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Flow cytometric investigations on *Pelargonium* × *crispum*: an estimation of nuclear DNA contents with two different internal standards

Durchflusszytometrische Untersuchungen bei *Pelargonium* × *crispum*: die Bestimmung von Gehalten der Kern-DNA mit zwei unterschiedlichen internen Standards

Summary

Seven *Pelargonium* × *crispum* cultivars, four breeding clones and the species *P. crispum* were analysed by flow cytometry. Tomato 'Stupické' (2C value = 1.96 pg) and cauliflower 'Korso' (2C value = 1.31 pg) were used as internal standards to estimate the DNA content of the samples. As expected, the 2C values of the investigated diploid and tetraploid genotypes discriminated significantly. Overall, the mean 2C values ranged from 0.97 to 2.18 pg DNA and formed three significantly different groups. The estimation of the DNA content of the cultivars and breeding clones was independent of the standard used with one exception; for *P. crispum* the estimated DNA contents differed significantly. The standards 'Stupické' and 'Korso' are equally appropriate for flow cytometric investigation of genotypes with 2C values < 1.8 pg like diploid genotypes of the section *Pelargonium*. Tomato 'Stupické' is a rather impractical standard for genotypes with 2C values of about 2 pg, due to the overlapping positions of the 2C values of sample and standard.

Key words: 1Cx value, 2C value, chromosome, cytotype, ploidy level

Zusammenfassung

Sieben *Pelargonium* × *crispum* Sorten, vier Zuchtklone und die Art *P. crispum* wurden mit der Durchflusszytometrie untersucht. Für die Bestimmung der DNA-Gehalte wurden die zwei internen Standards Tomate 'Stupické' (2C-Wert = 1.96 pg) und Blumenkohl 'Korso' (2C-Wert = 1.31 pg) verwendet. Wie erwartet, unterschieden sich die 2C-Werte der untersuchten diploiden Genotypen signifikant von denen der tetraploiden. Insgesamt variierten die 2C-Werte zwischen 0.97 und 2.18 pg DNA und bildeten drei signifikant unterschiedliche Gruppen. Die ermittelten DNA-Gehalte waren unabhängig vom verwendeten Standard mit einer Ausnahme: der DNA-Gehalt der Art *P. crispum* gemessen mit 'Korso' unterschied sich signifikant vom DNA-Gehalt, der mit 'Stupické' bestimmt wurde. Die Standards 'Stupické' und 'Korso' sind für durchflusszytometrische Untersuchungen von Genotypen mit 2C-Werten < 1.8 pg wie Diploide der Sektion *Pelargonium* gleichermaßen geeignet. Jedoch ist die Tomate 'Stupické' ein ungeeigneter Standard für Genotypen mit 2C-Werten von ca. 2 pg, weil sich die Positionen der 2C-Werte der Probe und des Standards überlappen.

Stichwörter: 1Cx-Wert, 2C-Wert, Chromosom, Ploidiestufe, Zytotyp

Introduction

Numerous karyological and cytological studies were conducted in the genus *Pelargonium* L'Hér. *Pelargonium* species have a great variation in basic chromosome numbers

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(x = 4, 8, 9, 10, 11, 17, 19) (Gibby and Westfold, 1983, 1986; van der Walt, 1985; Albers, 1988; Gibby et al., 1990, 1996). Ploidy levels range from diploid over tetra- and hexaploid up to octoploid (Gibby and Westfold, 1983, 1986; Albers and van der Walt, 1984; van der Walt, 1985; Gibby et al., 1990). Some species comprise cytotypes differing in ploidy (Gibby et al., 1990, 1996; Maggs et al., 1999). Moreover, species are divided into two groups according to the chromosome lengths: species with small (< 1.5 µm) or large chromosomes (> 1.5 µm) (Albers and van der Walt, 1984; Gibby and Westfold, 1986; Albers, 1988; Bakker et al., 2004; Weng et al., 2012).

Previous breeding research has shown that in *Pelargonium*, only intra- and intersubgeneric crossings between genotypes with the same basic chromosome number and same ploidy level give a viable fertile progeny (Horn, 1994; Plaschil et al., 2017). Consequently, it is crucial for successful breeding to know the basic chromosome number and the ploidy of the genotypes.

In contrast to the time-consuming counting of chromosomes, flow cytometry enables to investigate a large number of genotypes in a short time (Doležel et al., 2007; Leus et al., 2009). A comparison of unknown with known karyotypes makes the determination of the ploidy level possible. The usage of internal standards allows calculating the absolute value of nucleus DNA of the investigated genotypes from the flow cytometric histograms (Doležel et al., 2007). Therefore, flow cytometry is an appropriate tool for rapid estimation of the genome size, identification of ploidy levels, hybridisation, unreduced gametes, or somaclonal variation after in vitro culture (Leus et al., 2009; Sliwinska, 2018).

Until now, only few data about the genome size of *Pelargonium* species, gained by Feulgen cytophotometry (Greilhuber, 1988) and flow cytometric investigations (Weng et al., 2012; Nieuwenhuis, 2013; Leitch et al., 2019), were published. These studies used different internal standards like *Vinca minor* L. (Nieuwenhuis, 2013), *Arabidopsis thaliana* (L.) Heynh., and erythrocytes of *Oncorhynchus mykiss* Walbaum (Weng et al., 2012). The estimated DNA content (2C) ranged from 0.64 pg (*P. australe* Willd.) (Weng et al., 2012) to 16.2 pg (*P. radula*, syn. for *Pelargonium radens* H.E. Moore) (Greilhuber, 1988). Nieuwenhuis (2013) inspected the raw data of Weng et al. (2012) and noticed, that "the measurements made using *A. thaliana* as a control yielded 2C-value estimates roughly half as high as those made with trout erythrocytes as the control". The published averaged 2C values from both measurements (Weng et al., 2012) resulted in an underestimation of genome sizes (Nieuwenhuis, 2013) and are therefore ineligible for comparisons. In addition, *A. thaliana* seems to be an unsuitable internal standard for flow cytometry, because of its systemic endopolyploidy (Galbraith et al., 1991) and possibly difficult classification of the correct peaks in the histograms.

Based on these facts, the aim of the present experiment was to standardise the flow cytometry of *Pelargonium* and to identify appropriate internal standards in order to screen the very comprehensive *Pelargonium* gene pool of the Julius Kühn-Institut (JKI) consisting of wild species, cultivars, and experimental interspecific hybrids. For this purpose, a limited range of diploid and tetraploid *Pelargonium* × *crispum* plants was used as model plants for elaboration of a basic measurement report.

Materials and methods

Plant materials

Seven *P.* × *crispum* cultivars (Angel pelargoniums or Angels), four breeding clones and the species *P. crispum* were examined by flow cytometry. Their full and short names as well as the ploidy level are given in Table 1. All genotypes were maintained as a clone with at

Table 1. *Pelargonium* genotypes used for flow cytometry
Für die Durchflusszytometrie verwendete Pelargonium-*Genotypen*

Genotype	Short name	Ploidy level
P. crispum (P.J. Berg.) L'Hér.	*P. crispum*	2x
P. × *crispum* Mosquitaway™ 'Eva'	'Eva'	2x
P. × *crispum* Piccola™ 'Harlekin'	'Harlekin'	2x
P. × *crispum* Piccola™ 'Lavender Picotee'	'Lavender Picotee'	2x
P. × *crispum* Piccola™ 'Merlot'	'Merlot'	2x
P. × *crispum* Piccola™ 'Pink Picotee'	'Pink Picotee'	2x
P. × *crispum* Piccola™ 'Soft Pink'	'Soft Pink'	2x
P. × *crispum* pac® Angeleyes® 'Randy'	'Randy'	2x
Clone P 659	P 659	2x
Clone P 660	P 660	2x
Clone P 639	P 639	4x
Clone ZV 135	ZV 135	4x

least three plants and were cultured under greenhouse conditions.

The internal standards tomato (*Solanum lycopersicum* L.) 'Stupické' (Doležel et al., 1992) and cauliflower (*Brassica oleracea* L. subsp. *capitata* convar. *botrytis* var. *botrytis* L.) 'Korso' (JKI working collection) were cultivated in vitro on solid medium MS (Murashige and Skoog, 1962) supplemented with 0.2 mg l^{-1} 1-naphthalene acetic acid at 25 °C and 16 h light exposure and 8 h darkness.

Flow cytometric and statistical analysis

Three to four biological replications of each genotype were analysed with one or both internal standards (Table 2). *Pelargonium crispum*, the diploid cultivar 'Randy' and the tetraploid clone ZV 135 (Plaschil et al., 2015) were chosen for comparisons. Tomato 'Stupické' with its 2C value of 1.96 pg (Doležel et al., 1992) and cauliflower 'Korso' were used as internal standards to estimate the DNA content of the investigated samples. As preliminary work, the DNA content of 'Korso' was estimated using tomato 'Stupické' as internal standard.

The samples were prepared with the CyStain® PI Absolute P Kit (Sysmex), which consists of the nuclei extraction buffer and the staining solution buffer. To 20 ml staining buffer 1.5 ml propidium iodide (1 mg ml^{-1}, Sigma) and 60 µl ribonuclease A (1 mg ml^{-1}, Serva) were added (enough for 20 samples). In few cases, Galbraith buffer (Galbraith et al., 1983) was used instead of the Sysmex® Kit. In a 3 cm Petri dish with 500 µl of nuclei extraction buffer, pieces of petals or, more rarely, leaves of the *Pelargonium* samples were chopped together with a small amount of leaf material of the internal standard. Then 1 ml staining solution was added. This suspension was gently shaken by hand and poured through a Cell-Strainer Cap in a 5 ml Polystyrene Round Bottom Tube (BD Falcon). The measurement was carried out with the flow cytometer BD FACSCalibur (BD Biosciences). Using the analysis software BD CellQuest Pro (version 5.2.1, BD Biosciences), the 2C peaks were manually determined and the nuclear DNA contents (2C value) were calculated as following (Doležel et al., 2007):

$$\text{Sample 2C value (DNA pg)} = \text{Reference 2C value (pg)} \times \frac{\text{sample 2C mean peak position}}{\text{reference 2C mean peak position}}$$

Table 2. Averaged 2C and 1Cx values of the analysed *Pelargonium* genotypes depending on the used internal standard
Gemittelte 2C- und 1Cx-Werte der analysierten Pelargonium*-Genotypen in Abhängigkeit der verwendeten internen Standards*

Genotype	Standard	n	2C value (pg)[1]	SD (±)	1Cx value (pg)[1]	SD (±)
P. crispum	'Korso'	3	1.03 [a]	0.01	0.52 [a]	0.00
	'Stupické'	3	1.10 [b]	0.02	0.55 [b]	0.01
'Eva'	'Korso'	3	1.02 [a]	0.01	0.51 [a]	0.00
	'Stupické'	3	0.98 [a]	0.01	0.49 [a]	0.01
'Harlekin'	'Korso'	3	1.02 [a]	0.01	0.51 [a]	0.00
	'Stupické'	4	1.03 [a]	0.02	0.52 [a]	0.01
'Lavender Picotee'	'Korso'	3	1.00 [a]	0.01	0.50 [a]	0.00
	'Stupické'	3	0.99 [a]	0.01	0.49 [a]	0.00
'Merlot'	'Korso'	3	1.03 [a]	0.00	0.52 [a]	0.00
	'Stupické'	3	0.98 [a]	0.07	0.49 [a]	0.04
'Pink Picotee'	'Korso'	3	1.00 [a]	0.01	0.50 [a]	0.00
	'Stupické'	3	0.99 [a]	0.00	0.50 [a]	0.00
'Soft Pink'	'Korso'	4	1.01 [a]	0.01	0.51 [a]	0.00
	'Stupické'	3	1.01 [a]	0.01	0.50 [a]	0.00
'Randy'	'Korso'	3	1.01 [a]	0.01	0.50 [a]	0.01
	'Stupické'	4	1.02 [a]	0.02	0.51 [a]	0.01
P 659	'Korso'	3	1.00 [a]	0.02	0.50 [a]	0.01
	'Stupické'	3	0.97 [a]	0.02	0.49 [a]	0.01
P 660	'Korso'	3	1.03 [a]	0.02	0.52 [a]	0.01
	'Stupické'	3	1.01 [a]	0.02	0.50 [a]	0.01
P 639	'Korso'	3	2.18 [c]	0.02	0.54 [b]	0.00
ZV 135	'Korso'	4	2.15 [c]	0.03	0.54 [b]	0.01

n = number of biological replications analysed per genotype; [1]different letters indicate significant differences, Tukey's b test, α = 5%
n = Anzahl biologischer Wiederholungen per Genotyp; [1]*unterschiedliche Buchstaben zeigen signifikante Unterschiede, Tukey-B-Test,* α = 5%

In addition, the 1Cx values (basic genome size) were estimated by dividing the 2C value by ploidy (Leitch and Bennett, 2004; Greilhuber et al., 2005). All data were analysed with the statistical software Systat 13 (Germany) using the Tukey's b test, α = 5%.

Results

For the preparation of suspensions of intact nuclei we tested Galbraith (Galbraith et al., 1983) and Sysmex® lysis buffer. After staining with propidium iodide the quality of histogram peaks was obviously better with Sysmex® buffer (data not shown).

Seventeen biological replications of 'Korso' were analysed using 'Stupické' as internal standard. The estimated 2C values ranged from 1.27–1.35 pg DNA with a standard deviation (SD) of ± 0.03. The mean of the 2C value was 1.31 pg DNA and served as internal standard for the estimation of the *Pelargonium* samples.

All *Pelargonium* genotypes were analysed with the internal standard 'Korso' at least three times. In order to examine the influence of the internal standard on the measurement result, another standard tomato was tested. Therefore, eleven genotypes were additionally analysed with the internal standard 'Stupické'. The estimated 2C values were averaged per genotype and each standard (Table 2) and per genotype with both standards (Fig. 1), respectively. Since the ploidy was known, the 1Cx values were calculated (Table 2, Fig. 2). Significant differences in 2C DNA content and 1Cx values of the various *Pelargonium* genotypes were detected. As examples of the flow cytometric measurements, Figure 3 and 4 show the histograms of 'Pink Picotee' with the respective internal standard.

Except for *P. crispum*, the use of different standards had no influence on the results and no significant differences have been observed for both internal standards, 'Korso' and 'Stupické'. Overall, the mean 2C values ranged from 0.97 (P659 + 'Stupické') to 2.18 pg DNA (P639 + 'Korso') and formed three groups. The first group (0.97–1.03 pg) contains *P. crispum* + 'Korso' and following genotypes with both standards: the cultivars 'Eva', 'Harlekin', 'Lavender Picotee', 'Merlot', 'Pink Picotee', 'Soft Pink', 'Randy', and the clones P 660 and P 659. The second group includes *P. crispum* + 'Stupické' (1.10 pg) only, while the last group consists of the clones ZV 135 and P 639 (2.15 and 2.18 pg) (Table 2). Comparing the mean 2C values averaged over both standards, the genotypes split into three groups, because *P. crispum* was significantly different to each other diploid and tetraploid genotype (Fig. 1).

The mean 1Cx values, averaged over both standards are shown in Figure 2. The values ranged from 0.49 pg (P 659) to 0.54 pg (P 639, ZV 139). Regarding the 1Cx values two significantly different classes were revealed. The first class encompasses all diploid varieties and clones, whereas the species *P. crispum* as well as the tetraploid clones P 639 and ZV 135 belong to the second class.

Discussion

Flow cytometric measurements could be a powerful amendment for genotype characterization of cultivars and wild species of the highly variable genus *Pelargonium*. For this purpose, we introduce here two suitable internal standards with the aim flow cytometric measurement to make reliable for a broad *Pelargonium* gene pool.

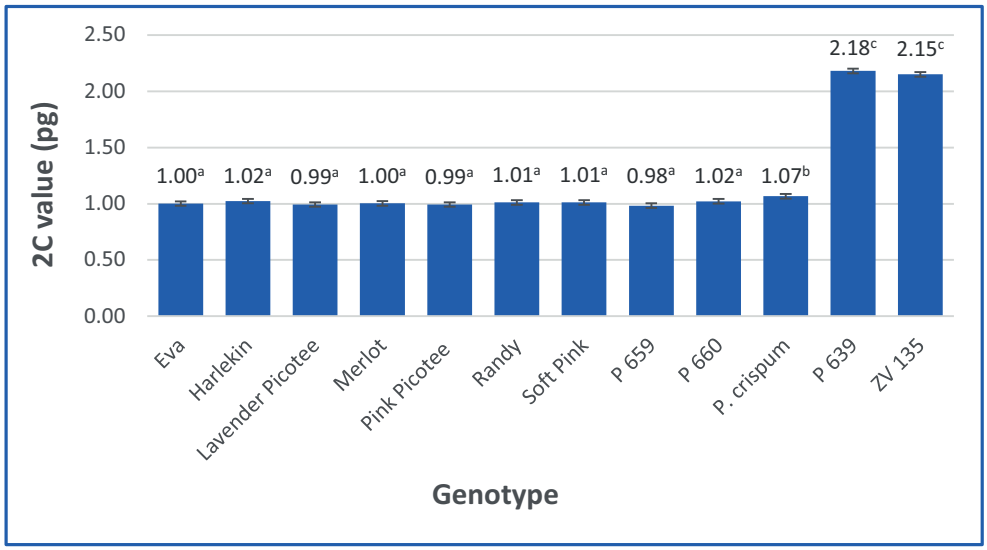

Fig. 1. Mean 2C values of the analysed *Pelargonium* genotypes averaged over both internal standards. Different letters indicate significant differences, Tukey's b test, α = 5%.
Mittelwerte der 2C-Werte der analysierten Pelargonium*-Genotypen, gemittelt über beide interne Standards. Unterschiedliche Buchstaben zeigen signifikante Unterschiede, Tukey-B-Test, α = 5%.*

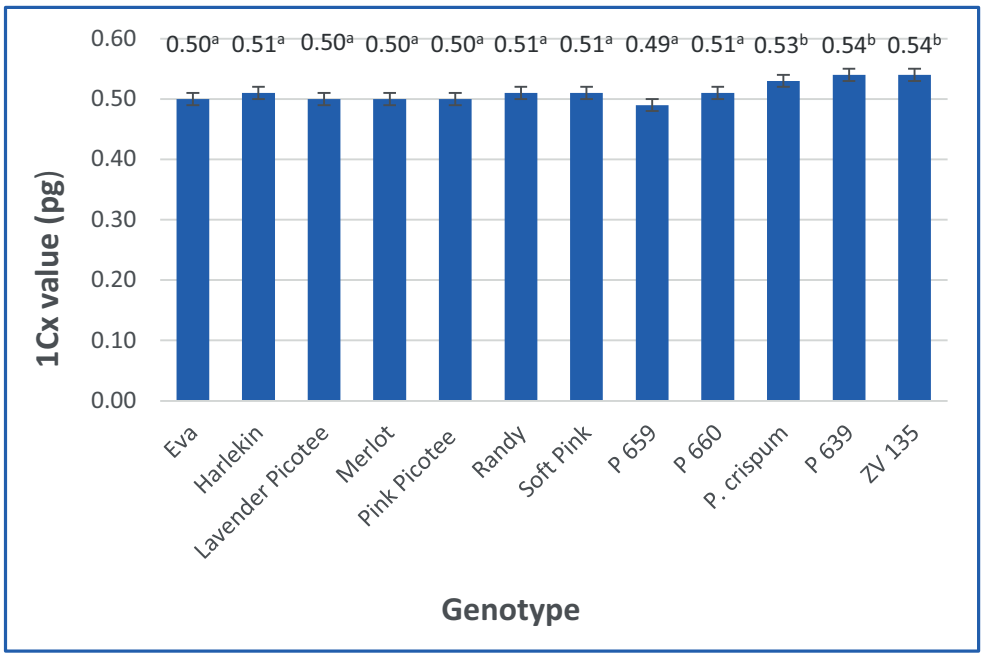

Fig. 2. Mean 1Cx values of the analysed *Pelargonium* genotypes averaged over both internal standards. Different letters indicate significant differences, Tukey's b test, α = 5%.
Mittelwerte der 1Cx-Werte der analysierten Pelargonium*-Genotypen, gemittelt über beide interne Standards. Unterschiedliche Buchstaben zeigen signifikante Unterschiede, Tukey-B-Test, α = 5%.*

Cauliflower is an easy accessible plant material. Our measurements showed that the 2C value estimation fits well to previous measurements of Arumuganathan and Earle (1991). They calculated the 2C value of four cauliflower cultivars with 1.30–1.37 pg using flow cytometry and nuclei from chicken red blood cells (2C = 2.33 pg) as internal standard. The present result of a mean 2C value = 1.31 pg for 'Korso' agrees with this analysis.

Comparing both internal standards of the current study, significantly different 2C values were only estimated for the genotype *P. crispum*. The use of 'Stupické' as internal standard lead to a higher 2C value (1.10 pg) than 'Korso' (1.03 pg) in *P. crispum*. These results may be traced back to secondary metabolites (Doležel and Bartoš, 2005, Doležel et al., 2007, Pellicer and Leitch, 2014) or a different age of the used plant tissue (Doležel et al., 2007). As expected, the mean 2C DNA content of the investigated diploid and tetraploid genotypes discriminated significantly with both internal standards.

It could be concluded, that both standards, 'Stupické' and 'Korso', are equally appropriate for flow cytometric investigation of genotypes with 2C values < 1.8 pg like diploid genotypes of the section *Pelargonium*. 'Stupické' is an unsuitable standard for genotypes with 2C values about 2 pg, because of the overlaid 2C peak positions. In our case, 'Korso' was reliable for the discrimination of diploid and tetraploid *P.* × *crispum* genotypes. *Pelargonium* genotypes with higher DNA contents need probably other internal standards like *Lactuca sativa* L. (2C = 6.61 pg, Barow and Meister, 2003) or *Pisum sativum* L. (2C = 9.09 pg, Doležel et al., 2007), because an ideal internal standard should have a genome size close to the target species (Doležel and Bartoš, 2005). As an example, Nieuwenhuis (2013) pointed out the negative influence on the results using an internal standard, like *A. thaliana*, with a genome size far from the target *Pelargonium* species.

Mean 1Cx values of the diploid *P.* × *crispum* do not show significant differences confirming the very narrow gene pool of Angel pelargoniums (Brawner, 2003; Plaschil et al., 2012, 2015). Spontaneous or induced autopolyploidisation like in clone P639 and ZV 135, respectively, resulted in genome upsizing in comparison to the diploid cultivars. However, there is no genome upsizing relative to the species *P. crispum*. In contrast, a 1Cx value downsizing was often reported after polyploidisation (Leitch and Bennett, 2004; Zenil-Ferguson et al., 2016). Nieuwenhuis (2013) described for the genus *Pelargonium* a decrease of 1Cx values with increasing ploidy levels during evolution. Maybe our result is traced back to the unknown origin of hybridisation of the clones and the short span after polyploidisation (Plaschil et al., 2012, 2015).

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Conflicts of interest

The authors declare no conflicts of interest.

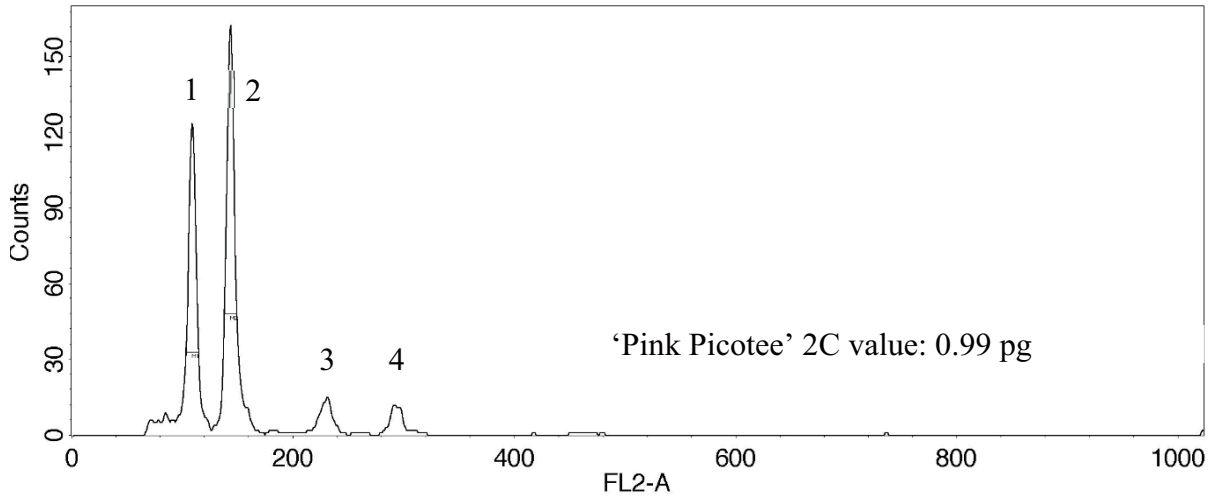

Fig. 3. DNA histogram shows two 2C peaks and two 4C peaks, which correspond to the nucleus DNA. 1: 2C peak sample 'Pink Picotee', 2: 2C peak internal standard cauliflower 'Korso', 3: 4C peak 'Pink Picotee', and 4: 4C peak 'Korso'.
Das DNA-Histogramm zeigt zwei 2C-Peaks und zwei 4C-Peaks, die mit der Kern-DNA korrespondieren. 1: 2C-Peak der Probe 'Pink Picotee', 2: 2C-Peak des internen Standards Blumenkohl 'Korso', 3: 4C-Peak von 'Pink Picotee' und 4: 4C-Peak von 'Korso'.

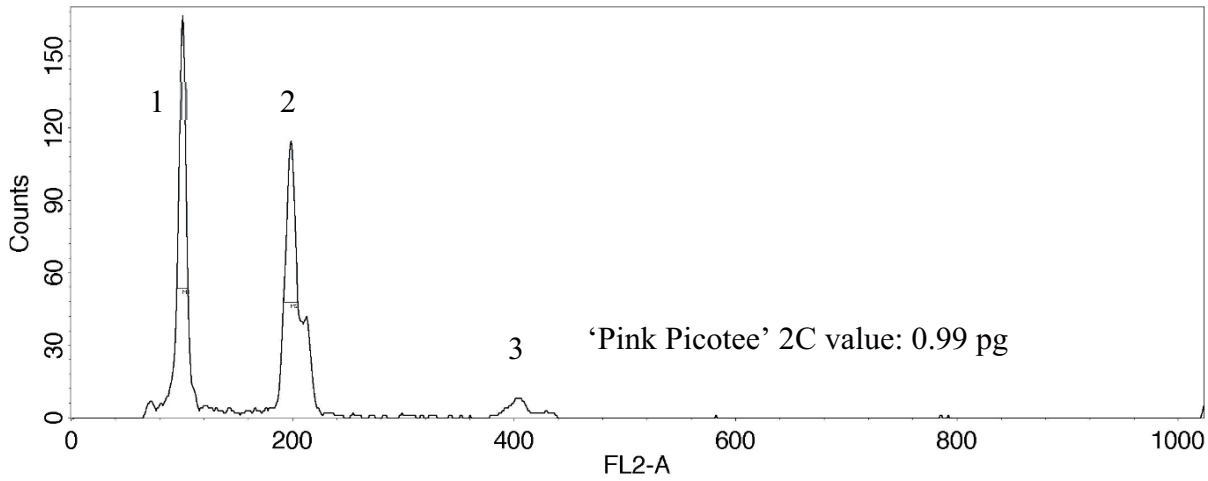

Fig. 4. DNA histogram shows two 2C peaks and one 4C peak, which correspond to the nucleus DNA. 1: 2C peak sample 'Pink Picotee', 2: 2C and 3: 4C peak internal standard tomato 'Stupické'.
Das DNA-Histogramm zeigt zwei 2C-Peaks und einen 4C-Peak, die mit der Kern-DNA korrespondieren. 1: 2C Peak der Probe 'Pink Picotee', 2: 2C- und 3: 4C-Peak des internen Standards Tomate 'Stupické'.

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