



Production of PMWaV-free pineapple plants using combined thermotherapy and etiolation during micropropagation

Mírian Piassi^{1,2} · Fernanda Nunes Peron² · Marcelo Piassi³ · Luiz Carlos Santos Caetano¹ · Andréa Ferreira Costa¹ · Patricia Machado Bueno Fernandes² · José Aires Ventura^{1,2}

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Abstract

Mealybug wilt of pineapple (MWP) is an economically critical viral disease affecting global pineapple production. In Brazil, MWP is associated with pineapple mealybug wilt-associated viruses (PMWaV-1, PMWaV-2, and PMWaV-3), which are primarily disseminated via vegetative propagation material, as infected plantlets often remain asymptomatic. In vitro propagation techniques provide a reliable approach to generate pathogen-free planting material. This study aimed to combine thermotherapy and etiolation with shoot tip culture to establish an optimized protocol for producing virus-free ‘Vitória’ pineapple plants. In vitro shoots, multiplied from axillary buds of MWP-symptomatic ‘Vitória’ pineapple plants, were subjected to heat treatments at 35, 37, and 39 (± 1) °C for 35 days, or incubated in continuous darkness for 30, 60, and 90 days to induce etiolation. Following thermotherapy or etiolation treatments, shoot tips (≈ 2.0 mm) were excised and cultured in vitro for 90 days prior to leaf sampling for viral detection by reverse transcription-quantitative PCR (RT-qPCR). Etiolation induction for 60 and 90 days resulted in the eradication of PMWaV-3. Thermotherapy at 39 (± 1) °C achieved the elimination of PMWaV-1 and PMWaV-3 across all tested samples, and PMWaV-2 in 67% of samples, with a shoot tip regeneration of 37%. Virus-free shoots were subsequently multiplied in vitro and acclimatized in a greenhouse before field transplantation, where they remained healthy over two production cycles. The developed protocol provides a valuable method for large-scale production of virus-free planting material, meeting growers’ demands in a cost-effective manner.

Key message

A protocol is presented for the micropropagation of pineapple cv. Vitória that efficiently produces virus-free plants, enabling the management of mealybug wilt of pineapple (MWP).

Keywords *Ananas comosus* · Virus eradication · Thermotherapy · Etiolation · Shoot tip culture

Abbreviations

MWP	Mealybug wilt of pineapple
PMWaV	Pineapple mealybug wilt-associated virus
ICTV	International Committee on Taxonomy of Viruses
MS	MS culture medium (Murashige and Skoog 1962)
BAP	6-Benzylaminopurine
RT	Room temperature
GR	Growth rate
H _f	Final height
H _i	Initial height
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

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✉ Mírian Piassi
mirian.piassi@incaper.es.gov.br

¹ Capixaba Institute of Research, Technical Assistance and Rural Extension (Incaper), Vitória, Espírito Santo, Brazil

² Biotechnology Core, Federal University of Espírito Santo (UFES), Vitória, Espírito Santo, Brazil

³ Federal Institute of Education, Science and Technology of Mato Grosso (IFMT), Barra do Garças, Mato Grosso, Brazil

HSP70h	Heat shock protein 70 homolog
RdRp	RNA-dependent RNA polymerase
Ct	Cycle threshold
RT-PCR	Reverse transcription-polymerase chain reaction
GB	Germoplasm Bank
SU	Survival
N	Node
SN	Shoot number

Introduction

The pineapple (*Ananas comosus* var. *comosus* (L.) Merr., Coppens & Leal) is one of the most economically and socially important tropical fruits, with a global production of 29.4 million tons in 2024 (FAOSTAT 2025). In addition to its importance as a food source, its cultivation employs thousands of people, contributing significantly to the agricultural sector in tropical and subtropical countries, both for large-scale producers and family farmers (FAOSTAT 2025; Maia et al. 2024). Mealybug wilt of pineapple (MWP) is the most economically important viral disease that affects pineapple production, transmitted by the mealybugs *Dysmicoccus brevipes* and *D. neobrevipes* (Sether et al. 1998; Sether and Hu 2002). It is caused by the Pineapple mealybug wilt-associated virus (PMWaV) complex (family *Closteroviridae*; genus *Ampelovirus*), for which six distinct viruses have been reported: PMWaV-1, PMWaV-2, PMWaV-3, PMWaV-5, PMWaV-6, and more recently, PMWaV-7 (Melzer et al. 2008; Sether et al. 2009; Larrea-Sarmiento et al. 2021, 2022; Massé et al. 2024). However, only PMWaV-1, PMWaV-2, and PMWaV-3 are included as species in the taxonomy by the International Committee on Taxonomy of Viruses - ICTV (Massé et al. 2024) and have been reported in Brazil (Peron et al. 2019; Vieira et al. 2024).

The most common symptoms of pineapple wilt are wilting, root drying, a red or yellow coloration on leaves, curling of the leaf margin toward the abaxial surface, and completely dry leaf tips (Model 2000; Ventura and Costa 2002; Larrea-Sarmiento et al. 2022; Vieira et al. 2024). Infected plants may fail to fruit or may produce an atrophied and withered fruit that is unsuitable for use in fresh consumption or processing (Peron et al. 2019; Ventura and Zambolim 2002). The etiology of the disease is complex and not yet fully understood. Factors such as environmental conditions, mealybug populations and plant genotype influence symptom expression, and due to these variations, plants may remain asymptomatic even when infected with PMWaV isolates (Dey et al. 2018). With the exception of a report from Australia, where PMWaV-2 was not found in high prevalence in some locations, and where

PMWaV-1 and PMWaV-3, associated with each other and other viruses, were linked to MWP symptoms (Gambley et al. 2008), the general consensus is that the severe symptoms are associated with PMWaV-2 infection and mealybug feeding (Sether et al. 2001, 2002; Peron et al. 2019). Specific genes in PMWaV-2 encode proteins that act as efficient suppressors of viral silencing mechanisms in the plant, explaining why this virus is particularly involved in the induction of MWP symptoms (Dey et al. 2015, 2018).

Because it propagates vegetatively, cultivated pineapple plants are called clones, and infected crowns, slips, suckers, or other propagules used for planting can spread preexisting diseases, often accumulating viruses in both quantity and diversity, which negatively affects plant growth and productivity (Larrea-Sarmiento et al. 2022; Massé et al. 2024). Phylogenetic analyses conducted by Massé et al. (2024) revealed no geographic structuring of the diversity of PMWaV-1, PMWaV-2, and PMWaV-3 isolates, corroborating the current hypothesis that these viruses have been disseminated worldwide primarily by human activity and infected propagative material. Disease control measures include the use of insecticides to control the vector and biotechnological processes such as plant tissue culture (Soler et al. 2025; Massé et al. 2026). However, despite the use of contact or systemic insecticides targeting mealybugs to control MWP, systematic applications of these chemicals reduce biodiversity, contaminate the environment and water resources, and pose a health risk to the general population (Soler et al. 2025).

On the other hand, in vitro cloning emerges as a promising solution for providing unlimited, high-quality propagation material, especially when the plantlet production process incorporates methodologies capable of inactivating and/or destroying viruses, enabling the production of virus-free plants (Bettoni et al. 2022; Pérez-Caselles et al. 2025; Massé et al. 2026). In addition to meeting the legislative requirements for propagation material regarding phytosanitary standards, virus-free micropropagated plantlets can be used as elite stock, restoring the vigor and productivity of cultivars, and serving as mother plants by producers, preventing vertical transmission of the virus (Macdiarmid et al. 2026). Therefore, their use has been growing worldwide as an efficient agricultural strategy for virus control (Wang et al. 2018; Ventura et al. 2019; Alavijeh et al. 2025). The availability of simplified in vitro techniques for obtaining virus-free shoot tips from different cultivars helps overcome previous obstacles to implementing cryopreservation technologies for long-term conservation (Bettoni et al. 2021). Among the techniques used to obtain virus-free plants are thermotherapy, etiolation, and meristem culture. Thermotherapy promotes viral inactivation and eventual elimination when the plant, or part of it, is maintained in

an incubator at temperatures high enough to inactivate the pathogen without damaging plant tissues (e.g., 35–38 °C) for one to five weeks (Torres et al. 2000). Etiolation can be effective in recovering virus-free plants because the rate of viral replication and/or movement does not keep pace with the accelerated elongation of the apex (Torres et al. 1998). Shoot tip culture, or meristem culture, consists of excising the apical meristematic dome of the plant along with one or two leaf primordia for in vitro establishment and micropropagation. The reduced size of the initial in vitro explant and rapid cell multiplication without vascular tissue formation are hypotheses explaining the absence of viral particles in this tissue. These techniques can be applied individually or in combination, with combined treatments increasing the success rate of producing virus-free plants (Bettoni et al. 2022; Alavijeh et al. 2025; Pérez-Caselles et al. 2025).

In pineapple, heat treatment can be used for virus elimination via direct immersion of crowns, slips, and suckers in hot water, using protocols such as hot water baths at 40–60 °C for 30–60 min (Ullman et al. 1991) or at 58 °C for 40 min (Sether et al. 2001). However, these methods may not be fully effective for viral eradication (Ullman et al. 1991) and are limited regarding the quantity of plantlets produced. Conversely, in vitro culture initiated with small shoot tips, generally 0.5 to 1.0 mm, has been successfully employed for viral eradication in pineapple (Sether et al. 2001; Hu et al. 2005; Souza et al. 2012; Silva et al. 2021). Nevertheless, this small size, required for virus elimination, hinders the handling and execution of the technique on a large scale, while also resulting in a high mortality rate (Silva et al. 2021). In this study, thermotherapy was incorporated into the procedure to allow for the use of larger shoot tips, aiming to facilitate in vitro handling and large-scale plantlet production. An efficient new methodology for producing healthy pineapple plantlets represents a crucial alternative for pineapple growers and disease management. The aim of this study was to develop efficient in vitro methods for eliminating the PMWaV viral complex (PMWaV-1, PMWaV-2, and PMWaV-3) by combining shoot-tip culture, thermotherapy, and etiolation, enabling the establishment of virus-free pineapple plants of the cultivar Vitória. This is the first study to address virus elimination in this cultivar.

Materials and methods

Plant material

Pineapple plants of the Vitória cultivar, exhibiting MWP symptoms and previously indexed via RT-qPCR for the *Pineapple mealybug wilt-associated virus* complex (PMWaV-1, PMWaV-2, and PMWaV-3), were micropropagated at

Incapér's Plant Tissue and Cell Culture Laboratory. To this end, axillary buds were introduced and multiplied for four subculture cycles in MS culture medium (Murashige and Skoog 1962) with 100 mg/L myo-inositol, 30 g/L sucrose, and supplemented with 2.0 mg/L 6-benzylaminopurine (BAP). The culture medium was solidified with 6.7 g/L agar and had its pH adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. Baby-food glass jars (cap. 200 mL) were used, with 30 mL of culture medium distributed per jar. The micropropagated plants were maintained in an incubation chamber with a light intensity of 30–50 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, a photoperiod of 16 h, and a temperature of 25 (± 2) °C (room temperature). Shoots with a height of 2.7–3.0 cm obtained from 18-week-old cultures were transferred to MS culture medium and subjected to thermotherapy and etiolation treatments.

Virus elimination treatments

For thermotherapy, in vitro shoots were kept in the dark in microbiological incubators (Fanem, Model 002-CB, SP, Brazil) at temperatures of 35, 37, and 39 (± 1) °C for 35 days. For etiolation, in vitro shoots remained in a germination chamber (SolidSteel, SSGFa, SP, Brazil) for 30, 60, and 90 days in the dark at a temperature of 25 (± 2) °C. To avoid accidental light exposure, each of the culture jars containing six shoots from the thermotherapy and etiolation treatments were wrapped with two layers of aluminum foil. The control, like the treatments, consisted of five flasks, each containing six shoots, which remained under the incubation chamber conditions described in “[Plant material](#)” (Materials and Methods). The variables analyzed were survival, initial and final shoot height, and node number (visualized due to the elongation of internodes characteristic of etiolation). Height was determined by measuring from the base of the shoot to its highest point. The height was used to calculate the growth rate (GR) using the equation $\text{GR} = H_f - H_i$, where H_f corresponds to the average final height and H_i to the average initial height. In the thermotherapy treatments, evaluations were also performed at 18 days. The number of nodes per plant was determined in the final evaluation of all treatments. Following thermotherapy and etiolation treatments, shoot tips from viable explants were excised (≈ 2.0 mm) under aseptic conditions inside a horizontal laminar flow chamber and inoculated onto the same establishment and multiplication medium described in the “[Plant material](#)” section (Materials and Methods). After 15 days of cultivation in an incubation chamber, under a light intensity of 30–50 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, a photoperiod of 16 h and a temperature of 25 (± 2) °C, the number of viable (non-necrotic and/or green) reintroduced shoot tips was evaluated, as well as the percentage of regeneration at 30 days (considering those

that resumed development, as evidenced by leaf growth and the presence of green coloration).

Plant sampling and virus indexing

Viral indexing was performed at the Biotechnology Core of Federal University of Espírito Santo (UFES), using specific primers for the viruses PMWaV-1, PMWaV-2 and PMWaV-3 (Table 1). For RT-qPCR three pineapple shoots of the Vitória cultivar were evaluated per treatment, obtained from 18-week-old cultures after the reintroduction of shoot tips. Total RNA was extracted from macerated liquid nitrogen leaf tissues (≈ 100 mg) using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Total RNA quality was measured by the A260/280 and A260/230 ratios on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Nucleic acids extracted using conventional protocols were treated with DNase (RQ1 RNase-Free DNase, Promega) according to the manufacturer's instructions. The cDNA was reverse-transcribed from 10 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cheshire, UK) and used as a template for real-time PCR. Reactions were performed in a final volume of 20 μ l containing 10 μ l of PCR Master Mix (Applied Biosystems) and virus-specific primers. The primers targeting the HSP70h gene of PMWaV-1, PMWaV-2, and PMWaV-3 were designed by Peron et al. (2019). RT-qPCR was performed using a 7500 Real-Time PCR System (software version 2.0.1; Applied Biosystems), according to the protocol of the Biotechnology Laboratory (UFES/BioTec). The reaction mixture was initially heated to 95 °C for 10 min; amplification occurred for 40 cycles of 15 s at 95 °C, followed by 1 min at 60 °C. The detection of PMWaV-1, PMWaV-2, and PMWaV-3 was determined based on the cycle threshold (Ct) values. Samples were considered positive for viral detection when at least one technical replicate showed a Ct value ≤ 34 and negative when both technical replicates showed Ct

values > 34 or undetermined Ct values. Thus, negative results indicate that the target viral RNA was not detected under the conditions of the RT-qPCR assay. A reaction containing no cDNA was included as negative control. As a validation experiment, following two production and field monitoring cycles, leaf samples from six pineapple plants were collected and analyzed via RT-PCR using a modified method with Trizol® (ThermoFisher, Massachusetts, USA), according to Carnielli et al. (2014).

Plants free of viruses and field establishment

Shoots subjected to thermotherapy at 39 (± 1) °C, analyzed by RT-qPCR, and found to be negative for the PMWaV viral complex were multiplied using the same medium and conditions described under the section “Plant material” (Materials and Methods), producing approximately 250 plantlets. Of these, 50 were deposited in the in vitro Germplasm Bank (GB) of Incaper (www.incaper.es.gov.br). The other micropropagated and virus-free ‘Vitória’ pineapple plants were rooted for 30 days in MS culture medium containing 100 mg/L of myo-inositol, 30 g/L of sucrose (pH adjusted to 5.8) and solidified with 6.7 g/L agar. Afterwards, they were planted in 128-cell styrofoam trays containing commercial agricultural substrate (Tropstrato HT®), cultivated in a greenhouse with 50% shading. Irrigation was carried out via micro-sprinklers twice daily for one minute. A foliar fertilizer (FORTH Solúveis®) was applied every 15 days. The trays containing the micropropagated plants were kept in a greenhouse for 90 days and then transplanted to nursery beds, where they remained for another three months, following the technical recommendations of Caetano and Ventura (2018). After this period, they were planted in the field, according to agronomic recommendations (Gomes et al. 2003) at the Incaper Experimental Farm in Bananal do Norte, ES, Brazil. After two production cycles and monitoring under field conditions, samples were collected from the base of the leaves of six random plants for indexing by

Table 1 Primers used for RT-PCR and RT-qPCR detection of PMWaV-1, PMWaV-2, and PMWaV-3 in pineapple samples

Assay	Virus	Direction	Primer sequences 5'-3'	Genome region	Amplicon size	References
RT-PCR	PMWaV-1	Sense	AGTGAAGTGTGGTGGATCCT	RdRp	281 pb	Gambley et al. 2009
		Antisense	TTTGTGTACGACTGTGCGCAAT			
	PMWaV-2	Sense	CATACGAACTAGACTCATACG	Hsp70h	609 bp	Sether et al. 2001, Gambley et al. 2008
		Antisense	CCATCCACCAATTTTACTAC			
	PMWaV-3	Sense	AGTTCACTGTAGATTTCGGA	Hsp70h	495 bp	Sether et al. 2005
		Antisense	ATTGATGGATGTGTATCG			
RT-qPCR	PMWaV-1	Sense	GCAGGCGGTAGTAAACGAA	Hsp70h	112 bp	Peron et al. 2019
		Antisense	AAGTGCCTCCTCCGAAATC			
	PMWaV-2	Sense	ACGGTACCAGCCGACTACA	Hsp70h	101 bp	Peron et al. 2019
		Antisense	CAGCGGTCGGTTCATTAC			
	PMWaV-3	Sense	TGACGTTGTCGGTGTGTTT	Hsp70h	110 bp	Peron et al. 2019
		Antisense	ACCACCGCCTGTACGTTTA			

Table 2 Survival percentage (SU), growth rate (GR) and node number (N) of ‘Vitória’ pineapple shoots in vitro cultured at 25 (± 2) °C or subjected to thermotherapy treatments in the absence of light (35, 37 and 39 °C), evaluated at 18 and 35 days of culture

Temperature (°C)	Incubation Period ^w				
	18 days		35 days		
	SU (%)	GR (cm) ^x	SU (%)	GR (cm) ^x	N (n°)
25 (± 2) ^y	100 a	0.7 b	100 a	1.5 b	1.0 b
35 (± 1)	100 a	3.3 a	100 a	4.6 a	1.5 a
37 (± 1)	100 a	0.2 c	90 a	0.4 c	1.0 b
39 (± 1)	100 a	0.1 c	73 a	0.1c	1.0 b
CV(%) ^z		22.3	20.9	21.6	10.9

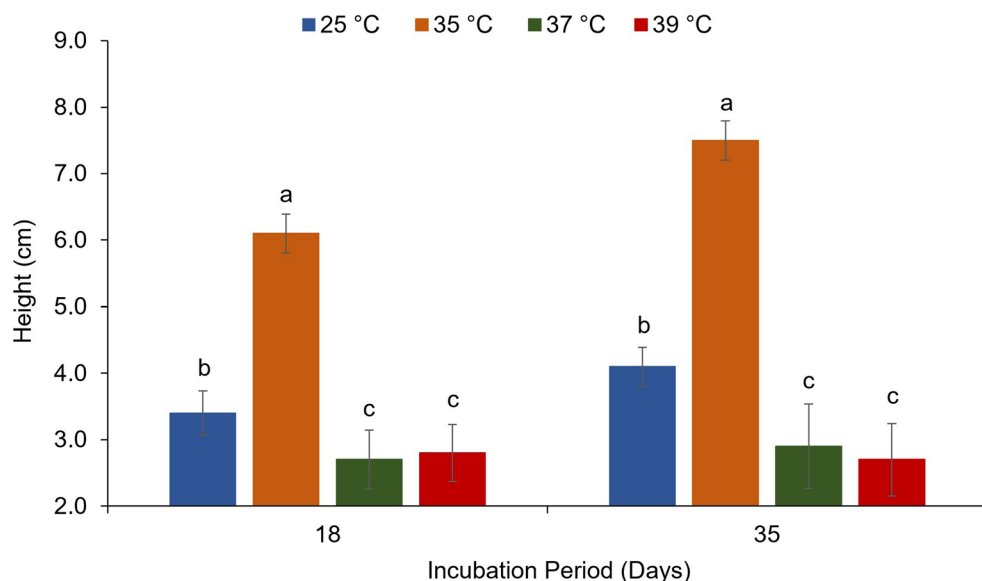
^w Means of five repetitions ($n=30$). Means followed by the same letter in the column do not differ statistically by Tukey's test ($p \leq 0.01$)

^x Height calculated by the difference between final and initial height

^y Room temperature

^z Coefficient of variation (%)

Fig. 1 Height of ‘Vitória’ pineapple shoots grown at 25 (± 2) °C, or subjected to thermotherapy treatments at 35, 37 and 39 (± 1) °C in incubators, in the absence of light. Columns represent means of five repetitions $\pm$ SD. Means followed by different letters, for each incubation period, differ statistically by Tukey's test ($p \leq 0.01$)



RT-PCR, using specific primers for PMWaV-1, PMWaV-2, and PMWaV-3 (Table 1). The accessions were also evaluated for the three viruses by RT-PCR, immediately after and one year after their deposition in the in vitro germplasm bank.

Experimental design and statistical analysis

The experiments were conducted in a completely randomized design (CRD) with seven treatments, and five replications (jars) each containing six shoots (replicates). Data normality was assessed using the Shapiro-Wilk test ($\alpha = 0.05$). Percentage data were arcsine-transformed prior to analysis to satisfy homoscedasticity assumptions. Analysis of variance (ANOVA) was performed and the means were compared using Tukey's test ($p \leq 0.01$). All statistical analyses were performed using the R software version 4.0.5 (R core Team 2025).

Results

Effect of heat treatment on shoot development

Thermotherapy treatment at 35 (± 1) °C did not affect the survival of pineapple shoots of the Vitória cultivar, and temperatures of 37 and 39 (± 1) °C did not affect survival at 18 days; however, after 35 days, they reduced the survival rate of the shoots to 90 and 73%, respectively (Table 2).

Shoots subjected to a temperature of 35 (± 1) °C, in the absence of light, for 35 days, showed a growth rate of 3.3 cm at 18 days and 4.6 cm at 35 days, significantly higher than shoots that remained under normal growth chamber conditions (0.7 cm at 18 days and 1.5 cm at 35 days), demonstrating that under these conditions the shoots exhibited etiolation, a typical developmental response of plants grown in the dark (Table 2; Fig. 1). This result is confirmed by the increase in the average number of nodes from 1.0 to 1.5 (Table 2) and by the “light green with white leaf bases”

coloration observed in 100% of the shoots, both characteristic of etiolated development (Fig. 2). In contrast, shoots subjected to treatments of 37 and 39 (± 1) °C, in the absence of light, for 35 days, showed growth inhibition without exhibiting etiolation.

Shoot tips after thermotherapy and clonal cleaning

After 15 days of culture, shoot tips reintroduced in vitro following heat treatments (Fig. 3) showed viability percentages of 67% and 47% at temperatures of 37 and 39 (± 1) °C, respectively, while those subjected at 25 (± 2) °C and 35 (± 1) °C exhibited 100% viability (Table 3). Necrosis, evidenced by dark coloration (brown or black), was identified as the cause of the loss of viability of the tips subjected to temperatures of 37 and 39 (± 1) °C. A curvature of the

base of the stem was observed in the shoots subjected to a temperature of 35 °C (Figs. 3A and B). At thirty days, the percentage of shoot tips that resumed growth (regeneration) was 100, 90, 67 and 37% of the initial 30 shoots for 25, 35, 37 and 39 (± 1) °C, respectively (Table 3).

Viral diagnosis demonstrated that maintaining shoots at 39 (± 1) °C for 35 days has the potential to eliminate PMWaV from pineapple plant tissues. Specifically, thermotherapy at 39 (± 1) °C eradicated PMWaV-1 and PMWaV-3 in all analyzed samples (6/6), as well as PMWaV-2 in two of the three analyzed samples. In contrast, all leaf samples from untreated control shoots maintained at 25 (± 1) °C remained infected with PMWaV-1 and PMWaV-2, with one sample also testing positive for PMWaV-3 (Table 3).

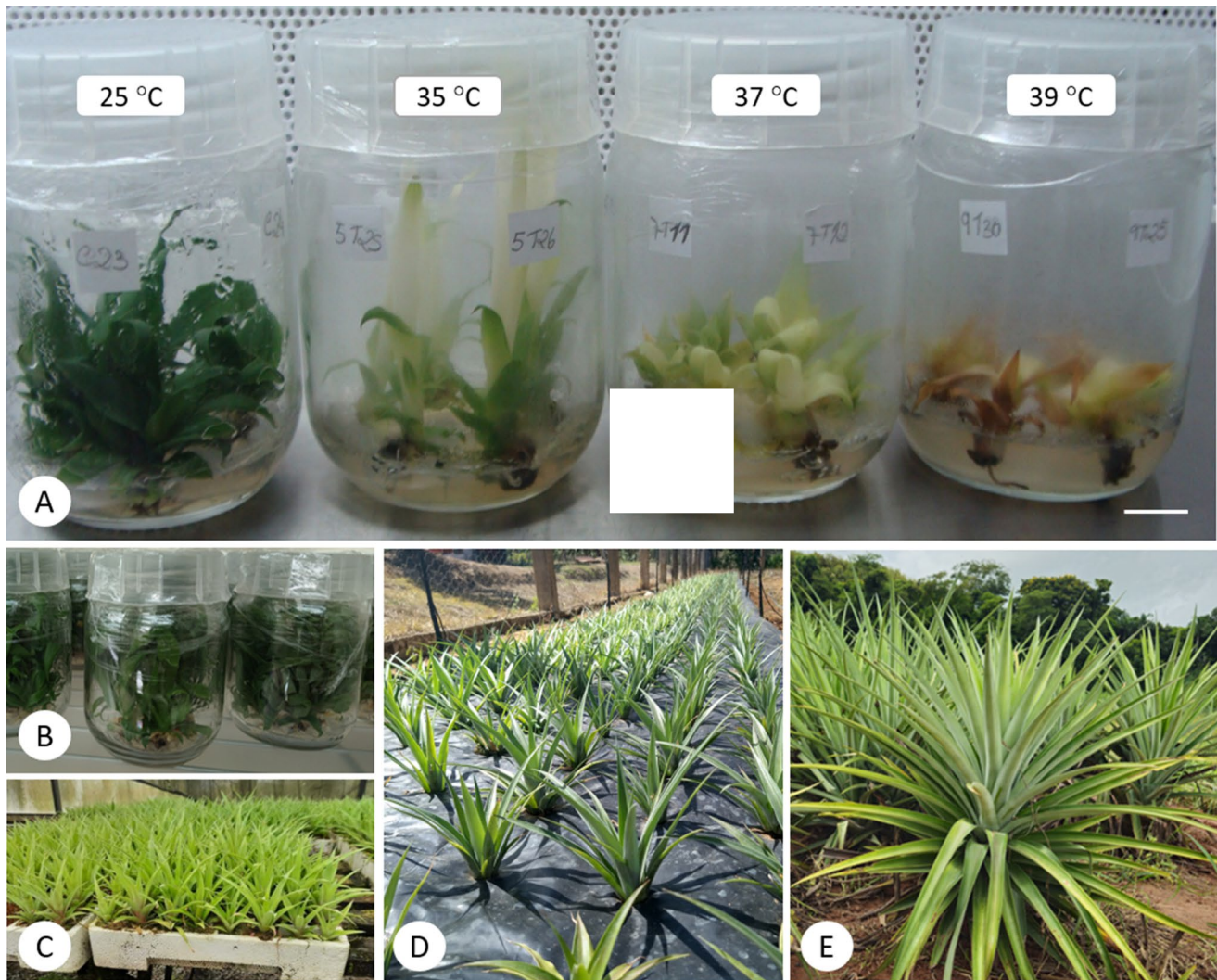


Fig. 2 In vitro eradication of viruses in pineapple, cv. Vitória. Shoots grown in incubation chamber (25 $\pm$ 2 °C) or subjected to thermotherapy treatments for 35 days in incubators, in the absence of light and temperatures of 35, 37 and 39 (± 1) °C (A). In vitro multiplication and

rooting of virus-free shoots (B). Development of virus-free plantlets in trays (C). Virus-free plantlets in the nursery (D). Healthy plants under field conditions at Incaper Experimental Farm Bananal do Norte, ES, Brazil, after virus cleaning (E). Bar = 10 mm

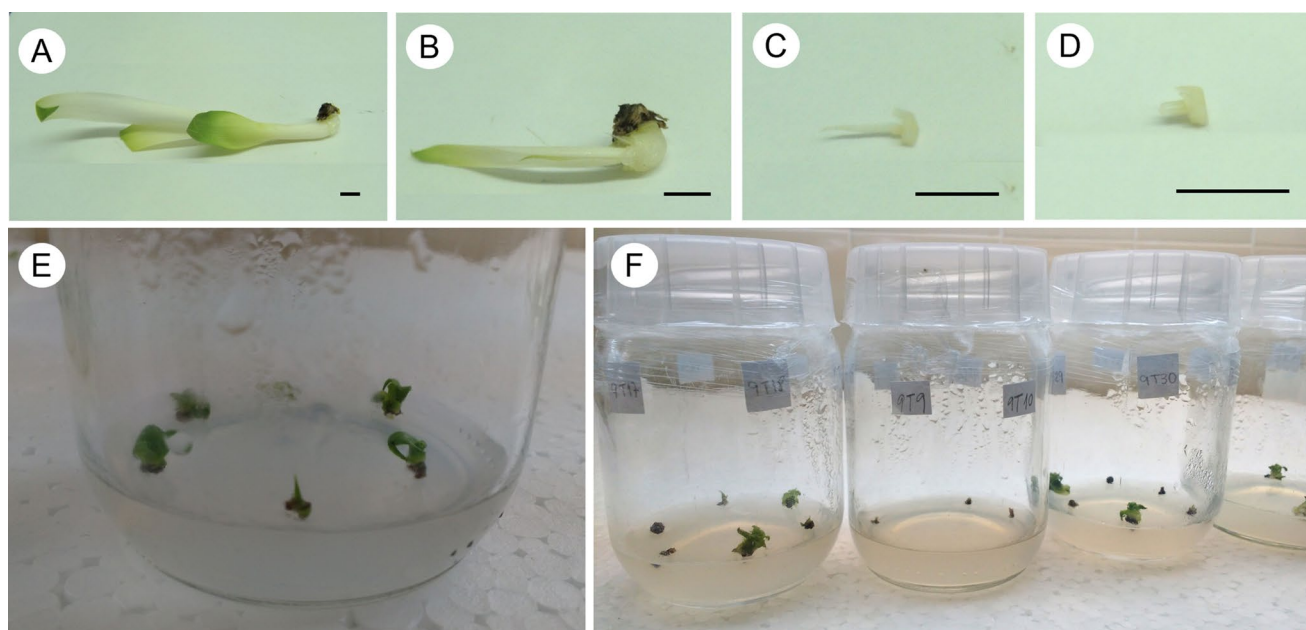


Fig. 3 Stages of shoot tips extraction from 'Vitória' pineapple shoots after thermotherapy treatment at 35 (± 1) °C (A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ D). Shoot tips that were subjected to thermotherapy at 35 (± 1) °C, after 15 days of reintroduction into MS culture medium + 2.0 mg/L of BAP

(E). Shoot tips that were subjected to thermotherapy at 39 (± 1) °C, after 30 days of reintroduction into MS culture medium + 2.0 mg/L of BAP (F). Bars: 5 mm

Table 3 Effect of heat treatment at different temperatures, for 35 days, on the viability (at 15 days) and regeneration (at 30 days) of shoot tips of 'Vitória' pineapple and viral infection (PMWaV-1, PMWaV-2, PMWaV-3)

Temperature (°C)	Viability (%) ^w	Regeneration (%) ^w	PMWaV-1	PMWaV-2	PMWaV-3
25 (± 2) ^x	100 a	100 a	+++ ^y	+++	+
35 (± 1)	100 a	90 a	+	+++	+
37 (± 1)	67 b	67 b	+	+++	++
39 (± 1)	47 b	37 b	-	+	-
CV(%) ^z	19.2	25.2			

^w Means of five repetitions ($n = 30$). Means followed by the same letter in the column do not differ statistically by Tukey's test ($p \leq 0.01$)

^x Room Temperature

^y Incidence: +++ = very strong positive test, ++ = strong positive test, + = weak positive test, - = negative test

^z Coefficient of variation (%)

Table 4 Growth rate (GR) and node number (N) of 'Vitória' pineapple shoots grown in vitro under 16 h photoperiod (Light/Dark: 16/8) or in dark (Light/Dark: 0/24) for 30, 60 and 90 days

Photoperiod (hours)		30 days ^w		60 days ^w		90 days ^w	
Light	Dark	GR (cm)	N (n°)	GR (cm)	N (n°)	GR (cm)	N (n°)
16	8	1.2 b	1.0 b	3.7 b	1.0 b	4.8 b	1.0 b
0	24	4.5 a	3.7 a	5.3 a	5.5 a	6.2 a	7.3 a
CV(%) ^x		10.8	10.8	18.8	12.8	9.2	7.9

^w Means of five repetitions ($n = 30$). Means followed by the same letter in the column do not differ statistically by Tukey's test ($p \leq 0.01$)

^x Coefficient of variation (%)

Effect of the dark period on shoot development

The survival of shoots induced to etiolate was 100% in all treatments, demonstrating that the period of darkness did not interfere with their viability. There was a significant increase ($p \leq 0.01$) in the growth of shoots subjected to the dark period, compared to those that remained under normal

culture room conditions. In the former, the growth rate was 4.5, 5.3, and 6.2 cm at 30, 60, and 90 days, respectively, compared to growth in the culture room, which was 1.2, 3.7, and 4.8 cm (Table 4). The means heights of shoots that remained in the dark for 30, 60, and 90 days were 7.4, 8.0, and 9.1 cm, respectively, and those that remained in the culture room for the same period showed averages of 3.8,

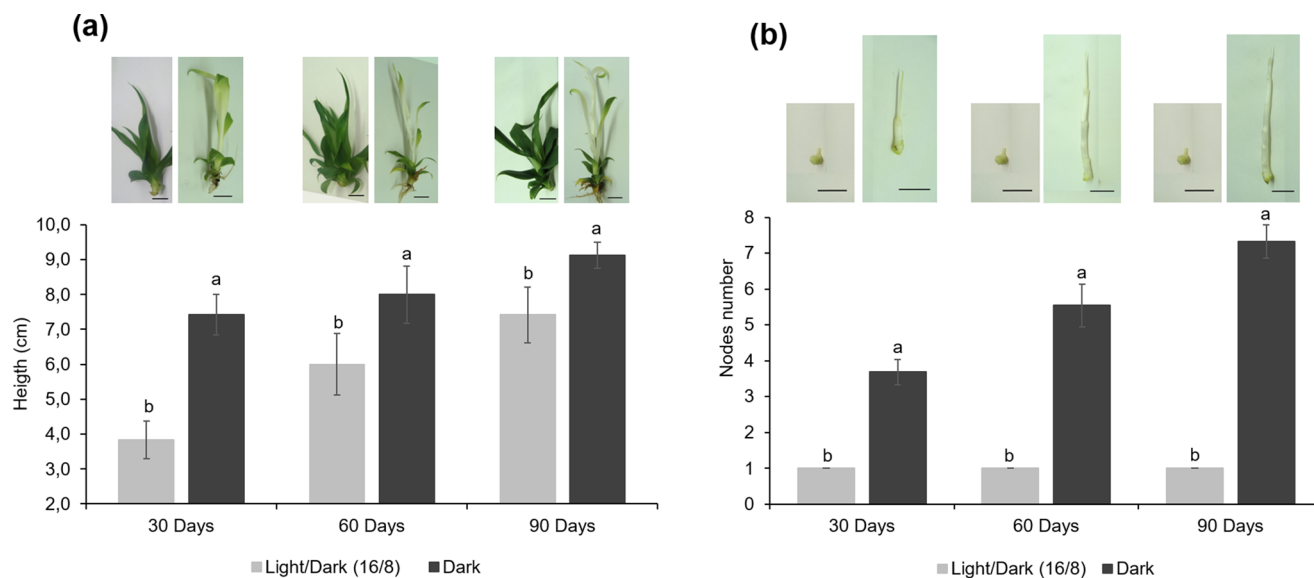


Fig. 4 Height **(a)** and node number **(b)** of ‘Vitória’ pineapple shoots grown in an incubation chamber, with light intensity of 30–50 $\mu\text{mol. m}^{-2}.\text{s}^{-1}$ and 16 h of light (Light/Dark: 16/8) or subjected to etiolation treatments for 30, 60 and 90 days in the absence of light (Dark),

all at temperature of 25 (± 2) °C (Room temperature). Columns represent means of five repetitions $\pm$ SD. Means followed by different letters within each incubation period differ statistically by Tukey’s test ($p \leq 0.01$). Bars: 10 mm

Table 5 Effect of different treatments for etiolation induction on the viability (at 15 days) and regeneration (at 30 days) of shoot tips of ‘Vitória’ pineapple and viral infection (PMWaV-1, PMWaV-2, PMWaV-3)

Dark period (days)	Viability (%) ^w	Regeneration (%) ^w	PMWaV-1	PMWaV-2	PMWaV-3
CON ^x	100 a	100 a	++ + ^y	+++	+
30	87 a	80 a	+	+++	+
60	97 a	93 a	++	+++	-
90	100 a	97 a	+++	+++	-
CV(%) ^z	16.1	23.3			

^w Means of five repetitions ($n=30$). Means followed by the same letter in the column do not differ statistically by Tukey’s test ($p \leq 0.01$)

^xCON - Control= 16/8 hours (light/dark)

^y Incidence: +++ = very strong positive test, ++ = strong positive test, + = weak positive test, - = negative test

^z Coefficient of variation (%)

6.0 and 7.4 cm (Fig. 4a). Etiolated growth was confirmed by the internode elongation, which allowed the visualization and counting of the nodes. With a longer period in the dark, there was an increase in the average number of nodes, reaching 3.7 nodes for etiolation for 30 days, 5.5 nodes for 60 days, and 7.3 nodes for 90 days (Table 4; Fig. 4b).

Shoot tips after etiolation induction and viral diagnosis

The shoot tips of ‘Vitória’ pineapple plants that were extracted after etiolation induction treatments and inoculated in a new culture medium showed viability percentages of 87% for 30 days in the dark and 97% for 60 days in the dark. Those subjected to darkness for 90 days showed 100% viability, as did those that remained in the culture room under a 16/8-hour photoperiod (light/dark) – control (Table 5). The green coloration observed in all shoots

indicated the absence of necrosis, with the loss related to handling errors during shoot tip extraction. At thirty days, the percentage of shoots that resumed growth (regeneration) was 100% for the control group and 80%, 93%, and 97% for the shoots kept in the dark for 30, 60, and 90 days, respectively.

Etiolated shoots at room temperature and kept in the dark for up to 90 days did not eliminate PMWaV-1 and PMWaV-2, but the PMWaV-3 was not detected at 60 and 90 days (Table 5). In the control shoots with 16/8 hours of photoperiod, the viral presence was very strong positive for PMWaV-1 and PMWaV-2, but weak positive for PMWaV-3, with the elimination of these viruses at the end of the 60-day treatment (Table 5). For PMWaV-2, the viral load was very high, and no effect of the treatment was observed for this virus, while for PMWaV-1, an intermediate behavior occurred, without, however, producing virus-free shoots.

The indexing results for the field-collected samples ($n=6$) and the in vitro samples ($n>10$) from the Incaper Active Germplasm Bank were negative for PMWaV-1, PMWaV-2, and PMWaV-3.

Discussion

MWP has been associated with declines in pineapple productivity in several producing regions (Moreno et al. 2023; Carrasco-Lozano et al. 2025; Ningrum et al. 2025). Therefore, the production of virus-free plantlets is considered a crucial first step for regaining high productivity and, consequently, the successful commercialization of this fruit. The protocol developed in this work, which subjected shoots to thermotherapy at $39 (\pm 1) ^\circ\text{C}$ for 35 days before extracting shoot tips of 2.0 mm in size and subsequent in vitro multiplication, proved promising for the production of pineapple plantlets free of the PMWaV viral complex.

Temperatures between 35 and $42 ^\circ\text{C}$ and exposure times of four to six weeks have been the most commonly used in thermotherapeutic treatments aimed at clonal cleaning. There is a consensus that the higher the temperature and the longer the exposure time, the greater the frequency of virus eradication; however, this comes at the expense of a lower survival rate of the plant material (Wang et al. 2018; Szabó et al. 2023). Therefore, the thermotherapy methodology needs to inactivate the virus while simultaneously allowing the plant material to survive, resulting in the production of virus-free plants. In our obtained results, a temperature of $35 (\pm 1) ^\circ\text{C}$ did not affect the viability of the explants; however, it was not able to inactivate or eliminate the PMWaV viruses, especially PMWaV-2. Although higher temperatures have been reported in the literature as being more effective in inactivating the virus (Torres et al. 2000; Acquadro et al. 2010; Lassois et al. 2013), several authors have reported better efficiency when using heat treatments at $35 ^\circ\text{C}$ combined with meristem/shoot tip culture, since the use of this temperature allows for higher survival and regeneration rates of the explants. In vitro cassava plants treated with thermotherapy at $35 ^\circ\text{C}$ for 42 days showed an 81% survival rate and 78% of plants free of *East African cassava mosaic virus* (EACMV) (Kidulile et al. 2018); for figs, although meristem culture eliminated 60 to 100% of the virus, stem segment culture associated with thermotherapy at $35 ^\circ\text{C}$ for 30 days proved more effective for shoot regeneration (40%) (Chalak et al. 2015). In these cases, the use of lower temperatures increased the plant survival rate and highlighted the potential of using combined clonal cleaning techniques in relation to the total number of virus-free plants produced. Regarding the etiolation observed in vitro-grown plants of 'Vitória' pineapple cultivar subjected to

$35 (\pm 1) ^\circ\text{C}$, internode elongation was not inhibited, which highlighted the possibility of combining these two clonal cleaning techniques, since, according to Torres et al. (1998), the rate of viral replication and/or movement does not follow the rapid apex elongation promoted by etiolation, giving the explant a greater chance of being free of pathogens. These authors also report that the combination of thermotherapy and etiolation techniques can increase the efficiency of obtaining virus-free plants. In our work, thermotherapy at $35 (\pm 1) ^\circ\text{C}$ with etiolation, followed by extraction of shoot tips, eliminated PMWaV-1 and PMWaV-3 viruses in 67% of the plants, but was not effective in inactivating/eliminating PMWaV-2, and this result may be associated with a higher viral load of this virus in the plants.

When comparing the results obtained in inducing etiolation in shoots of the 'Vitória' cultivar, other authors reported lower growth rates in explants of other pineapple cultivars when grown in the absence of light in a culture medium without growth regulators. For the hybrid pineapple PE X SC-52, kept in the dark for 35 days, Barboza and Caldas (2001) obtained shoot lengths of 4.8 cm, lower than the height of 7.4 cm obtained for the 'Vitória' pineapple; and for ornamental pineapples, kept in the dark for 60 days, lengths of 3.4 cm were observed in *Ananas comosus* var. *erectifolius*, and 6.2 cm in *Ananas comosus* var. *bracteatus* (Carvalho et al. 2009, 2012), also lower than the height of 8.0 cm presented by the 'Vitória' pineapple. Regarding the viability of the explants, it was observed that inducing etiolation for 30, 60, or 90 days did not interfere with the survival of the shoots. However, with accentuated elongation, a characteristic inherent to etiolation, the stem structure became very thin, facilitating breakage during the extraction of the shoot tips, which led to a loss of some shoots during handling.

Pineapple shoots of the Vitória cultivar did not show etiolation at temperatures of 37 and $39 (\pm 1) ^\circ\text{C}$, similar to that obtained by Kidulile et al. (2018), where the growth rate was reduced with increasing temperature in cassava explants treated with thermotherapy. Plants exposed to $28 ^\circ\text{C}$ (Control) grew normally, as did those exposed to $30 ^\circ\text{C}$, unlike those that remained at the higher temperatures of 35 and $40 ^\circ\text{C}$, which exhibited abnormal growth. The 67% viability percentage of pineapple shoots, cv. Vitória, subjected to a temperature of $37 ^\circ\text{C}$ for 35 days, can be considered high when compared to the result obtained at the same temperature, for 40 days, for potato, where survival percentages of 43% and 33% of plants free of *Potato virus Y* (PVY) were obtained (Mahmoud et al. 2009). For horseradish, all apical meristem explants subjected to $37 ^\circ\text{C}$ for 23 days survived and were 100% free of *Turnip Mosaic Virus* (TuMV) (Uchanski et al. 2004).

In our work, pineapple shoots, cv. Vitória, subjected to a temperature of $39 (\pm 1) ^\circ\text{C}$ for 35 days, had a survival rate

of 47%. This temperature was also used for ‘Idared’ apples for 6 days, with a survival rate of 62.5% and 60% of plants obtained free of *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV). However, there was no success in eliminating the same viruses for the ‘Sampiom’ apple cultivar subjected to the same conditions (Papstein et al. 2008). For the ‘Elektra’ and ‘Alexander Lucas’ pear cultivars, after in vitro thermotherapy of the culture at 39 °C for 5 to 10 days and the extraction of the apical meristem, 100% and 79.2% viable explants were obtained, with elimination percentages of 67% and 17% for *Apple stem pitting virus* (ASPV), respectively (Papstein et al. 2009). Even though pineapple has a narrower genetic base compared to the *Rosaceae* family, there may be variations in the results when different cultivars are used, by the aforementioned authors.

The results of this study demonstrated a trend toward greater success in eliminating PMWaV-1, PMWaV-2, and PMWaV-3 viruses with the use of higher temperatures. Thermotherapy at 39 (± 1) °C eliminated 100% of PMWaV-1 and PMWaV-3 viruses from the tested samples, and 67% of PMWaV-2 virus, the latter result being similar to that obtained in the clonal cleaning of ‘Elektra’ pears for *Apple stem pitting virus* (ASPV) (Papstein et al. 2009). PMWaV-2 has been directly associated with the incidence of pineapple wilt symptoms (Dey et al. 2015; Ningrum et al. 2025). In this study, it was the most difficult virus to eliminate, either due to an inherent characteristic of the virus itself or due to the presence of the highest viral load in the plants that originated the explants, since the selection was associated with the manifestation of MWP symptoms. With etiolation induction alone, without thermotherapy, no clonal cleaning was observed for PMWaV-1 and PMWaV-2 viruses in any of the dark incubation periods; however, negative tests were obtained for PMWaV-3 after etiolation for 30 and 60 days. This result, however, may be attributed to the low viral load in the original propagative material. In a study conducted by Pérez-Caselles et al. (2025) etiolation allowed the production of *Plum pox virus* (PPV) free apricot plants with low efficiency, but did not eliminate *Hop stunt viroid* (HSVd), which shows that although etiolation does not require specialized equipment and is simpler than thermotherapy, it may be less effective in eliminating some pathogens.

The successful viral elimination obtained with the application of thermotherapy at 39 (± 1) °C in shoots followed by shoot tip culture in this study demonstrated the feasibility of adopting protocols for extracting 2.0 mm shoot tips from pineapple plants. This would not only increase explant regeneration rates but also allow for a faster and easier procedure, enabling large-scale implementation. Using the method of extracting 0.5–1.0 mm shoot tips of from ornamental pineapple hybrids isolatedly, Silva et al. (2021) achieved clonal cleanup success in 43% of the plants

evaluated, citing that the recorded mortality was due to the oxidation of the shoot tips, which stemmed from their small size and possible lesions in the meristem region. Similarly to our study, Vivek and Modgil (2018) added thermotherapy to the procedure to achieve successful viral elimination even when using larger shoot tips. When these authors studied the application of thermotherapy to apple tree cuttings and the extraction of stem meristems of different sizes for clonal cleaning, they obtained low survival rates for meristems extracted at 0.2–0.4 mm. However, when these authors extracted meristems subjected to the same heat treatment (37–40 °C for 28 days), they observed that it was possible to achieve successful viral cleaning of the plant material, even in larger apical explants (0.6 mm), resulting in a higher survival rate of the shoots. This represents better efficiency in clonal cleaning, as it allows for greater speed in the extraction of stem tips, with greater regeneration success. With similar results, Betoni et al. (2022) add that thermotherapy can inhibit viral replication by increasing the rate of RNA degradation and reducing virus movement, which decreases the viral particle load and allows the use of larger meristems than those used for meristem culture without heat treatment.

The absence of viral infection after two field production cycles on the experimental farm highlighted the use of virus-free in vitro plants as a promising strategy to reduce viral load in pineapple cultivation. When comparing plots of conventional ‘Queen Victoria’ pineapple plants with plots formed with virus-free micropropagated plantlets, Massé et al. (2026) cited encouraging results, as they observed that four years after planting, 75% of the sampled plants remained virus-free, with infections limited to PMWaV-1 and PMWaV-3. Our results suggest, therefore, that the use of virus-free micropropagated plants can help prevent the introduction and spread of viruses associated with MWP, being an effective strategy for integrated disease management that combines the control of the mealybug with the production of healthy plantlets.

Conclusions

The production of virus-free propagative material is an important strategy for controlling viral diseases in cultivated plants, especially in vegetatively propagated species such as pineapple. This study showed that the combination of in vitro techniques using thermotherapy followed by shoot tip culture was more effective in eradicating PMWaV in infected pineapple plants, efficiently indexed by real-time PCR, yielding virus-free in vitro plants.

The effectiveness of eliminating the PMWaV complex viruses in micropropagated plants from 2.0 mm shoot tips, extracted from shoots kept at a temperature of 39 (± 1) °C

for 35 days, confirms the potential of adopting this protocol for clonal cleaning of pineapple plants of the Vitória cultivar. A temperature of 39 ($\pm$ 1) °C for 35 days allowed for the survival of 73% of the plant material and the total elimination of the PMWaV-1 and PMWaV-3 viruses, and of 67% of the tested samples for PMWaV-2. The addition of thermotherapy before extraction allowed the use of larger shoot tips ($\approx$ 2.0 mm), increasing the survival rate and eliminating the need for a stereomicroscope. This reduces the difficulty of handling in aseptic laminar flow chamber and the need for operational expertise, making the process easier, faster, and more economical. Therefore, the established protocol can enable the mass production of healthy pineapple plantlets.

The integrated protocol developed in this study allows for the micropropagation of PMWaV-free plants, with potential advantages over conventional propagation methods. It is useful for the conservation of genetic resources in breeding programs by providing virus-free material. It also allows for the planning of efficient PMWaV eradication programs and the supply of high-health stock plants, essential for disease management, and ensures productivity and fruit quality in the market.

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Author contributions All authors contributed to the study conception and design. MiP and JAV conceived the idea and design the experiments. Material preparation, data collection and analysis were performed by MiP; JAV, MaP and AFC assisted in data analysis and manuscript writing; LCSC provided support in nursery and field analysis; FNP and PMBF provided support in data collection and analysis in qPCR and RT-qPCR indexing. All authors read and approved the final version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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