Pak. J. Bot., 47(4): 1325-1331, 2015.

DNA CONTENTS OF EMBRYO STRUCTURES OF *BUTIA CAPITATA* GERMINATING *IN VITRO*

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Abstract

We determined the DNA contents and assessed the quality of the flow cytometry analyses of different structures of *Butia capitata* arising at various stages during the *In vitro* germination and growth of zygotene embryos, characterizing them both morphologically and anatomically. The experiments evaluated 16 organs and stages, with the embryo, cotyledon petiole and haustorium being evaluated at 8, 16 and 27 days, the primary root and leaf sheath at 16, 27 and 37 days, and the leaves after 37 days; the mature leaf was used as a control. DNA contents were found to vary according to the structures analyzed. After 27 days of *In vitro* culture, the cotyledon petiole, leaf sheath and radicle showed the lowest coefficients of variation and had the same amounts of DNA. The haustorium and cotyledon petiole had higher DNA values at eight days, but with coefficients of variation, indicating less reliability of these analyses. Anatomical sections of both structures after eight days showed idioblasts with large quantities of phenolic compounds that probably interfered with the analyses.

Key words: DNA, Embryo structures, *Butia capitata*, Zygotene embryos.

Introduction

Coquinho-azedo (*Butia capitata* Mart. Becc.) is a native palm of the *Cerrado* (neotropical savanna) biome that is used for many purposes in northern Minas Gerais State, Brazil (Lorenzi *et al*., 2010). The fruits can be consumed fresh or used to prepare juices, ice cream, and popsicles, or processed in the form of a pulp that is widely used and valued in the region (Faria *et al*., 2008). This species is generally found along riversides in areas with fertile soils. As these areas are favored for planting along the expanding agricultural frontiers in Brazil, its fruits are intensively harvested and this palm is becoming an endangered species**.**

The loss of species diversity is an obstacle to biodiversity conservation and the preservation of plants that can be domesticated for human uses (Jamnadass *et al.,* 2009). Studies focusing on the characterization and description of native species are therefore much needed, and biotechnology appears as a promising route for reproducing native species, especially *B. capitata*.

Flow cytometry is commonly used in tissue culture research to accompany cell DNA contents and the cell cycle, for ploidy analysis, for detecting haploid plants and di-haploid lines and inter-specific hybrids, to control ploidy stability and check aneuploidy, and in taxonomy (Ochatt, 2008, Dolezel & Greilhuber, 2010). The use of flow cytometry in taxonomic research focusing on the genus *Butia* will be important, as many difficulties have been encountered in characterizing its species using only morphological markers (Lorenzi *et al*., 2010). Flow cytometry also provides support for experiments focusing on somatic embryogenesis, chromosome duplication, somaclonal variation, and the effects of growth regulators.

Among the many factors that can influence flow cytometry analyses are the types of plant materials used and the extraction buffers and fluorochromes employed. The latter two criteria can best be examined during the testing of different protocols, while the types of plant material used must be standardized and characterized individually – as the chemical compositions of the tissues to be analyzed can interfere with flow cytometry parameters and mask the results. Bennett *et al*. (2008) reported that secondary metabolites such as anthocyanins could inhibit DNA staining and provide erroneous estimates of DNA contents. Loureiro *et al*. (2006) likewise noted that tannins and many other secondary metabolic compounds have this same masking capacity. Flow cytometry studies must therefore carefully define suitable plant materials and extraction techniques to ensure reliable results.

In order to examine the influence of phenolic compounds on DNA quantifications, Thiem & Sliwinska (2003) evaluated different tissues types in *Rubus chamaemorus*, but found no differences between them. Acclimatized plantlets of *Elaeis guineensis* from *In vitro* cultures, however, were found to have 3.701 pg of DNA, while friable callus had 3.201 pg of DNA (Rival *et al*., 1997).

Galbraith *et al*. (1983) proposed the use of leaf tissues for establishing DNA contents, although other plant parts should be evaluated to identify any alterations caused by chemical substances present in the leaves.

The aim of the present study was therefore to determine the DNA content of *Butia capitata* and assess the flow cytometry characteristics of different anatomical structures appearing at various stages during the *In vitro* germination of zygotic embryos, characterizing them both morphologically and anatomically.

Materials and Methods

The present study was conducted at the Tissue Culture Laboratory at the Federal University of Lavras. The material used was obtained near the town of Montes Claros in northern Minas Gerais State, Brazil. Bunches of ripened fruits with full yellow epicarps arising from the same plant were selected and similar numbers of fruits were harvested from each bunch. The fruits were depulped manually using a knife to obtain the pyrenes (endocarp + seed). The pyrenes were then washed in distilled water and dried in the shade, the endocarps subsequently cracked using a vice, and the seeds extracted.

The experiments were conducted using a completely randomized design with 16 seed organs and developmental stages, with five replicates each. The embryos, cotyledon petioles and haustoria were evaluated at 8, 16, 27 and 37 days, the primary roots and leaf sheaths at 16, 27 and 37 days, and the leaves at 37 days; mature plant leaves were used as controls. The choice of these sampling times was based on a study by Magalhães *et al*. (2012). The mature plant leaf used for extracting the nuclei was the youngest leaf (usually yellowish) inserted into the leaf sheath.

***In vitro* culture:** The seeds of *Butia capitata* were sterilized in a commercial sodium hypochlorite solution for 20 minutes and then washed three times in sterilized distilled water. The embryos were excised in a laminar flow hood, washed in distilled water, transferred to a commercial solution of 2.5% sodium hypochlorite for 10 minutes, rinsed three times in distilled water, and then held in distilled water for two hours before transfer to the culture medium.

Two hundred embryos were inoculated into test tubes containing 10 ml of MS medium (75% concentration) supplemented with 6g L^{-1} agar, 3 g L^{-1} activated charcoal, 30 g L^{-1} sucrose, 0.5 mg L^{-1} thiamine, 1 mg L^{-1} pyridoxine, and 0.5 mg L^{-1} nicotinic acid (Murashige & Skoog 1962, Ribeiro *et al*., 2010). The medium was adjusted to pH 5.7 and then sterilized by autoclaving at 120°C for 15 minutes. The embryos were kept at a constant temperature of 25 ± 2°C in the dark for the first thirty days, after which they were maintained under a 16-hour photoperiod (light intensity 42 W m^{-2}).

Thirty-seven days after *In vitro* inoculation the seedlings were removed at irregular intervals to analyze their structures and estimate their DNA contents and coefficients of variation (CV).

DNA content estimation by flow cytometry: Approximately 1 cm long portions of the structures to be tested, were removed and ground in 1 ml of cold Marie buffer (Marie and Brown, 1993) [50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM Na2.EDTA, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2) and 1% (w/v) polyvinylpyrrolidone-10 (PVP-10)] to extract their nuclei. The nuclei suspension was then aspirated through two layers of gauze using a plastic pipette and filtered through a 50 micron mesh. The nuclei were then stained by adding 25 µL of a propidium iodide (1mg/1mL) solution.

The same procedure was used to prepare the external standard (*Pisum sativum* with 9.09 pg). Ten thousand nuclei were evaluated for each sample using a logarithmic scale on a FACSCalibur cytometer (BD, Biosciences, San Jose, CA, USA). The histograms obtained with Cell Quest software were statistically analyzed using WinMDI 2.8 software (Scripps Research Institute, 2011). The nuclear DNA contents (pg) of the plants were estimated using the ratio of the fluorescence intensities of the G1 nuclei of the reference standard and the G1 nuclei of the samples and multiplying this ratio by the standard DNA reference. The DNA contents (pg) and coefficient of variations (CV) were subjected to analysis of variance and the means compared using the Scott-Knott test run on the Sisvar statistical program (Ferreira, 2011).

Biometric analyses: At each collection time, the lengths and diameters of the embryos, the cotyledon petioles, leaf sheaths, haustorium, roots and leaves were also measured using a digital pachymeter, and photomicrographs were taking using a Cannon digital camera A-650/S coupled to a Zeiss Stemi 2000-C microscope or a Zeiss Primo Star stereomicroscope. Ten repetitions were made for each structure. The terminology employed follows Panza *et al*. (2004) and Henderson (2006).

Seedling and embryo anatomy: To characterize the *B. capitata* material, samples were taken from 10 seedlings at 8 and 16 days, when idioblasts containing phenolic compounds were most abundant. The material was fixed in FAA50 (Johansen, 1940) for 24 hours, held under vacuum, dehydrated in an ethanol series, and subsequently stored in 70% ethanol (Jensen, 1962). Portions of the samples were embedded in methacrylate following the methodology proposed by Paiva *et al*. (2011). Cross- and longitudinal sections (6-7 µm) were obtained using a rotary microtome, stained with 0.05% toluidine blue at pH 6.8, and mounted with Entellan ® resin and a coverslip (O'Brien *et al*., 1964).

Photomicrographs were obtained using a Zeiss Stemi 2000-C optical microscope and a Zeiss Primo Star stereomicroscope, both fitted with a Cannon A-650 / S digital camera.

Results and Discussion

The DNA contents of the samples and their coefficients of variation demonstrated statistically significant differences at a 5% probability level by the Scott Knott mean test. The DNA contents of the samples varied according to the structures and the durations of *In vitro* culture. The observed variations reflected internal (intrinsic to the plant material) or external factors (related to the sample preparation methodology). These differences are expressed as coefficients of variation (CV = standard deviation/ mean) that generally ranged from 1 to 10% among the plant cells. According to Marie and Brown (1993), the CV is a basic criterion for validating cytological methods, with values between 1% and 2% indicating high-quality analyses, and 3% indicating routine values.

The CV is directly related to the quality of the samples, so that lower CV values indicate higher reliability of the analyses. The organs embryo, cotyledon petiole, root, and leaf sheath, the samples taken at 37 days, and adult plant leaves had the lowest CV's and therefore the most reliable DNA indices (Table 1). The haustoria samples at eight days have the highest CV, followed by the cotyledon petioles at 8 days and haustoria at 16 days – suggesting the influence of internal or external factors impairing sample quality (Table 1). These high CVs may be related to the production of secondary metabolites that bind to DNA – in a phenomenon designated as "self-tanning", which can cause variations in their apparent DNA contents (Greilhuber, 2007). Self-tanning can be due to the presence of secondary metabolites that interfere with fluorochrome binding, resulting in inaccuracies in genome size estimations. The species *Musa acuminata*, for example, contains significant quantities of phenolic compounds that affected the quality of *In vitro* plant analyses (Camolesi *et al*., 2007), as these compounds precipitated during sample preparation, making cytometer readings difficult.

Most of the genetic diversity found in seedlings obtained from tissue cultures (including changes in ploidy levels) result from damage caused by oxidative stress, the use of growth regulators, and the time period during which the material was maintained *In vitro* (various generations).

The lowest DNA contents were observed in the embryo, in leaves 37 days after inoculation of the adult plant, the cotyledon petioles (8, 16 and 37 days), roots (16 and 37 days), and in the leaf sheath (16 and 37 days) (Table 1). However, the ratios of these DNA structures at 16 days showed higher CV's, indicating poor accuracy.

The same amounts of DNA were observed in the cotyledon petiole and the primary root 27 days after *In vitro* inoculation, with the DNA values being intermediate to those mentioned previously. The CVs of these treatments were low, thus confirming their high reliability.

The highest DNA contents were observed after 8 days in the cotyledon petiole and haustorium – but their CVs were very high, indicating some interference in the DNA measurements. Primary root protrusion from the cotyledon (Fig. 1B) was observed in this phase, accompanied by intense production of secondary phenolic compounds (Fig. 1B, E). These substances are commonly found in the cotyledons of palm trees, with cells containing phenolic compounds forming a cone in the proximal region of the embryo (Sugimuma & Murakami, 1990, Panza *et al*., 2004). The presence of numerous idioblasts containing phenolic compounds external to the hood in *B. capitata* probably helps protects the plant against pathogens (Werker, 1997), and contributes to the protrusion of the root system. These phenolic compounds tend to intercalate with fluorochromes and affect their access to DNA, generating stoichiometric errors in DNA content estimates (Noirot *et al*., 2000). Thiem and Sliwinska (2003) noted the presence of phenolic compounds in the leaves of *Rubus chamaemorus*, leading them to modify the composition of the isolation buffer by adding the antioxidant PVP-10 (at concentrations of 0.5, 1 and 2%). Different quantities of DNA were encountered in different structures of *E. guieensis*, with *In vitro* calluses having 3.2 pg while acclimated seedlings and seeds contained approximately 3.7 pg (Rival *et al*., 1997). The DNA content of *A. aculeata* leaves was found to be approximately 5.81 pg (Abreu *et al*., 2011).

The haustorium of palm seeds expands to absorb the endosperm reserves. This organ does not expand *In vitro*, however, and degenerates as a natural consequence – as was observed by Magalhães *et al*., (2012) for *B. capitata* and by Ribeiro *et al*. (2012) for *Acrocomia aculeata*. Large quantities of phenolic compounds were observed in this structure, which probably affected the DNA readings and resulted in greater coefficients of variation.

Table 1. DNA contents and the coefficients of variation (CV) of the embryo, *In vitro* grown seedlings structures, and the mature leaves of *Butia capitata*.

Treatments	DNA (pg)	CV(%)
Embryo	3.99 a	0.59 a
Cotyledon petiole at 8 days	5.17 c	1.45 c
Haustorium at 8 days	5.03 c	1.81 d
Cotyledon petiole at 16 days	4.38 a	0.82 b
Primary root at 16 days	4.19 a	0.84 b
Leaf sheath at 16 days	4.03 a	0.87 b
Haustorium at 16 days	4.20 a	1.43 c
Cotyledon petiole at 27 days	4.61 b	0.59 a
Primary root at 27 days	4.55 b	0.63 a
Leaf sheath at 27 days	4.56 b	0.60 a
Haustorium at 27 days	4.65 b	1.58 c
Cotyledon petiole at 37 days	4.15 a	0.55 a
Primary root at 37 days	4.08 a	0.54 a
Leaf sheath at 37 days	4.23 a	0.42 a
Leaf at 37 days	4.05 a	0.55 a
Adult leaf	4.44 a	0.54 a

* Means followed by the same letter in any column belong to the same group and do not differ by the Scott-Knott test at a 5% level of probability

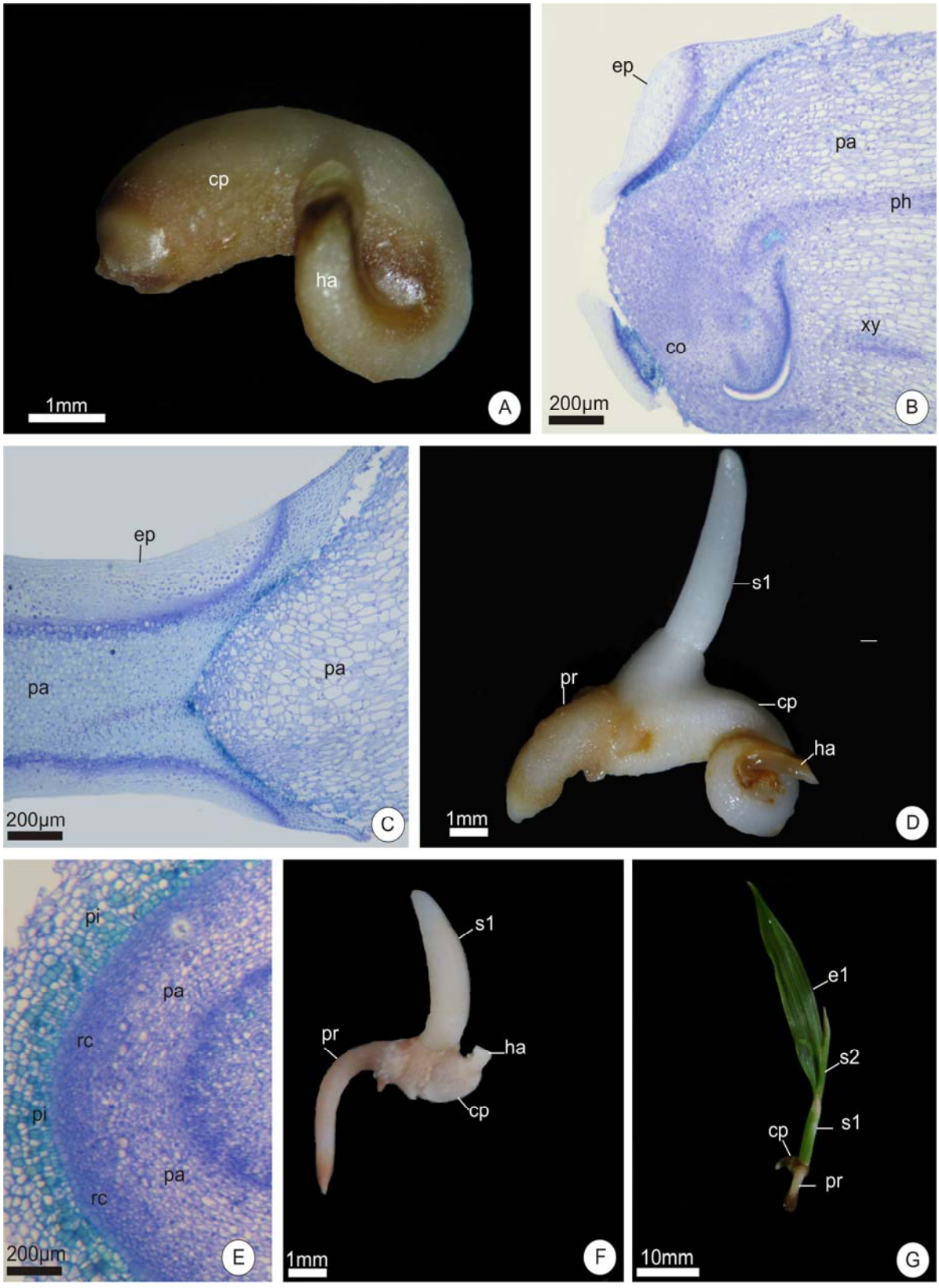

Fig. 1. Seedlings structures and *In vitro* germination of *Butia capitata*. Longitudinal sections (B, C, E), (A) seedling at 8 days; (B) base of the cotyledon petiole, with: phenolic idioblasts, and the root at 8 days, (C) haustorium at 8 days, (D) seedlings at 16 days; (E) root; (F) seedlings at 30 days (G) seedlings at 37 days. (b1: first leaf sheath; b2: second leaf sheath; co: root cap; cp: cotyledon petiole; cx: cortex, ep: epidermis; e1 eophyll; ha: haustorium, pa: parenchymal; ph: phloem; pi: phenolic idioblasts; pr: primary root; xy: xylem).

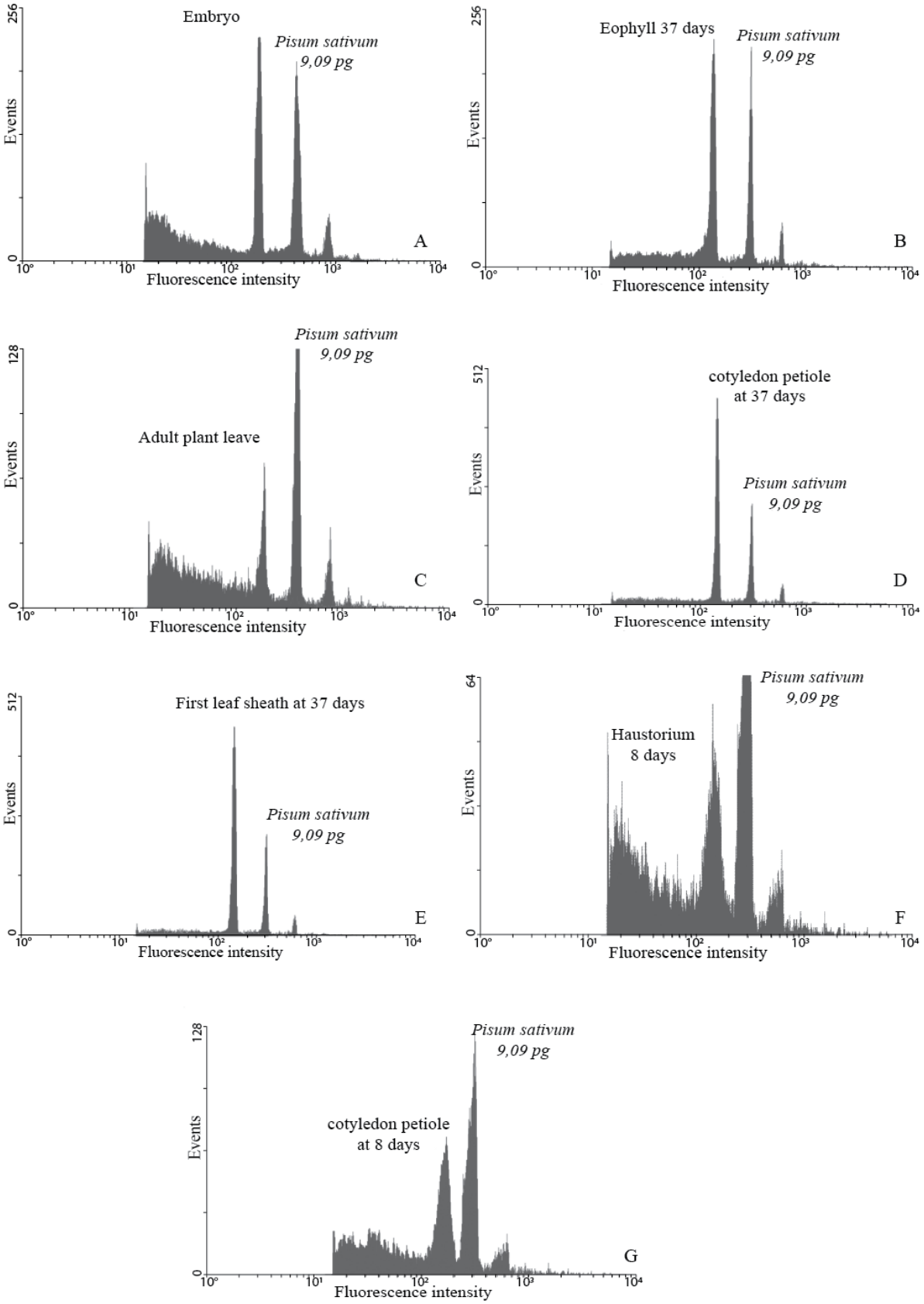

Fig. 2. Flow cytometry histograms for DNA quantification. A) embryo, (B) leaf at 37 (eophyll) days post cultivation, (C) adult plant leaf, (D) cotyledon petiole at 37 days, (E) firs leaf sheath at 37 days, (F) haustorium at 8 days; (G) petiole cotyledon at 8 days.

Differences were also observed in the DNA contents of these same structures over time. The cotyledon petiole showed 5.17 pg of DNA at 8 days after inoculation, 4.38 pg at 16 days, 4.61 pg after 27 days, and 4.15 pg after 37 days. A similar situation was observed with the haustorium, whose DNA content ranged from 4.20 pg at 16 days to 5.03 pg at 8 days. Histograms of DNA distributions always show variations, however, as DNA content measurements are not always precise (Loureiro & Santos, 2004). Differences may arise due to the fact that 2C nuclear DNA contents can be highly plastic in response to growing conditions (Msogoya *et al*., 2011). The DNA contents of the primary roots and leaf sheaths were more stable, however, and barely varied during the study period. The leaves of mature plants (both *In vitro* and at 37 days) and the embryos showed no significant differences in their DNA contents. Figure 2 presents the flow cytometric histograms of the DNA contents of the organs examined. The first peak in these histograms refers to the peak G1 interphase of the different structures analyzed, and the second peak refers to the G1 peak of the standard reference (*Pisum sativum*).

The histograms of the cotyledon petiole and leaf sheath treatments (Fig. 2D, E) showed extremely fine and clean peaks at 37 days (the thinner the peak, the smaller the CV), and while the histogram of the mature leaf showed a thin peak and low CV, it was saw-toothed and had an uneven format to the left of the peaks and was therefore of only intermediate quality (Fig. 2C).

The histograms of the haustorium and cotyledon petiole at 8 days showed the worst results, being very saw-toothed with very thick peaks, indicating low reliability of the results (Fig. 2F, G).

Most studies involving the standardization of protocols for flow cytometry DNA analyses have experimented with factors such as the types of DNA extraction buffers, doses of fluorochromes, extraction times of the samples, and the type of standard. It is also important that the plant material be well-characterized morphologically and anatomically, to help guarantee secure, reliable, and reproducible cytometry analyses.

Swelling of the cotyledon petiole was observed during the germination of *B. capitata*, as it expanded to approximately 6 mm; thereafter remaining stable, with little growth. This behavior was expected, as it is followed by the emission of the first leaf sheath and the primary root (Fig. 3). Similarly, the haustorium expanded in *In vitro* culture until approximately the 12th day, reaching approximately 3 mm, after which it began to degenerate (Figs. 3 and 4). There was an intense formation of idioblasts containing phenolic compounds at this time, and the tissue became darker due to the presence of these substances. The large quantities of phenolic compounds observed in this structure are likely to have a role in its degenerative phase. The root began to protrude after approximately 8 days, but could only be measured after 12 days. It had increased by 2 mm by the end of 16 days, and had reached over 4 mm in length after 37 days of cultivation. Increments in root diameters were also observed during the same period (Figs. 3, 4). The leaf sheath is responsible for protecting the growing first eophyll, and its emission occurred on about the twelfth day after cultivation, although it was only possible to accurately measure it from the sixteenth day onward. At the end of 37 days of cultivation the leaf sheaths were 14 mm long on the average. The seedling showed fully developed and expanded leaf blades after 37 days (Fig. 1G).

These results suggest, as proposed by Dolezel (1991), Greilhuber (2007), and Bennett *et al*. (2008), the presence of secondary metabolic compounds that can cause errors in DNA measurements employing flow cytometry. Greilhuber (1986, 1988) demonstrated the inhibitory effects of endogenous compounds (such as tannins) on DNA staining, and noted that secondary metabolites, such as high molecular weight (polyphenols), and even low molecular weight phenolic compounds such as quercetin, may reduce staining intensities in plants. Other hypotheses should be investigated more closely in the future, however. Bennett *et al*. (2008) suggested that both abiotic and biotic factors can lead to errors in DNA quantification because of genomic plasticity. Attempts to detect the existence (and extent) of variations in genome size and to prevent or minimize these changes have received considerable attention. Studies using flow cytometry must therefore take into account the type of material that will be analyzed, avoiding the use of darkly colored materials that concentrate secondary metabolic compounds; the study material must be also be carefully characterized morphologically to ensure reliable and repeatable results. The experimental conditions must also be monitored to improve standardization procedures and techniques for genome determinations (Wittmann, 2001).

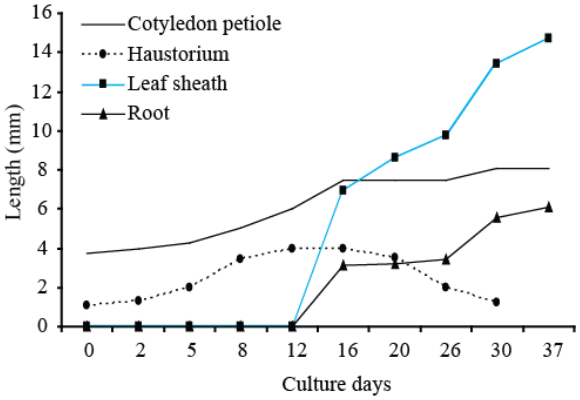

Fig. 3. Growth (length) of the organs of small sour-coconut seedling after 37 days of *In vitro* culture.

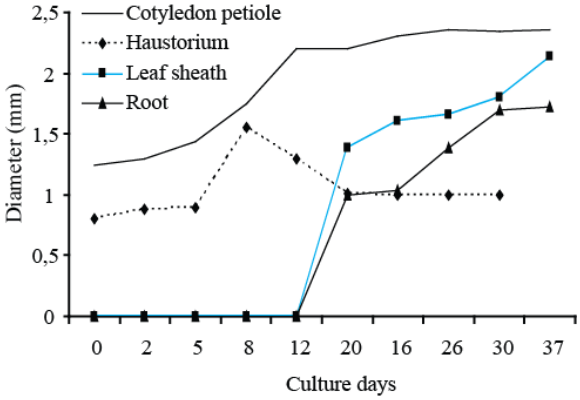

Fig. 4. Growth (diameter) of the organs of small sour-coconut seedling after 37 days of *In vitro* culture.

Conclusions

The DNA contents of different *Butia capitata* structures vary from 3.99 pg (embryo) to 5.17 pg (cotyledon petiole).

The DNA contents of the cotyledon petiole and haustorium varied during the evaluation period after the eighth day of *In vitro* culture, showing high coefficients of variation. The sheath, cotyledon petiole, and primary root have lower coefficients of variation and equivalent amounts of DNA, making these structures the most reliable organs for the analysis of *Butia capitata* tissues. Secondary metabolites appear to affect the quality of the samples.

Acknowledgments

Thanks to the Coordination of Improvement of Higher Education Personnel (CAPES) for granting the scholarship, and the National Council for Scientific and Technological Development (CNPQ) and the Research Foundation of the State of Minas Gerais (FAPEMIG) for funding this study. Denise MT Oliveira Federal University of Minas Gerais for their contribution to studies of anatomy.

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(Received for publication 20 April, 2014)