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RESEARCH PAPERS

Viral diagnosis and effectiveness of shoot tip culture for virus eradication in passion fruit (*Passiflora edulis* Sims) matrices used for hybrid seed production

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Highlights
- CABMV and a begomovirus were identified in samples of *Passiflora edulis* Sims
- The isolated application of the shoot tip culture technique provided a possibly virus-free propagule
- A micropropagation protocol for passion fruit buds was established

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KEYWORDS
Meristem culture;
CABMV;
LCV;
Begomovirus;
Detection;
Virus-free plant.

Abstract: The passion fruit crop is of significant importance in Brazil. However, fruit production nationwide has been declining due to viral infections. This study aimed to conduct a viral diagnosis and evaluate shoot tip culture for virus eradication in passion fruit (*Passiflora edulis* Sims) matrices used for hybrid seed production. Initially, branches from the CPMSC1, CPGA1, MR1, CPMGA2, and CPF1SSBR genotypes were collected in a greenhouse. RNA and DNA were extracted from the leaves for viral detection using RT-PCR and PCR with primers targeting genomic regions of cowpea aphid-borne mosaic virus (CABMV), lettuce chlorosis virus (LCV), and begomovirus. For viral diagnosis, it was observed that CABMV was detected in 100% of the samples from all tested matrices. Additionally, CPGA1 (17%) and CPMSC (11%) samples tested positive for begomovirus infection, while none of the samples were positive for LCV. For virus eradication, shoot tips (0.1 - 0.3 mm) with up to two primordial leaves were isolated and inoculated in an MS medium supplemented with 0.05 µM NAA, 0.44 µM BA, and 0.28 µM GA_3 to evaluate the effectiveness of shoot tip culture in eliminating CABMV from the previously

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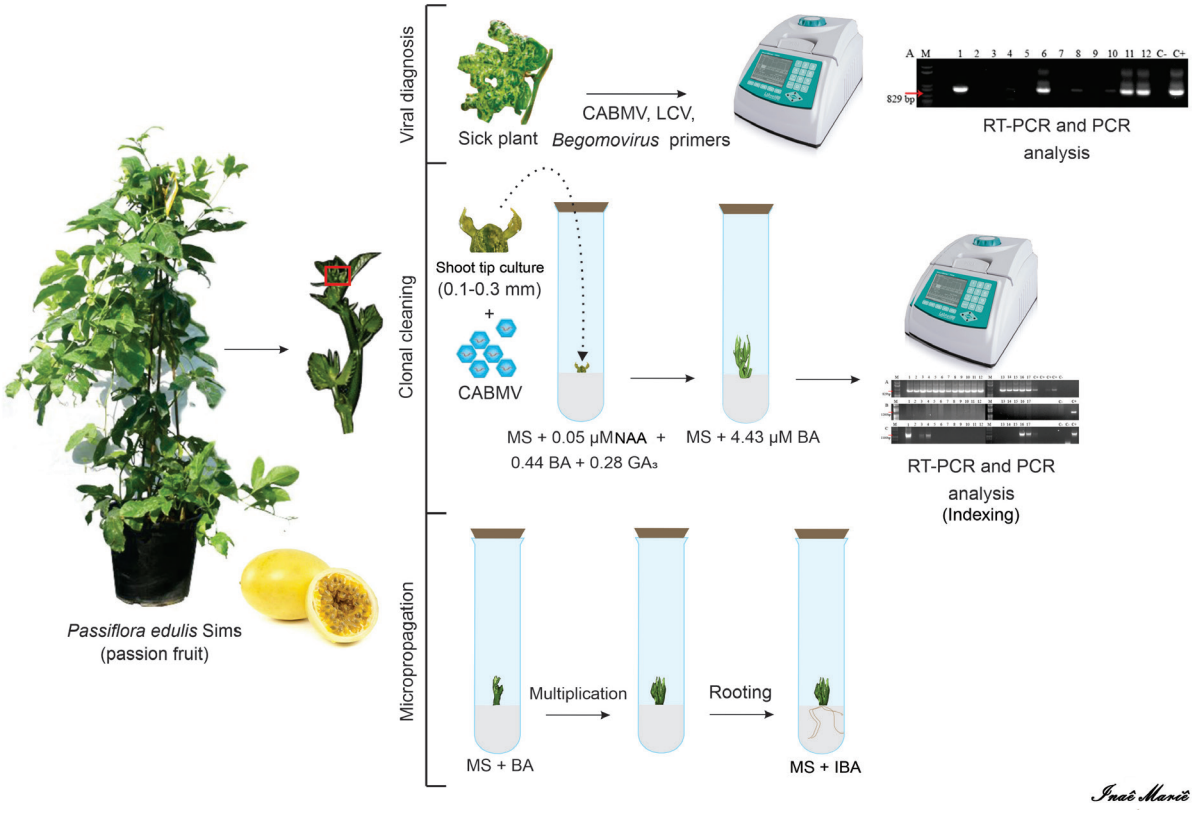
diagnosed matrices. After shoot tip regeneration, the explants were transferred to an MS medium containing 4.43 µM BA to stimulate bud proliferation and multiplication. Bud clusters obtained from the shoot tip culture were used for CABMV indexing by RT-PCR to confirm whether virus eradication had occurred. Viral eradication was only observed in one sample of the CPGA1 genotype. Subsequently, a multiplication protocol was established to increase the material upon regeneration, and using MS medium supplemented with 4.43 µM BA, shoot development of at least 6 mm was obtained after 30 days of cultivation. The material from the regenerated shoots was then used for subsequent multiplication, elongation, and rooting experiments. It was concluded that the culture of shoot tips is capable of providing virus eradication, although it requires optimization of the steps. Combining complementary techniques, such as thermotherapy, can enhance this eradication. Finally, the regenerated material from *in vitro* culture was used for the subsequent multiplication, elongation, and rooting experiments.

Graphical Abstract



Introduction

From the Passifloraceae family, passion fruit is a plant with a pantropical distribution covering 20 genera and around 500 species (Bernacci et al., 2020). Brazil is the primary center of diversity (Vidal et al., 2021; Fonseca et al., 2022), with about 157 native species, of which 87 are endemic (Bernacci et al., 2020). The genus *Passiflora*, from tropical America, is the most significant in the family (Oliveira & Ruggiero, 2005).

Passion fruit, particularly *Passiflora edulis* f. *flavicarpa* Degener, is one of the most important fruits in the country (Meletti, 2011). Its cultivation has advanced significantly over time. *P. edulis* fruit is abundant in antioxidants, flavonoids, anti-inflammatory, antibacterial, antifungal, antidiabetic, and anti-aging compounds present in all its parts, such as the seeds, bark, flower, and pulp (Jusuf et al., 2020; Gupta et al., 2022; Santos et al., 2022). Brazil leads the world in passion fruit production, with an annual yield of 697.8 thousand tons (Fonseca et al., 2022; Instituto Brasileiro de Geografia e Estatística, 2023). The Northeast region is the primary producer nationally. Meanwhile, the Distrito Federal achieves the highest yield (27.68 t/ha) due to the use of cultivars and effective management techniques (Meletti, 2011). However, cultivating passion fruit can be quite challenging due to its susceptibility to diseases caused by viruses, bacteria, and fungi. The necessity for frequent changes in cultivation areas arises from these pathogenic attacks (Meletti, 2011), often resulting in economic losses of up to 60% (Viana et al., 2014; Pallás et al., 2018; Singh et al., 2023).

The most common symptom observed in viral infections is a decrease in growth rate, leading to dwarfism or atrophy in the plant. Other noticeable symptoms typically appear on the

leaves, disrupting chlorophyll production and, consequently, photosynthesis. This results in the development of mosaics and ring spots, typical symptoms of systemic viruses, resulting in the emergence of light green, yellow, or white areas, mixed with the normal green of the leaf or fruit (mosaics). Ringspots are identified by chlorotic (yellow) or necrotic rings on leaves, stems, or fruits. Symptoms like leaf curling, yellowing, canker, and tumors are frequently observed (Mukhopadhyay, 2010).

Passion fruit woodiness disease (PWD), caused by potyviruses, is a major constraint for passion fruit cultivation. This viral disease has a significant impact on the yield and lifespan of plants, affecting all parts of the passion fruit plant (Cerqueira-Silva et al., 2014). In severe cases, it can lead to crop losses of up to 100% (Otipa et al., 2011). Through phylogenetic studies, it was possible to identify the cowpea aphid-borne mosaic virus (CABMV), a member of the genus *Potyvirus* in the family *Potyviridae*, as the primary causative agent of PWD, the most prevalent viral disease affecting passion fruit in Brazil (Nascimento et al., 2006). CABMV has a single-stranded, positive-sense RNA genome with approximately 10,000 nucleotides (Mink et al., 1994; Nascimento et al., 2004). Aphids act as vector for CABMV, with typical symptoms including mosaic patterns, wrinkling, deformation, and blistering on the leaf blade (Sampaio et al., 2008). Infected fruits display increased hardness, reduced size, and decreased pericarp, affecting fruit quality and hindering commercialization (Nascimento et al., 2006; Gomes et al., 2022). Furthermore, the current management and control methods for PDW are considered ineffective (Preisigke et al., 2021; Santos-Jiménez et al., 2022). These factors collectively reduce the orchards' lifespan, leading to significant economic losses for producers.

Mixed viral infections are common in nature and have distinct implications for plants, often resulting in more severe symptoms or mutual exclusion.Several emerging viruses have been identified in passion fruit crops worldwide, including in Brazil. These include *Passiflora edulis* symptomless virus (PeSV, genus *Roymovirus*, family *Potyviridae*), passion fruit chlorotic mottle virus (PCMoV, genus *Citlodavirus*, family *Geminiviridae*) (Fontenele et al., 2018), lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Closteroviridae*) (Vidal et al., 2021), cucurbit aphid-borne yellows virus (CABYV, genus *Polerovirus*, family *Solemoviridae*) (Vidal et al., 2018), as well as the more recent bean-associated cytorhabdovirus (BaCV, species *Cytorhabdovirus caricae*, genus *Cytorhabdovirus*, family *Rhabdoviridae*), and cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family Betaflexiviridae) (Vidal et al., 2023).

The presence of more severe symptoms in mixed infections has been documented in various studies. For example, studies involving potato virus X and potato virus Y in *Nicotiana tabacum* (Rochow & Ross, 1955) and potato virus Y (PVY, genus *Potyvirus*, family *Potyviridae*) and potato leafroll virus (PLRV, genus *Polerovirus*, family *Solemoviridae*) in potatoes (*Solanum tuberosum*) (Srinivasan & Alvarez, 2007) have shown this phenomenon. In the case of CABMV, its interaction with other viruses has been observed in bean cultivars. Combinations involving CABMV have been found to increase the occurrence of apical necrosis and plant death (Taiwo et al., 2007). Additionally, the presence of CABYV and CABMV were identified in passion fruit plants in the state of Bahia, Brazil (Vidal et al., 2018). Therefore, it is crucial to identify the agents responsible for causing diseases in plants to implement appropriate management strategies.

Currently, PCR is widely utilized in viral diagnosis. In passion fruit, PCR has been employed to identify DNA viruses, such as those belonging to the *Begomovirus* genus (Vaca-Vaca et al., 2016). Additionally, given the range of RNA viruses that affect passion fruit, RT-PCR is commonly used for diagnosing such viruses. For instance, RT-PCR was used to detect CAMBV in *Passiflora* species during studies on the genetic improvement of the crop (Gonçalves et al., 2018). In another study, RT-PCR was applied to passion fruit plants exhibiting symptoms of fruit hardening, using specific primers for the virus typically responsible for the disease in Japan. However, in this instance, the virus was not detected using the technique, requiring the genomic sequencing of the plants under investigation, which led to the identification of a potential new virus (Riska et al., 2019). In addition to transmission by vectors like aphids, viruses can also be transmitted through seeds and vegetative propagation (Kraus et al., 2010). In such cases, infected plants need to undergo cleaning before propagation or commercialization. Clonal cleaning methods are essential for producing plants free of pathogens. These methods include chemotherapy, thermotherapy, meristem/shoot tip cultures, and cryotherapy. A diagnosis must first be conducted to identify the disease-causing agent. Following the application of clonal cleaning methods, an indexing step is performed to verify the elimination of the pathogen. Typically, this indexing is carried out using ELISA and PCR techniques (Prammanee et al., 2011; Wang et al., 2018a; Bettoni et al., 2019; Ita et al., 2020; Kazemi et al., 2020). While meristem or shoot tip cultures followed by micropropagation are commonly utilized for clonal cleaning in different species, there is limited literature on the implementation of meristem culture in *Passiflora* species. One study accomplished clonal cleaning of *P. edulis* using shoot tip culture, yielding 26 acclimatized plants free from the tested virus (passion fruit woodiness virus - PWV). This highlights the possibility of conducting clonal cleaning exclusively through shoot tip culture (Prammanee et al. 2011).

For effective viral eradication, the meristem/shoot tip culture technique typically requires explant sizes within the range of 0.1 to 0.3 mm (Biricolti & Chiari, 1994; Retheesh & Bhat, 2010; Nerway et al., 2020; Kazemi et al., 2020). While some literature mentions the use of larger explant sizes (2 mm) for virus elimination, it is generally advised to associate shoot tip culture with other techniques, such as thermotherapy, when working with explants larger than 0.5 mm (Ramgareeb et al., 2010; Prammanee et al., 2011; Vivek & Modgil, 2018). Despite being a widely employed technique globally, the percentage of plant regeneration from meristems can be relatively low for some plant species due to factors like nutrient medium composition, concentration and combination of growth regulators, luminosity and growth temperature, explant size, and species type. Considering the challenges encountered by passion fruit producers due to viruses affecting this crop, this study aimed to conduct a viral diagnosis of passion fruit matrices through RT-PCR/PCR, with a primary focus on CABMV. Additionally, the efficacy of isolated shoot tip culture in eradicating the virus was assessed through RT-PCR/PCR indexing post-regeneration of the material *in vitro*.

Material and methods

All samples used in the experiments were collected in a greenhouse at the Plant Genetic Innovation Center of Embrapa Cerrados, located in Riacho Fundo II, Brasília, Distrito Federal, Brazil. Afterward, the collected material was transported over a distance of around 30 km to the Embrapa Genetic Resources and Biotechnology, where the experiments were carried out.

Genotypes used in the experiments

The genotypes used (CPMSC1, CPGA1, MR1, CPMGA2, CPF1SSBR) are parents of cultivars well established by Embrapa. The crossing of these five genotypes can result in cultivars called BRS Gigante Amarelo, BRS Sol do Cerrado, BRS Rubi do Cerrado, and BRS Ouro Vermelho (Embrapa Cerrados, 2019, 2020). At the time of collection, various symptoms were observed in the plants, including mosaic patterns of chlorosis, leaf wrinkling, and fruit deformity (Figure 1). Furthermore, many plants had dry branches, and the presence of insects and fungi throughout the plant. For this reason, these plants required a virus-free method of reproduction, as they were no longer able to reproduce through seeds. They had ceased producing fruit, and vegetative propagation was not viable due to the lack of growth in the plantlets.

Diagnosis's plant material

To identify viruses in passion fruit samples from a greenhouse, young leaves (around 9 cm) from symptomatic *P. edulis* Sims plants were initially collected with pruning shears and placed in labeled plastic bags. The samples were then transported to a laboratory, where they were stored at −80 °C and subsequently examined using PCR and RT-PCR to verify the presence of CABMV, LCV, and begomoviruses in the leaves of the five matrices under investigation.

Micropropagation and shoot tip culture plant material

To conduct a micropropagation experiment and another for virus eradication cleaning through shoot tip culture, branches around 15 centimeters long were collected and stored in a designated plastic bag, kept moist with water sprays to prevent drying during transportation. The branches were then moved from a greenhouse to the laboratories for immediate handling and inoculation. Multiple collections were done on various days to avoid prolonged storage of the material.

Detection of viruses

The screening of plants within the greenhouse aimed to detect CABMV, LCV, and begomoviruses. Nucleic acid

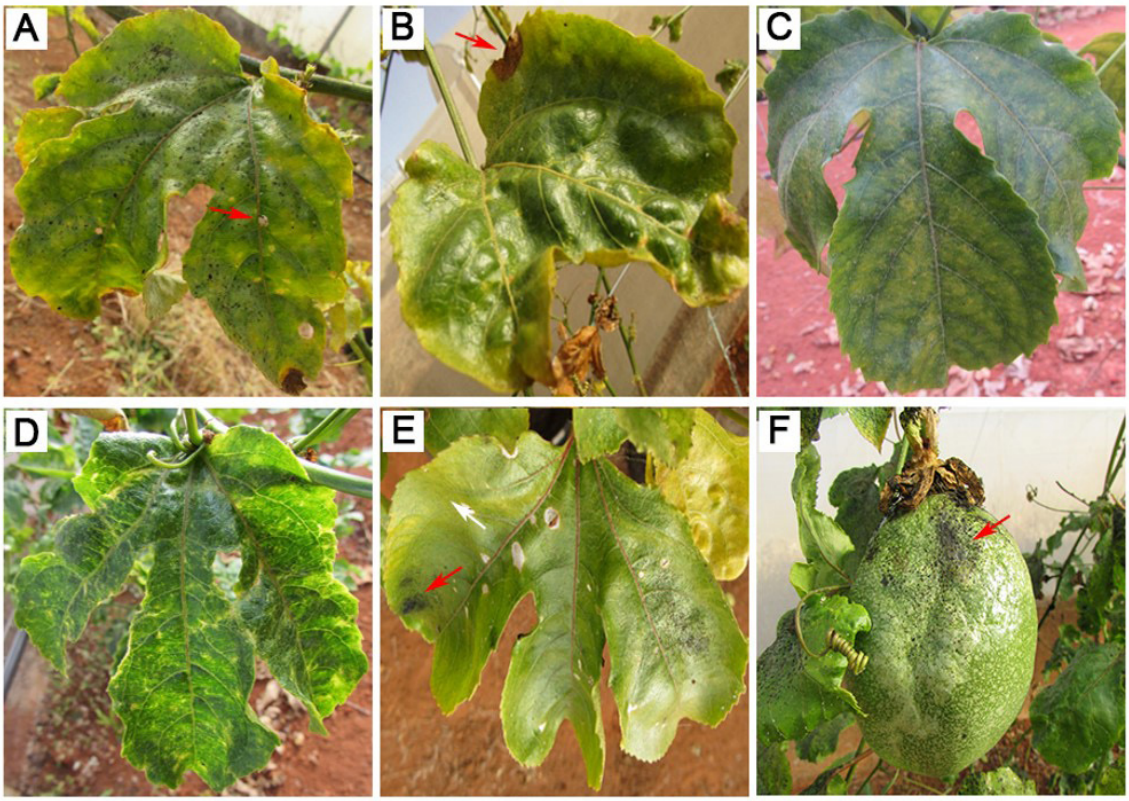


Figure 1. Symptoms and aggravating factors observed in different passion fruit (*Passiflora edulis* Sims) matrices used in viral diagnosis and virus eradication experiments. (A) CPMSC1 – leaf with the presence of fungus, mealybugs (red arrow), and yellowed edges; (B) CPGA1 – leaf showing wrinkling, yellowing edges and necrosis (red arrow); (C) MR1 – leaf with mosaic pattern; (D) CPMGA2 – leaf with yellow spots; (E) CPF1SSBR – presence of fungus (red arrow) and yellowing on the leaf (white arrow); (F) Fruit of genotype MR1 showing deformity and presence of fungus (red arrow) on the surface (color).

extraction involved obtaining three leaf discs from each accession (100 – 200 mg), excluding the leaf's central vein. The collected leaf discs were stored in a freezer at -80ºC. Subsequently, the leaf discs were macerated in liquid nitrogen, and the RNA and DNA extraction from the samples followed the Trizol® reagent (Invitrogen, Carlsbad, CA, USA), and Doyle & Doyle (1990) protocol, respectively. For the detection of CABMV and LCV, cDNA was synthesized with MMLV reverse transcriptase (RT) (Promega, USA) and random hexamer primer with total RNA, following the manufacturer's instructions.

To identify CABMV, a pair of primers, CABMV_CP_9250F and CABMV_CP_10115 R (Table 1), was developed using the CABMV isolate HQ880243 sequence (unpublished data). These primers target the virus's coat protein gene and were employed in PCR tests with Taq DNA Polymerase, recombinant (5 U/µL) (Invitrogen, Carlsbad, CA, USA), and extracted cDNA. The choice to use primers that encode the virus's protein coat was influenced by their proven effectiveness in previous research studies, demonstrating their specificity (Maciel et al., 2009; Melo et al., 2015). For LCV detection, RT-PCR was conducted under the same conditions as the previous assay, utilizing the primer LCV_RNA2_2793F and LCV_RNA2_3997R (Table 1), according to Vidal et al. (2021). To detect viral DNA, specifically for begomovirus, DNA was initially enriched for circular viral DNA using rolling circle amplification (RCA) with the Illustra TempliPhi amplification kit (GE Healthcare, Chicago, IL, USA). Then, the RCAs obtained were amplified in PCR assays using the PAL1v1978 and PAR1c496 (Table 1), these primer combinations are considered to be begomovirus universal primers, suitable for initial screening (Rojas, 1993). All DNA fragments were separated through electrophoresis in a 1% agarose gel and visualized under ultraviolet light. RT-PCR and RCA-PCR products were subjected to Sanger sequencing at Macrogen Inc. (South Korea) to confirm their identity. The obtained nucleotide sequences were analyzed and compared using the BLASTn tool available at the GenBank database.

Excision and establishment of the shoot tip for virus eradication

To start the clonal cleaning of the explants, the collected branches were trimmed to about 3 cm, and the outermost layers of the leaves were removed to aid handling in the laminar flow chamber and prevent *in vitro* contamination. Next, the shoots were dipped in 70% alcohol (v/v) for 1 minute, sodium hypochlorite (NaClO) (1% active chlorine), and three drops of Tween-20 for 15 minutes, followed by three rinses in distilled and autoclaved water.

The shoot tip, comprising the shoot apical meristem with up to two primordial leaves (0.1 – 0.3 mm), was transversely sectioned (Figure 2) using tweezers and scalpels. The explants were then introduced into test tubes (25 x 150 mm) containing 10 mL of culture medium. The culture medium consisted of MS salts (Murashige & Skoog, 1962) with 300 mg L^{-1} of activated charcoal and supplemented with 0.05 µM of 1-naphtaleneacetic acid (NAA), 0.44 µM 6-benzylaminopurine (BA), and 0.28 µM gibberellic acid (GA_3). To solidify the culture medium, 2.5 g L^{-1} of Phytagel® (Sigma) was added, following the protocol described by Fortes & Scherwinski-Pereira (2003), with modifications.

Following inoculation, the explants were incubated in darkness at 25±2ºC for seven days. Subsequently, they were exposed to the same temperature under a photoperiod of 16 hours and irradiation of 100 µmol m^{-2} s^{-1}, using "Daylight" LED lamps (Phillips). A completely randomized design consisting of five treatments (genotypes) with 22 repetitions each was utilized, with one explant per test tube. After 30 days, the explants were evaluated for survival percentage (greenish and swollen explants), number of primordial leaves, oxidation (explants showing total browning), and contamination. The data used to estimate the percentage of regeneration and oxidation were transformed by arc sine $\sqrt{x}/100$, while the number of primordial leaves was transformed by $\sqrt{x}$. Afterward, the transformed data underwent analysis of variance, and if significant differences were observed, means were compared using Tukey's test at a 5% probability level, utilizing the Sisvar statistical software.

Explants with a minimum of two visible buds were separated with the aid of a stereomicroscope, scalpel, and tweezers to propagate the material for indexing. The isolated bud aggregates were then transferred to a culture medium containing MS salts supplemented with 4.43 µM BA. To solidify the culture medium, 2.3 g L^{-1} of Phytagel® (Sigma) was added.

Establishment of microcuttings for *in vitro* multiplication

To evaluate a form of micropropagation for material from shoot tip culture, an experiment was conducted using microcuttings. In the lab, branches were trimmed to around

Table 1. Information regarding primer pairs utilized in diagnostic and indexing experiments in *Passiflora edulis* Sims.

Virus	Primer	Nucleotide Sequence	TmºC
Cowpea aphid-borne mosaic virus	CABMV_CP_9250F	GTGAGACGATAACTGTGGCGA	55 ºC
	CABMV_CP_10115R	CAGAAGATGTTGTGCTCCA	
Lettuce chlorosis virus	LCV_RNA2_2793F	AAGGTTCAGATCCGTTCATCTTGTA	60 ºC
	LCV_RNA2_3997R	CTTCCACGCATTCTCTGAATAAGTC	
Begomovirus	PAL1v1978	GCATCTGCAGGCCCACATYGTCTTYCCNGT	55 ºC
	PAR1c496	AATACTGCAGGGCTTYCTRTACATRGG	

*Tm = Melting temperature.

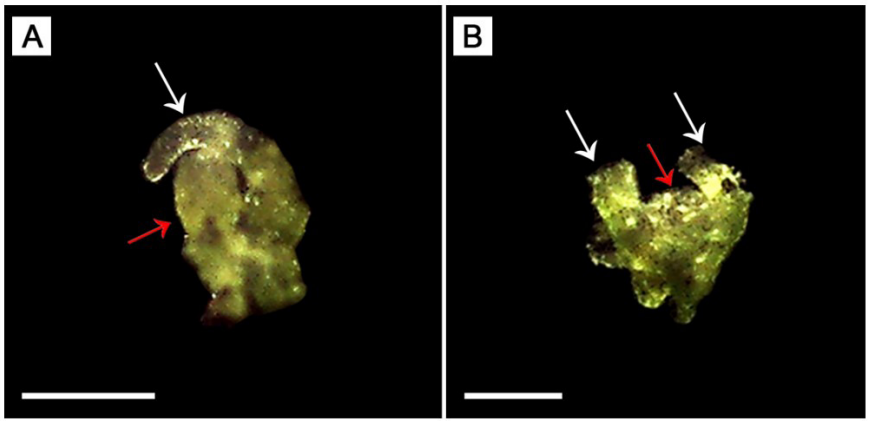


Figure 2. Shoot tip excised from passion fruit (*Passiflora edulis* Sims) matrices used to attempt virus eradication. (A) Explant (0.2 mm) containing only one primordial leaf (white arrow) and the apical stem meristem (red arrow); (B) Explant (0.1 mm) containing two primordial leaves (white arrows) and the apical stem meristem (red arrow). Bars = 0.25 mm.

3 cm, with all leaves removed, leaving only one axillary bud on each microcutting. Aseptic procedures were followed as previously described.

Using tweezers and scalpels, the material was further reduced into microcuttings measuring about 1 cm, which were then inoculated into test tubes with 10 mL of culture medium containing MS salts (Murashige & Skoog, 1962) supplemented with 4.43 µM of BA (Severin et al., 2011; Anand et al., 2012). To solidify the culture medium, 2.5 g L^{-1} of Phytagel® (Sigma) was added. The material was maintained at 25±2ºC, with 16 hours of photoperiod, and light irradiation of 100 µmol m^{-2} s^{-1} provided by "Daylight" LED lamps (Phillips). The experimental design was completely randomized with five treatments (genotypes) and 30 repetitions, with each repetition consisting of one explant. Evaluations were conducted every 15 days, considering the height of the buds and the development percentage (microcuttings with buds larger than 3 mm). After 45 days, the preexisting buds were separated from the microcuttings and transferred to test tubes containing 10 mL of the same culture medium used initially. The buds were kept under the same environmental conditions as in the previous experiment. The design was completely randomized in a 5 x 3 double factorial scheme, characterized by five genotypes and three subcultures, totaling 15 treatments. Each treatment consisted of 14 replicates, with one explant per experimental unit.

In total, three monthly subcultures were conducted, along with an evaluation at the end of each subculture. The explants were assessed for the number of buds per explant and contamination (%). Following the methodology described by Oliveira et al. (2008), the estimation of the number of shoots produced per explant was obtained by calculating the ratio between the number of subcultures (ns) and the average number of shoots produced per explant (ANS^{ns}), represented by estimated multiplication rate (Emr). These data allowed the deduction of the average number of plants obtained with the three subcultures. The data were transformed using $\sqrt{x}$.

For the further development of the shoot, various combinations of growth regulators and MS medium concentrations were tested. Propagules measuring approximately 5 mm in diameter from the *in vitro* multiplication of the CPMGA2 and MR1 genotypes were transferred to tubes containing 10 mL of MS (Murashige & Skoog, 1962) or MS medium with half the concentration of salts, both supplemented with 30 g L^{-1} of sucrose and BA at concentrations of 0, 0.1 and 1.0 µM, respectively, as proposed by Kawata et al. (1995). To solidify the culture medium, 2.5 g L^{-1} of Phytagel® (SIGMA) was added. The experimental design was completely randomized in a 2x2x3 triple factorial scheme, characterized by two genotypes, two concentrations of MS salts, and three different concentrations of BA, totaling 12 treatments, each with ten repetitions, with one explant per experimental unit. After 30 days, the explants were evaluated for height and oxidation/yellowing (%).

For shoot rooting, the propagules of the genotypes CPMSC1, CPGA1, MR1, and CPMGA2 were transferred to test tubes with 10 mL of culture medium. The medium consisted of MS salts (Murashige & Skoog, 1962) supplemented with indole-3-butyric acid (IBA) at different concentrations of 0, 1.2, 2.4, and 4.9 µM. To solidify the culture medium, 2.5 g L^{-1} of Phytagel® (Sigma) was added. The experimental design was completely randomized in a 4x3 double factorial scheme, characterized by four genotypes and three concentrations of IBA, totaling 12 treatments. Each treatment consisted of 10 replicates, each comprising one explant. After 45 days, the explants were evaluated for root length, number of roots, percentage of rooting, and oxidation.

The data from all experiments were subject to analysis of variance. When significant differences were observed, the means were compared using the Scott-Knott test at a 5% probability, using the Sisvar statistical software.

Results

Detection of viruses

In all the samples, CABMV was detected in the RT-PCR assay, and a fragment of approximately 829 bp was observed

(Figure 3A). One representative sample with a fragment of the expected size was selected and sequenced, and the presence of CABMV was confirmed. Considering that primers exhibit high specificity by binding to particular locations in the genome, their sensitivity may be impacted for the same reason. Using primers that target various regions of a pathogen's genome could serve as a solution to this issue. Nevertheless, sequencing the acquired material remains the optimal choice to eradicate any potential errors.

The alignment and evaluation of the identity between the sequenced isolate in this study and other CABMV viruses with sequences deposited in the NCBI database showed an identity of 97% with other CABMV isolates from passion fruit (GenBank accession numbers – KF725715, KF725308, and KF725707). LCV was not detected in any of the samples tested in the RT-PCR assay (data not shown). The begomovirus amplicons were identified with universal primers pAL1v1978 and pAR1c496 (Rojas, 1993) in three samples of the CPGA1 genotype, representing 17.6%, and in two samples of CPMSC1, representing 11.7% of the total samples collected (Figure 3B).

Excision and establishment of the shoot tip for clonal cleaning

With the establishment of the shoot tips in the medium, swelling of the explants was observed after approximately five days of cultivation for all genotypes (Figure 4A). This was followed by the greening of the explants and the appearance of new primordial leaves at 10 days (Figure 4B). After this period, the explants gradually increased in size until the last evaluation was conducted at 30 days (Figure 4C). However, the number of greenish explants decreased over time. The oxidation rate encompasses both explants that did not become greenish and those that became greenish and lost color in subsequent days.

At the end of the 30-day establishment period, the survival rate of the genotypes ranged from 22.7% to 68.1%, with the highest value attributed to genotype CPMGA2, despite statistically differing only from the CPMS1 genotype (Table 2). The analysis of variance did not detect a significant difference among the genotypes in terms of the mean number of primordial leaves per explant (Table 2). The oxidation rate ranged from 31.8% to 74.2%, with the highest value observed

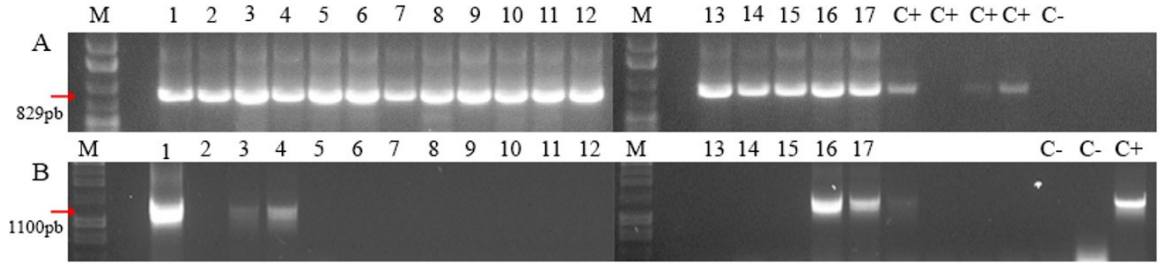


Figure 3. RT-PCR and PCR analysis for detecting cowpea aphid-borne mosaic virus (CABMV) and begomovirus in different genotypes of *Passiflora edulis* Sims. (A) Agarose gel electrophoresis of the PCR products of the CABMV coat protein gene with approximately 829 bp. M = Star 1kb DNA ladder (Cellco). Samples showing a bright band in the same height as the positive control are deemed positive for the virus. (B) Agarose gel electrophoresis of PCR products with begomovirus amplicons of approximately 1,100 bp. M = 1kb (1x biometric). C-: negative control. C+: positive control. Samples: 1-4 CPGA1; 5-7 MR1; 8-11 CPMGA2; 12-13 CPF1SSBR, and 14-17 CPMSC1.

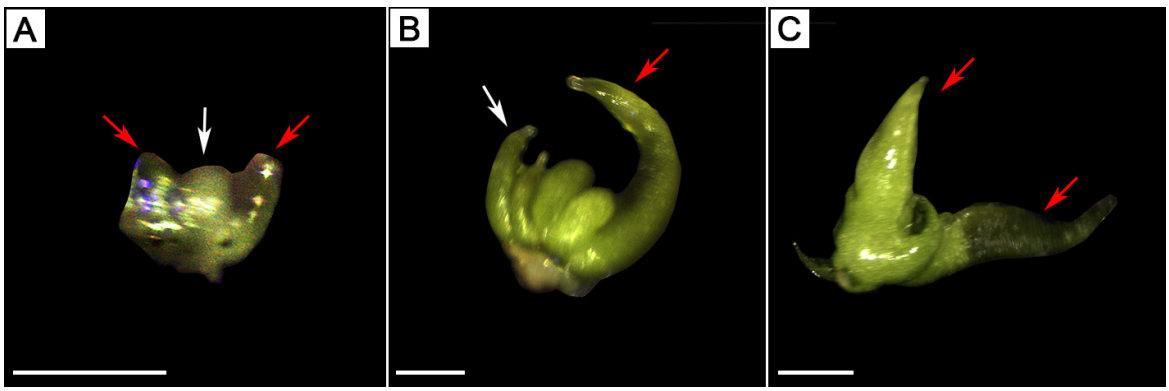


Figure 4. Apical/axillary shoot meristem regeneration phases of the CPGA1 genotype of *P. edulis* Sims grown in MS medium supplemented with different growth regulators. (A) Swollen explant after five days of cultivation, still presenting two primordial leaves (red arrow) and the apical stem meristem (white arrow); (B) Greenish explant with new primordial leaves after 15 days of cultivation, presenting a long leaf (red arrow) and other smaller leaves (white arrow); (C) Explant with more elongated primordial leaves (red arrow) after 30 days of cultivation. Bars = (A-B) 0.5 mm; (C) 1 mm.

in genotype CPMSC1 (Table 2). In general, the contamination rate was null in most genotypes, except for CPMSC1, which presented 4% of contamination.

To index the propagation material from the meristem culture, whole explants were collected from each of the genotypes, consisting of the micropropagated bud aggregates described above. Clearing the infection by CABMV, the prevalent virus in all tested samples, was unsuccessful, with the infection rate remaining at around 83%. These showed an amplified fragment of approximately 829 bp using the combination of primers CABMV_CP_9250F and CABMV_CP_10115R (Figure 5). On the other hand, in a sample of the CPGA1 genotype, such fragments were absent in samples 2, 3, and 5, indicating the possible elimination of the virus in these samples (Figure 5).

Establishment of microcuttings for *in vitro* multiplication

After microcutting inoculation, the development of pre-existing buds was observed from 15 days onwards in most matrices (Figure 6), although it was the CPMGA2 matrix that presented the most pronounced growth (Figure 6J-L). At 15 days, the CPGA1, MR1, and CPMGA2 matrices showed open leaves measuring approximately 1 cm (Figure 6E, H, K). At the end of the 30-day establishment period, most matrices presented multiple leaves measuring around 1 cm in the microcuttings (Figure 6C, F, I, L, O). However, the explants' yellowing was observed in this period, requiring the immediate transfer of the propagules to a new culture medium. The CPF1SSBR matrix showed little growth compared to the other genotypes (Figure 6M-O).

The CPGA1 matrix exhibited the highest average shoot height (1 cm), whereas the MR1 and CPF1SSBR matrices displayed the lowest values (0.6 cm) 30 days after inoculation in an MS medium containing BA. However, subsequent data analysis revealed no significant differences between genotypes concerning shoot height. The development percentage ranged from 71.4% to 90.4%, with the highest values observed in the CPMSC1 and CPF1SSBR genotypes and the lowest in genotype CPMGA2. Notably, although some genotypes exhibited greater shoot height, the number of explants demonstrating any development was comparatively lower. In general, the percentages of oxidation/yellowing and contamination did not affect the establishment of the genotypes. The material was transferred to a new medium after 30 days of cultivation to prevent explant losses attributed to yellowing.

Following the excision of the microcutting and inoculation in the same medium for multiplication purposes, a notable proliferation of buds was observed in most of the genotypes. From the initial subculture onward, clusters of buds emerged throughout the explant. These buds were isolated and inoculated into separate tubes for over 90 days (across three monthly subcultures). Results analysis revealed statistical variations among genotypes concerning the number of buds per explant. Regarding subcultures, each genotype exhibited an increase in multiplication rate, except for genotype CPF1SSBR (Table 3).

At the end of the last subculture (90 days), averages ranging from 1.4 to 6.3 buds/explant were recorded, with the highest values observed in the CPGA1, MR1, and CPGMA2 genotypes. However, no statistical differences were observed between the averages. The CPF1SSBR genotype exhibited the lowest average (1.4 buds/explant) (Table 3). The MR1 genotype displayed the

Table 2. Survival, oxidation, and mean number of primordial leaves per explant of five *Passiflora edulis* Sims matrices after 30 days in a medium containing NAA, BA, and GA_3.

Genotypes	Survival (%)	Oxidation (%)	Number of PL/E [(1)]
CPMSC1	22.7 b	74.2 a	2.1±0.1 a
CPGA1	40.9 ab	59.0 ab	2.3±0.1 a
MR1	40.9 ab	59.0 ab	2.1±0.1 a
CPMGA2	68.1 a	31.8 b	2.0±0.0 a
CPF1SSBR	40.9 ab	59.0 ab	2.0±0.0 a

[(1)] Mean number of primordial leaves per explant. Means ± standard error and percentages followed by the same letter do not differ from each other by the Tukey test, at 5% probability.

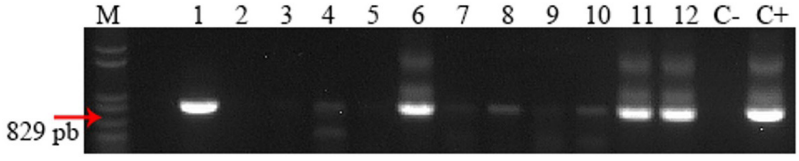


Figure 5. Indexing using RT-PCR for CABMV detection in *Passiflora edulis* Sims shoot tip culture, to confirm whether or not the virus has been eliminated. Agarose gel electrophoresis of PCR products of part of the cowpea aphid-borne mosaic virus (CABMV) viral capsid gene measuring approximately 829 bp. Samples showing a bright band in the same height as the positive control are deemed positive for the virus. M = Star 1kb DNA ladder (Cellco). Samples from meristem culture: 1 CPMSC1, 2-4 CPGA1, 5-7 MR1, 8-10 CPMGA2, and 11-12 CPF1SSBR.

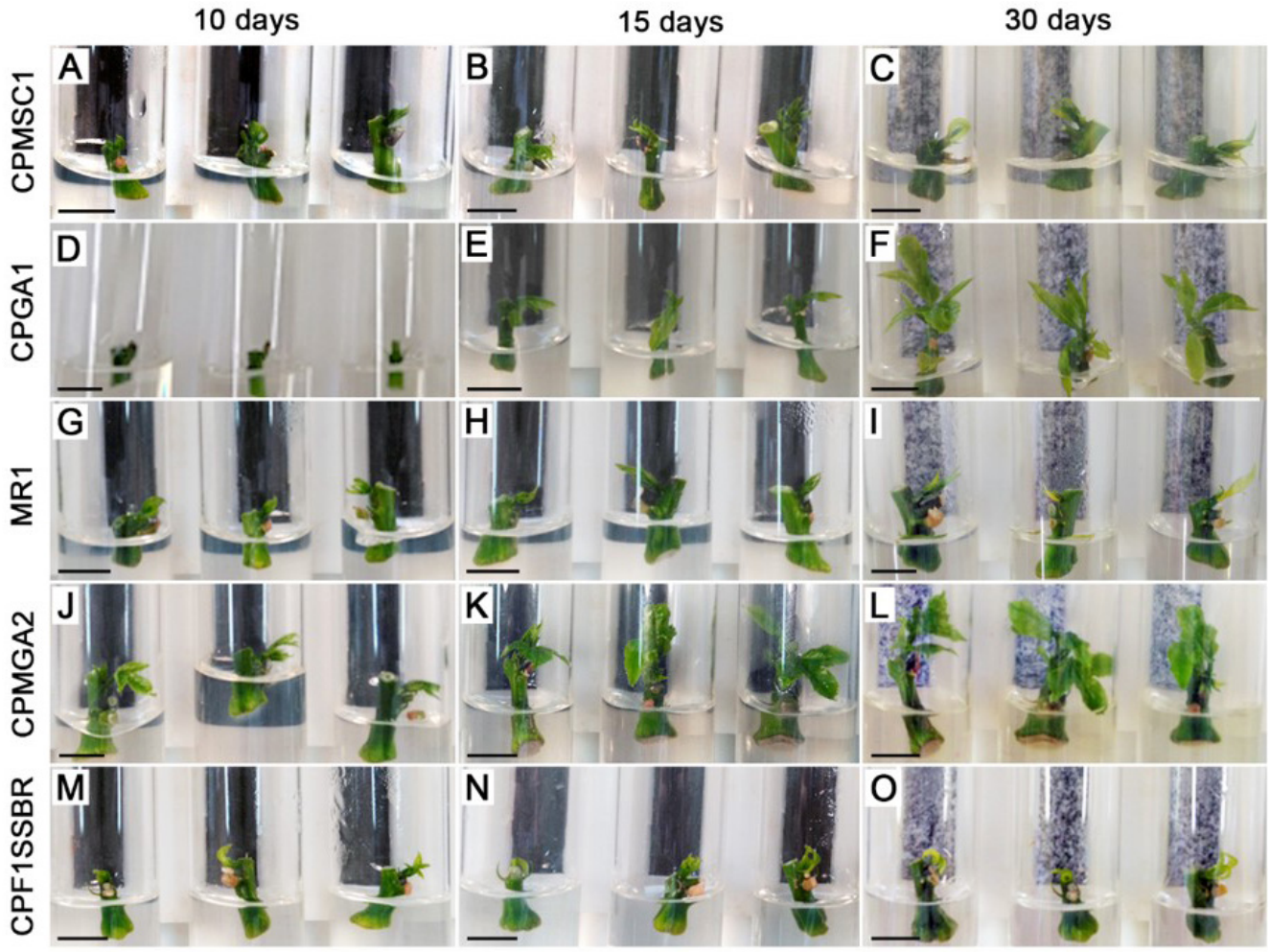


Figure 6. *In vitro* development of microcuttings of five *Passiflora edulis* Sims genotypes (CPMSC1, CPGA1, MR1, CPMGA2, and CPF1SSBR), observed 10, 15, and 30 days after inoculation in MS medium containing BA. Bars = 1 cm.

Table 3. Multiplication rate (buds/explant) of five genotypes of *Passiflora edulis* Sims after three monthly subcultures in MS medium supplemented 4.43 µM of BA.

Subcultures	Genotypes				
	CPMSC1	CPGA1	MR1	CPMGA2	CPF1SSBR
1	2.1±0.2 cA	2.6±0.2 bA	2.7±0.3 bA	2.8±0.2 bA	1.2±0.1 aA
2	3.5±0.3 bC	6.1±0.5 aB	8.2±0.5 aA	5.6±0.,5 aB	1.0±0.07 aD
3	5.7±0.4 aA	6.7± 0.4 aA	7.4±0.4 aA	6.7±0.4 aA	2.0±0.6 aB
Average	4.1±0.3 B	5.4±0.3 A	6.3±0.3 A	5.5±0.3 A	1.4±0.3 C
Emr	68	157	250	166	2.7

Means ± standard error followed by the same lowercase letter in the columns and uppercase letter in the lines do not differ by the Scott-Knott test at 5% probability. Emr = Estimated multiplication rate, each bud is believed to have the potential to transform into a plant.

highest estimated multiplication rate, suggesting the potential to obtain 250 buds with three subcultures. Each bud has the possibility of producing a plant (Table 3).

After 30 days of bud transfer to the elongation medium, no response was observed in the explants at any BA concentration or genotype, retaining the same characteristics as at time zero. Additionally, there was no observed oxidation or yellowing in the assessed period.

Given the lack of shoot development in previous experiments, the bud aggregates were transferred to a

medium containing IBA (indole-3-butyric acid) to induce rooting for consequent acclimatization. Root emission was initially observed 21 days after the transfer to the rooting medium. After 30 days, roots exceeding 2 cm were observed in various replicates of the matrices. By the end of the 45-day experiment, several replicates emitted roots larger than 2 cm, with emission occurring solely in the presence of callogenic structures. The CPGA1 matrix exhibited the highest rooting percentage at 20% with an IBA concentration of 1.2 µM by the end of the experiment. However, the IBA concentration of 2.4 µM provided 10% rooting in the CPMSC1, CPGA1, and MR1 matrices. Concerning the number of roots, the concentration of 2.4 µM of IBA provided the best results, ranging between 0.1 and 0.6 roots/explant.

Root length was most significant in the CPGA1 genotype (0.1 cm) at the concentration of 2.4 µM of IBA. However, the concentration of 1.2 µM of IBA provided the highest mean value among different matrices, totaling 0.09 cm. Generally, the percentage of oxidation remained below 5%, regardless of the genotype or IBA concentration. Following the rooting experiments, for pre-acclimatization, explants with emitted some roots were transferred to plastic cups containing a commercial substrate (*Bioplant Misturadora Agrícola* Ltda., MG, Brazil) and sand in a 3:1 (v/v) ratio. They were then maintained in a refrigerated biological incubator (Percival I-35LLVL, Bonne, Iowa), with a constant temperature of 25 °C and photoperiod of 16 h.

Discussion

The utilization of specific primers in the diagnosis of plant viruses is a well-documented practice in the literature (Sasi & Bhat, 2018; Kazemi et al., 2020; Fresnillo et al., 2022; Das et al., 2023). Certified PCR and RT-PCR, known for their sensitivity and accuracy, have been adapted for the diagnosis of various virus species in different crops, serving as a valuable tool for clonal cleanup validation (Das et al., 2023). Nevertheless, precise knowledge of genomic sequences is essential for designing specific primers for pathogen detection through molecular methods (Lau & Botella, 2017; Hariharan & Prasannath, 2021). Studies focused on identifying viruses in plants, particularly in commonly grown crops, aid in tracking their spread. This knowledge can assist in implementing targeted control measures against particular viruses, preventing financial losses associated with ineffective protocols. In the present study, primers that amplify specific regions of the CABMV coat protein gene were employed, similar to other studies. Melo et al. (2015) used such primers in phylogenetic studies involving 10 CABMV isolates collected from various crop fields in Nossa Senhora do Livramento, Bahia, Brazil. In another investigation, Maciel et al. (2009) used primers for the CAMBV coat protein gene for indexing infected *Passiflora* species. In addition to CABMV, LCV-specific primers, and universal begomovirus primers were also used. The infection of plants with LCV is well-documented in various plant species, including tomatoes (Zhang et al., 2017) and sugar beets (Wisler et al., 2007). More recently, LCV has been identified in *Catharanthus roseus* (Favara et al., 2020) and *Passiflora* spp. (Vidal et al., 2021) in Brazil. However, in our study, *P. edulis* samples did not test positive for LCV. Vaca-Vaca et al. (2016) utilized universal begomovirus primers and detected DNA-A and DNA-B fragments in *P. edulis* leaf samples in Colombia. Furthermore, begomovirus has been recently identified in *P. edulis* in India (Venkataravanappa et al., 2022) and China (Ye et al., 2022).

The detection of multiple viruses in *P. edulis* has been previously documented in Brazil. Fontenele et al. (2018) reported the identification of a new geminivirus co-infecting a passion fruit plant with CABMV in the Midwest region. More recently, Vidal et al. (2021) identified *Passiflora* spp. samples positive for LCV and CABMV. According to Vidal et al. (2021), the effects induced by LCV in passion fruit are unknown, and the presence of CABMV may mask LCV symptoms. The presence of multiple viruses in a plant can lead to more severe symptoms, as shown by Taiwo et al. (2007), in combinations of CABMV with other viruses. Therefore, studying more than one virus in plant research can aid in understanding these biological relationships and how to manage these infections.

In the establishment phase of the shoot tip with up to two primordial leaves (0.1 – 0.3 mm), the survival percentages (greenish and swollen explants) varied among the genotypes analyzed, ranging from 22.7% to 68.1%. Biricolti & Chiari (1994) reported a higher survival value of *Passiflora edulis* f. *edulis* meristems (95.6%) after 70 days in DKW medium (Driver & Kuniyuki, 1984) supplemented with different concentrations of BA, IBA, and GA_3. This difference may be attributed to variations in the culture medium and phytohormones used. Notably, Biricolti & Chiari (1994) used meristems that were 0.2-0.4 mm in length. According to Scherwinski-Pereira and Fortes (2004), the explant size can hamper survival and development *in vitro*. Tiwari et al. (2011), in their study with sugarcane (*Saccharum officinarum*), corroborated this hypothesis, showing that larger explants had a higher survival rate.

Nonetheless, studies conducted with other species' meristem and shoot tip cultures reported varying responsiveness percentages. Retheesh and Bhat (2010) achieved a response rate of 23.2%, characterized by enlargement and elongation when culturing the shoot tips of *Vanilla planifolia*. Azad et al. (2020) reported responsiveness ranging from 51 to 78% in apical meristems of *Solanum tuberosum* L., considering different varieties and combinations of growth regulators. These findings with varying percentages may support the hypothesis that the significant genetic diversity within the *Passiflora* genus can impact its *in vitro* response, as proposed by Faria et al. (2007). Therefore, research focusing on developing tailored establishment protocols is crucial to address this issue.

Following the indexing of samples from the shoot tip culture, viral cleaning was confirmed in only one sample, with weaker bands appearing in the agarose gel. However, the explants used for diagnosis were very premature, necessitating another round of indexing after further growth to eliminate false-negative suspicion. Prammanee et al. (2011) successfully obtained PWV-free plants using the shoot tip culture technique in *P. edulis*, indicating its efficiency for clonal cleaning in passion fruit. Agüero et al. (2013) applied the meristem culture technique to eliminate citrus leaf blotch virus (CLBV) and citrus tristeza virus (CTV) from

Nicotiana benthamiana and *Citrus aurantifolia* achieving successful elimination only in the case of CTV.

Viruses eradication through meristems and shoot tip cultures is a widely employed method for eliminating viruses in various plant species. Retheesh and Bhat (2010) reported the successful elimination of cucumber mosaic virus (CMV, *Cucumovirus*) and cymbidium mosaic virus (CymMV, *Potexvirus*) from *Vanilla planifolia* plants by inoculating meristems measuring approximately 0.1 to 0.25 mm in MS medium containing 0.45 µM of Thidiazuron (TDZ). Using this technique, Yao et al. (2022) achieved a virus-free rate of 73.3% in *Salvia miltiorrhiza* plants. Several other studies have demonstrated the efficiency of this technique in obtaining virus-free plants in diverse species, including *Piper nigrum* (Sasi & Bhat, 2018), *Musa* AAB (Tchatchambe et al., 2020), *Manihot esculenta* (Apio et al., 2021) and *Allium cepa* (Lou et al., 2023).

Apart from meristem size, considered a critical factor in virus elimination (Azad et al., 2020) and regenerative capacity (Scherwinski-Pereira & Fortes 2004), other factors such as the meristem's position in the plant and certain viruses' ability to invade the meristematic region can affect the success of eradicating the virus through meristem culture (Das et al., 2023). Despite the results obtained in the virus eradication attempt in this study, these findings can contribute to developing more effective future strategies, such as combining shoot tip culture with other techniques. Another strategy to enhance the chances of virus elimination is by combining the meristem/shoot tip culture technique with thermotherapy, subjecting infected plants to temperatures between 37 ºC and 40 ºC for two to three weeks (Vivek & Modgil, 2018), or around 50 ºC for 40 minutes (Ramgareeb et al., 2010), followed by later extraction. In a recent study, Nerway et al. (2020) compared the efficiency of shoot tip culture, electrotherapy, and chemotherapy for eliminating the dahlia mosaic virus (DMV) from plants of the genus *Dhalia*. According to the authors, 100% virus-free plants were obtained by placing shoot apices measuring around 0.2 and 0.3 mm in MS medium. Cryotherapy, on the other hand, stands out due to the high frequency of pathogen-free plants (Feng et al., 2013) and the possibility of using larger explants (Wang et al., 2018b).

In numerous studies, the use of BA is deemed essential for the proliferation of buds in various *Passiflora* spp., albeit with differences in concentration. Vieira et al. (2014) noted that the type of explant and the optimal growth regulator for bud proliferation in the *Passiflora* genus vary among species. Hypocotyl segments and BA were identified as most suitable for bud proliferation in *P. setacea*. However, variations within studies on the same species are evident. For instance, in investigations conducted with *P. edulis*, BA concentrations range between 4.43 µM (Soares et al., 2012) and 10 µM (Kawata et al., 1995). Moreover, the use of cytokinins, primarily BA, in *in vitro* elongation experiments with *Passiflora* explants is commonly reported. Prammanee et al. (2011) employed different concentrations of BA for the elongation of regenerated explants from apical meristem cultures of *P. edulis*, achieving successful root formation and acclimatization with shoots elongated at a concentration of 4.43 µM. Dornelas and Vieira (1994) reported the inefficiency of BA in the elongation of *Passiflora* spp., a challenge also highlighted by Biricolti and Chiari (1994), aligning with the observations in this study.

At the end of the experiment, the *in vitro* propagated propagules were subjected to a rooting experiment due to the challenges encountered in promoting shoot development in earlier trials. The observed rooting percentages in the present study were lower compared to findings in other studies. Isutsa (2004) achieved 100% *in vitro* rooting in *P. edulis* shoots in the presence of 24.5 µM of IBA. Shekhawat et al. (2015) reported 98% rooting in MS ½ medium supplemented with 9.8 µM of IBA. Regarding the number of roots, results were similar to those obtained by Goyal et al. (2015) using 4.9 to 9.8 µM of IBA. In comparison, Isutsa (2004) recorded an average of three roots per explant in his most effective treatment (24.5 µM IBA). Junghans et al. (2014) investigated the influence of the explant size (stem apex) on the rooting percentage in *P. edulis*. They found that the percentages of rooted explants for initial sizes of 1, 0.5, and 0.25 cm were 18%, 5%, and 0%, respectively, emphasizing the impact of explant size on *in vitro* development. These authors also highlighted the influence of genotype on various *in vitro* responses, aligning with the observations in this study. Effective rooting is a crucial stage in the *in vitro* cultivation of specific species, making studies detailing their methodologies essential for overcoming the challenges of this phase.

Negative results and future perspectives

Despite successful studies promoting virus eradication in plants using only shoot tip culture, it was not possible in this study to confirm virus elimination in samples that tested negative in the PCR indexing test. Additionally, several samples showed clearly positive results for the tested viruses. These findings highlight the potential influence of genetic variability, infection stage, or researcher experience on virus eradication outcomes. Nevertheless, these results support the consideration of utilizing techniques associated with shoot tip culture in future research.

Conclusion

While virus eradication was confirmed in only one sample of the CPGA1 genotype, improvements should be made to increase efficiency in genotypes that remained positive for CABMV after treatment. Effective management of explants, particularly during the multiplication of bud aggregates from shoot apices, is crucial for success. Combining meristem culture with techniques like thermotherapy and cryotherapy may further enhance the likelihood of obtaining virus-free material.

Several factors may have contributed to the lower efficiency observed, particularly the advanced infection stage in donor plants, which were not actively growing. For apical meristem tissue to remain virus-free, cell growth must outpace viral transmission. Etiolation of plantlets prior to collection could help mitigate this issue, although efforts to establish such plantlets have so far been unsuccessful.

The study demonstrated that microcuttings aimed at multiplying bud aggregates provided efficient in vitro

multiplication, especially for the CPGA1, MR1, and CPMGA2 matrices. In 30-day subcultures, exponential growth was observed, making this stage optimal for mass multiplication of these genotypes. However, the low rooting percentage and shoot development in bud aggregates suggest the need for further studies exploring different phytohormone concentrations. Additionally, research is required to improve elongation of aerial propagules in clustered bud states.

Conflict of interests

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