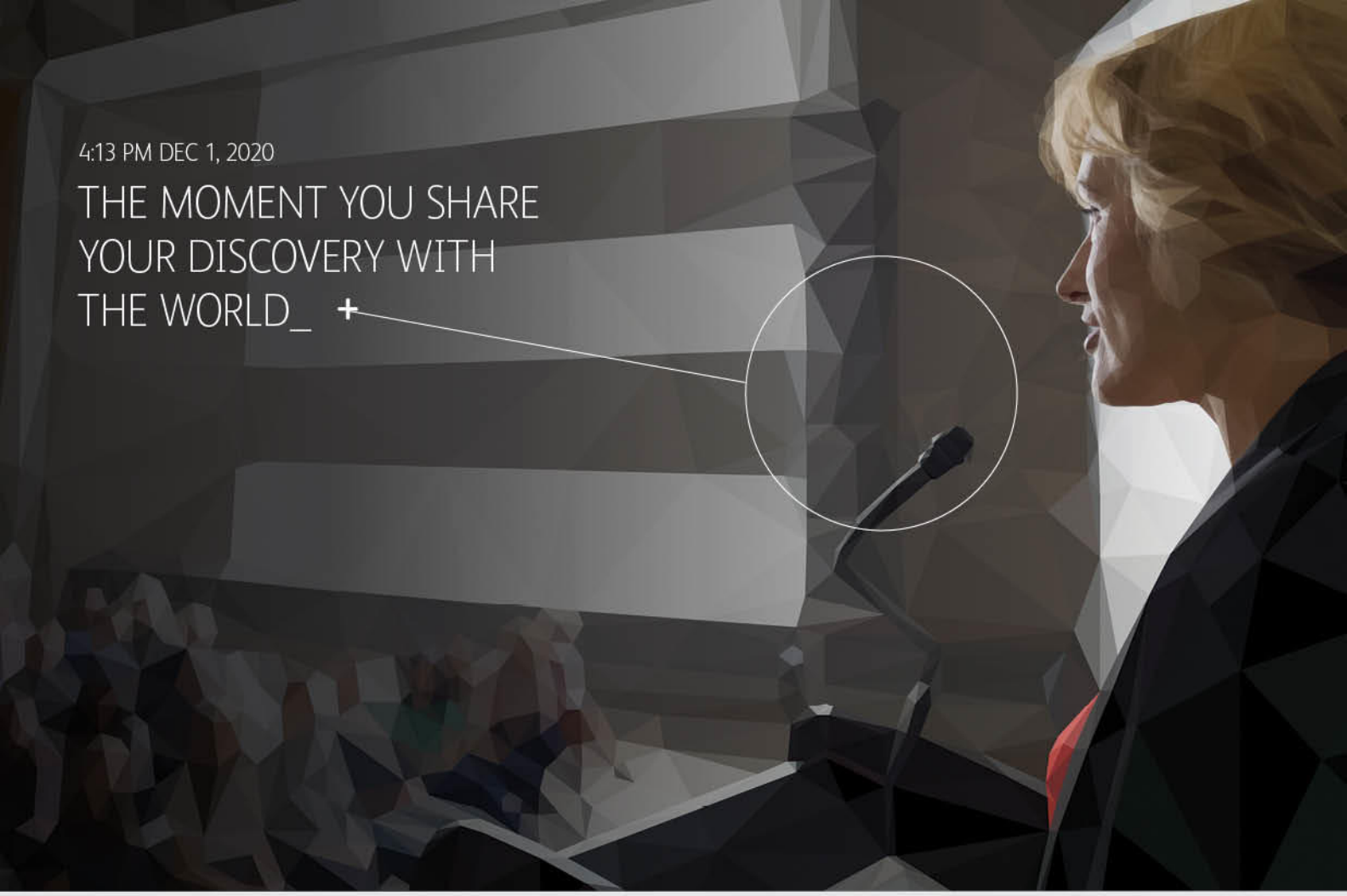

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Reliable DNA Ploidy Determination in Dehydrated Tissues of Vascular Plants by DAPI Flow Cytometry—New Prospects for Plant Research

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Background: Only fresh plant material is generally used for rapid DNA ploidy estimation by flow cytometry (FCM). This requirement, however, substantially limits convenient FCM application in plant biosystematics, population biology, and ecology. As desiccation is a routine way for sample preservation in field botany, potential utilization of dehydrated tissues of vascular plants in FCM research was examined.

Methods: Standard DAPI protocol was employed to evaluate the performance of 60 air-dried species, spanning more than 100-fold range of nuclear DNA amounts. Multiploid *Vaccinium* subg. *Oxycoccus* was selected as model taxon for detailed investigation and cytotype comparison.

Results: A majority of analyzed plants yielded distinct peaks with reasonable coefficients of variation after several months of storage at room temperature. Fluorescence intensity of nuclei isolated from desiccated tissues was highly comparable with that for fresh material, allowing reliable DNA ploidy estimation. Deep-freezer preservation substantially extended *Vaccinium* samples lifetime (at least to 3 years) and maintained high histogram resolution.

Conclusions: The introduced approach eliminates the need for fresh material in many vascular plants and thus opens new prospects for plant FCM. Convenient cytotype investigation in field research and retrospective ploidy determination in already herbarized samples are among the principal advantages. © 2006 International Society for Analytical Cytology

Key terms: DAPI; desiccated material; DNA ploidy; flow cytometry; herbarium vouchers; tissue preservation; *Vaccinium* subg. *Oxycoccus*; vascular plants

We have witnessed a rapid spread of flow cytometry (FCM) into various areas of plant sciences during the last two decades. The great popularity of this technique lies in its numerous advantages: (i) easy and convenient sample preparation that allows investigation of several dozens or even hundreds of samples per working day, (ii) high accuracy facilitating detection of minute variation in nuclear DNA amount, (iii) the possibility to investigate mitotically inactive cells from a broad variety of plant tissues (leaves, stems, roots, sepals, petals, seeds), (iv) non-destructiveness that opens a way for comprehensive investigation of rare and endangered species or seedlings in a very early ontogenetic stage, (v) rapid detection of mixed samples or endopolyploidy, and (vi) low operating costs (1). Without doubt, estimation of nuclear DNA content (either in relative or absolute units) is the most frequent application of FCM in plant research. Information gained about DNA ploidy level has been fruitfully utilized in plant biosystematics (reliable species determination in several closely related groups (2,3)), in breeding programmes (screening for suitable germplasm (4,5)), or in phytogeography and population biology (cytotype distribution at various spatial scales, insights into processes in diploid-polyploid sympatric populations (6–8)). Moreover, genome size data may also be beneficial in predicting various phenotypic characters and phenological and ecological behavior (9).

Fresh material is generally required for FCM acquisitions (10). This prerequisite, however, substantially limits the power of flow cytometry and precludes its comfortable utilization in plant biosystematics, population biology, and

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ecology. The majority of plant species must be analyzed within a rather short time interval because even turgor decrease may deteriorate the histogram resolution. The period might be extended by storing fresh moistened material in a refrigerator (for several days or a few weeks; pers. obs.). Two approaches have been proposed to overcome this limitation (11): (i) use of compact and portable flow cytometers operating in field conditions; (ii) use of fixed plant material (nuclei, cells, tissues).

There are two types of fixatives occasionally applied in FCM protocols working with vascular plants: formaldehyde and alcohol: acetic acid. Hülgenhof et al. (12) analyzed cereal nuclei fixed in a 1:1 mixture of 0.9% NaCl solution and ethanol, and concluded that combined staining (ethidium bromide + olivomycine) yielded symmetrical peaks with unimodal fluorescence distribution. Similarly, ethanol-fixed (70% or 95% EtOH) nuclei stained with DAPI were used for successful DNA content estimation in the graminoid genus *Paspalum* (13). Longer-term preservation of isolated nuclei was demonstrated by Chiatante et al. (14). Purified nuclei from meristematic pea root tissues could have been stored in 30% (v/v) glycerol at −20°C for several weeks without any appreciable loss of integrity. A similar protocol was adopted by Hopping (15) who found that nuclei of *Actinidia deliciosa* remained undamaged even after 9 months of storage in a freezer. However, their propidium iodide fluorescence intensity was about 5–7% lower than that of nuclei from fresh material. Although fixation generally did not increase coefficients of variation, aggregation of nuclei was often a side effect. An alternative approach was to analyze nuclei released from fixed cells or tissues. An effective but laborious and time consuming protocol involving ethanol: acetic acid cell fixation and enzymatic digestion of cell walls was reported for *Nicotiana tabacum* (16). Sgorbati et al. (17) were the first to demonstrate that mechanical chopping of formaldehyde-fixed leaves and roots in pea yielded a large number of nuclei suitable for DAPI flow cytometry. Nevertheless, only short-term storage of the fixed material (a few days or weeks) was realized, and the FCM performance of nuclei isolated from long-term-preserved tissues remains in need of a targeted study.

Herbarium voucher preparation (i.e. pressing and drying) is a conventional technique for material preservation in plant taxonomy, biosystematics, and ecology. As it is very simple, undemanding, and convenient, FCM investigation of dry samples would open new prospects for plant research and significantly increase the power of flow cytometry. Therefore, this study was targeted (i) to work out a procedure for DNA ploidy estimation in dry material and (ii) to find an effect of different drying and storing conditions as well as storing time on FCM measurements.

MATERIALS AND METHODS

Plant Material

Vaccinium subg. *Oxycoccus* (cranberry) was selected as the model taxon due to its considerable ploidy variation (2x, 4x, 5x, and 6x) that facilitates a comparative study of individual cytotype performance. In addition, our previous pilot investigation had revealed the feasibility of FCM analysis of 6-month-old herbarium vouchers. Plant material was collected in the field in October 2001 (2x + 6x – Šumava Mts.: peat bog Ždárecké rašeliniště 4x – Česká lípa: peat bog near Máchovo jezero lake; 5x – Šumava Mts.: peat bog Přední mlynářská sla't;) and immediately dried at four different regimes: (1) pressed in the desiccating cabinet at 40°C; (2) pressed at room temperature (ca. 20°C) with daily replacement of absorbent papers; (3) left freely at room temperature (ca. 20°C); and (4) left freely in the oven at 90°C. Desiccated plants were kept either at room temperature (RT) or in a deep freezer (−78°C; DF). FCM acquisitions were repeatedly performed over the course of 3 years in defined time intervals (once in 1–6 months). This design allowed us to examine the impact of different drying methods, different storage conditions, and the age of herbarium vouchers on histogram quality.

To gain information on FCM performance of a wider spectrum of vascular plants, 60 species representing 58 genera and 36 families were collected in October 2002 in the Botanical Garden of the Charles University, Prague. They included 3 spore-bearing taxa, 3 gymnosperms, 10 monocots, and 46 dicots. Their 2C-values ranged from 0.52 pg in *Holoschoenus romanus* to 55.1 pg in *Hippochaete ×moorei*, amounting to about a 106-fold difference. Plants were dried at 40°C (regime 1), stored at RT, and analyzed after 9 and 20 months.

Flow Cytometry

Sample preparation generally followed the procedure originally developed for human cells (18). Young intact leaves of analyzed plant(s) were chopped in 1 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20) with a new razor blade. About 0.5–1 cm^2 of the leaf tissue was used for fresh material; much greater quantities (ca. 5- to 10-fold) were essential to obtain sufficient numbers of nuclei from dehydrated tissues. The crude suspension was filtered through a 42 μm nylon filter and centrifuged at 150*g* for 5 min. The pellet was resuspended in 100 μl of fresh Otto I buffer and the samples were left for 30 min at RT. The staining solution consisted of 1 ml of Otto II buffer (0.4 M Na_2HPO_4. 12 H_2O) supplemented with DAPI (at final concentration 4 μg ml^{-1}) and β-mercaptoethanol (2 μg ml^{-1}). After 10 min of incubation at RT, fluorescence intensity was measured on a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp (HBO 100-long life). A simplified procedure without centrifugation was adopted for some species. In this procedure, only 0.5 ml of Otto I buffer was used for the nuclei isolation. The flow rate varied between 20 and 50 events per second, and the fluorescence of 5,000 particles was recorded. Histograms were evaluated using FloMax software (Partec GmbH, Münster, Germany). The Find peaks procedure was run, and coefficient of variation (CV) and maximum peak height were recorded. Analyses were mostly performed twice for each sample. The cytometer was adjusted so that the G0/G1 peak of the analyzed plant was located at channel 200 on

1024 linear scale. In *Vaccinium* subg. *Oxycoccus*, this channel corresponded to the position of tetraploid individuals. The same setting (identical gain) was adopted for all the cytotypes. To reduce nonspecific fluorescence signals, the lower threshold was always set to channel 70. Optimum instrument performance (to yield minimal CV, negligible background, symmetrical peaks, and a linear response) was checked on a daily basis using Partec UV-control calibration particles and fresh DAPI-stained *Pisum sativum* samples.

In the first step, specimens were analyzed without an internal standard to examine the afore-mentioned peak attributes. Except for *Vaccinium* subg. *Oxycoccus*, only histograms with CVs smaller than 10% and with a minimum peak height of 50 particles were accepted. Other histograms were discarded and corresponding samples were designated *no signal*. Simultaneous acquisitions with an internal standard of appropriate genome size (selected from the list of Doležel et al. (19)) were performed afterwards to test potential differences in DAPI fluorescence intensity between fresh and 20-month-old desiccated material. As the ploidy level of samples was always inferred from their nuclear DNA content, the term DNA ploidy level is adopted (20).

Statistical Analyses

Data were processed by the SAS 8.1 statistical package (SAS Institute, NC) using procedures UNIVARIATE (to test the data normality), ANOVA (to test between-cytotype temporal changes of two histogram attributes—CV and peak height), GLM (to evaluate the effect of selected factors—ploidy level, drying methods, storing conditions, and time of storage—on histogram attributes), and TTEST (to test differences of the afore-mentioned peak attributes between DF-stored and RT-stored samples at particular time).

RESULTS

FCM Performance of Dehydrated *Vaccinium* Samples

Herbarium vouchers of all cytotypes of *Vaccinium* sect. *Oxycoccus* released intact nuclei suitable for standard DAPI flow cytometry. The nuclei showed uniform fluorescence and produced distinct symmetrical peaks with reasonable CVs (Fig 1). For example, desiccated leaf tissues stored 9 months at RT yielded CV values largely comparable with those for fresh material (2.24–3.85% and 2.02–3.14%, respectively). In accordance with the expectation, recorded peak attributes had gradually changed with the ageing of the material: CVs had increased, peak heights had decreased, and background fluorescence had become more prominent. While the temporal change of the coefficient of variation (compared with the initial value for fresh material) did not differ among the four cytotypes ($P = 0.98$), peak height showed cytotype-specific alteration ($P = 0.02$). Hexaploid plants exhibited the most prominent decrease; after 36 months of storage, their peak height reached only 26.3% of the original size (in contrast to 37.5–51.4% in other cytotypes; see Fig. 1). Therefore, herbarium vouchers of high polyploids seem to have a shorter FCM lifetime than their

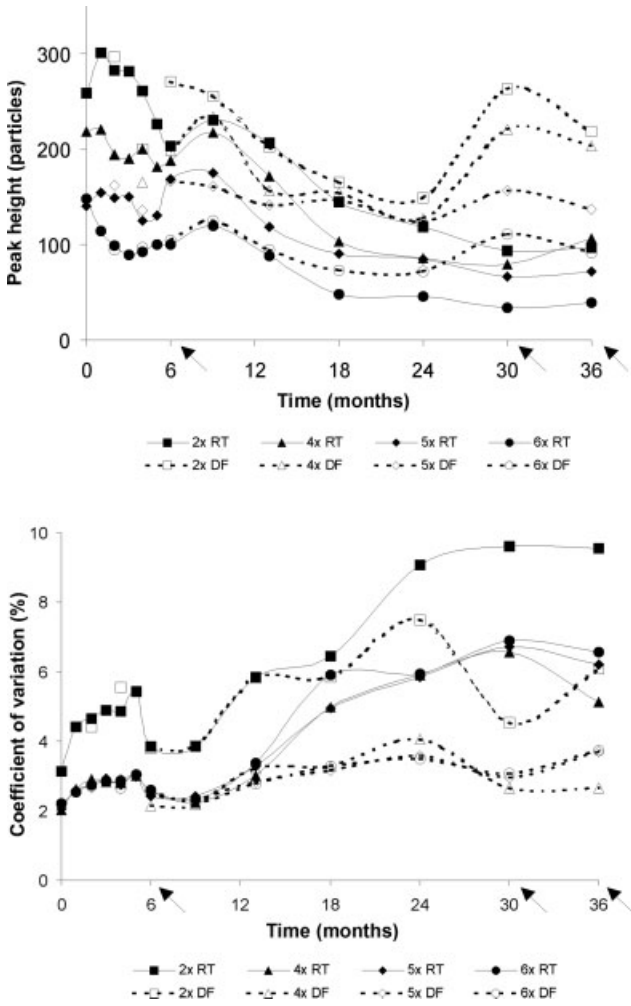

FIG. 1. Time-dependent variation of maximum peak height and coefficient of variation for particular cytotypes of *Vaccinium* subg. *Oxycoccus* stored at two different temperatures (room temperature; RT and deep freezer at −78°C; DF). Nuclei were mechanically isolated from dehydrated leaf tissues (herbarium vouchers) and stained with DAPI. The cytometer was adjusted so that the G0/G1 peak of the tetraploid cytotype was located at channel 200 on 1024 linear scale. Arrows indicate the recent replacement of the mercury arc lamp (otherwise the elapsed operation time varied between 50 and 150 h). Each point represents a mean from three runs (material dried at regimes 1, 2, and 3; see the text for explanation).

diploid or low polyploid counterparts because of reduced yield of intact nuclei. Indeed, investigation of several-year-old herbarium vouchers of various Ericaceae of known ploidy levels confirmed that diploid individuals produced distinct peaks more often than their polyploid counterparts of the same age (data not shown).

With the exception of destructive plant drying at a high temperature (regime 4), which gave no meaningful fluorescence signal, the mode of desiccation had little impact on peak quality in terms of both CV ($P = 0.75$), and maximum peak height ($P = 0.16$). Nevertheless, the essential requirement was a rapid desiccation process that precludes any tissue browning or even decaying accompanied by damage of nuclei. Attempts to use moistened leaf tissues (sunk either

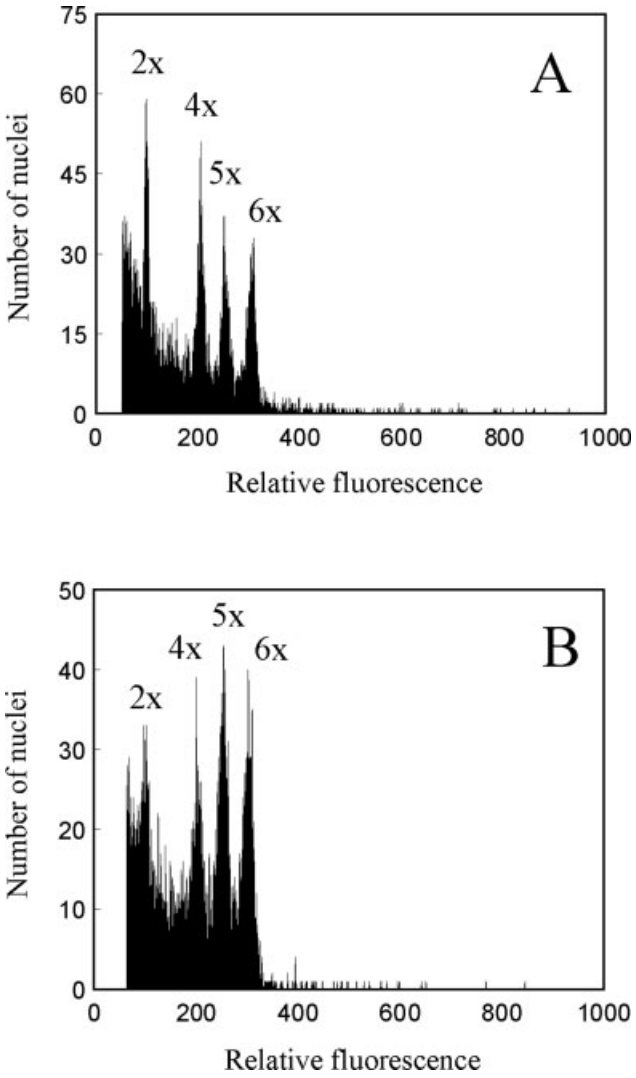

FIG. 2. Simultaneous analysis of DAPI-stained nuclei isolated from dehydrated leaf tissues (herbarium vouchers) of diploid (2x), tetraploid (4x), pentaploid (5x), and hexaploid (6x) cytotypes of *Vaccinium* subg. *Oxycoccus*. A: 9-month-old RT-stored material; B: 30-month-old DF-stored material.

in water or in Otto I buffer for various time intervals before sample preparation) did not lead to any histogram improvement. In contrast, peak attributes were sensitive to actual hardware settings, as inferred from the course of time series depicted in Figure 1. Although the instrument was always adjusted to yield optimal signal under given conditions, the recent replacement of the mercury arc lamp markedly increased the histogram resolution.

Naturally, a fundamental prerequisite for reliable DNA ploidy estimation in herbarium vouchers was the temporal stability of fluorescence intensity of dehydrated nuclei and therefore constant peak ratios between particular cytotypes. Simultaneous multi-ploidy analyses of dry material always gave peak ratios (nearly) identical to those from fresh tissue sessions (Fig. 2). Similarly, co-runs of nuclei isolated from fresh and dry leaves of the same cytotype always yielded a single peak, and the peak ratios remained (almost) unchanged at any combination of fresh and dry material (e.g. fresh diploid + dry tetraploid = dry diploid + fresh tetraploid). The maximum decrease of relative fluorescence of 3-year-old herbarized material of any *Vaccinium* cytotype (as compared to fresh tissues) did not exceed 6.5%.

Storage condition deserves special attention as this variable had a key effect on sample lifetime and both histogram CV and peak height ($P < 0.001$). DF-preservation considerably extended the storage time prior to successful FCM analyses and maintained high histogram resolution (compare the age of herbarium vouchers forming histograms in Fig. 2). Significant interactions between the time of storage and storage conditions ($P < 0.001$) allowed to perform further closer-insight test. After short storage time (1–13 months), recorded peak attributes were similar for both RT- and DF-kept vouchers (paired test, $P = 0.17–0.52$ and $P = 0.40–0.77$ for CV and peak height, respectively). With the time elapsed, however, the differences have become more prominent and after 18 months, the significance threshold was passed ($P < 0.001–0.03$ and $P = 0.002–0.03$ for CV and peak height, respectively). An illustrative example is the performance of 30-month-old tetraploid vouchers where peak quality for RT-stored samples (CV 6.57, maximum height 79 particles) markedly differs from that for DF-stored material (CV 2.66, height 221 particles) (see Fig. 1). Another significant finding associated with DF preservation was the extended stability of fluorescence intensity of dehydrated nuclei, showing virtually no shift in relative peak position after long-term storage. For instance, 3-year-old herbarium vouchers of the tetraploid RT-stored cytotype showed a 5.3% decline in relative nuclei fluorescence intensity, while DF-stored samples did not suffer from any fluorescence alteration. A representative histogram demonstrating constant fluorescence of a DF-preserved *Vaccinium* voucher is presented in Figure 3.

FCM Analysis of a Set of Dehydrated Vascular Plants

To achieve more representative and unbiased results, an estimation of relative nuclear DNA content was performed in herbarium vouchers of 60 species from 58 genera and 36 families. Surprisingly, a majority of the samples (86.7%) yielded distinct peaks after 9 months of storage at RT. Mean CV and maximum peak height were 5.00% (range 2.55–9.77%) and 144 particles (range 51–312), respectively (Table 1). Only eight species failed to produce acceptable histograms. Nevertheless, most of them posed serious problems already in fresh-tissue acquisitions probably due to high levels of secondary metabolites (e.g. *Jovibarba*: presence of organic acids; *Hedera*: tannins; and *Helianthemum*: mucilaginous compounds). After 20 months of storage, FCM investigation was still feasible in 43 out of 60 samples (71.7%). Naturally, peak attributes became worse; mean CV and maximum peak height were 6.42% (range 3.72–9.73%) and 94 particles (range 50–216), respectively (Table 1).

The use of an appropriate internal standard allowed us to examine a potential shift in nuclei fluorescence inten-

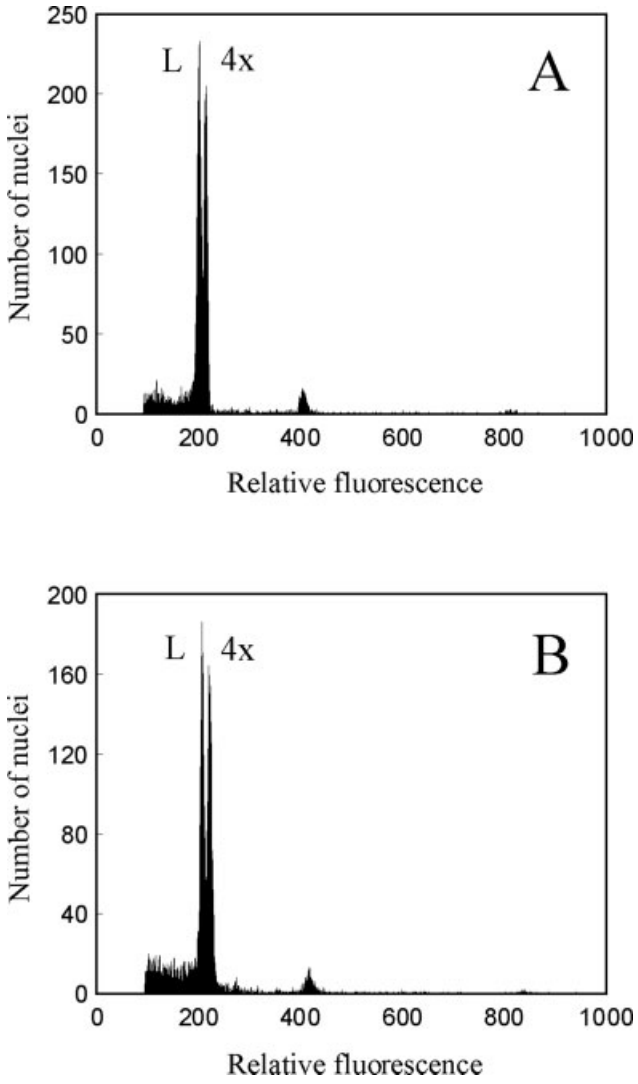

FIG. 3. Simultaneous analysis of DAPI-stained nuclei isolated from leaves of tetraploid *Vaccinium oxycoccus* (4x) and fresh *Lycopersicon esculentum* (L) as internal standard. A: Fresh *Vaccinium* plant (peak ratio 1.065, CV 1.40); B: 3-year-old herbarium voucher of *Vaccinium* preserved in deep freezer (peak ratio 1.072, CV 1.97).

sity between fresh and dry material. A certain decrease in DAPI fluorescence after tissue desiccation was often observed, ranging from 0.2 to 11.7% (mean 4.6%) in 20-month-old herbarium vouchers (Table 1). Nevertheless, the arbitrary threshold of 3% was not exceeded in nearly half of the samples, where the decrease may reflect rather instrumental drift than true variation. There was only a loose relationship between the fluorescence shift and either CV or maximum peak height.

DISCUSSION

DNA Ploidy Determination in Dehydrated Plant Material

Reliable estimation of DNA ploidy level in dehydrated samples of vascular plants (herbarium vouchers), using standard DAPI flow cytometry is demonstrated for the first time. This approach represents a new step in plant FCM and is believed to significantly enhance both the versatility and power of the technique. We are convinced that scientists of specializations involving cytotype investigation in field conditions (particularly plant taxonomists, biosystematists, and ecologists) will appreciate the feasibility of DNA ploidy determination in nonfresh samples as well as the simplicity of the protocol. Although this study was targeted on the investigation of herbarized wild plants, FCM measurements will likely be feasible also in various crop plants (e.g. grasses, alfalfa, etc.).

The requirement of living material has been repeatedly stressed as a crucial limitation for convenient FCM application in field botany (especially for expeditions to remote areas when rapid dispatch or maintenance of fresh samples becomes a difficult task (11)). Unlike routine investigation of fixed or frozen cells in animal FCM research (21), tissue preservation has only rarely been adopted in plant sciences (see a short notice about use of refrigerated, frozen or desiccated tissues in Dart et al. (22)). To deal with this issue, either transport of seeds instead of mature individuals, or commuting between the laboratory and the field was considered imperative. Our data demonstrated, however, that DNA ploidy estimation using FCM is feasible also in a large number of dehydrated vascular plant species, provided they were quickly and properly desiccated. Silicagel-dried samples are therefore just as suitable as traditional herbarium vouchers (pers. obs.). Genome size determination in dry material was previously successfully performed in up to 5-month-old vouchers of several mosses (23). These non-vascular plants are known to withstand long periods of drought without damage. Even intentional air desiccation did not have negative effects on nuclei integrity and subsequent propidium iodide fluorescence. Analogously, DAPI-stained nuclei released from dehydrated leaves of various vascular plants showed no signs of damage when evaluated by fluorescent microscopy in our study (data not shown).

The storage period of dry material we present, after which successful FCM analyses were achieved, is 36 months. This is significantly longer than the 9 months previously reported for samples treated with any chemical fixative (15). After 9 and 20 months of storage at RT, nearly 87 and 72% of the taxonomically-diverse herbarium vouchers, respectively, yielded acceptable histograms. Moreover, our pilot investigation revealed that a majority of examined vascular plants (incl. numerous taxa not presented in Table 1) produced distinct peaks shortly (several weeks) after their desiccation. This interval is considered long enough to allow the delivery of samples collected even in remote areas, and their subsequent DNA ploidy estimation. The maximum lifetime of dry leaf tissues to be analyzed by FCM seems highly species-specific. According to the current state of knowledge, it generally varies between 1 and 4 years for RT-stored material (the oldest successfully analyzed sample in our laboratory was a 6-year-old diploid voucher of *Empetrum nigrum*). Nevertheless, as noted earlier, the ploidy level and particularly

Table 1

Selected Peak Attributes (Coefficient of Variation and Maximum Peak Height) for DAPI-stained Nuclei Isolated from Fresh and Dehydrated Leaf Tissues (Herbarium Vouchers) of 60 Species from 58 Genera and 36 Families Stored at Room Temperature. The Cytometer was Adjusted so that the G0/G1 Peak of the Analysed Plant was Located at Channel 200 on 1024 Linear Scale. The Percentage Decrease in Nuclei Fluorescence Intensity of 20-Month-Old Dry Material (as Compared with the Fresh One) is Presented

		Fresh material		9 months old		20 months old		
Family	Species	CV (%)	Peak height	CV (%)	Peak height	CV (%)	Peak height	Fluorescence decrease (%)
Agavaceae	*Yucca filamentosa* L.	1.66	415	9.77	56	No signal	–	–
Apiaceae	*Petroselinum crispum* (Mill.) A. W. Hill	1.11	576	2.55	278	3.92	119	2.8
Apocynaceae	*Vinca major* L.	1.75	384	3.77	208	5.97	78	0.8
Aquifoliaceae	*Mahonia aquifolium* (Pursh) Nutt.	1.82	345	5.34	155	No signal	–	–
Araliaceae	*Hedera helix* L.	No signal	–	–	–	–	–	–
Aristolochiaceae	*Asarum europaeum* L.	1.71	418	4.83	137	5.95	96	7.1
Asparagaceae	*Asparagus tenuifolius* Lam.	2.12	253	3.34	225	5.74	79	4.7
Asteraceae	*Antennaria dioica* (L.) Gaertn.	2.13	350	8.89	65	No signal	–	–
	Bellis perennis L.	1.16	629	4.07	206	8.36	52	11.0
	Doronicum orientale Hoffm.	1.10	674	6.15	143	7.34	92	10.0
	Gnaphalium luteo-album L.	1.86	402	7.23	56	No signal	–	–
	Sonchus asper (L.) Hill	2.00	365	No signal	–	–	–	–
	Taraxacum sect. *Ruderalia*	1.40	450	8.87	74	No signal	–	–
Berberidaceae	*Berberis thunbergii* DC.			No signal	–	–	–	–
Brassicaceae	*Aurinia saxatilis* (L.) Desv.	2.05	352	3.60	146	3.72	130	0.8
	Iberis sempervirens L.	2.40	221	3.65	159	5.99	86	0.5
Buxaceae	*Pachysandra terminalis* Siebold & Zucc.	1.74	361	6.70	98	8.56	73	10.2
Cannaceae	*Canna indica* L.	1.56	412	No signal	–	–	–	–
Caryophyllaceae	*Dianthus gratianopolitanus* Vill.	1.66	351	3.57	188	4.26	145	3.2
	Minuartia verna (L.) Hiern. agg.	1.91	290	3.80	154	3.99	126	2.0
Cistaceae	*Helianthemum nummularium* (L.) Mill.	No signal	–	–	–	–	–	–
Crassulaceae	*Jovibarba globifera* (L.) J. Parn.	2.84	77	No signal	–	–	–	–
Cupressaceae	*Juniperus communis* L.	1.36	432	4.36	142	4.10	181	2.3
Cyperaceae	*Cladium mariscus* (L.) Pohl	2.60	289	6.04	95	7.68	58	0.9
	Holoschoenus romanus (L.) Fritsch	2.66	279	3.34	192	8.00	72	0.6
Equisetaceae	*Hippochaete* ×*moorei* (Newman) H. P. Fuchs	2.00	396	3.14	235	3.78	216	11.7
Ericaceae	*Calluna vulgaris* (L.) Hull	3.21	199	5.15	83	7.46	88	3.5
	Empetrum hermaphroditum Hagerup	2.14	237	5.26	100	5.78	126	1.6
	Rhododendron - evergreen cultivar	3.93	132	4.88	108	5.88	96	2.1
	Vaccinium myrtillus L.	2.82	174	4.12	112	5.97	103	2.9
Fabaceae	*Sarothamnus scoparius* (L.) W.D.J. Koch	2.38	321	3.39	178	7.33	50	2.9
	Trifolium hybridum L.	1.93	406	4.14	142	4.82	117	0.6
	Trifolium medium L.	1.84	368	6.00	109	6.03	82	0.3
	Ulex europaeus L.	1.49	526	3.30	161	4.80	124	7.7
Lamiaceae	*Ajuga reptans* L.	1.52	350	2.70	312	8.45	79	3.5
	Lavandula angustifolia Mill.	2.02	277			8.91	55	4.3
	Stachys germanica L.	2.70	272	3.39	217	4.67	160	1.9
Myrtaceae	*Myrtus communis* L.	4.93	132	No signal	–	–	–	–
Pinaceae	*Pinus sylvestris* L.	2.46	194	No signal	–	–	–	–
Plantaginaceae	*Plantago lanceolata* L.	1.77	360	7.33	113	No signal	–	–
	Plantago major L.	1.95	401	7.67	117	9.73	53	7.8
Poaceae	*Avenula* sp.	2.59	199	3.19	208	6.69	79	9.5
	Phalaris arundinacea L.	1.08	698	4.84	137	7.97	74	7.9
	Poa annua L.	1.39	436	3.56	156	9.43	51	1.9
	Festuca rupicola Heuffel	2.25	178	3.89	138	8.17	60	3.4
Polygonaceae	*Rumex obtusifolius* L.	1.51	419	6.01	78	7.33	60	5.4
Polypodiaceae	*Phyllitis scolopendrium* (L.) Newman	1.58	493	4.38	144	4.32	127	9.3
	Polypodium vulgare L.	1.27	485	2.58	275	3.74	163	4.0
Primulaceae	*Lysimachia nummularia* L.	1.61	371	8.61	51	7.97	82	7.3
Ranunculaceae	*Helleborus purpurascens* Waldst. & Kit.	1.41	434	4.82	141	6.39	88	9.8
	Ranunculus repens L.	1.11	682	5.86	97	No signal	–	–
Rosaceae	*Filipendula vulgaris* Moench	2.04	195	6.27	112	7.70	82	4.4
	Fragaria ×*magna* Thuill.	7.56	82			6.13	58	0.2
	Potentilla tabernaemontani Asch.	7.75	63	5.29	146	No signal	–	–
Rubiaceae	*Galium album* Mill.	1.95	333	5.39	103	8.71	62	8.2
Salicaceae	*Salix rosmarinifolia* L.	3.00	216	5.29	131	7.85	95	4.8
Saxifragaceae	*Saxifraga paniculata* Mill.	2.33	255	4.04	158	4.92	79	0.5
Scrophulariaceae	*Linaria genistifolia* (L.) Mill.	2.56	291	5.21	134	No signal	–	–
Smilacaceae	*Smilax aspera* L.	1.96	354	5.25	136	6.15	75	2.0
Taxaceae	*Taxus baccata* L.	1.54	413	4.99	106	5.26	78	10.0

the storage conditions may significantly alter the sample performance. To attain optimum results and extend the voucher lifetime, dehydrated material should be kept in a deep freezer.

Although the estimation of DNA ploidy was successful in a large proportion of dry samples, herbarium vouchers of some vascular plant species failed to yield any meaningful FCM output. Unfortunately, every attempt to find one or more characters that would predict the feasibility and accuracy of FCM assays was in vain. Plants with rigid leathery leaves, covered with a wax layer, and therefore resistant to water stress, are more likely to yield superior FCM profiles. On the contrary, species rich in secondary metabolites (polyphenols, organic acids, etc.) or rapidly loosing their turgor are bound to produce low-quality histograms or even give no meaningful signal. Generally, histogram quality (inferred from the peak height and CV value) showed continuous variation with significant differences between particular genera and families (Table 1). Importantly, FCM analyses were not negatively influenced by standard anti-infestation treatments occasionally applied in herbaria, such as pyrethroide gassing (pers. obs.).

The selection of alternative plant tissues instead of traditional leaves may improve the efficiency of FCM acquisitions. For instance, nuclei isolated from the stems of dehydrated *Pimpinella* species constituted superior peaks in comparison with their leaf counterparts (pers. obs.). Similarly, seeds might be used for DNA ploidy determination in older vouchers (24), as they usually have a longer FCM lifetime than vegetative tissues. Care must, nonetheless, be taken when interpreting these histograms as: (i) nuclei of two different ploidy levels (embryo + endosperm) may co-occur in the seed (25); (ii) a large proportion of embryonic nuclei might be arrested in the G2 phase of the cell cycle, leading to an incorrect DNA ploidy estimation (26); (iii) the ploidy level of progenies (seeds) might differ from that of maternal plants (due to heteroploid hybridization, involvement of nonreduced gametes, or haploid parthenogenesis). Therefore, seed-based acquisitions should better be avoided in groups with versatile breeding systems (e.g. *Hieracium* subg. *Pilosella*) that can easily generate progenies of several different ploidy levels (27).

Comparison with Other Methods

Although the estimation of nuclear DNA content in nonfresh material has been a continuously debated issue of plant FCM (11), only a few studies have taken the challenge and implemented nuclei, cell, or tissue preservation using nonadditive (alcohol: acetic acid) or additive (formaldehyde) fixatives (12–17). Fixation is undoubtedly a very helpful approach, but several weak or questionable points must be taken into account: (i) fixed nuclei often tend to clump; (ii) fluorescence intensity of DNA dyes might be altered by the fixation procedure (28); (iii) non-additive fixatives are known to release tannins from vacuoles that may subsequently interfere with quantitative DNA staining (29); (iv) some protocols are so demanding that their completion outside the laboratory is hardly feasible (16); (v) only short-term storage of the fixed material has mostly been realized. For these reasons, FCM investigation of chemically-fixed samples has gained only little attention from field-oriented plant researchers.

On the contrary, our protocol exploiting desiccated samples eliminates some of the afore-mentioned inconveniences. Voucher preparation by air drying is perfectly simple and can be realized virtually anywhere. Nuclei from dehydrated tissues are isolated just before the FCM acquisition, and therefore, any aggregation is avoided. In addition, air-dried samples retain their ability to allow successful FCM analyses longer than fixative-preserved ones. Indeed, no other method has permitted ploidy determination in several-year-old vouchers of vascular plants. Considering the methodological simplicity of DNA ploidy estimation in dehydrated plant tissues, it is almost incredible that such a beneficial approach has been neglected up to now. A possible explanation lies in the only recent spread of FCM into plant disciplines routinely working with herbarized material (taxonomy, systematics, and ecology).

A certain decrease in DAPI fluorescence intensity (0.2–11.7%) often occurred in nuclei isolated from long-term-stored samples (Tab. 1). An identical effect was previously reported also for propidium-iodide-stained nuclei of *Actinidia deliciosa* preserved for 9 months in 30% glycerol at −18°C (15). The nature of the observed fluorescence change, however, remains unknown. It could possibly be caused by altered accessibility of fluorochromes to DNA, nucleic acid loss, or some other reason. Despite a certain shift in fluorescence, the reliability of DNA ploidy estimation in desiccated material is not compromised. In low polyploids, the rise of relative DNA content between successive cytotypes is sufficiently high to obscure a potential few-percent decrease due to long-term storage. More care should be given to the discrimination of high polyploids because the relative between-cytotype difference in DNA amount decreases with increasing ploidy level. Such samples should preferably be analyzed shortly after desiccation while there is virtually no decline in fluorescence intensity. Similarly, DF-preservation seems to be an efficient way of ensuring unchanged nuclei fluorescence. Anyway, absolute genome size determination using DNA intercalators in dehydrated plant material should be avoided (because of both fluorescence decrease and higher CVs).

Prospects

Various lines of plant research (particularly taxonomy, population biology, and ecology) should benefit from the introduced way of easy and reliable DNA ploidy estimation in dehydrated material. There are only two basic prerequisites for FCM investigation of nonfresh samples: rapid desiccation and DF-preservation (if longer-term storage is required). Naturally, not all vascular plants are able to withstand this treatment without apparent damage. Most of them, nonetheless, yield distinct FCM histograms. The described approach significantly simplifies the transport of samples from remote areas, facilitates retrospective DNA ploidy determination in already herbarized samples,

and allows to postpone analyses if the capacity of a laboratory is saturated.

There are numerous issues concerning FCM investigation of dry samples that need to be solved in the future: (i) What is the origin of nuclei released from dehydrated tissues; is it the whole body or just some particular layer? (ii) Are there any sample attributes allowing to predict the feasibility and quality of FCM assays? (iii) What is the cause of the nuclei fluorescence decrease in long term-stored samples? (iv) Which sample features influence the degree of fluorescence decrease (our preliminary data indicate higher fluorescence stability in plants with low nuclear DNA content)? (v) How long may samples be stored without apparent deterioration in various conditions? (vi) May other isolation and staining buffers improve the histogram quality? We believe that our work will stimulate further research into the utilization of nonfresh samples in plant FCM.

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