Universidade de Aveiro Departamento de Biologia
2007

João Carlos Mano Loureiro

APLICAÇÃO DA CITOMETRIA DE FLUXO AO ESTUDO DO GENOMA VEGETAL

FLOW CYTOMETRIC APPROACHES TO STUDY PLANT GENOMES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Conceição Santos, Professora Associada do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Professor Doutor Jaroslav Doležel, Professor Associado da Universidade Palacky, Olomouc, República Checa e Chefe do Laboratório de Citogenética Molecular e Citometria do Instituto de Botânica Experimental, Olomouc, República Checa.

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agradecimentos / acknowledgements

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palavras-chave

ácido tânico, base de dados FLOWER, citometria de fluxo, compostos citosólicos, espécies lenhosas, estabilidades do nível de ploidia, genoma vegetal, hibridização *in situ*, nível de ploidia, tamanho do genoma, tampões de isolamento nuclear

resumo

Hoje em dia, a aplicação da citometria de fluxo (CMF) no estudo do genoma vegetal consiste maioritariamente em análises do nível de ploidia e em estimativas do conteúdo em ADN nuclear. Esta Tese de Doutoramento tem como objectivo aplicar a CMF e outras técnicas citológicas, como a microscopia de fluorescência e a hibridização *in situ*, no estudo do genoma vegetal.

O primeiro Capítulo introduz a CMF, nomeadamente o seu funcionamento, aspectos metodológicos da análise do conteúdo em ADN nuclear em plantas, e aplicações da CMF na biologia vegetal.

No Capítulo II são apresentados dois estudos sobre a estabilidade do nível de ploidia durante o processo de cultura *in vitro* de sobreiro (Capítulo II.1) e zimbro (Capítulo II.2), duas espécies lenhosas de elevada importância económica e ecológica.

No Capítulo III são apresentados os resultados das análises de conteúdo em ADN nuclear efectuadas em três grupos de espécies vegetais. No Capítulo III.1 a CMF foi utilizada para estimar pela primeira vez o tamanho do genoma nuclear de cultivares de oliveira e zambujeiro; a variação intraspecífica como tópico de elevado interesse é aqui discutida tendo em consideração as boas práticas. No Capítulo III.2 foi analisado o conteúdo em ADN nuclear de três espécies de Ulmaceae da Península Ibérica que contêm compostos mucilaginosos, conhecidos por interferirem com as análises por CMF; é também apresentado um protocolo eficiente que previne o efeito negativo deste compostos e permite uma correcta análise do tamanho do genoma de espécies com problemas similares. No Capítulo III.3 é apresentado um estudo extensivo de CMF e citogenética molecular realizado em espécies de festuca da Península Ibérica.

Considerando os resultados obtidos nos Capítulos II e III, no Capítulo IV foram efectuados estudos inovadores de índole metodológica. No Capítulo IV.1 é apresentada a primeira comparação sistemática de tampões de isolamento nuclear, enquanto no Capítulo IV.2 é estudado o efeito negativo do ácido tânico, um composto fenólico conhecido por interferir com os núcleos vegetais e com as estimativas de conteúdo em ADN. De acordo com os resultados obtidos nestes estudos, no Capítulo IV.3 foram testados dois novos tampões de lise nuclear em 37 espécies vegetais.

No Capítulo V é introduzida a base de dados “Plant DNA Flow Cytometry (FLOWER)”. Esta base de dados contém informações retiradas de mais de 700 publicações na área da CMF vegetal, disponibilizando-as de uma forma atraente e permitindo a realização de análises quantitativas respeitantes a muitos tópicos importantes.

Finalmente, no Capítulo VI são apresentadas as conclusões da presente Tese de Doutoramento, com especial incidência nas boas práticas necessárias para estimar o conteúdo em ADN nuclear em plantas e nas direcções futuras da CMF no estudo do genoma vegetal.

keywords

cytosolic compounds, DNA ploidy level, flow cytometry, FLOWER database, genome size, *in situ* hybridization, nuclear isolation buffers, plant genomes, ploidy stability, tannic acid, woody plant species.

abstract

Nowadays, the application of flow cytometry (FCM) to study plant genomes is mostly focused on DNA ploidy level analyses and nuclear DNA content estimations. The objective of this PhD Thesis is to applly FCM and related techniques, as fluorescence microscopy and *in situ* hybridization, to study plant genomes.
The first chapter introduces FCM, namely the functioning of this technique, the methodological aspects of the analysis of nuclear DNA content in plants, and the applications of FCM in plant sciences.
In Chapter II, studies of ploidy stability of the *in vitro* culture process of two economically important woody plant species, *Quercus suber* (Chapter II.1) and *Juniperus phoenicea* (Chapter II.2), are presented.
In Chapter III, three studies regarding nuclear DNA content analyses in plant species are given. In Chapter III.1 the genome size of olive cultivars and wild olive was estimated for the first time using FCM; the hot topic of intraspecific variation is discussed here with respect to best practices. In Chapter III.2, the nuclear DNA content of three Ulmaceae species, containing mucilaginous compounds that interfere with FCM analysis, is analysed; an efficient protocol that circumvented this problem and enabled reliable genome size estimations in these species is presented. A through flow cytometric and molecular cytogenetic study on fescue species of the Iberian Peninsula is performed in Chapter III.3.
In light with the results obtained in Chapters II and III, innovative methodological studies were performed in Chapter IV. In Chapter IV.1 the first systematic comparison of lysis buffers is presented, while in Chapter IV.2 the negative effect of tannic acid, a common phenolic compound known to interfere with plant nuclei and DNA content estimations, is analysed. According with the results obtained in these studies, in Chapter IV.3 two new lysis buffers were tested with a set of 37 plant species.
In chapter V the Plant DNA Flow Cytometry Database (FLOWER) is introduced. The FLOWER database collects information from more than 700 publications in this area of FCM, and makes it accessible in one user-friendly design that enables quantitative analysis of many important topics.
Finally in Chapter VI the conclusions of the present PhD Thesis are presented with special focus on a compilation of best practices for nuclear DNA estimation using FCM and the future directions on the use of this technique to study plant genomes.

"A scientist is happy, not in resting on his attainments but in the steady acquisition of fresh knowledge." - Max Planck

“In my head nothing is lost, everything is transformed.” - João Loureiro adapted from the Law of Conservation of Mass, Antoine Lavoisier.

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Abbreviations:

2,4-D – dichlorophenoxyacetic acid;

ADC – analogue-to-digital converter;

AFLP – amplified fragment length polymorphism;

BAP – 6-benzylaminopurine;

BF – background factor;

BP – band-pass;

bp – base pairs;

BrdU – 5-bromo-2'-deoxyuridine;

CV – coefficient of variation;

DAPI – 4',6-diamidino-2-phenylindole;

DI – DNA fluorescence index;

DIG – digoxigenin;

DKW – Driver and Kuniyuki medium;

DTT – dithiothreitol;

EB – ethidium bromide;

EDTA – ethylenediaminetetraacetic acid;

FACS – fluorescence activated cell sorter;

FCM – flow cytometry;

FCSS – flow cytometric seed screen;

FISH – fluorescent *in situ* hybridization;

FL – fluorescence light;

FPCV – full peak coefficient of variation;

FS – forward scatter;

GFP – green fluorescent protein;

GISH – genomic *in situ* hybridization;

HEPES – 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid;

HO – Hoechst;

IAA – indole-3-acetic acid;

IBA – indole-3-butyric acid;

KIN – kinetin;

laser – light amplification by stimulated emission of radiation;

log – logarithmic;

LP – long-pass;

MOPS – 4-morpholinepropane sulfonate;

MS – Murashige and Skoog medium;

MSWH – MS medium without growth regulators;

NAA – naphthaleneacetic acid;

OM – Rugini olive medium;

PBS – phosphate buffered saline;

PCR – polymerase chain reaction;

pg – picograms;

PI – propidium iodide;

PVP – polyvinyl pyrrolidone;

RAPD – random amplification of polymorphic DNA;

RBC – red blood cells;

RFLP – restriction fragment length polymorphism;

SD – standard deviation;

SE – somatic embryos;

SP – short-pass;

SS – side scatter;

SSR – simple sequence repeats;

TA – tannic acid;

TRIS – tris-(hydroxymethyl)-aminomethane;

UPGMA – unweighted pair group method with arithmetic mean;

WPM – woody plant medium;

YF – yield factor.

Chapter I

Plant flow cytometry – a general introduction

Chapter partially published as an original article in a non indexed national journal:
Loureiro J, Santos C (2004) Aplicação da citometria de fluxo ao estudo do genoma vegetal. Boletim de Biotecnologia **77**:18-29 (in Portuguese).

Flow cytometry – definition and brief history

Cytometry is a process in which physical and/or chemical characteristics (light scatter, fluorescence) of single particles (biological or non biological) are measured. In flow cytometry (FCM), the measurements are made as particles pass one by one through the interrogation point, in a narrow liquid stream (Shapiro 2004). The measurement in flow enables high speed analyses (10^2 – 10^3 particles/s) and random selection of particles from the whole population without any bias (Doležel 1997).

It is believed that the first description of a FCM device dates back to 1934, when Moldavan suggested counting cells in a fluid stream (Bennett and Leitch 2005*a*). However, it seems that Moldavan failed to develop a functional apparatus, and only thirteen years later, after demands of the US Army (in the World War II period) for the development of an equipment that could rapidly detect bacteria in aerosols, Gucker and co-workers (Gucker *et al.* 1947) developed what is generally recognized as the first flow cytometer used for observation of biological cells. In this instrument a sheath of filtered air was flowing through a dark-field illuminated chamber (Shapiro 2004). The attempt of several industrial organizations to build up similar apparatus resulted, in early 1950s, in an alternative flow-based method for cell counting. In 1953, Crosland-Taylor adapted the sheath-flow principle for counting of red blood cells in saline solutions, and in 1956, Wallace Coulter developed the first instrument that proved effective in the counting and sizing of blood cells (Shapiro 2007). This electrical engineer reasoned that, due to the poor conductivity of cells when compared with saline solutions, cells suspended in such a solution and passing one by one through a small orifice (< 100 µm), would be detectable by a change in electrical impedance of the orifice, proportional to the cell volume, and that resulted in a voltage pulse (Shapiro 2004). The Coulter counter (Coulter Electronics, now Beckman Coulter®, Hialeah, FL, USA) was soon widely adopted in clinical laboratories and current instruments are still based in this principle.

In the following decade with the need to extract cells with known measured characteristics, the first flow sorters were developed. A flow sorter is a flow cytometer that uses electrical and/or mechanical means to divert and collect particles with measured characteristics that fall within a user-selected range of values (Shapiro 2004). The most popular system was that of Mack Fulwyler (Robinson 2005) who, working at the Los Alamos National Laboratory, adapted the then recently developed ink jet printer technology, *i.e.*, after the passage through the cytometer measuring system, the saline stream was broken into droplets, and those that contained the particles of interest were

electrically charged at the droplet break-off point; the charged droplets were then deflected into a collection tube by an electric field, while the uncharged ones were directed to a waste compartment (Shapiro 2007).

The late 1960s saw the introduction of fluorescence measurement (*i.e.*, emission of longer wavelengths when electrons return from the excited state to the ground state) to FCM, which broadened the range of applications of this technique and enabled the spread of multiparameter analyses. The first commercial fluorescence flow cytometer, the Cytofluorograph (Bio/Physics Systems, Mahopac, NY, USA), appeared in 1970 and since that time more and more powerful apparatus have been developed, mainly by two manufacturers, Becton-Dickinson (now BD Biosciences®, San Jose, CA, USA) and Coulter Electronics (now Beckman Coulter®). Particularly important was the development in 1974 of the first commercial cell sorter, coined Fluorescence Activated Cell Sorter (FACS), an acronym still in use nowadays, although it is sometimes erroneously applied to instruments without sorting capabilities.

In the last decades, advances in technology led to the development of smaller apparatus, *i.e.*, bench-top analysers with basic components fit for a routine use in laboratories, and more complex instruments capable of measuring many properties from particles at increasingly higher rates and sensitivity, available in a relatively small community of academic, government and industrial laboratories (Suda 2004).

In recent years this area also experienced the development of a continuously expanding array of reagents, fluorescent labels and highly sophisticated data analysis procedures, which make FCM an exceptionally effective, accurate, and precise technique to obtain unbiased and quantitative information from single cells. Its principal disadvantages being the complexity and cost of the apparatus (Shapiro 2007). All the potentialities of FCM spread its application to various fields of biological sciences, being routinely used in clinical diagnostics, biotechnology, and basic and applied research.

Instrumentation and functioning

A typical flow cytometer consists of fluidic, optical, electronic, computational and mechanical features (Sklar 2005).

The **fluidic system**, by means of hydrodynamic focusing, generates a stable fluid stream in which particles are confined into a narrow central core and delivered one at a time along the same path to the focal point of the light source. The flow chamber (= flow cell) represents the central part of the instrument and is considered the most important fluidic component (Fig. I.1). The flow cell type considered here as an example, *i.e.*, the

"enclosed stream", contains a rectangular channel with a sensing area at its centre. A pressurized stream of sheath fluid (either water or saline solution) enters the channel at the lower end and flows upwards. While the sheath stream is flowing through the channel, a stream of sample is injected into the middle of the sheath stream. The sheath stream surrounds, but does not mix with the sample stream and, due to a greater pressure of the sheath stream, the sample stream is focused, so that the particles flow through the laser beam in a single file, *i.e.*, one at a time (Anonymous 2002). Typical stream velocities are between 1 and 10 m/s, resulting in analyses of 100 to 1,000 cells/s (Doležel 1991).

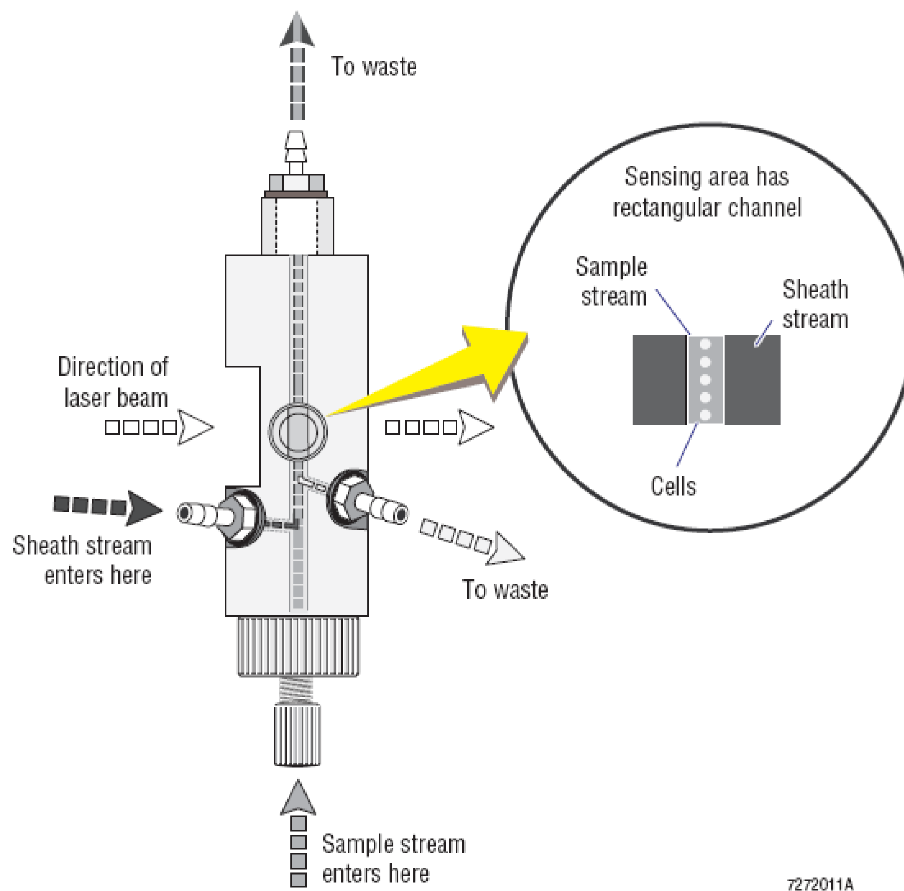

Fig. I.1 Diagram of an "enclosed stream" flow chamber (adapted from Anonymous 2002).

The "enclosed stream" configuration described above is used in most bench-top flow cytometers and operates with velocities lower than those of the "jet-in-air" design, which may result in higher detection sensitivity. The "jet-in-air" configuration is common in most cell sorters. In this system, the sample stream exits from the flow chamber into open air by orifices of narrow diameter (thus the high speed), where it is intersected by the light beam. In the "jet-on-open surface" design, particles are measured in a liquid stream while it is

flowing on a glass surface (Suda 2004). This configuration is mostly found in some arc lamp based instruments.

The main functions of the **optical system** are to allow particles to be illuminated by one or more light sources and to resolve and direct the subsequent scattered light and multiple fluorescence signals to individual detectors (Sklar 2005). Therefore the optical system can be divided in two main operational units: light source and detector system (Fig. I.2).

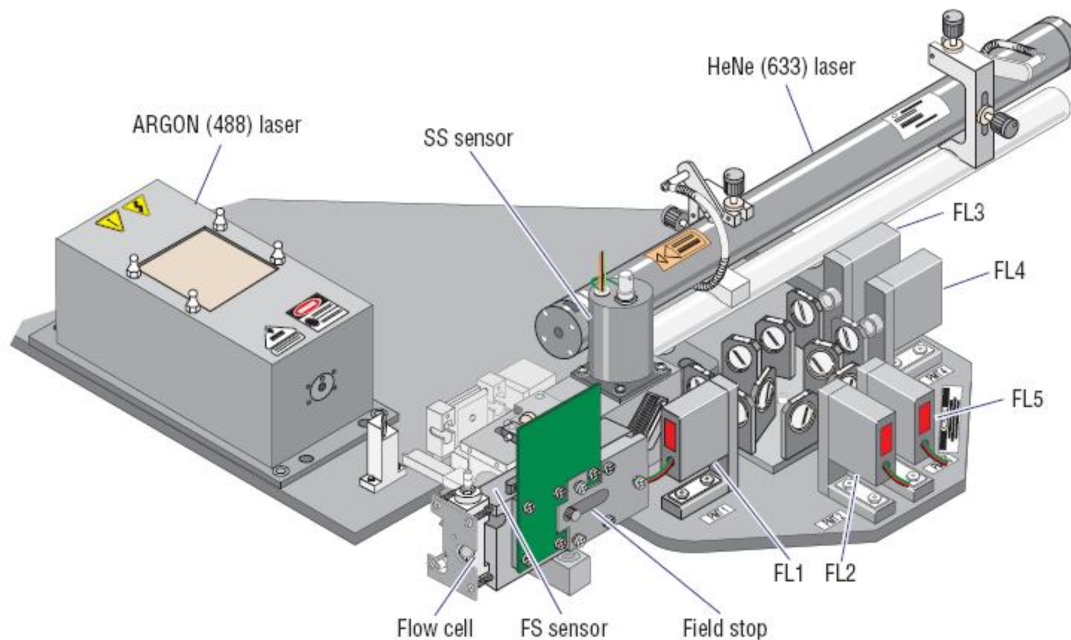

Fig. I.2 Optical system of the FC500 flow cytometer from Beckman Coulter® with two light sources, two light scatter sensors (FS and SS) and five fluorescence light (FL) sensors (adapted from Anonymous 2002).

Two types of illumination are found in most commercial flow cytometers: lasers and/or arc lamps.

Arc lamps, especially the high pressure mercury arc lamps, are a common, relatively inexpensive source of fluorescence excitation light that delivers a continuous, broad spectrum with several intense lines. With the appropriate selection of filters, light with a wide range of wavelengths can be selected to excite a variety of fluorescent stains (Peters 1979). Despite having a shorter lifetime (up to 400 h) than lasers (up to 5,000 h), the low cost and easy maintenance of arc lamps are among their principal advantages (Suda 2004). However, if for strongly fluorescent objects, arc lamp illuminators can yield fluorescence intensity histograms of equal resolution to laser-illuminated systems, for weakly fluorescent particles, high resolution can only be achieved using high power lasers (Peters 1979). In plant sciences arc lamps are mainly used for dyes excited in the UV region and are well suited for less demanding ploidy level estimations.

Lasers (acronym for "light amplification by stimulated emission of radiation") produce a stable, bright, narrow beam of monochromatic light at specific wavelengths (Carter and Ormerod 2000). The emission produced by lasers is confined to a very small solid angle, making it possible to focus almost all the energy of the beam to a circular or elliptical spot (higher sensitivity) (Shapiro 2004). However, they only emit distinct spectral lines over a narrow range and will not optimally excite some of the commonly used stains (Peters 1979). Most of the bench-top flow cytometers are equipped with an air-cooled argon ion laser tuned at 488 nm (it is the case of the flow cytometer available at the Department of Biology of University of Aveiro, Portugal). Helium-neon (the most common emits red light at 633 nm) and helium-cadmium (emitting either in the blue, 442 nm, or in the UV, 325 nm) lasers are often available in more complex systems, which are usually equipped by more than one laser (*e.g.*, Fig. I.2). The more recent, red diode (emitting at 635 nm) and solid state lasers (emitting at violet, blue or green light) are more compact and are characterized by lower optical noise levels than argon ion and helium-neon lasers. Therefore in some recent apparatus these lasers are frequently used (*e.g.*, CyAn™ ADP Analyser from DakoCytomation®, Fort Collins, CO, USA; CyFlow®SL instruments from Partec®, Münster, Germany). With direct benefits in plant sciences, namely for genome size measurements using propidium iodide (PI), Partec® provides in some of their cytometers, the novel solid green state crystal laser (Nd:YAG). The advantage of this laser is that it generates light at 532 nm, which is close to the optimal excitation wavelength for PI (in contrast with most lasers which emit at 488 nm, see below the fluorescence spectrum of PI), resulting in a more sensitive analysis with lower coefficient of variation (CV) values. The main disadvantages of lasers are their wavelength specificity (if other wavelengths are needed, other lasers must be used) and high cost (Suda 2004).

To measure fluorescence light pulses, the optical detector system is focused at the intersection of the excitation light beam with the sample stream. The optical part of a flow cytometer has the following purposes: focusing of the excitation light, selection of required wavelengths, and collection of the output light with respective delivery to photodetectors (= photo sensors).

To achieve identical illumination of particles that may not follow the exact same path within the liquid stream, the laser beam must be focused with cross-cylindrical lenses before it reaches the sample stream (Fig. I.3). The first lens controls the width of the beam; the second, the height. The resulting elliptical beam is perpendicular to the sample stream and focused on the sensing area of the flow cell (Anonymous 2002). Some "jet-in-air" systems use spherical beams instead of elliptical ones.

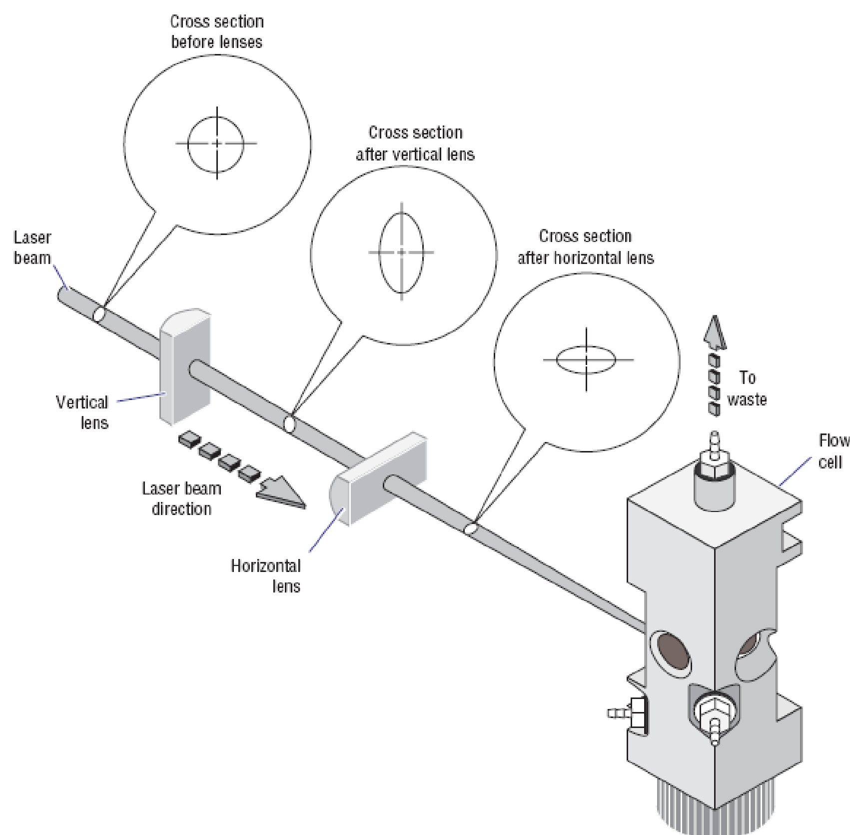

Fig. I.3 Laser beam shaping (adapted from Anonymous 2002).

As particles of the sample stream are illuminated by the elliptical laser beam, they scatter the laser light and emit fluorescent light from fluorescent dyes attached to them (if provided or in the case of autofluorescent particles). The light scattered at narrow angles to the axis of the laser beam is called forward scatter (FS), and the light scattered at about a 90º angle to the axis of the laser beam is denominated side scatter (SS). While FS gives an indication of the size of the particle, SS provides information about the internal granularity and surface roughness. Besides FS and SS, particles may emit fluorescent light (FL) at all angles to the axis of the laser beam (Fig. I.2). As this light may consist of various colours, it must be separated into specific wavelengths before it reaches the sensors (Suda 2004). Different types of optical filters are used for that purpose (Fig. I.4): long-pass (LP), transmit light above a specific wavelength; short-pass (SP), transmit light below a specific wavelength; band-pass (BP), transmit light within a certain range of wavelengths; and dichroic mirrors (D), selectively pass light of a small range of wavelengths while reflecting others (Anonymous 2002). The position of the dichroic filters (at a 45º angle) is usually designed to reduce the number of optical surfaces that

fluorescence light must pass to reach the sensors. In more sophisticated apparatus the optical filters can be interchanged without the need to realign the optical system.

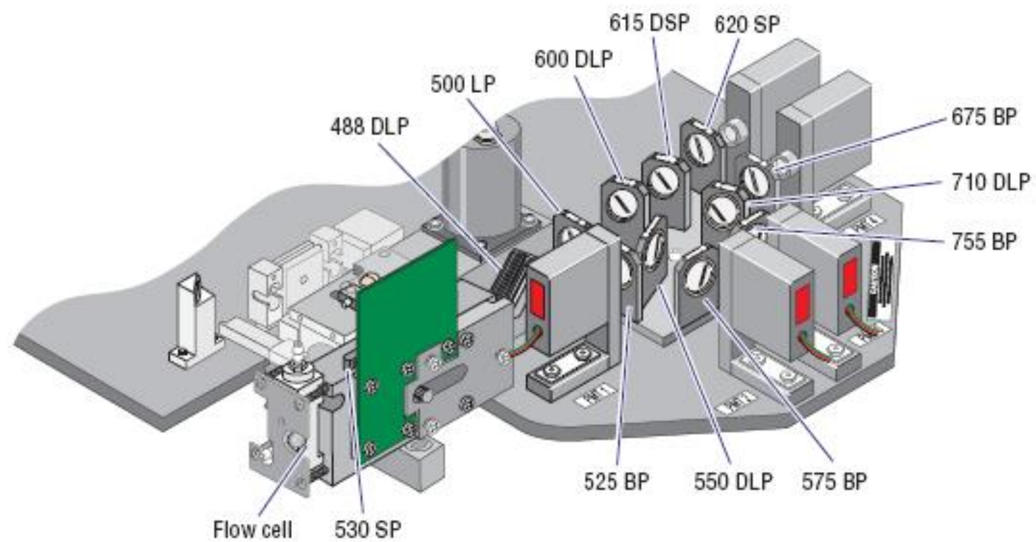

Fig. I.4 Standard filter configuration of the FC500 flow cytometer from Beckman Coulter® (adapted from Anonymous 2002).

The output light from illuminated particles is then collected by photo sensors, either photodiodes (FS) or photomultiplier tubes (SS and FL). As each particle passes through the laser beam, these sensors generate electric current pulses proportional to the intensity of the incoming light pulses. The **electronic system** of the cytometer amplifies, conditions, integrates, and analyzes these pulses (Anonymous 2002). An increasing amount of the electronics has become computer based, with the latest cytometers presenting large-scale integrated circuits, microprocessors, microcontrollers, and digital signal-processing chips. Some voltage pulses must be amplified so that the characteristics of the particles can be measured. Most systems enable an increase of the gain to linearly amplify the signals, and to logarithmically transform the linear data (Anonymous 2002). Most of the nuclear DNA content measurements should be made using the linear amplification. Before storage, the held voltages from the analogue circuitry are digitized. The analogue-to-digital converter (ADC) translates the continuous voltage analogue range into a discrete scale which can be represented by a binary number. In most systems ADCs providing 10-bit resolution are found. These will divide the scale into 1024 channels, with voltages being represented by a binary number between 0 and 1023 (Carter and Ormerod 2000). Recent advances in ADC technology led to large increases in the performance, and decreases in the price, with BD Bioscience® offering high-speed

digitization systems, and DakoCytomation® and Partec® enabling high-resolution digital data analysis (Shapiro 2007).

The **computational system** is directed at post-acquisition data display and analysis, including the latest possibility of software compensation. Data can be stored in the form of a histogram (one- or two- parameters) or in a "list mode", where the measurements of each individual particle are recorded (Doležel 1991). After storage, several approaches can be used to reanalyze the data: region creation and assignment; gating, *i.e.*, specification that only certain particles are to be analysed; and histogram overlay, for a better visual comparison of the data. Also, most software enables basic statistics that are useful for results interpretation, and include mean, mode and peak channel position, and full and half peak CV.

Mechanical components are now being integrated with flow cytometers. These include automated sample loaders (= carousel) and coordinated cloning trays for precise collection of droplets in flow sorters.

Flow cytometry and plant sciences

Plants differ from animals in a few general ways, one of which is the presence of a cellulosic cell wall. As plants comprise complex three-dimensional tissue architectures of interlinked cells, it seemed that FCM, which requires samples in the form of single-particle suspensions, was not applicable to higher plants (Galbraith 2004). However, the first report that used this technique was already published in 1973 (Heller 1973). The author used an Impulsecytophotometer from Partec® to analyse fluorescence signals from *Vicia faba* (Fabaceae) nuclei prepared from fixed tissues after enzymatic treatments with pectinase and pepsin. The methodology employed was laborious and time consuming, and the fact that the paper was written in German may have hampered an higher impact in plant community (Doležel and Bartoš 2005).

It was only approximately ten years later that FCM started to be applied more frequently in plant sciences. After discarding the possibility of using intact cells for estimating DNA content (the rigid cell wall is autofluorescent and confers an irregular shape to cells that disturbs the fluid stream), researchers focused on **protoplasts** (the cell wall being removed using hydrolytic enzymes) that are spherical and behave regularly within the flow stream (Puite and Tenbroeke 1983). However, the low permeability of the plasma membrane, the autofluorescence of cytoplasm (and in particular of chlorophyll), and the "off-centre" position of the nucleus, compromised the resolution of histograms of relative DNA content (Galbraith 1990). If the first two reasons could be circumvented by

fixation of protoplasts with ethanol-acetic acid, the non-identical localization of the nucleus remained a problem. Therefore, efforts were done to isolate **intact nuclei** for FCM analyses.

In 1983, two different methods to isolate intact nuclei were proposed: Puite and Tenbroeke (1983) obtained intact nuclei after protoplast lysis in the presence of non-ionic detergents, while Galbraith *et al.* (1983) prepared a suspension of nuclei by chopping a small amount of fresh tissue in a hypotonic buffer supplemented with a non-ionic detergent. Despite leading to very good histograms of DNA content, the first approach was laborious, time consuming and inapplicable to some species and certain types of tissues (Doležel and Bartoš 2005). Therefore few researchers have followed this method thereafter (Bergounioux *et al.* 1988, 1992; Hülgenhof *et al.* 1988; Ulrich *et al.* 1988; Ulrich and Ulrich 1991). On the other hand, the ingenious method of David Galbraith and co-workers was simple, convenient, rapid and capable of providing histograms of high quality for many plant species. This method largely stimulated the application of FCM in plant sciences and it remains the main (523 citations as of March 2007) and most reliable procedure for nuclear isolation from plant tissues (Doležel and Bartoš 2005).

The interest to study **pollen grains** (= microgametophytes), especially for the analysis of the frequency of 2n pollen formation (important in microevolution studies of several vascular plant groups), has led to development of several strategies to isolate and stain nuclei from microgametophytes for FCM analysis (Suda 2004). The most popular methodology is an adaptation of the chopping procedure of Galbraith *et al.* (1983) and consists of staining nuclei with a fluorochrome capable to pass trough the wall of both dry and fresh mature pollen grains [*e.g.*, 4',6-diamidino-2-phenylindole (DAPI)] with subsequent chopping for nuclear release (Bino *et al.* 1990). This methodology produced interpretable histograms and proved to be feasible for ploidy level estimation, including identification of unreduced pollen grains, in some plant species (*e.g.*, Misset and Gourret 1996; Sugiura *et al.* 1998; Mishiba and Mii 2000; Pichot and El Maâtaoui 2000). Recently, Pan *et al.* (2004) developed a simple method for isolating pollen nuclei from *Brassica napus* (Brassicaceae) and *Triticum aestivum* (Poaceae) using ultrasonic treatment. However, the success of both methods may depend on the resistance (thickness and number of apertures) of the outer membrane of the mature pollen grain, the exine, and on a possibility to avoid the autofluorescence of pollen wall constituents (*e.g.*, sporopolenins). For example, in pollen grain analysis in *Oxalis pes-caprae* (Oxalidaceae) no nuclei could be isolated using any of the procedures, and exaggerated noise signals were only observed (J. Loureiro 2006, pers. obs.).

Few studies so far analysed cellular organelles other than nuclei. **Chloroplasts**, the only organelles naturally autofluorescent in higher plants, were studied by Schroder and Petit (1992) who characterized the integrity of chloroplasts isolated from *Spinacia oleracea* (Amaranthaceae) and distinguished intact chloroplasts from thylakoid membranes and from various chloroplasts membrane subfractions (Galbraith 2004). The characteristics of chlorophyll fluorescence of mesophyll and bundle-sheath thylakoids were investigated in *Zea mays* (Poaceae; Pfündel *et al.* 1996), with further sorting of thylakoids from each tissue (Pfündel and Meister 1996). Flow cytometry analysis of **mitochondria** can be resumed to the detection of glycosil resides at the mitochondrial surface by using lectins (Petit *et al.* 1986), and to the use of rhodamine 123 to monitor the mitochondrial membrane potential (Petit 1992). Also, recent studies of the apoptosis process in plants using FCM were focused on the role of this organelle (Weir *et al.* 2003).

In the past twenty years a good deal of progress has been made in the development of methodologies for flow analysis and sorting of plant **chromosomes** (Galbraith 2004). Although they may be applicable to species with a low number of chromosomes and of different sizes, many studies so far used FCM with success. After the initial selection of species with small chromosome numbers (de Laat and Blaas 1984), the latest studies focused on the isolation and flow sorting of many pairs of chromosomes (as many as possible) from important crop species that include *T. aestivum*, *Pisum sativum* (Fabaceae), *Z. mays*, *Hordeum vulgare* (Poaceae), *Cicer arietinum* (Fabaceae) (for a review see Doležel *et al.* 2007*a*). Flow-sorted chromosomes have particular importance in physical mapping studies, for the localization of specific DNA sequences and for preparation of large-insert DNA libraries.

Contrarily to what occurred in human and animal FCM, where novel markers were developed almost since the primordial times of this technique, only recently this started to occur in plant FCM. Iona Weir has pioneered the development of FCM protocols for analyzing **apoptosis** and programmed cell death in plants by using and adapting the procedures already in vogue in animals (for a review see Weir 2001). Also, David Galbraith and co-workers have put considerable effort into the development of molecular markers that are able to identify any plant cell type of interest (Galbraith 2004). From all the studied markers, the green fluorescent protein (GFP) appeared particularly suited for the analysis of **plant gene expression** in individual cells and/or organelles using FCM and flow sorting (Galbraith 2007).

Analyses of nuclear DNA content

Estimation of DNA content in cell nuclei is one of the most important applications of FCM in plant sciences. As stated above, preparation of suspensions of intact nuclei for analyses of nuclear DNA content has been almost universally performed following the method of Galbraith *et al.* (1983) (Fig. I.5). In this procedure, the nuclei are released into a nuclear isolation buffer by mechanical homogenization (chopping) with a sharp razor blade (or scalpel) of a small amount of fresh plant tissue (Doležel and Bartoš 2005). The nuclear suspension is then sieved using a nylon mesh to remove large debris and nuclei stained with DNA specific fluorochrome. As FCM analyses relative fluorescence intensity, absolute estimations of nuclear DNA content require the use of a reference standard of known genome size.

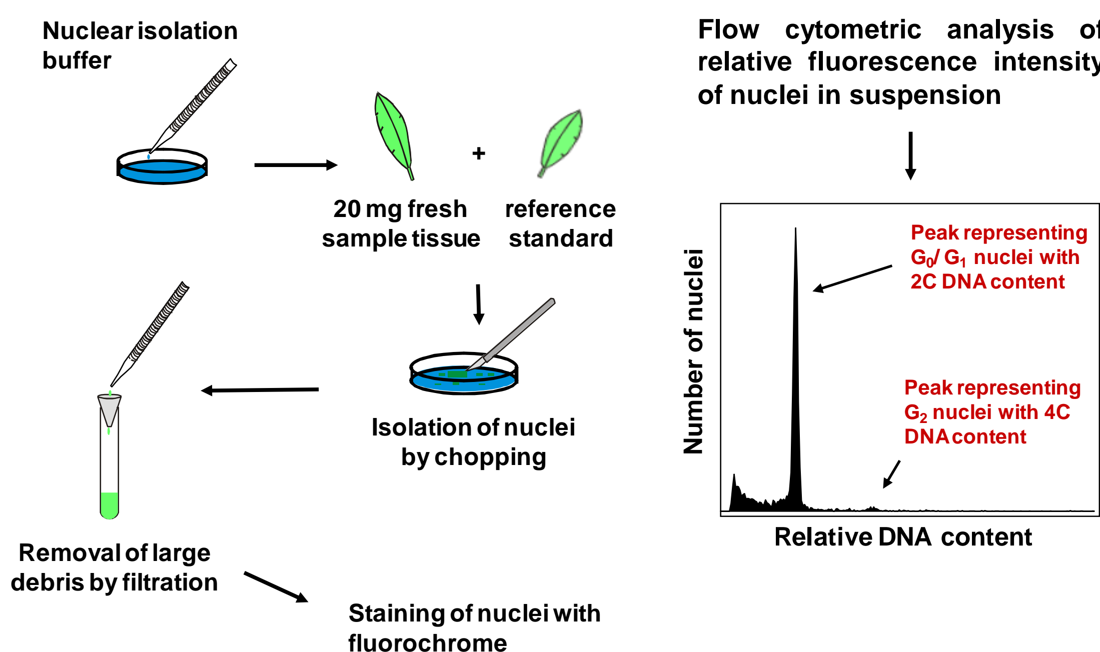

Fig. I.5 Diagram of the sample preparation procedure for FCM DNA measurements developed by Galbraith *et al.* (1983). Figure adapted from the website http://www.ueb.cas.cz/Olomouc1/LMCC/lmcc.html.

Few studies so far used a homogenisation procedure other than chopping to release nuclei from plant tissues for DNA FCM analyses: Engelen-Eigles *et al.* (2000) macerated endosperm of *Z. mays* with a flattened probe, while Wan *et al.* (1991) and Cuadrado *et al.* (2004) released the nuclei by grounding the tissue with a homogeniser. Recently, Bueno *et al.* (2003), when analysing the ploidy stability of *Quercus suber* embryos, assayed three different homogenisation methods – using a Potter S homogenizer (three pulses of 15 s each), manual homogenisation with a Teflon® pistil, and chopping with a razor blade – and verified that satisfa ctory results were only obtained when samples were prepared by chopping.

In the following section, issues related with sample preparation for FCM DNA measurements will be introduced, with attention given to plant material, including sample storage, nuclear isolation buffers, fluorochromes, calibration and standardization, data output and interpretation, and associated methodological problems.

Plant material

Theoretically, any tissue containing intact nuclei is suitable for measurement of nuclear DNA content with FCM (Greilhuber *et al.* 2007). However, as it will be discussed below, there are several problems associated with the isolation of nuclei suitable for analysis.

Generally, it is recommended the use of fresh almost fully expanded leaves. However, if results are unsatisfactory, the utilization of other tissues (especially colourless ones) should be considered. Any tissue should be intact, parasite- and disease- free and, if possible, the plants that are compared should be cultivated under the same conditions (especially the light regime) to eliminate the potential negative effect of different levels of cytosolic compounds.

The quantity of plant material needed depends on the tissue and should be determined empirically. Typically, 20 to 100 mg of leaf material per sample is enough in most species to obtain a sample flow rate of 50 to 100 particles/s. Generally, while in fleshy tissues the amount of material required to release a sufficient number of nuclei needs to be increased (Chapter IV.1), in seeds and compact *in vitro* cultured *callus*, the opposite occurs (J. Loureiro 2005, pers. obs.). Also, in some species that contain cytosolic compounds, large quantities of material may not be the best strategy (J. Doležel 2006, pers. comm.), despite that a compromise with the chopping intensity is usually the key to prepare a sample with the appropriate number of nuclei and the lowest possible quantity of interfering compounds.

Fresh leaf material may be transported or sent by post wrapped in moistened paper tissue and enclosed in a plastic bag (Doležel and Bartoš 2005). Low temperatures should be maintained during transportation. For example, more than 320 samples of *Dioscorea* spp. were brought in these conditions from Nigeria and arrived in a good state of conservation (J. Loureiro 2006, pers. obs.). Alternatively, if the material starts to deteriorate quickly, and the laboratory has no capacity to perform the analysis in the required period of time, nuclei can be fixed after isolation with ethanol, ethanol : acetic acid fixatives (Hülgenhof *et al.* 1988; Ozias-Akins and Jarret 1994; Jarret *et al.* 1995) or glycerol (Chiatante *et al.* 1990; Hopping 1993). Fixed nuclei may be maintained for a prolonged period of time before analysis. However, long term storage of nuclei in glycerol (up to 9 months in a freezer) decreased PI fluorescence intensity of nuclei of *Actinidia*

deliciosa (Actinidiaceae) by 5-7% when compared with fresh material, indicating some changes in chromatin structure (Hopping 1993). Therefore, fixed nuclei may only be suitable for DNA FCM applications where dyes with lower susceptibility to the state of chromatin (*e.g.*, DAPI) can be used. Moreover, even after shorter periods of storage, substantial loss (Hopping 1993) and clumping of nuclei was often observed (Doležel 1991).

As an alternative to fresh tissues, nuclei could be released from fixed cells or tissues. Unfortunately, fixed material has long been considered inappropriate for absolute DNA measurements using FCM. In modern cytogenetics two main types of fixatives can be found: non-additive (by ethanol-acetic acid) and additive (by formaldehyde). Few authors have used non-additive fixatives (Pfosser 1989; Pfosser *et al.* 1990; Setter and Flannigan 2001), the main reason being the protocol that involves enzymatic digestion of cell walls, hence it is laborious and time consuming. Also, the use of pectinases and cellulases for digestion may result in a decreased fluorescence intensity of the nuclei (Doležel 1991). Finally, it seems highly probable that DNA staining by intercalating dyes, as ethidium bromide (EB) and PI, can be disturbed by this type of fixation, either by a direct modification of the chromatin structure by the fixative or by the release of tannins from vacuoles that strongly bind to chromatin and interfere with quantitative DNA staining, as shown for the Feulgen reaction (Greilhuber 1988). This last argument is the main reason why formaldehyde fixation cannot be used for absolute nuclear DNA estimations (Overton and McCoy 1994). Even though the same authors were able to completely reverse the effect of formalin by re-suspending formalin-fixed human cells in PBS and heating them at 75ºC for at least 1 h prior to staining with PI, in *P. sativum* and *Glycine max* (Fabaceae) leaf tissue fixed in 4% formaldehyde and stored for 24 h, it was verified that phosphate buffered saline (PBS) and heating did not restore completely the fluorescence of nuclei (Rodriguez *et al.* 2005). Although not recommended, Cuadrado *et al.* (2004) used roots fixed in 1% formaldehyde prepared in tris-(hydroxymethyl)-aminomethane (TRIS) buffer as plant material for PI genome size estimation in *Saccharum officinarum* x *S. spontaneum* (Poaceae) cultivars. For relative DNA measurements, mostly using DAPI, Sgorbati *et al.* (1986) demonstrated that a short fixation (up to one week) in formaldehyde yielded a large number of nuclei (in 3-12 times higher quantity than in fresh material) suitable for FCM. This procedure was further used by Sgorbati and co-workers in several other ploidy level studies (Bracale *et al.* 1997; Citterio *et al.* 2002; Sgorbati *et al.* 2004). As there certainly are many fixed cytological samples stored in botany laboratories all over

the world, the development of routine methodologies for this kind of material is of high importance in plant FCM.

Frozen material has also been used in several works either for absolute (Grattapaglia and Bradshaw 1994; Cros *et al.* 1995; Nagl and Treviranus 1995) or relative nuclear DNA content estimations (Nsabimana and van Staden 2006; Suda and Trávníček 2006). For the latter purpose, and using herbarium material, Suda and Trávníček (2006) found that the longevity of samples was significantly prolonged by their storage in deep freezer at -78ºC with clear differences in DNA peak quality between vouchers kept at room temperature and in deep freezer. However, as the suitability of this storage strategy for genome size estimations remains unstudied, its use should be considered with caution.

During the last decade, several investigations used dry seed material for determination of nuclear DNA content by FCM with considerable success (*e.g.*, Bino *et al.* 1993; Matzk *et al.* 2000, 2001; Śliwińska *et al.* 2005). If it may seem surprising that chromatin from dormant tissue can be easily stained with fluorochromes, it may also be possible that dry cells release less nucleases into the nuclear homogenate than turgid cells from soft tissue and that certain dry organs contain less cytosolic compounds (Greilhuber *et al.* 2007). However, for optimal results it is important that the dry tissue is first crushed and then immediately stained in the buffer (Matzk 2007).

Recent work by Suda and Trávníček (2006) showed the feasibility of performing DNA ploidy estimations on up to 3-years-old (at least) herbarium material (prepared by pressing and drying) of certain plant groups. Most of the analyzed plants yielded distinct peaks after several months of storage at room temperature with fluorescence intensity of nuclei isolated from desiccated tissues and stained with DAPI being highly comparable with that of fresh material. Also for relative DNA measurements, Suda and co-workers already use routinely and with substantial success silica gel-dried material (J. Suda 2006, pers. comm.). As desiccation is a routine way of sample preservation in field botany, the possibility of using dehydrated tissues opens new and promising prospects for plant DNA FCM.

Nuclear isolation buffers

As mentioned above, in the method of Galbraith *et al.* (1983) the nuclei are released into a nuclear isolation buffer by mechanical homogenization of a small amount of fresh tissue. The composition of the buffer is crucial for the release of intact nuclei free of adhering cytoplasm in sufficient quantities, maintenance of nuclear stability and prevention of nuclear aggregation. It also has an important function in DNA protection against degradation by endonucleases, in stoichiometric staining and in nuclear protection against

the negative effect of cytosolic compounds (Doležel and Bartoš 2005). Since the very beginning of plant DNA FCM, several laboratories developed their own buffer formulas. The current version of the FLOWER database (http://flower.web.ua.pt/), described in Chapter V, lists 27 lysis buffers with different chemical composition.

Most buffers work at a near neutral pH (from 7.0 to 8.0) and are based on organic buffer substances such as 4-morpholinepropane sulfonate (MOPS; Galbraith *et al.* 1983; Bino *et al.* 1993), TRIS (Doležel *et al.* 1989; Rayburn *et al.* 1989; Pfosser *et al.* 1995) and 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid (HEPES; de Laat and Blaas 1984; Arumuganathan and Earle 1991). Chromatin stabilizers, as Mg^{2+} or spermine, and chelators, such as ethylenediaminetetraacetic acid (EDTA; metal chelator) and citrate (mild chelating agent), are usually added. Given the counteracting effects of both components, one must only be thoughtful to not use metal chelators and Mg salts in the same buffer (Galbraith 2007). The composition of the ten most popular nuclear isolation buffers in plant DNA FCM is given in Table V.1.

The usefulness of some of the buffers is sometimes difficult to evaluate as their performance was not analysed thoroughly, nor compared with other buffers. In Chapter IV.1 the first systematic comparison of lysis buffers is presented. Here the performance of four of most common buffers differing in chemical composition – Galbraith's (Galbraith *et al.* 1983), LB01 (Doležel *et al.* 1989), Otto's (Otto 1992; Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser *et al.* 1995) – was compared using seven plant species, whose tissues differ in structure and chemical composition. The same group of buffers was also studied in the FCM and microscopic analysis of the effect of tannic acid, a common phenolic compound, on plant nuclei and estimation of DNA content (Chapter IV.2).

Given the diversity of plant tissues, including the immense variety of intrinsic chemical compounds, it is no surprise that a universal buffer has not been developed so far (Doležel and Bartoš 2005). However, considering the results of Chapters IV.1 and IV.2 we elected to develop two nuclear isolation buffers that could be suitable for a broad range of plant tissues. The nuclear isolation buffer (NIB) and woody plant buffer (WPB) are presented and evaluated in Chapter IV.3.

Filtration

The almost universal method for filtration of the nuclear homogenate consists on the use of a nylon mesh with a pore size ranging from 20 to 80 µm. This will remove cell fragments and large debris. Recently, Lee and Lin (2005) suggested an alternative approach that could supposedly be used on recalcitrant tissues, particularly those that contain calcium oxalate crystals. These authors designed a cotton column that after

wetting in the lysis buffer was rolled and inserted in the middle of a 5 mL pipette tip. This method apparently reduced the percentage of contaminants and, by this way, purified the nuclear suspension. However a 50% decrease in the yield of nuclei was also observed.

Fluorochromes

After sample homogenization, nuclei must be stained with a fluorochrome, which should bind specifically and stoichiometrically to DNA. Fluorescent stains can be divided according to the binding mode to DNA as follows: (i) dyes that quantitatively intercalate into double-stranded nucleic acids, and (ii) base specific minor grove-binding dyes with A-T or G-C preference. The fluorochromes used so far in plant DNA FCM according to the FLOWER database (Chapter V) are listed in Table I.1. An analysis of the usage frequency of fluorochromes, along with an explanation for the obtained results, is presented in Chapter V.

Table I.1 Fluorochromes used in plant DNA flow cytometry. Information on the binding mode and excitation and emission wavelengths of each stain is also given.

Fluorochrome	Primary biding mode	Wavelength (nm)	
		Excitation	Emission
Propidium iodide (PI)	Intercalation	530 (blue-green)	605 (red)
Ethidium bromide (EB)	Intercalation	540 (blue-green)	615 (red)
Acridine orange	Intercalation	502 (blue)	525 (green)
DAPI	A-T specific	365 (UV)	450 (blue)
Hoechst 33258	A-T specific	365 (UV)	465 (blue)
Hoechst 33342	A-T specific	360 (UV)	460 (blue)
Chromomycin A3	G-C specific	445 (violet-blue)	570 (green)
Mithramycin	G-C specific	445 (violet-blue)	575 (green)
Olivomycin	G-C specific	440 (violet-blue)	560 (green)

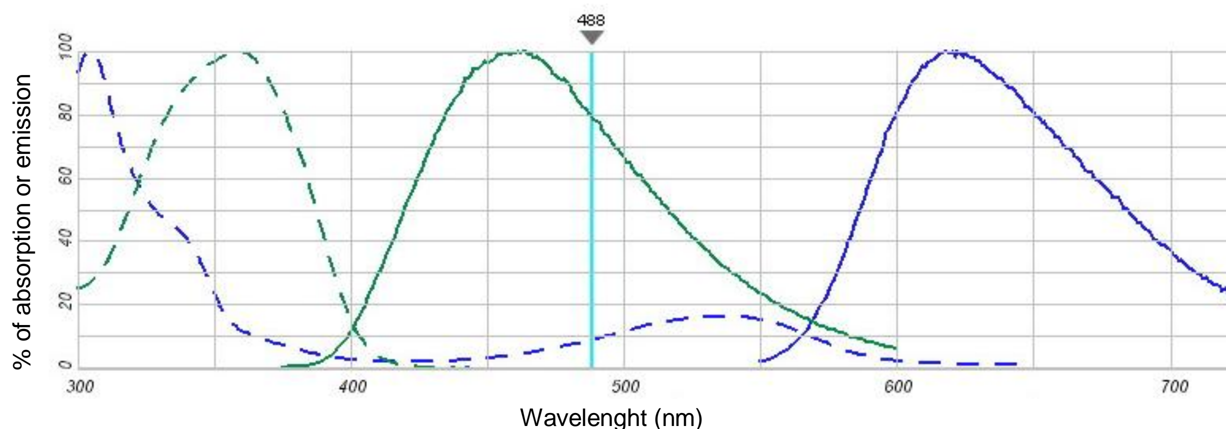

Fig. I.6 Absorption (dashed lines) and fluorescence emission (solid lines) spectra of DAPI (green) and PI (blue) bound to DNA (adapted from the Invitrogen® spectral viewer, http://probes.invitrogen.com/resources/spectraviewer/).

For absolute DNA measurements it is important to use intercalating dyes (PI and EB), as these fluorochromes are not affected by DNA base composition (Doležel *et al.* 1992). PI and EB are usually excited by a 488 nm argon laser, which is not the optimal excitation wavelength for both dyes (Table I.1 and Fig. I.6), thus affecting the accuracy and resolution of the analyses (Suda 2004). To overcome this weakness, some recent instruments of Partec are equipped with the Nd:YAG laser that emits at 532 nm, which is almost optimal for excitation of PI and EB. Furthermore, for maximal fluorescence and highest resolution, EB and PI should be added at near neutral pH (between 7.2 and 7.4) in a buffer that provides ionic strength (Le Pecq and Paoletti 1967), and at saturating concentrations (Doležel and Bartoš 2005). Even though both dyes stain DNA above pH 4, higher resolution is usually obtained at higher pH (pH below 7 enhances the activity of endonucleases). Although Barre *et al.* (1996) suggested that the optimal dye concentration should be determined for each given pair of species, concentrations between 50 and 150 µg mL^{-1} are usually found appropriate (Greilhuber *et al.* 2007; Chapter IV.2). An incubation period of 5-10 min is usually adequate for saturating the DNA sites, and longer staining times (*e.g.*, 60 min) often lead to a decrease of fluorescence (see Chapter IV.2) and/or to increased levels of background debris (Barre *et al.* 1996). As PI and EB bind to double-stranded RNA, a pre-treatment with RNase is necessary for meaningful DNA measurements. If in leaf tissues the addition of RNase may not be so critical, for tissues undergoing high levels of protein synthesis, such as meristems and seeds, the treatment with RNase is essential (Doležel and Bartoš 2005). Unfortunately, EB and PI are sensitive to the chromatin structure, which implies that changes in chromatin condensation (due to growth state, tissue type and/or cytosolic compounds) might affect DNA content estimations. As discussed above, this susceptibility is the main reason why tissue fixation is not recommended for absolute DNA content estimations. For reasons that remain unclear, PI has been considered superior to EB (Crissman *et al.* 1976), which together with a lower toxicity, makes this fluorochrome the preferred intercalating dye for staining plant nuclear DNA (for quantitative data see Chapter V).

Despite excited in the UV range with mercury arc lamps (Table I.1 and Figure I.6), fluorochromes with A-T specificity (DAPI and Hoechst dyes) are highly popular for relative DNA measurements, with DAPI clearly being the preferred dye (Chapter V). This is probably because a comparison study between both dyes revealed that DAPI provided histograms with higher fluorescence intensity and resolution (Suda 2004). Contrarily to what occurs with intercalating dyes, the binding of DAPI to DNA is not influenced by the state of chromatin condensation, which can result in histograms with higher resolution.

DAPI (at non saturating concentrations ranging from 1 to 10 μg mL^{-1}) and Hoechst staining of DNA are usually carried at pH 7.0.

Mithramycin, together with other fluorescent antibiotics (chromomycin A3, olivomycin) constitute the set of fluorochromes that bind to the G-C rich regions of DNA. These dyes are optimally excited at about 440 nm (Table I.1), and due to lower resolution of histograms as compared to A-T specific dyes (Ulrich *et al.* 1988), their use in plant DNA FCM is almost restricted to studies of genomic base composition. Mithramycin, the most used G-C specific stain, is typically used in concentrations ranging from 50 to 100 μg mL^{-1} (Ulrich *et al.* 1988) and its optimal staining is achieved in the pH range 5-9 (Doležel 1991). Also, the presence of magnesium ions is fundamental for formation of complexes between G-C dyes and DNA, with the concentration of $MgCl_2$ being important for a higher fluorescence intensity and histogram resolution (Doležel 1991). As all base specific dyes only bind to double-stranded DNA the addition of RNase is not necessary.

The stain can either be already present in the nuclei isolation buffer or be added after nuclear homogenization and filtration. While some time can be saved in the first approach, it also increases the probability of skin and laboratory contamination of the sample and the number of disposables that would need to be treated as toxic waste (Greilhuber *et al.* 2007). Therefore the second strategy is highly preferable in most situations.

Stained samples can be maintained at room temperature in buffers containing metal chelators (more suitable temperature for the activity of RNase). On the other hand, samples prepared in buffers without these components and containing divalent cations should be incubated at ice-cold temperature in order to decrease nuclease activity (Doležel 1991).

Calibration and standardization

In addition to a careful employment of sample's preparation and staining methods, the instrument must also be perfectly adjusted for obtaining of high resolution analyses. The instruments precision can be either monitored by fluorescently labelled microspheres or by stained nuclei from animal cells (trout red blood cells (RBC), chicken RBC or human leukocytes) (Doležel 1991). In our laboratory, when none of these calibration standards is available, we use freshly prepared nuclei of the "plant gold" reference standard (Greilhuber *et al.* 2007), *P. sativum*, isolated in the nuclear isolation buffer that is going to be used in that working day. In all cases, only when CV values below 2-2.5% are achieved the FCM analysis is started.

As FCM analyses relative fluorescence intensity, absolute DNA measurements always require the use of a reference standard, whose genome size is known. The genome size or ploidy level of an unknown sample is determined by comparing its peak position with that of the standard (Suda 2004). Standardization can be accomplished by several ways. In the external standardization approach nuclei from both sample and standard are prepared and measured separately. Relative peak position of the reference standard is checked before or after each sample run (Suda 2004). In this procedure, nonetheless the instrument settings are kept constant throughout the analysis, measurements may be affected by random instrument drift and by a non-identical sample preparation and staining (Doležel and Bartoš 2005). External standardization is only acceptable when the demands of precision are not high, as in DNA ploidy screening (Greilhuber *et al.* 2007). Disadvantages of this approach are easily overcome by internal standardization, in which nuclei of the sample and standard are isolated, stained and analysed simultaneously (Doležel 1991). By this way, identical conditions for the sample and the standard are as far as possible guaranteed. Some authors have used a compromise between these two approaches, named “pseudo-internal” standardization, in which the isolation of sample and standard nuclei is made independently before mixing and analysing (Price and Johnston 1996; Price *et al.* 1998; Johnston *et al.* 1999). In this procedure, only the errors associated with the instrument are eliminated, because the analyses are still subject to bias by the variation in nuclei isolation and staining (Doležel and Bartoš 2005). Data on standardisation and standard use can be easily excerpted from the FLOWER database (Chapter V).

Usually, one internal reference standard is used per sample. However, when the occurrence of aneuploidy is expected, a second reference point (*e.g.*, plant of the same species with known chromosome number, usually diploid) may be used to improve the sensibility of the assay (Suda 2004). This strategy was successfully employed in the study of DNA ploidy stability of the embryogenic process of *Cyphomandra betacea* (Tamaricaceae), in which several aneuploidy events were easily detected (L. Currais *et al.* 2005, unpublished data). Here, besides sample and reference standard material (*P. sativum*), leaf tissue from a diploid individual of *C. betacea* (tamarillo) was added (Fig. I.7).

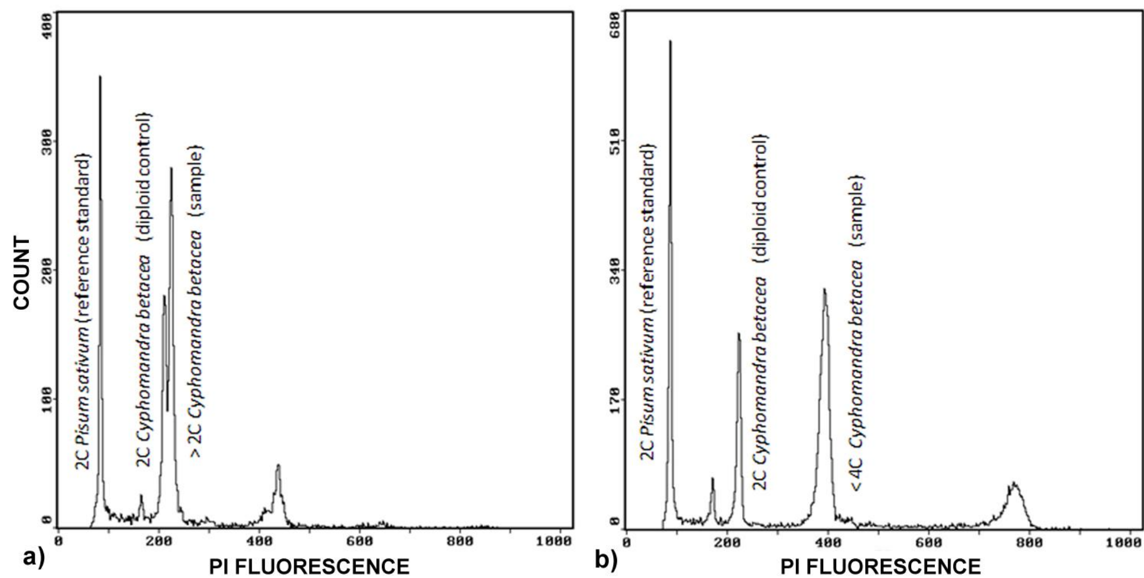

Fig. I.7 Ploidy stability analyses of the embryogenic process of *Cyphomandra betacea* (tamarillo). Histograms of PI fluorescence (relative units) of nuclei isolated from an unknown sample of tamarillo, from *Pisum sativum* cv. Ctirad (reference standard) and tamarillo diploid plant (secondary reference point): a) hyperdiploid (2n > 2x) individual; b) hypotetraploid (2n < 4x) individual (L. Currais *et al.* 2005, unpublished data).

According with Greilhuber *et al.* (2007), the selection of the most appropriate standard species should be guided by several criteria: biological similarity, genome size, nature of the standard, availability, cytological homogeneity, accessibility and reliability of C-values. An ideal DNA reference standard should be biologically similar (*i.e.*, the use of animal standards, as chicken RBC and human leucocytes is not advisable as the concept of internal standardization is violated) and have a different genome size from the unknown sample, but not too different to avoid the risk of nonlinearity and offset errors (Doležel and Bartoš 2005). Also, it should be free of cytosolic compounds and its preparation should be easy and result in narrow DNA peaks. The chromatin status of the standard and the sample should be similar; this may be assured by using the same type of tissue (*e.g.*, leaves) at a similar growth state. Finally, the standard should be genetically stable with constant and reliable genome size estimation, permanently accessible to other researchers in sufficient quantities, seed-propagated, easy to multiply and cytologically homogeneous (Greilhuber *et al.* 2007). All these requirements are difficult to satisfy and, consequently, many different standards have been employed so far (for a list of the most used reference standards see Table V.2). The definition of a unified set of cross-calibrated reference standards distributed at appropriate genome size intervals is a current topic in plant DNA FCM, and although two lists of reference species (in each study calibrated with

a primary reference standard) have already been presented (Doležel *et al.* 1998; Johnston *et al.* 1999), a full consensus is yet to be reached. In our laboratory the set of reference species proposed by Doležel *et al.* (1998) was chosen (Table I.2). A thorough discussion of all the problems associated with reference standards is given in Chapter V, taking in consideration the quantitative data given by the FLOWER database there presented.

Table I.2 DNA reference standards available from the Laboratory of Molecular Cytology and Cytometry, Olomouc, Czech Republic as used in our laboratory.

Species	Cultivar	2C DNA content (pg)[1]	Reference
Vicia faba (Fabaceae)	'Inovec'	26.90	Doležel *et al.* 1992
Secale cereale (Poaceae)	'Dankovské'	16.19	Doležel *et al.* 1998
Pisum sativum (Fabaceae)	'Ctirad'	9.09	Doležel *et al.* 1998
Zea mays (Poaceae)	'CE-777'	5.43	Lysák and Doležel 1998
Glycine max (Fabaceae)	'Polanka'	2.50	Doležel *et al.* 1994
Lycopersicon esculentum (Solanaceae)	'Stupické'	1.96	Doležel *et al.* 1992
Raphanus sativus (Brassicaceae)	'Saxa'	1.11	Doležel *et al.* 1992

[1] Nuclear DNA content was established using human male leukocytes (2C = 7.0 pg DNA; Tiersch *et al.* 1989) as a primary reference standard.

Quality control and data presentation

The required number of nuclei for each analysis is typically set at 5,000 or 10,000 counts. However, as the analysis also includes the debris background, higher count values may be needed in some situations. Greilhuber *et al.* (2007) has shown that relevant peaks should represent at least 1300 nuclei, even though higher counts resulted in more stable and defined peak positions.

Cytometric data can be either presented in the form of 2-D histograms (one parameter analysis) or 3-D cytograms (two parameter analyses, also presented in 2-D). The histogram is usually used to present FL data (Fig. I.8a), with the horizontal axis presenting the intensity of the FL signal and the vertical axis the number of particles with a given range of intensity (Suda 2004). Cytograms are scarcely used in plant sciences; however in our laboratory we routinely analyse the following cytograms: FS *vs.* SS both in logarithmic (log) scale (the usefulness of this cytogram is debated in Chapter IV.1; Fig. I.8b), FL *vs.* time (to monitor the fluorescence stability of nuclei over time; Fig. I.8c), SS in log scale *vs.* FL (to monitor the possible effect of cytosolic compounds, for details see Chapter IV.2; Fig. I.8d), and FL pulse integral *vs.* FL pulse height (to evaluate and

eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets; Fig. I.8e).

Usually a discriminator (= threshold) is set at a FL value of 50 to eliminate particles with autofluorescence and/or with low fluorescence values, and the mean position of the first G_0/G_1 peak (representing the species with lowest genome size), either from the sample or the reference standard, should be located on (almost) the same fluorescence channel (*i.e.*, voltage and gain should be kept constant throughout an experiment), usually not below channel 200 in a 1,024 channels scale. Also, peaks of both the internal standard and the sample should be symmetrical and of approximately the same height (Suda 2004).

The creation of regions, besides enabling the gating of particles of interest, is in some software (*e.g.*, SYSTEM II from Beckman-Coulter®) fundamental to excerpt statistical data from the graphics. The following regions are routinely defined in our laboratory: in the FL histogram, linear regions are defined for each peak (Fig. I.8a); in the FS *vs.* SS cytogram (Fig. I.8b), a region is defined around the population of nuclei; in the FL pulse integral *vs.* FL pulse height cytogram (Fig. I.8e), a region is defined around individual nuclei. The region created in the last cytogram is used to eliminate doublets of 2C that can be erroneously assessed as 4C. In some cases, a region around pure nuclear fractions can be defined in the SS *vs.* FL cytogram (Fig. I.8d), and thus avoid physical purification procedures before the analysis.

A typical DNA FL histogram comprises a prominent peak corresponding to nuclei in the G_0/G_1 phase of the cell cycle (with a 2C DNA content), a small peak that correspond to nuclei in the G_2 phase (with a 4C DNA content) and some signals in between that correspond to nuclei in the S phase (nuclei synthesising new DNA) (Fig. I.5). As there are many sources of variation in the FCM analyses (the most important of them were discussed above), wider peaks and background debris are usually observed (Suda 2004), thus the quality of a nuclear suspension is best evaluated by analysing the histogram of relative nuclear DNA content, which unfortunately is not always included in plant DNA FCM publications (Chapter V). Histograms of good quality should contain a low background debris and symmetrical G_0/G_1 and G_2 peaks with low variation (Doležel and Bartoš 2005).

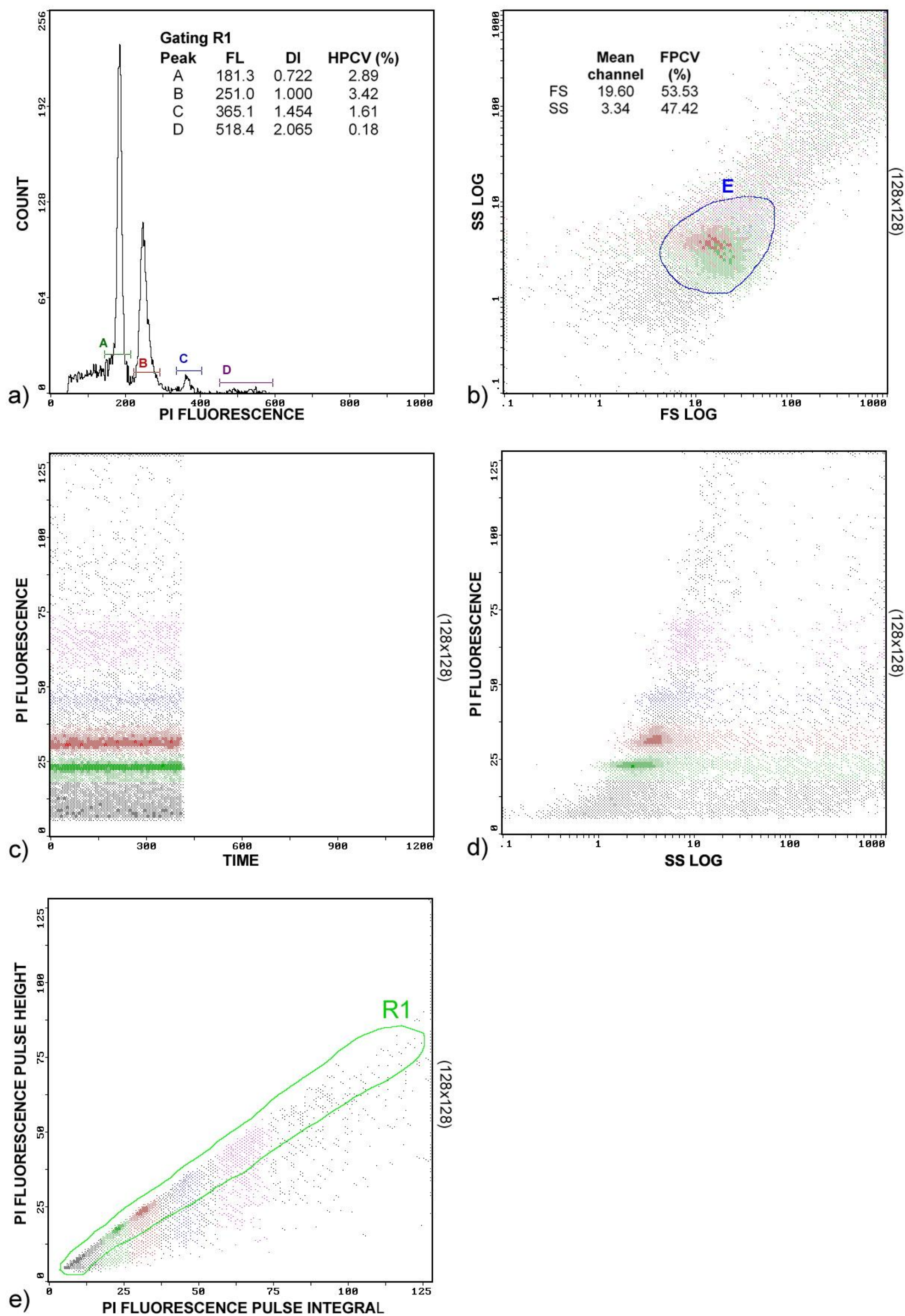

Fig. I.8 Histogram of PI fluorescence (a, in relative units) and cytograms of FS (FS LOG) *vs.* SS (SS LOG) (b, in relative units), PI fluorescence *vs.* time (in seconds; c), SS LOG *vs.* PI fluorescence (d) and PI fluorescence pulse integral *vs.* PI fluorescence pulse height (e) of nuclei of *Quercus suber* (Fagaceae; peaks: A - G_0/G_1 nuclei, coloured in green, and C - G_2 nuclei coloured in blue) and *Glycine max* cv. Polanka (as internal reference standard with peaks: B - G_0/G_1 nuclei, coloured in red, and D - G_2 nuclei, coloured in purple) simultaneously isolated in WPB buffer and stained with PI. Mean FL channel, DNA index (mean channel of *Q. suber* / mean channel of *G. max*) and CV values of each peak are given in the histogram a) and mean channel of FS and SS and CV of each parameter are given in cytogram b) (adapted from Santos *et al.* 2007).

The peak variation is usually given by the half peak coefficient of variation (*i.e.*, ratio between the standard deviation and the mean). Unlike the standard deviation this parameter does not depend on the peak mean, thus it enables a direct comparison of peaks at different channel positions (Doležel and Bartoš 2005). Usually CV values between 1% and 10% are obtained. Although under certain conditions, CV values lower than 1% may be obtained (0.53% in Doležel and Göhde 1995), in most cases CV values below 3% are the goal to reach (Marie and Brown 1993). In some "difficult" species (species containing elevated quantities of cytosolic compounds and species with low genome size, see Chapter IV.3) this threshold is hard to achieve and CV values below 5% are considered acceptable (Galbraith *et al.* 2002). Besides being dependent on the plant material, this precision measure is also influenced by the fluorochrome (DAPI is usually the stain that provides histograms with higher resolution) and by the sample preparation, including the lysis buffer chemical composition (see Chapter IV.1). It should be noted that CV values lower than half of the difference between DNA contents of samples is fundamental for their discrimination. As an example, a CV value of 3% would permit detection of a 6% difference in DNA content between two samples (Suda 2004). Like the FL histograms, the CV values obtained in each experiment should be reported in the final publication (for a frequency analysis of published CV values see Chapter V). In chapter IV.2 the use of the full peak coefficient of variation (FPCV) as a measure of variation of scatter parameters is suggested, with homogeneous populations of nuclei presenting FPCV values below 50%.

The CV of DNA peaks is a measure of precision of the FCM analysis, but the reproducibility of a given experiment is only assessed by replicate measurements. Independent replications should be carried out, with their number varying according to the objective of the study. For genome size estimation, and when possible, it is generally assumed that a minimum of three plants should be analysed (Greilhuber and Obermayer 1997), each of them three times on different days (Suda 2004). The effect of performing the experiment in different days is analysed in Chapter IV.I. Furthermore, if slightly but significant different samples are detected during an experiment, the difference should be confirmed by re-comparing these samples in independent assays (Greilhuber *et al.* 2007) (as an example, see the study of genome size in *Festuca summilusitana* in Chapter III.3). If one narrow and symmetrical DNA peak is obtained, it is highly probable that the apparent difference is just an artefact.

Problems and limitations of plant flow cytometry

Despite being the most appropriate method for DNA content measurement, FCM also presents some drawbacks. The most serious and recent problem, concerns the nuclear isolation and staining procedures in some vascular plants. The tissue of several of those species contains cytosolic compounds that are released during the nuclear isolation and that may interfere with the staining procedure, namely by affecting the accessibility of the fluorochrome to nuclear DNA, and the scatter properties of particles by an aggregation of minor particles with nuclei. Although the interference with fluorescence intensity of nuclei has been recognized for some time in cytophotometry (Greilhuber 1986), only after the works of Noirot *et al.* (2000) and Price *et al.* (2000) this effect was seriously considered in the FCM area (Greilhuber *et al.* 2007). The effect on nuclei scatter properties is first described in Chapter IV.2.

Even though the chemical nature of cytosolic compounds is still scantly explored (for a small overview of the current knowledge see section 4.6 of Greilhuber *et al.* 2007), phenolic compounds have been already identified as prime candidates (Noirot *et al.* 2003). Phenols are present in many plant tissues, especially in woody species, and if in some cases their negative effect can be efficiently counteracted by antioxidants (*e.g.*, ß-mercaptoethanol (Doležel *et al.* 1994); metabisulfite (Zoldoš *et al.* 1998, Chapter IV.3) and polyvinyl pyrrolidone (PVP) (Bharathan *et al.* 1994; Yokoya *et al.* 2000), in other situations where the concentration of cytosolic compounds is supposedly higher, the interference can lead to biased estimations of nuclear DNA content (Noirot *et al.* 2000, 2002, 2003, 2005) and skewed DNA peaks (see Chapter IV.2). Indeed, in our early work in FCM there were many difficulties related with cytosolic compounds, as the analysis of the ploidy stability of the somatic embryogenic process of two economically important woody plant species, *Eucalyptus globulus* (Myrtaceae; Pinto *et al.* 2004) and *Quercus suber* (Fagaceae; Chapter II.1), was clearly affected by phenols. Pinto *et al.* (2004) addressed these problems and proved the presence of interfering cytosolic compounds by performing the test for inhibitors suggested by Price *et al.* (2000). This test is based on the observation that inhibitors besides interfering with sample nuclei, also interact with the nuclei of reference standard. Therefore, if the fluorescence intensity of standard nuclei isolated alone is compared with that of standard nuclei isolated together with the sample, and the fluorescence of the co-chopped standard is lower than the lone-chopped standard, it is likely that there is a negative effect of the released inhibitor (Greilhuber *et al.* 2007).

The results of these works and the need to have a better understanding of the negative interference of cytosolic compounds stimulated us to investigate in detail the effect of a common phenolic compound, tannic acid, on *P. sativum* and *Z. mays* nuclei and on estimation of DNA content. The results of this work are presented in Chapter IV.2. Without going into the details, it is interesting to note that the tannic acid effect described there was also observed for some of the species analysed in Chapter IV.3 and in pigmented leaves of *Rumex pulcher* (Polygonaceae) that were co-chopped with *P. sativum* (Greilhuber *et al.* 2007).

Another class of phenolic compounds that deserves attention are the coumarins. Walker *et al.* (2006) found that differences in genome size of *Bituminaria bituminosa* (Fabaceae) between cold and hot seasons were artefactual and due to variation in the concentration of furanocoumarins, which are known to accumulate at greater extent at higher temperatures. Also, flavonoids, as the anthocyanin cyaniding-3-rutinoside, were shown to act as fluorescence inhibitors in *Euphorbia pulcherrima* (Euphorbiaceae; S. Johnston *et al.* 2005, pers. comm.). These authors showed, that this compound, which is present in red bracts but absent in green leaves, was responsible for the significant differences on C-value obtained between both organs.

Mucilaginous compounds are also a problem in some plant species (Lanaud *et al.* 1992; Morgan and Westoby 2005; Talent and Dickinson 2005). Besides hampering the release of nuclei, these compounds confer the nuclear suspension a high viscosity, which makes the filtration step difficult to accomplish and consequently outcomes in the recovery of a low volume of nuclear homogenate. Morgan and Westoby (2005), when studying the relationship between nuclear DNA content and leaf life span and leaf mass area, found for many species of Myrtaceae and Rutaceae that the ineffective nuclear isolation was due to mucilaginous compounds. These authors tried to circumvent this problem by washing the tissues with PVP in MOPS buffer before staining, by using different tissues, by varying the amount of detergent and by using a filter with smaller pore size, but none of these strategies resulted in a more successful isolation of nuclei. The negative interference of mucilaginous compounds is also a topic of discussion in this PhD Thesis, as leaves of some of the species of Ulmaceae studied in Chapter III.2 presented high levels of these compounds, which were shown to clearly interfere with the estimation of genome size. Studies on the genome size of *Pterocarpus indicus* (Fabaceae) and *P. santalinoides*, conducted in our laboratory, also revealed the presence of high concentrations of these compounds in leaf tissue (Loureiro *et al.* 2006).

In addition to these pitfalls, other drawbacks of plant FCM have long been identified, *i.e.*, the requirement of fresh plant material and the limited sensitivity of FCM for detecting low level aneuploidy. As discussed above, despite some nice prospects on the use of herbarium vouchers for ploidy level estimation have been recently opened, the possibility of using fixed material remains limited still. Also, as there is no general agreement on a set of reference standards, and some significant differences have been detected among laboratories, especially when estimates were obtained using instruments with different light source and flow chamber (Doležel *et al.* 1998), discrepancies in plant genome size data obtained in different laboratories should be regarded with caution.

Advantages of flow cytometry

FCM has several important advantages over related techniques, as chromosome counting and microdensitometry:

- Sample preparation is easy, convenient and rapid. The protocol is easy to reproduce and it lasts approximately 10 min, including a 5 min incubation period. As FCM analyses are made at high speed, usually after 5 min, data from the required number of nuclei are already acquired. This allows the analysis of many samples per working day (my personal record is 76 samples).
- Only small amounts of plant material are usually required for sample preparation;
- A variety of plant tissues can be used for sample preparation, *i.e.*, roots, stems, petals, seeds, fruits, among others, without a need for mitotically active cells.
- It enables the detection of subpopulations, which is particularly important in endopolyploidy and mixoploidy studies and in the detection of minor cytotypes.
- After the equipment is purchased (the main expense), the demand in consumables is relatively small, which results in relatively low running costs.

Related and complementary methods

The approaches used for estimation of nuclear DNA content of a given organism can be divided into analysis of DNA extracted from a large number of cells, and measurement of individual nuclei (Doležel and Bartoš 2005). The first approach includes the chemical analysis and reassociation kinetics. These methods are laborious, time consuming, not particularly accurate and hard to interpret in terms of C-values. Therefore, almost all the modern technologies for measurements of nuclear DNA content use single nuclei (Bennett and Leitch 2005*a*).

Contrarily to the measurement in FCM, in static cytometry nuclei are fixed on a solid surface and the DNA content amount can be estimated either from the fluorescence intensity (fluorometry) similarly to FCM, or from the optical density of the stained region (densitometry) (Suda 2004). The latter technique is certainly the most popular, and it involves staining of the DNA with the Feulgen reaction. While, in less sophisticated systems (microdensitometers or microspectrophotometers) the measurement has been done in many small areas across the object and the values integrated, in more recent instruments (*i.e.*, image cytometry) the photometer has been replaced by video or digital cameras and the optical density is determined by estimating grey level values of pixels of a grabbed image (Vilhar *et al.* 2001).

Doležel *et al.* (1998) already showed that, when best practices are followed, FCM results agreed well with those obtained using Feulgen densitometry. This, together with the fact that densitometry techniques are laborious and time consuming resulted in a progressive shifting in the preference of researchers towards FCM (Bennett and Leitch 2005*a*). The only marked advantage of Feulgen densitometry is the possibility of using fixed plant material.

In most cases the use of flow cytometry does not remove the need for cytological work on the studied species, namely the chromosome counting of individual plants (Bennett and Leitch 2005*a*). More advanced molecular cytogenetic techniques, as fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH), can also be very useful, especially for phylogeny studies. Together with FCM, these methodologies were employed in the study of 14 *taxa* of *Festuca* sp. (Poaceae) from Iberian Peninsula (Chapter III.3).

Applications of flow cytometry in plant sciences

Estimation of DNA amount, either in relative or absolute units, is undoubtedly the most common application of FCM in plant sciences. Frequency analyses of the main objectives of FCM studies are given in Chapter V.

Relative nuclear DNA content

Ploidy analysis is the simplest use of DNA FCM in plants, with applications ranging from breeding and seed quality testing to taxonomy and population biology (Galbraith *et al.* 2005). As FCM analyses nuclear DNA content and not the number of chromosomes, in 1984 the Committee on Nomenclature of the Society of Analytical Cytology (Hiddemann *et al.* 1984) suggested that results of cytometric analyses should be differentiated from data

obtained by cytogenetic techniques. The term "DNA aneuploidy" is recommended to describe a sample with abnormal DNA content. Recently, Suda *et al.* (2006) reinforced this position and appealed for consistent terminology in plant sciences and suggested the use of the prefix "DNA" (*i.e.*, DNA ploidy, DNA aneuploidy) when referring to cytometric data. As an example, DNA diploid describes a sample with nuclear DNA content corresponding to a sample with a diploid number of chromosomes determined using a conventional chromosome counting.

Ploidy level screening

The knowledge of the ploidy level has important outcomes in biotechnology, breeding and seed production, and biosystematics.

In the field of plant biotechnology, FCM is usually applied to analyse the genetic stability of *in vitro* cultures and the DNA ploidy level of regenerated plants (Brutovská *et al.* 1998; Endemann *et al.* 2002; Pinto *et al.* 2004; Leal *et al.* 2006). FCM is an important tool in this area, given that the estimation of ploidy level using chromosome counting is many times difficult to perform, as *in vitro* cultures are usually characterized by low mitotic activity (Doležel 1997). Most of the studies focused on the effect of culture conditions and ageing in the ploidy stability of regenerants, in order to certify the suitability of a protocol for large-scale "true-to-type" propagation of selected genotypes. This is indeed one of the main applications of FCM in our laboratory, which results from the long-term interest on the micropropagation of plants with economical importance, such as *E. globulus* (Pinto *et al.* 2002*a*), *Q. suber* (Pinto *et al.* 2002*b*), *Juniperus phoenicea* (Cupressaceae; Brito 2000), *Ulmus minor* (Ulmaceae; Conde *et al.* 2004), among other, for which routine micropropagation protocols have already been established. In Chapter I, the analysis of the ploidy stability during culture *in vitro* of two of those species, *Q. suber* and *J. phoenicea*, is presented. The analysis of the ploidy level is also important in genetic transformation experiments, namely for selecting suitable explants and for minimizing the formation of polyploid transformants, which are often obtained (Chen *et al.* 2001; Ellul *et al.* 2003).

In many species, genetic improvement by conventional methods is time consuming and limited by the space for field experiments. The production of haploids increases the efficiency of selection and opens the possibility for genetic studies and breeding (Hofer and Grafe 2003). Haploids are typically obtained from anther, microspore, and ovary (gynogenesis) *in vitro* cultures and subsequently subjected to treatments with polyploidizing agents (antimitotic substances, as colchicine and oryzalin) for chromosome

doubling, thus producing fertile homozygous lines. Spontaneous dihaploids can also be obtained although usually at lower extent. FCM appears as the ideal technique to rapidly screen the ploidy level of high numbers of regenerants at an early developmental stage (Geoffriau *et al.* 1997; Hofer and Grafe 2003; Lotfi *et al.* 2003; Weber *et al.* 2005; Supena *et al.* 2006).

Whereas in some *in vitro* culture studies the goal is to obtain "true-to-type" regenerants, in others (*e.g.,* Eeckhaut *et al.* 2004) the objective is to produce polyploid varieties/cultivars that may gain new characteristics of economical interest (particularly important in ornamental plants), regain fertility or prevent hybrid sterility, after interspecific crosses between plants of different ploidy levels. Contrarily, another interest can be the production of sterile triploid plants (from a cross between diploids with induced tetraploids) for cultivation in park roads and sidewalks (Vainola 2000) and for horticultural purposes. As in doubled haploid production, polyploidization is usually achieved using antimitotic agents, and regenerants are efficiently screened by FCM (Awoleye *et al.* 1994; van Duren *et al.* 1996; de Carvalho *et al.* 2005; Escandon *et al.* 2005). By other way, in natural conditions, the increase of the ploidy level is often associated with apomixis, *i.e.*, the asexual reproduction through seeds (Holm and Ghatnekar 1996; Bantin *et al.* 2001). An efficient screen for reproductive pathways, in which apomixis is included, based on FCM analysis is a current topic and will be given special attention in a separate section bellow.

In some crops (*e.g.*, *Beta vulgaris*, Amaranthaceae), ploidy is an important parameter of seed lot quality (de Laat *et al.* 1987). To further speed up the analysis, high numbers of individual plants can be pooled in one sample (de Laat *et al.* 1987; Dimitrova *et al.* 1999). However, for a more accurate detection of rare seed contamination or plants of undesirable ploidy level, the analysis of individual plants is necessary (Galbraith *et al.* 2005).

Interspecific hybridization is often applied to transfer desired characters from one species to another. If parental species differ enough in their nuclear DNA amounts, FCM can detect interspecific hybrids according with their intermediate DNA values (Doležel 1997). This is one of the most common applications of FCM in breeding programs and has been already applied to detect and characterize hybrid plants in *Allium* spp. (Alliaceae; Keller *et al.* 1996), *Solanum* spp. (Solanaceae; Dolnicar and Bohanec 2000; Szczerbakowa *et al.* 2003; Trabelsi *et al.* 2005) and *Citrus* spp. (Rutaceae; Scarano *et al.* 2003; Khan and Grosser 2004) among other genera. Similarly, the karyological stability of somatic hybrids produced by protoplast fusion can also be assessed by FCM (Oberwalder

et al. 1998; Ilcheva *et al.* 2001; Binsfeld and Schnabl 2002). As in other applications, FCM enables the screening of large numbers of progenies at an early stage.

As a resume, research and industrial applications of FCM DNA ploidy level include:

- Control of ploidy stability (*e.g.*, micropropagation *in vitro* or conformity of seed lots);
- Screening for haploid plants (*e.g.*, production of dihaploids);
- Screening for diploid plants (*e.g.*, spontaneously occurring dihaploids and selection of diploid plants after genetic transformation);
- Screening for triploid plants (*e.g.*, hybrids with low seed production);
- Screening for novel ploidy levels (*e.g.*, production of tetraploids);
- Screening for interspecific hybrids.

Besides the above referred applications, ploidy level screening is also very useful in taxonomy and biosystematics. FCM has been used successfully in the analysis of the ploidy status of many accessions of cultivated species. Some examples of ploidy screening in germplasm accessions include *Medicago sativa* (Fabaceae; Brummer *et al.* 1999), *Bromus* spp. (Poaceae; Tuna *et al.* 2001), *Dioscorea alata* (Dioscoreaceae; Egesi *et al.* 2002) and *Musa* spp. (Musaceae; Bartoš *et al.* 2005; Nsabimana and van Staden 2006; Pillay *et al.* 2006). In a collaboration work with our laboratory, germplasm characterization of more than 300 accessions of *Dioscorea* spp. was undertaken using FCM (J. Obidiegwu *et al.* 2006, unpublished data). Also, the use of FCM permitted the discovery of new cytotypes in several plant species (Lysák and Doležel 1998; Suda 2002; Weiss *et al.* 2002) and the development of large-scale studies of population cytotype structure (Husband and Schemske 1998; Burton and Husband 1999; Hardy *et al.* 2000; Baack 2004; Suda *et al.* 2004). These studies are aimed at understanding the overall distribution pattern of cytotypes (sympatry *vs.* parapatry), the evolutionary forces governing the cytotype co-existence, inter- *vs.* intra-cytotype competition, and/or mechanism of reproductive isolation (Suda 2004). Also, as insights on the origin and establishment of new polyploid species may be gained by studying the distributions of ancestral and derivative cytotypes at multiple spatial scales, these studies present high importance in current biosystematics. In our laboratory, FCM was efficiently applied to determine DNA ploidy level of *O. pes-caprae*, a widespread invasive weed in the Mediterranean climate region that presents different ploidy levels (tetraploid and pentaploid) associated with given morphotypes (short- and long-styled) or sterile floral form (Castro *et al.* 2007*b*) (Fig. I.9). In this study, more than 350 individuals from 52

populations in the Western Area of the Mediterranean Region were rapidly screened for ploidy level using FCM .

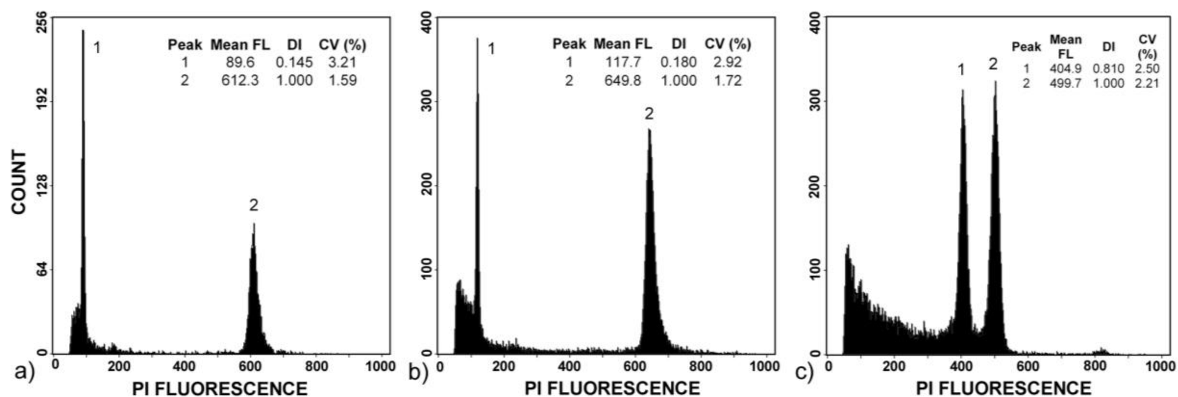

Fig. I.9 Histograms of relative PI fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA, internal reference standard) and *Oxalis pes-caprae*: a) long-styled morph; b) short-styled morph. In histogram c), a short-styled individual was used as an internal standard instead of *P. sativum*. In the three histograms the following peaks are marked: 1 – nuclei at G_0/G_1 phase of sample; 2 – nuclei at G_0/G_1 phase of internal standard. The mean channel number (Mean FL), DNA index (DI, *i.e.*, mean channel number of sample / mean channel number of reference standard) and coefficient of variation value (CV, %) of each G_0/G_1 peak are also given (adapted from Castro *et al.* 2007*a*).

Aneuploidy and B chromosomes

The accuracy of FCM to detect small differences in nuclear DNA content generally requires the application of internal standardization with a small difference in genome size between sample and reference standard (if needed, two different standards can be applied to improve the sensibility of the analysis, Fig. I.7), the lowest possible CV of DNA peaks, and an equal proportion of nuclei within sample and standard peaks (Roux *et al.* 2003). Bashir *et al.* (1993) were the first to use FCM to evaluate aneuploid plant populations, by detecting the presence of a pair of rye chromosomes in wheat-rye addition lines. Two years later, Pfosser *et al.* (1995) were able to detect very small differences of 1.84% in wheat DNA content that resulted from the addition of a pair of rye chromosome arms to the wheat genome. In *Humulus lupulus* (Cannabaceae), Šesek *et al.* (2000) were able to detect one extra chromosome. However, if in the referred studies the case species presented big chromosomes, Roux *et al.* (2003) was able to detect aneuploidy in triploid *Musa,* whose chromosomes are small. In this study, *in vitro* mutagenesis was used to generate aneuploid plants with minus one or two chromosomes. Subsequent characterization by DNA FCM and chromosome counting revealed a perfect agreement between both methods. In our laboratory promising results were obtained in the analyses of the ploidy stability of *in vitro* cultured plants of *C. betacea*, with a clear identification of

aneuploid individuals (Fig. I.7). Despite the nice works discussed above, which show the reliability and sensitivity of FCM to detect aneuploidy, in some cases the occurrence of a low level aneuploidy can be difficult to prove without a confirmation by chromosome counting.

FCM analysis of B chromosomes (= accessory, supernumerary) is almost at its beginning. Due to their small sizes, the detection of accessory chromosomes is sometimes complex, and their presence is usually associated with intraspecific variation in genome size (*e.g.*, Huff and Palazzo 1998). However, recent studies in *Boechera holboellii* (Brassicaceae), revealed a bimodal distribution of nuclear fluorescence related with the presence or absence of B chromosomes (Sharbel *et al.* 2004).

Sex determination in dioecious plants

In breeding and production of dioecious crops, the identification of plant sex at an early stage of ontogenesis can be of high importance (Galbraith *et al.* 2005). If there is sufficient heteromorphism in DNA content of sex chromosomes (small X and large Y) and if very high resolution analyses are achieved (even higher than for aneuploidy), FCM can be applied with success, as shown by the studies of Costich *et al.* (1991) and Doležel and Göhde (1995). Although Costich *et al.* (1991) were the first to demonstrate the feasibility of FCM for such purposes, they used an “indirect” sex comparison, with male and female individuals being processed separately and their DNA amounts further compared. Doležel and Göhde (1995), in *Silena latifolia* (Caryophyllaceae), presented a more elegant approach as samples of both sexes were analysed simultaneously, with the resulting histogram presenting two non-overlapping peaks (CV values as low as 0.53% were obtained). A different approach, based in differences in nucleolar heterochromatin between female and male individuals, has been demonstrated for *Phoenix dactylifera* (Arecaceae) using dual staining of nuclei (as in studies of base composition, see below) and subsequent analysis by FCM (Siljak-Yakovlev *et al.* 1996).

Endopolyploidy, mixoploidy and similar phenomena

Endoreduplication (= endopolyploidization), which consists of repeated cycles of DNA synthesis without occurrence of cell divisions, is a common phenomenon in differentiated cells of seed plants (Smulders *et al.* 1995; Śliwińska and Lukaszewska 2005). This process leads to the presence of cells with various ploidy levels in an organ, *i.e.*, polysomaty (Śliwińska and Lukaszewska 2005). FCM is considered the ideal technique to

reliably detect and characterize this phenomenon and since the pioneer work of de Rocher *et al.* (1990) the number of studies on endopolyploidy continuously increased.

The patterns of polysomaty within a species are usually different in various organs and correlated with the developmental stage (de Rocher *et al.* 1990; Gilissen *et al.* 1993; Lee *et al.* 2004; Barow 2006). Although it is a common and frequently studied phenomenon, the biological significance of endopolyploidy is not yet understood (Mishiba and Mii 2000; Śliwińska and Lukaszewska 2005). Some correlations between systematics, organ, life strategy, genome size, cell size and nuclear volume were already found (Lemontey *et al.* 2000; Kudo and Kimura 2002; Barow and Meister 2003). Barow and Meister (2003) suggested that phylogenetic position is the major factor determining the degree of endopolyploidy within a species, while organ type, life cycle and nuclear DNA content presented a minor but also important effect on endoreduplication. So far, systemic endopolyploidy was mainly described in species with small genomes including *Arabidopsis thaliana* (Brassicaceae; Galbraith *et al.* 1991), *Beta vulgaris* (Śliwińska and Lukaszewska 2005), *Brassica oleracea* (Kudo and Kimura 2002), *Cucumis sativus* (Cucurbitaceae; Gilissen *et al.* 1993), *Lycopersicon esculentum* (Smulders *et al.* 1995), and several species of Orchidaceae (Jones and Küehnle 1998; Fukai *et al.* 2002; Lim and Loh 2003; Yang and Loh 2004) and succulents (de Rocher *et al.* 1990; Mishiba and Mii 2000). Also, recent work by Barow and Meister revealed the occurrence of polysomaty in several organs of 33 out of 54 seed plant species, belonging to 10 angiosperm families (Barow and Meister 2003). In our laboratory, FCM was successfully applied in the evaluation of polysomaty in various organs of *Polygala vayredae* (Polygalaceae) and *P. calcarea*, two congeneric species differing in their distribution range. In this study, different patterns of endopolyploidy were observed according with the species, organ and developmental stage (Castro *et al.* 2007*b*).

Contrarily to chromosome counting, where traditionally only one histological layer is analysed in a root tip meristem, all three histological layers (LI, LII and LIII) can be studied using FCM. Therefore, it is possible to detect differences in ploidy level among layers, *i.e.*, mixoploidy (= chimerism). In the screening of ploidy level of *Rhododendron* spp. (Ericaceae), de Schepper *et al.* (2001*a*) detected one cultivar that was a cytochimera, with two mixoploid (2x + 4x) histological layers and one tetraploid. Cytochimeras were also found in *Hosta* (Agavaceae; Zonneveld and van Iren 2000) and in colchicine induced *in vitro* cultured tissue of *Musa* spp. (Roux *et al.* 2001).

De Schepper *et al.* (2001*b*) also discovered that differences in petal edges colour of the variegated flowers of cultivated azaleas, were correlated with a somatic mosaic (= somatic

polyploidy) phenomenon, *i.e.*, petal edges proved to be tetraploids, while the rest of the flower tissue was diploid. In addition neither the flower colour pattern nor the ploidy differences were found to have a chimeric origin.

Recently, FCM was also very useful to clarify the postulate of "cryptopolyploidy", *i.e.*, an intrinsically polyploid but numerically non-polyploid structure of chromosome complements (Greilhuber and Obermayer 1999; Obermayer and Greilhuber 2006). In both these works, the differences in DNA content that were obtained were far from the expected values if the concept of "cryptopolyploidy" was correct.

Cell cycle

As the nuclear DNA content reflects the position of a cell within the cell cycle, FCM can also be used to study cell cycle kinetics (Doležel 1991). Some of the methods (*e.g.*, graphical, nonparametric curve-fitting) that are employed to deconvolve DNA content histograms are relatively simple, but also lack resolution. In addition, as fluorescence histograms are static, no information on nuclei progression through the cell cycle is obtained. Furthermore, the method that is used to isolate nuclei precludes the detection of M phase cells and the reliability of the analysis is dependent on CV values of DNA peaks, on debris background, and on the proportion of nuclear doublets (Galbraith *et al.* 2005). Despite of the referred limitations these methods have been used to study the cell cycle of many plant tissues and organs (Doleželová *et al.* 1992; Śliwińska *et al.* 1999; Sandoval *et al.* 2003; Śliwińska 2003). FCM has also been used to evaluate the extent of cell cycle synchronization and the position of a synchronized population within the cell cycle (Peres *et al.* 1999). These studies enabled the analysis of cell cycle regulation and the effect of several compounds on cell cycle progression, as demonstrated by the studies of Roudier *et al.* (2000) and Binarová *et al.* (1998), respectively.

The precision of cell cycle analysis can be improved if multiparametric analyses are employed. This is an almost unexplored area in plant sciences, which enables the distinction between cycling and quiescent cells and between cells in G_2 and M phase of the cell cycle. The most popular method is based on the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into newly synthesized DNA in place of thymidine (Galbraith *et al.* 2005). BrdU can be detected either by using specific antibodies (Lucretti *et al.* 1999), the preferred method due to the use of short labelling pulses of BrdU, or based on quenching the fluorescence of the DNA dye Hoechst 33258 (Trehin *et al.* 1998). Preliminary studies using the first approach are presented in Galbraith *et al.* (2005). For a review on the use of FCM to study cell cycle in plants see Pfosser *et al.* (2007).

Absolute nuclear DNA content

Genome size

The knowledge of genome size is important in many areas of plant research. However, before focusing on the significance of genome size studies it is important to introduce the current terminology and nomenclature for C-values.

Several definitions were already given to the "C" symbol. First introduced as the "constant" DNA content (*i.e.*, the amount of DNA that was characteristic of a particular genotype) by Swift (1950), this symbol was later defined by Bennett and Smith (1976) as the "DNA content of the unreplicated haploid chromosome complement". Bennett *et al.* (1998) restricted the term "genome size" to the DNA content of the monoploid chromosome set, while "C-value" referred to the DNA content of the complete chromosome complement. Whereas, C-value equals the unreplicated genome size in diploid species, in polyploid species nuclear C-value always exceeds genome size. These concepts lacked a general acceptance within the scientific community and recently a consistent terminology was presented by Greilhuber *et al.* (2005). The adjectives "monoploid" and "holoploid" were added to genome size to distinguish between monoploid genome, *i.e.*, the single genome with *x* chromosomes, of which there are two per unreplicated nucleus in a diploid individual and several in a polyploid, and the complete, which is the holoploid genome. C-value is the abbreviation for the holoploid genome and Cx-value for the monoploid genome, with x referring to the basic chromosome number *x*). Quantitative data are given with numerical prefix as 1C-, 2C-, 1Cx-, 2Cx-values, and so on (Greilhuber *et al.* 2005).

Flow cytometric estimation of nuclear genome size should involve simultaneous measurement (internal standardization) of specimen and reference standard nuclei. The genome size of the sample is then calculated by the ratio between the mean channel position of G_0/G_1 peaks of the sample and the standard, multiplied by the nuclear DNA content of the reference standard.

Both monoploid and holoploid genome sizes can be expressed in mass units (usually picograms, pg), or as number of base pairs (usually Mbp). Conversion factors for both units were recently corrected by Doležel *et al.* (2003) and are as follows:

$$\text{DNA content (bp)} = (0.978 \times 10^9) \times \text{DNA content (pg)}$$

$$\text{DNA content (pg)} = \text{DNA content (bp)} / (0.978 \times 10^9)$$

The current state of knowledge regarding higher plant genome sizes is resumed in Table I.3. Since 1950, more than 10,000 quantitative estimates of plant C-values were made, covering no less than 4,500 plant species (Bennett and Leitch 2005*a*). Along the

years, Bennett and colleagues compiled large lists of published and unpublished C-values in a total of more than 4,000 angiosperm species (1.8% of the angiosperm flora) collected from 465 original publications (Bennett and Smith 1976, 1991; Bennett *et al.* 1982, 2000*a*; Bennett and Leitch 1995, 1997, 2005*b*). In order to fill phylogenetic gaps, recent efforts have been focused on increasing the familiar representation in angiosperms (see key recommendation 1 of the Second Plant Genome Size Workshop, Kew, 2003) (Hanson *et al.* 2001*a*, 2001*b*, 2003, 2005; Leitch and Hanson 2002). Despite of these efforts, the goal of 75% familial representation by 2008/2009 is still far from being reached (M.D. Bennett 2005, pers. comm.).

Table I.3 Descriptive statistics (minimum – min., maximum – max., mean, mode and range) of 1C DNA values in major plant groups, together with the level of species representation of C-values data using the latest release of the Plant DNA C-values database (Bennett and Leitch 2005*c*; adapted from Leitch and Bennett 2007).

Plant group	Min. (pg)	Max. (pg)	Mean (pg)	Mode (pg)	Range (max./min.)	No. species with DNA C-values	No. species recognized	Species representation (%)
Bryophytes	0.085	6.42	0.54	0.45	74	176	≈ 18,000	≈ 1.0
Pteridophytes	0.16	72.68	12.93	7.10	454	67	≈ 11,900	≈ 0.6
Gymnosperms	2.25	32.20	16.99	9.95	14	207	≈ 730	≈ 28.4
Angiosperms	0.065	127.40	6.30	0.60	1,960	4,427	≈ 250,000	≈ 1.8

If it is clear that the information on C-values in angiosperms has been continuously compiled and updated, the same cannot be said for the other plant groups, as it was not until 1998 that the first reference list for a non-angiosperm group was published (Murray 1998). This compilation presents estimates for 117 gymnosperm species, which together with values from recent works that include the completion of familial representation (Leitch and Hanson 2002), makes gymnosperms the plant group with the highest percentage of species representation (Table I.3).

The scenario on the other major plant groups, *i.e.*, pteridophytes and bryophytes, is rather poor (Table I.3). In pteridophytes (clubmosses, ferns and horsetails), a list of estimates of DNA amounts was first compiled by Bennett and Leitch (2001), which included DNA C-values for only 48 species. Since then, only the works of Obermayer *et al.* (2002) and Hanson and Leitch (2002) focused on filling some of the phylogenetic gaps on C-values data on this group. In bryophytes (mosses, liverworts and hornworts), no list has been made so far, although the exhaustive study of Voglmayr (2000), where estimations of genome size for 138 different *taxa* are given, is a good approximation of a

compilation of C-values in this group. Unfortunately, a few other studies were targeted to bryophytes (*e.g.,* Temsch *et al.* 1998).

Due to the high number of C-value estimations (compiled in an already high number of lists) that started to be available and to the difficulty in determining whether an estimate for a given species was already listed, in 1997, M.D. Bennett and I. Leitch decided to provide data in a user-friendly database and to make it available in an internet webpage (http://www.rbgkew.org.uk/cval/homepage.html) (Bennett and Leitch 2005*c*). The current release of this database contains estimates of DNA content for 5,150 species comprising 4,427 angiosperms, 207 gymnosperms, 67 pteridophytes, 176 bryophytes and 253 algae.

From the basic descriptive statistics provided in Table I.3 it is possible to observe a remarkable genome size variation in higher plants, with 1C-values ranging from 0.065 pg in *Genlisea aurea* and *G. margaretae* to 127.4 pg in *Fritillaria assyriaca*. The largest range in C-values is found in angiosperms, while the lowest is verified in gymnosperms. However, caution is needed as the known C-values may not be totally representative (see species representation in Table I.3).

The C-value information provided by the Plant DNA C-values database has a direct practical utilization in modern molecular research, namely for an appropriate planning of the construction of genomic DNA libraries (*i.e.*, estimation of the number of clones needed to achieve a desired genome coverage), and of amplified fragment length polymorphism (AFLP; Fay *et al.* 2005) and microsatellites experiments (Garner 2002). It is also important to identify candidate species for complete genome sequencing. The database also enables large-scale comparative analyses of genome size, with applications in the fields of ecology, evolution, genomics and conservation (Bennett and Leitch 2005*a*). In the works of C. Knight and colleagues, correlations between genome size (using a set of C-values from the database) and several environmental factors (Knight and Ackerly 2002), and between genome size and seed mass (Beaulieu *et al.* 2007), were inspected. Also, Leitch and Bennett (2004) used data from 3021 angiosperm species to provide insights of the evolution of C-value in polyploid species. In the conservation area, Vinogradov (2003) and Knight *et al.* (2005) showed a negative relationship between genome size and species richness in different plant groups, suggesting that plants with large genomes may be more susceptible to extinction. Finally, large-scale analyses were conducted across the angiosperm phylogeny, by superimposing DNA values on rigorous phylogenetic trees (Leitch *et al.* 1998; Soltis *et al.* 2003). These works showed that ancestral angiosperms had very small genome [≤ 1.4 pg, according to the genome size categories defined by Soltis *et al.* (2003)] and that very large genomes (≥ 35.0 pg) represent a derived condition

that evolved independently more than once during the evolution of angiosperms, namely in monocots and in Santalales clades. Recently, Leitch *et al.* (2005) extended the analysis to other land plant groups and provided insights into the genome size of ancestral species (very small genome sizes in angiosperms and bryophytes, and intermediate, *i.e.*, > 3.5 and < 14.0 pg, in gymnosperms and most monilophytes) and evidence for a dynamic, bidirectional genome size evolution (*i.e.*, either increases or decreases of genome size can take place during evolution). For a summary of the results see Bennett and Leitch (2005*a*). Although at a lower scale, examination of the genome size variation was already done within some individual families of angiosperms (*e.g.*, Obermayer *et al.* 2002; Wendel *et al.* 2002), and among endemic insular angiosperms (Suda *et al.* 2003, 2005). In the studies of Suda and co-workers, it was shown that endemic genera from Macaronesia possess very small DNA amounts, plausibly by a selection towards genomes that rapidly adapt to islands environment by a fast individual development.

It has already been mentioned that genome size data is important in the prediction of several phenotypic characters, phenology, and ecological behaviour, which are explained by the nucleotype concept [*i.e.*, conditions of the nuclear DNA, as its amount, that affect the phenotype independently of its encoded informational content (Bennett 1971)]. Among the correlations already found between genome size and a wide range of characters at nuclear, cellular and tissue level, positive relationships were discovered between total amount of DNA and: minimum nuclear volume (Baetcke *et al.* 1967), total somatic chromosome volume (Bennett *et al.* 1983), duration of mitosis and meiosis (Bennett 1977), seed size and volume (Bennett 1972), among other characters (for a review see (Bennett and Leitch 2005*a*). Genome size variation can also have consequences at the whole plant level, influencing many aspects of plant development. Therefore, the influence of genome size also extends to ecological and environmental issues. The relationship between genome size and duration of meiosis, implied a correlation between DNA amount and minimum generation time in herbaceous plants, with ephemerals generally presenting the smallest genomes, followed by annual and facultative perennials, whilst obligate perennials possess higher DNA amounts (Bennett 1972). Also, Bennett *et al.* (1998) suggested that a small genome is a requirement for weediness. Other important ecological implications include frost sensitivity (positive correlation; MacGillivray and Grime 1995) and plant phenology (negative correlation; Grime *et al.* 1985). Nevertheless, and notwithstanding the importance of these trends, it should be noted that ecological variables are not a general property of all plant groups, and no similar relationships were found in numerous *taxa* (Suda 2004).

Genome size studies can also be important in the field of taxonomy and systematics. In some related species with the same number of chromosomes but different genome size, FCM can be used for species recognition [*e.g.*, subspecies delimitation in *Crepis foetida* (Asteraceae; Dimitrova *et al.* 1999)]. The usefulness of FCM for *taxa* delimitation and for analyses of interspecific variation has been documented in *Petunia* spp. (Solanaceae; Mishiba *et al.* 2000), *Hydrangea* spp. (Hydrangeaceae; Cerbah *et al.* 2001), *Artemisia* spp. (Asteraceae; Torrell and Vallès 2001), *Cistus* spp. (Cistaceae; Ellul *et al.* 2002), *Cirsium* spp. (Asteraceae; Bureš *et al.* 2004), *Carthamus* spp. (Asteraceae; Garnatje *et al.* 2006), among other genera. At a specific level the interest is usually focused on the stability of nuclear genome, namely among geographically isolated populations. Small, negligible differences in nuclear genome size have been observed in *Pisum sativum* (Baranyi and Greilhuber 1995), *Sesleria albicans* (Poaceae; Lysák *et al.* 2000), *Abies fraseri* (Pinaceae; Auckland *et al.* 2001) and *Allium cepa* (Bennett *et al.* 2000*b*). However, these studies contrast with reports where intraspecific variation in genome size has been documented (Rayburn *et al.* 1989; Michaelson *et al.* 1991; Rayburn *et al.* 2004). Therefore, while some argue for a large plasticity of nuclear genome, others claim for a more stable genome size within species. In reality, the growing number of reports that do not confirm the intraspecific variation reported in original publications [see list of "blunder-killing" papers of Suda (2004) and the reviews by Greilhuber (1998, 2005)] has shifted the pendulum towards the stability side, and without eliminating the possibility of occurrence [see recent work by Šmarda and Bureš (2006)] it seems that this phenomenon is much less common than previously assumed. In many of the papers that were later disproved, best practice methodologies were not followed and apparent variation of genome size could be due to instrumental or methodological errors, interference of cytosolic compounds with DNA staining, differences between laboratories, and/or taxonomy heterogeneity of the material under investigation (Doležel *et al.* 2007*b*). In chapter III, three nuclear DNA content studies are presented, which include analyses at inter- (Chapter III.1 and III.3) and/or intra-specific level (Chapter III.2).

Other interesting applications of genome size in taxonomy include the assessment of genomic constitution in allopolyploid *taxa* (*i.e.*, polyploid plants that combine genomes from at least two different parental species) (Lee *et al.* 1997; Lysák *et al.* 1999) and the detection of hybrid individuals from homoploid crosses (Jeschke *et al.* 2003; Morgan-Richards *et al.* 2004).

DNA base composition

The proportion of A-T and G-C base pairs provides further information about genome organization. The base composition varies among plant families, but usually presents high similarity at lower taxonomic levels (Barow and Meister 2002). The FCM estimation of DNA base content is based on the comparison of fluorescence intensities of nuclei stained with intercalating and base-specific dyes (Schwencke *et al.* 1998; Kamaté *et al.* 2001; Bogunic *et al.* 2003; Siljak-Yakovlev *et al.* 2005). All these studies as well as many others, followed the model developed by Godelle *et al.* (1993) that assumed a linear correlation between DAPI and A-T base proportion, despite that the exact mode of binding of this fluorochrome still is poorly known. Indeed, recent studies revealed that the referred model presented a rather simplistic approach, as it did not consider the effect of non-randomness of base sequences on dye binding (Barow and Meister 2002), and some incorrectness, as the number of consecutive bases necessary to bind a dye molecule (Meister 2005). For an updated overview on this topic see Meister and Barow (2007).

Reproductive mode screening

The knowledge of the reproductive process implicated in embryo and seed formation is important in plant breeding, seed industry, and evolutionary and biosystematics studies. An overwhelming advance in this area was triggered by the development of a reliable and efficient protocol [after modifications of the chopping procedure of Galbraith *et al.* (1983)] for screening the reproductive mode using FCM in mature seeds (Matzk *et al.* 2000). Taking into consideration the type of male and female gametes (reduced *vs.* unreduced), and the origin of embryo (zygotic *vs.* parthenogenetic) and endosperm (pseudogamous *vs.* autonomous route), 12 different pathways of seed formation can theoretically be reconstructed (Matzk 2007). Since its release, the protocol for FCM seed screen (FCSS) has been used to characterize the mode of reproduction of *Hypericum* spp. (Clusiaceae; Matzk *et al.* 2001), *Coprosma* spp. (Rubiaceae; Heenan *et al.* 2002), *Poa pratensis* (Poaceae; Stephens *et al.* 2006), among other species. Recently, most studies are interested in the discrimination of apomictic mutants in sexual plants, in quantifying the ratio between apomictic and sexual progeny in species with facultative apomixis, and in assessing the contribution of unreduced gametes (Doležel *et al.* 2007*b*). For a review of this novel and interesting topic see Matzk (2007).

Genotoxicity assays

Another attractive application of FCM is the analysis of the effect of genotoxic compounds on nuclear DNA content. McMurphy and Rayburn (1993) reported changes in nuclear DNA content of *Zea mays* seedlings grown in soil contaminated with coal fly ash. These changes were due to higher fluorescent intensities of nuclei isolated from seedlings grown in contaminated soils when compared to nuclei from control plants. Increases in CV values of G_0/G_1 peaks and in the frequency of G_2 nuclei were also observed in fly ash treatments. Also in *Z. mays*, Biradar *et al.* (1994) observed changes in nuclear DNA after exposure to the fungicide Triticonazole. More recently, Citterio *et al.* (2002), proposed a new strategy, based on FCM and AFLP, to assess soil genotoxicity. Using *Trifolium repens* (Fabaceae) plants grown in soils containing heavy metals these authors detected a decrease in DNA index (due to a loss of fluorescence in sample and standard nuclei) with the increase of chromium concentrations. In parallel, an increase of debris background was observed in higher concentrations of chromium. Histograms presented in this study are very similar to those presented in Chapter IV.2. This indicates that the changes in DNA content were not due to genotoxic effect of chromium, but rather due to higher production of cytosolic compounds in roots and shoots, whose production was induced by chromium exposure, and which affected nuclei fluorescence. Therefore, caution and awareness of this phenomenon is very important to certify whether the reported differences are real or artefactual. Also, no ploidy alterations were detected after exposure of *T. repens* plants to soil contaminated with two different polycyclic aromatic hydrocarbons (Aina *et al.* 2006). Our experience in this field points in this direction, as the genotoxic assays already developed in our laboratory using different species, tissues and heavy metals, up to this moment failed to reveal any differences in the ploidy level and/or in the CV values of DNA peaks (*e.g.*, Monteiro *et al.* 2005*a*, 2005*b*).

FLOWER database

In Chapter V, a plant DNA FCM database (FLOWER) is presented. This database intends to be a comprehensive, easily accessible and user-friendly source of information on plant FCM articles, as well as a platform for developing quantitative analyses of particular aspects important in FCM practice. The usefulness of the database is demonstrated by presenting and discussing the results of the analyses made for the most relevant FCM parameters.

First plant FCM book

As pointed out throughout this chapter, many reviews of particular topics in plant FCM can be found in the recently published book “Flow Cytometry with Plant Cells – Analysis of Genes, Chromosomes and Genomes” (Fig. I.10). This is the first book on the applications of FCM to plants, and is prepared in a comprehensive and instructive way, covering nearly all the fields of the actual research of FCM in plant sciences. It also presents many practical advices that are helpful for beginners and even for more experienced users. Therefore, its use as primary source of updated and reviewed information is highly recommended.

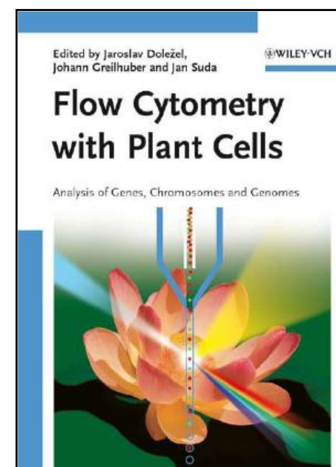

Fig. I.10 Cover of the first book dedicated to the applications of FCM in plant sciences.

Objectives of this PhD Thesis

The aim of this PhD Thesis was to apply FCM and related techniques to study plant genomes. It was intended to explore in detail the potentialities of FCM for DNA ploidy level and genome size estimations, with particular attention given to the study of recalcitrant woody plant species (Chapters II and III). It was also a goal to develop innovative methodological studies concerning one of the most unexplored subjects in plant FCM, *i.e.*, nuclear isolation buffers, as well as one of the current hot topics, which is the interference of cytosolic compounds on plant nuclei and nuclear DNA content estimations (Chapter IV). Finally, it was an objective to collect, compile and analyse the published data on plant FCM, and make the obtained information available in one user friendly database to the plant FCM community (Chapter V).

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Chapter II

Ploidy stability analyses in woody plant species

II.1 Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry

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Loureiro J, Pinto G, Lopes T, Doležel J, Santos C (2005) Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. Planta **221**:815-522.

Abstract

Flow cytometry analyses were used to verify the ploidy stability of *Quercus suber* L. (Fagaceae) somatic embryogenesis process. Leaf explants of two adult cork oak trees (QsG0 and QsG5) of the North of Portugal were inoculated on MS medium with 2,4-D and zeatin. After 3 months, *calli* with embryogenic structures were isolated and transferred to fresh MS medium without growth regulators, and somatic embryo evolution was followed. Morphologically normal somatic embryos (with two cotyledons) and abnormal somatic embryos (with one or three cotyledons) were used in this assay. Flow cytometry combined with propidium iodide staining was employed to estimate DNA ploidy levels and nuclear DNA content of somatic embryos and leaves from mother plants. No significant differences ($P \leq 0.05$) were detected among embryos, and between the embryos and the mother plants. Also, after conversion of these embryos, no significant morphological differences were observed among the somatic embryo derived plants. These results and further studies using converted plantlet leaves and embryogenic *callus* tissue indicate that embryo cultures and converted plantlets were stable with regard to ploidy level. As no major somaclonal variation was detected our primary goal of "true-to-type" propagation of cork oak using somatic embryogenesis was assured at this level. The estimation of the 2C nuclear DNA content for this species is similar to the previously obtained value.

Keywords flow cytometry; ploidy stability; nuclear DNA content; ploidy changes; *Quercus* L.; somatic embryogenesis

Introduction

The genus *Quercus* (Fagaceae) comprises more than 600 species, most of them trees, characterised by their fruits (acorns). Some of the species of this genus are of outstanding economical and forestry importance (Bueno *et al.* 2000). Cork oak (*Quercus suber* L.) is an abundant species in the Atlantic and West Mediterranean countries where it is an important component of Mediterranean ecosystems (Pinto *et al.* 2002). In these countries and particularly in Portugal where it is used for production of more than 50% of the cork used worldwide, the cork transformation industry is of great economical importance (Pinto *et al.* 2001). Although in recent data cork oak is still the second predominant forestry species in Portugal, where it occupies approximately 800,000 ha (Direcção Geral de Florestas 2001), this species is threatened and was recently given a protection status by FAO (1996). Unfortunately, during 2003 and 2004, huge areas of cork oak plantations were destroyed by fire with dramatic impacts at both environment and social/economical levels in Portugal. Therefore, it is urgent to develop and monitor efficient methodologies of regeneration of selected genotypes for both germplasm preservation and reforestation.

In vitro propagation methodologies, such as somatic embryogenesis, present some interesting alternative approaches to the traditional propagation techniques as the latter has some serious constraints. Somatic embryogenesis is frequently regarded as the best system for propagation of superior genotypes (Kim 2000) mostly because both root and shoot meristems are present simultaneously in somatic embryos (SE). Somatic embryogenesis has been reported for some *Quercus* species [for a review see Wilhelm (2000)] however it is still consensual that this is a difficult genus to work with. For *Q. suber* most of the somatic embryogenesis studies (Hernandez *et al.* 1999; Toribio *et al.* 1999; Hornero *et al.* 2001; Pinto *et al.* 2001) were accomplished with success using juvenile materials. The use of juvenile material, however, may, in the near future, hamper the integration of this technique in breeding/improvement programs where selection is focused in adult plants (Pinto *et al.* 2002). This major limitation has been recently overcome by Pinto *et al.* (2002) that presented a reproducible protocol for somatic embryogenesis induction from leaves of a 60-year-old cork oak tree, including plant conversion. This report opens new perspectives for breeding programs of selected cork oak genotypes supported by the fact that no morphological abnormalities have been found up till now in SE derived plants.

Somatic embryogenesis has long been regarded as a stable system. However, in other *Quercus* spp. there is evidence that somatic embryogenesis can have a multicellular origin that increases the risk for somaclonal variation (Wilhelm 2000). Recent findings in

Q. robur (Wilhelm *et al.* 1999; Endemann *et al.* 2001) demonstrate that several years of continuous culture can result in tetraploidy. Therefore to minimize the risk for genetic variation a rapid screening for possible changes has to be applied to assure our primary goal of “true-to-type” propagation.

Random amplification of polymorphic DNA (RAPD) markers for genetic stability analysis were used for *Q. suber* (Gallego *et al.* 1997) and *Q. serrata* (Thakur *et al.* 1999; Ishii *et al.* 1999) SE but no aberrations were detected in the banding pattern. However, several studies in conifers indicated that these types of markers may not be ideal for assessing the mutability of the cell lines and derived plants, as compared to markers such as simple sequence repeats (SSRs) or microsatellites (Isabel *et al.* 1996; Fourré *et al.* 1997). Besides this approach, other methodologies focused on ploidy changes must also be routinely used to assay genetic stability. The use of flow cytometry (FCM) for nuclear DNA content analysis is an excellent alternative for assessing clonal fidelity. However, few reports have used this technique to assay somaclonal variation in woody plants (*e.g.*, Bueno *et al.* 1996; Endemann *et al.* 2001; Conde *et al.* 2004; Pinto *et al.* 2004) and there is only one report concerned to *Q. suber* embryos derived from gametic embryogenesis (Bueno *et al.* 2000).

Flow cytometry, has been presently used in plant sciences, mostly focused on nuclear DNA content, ploidy and cell cycle analyses (*e.g.*, Trehin *et al.* 1998; El Maâtaoui and Pichot 1999; Moscone *et al.* 2003; Conde *et al.* 2004; Pinto *et al.* 2004). These analyses are based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence of stained nuclei (Doležel 1991). In most plants, analyses of relative DNA content of nuclei isolated from young tissues yields a histogram showing a dominant peak corresponding to nuclei at the G_0/G_1 phase of the cell cycle and a minor peak corresponding to G_2 nuclei. To estimate ploidy levels, the position of the G_0/G_1 peak on a histogram of an unknown sample is compared to that of a reference plant with known ploidy (Doležel 1997). In comparison with chromosome counting, FCM presents several important advantages like: convenience (sample preparation is easy), rapid output (dozens of samples can be prepared and analysed in one working day), non requirement of dividing cells and it needs only few milligrams of tissue (Doležel 1997).

In the investigation reported here, FCM analyses were used to verify the ploidy stability of *Q. suber* somatic embryogenesis process by screening for chromosomal changes during different stages of the process and among SE showing morphological abnormalities. To accomplish this goal, *in vitro* morphologically normal SE (with two cotyledons, SE2) and abnormal SE (with one cotyledon, SE1 or three cotyledons, SE3)

were screened and compared among themselves and with leaves of the adult plant from which they were obtained. To complement these analyses, we estimated the ploidy level of embryogenic *callus* tissue and converted plantlets and compared the obtained values with the ones found previously.

Materials and methods

Induction of somatic embryogenesis

Cuttings collected during the months of May and June from two adult *Q. suber* L. trees (QsG0 and QsG5) in the North of Portugal (Fig. II.1.1a) were treated as described by Pinto *et al.* (2002). Embryogenic *calli* were induced in accordance with the protocol established by Pinto *et al.* (2002). Briefly, explants were placed on Murashige and Skoog (1962) (MS) medium with 30 g L^{-1} sucrose, 3 g L^{-1} gelrite, pH adjusted to 5.8 and supplemented with 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.0 µM zeatin, in the dark at 24 ± 1ºC to induce somatic embryogenesis. After 3 weeks, cultures were transferred to a photoperiod of 16 h and exposed to a light intensity of 98 ± 2 µmol m^{-2} s^{-1}. Three months later, *calli* were transferred to the same induction medium and when somatic embryos (SE) were present, the embryogenic *calli* were isolated and transferred to fresh MS medium without growth regulators (MSWH) and SE evolution was followed.

When SE reached the cotyledonal stage morphological abnormalities could be observed (*e.g.*, the number of cotyledons). Therefore, two strategies were adopted: (1) normal and abnormal SE were isolated for embryo germination and/or conversion and (2) the same types of SE were isolated for ploidy stability analysis by FCM. As SE converted and plants were obtained, leaves from these were collected and ploidy analyses were performed. Also, and in order to evaluate if somatic changes occur during early embryogenesis process, SE *calli* samples were also analysed by FCM.

Calli used in the stability assay that belong to QsG0 genotype were approximately 4-year-old or less and *calli* of QsG5 genotype were approximately 1-year-old. During the whole experiment *calli* were subcultured on the same medium every 4 weeks. All chemicals used in these experiments were purchased from Sigma (St. Louis, MO, USA).

Laser flow cytometry analysis

Nuclear suspensions from adult plant leaves (Fig. II.1.1a), embryogenic *callus* tissue (Fig. II.1.1b), SE (Fig. II.1.1c–e) and converted plantlet leaves (Fig. II.1.1f–g) were prepared according to Galbraith *et al.* (1983). In brief, to release nuclei from the cells, one half of a young leaf (1 cm^2 to 2 cm^2), cotyledonal embryos of approximately 5 mm size or 50 mg of embryogenic *callus* tissue were chopped with a sharp razor blade together with a young

leaf of the internal reference standard *Glycine max* cv. Polanka (Fabaceae; 2C = 2.5 pg DNA) in Marie's isolation buffer (50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM EDTA.Na_2, 50 mM sodium citrate, 0.5% (v/v) Tween 20, 50 mM HEPES, pH 7.2; Marie and Brown 1993). The suspension of nuclei was then filtered through a 50 µm nylon filter to remove fragments and large tissue debris. Then 50 µg mL^{-1} of propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 µg mL^{-1} of RNase (Sigma) were added to the samples. PI was added to stain the DNA and RNase to eliminate the RNA and prevent the binding of PI to RNA. Samples were analysed within a 10-min period in a Coulter EPICS-XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter and converted on 1,024 ADC channels. Prior to analysis, the instrument was checked for linearity with fluorescent check beads (Beckman Coulter®) and the amplification was adjusted so that the peak corresponding to *Q. suber* nuclei was positioned approximately at channel 200. This setting was kept constant. The results were obtained in the form of two histograms: linear-fluorescence light scatter (FL) and FL pulse integral *vs.* FL pulse height. In the latter cytogram an "interest zone" was defined to discriminate doublets (events with a higher pulse area but the same pulse height as single nuclei), partial nuclei, nuclei with associated cytoplasm and other debris (Brown *et al.* 1991). By this way, we assumed that only single nuclei were included in the FL DNA histogram.

The relative DNA content of at least ten morphologically normal SE, at least 15 abnormal SE (SE1 or SE3) and at least eight adult plant leaves were measured per genotype and given as an index relative to the internal standard (*G. max*). The nuclear DNA content of two embryogenic *calli* and of leaves from five converted plantlets was also assayed, and the values obtained were compared with the ones obtained for SE and adult plant leaves. At least 5,000 – 10,000 nuclei were analysed per sample.

The nuclear genome size of *Q. suber* was calculated according to the following formula:

$$Q.\ suber\ \text{2C nuclear DNA content (pg)} = \frac{Q.\ suber\ \ G_0/G_1\ \text{peak mean}}{G.\ max\ \ G_0/G_1\ \text{peak mean}} \times 2.50$$

Conversion of mass values into base-pair numbers was done according to the factor 1 pg = 978 Mbp (Doležel *et al.* 2003).

Statistical analysis

Statistical analyses were performed using a one-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA) to analyse possible differences among morphologically normal SE2 and abnormal SE (SE1 or SE3), and among mother plant leaves, embryogenic *callus* tissue, SE and converted plantlet leaves.

Results

Morphological characterisation

Embryogenic *calli* of cork oak were compact and showed somatic embryogenic structures, mostly at globular stage (Fig. II.1.1b). The embryogenic lines used in this study had low levels of phenolisation and this characteristic was stable with time.

Cotyledonal somatic embryos (SE) derived from these embryogenic *calli* had large and compact green cotyledons, but a high number usually represented one, three or more cotyledons (Fig. II.1.1c–e). Nevertheless, no morphological differences could be observed within the converted plants (Fig. II.1.1f–g). These plants had well developed roots and shoots, but generally in leaves (even in younger ones used in the FCM studies) a browning reaction could be observed immediately after cutting.

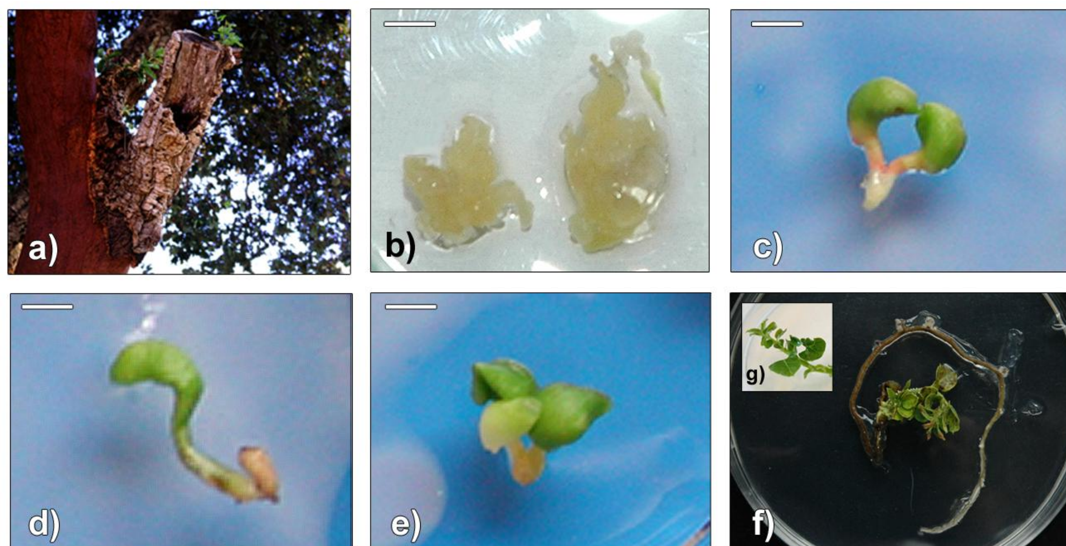
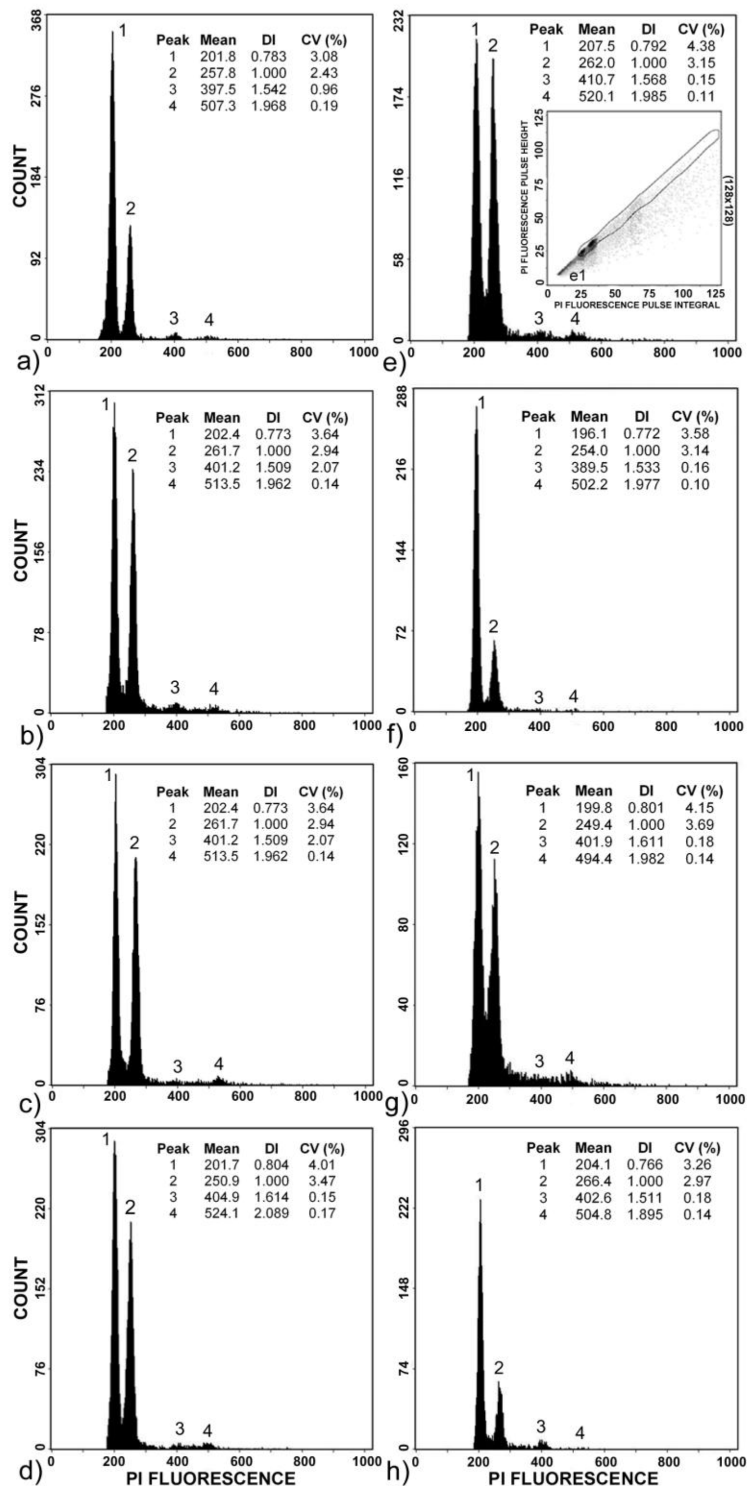


Fig. II.1.1 Somatic embryogenesis and plant regeneration in *Quercus suber* L. a) Mother plant of genotype QsG0; b) embryogenic *callus* tissue; c) dicotyledonal somatic embryo with large and compact green cotyledons; d) abnormal somatic embryo with one green cotyledon; e) abnormal somatic embryo with three green cotyledons; f,g) examples of plantlets from somatic embryo conversion. *Bars* = 3 mm.

Flow cytometric analyses

The linear FL histograms (Fig. II.1.2a–h) of relative nuclear DNA content of leaves of mother plants and SE showed distinct G_0/G_1 peaks with coefficients of variation (CV) ranging from 2.6% to 6.0% for leaves of the mother plants, from 2.7% to 5.2% for SE2, from 2.6% to 4.2% for SE1 and from 3.1% to 5.5% for SE3.

Fig. II.1.2 Histograms of relative PI fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Glycine max* cv. Polanka (2C = 2.50 pg DNA, as internal reference standard) and *Quercus suber* L.: a) leaves of the mother plant of genotype QsG0; b) somatic embryo with two cotyledons of genotype QsG0; c) somatic embryo with one cotyledon of genotype QsG0; d) somatic embryo with three cotyledons of genotype QsG0; e) leaves of the mother plant of genotype QsG5; f) somatic embryo with two cotyledons of genotype QsG5; g) somatic embryo with one cotyledon of QsG5; h) somatic embryo with three cotyledons of genotype QsG5. In all histograms four peaks were observed: peak 1 – nuclei at G_0/G_1 phase of *Q. suber*; peak 2 – nuclei at G_0/G_1 phase of *G. max*; peak 3 – nuclei at G_2 phase of *Q. suber*; peak 4 – nuclei at G_2 phase of *G. max*. e) Inset represents a relative PI fluorescence pulse integral *vs.* relative PI fluorescence pulse height cytogram where a gating region (e1) was defined to exclude doublets and particles of plant debris (present in the bottom left corner of the cytogram). A similar gating region was applied to all PI fluorescence histograms. The mean channel number (Mean), DNA index (DI) and coefficient of variation (CV, %) of each DNA peak are also given.



Embryogenic *callus* tissue had CV values ranging from 3.8% to 4.6% (Fig. II.1.3a) and leaves from converted plantlets from 3.1% and 4.0% (Fig. II.1.3b). As expected, throughout the experiment, the internal standard, *G. max*, had a tight 2C distribution (mean CV = 3.0%; Figs. II.1.2 and II.1.3).

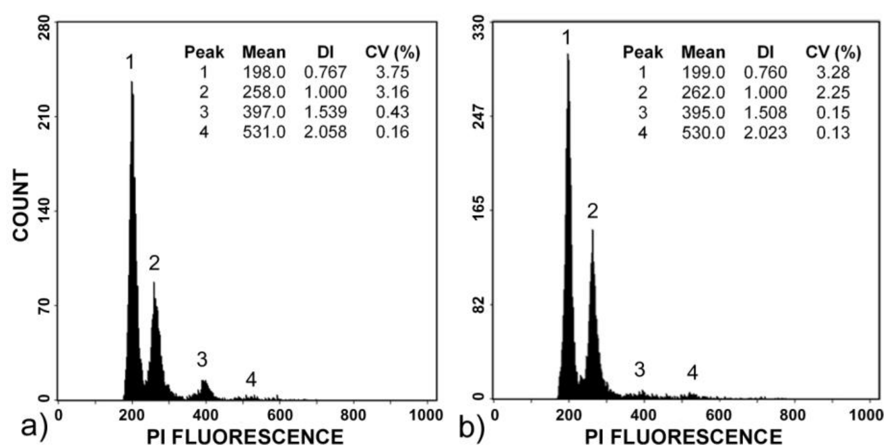


Fig. II.1.3 Histograms of PI fluorescence (in relative units) obtained after simultaneous analysis of nuclei isolated from *Glycine max* cv. Polanka (2C = 2.50 pg DNA, as internal reference standard) and *Quercus suber*: a) embryogenic *callus* tissue of QsG0; b) leaves of converted plantlets of QsG0. In all histograms four peaks were observed: peak 1 – nuclei at G_0/G_1 phase of *Q. suber*; peak 2 – nuclei at G_0/G_1 phase of *G. max*; peak 3 – nuclei at G_2 phase of *Q. suber*; peak 4 – nuclei at G_2 phase of *G. max*. The mean channel number (Mean), DNA index (DI) and coefficient of variation (CV, %) of each DNA peak is also given.

The mean nuclear DNA fluorescence index (DI = $2C_{Q.\ suber}/2C_{G.\ max}$) for *Q. suber* QsG0 ranged from 0.780 (SE3) to 0.805 (mother plant leaves) and for QsG5 from 0.775 (SE2) to 0.805 (mother plant leaves, Table II.1.1).

Table II.1.1 Nuclear DNA content of mother plant leaves (MPL), apparently normal somatic embryos (SE) with two cotyledons (SE2), SE with one cotyledon (SE1) and SE with three cotyledons (SE3)[1].

Genotype	Plant material source	DNA index	SD	Nuclear DNA content (pg/2C)	SD	1C genome size (Mbp)[2]	CV (%)	R	one-way ANOVA
QsG0	MPL	0.805	0.0208	2.01	0.052	983	4.16	8	n.s.
	SE1	0.783	0.0178	1.96	0.045	958	3.47	15	n.s.
	SE2	0.782	0.0190	1.96	0.048	958	3.66	16	n.s.
	SE3	0.780	0.0219	1.95	0.055	954	3.55	9	n.s.
QsG5	MPL	0.805	0.0125	2.01	0.031	983	4.80	13	n.s.
	SE1	0.789	0.0141	1.97	0.036	963	2.98	4	n.s.
	SE2	0.775	0.0105	1.94	0.026	949	3.66	7	n.s.
	SE3	0.781	0.0100	1.95	0.025	954	3.72	7	n.s.

[1] The values are given as mean and standard deviation of the mean (SD) of DNA index relative to the internal standard *Glycine max* cv. Polanka, as a mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as 1C genome size of *Quercus suber*. The mean coefficient of variation (CV) of the *Q. suber* 2C nuclei in histograms and the number of replicates (R) are also given. n.s. – not significantly different.

[2] 1 pg DNA = 978 Mbp (Doležel *et al.* 2003).

This 3% variation did not interfere with the assignment of peaks to 2C and 4C level values and if tetraploid levels were to be observed a DI of approximately 1.600 is to be expected. Also, statistical analysis (one-way ANOVA) showed no significant differences for $P \leq 0.01$ among different groups. Further studies using embryogenic *callus* tissue and converted plantlet leaves revealed similar results (Table II.1.2).

Table II.1.2 Nuclear DNA content of embryogenic *callus* tissue (ECT) and converted plantlet leaves (CPL)[1].

Plant material source	Index	SD	Nuclear DNA content (pg/2C)	SD	1C Genome size (Mbp)[2]	CV (%)	R	one-way ANOVA
ECT	0.766	0.0027	1.91	0.007	936	4.20	2	n.s.
CPL	0.753	0.0042	1.88	0.010	921	3.46	5	n.s.

[1] The values are given as mean and standard deviation of the mean (SD) of DNA index relative to the internal standard *Glycine max* cv. Polanka, as a mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as 1C genome size of *Quercus suber* L. The mean coefficient of variation (CV) of the *Q. suber* 2C nuclei in histograms and the number of replicates (R) are also given. n.s. – not significantly different.

[2] 1 pg DNA = 978 Mbp (Doležel *et al.* 2003).

The determination of the nuclear DNA content of *Q. suber* in absolute units ranged from 1.88 pg/2C to 2.01 pg/2C and these estimates were reproducible for a given sample source (Tables II.1.1 and 2).

Discussion

Morphological characterisation

The high compactness of embryogenic tissues in cork oak is consistent with the description for other species (*e.g.*, *Eucalyptus globulus*, Myrtaceae; Pinto *et al.* 2004) and it differs from undifferentiated *calli* that are in general much more friable and have large and vacuolated cells (Pinto *et al.* 2002). Also, and although the embryogenic lines used in this study had low levels of phenolisation, this may be a recurrent phenomenon in other embryogenic lines. Cotyledonal SE in cork oak may reach high dimensions (up to 15 mm) and have one, two or more, large cotyledons (up to 10 mm), suggesting an abnormal development (Pinto *et al.* 2002). Nevertheless, the conversion of these abnormal SE was similar to the normal dicotyledonal SE (Pinto *et al.* 2002).

Flow cytometry analysis

The CV value is very important in FCM studies and some authors (*e.g.*, Marie and Brown 1993; Galbraith *et al.* 2002) consider it an elementary criterion that reflects the quality of the applied methodology. Marie and Brown (1993) referred a range of 1–2% for top quality

analysis in plant cells and 3% as a routine value. On the other hand, Galbraith *et al.* (2002) suggested a CV of less than 5% as the acceptance criterion. Although some values are slightly higher than these, one must take in consideration that *Q. suber* is a recalcitrant species and till now, as in most woody plants, it is very difficult to obtain these recommended values. Zoldoš *et al.* (1998) obtained for leaves of *Q. suber* a mean CV value of 4.1% and these authors justified these values with the excessive browning of the samples, degradation of nuclei and pH instability. Endemann *et al.* (2001) working with *Quercus robur* L. and using 4,6-diamidino-2-phenylindole (DAPI) staining, obtained CV values ranging from 1.5% to 2.5% for immature, translucent SE and 2.5% to 4.5% for mature, opaque SE.

Reports using 4'6-diamidino-2-phenylindole (DAPI) instead of PI showed apparently lower CV values, permitting high-resolution analysis (Doležel and Göhde 1995). Also, while Zoldoš *et al.* (1998) using PI found CV values ranging from 3.3% to 6.9% for oak species, Endemann *et al.* (2001) using DAPI had lower CV values (a maximum value of 4.5%) supporting the idea that the use of DAPI seems to give lower CV values. However, the use of DAPI (an A-T base specific dye) to estimate nuclear DNA content must be regarded very carefully and has been even questioned by Doležel (1991) and Marie and Brown (1993).

Although in histograms a high background noise was frequently found in the lower channel numbers, this occurrence is not unusual in some plant species (Kudo and Kimura 2001) and this problem is ascribed to broken cells damaged during the extraction procedure or to autofluorescence of chloroplast in the cytosol (Emshwiller 2002).

The results reported here strongly indicate that all the samples analysed have the same ploidy level and that no polyploidy was observed in embryogenic *callus* tissue (a tissue that is currently pointed out in literature as potentially instable) and in SE. No polyploidy occurred when SE were germinated and converted to plantlets. Therefore we believe that there is relative ploidy stability at this level during the embryogenic process used for *Q. suber*. No significant differences ($P \leq 0.01$) were also found between SE2 and abnormal SE (SE1 or SE3). These morphological abnormalities did not reflect major genetic differences and these SE were germinated and converted into plantlets showing no morphological/genetic changes (Fig. II.1.1f). The stability reported here was not described for other species where polyploidization was found during somatic embryogenesis (Bueno *et al.* 1996; Kubalàková *et al.* 1996; Tremblay *et al.* 1999; Kudo and Kimura 2001; Endemann *et al.* 2001). Endemann *et al.* (2001) found in *Q. robur* that tetraploidy occurred in 8% of the clones tested over a culture period of 7 years. In *Q.*

canariensis, tetraploidy occurred in SE after 14 months of continuous subculture (Bueno *et al.* 1996). With regard to chromosomal instability in plant *callus* culture, polyploidy is the most commonly observed genetic change (Geier 1991). Endemann *et al.* (2001) referred that prolonged time in culture or any unfavourable condition or substance that affects or blocks plant metabolism, growth or development may result in polyploidization. In our case, despite the prolonged time in culture (at least 1 year for QsG5 and at least 4 years for QsG0) no polyploidization was detected. We find that the protocol developed by Pinto *et al.* (2002) combines a simple methodology with a low hormone system that decreases the risk for somaclonal variation. Genetic analysis by microsatellites using the same material also showed high levels of stability and support our findings (Lopes *et al.* 2003).

Although no polyploidization was detected in *Q. suber in vitro* cultures, the CV values obtained could mask the possible occurrence of small differences in nuclear DNA content, as FCM only allows to measure large differences in genome size (up to 1%; Pfosser *et al.* 1995). Therefore the presence of a low level aneuploidy should not be totally excluded and complementary studies, such as chromosome counting analysis, to evaluate this situation are presently being conducted in our laboratory (data not shown). Recently, Roux *et al.* (2003), combining FCM and chromosome counting, showed that FCM could rapidly detect aneuploidy situations in *Musa* sp. Moreover, the absence of major ploidy changes does not exclude that genetic differences such as DNA polymorphism are present. We have recently evaluated microsatellite instability in this material and found a small percentage of variance only in QsG5 (Lopes *et al.* 2003). Despite no significant differences were detected in the ploidy level, the higher DI and CV values found in some tissues (*e.g.*, leaves of the mother plants) may suggest that, as in other plant species—*e.g.*, *Pinus* spp. (Pinaceae; Greilhuber 1988), *Helianthus annuus* (Asteraceae; Price *et al.* 2000), *Coffea liberica* var. *dewevrei* (Rubiaceae; Noirot *et al.* 2000, 2002, 2003) and *E. globulus* (Pinto *et al.* 2004) — some tissues contain compounds that may affect the analysis. Similarly to what was reported in *Quercus* species (Favre and Brown 1996; Zoldoš *et al.* 1998), some browning and tanning also occurred in leaves of the mother plants and of SE-derived plants. Browning and tanning may substantially interfere with the analysis (Favre and Brown 1996). Supporting this hypothesis, Noirot *et al.* (2000) highlighted cytosolic effects of dye accessibility to DNA in coffee plant cells, and that cytosolic compounds can bias nuclear DNA content estimates by up to 20%. Also associated with these differences may be the fact that PI is a DNA intercalator sensitive to chromatin structure and that chromatin structure may change between leaves and somatic embryos.

These DNA values obtained are, in general, slightly higher than the previously published values for this species. Zoldoš *et al.* (1998) obtained a nuclear DNA content of 1.91 pg/2C in cork oak leaves. This difference is however, normal among laboratories due to the use of different methodologies, as sample processing and buffer composition, and to the use of different standards – *e.g.*, Zoldoš *et al.* (1998) used *Petunia hybrida* (Solanaceae, 2C = 2.85 pg DNA).

Conclusions

The main goal of the present work was fully accomplished as we succeeded in verifying, by using FCM that no major ploidy changes occurred during the somatic embryogenesis process of *Q. suber*, despite the age (1 to 4-years-old) of the cultures. The findings support the conclusion that the protocol of Pinto *et al.* (2002) may be a reliable one for large scale micropropagation of this species.

This report also shows that: (1) no major ploidy differences were detected between SE and the mother plant from which they were obtained; (2) no ploidy differences were detected between SE2 and abnormal SE (SE1 or SE3); (3) analyses of embryogenic *callus* tissue and converted plantlet leaves sustained the previously obtained results; (4) homogeneity is found within and between sample sources; (5) the higher DI and CV values found in leaves of mother plant may be due to the presence of secondary compounds that affect the FCM analysis; (6) although slightly higher than the previously obtained values for this species, the nuclear DNA content estimations must be aware of inter-laboratory differences.

We, therefore consider the FCM technique as a valuable method to assess a “true-to-type” propagation in cork oak, in addition to other more traditional molecular biology techniques (*e.g.*, microsatellites, AFLP, DNA methylation).

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II.2 Analysis of the ploidy stability of *Juniperus phoenicea* L. micropropagation process using flow cytometry

Chapter section published as original article in SCI journal:
Loureiro J, Capelo A, Brito G, Rodriguez E, Silva S, Pinto G, Santos C (2006) Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. Biologia Plantarum **51**:7-14.

Abstract

We report here the successful micropropagation of adult *Juniperus phoenicea* L. (Cupressaceae) with respective ploidy stability studies. Microcuttings with axillary buds were grown on five media supplemented with different growth regulator combinations. Best elongation rates were achieved on Driver and Kuniyuki (DKW) medium supplemented with kinetin alone or with naphthaleneacetic acid (NAA), while Rugini olive (OM) medium stimulated the development of new branches. Shoots growing on Murashige and Skoog (MS) medium browned and showed necrotic zones. Shoots of second to fourth subcultures usually had higher elongation rates than those of the first culture. For rooting assays, half strength DKW and OM media, different concentrations of growth regulators, auxin continuous exposure *vs.* dipping and the type of solid matrix were assessed. During rooting assays, two morphotypes were observed with one type having well developed internodes and the other showing hyperhydratation and no internode development. High rooting rates (40%) were only obtained in the first morphotype shoots exposed for 5 min to 2.4 µM indole-3-butyric acid and then transferred to OM medium without growth regulators. Plants were acclimatized in pots containing a mixture of peat:*Perlite* (3:2) in greenhouse with progressive reduction of relative humidity. A flow cytometric (FCM) screening for major ploidy changes revealed no differences among the morphotypes and between them and the mother plant. Also the nuclear DNA content of this species was estimated for the first time using FCM (2C = 24.71 pg DNA).

Keywords *in vitro* culture; nuclear DNA content; plant acclimatization; rooting studies

Introduction

The genus *Juniperus* (Cupressaceae) has about 75 to 80 species and is the second largest genus of conifers. *Juniperus* species are widely distributed in semiarid regions where they are used extensively for landscaping, wood and medicinal purposes. *Juniperus phoenicea* L. is native to some regions of the Mediterranean basin, Canary Islands and North Africa. Recently the taxonomic position of this species was questioned (for details see Rivas-Martínez *et al.* 1993) and this misleading taxonomy data requires urgent clarification.

The lack of pollination, low pollen viability and/or embryo degeneration are important causes for reduced seed production in *Juniperus* (Ortiz *et al.* 1998). Also, species that grow upright are more recalcitrant than prostrate ones (Hartman *et al.* 1990). Dirr and Heuser (1987) reported that cuttings of some *Juniperus* species could be rooted using up to 4.5% indole-3-butyric acid (IBA). Also, Hartman *et al.* (1999) reported propagation of *J. virginiana* and *J. procumbens* by cuttings and of *J. virginiana* and *J. chinensis* by seed germination, but highlighted that the success of rooting may be lower than 10% when adult trees are used as mother plants (Edson *et al.* 1996). Unfortunately, *J. phoenicea* is not efficiently propagated by traditional methods and results are extremely inconsistent and not reproducible (Brito 2000). For example, and similarly to other *Juniperus* species, *J. phoenicea* does not have a high rate of plant production through seed germination (Ortiz *et al.* 1998).

Therefore, micropropagation should be performed in order to improve the propagation of *Juniperus* species. Contrarily to *Pinus* (*e.g.*, Gomez and Segura 1995, 1996; Andersone and Ievinsh 2005), few information is available in *Juniperus* species. Gomez and Segura (1995) reported the proliferation of *J. oxycedrus* by axillary shoot proliferation and later found some morphogenic capacity in *calli* derived from single cell culture of the same species (Gomez and Segura 1996). More recently, Shanjani (2003) highlighted the importance of nitrogen on *callus* induction and plant regeneration of *J. excelsa*, and new media formulation based on the explant mineral composition are being proposed as a new strategy for highly recalcitrant species (*e.g.*, Gonçalves 2004). Alternatively, a new method involving germination of *in vitro* cultured embryos on Murashige and Skoog (1962) medium (MS) was proposed to improve germination and propagation of *J. oxycedrus* ssp. *oxycedrus* and ssp. *macrocarpa* (Cantos *et al.* 1998).

Due to the possible occurrence of somaclonal variation, the analysis of the ploidy stability of micropropagated plants is of particular importance. These types of studies in conifers are rare and the ones using flow cytometry (FCM) are even rarer. Gajdošová *et*

al. (1995) used this technique to analyse the genetic stability of embryogenic *calli* of silver fir and its hybrids and Libiaková *et al.* (1995) analysed the genetic stability of *Abies concolor* × *Abies grandis* (Pinaceae) *calli* and *in vitro* regenerated shoots. FCM has the advantages that nuclei are analysed individually and at high speed, large populations can be measured in a short time and the presence of subpopulations can be detected (Shapiro 2004). Also, since there is no need to employ tissues with dividing cells and it is easy and rapid to prepare samples, FCM became the preferred method to perform this type of analyses (Doležel and Bartoš 2005).

This investigation describes for the first time a reliable protocol for *in vitro* regeneration and acclimatization of *J. phoenicea* plants derived from an adult field tree. The ploidy stability of plantlets derived from the micropropagation protocol and from rooting assays was assessed using FCM and the nuclear DNA content of this species was determined for the first time using leaves of the mother field tree.

Materials and methods

In vitro culture establishment

Cuttings (15 - 20 cm long) from terminal branches of adult trees (20-year-old) from Porto Santo Island, collected in the spring, were used as source of microcuttings for *in vitro* studies. Half of the cuttings were treated immediately and the other half was preconditioned in a greenhouse (22 ± 1ºC, 16 h photoperiod with irradiance of 400 µmol m^{-2} s^{-1}) for at least one week with periodic sprays of a fungicide solution: 0.75 g mL^{-1} Derosal (Hoescht and Schering AgrEvo, Berlin, Germany) and 1.5 mg L^{-1} Previcur^{N} (Hoescht and Schering AgrEvo). Cuttings were washed in tap water for 10 min, decontaminated in ethanol 70% (v/v) for 1 min and then immersed two times (10 min each) in 200 mL of a commercial bleach solution (2.5 - 3.0% available chloride) containing 5 drops of Teepol (Cruz Verde, Lisboa, Portugal). Cuttings were then rinsed in a sterilised fungicide solution of 1 g L^{-1} Benlate (Rhône-Poulenc, Lisboa, Portugal) for 10 min and washed in sterile water three times (5 min each).

To test the influence of medium composition and growth regulators on shoot propagation, decontaminated initial explants (1 cm tall cuttings with one axillary bud) were placed on different agar media: Driver and Kuniyuki medium (DKW; Driver and Kuniyuki 1984), MS medium, Olive medium (OM; Rugini 1984), Schenk and Hildebrandt medium (SH; Schenk and Hildebrandt 1972) or woody plant medium (WPM; McCown and Lloyd 1981), supplemented with different growth regulators (Table II.2.1). Each treatment consisted of ten 400 mL flasks (with 50 mL medium) containing five explants each, giving

a total of 50 explants. Cultures were incubated in a growth chamber at 22 ± 1ºC, with a 16 h photoperiod and irradiance of 98 µmol m^{-2} s^{-1}.

The influence of culture cycle on shoot multiplication was evaluated by comparing shoots response in different culture periods: 1st culture (shoots derived from the field mother plant), 2nd culture (shoots derived from 2-month-old shoots of the 1st culture) and so on.

Shoot survival and morphological characters as shoot length, number of shoots per explant and number of branches per shoot were evaluated in each culture period. *Callus* production was also evaluated. Treatments were repeated two times in independent experiments.

Rooting studies and plant acclimatization

Shoots (2 - 3 cm long) were transferred to different rooting conditions: various basal culture medium, type and concentration of growth regulator, continues exposure to auxin *vs.* auxin dipping for 1 min, 5 min or 1 h, and composition of the solid matrix (Table II.2.2). Cultures were incubated under the conditions described previously for shoot culture. Each treatment consisted of eight 400 mL flasks containing 5 explants each, giving a total of 40 explants. When roots were at least 2 cm long, *in vitro* plantlets were transferred to pots with sterilised mixture of peat:*Perlite* (3:2) and treated with a fungicide solution: 0.75 g mL^{-1} Derosal and 1.5 mg L^{-1} Previcur^{N}. After that, they were transferred to a greenhouse and grown at 22 ± 1ºC, with a 16 h photoperiod and an irradiance of 400 µmol m^{-2} s^{-1}, where they were acclimatized to progressive decreasing of relative humidity.

Ploidy stability analysis using flow cytometry

Nuclear suspensions from micropropagated and mother plant leaves were prepared according to Galbraith *et al.* (1983). In short, nuclei were released from cells by chopping the tissue with a razor blade in a Petri dish containing 1 mL of Tris.$MgCl_2$ buffer (200 mM TRIS, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5; Pfosser *et al.* 1995). The buffer was supplemented with 1% (w/v) PVP-10 to reduce the possible influence of secondary metabolites on propidium iodide (PI) staining. The suspension of nuclei was then filtered through a 50 µm nylon filter and 50 µg mL^{-1} of PI (Fluka, Buchs, Switzerland) and 50 µg mL^{-1} of RNase (Sigma, St. Louis, MO, USA) were added to the samples to stain the DNA. Samples were analysed within a 10 min period in a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Integral fluorescence and fluorescence pulse height and

width emitted from nuclei were collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter and converted on 1,024 ADC channels. Prior to analysis, the instrument was checked for linearity with Flow-Check fluorospheres (Beckman Coulter®). Doublets, partial nuclei, nuclei with associated cytoplasm and other debris were discriminated using a specific gating region defined in a linear-fluorescence light scatter (FL) pulse integral *vs.* FL pulse height cytogram. Leaves of *Secale cereale* cv. Dankovské (Poaceae; 2C = 15.95 pg DNA; Doležel *et al.* 1998) were used as an internal reference standard. At least 5,000 nuclei were analysed per sample.

To estimate the ploidy level, the position of the G_0/G_1 peak of the sample on a histogram was compared with the internal reference plant with known ploidy. The size of the nuclear genome of *J. phoenicea* was estimated according to the following formula:

$$\textit{J. phoenicea}\ \text{2C nuclear DNA content (pg)} = \frac{\textit{J. phoenicea}\ G_0/G_1\ \text{peak mean}}{\textit{S. cereale}\ G_0/G_1\ \text{peak mean}} \times 15.95$$

Conversion into base-pair numbers was performed using the following factor: 1 pg = 978 Mbp (Doležel *et al.* 2003).

The relative nuclear DNA content of four micropropagated plantlets obtained in OM medium was estimated and compared with the values obtained from five replicates of the adult mother field tree. To assay the possible influence of different rooting conditions on explants ploidy stability, the relative nuclear DNA content of at least one explant in OM medium from each rooting condition was estimated.

Statistical analysis

Data from *in vitro* culture and rooting studies were averaged from two independent analyses and were analysed using the one-way analysis of variance (ANOVA). A multiple comparison Tukey-Kramer test was applied when necessary to determine exactly which groups were different. Statistical analyses of data from ploidy stability assays were performed using an unpaired t-test (for comparison between micropropagated plantlets and the adult mother plant) and a two-way ANOVA (for comparison between different rooting conditions – auxinic dipping and composition of solid matrix – and detection of possible interactions between them). All statistical studies were performed using SigmaStat for Windows (Version 3.1, SPSS Inc., Richmond, CA, USA).

Results and discussion

In vitro culture establishment

Higher surviving rates (58% from a total of 30 explants) were obtained with explants derived from preconditioned cuttings (that were periodically treated with a fungicide solution) than with explants derived directly from the field (22% from a total of 30 explants). The strategy of greenhouse preconditioning of field cuttings to prevent *in vitro* infections was already applied in micropropagation studies of other field trees with similar success (Pinto *et al.* 2002). Simultaneously, the method used for explants disinfection was successful.

Explants growing on DKW, OM and SH media presented, in the first culture, similar surviving rates ($P \leq 0.05$) that were slightly higher than those of explants growing on WPM ($P \leq 0.1$; data not shown) and higher than the surviving rates of explants on MS medium, which was more evident during the 2nd to 4th subcultures. In fact, with the exception of MS and SH media, survival rates increased considerably in subsequent subcultures (76 - 100%). On MS medium, shoots browned and showed apical necrosis that eventually spread during subcultures to the whole shoot leading to its death. Therefore, this medium was not suitable to establish *in vitro* shoot cultures in this species (Fig. II.2.1a). On SH medium some of the shoots dyed, while the surviving ones showed a light green colour and new growing zones.

No significant differences were obtained among different growth regulator combinations in regards of shoot survival rates; 100% survival rates were obtained on DKW medium with 0.93 µM kinetin (KIN) or with 0.45 µM 6-benzylaminopurine (BAP) and on OM medium with 2.74 µM naphthaleneacetic acid (NAA) and 1.86 µM KIN.

Independently of the subculture, some morphologic differences (shoot length, number of shoots per explant and number of branches per shoot) were observed. Shoots grown on MS medium showed the lowest survival, lowest number of shoots per explant and a reduced number of branches per shoot ($P \leq 0.05$; Table II.2.1). Shoots grown on WPM also had low number of shoots per explants and low branching, but elongation was in general higher than on MS medium ($P \leq 0.05$) while oxidation was lower (Table II.2.1). On DKW medium explants had higher elongation rates when compared to all the other media ($P \leq 0.05$, Table II.2.1), presenting well developed branches and green leaves (Fig. II.2.1b). In this medium the number of shoots per explant and the number of branches per shoot were among the highest values obtained. Only on OM medium better results were obtained concerning these two characters ($P \leq 0.05$). On this medium, high amounts of tissue developed at the base of the shoots although elongation was significantly lower

than on DKW ($P \leq 0.05$). The most outstanding feature of shoots developed on OM medium is that they looked completely different from shoots developed on other media: internodes were almost absent, and shoots had a high amount of branches with lots of small, rolled, and dark green leaves (Fig. II.2.1c).

Fig. II.2.1 *Juniperus phoenicea* shoot culture on: a) MS medium (arrows are pointing out necrotic regions, *bar* = 0.5 cm); b) DKW medium (*bar* = 0.5 cm); and c) OM medium (*bar* = 0.5 cm). Shoot morphotypes were observed during the same rooting conditions (shoots exposed for 5 min to 2.4 µM IBA and transferred to OM medium without growth regulators): d) morphotype 1 with well developed branches and green leaves (*bar* = 0.5 cm); e) morphotype 2 with high vitrification, short internodes and light green leaves (*bar* =0.5 cm); f) 4-month-old morphotype I plantlet with well developed roots (*bar* = 0.5 cm); g) and h) plantlets during acclimatization on peat:*Perlite* (3:2) in greenhouse for 3 days (g, *bar* = 1.0 cm) or 3 months (h, arrows are pointing out new leaves, *bar* = 0.5 cm). i) Typical *callus* formed on OM medium (see root development, *bar* = 1.0 cm).

Table II.2.1 Effect of basal media and growth regulator combinations on *Juniperus phoenicea* survival rate (%), mean shoot length (cm), number of shoots per explant and number of formed branches per explant during 1st culture and at the end of 4th subculture (mean shoot length at the beginning of every culture = 1.3 ± 0.3 cm). In each parameter the same letter(s) (a, b or c) means that no significant differences were detected according to the one-way analysis of variance and to the multiple comparison Tukey-Kramer test ($P \leq 0.05$; from two independent experiments, n = 30). Last column refers to the capacity of different media to produce and maintain *calli* (- no production; + low production; ++ good production). N.D. - not determined.

Medium	Growth regulators (μM)	Shoot survival (%)		Shoot length (cm)		Number of shoots per explant		Number of branches per shoot		*Callus*
		1st	4th	1st	4th	1st	4th	1st	4th	
DKW	0.93 KIN	70 ± 13a	100 ± 0c	4.5 ± 0.8bc	5.4 ± 0.5bc	4.2 ± 0.8b	4.5 ± 1.2b	27.4 ± 7.1b	32.6 ± 14.2b	-
	0.45 BAP	72 ± 10a	100 ± 0c	3.5 ± 0.5ab	4.8 ± 0.3b	4.7 ± 1.3b	3.1 ± 0.7b	23.7 ± 9.4b	35.4 ± 18.4b	-
	2.74 NAA + 1.86 KIN	63 ± 11a	93 ± 2b	4.8 ± 0.6c	6.1 ± 0.8c	5.3 ± 2.5bc	3.3 ± 1.3b	39.1 ± 10.5b	38.4 ± 18.4b	+
	2.74 NAA + 0.45 BAP	70 ± 14a	90 ± 6b	3.8 ± 0.8ab	3.8 ± 0.8b	4.1 ± 1.0b	4.2 ± 0.9b	24.0 ± 11.8b	39.6 ± 15.7b	-
	2.74 NAA + 0.90 BAP	68 ± 15a	92 ± 4b	4.0 ± 0.4b	4.1 ± 0.2b	4.8 ± 1.1b	4.8 ± 1.1b	22.4 ± 6.0b	37.6 ± 11.1b	-
MS	0.93 KIN	60 ± 18a	66 ± 2a	2.5 ± 0.9a	2.7 ± 0.9a	1.0 ± 0.0a	1.1 ± 0.3a	18.6 ± 14.2ab	15.4 ± 6.4a	-
	0.45 BAP	55 ± 16a	51 ±14 a	2.1 ± 0.6a	2.8 ± 1.1a	1.1 ± 0.2a	1.3 ± 0.6a	11.7 ± 7.3a	19.3 ± 8.5a	-
OM	0.93 KIN	65 ± 16a	94 ± 2b	3.2 ± 0.1ab	3.4 ± 0.2ab	8.3 ± 1.8c	8.3 ± 1.8c	43.0 ± 17.5bc	45.4 ± 10.1bc	-
	0.45 BAP	70 ± 21a	90 ± 7b	2.2 ± 0.5a	3.1 ± 0.1ab	7.2 ± 2.0c	7.6 ± 2.0c	52.2 ± 20.1c	61.6 ± 2.4c	-
	2.74 NAA + 1.86 KIN	72 ± 10a	100 ± 0c	3.0 ± 0.4ab	4.0 ± 0.5ab	9.4 ± 3.0c	9.4 ± 3.0c	49.5 ± 12.1c	59.3 ± 21.7c	++
	2.74 NAA + 0.45 BAP	65 ± 12a	95 ± 2b	2.6 ± 0.2a	3.0 ± 0.7ab	8.5 ± 0.5c	8.5 ± 0.5c	38.7 ± 11.6bc	33.4 ± 13.5b	++
	2.74 NAA + 0.90 BAP	72 ± 8a	90 ± 4b	2.8 ± 0.5a	2.9 ± 0.3ab	6.2 ± 0.3bc	6.2 ± 0.3bc	54.2 ± 11.3c	45.6 ± 9.6bc	++
SH	0.93 KIN	58 ± 15a	45 ± 12a	2.1 ± 1.0a	N.D.	N.D.	N.D.	N.D.	N.D.	-
	0.45 BAP	70 ± 10a	61 ± 2a	1.0 ± 0.8a	2.3 ± 0.2a	0.5 ± 0.2a	1.4 ± 0.23a	N.D.	N.D.	-
WPM	0.93 KIN	64 ± 20a	85 ± 6b	3.8 ± 0.4b	4.3 ± 0.5b	2.7 ± 0.6ab	1.1 ± 0.2a	21.6 ± 7.3a	17.3 ± 6.6a	-
	0.45 BAP	65 ± 17a	90 ± 10b	3.4 ± 0.5ab	3.8 ± 0.6b	2.1 ± 0.3ab	2.1 ± 1.0a	19.7 ± 7.1a	15.8 ± 3.4a	-
	2.74 NAA + 0.86 KIN	70 ± 22a	90 ± 0b	2.0 ± 0.2a	2.8 ± 0.1a	2.8 ± 0.4b	2.3 ± 0.8ab	14.9 ± 12.9a	21.2 ± 5.9a	-
	2.74 NAA + 0.45 BAP	67 ± 10a	88 ± 6b	2.6 ± 0.6a	2.9 ± 0.6a	2.5 ± 0.4ab	1.7 ± 1.1a	16.5 ± 5.8a	11.1 ± 3.5a	-
	2.74 NAA + 0.90 BAP	62 ± 24a	76 ± 10b	3.4 ± 0.5ab	3.9 ± 0.5bc	2.5 ± 0.8b	1.5 ± 1.3a	11.3 ± 4.7a	19.6 ± 5.2a	-

In *J. oxicedrus* the best medium for culture establishment was a modified SH medium without growth regulators or supplemented with BAP (Gomez and Segura 1995). This medium was therefore tested in our studies to micropropagate *J. phoenicea*. However, in this species SH medium led to heterogeneous response with some shoots dying during subsequent subcultures (Table II.2.1).

Concerning the different growth regulator combinations tested no significant differences ($P \leq 0.05$, Table II.2.1) were detected in most cases. An exception was observed in DKW medium where the combination of 2.74 µM NAA and 1.86 µM KIN produced the best elongation rates and a good number of shoots per explant and branches per shoot.

In general higher shoot lengths and number of branches per shoot were observed during subsequent subcultures. This may be explained by the fact that, during 1st culture, growth could be limited by stress imposed by the decontamination process and that shoots need to adjust to the *in vitro* conditions as they derived from stems of field plants. By other way, shoots in the 2nd to 4th subcultures were already adapted and able to maximise growth by using the conditions supplied by the *in vitro* culture, and frequently, new formed regions (evident by their light green colour) could be observed at the top of the branches.

Rooting studies and plant acclimatization

For rooting experiments two strategies were adopted concerning shoots exposure to auxins [indole-3-acetic acid (IAA), IBA and NAA]: continuous exposure or dipping for 1 min, 5 min or 1 h (Table II.2.2). These studies were performed on half strength DKW (½ DKW) and OM medium as these were the media where the best results were obtained previously. On ½ DKW medium, rooting was only observed when IBA was chosen. For this reason IBA was the growth regulator in the OM medium rooting assays. Concerning the continuous auxin exposure, rooting was only observed on ½ DKW medium with 4.1 µM IBA (Table II.2.2). On this medium root primordia were observed in 6% of shoots and 4 weeks after transfer to rooting medium (Table II.2.2). When shoots were exposed for 1 h to 2.5 mM IBA, roots appeared in 14% of them and a 1 min dipping induced roots in 6% of the shoots.

Table II.2.2 Effects of medium (½ DKW or OM), different concentrations of growth regulators (IAA, IBA or NAA), type of exposure to growth regulators (continuous, 1 or 5 min dipping or 1 h exposure) and composition of solid matrix (agar or peat:*Perlite;*3:2) on rooting of *Juniperus phoenicea* explants (n = 40).

Medium	Growth regulator (µM)	Exposure	Solid matrix	Rooting (%)	Number of roots per explant	Root length (cm)
½ DKW	no regulators	-	agar	0	-	-
OM	4.1 IBA	continuous	agar	6.0	1.4	0.8
	2.5 IBA	1 min dipping	agar	6.0	1.0	0.2
	2.5 IBA	1 h exposure	agar	14.0	1.3	1.3
	2.4 IBA	1 min dipping	agar	10.0	2.0	2.0
	2.4 IBA	5 min dipping	agar	40.0	3.0	2.0
	2.4 IBA	1 min dipping	peat:*Perlite*	10.0	1.0	1.0
	2.4 IBA	5 min dipping	peat:*Perlite*	0	-	-

On OM medium, dipping in 2.4 µM IBA induced higher percentages of rooting (10% for 1 min exposure and 40% for 5 min exposure). These values were higher in agar medium when compared to the same conditions on a peat:*Perlite* mixture (n = 20).

Comparing with previous results in the same genus, this species seems to have different rooting abilities. For example, rooting of *J. scoppulorum* reached 82% (Dirr and Heuser 1987). However, this macropropagation depends on the species/ecotype, and is reduced when adult trees are used. Wagner *et al.* (1994) obtained less than 10% rooting in cuttings from 12-year-old mother plants. Gomez and Segura (1995) reported low rooting capacity of *in vitro* shoots of *J. oxycedrus*, and the genotypes of *J. phoenicea* from Porto Santo Island were for a long time extremely recalcitrant to both *ex vitro* and *in vitro* rooting (Brito 2000). Some of the reasons for this difficulty may be the peculiar environmental conditions of the Island together with the genotype/ecotypes used.

Finally, two morphotypes were observed: one group (approx. 70%) defined as “Morphotype I” had apparently normal shoots with green leaves, well developed internodes and a large number of branches (Fig. II.2.1d); the other group (app. 30%), defined as “Morphotype II”, was characterized by frequent hyperhydratation and short/almost absent internodes (Fig. II.2.1e). Rooting was only achieved in shoots of the “Morphotype I“ (Fig. II.2.1f).

Plant acclimatization in greenhouse conditions is still in course and restricted to few plants but, in “Morphotype I“, the acclimatisation is up to the moment 70% successful (Fig. II.2.1g and II.2.1h).

When developing a plant regeneration protocol, *callus* formation should be avoided. However, among the 19 media combinations tested some led to the production of *callus*

tissue. This is the first report on *callus* production in *J. phoenicea* (Fig. II.2.1I). *Callus* production may be important for studies of indirect morphogenesis (*e.g.*, somatic embryogenesis) – that are presently occurring in our laboratory – or for studies of production of secondary metabolites. An interesting example is the production of high contents of podophyllotoxin (a strong anti-tumour agent) in *callus* tissue from leaves of *J. chinensis* on SH medium supplemented with 16.2 µM NAA and 0.93 µM KIN (Muranaka *et al.* 1998). Other growth regulator combinations as 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.93 µM KIN or 5.4 µM NAA and 0.93 µM KIN also originated good *calli* production although not so efficiently (Muranaka *et al.* 1998). Also, Gomez and Segura (1995, 1996) achieved *callus* formation from adult *J. oxycedrus* plants in SH medium with 2,4-D. Our results with *J. phoenicea* confirm that, similarly to *J. chinensis* and among the combinations tested, NAA plays an important role on *callus* production as the media that induced *callus* had this growth regulator (Table II.2.1). On the other hand, stems showed to be, in this species, a good explant source for *callus* production.

Ploidy stability

FCM analyses were applied to study the ploidy stability of *J. phoenicea* plantlets derived from the micropropagation protocol and from rooting assays. In *J. phoenicea* to assure that a sufficient number of nuclei were obtained the amount of material per sample had to be increased.

Despite being a woody plant species, which are recalcitrant to this type of analyses (Chapter II.1), the mean coefficient of variation (CV) values obtained (Table II.2.3 and 4) were below 5.0% that is considered the acceptance criterion by Galbraith *et al.* (2002). As expected, the internal standard *S. cereale* had a lower 2C distribution (mean CV = 3.0%; Fig. II.2.2). The development of a protocol that gives low CV values is of crucial importance (this issue is fully addressed by Pinto *et al.* 2004) as it is a measure of the precision of the analysis. Unfortunately, in many reports this information is not provided and this difficult results interpretation. There is usually a correlation between higher CV values and higher background noise, especially in lower channel numbers (Emshwiller 2002). This applies to this case where some background noise was observed (Fig. II.2.2). Despite that, the FCM protocol established here for *J. phoenicea* provided good results demonstrating its reliability and potential use in other *Juniperus* species.

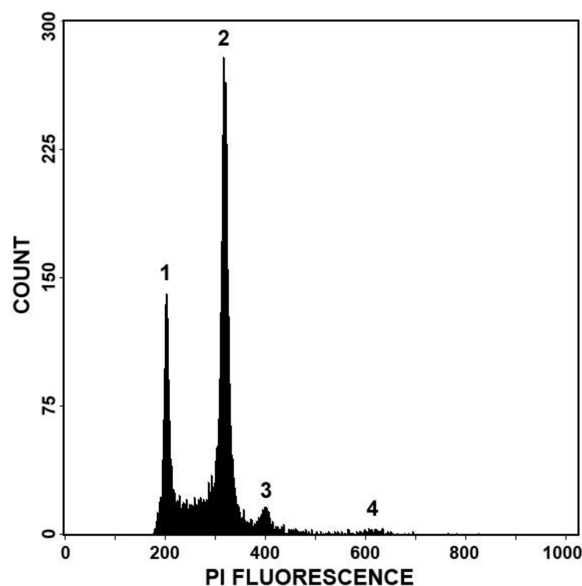
Table II.2.3 Nuclear DNA content of micropropagated plantlets and adult mother field tree from which they were obtained. Means ± SD, CV - coefficient of variation (%). The differences were not significant at $P \leq 0.05$; $n = 4$ for micropropagated plantlets and $n = 5$ for trees. For details see materials and methods.

Plant material	Nuclear DNA content (pg/2C)	1C genome size (Mbp)	CV (%)
Plantlets	24.46 ± 0.645	11,961	3.46
Adult mother tree	24.71 ± 0.178	12,083	2.44

Table II.2.4 Nuclear DNA content of *Juniperus phoenicea* morphotypes cultivated on OM medium and dipped in 5.0 µM IBA for 1 or 5 min before rooting on agar or peat:*Perlite* (3:2). Means ± SD, CV - coefficient of variation (%). The differences were not significant at $P \leq 0.05$; $n = 1 - 3$. For details see materials and methods.

Morphotype	Solid matrix	Dipping (min)	Nuclear DNA content (pg/2C)	CV (%)	*n*
1	agar	1	24.94 ± 0.257	3.62	3
		5	25.03 ± 0.231	3.38	3
	peat:*Perlite*	1	25.09	4.45	1
		5	24.48	4.24	1
2	agar	1	24.61	2.29	1
		5	24.86 ± 0.420	2.58	2
	peat:*Perlite*	1	24.25	3.02	1
		5	25.03 ± 0.101	2.87	2

Fig. II.2.2 Histogram of PI fluorescence intensity (in relative units) obtained after simultaneous analysis of nuclei isolated from *Secale cereale* (2C = 15.95 pg DNA, as internal reference standard) and *Juniperus phoenicea* leaves of the adult mother plant. Four peaks were observed in the histogram: 1 - nuclei at G_0/G_1 phase of *S. cereale*; 2 - nuclei at G_0/G_1 phase of *J .phoenicea*; 3 - nuclei at G_2 phase of *S.cereale*; 4 - nuclei at G_2 phase of *J. phoenicea*.



Considering the *in vitro* culture protocol used to establish *J. phoenicea*, the mean nuclear DNA fluorescence index (DI = $2C_{J.\ phoenicea}$ / $2C_{S.\ cereale}$) obtained for the micropropagated plantlets (DI = 1.533) was not significantly different ($P \leq 0.05$) to the one obtained for the mother field plant (DI = 1.549). These results strongly indicate that the micropropagation protocol described here apparently does not induce major ploidy changes. Nevertheless, the CV values obtained could mask the possible occurrence of small differences in nuclear DNA content (aneuploidy and DNA polymorphism) and therefore complementary studies, such as chromosome counting and microsatellites, to evaluate this situation, are presently being conducted in our laboratory.

To assay the possible influence of different rooting conditions on explants ploidy stability, the relative nuclear DNA content of plantlets from each morphotype was assessed (Table II.2.4). The DI values obtained for each set of conditions were very similar ($P \leq 0.05$). Also, no statistically significant differences were detected between morphotypes, revealing that the morphologic differences did not reflect major genetic changes. Up to the moment, all micropropagated plants that were acclimatized showed a normal development. Nevertheless, more refine techniques (as microsatellites to assess DNA mutations and DNA methylation to evaluate the influence of epigenetic changes) are currently being performed in our laboratory.

The determination of nuclear DNA content of *J. phoenicea* in absolute units was performed using the adult field tree and the obtained value was 2C = 24.71 $\pm$ 0.178 pg (1C genome size = 12,083 Mbp). This is the first report for this species and the obtained value is similar to the values obtained by Dhillon (1987) using Feulgen staining and microspectrophotometry and Hizume *et al.* (2001) using FCM, for other *Juniperus* species (*J. virginiana*, *J. conferta* and *J. rigida*), and as reported above FCM may also be used as an important tool for taxonomic studies in this genus.

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Chapter III

Nuclear DNA content analyses in plant species

III.1 Nuclear DNA content estimations in wild olive (*Olea europaea* L. ssp. *europaea* var. *sylvestris*) and Portuguese cultivars of *O. europaea* using flow cytometry

Chapter section published as a short communication in SCI journal:
Loureiro J, Rodriguez E, Costa A, Santos C (2007) Nuclear DNA content estimations in wild olive (*Olea europaea* L. ssp. *europaea* var. *sylvestris*) and Portuguese cultivars of *O. europaea* using flow cytometry. Genetic Resources and Crop Evolution 54:21-25.

Abstract

Olive tree (*Olea europaea* L.; Oleaceae) is an economically important woody fruit crop widely distributed in the Mediterranean regions. In this work the genome size of six Portuguese cultivars of olive (*O. europaea* ssp. *europaea* var. *europaea*) and wild olive (*O. europaea* spp. *europaea* var. s*ylvestris*) was estimated for the first time. The nuclear DNA content of *O. europaea* cultivars ranged between 2.90 ± 0.020 pg/2C and 3.07 ± 0.018 pg/2C and the genome size of wild olive was estimated as 3.19 ± 0.047 pg/2C DNA. These results suggest a low intraspecific variation at least among the studied cultivars and between them and wild olive. This is not in accordance with previous results in some Italian cultivars where high genome size heterogeneity was found. The methodology presented here seems appropriate for genome size estimations within this genus and opens good perspectives for a large screening of estimation of nuclear DNA content among *O. europaea* cultivars and *Olea* species that could clarify this issue.

Keywords flow cytometry; genome size; intraspecific variation; nuclear DNA content; *Olea europaea*; wild olive

Introduction

The genus *Olea* L. (Oleaceae) consists of around 30 species distributed in Europe, Asia, and Africa. Olive tree (*Olea europaea* L.) is a woody fruit crop widely distributed in the Mediterranean regions and comprises several economically important cultivars and wild olive genotypes. The Mediterranean basin provides ideal conditions for olive growing, and supplies more than 97% of the world production of olive (Rugini 1995). Despite of great economical importance, little attention was given to the cytogenetics of this species (2n = 46 chromosomes) with most of the studies concerning the genetic diversity of this species using molecular markers (*e.g.*, Gemas *et al.* 2004).

Despite being a useful descriptor for characterization of plant genetic resources (*e.g.*, Jarret *et al.* 1994), nuclear DNA content of olive cultivars was only determined for the first time by Rugini *et al.* (1996), who used Feulgen cytophotometry to estimate the 2C nuclear DNA content of cvs. 'Frantoio' and 'Leccino'. More recently and using the same technique, Bitonti *et al.* (1999) estimated the genome size of cvs. 'Dolce Agogia' and 'Pendolino'. The results of these studies indicated a high genome size intraspecific variation among the studied Italian cultivars. Bitonti *et al.* (1999) also analysed the genome size of other *Olea* species and verified that it was considerably lower than the genome size of *O. europaea* cultivars.

In recent years, flow cytometry (FCM) became the dominant technique for genome size and DNA ploidy level analyses in plant sciences (for a review of its applications see Doležel 1997), mainly because of its rapidity, ease and accuracy, which is unsurpassed by other classical methodologies as Feulgen microspectrophotometry (Doležel and Bartoš 2005).

Until now, this technique was never applied in the study of nuclear DNA content of *O. europaea* and its cultivars. In this study, FCM was applied to estimate for the first time the genome size of six economically important Portuguese cultivars of *O. europaea* ssp. *europaea* var. *europaea* and wild olive (*O. europaea* ssp. *europaea* var. *sylvestris*). Our goal was to verify the occurrence of intraspecific variation within this species and compare the obtained values with previous ones, already available for other cultivars of this species.

Material and methods

Plant material

Plant material of six *O. europaea* cultivars was collected from healthy mature field trees (Table III.1.1) of the Germplasm collection of Quinta da Nossa Senhora de Lurdes, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. The wild olive plant material was obtained from a field tree in Porto de Mós, Portugal. Young leaves were collected and kept in moistened paper for a maximum period of 2 days until FCM analysis.

Nuclear DNA content analyses

Nuclear suspensions were obtained from leaves following the protocol developed by Galbraith *et al.* (1983). In brief, nuclei were released from cells by chopping half a young leaf (1 to 2 cm^2) of both *O. europaea* and *Pisum sativum* cv. Ctirad (internal reference standard with 2C = 9.09 pg of DNA, Doležel *et al.* 1998; kindly provided by Jaroslav Doležel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic), in a glass Petri dish containing 1 mL of LB01 nuclear isolation buffer (15 mM TRIS, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl and 0.1% (v/v) Triton X-100, pH 7.5; Doležel *et al.* 1989). The buffer had to be supplemented with 15 mM of ß-mercaptoethanol to reduce the interaction between secondary compounds and propidium iodide (PI) staining. Also, the chopping of *O. europaea* leaves was quick (30 s) and not very intense. The nuclear suspension was then filtered through a 50 μm nylon filter and nuclei were stained with 50 μg mL^{-1} PI (Fluka, Buchs, Switzerland). Also, 50 μg mL^{-1} RNase (Sigma, St Louis, USA) was added to nuclear suspension to prevent staining of double stranded RNA. Samples were kept on ice and analysed within a 10 min period in a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. Doublets, partial nuclei, nuclei with associated cytoplasm and other debris were removed from analysis using a specific gating region defined in a linear-fluorescence light scatter (FL) pulse integral *vs.* FL pulse height cytogram. For each sample at least 5,000-10,000 nuclei were analysed.

The nuclear genome size of *O. europaea* was estimated according to the following formula:

O. europaea 2C nuclear DNA content (pg) =

$$\frac{O.\ europaea\ \mathrm{G_0/G_1\ peak\ mean}}{P.\ sativum\ \mathrm{G_0/G_1\ peak\ mean}} \times 9.09$$

Conversion into base-pair numbers was performed using the following factor: 1 pg = 978 Mbp (Doležel *et al.* 2003).

Three field trees were analysed for each *O. europaea* cultivar and two replicates for each tree were performed in two different days to avoid possible instrumental drift. One field tree of wild olive was analysed five times in two different days.

Statistical analyses

Genome size data was analysed using a one-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA) procedure. A Tukey-Kramer multiple comparison test was used for pair-wise comparison.

Results and discussion

The determination of the nuclear DNA content of *O. europaea* Portuguese cultivars ranged between 2.90 ± 0.020 pg/2C (cv. Verdeal) and 3.07 ± 0.018 pg/2C (cv. Santulhana). The genome size of wild olive was estimated as 3.19 ± 0.047 pg/2C DNA (Table III.1.1). Statistical analyses revealed significant differences between wild olive and all tested cultivars and, with the exception of cv. Cobrançosa, between cv. Verdeal and the remaining cultivars. Nevertheless, the difference between extreme values of genome size is only of 3.3% for the Portuguese cultivars, and only of 6.9% between wild olive and cv. Verdeal. It is therefore difficult to attest with certainty that these differences are real and not derived from technique related variations. In spite of that, previous estimations of genome size within this species using the Feulgen cytophotometry technique, revealed high nuclear DNA content heterogeneity among cultivars (Rugini *et al.* 1996; Bitonti *et al.* 1999). In these works, the genome size of several Italian cultivars of olive was estimated and the obtained values ranged from 3.90 pg/2C (cv. Dolce Agogia) to 4.66 pg/2C (cv. Pendolino), which accounts for a 16.3% difference. Bitonti *et al.* (1999) found that these DNA content determinations were positively correlated with the copy number of DNA repeats in the genomes studied, indicating that these genomes may differ in their amount of repetitive DNA.

Table III.1.1 Nuclear DNA content of *Olea europaea* ssp. *europaea* var. *europaea* cultivars and wild olive (*O. europaea* ssp. *europaea* var. *sylvestris*). The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as mean of the 1C genome size in Mbp. The range of values (min. – minimum, max. – maximum) obtained for each cultivar and wild olive is also presented. The mean coefficient of variation (CV, %) of the 2C peak, the number of genotypes tested (*n*) and the number of replicates made per genotype (R) are also given.

Subspecies and variety	Cultivar	Nuclear DNA content (pg/2C)				1C genome size (Mbp)[2]	CV (%)	*n* (R)
		Mean[1]	SD	Min.	Max.			
europaea var. *europaea*	'Redondal'	3.04a	0.042	2.96	3.09	1,488	3.83	3 (2)
europaea var. *europaea*	'Santulhana'	3.07a	0.018	3.06	3.11	1,500	3.18	3 (2)
europaea var. *europaea*	'Negrinha'	3.07a	0.013	3.06	3.09	1,502	2.76	3 (2)
europaea var. *europaea*	'Madural'	3.05a	0.012	3.03	3.07	1,492	3.16	3 (2)
europaea var. *europaea*	'Verdeal'	2.97b	0.020	2.95	3.00	1,453	2.66	3 (2)
europaea var. *europaea*	'Cobrançosa'	3.03ab	0.035	2.96	3.07	1,480	2.93	3 (2)
europaea var. *sylvestris*	-	3.19c	0.047	3.12	3.24	1,558	3.32	1 (5)

[1] Means followed by the same letter are not statistically different according to the multiple comparison Tukey-Kramer test at $P \leq 0.05$.

[2] 1 pg DNA = 978 Mbp according to Doležel *et al.* (2003).

Genuine intraspecific variation involving differences in heterochromatic sequences are known but rather rare. An example of this was described in the subspecies pair *Scilla bithynica* spp. *bithynica* (Colchicaceae), which presents many large C-bands and a 1C genome size of 29.2 pg and *S. bithynica* ssp. *radkae*, with few small C-bands and a 1C genome size of 22.9 pg of DNA (Greilhuber 1998). On the other hand, several reports concerning intraspecific variation that suggested a rather plastic genome were further rebutted after reinvestigation of the original material (for a review see Suda 2004). In most of these cases, the supposed intraspecific variation was a result of technical artefacts and/or *taxon*omic errors (Greilhuber *et al.* 1998). Methodological errors that cause non-stoichiometric binding of the fluorochrome are often responsible for such variations. As an example, hot hydrolysis (1 M HCl, 60ºC), which is not recommended because the staining optimum lasts for a very short time, is often used by many authors (*e.g.*, Cavallini *et al.* 1996; Bitonti *et al.* 1999).

An interesting case of a supposed plastic genome, which was further disproved, is sunflower, *Helianthus annuus* (Asteraceae): Cavallini *et al.* (1996) using Feulgen cytophotometry, found a 1.58-fold genome size variation between lines of *H. annuus* and

suggested a correlation between these nuclear DNA changes and the degree of methylation and amount of repetitive DNA. Also in this species, Johnston *et al.* (1996) and Price and Johnston (1996) using FCM, found a 287% intraspecific difference and claimed that the genome size of sunflower varied with light quality and quantity. Nevertheless, subsequent re-evaluation by Price *et al.* (2000) revealed that these differences were caused by a technical artefact caused by the release of cytosolic compounds during the nuclei isolation procedure. These compounds interfered with the analysis and their levels were influenced by the quantity and quality of the light. As cytosolic compounds, like phenols, are common, at least in woody plant species, intraspecific genome size variation must be interpreted with caution, until an independent confirmation is made.

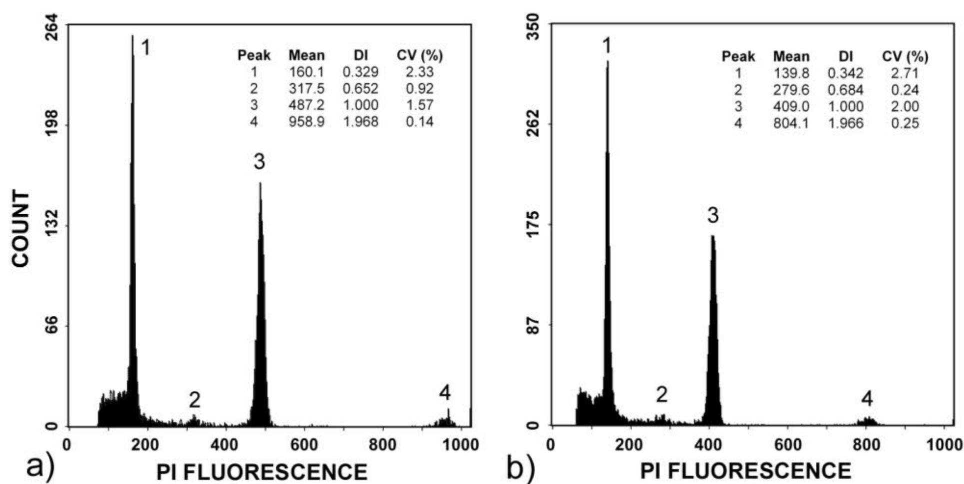

Fig. III.1.1 Histograms of relative PI fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA, as internal reference standard) and *Olea europaea*: a) ssp. *europaea* var. *europaea* cv. Verdeal; and b) ssp. *europaea* var. *sylvestris*. In both histograms four peaks were observed: 1 – nuclei at G_0/G_1 phase of *O. europaea*; 2 – nuclei at G_2 phase of *O. europaea*; 3 – nuclei at G_0/G_1 phase of *P. sativum*; 4 – nuclei at G_2 phase of *P. sativum*. The mean channel number (Mean), DNA index (DI, *i.e.*, mean channel number of sample / mean channel number of reference standard) and coefficient of variation (CV, %) value of each DNA peak are also given.

Olive trees, as other woody plants, appeared to be difficult species to isolate nuclei in proper conditions for FCM estimation of nuclear DNA content. In initial experiments this was the case, as nuclei isolated in LB01 buffer without protectants such as ß-mercaptoethanol, metabisulfite or polyvinyl pyrrolidone (PVP-10) were very instable and histograms of relative fluorescence intensity presented peaks with poor resolution (coefficient of variation, CV > 5.0%), suggesting the possible interference of cytosolic compounds (data not shown). The addition of ß-mercaptoethanol (an anti-oxidant used to suppress the interference of secondary products, Galbraith *et al.* 2002), and the reduction

of tissue chopping intensity (to decrease the release of cytosolic compounds), were fundamental to obtain nuclear fluorescence stability and peaks with good CV values (Fig. III.1.1, Table III.1.1). Also, very low background noise was obtained in histograms, leading to the conclusion that the followed methodology provided unbiased estimations of nuclear DNA content for this species. This procedure has therefore the potential to be used in the genome size estimation of other *O. europaea* cultivars and olive species.

Taking in consideration the low heterogeneity of the nuclear DNA content estimations found in this work, and the contrary data obtained previously for Italian cultivars of olive, which point in the direction of intraspecific variation, it would be interesting to estimate the genome size of a vast number of olive cultivars [there are up to 2,600 identified cultivars (Rugini and Lavee 1992)] and related species to ascertain which case is correct. If the intraspecific variation is confirmed, FCM and other genome characters can be used to easily identify some *Olea* cultivars and help understanding the relationships between species within *Olea* genus and the phylogenesis of cultivated olive.

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III.2 Genome size estimations on *Ulmus minor* Mill., *Ulmus glabra* Huds. and *Celtis australis* L. using flow cytometry

Chapter section submitted as short research paper in SCI journal:
Loureiro J, Rodriguez E, Gomes A, Santos C (2006) Genome size estimations on *Ulmus minor* Mill., *Ulmus glabra* Huds. and *Celtis australis* L. using flow cytometry. Plant Biology (*in press*)

Abstract

The Ulmaceae family is composed by nearly 2,000 species widely distributed in the northern hemisphere. Despite their wide distribution area, there are only four native species in the Iberian Peninsula. In this work the genome size of three of those species (*Ulmus minor*, *U. glabra* and *Celtis australis*) was estimated using flow cytometry. The nuclear DNA content of *C. australis* was estimated as 2.46 ± 0.061 pg/2C, of *U. minor* as 4.25 ± 0.158 pg/2C and *of U. glabra* as 4.37 ± 0.103 pg/2C of DNA. No statistically significant differences were detected among individuals of the same species. These species revealed to be problematic for flow cytometric analyses, due to the release of mucilaginous compounds into the nuclear suspension. Despite that, the modified protocol here presented ensured high quality analyses (low coefficient of variation and background debris and nuclear fluorescence stability), opening good perspectives on its application to estimate the genome size of species with similar problems.

Keywords C*eltis australis* L.; flow cytometry; genome size; mucilaginous compounds; nuclear DNA content; *Ulmus glabra* Huds.; *Ulmus minor* Mill.

Introduction

The Ulmaceae family is composed by approximately 2,000 species, many of which are economically important as a noble source of wood and as ornamental trees. This family is widely distributed within tropical, sub-tropical and temperate regions of the northern hemisphere (Richens 1983). In Iberian Peninsula there are only four recognized species of Ulmaceae: *Celtis australis* L., *Ulmus minor* Mill., *U. glabra* Huds. and *U. laevis* Pall (Navarro and Castroviejo 1995). While the former three species are widely distributed throughout this region, *U. laevis* is only found in Soria and Navarra provinces, where it was naturalized (Navarro and Castroviejo 1995). Unfortunately, the number of Ulmus trees, commonly known as elms, has been decreasing since the beginning of the 20th century due to the Dutch elm disease (Stipes and Campana 1981). Unfortunately, breeding programmes dedicated to the development of resistant cultivars or hybrid species have been, up to the moment, unable to totally eradicate the disease.

Despite of their high economical importance, cytogenetic studies, which serve as the basic information for future genomic efforts, were scarcely made in species belonging to Ulmaceae. In what concerns genome size estimations only three nuclear DNA content values were found in the literature (Bennett and Leitch 2005*a*): 2C DNA = 1.36 pg for *Holoptelea integrifolia* Planch. (D. Ohri 2002, pers. comm.), 2C DNA = 2.15 pg for *U. glabra* (S.R. Band 1984, pers. comm.), and 2C DNA = 2.70 pg for *C. australis* (Ohri and Kumar 1986). All these estimations were performed using Feulgen microdensitometry. The popularity of this technique has been decreasing over time, as it is laborious and time consuming. On the other hand, flow cytometry (FCM), a faster, easier and more accurate system, became gradually the preferred technique for genome size estimations and DNA ploidy level analyses in plants (Bennett and Leitch 2005*b*). In most plants, analyses of relative DNA content of nuclei isolated from young tissues yield histograms with good resolution presenting a dominant peak corresponding to nuclei at the G_0/G_1 phase of the cell cycle and a minor peak corresponding to G_2 nuclei (Doležel and Bartoš 2005). However, as it in shown in Chapter IV.1 in a systematic nuclear isolation buffers comparison, some plant species are more recalcitrant to analyse than others, with a diverse array of problems prone to affect the FCM analysis. In that study it was verified that *C. australis* leaf tissue presented mucilaginous compounds, which according to the buffer, interfered with the analysis, namely by affecting histograms overall quality and nuclear fluorescence stability.

In this study the nuclear DNA content of three Ulmaceae species was estimated for the first time using FCM. Due to the problems verified in isolating nuclei from those species, it

became an objective to develop a reliable protocol to isolate nuclei in good conditions from tissues containing mucilaginous compounds.

Materials and Methods

Leaves of four *C. australis* mature trees and fruits (samaras) of three *U. minor* and one *U. glabra* tree located in Aveiro region (Portugal) were used as plant material for nuclear DNA content estimations.

Nuclear suspensions were obtained from approximately 30 mg of plant material following the protocol developed by Galbraith *et al.* (1983), with some modifications. Plant tissues were placed in a Petri dish together with 50 mg of *Zea mays* cv. CE-777 leaves (internal reference standard with 2C = 5.43 pg DNA; Lysák and Doležel 1998; kindly provided by Prof. Jaroslav Doležel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic) and one mL of the selected nuclear isolation buffer. Four nuclear isolation buffer were tested in preliminary experiments: Galbraith's buffer (Galbraith *et al.* 1983), LB01 (Doležel *et al.* 1989), Tris.$MgCl_2$ (Pfosser *et al.* 1995) and Otto's buffers (Otto 1992; Doležel and Göhde 1995). From these buffers, only Tris.$MgCl_2$ provided good results with the three species. Nevertheless, the lysis buffer had to be supplemented with polyvinyl pyrrolidone (PVP), to a final chemical composition of 0.2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, 1% (w/v) PVP-10, pH 7.5. Also, to minimize the release of cytosolic compounds, the chopping was quick (about 30 s) and not very intense and a two minute incubation period in the buffer before filtration was necessary to reduce the viscosity of the sample and increase nuclei yield. The nuclear suspension was filtered through a 50 μm nylon mesh and nuclei were stained with 50 μg mL^{-1} propidium iodide (PI, Fluka, Buchs, Switzerland). Also, 50 μg mL^{-1} RNase (Sigma, St. Louis, MO, USA) was added to the nuclear homogenate to avoid PI staining of double stranded RNA. Samples were kept on ice and analysed after a 5 min incubation period using a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm and PI fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. The results were acquired using the SYSTEM II software (v. 3.0, Beckman Coulter®) in the form of four graphics: cytogram of forward scatter (FS) *vs.* side scatter (SS) both in logarithmic (log) scale; fluorescence integral (FL) histogram; FL *vs.* time cytogram; and FL pulse integral *vs.* FL pulse height cytogram. The first cytogram was used to scan for possible effects of cytosolic compounds, as recommended in Chapter IV.2. In the latter cytogram, a region

was defined to discriminate nuclei doublets, partial nuclei, nuclei with associated cytoplasm and other debris (Brown *et al.* 1991). In each sample at least 5,000 nuclei were analysed.

The nuclear genome size of each Ulmaceae species was estimated according to the following formula:

Sample 2C nuclear DNA content (pg) =

$$\frac{\textit{Sample}\ \ G_0/G_1\ \text{peak mean}}{\textit{Z. mays}\ \ G_0/G_1\ \text{peak mean}} \times 5.43$$

Conversion into base-pair numbers was performed using the factor: 1 pg = 978 Mbp (Doležel *et al.* 2003). At least three replicates were performed per genotype in three different days, to avoid instrumental fluctuations.

Genome size data was analyzed using the one-way ANOVA procedure and the Tukey-Kramer multiple comparison test was applied for pair-wise comparison (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA).

Results and discussion

Nuclear DNA content estimations for each species are presented in Table III.2.1. The genome size of *C. australis* was estimated as 2.46 ± 0.061 pg/2C, of *U. minor* as 4.25 ± 0.158 pg/2C and of *U. glabra* as 4.37 ± 0.103 pg/2C (Fig. III.2.1). Statistical analyses did not reveal significant differences among trees of the same species and between *U. minor* and *U. glabra* ($P \leq 0.05$). Statistically significant differences were detected between *C. australis* and the remaining species ($P \leq 0.05$). *Ulmus minor* and *U. glabra* presented almost two times the 2C value of *C. australis* and very similar values between them, which indicate possible genome stability within this genus.

If we compare these results with previous determinations in these species, it is worth notice that whereas the value obtained for *C. australis* is similar to the previous estimation obtained using Feulgen microdensitometry (2C DNA = 2.70 pg; Ohri and Kumar 1986; Table III.2.1), the value that we obtained for *U. glabra* is very different from the one obtained by S.R. Band (1984, pers. comm.) using the Feulgen technique. Indeed our estimation is more than the double of the previous determination for this species. Unfortunately, this estimation has never been published in a scientific journal, being only available in the Kew Plant DNA C-values database (Bennett and Leitch 2005*a*) where

information on methodological procedures is scarce, thus making any comparison of 2C values a difficult task.

Table III.2.1 Nuclear DNA content of *Ulmus minor*, *U. glabra* and *Celtis australis*. The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as mean of the 1C genome size in Mbp. The mean coefficient of variation (CV, %) of the G_0/G_1 peak is also given, the number of genotypes (*n*) and number of replicates per genotype (R) are also given. Nuclear DNA content, applied methodology and original reference of previous estimations are also provided [values were collected from the Kew Plant DNA C-values database (Bennett and Leitch 2005*a*); Fe – Feulgen microdensitometry]. Nuclear DNA content mean values followed by the same letter (*a* or *b*) are not statistically significant according to the Tukey-Kramer pair-wise comparison test at $P \leq 0.05$.

Species	Nuclear DNA content (pg/2C)		1C genome size (Mbp)[1]	CV (%)	*n* (R)	Previous estimations		
	Mean	SD				Nuclear DNA content (pg/2C)	Method	Reference
Celtis australis	2.46a	0.018	1201	3.51	4 (5)	2.70	Fe	Ohri and Kumar 1986
Ulmus minor	4.25b	0.090	2078	4.18	3 (4)	-	-	-
Ulmus glabra	4.37b	0.103	2136	3.78	1 (3)	2.15	Fe	Band 1984 (pers. comm.)

[1] 1 pg DNA = 978 Mbp according to Doležel *et al.* (2003).

The differences observed between this work and previous studies may be explained by the interference of cytosolic compounds that are released upon chopping. Greilhuber (1988) already showed that tannins were among the compounds that can affect nuclear DNA content determinations using Feulgen cytophotometry. This interference was also verified in FCM studies, as shown by the works of Noirot *et al.* (2000, 2003) in coffee trees, and more recently by the thorough study on the effect of tannic acid on plant nuclei presented in Chapter IV.2. In these studies the negative effect of cytosolic compounds, *i.e.*, serious decrease of sample's quality and nuclear fluorescence, together with instrument clogging has been demonstrated, ultimately resulting in flawed data. Rodriguez *et al.* (2005) in a preliminary work with these species detected the release of mucilaginous compounds when plant material (either leaves or fruits) were chopped for FCM analysis. In our work, these compounds were present in higher concentrations in leaves of *Ulmus* species and caused a constant decrease of nuclear fluorescence over time (Fig. III.2.1d) in both sample and standard. This decrease lead to a shift of both peaks towards the left, with nuclei of the reference standard (*Z. mays*) loosing more fluorescence than those of the sample; and to DNA peaks broadening, ensuing unacceptable coefficient of variation

(CV) values in both species (Fig. III.2.1e). The difference in peak position between sample and reference standard (DNA index, DI) was calculated from mean values, as the use of modal values revealed similar differences to those obtained with mean values (data not shown). According with the results presented in Chapter IV.2, leaves of *Ulmus* seem to contain a very high concentration of cytosolic compounds, with the prime effect being an immediate decrease of nuclear fluorescence that in a way masked the negative influence of debris coating, here having less importance.

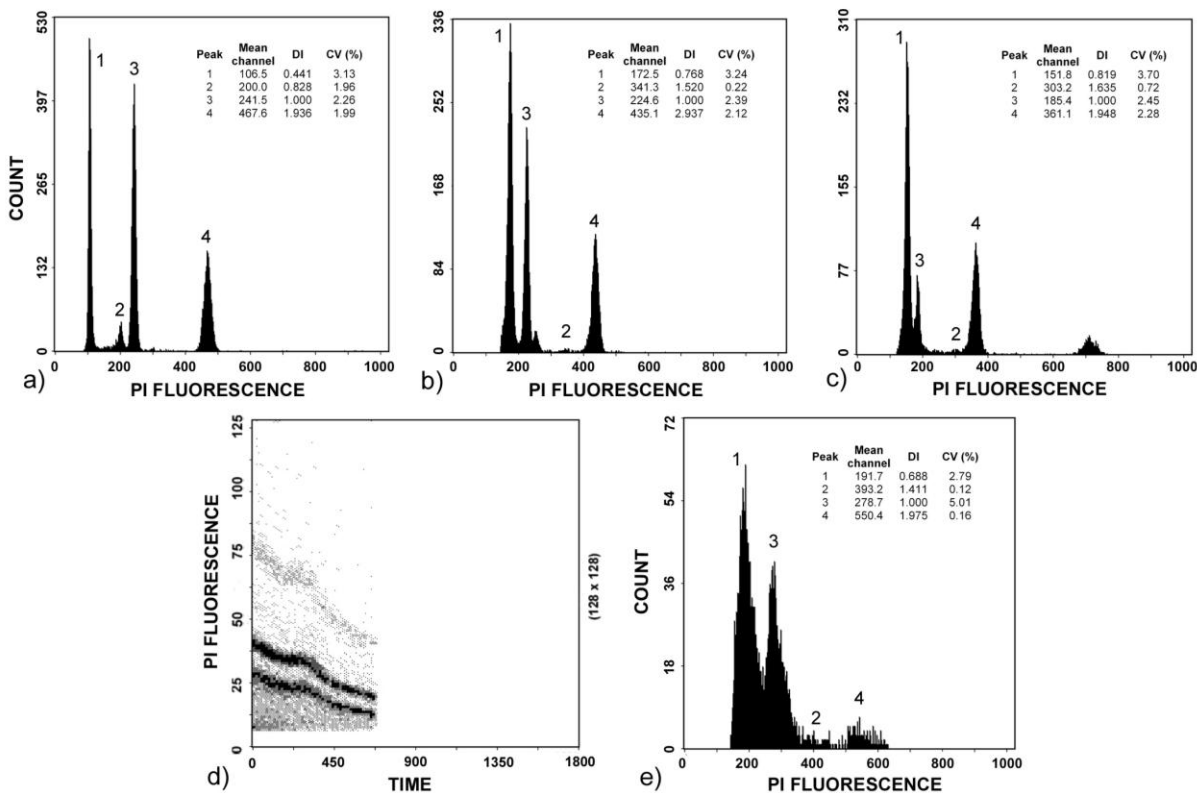

Fig. III.2.1 Histograms of relative fluorescence intensity (PI fluorescence) obtained after simultaneous analysis of nuclei of *Zea mays* cv. CE-777 (2C = 5.43 pg DNA, as an internal reference standard) and: a) *Celtis australis*; b) *Ulmus minor*; and c) *U. glabra* isolated in a modified Tris.$MgCl_2$ buffer. A cytogram of PI fluorescence *vs.* time (in seconds, d) and a histogram of relative PI fluorescence intensity (e) of nuclei of the internal standard and *Ulmus glabra* isolated in Galbraith's buffer are also given. Please notice that in these graphics, nuclear fluorescence was not stable with serious consequences in the quality given by the coefficient of variation (CV, %) and in the DNA index (DI, *i.e.*, ratio between the mean channel position of sample and standard G_0/G_1 peaks) of G_0/G_1 peaks. In any histogram four peaks were visible: 1 – nuclei at G_0/G_1 phase of sample; 2 – nuclei at G_2 phase of sample; 3 – nuclei at G_0/G_1 phase of *Z. mays*; 4 – nuclei at G_2 phase of *Z. mays*. The mean channel position (Mean channel) of each DNA peak is also given.

In an attempt to overcome this problem, four buffers were tested to detect which one dealt better with mucilaginous compounds. Tris.$MgCl_2$ was the only buffer providing good results (CV values < 5.0%) with these species. This is in accordance with the results

obtained in Chapter IV.1 for *C. australis* leaves. These authors hypothesised that the higher detergent concentration in Tris.$MgCl_2$ buffer was important to reduce sample viscosity and minimize the negative effect of mucilaginous compounds. As expected, in our work the fluorescence of nuclei isolated from *C. australis* leaves was stable over time and DNA histograms presented low debris background and relatively low CV values (below 4.0%, Fig. III.2.1a). However, leaves of *Ulmus* species still provided mediocre results. As a solution and following the suggestion made by Suda (2004), an alternative tissue with lower concentration of mucilaginous compounds was chosen. Therefore, nuclear DNA estimations of *U. minor* and *U. glabra* were done using the samaras, which provided satisfying results, *i.e.*, DNA peaks with relatively low CV values (below 4.0% in *U. glabra* and below 5.0% in *U. minor*) and debris background, and nuclei presenting high fluorescence stability (Fig. III.2.1b and c). This approach enabled the FCM analysis of these recalcitrant species with mean genome size estimations having low standard deviation values (Table III.2.1). Also, no statistically significant differences were observed among days of analyses.

The approach here presented, *i.e.*, testing different nuclear isolation buffers and plant tissues to determine which combination provides better results, should be faced as a routine procedure for analysing new plant material. In this case, this approach proved to be an efficient strategy to obtain a reliable procedure for DNA FCM studies in Ulmaceae species, opening perspectives for the future application of this technique to estimate the genome size of other species belonging to this family, including hybrid detection, and to study other plant species with similar problems.

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III.3 Flow cytometric and cytogenetic analyses of Iberian Peninsula *Festuca* spp.

Chapter section submitted as original article to SCI journal:
Loureiro J, Kopecký D, Castro S, Santos C, Silveira P (2006) Flow cytometric and cytogenetic analyses of Iberian Peninsula *Festuca* spp. Plant Systematics and Evolution (*submitted*)

Abstract

Festuca L. (Poaceae) presents an important diversification centre in Iberian Peninsula. We used chromosome counting, fluorescence (FISH) and genomic *in situ* hybridization (GISH), and FCM to clarify the taxonomic position of several *taxa*, to search for phylogenetic relationships and to assess the extent and pattern of genome variation in fescues. The chromosome number of *Festuca duriotagana* is determined for the first time and new ploidy level estimations are given for *F. rothmaleri* and *F. summilusitana*. In the latter species, besides the reported decaploid level, dodecaploidy was found in some populations, which points to the existence of an unrecognized *taxon*. For each section, a decrease of genome size with increase of polyploidy was observed. In general, *in situ* hybridization techniques failed to reveal phylogenetic relationships among the selected species. In FISH, a variation in the number of rDNA sites was observed in some species. GISH results indicate that *F. henriquesii* is not a progenitor of the studied polyploid species.

Keywords fescues; *Festuca*; flow cytometry; genome size; Iberian Peninsula; *in situ* hybridization; karyology; molecular cytogenetics

Introduction

Festuca L. (Poaceae) is one of the most complex and numerous genus of Poaceae. In the Iberian Peninsula there is an important diversity centre of approximately 100 *taxa* (Cebolla Lozano and Rivas Ponce 2003). From these, 24 *taxa* distributed through six sections are recognized in the Portuguese continental flora (Franco and Rocha Afonso 1998). These include five species (*Festuca brigantina* Markgr.-Dann., *F. duriotagana* Franco & R. Afonso, *F. elegans* Boiss., *F. henriquesii* Hack. and *F. summilusitana* Franco & R. Afonso) listed in the European Community Habitats Directive 92/43/EEC – Annex II, where they are referred as species of community interest whose conservation requires the designation of special areas. Moreover, *F. brigantina* ssp. *brigantina*, *F. duriotagana* and *F. henriquesii* are endemic from Portugal continental region. The close morphological similarities among fescues and the occurrence of a high number of ecotypes/varieties that resulted from specific genetic variability and different environmental conditions, leads to difficulties in the identification and classification of species from this genus (de la Fuente *et al.* 1997; Huff and Palazzo 1998). Due to its high complexity, Iberian Peninsula species of *Festuca* have been subject of intensive taxonomic and karyologic studies (*e.g.*, de la Fuente *et al.* 2001; Ortúñez and de la Fuente 2004). Many of these studies used both approaches, as for this genus the number of chromosomes has been regarded an important tool in delimitation of new species, which sometimes are not so easily separated using morphoanatomic analyses (de la Fuente and Ortúñez 2000).

Besides chromosome number, their morphology can also be an important character in the study of relationships among species (Harper *et al.* 2004). Fluorescence *in situ* hybridisation (FISH) is a powerful tool that enables the visualization of specific DNA sequences on chromosomes squashed in a microscope slide. Ribosomal DNAs (rDNAs) are among the most used DNA sequences for FISH, as their sites differ between closely related species (both in number of sites and in their position), while the order of most genes remains conserved during evolution (Harper *et al.* 2004). FISH with rDNAs has been applied to study the phylogeny of some of the most economically important fescue species (*e.g.*, *F. arundinaceae* Schreb., *F. pratensis* Huds. and *F. arundinaceae* var. *glaucescens* Boiss.; Thomas *et al.* 1997). Also, Harper *et al.* (2004) used FISH to study the rDNA patterns on chromosomes of *F. scariosa* (Lag.) Asch. & Graebn. (section *Scariosae*) and four diploid species of section *Montanae* in order to search for diploid progenitors of polyploid species.

Genomic *in situ* hybridization (GISH) is a modified technique of FISH that, in hybrids or polyploid plants, enables the visualization of chromosomes of different genomes with

different colours (Raina and Rani 2001). This method is based on hybridization of total genomic DNA with chromosomal DNA fixed on a microscopic slide (Schwarzacher *et al.* 1989). In *Festuca*, despite having been already used with phylogenetic purposes in the discrimination of ancestral progenitor genomes of allohexaploid *F. arundinacea* (Humphreys *et al.* 1995), most of the studies concerned hybrids of *Lolium* x *Festuca* (*e.g.*, Kopecký *et al.* 2005*a*, 2005*b*; Kopecký *et al.* 2006; Kosmala *et al.* 2006).

Estimations of nuclear DNA content may provide useful information in phylogenetic relationships analyses. Furthermore, it can also provide important knowledge on the complexity of a genome (Doležel 1997). As it was shown by Bennett and Leitch (2005), genome size is still unknown for about 98% of angiosperm species. Until now, most of the estimations of nuclear DNA content of fescues were determined using Feulgen microdensitometry (Bennett *et al.* 1982; Grime and Mowforth 1982; Schifino and Winge 1983; Seal 1983; Ceccarelli *et al.* 1992) and only recently, flow cytometry (FCM), has been used for genome size estimations within this genus: Huff and Palazzo (1998) estimated the nuclear DNA content of 10 fine fescue species and Arumuganathan *et al.* (1999) estimated the genome size of *F. arundinacea* and *F. longifolia* Thuill.

The objective of this work was to study the cytology of several fescue species. For that, we intended to determine for the first time the chromosome number of *F. duriotagana*, clarify the chromosome number of *F. ampla* Hack. and confirm the ploidy level of the remaining species, especially *F. summilusitana*. Also, molecular cytogenetic techniques of FISH and GISH were applied in wild species of *Festuca* to search for phylogenetic relationships, namely the progenitor character of *F. henriquesii*, the only diploid species from West Iberian Peninsula. Finally, it was our goal to use FCM to estimate the nuclear DNA content of 14 *taxa* (from which 11 are first estimations) and by this way to contribute to the assessment of the extent and pattern of genome size variation within this genus.

Material and methods

Plant material

Plant and seed samples of 14 *taxa* of *Festuca* were collected from several field locations in Portugal (Table III.3.1). Field collected and seed germinated plants were potted and maintained in a greenhouse at 22 ± 1ºC, photoperiod of 16 h and light intensity of 530 ± 2 µmol m^{-2} s^{-1}. Voucher specimens were collected and kept in the Herbarium of the University of Aveiro (AVE).

Table III.3.1 Scientific name and localities of sample collection of the *Festuca* species studied in this work. Voucher specimens were kept in the Herbarium of the University of Aveiro (AVE).

Species	Localities
Festuca L. subgen. *Festuca*	
Section *Subbulbosae* (Nyman) Hack.	
F. paniculata (L.) Schinz & Thell. subsp. *multispiculata* Rivas Ponce & Cebolla	Bragança: Serra da Nogueira, summit with 1218 m a.s.l. between Castelinho and Nogueira, 29TPG72. Coimbra: Serra do Açor, Mata da Margaraça, 29TNE9252, c. 600 m.
Section *Festuca*	
F. henriquesii Hack.	Guarda: Serra da Estrela, near Torre, 29TPE16, c. 1970 m.
F. ampla Hack. ssp. *ampla*	Coimbra: Serra do Açor, Covanca, 29TPE0049, c. 900 m.
F. ampla Hack. ssp. *transtagana* Hack.	Faro: Foia, 29SNB33, c. 900 m.
F. brigantina Markgr.-Dann. ssp. *brigantina*	Bragança: Mosqueiro, 29TPG72, c. 1070 m.
F. summilusitana Franco & Rocha Afonso	Bragança: Serra da Nogueira, summit with 1218 m altitude between Castelinho and Nogueira, schist, 29TPG72. Vila Real: Serra do Marão, Senhora da Serra, schist, 29TNF96, c. 1415 m. Aveiro: Serra do Caramulo, Caramulinho, granite, 29TNE68, c. 1050 m. – population C1 Aveiro: Serra do Caramulo, near Cruzinha, schist, 29TNE7195, c. 1000 m. – population C2 Aveiro: Serra do Caramulo, near Monteteso, schist, 29TNE7094, c. 900 m. – population C3 Aveiro: Serra da Freita, 1 km N of Albergaria da Serra, schist, 29TNF62, c. 1000 m. Coimbra: Serra da Lousã, Castelo do Trevim, schist, 29TNE 7038, c. 1200 m. – population L1 Coimbra: Serra da Lousã, Penedos de Góis, quartzite, 29TNE7439, c. 1050 m. – population L2 Coimbra: Serra da Lousã, Santo António das Neves, schist, 29TNE7137, c. 1150 m. – population L3 Coimbra: Serra da Lousã, c. 750 m S of Castelo do Trevim, schist, 29TNE7037, c. 1090 m. – population L4 Guarda: Serra da Estrela, near Torre, granite, 29TPE16, c. 1960 m. – population E1 Guarda: Serra da Estrela, between Sabugueiro and Penhas Douradas, granite, 29TPE17, c. 1200 m. – population E2 Guarda: Serra da Estrela, Poço do Inferno, granite, 29TPE27, c. 1100 m. – population E3 Guarda: Serra da Estrela, Gouveia, between Santinha and Santiago, schist, 29TPE28, c. 1415 m. – population E4
Section *Eskia* Willk.	
F. elegans Boiss. ssp. *merinoi* (Pau) Fuente & Ortúñez	Coimbra: Serra do Açor, Cabeço da Fonte de Espinho, 29TNE9452, c. 1000 m. Guarda: Serra da Estrela, Manteigas, c. 1100 m.
Section *Aulaxyper* Dumort.	
F. nigrescens Lam. ssp. *microphylla* (St.-Yves) Markgr.-Dann.	Coimbra: Serra do Açor, Cabeço da Fonte de Espinho, 29TNE9452, c. 1000 m.
F. rubra L. ssp. *litoralis* (G. F. W. Meyer) Auquier	Viana do Castelo: Foz do Rio Neiva, 29TNG10, c. 1 m
F. rubra L. ssp. *pruinosa* (Hack.) Piper	Viana do Castelo: near Farol de Montedor, 29TNG12, c. 2 m
F. rubra L. ssp. *rubra*	Coimbra: Serra do Açor, Casais de São Pedro, 29TPE0052, c. 1250 m.
F. rothmaleri (Litard.) Markgr.-Dann.	Coimbra: Serra do Açor, near Castanheira, 29TNE9449, c. 900 m. Guarda: Serra da Estrela, near Lagoa Comprida, 29TPE16, c. 1600 m.
F. duriotagana Franco & Rocha Afonso var. *barbata*	Portalegre: Barragem de Belver, 29SND87, c. 40 m.
Festuca L. subgen. *Schenodorus* (P. Beauv.) Peterm.	
Section *Schenodorus*	
F. arundinaceae Schreb. ssp. *mediterranea* (Hack.) K.Richt.	Coimbra: Serra do Açor, Fajão, margins of Rio Ceira, 29TNE9146, c. 475 m.

Chromosome preparations and counting

When available, seeds were germinated in Petri dishes on wet filter paper and the obtained seedlings were planted in 30 mm pots in the greenhouse. Well developed plantlets were transferred into a hydroponic culture with an aerated solution of 0.9 g L^{-1} Hydroponex (Hu-Ben, Čerčany, Czech Republic) at 25 ± 2ºC. In some cases, field collected plants were transferred directly into hydropony. Mitotic metaphase spreads were prepared from root tips according with the protocol of Masoudi-Nejad *et al.* (2002).

For chromosome counting, chromosomes on a slide were counterstained with 1.5 µg mL^{-1} 4',6-diamidino-2-phenylindole (DAPI) made in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). Slides were evaluated with an epifluorescence Olympus AX70 microscope (Center Valley, PA, USA). Images were acquired with a Cooke SensiCam B/W camera (Eugene, OR, USA) and processed using ScionImage (Frederick, MD, USA) and Adobe Photoshop (San Jose, CA, USA) software.

Fluorescence in situ *hybridization with rDNA probes*

For FISH analyses, chromosomes of species from sections *Festuca* (*F. henriquesii*, *F. ampla* ssp. *ampla*, *F. brigantina* and *F. summilusitana*), *Aulaxyper* (*F. rubra* ssp. *litoralis* and *F. duriotagana*) and *Subbulbosae* (*F. paniculata* ssp. *multispiculata*) were hybridized with biotin labelled DNA clone pTa71 (Gerlach and Bedbrook 1979) containing a 9 kb EcoRI fragment of wheat ribosomal DNA and carrying the 18S-5.8S-26S cluster of ribosomal DNA genes (referred from here as 45S rDNA), and with digoxigenin (DIG) labelled probe for 5S rDNA. This probe was prepared using the polymerase chain reaction (PCR) with a pair of specific primers (RICRGAC1, RICRGAC2), which amplify 303 bp in rice (Fukui *et al.* 1994), using rice genomic DNA as a template. *In situ* hybridization was performed according to the protocol of Masoudi-Nejad *et al.* (2002). Sites of probe hybridization were detected by biotin-streptavidin-Cy3 conjugate (Amersham, Piscataway, NJ, USA) and fluorescein-conjugated anti-DIG antibody (Roche, Indianapolis, IN, USA). Chromosomes were counterstained and evaluated using the methodology described above.

Genomic in situ hybridization

In GISH experiments, total genomic DNA of *F. henriquesii* was labelled with biotin according to manufacturer's instructions (Biotin-Nick Translation Kit, Roche) and used as a probe on *F. ampla* ssp. *ampla*, *F. brigantina*, *F. summilusitana*, *F. paniculata* ssp. *multispiculata*, *F. rubra* ssp. *litoralis* and *F. duriotagana* chromosomes. Salmon sperm

DNA was used as blocking DNA. *In situ* hybridization and detection was done as described for FISH. Some slides where FISH was already applied were also analysed by GISH. For this, slides were washed according with the protocol described by Schwarzacher and Heslop-Harrison (2000).

Genome size estimations using flow cytometry

For FCM analyses of genome size, leaves of field collected or seed germinated plants were used as plant material. Nuclear suspensions were prepared according to Galbraith *et al.* (1983), by chopping 200 mg of leaf tissue of *Festuca* sp. and 50 mg of internal standard leaves with a razor blade in a glass Petri dish containing 1.5 mL of Tris.$MgCl_2$ nuclear isolation buffer (200 mM TRIS, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) and Triton X-100, pH 7.5; Pfosser *et al.* 1995) supplemented with 1% (w/v) polyvinyl pyrrolidone (PVP-10). With exception of *F. ampla*, *Pisum sativum* cv. Ctirad (Fabaceae; 2C = 9.09 pg of DNA; Doležel *et al.* 1998) was used as an internal reference standard for all the studied species. In *F. ampla*, *Z. mays* cv. CE-777 (Poaceae; 2C = 5.43 pg of DNA; Doležel *et al.* 1998) was chosen as reference standard. In *Festuca* sp., in order to obtain a good number of nuclei per mL, the quantity of plant material and chopping intensity had to be increased. Nuclear suspension was then filtered through an 80 μm nylon filter and 50 μg mL^{-1} of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 μg mL^{-1} of RNase (Fluka, Buchs, Switzerland) were added to the samples to stain the DNA and avoid staining of double stranded RNA, respectively. Samples were kept on ice and analysed within a 10 min period in a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer equipped with a 488 nm air-cooled argon-ion laser. Integral fluorescence and fluorescence pulse height and width emitted from nuclei were collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. Prior to analysis, the instrument was checked for linearity with fluorescent beads (Beckman Coulter®). Doublets, partial nuclei, nuclei with associated cytoplasm and other debris were removed from analysis using a specific gating region defined in a linear fluorescence (FL) pulse integral *vs.* FL pulse height. At least 5,000 nuclei were analysed per sample. For each species, at least five individuals were analysed in three different days to avoid errors due to instrumental drift.

The holoploid genome size (2C; *sensu* Greilhuber *et al.* 2005) of *Festuca* species was estimated according to the following formula:

2C nuclear DNA content (pg) =

$$\frac{\textit{Festuca}\ \ G_0/G_1\ \text{peak mean}}{\text{reference standard}\ \ G_0/G_1\ \text{peak mean}} \times \text{nuclear DNA content of reference standard}$$

The monoploid genome size (2Cx; *sensu* Greilhuber *et al.* 2005) of all species was also calculated in mass values (pg) and Mbp (1 pg = 978 Mbp; Doležel *et al.* 2003).

Statistical analysis

For each species, within and between populations (when applicable) differences in nuclear DNA content were analysed and compared using either a t-test or a one-way ANOVA. When necessary, a multiple comparison Tukey-Kramer test was applied to determine exactly which groups presented significantly different values. In sections *Festuca* and *Aulaxyper*, linear regression analyses were performed between mean nuclear DNA content and chromosome numbers of each species. All statistical studies were performed using SigmaStat for Windows (Version 3.1, SPSS Inc., Richmond, CA, USA).

Results

Chromosome counting

The chromosome numbers determined in this study are presented in Table III.3.2. With the exception of *F. summilusitana*, where besides the reported decaploid ploidy level (2*n* = 10*x* = 70) a new chromosome number has been found (2*n* = 12*x* = 84, Fig. III.3.1A), the observed chromosome counts were generally in agreement with previously reported estimations (Table III.3.2). A wide range of ploidy levels was observed, from 14 chromosomes in *F. henriquesii* to 70 and 84 chromosomes in *F. summilusitana*. The analysed individuals of *F. ampla* ssp. *ampla* and *F. ampla* ssp. *transtagana* were all tetraploids (2*n* = 4*x* = 28). The number of chromosomes of *F. duriotagana* (2*n* = 10*x* = 70) was counted for the first time. No B chromosomes were observed in the studied species.

Table III.3.2 Observed (obs., by chromosome counting), estimated (est., using the linear regression analysis) and reported (rep.) chromosome numbers of the *Festuca* species studied in this work.

		Chromosome number (2n)			
Species	Distribution	Obs.	Est.	Rep.	Reference
F. paniculata ssp. *multispiculata*	Iberian Peninsula	28	-	28	Devesa *et al.* 1990
F. henriquesii	Portugal	14	14	14	Ferrero and de la Fuente 1996; de la Fuente *et al.* 2001
F. ampla ssp. *ampla*	Iberian Peninsula and North Africa	28	28	28	Ortúñez and de la Fuente 1995
				28, 42	de la Fuente *et al.* 2001
				56	Malik and Thomas 1966
F. ampla ssp. *transtagana*	Iberian Peninsula	-	28	-	-
F. brigantina ssp. *brigantina*	Portugal	56	56	56	Ferrero and de la Fuente 1996; de la Fuente *et al.* 2001
F. summilusitana	Iberian Peninsula	70, 84	70, 84	42	Ortúñez and de la Fuente 1995
				70	de la Fuente and Ortúñez 2000; de la Fuente *et al.* 2001
F. elegans ssp. *merinoi*	Iberian Peninsula	-	-	28	Ferrero and de la Fuente 1996; Ortúñez and de La Fuente 2004
F. nigrescens ssp. *microphylla*	Europe	-	42	42	de la Fuente *et al.* 1997
F. rubra ssp. *litoralis*	Europe	42	42	42	Kerguélen 1975
F. rubra ssp. *pruinosa*	Worldwide	42	42	42	Kerguélen 1975
F. rubra ssp. *rubra*	Worldwide	-	56	42	Seal 1983
				42, 56	Konarska 1974
				56	Huff and Palazzo 1998
F. rothmaleri	Iberian Peninsula	-	42	42	Devesa *et al.* 1990
				56	Al-Bermani *et al.* 1992
F. duriotagana	Portugal	70	70	-	-
F. arundinaceae ssp. *mediterranea*	Worldwide	-	-	42	Queirós 1973

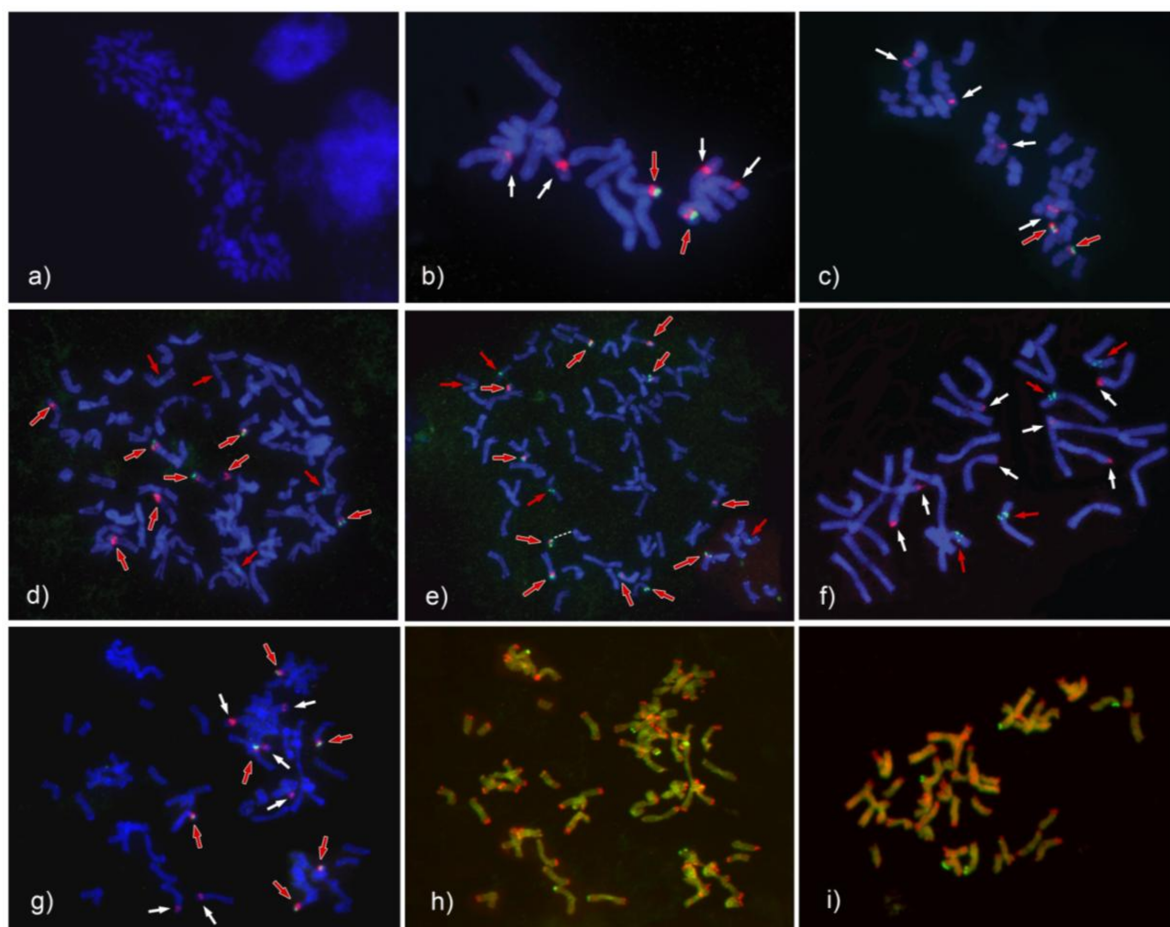

Fig. III.3.1 Cytogenetic analysis of fescue species: a) Metaphase spread of *F. summilusitana* from population C1 ($2n = 12x = 84$); b-g) FISH on metaphase plates with probe for 45S rDNA (red colour) and probe for 5S rDNA (green colour) in *F. henriquesii* (b), *F. ampla* ssp. *ampla* (c), *F. summilusitana* from Serra do Caramulo (d), *F. summilusitana* from Serra do Caramulo (e), *F. paniculata* ssp. *multispiculata* (f), *F. duriotagana* (g); h-i) GISH on mitotic metaphase plates of *F. duriotagana* (h) and *F. rubra* ssp. *litoralis* (i). White thin arrows point for chromosomes carrying 45S rDNA, red thin arrows for chromosomes with the 5S rDNA and red arrows with white contour (thicker) for chromosomes carrying both 45S and 5S rDNAs. Total genomic DNA of *F. henriquesii* was labelled with biotin and used as a probe (green colour); salmon sperm DNA was used as block. a-j) Chromosomes were counterstained using DAPI (blue colour).

Nuclear DNA content estimations

The 2C nuclear DNA content of 14 *taxa* of *Festuca* was determined using FCM (Table III.3.3). Fluorescence histograms (Fig. III.3.2) of relative nuclear DNA content showed distinct G_0/G_1 peaks with coefficients of variation (CV) usually below 4.0% for fescue. In 83.5% of the estimations, the CV value was below 3.0%, and only in 1.6% of the cases these values were above 4.0%. *Festuca* species mean holoploid genome sizes (2C) ranged from 5.86 pg/2C in *F. henriquesii* to 23.77 pg/2C in *F. summilusitana* (Table III.3.3). With the exception of *F. summilusitana*, low standard deviations of 2C nuclear

DNA content were obtained (< 2.5%), with no statistically significant differences being observed among populations ($P \leq 0.05$), revealing a high homogeneity of the values within each species. The analysis of the monoploid genome sizes (2Cx) revealed interesting results (Table III.3.3): usually within each section, a decrease of the 2Cx value was observed with the increase of polyploid level (*e.g.*, diploid species *F. henriquesii* presented a 2Cx value of 2.93 pg, while octoploid species *F. brigantina* presented a 2Cx value of 2.14 pg). Also, the 2Cx value for species with the same ploidy level seemed to differ between sections. Despite the analyses included only one species, *taxa* belonging to sections *Subbulbosae* and *Eskia* presented a 2Cx value of 3.75 pg and 3.09 pg, respectively, and seemed to have a higher monoploid genome size (*i.e.*, chromosomes of a basic set are bigger, on average) than *taxa* belonging to sections *Festuca* and *Aulaxyper*. From these sections, *Aulaxyper* seems to have *taxa* with the smallest chromosomes, on average. The only *taxon* from section *Schenodorus*, *i.e.*, *F. arundinaceae* ssp. *mediterranea*, had a 2Cx value of 2.67 pg.

Table III.3.3 Nuclear DNA content estimations of *Festuca* species studied in this work[1].

		Nuclear DNA content							
			2C range						
Species	No. of populations	2C (pg)	Min.	Max.	Dif. Pop.	2Cx (pg)	2Cx (Mbp)[2]	CV (%)	*n*
F. paniculata ssp. *multispiculata*	2	15.02 ± 0.382	14.21	15.62	n.s.	3.75	3,671	2.78	10
F. ampla ssp. *ampla*	1	8.74 ± 0.183	8.47	8.93	-	2.18	2,136	2.55	5
F. ampla ssp. *transtagana*	1	9.16 ± 0.381	8.27	9.52	-	2.29	2,239	2.96	10
F. henriquesii	1	5.86 ± 0.226	5.50	6.10	-	2.93	2,864	2.44	10
F. brigantina	1	17.08 ± 0.249	16.78	17.38	-	2.14	2,087	2.47	5
F. summilusitana	14	23.77 ± 1.584	20.50	27.19	s.	2.38	2,325	2.48	129
F. elegans ssp. *merinoi*	2	12.31 ± 0.148	12.20	12.74	n.s.	3.09	3,011	1.90	10
F. nigrescens ssp. *microphylla*	1	13.80 ± 0.104	13.65	13.94	-	2.30	2,249	2.11	5
F. rubra ssp. *litoralis*	1	13.19 ± 0.257	12.98	13.55	-	2.20	2,149	2.70	5
F. rubra ssp. *pruinosa*	1	12.69 ± 0.073	12.62	12.78	-	2.11	2,068	2.36	5
F. rubra ssp. *rubra*	1	17.66 ± 0.305	17.29	18.09	-	2.21	2,159	3.07	5
F. rothmaleri	2	13.66 ± 0.203	13.42	14.05	n.s.	2.28	2,227	2.13	10
F. duriotagana	1	20.66 ± 0.186	20.41	20.81	-	2.07	2,021	1.90	5
F. arundinaceae ssp. *mediterranea*	1	15.94 ± 0.311	15.56	16.38	-	2.67	2,598	2.30	5

[1] The values are given as mean and standard deviation of the mean of the holoploid nuclear DNA content (2C in pg) of individuals of each species. The 2C range is defined by the minimum (Min.) and maximum (Max.) value obtained for each species. Differences among populations (Dif. Pop.) were analysed using a t-test or a one-way ANOVA (n.s. – not significantly different; s. – significantly different at $P < 0.05$). The monoploid nuclear DNA content (2Cx) in mass values (pg) and Mbp, the mean coefficient of variation (CV, %) and the number of analysed individuals (*n*) are also provided for each species. [2]1 pg DNA = 978 Mbp (Doležel *et al.* 2003).

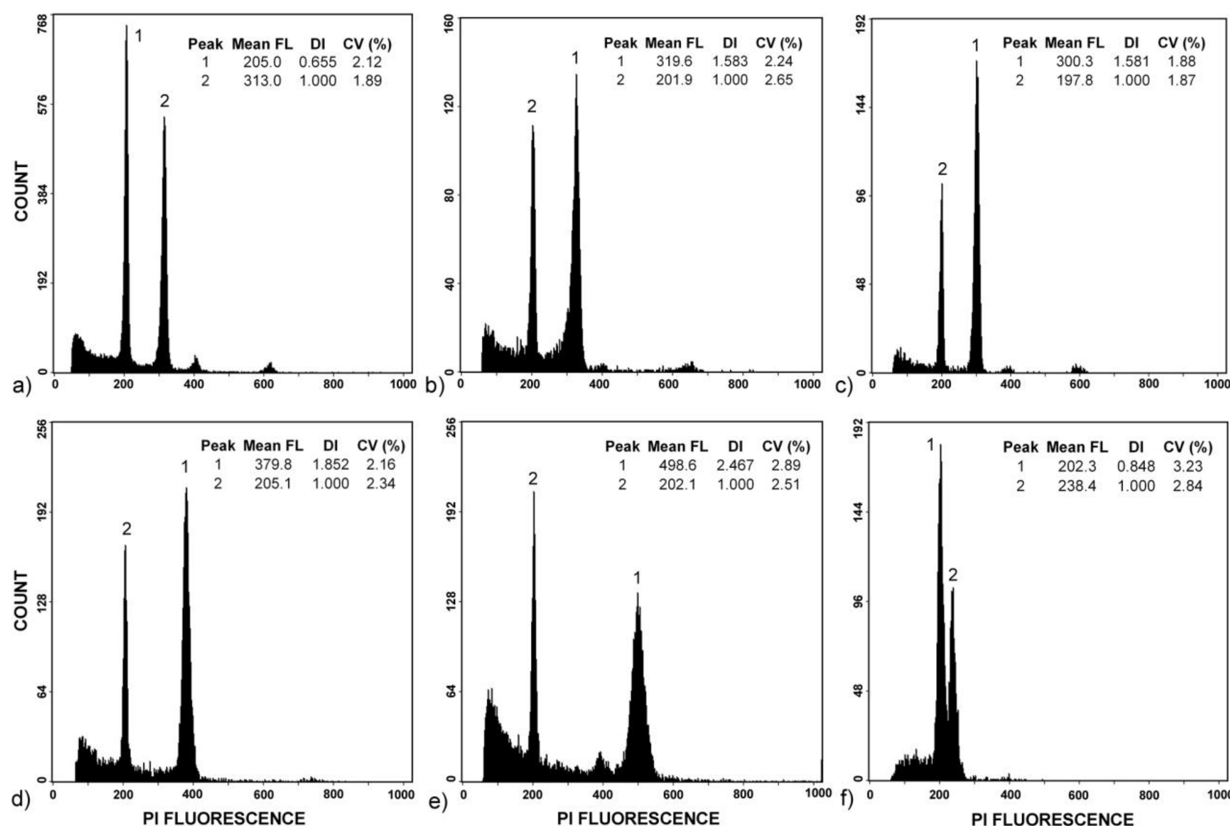

Fig. III.3.2 Flow cytometric histograms of relative PI fluorescence intensity obtained after simultaneous analysis of nuclei isolated from the internal reference standard and *Festuca*: a) *F. henriquesii* (2*n* = 2*x* = 14); b) *F. ampla* ssp. *ampla* (2*n* = 4*x* = 28); c) *F. rothmaleri* (2*n* = 6*x* = 42); d) *F. brigantina* (2*n* = 8*x* = 56); and e) *F. summilusitana* (2*n* = 10*x* = 70). In these histograms the following peaks are marked: 1 – nuclei at G_0/G_1 phase of sample; 2 – nuclei at G_0/G_1 phase of internal standard (*Pisum sativum* cv. Ctirad with 2C = 9.09 pg DNA in histograms a) and c-e), and *Zea mays* cv. CE-777 with 2C = 5.43 pg DNA in histogram b). Histogram f) was obtained after simultaneous analysis of nuclei isolated from *F. summilusitana* collected in Serra do Marão (peak 1, 2*n* = 10*x* = 70) and *F. summilusitana* from population C1 (peak 2, 2*n* = 12*x* = 84). The mean channel number (Mean FL), DNA index (DI, i.e., mean channel number of sample / mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given.

Within sections *Festuca* and *Aulaxyper*, the observed chromosome numbers were highly positively correlated with the nuclear DNA content estimations (Fig. III.3.3). Regression coefficients (R^2) of 0.9929 and 0.9781 were obtained for sections *Festuca* and *Aulaxyper*, respectively. Linear regression analyses (y = 3.3183x - 3.0554, section *Festuca*; y = 3.6429x - 6.6568, section *Aulaxyper*) were used to estimate the DNA ploidy level of fescue species within each section. With exception of *F. rothmaleri*, these estimations were in agreement with our observations and with previously reported values. A tetraploid level was estimated for *F. ampla* ssp. *transtagana* (2n = 4x = 28).

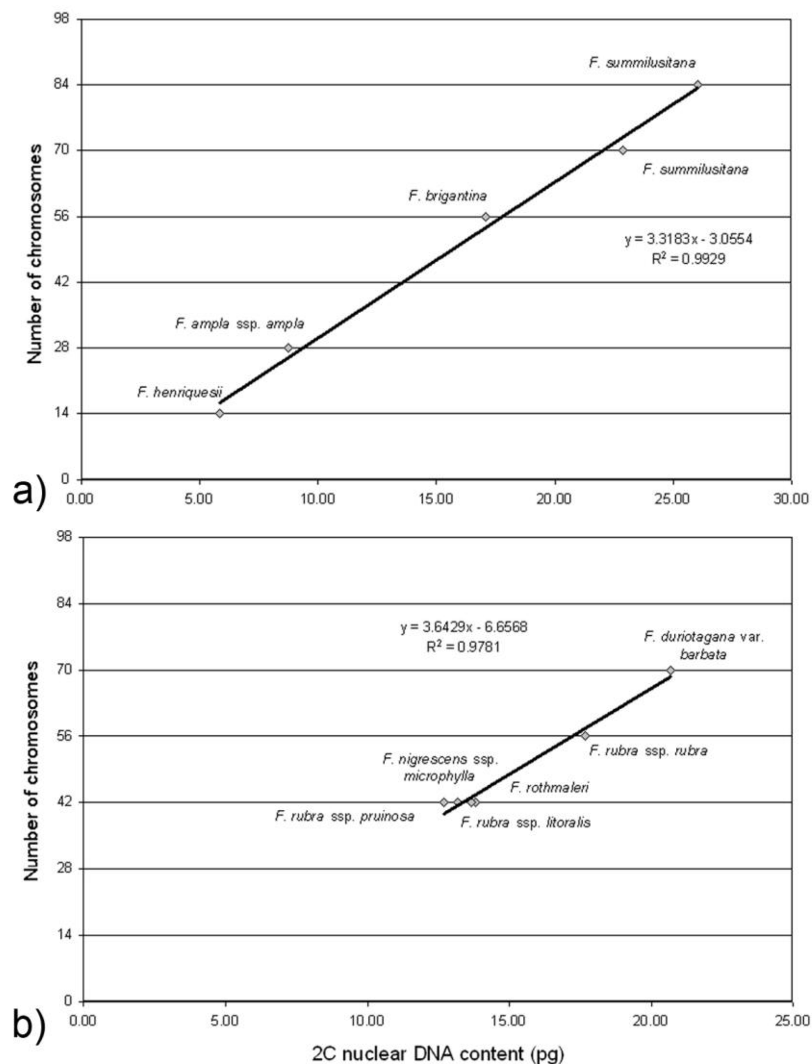

Fig. III.3.3 Linear regression analyses between mean nuclear DNA content and chromosome number of species belonging to sections *Festuca* (a) and *Aulaxyper* (b). The linear regression equation and coefficient (R^2) are provided in each graphic.

For each species, differences in nuclear DNA content among populations were not statistically significant (Table III.3.3). The only exception was observed in *F. summilusitana* where two ranges of non overlapping DNA values were obtained: plants growing on schist substrate presented 2C nuclear DNA estimations ranging from 20.50 to 24.00 pg (mean 2C value = 22.69 ± 0.617 pg of DNA) while plants from granite presented 2C values ranging from 24.02 to 27.19 pg (mean 2C value = 25.67 ± 0.754 pg of DNA) (Table III.3.4). These results were in accordance with the two observed chromosome numbers for this species ($2n = 10x = 70$ and $2n = 12x = 84$), with the lower values corresponding to individuals presenting the decaploid ploidy level and the higher ones, to dodecaploid individuals.

Table III.3.4 Nuclear DNA content estimations in *Festuca summilusitana*[1].

Population	Type of substrate	Nuclear DNA content			*n*
			2C range		
		2C (pg)	Min.	Max.	
Bragança: Serra da Nogueira	S	22.34 ± 0.431ab	21.80	22.89	5
Vila Real: Serra do Marão	S	22.39 ± 0.379ab	21.86	22.93	5
Aveiro: Serra do Caramulo – C1	G	25.42 ± 0.625c	24.81	26.57	6
Aveiro: Serra do Caramulo – C2	S	22.66 ± 0.286ab	22.30	23.06	9
Aveiro: Serra do Caramulo – C3	S	23.36 ± 0.684b	22.63	24.00	3
Aveiro: Serra da Freita	S	23.23 ± 0.425b	22.59	23.62	5
Coimbra: Serra da Lousã – L1	S	22.93 ± 0.614ab	21.38	23.39	9
Coimbra: Serra da Lousã – L2	S	23.06 ± 0.517ab	22.08	23.70	7
Coimbra: Serra da Lousã – L3	S	23.25 ± 0.444b	22.44	23.80	7
Coimbra: Serra da Lousã – L4	S	23.07 ± 0.251ab	22.69	23.29	5
Guarda: Serra da Estrela – E1	G	25.37 ± 0.523c	24.02	26.24	30
Guarda: Serra da Estrela – E2	G	26.78 ± 0.318d	26.40	27.18	5
Guarda: Serra da Estrela – E3	G	26.72 ± 0.367d	26.35	27.19	5
Guarda: Serra da Estrela – E4	S	22.28 ± 0.561a	20.50	23.44	28
	S	**22.69 ± 0.617**	**20.50**	**24.00**	**83**
	G	**25.67 ± 0.754**	**24.02**	**27.19**	**46**

[1] The values are given as mean and standard deviation of the mean (SD) of the nuclear DNA content (2C in pg) for individuals of each population. Means followed by the same letters (a, b, c or d) are not significantly different at $P \leq 0.05$ according to the Tukey-Kramer multiple comparison test. The DNA range, defined by the minimum (Min.) and maximum (Max.) value obtained for individuals in each population and the number of analysed individuals (*n*) are also provided. For each population the type of substrate where fescue individuals were growing is given (S – schist, G – granite). In bold, the mean and SD values of nuclear DNA content, the DNA range and the number of individuals according with the type of substrate are given. Please note that there are no overlapping values between individuals of each type of substrate.

Fluorescence in situ *hybridization*

Chromosome morphology analysis using FISH was successfully performed for some of the species (*F. henriquesii*, *F. ampla* ssp. *ampla* and *F. brigantina*), where well defined patterns of rDNAs signals were obtained. For others (*F. summilusitana*, *F. rubra* ssp. *litoralis*, *F. duriotagana* and *F. paniculata* ssp. *multispiculata*), the application of this technique was less successful, as a variable number of rDNA signals was obtained with doubts remaining on their exact number. For *F. henriquesii*, three pairs of chromosomes carrying 45S rDNA were found, one of which was linked with 5S rDNA (Figure III.3.1b). In *F. ampla* ssp. *ampla*, a similar pattern to *F. henriquesii* was obtained (Figure III.3.1c). In *F. brigantina* four pairs of chromosomes carried both 45S and 5S rDNAs and one pair contained only the 5S rDNA locus. For these three *taxa*, the location and number of the signals were reproducible. In *F. summilusitana*, while one chromosome pair with 5S rDNA

was consistently found, a variable number of chromosomes carrying both 45S and 5S rDNAs (8 to 11 signals; Fig. III.3.1d and e, respectively) was observed. A similar variability was found in *F. paniculata* ssp. *multispiculata*, where two pairs of chromosomes with 5S rDNA were constantly found, and a variable number of 6 to 8 chromosomes containing the 45S rDNA was obtained (*e.g.*, Fig. III.3.1f, where seven 45S rDNA signals can be observed). In most of the spreads of *F. duriotagana*, three pairs of chromosomes carried 45S and 5S rDNAs and another three contained only the 45S rDNA locus (Fig. III.3.1g). Nevertheless, in some cases, a different pattern of signals was observed: two pairs of chromosomes carrying 45S and 5S rDNAs, two pairs carrying only the 45S and another two containing only the 5S locus. In *F. rubra* ssp. *pruinosa*, whereas the most common pattern consisted on one pair of chromosomes carrying both 45S and 5S rDNAs, another pair containing 5S rDNA and two chromosome pairs carrying the 45S rDNA, in some spreads the 45S signal was only found in three chromosomes.

Genomic in situ *hybridization*

Genomic *in situ* hybridization with hybridization of total genomic DNA of *F. henriquesii* on chromosome spreads of *F. ampla* ssp. *ampla*, *F. brigantina*, *F. summilusitana*, *F. paniculata* ssp. *multispiculata*, *F. rubra* ssp. *litoralis* and *F. duriotagana*, was in most cases unsuccessful. While it was clear that no hybridization occurred with chromosomes of *F. paniculata* ssp. *multispiculata*, in other species a specific number of unexpected and highly localised hybridization signals was obtained (Fig. III.3.1h and I), with no evident hybridization in the remaining parts of the chromosomes.

Discussion

Cytologically, *Festuca* is a diversified genus that presents ploidy levels ranging from diploid ($2n = 2x = 14$) to dodecaploid ($2n = 12x = 84$), with about 74% of the species occurring as polyploids (Seal 1983). Eurasia appears as a primary diversification centre of this genus, with the occurrence of a higher percentage of species with lower ploidy levels (especially, diploid and tetraploid; Dubcovsky and Martínez 1992). Nevertheless, whereas this is clearly evident for species belonging to the Spanish flora with a predominance of the diploid level in most sections (mostly distributed in the eastern half of Iberian Peninsula) (de la Fuente and Ortúñez 2001; de la Fuente *et al.* 2001), in the Portuguese continental Flora only one diploid species is recognized, *i.e.*, *F. henriquesii*. The diploid level of this species was confirmed by our work and, as suggested for other diploid species, it seems that this *taxon* presents ancient and stable characters (Ferrero and de la

Fuente 1996; de la Fuente *et al.* 2001). Ortúñez and de la Fuente (2004) suggested a correlation between ploidy level and geographical distribution, with diploid species being orophilous and having a restricted distribution in the Iberian Peninsula, whereas species of higher ploidy levels present a wider distribution area. This is the case of *F. henriquesii*, an endemic *taxa* from Serra da Estrela and Serra do Gerês, Peneda and Barroso (Ferrero and de la Fuente 1996). Nevertheless, species from section *Festuca* with higher ploidy levels, as *F. summilusitana*, are also restricted to a reduced area of distribution.

Two ploidy levels, tetraploid and hexaploid, are currently assigned to *F. ampla*, with the lower level corresponding to populations in the boundaries of distribution and the higher one to populations in the central area (de la Fuente *et al.* 2001). However, in our work we were only able to detect tetraploid individuals, despite having analysed one population in each of the suggested areas of distribution. The chromosome number of *F. rothmaleri* estimated in our work (2n = 6x = 42) differed from the reported ploidy level for this species, *i.e.*, octoploid (Al-Bermani *et al.* 1992). Little information on the studied specimens was provided by the authors and in light with the data provided in the study of de la Fuente *et al.* (2001), where hexaploid specimens of *F. ampla* were recognized as *F. rothmaleri*, it seems that the occurrence of the hexaploid level in *F. rothmaleri* should not be discarded. Nevertheless, further studies should be done to confirm the ploidy level of this species.

The chromosome number of *F. summilusitana* has also been subject of constant updates and rectifications over the years. This species after being first identified as hexaploid (Ortúñez and de la Fuente 1995) was recently given a decaploid level by de la Fuente and Ortúñez (2000) and de la Fuente *et al.* (2001). In our work, two ploidy levels were obtained both by chromosome counting and FCM estimation: decaploid ($2n = 2x = 70$) and dodecaploid ($2n = 2x = 84$). Whereas our results are in accordance with those of de la Fuente *et al.* (2001) for the populations of Serra do Marão, Serra da Nogueira and Serra da Estrela ($2n = 10x = 70$), for the last locality in populations of granite substrate a dodecaploid level was found. Dodecaploid populations were also found in granite substrate of Serra do Caramulo. The occurrence of such a high ploidy level in *Festuca* genus is almost new, as until now it was only reported for *F. gamisansii* ssp. *gamisansii* (Portal 1999). Nevertheless, the accuracy of this estimation was questioned by Foggi *et al.* (2005).

The chromosome number of *F. duriotagana* was first determined in this work ($2n = 10x = 70$) and although such a high ploidy level has already been reported for another species of this section (*F. nevadensis*; Galland 1988), it can be an important character to clarify

the *taxon*omic position of this *taxon*. Some authors suggested the inclusion of individuals of *F. duriotagana* on the plastic variability of *F. ampla* (section *Festuca*; Anonymous 2006), while others had no doubt on classifying it as a separate *taxa* of section *Aulaxyper* (Al-Bermani *et al.* 1992; Cebolla Lozano and Rivas Ponce 2003). The chromosome number determined in this work seems to sustain the latter hypothesis, as such a high number of chromosomes have never been found for individuals of *F. ampla*.

Flow cytometry was only sporadically used for estimating the genome size of fescue species (Huff and Palazzo 1998; Arumuganathan *et al.* 1999). Huff and Palazzo (1998) studied the nuclear DNA content of 10 fescue species belonging to sections *Festuca* and *Aulaxyper* and also obtained a positive correlation between DNA contents and chromosome numbers. In species of the same section and for each ploidy level, mean DNA content estimations obtained by Huff and Palazzo (1998) were generally lower (up to 17%) than those obtained in our work. Differences in nuclear DNA estimations among laboratories are commoner than expected (Doležel *et al.* 1998) and can be justified by the use of different methodologies and reference standards [*e.g.*, Huff and Palazzo (1998) used chicken red blood cells with 2.33 pg/2C of DNA as reference standard]. In a recent study by Šmarda and Bureš (2006) intraspecific DNA content variability in *F. pallens* has been documented with authors suggesting that the differences in relative genome size were correlated with geographical coordinates. Differences in the amount of DNA content among populations were also observed by Huff and Palazzo (1998). These authors suggested that B chromosomes could be responsible for some of the reported variation. With exception of *F. summilusitana*, our data was highly homogeneous among populations. The supposed intraspecific variability observed in *F. summilusitana* was seen to be related with the occurrence of two ploidy levels in this species. The apparent preference of each “conspecific” ploidy race to a particular type of substrate (granite and schist) and the importance of the chromosome number in species delimitation within this genus suggests a possible separation of *F. summulistana* in two independent *taxa*. Morphoanatomic studies, necessary to test this hypothesis are already under development in our laboratory.

The analysis of the monoploid genome size revealed that, usually, species with higher ploidy levels presented a reduction in DNA amount relative to species with lower ploidy levels. A similar observation was obtained by Seal (1983) who measured the nuclear DNA content of several *Festuca* species using Feulgen densitometry. Recently, the loss of DNA after polyploid formation, *i.e.*, genome downsizing, was suggested as a widespread phenomenon of considerable biological significance (Leitch and Bennett 2004). Possible

explanations for this phenomenon include the elimination of non-coding DNA sequences during polyploid formation (*e.g.*, Shaked *et al.* 2001). Seal (1983) also compared for each ploidy level the mean DNA contents within each section and observed differences among *taxa* from four sections. As in our work, this author observed that species from section *Schenodorus* presented higher mean DNA content than species from section *Aulaxyper*. Despite the interesting results obtained here regarding genome downsizing in polyploid species of *Festuca* and the remarkable nuclear DNA content differences obtained among sections of this genus, an extensive survey is still required to fully understand the patterns of genome size evolution in this genus.

Until this moment only three diploid species have been identified as progenitors of polyploid fescue species. *F. pratensis*, as a progenitor of hexaploid *F. arundinaceae*, was the first species to be identified, by means of restriction fragment length polymorphism (RFLP; Xu and Sleper 1994) and GISH (Humphreys *et al.* 1995). Recently, Harper *et al.* (2004) used FISH to confirm the ancestor character of *F. scariosa* and suggested that *F. altissima* was a good candidate for some polyploid species from section *Schenodorus*. The ancient and paleoendemic character of the diploid species *F. henriquesii*, suggested that it could be a good candidate for an ancestor of some polyploid species from section *Festuca*, where it is included. Indeed, FISH analyses seemed to point out in that direction as the number and distribution of rDNA sites were very similar between *F. henriquesii* and *F. ampla*. In species with higher ploidy levels, *i.e.*, *F. brigantina* and *F. summilusitana*, this was not so evident, as the two pairs of chromosomes carrying the 45S rDNAs found in *F. henriquesii* were absent. The pattern of rDNA sites was similar between *F. brigantina* and *F. summilusitana*, with the only exceptions being some *F. summilusitana* individuals that presented a higher and variable number of chromosomes carrying both 45S and 5S rDNAs. Variation in the number and position of rDNA sites has been already observed in plants, but most of the works reported changes in the position of 45S and 5S rDNAs, and not in their number (*e.g.*, Schubert and Wobus 1985). Doubts remained on whether this variation was real or if it was due to technical problems of the FISH procedure. Even so, it was evident that species from *Festuca* section presented different rDNA patterns from those of *Subbulbosae* and *Aulaxyper*. Chromosomes of *F. paniculata* ssp. *multispiculata* were clearly larger than those of other species which is in accordance with the FCM data. The number and distribution of rDNA sites was also very different from any other species, as no chromosomes carrying both rDNAs were observed. A variable number of 45S rDNAs was observed, with most of the genotypes being heterozygous with an extra seventh site on one chromosome. An odd number of 45S rDNA was also observed in

Lolium perenne and *L. rigidum* var. *rigidum*, but doubts remained on the nature of these additional sites (Thomas *et al.* 1996). Some variation on the number of rDNA sites was also observed in *F. rubra* ssp. *litoralis* and *F. duriotagana*. This and the high number of chromosomes present in some of the species made the analysis and interpretation of the results a difficult task, and one could consider that in this case the application of FISH methodology to wild species of *Festuca* has not been as successful as expected. Nevertheless, this method has been of utmost importance in phylogeny studies of some fescue species (Thomas *et al.* 1997) and it was already applied with success in wild diploid species from section *Montanae* (Harper *et al.* 2004).

The application of GISH to study possible phylogenetic relationships in wild fescue species revealed some unexpected results. An unpredictable and without explanation homology with whole chromosomes, excluding the telomeric regions, was found when total genomic DNA of *F. henriquesii* was hybridized with *F. ampla* ssp. *ampla* chromosomes. With the exception of *F. paniculata* ssp. *multispiculata* chromosomes, to which there was no hybridization, hybridization with other species resulted in a specific pattern of highly localised hybridization signals, possibly related to heterochromatin. Takahashi *et al.* (1999) also obtained highly localized signals on maize chromosomes, when genomic DNA from four wild relatives was used as a probe. The lack of hybridization in *F. paniculata* ssp. *multispiculata* seems to confirm the previously established separation of these two species in separate and distant sections. Despite of some uncertainties, it seems that either the studied fescue species evolved so much that there are very few homologous zones remaining between them and *F. henriquesii*, or that this species is not an ancestral progenitor of the studied polyploid *Festuca* species. Therefore, further phylogenetic studies should focus on the remaining diploid species present in the Iberian Peninsula.

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Chapter IV

Optimization of methodologies used in the isolation of plant nuclei for flow cytometry

IV.1 Comparison of four nuclear isolation buffers for plant DNA flow cytometry

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Abstract

Background and Aims DNA flow cytometry requires preparation of suspensions of intact nuclei, which are stained using a DNA-specific fluorochrome prior to analysis. Various buffer formulas were developed to preserve nuclear integrity, protect DNA from degradation and facilitate its stoichiometric staining. Although nuclear isolation buffers differ considerably in chemical composition, no systematic comparison of their performance has been made until now. This knowledge is required to select the appropriate buffer for a given species and tissue.

Methods Four common lysis buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$) were used to prepare samples from leaf tissues of seven plant species (*Sedum burrito*, *Oxalis pes-caprae*, *Lycopersicon esculentum*, *Celtis australis*, *Pisum sativum*, *Festuca rothmaleri* and *Vicia faba*). The species were selected to cover a wide range of genome sizes (1.30 – 26.90 pg per 2C DNA) and a variety of leaf tissue types. The following parameters were assessed: forward (FS) and side (SS) light scatters, fluorescence of propidium iodide-stained nuclei, coefficient of variation of DNA peaks, presence of debris background and the number of nuclei released from sample tissue. The experiments were performed independently by two operators and repeated on three different days.

Key results Clear differences among buffers were observed. With the exception of *O. pes-caprae*, any buffer provided acceptable results for all species. LB01 and Otto's were generally the best buffers, with Otto's buffer providing better results in species with low DNA content. Galbraith's buffer led to satisfactory results and Tris.$MgCl_2$ was generally the worst, although it yielded the best histograms in *C. australis*. A combined analysis of FS and SS provided a “fingerprint” for each buffer. The variation between days was more significant than the variation between operators.

Conclusions Each lysis buffer tested responded to a specific problem differently and none of the buffers worked best with all species. These results expand our knowledge on nuclear isolation buffers and will facilitate selection of the most appropriate buffer depending on species, tissue type and the presence of cytosolic compounds interfering with DNA staining.

Key words Angiosperms; flow cytometry; genome size; lysis buffers; nuclear DNA content; nuclear isolation buffers; propidium iodide; stoichiometric error

Introduction

Flow cytometry (FCM) was developed in the 1950s but its application to plant sciences was delayed until the late 1980s when it became an important technique for estimation of nuclear DNA content, determination of DNA ploidy level and cell cycle analysis (Galbraith 2004; Shapiro 2004; Bennett and Leitch 2005). This delay was mainly due to problems with the preparation of suspensions of intact nuclei from thick tissues consisting of cells with a rigid cell wall. In his pioneering work, Heller (1973) used hydrolytic enzymes to digest cell walls and release nuclei from fixed tissues. The method was time consuming and rarely followed by others. Among later investigators, Ulrich and Ulrich (1986), Ulrich *et al.* (1988) and Bergounioux *et al.* (1988, 1992) employed a modified approach in which cell nuclei were released after hypotonic lysis of intact protoplasts. In addition to being time consuming, the need for intact protoplasts limited the application of this protocol to some species and certain types of tissues. As an alternative, Galbraith *et al.* (1983) developed a rapid and convenient method for isolation of plant nuclei by chopping plant tissues in a lysis buffer. Since then, this has been the main and most reliable method for nuclear isolation in plant FCM.

In addition to releasing nuclei from intact cells, lysis buffers must ensure the stability of nuclei throughout the experiment, protect DNA from degradation and facilitate stoichiometric staining. Since the late 1980s, some laboratories have developed their own buffer formulas. As a result, about 25 different lysis buffers are known, although only eight are commonly used. Their chemical composition varies, but it usually includes chromatin stabilizers (*e.g.*, $MgCl_2$, $MgSO_4$, spermine); chelator agents [*e.g.*, ethylenediaminetetraacetic acid (EDTA), sodium citrate] to bind divalent cations, which serve as nuclease cofactors; inorganic salts (*e.g.*, KCl, NaCl) to achieve proper ionic strength; organic buffers [*e.g.*, 3-(N-morpholino) propanesulfonic acid (MOPS), tris-(hydroxymethyl)-aminomethane (TRIS) and 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid (HEPES)] to stabilize the pH of the solution (usually set between 7.0 and 8.0); and non-ionic detergents (*e.g.*, Triton X-100, Tween 20) to release nuclei, disrupt chloroplasts as fluorescent entities, remove and hinder cytoplasmatic remnants from nuclei surface, and decrease the aggregation affinity of nuclei and debris (Coba de la Peña and Brown 2001; Doležel and Bartoš 2005).

Given the different chemical composition and diversity of plant tissues, it may be expected that each buffer would perform differently. This problem was exacerbated by the recent observation of the presence of cytosolic compounds that are released during nuclei isolation. These compounds interact with nuclear DNA and/or the fluorochrome, affecting

sample quality and causing stoichiometric errors in DNA staining (Noirot *et al.* 2000, 2003; Pinto *et al.* 2004; Walker *et al.* 2006; Chapter IV.2). In Chapter IV.2 it was observed that nuclei of *Pisum sativum* and *Zea mays* responded differently to tannic acid, a common phenolic compound in plants, when isolated in different nuclear isolation buffers. However, no systematic comparison of lysis buffers has been made until now.

We set out to compare four of the most common buffers that differ in chemical composition: Galbraith's buffer (Galbraith *et al.* 1983), LB01 (Doležel *et al.* 1989), Otto's buffer (Otto 1992; Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser *et al.* 1995). We evaluated light scatter and fluorescence properties of nuclei in suspension, the presence of debris background and the number of nuclei released from sample tissue. Samples were prepared from leaf tissue of seven plant species that cover a wide range of genome sizes (1.30 – 26.90 pg per 2C DNA) and whose tissues differ in structure and chemical composition (Table IV.1.1). *Pisum sativum* (Fabaceae), *Lycopersicon esculentum* (Solanaceae) and *Vicia faba* (Fabaceae) are common DNA reference standards for FCM; *Sedum burrito*, a species from Crassulaceae, has fleshy leaves with many reserve substances; *Oxalis pes-caprae* (Oxalidaceae) has an acidic cell sap (Castro *et al.* 2007); *Celtis australis* (Ulmaceae) releases mucilaginous compounds after tissue homogenization (Rodriguez *et al.* 2005*a*; Chapter III.2); and *Festuca rothmaleri* (Poaceae) has rigid leaves that are difficult to chop (Chapter III.3). The effect of instrumental drift and operator were also evaluated. The main goal of the study was to provide data that facilitate selection of the appropriate buffer and to propose strategies to minimize common problems in plant DNA flow cytometry.

Table IV.1.1 Nuclear DNA content of the seven plant species used in this study.

Species	Family	Nuclear DNA content (pg/2C)	Reference
Sedum burrito	Crassulaceae	1.30	This work
Oxalis pes-caprae	Oxalidaceae	1.37	Castro *et al.* 2007
Lycopersicon esculentum cv. Stupické	Solanaceae	1.96	Doležel *et al.* 1992
Celtis australis	Ulmaceae	2.46	Rodriguez *et al.* 2005*a*; Chapter III.2
Pisum sativum cv. Ctirad	Fabaceae	9.09	Doležel *et al.* 1989
Festuca rothmaleri	Poaceae	13.67	Chapter III.3
Vicia faba cv. Inovec	Fabaceae	26.90	Doležel *et al.* 1992

Materials and methods

Plant material

Plants of *Lycopersicon esculentum* 'Stupické', *Pisum sativum* 'Ctirad' and *Vicia faba* 'Inovec' were grown from seeds. Plants of *Festuca rothmaleri* and *Oxalis pes-caprae* were kindly provided by Prof. Paulo Silveira and Dr. Sílvia Castro (Department of Biology, University of Aveiro, Portugal), respectively. Plants of *Sedum burrito* were obtained from Flôr do Centro Horticultural Centre (Mira, Portugal). All plants were maintained in a greenhouse at 22 ± 2°C, with a photoperiod of 16 h and a light intensity of 530 ± 2 μmol m^{-2} s^{-1}. Leaves of *Celtis australis* were collected directly from field-growing trees in Aveiro, Portugal.

Sample preparation

Approximately 40–50 mg of young leaf tissue was used for sample preparation. The amount of material required to release a sufficient number of nuclei in *S. burrito* had to be increased to approximately 500 mg due to the fleshy nature of the leaves. Nuclei suspensions were prepared according to Galbraith *et al.* (1983). Four common nuclear isolation buffers (Doležel and Bartoš 2005) were used to prepare samples (Table IV.1.2). One millilitre of nuclei suspension was recovered and filtered through a 50 μm nylon filter to remove cell fragments and large debris. Nuclei were stained with 50 μg mL^{-1} propidium iodide (PI; Fluka, Buchs, Switzerland), and 50 μg mL^{-1} RNase (Sigma, St Louis, MO, USA) was added to the nuclear suspension to prevent staining of double-stranded RNA. Samples were incubated on ice and analysed within 10 min.

Table IV.1.2 Four nuclear isolation buffers most frequently used in plant DNA flow cytomety.

Buffer	Composition[1]	Reference
Galbraith	45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0	Galbraith *et al.* 1983
LB01	15 mM TRIS, 2 mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0[2]	Doležel *et al.* 1989
Otto[3]	Otto I: 100 mM citric acid, 0.5% (v/v) Tween 20 (pH 2-3) Otto II: 400 mM $Na_2PO_4.12H_2O$ (pH 8-9)	Otto 1992; Doležel and Göhde 1995
Tris.$MgCl_2$	200 mM TRIS, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5	Pfosser *et al.* 1995

[1] Final concentrations are given

[2] The buffer formula contains 15 mM ß-mercaptoethanol. However, as the other buffers were used without additives suppressing the negative effect of phenols and other cytosolic compounds, LB01 was used without ß-mercaptoethanol in this study.

[3] pH of the buffers is not adjusted. The nuclei are isolated in Otto I buffer; DNA staining is done in a mixture of Otto I and Otto II (1:2) with a final volume of 1 mL.

Flow cytometric analyses

Samples were analysed in a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. The results were acquired using the SYSTEM II software version 3.0 (Beckman Coulter®). Prior to analysis, the instrument was checked for linearity with fluorescent beads (Beckman Coulter®), and the amplification settings were kept constant throughout the experiment.

The following parameters were evaluated in each sample: forward light scatter (FS, to estimate relative size of particles), side light scatter (SS, to estimate relative optical complexity of particles), relative fluorescence intensity of PI stained nuclei (FL), half peak coefficient of variation (CV) of the G_0/G_1 peak (to estimate nuclei integrity and variation in DNA staining), a debris background factor (DF, to assess sample quality) and a nuclear yield factor (YF, to compare the amount of nuclei in suspension independently of the amount of leaf tissue used).

CV (%) was calculated using the following formula:

$$\text{Half peak CV (\%)} = \frac{42.46 * \text{width of peak at half the peak height}}{\text{peak position}}$$

DF (%) was calculated as follows:

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

DF increases as debris increases.

YF (nuclei s^{-1} mg^{-1}) was calculated using the formula:

$$\text{YF (nuclei s}^{-1}\text{ mg}^{-1}\text{)} = \frac{\text{Total number of intact nuclei / number of seconds of run (s)}}{\text{Weight of tissue (mg)}}$$

The flow rate was defined as low and was kept constant throughout the experiment.

The analysis was performed on three different days and by two operators (labelled here as A and B). Five replicates were performed per operator for each buffer and in each replicate at least 5,000 nuclei were analysed. Histograms of FL obtained with the best and worst performing buffers were overlaid using WinMDI software (Trotter 2000, Fig. IV.1.1).

For *S. burrito*, nuclear genome size was estimated using *L. esculentum* cv. Stupické (2C = 1.96 pg DNA; Doležel *et al.* 1992) as internal reference standard according to the following formula:

S. burrito 2C nuclear DNA content (pg) =

$$\frac{S.\,burrito\ \ G_0/G_1\ \text{peak mean}}{L.\,esculentum\ \ G_0/G_1\ \text{peak mean}} \times 1.96$$

Conversion of mass values into base-pair numbers was achieved according to the factor 1 pg = 978 Mbp (Doležel *et al.* 2003).

Statistical analyses

Statistical analyses were performed using a three-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA) to assess for differences among buffers and dates and between operators. When treatments were significantly different, a Holm–Sidak multiple comparison test was used for pair-wise comparison. Hierarchical cluster analyses were performed using NCSS 2004 (Hintze 2004). Dendrograms highlighting dissimilarities among buffers and between operators were obtained using FS, SS, FL, CV, BF and YF. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) was followed in each species as it yielded the highest co-phenetic correlation coefficient.

Results

With the exception of *O. pes-caprae*, for which measurable samples were only obtained with Otto's and Galbraith's buffers, all buffers yielded acceptable histograms with all species tested. In any analysis, it was possible to isolate a reasonable number of nuclei (approximately 20–60 nuclei/s in a low-speed configuration), and obtain well-defined histograms with DNA peaks presenting acceptable CV values (< 5.0%; Galbraith *et al.* 2002; Fig. IV.1.1, Table IV.1.3). Table IV.1.3 indicates the best performing buffer(s) for each species. The selection criteria were the highest FL and YF values, and the lowest CV and DF values.

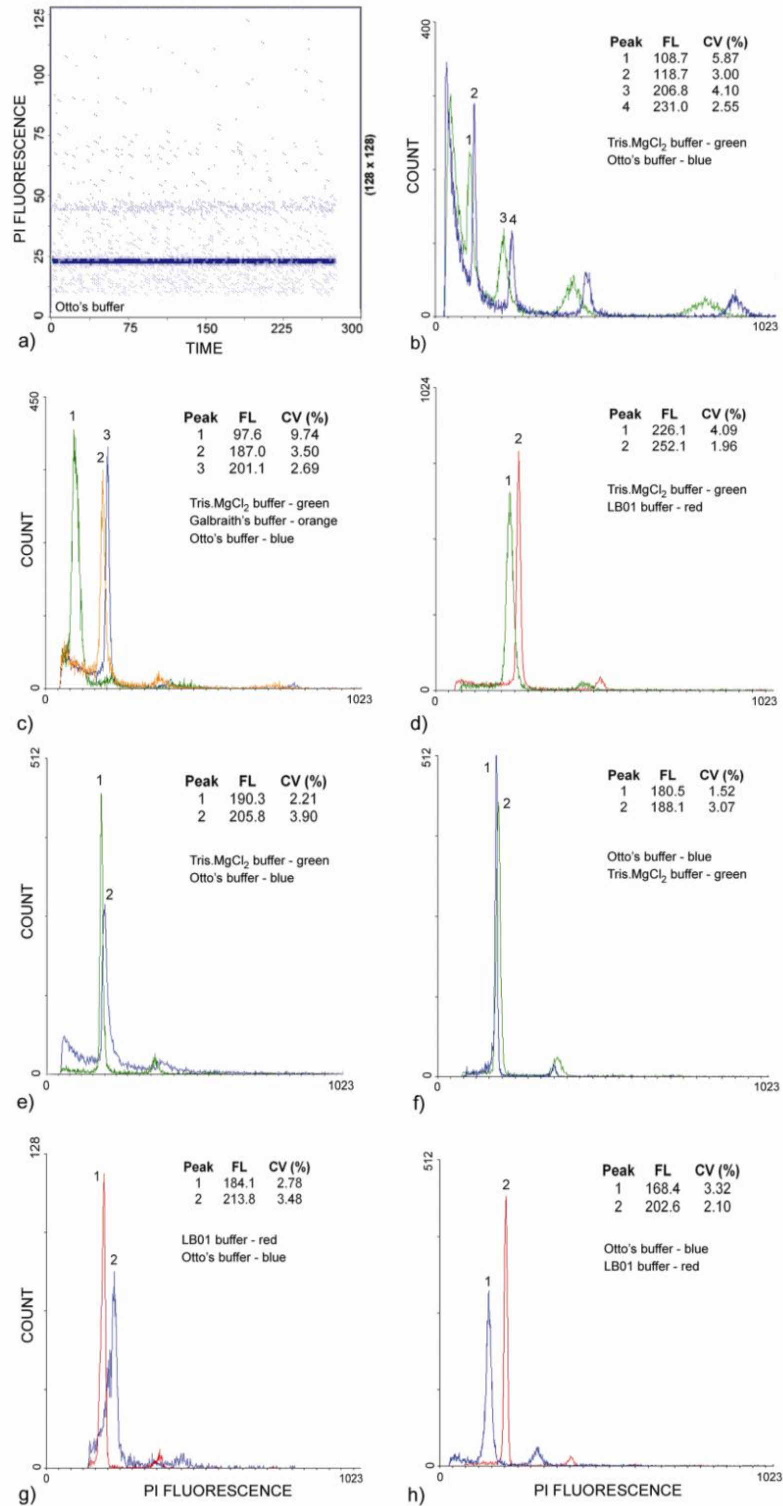

Fig. IV.1.1 Cytogram of fluorescence intensity (PI, relative fluorescence) *vs.* time of *Pisum sativum* nuclei isolated with Otto buffers (a) and histograms of PI relative fluorescence (b–h), which show overlays of distributions obtained with the best and worst performing buffer for each species: b) *Sedum burrito*; c) *Oxalis pes-caprae*; d) *Lycopersicon esculentum*; e) *Celtis australis*; f) *Pisum sativum*; g) *Festuca rothmaleri*; h) *Vicia faba*. Relative mean channel numbers (FL) and coefficients of variation (CV, %) of G_0/G_1 peaks are given. Four lysis buffers were compared: LB01 (red), Galbraith's (orange), Tris.$MgCl_2$ (green) and Otto's (blue).

Table IV.1.3 Flow cytometric parameters assessed in each species[1].

Species	Buffers	FS (channel units)		SS (channel units)		FL (channel units)		CV (%)		BF (%)		YF (nuclei s^{-1} mg^{-1})	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sedum burrito	LB01	57.66a	8.724	11.37a	2.448	110.9a	5.93	4.48a	0.407	69.15a	24.903	0.06a	0.004
	Galbraith	41.06b	5.171	5.36b	2.299	112.1a	2.57	3.59ab	0.396	60.02a	16.631	0.05b	0.004
	Tris.$MgCl_2$	57.50a	8.556	13.34a	2.670	103.0b	6.19	5.61c	1.000	68.85a	13.874	0.05ab	0.004
	Otto	**8.01c**	**2.179**	**2.58c**	**0.787**	**121.4c**	**3.93**	**3.27b**	**0.288**	**65.96a**	**6.33**	**0.05ab**	**0.006**
Oxalis pes-caprae	LB01	-	-	-	-	-	-	-	-	-	-	-	-
	Galbraith	171.33a	36.404	19.75a	6.920	178.5a	5.21	4.14a	0.778	7.05a	4.977	1.01a	0.364
	Tris.$MgCl_2$	-	-	-	-	-	-	-	-	-	-	-	-
	Otto	**26.32b**	**4.933**	**3.90b**	**0.641**	**200.7b**	**6.24**	**3.03b**	**0.415**	**8.96a**	**5.908**	**0.62b**	**0.125**
Lycopersicon esculentum	**LB01**	**15.42a**	**2.125**	**2.97a**	**0.494**	**257.9a**	**11.50**	**2.88a**	**0.880**	**21.21a**	**14.137**	**2.47a**	**1.512**
	Galbraith	21.53b	3.752	3.40a	0.713	208.5b	49.61	2.87a	0.712	16.87a	10.036	1.69ab	0.814
	Tris.$MgCl_2$	17.48a	4.970	4.76b	1.562	216.7b	27.27	3.70b	0.836	20.52a	12.918	1.72ab	1.745
	Otto	**3.68c**	**1.537**	**1.95c**	**0.766**	**269.9a**	**11.17**	**2.18c**	**0.266**	**28.49b**	**13.254**	**0.91b**	**0.617**
Celtis australis	**LB01**	**28.47a**	**4.847**	**11.45a**	**3.481**	**182.1a**	**13.13**	**3.02a**	**0.315**	**7.66a**	**3.565**	**0.50a**	**0.224**
	Galbraith	25.57a	1.842	4.54b	0.637	171.3b	7.76	2.87a	0.259	8.27a	3.445	0.35a	0.162
	Tris.$MgCl_2$	**41.71b**	**6.944**	**16.08c**	**2.157**	**180.3a**	**10.44**	**2.85a**	**0.419**	**9.10b**	**5.085**	**0.49a**	**0.291**
	Otto	8.20c	1.404	7.25b	1.787	192.8c	8.23	3.46b	0.405	23.96c	4.615	0.49a	0.200
Pisum sativum	**LB01**	**47.54a**	**1.550**	**10.87a**	**3.078**	**183.5a**	**5.06**	**2.81a**	**0.461**	**7.19a**	**2.823**	**2.33a**	**0.490**
	Galbraith	51.97a	3.624	5.29b	1.155	178.6a	3.36	3.02ab	0.465	6.34a	1.765	2.30a	0.876
	Tris.$MgCl_2$	59.37b	4.826	16.74c	2.914	177.7a	11.86	3.29b	0.515	6.39a	2.781	2.59a	0.894
	Otto	**5.72c**	**0.993**	**4.79b**	**1.083**	**190.1a**	**6.06**	**1.94c**	**0.180**	**9.85b**	**2.668**	**1.15b**	**0.367**
Festuca rothmaleri	**LB01**	**57.35a**	**1.825**	**8.57a**	**0.799**	**196.1a**	**6.07**	**3.24a**	**0.452**	**14.23ab**	**3.283**	**0.42a**	**0.182**
	Galbraith	61.03a	2.250	6.84b	0.649	182.7b	7.15	3.33ab	0.459	15.07a	1.974	0.27b	0.131
	Tris.$MgCl_2$	69.85a	1.603	17.72c	0.815	185.1b	8.06	3.66ab	0.312	17.30b	5.701	0.54a	0.195
	Otto	13.84b	2.846	8.55a	1.603	210.9c	6.77	3.76b	0.613	23.86c	12.992	0.11c	0.066
Vicia faba	**LB01**	**104.82a**	**6.021**	**11.61a**	**1.742**	**201.5a**	**4.45**	**2.40a**	**0.178**	**6.36a**	**2.326**	**0.87a**	**0.290**
	Galbraith	**114.55b**	**2.787**	**8.58b**	**1.306**	**196.9a**	**4.88**	**2.41a**	**0.148**	**5.80a**	**0.806**	**0.82a**	**0.235**
	Tris.$MgCl_2$	115.45b	5.194	20.36c	1.443	191.5a	9.10	2.91b	0.272	6.85a	4.609	1.30b	0.345
	Otto	21.56c	8.721	14.05a	7.589	184.2a	16.92	2.22a	0.532	4.35a	1.751	0.45c	0.186

[1] The values are given as mean and standard deviation of the mean (SD) of forward scatter (FS, channel units), side scatter (SS, channel units), fluorescence (FL, channel units), coefficient of variation of G_0/G_1 DNA peak (CV, %), background factor (BF, %) and nuclear yield factor (YF, nuclei s^{-1} mg^{-1}). Means followed by the same letter (a, b or c) are not statistically different according to the multiple comparison Holm-Sidak test at $P \leq 0.05$. Buffer(s) that performed best in each species are shown in bold.

Sedum burrito

This species was investigated because of its expected small genome size and fleshy leaves. FCM analysis revealed the occurrence of polysomaty, as demonstrated by the presence of discrete populations of nuclei with DNA contents of 2C, 4C, 8C, 16C and higher. In order to observe a higher number of endopolyploidy levels, instrument gain was set such that the 2C peak was approximately on channel 100. The 2C nuclear DNA content was estimated as 1.30 ± 0.09 pg (2C = 1,271 Mbp); this is the first estimate for this species (Table IV.1.1). Comparative analysis of the four buffers revealed that Otto's was the best in this species. In general, YF values were low (the lowest values from all the test species), whereas DF values were high (approx. 65.0%; Table IV.1.3, Fig. IV.1.1b).

Oxalis pes-caprae

Only Otto's and Galbraith's buffers provided acceptable results with this species. Otto's was clearly and significantly better than Galbraith's (Fig. IV.1.1c), with higher mean FL intensities and lower CV values (Table IV.1.3). Figure IV.1.1c also shows the histogram obtained after nuclear isolation with Tris.$MgCl_2$ buffer. In this case a G_0/G_1 peak with an unacceptable CV value (9.74%) and considerable loss of fluorescence was obtained. A similar result was obtained for nuclei isolated with LB01.

Lycopersicon esculentum

Acceptable results were obtained with all four buffers. Two buffer groups with statistically significant differences in FL were obtained. Samples prepared with Galbraith's and Tris.$MgCl_2$ buffers yielded lower mean FL values than their counterparts prepared with LB01 and Otto's buffers (Fig. IV.1.1d). The FL values were highly heterogeneous among buffers. Otto's and LB01 were the best buffers, with Otto's providing lower CV values and higher FL, but lower YF and higher BF than LB01 (Table IV.1.3).

Celtis australis

Low CV values (<3.0%) and low DF (<10.0%) were observed for this species. Nevertheless, it was not easy to obtain sufficient nuclei and the second lowest YF values were observed in this species. With regard to FL, only LB01 and Tris.$MgCl_2$ buffers were not statistically different, with nuclei isolated from Galbraith's buffer presenting the lowest mean FL and Otto's the highest mean FL. All parameters combined, Tris.$MgCl_2$ and LB01 were the best buffers, as nuclei in Tris.$MgCl_2$ presented the lowest CV values and similar FL intensity and YF values as in LB01 (Table IV.1.3, Fig. IV.1.1e).

Pisum sativum

In this species, all buffers performed reasonably well. The lowest FL intensities were obtained for nuclei isolated with Tris.$MgCl_2$, although no statistically significant differences were observed among the tested buffers. The best buffer for this species was Otto's (Table IV.1.3, Fig. IV.1.1f). Among the investigated species, *P. sativum* was the one with the highest YF.

Festuca rothmaleri

With the exception of CV values and YF, overall results for this species were satisfying with all four buffers tested. No statistically significant differences were found regarding the FL of nuclei isolated in Tris.$MgCl_2$ or Galbraith's buffers, as nuclei from both buffers presented low FL values. The best buffer for this species was LB01 (Table IV.1.3, Fig. IV.1.1g).

Vicia faba

This species gave the lowest CV and DF values among those tested. Generally, the results were very similar to those obtained for *P. sativum*. FL was similar for all the buffers, and no statistically significant differences were observed. Interestingly, *Vicia faba* was the only species for which Otto's was not the buffer with the highest FL (Fig. IV.1.1h). Despite low CV values of DNA peaks, this buffer gave the worst results, with the G_0/G_1 peak shifted towards the lower channels. This was due to fluorescence instability, which decreased over time. In all other species and with the remaining buffers, FL was stable after 10 min of incubation with PI (Fig. IV.1.1a). Results obtained with LB01 and Galbraith's buffers were similar and the best for this species (Table IV.1.3).

Analysis of FS and SS

Generally, FS and SS values differed considerably among the test buffers. Nevertheless, in most of the species, analysis of scatter parameters revealed that two of the four buffers were more similar than the others, and no statistically significant differences were observed between them. Interestingly, and with the exception of *S. burrito*, buffers that had similar FS mean values were not those that had similar SS values. This can be seen on cytograms of FS *vs.* SS obtained in *P. sativum* (Fig. IV.1.2). In this species and for FS, no statistically significant differences were observed between LB01 and Galbraith's buffers; for SS, no difference was observed for Galbraith's and Otto's buffers. Interestingly,

simultaneous analysis of FS and SS resulted in a species-specific pattern that could be used as a “fingerprint” of each buffer.

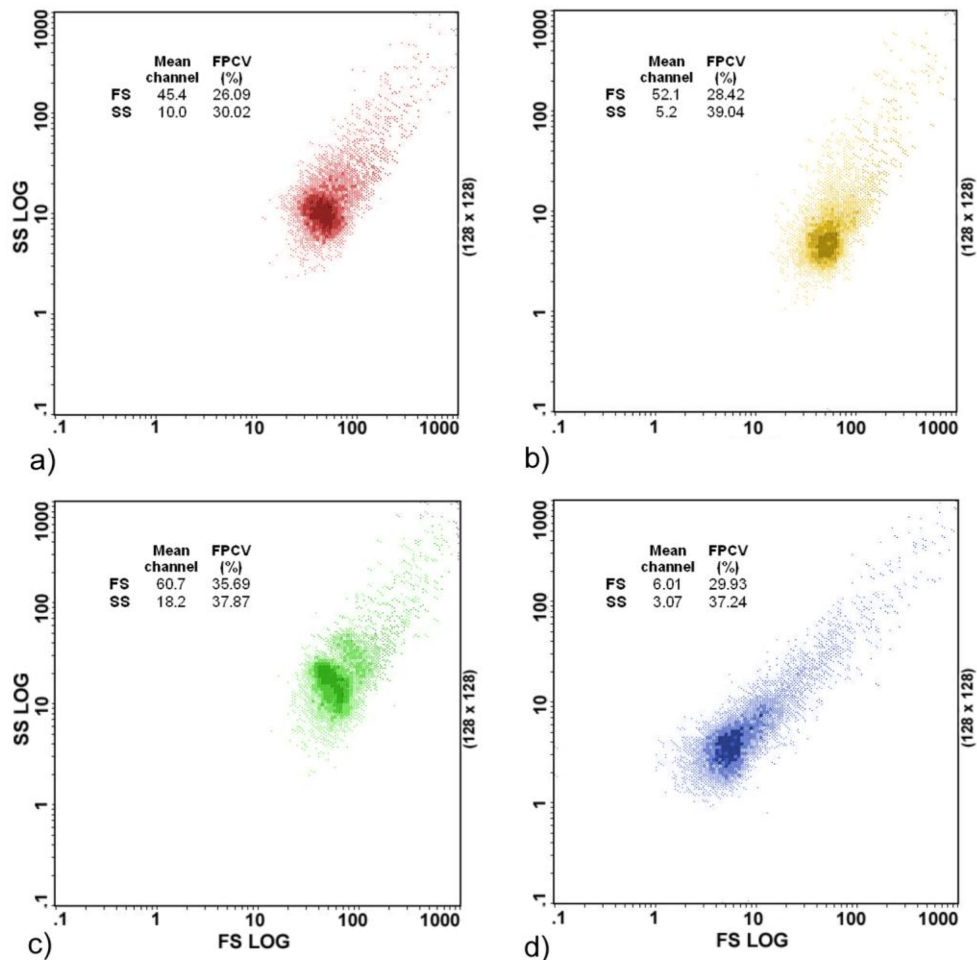

Fig. IV.1.2 Cytograms of forward scatter (logarithmic scale, FS LOG) *vs.* side scatter (logarithmic scale, SS LOG) obtained after the analysis of *Pisum sativum* nuclei isolated with four lysis buffers: a) LB01; b) Galbraith's; c) Tris.$MgCl_2$; and d) Otto. The mean channel number (Mean channel) and full peak coefficient of variation (FPCV, %) are given for both parameters. Note that the patterns of distributions are characteristic for each buffer.

Effect of operator and date of analysis

In most cases, no statistically significant differences were observed between operators and dates of analysis. Operators provided more homogeneous results than dates; in the former, statistically significant differences were observed only for BF and YF in more than one species. Significant differences between the dates of analysis were detected in some species for FS, SS, CV and BF. With regard to FL, one of the most important parameters in FCM analyses, significant among-day differences were detected only in *C. australis*,

and differences between operators occurred only in *L. esculentum*. The two species more susceptible to differences were *L. esculentum* and *V. faba* (Table IV.1.4).

Table IV.1.4 Three-way ANOVA analysis of the dates (D) and operators (O) for the parameters evaluated on each species (forward scatter, FS; side scatter, SS; fluorescence, FL; coefficient of variation of the G_0/G_1 DNA peak, CV; background factor, BF; and nuclear yield factor, YF). n.s. – not significantly different; s. – significantly different at $P \leq 0.05$.

Species	FS		SS		FL		CV		BF		YF	
	D	O	D	O	D	O	D	O	D	O	D	O
S. burrito	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	s.	n.s.	n.s.	n.s.
O. pes-caprae	s.	n.s.	s.	n.s.	n.s.	n.s.	s.	n.s.	n.s.	n.s.	n.s.	n.s.
L. esculentum	s.	n.s.	n.s.	n.s.	n.s.	s.	s.	n.s.	s.	s.	s.	s.
C. australis	n.s.	n.s.	n.s.	n.s.	s.	n.s.	n.s.	n.s.	s.	n.s.	n.s.	n.s.
P. sativum	n.s.	n.s.	s.	n.s.	n.s.	n.s.	s.	n.s.	s.	n.s.	n.s.	n.s.
F. rothmaleri	s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	s.	n.s.	n.s.
V. faba	n.s	s.	s.	s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	s.	s.

Hierarchical cluster analysis

With the exception of the results for *S. burrito*, the four buffers fell into two highly dissimilar and consistent clusters; one with Otto's buffer and the other with the remaining buffers. In *S. burrito* (Fig. IV.1.3a), one cluster was formed with Otto's and Galbraith's buffers, while the other comprised Tris.$MgCl_2$ and LB01 buffers. In *C. australis* (Fig. IV.1.3c), Galbraith's buffer was more similar to LB01 and Tris.$MgCl_2$ buffers than to Otto's buffer; in addition, "Tris.$MgCl_2$ A" was more similar to LB01 than to "Tris.$MgCl_2$ B". In *P. sativum*, "Galbraith's A" was more related to LB01 than to "Galbraith's B" (Fig. IV.1.3d), whereas for *F. rothmaleri*, "LB01 A" was more similar to Galbraith's than to "LB01 B" (Fig. IV.1.3e). In *L. esculentum*, two groups were formed from the second cluster: Tris.$MgCl_2$ and "Galbraith A" formed one group and LB01 and "Galbraith B" formed the other (Fig. IV.1.3b). In *V. faba* (Fig. IV.1.3f), LB01 and Galbraith's formed one group owing to greater similarities between operators than within each buffer. As previously stated, in *O. pes-caprae* only two buffers (Otto's and Galbraith's) provided acceptable results, with considerable dissimilarities between them (data not shown).

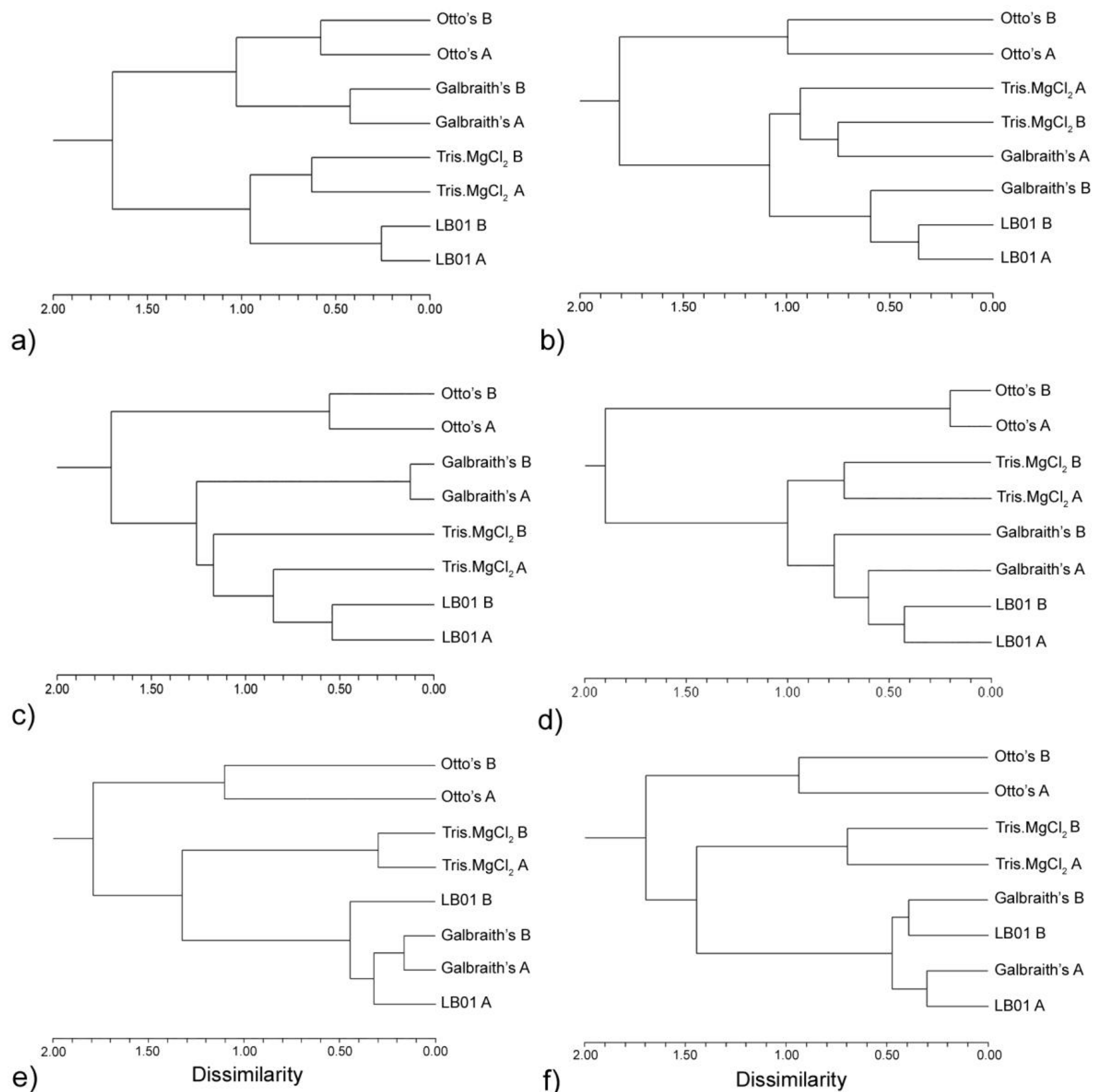

Fig. IV.1.3 Dendrograms obtained after hierarchical cluster analysis of the following species: a) *Sedum burrito*; b) *Lycopersicon esculentum*; c) *Celtis australis*; d) *Pisum sativum*; e) *Festuca rothmaleri*; and f) *Vicia faba*, according to the parameters FS, SS, FL, CV, BF and YF. With the exception of *S. burrito*, the four buffers fell into two highly dissimilar clusters of the same buffers.

Discussion

Four nuclear isolation buffers were used with a set of species that were chosen to represent different types of leaf tissues and different nuclear DNA content (1.30 – 26.90 pg per 2C DNA). As expected, popular DNA reference standards (*P. sativum*, *V. faba* and *L. esculentum*) were easy to work with. Nevertheless, not all buffers worked well with *L. esculentum*, possibly owing to the presence of cytosolic compounds. However, as the aim

of the study was to compare the performance of basic buffer formulas, the use of additives that could counteract the negative effects of cytosol was avoided.

Overall, the best results were obtained with *P. sativum*. As its 2C nuclear DNA content is in the middle of the known range of genome sizes in plants, this observation underlines its position as one of the best standards for plant DNA FCM. By contrast, *Sedum burrito* was the most difficult species to analyse due to low DNA content, occurrence of polysomaty and high leaf water content, which hampered sample preparation and analysis. Moreover, its tissues may contain tannins (J. Greilhuber 2006, pers. comm.).

In *O. pes-caprae*, cytosol of which is highly acidic (pH < 3.0), measurable samples could be prepared using only Otto's and Galbraith's buffers, with Otto's being highly superior. This is in accordance with the results of Emshwiller (2002) who analysed ploidy levels in *Oxalis*. After testing LB01, $MgSO_4$ and Otto's buffers, she obtained measurable samples only with Otto's. The former two buffers failed presumably as a result of the acidic cell sap, which may have exceeded the buffering capacity of LB01 and $MgSO_4$.

Celtis australis was the only woody plant species included in the present study, and was chosen because of the presence of mucilaginous compounds (Rodriguez *et al.* 2005*a*; Chapter III.2), which increase sample viscosity, restrain nuclei release and cause their clumping. Interestingly, this was the only species for which Tris.$MgCl_2$ was the best performing buffer. This was probably because of a higher concentration of the non-ionic detergent, which suppressed the effect of mucilaginous compounds. Leaf tissues of *F. rothmaleri* were particularly hard and difficult to chop. In addition, preliminary experiments with this species revealed the presence of cytosolic compounds, which would be expected to interfere with DNA staining. However, given the pattern of FS and SS obtained, the so-called “tannic acid effect” (Chapter IV.2) was absent, indicating that these compounds were released at low concentration or not at all.

In order to compare the performance of nuclear isolation buffers, a set of parameters was carefully selected to evaluate sample quality. Furthermore, stability of fluorescence and light scatter properties of isolated nuclei over time were confirmed. Among the parameters chosen, the coefficient of variation of DNA peaks (CV) is of major importance. Galbraith *et al.* (2002) considered 5.0% as the maximum acceptable CV value in plant DNA flow cytometry. With the exception of Tris.$MgCl_2$ buffer when used with *S. burrito*, all mean CV values obtained herein were below this limit. Ideally, a nuclei sample should be free of cell and tissue debris. Emshwiller (2002) noted a correlation between CV and background noise. In the present study, this correlation was found only in some species (*e.g.*, *F. rothmaleri*). Rather, the results here suggest that the extent of background debris

is determined by the buffer itself. For example, a higher detergent concentration in a buffer could lead to chloroplast lysis and consequently decrease the number of fluorescent particles contributing to debris signals (Coba de la Peña and Brown 2001).

LB01 buffer provided very good results, with the exception of *O. pes-caprae* and *S. burrito*, low CV, high FL and YF values were obtained with this buffer. High nuclei FL intensities obtained with this buffer were definitely an advantage as compared with Tris.$MgCl_2$ and Galbraith's buffers. Galbraith's buffer seems well balanced, as acceptable results were achieved in all species. Surprisingly, the buffer gave reasonable results also with *O. pes-caprae*, which is characterized by highly acidic cytosol. The presence of MOPS in the buffer may shed light on these findings as it has a pKa of 7.2 and a better buffering capacity than TRIS with a pKa of 8.1. A disadvantage of this buffer was the low fluorescence intensity of nuclei. Collectively, Tris.$MgCl_2$ was the worst performing buffer. Nevertheless, it provided the best results for *C. australis*. This may have been due to the higher concentration of non-ionic detergent (Table IV.1.1), which counteracted the agglutinating effect of mucilaginous compounds and decreased sample viscosity. As with Galbraith's buffer, low FL values were obtained with Tris.$MgCl_2$.

Otto's buffer is unique in that sample preparation involves two steps. The results obtained with Otto's buffer were excellent in many species, especially those with lower nuclear DNA content. Doležel and Bartoš (2005) highlighted the quality of this buffer, which is known to provide DNA content histograms with unequalled resolution. One explanation for this may be that citric acid improves chromatin accessibility and “homogenizes” chromatin structure, eliminating differences in staining intensity among nuclei with the same DNA content but different chromatin state. This could explain the highest FL and lowest CV values, as observed here. Another characteristic of this buffer was that light scatter values (especially FS) were significantly lower than those of other buffers. This may be explained by the action of citric acid, which causes nuclei fixation (Doležel and Bartoš 2005). It is noteworthy that the pattern of FS *vs.* SS distribution was similar to that obtained when analysing nuclei fixed with formaldehyde (Rodriguez *et al.* 2005*b*). Although providing excellent results, this buffer gave the highest BF and lowest YF values. The former can be explained by nuclei instability after the addition of Otto II. After this step, nuclei deteriorate rapidly in some species (J. Doležel 2005, unpublished results). The lowest YF values could be due to the detergent (Tween 20), which is weaker than Triton X-100, thus having a lower capacity to release nuclei.

An important consequence of the observation of different fluorescence values obtained with the different buffers is that different fluorescence ratios may be expected with the

same species pair if the samples are prepared in different buffers. In fact, Doležel *et al.* (1998) observed differences in peak ratios obtained in different laboratories. Further studies are needed to confirm this observation, as it may have important consequences for estimation of genome size.

The present results showed that well-defined populations of nuclei could be observed on cytograms of FS *vs.* SS. With the exception of *S. burrito*, for which LB01 and Tris.$MgCl_2$ buffers did not present statistically significant differences for both parameters, the analysis of FS and SS provided a "fingerprint" pattern for each buffer. In Chapter IV.2 it was showed that these parameters were sensitive to the presence of tannic acid, a cytosolic compound common in plants, and recommended the analysis of light scatter to verify suitability of particular samples for plant DNA FCM.

As nuclei samples are prepared manually, it was important to assess the effect of date of analysis and operator on sample quality variation. Date was found to be more significant than the effect of different operators, especially for FS, SS, CV and DF. YF, which depends on the way the sample is chopped and hence on the operator, did not vary. It was expected that FL, which is a primary source of data in FCM analysis, would not depend on the operator or the day of chopping. This was confirmed in all species except *L. esculentum* and *C. australis*. In the former species, a significant variation was obtained between operators. These differences could be explained by variable results obtained with Galbraith's buffer. In *C. australis*, significant differences were obtained among dates. In this case, significant differences were due to results obtained on one single day. Emshwiller (2002) found significant differences when multiple preparations from the same plant were run on different days. These differences and those found in the present study were probably due to instrument drift (Kudo and Kimura 2001). To avoid this type of error, several authors have recommended that each measurement be repeated at least three times on three different days (Suda 2004; Doležel and Bartoš 2005).

This is the first study that has systematically compared nuclear isolation buffers for DNA FCM. The results show that none of the buffers works best with all species, and statistically significant differences in sample quality were observed among the four buffers. The results obtained with different species and contrasting types of leaf tissues can serve as guidelines in buffer selection. Nevertheless, it is recommended that a range of buffers be tested when working with a new species and tissue type. Once the best buffer has been identified, additives should be tested if required to suppress negative effects of phenols and other cytosolic compounds.

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IV.2 Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content

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Abstract

Background and Aims Flow cytometry (FCM) is extensively used to estimate DNA ploidy and genome size in plants. In order to determine nuclear DNA content, nuclei in suspension are stained by a DNA specific fluorochrome and fluorescence emission is quantified. Recent studies have shown that cytosolic compounds may interfere with binding of fluorochromes to DNA, leading to flawed data. Tannic acid, a common phenolic compound, may be responsible for some of the stoichiometric errors, especially in woody plants. In this study, the effect of tannic acid on estimation of nuclear DNA content was evaluated in *Pisum sativum* and *Zea mays*, which were chosen as model species.

Methods Nuclear suspensions were prepared from *P. sativum* leaf tissue using four different lysis buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$). The suspensions were treated with tannic acid (TA) at 13 different initial concentrations ranging from 0.25 to 3.50 mg mL^{-1}. After propidium iodide (PI) staining, samples were analysed using FCM. In addition to the measurement of nuclei fluorescence, light scatter properties were assessed. Subsequently, a single TA concentration was chosen for each buffer and the effect of incubation time was assessed. Similar analyses were performed on liquid suspensions of *P. sativum* and *Z. mays* nuclei that were isolated, treated and analysed simultaneously. FCM analyses were accompanied by microscopic observations of nuclei suspensions.

Key results TA affected PI fluorescence and light scatter properties of plant nuclei, regardless of the isolation buffer used. The least pronounced effects of TA were observed in Tris.$MgCl_2$ buffer. Samples obtained using Galbraith's and LB01 buffers were the most affected by this compound. A newly described “tannic acid effect” occurred immediately after the addition of the compound. With the exception of Otto's buffer, nuclei of *P. sativum* and *Z. mays* were affected differently, with pea nuclei exhibiting a greater decrease in fluorescence intensity.

Conclusions A negative effect of a secondary metabolite, TA, on estimation of nuclear DNA content is described and recommendations for minimizing the effect of cytosolic compounds are presented. Alteration in light scattering properties of isolated nuclei can be used as an indicator of the presence of TA, which may cause stoichiometric errors in nuclei staining using a DNA intercalator, PI.

Keywords cytosolic compounds; dye accessibility; genome size; flow cytometry; nuclear DNA content; *Pisum sativum*; propidium iodide; tannic acid; *Zea mays*

Introduction

Flow cytometry (FCM) is a powerful technique that was originally developed to count blood cells (Shapiro 2004). With improvements to equipment and methodologies, FCM has been adapted to many areas of biology including plant sciences (Doležel 1997). Since the introduction of FCM to plant studies, estimation of DNA ploidy levels and determination of nuclear genome size have been the two most frequent applications (Bennett and Leitch 2005). Owing to its ease, rapidity and accuracy, FCM has been an attractive alternative to traditional methods such as Feulgen microspectrophotometry (Doležel and Bartoš 2005). When analysing nuclear genome size, many authors have noted its variation within species (Price and Johnston 1996*a*; Rayburn *et al.* 1997, 2004; Ellul *et al.* 2002). However, the occurrence of this phenomenon and its extent remains a matter of discussion, as contradictory results have been obtained by different authors when analysing the same materials (Greilhuber and Obermayer 1997; Obermayer and Greilhuber 1999; Price *et al.* 2000; Suda 2004).

Reliable estimates of nuclear DNA content require proportionality between the digitized fluorescence signal and DNA content. This depends on several factors such as stoichiometry of dye binding to DNA, accessibility of DNA to the fluorochrome, fluorescence absorption and linearity of the instrument amplification system (Bagwell *et al.* 1989). The accessibility of nuclear DNA to fluorochromes has recently been a topic of major concern as it was found that cytosolic compounds could interfere with fluorescent staining of nuclei in suspension (Noirot *et al.* 2000, 2002, 2003, 2005; Price *et al.* 2000; Pinto *et al.* 2004; Walker *et al.* 2006). These observations indicated that FCM can produce flawed data if the effect of cytosolic compounds is ignored.

Price *et al.* (2000) speculated that inhibitors that decrease dye fluorescence of nuclei were common in plants. The authors did not point to a specific compound but suggested involvement of one or more of the numerous secondary metabolites. Noirot *et al.* (2000), working with coffee trees, revealed negative effects of cytosol on accessibility of DNA to propidium iodide (PI), and showed that cytosolic compounds could bias nuclear DNA content estimates by up to 20%. More recently, Noirot *et al.* (2003) identified two compounds that influenced PI fluorescence of petunia nuclei: caffeine and chlorogenic acid (a precursor of polyphenols). Whereas caffeine increased PI accessibility to petunia DNA, chlorogenic acid significantly decreased petunia nuclei fluorescence.

Tannic acid (TA) is a common phenolic compound, frequently accumulated in various tissues of plants belonging to diverse taxonomic groups, especially woody species. Several authors have claimed that TA might be responsible for stoichiometric errors in

genome size estimations: Greilhuber (1986) showed that in DNA cytophotometry, tannins could interfere with the Feulgen reaction to an extent that makes it worthless as a quantitative method; using FCM, Favre and Brown (1996) and Zoldoš *et al.* (1998) encountered difficulties in measuring nuclear DNA content in *Quercus* species (Fagaceae) and suggested that they were due to the presence of TA. In addition, in Chapter II.1 it was speculated that tannins were responsible for higher DNA content estimates obtained for *ex vitro* leaves of *Quercus suber* as compared with *in vitro* material.

The main objectives of this study were: (1) to test the effect of TA on PI fluorescence and light scattering properties of nuclei of *Pisum sativum* (Fabaceae) and *Zea mays* (Poaceae); (2) to evaluate the effect of different nuclear isolation buffers on the interaction of TA with nuclei in suspension; (3) to evaluate the kinetics of the interaction of TA with nuclei samples; (4) to test if nuclei of two different species, *P. sativum* and *Z. mays*, are affected similarly by TA when isolated, processed and analysed simultaneously; (5) to identify diagnostic patterns that can be used to recognize the action of cytosolic compounds; and (6) to propose recommendations on how to minimize the negative effects of cytosolic compounds on estimation of nuclear DNA content using FCM.

Materials and methods

Plant material

Plants of garden pea (*Pisum sativum* L.) ‘Ctirad’ and maize (*Zea mays* L.) ‘CE777’ were grown from seeds in a greenhouse at 22 ± 2°C, with a photoperiod of 16 h and a light intensity of 530 ± 2 μmol m^{-2} s^{-1}.

Sample preparation

Nuclei suspensions were obtained after chopping approximately 350 mg of leaf tissue according to Galbraith *et al.* (1983). Four of the most popular nuclear isolation buffers (Chapter IV.1) were used to prepare samples: Galbraith's buffer (Galbraith *et al.* 1983), LB01 (Doležel *et al.* 1989), Otto's buffers (Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser *et al.* 1995). ß-Mercaptoethanol was not included in LB01, as this study intended to compare basic buffer formulas without any additives that could modulate the action of tannic acid. Otto I and Otto II buffers were mixed in a 1:2 ratio. Nuclear suspension was filtered through a 50 μm nylon filter and RNase A (Fluka, Buchs, Switzerland) at a concentration of 50 μg mL^{-1} was added to each sample. Samples were kept on ice until analysis.

Staining of nuclei

The effect of PI concentration and staining time was analysed in order to determine a saturating concentration to be used in subsequent experiments. Four mL of nuclear suspension was prepared from approximately 350 mg of pea leaves. The homogenate was divided into eight aliquots (0.5 mL each) and PI (Fluka) was added to achieve the following concentrations: 10, 25, 50, 75, 100, 150, 250 and 350 μg mL^{-1}. Samples were analysed 0, 5, 10 and 60 min after incubation with PI. Each sample was measured for exactly 2 min. In all further experiments, nuclei were stained with 150 μg mL^{-1} of PI (optimal concentration).

Flow cytometry measurements

Nuclear samples were analysed using a Coulter Epics XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. PI fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. Prior to analysis the instrument was checked for linearity with Flow-Check fluorospheres (Beckman Coulter®, Hialeah, FL, USA). The amplifier system was set to a constant voltage and gain throughout the experiments. The results were acquired using the SYSTEM II software version 3.0 (Beckman Coulter®) in the form of six graphics: fluorescence pulse integral (FL); FL *vs.* fluorescence pulse height; forward scatter (FS) *vs.* side scatter (SS), both in logarithmic (log) scales; SS in log scale *vs.* FL; FL *vs.* time; and SS in log scale *vs.* time. FS is proportional to particle size. However, owing to its response similarity with SS, it was not considered during data evaluation. The coefficients of variation (CV) were calculated for SS (SS-CV, %) and FL (FL-CV, %).

Fluorescence microscopy

Nuclear suspensions were evaluated in a Nikon Eclipse 80i fluorescence microscope (Nikon Corporation, Kanagawa, Japan) using the G-2A filter cube. Digital photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Wetzlar, Germany).

The effect of tannic acid on P. sativum

<u>Effect of different concentrations.</u> Seven mL of nuclear suspension was divided into 14 aliquots (0.5 mL each), which were treated with TA (Fluka) made in H_2O at: 0.0 (control), 0.25, 0.30, 0.40, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50 and 3.50 mg mL^{-1}. A

0.25 mL aliquot of TA solution was added to each sample (final concentrations: 0.0, 0.083, 0.100, 0.133, 0.167, 0.250, 0.333, 0.417, 0.500, 0.583, 0.667, 0.750, 0.833 and 1.167 mg mL^{-1}) and left to incubate for 15 min on ice. The sample was then stained with PI for 5 min on ice and analysed for 2 min. The experiment was replicated three times for each buffer.

Effect of incubation time. For each nuclear isolation buffer, a different concentration of TA was chosen based on the results of previous experiments. The nuclear suspension (4 mL) was divided into four 1 mL aliquots. Three TA treatments were applied (TA1, TA2 and TA3): TA1 and TA2 treatments consisted of adding 0.5 mL of TA solution to the sample. In the TA1 treatment, three 0.5 mL aliquots were stained with PI for 5 min and analysed immediately. In TA2, samples were incubated with TA for 15 min prior to staining with PI. TA1 and TA2 samples were analysed for a period of 2 min. In TA3, three 0.33 mL aliquots were taken, stained by PI for 5 min and analysed for 60 s. Measurement was then halted and 0.16 mL of TA solution was added to the sample. The measurement was resumed in order to follow the kinetics of the addition of TA. The order in which the samples were measured was randomized within treatments to avoid a systematic error. This experiment was replicated twice for each buffer on different days.

Simultaneous analysis of P. sativum *and* Z. mays *nuclei.*

This experiment was designed to determine if nuclei from two different species are affected by TA in the same way. Two experiments were carried out exactly as the two experiments above, except that instead of using 350 mg of pea leaf tissue, 175 mg of *Z. mays* leaves and 175 mg of *P. sativum* leaves were chopped simultaneously in a Petri dish. In the second experiment, a particular concentration of the TA solution was chosen for each buffer.

Statistical analyses

Statistical analyses were performed using a two-way ANOVA (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA). When treatments were significantly different, a Holm–Sidak multiple comparison test was used for pair-wise comparison. To calculate the optimal PI concentration, logarithmic and hyperbolic regression analyses were performed (GraphSight version 2.0.1, Belarus). A one-way ANOVA was performed to analyse differences among concentrations of PI and incubation times with this fluorochrome.

Results

The effect of PI concentration and staining time

The effect of PI concentration on nuclear fluorescence was computed for each staining period and for each buffer. The logarithmic fitting, despite not being perfect, provided better regression coefficients than other methods, such as hyperbolic fitting (data not shown). These results show that the addition of 150 µg mL^{-1} of PI was saturating (Fig. IV.2.1). The increase of nuclear fluorescence was not statistically significant at higher PI concentrations. For practical convenience, a 5 min staining period was chosen (Fig. IV.2.2). Statistically significant differences were observed only for the 60 min incubation period with PI in nuclei isolated with Tris.$MgCl_2$ buffer. In this case, nuclei fluorescence decreased.

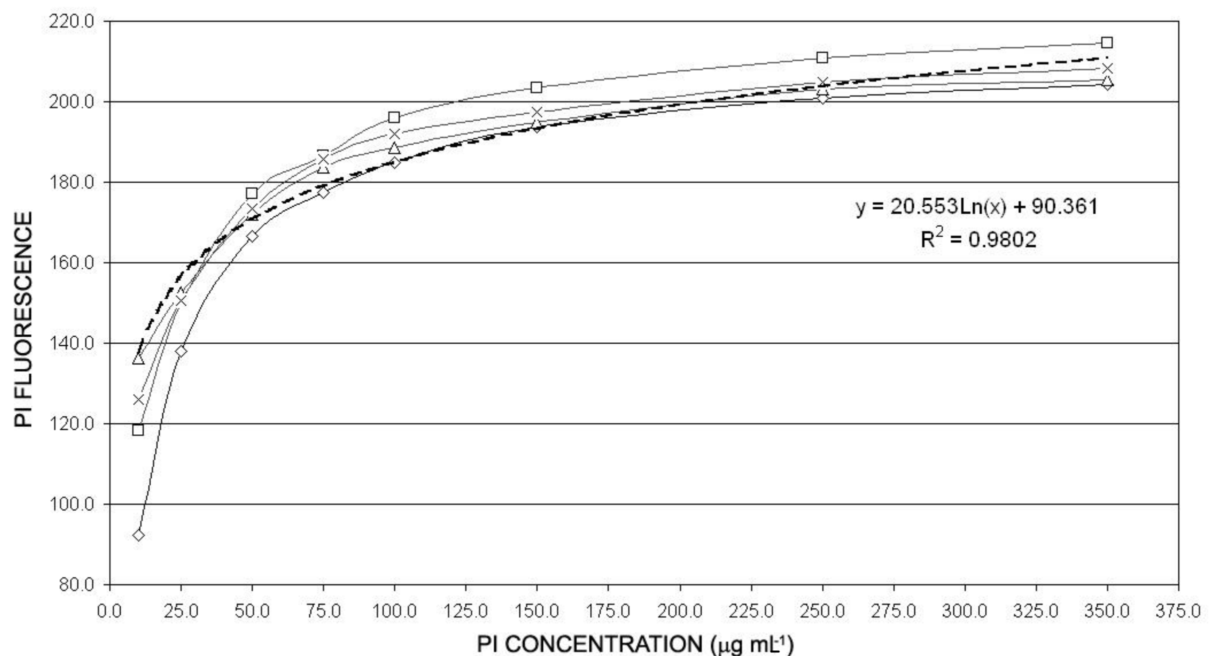

Fig. IV.2.1 Effect of propidium iodide (PI) concentration (µg mL^{-1}) on fluorescence intensity (mean channel number) of *Pisum sativum* nuclei isolated with four different nuclear isolation buffers: LB01 (squares), Tris.$MgCl_2$ (crosses), Galbraith's (diamonds), Otto's (triangles). The logarithmic fitting with the respective coefficient of regression (R^2) of the values obtained with Otto's buffer are also presented (dashed line).

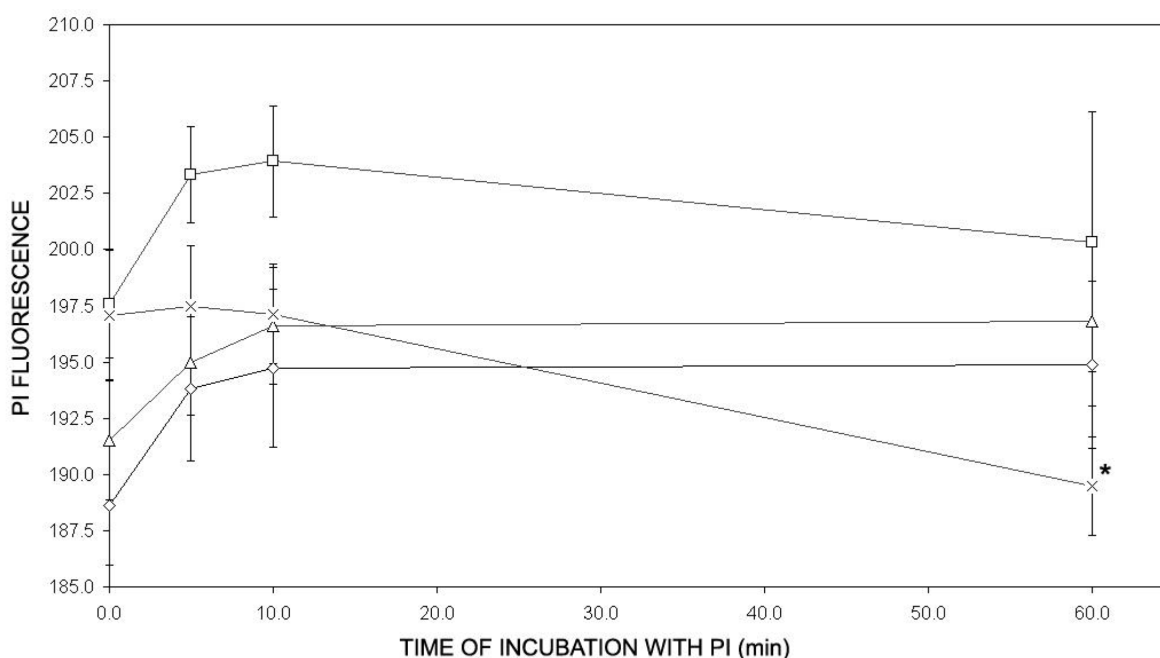

Fig. IV.2.2 Effect of incubation time (min) with propidium iodide (PI) on fluorescence intensity (mean channel number) of *Pisum sativum* nuclei isolated with four different nuclear isolation buffers: LB01 (squares), Tris.$MgCl_2$ (crosses), Galbraith's (diamonds), Otto's (triangles). The asterisk indicates a statistically significant difference between incubation times according to the multiple comparison Holm–Sidak test at $P \leq 0.01$.

The effect of TA on P. sativum *nuclei*

Effect of different concentrations. A detailed description of the action of TA will focus on nuclei isolated with Tris.$MgCl_2$ – a buffer on which nuclei were least affected (Table IV.2.1, Fig. IV.2.3). In the absence of TA in nuclei suspension, relatively low SS-CV (30.00 – 40.00%) and FL-CV (<2.50%) values were obtained (Table IV.2.1). Up to a TA concentration of 0.50 mg mL^{-1}, no significant effect on SS, SS-CV, FL or FL-CV was detected. At 0.50 mg mL^{-1} TA, an increase in SS and SS-CV was observed. At a concentration of 0.75 mg mL^{-1} and in particular at 1.00 mg mL^{-1}, the presence of two new populations of particles was detected on the SS *vs.* FL cytogram (arrows on Fig. IV.2.3f and IV.2.3i). These particles were characterized by high SS and SS-CV values (Table IV.2.1). As will be shown later, similar populations of particles characterized by high SS and SS-CV were also observed with other buffers albeit at different TA concentrations (Table IV.2.2). Hereafter, this phenomenon is termed the “tannic acid effect”.

Table IV.2.1 Analysis of SS, SS-CV, FL, FL-CV (mean and standard deviation of the mean) of particles in liquid suspensions prepared from *P. sativum* leaf tissue with four nuclei isolation buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$) and exposed to different concentrations of tannic acid (TA). SS and FL are shown as channel numbers and SS-CV and FL-CV in %. The incipient concentrations for TA effect are highlighted in grey. Means followed by the same letter are not significantly different at $P \leq 0.05$ according to the Holm-Sidak multiple comparison test.

TA (mg mL^{-1})	Galbraith's buffer				LB01 buffer				Otto's buffer				Tris.$MgCl_2$ buffer			
	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)
CTRL	4.61 ± 0.290a	40.71 ± 1.887a	211.0 ± 3.81a	2.88 ± 0.476a	8.43 ± 0.580a	37.66 ± 7.955a	210.6 ± 4.60a	2.33 ± 0.103a	7.36 ± 0.318a	35.41 ± 3.764a	208.1 ± 9.41a	1.48 ± 0.046a	13.33 ± 0.961a	34.29 ± 2.402a	194.0 ± 9.37acd	2.21 ± 0.854a
0.25	10.06 ± 6.739a	92.91 ± 39.858ab	210.0 ± 8.95a	3.01 ± 0.842a	23.63 ± 15.519a	91.18 ± 24.770b	215.3 ± 4.07a	2.64 ± 0.327a	8.33 ± 0.401ab	34.99 ± 3.168a	201.7 ± 13.57a	1.50 ± 0.097a	12.97 ± 1.021a	34.96 ± 2.330a	211.9 ± 10.87abc	2.41 ± 0.435a
0.30	26.53 ± 9.151a	138.62 ± 1.146bc	205.7 ± 10.81a	3.20 ± 0.635a	44.40 ± 8.879ab	120.13 ± 4.246b	217.3 ± 1.22ab	3.71 ± 0.544ab	9.19 ± 1.098ab	39.97 ± 6.820a	198.5 ± 16.06a	2.17 ± 0.583a	13.07 ± 0.945a	35.51 ± 3.115a	213.4 ± 8.24abc	2.38 ± 0.297a
0.40	59.50 ± 28.705b	149.55 ± 16.200c	207.5 ± 10.75a	3.98 ± 1.631ab	71.03 ± 14.490b	116.72 ± 1.991b	226.8 ± 5.17b	3.78 ± 0.887ab	10.70 ± 0.100ab	44.35 ± 6.376a	200.6 ± 9.11a	1.73 ± 0.104a	13.70 ± 1.709ab	36.75 ± 2.627a	214.2 ± 5.54ab	2.47 ± 0.340a
0.50	67.63 ± 10.504b	131.30 ± 9.893c	206.1 ± 11.43a	4.71 ± 1.206ab	87.43 ± 20.144b	106.13 ± 3.482b	228.1 ± 5.10b	4.98 ± 0.725b	14.27 ± 1.464ab	53.03 ± 10.260a	197.7 ± 8.46a	1.91 ± 0.166a	22.23 ± 15.667ab	57.92 ± 38.559ab	214.9 ± 12.33ab	2.46 ± 0.269a
0.75	78.87 ± 8.411b	126.09 ± 1.209c	186.7 ± 18.76a	5.52 ± 1.959ab	141.37 ± 46.012b	101.56 ± 3.583b	227.9 ± 3.35b	4.34 ± 0.872b	59.23 ± 18.451bc	103.35 ± 11.866bc	194.1 ± 6.72a	2.79 ± 0.630a	27.00 ± 15.186ab	75.17 ± 27.019ab	216.1 ± 13.14ab	3.01 ± 0.252a
1.00	86.70 ± 1.311b	121.59 ± 7.796c	150.8 ± 30.78b	8.27 ± 4.880b	160.35 ± 27.648b	98.06 ± 3.246b	227.6 ± 0.28b	4.80 ± 0.757b	84.37 ± 34.480bc	93.94 ± 22.399bc	185.4 ± 10.79ab	4.92 ± 1.038b	32.70 ± 9.009ab	91.91 ± 11.383b	213.9 ± 6.02ab	3.15 ± 0.577a
1.25	No pea nuclei peaks were distinguishable				No pea nuclei peaks were distinguishable				108.13 ± 34.872bc	75.35 ± 24.578ca	163.9 ± 18.77b	5.51 ± 0.631b	45.70 ± 16.838b	91.11 ± 11.383b	228.8 ± 16.30b	3.24 ± 1.52a
1.50	-	-	-	-	-	-	-	-	No pea nuclei peaks were distinguishable				30.70 ± 7.808ab	89.89 ± 15.428b	203.8 ± 7.53ab	3.05 ± 0.520a
1.75	-	-	-	-	-	-	-	-	-	-	-	-	34.63 ± 10.571ab	95.95 ± 15.298b	195.2 ± 15.37a	3.34 ± 1.09a
2.00	-	-	-	-	-	-	-	-	-	-	-	-	34.97 ± 10.702ab	91.62 ± 14.44b	191.1 ± 2.14a	3.35 ± 0.46a
2.25	-	-	-	-	-	-	-	-	-	-	-	-	29.07 ± 6.521ab	91.14 ± 18.766b	181.6 ± 3.75c	3.28 ± 0.921a
2.50	-	-	-	-	-	-	-	-	-	-	-	-	29.33 ± 6.300ab	82.81 ± 18.860b	168.6 ± 11.40cd	3.45 ± 0.681a
3.50	-	-	-	-	-	-	-	-	-	-	-	-	No pea nuclei peaks were distinguishable			

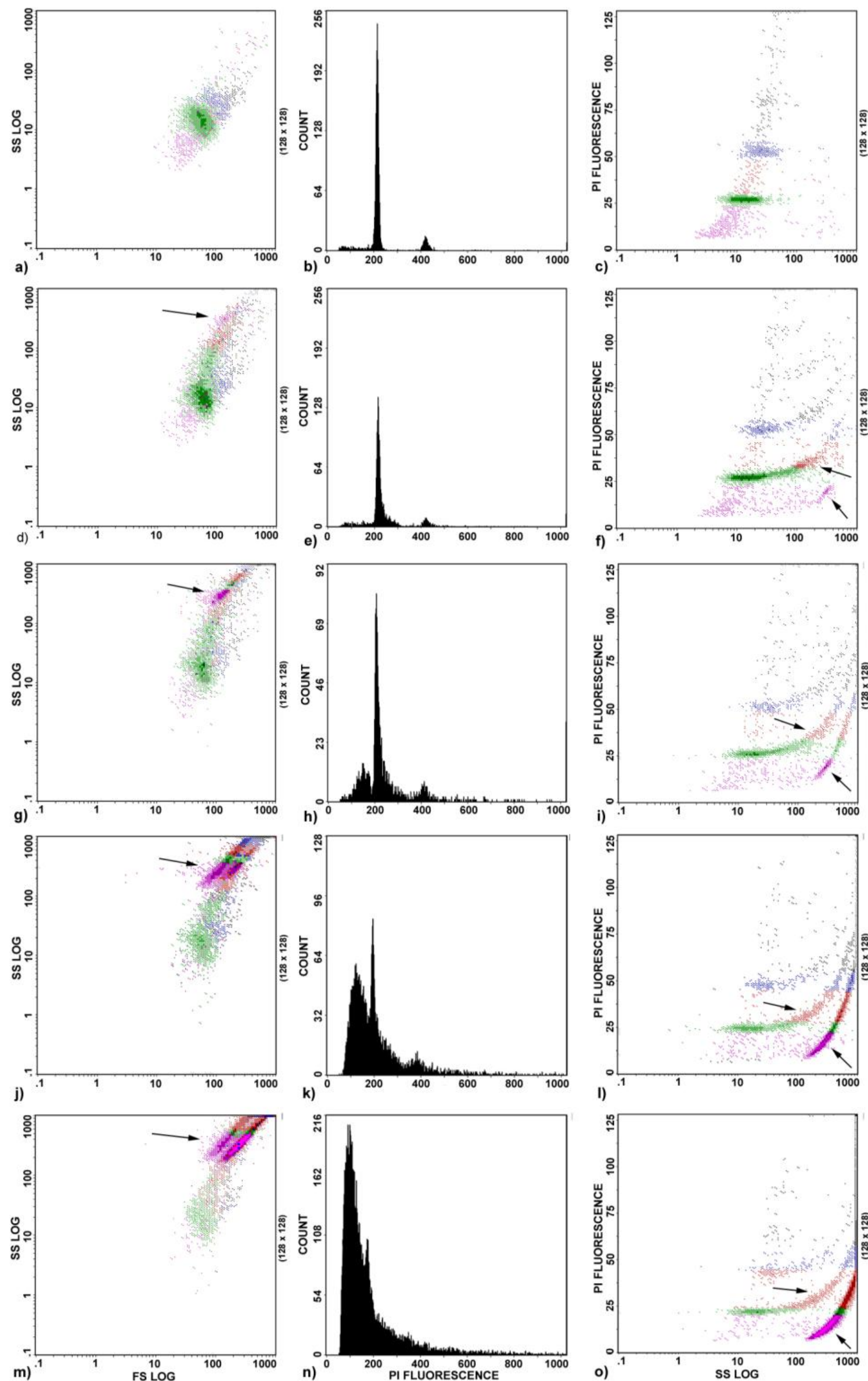

Fig. IV.2.3 The effect of tannic acid (TA) on propidium iodide (PI) fluorescence and light scattering properties of *Pisum sativum* nuclei in suspension. Nuclear suspensions were obtained with Tris.$MgCl_2$ buffer and incubated for 15 min with TA at: a-c) 0.00 mg mL^{-1}; d-f) 0.75 mg mL^{-1}; g-i) 1.00 mg mL^{-1}; j-l) 1.75 mg mL^{-1}; and m-o) 2.50 mg mL^{-1}. The results are displayed as forward scatter (logarithmic scale, FS LOG) *vs.* side scatter (logarithmic scale, SS LOG) cytograms (a, d, g, j and m), PI fluorescence intensity histograms (b, e, h, k and n) and SS log *vs.* PI cytograms (c, f, i, l and o). Arrows indicate new populations that appeared after the addition of particular TA concentrations.

Table IV.2.2 Concentration of tannic acid (TA, mg mL^{-1}) at which the TA effect (significant change in SS and SS-CV) occurs, at which an increase or decrease in fluorescence intensity is observed and at which no nuclei peaks can be discriminated from the debris background (n.s. – non significant changes were observed at $P \leq 0.05$).

Buffer	Analysis of *P. sativum*			Simultaneous analysis of *Z. mays* and *P. sativum*			
	SS and SS-CV	FL (loss, -; gain, +)	No peaks are visible	SS and SS-CV	FL (loss, -; gain, +)	FL ratio	No peaks are visible
Galbraith's	0.25-0.30	0.75 (-)	1.25	0.50-0.75	0.75 (-)	0.50-0.75	1.25
LB01	0.25-0.30	0.40 (+)	1.25	0.30	0.40 (+)	0.40	0.75
Otto's	0.75	1.00 (-)	1.50	1.00	0.30 (-)	n.s.	1.25
Tris.$MgCl_2$	1.00	2.25 (-)	3.50	1.75	2.25 (-)	1.75	3.50

At up to 0.75 mg mL^{-1} TA, no clear effect on FL was observed. In two of three replications, the presence of nuclei with slightly higher fluorescence was detected. Microscopic analyses of samples revealed a tendency to precipitation, and in addition to single nuclei (as observed at lower concentrations of TA; Fig. IV.2.4a and IV.2.4b) some nuclei were observed to which weakly fluorescent particles were attached (Fig. IV.2.4c and IV.2.4d). This apparently resulted in higher FL and consequently higher FL-CV values (3.00–3.25%). At the same time, a second subpopulation of particles with higher SS but lower FL started to appear. Microscopic observation of samples revealed the presence of a new subpopulation consisting of clumps of weakly fluorescent particles that were not attached to nuclei, thus having relatively high optical complexity and lower FL than nuclei (Fig. IV.2.4c and IV.2.4d).

With increasing concentrations of TA (1.25, 1.50, 1.75 and 2.00 mg mL^{-1}), the "tannic acid effect" became more pronounced as the number of events present in the new subpopulations (characterized by high SS and SS-CV) increased continuously and the proportion of "clean" nuclei declined (Fig. IV.2.3j and IV.2.3m). No significant decrease in fluorescence of PI-stained nuclei was observed up to a TA concentration of 1.75 mg mL^{-1} (Fig. IV.2.3k). Only at 2.00 mg mL^{-1} TA did a significant decrease of nuclei FL of about 15.0% occur. At this point, the proportion of "clean" nuclei in the suspension was low (<10.0%). At concentrations of 2.25 and 2.50 mg mL^{-1} TA, the fraction of "clean" nuclei in the suspension continued to decrease, and it was difficult to discriminate nuclei peaks from the background debris. This was clearly due to the presence of the two new subpopulations of particles (Fig. IV.2.3m–o). Fluorescence of the remaining "clean" nuclei was considerably lower when compared with controls. In samples treated with the highest concentration of TA (3.50 mg mL^{-1}), no peaks representing pea nuclei could be discriminated from the background.

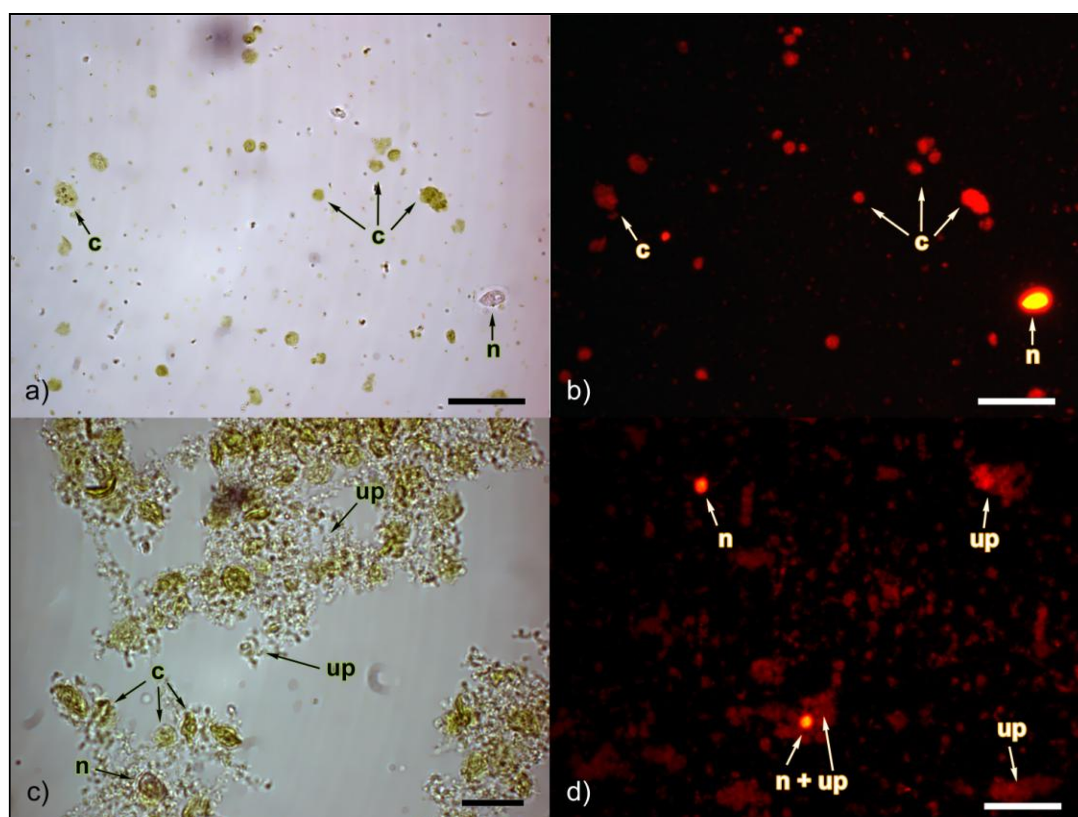

Fig. IV.2.4 Micrographs of nuclei suspensions prepared with Tris.$MgCl_2$ buffer: a) bright-field image of control without tannic acid (TA, *bar* = 20 µm, magnification 400x); b) fluorescence image of control without TA (*bar* = 20 µm, magnification 400x); c) bright-field image after addition of TA (*bar* = 10 µm, magnification 1000x); d) fluorescence image after addition of TA (*bar* = 20 µm, magnification 400x). n – nuclei, c – chloroplasts, up – unspecific particles. In contrast to control samples, clumps of various particles (up) can be observed after the addition of TA. Fluorescence images were over-exposed to observe particles with lower fluorescence.

Other nuclear isolation buffers. When compared with the Tris.$MgCl_2$ buffer, the remaining three buffers that were used to isolate *P. sativum* nuclei were more affected by TA, and lower concentrations of this compound were sufficient to induce the TA effect (Tables IV.2.1 and IV.2.2).

In Galbraith's buffer, 0.25 mg mL^{-1} TA caused a doubling of SS values with SS-CV of 92.0%, a value observed with the Tris.$MgCl_2$ buffer only at 0.75 mg mL^{-1} TA. As in Tris.$MgCl_2$, nuclei fluorescence was only affected at higher concentrations of TA (0.75 and 1.00 mg mL^{-1}). At those concentrations, FL of nuclei was lower by 11.5 and 28.5%, respectively, when compared with the untreated control, and FL-CV values were above 5.0%. For a TA concentration of 1.25 mg mL^{-1}, peaks of pea nuclei could not be distinguished from the background (Table IV.2.1).

The use of LB01 buffer gave results similar to those obtained with Galbraith's buffer, as nuclei were affected with the addition of the same concentration of TA (0.25 mg mL^{-1}). In addition, no peaks were visible after the addition of 1.25 mg mL^{-1} TA. Nevertheless and contrarily to what was observed for all the other buffers, nuclear fluorescence increased with increasing TA concentration. A significant fluorescence increase was obtained after the addition of 0.30 mg mL^{-1} TA. At 0.50 mg mL^{-1} TA, a 7.7% difference, as compared with control, was observed (Table IV.2.1).

Nuclear suspension in Otto's buffer was less affected by TA than in Galbraith's and LB01 buffers, but more than in Tris.$MgCl_2$. The TA effect was observed at a concentration of 0.75 mg mL^{-1}, as in the Tris.$MgCl_2$ buffer. However, in contrast to what was observed with other three buffers, PI fluorescence decreased even with the addition of TA at the lowest concentration. Nevertheless, a statistically significant decrease of fluorescence was observed only at 1.00 mg mL^{-1} TA. At this concentration the FL-CV increased significantly, reaching values of 5.0%. At a TA concentration of 1.25 mg mL^{-1}, nuclei fluorescence decreased by 21.0%, and this was the highest TA concentration at which nuclei peaks could be distinguished (Table IV.2.1).

Effect of incubation time. Following the concentration tests performed with each buffer, a particular concentration of TA was chosen (Table IV.2.3) to test whether incubation time was of importance. In Galbraith's and LB01 buffers the time of incubation had no marked effect; in both TA1 and TA2 experiments, the TA effect occurred, and extremely high SS and SS-CV values were obtained. Although statistically significant differences between TA1 and TA2 were observed in SS and SS-CV, in practice, nuclei from both experiments were severely affected by the addition of TA (Table IV.2.3). The FL-CV value was also higher in these treatments compared with the control value. As expected, and similarly to the results obtained in the concentration tests, nuclei fluorescence decreased significantly after the addition of 0.50 mg mL^{-1} TA in Galbraith's buffer. By contrast, in LB01, fluorescence increased significantly with the addition of the same TA concentration. In the TA3 experiment, an immediate increase in SS and SS-CV was observed in both buffers when adding TA after running the sample for 60 s.

In Otto's buffer, the time of incubation was an important factor, as statistically significant increases of SS and SS-CV were observed in TA2 as compared with TA1. Interestingly, a decrease in fluorescence was observed in both experiments. The change in SS, SS-CV and FL occurred immediately after the addition of TA.

Table IV.2.3 The effect of incubation time with tannic acid (TA) on plant nuclei. SS, SS-CV, FL, and FL-CV (mean and standard deviation of the mean) were analysed in liquid suspensions prepared from *P. sativum* leaf tissue with four nuclei isolation buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$), exposed to TA at a particular initial concentration (c_i), and incubated for different periods of time (CTRL – no incubation with TA; TA1 – incubation with TA for 5 min; TA2 – incubation for 20 min with TA). In TA3, samples were run for 60 s without TA (TA3bta) and, after a pause, TA was added to samples (TA3ata). SS and FL are shown as channel means and SS-CV and FL-CV in %. Means followed by the same letter are not significantly different according to the Holm-Sidak multiple comparison test at $P \leq 0.05$.

Experiment	Galbraith's buffer (c_i = 0.50 mg mL^{-1})				LB01 buffer (c_i = 0.50 mg mL^{-1})				Otto's buffer (c_i = 0.75 mg mL^{-1})				Tris.$MgCl_2$ buffer (c_i = 1.00 mg mL^{-1})			
	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)	SS (channel)	SS-CV (%)	FL (channel)	FL-CV (%)
CTRL	4.34 ± 0.677a	57.53 ± 17.587a	201.3 ± 7.94a	2.36 ± 0.210a	6.25 ± 3.275a	37.79 ± 3.440a	220.1 ± 5.86a	2.68 ± 0.420a	2.55 ± 0.244a	34.30 ± 2.521a	220.3 ± 3.53a	1.64 ± 0.201a	14.77 ± 0.462a	31.80 ± 1.744a	217.5 ± 6.60a	2.43 ± 0.067a
TA1	39.25 ± 17.514b	149.36 ± 12.592b	191.5 ± 5.85b	4.27 ± 2.048b	55.60 ± 27.70b	127.20 ± 9.12b	235.5 ± 5.96b	4.03 ± 1.050b	4.44 ± 0.181b	43.01 ± 8.187b	169.02 ± 6.892b	2.23 ± 0.253b	14.27 ± 0.289a	31.33 ± 0.652a	194.2 ± 3.47b	3.11 ± 0.247b
TA2	49.67 ± 22.554c	131.95 ± 9.263c	196.9 ± 3.07c	3.77 ± 1.149a	61.92 ± 31.867c	113.67 ± 4.166c	233.0 ± 2.24b	4.60 ± 0.907b	43.81 ± 8.187c	60.06 ± 3.926c	171.3 ± 6.66b	2.30 ± 0.184b	35.17 ± 7.051b	94.16 ± 5.179b	221.7 ± 4.68a	3.18 ± 0.217b
TA3bta	8.51 ± 4.468a	65.53 ± 0.963a	199.8 ± 10.92a	2.60 ± 0.626a	4.59 ± 2.257a	48.93 ± 2.017a	225.6 ± 6.84a	2.55 ± 0.428a	2.12 ± 0.222a	45.59 ± 5.720a	224.1 ± 2.83a	1.79 ± 0.271a	13.80 ± 0.265a	31.70 ± 0.721a	226.1 ± 3.44a	2.48 ± 0.127a
TA3ata	30.14 ± 33.660b	103.08 ± 32.271b	188.8 ± 6.33a	3.24 ± 1.210a	40.15 ± 21.116b	133.82 ± 16.520b	243.5 ± 3.94b	3.49 ± 1.601b	9.99 ± 6.372b	81.70 ± 23.239b	179.8 ± 6.63 b	2.25 ± 0.356b	16.17 ± 0.603b	30.70 ± 0.722a	228.5 ± 0.42a	2.42 ± 0.095a

In Tris.$MgCl_2$, a similar result to that obtained with Otto's buffer was verified, *i.e.*, the TA effect was observed only after 15 min of incubation. Compared with data for control nuclei, SS remained the same but FL was lower by 10.0% in the TA1 experiment. This was not the case for TA2, in which both SS and SS-CV increased significantly but the fluorescence intensity of nuclei was similar to the values obtained in the control. No significant differences were detected in any SS properties, FL and FL-CV in TA3.

Simultaneous analysis of P. sativum *and* Z. mays *nuclei*

To test if two different species were affected in the same way by TA, nuclei from *Zea mays* and *Pisum sativum* were isolated, treated and analysed simultaneously. In order to compare the response of the two species, three ratios were computed: SS ratio (*Z. mays* mean SS / *P. sativum* mean SS), SS-CV ratio (*Z. mays* mean SS-CV / *P. sativum* mean SS-CV) and FL ratio (*Z. mays* mean FL / *P. sativum* mean FL).

Effect of different concentrations. In Galbraith's buffer, the addition of 0.40 mg mL^{-1} TA increased the *Z. mays* SS-CV. The increase was only statistically significant at 0.75 mg mL^{-1} TA. With increasing concentrations of TA, the SS and SS-CV from both species increased similarly and the FL ratio remained constant until a TA concentration of 0.75 mg mL^{-1}. Thereafter, a statistically significant increase in the FL ratio was observed. This was due to a more pronounced decrease of fluorescence observed in *P. sativum* nuclei than in *Z. mays*. As in the single species analysis, no nuclei peaks could be observed after addition of 1.00 mg mL^{-1} TA (Table IV.2.4).

In LB01 buffer, the TA effect occurred after treatment with 0.30 mg mL^{-1} TA. With increasing TA concentrations, both SS and SS-CV ratios increased. This was due to a more rapid increase of these parameters in *Z. mays* than in *P. sativum*. The FL ratio also increased with increasing concentrations of TA, but in line with the results obtained with *P. sativum* alone, the increase was due to a fluorescence gain that was higher in *Z. mays* than in *P. sativum* (Table IV.2.4).

Otto's buffer was the only one in which the ratios between SS, SS-CV and FL in *Z. mays* and *P. sativum* did not change significantly with changing concentrations of TA. With regard to the FL ratio, nuclei from both species lost a similar amount of fluorescence. The TA effect occurred after the addition of 1.00 mg mL^{-1} TA, and even at 1.25 mg mL^{-1} TA it was not possible to discriminate between nuclei peaks and background noise (Table IV.2.4).

Table IV.2.4 Analysis of *Zea mays* SS-CV (%), SS ratio, SS-CV ratio and FL ratio (mean and standard deviation of the mean) of particles in liquid suspension prepared from *P. sativum* and *Z. mays* leaf tissue with four different buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$) and exposed to different concentrations of tannic acid (TA). Three ratios were calculated: SS ratio (*Z. mays* mean SS / *P. sativum* mean SS), SS-CV ratio (*Z. mays* mean SS-CV / *P. sativum* mean SS-CV) and FL ratio (*Z. mays* mean FL / *P. sativum* mean FL). The incipient concentrations for TA effect are highlighted in grey. Means followed by the same letter are not significantly different according to the Holm-Sidak multiple comparison test at $P \leq 0.05$.

TA (mg mL^{-1})	Galbraith's buffer				LB01 buffer				Otto's buffer				Tris.$MgCl_2$ buffer			
	Zea mays SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio
CTRL	37.86 ± 9.359a	0.808 ± 0.1742a	1.002 ± 0.3772a	0.600 ± 0.007a	37.78 ± 6.578a	0.748 ± 0.0830ab	0.953 ± 0.0868a	0.613 ± 0.002a	41.00 ± 5.735ab	0.572 ± 0.1237a	1.041 ± 0.2013a	0.598 ± 0.0098a	38.66 ± 0.714a	0.766 ± 0.1347a	1.096 ± 0.1054a	0.602 ± 0.0039a
0.25	40.17 ± 8.892a	0.806 ± 0.0950a	1.020 ± 0.2176a	0.600 ± 0.011a	59.40 ± 22.558a	0.732 ± 0.1080a	1.004 ± 0.1468a	0.614 ± 0.003a	39.32 ± 5.645a	0.573 ± 0.1144a	1.108 ± 0.2313a	0.593 ± 0.0045a	39.17 ± 0.623a	0.812 ± 0.1763ab	1.020 ± 0.1098a	0.608 ± 0.0062ab
0.30	43.01 ± 11.288a	0.835 ± 0.1544a	1.106 ± 0.2255a	0.604 ± 0.010a	101.64 ± 25.449b	0.852 ± 0.2038ab	1.162 ± 0.0915a	0.618 ± 0.001a	38.73 ± 2.475a	0.754 ± 0.3479a	0.958 ± 0.0589a	0.592 ± 0.0012a	37.59 ± 2.209a	0.797 ± 0.1545a	0.988 ± 0.0993a	0.604 ± 0.0043a
0.40	70.56 ± 47.319a	0.869 ± 0.1943a	1.084 ± 0.1857a	0.605 ± 0.006a	132.74 ± 6.432b	0.922 ± 0.0479bc	1.156 ± 0.1418b	0.633 ± 0.003b	40.95 ± 5.327ab	0.594 ± 0.0657a	1.118 ± 0.2333a	0.591 ± 0.0036a	38.07 ± 2.425a	0.776 ± 0.1280a	0.997 ± 0.1007a	0.609 ± 0.0058ab
0.50	75.37 ± 58.957a	0.810 ± 0.1538a	1.032 ± 0.3321a	0.607 ± 0.009ab	114.46 ± 6.174b	1.070 ± 0.1294c	1.190 ± 0.1335b	0.646 ± 0.009b	43.59 ± 5.069ab	0.642 ± 0.0272a	1.099 ± 0.1008a	0.594 ± 0.0020a	38.44 ± 2.615a	0.777 ± 0.1208a	1.027 ± 0.0178a	0.609 ± 0.0034ab
0.75	149.57 ± 14.284b	0.724 ± 0.0697a	1.089 ± 0.0858a	0.618 ± 0.006b	No peaks were distinguishable				53.79 ± 15.875ab	0.628 ± 0.1287a	0.874 ± 0.1634a	0.592 ± 0.0064a	38.57 ± 2.116a	0.772 ± 0.1207a	1.001 ± 0.0525a	0.612 ± 0.0039ab
1.00	145.91b	0.955a	1.112a	0.659c	-	-	-	-	66.17 ± 7.353b	0.894 ± 0.0613a	1.000 ± 0.0337a	0.600 ± 0.0252a	39.82 ± 1.575a	0.759 ± 0.1212a	1.075 ± 0.1378a	0.612 ± 0.0012ab
1.25	No peaks were distinguishable				-	-	-	-	No peaks were distinguishable				43.04 ± 9.322a	0.753 ± 0.1144a	1.060 ± 0.0373a	0.614 ± 0.0033ab
1.50	-	-	-	-	-	-	-	-	-	-	-	-	55.00 ± 27.740ac	0.792 ± 0.1758a	1.128 ± 0.0627a	0.616 ± 0.0021ab
1.75	-	-	-	-	-	-	-	-	-	-	-	-	92.86 ± 17.295bc	0.887 ± 0.1224bc	1.164 ± 0.1466a	0.622 ± 0.0051b
2.00	-	-	-	-	-	-	-	-	-	-	-	-	91.790 ± 5.388bc	0.828 ± 0.0464bc	1.141 ± 0.0145a	0.615 ± 0.0086ab
2.25	-	-	-	-	-	-	-	-	-	-	-	-	88.21c	0.888c	1.168a	0.614ab
2.50	-	-	-	-	-	-	-	-	-	-	-	-	82.46c	0.865bc	1.120a	0.612ab
3.50	-	-	-	-	-	-	-	-	-	-	-	-	No peaks were distinguishable			

In Tris.$MgCl_2$ buffer, the TA effect occurred after the addition of 1.75 mg mL^{-1} TA. At this concentration a statistically significant increase was observed in the SS ratio, with the increase in SS being more pronounced in *Z. mays* than in *P. sativum*. At this concentration also the FL ratio increased significantly, because *P. sativum* nuclei lost more fluorescence than those of *Z. mays*. No nuclei peaks could be observed when 3.50 mg mL^{-1} TA was added to the samples (Table IV.2.4).

Effect of incubation time. For Galbraith's buffer, a significant increase in *Z. mays* SS-CV was observed, but no significant differences were detected in the SS, SS-CV and FL ratios, either in TA1 or in TA2. The addition of TA after running samples for 60 s (experiment TA3) confirmed these results (Table IV.2.5).

In LB01, the addition of 0.40 mg mL^{-1} TA was sufficient to induce the TA effect in both TA1 and TA2 experiments, significantly increasing the SS, SS-CV and FL ratios. Interestingly, SS, SS-CV and FL ratios were not affected in TA3 (Table IV.2.5).

In Otto's buffer, a longer incubation time with TA led to a higher SS-CV in *Z. mays* and affected nuclei SS values from both species differently. As expected from the concentration tests, no significant differences were detected in the SS-CV and FL ratios. An unexpected result was obtained in TA3 as no peaks could be distinguished immediately after the addition of TA (Table IV.2.5).

For Tris.$MgCl_2$, the addition of 2.00 mg mL^{-1} TA caused an increase in the *Z. mays* SS-CV, in both TA1 and TA2. This was statistically significant only for TA2. The SS ratio was not affected in both experiments and the SS-CV ratio was only affected when samples were incubated for 15 min with TA. In TA1 and TA2, the FL decreased in both species, although, as the decrease was higher in *P. sativum*, the FL ratio increased. In TA3, a significant increase was only detected for the *Z. mays* SS-CV and SS ratio (Table IV.2.5).

The results obtained with all the buffers and in all experiments were reproducible, and no statistically significant differences were obtained between replicates.

Table IV.2.5 Analysis of *Zea mays* SS-CV (%), SS ratio, SS-CV ratio and FL ratio (mean and standard deviation of the mean) of particles in liquid suspension prepared from *P. sativum* and *Z. mays* leaf tissue with four different buffers (Galbraith's, LB01, Otto's and Tris.$MgCl_2$) exposed to a specific initial concentration (c_i) of tannic acid (TA) and incubated for different periods of time (CTRL – no incubation with TA; TA1 – incubation with TA for 5 min; TA2 – incubation for 20 min with TA). In TA3 samples were run for 60 s without TA (TA3bta) and, after pause, TA was added to samples (TA3ata). Means followed by the same letter are not significantly different according to the Holm-Sidak multiple comparison test at $P \leq 0.05$.

Experiment	Galbraith's buffer (c_i = 0.50 mg mL^{-1})				LB01 buffer (c_i = 0.40 mg mL^{-1})				Otto's buffer (c_i = 1.00 mg mL^{-1})				Tris.$MgCl_2$ buffer (c_i =2.00 mg mL^{-1})			
	Zea mays SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio	*Zea mays* SS-CV (%)	SS ratio	SS-CV ratio	FL ratio
CTRL	38.22 ± 4.079a	0.811 ± 0.0789a	1.143 ± 0.1850a	0.612 ± 0.0017a	36.57 ± 3.795a	0.670 ± 0.0254a	1.009 ± 0.0681a	0.617 ± 0.0052a	31.61 ± 3.261a	0.617 ± 0.0206a	1.113 ± 0.0790a	0.607 ± 0.0013a	39.60 ± 4.122a	0.652 ± 0.0227a	1.119 ± 0.0452a	0.603 ± 0.0151a
TA1	78.98 ± 37.786b	0.798 ± 0.1410a	1.100 ± 0.0914a	0.617 ± 0.0032a	106.43 ± 13.787b	0.773 ± 0.0712b	1.202 ± 0.0544b	0.624 ± 0.0028b	51.99 ± 7.125b	0.674 ± 0.0107b	1.218 ± 0.0885a	0.601 ± 0.0069a	66.75 ± 30.047a	0.725 ± 0.1369a	1.167 ± 0.0819a	0.632 ± 0.0044b
TA2	56.60 ± 21.179c	0.760 ± 0.0197a	1.226 ± 0.2146a	0.616 ± 0.0025a	112.15 ± 16.235b	0.807 ± 0.1304b	1.187 ± 0.0936b	0.624 ± 0.0041b	78.16 ± 15.923c	0.709 ± 0.0418c	1.152 ± 0.0851a	0.599 ± 0.0181a	91.00 ± 5.005b	0.779 ± 0.0428a	1.279 ± 0.0978b	0.634 ± 0.0048b
TA3bta	27.72 ± 12.054a	0.965 ± 0.1064a	1.045 ± 0.1038a	0.613 ± 0.0103a	39.68 ± 8.771a	0.805 ± 0.0488a	1.158 ± 0.0525a	0.612 ± 0.0033a	37.74 ± 4.924	0.629 ± 0.0518	1.105 ± 0.1463	0.613 ± 0.0053	41.23 ± 2.128a	0.665 ± 0.0368a	1.165 ± 0.0476a	0.604 ± 0.0135a
TA3ata	29.51 ± 13.619a	1.049 ± 0.1270a	0.988 ± 0.1480a	0.614 ± 0.0079a	86.27 ± 21.721b	0.798 ± 0.0548a	1.067 ± 0.0861b	0.611 ± 0.0021a	No peaks were distinguishable				72.56 ± 9.478b	0.951 ± 0.1197b	1.110 ± 0.0937a	0.606 ± 0.0212a

Discussion

The negative role of cytosolic compounds on estimation of genome size using FCM in plants has been formally studied only recently (Noirot *et al.* 2000, 2002, 2003, 2005; Price *et al.* 2000; Walker *et al.* 2006). Most of the studies were performed using crude tissue homogenates without analysing specific effects of individual chemical species. The only exception was the work of Noirot *et al.* (2003), which showed that the presence of chlorogenic acid (a precursor of polyphenols) induced a significant decrease in fluorescence of *Petunia hybrida* (Solanaceae) nuclei. These authors also evaluated the effect of caffeine and found that it caused a significant increase on PI fluorescence.

The negative effect of TA, a common phenolic compound, which is frequently accumulated in various plant tissues, was discovered by Greilhuber (1988) who observed that tannins caused stoichiometric errors in Feulgen staining by limiting the access of Schiff's reagent to DNA. The author suggested interaction of tannins with nuclear proteins. In contrast, Price *et al.* (2000) found that soluble tannins isolated from *Helianthus annuus* (Asteraceae) leaves did not inhibit PI fluorescence of pea nuclei isolated in Galbraith's buffer, despite increasing the variance of the peaks. The results herein do not confirm this conclusion and show a strong effect of TA on relative fluorescence intensity of PI stained nuclei. Furthermore, we show for the first time that a cytosolic compound can change light scattering properties of particles in a tissue homogenate.

An increase in SS and a high SS-CV were found to be diagnostic for the presence and action of TA, and the term “tannic acid effect” is given to this phenomenon. The effect is due to the appearance of two new populations of particles in the nuclear suspension, which are characterized by particular side scatter and fluorescence intensity properties. In samples prepared from tissues that are relatively free of cytosolic compounds, a homogeneous population of nuclei with relatively low-scatter CV values (<50.00%) should be observed on a cytogram of FS *vs.* SS. These results imply that, in addition to analysing FL and presenting FL histograms, researchers should also analyse and present the FS *vs.* SS in log scale and/or SS in log scale *vs.* FL cytograms, with the respective CV values obtained for each parameter. When the TA effect is observed, estimates of genome size may be compromised and the results should be interpreted with caution. As the TA effect usually occurs before a change in nuclei fluorescence is observed, it appears to be a safe parameter to identify “problematic” samples.

The increase in nuclei SS observed at higher concentrations of TA was most probably due to the occurrence of precipitates induced by TA. These and the complex structure of TA led to nuclei aggregation. Greilhuber (1986) observed that fixed tannins adhered

tenaciously to cellular structures, particularly to chromatin, strongly interfering with the Feulgen reaction. Other studies also described the effect of TA on protein precipitation (Giner Chavez *et al.* 1997) and membrane aggregation (Simon *et al.* 1994).

The TA effect describes the occurrence of two new and distinct populations of particles in a tissue homogenate. The first consists of nuclei to which weakly fluorescent particles were attached. This population differed from the population of single nuclei by slightly higher FL and higher SS values. The second population was characterized by higher optical complexity and lower FL. This population did not include nuclei and comprised various clumps of weakly fluorescent particles. Thus, at the time the TA effect occurs, three subpopulations of particles are detected by FCM: single nuclei, aggregates of nuclei with unspecific particles, and aggregates of diverse particles devoid of nuclei.

The TA effect was described in this study based on the use of a chemically defined compound. Unfortunately, little is known about the amount of TA naturally occurring in leaves and the amount of TA released after nuclear isolation procedures. Some tissues or plants may differ 100-fold in their polyphenol content. A range of treatments was therefore used from low concentrations with no detectable effect to very high concentrations that made the analyses of DNA content impossible. It will be important to prove that a similar effect is observed when analysing samples from plant tissues containing cytosolic compounds.

Price and Johnston (1996*b*) analysed DNA content in sunflower, which was later shown to contain compounds interfering with DNA staining (Price *et al.* 2000). The authors observed increased variation in DNA peaks on FL histograms and the presence of elevated levels of background debris. In order to improve the resolution of histograms of DNA content, they suggested a gating to discriminate populations of particles with optical properties different from nuclei. However, the results here indicate that even fluorescence from apparently normal populations of nuclei may be affected. Furthermore, Pinto *et al.* (2004) and in Chapter II.1, when working with two woody plants rich in phenolic compounds, *Eucalyptus globulus* (Myrtaceae) and *Quercus suber*, respectively, obtained similar FS and SS patterns. In both species, it was difficult to obtain satisfactory results for mature field leaves as FL histograms were very similar to those obtained in this work. It was also clear that nuclei from target and standard species lost a great deal of fluorescence.

Previous studies on the effect of cytosolic compounds did not analyse the effect of nuclear isolation buffer (Noirot *et al.* 2000, 2002, 2003; Price *et al.* 2000). It was established here that different nuclear isolation buffers gave samples variable resistance

to the negative effect of TA. However, the reasons underlying these differences are not clear. Nuclear suspensions made in LB01 and Galbraith's buffers were more susceptible to TA than those in Otto's and Tris.$MgCl_2$ buffers. Despite being vulnerable to lower concentrations of TA, nuclei isolated with LB01 maintained or even gained fluorescence (although this may be due to the presence of nuclei with attached fluorescent particles). This may be explained by the presence of spermine, which seems to be a better chromatin stabilizer than $MgCl_2$ used in both Galbraith's and Tris.$MgCl_2$ buffers. Otto's buffers do not contain a chromatin stabilizer and the presence of citric acid, which improves chromatin accessibility (Doležel and Bartoš 2005), does not prevent the continuous loss of fluorescence. It is also possible that the higher tendency to clumping in LB01 and Galbraith's buffers in the presence of TA was due to the lower concentration of Triton X-100, which is included to disperse chloroplasts and decrease the tendency of nuclei and cytoplasmatic debris to aggregate (Doležel and Bartoš 2005). Otto's buffer contains the weakest detergent, Tween 20, despite being at the same concentration as Triton X-100 in the Tris.$MgCl_2$ buffer. One possibility may be to use non-ionic detergents at higher concentrations to release chlorophyll from plastids and decrease fluorescence of debris.

In order to avoid the effect of antioxidants, LB01 buffer was used without ß-mercaptoethanol. Because other buffers do not contain antioxidants, the results were not influenced by these compounds. Other precautions were also taken to obtain reproducible results. Following Noirot *et al.* (2000), a greater amount of plant tissue was chopped in appropriate volumes of nuclear isolation buffer to minimize possible intersample variations resulting from chopping time and intensity, and material quantity. Suspensions of plant nuclei thus obtained were then divided into aliquots and subjected to different treatments. In addition, the staining was done with PI at saturating concentrations.

The findings of this study are relevant to standardization procedures. Internal standardization is commonly regarded to be the best procedure for genome size estimation, avoiding errors due to instrument instability and variation in sample preparation and staining. With the aim to eliminate the negative effect of cytosolic compounds, Price *et al.* (2000) and Noirot *et al.* (2000, 2003, 2005) recommended internal standardization, the rationale being that the standard and sample nuclei are influenced to the same extent. However, there is no substantial proof that cytosolic compounds affect staining of nuclei from different species in the same way. When measuring DNA content in *Gossypium* (Malvaceae), Hendrix and Stewart (2005) used Galbraith's buffer to prepare nuclei suspension and obtained different effects when *Oryza sativa* (Poaceae) was used instead

of *Hordeum vulgare* (Poaceae) as internal standard, usually with an increase in fluorescence. The results here show that with the exception of Otto's buffer, nuclei from *P. sativum* were more affected by TA than those of *Z. mays*, leading in some cases to more than a 9.0% stoichiometric error.

In order to counteract the negative effect of cytosolic compounds on DNA staining, it is important to ascertain whether the interaction with DNA occurs immediately or if it develops with time. The results of the current study clearly show that the reaction is very rapid. This explains the results of Noirot *et al.* (2003, 2005), who were not able to eliminate the negative effect of cytosol by centrifugation, dilution and heat treatments of tissue homogenates.

The results demonstrate the importance of nuclear isolation buffer for accurate estimation of genome size. In addition to testing various buffers, selection of tissues with lower or no phenolic compounds should enable unbiased estimations (Suda 2004). If no tissue and buffer combination provides acceptable results, the inclusion of buffer additives counteracting the negative action of cytosol is imperative. The most frequently used compounds are: ß-mercaptoethanol, a powerful and popular reducing agent, but which is forbidden for health reasons in many laboratories; metabisulfite, a more user-friendly antioxidant that is being used instead of ß-mercaptoethanol; and polyvinyl pyrrolidone (PVP-10), a commonly used tannin-complexing agent. Preliminary results with these protectants show that PVP-10 can suppress the TA effect (data not shown). If the TA effect continues to be present even after using additives, the quantity of plant material and chopping intensity should be reduced.

In conclusion, the present study has shown that cytosolic compounds, such as TA, interact with nuclei, potentially causing errors in estimation of nuclear DNA content. It is recommended that the presence of the TA effect is checked by analysing light scattering properties of particles in tissue homogenates. If the TA effect is observed, other buffers should be tested for tissue homogenization as well as various buffer additives. Although there are no ideal solutions, these recommendations, and the results presented here, should contribute to obtaining unbiased estimates of nuclear genome size using FCM.

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IV.3 Two new nuclear isolation buffers for plant DNA flow cytometry – examination with 37 species

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Abstract

Background and Aims After the initial boom in the application of flow cytometry in plant science in late 1980s and early 1990s, which was accompanied by development of many nuclear isolation buffers, only a few efforts were made to develop new buffer formulas. In this work we utilized recent data on the performance of nuclear isolation buffers to develop two nuclear isolation buffers, NIB and WPB, for plant DNA flow cytometry.

Methods The new NIB and WPB buffers were used to prepare samples for flow cytometry of nuclear DNA content in a set of 37 plant species that included herbaceous and woody *taxa* with leaf tissues differing in structure and chemical composition. The following parameters of isolated nuclei were assessed: forward and side light scatter, propidium iodide fluorescence, coefficient of variation of DNA peaks, quantity of debris background, and the number of particles released from sample tissue. Nuclear genome size of 30 selected species was also estimated using a buffer that performed best in a given species.

Key results In the “easy” species, the use of both buffers resulted in high quality samples. The samples obtained with the NIB buffer usually resulted in DNA histograms with higher or similar resolution than those prepared in WPB buffer. In more recalcitrant tissues, such as those coming from woody plants, WPB buffer performed better, while, in some cases, NIB buffer failed to provide acceptable results. Improvement in resolution of DNA content histograms was achieved in most of the analysed species in comparison with previously published buffers.

Conclusions WPB is a reliable buffer, suitable also for the analysis of problematic tissues/species. Although NIB buffer failed with some plant species, it provided high quality DNA histograms in “easy” ones, and its use is recommended in similar situations. We suggest that both buffers are used regularly in the future. Our results indicate that even with a broad range of species, either buffer is suitable for preparation of high quality suspensions of intact nuclei suitable for DNA flow cytometry.

Keywords flow cytometry; genome size; lysis buffers; NIB; nuclear DNA content; nuclear isolation buffer; propidium iodide; woody plant buffer; WPB.

Introduction

Since the introduction of flow cytometry (FCM) to plant sciences in the 1980s, estimation of nuclear DNA content has been the major application of FCM in research, breeding and production (Doležel and Bartoš 2005). The spread of the method was encouraged by the relative simplicity of sample preparation, which typically involves mechanical homogenization of plant tissues in a nuclear isolation buffer (Galbraith *et al.* 1983). The buffer should facilitate isolation of intact nuclei free of adhering cytoplasmic debris, maintain nuclei stability in liquid suspension and prevent their aggregation. It ought to protect nuclear DNA from degradation and provide an appropriate environment for specific and stoichiometric staining of nuclear DNA, including the minimization of negative effects of some cytosolic compounds on DNA staining.

With the aim to fulfil these needs and to analyse nuclear DNA content with the highest resolution, many laboratories developed their own nuclear isolation buffer formulas. The current release of the FLOWER database (http://flower.web.ua.pt/) lists 27 lysis buffers with different chemical composition (Loureiro *et al.* 2007; Chapter V). The usefulness of some of the buffers is difficult to judge as their performance was not analysed thoroughly, nor it was compared with other buffers. However, there are some exceptions and concern mainly the most popular buffers. Thus, de Laat *et al.* (1987) compared their buffer with a commercial solution, analysing coefficient of variation (CV) of DNA peaks and the amount of debris background. Doležel *et al.* (1989) introduced the LB01 buffer by analysing the nuclear DNA content of leaves and *in vitro* cultured *calli* of several plant species. Arumuganathan and Earle (1991*a*) proposed a buffer containing $MgSO_4$ and used it for genome size estimation in over 100 plant species (Arumuganathan and Earle 1991*b*). Marie and Brown (1993) tested their new buffer in approximately 70 plant species. Ulrich and Ulrich (1991) and Doležel and Göhde (1995) showed the usefulness of the so called Otto buffers (Otto 1990) for high resolution analyses of DNA content. Finally, Pfosser *et al.* (1995) tested Tris.$MgCl_2$ buffer by evaluating the sensitivity of DNA flow cytometry to detect aneuploidy in wheat.

A systematic comparison of nuclear isolation buffers was done only recently (Chapter V.2). In this study four of the most common buffers differing in chemical composition [Galbraith (Galbraith *et al.* 1983), LB01, Otto and Tris.$MgCl_2$ buffers] were compared. Among other, these results confirmed the, until then, empirically known fact, *i.e.*, due to the diversity of plant tissues in structure and chemical composition, no single buffer works well with every species (Doležel and Bartoš 2005). Nonetheless, in Chapter IV.1 it was showed that some lysis buffers consistently yielded better results than others, at least with

"easy" species. The same set of buffers was evaluated in a study on the effect of tannic acid, a common phenolic compound, on isolated plant nuclei and estimation of DNA content (Chapter IV.2). The study revealed that tannic acid affected fluorescence and light scatter properties of nuclei in suspension regardless of the isolation buffer. However, the extent of the negative effect of tannic acid was different for each buffer.

Considering the results presented in Chapters IV.1 and IV.2, we elected to develop nuclear isolation buffers suitable for a broader range of plants. In this study we report on two nuclear isolation buffers: nuclear isolation buffer (NIB), and woody plant buffer (WPB), and evaluate them using the same set of plant species and the same parameters as used in Chapter IV.1: light scatter and fluorescence properties of isolated nuclei, presence of debris background, CV of DNA peaks, and the number of nuclei released from sample tissues. In order to achieve a more thorough evaluation of the buffers performance a wide set of plant species representing 37 *taxa* (belonging to 24 different families), including herbaceous and woody plant species with tissues differing in structure and chemical composition, were analyzed. Moreover, the genome size of these *taxa* was estimated using a buffer that performed better in a given species, out of them 10 being new estimations.

Materials and methods

Plant material

Plants of *Coriandrum sativum* (Apiaceae), *Lycopersicon esculentum* 'Stupické' (Solanaceae), *Pisum sativum* 'Ctirad' (Fabaceae) and *Vicia faba* 'Inovec' (Fabaceae) were grown from seeds. Plants of *Festuca rothmaleri* (Poaceae), *Oxalis pes-caprae* (Oxalidaceae) and *Pterospartum tridentatum* (Fabaceae) were kindly provided by Prof. Paulo Silveira, Dr. Sílvia Castro and Eng. Armando Costa (Department of Biology, University of Aveiro, Portugal), respectively. Plants of *Olea europapaea* (Oleaceae), *Quercus robur* (Fagaceae), *Saintpaulia ionantha* (Gesneriaceae) and *Vitis vinifera* (Vitaceae) were available from previous works made in our Laboratory at University of Aveiro. Plants of *Sedum burrito* (Crassulaceae) were obtained from Flôr do Centro Horticultural Centre (Mira, Portugal). All plants were maintained in a greenhouse at 22 ± 2ºC, with a photoperiod of 16 h and a light intensity of 530 ± 2 µmol m^{-2} s^{-1}. Leaves from the remaining *taxa* were collected directly from field-grown individuals in Aveiro and Oporto districts, Portugal, and either analysed immediately or maintained in a refrigerator on a moistened paper for a maximum period of two days until use.

Sample preparation

In each *taxon*, 40-50 mg of young leaf tissue was used for sample preparation. However, in *S. burrito* the quantity of leaf material required to release sufficient number of nuclei had to be increased to approximately 500 mg (Chapter IV.1). Nuclear suspensions were prepared according to Galbraith *et al.* (1983) using the NIB and WPB isolation buffers, NIB and WPB (Table IV.3.1). In each case, one mL of buffer solution was added to a Petri dish containing the plant tissue, which was chopped using a sharp razor blade for approximately 60 s. For genome size estimations, the buffer that performed better in a particular species was chosen and leaf tissue was chopped simultaneously with that of appropriate DNA reference standard (Table IV.3.3). The resulting nuclear suspension was filtered through 80 µm nylon filter to remove large debris. Nuclei were stained with 50 µg mL^{-1} propidium iodide (PI; Fluka, Buchs, Switzerland), and 50 µg mL^{-1} RNase (Fluka) was added to nuclear suspension to prevent staining of double stranded RNA. Samples were incubated on ice and analyzed within 10 min.

Table IV.1.3 Chemical composition of the NIB and WPB nuclei isolation buffers.

Buffer	Composition[1]
NIB	0.5 mM spermine.4HCl, 30 mM sodium citrate.$3H_2O$, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0.5% (v/v) Triton X-100, pH 7.0
WPB	0.2 M Tris.HCl, 4mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% (w/v) PVP-10, 1% (v/v) Triton X-100, pH 7.5

[1]Final concentrations are given. Both buffers should be stored in aliquots at 4 ºC. MOPS – 4-morpholinepropane sulfonate; TRIS – tris-(hydroxymethyl)-aminomethane; EDTA – ethylenediaminetetraacetic acid.

Flow cytometric analyses

Samples were analyzed with a Coulter EPICS XL (Beckman Coulter®, Hialeah, FL, USA) flow cytometer equipped with an air cooled argon-ion laser tuned to 15 mW and operating at 488 nm. Fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. The results were acquired using the SYSTEM II software (version 3.0, Beckman Coulter®). The instrument settings (amplification and sample rate) were kept constant throughout the experiment. For each species analysed in Chapter IV.1, the instrument settings in the present work were the same as in Chapter IV.1.

For each sample, the following parameters were analysed: forward scatter (FS) as a measure of particle's relative size, side scatter (SS) as a measure of particle's relative optical complexity, relative fluorescence intensity of PI stained nuclei (FL), half peak coefficient of variation (%) of the G_0/G_1 peak as a measure of nuclear integrity and variation in DNA staining, a debris background factor (DF in %) as a measure of sample

quality, and a nuclear yield factor (YF in nuclei s^{-1} mg^{-1}) in order to compare the quantity of nuclei in suspension independently of the amount of sample tissue used. The latter three parameters were determined according to Chapter IV.1. Histograms of FL obtained with each buffer were overlaid using WinMDI software (Trotter 2000; Fig. IV.3.1).

In each species, five replicates were performed on three different days for each buffer. In each replicate at least 5,000 nuclei were analyzed.

For genome size estimations, three replicates on three different days were made using the buffer that performed better in a given species. Nuclear DNA content of each species was calculated according to the formula:

$$\text{Sample 2C nuclear DNA content (pg)} = \frac{\text{sample } G_0/G_1 \text{ FL mean}}{\text{reference standard } G_0/G_1 \text{ FL mean}} \times \text{2C nuclear DNA content of reference standard}$$

Conversion of mass values into number of base pairs was done according to the factor 1 pg = 978 Mbp (Doležel *et al.* 2003).

Statistical analyses

Differences between buffers for each parameter were analysed using a t-test (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA).

Results

Chemical composition of NIB and WPB nuclear isolation buffers

The chemical compositions of NIB and WPB buffers are based on the observations from our previous studies obtained in Chapters IV.1 and IV.2, and were developed in a series of preliminary tests. The NIB buffer includes components of popular buffers: LB01 (spermine, KCl and NaCl), Galbraith (MOPS and sodium citrate) and Tris.$MgCl_2$ (higher concentration of Triton X-100) buffers. The WPB buffer is mostly based on Tris.$MgCl_2$ buffer (Tris.HCl, $MgCl_2.6H_2O$ and Triton X-100 in higher concentration) in which the least pronounced effects of tannic acid were observed (Chapter IV.2), with addition of components of LB01 (NaCl and $EDTA.Na_2.2H_2O$), metabisulfite and pyrrolidone (PVP-10).

Performance of nuclear isolation buffers

Testing the two new buffers with 37 plant species revealed pronounced differences (Table IV.3.2). Out of the seven species that were analyzed in Chapter IV.1 (highlighted in Table IV.3.2), the use of both buffers resulted in good DNA content histograms in *F. rothmaleri*,

O. pes-caprae and *S. burrito*, and very good histograms in *L. esculentum*, *P. sativum* and *V. faba*. The only exception was *Celtis australis* in which measurable samples were only obtained with the WPB buffer. Out of the remaining 30 *taxa*, the NIB buffer yielded acceptable histograms in only 15 of them (*i.e.*, 50% success rate), while the WPB buffer worked well with all 30 species. Whereas the NIB performed better than WPB in 57.1% of the original set of seven species (Chapter IV.1), in the remaining 15 taxa where both buffers worked well, it was only better in *Allium triquetrum* (Alliaceae) and *Euphorbia peplus* (Euphorbiaceae). The better performing buffer was usually characterized by higher FL and YF and lower CV and BF values.

The yield factor (YF) was the parameter where more statistically significant differences were detected between both buffers (47.6% of the species). With exception of *E. peplus*, the observed differences were due to higher yield observed with WPB. Also, when statistically significant differences were observed for the FL parameter (*i.e.*, in 42.8% of the cases), those were due to a higher fluorescence of nuclei isolated with WPB than with NIB.

In 18 species, the CV values were lower than 3.0%, while in the remaining ones the values ranged from 3.0 to 5.0%. The lowest CV values were observed after analyzing *A. triquetrum* nuclei isolated with the WPB buffer (mean CV value = 1.79%). Statistical analysis revealed that in contrast to YF and FL, CV values were more homogenous between buffers, with significant differences between both buffers being only detected in four species. Major differences in CV were detected in *Ilex aquifolium* (Aquifoliaceae; 2.57% and 4.10% for WPB and NIB, respectively), and in *V. vinifera* (Vitaceae; 3.57% and 4.77% for WPB and NIB, respectively). Despite significant differences were detected in the remaining two species, *O. europaea* and *Magnolia* x *soulangiana* (Magnoliaceae), the CV values were low (< 3%) with any buffer.

When evaluating the BF, significant differences between lysis buffers were only observed in five species, *C. sativum*, *Magnolia* x *soulangiana*, *O. europaea*, *P. sativum* and *V. faba*. With the exception of *Magnolia* x *soulangiana*, samples isolated with the NIB buffer exhibited higher debris background. Although the BF values differed in *Magnolia* x *soulangiana*, *P. sativum* and *V. faba*, they were among the lowest values obtained in this study. Contrarily, the species with the highest background debris were *Tamarix Africana* (Tamaricaceae), *E. peplus*, *Chamaecyparis lawsoniana* (Cupressaceae) and *Salix babylonica* (Salicaceae), with values being usually higher than 30%. In most of the other species, BF values ranged between 10 and 20%.

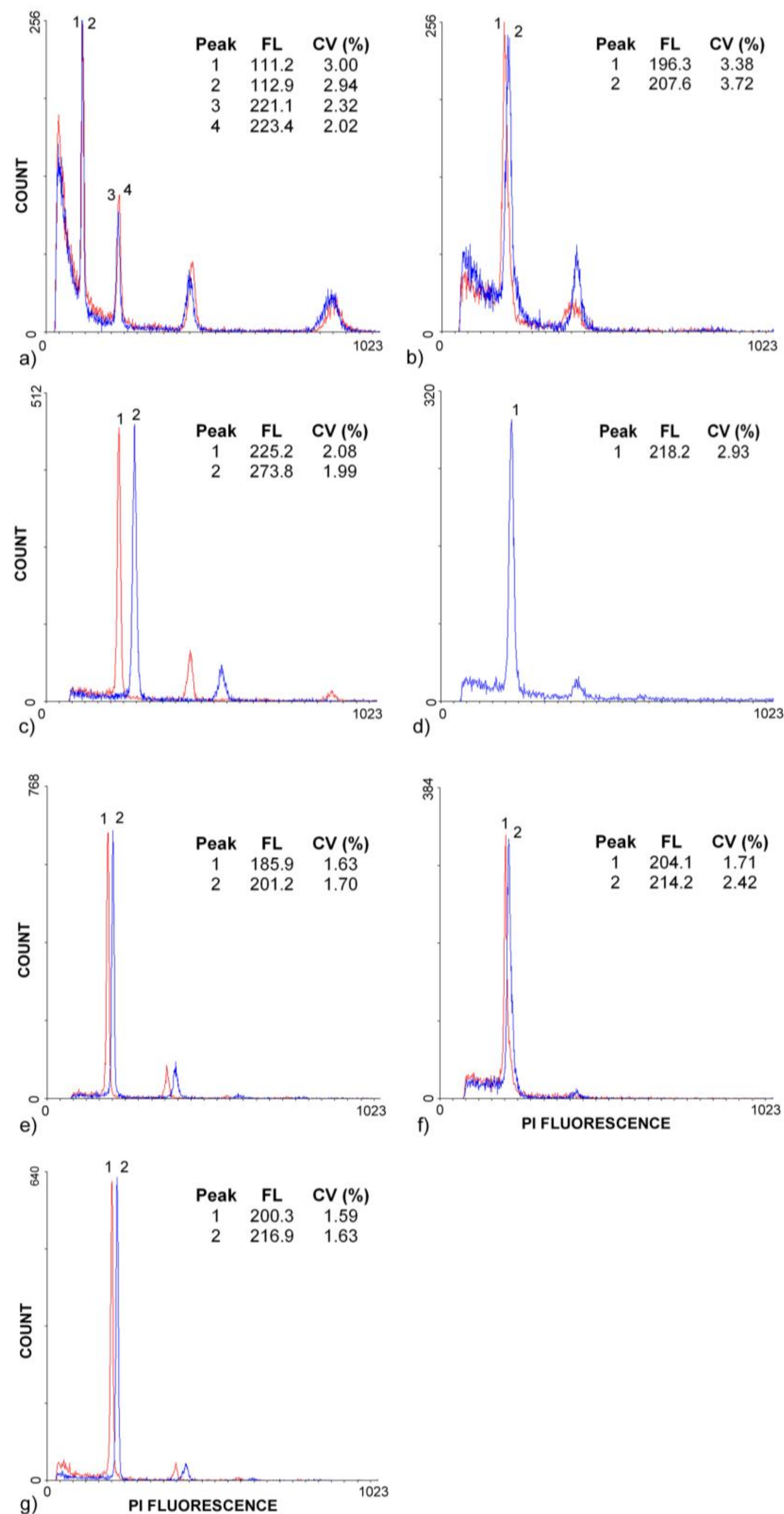


Fig. IV.3.1 Histograms of relative fluorescence intensities (PI fluorescence, relative units) with overlays of distributions obtained with the nuclei isolation buffer (NIB, red) and the woody plant buffer (WPB, blue) for each species: a) *Sedum burrito*; b) *Oxalis pes-caprae*; c) *Lycopersicon esculentum*; d) *Celtis australis*; e) *Pisum sativum*; f) *Festuca rothmaleri*; g) *Vicia faba*. Mean channel numbers (FL) and coefficients of variation (CV, %) of G_0/G_1 DNA peaks are given.

Table IV.3.2 Buffers comparison test. Flow cytometric parameters assessed in each species[1].

Taxa	G.t.	Buffer	FS (channel units)		SS (channel units)		FL (channel units)		CV (%)		BF (%)		YF (nuclei s^{-1} mg^{-1})	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acer negundo	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	29.16	3.050	26.14	10.235	234.3	17.16	3.24	0.346	16.45	1.621	0.79	0.267
Actinidia d eliciosa	H	NIB	5.85a	2.532	10.56a	2.292	197.1a	10.28	3.02a	0.186	14.00a	4.151	0.45a	0.174
		WPB	16.60b	3.932	13.74a	3.015	210.2a	8.45	2.76a	0.365	12.22a	0.817	0.84b	0.563
Allium triquetrum	H	NIB	9.02a	1.494	5.12a	1.749	194.9a	2.56	1.79a	0.379	12.47a	2.392	0.22a	0.082
		WPB	12.88b	1.527	4.77a	1.189	194.2a	3.24	2.14a	0.293	10.51a	2.075	0.35b	0.073
Aloysia triphylla	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	10.13	1.127	6.88	0.782	203.2	11.60	2.93	0.373	25.68	6.132	0.63	0.114
Chamaecyparis lawsoniana	W	NIB	24.72a	7.405	33.82a	20.371	193.1a	4.80	2.56a	0.248	36.18a	5.533	0.10a	0.039
		WPB	14.88b	1.472	16.82a	0.642	192.8a	3.09	2.48a	0.169	35.26a	1.369	0.13a	0.054
Celtis australis	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	54.25	16.273	9.59	3.952	208.0	13.77	2.99	0.383	21.31	4.762	0.30	0.204
Citrus limon	W	NIB	2.29a	1.860	3.34a	0.564	171.3a	5.43	3.62a	0.256	16.50a	3.859	1.00a	0.387
		WPB	4.15a	1.887	3.45a	0.689	170.6a	8.06	3.75a	0.304	12.09a	2.665	1.13a	0.298
Citrus sinensis	W	NIB	1.44a	2.054	6.00a	3.903	174.9a	7.04	3.75a	0.344	18.00a	6.445	0.89a	0.259
		WPB	4.36a	2.142	10.83a	3.130	192.1b	14.55	4.29a	1.252	17.28a	6.572	1.30b	0.245
Coriandrum sativum	H	NIB	19.52a	2.578	18.90a	4.257	206.1a	4.32	2.69a	0.697	26.56a	9.429	0.89a	0.395
		WPB	18.92a	6.038	29.58b	7.990	199.8a	7.05	2.13a	0.118	12.84b	1.477	2.16b	0.720
Diospyros kaki	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	21.28	6.424	13.96	6.413	205.7	4.78	2.09	0.200	26.36	3.922	0.68	0.244
Euphorbia peplus	H	NIB	15.10a	3.491	15.36a	6.502	202.2a	3.01	3.66a	0.134	36.12a	4.499	2.74a	0.761
		WPB	9.72b	0.927	12.26a	1.948	221.1b	4.87	4.00a	0.577	38.80a	2.431	2.11b	0.410
Festuca rothmaleri	H	NIB	12.76a	2.201	7.91a	1.018	205.4a	14.69	2.59a	0.595	9.67a	1.966	0.21a	0.149
		WPB	15.52a	6.115	15.03b	3.495	209.5a	8.69	3.25a	0.752	10.33a	5.457	0.59b	0.349
Ficus carica	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	20.60	5.003	8.77	3.494	214.9	4.56	4.16	0.307	26.98	2.159	0.46	0.114
Forsythia x *intermedia*	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	44.42	2.307	26.62	8.784	198.2	10.71	2.70	0.362	10.00	1.011	1.07	0.342
Ginkgo biloba	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	10.84	2.140	20.42	4.354	196.6	8.80	2.35	0.388	19.98	5.064	0.32	0.223
Ilex aquifolium	W	NIB	15.03a	1.556	20.70a	6.290	194.0a	19.93	4.10a	0.913	18.48a	4.639	1.01a	0.722
		WPB	9.50b	1.650	22.32a	15.576	271.4b	13.65	2.57b	0.343	19.16a	2.618	1.20a	0.632
Laurus nobilis	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	19.88	7.225	3.65	1.443	235.5	5.40	2.35	0.597	25.78	4.959	0.84	0.556
Lycopersicon esculentum	H	NIB	7.61a	1.097	1.14a	0.395	232.9a	6.68	2.31a	0.490	15.16a	1.849	1.00a	0.456
		WPB	11.53b	1.299	1.72a	0.780	264.8b	7.87	2.23a	0.144	14.36a	1.503	1.33a	0.403
Magnolia x *soulangiana*	W	NIB	28.60a	2.330	22.13a	3.917	141.3a	9.48	2.90a	0.809	4.13a	1.251	0.90a	0.176
		WPB	28.58a	2.351	24.60a	2.127	199.5b	4.00	1.80b	0.121	9.26b	2.383	0.80a	0.215
Malus x *domestica*	W	NIB	8.83a	2.453	26.94a	13.226	202.8a	2.32	3.70a	0.655	17.98a	0.335	1.10a	0.375
		WPB	9.84a	2.072	15.14a	2.304	214.2b	5.14	3.24a	0.455	16.82a	3.804	1.89a	0.815
Olea europaea ssp. *europaea*	W	NIB	16.56a	4.368	11.26a	4.033	178.1a	10.53	2.97a	0.562	23.72a	3.025	0.16a	0.056
		WPB	12.38a	0.968	12.22a	1.657	210.3b	5.02	2.18b	0.259	17.28b	1.299	0.33b	0.097
Oxalis pes-caprae	H	NIB	54.50a	19.493	9.00a	5.103	198.7a	11.68	3.29a	0.292	8.94a	2.480	0.68a	0.162
		WPB	66.19a	34.878	11.34a	5.474	206.3a	4.97	3.80a	1.061	11.94a	3.991	0.64a	0.150
Papaver rhoeas	H	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	19.98	3.333	20.28	6.257	199.2	10.11	2.65	0.526	23.00	14.392	0.24	0.096
Pinus pinea	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	57.58	10.570	98.14	28.467	185.6	12.45	3.09	0.300	22.88	5.228	0.03	0.024
Prunus domestica	W	NIB	4.25a	0.978	3.76a	0.525	189.9a	1.74	4.35a	0.457	21.98a	3.127	1.64a	0.375
		WPB	10.45b	2.271	6.04b	1.376	204.6a	4.99	4.24a	0.528	22.98a	4.757	1.82a	0.423
Pisum sativum	H	NIB	12.59a	2.431	4.50a	2.431	185.1a	4.25	1.79a	0.232	11.98a	2.618	0.62a	0.226
		WPB	32.34b	2.951	6.49a	2.951	195.5b	5.71	1.92a	0.180	7.39b	1.214	1.15b	0.285
Prunus persica	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	8.87	2.124	8.38	2.164	219.0	8.13	4.91	0.700	21.22	4.985	1.31	0.462
Pterospartum tridentatum	H	NIB	31.84a	12.800	45.86a	15.990	196.8a	6.94	3.25a	0.764	29.40a	2.176	1.18a	0.775
		WPB	12.38b	2.655	13.82b	1.630	201.5a	5.89	2.71a	0.320	29.38a	3.314	0.87a	0.641
Pyrus communis	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	8.26	2.985	14.59	3.965	203.0	5.12	3.20	0.361	18.26	3.238	1.66	0.234

Continued

Table IV.3.2 Continued[1].

Taxa	G.t.	Buffer	FS (channel units)		SS (channel units)		FL (channel units)		CV (%)		BF (%)		YF (nuclei s^{-1} mg^{-1})	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Quercus robur	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	13.96	1.754	19.86	0.896	212.7	8.29	2.76	0.747	26.64	5.194	0.56	0.410
Rosa sp.	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
		WPB	20.44	3.989	12.17	4.414	200.1	5.48	2.46	0.347	18.98	2.491	1.52	0.581
Saintpaulia	H	NIB	-	-	-	-	-	-	-	-	-	-	-	-
ionantha		WPB	12.25	1.756	22.76	3.890	204.7	5.22	3.42	0.312	22.32	1.988	0.47	0.167
Salix	W	NIB	10.80a	3.908	8.62a	4.985	192.9a	8.97	3.45a	0.178	32.52a	3.858	1.30a	0.456
babylonica		WPB	6.53b	0.537	4.24a	1.911	194.0a	7.64	3.17a	0.432	26.24a	5.262	1.53a	0.878
Sedum burrito	C	NIB	8.34a	1.903	0.55a	0.340	114.0a	3.21	3.00a	0.505	54.64a	6.941	0.10a	0.026
		WPB	12.81b	0.919	0.58a	0.162	113.1a	4.23	3.24a	0.323	49.96a	11.967	0.09a	0.043
Tamarix	W	NIB	-	-	-	-	-	-	-	-	-	-	-	-
africana		WPB	26.60	3.106	22.88	4.913	208.8	5.50	2.75	0.277	39.00	4.463	0.96	0.313
Vicia faba	H	NIB	36.84a	7.264	4.46a	1.038	202.3a	4.74	1.60a	0.232	7.25a	2.436	0.21a	0.120
		WPB	74.35b	11.331	6.49b	1.330	212.3b	5.19	1.72a	0.187	6.45b	1.250	1.03b	0.348
Vitis vinifera	W	NIB	7.33a	3.091	3.99a	1.949	206.6a	6.21	4.77a	0.422	27.46a	8.234	0.65a	0.207
		WPB	4.32a	0.899	2.67a	1.440	213.1a	8.25	3.57b	0.202	21.86a	6.917	1.29b	0.494

[1] Values are given as mean and standard deviation of the mean (SD) of forward scatter (FS, channel units); side scatter (SS, channel units); fluorescence (FL, channel units); coefficient of variation of G_0/G_1 DNA peak (CV, %); background factor (BF, %) and yield factor (YF, %). Means followed by the same letter (a or b) are not statistically different according to a t-test at $P \leq 0.05$. The seven species that were analyzed in Chapter IV.1 are highlighted and the buffer chosen for the genome size estimations in each species is shown in bold type. G.t. – growth type.

Nuclei isolated with any buffer differed more in FS than in SS. Out of the 21 species where both buffers worked well, FS values were significantly different in 11 species, while only in five species this was observed for SS. *P. tridentatum*, *Prunus domestica* (Rosaceae) and *V. faba* were the only species with statistical significant differences between buffers, for both parameters. No visible correlation between buffer type and nuclei relative size and complexity could be observed.

Estimation of nuclear genome size

Table IV.3.3 list C-values for 30 species as determined in this study, five of which are first estimations using FCM and 10 are new estimates. The buffer that performed better with each species was selected to estimate its genome size.

As expected, the mean CV values of DNA peaks (Table IV.3.3) were generally within the range of values obtained in the first part of the study (Table IV.3.2, Fig. IV.3.2). Also, the standard deviation (SD) values were very low, with only one species presenting a SD value higher than 4% (*Rosa* sp., Rosaceae; 4.06%), revealing that the three replicates performed per species in three different days yielded highly homogenous estimations of nuclear DNA content.

Table IV.3.3 Genome size estimations in selected plant species[1].

		Nuclear DNA content (pg/2C)						
		This work				Previous estimations		
Species	Family	2C (pg)	1C (Mbp)[2]	CV (%)	Stand.	pg/2C	Method	Reference
Acer negundo L.	Aceraceae	1.07 ± 0.033	525	3.14	*L.e.*	N.D.		
Actinidia deliciosa (A. Chev.) C.F. Liang & A.R. Furguson	Actinidaceae	4.80 ± 0.058	2,349	2.77	*P.s.*	4.45	FCM:PI	Hopping 1994
						3.97	FCM:PI	Ollitrault *et al.* 1994*b*
Allium triquetrum L.	Liliaceae	38.15 ± 0.381	18,655	2.02	*V.f.*	36.30	Fe	Jones and Rees 1968
						39.30	Fe	Labani and Elkington 1987
Aloysia triphylla (L'Hér.) Britton	Verbenaceae	1.47 ± 0.014	720	2.79	*L.e.*	N.D.		
Chamaecyparis lawsoniana (Murr.) Parl.	Cupressaceae	21.01 ± 0.151	10,274	2.95	*V.f.*	23.05	FCM:PI	Hizume *et al.* 2001
						30.10	Fe	Ohri and Khoshoo 1986
Citrus limon (L.) Burm. f.	Rutaceae	0.84 ± 0.005	409	3.74	*L.e.*	0.80	FCM:PI	Ollitrault *et al.* 1994*a*
						0.77, 0.80	FCM:PI	Kayim *et al.* 1998
						0.77–1.15	FCM:PI	Iannelli *et al.* 1998
						1.24, 1.30	FCM:PI	Capparelli *et al.* 2004
Citrus sinensis (L.) Osbeck	Rutaceae	0.87 ± 0.003	425	4.02	*L.e.*	0.75	FCM	Ollitrault *et al.* 1994*a*
						0.76, 0.85	FCM	Kayim *et al.* 1998
						0.76, 0.82	FCM	Arumuganathan and Earle 1991*b*
						1.20	Fe	Nagl *et al.* 1983
						1.24	Fe	Guerra 1984
Coriandrum sativum L.	Apiaceae	5.08 ± 0.105	2,483	2.60	*P.s.*	4.10	Fe	Olszewska and Osiecka 1983
						7.65, 9.55	Fe	Das and Mallick 1989
						8.85, 9.45	Fe	Chattopadhyay and Sharma 1990
Diospyros kaki L.f.	Ebenaceae	5.08 ± 0.002	2,482	2.27	*P.s.*	N.D.		
Euphorbia peplus L.	Euphorbiaceae	0.69 ± 0.004	335	4.50	*L.e.*	N.D.		
Ficus carica L.	Moraceae	0.73 ± 0.030	356	4.20	*L.e.*	1.41	Fe	Ohri and Khoshoo 1987
Forsythia x *intermedia* Zabel	Oleaceae	2.01± 0.007	985	3.22	*G.m.*	N.D.		
Ginkgo biloba L.	Ginkgoaceae	22.85 ± 0.154	11,172	2.48	*V.f.*	19.50	FCM:EB	Marie and Brown 1993
						21.60	FCM:PI	Barow and Meister 2002
						19.86	Fe	Ohri and Khoshoo 1986
Ilex aquifolium L.	Aquifoliaceae	1.93 ± 0.038	944	2.89	*G.m.*	N.D.		
Laurus nobilis L.	Lauraceae	6.50 ± 0.089	3,215	2.26	*Z.m.*	6.10	FCM :PI	Zonneveld *et al.* 2005
Magnolia x *soulangiana* Soul. Bod.	Magnoliaceae	9.83 ± 0.002	4,806	2.43	*Z.m.*	11.95	Fe	Nagl *et al.* 1977
						14.20	Fe	Olszewska and Osiecka 1983
Malus x *domestica* (Borkh.) Borkh.	Rosaceae	1.56 ± 0.023	765	3.39	*L.e.*	1.50–2.86[3]	FCM:PI	Dickson *et al.* 1992
						1.52–2.48[3]	FCM:PI	Tatum *et al.* 2005
Olea europaea L. ssp. *europaea*	Oleaceae	3.24 ± 0.020	1,583	3.80	*P.s.*	4.40, 4.52	Fe	Rugini *et al.* 1996
						3.90–4.66	Fe	Bitonti *et al.* 1999
						2.97–3.07	FCM:PI	Loureiro *et al.* 2007*b*

Continued

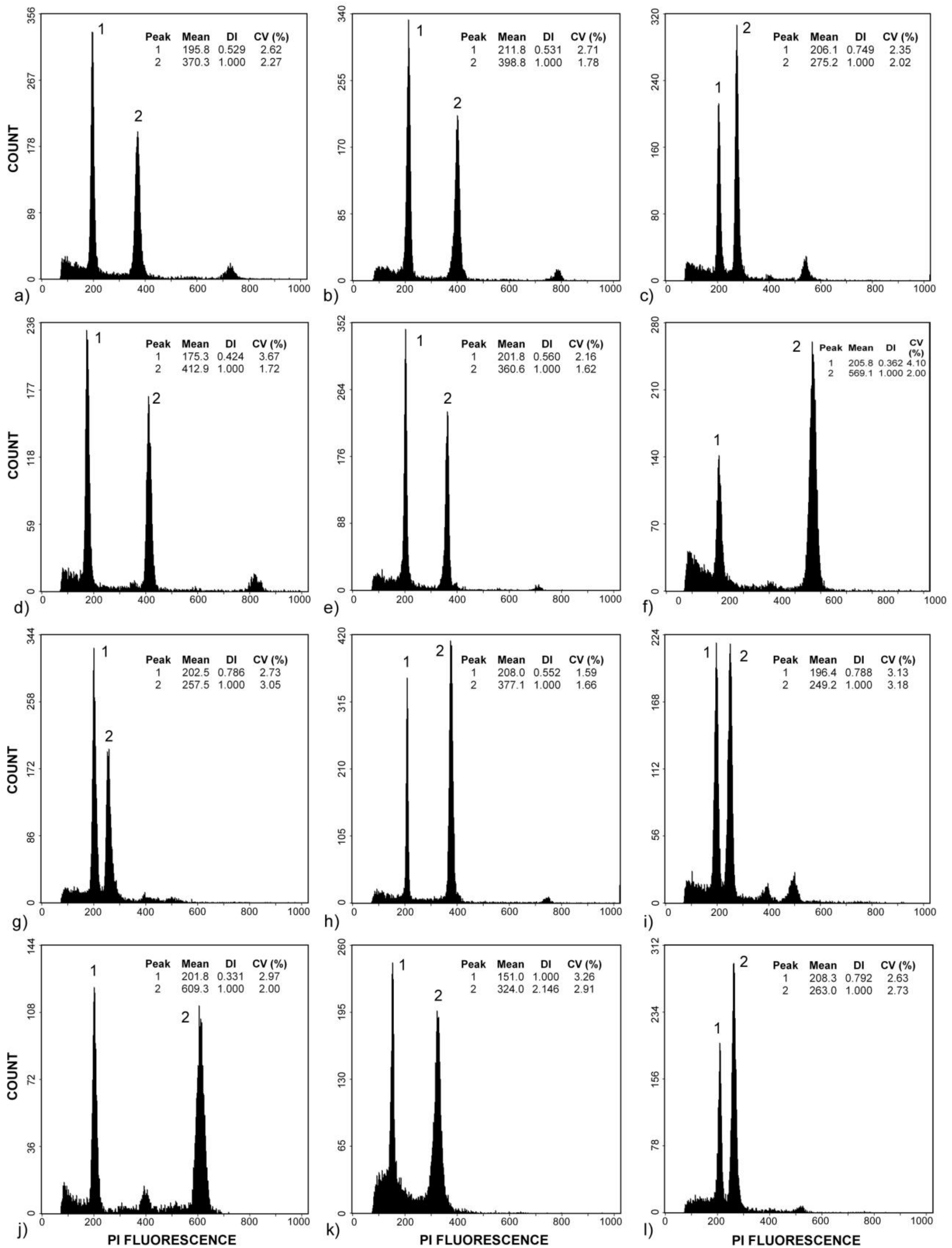
Table IV.3.3 Continued[1].

Species	Family	Nuclear DNA content (pg/2C)						
		This work				Previous estimations		
		2C (pg)	1C (Mbp)[2]	CV (%)	Stand.	pg/2C	Method	Reference
Papaver rhoeas L.	Papaveraceae	11.00 ± 0.076	5,378	1.95	*Z.m.*	5.20	Fe	Nagl *et al.* 1983
						5.25	Fe	Bennett and Smith 1976
						7.14	Fe	Srivastava and Lavania 1991
Pinus pinea L.	Pinaceae	56.09 ± 1.826	27,429	3.34	*V.f.*	60.80	FCM:PI	Grotkopp *et al.* 2004
Prunus domestica L.	Rosaceae	0.66 ± 0.010	323	4.10	*L.e.*	0.61	FCM:PI	Arumuganathan and Earle 1991*b*
Prunus persica (L.) Batsch	Rosaceae	0.62 ± 0.010	303	4.30	*L.e.*	0.54, 0.55	FCM:PI	Arumuganathan and Earle 1991*b*
						0.54, 0.55	FCM:PI	Dickson *et al.* 1992
						0.57–0.64	FCM:PI	Baird *et al.* 1994
Pterospartum tridentatum (L.) Willk.	Fabaceae	4.64 ± 0.050	2,269	2.92	*Z.m.*	N.D.		
Pyrus communis L.	Rosaceae	1.24 ± 0.028	605	3.00	*L.e.*	1.03, 1.11	FCM:PI	Arumuganathan and Earle 1991*b*
						1.11	FCM:PI	Dickson *et al.* 1992
Quercus robur L.	Fagaceae	1.98 ± 0.057	968	2.88	*G.m.*	1.85	FCM:EB	Favre and Brown 1996
						1.90	FCM:EB	Zoldoš *et al.* 1998
Rosa sp.	Rosaceae	2.46 ± 0.104	1,204	2.89	*Z.m.*	0.78–3.04[3]	FCM:PI	Yokoya *et al.* 2000
						0.20–1.65[3]	FCM:PI	Dickson *et al.* 1992
						0.25–1.30[3]	Fe	Bennett and Smith 1976
						2.85*	Fe	Greilhuber 1988
Saintpaulia ionantha Wendl.	Gesneriaceae	1.50 ± 0.017	732	3.41	*L.e.*	N.D.		
Salix babylonica L.	Salicaceae	1.61 ± 0.008	786	2.65	*L.e.*	N.D.		
Tamarix africana Poir.	Tamaricaceae	3.30 ± 0.026	1,612	2.66	*Z.m.*	N.D.		
Vitis vinifera L.	Vitaceae	1.19 ± 0.025	583	2.86	*L.e.*	1.00	FCM:PI	Arumuganathan and Earle 1991*b*
						1.60	FCM:HO	Faure and Nougarede 1993
						0.86-1.00	FCM	Lodhi and Reisch 1995
						1.17–1.26	FCM:PI	Leal *et al.* 2006

[1] Values are given as mean and standard deviation of the mean of nuclear DNA content in mass values (2C, pg) and base pairs (1C, Mbp). The coefficient of variation (CV, %) of G_0/G_1 peaks and the reference standard used to estimate the genome size in each species are also given. For each species, previous 2C nuclear DNA content estimations together with the used methodology (Fe – Feulgen microdensitometry; FCM – flow cytometry; PI – propidium iodide; EB – ethidium bromide; HO – Hoechst 33342) and original reference are also provided. *L.e.*, *Lycopersicon esculentum* cv. Stupické (2C = 1.96 pg DNA; Doležel *et al.* 1992); *G.m.*, *Glycine max* cv. Polanka (2C = 2.50 pg DNA; Doležel *et al.* 1994); *Z.m.*, *Zea mays* cv. CE-777 (2C = 5.43 pg DNA; Lysák and Doležel 1998); *P.s.*, *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA; Doležel *et al.* 1998); *V.f.*, *Vicia faba* cv. Inovec (2C = 26.90 pg DNA; Doležel *et al.* 1992). N.D. – not determined.

[2] 1 pg = 978 Mbp (Doležel *et al.* 2003).

[3] These values reflect differences in the ploidy level of individuals.



a)
Peak Mean DI CV (%)
1 195.8 0.529 2.62
2 370.3 1.000 2.27
b)
Peak Mean DI CV (%)
1 211.8 0.531 2.71
2 398.8 1.000 1.78
c)
Peak Mean DI CV (%)
1 206.1 0.749 2.35
2 275.2 1.000 2.02
d)
Peak Mean DI CV (%)
1 175.3 0.424 3.67
2 412.9 1.000 1.72
e)
Peak Mean DI CV (%)
1 201.8 0.560 2.16
2 360.6 1.000 1.62
f)
Peak Mean DI CV (%)
1 205.8 0.362 4.10
2 569.1 1.000 2.00
g)
Peak Mean DI CV (%)
1 202.5 0.786 2.73
2 257.5 1.000 3.05
h)
Peak Mean DI CV (%)
1 208.0 0.552 1.59
2 377.1 1.000 1.66
i)
Peak Mean DI CV (%)
1 196.4 0.788 3.13
2 249.2 1.000 3.18
j)
Peak Mean DI CV (%)
1 201.8 0.331 2.97
2 609.3 1.000 2.00
k)
Peak Mean DI CV (%)
1 151.0 1.000 3.26
2 324.0 2.146 2.91
l)
Peak Mean DI CV (%)
1 208.3 0.792 2.63
2 263.0 1.000 2.73
COUNT
PI FLUORESCENCE

The species studied in this work represent a wide range of genome sizes, ranging from 0.62 pg DNA/2C in *Prunus persica* to 56.09 pg DNA/2C in *Pinus pinea* (Pinaceae). Following the genome size classes defined by Soltis *et al.* (2003), most of the species studied in this work (63.3%) belong to the “small” (>1.4 to ≤3.5 pg) or “very small” (≤1.4 pg) categories. In seven species (23.3%) “intermediate” (>3.5 to ≤14.0 pg) genome sizes were found and only four species (13.4%) are characterized by “large” (>14.0 to ≤35.0 pg) or “very large” (>35.0 pg) genome sizes. While in some species our assessments were in close agreement with previous reports considerable differences were observed in other cases with most of the discrepancies concerning the results obtained with Feulgen microdensitometry (Table IV.3.3).

Discussion

After the initial period of development of plant DNA flow cytometry in the late 1980s and early 1990s, less attention was paid to the improvement of nuclear isolation buffers. Our recent studies (Chapters IV.1 and IV.2) provided quantitative data on the performance of the most popular isolation buffers and showed that none of them worked well with all species that represented different types of leaf tissues and different nuclear genome sizes. It was also clear that the chemical composition was important to cope with the negative effect of tannic acid, a frequent component of plant cytosol. The results of these studies prompted us to develop new buffers.

These NIB and WPB buffers described in this work provided good results for many of the set of 37 species. However, while it was possible to isolate nuclei in good condition from any species using the WPB buffer, the success of NIB buffer seemed highly dependent on the growth type, as in most woody plants this buffer failed. On the other hand, in “easy” species this buffer resulted in samples of similar or higher quality than those obtained with WPB.

Fig. IV.3.2 (left) Histograms of relative fluorescence intensities (PI fluorescence, relative channel numbers) obtained after simultaneous analysis of nuclei isolated from sample and internal reference standard, using the best performing buffer (see Table IV.3.2). Key: a) *Acer negundo*; b) *Actinidia deliciosa*; c) *Aloysia triphylla*; d) *Citrus limon*; e) *Diospyros kaki*; f) *Ficus carica*; g) *Ilex aquifolium*; h) *Magnolia* x *soulangiana*; i) *Malus* x *domestica*; j) *Pinus pinea*; k) *Prunus domestica*; l) *Quercus robur*. The following reference standards were used: a, c, d, f, i and k) *Lycopersicon esculentum* cv. Stupické (2C = 1.96 pg DNA); g and l) *Glycine max* cv. Polanka (2C = 2.50 pg DNA); h) *Zea mays* cv. CE-777 (2C = 5.43 pg DNA); b and e) *Pisum sativum* cv. Ctirad (2C = 9.09 pg DNA); j) *Vicia faba* cv. Inovec (2C = 26.90 pg DNA). Mean channel numbers (Mean), DNA index (DI, defined as the mean channel number of sample / mean channel number of reference standard) and coefficients of variation (CV, %) of G_0/G_1 DNA peaks are given.

Woody plants are considered recalcitrant for DNA FCM as their tissues often contain cytosolic compounds that interfere with fluorescent staining of nuclear DNA (Noirot *et al.* 2000, 2005; Chapter IV.2). This was the case of most of the species where NIB was ineffective, as an effect similar to the "the tannic acid effect" described in Chapter IV.2 was observed on the SS *vs.* FL cytograms (Figure IV.3.3). The addition of metabisulfite (an antioxidant) and PVP-10 (a tannin-complexing agent) to WPB buffer seemed essential for its success in species where NIB failed and for the overall good performance of the buffer. Metabisulfite, PVP-10, and other compounds with similar properties (*e.g.*, ß-mercaptoethanol, ascorbic acid) were previously used to counteract the negative interference of cytosolic compounds on nuclei fluorescence in oak (Zoldoš *et al.* 1998), rose (Yokoya *et al.* 2000) and olive (Chapter III.1). Antioxidants keep phenolics in a reduced state, enabling the reversibility of the free hydrogen bonds and its resolution by an added competitor (usually PVP-10 or PVP-40; Greilhuber *et al.* 2007).

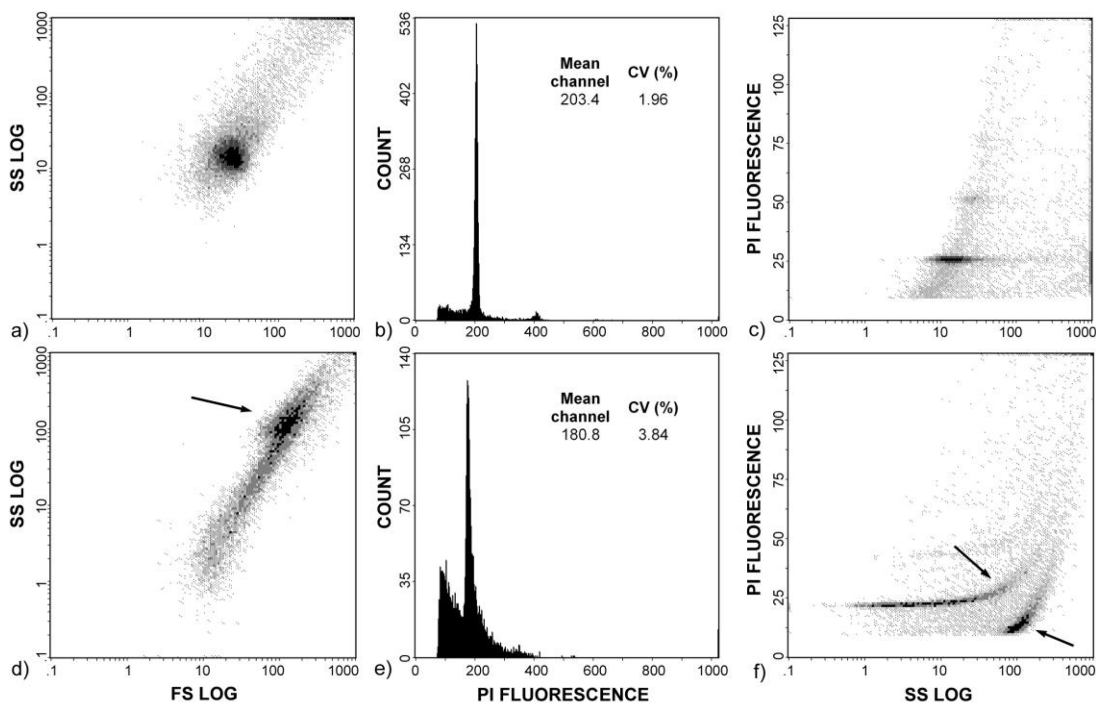


Fig. IV.3.3 Forward scatter (logarithmic scale, FS LOG) *vs.* side scatter (logarithmic scale, SS LOG) cytograms (a and d), histograms of PI fluorescence intensity (PI fluorescence, relative channel numbers) (b and e) and SS log *vs.* PI fluorescence cytograms (c and f) of nuclear suspensions of *Rosa* sp. obtained with WPB buffer (a-c) and NIB buffer (d-f). An effect similar to the "tannic acid effect" (Chapter IV.2) was observed in nuclear suspensions obtained with NIB buffer. Arrows indicate additional populations that appeared when nuclei were isolated with NIB buffer. Mean channel numbers (Mean channel) and coefficients of variation (CV, %) of G_0/G_1 peaks are given.

Generally, the NIB and WPB buffers yielded better results than the four buffers tested in Chapter IV.1. This was evident for the CV parameter, as in most species an improvement of peak resolution was achieved. A positive effect on nuclei fluorescence and background debris was also observed with both buffers. Surprisingly, in *Celtis australis* measurable samples were only obtained with WPB. Although NIB buffer has the same concentration of Triton X-100 as Tris.$MgCl_2$ buffer (the best buffer for this species in Chapter IV.1) it was not able to surpass the negative effect of mucilaginous compounds. Interestingly, both NIB and WPB seem to exhibit good buffering capacity, as they were suitable for isolation of nuclei from leaf tissues of *Oxalis pes-caprae*, that are characterized by highly acidic cell sap (Chapter IV.1). The only apparent drawback of NIB and WPB buffers was that for some species (especially in the "easy" ones) rather low YF values were obtained. This was unexpected as the concentration of Triton X-100 in both buffers was increased as compared with LB01 and Galbraith buffers. However, this drawback can be compensated by using higher amount of sample tissue.

Despite their commonness and/or economical interest, DNA content has not been analysed by FCM in 13 out of the 37 species used in this study. Moreover, in *Chamaecyparis lawsoniana* (Hizume *et al.* 2001), *Ginkgo biloba* (Ginkgoaceae; Marie and Brown 1993; Barow and Meister 2002), *Laurus nobilis* (Lauraceae; Zonneveld *et al.* 2005) and *Prunus domestica* (Arumuganathan and Earle 1991*b*), the published reports do not include DNA content histograms and data on CV values, making any comparison of buffers performance impossible. For the remaining species only indirect comparisons can be made as the experimental conditions in each work are unlike the ones followed here. However, judging from published CV values and DNA content histograms, with exception of *Pinus pinea*, the buffers described in the present work provided better (*e.g.*, *Quercus robur*; *Malus* x *domestica*, Rosaceae; *Diospyros kaki*, Ebenaceae) or similar (*e.g.*, *Olea europaea*, *Vitis vinifera*) results. Particularly interesting are the high resolution histograms obtained in *Q. robur* using the WPB buffer. Leaves of this and other species from this genus contain phenolic compounds that interfere with fluorescent staining of nuclear DNA (Zoldoš *et al.* 1998; Chapter II.1). In order to estimate genome size in seven *Quercus* species, including *Q. robur*, Zoldoš *et al.* (1998) modified the Galbraith's buffer by adding metabisulfite. In their study, CV values ranged from 4.2 to 6.9% for *Q. robur* while in our work, mean CV values under 3% and low BF values (< 20%) were achieved. In *P. pinea*, the NIB and WPB buffers resulted in CVs around 3%, *i.e.*, higher than those obtained by Grotkopp *et al.* (2004) who used a modified Galbraith buffer to obtain CV values typically under 2%. It should be noted, however, that we used fine needles to prepare nuclear

suspensions, while Grotkopp *et al.* (2004) used megagametophyte, from which it is easier to prepare nuclear suspensions.

In addition to the comparison of two new nuclei isolation buffers, this work provided data on nuclear DNA content in 30 plant species. We noted that samples prepared from species with small genome sizes (< 1.0 pg/2C DNA) exhibited higher CV values. Even in "easy" species, a negative relationship between genome size and BF was observed (*e.g.*, *Sedum burrito* and *Euphorbia peplus*). This was clearly due to the presence of other particles than intact nuclei in the samples (Galbraith *et al.* 2002). These include autofluorescent chlorophyll, nuclei fragments and non-specifically stained cellular debris, which contribute to the background distribution over which nuclear DNA content distribution is superimposed. Debris attached to isolated nuclei then increases the variation of nuclei fluorescence intensity (Chapter IV.2).

For the 20 species whose genome size was estimated before, better agreement was observed for previous results that were obtained by FCM as compared to those obtained by Feulgen microdensitometry. This was the case of *Coriandrum sativum*, where our estimate of 5.08 pg DNA (2C) differs from earlier estimates using the Feulgen technique that ranged from 7.65 to 9.55 pg (Das and Mallick 1989; Chattopadhyay and Sharma 1990). Our estimates of C-values are also lower than Feulgen-based estimates for *Magnolia* x *soulangiana* and *C. lawsoniana* (Nagl *et al.* 1977; Olszewska and Osiecka 1983; Ohri and Khoshoo 1986). However, our estimate for the latter species is similar to that of Hizume *et al.* (2001) who used FCM. On the other hand, we determined 2C = 11.00 pg DNA for *Papaver rhoeas* (Papaveraceae), which is the double of that obtained by Nagl *et al.* (1983), Bennett and Smith (1976) and Srivastava and Lavania (1991) using the Feulgen procedure. Other noteworthy difference concerns *Ficus carica* (Moraceae), in which our estimate of 2C value is only half of that determined by Feulgen microspectrophotometry (Ohri and Khoshoo 1987).

The differences between FCM and Feulgen densitometry are rather unexpected as Doležel *et al.* (1998) showed a close agreement between both methods. However, as noted by these authors, there are many critical points of the Feulgen procedure (*e.g.*, fixation, slide preparation and storage, acid hydrolysis) which determine its precision. Also, as it was already discussed here for FCM, staining inhibitors can also be an important source of staining error in Feulgen microdensitometry, as shown by Greilhuber (1988). Some differences between FCM estimates of genome sizes in different laboratories may be explained by the use of different reference standards, sample preparation and staining protocols, and flow cytometers (Doležel *et al.* 1998).

This work reports the first estimates of genome size in 10 plant species. As most of the families to which these species belong are poorly represented at the genus or species level in the Plant DNA C-values database (Bennett and Leitch 2005). The estimates for *Acer negundo* (Aceraceae), *Aloysia triphylla* (Verbenaceae), *Forsythia* x *intermedia* (Oleaceae), *Pterospartum tridentatum* (Fabaceae), *Saintpaulia ionantha* (Gesneriaceae) are at the lower limit of the known range of C-values for each family. Contrarily, our C-value for *Salix babylonica* (Salicaceae) is near the upper limit of the known range of C-values in *Salix* sp. Our estimates for *Ilex aquifolium* and *E. peplus* are the lowest C-values so far in Aquifoliaceae and in the *Euphorbia* genus, respectively. By contrast, our genome size estimation for *Diospyros kaki* (Ebenaceae) is the highest among the three species of *Diospyros* already analysed. Finally, our C-value for *T. africana* is close to that of Zonneveld *et al.* (2005) for *Tamarix tetandra*, which was until now the only species analysed in Tamaricaceae.

In conclusion, our results show that in species relatively free of cytosolic compounds, NIB buffer provides similar and in some cases better results than the WPB buffer, and may be preferred. When more problematic tissues are analysed, NIB buffer usually performs poorer than WPB, which is more suitable for “difficult” samples characterised for example by the presence of phenolics and mucilaginous compounds. When compared with other nuclei isolation buffers, the use of WPB results in improved histogram quality. Therefore it is recommended as the first choice when problematic tissues/species are used for DNA flow cytometry.

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Chapter V

FLOWER: A dynamic internet database of plant DNA flow cytometry

Chapter submitted as an original article to SCI journal:
Loureiro J, Rodriguez E, Suda J, Doležel J, Santos C (2007) FLOWER: A dynamic internet database of plant DNA flow cytometry. Cytometry *(submitted).*

Abstract

Background: The last two decades witnessed a burst of applications of flow cytometry (FCM) in plant sciences, accompanied by an ever-increasing number of research articles published in various journals. As a consequence, it becomes more and more difficult to follow all published work. This situation calls for a comprehensive, easily accessible and user-friendly database, in which up-to-date FCM data would be stored. To fill this gap, we built FLOWER, a dynamic internet database of plant DNA flow cytometry. This paper introduces the database and demonstrates its options and usefulness.

Methods: Available articles dealing with flow cytometric analysis of DNA content (~780 references) were carefully excerpted for methodology- and instrumentation-related details and available data were uploaded to an internet database. This dynamic webpage offers a number of searchable and output fields and is freely accessible to the public at http://flower.web.ua.pt/.

Results: Quantitative data on nuclear isolation buffers, methods of standardization (including the choice of reference standards), the use of DNA fluorochromes, and measures for estimation of the results quality were obtained. The database was used to evaluate the methods used by the authors in relation to the best practice guidelines and recommendations. Moreover, off-line frequency analyses and a survey of global trends in plant DNA FCM were made based on the information on taxonomic representation, objective(s) of the studies, type(s) of flow instrument(s), scientific journals, and countries of authors' origin.

Conclusions: The first comprehensive database of plant DNA flow cytometry, FLOWER, was developed as a free search and survey tool, which facilitates easy evaluation of methodological steps, formulation of additional recommendations, and identification of research gaps and hot topics. We expect that the database will become a primary reference source for plant DNA FCM and will stimulate further use of this high-throughput technique in plant sciences.

Keywords cell cycle; database; DNA ploidy level; flow cytometry; FLOWER; fluorochrome; genome size; internet; nuclear isolation buffer; standardization.

Introduction

With its ability to measure light scatter and fluorescence properties of microscopic particles and sort desired subpopulations at high speed and purity, flow cytometry (FCM) is a powerful analytical and preparative tool (Shapiro 2004). Although originally developed for the analysis of human and animal cells, the technique has also been successfully applied in plant research, breeding and industry already since the late 1980s (Loureiro *et al.* 2007). Determination of nuclear DNA content soon became the leading application with a number of practical uses, including estimation of DNA ploidy level and genome size, cell cycle analysis, and reconstruction of reproduction pathways (Doležel and Bartoš 2005). As expected, the extensive use of DNA flow cytometry in plant sciences has been accompanied by a continuous growth in the number of articles published over the years (Fig. V.1).

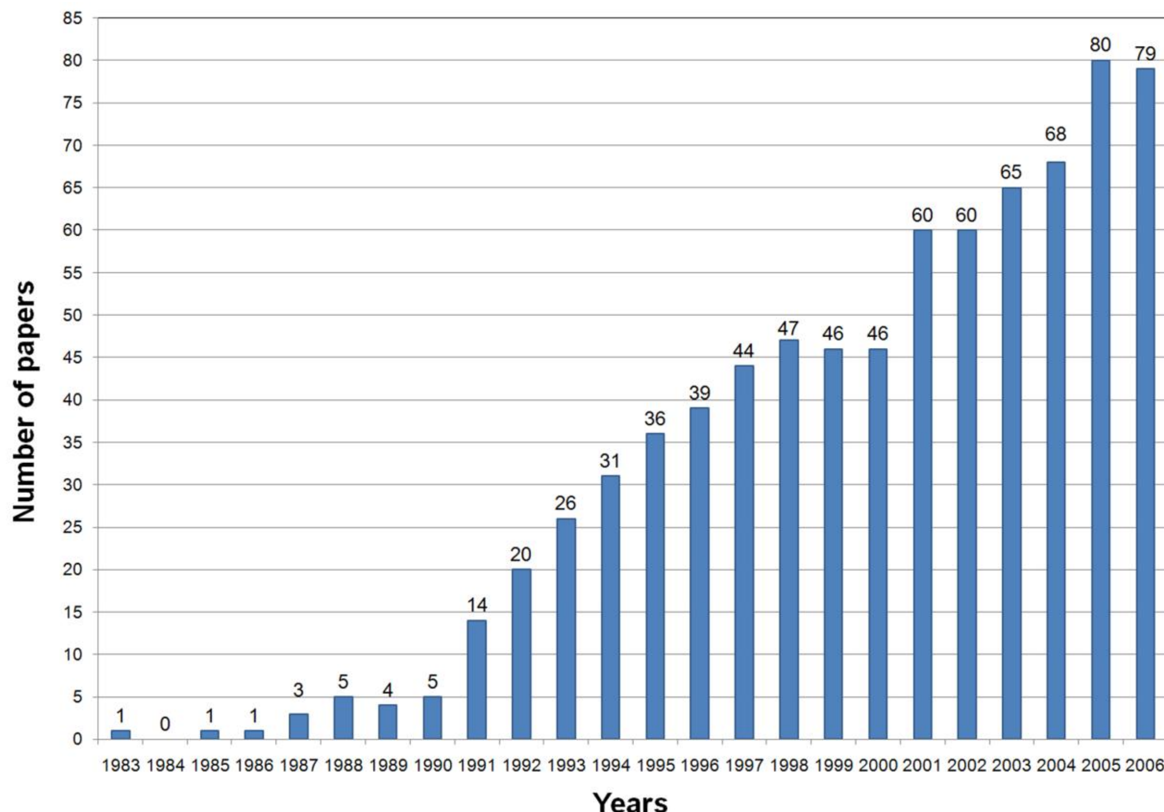


Fig. V.1 Year distribution of the articles included in the Plant DNA Flow Cytometry (FLOWER) database.

The popularity of DNA FCM in plant biology stems, among other reasons, in simple and rapid sample preparation, possibility to address a wide range of challenging questions, and reasonable running costs. However, apparent simplicity of FCM assays may in some cases tempt to excessive relaxation of quality measures, resulting in biased measurements, particularly in genome size studies where non-confirmed intraspecific

variation of genome size were reported (Greilhuber 2005). Together with difficulties caused by cytosolic compounds, which may interfere with nuclei isolation and/or DNA staining (Noirot *et al.* 2003, 2005; Chapter IV.2), these difficulties may discourage some researchers from applying DNA FCM in their work.

Alarmed by this situation, researchers engaged in genome size projects took several actions to settle the problems and put a set of essential quality standards into practice. A list of recommendations was approved at the Plant Genome Size Workshops (held in the Royal Botanic Gardens, Kew in 1998 and 2003, and at the International Botanical Congress, Vienna in 2005). Proper choice of nuclear isolation buffers, reference standards, and DNA fluorochromes, along with a constant alertness of potential negative effects of cytosolic compounds, received a special attention (see http://www.rbgkew.org.uk/cval/pgsm/ for further details).

The guidelines were largely based on the hands-on experience of the authorities attending these meetings. However, quantitative data, which could support the decisions, were lacking by that time. Specifically, there was no exhaustive survey of literature on DNA FCM, and an ever-increasing number of articles deterred most users from performing extensive comparisons on a self-help basis.

As a response to these needs, we built and released a Plant DNA Flow Cytometry Database (FLOWER), which is freely accessible, in a dynamic webpage format, on the internet (http://flower.web.ua.pt/). The database is regularly updated and contains about 780 entries at time of writing this chapter (March 2007). The FLOWER database is intended as a first-hand source of information for FCM users working with plant cells, by providing bibliographic records together with relevant data on taxonomy, methodology, and instrumentation. In addition, it also allows off-line frequency analyses of various parameters, including year trends, easy scrutiny of adopted protocols, and identification of research gaps and other hot topics.

The aim of this paper is to introduce the FLOWER database to the growing community of FCM users and demonstrate its scientific potential and usefulness. Quantitative analyses of selected key parameters are presented, and the findings confronted with the best practice recommendations.

Materials and methods

Hardcopies of articles included in the FLOWER database were gathered, on a long-term basis, in both conventional and online libraries and by requesting reprints from corresponding authors (to whom we are much indebted). At the time of writing (March 2007), the number of articles reached 780, including three book sections and 777 journal articles. The database is regularly updated by the authors who appreciate receiving newly published papers relevant to plant DNA flow cytometry.

The following data were excerpted from each article: author(s), title, year of publication, bibliographic details (publication name, volume, and pagination), countries of authors' origin, nuclear isolation buffer(s) and their modification(s), DNA fluorochrome(s), taxonomic details on experimental objects (plant group, family, species, and growth type), main and secondary objective(s) of the studies, method(s) of standardization, reference standard(s) used (type, species and cultivar, and assigned 2C nuclear DNA content), flow cytometer(s) (brand and model), coefficients of variation of DNA peaks (whether published or not, and the range of values), DNA histograms (presence or absence in the publication), and herbarium voucher (availability or absence). A link to the Plant DNA C-values database (http://www.rbgkew.org.uk/cval/homepage.html) is achieved by providing, in the detailed output screen, the reference number given by the Kew authorities.

Originally built as a Microsoft Office Excel (Redmond, WA, USA) datasheet, the database was later transformed into a dynamic webpage format, running under w3nexdb platform (http://www.aconet.cz), and made accessible on the Internet (homepage: http://flower.web.ua.pt/). Its basic structure (searchable and output fields) is presented in Figure V.2. Searching is possible either by typing free text (for Family, Genus + Species, and Author(s) fields) or by pre-defined options (for the remaining fields).

Frequency analyses and descriptive statistics were performed off-line using Microsoft Office Excel with the aid of an automatic filter tool. Journal Citation Reports and Essential Science Indicators, the analytical tools provided by the ISI Web of Knowledge™ (http://isiknowledge.com/), were used for result comparison in some cases.

A

Plant FCM Database

1
Family
Species
Author(s)

2
Year >= 2005 OR = -----
Country CZ OR -----
Buffer ----- OR -----
Fluorochrome ----- OR -----
* Group ----- OR -----
** Growth Type ----- OR -----
Objective ----- OR -----
Type of Standardization ----- OR -----
Standard Type ----- OR -----
Plant Standard ----- OR -----
Cytometer ----- OR -----
*** CV ----- Hist -----

RESET SUBMIT QUERY

Records 1-12 from 12

B

Title	Year	Country	Buffer	Family	Growth	Objective
A modified ploidy levels	2006	CZ	Tris.MgCl2	ASTERACEAE	H	Ploidy level
Assessment flow cytometry	2005	PT/CZ	Maries	FAGACEAE	W	Genome size & Ploidy level
Chromosome number Pilosella	2005	CZ/SLK	Otto	ASTERACEAE	H	Ploidy level
Effect Silene latifolia	2005	CZ	Otto	CARYOPHYLLACEAE	H	Ploidy level
Genome size their hybrid	2005	CZ	Otto	POACEAE	H	Genome size & Ploidy Level
Genome size flora completed	2005	CZ	Otto	VA	H & W	Genome size
Morphological Slovakia	2005	CZ/SLK	Otto	ORCHIDACEAE	H	Genome size & Ploidy level
Nuclear DNA Taraxacum	2005	CZ	Otto	ASTERACEAE	H	Genome size
Nuclear taxonomic implications	2005	CZ	Otto	MUSACEAE	H	Genome size
Ploidy level...fescues	2005	CZ/GER	Otto	POACEAE	H	Ploidy level
Ploidy specimens	2006	CZ	Otto	POACEAE	H	Ploidy level
Reliable DNA plant research	2006	CZ	Otto	VA	H & W	Ploidy level

C

Plant FCM Database

Genome size their hybrid 5 / 12

	Field	Value
	Author(s)	Mahelka, Krahulec
	Title	Genome size their hybrid
	Year	2005
	Country	CZ
	Buffer	Otto
	Buffer Modifications	2-mercaptoethanol
	Fluochrome	PI, DAPI
	Species	Elytrigia spp.
*	Group	A
	Family	POACEAE
**	Growth Type	H
	Objective	Genome size & Ploidy Level
	Type of Standard	I
	Standard Type	P
	Plant Standard	Vicia faba cv. Inovec
	ST. 2C	26,9
	Cytometer	P - PA II
***	CV	YES
***	Hist	YES
	Kew	N
	CV Range	A1-B

Fig. V.2 Screen preview of the FLOWER database (http://flower.web.ua.pt/): a) searchable fields (1 – fields with free text search, 2 – fields with pre-defined options); b) output fields; c) detailed output fields for a selected entry. Available options: * bryophyte / lycophyte / monilophyte / gymnosperm / angiosperm; ** herbaceous / woody / other; *** presence or absence.

Results and discussion

Taxonomic representation

Investigations into the taxonomic representation (from the Division to the Species level) of analyzed plants showed that angiosperms, the group with the highest number of recognized species (about 250,000), also predominated in the FCM studies (92.7% of all publications). Gymnosperms were assayed in 4.8% of articles stored in the FLOWER database. Nevertheless, due to a much lower number of recognized species (~ 730), better representation of gymnosperms was actually achieved. Other major taxonomic groups, *i.e.*, lycophytes (~ 900 recognized species), monilophytes (~ 9,000 recognized species), and bryophytes (~ 18,000 recognized species), only accounted for 2.5% of the database entries, suggesting a rather poor coverage of these plant groups.

Angiosperm families with economic value clearly lead the list of favourite plant groups, with Poaceae (17.0% of angiosperm references), Brassicaceae (8.5%), Fabaceae (7.9%), Solanaceae (7.8%), and Asteraceae (5.8%) collectively accounting for nearly half of the angiosperm database entries. Pinaceae mirrored this scenario in gymnosperms (77.8% of corresponding FCM references) and the genus *Pinus* was itself analyzed in 42.4% of gymnosperm studies.

The majority of FCM articles (77.1%) investigated herbaceous species while woody counterparts were involved in 20.3% of the studies. Other recognized growth types (*i.e.*, succulents, spore-bearing vascular plants, and bryophytes) were largely neglected and their contribution hardly reached 2.6%. These results are not surprising as herbaceous *taxa* definitely surpass in the number any other growth type. In addition, they are generally considered less challenging for flow cytometric analysis of nuclear DNA content than woody plants, which often pose serious problems due to the presence of cytosolic compounds interfering with DNA staining.

Nuclear isolation and staining buffers

Current FCM protocols are mostly based on the breakthrough methodological development of Galbraith *et al.* (1983), who accomplished nuclei release by simple tissue chopping in an appropriate isolation buffer. The role of nuclear isolation buffers is to facilitate release of intact nuclei free of cytoplasmic remnants, assure their stability for a necessary period of time, protect DNA from degradation by endonucleases, and provide environment for specific and stoichiometric DNA staining (Greilhuber *et al.* 2007). To meet these requirements, a well-balanced chemical composition of lysis buffers is of crucial importance. Given the diversity of plant tissues, including the range of intrinsic chemical

compounds, it is perhaps not surprising that there is no universal buffer that suits all types of samples (Doležel and Bartoš 2005) and the situation is unlikely to change in future. A different performance of the four most popular lysis buffers was recently demonstrated in Chapter IV.1, where, along with changes in DNA peak quality, shifts in fluorescence intensities, depending on particular species-buffer combination, were observed. This implies that nuclear isolation buffers play a pivotal role in FCM protocols and, consequently, are among the most thoroughly explored fields in the FLOWER database.

The literature survey showed that no less than 27 different lysis buffers, including commercial ones, have been developed and used with plant samples (NB: all buffers based on TRIS and $MgCl_2$ were merged into a single item, as only minor modifications were noticed; two commercial buffers were also grouped together). The chemical composition of the top ten non-commercial buffers along with their frequency of use is given in Table V.1.

Table V.1 Nuclear isolation buffers used in plant DNA flow cytometry. Frequency of use (%), chemical composition, and original references of the ten most popular non-commercial solutions are given

Nuclear isolation buffers[1]	Frequency of use (%)	Chemical composition[2]	Original reference(s)
Galbraith's	18.3%	45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0	Galbraith *et al.* 1983
$MgSO_4$	12.5%	9.53 mM $MgSO_4.7H_2O$, 47.67 mM KCl, 4.77 mM HEPES, 6.48 mM DTT, 0.25% (v/v) Triton X-100, pH 8.0	Arumuganathan and Earle 1991
Otto's[3]	10.6%	Otto I: 100 mM citric acid, 0.5% (v/v) Tween 20 (pH approx. 2-3); Otto II: 400 mM $Na_2PO_4.12H_2O$ (pH approx. 8-9)	Doležel *et al.* 1989
LB01	10.2%	15 mM TRIS, 2 mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 15 mM ß-mercaptoethanol, 0.1% (v/v) Triton X-100, pH 7.5	Otto 1990; Doležel and Göhde 1995
Tris.$MgCl_2$[4]	6.5%	200 mM TRIS, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5	Pfosser *et al.* 1995
Baranyi's[3]	3.7%	Baranyi solution I: 100 mM citric acid monohydrate, 0.5% (v/v) Triton X-100 Baranyi solution II: 400 mM $Na_2PO_4.12H_2O$, 10 mM sodium citrate; 25 mM sodium sulfate	Baranyi and Greilhuber 1995
Bergounioux's	3.4%	«Tissue culture salts» supplemented with 700 mM sorbitol, 1.0% (v/v) Triton X-100, pH 6.6	Bergounioux *et al.* 1986
Rayburn's	3.0%	1 mM hexylene glycol, 10 mM TRIS, 10 mM $MgCl_2$, 0.5% (v/v) Triton X-100, pH 8.0	Rayburn *et al.* 1989
Bino's	3.0%	200 mM mannitol, 10 mM MOPS, 0.05% (v/v) Triton X-100, 10 mM KCl, 10 mM NaCl, 2.5 mM DTT, 10 mM spermine.4HCl, 2.5 mM $Na_2EDTA.2H_2O$, 0.05% (w/v) sodium azide, pH 5.8	Bino *et al.* 1993
de Laat's	2.7%	15 mM HEPES, 1 mM EDTA $Na_2.2H_2O$, 0.2% (v/v) Triton X-100, 80 mM KCl, 20 mM NaCl, 15 mM DTT, 0.5 mM spermine.4HCl, 300 mM sucrose, pH 7.0	de Laat and Blaas 1984
Commercial buffers	16.0%	Partec's® commercial buffers; Chemunex® commercial buffer	
Other buffers	10.0%		

[1] When no designation was provided in the original reference, buffers were usually named after the primary author or after a characteristic chemical compound.

[2] Final concentrations are given (MOPS – 4-morpholinepropane sulfonate; DTT – dithiothreitol; TRIS – tris-(hydroxymethyl)-aminomethane; EDTA – ethylenediaminetetraacetic acid; HEPES – 4-(hydroxymethyl)piperazine-1-ethanesulfonic acid). For details on buffer preparation see the original reference(s).

[3] pH of the buffers is not adjusted.

[4] The first recipe and reference for Tris.$MgCl_2$ is presented. Several minor modifications have been made so far, but the basic composition, nonetheless, remains stable.

Galbraith's buffer is clearly the leading one (with an 18.3% incidence), followed by commercial buffers (16.0%). Three more buffers passed the ten-percent threshold: $MgSO_4$ (12.5%), Otto's (10.6%), and LB01 (10.2%). Together with Tris.$MgCl_2$ buffers (6.5%), these six most popular solutions accounted for nearly three-quarters of all FCM publications. The next five buffers were used in 15.8% of studies, while the remaining 16 buffers accounted for only 10.0% of the database entries.

Insights into the temporal variation in the use of nuclear isolation buffers revealed the increasing contribution of the six most popular solutions (Fig. V.3, lines). Indeed, the remaining 21 buffers were only applied in 19.7% of FCM works published during the last five years. A relative frequency of use of individual buffers has also been changing over time. The importance of Galbraith's buffer slightly decreased in the last decade, despite remaining the third most popular buffer nowadays (Fig. V.3, bars). A similar scenario also concerns both LB01 and $MgSO_4$ buffers, with the latter experiencing a more serious decline. On the contrary, commercial buffers have become increasingly popular since 1990s and they are the most widely used solutions in contemporary FCM. Their principal advantage apparently consists in availability as ready-to-use kits. Considering the ease of preparation of non-commercial buffers, we speculate that newcomers in plant FCM are the main customers. If correct, this may also indicate that a growing number of research groups are using, at least occasionally, FCM in their projects. Finally, the popularity of Otto's and Tris.$MgCl_2$ buffers has also been increasing over time, although the overall patterns are different. Otto's buffers were largely neglected for nearly one decade (first results with animal cells were published in 1990 (Otto 1990), after which they experienced a dramatic increase, and currently occupy the third position. Such a delay is rather surprising as already in 1995, Doležel and Göhde (1995) documented on plant samples that this methodology yielded DNA histograms with very high resolution. The comparative experimental study presented in Chapter IV.1 further supported this opinion and showed that Otto's buffers were among the best performing solutions. By contrast, Tris.$MgCl_2$ (in its original composition; Pfosser *et al.* 1995) was generally found to be the least suitable buffer. In this respect, a certain temporal increase in the use of lysis buffers based on Tris.$MgCl_2$ is interesting and may possibly be related to attempts in improving their performance by slightly modifying its composition.

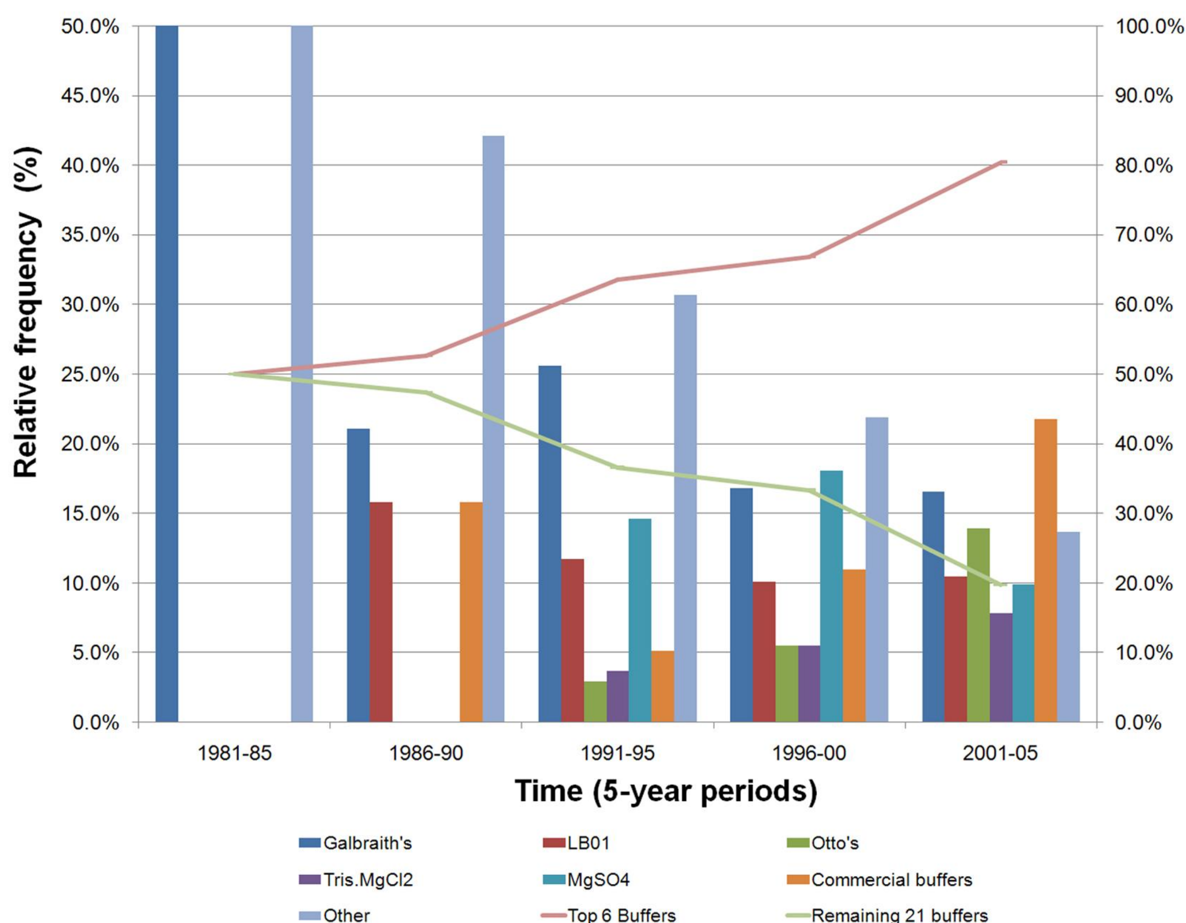


Fig. V.3 Relative frequencies of nuclear isolation buffers used in plant DNA flow cytometry through the years (%). Data were grouped into 5-year periods.

Generally, the use of individual buffers is tightly correlated with a particular geographic region and more than 50% of hits often come from only three countries. Galbraith's buffer has been mainly used in the USA (22.5%; the country of its origin), France (16.6%), and New Zealand (12.8%). This solution actually has one of the largest geographical coverage, being applied in more than 20 different countries. Its success plausibly stems from both a well-balanced constitution (Chapter IV.1) and a long-term appearance on FCM stage (since 1983). Commercial buffers are preferentially used in Japan (19.7%), a country with rapidly developing FCM facilities (81.3% of all articles were published after the year of 2000), Poland (16.9%), and the USA (11.3%). A highly localized use was observed for $MgSO_4$ buffer; nearly half of the users resided in the USA (47.0%), the country where it had been developed, being only distantly followed by Finland (9.8%). A certain analogy, though less pronounced, concerned LB01 and Otto's buffers, with both predominating in the Czech Republic (28.0% and 31.0% of database hits, respectively). Once again, the discrepancy is plausibly driven by the buffer's origin – LB01 was

developed in the Czech Republic (Doležel *et al.* 1989) and Otto's procedure was adapted to fresh plant tissues in a joint work of Czech and German researchers (Doležel and Göhde 1995). Because these buffers usually provide superior results with a number of plant species (Doležel and Bartoš 2005; Chapter IV.1), one may wonder for such a restricted geographic use. Surveying the buffer data, a clear tendency appears throughout the publication history of a particular researcher and/or research group and it concerns the preferential use of one or a few lysis buffers, irrespective of their quality and/or species adequacy. Perhaps the most striking example of this is Rayburn's buffer (Rayburn *et al.* 1989); developed in 1989, it has only been used twice (out of 25 hits) outside the original laboratory. It should, however, be noted that sticking to a particular methodology may have important consequences on the quality of results, as clearly showed in Chapter IV.1.

There were no obvious preferences in buffer selection with respect to plant growth type. Galbraith's buffer predominated in research on both herbaceous and woody species. A certain disproportion was encountered in LB01; while it was the fifth most used buffer with herbaceous species, it moved ahead to the second position when woody plants were involved. The more frequent choice of LB01 with woody species may be related to the inclusion of ß-mercaptoethanol (an antioxidant agent) in the buffer formula. This compound is usually added in order to counteract negative effects of cytosolic compounds, which generally occur at higher concentrations in woody species. Interestingly, original buffer composition was altered (*e.g.*, by addition of reducing agents and/or polyvinyl pyrrolidone of low molecular weights) in only 15.3% of works dealing with herbaceous species, with this proportion increasing to 33.9% in studies on woody *taxa*.

The above-mentioned insights into the use and performance of nuclear isolation buffers clearly showed that testing several different buffers is always advisable prior to routine FCM analyses in order to determine optimal nuclear isolation and staining solutions for a given species and tissue type and guarantee accuracy of measurements

Standardization and Standards

As flow cytometers only provide relative fluorescence values, correct interpretation of the results requires calibration against a reference material with known fluorescence properties. Genome size (C-/Cx-values) or DNA ploidy level of an analyzed plant is then determined by comparing sample and standard fluorescence intensities. DNA reference standards can be either external or internal. In the former approach, sample and standard nuclei are processed separately, which, however, may introduce serious bias for several reasons (Greilhuber et al. 2007). Non-identical conditions of nuclei isolation and DNA

staining, as well as instrument fluctuations are largely eliminated by internal standardization, which involves simultaneous nuclei isolation, staining, and analysis. Accordingly, internal standardization is the recommended practice, and was followed by most researchers (58.6% of articles stored in the FLOWER database). External standardization was adopted in much smaller fraction of publications (6.9%) and both approaches were used in only 1.0% of the studies. In 31.6% of the FCM works, no reference point was used. These figures change dramatically if the two main applications, estimation of genome size and DNA ploidy determination, are considered separately. While internal standardization clearly predominates in the former (91.9% internal *vs.* 7.5% external), ploidy-based studies show a different pattern (47.8% internal, 8.0% external, and 43.8% no standardization).

Several criteria should guide the selection of appropriate reference species, including biological similarity, reasonable proximity of genome size, easy availability, cytological stability, low level of secondary metabolites, and reliability of estimated C-value (Greilhuber *et al.* 2007). Although some attempts have been made to establish a set of primary references species, the discussions have not lead to a general agreement and a high number of different standards have been employed along the years.

Plant and animal reference standards were selected in 72.6% and 27.4% of studies, respectively. However, the suitability of animal standards has been questioned since 1997 when the first Angiosperm Genome Size Workshop was held in Kew. In particular, this alertness concerned fixed chicken red blood cells (chicken RBCs), which have been by far the most common animal standard (74.0% of references). A lack of agreement on chicken genome size and its stability across different breeding lines were among the principal reservations risen (Johnston *et al.* 1999). Our findings using the FLOWER database support this scepticism and show that published chicken 2C-values varied from 1.88 pg (Chen *et al.* 2002) to 2.50 pg (Iannelli *et al.* 1998), with the most common value being 2.33 pg (87.3% of references). Importantly, the use of chicken RBCs as a reference point for plant samples has dramatically decreased over the years, plausibly in response to continuous warnings (Fig. V.4).

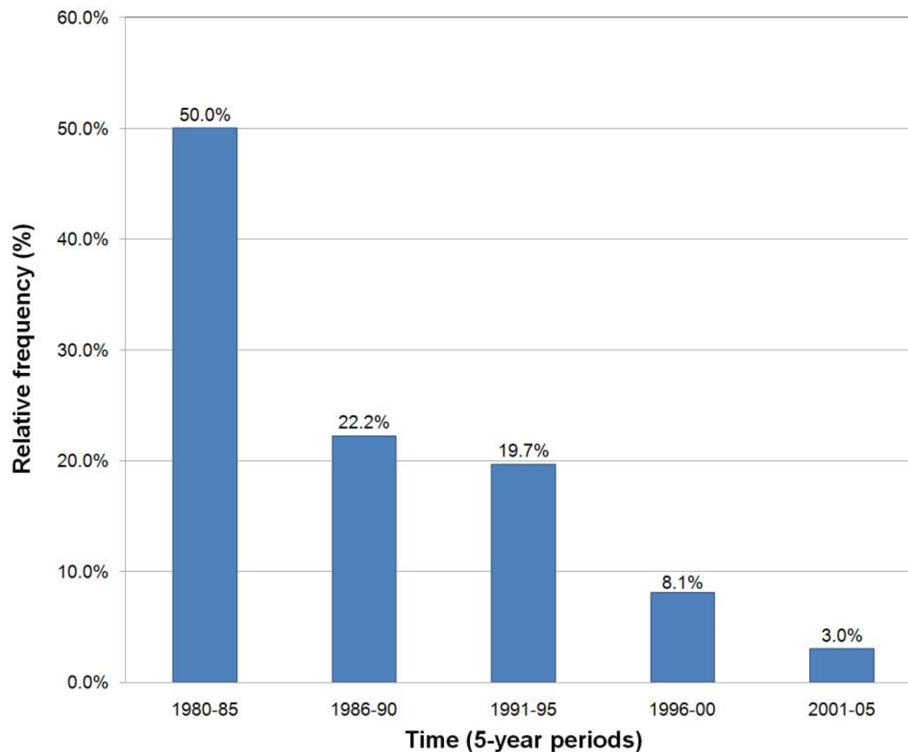


Fig. V.4 Relative frequencies of the use of chicken red blood cells as reference standard in genome size estimations over the years (%). Data were grouped into 5-year periods.

However, neither the choice of suitable plant reference material is trouble-free. Different species must be selected in order to cover adequately the whole range of plant genome sizes (differing about 2,000-fold). Unfortunately, there is little agreement not only on the set of primary plant standards but also on the 2C-values assigned to them. This is well illustrated by Table V.2, which summarizes the ranges of 2C values for the 16 most commonly used reference species, including *Pisum sativum* (Fabaceae; 15.7% of the database hits), *Hordeum vulgare* (Poaceae; 11.8%), and *Petunia hybrida* (Solanaceae; 10.7%). Along with variation in assigned 2C-values, one may also recognize that some reference standards are rather redundant because their genome size approaches another, perhaps more suitable, species. *Pisum sativum* and *Hordeum vulgare* are representative examples. Actually, the former species has been considered an ideal candidate for a “plant gold standard” due to intermediate 2C-value among angiosperms (Doležel and Bartoš 2005), stability of nuclear genome size (Baranyi and Greilhuber 1995, 1996), low or null concentration of interfering cytosolic compounds, easy availability, fast germination, and similar response to different nuclear isolation buffers (Chapter IV.1).

Not only the choice of reference species but also the cultivar selection may be controversial. For instance, no less than seven different cultivars have been used in each of the two most popular reference plants, *P. sativum* and *H. vulgare*. Even more

important, with consequences on the accuracy of genome size estimates, are the incongruities found in the 2C-values assigned by different researchers to the same reference cultivar. Four different values, differing up to by 18.4%, exist for *P. sativum* cv. Minerva Maple: 2C = 8.22 pg (n = 3; first cited in Joyner *et al.* 2001), 2C = 9.56 pg (n = 6; Price *et al.* 1998), 2C = 9.64 pg (n = 1; Johnston *et al.* 1999), and 2C = 9.73 pg (n = 7; Leitch *et al.* 2001). There is no doubt that this variation is artefactual as a worldwide stability of genome size of pea was repeatedly confirmed (Baranyi and Greilhuber 1995, 1996; Baranyi *et al.* 1996).

Table V.2 The most popular plant DNA reference standards (without cultivar distinction) used for FCM estimation of genome size.

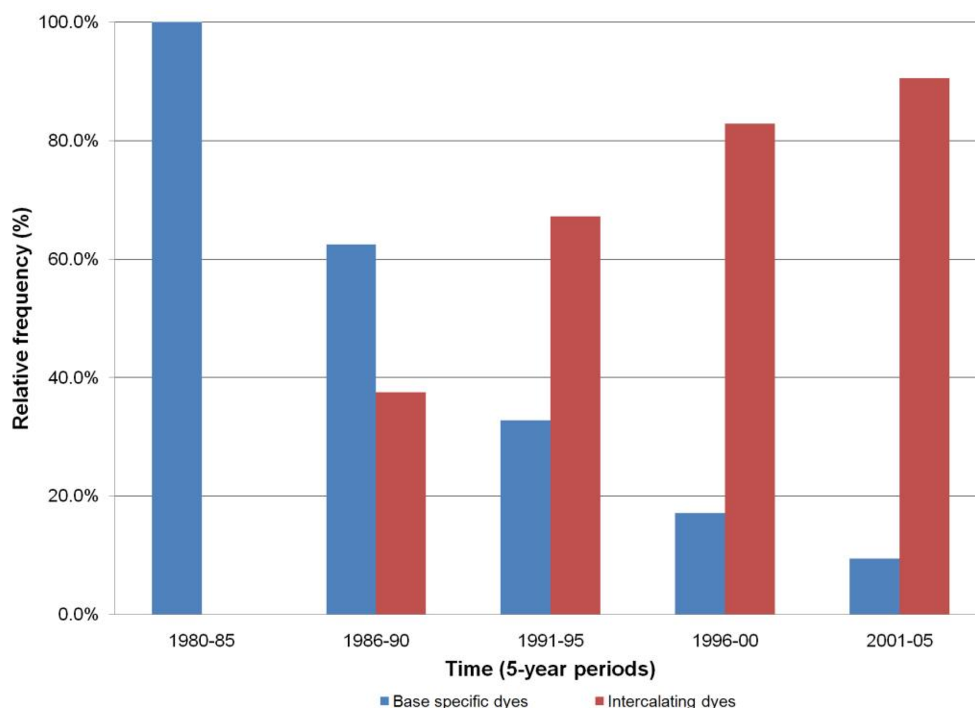
Plant DNA reference standards	Range of assigned 2C values (Min-Max (pg))	Variation (%) in the range of assigned 2C values	Nº of papers	Frequency of use (%)
Arabidopsis thaliana (L.) Heynh.	0.14 - 0.47	128.6	7	1.3%
Oryza sativa L.	0.89 - 1.20	34.8	14	3.8%
Vigna radiata (L.) R. Wilczek	1.06	-	4	2.2%
Raphanus sativus L.	1.11	-	8	1.6%
Lycopersicon esculentum Mill.	1.96 - 2.01	2.6	29	6.4%
Trifolium repens L.	2.07	-	2	1.9%
Glycine max Merr.	2.27 - 2.78	18.9	21	6.1%
Petunia hybrida Vilm.	2.85 - 3.35	17.5	39	11.1%
Zea mays L.	5.00 - 5.47	9.4	31	8.6%
Pisum sativum L.	8.22 - 9.73	20	57	15.0%
Hordeum vulgare L.	9.81 - 11.26	14.8	43	12.7%
Secale cereale L.	15.58 - 16.80	7.8	8	1.6%
Agave americana L.	15.9	-	1	2.2%
Vicia faba L.	25.40 - 26.90	3.7	11	2.5%
Triticum aestivum L.	30.90 - 34.85	12.8	20	6.1%
Allium cepa L.	33.50 - 34.89	4.1	12	4.1%
Other species	-	-	44	12.7%

Fluorochromes

Three groups of nucleic acid-selective fluorochromes are generally distinguished according to their mode of binding: (i) intercalating dyes (*e.g.*, ethidium bromide, EB, and propidium iodide, PI), (ii) base-specific minor grove-binding dyes with A-T preferences (*e.g.*, Hoechst dyes and DAPI), and (iii) base-specific dyes with G-C preferences (*e.g.*, chromomycin, mithramycin, and olivomycin). As first pointed out by Doležel *et al.* (1992)

and later experimentally confirmed in an extensive inter-laboratory comparison (Doležel *et al.* 1998), genome size estimation in absolute units requires intercalating dyes while the rules for fluorochrome selection are much less stringent if DNA ploidy is screened.

The FLOWER database showed that PI (45.1% of hits) and DAPI (39.7%) clearly dominated in plant DNA flow cytometry studies. Other fluorescent dyes collectively accounted for only 15.2% of articles, and their more extensive use usually dated back to early experiments. The popularity of DAPI in DNA ploidy and base composition studies is likely driven by its low sensitivity to chromatin structure that results in histograms with high resolution, relatively low toxicity, and possibility to be excited by UV wavelengths from relatively cheap arc lamps. In genome size estimations, PI with 72.4% incidence was only distantly followed by other intercalating dye, EB (9.4% of articles). The disproportion may be related to lower toxicity of PI and, perhaps more importantly, to conviction that PI produces histograms with higher level of resolution (Shapiro 2004; Doležel and Bartoš 2005), although no targeted study with plant material has so far been performed. Although not in accordance with the best practice rules, base-specific dyes were applied in 13.8% of plant genome size studies. This violation occasionally occurs even in contemporary FCM studies, but continuous temporal decrease in the use of base-specific dyes for DNA



content estimation in absolute units provides ground for optimism (Fig. V.5).

Fig. V.5 Relative contributions of base-specific and intercalating dyes in genome size estimations over the years (%). Data were grouped into 5-year periods.

Quality measures of DNA flow cytometric analyses

To assess the quality of analyses of nuclear DNA content, coefficient of variation (CV) of DNA peaks and overall fluorescence profile as appearing on DNA histograms are among the most widely accepted criteria, and should ideally be included in every publication. However, detailed literature survey revealed that the state of affairs in plant sciences is far from optimum, and CV values and DNA histograms were only presented in 30.4% and 66.6% of all articles, respectively. CV values were more common in genome size (44.1%) than in ploidy (22.5%) studies whereas DNA histograms showed an opposite trend (59.3% *vs.* 70.4% of corresponding references). Although higher figures were expected for both FCM applications, the differences may have justifiable reasons: low CV values are mandatory for accurate genome size estimates while certain relaxation is tolerable in DNA ploidy analyses. On the contrary, ploidy differences are most easily evaluated visually from DNA histograms.

The FLOWER database also allowed insights into the range of CV values of DNA peaks. High-quality histograms (CV values < 3.0%) were obtained in 34.0% of publications, and 38.6% of articles reported CV values between 3.0% and 5.0%. Rather high CV values (5.0 – 10.0%) were obtained in 22.8% of studies, while an unacceptable level of resolution (CV values > 10.0%) only concerned 4.6% of the references. When CV values were provided, our calculations show that more than 70% of articles meet the five-percent threshold, a value advocated by Galbraith *et al.* (2002).

Objectives of FCM studies

Estimations of DNA ploidy level and nuclear DNA amount are by far the most common applications of DNA flow cytometry in plants, accounting for 51.2% and 36.4% of references, respectively. Quite surprisingly and unlike human and animal research, cell cycle studies with plant cells are still scarce and this topic has only been covered in 6.0% of the database entries. Nonetheless, multiparametric FCM analyses of cell cycle kinetics represent a promising field of current biology and perhaps we will live to see a significant growth of publications in near future (Galbraith *et al.* 2005). Other minority applications, including estimation of base composition, sex determinations in dioecious plants, and technical and standardization experiments, accounted for the remaining 6.4% of the FCM articles.

Instrumentation

Relative contribution of different FCM manufacturers and popularity of particular flow cytometer models are other subjects that can easily be assessed with the aid of the FLOWER database. Distinct features of plant material together with somewhat different requirements imposed on flow instruments are reflected by differences in the importance of individual brands when compared to biomedical sciences, in which Becton Dickinson® (San Jose, CA, USA) and Beckman Coulter® (Hialeah, FL, USA) lead the way (Dubelaar and Jonker 2000). In plant sciences, nearly half of research groups (46.4%) used Partec® (Münster, Germany) flow cytometers. Beckman-Coulter® and Becton-Dickinson® occupied the second and third positions with 28.7% and 19.2% of hits, respectively. The remaining 5.7% of equipments comprised products from Leitz, Phywe, and Ortho Instruments (all discontinued), and more recent offerings by Bio-Rad® (Hercules, CA, USA; now acquired by Apogee flow systems®, Hertfordshire, UK) and Dako® (Glostrup, Denmark).

The prominent position of Partec® may be related either to a targeted customization of cytometers to plant samples and/or to a relatively lower price of their products. As project budgets in plant sciences are generally smaller than in other fields where FCM is routinely employed (*e.g.*, clinical studies), price is undoubtedly a significant criterion guiding purchase decisions. Based on our personal experience we speculate that research groups that use flow cytometry on a daily basis stick to Partec® instruments while occasional users rather collaborate with teams from clinical laboratories where Beckman-Coulter® and Becton-Dickinson® instruments predominate.

The search for the most successful (or better the most intensively used) model per manufacturer showed that CA-II flow cytometer (released in 1987) got primacy among the Partec's® products, Epics V (1977) among the Beckman Coulter's®, and FACScan (1991) among the Becton Dickinson's® offerings. Notwithstanding that all these models were released many years ago and have already been discontinued and substituted by newer and more sophisticated instruments, they still seem to be used extensively, at least on plant oriented studies.

Target journals

Researchers will likely find useful a comprehensive list of scientific journals where plant FCM contributions are being published. This database field may facilitate a better targeting of the most appropriate journal, based on its scope and performance metrics, including impact factor. Table V.3 summarizes the ten most popular periodicals with respect to the number of plant FCM studies. Although fully dedicated to flow cytometry, the journal Cytometry lags behind; plausibly, plant researchers regard it overly technical or

being a little beyond scope as it is absent from the Plant Science journal list provided by the ISI Web of Knowledge™.

Table V.3 The ten most popular scientific journals in plant DNA flow cytometry.

Scientific journal	Nº of papers	Frequency (%)
Annals of Botany	64	8.2%
Plant Cell Reports	59	7.6%
Theoretical and Applied Genetics	53	6.8%
Plant Cell Tissue and Organ Culture	41	5.3%
Plant Science	41	5.3%
Euphytica	37	4.7%
Plant Systematics and Evolution	25	3.2%
American Journal of Botany	20	2.6%
Crop Science	19	2.4%
Genome	18	2.3%
Other journals	402	51.6%

What kinds of studies are published in the preferred journals? The Annals of Botany (AoB) has a broad scope, although articles focused on nuclear DNA content estimation clearly prevail. The Plant Science (PS) also publishes a wide range of topics, including both genome size and DNA ploidy studies. The latter investigations, especially in conjunction with micropropagation, *in vitro* culture and transformation assays, are more common in the Theoretical and Applied Genetics (TAG), Plant Cell Reports (PCR), and Plant Cell, Tissue and Organ Culture (PCTOC). Finally, FCM works related to plant breeding fit best in the scope of the Euphytica (EU).

With the exception of TAG, there has been an increasing number of FCM articles published in the six leading journals introduced above (Fig. V.6). The decrease encountered in the last six years for TAG may be related to a certain shift in its scope, now being more focused on molecular biology. Consequently, FCM authors may have been pushed to submit their contributions to alternative periodicals, such as PCR and PCTOC. Actually, these two journals have experienced the highest increase in the number of plant FCM articles since 2000 (up to three-fold increase in PCTOC).

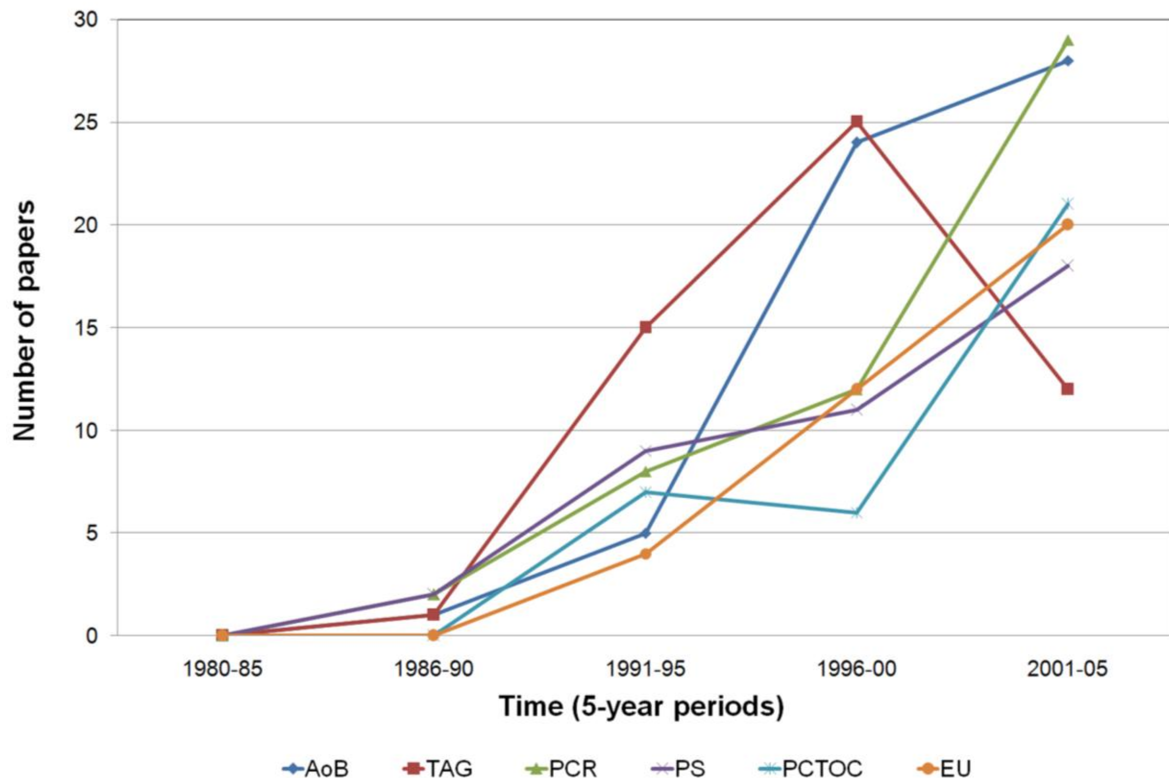


Fig. 6. Number of papers dealing with plant DNA flow cytometry published in the six most popular scientific journals, over the years. Data were grouped into 5-year periods. AoB – Annals of Botany; TAG – Theoretical and Applied Genetics; PCR – Plant Cell Reports; PS – Plant Science; PCTOC – Plant Cell, Tissue and Organ Culture; EU – Euphytica.

Country of origin of FCM researchers

To complete the frequency analyses of relevant fields in the FLOWER database, Table V.4 summarizes the top 20 countries of author(s) origin(s). As expected, majority of plant FCM researchers resided in the USA (16.6%), followed by France (11.6%), the Czech Republic (7.8%), Japan (7.4%), and Germany (5.9%). Actually, these five countries collectively account for about half of the DNA FCM articles. Nonetheless, the position of leading countries is altered if absolute article counts are weighted by the total number of papers published, per country, in plant and animal sciences in the last decade (1996-2006). The USA, Japan, Germany, United Kingdom, and Canada experienced the most dramatic shift downwards while the rank of Slovenia, Slovakia, Portugal, and Finland changed considerably in the opposite direction. It may be worth noting that the Czech Republic retained its forefront position.

Table V.4 Twenty leading countries with respect to author(s) origin.

Country	Nº of papers	Frequency of use (%)	Weighted frequency (%)[1]	Rank	Weighted rank
United States of America	161	16.6%	0.006%	1	18
France	113	11.6%	0.021%	2	9
Czech Republic	76	7.8%	0.147%	3	1
Japan	72	7.4%	0.009%	4	15
Germany	57	5.9%	0.008%	5	16
The Netherlands	53	5.5%	0.024%	6	7
Austria	36	3.7%	0.044%	7	5
Italy	36	3.7%	0.010%	8	14
New Zealand	34	3.5%	0.069%	9	3
People's Republic of China	31	3.2%	0.007%	10	17
Great Britain	29	3.0%	0.004%	11	20
Poland	27	2.8%	0.023%	12	8
Spain	27	2.8%	0.010%	13	13
Canada	21	2.2%	0.005%	14	19
Belgium	16	1.6%	0.014%	15	12
Slovenia	15	1.5%	0.091%	16	2
Finland	15	1.5%	0.018%	17	10
Portugal	11	1.1%	0.027%	18	6
Slovakia	10	1.0%	0.045%	19	4
Mexico	10	1.0%	0.018%	20	11

[1] The weighted frequency was calculated, per country, as a ratio between the total number of FCM references and the number of articles in plant & animal sciences published in the past decade (1996-2006).

Conclusion

The usefulness and scientific potential of the FLOWER database as a comprehensive and easily accessible source of information on plant DNA flow cytometry (search tool) along with a platform for performing quantitative analyses of relevant parameters of FCM practice (survey tool) has been demonstrated in this manuscript. A possibility to determine major gaps in the current research as well as to assess how the best practice recommendations are followed opens up new possibilities for a better targeting of further scientific goals and more straightforward decision-making on FCM methodology. The availability of the interactive FLOWER database on the Internet (http://flower.web.ua.pt/) guarantees both accessibility to plant FCM user's worldwide and to regularly updated data. We expect that this platform will stimulate further growth of DNA flow cytometry in plant sciences.

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Chapter VI

Plant flow cytometry – concluding remarks

Conclusions

As continuously highlighted in this PhD Thesis, FCM is a very powerful technique that, since early 1980s, has been increasingly applied in the study of plant genomes. However, it is my conviction that many of its potentialities, mostly those that imply multiparametric analyses, remain scarcely explored (see cell cycle studies, for example). The concretization of such studies together with the development of more compact low-cost instruments will certainly broaden the spectrum of FCM users in plant sciences making it a routine technique in many laboratories of plant research and in industry.

Ploidy level analyses and genome size studies, the two most common applications of FCM, have been successfully explored in Chapters II and III, respectively. These studies highlighted some of the current drawbacks of FCM, in which the negative effect of cytosolic compounds on isolation of plant nuclei and staining their DNA appears as one of the hottest topics. Methodological studies, focused mostly on sample preparation were investigated in Chapter IV. Although these reports provide new insights into the interference of cytosolic compounds, namely tannic acid, many efforts still need to be done in order to improve the current methodology and control the effects of these compounds. The improvement of lysis buffers is one of the possible solutions and some interesting and promising results were obtained with two new buffers (Chapter IV.3). However, at a basic level it is of outmost importance that researchers are aware of this source of error and risk of biased data production. This information is especially important for inexperienced FCM users that are often mislead and deluded by the apparent ease of sample preparation and by the rapidity by which data can be obtained, which may result in the production of artefacts. Some of the quantitative analyses of published data, which are presented in Chapter V, are also important to evaluate the reliability of published results and to check how the requirement for the best practice was met, contributing to the discussion on good practices, to the formulation of recommendations and to the identification of other hot topics in the field of plant FCM.

Best practices for nuclear DNA content using flow cytometry

Despite many recommendations were given throughout this Thesis and even though a similar compilation of best practices has already been done by Suda (2004), I still believe that as a resume of this work it is appropriate to list several suggestions that if followed will increase the probability of performing reliable estimations of nuclear DNA content. However, having this knowhow does not imply that researchers forget the “know-why”.

Plant material	○ Use fresh almost full expanded, intact, parasite- and disease-free leaves as starting material of both sample and reference standard. ○ For comparative studies, cultivate plants under the same conditions to avoid a possible negative effect of different levels of cytosolic compounds. ○ Sample and standard tissues should have a similar metabolic and developmental state. ○ Herbarium vouchers of the sample material should be kept in recognized herbaria.
Buffers	○ Test a range of different buffers when working with a new species and tissue type to elect the one that performs best.
Fluorochrome	○ For absolute nuclear DNA estimations use propidium iodide at saturating concentrations. For relative nuclear DNA estimations and when higher resolution is needed (*e.g.*, aneuploidy, sex determination in dioecious species), DAPI can be used. ○ Add the stain after nuclear homogenization and filtration instead of including it directly in the nuclear isolation buffer. This avoids the risk of contamination by genotoxic DNA fluorochromes.
Calibration and Standardization	○ Monitor on a daily basis the instrument performance. ○ Use internal standardization as a routine practice. ○ Select a set of reference standards to work with. My suggestion is to use the reference standards provided by the Laboratory of Molecular Cytogenetics and Cytometry, Olomouc, CZ, as seeds are provided in large quantities at a cost-free basis. ○ Choose a reference standard that has a different genome size from the sample, but not too different to avoid the risk of nonlinearity and offset errors. ○ If possible, use the same internal standard for all *taxa* belonging to the genus (or group) under analysis.
Quality control and data presentation	○ Routinely analyse the following graphics: FL in linear scale, FS *vs.* SS both in logarithmic (log) scale (buffers “fingerprint”), FL *vs.* time (monitoring of nuclear FL stability), SS in log scale *vs.* FL (monitor the effect of cytosolic compounds), FL pulse integral *vs.* FL pulse height (evaluate and eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets). ○ Run samples at a rate of 30 to 100 particles/s. ○ Position the leftmost DNA peak at approximately channel 200 (on a 1,024 channels scale), and keep the instrument voltage and gain settings constant throughout the experiment. ○ Analyse at least 1,300 nuclei per relevant peak. ○ Peaks of the sample and standard nuclei should be symmetrical and have approximately the same height.

Quality control and data presentation	o	The coefficient of variation of G_0/G_1 peaks should be as low as possible. CV values below 3.0% should be obtained routinely, although higher values (>3 and <5 %) may be acceptable when working with species possessing very small nuclear DNA content or recalcitrant tissues. Discard samples that present CV values above 5.0% and repeat sample preparation using modified conditions.
	o	When possible and applicable, at least three individuals per *taxon*/treatment should be analysed.
	o	Each measurement should be repeated at least three times on different days, and, if possible, using at least two different operators.
	o	In a publication always present CV values, discuss their range, and provide a representative DNA histogram(s).
Cytosolic compounds	o	Be aware for the possible interference of cytosolic compounds.
	o	Browning and/or precipitation of nuclear suspension are common symptoms of the presence of phenolic compounds.
	o	The presence of the described "tannic acid effect" should be checked by analyzing light scatter properties of particles. The test for inhibitors suggested by Price *et al.* (2000) can be another approach for detecting the negative interference of cytosolic compounds.
	o	When detected, various nuclear isolation buffers should be tested to detect if there is one that is less affected by the cytosolic compounds. Additionally, the selection of different tissues with lower or no cytosolic compounds (*e.g.*, cotyledons, leaf petioles, young stems) can also be a good strategy to produce unbiased estimations. If no buffer and tissue combinations provide acceptable results, buffer additives (*e.g.*, metabisulfite, PVP-10) should be included in the lysis buffer. If even so the effect persists, the quantity of plant material and chopping intensity should be reduced.
	o	For purposes of ploidy level estimation, fluorochromes that are less affected by the state of chromatin condensation (*e.g.*, DAPI) should be tested.
Intraspecific variation	o	Confirm true variability in nuclear DNA content by simultaneously analysing samples that supposedly differ in genome size. If two peaks or one bifurcated peak are detected, it means that the observed differences in DNA amount are most probably true.
	o	The use of two reference standards is another useful approach.
	o	When intraspecific variation is suspected, chromosome counting should be performed to verify if aneuploidy or the presence of B chromosomes are responsible for the observed differences.
	o	Finally, carefully and critically analyse and discuss published FCM literature.

Future directions on the use of flow cytometry to study plant genomes

Three different lines of FCM future research in plant sciences can be distinguished: 1) technical related challenges, which are necessary for a wider and even more successful implementation of this technique in plant sciences; 2) application related challenges; and 3) general challenges.

Among the technical challenges, the early identification of cytosolic compounds (*e.g.*, phytochemical tests for discarding problematic samples), the knowledge of the mechanisms and extent of their action, and the search for solutions to overcome this problem appear as urgent and important topics of future research.

Another methodological issue that deserves future investigation is the development of routine techniques for applying FCM to conserved (fixed or dried) plant material. This will open the possibility of analysing thousands of fixed samples stored in botany laboratories all over the world. Although the recent success in the analysis of dried materials (either herbarium vouchers or silica gel dried samples) opens new perspectives for analyzing field-collected samples, currently only relative DNA estimations can be done using these materials as acceptable histograms of DNA content can only be obtained after staining with DAPI (PI fails to deliver histograms with acceptable quality; J. Suda 2005, pers. comm.). Therefore the evaluation of novel intercalary stains that bind specifically and stoichiometrically to DNA may be a useful approach to follow. Marie *et al.* (1996) applied with success nuclei acid dyes, YOYO-1, YO-PRO-1 and PicoGreen, in the FCM analysis of marine prokaryotes. Similar studies in plant material are needed and should evaluate the suitability of these fluorochromes using current procedures of nuclear isolation, as well as their sensitivity to chromatin structure and their susceptibility to the negative effect of cytosolic compounds.

Also, the development of low-cost, compact and robust flow cytometers is expected in a near future and will enable the development of FCM analyses directly in the field. However there are some problems related with this possibility. For careful and methodological FCM analyses, besides the transportation of the flow cytometer, a vast array of material and reagents (*e.g.*, several lysis buffers to obtain the optimal protocol of sample preparation) should also be taken to the field. Additionally, in some applications of FCM in plant sciences (*e.g.*, genome size of species that were never analysed) a set of plant of reference standards should also be brought. Therefore, these cytometers should only find a direct application in the ploidy screening of very large numbers of individuals (representing a limited number of species), whose FCM protocol is already well established.

The enhancement in resolution of histograms of DNA content is an expected outcome of the improvement of technical related challenges, and will permit, among other applications, the routine detection of aneuploidy.

As a powerful tool for the study of plant genomes it is expected that new FCM applications continue to appear in plant sciences. However, for some of the established applications of FCM there are some challenges that still need to be achieved. Much work remains to be done with respect to the knowledge of plant genome sizes, namely for achieving the targets set at the Second Plant Genome Size Workshop (held at the Royal Botanic Gardens, Kew in September 2003). Unfortunately, the review of the progress made in 2005 (M.D. Bennett *et al.* 2005, pers. comm.) was way beyond expectations and considerable effort needs to be done to accomplish the targets set for 2008. This information is essential to study evolutionary trends in genome size evolution and its biological role, among other studies.

The study of the cell cycle, including endopolyploidization and apoptosis, is still in its beginning. It is expected that in the future, FCM and cell sorting will enable the monitoring of cell cycle dynamics in specific cell types, by following the expression of fluorescent proteins using multiparametric analyses.

In ploidy level estimations using FCM it is expected that future work continue to explore the differences in ploidy level and/or genome size at various geographical scales. It is also likely that FCM will help in the understanding of the timing and rate of polyploid formation in natural populations, by enabling estimates of ploidy level in pollen and frequencies of polyploid progeny.

A possibility to determine the mode of reproduction by analyzing nuclear isolation from mature seeds (so call flow cytometric seed scan, FCSS) opened new avenues for discrimination of apomictic mutants in sexual plants, quantification of the apomictic/sexual progeny ratio in species with facultative apomixis, and the assessment of the contribution of unreduced gametes. Thus the FCSS has a great potential in breeding programmes as well as in plant systematics.

Finally and as a general challenge, it is of outmost importance that the plant FCM community agrees on a list of standard plant species for the whole range of C-values. For that a collaboration between the most important laboratories in plant FCM is needed, as done in the work of Doležel *et al.* (1998). Such an agreement would enable a more accurate comparison of data obtained by different research teams.

Future personal expectations

In accordance with the challenges that were highlighted above, there are two main topics that deserve my particular interest and whose investigation I hope to carry in the future. First, and as an extension of some of the work developed in this Thesis, I remain interested in some technically related issues, namely those concerning the negative effect of cytosolic compounds on plant nuclei and nuclear DNA estimations. For a complete understanding of the mechanisms underlying the negative effect of these compounds, and for the finding of appropriate solutions to surpass their action, it is my belief that a joint collaboration with biochemists is fundamental and will be hopefully ensued. I also have great expectations on the possibility of using novel intercalating fluorochromes for plant DNA FCM studies. In fact, I am going to initiate these investigations in the near future. These methodologically targeted investigations will surely contribute to the increased use of FCM in plant sciences.

Last but not least, it is my desire to continue applying FCM to estimate the ploidy and genome size in various plant species, namely with the aim to understand the mechanisms of adaptive evolution through polyploidy. In that sense, there are good prospects in studying genome size variation in groups with adaptive radiation (*e.g.*, Oxalidaceae and Irididaceae families from Cape flora, South Africa) and superimposing genome size data on phylogenetic trees in search of evolution patterns.

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