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# Strategies for vegetative propagation and viral cleaning of a miniature ornamental pineapple hybrid

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**ABSTRACT.** This study assessed and compared different methods for vegetative propagation of a miniature ornamental pineapple hybrid (ORN-MUT), seeking to determine the best method for production of plantlets, as well as for removal of the PMWaV viral complex from plants cultured *in vitro*, for production of healthy parent plants. Pineapple wilt is a disease that can cause large economic and is caused by a viral complex called *Pineapple mealybug wilt-associated virus* (PMWaV). For this, four propagation methods were evaluated (conventional, stem sectioning, micropropagation and etiolation of nodal segments). The time necessary for each method and the number of plants formed were assessed. Stem tips (0.5 mm) were cultured and indexed for three PMWaV types. Conventional propagation produced 17 plantlets per plant in 566 days, stem sectioning produced 2.3 plantlets per stem in 591 days, while the conventional micropropagation technique produced 1,284 plants after four subcultures in 778 days. Stems etiolated for 60 days showed peak production in the second subculture, with 1,224 plants. This method required 883 days to obtain plants with ideal size for transplantation to the field. In turn, stems etiolated for 120 days produced 935 plants at the end of four subcultures, with peak output in the third subculture, in which the plants could be cultivated in the field after 943 days. Conventional micropropagation and etiolation for 60 days were the best methods for production of plantlets of the ORN-MUT hybrid. The results of this work showed that the cultivation of shoot tips is an efficient strategy to remove the PMWaV complex and obtain healthy mother plants and can be a useful tool for other varieties of pineapple.

**Keywords:** *Ananas comosus* (L.) Merrill; etiolation of nodal segments; indexation; conventional micropropagation; conventional propagation; stem sectioning.

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## Introduction

Ornamental pineapple plants are sold in the Brazilian flower market and exported to other countries, like Netherlands, Germany, Portugal and the United States (Carvalho, Braga, Santos, & Morais, 2005). The growing interest of consumers for ornamental varieties has prompted the development of new pineapple hybrids tailored for various floriculture categories, enabling the offer of plants with novel traits in the Brazilian market (Souza et al., 2012a; Souza, Costa, Santos-Serejo, & Souza, 2014).

Among them, a hybrid called ORN-MUT has desirable traits for sale as a potted plant. These include small size, attractive pink fruits without spines, and mainly recurrent flowering on the fruit crown, formed after the plant's complete cycle, which winds up generating a miniature pineapple plant. This hybrid has great potential for ornamental market as potted plant, for its beauty as well as for its compact size, very rare in pineapple plants.

Pineapple plants are generally propagated vegetatively by means of plantlets (Jiménez, Sanewski, Reinhart, & Bartholomew, 2018). Besides the risk of disseminating pests and diseases, propagation of pineapple plants by the conventional method is slow and uneven, requiring a long period for establishment of a new planting area (Souza et al., 2013).

Other methods of vegetative propagation, like stem sectioning and *in vitro* culture, are well known and can improve the production of disease-free plantlets in less time (Reinhardt, Matos, Cunha, & Lima, 2013). Nevertheless, irrespective of the propagation method used to produce plantlets of any pineapple genotype, the health of the parent plant must be guaranteed.

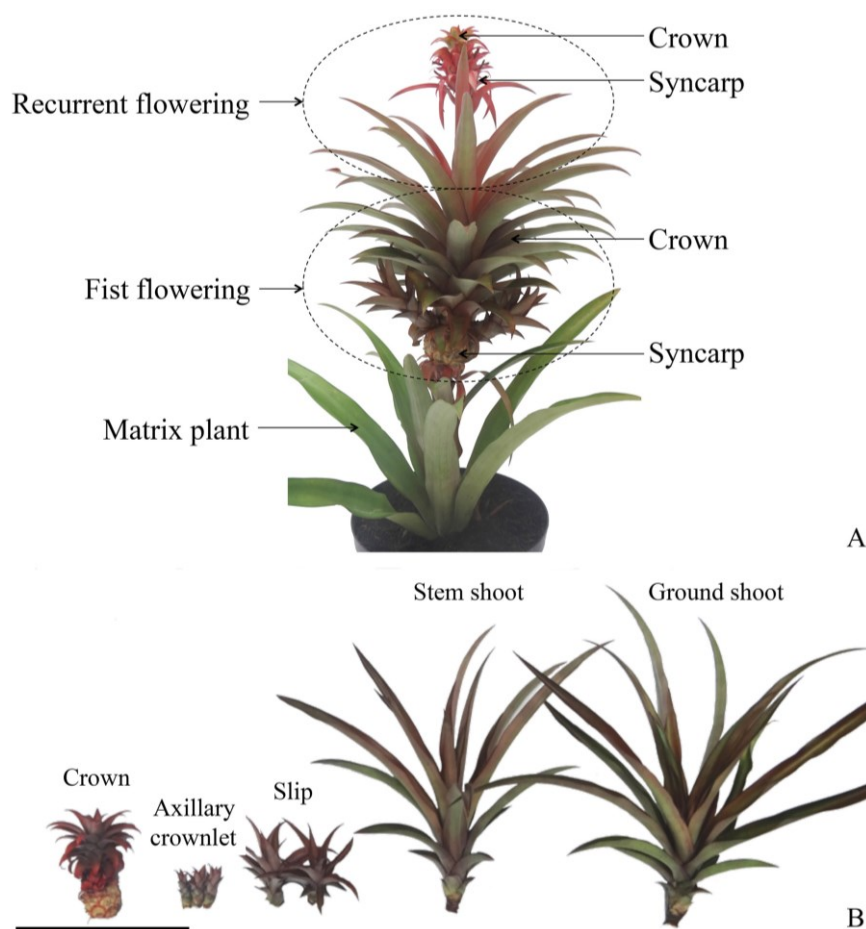
Pineapple wilt, caused by a viral complex called *Pineapple mealybug wilt-associated virus* (PMWaV 1-3), transmitted by the mealybug *Dysmicoccus brevipes* (Sether, Melzer, Busto, Zee, & Hu, 2005), is a disease that can cause large economic losses. The pineapple's vegetative propagation system is another way of spreading the virus and can result in a significant impact on the production of contaminated seedlings for new plantings. Concealed damages are a major concern, because in some cases contaminated plants do not present symptoms, which are only manifested when the plant infected by the virus is also colonized by the vector mealybug (Sether & Hu, 2002). This makes the selection of seedlings in the field for planting difficult.

The hybrid ORN-MUT has been shown to be susceptible to pineapple wilt by visual and molecular analysis of symptomatic plants in the field. The *in vitro* cultivation of shoot tips is a strategy with demonstrated efficacy in removing and cleaning the PMWaV complex in wild pineapple varieties (Botella, Hamill, Ko, Matsumoto, & Souza, 2018). The success of viral cleaning is highly correlated with the final size of the stem apex. The smaller the apex size, the greater the efficiency in viral cleaning and also the lower the chance of survival (Biswas, Hossain, & Islam, 2007).

The aim of this study was to evaluate and compare different methods of vegetative propagation (conventional propagation, stem sectioning, conventional micropropagation and etiolation of nodal segments) of the miniature ornamental pineapple hybrid ORN-MUT, to determine the best method for seedlings production, as well as for removal and cleaning of the viral complex (PMWaV) from plants cultivated *in vitro*, for production of healthy parent plants.

### Material and methods

The following methods for vegetative propagation of the miniature ornamental pineapple hybrid ORN-MUT were analyzed (Figure 1A): conventional propagation; propagation by stem sectioning; conventional micropropagation; and micropropagation by etiolation. For each method, 20 plants or plantlets were used as the starting point for the multiplication step.



**Figure 1.** A) ORN-MUT hybrid with complete flowering, highlighting the 'new plant' formed (recurrent flowering). B) region of the plant from which each type and size of conventional bud was obtained from the miniature ornamental pineapple hybrid ORN-MUT. Bars: 12 cm.

### Conventional propagation

After fruiting, the stems with fruit were maintained in the field for three months, after which the number of plantlets were counted originating from ground shoots, stem shoots, slips, crowns and slip-crowns, removing each type of material from the soil and separating the plantlets by type (Figure 1B), as described by Jiménez et al. (2018). For this evaluation, we considered the two flowering steps, that of the plant and of the crown (recurrent flowering).

### Stem sectioning

The stem sectioning was performed just after the first fruiting, without considering recurrent flowering. The plants were removed from the field and the roots, peduncle and leaves were carefully removed to expose the stem with buds. On this occasion, the length and diameter of each stem were measured and then they were sectioned longitudinally, immersed in a fungicide solution for five min. and cultivated in a commercial substrate (Vivato®) in production beds. The number of plantlets emerged was counted after 40 days. These were removed when they reached length of 10 cm and replanted in trays containing the same substrate in a greenhouse.

### Conventional micropropagation

Seven plants were retrieved from the field for *in vitro* establishment of the ORN-MUT hybrid by conventional micropropagation. The leaves were removed from the stalk, which was washed with commercial detergent before excision of the buds and subsequent disinfestation in a laminar flow chamber under aseptic conditions.

The disinfestation consisted of treatment of the plantlets in a 70% (v v<sup>-1</sup>) ethanol solution for 5 min. followed by immersion in a sodium hypochlorite solution at 2.0 to 2.5% for 20 min. and washing in autoclaved distilled water three times for one min. After this procedure, the plantlets were reduced by removing the excess tissues and were placed in test tubes containing 15 mL of MS nutritive medium (Murashige & Skoog, 1962) supplemented with 3% sucrose and solidified with 2.4 g L<sup>-1</sup> of Phytigel® autoclaved at 120°C for 20 min., with pH adjusted to 5.8. The tubes were kept in a growth room with controlled conditions of temperature (27 ± 1°C), photoperiod (16 hours fluorescent light) and photon flux density (40 µmol m<sup>-2</sup> s<sup>-1</sup>). After 45 days, the following parameters were evaluated: total number of buds, percentage of contaminated buds, percentage of oxidized buds; and percentage of surviving buds.

To start production of plantlets by conventional micropropagation, 20 plants were transferred to a multiplication medium composed of MS salts and vitamins, supplemented with 3% sucrose, 0.5 mg L<sup>-1</sup> of BAP, 0.2 mg L<sup>-1</sup> of ANA and solidified with 2.4 g L<sup>-1</sup> of Phytigel®, with pH adjusted to 5.8 before autoclaving at 120°C for 20 min. Four successive subcultures were performed, with intervals of 45 days, under the same incubation conditions, and the number of shoots formed after each period was counted.

### Micropropagation by etiolation of nodal segments

Two etiolation periods were analyzed, 60 and 120 days, during which 20 plants were incubated in the dark, for a total of 40 plants. For this purpose, the leaves were cut from plants after the fourth subculture by conventional micropropagation, reducing the size of the plants to approximately 2 cm. These plants were then established individually in test tubes containing MS medium supplemented with 3% sucrose, 1.0 mg L<sup>-1</sup> of GA<sub>3</sub>, solidified with 2.4 g L<sup>-1</sup> of Phytigel®, with pH adjusted to 5.8 before autoclaving at 120°C for 20 min., following methodology of (Souza, Canto, Souza, & Costa, 2010). The tubes were transferred to the growth room at 27 ± 1°C and kept in the dark for 60 and 120 days, with 20 plants (repetitions) for each interval, as described. At the end of each etiolation period, the stem length (cm) and number of internodes were determined.

For the multiplication test, the roots and stem tips were removed to break the apical dominance and thus induce growth of axillary shoots (Barboza & Caldas, 2001; Carvalho, Pinheiro, Dias, & Moraes, 2009). Then the entire etiolated stems were transferred to pots (13 height and 13 cm diameter) containing the same multiplication medium described for conventional micropropagation. The plants were kept under the same incubation conditions in the growth chamber. Four successive subcultures were performed, at intervals of 45 days, with determination of the number of shoots formed after each period.

### Statistical analysis

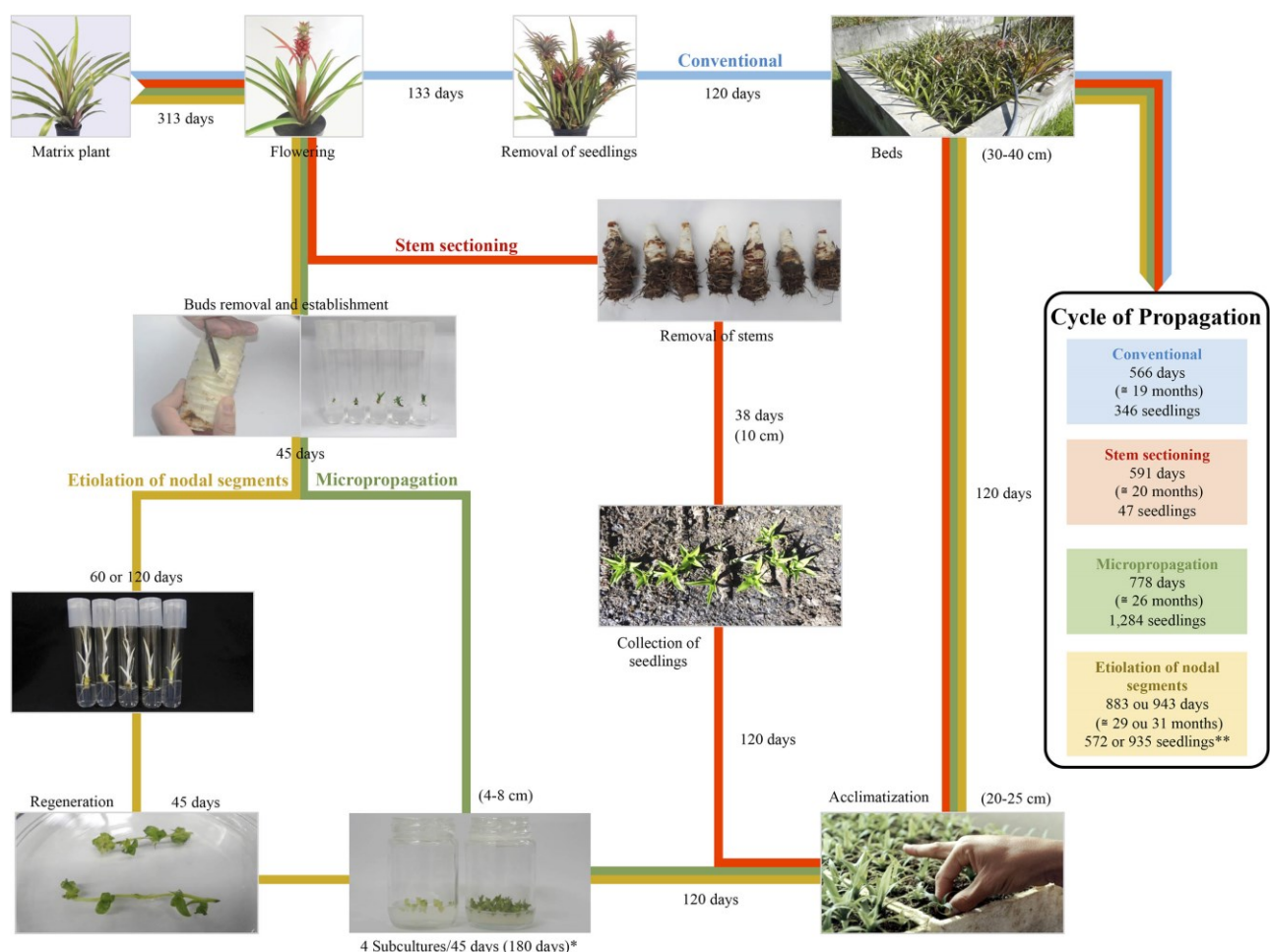
The following descriptive statistics for the conventional propagation and stem sectioning methods were calculated: mean, minimum, maximum, standard deviation and coefficient of variation. The design was completely randomized with 20 replicates per treatment. The data were submitted to analysis of variance and the means were compared by the Tukey test ( $p < 0.01$ ) in the case of significant values of the F-test, using the SAS statistical program (SAS Institute, 2010).

The propagative potential of the *in vitro* methods was measured by calculating the geometric growth rate (TCG) between two successive subcultures and between the first and last subculture, according to the method described by Silva et al. (2016). The design was completely randomized with 20 replicates per treatment.

A Poisson log-linear regression model was fitted to the data on number of shoots from each of the *in vitro* propagation methods studied (conventional micropropagation, micropropagation by etiolation in 60 days and 120 days), considering the number of shoots as the dependent variable and the data from the subcultures as independent variables. The data analysis was performed with the Statistica 7.0 software (Statsoft, 2005).

### Summary for experiment

A diagram, summarizing the main steps of each method, the number of plantlets obtained, as well as the time (in days) of each system (Figure 2). Each method is represented by a color. The number of days presented for the *in vitro* propagation methods in the diagram considers the complete cycle of the principal plant (without recurrent flowering), not only the multiplication step.



**Figure 2.** Production cycle, number of days and quantity of plantlets produced by different propagation methods of the miniature ornamental pineapple hybrid ORN-MUT. Conventional propagation (blue line); Stem sectioning (red line); Conventional micropropagation (green line); Etiolation of nodal segments (yellow line). \*Four subcultures performed at 45 days intervals, for a total of 180 days. \*\*Etiolation performed for periods of 60 and 120 days, respectively.

### Indexation for wilt virus (PMWaV) and shoot tip cultures

The culturing of shoot tips to remove and clean the *Pineapple mealybug wilt-associated virus* (PMWaV) was performed with ten plants from micropropagation. Shoot tips were excised with maximum length of 0.5 mm and cultured in test tubes (one tip per tube) containing 5 mL of MS culture medium supplemented with 3% sucrose and solidified with 2.4 g L<sup>-1</sup> of Phytigel®, with pH adjusted to 5.8 before autoclaving at 120°C for 20 min. The tubes were transferred to a growth room at 27 ± 1°C, photoperiod of 16 hours fluorescent light and photon flux density of 40 µmol m<sup>-2</sup> s<sup>-1</sup>. This stem tip cultures were maintained for 180 days for emergence and growth of plants, to verify the best size for removal of leaves for indexation, as well as to count the number of surviving and dead tips.

### Extraction of total RNA

The total RNA was extracted according to the method described by Gambino, Perrone, and Gribaudo (2008). Approximately 1.0 mL of leaf tissue was macerated with liquid nitrogen and transferred to 2.0 mL microtubes, to which 800 µL of extraction buffer solution was added (CTAB 2.0%, NaCl 2.0 mol L<sup>-1</sup>, Tris-HCl 100 mmol L<sup>-1</sup> at pH 8.0, EDTA 25 mmol L<sup>-1</sup>, PVP-40 2.5% and β-mercaptoethanol 2.0%) after preheating the tubes in a water bath at 65°C. The tubes were then homogenized in a vortex. After this procedure, 800 µL of chloroform and isoamyl alcohol (24:1) was added to each tube, and they were centrifuged at 11,000 x g for 10 min. at 4°C. The supernatant was transferred to 1.5 mL microtubes. Lithium chloride (LiCl 3 mol L<sup>-1</sup>) was added to the microtube in the same volume as the supernatant. The samples were then incubated in an ice bath for 30 min. and subsequently centrifuged at 21,000 x g for 20 min. at 4°C. To the precipitate, 500 µL of SSTE (Tris-HCl 10 mmol L<sup>-1</sup> pH 8.0, EDTA 1.0 mmol L<sup>-1</sup>, SDS 1.0% and NaCl 1.0 mol L<sup>-1</sup>) and 500 µL of chloroform and isoamyl alcohol (24:1) were added. The contents of the tubes were homogenized and then centrifuged at 11,000 x g for 10 min. at 4°C. The total RNA was precipitated with 0.7 volumes of cold isopropanol and centrifuged immediately at 21,000 x g for 15 min. at 4°C. Then the total RNA was washed with sterile 70% ethanol and centrifuged at 13,000 rpm for 10 min. The samples obtained were placed in an oven at 37°C for 15 min. and resuspended in sterile deionized DEPC (diethyl pyrocarbonate).

### Detection of types of PMWaV by reverse transcription followed by polymerase chain reaction (RT-PCR)

Reverse transcription (RT) consists of two consecutive steps. The first involves synthesis of complementary DNA (cDNA). For this purpose, 1.0 µL of dNTP 10 mmol L<sup>-1</sup>, 1.0 µL of the primer random hexamer 6 bp, 1.0 µL of primer PMWaV-1 R, 1.0 µL of primer PMWaV-2 R, 1.0 µL of primer PMWaV-3 R, 4.0 µL template RNA and 1.0 µL of sterile deionized water were placed in 50 µL PCR microtubes. The mixture was incubated at 70°C for 10 min. in a thermocycler, followed by 4°C for 2 min. Then 2.0 µL of M-MLV 10x buffer, 0.5 µL of RNase Out®, 6.5 µL of sterile deionized water and 1.0 µL of the enzyme M-MLV reverse transcriptase were added, obtaining a total reaction volume of 20 µL. The final mixture was incubated at 37°C for 50 min. in the thermocycler, followed by 10 min. at 80°C to inactivate the enzyme. In the s step, 3.0 µL of the recently synthesized cDNA, 1.0 µL of primer F 10 nmoL, 1.0 µL of primer R 10 nmoL, 1.0 µL of dNTP 2.5 mmol L<sup>-1</sup>, 2.5 µL of *Taq* DNA polymerase PCR buffer (10x), 1.5 µL of MgCl<sub>2</sub> 50 mmol L<sup>-1</sup>, 14.8 µL of sterile deionized water and 0.2 µL of *Taq* polymerase 5 U µL<sup>-1</sup> were placed in microtubes, obtaining a reaction volume of 25 µL.

Three reaction tubes were prepared for each sample, the first containing specific primers for PMWaV-1, the second for PMWaV-2 and the third for PMWaV-3. The amplification process by polymerase chain reaction (PCR) consisted of initial denaturing at 94°C for 4 min., followed by 38 cycles involving sequential steps of denaturing (94°C/45"), annealing of the primers (48°C/40") and extension (72°C/1'30"). The result of the RT-PCR was obtained by electrophoreses on 1.5% agarose gel, in TBE buffer, at 80 V for at least 2.5 hours and was revealed with a transilluminator. Although the tissue culture laboratory already had positive and negative controls for each viral strain studied, we also used a sample from a plant cultivated in the field to confirm the existence of the virus.

## Results and discussion

### Strategies for vegetative propagation

We observed significant differences in the number of plantlets produced and the time (in days) to obtain the plantlets by the different propagation methods.

Conventional propagation (Figure 2-red line) produced an average of 17 plantlets per plant in a period of 566 days (about 19 months). During this time, the plantlets remained in beds until reaching a height of 40 cm, adequate for cultivation in the field. A drawback was the difficulty of obtaining uniform plantlets by the conventional, method, since they came from different parts of the parent plant. Most of the plantlets obtained were of the stem shoot type, followed by axillary crownlet (Table 1).

The stem sectioning method produced an average of 2.3 plantlets per stem and a total of 47 plantlets at the end of the experiment. The stems were very short and thin, with average length of 13 and diameter of 3 cm, impairing the emergence of shoots.

From the seven plants selected for *in vitro* establishment of the hybrid, it was possible to excise 138 buds for inoculation in culture medium after the disinfestation procedure. Of these, 39.2 suffered from fungal contamination, 25.4 from bacterial contamination, and 3.62% were oxidized, leaving 44 buds to perform the study.

After the four subcultures, a total of 1,284 plants were obtained by the conventional micropropagation method (Table 2). The growth rate indicated peak production in the fourth subculture (Table 2), a result not differing greatly from the findings of other studies that in general this peak is attained in the third or fourth subculture for many genotypes.

The peak production after 60 days of etiolation occurred after the second subculture, obtaining 1,224 plants (Table 2). There was a significant decline in the number of plants obtained at the end of the four subcultures, mainly between the third and fourth (Table 2).

**Table 1.** Number of plantlets obtained per plant from conventional propagation of the miniature ornamental pineapple hybrid ORN-MUT.

Types of plantlets/plant	Mean*	Minimum	Maximum	S**
Ground shoot	0.70 d	0.00	3.00	0.86
Stem shoot	6.40 a	2.00	12.00	3.03
Slip	2.15 cd	0.00	9.00	2.92
Crown	2.75 bc	1.00	5.00	1.16
Axillary crownlet	5.30 ab	0.00	11.00	3.01
Mean	3.46			
CV (%)	51.02			

\*Means followed by the same letter in the column do not differ statistically from each other by the Tukey test at 1% probability ( $p < 0.01$ ). \*\*Standard deviation.

**Table 2.** Total number of shoots in the four subcultures for each of the *in vitro* propagation methods used and geometric growth rate among the subcultures of the miniature ornamental pineapple hybrid ORN-MUT.

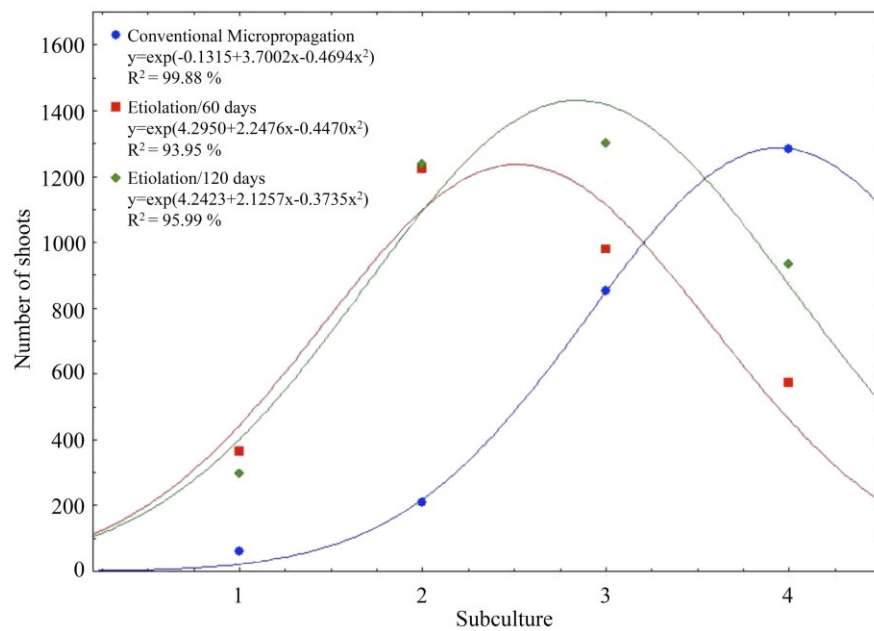
<i>In vitro</i> methods	Subcultures (S) – shoot number					Geometric growth rate			
	S0	S1	S2	S3	S4	S0-S1	S1-S2	S2-S3	S3-S4
Conventional Micropropagation/ 45 days	20*	60	208	852	1,284	2.47	2.80	3.18	0.92
Micropropagation/ Etiolation/ 60 days	114**	365	1,224	980	572	2.62	2.73	-0.49	-1.19
Micropropagation/ Etiolation/ 120 days	221*	296	1,238	1,302	935	0.65	3.23	0.11	-0.73

\*Considering 20 initial explants. \*\*The initial subculture for micropropagation by etiolation and total number of nodes (axillary buds) formed from 20 plants during the etiolation period.

In turn, the treatment for 120 days produced 935 plants in the last of the four subcultures, with peak production in the third subculture (1,302 plants). Although the number of starting explants (internodes) was greater, there was no correlation with the number of shoots, but the propagative potential was slightly higher than the treatment for 60 days of etiolation. In other words, longer etiolation time, despite increasing the number of internodes, did not affect the final production of shoots considering the prolonged time required.

The Poisson log-linear regression model clearly demonstrated the difference between the *in vitro* propagation methods in terms of number of shoots produced and subcultures evaluated, as well as the behavior of the plans during the subcultures (Figure 3).



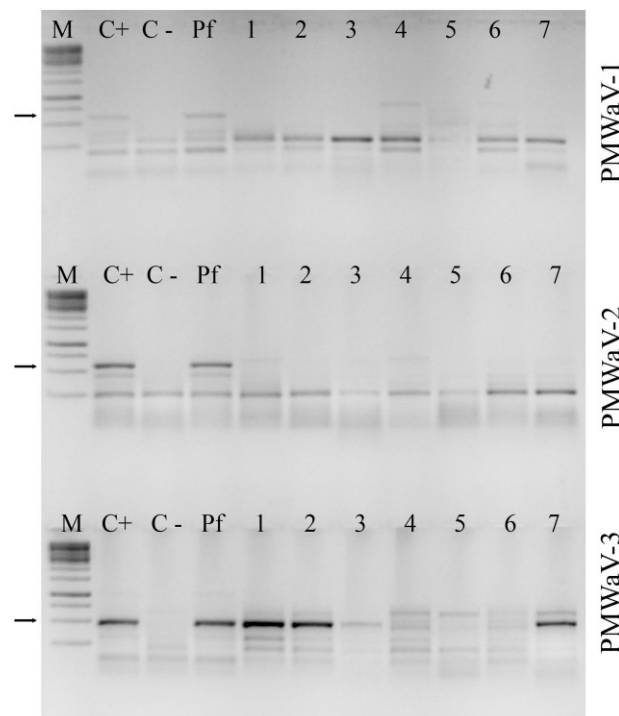


**Figure 3.** Number of shoots produced and estimated by the Poisson log-linear regression model in function of the subcultures for different *in vitro* propagation methods of the miniature ornamental pineapple hybrid ORN-MUT.

### Viral cleaning

With respect to the cultivation of the shoot tips and viral cleaning of the miniature hybrid ORN-MUT, 70% of the plants survived and developed satisfactorily to enable indexation analyses. The mortality recorded was due to oxidation of the shoot tips, in function of the small size and possible injuries in the meristem region. Of the surviving shoot tips, viral cleaning was achieved with 43% (3 of 7 tips) of the ORN-MUT plants evaluated.

The indexation allowed identifying three plants (4, 5 and 6) free of all three viral types (PMWaV- 1, 2 and 3) that cause pineapple wilt. The plant from cultivation in the field and the positive control presented fragments corresponding to the virus's size (592 pb), used as a standard 1 Kb marker (Ludwig; Figure 4).



**Figure 4.** Indexation of plants of the miniature ornamental pineapple hybrid ORN-MUT in relation to the three types of *Pineapple mealybug wilt-associated virus* (PMWaV-1, PMWaV-2 and PMWaV-3). M) 1 kb molecular weight marker; C+) positive control, plant contaminated with the three viral strains; C-) negative control, plant free of virus; Pf) field plant of ORN-MUT utilized as initial explant for *in vitro* culture; 1-7) *In vitro* clones after micropropagation and crown tip culture of ORN-MUT.



### Strategies for vegetative propagation

Evaluation of the propagation potential of the ORN-MUT hybrid by the conventional method was important to learn the capacity for tillering and production of plantlets of this ornamental variety, aiming to select the most developed, vigorous and healthy individuals for planting in the field. In general, the slip plantlets are chosen most often because they are easy to collect, usually have good vigor and intermediate culture cycle between crown and ground shoot plantlets. The ground shoot plantlets, although having greater development, shorter production cycle and earlier natural flowering, have low yield and are difficult to remove from the plant, besides having non-uniform size and weight. The stem shoot plantlets have low yield and are not often used for commercial cultivation. Those from the crown, in turn, have more uniform size and weight but less vigor and a longer planting-collection cycle, only being used in regions where the fruit is processed industrially (Jiménez et al., 2018).

One of the factors that limits conventional propagation of pineapple plants is poor phytosanitary quality of the plantlets, hindering expansion of the crop into new areas and demanding more intensive management to minimize the proliferation of pests and diseases, such as fusariosis (*Fusarium guttiforme*, Nirenberg & O'Donnell; Jiménez et al., 2018) or pineapple wilt (*Pineapple mealybug wilt-associated virus* – PMWaV; Souza et al., 2013).

Therefore, we expected the production of plantlets by stem sectioning to be a more efficient method for propagation of the miniature hybrid ORN-MUT. The low production of plantlets by this method might have been related to the hybrid's small size, because of its selection for this trait as a potted plant. Nevertheless, the technique can be complementary to the conventional method, after obtaining plantlets, so it should not be neglected. According to Jiménez et al. (2018), stems of very small pineapple plants have low shoot production rates due to the slower vegetative development and long period for the plantlets to attain suitable size, compromising the yield of the sections. However, stem sectioning has been used successfully for other pineapple varieties, especially for food use.

The physiological state of the plant is an important determinant for the success of this method. The stem should be obtained soon after collection of the fruit, when the emission of ground shoots intensifies, because any delay can mean reduced vigor. We respected this principle, in particular by only using plants after the first fruiting cycle, not those from recurrent flowering. However, another hypothesis for such low yield can be related to the endogenous hormonal balance of a plant that has been developed for new flowering, as the case of this mutant. At the end of fruiting, the plant's physiology is focused on tillering, for the plant to flower again, which can alter its hormonal balance (Mercier, 2004).

The *in vitro* growth rate of ORN-MUT by conventional micropropagation was similar to that of various other genotypes from the *in vitro* Pineapple Active Germplasm Bank, which have shown geometric growth rates in the first three subcultures, followed by a decline in the subsequent subcultures, resulting from the reduced propagative potential (Silva et al., 2016). This type of behavior during micropropagation was noted by use of the geometric growth rate, which was employed for the first time for *Neoglasiovia variegata* (Arruda) Mez, another bromeliad (Silveira et al., 2009). The geometric growth rate more precisely reflects the biological behavior of treatments than the number of shoots obtained, and hence the propagative potential of the species evaluated (Silva et al., 2016).

The accentuated decline after the third (60 days) and fourth (120 days) subculture in the *in vitro* production of etiolated plants was mainly due to the loss of many shoots which at the start of the subculturing appeared to be healthy, but that passed through an intense oxidation process that caused the death of a significant portion of the shoots produced. A process similar to oxidation was observed in the 'Xavante' variety of blackberry, which might have been caused by the interaction of the time spent by the parent plants in the absence of light with the reduction of MS salts in the culture medium (Pelizza et al., 2016). However, many researchers have found the absence of light to be a strategy to reduce oxidation processes during *in vitro* cultivation, revealing the need for additional studies to understand the causes that led to such high oxidation in this experiment. This high contamination rate in the establishment phase revealed the need for adjustments in the disinfection protocol used, even though it had been found adequate for various other pineapple genotypes (Moraes, Almeida, & Cazé Filho, 2007; Souza, Souza, Silva, Souza, & Costa, 2012b; Souza et al., 2013; Alves, Londe, Melo, & Rodrigues, 2014).

The three *in vitro* propagation methods presented peak production at distinct moments, but in all of them the propagative potential declined with time, a result also reported in other studies with pineapple (Hamad & Taha, 2008) as well as other species, such as banana plants (Mendes, Filippi, Demétrio, &

Rodrigues, 1999). The causes of this decline can be varied, but the duration of the interval between subcultures seems to be relevant. Very brief intervals can favor the subculture of plantlets that are still relatively unproductive.

Conventional micropropagation and micropropagation by etiolation of nodal segments are methods that have been previously studied for production of pineapple plantlets. Various protocols have been described in the literature (Kiss, Kiss, Gyulai, & Heszky, 1995; Guerra, Dal Vesco, Pescador, Schuelter, & Nodari, 1999; Barboza & Caldas, 2001; Carvalho et al., 2005; 2009; Souza et al., 2010; 2012b; Santos, Barbosa, Vieira, & Carvalho, 2015; Reinhardt et al., 2018; Taha, Ibrahim, Gaafar, Zaied, & Shamma, 2019), but no previous study has compared the techniques *in vitro*, as done in this work.

Of particular note was the smaller production of plantlets by the *ex vitro* methods, even though, as mentioned, the result for stem sectioning fell short of that observed for other varieties, probably due to the mutation.

With respect to the *in vitro* methods evaluated, conventional propagation and etiolation for 60 days were efficient to obtain a large number of plantlets in a short tie interval. The period between the bud establishment phase and peak shoot production during the subcultures was very similar for these methods. The conventional micropropagation took 225 days to obtain production of 1,284 plants, which coincided with the fourth subculture. In route with etiolation for 60 days, there was only a difference of 15 days in relation to conventional micropropagation, requiring 240 days to obtain a peak production of de 1,224 plants in the second subculture. The employment of etiolation for 120 days, although presenting peak production of 1,302 plants in the third subculture (greater than the other *in vitro* treatments), required 345 days to obtain a large number of plantlets, i.e., four months longer than the other methods, and hence relatively inefficient.

### Viral cleaning

The plants of this hybrid were infected with all three viral types, although the removal of PMWaV-3 was more difficult. No previous study has investigated this matter, but a similar result was found in preliminary studies (data not shown) in relation to PMWaV-3. The culture of shoot tips with small sizes can be an efficient strategy for viral cleaning of pineapple plants. Souza et al. (2010), studying the cultivar BRS Ajubá (food use), observed significant results: the technique removed the virus from half the plants. In turn, Botella et al. (2018) obtained a PMWaV removal rate of 90% of accessions of *Ananas comosus* L.

The success of viral cleaning is strongly correlated with the size of the shoot tip. The smaller the tip, the more efficient the viral cleaning tends to be, but the lower the chance of survival is (Biswas et al., 2007). The wilt virus is preferably located in the phloem tissues, which are absent in the region of the meristematic dome (Wang & Valkonen, 2009; Niehl & Heinlein, 2011). Examination of histological sections of pineapple shoot tips has demonstrated that the meristematic dome region has few or no vessels, with dense cytoplasm cells, and as the distance from the dome increases, the tissues start to become differentiated into transmitting tissues (xylem and phloem) and parenchymatic tissues (Souza et al., 2016).

Therefore, for cleaning and survival of the shoot tips, the meristematic dome should be left intact, with only 1 to 2 primordial leaves and with size between 0.5 and 1.0 mm, in which case it is possible to obtain healthy plants free of PMWaV (Botella et al., 2018).

The viral cleaning method based on *in vitro* culture of shoot tips has been described for various species, associated with other techniques, such as thermotherapy and cryotherapy (Sether et al., 2001; Wang, Cuellar, Rajamäki, Hirata, & Valkonen, 2008; Souza et al., 2016). Therefore, this technique can be used as a complementary strategy with other methods of removing the PMWaV complex from pineapple plants.

### Conclusion

Conventional micropropagation and etiolation of nodal segments were efficient *in vitro* methods to produce many uniform plantlets and were superior in comparison to another methods;

Conventional micropropagation and etiolation of nodal segments with period of 60 days were superior to etiolation with a period of 120 days, specifically in relation to the time necessary to obtain plantlets;

The culture of shoot tips is an efficient strategy to clean the PMWaV complex from pineapple plants and obtain healthy parent plants and can be applied to another pineapples varieties.

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