Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v38i1.26433

Univariate and multivariate statistical tools for *in vitro* conservation of citrus genotypes

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ABSTRACT. This study aimed to evaluate the influence of the growing environment on the *in vitro* conservation of citrus genotypes obtained from the Active Citrus Germplasm Bank of Embrapa Cassava and Fruit. The study used multivariate statistic tools in order to improve the efficiency in the analysis of the results. Approximately 1-cm of length microcuttings from plantlets derived from ten genotypes previously cultured *in vitro* were inoculated in test tubes containing 20 mL of WPM culture medium supplemented with 25 g L^{-1} sucrose, solidified with 7 g L^{-1} agar and adjusted to a pH of 5.8, and maintained under three environmental conditions for 180 days. The experiment was carried out in a completely randomized design in a split-plot in the space, with 15 replications. The results indicate that the principal component analysis is an effective tool in studying the behavior of different genotypes conserved under different *in vitro* growing conditions. The growing conditions of 22±1°C, a light intensity of 10 µmol $m^{-2}.s^{-1}$ and a 12 hours photoperiod was the most adequate for reducing the growth of *in vitro* conserved plants, increasing the subculture time interval while keeping the plants healthy.

Keywords: multivariate analysis of variance, partial correlation, principal component analysis, germplasm, *Citrus* spp.

Ferramentas estatísticas multivariadas para a conservação *in vitro* de genótipos de citros

RESUMO. Este estudo teve por objetivo avaliar a influência do ambiente de cultivo para a conservação *in vitro* de genótipos de citros oriundos do Banco Ativo de Germoplasma da Embrapa Mandioca e Fruticultura. O estudo utilizou ferramentas estatísticas multivariadas a fim de melhorar a eficiência na análise dos resultados. Microestacas de plântulas de dez genótipos previamente cultivadas *in vitro*, com aproximadamente 1 cm, foram inoculadas em tubos de ensaio com 20 mL do meio de cultura WPM suplementado com 25 g L^{-1} de sacarose, solidificado com 7 g L^{-1} de ágar e pH ajustado em 5,8, e mantidas em três ambientes de cultivo por 180 dias. O experimento foi instalado em um delineamento inteiramente casualizado em esquema de parcela subdividida no espaço, com 15 repetições. Os resultados indicam que a análise de componentes principais é uma ferramenta eficiente para estudar o comportamento de genótipos conservados em diferentes ambientes de cultivo *in vitro*. O ambiente de cultivo com 22±1ºC, intensidade luminosa de 10 µmol m^{-2} s^{-1} e 12h de fotoperíodo é o mais indicado para reduzir o crescimento das plantas conservadas *in vitro*, prolongando o tempo de subcultivo e mantendo as plantas sadias.

Palavras-chave: análise de variância multivariada, correlação parcial, análise de componentes principais, germplasm, *Citrus* spp.

Introduction

Several biotechnological strategies involving the *in vitro* culture of cells, tissues and plant organs have been developed and refined as alternatives to conventional conservation strategies. These techniques overcome various limitations that are inherent to conventional *ex situ* and *in situ* conservation methods and allow for the pest-free exchange of plant genetic resources (Moosikapala & Te-Chato, 2010; Scherwinski-Pereira & Costa, 2010). Because of the threat of Huanglongbing (HLB) disease, which is caused by *Candidatus liberibacter* spp. and has had devastating effects on the global citrus industry, the removal of citrus collections from open field conditions is essential. Recourses, such as *in vitro* conservation, are alternative approaches for establishing backup collections and may ensure the availability of healthy material for storage and exchange.

This conservation technique involves maintaining the plants under slow growing

laboratory conditions, which is accomplished with periodic subcultures and has been successfully used for several other crops (Divakaran, Babu, & Peter, 2006; Lata, Moraes, Bertoni, & Pereira, 2010; Rani & Dantu, 2012; Srivastava, Purshottam, Srivastava, & Misra, 2013). For large collections, however, these periodic subcultures become labor intensive, and there is an associated risk of somaclonal variation (Gaafar & Saker, 2006; Israeli, Lahav, & Reuveni, 1995; Negri, Tosti, & Standardi, 2000; Souza et al., 2009; Reuveni & Israeli, 1990).

One method to circumvent these issues is to increase the time interval between subcultures, which would reduce cost and risk like contamination or somaclonal variation. Various strategies can be used to accomplish this task, such as reducing the temperature, light intensity, photoperiod and modifying the culture medium. Osmotic agent supplements, such as mannitol and sorbitol, and growth inhibitors that interfere with the metabolic pathways of the plant and affect the plant's growth rate also can be used (Balch, Reyes, & Carrillo, 2012; García, Malaurie, Viltres, & Campos, 2008; Gopal & Chauhan, 2010; Lata et al., 2010; Moosikapala & Te-Chato, 2010; Scherwinski-Pereira & Costa, 2010).

WPM (Wood Plant Medium) (Lloyd & McCown, 1980) has shown promising results as a culture medium for the *in vitro* establishment of citrus active germplasm banks at Embrapa Cassava and Fruit Crops (Souza et al., 2011), but other improvements to the protocol still need to be made. The diversity of species and related genera makes the use of a single culture condition difficult and further studies are required.

The first factor tested in the *in vitro* conservation of a species is typically a reduction in growth room temperature. Each species has a temperature limit at which growth restriction is observed without causing physiological damage to the plant. In general, tropical fruit tree species are more sensitive at storage temperatures below 15°C than species from temperate zones. Temperate crops, such as raspberries, blackberries, apples and pears can be conserved with relative success at temperatures above 4°C (Scherwinski-Pereira & Costa, 2010). Therefore, the successful conversation of various plant species appears to depend on the plant's sensitivity to low temperatures, and this has been the primary factor investigated in the conservation of various plant species (Ahmed, Anjum, Shah, & Hamid, 2010; Capuana & Lonardo, 2013; Islam, Leunufna, Dembele, & Keller, 2003; Negash, Krens, Schaart, & Visser, 2001).

To reduce the growth rate and increase the interval period between subcultures, reported that the temperature for *in vitro* conservation should be approximately 20ºC for tropical species that cannot tolerate temperatures lower than 20ºC. The species from subtropical and temperate climates can be maintained at lower temperatures ranging from 10 to 15°C (Matsumoto, Cardoso, & Santos, 2010).

Light emissions are another factor that should be investigated in *in vitro* germplasm conservation. There are three main factors that should be assessed: photoperiod, irradiance and spectral composition. The photoperiod should be adjusted beforehand to 12:12, 14:10 or 16:8 (ratio of light/dark hours) (Morini, Marzialetti, & Babuiere, 1991). Matsumoto, Cardoso and Santos (2010) reported that a decrease in light intensity can reduce plant growth. However, they also reported that this decrease can not be too drastic because it may cause etiolation and prevent chlorophyll activation, which can result in a very severe reduction in growth and plant death.

In the *in vitro* conservation studies the univariate statistics techniques to assess the efficiency of the tested treatments are commonly used (García et al., 2008; Gopal & Chauhan, 2010; Rakosy-Tican, Bors, & Szatmari, 2012; Rani & Dantu, 2012; Srivastava et al., 2013). However, the study of the variables alone may not be sufficient to model the biological phenomenon, since some important information can be lost if dependency relationships between variables are ignored.

Thus, multivariate analysis may be a differential and an efficient tool, since it considers the simultaneous evaluation of many traits. This analysis makes the correlations between traits and provides inferences on a known level of significance (Johnson & Wichern, 2007). Multivariate analysis techniques have been little exploited in studies involving *in vitro* conservation due to the complexity of their calculations and lack of knowledge by the researchers.

The aim of this study was to evaluate the effect of the culture environment on the *in vitro* conservation of different citrus genotypes. A complementary goal was to evaluate the use of univariate and multivariate statistical tools and principal component analysis techniques for clustering genotypes that demonstrate similar behaviors under the different culture conditions used.

Material and methods

Microcuttings without leaves and approximately 1 cm of length and with one or two lateral buds

were used and were derived from *in vitro* plants with the following genotypes: lemon trees 'Cravo' clone Common (*Citrus limonia* Osbeck), 'Volkameriano' clone Catânea 2 (*C. volkameriano* V. Ten. & Pasq.) and 'Rugoso Mazoe' (*C. jambhiri* Lush.); citrange trees 'Carrizo' (*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.) and 'Troyer' (*C. sinensis* (L.) Osbeck x *P. trifoliata* (L.) Raf.); orange trees 'Azeda' clone Narrow Leaf (*C. aurantium* L.) and 'Azeda' clone Common (*C. aurantium* L.); citrumelo trees 'Swingle' (*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.); and hybrids TSKC x CTSW 03 [*C. sunki* hort. ex Tanaka x (*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.)] and TSKC x (TR x LCR) 059 [*C. sunki* hort. ex Tanaka x (*P. trifoliata* (L.) Raf. x *C. limonia* Osbeck)]. These microcuttings were cultured *in vitro* at the Tissue Culture Laboratory of Embrapa Cassava and Fruit Crops, Cruz das Almas, Bahia, Brazil.

In the a laminar flow hood the microcuttings were inoculated in test tubes containing 20 mL of WPM culture medium supplemented with 25 g L^{-1} sucrose. The culture media were solidified with 7 g L^{-1} agar and had the pH adjusted to 5.8 prior to autoclaving. The microcuttings were maintained under the following culture conditions: a) environment 1 - climate-controlled chamber with the temperature set at 17±1°C, photon flux density of 20 µmol m^{-2} s^{-1} and a 16 hours photoperiod ; b) environment 2 - room temperature set at 22±1°C, photon flux density of 10 µmol m^{-2} s^{-1} and a 12 hours photoperiod; and c) environment 3 - room temperature set at 27±1°C, photon flux density of 30 µmol m^{-2} s^{-1} and a 16 hours photoperiod.

The experiment was conducted in a completely randomized design in a split-plot in the space. The plots were formed by three cultivation environments and the subplots by ten genotypes of citrus with 15 replicates. Each experimental unit consisted of a test tube containing a micropiles.

At 180 days of *in vitro* culture, the following variable were analyzed: plant height in cm (PH), number of green leaves (NGL), number of senescent leaves (NSL), number of microcuttings (NM) and plant dry mass in g (PDM).

The following descriptive statistics were calculated for the data obtained: mean, minimum and maximum values, standard deviation and coefficient of variation (CV). Data were also analyzed using the analysis of variance F-test. The variables number of green leaves, number of senescent leaves and number of microcuttings were transformed to $\sqrt{x+0.5}$ to meet the assumptions of the analysis of variance. The means for the different environments were compared using the Tukey's test at a 5% probability, and the means for the genotypes were grouped using the Scott-Knott test at a 5% probability.

The multivariate approach was used, considering the statistical model of completely randomized design in a split plot design in order to obtain the estimate of the partial correlation coefficients between the variables based on the residual sum of squares and cross products matrix. This correlation measures the association between variables while disregarding the effect of treatments (Hair Jr., Black, Babin, & Anderson, 2009). The relative contribution of each variable was calculated using the criteria described by Singh (1981), and a principal component analysis was used to cluster the genotypes as a function of the variables evaluated for each culture environment.

Statistical analyses were performed using the statistical program SAS - Statistical Analysis System (SAS, 2004a), Statistica (SAS, 2004b) and Genes (Cruz, 2014).

Results and discussion

The correlation coefficient calculated for plant height and number of green leaves was low, positive and significant ($r = 0.25^{**}$) and demonstrates that conserving materials of a smaller size and with green leaves is possible (Table 1). In the *in vitro* genebank of citrus at Embrapa Cassava and Fruits when the plant has 80% of green leaves, this condition is considered satisfactory since the plants with these characteristics have been presented good regenerative capacity (data not show).

Table 1. Partial correlation coefficients for plant height in cm (PH), number of green leaves (NGL), number of senescent leaves (NSL), number of microcuttings (NM) and plant dry mass in g (PDM) for citrus genotypes grown in different *in vitro* conservation environments.

Variables	NGL	NSL	NM	PDM
PH	0.25^{**}	-0.07^{ns}	0.50^{**}	0.63^{**}
NGL		-0.13^{*}	0.31^{**}	0.43^{**}
NSL			-0.02^{ns}	-0.05^{ns}
NM				0.45^{**}

**and *significant at 1 and 5%, respectively, using a t-test. ^{ns}non-significant at 5% probability.

As expected, a significant negative correlation between the NGL and NSL was observed. In regards color, Santos et al. (2012) reported that greener plants should be considered, even if they are a little larger than the desired size.

There was also a moderate correlation between the PH and NM (0.50^{**}), and a high correlation between the PH and PDM (0.63^{**}). These results allowed us to disregard the NM and PDM variables

in this study because the same behavior can be observed through the PH variable alone.

Table 2 is a summary of the analysis of variance results. There was a significant effect in the Environment x Genotype interaction for all of the variables tested ($p < 0.05$) except the NGL, for which only the environment was significant.

Table 2. Summary of analysis of variance results for plant height in cm (PH), number of green leaves (NGL), number of senescent leaves (NSL), number of microcuttings (NM) and plant dry mass in g (PDM) of citrus genotypes grown in different *in vitro* conservation environments.

Source of Variation	DF	Mean Square				
		PH	NGL	NSL	NM	PDM
Environment	2	17.13**	25.19**	0.22ns	0.24**	0.0013**
Error a	42	0.57	0.37	0.17	0.03	0.0001
Genotype	9	11.57**	0.63ns	0.75**	0.52**	0.0019**
Env. x Gen.	18	3.03**	0.59ns	0.67**	0.12**	0.0005**
Error b	354	0.51	0.36	0.18	0.03	0.0001
CV (%)		28.07	22.60	42.78	13.52	40.14
Overall mean		2.54	7.06	0.69	1.37	0.03

DF: degrees of freedom. ** and *significant at 1 and 5%, respectively, using a t-test. nsnon-significant at 5% probability.

The interaction results are shown in Table 3. For plant height, the three environments promoted different behaviors according to genotype. Plants were taller in the environment with a temperature of 27±1°C, a photon flux density of 30 μmol m^{-2} s^{-1} and a 16 hours photoperiod. The lowest height values were obtained for the samples cultured in the climate-controlled chamber at a temperature of 17±1°C with a photon flux density of 20 μmol m^{-2} s^{-1} and a 16 hours photoperiod.

The plants grown under this condition had yellow leaves and an unhealthy appearance (Figure 1a), which affects plant growth and accelerates the leaf fall, as shown by the NSL values (Table 3). Culturing samples at a room temperature of 22±1°C, photon flux density of 10 μmol m^{-2} s^{-1} and a 12 hours photoperiod yielded overall lower plant heights than culturing samples at a room temperature of 27±1ºC, photon flux density of 30 μmol m^{-2} s^{-1} and a 16 hours photoperiod. There were some specific genotypes, however, that showed no significant difference in height between these two culture conditions. The differences observed in the number of green leaves for each environment should also be considered (Table 4) because this variable determines which conservation condition should be used. In addition to the NGL, the physiological condition of the leaves was also taken into consideration (Figure 1b).

Senescence is an important indicator of successful *in vitro* germplasm conservation because it is an accurate reference of the plant aging process *in vivo* and *in vitro*. Determining the degree of senescence that should be used to establish the appropriate timing for subculture, however, is difficult. It is difficult to determine the most effective subculture interval that will not negatively affect the subsequent plant regenerations (Canto et al., 2004) using senescence results. This issue has made subculturing large *in vitro* collections and establishing new germplasms a challenge.

Table 3. Mean values for plant height in cm (PH), number of senescent leaves (NSL), number of microcuttings (NM) and plant dry mass in g (PDM) of citrus genotypes grown in different *in vitro* conservation environments.

Genotype Number	Genotype	Environment		
		1	2	3
	Plant height (cm)			
1	LCCC	1.32 Bb	1.99 bA	2.39 cA
2	LVCC2	1.99 bB	2.07 bB	2.88 cA
3	CC	2.30 aC	3.67 aB	4.53 aA
4	LRM	2.37 aA	2.25 bA	2.16 cA
5	TSKC x CTSW 03	2.23 aA	2.49 bA	2.46 cA
6	OACNL	1.68 bB	2.20 bAB	2.61 cA
7	CT	2.47 aB	3.76 aA	3.83 bA
8	OACC	1.87 bA	2.17 bA	2.45 cA
9	TSKC x (TR x LCR) 059	2.44 aB	3.26 aA	2.25 cB
10	CS	2.66 aA	2.19 bA	2.64 cA
	Mean value	2.13	2.61	2.82
	Number of senescent leaves			
1	LCCC	1.62 aA	0.13 bB	0.13 bB
2	LVCC2	0.07 bA	0.27 bA	0.13 bA
3	CC	0.93 aAB	1.14 aA	0.27 bB
4	LRM	0.07 bA	0.27 bA	0.57 bA
5	TSKC x CTSW 03	1.40 aA	1.20 aA	1.07 aA
6	OACNL	0.50 bA	0.54 bA	0.20 bA
7	CT	0.79 bAB	1.27 aA	0.13 bB
8	OACC	2.00 aA	1.36 aA	0.29 bB
9	TSKC x (TR x LCR) 059	0.43 bB	0.40 bB	1.92 aA
10	CS	0.31 bB	0.42 bB	1.47 aA
	Mean value	0.81	0.70	0.62
	Number of microcuttings			
1	LCCC	1.00 aA	1.07 cA	1.20 bA
2	LVCC2	1.13 aA	1.27 cA	1.40 bA
3	CC	1.20 aB	2.36 aA	2.53 aA
4	LRM	1.33 aA	1.27 cA	1.07 bA
5	TSKC x CTSW 03	1.13 aA	1.13 cA	1.27 bA
6	OACNL	1.00 aA	1.00 cA	1.13 bA
7	CT	1.36 aB	2.13 aA	2.20 aA
8	OACC	1.27 aA	1.00 cA	1.21 bA
9	TSKC x (TR x LCR) 059	1.29 aA	1.67 bA	1.38 bA
10	CS	1.46 aA	1.08 cA	1.20 bA
	Mean value	1.22	1.40	1.46
	Plant dry mass (g)			
1	LCCC	0.02 bA	0.02 bA	0.02 bA
2	LVCC2	0.02 bA	0.03 bA	0.03 bA
3	CC	0.03 aB	0.05 aA	0.05 aA
4	LRM	0.03 aA	0.02 bA	0.03 bA
5	TSKC x CTSW 03	0.02 bA	0.03 bA	0.03 bA
6	OACNL	0.02 bA	0.03 bA	0.02 bA
7	CT	0.03 aB	0.04 aA	0.04 aA
8	OACC	0.03 aA	0.03 bA	0.03 bA
9	TSKC x (TR x LCR) 059	0.03 aB	0.04 aA	0.02 bB
10	CS	0.02 bA	0.02 bA	0.03 bA
	Mean value	0.03	0.03	0.03

Means followed by the same lowercase letters in the column are part of the same group as assessed using the Scott-Knott test at 5% significance and means followed by the same uppercase letters are not significantly different from each other as assessed using the Tukey's test at 5% significance. LCCC (lemon tree 'Cravo' clone Common); LVCC2 (lemon tree 'Volkameriano' clone Catânea 2); CC (citrange 'Carrizo'); LRM (lemon tree 'Rugoso Mazoe'); TSKC x CTSW 03 (tangerine tree 'Sunki' common x citrumelo 'Swingle'); OACNL (orange tree 'Azeda' clone Narrow Leaf); CT (citrange 'Troyer'); OACC (orange tree 'Azeda' clone Common); TSKC x (TR x LCR) 059 [tangerine tree 'Sunki' common x (*Poncirus trifoliata* (L.) Raf. x lemon tree 'Cravo')]; CS (citrumelo 'Swingle').

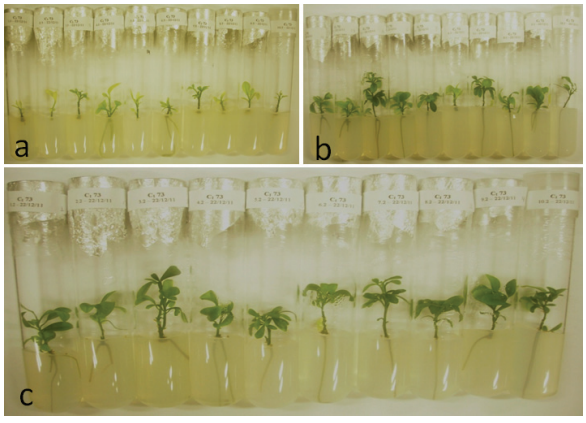

Figure 1. Plants from left to right lemon tree 'Cravo' clone Common, 'Volkameriano' clone Catânea 2, citrange 'Carrizo', lemon tree 'Rugoso Mazoe', TSKC x CTSW 03, orange tree 'Azeda' clone Narrow Leaf, citrange 'Troyer', orange tree 'Azeda' clone Common, TSKC x (TR x LCR) 059 and citrumelo 'Swingle' cultured *in vitro* in a climate-controlled chamber at 17±1ºC photon flux density of 20 µmol m^{-2} s^{-1} and a 16 hours photoperiod (a), in a room at 22±1ºC, photon flux density of 10 µmol m^{-2} s^{-1} and a 12 hours photoperiod (b) and at 27±1°C, photon flux density of 30 µmol m^{-2} s^{-1} and a 16 hours photoperiod (c) for 180 days.

Table 4. Mean values for the number of green leaves of the citrus genotypes calculated for the different *in vitro* conservation environments.

Environment	Mean
1	4.61 c
2	7.50 b
3	8.93 a

Means followed by the same letter are not significantly different as assessed using the Tukey's test at 5% significance.

As for the number of microcuttings there was no significant difference between the cultures at 22±1°C and 27±1°C for any of the genotypes studied when the number of microcuttings differed. Therefore, this variable was considered secondary when selecting the best growth environment for the *in vitro* conservation of these genotypes. Similar PDM results were observed as shown by the correlation between PH and number of microcuttings and PDM.

The variables that appeared to be most affected based on the correlations between the original variables and the principal components and could be used to identify the different behaviors of the genotypes are shown in Table 5. The variables that were most affected among genotypes within the principal component 1 in the climate-controlled chamber culture environment (17±1°C) were PH (39.98%), NM (34.53%) and NSL (15.43%). The variables that were the most affected in the room environments at 22±1ºC and 27±1ºC were PH (27.10 and 31.38%), PDM (27.96 and 31.06%) and NM (26.43 and 29.35%) (Table 6). For the principal component 2, the NGL was the variable most affected for all of the culture environments. When both principal component 1 and 2 aretaken into consideration, the variables that were most affected and could be used to identify the different behaviors of the genotypes in the different *in vitro* culture environments were NGL and PH.

Table 5. Sensitivity of the variable results based on the correlations between the original variables and the principal components of the *in vitro* culture of citrus genotypes in different *in vitro* conservation environments.

Variables	Environment 1		Environment 2		Environment 3	
	PC1 (46.16%)	PC2 (30.00%)	PC1 (68.82%)	PC2 (21.86%)	PC1 (61.74%)	PC2 (27.30%)
PH	39.98	0.54	27.10	0.80	31.38	0.80
NGL	1.59	48.62	10.40	44.97	0.10	59.42
NSL	15.43	11.10	8.11	53.89	8.11	37.66
NM	34.53	2.17	26.43	0.30	29.35	1.92
PDM	8.47	37.58	27.96	0.04	31.06	0.19

PH (plant height); NGL (number of green leaves); NSL (number of senescent leaves), NM (number of microcuttings) and PDM (plant dry mass). PC1 (principal component 1) and PC2 (principal component 2).

Table 6. Relative contribution of variables to diversity based on the criteria described by Singh (1981), in each environment, for plant height in cm (PH), number of green leaves (NGL), number of senescent leaves (NSL), number of microcuttings (NM) and plant dry mass in g (PDM) for the citrus genotypes grown in different *in vitro* conservation environments.

Variables	Environment 1		Environment 2		Environment 3	
	Sij	Sij (%)	Sij	Sij (%)	Sij	Sij (%)
PH	15.35	12.12	42.26	17.97	52.36	21.77
NGL	68.93	54.45	150.31	63.92	128.78	53.53
NSL	40.21	31.76	20.93	8.90	37.46	15.57
NM	2.10	1.66	21.65	9.21	21.97	9.13
PDM	0.00	0.00	0.01	0.00	0.01	0.00

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

The first and second components combined represented 76.16, 90.68 and 89.04% of the total variance in the 17±1°C, 22±1°C and 27±1°C environments, respectively.

The principal component analysis grouped the genotypes that demonstrated similar growth behaviors in the different culture environments used. Based on this analysis, the different citrus genotypes formed five distinct groups in the climate-controlled chamber at 17±1ºC and the rooms at 22±1ºC and 27±1ºC (Figure 2). These groups differed between each environment, which illustrates the different responses each genotype had to the different culture environments. In the room environments at 22±1ºC and 27±1ºC, genotypes 3 (citrange 'Carrizo') and 7 (citrange 'Troyer') were grouped together. Both genotypes demonstrated greater growth according to the variable results analyzed (Table 3), and this growth behavior may be the reason these genotypes formed a separate individual group under these environmental conditions. Genotypes 5, 6 and 8 were grouped together under the culture conditions at 22±1°C

and at 27±1°C. These genotypes responded differently to the culture environment. Genotype 8 had a higher NSL value, which differed from the results observed for the other genotypes and indicates that this genotype is more susceptible to senescence in this culture environment.

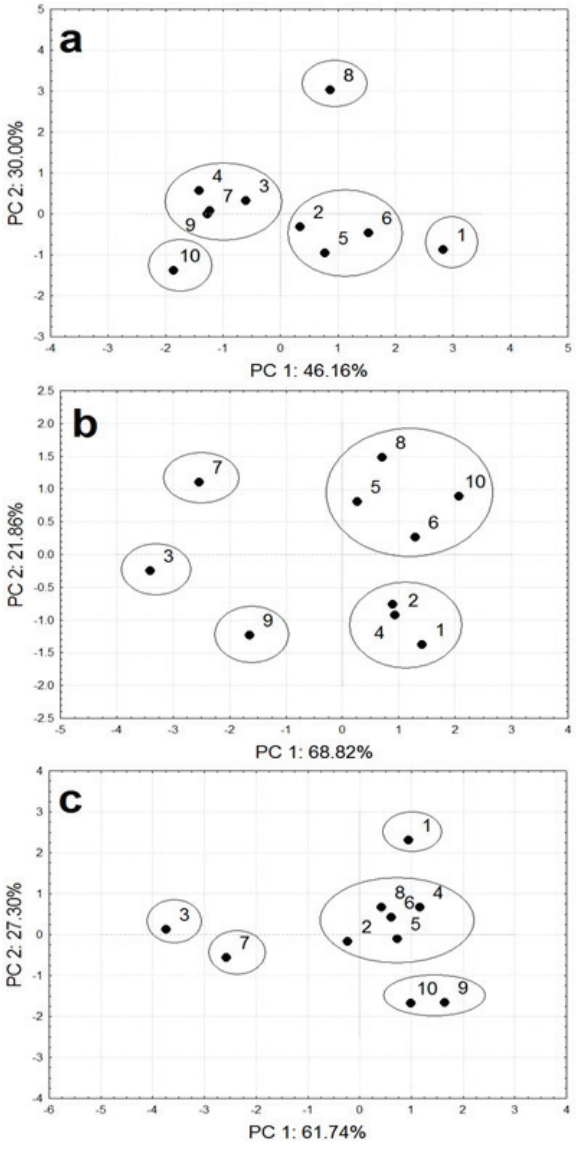

Figure 2. Scatterplots of the principal component 1 and 2 scores for the *in vitro* culture of citrus plants in the following environments: climate-controlled chamber at 17±1°C, photon flux density of 20 µmol m^{-2} s^{-1} and a 16 hours photoperiod (a), room at 22±1°C, photon flux density of 10 µmol m^{-2} s^{-1} and a 12 hours photoperiod (b), and room at 27±1ºC, photon flux density of 30 µmol m^{-2} s^{-1} and a 16 hours photoperiod (c). The numbers 1 to 10 corresponds to the 10 citrus genotypes listed in Table 3 in the same order.

These results suggest that using the principal component analysis technique may improve the management strategies of *in vitro* collections because it facilitates subculture planning based on behavior similarities between genotypes. Using this tool to analyze a large number of accessions, including different species and genera, should significantly improve the efficiency of collection management.

As for the differences observed between the different genotypes based on the criteria described by Singh (1981) (Table 6), the variables that most accurately represented the growth behavior were NGL and NSL at 17±1°C and NGL and PH at 22±1°C and 27±1°C.

Temperature is one of the primary factors taken into consideration when optimizing the *in vitro* conservation conditions for minimal growth because every species has an optimal culture temperature and light intensity for development.

Reducing the temperature is a common strategy used for tropical and subtropical species because they typically demonstrate significant and unique reductions in plant metabolism (Normah, Chin, & Reed, 2013). These type of responses have been observed in pineapple (Canto et al., 2004; Souza et al., 2006), cassava (Souza et al., 2009), sugarcane (Lemos, Ferreira, Alencar, Albuquerque, & Ramalho Neto, 2002), grapevine (Silva, Luis, & Scherwinski-Pereira, 2012), banana (Oliveira, Silva, Silva, & Silveira, 2000) and potato (Conceição, Fortes, & Silva, 1999).

According to Lemos, Ferreira, Alencar, Albuquerque and Ramalho Neto (2002), very low (12°C) or very high (25°C) temperatures promote the yellowing and death of explants and are therefore inadequate for the maintenance of viable sugarcane explants conserved *in vitro*. These authors emphasized that the use of excessively low temperatures during *in vitro* culturing may reduce the activity of important enzymes, which would compromise the overall metabolism of the plant. The ideal temperature is dependent on the species of interest, and multiple studies should be conducted when starting a new *in vitro* collection.

Tropical and subtropical plants typically survive in temperatures reduced to between 15 and 20°C (Souza et al., 2009). The results obtained for the citrus plants in this study, however, showed that a temperature of 17°C was too low. The development of the conserved plants was impaired at this temperature.

A pineapple collection with 230 accessions is maintained at a temperature of 22°C and a 12 hours photoperiod. A reduction in the metabolism of the conserved plants was achieved by reducing the temperature and the amount of MS salts used (Souza et al., 2006).

Likewise, accessions of vetiver [*Chrysopogon zizanioides* (L.) Roberty] were conserved under a regime of slow growth for a period of 270 days by reducing the concentration of MS salts to 25% of the normal concentration and the temperature to 18°C. For many species, a combination of different factors must be altered to obtain satisfactory results (Santos et al., 2012).

In this study, a reduction in heigt was observed in a majority of the genotypes by culturing them in at a temperature of 22°C, photon flux density of 10 μmol m^{-2} s^{-1} and a 12 hours photoperiod and with WPM as the basal medium. These results indicate that the plants were well conserved for six months and suggest that a longer subculture interval is possible. The *in vitro* introduction and maintenance of approximately 30 accessions into the citrus Active Germplasm Bank (Banco Ativo de Germoplasma - BAG) at the Embrapa Cassava and Fruit Crops (data not shown) under these conditions demonstrates the potential of achieving preserving samples for longer than six months. Some accessions were preserved for as long as one year under these temperature and photoperiod conditions without needing to be subcultured. These plants are still under evaluation, and the data obtained could support the results observed in this study as well as provide new insights given that the number of accessions is much higher.

Similar results have been reported by Silva, Luis and Scherwinski-Pereira (2012), who showed that grapevine genotypes can be stored *in vitro* for six months in WPM medium at 20°C light intensity of 38 μmol m^{-2} s^{-1} and a 12 hours photoperiod. In addition, Camillo, Scherwinski-Pereira, Vieira and Peixoto (2009) reported that a temperature of 20°C light intensity of 30 mmol m^{-2} s^{-1} and a 12 hours photoperiod combined with WPM/2 culture medium provided conditions in which *Cochlospermum regium* explants could be maintained and conserved under a minimal *in vitro* growth regimen. Silva and Scherwinski-Pereira (2011) also observed that 20°C, 12 hours photoperiod with a fluorescent light of approximately 38 μmol s^{-1} m^{-2} photon flux, was an effective temperature for the *in vitro* conservation of *Piper aduncum* and *P. hispidinervum* shoots.

Studies on *in vitro* conservation have shown promising results that suggest longer intervals between subcultures is possible. In sugarcane, Lemos et al. (2002) reported that it is possible to conserve microplants under slow-growth conditions for 12 months when they are maintained at a temperature of 15°C light intensity of 50 μmol m^{-2} s^{-1} and a 16 hours photoperiod.

Oliveira et al. (2000) found that the interval between the transfer of diploid banana accessions conserved *in vitro* should be 180, 360 and 450 days, when they are maintained at a mean temperature of 26, 22 and 17°C, respectively, 2000 lux light intensity, 16 hours photoperiod. According to the Consultative Group on International Agricultural Research (Consultative Group for International Agricultural Research [CGIAR], 2012), the most suitable growth conditions for the *in vitro* conservation of banana are a temperature of 16±1°C, a photon flux density of 25 μmol m^{-2} s^{-1} and a 24 hours photoperiod. For cassava, the most suitable conditions are a temperature of 18-24°C, a photon flux density of 18.5 μmol m^{-2} s^{-1} and a 12 hours photoperiod.

Studies on tissue cultures from multiple crops, including citrus, show that the morphogenetic responses of plants cultured *in vitro* are influenced by genotype, the type of explant and the culture medium (Costa, Souza, & Almeida, 2006). Marin and Duran-Vila (1991) used a micropropagation protocol to conserve the germplasm of *Citrus* and related genera *in vitro* at a temperature of 26±1°C, a 16 hours photoperiod and a light intensity of 40 μmol m^{-2} s^{-1} and performed only one or two subcultures per year. They concluded that the protocol could easily be applied to many species and parental cultivars of citrus while waiting for the development of better techniques. These conditions of a higher temperature, light intensity and photoperiod normally accelerate the metabolic processes in plants, which would reduce the interval period between subcultures.

Currently, there is no standard procedure for all of the genotypes of all species. For the successful management of large collections, a protocol must be developed that favors the largest possible number of accessions.

Conclusion

The variables that most helped explain the observed variability in the behavior of citrus genotypes under different *in vitro* culture conditions were the number of green leaves and plant height.

The culture environment at 17±1°C, a photon flux density of 20 μmol m^{-2} s^{-1} and a 16 hours photoperiod was unsuitable for the *in vitro* culture of these citrus genotypes.

The best conditions for reducing plant metabolism were a room environment at 22±1°C, a photon flux density of 10 μmol m^{-2} s^{-1} and a 12 hours photoperiod.

The principal component analysis is an effective tool for studying the behavior of citrus genotypes being conserved in different *in vitro* culture environments.

Acknowledgements

We would like to thank Embrapa Cassava and Fruit Crops for providing the physical infrastructure and financial support that was essential for conducting these studies.

We would also like to thank the Brazilian Federal Agency for Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Capes) for the financial aid awarded scholarships.

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Received on January 29, 2015.
Accepted on April 13, 2015.