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Flow cytometry – a modern method for exploring genome size and nuclear DNA synthesis in horticultural and medicinal plant species

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ABSTRACT

Flow cytometry (FCM) has been used for plant DNA content estimation since the 1980s; however, presently, the number of laboratories equipped with flow cytometers has significantly increased and these are used extensively not only for research but also in plant breeding (especially polyploid and hybrid breeding) and seed production and technology to establish seed maturity, quality and advancement of germination. A broad spectrum of horticultural and medicinal species has been analyzed using this technique, and various FCM applications are presented in the present review. The most common application is genome size and ploidy estimation, but FCM is also very convenient for establishing cell cycle activity and endoreduplication intensity in different plant organs and tissues. It can be used to analyze plant material grown in a greenhouse/field as well as *in vitro*. Due to somaclonal variation, plant material grown in tissue culture is especially unstable in its DNA content and, therefore, FCM analysis is strongly recommended. Horticultural species are often used as internal standards in genome size estimation and as models for cytometrically studied cytotoxic/anticancer/allelopathic effects of different compounds. With the growing interest in genome modification, increased application of FCM is foreseen.

Keywords: cell cycle, endoreduplication, *in vitro* culture, plant breeding, ploidy, seed technology, taxonomy

Abbreviations:
FCM – flow cytometry, GS – genome size

INTRODUCTION

Flow cytometry (FCM), commonly applied in multiparametric analysis in medicine, is most frequently used in plant sciences to establish only one parameter, the nuclear DNA content. Because of its advantages, such as high throughput, accuracy and resolution, negligible destructiveness to plants, as well as low operating cost per sample, it has replaced other methods of ploidy and/or genome size (GS) estimation in a large number of laboratories (Vrána et al., 2014). A few years ago, Bennett and Leitch (2011) reported that the proportion of all plant GS estimates using FCM was 85%, and, taking into account the recent popularity of this method, it is likely that this figure is significantly higher today.

FCM analyzes the optical properties of single particles/cells within a suspension. In the case of

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plants, these particles are usually nuclei isolated by chopping an intact tissue/organ and stained with a fluorescent dye that intercalates or binds to DNA. Cells/nuclei pass in a fluid stream through a flow cell, intersect the focus of an intense light source (typically a laser), and absorb light. This light produces fluorescent signals, which are displayed as a histogram and/or a dot plot (Fig. 1; Vrána et al., 2014). A cytometer can be additionally equipped with light scatterers for side scatter (SSC) and forward scatter (FSC). SSC provides information on cell complexity and FSC on cell size. Some cytometers (sorters) can isolate particles of interest based on their physical or fluorescent parameters. The most common fluorochromes used for plant DNA staining are propidium iodide (PI; intercalating dye) and 4',6-diamidino-2-phenylindole (DAPI; a dye that binds to AT-rich regions of DNA).

Plant material typically used for FCM analysis is fresh young leaves, but any plant part containing intact nuclei can be used. In cases when fresh material is not available, dry leaves or seeds can be used, although these require some modifications in sample preparation and/or experience in the interpretation of histograms (Sliwinska et al., 2005; Suda and Trávníček, 2006; Razafinarivo et al., 2012).

Even though nuclear sample preparation and FCM analysis of plant material are relatively easy and fast (Galbraith et al., 1983), there are some 'difficult' species in which the cytosol contains compounds (secondary metabolites) that inhibit DNA staining and cause a stoichiometric error in FCM estimation of nuclear DNA content (Noirot et al., 2000). These compounds, mostly polyphenols, are present in some annual horticultural species, many shrubs and trees, and in most medicinal species. *Helianthus annuus* was one of the first species in which compounds were found to bias FCM measurement by interfering with PI intercalation into the DNA helix and/or with fluorescence (Price at al., 2000). The researchers proposed a special test for detecting the presence of staining inhibitors, which should be performed for any plant material that may contain such compounds. Modifications of the sample preparation procedure, such as changing the composition of the nuclei isolation buffer, incubation of the sample on ice for 30-60 min. before analysis, and/or spinning and resuspending the isolated nuclei, should be performed individually for any plant material containing staining inhibitors to obtain reliable results of FCM analysis. Loureiro et al. (2007), comparing two different nuclei isolation buffers for FCM analysis of a number of horticultural species, found that the one containing

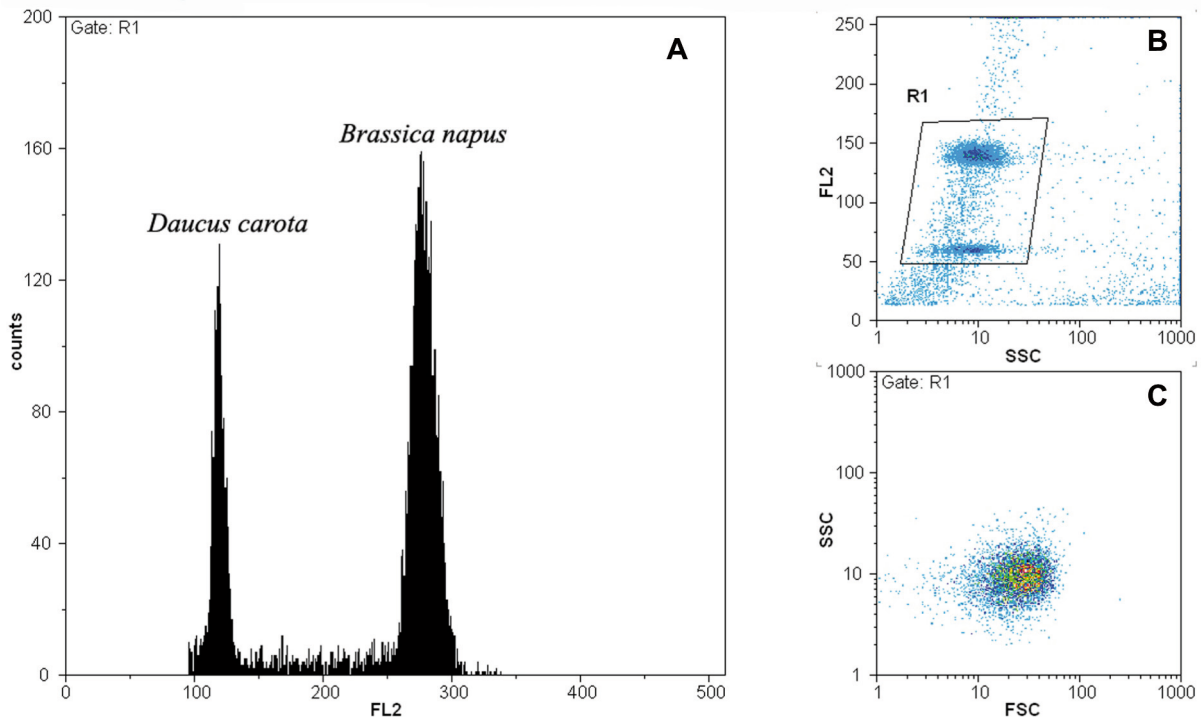

Figure 1. Histogram of PI fluorescence intensity (FL2; A) and dot plots on side scatter (SSC) versus PI fluorescence (B) and forward scatter (FSC) versus SSC (C) in the nuclei of young leaves of *Daucus carota* (sample) and *Brassica napus* (internal standard, 2C = 2.18 pg)

1% polyvinylpyrrolidone (PVP-10) was more suitable for recalcitrant samples characterized by the presence of phenolic and mucilaginous compounds. Modifications used successfully for 11 woody Rosaceae species included the addition of PVP-10 and/or β-mercaptoethanol at different concentrations to the nuclei isolation buffer (Jedrzejczyk and Sliwinska, 2010). Sadhu et al. (2016) established the nuclear DNA content of 24 'difficult' species of the Zingiberaceae family, containing different active metabolites such as flavonoids, phenolic acids, and essential oils, using a buffer with compounds that stabilised pH (MOPS) and chromatin (spermine), chelated divalent cations (EDTA), and with antioxidants PVP and β-mercaptoethanol. In addition to the staining inhibitors, mucilages, which are polysaccharides present in some plant tissues, make sample preparation difficult; they attract nuclei and cause their clumping. GS estimation of five species belonging to the family Hyacinthaceae, characterized by high mucilage content, was established after modifications to the frequently used Galbraith's isolation buffer (Galbraith et al., 1983), by increasing pH and detergent concentration, and replacing sodium citrate with citric acid (Nath et al., 2014). Optimized protocols for sample preparation of 13 *Viola* and *Hylocereus* species, whose tissues are also rich in polysaccharides, were proposed by Cires et al. (2011) and Li et al. (2017), respectively. An alternative to modifying the sample preparation procedure is to find tissues/plant parts free of undesirable compounds, for example a seed (Sliwinska et al., 2005; Jedrzejczyk and Sliwinska, 2010). Since not all researchers are aware of the above problems, the results of nuclear DNA content estimation should be regarded with caution when no modifications to standard preparation procedures have been applied for species known to be 'difficult'.

Despite the difficulties in FCM analysis of some species, the method is widely used in taxonomy, ecology, evolutionary biology, polyploid and hybrid breeding, seed biology and technology, and *in vitro* cultures. In this review, recent literature (from the year 2000 to the present) on the applications of FCM in the analysis of the DNA content of horticultural and medicinal species is presented.

GENOME SIZE AND PLOIDY ESTIMATION

The nuclear genome is defined as the chromosome complement and its DNA, characteristic of a particular organism (Greilhuber et al., 2005). Knowledge of its size is important for the identification of species, verification of their taxonomic position, and identification of plant material cultured *in vitro*, the genome of which has been changed by somaclonal variation. It is an essential starting point for projects involving genome sequencing, for optimizing some molecular biology methods such as microsatellites and amplified fragment length polymorphism (AFLP), and for determining the number of clones in a genomic library (Leitch and Bennett, 2007). It is also used for studying the role of the C-value (DNA content of a holoploid genome with chromosome number *n*; Greilhuber et al., 2005) in plant growth and development, and plant responses to environmental stresses. GS can be expressed in pg or Mbp (1 pg = 978 Mpb; Doležel et al., 2003).

For flow cytometric GS measurements, an internal reference standard (a plant with known nuclear DNA content, processed together with the sample) is needed (Fig. 1; Vrána et al., 2014). In many laboratories, horticultural species such as *Petunia hybrida*, *Glycine max*, *Allium cepa*, and *Pisum sativum*, which possess a stable genome size, serve as internal standards. Although FCM makes it relatively easy to establish nuclear DNA content, to date the GS of only about 3% of angiosperms has been reported. For important horticultural species, such as *Brassica oleracea*, *Daucus carota*, *A. cepa*, *Cucumis sativus* and many others, this characteristic was initially established in the 1970s and 1980s using Feulgen cytophotometry, and then re-established at the turn of the 20th and 21st centuries using FCM (Bennett and Leitch, 2012; Tab. 1).

Taxonomy, evolution, and ecology

The taxonomy of a number of families is complex, with species expressing strong morphological uniformity or few cytotypes (differing in ploidy or chromosome structure). FCM, often combined with molecular methods, is a good tool for the establishment/verification of the systematic position of a species. One of the families of great interest in terms of nuclear DNA content is the Orchidaceae, with almost 170-fold variation in GS; 1C-value varies from 0.33 pg in *Trichocentrum maduroi* to 55.4 pg in *Pogonia ophioglossoides* (Leitch et al., 2009). Recent reports on GSs have provided information to fill the phylogenetic gaps in this family (Lin et al., 2001; Bory et al., 2008; Rupp et al., 2010; Moraes et al., 2012; Jersáková et al., 2013; Těšitelová et al., 2013). With the aim of providing information for taxonomic studies, Favoreto et al. (2012) established the DNA content for 14

Table 1. Genome sizes of major horticultural species established by Feulgen cytophotometry and re-established by flow cytometry (Plant DNA C-value Database; Bennett and Leitch, 2012)

Genus/species		2C DNA content (pg)
Latin name	Common name	
Allium cepa L.	Onion	28.73-34.80
A. porrum L.	Leek	45.30-65.30
A. sativum L.	Garlic	31.45-35.70
A. schoenoprasum L.	Chives	15.25-32.70
Asparagus officinalis L.	Asparagus	2.70-6.67
Aster L.	Aster	3.25-21.43
Beta vulgaris L.	Beetroot	1.50-2.60
Brassica oleracea L.	Cauliflower/Cabbage	1.25-1.80
B. rapa L.	Chinese Cabbage	1.08-1.60
Capsicum annuum L.	Pepper/Chili	5.50-10.85
Cucumis melo L.	Melon	1.05-2.50
C. sativus L.	Cucumber	0.75-2.10
Daucus carota L.	Carrot	1.00-4.00
Lactuca sativa L.	Lettuce	5.30-6.61
Petroselinum crispum (Mill.) Nyman ex A.W. Hill	Parsley	3.95-8.60
Phaseolus coccineus L.	Runner Bean	1.35-3.50
P. vulgaris L.	Common Bean	1.00-3.70
Pisum sativum L.	Pea	7.60-11.85
Prunus persica L.	Peach	0.55-0.66
Raphanus sativus L.	Radish	0.90-2.90
Rosa L.	Rose	0.25-3.05
Solanum melongena L.	Eggplant	1.95-2.50
Spinacia oleracea L.	Spinach	1.65-2.69
Tulipa L.	Tulip	24.70-120.90
Vicia faba L.	Broad Bean	22.85-54.80
Zea mays L.	Maize	4.7-12.6

Bromeliaceae species and produced a dendrogram for members of the Pitcairnioideae, Tillandsioideae, and Bromelioideae. Their report was supplemented by Gitaí et al. (2014), who established the GS of 28 species from the family Bromeliaceae. Other horticultural genera on which systematic classification studies have been supported by GS measurements are the vegetables: *Lactuca*, *Capsicum*, *Daucus* (Koopman, 2000; Doležalova et al., 2002; Moscone et al., 2003; Tavares et al., 2014; Nowicka et al., 2016), ornamentals: *Rosa*, *Galanthus*, *Hemerocallis*, *Agapanthus*, *Lilium*, *Passiflora*, *Nerine*, *Dahlia*, *Fuchsia*, *Hydrangea*, *Nasturtium*, *Penstemon*, *Anthurium*, *Tulipa*, *Drimia*, *Cotoneaster* (Yokoya et al., 2000; Saito et al., 2003; Zonneveld and Duncan, 2003, 2006; Zonneveld et al., 2003; Souza et al., 2004; Marasek et al., 2005; Temsch et al., 2008; Talluri and Murray, 2009; Morozowska et al., 2010; Mortreau et al., 2010; Broderick et al., 2011; Bliss and Suzuki, 2012; dos Santos et al., 2014; Abedi et al., 2015; Nath et al., 2015; Rothleutner et al., 2016), and orchard crops: *Ribes*, *Malus*, *Crataegus*, *Mespilus*, and *Olea* (Chiche et al., 2003; Talent and Dickinson, 2005; Tatum et al., 2005; Brito et al., 2008). Although it is usually difficult to conduct FCM analyses on medicinal and culinary species (see Introduction), assessment of 2C was recently completed for: *Curcuma*, *Salvia*, *Gentiana*, *Thymus*, *Ocimum*, and 66 traditional Chinese medicinal species (Leong-Škorničková et al., 2007; Maksimović et al., 2007; Mishiba et al., 2009; Carović-Stanko et al., 2010; Koroch et al., 2010; Mahdavi and Karimzadeh, 2010; Zhang et al., 2013; Rewers and Jedrzejczyk, 2016). The collected C-values can be useful not only for taxonomic purposes but also for species

identification, germplasm conservation, and breeding.

Estimation of nuclear DNA content has also been used to resolve evolutionary questions. One of the interesting families, which includes not only the model species *Arabidopsis thaliana* but also important vegetables as well as ornamental and medicinal species, is the Brassicaceae. Based on the C-values of 185 Brassicaceae taxa, Lysak et al. (2009) concluded that GS in this family had not changed substantially over time and most likely evolved passively. They also suggested that there was a paradox between the maintenance of a narrow range of small GSs over a long evolutionary time and the evidence of genomic changes that could have led to genome obesity. Their study confirmed earlier observations by Johnston et al. (2005), who, after measuring the DNA content of 34 Brassicaceae species and sequencing the ITS region (internal transcribed spacer region of DNA between the small and large ribosomal RNA genes) for 23 taxa, had found that despite the evidence of species with decreases and increases in GS, including polyploidization, the evolution of this family's GS had a conservative nature. Polyploidy, and also dysploidy (an increase or decrease in chromosome number as a result of structural rearrangements) as mechanisms of genome evolution have been suggested for *Ocimum*, Orchidaceae, Bromeliaceae, *Colchicum*, and Iridaceae (Bory et al., 2008; Carović-Stanko et al., 2010; Rupp et al., 2010; Trávníček et al., 2011; Moraes et al., 2012, 2015; Jersáková et al., 2013; Fridlender et al., 2014; Gitaí et al., 2014; Yeh et al., 2015).

Establishment of the DNA content for 40 *Malus* genomes had led to the conclusion that polyploids, especially allopolyploids, could easily withstand the loss of certain amounts of DNA (Tatum et al., 2005). A tendency towards downsizing of the monoploid *Malus* genome with increased ploidy was observed. A similar tendency was found in *Fuchsia*, where polyploid species have considerably lower 1Cx-values (DNA content of a monoploid genome with chromosome base number *x*; Greilhuber et al., 2005) than diploid ones (Talluri and Murray, 2009). The GS of *Anthurium*, which generally does not correlate with the chromosome count and phylogenetic relationships, in addition to polyploidization and chromosome reorganization or loss, depends on the amplification and reduction of repetitive elements (Bliss and Suzuki, 2012). Similarly, in the legume tribe *Fabeae*, the accumulation of repetitive DNA accounts for 85% of the genome size differences between the species (Macas et al., 2015). Also, as was concluded from the combined analyses of GS, fluorescence *in situ* hybridization (FISH), and BAC clone sequencing, genome evolution of *Eucalyptus* seems to be driven by local expansion of repetitive heterochromatin clusters (Ribeiro et al., 2016). Repetitive DNA can also be responsible for intraspecific variation. The 2C-value variation among cultivars of *Curcuma longa* was suggested to be due to different transposon expression and integration (Basak et al., 2017). In turn, the amount of DNA could provide information on the number of (retro)transposon copies present in a certain genotype, as determined for *Iris* species and hybrids using FCM together with dot blot hybridization and a genomic library screen, for the estimation of the copy number of IRRE (*IR*is *R*etro*E*lement; Kentner et al., 2003).

Traditionally, ploidy is estimated by chromosome counting. However, this method needs time-consuming microscopic slide preparation, and counting can be difficult and unreliable, especially if the chromosomes are small and/or numerous. This method can be replaced by FCM if there is a plant of the same species of known ploidy available (a so-called external standard, processed separately from the sample), or if the Cx-value is known. An example of the first approach was used in establishing the ploidy of three *Fragaria* species and of a naturally occurred putative hybrid found in Germany, where the octoploid cultivated *F.* × *ananassa* served as an external standard (Nosrati et al., 2011). After checking its constancy among related specimens and making a comparison with established and previously published DNA amounts, the Cx-value was used to assign ploidy to 74 *Rosa* species (Roberts et al., 2009). Among the studied species, 2*x*, 4*x*, 5*x*, 6*x*, 7*x*, and 8*x* roses were found, which verified earlier reports.

Chromosome counting in shrubs and trees can be especially problematic due to the difficulties in obtaining appropriate tissues in which to view mitotic figures. Talent and Dickinson (2005), measuring the nuclear DNA content of *Crataegus* and *Mespilus* species, confirmed that FCM can be more precise for establishing ploidy. To previously published ploidies, 2*x*, 3*x*, 4*x*, and 6*x*, they added evidence for 5*x* in *Crataegus*. Interesting ecological observations were made on the ploidy in *Aster amellus* in about 300 populations; each individual population consisted of only one basic ploidy (diploid or hexaploid), while only one mixed-ploidy population was detected (Mandáková

and Münzbergová, 2006; Castro et al., 2012). Surprisingly, no intermediate tetraploid cytotype was found, which speaks for a lack of hybridization between the cytotypes, despite the close proximity of the populations of different ploidy.

Some researchers claim that there is a relationship between GS and environmental conditions. For example, Knight and Ackerly (2002) examined variation in GS for over 400 plant species in California in relation to the mean July maximum temperature, January minimum temperature, and annual precipitation, and concluded that species with small GS predominate in all environments, while those with large genomes occur at intermediate July maximum temperatures, and decline in frequency at both extremes of the July temperature gradient, and with decreasing annual precipitation. Another example are species of *Lilium*, possessing giant genomes, in which GS correlates negatively with annual temperatures and precipitation (Du et al., 2017).

Plant breeding

The flow cytometer has of late become an essential piece of equipment for modern plant-breeding companies because knowledge of plant DNA content is necessary in many breeding programmes/technologies, e.g. those directly involving ploidy change in polyploid breeding. Polyploidization is usually accompanied by increased cell size, which leads to alterations in morphology that are favourable for horticultural plants. Polyploids are usually characterized by larger organs, an increased width-to-length ratio of leaves, and their deeper green colour or more compact growth habit (Shao et al., 2003). Induction of polyploidy is often applied to floral crops because plants with a higher ploidy usually produce larger flowers than diploids, their flowering period is longer, and sometimes their flowers have a deeper colour. Polyploids (usually tetraploids) are most often obtained *in vitro* or *ex vitro* by treatment with an antimitotic agent, and plants of desirable ploidy are selected using FCM. This approach was successfully performed for 3 *Hibiscus* species, *Rhododendron* hybrid *R.* × *kosterianum*, *Syringa vulgaris* × *S. pinnatifolia*, a few *Rosa* species, *Gerbera jamesonii*, *Hemerocallis* × *hybrida*, and *Petunia axillaris* (Rose et al., 2000; Väinölä, 2000; Van Huylenbroeck et al., 2000; Allum et al., 2007; Gantait et al., 2011; Crespel et al., 2015; Podwyszyńska et al., 2015; Regalado et al., 2017). The induction of artificial polyploidy can also increase the production of important medicinal compounds and for this reason was introduced into *Ocimum basilicum*, *Thymus persicus*, and *Trachyspermum ammi* (Omidbaigi et al., 2010; Tavan et al., 2015; Noori et al., 2017). However, the amount of nuclear DNA does not always correlate with the size of particular organs. Unexpectedly, among 17 *Cucurbita* sp. (*C. pepo* and *C. maxima*) cultivars, which encompassed the whole range of fruit types reported for pumpkin, both the miniature and jumbo types possessed the smallest genomes and cell size (Tatum et al., 2006).

FCM is also helpful in identifying intra- and interspecific hybrids in hybridization breeding. The advantage of FCM over observations of the morphological characteristics of mature hybrids is the possibility of performing DNA content measurements at the early stages of seedling development, or even using seeds. Knowing the ploidy is especially important for species like *Musa* ssp., cultivars of which are diploid, triploid or tetraploid, for the management of germplasm and breeding programmes (Nsabimana and van Staden, 2006). In *Musa*, in addition to different ploidies, aneuploids (plants with an under- or over-representation of one or more chromosomes) occur among the triploid populations, which are undesirable during vegetative propagation but can be used for gene isolation and mapping (Roux et al., 2003). It is difficult to detect them using FCM, but Roux et al. (2003) developed a reliable protocol based on the DNA index, which allowed determination of the percentage of DNA content in a plant of unknown ploidy in relation to a triploid one. Aneuploids also occurred after crossing diploid and triploid *Asparagus officinalis*, among them trisomics, which are useful for genetic studies (Ozaki et al., 2004). Similarly, aneuploids have been detected within the progenies of 3*x*-2*x* and 3*x*-4*x* backcrosses of *Lilium* hybrids (Lim et al., 2003). For some aneuploid plants, FCM has been combined with microscopic chromosome counting to confirm the exact chromosome number. Somatic aneuploidy also occurred in *Vanilla* hybrids; however, their pollen grains, in contrast to those of *A. officinalis* and *Lilium* hybrids, had a euploid chromosome number (Lepers-Andrzejewski et al., 2011).

The objective of interspecific hybridization is to transfer valuable traits for quality or biotic/abiotic resistance from wild to cultivated species, as well as to broaden the genetic diversity of the obtained breeding material, or to obtain hybrid plants with new, commercially interesting combinations of traits. Kamiński et al. (2016) introduced cytoplasmic

male sterility (CMS), a trait desirable in *Brassica* breeding, from CMS *B. napus* var. *oleifera* into *B. rapa* inbred lines. After confirming cytometrically and morphologically the interspecific character of the hybrids, a valuable breeding line of CMS *B. rapa* genotypes was obtained.

After measuring the DNA content of the parental species and of four artificial hybrids between different *Fuchsia* species, it was found that the amount of DNA in the hybrids was intermediate between those of the parental species (Talluri and Murray, 2009), which confirmed that this characteristic could be used to quickly identify them. Similarly, after crossing several *Rhododendron* species and hybrids, differing in flower colour, plants of intermediate DNA content were obtained (Eeckhaut et al., 2003). Likewise, artificially produced interspecific *Dahlia* hybrids possessed intermediate C-values close to those expected in relation to the DNA content of their parents (Temsch et al., 2008). In contrast, out of nine interspecific hybrids between four *Capsicum* species, only one, (*C. chinense* × *C. baccatum*)F_1, possessed a nuclear DNA content intermediate between and different from both parents. In some hybrids, it was similar to the mother species, and naturally, if both parent species had a similar genome size, the hybrid was identical in DNA content and had first to be identified using biometric characteristics after it produced fruits (Olszewska et al., 2007). Knowledge of DNA content has been important for the breeding of *Sarcococca* spp.; after performing a number of interspecific crosses between 19 species and cultivars, Denaeghel et al. (2017) concluded that neither different ploidies nor genome size was a complete barrier to hybridization. Broad scale FCM surveys applied to crosses of *Vaccinium* species differing in ploidy has led to the conclusion that *V. corymbodendron* either possesses no ploidy barriers to hybridization or possesses genomic dosage factors that differ from species belonging to the section *Cyanococcus* (Ehlenfeldt and Ballington, 2017). The performed crosses demonstrated that it was possible to combine 3*x V. corymbodendron* germplasm with 4*x*, 6*x*, and 8*x* germplasm, which opened up new possibilities for the breeding of this species.

Estimations of nuclear DNA content and ploidy are also important for rootstock breeding, especially of *Citrus* (Seker et al., 2003). In this genus, the rootstock is obtained from seeds containing one zygotic and several somatic embryos, which can lead to partial apomixis and polyploidization of the offspring. Thus, the production of true-to-type *Citrus* rootstock requires establishing the DNA content. Also, somatic embryos occur in *A. officinalis*, and FCM analysis of juvenile phylloclades have revealed that some of them are apomictic haploids; after chromosome doubling such plants can be a valuable breeding material (Zenkteler et al., 2012). Analysis of the ploidy of seedlings does not always provide accurate information on the pathway by which reproduction of the plant occurs. However, it can be easily determined by FCM using seeds, by establishing the ploidy of the embryo and endosperm (Matzk et al., 2000). Studies by Matzk et al. (2000), among others, confirmed that the mode of reproduction of horticultural species such as *A. cepa*, *Beta vulgaris*, *B. napus,* and *Zea mays* was obligate sexual. Using this approach, various kinds of apomixis were detected in populations of fruit crops such as *Malus* and *Rubus* (Kron and Husband, 2009; Šarhanová et al., 2012), ornamental species *Crataegus* and *Rudbeckia bicolor* (Talent and Dickinson, 2007; Musiał et al., 2012), herbs *Hypericum perforatum* and *Arnica cordifolia* (Matzk et al., 2001; Kao, 2007), and different species of *Allium* (Specht et al., 2001). FCM was also used in supporting microscopic and/or molecular studies on reproductive strategies of *Aronia melanocarpa*, *Townsendia hookeri*, and *Sorbus* spp. (Persson Hovmalm et al., 2004; Thompson et al., 2008; Meyer et al., 2014; Hajrudinović et al., 2015).

IN VITRO CULTURES

FCM is broadly used in tissue culture of horticultural and medicinal plants (Tab. 2). It is well known that somaclonal variation occurs during *in vitro* culturing of plant material (for review see Bairu et al., 2011). The cause of this variation can be mutations, chromosome rearrangements and aberrations, or polyploidization. Somaclonal variation often changes the morphology of the plants produced *in vitro* and therefore can be detected visually; however, for more precise analysis both molecular methods and FCM are currently applied. Micropropagation, commonly used for the production of uniform material (clones), has to guarantee genetic stability of the final product, and one of its indicators is ploidy/GS. To verify this, chromosome counting, and lately more commonly, FCM is used (Tab. 2). Ploidy instability is especially likely when plantlets are regenerated *via* callus. In this tissue, a considerable number of cells with higher than 2C DNA content often occur, and they can initiate regeneration of polyploid plants (Thiem

Table 2. Use of FCM in tissue cultures of horticultural and medicinal species. M, micropropagation; H, haploid/double haploid production; P, polyploid production; Pr, protoplast culture/fusion; Er – hybridization with embryo rescue; T, transformation; AC, anticancer compound testing

Genus/species	Type of culture	Reference
Actinidia deliciosa	M, P	Góralski et al., 2005
Aeschynanthus radicans	M	Cui et al., 2009
Allium cepa	H	Martínez et al., 2000; Alan et al., 2007
Anthurium andreanum	M	Gantait and Sinniah, 2011
Asparagus officinalis	P	Carmona-Martín et al., 2015; Regalado et al., 2015
Bacopa monnieri	M	Largia et al., 2015
Capsicum	H	Olszewska et al., 2014; Nowaczyk et al., 2015
Carica papaya	P	Clarindo et al., 2008
Chaenomeles japonica	P	Stanys et al., 2006
Chrysanthemum	M	Lema-Rumińska and Śliwińska, 2009, 2015; Naing et al., 2013
Citrus	H, P, Pr, Er, T	Cabasson et al., 2001; Navarro et al., 2003; Wu and Mooney, 2002; Scarano et al., 2003; Fanciullino et al., 2005; Guo and Grosser, 2005; Guo et al., 2006; Aleza et al., 2009, 2010; Grosser and Gmitter Jr, 2011; Cardoso et al., 2014
Cocos nucifera	M	Sandoval et al., 2003
Colocynthis citrullus	M	Ntui et al., 2009
Copiapoa tenuissima	M	Lema-Rumińska, 2011
Coriandrum sativum	M	Ali et al., 2017
Cucumis	M, H, T, Er	Gémes-Juhász et al., 2002; Lotfi et al., 2003; Akasaka-Kennedy et al., 2004; Nuñez-Palenius et al., 2006; Sebastiani and Ficcadenti, 2016
Cucurbita	M, Er	Ananthakrishnan et al., 2003; Lee et al., 2003; Šiško et al., 2003
Cyclamen	M, Pr	Schmidt et al., 2006; Borchert et al., 2007; Prange et al., 2012
Cymbidium	M	Teixeira da Silva et al., 2006
Daucus carota	H, T	Deroles et al., 2002; Kiszczak et al., 2011
Dianthus acicularis	M	Shiba and Mii, 2005
Eryngium	M	Thiem et al., 2013; Kikowska et al., 2014, 2016
Eucalyptus globulus	M	Pinto et al., 2004, 2011
Eulophia nuda	M	Nanekar et al., 2014
Fragraria × ananassa	M	Forni et al., 2001
Gentiana	M, Pr	Rybczyński et al., 2007; Fiuk and Rybczyński, 2008; Mikuła et al., 2008; Tomiczak et al., 2015, 2016, 2017
Inula verbascifolia	M	Sliwinska and Thiem, 2007
Ipomoea batatas	T	Deroles et al., 2002
Lathyrus	M	Ochatt et al., 2013
Lilium × formolongi	Pr	Horita et al., 2003
Malus domestica	Pr, Er, T	Maddumage et al., 2002; Gonai et al., 2006
Manihot eslulenta	P	Zhou et al., 2017
Musa	M	Roux et al., 2001; Escobedo-GraciaMedrano et al., 2014
Oenothera paradoxa	M	Sliwinska and Thiem, 2007
Olea	M	Lopes et al., 2009; Brito et al., 2010
Paris polyphyla	AC	Yan et al., 2009
Passiflora cincinnata	M	Pinto et al., 2010
Pelargonium	Pr, T	Nassour et al., 2003; Hassanein and Dorion, 2005; Hassanein et al. 2005
Physalis ixocarpa	H	Escobar-Guzmán et al., 2009
Pisum sativum	H	Ribalta et al., 2012
Plantago asiatica	M	Makowczyńska et al., 2008

Table 2 continued.

Genus/species	Type of culture	Reference
Primula	Er	Kato et al., 2001
Prunus	H, P	Peixe et al., 2004; Vujović et al., 2012
Pueraria lobata	M	Sliwinska and Thiem, 2007
Punica granatum	P	Shao et al., 2003
Pyrus	M, H, Er	Bouvier et al., 2002; Kadota and Niimi, 2002; Sun et al., 2011; Gonai et al., 2006
Raphanus sativus	M	Curtis et al., 2004
Rosa	Er	Abdolmohammadi et al., 2014
Rubus chamaemorus	M	Thiem and Sliwinska, 2003; Sliwinska and Thiem, 2007
Scutellaria	AC	Parajuli et al., 2009
Solanum	M, H, Pr, T	Collonnier et al., 2001; Bartoszewski et al., 2003; Ellul et al., 2003; Seguí-Simarro and Nuez, 2007; Xing et al., 2010; Szczerbakowa et al., 2011
Solidago	M	Sliwinska and Thiem, 2007
Spathiphyllum wallisii	P	Eeckhaut et al., 2004
Teuricum scorodonia	M	Makowczyńska et al., 2016
Tricyrtis hirta	M	Nakano et al., 2006
Ullucus tuberosus	P	Viehmannová et al., 2012
Vigna subterranea	M	Koné et al., 2007
Viola uliginosa	M	Slazak et al., 2015
Vitis vinifera	M, H, P	Lima et al., 2003; Leal et al., 2006; Yang et al., 2006; Prado et al., 2010
Ziziphus jujuba	P	Gu et al., 2005; Shi et al., 2015

and Sliwinska, 2003; Makowczyńska et al., 2008; Slazak et al., 2015; Tomiczak et al., 2016).

Polyploidization *in vitro* by the application of antimitotic compounds has already been mentioned in the previous section. Another interesting way of obtaining polyploids, using *in vitro* cultures, was detailed by Góralski et al. (2005). They used the endosperm of *Actinidia deliciosa* seeds as explant material and, after callus formation, obtained triploid plants, which traditionally are produced by crossing diploid and tetraploid plants. Equally important as producing polyploids is haploid and double-haploid production, which can shorten the time to obtain homozygous lines for hybrid/heterosis breeding by a few years (Gałązka and Niemirowicz-Szczytt, 2013). Haploids have lately been produced *via* andro- or gynogenesis in such horticultural species as *A. cepa*, *Capsicum* ssp., *C. melo*, *C. sativus*, *D. carota*, *Physalis ixocarpa*, *P. sativum*, *Prunus* ssp., *Pyrus* ssp., *S. lycopersicum*, *V. vinifera* (Tab. 2). In all those species, the ploidy of the produced plants was checked using FCM. It is worth mentioning that FCM is a unique method for detecting mixoploidy during (double) haploid production.

Biotechnological methods are also applied to produce interspecific hybrids, and, as in traditional hybridization, FCM is often helpful in verifying the hybrid status of the resultant material. Two main *in vitro* techniques, embryo rescue and protoplast fusion, are used to cross species that otherwise cannot be successfully crossed. Gonai et al. (2006), after pollinating pistils of *Pyrus pyrifolia* with pollen of *Malus domestica*, cultured the fertilized embryos *in vitro* and obtained intergeneric shoots with a DNA content intermediate between the parents. Embryo rescue was also used to obtain hybrids between different *Cucurbita* species, and in all of them the relative nuclear DNA content confirmed successful hybridization (Šiško et al., 2003). In contrast, after embryo culture and regeneration of a hybrid between *C. sativus* and a wild *Cucumis* species, *C. hystrix*, about 7% of the obtained plants possessed double the expected amount of DNA and were considered as chromosome-doubled F_1 hybrids (Chen et al., 2002). Another unexpected genome composition occurred after crossing *Primula sieboldii* with *P. obconica* and rescuing the hybrid embryos (Kato et al., 2001). In addition to normal diploid hybrids, two types of triploids were found, of which four had two genomes of *P. sieboldii* and

one of *P. obconica*, and one had one genome of *P. sieboldii* and two of *P. obconica*. These examples illustrate well the necessity of checking ploidy/GS after using *in vitro* cultures for zygotic interspecific hybridization. However, the variation in ploidy and genome composition of the obtained material is much higher when using somatic hybridization (protoplast fusion). This technique has been applied extensively to *Citrus* breeding for ploidy manipulation, mostly to generate allotetraploids and autotetraploids (as by-products) for interploid crosses, e.g. to obtain seedless triploids or to provide tetraploid somatic hybrid rootstock (for review see Grosser and Gmitter Jr., 2011). Somatic hybridization has also been used for this genus to manipulate chloroplast and mitochondrial genomes, using *C. deliciosa* as an embryogenic parent and *C. sinensis* or *C. paradisi* as a mesophyll parent (Cabasson et al., 2001). After analyzing the nuclear DNA content and nuclear and mitochondrial DNA restriction fragment length polymorphism (RFLP), the regenerated diploid plants were assumed to be alloplasmic hybrids, or cybrids. *Solanum*, too, is a genus often subjected to protoplast fusion with the objective of producing synthetic allopolyploids. Somatic hybrids between *S. melongena* and *S. aethiopicum* were produced by electrofusion of protoplasts in order to transfer resistance against *Ralstonia solanacearum* from *S. aethiopicum* (Collonnier et al., 2001). Cytometric analysis of the ploidy of the regenerated plants revealed that as much as 75% of the regenerants were diploids instead of the expected tetraploids, and only the remaining 25%, considered as putative hybrids, were subjected to molecular analyses to verify their hybrid status. Szczerbakowa et al. (2011) performed repeated fusion of protoplasts of 7*x* and 8*x* interspecific hybrids *S. nigrum* + *S. tuberosum* and diploid *S. tuberosum* clones in order to enlarge the *S. tuberosum* input into the hybrid genome. Unfortunately, the newly synthetized allopolyploids possessed only slightly more nuclear DNA than the parental hybrids, instead of the expected addition of the entire *S. tuberosum* genome, and the amount of DNA was different in different hybrids. Studies by Tomiczak et al. (2017) confirmed the occurrence of variations in DNA content in calli and plants after electrofusion of cell suspension-derived protoplasts of diploid *Gentiana kurroo* and leaf mesophyll-derived protoplasts of tetraploid *G. cruciata*. They reported that polyploidization occurred at the early stage of post-fusion culture, and during the subsequent four years of *in vitro* culture gradual elimination of nuclear DNA, mixoploidy, and high genetic instability were observed.

The amount of DNA transferred during genetic transformation is too small to be detected by FCM. However, in some transformation experiments the method is used for ploidy estimation. Indeed, the occurrence of diploid and tetraploid transformants of *C. melo*, *Pelargonium* spp., and *S. lycopersicum* (Bartoszewski et al., 2003; Ellul et al., 2003; Akasaka-Kennedy et al., 2004; Hassanein et al., 2005) confirm the recommendation to check the ploidy of transgenic plants before using them in further research or introducing into a breeding programme. As in micropropagation and somatic hybrid production, this especially applies when callus or protoplasts are used for transformation. Guo and Grosser (2005) confirmed this when they fused mesophyll protoplasts of *gfp*-transformed *C. sinensis* with embryogenic callus-derived protoplasts of *C. reticulate* to visually screen somatic hybrids. GFP-expressing embryoids obtained *via* hybrid callus were verified by FCM and simple sequence repeat (SSR) analyses to be diploid cybrids or tetraploid somatic hybrids. After the transformation of *M. domestica* protoplasts with *gfp*, another ability of FCM, counting of protoplasts/cells, was used (Maddumage et al., 2002). This made it possible to determine the efficiency of polyethylene glycol-mediated transformation at different temperatures. Deroles et al. (2002) used yet another application of cytometry, estimation of nuclear DNA condensation, to determine if this was connected with the efficiency of transformation of *D. carota* cell cultures; such a relationship, however, was not found.

THE CELL CYCLE AND ENDOREDUPLICATION

Proliferating cells pass through four phases during the cell cycle: G_1, the period of cell growth during which the nucleus possesses a 2C DNA content; S, where DNA replication takes place, which results in a doubling of DNA content to 4C; G_2, a second growth period during which a nucleus retains its 4C DNA content; and M, mitosis, when DNA is equally divided into two daughter nuclei. Cells that leave the cell cycle enter the quiescent G_0 state usually from the G_1 phase, and thus they possess 2C DNA (Fig. 2A). Differentiating cells of some plant tissues/organs (e.g. endosperm, cotyledons, vascular tissues) go through repeated rounds of DNA replication that are not followed by mitosis (endocycles), resulting in endopolyploid (>4C) cells

(Fig. 2B). This process is called endoreduplication and occurs in most angiosperms (for review see Breuer et al., 2014). Since both the mitotic cell cycle and endoreduplication are characterized by changes in nuclear DNA content, they can be studied by FCM (Fig. 2).

Seed science and technology

One of the most common applications of studying the cell cycle by FCM is to follow the physiological status of seeds during development, maturation, and germination/priming (Sliwinska, 2009 and references therein). Studies, performed mostly on *C. annuum*, *S. lycopersicum*, and *B. vulgaris* seeds, have revealed that the ratio between 4C (G_2) and 2C (G_0/G_1) cells increases when the cell cycle is activated, which occurs at the early stages of seed development and during Phase II of germination (the phase that precedes radicle protrusion). In contrast, during seed maturation, when the cell cycle ceases, the proportion of G_2 cells decreases, and in some species only G_0/G_1 cells are present in dry mature seeds (e.g. *Cichorium endiva* or *Lactuca sativa*). Therefore, the 4C/2C ratio (the proportion between the number of nuclei with 4C DNA and the number of nuclei with 2C DNA) has been recommended to seed producers as a helpful marker in establishing optimal seed harvest time and for monitoring the progress of seed priming/conditioning (post-harvest hydration treatments of seeds to improve germination or seedling growth). This ratio has also been found to be a good indicator of deterioration and the extent of enhancement of deteriorated *Lens culinaris* seeds by matriconditioning (Sadowski and Sliwinska, 2007). In studies on the quality of *G. max* seeds harvested from nematode (*Meloidogyne javanica)*-infected mother plants, estimation of the 4C/2C ratio revealed that during germination the cell cycle was activated more slowly in these seeds than in those from uninfected plants (Forti et al., 2015). This confirmed the inhibitory effect of plant nematode infection on subsequent germination of the progeny.

In the seeds of some species, in addition to 2C and 4C cells there occur those with a higher DNA content (endopolyploid). During development, endoreduplication occurs mostly in the endosperm, but also in the embryo (with different intensities in different tissues/organs, depending on the species). The most extensively studied by FCM is the endosperm of *Z. mays* (Dilkes et al., 2002; Dante et al., 2014, and references therein). In this species (and also in other cereals), high mitotic activity is observed at the beginning of endosperm development; however, at later stages, mitosis ceases and the number of nuclei undergoing endoreduplication greatly increases; the DNA content in some can reach 768C. The starchy endosperm cells of *Z. mays* subsequently undergo programmed cell death (PCD), and the DNA content cannot be measured by FCM because of the destruction of nuclei. A different pattern of endoreduplication occurs in *C. sativus*. The DNA content in endosperm cells of this species reaches its maximum, 96C, about three weeks after pollination, and then the proportion of cells of high ploidies gradually decreases (Rewers et al., 2009). At harvest time, only 3C and 6C living cells are present, and those with a higher DNA content probably underwent PCD during seed development, like in *Z. mays*. In *C. sativus* embryos there is much lower endoreduplication (up to 16C) than in the endosperm. However, in some species belonging

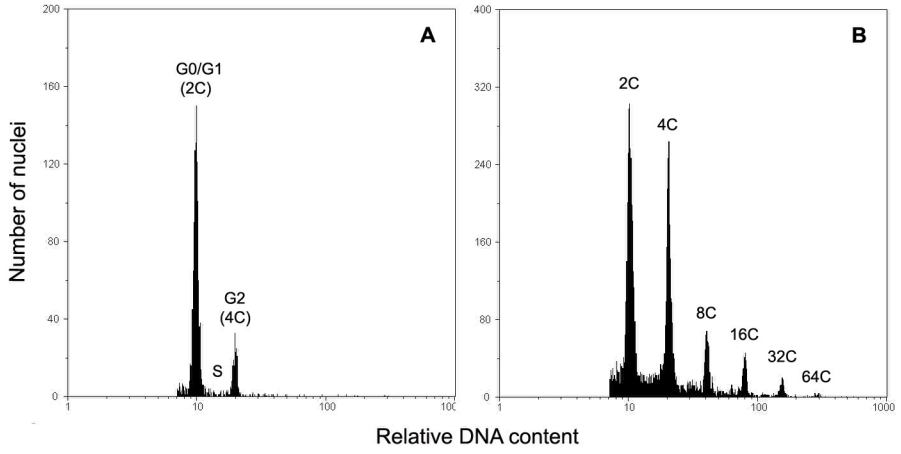

Figure 2. Histograms of relative DNA content in the nuclei of *Arabidopsis thaliana* young leaf (A) and hypocotyl (B); peaks correspond to the particular phases of mitotic cycle (A) or endoreduplication state (B)

to the Fabaceae family, where the endosperm is totally resorbed during embryo development, the cotyledons become the major storage organs, and they contain large, highly endopolyploid cells (Pal et al., 2004; Rewers and Sliwinska, 2012). The ploidy of some cotyledon cells of *Phaseolus vulgaris* at harvest can reach 128C. Endoreduplication intensity in the Fabaceae cotyledons depends on their type (persistent, non-persistent, haustorial) and can mark seed maturity. In contrast to the highly polysomatic (possessing somatic cells of different ploidies, including endopolyploid ones) species of the Fabaceae family, during the development of seeds of the orchid, *Phalaenopsis aphrodite* subsp. *formosana,* the cells possess, almost exclusively, 2C or 4C DNA, with only a small proportion (0.4-3.5%) of 8C cells (Jean et al. 2011).

During germination, two strategies seem to drive axis elongation and the protrusion of radicle through the seed coat. In polysomatic species, it is supposed to be endopolyploidization of cells (which coincides with their elongation; Sliwinska et al., 2009), and in non-polysomatic (possessing no endopolyploid cells) cell divisions. Indeed, during Phase II of germination, i.e. before radicle emergence from the seed, in most of the studied polysomatic species the proportion of endopolyploid cells increases, and the intensity of endoreduplication is different in different regions of the embryo axis (Rewers and Sliwinska, 2014). In species with epigeal seedling establishment (i.e. the cotyledons emerge above the ground following germination), such as *P. vulgaris* and *C. sativus*, endoreduplication during germination is highest in the transition zone (the region where the hypocotyl and radicle are joined), and in hypogeal species (i.e. the cotyledons remain below the ground after germination), e.g. *P. sativum* and *L. culinaris*, in the hypocotyl. For polysomatic species, the 4C/2C ratio is not recommended as a marker of seed maturity and germination/priming; instead, the $\sum$>2C/2C ratio, which includes also endopolyploid cells, should be used. Another coefficient for establishing endopolyploidy, proposed by Barow and Meister (2003), is the cycle value, which indicates the mean number of endoreduplication cycles per nucleus.

Plant growth and development

Endopolyploidy usually coincides with an increase in cell size and is, therefore, typical of storage cells and those expressing high metabolic activity; it is species- and organ/tissue-specific (Tab. 3). It occurs during development of such organs as leaves, flowers, fruits, and roots. Barow and Meister (2003), studying endopolyploidization in different organs of 54 seed plant species, including horticultural ones, concluded that the major factor determining the intensity of endoreduplication is the taxonomic position of the species. However, life cycle, genome size, and organ type are also important. They also suggested that endopolyploidization is a means of growth acceleration of a plant species growing in niches where there is a requirement for fast development. It has been suggested that growth by endoreduplication is more economical than that involving mitotic divisions because it does not require cytoskeleton rearrangements, chromosome segregation, or disruption and re-synthesis of the nuclear envelope (Pirrello et al., 2014). Thus, controlling endoreduplication can be agro-economically important. However, its functional role in plant growth and development is still vague, and FCM should be helpful in deciphering it.

An increase in endoreduplication during development is evident already in the young seedling. In *B. oleracea*, polysomaty was observed in the hypocotyl, cotyledons, and root as early as immediately after radicle protrusion, and during first leaf production the proportion of endopolyploid cells in these organs considerably increased, although not to the same extent in different organs (Kudo and Kimura, 2001a). FCM analysis of *B. oleracea* seedling leaves revealed that while in the youngest leaves only 2C and 4C cells were present, in mature leaves some cells of the midrib and petiole had undergone one endocycle and possessed 8C DNA. Similarly, progressive endoreduplication was observed during seedling development of *Allium fistulosum*, *Raphanus sativus*, and *Spinacia oleracea* (Kudo and Kimura, 2002a; Kudo et al., 2003, 2004).

For ornamental plants, the size of the flowers is a commercially-important trait. Agulló-Antón et al. (2013) showed a positive correlation between endopolyploidy, cell size and petal size of *Dianthus* and suggested that this could be used in breeding programmes aimed at obtaining cultivars with large flowers. Different parts of the flowers of a number of orchid species have been cyto-metrically analyzed (Tab. 3). Since orchids are currently mostly propagated by tissue culture, they are exposed to somaclonal variation, which can result in morphological changes to their flowers. Analysing somaclonal variants of *Phalaenopsis*, Lee et al. (2016) found that the degree of endoreduplication in the petals and flower stalks

Table 3. Endopolyploidy in different organs/tissues of horticultural species as established by FCM

Genus/species	Organ/tissue	Highest DNA content	Reference
Allium fistulosum	Leaf	16C	Kudo et al., 2003
Brassica oleracea	Shoot tip	4C	Kudo and Kimura, 2001a, 2001b, 2002b
	Leaf	16C	
	Cotyledon	16C	
	Hypocotyl	16C	
	Petal	32C	
	Sepal	16C	
	Carpel	8C	
	Root	16C	
Capsicum annuum	Leaf	4C	Ogawa et al., 2010; Olszewska et al., 2014
	Fruit	256C	
Cymbidium	Leaf	4C	Fukai et al., 2002
	Petal	16C	
	Ovary	8C	
	Pedicel	16C	
	Stalk	16C	
	Rhizome	16C	
Delosperma cooperi	Leaf	8C	Braun and Winkelmann, 2016
	Internode	8C	
	Petaloid staminodes	16C	
	Sepal	16C	
	Root	8C	
Dianthus	Leaf	32C	Agulló-Antón et al., 2013
	Petal	32C	
	Sepal	32C	
Hylocereus undatus	Flower	16C	De Menezes et al., 2016
	Fruit	16C	
Lampranthus	Leaf	16C	Braun and Winkelmann, 2016
	Cotyledon	16C	
	Internode	16C	
	Petaloid staminodes	16C	
	Sepal	16C	
	Root	16C	
Malus × domestica	Leaf	4C	Malladi and Hirst, 2010
	Fruit	8C	
Phalaenopsis	Protocorm	16C	Jean et al., 2011; Lee et al., 2016
	Leaf	8C	
	Petal	16C	
	Flower stalk	16C	
Pisum sativum	Cotyledon	32C	Lagunes-Espinoza et al., 2000; Rewers and Sliwinska, 2014
	Hypocotyl	16C	
	Root	8C	
	Pod wall	16C	
Portulaca grandiflora	Leaf	64C	Mishiba and Mii, 2000
	Sepal	64C	
	Petal	32C	
	Filament	32C	
	Anther	16C	
	Stigma + style	16C	

Table 3 continued.

Genus/species	Organ/tissue	Highest DNA content	Reference
Raphanus sativus	Shoot tip	4C	Kudo and Kimura, 2002a
	Leaf	8C	
	Cotyledon	16C	
	Hypocotyl	32C	
	Root	32C	
Solanum lycopersicum	Sepal	32C	Cheniclet et al., 2005; Pirrello et al., 2014
	Ovary	8C	
	Fruit	512C	
Spathoglottis plicata	Leaf	8C	Yang and Loh, 2004
	Root	8C	
Spinacia oleracea	Leaf	64C	Kudo et al., 2004
	Cotyledon	32C	
	Hypocotyl	32C	
	Root	32C	
Vanda	Leaf	32C	Lim and Loh, 2003
	Stem	2C	
	Petal	2C	
	Sepal	2C	
	Pedicel	2C	
	Aerial root	16C	
	Terrestrial root	32C	
Vanilla plantifolia	Young leaf	16E*	Brown et al., 2017
	Mature leaf	64E	
	Stem	64E	
	Seed	64E	

*Due to partial endoreduplication, C-value was replaced by E-value according to endoreduplication state

was higher in the variants than in control plants, and this corresponded with the diameter of the petal and the thickness of different organs, traits potentially desirable in orchid breeding. When applying FCM to analyse orchids, one must be aware, however, that in some Orchidaceae species partial endoreduplication occurs, which makes interpretation of the histograms challenging (Brown et al., 2017).

One of the best recognized endoreduplication patterns in a fruit is that of *S. lycopersicum* (Pirrello et al., 2014, and references therein). During development, pericarp cells undergo intensive endoreduplication, reaching as high as 512C DNA (Cheniclet et al., 2005). Studies of the fruits of 20 *S. lycopersicum* lines displaying a wide fruit weight range revealed a strong positive correlation between endopolyploidy, cell size and fruit weight. Surprisingly, in the pericarps of all the studied lines, a similar cell layer number and cell size occurred at anthesis, which suggests that polyploidy-associated cell expansion is the mechanism determining the final fruit weight. Ogawa et al. (2010) performed a similar experiment on the fruits of 12 *Capsicum* genotypes that differed in size and weight. Endoreduplication up to 256C was found in the pericarp, and the number of endocycles correlated with pericarp thickness, fruit diameter and fresh weight. Endoreduplication also plays an important role in the development of *C. sativus* fruit (Fu et al., 2010). The recognition of the controlling mechanism for fruit growth can be useful in plant breeding and our understanding of the establishment of fleshy fruit quality traits (Pirrello et al., 2014).

Studies have also been conducted on the influences of some environmental factors on endoreduplication. The importance of light in controlling endoreduplication intensity has been investigated the most thoroughly. Light, or its absence, appears to be a crucial factor for the regulation of endoreduplication intensity in the hypocotyl of polysomatic horticultural species such as *R. sativus*, *C. sativus*, *S. lycopersicum*, and *S. oleracea*; in dark-grown hypocotyls, enhanced

endopolyploidization accompanies their elongation (Amijima et al., 2014; Tanaka et al., 2016). In *B. oleracea* hypocotyls grown in darkness, about 10% of cells underwent an additional endocycle (reaching 32C), while in light-grown hypocotyls, cells with such a high DNA content were not detected (Kudo and Mii, 2004). In contrast, in the cotyledons of *R. sativus* and *C. sativus*, which expanded more in light than in darkness, the proportion of endopolyploid cells was higher in the light-grown seedlings (Tanaka et al., 2016). Low temperature decreased the growth rate and the rate of transition from a lower to higher C-value during the development of flowers of two orchid species (Lee et al., 2007). However, the distribution of the cells with different ploidies in fully-developed flowers was similar regardless of the growth temperature, and the authors concluded that the longer growth period 'compensated' for the decreased endoreduplication transition rate at low temperature. Another stress growth condition, water deficit, inhibited both cell division and endoreduplication in developing endosperms of *Z. mays* kernels (Setter and Flannigan, 2001).

Effect of cytotoxic compounds/elements

Another application of FCM is studying the effect of different cytotoxic compounds, often demonstrating allelopathic or anticancer activity, on the cell cycle or induction of PCD. The common strategy is to treat root meristems of a model plant with an extract of a plant species showing such activity and then study its effect by combined FCM and microscopic analyses. Using this strategy, Sánchez-Moreiras et al. (2008) found that benzoxazolin-2(3H)-one, an allelopathic compound produced by *Secale cereale*, retarded the cell cycle in *L. sativa* root meristems by blocking the G_2/M checkpoint. Similar effects on *A. cepa* root tips were induced by extracts of the medicinal plants *Rhodiola rosea*, *Taxus baccata*, and *T. brevifolia* (Majewska et al., 2000, 2003, 2008). Roots of *A. cepa* were also used to determine the mode of action of cyanamide, an allelochemical produced by *Vicia villosa* (Soltys et al., 2011). This compound caused changes such as inhibition of the cell cycle and modifications to the arrangement of the cytoskeleton, which, in turn, decreased *A. cepa* root growth rate. In the cyanamide-treated roots of *Z. mays,* in addition to a reduction in the frequency of cell divisions, the proportion of endopolyploid cells increased (Soltys et al., 2014). Application of plant hormones (usually to *in vitro* cultures) can also influence the cell cycle and endoreduplication. Treatment of *Doritaenopsis* cell suspension with 2,4-dichlorophenoxyacetic acid (2,4-D) caused an arrest of the cell cycle at G_2 phase and increased the proportion of endopolyploid cells, especially those with 8C DNA (Mishiba et al., 2001).

Anti-cancer activities of compounds produced by plants can also be studied by direct treatment of human/animal cells and FCM-based analysis of apoptosis and the cell cycle. Such studies have been performed on the anti-cancer effects of *Scutellaria* extracts using human malignant gliomas, breast carcinomas, and prostate cancer cells (Parajuli et al., 2009), and the lung adenocarcinoma mouse cell line has been used to study the cytotoxicity of steroid saponins produced by *Paris polophylla* (Yan et al., 2009).

FCM makes it also possible to study the effects of heavy metals on the cell cycle and endoreduplication. Fusconi et al. (2006) treated *P. sativum* roots with cadmium (Cd) and observed a progressive reduction in 2C and an increase in 4C nuclei, while the proportion of 8C nuclei was stable. This coincided with the blocking of mitosis and consequently an decrease in root growth.

CONCLUSIONS

FCM is broadly used for DNA content estimation in horticultural and medicinal species because it is fast, relatively cheap, accurate and convenient, and does not require any special preparation or a large amount of plant material. Even if a laboratory is not equipped with a flow cytometer, fresh material, which is the most suitable for analysis, can be sent by courier to an FCM laboratory and arrive within 1-2 days. Dry material, which is also useful for DNA content estimation, can be sent over any distance and stored without concern for its deterioration; this makes FCM analysis easily available for any laboratory. The necessity to establish DNA content has become obvious not only to researchers but also to plant breeders and seed producers. The most common application of FCM is establishing genome size and ploidy, which is helpful in studying species taxonomy, evolution and ecology, as well as being invaluable for polyploid and hybrid breeding. It is also important when biotechnological methods, such as micropropagation, haploid production, somatic hybridization, and transformation are used. Somaclonal variation that occurs in tissue cultures causes genetic instability of the plant material produced in these artificial conditions, and FCM is one of the methods which can detect changes in DNA at any stage of culturing, even

very early ones. Since genome size can relate to environmental conditions, its determination can be informative in studies of global climate change. Not only ploidy, but also the distribution of cells with different ploidies in certain organs/tissues is important for broadening our knowledge of plant genetics and physiology. Monitoring the cell cycle and endoreduplication by FCM is used to follow seed development, maturation, and advancement of germination as well as plant growth. Knowledge on the control of endoreduplication intensity in horticultural plants can potentially be used to improve commercially important traits such as the size of flowers or fleshy fruits. In medicinal plants, FCM can be applied to increase the production of desirable bioactive compounds, including their production in bioreactors. This technology also provides a useful approach in the study of allelopathic or anticancer effects of cytotoxic compounds. With the development of new ways to make genome modifications, FCM will doubtless be of increasing importance in the coming years.

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