

Cleaning cassava genotypes infected with cassava frogskin disease via *in vitro* shoot tip culture

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Genet. Mol. Res. 16 (2): gmr16029556

Received November 29, 2016

Accepted April 27, 2017

Published May 31, 2017

DOI <http://dx.doi.org/10.4238/gmr16029556>

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ABSTRACT. This study aimed to develop a methodology for eliminating cassava frogskin disease (CFSD) from *in vitro* shoot tip culture by associating thermotherapy and tetracycline. Cuttings from different accessions (BGM0232, BGM0315, BGM0464, BGM584, BGM0841, and BGM1342), infected with CFSD according to visual inspection of the disease symptoms, were used for cleaning. To verify the absence of other diseases, the plants were indexed for Cassava common mosaic virus - CsCMV (by ELISA) and Cassava vein mosaic virus - CsVMV (by polymerase chain reaction, PCR), proving that the accessions were free of these viruses, except for BGM0315 and BGM0464, which were infected with CsVMV. Subsequently, the cuttings were submitted to different tetracycline concentrations for 3 min, and then subjected to thermotherapy under different temperatures (35°, 38°, 40°, 45°, and 55°C). Shoots of 2 cm were harvested, and their surfaces were sterilized in a laminar flow chamber. Subsequently, the shoot tips of different sizes were removed (0.2, 0.4, 0.5, and 1.0

mm) for inoculation in a culture medium with tetracycline at the same concentrations in which the cuttings were dipped. After 60 days of cultivation, the explants were transferred to a multiplication medium without antibiotics. Thirty days after the transfer, the viability of the regenerated plants was evaluated, which were then acclimatized for 70 days in a greenhouse and transferred to the field. After 7 months, a visual analysis of the symptomatic roots and a PCR analysis were held to prove the elimination of CFSD and CsVMV from the accessions infected with these viruses (BGM0315 and BGM0464), respectively. Most of the treatments resulted in 100% cleaning of CFSD-infected plants. From accessions that were also infected with CsVMV, only 2% of the plants remained infected, also demonstrating the cleaning efficiency of this protocol for this disease.

Key words: *Manihot esculenta* Crantz; Cassava frogskin disease; Thermotherapy; Tetracycline

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), one of the main crops for human and animal consumption, has great economic and social importance for many countries. Additionally, some traits, such as its drought tolerance, make this species appropriate for cultivation in different regions, with wide adaptation to different climate and soil conditions (Lenis et al., 2006; Foloni et al., 2010) and tolerance to pests and diseases (Venturini et al., 2015). In addition, Oliveira et al. (2015) emphasize its relatively stable productivity in different environments and flexibility at harvest. However, globally, the main components related to impairment of cassava production are soil and climatic growing conditions and the quality of cuttings, which cause unevenness in plant development and result in production losses (Mattos et al., 2006). Other biotic constrains, such as mites, insects, and many diseases as root rot, cassava bacterial blight, Cassava vein mosaic virus (CsVMV), Cassava common mosaic virus (CsCMV), and cassava frogskin disease (CFSD), have caused severe restrictions on cassava production in Latin America (Calvert and Thresh, 2002; López et al., 2006; Carvajal-Yepes et al., 2014; Souza et al., 2014). The CFSD directly affects the starch, root quality and thus crop yield of cassava (Calvert and Thresh, 2002; Alvarez et al., 2009).

The etiology of CFSD is complex because some authors attribute the disease to different species of viruses, such as Cassava frogskin-associated virus (CsFSaV; Reoviridae/Oryzavirus), Cassava torrado-like virus (CsTLV; Secoviridae/Torradovirus), Cassava new alphaflexivirus (CsNAV; Alphaflexiviridae/Potexvirus), and Cassava polero-like virus (CsPLV; Luteoviridae/Polerovirus) (Carvajal-Yepes et al., 2014), and also with phytoplasma belonging to the 16SrIII-L (Alvarez et al., 2009; Oliveira et al., 2014) and 16SrIII-A (Souza et al., 2014) subgroups.

Plants infected with this disease usually have no symptoms on the aerial part; while the tuberous root exhibited a woody appearance, thickened cork-like peel with opaque aspect, and coalescent lip-like slits in a honeycomb pattern; whereas severe affected plants do not show formation of tuberous roots, due to the absence of starch accumulations (Calvert and Thresh, 2002; Alvarez et al., 2009; Carvajal-Yepes et al., 2014). Calvert and Thresh (2002) pointed out

that, in some situations, certain genotypes of cassava mosaic symptoms can develop on leaves and stems near the ground can be slightly swollen.

Controlling CFSD involves the removal of diseased plants, the sanitization of tools with sodium hypochlorite, the use of healthy cuttings, the establishment phytosanitary quarantine and the adoption of measures to prevent its spread, especially to areas free of the disease (Calvert and Thresh, 2002; Alvarez et al., 2009). As cassava is a vegetatively propagated plant, the accumulation of phytoplasma and/or virus in cuttings is recurrent, resulting in losses in root yield that can reach 90% (Alvarez et al., 2009) and, in extreme cases, 100% (Oliveira EJ, unpublished results). An important strategy to control CFSD in infected plants is to clean these materials via meristem culture, thermotherapy, antibiotics, cryotherapy, and micrografting (Milošević et al., 2012). According to Panattoni et al. (2013), thermotherapy has been the most commonly applied technique in cleaning protocols for plants infected by systemic disease induced by viruses belonging to 13 families since 1991, being the use of treatments in association the most reliable strategy.

According to Menezes Júnior (2011), thermotherapy associated with *in vitro* cultivation helps reduce systemic pathogens, such as viruses. The cultivation of the infected plants at high temperatures significantly reduces virus replication by interrupting nucleic acid synthesis, and the same occurs with other systemic pathogens, such as phytoplasma. However, the optimal temperature for inhibiting this replication is highly variable in different pathogens, although thermotherapy combined with meristem culture may improve the efficiency of pathogen elimination (Milošević et al., 2012). Moreover, chemotherapy with the use of ribavirin, ditiouracil, tetracycline, cefotaxime, and erythromycin in association with the cultivation of meristems and apex shoots and in combination with thermotherapy has also been promising for the elimination of pathogens (Nascimento et al., 2003; Panattoni et al., 2007, 2013).

To Milošević et al. (2012), the pathogen distribution in apexes may vary depending on the host-pathogen interaction in regard to infection degree making it necessary to investigate the appropriate explant size to completely eliminate the pathogen and not interfere with the plant regeneration. There are reports in the literature on the use of meristem or shoot tips that are 0.2 to 0.7 mm long for pathogen eradication (Singh et al., 2007; Panattoni et al., 2013; Mwangangi et al., 2014).

According to Wang and Valkonen (2008), phytoplasma restriction in young tissues occurs because the phloem vessel elements are developing and therefore do not allow the passage or movement of such pathogens. Therefore, the *in vitro* culture of apexes and meristems has been used for cleaning infected plants with virus, bacteria, and phytoplasmas (Singh et al., 2007; Banerjee et al., 2010). Moreover, these authors reported that the size of apexes can influence this cleaning process, as phytoplasma was detected in sweet potato apexes of 1.0 and 1.5 mm, but not in 0.5-mm apexes. In meristem cultivation, another strategy for virus and phytoplasma control is cleaning plants with the use of antiviral substances and antibiotics, respectively. According to Panattoni et al. (2013), viral chemotherapy is based on the assumption that the nucleic acid synthesis of the virus may be inhibited by antiviral molecules. The success of explant *in vitro* cultures in culture medium with antiviral substances and antibiotics has been reported in several papers (Gribaudo et al., 2007; Singh et al., 2007; Mwangangi et al., 2014).

Milošević et al. (2012) emphasize that virus elimination is most effective when different techniques (meristem culture, thermotherapy, cryotherapy, and chemotherapy) are used jointly. Therefore, a combination of cleaning techniques may constitute an efficient alternative

for the disposal of CFSD-infected cassava plants, since the etiology of this disease has not been fully confirmed. Hence, *in vitro* culture of shoot tips, associated with thermotherapy and chemotherapy, may be an effective strategy in eliminating viruses or phytoplasma in cassava plants infected with CFSD, which this study focused on. In other studies, the addition of cleaning techniques has shown promising results for virus and phytoplasma elimination (Senula et al., 2000; Chalak et al., 2005; Mwangangi et al., 2014).

Considering the lack of reports on CFSD-resistant cultivars and the difficulty in recognizing the symptoms of infected plants before harvest, as well as the significant reduction in tuberous root productivity and quality, it is necessary to develop cleaning strategies for currently infected cultivars. The objective of this study was to develop a frogskin elimination methodology based on cultivating *in vitro* shoot tips of infected cassava plants associated with thermotherapy and the use of tetracycline.

MATERIAL AND METHODS

Plant material

Cuttings from CFSD-infected plants of accessions ‘Cidade Rica’ (BGM0232), ‘Riqueza II’ (BGM0584), ‘Mandioca Lagoa’ (BGM1342), ‘CM-425/7’ (BGM0315), ‘Sabará’ (BGM0464), and ‘Sauma’ (BGM0841) that originated from the Cassava Active Germplasm Bank (BAG-Cassava) of Embrapa Mandioca e Fruticultura were used for this analysis. To ensure that these mother plants had no other disease, indexation was carried out using ELISA and polymerase chain reaction (PCR) for the Cassava common mosaic virus - CsCMV (Mowat and Dawson, 1987) - and Cassava vein mosaic virus - CsVMV (Calvert et al., 1995) - respectively. The diagnosis showed that all accessions were free of CsCMV, but the BGM0315 and BGM0464 were infected with CsVMV.

Treatments used in thermotherapy

The cuttings taken from CFSD- and CsVMV-infected plants were germinated in plastic pots containing the commercial substrate (Vivatto, Technes Agrícola Ltda.) and subjected to the thermotherapy process using climate chambers with the following temperatures:

1) $55^{\circ} \pm 1^{\circ}\text{C}$ during the day and $38^{\circ} \pm 1^{\circ}\text{C}$ overnight; 2) $45^{\circ} \pm 1^{\circ}\text{C}$ during the day and $35^{\circ} \pm 1^{\circ}\text{C}$ overnight; 3) $40^{\circ} \pm 1^{\circ}\text{C}$ during the day and $32^{\circ} \pm 1^{\circ}\text{C}$ overnight; 4) $38^{\circ} \pm 1^{\circ}\text{C}$ during the day and $32^{\circ} \pm 1^{\circ}\text{C}$ overnight, with two 50°C peaks for 1 h (a peak early in the morning and another in the early afternoon); 5) $38^{\circ} \pm 1^{\circ}\text{C}$ during the day and $32^{\circ} \pm 1^{\circ}\text{C}$ overnight, and the cuttings were subjected to a pretreatment in water at 55°C for 10 min; and 6) $35^{\circ} \pm 1^{\circ}\text{C}$ throughout the time.

The photoperiod used in the acclimatized chambers was 16-h light for a period of 3 to 4 weeks.

Antibiotic treatments

Initially, some preliminary tests were conducted to adjust tetracycline concentrations to prevent phytotoxic effects on the development of cassava plants:

- First test: three cassava accessions [‘Cidade Rica’ (BGM0232), ‘Riqueza II’

(BGM0584), and 'Mandioca Lagoa' (BGM1342)], three shoot tip sizes (0.2, 0.5, and 1.0 mm) and three tetracycline concentrations (0, 100, and 400 mg/L).

- Second test: three cassava accessions (BGM0232, BGM0584, and BGM1342), two microcutting sizes (5 and 10 mm) and three tetracycline concentrations (0, 100, and 400 mg/L).

- Third test: three cassava accessions (BGM0232, BGM0584, and BGM01342) and seven tetracycline concentrations (0, 1.25, 2.5, 5, 10, 20, and 40 mg/L), using a shoot tip of 0.2 mm.

- Fourth test: three cassava accessions ['CM-425/7' (BGM0315), 'Sabará' (BGM0464), and 'Sauma' (BGM0841)], two shoot tip sizes (0.2 and 0.4 mm) and four tetracycline concentrations (0, 5, 10, and 15 mg/L).

In all of the experiments, a completely randomized factorial design with 15 to 30 repetitions per treatment was used.

Growing conditions

After adjusting the temperature and antibiotic concentrations, cuttings from accessions BGM0315 and BGM0464 were immersed in pots containing water with different tetracycline concentrations (0, 5, 10, and 15 mg/L) for 3 min, then transferred to polyethylene pots containing commercial substrate (Vivatto, Technes Agrícola Ltda.) and maintained at a constant temperature of $35^{\circ} \pm 1^{\circ}\text{C}$.

The shoots originating from the cuttings were kept in a climatized chamber and cut at approximately 2 cm in length, placed in the container with distilled water and then subjected to sanitization in a laminar flow chamber by immersion in 50% ethanol for 3 min and sodium hypochlorite (0.25%) for 3 min followed by triple washing in autoclaved distilled water.

After sanitization, the apexes of 0.2 and 0.4 mm were extracted. The explants were transferred to a growing cassava medium containing MS salts (Murashige and Skoog, 1962) supplemented with 1 mg/L thiamine, 100 mg/L inositol, 0.02 mg/L NAA (naphthaleneacetic acid), 0.04 mg/L BAP (benzylaminopurine), 0.05 mg/L GA_3 (gibberellic acid) and 20 g/L sucrose; solidified with 2.4 g/L Phytigel™ (Sigma-Aldrich, Co.), with pH set to 5.8 and different concentrations of tetracycline (0, 5, 10, and 15 mg/L); and then placed in a growth room with a temperature of $27^{\circ} \pm 1^{\circ}\text{C}$, a 16-h photoperiod, and photon flow density of $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 60 days.

After 60 days of establishing phase, the calluses formed on the basis of the apex were removed within a laminar flow chamber, and the explants transferred to test tubes containing the multiplication medium composed by the minerals from MS media supplemented with 0.01 mg/L NAA, BAP and GA_3 , each, 20 g/L sucrose and 2.4 g/L Phytigel™ (Sigma-Aldrich, Co.) without tetracycline. The pH of the medium and the cultivation conditions were the same used during the establishment phase.

Plant evaluation and acclimatization

After 30 days of culture, the viability of the plants within each treatment was evaluated using the following parameters: plant height (PH), in cm; number of green leaves (NGL); number of microcuttings (NM) with about 1 cm, and number of roots (NR). Acclimatization was carried out by washing the roots for removal of the culture medium; then, the plants were transferred to plastic bags containing a mixture of the autoclaved commercial substrate (Vivatto, Technes Agrícola Ltda.), organic soil and coconut coir (1:1:1). The plants were then

watered and covered with plastic cups to avoid water loss. The plastic cups were removed when the plants reached about 8 cm.

Collecting leaf samples and field planting

After 55 days of acclimatization in the greenhouse, plant leaf samples of each repetition of all treatments were packed in aluminum foil, identified, transferred to a cooler with ice and then stored at -80°C in an ultrafreezer to perform indexing. After 70 days of acclimatization, the seedlings were planted in the field.

Planting was carried out in two randomized blocks; each experimental plot consisted of four to five plants, depending on the number of surviving plants on each treatment. Fertilization was carried out according to the soil analysis and hoeing for controlling weeds.

Symptomatic analysis of plants in the field and molecular analysis to evaluate protocol effectiveness

Seven months after planting in the field, plants were harvested to evaluate the CFSD symptoms and thus verify the efficiency of the protocol used for CFSD elimination through visual analysis for the presence or absence of disease symptoms in the roots (no formation of tuberous roots, fibrous roots, and cork epidermis and the presence of lip-like slits).

Besides visual analysis in the field, PCR was performed for detection of CsVMV (Calvert et al., 1995) in the plants derived from accessions BGM0315 and BGM0464 (originally infected with CFSD and CsVMV). For this, DNA was extracted from leaves collected from all plants of these genotypes from each cleaning treatment using the Doyle and Doyle (1987) protocol.

Data analysis

The data of explant viability in the treatments (accession, tetracycline concentrations, and shoot tip sizes) were subjected to analysis of variance. The averages of different treatments were compared by F and Tukey tests at 5% probability. Since the objective was to define the best tetracycline concentration and not analyze the gap between them, polynomial regression models were not used. The parameters NGL, NM, and NR were converted to $\sqrt{x + 0.5}$ in order to meet the assumptions of analysis of variance. All statistical analyses were performed using the Statistical Analysis System (SAS) software (SAS Institute, 2004).

RESULTS AND DISCUSSION

Treatments used in thermotherapy

All five treatments above 35°C temperatures adversely affect the sprouting, resulting in the death of cuttings and, therefore, the shoot development. Moreover, temperature treatment at $35^{\circ} \pm 1^{\circ}\text{C}$ did not cause the death of cuttings and allowed the proper shoot development and, therefore, the temperature used in all thermotherapy stages.

According to Panattoni et al. (2013) temperatures most frequently used in studies published on thermotherapy from 1991 to 2010 were set between 35° and 38°C . Moreover,

the growing meristem tips (1 mm) in cassava treated by thermotherapy at higher temperatures (38°C for 21 days) resulted in 68.8% of regenerated plants and were 84.4% free from Cassava brown streak virus (CBSV) (Mwangangi et al., 2014). The fact that these sprouts do not develop at higher temperatures may be due to increased sensitivity of the accessions used in these conditions. Moreover, even using lower temperatures (35°C), there are reports of success in pathogens cleaning, such as potato clones whose increase in temperature decreased the survival rate of the explants, and 35° ± 1°C gave the lowest survival percentage of meristems (20.47%), but a higher rate of virus-free plants (43.79%) (Ali et al., 2013). Another example, from a garlic culture, showed that treatment at 55°C for 5 min resulted in 50% bulbil survival, without removing viruses, whereas bulbil treatment at 36°C for 40 days allowed complete survival of the meristems and high-rate virus-free plants (84%) (Senula et al., 2000).

Antibiotic treatments

In the initial experiments with tetracycline concentrations of 0, 100, and 400 mg/L, 0.2 to 1.0 mm stem apices were used in the first test and 5- and 10-mm microcuttings were used in the second test. In all these experiments, there was phytotoxicity and no plant regeneration in the doses used, even with a 50times increase in the explants' size (0.2 to 10 mm). Therefore, the initial doses used were very high to start the *in vitro* cleaning process of accessions infected with CFSD. Using lower tetracycline concentrations (between 0 and 40 mg/L), the third test showed higher viability and regeneration capacity of smaller explants (0.2 mm) to 10 mg/L tetracycline. However, from 20 mg/L there was lower development and death of explants, and the concentration of 40 mg/L resulted in the death of all apices. Other studies have also demonstrated the toxicity symptoms caused by antibiotics in explants from different plant species. In potato *in vitro* culture, Pereira and Fortes (2003) reported severe phytotoxic effects on the growth and explant multiplication rate as a function of increasing concentrations (0, 32, 64, 128, 256, 512, and 1024 mg/L) of chloramphenicol, streptomycin, and tetracycline in the culture medium. In the study of Chalak et al. (2005), no plant was regenerated from 1-cm microcuttings subjected to treatments with 50, 100, and 150 µg/mL oxytetracycline for phytoplasma cleaning (*Candidatus* Phytoplasma phoenicium) in almond tree varieties. Gribaudo et al. (2007) also observed phytotoxic effects in vine explants using oxytetracycline and showed that auxiliary buds were greatly affected when grown in a culture medium with 100 mg/L oxytetracycline.

Observations made during the first three preliminary tests with tetracycline were decisive for concentrations that allow plant regeneration to be evaluated for the CFSD elimination. Therefore, considering the high phytotoxicity observed in tetracycline dosages above 20 mg/L, a fourth experiment was carried out at the concentrations of 0, 5, 10, and 15 mg/L. With this reduction in the tetracycline dose, a better development of explants occurs, making it possible to assess the behavior of cassava accessions *in vitro* culture of shoot tips (Table 1).

To BGM0315, the higher PH values were found at tetracycline concentrations of 5 and 15 mg/L, using 0.4 and 0.2 mm apical stems, respectively. For the accession BGM0464, no difference was found in PH between tetracycline concentrations and shoot tip sizes. Although there was no difference between the tetracycline concentrations of accession BGM0841, a difference was found between the shoot tip sizes in the absence and presence of 5 mg/L of the antibiotic, with higher values for the PH size of 0.2 mm (Figure 1).

Table 1. Summary of analysis of variance for plant height (PH) in cm, number of green leaves (NGL), number of microcuttings (NM), and number of roots (NR) of cassava plants from two sizes of shoot tips (0.2 and 0.4 mm) of accessions BGM0315, BGM0464, and BGM0841, cultivated *in vitro* at different tetracycline concentrations (0, 5, 10, and 15 mg/L).

Source of variation	d.f.	Mean square			
		PH	NGL	NM	NR
Accessions (AC)	2	17.66*	13.80**	0.94**	0.29 ^{ns}
Shoot tips (ST)	1	0.97 ^{ns}	3.28**	0.36 ^{ns}	2.43*
Tetracycline concentration (TC)	3	14.57*	0.84 ^{ns}	0.23 ^{ns}	0.21 ^{ns}
AC x ST	2	19.12*	0.56 ^{ns}	1.02**	1.20*
AC x TC	6	16.40**	2.15**	0.13 ^{ns}	0.85*
ST x TC	3	8.17 ^{ns}	1.95**	0.15 ^{ns}	0.67 ^{ns}
AC x ST x TC	6	15.64**	0.79 ^{ns}	0.36*	0.62 ^{ns}
Error	356	5.41	0.42	0.14	0.37
Coefficient of variation (%)		79.61	24.09	25.43	51.10
General average		2.92	7.29	1.82	1.29

**,*Significant at 1 and 5% probability by the F-test. ^{ns}Not significant at 5% probability.

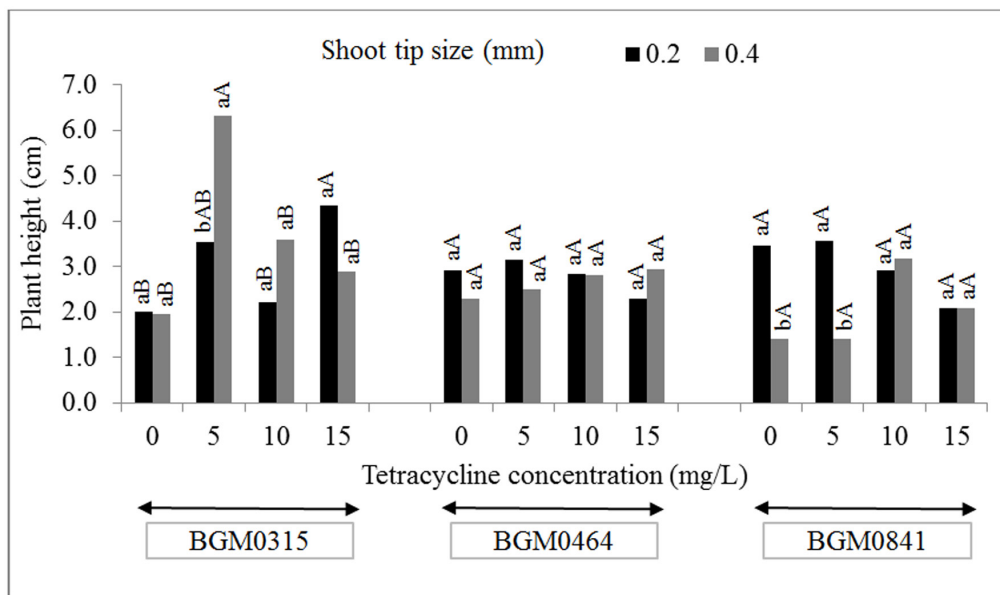


Figure 1. Average plant height values (cm) of cassava from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, evaluated after 30 days of subculture. Means followed by the same lowercase letters do not differ by the F-test ($P < 0.05$) by comparing apex size in each tetracycline concentration, while the same uppercase letters do not differ by the Tukey test ($P < 0.05$), by comparing different concentrations of tetracycline in every apex size for each accession.

The PH assessment is considered important in pathogen cleanup activities because smaller shoots cannot survive during acclimatization, given that cassava is highly sensitive during this step, especially in light of the changes in temperature and relative humidity that occur during the acclimatization phase. In this context, BGM0315 plants from the 0.4-mm apices, grown in medium containing 5 mg/L tetracycline, would be most likely to survive in the acclimatization phase. Moreover, larger plants are more suitable for micropropagation because they increase the proliferation rate and can accelerate the clonal propagation of free-frogskin materials.

Other authors have reported an increased survival rate of cassava plants at a concentration of 10 mg/L ribavirin, compared with other treatments (20 and 30 mg/L), and this concentration was also the most effective in eliminating CBSV (Mwangangi et al., 2014). Senula et al. (2000) also added ribavirin (50 or 100 mg/L) in the culture medium and observed a reduction in plant regeneration and a significant increase in elimination of viruses in garlic (from 19.6 to 59.3%). However, the concentration of 100 mg/L ribavirin caused deformation and hyperhydration in 90 to 100% of plants from larger explants (1 mm), making them unsuitable, while there was no regeneration in the smaller explants (0.3 mm).

For the NGL assessment, the highest averages were observed in BGM0464, with different behavior in relation to tetracycline concentrations (Figure 2). For the BGM0315 accession, the lower NGL was found at a concentration of 10 mg/L, while for BGM0464 and BGM0841, the lowest NGL occurred in the absence of tetracycline, thereby indicating that the antibiotic was favorable to NGL development in these accessions (Figure 2). As the leaves are responsible for energy uptake and organic compound synthesis, more leaves will result in a higher fruit set rate in the acclimatization phase and therefore in the field.

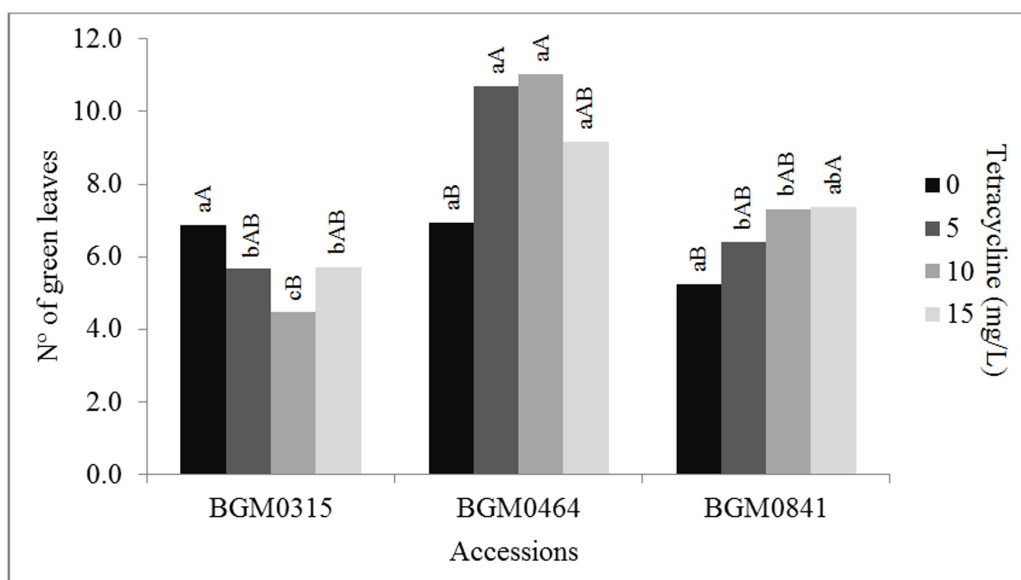


Figure 2. Average number of plant green leaves from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, 30 days after subcultivation. Means followed by the same letters do not differ by the Tukey test ($P < 0.05$); lowercase letters compare accession at each tetracycline concentration while uppercase letters compare different tetracycline concentrations in each accession.

Regarding the apex size due to the tetracycline concentration, no significant differences were observed between the antibiotic concentrations in the NGL characteristic. Only a variation was observed between the two apex sizes at concentrations of 10 and 15 mg/L, with higher amounts of live leaves present in the plants from 0.04-mm explants (Figure 3). Banerjee et al. (2010) suggested that the explants' size can significantly influence the survival rate and success of the culture clonal cleaning. Furthermore, they mentioned that among the three methods used for phytoplasma disposal (thermotherapy, tissue culture, and chemotherapy),

the most widely used for the disposal of such pathogens is the culture of the apical meristem, which alone can promote systemic pathogen elimination because meristematic tissues are not vascularized. As an example, Milošević et al. (2011) had a cleaning efficiency at 80% of the meristems (0.3 to 1.5 mm) of *Impatiens hawkeri* Bull. infected with the Tomato spotted wilt virus (TSWV). Then, Milošević et al. (2012) demonstrated 75% cleaning efficiency in *Impatiens walleriana* plants infected with TSWV when using only meristem cultivation. Therefore, the above authors also reported that the effectiveness of viral cleaning depends on the genotype, and the risk of failing to clean also increases with the explant size.

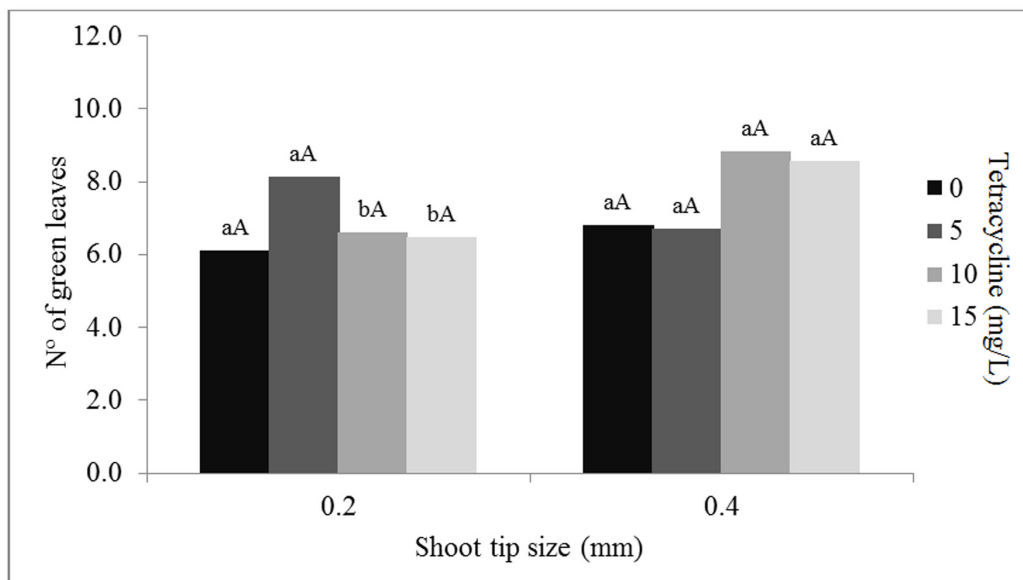


Figure 3. Average number of green leaves from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, 30 days after subcultivation. Means followed by the same lowercase letters do not differ by the F-test ($P < 0.05$), by comparing apex size in each tetracycline concentration, while the same uppercase letters do not differ by the Tukey test ($P < 0.05$), by comparing different tetracycline concentrations in each apex size.

Alam et al. (2013) successfully used meristems with sizes ranging from 0.3 to 0.5 mm in virus elimination, since they could not find surviving sweet potato plants when using meristems smaller than 0.3 mm. Mwangangi et al. (2014) evaluated, among other factors, the effect of shoot tip sizes (0.5, 1, 2, and 10 mm) in eliminating CBSV in cassava plants. As a result, the authors mention that 63% of the plants regenerated from established shoot tips of 0.5 mm, compared to 88% of the 2-mm shoot tips, underscoring that the greater the apex size the greater the number of regenerated plants, and the number of virus-free seedlings was inversely proportional to the explants' size.

In Senula et al.'s (2000) study, after culturing *in vitro* garlic meristematic apices (0.3 to 0.8 mm), the *in vitro* plant regeneration rate ranged from 1 to 80% depending on the genotype, and the viruses were eliminated in 85 to 95% of these plants. In the genotype, the survival of the explants was influenced by meristem size, since explants of approximately 1 mm showed

90 to 100% regeneration, while smaller meristems (one leaf primordium) developed more slowly; there was no regeneration in six genotypes. In other studies, the virus elimination frequency by shoot tip cultivation decreased from 31 to 12% when the size of apices increased from 0.3 to 1.5 mm (Yin et al., 2011). For Parmessur et al. (2002), meristematic apices larger than 1 mm were more likely to remain infected, which did not occur in meristems smaller than 0.3 mm. As not all meristem tips are virus free, Parmessur et al. (2002) emphasized the need for more sensitive diagnostic tools for indexing plants.

For the NR assessment, a significant difference was observed between accessions only at a concentration of 10 mg/L tetracycline, and the BGM0464 showed less NR (Figure 4). Such accession is probably more sensitive to tetracycline, given that the use of antibiotics can result in phytotoxicity inhibiting plant growth and development.

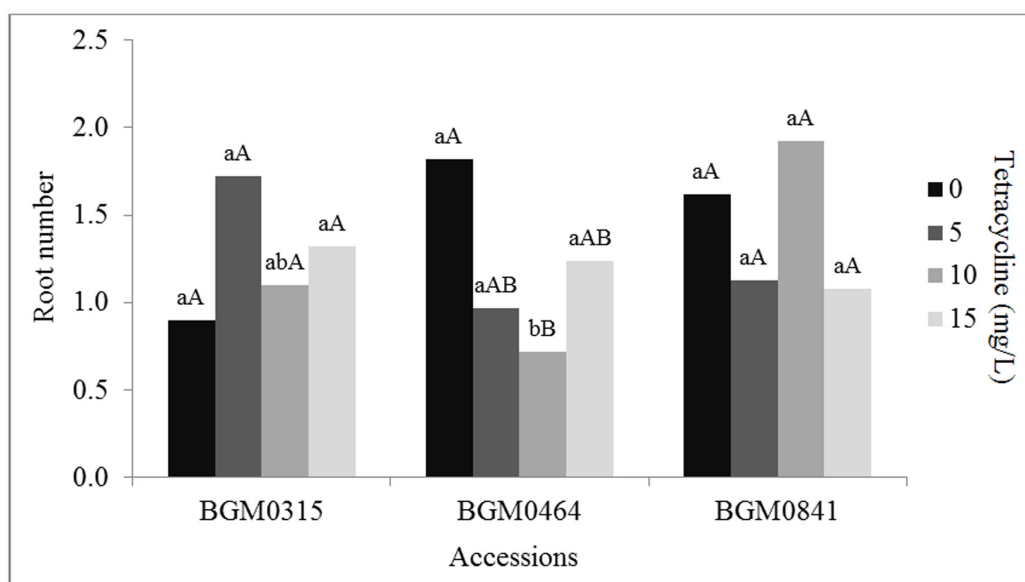


Figure 4. Average number of cassava roots from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, 30 days after subcultivation. Means followed by the same letters do not differ by the Tukey test ($P < 0.05$); lowercase letters compare accessions at each tetracycline concentration and uppercase letters compare different tetracycline concentrations in each accession.

Considering the different cassava accessions due to the shoot tip size, there was no difference between accession and apex sizes for NR, except for BGM0841, which showed lower NR in 0.4 mm (Figure 5). This could be related to the fact that apices with 0.2 mm are naturally free from pathogens. However, many studies have shown the presence of endophytic microorganisms in several species grown *in vitro*, and this interaction between plants and microorganisms can stimulate plant growth (Esposito-Polesi, 2011).

Regarding the NM, there is a variation between the apex sizes at a concentration of 10 mg/L tetracycline for accession BGM0315 with lower NM in the 0.2 mm. For BGM0464 and BGM0841, there was only a difference between apex sizes in the production of microcuttings

at a concentration of 5 mg/L, with higher NM at 0.2 mm (Figure 6). These results show an example of what happens to other cultures and the difference on morphogenetic responses among genotypes for some traits (Nogueira et al., 2001).

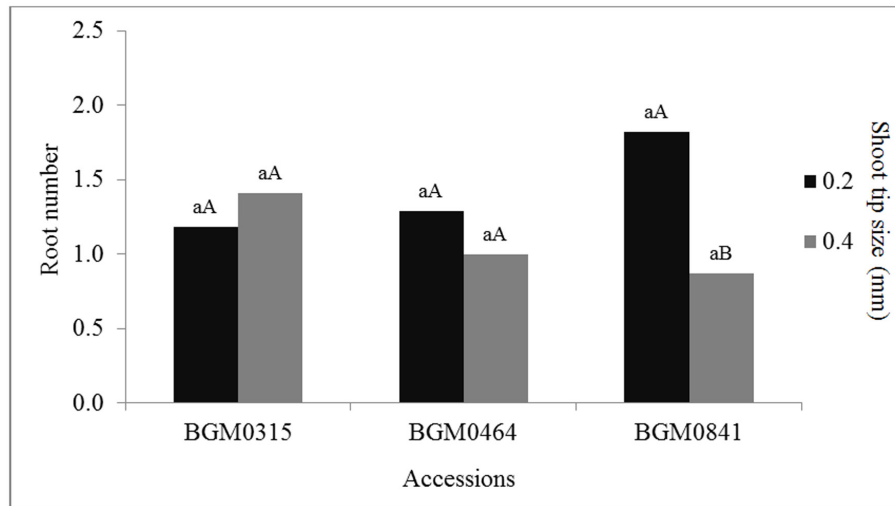


Figure 5. Average number of cassava roots from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, 30 days after subcultivation. Means followed by the same lowercase letters do not differ by the Tukey test ($P < 0.05$), by comparing accessions in each apex size, while the same uppercase letters do not differ by the F-test ($P < 0.05$), by comparing different apex size for each accession.

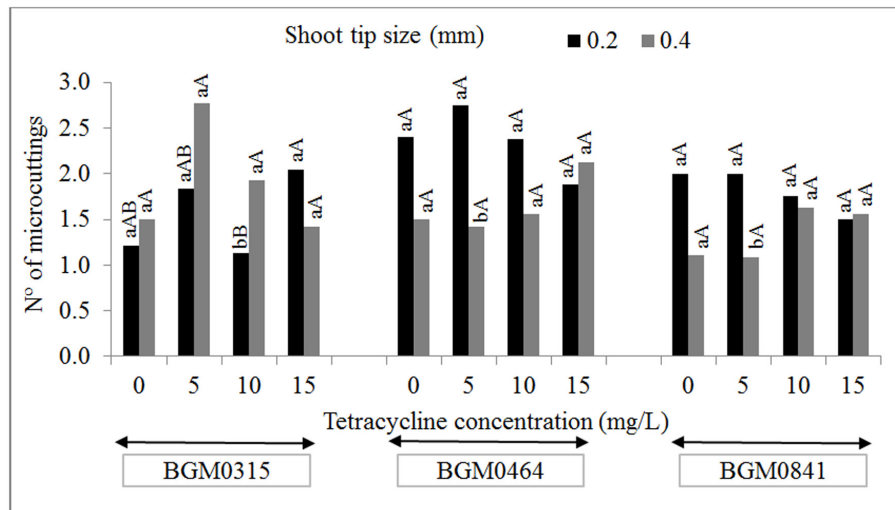


Figure 6. Average number of microcuttings from two shoot tip sizes of accessions BGM0315, BGM0464, and BGM0841 cultivated *in vitro* in tetracycline concentrations for 60 days, 30 days after subcultivation. Means followed by the same lowercase letters do not differ by the F-test ($P < 0.05$) by comparing apex size in each tetracycline concentration, while the same uppercase letters do not differ by the Tukey test ($P < 0.05$), by comparing different concentrations of tetracycline in every apex size for each accession.

In general, we observed different behavior between the accessions used in this study regarding the *in vitro* cultivation of shoot tip in culture medium with tetracycline. The antibiotic concentrations used in the fourth trial did not affect the regenerative capacity of the explants. Furthermore, there were no major differences regarding shoot tip size in plant development, making it possible to identify some variations between treatments on the basis of antibiotic concentrations and the morphogenetic potential of the genotypes.

Positive and negative effects of the antibiotics in culture medium vary depending on the phytotoxicity as well as the species and type of plant material used. In potatoes, for example, Pereira and Fortes (2003) reported that ampicillin was the only antibiotic that did not affect the survival and development of shoots for the multiplication, even at the highest concentration (1.024 g/L), and after a 21-day culture, there was about 100% survival explants. Pereira and Fortes (2003) emphasized that the 1 antibiotics, chloramphenicol, streptomycin, and tetracycline, negatively affected the *in vitro* development of potato, causing a significant decrease in shoot height and multiplication rate along with the increase of their concentrations in the culture medium. Moreover, Ćurković-Perica and Ježić (2010) reported that in *Catharanthus roseus* (L.) G. Don, none of the quercetin concentrations (10, 100, or 1 mM) showed efficient cleaning of plants infected with phytoplasma. Kamińska et al. (2005) also found that in roses the antibiotics baytril and gentamicin were phytotoxic at concentrations of 25 and 50 mg/L, and, furthermore, have not been effective to eliminate phytoplasma.

Symptomatic and molecular analyses of plants under field conditions to assess protocol efficiency

The plants obtained from the CFSD cleaning treatments were planted in the field and were evaluated for the presence of disease symptoms at 7 months. After harvesting and visual analysis of the roots, it was proven that the efficiency of disease elimination in plants by the association of the tissue culture with thermotherapy and chemotherapy occurred, since for most treatments there was 100% cleaning of infected plants (Table 2). However, the results showed that even without the presence of the tetracycline antibiotic in the culture medium during the *in vitro* culture phase, the plants from accessions BGM0315, BGM0464, and BGM0841 showed no symptoms of the disease in the roots. Thus, the *in vitro* 0.2 or 0.4 mm cultivation apexes were effective in eliminating CFSD in infected plants, even without the use of antibiotics in the culture medium.

In accession BGM0315, plants were identified with symptoms of CFSD in the root treatments - 0.2 mm apical grown in culture medium containing tetracycline 5 mg/L (10% of infected plants), shoot tip 0.4 mm without tetracycline (12.5% of infected plants) - in the presence of 5 mg/L (20.0% of infected plants) and 15 mg/L tetracycline (12.5% of infected plants) (Table 2). These results suggest that for the BGM0315 genotype, the CFSD cleaning process is less efficient in comparison with other accessions, which may be related to morphogenetic differences between this genotype and the others, as well as the existence of a higher pathogenic on the shoot tips of this accession. In future studies, BGM0315 should be evaluated with higher concentrations of tetracycline or other antibiotics to verify cleaning efficiency.

Besides CFSD, we also verified the effectiveness of cleaning CsVMV in the accessions BGM0315 and BGM0464. From a total of 143 plants analyzed, only three (2%) had fragments associated with CsVMV in PCR. Positive samples were taken from the treatments: BGM0315 - 0.4 mm shoot tip cultured in the tetracycline concentration at 5 mg/L; and BGM0464 - 0.4

mm shoot tip grown in tetracycline concentrations at 10 and 15 mg/L. Therefore, the shoot tip of 0.2 mm was more efficient in eliminating CsVMV than the one of 0.4 mm. The results show that the protocol used, besides being effective in eliminating the frogskin disease, was also efficient in eliminating the CsVMV. The efficiency of cleaning CsVMV was much higher than that reported in sweet potato culture for Sweet potato feathery mottle virus (SPFMV; Potyvirus) and Sweet potato mild mottle virus (SPMMV), as well as for complex infection in which approximately 85% of plants were cleaned with the use of meristem at 0.3 to 0.5 mm (Alam et al., 2013).

Table 2. Efficiency of cassava frogskin disease (CFSD) cleaning in *in vitro* shoot tip culture, after thermotherapy and tetracycline treatments, evaluated 7 months after cultivation in field conditions.

Accession	Apex sizes (mm)	Tetracycline concentrations (mg/L)	Total number of plants (field)	Plants with no CFSD root symptoms (%)
BGM0315	0.2	0	9	100.0
		5	10	90.0
		10	8	100.0
		15	8	100.0
	0.4	0	8	87.5
		5	10	80.0
		10	8	100.0
		15	8	87.5
BGM0464	0.2	0	10	100.0
		5	10	100.0
		10	10	100.0
		15	10	100.0
	0.4	0	5	100.0
		5	8	100.0
		10	10	100.0
		15	10	100.0
BGM0841	0.2	0	10	100.0
		5	10	100.0
		10	10	100.0
		15	10	100.0
	0.4	0	8	100.0
		5	5	100.0
		10	10	100.0
		15	10	100.0

Mwangangi et al. (2014) had 88.2, 72.0, and 66.7% CBSV-free cassava plants from the 0.5-, 1.0-, and 2.0-mm meristem tips, respectively. These authors reported that higher percentages of virus-free plants were obtained due to the increased concentrations of ribavirin and salicylic acid antiviral substances. Furthermore, they found that thermotherapy treatment (38°C) combined with the use of a 1-mm shoot tip resulted in 68.8% of regenerated plants and 84.4% plant-free viruses.

Singh et al. (2007) submitted explants derived from shoot tip (>0.5 mm) of *Catharanthus roseus* L. (G.) Don infected with phytoplasma to different concentrations of oxytetracycline (25, 50, 75, and 100 mg/L) for 2 weeks followed by six subcultures in medium without antibiotics. These authors reported that 50% of infected plants from explants and cultured in medium containing 75 mg/L oxytetracycline antibiotic were free of phytoplasma and remained after 3 years.

The CFSD cleaning efficiency was only analyzed based on the symptoms under field conditions, whereas the molecular diagnosis currently available for indexing has shown inconsistent results in cassava accessions known to be infected based on visual analysis of field

symptoms. On the other hand, some authors have shown success in molecular diagnosis of phytoplasmas, such as Gribaudo et al.'s (2007) sample in plant matrices and micropropagated vines using nested-PCR and RFLP with 100% cleaning efficiency *in vitro* culture of axillary buds in culture medium with oxytetracycline.

Mwangangi et al. (2014) used RT-PCR to confirm the deletion of the CBSV in infected cassava plants, and the analysis showed that thermotherapy (38°C) in combination with the apex meristem cultivation resulted in 84% of the virus-free plants. The PCR technique has also been used for molecular diagnosis of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) in cassava (Nkaa et al., 2013).

Despite the aspects and techniques covered in other studies, Alam et al. (2013) mentioned that serological methods or other molecular diagnostics impact on cost-effectiveness analysis. Therefore, these authors also evaluated the efficiency of clonal cleaning from the visual analysis of the virus symptoms in sweet potato plants from meristem culture grafted indicator plants. Although it was not possible to perform molecular diagnoses, and thus using only visual data symptoms present in 7-month growth plants, this study presents important results for the supply of healthy propagation material, maintaining the sustainability and competitiveness of the cassava agribusiness. According to Fox (1997), no pathogens or disease identification method is comparable in reliability and simplicity; therefore, different diagnosis methods for plant diseases are often used in a complementary manner and not as an alternative.

CONCLUSIONS

Tetracycline at concentrations of 5 to 15 mg/L does not adversely affect the regeneration capacity of explants, being favorable to plant growth *in vitro* for most treatments. Cassava explants subjected to *in vitro* culture associated with thermotherapy and chemotherapy are effective in viral cleaning of CsVMV and CFSD.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors thank FAPESB, CAPES, and CNPq for the financial assistance and scholarship support.

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