HortScience 36(1):125–127. 2001.

Flow Cytometric Analysis of Ploidy in *Rhododendron* (subgenus *Tsutsusi*)

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Additional index words. flow cytometry, histogenic layer, Ericaceae

Abstract. **Ploidy level was determined for six species and 88 cultivars of the *Rhododendron* subgenus *Tsutsusi*. High-resolution flow cytometry of nuclear DNA was performed on macerated plant tissue. All plants analyzed were diploid (2n = 26) with the exception of 'Euratom', 'Euratom Orange', and 'Red Wing', which were triploid (3n = 39), and 'Casablanca Tetra', which was found to be a cytochimera: mixoploid (2n + 4n) in the LI and LII, but tetraploid in the LIII. The described method has proven to be useful in screening a large population of rhododendrons. Analysis of different organs and plant tissues was easily accomplished through flow cytometry, and has proven useful in determining the ploidy of different histogenic layers.**

The genus *Rhododendron*, consisting of ≈800 species, belongs to the heather family (Ericaceae), and contains eight subgenera (Chamberlain and Rae, 1990; Cullen, 1991). The subgenus *Tsutsusi* comprises 66 species, with spring leaves semi-evergreen and summer leaves persistent. The many hybrids are generally known as evergreen azaleas (Cox and Cox, 1997). Huge numbers are grown as pot plants for home decoration, making it one of Belgium's most popular ornamentals. Within the evergreen azaleas, four more or less well-defined groups of cultivated azaleas are distinguished: Belgian pot azaleas, Hirado, Kurume, and Satsuki azaleas.

Belgian pot azaleas have been created from a relatively narrow genetic base of collector's material, from botanical gardens and private collections brought from the Far East. *Rhododendron simsii* Planch. subgenus *Tsutsusi*, the species accepted as the primary ancestor, originates from hilly areas in China (Chang Jiang Valley), Thailand, Laos, and Myanmar. Apart from *R. simsii*, at least three other species from the *Tsutsusi* subgenus [*R. indicum* (L.) Sweet, *R. mucronatum* (Blume) G. Don and *R. scabrum* G. Don], from Southeast Asia and Japan, may have contributed (Heursel, 1999).

The efficiency of a breeding program is strongly enhanced by the knowledge of the ploidy level of the population. Taxonomists use nuclear DNA content to help determine the (phylogenetic) evolution of plant species. Estimations of nuclear genome sizes are also important for the mapping of plant genomes and the development of strategies for isolation of plant genes (Arumuganathan and Earle, 1991a). Conventionally, microscopic chromosome counting and Feulgen scanning microspectrophotometry, using meristematic mitotic cells, have been conducted to determine chromosome number in plants (Bennet and Smith, 1976; Price, 1988; Price et al., 1980). However, these methods are time-consuming and laborious. Nuclear DNA content of rhododendrons is difficult to estimate with these techniques because of the small chromosome size in *Rhododendron* (McAllister, 1993). Furthermore, chromosome counts can be conducted only during a short, difficult-to-predict period of time during the growing season (Heursel, personal communication). Flow cytometric analysis offers a valuable and rapid alternative to traditional chromosome counts.

Flow cytometry involves the analysis of fluorescence and light-scattering properties of single particles during their passage within a narrow, precisely defined, liquid stream (Dolezel, 1991). Heller (1973) was the first to use this technique for DNA analysis in plant cells. The technique is now widely used for many plant species (Arumuganathan and Earle, 1991b; Baert et al., 1992; Bennet and Leitch, 1995; Dolezel, 1997; Galbraith, 1990). Compared with conventional chromosome counting, the main advantages of flow cytometry are its accuracy, convenience, simplicity, low cost, and rapidity (De Laat et al., 1987; Dolezel, 1997; Galbraith, 1990; Galbraith et al., 1983).

All previous ploidy analyses of the genus *Rhododendron* have employed conventional chromosome counting (Heursel and De Roo, 1981; Janaki Ammal et al., 1950; McAllister, 1993; Pryor and Frazier, 1970). The basic diploid chromosome number in *Rhododendron* is 26. Some lepidote species of the genus are highly polyploid, whereas the elepidote

Table 1. Cultigens of *Rhododendron* subjected to flow cytometry.

R. simsii	Doctor Heimann	Julia	Pink Dream
R. indicum	Donatine	Kingfisher	Professor Wolters
R. scabrum	Dorothy Gish	Kirin	Red Wing[z]
R. mucronatum	Eclaireur	Knut Erwen	Reinhilde
R. kiusianum	Elsa Kaerger	Kosmos	Reinhold Ambrosius
R. noriakianum	Eric Danneberg	Lara	Rex
Ademurasaki	Directeur Van Slycken	Laura Ashley	Roi Leopold
Ambrosiana	Etoile de Belgique	Leopold Astrid	Rosali
Adventsglocke	Euratom[z]	Marianne Haerig	Sachsenstern
Anja	Euratom Orange[z]	Mevr. A. Heungens	Sayonara
Aline	Flamenco	Mevr. E. Troch	Schuman
Apollo	Friedhelm Scherrer	Mme Bourlard	Sima
Avenir	Gerda Keessen Wit	Mme P.B. Van Acker	Solitaire
Aquarell	Gilbert Mullaie	Mme Petrick	Spreeperle
Bertina	Gilles	Mistral	Stewartstownean
Blue Danube	Glaser Nummer 10	Niobe	Tamira
Carmen	Gloire de St. Georges	Nordlicht	Tempérance
Casablanca Tetra[y]	Heide Hanisch	Omurasaki	Terra Nova
Christine	Heiwa-no-Hikari	Ostalett	Vervaeneana
Coelestine	Hellmut Vogel	Otto	Weinachtsblume
Comtesse de Kerkhove	Herrmann's Superba	Palestrina	Werner Muckel
Concinna	Hexe	Panfilia	Phoenix
Cyriel Buysse	Inga	Paul Schaeme	
Doberlug	James Belton	Perle de Swijnaerde	

[z]Triploid cultivars (3n = 39) of the *Rhododendron* collection of the Dept. of Plant Genetics and Breeding.
[y]Cytochimera: mixoploid (2n/4n) in the LI and LII histogenic layer; tetraploid (4n = 52) in the LIII histogenic layer.

Received for publication 28 Dec. 1999. Accepted for publication 30 May 2000. This research was funded by a grant of the Fonds Wetenschappeliijk Onderzoek, Dienst Wenteneschap Techniek Cultuur/Inter-University Attraction Poles. We are indebed to Hilde Carlier for her excellent technical assistance and sincerely thank Jaroslav Dolexel for his informative remarks and the critical reading of the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.
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species are mostly diploid (Janaki Ammal, 1950). The objective of this research was to assess the ploidy level of the *Tsutsusi* collection maintained by the Dept. of Plant Genetics and Breeding at Melle, Belgium. Therefore, 88 *Tsutsusi* cultivars and six *Tsutsusi* species (*R. mucronatum, R. scabrum, R. indicum, R. simsii, R. kiusianum* Makino, and *R. noriakianum* Suzuki), hereafter collectively referred to as cultigens, were subjected to flow cytometric ploidy analysis.

Materials and Methods

Plant material. Of the 300 cultigens of *Rhododendron* present in the collection of the Dept. of Plant Genetics and Breeding, six *Tsutsusi* species and 88 cultivars were analyzed (Table 1).

Preparation of suspensions of nuclei. Generally, analyses were performed on young leaves obtained from plants grown in the greenhouse. For some cultigens, flower petals and root tips were also analyzed.

Measurements were performed on nuclei isolated from 5-mm^2 discs of leaf tissue and each cultigen was analyzed in triplicate. Because of the clonal nature of asexually propagated azaleas, plants were randomly harvested within each cultivar. The leaf discs were chopped with a sharp razor blade at room temperature, essentially according to Galbraith et al. (1983). Because of the woody nature of *Rhododendron*, two different procedures were tested to optimize the preparation of nuclear suspensions. First, leaf discs were chopped in 400 µL of solution containing 0.1 M citric acid and 0.5% Tween-20® (pH ≈2.5) (Otto, 1990). The samples were then passed through a 100-µm nylon filter. The residue on the filter was washed with 700 µL of a solution containing 0.4 M Na_2HPO_4 and 2 $mg \cdot L^{-1}$ of 4´, 6-diamidino-2-phenylindole (DAPI) (pH ≈8.5) (Otto, 1990). The nuclear suspension was then analyzed.

Alternatively, samples were macerated with the LB01 buffer (Dolezel et al. 1989), containing 15 mM Tris-Cl (pH 7.5), 2 mM disodium ethylenedinitrilo tetraacetic acid (EDTA), 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, and 0.1% Triton X-100®. The subsequent filtration and wash steps of the extraction method were as described by Dolezel et al. (1989).

Flow cytometric analysis. For flow cytometry, the PARTEC Cell Analyser II (Partec, Münster, Germany) was used, equipped with the DPAC software (Data Pool Application for CA-II; Partec, Münster, Germany). After filtration, the nuclear suspensions were passed through the flow chamber, which was filled with a sheath fluid (deionized water). The nuclei traversed the focus of an intense beam of light, produced by the high-pressure mercury vapor damp lamp. At a wavelength of 365 nm, the nuclei, stained with DAPI, fluoresce. The excitation light is collected by a lens and converted to pulses of electric current by a photomultiplier. The electronic signals are then digitized and the binary data are stored as one-dimensional histograms (256 channel positions). The fluorescence intensity is correlated directly with the amount of DNA that is stained with DAPI. Flow cytometry is a relative method of analysis. To calibrate the output, the first sample introduced in the flow cytometer is the external standard, which, in this study, was the azalea cultivar Nordlicht, a confirmed diploid (Heursel and De Roo, 1981). The voltage of the photomultiplier was adjusted in order to fix the signal of the diploid standard at channel position 50. During the course of the flow cytometric measurements of cultigens of unknown ploidy, the external standard was analyzed every 12 samples. This enabled us to determine if peak positions, i.e., diploid azalea signals at channel position 50, triploid and tetraploid signals at positions 75 and 100, respectively, were stable.

Results and Discussion

The objective of introducing flow cytometry in *Rhododendron* is to be able to analyze quickly large numbers of samples. Because of the woody nature of *Rhododendron*, the preparation of nuclear suspensions is of critical importance in the flow cytometric procedure. The quality of the histograms (Fig. 1) produced following lysis in 0.1 M citric acid/0.5% Tween-20® or with the LB01 buffer (Dolezel et al. 1989) was comparable, and the CV was <5%. Because the first procedure is less labor-intensive, all further experiments were conducted in this way. Standard flow cytometry of diploid tissue resulted in a histogram with two peak regions. The first region consists of cells in the G0/G1 phase of the cell cycle corresponding to the 2C DNA content. The second, smaller peak, at twice the channel position of the 2C peak, consists of cells in G2 or M phases. Cells between the two peaks are at some point in the S phase. The surface area of the peaks is correlated with the number of cells that excite at the same fluorescence intensity, thus containing the same amount of DNA. Fig.1 shows the histogram of a leaf sample of a diploid azalea genotype.

All plants included in this study were determined to be diploid (2n = 26), with the exception of four genotypes: 'Euratom', 'Euratom Orange', 'Red Wing', and 'Casablanca Tetra'. Flow cytometric analysis of roots, leaves, and petals revealed that 'Euratom', 'Euratom Orange', and 'Red Wing' were triploid (3n = 39), with a peak at position 75, which is in agreement with earlier reports (Heursel and De Roo, 1981; Pryor and Frazier, 1970) (Fig. 1). The cultivars Euratom and Red Wing arose from a cross of 'Hexe' x 'Apollo' and 'Hexe' x 'Willem van Oranje', respectively. 'Hexe' and 'Apollo' are diploid. 'Willem van Oranje' is no longer available and there are no reports on its ploidy level. However, the parents of 'Willem Van Oranje' are 'Mme Petrick' and 'Apollo', both of which were found to be diploid. This suggests that 'Willem Van Oranje' is probably a diploid as well. The origin of the triploid state of 'Euratom' and 'Red Wing' remains uncertain. As both cultivars are descendants of 'Hexe', 'Hexe' may produce unreduced gametes (Heursel and De Roo, 1981; Pryor and Frazier, 1970).

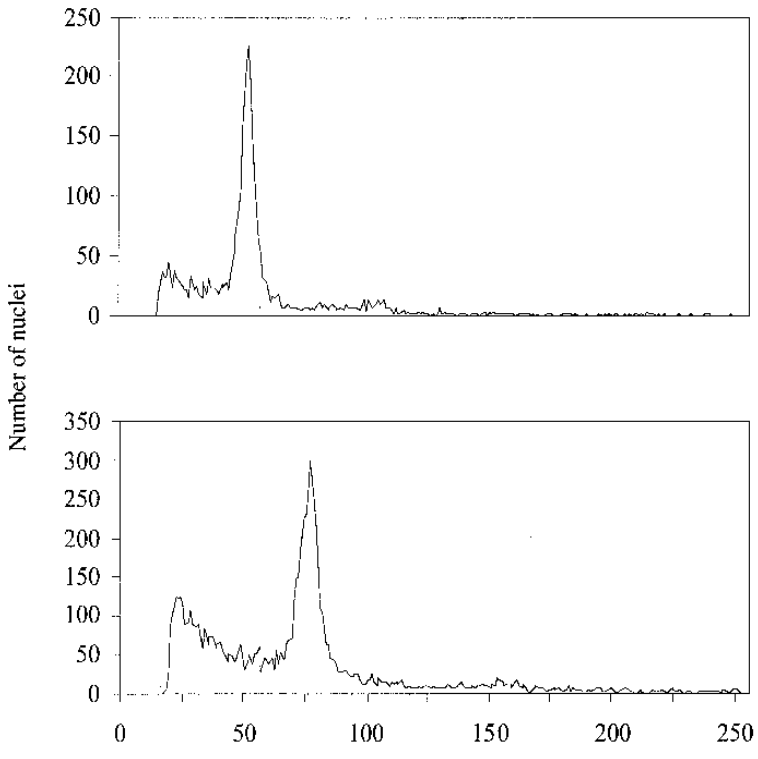

Fig. 1. Histograms of relative DNA content obtained after flow cytometric analysis of nuclei isolated from (**A**) leaf tissue of the diploid azalea 'Nordlicht' (channel number 50), and (**B**) petals of the triploid azalea cultivar Euratom (channel number 75).

'Casablanca Tetra' was previously reported to be tetraploid (Heursel and De Roo, 1981). Flow cytometric measurements on young leaves revealed both diploid and tetraploid nuclei, at positions 50 and 100, respectively (Fig. 2). In order to be able to explain this result, we decided to measure the ploidy in different organs and histogenic layers. Samples were prepared from sepals, petals, and root tips. The different histogenic layers were sampled as follows. Petals arise by divisions predominantly in the LI and LII (Irish, 1998). Epidermal peelings of the flower petals represent the LI layer, while the subepidermal layer originates from the LII. The LIII layer was analyzed by measuring ploidy in root tips. Sepal, petal, and leaf samples resulted in the same DNA histograms, with peaks at both the diploid and tetraploid positions (Fig. 2). Similar results were obtained from flow cytometric analysis of epidermal peelings (LI) and subepidermal cells (LII) of the petals (Fig. 2). Apparently the LI and LII histogenic layers of 'Casablanca Tetra' are mixoploid, i.e., a mosaic of diploid and tetraploid cells. Histograms obtained from the analysis of root tips, however, revealed only tetraploid nuclei (Fig. 2). Because their investigations of chromosome number were carried out using meristematic cells in young root tips, Heursel and de Roo (1981) proposed that 'Casablanca Tetra' was tetraploid. We show that 'Casablanca Tetra' is a cytochimera with a mixoploid LI and LII layer, and an entirely tetraploid LIII layer.

High-resolution flow cytometry of nuclei stained with DAPI has proven very useful for rapid ploidy determination of a large number of cultigens of the genus *Rhododendron*. Ploidy analysis of different organs and plant tissues was accomplished easily using flow cytometry, allowing the determination of ploidy level of different histogenic layers. This information is of interest as an additional tool in phylogenetic studies in *Rhododendron*.

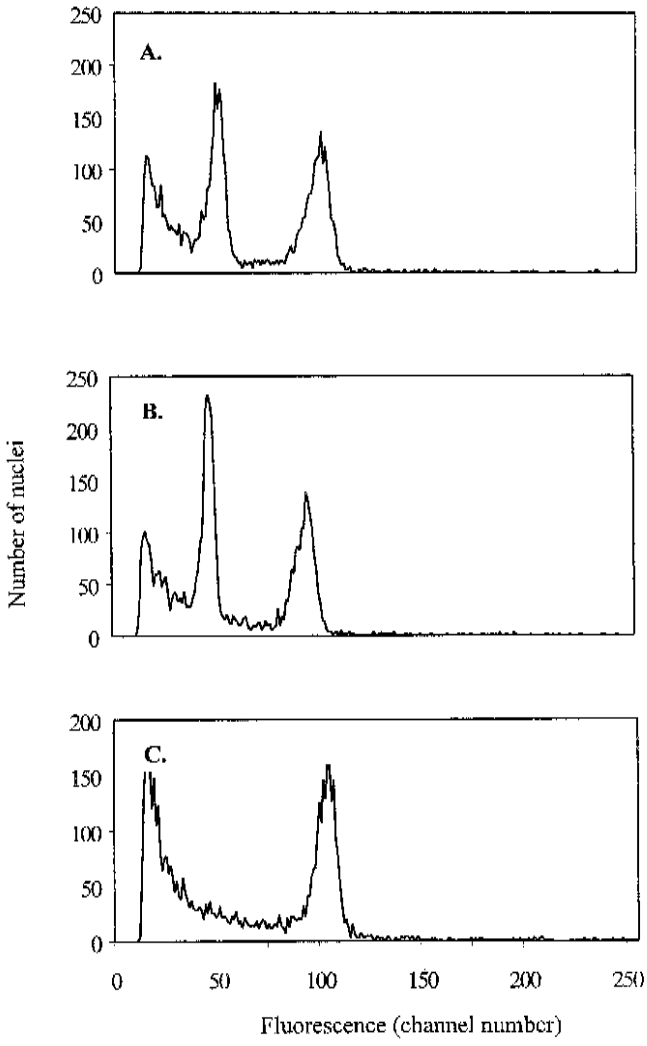

Fig. 2. Comparison of the histograms obtained after flow cytometric analysis of young leaves, sepals, and petals (**A**), epidermal peelings (**B**), and roots (**C**) of the azalea cultivar Casablanca Tetra. The peaks at channel number 50 and 100 represent 2C and 4C nuclei, respectively.

Literature Cited

Arumuganathan K. and E.D. Earle. 1991a. Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rptr. 9:229–233.

Arumuganathan K. and E.D. Earle. 1991b. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rptr. 9:208–218.

Baert, J., D. Reheul, E. Van Bockstaele, and A. De Loof. 1992. A rapid method for estimating persistence of tetraploid perennial ryegrass in pasture mixtures with diploid perennial ryegrass. Biol. Plant. 34:381–385.

Bennet, M.D. and I.J. Leitch. 1995. Nuclear DNA amount in angiosperms. Ann. Bot. 76:113–176.

Bennet, M.D. and J.B. Smith. 1976. Nuclear DNA amount in angiosperms. Phyl. Trans. Royal Soc. London, Series B 274:227–274.

Chamberlain, D.F. and S.J. Rae. 1990. A revision of *Rhododendron* IV subgenus *Tsutsusi*. Edinburgh J. Bot. 47:89–200.

Cox P. and K. Cox. 1997. The encyclopedia of *Rhododendron* species. Glendoick Publ., Glendoick Gardens Ltd., Glencarse, Scotland.

Cullen, J. 1991. The logic of *Rhododendron* classification. Rpt. Royal Hort. Soc. London, 44:14–19.

De Laat, A.M.M., W. Göhde, and M.J. Vogelzang. 1987. Determination of ploidy of single plants and plant populations by flow cytometry. Plant Breeding 99:303–307.

Dolezel, J. 1991. Flow cytometric analysis of nuclear DNA content in higher plants. Phytochem. Anal. 2:143–154.

Dolezel, J. 1997. Application of flow cytometry for the study of plant genomes. J. Appl. Genet. 38:285–302.

Dolezel J., P. Binarova, and S. Lucretti. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Plant. 31:113–120.

Galbraith, D.W. 1990. Flow cytometric analysis of plant genomes. Methods in Cell Biol. 33:549–561.

Galbraith D.W., K.R. Harkins, J.M. Maddox, N.M. Ayres, D.P. Sharma, and E. Firozabady. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051.

Heller, F.O. 1973. DNA-Bestimmüngen an Keimwurzellen von *Vicia faba* mit Hilfe der Impulszytophotometrie. Berl. Deutsch Bot. Ges. 86:437–441.

Heursel, J. 1999. Azalea's oorsprong, veredeling en cultivars. Lannoo-Terra, Tielt, Belgium.

Heursel, J. and R. De Roo. 1981. Polyploidy in evergreen azaleas. HortScience 16:765–766.

Irish, V.F. 1998. Floral development in *Arabidopsis*. Plant Physiol. Biochem. 36:61–68.

Janaki, Ammal, E. 1950. Polyploidy in the genus *Rhododendron*, p. 92–98. In: The rhododendron year book 1950. Royal Hort. Soc. London.

Janaki Ammal, E., I. Enoch, and M. Bridgewater. 1950. Chromosome numbers of species of *Rhododendron*. p. 78–91. In: The rhododendron year book 1950. Royal Hort. Soc. London.

McAllister, H.A. 1993. Chromosome numbers in rhododendrons, p. 24–30. In: Rhododendrons with camellias and magnolias 1993. Royal Hort. Soc. London.

Otto, F. 1990. DAPI staining of fixed cells for high resolution flow cytometry of nuclear DNA, p. 105–110. In: Z. Darzynkiewicz and H.A. Crissman (eds.). Methods in cell biology, Vol. 33, Academic Press, New York.

Price, H.J. 1988. DNA content variation among higher plants. Ann. Missouri Bot. Garden 75:1248–1257.

Price, H.J., K. Bachmann, K.L. Chambers, and J. Riggs. 1980. Detection of intraspecific variation in nuclear DNA content in *Microseris douglasii*. Bot. Gaz. 141:195–198.

Pryor, R. and L. Frazier. 1970. Triploid azaleas of the Belgian-Indian series. HortScience 5:114–115.