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Research Article

Leaves and Seeds as Materials for Flow Cytometric Estimation of the Genome Size of 11 Rosaceae Woody Species Containing DNA-Staining Inhibitors

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The presence of some secondary metabolites in the cell cytosol can cause a stoichiometric error in the flow cytometric estimation of nuclear DNA content. There is no fully reliable method to completely eliminate the effect of these compounds on nuclei fluorescence, and therefore using plant organs/parts free of staining inhibitors is recommended. Eleven species of Rosaceae with high concentrations of propidium-iodide-staining inhibitors were studied to check the possibility of using seeds instead of leaves for genome size estimation. Despite optimizing the concentration and composition of antioxidants in nuclei-isolation buffer for each species, the effect of cytosolic compounds present in the leaves could not be avoided entirely. None of the seeds of the studied species contained inhibitors, and they produced histograms of good quality. The genome size of the studied species ranged from 1.15 to 3.17 pg/2C; for 10 species the DNA content was estimated for the first time.

1. Introduction

The Rosaceae family is economically very important because many of the species are cultivated for their fruits (e.g., *Malus*, *Pyrus*, *Prunus*, *Fragaria*, and *Rubus*) or have ornamental value (*Rosa*). It has been well studied, although the systematic position and evolution of many taxa are still not clear. Knowledge of the genome size would be helpful in their classification. Within the Rosaceae, polyploidy series from diploid to 12-ploid or higher occur [1, 2]. Since the chromosomes are small and often numerous, ploidy estimation by chromosome counts is difficult. In addition, microscopic chromosome counting is time-consuming and limited to a few tissues. Therefore, flow cytometry (FCM) is a more convenient alternative for establishing the ploidy/genome size of Rosaceae species.

Flow cytometry, a fast and accurate method for the estimation of DNA content, has become the predominant technique for establishing plant genome size [3, 4]. The most common procedure of sample preparation involves chopping plant organs/tissues (mainly leaves) in a nuclei-isolation buffer and measuring the fluorescence of a fluorochrome intercalated into the DNA double helix [5]. The composition of the isolation buffer is critical for accurate FCM measurements. Besides facilitating the isolation of intact nuclei, it should maintain their stability, prevent their aggregation, protect DNA from degradation, and provide an appropriate environment for specific and stoichiometric staining of nuclear DNA [6]. During the last decade, one of the most challenging problems faced by FCM users has been adjusting the buffer composition to the requirements of specific species, especially if they contain staining inhibitors such as phenols, caffeine, and other secondary metabolites in the cytosol of their leaf cells [6–10]. Many species of the Rosaceae family, especially woody plants, belong to this "difficult" group [1, 11, 12]. Special and more time-consuming procedures have been applied to analyze their DNA content by flow cytometry, involving two-step nuclei extraction and a staining protocol, and/or optimizing buffer composition by the addition of antioxidants [1, 2, 13–15].

As shown before [6, 10, 11, 16] and in the present paper, the buffer composition as well as the antioxidant choice and concentration should be optimized individually, which involves additional, often multiple and time-consuming steps before genome size estimation. Omitting such steps can result in inaccurate estimation of the nuclear DNA content. Thus, a simple procedure for sample preparation of these difficult species would speed up analysis of their genome size as well as of the genome composition of allopolyploid/hybrid species.

Despite many studies, there is little information on the mode of action of the staining inhibitors and no universal method to completely avoid their effects on DNA content estimation ([4, 17] and references therein). Inhibition probably involves the intercalation of secondary metabolites into DNA and/or their direct reaction with the dye molecule that interferes with its fluorescence [7, 10]. The addition of antioxidants, such as polyvinylpyrrolidone, β-mercaptoethanol, or dithiothreitol to a buffer, although helpful, does not always guarantee the correct measurement of DNA content, especially if added in too low concentration ([11, 18], this study). Since the composition and concentration of secondary metabolites is different in different species/tissues, even buffers currently developed for "difficult" species; for example, woody plant buffer (WPB: [6]) cannot be considered as suitable for all of them. Moreover, for the leaves or even leaf buds of some species that contain a lot of secondary metabolites or/and mucilage, flow cytometry has yielded largely uninformative results (e.g., *Polystachia*, *Ulmus*, *Betula*, *Thymus, Rhododendron, Drosera, Viburnum* [17, 19, 23], Jedrzejczyk and Sliwinska, unpublished results). Therefore, using a plant organ/part that is free of inhibitors is a better alternative for genome size estimation. For example, for *Betula*, young, winter-dormant twigs have been utilized [19]. Our previous reports showed that leaves can be replaced by seeds for genome size estimation [18, 20, 21]. The mature embryos of orthodox seeds (e.g., seeds that acquire desiccation tolerance during development and may be stored in the dry state) usually contain most of their cells arrested in the G_0/G_1 phase of the cell cycle (for review see [22]) and thus are suitable for establishing the 2C-value. The presence of nuclei of different ploidies (endosperm or endoreduplicated embryo nuclei) in some seeds can be overcome by the isolation and utilization of a part where this has not occurred (usually the radicle).

It is generally assumed that DNA content estimates by flow cytometry become more reliable as histogram quality improves, with CV and debris measures typically regarded as the best measures of quality [3, 6, 23]. Previous reports [18] have shown that DNA content measures may differ between seeds and leaves but we present for the first time an analysis of the relative sample quality of seed and leaf tissue. The Roseaceae was selected for this study to test the idea that seeds may be a good alternative to leaves in difficult plant species.

The aim of the present study was to find if the seeds of the 11 Rosaceae woody species containing staining inhibitors in the leaf cytosol are free of such compounds, to compare the quality of the FCM histograms of the leaf and seed nuclei, and determine the genome size of those two materials to check their suitability. We tried to neutralize the effect of the staining inhibitors present in leaves by addition of antioxidants, in concentration and composition established individually for each species. However, in most cases this did not improve the histogram quality parameters to the level of those of the inhibitor-free tissue of the seeds. The effect of the antioxidants on propidium iodide (PI) fluorescence of the nuclei isolated from inhibitor-free tissue (seeds) was studied also. We suggest that using seeds allows the application of a standard one-step protocol to prepare a nuclear sample, without the necessity for time-consuming optimization of buffer composition and the risk of incomplete suppression of inhibitor activity by antioxidants. To the best of our knowledge, for 10 out of the 11 species this also is the first report on their genome size.

2. Materials and Methods

2.1. Plant Material. Nuclear DNA content was estimated in the leaves and seeds of 11 species of trees and shrubs belonging to the Rosaceae family (Table 1). All the species except *Prunus padus* (Prunoidae; $x = 8$) belong to the Spiraeoideae ($x = 17$) subfamily. Plant material (depending on availability, collected from one to three specimens of each species) originated from the Botanical Garden of the Kazimierz Wielki University in Bydgoszcz, Poland. Each studied specimen is recorded in the garden database. Fruits were collected in October-November 2007 and 2008; seeds were removed, dried, and stored at 4°C until the following summer, to be analyzed at the same time as the leaves. Young, fully developed leaves were collected in August and analyzed within 2-3 days; during this period they were stored in humid filter paper at 4°C. *Petunia hybrida* (P × Pc6; 2.85 pg/2C: [24]) and *Zea mays* (CE-777; 5.43 pg/2C: [25]) were used as internal standards.

The whole seed (including seed coat) of *Amelanchier stolonifera*, *Crataegus coccinea, Malus floribunda, P. padus*, and *Sorbus intermedia*, half seed with the radicle (including seed coat) for all species of *Cotoneaster* genus, and the radicle of *Pyrus elaeagrifolia* were used to prepare flow cytometric samples. For internal standards, a single radicle tip (*Z. mays*) or 30–40 whole seeds (*P. hybrida*) were co-chopped with the target species seed or seed part. For species of the *Cotoneaster* genus, 2 or 3 leaves were included in a sample, and for the other species, and the internal standards, leaf blade fragments of about 0.5–1 cm^2 were included. Single samples contained either nuclei from leaves or from seeds of the target species and the internal standard.

2.2. Flow Cytometry. The test for the presence of PI-staining inhibitors in the leaves and seeds of all species was performed following the protocol of Price et al. [7]. For nuclei isolation, Galbraith's buffer [5], supplemented with PI (50 μg/mL) and ribonuclease A (50 μg/mL), was used. PI fluorescence of two samples was compared: sample 1 that contained nuclei isolated from the leaf/leaves or the radicle tip/seeds of an internal standard (*Z. mays* or *P. hybrida*, depending on the species: Table 2), and sample 2 that contained nuclei

Table 1: Presence of staining inhibitors in leaves and seeds of trees and shrubs belonging to the Rosaceae family. SH: shrub; TR: tree.

Species name		Life habit	Presence of inhibitors	
Latin	Common		Leaves	Seeds
Amelanchier stolonifera Wieg.	Running Serviceberry	SH	+	—
Cotoneaster dammeri "Royal Carpet"	Bearberry Cotoneaster	SH	+	—
Cotoneaster divaricatus Rehd. et Wils.	Spreading Cotoneaster	SH	+	—
Cotoneaster hjelmqvistii Flinck & Hylmö	Hjelmqvist's Cotoneaster	SH	+	—
Cotoneaster horizontalis Decne. "Variegatus"	Rockspray (Herring Bones) Cotoneaster	SH	+	—
Cotoneaster veitchii G. Klotz	Many-flowered Cotoneaster	SH	+	—
Crataegus coccinea L.	Scarlet Hawthorn	TR	+	—
Malus floribunda Sieb. ex Van Houtte	Japanese Crabapple	TR	+	—
Prunus padus L.	Bird Cherry	TR	+	—
Pyrus elaeagrifolia Pall.	Oleaster-leafed Pear	TR	+	—
Sorbus intermedia (Ehrh.) Pers.	Swedish Whitebeam	TR	+	—

Table 2: 2C DNA content of leaves and seeds of trees and shrubs belonging to the Rosaceae family.

Species name	Internal standard*	Buffer**	2C DNA (pg, mean ± SD)	
			Leaves	Seeds
Amelanchier stolonifera	2	B	2.59 ± 0.02 a***	2.55 ± 0.01 b
Cotoneaster dammeri	2	C	1.41 ± 0.01^{ns}	1.41 ± 0.02
Cotoneaster divaricatus	2	C	2.78 ± 0.01 a	2.73 ± 0.02 b
Cotoneaster hjelmqvistii	2	C	2.74 ± 0.03 a	2.70 ± 0.01 b
Cotoneaster horizontalis	2	C	2.77 ± 0.03^{ns}	2.77 ± 0.02
Cotoneaster veitchii	2	C	2.67 ± 0.02 a	2.63 ± 0.02 b
Crataegus coccinea	2	B	3.16 ± 0.02^{ns}	3.17 ± 0.02
Malus floribunda	1	B	1.45 ± 0.01^{ns}	1.44 ± 0.01
Prunus padus	1	B	1.15 ± 0.02^{ns}	1.15 ± 0.02
Pyrus elaeagrifolia	1	A	1.15 ± 0.01^{ns}	1.15 ± 0.02
Sorbus intermedia	2	B	2.82 ± 0.01^{ns}	2.81 ± 0.02

*1: *Petunia hybrid*; 2: *Zea mays*'; **A: Galbraith's + 1% (w/v) PVP-10; B: Galbraith's + 1.5% (w/v) PVP-10; C: Galbraith's + 2% (w/v) PVP-10 + 15 mM β-mercapthoethanol; ***2C-values in leaves and seeds of the certain species (in lines) followed by different letters are significantly different at $P = .05$ (Student's t-test); ns: no significant difference.

released simultaneously from the leaf or seed(s)/seed part of a target species and an internal standard. To prepare a sample, selected plant parts were chopped with a sharp razor blade in a plastic Petri dish with 1 mL of the buffer. The suspension was passed through a 50 μm mesh nylon filter. Samples prepared from leaves were analyzed after about 10 minutes of incubation on ice, and samples prepared from seeds after 20–30 minutes [18]. For each sample, fluorescence in at least 7000 nuclei (across all peaks) was measured using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer, equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatters. Analyses were performed on five replicates. Histograms were analyzed using the FloMax (Partec GmbH, Münster, Germany) software.

For establishing the concentration and combination of antioxidants that stabilize PI fluorescence in leaf samples, a test for inhibitors was repeated using Galbraith's buffer with the addition of 1% (w/v) polyvinylpyrrolidone (PVP-10; buffer A). For samples from species which after application of buffer A still showed decreased fluorescence of the nuclei of the internal standard, the concentration of PVP in the buffer was increased to 1.5% (w/v) (buffer B) and the inhibitor test performed again. For *Cotoneaster* species, which still showed the inhibition effect on PI fluorescence when buffer B was applied, the combination of 2% (w/v) PVP and 15 mM β-mercaptoethanol (buffer C) was applied. A sample was

Table 3: Effect of antioxidants on flow cytometric estimation of 2C DNA content in seeds of selected species belonging to the Rosaceae family.

Species name	2C DNA (pg, mean ± SD)	
	Buffer without antioxidants	Buffer with antioxidants*
Amelanchier stolonifera	2.56 ± 0.01^{ns}	2.55 ± 0.01
Cotoneaster divaricatus	2.74 ± 0.01^{ns}	2.73 ± 0.01
Crataegus coccinea	3.18 ± 0.02^{ns}	3.17 ± 0.01
Malus floribunda	1.45 ± 0.01^{ns}	1.44 ± 0.00
Prunus padus	1.15 ± 0.01^{ns}	1.15 ± 0.01

*For a certain species the buffer as stated in Table 2 was applied; ns: no significant difference between the values in lines (Student's t-test, $P = .05$).

prepared and analyzed as described above. Analyses were performed on five replicates.

An additional test was performed to study the effect of the presence of antioxidant(s) in a buffer on the estimation of the 2C-value in seeds of selected species (Table 3). The nuclei were isolated using Galbraith's buffer without any antioxidant and buffer supplemented with antioxidant/combination of two antioxidants suitable for the certain species, as established in the previous experiment (Table 2). Measurements were performed on five replicates, using the same procedure of sample preparation and analysis as in the previous two experiments. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of the target species/internal standard on the histogram of fluorescence intensities.

For measurements of genome size in the leaves and seeds, selected plant parts of the target species and of the internal standard were chopped simultaneously using buffer A, B, or C (Table 2). Analyses were performed on 10 replicates, using the same procedure as in the previous experiments. In addition to nuclear DNA content, a debris background factor (DF) before the application of signals gating was calculated according to the following equation [10]:

$$\mathrm{DF} = \frac{\text{Total number of particles} - \text{Total number of intact nuclei}}{\text{Total number of particles}} \times 100\%. \quad (1)$$

The results were estimated using a one-way analysis of variance and a Student's t-test ($P = .05$).

3. Results and Discussion

Many species, especially woody and medicinal plants, produce numerous secondary metabolites that interfere with the staining of DNA by intercalating fluorochromes and thus cause a stoichiometric error in DNA content measurements [1, 6, 11, 12, 16, 26, 27]. The addition of antioxidants to the isolation buffer in many cases seems to have no measurable effect on reducing this interference ([7, 18, 27], this paper). Additionally, the quantity of inhibitor(s) is apparently environmentally regulated [28, 29], which makes this method unreliable even if the optimal antioxidant concentration for certain species appears to have been established. Therefore, using tissues that contain such compounds for flow cytometric estimation of DNA content should be avoided. In the present research, a test for the presence of staining inhibitors showed that while the leaves of all the studied species contained compounds that biased the fluorescence of the internal standard nuclei (basing on our experience, the shift of the G_0/G_1 peak of the internal standard by at least three channels on the 512-channel scale was considered as indicative of the presence of staining inhibitors in the cytosol of the cells of the target species), their seeds were free of them (Table 1). Nevertheless, to avoid conducting instrument calibration between analyses of different samples, the same buffers (containing PVP or PVP and β-mercaptoethanol, marked as A, B, or C) were used for both leaves and seeds. This was appropriate, because experiments using seeds of five species showed that there were no statistically significant differences between the 2C-values of their nuclei isolated with and without antioxidants in the buffer (Table 3, Figure 1). This conclusion is important, since in some laboratories buffers containing antioxidants are used for nuclei isolation even if the plant material does not contain secondary metabolites.

The histograms of the nuclei isolated from seeds were of better quality than those from leaves (Figures 1 and 2, Table 4). In some seed nuclei samples, especially in those containing PVP at high concentrations and β-mercaptoethanol in the buffer, an additional population of particles appeared (Figures 1(e) and 1(f), arrows); this was eliminated by gating and did not bias DNA content measurements. The signals probably corresponded to complexes of the PI with the antioxidant(s). For all the species but *P. padus*, the CV of the G_0/G_1 peak of the target species seed nuclei was statistically lower than in leaves (Table 4). Also the background debris level (expressed as DF) in most cases was lower when seeds were used for sample preparation instead of leaves (except *C. dammeri*, *C. divaricatus*, *C. veitchii*, and *P. padus*). In the leaves of some species, without the addition of antioxidants it was hard even to distinguish the peaks, and/or the peaks were shifted due to a decrease in fluorescence (Figure 2(a)). Even after supplementing the isolation buffer with antioxidants, the extent of debris in the leaf samples was obvious when compared to those from seeds; in some samples it was over 80% before gating was applied. The SSC and FSC showed the presence of additional particle populations in almost all samples of leaf nuclei (Figures 2(b) and 2(f), arrows), similar to the "tannic acid effect" [10]. They most probably resulted from aggregates of nuclei or parts of nuclei with unspecific particles and of diverse particles devoid of nuclei (inhibitors, antioxidants) with PI stain. We show that in the studied here species there is an association between the presence of inhibition effects and lower sample quality, even in cases in which DNA content measures do not differ. However, while seed histograms quality was higher than the one of leaf histograms in most species when using our protocols, in some cases it might be

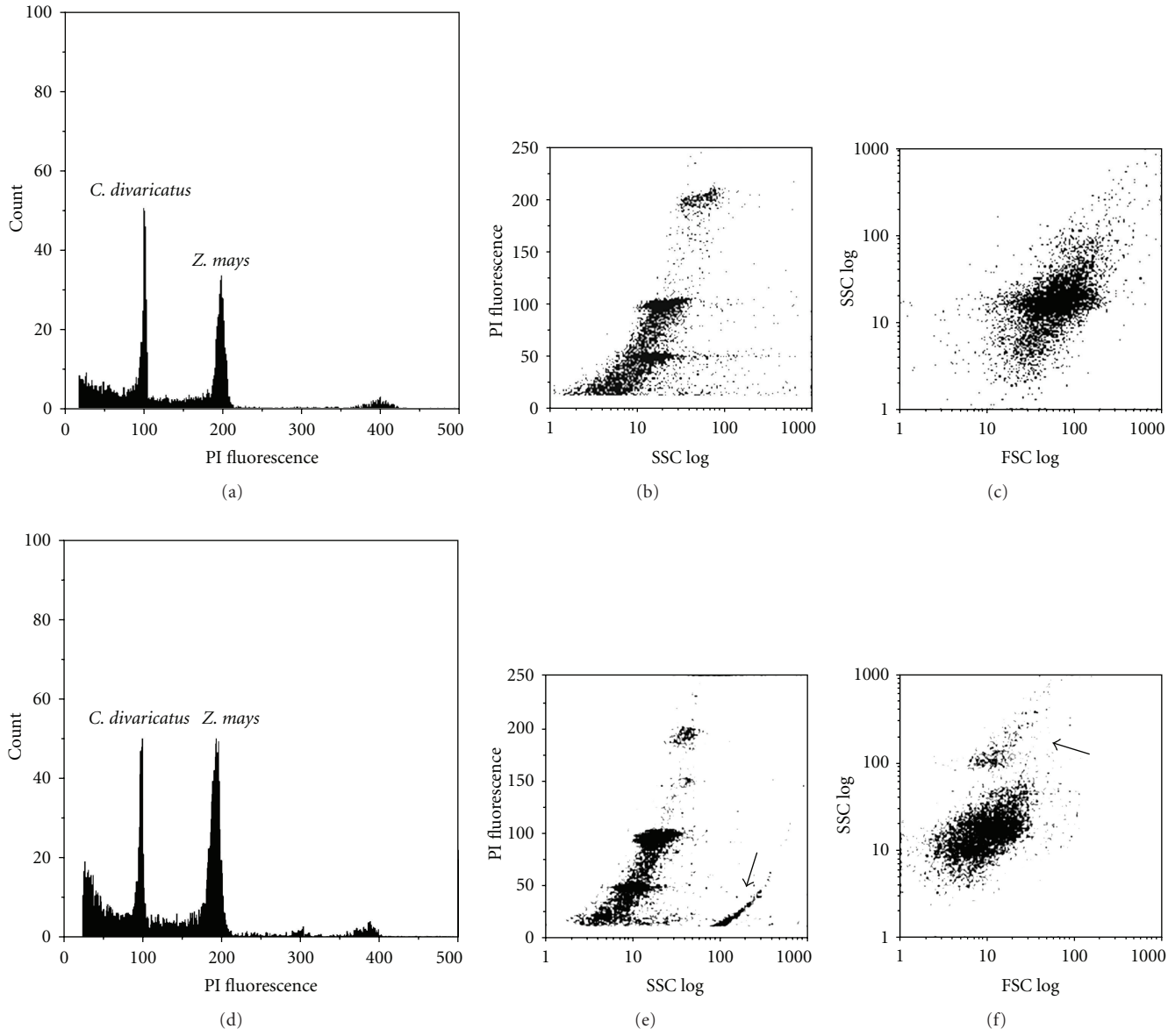

Figure 1: Histograms of PI fluorescence intensity (a, d) and dot plots on side scatter (SSC) versus PI fluorescence (b, e) and forward scatter (FSC) versus SSC (c, f) in the nuclei of seeds of *C. divaricatus* and *Z. mays* (internal standard) isolated using Galbraith's buffer (a–c) and Galbraith's buffer supplemented with 2% (w/v) PVP-10 and 15 mM β-mercaptoethanol. *C. divaricatus* does not possess the 4C nuclei that coincide with the 2C peak of *Z. mays*. Arrows indicate fluorescent particles without nuclei.

possible to achieve improved results from leaves using other refinements, notably other buffers and/or younger leaf tissue.

The 2C-values obtained for the leaves and seeds were not statistically different for seven out of 11 species (Table 2). However, in the leaves of *A. stolonifera* and three *Cotoneaster* species, the 2C-values were higher than in the seeds. A similar tendency was observed previously for *M. coronaria* [30] and *Eucalyptus globulus* [31]. However, in *Helianthus annuus* and *Brassica napus* a higher 2C-value was estimated in the seeds than those in the leaves [18]. Probably the material studied here contained interfering staining inhibitors that they could not be completely eliminated by addition of antioxidants, and thus the overestimated values were due to the reduction of the fluorescence of the internal standard nuclei. Another explanation of the differences between the 2C-values in leaves and seeds could be due to a different chromatin structure in those two organs. Differences in the chromatin condensation were previously observed in different tissues/organs of *Z. mays* [32, 33]. However, in the present experiments it is not likely that the lower estimation of DNA content in some seeds is due to differences in chromatin structure; such differences would be either present in or absent from all species and influence all the measurements, which was not the case. Also, taking into consideration that the DF was similar for both leaves and seeds in two out of four species for which the differences

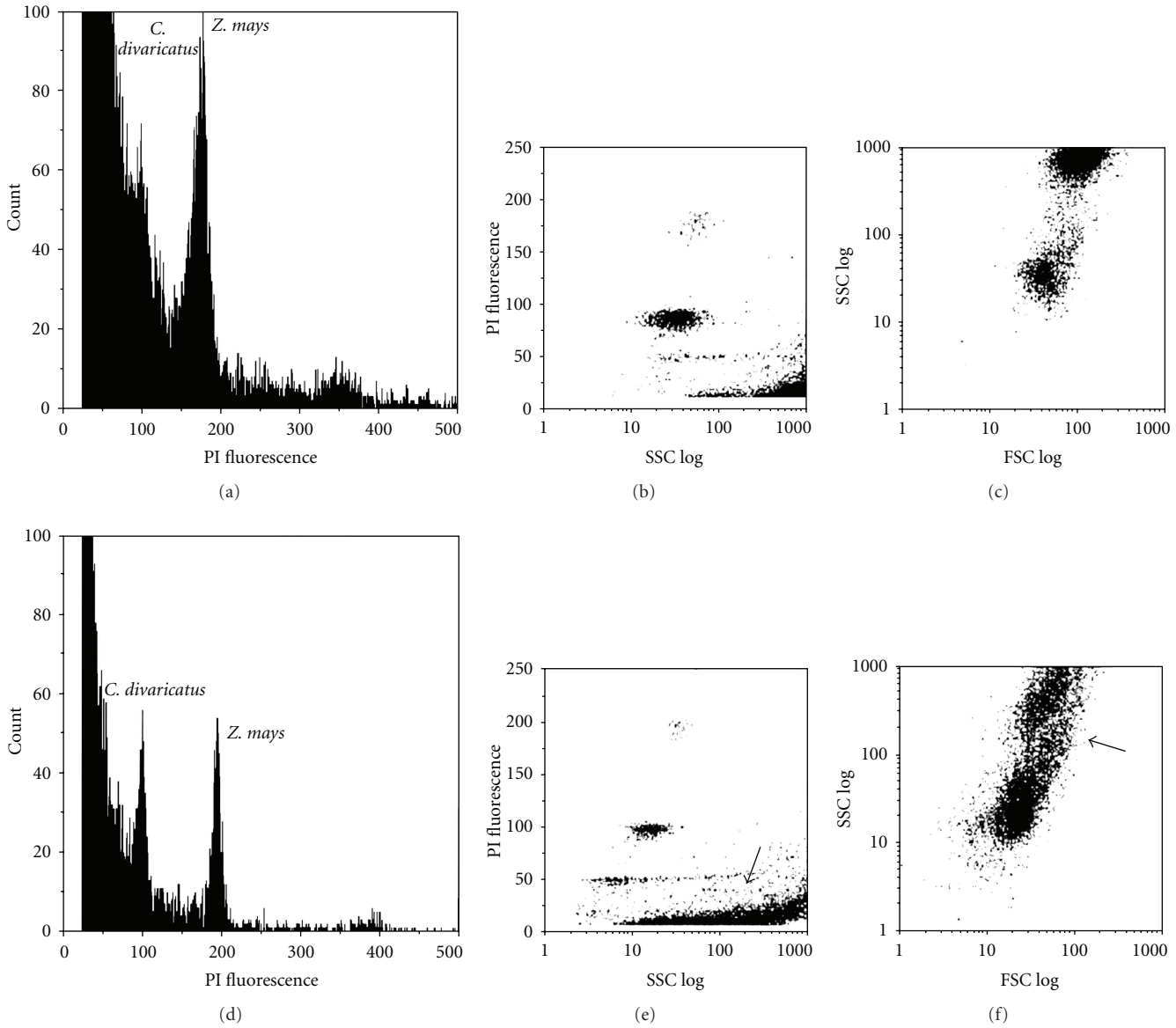

Figure 2: Histograms of PI fluorescence intensity (a, d) and dot plots on side scatter (SSC) versus PI fluorescence (b, e) and forward scatter (FSC) versus SSC (c, f) in nuclei of leaves of *C. divaricatus* and *Z. mays* (internal standard) isolated using Galbraith's buffer (a–c) and Galbraith's buffer supplemented with 2% (w/v) PVP-10 and 15 mM β-mercaptoethanol. *C. divaricatus* does not possess the 4C nuclei that coincide with the 2C peak of *Z. mays*. Arrows indicate additional populations of particles (see text for explanation).

in 2C-values occurred, it does not also seem that these were because of difficulties in measuring peak positions due to too much debris.

The 2C-values in the Rosaceae ranges from 0.20 to 7.30 pg [34]. Out of four subfamilies, a relatively large genome size occurs in the Spiraeoideae, which supports the polyploidy origin of this subfamily [1]. The genome size of the species studied here, most of which belong to Spiraeoideae, range from 1.15 pg/2C to 3.17 pg/2C. Based on the literature [1, 2, 14, 15, 30, 35], which provides 2C-values and/or ploidies for Spiraeoideae species related to those studied here, it can be assumed that they are mostly tetraploid, except for *Cotoneaster dammeri, M. floribunda,* and *P. elaeagrifolia*, which are diploid. Although cross-pollination between individuals with different ploidy levels is possible in the Rosaceae, no differences in ploidy were observed here between leaves and seeds originating from the same tree/shrub. Nevertheless, in seeds of species where there is a polyploidy series it is possible that hybridization between plants of different ploidies has occurred, and that the DNA content in the embryo may not correspond to that of the mother plant. In such cases, the leaves of the parent should be analyzed as a ploidy control.

Only for one species studied here, *M. floribunda* was the genome size previously established. Our values, 1.45 pg/2C in the leaf and 1.44 pg/2C in the seed (Table 2), are very close to

Table 4: Quality of the histograms of nuclei isolated from leaves and seeds of trees and shrubs belonging to the Rosaceae family. CV: coefficient of variation for the G_0/G_1 peak; DF: debris background factor.

Species name	CV of target species (%, mean ± SD)		CV of internal standard (%, mean ± SD)		DF (%, mean ± SD)	
	Leaves	Seeds	Leaves	Seeds	Leaves	Seeds
Amelanchier stolonifera	6.54 ± 0.52 a*	4.43 ± 0.43 b	4.09 ± 0.41 a	4.63 ± 0.31 b	57.8 ± 4.3 a	22.2 ± 3.6 b
Cotoneaster dammeri	5.73 ± 0.68 a	3.83 ± 0.39 b	2.82 ± 0.37^{ns}	3.12 ± 0.30	78.6 ± 8.7^{ns}	67.0 ± 18.5
Cotoneaster divaricatus	4.52 ± 0.21 a	3.35 ± 0.70 b	3.23 ± 0.51^{ns}	3.36 ± 0.45	83.1 ± 14.4^{ns}	78.0 ± 4.9
Cotoneaster hjelmqvistii	4.58 ± 0.54 a	4.04 ± 0.25 b	3.53 ± 0.41 a	3.98 ± 0.31 b	56.2 ± 10.3 a	38.8 ± 3.5 b
Cotoneaster horizontalis	5.66 ± 0.50 a	3.95 ± 0.40 b	4.17 ± 0.56 a	3.64 ± 0.33 b	73.9 ± 4.3 a	40.6 ± 2.8 b
Cotoneaster veitchii	4.52 ± 0.57 a	3.40 ± 0.76 b	3.25 ± 0.52^{ns}	3.51 ± 0.58	75.6 ± 13.2^{ns}	66.1 ± 22.4
Crataegus coccinea	5.68 ± 0.59 a	4.06 ± 0.35 b	4.46 ± 0.48 a	3.42 ± 0.52 b	47.1 ± 8.8 a	31.6 ± 8.5 b
Malus floribunda	5.84 ± 0.74 a	4.45 ± 0.25 b	3.78 ± 0.41^{ns}	3.79 ± 0.41	45.2 ± 6.3 a	30.1 ± 3.1 b
Prunus padus	5.94 ± 0.42^{ns}	5.95 ± 0.45	3.60 ± 0.39^{ns}	3.64 ± 0.40	52.8 ± 10.2^{ns}	53.9 ± 7.8
Pyrus elaeagrifolia	5.86 ± 0.71 a	4.82 ± 0.17 b	3.81 ± 0.36 a	3.36 ± 0.19 b	44.2 ± 6.9 a	25.5 ± 8.8 b
Sorbus intermedia	5.78 ± 0.42 a	4.36 ± 0.46 b	3.92 ± 0.30 a	4.28 ± 0.36 b	53.4 ± 9.26 a	34.2 ± 6.0 b

*Values for leaves and seeds of the certain species and for the certain parameter (in lines) followed by different letters are significantly different at $P = .05$ (Student's t-test); ns: no significant difference.

those reported by Tatum et al. (1.46 pg: [15]). Polyploidy in the *Crataegus* genus has been extensively studied [2, 13, 14]; *C. coccinea* is reported to be tetraploid, and the DNA content for 4*x Crataegus* ranges from 2.74 to 3.34 pg/2C. Estimations obtained here, 3.16 pg (for leaves) and 3.17 pg (for seeds), fall within this range. There are only single estimations of the genome sizes of species of the genera *Amelanchier, Cotoneaster, Pyrus,* and *Sorbus*, but not of those reported here [34]. Thus, our measurements provide 10 new entries to the plant C-value database.

Presently, seeds are not often used for genome size estimation; besides their many advantages they also have some disadvantages. They are convenient because they can be transported and stored dry with no distance or time limit and analyzed at a convenient time. For example, using seeds for genome size estimation has been proposed for desert plants, especially for those that do not develop leaves [20]. However, there can be some confusion in the interpretation of FCM results obtained from seeds due to the presence of cells with a higher than 2C DNA content [21, 22]. Nevertheless, if the whole seed is not suitable for use, it is usually possible to isolate a tissue or region in which most of the cells are arrested in the G_0/G_1 phase of the cell cycle (possessing 2C DNA). This may require a knowledge of seed biology and/or additional experiments to find the most suitable tissue for a particular seed type/species. If dissection of the radicle is needed, sample preparation involves additional time and precision; for small seeds, the use of a microscope may be required. Seeds are usually produced in abundance, although they may be rare or absent in some populations and years, and thus not available for DNA content measurement. Also, they have to be used with caution because in some families hybridization between species may occur, the Rosaceae being an example [30].

Within the Rosaceae family, seeds of *Cotoneaster* have been used previously for flow cytometric DNA content measurement for ploidy estimation, using external standardization and a two-step procedure, involving centrifugation [13, 14]. As shown here, this complex procedure can be successfully replaced by a simple and reliable one-step standard protocol, using the whole seed, the dissected radicle, or part of the seed containing the radicle. Such material is suitable for analysis without any other special pretreatment for sample preparation.

Our results demonstrate that the use of seeds may greatly simplify protocols in cases in which difficult leaf tissue requires special procedures. However, the simplification of protocols is one consideration among many. The final choice of tissue will depend on a variety of factors, including tissue availability, storage and transport concerns, the ease with which paternal DNA content can be inferred from that of progeny, requirements for sampling nonreproductive individuals, and the relative difficulty of seed and leaf preparation, both of which may vary considerably.

In conclusion, in the Rosaceae species containing staining inhibitors in the leaf cytosol, their seeds, which are free of such compounds, are a suitable alternative material for flow cytometric estimation of DNA content. The presence of staining inhibitors should be tested for each species, especially woody ones, regardless of the plant material used for measurement, and the composition of the nuclei-isolation buffer should be optimized for individual tissues/species. However, even after the addition of antioxidants, a stoichiometric error in the flow cytometric estimation of DNA content in "difficult" species can occur, and thus using plant parts that contain compounds which affect nuclei fluorescence should be avoided.

Abbreviations

FCM: Flow cytometry
PI: Propidium iodide
PVP: Polyvinylpyrrolidone
SSC: Side scatter
FSC: Forward scatter
DF: Debris background factor.

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