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Methods of internal standards' preservation for genome size assessments: a comparative study

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Abstract. Assessing genome size in plant species using flow cytometry requires fresh plant material from both the target species and appropriate internal standards. The use of fresh material from the standards is sometimes difficult. For this reason, a research about three preservation methods and their results when using the plants in flow cytometry has been conducted. We have focused on four of the most used internal standards in flow cytometry to estimate the nuclear DNA amount. Our results pointed out that the best method of conservation was lyophilisation. The conservation method based on drying with silica gel is more advisable to establish the ploidy level than to provide an absolute value of nuclear DNA content. Finally, ultrafreezing is not an appropriate preservation method.

Keywords: flow cytometry, freezing, genome size, internal standards, lyophilisation, silica gel preservation.

INTRODUCTION

The genome size, also named the nuclear DNA amount or 2C value, is a parameter that can be related to many other characteristics of an organism and its environment (Wakamiya et al. 1993; Wang et al. 2021). The term 'C-value' was coined by Swift (1950), the 'C' accounting for 'constancy' and referring to the DNA content of an unreplicated haploid chromosome complement. Later, several studies have established the influences of environmental factors on the variation of this parameter, although it was initially proposed as a constant of the organism and even of the species (Greilhuber et al. 2005). Other studies pointed out that some genuine variation exists, even within a species (Šmarda and Bureš 2010; Díez et al. 2013; Kolář et al. 2017; Boutte et

al. 2020; Becher et al. 2021), and that this can be due to intrinsic (ploidy level, aneuploidy, recombination rates, tandem repeats) or extrinsic (altitude, latitude, soil type, etc.) factors. Genome size information is crucial as the base to perform whole genome sequencing (WGS) and thus further research on this parameter, still unknown for a huge percentage of plant species, is needed (Pellicer et al. 2022). Despite there are many studies devoted to the importance of protocols and their effect on the accuracy of measurements, often highlighting the constancy of the value, the relative proximity to the genome size of the studied plants, the absence of some interfering cytosol metabolites or the possibility of producing low coefficients of variation in the measurements (Jedrzejczyk and Sliwinska 2010; Suda and Leitch 2010; Temsch et al. 2022), very few studies have been performed focused on methods of preservation and storage of plant material (Čertner et al. 2021; Tang et al. 2023), and even less on this aspect applied to internal standards.

The most commonly used technique to estimate genome size is flow cytometry (FCM), which allows estimating the amount of nuclear DNA by the relationship of the intensity of the measured fluorescence of the studied plants and the internal standard (Doležel and Bartos 2005; Hare and Johnston 2011). The quantification of the DNA of plant cells by flow cytometry basically requires fresh plant material from the target species and internal standards, and this need complicates the transfer and the storage of the samples (Doležel and Bartos 2005). The possibility of using fresh material from the standards is sometimes difficult, because the plant is not always available in its optimal state when the collected samples are analysed, although cultivation makes it possible in many cases. The same, and more complicated when dealing with wild plants, goes for the target plants, either because they were collected long time ago and are pending to be processed or because of slow processing. Storing plants in a conventional cold room at 4 °C, or freezing them at -18 °C, does not prevent their degradation, at least after a more or less long period of time. The temperature above 4 °C causes the breaking of the hydrogen bonds between the nitrogenous bases. Even environmental water can separate the two DNA chains by hydrolysis. A good conservation system would be necessary to avoid problems arising from the poor condition of the target plants and standards when there are delays in their processing that may be due to different reasons.

Facing the difficulty to standardize a preservation method for the great diversity of existing species, here we have focused on four of the most used internal standards in flow cytometry to assess the nuclear DNA amount of plants: *Lycopersicon esculentum, Petunia hybrida, Pisum sativum*, and *Triticum aestivum*, with the aim of testing different preservation methods, lyophilisation (also known as freeze drying), drying in silica gel and freezing at ultra-low temperature, and comparing the results obtained.

MATERIALS AND METHODS

Plant material and preservation treatments

Fresh material from leaves of tomato [*Lycopersicon esculentum* Mill. 'Montfavet 63/5' (2C=1.99 pg, Marie and Brown 1993)], petunia [*Petunia hybrida* Vilm. 'PxPc6' (2C=2.85 pg, Marie and Brown 1993)], common pea [*Pisum sativum* L. 'Express long' (2C=8.37 pg, Marie and Brown 1993)] and wheat [*Triticum aestivum* L. 'Triple Dirk' (2C=30.9 pg, Marie and Brown 1993)] were obtained from seeds and grown in a greenhouse at the Faculty of Pharmacy and Food Sciences (University of Barcelona), and treated with three conventional preservation systems usually used at the laboratories: lyophilisation, drying in silica gel and freezing at ultra-low temperature. Freezing was performed at -85 °C in a freezer Sanyo Electric Corporation (Moriguchi City, Osaka, Japan). The lyophilisation was carried out with a lyophiliser, FTS Systems Inc. (Stone Ridge, New York, USA) preserving the plant material into airtight glass jars by following the steps: 1) 2 minutes in liquid nitrogen 2) immersion of the sample in crushed ice and, 3) lyophilisation during 48 hours under vacuum < 500 mTorr. For the silica gel drying, the samples were kept in silica gel at room temperature for one month before the measurement (time 0).

The water percentages were obtained by drying in an oven until constant weight.

Flow cytometry assessments

Pisum sativum was used as standard to assess the genome size of *Triticum aestivum, Petunia hybrida* and *Lycopersicon esculentum,* while *Petunia hybrida* was the standard chosen to establish the nuclear DNA amount of *Pisum sativum*. Five individuals by each of the three treatments of the four standards and two samples of each individual were measured. To carry out the measurements, the standard with a DNA content closest to the theoretical value of the target standard has been chosen. When used as internal standards in this research, the leaf materials were always fresh.

An amount of 40-50 mg of young leaf tissue was used for sample preparation. Leaf material of each inter-

nal standard studied together with the leaf material of the plant used here as internal standard, as mentioned above, was submerged in 1,200 µL of isolation buffer LB01 (Doležel et al. 1989), supplemented with 100 µg/mL of ribonuclease A in a Petri dish, and mechanically chopped using a razor blade. The extract obtained was filtered to 20 µm pore nylon mesh, and stained with 36 µL of propidium iodide (1 mg/mL). Samples remained on ice until analysis.

The DNA measurements were carried out in a flow cytometer Epics XL (Coulter Corporation, Hialeah, Florida, USA). The cytometer used has an air-cooled argon-ion laser tuned at 15 mW and 488 nm of wave length excitation with forward scatter (FS) and side scatter (SS). FS measures the particle size and SS measures the particle complexity. Fluorescence was collected to 620 nm band pass filter (red). Two replicates of five different leafs of each plant were analysed. Acquisition was automatically stopped at 8,000 nuclei counts. The results were acquired by the System II Software version 3.0 (Coulter Electronics). Prior to analysis, the instrument was checked with standard fluorescent beads (Coulter Electronics).

For all the target plants, measurements were carried out immediately after the treatment (t0) and after six months (t6) in the same individual.

Statistical analyses

The variation of the cytometric measurements is usually expressed as the half peak coefficient of variation (HPCV). This parameter, expressed in percentage, indicates the dispersion of fluorescence intensities as the ratio of the standard deviation to the mean measured at 50% peak height. In addition, the mean, standard deviation, coefficient of variation (CV) and confidence interval (CI) were also calculated for each standard and treatment. A paired sample t-test was carried out to determine whether the mean difference between the GS values between t0 and t6 is zero for each treatment and standard. Previously, a Shapiro-Wilk test of normality was carried out for all samples.

Statistical analyses were performed using XLSTAT 2023.1.1 (Lumivero 2023), Excel 16.0.4266 by Microsoft Office (Microsoft Corporation).

RESULTS AND DISCUSSION

The results of descriptive statistics are included in the Table 1. No genome size measurements have been obtained for *Triticum aestivum* lyophilized for six months.

The calculated parameters (mean, SD, CV and CI) reveal a great dispersion of values in the assessments of genome size of the standards. In fact, in many cases the established value of the standard (Marie and Brown 1993) does not fall within the confidence interval calculated from the average and standard deviation of each subset of data. In the lyophilized material, only in *Pisum sativum* (t6) and *Lycopersicon esculentum* (t0) the established values for both internal standards fall outside the interval, while for the remaining treatments (silica gel and freezing), almost all standards values fall out, excepting for the frozen material of *Petunia hybrida* (t6) and *Lycopersicon esculentum* (t0) (Table 1). These results point out that the best treatment to preserve samples is the lyophilisation, while silica gel preservation is the least recommended, despite the results for frozen wheat are missing, probably due to DNA degradation during the freezing process.

Results of p-values from the Paired t-test are displayed in Table 2. Shapiro-Wilk test reveals that 2C values of most data subsets follow a normal distribution. P-values below 0.05 (Table 2) indicate that there are statistically significant differences between the genome size measurements at t0 and t6 into the same standard and treatment. In this case, the different standards behave differently over time, which allows us to assert that some characteristics of the plant species and, in particular, of their leaves, influence their state of conservation. Thus, we note that *Triticum aestivum* and *Pisum sativum*, with a lower water content (74.35% and 80.22%, respectively) are the species showing less genome size variation over time, while *Petunia hybrida* (87.97%) and *Lycopersicon esculentum* (85.53 %) are more time sensitive.

Although a considerable effort has been dedicated to defining the characteristics of the internal standards employed in quantifying DNA, one of the relevant ones being the easy availability (Temsch et al. 2022), the studies on the preservation of plant material for flow cytometry are very scarce. Some authors (Tang et al. 2023) have investigated for alternative solutions such as the use of spores and pollen as internal standards. These authors claim that these standards are ready-to-use, easy to handle, and include long-term storage compared to traditional fresh leaf standards.

Čertner et al. (2021) have analysed the advantages and limitations of different strategies and material storage, but they have not specifically focused on internal standards. The authors conclude that frozen plants can be stored for up to months or years, and silica gel-dried material for up to two years, in both cases only for ploidy level determinations. In fact, these authors only consider as viable materials for genome size estimation

Table 1. Descriptive statistics for the 2C values of the internal standards immediately after the treatment (t0) and after six months preserved (t6).

	Triticum aestivum	*Petunia hybrida*	*Pisum sativum*	*Lycopersicon esculentum*	
2C (pg)	30.9	2.85	8.37	1.99	
H_2O content (%)	74.35	87.97	80.22	85.53	
	t0				
Mean (x̄)±SD	30.914±0.671	2.882±0.028	8.321±0.164	2.092±0.076	
Coefficient of variation (CV)	2.171	0.970	1.976	3.651	
Confidence interval (CI)	[30.080; 31.747]	[2.847; 2.917]	[8.117; 8.525]	[1.997; 2.187]*	Lyophilised
	t6				
Mean (x̄)±SD	31.537±0.596	2.903±0.073	8.050±0.149	1.963±0.050	
Coefficient of variation (CV)	1.890	2.515	1.846	2.569	
Confidence interval (CI)	[30.797; 32.278]	[2.812; 2.994]	[7.866; 8.235]*	[1.900; 2.026]	
	t0				
Mean (x̄)±SD	31.841±0.523	3.144±0.024	7.979±0.171	2.037±0.031	
Coefficient of variation (CV)	1.644	0.779	2.140	1.523	
Confidence interval (CI)	[31.191; 32.491]*	[3.113; 3.174]*	[7.767; 8.191]*	[1.998; 2.075]*	Silica gel-preserved
	t6				
Mean (x̄)±SD	32.004±0.206	2.587±0.164	8.044±0.112	1.404±0.131	
Coefficient of variation (CV)	0.643	6.352	1.396	9.332	
Confidence interval (CI)	[31.749; 32.260]*	[2.383; 2.791]*	[7.905; 8.184]*	[1.242; 1.567]*	
	t0				
Mean (x̄)±SD	34.165±0.362	2.690±0.029	7.424±0.056	2.092±0.091	
Coefficient of variation (CV)	1.059	1.088	0.752	4.353	
Confidence interval (CI)	[33.716; 34.615]*	[2.653; 2.726]*	[7.354; 7.493]*	[1.979; 2.205]	Frozen
	t6				
Mean (x̄)±SD	missing	2.784±0.233	7.680±0.356	2.412±0.112	
Coefficient of variation (CV)	missing	8.360	4.634	4.645	
Confidence interval (CI)	missing	[2.495; 3.074]	[7.238; 8.122]*	[2.273; 2.551]*	

Table 2. p-values from the paired t-test. *Statistically significant differences in 2C values between immediately after the treatment (t0) and after six months preserved (t6).

	Triticum aestivum	*Petunia hybrida*	*Pisum sativum*	*Lycopersicon esculentum*
Llyophilised	p=0.222	p=0.558	p=0.070	p=0.023*
Silica gel preserved	p=0.537	p=0.002*	p=0.539	p=0.000*
Freezing	-	p=0.414	p=0.198	p=0.017*

in absolute fresh tissue units freshly germinated seedlings (which consist also of fresh tissue), and glycerol-preserved nuclei, with silica gel-desiccated tissue, dry seeds, frozen tissue and chemically-fixed tissue as apt for ploidy level determination. Concerning dried material, this agrees with the evidences of usefulness of such tissues for ploidy level determination brought by Suda and Trávníček (2006). Indeed, Sliwinska et al. (2021) recommend to avoid preserved (herbarium vouchers, silica gel-dried tissue, frozen tissue) or fixed samples for a robust genome size assessment in plants. Bourge et al. (2018) state that measurements in dried samples could be less precise and even need correction factors. Conversely, Wang and Yang (2016) affirm that desiccated tissues that remain green (without brown or yellow marks), and stored at -80 °C for less than six months are suitable for genome size estimations with absolute values.

According to the present results, we can add lyophilisation, which is not included in any of previous mentioned studies, as another preservation method allowing nuclear DNA content assessment in absolute units, and confirm the non-suitability of silica gel-desiccated and frozen materials for such precise estimations, although they can be appropriate for ploidy level determination.

Concluding remarks

The optimal conservation method among the three evaluated is lyophilisation. At the initial time point, the values align closely with those of the fresh material, and even after six months, there are no noteworthy differences between both values. Lyophilisation emerges as a more convenient and cost-effective option for sample preservation, when compared to the commonly used cryogenic technique involving liquid nitrogen in situ (Čertner et al. 2021), which is not always practical in field work.

Drying with silica gel stands as a generally acceptable conservation method. However, its effectiveness diminishes in tissues with higher water content, as evidenced by an increase in the coefficient of variation of results. This technique is better suited for determining ploidy levels than providing an absolute DNA content value.

On the other hand, ultrafreezing proves to be the least effective preservation method among the three tested. This inferiority is likely attributed to the freezing process allowing ample time for structural water to degrade a portion of the DNA.

In summary, lyophilisation stands out as the best choice, offering comparable values to fresh material at time 0 and maintaining consistency over a 6-month period. The current results allow to add lyophilisation to the other systems of preservation (apart from using fresh tissue, which is ideal whenever possible) proposed to date to estimate genome size, whereas freezing and silica gel-drying are confirmed to be useful to preserve plant materials for ploidy level establishment, but not for nuclear DNA content assessment in absolute units.

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