Occurrence of Polyploidy in Rhododendron luteum Sweet, Hardy Ghent, and Rustica Hybrids

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Abstract

By means of flow-cytometric measurements, *Rhododendron luteum* Sweet, Pentanthera, Hardy Ghent, and Rustica hybrids were found to be polyploid. For *R. luteum* this was in contrast with earlier publications. Most of the hybrids tested were tetraploid though triploidy seemed to occur regularly. Diploidy did not occur. The ploidy level of other Pentanthera species matches literature data. The results imply a strong relationship between Hardy Ghent and Rustica hybrids and tetraploid Pentanthera species. The discovery of polyploids offers multiple perspectives for further breeding, including interspecific hybridization and optimization of ploidy-level-influenced growth vigor.

Introduction

Polyploidy is a very useful tool in plant breeding, since it may allow plant breeders to overcome barriers to hybridization (dissolving interploidy blocks), to restore hybrid fertility by the creation of allopolyploids, to develop sterile cultivars (meiosis being prevented by complications due to the presence of multiple homologous chromosomes), to enhance pest resistance and disease tolerance in allopolyploids by the additive effect of defense chemicals inherited by both parents, or to create larger plants with an enhanced vigor (Stebbins, 1971). The connection between ploidy level and plant cell size has not fully been elucidated yet, though ploidy level clearly exerts an important control on cell size (Kondorosi *et al.*, 2000). Sometimes chimeras occur; if the chimerism is periclinal, histogenic layers and the resulting somatic tissues show different ploidy levels since leaves originate from the LI + LII, anther filaments from the LII, and roots from the LIII layer (Tilney-Basset, 1986).

Polyploidy has been realized in several ornamental crops such as *Alstroemeria* (Lu and Bridgen, 1997), *Syringa* (Rose *et al.*, 2000a), *Buddleja* (Rose *et al.*, 2000b), *Cyclamen persicum* (Takamura and Miyajima, 1996) and *Rosa* (Roberts *et al.*, 1990). Polyploidization attempts in *Rhododendron* are numerous (Paden, 1990; Eiselein 1994a, b; Kehr, 1996; Väinölä, 2000; Eeckhaut *et al.*, 2001).

Ploidy analysis by conventional chromosome counting is time consuming and laborious (Martens and Reisch, 1988; Owen and Miller, 1993). Flow cytometry offers a valuable, rapid, simple, accurate and fairly cheap alternative; it involves the analysis of fluorescence and light-scattering properties of single particles during their passage within a narrow, precisely defined, liquid stream (Galbraith *et al.*, 1983; Dolezel *et al.*, 1989; Dolezel, 1991). Heller (1973) was the first to use this technique for DNA analysis in plant cells; later on it was used for differ-

Partial Glossary—Genetics Terms

Chimera. A plant or plant organ consisting of tissues of more than one genetic composition and origin (*Hortus Third*, p. 1212). A **periclinal chimera** would be one on the surface of a plant.

Ghent, Belgium (**Gent**, in Dutch) has been a center for azalea hybridizing in Europe since the early 1800s (Galle, p. 81).

Ghent hybrids (in Holland sometimes known as the **Pontic hybrids**). A consolidation of a number of deciduous azalea hybrid groups developed in Belgium, including the Mortier hybrids, (crosses of the Flame and Pinxterbloom azaleas—*Rhododendron calendulaceum* x *R. periclymenoides*); the Ornatum hybrids (crosses of *R. calendulaceum, R. viscosum,* and *R. luteum;* the Viscosepalum hybrids (*R. molle* x *R. viscosum*); and M.L. Verschaffelt's crosses of Mortier's seedlings with Ornatums, Pontic azalea *(R. luteum),* and Flame and Pinxterbloom azaleas. In 1850 there were 500 named Ghent hybrids; today, 100 are listed, with perhaps 25 commonly grown (Galle, p. 81).

Heterochromatic. Having or consisting of different or contrasting colors.

Histogenic. Of or pertaining to the process of tissue development and differentiation.

Homologous. Corresponding in basic type of structure and deriving from a common primitive origin.

Ploidy. Chromosomes, through which heredity is transmitted by way of genes, occur within the nucleus of each living cell of the plant body in sets which are known technically as genomes. Ploidy, as used in *Hortus Third,* refers to the degree of duplication of genomes or of individual chromosomes making up the genome. Normally each vegetative cell of the plant body contains two genomes and the plant is known as a **diploid**. Continued duplication of genomes leads to

the formation of **polyploid** plants with three (**triploid**), four (**tetraploid**), etc. sets of chromosomes. If the genomes are dissimilar, derived from parents belonging to different species, the plant is an **allopolyploid**. Many species of plants are known or thought to be of polyploid origin, but the details of their origin are lost in antiquity, and for all practical purposes they are considered to be normal diploids. While the plant breeder needs acquaintance with the complexities and subtleties of polyploidy, especially as these relate to sterility and the expression of desired traits, most horticulturists needs only recognize that polyploidy, and in particular allopolyploidy, is a widespread phenomenon in the plant kingdom.... In the horticultural trade, polyploidy tends to be publicized only in those instances where it confers desirable characteristics such as gigantism of floral parts, increased vigor, or adaptability to a wide range of soils and climate. (Hortus Third, p. 887)

Related terms: **Aneuploidy**. Characterizing an organism that does not contain an exact multiple of the basic set of chromosomes. (Helms, Ted, 2000, NDSU). **Mixoploidy**. Containing cells with chromosome numbers that deviate from the normal tetraploid. (S. Jelenic et. al., p. 14).

Rustica Flora Pleno hybrids, were introduced into Belgium about 1890 by Charles Vuylsteke, who acquired them from Louis de Smet, also of Belgium. Their origin is probably unknown, possibly Double Ghent azalea crosses with the Japanese azalea (Galle, p. 89).

Somatic. Of or pertaining to any of the cells of an organism that become differentiated into the tissues, organs, etc.

Transposon. A segment of DNA that moves to a new location in a chromosome, or to another chromosome or cell, and alters the existing genetic instructions, sometimes producing significant changes.

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ent plant species (Galbraith, 1990; Arumuganathan and Earle, 1991; Bennett and Leitch, 1995).

The genus *Rhododendron* (± 1000 species) is divided into eight subgenera, the four most important subgenera being Tsutsusi (evergreen azaleas except Brachycalyx section), Pentanthera (deciduous azaleas), Rhododendron (lepidote *Rhododendrons*) and Hymenanthes (elepidote *Rhododendrons*) (Chamberlain *et al.,* 1996). Hymenanthes are evergreen and have large non-scaly leaves; Rhododendrons (subgenus) have smaller scaly leaves, are usually evergreen, but occasionally are semi-deciduous. Species and hybrids of these two subgenera comprise what gardeners refer to loosely as rhododendrons, while Pentanthera and Tsutsusi comprise the azaleas. The Pentanthera subgenus is confined to 30 species divided over four sections. The deciduous azaleas are generally taller, upright plants with less branching than in the evergreen species. The leaves are often much larger than those of the evergreen species, and they are usually not glossy. The flowers of most species have relatively long, narrow tubes. Colors include white and a variety of tones, from pale to strong, in the yellows, oranges, yellowish pinks, reds, pinks, purples, and purplish pinks (Voss, 2001).

Among deciduous azaleas, many hybrid groups have been created since the 19th century (De Raedt and De Groote, 2000). In Gent, between 1804 and 1834, a baker named P. Mortier began producing the Ghent hybrid azaleas (the so-called *Azalea mortieriana*). He used three American species: *R. calendulaceum, R. periclymenoides,* and *R. speciosum* and the scented *R. luteum* from around the Black Sea to produce some robust, sweetly scented, and hardy plants. Other species possibly involved in the development of Hardy Ghent are *R. prinophyllum, R. viscosum, R. canescens, R. flammeum, R. occidentale,* and *R. molle.* The Double Ghent hybrids originated about 1853 near Frankfurt and were mainly propagated by Louis Van Houtte. In 1888 Charles Vuylsteke introduced double forms called the Rustica hybrids (probably Double Ghent hybrids x *R. molle* ssp. *japonicum*) that were originally bred by Louis Desmet. Louis Van Houtte also introduced Mollis hybrids, a complex group derived mainly from *R. molle* ssp. *japonicum* but confusing due to the vicissitudes of the names of the Chinese and Japanese azaleas and the early history of their introduction and breeding. The Mollis hybrids are generally not as hardy as the Ghent, due to their *R. molle* parentage, and are best grown on their own roots. Knap Hill hybrids were developed by Anthony Waterer and son by crossing Ghent Hybrids with *R. molle* var. *molle, R. occidentale,* and other species.

The first chromosome study of rhododendrons was published by Sax in 1930. The chromosome number of 360 *Rhododendron* species has been determined by counting (Janaki Ammal *et al.,* 1950; McAllister, 1993).

The basic chromosome number (x) within the genus is 13. Different species were found to be polyploid; however, species that are classified within the Pentanthera subgenus were found to be diploid, except for the tetraploids *R. calendulaceum* and *R. canadense.* The aim of this research was a flow cytometrical control of the ploidy level of Hardy Ghent and Rustica hybrids and their presumable ancestors (if available).

Materials and methods

Plant material

Species: In the literature, the only Pentanthera species described as tetraploids are *R. calendulaceum* Torr. and *R. canadense* Torr. (Janaki Ammal *et al.,* 1950). Therefore *R. calendulaceum* leaf material was used in control samples. Species that were collected for ploidy measurement included *R. luteum* Sweet (gathered from three different and independent sources), *R. prinophyllum* Millais, *R. viscosum* Torr., *R. occidentale* A. Gray, *R. canescens* Sweet and *R. periclymenoides* Shinners. Besides leaves, flower (anther filaments) and root material was harvested and flow cytometric measurements were performed in order to detect possible (periclinal) chimerism.

Hybrids: Nuclear suspensions from leaf material of Hardy Ghent 'Nancy Waterer', 'Unique', 'Narcissiflorum', 'Jozef Baumann', 'Maja', 'Rosetta', 'Mina Van Houtte', 'Daviesii', 'Semiramis', 'Quadricolor', 'Souvenir du President Carnot', 'Marie Verschaffelt', 'Batholo Lazarri', 'Guelder Rose', 'Gloria Mundi', 'Coccinea Major', 'Raphaël De Smet', 'General Trauff', 'Graff von Meran', 'Goldlack', and 'Van Houtte Flore Pleno' and Rustica hybrids 'Norma', 'Phebe', 'Fenelon' and 'Racine' were tested.

Ploidy measurement

To determine the ploidy level, a flow cytometer, Partec PAS (particle analyzing system) III, was used as described by De Schepper *et al.* (2001). From young leaves on control plants (with known ploidy level) and from plants with the unknown ploidy level, 5 mm discs were punched. The sample was chopped with a sharp razor blade in 250 µl buffer solution, containing 0.1 M citric acid and 0.5 % Tween 20 (pH ± 2.5) for the isolation of the nuclei. The chopped sample was passed through a nylon filter of 100 µm mesh size. Afterwards, 500 µl of the second buffer, containing 0.4 M Na_2HPO_4 and 2 mg/l DAPI (4', 6'-diamidino-2-phenylindol) (pH ± 8.5) was passed through the filter to get the staining. The pH of the mixture of both buffers was 7, which is the optimal pH for the staining with DAPI (Otto, 1990).

After filtration, the nuclear suspensions were passed through the flow chamber, filled with a sheath fluid (de-ionized water). The nuclei traversed the focus of an intense beam of light, produced by a high-pressure mercury vapor damp lamp. At a wavelength of 365 nm the nuclei, stained with DAPI, fluoresced. The excitation light was collected by a lens and converted to pulses of electrical current by a photomultiplier. The electronic signals were then digitized and the binary data stored as one-dimensional histograms. The fluorescence intensity is linearly correlated with the amount of DNA that was stained with DAPI.

The first sample measured in the flow cytometer was the external standard (control plant). The use of flow cytometry is a relative measurement; the first sample should gauge the apparatus for the plants to measure. The voltage of the photomultiplier, which transfers the DAPI-fluorescence (depending on the DNA content) into an electrical current, was adjusted in such a way that a diploid peak (standard) is fixed at position 100. A haploid peak will in that case occur at position 50, a tetraploid peak at position 200. The measurement of the external standard was repeated after every 10-12 measurements of plants with unknown ploidy level, to correct the voltage of the photomultiplier if necessary. Peak shifts were corrected by adding an internal standard (a diploid *Lolium multiflorum* 'Bellem'—Italian ryegrass—nuclear suspension) to the samples. Each plant was measured until two unequivocal results were obtained (two samples were not always sufficient due to occasional high nuclear suspension viscosity, resulting in broad and indefinable peak patterns).

Results

Species: With the exception of *R. prinophyllum,* root material was hard to measure and gave unclear results. The determination of nuclear DNA content of leaves and filaments was easier to establish, though multiple measurements were required to obtain the results. Especially *R. viscosum* nuclear suspensions seemed to inhibit sheath flow by a high viscosity. Results are presented in Table 1 and Figure 1. All *R. luteum* and *R. calendulaceum* samples were found to be tetraploid. All other species were diploid as expected.

Hybrids: All genotypes were compared to the tetraploid *R. calendulaceum* standard. Sixteen Hardy Ghent hybrids and two Rustica hybrids turned out to be tetraploid, whereas five Hardy Ghent hybrids and two Rustica hybrids were found to be triploids (Table 2; Figure 2). The triploid hybrids are 'Mina Van Houtte', 'Daviesii',

Table 1. Ploidy Measurement of Pentathera Species
(° flowers not present * measurement unclear)

Species	2n (leaves)	2n (anther filaments)	2n (roots)
R. calendulaceum	4x	4x	*
R. luteum	4x	4x	*
R. prinophyllum	2x	2x	2x
R. viscosum	2x	2x	*
R. occidentale	2x	2x	*
R. canescens	2x	°	*

'Quadricolor', 'Gloria Mundi', 'Van Houtte Flore Pleno', 'Norma' and 'Phebe'. Ploidy peaks were found to shift during successive measurements towards higher ploidy levels. In order to avoid the need for continuous calibration between every measurement by an external standard sample (*R. calendulaceum*) an internal standard (a *Lolium multiflorum* 'Bellem' nuclear suspension) was added to each sample (Figure 2). The ratio between the sample peak fluorescence (SPF) and the grass peak fluorescence (GPF), as interpreted from the X-axis, compared to the ratio between the *R. calendulaceum* peak and the grass peak fluorescence, gave a more trustworthy indication of the ploidy level of the sample. Results, however, were found to be similar to those results obtained after measuring without internal standards (Table 2). Two groups can clearly be distinguished: the largest group has a SPF/GPF ratio of 0.68-0.80, comparable to (or slightly higher than) the *R. calendulaceum* SPF/GPF ratio of 0.70, whereas the ratios of the smallest group vary between 0.49 and 0.58. The ratios of the smallest group are 70-83% of the *R. calendulaceum* ratio; therefore, it may be assumed that the smallest group contains triploids and the largest group holds tetraploids.

Discussion

Our results imply a very strong influence of tetraploid species during the development of Hardy Ghent and Rustica hybrids. The botanical species *R. luteum* appears to be tetraploid, which is contradictory to previous results obtained by chromosome counting (Janaki Ammal *et al.*, 1950). This was observed on samples collected on three different genotypes (seedlings growing at different places). This also explains why most of Hardy Ghent and Rustica hybrids, measured in this experiment and derived from crosses involving *R. luteum* and *R. calendulaceum*, are tetraploid. Mixoploidy was not observed. Since most hybrids had already been created in the 19th century, exact parental data usually are missing. We suspect that molecular analysis would reveal a closer relationship of the hybrid groups with *R. calendulaceum* and *R. luteum* (and *R. canadense* and other possible tetraploids) than with diploid botanical species. This is especially important regarding *R. luteum*, which has so far incorrectly been described as a diploid species, and has probably been underestimated in the past. All other measured species were diploid, which was in agreement with earlier publications. A thorough ploidy screening for all 30 Pentanthera species and other hybrid groups is highly recommended, to find out whether even more species are polyploid. Considering ploidy levels, most *Rhododendron* breeders still directly or indirectly rely on the data presented by Janaki Ammal (1950) that appear not entirely trustworthy, most probably due to the unavailability of flow cytometry as an accurate, quick, and reliable screening tool at the time.

Some hybrids are triploid, indicating the presence of diploid species in their ancestry, which did not establish an aberrant morphology or growth vigor compared to the tetraploids. Apart from *R. luteum*, Pentanthera species that were tested and that are possibly involved in the creation of the hybrids were diploid. These data suggest that the origin of tetraploid hybrids is confined to tetraploid species (*R. luteum* and *R. calendulaceum*) whereas triploid hybrids were derived from crosses with diploid species. When regarding the ancestry of the triploid hybrids, some interesting data are revealed. R. 'Daviesii' is often described as a *R. viscosum* x *R. molle* seedling. *R. molle* had no part in our experiments but is described as diploid. Probably 'Daviesii' results from an interploidy pollination and therefore the *viscosum* x *molle* parentage appears questionable (unless

Table 2. SPF/GPF* Ratios and Ploidy Level for All Hardy Ghent and Rustica Hybrids Examined

(SPF: sample peak fluorescence GPF: grass peak fluorescence)

Hybrid	Ratio SPF/GPF	2n (leaf)	Hybrid	Ratio SPF/GPF	2n (leaf)
R. calendulaceum (2n=4x)	0.70	4x	'Batholo Lazarri'	0.72	4x
			'Guelder Rose'	0.75	4x
'Nancy Waterer'	0.75	4x	'Gloria Mundi'	0.58	3x
'Unique'	0.68	4x	'Coccinea Major'	0.76	4x
'Narcissiflorum'	0.74	4x	'Raphaël De Smet'	0.74	4x
'Jozef Baumann'	0.75	4x	'General Trauff'	0.72	4x
'Maja'	0.76	4x	'Graff von Meran'	0.72	4x
'Rosetta'	0.75	4x	'Goldlack'	0.71	4x
'Mina Van Houtte'	0.54	3x	'Van Houtte Flore Pleno'	0.55	3x
'Daviesii'	0.55	3x			
'Semiramis'	0.77	4x			
'Quadricolor'	0.50	3x	'Norma'	0.51	3x
'Souvenir du Pres. Carnot'	0.71	4x	'Phebe'	0.49	3x
			'Fenelon'	0.70	4x
'Marie Verschaffelt'	0.76	4x	'Racine'	0.80	4x

R. molle is not a diploid). 'Gloria Mundi' is suspected to be a cultivated *R. calendulaceum* type; however, in that case we would have expected a tetraploid ploidy level.

Unlike members of the other subgenera, Pentantheras occur naturally at distinct places, widely scattered over the globe: the Caucasian region, Eastern North America, Western North America, and the Far East. As a result, Pentantheras do not seem to form a distinct group like Tsutsusi, Hymenanthes, or Rhododendron on morphological or on molecular levels (Kron, 1993; 2000). Polyploidization has occurred at at least two developmental centers: the Caucasian region *(R. luteum)* and Eastern North America *(R. calendulaceum)*. This is not surprising, since recurrent formation of polyploidy has been described in numerous crops (Leitch and Bennett, 1997; Soltis and Soltis, 2000). Gene silencing, gene diversification, and/or chromosomal translocation can be assumed to have influenced subsequent species development since they are the usual consequences of polyploid formation (Soltis and Soltis, 1993). Upon allopolyploid formation, dormant transposons may be activated by a genomic shock due to a difference in repetitive elements between two parental genomes (McClintock, 1984); this causes an expansion of heterochromatic knobs leading to increased chromosome length (Comai, 2000). This may be an explanation for the slightly increased ploidy level (> 4x) that was found in most tetraploids, compared to *R. calendulaceum.* Possibly hybridization with other tetraploids (creating the Hardy Ghent hybrid group) caused limited knob formation, thus extending the nuclear genome slightly. Aneuploidy due to chromosomal addition appears unlikely because of complete plant fertility.

The higher ploidy level of certain groups of deciduous azaleas can be part of the explanation for crossing incongruity with evergreen azaleas (Tsutsusi), which mostly are diploid (De Schepper *et al.,* 2001). However, crossing barriers occurs to the same extent when tetraploid Tsutsusi genotypes are pollinated by *R. luteum* or Hardy Ghent hybrids (Eeckhaut, data unpublished).

Bilateral crosses are impeded by Tsutsusi pollen tube growth inhibition in the Pentanthera style (Ureshino *et al.,* 2000). The extent to which this inhibition is caused by ploidy differences, is unknown. However, Rouse (1993) describes a higher congruity of Tsutsusi species with (diploid) *R. occidentale.* Therefore, pollination of *R. luteum* or other tetraploids with diploid pollen might be a means to circumvent tube growth problems and to induce seed set. Tetraploid pollen donors can be created through chromosome doubling as described in Vaïnola (2000) or Eeckhaut *et al.* (2001). Next to the efficiency improvement (or establishment) of deciduous x evergreen azalea crossing, the data obtained in this research may also be of interest for azaleodendron breeding. The very confined number of azaleodendrons obtained so far (Salley and Greer, 1992) might be caused by different ploidy levels; chromosome doubling of rhododendrons or selection of tetraploid rhododendrons may enhance breeding efficiency significantly.

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Erik is chief of staff, while Leen and Tom are performing scientific research. The DvP-CLO is a Flemish (Dutch-speaking part of Belgium) governmental research institution. Erik manages about 100 people, 30 of whom are academics.

Tom's Ph.D. research project covered interspecific breeding between Beligh pot azaleas and yellow flowering species belonging to different subgenera or sections of Rhododendron (Hymenanthes, Rhododendron, Pentathera, Vireya). He is now performing interspecific crosses between wild and cultivated roses.

Leen has practical experience with flow cytometry, and she assisted in interpretation of the ploidy histograms for this paper. She is currently working on a project regarding development of bioassays to determine resistance of roses against several fungal pathogens.

***Albert De Raedt** provided the research team with the plant material. For the past 20 years he has collected many deciduous azaleas, especially Hardy Ghent and Rustica hybrids, in his magnificent garden. Here, he maintains an extensive gene pool that he is willing to "share" with the researchers on this project, and the team has collaborated on several occasions. In 2000 he published a monograph on the Hardy Ghent azaleas.*

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Figure 1. Flow-cytometrical Patterns of *R. Calendulaceum, R. Luteum* and *R. Prinophyllum* Leaves, Anther Filaments, and Roots.
The sample peak is indicated by arrow. Peaks on the extreme left are caused by debris fluorescnce (d).
X-axis: fluorescence (channel number); Y-axis: number of nuclei.

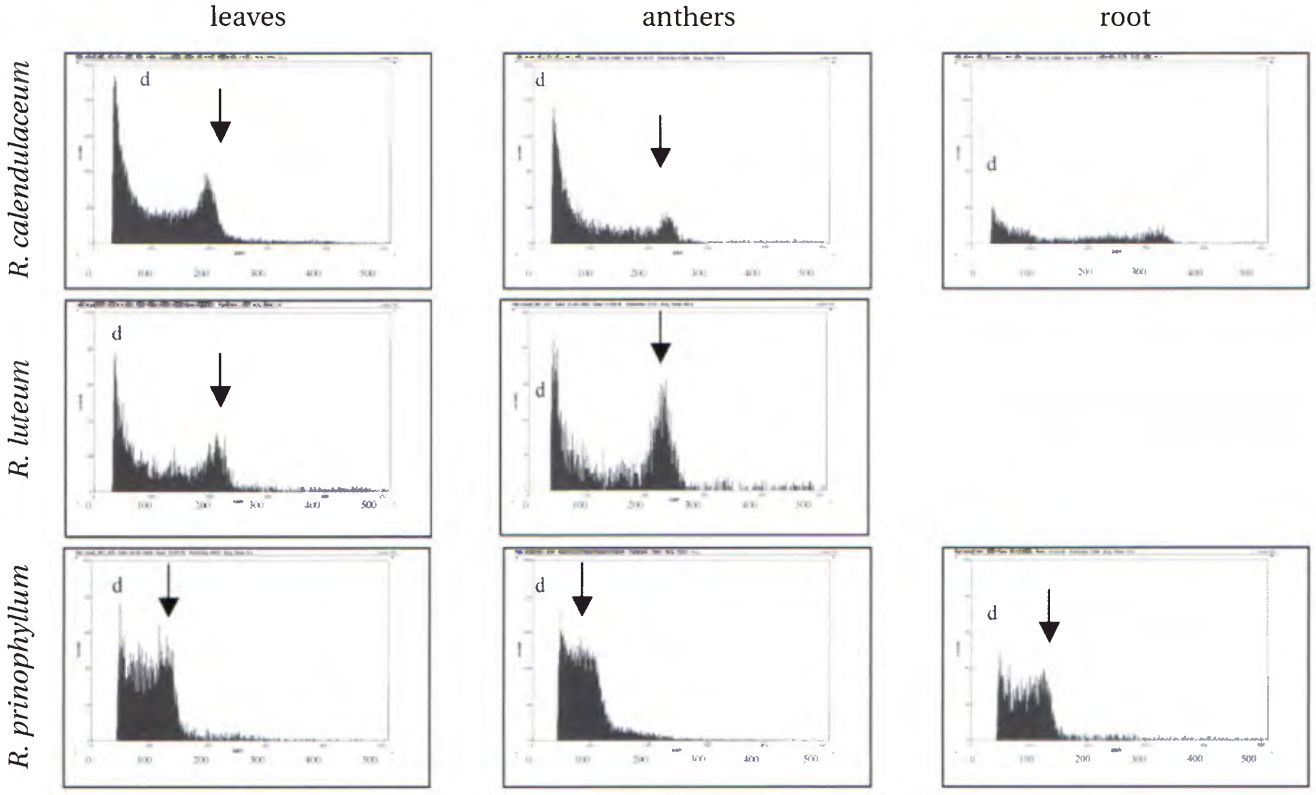

Figure 2. Flow-cytometrical Patterns of *R. Calendulaceum, R.* 'Jozef Baumann', and *R.* 'Daviesii', Without and With the Application of an Internal *Lolium* Standard (IS)
The sample peak is indicated by the arrow. Peaks on the extreme left are caused by debris fluorescnce (d).
X-axis: fluorescence (channel number); Y-axis: number of nuclei.