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BIOLOGY

In vitro conservation of 'Florida Rough' lemon plants

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ABSTRACT: *The establishment of minimum growth conditions is essential for in vitro germplasm conservation. Changes to the basic medium and carbon source concentrations are important factors for reducing plant growth in vitro. This study adjusted a protocol for the in vitro conservation of 'Florida Rough' lemon plants. Microcuttings (approximately 1 cm) from plants that were previously cultivated in vitro were inoculated into test tubes with 10 mL of woody plant medium (WPM) at different concentrations (1/1, 1/2 and 1/4) and supplemented with 0, 12.5, 25 and 50 g.L^{-1} of sucrose, solidified with 7 g.L^{-1} agar and adjusted to pH 5.8. The experiment was completely randomized in a 3 x 4 factorial design with 15 replications and was maintained under controlled conditions for 360 days. After this period the plant height in cm (PH), the plant dry mass in g (PDM) and the 21 numbers of green leaves (NGL), senescent leaves (NSL) and microcuttings (NM) were evaluated. The variables that best explained the observed behavior of the 'Florida Rough' lemon plants were NGL and PH, with values of 61.63 and 35.08%, respectively. The original concentration of the WPM with the addition of 25 g. L^{-1} of sucrose yielded the best 'Florida Rough' lemon plant growth reduction in vitro while maintaining the physiologically health of the plants.*
Key words: *Citrus spp., germplasm conservation, multivariate analysis, plant height, numbers of green leaves.*

Conservação *in vitro* de plantas de limoeiro 'Rugoso da Florida'

RESUMO: *O estabelecimento de condições de crescimento mínimo é fundamental para a conservação in vitro de germoplasma. Alterações nas concentrações do meio básico, na fonte de carbono, são fatores importantes na desaceleração do crescimento de plantas in vitro. Este trabalho teve como objetivo ajustar um protocolo de conservação in vitro de plantas do limoeiro 'Rugoso da Flórida'. Microestacas de plantas previamente cultivadas in vitro, com aproximadamente 1 cm, foram inoculadas em tubos de ensaio contendo 10 mL do meio de cultura WPM em diferentes concentrações (1/1, 1/2 e 1/4), suplementado com 0; 12,5; 25 e 50 g.L^{-1} de sacarose, solidificado com 7 g.L^{-1} de ágar e o pH ajustado em 5,8. O experimento foi instalado em delineamento inteiramente casualizado com 15 repetições, em esquema fatorial 3 x 4, e mantido sob condições controladas durante 360 dias. Após este período, foram avaliadas altura de planta em cm (AP), número de folhas verdes (NFV), número de folhas senescentes (NFS), número de microestacas (NM) e massa da planta seca em g (MPS). As variáveis que mais contribuíram para explicar a diferença existente no comportamento das plantas do limoeiro 'Rugoso da Flórida' no meio de cultura WPM foram NFV e AP com 61,63 e 35,08%, respectivamente. O meio de cultura WPM na sua concentração original com a adição de 25 g.L^{-1} de sacarose promoveu os melhores resultados na redução do crescimento das plantas in vitro do limoeiro 'Rugoso da Flórida', mantendo-as fisiologicamente sadias.*
Palavras-chave: *Citros, conservação de germoplasma, análise multivariada, altura da planta, número de folhas verdes.*

INTRODUCTION

Citrus genetic resources are fundamental for the genetic improvement of these species because they include the genotypes of wild-type, lineage, obsolete and modern varieties that contain useful characteristics for developing new scion cultivares and rootstocks. To ensure the use of this important gene pool, it is necessary to establish germplasm collections that can be efficiently and safely stored.

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Currently, citrus conservation is achieved by maintaining banks under field conditions. However, in the field, germplasm conservation is expensive due to the amount of labor required during initial planting, subsequent replanting, weeding, fertilization and treatment to prevent disease and remove pests (PANIS et al., 2020). In addition, germplasms that are conserved under field conditions are vulnerable to a number of factors, such as pests, adverse environmental conditions and anthropogenic activities when these factors are combined with the high maintenance, land area, labor and management costs, this form of conservation is expensive (RAJASEKHARAN & RAMANATHA RAO, 2019).

Thus, biotechnology offers several advantages and possibilities that could serve as complements or alternatives to traditional citrus conservation methods. For example, *in vitro* conservation is a technique that maintains plants in a laboratory under slow growth with periodic subcultures (PANIS et al., 2020; JOSHI, 2017). Slow plant growth maximizes the interval between subcultures without affecting the viability of the culture, which facilitates collection management. Other advantages of *in vitro* conservation include reductions in the physical space that is required to maintain a large collection, the ease of germplasm exchange, and the protection of the conserved germplasms against biotic and abiotic factors (SCHERWINSKI-PEREIRA & COSTA, 2010).

However, despite these advantages, *in vitro* conservation requires skilled labor. In addition, depending on the growth rates of the conserved plants, short time-interval subculture production can be laborious, costly and can favor somaclonal variations. Therefore, studies that focus on the *in vitro* conservation of plant species seek minimum plant growth conditions by reducing plant metabolism.

Various strategies can be used to minimize plant growth and reduce plant metabolism, including reducing temperature and light intensity or changing the culture media by adding supplemental osmotic agents (such as mannitol and sorbitol) or growth inhibitors (PANIS et al., 2020; SCHERWINSKI-PEREIRA et al., 2010; CARVALHO et al., 2016). These strategies have been applied to different species and have yielded good results (MUNÕZ et al., 2019; SEDLAKA et al. 2019).

Osmotic agents, such as sucrose, sorbitol and mannitol, act as growth retardants when added to culture media by causing osmotic stress in plants, reducing the water potential and restricting water availability for explants (LÉDO et al., 2007). Sucrose is among the most commonly used carbohydrates in nutrient media. Sucrose is essential for plant growth because photosynthesis is limited in plants or explants that are cultivated *in vitro* (MURASHIGE & SKOOG, 1962). Thus, the sucrose concentration in culture media can significantly alter plant metabolism.

Several basic media formulations have been used in *in vitro* cultures for germplasm conservation. Although, there is no standard formula, modified and diluted MS media (MURASHIGE & SKOOG, 1962) have been used for the *in vitro* conservation of various species (LÉDO et al., 2007; CHAIDIR et al., 2019). However, the MS medium is not efficient for many woody species because media with more dilute macronutrient concentrations produce better plant growth and development (GRATTAPAGLIA & MACHADO, 1998).

For example, woody plant medium (WPM), (MCCOWN & LLOYD, 1981) contains 25% of the nitrate and ammonia ion levels that are found in the MS medium and contains higher levels of sulfate and potassium ions. Thus, WPM has been widely and successfully used for the micropropagation of woody species. In addition, WPM cultures yielded promising results for storing *Amburana cearensis* genotypes for 10 months (ALVIM et al., 2020) and for maintaining and conserving *Cochlospermum regium* explants under minimum *in vitro* growth conditions (CAMILLO et al., 2009).

Currently, this basic medium is being used to establish an active *in vitro* citrus fruit germplasm bank of Embrapa Cassava and Fruits (Embrapa Mandioca e Fruticultura**)**, (SOUZA et al., 2009). Although, the results are positive, improvements are required because *in vitro* citrus conservation is used for many species and related genera, which makes it difficult to establish optimal conditions for the cultivation of all genotypes.

Consequently, this study adapted a protocol for the *in vitro* conservation of Florida Rough lemon (*Citrus jambhiri* Lush.).

MATERIALS AND METHODS

Mature fruits were collected from 'Florida Rough' lemon trees that were grown in the experimental citrus field of Embrapa Cassava and Fruits at Cruz das Almas, Bahia. The fruits were taken to the Laboratory of Tissue Culture, washed with running water and sliced crosswise. Next, the seeds were extracted, washed with detergent in running water, left to dry at room temperature and subsequently stripped of their external integument, the testa.

These seeds were brought to a laminar flow hood and subjected to an asepsis process by immersion in 70% ethanol for 5 minutes. Next, the seeds were treated with an aqueous sodium hypochlorite solution that contained 1% active chlorine and 3 drops of Tween-20 detergent for 20 minutes, followed by 3 rinses in sterile distilled water. After disinfection, the seeds were inoculated into test tubes that contained approximately 10 mL of WPM that had been supplemented with 25 $g.L^{-1}$ sucrose, solidified with 7 $g.L^{-1}$ agar and adjusted to pH 5.8 prior to autoclaving. The tubes were kept in a growth chamber at 27 ± 1 ºC, with a light flux density of 30 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 16 hours for 45 days. These tubes served as the explant sources when setting up the experiments.

Next, microcuttings of these plants (approximately 1 cm in size) were inoculated into test tubes that contained 10 mL of WPM at different concentrations (1/1, 1/2 and 1/4), which had been supplemented with 0, 12.5, 25 and 50 $g.L^{-1}$ sucrose, solidified with 7 $g.L^{-1}$ agar and adjusted to pH 5.8 prior to autoclaving. Next, they were kept in a conservation room with a temperature of 22 ± 1 ºC, a photoperiod of 12 h and a photon flux density of 20 $\mu mol\ m^{-2}.\ s^{-1}$.

After 360 days, the plant height in cm (PH), the plant dry mass in g (PDM) and the numbers of green leaves (NGL), senescent leaves (NSL) and number of microcuttings (NM), with approximately 1 cm in length were evaluated.

The experiment was conducted in a completely randomized design with a 3 x 4 factorial scheme for the WPM (1/1, 1/2 and 1/4) and sucrose (0, 12.5, 25 and 50 $g.L^{-1}$) concentrations. In addition, 15 replications were conducted. Each experimental plot was established with a test tube that contained 1 microcutting. Next, the data were subjected to an analysis of variance F-test. For this experiment we considered WPM concentration as a qualitative factor since we evaluated the conservation of *in vitro* plants under condition of high, medium, and low salt concentrations. The average WPM concentrations were compared using the Tukey's test at a probability of 5%. Polynomial regression models were used for the average sucrose concentrations. The NGL, NSL and NM variables were transformed using the square root of (x + 0.5) to meet the assumptions of the analysis of variance. The data obtained were submitted to the test of Shapiro-Wilks normality before and after data transformation.

In addition, a MANOVA was performed to verify the simultaneous effects of the treatments on the variables. The significances of the treatments were tested against the Wilks's criterion according to JOHNSON & WICHERN (1992). Based on the matrix of the sums of squares and products that were obtained from the MANOVA, the partial correlation coefficients were calculated. In addition, a multicollinearity diagnosis was performed according to the criterion of MONTGOMERY & PECK (1981). Furthermore, the criteria presented by SINGH (1981) were used to calculate the relative contributions of each variable in the multivariate analysis. Statistical analyses were conducted with SAS (SAS, 2004) and Genes (CRUZ, 2014).

RESULTS AND DISCUSSION

Plants that were kept in different sucrose and WPM concentrations exhibited different behaviors that depended on the treatment and the considered variables (Figure 1).

The variables that best explained the observed behavior of the 'Florida Rough' lemon plants were NGL and PH, with values of 61.63 and 35.08%, respectively according to SINGH (1981), (Table 1). Both variables were fundamental for the establishment of efficient *in vitro* conservation protocols. A reduced *in vitro* plant growth rate, which is the goal of conservation, is desirable because it indicates reduced metabolism that is not the result of other events, such as toxicity, which can occur in other treatment methods. In contrast, this *in vitro* reduction must correspond with plants with good leaf areas, which ensures their regeneration at the end of the conservation period. This discussion clearly indicates the importance of a significant NGL. Thus, results obtained from the criteria of Singh are a positive indicator relative to this study.

The significant effects of the sucrose interactions with WPM on PH, NM and PDM are shown in table 2. A significant difference occurred regarding the isolated factors in the NGL. However, no significant effects occurred regarding the NSL. The coefficients of variation (CV) varied from 13.21 to 41.41% for NM and PDM, respectively. The field tissue culture experiments generally resulted in higher CV, which indicated that the response data distribution did not follow the assumption of normality, despite maintaining control over the growth conditions (temperature, light and photoperiod).

These results led to high CV and required data transformation for interpretation (MIRANDA-FONTAIÑA & FERNÁNDEZ-LÓPEZ, 2009). In the *in vitro* conservation of mangabeira (*Hancornia speciosa*), SÁ et al. (2011) found CV that ranged from 35.65 to 112.44%. In contrast, in the *in vitro*

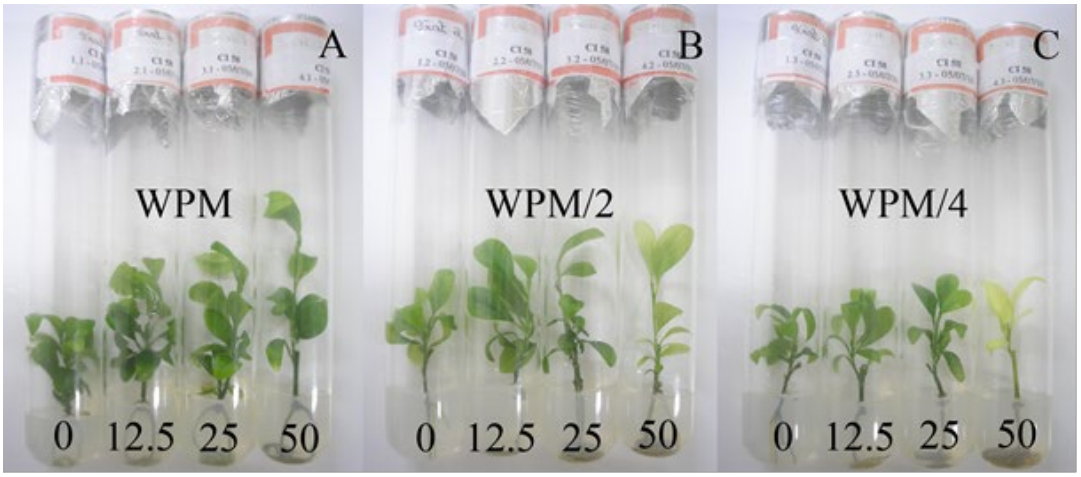

Figure 1 - 'Florida Rough' lemon plants following 360 days of *in vitro* conservation in WPM at the normal (A), 1/2 (B) and 1/4 (C) WPM concentrations and with sucrose does of 0, 12.5, 25 and 50 $g.L^{-1}$, respectively. Photo: Antonio da Silva Souza.

propagation of the Heliconia champneiana cv. Splash, RODRIGUES et al., (2018) reorted CV between 11.09 and 38.97%. These studies both confirmed trends of increasing CV for this type of. In addition, these studies confirmed the need for efficient evaluation criteria. Beyond the environmental conditions, factors such as genetic material variability and plant physiological stage, which gave birth to explant type should be considered when interpreting the CV (WERNER et al., 2013).

Regarding the MANOVA, a significant effect was observed for all variables according to the Wilks's criterion when the variables were considered together (Λ; Table 2).

One advantage of the multivariate extension method relative to the traditional univariate method is the potential estimation of the partial correlation matrix from the sum of squares matrix and the residual product (CARVALHO et al., 2014). The partial correlation establishes a degree of association between 2 variables, which eliminates the treatment effects. In general, the information provided by univariate modeling are covered by multivariate extension, without; however, take into account a level of significance set and the performance of the correlations between variables. MARDIA (1971) stated that the MANOVA is robust for data that don't meet the normality assumption, which indicates that the MANOVA is a suitable tool for tissue culture studies.

Tables 3 and 4 show the polynomial regression models and the average NGL values, respectively. Fitting with the first and second-degree models resulted in R^2 values of 54.14 to 98.89% (Table 3).

A linear behavior was observed between increasing PH and increasing sucrose concentrations when WPM concentrations were 1/1 and 1/2. However, at WPM concentrations of 1/4 WPM, no significant differences were observed between the tested sucrose doses. These findings suggested that the interactions between these 2 factors (WPM and sucrose) limit plant development (Table 3). A more pronounced increase in PH was observed with increasing sucrose dose when a WPM concentration of 1/1 ($\hat{b}$ =0.07) was used rather than a WPM concentration of 1/2 ($\hat{b}$ =0.02). This result indicated that the basic medium components affected plant performance more than the sucrose dose. In addition, the results indicated that the absence of sucrose in the medium resulted in the lowest PH, regardless of the WPM concentration (Table 3). The WPM concentration of 1/2 was initially considered as optimal because reduced growth rate corresponds with metabolic reduction.

In contrast, although, the interactions between the factors were not significant for NGL, a first-degree descending model was established. This

Table 1 - Relative contributions of the diversity variables (Sij) according to the criterion of SINGH et al. (1981) for plant height in cm (PH), plant dry mass in g (PDM) and the numbers of green leaves (NGL), senescent leaves (NSL) and microcuttings (NM) of 'Florida Rough' lemon plants as a function of the WPM (1/1, 1/2 and 1/4) and sucrose (0, 12.5, 25 and 50 $g.L^{-1}$) concentration after 360 days of conservation (temperature of 22 ± 1 ºC, a photoperiod of 12 h and a photon flux density of 20 µmol $m^{-2}.s^{-1}$).

Variable	Sij	Sij (%)
PH	136.05	35.08
NGL	239.02	61.63
NSL	6.09	1.57
NM	6.44	1.66
PDM	0.22	0.06

S.j contribution of the variable *x* for the value of the Mahalanobis distance between the genotypes *i* and *i*.

model indicated that sucrose concentration was not a decisive factor for increasing NGL. However, the highest values for this variable were reported in the absence of a carbohydrate source (Table 3). This finding highlights the role that the basic medium plays in plant matter development. In this case, the relevant growth parameters are maintained and results in a good number of green leaves, but significant reductions in plant metabolism are observed (Table 4). However, although the results showed that the plants that were conserved in the absence of sucrose produced the highest NGL, the research was only conducted over a 12-month period. Thus, the absence of a carbon source could have negative consequences for later timepoints.

None of the major *in vitro* collections housed in institutions, such as the Biodiversity in Belgium, the International Center for Tropical Agriculture (ICTA) in Colombia, or the International Institute of Tropical Agriculture (IITA) in Nigeria, maintain their collections in the absence of sucrose or another carbon source. In the Biodiversity collection of banana plants (for which the average subculture interval is 12 months), 30 $g.L^{-1}$ of sucrose were used. In contrast, sucrose concentrations of 20 and 30 $g.L^{-1}$ are used for cassava with subculture intervals of 4 to 19 months at the ICTA and IITA, respectively (CGIAR, 2012).

Thus, a concentration of 25 $g.L^{-1}$ of sucrose was chosen as the optimum sucrose concentration. This concentration was chosen by comparing the

Table 2 - Summary of the univariate and multivariate analyses of variance for plant height in cm (PH), plant dry mass in g (PDM) and the numbers of green leaves (NGL), senescent leaves (NSL) and microcuttings (NM) of 'Florida Rough' lemon plants maintained *in vitro* under different concentrations of sucrose and WPM after 360 days of conservation (temperature of 22 ± 1 ºC, a photoperiod of 12 h and a photon flux density of 20 µmol $m^{-2}.s^{-1}$).

Sources of variation	DF	----------Mean Square----------					Λ
		PH	NGL	NSL	NM	PDM	
Sucrose	3	23.9901^{**}	1.1366^{**}	0.0588^{ns}	0.1167^{*}	0.0553^{**}	0.4256^{**}
WPM	2	20.4841^{**}	1.5227^{**}	0.0799^{ns}	0.0312^{ns}	0.0264^{**}	0.7781^{**}
Suc. x WPM	6	9.0674^{**}	0.1451^{ns}	0.1258^{ns}	0.0876^{*}	0.0098^{**}	0.6027^{**}
Error	164	1.4143	0.2539	0.1020	0.0325	0.0020	
CV (%)		27.94	15.44	37.87	13.21	41.41	
Overall average		4.2563	10.4318	0.3125	1.3977	0.1093	

DF, degrees of freedom. MS, Mean Square. ** and *, significant at 1 and 5% probability, respectively, for the F test. ^{ns}, not significant at a probability of 5%. Λ, value calculated with the Wilks's criterion.

Table 3 - Regression equations, coefficients of determination, optimal doses and estimated values of plant height (cm), plant dry mass (g) and the numbers of green leaves and microcuttings of the 'Florida Rough' lemon tree as a function of the WPM and sucrose ($g.L^{-1}$) concentrations.

WPM Concentration	Equation	R^2 (%)	Optimal Dose	Estimated Value
----------Plant height----------				
1/1	$\hat{y}^{**} = 3.1361 + 0.0715x$	98.89	25.00	4.92
1/2	$\hat{y}^{*} = 3.9946 + 0.0208x$	54.14	25.00	4.51
1/4	$\hat{y}^{ns} = 3.60$	-	25.00	3.60[1]
----------Number of green leaves----------				
1/1; 1/2; 1/4	$\hat{y}^{**} = 11.3900 - 0.0429x$	77.22	25.00	10.32
----------Number of microcuttings----------				
1/1	$\hat{y}^{*} = 0.9268 + 0.0387x - 0.0005x^2$	80.46	38.70	1.68
1/2	$\hat{y}^{ns} = 1.47$	-	0.00	1.47[1]
1/4	$\hat{y}^{ns} = 1.37$	-	0.00	1.37[1]
----------Plant dry mass----------				
1/1	$\hat{y}^{**} = 0.0560 + 0.0028x$	87.48	50.00	0.20
1/2	$\hat{y}^{**} = 0.0944 + 0.0013x$	83.01	50.00	0.16
1/4	$\hat{y}^{ns} = 0.09$	-	0.00	0.09[1]

** and *, significant at 1 and 5% of probability, respectively, for the F-test. ns, non-significant at a probability of 5%. [1]Based on the average observed values.

NGL results (Table 3) that were obtained at a sucrose concentration of 25 $g.L^{-1}$ (10.32) with the NGL results that were obtained in the absence of sucrose (11.39). In addition, previous reports from large collections were considered. The PH at this concentration (4.92 cm) did not compromise the expected results. This finding highlights the potential of using regression equations for obtaining estimated values from doses that are considered optimal. This method could become a very useful tool for managing collections and for establishing appropriate treatments.

For sweet potato landraces, positive effects were obtained with the use of sucrose (2%) as a carbon source and the use of an osmotic regulator to maintain the viability of the explants conserved *in vitro* (VETTORAZZI et al., 2017).

According to PACHECO et al. (2016), salt concentrations are often reduced in a basic medium for slow growth conservation. Accordingly, AHMED & ANJUM (2010) observed good survival rates with reduced salt concentrations for a period of 6 months when studying pear (*Pyrus* sp.) cultures. After 6 months, budding death was observed due to the insufficient nutrient concentrations in the culture medium. Thus, longer periods of conservation might require higher salts and vitamin concentrations in the basic medium to ensure good survival rates and regeneration.

No significant differences were observed between the sucrose concentrations at WPM concentrations of 1/2 and 1/4 for NM. The largest NM was obtained at the optimal sucrose concentration of 38.70$g.L^{-1}$, which was estimated from the equation that was formulated from using the complete WPM and from the second-degree equation for the 1.68 microcuttings (Table 3).

Regarding the PDM, the highest results were obtained at a sucrose concentration of 50 $g.L^{-1}$ at WPM concentrations of 1/1 and 1/2 and with the values that were estimated with the regression equations for 0.20 g and 0.16 g, respectively (Table 3). This result was expected because the greatest PH values were observed at this concentration.

The condition number (CN) for the multicollinearity diagnosis was 3.79, which is a weak multicollinearity in the correlation matrix according to the classification system of MONTGOMERY & PECK (1981). This CN indicated the possibility of obtaining a reliable estimate in biological terms. The presence of multicollinearity (albeit low) in the specific case of the present study could be due to the correlation between PH and PDM. Thus, PH and PDM could generate similar information.

In addition, a weak correlation occurred between PH and NGL ($r = 0.17^{*}$). This correlation

Table 4 - Average values of the numbers of green leaves for 'Florida Rough' lemon tree as a function of WPM concentration.

WPM	Average
1/1	11.1897 a
1/2	10.7931 a
1/4	9.3500 b

Averages followed by the same lowercase letter did not differ according to the Tukey's test at a probability of 5%.

enabled the generation of plants with less height, but with an appropriate number of green leaves (Table 5), which are effective conditions for *in vitro* conservation. There was no significant correlation (r = -0.13ns) between PH and NM, in which indicated that the numbers of microcuttings were not high in the plants with increased height (Table 5). This finding is related to the fact that citrus plants grown *in vitro* have shorter distances between their internodal segments, which reduces the number of microcuttings per plant. Therefore, this variable is important.

This result is an important aspect of *in vitro* plant conservation. The correlated variables must be considered in the evaluations to establish minimum growth conditions while maintaining plant viability. These variables vary for different species and depend on the genetics, *in vitro* growth dynamics and criteria adopted by the investigator.

The color of the plant leaves was also observed under the different treatments. As the sucrose concentration increased and the WPM concentration decreased, the plants had greater numbers of yellow leaves. This finding indicated that high sucrose concentrations could cause toxic effects in plants when the concentrations of the other culture medium components are lower (Figure 1). The toxic effects of *in vitro* conservation experiments are frequent due to the use of high concentrations of osmotic regulators (ZAYOVA et al., 2017; SANGHAMITRA et al., 2019).

The results indicated that it is possible to conserve 'Florida Rough' lemon plants *in vitro* without subculturing for a period of 12 months by using normal concentrations of WPM. However, further studies with other genotypes are necessary for defining the protocol for *in vitro* citrus species conservation because variations may occur between plants that are maintained *in vitro* within the same genotype.

MARIN & DURAN-VILA (1991) proposed a micropropagation protocol for *in vitro* citrus germplasm conservation. The subculture cycle in this proposed protocol varied from 8 to 12 months and included multiple operations (nodal segment culture, shoot rooting and rooted shoot elongation) that permitted the maintenance of regenerated citrus plants *in vitro* for up to 12 months without subculture. However, some growth regulators were added to the standard MS culture medium. During subculturing, these additives increase the risk of somaclonal variations in the plants.

Table 5 - Partial correlation coefficients for plant height in cm (PH), plant dry mass in g (PDM) and the numbers of green leaves (NGL), senescent leaves (NSL) and microcuttings (NM) of 'Florida Rough' lemon plants as a function of the different WPM and sucrose concentrations.

	NGL	NSL	NM	PDM
PH	0.17^{*}	-0.15^{ns}	-0.13^{ns}	0.36^{**}
NGL		-0.17^{*}	0.39^{**}	0.39^{**}
NSL			0.14^{ns}	-0.18^{*}
NM				0.12^{ns}

** and *, significant at 1 and 5%, respectively, in a t-test. ^{ns}, not significant at a probability of 5%.

Nevertheless, SOUZA et al. (2009) used a protocol with no added growth regulators to maintain citrus and related genotypes with high polyembryony for a period of 18 months without subculture.

Despite the promising results obtained by these authors, the demand for modifications to the current protocol mainly result from the different species and the related genera that comprise the citrus germplasm under field conditions (which must be established *in vitro* to create a backup).

CONCLUSION

The main variables that contributed to the observed variability in response to the treatment effects during the *in vitro* conservation of 'Florida Rough' lemon plants were NGL and PH.

It is possible to conserve 'Florida Rough' lemon plants *in vitro* without subculturing for a period of 12 months by using normal concentrations of WPM with 25 g. L^{-1} of sucrose.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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