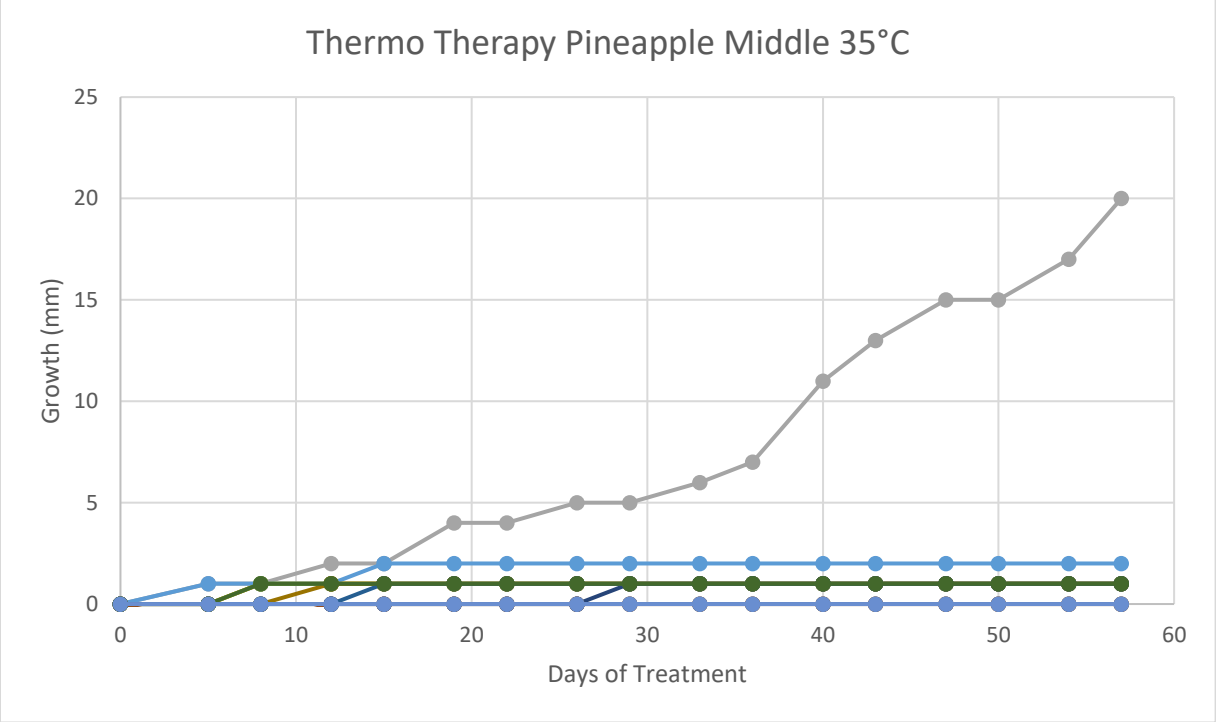


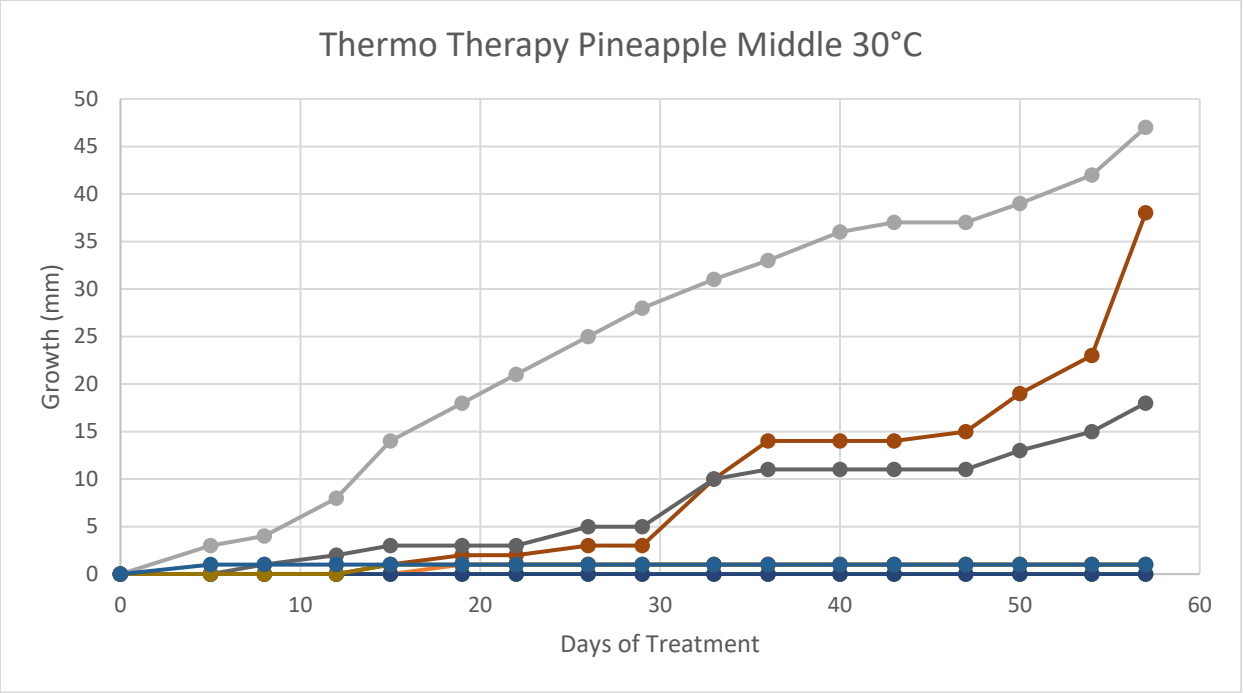
Fig. 4.8. Mana Ulu taro positive for TaVCV.

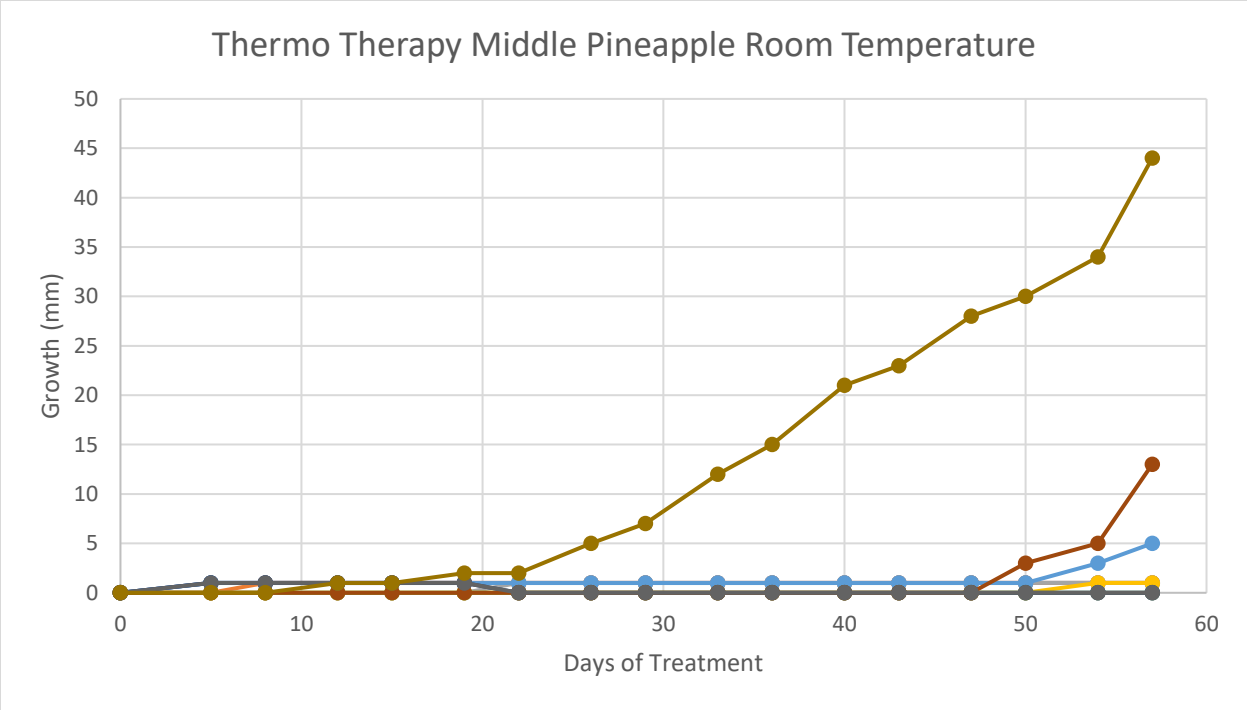
APPENDICES

Appendix: Thermo therapy treated pineapple middle plant heights plotted against temperature treatment.






Thermo Therapy Pineapple Middle 30°C
Growth (mm)
Days of Treatment
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60


Thermo Therapy Middle Pineapple Room Temperature
Growth (mm)
Days of Treatment
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60

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