J. Amer. Soc. Hort. Sci. 149(3):131–141. 2024. https://doi.org/10.21273/JASHS05376-24

Flow Cytometry for Estimating Plant Genome Size: Revisiting Assumptions, Sources of Variation, Reference Standards, and Best Practices

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Keywords. accuracy, buffers, DNA content, error, fluorochromes, plant reference standards, precision, secondary metabolites

Abstract. Flow cytometry has been widely used to estimate relative and absolute genome sizes (DNA contents) of plants for more than 50 years. However, the accuracy of these estimates can vary widely because of many factors, including errors in the genome size estimates of reference standards and various experimental methods. The objectives of this study were to reassess genome sizes of commonly used reference standards and quantify sources of variation and error in estimating plant genome sizes that arise from buffers, confounding plant tissues, tissue types, and plant reference standards using both 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) fluorochromes. Five separate studies were performed to elucidate these objectives. Revised estimates of genome sizes of commonly used plant reference standards were determined using human male leukocytes as a primary standard with an updated genome size (6.15 pg; 12.14% lower than that of earlier studies) using both DAPI and PI fluorochromes. Comparison of six different buffers (Galbraith's, LB01, MB01, MgSO4, Otto's, and Sysmex) resulted in variations in genome size estimates by as much as 18.1% for a given taxon, depending on the buffer–fluorochrome combination. The addition of different confounding plant tissues (representing 10 diverse taxa and associated secondary metabolites) resulted in variations in genome size estimates by as much as 10.3%, depending on the tissue–fluorochrome combination. Different plant tissue types (leaf color/exposure and roots) resulted in a variation in genome size estimates of 10.7%, independent of the fluorochrome. The selection of different internal reference standards introduced an additional variation in genome size estimates of 5.9%, depending on the standard–fluorochrome combination. The choice of fluorochrome (DAPI vs. PI) had one of the largest impacts on genome size estimates and differed by as much as 32.9% for *Glycine max* 'Polanka' when using human male leukocytes as an internal standard. A portion of this variation (~10.0%) can be attributed to the base pair (bp) bias of DAPI and variations in Guanine-Cytosine (GC):Adenine-Thymine (AT) ratios between the sample and standard. However, as much as 22.9% of the variation in genome size estimates may result from how effectively these fluorochromes stain and report the genome. The combined variation/error from all these factors (excluding variation from bp bias for different fluorochromes, and assuming variations from confounding tissues and tissue types to both result from secondary metabolites) totaled 57.6%. Additional details of how selected factors impact accuracy, precision, and the interaction of these factors are presented. Overall, flow cytometry can be precise, repeatable, and extremely valuable for determining the relative genome size and ploidy of closely related plants when using consistent methods, regardless of fluorochrome. However, accurate determination of the absolute genome size by flow cytometry remains elusive, and estimates of genome size using flow cytometry should be considered gross approximations that may vary by ±29% or more as a function of experimental methods and plant environments. Additional recommendations of best practices are provided.

Received for publication 13 Jan 2024. Accepted for publication 24 Feb 2024. Published online 17 Apr 2024.
This research was funded by the NC Agricultural Research Service, Raleigh, NC, USA. This paper is a portion of a thesis submitted by JN in fulfilling a degree requirement. TGR and JN designed the experiments, completed the statistical analyses, and wrote the initial draft of the manuscript. JN performed the experiments and data collection. NL provided technical assistance. HC participated in the presentation and discussion of the results. All authors reviewed and made suggestions for revising the manuscript. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee of warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.
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Flow cytometry was first used for plants in Germany (Heller 1973) to measure the genome size of *Vicia faba*. However, the complex enzyme-based methodology made it difficult to replicate, and the application did not gain widespread recognition until the 1980s. Galbraith et al. (1983) successfully streamlined the protocol by using mechanical homogenization. Flow cytometry-based estimations of genome size have been used to determine ploidy, support genome sequencing and taxonomic studies, estimate guanine-cytosine (GC):adenine-thymine (AT) contents, and determine the sex of dioecious plants (Moreno et al. 2018; Šmarda et al. 2012). Although the use of plant flow cytometry to estimate the relative genome size and ploidy is widely accepted, the accuracy of flow cytometry for estimating the absolute genome size in plants is more difficult to validate. The use of

fluorochromes to stain DNA and measure genome sizes is complicated by many factors, including interferences from secondary metabolites, buffer effects, fluorochrome affinities and staining efficacy, chromatin composition and architecture, and other factors that can interfere with fluorescence.

Fluorochrome effects. A diverse range of fluorochromes have been used in plant flow cytometry. How effectively these dyes stain the entire genome, the impact of chromatin on staining efficacy, and how the fluorescence of these dyes is affected by buffers and diverse plant metabolites are largely unknown. Significant variations in the estimated plant genome size have been observed when different fluorochromes were used (Doležel et al. 1992); these were attributed to differences in staining, including base pair (bp) biases and incubation time. Although many stains have been used for plant flow cytometry, propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) are more commonly used. Because DAPI preferentially stains adenine and thymine, variations in the bp composition between the sample and internal standard can result in an error/bias (Doležel et al. 1992). Therefore, it is often suggested that intercalating stains like PI more accurately determine the absolute genome size (Doležel et al. 2003) because GC/AT contents vary considerably across vascular plants (Galbraith et al. 2021). Although the concern regarding bp bias is important, other fluorochrome characteristics including the efficacy of staining densely packed chromatin and the sensitivity of fluorescence to interfering metabolites also impact the accurate estimation of genome size (Darzynkiewicz and Traganos 1988; Darzynkiewicz et al. 1984).

Buffers effects. Buffers are essential components for both nuclei isolation and staining when preparing samples for flow cytometry. Isolation and staining buffers can be separate or combined. Buffer components may include surfactants, isotonic osmotic agents, pH buffers, phenolic binding ingredients [e.g., polyvinylpyrrolidone (PVP)], compounds that help preserve DNA (e.g., spermine), and enzymes that break down RNA or proteins. Because of the diversity of tissue types, morphology, and metabolite composition, buffers have been optimized for different applications (Loureiro et al. 2006b). As many as 28 buffers are commonly used (Doležel et al. 2007). The development of woody plant buffer (WPB) was shown to more effectively produce acceptable results for problematic tissues when compared with general purpose buffer (GPB) when screened across 37 taxa (Loureiro et al. 2007a). Otto's buffers I and II were found to work particularly well with more acidic plant cells (Emshwiller 2002). Sadhu et al. (2016) developed the novel buffer MB01 that reduced the degradation and adhesion of nuclei in Zingiberaceae species. In some cases, Otto's buffers I and II were best for determining genome sizes of plants that have smaller genome sizes (Loureiro et al. 2006b). Although optimizing buffers for problematic species is often warranted, research has shown that buffers can interact with plant metabolites and fluorochromes to impact genome size estimates (Loureiro et al. 2006b).

Plant tissue and metabolite effects. Diverse secondary metabolites in plants may vary substantially between plant taxa and tissues. The presence of these compounds can lead to errors when estimating the plant genome size using flow cytometry (Bennett et al. 2008; Loureiro et al. 2006a; Noirot et al. 2003; Price et al. 2000; Sharma et al. 2019). Substances such as anthocyanins (Bennett et al. 2008), caffeine (Noirot et al. 2003), chlorogenic acid (Noirot et al. 2003), coumarins (Sastry et al. 1993), ellagic acid (Dixit and Gold 1986), tannic acid (Loureiro et al. 2006a) and other unidentified compounds (Price et al. 2000) have been shown to interact with fluorochromes and nuclear DNA, leading to either an underestimation or overestimation of the genome size (Sharma et al. 2019). Some cytosolic compounds can interfere with stains, causing quenching, or impact DNA condensation and DNA accessibility. Stain interactions with cytosolic compounds can result in as much as 20% error when determining genome size (Noirot et al. 2000). In some cases, caffeine may prevent PI from binding with the DNA by competition or by forming complexes with the intercalating dye (Greilhuber 1988). This interaction between PI × caffeine usually leads to lower genome size estimates; however, when caffeine forms complexes with phenolic compounds, the genome size estimates may be higher (Noirot et al. 2003). Tannic acids, in particular, can impact genome size estimations and may interact with the nuclei isolation and staining buffers (Loureiro et al. 2006a). When working with taxa containing high levels of tannic acid or other phenolics, the addition of polyvinylpyrrolidone (PVP), chelating agents, and antioxidants may be appropriate (Loureiro et al. 2021). Additionally, anthocyanin pigments have been shown to affect the results of PI staining, and it has been suggested that some intraspecific variations in genome sizes observed in the literature may be related to anthocyanin levels in the different tissues sampled (Bennett et al. 2008).

Tissue type effects. The choice of plant tissue has been shown to impact results when estimating the plant genome size with flow cytometry. Tissue effects on genome size estimations can be attributed to the organ that the tissue comes from, whether that tissue is alive, dead, or fixed, and the cytosolic compounds found in the tissue. Different secondary metabolites found in varying types and amounts across plant tissues can also result in tissue-specific interference. Leaf lamina tissue is commonly sampled in flow cytometry protocols because of its accessibility (Doležel et al. 2007; Pellicer et al. 2021); however, studies also use floral tissues, vegetative buds, and root tissue. Michaelson et al. (1991) found that genome size estimates of *Helianthus annuus* vary as much as 48% among leaves from different nodes of the same plant when using PI. Price et al. (2000) later provided evidence that this variation was attributable to environmentally induced inhibitors that interfere with intercalation and/or fluorescence of PI (Price et al. 2000). Although the tissue type is often selected to maximize efficient nuclei extraction, characteristics of different tissues may impact DNA staining, fluorescence, or both.

Reference standard effects. Because flow cytometers measure relative fluorescence, it is essential to include a reference standard with a known genome size to calculate the genome size for an unknown. The selection of an appropriate reference standard is essential to the accurate calculation of the plant genome size. Early applications of flow cytometry to estimate plant genome sizes often used animal nuclei as reference standards, primarily because these standards were both well-researched and readily available. However, animal nuclei are generally no longer recommended as internal standards because they may interact differently than plant nuclei with various factors, including buffers, fluorochromes, and plant metabolites (Temsch et al. 2022). Additionally, the use of internal standards with similar but distinct genome sizes is often recommended to protect against the nonlinearity of fluorescence photomultipliers and extrapolation errors (Bagwell et al. 1989; Suda and Leitch 2010). Therefore, the

available animal standards also did not offer adequate coverage of the genome size variation present in plants, which is currently known to have a lower limit of 0.14 pg for *Genlisea tuberosa* (Fleischmann et al. 2014) and an upper limit of 304.46 pg for *Paris japonica* (Pellicer et al. 2010). As a result, the use of diverse plant reference standards has been recommended (Doležel and Greilhuber 2010; Johnston et al. 1999; Price et al. 1980).

Other issues exist when genome size estimates of these reference standards are based on different methods and/or various estimates used for the genome sizes of the primary standards (Loureiro et al. 2007b). *Pisum sativum* 'Ctirad', one of the most used reference standards, has published genome size estimates ranging from 8.20 to 9.60 pg (17.1% variation), depending on methods (Doležel and Greilhuber 2010). Part of the variations in genome size estimates of reference standards comes from variations in genome size estimates of primary standards. The best estimate of the true genome size of male human leukocytes has changed over time, ranging from 7.0 pg (Tiersch et al. 1989) to 6.41 pg (Piovesan et al. 2019). Genome size values for a standard set of commonly used reference standards were provided by Doležel et al. (1992, 1998) and based on human male leukocytes, with an estimated genome size of 7.0 pg. As a result, many plant genome sizes that were determined using these plant reference standards with genome sizes based on outdated values for human male leukocytes have inflated estimates of genome size.

Defining and calculating accuracy and precision. Accuracy and precision are terms that are commonly used to describe the degree of correctness or exactness of a measurement (National Institute of Standards and Technology 2023). Although definitions can vary, when used here, accuracy (bias) refers to the degree to which a measurement or estimate agrees with the true value, if that value is known. Accuracy is commonly expressed as a percentage of the error between the measured value and true value. Precision (repeatability), when used here, refers to the degree of reproducibility or agreement between multiple measurements or estimates of the same quantity under the same conditions. Precision is often expressed as a measure of variance among measurements (e.g., mean absolute deviation, *SD*, or *SEM*).

In the context of plant flow cytometry, accuracy and precision are important for obtaining reliable and meaningful data. If the data are inaccurate, then they will not reflect the true genome size of the measured cells. If the data are not precise, then they will not be reliable or repeatable.

The overall objectives of this study were to reassess genome sizes of commonly used reference standards and quantify variability and error in estimating plant genome sizes that arise from different extraction buffers, reference standards, confounding plant tissue, and tissue types within a plant using both DAPI and PI fluorochromes.

Materials and Methods

General flow cytometry. All plant material was grown onsite, and fresh tissue was used. Plant tissue samples were prepared by co-chopping leaf disks (0.26 cm^2) or terminal roots (3 cm long) with a reference standard using a double-edged razor blade in a petri dish containing 500 μL of extraction buffer. Then, samples were filtered through 50-μm nylon mesh and stained using 2.0 mL of staining buffer containing either DAPI or PI. PI samples were incubated at room temperature for 60 min before running. Nuclei fluorescence was determined using a flow cytometer (Quantum P; QuantaCyte, Mullica Hill, NJ, USA), sampling >5000 nuclei per sample/standard combination, and performing analyses using CyPad software (QuantaCyte version 1.3). Sysmex (Sysmex Corporation, Kobe, Hyogo, Japan) cleaning solution and Sysmex decontamination solution (Sysmex Corporation) were used to clean the cytometer between each run. The 2C DNA contents were calculated as follows: 2C = DNA content of standard × (mean fluorescence value of sample ÷ mean fluorescence value of the standard).

Expt. 1: Estimating genome sizes of plant reference standards using human male leukocytes as an internal standard and either DAPI or PI fluorochromes. Reference plant standards included *G. max* 'Polanka', *P. sativum* 'Ctirad', *Raphanus sativus* 'Saxa', *Secale cereale* 'Dankovske', *Vicia faba* 'Inovec', and *Zea mays* CE-777, which were graciously provided by J. Doležel. Leukocytes, drawn from a male laboratory member, and isolated by Zen-Bio Inc. (Durham, NC, USA) as their human peripheral blood mononuclear cells product. Plant tissues were chopped in a petri dish containing 500 μL of CyStain Precise P (Sysmex Corporation) extraction buffer or CyStain Absolute P (Sysmex Corporation) extraction buffer as DAPI and PI, respectively. Then, 12 μL of fresh suspended human leukocytes was added to the suspended plant nuclei and vortexed. The mixed nuclei and buffer were stained using 2.0 mL of staining buffer containing either DAPI (CyStain Precise P; Sysmex Corporation) or PI (CyStain Absolute P; Sysmex Corporation). The experiment had a split block design with fluorochrome as the main factor and the reference standard as the sub-factor. A complete factorial set of all six plant standards and two fluorochromes was processed on a given day and treated as a complete block, with seven replicates blocked over 7 d.

Updated estimate of the male human genome size. The Telomere-to-Telomere Consortium (Nurk et al. 2022) recently compiled a complete 3,054.815472-Mbp monoploid sequence of the human female genome, providing a diploid holoploid size of 6,109.630944 Mbp. Correcting for the smaller Y chromosome in the male genome reduced the size slightly by 1.5514% (Piovesan et al. 2019), thus providing a size of 6,014.8461295 Mbp. A genome size of 6.150413 pg was estimated by converting from bp to mass using GC content for the male genome of 40.91% (Piovesan et al. 2019) and relative weights of nucleotide pairs for AT of 615.3830 atomic mass unit (AMU) and GC of 616.3711 AMU (Doležel et al. 2003) with the following formula: (GC bp × 616.3711 AMU) + (AT bp × 615.3830 AMU) = AMU human male genome (Doležel et al. 2003). Further converting to pg was performed by using the following formula: 1 pg = 1.660539×10^{-12} pg/1 AMU (Mohr and Taylor 2000).

Expt. 2: Effects of buffers and fluorochrome on the estimate of the genome size. Seven flow cytometry nuclei isolation and staining buffer combinations were used to estimate *G. max* 'Polanka' and *P. sativum* 'Ctirad' genome sizes with DAPI and PI fluorochromes. Otto's I and II (combination) (Doležel and Göhde 1995), LB01 (Doležel et al. 1989), MB01 (Sadhu et al. 2016), $MgSO_4$ (Arumuganathan et al. 1991), and Galbraith's buffer (Galbraith et al. 1983) were made in the laboratory. In addition, nuclei isolation buffers and stain buffer combinations of CyStain™ ultraviolet Precise P Nuclei Extraction Buffer and CyStain™ ultraviolet Precise P Staining Buffer (Sysmex Corporation) as DAPI and CyStain™ PI Absolute P Nuclei

Extraction Buffer and CyStain™ PI Absolute P Staining Buffer (Sysmex Corporation) as PI were included. *P. sativum* 'Ctirad' and *G. max* 'Polanka' were chosen for comparison because of their similar AT:CG ratios (~3% difference) (National Center for Biotechnology Information, 2023a, 2023b) to minimize bp bias when comparing DAPI and PI fluorochromes. Plant standard tissues were co-chopped in petri dishes with 500 μL of each extraction buffer with no fluorochrome. Then, nuclei were stained using 2.0 mL of the matching staining buffer with either 4 μg/mL DAPI or 50 μg/mL PI, except for the Sysmex buffers, which have proprietary concentrations. For staining buffers containing PI, the enzyme RNaseA was included at a concentration of 50 μg/mL. The experiment had a split block design with fluorochrome as the main factor and buffer as the sub-factor. A complete factorial set of all seven buffers and two fluorochromes was processed on a given day and treated as a complete block with five replicates blocked over 5 d.

Expt. 3: Effects of confounding plant tissue and fluorochrome on the estimate of the genome size. Ten confounding taxa were chosen from a diverse range of plant families with varied secondary metabolites, including *Ginkgo biloba*, *Thuja* 'Green Giant', *Illicium* 'NCIH1', *Magnolia virginiana* 'Jim Wilson', *Miscanthus sinensis* 'Gracillimus', *Arundo donax*, *Cannabis sativa, Rosa chinensis* 'Mutabilis', *Pterocarya fraxinifolia*, and *Camellia* 'Ashton's Supreme'. *P. sativum* 'Ctirad' and *G. max* 'Polanka' were used as standard pairs. Standard pairs and confounding plant tissue were co-chopped in a petri dish containing 500 μL of extraction buffer CyStain Precise P (Sysmex Corporation) or CyStain Absolute P (Sysmex Corporation) for DAPI and PI, respectively. Then, nuclei were stained using 2.0 mL of staining buffer containing DAPI (CyStain Precise P; Sysmex Corporation) or PI (CyStain Absolute P; Sysmex Corporation). The experiment had a split block design with fluorochrome as the main factor and confounding tissue as the sub-factor. A complete factorial set of all 10 confounding plant tissues and two fluorochromes were processed on a given day and treated as a complete block with six replicates blocked over 6 d.

Expt. 4: Effects of plant tissue type and fluorochrome on the estimate of the genome size of *Berberis thunbergii* 'NCBT2'. Containerized specimens of *B. thunbergii* 'NCBT2' Sunjoy Neo® were used to sample tissue with *P. sativum* 'Ctirad' as an internal standard. Three *Berberis* tissue types were sampled: roots, red leaves (exposed to full sun), and yellow leaves (shaded leaves from the canopy interior). *Berberis* tissue samples and plant standard tissues were co-chopped in a petri dish containing 500 μL of extraction buffer CyStain Precise P (Sysmex Corporation) or CyStain Absolute P (Sysmex Corporation) as DAPI and PI, respectively. Then, nuclei were stained using 2.0 mL of staining buffer containing DAPI (CyStain Precise P; Sysmex Corporation) or PI (CyStain Absolute P; Sysmex Corporation). The experiment had a split block design with fluorochrome as the main factor and tissue type as the sub-factor. A complete factorial set of all three tissue types and two fluorochromes was processed on a given day and treated as a complete block with six replicates blocked over 6 d.

Expt. 5: Effects of the internal plant reference standard and fluorochrome on the estimate of the genome size. Genome sizes for both *P. sativum* 'Ctirad' and *Z. mays* 'CE-777' were estimated using different internal plant reference standards in the following (sample/reference) combination pairs: *P. sativum* 'Ctirad' and *S. cereale* 'Dankovske'; *P. sativum* 'Ctirad' and *Z. mays* 'CE-777'; *Z. mays* 'CE-777' and *P. sativum* 'Ctirad'; and *Z. mays* 'CE-777' and *G. max* 'Polanka'. Sample and reference tissues were co-chopped in a petri dish containing 500 μL of extraction buffer CyStain Precise P (Sysmex Corporation) or CyStain Absolute P (Sysmex Corporation) for DAPI and PI stains, respectively. Then, nuclei were stained using 2.0 mL of staining buffer containing DAPI (CyStain Precise P; Sysmex Corporation) or PI (CyStain Absolute P; Sysmex Corporation). The experiment had a split block design with fluorochrome as the main factor and sample/reference combination as the sub-factor. A complete factorial set of all four sample/reference and two fluorochromes was processed on a given day and treated as a complete block with six replicates blocked over 6 d.

Statistics. An analysis of variance was completed for each experiment using SAS (SAS version 9.4; SAS Institute, Cary, NC, USA) PROC MIXED for the split-plot design. When the Levene test for unequal variance between fluorochromes was significant, PROC MIXED was also used to adjust for heteroscedasticity using the DDFM = SATTERTHWAITE option in the model statement and GROUP = fluorochrome in the REPEATED statement. When appropriate, individual mean comparisons were conducted using LSMEANS/DIFF.

Results

Expt. 1: Estimating genome sizes of plant reference standards using human male leukocytes as an internal standard and either DAPI or PI fluorochromes. An analysis of variance showed that the genome size of the plant standards varied as a function of fluorochrome, taxa, and an interaction between the two ($P < 0.01$). The difference in the estimated genome size between the two fluorochromes varied depending on the taxa (Table 1). Genome size values determined using PI were generally lower than those reported previously (Doležel et al. 1992, 1998; Lysák and Doležel 1998). The difference in genome size estimates determined by DAPI and PI varied from 11.7% for *P. sativum* 'Ctirad' to 32.9% for *G. max* 'Polanka'. The measurement precision (repeatability) was relatively high for any given reference standard, with the *SEM* ranging from 0.01 to 0.16 pg for DAPI and from 0.01 to 0.28 pg for PI, and it was always less than 2% of the mean.

Expt. 2: Effects of buffers and fluorochrome on the estimate of the genome size. Regardless of whether the genome size of *P. sativum* 'Ctirad' was estimated using *G.* 'Polanka' as a reference standard or the reciprocal, there was a significant interaction between extraction buffer × fluorochrome on the estimate of the plant genome size. These effects demonstrated that the use of different buffers influenced the estimate of the plant genome size, but the degree of influence depended on which fluorochrome was used. Genome size estimates determined with different buffers varied as much as 18.1% when using DAPI and 14.1% when using PI (Table 2). Estimates of genome size were generally higher when using DAPI than when using PI, but the degree of difference varied by buffer. Precision, as measured by the *SEM*, for any given buffer/sample combination ranged from 0.01 to 0.08 pg for DAPI and 0.02 to 0.21 pg for PI, and it was always less than 3% of the mean.

Table 1. Calculated 2C genome sizes of six common plant standards measured using either 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) fluorochromes and human male leukocytes (6.150413 pg, estimated genome size) as an internal standard.

Plant standard	Fluorochrome[i]	Estimated 2C genome size [mean ± *SEM* (pg)][ii,iii]	Previously published 2C genome size with PI (pg)
Glycine max 'Polanka'	DAPI	2.79 ± 0.03*	N/A[x]
Pisum sativum 'Ctirad'	DAPI	8.66 ± 0.05*	N/A
Raphanus sativus 'Saxa'	DAPI	1.24 ± 0.01*	N/A
Secale cereale 'Dankovske'	DAPI	11.16 ± 0.13*	N/A
Vicia faba 'Inovec'	DAPI	26.68 ± 0.16*	N/A
Zea mays CE-777	DAPI	4.02 ± 0.07*	N/A
G. max 'Polanka'	PI	2.10 ± 0.02*	2.50[v]
P. sativum 'Ctirad'	PI	7.75 ± 0.05*	9.09[vi]; 8.02[ix]
R. sativus 'Saxa'	PI	0.94 ± 0.01*	1.11[iv]; 0.98[viii]
S. cereale 'Dankovske'	PI	13.58 ± 0.11*	16.19[vi]
V. faba 'Inovec'	PI	21.43 ± 0.28*	26.90[iv]; 23.80[ix]
Z. mays CE-777	PI	5.04 ± 0.08*	5.43[vii]

[i] CyStain Precise P (Sysmex Corporation, Kobe, Hyogo, Japan) or CyStain Absolute P (Sysmex Corporation) staining buffers were used as DAPI and PI, respectively.
[ii] Values are means (n = 7) ± *SEM*.
[iii] *Indicates that the two means for the estimated 2C genome size determined for the same plant standard using the two fluorochromes were significantly different ($P < 0.01$).
[iv] Doležel et al. (1992).
[v] Doležel et al. (1994).
[vi] Doležel et al. (1998).
[vii] Lysák and Doležel (1998).
[viii] Šmarda et al. (2014).
[ix] Veselý et al. (2012).
[x] N/A indicates that there is no previously published genome size for the relevant plant standard using the fluorochrome DAPI.

Expt. 3: Effects of confounding plant tissue/metabolites and fluorochrome on the estimate of the genome size. When estimating the genome size for *G. max* 'Polanka' using *P. sativum* 'Ctirad' as an internal standard, there was a significant interaction between fluorochrome × confounding plant tissue ($P < 0.01$) for both genome size and percent error. Genome size estimates for a given sample ranged by 8.0% when using DAPI and 10.3% when using PI, depending on which confounding tissues were present (Table 3). Accuracy, measured as % error compared with the control for DAPI and PI, varied in magnitude and direction, depending on which confounding plant tissue was present. In some cases, the confounding tissue

Table 2. Genome size (2C) for *Glycine max* 'Polanka' and *Pisum sativum* 'Ctirad' as a function of extraction buffer and either 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) fluorochromes.

Buffer	Fluorochrome	Estimated 2C genome size [mean ± *SEM* (pg)] for *G. max* 'Polanka'[i,ii]	Estimated 2C genome size [mean ± *SEM* (pg)] for *P. sativum* 'Ctirad'[ii,iii]
Galbraith's	DAPI	3.18 ± 0.04[a]	7.61 ± 0.11[a]
LB01	DAPI	3.18 ± 0.01[a]	7.59 ± 0.02[a]
MB01	DAPI	3.11 ± 0.02[a]	7.77 ± 0.06[a]
MgSO4	DAPI	3.18 ± 0.03[a]	7.61 ± 0.06[a]
Otto's	DAPI	2.91 ± 0.03[b]	8.31 ± 0.08[b]
Sysmex[iv]	DAPI	2.70 ± 0.01[c]	8.96 ± 0.04[c]
Galbraith's	PI	2.13 ± 0.02[B]	7.65 ± 0.07[AB]
LB01	PI	2.05 ± 0.02[BC]	7.93 ± 0.09[BC]
MB01	PI	1.96 ± 0.05[C]	8.32 ± 0.21[D]
$MgSO_4$	PI	2.03 ± 0.05[C]	8.03 ± 0.18[CD]
Otto's	PI	2.23 ± 0.02[A]	7.29 ± 0.07[A]
Sysmex[v]	PI	1.99 ± 0.02[C]	8.20 ± 0.08[CD]

[i] Determined with *P. sativum* 'Ctirad' as an internal standard with an estimated genome size of 8.66 pg when using DAPI and 7.75 pg when using PI.
[ii] Values are means (n = 5) ± *SEM*. Values followed by different letters within a column and fluorochrome are significantly different ($P \leq 0.05$). Lowercase letters represent comparisons within the DAPI fluorochrome. Uppercase letters represent comparisons within the PI fluorochrome.
[iii] Determined with *G. max* 'Polanka' as an internal standard with an estimated genome size of 2.79 pg when using DAPI and 2.10 pg when using PI.
[iv] Sysmex Precise P proprietary buffer kit (Sysmex Corporation Kobe, Hyogo, Japan) was used with DAPI.
[v] Sysmex Absolute P proprietary buffer kit (Sysmex Corporation) was used with PI.

Table 3. Genome size (2C), accuracy (% error), and precision [standard error of the mean (*SEM*) and mean absolute deviation (MAD)] of estimated 2C genome size for *Glycine max* 'Polanka' and *Pisum sativum* 'Ctirad' processed with confounding tissue from diverse plants using either 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) fluorochromes.

Confounding plant tissue	Fluorochrome	2C genome size [mean ± *SEM* (pg)] for *G. max* 'Polanka'[i]	Error (%) ± *SEM*	MAD [mean ± *SEM* (pg)]	2C genome size [mean ± *SEM* (pg)] for *P. sativum* 'Ctirad'[ii]	Error (%) ± *SEM*	MAD [mean ± *SEM* (pg)]
Arundo donax	DAPI	2.66 ± 0.04[iii]	−1.05 ± 1.32	0.07 ± 0.01	9.10 ± 0.12	1.04 ± 1.34	0.25 ± 0.05
Camellia 'Ashton's Supreme'	DAPI	2.61 ± 0.03	−2.73 ± 1.09	0.06 ± 0.01	9.26 ± 0.10	2.76 ± 1.15	0.23 ± 0.02
Cannabis sativa	DAPI	2.68 ± 0.02	−0.24 ± 0.63	0.03 ± 0.01	9.02 ± 0.06	0.16 ± 0.65	0.09 ± 0.04
Control	DAPI	2.69 ± 0.04	0.00 ± 1.46	0.07 ± 0.02	9.01 ± 0.13	0.00 ± 1.45	0.23 ± 0.08
Ginkgo biloba 'Mariken'	DAPI	2.70 ± 0.05	0.60 ± 1.95	0.09 ± 0.03	8.96 ± 0.17	−0.52 ± 1.92	0.31 ± 0.10
Illicium 'NCIH1'	DAPI	2.69 ± 0.06	0.19 ± 2.24	0.12 ± 0.0	9.00 ± 0.20	−0.05 ± 2.17	0.39 ± 0.09
Magnolia virginiana 'Jim Wilson'	DAPI	2.82 ± 0.03	5.02 ± 1.06	0.06 ± 0.01	8.57 ± 0.09	−4.83 ± 0.97	0.18 ± 0.03
Miscanthus sinensis 'Gracillimus'	DAPI	2.76 ± 0.03	2.69 ± 1.03	0.05 ± 0.02	8.77 ± 0.09	−2.68 ± 0.98	0.16 ± 0.05
Pterocarya fraxinifolia	DAPI	2.69 ± 0.02	0.15 ± 0.83	0.04 ± 0.02	8.99 ± 0.07	−0.23 ± 0.81	0.13 ± 0.04
Rosa chinensis 'Mutabilis'	DAPI	2.60 ± 0.03	−3.26 ± 1.11	0.06 ± 0.01	9.31 ± 0.11	3.32 ± 1.18	0.22 ± 0.05
Thuja 'Green Giant'	DAPI	2.72 ± 0.05	1.47 ± 1.78	0.09 ± 0.02	8.88 ± 0.15	−1.40 ± 1.70	0.30 ± 0.07
A. donax	PI	1.95 ± 0.02	−0.69 ± 0.99	0.04 ± 0.01	8.35 ± 0.08	0.69 ± 0.99	0.15 ± 0.04
C. 'Ashton's Supreme'	PI	2.10 ± 0.02	7.15 ± 0.96	0.04 ± 0.01	7.74 ± 0.07	−6.68 ± 0.84	0.14 ± 0.03
C. sativa	PI	1.97 ± 0.01	0.47 ± 0.61	0.02 ± 0.01	8.25 ± 0.05	−0.50 ± 0.60	0.07 ± 0.04
Control	PI	1.96 ± 0.02	0.00 ± 1.00	0.04 ± 0.01	8.29 ± 0.08	0.00 ± 1.00	0.16 ± 0.04
G. biloba 'Mariken'	PI	1.92 ± 0.02	−2.24 ± 1.08	0.04 ± 0.01	8.48 ± 0.09	2.30 ± 1.12	0.19 ± 0.03
I. 'NCHI1'	PI	1.91 ± 0.02	−2.98 ± 0.81	0.03 ± 0.01	8.54 ± 0.07	3.06 ± 0.89	0.12 ± 0.05
M. virginiana 'Jim Wilson'	PI	2.01 ± 0.02	2.47 ± 1.03	0.04 ± 0.01	8.09 ± 0.08	−2.41 ± 0.98	0.17 ± 0.03
M. sinensis 'Gracillimus'	PI	1.95 ± 0.01	−0.78 ± 0.68	0.03 ± 0.01	8.35 ± 0.06	0.76 ± 0.68	0.11 ± 0.03
P. fraxinifolia	PI	2.03 ± 0.02	3.15 ± 0.76	0.03 ± 0.01	8.04 ± 0.06	−3.07 ± 0.73	0.10 ± 0.04
R. chinensis 'Mutabilis'	PI	1.96 ± 0.02	−0.21 ± 0.91	0.03 ± 0.01	8.31 ± 0.07	0.21 ± 0.89	0.13 ± 0.06
T. 'Green Giant'	PI	1.93 ± 0.02	−1.97 ± 0.82	0.03 ± 0.01	8.46 ± 0.07	2.00 ± 0.85	0.14 ± 0.03

[i] Determined with *P. sativum* 'Ctirad' as an internal standard with a genome size of 8.66 pg when using DAPI and 7.75 pg when using PI.
[ii] Determined with *G. max* 'Polanka' as an internal standard with a genome size of 2.79 pg when using DAPI and 2.10 pg when using PI.
[iii] All values are means (n = 6) ± *SEM*.

drove genome size estimates consistently higher (e.g., *M. virginiana* 'Jim Wilson' and *P. fraxinifolia*) or lower (e.g., *A. donax* and *R. chinensis* 'Mutabilis'), regardless of which fluorochrome was used; in other cases, the confounding tissue drove genome size estimates in opposite directions, depending on the fluorochrome (e.g., *C.* 'Ashton's Supreme', *C. sativa*, *G. biloba* 'Mariken', *I.* 'NCIH1', *M. sinensis* 'Gracillimus', and *T.* 'Green Giant'), demonstrating differential effects. The range of error was more pronounced for PI (−2.98% to 7.15%) than for DAPI (−3.26% to 5.02%). Precision, measured as the mean absolute deviation (MAD), was only influenced by the fluorochrome ($P < 0.01$), and it was greater (lower MAD) for PI ($\bar{X} = 0.03$) than for DAPI ($\bar{X} = 0.07$). Precision, as measured by the *SEM*, ranged from 0.02 to 0.06 pg for DAPI and from 0.01 to 0.02 pg for PI, and it was always less than 3% of the mean.

When estimating the genome size for *P. sativum* 'Ctirad' using *G. max* 'Polanka' as an internal standard, there was also a significant interaction between fluorochrome × confounding plant taxon ($P < 0.01$) for both genome size and percent error. Genome size estimates were 8.6% when using DAPI and 10.3% when using PI, depending on the presence of different confounding tissues (Table 3). Accuracy (% error) also varied in magnitude and direction, depending on the confounding tissue that was present, but the direction of error was generally the opposite of that when *P. sativum* 'Ctirad' was the internal standard compared with *G. max* 'Polanka'. Precision (MAD) varied as a function of both fluorochrome ($P < 0.01$) and confounding plant taxon ($P < 0.05$), but with no interaction. Again, precision was higher (lower MAD) for PI ($\bar{X} = 0.13$) than for DAPI ($\bar{X} = 0.23$). Precision, as measured by the *SEM*, ranged 0.06 to 0.20 pg for DAPI and from 0.05 to 0.09 pg for PI, and it was always less than 3% of the mean.

Regardless of the sample/standard combinations, estimates of the genome size were generally higher when using DAPI than PI, but the degree of difference varied as a function of the confounding plant tissue.

Expt. 4: Effects of plant tissue type and fluorochrome on the estimate of the genome size of *Berberis thunbergii* 'NCBT2'. Both tissue type and fluorochrome had significant effects ($P < 0.01$) on the genome size estimation with no interaction. Regardless of tissue type, genome size estimates were higher when using DAPI (main effect mean of 3.32 pg) than when using PI (main effect mean of 2.80 pg), with a difference of 18.6% (Table 4). Regardless of fluorochrome, genome size estimates for different tissue types were all significantly different from one another ($P < 0.01$), with the highest for red leaves (main effect mean of 3.22 pg), followed by that for yellow

Table 4. Genome size (2C) for *Berberis thunbergii* 'NCBT2' as a function of tissue type (unshaded red leaves, shaded yellow leaves, and roots) using either 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) fluorochromes with *Pisum sativum* 'Ctirad' as an internal standard.

Fluorochrome	Tissue type	2C genome sizes [mean ± *SEM* (pg)][i,ii]
DAPI	Red leaves	3.54 ± 0.04[a]
DAPI	Yellow leaves	3.26 ± 0.05[b]
DAPI	Roots	3.16 ± 0.02[b]
PI	Red leaves	2.90 ± 0.04[A]
PI	Yellow leaves	2.86 ± 0.05[A]
PI	Roots	2.65 ± 0.04[B]

[i] Values are means (n = 6) ± *SEM*.
[ii] Values followed by different letters are significantly different ($P \leq 0.05$). Lowercase letters represent comparisons within the DAPI fluorochrome. Uppercase letters represent comparisons within the PI fluorochrome.

leaves (main effect mean of 3.06 pg), and the lowest for roots (main effect mean of 2.91 pg), with a difference of 10.7%. Precision, as measured by the *SEM*, ranged from 0.02 to 0.05 pg for DAPI and from 0.04 to 0.05 pg for PI, and it was always less than 2% of the mean.

Expt. 5: Effects of the internal plant standard and fluorochrome on the estimate of the genome size. There was a significant effect of the internal plant standard, fluorochrome, and interaction between the two when estimating genome size ($P < 0.05$). Estimates of the genome size of *P. sativum* 'Ctirad' varied between the two internal standards (*Secale cereale* 'Dankovske' and *Zea mays* CE-777) by 5.24% when using DAPI and by 5.87% when using PI (Table 5). Estimates of the genome size of *Z. mays* CE-777 did not differ between the two internal standards (*G. max* 'Polanka' and *P. sativum* 'Ctirad'), regardless of fluorochrome.

Accuracy (% error) and precision (MAD) were both significantly affected ($P < 0.01$) by the internal standard, but not by fluorochrome or an interaction between the internal standard and fluorochrome. Estimating the genome size of *P. sativum* 'Ctirad' using *S. cereale* 'Dankovske' as an internal standard resulted in an underestimate of genome size (compared with using human male leukocytes) (Table 1), lower % error, and similar precision (similar MAD) compared with using *Z. mays* CE-777 as an internal standard that overestimated the genome size. When estimating the genome size of *Z. mays* CE-777, using either *G. max* 'Polanka' or *P. sativum* 'Ctirad' as internal standards, both resulted in underestimating genome sizes with similar accuracy and precision. Precision, as measured by the *SEM*, ranged from 0.03 to 0.10 pg for DAPI and from 0.04 to 0.10 pg for PI, and it was always less than 2% of the mean.

Discussion

Expt. 1: Estimating genome sizes of plant reference standards using human male leukocytes as an internal standard and either DAPI or PI fluorochromes. Genome sizes determined using PI were 3.5% to 25.5% lower than those reported previously (Doležel et al. 1992, 1998; Lysák and Doležel 1998). In part, these lower values likely resulted from our use of a revised (12.14% lower) estimate of the human male leukocyte genome size of 6.15 pg compared with 7.0 pg used previously.

The calculated genome sizes of the plant standards measured by DAPI and PI were significantly different (Table 1). For many of the plant standards, the DAPI-determined genome sizes were larger than the PI-determined measurements, except for *Zea mays* CE-777 and *Secale cereale* 'Dankovske', which were lower when using DAPI. Considering the potential for bp bias when using DAPI, the lower values for these grasses when using DAPI could be explained by the lower AT content generally found in monocot genomes compared to that found in dicots (Singh et al. 2016). The larger genome size estimates found with DAPI for the remaining plant standards suggested that DAPI may stain a greater portion of the sample nuclear DNA than that stained by PI. It is noteworthy that the difference in the genome size estimates determined by DAPI and PI varied from 11.7% for *P. sativum* 'Ctirad' to 32.9% for *G. max* 'Polanka', even though these two species have very similar GC contents (37.5% and 35.0%, respectively) (National Center for Biotechnology Information 2023a, 2023b). Considering that humans have a genome AT content of 59.1% and *G. max* has a genome AT content of 65.0% (a difference of 10.0%), we would expect the bp bias of DAPI to overestimate the genome size of *G. max* by a similar percentage, but values for DAPI were 32.9% higher than those for PI for *G. max* 'Polanka', indicating that 22.9% of the difference was caused by factors other than bp composition.

The efficacy of different fluorochromes to penetrate and stain the entire genome is one of the greatest unknowns when using

Table 5. Genome size (2C), accuracy (% error), and precision (mean absolute deviation) for *Pisum sativum* 'Ctirad' and *Zea mays* CE-777 when using different internal standards and either 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) fluorochromes.

Sample	Internal standard[i]	Fluorochrome	2C genome size of sample [mean ± *SEM* (pg)][ii]	Error (%) ± *SEM*	MAD [mean ± *SEM* (pg)]
P. sativum 'Ctirad'	*Secale cereale* 'Dankovske'	DAPI	8.59 ± 0.10[c]	−0.80 ± 0.82[a]	0.14 ± 0.05[a]
P. sativum 'Ctirad'	*Z. mays* CE-777	DAPI	9.04 ± 0.10[d]	4.40 ± 0.83[b]	0.16 ± 0.04[a]
P. sativum 'Ctirad'	*S. cereale* 'Dankovske'	PI	7.67 ± 0.07[a]	−1.01 ± 0.72[a]	0.14 ± 0.03[a]
P. sativum 'Ctirad'	*Z. mays* CE-777	PI	8.12 ± 0.10[b]	4.81 ± 1.23[b]	0.22 ± 0.05[a]
Z. mays CE-777	*Glycine max* 'Polanka'	DAPI	3.88 ± 0.03[A]	−3.45 ± 0.84[A]	0.09 ± 0.01[A]
Z. mays CE-777	*P. sativum* 'Ctirad'	DAPI	3.85 ± 0.03[A]	−4.17 ± 0.75[A]	0.07 ± 0.02[A]
Z. mays CE-777	*G. max* 'Polanka'	PI	4.80 ± 0.04[B]	−4.87 ± 0.59[A]	0.06 ± 0.02[A]
Z. mays CE-777	*P. sativum* 'Ctirad'	PI	4.82 ± 0.07[B]	−4.48 ± 0.12[A]	0.13 ± 0.03[A]

[i] Genome size values for internal standards were taken from Table 1 when using the same fluorochrome.
[ii] Values are means (n = 6) ± *SEM*. Values followed by different letters within a column are significantly different ($P \leq 0.05$). Lowercase letters represent comparisons for *P. sativum* 'Ctirad' samples. Uppercase letters represent comparisons for *Z. mays* CE-777 samples.

flow cytometry to estimate genome sizes. DNA in plant cells can be tightly packaged with DNA–histone complexes forming nucleosomes and chromatin that are further coiled into 30-nm fibers to various degrees, depending on the plant, stage of the cell cycle, and transcriptional state (Annunziato 2008). Different fluorochromes may interact differently with these chromatin structures, and different plant species have vastly different gross organizations of chromatin and ratios of heterochromatin to euchromatin (Nagl 1979, 1982), which can also vary between tissues (Probst and Scheid 2015). Research of mammalian leukemia nuclei showed that using an acid pretreatment to denature histones resulted in more effective DNA staining (Darzynkiewicz and Traganos 1988; Darzynkiewicz et al. 1984). Following acid pretreatment, staining with PI increased 103% to 107%, whereas staining with DAPI increased by 43% to 45%, indicating that DAPI stained a much greater portion of the DNA before acid treatment. Darzynkiewicz et al. (1984) further emphasized that regardless of dye, a large portion of nuclear DNA that is in native chromatin is unstainable, and the portion that is stainable is not representative of the absolute DNA content per cell. Denaturing histones in plant cells can be more difficult than denaturing histones in mammalian cells because of the presence of cytosolic compounds (Ledvinová et al. 2018). It would be highly desirable to develop approaches to quantify and minimize this source of error. In addition to staining efficacy, the lower genome size estimates determined using PI may have resulted from plant metabolites interfering with the fluorescence of PI more than they interfered with DAPI. Because of these issues and unknowns, the assumption that PI is a preferred and more accurate fluorochrome for estimating plant genome size, simply because it is intercalating, is highly questionable.

The use of updated values for genome sizes of these plant reference standards (Table 1) is recommended. Furthermore, using values for plant reference standards determined with the same fluorochrome as those used in practice is recommended.

Expt. 2: Effects of buffers and fluorochrome on the estimate of the genome size. Genome size estimates varied as much as 18.1% as a function of the combination of fluorochrome and extraction buffer (Table 2). This interaction suggests that different buffer components can also interact with fluorochromes or the DNA structure, resulting in significantly different genome size estimates. This variation was greater when using DAPI than PI. Loureiro et al. (2006b) compared four buffers with seven plant species using PI and found that the sample relative fluorescence varied from 6.9% to 29.5% for a given species, depending on the buffer. Wang et al. (2015) found that 7% to 21% of the genome size variation observed during their work with the genus *Primulina* was attributed to buffer effects.

Expt. 3: Effects of confounding plant tissue/metabolites and fluorochrome on the estimate of the genome size. Depending on the combination of confounding plant tissue and fluorochrome, genome size estimates varied by as much as 8.0% to 8.6% when using DAPI and 10% to 10.3% when using PI, indicating that both fluorochromes are susceptible to substantial errors induced by these confounding plant tissues, with PI being more susceptible than DAPI. The significant interaction between fluorochrome × confounding plant taxon indicated that the fluorochromes responded differentially to the various confounding tissues. When using DAPI, the addition of *C.* 'Ashton's Supreme', *R. chinensis* 'Mutabilis', and *M. virginiana* 'Jim Wilson' resulted in genome size errors of 2.8%, 3.3%, and 5.0%, respectively. When using PI, the addition of *I.* 'NCIH1', *P. fraxinifolia*, and *C.* 'Ashton's Supreme' resulted in genome size errors of 3.1%, 3.2%, and 7.2%, respectively. In a similar study that compared mixed and unmixed extracts, including selected combinations of coffee (*Coffea* spp.), rice (*Oryza sativa*), oil palm (*Elaeis guineensis*), cacao (*Theobroma cacao*), yam (*Dioscorea alata*), and petunia (*Petunia hybrida*) with PI fluorochrome, differences in genome sizes of mixed samples varied by as much as 20% (yam/petunia) when compared with those of unmixed samples (Noirot et al. 2000). Noirot et al. (2002) also noted that the fluorescence peak location of petunia varied by 19.1% with different coffee cytosols when using PI, whereas they only varied by 6% when using DAPI.

The confounding plant tissues included in this study were selected because of their taxonomic and biochemical diversity. Based on this study, it is not possible to know which physical or chemical properties of the confounding tissues impacted the fluorochrome binding or fluorescence either directly or indirectly (e.g., pH changes). However, it is known that *Rosa*, *Magnolia*, and the Juglandaceae (e.g., *Pterocarya* spp.) have diverse polyphenolics (Ebrahimzadeh et al. 2009; Thakur and Sidhu 2013; Wang et al. 2023). Noirot et al. (2002) showed that the addition of 0.5% chlorogenic acid (a precursor of polyphenols) decreased petunia PI nuclei fluorescence by 5%. In a study that compared the effects of tannic acid and different buffers on DNA content estimates, Loureiro et al. (2006a) found that the addition of 1 mg/mL of tannic acid reduced the PI fluorescence of pea (*P. sativum*) nuclei by 28.5% when using Galbraith's buffer, but that it increased fluorescence by 8.1% when using LB01 buffer. *Camellia* spp. are known to have both complex phenolics and variable levels of caffeine (Huang et al. 2013; Nagata and Sakai 1984) that can affect the fluorescence of PI by limiting the accessibility of the dye, possibly because of changes to the chromatin structure (Noirot et al. 2003). Other confounding tissues, including *Illicium* spp., that have diverse bioactive metabolites, including prenylated C6–C3 compounds, neolignans, and secoprezizaane-type sesquiterpenes that are found exclusively in *Illicium* (Liu et al. 2009), had minimal effects on genome size estimates. Similarly, *C. sativa*, which contains diverse cannabinoids (Lal et al. 2021), had little effect on estimates of the genome size. Price et al. (2000) found that combining extracted nuclei from independently chopped *Helianthus annuus* and *P. sativum* 'Minerva Maple' at different ratios and then staining with PI resulted in a 17% variation in genome size estimates for *H. annuus*. However, this error was largely negated when tissues were co-chopped. An investigation of fluorescence inhibition by sampling red bracts and green leaves from *Euphorbia pulcherrima* co-chopped with *P. sativum* 'Minerva Maple' as a reference standard found that red bracts had 2.8% to 6.9% lower genome sizes than green leaves, apparently because of anthocyanins (Bennett et al. 2008).

Expt. 4: Effects of the plant tissue type and fluorochrome on the estimate of the genome size of *Berberis thunbergii* 'NCBT2'. The higher values of genome sizes when using DAPI compared with the use of PI were consistent with our other results for dicots. In this case, genome sizes determined with DAPI were, on average, 18.6% higher than the values determined with PI. The variation in genome size estimates among tissue types (10.7%) could have resulted from different pigments and metabolites interacting with the fluorochromes. *B. thunbergii* 'NCBT2' is unusual because leaves that are fully exposed to the sun develop a brilliant red color (probably because of anthocyanins), whereas leaves in the shade maintain a golden yellow color (Fig. 1). Bennett et al. (2008) found that red bract tissue

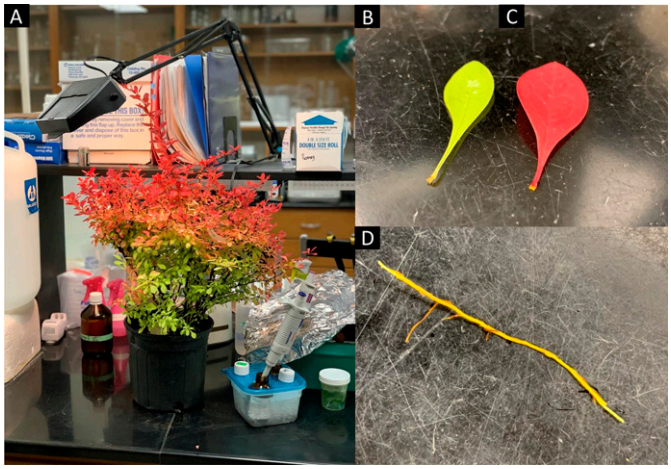

Fig. 1. *Berberis thunbergii* 'NCBT2' Sunjoy Neo® including a container-grown plant (**A**), yellow leaves (**B**), red leaves (**C**), and root tissue (**D**).

from *E. pulcherrima* had as much as 6.9% lower genome size estimates compared with those of green leaves (likely because of the anthocyanin cyanidin-3-rutinoside); however, we found that red leaves had higher estimated genome sizes than yellow leaves or roots. The inner roots of most *Berberis* spp. (and many other Berberidaceae) are bright yellow because of the presence of the alkaloid berberine, which is known to bind to nucleic acids (Díaz et al. 2009). The presence of berberine may have contributed to the lower genome size estimates for the roots.

Expt. 5: Effects of internal plant standards and fluorochrome on the estimate of the genome size. The estimates for genome size, which sometime differ, when using the same fluorochrome but different internal standards indicate that there can be complicated interactions among the samples and internal standards (Table 5). The use of both secondary and primary (in this case, human leucocytes) standards is expected to introduce some potential error. However, these errors vary depending on the specific combination of sample and internal standard, and they differed by as much as 5.9% in this rather limited study of different sample/standard combinations. Doležel et al. (1998) found that the use of *P. sativum* 'Ctirad' as a standard led to consistently higher measurements by as much as 5.4% compared to measurements obtained using *Allium cepa* 'Alice'. During a more extensive study that compared eight different plant reference standards using published genome size estimates for those reference standards and PI fluorochrome, genome size estimates of samples varied by as much as 44.4%, depending on which reference standard was used (Praça-Fontes et al. 2011).

Sources and magnitude of variation/error. The most recent estimate of the genome size of human male leukocytes, used as a primary standard, to estimate genome sizes of secondary plant standards, is now 12.1% lower than the values reported previously (see Materials and Methods). In addition to this error, the selection of the plant reference standard introduced additional variation in genome size estimates of 5.9% (Expt. 5). The use of different extraction buffers resulted in variations in genome size estimates of as much as 18.1% for a given taxa, depending on the buffer/fluorochrome combination (Expt. 2). The addition of different confounding plant tissues resulted in variations in genome size estimates by as much as 10.3%, depending on the tissue/fluorochrome combination (Expt. 3). Different plant tissue types (leaf color/exposure and roots) also resulted in variations in genome size estimates of 10.7%, independent of fluorochrome (Expt. 4). Because the variations caused by different plant tissue types are most likely attributable to variations of secondary metabolites, Expts. 3 and 4 may highlight the same error and are not additive. The choice of fluorochrome (DAPI vs. PI) had one of the greatest impacts on variations in genome sizes and differed by as much as 32.9% for *G. max* 'Polanka' when using human male leukocytes as an internal standard. A portion of this variation (~10.0%) can be attributed to the bp bias of DAPI and variation in AT:CG ratios between the sample and standard, with 22.9% of the variation in genome size estimates resulting from how effectively these fluorochromes stain and report the genome. Excluding variations from the revised genome size estimates for human male leukocytes and potential variations from bp bias, the combined experimental variation/error from different reference standards (5.9%), buffers (18.1%), confounding tissues and metabolites (10.7%), and fluorochromes (22.9%) was 57.6%. During a comparison of four laboratories that all used PI fluorochrome, Doležel et al. (1998) found that estimates of genome size varied by as much as 50% for *G. max* 'Polanka'. In conclusion, because of variations in plant tissues and experimental methods, estimates of absolute plant genome sizes using flow cytometry are only reliably accurate to ±29%, but probably less than that, as this study only evaluated a limited range of selected variables.

Conclusions

The determination of relative plant genome sizes by flow cytometry can be very precise and repeatable (as evidenced by low MADs and *SEM*s) and extremely valuable for determining the relative genome size and ploidy of closely related plants when using consistent methods.

However, the accurate determination of the absolute genome size by flow cytometry remains elusive. Estimates of the genome size using flow cytometry should be considered approximations (±29% or more) and relative to an internal standard under specific experimental conditions as influenced by extraction buffer, fluorochrome, confounding plant tissue/metabolites, tissue type, and other factors that could interact with binding and fluorescence of different fluorochromes. Because of the fundamental unknown of how effectively different fluorochromes stain the entire genome and the differential sensitivity of PI and DAPI to buffers, confounding tissues/metabolites, internal standards, and tissue types, the assumption that PI is a preferred and more accurate fluorochrome for estimating the plant genome size, simply because it is intercalating, is highly questionable. In fact, the evidence presented here suggests that PI may underestimate the plant genome size by as much as 23% compared with DAPI. Because of revisions in the calculated genome size of human leukocytes, which has historically been used as a primary standard to estimate genome sizes of secondary plant standards, many earlier estimates of the plant genome size may be overestimated by ~12.1% and should be recalculated if the best estimate of the absolute genome size is desired. We recommend adopting a revised genome size value of human male leukocytes of 6.150413 pg. Because of the idiosyncrasies and unknown potential for differential genome staining among different fluorochromes, the genome sizes of secondary plant standards should be determined with the same fluorochrome used for measuring unknowns. For example, when using DAPI to estimate the genome size, genome

size estimates for the internal standard should also be determined with DAPI (Table 1). When reporting and comparing plant genome size estimates, it is important to identify the tissue type, buffers, fluorochrome, and internal standard (and associated genome size).

References Cited

Annunziato A. 2008. DNA packaging: Nucleosomes and chromatin. New Educator. 1(1):26.

Arumuganathan K, Slattery JP, Tanksley SD, Earle ED. 1991. Preparation and flow cytometric analysis of metaphase chromosomes of tomato. Theor Appl Genet. 82:101–111. https://doi.org/10.1007/BF00231283.

Bagwell CB, Baker D, Whetstone S, Munson M, Hitchcox S, Ault KA, Lovett EJ. 1989. A simple and rapid method for determining the linearity of a flow cytometer amplification system. Cytometry A. 10:689–694. https://doi.org/10.1002/cyto.990100604.

Bennett MD, Price HJ, Johnston JS. 2008. Anthocyanin inhibits propidium iodide DNA fluorescence in *Euphorbia pulcherrima*: Implications for genome size variation and flow cytometry. Ann Bot. 101(6):777–790. https://doi.org/10.1093/aob/mcm303.

Darzynkiewicz Z, Traganos F, Kapuscinski J, Staiano-Coico L, Melamed MR. 1984. Accessibility of DNA *in situ* to various fluorochromes: Relation to chromatin changes during erythroid differentiation of Friend leukemia cells. Cytometry A. 5:355–363. https://doi.org/10.1002/cyto.990050411.

Darzynkiewicz Z, Traganos F. 1988. Unstainable DNA in cell nuclei. Comparison of ten different fluorochromes. Anal Quant Cytol Histol. 10(6):462–466.

Díaz MS, Freile ML, Gutiérrez MI. 2009. Solvent effect on the UV/Vis absorption and fluorescence spectroscopic properties of berberine. Photochem Photobiol Sci. 8(7):970–974. https://doi.org/10.1039/b822363g.

Dixit R, Gold B. 1986. Inhibition of Nmethyl-N-nitrosourea-induced mutagenicity and DNA methylation by ellagic acid. Proc Natl Acad Sci USA. 83:8039–8043. https://doi.org/10.1073/pnas.83.21.8039.

Doležel J, Bartoš J, Voglmayr H, Greilhuber J. 2003. Letter to the editor. Cytometry A. 51A:127–128.

Doležel J, Binarová P, Lcretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant. 31(2):113–120. https://doi.org/10.1007/BF02907241.

Doležel J, Doleželová M, Novák FJ. 1994. Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). Biol Plant. 36:351–357. https://doi.org/10.1007/BF02920930.

Doležel J, Göhde W. 1995. Sex determination in dioecious plants *Melandrium album* and *M. rubrum* using high-resolution flow cytometry. Cytometry A. 19(2):103–106. https://doi.org/10.1002/cyto.990190203.

Doležel J, Greilhuber J, Lucretti S, Meister A, Lysák MA, Nardi L, Obermayer R. 1998. Plant genome size estimation by flow cytometry: Inter-laboratory comparison. Ann Bot. 82:17–26. https://doi.org/10.1093/oxfordjournals.aob.a010312.

Doležel J, Greilhuber J, Suda J. 2007. Flow cytometry with plants: An overview, p 41–65. In: Doležel J, Greilhuber J, Suda J (eds). Flow cytometry with plant cells: Analysis of genes, chromosomes and genomes. Wiley-VCH Verlag GmbH & Co., Weinheim, Germany. https://doi.org/10.1002/9783527610921.ch3.

Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol Plant. 85(4):625–631. https://doi.org/10.1111/j.1399-3054.1992.tb04764.x.

Doležel J, Greilhuber J. 2010. Nuclear genome size: Are we getting closer? Cytometry A. 77(7):635–642. https://doi.org/10.1002/cyto.a.20915.

Ebrahimzadeh MA, Nabavi SF, Nabavi SM. 2009. Essential oil composition and antioxidant activity of *Pterocarya fraxinifolia.* Pak J Biol Sci. 12(13):957. https://doi.org/10.3923/pjbs.2009.957.963.

Emshwiller E. 2002. Ploidy levels among species in the '*Oxalis tuberosa* Alliance' inferred by flow cytometry. Ann Bot. 89(6):741–753. https://doi.org/10.1093/aob/mcf135.

Fleischmann A, Michael TP, Rivadavia F, Sousa A, Wang W, Temsch EM, Greilhuber J, Müller KF, Heubl G. 2014. Evolution of genome size and chromosome number in the carnivorous plant genus *Genlisea* (Lentibulariaceae), with a new estimate of the minimum genome size in angiosperms. Ann Bot. 114(8):1651–1663. https://doi.org/10.1093/aob/mcu189.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Sci. 220(4601):1049–1051. https://doi.org/10.1126/science.220.4601.1049.

Galbraith D, Loureiro J, Antoniadi I, Bainard J, Bureš P, Cápal P, Castro M, Castro S, Čertner M, Čertnerová D, Chumová Z, Doležel J, Giorgi D, Husband BC, Kolář F, Koutecký P, Kron P, Leitch IJ, Ljung K, Lopes S, Lučanová M, Lucretti S, Ma W, Melzer S, Molnár I, Novák O, Poulton N, Skalický V, Sliwinska E, Šmarda P, Smith TW, Sun G, Talhinhas P, Tárnok A, Temsch EM, Trávníček P, Urfus T. 2021. Best practices in plant cytometry. Cytometry A. 99(4):311. https://doi.org/10.1002/cyto.a.24295.

Greilhuber J. 1988. "Self-tanning"–a new and important source of stoichiometric error in cytophotometric determination of nuclear DNA content in plants. Plant Syst Evol. 158:87–96. https://doi.org/10.1007/BF00936335.

Heller FO. 1973. DNA measurement of *Vicia faba* L. with pulse cytophotometry. Ber Dtsch Bot Ges. 86(5–9):437–441.

Huang H, Tong Y, Zhang QJ, Gao LZ. 2013. Genome size variation among and within *Camellia* species by using flow cytometric analysis. PLoS One. https://doi.org/10.1371/journal.pone.0064981.

Johnston JS, Bennett MD, Rayburn AL, Galbraith DW, Price HJ. 1999. Reference standards for determination of DNA content of plant nuclei. Am J Bot. 86(5):609–613. https://doi.org/10.2307/2656569.

Lal S, Shekher A, Narula AS, Abrahamse H, Gupta SC. 2021. Cannabis and its constituents for cancer: History, biogenesis, chemistry and pharmacological activities. Pharmacological Res. 163:105302. https://doi.org/10.1016/j.phrs.2020.105302.

Ledvinová D, Mikulášek K, Kuchaříková H, Brabencová S, Fojtová M, Zdráhal Z, Lochmanová G. 2018. Filter-aided sample preparation procedure for mass spectrometric analysis of plant histones. Front Plant Sci. 9:1373. https://doi.org/10.3389/fpls.2018.01373.

Liu Y, Su X, Huo C, Zhang X, Shi Q, Gu Y. 2009. Chemical constituents of plants from the genus *Illicium.* Chem Biodivers. 6(7):963–989. https://doi.org/10.1002/cbdv.200700433.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2006a. Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. Ann Bot. 98(3):515–527. https://doi.org/10.1093/aob/mcl140.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2006b. Comparison of four nuclear isolation buffers for plant DNA flow cytometry. Ann Bot. 98(3):679–689. https://doi.org/10.1093/aob/mcl141.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2007a. Two new nuclear isolation buffers for plant DNA flow cytometry: A test with 37 species. Ann Bot. 100(4):875–888. https://doi.org/10.1093/aob/mcm152.

Loureiro J, Suda J, Doležel J, Santos C. 2007b. FLOWER: A plant DNA flow cytometry database, p 423–438. Flow cytometry with plant cells: analysis of genes, chromosomes, and genomes. https://doi.org/10.1002/9783527610921.ch18.

Loureiro J, Kron P, Temsch EM, Koutecký P, Lopes S, Castro M, Castro S. 2021. Isolation of plant nuclei for estimation of nuclear DNA content: Overview and best practices. Cytometry A. 99(4):318–327. https://doi.org/10.1002/cyto.a.24331.

Lysák MA, Doležel J. 1998. Estimation of nuclear DNA content in *Sesleria* (Poaceae). Caryologia. 51(2):123–132. https://doi.org/10.1080/00087114.1998.10589127.

Michaelson MJ, Price HJ, Johnston JS, Ellison JR. 1991. Variation of nuclear DNA content in *Helianthus annuus* (Asteraceae). Am J Bot. 78(9):1238–1243. https://doi.org/10.1002/j.1537-2197.1991.tb11416.x.

Mohr PJ, Taylor BN. 2000. CODATA recommended values of the fundamental physical constants. Rev Mod Phys. 72(2):351. https://doi.org/10.1103/RevModPhys.72.351.

Moreno R, Castro P, Vrána J, Kubaláková M, Cápal P, García V, Gil J, Millán T, Doležel J. 2018. Integration of genetic and cytogenetic maps and identification of sex chromosome in garden asparagus (*Asparagus officinalis* L.). Front Plant Sci. 9:1068. https://doi.org/10.3389/fpls.2018.01068.

Nagata T, Sakai S. 1984. Differences in caffeine, flavanols and amino acids contents in leaves of cultivated species of *Camellia.* Jpn J Breed. 34(4):459–467. https://doi.org/10.1270/jsbbs1951.34.459.

Nagl W. 1979. Condensed interphase chromatin in plant and animal cell nuclei: Fundamental differences. In: Nagl W, Hemleben V, Ehrendorfer F (eds). Genome and chromatin: Organization, evolution, function. Springer, Vienna, Austria. https://doi.org/10.1007/978-3-7091-8556-8_20.

Nagl W. 1982. Condensed chromatin: Species-specificity, tissue-specificity, and cell cycle-specificity as monitored by scanning cytometry, p 171–218. In: Nicolini C (ed). Cell growth. Plenum, New York, NY, USA. https://doi.org/10.1007/978-1-4684-4046-1_9.

National Center for Biotechnology Information. 2023a. *P. sativum* annotation Release 100 - GCF_024323335.1. Bethesda, MD, USA: National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_000219495.3/. [accessed 8 Sep 2023].

National Center for Biotechnology Information. 2023b. *G. max* annotation release 108 - GCF_000004515.6. Bethesda, MD, USA: National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_030864155.1/. [accessed 8 Sep 2023].

National Institute of Standards and Technology. 2023. Technical Note 1297: Appendix D1. Terminology. https://www.nist.gov/pml/nist-technical-note-1297/nist-tn-1297-appendix-d1-terminology#d12.

Noirot M, Barre P, Duperray C, Louarn J, Hamon S. 2003. Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: Consequences on genome size evaluation in coffee tree. Ann Bot. 92(2):259–264. https://doi.org/10.1093/aob/mcg139.

Noirot M, Barre P, Louarn J, Duperray C, Hamon S. 2000. Nucleus–cytosol interactions—a source of stoichiometric error in flow cytometric estimation of nuclear DNA content in plants. Ann Bot. 86(2):309–316. https://doi.org/10.1006/anbo.2000.1187.

Noirot M, Barre P, Louarn J, Duperray C, Hamon S. 2002. Consequences of stoichiometric error on nuclear DNA content evaluation in *Coffea liberica* var. *dewevrei* using DAPI and propidium iodide. Ann Bot. 89:85–89. https://doi.org/10.1093/aob/mcf056.

Nurk S, Koren S, Rhie A, Rautiainen M, Bzikadze AV, Mikheenko A, Vollger MR, Altemose N, Uralsky L, Gershman A, Aganezov S. 2022. The complete sequence of a human genome. Sci. 376(6588):44–53. https://doi.org/10.1126/science.abj6987.

Pellicer J, Fay MF, Leitch IJ. 2010. The largest eukaryotic genome of them all? Bot J Linn Soc. 164(1):10–15. https://doi.org/10.1111/j.1095-8339.2010.01072.x.

Pellicer J, Powell RF, Leitch IJ. 2021. The application of flow cytometry for estimating genome size, ploidy level endopolyploidy, and reproductive modes in plants, p 325–361. Molecular Plant Taxonomy: Methods and Protocols. https://doi.org/10.1007/978-1-0716-0997-2_17.

Piovesan A, Pelleri MC, Antonaros F, Strippoli P, Caracausi M, Vitale L. 2019. On the length, weight and GC content of the human genome. BMC Res Notes. 12(1):1–7. https://doi.org/10.1186/s13104-019-4137-z.

Praça-Fontes MM, Carvalho CR, Clarindo WR, Cruz CD. 2011. Revisiting the DNA C-values of the genome size-standards used in plant flow cytometry to choose the "best primary standards". Plant Cell Rep. 30:1183–1191. https://doi.org/10.1016/j.pbi.2015.05.011.

Price HJ, Hodnett G, Johnston JS. 2000. Sunflower (*Helianthus annuus)* leaves contain compounds that reduce nuclear propidium iodide fluorescence. Ann Bot. 86(5):929–934. https://doi.org/10.1006/anbo.2000.1255.

Price ML, Hagerman AE, Butler LG. 1980. Tannin content of cowpeas, chickpeas, pigeon peas, and mung beans. J Agr Food Chem. 28(2):459–461. https://doi.org/10.1006/anbo.2000.1255.

Probst AV, Scheid OM. 2015. Stress-induced structural changes in plant chromatin. Curr Opin Plant Biol. 27:8–16. https://doi.org/10.1016/j.pbi.2015.05.011.

Sadhu A, Bhadra S, Bandyopadhyay M. 2016. Novel nuclei isolation buffer for flow cytometric genome size estimation of Zingiberaceae: A comparison with common isolation buffers. Ann Bot. 118(6):1057–1070. https://doi.org/10.1093/aob/mcw173.

Sastry SS, Spielmann HP, Hearst JE. 1993. Psoralens and their application to the study of molecular biological processes. Adv. Enzymol. 66:85–148. https://doi.org/10.1002/9780470123126.ch3.

Sharma S, Kaushik S, Raina SN. 2019. Estimation of nuclear DNA content and its variation among Indian Tea accessions by flow cytometry. Physiol Mol Biol Plants. 25:339–346. https://doi.org/10.1007/s12298-018-0587-3.

Singh R, Ming R, Yu Q. 2016. Comparative analysis of GC content variations in plant genomes. Trop Plant Biol. 9:136–149. https://doi.org/10.1007/s12042-016-9165-4.

Šmarda P, Bureš P, Šmerda J, Horová L. 2012. Measurements of genomic GC content in plant genomes with flow cytometry: A test for reliability. New Phytol. 193(2):513–521. https://doi.org/10.1111/j.1469-8137.2011.03942.x.

Šmarda P, Bureš P, Šmerda J, Horová L, Leitch I, Mucina L, Pacini E, Tichý L, Grulich V, Rotreklová O. 2014. Ecological and evolutionary significance of genomic GC content diversity in monocots. Proc Natl Acad Sci USA. 111:E4096–E4102. https://doi.org/10.1073/pnas.1321152111.

Suda J, Leitch IJ. 2010. The quest for suitable reference standards in genome size research. Cytometry A. 77A:717–720. https://doi.org/10.1002/cyto.a.20907.

Temsch EM, Koutecký P, Urfus T, Šmarda P, Doležel J. 2022. Reference standards for flow cytometric estimation of absolute nuclear DNA content in plants. Cytometry A. 101(9):710–724. https://doi.org/10.1002/cyto.a.24495.

Thakur S, Sidhu MC. 2013. Phytochemical screening of leaves and seeds of *Magnolia grandiflora* L. Pharm Lett. 5(4):278–282.

Tiersch TR, Chandler RW, Wachtel SS, Elias S. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry A. 10:706–710. https://doi.org/10.1002/cyto.990100606.

Veselý P, Bureš P, Šmarda P, Pavlick T. 2012. Genome size and DNA base composition of geophytes: The mirror of phenology and ecology? Ann Bot. 109:65–75. https://doi.org/10.1093/aob/mcr267.

Wang J, Liu J, Kang M. 2015. Quantitative testing of the methodology for genome size estimation in plants using flow cytometry: A case study of the *Primulina* genus. Front Plant Sci. 6:354. https://doi.org/10.3389/fpls.2015.00354.

Wang P, Hu M, Wang L, Qu J, Liu Y, Li C, Liu Z, Ma C, Kang W. 2023. Chemical constituents and coagulation effects of the flowers of *Rosa chinensis* Jacq. J Future Foods. 3(2):155–162. https://doi.org/10.1016/j.jfutfo.2022.12.006.