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Use of multivariate analysis to evaluate the effect of sucrose on *in vitro* cassava conservation

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The aim of this work was to evaluate the effect of sucrose to reduce the *in vitro* growth of cassava plants using multivariate statistical tests. Cassava conservation has a relevant role as an auxiliary strategy for preservation and genetic breeding. Micro-cuttings of cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 from the Active Germplasm Bank of the Embrapa Cassava and Fruits were tested with five different concentrations of sucrose (0, 14.6, 29.2, 43.8, 58.5 mM) and the following variables were evaluated: plant height (cm), total number of leaves, number of senescent leaves, number of micro-cuttings, size of callus, number of roots and plant vigor. The data were submitted to analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA). According to the criterion of Singh, the variable that contributed most to explain the behavior of cassava plants under the conditions studied was the number of leaves, with 36.52%, while the vigor of the plants showed the lowest contribution (0.66 %). The best concentration for *in vitro* cassava conservation was 58.45 mM of sucrose, based on the number of viable plants after incubation.

Key words: Biotechnology, germplasm conservation, tissue culture, *Manihot esculenta*, genetic resources.

INTRODUCTION

The genetic diversity of cassava in Brazil provides a wide genetic base for breeding programs in the tropics. This variability covers a group of genes that confer resistance to major pests and diseases that affect the crop and enables adaptation to different edaphoclimatic conditions (Albuquerque et al., 2009).

Genetic erosion in cassava is mainly caused by biotic and abiotic stresses, which along with the expansion into new agricultural frontiers is a fact that should not be ignored in genetic breeding program, to avoid jeopardizing present and future actions. Therefore, efforts are required to conserve this germplasm (Fukuda et al., 2002; Rival and Mckey, 2008).

In vitro conservation comprises maintenance of micro controlling different growing conditions, such as temperature, photosynthetically active radiation, photoperiod and

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Table 1. List of cassava accessions, common name, origin and collection site.

Accessions	Common Name	Origin	Collection Site
BGM 264	Cigana	EAUFBA	Amargosa-BA, Brazil
BGM 265	Panguá	IPEAL	Santa Teresinha-BA, Brazil
BGM 1037	Casco de Burro	CENARGEN	Aurora do Norte-TO, Brazil
BGM 1282	Macaxeira-Cara	CENARGEN/CNPMF	Ouricuri-PE, Brazil

Source: *Embrapa Cassava and Fruits* (2003).

concentrations of osmotic retardants and hormones in the culture medium (Canto et al., 2004), therefore extending the interval between subcultures. This reduces the need for labor and reagents and as a result decreases the risks of fungal and/or bacterial contamination (Lemos et al., 2002).

Plants kept *in vitro* do not perform photosynthesis enough to guarantee the development of explants, it is necessary to add a carbon source such as a sugar to the culture medium. The sugar most commonly used in nutritive media is sucrose because it is easily metabolized by many species (De Conti et al., 2011). Reducing the amount of sucrose in the culture medium results in lower explant growth.

Usually, univariate statistical methods are used in studies of *in vitro* conservation to evaluate the effectiveness of treatments. However, studying the variables separately may not be enough to model biological phenomena, since important information is lost by disregarding the existing correlations between variables. Multivariate analysis involves considering all relevant variables at the same time in the theoretical interpretation of data (Neto, 2004). This enables evaluating a set of characteristics by taking into account their correlations, leading to inferences about the set of effects of characteristics at a set level of significance (Johnson and Wichern, 1992). However, multivariate analysis techniques have only infrequently been used in studies involving *in vitro* conservation (Carvalho, 2014; Franco et al., 2007).

The aim of this work was to evaluate the effect of sucrose in reducing the *in vitro* growth of cassava plants using multivariate statistical tests, aimed at more efficient study of *in vitro* conservation of cassava germplasm.

MATERIALS AND METHODS

Plant material

Four cassava cultivars were selected from different regions of Brazil, from experimental fields of the Active Germplasm Bank of the Embrapa Cassava and Fruits. Table 1 shows the accessions, common name, origin and collection site.

In vitro plant growth

The plants of the accessions from Table 1 were used as initial explants for conservation experiments. Excised meristems from plants kept in a greenhouse were decontaminated and inoculated in 4S medium containing MS (Murashige and Skoog, 1962) salts supplemented with 1 mg L^{-1} of thiamine, 100 mg L^{-1} of inositol, 0.02 mg L^{-1} of naphthalene acetic acid (NAA), 0.04 mg L^{-1} of benzylamino-purine (BAP), 0.05 mg L^{-1} of gibberellic acid (GA_3), 20 g L^{-1} of sucrose, solidified with 2.4 g L^{-1} of Phytagel® (SIGMA, USA), with pH adjusted to 5.8, at a temperature of 27 ± 1ºC, 16 h photoperiod and photon flux density 30 µmol $m^{-2}s^{-1}$ for 30 days (establishment phase).

The multiplication phase was conducted with three subcultures at intervals of 45 days using as explants microcuttings of 1.0 cm inoculated in 17 N medium (CIAT, 1984), composed of 1/3 of the macro and micronutrients of MS medium supplemented with 0.35 mg L^{-1} of thiamine, 35 mg L^{-1} of inositol, 0.01 mg L^{-1} of NAA, 0.01 mg L^{-1} of BAP, 0.01 mg L^{-1} of GA3, 20 g L^{-1} of sucrose, 2.4 g L^{-1} Phytagel ®, with pH adjusted to 5.8. The incubation was carried out in a growth room with 16-h photoperiod, temperature of 26 ± 1°C and photon flux density of 30 µmol.$m^{-2}.s^{-1}$.

In vitro conservation

Microcuttings (1.0 cm length) from plants obtained in the previous step were inoculated in basic culture medium “8S” (CIAT, 1984). The medium consisted of mineral salts and vitamins from "MS" supplemented with NAA (0.01 mg L^{-1}), BAP (0.02 mg L^{-1}), GA_3 (0.1 mg L^{-1}), with pH adjusted between 5.7 and 5.8, solidified with 2 g L^{-1} of Phytagel®. The plants were stored in a slow growth room with a temperature of approximately 22°C, having a 12-hphotoperiod and photon flux density of 30 µmol $m^{-2}s^{-1}$. The sucrose concentrations used were based on the results obtained by Macia (2011).

The effect of four sucrose concentrations was evaluated against a control treatment without sucrose in the culture medium. The evaluated concentrations were 14.6, 29.2, 43.8 and 58.5 mmol L^{-1}. Evaluations were performed at 30, 60, 120 and 330 days after incubation. Then after 330 days in the preservation medium, the surviving plants were transplanted and cultured in 17 N growth medium. The plant viability was assessed by calculating the regeneration rate (%) per accession and treatment.

Variables

The following variables were evaluated: plant height (PH) in cm, total number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC), number of roots (NR), root length (RL) in cm, size of callus (CS), using the scale 0 (absent), 1 (small), 2 (medium) and 3 (large), and plant vigor (V), using the scale 3 (totally green plant), 2 (slightly yellowish plant), 1 (very yellowish plant) and 0 (dead plant).

Statistical analysis

A completely randomized model was used in split plots in time to

Table 2. Summary of analysis of variance (ANOVA) for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), callus size (CS), number of microcuttings (NMC), number of roots (NR), root length (RL) and vigor (V) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282.

SV	DF	MS							
		PH	NL	NSL	CS	NMC	NR	RL	V
Acession	3	223.74**	23.41**	17.20**	4.29**	5.77**	9.87**	73.14^{ns}	1.28^{ns}
Sucrose	4	641.62**	43.26**	31.73**	3.40**	24.86**	28.76**	318.74**	0.18^{ns}
Access*Suc	12	127.84**	7.90**	5.62**	2.37**	4.44**	4.04**	57.47^{ns}	0.41^{ns}
Error A	76	14.91	1.93	1.07	0.30	0.52	0.64	22.30	0.19
Days	4	468.71**	62.03*	116.36**	3.04*	20.90**	38.35*	224.34**	25.36*
Error B	16	2.31	0.25	0.23	0.06	0.12	0.16	3.86	0.07
Access*Days	12	28.94**	1.41^{ns}	2.50**	0.08^{ns}	0.57**	1.03**	24.78**	0.27^{ns}
Suc*Days	16	35.52**	2.25**	2.60**	0.06^{ns}	1.30**	3.13**	30.76**	0.13^{ns}
Access*Suc*Daysem	48	9.78**	0.94**	1.02**	0.12**	0.34**	0.51**	10.79^{ns}	0.19**
Error C	1054	4.00	0.44	0.32	0.05	0.14	0.25	5.91	0.08
CV (%)		41.97	30.52	32.60	14.99	19.93	36.04	22.07	19.24
Mean		4.76	5.51	3.72	2.08	3.62	2.11	11.01	1.99

*significant at 5% by the F-test. ^{ns}not significant at 5%; SV, source of variation; DF, degree of freedom; MS, mean square.

Table 3. Relative contribution of the variables to diversity according to the criterion of Singh (1981) for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), callus size (CS), number of microcuttings (NMC), number of roots (NR) and vigor (V) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 for different concentrations of sucrose.

Variable	Sij	Sij (%)
PH	6846.15	7.12
NL	35132.47	36.52
NSL	32344.24	33.62
CS	631.84	0.66
NMC	12385.41	12.87
NR	8657.44	9.00
V	207.87	0.22

Sij, Measure of the relative importance of character j in canonical variables based on the Mahalanobis distance i.

perform analysis of variance. In the plots, four accessions and five concentrations of sucrose were analyzed, while in the subplots four evaluation times were considered (30, 60, 120 and 330 days after incubation) along with their respective interactions with the plots factors. Five replications per treatment were used, each consisting of three plants. The experimental plot was formed by a test tube containing one microcutting. The data on number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC) and number of roots (NR) were transformed to $(x+0.5)^{1/2}$ to satisfy the assumptions for the analysis of variance. All the assumptions of ANOVA were tested. In ANOVA, F-test criterion was used to test the significance of the treatments. The analyses were performed with the aid of the SAS program (SAS Institute Inc., 2000).

Multivariate analysis of variance (MANOVA) was also performed to check the effect of the treatments regarding the variables. According to Johnson and Wichern (1992), the Wilks criterion was used to test the significance of the treatments. Based on the matrix of the sums of squares and products obtained from MANOVA, the partial correlation coefficients were calculated, and multicollinearity diagnosis was performed according to the criterion of Montgomery and Peck (1981). For the calculation of the relative contribution of each variable in the multivariate analysis, the criterion of Singh (1981) was used.

RESULTS AND DISCUSSION

The analysis of variance (ANOVA) indicated different treatment effects of sucrose, accession and evaluation time on the dependent variable, according to the treatment and the variable considered (Table 2).

It is necessary to emphasize that, since the evaluations are made in the same experimental plot, it was necessary to include two new components in the model (errors a and b). This is because, of course, there is no randomization of the evaluation times in different repetitions, that is, measures were taken at the same time on the same plots. Consequently, there is no independence of measurements taken over time. Thus, it is suggested to perform analysis of variance with three experimental errors (Ramalho et al., 2000).

As indicated by the criterion of Singh (1981), the variables that contributed most to explain the behavior of cassava plants under the established conditions were the number of leaves (NL) and number of senescent leaves (NSL), with 36.52 and 33.62%, respectively, while callus size (CS) and plant vigor (V) showed the lowest contribution with 0.66% and 0.22%, respectively (Table 3).

This analysis allows the selection of variables that are important for *in vitro* conservation studies, enabling the management of data and subsequent analyses.

Table 4 shows that partial correlation coefficients, obtained by multivariate analysis (MANOVA), allowed an

Table 4. Partial correlation coefficients for plant height (PH), number of leaves (NL), number of senescent leaves (NSL), number of microcuttings (NMC) and number of roots (NR) in cassava accessions BGM 264, BGM 265, BGM 1037 and BGM 1282 for different concentrations of sucrose.

Variables	NL	NSL	NMC	NR
PH	0.65**	0.62**	0.78**	0.52**
NL		0.80**	0.79**	0.59**
NSL			0.71**	0.53**
NMC				0.57**

** Significant at 1% by the t-test.

even more detailed analysis, as was performed with the most important variables of the study (PH, NL, NSL and NMC) according to the criterion of Singh (1981). The highest correlations were obtained between NL and NSL (0.80**), NL and NMC (0.79**) and PH and NMC (0.78**), involving conservation impact variables that express the development of plants under the established conditions. The partial correlation coefficient shows how the behavior of a variable is related to another and in some cases allows the elimination of one of them, since their behavior can be predicted by the other. In cases where the measurement of a given variable is laborious or can be destructive, the high correlation with another variable may eliminate the need for these measurements.

The condition number of Montgomery and Peck (1981) refers to the ratio between the highest and lowest values obtained from the principal component analysis; if the condition number is less than 100, it is refer to a low multicollinearity. The condition number from the multicollinearity diagnosis obtained in this study was 51.54, classified as weak multicollinearity in the correlation matrix according to the classification of Montgomery and Peck (1981), so it was possible to obtain a reliable estimate in biological terms. In this work, NL, NSL and PH showed high correlation with the number of microcuttings (NMC), facilitating evaluation of the results obtained on the efficiency of treatments in reducing the metabolism of plants and their subsequent viability. These variables are considered to be of high relevance to *in vitro* conservation studies of cassava and the statistical tools used in this work confirmed this importance.

An advantage of multivariate extension is the possibility to estimate the partial correlation matrix obtained from the sum of squares and the product of residuals when compared with the traditional univariate method. The partial correlation establishes the degree of association between two variables, eliminating the effect of the treatments. When the number of repetitions is large, Ito and Schull (1964) and Korin (1972) observed that the tests used in multivariate analysis of variance are not greatly influenced by the heterogeneity of variance and covariance matrices. Mardia (1971) concluded that multivariate analysis of variance is robust and its ability to deal with non-normality makes it a more suitable tool for tissue culture work.

In the search for a suitable condition that can accommodate the largest possible number of *in vitro* cassava accessions with the most standardized behavior possible, the elimination of two variables (CS and V) implies a significant reduction of work.

In a given study, the importance of a variable will depend on the species and the objective. For example, in citrus conservation the number of microcuttings is not a determining factor, as evidenced by studies by Carvalho, (2014). In cassava, this variable is essential because several microcuttings are obtained from the same plant and the increase in the number of microcuttings will depend on the treatment. It is quite complicated to break the apical dominance of cassava *in vitro* and the replication depends on the number of microcuttings. Vidal (2009), studying *in vitro* cassava cultivation, found a strong positive correlation (0.71), similar to the one obtained in this work (0.78), between plant height and number of microcuttings. Also, Macia (2011) suggests that these two variables may present good correlation for *in vitro* cassava conservation.

The results obtained in this work are consistent with those obtained by Londe et al. (2012), when evaluating sucrose's effect on growth rate. They noted that the 58 mM concentration produced greater plant height. Macia (2011) tested different sucrose concentrations on *in vitro* cassava conservation (29, 58, 116 and 232 mM) and observed the best results in the same concentration. The plants showed a reduction of cellular metabolism without compromising their viability after the conservation period.

Regarding the difference in behavior among the four accessions, BGM 1282 presented the lowest values for all variables. The other three cultivars (BGM 264, BGM 265 and BGM 1037) showed similar performance for PH, NL, NSL and NMC variables in the concentration of 43.8 mM (15 gL^{-1}). Regarding the management of *in vitro* germplasm banks, this result can be considered encouraging since one of the major difficulties found is exactly the different behaviors observed among the

Table 5. Number of plants surviving after 330 days (NPSO), number of cultured microcuttings (NMC), number of viable plants (NVP) and plant viability (PV) of different cassava accessions after preservation in different sucrose concentrations.

Sucrose (mM)	NPSO	NMC	NVP	PV%
0.0	10	10	0	0
14.6	8	8	0	0
29.2	2	2	0	0
43.8	7	19	5	26
58.5	5	25	22	88
Accessions				
BGM 264	4	26	5	19
BGM 265	17	24	18	75
BGM 1037	2	6	4	67
BGM 1282	6	8	0	0

genotypes preserved.

The maintenance of plant viability after the incubation period is one of the most important aspects of *in vitro* conservation. For this purpose, the metabolism reduction cannot cause a loss of viability. This work showed that the loss of plants in BGM 264 begins from 120 days of culture, similar to BGM 265, regardless of sucrose concentration. The first dead plants of BGM 1037 and BGM 1282 appeared at 30 days of conservation, confirming one of the problems for *in vitro* cassava conservation.

Table 5 shows, in general, the results obtained separately by treatment and accessions, confirming that the survival rates are directly related to the best treatment for the conservation of these four accessions. Despite the encouraging results with the concentration of 43.8 mM, only one accession (BGM 265) presented viable plants at the end of 330 days of cultivation, with a 39% viability rate. For BGM 264 and BGM 1037, viable plants were obtained after the same conservation period with the higher sucrose concentration, 90% and 100% in that order, although there were only four BGM 1037 plants tested. These results can be considered promising since for cassava the maximum time of conservation is 270 days (IITA, 2002).

Table 5 shows the drastic reduction in metabolism observed in accession BGM 1282, which made renewed growth after incubation impossible, making clear the need for adjustments in the conservation of this material with higher concentrations of sugar. Once again, these results emphasize the strong genotype-dependence observed in the *in vitro* behavior of cassava varieties and how limiting and determining the carbohydrate source can be.

Conclusion

Multivariate analysis can be considered an efficient tool for studies of *in vitro* cassava conservation, and the sucrose concentrations of 14.6 and 29.2 mM reduced the metabolism of cassava plants *in vitro*, but made it impossible to regenerate them. The best concentration for *in vitro* cassava conservation is 58.5 mM of sucrose.

Conflict of interests

The authors did not declare any conflict of interest.

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Abbreviations: PH, Plant height; **NL,** total number of leaves; **NSL,** number of senescent leaves; **NMC,** number of micro-cuttings; **NR,** number of roots; **RL,** root length; **CS,** size of callus; **V,** plant vigor.

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