Faculteit Landbouwkundige en
Toegepaste Biologische Wetenschappen

Academiejaar 2002-2003

PLOIDY BREEDING AND INTERSPECIFIC HYBRIDIZATION IN *SPATHIPHYLLUM* AND WOODY ORNAMENTALS

PLOÏDIEVEREDELING EN INTERSPECIFIEKE HYBRIDISATIE BIJ *SPATHIPHYLLUM* EN HOUTACHTIGE SIERGEWASSEN

door

ir. Tom EECKHAUT

Thesis submitted in fulfillment of the requirements for the degree
of Doctor (Ph.D.) in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad van
Doctor in de Toegepaste Biologische Wetenschappen

op gezag van

Rector: **Prof. Dr. A. DE LEENHEER**

Decaan:
Prof. Dr. ir. H. VAN LANGENHOVE

Promotoren:
Prof. Dr. ir. E. VAN BOCKSTAELE
Prof. Dr. ir. P. DEBERGH

De promotoren:

Prof. Dr. ir. E. Van Bockstaele

Prof. Dr. ir. P. Debergh

De auteur

ir. Tom Eeckhaut

"Plant breeders are applied evolutionists, even if they have hardly noticed the fact"

N.W. Simmonds

Woord vooraf

De voorbije maanden heeft het ineenflansen van dit boekje mij in niet geheel onaanzienlijke mate geconfronteerd met ervaringen die mij al jarenlang in hoge mate boeien: dokkeren over kasseien, beklimmen van slopende hellingen, racen tegen de klok en afronden met een nijdig sprintje. Dat ik uiteindelijk de streep haal is in de eerste plaats te danken aan de rugwind die mij steeds royaal werd verstrekt door een hele groep mensen.

Allereerst wens ik een dankwoord te richten tot mijn beide promotoren. Prof. Pierre Debergh liet mij als student kennismaken met weefselteelt en toont zich nu reeds 7 jaar lang een gedroomd wetenschappelijk leermeester. Na mijn studies bood hij mij de kans 2 jaar verder in het Laboratorium voor Tuinbouwplantenteelt te blijven werken. Ook hierna bleef zijn interesse in mijn doen en laten constant en kon ik steeds op zijn feedback rekenen, die altijd van grote waarde bleek. Voor dit alles ben ik hem veel dank verschuldigd. Mijn tweede 'ploegleider', Prof. Erik Van Bockstaele, ben ik eveneens heel dankbaar voor zijn aanmoedigingen om voort te werken aan dit werkstuk, de kansen die hij mij bood om mijn vorig werk af te ronden en zijn managementscapaciteiten, die mij toelieten om ondanks het doorlopen van meerdere projecten een hoge graad van continuïteit in mijn werk te integreren, die uiteindelijk het schrijven van dit doctoraat hebben mogelijk gemaakt. Tevens ben ik hem dankbaar voor de interesse die hij steeds voor mijn vorderingen aan de dag legde, ondanks de voor velen onoverzichtelijke mozaïek aan projecten en werkzaamheden waarover hij de dagelijkse hoede heeft.

Een speciaal woord van dank behoud ik ook voor aan mijn oudere en wijzere (?:-) collega's, die mij van den beginne lieten meegenieten van een schat aan ervaring en know-how. Stefaan Werbrouck, die mij als student reeds begeleidde en wiens deur tot op de dag van vandaag wijd openstaat; Johan Van Huylenbroeck, die zovele van mijn teksten grondig doornam en bij wie ik steeds terechtkan voor de soms broodnodige goede raad; Jan De Riek, voor de hulp bij de verwerking van de moleculaire analyses en veelvuldige hard- en softwareproblemen; Gilbert Samyn en Sandra De Schepper, bij wie ik steeds mocht aankloppen voor discussie of advies; en verder ook aan Els Coart, Hilde Muylle en Veerle Lamote, wiens praktische tips tijdens deze doctoratitis-periode van goudwaarde waren.

Een grote "dank u" aan Paul Bruynseels die honderden liters medium voor mij heeft gemaakt en een hele hoop vaak minder aangename klussen steeds met de glimlach heeft geklaard, en aan Romain Uytterhaegen, die als geen ander een schat aan praktische kennis over azaleaveredeling bezit en mij voorzag van hopen praktische tips. Ook Jochen Dendauw, Ronald Van Den Oord, Kristien Janssens, Veerle Cools, Hilde Carlier en Laurence Desmet wens ik te bedanken voor de veelvuldige technische bijstand. Verder ben ik ook een woord van dank verschuldigd aan Patrick Vandenhoeck, Nele Van Moer, Wouter Tailleux en Stijn Op De Beeck, mijn stagiairs en thesisstudenten.

Mijn bureaugenotes Leen Leus, Katrien Maes, Myriam Van Quickenborne, Katrijn Van Laere, Hilde Dhuyvetter en Claire Piette, die een exponent vormden van de relatieve absentie van het Y-chromosoom in mijn dagelijkse omgeving, bedank ik bij deze voor de babbels van zowel wetenschappelijke als ontspannen aard die bijdroegen tot de goede sfeer. Tevens

bedankt Leen, voor alle wetenschappelijke en technische ondersteuning bij de ploïdiemetingen. Uiteraard mag ik ook diegenen niet vergeten die door gezamenlijke quiz-, squash- en loopactiviteiten tussen en na de uren bijdroegen aan een aangename werkomgeving; ik kan niet iedereen bij naam vermelden maar ik maak toch een uitzondering voor Thea Versluys en Pol Vanneste, de harde kern van de universitaire kaartclub:-).

Verder gaat mijn dank ook uit naar de leden van de lees-en examencommissie voor het doornemen van dit manuscript, hun kritische opmerkingen en hun aanwezigheid op de openbare verdediging.

Dankjewel ook aan alle familieleden, vrienden, kennissen, studiegenoten en de talloze (ex-) collega's die ik hier niet bij naam vermeld heb maar waarbij ik de voorbije jaren terecht kon. Last but certainly not least is ook een dankwoord aan mijn ouders en broer onontbeerlijk. Zij zijn degenen geweest die mij steeds gestimuleerd hebben het beste uit mijzelf te halen en degenen die mij in de moeilijke periodes opnieuw wisten te relanceren. Klaar voor een volgende etappe, want de beste garantie voor de dag van morgen is die van vandaag.

Content

Samenvatting

Bij een aantal sierplanten werden *in vitro* technieken uitgewerkt om de productie van haploïden, polyploïden en interspecifieke hybriden mogelijk te maken. De toepassingsmogelijkheden van de gebruikte technieken en het mogelijk nut van de bekomen planten verschillen naargelang de plantensoort. In totaal werden 4 genera in het onderzoek betrokken, die zowel monocotylen als dicotylen en bloeiende kamerplanten evenals houtachtige siergewassen omvatten.

Ovulencultuur van *Spathiphyllum wallisii* liet toe homozygote genotypen te ontwikkelen. De efficiëntie lag eerder laag; de reactie van de ovulen op het inductiemedium was sterk cultivar-afhankelijk. Ploïdie- en AFLP-analyses lieten een ondubbelzinnige bevestiging van homozygotie toe, hoewel er geen waarneembare inteeltdepressie optrad. Er dienen echter meer homozygoten (vertrekkend van verschillende cultivars) ontwikkeld te worden om algemene conclusies te formuleren. Verdubbeling van het (haploïd) genoom bleek spontaan op te treden. Een aantal *in vitro* technieken (gebaseerd op verschillende types uitgangsmateriaal) om tetraploïde *Spathiphyllums* te induceren werden uitgewerkt; als meest efficiënte kwam de behandeling van secundaire somatische embryo's (oorspronkelijk geïnduceerd op antherenfilamenten) naar voor. De combinatie van ovulencultuur en polyploïdisatie laat de ontwikkeling van autotriploïden via zaad toe.

De inductie van polyploïdie bij de vlinderstruik (*Buddleja*) lag heel hoog. Interspecifieke hybriden tussen *B. davidii* en *B. weyeriana* werden ontwikkeld en getest m.b.v. AFLP; hoewel volop hybriden gevormd werden, waren de meeste combinaties van ouderplanten weinig fertiel. Uit het onderzoek bleek verder dat *B. weyeriana* niet triploïd is; totnogtoe kon geen definitief uitsluitsel geformuleerd worden betreffende de genomische samenstelling van deze allopolyploid. De relatief eenvoudige induceerbaarheid van polyploïden en interspecifieke hybriden, gekoppeld aan een hoge groeikracht, bieden uitzicht op verder onderzoek naar intergenoom interacties.

Vergeleken met *Buddleja*, lag de efficiëntie van polyploïdie-inductie bij potazalea (*Rhododendron simsii* hybriden) eerder laag. Nochtans konden een aantal volledig tetraploïde planten geregenereerd worden. De gewijzigde morfologie biedt mogelijk commerciële perspectieven. Tevens werd een techniek ontwikkeld om de creatie van amfidiploïden na interspecifieke hybridisatie mogelijk te maken. Zowel genotypen uit de Hymenanthes-, Pentanthera-, als Rhododendron- (inclusief Vireya) subgenera werden gebruikt in interspecifieke bestuivingsexperimenten met potazalea, met als doel het inkruisen van biosynthesegenen voor gele of oranje bloempigmenten. Incongruentie trad op verschillende niveaus op, zowel vóór als ná de bevruchting van de eicel; bovendien was albinisme na sommige combinaties verantwoordelijk voor 100% uitval. *In vitro* cultuur van onrijp zaad verhoogde de efficiëntie van de kruisingen. Een aantal groeikrachtige hybriden werd ontwikkeld na kruisingen met Hymenanthes-, Rhododendron- en Vireya-cultivars. Microsatellieten bleken de meest efficiënte merkers voor ouderschapsanalyse. Bloei werd nog niet waargenomen.

Interspecifieke hybridisatie was ook succesvol na *Hibiscus syriacus* x *H. paramutabilis* kruisingen. Een aantal hexaploïde hybriden werd ontwikkeld. Bloei is reeds opgetreden. Deze genotypen zullen gebruikt worden als prebreeding-materiaal met het oog op de verrijking van het bestaande *H. syriacus*–assortiment, waarin introgressie van de grotere groeikracht van *H. paramutabilis* beoogd wordt. Op dat punt zijn de ontwikkelde hybriden uiteenlopend.

Morfologische merkers konden niet steeds aangewend worden na interspecifieke hybridisatie, aangezien veel van de ontwikkelde hybriden matromorf waren. Dit was vooral het geval voor *Rhododendron* en *Buddleja*, terwijl *Hibiscus*-hybriden een eerder intermediaire habitus vertoonden. Via AFLP werd een ouderschapsanalyse uitgevoerd. Morfologische gevolgen van polyploïdisatie waren zowel duidelijk bij *Spathiphyllum*, *Rhododendron* als *Buddleja*, en bevestigen de in de literatuur beschreven 'typische' kenmerken van polyploïden. Tevens was de hogere efficiëntie van dinitroaniline-herbiciden voor de productie van polyploïden vergeleken met colchicine opmerkelijk.

Summary

We elaborated *in vitro* techniques for a number of ornamental crops in order to establish the production of haploids, polyploids and interspecific hybrids. The possibilities to apply these techniques and the horticultural relevance of the new genotypes are evidently dependent on the species used. Altogether 4 different genera were involved in the experiments, including monocots as well as dicots, and flowering indoor plants as well as woody ornamentals.

Culture of *Spathiphyllum wallisii* ovules allowed the development of homozygous genotypes. Efficiency was rather low and the reaction of the ovules was to a large extent dependent on the cultivar. Ploidy and AFLP-analyses unequivocally confirmed the homozygous state of the plantlets. There was no visible inbreeding depression, but more doubled haploids derived from different cv's should be created before generalizing this observation. Chromosome doubling in haploids appeared to occur spontaneously. Starting from various tissue types, different polyploidization techniques were attempted in *Spathiphyllum*; treatment of secondary somatic embryos (originally induced on anther filaments) turned out to be most efficient. Theoretically, the combination of ovule culture and polyploidization allows the development of autotriploids through seed, after the selection of the best fitting diploid and tetraploid parents.

Polyploidy induction in the butterfly bush (*Buddleja*) through seedling treatment was very high. Interspecific hybrids between *B. davidii* and *B. weyeriana* were developed and tested through AFLP. Many hybrids were derived from these crosses, although most parent combinations did not result in viable seedlings. Moreover, ploidy screening revealed the non-triploid status of *B. weyeriana*. The genomic constitution of this allotriploid remains unclear so far. Thanks to the relatively simple induction of polyploids and interspecific hybrids and the high growth vigor prospects for further research of intergenomic interactions are evident.

Compared to *Buddleja* polyploidization efficiency in pot azalea (*Rhododendron simsii* hybrids) was rather low. Nevertheless, a number of fully tetraploid plants were regenerated. The altered morphology may be commercially interesting; at the same time the technique may aid in the creation of amphidiploid interspecific hybrids. Interspecific pollination experiments with pot azalea, aiming to introduce genes involved in the biosynthesis of yellow or orange flower pigments, included crossing partners from the Hymenanthes, Pentanthera and Rhododendron (including Vireya) subgenera. Incongruity was observed at different levels, both pre- and postzygotic. Moreover, albinism was responsible for 100% mortality after combinations of specific groups. *In vitro* culture of immature seeds clearly increased crossing efficiency. Hymenanthes, Rhododendron and Vireya genotypes could be hybridized with azalea. Microsatellites were the most efficient markers for paternity testing. Flowering was not observed yet.

Interspecific hybridization was also successful after crossing *Hibiscus syriacus* x *Hibiscus paramutabilis*, resulting in tetraploid and hexaploid hybrids. Flowering was evaluated. The hybrids will be used as prebreeding material for the enlargement of the available *H. syriacus* assortment, aiming for introgression of the growth vigor of *H. paramutabilis*. The hybrids obtained so far show divergent growth characteristics.

Visual distinction of hybrids was not always possible since most were matromorph, especially in *Rhododendron* and *Buddleja*, while *Hibiscus* hybrids showed intermediate characteristics. AFLP-analyses were performed to reveal parenthood. Morphological consequences of polyploidization are obvious in *Spathiphyllum*, *Rhododendron* and *Buddleja*, and were consistent with 'typical' polyploid traits described in literature. In all species, the higher efficiency of dinitroaniline herbicides in producing polyploids compared to colchicine is remarkable.

List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	isopentenyladenosin
A	adenin
ABA	abscisic acid
AFLP	Amplified Fragment Length Polymorphism
B5	Gamborg (1968) medium
BA	N_6-benzyladenin
BMB	basal medium *Buddleja*
BMH	basal medium *Hibiscus*
BMR	basal medium *Rhododendron*
BMS	basal medium *Spathiphyllum*
Bp	base pairs
C	cytosin
COL	colchicine
CTAB	alkyltrimethylammoniumbromid
cv	cultivar
cv's	cultivars
DAPI	4', 6-diamidino-2-phenylindol
DMSO	dimethylsulfoxide
E-	primer homologous with EcoRI adaptor
EDTA	ethylenediaminetetraacetic acid
EtOH	ethylalcohol
FDA	fluorescein diacetic acid
FDR	first division restitution
G	guanin
GISH	genomic in situ hybridization
Hy	Hymenanthes subgenus
IMA	imazalil
KIN	kinetin
LI	histogenic layer 1
LII	histogenic layer 2
LIII	histogenic layer 3
LDPE	low density polyethylene
LSD	least significant difference
M-	primer homologous with MseI adaptor
MI	mitosis inhibitors
MS	Murashige & Skoog (1962) medium
NAA	α-naphtylacetic acid
NN	Nitsch & Nitsch (1969) medium
ORY	oryzalin
PAD	plants with artificially doubled chromosome number
PAGE	polyacrylamid gel electrophoresis
PAR	photosynthetic active radiation
PCR	polymerase chain reaction
Pe	Pentanthera subgenus
PIC	picloram
PRO	prochloraz
Rh	Rhododendron section (in Rhododendron subgenus)
SDR	second division restitution
SE	somatic embryogenesis
se	somatic embryos
SSR	simple sequence repeats (microsatellites)
T	thymin
TDZ	thidiazuron
TRI	trifluralin
TRIF	triflumizol
Ts	Tsutsusi subgenus
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
Vi	Vireya section (in Rhododendron subgenus)
WPM	Woody Plant (1980) medium
Z	zeatin

Introduction

Breeders of ornamentals are constantly in search of new technologies that may offer a valuable aid in cost reduction, product quality enhancement and assortment enlargement. Plant tissue culture comprises a set of *in vitro* techniques, methods and strategies. It has been exploited to create genetic variability to improve crop plants and their health status and to preserve germplasms (Taji *et al*., 2002).

In vitro clonal micropropagation is a widely used method to multiply numerous herbaceous and woody plant species (Van Bockstaele, 2001; Debergh, 2002). The technique is used to produce plants under controlled climatic conditions in culture containers on a medium containing all inorganic and organic nutrients, a carbon source and growth regulators (George, 1993). It therefore offers a powerful tool for different breeding purposes, allowing to keep the plants in a highly controlled environment and to grow new plants starting from isolated organs or undifferentiated tissue. *In vitro* techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in breeding lines (Brown & Thorpe, 1995). Apart from micropropagation, the most important *in vitro* applications are somaclonal and gametoclonal variation, pathogen eradication, germplasm conservation, synthetic seed production through somatic embryogenesis, haploid breeding, polyploid breeding and interspecific hybridization. The latter three are evaluated in more detail in the literature review (chapter 1). Chapter 2 provides a description of the general materials and methods that were applied in this research.

The relative importance of flowering indoor plants (incl. *Spathiphyllum*), pot azalea and ornamental shrubs (incl. *Hibiscus* and *Buddleja*) within the Belgian ornamental sector is evident from figures published by EROV. All three groups show a positive and increasing trade balance. Export values of 52 million € (flowering indoor plants), 39 million € (pot azalea) and 50 million € (ornamental trees) were noted, respectively corresponding with 19.8%, 14.9% and 19.1% of the total Belgian export of ornamentals (Grillaert, 2003).

In vegetatively propagated ornamentals, consisting of complex hybrids, clonal propagation by seeds is a demand. Haploid breeding allows the creation of homozygous parent plants for an F_1-hybrid generation, with a homogeneous morphology (apart from environmental influences) due to an identical genotype, and in this way the multiplication of desired genotypes through seed becomes a reality. *Spathiphyllum* is very heterozygous and is frequently sold as seedlings. Tissue culture approaches can contribute to a significant product quality enhancement. Compared to micropropagated genotypes *in vitro*, approaches to produce F_1-hybrids would be substantially cheaper. Theoretically, all haploid cells can be used for haploid production; since microspores of *Spathiphyllum* were found to be recalcitrant, emphasis was put on gynogenesis (chapter 3).

Polyploidy is often associated with enhanced growth vigor, larger flowers or other traits of major importance for ornamental crops. Polyploidy induction is wanted in the genera *Spathiphyllum*, *Rhododendron* and *Buddleja*. Different techniques were evaluated to find the most efficient chromosome doubling protocol. In the herbaceous crop *Spathiphyllum wallisii*, polyploidization through somatic embryogenesis and adventitious shoot formation was

attempted (chapter 4). Along with haploid breeding technology, chromosome doubling would enable the creation of triploid F_1 hybrids with increased growth vigor, as is the case with other triploids, which can be synthesized through seed. The aim of polyploidy induction in the woody ornamentals *Buddleja davidii* and *Rhododendron simsii* was to obtain plants with altered morphologic features and to develop appropriate technology for the development of synthetic allopolyploid hybrids. *Buddleja* seedlings were treated with different mitosis inhibitors (chapter 5); for azalea, alternative procedures were also tested (chapter 7).

Interspecific hybridization is a means of transferring genes within related species; as such, it offers an alternative for genetic modification. Growth and development of the "alien" pollen tube can be impeded in the female style; moreover, after fertilization embryo development can be arrested by malformation of nurse tissue (mostly endosperm). "Embryo rescue" is an *in vitro* technique allowing more hybrid embryos to develop on an adapted medium. In chapter 5, *Buddleja davidii* breeding through interspecific hybridization is discussed. Like azalea and *Hibiscus*, *Buddleja* is a woody ornamental, but a far more vigorous grower, and therefore considered suitable for the establishment of model techniques. The backcrossing of *B.* x*weyeriana* to *B. davidii* was performed in order to allow introgression of yellow flower petals in *B. davidii*. Within *Rhododendron* the obtention of pot azaleas with new floral traits could benefit from embryo rescue. Indeed, classical breeding is strongly hampered by incongruity between parents belonging to different subgenera. However, since the genus *Rhododendron* is a giant jigsaw puzzle of hundreds of species and thousands of hybrids, finding the most suitable partners is at least as important as creating the best possible *in vitro* environment for embryo maturation. This is described in chapter 6. The objective of interspecific breeding within *Hibiscus* was to introduce enhanced growth vigor into *Hibiscus syriacus* through crossings with *H. paramutabilis* (chapter 8). In chapter 9 at last, general conclusions are drawn and perspectives for future breeding strategies are formulated.

1. Literature review

1.1. Haploid induction

For the induction of haploids, *in vitro* techniques are much more efficient than interspecific hybridization or *in vivo* treatments with plant growth regulators, temperature or irradiation (Atanassov *et al*., 1995). From literature it is evident that the induction of (doubled) haploids is largely restricted to a limited number of families: Solanaceae, Brassicaceae, Cucurbitaceae, Asteraceae, Poaceae, Liliaceae (Keller & Korzun, 1996). In several ornamentals the production of haploids has been attempted (De Jeu, 2000): *Tulipa* (Van den Bulk *et al*., 1994), *Lilium* (Han *et al.,* 1997), *Gerbera* (Meynet & Sibi, 1984; De Wit *et al*., 1990; Miyoshi & Asakura, 1996). Three main *in vitro* methods are frequently used to generate haploids (Bajaj, 1990): culture of excised ovaries and ovules, embryo culture after wide pollination, and culture of excised anthers and microspores. Pierik (1999) also mentions chemical treatments, temperature shocks or irradiation, and frequently environmental stress conditions are applied along with microspore or anther culture.

In vitro culture of nonfertilized ovaries is a unique possibility for obtaining haploids from male sterile plants; moreover, female gametophytes are alternative sources for the recovery of haploids if anthers or microspores do not respond to culture conditions or produce calli with low morphogenic potential. The frequency of regeneration of green plants from female gametophytes is higher than from males (Mukhambetzhanov, 1997), but in general the recovery of haploid plantlets is lower since ovaries or ovules often only increase their size by cell proliferation of somatic tissues and embryo sac haploid elements do not frequently show embryogenic activity.

Rangan (1984) described gynogenesis as "the further development *in vitro* of the egg cell or another haploid nucleus of the embryo sac mother cell, without fertilization". In other words, haploid production is the result of abnormal development of the female gametophyte and subsequent embryo or callus formation. Generally only one gynogenetic structure develops per embryo sac from the egg cell, synergids or antipodal cells, whereas no embryos appear in ovules containing autonomous endosperm, making the development of embryo and endosperm competitive (Mol, 1999). The first haploid calli were obtained from unfertilized ovaries of *Ginkgo biloba* by Tulecke (1964). Many factors limit the development of haploid plantlets through ovary or ovule culture: genotype, developmental stage of the gametophyte, nutrient medium composition and culture conditions (Ferrie *et al*., 1995).

The plant genotype seems to be one of the most important parameters in gynogenesis. Empirically selected conditions for obtaining haploids from a cultivar or species usually prove to be not adequate for another one. This implies that genotypes of different origin should be considered in all experiments. Genes responsible for differences between species suitable or non-suitable for gynogenesis have not been identified yet. In general, the developmental stage of the embryo sac mother cell seems to be less important (Cappadocia *et al.,* 1988). Apparently the development of a female gametophyte is less developmental stage dependent than for male gametophytes, where the uninuclear stage is optimal. However, according to Mukhambetzhanov (1997) the late stages of embryo sac development are most suitable for the cultivation of nonfertilized ovaries or ovules of the majority of plant species.

Most authors who have been using gynogenesis apply growth regulators (auxins and cytokinins); no general recommendations can be formulated, although higher concentrations of growth regulators may be responsible for the induction of calli on somatic tissues and thus inhibit embryogenesis from haploid cells. Some rare reports mention the beneficial effect of physical stress on gynogenesis (Keller & Korzun, 1996). Because ovules are small and fragile, they are most often isolated from a precultured ovarium and transferred to a fresh medium. Ovary and ovule cultures of most species are kept in the dark. Haploid plantlets are formed indirectly (through formation of callus and organogenesis, e.g. *Gerbera*), or directly, without callus formation (e.g. tobacco) (Keller & Korzun, 1996; Mukhambetzhanov, 1997; Metwally *et al*., 1998). In case spontaneous diploidization doesn't occur, colchicine or other mitotic disrupters (Van Tuyl *et al*., 1992; Hansen & Andersen, 1996; Rao & Suprasana, 1996; Hansen *et al*., 2000) are used.

Gynogenetic haploids have been derived in the following plant species: *Beta vulgaris* (Van Geyt *et al*., 1987; Doctrinal *et al*., 1989; Lux *et al*., 1990; Ferrant & Bouharmont, 1994; Hansen *et al*., 2000), *Cucumis melo* (Cuny *et al*., 1993; Ficcadenti *et al*., 1999), *Allium cepa* (Keller, 1990; Campion & Alloni, 1990; Campion *et al*., 1992; Bohanec *et al*., 1995; Geoffriau *et al*., 1997b), *Allium porrum* (Schum *et al*., 1993), *Melandrium album/Silene alba* (Mol, 1992), *Triticum durum* (Sibi *et al*., 2001), *Triticum aestivum* and *Nicotiana tabacum* (Zhu *et al*., 1979), *Hordeum vulgare* (Wang & Kuang, 1981), *Zea mays* (Uchimaya *et al*., 1971), *Gossypium hirsutum* (Pallares, 1984), *Helianthus annuus* (Hongyuan *et al*., 1986), *Oryza sativa* (Chang *et al*., 1986), *Solanum tuberosum* (Hvilkovskaya, 1982) and *Cucurbita pepo* (Shalaby, 1996; Metwally *et al*., 1998).

Gynogenesis can often be induced by pollination with irradiated pollen or pollen from related species (Bohanec, 1994). There are multiple applications for irradiation in plant breeding (Ahloowalia, 1998). One of them is the inhibition of the division of the generative nucleus, thus preventing the formation of sperm nuclei. Although this prevents the creation of a diploid embryo, the "pollination and fertilization stimuli" are still present because the vegetative nucleus (being less dense) remains relatively unaffected by the irradiation, allowing the germination and the growth of the pollen tube from stigma to ovule. These stimuli can induce a parthenogenetic response, which is already described in a number of species. It is important to find the appropriate radiation dose for preventing the division of the generative nucleus but not arresting the growth of the pollen tube through the pistil. For *Petunia hybrida*, *Cucumis melo*, *Cucumis sativus*, *Malus domesticus*, *Pyrus communis* and *Rosa hybrida* the doses used were at least 300 Gray (Raquin, 1985; Sauton & Dumas de Vaulx, 1987; Rode & Dumas de Vaulx, 1987; Zhang & Lespinasse, 1991; Falque *et al*., 1992; Bouvier *et al*., 1993; Cuny *et al*., 1993; Meynet *et al*., 1994; Maluszynski *et al*., 1996, Hofer & Lespinasse, 1996; Todorova *et al*., 1997; Sato *et al*., 2000, Wenslaff & Lyrene, 2000). *In vitro* regeneration from ovaries, ovules or embryos increases the yield of induced haploids.

Pollination with pollen of related species can induce embryo development but later on elimination of the chromosomes of the wild species can occur, causing the development of a haploid plantlet. This technique is widely used in barley (*Hordeum vulgare* x *Hordeum bulbosum*) (Kasha & Kao, 1970), wheat (Ahmad & Comeau, 1990), oat (Riera-Lizarazu *et al*.,

1996) and potato (*Solanum tuberosum* x *Solanum phureja*) (Rowe, 1974). DNA-sequence elimination is one of the major responses of the wheat genome to wide hybridization (Shaked *et al*., 2001). Also pollen of a triploid cv is able to induce parthenogenesis in *Citrus* (Germana & Chiancone, 2001).

Unlike gynogenesis, androgenesis can be triggered by many physical factors (Wang & Chen, 1986; Rode & Dumas de Vaulx, 1987; Chaboud & Perez, 1992; Chen, 1992; Cuny *et al*., 1993; Cao *et al*., 1994; Meynet *et al*., 1994; Coumans & Zhong, 1995; Hu *et al*., 1995; Dunwell, 1996; Rihova & Tupy, 1996; Sopory & Munshi, 1996; Touraev *et al*., 1996; Dohya *et al*., 1997, Hu & Kasha, 1997): temperature, irradiation, pressure, CO_2-treatment, centrifugation are considered as main environmental triggers; of course, also genotype, developmental stage of the microspores and medium composition can provoke or prevent microspore reaction.

1.2. Polyploid induction

Polyploidy can arise naturally due to somatic mutation (a disruption in mitosis) resulting in chromosome doubling in (a) meristematic cell(s) that will subsequently produce a polyploid shoot, or due to the merger of unreduced gametes (egg cells or pollen that have undergone First or Second Division Restitution (FDR/SDR). Goldblatt (1980) suggests that 30-70% of flowering plants are of polyploid origin; more recently, 95% of pteridophytes and up to 80% of angiosperms were estimated to be polyploid (Masterson, 1994). The stonecrop *Sedum suaveolens* has the highest chromosome number of any angiosperm (2n ~ 640 ~ 80-ploid) (Uhl, 1978) and the fern *Ophioglossum pycnostichum* the highest among all plants (2n ~ 1260 ~ 84-ploid) (Löve *et al*., 1977). Many plant species, particularly the cultivated ones, such as potato, coffee, banana, wheat, cotton, alfalfa, sugarcane, maize etc., have gained polyploid genomes or undergone polyploidization events during their evolution (Ayala *et al*., 2000). Polyploids tend to be more self-fertile or apomictic; this can provide benefits in stressful environments that compromise normal generative multiplication. Molecular data for recurrent creation of polyploids have been published (Leitch & Bennett, 1997; Soltis & Soltis, 2000); nearly all polyploid species examined are polyphyletic. Also cytoplasmic diversity due to reciprocal origins has been revealed.

Polyploids can be autopolyploid (more than 2 homologous chromosomes of 1 single species) or allopolyploid (chromosomes descending from different species). Synthetic polyploids, especially allopolyploids, are known to be substantially less stable than natural polyploids (Comai, 2000). On the other hand, allopolyploidy can be favored by heterosis effects generated by the combination of homoeologous genes; moreover, their original instability, although usually deleterious, might generate sufficient phenotypic variation for the exploitation of new ecological niches. Polyploidization is estimated to be responsible for 2-4% of angiosperm speciation (Otto & Whitton, 2000).

Autopolyploids have three important attributes compared to their diploid progenitors: enzyme multiplicity, increased heterozygosity and increased allelic diversity. Polysomic inheritance is the basis for the genetic attributes of polyploids, thus providing strong arguments for their success in nature, whereas in allopolyploids this increased biochemical diversity is achieved via ‘fixed heterozygosity’ which results from the addition of divergent genomes. This

'enzyme multiplicity' (Soltis & Soltis, 1993) may well extend the range of environments in which the plant can grow (Roose & Gottlieb, 1976). Because of this increased flexibility, polyploids are generally better colonizers than diploids (Soltis & Soltis, 2000). A disproportionate larger number of polyploids are found in cold, dry regions.

Myriads of genetic interactions are made possible by polyploidy (Osborn *et al*., 2003), that are reflected in three primary possibilities for the evolutionary fate of duplicated genes: functional diversification, decay and silencing, and retention of the original function (Wendel, 2000). Subsequent evolution of polyploid genomes include gene silencing, gene and genome diversification (Soltis & Soltis, 1993). Gene silencing suppresses the expression of additional copies of genes in the polyploid nucleus. As a result the plant may essentially behave like a diploid. A suggestion is that multiple copies of a sequence trigger epigenetic phenomena, termed homology-dependent or repeat-induced gene silencing. Sometimes one complete parental set is silenced, called nucleolar dominance (Pikaard, 1999). One of the more intriguing aspects of nucleolar dominance is the observation that it may be complete in vegetative tissues whereas homoeologous rRNA-genes are co-expressed in floral organs, thus demonstrating the (tissue-specific) reversibility of epigenetic gene silencing.

Homology dependent gene silencing can not only result from epigenetic interactions between homoeologous genes, but also from interactions between a gene and related heterochromatic repeats (predominantly composed of transposable elements), who are activated (demethylated) by a genomic shock and silence expression of genes bearing similar sequences, on transcriptional or post-transcriptional level. Comai (2000) reports synthetic allopolyploids to be phenotypically hypersensitive to demethylation; this may lead to gene activation but also to downregulation due to demethylation on a negative regulator of transcription. Possibly gene inactivation through methylation is a defense response against invasive transposons (Matzke & Matzke, 1998). It appears that methylation and histone deacetylation act as partners in the enforcement of rRNA-gene silencing (Chen & Pikaard, 1997).

Nascent polyploids are not always characterized by epigenetic processes and/or genetic changes; some seem to be genomically quiescent (Liu & Wendel, 2002). Rapid genomic changes or epigenetic effects upon allopolyploidization as observed in wheat (Feldman *et al*., 1997; Kashkush *et al*., 2002), rice (Liu & Wendel, 2000), *Brassica* (Song *et al*., 1995) and *Arabidopsis* (Comai *et al*., 2000) seem to be lacking in cotton (Liu *et al*., 2001) and *Spartina anglica*, a Poaceae species (Baumel *et al*., 2002).

The extra copies of essential genes can mutate and diverge, resulting in new traits without compromising essential functions. Genomic change may occur very rapidly following polyploid formation at all levels of the genome, from the chromosome to the DNA-sequence (Leitch & Bennett, 1997). GISH identified considerable structural rearrangements upon polyploid formation. It appears that some of these chromosomal translocations are random whereas others are species-specific (occurring in every independent polyploid population). Possibly, these specific changes must occur in the nuclear genome to restore fertility and nucleocytoplasmic compatibility. One could say that karyotypic stability is achieved at the expense of the evolutionary flexibility provided by unhindered recombination between the

parental genomes. Rearrangements in noncoding genomic DNA, as in wheat (Ozkan *et al*., 2001) may contribute to such a stabilization process. In allotetraploid tobacco, one recombinant chromosome is common to all genotypes and may represent a species-specific chromosome translocation (Parokonny & Kenton, 1995).

"Meiotic drive" refers to the subversion of meiosis by a locus that causes its own preferential segregation, often by causing chromosomal breaks upon absence of these loci in the gametes. Activation of dormant transposons may be caused 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). Interspecific crosses increase retroelement insertional activity (Liu & Wendel, 2000); since polyploid genomes contain duplicates of all genes they are assumed to be buffered from the deleterious consequences of transposition (Matzke *et al*., 1999). Retrotransposon-mediated massive heterochromatin expansion may cause dramatic genomic remodeling in the hybrid genome and hence rapid differentiation from the parental genomes (O'Neill *et al*., 1998). IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposons-microsatellite amplified polymorphism) are DNA-based markers that allow investigation of genetic variation related to the distribution and activity of retrotransposons (Kalendar *et al*., 1999).

Normally, upon homoeologous recombination in interspecific hybrids DNA-mismatch repair is initiated when a mutS-like protein binds to a mismatched base pair or displaced loop in double stranded DNA (Kolodner & Marsischky, 1999), leading to replacement of a large patch of single stranded DNA containing the mismatch. *Salmonella* mutants deficient in mismatch repair are capable of conjugational recombination with *E. coli* (Rayssiguier *et al*., 1989). However, if an excessive number of mismatches are formed, mutS-protein could be depleted, allowing homoeologous DNA-strands to continue the recombination process (Humbert *et al*., 1995). Collapse of mismatch repair would explain genomic instability in allopolyploids leading to formation of acentric and dicentric chromosomes or smaller restriction fragments.

Chromosomal repatterning (translocation) has occurred in many allopolyploids; once stabilized the nuclear genome in *Brassica* usually is more similar to that of the parental diploid that contributed the cytoplasm (Song *et al*., 1995), whereas contradictory results were obtained for wheat (Ozkan *et al*., 2001). It can be concluded that upon the formation of an allopolyploid a selection pressure is established, sometimes pushing chromosomal rearrangements towards the side of the cytoplasm donating parent. Nuclear genomes of natural allopolyploids are considerably more distant from the progenitor diploid species than are those of the synthetic allopolyploids. *Brassica* polyploids containing parental genomes from widely divergent diploid species underwent more extensive genomic change than those containing more similar parental genomes (Song *et al*., 1995). Genome divergence was quite rapid, being detectable five generations after polyploid formation. It seems that genomic changes occur in a burst after allopolyploidization. Polyploids should not be considered as evolutionary 'dead-ends', but, on the contrary, as highly dynamic systems with an improved colonizing ability thanks to their capability of undergoing considerable evolutionary change.

Kahskush *et al.* (2002) mimicked the natural origin of wheat (probably the best studied allopolyploid) by the creation of a synthetic hybrid using 2 diploid progenitors that were highly related to wheat ancestors. They found that gene loss and methylation related silencing already occurred in the F_1 generation, and that the functions of the genes involved were very divergent. On the other hand, all activated genes were retroelements. Their findings are corresponding with earlier research that reported DNA-sequence elimination as an immediate response to allopolyploidization in wheat (Ozkan *et al*., 2001; Shaked *et al*., 2001). Moreover, this elimination started earlier in those synthetic hybrids that resembled most naturally occurring allopolyploids, which suggests they may be part of a stabilizing mechanism (Feldman *et al*., 1997). This would explain the rapid and "directed" sequence elimination in many allopolyploids.

In a nutshell, genome evolution in (allo)polyploids comes to cytonuclear stabilizations, chromosomal structural evolution, rapid (non-Mendelian) genomic changes and inter-genomic invasions, that are non-mutually exclusive and may be reinforced or influenced by superimposed epigenetic effects. Therefore, the observation of non-additivity of some hybrids compared to its parents is not surprising (Wendel, 2000). While gene loss is evidently irreversible, gene silencing is potentially reversible; in addition, methylated genes may become hot spots for future mutations.

Polyploids usually show different morphology compared with diploids, the amount of differences being very dependent on the plant species, the degree of heterozygosity, the ploidy level, and the mechanism relating to gene silencing, gene interactions, gene dose effects and regulation of specific traits and processes (Tal, 1980; Lewis, 1980; Levin, 1983; Dhawan & Lavania, 1996; Leitch & Bennett, 1997). There is growing evidence that size and morphology of cells are linked to their DNA-content (Kondorosi *et al*., 2000), the cell volume being positively correlated with the degree of polyploidy, while cell shape is rather defined by morphogenic parameters such as hormone gradients, light, the actin cytoskeleton, the cell wall and the neighbouring cells. Yeast cells expressing low levels of G_1 cyclins delay the entry point into the cell cycle and therefore achieve a greater cell size during the G_1 phase; the repression of these cyclins is ploidy-dependent (Galitski *et al*., 1999). Polyploidy alone can thus effect the expression of certain genes. In maize, the ploidy level can either increase or decrease expression, depending on the particular gene (Guo *et al*., 1996). Multiplication of the genome has been proposed to increase metabolic activity, rRNA-synthesis and transcriptional activity (Baluska & Kubica, 1992; Hieter & Griffiths, 1999). Members of multigene families such as *ccs52*, a plant homolog of the anaphase-promoting complex activators that are involved in mitotic cyclin degradation and whose overexpression leads to the arrest of cell division and induces endoreduplication and cell enlargement (Cebolla *et al*., 1999) appears to be conserved in plants and might therefore be involved in the morphogenesis of different cell types. Allopolyploidy may have an even greater effect on gene regulation because the incompatibilities between different genomes are more disruptive than changes caused by additional copies of the same genome.

From a horticultural point of view, one of polyploids greatest values is that they can have much larger and heavily textured flowers (which might also last longer); flowering might also be delayed and/or prolonged. Polyploidization leads to thicker leaves and stems, a deeper green

color, increased width-to-length ratio of leaves and a more compact growth habit. The higher heterozygosity level might contribute to heterosis. Negative side effects of polyploidization might be infertility (however, this can be a desired trait), brittle wood, watery fruit, stunting and malformation. Polyploidy disrupts self-incompatibility in many species (De Nettancourt, 1997).

Polyploidy is a very useful tool in plant breeding, since it may allow to overcome barriers to hybridization (dissolving interploid 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 homoeologous 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).

Colchicine, an allelopathic compound produced by *Colchicum autumnale*, is known to inhibit the formation of spindle fibers and effectively arrest mitosis at the anaphase stage (Blakeslee, 1937; Reese, 1950; Hancock, 1997). Since chromosomes have already multiplied but cell division is arrested, polyploid cells are created. However, colchicine is a carcinogenic compound and generally less efficient than modern alternatives. During the seventies, nitrous oxide was the first applied alternative (Taylor *et al*., 1976); nowadays, there is a tendency towards the use of herbicides with a similar mode of action, such as the dinitroanilines oryzalin and trifluralin (Morejohn *et al*, 1987; Verhoeven, 1990; Vaughn & Lehnen, 1991; Van Tuyl *et al*., 1992, Rao & Suprasana, 1996; Hansen & Andersen, 1996), the phosphoric amides such as amiprophos methyl (Hansen *et al*., 2000) and the carbamate herbicides like chlorpropham and propham. Other widely used mitotic disrupter herbicides include dithiopyr and pronamide. All of these compounds block mitosis in a similar way, by preventing the formation of the microtubules of the mitotic spindle along which chromosomes are separated (Morejohn *et al*., 1987).

The more detailed mode of action of the aforementioned herbicides is described by Vaughn (2000). Natural tolerance against these compounds is found in Apiaceae (Vaughan & Vaughn, 1988); moreover, pretreatment with taxol also provides resistance in other plant species thanks to its promotion of microtubule stability (Vaughn & Vaughan, 1990). Antimitotic herbicides are mostly used in lower doses then colchicine, are more effective and less toxic for humans (Vaughn & Vaughan, 1988). *In vivo* material appears to be less sensitive than *in vitro* material; surfactants, wetting agents and other carriers like DMSO sometimes enhance efficiency (Kehr, 1996b).

Since polyploidy is able to change many morphological properties, it is evident that polyploidy can be verified by a number of tests. Pure morphological testing includes measuring leaf, flower, or fruit size, or (more time consuming) pollen size, the number of chloroplasts per guard cell (Solov'eva, 1990), or the size of guard cells or stomates, all of which are positively correlated to the increase of the ploidy number. The only accurate way to determine the exact ploidy level is to count the chromosome number. However, it should be considered that polyploids can be cytochimeras. Chimeras vary in different types of tissue (Tilney-Basset, 1986). Meristems are typically composed of 3 histogenic layers: LI (epidermal layer), LII (cortical/mesophyl layer) and LIII (corpus layer); root tip chromosome counting only provides information on the LIII layer, stomatal count on the LI layer, while for

reproductive purposes the LII layer (from which gametes develop) is the most important. The LII layer ploidy level is reflected in pollen size and chromosome counts from reproductive tissue (e.g. anthers). In periclinal ploidy chimeras, histogenic layers and the resulting somatic tissues show different ploidy levels. Flow cytometry results are relative; most often a complete leaf is measured, which allows to distinguish cytochimeras from tetraploids by the occurrence of 2 cell types with a different ploidy level.

Polyploidization has been realized in several ornamental crops, such as: *Rhododendron* (Väinölä, 2000), *Alstroemeria* (Lu & Bridgen, 1997), *Syringa* (Rose *et al*., 2000a), *Buddleja* (Rose *et al*., 2000b), *Cyclamen* (Takamura & Miyajima, 1996) and *Rosa* (Roberts *et al*., 1990). Numerous examples of chromosome doubling *in vitro* can be retrieved from literature. Most often colchicine is used, though oryzalin is frequently suggested as a more efficient alternative; polyploidy is induced starting from different types of explants, like shoot tips (Hamill *et al*., 1992; Bouvier *et al*., 1994; Van Duren *et al*., 1996; Adaniya & Shirai, 2001), axillary node cuttings (Awoleye *et al*., 1994), buds (Chakraborti *et al*., 1998; Notsuka *et al*., 2000), ovules (Gmitter & Ling, 1991), callus (Roy *et al*., 2001) or complete plantlets (Geoffriau *et al*., 1997a).

1.3. Interspecific hybridization

The difficulty of creating interspecific hybrids increases along with the phylogenetic distance between the parents (Sharma, 1995). As suggested by Hogenboom (1973; 1975) breeding incompatibility between plants may be controlled by either of two major mechanisms, incompatibility or incongruity. Incompatibility has developed numerous times during the evolution of flowering plants (Franklin-Tong & Franklin, 2000). It is typically involved in crosses between closely related genotypes that possess at least some elements of an S-gene controlled self- or inter-incompatibility system. Incompatibility is a natural defense against inbreeding, as only the growth of "self" pollen is arrested, which may occur at various stages between pollination and fertilization (Wheeler *et al*., 2001) or even afterwards (Williams *et al*., 1984). This is determined by the nature of the incompatibility system (Newbegin *et al*., 1993) that may be dependent on the pollen genotype (gametophytic) or on the pollen parent genotype (sporophytic). Different flower types within a single species may cause heteromorphic incompatibility; although it is mostly controlled by one single gene, systems governed by two or more loci have been described, e.g. in Poaceae (Gaude & McCormick, 1999).

Incongruity is typically seen in crosses where separate evolution of the partners (being different species) has led to a breakdown in control of pollen tube behavior in the pistil. It may therefore be considered as an isolating mechanism developed as a by-product of evolutionary divergence (Hogenboom, 1975). The development of the hybrid genome may also be hampered or even arrested by multiple barriers in a later stage (Table 1.1). An alternative model proposed by de Nettancourt (1977; 1997) contrasts with incongruity since it suggests that cross-hybridization is prevented by an active process that inhibits what would otherwise be a compatible pollination. Although these kind of responses have been observed within several families (Wheeler *et al*., 2001), their extent remains unclear so far.

Incongruity can be caused by both pre- and postfertilization barriers; incompatibility mainly shows up before gamete fusion (Raghavan, 1997). Whereas incompatibility promotes outbreeding, incongruity limits the possibilities for species hybridization (McCubbin & Kao, 1996). Incompatibility nor incongruity are caused by sterility (non-viability of the gametes, Knox *et al*., 1986). A connection between the underlying mechanisms behind incongruity and self-incompatibility has been suggested by the so-called SI x SC rule, stipulating that pollen from self-compatible species is often rejected by pistils of self-incompatible species, whilst the reciprocal crosses are viable. However, there are exceptions to this rule (Wheeler *et al*., 2001). Up to this time, several techniques have been used to overcome fertilization barriers: the application of exogenous growth substances and immunosuppressants, embryo rescue, intraovarian pollination *in vivo* and *in vitro*, *in vitro* fertilization of ovules, and somatic cell hybridization (Zenkteler, 1990). Six main hybridization barriers may be distinguished (Table 1.1) in incongruous crosses.

Table 1.1. Different manifestations of incongruity after interspecific pollination.

	incongruity type	**possible solution**
prezygotic	pollen tube growth inhibition	prefertilization treatments
postzygotic	absence of endosperm, no seed germination	embryo rescue
	hybrid albinism	bilateral crosses; 'bridge' plants
	lack of growth vigor ('hybrid vigor')	other crosses
	hybrid breakdown	other crosses
	sterility	allopolyploidization

1.3.1. Prezygotic incongruity

The exchange of signals between females and males mediates successful fertilization and is one of the central themes in biology. Gametophytes of angiosperms (producing egg cells and sperm cells) play critical roles in the fertilization. Pollination can be divided into characteristic stages that include: (a) pollen hydration and germination; (b) growth of the pollen tube through the stigma, style and ovary; (c) pollen tube guidance to the ovule micropyle; and (d) delivery of the sperm to the embryo sac (Wilhelmi & Preuss, 1997). The time required for this series of events varies considerably depending on the species, from a few hours to several months. An overview of the different stages is shown in Fig. 1.1.

The influence of phytohormonal compounds on signaling has not been unraveled, though interesting data are already available (Baker *et al*., 1997; Katsukawa *et al*., 2000). Phytohormones produced in the fertilized ovules also affect fruit set, seed set and abscission (Pickersgill *et al*., 1993; Van Creij *et al*., 1997). ABA-concentrations increase fast in incompatibly pollinated flowers and slowly in unpollinated ones, while decreasing after compatible pollinations. Auxin concentrations increased after compatible pollination. Ethylene levels remain relatively unaffected after compatible pollinations but increase after incompatible pollinations or non-pollination. ABA and ethylene cause abscission of incompatibly or non-pollinated flowers. A practical application might be the use of ethylene antagonists (CO_2, Ag, …) for the realization of incompatible/incongruous crosses (if at least the increase of ethylene levels is one of the causes of incongruity – and not one of the

consequences). On the other hand, in *Nicotiana* there appears to be a correlation between ethylene production after pollination of mature pistils and pollen growth. The production of ethylene differs depending on the pollen used; in some cases ethephon can minimize the arrest of incongruous pollen tubes inside the style (Sanchez & Mariani, 2002). Ethylene may inhibit both incompatibility (by decreasing the synthesis of S-products) and incongruity (by stimulating pollen growth in senescent flowers) (Williams & Webb, 1987). From this data it appears that ethylene might have a dual effect: a promotion of the growth of incongruous pollen, but also a triggering of abscission after incongruous pollination, thus limiting the possibility for the sperm nuclei to reach the ovules.

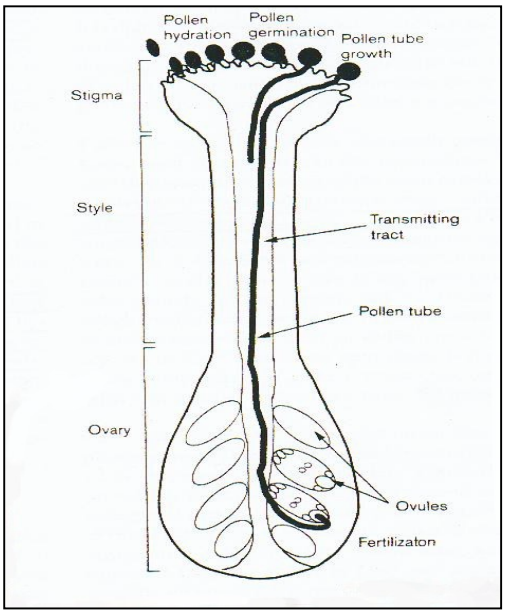


Figure 1.1. Generalized diagram of a pistil and the pollination route (Franklin-Tong, 1999).

Irradiated mentor pollen, which is sterile, may nevertheless have a stimulatory function (Stettler, 1968) and is therefore often applied in interspecific pollinations, upon mixing with incongruous pollen (Villar & Gaget-Faurobert, 1997). Cut-style pollination is often suitable for hybridization between parents with relatively different pistil lengths since pollen tube growth and development are often impeded in (too) short or long pistils (Williams & Rouse, 1988); it was applied successfully in different interspecific pollination in ornamental genera, like *Vriesea* (Vervaecke, 2002), *Tulipa* (Van Creij *et al*., 1997) and *Lilium* (Van Tuyl *et al*., 1991; Janson, 1993). The style can be completely removed in order to pollinate the ovules directly (placental pollination). Another method is style grafting where a compatible style is grafted on an incompatible ovary (Van Tuyl *et al*., 1991). The technique is referred to as 'ovary grafting' when next to the donor style (a part of) the donor ovary is grafted on the ovary of the seed parent. In lilies, hormone treatment of the style was more efficient than ovary grafting, placental pollination, mentor pollination and warm water treatment to circumvent the inhibition of ovule penetration (Van Creij *et al*., 1997). A rather marginal technique is hexane treatment of the stigmatic surface (Whitecross & Willing, 1975). *In vitro* fertilization and protoplast fusion do not require *in vivo* pollen tube growth but mostly confront the breeder with complicated technical problems or poor regeneration of the fusion products (Brown & Thorpe, 1995).

1.3.2. Postzygotic incongruity

1.3.2.1. *Absence of seed production or germination*

Ploidy differences between parent plants might be one of the main incongruity barriers according to Badger (1988) by causing a 'triploid block' resulting in the malformation of endosperm and the inhibition of germination. Endosperm absence or retarded development is often the cause of spontaneous abortion of hybrid embryos after interspecific pollinations (Van Tuyl *et al*., 1991; Pickersgill *et al*., 1993; Sharma, 1995). Johnston *et al.* (1980) proposed the endosperm balance number theory to explain the basis for normal seed production but currently there is no consensus on the requirements for the formation of hybrid endosperm. Fertilization-independent endosperm and fertilization-independent seed *Arabidopsis* mutants have been isolated in which an endosperm-like tissue arises from the central cell in the absence of fertilization, confirming the degree of autonomy of the central cell at this stage (Ohad *et al*., 1996; Chaudbury *et al*., 1997). These mutants suggest that endosperm development is probably triggered by a combination of maternal factors, which are interpreted differently by the central cell and the egg cell (Slocombe *et al*., 1999). Starch is deposited in modified plastids, the amyloplasts; loci responsible for the major enzymes involved in starch biosynthesis have been identified (Gallusci *et al*., 1996).

Using *in vitro* media, which replace the endosperm and allow maturation of the hybrid embryo, can solve the lack of endosperm. This would not only allow to rescue seedlings from more crosses, but also to rescue more seedlings per cross. Next to rescuing hybrid (or haploid) embryos the main applications of embryo culture are solving the problems of low seed set, seed dormancy, slow germination, germination of obligatory parasites, shortening the breeding cycle and vegetative propagation (Zenkteler, 1990; Pierik, 1999). The main factors affecting its success are genotype, developmental stage of the embryo at isolation, growth conditions of the motherplant, composition of the nutrient media, oxygen, light and temperature (Pierik, 1999). The term "embryo rescue" is restricted to only those cases where the embryos, if not rescued are endangered and would not form seedlings. There are heterotrophic and autotrophic stages in embryo development. In the heterotrophic stage, the embryo (called proembryo) is smaller and usually requires growth regulators (Raghavan, 1980). During this latter phase, up to the heart stage, embryos fully depend on the endosperm and other maternal tissues of the ovules. Hybrid embryos which start to degenerate at the heterotrophic phase, after isolation and culture in artificial media, die soon after transfer (Zenkteler, 1990).

'Embryo rescue' therefore offers a promising tool for interspecific breeding as an aid in hybridization by sexual means (Zenkteler, 1990; Pickersgill *et al*., 1993). Hybridization through this technique has proven possible on *Capsella* (Monnier, 1988), *Tulipa* (Van Creij *et al*., 1997), *Lilium* (Asano & Myodo, 1977; Arzate-Fernandez *et al*., 1998; Van Tuyl & Van Holsteyn, 1996), *Arachis* (Feng *et al*., 1996), *Alstroemeria* (De Jeu & Jacobsen, 1995; Burchi *et al*., 1998), *Camellia* (Hwang *et al*., 1992), *Nicotiana* (Reed & Collins, 1978; Nikova & Zagorska, 1990), *Phaseolus* (Mok *et al*., 1978), *Cajanus* (Mallikarjuna & Moss, 1995) and within the Brassicaceae (Inomata, 1978; Quazi, 1988; Gundimeda *et al*., 1992; Thierfelder *et al*., 1992; Lelivelt, 1993; Li *et al*., 1995; Momotaz *et al*., 1998); reviews have been published

by Sharma (1995), Sharma *et al*. (1996) and De Jeu (2000). 'Embryo rescue' can be performed in one step (embryo culture) or two steps (*in ovulo* culture + embryo culture). The most applied culture methods are ovary slice culture, ovule with placenta culture, single ovule culture, and embryo sac culture (Van Tuyl & De Jeu, 1997; Chi, 2002). Monnier (1988) has shown that the success of an embryo rescue medium partly depends on sugar, Fe and Ca-concentration. Immature embryos are known to require higher osmotic strength (lower potential) of the medium compared to the relatively mature ones. High osmotic strength of the medium prevents precocious germination of young embryos and supports normal embryonic growth. Therefore sugar concentration is at least as important for the osmolarity of the medium than for providing nutrition, at least when culturing immature embryos (Sharma *et al*., 1996). Though embryo rescue is not always required to obtain hybrids it might improve crossing efficiency significantly compared to natural crosses (Faure *et al*., 2002).

1.3.2.2. *Hybrid albinism*

Incompatibility between the plastome and nuclear genome inhibits chloroplast development and chlorophyll formation. Heavy chlorosis may very well cause inviability of the hybrid seedlings (Przywara *et al*., 1989; 1996; Badami *et al*., 1997; Ha *et al*., 1998) and is occurring after interspecific hybridization in many genera (Yao & Cohen, 2000). Affected plants are either white (total albinism), either having extended white sectors (variegated) or either becoming green with ageing (virescent). *Zantedeschia* is a model crop for researching plastid DNA-inheritance after interspecific crosses. Yao *et al*. (1994; 1995) have found that the use of bridge plants may help in obtaining viable hybrids between 2 sections of this genus. *In vitro* embryo rescue experiments (Yamaguchi, 1986) on *Rhododendron* reveal that, although more seedlings were obtained, the relative number of albinos increased.

All plastids develop from proplastids according to the requirements of each differentiated cell, and which type is present is determined in large part by the nuclear genome (Gruissem, 1989). Proplastids are present in meristematic cells and can develop into specialized plastid types such as chloroplasts, amyloplasts or chromoplasts. Chloroplast development is associated with significant morphogenic and molecular changes, the most visible being the accumulation of chlorophyll and the assembly of the complex thylakoid membrane system. In darkness, proplastids normally develop into etioplasts, which are characterised by the presence of one or several prolamellar bodies (Ryberg *et al*., 1993). Following the transfer of dark-grown leaves to light, the regular structure of the prolamellar bodies gradually disappears, and the protothylakoids of the etioplastids are transformed into thylakoids and the membranes start to overlap, forming typical grana.

According to Yao & Cohen (2000) hybrid *Zantedeschia* albinos do not develop prolamellar bodies in their etioplasts in darkness and do not form grana in light, which indicates that the block to chloroplast development occurs before or during the development of etioplasts. They propose a multiple gene control of plastome-genome incompatibility and plastid DNA-inheritance, based upon backcrosses that show a higher compatibility as the number of genes from the parent contributing the plastids increases.

1.3.2.3. *Lack of growth vigor*

Lack of growth vigor is often correlated with low chloroplast content. However, also growth of green seedlings may get arrested due to the incompatibility between both parental genomes and the formation of non-functional (consisting of hybrid subunits) proteins. Lack of growth vigor can not be resolved and therefore needs to be prevented to the largest possible extent by other parent plant combinations or a larger quantity of pollinations. Hybrid weakness, dwarfism and inviability have been reported in many plant genera: according to Levin (1978) these effects are caused by genome disharmony and incompatible development cues, the deleterious, complementary action of one or a few genes, and/or cytoplasmic effects.

1.3.2.4. *Hybrid breakdown*

Pollination with pollen of wild but closely related species can induce the development of an embryo but elimination of the chromosomes of the wild species, causing the developing plantlets to be haploid (see 1.1) (Kasha & Kao, 1970; Rowe, 1974). Hybrid breakdown has not been described in the genera *Rhododendron, Hibiscus* or *Buddleja*.

1.3.2.5. *Hybrid sterility*

Errors in chromosome segregation at meiosis may cause F_1 sterility (Heslop-Harrison, 1999). One of the most striking consequences of a disturbed synapsis is the abortion of the malformed embryo sac in many intersubgeneric hybrids, causing female sterility (Bouharmont *et al*., 1988). Microspores resulting from a disturbed meiosis usually are non-viable (Van Tuyl & De Jeu, 1997; Shamina *et al*., 1999).

Embryo rescue may need to be combined with allopolyploidization to result in fertile offspring (Chen *et al*., 2002), which can be used as pre-breeding material. Non-homologous chromosomes can prevent the synthesis of functional gametes, which should then be resolved by allopolyploidization. It should be kept in mind that mitotic allopolyploids produce homogenous gametes, which increases the predictability of the F_2, but on the other hand limits the expression of recessive genes that are only present in the chromosomes of one of both parental species. Only meiotic allopolyploidization (after the fusion of 2n gametes) may result in introgression. The occurrence of mismatches is prevented by so-called "mismatch repair" genes which prevent non-homologous chromosomes to cross-over (Hunter *et al*., 1996). Even when chromosome pairing is able to proceed, low F_1 fertility may be controlled by sterility genes (Ha *et al*., 1998).

1.4. *Spathiphyllum wallisii* Regel

1.4.1. Taxonomy (Huxley *et al*., 1999)

Division: Magnoliophyta
Class: Monocotyledonae
Subclass: Arecidae
Order: Arales
Family: Araceae

1.4.2. Plant description

Spathiphyllum shoots end in a protogynous inflorescence: a fleshy axis covered with sessile hermaphrodite flowers. This spadix is enveloped by a white spathe. The first inflorescence appears after the formation of 8-10 fully grown leaves; the development of two more leaves is required for the following inflorescence. Each ovary is surrounded by anthers and tepals (Fig. 1.2) and contains 8-10 ovules. The berries contain only few seeds. Most cv's are *S. wallisii* Regel or *S. floribundum* (Linden & André) N.E.Br. hybrids. *S. wallisii* has conical ovaries and a concave, erect spathe whereas *S. floribundum* spadices are nearly smooth and spatha are flat and spreading (Walters *et al*., 1996).

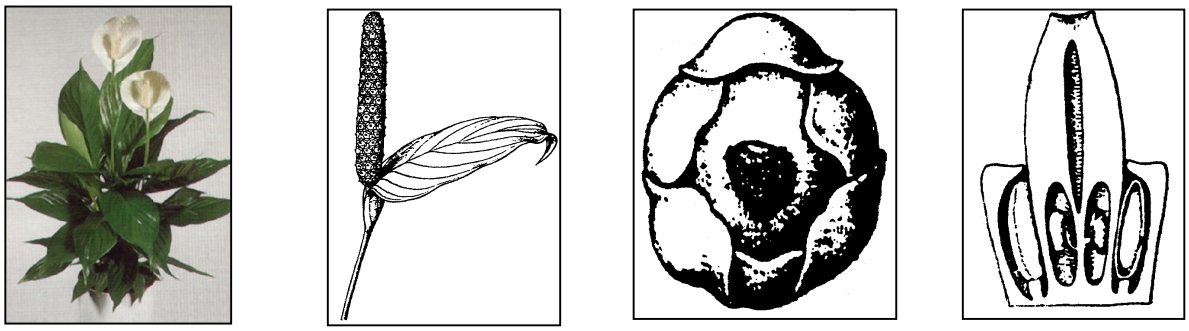


Figure 1.2. *Spathiphyllum*: plant, spadix and spathe, ovary and tepals, positioning of ovules and anthers (Buzgó, 1996).

1.4.3. Ploidy breeding

Only few data are available on the breeding of haploids in the family Araceae (Ko *et al*., 1996). It has become clear that *Spathiphyllum* is very recalcitrant to androgenesis (Eeckhaut, 1999). Haploid induction should therefore primarily be attempted through culture of isolated ovules. Nevertheless, anther culture might be interesting for the induction of somatic embryos developing on filaments.

A prerequisite for genetic manipulation, mutation induction or ploidy manipulation of the ornamental crop *Spathiphyllum* is the availability of a system that allows a target cell to regenerate into a new plant. Induction of somatic embryos could offer a powerful tool to reach this goal. The most striking example of flexibility in the initiation of plant embryogenesis is found in carrot cell suspensions, in which single cells develop into an embryo in completely synthetic media as first observed by Reinert (1959). In such cells, the somatic embryo receptor-like kinase (SERK) gene is expressed during the first few cell divisions and is turned off at the globular stage. It encodes a leucine-rich repeat transmembrane receptor kinase which may transduce a signal conferring an embryo fate to plant cells (Schmidt *et al*.,1997; Mordhorst *et al*., 1998). The term "embryogenic cell" is restricted to those cells that have completed the transition from a somatic state to one in which no further exogenously applied stimuli such as the application of growth regulators are necessary to produce the somatic embryo (De Jong *et al*., 1993).

An embryo, whether zygotic or somatic in origin, is essentially bipolar with root and shoot primordial structures and has separate vascular bundles, unlike adventitious shoots. From bottom to top the body pattern of an embryo consists of the embryonic root, including the root cap and the root meristem, hypocotyls, cotyledon(s) and the shoot apical meristem. In radial fashion, from the outside to the inside, the protoderm, ground meristem and procambium are the main tissue types (Jürgens, 1995). Early divisions in somatic embryos are less regular than those in their zygotic counterparts (Mordhorst *et al*., 1997; Vroemen & De Vries, 1999). After appropriate culture manipulations (usually involving auxins in combination with cytokinins) somatic embryos can develop from almost any part of the plant body. Competent cells may belong to several morphologically different types. While the cellular origin of somatic embryos is quite different from zygotic ones, both the radial and the apical-basal patterns form in correct fashion (Mordhorst *et al*., 1997).

Somatic embryogenesis has been reported in the family Araceae. In *Anthurium scherzerianum* leaf pieces were used as explants (Hamidah *et al*., 1997); in *Anthurium andreanum* laminas (Matsumoto *et al*., 1996); in *Pinellia pedatisecta* seeds (Wu *et al*., 1996) and in *Xanthosoma sagittifolium* stem apices (Gomez *et al*., 1992). Anther filaments proved to contain sensitive tissue for the induction of somatic embryos. This has been described for a variety of species, such as *Aesculus hippocastanum* (Jorgensen, 1989; Kiss *et al*., 1992; Radojevic, 1995; Capuana & Debergh, 1997), *Rosa hybrida* (Noriega & Sondahl, 1991; Firoozabady *et al*., 1994, Ibrahim & Debergh, 2001), *Hibiscus syriacus* (Lee *et al*., 1991), *Vitis vinifera* (Regner *et al*., 1996) and *Theobroma cacao* (Alemanno *et al*., 1996, Silva & Debergh, 2001). Somatic embryogenesis on anther filaments of *Spathiphyllum* has first been described by Werbrouck *et al*. (2000); data are lacking for other Araceae genera. Chen & Kuehnle (1999) have described somatic embryogenesis on etiolated *Spathiphyllum* internodes, but only for 1 genotype.

Werbrouck & Debergh (1995;1996) have described an alternative regeneration system for *Spathiphyllum wallisii*, using *in vitro* shoot bases that produced clusters of tiny shoots when exposed to a combination of cytokinins and imidazole fungicides. As this system proved to be efficient and trustworthy, its possible use as a regeneration tool is obvious.

Within the family Araceae, polyploidization was performed in the genus *Zantedeschia* (Cohen & Yao, 1996; Yao & Cohen, 1996) to allow hybridization between winter dormant and non-dormant species. The tetraploids could also be used in a breeding program to yield triploid plants. In *Xanthosoma* tetraploidy was achieved as an intermediate step in a breeding program for root-rot resistant cv's (Esnard *et al*., 1993). No reports are available on polyploidization of *Spathiphyllum*. The basic chromosome number of *S. wallisii* is 15 (Marchant, 1973). Polyploid *Spathiphyllum* cultivars, like 'Sensation' (2n = 3x, origin unknown), however show significantly enhanced growth vigor compared to diploids. Therefore it would be interesting to develop *in vitro* techniques allowing to create tetraploids. Next to their own ornamental value (Different leaf/flower morphology? Increased growth vigor?) they can be used to create triploids. Triploid plants can - dependent on the species - hardly or impossibly be used as breeding material and therefore offer an interesting plant breeders protection. Combined with the induction of haploids, polyploidization techniques would allow clonal propagation of triploid F_1 hybrids through seed.

1.5. *Buddleja davidii* Franch.

1.5.1. Taxonomy (Bean, 1989a)

Division: Magnoliophyta
Class: Dicotyledonae
Subclass: Asteridae
Order: Gentianales
Family: Loganiaceae

1.5.2. Plant description (Bean, 1989a)

Buddleja davidii (Fig. 1.3.) is the most important *Buddleja* species suitable for temperate gardens. It is a multi-stemmed and spineless shrub reaching a height of 2 to 4 m. The plant is deciduous to semi-deciduous when mature, but seedlings tend to retain their leaves. Hundreds of honey-scented white, pink, purple or blue flowers are borne in panicles (10-30 cm long) in early summer. The small (3 mm) flowers are hermaphrodite; *B. davidii* starts flowering and fruiting after 1 year, although some panicles may be present within the first year. About 50 cultivars are available. *Buddleja globosa* Hope has orange scented flowers in small spherical clusters.

Figure 1.3. *Buddleja davidii*: plant, panicle and single flower and *Buddleja* x*weyeriana* 'Sungold' panicle (right).

1.5.3. Polyploidy induction and interspecific hybridization

The high amount of genetic variability present in the genus *Buddleja* creates considerable opportunities to develop new and novel cultivars through controlled breeding. Initial efforts focused on dwarfness, silver-gray leaf color, flower color and branched-panicle (inflorescence) architecture (Tobutt, 1992). Hybridization has already been performed within this genus a few decennia ago and seems to offer great further potentials (Moore, 1949; Moore, 1952). Today already a few clones derived from interspecific hybridization are available in the commercial trade. The aims of our breeding work in *Buddleja* are to induce an altered morphology in *Buddleja davidii*; more specifically dwarf forms, improved flower size and/or yellow color, and to develop sterile *Buddleja* hybrids to limit the invasiveness of the butterfly bush. To reach this goal different breeding strategies were followed.

Van de Weyer (1920) hybridized *B. globosa* x *B. davidii*, resulting in *B.* x*weyeriana*, with an intermediate habitus. The number of seedlings obtained though was not very high and most hybrids were sterile. This explains why backcrossing of *B.* x*weyeriana* with *B. davidii* was found problematic. *Buddleja davidii* is reported to have 76 chromosomes whereas *B. globosa*

has 38 (Moore, 1947). The ploidy level of the *B.* x*weyeriana* 'Sungold' (obtained through mutation within an F_2 generation of *B. globosa* x *B. davidii*) hybrid is logically expected to be intermediate. Tetraploidy induction in the genus *Buddleja* might be useful to aid in interspecific breeding (either to equalize the ploidy number of the parent plants, either to double the ploidy number of the hybrid allowing meiosis).

Tetraploid *Buddleja globosa* could already be obtained by application of colchicine to nodal sections (Rose *et al*., 2000). Those showed remarkable morphological differences compared to diploids. Tetraploids have shorter internodes resulting in more compact plants. The leaves are broader, thicker and more crinkled. Complete tetraploids could be visually distinguished from diploids. Tetraploids appeared to be more susceptible to frost. Flowering seems delayed; although size of the flowers did not differ, but the shape of the tetraploid flowers was more elliptical, rather than spherical. Anthers developed poorly.

1.6. *Rhododendron* sp.

1.6.1. Taxonomy (Chamberlain *et al*., 1996)

Division: Magnoliophyta
Class: Dicotyledonae
Subclass: Dilleniidae
Order: Ericales
Family: Ericaceae

1.6.2. Plant description

The five-lobed structure of a *Rhododendron* flower is shown in Fig. 1.4. A single ovary contains several hundreds of ovules in different developmental stages. *R. simsii* Planch. is the main ancestor of Belgian pot azaleas (*R. simsii* hybrids, formerly known as *Azalea indica*). Remarkable *R. simsii* features are the widely funnel-shaped large corolla and the inflorescence, in clusters of 2-3 or sometimes up to 5-6 flowers. Leaves are evergreen or dimorphic (spring leaves often deciduous, summer leaves persistent). Inflorescences are terminal; the calyx is 5-lobed; the corolla and (usually) 10 stamens are shorter than the gynoecium (Davidian, 1995).

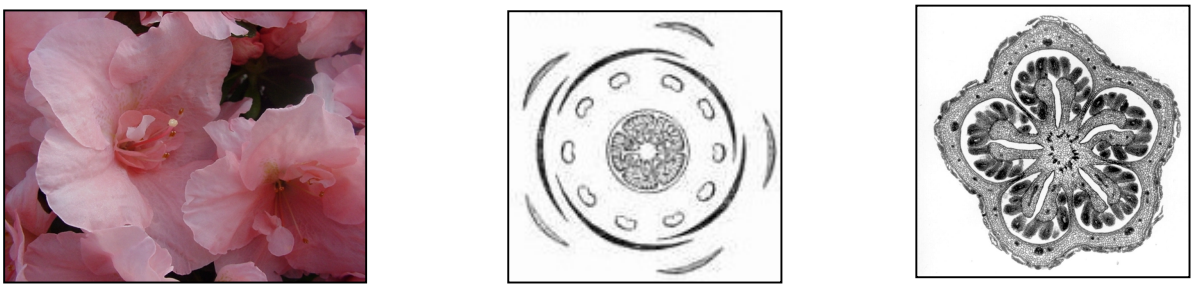


Figure 1.4. The *Rhododendron* flower: *R. simsii* 'Linda', flower diagram, ovary structure (Williams *et al.*, 1990).

1.6.3. The genus *Rhododendron*

The genus *Rhododendron* (± 1000 species) is divided into 8 subgenera (Table 1.2), the 4 most important subgenera being Tsutsusi (evergreen azaleas except Brachycalyx section), Pentanthera (deciduous azaleas), Rhododendron (lepidote *Rhododendron*s) and Hymenanthes (elepidote *Rhododendron*s) (Chamberlain *et al*., 1996). Because of their ornamental importance, all of these subgenera have been discussed, revised and/or classified by many authors (Spethmann, 1987; Chamberlain & Rae, 1990; Cullen, 1991; Cox & Cox, 1995; 1997). The greatest natural gene center, with more than 300 species, is in Asia in an area ranging from Nepal along the line of the Himalayas into northern Myanmar and the provinces of Yunnan and Szechwan in South-West China (Leach, 1961). *Rhododendrons* exist over 50 million years (Irving, 1993). Their present distribution on the globe is mainly affected by three physical factors: global climate change, the drift of continents and the rise of mountains. Hymenanthes are evergreen and have large non-scaly leaves; Rhododendrons (subgenus) have smaller scaly leaves, are usually evergreen, but occasionally semi-deciduous. Species and hybrids of these 2 subgenera comprise what gardeners refer to loosely as "rhododendrons", while Pentanthera and Tsutsusi comprise the "azaleas".

Table 1.2. The genus *Rhododendron* (Chamberlain *et al*., 1996).

SUBGENUS	SECTION	COMMENTS
Rhododendron	Rhododendron	27 subsections, 211 species, scaled leaf
	Vireya	7 subsections, 310 species, 'Malaysian' species
	Pogonanthum	21 species
Hymenanthes	Ponticum	24 subsections, 302 species, unscaled leaves, garden hybrids with large flowers
Pentanthera	Pentanthera	23 species, deciduous azaleas
	Rhodora	2 species
	Viscidula	1 species
	Sciadorhodion	4 species
Tsutsusi	Tsutsusi	94 species, evergreen azaleas
	Brachycalyx	23 species, deciduous azaleas
Azaleastrum	Azaleastrum	11 species
	Choniastrum	19 species
Therorhodion	-	2 species
Mumeazalea	-	1 species
Candidastrum	-	1 species

Diverse morphological characteristics are encountered in the various species of *Rhododendron*. The flowers may be rotate-campanulate, campanulate, funnel-campanulate, funnel-shaped, or tubular-campanulate, and the number of stamens is usually five to ten. The foliage of *Rhododendron* reflects the climate and altitude of their origin, the smallest scaly leaves being found at higher elevations. Rhododendrons occur in all continents of the northern hemisphere. Most species are found in China and the Himalaya, while tropical species (Vireyas) grow mainly in Malaysia and Indonesia. The genus does also occur in northeastern Asia, the Caucasus and North America.

The plants belonging to the subgenera Pentanthera (deciduous) and Tsutsusi and Azaleastrum (evergreen) are called azaleas. An azalea can be a shrub or a small tree. It may be evergreen or deciduous, and the inflorescences may be single- or multiflowered. Typically, the evergreen azaleas are much-branched shrubs, as broad as high, and clothed with medium- to dark-green, somewhat glossy, entire (non serrate or lobed) leaves. In winter, the foliage of some varieties takes on a bronzy or deep purplish coloration. While form and foliage are attractive year around, popular appreciation focuses on the often spectacular floral display in the spring. In the cultivated azaleas, flower form ranges from single to fully double, and color patterns may be solid, flecked, striped, sectored, or picotee. Colors include white, yellowish pinks, reds, pinks, purples, and purplish pinks.

The deciduous azaleas are generally taller, upright plants with less branching than in evergreen species. And, of course, in winter they present only the pattern of their stems and branches. 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). Evergreen azaleas are not truly ‘evergreen’ in the usual sense of the word. Their foliage is dimorphic. Spring leaves appear, distributed along the branches, at, or just after, flowering time. They fall in autumn, often turning yellow before they do so. Other leaves unfold in early summer; they are thicker, smaller, more leathery, and darker than the spring leaves and crowded at the tip of the branches.

In the wild, the height of familiar species of the evergreen azaleas range from prostrate to 3 m; their leaf-blade lengths, from 0.3 cm to 8 cm; and corolla length (receptacle to petal tip) from 0.6 to 6 cm. Flower buds commonly produce 1 to 4 flowers, depending on the species. In the deciduous azaleas, height ranges from about 1 to 10 m and leaf-blade length from 3.4 to 12 cm. A flower bud may produce as few as 3 or up to 24 flowers, depending on the species. There are, of course, many other differences among the species in both the evergreen and deciduous groups. The species and cultivars of azalea, though widely adaptable, do exhibit certain differences in their cultural requirements. Cold hardiness, heat tolerance, the composition and pH of soil or other growing medium must be considered. Galle (1987) states that azaleas perform best when the pH of the medium is between 4.5 and 6.0 (acidic). As with other groups of plants, a wide range of growth rates is encountered, partly reflecting the genetic make-up of the plants, and partly depending on climate, soil, availability of water, etc.

1.6.4. Breeding history

Within each subgenus, Rhododendrons generally hybridize readily. Among commercially available *Rhododendron* genotypes, many hybrid groups can be distinguished. Garden rhododendrons (Hymenanthes and Rhododendron subgenera) are *arboreum*, *campylocarpum*, *catawbiense*, *caucasicum*, *discolor*, *fortunei*, *griffithianum*, *impeditum*, *insigne*, *maximum*, *metternichii*, *oreodoxa*, *ponticum*, *repens*, *smirnowii*, *wardii*, *williamsianum* or *yakushimanum* hybrids (Berg & Heft, 1991; Davidian, 1982; 1989; 1992). According to Kron (2000) garden Rhododendrons are more closely related to evergreen azaleas than deciduous azaleas are.

Deciduous and evergreen azalea hybrids were developed late in the 19th century. Azaleas first found their way into cultivation in Japan (Fairweather, 1988). The cultivation of Rhododendrons in Europe as garden plants started around 1800, after the introduction of American species (Cox, 2000). Among deciduous azaleas lots of hybrid groups have been created (De Raedt & 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* Torrey, *R. nudiflorum* Torrey and *R. speciosum* Sweet and the scented *R. luteum* Sweet from around the Black Sea to produce some robust, sweetly scented, and hardy plants. Other species possibly involved in the development of 'Hardy Ghents' are *R. periclymenoides* Michx., *R. prinophyllum* Millais, *R. viscosum* Torrey, *R. canescens* Sweet, *R. flammeum* Sarg., *R. occidentale* Gray and *R. molle* Don. 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. japonicum* Suringar), originally bred by Louis Desmet. Louis Van Houtte also introduced Mollis Hybrids, a complex group derived mainly from *R. 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 Ghents, due to their *R. molle* parentage, and perform best on their own roots. Knap Hill hybrids at last were developed by Anthony Waterer and son by crossing Ghent Hybrids with *R. molle, R. occidentale* and other species. On a molecular as well as morphological level, Pentantheras do not seem to form a distinct group like Tsutsusi, Hymenanthes or Rhododendron do (Kron, 1993; 2000).

Actually known Belgian pot azaleas have been hybridized starting from 4 Tsutsusi species: *R. simsii* Planch., *R. indicum* Sweet, *R. scabrum* Don and *R. mucronatum* Don. *R. simsii*, the only Chinese ancestor, is responsible for early flowering and sport induction capability of pot azaleas; *R. indicum* caused the semi-spherical habitus of the hybrids, while the other 2 Japanese species passed on the genes responsible for purple color and larger flowers (Heursel, 1999). Pot azaleas should not be mixed up with *R indicum* (Satsuki), *R. scabrum* (Hirado) or *R. kiusianum* Makino/*R. kaempferi* Wils. (sometimes referred to as *R. obtusum* Planch.) (Kurume) hybrids, which all belong to the subgenus Tsutsusi.

Pot azaleas have mainly been bred in Belgium and Germany. German breeders have been very successful thanks to the emphasis they put on the reduction of production costs, the spreading and increase of the flowering period. At this very moment, 'Hellmut Vogel' and its

sports represent over 60% of the absolute number of pot azaleas grown in Belgium. This cultivar, bred by Otto Stahnke, advanced the flowering season by 4 months, allowing the availability of pot azaleas from August 15.

Although a lot of research has been performed on floral traits as color, corolla size, stamen morphology and pigment composition (Santamour & Pryor, 1973; Pryor, 1973; Harborne, 1980; Heursel, 1987; Heursel & Garretsen, 1989) few ornamental features have been genetically characterized. In the mean time DNA-fingerprinting has shown its use in *Rhododendron* phylogeny, cultivar identification and breeding (Iqbal *et al*., 1994; Kobayashi *et al*., 1995; 1996; 1998; 2000; De Riek *et al*., 1997; 1999; 2000; Kron, 1998; 2000). Mertens (2000) developed a transformation protocol using *Agrobacterium tumefaciens* to assist conventional breeding strategies. Nevertheless, molecular research on pot azaleas is not evident because of the long generation period (3 years from pollination to flowering seedlings), the need of space and their woody nature. A lot of *in vitro* research on pot azalea and other Rhododendrons has already been performed, involving micropropagation, shoot induction and regeneration, somaclonal variation and ovule culture (Anderson, 1975; Preil & Engelhardt, 1977; Anderson, 1980; Meyer, 1982; McCown & Lloyd, 1983; Anderson, 1984; Ettinger & Preece, 1985; Dai *et al*., 1987; Pogany & Lineberger, 1990; Iapuchino *et al*., 1992; Preece *et al*., 1993; Mertens & Samyn, 1994; Mertens *et al*., 1996; Brand & Kiyomoto, 1997; Michishita *et al*., 2001; Samyn *et al*., 2002).

New pot azalea cultivars are seedlings (48%) or sports from existing cultivars (52%). A sport was defined by Pratt (1983) as "an individual exhibiting in whole or in part a sudden spontaneous deviation beyond the normal limits of individual variation usually as a result of mutation, especially of somatic tissue". The induction of sports has been studied on a molecular level (De Schepper, 2001). The main advantage of a sport is that the selection from seedling to a new cultivar is avoided, whereas growth characteristics are identical to the original genotype.

In 2000, in Belgium pot azaleas were grown on 426 ha (212 ha under glass). The number of specialized nursing companies (concentrated around Gent) was 279, with a mean area of 1.5 ha. Yearly, Belgium produces about 40 million pot azaleas being 50% of the European production and accounting for 58 million € or 12% of total Belgian ornamental production (figures: Nationaal Instituut voor de Statistiek, www.statbel.fgov.be & Centrum voor Landbouweconomie, www.clecea.fgov.be). However, growers as well as consumers require more variation; for an ornamental flower color is of utmost importance. So far, no yellow, orange or blue azaleas could be created.

1.6.5. Pigmentation

Flower color is caused by three different pigment groups: chlorophylls, flavonoids and carotenoids (Mol *et al*., 1998). Chlorophyll is located in chloroplasts. Like carotenoids, which are found within chromoplasts, chlorophyll is lipid soluble. Flavanoids are located within the vacuoles and are water-soluble (Spethmann, 1979; Wagner, 1979). Very little is known about the biochemistry of carotenoids and chlorophylls as related to flower color. On the other hand, a lot of information is known about flavonoid biochemistry and flower color thanks to

numerous biochemical studies (De Loose, 1969; 1979; De Cooman *et al*., 1993). Flavonoids can be subdivided into anthocyanins, flavonols, aurones, chalkones and gossypetins (Griesbach, 1987). Virtually all the genes that encode the enzymes of biosynthesis have been isolated (Holton & Cornish, 1995). Flower color is the result of a mix of the three pigment classes in different proportions. For example, the black crimson *R. sanguineum* flowers combine anthocyans in their epidermis with chloroplasts in their subepidermal layers (Halligan, 1986).

Anthocyans and flavonols are the flower pigments responsible for petal color of evergreen azaleas (Fig. 1.5 & 1.6). Flavonols are copigments that shift the red anthocyan color slightly towards blue (Holton *et al*., 1993). Like anthocyans, they are derived from dihydroflavonols, via flavonol synthase. Anthocyans are absent in white flowers; however, in the vacuoles of some of these flowers flavonols are present (Heursel, 1999). In most rhododendrons pH of the vacuoles of petal cells is buffered, so soil pH has no effect on flower color. In general, the pH of *Rhododendron* flowers is predominantly under genetic control with very little environmental interaction. In addition, aging does not change flower color by pH increase (often correlated with 'blueing') though it might affect color intensity. Furthermore, pigment synthesis and breakdown can be influenced by light and temperature, and traces of metals (Pecherer, 1992). The shape of the cells that accumulate anthocyanin pigments influences their optical properties and thereby the colour that is perceived (Mol *et al*., 1998).

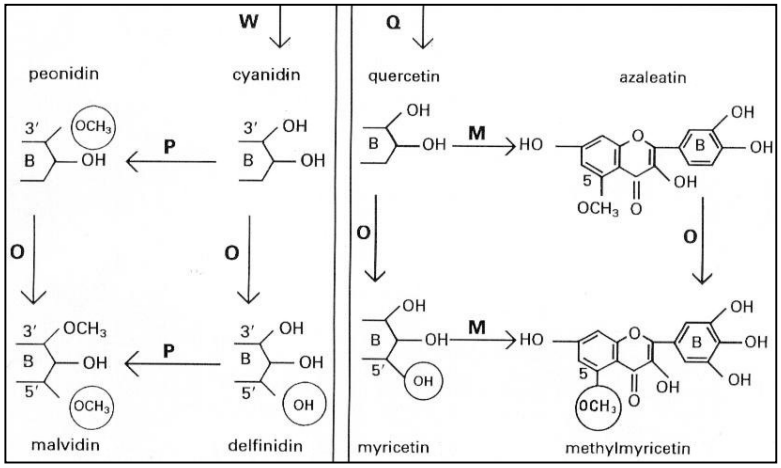
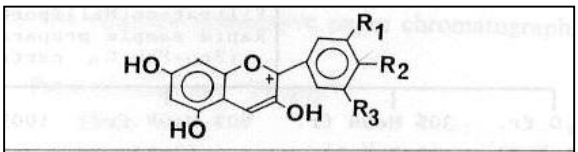
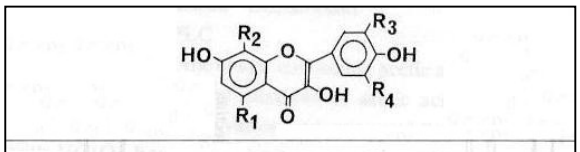


Figure 1.5. Flavonoids in pot azalea (Heursel & Horn, 1977).



Compound	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	OH	H
Petunidin	OCH_3	OH	OH
Pelargonidin	H	OH	H
Peonidin	OCH_3	OH	H
Malvidin	OCH_3	OH	OCH_3



Compound	R_1	R_2	R_3	R_4
5-O-methylmyricetin	OCH_3	H	OH	OH
Gossypetin	OH	OH	OH	H
Myricetin	OH	H	OH	OH
5-O-methylquercetin	OCH_3	H	OH	H
Quercetin	OH	H	OH	H
5-O-methylkaempferol	OCH_3	H	H	H
Kaempferol	OH	H	H	H

Figure 1.6. Main anthocyans (left) and flavonols (right) in the genus *Rhododendron* (Arisumi *et al.*, 1985).

Anthocyans occur in plant cells in combination with a variety of sugars; the kind and number of associated sugars will also affect flower color (e.g. gossypetin 3-galactoside occurring as a pigment in yellow flowers and gossypetin 3-rhamnoside in orange flowers). In 1977, Heursel & Horn proposed a model for pigment inheritance within pot azalea, ruled by six loci (Fig 1.5). White is homozygous recessive (ww). A gene Q is responsible for the synthesis of quercetin, O oxidizes position 5', M methylates position 5, P methylates 3' and 5' position and G is responsible for 5' glycosylation of the anthocyanidins.

Color phenotypes of pot azalea are described on the basis of the Royal Horticultural Society Color Chart (1966). The occurrence of flavonoids is very genotype specific, in such a degree that it can be used as a tool for biochemical identification (Kunishige & Kobayashi, 1980; Van Sumere *et al*., 1985). Cyanidin and peonidin cause red flower color; malvidine is a purple pigment; flavonols cause a blueish effect in the presence of anthocyanins but are synthesized in a too low amount to show yellow flower colors when no anthocyanins are present. Delfinidin does not occur often in cultivars since it yields a rather unattractive carmine-purple color.

Flavonoids, lacking in pot azalea but occurring in other *Rhododendron* species, are pelargonidin (orange-red), kaempferol (copigment) and, most important, gossypetin (yellow or orange). Apparently, the hydroxylase needed for the synthesis of gossypetin is missing in pot azalea. On the other hand, the presence of pelargonidin and kaempferol is not stable, probably because of a too high activity of flavanon 3-hydroxylase. Gossypetin appears to be the flavonol most important for the induction of yellow flowers in *Rhododendron* (Arisumi *et al*., 1985). Although flavonols occur in pot azaleas, gossypetin is lacking within the subgenus Tsutsusi. The competition of gossypetin with other flavonols can decrease the intensity of the yellow flower color. In other words, when breeding for yellow flowering plants, one should not only aim to increase the total amount of flavonols but also the relative amount of gossypetin among these flavonols. Arisumi *et al*. (1985) assume that methylation and hydroxylation in most *Rhododendron* species are not regulated by a single gene (because of the high complexity and variation within methylation and hydroxylation patterns).

The subgenus Pentanthera comprises the only yellow flowering azaleas. Their yellow (or orange) flower color is due to the presence of carotenoids (Santamour & Dumuth, 1978). Carotenoids do not occur in the vacuoles, but in particular organelles, the chromoplasts. Little is known about their biosynthesis and exact mode of action (Cunningham & Gantt, 1998; De Keyser *et al*., 2001). Therefore, although chromoplasts are inherited maternally, we can not exclude that their synthesis is partly regulated by the nuclear genome (and that way, also by the pollen parent). The main carotenoid pigments in yellow flower petals (corollas) of *Rhododendron* species and cultivars are β-carotene, prolycopene, α-carotene-5,6-epoxide, lutein, and lutein-5,6-epoxide. Next to Pentanthera, only some tropical Rhododendrons (Vireyas) synthesize carotenoids as main flower pigments although they also occur in gossypetin producing species like *R. wardii* Smith (Spethmann, 1979). The yellow flower color of Hymenanthes and Rhododendron (except Vireya) species and hybrids is caused by flavonols.

Since the absolute amount of flavonols produced by Tsutsusi species (evergreen azaleas) is quite low and gossypetin nor carotenoids are synthesized, yellow flowers do not occur within this group. However, the presence of flavonols is important to create an anthocyan/flavonol copigmentation. Depending on the type of anthocyan and flavonol, and their relative quantities, a color shift towards blue is caused by this copigmentation. The color originating from this copigmentation is called 'carmine red' (Holton *et al*., 1993). Blue flowers are difficult to retrieve within the genus; however, some hybrids of *R. augustinii, impeditum, hippophaeoides* and *russatum* approximate this color. As for carmine red, blue is caused by an anthocyan/flavonol copigmentation. This blue copigmentation is very pH-dependent and the pH of azalea cells is too low.

1.6.6. Interspecific hybridization

In the past, numerous efforts have already been undertaken to breed an evergreen yellow flowering azalea (Pryor, 1973; Wada, 1974; Jaynes, 1976; Kehr, 1977; 1987, Badger, 1988, Dermul, 1989). Next to pre-fertilization incongruity also postzygotic abortion, albinism and low hybrid vigor occur (Longly, 1994). Although after many interspecific *Rhododendron* pollinations pollen tube growth appears hampered, it is rarely totally inhibited (Okonkwo & Campbell, 1990; Williams & Rouse, 1990; Rouse, 1993), suggesting that most incongruity problems have a postzygotic nature. Preil and Ebbinghaus (1985) were able to cross azalea (seed parent) with a limited number of wild *Rhododendron* species. However, most pollinations didn't result in fruit development or germinating seeds. The hybrids obtained withered away soon or didn't show yellow flowers.

An overview of the reproductive biology of *Rhododendron* is presented by Williams *et al*. (1990). Most species are self-compatible and no genetic pollen-style incompatibility systems have been recorded in the genus. However, interspecific incongruity between plants of different subgenera is a major problem for breeders (Heyting, 1970). Rouse (1993) has performed a lot of interspecific pollinations within *Rhododendron* (mainly Vireyas). Results show that incongruity barriers can (and will) occur at any time from pollination till flowering. However, the creation of azaleodendrons (the product of a cross between an "azalea" and a "rhododendron") is not impossible (Rouse *et al*., 1988). The first azaleodendron was reported in 1936 (Bowers). 'Lilian Queen', 'Martine', 'Ria Hardijzer' and 'Hardijzers Beauty' are supposed hybrids between *R. racemosum* Franch. and an evergreen azalea (Salley & Greer, 1992). Of course, next to incongruity barriers also environmental circumstances should be taken into account, like temperature (Kho & Baer, 1973), flower age (Werbrouck, 1987) and differences in style length between parents (Williams & Rouse, 1988). It is evident that pollen germination and guidance through the style is very complex and dependent on a wide variety of signaling. Different species have developed different guidance systems, thus inhibiting interspecific crosses. Fertilization can be inhibited at several stages after pollination (Fig. 1.7).

This "incompatibility" can be caused by different flower morphology (Williams & Rouse, 1988; Williams & Rouse, 1990). Prezygotic incongruity totally inhibits interspecific hybridization after Pentanthera x Tsutsusi (Ureshino *et al*., 2000), Pentanthera x Vireya, Tsutsusi x Vireya and (non-Vireya) Rhododendron x Vireya pollinations (Rouse, 1993). Moreover, in some cases pollen growth can be delayed to such an extent that embryo sacs

have degenerated by the time sperm cells arrive at the micropyle, as was suggested for some Rhododendron x Tsutsusi crosses (Longly, 1994); the frequency of this phenomenon probably increases as style length of both parents differs more, since pollen tube growth rates usually decrease as pistil length increases (Williams & Rouse, 1990).

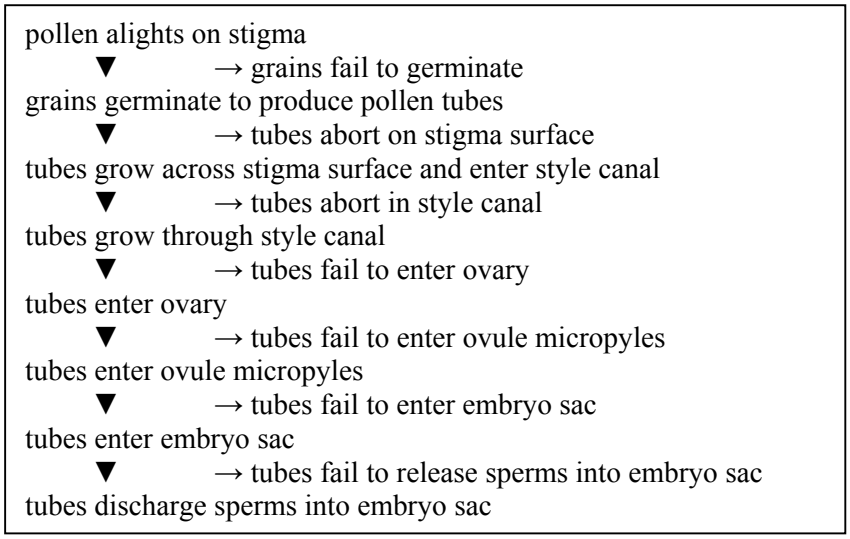


Figure 1.7. Inhibition of fertilization after incompatible interspecific *Rhododendron* crosses at different stages (Williams *et al*., 1982).

During interspecific *Rhododendron* breeding, albinism occurs rather frequent, even after intrasubgeneric pollinations (Michishita *et al*., 2002). In Japan, evergreen *R. indicum* hybrids could be crossed with deciduous azaleas thanks to embryo rescue techniques, provided the evergreen azalea was the seed parent (Ureshino *et al*., 1998; Ureshino *et al*., 1999; Miyajima *et al*., 2000). It appeared that after a three-way cross more seedlings inherited plastid-DNA from the pollen donor than after a control cross. The reason for this phenomenon remains unclear, however the inheritance of plastid-DNA from pollen donors has been described before (Hagemann, 1992; Yao *et al*., 1994). Using a rapid screening method through DAPI fluorescence, Corriveau & Coleman (1988) detected putative plastid-DNA in the generative and/or sperm cells of pollen from 43 species (out of 235 species tested). One of these 43 species was *Rhododendron maximum* Linn. (the only Ericaceae species in the test). Biparental influences on plastid development had already been proposed by Noguchi (1932). The occurrence of albinism is often linked to the inheritance of plastid-DNA and therefore to application of either parent as pollen donor or receptor. According to Krebs (1997) the inhibition of synapsis is mainly a problem in intersubgeneric crosses.

1.6.7. Breeding of tetraploid *Rhododendron simsii* hybrids

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 tetraploid; however, species that are classified within the subgenus Tsutsusi were found to be diploid. A screening of commercial evergreen azalea cultivars by Heursel and De Roo (1981) revealed only one triploid 'Euratom', all other cultivars being diploid. Similar results were obtained by Pryor and Frazier (1970). The first

successful chromosome doubling of *Rhododendron* sp. by colchicine was reported by Pryor and Frazier (1968). *In vivo* polyploidy induction on *Rhododendron* seedlings has been studied by Kehr (1996a; 1996b). Paden (1990) was able to induce tetraploidy through incubation of *in vitro* shoots of *Rhododendron* (garden) hybrids in liquid medium containing colchicine; afterwards the ploidy level was determined in an effective way by counting the chloroplasts in the epidermal guard cells. Of course, the risk of creating cytochimeras instead of tetraploids is present; moreover, colchicine might induce significant physiological effects that could impact somatic characteristics (effect on hormonal balance, point mutations, changes in chromosomal organelles, changes in chloroplast bodies) (Eiselein, 1994a; 1994b). More recently, Vaïnölä (2000) proved the higher efficiency of oryzalin compared to colchicine within the genus *Rhododendron*. Attempts to double the ploidy number of evergreen pot azaleas have started more then a decade ago (Vanoverschelde, 1989). The value of tetraploid azaleas might be purely ornamental (larger or longer lasting flowers, improved habitus); on the other hand, polyploidization could be a useful tool to overcome crossing barriers between incongruous *Rhododendron* species. Moreover, tetraploid azaleas can be crossed with diploids to breed triploid plants (with possibly optimized growth vigor). Recently, somatic polyploidy has been detected in picotee flowers, like 'Marcella', 'Koningin Fabiola' and 'Starlight' (De Schepper, 2001); fully tetraploid plants could be regenerated *in vitro* from these flowers (De Schepper *et al*., 2003).

1.7. *Hibiscus* sp.

1.7.1. Taxonomy (Bean, 1989b)

Division: Magnoliophyta
Class: Dicotyledonae
Subclass: Dilleniidae
Order: Malvales
Family: Malvaceae

1.7.2. Plant description (Bean, 1989b)

The calyx mostly is bell-shaped, 5-lobed, sometimes prominently 10-veined, the median vein to each lobe usually bearing a raised gland. The 5 petals are mostly longer than the calyx, white to yellow, red, purplish or rarely bluish, generally with a basal maroon spot. Stamens are united in a tubular column, mostly much longer than the petals, the style is usually 5-branched at the apex, sometimes only 5-lobed; the fruit is a capsule with 5 compartments, each with 3 or more seeds (Fig. 1.8).

Figure 1.8. The *Hibiscus* flower: *H. syriacus* 'Red Heart', *H. paramutabilis*, pollen and style.

1.7.3. Interspecific hybridization

In the genus *Hibiscus* a few species, including *H. syriacus* L., *H. sinosyriacus* Bailey and *H. paramutabilis* Bailey are natural to temperate regions of the world. All three are native to China (Bates, 1965). Among these species, breeding work is mainly done in *H. syriacus* (althea or rose of Sharon). About 40 different cultivars (with varying flower color and shape) are commonly in culture in Europe and a lot more genotypes are present in different collections (Yu & Yeam, 1972; Van de Laar, 1997; Van Huylenbroeck *et al.*, 2000). Breeding work has also resulted in creating polyploid cultivars, which in general have larger flowers and a longer flowering period (Egolf, 1970; 1981; Van Huylenbroeck *et al*., 2000). Morphologically and genetically *H. syriacus* is clearly discriminated from *H. paramutabilis* (Bates, 1965; Van Huylenbroeck *et al*., 2000). The latter grows more vigorously, has larger round-shaped leaves and flower diameter is increased. These characteristics are interesting to be transferred into *H. syriacus*. Within the genus, different chromosome numbers (x) have been reported (Skovsted, 1941; Darlington & Wylie, 1955). *H. syriacus* is described as a tetraploid (2n = 4x = 80). *H. paramutabilis* has 82 chromosomes (Niimoto, 1966). Attempts to create interspecific hybrids between *H. syriacus* and *H. rosa-chinensis*, especially to introduce new flower colors and forms, were not successful so far (Yu *et al*.,1976; Paek *et al*, 1989a).

Our goal was to hybridize *H. syriacus* cultivars with *H. paramutabilis* in order to increase the genetic variability within *H. syriacus*. Although recently reports have been published on the hybridization of these species and the possible strategies to increase the fertility of the hybrids (Kyung & Kim, 2001a; 2001b; Kyung *et al*., 2001a; 2001b) the best hybridization strategy and the inheritance of morphological traits remains unclear. Moreover, the breeding of triploid/hexaploid hybrids was apparently not attempted so far, though triploidy could have its influence on the segregation of flower colors (Kim *et al*., 1999) and other traits (Shim *et al*., 1993).

Embryo rescue in *H. syriacus* has already successfully been performed following (intraspecific) diploid x tetraploid pollination (Kim *et al*., 1996a; 1996b). It can also be applied in breeding programs of other *Hibiscus* species (Ahmed, 1992). Literature on ovule and embryo culture is available as well (Paek *et al*., 1989b; 1989c). Although somatic hybridization of *H. syriacus* protoplasts with other *H.* protoplasts could be established (Paek *et al*., 1989a) and regeneration of *Hibiscus syriacus* protoplasts is possible (Song & Park, 1998; 1999), so far the regeneration of hybridized protoplasts remains impossible.

2. General materials and methods

2.1. Plant material

2.1.1. General environmental conditions

Mother plants were cultivated in greenhouses at 18-20 °C and 65% relative humidity, or outdoors, in containers or in open ground (Table 2.1.) Containers were plastic unless mentioned otherwise. Normal nursery practices for watering, ventilation, fertilization and pest control were followed. Pollination, preceded by emasculation (except in *Hibiscus*), was performed in an isolated greenhouse by rubbing anthers releasing pollen over a susceptible stigma of a fully opened flower; more precise information on pollination is presented in the relevant chapters.

After careful removal of the agar, plantlets (single shoots) were acclimatized for 12 weeks after rooting *in vitro* (on basal medium as in Table 2.2. except when mentioned otherwise) in plastic seedling trays, in a fog unit of the greenhouse (18-20 °C, 16h photoperiod); after 3 weeks they were transferred to a multi-layer growing room (Pieters *et al*., 1989) (21 ± 1 °C, relative humidity 75-80%, CO_2-concentration between 350 and 450 ppm). During the last 9 weeks and after acclimatization plantlets were fertilized regularly with Flory 3 (NPK 15-11-15, 0.5 g/l). Substrates were 100% sphagnum peat, pH 5.0 –5.5, Snebbout n.v. Structural soil culture substrate 9A, pH 5.0 – 5.5 (substrate 1); 100% sphagnum peat, pH 5.4 – 5.8, 1.25 kg fertilizer mix in the ratio 14N:16P:18K Snebbout n.v. Structural soil culture substrate 6A (substrate 2); 100% sphagnum peat, pH 4.0-4.5, containing 60% organic matter, Neuhaus Humin soil culture substrate (substrate 3) or sphagnum peat 4: coconut fibre 1, pH 4, containing 125 g spore elements, EGO soil culture substrate (substrate 4).

General information on ploidy level, cv's and nursery practices for every genus are presented in Table 2.1. More detailed information is provided in the respective chapters.

2.1.2. *In vitro* initiation

Spathiphyllum inflorescences were cut when the spathe was still tightly closed. Immature seeds or embryos of *Buddleja*, *Rhododendron* and *Hibiscus* were isolated after sterilization of the fruits; mature seeds were sterilized directly.

Preceding *in vitro* initiation, *in vivo* material was rinsed in 70% ethanol, sterilized for 15 min in a 10 % NaOCl solution with 0.005% teepol and finally rinsed 3 times in autoclaved water. Tissue culture plants were subcultured every 6 weeks unless mentioned otherwise.

2.2. *In vitro* media compositions

The basal media for micropropagation of *Spathiphyllum wallisii* Regel (BMS), *Buddleja davidii* Franch. (BMB), *Rhododendron* sp. (BMR), and *Hibiscus* sp. (BMH) are presented in Table 2.2.

Table 2.1. Ploidy level, cv's used and nursery practices per genus (C# indicates relevant chapter for detailed information). NR = not relevant.

	Spathiphyllum	***Buddleja***	***Rhododendron***	***Hibiscus***
x	15	19	13	20
cv's	Deroose Plants NV: 'Daniël' (D), 'Stefanie' (D), 'Alfa' (D) Microflor NV: 'Speedy' (D)	DvP-CLO collection: *B. davidii* 'Royal Red', 'Ile de France', 'Nanho Purple' (all T) *B.* x*weyeriana* 'Sungold' (C5)	DvP-CLO collection, private gardens and commercial nurseries (C6)	DvP-CLO collection: *H. syriacus* 'Melwhite' (T), 'Oiseau Bleu' (T), 'Red Heart cv' (O),'Purple cv' (O) *H. paramutabilis* (2n=82)
standard mother plant cultivation	greenhouse conditions	outdoors in containers or open-ground	greenhouse conditions (Tsutsusi, Vireya) or outdoors (Hymenanthes, Rhododendron, Pentanthera)	outdoors in containers or open-ground
substrate	Acclimatization: substrate 1 Cultivation: substrate 2	Acclimatization: substrate 1 Cultivation: substrate 2	Acclimatization and seed germination: substrate 3 (sifted) Cultivation: substrate 4	Acclimatization: substrate 1 Cultivation: substrate 2
emasculation	NR	upon flower opening	C6	no
pollination	NR	fresh pollen	fresh pollen (on receptive stigma)	fresh pollen
flower induction	no	no	greenhouse conditions	no
***in vitro* inocula**	ovaries (C3), anther filaments (C4)	embryos (C5)	immature seeds (C6), seeds (C7)	embryos (C8)
***in vitro* subcultures**	ovules, embryos (C3/C4) adventitious shoots (C4)	seedlings (C5)	seedlings (C6/C7), adventitious shoots (C6/C7)	seedlings (C8)

D, T, O: diploid, tetraploid, octoploid.

Table 2.2. Basal media composition for *Spathiphyllum wallisii*, *Buddleja davidii*, *Rhododendron* sp. and *Hibiscus* sp.

	***Spathiphyllum wallisii* (BMS)**	***Buddleja davidii* (BMB)**	***Rhododendron* sp. (BMR)**	***Hibiscus* sp. (BMH)**
macroelements	MS	B5	WPM	½ MS
microelements	NN	B5	WPM	MS
vitamins	555 µM myo-inositol 0.89 µM thiamine HCl	B5	WPM	MS
Fe complex	MS	B5	WPM	MS
C-source	167 µM sucrose	111 µM sucrose	111 µM sucrose	167 µM sucrose
agar	3 g/l Roth + 4g/l MC29	7 g/l MC29	7 g/l MC29	7 g/l MC29
pH	5.8	5.8	5.4	5.8

MS: Murashige & Skoog (1962), NN: Nitsch & Nitsch (1969), B5: Gamborg (1968), WPM: Lloyd & McCown (1980).

The media were autoclaved (121 °C, 30 min, 500 hPa). Petri dishes (ϕ = 5.5 cm) were filled with 10 ml medium and sealed with LDPE (low density polyethylene) foil. Meli-jars (De Proft *et al*., 1985) contained 100 ml medium/jar. Cultures were maintained at 23 ± 2 °C under a 16 h photoperiod at 40 µMol m^{-2} s^{-1} PAR, supplied by cool white fluorescent lamps (OSRAM L36W/31), unless mentioned otherwise. Specific media compositions and methods are elaborated in the respective chapters.

2.3. Application of mitosis inhibitors

Mitosis inhibitors (MI) used in this study are presented in Fig. 2.1. Appropriate ORY (oryzalin) and TRI (trifluralin) solutions were prepared based respectively on commercial herbicides Surflan and Treflan (DowElanco ™) (both containing 48% a.i.). Colchicine (COL) was available in crystallized form.

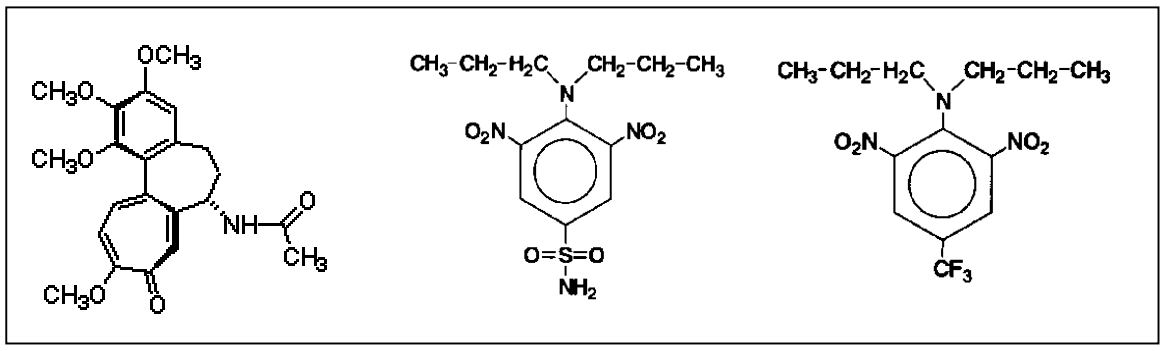


Figure 2.1. COL (left), ORY (middle) and TRI (right).

For treating seedlings, DMSO (0.5%) and the surfactant Teepol (0.05%) were added to all solutions; the surfactant "wets" the epidermal cells surface while DMSO increases cell permeability, thus jointly increasing the effectiveness of the solution (Kehr, 1996a). MI-solutions were daily administered between the cotyledons.

For all other applications MI-solutions were filter sterilized (0.22 μm) and subsequently added to autoclaved medium. Specifications on dilutions, materials and methods are provided in Chapters 4,5 and 7.

2.4. Characterization of prefertilization barriers

Characterization of prefertilization barriers was performed as described by Cuevas *et al.* (1994), Ureshino *et al.* (2000) and Vervaeke (2001). Pistils were harvested 24, 48, 72 or 96 h after pollination and fixed in FAA (formalin: 70% ethanol: acetic acid = 1:18:1) for 24 h, after which they were transferred to 70% ethanol for storage at 5° C until fixed and observed. Fixing the pistils consists of rinsing in tap water and soaking in 8N sodium hydroxide for 24 h to soften the tissue. The softened pistils were washed 3 times in distilled water to remove sodium hydroxide, and subsequently stained with a 0.1% solution of water-soluble aniline blue dye in 0.1 N tri-potassium phosphate for 3 h at room temperature. The stained materials were then squashed on a glass slide under a cover glass and observed with a fluorescent photomicroscope (LEICA DM IRB/MPS 52).

2.5. Irradiation

The X-ray source was a 15 MeV electron accelerator (Fig. 2.2) (Mondelaers *et al*., 1996), which supplied the electrons with 20 kW and an (adjustable) energy of 3 to 15 MeV. The material could be 'hit' with the electron beam directly, or the electrons could be converted into X-rays. For our purposes electrons were stopped in a 5 mm x 10 mm tantalium converter, embedded in graphite, that released part of the kinetic energy as X-rays.

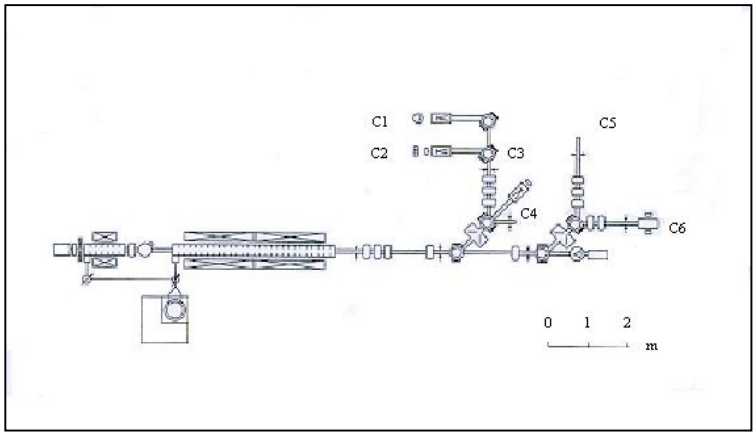


Figure 2.2. The electron accelerator. The electrons are accelerated and deflected to the C1, C2 or C5 exit; C3, C4 and C6 are exits for X-rays (converted from kinetic energy of the electron beam) (after Mondelaers *et al*., 1996).

2.6. Determination of leaf morphological indexes

Leaf length over width (L/B), angle of leaf basis (α) and relative width of the mid lobe (M/B) were determined as shown in Fig 2.3 as described by Van Huylenbroeck *et al.* (2000).

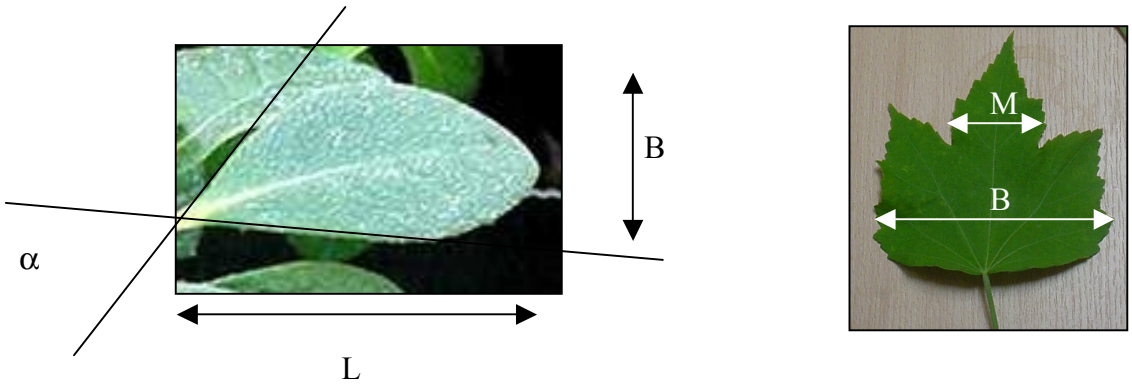


Figure 2.3. Determination of L/B and M/B indices and angle of leaf basis.

2.7. Flow cytometrical ploidy measurement

Ploidy measurement by conventional chromosome counting is time consuming and laborious (Martens & Reisch, 1988; Owen & Miller, 1993). Flow cytometry, which offers a valuable, rapid, simple, accurate and fairly cheap alternative, 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) (Fig. 2.4.). Heller (1973) was the first to use this technique for DNA-analysis in plant cells; later on it was used for different plant species (Galbraith, 1990; Arumuganathan & Earle, 1991; Baert *et al.*, 1992; Bennett & Leitch, 1995).

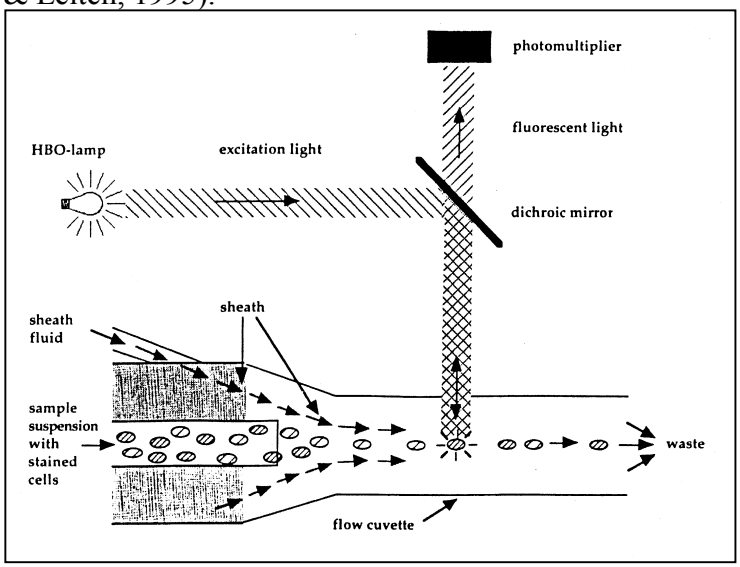


Figure 2.4. Hydrodynamic focusing: the chromosome suspension is injected into a cuvette filled with a sheath fluid, that is moving with a greater velocity and forces the sample into a very narrow (10 µm), laminar flowing stream without mixing both streams (Partec, 1999).

Flow cytometry was performed using a Partec PAS III as described by De Schepper *et al*. (2001). Young leaf samples were taken from one plant with a known ploidy level and the plants with the unknown ploidy level. Discs of 5 mm diameter were punched out of the youngest leaves. 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. Afterwards, 500 µl of

the second buffer, containing 0.4 M Na_2HPO_4 and 2 mg/l DAPI (pH ± 8.5) was passed through the filter before staining. The mixture of the two buffers gave pH ± 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 light beam, 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 were 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). Flow cytometry is a relative measurement, the first sample gauges the apparatus. 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) was fixed at position 100 (channel number), unless mentioned otherwise. Haploid peaks occurred at position 50, tetraploid peaks at position 200. The measurement of the external standard was repeated after every 10 measurements to correct the voltage of the photomultiplier if necessary.

2.8. DNA-isolation

Young leaf material was harvested from acclimatized plants. At harvest plant material was immediately immersed in liquid nitrogen and subsequently lyophilized for 48 h. The dry material was vacuum-packed for storage at –20°C until DNA-extraction. Stored material was ground using a Culatti mechanical mill. To 25-75 mg of lyophilized grounded tissue, 10 ml of 50 mM Tris-HCl buffer (pH 8) containing 5 mM EDTA, 350 mM sorbitol, 0.1 % β-mercaptoethanol, 10 % Polyethylene Glycol (MW 8000) and 0.001 % Bovine Serum Albumine were added. This mixture was homogenized for 30 s by inverting the tubes and then filtered through Miracloth (Calbiochem) to remove coarse cell debris. The nuclei fraction was pelleted by centrifugation (2500 g, 5 min, 4°C). To the nuclei fraction, 1 ml CTAB extraction buffer (100 mM Tris-HCl pH 8, containing 2 % CTAB, 20 mM EDTA, 1.4 M NaCl, 0.5 mM $Na_2S_2O_5$, 0.4 % β-mercaptoethanol and 1 % polyvinylpyrrolidone MW 40000) and RNAse (10 U) were added (Doyle & Doyle, 1987). Samples were incubated for 40 min at 65°C. Subsequently, samples were homogenized with 1 ml chloroform/isoamylalcohol (24/1) and centrifuged for 15 min at 10000 g. The supernatant was transferred to a fresh tube and the DNA precipitated with 1 ml of ice cold (-20°C) isopropanol. After centrifugation (5000 g; 15 min), the pellet was washed with EtOH (76 %) + 0.2 M NaOAc, dried and dissolved in water. DNA-concentration and quality were constantly checked relative to a standard series of viral lambda-DNA after electrophoresis on a 1.5 % TAE buffered agarose gel.

2.9. AFLP molecular analysis

AFLP (Amplified Fragment Length Polymorphism) is based on the selective amplification of restriction fragments from a total restriction digest of genomic DNA (Vos *et al*., 1995). It was

performed using the commercially available kit from Perkin-Elmer Biosystems for fluorescent fragment detection (Perkin-Elmer, 1995; Roldan-Ruiz *et al.*, 1997). EcoRI and MseI (Gibco BRL) were used for DNA-digestion. Two adaptors, one for the EcoRI ends and one for the MseI ends were ligated to the restriction fragments by adding 25 µl of a mix containing 50 pmol MseI adaptor, 5 pmol EcoRI adaptor, 10 mM Tris-HCl, 10 mM MgOAc, 8 mM ATP, 50 mM dithiotreitol (pH 7.5) and 1.4 U DNA-ligase. After 2h incubation at 37 °C, ligation mixtures were diluted to 10% of their initial concentration. Subsequently, preamplification was performed using primers complementary to the EcoRI and MseI adaptors (extended with 1 additional selective 3' nucleotide). PCR reactions were carried out in a 50 µl mix of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 ng of each primer, 1 U Taq DNA-polymerase and 5 µl of the ligation mixture dilution, in a Hybaid Omni Gene Cycler. Again, preamplification products were diluted to 10% of their initial concentration. The actual PCR fluorescent selective amplification mix was composed of 3 µl diluted preamplification, 1 µl EcoRI primer at 1 µM (fluorescent labeled), 1 µl MseI primer at 5 µM and 15 µl AFLP Core Mix.

After the PCR reaction, the samples were denatured by adding 20 µl of formamide buffer (5 formamide: 1 25mM EDTA + 50 mg/ml dextrane) and heating for 3 min at 90°C. For PAGE, 1.5 µl of each sample was loaded on 5 % acrylamide/bisacrylamide 19/1, 7.5 M urea and 1 X Tris-borate-EDTA (TBE) buffer (10 X TBE was composed of 213 mM Tris-HCl, 222 mM H_3BO_3 and 5mM EDTA pH 8). GS-500-ROX labeled size standard (Perkin-Elmer) was loaded on each lane in order to facilitate the automatic analysis of the gel and the sizing of the fragments. During analysis with an ABI 377 Prism 377 DNA-sequencer, the fluorescent signal in each lane was recorded continuously. For each fluorescent dye electropherograms were extracted. GENESCAN 2.1 was used to estimate detection time, signal peak height and surface for each fragment. Sizing of the fragments was performed by the Genescan software module by interpolation to the internal lane standard according to the Local Southern algorithm. Only the fragments between 70 bp and 500 bp were considered.

Multivariate analysis is performed using Jaccard and Simple Matching similarity indices, that reflect mutual similarity of 2 genomes and are respectively calculated by

$$\text{Jaccard} = \frac{C}{A + B + C} \quad \text{and Simple Matching} = \frac{C + D}{A + B + C + D}$$

with A = number of fragments uniquely present in genome 1, B = number of fragments uniquely present in genome 2, C = number of fragments present in both genomes, D = number of fragments absent in both genomes. These indices are commonly used tools for biodiversity evaluation (Karp *et al*., 1997). As a rule, data obtained from all primer combinations were taken as a whole for similarity index calculations and proceeding representations.

A phylogenetic tree (dendrogram) was constructed using clustering with the Unweighted Pair Group Method with Arithmatic Mean (UPGMA). The dendrogram is built stepwise by clustering the 2 most similar genotypes in a subsequent manner, each time reducing the number of groups by 1 unit, finally resulting into a "tree" (Sneath & Sokal, 1973).

2.10. SSR molecular analysis

The SSR or microsatellite is a codominant technique, involving the use of specific primers to PCR-amplify genomic regions containing tandems of short sequence motifs of 1-6 nucleotides (SSR loci). Polymorphisms at microsatellite loci arise due to changes in the number of repeats, insertion of motifs and imperfect motifs, variations in the adjoining non-repetitive region or to interruption of perfect repeats (Davierwala *et al*., 2000; Holton, 2001).

Upon DNA-isolation, PCR amplifications were performed using the Geneamp PCR reagent kit of Applied Biosystems. The reaction volume was a 20 µl mix of 25 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.075 µM forward and reverse primer (1 primer being fluorescent labeled), 2.5 µg Bovine Serum Albumin and 1 U Taq polymerase.

After the PCR reaction, the samples were denatured by adding 20 µl of formamide buffer and heating for 3 min at 90°C. PAGE and extraction of electropherograms were as described in 2.9.

2.11. Statistical analysis

For the statistical analysis SPSS 10.1 for Windows was used. A one way or multifactorial analysis of variance (ANOVA) was conducted on the data obtained; if significant differences occurred means were separated by the LSD (P=0.05) method.

3. Creation of homozygous *Spathiphyllum wallisii* through gynogenesis

3.1. Introduction

Spathiphyllum is an ornamental which can be very easily propagated *in vitro* (Fonnesbech & Fonnesbech, 1979). However, actually most commercial plants are very heterogeneous seedlings (3-5/container). F_1-hybrids, having the same genotype, would show a more homogeneous morphology and flowering time. For the induction of F_1-hybrids the creation of homozygous parental plants is required. Since classical breeding would require 7 generations of self-fertilization, it is recommended to develop homozygous parents through *in vitro* culture of haploid cells (egg cells or microspores). Afterwards, the combination ability of the homozygous plants can be determined to find the most suitable parents for F_1-hybrid breeding.

In preliminary experiments (Eeckhaut, 1999), we experienced that *Spathiphyllum wallisii* was very recalcitrant towards androgenesis. The only Araceae species known to be amenable to androgenesis is *Zantedeschia aethiopica* (L.) Spreng. (Ko *et al*., 1996). For that reason gynogenesis, an alternative for androgenesis, was attempted. A two-step protocol, involving subsequent ovary and ovule culture, was examined based on multiple genotypes, and refined for a single genotype. Possible doubled haploids were screened flow cytometrically and by AFLP-analyses.

3.2. Specific materials and methods

3.2.1. Ovary/ovule culture and embryo conversion

General mature plant nursery practices were as described in Table 2.1. Inflorescences were harvested 2 days before the opening of the spathe. Inflorescences were sterilized (2.1.); ovaries were isolated and placed on different ovary culture media (3.2.1.1.; 3.2.1.2). Ovules were dissected from ovaries and subcultured on ovule culture media (3.2.1.1.; 3.2.1.2). Both culture types were maintained at 23 ± 2 °C in the dark. Resulting embryos were converted on BMS in Meli-jars under a 16 h photoperiod. After 6 months plants were acclimatized (2.1).

3.2.1.1. *Genotype influence*

Ovaries of ‘Daniël’ (200 ovaries/treatment), ‘Stefanie’ (160 ovaries/treatment) and ‘Alfa’ (120 ovaries/treatment) were cultivated on ovary medium A: BMS (2.2.) + 0.25 µM TDZ + 1.6 µM PIC + 0 or 15 µM IMA (imazalil, Fungaflor ™) (20 ovaries/petri dish). After 4 weeks swollen ovules were isolated and transferred to ovule medium A: BMS + 0.8 µM PIC + 0, 1, 2 or 4 µM TDZ (10 ovules/petri dish). Altogether, this experiment comprised 2 (ovary media A) x 4 (ovule media A) = 8 treatments. Embryos were converted and the first leaflets were used for flow cytometrical analysis (2.7.).

3.2.1.2. *Development of a suitable sequence of media*

Ovaries of ‘Stefanie’ (the most reactive genotype in preliminary experiments, 3.3.1.) were cultivated on ovary medium B: BMS containing 1.6 µM PIC + 10 µM BA, 0.5 µM or 1µM TDZ or (0.5 µM TDZ + 10 µM Z) in combination with 13 µM IMA, 13 µM PRO, 13 µM TRIF or no fungicide (30 ovaries/treatment). After 6 weeks swollen ovules were isolated from the ovaria and put on ovule medium B: BMS + 0, 5, 10 or 20 µM Z. For each of the 16 (ovary media B) x 4 (ovule media B) = 64 treatments, 15 ovules were transferred. The resulting embryo clusters were cut in pieces (± 50 mm^3) and placed on BMS + 10 µM BA + 0.02 µM NAA for multiplication, after which they were converted. Flow cytometry was applied as described in 2.7. The device was gauged at 50 channel numbers using a diploid reference.

3.2.2. AFLP-analysis

3.2.2.1. *Genotype influence*

Preamplification and amplification were performed using random primer combinations E-ACA + M-CTG (code: 1), E-ACC + M-CAG (2), E-ACG + M-CTG (3), E-ACT + M-CAT (4), E-ACT + M-CTT (5), E-AGC + M-CTA (6), E-AGG + M-CAA (7), E-AGG + M-CTT (8), E-ACC + M-CAT (9).

3.2.2.2. *Development of a suitable sequence of media*

The following combinations were selected (based on 3.3.1): E-ACC + M-CAG (2), E-ACG + M-CTG (3), E-ACT + M-CAT (4) and E-ACA + M-CTC (10).

3.3. Results

3.3.1. Genotype influence

3.3.1.1. *Ovary and ovule culture*

Ovaries of all cv’s were equally swollen on both media after 4 weeks (Fig. 3.1.), upon which 80 to 150 white enlarged ovules were isolated (Table 3.1.). Some formed embryo clusters after 6 weeks on ovule medium A. Single embryos were never observed. The exact effect of both ovary and ovule media on ovule embryogenesis is presented in Tables 3.1 and 3.2. In ovule medium of ‘Daniël’ and ‘Stefanie’ embryo clusters were rarely observed.

The presence of TDZ in the ‘Alfa’ ovule medium A was required for embryo induction and increasing concentrations improved embryogenesis. No significant interaction with the presence of IMA in ovary medium A was found. Adding IMA was not statistically relevant. Because of the low embryogenic capacity in ovule cultures of ‘Daniël’ and ‘Stefanie’ statistical testing was only possible with the data of ‘Alfa’ ovules.

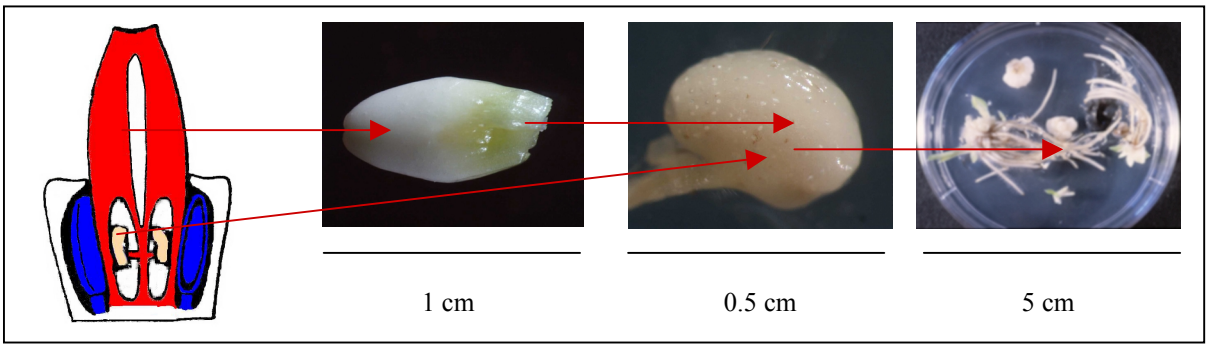


Figure 3.1. Different steps in ovule culture of *Spathiphyllum wallisii* 'Alfa': flower (left), ovary (middle left), ovule (middle right), ovule embryogenesis (right).

Table 3.1. Number of **e**mbryogenic *Spathiphyllum wallisii* ovules after a 4 week ovary culture and a 6 week ovule culture period.

Genotype	IMA in ovary medium A (µM)	TDZ in ovule medium A (µM)	# ovules subcultured	# embryogenic ovules
'Daniël'	**0**	**0**	150	0
		1	150	0
		2	150	0
		4	150	0
	15	**0**	150	0
		1	150	0
		2	150	10
		4	150	0
'Stefanie'	**0**	**0**	120	0
		1	120	4
		2	120	0
		4	120	0
	15	**0**	120	0
		1	120	0
		2	120	0
		4	120	1
'Alfa'	**0**	**0**	80	0
		1	80	16
		2	80	14
		4	80	14
	15	**0**	80	0
		1	80	3
		2	80	8
		4	80	21

Table 3.2. Effect of IMA (in the ovary medium A) and TDZ (in the ovule medium A) on the yield of embryogenic clusters on *Spathiphyllum wallisii* 'Alfa' ovules. Means followed by the same symbol are not significantly different (LSD 95%).

		# ovules forming embryo clusters / 10 explants
IMA in ovary medium A (µM)	**0**	1,37 a
	15	1,00 a
TDZ in ovule medium A (µM)	**0**	0,00 a
	1	1,19 ab
	2	1,37 b
	4	2,19 b
	IMA	NS
	TDZ	*
	IMA x TDZ	NS

NS, *: Non-significant or significant at $P = 0.01$ using analysis of variance.

3.3.1.2. *Flow cytometrical and molecular analysis*

Only 1 'Daniël', 3 'Stefanie' and 27 'Alfa' embryo clusters converted into plantlets. Upon acclimatization, they developed into normal plants, with no visual phenotypic differences from the mother plant. Flow cytometry revealed the diploid nature of the plants originating from all 31 converted embryo clusters.

AFLP-analyses elucidated that not every primer combination was appropriate. The 5 relevant primer combinations (1, 2, 3, 4 and 6) showed that 1 plant of the 'Stefanie' group ('S3') was distinct from the mother plant (Fig. 3.2.). It originated from an ovary that was not exposed to IMA and an ovule that was subject to 1 µM TDZ. Other 'Stefanie' progenies ('S1', 'S2') electropherograms were identical to the one of 'Stefanie' for all primer combinations. All 'Alfa' and 'Daniel' descendants were mutually identical to their respective progenitors (AFLP-patterns not shown).

3.3.2. Development of a suitable ovary and ovule media sequence

3.3.2.1. *Ovary and ovule culture*

Ovaries of 'Stefanie' were swollen after 4 weeks on ovary medium B (3.2.1.2.) (like in Fig. 3.1.), and the white and enlarged ovules were isolated and subcultured on ovule medium B. Nearly all further development was arrested as most ovules turned black and degenerated. Only 1 out of 15 ovules, isolated from ovaria cultured on ovary medium B containing 13 µM TRIF + 1µM TDZ, and subsequently transferred to Z-free ovule medium B, manifested secondary embryogenesis 6 weeks after transfer. The resulting embryo cluster was subcultured, converted and acclimatized. The plants were referred to as 'S4' and grew as vigorously as the original 'Stefanie' genotype (Fig. 3.3).

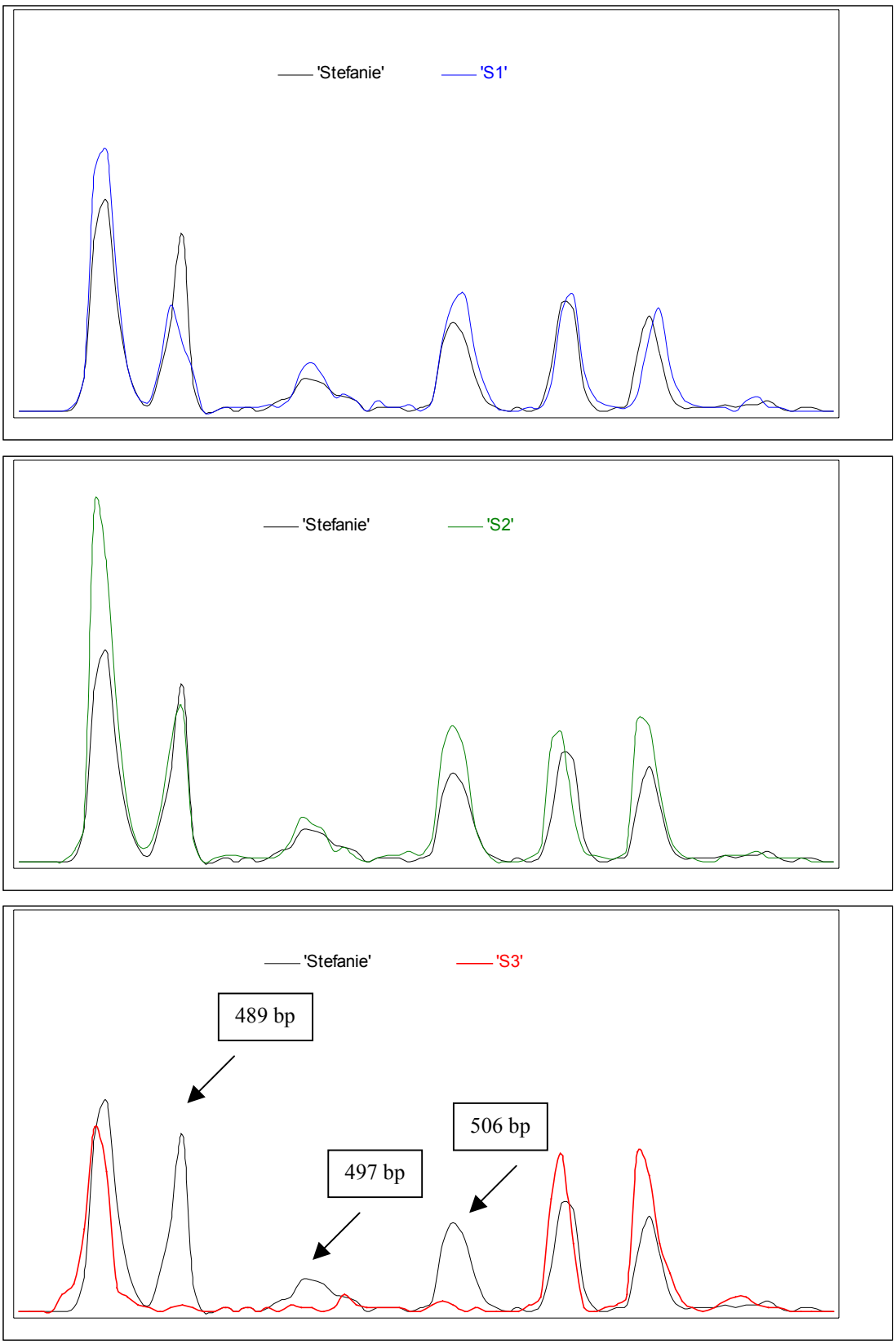


Figure 3.2. Electropherogram obtained after AFLP-analysis (primer combination E-ACA + M-CTG) of 2 *Spathiphyllum wallisii* plants originating from maternal tissue ('S1' and 'S2') and 1 doubled haploid plant ('S3') from ovule culture A, all compared to the heterozygous mother plant 'Stefanie'.

Figure 3.3. The converted *Spathiphyllum wallisii* doubled haploid (left) and 'Stefanie' (right).

3.3.2.2. *Flow cytometrical and molecular analysis*

Ploidy measurement on the first developed leaflet from the converting embryo cluster revealed 'S4' was haploid (Fig. 3.4.). However, flow cytometry of leaves of acclimatized plants from the same origin only showed a diploid peak.

After selective amplification, the primer combinations E-ACC + M-CAG (2) and E-ACT + M-CAT (4) yielded an appropriate number of distinct fragments (Fig. 3.5.), showing that the AFLP-fragments of the plant obtained through ovule culture were a subset of the AFLP-fragments of the mother plant 'Stefanie'.

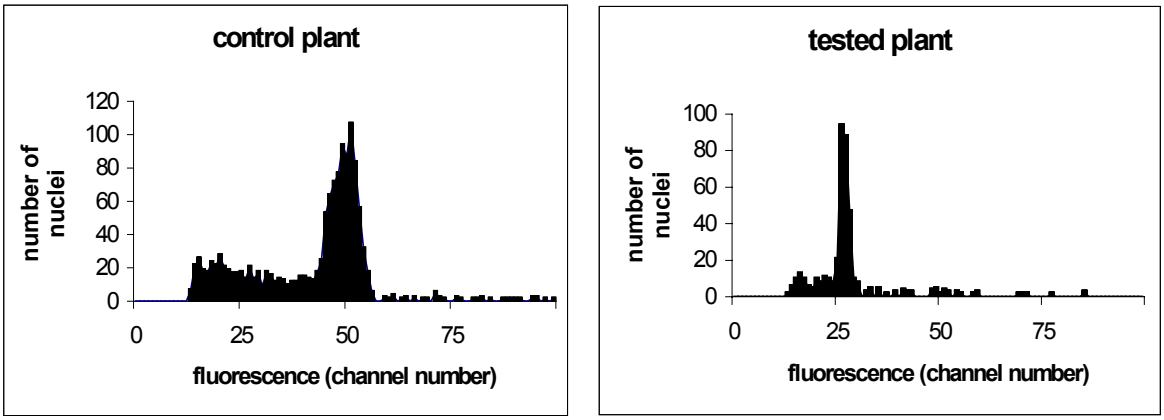


Figure 3.4. Histograms obtained after flow cytometrical analysis of a haploid *Spathiphyllum wallisii* 'Stefanie' plantlet (right) obtained through ovary culture on BMS + 13 µM% TRIF + 1 µM TDZ followed by ovule culture on BMS.

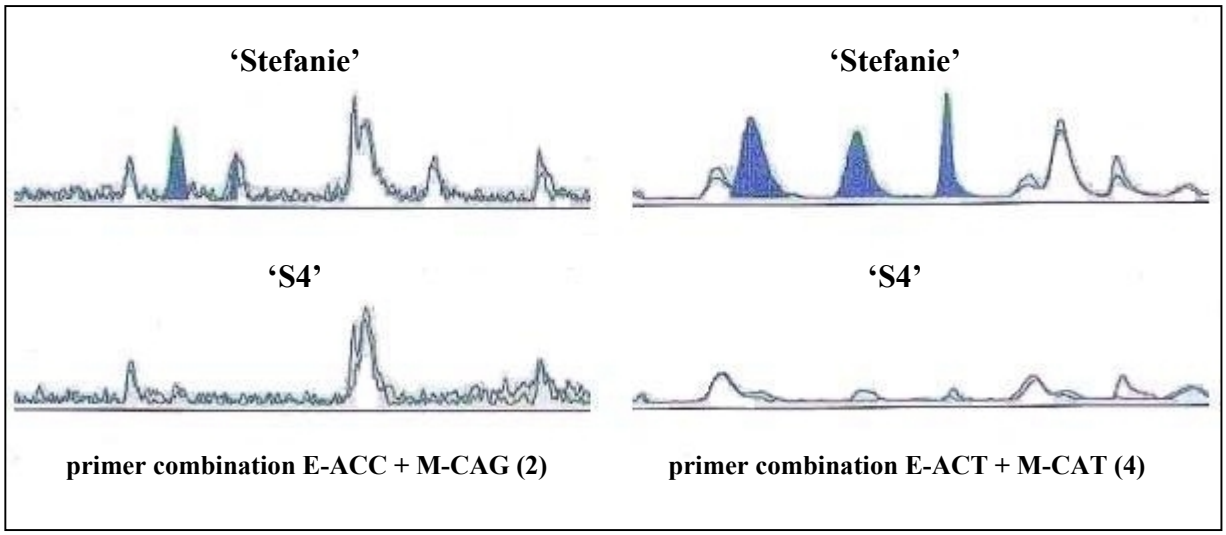


Figure 3.5. AFLP-pattern of *Spathiphyllum wallisii* 'Stefanie' and 'S4'. For both primer combinations fragments from 390 to 450 bp are shown. DNA-fragments lacking in 'S4' are indicated as hatched peaks.

3.4. Discussion

For obvious reasons, the number of gametes (and the embryogenic potential) in ovule culture is significantly less than in microspore culture. Moreover, during culture of isolated microspores the proliferation of surrounding diploid tissue is impossible. In a series of preliminary experiments, covering a wide range of stress treatments, genotypes, culture techniques and media compositions, somatic development of microspores could not be induced (Eeckhaut, 1999). Although microspores remained viable, as shown by FDA staining, cell division and subsequent embryo development were never observed. All our preliminary experiments with microspore cultures were based on literature data on crops from other families (Wang & Chen, 1986; Rode & Dumas de Vaulx, 1987; Chaboud & Perez, 1992; Chen, 1992; Cuny, 1993; Cao *et al*., 1994; Meynet *et al*., 1994; Coumans & Zhong, 1995; Hu *et al*., 1995; Dunwell, 1996; Rihova & Tupy, 1996; Sopory & Munshi, 1996; Touraev *et al*., 1996; Dohya *et al*., 1997, Hu & Kasha, 1997) since microspore culture was never established in Araceae. Although successful *Zantedeschia* anther culture has been described (Ko *et al*., 1996) possible haploid formation from *Spathiphyllum* anthers was impeded by multiple somatic embryo formation on the filaments (Werbrouck *et al*., 2000). Indeed, because of small total size of the anthers (± 2 mm) the embryogenic filaments were almost impossible to detach.

In a first experiment it was evaluated which of the 3 *Spathiphyllum* genotypes was most appropriate for haploid induction. The *Spathiphyllum wallisii* cv's used in this study were created by the same breeder and show high consanguinity; in spite of this genotype influences were decisive for the reaction of isolated ovules *in vitro*. 'Alfa' ovules easily reacted by somatic embryogenesis on a medium enriched with TDZ (Table 3.1.). TDZ was preferred over Z and other cytokinins since it has a stronger cytokinin-like activity, although it is probably active on another level than Z, it is reported to minimize cytokinin oxidase activity (Hare & Van Staden, 1994a, b). All 'Alfa' derived plantlets were somaclonal regenerants.

‘Daniël’ and ‘Stefanie’ ovules were significantly less embryogenic. One ‘Stefanie’ ovule yielded a doubled haploid (Fig. 3.2.).

Because of substantial genotype interaction, in a follow-up experiment, ovaries (and, subsequently, ovules) of ‘Stefanie’, the only responsive genotype was placed on different media sequences to establish the most efficient doubled haploid induction and regeneration protocol for this 1 genotype. In the ovule medium, TDZ was replaced by Z to avoid high division rates of somatic cells, as on ‘Alfa’ ovules during the first experiment. Again, 1 doubled haploid was retrieved (Fig. 3.5.). This time, somatic tissue embryogenesis did not interfere with haploid development, probably owing to the omission of TDZ. Cytokinins were not required for embryogenesis as ovules were only reactive on Z-free medium.

During the experiments ovary media were enriched with different imidazole fungicides (IMA, PRO and TRIF). Those were included because, at least in Araceae, they have phytohormonal side effects which enhance adventitious organogenesis (Werbrouck & Debergh, 1996; Werbrouck *et al.*, 1996). Moreover, in previous experiments (data not presented) IMA was shown to improve the swelling of ovules within the ovaries, thus facilitating their isolation. During the first experiment, IMA caused no statistically significant effects or interactions with regard to ‘Alfa’ ovule embryogenesis (Table 3.2.); again, better ovule swelling during ovary culture was obvious. Probably this swelling was merely caused by integument expansion. Relatively high LSD intervals were noticed during statistical processing owing to large differences between petri dishes containing the same medium. Embryogenesis in separate dishes seemed to be a self-intensifying process.

Only 1 out of 2 haploids (‘S4’) could be identified by flow cytometry (Fig. 3.4.) and only at a very young stage. This suggests that some cells of the young haploid embryo cluster doubled spontaneously and formed more vigorous plantlets able to acclimatize. Theoretically, diploid tissue can originate from somatic cells as well as from spontaneously doubled haploids. A molecular tool was required for the elucidation of their origin.

AFLP-analysis with particular primer combinations was effective to distinguish plants which originated from maternal tissue from the doubled haploids. AFLP is not codominant and therefore may appear less suitable for distinguishing homozygous from heterozygous plants; however, the use of codominant markers like microsatellites was restricted since no suitable primers for *Spathiphyllum* were available (no priory sequence information was required for AFLP primer construction). Multiple primer combinations were included to allow better analysis of the offspring. In combination with ploidy analysis on the first emerging leaflet, AFLP was efficient in selecting homozygous genotypes. However, not every primer combination was suitable.

Altogether, 2 doubled haploid genotypes were obtained from ‘Stefanie’ ovules. Since *Spathiphyllum wallisii* cv’s are heterozygous plants, the absence of a severe inbreeding depression (Fig. 3.3.) was remarkable; however, because only 2 plants were obtained (from the same cv) general conclusions about inbreeding depression after *Spathiphyllum* ovule culture should not be drawn. The plants developed normal spadices. In eventual further

studies research should be extended towards other genotypes, preferably from different origins.

The final aim of these experiments was to develop appropriate technology for the synthesis of *Spathiphyllum wallisii* F_1 hybrids through seed. Since homozygous plants synthesize only one gametal genotype (identical to either chromosomal set of the parent) a doubled haploid x doubled haploid cross would result in a fully identical offspring. Upon establishment of an efficient protocol, a large number of homozygous plants derived from selected maternal genotypes can be created. The next step would be to determine General and Single Combining Abilities of the doubled haploids to determine the best parental combination for the F_1 seed production. Although doubled haploids could so far only be derived from 1 single cultivar, this may be considered a first main step towards the establishment of such a protocol.

4. Development of tetraploid *Spathiphyllum wallisii*

4.1. Introduction

For a large group of botanical ornamentals (orchids, lily, *Gladiolus*…) and others (wheat, cotton, *Lolium*, *Malus*, *Fragaria*…) an enhanced ploidy level improves morphological properties, like larger flowers or faster growth. Moreover, tetraploids can be used to generate triploids after crossing with a diploid.

In vitro shoot bases of *Spathiphyllum wallisii,* when exposed to a BA/IMA combination, produce a cluster of tiny shoots, probably due to gibberellin (Werbrouck & Debergh, 1995;1996, Werbrouck *et al*., 1996) and brassinosteroid biosynthesis inhibition by imazalil (Werbrouck *et al.*, 2003) This was observed on all genotypes studied. We considered the application of this regeneration system for polyploid induction.

Somatic embryogenesis has a wide variety of potential applications: rapid multiplication, mutation induction and genetic modification. Unlike its relative *Anthurium* (Matsumoto *et al*., 1996; Hamidah *et al*., 1997), *Spathiphyllum wallisii* leaves do not form somatic embryos. On the other hand, its anther filaments form somatic embryos abundantly when exposed to an appropriate *in vitro* medium. Embryogenesis on *S. wallisii* anther filaments can be induced by a wide variety of auxin/cytokinin combinations (Werbrouck *et al*., 2000). These somatic embryos can be converted quite easily. In this study somatic embryogenesis was optimized, as a regeneration tool for the breeding of tetraploid *Spathiphyllum wallisii* cv's. The most appropriate auxin and cytokinin concentration to yield embryogenesis were determined. Special attention was paid to the developmental stage of the anthers. We also tested the induction of somatic embryos on media enriched with an antimitotic agent (MI): COL (colchicine), ORY (oryzalin) or TRI (trifluralin), and examined the efficiency of tetraploidy induction during both primary and secondary embryogenesis.

Our ultimate goal is to produce triploid *S. wallisii* F_1-hybrids through seed. Therefore a homozygous tetraploid should be crossed with a homozygous diploid. The creation of homozygous plants can be established through gynogenesis (Eeckhaut *et al*., 2001b; Chapter 3).

Different cv's were used in a series of experiments. Since both adventitious shoot formation and somatic embryogenesis are non-genotype specific events (Werbrouck & Debergh, 1995;1996, Werbrouck *et al*., 2000), the starting material was chosen based on flower or subculture availability.

4.2. Specific materials and methods

4.2.1. Overall practices

Spathiphyllum wallisii 'Speedy' mother plants were grown under greenhouse conditions (as in 2.1.). *Spathiphyllum wallisii* 'Daniel' *in vitro* plants were available from routinely maintained cultures (BMS as in 2.2. + 2.2 µM BA) at 6 weeks intervals. Anthers (with filament) and

somatic embryo clusters were cultured under dark conditions at 20 ± 2 °C. Conversion of embryos and adventitious shoot growth were performed in Meli-jars on BMS at 23 ± 2 °C under a 16 h photoperiod at 40 µMol m^{-2} s^{-1} PAR. Primary and secondary embryos were induced in petridishes (ϕ = 5.5 cm) that were taped with LDPE foil. COL, ORY and TRI (2.3.) aqueous dilutions were filter sterilized (0.22µm) and added to the appropriate media after autoclaving. COL was used in a shock-treatment (1 or 2 days) in liquid medium. The other MIs remained in the culture medium during the whole duration of a subculture cycle.

4.2.2. Adventitious shoot formation

Two hundred and ten 'Daniel' shoots (± 3 cm) were put on BMS + 10 µM BA + 15 µM IMA (imazalil). Five jars each (6 shoots/jar) were enriched with 3 or 10 µM ORY or TRI; the shoots remained on these media for the total culture period of 12 weeks. The remaining 90 shoots were put on BMS + 10 µM BA + 15 µM IMA. After 1 week, 30 shoots of the latter group were incubated in vessels with liquid BMS + 100 µM COL during 16h, on a rotary shaker (40 rpm). Subsequently the shoots were inoculated on BMS + 20 µM BA + 15 µM IMA (100 ml/vessel, 6 shoots/ jar). After 3 more weeks, the same procedure was performed with 30 other shoots. After 12 weeks, the swollen shoot bases, with numerous adventitious shoots, were cut into smaller shoot clusters and grown on BMS for elongation and root induction during 8 weeks. Acclimatization and flow cytometry were as in 2.1. and 2.7., respectively.

4.2.3. Optimization of somatic embryogenesis

4.2.3.1. *Optimal NAA- and TDZ-concentration*

BMS was supplemented with 0, 5, 10 or 15 µM NAA and 0, 1, 3 or 9 µM TDZ. On every medium 20 freshly sterilized (2.1.) and dissected anthers of 'Daniel' were placed (20 anthers/ petri dish x 10 petri dishes/treatment x 16 treatments = 3200 anthers).

4.2.3.2. *Effect of developmental stage*

Inflorescences with closed spatha were classified in three stages: (1) pale green spadix, non-bulging ovaria; (2) white spadix, slightly bulging ovaria; (3) white spadix, bulging ovaria, spathe almost open. Anthers of *Spathiphyllum* 'Daniel' were sterilized, dissected and placed on BMS supplemented with 3 µM TDZ and 10 µM NAA. The spadix was divided in 3 zones: top, middle and base (with flowers ripening from base to top) (20 anthers/petri dish x 6 petri dishes/ (stage x zone) x 3 stages x 3 zones = 1080 anthers).

4.2.4. Induction of primary somatic embryos

One thousand one hundred anthers of *Spathiphyllum wallisii* 'Speedy' (20 anthers/petri dish, 5 petri dishes/treatment, 11 treatments) were sterilized and dissected. A first group of 8 x 100 anthers were placed on BMS + 10 µM 2,4-D + 2.5 µM TDZ enriched with 1, 3, 10 or 30 µM TRI or ORY. Another 2 x 100 anthers were kept in a 100 µM COL (in liquid BMS) solution for 4 or 16 h, and subsequently transferred to BMS + 10 µM 2,4-D + 2.5 µM TDZ. The

remaining 100 anthers were put on BMS + 10 µM 2,4-D + 2.5 µM TDZ as a control. Primary embryos were converted. Flow cytometry (2.7.) was performed on the first leaflets.

4.2.5. Induction of secondary somatic embryos

We inoculated 1000 'Speedy' anthers (20 per petri dish, 50 dishes) on BMS + 10 µM 2,4-D + 2.5 µM TDZ. The 544 anthers forming primary embryos after 6 weeks (Fig. 4.1) were divided into 4 groups of 136 explants (8 petridishes x 17 anthers/petridish). The first (control) group was transferred to BMS + 10 µM 2,4-D + 0.2 µM TDZ. The second group was transferred to the same medium complemented with 10 µM ORY and the third group to the same but enriched with 10 µM TRI. The last 136 explants were incubated in liquid medium + 100 µM COL for 16 h and subsequently transferred to BMS + 10 µM 2,4-D + 0.2 µM TDZ. Secondary embryos were converted and flow cytometrical analysis (2.7.) was performed.

4.3. Results

4.3.1. Adventitious shoot formation

In the shoot induction medium, clusters of tiny adventitious shoots were induced at the base of the main shoot. Toxicity effects of either treatment are presented in Table 4.1. The development of adventitious shoots (or in a later stage, their survival) was significantly negatively influenced by the presence of ORY or TRI. There was no significant difference between both products, but shoot formation decreased as the ORY- or TRI-concentration increased. A shock treatment with COL was neither advantageous nor detrimental to the formation of shootlets. Tetraploids or mixoploids were not observed.

Table 4.1. Shoot formation (/Meli-jar) on *Spathiphyllum wallisii* 'Daniel' explants after treatment with different mitosis inhibitors, on BMS + 10 µM BA + 15 µM IMA. Means followed by the same symbol are not statistically different (LSD 95%).

treatment	# shoots
control	80.4 cd
3 µM ORY	56.8 b
3 µM TRI	66.6 bc
10 µM ORY	38.4 a
10 µM TRI	36.4 a
100 µM COL after 1 week	87.6 d
100 µM COL after 4 weeks	72.2 bcd

4.3.2. Optimization of somatic embryogenesis

4.3.2.1. *Optimal NAA- and TDZ-concentration*

The filaments swelled and started to form somatic embryos at their base (Fig. 4.1). Results were scored after 6 weeks and are presented in Table 4.2. It is evident that TDZ and NAA were required to induce embryogenesis. Their presence and mutual interaction were statistically significant for embryo formation. The exact concentration of TDZ was not

critical; 10 and 15 µM NAA were significantly more beneficial for somatic embryogenesis than 5 µM NAA.

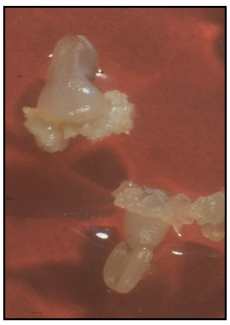


Figure 4.1. Somatic embryogenesis at *Spathiphyllum wallisii* 'Daniel' filament bases.

Table 4.2. Effect of NAA and TDZ on *Spathiphyllum wallisii* 'Daniel' anther filaments: total number of anthers (/800) forming se. Treatments indicated with the same symbol are not significantly different (LSD 95%).

	# anthers forming somatic embryos
0 µM NAA	0 a
5 µM NAA	293 b
10 µM NAA	370 c
15 µM NAA	358 c
0 µM TDZ	0 a
1 µM TDZ	357 b
3 µM TDZ	349 b
9 µM TDZ	315 b
NAA	*
TDZ	*
NAA x TDZ	*

*: significant at $P = 0.05$ using analysis of variance.

4.3.2.2. *Effect of developmental stage*

There was no significant interaction between inflorescence stage and anther position. Stage 1 anthers gave significantly more somatic embryos than anthers of stage 2 or stage 3 (Fig. 4.2.). Stage 2 anthers were at their turn significantly more sensitive for the formation of se than stage 3 anthers. The younger the anthers, the more sensitive their filament cells were for the induction of somatic embryos. As Fig. 4.3. shows, also the effect of the position of the anthers within the spadix cannot be neglected. The higher the position of the anthers within a spadix, the younger they are. This can be observed by the pollen stage (results not shown). Even within a single spadix, younger (less developed) anthers yielded significantly more somatic embryos.

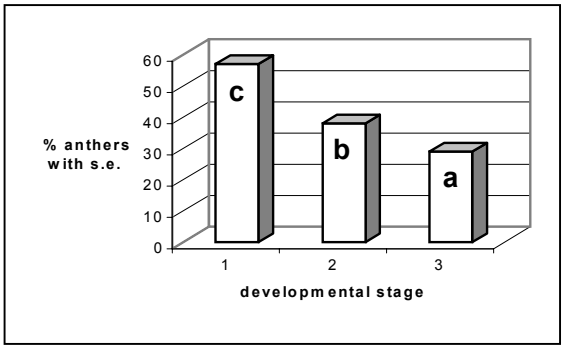


Figure 4.2. Effect of developmental stage of anthers on the formation of somatic embryos on *Spathiphyllum wallisii* 'Daniel' anther filaments, on BMS + 3 µM TDZ and 10 µM NAA. Means represented by the same letter are not significantly different (LSD 95 %).

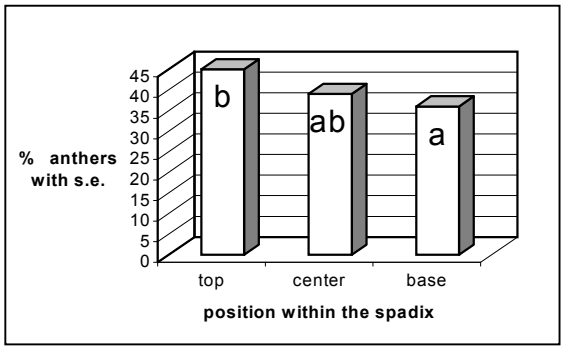


Figure 4.3. Effect of the anther position within the spadix on the formation of somatic embryos on *Spathiphyllum wallisii* 'Daniel' filaments, on BMS + 3 µM TDZ and 10 µM NAA. Means represented by the same letter are not significantly different (LSD 95%).

4.3.3. Tetraploidy induction during primary embryogenesis

Results are presented in Table 4.3. Primary embryos were only observed on somatic (diploid) filament tissue. The MIs significantly limited the number of filaments showing an embryogenic response, whatever the treatment. Especially COL was very toxic. TRI was significantly less toxic than the related compound ORY. TRI at 1 µM formed less embryos than at 3 or 10 µM but this was probably due to abundant embryogenesis on the reactive explants, depriving nutrients for other anthers in the same petri dish. The number of regenerated tetraploids was low: 1 for 3 µM and 10 µM TRI respectively, making the final efficiency of this technique 2 tetraploids/1000 anthers over all treatments.

4.3.4. Tetraploidy induction during secondary embryogenesis

After 6 weeks, the primary embryos yielded clusters of secondary embryos as visualized in Fig. 4.4., that could be easily converted after 8 weeks on BMS. As shown in Table 4.4 the addition of MIs influenced the number of plantlets that were regenerated, but the difference was not always significant, again COL was most toxic. TRI did not affect conversion in a negative way, and 5.5 % (8/146) of the plantlets were tetraploids. The ORY treatment was almost as effective for the production of tetraploids (4.7 % or 4 tetraploids out of 85 obtained plantlets), but yielded significantly less plantlets when compared to TRI. Apparently ORY is more toxic than TRI. The COL-treatment was the least efficient; only 2.5 % were tetraploid.

Table 4.3. Primary embryogenesis on *Spathiphyllum wallisii* 'Speedy' anthers on BMS + 10 µM 2,4-D + 2.5 µM TDZ, influenced by antimitotic agents. Results followed by the same symbol are not significantly different (LSD 95%).

Treatment	% anthers showing primary embryogenesis
Control	51 d
1 µM ORY	7 ab
3 µM ORY	8 ab
10 µM ORY	7 ab
30µM ORY	4 ab
1 µM TRI	7 ab
3 µM TRI	21 c
10 µM TRI	11 b
30 µM TRI	7 ab
100 µM COL, 4h	0 a
100 µM COL, 16h	0 a

1 cm

Figure 4.4. Clusters of *Spathiphyllum wallisii* 'Speedy' secondary embryos.

Table 4.4. Tetraploidy induction in *Spathiphyllum wallisii* 'Speedy' through exposure of primary embryos developed on anther filaments, to mitosis inhibitors ORY, TRI or COL on BMS + 10 µM 2,4-D + 2.5 µM TDZ. Results followed by the same symbol are not significantly different (LSD 95%).

	# anthers with primary embryos	# plantlets obtained	# tetraploids
Control	136	122 bc	0 a
10 µM ORY	136	85 ab	4 ab
10 µM TRI	136	146 c	8 b
100 µM COL	136	79 a	2 a

Tetraploid plants could easily be acclimatized. When correlating the results of the ploidy level determinations (Fig. 4.5.) with the morphology of the plants, it was obvious that most tetraploids could be visually selected by their round and thick leaves (Fig. 4.6.). Tetraploids were fertile and could be crossed with diploids (data not presented).

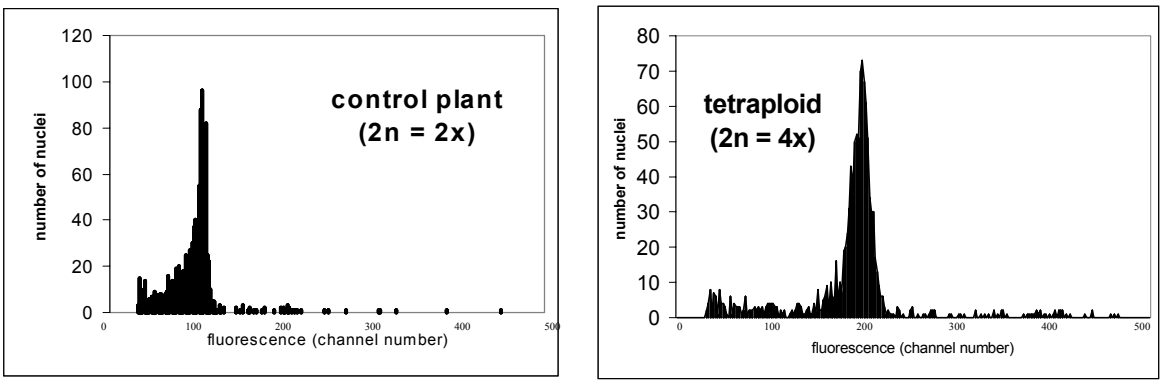


Figure 4.5. Flow cytometrical analysis of a diploid (left) and a tetraploid (right) *Spathiphyllum* wallisii 'Speedy' plant.

Figure 4.6. Morphology of diploid (left) and tetraploid (right) *Spathiphyllum wallisii* 'Speedy' leaves and flowers.

4.4. Discussion

Adventitious shoot formation, though being a suitable regeneration tool, does not appear to be as efficient as somatic embryogenesis for polyploidy induction (4.3.1). Growing shoots is very labor intensive and efficiency will probably never be sufficient; moreover, after eventual polyploidization we can expect mixoploidy to occur since many shootlets probably arose from multiple cells. Toxicity effects of 3 and 10 µM ORY or TRI remain limited (Table 4.1.), which is in accordance with observations of somatic embryogenesis on anther filaments. Evidently, cultivar differences prohibited a full comparison between both methods.

Since somatic embryos are able to emerge from one single cell, they can be used for several purposes. The type and concentration of cytokinins and auxins in the induction medium were not critical, though their combined presence was required for interaction with embryogenesis (Table 4.2.). The younger the anther, the more embryos were formed (Fig. 4.2; Fig. 4.3). The embryos could easily be converted into plantlets on BMS.

From our experiments it is evident that the regeneration capacity of primary embryos is strongly impeded by the presence of MIs, especially COL (Table 4.3.). As their concentration

increased, embryogenesis decreased, probably due to hampering of cell division, which is a logical consequence of the use of mitosis arresting compounds. As a result the induction of polyploid *Spathiphyllum wallisii* plants through secondary somatic embryogenesis was more performant (Table 4.4.). Secondary somatic embryogenesis is quite efficient, and the best treatments (using 10 µM ORY or TRI) yielded an average of 5% polyploid plants. Moreover, the technique is quite simple as the agent could be introduced into the culture medium and did not require shock therapy.

Tetraploids could be visually distinguished from diploids, but flow cytometry offered a valuable, rapid and more objective screening tool (Fig. 4.5.). The obtained tetraploid *Spathiphyllum wallisii* 'Speedy' plants showed an altered morphology compared to their diploid counterpart (Fig. 4.6.), though they did not exhibit an increased growth vigor; this may well be realized at triploid level, after crossing with a diploid. At the tetraploid level, a higher heterozygosity level (after crossing with other tetraploids) may be required to reveal the actual growth potential. Based upon morphological parameters, tetraploids have an intrinsic value on top of their possible application in breeding programs for the production of triploids. Small scale experiments (data not shown) have shown a very smooth growth of triploid embryos (after tetraploid x diploid crossings) *in vitro*; problems may occur *in vivo* due to the reduced formation of endosperm. Combined with the techniques described in Chapter 3, the production of triploid F_1 *Spathiphyllum wallisii* hybrids through seed is theoretically possible.

5. Creation of octoploid *Buddleja davidii* and interspecific *Buddleja* hybrids

5.1. Introduction

Rose *et al*. (2000b) already succeeded in manipulating the chromosome number of *B. globosa* in view of breeding experiments with the amphitetraploid *B. davidii*. They noticed considerable changes in morphology and sterility, including shorter internodes, broader, thicker and more crinkled leaves, greater frost susceptibility, elliptical inflorescences and poor anther development. The opportunities for the development of dwarf and/or sterile *B. davidii* hybrids are therefore evident. As reported by Moore (1947) chromosome numbers in this genus are already high; *B. globosa*, bearing the lowest chromosome number, is an amphidiploid with n = 7+12 (Darlington & Wylie, 1955).

B. x*weyeriana* has been created after crossing *B. globosa* x *B. davidii* (Van De Weyer, 1920) and is a commonly propagated *Buddleja* interspecific hybrid. *B.* x*weyeriana* has yellow flowers, but with a highly aberrant morphology compared to *B. davidii*. There are no reports on micropropagation of *B. davidii*, *B.* x*weyeriana* or *B. globosa*.

Our research to induce polyploidy in *Buddleja davidii* served 2 main targets: (i) to establish an efficient chromosome doubling procedure, allowing rapid multiplication of the (eventually) obtained polyploid genotypes, and (ii) to create genotypes with a different morphology, to increase the number of available cv's within the *B. davidii* assortment. Developing sterile genotypes to control undesired invasiveness and facilitating gene introgression by crossing parents with identical chromosome numbers may theoretically be supplementary reasons for polyploidization, but they were not considered in this work.

Through *in vitro* techniques we attempted to realize many *B. davidii* x *B.* x*weyeriana* backcrosses, to limit *B. globosa* gene introgression and to obtain a yellow-flowered hybrid with a *B. davidii*-like morphology. We tested the resulting seedlings through AFLP-analysis.

5.2. Specific materials and methods

5.2.1. Plant material

Buddleja davidii cv's 'Royal Red', 'Ile de France' and 'Nanho Purple' are tetraploids, whereas *B.* x*weyeriana* 'Sungold' is assumed to be triploid (preliminary measurements, unpublished). *B.d.* cv's originated from intraspecific crosses. 'Sungold' is a mutant which originated from an F_2-generation, after an interspecific *B. globosa* x *B. davidii* cross (Van De Weyer, 1920). Nursery practices were as described in 2.1. Emasculation was performed upon opening of the flowers by removing the petals (with attached stamina) and pollinated in an insect free greenhouse. Only fresh pollen, collected from fully opened flowers, was used for pollination.

5.2.2. Polyploidy induction

Embryos from freely pollinated 'Royal Red', 'Ile de France' and 'Sungold' flowers were dissected from sterilized (as described in 2.1) mature seeds and put on BMB (2.2). Seedlings were treated when cotyledons had emerged, as described in 2.3. We applied 5 µl of 3 mM and 0.3 mM oryzalin (ORY) or trifluralin (TRI) solutions between the cotyledons during 3 consecutive days. Per treatment 120 ('Royal Red'), 60 ('Ile de France') and 30 ('Sungold') seeds were sown (10/petri dish). The ploidy level of surviving plantlets was measured by flow cytometry (2.7). Afterwards, plants were acclimatized in the greenhouse as described in 2.1.

5.2.3. Interspecific hybridization

In our experiments, the most widespread *B.* x*weyeriana*, 'Sungold', was combined with *B. davidii* 'Nanho Purple', 'Royal Red' and 'Ile de France' (producing the largest amount of pollen). Before pollination, the seed parents were emasculated. Altogether, 344 *B. davidii* x *B.* x*weyeriana* and 354 *B.* x*weyeriana* x *B. davidii* pollinations were performed. Prefertilization barriers were characterized as described in 2.4. Immature seeds were germinated *in vitro* 6 weeks after pollination on BMB without growth regulators and subcultured every 5 weeks. Four AFLP primer combinations (chosen randomly) were tested as described in 2.8. and 2.9.: E-ACA + M-CTG, E-ACT + M-CAT, E-AGG + M-CTT and E-ACG + M-CAA. Flow cytometrical measurements of the assumed hybrids (as in 2.7.) were performed in 5 replicates allowing statistical analysis (2.11.). Morphological parameters were determined as in 2.6.; an official color chart (Royal Horticultural Society, 1996) was followed for petal color determination.

5.3. Results

5.3.1. Polyploidy induction

The highest TRI and ORY concentration had a toxic effect on seedling survival (Table 5.1). TRI at 0.3 mM was more toxic than ORY at 0.3 mM (39% versus 61% survival). 'Sungold' seedlings only survived after 0.3 mM ORY treatment (18/30). Cultivar effects nor interactions with MI or concentration are significant. The exact MI applied is less significant than its concentration.

Upon flow cytometry, mixoploids showed 2 peaks: at the original and at the doubled level. Table 5.1 shows that the number of mixoploids was not cultivar dependent, other parameters interfered. Double and triple interactions were significant for the yield of mixoploids. When used at 0.3 mM, ORY induced most mixoploids (12.4% over all cv's).

Both ORY and TRI could be used in a very efficient way to produce PADs (plants with artificially doubled chromosome number that are non-mixoploid) from *B. davidii* and *B.* x*weyeriana* genotypes, starting from mature seed. ORY yielded most PADs, especially when used at 0.3 mM (69 PADs/128 survivors). The number of PADs was cultivar, chemical and concentration dependent (Table 5.1.); moreover, there was a significant interaction between all pairs of parameters, although the triple interaction was not significant for polyploidy

induction. ‘Royal Red’ seedlings appeared to be more amenable to polyploidy induction than the other seedlings.

The number of mixoploids obtained was smaller than the number of PADs, except for ‘Sungold’. For these seedlings, the application of 0.3 mM ORY was the only successful treatment. Flow cytometrical control after complete acclimatization revealed that mixoploidy was not persistent in ‘Sungold’: 1 plant had fully doubled its DNA-content, whereas the remainder had reverted towards the original status. As opposed to ‘Sungold’, mixoploid *B. davidii* hybrids had retained both ploidy levels after full acclimatization. Only 5 ‘Royal Red’ and 2 ‘Sungold’ PADs survived, which was a too small number for thorough morphological comparisons; 1 ‘Sungold’ PAD displayed white petal colors whereas flowers of all other plants were purple to violet (RHS colors 80-82) or violet (RHS colors 87-88). Immediately after acclimatization, the rounder leaf of some plantlets was obvious (Fig. 5.1.), as were the shorter internodes of others; nevertheless, not every mixoploid or PAD could be visually distinguished from their respective progenitors. Indeed, a broad (L/B ± 2) as well as a narrow (L/B ± 4) leaf type were observed on different genotypes, but the broad leaf type was not always persistent after transplant to soil.

Table 5.1. Survival rate and yield of mixoploids (2x/4x) and PAD in seedlings of 3 *Buddleja* cv’s after application of different mitosis inhibitors (MI) at different concentrations as determined by flow cytometry. Means followed by the same symbol are not statistically different (LSD 95%).

	survival rate (% of total # seedlings)	**mixoploids (% of total # seedlings)**	**PADs (% of total # seedlings)**
‘Royal Red’	29.4	3.8	11.9 b
‘Ile de France’	30.0	3.8	5.8 a
‘Sungold’	15.0	8.4	3.4 a
oryzalin	33.3 b	7.1 b	16.2 b
trifluralin	21.9 a	1.4 a	1.4 a
3 mM	5.2 a	1.4 a	1.9 a
0.3 mM	50.0 b	7.1 b	15.7 b
cultivar	NS	NS	**
MI	**	***	***
concentration	***	***	***
cultivar x MI	NS	*	**
cultivar x concentration	NS	*	***
MI x concentration	**	***	***
cultivar x MI x concentration	NS	**	NS

NS, *, **, *** : not significant or significant difference at P = 0.05, 0.01 or 0.001 respectively.

5.3.2. Interspecific hybridization

In neither of the pollinated pistils pollen tube growth was hampered. Both *B. davidii* ‘Ile de France’ and ‘Royal Red’ pollen tubes reached *B.* x*weyeriana* ‘Sungold’ ovules; the reverse

cross was impeded neither. Table 5.2 shows that only one seed parent, 'Royal Red', was appropriate for a backcross with *B.* x*weyeriana*. No embryos could be harvested after an 'Ile de France' x 'Sungold' pollination, and 'Nanho Purple' x 'Sungold' embryos were not viable.

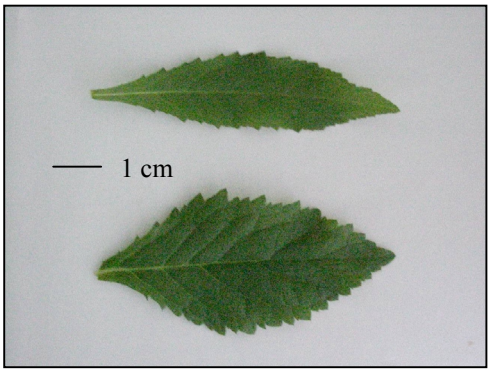


Figure 5.1. Typical tetraploid *Buddleja davidii* 'Royal Red' leaf (up) versus an octoploid leaf (down).

Table 5.2. Embryos and seedlings rescued after *B. davidii* x *B.* x*weyeriana* and *B.* x*weyeriana* x *B. davidii* pollination.

Seed parent	Pollen parent	# pollinated flowers	# embryos	# acclimatized plants
'Royal Red'	'Sungold'	69	118	80
'Nanho Purple'	'Sungold'	141	15	0
'Ile de France'	'Sungold'	134	0	0
'Sungold'	'Royal Red'	72	0	0
'Sungold'	'Nanho Purple'	168	16	7
'Sungold'	'Ile de France'	114	0	0

Plants were transferred to soil and grew vigorously. Albinism and hybrid vigor were not observed. Leaf type and growth habit of the hybrids were matromorph. Apart from 2 genotypes with RHS color 87C, 'Royal Red' x 'Sungold' seedlings all had purple/violet petals (RHS colors 80-82), whereas 'Sungold' x 'Nanho Purple' seedlings displayed paler and violet flower colors (RHS colors 87-88). (Fig. 5.2.). The latter combination clearly produced less pollen. Pollen size was homogeneous in every hybrid observed.

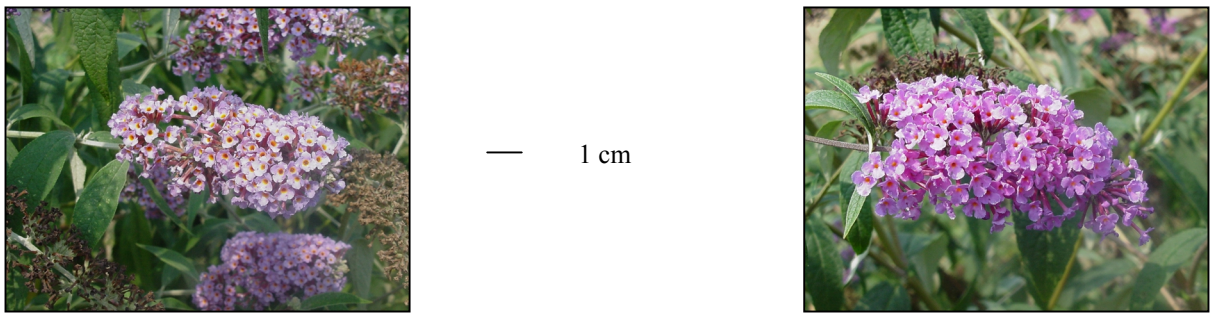


Figure 5.2. *B.w.* 'Sungold' x *B.d.* 'Nanho Purple' (left) and *B.d.* 'Royal Red' x *B.w.* 'Sungold' (right).

Two 'Royal Red' x 'Sungold' and 2 'Sungold' x 'Nanho Purple' seedlings were subjected to AFLP-analysis along with their assumed parent plants. Primer combinations E-ACA + M-CTG and E-ACG + M-CAA yielded most polymorphisms. By calculating the pair wise genetic similarities, using as well the Jaccard as the Simple Matching similarity coefficients,

the genetic conformity between the parent plants and the hybrids was determined (Table 5.3), and are represented in a dendrogram (Fig. 5.3).

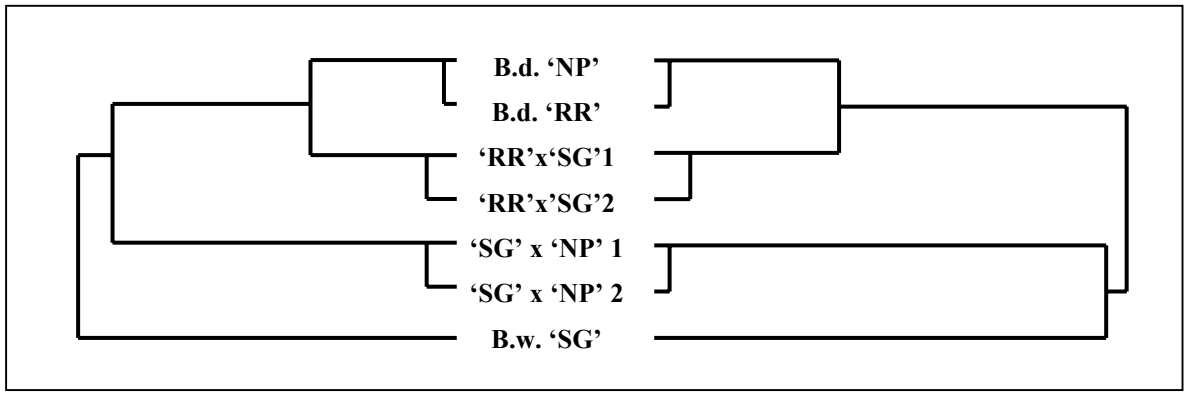


Figure 5.3. Dendrogram showing the genetic relatedness of interspecific *Buddleja* hybrids and their parents tested by AFLP. The dendrogram was constructed using UPGMA-clustering based on a genetic similarity matrix containing the Jaccard (left) and Simple Matching (right) similarity coefficients between pairs of plants.

Both coefficients resulted in very similar proximity matrices as shown in Table 5.3. Nevertheless, the Jaccard-based dendrogram implicates that 'SG' x 'NP' seedlings are more related to the pollen parent than to the seed parent, whereas the Simple Matching-based dendrogram indicates the reverse (Fig. 5.3.). Similarities between seed parent and progeny were smaller after pollination of *B.* x*weyeriana* than after pollination of *B. davidii*.

Table 5.3. Proximity matrices representing pair wise Jaccard and Simple Matching similarity coefficients between *Buddleja* cvs and their alleged hybrids, obtained through AFLP-analysis ('NP' = *B.d.* 'Nanho Purple', 'RR' = *B.d.* 'Royal Red', 'SG' = *B.w.* 'Sungold').

genotype	*B.d.* 'NP'	*B.d.* 'RR'	'RR' x 'SG' 1	'RR' x 'SG' 2	*B.w.* 'SG'	'SG' x 'NP' 1	'SG' x 'NP' 2
Jaccard							
B.d. 'NP'	1.000	.961	.892	.873	.766	.802	.792
B.d. 'RR'	.961	1.000	.885	.879	.748	.795	.785
'RR'x'SG'1	.892	.885	1.000	.946	.748	.763	.747
'RR'x'SG'2	.873	.879	.946	1.000	.724	.750	.734
B.w. 'SG'	.766	.748	.748	.724	1.000	.759	.760
'SG' x 'NP' 1	.802	.795	.763	.750	.759	1.000	.952
'SG' x 'NP' 2	.792	.785	.747	.734	.760	.952	1.000
Simple Matching							
B.d. 'NP'	1.000	.962	.893	.874	.767	.802	.792
B.d. 'RR'	.962	1.000	.887	.881	.748	.796	.786
'RR'x'SG'1	.893	.887	1.000	.950	.755	.764	.748
'RR'x'SG'2	.874	.881	.950	1.000	.730	.752	.736
B.w. 'SG'	.767	.748	.755	.730	1.000	.777	.780
'SG' x 'NP' 1	.802	.796	.764	.752	.777	1.000	.959
'SG' x 'NP' 2	.792	.786	.748	.736	.780	.959	1.000

Flow cytometrical control was performed on seed and pollen parents, both hybrid groups and *Buddleja globosa*. It revealed that *B.* x*weyeriana* 'Sungold' appeared pentaploid. When included in the same sample, 'Sungold' and 'Nanho Purple' were responsible for the appearance of (most often) clearly separated peaks. This was noticed as well with as without the use of tetraploid *Lolium perenne* 'Merlinda' as an internal standard (Table 5.4; Fig. 5.4.).

Table 5.4. SPF/GPF (*100) ratios after flow cytometrical analysis of *B. davidii* 'Nanho Purple', *B.* x*weyeriana* 'Sungold', their hybrids and *B. globosa*. Means indicated by the same symbol are not significantly different (LSD 95%) (* indicates mixed sample 'Nanho Purple' + 'Sungold').

sample	SPF/GPF x 100	
'Nanho Purple'	41,76	c
'Sungold'	52,49	f
'Nanho Purple' *	39,80	b
'Sungold' *	50,77	e
'Royal Red' x 'Sungold' hybrids	42,81	c
'Sungold' x 'Nanho Purple' hybrids	48,08	d
B. globosa	31.16	a

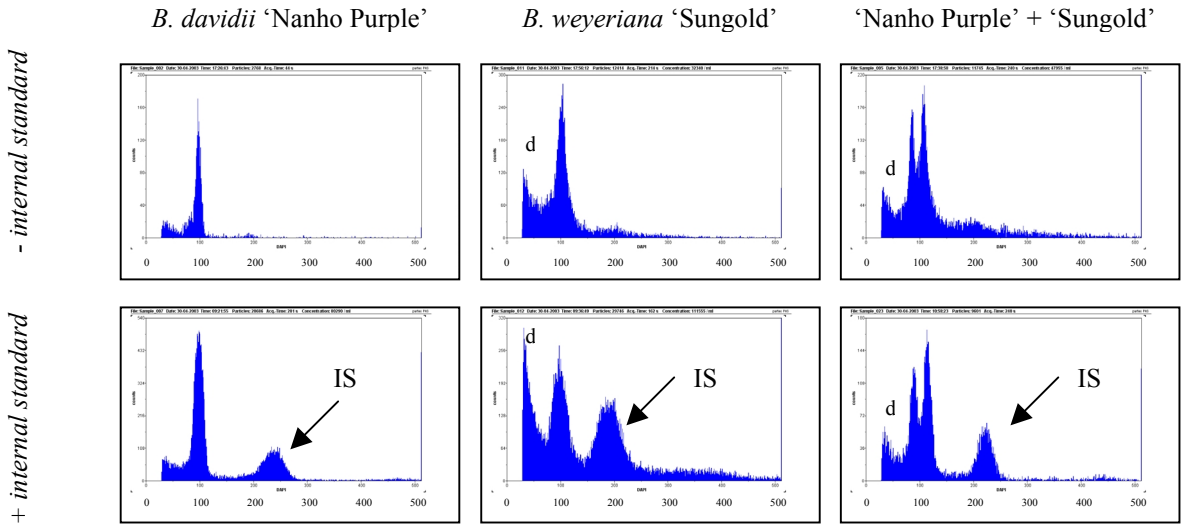


Figure 5.4. Flow cytometrical patterns of *B. davidii* 'Nanho Purple', *B.* x*weyeriana* 'Sungold' and a mixed sample, without and with the application of an internal *Lolium* standard (IS, indicated by arrow). Peaks on the extreme left are caused by debris fluorescence (d). X-axis: fluorescence (channel number); Y-axis: number of nuclei.

Significant differences could evidently better be observed when an internal standard was applied, reducing the standard deviation. The difference between both species was in that case very obvious. Intriguingly, the *B. davidii* peak shifted towards the left when measured together with 'Sungold', causing the separated peak pattern. When measured with an internal grass standard, 'Sungold' also remained at its original position but caused a shift of the grass peak to the left, causing a higher sample peak fluorescence (SPF)/grass peak fluorescence (GPF) ratio compared to *B. davidii* 'Nanho Purple'. The mean SPF/GPF value of the acclimatized hybrids (7 'Sungold' x 'Nanho Purple' and 80 'Royal Red' x 'Sungold' seedlings) is also represented in Table 5.4, showing it was only significantly different from

the maternal genotype in 'Sungold' x 'Nanho Purple'. Without formulating immediate conclusions regarding the cause of these ploidy differences, it is evident that 'Sungold' is not a triploid and has a DNA-content even slightly higher than the tetraploid *B. davidii* 'Nanho Purple'.

5.4. Discussion

Buddleja x*weyeriana*, an interspecific hybrid of *B. globosa* x *B. davidii* (Van De Weyer, 1920), is a model system for studying the behavior of very recently developed interspecific hybrids upon backcross and polyploidization experiments. At the same time know-how can be developed for the creation of synthetic interspecific and/or polyploid (and possibly sterile) *Buddleja* genotypes for horticultural use.

We compared our results to those obtained by Rose *et al*. (2000b) on tetraploidy induction in nodal sections of *B. globosa* with COL. Regarding the number of octoploids obtained as well as the relatively low number of mixoploids, polyploidization of *B. davidii* seedlings by ORY or TRI is as efficient (Table 5.1.). Mixoploids are possibly periclinal cytochimeras (1 or 2 of the 3 histogenic layers are fully polyploid). If the LII layer is tetraploid, a chimera will develop diploid gametes (Tilney-Basset, 1986). Although the purpose of this experiment was the induction of plants with a fully doubled chromosome set, the number of mixoploids obtained should also be considered. Indeed, they as well are an indication for the efficiency of the MIs.

Rose *et al*. (2000b) attempted polyploidization on nodal explants, which offers an advantage over seedlings as they represent 1 single genome. However, applying COL on nodal explants was not as efficient as our ORY- or TRI-treatments of seedlings. Moreover, the survival rate of the nodal explants was lower and the procedure looks more labor intensive. The absolute number of PADs obtained is very high, the best treatment resulting in ± 50% survivors that were solid octoploids (Table 5.1.). Comparable success rates on other genera could not be retrieved from literature.

Not every PAD or mixoploid has an altered morphology; some exhibit dwarf growth or aberrant leaf characteristics (Fig. 5.1). Apparently, morphological consequences of chromosome doubling in *B. davidii* are similar, but more limited, than after polyploidization of *B. globosa* (Rose *et al.*, 2000). This might be due to the higher ploidy level of *B. davidii*, which has twice the chromosome number of *B. globosa*.

The backcross *B.* x*weyeriana* x *B. davidii* can be achieved through *in vitro* embryo rescue. Since Van De Weyer (1920) reported that yellow flower color is a recessive trait, occurring only in the second generation of a *B. globosa* x *B. davidii* cross, the creation of an F_2 might be required to obtain yellow flowering hybrids. Prezygotic barriers were not observed, as expected, since *B.* x*weyeriana* 'Sungold' is an interspecific hybrid itself, descending from *B. davidii*. However, as a result of a reduced congruity, only 2 out of 6 parent combinations yielded viable F_1 seedlings (Table 5.2.). Flowers of both hybrid groups were mutually different (Fig. 5.2.). Reverse crosses were unsuccessful. Albinism and reduced vigor were absent and *in vitro* germination was not problematic. The number of polymorphisms that

could be detected through AFLP was limited since *B.* x*weyeriana* and *B. davidii* are closely related. The analysis clearly proved that no seedling resulted from self-fertilization; all were the result of an interspecific cross (Fig. 5.3; Table 5.3.). Seedlings of the same cross were mutually very similar, as are both *B. davidii* cultivars, whereas the genetic distance between seed parent and offspring is substantially larger.

Ploidy screening yielded intriguing results, shedding a new light upon the origin and genotype of *B.* x*weyeriana* 'Sungold', originated from a *B. globosa* x *B. davidii* F_2 generation. First of all, it appears to be not a triploid. Its position on a flow cytometrical histogram is not different from the position of the tetraploid *B. davidii* 'Nanho Purple', whose ploidy level in turn is identical to other *B. davidii* genotypes 'Nanhoensis', 'Pink Delight', 'Nanho Blue', 'Ile de France' and 'Royal Red' (data not presented). Therefore 'Sungold' can be assumed to be a tetraploid. This is contradictory to the assumption that this hybrid has a nuclear DNA-content intermediate to its parents *B. globosa* and *B. davidii*.

When included in a mixed sample for flow cytometry analysis (with 'Nanho Purple' or an internal grass standard), 'Sungold' peaks kept their place while other peaks shifted to the left (compared to control samples) (Fig. 5.4.). Hybrids showed a DNA-level reasonably resembling that of the seed parent, though a minor shift towards more intermediate levels could be detected, especially in 'Sungold' x 'Nanho Purple' hybrids (Table 5.4.). Three main hypotheses can be deducted from these findings:

i) Regarding the higher c-value of the *globosa* chromosome set (Table 5.4) 'Sungold' possibly is a (near-?)tetraploid (2n = 4x = GGDD, with G representing a *B. globosa* chromosome set and D a *B. davidii* set). In that case it would have originated either after selfing, from diploid or near-diploid gametes (GD) produced by the triploid F_1 ancestor (2n = 3x = GDD), either after fusion of an unreduced gamete of the F_1 ancestor and a normal *globosa* gamete (G) after backcrossing, possibly involving homoeologous recombination between G and D genomes. 'Sungold' in both cases is tetraploid (GGDD), but would appear pentaploid compared to the tetraploid *B.* x*weyeriana* because the c-value of 2 G chromosome sets more or less matches the c-value of 3 D chromosome sets (Table 5.4.).

The formation of diploid gametes by triploids is far from unusual (Otto & Whitton, 2000). Although they were not observed in *Buddleja* so far, unreduced gametes occur in most plant species (Harlan & de Wet, 1975, Veilleux, 1985). Moreover, FDR, SDR and IMR gametes allow homoeologous recombination between different genomes present in the F_1 (Mendiburu & Peloquin, 1977; Karlov *et al*., 1999; Lim *et al*., 2003). Unreduced gametes also occur in doubled haploids and interspecific hybrids (Crespel *et al*., 2002; Lim *et al*., 2003), possibly owing to negative selection of nonfunctional 'normal' meiotic products. At least in autotriploids there is evidence for the production of triploid gametes (Brandham, 1982); though systematical reviews for allotriploids seem to be lacking, there are several examples among crop plants in which allotriploids have been successfully used as parents and the resulting progenies have been used in backcrossing programs (Lim *et al*., 2003). Especially in banana and plantain balanced triploid gametes occur in high frequencies (Shepherd, 1999).

However, this theory does not satisfactory explain the c-value difference between both groups of interspecific hybrids (theoretically, all having genotype GDDD). A possible explanation for this anomaly is the interference of secondary metabolites (Dolezel, 1991),

whose synthesis would be maternally inherited, causing a shift of 'Sungold' and 'Sungold' x 'Nanho Purple' ploidy patterns towards higher levels, or alternatively, a shift towards lower ploidy levels of all other genomes included in the same sample, as was observed (Fig. 5.4.).

ii) 'Sungold' is a pentaploid (2n = 5x = GGDDD). The original F_1, that was selfed to obtain the 'Sungold' ancestor, was triploid (2n = 3x = GDD). Pentaploidy could have arisen after the formation of unreduced gametes GDD, that fused with diploid gametes GD after selfing to result in a zygote GGDDD. Homoeologous recombination, rapid chromosome translocation, loss of DNA-sequences, karyotypic stabilizing, epigenetic effects and/or the loss of sterility genes allowed the loss of sterility of the original F_2 leading to the 'mutant' 'Sungold'. 'Sungold' would then produce gametes with unequal chromosome numbers, GDD egg cells and GD pollen. Adapted forms of meiosis resulting in unequal chromosome number between macrospores and microspores have been described in few reproductive systems: *Rosa canina* (Täckholm, 1920), *Leucopogon juniperus* (Smith-White, 1955), *Andropogon ternatus* (Normann & Quarin, 1987), all of which form univalents as well as bivalents during meiosis.

Still following this hypothesis, 'Sungold' x *B. davidii* and *B. davidii* x 'Sungold' pollinations would result in GDDDD and GDDD offspring, respectively. This would explain the ploidy difference between both groups of seedlings. It would also account for the minor ploidy shift between 'Sungold' (GGDDD) and 'Sungold' x 'Nanho Purple' (GDDDD) on the one hand and for the difference between 'Royal Red' (DDDD) and 'Royal Red' x 'Sungold' (GDDD) on the other hand, taking into consideration that the *globosa* genome is slightly larger than that of *davidii* (Table 5.4.).

Criticism on this hypothesis may include the occurrence of the 'Sungold' peak at a rather tetraploid level in ploidy histograms and the relative unlikeliness of the rapid establishment of a meiotic system resulting in gametes with different ploidy levels. However, fertility restoring genome alterations in interspecific hybrids are known to be a bottleneck which is fairly quickly overcome in many genera (Parokonny & Kenton, 1995; Song, 1995; Leitch & Bennett, 1997, Ozkan *et al*., 2001). Therefore it is not improbable it should have occurred in the pentaploid 'Sungold' ancestor. In addition, at this point the system still seems to be highly inefficient when considering the genotype dependent efficiency of the crosses that were performed and may therefore be considered as 'still evolving'.

iii) Considering the apparent lack of 2n gamete formation in *Buddleja* on other ploidy levels, another option is that 'Sungold' does not dispose over a special meiotic system, but instead produces gametes ranging from diploid to triploid. A strong selection is made on the seed parent, allowing either preferential fertilization either preferential further development of seeds leading to an appropriate embryo/endosperm ploidy ratio; this is comparable to observations in *Lilium* hybrids (Lim *et al*., 2003). 'Sungold' x 'Nanho Purple' would result in ratios varying from (2+2)/(4+2) = 1/1.5 to (3+2)/(6+2) = 1/1.6 and 'Royal Red' x 'Sungold' would in turn create ratios from (2+2)/(4+2) = 1/1.5 to (2+3)/(4+3) = 1/1.4. In both cases there would be a tendency towards the lowest ratio. However, differences between ratios remain limited so one can wonder whether they are able to induce a selection as strong as proposed in this model. Theoretically, different pollen ploidy levels could be associated with different pollen sizes, but only one pollen type was found per plant.

Based on the available data, the exact ancestry and genotype of 'Sungold' can not be fully elucidated. However, intriguing results have been revealed; 'Sungold' is not triploid, is not sterile and can be backcrossed to *B. davidii* in both directions using embryo rescue, though efficiency remains low. We suspect that in the hybrids studied, chromosome counting and GISH analysis may be required to respectively reveal the exact chromosome number and genome rearrangements. Because of its hybridizing history, its allopolyploid nature and its vigorous growth the genus *Buddleja* offers an intriguing model system for allopolyploid evolution, despite its high chromosome numbers.

6. Interspecific crosses in the genus *Rhododendron*

6.1. Introduction

Interspecific breeding experience with Tsutsusi hybrids has mainly been gathered by Preil & Ebbinghaus (1985) and Rouse (1993). Rouse concentrated on Vireya crosses whereas Preil & Ebbinghaus used genotypes from either subgenus in unilateral crosses. Neither was successful in obtaining yellow flowering hybrids. Ureshino *et al*. (1998) succeeded in obtaining yellow hybrids after three-way Tsutsusi x Pentanthera crosses, but all seedlings turned out to be (semi)deciduous and not vigorous. Pigment biosynthesis and its inheritance within *R. simsii* hybrids have mainly been described by Heursel (1987; 1999) and De Loose (1969; 1970). In earlier crossing experiments with pot azalea, especially *R. keiskei* Miq., *R. lutescens* Franch. and *R. burmanicum* Hutch. were used as breeding partners (Longly, 1994).

This chapter reports the results of interspecific pollinations made within the genus *Rhododendron* between yellow, orange or blue flowering botanical species or hybrids from the Hymenanthes, Pentanthera or Rhododendron (including Vireya) subgenera and Tsutsusi (mainly *R. simsii*) hybrids. The objective of this series of experiments was to select possible parent plants for the breeding of pot azaleas with new petal colors (yellow, orange or blue). *Rhododendron* is a very large genus and taxonomic classifications are not at all settled. We selected a limited number of genotypes for our breeding experiments.

Taking into account all possible barriers to successful hybridization, a stepwise experimental set-up was established. Preceding actual pollinations, ploidy screening was performed on all genotypes. 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). Different species are polyploid; Pentanthera species are diploid, except for the tetraploids *R. calendulaceum* Torrey and *R. canadense* Torrey. Our research included flow cytometrical control of the ploidy level of hardy Ghent and Rustica hybrids and their presumable ancestors (when available).

Pollen tube staining was performed to detect possible prefertilization barriers, which apparently inhibit interspecific *Rhododendron* hybridization (Rouse, 1993; Ureshino, 2000); in a follow-up experiment we tried to overcome these barriers. Ovule culture was performed to prevent possible abortion and to promote germination. Therefore we optimized the germination medium for immature *Rhododendron* seeds. This part of our research was based on similar work by Preil & Ebbinghaus (1985) and Longly (1994). The efficiency of ovule culture was compared to *in vivo* sowing. Finally, both SSR-and AFLP-markers were used to characterize the exact descent of the seedlings and to select the best suitable molecular screening tool.

6.2. Specific materials and methods

6.2.1. Plant material

Different pot azalea cultivars and seedlings were used (Table 6.1.) along with 1 (tetraploid) *R. obtusum* Planch. hybrid, 1 *R. simsii* x *R. noriakianum* T. Suzuki hybrid, 1 *R. kaempferi* Wils.

x *R. simsii* hybrid and 2 triploid *R. simsii* x *R. obtusum* hybrids. Figs. 6.1.A. and 6.1.B. provide an overview of the 19 wild species and 19 cultivated hybrids used in our experiments. The ancestry of the Hymenanthes, Pentanthera and Rhododendron hybrids in the breeding program is given in Table 6.2. Yellow or orange flowering genotypes from Fig 6.1.B were crossed with white pot azaleas; purple pot azaleas were combined with blue or lavender flowering plants. General nursery practices were as in 2.1.

All Tsutsusi genotypes were present in the CLO-DvP collection; Pentanthera genotypes were either present in the CLO-DvP collection or were gathered from a private garden (except *R. luteum* leaf, flower and root material that was collected from 3 independent sources); Rhododendron, Vireya and Hymenanthes plants were bought from commercial nurseries.

6.2.2. Ploidy screening

6.2.2.1. *General screening*

Ploidy screening was performed on all Hymenanthes, Pentanthera and Rhododendron genotypes used as breeding partners for pot azalea, as described in 2.7. A diploid *R. simsii* hybrid was used as a standard.

6.2.2.2. *Pentanthera species*

R. calendulaceum Torrey leaf material was used in control samples. Species collected for ploidy measurements included *R. luteum* Sweet*, R. prinophyllum* Millais*, R. viscosum* Torrey*, R. occidentalis* Gray*, R. canescens* Sweet and *R. periclymenoides* Shinners. Besides leaves, anther filaments and root material were used to detect possible (periclinal) chimerism.

6.2.2.3. *Pentanthera hybrids*

Nuclear suspensions from leaf material of hardy Ghents 'Nancy Waterer', 'Unique', 'Narcissiflora', '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 measured.

Peak shifts were corrected by adding an internal standard (a diploid *Lolium multiflorum* 'Bellem' nuclear suspension, that displays high-quality peaks suitable as a reference) to the samples. Each plant was measured until 2 unequivocal results were obtained.

6.2.3. Pollinations

Seed parents were emasculated by removal of the anthers, mostly upon flower opening. The risk of self-pollination appeared high in *R. valentinianum*, *R.* 'Goldsworth Orange', *R.* 'Hillier's form' (high pollen production) and *R.* 'Jingle Bells' (cleistogamy), which were therefore emasculated before the opening of the flowers. Whenever possible, every genotype

was used both as seed and as pollen parent. Also *R.* 'Jingle Bells' and *R.* 'Nancy Evans', exhibiting 'hose-in-hose' flower types (colored sepals) were used as seed parents.

Subgenus Hymenanthes (Hy)

Section Ponticum

Subsection Campylocarpa

R. wardii Smith (10D), 'Nancy Evans' ° (10C)

Subsection Neriiflora

R. dichroanthum Diels subsp. *scyphocalyx* (Balf. & Forrest) Cowan *, *R. citriniflorum* Balf. *, *R. sanguineum* Franch. *haemalum* (Balf. & Forrest) Chamb. *, 'Goldsworth Orange' (13C), 'Jingle Bells' (16B middle, 39B margin), 'Kupferberg' (23C)

Subsection Thomsonia

'Viscy' (12D)

Subgenus Pentanthera (Pe)

Section Pentanthera

Subsection Pentanthera

R luteum Sweet (15A), 'Hollandia' (12C), 'Nancy Waterer' (15A), 'Unique' (33C)

Subgenus Rhododendron

Section Rhododendron (Rh)

Subsection Boothia

R. chrysodoron Tagg ex Hutch. (3A), *R. megeratum* Balf. & Forrest (7A), *R. sulfureum* Franch. (5A)

Subsection Cinnabarina

R. cinnabarinum Hook. subsp. *xanthocodon* (Hutch.) Cullen *, *R. cinnabarinum* Hook subsp. *concatenans* Hutch. *

Subsection Lapponica

'Azurwolke' (90C), 'Blaufeder' (83B), 'Habashan' (86C), 'Hillier's Form' (85B), 'Yellow Hammer' (8B)

Subsection Maddenia

R. leptocladon Dop (5D), *R. burmanicum* Hutch. (4B) °, *R. valentinianum* Hutch. (13A)

Subsection Tephropepla

R. xanthostephanum Merr. (5C), 'Euan Cox' ° (3C)

Subsection Triflora

R. lutescens Franch. *, 'Shamrock' ° (4D), 'Golden Bee' ° (3B)

Subsection Uniflora

'Curlew' (5C), 'Chikor'° (4D)

Section Vireya (Vi)

Subsection Pseudovireya

R. kawakamii Hayata (15C)

Subsection Euvireya

R. laetum Smith (15A), *R. macgregoriae* F.Muell. (15B), *R. aequabile* Blume *, 'Sunny' (13B middle, 29B margin)

Figure 6.1.A. Wild species and cultivated hybrids from the Hymenanthes, Pentanthera and Rhododendron subgenera used in our breeding program with pot azalea (° no pollen available, * only pollen available). Petal colors are indicated between brackets following the Royal Horticultural Society Color Chart (1966).

R. wardii

'Nancy Evans'

R. dichroanthum scyphocalyx

'Goldsworth Orange'

'Jingle Bells'

'Kupferberg'

R. citriniflorum

R. sanguineum haemalum

'Viscy'

R. luteum

'Hollandia'

'Nancy Waterer'

'Unique'

R. chrysodoron

R. megeratum

R. sulfureum

R. cinnabarinum xanthocodon

R. cinnabarinum concatenans

R. leptocladon

R. burmanicum

R. valentinianum

'Azurwolke'

'Blaufeder'

'Habashan'

'Hillier's Form'

'Yellow Hammer'

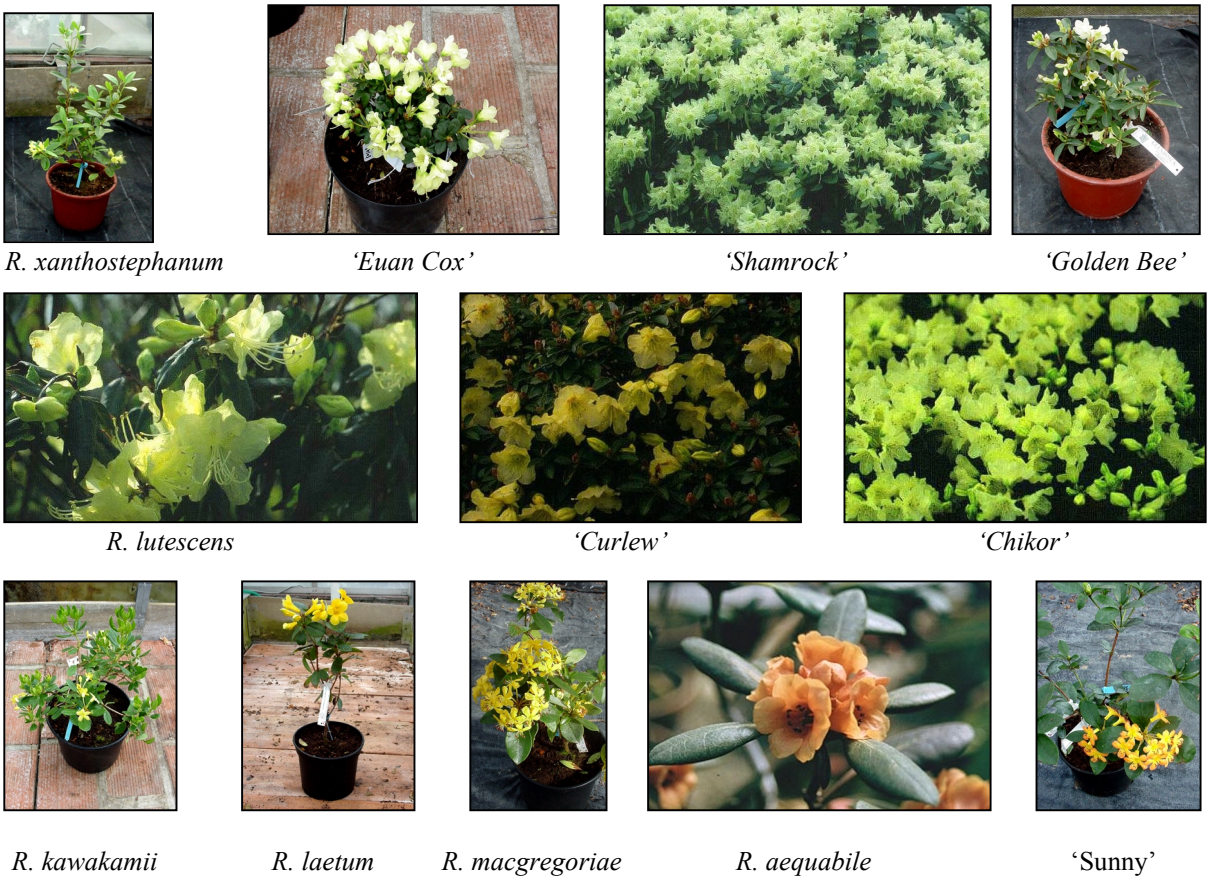


Figure 6.1.B. Wild species and cultivated hybrids from the Hymenanthes, Pentanthera and Rhododendron subgenera used in our breeding program with pot azalea.

Table 6.1. Tsutsusi genotypes (Ts) used in our breeding program.

Abbreviation	Tsutsusi genotype	Remarks
CV1	'Dogwood'	*R. simsii* hybrid, 2n = 2x, white
CV2	'Dame Blanche'	*R. simsii* hybrid, 2n = 2x, white
CV3	'Mevrouw Gerard Kint Wit'	*R. simsii* hybrid, 2n = 2x, white
CV4	'Mont Blanc'	*R. simsii* hybrid, 2n = 2x, white
CV5	'Gerda Keessen' (petal margin regenerant)	*R. simsii* hybrid, 2n = 4x, white
CV6	'Mevrouw Jozef Heursel'	*R. simsii* hybrid, 2n = 2x, purple
CV7	'Starlight' (petal margin regenerant)	*R. simsii* hybrid, 2n = 4x, white
CV8	'Aline'	*R. simsii* hybrid, 2n = 2x, white
CV9	'Casablanca tetra'	*R. obtusum* hybrid, 2n = 4x, white
SL1	'Firmin De Waele' x 'Dame Blanche'	*R. simsii* hybrid, 2n = 2x, white
SL2	'Mevrouw Andre Heungens' x 'Mistral' (1)	*R. simsii* hybrid, 2n = 2x, white
SL3	'Mevrouw Andre Heungens' x 'Mistral' (2)	*R. simsii* hybrid, 2n = 2x, white
SL4	'Phoenix' x 'Mistral'	*R. simsii* hybrid, 2n = 2x, white
SL5	'Phoenix' x 'Casablanca tetra' (1)	*R. simsii* x *R. obtusum* hybrid, 2n = 3x, purple
SL6	'Phoenix' x 'Casablanca tetra' (2)	*R. simsii x R. obtusum* hybrid, 2n = 3x, purple
SL7	'Madame Roggeman' x *R. noriakianum*	*R. simsii* x *R. noriakianum* hybrid, 2n = 2x, purple
SL8	*R. kaempferi* x 'Mevrouw Andre Heungens'	*R. kaempferi x R. simsii* hybrid, 2n = 2x, white

Table 6.2. Hybrid origin of the Hymenanthes, Pentanthera and Rhododendron hybrids used in our breeding program with Tsutsusi genotypes (Salley & Greer, 1992).

Hybrid name	Hybrid origin
'Azurwolke' ('Blue Cloud')	*russatum x {(intricatum x fastigiatum) x augustinii)}*
'Blaufeder'	*?*
'Chikor'	*rupicola chryseum x ludlowii*
'Curlew'	*ludlowii x fletcherianum*
'Euan Cox'	*hanceanum x ludlowii*
'Golden Bee'	*(keiskei x ?) x mekongense melinanthum*
'Goldsworth Orange'	*dichroanthum x fortunei discolor*
'Habashan'	*hippophaeoides x ?*
'Hillier's form'	*?*
'Hollandia'	hardy Ghent hybrid
'Jingle Bells'	*(dichroanthum x griersonianum) x (campylocarpum x fortunei discolor)*
'Kupferberg'	*dichroanthum x ?*
'Nancy Evans'	*(Goldsworth Orange x wardii) x {(dichroanthum x decorum) x [(griffithianum x arboreum) x griffithianum]}*
'Nancy Waterer'	hardy Ghent hybrid
'Shamrock'	*keiskei x hanceanum*
'Sunny'	*christianae x macgregoriae*
'Unique'	hardy Ghent hybrid
'Viscy'	*{[(griffithianum x catawbiense) x (brookeanum x lobbii)] x (campylocarpum x ?)} x viscidifolium*
'Yellow Hammer'	*sulfureum x flavidum*

6.2.4. Pollen tube staining

6.2.4.1. *Effect of pollination type*

Prefertilization barriers were studied by pollen tube staining as described in 2.4. Eight combinations of subgenera (Ts x Hy, Hy x Ts, Ts x Pe, Pe x Ts, Ts x Rh, Rh x Ts, Ts x Vi and Vi x Ts) were established. For every subgenus combination, 3 parent combinations were chosen (depending on flower availability). For every parent combination, 4 flowers were harvested: 2 styles were fixed after 4 days, the remaining 2 after 4 weeks.

6.2.4.2. *Effect of prefertilization treatments*

Diverse prefertilization treatments were applied in an attempt to promote pollen tube growth after Pe x Ts pollinations. *R. luteum* and the hardy Ghent hybrid 'Unique' were pollinated with *R. simsii* 'Dogwood' or *R. kiusianum* Makino pollen. Four styles were fixed after every treatment, 4 days after pollination. The effect of the treatments was considered through fruit formation (and following ovule development) and pollen tube staining observation (2.4.). Tube growth rate was quantified by measuring the relative distance between the stigma and the ovary covered by the tubes. The following prefertilization treatments were applied:

• pollination with mentor pollen: pollen from a congruous Pentanthera-genotype was irradiated with 100, 300, 600 or 1000 Gray (as described in 2.5.), and applied on the stigma along with incongruous Tsutsusi pollen. The effects of this treatment were evaluated based only on fruit and/or ovule development.
• cut-style/stigma exudates: the style of the seed parent was shortened until it matched the pollinator style; the cut surface was moistened with stigma exudate of another Pentanthera genotype.
• phytohormones: GA_3, NAA en BAP were diluted to 57.7 mM, 107.4 mM and 88.8 mM, respectively, and 100 µl of the dilution was applied on the stigma before pollination.

6.2.5. *In vitro* experiments

6.2.5.1. *Development of a suitable germination medium*

Since seeds cultured *in vitro* are immature, and germination is expected to be inhibited by dormancy, the use of gibberellins is probably required to optimize germination. The optimal GA_3 concentration was determined. After autoclaving, BMR (basal *Rhododendron* medium, see 2.2.) was supplemented with 0, 29, 58, 145 or 289 µM GA_3 (filter sterilized). On each medium 120 immature *R. simsii* 'Dogwood Bont' x 'Kingfisher' seeds (ovaries harvested 3 months after pollination) were initiated (2.2.) in 6 petri dishes. The number of germinated seeds was counted after 4 weeks.

6.2.5.2. *Ovule culture after interspecific crosses*

The selected germination medium for immature seeds/fertilized ovules was BMR + 145 µM GA_3 (see 'Results' section); seedlings were micropropagated on BMR + 4.5 µM 2iP (according to Ureshino *et al*., 1998). The rooting medium consisted of 1/10 strenght MS macro and micro salts (Murashige & Skoog, 1962), 114 µM sucrose, 11.4 µM IAA, 2.5 g/l activated charcoal and 7 g/l MC29 agar (pH 5.7) (according to Mertens *et al*., 1996). Four to six months after pollination (2 months when the seed parent was a Vireya genotype), fruits were harvested. Sterilization procedures were as described in 2.1. Immature seeds were dissected from the ovaries and initiated on germination medium. Upon germination, seedlings were transferred to BMR; before acclimatization (2.1.), they were counted, multiplied on multiplication medium and transferred to rooting medium. One year after pollination the number of vigorous (>1 cm, no albino leaves) survivors was counted to determine the final efficiency of each subgenus combination.

6.2.6. *In vivo* sowing

Five seed capsules of *R. burmanicum* x CV2 and 'Sunny' x SL2 were harvested respectively 9 and 3 months after pollination. Seeds were sown *in vivo* in the greenhouse, in ceramic recipients (conditions as in 2.1.); germination rates were scored after 2 months.

6.2.7. Determination of parenthood

Ploidy analyses (2.7.) were used for paternity testing when the chromosome number of both parents was unequal. A representative group of seedlings (> 2 cm, 2 plants / parent combination if available) was selected for AFLP- and SSR-analysis. Three AFLP primer combinations were applied: E-ACT + M-CTA, E-AAG + M-CTA and E-ACT + M-CAT (based on De Riek *et al*., 2000), allowing the construction of Jaccard or Simple Matching based proximity matrices and dendrograms (as described in 2.9.). Two SSR primer sets were used, that were developed at DvP (Maertens, 2000; De Riek *et al*., personal communication): AZA 2.40 (forward primer 5' TGC TTG CTT TTG CGT TCT T 3', reverse primer 5' GCC CAT AGG AGG ATT ACT TGC 3', repeat motif $AC_{10}TC_8AC_{17}$) and AZA 2.56 (forward primer 5' CAA TGG GTT GGG ACT TGC 3', reverse primer 5' GAC TAC AGC TTT CTG ACG TTA CTC C 3', repeat motif TG_{15}) as in 2.10.

6.3. Results

6.3.1. Ploidy screening

6.3.1.1. *Breeding material*

Results are presented in Table 6.3. Measured as well as literature based values are presented. The ploidy level of the hybrids was not available in literature; therefore the chromosome number of their seed parents (Table 6.2.) was taken as a standard.

6.3.1.2. *Pentanthera species*

Results are presented in Table 6.4 and Fig. 6.2. Root material (except of *R. prinophyllum*) was hard to measure and gave unclear results. Nuclear DNA-content of leaves and anthers was easier to determine, though multiple measurements were required to yield unequivocal results. Especially nuclear suspensions *R. viscosum* inhibited sheath flow, because of their high viscosity. All *R. luteum* and *R. calendulaceum* samples were found to be tetraploid. All other species were diploid.

6.3.1.3. *Pentanthera hybrids*

Sixteen hardy Ghent hybrids and 2 Rustica hybrids were tetraploid, whereas 5 hardy Ghent hybrids and 2 Rustica hybrids were found to be triploids (Table 6.5; Fig. 6.3). Ploidy peaks were 'shifting' during successive measurements towards higher ploidy levels. 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 similar to those obtained after measuring without internal standards (Table 6.5). Two groups could 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 varied between 0.49 en 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.

Table 6.3. Ploidy levels of Hy, Pe and Rh species and hybrids applied in our breeding program with *R. simsii* hybrids (SG = subgenus, - only pollen available, * not described by Janaki Ammal *et al.* (1950), / parent plants have different ploidy levels).

SG	Tested species or hybrid	2n (measured)	2n (literature)	SG	Tested species or hybrid	2n (measured)	2n (literature)
Hy	*R. wardii*	2x	2x	Rh	*R.* 'Blaufeder'	4x	*
	R. 'Nancy Evans'	2x	2x		*R.* 'Habashan'	2x	2x
	R. dichroanthum scyphocalyx	-	2x		*R.* 'Hillier's form'	4x	*
	R. 'Goldsworth Orange'	2x	2x		*R.* 'Yellow Hammer'	7x – 8x	2x/6x
	R. 'Jingle Bells'	2x	2x		*R. leptocladon*	2x	*
	R. 'Kupferberg'	2x	2x		*R. burmanicum*	2x	*
	R. citriniflorum	-	2x		*R. valentinianum*	2x	2x
	R. sanguineum haemalum	-	2x		*R. xanthostephanum*	2x	2x
	R. 'Viscy'	2x	2x		*R.* 'Euan Cox'	2x	2x
					R. 'Shamrock'	2x	2x
Pe	*R. luteum*	> 4x	2x		*R.* 'Golden Bee'	2x	2x
	R 'Hollandia'	> 4x	2x/4x		*R. lutescens*	2x	2x
	R. 'Nancy Waterer'	> 4x	2x/4x		*R.* 'Curlew'	2x	2x
	R. 'Unique'	> 4x	2x/4x		*R.* 'Chikor'	2x	2x
Rh	*R. chrysodoron*	2x	2x	Vi	*R. kawakamii*	2x	*
	R. megeratum	2x	2x		*R. laetum*	4x	*
	R. sulfureum	2x	*		*R. macgregoriae*	4x	*
	R. cinnabarinum concatenans	-	6x		*R. aequabile*	-	*
	R. cinnabarinum xanthocodon	-	6x		*R.* 'Sunny'	4x	*
	R. 'Azurwolke'	4x	4x				

Table 6.4. Flow cytometrical ploidy measurement of Pentanthera species (- flowers not available, * 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	-	*
R. periclymenoides	2x	2x	*

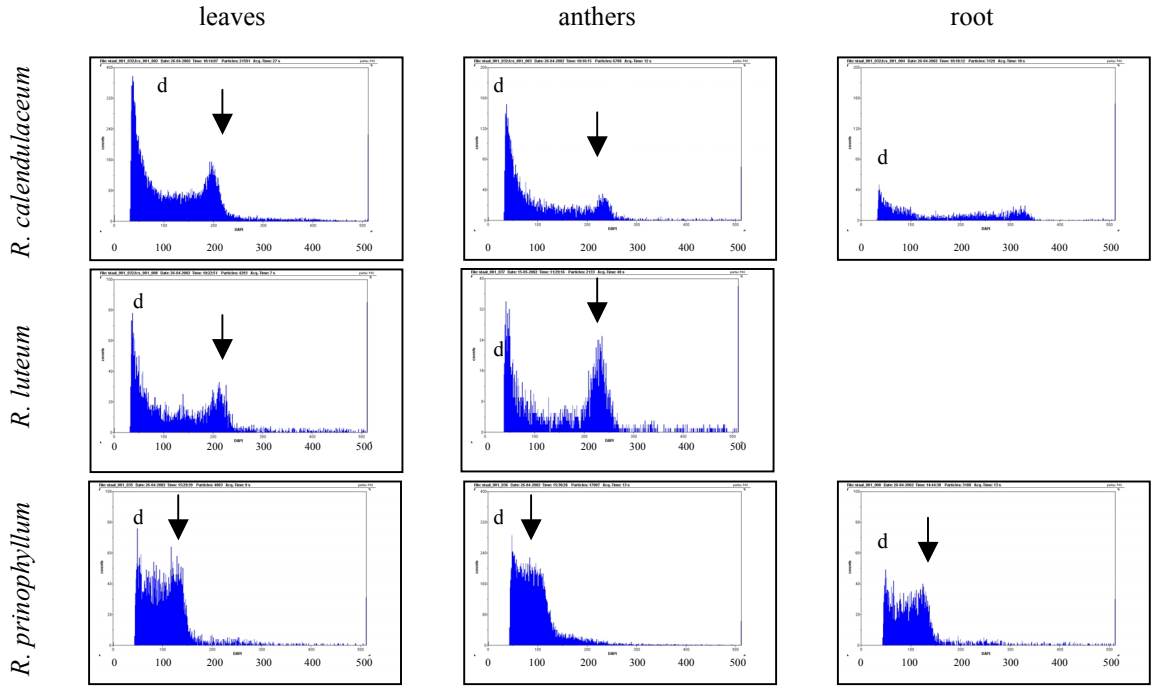


Figure 6.2. Flow cytometrical patterns of *R. calendulaceum*, *R. luteum* and *R. prinophyllum* leaves, anther filaments and roots (sample peak indicated by arrow). Peaks on the extreme left are caused by debris fluorescence (d). X-axis: fluorescence (channel number); Y-axis: number of nuclei.

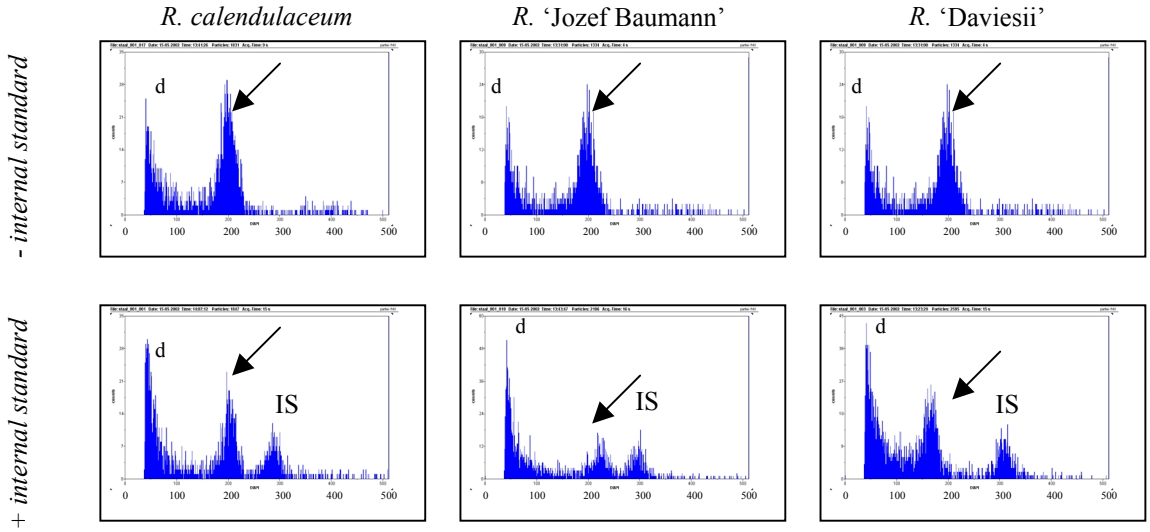


Figure 6.3. Flow cytometrical patterns of *R. calendulaceum*, *R.* 'Jozef Baumann' and *R.* 'Daviesii' (sample peak indicated by arrow), without and with the application of an internal *Lolium* standard (IS). Peaks on the extreme left are caused by debris fluorescence (d). X-axis: fluorescence (channel number); Y-axis: number of nuclei.

Table 6.5. SPF/GPF ratios after flow cytometrical analyses and ploidy level for hardy Ghent and Rustica hybrids (R) (subgenus Pentanthera) examined.

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
'Narcissiflora'	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	'Norma' (R)	0.51	3x
'Quadricolor'	0.50	3x	'Phebe' (R)	0.49	3x
'Souvenir du Pres. Carnot'	0.71	4x	'Fenelon' (R)	0.70	4x
'Marie Verschaffelt'	0.76	4x	'Racine' (R)	0.80	4x

6.3.2. Pollen tube staining

6.3.2.1. *Effect of pollination type*

Table 6.6 shows a lack of pollen tube growth (Fig. 6.4.), combined with early abortion, after Pe x Ts and Ts x Vi crosses. Hy pollen tubes grew slowly in Ts styles, whereas Ts pollen tubes reached the ovary of Hy flowers quickly. Pollen tube growth after interspecific Ts x Rh and Rh x Ts pollinations was delayed but most often not inhibited. In *R. kawakamii*, which belongs to the Pseudovireya section, Tsutsusi pollen tube growth was inhibited whereas Euvireya styles were able to guide the same pollen to the ovary. Similar reactions within groups were recorded in 4 combinations: Hy x Ts and Ts x Pe (rapid and complete pollen tube growth) and Pe x Ts and Ts x Vi (complete and near immediate growth arrest after germination). Differences within groups were most striking after Ts x Hy pollinations.

The fruits which aborted after 4 weeks showed no or limited pollen tube growth after 4 days, although not every parent combination showing limited pollen tube growth after 4 days resulted in spontaneous abortion. Finally, all non aborted flowers allowed pollen germination and growth through the style up to the ovary.

6.3.2.2. *Effect of prefertilization treatments*

Pollen tube growth of Pentanthera was not hampered in Tsutsusi styles, whereas there was no pollen tube growth at all after the reverse cross (as shown in 6.3.2.1). Moreover, pollen tube growth was not initiated on cut styles or after BAP treatment. A minor growth of pollen tubes after auxin or gibberellin treatment of the stigma was observed (Table 6.7). The growth was significantly higher after GA_3 treatment. However, subsequent fruit or ovule development were not observed.

Table 6.6. Pollen tube growth after interspecific *Rhododendron* pollination (*growth limited to upper half of the style, -fruits aborted).

Pollination type	Parent combination	Tube growth (4 days)	Tube growth (4 weeks)
Ts x Hy	SL1 *x R. wardii*	No	-
	SL1 x *R.* 'Goldsworth Orange'	Yes*	-
	SL1 x *R.* 'Jingle Bells'	Yes*	Yes
Hy x Ts	*R.* 'Nancy Evans' x CV2	Yes	Yes
	R. 'Goldsworth Orange' x CV1	Yes	Yes
	R. 'Jingle Bells' x CV1	Yes	Yes
Ts x Pe	SL2 x *R. luteum*	Yes	Yes
	SL1 x *R.* 'Nancy Waterer'	Yes	Yes
	CV3 x *R.* 'Hollandia'	Yes	Yes
Pe x Ts	*R. luteum* x SL1	No	-
	R. luteum x CV2	No	-
	R. 'Nancy Waterer' x CV2	No	-
Ts x Rh	SL2 x *R. chrysodoron*	Yes	Yes
	SL4 x *R. valentinianum*	No	Yes
	CV8 x *R.* 'Curlew'	Yes*	Yes
Rh x Ts	*R. sulfureum* x SL2	Yes*	-
	R. burmanicum x CV2	Yes	Yes
	R. 'Golden Bee' x CV1	Yes	Yes
Ts x Vi	SL1 x *R. laetum*	No	-
	CV5 x *R. macgregoriae*	No	-
	CV9 x *R.* 'Sunny'	No	-
Vi x Ts	*R. kawakamii* x CV1	No	-
	R. laetum x CV1	Yes	Yes
	R. 'Sunny' x SL2	Yes	Yes

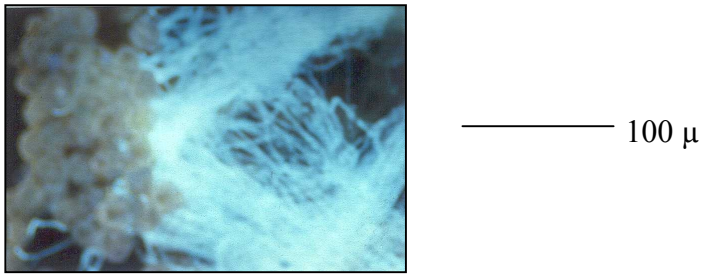


Figure 6.4. Pollen tube staining in a *Rhododendron* pistil.

Table 6.7. Prefertilization treatments after Pe x Ts pollinations: % distance stigma-ovary covered by pollen tubes after 4 days. Means indicated by the same symbol are not statistically different (LSD 95%) (CS/SE = cut-style + stigma exudate).

Seed parent	Pollen parent	% distance
R. 'Dogwood'	*R. luteum*	100 d
R. luteum	*R.* 'Dogwood'	0 a
R. luteum	*R.* 'Dogwood' + CS/SE	0 a
R. luteum	*R.* 'Dogwood' + BAP	0 a
R. luteum	*R.* 'Dogwood' + GA_3	61 c
R. luteum	*R.* 'Dogwood' + NAA	24 b
R. kiusianum	*R.* 'Unique'	100 D
R. 'Unique'	*R. kiusianum*	0 A
R. 'Unique'	*R. kiusianum* + CS/SE	0 A
R. 'Unique'	*R. kiusianum* + BAP	0 A
R. 'Unique'	*R. kiusianum* + GA_3	35 C
R. 'Unique'	*R. kiusianum* + NAA	8 B

6.3.3. Development of a suitable germination medium for immature seeds

Results are presented in Fig. 6.5. The optimal GA_3 concentration was 145 µM which gave significantly higher germination rates than all other treatments except 289 µM. However, 29 µM was already sufficient to increase the germination rate significantly.

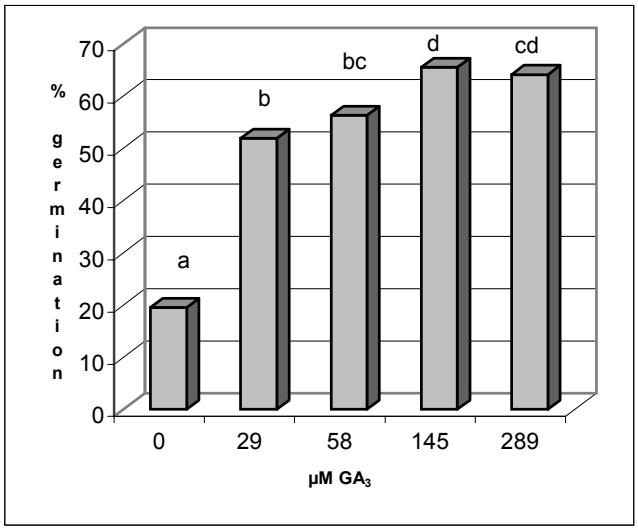


Figure 6.5. Germination rate of *R. simsii* 'Dogwood Bont' x 'Kingfisher' seeds 3 months after pollination on BMR + GA_3.

6.3.4. Ovule culture

From 2361 pollinated flowers, 349 (14.78 %) did not abort before *in vitro* initiation. From the 13549 ovules cultured *in vitro*, 1488 (10.98 %) germinated, 319 (21.44 %) developed into

green seedlings. Germination started at very divergent moments, but was mostly observed after 3 weeks. Tables 6.8 – 6.15 provide a detailed overview of the results per subgenus combination. In 6.3.4.9. a general overview on ovule culture is provided.

6.3.4.1. *Tsutsusi x Hymenanthes*

Table 6.8. shows that many SL1 x *R.* 'Jingle Bells' ovules could be isolated, but their germination efficiency was very low. Four out of 7 combinations did not result in fruit setting; 2 combinations yielded green seedlings but the number of albinos was higher. Vigorously growing seedlings were not obtained.

Table 6.8. Interspecific *Rhododendron* crosses: results from Ts x Hy pollinations. Ovules were initiated after 4-6 months on BMR + 145 µM GA_3 and the # vigorous seedlings was counted after 1 year.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
SL1	*R. wardii*	20	0	-	-	-	-
SL1	*R. dichroanthum scyphocalyx*	6	3	20	3	3	0
SL1	*R.* 'Goldsworth Orange'	35	0	-	-	-	-
SL1	*R.* 'Jingle Bells'	27	14	1135	7	0	-
SL1	*R. citriniflorum*	8	0	-	-	-	-
SL1	*R. sanguineum haemalum*	6	1	24	0	2	0
SL1	*R.* 'Viscy'	12	0	-	-	-	-

6.3.4.2. *Hymenanthes x Tsutsusi*

Only 3 out of 10 parent combinations resulted into complete fruit abortion, but non aborted fruits were empty in 3 other combinations. Green seedlings were obtained from 4 combinations (Table 6.9), although virescence was prominent in 2 combinations. Despite this chlorosis, 3 combinations yielded vigorous plantlets.

6.3.4.3. *Tsutsusi x Pentanthera*

Despite low germination rates and occasional setting of empty fruits (2/10 combinations), a majority (8/10) of the pollinations yielded seedlings (Table 6.10) but those were all albino (Fig. 6.6). Because of the tetraploid DNA-level of *R. luteum* and its hybrids, the interspecific character of the seedlings could be verified through flow cytometry (6.3.6.).

Table 6.9. Interspecific *Rhododendron* crosses: results from Hy x Ts pollinations (* first true leaves show heavy chlorosis). Ovules were initiated after 4-6 months on BMR + 145 µM GA_3 and the # vigorous seedlings was counted after 1 year.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
R. wardii	CV1	3	2	0	-	-	-
R. wardii	SL1	3	0	-	-	-	-
R.'Nancy Evans'	CV2	21	12	105	5	20*	0
R.'Nancy Evans'	CV1	20	15	319	20	36*	2
R. 'Goldsworth Orange'	CV1	41	31	332	84	105	28
R. 'Jingle Bells'	CV1	33	22	5	0	2	2
R. 'Jingle Bells'	CV2	12	0	-	-	-	-
R. 'Kupferberg'	CV2	14	13	0	-	-	-
R. 'Kupferberg'	CV1	14	11	0	-	-	-
R. 'Viscy'	CV1	18	0	-	-	-	-

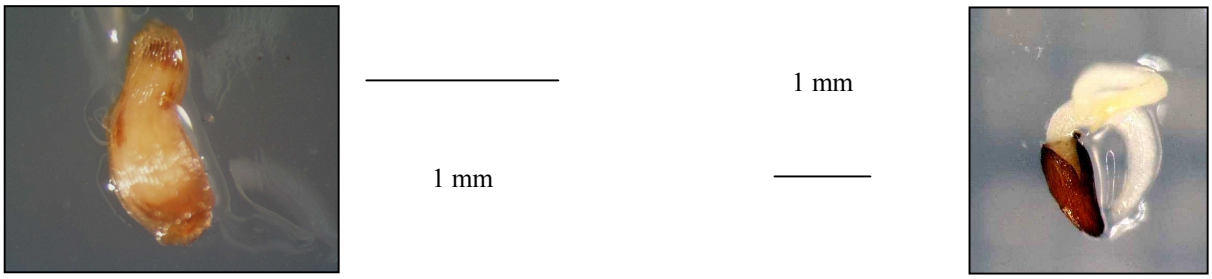


Figure 6.6. Seed and albino seedling obtained after Ts x Pe pollination: CV3 x *R. luteum*.

Table 6.10. Interspecific *Rhododendron* crosses: results from Ts x Pe pollinations. Ovules were initiated after 4-6 months on BMR + 145 µM GA_3.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
CV1	*R. luteum*	33	18	14	3	0	-
SL2	*R. luteum*	89	21	105	40	0	-
SL3	*R. luteum*	21	4	26	2	0	-
SL4	*R. luteum*	37	22	224	3	0	-
SL7	*R. luteum*	32	9	0	-	-	-
CV3	*R. luteum*	61	1	0	-	-	-
SL1	*R. luteum*	41	26	1476	7	0	-
SL1	*R.* 'Nancy Waterer'	103	38	554	46	0	-
CV4	*R.* 'Nancy Waterer'	27	24	2679	39	0	-
CV3	*R.* 'Hollandia'	65	7	68	2	0	-

6.3.4.4. *Pentanthera x Tsutsusi*

This subgenus combination is incongruous due to pollen tube growth inhibition (Table 6.6) and did not yield fruits (Table 6.11).

Table 6.11. Interspecific *Rhododendron* crosses: results from Ts x Pe pollinations.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
R. luteum	SL1	15	0	-	-	-	-
R. luteum	CV2	17	0	-	-	-	-
R. 'Nancy Waterer'	CV2	7	0	-	-	-	-
R. 'Nancy Waterer'	SL1	5	0	-	-	-	-

6.3.4.5. *Tsutsusi x Rhododendron*

Out of 31 parent combinations, 9 set fruit and 6 provided fertilized ovules that could be cultured *in vitro* (Table 6.12), 5 of which produced green plantlets. Especially upon pollination with *R. lutescens* the number of seedlings was high, but those lacked growth vigor and withered during the first year after pollination. Finally only the SL2 x *R. chrysodoron* pollination yielded vigorous plants.

6.3.4.6. *Rhododendron x Tsutsusi*

We evaluated 38 combinations (Table 6.13): 16 yielded fruits, although those were empty after 5 combinations. Solid albinos were rare, but virescence was observed frequently. Mainly the 3 most successful combinations, all having *R. burmanicum* as seed parent, were hit by severe chlorosis once the first 'true' leaves appeared (Fig. 6.7). Out of 10 combinations producing green seedlings, 5 yielded vigorous plantlets after 1 year.

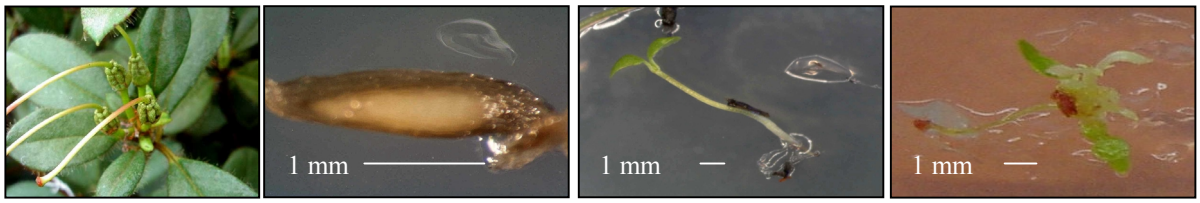


Figure 6.7. *R. burmanicum* x CV1 interspecific pollination: seed capsules, seed partially filled with endosperm, seedling, appearance of albino 'true' leaves.

Table 6.12. Interspecific *Rhododendron* crosses: results from Ts x Rh pollinations (* first true leaves show heavy chlorosis). Ovules were initiated after 4-6 months on BMR + 145 μM GA_3 and the # vigorous seedlings was counted after 1 year.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
SL4	*R. chrysodoron*	3	1	0	-	-	-
SL2	*R. chrysodoron*	27	2	80	0	32	16
SL2	*R. megeratum*	7	0	-	-	-	-
SL2	*R. sulfureum*	16	0	-	-	-	-
SL4	*R. sulfureum*	13	0	-	-	-	-
SL1	*R. sulfureum*	6	0	-	-	-	-
SL1	*R. cinnabarinum xanthocodon*	5	0	-	-	-	-
SL1	*R. cinnabarinum concatenans*	11	0	-	-	-	-
SL1	*R.* 'Azurwolke'	5	0	-	-	-	-
CV5	*R.* 'Azurwolke'	12	3	120	0	1	0
CV6	*R.* 'Azurwolke'	5	0	-	-	-	-
CV9	*R.* 'Azurwolke'	6	0	-	-	-	-
SL5	*R.* 'Azurwolke'	3	0	-	-	-	-
SL5	*R.* 'Blaufeder'	2	0	-	-	-	-
SL4	*R.* 'Blaufeder'	4	0	-	-	-	-
CV6	*R.* 'Blaufeder'	3	0	-	-	-	-
CV6	*R* 'Habashan'	2	0	-	-	-	-
SL7	*R* 'Habashan'	16	0	-	-	-	-
CV7	*R* 'Habashan'	5	0	-	-	-	-
CV5	*R* 'Hillier's form'	5	3	159	0	0	-
CV9	*R* 'Hillier's form'	4	0	-	-	-	-
SL5	*R* 'Hillier's form'	19	0	-	-	-	-
SL4	*R* 'Hillier's form'	8	0	-	-	-	-
SL2	*R.* 'Yellow hammer'	2	0	-	-	-	-
SL2	*R. leptocladon*	14	0	-	-	-	-
SL2	*R. valentinianum*	8	0	-	-	-	-
SL4	*R. valentinianum*	35	1	0	-	-	-
SL4	*R. xanthostephanum*	6	1	0	-	-	-
SL1	*R. lutescens*	16	7	378	0	198*	0
SL1	*R.* 'Curlew'	3	1	44	1	8	0
CV8	*R.* 'Curlew'	8	4	208	0	10	0

6.3.4.7. *Tsutsusi x Vireya*

This subgenus combination is incongruous due to pollen tube growth inhibition (Table 6.6) and did not yield fruits (Table 6.14).

Table 6.13. Interspecific *Rhododendron* crosses: results from Rh x Ts pollinations (* first true leaves show heavy chlorosis). Ovules were initiated after 4-6 months on BMR + 145 μM GA_3 and the # vigorous seedlings was counted after 1 year.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
R. chrysodoron	SL2	16	6	496	0	8	5
R. megeratum	SL2	6	0	-	-	-	-
R. sulfureum	SL2	40	0	-	-	-	-
R. 'Azurwolke'	CV6	7	0	-	-	-	-
R. 'Azurwolke'	CV9	8	2	115	0	8	0
R. 'Azurwolke'	SL1	13	3	20	0	10	10
R. 'Azurwolke'	SL6	12	5	285	0	3	0
R. 'Azurwolke'	CV1	4	0	-	-	-	-
R. 'Blaufeder'	CV1	3	0	-	-	-	-
R. 'Blaufeder'	CV6	8	0	-	-	-	-
R. 'Habashan'	CV6	7	0	-	-	-	-
R. 'Habashan'	SL7	32	0	-	-	-	-
R. 'Habashan'	SL2	6	0	-	-	-	-
R. 'Hillier's form'	CV9	5	0	-	-	-	-
R. 'Hillier's form'	CV6	5	0	-	-	-	-
R. 'Yellow Hammer'	CV4	7	0	-	-	-	-
R. 'Yellow Hammer'	SL2	34	7	0	-	-	-
R. leptocladon	SL2	17	2	50	0	0	-
R. burmanicum	SL2	4	0	-	-	-	-
R. burmanicum	SL1	3	0	-	-	-	-
R. burmanicum	CV2	121	26	1221	0	150*	0
R. burmanicum	SL8	43	15	702	38	20, 232*	18
R. burmanicum	CV1	52	11	1100	100	169*	0
R. valentinianum	CV1	2	2	350	0	2	0
R. valentinianum	SL2	19	0	-	-	-	-
R. xanthostephanum	SL2	18	4	500	0	46	32
R. xanthostephanum	CV1	4	0	-	-	-	-
R. xanthostephanum	SL1	11	0	-	-	-	-
R. 'Euan Cox'	CV1	26	6	2	0	1	1
R. 'Euan Cox'	CV2	26	0	-	-	-	-
R. 'Shamrock'	CV2	32	5	0	-	-	-
R. 'Golden Bee'	CV1	42	0	-	-	-	-
R. 'Golden Bee'	SL1	16	4	0	-	-	-
R. 'Golden Bee'	SL2	33	7	0	-	-	-
R. 'Curlew'	SL3	29	5	0	-	-	-
R. 'Chikor'	SL2	17	0	-	-	-	-
R. 'Chikor'	CV1	21	0	-	-	-	-
R. 'Chikor'	CV2	87	0	-	-	-	-

Table 6.14. Interspecific *Rhododendron* crosses: results from Ts x Vi pollinations.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
SL1	*R. kawakamii*	3	0	-	-	-	-
SL1	*R. laetum*	31	0	-	-	-	-
SL2	*R. laetum*	9	0	-	-	-	-
CV5	*R. macgregoriae*	19	0	-	-	-	-
CV9	*R. macgregoriae*	7	0	-	-	-	-
SL3	*R. macgregoriae*	2	0	-	-	-	-
SL4	*R. macgregoriae*	9	0	-	-	-	-
SL1	*R. macgregoriae*	13	0	-	-	-	-
SL1	*R. aequabile*	7	0	-	-	-	-
CV7	*R.* 'Sunny'	2	0	-	-	-	-
SL2	*R.* 'Sunny'	2	0	-	-	-	-
SL7	*R.* 'Sunny'	5	0	-	-	-	-
CV9	*R.* 'Sunny'	8	0	-	-	-	-
SL4	*R.* 'Sunny'	3	0	-	-	-	-

6.3.4.8. *Vireya x Tsutsusi*

Eleven different pollinations were performed (Table 6.15). *R. laetum* x SL2 seeds did not germinate; 2 of the 3 combinations including *R.* 'Sunny' produced seedlings, and chlorosis was not observed. 'Sunny' is tetraploid, allowing hybrid screening by flow cytometry (6.3.6.).

Table 6.15. Interspecific *Rhododendron* crosses: results from Vi x Ts pollinations. Ovules were initiated after 4-6 months on BMR + 145 µM GA_3 and the # vigorous seedlings was counted after 1 year.

seed parent	pollen parent	# flowers pollinated	# fruits not aborted	# ovules initiated	# albino seedlings	# green seedlings	# vigorous survivors
R. kawakamii	SL1	8	0	-	-	-	-
R. kawakamii	CV1	31	0	-	-	-	-
R. laetum	CV1	14	5	0	-	-	-
R. laetum	SL2	10	1	50	0	0	-
R. macgregoriae	SL2	65	0	-	-	-	-
R. macgregoriae	CV4	12	0	-	-	-	-
R. macgregoriae	CV1	21	0	-	-	-	-
R. macgregoriae	CV9	49	0	-	-	-	-
R. 'Sunny'	CV9	23	5	0	-	-	-
R. 'Sunny'	SL2	39	11	550	0	23	8
R. 'Sunny'	SL7	8	5	40	0	2	0

6.3.4.9. *General overview*

The direction of the cross was of utmost importance for abortion, germination and albinism. The rate of abortion of pollinated flowers was a reflection of prezygotic barriers (Table 6.6). Hy x Ts and Ts x Pe crosses yielded a relatively high number of fruits whereas Pe x Ts and Ts x Vi did not yield fruits at all, the other combinations having an intermediate efficiency. On

average, an immature fruit yielded 38.83 ovules that could be isolated, but this efficiency was higher in Ts x Hy and lower in Hy x Ts and Rh x Ts crosses. *In vitro* germination rates of fertilized ovules on BMR + 145 µM GA_3 (which are ± 65% after control pollinations as indicated in Fig. 6.5.) were > 20% in Hy x Ts and Ts x Rh and < 5% in Vi x Ts, Ts x Hy and Ts x Pe combinations. Total albinism was observed after Ts x Pe crosses (Table 6.10). Other manifestations of chloroplast deficiency, mainly virescence, were obvious in all Ts combinations with Hy and Rh but not in Vi x Ts crosses. Seedling vigor is obviously reduced in most subgenus combinations, except for non-virescent Rh x Ts seedlings that were relatively vigorous growers. Throughout all ovule culture experiments, no unequivocal effects of the exact Tsutsusi genotype involved on efficiency were observed. The number of vigorous seedlings obtained after 1 year was higher if the Ts genotype was the pollen parent. Of course, this figures do not represent final efficiencies as the number of actual intersubgeneric hybrids remained to be determined.

6.3.5. *In vivo* sowing

The 5 *R. burmanicum* x CV2 seed capsules didn't yield viable seedlings. All seedlings were albino or pale green, and withered after germination. Five green seedlings were yielded from *in vivo* sowing of 'Sunny' x SL2 mature seed capsules. Growth vigor was low for both *in vivo* and *in vitro* seedlings.

6.3.6. Determination of parenthood

Vigorously growing survivors (except 'Nancy Evans' x CV 1) were selected from successfully acclimatized plantlets (Fig. 6.8; Table 6.16). All seedlings had a representative habitus, except the *R.* 'Goldsworth Orange' x CV1 and *R. chrysodoron* x SL2 samples that were, within their respective cross, the only ones that yielded sufficient leaf material for DNA-isolation.

At a young stage, all seedlings showed maternal leaf morphology and growth habit, except for *R.* 'Euan Cox' x CV1. That was a vigorous grower whereas its seed parent had a dwarf habitus. None of the seedlings developed mature leaves or flowers suitable for scoring morphological markers as described in 2.6. For that reason, flow cytometrical and molecular analysis (Table 6.16) were performed. Fig. 6.8 reflects differences in growth vigor between seedlings.

6.3.6.1. *Ploidy screening*

Ploidy screening was applied when both parent plants had different DNA-contents, as was the case for seedlings obtained after Ts x Pe (Fig. 6.9) and Vi x Ts (Fig. 6.10) pollinations. The seedling ploidy levels were all intermediate, indicating that the seedlings were interspecific hybrids.

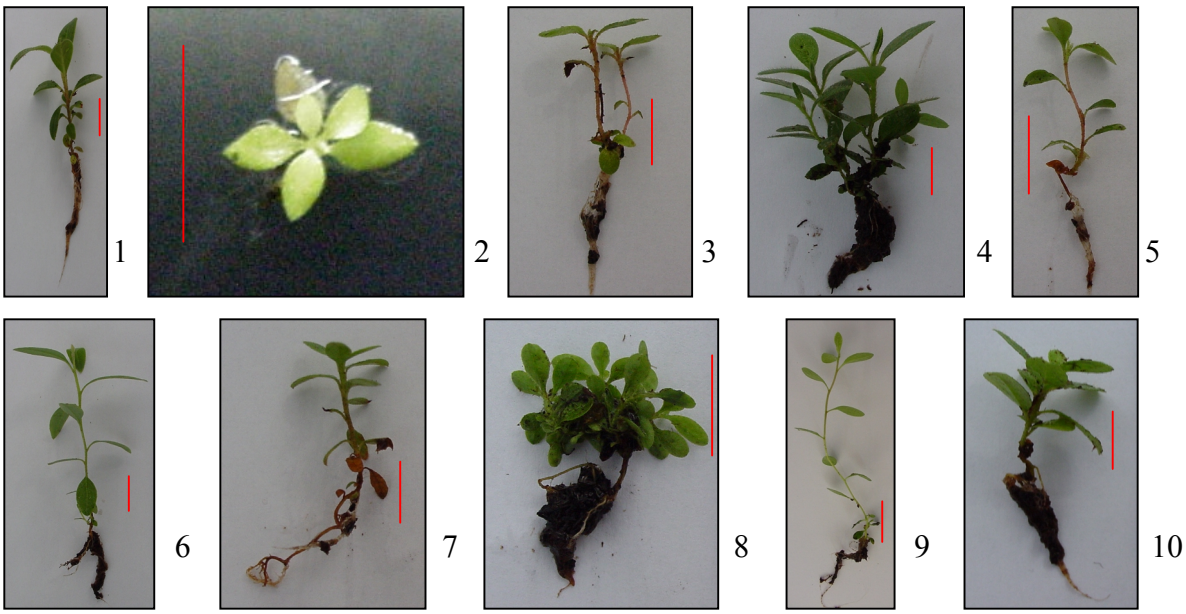


Figure 6.8. Seedlings originating from intersubgeneric *Rhododendron* crosses, 1 year after pollination (bar represents 1 cm): 1 = *R.* 'Goldsworth Orange' x CV1, 2 = *R.* 'Nancy Evans' x CV1, 3 = *R.* 'Jingle Bells' x CV1, 4 = SL2 x *R. chrysodoron,* 5 = *R. chrysodoron* x SL2, 6 = *R. xanthostephanum* x SL2, 7 = *R.* 'Azurwolke' x SL1, 8 = *R. burmanicum* x SL8, 9 = *R.* 'Euan Cox' x CV1, 10 = *R.* 'Sunny' x SL2.

Table 6.16. Selection of representative seedlings among reputed hybrids for SSR- and AFLP-screening after intersubgeneric *Rhododendron* crosses.

subgenus combination	parent combination	# plants tested through AFLP	# plants tested through SSR
Hy x Ts	*R.* 'Goldsworth Orange' x CV1	2	2
	R. 'Nancy Evans' x CV1	-	2
	R. 'Jingle Bells' x CV1	-	2
Ts x Rh	SL2 x *R. chrysodoron*	2	2
Rh x Ts	*R. chrysodoron* x SL2	1	1
	R. xanthostephanum x SL2	1	2
	R. 'Azurwolke' x SL1	2	2
	R. burmanicum x SL8	-	2
	R. 'Euan Cox' x CV1	-	1
Vi x Ts	*R.* 'Sunny' x SL2	1	2

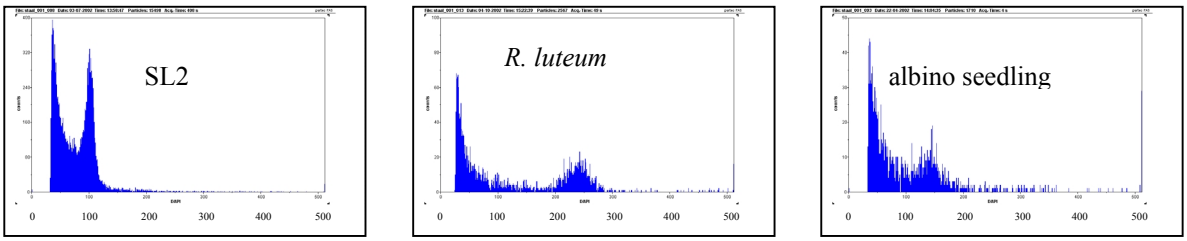


Figure 6.9. Flow cytometrical analysis of SL2, *R. luteum* and an albino seedling obtained after SL2 x *R. luteum* pollination (X-axis: fluorescence, Y-axis: number of nuclei).

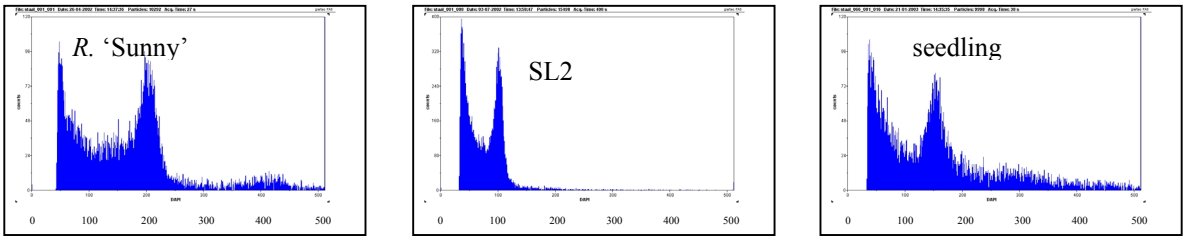


Figure 6.10. Flow cytometrical analysis of 'Sunny', SL2 and a seedling obtained after 'Sunny' x SL2 pollination (X-axis: fluorescence, Y-axis: number of nuclei).

.

6.3.6.2. *Molecular analysis*

AFLP-data were interpreted on electropherograms (exemplified in Fig. 6.11), proximity matrices (Table 6.17) and dendrograms (Fig. 6.12). SSR-data were interpreted on electropherograms (exemplified in Fig. 6.13) and are presented in Table 6.18.

• *AFLP*

DNA-fragments of seedlings and seed parents represented in the electropherograms were largely concurrent. The 'Sunny' x SL2 seedling showed a profile that was obviously aberrant since it included various fragments lacking in 'Sunny' but present in the paternal genotype (Fig. 6.11), indicating that this seedling arose from an interspecific cross. However, for quantitative data concerning the mutual consanguinity of all genotypes tested, the proximity matrices (Table 6.17) were considered.

Jaccard as well as Simple Matching similarity indices were calculated, both providing the same result. Most of the seedlings showed a high degree of relationship towards the seed parent and eventual full sibs but not to their reputed pollen parent. The 'Sunny' x SL2 seedling was diagnosed as an exception, because it showed a relatively low similarity to the seed parent. The only plants that showed a higher affinity with SL2 than this seedling did were other Ts types. Mutual relationships between all genotypes were visualized in a dendrogram (Fig. 6.12) and reflected the same pattern: the Vireya x azalea pollination resulted in an interspecific hybrid whereas no proof for the hybrid character of the other seedlings could be deducted.

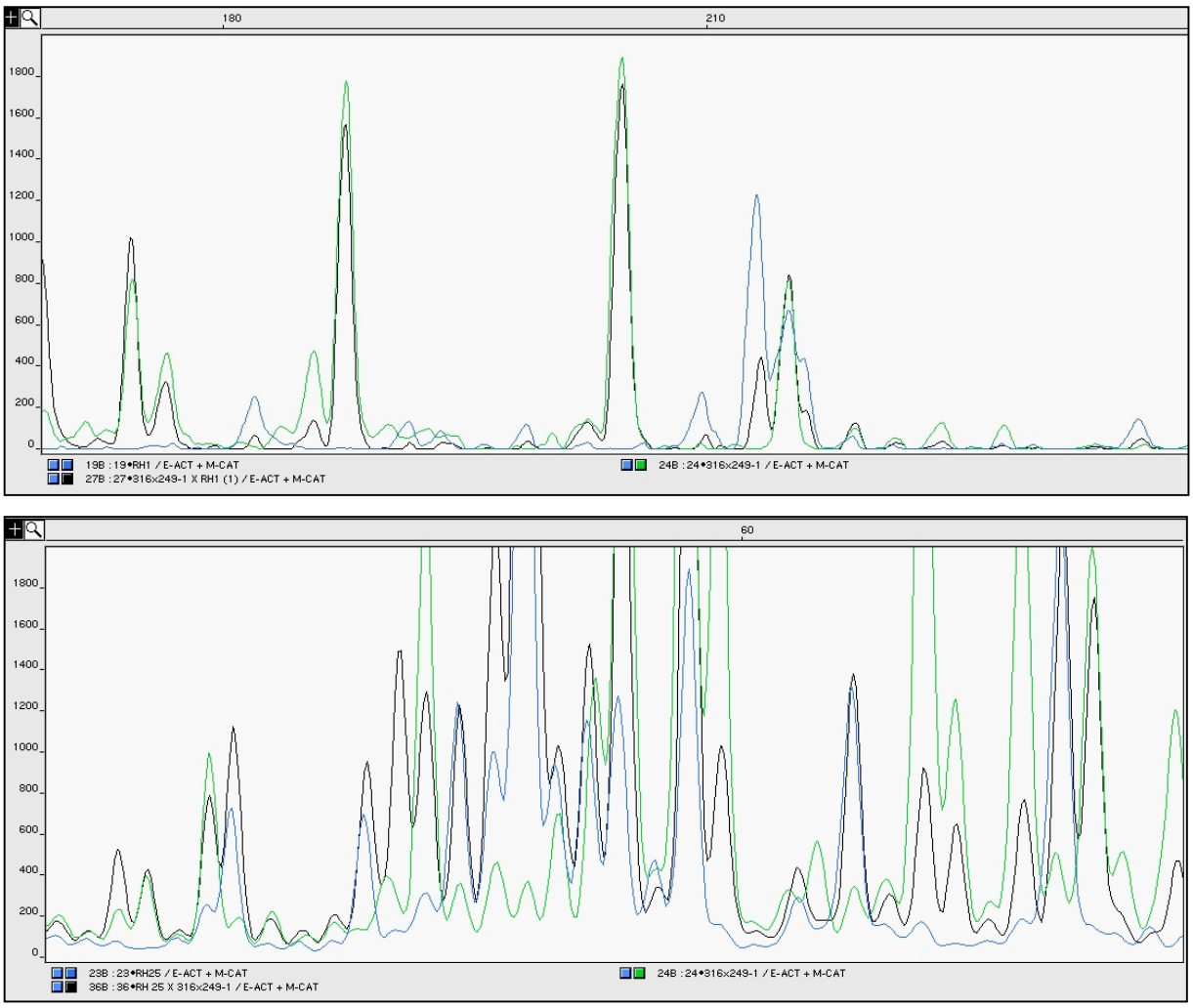


Figure 6.11. Electropherograms obtained after selected restriction fragment amplification by E-ACT and M-CAT of *R. chrysodoron* (blue), SL2 (green) and their assumed hybrid (black) (top) and of *R.* 'Sunny' (blue), SL2 (green) and their assumed hybrid (black) (bottom) (X-axis: bp length, Y-axis: fluorescence).

• *SSR*

Unlike AFLP, SSR yielded conclusive evidence for the interspecific character of half of the seedlings that were tested. Mendelian inheritance of uniquely maternal alleles due to self pollination was observed in SL2 x *R. chrysodoron* –1, SL2 x *R. chrysodoron* –2, *R. chrysodoron* x SL2, *R. xanthostephanum* x SL2 –1, *R. xanthostephanum* x SL2 –2, *R.* 'Azurwolke' x SL1 –1, *R.* 'Azurwolke' x SL1 –2, *R.* 'Goldsworth Orange' x CV1 –1 and *R.* 'Goldsworth Orange' x CV1 –2 (Table 6.19). On the other hand, electropherograms yielded obvious proof for the inheritance of paternal alleles in *R.* 'Jingle Bells' x CV1 -1, *R.* 'Jingle Bells' x CV1 -2, *R.* 'Nancy Evans' x CV1 –1, *R.* 'Nancy Evans' x CV1 –2, *R. burmanicum* x SL8 –1, *R. burmanicum* x SL8 –2 and *R.* 'Euan Cox' x CV1 (Table 6.18; Fig. 6.13). This verifies their interspecific status. *R.* 'Sunny' x SL2 –1 and *R.* 'Sunny' x SL2 –2 were peculiar since they contained alleles that are neither maternal or paternal. Without drawing immediate conclusions concerning the origin of this phenomenon, it could be stated that both seedlings did not arise from accidental self-pollination and were therefore very probably interspecific hybrids. In *R. burmanicum*, *R.* 'Euan Cox', *R. burmanicum* x SL8 –1, *R. burmanicum* x SL8 –

2, *R.* 'Euan Cox' x CV1, *R.* 'Sunny' and *R.* 'Sunny' x SL2 –2 electropherograms a fragment of 309 bp was visualized that is associated with another locus, probably in a homozygous state in the parent plants and in a heterozygous state in the seedlings.

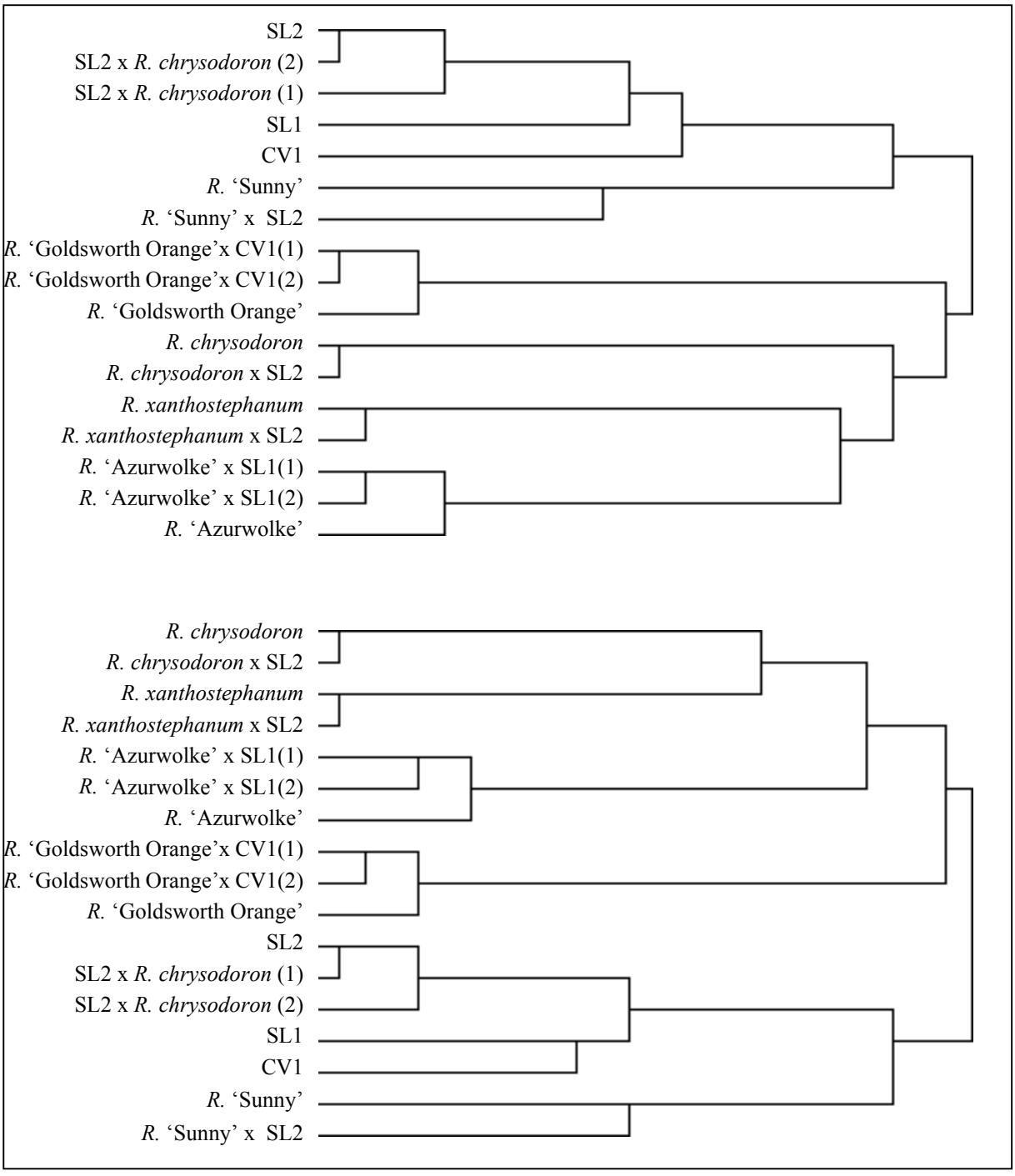


Figure 6.12. Dendrogram showing the genetic relatedness of all *Rhododendron* parent plants and seedlings tested by AFLP. The dendrogram was constructed using UPGMA-clustering based on a genetic similarity matrix containing the Jaccard (up) and Simple Matching (down) similarity coefficients between pairs of plants.

Table 6.17. Proximity matrices with pair wise Jaccard and Simple Matching similarity coefficients obtained after AFLP-analysis of intersubgeneric *Rhododendron* crosses.

genotype	SL2	H1	H1	SL1	R1	H3	R2	H4	H5	R3	H6	H7	R4	H8	R5	H9	CV1
Jaccard																	
SL2	1.000	.671	.719	.570	.285	.285	.304	.323	.310	.319	.325	.313	.344	.433	.258	.279	.497
SL2 x *R. chrysodoron* (H1)	.671	1.000	.628	.488	.306	.314	.299	.325	.320	.317	.295	.285	.301	.407	.244	.269	.482
SL2 x *R. chrysodoron* (H2)	.719	.628	1.000	.538	.283	.283	.307	.298	.303	.301	.318	.295	.338	.433	.238	.267	.504
SL1	.570	.488	.538	1.000	.307	.319	.297	.329	.328	.315	.322	.309	.314	.411	.285	.302	.532
***R. chrysodoron* (R1)**	.285	.306	.283	.307	1.000	.704	.369	.368	.352	.358	.340	.330	.298	.336	.396	.376	.289
***R. chrysodoron* x SL2 (H3)**	.285	.314	.283	.319	.704	1.000	.341	.365	.346	.371	.348	.338	.308	.337	.381	.373	.300
***R.* 'Azurwolke' (R2)**	.304	.299	.307	.297	.369	.341	1.000	.650	.649	.329	.304	.298	.351	.333	.412	.360	.306
***R.* 'Azurwolke' x SL1 (H4)**	.323	.325	.298	.329	.368	.365	.650	1.000	.689	.326	.329	.353	.347	.382	.410	.414	.335
***R.* 'Azurwolke' x SL1 (H5)**	.310	.320	.303	.328	.352	.346	.649	.689	1.000	.347	.344	.351	.333	.362	.401	.412	.330
***R.* 'Goldsworth Orange' (R3)**	.319	.317	.301	.315	.358	.371	.329	.326	.347	1.000	.669	.670	.358	.358	.338	.313	.321
***R.*'Goldsworth Orange' x CV1 (H6)**	.325	.295	.318	.322	.340	.348	.304	.329	.344	.669	1.000	.703	.360	.380	.317	.345	.301
***R.*'Goldsworth Orange' x CV1 (H7)**	.313	.285	.295	.309	.330	.338	.298	.353	.351	.670	.703	1.000	.352	.342	.321	.332	.301
***R.* 'Sunny' (R4)**	.344	.301	.338	.314	.298	.308	.351	.347	.333	.358	.360	.352	1.000	.554	.320	.296	.333
***R.* 'Sunny' x SL2 (H8)**	.433	.407	.433	.411	.336	.337	.333	.382	.362	.358	.380	.342	.554	1.000	.331	.338	.395
***R. xanthostephanum* (R5)**	.258	.244	.238	.285	.396	.381	.412	.410	.401	.338	.317	.321	.320	.331	1.000	.689	.284
***R. xanthostephanum* x SL2 (H9)**	.279	.269	.267	.302	.376	.373	.360	.414	.412	.313	.345	.332	.296	.338	.689	1.000	.322
CV1	.497	.482	.504	.532	.289	.300	.306	.335	.330	.321	.301	.301	.333	.395	.284	.322	1.000
Simple Matching																	
SL2	1.000	.821	.850	.749	.514	.516	.483	.490	.476	.519	.516	.514	.528	.610	.483	.510	.692
SL2 x *R. chrysodoron* (H1)	.821	1.000	.800	.699	.557	.566	.498	.512	.509	.538	.503	.505	.502	.601	.491	.523	.693
SL2 x *R. chrysodoron* (H2)	.850	.800	1.000	.735	.531	.533	.503	.479	.486	.519	.526	.514	.538	.624	.483	.517	.709
SL1	.749	.699	.735	1.000	.556	.568	.493	.514	.514	.533	.530	.528	.514	.603	.531	.552	.730
***R. chrysodoron* (R1)**	.514	.557	.531	.556	1.000	.862	.589	.575	.561	.598	.570	.571	.519	.549	.655	.641	.537
***R. chrysodoron* x SL2 (H3)**	.516	.566	.533	.568	.862	1.000	.563	.573	.556	.610	.578	.580	.531	.551	.643	.639	.549
***R.* 'Azurwolke' (R2)**	.483	.498	.503	.493	.589	.563	1.000	.784	.784	.521	.483	.488	.526	.497	.627	.582	.502
***R.* 'Azurwolke' x SL1 (H4)**	.490	.512	.479	.514	.575	.573	.784	1.000	.808	.503	.497	.533	.509	.538	.613	.620	.519
***R.* 'Azurwolke' x SL1 (H5)**	.476	.509	.486	.514	.561	.556	.784	.808	1.000	.528	.514	.533	.495	.517	.606	.620	.516
***R.* 'Goldsworth Orange' (R3)**	.519	.538	.519	.533	.598	.610	.521	.503	.528	1.000	.815	.821	.552	.544	.577	.556	.538
***R.*'Goldsworth Orange' x CV1 (H6)**	.516	.503	.526	.530	.570	.578	.483	.497	.514	.815	1.000	.838	.545	.557	.545	.577	.507
***R.*'Goldsworth Orange' x CV1 (H7)**	.514	.505	.514	.528	.571	.580	.488	.533	.533	.821	.838	1.000	.547	.528	.561	.575	.519
***R.* 'Sunny' (R4)**	.528	.502	.538	.514	.519	.531	.526	.509	.495	.552	.545	.547	1.000	.713	.540	.519	.533
***R.* 'Sunny' x SL2 (H8)**	.610	.601	.624	.603	.549	.551	.497	.538	.517	.544	.557	.528	.713	1.000	.542	.552	.587
***R. xanthostephanum* (R5)**	.483	.491	.483	.531	.655	.643	.627	.613	.606	.577	.545	.561	.540	.542	1.000	.854	.530
***R. xanthostephanum* x SL2 (H9)**	.510	.523	.517	.552	.641	.639	.582	.620	.620	.556	.577	.575	.519	.552	.854	1.000	.571
CV1	.692	.693	.709	.730	.537	.549	.502	.519	.516	.538	.507	.519	.533	.587	.530	.571	1.000

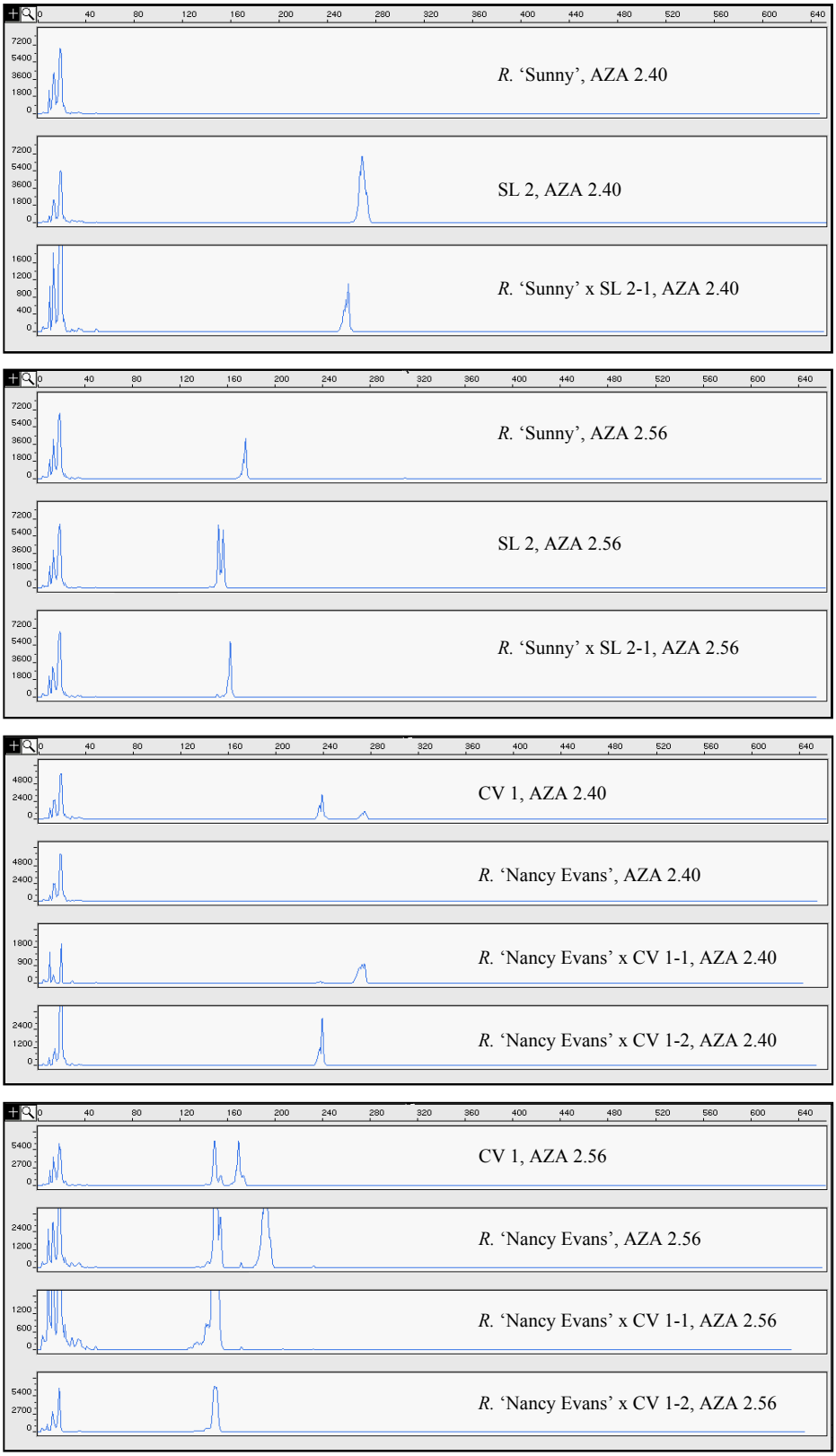


Figure 6.13. DNA fragments obtained after the application of SSR primer sets AZA 2.40 (1st and 3rd plot) and AZA 2.56 (2nd and 4th plot) on total genomic DNA of *R.* 'Sunny' x SL 2-1 (upper 2 electropherograms) and *R.* 'Nancy Evans' x CV 1-1 and *R.* 'Nancy Evans' x CV 1-2 (lower 2 electropherograms) and their respective seed and pollen parents (X-axis: bp length; Y-axis: fluorescence).

Table 6.18. PCR products obtained (in bp) after application of SSR primer sets AZA 2.40 (upper row, capitals) and AZA 2.56 (lower row) on intersubgeneric *Rhododendron* hybrids. Double figures represent heterozygous loci, single figures represent homozygous loci (* corresponding with other locus). A/a = azalea alleles, R/r = rhododendron alleles, X/x = unknown alleles.

alleged cross	seedling	seed parent	pollen parent
SL2 x *R. chrysodoron* –1	A1	A1	-
	a1, a2	a1, a2	r1
SL2 x *R. chrysodoron* -2	A1	A1	-
	a1	a1, a2	r1
R. chrysodoron x SL2	-	-	A1
	r1	r1	a1, a2
R. xanthostephanum x SL2 -1	-	-	A1
	r2	r2	a1, a2
R. xanthostephanum x SL2 -2	-	-	A3, A4
	r2	r2	a4, a5
R. 'Azurwolke' x SL1 -1	-	-	A2
	r3, r4	r3, r4	a3
R. 'Azurwolke' x SL1 -2	-	-	A2
	r3, r4	r3, r4	a3
R. 'Goldsworth Orange' x CV1 -1	-	-	A3, A4
	r5, r6	r5, r6	a4, a5
R. 'Goldsworth Orange' x CV1 -2	-	-	A3, A4
	r5	r5, r6	a4, a5
R. 'Sunny' x SL2 -1	X1	-	A1
	x1	r7, r8*	a1, a2
R. 'Sunny' x SL2 -1	X2	-	A1
	x1, r7, r8*	r7, r8*	a1, a2
R. 'Jingle Bells' x CV1-1	A3	-	A3, A4
	r10, a5	r9, r10	a4, a5
R. 'Jingle Bells' x CV1-2	A3	-	A3, A4
	r10, a4	r9, r10	a4, a5
R. 'Nancy Evans' x CV1 -1	A4	-	A3, A4
	r11, a4	r11, r12	a4, a5
R. 'Nancy Evans' x CV1 -2	A3	-	A3, A4
	r11, a4	r11, r12	a4, a5
R. burmanicum x SL8 -1	A5	-	A5, A6
	r13, a6, r8*	r13, r8*	a6
R. burmanicum x SL8 -2	A5	-	A5, A6
	r13, a6, r8*	r13, r8*	a6
R. 'Euan Cox' x CV1	A4	-	A3, A4
	r14, a5, r8*	r14, r15, r8*	a4, a5

6.3.7. General overview

Table 6.19 provides a general overview of successful and unsuccessful pollinations subject to molecular analysis. Abortion percentages were concurrent with the subgenus combination, all

3 Hy x Ts pollinations causing less abortions then others; obviously low or high abortion rates were not associated with the occurrence of intersubgeneric hybrids. The 2 combinations with the lowest number of harvested ovules per fruit yielded interspecific hybrids, as did the 2 combinations with the lowest percentage of vigorous seedlings. At least in these combinations, low ovule development or seedling vigor were positive markers for the occurrence of hybrids. In 2 out of 3 cases, albinism was linked to an interspecific character. Only the combination of high ovule development and absence of total albinism yielded interspecific hybrids after *R.* 'Sunny' x SL2 pollination. Except for this latter combination there was a positive correlation between more obvious occurrence of postzygotic barriers and the actual creation of interspecific hybrids. From a quantitative point of view these barriers of course limited the number of hybrid seedlings.

Table 6.19. Overview of interspecific *Rhododendron* seedlings obtained, including method(s) of detection (* indicates supplementary indication through non-matromorph morphology) (SG = subgenus).

SG x SG	pollination	abortion %	# ovules / fruit	albinism %	non-vigor %	detection of hybrids
Hy x Ts	*R.* 'Nancy Evans' x CV1	25	21.27	35.71	94.74	yes (SSR)
	R. 'Jingle Bells' x CV1	33.33	0.23	0	0	yes (SSR)
	R. 'Goldsworth Orange' x CV1	24.99	10.71	44.44	73.33	no
Ts x Rh	SL2 x *R. chrysodoron*	92.59	40	0	50	no
Rh x Ts	*R. chrysodoron* x SL2	62.5	82.67	0	37.5	no
	R. 'Azurwolke' x SL1	69.23	6.67	0	0	no
	R. burmanicum x SL8	65.12	46.8	13.1	92.86	yes (SSR)
	R. xanthostephanum x SL2	77.78	125	0	30.43	no
	R. 'Euan Cox' x CV1	76.92	0.33	0	0	yes (SSR)*
Vi x Ts	*R.* 'Sunny' x SL2	71.79	50	0	65.22	yes (flow cytometry, AFLP, SSR)

Table 6.20. presents a summary of all prezygotic and postzygotic barriers met from pollination onward until molecular confirmation of the interspecific status of the seedlings, and allows to formulate general conclusions about the final efficiency of each subgenus combination. Extrapolation of the results from the seedlings screened at the molecular level gives a final efficiency of 4 vigorously growing and non-chlorophyll deficient intersubgeneric hybrids per 179 Hy x Ts pollinations (2 *R.* 'Nancy Evans' x CV1 + 2 *R.* 'Jingle Bells' x CV1). For Rh x Ts and Vi x Ts, these efficiencies amounted to 19 hybrids / 836 pollinations (18 *R. burmanicum* x SL8 + 1 *R.* 'Euan Cox' x CV1) and 8 hybrids / 280 pollinations (8 *R.* 'Sunny' x SL2), respectively. All other subgenus combinations showed a zero efficiency. Altogether, 31 green and vigorous hybrid seedlings were created from 2361 interspecific pollinations.

These figures are given with reserve since *R.* 'Goldsworth Orange' x CV1 and *R. chrysodoron* x SL2 plantlets were not representative for the whole of seedlings yielded by these crosses. The extrapolation of the (negative) results is therefore maybe inappropriate. Possibly some

non vigorous *R.* 'Goldsworth Orange' x CV1 and *R. chrysodoron* x SL2 are interspecific hybrids, and the efficiency of Hy x Ts and Rh x Ts crosses is underestimated.

Table 6.20. Efficiency of intersubgeneric *Rhododendron* pollinations after ovule culture.

pollination type	# flowers pollinated	# fruits not aborted	# ovules initiated	# ovules germinated	# green seedlings	# vigorous survivors	# hybrids / # plants tested
Ts x Hy	114	18	1179	15	5	0	-
Hy x Ts	179	58	756	270	161	32	4 / 6
Ts x Pe	509	160	5146	142	0	-	-
Pe x Ts	44	0	-	-	-	-	-
Ts x Rh	279	20	989	250	51	16	0 / 1
Rh x Ts	836	76	4841	787	98	66	3 / 9
Ts x Vi	120	0	-	-	-	-	-
Vi x Ts	280	17	640	25	25	8	2 / 2
Total	2361	349	13551	1489	340	122	9 / 18

6.4. Discussion

6.4.1. Ploidy screening of the mother plants

The botanical species *R. luteum* appeared to be tetraploid (Table 6.4.; Fig. 6.2.), which is contradictory to previous results obtained by chromosome counting (Janaki Ammal *et al*., 1950). This explains why hardy Ghent hybrids, measured in this experiment (Fig. 6.3.; Table 6.5.) and derived from crosses involving *R. luteum* and *R. calendulaceum* (2n = 4x), are tetraploid. Since most hybrids have already been created in the 19th century, exact parental data are usually missing. As a consequence, the contribution of *R. luteum*, which has so far incorrectly been described as a diploid species, has probably been underestimated in the past. A thorough ploidy screening of all 30 Pentanthera species and other hybrid groups is highly recommended, to find out whether even more species are polyploid. For ploidy levels, most *Rhododendron* breeders still directly or indirectly rely on the data presented by Janaki Ammal *et al.* (1950). Due to the unavailability of flow cytometry as an accurate, quick and reliable screening tool in those days their data should be interpreted with caution.

Unlike the other subgenera, Pentantheras occur naturally at different places, widely scattered over the globe: the Caucasian region, Eastern North America, Western North America and the Far East. Therefore Pentantheras do not seem to form a distinct group, like Tsutsusi, Hymenanthes or Rhododendron, as well on the morphological as on the molecular level (Kron, 1993; 2000). Polyploidization occurred at least at 2 developmental centers: the Caucasian region (*R. luteum*) and Eastern North America (*R. calendulaceum*). This is not surprising since recurrent and mutually independent formation of polyploidy has been described in numerous crops (Leitch & Bennett, 1997; Soltis & Soltis, 2000). Gene silencing, gene diversification and/or chromosomal translocation, being the usual consequences of polyploid formation (Soltis & Soltis, 1993), can be assumed to have influenced subsequent species development. 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 heterochromatin ‘knobs’ leading to increased chromosome length (Comai, 2000). This may explain the slightly increased ploidy level (> 4x) found in *R. luteum* and its hybrids. Aneuploidy due to chromosomal addition appears unlikely because of complete plant fertility.

Chromosome numbers of subgenus Rhododendron genotypes were not always consistent with literature data (Janaki Ammal *et al*., 1950), but differences were less common than in Pentanthera (6.4.1.). The nuclear DNA-amount of the hybrid ‘Yellow Hammer’ was slightly higher than in the hexaploid *R. flavidum*, one of its parents (Table 6.3). However, the other parent, *R. sulfureum*, was diploid. Possibly, the hybrid which we would expect to be tetraploid has doubled its nuclear DNA-level spontaneously from 2n = 4x to 2n = 8x; another possible cause is the fertilization of unreduced *R. flavidum* gametes (2n = 7x).

6.4.2. Characterization of intersubgeneric crossing barriers

Six main incongruity barriers can be distinguished after interspecific pollinations (Table 1.1), most could be observed during our pollination experiments. Genotype combinations resulting in incongruous reactions, evidently provide less opportunities towards the creation of (commercially) interesting hybrids; on the other hand, incongruous reactions (in Hy x Ts, Ts x Hy, Ts x Pe, Rh x Ts and Ts x Rh) are strong indications for interspecific seedling character. Hybrid breakdown, resulting in haploids, was not found and is so far not described in the genus *Rhododendron*. Each other barrier is discussed stepwise, in order of appearance.

6.4.2.1. *Prezygotic barriers*

After crosses between Tsutsusi genotypes and plants of other subgenera, severe pollen tube growth inhibition (prezygotic incongruity) was only observed after Pe x Ts and Ts x Vi pollinations (Table 6.6.). This was already observed by Rouse (1993) and Ureshino *et al.* (2000). In *R. kawakamii* (Pseudovireya section), Ts pollen tube growth was inhibited whereas Euvireya styles were able to guide the same pollen into the ovary. Pollen tube growth after interspecific crosses of evergreen azalea with Rh or Hy was not arrested, but was slowed down occasionally, which increases the odds for degeneration of the embryo sac before pollen tube arrival. A ‘hose-in-hose’ flower morphology, associated with female sterility in Tsutsusi (Heursel, 1999), probably at the prezygotic level, does not inhibit seed formation in ‘Nancy Evans’ (Hymenanthes).

The extent to which pollen tube growth inhibition is caused by ploidy differences, is unknown. Rouse (1993) describes a higher congruity of Tsutsusi species with (diploid) *R. occidentale*. However, based on positive results after interploidy crosses performed in this research, a ploidy determined inhibition of pollen tube growth in style tissue appears unlikely, since a matching ploidy level between parent plants was not an absolute requirement for the creation of hybrids. All data gathered, it becomes evident that bi-directional pollination was absolutely required to get a proper idea of crossing congruity.

Stigma treatment with plant growth regulators may not only promote tube growth but can also affect seed formation (Katsukawa *et al*., 2000). When applied on *Rhododendron* stigmas, phytohormones were not able to guide sperm nuclei to the ovary and into the ovules. Also other prefertilization treatments after Pe x Ts pollinations remained unsuccessful, as they did not even stimulate early pollen tube growth as observed after GA_3 or NAA treatment of the style (Table 6.7.). Since 6 out of the 8 subgenus combinations showed no or limited pollen tube growth inhibition, and prefertilization barriers could (probably) not be overcome, we focused on postfertilization incongruity problems in the 6 remaining subgenera combinations.

6.4.2.2. *Absence of seed germination*

For most combinations seed germination was low but could be optimized *in vitro* by adding 145 µM GA_3 (Fig. 6.5.; Tables 6.8.– 6.15.). Though this is a very high concentration, it is not surprising since it was already applied for ovule culture after Ts x Pe pollinations (Ureshino *et al*., 1998). Ovule culture may also aid in shortening the period between pollination and sowing after congruous pollinations, yielding a considerable gain of time (up to 6 months) for crops like azalea (Michishita *et al.*, 2001).

Next to medium composition, the main parameter for success in an 'embryo rescue' protocol is the developmental stage of the fertilized ovules. It should precede spontaneous abortion of the fruits; however, the longer the fruits remain on the seed parent, the further the seed will develop. However, after incongruous pollination development can be expected to be slower than in congruous crosses. In our experiments, abortion of fertilized flowers was found to be associated with the subgenus combination (Table 6.20). Ureshino *et al*. (1998) recommend to start ovule culture 4-6 months after pollination. Since many flowers abort before fertilization occurs, within a few weeks (Table 6.6.; Longly, 1994), and subsequently the abortion rates decrease quickly during the following months, the same initiation time (5 months ± 1 month) was applied in our crosses. An exception was made for Vi x Ts pollinations, since Vireya seeds are already mature 3 months after pollination. *In vitro* culture of fertilized Vireya ovules was therefore started after 2 months. The germination efficiency *in vivo* was obviously much lower than *in vitro*, although probably the efficiency of the latter will decrease during acclimatization of chlorotic hybrids.

6.4.2.3. *Hybrid albinism*

In absolute terms, relatively high numbers of green seedlings were obtained, which however arose from a confined number of crosses. Total albinism only occurred in Ts x Pe seedlings (Fig. 6.6.), in accordance to literature (Ureshino *et al*., 1998; Ureshino *et al*., 1999; Miyajima *et al*., 2000). Heavy virescent chlorosis was observed in many seedlings after different types of pollination (Hy x Ts, Ts x Hy, Rh x Ts and Ts x Rh) leading to severe growth inhibitions (Fig. 6.7.). Within the same subgenus combination albinism rates could diverge substantially. Albinism was evidently correlated with acclimatization problems. Crosses resulting into a majority of seedlings with decreased growth vigor, due to partial albinism, were interspecific hybrids.

6.4.2.4. *Lack of growth vigor*

Growth vigor was dependent on the exact parental combination (Fig. 6.8.). Nevertheless, hybrid weakness as described by Levin (1978) was obvious in many combinations. Especially within *R.* 'Goldsworth Orange' x CV1 and *R. chrysodoron* x SL2 seedlings, considerable growth variations were observed. Because of decreased growth vigor of most seedlings, flowering and subsequent fertility research will probably be delayed.

6.4.3. Screening of putative hybrids

Since nearly all seedlings were matromorph (Fig. 6.8.), parenthood determination based upon morphological characteristics was not appropriate. Vi x Ts and Ts x Pe seedling ploidy levels were intermediate between parents, proving their interspecific character (Fig. 6.9.; Fig. 6.10.). Therefore, for other subgenera combinations molecular screenings through AFLP and SSR were applied (Table 6.16.). In some combinations self-pollination was not a valid explanation for fruit development on this seed parent, because of male sterility (*R.* 'Nancy Evans', *R. burmanicum*).

High rates of ovule germination are not necessarily associated with high production of hybrids as they may also be caused by (pseudo)fertilization with undesired pollen. Flowers were carefully emasculated before pollination but accidental pollination by neighbouring flowers (self-pollination) could not be entirely excluded. Indeed, many Rh x Ts, Ts x Rh and Hy x Ts combination tested revealed AFLP-profiles that were not in accordance with what could be expected from true interspecific hybrids, but instead insinuated self-pollination (Fig. 6.11.). Jaccard similarity indices were obviously lower than Simple Matching similarity indices owing to many 'double zeros' (DNA-fragments lacking in both profiles mutually compared but occurring in others), indicating that the group of genotypes examined was very heterogeneous (Table 6.17.). Dendrograms clearly showed the clustering of different subgenera (Fig. 6.12.), with each alleged hybrid belonging to the same subgenus as its seed parent. The 'Sunny' seedling (Vi x Ts) showed less resemblance to its mother and was the only interspecific hybrid in this analysis.

SSR-analysis revealed the interspecific character of seedlings obtained from 5 different parental combinations belonging to 3 different subgenus combinations (Fig. 6.13.; Table 6.18.). AFLP and ploidy analyses only showed that the Vi x Ts seedlings were hybrids. Compared to the AFLP-analysis, the SSR-analysis included more seedlings; those were diagnosed as interspecific genotypes. On the other hand, SSR confirmed the hypothesis of self-pollination as the origin of all seedlings tested through AFLP, except *R.* 'Sunny' x SL2-1 (Table 6.18.). The negative results of both *R.* 'Goldsworth Orange' x CV1 and SL2 x *R. chrysodoron* samples may possibly be explained by the fact that they are not representative for the other seedlings within their respective cross. Hy x Ts and Ts x Rh pollination efficiencies may therefore be underestimated; however, further development of other *R.* 'Goldsworth Orange' x CV1 and *R. chrysodoron* x SL2 plantlets is improbable since growth was arrested upon acclimatization. This enforces our suspicion that they are hybrids. On the other hand, they are of no practical use since consistent growth and development is inhibited. As a consequence, the final number of vigorous green interspecific seedlings is not affected.

From the previous discussion, it is obvious that both AFLP and SSR are suitable molecular markers for the detection of interspecific *Rhododendron* hybrids (Table 6.19.). However, differences between both techniques are substantial. The drawbacks of SSR are high developmental costs, species specificity and the possible occurrence of zero alleles (owing to mutation in the primer annealing sequences) and stutter bands (artifacts produced by DNA polymerase slippage, mostly +1 or –1 repeat), interfering with the results. However, these weaknesses are out of proportion to its benefits: a relatively simple PCR assay, easy interpretation of the results and the codominant character (the observed peaks are directly coherent with the presence of an allele and reflect their length, determined by the number of repeats between the primer annealing sites). AFLP offers the advantage of a broader applicability because of non-specificity, but the interpretation of the results is more complicated since it is dominant and the presence or absence of a certain DNA fragment is not always totally clear. Because of easier unambiguous interpretation, SSR is preferable over AFLP during this research. Fragments possibly originating from polymerase 'stuttering' were not observed. However, when using the AZA 2.56 primer combination, electropherograms of *R. burmanicum*, *R.* 'Euan Cox', *R. burmanicum* x SL8 –1, *R. burmanicum* x SL8 –2, *R.* 'Euan Cox' x CV1 and *R.* 'Sunny' x SL2 –2 revealed an additional (3rd) fragment of 309 bp (r8), although most of the plants were diploid (Table 6.18.). It is probably associated with another locus and was not considered in our interpretation of the results. This fragment is probably typical for genotypes in the subgenus Rhododendron (including Vireya).

SSR-analysis was performed on samples from 10 different origins, 5 of which yielded unambiguous negative results (no hybrids) and 4 of which yielded unambiguous positive results (hybrids). The only combination resulting into an equivocal allele pattern, for both seedlings examined, was *R.* 'Sunny' x SL2 (Table 6.18). Provided the minor 309 bp peak (r8 allele) is disregarded for reasons mentioned above, we would expect both seedlings to inherit the r7 maternal allele (homozygous in *R.* 'Sunny') and either the a1 or a2 paternal allele (heterozygous in SL2) after AZA 2.56 primer set application. However, only 1 seedling inherited the maternal fragment, whereas none of the aforementioned paternal fragments was present in either seedling. Primer set AZA 2.40 yielded a second anomaly, since the 269 bp paternal fragment corresponding with the A1 allele, although homozygous, was not passed on to the seedlings. Moreover, both seedlings contained fragments that were not present in either alleged parent: X1 and X2 (after AZA 2.40 application) and x1 (after AZA 2.56 application). However, since the pollinator was a diploid, as revealed by ploidy analysis, and the only diploid Rhododendrons bearing flowers at the same time, as *R.* 'Sunny', were diploid evergreen azaleas, the seedlings must truly be intersubgeneric hybrids.

An acceptable explanation for these observations is that the pollen parent was not SL2, but another Tsutsusi genotype, either due to accidental wild-pollination or to mislabeling of the plant. The supposed pollen parent would bear both the (AZA 2.40) X1 and X2 alleles (heterozygous alleles on the same locus) and the (AZA 2.56) x1 fragment. Of course, this does not account for the absence of the r7 maternal allele in one of the seedlings. The most plausible explanation for its absence would be a mutation in the primer annealing sequence of this seedling, resulting into a so-called "zero-allele" that evidently was not amplified through PCR and visualized on a gel or an electropherogram. Indeed, additional SSR analysis (data

not provided) revealed all X alleles to be inherited from SL7 (which was the only pollinator tested that induced fruit set on 'Sunny', apart from SL2). The equivocal results on the 'Sunny' x Tsutsusi seedlings were caused by a combination of a "zero-allele" and mislabeling of the plant material. Anyhow, it is beyond doubt that the seedlings obtained are true Vireya x Tsutsusi hybrids.

6.4.4. General conclusions

Literature on interspecific breeding within the genus *Rhododendron* mainly concerns pollinations between evergreen and deciduous azaleas. Our findings were that Ts x Pe pollinations resulted in non-viable albinos and that Tsutsusi pollen growth was arrested at the top of Pentanthera styles. This coincides with all published data (Heyting, 1970; Pryor, 1973; Jaynes, 1976; Ureshino, 1998). In our research, positive and unambiguous proof for intersubgeneric hybridization was obtained after molecular screening of *R.* 'Nancy Evans' x CV1, *R.* 'Jingle Bells' x CV1, *R. burmanicum* x SL8 and *R.* 'Euan Cox' x CV1. In other words, both Rh as Ts genotypes can be included in a breeding program with pot azalea, though only a minority of combinations is successful (Table 6.20.). According to Kron (2000) garden Rhododendrons are more closely related to evergreen azaleas than deciduous azaleas are, which could provide an explanation for the relative success of crosses with Rh or Hy, compared to crosses including Pe. Hybrids were only obtained if evergreen azaleas were used as pollen donors instead of seed parents. This explains the lack of success in the research of Preil & Ebbinghaus (1985) who carried out Ts x Hy, Ts x Pe and Ts x Rh pollinations on a larger scale, but did not include reciprocal crosses. SL2 x *R. chrysodoron* and *R.* 'Goldsworth Orange' possibly yielded interspecific hybrids, that could not be screened by AFLP or SSR. However, those plantlets exhibited a highly decreased growth vigor, and therefore offer no potential for breeding programs and/or commercial propagation.

So far, Vireyas have been relatively neglected in interspecific breeding experiments with Tsutsusi genotypes, except by Rouse (1993) who obtained fairly promising results. This was confirmed by our creation of interspecific Vi x Ts hybrids, whose interspecific character was reflected in ploidy, AFLP- and SSR-analyses. Not all Vireya genotypes used in this study were suitable seed parents. Vireyas are definitely worthwhile further investigation since they offer numerous advantages: they include hundreds of species and hybrids, showing a very wide variation in flower texture and colour, and allow fast maturation of seeds. Moreover, like pot azalea many are suitable indoor plants. The taxonomic classification of the Vireya group as a lower part of the subgenus Rhododendron by Chamberlain (1996) is not appropriate, according to literature (Williams *et al*., 1990; Rouse, 1993) and our own data.

In a nutshell, it can be concluded that the creation of an F_1 generation, probably sufficiently vigorous for flower induction, is possible with different types of subgenus combinations within the genus *Rhododendron*. Interspecific hybrids could be created after Hy x Ts, Rh x Ts and Vi x Ts pollination; Ts x Pe, Pe x Ts, Ts x Hy and Ts x Vi combinations did not yield viable seedlings. Also Ts x Rh crosses are most probably inadequate (Table 6.20.) The final quantitative results may be misleading when considering the efficiency of separate subgenera combinations since those are composed of few successful genotype combinations.

Nonetheless, it is obvious that future fertility research should focus on the 3 successful subgenus combinations mentioned above.

The creation of an F_2 generation will probably be required to elucidate the inheritance and expression of yellow flower colour genes, since not all biosynthesis pathways have been described (Heursel & Horn, 1977; Griesbach, 1987; Spethmann, 1989; Cunningham & Gantt, 1998; De Keyser *et al*., 2001). The possible contribution of the interspecific genotypes created during this research towards the breeding of an evergreen azalea will have to be determined after flowering and fertility research, including further crosses. Fertility research is required to determine the best possible strategy for introgression of the yellow pigment genes. As chromosome doubling (the creation of allopolyploids after hybridization) does not stimulate homoeologous recombination, in a first stage the possible synthesis of unreduced gametes will have to be examined since FDR, SDR or IMR gamete formation does allow homoeologous recombination between the different genomes in an allopolyploid (Mendiburu & Peloquin, 1977; Karlov *et al*., 1999).

7. Development of tetraploid *Rhododendron simsii* hybrids

7.1. Introduction

The effects of polyploidization on the habitus of *Rhododendron simsii* hybrids has not been studied yet. Although polyploidy research has been performed within the genus *Rhododendron* (Kehr, 1987; Vanoverschelde, 1989; Väinölä, 2000), the possible interest of polyploids in *R. simsii* hybrids breeding is still unknown since nearly all available cultivars are diploid (Heursel & De Roo, 1981; De Schepper, 2001).

Our objectives were twofold: (i) to establish an efficient chromosome doubling procedure, allowing rapid multiplication of the (eventually) obtained polyploid genotypes, and (ii) to characterize morphological consequences of polyploidy in pot azalea. Our research included different techniques and explant types to compare their mutual efficiency.

7.2. Specific materials and methods

7.2.1. Plant material

All cv's were diploids. 'Mevrouw Marcel Van Belle' and 'Alexander' were grown in a greenhouse under normal nursery circumstances (2.1.) and flowered simultaneously. 'Laura Ashley' and 'Madame Troch' explants (2 cm shoots) were taken from routinely maintained *in vitro* stock cultures on BMR + 4.5 µM 2iP. Plants were acclimatized as in 2.1.

7.2.2. Treatment of *in vitro* seedlings

Seeds ('Mevrouw Marcel Van Belle' x 'Alexander') were sterilized as described in 2.1. and sown on BMR (basal *Rhododendron* medium, 2.2.) in petri dishes (ϕ = 5.5 cm); after emergence of the cotyledons seedlings were treated with a COL (colchicine) (1.25 mM or 6.25 mM), ORY (oryzalin) or TRI (trifluralin) (0.3 mM or 1.5 mM) solution during either 3 or 7 consecutive days as described in 2.3. Per treatment 100 (lowest concentration of each agent, 3 days application) or 25 seeds (all other treatments) were sown. Upon completion of seedling treatment, seedlings were transferred to BMR and subsequently subcultured every 6 weeks. Flow cytometry (2.7.) was performed on surviving seedlings.

7.2.3. Direct sowing on induction medium

Seeds ('Mevrouw Marcel Van Belle' x 'Alexander') were sterilized as described in 2.1. and sown on BMR enriched with 0, 0.3, 1, 3 or 10 µM TRI or ORY in petri dishes (ϕ = 5.5 cm) and maintained for 6 weeks. Per treatment 25 seeds were sown. Seedlings were subcultured periodically every 6 weeks on BMR. After 12 weeks, the germination of the seeds was evaluated and the ploidy level of the surviving seedlings was tested with flow cytometry (2.7.).

7.2.4. Application of mitosis inhibitors in multiplication medium

'Laura Ashley' and 'Madame Troch' shootlets were multiplied on BMR (2.2.) + 24.6 µM 2iP + 0, 0.5, 1, 2, 5 or 10 µM ORY (2 cm shoots, 18 explants/treatment) in Meli-jars. After 12 weeks, the formation of new shootlets and their ploidy level were evaluated (2.7.).

7.2.5. Determination of leaf and flower morphological indices

Leaf length over width (L/B) and angle of leaf basis (α) were determined (2.6.) on 5 fully developed leaves of acclimatized plants. Thickness of 4 petal margins, 4 petal central zones (originating from different flowers) and 4 leaf laminas was measured using a micrometer (Mauser, CDI-Chicago).

7.3. Results

7.3.1. Treatment of *in vitro* seedlings

As shown in Table 7.1, increasing concentrations of COL, ORY and TRI caused a higher mortality among the seedlings, but this tendency was not significant. COL was significantly less toxic than both herbicides. However, only ORY and TRI were able to induce tetraploidy or mixoploidy. An increase in the duration of the treatment was not significantly less toxic than an increase in the concentration of the mitotic agent applied. All tetraploids and most mixoploids were obtained through a 3 day treatment with 0.3 mM ORY or TRI. Fig. 7.1 shows typical flow cytometrical histograms of diploid, tetraploid and mixoploid (2n = 2x/4x) *Rhododendron simsii* plantlets.

Table 7.1. Mixoploids and tetraploids obtained through treatment of *in vitro Rhododendron simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seedlings with COL, ORY or TRI after 12 weeks. Results followed by the same symbol are not significantly different (LSD 95%).

treatment	% dead seedlings	# tested seedlings	2x/4x mixoploids	tetraploids
COL 1.25 mM 3d	10 a	90	0	0
COL 6.25 mM 3d	40 bc	15	0	0
COL 1.25 mM 7d	24 ab	19	0	0
ORY 0.3 mM 3d	62 cd	38	9	2
ORY 1.5 mM 3d	72 d	7	1	0
ORY 0.3 mM 7d	68 d	8	1	0
TRI 0.3 mM 3d	64 d	36	17	4
TRI 1.5 mM 3d	84 d	4	1	0
TRI 0.3 mM 7d	76 d	6	1	0

7.3.2. Direct sowing on induction medium

Results are presented in Table 7.2. Only 1 mixoploid and 1 tetraploid were induced. Germination was not strikingly inhibited by either treatment.

7.3.3. Application of mitosis inhibitors in multiplication medium

There was a significantly negative correlation between the ORY concentration in the multiplication medium and the number of new axillary shoots that developed (Table 7.3).

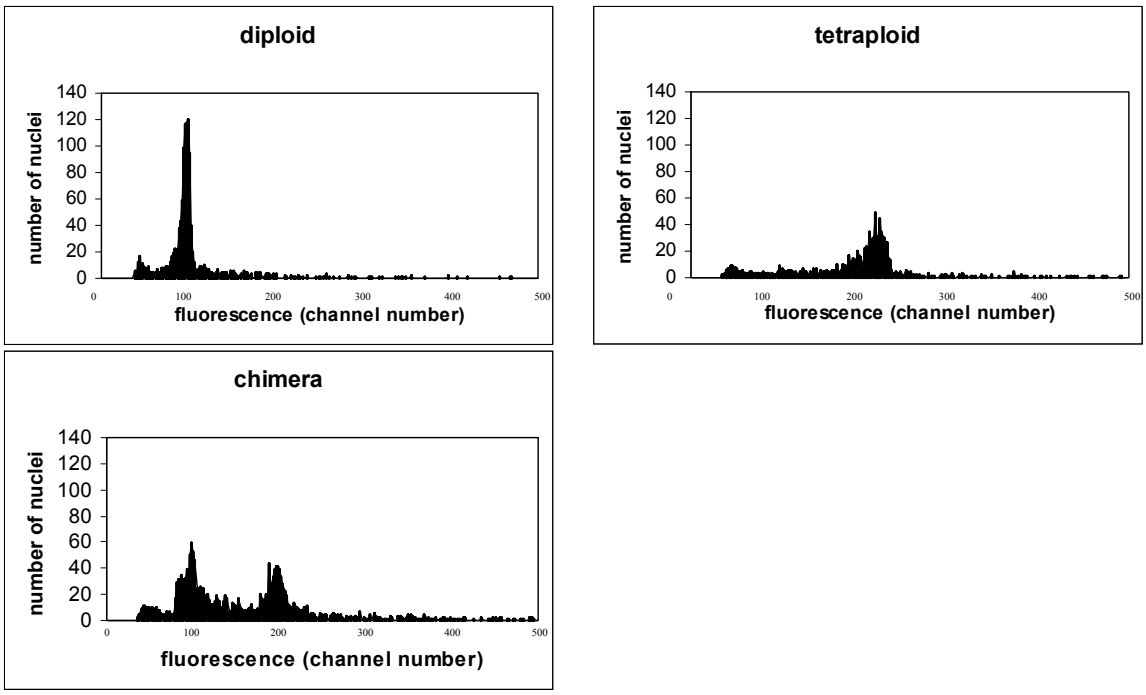


Figure 7.1. Typical flow cytometrical pattern of a diploid, a tetraploid and a mixoploid *Rhododendron simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seedling, obtained after MI application between the cotyledons.

Table 7.2. Mixoploids and tetraploids obtained after direct sowing of *Rhododendron simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seeds on induction medium, enriched with ORY or TRI after 12 weeks.

treatment	**% non-germinating seeds**	**tested seedlings**	**2x/4x mixoploids**	**tetraploids**
control	4	-	-	-
ORY 0.3 µM	12	22	0	0
ORY 1 µM	12	22	0	0
ORY 3 µM	8	23	1	0
ORY 10 µM	16	21	0	0
TRI 0.3 µM	8	23	0	0
TRI 1 µM	8	23	0	0
TRI 3µM	20	20	0	0
TRI 10 µM	12	22	0	1

This correlation was more striking in 'Madame Troch' than in 'Laura Ashley'. Many of the shoots induced showed severe growth inhibition and could not be tested. None of the ORY concentrations applied was able to induce a fully tetraploid new shoot, however mixoploids did develop.

7.3.4. Determination of leaf and flower morphological indices

Altogether 7 tetraploids were created, all from treatment of 'Marcel Van Belle' x 'Alexander' seedlings (Tables 7.1. & 7.2.). One single tetraploid exhibited an altered habitus while still *in vitro*: its leaves were smaller and rounder and the plantlet grew slower than a diploid (Fig. 7.2). Later on (upon acclimatization), its growth was as vigorous as for other seedlings.

Table 7.3. Mixoploid and tetraploid axillary shoots obtained after *in vitro* multiplication of *Rhododendron simsii* 'Madame Troch' and 'Laura Ashley' on BMR + 24.6 µM 2iP enriched with ORY after 12 weeks. Means followed by the same symbol are not significantly different (LSD 95%).

'Madame Troch'	**induced shootlets/ explant**	**tested shoots**	**2x/4x mixoploids**	**tetraploids**
ORY 0 µM	4.11 c	0	0	0
ORY 0.5 µM	2 b	12	1	0
ORY 1 µM	1.11 a	4	3	0
ORY 2µM	0.83 a	2	0	0
ORY 5 µM	0.61 a	3	0	0
ORY 10 µM	0.5 a	1	0	0

'Laura Ashley'	**induced shootlets/ explant**	**tested shoots**	**2x/4x mixoploids**	**tetraploids**
ORY 0 µM	2.83 c	0	0	0
ORY 0.5 µM	2 bc	9	0	0
ORY 1 µM	2.17 bc	12	1	0
ORY 2µM	1.83 bc	9	2	0
ORY 5 µM	0.67 ab	4	0	0
ORY 10 µM	0.17 a	1	0	0

Figure 7.2. Tetraploid *R. simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seedling (left) showing an aberrant leaf morphology compared to a diploid control seedling (right).

Five tetraploid 'Mevrouw Marcel Van Belle' x 'Alexander' and 12 mixoploid seedlings were successfully acclimatized. Growth was not hampered by higher or mixed ploidy levels and the plants did not exhibit dwarfism. Flowering was induced on 3/5 tetraploids and 7/12 mixoploids (Fig. 7.3). Anther development was very poor and anthesis was next to absent, causing a reduced fertility. One tetraploid stem (seedling 7) was broken due to its very brittle

wood after determination of L/B and α indices (7.3.4.1), leaving no mature leaves for lamina thickness determination (7.3.4.2).

Figure 7.3. Flower induction on *Rhododendron simsii* 'Mevrouw Marcel Van Belle' (left), 'Alexander' (middle left), a tetraploid seedling (middle right) and a mixoploid seedling (right).

7.3.4.1. *L/B and α indices*

In a young stage, the leaf habitus of a tetraploid azalea were not different from diploids or mixoploids; however, mature leaves tended to show a lower L/B index (Fig. 7.4). Measurements proved that these differences were statistically significant (Table 7.4). Whereas L/B for tetraploids amounted to 1.5 – 1.7 the parent plants and mixoploids index was larger than 1.8. Tetraploid leaves were wider than diploid leaves and also their leaf basis angle was broader, though variation within a single plant was more substantial for the latter parameter.

Table 7.4. L/B and α index of tetraploïd and mixoploid *R. simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seedlings. Means indicated by the same symbol are not statistically different (LSD 95%).

	Ploidy	**L/B index**		**Leaf basis angle**	
'Mevr Vanbelle'	2x	2,25	h	62° 24'	a
'Alexander'	2x	2,24	gh	61° 48'	a
seedling 1	4x	1,57	a	93°	hi
seedling 2	4x	1,67	ab	84° 36'	fgh
seedling 3	2x/4x	2,21	fgh	65° 36'	ab
seedling 4	2x/4x	2,06	defgh	72° 48'	bcde
seedling 5	2x/4x	1,84	bc	79° 36'	defg
seedling 6	2x/4x	1,99	cde	81° 48'	efg
seedling 7	4x	1,63	a	95°	i
seedling 8	4x	1,53	a	86° 24'	fghi
seedling 9	2x/4x	1,90	cd	67°	abc
seedling 10	2x/4x	1,91	cde	79° 36'	defg
seedling 11	2x/4x	2,09	efgh	61° 12'	a
seedling 12	2x/4x	2,02	cdef	74°	bcde
seedling 13	2x/4x	2,01	cde	81° 36'	efg
seedling 14	2x/4x	2,06	defg	70° 48'	abcd
seedling 15	2x/4x	1,86	c	77°	cdef
seedling 16	2x/4x	1,85	bc	80° 12'	defg
seedling 17	4x	1,60	a	88°	ghi

7.3.4.2. *Leaf lamina, petal margin and petal central zone thickness*

Although Table 7.5. indicates that tetraploid leaves were thicker than diploid leaves, statistically significant differences between all mixoploids and tetraploids were not observed, owing to a large variation within mixoploids.

The number of plants with floral development was limited, therefore the results on flower morphology are less reliable. Tetraploid petal margins were obviously not thicker than diploid ones. On the contrary, petal central zones were strikingly thicker when tetraploid. Indeed, all tetraploid flowers exhibited a significant increase in comparison to all mixoploids and diploids, apart from the mutual similarity between seedling 16 (2x/4x) and seedling 17 (4x) (Table 7.5).

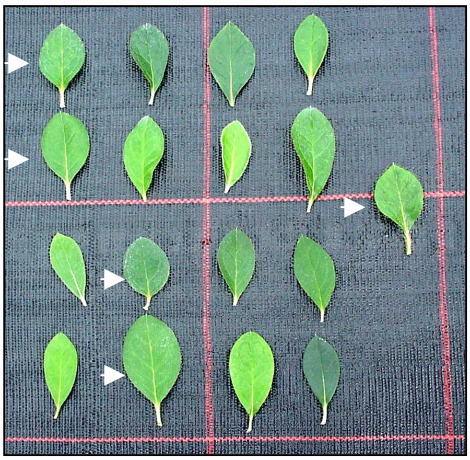


Figure 7.4. Typical mature leaves of solid tetraploid (indicated) and mixoploid *R. simsii* 'Mevrouw Marcel Van Belle' x 'Alexander' seedlings.

Table 7.5. *Rhododendron simsii* leaf lamina, petal margin and petal central zone thickness. Means indicated by the same symbol are not statistically different (LSD 95%).

	Ploidy	**Leaf lamina thickness (mm)**		**Petal margin thickness (mm)**		**Petal central zone thickness (mm)**	
'Mevr Vanbelle'	2x	0.123	ab	0.124	bc	0.453	cde
'Alexander'	2x	0.137	abc	0.102	ab	0.264	a
seedling 1	4x	0.168	def				
seedling 2	4x	0.196	f	0.128	bcd	0.772	g
seedling 3	2x/4x	0.149	bcd				
seedling 4	2x/4x	0.180	ef				
seedling 5	2x/4x	0.168	def	0.130	cd	0.327	ab
seedling 6	2x/4x	0.126	ab				
seedling 8	4x	0.171	def	0.154	de	0.809	g
seedling 9	2x/4x	0.143	abcd	0.145	cde	0.496	de
seedling 10	2x/4x	0.135	abc				
seedling 11	2x/4x	0.171	def	0.138	cde	0.387	bc
seedling 12	2x/4x	0.124	ab				
seedling 13	2x/4x	0.121	a	0.089	a	0.368	b
seedling 14	2x/4x	0.160	cde	0.172	e	0.437	cd
seedling 15	2x/4x	0.144	abcd	0.124	bc	0.511	e
seedling 16	2x/4x	0.163	cde	0.132	cd	0.649	f
seedling 17	4x	0.169	def	0.140	cde	0.602	f

7. 4. Discussion

Based on the results from Tables 7.1, 7.2 and 7.3 we can conclude that treatment of *in vitro* seedlings (as first described by Kehr, 1996a) was more efficient than direct sowing or *in vitro* multiplication to induce tetraploidy in azalea *in vitro*. However, compared to the 'direct sowing' method it was more labor consuming. Moreover, with both methods polyploidy was induced from an 'unknown' genotype (a seedling). It would also be interesting to induce

polyploidy starting from a commercially available cultivar; not only would this allow a better comparison between diploid and tetraploid, but the tetraploid would also have a greater chance of having commercial value (since it can inherit many beneficial characteristics from its original counterpart). On the other hand, it must be stated that this is far from evident since tetraploids may also show severe growth or yield repression compared to their diploid progenitors. This repression can only be abolished through extensive selection after crossing at the tetraploid level (Levin, 1983; Leitch & Bennett, 1997). In our experiments, no tetraploids could be developed on simple multiplication medium enriched with ORY (Table 7.3). Possibly some tetraploid shoots did develop, but were inhibited by more vigorously growing diploid shoots. Since mixoploids developed, it was clear that ORY doubled the chromosome content of some cells.

Mixoploids (2x/4x) find their origin in more than one cell. At least one of the original cells was diploid, at least one was tetraploid. The number of mixoploids provided a good indication of the efficiency of the treatments. As was already stated, 'direct sowing' is not efficient (Table 7.2). Both other treatments induced the development of a larger group of mixoploids than of tetraploids. In treatment 1, mixoploid tissue developed from meristem cells; in treatment 3 from stem tissue. Morphological consequences of mixoploidy were small (Table 7.4; Table 7.5; Fig. 7.3; Fig. 7.4).

Through additional flow cytometrical analysis on anther filaments (L2 layer) and (a second analysis of) leaf tissue (L1+L2 layer) (data not included) the chimerical nature of the mixoploids was considered. None of the chimeras, when grown into flowering plants, had shifted into solid diploids or tetraploids. Filaments were mixoploid as well.

Regarding the chemicals used it may be concluded that ORY and TRI were far more efficient than COL (Table 7.1.). Based on the results with the seedling treatments, COL was omitted in further experiments because of its lower efficiency. Similar results were already presented by Vaïnola (2000), though TRI was not used in the latter study. Since COL also affects other physiological processes (Eiselein 1994a; 1994b), probably to a larger extent than dinitroanilines, it should not be preferred over its alternatives. Vaïnola (2000) too reported a higher degree of mixoploidy than tetraploidy, a slower initial growth vigor of tetraploids and an increasing mortality rate among explants by an increasing concentration (being less affected by the duration of exposure). However, it should be kept in mind that Vaïnola's research was not performed on Tsutsusi genotypes.

Apart from 1 single plantlet (Fig. 7.2), most mixoploids and tetraploids were not strikingly different from common diploids *in vitro*. Rapid visual separation was therefore not possible. Morphological consequences of polyploidization are however fully revealed after acclimatization. Roose & Gottlieb (1976) and Leitch & Bennett (1997) reported a lower L/B index to be associated with higher ploidy levels, as observed in this study (Table 7.4.; Fig. 7.4.). Kehr (1996a) also mentioned effects on flower and leaf morphology, reduced fertility and brittle wood, all of which were observable. De Schepper (2001) was able to distinguish somatic polyploidy within the same flower petals of a picotee *Rhododendron simsii* bud sport, correlated with increased petal width. All these data are in accordance with our observations on the consequences of polyploidy on flower and leaf morphology (Fig. 7.3; Fig. 7.4; Table

7.4.; Table 7.5.). A more compact growth habit, a prolonged flowering period or a deeper green leaf color on the other hand were not observed. Chlorophyll contents and number of epidermal guard cells in tetraploids remain to be considered as they are possibly dependent on ploidy level (Paden, 1990).

Tetraploidy induction may prevent hybrid sterility after interspecific crosses. Although many authors consider tetraploids as an intermediate station for the breeding of sterile triploids, few triploid Tsutsusi genotypes (e.g. ‘Euratom’) have been created so far, providing few evidences for a disrupted meiosis.

8. Interspecific crosses in the genus *Hibiscus*

8.1. Introduction

Hybridization efforts between *H. syriacus* and *H. rosa-chinensis* L. (a potentially large source of genetic variability) were unsuccessful so far. Earlier research (Van Huylenbroeck *et al*., 2000; Kyung & Kim, 2001a; 2001b; Kyung *et al*, 2001a; 2001b) indicated that the prospects for introducing *H. paramutabilis* genes appeared more favourable.

We tried to hybridize tetraploid and octoploid *H. syriacus* cultivars with *H. paramutabilis* to increase the genetic variability within *H. syriacus* breeding lines. Our ultimate goal is the introgression of growth vigour genes into the *H. syriacus* genome. The hybrid character of the obtained seedlings was tested by flow cytometry, morphological parameters and AFLP-analysis.

8.2. Specific materials and methods

8.2.1. Plant material

Four different *Hibiscus syriacus* cultivars (2n = 4x 'Melwhite' and 'Oiseau Bleu' and 2n = 8x 'Red Heart cv' and 'Purple cv') were used for making a series of reciprocal crosses with *Hibiscus paramutabilis* (2n = 4x + 2). General nursery practices as in 2.1. were followed. Both octoploid cultivars were developed at the Sung Kyun Kwan University, Korea (Shim *et al*., 1993). Hand pollination was done under controlled environmental conditions in a plastic greenhouse that was kept insect free. Fresh pollen was collected on mature flowers. Altogether, 155 *H. syriacus* x *H. paramutabilis* and 23 *H. paramutabilis* x *H. syriacus* pollinations were performed.

8.2.2. Study of prezygotic incongruity

Procedures as described in 2.4. were followed. Pollinations between 'Oiseau Bleu' (2 x 4 flowers), 'Melwhite' (2 x 4 flowers) or 'Red Heart cv' (2 x 2 flowers) and *H. paramutabilis* were performed in both ways. One day after pollination, styles were collected and pollen tubes were fixed.

8.2.3. Embryo rescue

Six to ten weeks after pollination the seeds from the crosses which resulted in fruit setting were sterilized (2.1.); embryos were dissected and germinated *in vitro* on BMH (Basal medium *Hibiscus*, 2.2.) in petri dishes. Seedlings were transferred to Meli-jars after the formation of the first true leaves. Plants were subcultured every 6 weeks. Eight months after embryo initiation the converted plantlets were transferred to the greenhouse.

8.2.4. Determination of parenthood

The interspecific character of the generated seedlings was checked by comparing ploidy level, morphological parameters and AFLP-data of the parent plants with the hybrid offspring. Leaf parameters were determined as described in 2.6. The following leaf indices were determined on 5 fully developed leaves: leaf length over width (L/B), angle of leaf basis (α) and relative width of the mid lobe (M/B). Five AFLP primer combinations were tested as described in 2.8 and 2.9: E-AAC + M-CAT; E-ACA + M-CTG; E-AAG + M-CTA; E-ACC + M-CAT and E-AGC + M-CTA (randomly chosen) The number of markers uniquely present in each of the crossing parents was checked for segregation in the offspring. Supplementary ploidy analyses (as in 2.7.) were performed.

8.3. Results

8.3.1. Study of prezygotic incongruity

Pollen tube growth in the style of the seed parent was not arrested after either pollination. Obviously, prezygotic barriers are not present in *H. paramutabilis* x *H. syriacus* and *H. syriacus* x *H. paramutabilis* crosses.

8.3.2. Embryo rescue

An overview of the different crosses is given in Table 8.1. When used as seed parent, *H. paramutabilis* failed to set fruits. However, when pollinated by *H. paramutabilis*, *H.s.* 'Oiseau Bleu' and *H.s.* 'Red Heart cv' reacted by fruit setting. Of *H.s.* 'Oiseau Bleu' 41 flowers were pollinated, resulting in 7 fruits. After pollination of 48 *H.s.* 'Red Heart cv' flowers, 5 fruits were harvested. Fruits containing swollen (=fertilized) ovules were not observed on *H.s.* 'Melwhite' or *H.s.* 'Purple cv'.

Out of the collected fruits, 26 *H.s.* 'Red Heart cv' x *H. paramutabilis* embryos and 45 *H.s.* 'Oiseau Bleu' x *H. paramutabilis* embryos were isolated and initiated *in vitro*. Although the embryos were isolated 6 to 10 weeks after pollination, their developmental stage varied strongly from the globular and heart-shaped stage up to the torpedo-shaped and cotyledonary one (Fig 8.1).

Germination occurred within 2-3 weeks; however, the first true leaves were only formed after 4-5 months *in vitro*. Finally, 5 *H.s.* 'Red Heart cv' x *H. paramutabilis* embryos and 7 *H.s.* 'Oiseau Bleu' x *H. paramutabilis* embryos could be converted (Table 8.1). Of these respectively 1 and 6 plants were successfully acclimatized and transferred to the greenhouse.

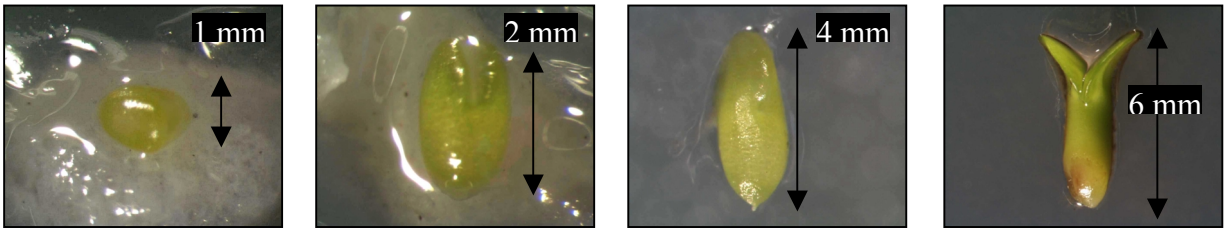


Figure 8.1. Globular, heart-shaped, torpedo-shaped and cotyledonary stage of *Hibiscus syriacus* x *H. paramutabilis* embryos.

Table 8.1. Overview of the different crosses between *Hibiscus syriacus* cultivars and *Hibiscus paramutabilis* with number of harvested fruits, collected embryos and converted seedlings (* between brackets the number of converted seedlings that were successfully transferred to the greenhouse).

♀	♂	# pollinated flowers	# fruits	# embryos	# converted seedlings
H.s. 'Oiseau Bleu'	*H. paramutabilis*	41	7	45	7 (6)*
H.s. 'Melwhite'	*H. paramutabilis*	36	0	-	-
H.s. 'Red Heart cv'	*H. paramutabilis*	48	5	26	5 (1)*
H.s. 'Purple cv'	*H. paramutabilis*	30	0	-	-
H. paramutabilis	*H.s*. 'Oiseau Bleu'	2	0	-	-
H. paramutabilis	*H.s.* 'Melwhite'	8	0	-	-
H. paramutabilis	*H.s*. 'Red Heart cv'	5	0	-	-
H. paramutabilis	*H.s.* 'Purple cv'	8	0	-	-

Determination of ploidy level showed that all seedlings from *H.s*. 'Oiseau Bleu' x *H. paramutabilis* were tetraploid, while the *H.s*. 'Red Heart cv' x *H. paramutabilis* seedling was hexaploid, indicating that this seedling was a true hybrid between the octoploid *H.s*. 'Red Heart cv' and the tetraploid *H. paramutabilis* (Table 8.2).

Table 8.2. Ploidy level and leaf indices for the *Hibiscus* parent plants and the generated seedlings (n=5) (* OISPAR = seedling from *H.s*. 'Oiseau Bleu' x *H. paramutabilis,* REDPAR = seedling from *H.s*. 'Red Heart cv' x *H. paramutabilis*). Means indicated by the same symbol are not statistically different (LSD 0.05).

Plant *	Ploidy	L/B	α (°)	M/B
H.s. 'Oiseau Bleu'	2n = 4x	1.55 c	118.4 ab	0.37 a
H.s. 'Red Heart cv'	2n = 8x	2.03 d	91.8 a	0.58 c
H. paramutabilis	2n = 4x	0.99 a	255.3 f	0.60 c
OISPAR-1	2n = 4x	1.05 ab	209.0 e	0.44 b
OISPAR-2	2n = 4x	1.12 b	161.0 cd	0.45 b
OISPAR-3	2n = 4x	0.99 a	177.0 de	0.42 ab
OISPAR-4	2n = 4x	1.14 b	180.4 de	0.44 b
OISPAR-5	2n = 4x	1.07 ab	186.4 de	0.45 b
OISPAR-6	2n = 4x	1.10 ab	129.6 bc	0.55 c
REDPAR-1	2n = 6x	1.05 ab	208.0 e	0.46 b

8.3.3. Leaf morphology

In a young stage, leaf morphology of the hybrid seedlings was in general more comparable to that of the male parent *H. paramutabilis* than to *H. syriacus*. Later on, leaves acquired a more intermediate habitus (Fig 8.2).

All hybrid seedlings had broad leaves, as expressed by a low leaf length over width (L/B) index. This is a typical characteristic for *H. paramutabilis* and *H. sinosyriacus* (Van Huylenbroeck *et al*., 2000). For most seedlings leaf angle and relative width of the mid lobe were intermediate to the parent plants (Table 8.2). Only seedling OISPAR-6 had leaves with a

narrow leaf angle, typical for most *H. syriacus* cultivars (Van Huylenbroeck *et al*., 2000). Growth vigour of all seedlings was very strong. Especially OISPAR-1 and OISPAR-2 showed a plant growth comparable to *H. paramutabilis*.

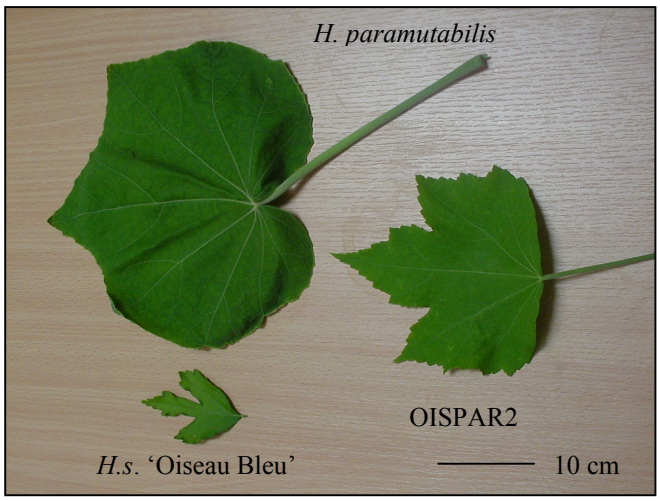


Figure 8.2. Leaf morphology of the assumed *H. syriacus* x *H. paramutabilis* hybrid 'OISPAR2' and both parents.

So far, we only observed flowers on OISPAR-2. Flowers had a very light purplish colour with a purplish-red centre and a diameter comparable to *H. paramutabilis* flowers. *H.s.* 'Oiseau Bleu' flowers were violet-blue with a purplish-red centre, while *H. paramutabilis* had white flowers with a red centre. Compared to both parent plants petals of the hybrid were clearly separated from each other (Fig. 8.3).

Figure 8.3. Flower morphology of *H.s.* 'Oiseau Bleu', *H. paramutabilis* and OISPAR2.

8.3.4. AFLP-analysis

The 3 crossing parents used appeared to be very polymorphic. In total, the 5 primer combinations used generated 835 markers that were polymorphic between at least 2 of the 3 crossing parents. *H. paramutabilis* showed 302 completely unique markers; only 61 markers were common with *H. s.* 'Oiseau Bleu' and 57 with *H. s.* 'Red Heart cv'. *H. s.* 'Oiseau Bleu' could be characterised by 146 unique markers and *H. s.* 'Red Heart cv' by 79 markers. *H. s.* 'Oiseau Bleu' and *H. s.* 'Red Heart cv' shared 190 markers that differentiated them from *H. paramutabilis*. These data clearly exemplified the species differences between *H. paramutabilis* and *H. syriacus* and confirmed the data presented by Van Huylenbroeck *et al.* (2000). In Table 8.3 the appearance of AFLP marker bands that were uniquely present in one of the crossing parents is given for the tested offspring. Here we checked in what way markers

that are unique to each of the crossing partners reappear in the offspring enabling to control the effectiveness of the crossing. Table 8.3 clearly demonstrates that an equal share of the markers can be traced back to each of the crossing parents indicating the true hybrid nature of the offspring.

Table 8.3. Distribution of AFLP-markers that are unique to one of the crossing parents after interspecific *H. syriacus* x *H. paramutabilis* pollinations.

	# markers segregating for *H. syriacus* parent		**# markers segregating for *H. paramutabilis* parent**	
Plant	**Unique to *H. s.* parent**	**In offspring plant**	**Unique to *H. p.* parent**	**In offspring plant**
OISPAR-1	336	162	359	177
OISPAR-2	336	157	359	173
OISPAR-3	336	159	359	167
OISPAR-4	336	183	359	177
OISPAR-5	336	153	359	170
OISPAR-6	336	181	359	140
REDPAR-1	359	177	363	199

Fig. 8.4 gives an overall view on the genetic relationships between the plants analysed by AFLP-markers. By calculating the pair wise genetic similarities (Jaccard similarity coefficient) the genetic conformity between the parent plants and the hybrids was determined and represented in a dendrogram.

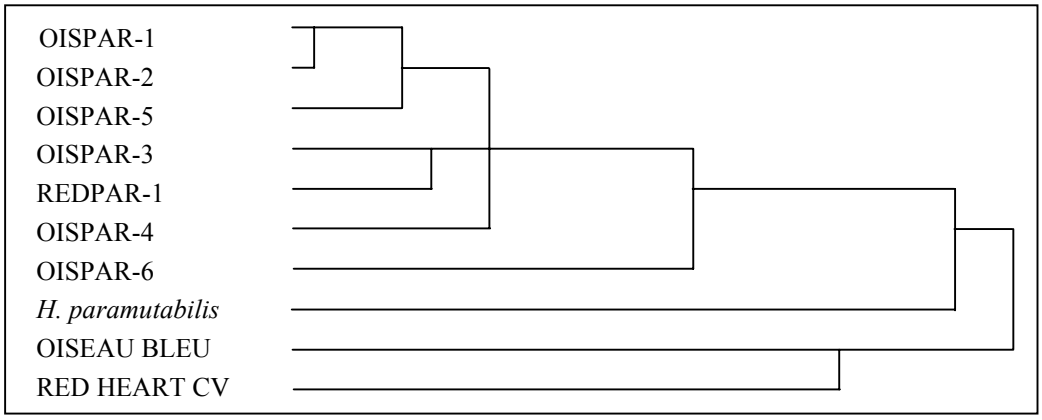


Figure 8.4. Dendrogram showing the genetic relatedness of all interspecific *Hibiscus syriacus* x *H. paramutabilis* seedlings tested by AFLP. The dendrogram was constructed using UPGMA-clustering based on a genetic similarity matrix containing the Jaccard similarity coefficients between pairs of plants.

The group of hybrids came forward as a separate identity that was genetically different from both *H. syriacus* and *H. paramutabilis*. The hybrids were all more related to one other than to one of the crossing parents. REDPAR-1, although derived from a different crossing and being a hexaploid, was also clustered within the group of the tetraploid hybrids derived from *H. s.* 'Oiseau Bleu'. This result could partly be influenced by the fact that AFLP-markers behave as dominant markers only differentiating between absence or presence of the allele.

8.4. Discussion

Though there is no absolute incongruity between *H. syriacus* and *H. paramutabilis*, crossing compatibility is probably only unilateral, using *H.s*. as seed parent (Table 8.1.). Nevertheless, pollen tube growth was not arrested or delayed after either pollination. This was contradictory to reports that mention a slower pollen tube growth after incompatible crosses and a lower number of pollen tubes in the style compared to compatible crosses (Kyung & Kim, 2001a; 2001b).

AFLP and morphological analyses showed *H. paramutabilis* to be clearly distinct from *H. syriacus* (Van Huylenbroeck *et al*., 2000); however, phylogeny gaps between *H. syriacus* and *H. paramutabilis* on the one hand are probably less substantial than between *H. syriacus* and *H. rosa-chinensis* or other tropical *Hibiscus* species (which offer a larger potential for introgression of new traits into *H. syriacus* cv's) on the other hand. *H. syriacus* and *H. paramutabilis* likely outlived a longer common evolution, being native to the same temperate region. This may account for the relative lack of fertilization barriers that occurred after pollination. Hybrid albinism, hybrid vigor and hybrid breakdown were not observed. Fertility of the hybrids could not be determined due to lack of flowers during the first flowering season.

Embryo development (Fig. 8.1.) was slower than during intraspecific *H.s.* crosses, making embryo rescue preferable over *in vivo* sowing. This was in accordance with Song *et al*. (1998), who stated that the embryos obtained in intraspecific crosses in *H. syriacus* normally grow much faster; the torpedo stage is already reached 16 days after pollination. Since embryos could be isolated from the ovules easily, ovule culture seemed useless and was not attempted. From the isolated embryos only some of the torpedo-shaped and cotyledonary ones developed into plantlets. The same phenomenon has been described in the genus *Actinidia* (Hirsch *et al*., 2001).

The hybrids had intermediate characteristics and could be distinguished from either parent on both morphological (Table 8.2.; Fig. 8.2.; Fig. 8.3.) and molecular basis (Table 8.3.; Fig. 8.4.). These results proved that interspecific hybrids between *H. syriacus* and *H. paramutabilis* were obtained. Moreover, for REDPAR-1 (2n=6x) this was confirmed through flow cytometrical ploidy analysis (Table 8.2.). Both growth vigour and leaf morphology (Fig. 8.3.) of *H. paramutabilis* could partly be introduced in the seedlings.

Further observations of plant development and flowering are still required. The hybrid plants will be included in the ongoing breeding programs with *H. syriacus*; whether they are suitable prebreeding material for the development of new cv's remains to be determined. Based upon the observation of flowers and similar work by Kyung & Kim (2001a; 2001b) and Kyung *et al*. (2001a; 2001b) the hybrids have an extensive potential in this respect.

Since regeneration of fused *Hibiscus* protoplasts is so far impossible, interspecific breeding should focus further on overcoming crossing barriers between different species. As during our research incongruity barriers appeared to be insufficient to prevent hybridization, it can be assumed that the formation of hybrid embryos is likely to occur after several interspecific

Hibiscus crosses, although their development may be hampered *in vivo*. In the first place, other breeding partners for *H. syriacus*, including species native to temperate regions (like *H. sinosyriacus*) should be involved to find out whether incongruity barriers are as confined as towards *H. paramutabilis*. Second, when aiming for introgression of a variety of novel flower traits, crossing barriers with tropical *H.* species must be characterized to develop the best possible fertilization and culturing techniques.

9. General discussion, conclusions and perspectives

9.1. Haploid induction

Three main *in vitro* methods are frequently used to generate haploids (Bajaj, 1990): culture of excised ovaries and ovules, embryo culture after wide pollination, and culture of excised anthers and microspores. Androgenesis starting from microspores is the most recommendable way to develop haploids since they do not include maternal tissue that can proliferate, and gametes are abundantly present. Gynogenesis and embryo rescue are usually applied when microspore cultures do not yield embryos, as was the case for all *Spathiphyllum wallisii* cv's tested.

Ferrie *et al.* (1995) mentioned that plant genotype, developmental stage of the gametophyte, nutrient medium composition and culture conditions influence gynogenesis. However, regarding the importance of the developmental stage of the embryo sac, different opinions are formulated (Cappadocia *et al*., 1988; Mukhambetzhanov, 1997). Relatively less attention has been given towards the importance of medium composition and culture conditions. Most authors only agree that the plant genotype is of major importance.

The importance of the genotype is confirmed by our findings on *Spathiphyllum wallisii* (Chapter 3). Ovule culture (Fig. 3.1.) only succeeded for 1 genotype, although other genotypes tested were half-sibs or closely related. When supplied to ovary media, IMA improved the isolation of the ovules. Cytokinins should be avoided in ovule cultures because of their induction of somatic cell proliferation. Developmental stage of the embryo sac and stress conditions were not included in this research as main parameters.

The most remarkable finding on the (spontaneously) doubled haploid plants was the apparent lack of inbreeding depression (Fig. 3.3.). Because of the limited number of plants obtained these results can not be generalized. Doubled haploids were morphologically not aberrant from the original genotypes, and combined data from flow cytometry and molecular fingerprinting were required to distinct both. Flow cytometry revealed instant and spontaneous chromosome doubling immediately after plant conversion (Fig. 3.4.). Since *S. wallisii* is very heterozygous, we expected many EcoRI and MseI restriction sites of the original genotype to be absent in the doubled haploid, as a result of which less amplified DNA-fragments are expected after AFLP. This was reflected in the electropherograms (Fig. 3.2; Fig. 3.5.).

Preliminary experiments (data not presented) were also performed using irradiated pollen as a stimulus for haploid induction. More extended research is required to yield conclusive results; but also this technique appears to have potential for the creation of doubled haploids. The main advantage over 'normal' gynogenesis would be a possibly less genotype dependent parthenogenic response, though we may in turn expect other parameters to become more important.

As a final conclusion it can be stated that although the creation of homozygous *Spathiphyllum wallisii* plants through gynogenesis via ovule culture was possible, the establishment of a

protocol suitable for a large group of cultivars will probably be impossible due to a very significant genotype influence. Although haploid breeding through microspore culture is a far more elegant system, it could so far not be applied for many ornamental crops, including *S. wallisii*.

9.2. Polyploid induction

Though estimates on the amount of natural angiosperm polyploids are very divergent, their evolutionary success is obvious. In fact, polyploidy appears to be the rule rather than the exception. However, 'ancient' polyploids have gone through an extensive process of diploidization and behave like diploids at chromosomal and genomic level due to genomic rearrangements and epigenetic effects (Leitch & Bennett, 1997; Wendel, 2000; Liu & Wendel, 2002). Many species that we regard as diploids, are actually amphipolyploids. For convenience's sake, in this work *Spathiphyllum wallisii* (n=15), *Rhododendron simsii* (n=13) and *Buddleja globosa* (n=19) were considered diploid, whereas *Buddleja davidii* (n=38) was regarded as tetraploid. Contradictory to the herbaceous crop *Spathiphyllum*, *Rhododendron* and *Buddleja* hybrids are woody ornamentals, which have a relatively low rate of polyploidization in nature, possibly through formation of wood fibers in the cambium that constrains (ploidy related) cell size changes (Otto & Whitton, 2000).

The starting material is of utmost importance for the review of the polyploidization techniques applied, and depends on the exact aim of the experiment. For *Spathiphyllum* breeding (Chapter 4), our ultimate goal was to establish an efficient regeneration protocol for tetraploid tissue as an intermediate step towards the creation of triploids. Therefore the starting material was an existing cv. The main objective in *Rhododendron* (Chapter 7) and *Buddleja* (Chapter 5) breeding was to evaluate morphological consequences of polyploidization; therefore chromosome doubling of many different genotypes (seedlings) was attempted. In *Rhododendron*, the efficiency of several techniques was compared, resulting in a higher percentage of tetraploidy after treatment of seedlings (Table 7.1.). Polyploidization rates were exceptionally high in *Buddleja* (Table 5.1.), presumably because it is a more vigorous grower and cells with doubled ploidy level retain a relatively high capacity to divide and grow, whereas 'Growth competition' with diploid cells is higher in *Spathiphyllum* and especially azalea. The success of polyploidization protocols is therefore dependent on their regeneration efficiency.

Throughout all experiments flow cytometry was applied as the only method for ploidy determination (Fig. 4.5.; Fig. 5.4.; Fig. 7.1.). Although results are relative, and should therefore be handled with caution, flow cytometry is a rapid, trustworthy and elegant alternative for the classical counting of chromosomes. Apart from the fact it is rather difficult to establish suitable protocols for chromosome counting, especially for species with high chromosome numbers, and that all published protocols are usually suitable for a very limited number of species, flow cytometry is universally applicable for ploidy determination of a very wide range of plant species (Galbraith, 1990; Arumuganathan & Earle, 1991; Dolezel, 1991; Baert *et al*., 1992). Other polyploidy detection systems are the study of stomatal guard cells, pollen, leaf or flower morphology. However, morphological consequences of polyploidization in *Spathiphyllum*, *Buddleja* and *Rhododendron* were still to be determined at the start of our

experiments. Morphology-based polyploidy detection was therefore not applicable. During our experiments flow cytometry has always shown to be reliable; however, since the exact chromosome number remains unknown careful interpretation of the results is required. It should be kept in mind that the method gives no indications on x (ploidy number) but on c (the physical mass of the genome). For that reason measurements of plants with extended chromosomes, although having the same number, show slightly different results compared to a control plant. In addition, DAPI, the agent responsible for the fluorescence reaction, only binds to A-T nucleotide pairs in the DNA (Otto, 1990). Differences in AT/GC ratios between different genomes can for that reason have an important repercussion on fluorescence observations. *Buddleja globosa* 'Sungold', and its descendants after pollination with *B. davidii*, exemplify the gain of ambiguous results, since 3 possible explanations can be retained for the higher fluorescence of 'Sungold' DNA compared to *B. davidii* (Table 5.4.; Fig. 5.4.). Tetraploidy as well as (near-)pentaploidy, probably arisen after gamete selection or 'meiotic drive', may provide plausible explanations for our observations. Measurements for *Spathiphyllum* and *Rhododendron* were unequivocal.

'Chimerism' holds the stable coexistence of different genotypes within one plant (Tilney-Basset, 1986). The creation of (undesired) ploidy chimeras therefore gives an indication on the efficiency of the polyploidization method and the antimitotic agent applied. Since genotypes bearing 2 different ploidy levels often shifted into solid non-mixoploid plants (most often returning to a complete diploid status), especially in *Buddleja*, the appellation "mixoploid" is more appropriate. In *Spathiphyllum*, mixoploids were not retrieved after regeneration of secondary somatic embryos that were treated with MIs (Table 4.4.). On the other hand, many mixoploids were obtained after treatment of seedlings of the woody ornamentals *Buddleja* and *Rhododendron* (Tables 5.1.; 7.1.; 7.2.; 7.3.), probably due to inhibition of the growth of nascent tetraploid cells in the meristem, whereas growth vigor of neighbouring cells was unaffected, allowing further development from 2 different cell types. Interestingly, mixoploids in pot azalea were more stable than in *Buddleja*, and showed intermediate characteristics. Mixoploids can eventually be used in follow-up breeding programs, provided that the histogenic L_2-layer from which gametes develop is fully tetraploid.

As mitotic disrupter herbicides work in the same way as COL (colchicine), attempts have been made to use them as more efficient and less toxic alternatives to induce tetraploidy in plants. A number of successful attempts have been made with oryzalin, including the doubling of anther-derived maize cultures (Wan *et al*., 1991), beet (Hansen *et al*., 2000) and onion ovule cultures (Geoffriau *et al*., 1997) and haploid apple shoots (Bouvier *et al*., 1994). In all these cases ORY (oryzalin) or TRI (trifluralin) were more efficient than COL. Seedlings of a number of different ornamental species became tetraploid using either ORY or TRI, including species that were recalcitrant to DNA-duplication during COL treatment (Vaughn, 2000). The higher efficiency of ORY and TRI compared to the 'traditional' COL in other plant species is confirmed by our own results. Both herbicides were twice as efficient as COL for the induction of polyploids in *Spathiphyllum* (Table 4.4.); in pot azalea, COL was not efficient at all (Table 7.1.). In course of time, as this difference in efficiencies was observed in all experiments, COL was omitted in further tests. ORY and TRI are only 2 alternatives for COL and belong to the chemical class of dinitroanilines. Next to dinitroanilines, also benzoic

acid, phosphoroamidates and pyridazines are known to interact with microtubule assembly, and could therefore be applied as COL alternatives (Vaughn, 2000). All herbicides mentioned offer the advantage of a higher stability, a lower dose required and a lower toxicity to humans over COL. The higher stability allows lasting exposure compared to colchicine, that is only applicable in relatively short shock treatments. All results obtained in this work encouraged the use of herbicides for *in vitro* induction of polyploids and the avoidance of COL.

The altered morphology of polyploids, probably caused through DNA-level mediated changes at cellular level (Kondorosi *et al*., 2000) offers plenty of motives for polyploid breeding of ornamentals. As mentioned in chapter 1, possible consequences of polyploidy are altered leaf, flower and pollen morphology and affected growth habit. A decreased length/width ratio was observed in all plant species examined and was statistically analyzed for *Rhododendron* (Table 7.4.). Also flower morphology in *Spathiphyllum* and *Rhododendron* was clearly influenced by the ploidy level (Fig. 4.6.; Fig. 7.3.; Table 7.5.). Pollen morphology was not considered. Growth vigor remained rather untouched; probably an eventual decrease is only visualized at higher ploidy levels. It should be mentioned that results were quite divergent when using seeds as starting material; leaf and flower morphology were highly different within polyploidized plants and sometimes hardly aberrant between plants with different ploidy levels, making it difficult to correlate polyploidy with 'typical' morphological properties for a particular species (Table 7.4.; Table 7.5.). In some cases, also growth vigor was negatively influenced but as mentioned above this was rather exceptional. Delay of flowering was never observed. Reduced fertility was noticed in *Rhododendron* but not in *Spathiphyllum*; however, in the latter relatively few genotypes were tested and general conclusions are therefore hard to be drawn.

Considering the 'typical' polyploid characteristics observed in *Rhododendron*, *Spathiphyllum* and *Buddleja* but also described in other species (Goldblatt, 1980; Tal, 1980; Levin, 1983; Leitch & Bennett, 1997) the possible horticultural application of polyploids for ornamental purposes is obvious. For the creation of dwarf growth it seems recommendable to pass through several polyploidization cycles to induce higher ploidy levels. Optimal growth vigor is reached at the so-called 'ploidy optimum', but this evidently is highly dependent on the exact species. The creation of tetraploids can be an intermediate step towards the breeding of triploids; although 'triploid block' may occur because of incompatibilities between endosperm and embryo ploidy level. In our research, the creation of polyploids itself and/or the determination of morphological consequences was emphasized, rather than the establishment of further breeding programs.

Considering the options for further breeding we should not only focus on the creation of autopolyploids. Allopolyploidy is a common phenomenon in (natural) interspecific hybrids and is often induced in synthetic hybrids to restore fertility. Polyploid breeding is therefore strongly linked to the creation of interspecific hybrids (see further), and the development of an efficient technique for autopolyploid chromosome doubling is able to contribute to the development of fertile interspecific hybrids. If necessary, chromosomes can already be doubled at seedling stage although ORY treatment would probably be deleterious for many seedlings, whatever the species. It may be concluded that the polyploidization techniques described in this work offer possibilities for further autopolyploidy breeding as well as for

doubling of interspecific hybrids (when appropriate), although efficiency remains to be improved, especially in *Rhododendron*.

9.3. Interspecific hybridization

Interspecific and intergeneric crosses allow to introduce new genetic variation into cultivated plants. Van Tuyl & De Jeu (1997) considered interspecific hybridization to be the most important source of genetic variation in ornamentals. The introgression of genes may be part of a breeding program towards disease resistance or morphology alteration (Uhlinger, 1982). Different barriers have been met from the first attempts on (Stebbins, 1958). *In vitro* methods allow to develop an integrated procedure for overcoming fertilization barriers, since environmental conditions can be controlled and optimized, allowing maximum repeatability of the experiments. In this work, emphasis was put on *in vitro* 'embryo rescue' as a tool for circumventing postzygotic barriers, mostly due to endosperm malformation; *in vitro* pollination and fertilization was not attempted in *Buddleja* (Chapter 5), *Rhododendron* (Chapter 6) or *Hibiscus* (Chapter 8). As Hermsen (1984) quotes "the statement, that two species are not crossable, is controversial unless a broad genetic variation of the parental species has been used and the cross combinations have been carried out on a large scale under a wide range of environmental conditions". Incongruity is determined by both genetic and environmental factors, and the variation within genotypes and environmental conditions should therefore be substantial.

Fig. 9.1. represents different barriers that may be met after performing an interspecific pollination. After choosing an appropriate combination of parental genotypes, preliminary to the actual pollination, the possible existence of prezygotic barriers can be verified by microscopic evaluation of pollen tube growth in test crosses (Fig. 6.4.). In *Hibiscus* and *Buddleja*, no such impedances were found; in *Rhododendron* they were present (Table 6.6.), as could partly be expected based on literature data (Rouse *et al*., 1993; Ureshino *et al*., 2000). Figure 9.1. enumerates the most common treatments to deal with prezygotic barriers, some of which were attempted in *Rhododendron* crosses, but without success (Table 6.7.). Other treatments were technically unavailable or did not seem opportune at this time. Perhaps emphasis should focus towards environmental effects. Sullivan (1989) reported on the beneficial effect of high temperatures and humidity levels, although he did not provide quantitative data and was rather aiming to increase the efficiency of congruous pollinations. Ploidy differences may be responsible for a disturbed embryo-endosperm equilibrium and thus totally inhibit the development of hybrids; however, in our research this was not the case. On the contrary, different nuclear DNA-contents provided a possibility for hybrid screening owing to intermediate DNA-content in the hybrid, as was the case in *Rhododendron* (Fig. 6.9; Fig. 6.10.).

Spontaneous abortion was mainly a problem after *Hibiscus* and *Rhododendron* interspecific crosses, as was lack of germination of the immature seeds (Table 6.20.; Table 8.1.). The latter was probably due to endosperm malformation, although this was not quantified. The importance of 'genic balance' to endosperm development is in fact known to be highly species dependent (Johnston *et al*., 1980). Endosperm development was certainly not totally inhibited (Fig. 6.6; Fig. 6.7.) but the formation of viable seeds was significantly reduced. This

urged the introduction of an *in vitro* step to save hybrid embryos from abortion and/or to allow further maturation. Neither *Buddleja*, *Rhododendron* or *Hibiscus* requested the use of complex media, but germination efficiency in *Rhododendron* nevertheless was clearly increased by 'embryo rescue' after interspecific pollination (Tables 6.8 – 6.15.).

Albinism, whether total, variegated or virescent, may be a major barrier for the creation of viable hybrids. Although it was no obstacle during the experiments with *Buddleja* or *Hibiscus*, it obviously was after several intersubgeneric *Rhododendron* combinations (Fig. 6.6.; Fig. 6.7.; Table 6.20.). This agreed with available literature; however, the latter concentrates mainly on deciduous azaleas, whereas relatively few attention is given towards virescent albinism that occurs after other genotypic combinations. Since albinism is caused by nucleocytoplasmatic incompatibility, the most obvious approach would be to perform the reciprocal cross; however, this may be hampered by other barriers, even earlier in the fertilization process, as is the case in Pentanthera x Tsutsusi pollinations (Table 6.6.). Albinism is evidently associated with low autotrophy and slow growth, and therefore represents a major obstacle towards the creation of viable interspecific hybrids in many crops. Decreased 'hybrid' vigor, not due to inhibited chloroplast development but rather to imperfect assembly of protein subunits or unbalanced new gene combinations, causing multiple and diverse aberrations, seemed absent in *Hibiscus* and *Buddleja*; in *Rhododendron*, the inhibition of growth vigor is obvious in some crosses but absent in others (Fig. 6.8.)

Confirmation of the hybrid genotype in both *Buddleja* and *Hibiscus* could fluently be performed by the use of AFLP-markers (Fig. 5.3.; Table 5.3.; Table 8.3.; Fig. 8.4.) that are obviously more applicable than morphological markers, which could only be used in *Hibiscus* (Table 8.2.; Fig. 8.2.; Fig. 8.3.). AFLP is known as a fast and reliable molecular marker technique for assessing genetic conformity for both the study and conservation of natural diversity and for the characterization of breeding gene pools, e.g. in soybean (Maughan *et al*., 1996), lettuce (Hill *et al*., 1996), tea (Paul *et al*., 1997), sunflower (Hongtrakul *et al*., 1997), maize (Ajmone Marsan *et al*., 1998), *Allium* (De Clercq *et al*., 1999) and azalea (De Riek *et al*., 1999), and during this study demonstrated its efficiency in paternity testing. Over many other molecular markers, it offers the advantage that no priory sequence information is required. Moreover, the restriction fragments amplified by a single primer combination often yield several DNA-polymorphisms.

In *Rhododendron* however, positive results from AFLP-analysis were only obtained for Vireya x Tsutsusi crosses (Fig. 6.11.; Fig. 6.12.; Table 6.17.). AFLP proximity matrices were strikingly similar to the seed parent matrices. SSR-analysis provided proof for the interspecific character of half of the seedlings tested (Table 6.18.; Fig. 6.13.). Most of those hybrids were not tested through AFLP-analysis. However, it is quite obvious that AFLP is more time-consuming, as well for the practical procedure as for the interpretation of data. Moreover, comparison of seed parent, pollen parent and progeny through SSR-electropherograms directly visualizes Mendelian inheritance of the fragments, thanks to the codominant character of the technique. Provided suitable SSR primer combinations are available, SSR therefore is preferable over AFLP for molecular screening of interspecific hybrids.

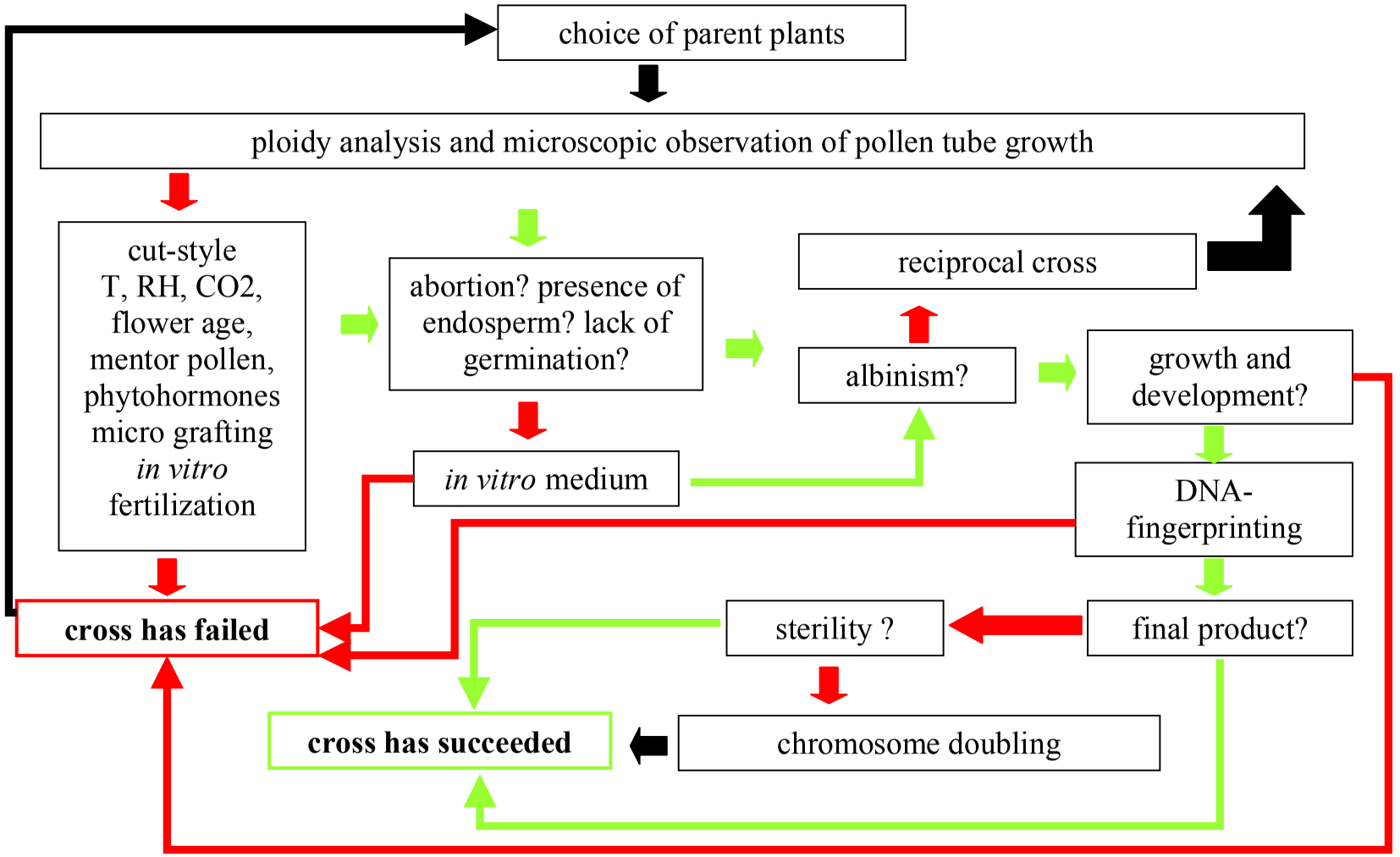


Figure 9.1. Possible prefertilization and postfertilization barriers that occur due to incongruity after interspecific pollination (→ no barrier, → barrier)

F_1-sterility of interspecific hybrids is very common and may, among others, be the consequence of reduced chromosome pairing during meiosis. Breeding at polyploid levels is widely used in interspecific hybridization programmes of many ornamental crops such as *Alstroemeria*, *Dendranthema*, *Freesia*, *Gladiolus* and *Lilium* (Van Tuyl & De Jeu, 1997). Crossability between parents may be improved in the process of plant breeding by equalizing their functional ploidy level. Allopolyploids may function as fertile bridges for gene introgression into the cultivar assortment (Hermsen, 1984).

Two major techniques can be applied in order to create allopolyploids. Somatic (mitotic) chromosome doubling may allow homologous pairing of chromosomes and thus restore fertility. The degree of fertility of interspecific *Buddleja*, *Hibiscus* and *Rhododendron* hybrids remains to be determined. However, since most incongruous reactions were noted in *Rhododendron*, F_1 sterility can be expected to create major obstacles for further breeding.

To overcome these problems, somatic chromosome doubling techniques have already been developed (9.2.; Chapters 4, 5 & 7). However, gametes originating from allopolyploids have been created after homologous pairing of chromosomes and therefore do not allow gene introgression from homoeologous chromosomes.

Evidently, 2n gametes would provide a better tool for allopolyploidization since FDR, SDR and IMR allow homoeologous recombination (Mendiburu & Peloquin, 1977; Karlov *et al*., 1999). Unreduced gametes occur in most plant species (Harlan & de Wet, 1975; Veilleux, 1985; Lamote, 2002) and also in doubled haploids and interspecific hybrids (Crespel *et al*., 2002; Lim *et al*., 2003). In *Lilium*, the formation of 2n gametes is dramatically increased upon hybrid formation (Van Tuyl *et al*., 1989), a phenomenon that is also observed in other crops (Van Tuyl & De Jeu, 1997). Therefore 2n gamete formation would provide a very convenient tool for efficient introgression of desired genes in F_2 interspecific hybrids. No specific literature data on 2n gametes in *Rhododendron*, *Buddleja* or *Hibiscus* could be obtained.

From chapter 1, it is obvious that a myriad of genetic and epigenetic reactions may be induced after allopolyploid formation. Their characterization was no part of the intention of this work; however, it is quite obvious that, next to 'normal' genome additivity or gene introgression, karyotypic stabilization owing to rapid chromosomal repatterning, translocations, sequence elimination, transposon activity and epigenetic effects may largely affect phenotypic behavior of the hybrids obtained, possibly yet at the diploid level. The extent to which these phenomena act is very dependent on the plant species and remains to be determined for the crops involved in this work. Anyhow, the creation of interspecific hybrids, along with chromosome doubling technology, offers extended opportunities for ornamental breeders. Incomprehensibly, relatively few research has been performed on this topic during the last few years. A better understanding of allopolyploid/interspecific hybrid associated phenomena would undoubtedly lead to substantial innovation in the commercial assortment of many ornamentals. *In vitro* techniques can play an important part in improving the overall efficiency of these processes in many crops.

Appendix

The present thesis was partly redrafted after the following papers.

EECKHAUT T, SAMYN G, VAN BOCKSTAELE E (2001a). *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. Acta Horticulturae, 572: 43-49.

EECKHAUT T, WERBROUCK S, DENDAUW J, VAN BOCKSTAELE E, DEBERGH P (2001b). Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell, Tissue and Organ Culture, 67: 181-189.

EECKHAUT T, WERBROUCK S, LEUS L, VAN BOCKSTAELE E, DEBERGH P. Polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. Submitted to Plant Cell Tissue and Organ Culture.

EECKHAUT T, LEUS L, DE RAEDT A, VAN BOCKSTAELE E. Occurrence of polyploidy in *Rhododendron luteum* Sweet, hardy Ghent and Rustica hybrids. Submitted to South African Journal of Botany.

WERBROUCK S, EECKHAUT T, DEBERGH P (2000). Induction and conversion of somatic embryogenesis on the anther filament of *Spathiphyllum* Schott. Acta Horticulturae, 520: 263-269.

References

ADANIYA S, SHIRAI D (2001). *In vitro* induction of tetraploid ginger (*Zingiber officinale* Roscoe) and its pollen fertility and germinability. Scientia Horticulturae, 88: 277-287.

AHLOOWALIA B (1998). Radiation techniques in crop and plant breeding. Quarterly Journal of the International Atomic Energy Agency, 40: 37-39.

AHMAD F, COMEAU A (1990). Wheat X pearl millet hybridization: consequence and potential. Euphytica, 50: 181-190.

AHMED S (1992). Recovery of fertile backcross derivatives in a sterile interspecific hybrid of *Hibiscus* (in Bangladesh). Plant Tissue Culture, 2: 35-39.

AJMONE MARSAN P, CASTIGLIONI P, FUSARI F, KUIPER M, MOTTO M (1998). Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. Theoretical and Applied Genetics, 96: 219-227.

ALEMANNO L, BERTHOULY M, MICHAUX-FERRIERE N (1996). Histology of somatic embryogenesis from floral tissues of cocoa. Plant Cell, Tissue and Organ Culture, 46: 187-194.

ANDERSON W (1975). Propagation of *Rhododendrons* by tissue culture: Part I. Development of a culture medium for multiplication of shoots. Proceedings of the International Plant Propagation Society, 25: 129-135.

ANDERSON W (1980). Tissue culture propagation of red and black raspberries, *Rubus idaeus* and *Rubus occidentalus*. Acta Horticulturae, 112: 13.

ANDERSON W (1984). A revised tissue culture medium for shoot multiplication of *Rhododendron*. Journal of the American Society for Horticultural Science, 109: 343-347.

ARISUMI K, SAKATA Y, MIYAJIMA I (1985). Studies on the flower colours in *Rhododendron*. Memoirs of the Faculty of Agriculture, Kagoshima University, 21: 133-147.

ARUMUGANATHAN K, EARLE E (1991). Estimation of nuclear DNA content of plants by flow cytometry. Plant Molecular Biology Reports, 9: 229-233.

ARZATE-FERNANDEZ A, NAKAZAKI T, TANISAKA T (1998). Production of diploid and triploid interspecific hybrids between *Lilium concolor* and *L. longiflorum* by *in vitro* ovary slice culture. Plant Breeding, 117: 479-484.

ASANO Y, MYODO H (1977). Studies on crosses between distantly related species of lilies: the culture of immature hybrid embryos. Journal of the Japanese Society for Horticultural Science, 46: 267-273.

ATANASSOV A, ZAGORSKA N, BOYADJIEV P, DJILIANOV D (1995). *In vitro* production of haploid plants. World Journal of Microbiology & Biotechnology, 11: 400-408.

AWOLEYE F, VAN DUREN M, DOLEZEL J, NOVAK FJ (1994). Nuclear DNA content and *in vitro* induced somatic polyploidisation cassava (*Manihot esculenta* Crantz) breeding. Euphytica, 76: 195-202.

AYALA F, FITCH W, CLEGG M (2000). Variation and evolution in plants and microorganisms: toward a new synthesis 50 years after Stebbins. Proceedings of the National Academy of Sciences, 97: 6941-6944.

BADAMI P, MALLIKARJUNA N, MOSS J (1997). Interspecific hybridization between *Cicer arietinum* and *C. pinnatifidum*. Plant Breeding, 116: 393-395.

BADGER B (1988). In search of a yellow evergreen azalea (how to hybridize for a yellow evergreen azalea). Journal of the American Rhododendron Society, 42: 74-79.

BAERT J, REHEUL D, VAN BOCKSTAELE E, DE LOOF A (1992). A rapid method by flow cytometry for estimating persistence of tetraploid perennial ryegrass in pasture mixtures with diploid perennial ryegrass. Biologia Plantarum, 34: 381-385.

BAKER R, HASENSTEIN K, ZAVADA M (1997). Hormonal changes after compatible and incompatible pollination in *Theobroma cacao* L. HortScience, 32: 1231-1234.

BAJAJ Y (1990). *In vitro* production of haploids and their use in cell genetics and plant breeding. In: Bajaj Y (ed) Biotechnology in Agriculture and Forestry. Springer, Berlin, Vol 12: 3-44.

BALUSKA F, KUBICA S (1992). Relationship between the content of basic nuclear proteins, chromatin structure, rRNA trancription and cell size in different tissues of the maize root apex. Journal of Experimental Botany, 252: 991-996.

BATES D (1965). Notes on the cultivated Malvaceae. 1. *Hibiscus*. Baileya, 13: 57-130.

BAUMEL A, AINOUCHE M, KALENDAR R, SCHULMAN A (2002). Retrotransposons and genomic stability in populations of the young allopolyploid species *Spartina anglica* C.E. Hubbard (Poaceae). Plant Molecular Biology, 19: 1218-1227.

BEAN W (1989a). Trees & shrubs hardy in the British Isles: 8th Edition, Volume I: A-C. John Murray, London, 845 pp.

BEAN W (1989b). Trees & shrubs hardy in the British Isles: 8th Edition, Volume II: D-M. John Murray, London, 784 pp.

BENNETT M, LEITCH I (1995). Nuclear DNA amount in angiosperms. Annals of Botany, 76: 113-176.

BERG J, HEFT L (1991). *Rhododendron* und immergrüne Laubgehölze. Ulmer, Stuttgart, 272 pp.

BLAKESLEE A (1937). Dédoublement du nombre de chromosomes chez les plantes par traitement chimique. Comptes Rendus de l' Academie des Sciences, 205: 476-479.

BOHANEC B (1994). Induction of gynogenesis in agricultural crops: a review. Proceedings of the International Colloquium on Impact of Plant Biotechnology on Agriculture, Rogla, Slovenia, December 5th-7th, 1994: 43-55.

BOHANEC B, JAKSE M, IHAN A, JAVORNIK B (1995). Studies of gynogenesis in onion: induction procedures and genetic analysis of regenerants. Plant Science, 104: 215-224.

BOUHARMONT J, DABIN P, HEURSEL J (1988). Studie van het geslachtelijk voortplantingssysteem bij azalea en *Rhododendron*. Landbouwtijdschrift, 41: 1117-1127.

BOUVIER L, FILLON F, LESPINASSE Y (1994). Oryzalin as an efficient agent for chromosome doubling of haploid apple shoots *in vitro*. Plant Breeding, 113: 343-346.

BOUVIER L, ZHANG Y & LESPINASSE Y (1993). Two methods of haploidization in pear, *Pyrus communis* L.: greenhouse seedling selection and in situ parthenogenesis induced by irradiated pollen. Theoretical and Applied Genetics, 87: 229-232.

BOWERS C (1936). Rhododendrons and azaleas. The MacMillan Company, New York, 525 pp.

BRAND M, KIYOMOTO R (1997). The induction of tissue proliferationlike characteristics in *in vitro* cultures of *Rhododendron* 'Montego'. HortScience, 32: 989-994.

BRANDHAM P (1982). Inter-embryo competition in the progeny of autotriploid *Aloineae* (Liliaceae). Genetica, 59: 29-42.

BROWN D, THORPE T (1995). Crop improvement through tissue culture. World Journal of Microbiology & Biotechnology, 11: 409-415.

BURCHI G, MERCURI A, BIANCHINI C, BREGLIANO R, SCHIVA T (1998). New interspecific hybrids of *Alstroemeria* obtained through *in vitro* embryo-rescue. Acta Horticulturae, 508: 233-235.

BUZGO M (1996). Euglossinenbestäubung bei *Spathiphyllum*. Briefe aus dem Botanischen Garten Zürich, 30: 1-2.

CAO M, LI Y, LIU F, DORE C (1994). Embryogenesis and plant regeneration of pakchoi (*Brassica rapa* L. ssp. *chinensis*) via *in vitro* isolated microspore culture. Plant Cell Reports, 13: 447-450.

CAMPION B, ALLONI C (1990). Induction of haploid plants in onion (*Allium cepa* L.) by *in vitro* culture of unpollinated ovules. Plant Cell Tissue and Organ Culture, 20: 1-6.

CAMPION B, AZZIMONTI M, VIANI E, SCHIAVI M, FALAVIGNA A (1992). Advances in haploid plants induction in onion (*Allium cepa* L.) through *in vitro* gynogenesis. Plant Science, 86: 97-104.

CAPPADOCIA M, CHRETIEN L, LAUBLIN G (1988). Production of haploids in *Gerbera jamesonii* via ovule culture. Canadian Journal of Botany, 66: 1107-1110.

CAPUANA M, DEBERGH P (1997). Improvement of the maturation and germination of horse chestnut somatic embryos. Plant Cell Tissue and Organ Culture, 48: 23-29.

CEBOLLA A, VINARDELL J, KISS E, OLAH B, ROUDIER F, KONDOROSI A, KONDOROSI E (1999). The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy-dependent cell enlargement in plants. EMBO Journal, 18 : 4476-4484.

CHABOUD A, PEREZ R (1992). Generative cells and male gametes: isolation, physiology, and biochemistry. In: Russell S, Dumas De Vaulx C (eds) Sexual reproduction in flowering plants. International Review of Cytology, 140. San Diego, Academic Press: 205-229.

CHAKRABORTI S, VIJAYAN K, ROY B, QADRI S (1998). *In vitro* induction of tetraploidy in mulberry (*Morus alba* L.). Plant Cell Reports, 17: 799-803.

CHAMBERLAIN D, HYAM G, ARGENT G, FAIRWEATHER G, WALTER KS (1996). The genus *Rhododendron*. Its classification & synonymy. Royal Botanical Garden Edinburgh, 181 pp.

CHAMBERLAIN D, RAE S (1990). A revision of *Rhododendron* IV subgenus Tsutsusi. Edinburgh Journal of Botany, 42: 89-200.

CHANG Z, HONGYUAN Y, HUIQIAO T, ZHONGLAI L, HUA Y (1986). *In vitro* culture of unpollinated ovaries in *Oryza sativa* L. In: Hu H, Yang H (eds) Haploids of higher plants *in vitro*. China Academic Publishers, Beijing: 165-181.

CHAUDHURY A, MING L, MILLER C, CRAIG S, DENNIS E, PEACOCK W (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences, 94: 4223-4228.

CHEN F, KUEHNLE A (1999). Micropropagation of *Spathiphyllum* cv. Sensation from in vitro etiolated internode explants. In: XVI International Botanical Congress, St Louis, Book of abstracts, nr 2383.

CHEN J, STAUB J, ADELBERG J, LEWIS S, KUNKLE B (2002). Synthesis and preliminary characterization of a new species (amphidiploid) in *Cucumis*. Euphytica, 123: 315-322.

CHEN L (1992). Studies on increasing the induction frequency of embryoids in anther culture of citrus. Scientia Agricultura Sinica, 25: 90-91.

CHEN Z, PIKAARD C (1997). Epigenetic silencing of RNA polymerase I transcription : a role for DNA methylation and histone modification in nucleolar dominance. Genes and Development, 11 : 2124-2136.

CHI H (2002). The efficiencies of various embryo rescue methods in interspecific crosses of *Lilium*. Botanical Bulletin of Academica Sinica Taipei, 43: 139-146.

COHEN D, YAO J (1996). *In vitro* chromosome doubling of nine *Zantedeschia* cultivars. Plant Cell Tissue and Organ Culture, 47: 43-49.

COMAI L (2000). Genetic and epigenetic interactions in allopolyploid plants. Plant Molecular Biology, 43: 387-399.

COMAI L, TYAGI A, WINTER K, HOLMES-DAVIS R, REYNOLDS S, STEVENS Y, BYERS B (2000). Phenotypic instability and rapid gene silencing in newly formed *Arabidopsis* allotetraploids. The Plant Cell, 12: 1551-1567.

CORRIVEAU J, COLEMAN A (1988). Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. American Journal of Botany, 75: 1443-1458.

COUMANS M, ZHONG D (1995). Doubled haploid sunflower (*Helianthus annuus*) plant production by androgenesis: fact or artifact? Part 2. *In vitro* isolated microspore culture. Plant Cell, Tissue and Organ Culture, 41: 203-209.

COX K (2000). Rhododendrons en azalea's. Schuyt en co, Haarlem, 128 pp.

COX P, COX K (1995). Encyclopedia of *Rhododendron* hybrids. Batsford Ltd, London, 318 pp.

COX P, COX K (1997). Encyclopedia of *Rhododendron* species. Glendoick Publishing, Perth, 396 pp.

CRESPEL L, GUDIN S, MEYNET J, ZHANG D (2002). AFLP-based estimation of 2n gametophytic heterozygosity in two parthenogenetically derived dihaploids of *Rosa hybrida* L. Theoretical and Applied Genetics, 104: 451-456.

CUEVAS J, RALLO L, RAPOPORT HF, LAVEE S, KLEIN I (1994). Staining procedure for the observation of folive pollen tube behaviour. Acta Horticulturae, 356: 264-267.

CULLEN J (1991). The logic of *Rhododendron* classification. Rhododendrons (1992). The Royal Horticultural Society London, 44: 14-19.

CUNNINGHAM F, GANTT E (1998). Genes and enzymes of carotenoid biosynthesis in plants. Annual Reviews of Plant Physiology and Plant Molecular Biology, 49: 557-583.

CUNY F, GROTTLE M, DUMAS DE VAULX R, RIEU A (1993). Effects of gamma irradiation of pollen on parthenogenetic haploid production in muskmelon (*Cucumis melo* L). Environmental and Experimental Botany, 33: 301-312.

DAI C, LAMBETH V, TAVEN R, MERTZ D (1987). Micropropagation of *Rhododendron prinophyllum* by ovary culture. HortScience, 22: 491-493.

DARLINGTON C, WYLIE A (1955). Chromosome atlas of flowering plants. University Press, Aberdeen, 519 pp.

DAVIDIAN H (1982). The *Rhododendron* species, Vol I. Lepidotes. Timber Press Portland, Oregon, 431 pp.

DAVIDIAN H (1989). The *Rhododendron* species, Vol II. Elepidote species, series Arboreum-Lacteum. Batsford Ltd, London, 344 pp.

DAVIDIAN H (1992). The *Rhododendron* species, Vol III. Elepidotes continued. Timber Press Portland, Oregon, 381 pp.

DAVIDIAN H (1995). The *Rhododendron* species, Vol IV. Azaleas. Timber Press Portland, Oregon,184 pp.

DAVIERWALA A, RAMAKRISHNA W, RANJEKAR P, GUPTA V (2000). Sequence variations at a complex microsatellite locus in rice and its conservation in cereals. Theoretical and Applied Genetics, 101: 1291-1298.

DEBERGH P (2002). Biotechnology plant. Opleidingsonderdeel, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Gent, 221 pp.

DE CLERCQ H, BAERT J, VAN BOCKSTAELE E (1999). Breeding potential of Belgian landraces of leak (*Allium ampeloprasum* var. *porrum*). Euphytica, 106: 101-109.

DE COOMAN L, EVERAERT E, FACHE P, VANDECASTEELE K, VAN SUMERE C (1993). Flavonoid biosynthesis in petals of *Rhododendron simsii*. Phytochemistry, 33: 1419-1426.

DE JEU M (2000). *In vitro* techniques for ornamental breeding. Acta Horticulturae, 508: 55-60.

DE JEU M, JACOBSEN E (1995). Early postfertilization ovule culture in *Alstroemeria L*. and barriers to interspecific hybridization. Euphytica, 86: 15-23.

DE JONG A, SCMIDT E, DE VRIES S (1993). Early events in higher-plant embryogenesis. Plant Molecular Biology, 22: 367-377.

DE KEYSER E, DENDAUW J, DE RIEK J, VAN BOCKSTAELE E (2001). Characterization of carotenoid biosynthesis in *Rhododendron luteum*. Mededelingen van de Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, 66: 439-441.

DE LOOSE R (1969). The flower pigments of the Belgian hybrids of *Rhododendron simsii* and other species and varieties from *Rhododendron* subseries *obtusum*. Phytochemistry, 8: 253-259.

DE LOOSE R (1979). Characterisation of *Rhododendron simsii* Planch. Cultivars by flavonoid & isozyme markers. Scientia Horticulturae, 11: 175-182.

DE NETTANCOURT D (1977). Incompatibility in angiosperms. Springer, Berlin, 230pp.

DE NETTANCOURT D (1997). Incompatibility in angiosperms. Sexual Plant Reproduction, 10: 185-199.

DE PROFT M, MAENE L, DEBERGH P (1985). Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. Physiologia Plantarum, 65: 375-379.

DE RAEDT A, DE GROOTE S (2000). De harde Gentse. Geers Offset, Oostakker, 189 pp.

DE RIEK J, DENDAUW J, MERTENS M, DE LOOSE M, HEURSEL J, VAN BOCKSTAELE E (1999). Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas. Theoretical and Applied Genetics, 99: 1155-1165.

DE RIEK J, DE SCHEPPER S, MERTENS M, VAN BOCKSTAELE E, HEURSEL J, DE LOOSE M (1997). The application of RAPD markers for the identification of *Rhododendron* species. Archives International Physiology Biochemistry, 104: 9.

DE RIEK J, MERTENS M, DENDAUW J, HEURSEL J, VAN BOCKSTAELE E, DE LOOSE M (2000). The use of fluorescent AFLP to assess genetic conformity of a breeders collection of *R. simsii* hybrids. Acta Horticulturae 508: 99-104.

DERMUL D (1989). Etude d'un croisement interspecifique dans le genre *Rhododendron*: *R. simsii Planch.* x *R. lutescens* Franchet. Afstudeerwerk, Faculte des sciences, Universite Catholique de Louvain.

DE SCHEPPER S (2001). Molecular analysis of the induction of sports in azalea (*Rhododendron simsii* hybrids). Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences, Cell and Gene Biotechnology, UG, 173 pp.

DE SCHEPPER S, LEUS L, MERTENS M, VAN BOCKSTAELE E, DEBERGH P, DE LOOSE M (2001). Flow cytometric analysis of ploidy in Rhododendron (subgenus Tsutsusi). HortScience, 36: 125-127.

DE SCHEPPER S, LEUS L, EECKHAUT T, VAN BOCKSTAELE E, DEBERGH P, DE LOOSE M (2003). Somatic polyploid picotee flowers: new roads to tetraploidy for Belgian pot azaleas. Plant Cell Tissue and Organ Culture, accepted for publication.

DE WIT J, ESENDAM H, STRACERS L, AAPOLA A, ELOMAA P, HONKANEN J, LJUNGBERG H, SEPPÄNEN P, TÖRMÄLÄ T (1990). Production of haploid *Gerbera* clones through ovule culture. Poster Symposium EUCARPIA Section Ornamentals 10-14 November 1990, Wageningen, The Netherlands.

DHAWAN O, LAVANIA U (1996). Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica, 87: 81-89.

DOCTRINAL M, SANGWAN R & SANGWAN-NORREEL B (1989). *In vitro* gynogenesis in *Beta vulgaris* L. Effect of plant growth regulators, temperature, genotypes and season. Plant Cell Tisssue and Organ Culture, 17: 1-12.

DOHYA N, MATSUBARA S, MURAKAMI K (1997). Callus formation and regeneration of adventitious embryos from celery microspores by anther and isolated microspore cultures. Journal of the Japanese Society for Horticultural Science, 65: 747-752.

DOLEZEL J (1991). Flow cytometric analysis of nuclear DNA content in higher plants. Phytochemical analysis, 2: 143-154.

DOLEZEL J, BINAROVA P, LUCRETTI S (1989). Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum, 31: 113-120.

DOYLE J, DOYLE JL (1987). Genomic Plant DNA preparation from fresh tissue – CTAB method. Phytochemistry Bulletin, 19: 11.

DUNWELL J (1996). Microspore culture. In: Mohan S, Sopory S, Veilleux R (eds) Current plant science and biotechnology in agriculture: *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, vol 1: 205-216.

EECKHAUT T (1999). Studiecentrum voor *in vitro* veredeling van sierplanten. Overeenkomst nr. D 1/2- 5816A. Verslag van de werkzaamheden van 01/02/98 tot 01/09/99 (2e conventie).

EECKHAUT T T, SAMYN G, VAN BOCKSTAELE E (2001a). *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. Acta Horticulturae, 572: 43-49.

EECKHAUT T, WERBROUCK S, DENDAUW J, VAN BOCKSTAELE E, DEBERGH P (2001b). Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell, Tissue and Organ Culture, 67: 181-189.

EGOLF D (1970). *Hibiscus syriacus* 'Diana' a new cultivar (Malvaceae). Baileya, 17: 75-78.

EGOLF D (1981). Meet 'Helene' a new *Hibiscus syriacus*. American Nurseryman, 154: 11-67.

EISELEIN J (1994a). An improved chromosome staining method applied to the study of colchicine effects in *Rhododendron*. Journal of the American Rhododendron Society, 48: 143-146.

EISELEIN J (1994b). A study of chromosome yields and growth responses in colchicine treatment. Journal of the American Rhododendron Society, 48: 205-209.

ESNARD J, FERWERDA F, RIVERA-AMADOR E, HEPPERLY P (1993). Induction of tetraploidy in the tanier cultivar 'Inglesa' (*Xanthosoma sagittifolium* (L.) Schott). Plant Breeding, 111: 335-338.

ETTINGER T, PREECE J (1985). Aseptic micropropagation of *Rhododendron* P.J.M. hybrids. Journal of Horticultural Science, 60: 269-274.

FAIRWEATHER C (1988). Azaleas. Century Hutchinson Ltd, London, 96 pp.

FALQUE M, KODIA A, SOUNIGO O, ESKES A, CHARRIER A (1992). Gamma-irradiation of cacao (*Theobroma cacao* L.) pollen: effect on pollen grain viability, germination and mitosis and on fruit set. Euphytica, 64: 167-172.

FAURE N, SERIEYS H, KAAN F, BERVILLE A (2002). Partial hybridization in crosses between cultivated sunflower and the perennial *Helianthus mollis*: Effect of *in vitro* culture compared to natural crosses. Plant Cell Reports, 20: 943-947.

FELDMAN M, LIU B, SEGAL G, ABBO S, LEVY A, VEGA J (1997). Rapid elimination of low-copy DNA sequences in polyploid wheat: a possible mechanism for differentiation of homoelogous chromosomes. Genetics, 147: 1381-1387.

FENG Q, STALKER H, PATTEE H (1996). Plant recovery of selfs and interspecific hybrids of *Arachis* by *in vitro* culture of peg tips. Crop Science, 36: 1660-1666.

FERRANT V, BOUHARMONT J (1994). Origin of gynogenetic embryos of *Beta vulgaris* L. Sexual Plant Reproduction, 7: 12-16.

FERRIE A, PALMER C, KELLER W, THORPE T (1995). Haploid embryogenesis. In: Thorpe T (ed). *In vitro* embryogenesis in plants. Kluwer Academic Publishers, Dordrecht: 309-344.

FICCADENTI N, SESTILI S, ANNIBALI S, DI MARCO M, SCHIAVI M (1999). *In vitro* gynogenesis to induce haploid plants in melon *Cucumis melo* L. Journal of Genetics and Breeding, 53: 255-257.

FIROOZABADY E, MOY Y, COURTNEY-GUTTERSON N, ROBINSON K (1994). Regeneration of transgenic rose (*Rosa hybrida*) plants from embryogenic tissue. Bio/Technology, 12: 609-613.

FONNESBECH M, FONNESBECH A (1979). *In vitro* propagation of *Spathiphyllum.* Scientia Hort, 10: 21-25.

FRANKLIN-TONG V (1999). Signaling and the modulation of pollen tube growth. Plant Cell, 11: 727-738.

FRANKLIN-TONG V, FRANKLIN F (2000). Self-incompatibility in *Brassica*: the elusive pollen S gene is identified! Plant Cell, 12: 305-308.

GALBRAITH D, HARKINS K, MADDOX J, AYRES N, SHARMA D, FIROOZABADY E (1983). Rapid flow cytometrical analysis of the cell cycle in intact plant tissues. Science, 220: 1049-1051.

GALBRAITH D (1990). Flow cytometric analysis of plant genomes. Methods in Cell Biology, 33: 549-561.

GALITSKI T, SALDANHA A, STYLES C, LANDER E, FINK G (1999). Ploidy regulation of gene expression. Science, 285: 251-254.

GALLE F (1987). Azaleas. Timber Press Portland, Oregon, 519 pp.

GALLUSCI P, VAROTTO S, MATSUOKO M, MADDALONI M, THOMPSON R (1996). Regulation of cytosolic pyruvate, orthophospate dikinase expression in developing maize endosperm. Plant Molecular Biology, 31: 45-55.

GAMBORG O, MILLER R, OJIMA K (1968). Nutrient requirements of suspension cultures of soybean roots cells. Experimental Cell research, 50: 151-158.

GAUDE T, MCCORMICK S (1999). Signaling in pollen-pistil interactions. Seminars in Cell and Developmental Biology, 10: 139-147.

GEOFFRIAU E, KAHANE R, BELLAMY C, RANCILLAC M (1997a). Ploidy stability and *in vitro* chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). Plant Science, 122: 201-208.

GEOFFRIAU E, KAHANE R, RANCILLAC M (1997b). Variation of gynogenesis ability in onion (*Allium cepa* L.). Euphytica, 94: 37-44.

GEORGE E (1993). Plant propagation by tissue culture. Part 1. The technology. Exegetics Ltd, London, 574 pp.

GERMANA M, CHIANCONE B (2001). Gynogenetic haploids of *Citrus* after *in vitro* pollination with triploid pollen grains. Plant Cell Tissue and Organ Culture, 66: 59-66.

GMITTER F, LING X (1991). Embryogenesis *in vitro* and nonchimeric tetraploid plant recovery from undeveloped *Citrus* ovules treated with colchicine. Journal of the American Society for Horticultural Science, 116: 317-321.

GOLDBLATT P (1980). Polyploidy in angiosperms. In: Lewis W (ed) Polyploidy. Plenum press, New York: 219-239.

GOMEZ L, VALVERDE R, ARIAS O, THORPE T (1992). Regeneration of *Xanthosoma sagittifolium* by somatic embryogenesis. Agronomica Costarricense, 16: 219-223.

GRIESBACH R (1987). *Rhododendron* flower color: genetic/cultural interactions. Journal of the American Rhododendron Society, 41: 20-21.

GRILLAERT G (2003). De sierteelt klimt verder uit het dal. Verbondsnieuws, 47: 15-16.

GRUISSEM W (1989). Chloroplast gene expression: how plants turn their plastids on. Cell, 56: 161-170.

GUNDIMEDA H, PRAKASH S, SHIVANNA K (1992). Intergeneric hybrids between *Enarthrocarpus lyratus*, a wild species, and crop brassicas. Theoretical and Applied Genetics, 83: 655-662.

GUO M, DAVIS D, BIRCHLER J (1996). Dosage effects on gene expression in a maize ploidy series. Genetics, 142: 1349-1355.

HA S, MAEKAWA M, KITA F, KINOSHITA T (1998). Cytological relationships among *Eumelilotus* species analysed using cytological observations of interspecific hybrids obtained by *in vitro* culture. Euphytica, 101: 17-22.

HAGEMANN R (1992). Plastid genetics in higher plants. In: Herrman R (ed) Cell organelles. Springer, New York: 65-96.

HALLIGAN J (1986). Breeding for a new type of blue *Rhododendron*. Journal of the American Rhododendron Society, 40: 164-165.

HAMIDAH M, KARIM AGA, DEBERGH P (1997). Somatic embryogenesis and plant regeneration in *Anthurium scherzerianum*. Plant Cell, Tissue and Organ Culture, 48: 189-193.

HAMILL S, SMITH M, DODD W (1992). *In vitro* induction of banana autotetraploids by colchicine treatment of micropropagated diploids. Australian Journal of Botany, 40: 887-896.

HAN D, NIIMI Y, NAKANO M (1997). Regeneration of haploid plants from anther cultures of the Asiatic hybrid lily 'Connecticut King'. Plant Cell Tissue and Organ Culture, 47: 153-158.

HANCOCK J (1997). The colchicine story. HortScience, 32: 1011-1012.

HANSEN N, ANDERSEN S (1996). *In vitro* chromosome doubling potential of colchicine, oryzalin, trifluralin and APM in *Brassica napus* microspore culture. Euphytica, 88: 159-164.

HANSEN N, GERTZ A, JOERSBO M, ANDERSEN S (2000). Chromosome doubling *in vitro* with amiprophos-methyl in *Beta vulgaris* ovule culture. Acta Agriculturae Scandinavica, Section B : Soil and Plant Science, 50: 89-95.

HARBORNE J (1980). Flavonoid pigments as both taxonomic & phylogenetic markers in the genus *Rhododendron*. In: Luteyn J, O'Brien M (eds) Contributions towards a classification of Rhododendron. New York Botanical Gardens, New York: 145-160.

HARE P, VAN STADEN J (1994a). Cytokinin oxidase: biochemical features and physiological significance. Physiologia Plantarum, 91: 128-136

HARE P, VAN STADEN J (1994b). Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant and Cell Physiology, 35: 1121-1125.

HARLAN J, DE WET J (1975). On Ö. Winge and a prayer: the origins of polyploidy. Botanical Reviews, 41: 361-390.

HELLER F (1973). DNA-Bestimmungen an Keimwurzellen von *Vicia faba* mit Hilfe der Impulszytophotometrie. Berichten der Deutschen Botanischen Gesellschaft, 86: 437-441.

HERMSEN J (1984). Nature, evolution, and breeding of polyploids. IOWA State Journal of Research, 58: 461-474.

HESLOP-HARRISON Y (1999). Aspects of the cell biology of pollination and wide hybridization. In: Cresti M, Cai G, Moscatelli A (eds). Fertilization in higher plants (molecular and cytological aspects). Springer, Berlin: 139-144.

HEURSEL J (1987). Inheritance of flower colours and breeding of evergreen azaleas. Journal of the American Rhododendron Society, 41: 141-145.

HEURSEL J (1999). Azalea's : oorsprong, veredeling en cultivars. Lannoo, Tielt, 192 pp.

HEURSEL J, DE ROO R (1981). Polyploidy in evergreen azaleas. HortScience, 16: 765-766.

HEURSEL J, GARRETSEN F (1989). Inheritance of corolla size, number of stamens & percentage of plants with petaloid stamens in evergreen azaleas (*Rhododendron* Subsect. *obtusa*). Plant Breeding, 103: 304-309.

HEURSEL J, HORN W (1977). A hypothesis on the inheritance of flower colours and flavonoids in *Rhododendron simsii* Planch. Zeitschrift für Pflanzenzüchtung, 79: 238-249.

HEYTING J (1970). Hybrids between elepidote and lepidote rhododendrons. Quarterly Bulletin of the American Rhododendron Society, 24: 97-98.

HIETER P, GRIFFITHS (1999). Polyploidy-more is more or less. Science, 285: 210-211.

HILL M, WITSENBOER H, ZABEAU M, VOS P, KESSELI R, MICHELMORE R (1996). PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. Theoretical and Applied Genetics, 93: 1202-1210.

HIRSCH A, TESTOLIN R, BROWN S, CHAT J, FORTUNE D, BUREAU J, DE NAY D (2001). Embryo rescue from interspecific crosses in the genus *Actinidia*. Plant Cell Reports, 20: 508-516.

HOFER M, LESPINASSE Y (1996). Haploidy in apple. In: Jain S, Sopory S, Veilleux R (eds) *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, vol 3: 261-276.

HOGENBOOM N (1973). A model for incongruity in intimate partner relationships. Euphytica, 22: 219-233.

HOGENBOOM N (1975). Incompatibility and incongruity: two different mechanisms for the non-functioning of intimate partner relationships. Proceedings of the Royal Society of London, 188: 361-375.

HOLTON R (2001). Plant genotyping by analysis of microsatellites. In: Henry R (ed) Plant genotyping: the DNA fingerprinting of plants. Oxford University Press, Wallingford: 15-27.

HOLTON T, BRUGLIERA F, TANAKA Y (1993). Cloning and expression of flavonol synthase from *Petunia hybrida*. The Plant Journal, 4: 1003-1010.

HOLTON T, CORNISH E (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell, 7: 1071-1083.

HONGTRAKUL V, HUESTIS G, KNAPP S (1997). Amplified fragment length polymorphisms as a tool for DNA fingerprinting of sunflower germplasm: genetic diversity among inbred lines. Theoretical and Applied Genetics, 95: 400-407.

HONGYUAN Y, CHANG Z, DETIAN C, HUA Y, YAN W, XIAOMING C (1986). *In vitro* culture of unfertilised ovules in *Helianthus annuus* L. In: Hu H, Yang H (eds) Haploids of higher plants *in vitro*. China Academic Publishers, Beijing: 182-191.

HU T, KASHA K (1997). Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary co-culture. Plant Cell Reports, 16: 520-525.

HU T, ZIAUDDIN A, SIMION E, KASHA K (1995). Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media: I. Effects of pretreatment, isolation methods, and hormones. *In vitro* Cellular and Developmental Biology of Plants, 31: 79-83.

HUMBERT O, PRUDHOMME M, HAKENBECK R, DOWSON C, CLAVERYS J (1995). Homeologous recombination and mismatch repair during transformation in Streptococcus pneumoniae: saturation of the Hex mismatch repair system. Proceedings of the National Academy of Sciences, 92: 9052-9056.

HUNTER N, CHAMBERS S, LOUIS E, BORTS R (1996). Mismatch repair is involved in meiotic sterility of a yeast hybrid. EMBO Journal, 15:1726-1733.

HUXLEY A, GRIFFITHS M, LEVY M (1999). The new Royal Horticultural Society Dictionary of Gardening, Volume IV. MacMillan, London, 3336 pp.

HVILKOVSKAYA B (1982). Callus formation and plant regeneration from explants of mono (2n=x=12) and dihaploid (2n=x=24) plants of Solanum tuberosum. Cytologiya i Genetica, 17: 12-19.

HWANG Y, OKUBO H, FUJIEDA K (1992). Pollen tube growth, fertilization and embryo development of *Camellia japonica* L. x *C. chrysantha* (Hu) Tuyama. Journal of the Japanese Society for Horticultural Science, 60: 955-961.

IAPUCHINO G, MCCULLOCH S, CHEN T (1992). Adventitious shoot formation from leaf explants of *Rhododendron.* Plant Cell, Tissue and Organ Culture, 30: 237-241.

IBRAHIM R, DEBERGH P (2001) Factors controling high efficiency adventitious bud formation and plant regeneration from *in vitro* leaf explants of roses (*Rosa hybrida* L.). Scientia Horticulturae, 88: 41-57.

INOMATA N (1978). Production of interspecific hybrids between *Brassica campestris* and *Brassica oleracea* by culture *in vitro* of excised ovaries. Part 2. Effects of coconut-milk and

casein hydrolysate on the development of excised ovaries. Japanese Journal of Genetics, 53: 1-12.

IQBAL M, PADEN D, RAYBURN A (1995). Assessment of genetic relationships among Rhododendron species, varieties and hybrids by RAPD analysis. Scientia Horticulturae, 63: 215-223.

IRVING E (1993). Concerning the origin and distribution of Rhododendrons. Journal of the American Rhododendron Society, 47, 139-145.

JANAKI AMMAL E, ENOCH I, BRIDGWATER M (1950). Chromosome numbers in species of *Rhododendron*. The *Rhododendron* Yearbook. Royal Horticultural Society: 78-91.

JANSON J (1993). Placental pollination in *Lilium longiflorum* Thunb. Plant Science, 99: 105-115.

JAYNES R (1976). F1 crosses of evergreen and deciduous azaleas and other wide crosses of *Rhododendron*. Quarterly Bulletin of the American Rhododendron Society, 30: 44-48.

JOHNSTON S, DEN NIJS T, PELOQUIN S, HANEMAN R (1980). The significance of gene balance to endosperm development in interspecific crosses. Theoretical and Applied Genetics, 57: 5-9.

JORGENSEN J (1989). Somatic embryogenesis in *Aesculus hippocastanum* L. by culture of filament callus. Journal of Plant Physiology, 135: 240-241.

JÜRGENS G (1995). Axis formation in plant embryogenesis: cues and clues. Cell, 81: 467-470.

KALENDAR R, GROB T, REGINA M, SUONIEMI A, SCHULMAN A (1999). IRAP and REMAP: two new retrotransposon-based DNA fingerprinting techniques. Theoretical and Applied Genetics, 98: 704-711.

KARLOV G, KHRUSTALEVA L, LIM K, VAN TUYL J (1999). Homeologous recombination in 2n gamete producing interspecific hybrids of Lilium (Liliaceae) studied by genomic in situ hybridization (GISH). Genome, 42: 681-686.

KARP A, EDWARDS K, BRUFORD M, VOSMAN B, MORGANTE M, SEBERG O, KREMER A, BOURSOT P, ARCTANDER P, TAUTZ D, HEWITT G (1997). Molecular Technologies for Biodiversity Evaluation: Opportunities and Challenges. Nature Biotechnology, 15: 625-628.

KASHA K, KAO K (1970). High frequency haploid production in barley (*Hordeum vulgare* L.). Nature, 225: 874-876.

KASHKUSH K, FELDMAN M, LEVY A (2002). Gene loss, silencing and activation in a newly synthesized wheat allotetraploid. Genetics, 160: 1651-1659.

KATSUKAWA K, MORI G, IMANISHI H (2000). Effect of applying naphthaleneacetic acid (NAA) onto stigma on fruit set and seed formation in interspecific hybrids between *Lycoris* species. Journal of the Japanese Society for Horticultural Science, 69: 224-226.

KEHR A (1977). Azaleodendron breeding. Quarterly Bulletin of the American Rhododendron Society, 31: 226-232.

KEHR A (1987). Hybridizing azaleas. In: Galle F (ed) Azaleas. Timber Press, Portland, Oregon, 519 pp.

KEHR A (1996a). Polyploids in *Rhododendron* breeding. Journal of the American Rhododendro*n* Society, 50: 215-217.

KEHR A (1996b). Woody plant polyploidy. American Nurseryman, 183: 38-47.

KELLER J (1990). Culture of unpollinated ovules, ovaries, and flower buds in some species of the genus *Allium* and haploid induction via gynogenesis in onion (*Allium cepa* L.). Euphytica, 47: 241-248.

KELLER J, KORZUN L (1996). Ovary and ovule culture for haploid production. In: Mohan S, Sopory S, Veilleux R (eds) Current plant science and biotechnology in agriculture: *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, vol 1: 217-235.

KHO Y, BAER J (1973). Improving the cross *Rhododendron impeditum* x *Rhododendron* 'Elizabeth' by temperature treatment. Euphytica, 22: 234-238.

KIM J, HAM D, LIM H, LEE K (1996a). Cross compatibility, seed germinility, and embryo rescue following 2x X 4x crosses in *Hibiscus syriacus*. Journal of the Korean Society for Horticultural Science, 37: 713-718.

KIM J, HAM D, LIM H, LEE K (1996b). Development of immature embryo and abnormal endosperm after reciprocal crosses between diploids and tetraploids in *Hibiscus syriacus*. Journal of the Korean Society for Horticultural Science, 37: 462-467.

KIM J, KIM Y, KYUNG H (1999). Segregation in flower colors on F1 hybrids derived from diploid x tetraploid crosses in *Hibiscus syriacus* L. Journal of the Korean Society for Horticultural Science, 40: 107-111.

KISS J, HESZKY L, KISS E, GYULAI G (1992). High efficiency adventive embryogenesis on somatic embryos of anther, filament and immature proembryo origin in horse-chestnut (*Aesculus hippocastanum* L.) tissue culture. Plant Cell, Tissue and Organ Culture, 30: 59-64.

KNOX R, WILLIAMS E, DUMAS DE VAULX C (1986). Pollen, pistil and reproductive function in crop plants. In : Janick J (ed). Plant Breeding Reviews. AVI Publishing Co, Westport, vol 4: 9-79.

KO J, KIM Y, EUN J (1996). Embryogenesis and plant regeneration by the anther culture of *Zantedeschia aethiopica* spp. Journal of the Korean Society for Horticultural Science, 37: 468-474.

KOBAYASHI N, AKABANE M, HANDA T, TAKAYANAGI K (1996). Inheritance of morphological characters and RAPD markers in intersubgeneric hybrids of azalea (*Rhododendron kiusianum* Makino x *R. indicum* (L.) Sweet) x *R. japonicum* (A. Gray) Suringer f. *flavum* Nakai. Journal of the Japanese Society for Horticultural Science, 65: 145-153.

KOBAYASHI N, HANDA T, TAKAYANAGI K, ARISUMI K (2000). Chloroplast DNA polymorphisms and morphological variation in Japanese wild azaleas, the origin of evergreen azalea cultivars. Acta Horticulturae, 521: 173-177.

KOBAYASHI N, KAWASHIMA S, HANDA T, TAKAYANAGI K, ARISUMI K, CONSIDINE J, GIBBS H (1998). Introgression in Japanese evergreen azaleas (*Rhododendron kiusianum* and *R. kaempferi*). Acta Horticulturae, 454: 325-328.

KOBAYASHI N, TAKEUCHI R, HANDA T, TAKAYANAGI (1995). Cultivar identification of evergreen azalea with RAPD method. Journal of the Japanese Society for Horticultural Science, 64: 611-616.

KOLODNER R, MARSISCHKY G (1999). Eukaryotic DNA mismatch repair. Current Opinions in Genetics and Development, 9: 89-96.

KONDOROSI E, ROUDIER F, GENDREAU E (2000). Plant cell-size control: growing by ploidy? Current Opinion in Plant Biology, 3: 488-492.

KREBS S (1997). A cytogenetic study of sterile Rhododendron hybrids. Journal of the American Rhododendron Society, 51: 70-74.

KRON K (1993). A revision of *Rhododendron* section *Pentanthera*. Edinburgh Journal of Botany, 50: 249-364.

KRON K (1998). Using DNA sequences to estimate evolutionary relationships among rhododendrons and azaleas. Journal of the American Rhododendron Society, 52: 70-72.

KRON K (2000). Evolutionary relationships of azaleas and rhododendrons. In: Jacobs R(ed) Jaarboek van de Belgische Dendrologische Vereniging. Bonheiden: 30-40.

KUNISHIGE M, KOBAYASHI Y (1980). Chromatographic identification of Japanese azalea species and their hybrids. In Luteyn J, O'Brien M (eds) Contributions towards a claasification of Rhododendron. New York Botanical Gardens, New York: 277-287.

KYUNG H, KIM J (2001a). Effect of flowering season on the compatibilities of interspecific single, three way, and double crosses among *Hibiscus syriacus*, *H. sinosyriacus*, and interspecific hybrids (*H. syriacus* x *H. paramutabilis)*. Journal of the Korean Society for Horticultural Science, 42: 568-574.

KYUNG H, KIM J (2001b). Relationships between pistil receptivity and cross compatibility in interspecific single, three way, and double crosses among *Hibiscus syriacus*, *Hibiscus sinosyriacus*, and interspecific hybrids (*H. syriacus* x *H. paramutabilis*). Journal of the Korean Society for Horticultural Science, 42: 561-567.

KYUNG H, PARK S, KIM J (2001a). Effects of bud and old-flower pollination on the interspecific single and three way cross compatibilities between cultivar groups of *Hibiscus syriacus* and *H. sinosyriacus*. Journal of the Korean Society for Horticultural Science, 42: 575-580.

KYUNG H, PARK S, KIM J (2001b). Embryo abortion and germination inhibition in interspecific single, three way, and double crosses among *Hibiscus syriacus*, *Hibiscus sinosyriacus*, and interspecific hybrids (*H. syriacus* x *H. paramutabilis*). Journal of the Korean Society for Horticultural Science, 42: 737-742.

LAMOTE V, BAERT J, ROLDAN-RUIZ I, DE LOOSE M, VAN BOCKSTAELE E (2002). Tracing of 2n egg occurrence in perennial ryegrass (*Lolium perenne* L.) using interploidy crosses. Euphytica, 123: 159-164.

LEACH D (1961). Rhododendrons of the world. Charles Scribner's Sons, New York, 544 pp.

LEE B, KIM Y, LEE S, SONG W, .KIM T (1991). Plant regeneration from explants from mature trees of *Hibiscus syriacus* (cv. bulsae) via somatic embryogenesis and organogenesis. Research Report of the Institute of Forest Genetics, 27: 85-90.

LEITCH I, BENNETT M (1997). Polyploidy in angiosperms. Trends in Plant Science, 2: 470-476.

LELIVELT C (1993). Studies of pollen grain germination, pollen tube growth, micropylar penetration and seet set in intraspecific and intrageneric crosses within three Cruciferae species. Euphytica, 67: 185-197.

LEVIN D (1978). The origin of isolating mechanisms in flowering plants. Evolutionary Biology, 11: 185-197.

LEVIN D (1983). Polyploidy and novelty in flowering plants. American Naturalist, 122: 1-25.

LEWIS D, CROWE L (1958). Unilateral interspecific incompatibility in flowering plants. Heredity, 12: 233-256.

LEWIS W (1980). Polyploidy in species populations. In: Lewis W (ed) Polyploidy: biological relevance. Plenum Press, New York, 103-144.

LI Z, LIU H, LUO P (1995). Production and cytogenetics of intergeneric hybrids between *Brassica napus* and *Orychophragmus violaceus*. Theoretical and Applied Genetics, 91: 131-136.

LIM K, RAMANNA S, DE JONG J, JACOBSEN E, VAN TUYL J (2001). Indeterminate meiotic restitution (IMR): a novel type of meiotic restitution mechanism detected in interspecific lily hybrids by GISH. Theoretical and Applied Genetics, 103: 219-230.

LIM K, RAMANNA S, JACOBSEN E, VAN TUYL J (2003). Evaluation of BC2 progenies derived from 3x-2x and 3x-4x crosses of Lilium hybrids: a GISH analysis. Theoretical and Applied Genetics, 106: 568-574.

LIU B, BRUBAKER C, CRONN R, WENDEL J (2001). Polyploid formation in cotton is not accompanied by rapid genomic changes. Genome, 43: 874-880.

LIU B, WENDEL J (2000). Retroelement activation followed by rapid repression in interspecific hybrid plants. Genome, 43: 874-880.

LIU B, WENDEL J (2002). Non-mendelian phenomena in allopolyploid genome evolution. Current Genomics, 3: 1-17.

LLOYD G, MCCOWN B (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proceedings of the International Plant Propagation Society, 30: 421.

LONGLY B (1994). Centre d'étude de la reproduction vegetale. Rapport 1992-1994, Convention D1/4-3309/5489A.

LÖVE A, LÖVE D, PICHI SERMOLLI R (1977). Cytotaxonomical atlas of the pteridophyta. Cramer, Vaduz, 122 pp.

LU C, BRIDGEN M (1997). Chromosome doubling and fertility study of *Alstroemeria aurea* x *A. caryophyllaea*. Euphytica, 94: 75-81.

LUX H, HERRMANN L, WETZEL C (1990). Production of haploid sugar beet (*Beta vulgaris* L.) by culturing unpollinated ovules. Plant Breeding, 104: 177-183.

MAERTENS L (2000). Ontwikkelen van microsatellietmerkers bij azalea en raaigras. Eindwerk voorgedragen tot het behalen van het diploma van Industrieel Ingenieur in Chemie, optie Biochemie, 99pp.

MALLIKARJUNA N, MOSS J (1995). Production of hybrids between *Cajanus platycarpus* and *Cajanus cajan*. Euphytica, 83: 43-46.

MALUSZYNSKI M, SZAREJKO I, SIGURBJÖRNSSON B (1996). Haploidy and mutation techniques. In: Mohan J, Sopory S, Veilleux R (eds) *In vitro* haploid production of higher plants. Kluwer Academic Publishers, Dordrecht, vol 1: 67-93.

MARCHANT C (1973). Chromosome variation in Araceae: V. Acoraceae to Lasieae. Kew Bulletin, 28: 199-210.

MARTENS M, REISCH B (1988). An improved technique for counting chromosomes in grapes. HortScience 23: 896-899.

MASTERSON J (1994). Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science, 264: 421-424.

MATSUMOTO T, WEBB D, KUEHNLE A (1996). Histology and origin of somatic embryos derived rom *Anthurium andreanum* Linden ex Andre lamina. Journal of the American Society for Horticultural Science, 121: 404-407.

MATZKE M & MATZKE A (1998). Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. Cellular and Molecular Life Sciences, 54: 94-103.

MATZKE M, MITTELSTEN SCHEID O, MATZKE A (1999). Rapid structural and epigenetic changes in polyploid and aneuploïd genomes. Bioessay, 21: 761-767.

MAUGHAN P, SAGHAI MAROOF M, BUSS G, HUESTIS G (1996). Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance and near-isogenic line analysis. Theoretical and Applied Genetics, 95: 400-407.

MCALLISTER H (1993). Chromosome numbers in rhododendrons. In: Rhododendrons with Camellias and Magnolias. Royal Horticultural Society, London: 24-30.

MCCLINTOCK B (1984). The significance of responses of the genome to challenge. Science, 226: 792-801.

MCCOWN B, LLOYD G (1983). A survey of the response of *Rhododendron* to *in vitro* culture. Plant Cell, Tissue and Organ Culture, 2: 77-85.

MCCUBBIN A, KAO T (1996). Self-incompatibility and pollen rejection in angiosperms: In: Mohan S, Sopory S, Veilleux R (eds) Current plant science and biotechnology in agriculture: *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht: 225-253.

MENDIBURU A, PELOQUIN S (1977). The significance of 2n gametes in potato breeding. Theoretical and Applied Genetics, 49: 53-61.

MERTENS M (2000). Inheritance of foreign genes in transgenic azalea generated by *Agrobacterium*-mediated transformation. Acta Horticulturae, 521: 127-131.

MERTENS M, SAMYN G (1994). Regeneration and somaclonal variation of *Rhododendron simsii* offer prospects to the obtainment of commercially valuable sports. Mededelingen FLTBW RUG, 59: 2495-2497.

MERTENS M, WERBROUCK S, SAMYN G, BOTELHO DOS SANTOS MOREIRA DA SILVA H, DEBERGH P (1996). *In vitro* regeneration of evergreen azaleas from leaves. Scientia Hort, 45: 231 - 236.

METWALLY E, MOUSTAFA S, EL SAWY B, HAROUN S, SHALABY T (1998). Production of haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita pepo*. Plant Cell Tissue and Organ Culture, 52: 117-121.

MEYER M (1982). *In vitro* propagation of *Rhododendron catawbiense* from flower buds. HortScience, 17: 891-892.

MEYNET J, BARRADE R, DUCLOS A, SIADOUS R (1994). Dihaploid plants of roses (*Rosa* X *hybrida*, cv 'Sonia') obtained by parthenogenesis induced using irradiated pollen and *in vitro* culture of immature seeds. Agronomie, 3: 169-175.

MEYNET J, SIBI M (1984). Haploid plants from *in vitro* culture of unfertilized ovules in *Gerbera jamesonii*. Zeitschrift für Pflanzenzüchtung, 93: 78-85.

MICHISHITA A, URESHINO K, MIYAJIMA I (2001). Shortening the period from crossing to the seedling stage through ovule culture of interspecific crosses of azalea (*Rhododendron* spp.). Journal of the Japanese Society for Horticultural Science, 70: 54-59.

MICHISHITA A, URESHINO K, MIYAJIMA I (2002). Plastome-genome incompatibility of Rhododendron serpyllifolium (A. Gray) Miq. to evergreen azalea species belonging to series Kaempferia. Journal of the Japanese Society for Horticultural Science, 71: 375-381.

MIYAJIMA I, URESHINO K, KOBAYASHI N, AKABANE M (2000). Flower color and pigments of intersubgeneric hybrid between white-flowered evergreen and yellow-flowered deciduous azaleas. Journal of the Japanese Society for Horticultural Science, 69 : 280-282.

MIYOSHI K, ASAKURA N (1996). Callus induction, regeneration of haploid plants and chromosome doubling in ovule cultures of pot gerbera (*Gerbera jamesonii*). Plant Cell Reports, 16: 1-5.

MOK D, MOK M, RABAKOARIHANTA A (1978). Interspecific hybridization of *Phaseolus vulgaris* hybrid parent with *Phaseolus lunatus* hybrid parent and *Phaseolus acutifolius* hybrid parent. Theoretical and Applied Genetics, 52: 209-215.

MOL J, GROTEWOLD E, KOES R (1998). How genes paint flowers & seeds. Trends in Plant Science, 3: 212-217.

MOL R (1992). *In vitro* gynogenesis in *Melandrium album*: from parthenogenetic embryos to mixoploid plants. Plant Science, 81: 261-269.

MOL R (1999). Embryological aspects of *in vitro* gynogenesis in plant organ cultures. Acta Biologica Cracoviensia, 41: 67-74.

MOMOTAZ A, KATO M, KAKIHARA F (1998). Production of intergeneric hybrids between *Brassica* and *Sinapis* species by means of embryo rescue techniques. Euphytica, 103: 123-130.

MONDELAERS W, VAN LAERE K, GOEDEFROOT A, VAN DEN BOSSCHE K (1996). The Gent University 15 MeV high-current linear electron accelerator facility. Nuclear Instruments and Methods in Physics Research, 368: 278-282.

MONNIER M (1988). Culture d'embryons et d'ovules / embryogénèse zygotique et somatique. In: Zrÿd J (ed) Cultures de cellules, tissus et organes vegetaux. Presses polytechniques romandes, Lausanne: 105-134.

MOORE R (1947). Cytotaxonomic studies in the Loganiaceae. I. Chromosome numbers and phylogeny in the Loganiaceae. American Journal of Botany, 34: 527-538.

MOORE R (1949). Cytotaxonomic studies in the Loganiacae. III. Artificial hybrids in the genus *Buddleia* L. American Journal of Botany, 36: 511-516.

MOORE R (1952). An interspecific hybrid in *Buddleia* . Journal of Heredity, 43: 41-45.

MORDHORST A, TOONEN M, DE VRIES S (1997). Plant embryogenesis. Critical Reviews in Plant Sciences, 16: 535-576.

MORDHORST A, VOERMAN K, HARTOG M, MEIJER E, VAN WENT J, KOORNNEEF M, DE VRIES S (1998). Somatic embryogenesis in *Arabidopsis thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. Genetics, 149: 549-563.

MOREJOHN L, BUREAU T, MOLE-BAJER T, BAJER A, FOSKET D (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. Planta, 172: 252-264.

MUKHAMBETZHANOV S (1997). Culture of nonfertilized female gametophytes *in vitro.* Plant Cell, Tissue and Organ Culture, 48: 111-119.

MURASHIGE T, SKOOG F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

NEWBEGIN E, ANDERSON M, CLARKE A (1993). Gametophytic self-incompatibility systems. Plant Cell, 5: 1315-1324.

NIIMOTO D (1966). Chromosome numbers of some *Hibiscus* species and other Malvaceae. Baileya, 14: 29-34.

NIKOVA V, ZAGORSKA N (1990). Overcoming hybrid incompatibility between *Nicotiana africana* Merxm. and *Nicotiana tabacum* and development of cytoplasmically male sterile tobacco forms. Plant Cell, Tissue and Organ Culture, 23: 71-75.

NITSCH J, NITSCH C (1969). Haploid plants from pollen grains. Science, 163: 85-87.

NOGUCHI Y (1932). Studies on the species crosses of Japanese *Rhododendron*. I. On the crossability between various species and the cotyledon color of F1 seedlings. Japanese Journal of Botany, 6: 103-104.

NORIEGA C, SONDAHL M (1991). Somatic embryogenesis in hybrid tea roses. Bio/Technology, 9: 991-993.

NORRMANN G, QUARIN C (1987). Permanent odd polyploidy in a grass (*Andropogon ternatus*). Genome, 29: 340-344.

NOTSUKA K, TSURU T, SHIRAISHI M (2000). Induced polyploid grapes via *in vitro* chromosome doubling. Journal of the Japanese Society for Horticultural Science, 69: 543-551.

OHAD N, MARGOSSIAN L, HSU Y, WILLIAMS C, REPETTI P, FISCHER R (1996). A mutation that allows endosperm development without fertilization. Proceedings of the National Academy of Sciences, 93: 5319-5324.

OKONKWO C, CAMPBELL R (1990). Pollen tube growth in pistils of *Rhododendron* following intra- and interspecific pollinations. Genetics and Breeding, 23: 9-15.

O'NEILL R, O'NEILL M, GRAVES J (1998). Undermethylation associated with retroelement activation and chromosome remodeling in an interspecific mammalian hybrid. Nature, 393: 68-72.

OSBORN T, PIRES J, BIRCHLER J, AUGER D, CHEN Z, LEE H, COMAI L, MADLUNG A, DOERGE R, COLOT V, MARTIENSSEN R (2003). Understanding mechanisms of novel gene expression in polyploids. Trends in Genetics, 19: 141-147.

OTTO F (1990). DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewicz Z, Crissman H (eds) Methods in Cell Biology. Academic Press, New York, vol 33: 105-110.

OTTO S, WHITTON J (2000). Polyploid incidence and evolution. Annual Reviews in Genetics, 34: 401-437.

OWEN H, MILLER A (1993). A comparison of staining techniques for somatic chromosomes of strawberry. HortScience, 28: 155-156.

OZKAN H, LEVY A, FELDMAN M (2001). Allopolyploidy-induced rapid genome evolution in the wheat (*Aegilops-Triticum*) group. Plant Cell, 13: 1735-1747.

PADEN D (1990). Doubling chromosomes with colchicine treatment *in vitro* as determined by chloroplast number in epidermal guard cells. Journal of the American Rhododendron Society, 44: 162-165.

PAEK K, HWANG J, JONG S, PARK S (1989a). Somatic hybridisation by protoplast fusion in *Hibiscus syriacus* and *Hibiscus rosachinensis*. Korean Journal of Breeding, 21: 95-102.

PAEK K, HWANG J, JONG S, PARK S (1989b). *In vitro* pollination, ovule culture of *Hibiscus syriacus* and differentiation of adventitious bud and somatic embryo from callus. Journal of the Korean Society for Horticultural Science, 30: 145-156.

PAEK K, SON Y, HWANG J, JONG S, PARK S (1989c). Plant regeneration in tissue cultures initiated from immature embryos of *Hibiscus syriacus* L. Korean Society of Plant Tissue Culture, 16: 93-104.

PALLARES P (1984). First results from *in vitro* culture of unfertilized cotton ovules (*Gossypium hirsutum*). Coton et Fiberes Tropicales, 39: 145-152.

PANDEY K (1981). Evolution of unilateral incongruity in flowering plants: further evidence in favour of twin specificities controling intra- and interspecific incompatibility. New Phytologist, 89 : 705-728.

PAROKONNY A, KENTON A (1995). Comparative physical mapping and evolution of the *Nicotiana tabacum* L. karyotype. In : Brandham P, Bennett M (eds) Kew Chromosome Conference. Royal Botanic Gardens, Kew : 301-320.

PARTEC (1999). PAS III (Particle Analyzing System) manual.

PAUL S, WACHIRA F, POWELL W, WAUGH R (1997). Diversity and genetic differentiation among populations of Indian and Kenyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. Theoretical and Applied Genetics, 94: 255-263.

PECHERER B (1992). The color of rhododendron flowers. Journal of the American Rhododendron Society, 46: 200-202.

PERKIN-ELMER (1995). AFLP™ Plant Mapping Kit: Protocol.

PICKERSGILL B, HAYWARD M, BOSEMARK N, ROMAGOSA I (1993). Interspecific hybridization by sexual means. In: Hayward M, Bosemark N, Romagosa I (eds) Plant Breeding: principles and prospects, Plant Breeding Series. Kluwer Academic Publishers, Dordrecht, vol 1: 63-78.

PIERIK R (1999). *In vitro* culture of higher plants. Kluwer Academic Publishers, Dordrecht, 360 pp.

PIETERS B, BOESMAN G, DEBERGH P, LEMEUR R (1989). Control of flowering in *Calathea crocata* in multi-layer cells. Acta Horticulturae, 246: 113-120.

PIKAARD C (1999). Nucleolar dominance and silencing of transcription. Trends in Plant Science, 4: 478-483.

POGANY M, LINEBERGER R (1990). Phenotypic variation during micropropagation of the chimeral *Rhododendron* 'President Roosevelt'. Plant Cell Tissue and Organ Culture, 21: 201-209.

PRATT C (1983). Somatic selection & chimeras. In: Moore J, Janick J (eds) Methods in Fruit Breeding. Purdue University Press, West Lafayette: 172-185.

PREECE J, IMEL M, SHEVADE A (1993). Regeneration of *Rhododendron* PJM group plants from leaf explants. Journal of the American Rhododendron Society, 47: 68-71.

PREIL W, EBBINGHAUS R (1985). Bastardierungen von Topfazaleen (*Rhododendron simsii*) mit anderen *Rhododendron*-Arten. Rhododendron und immergrüne Laubgehölze, Jahrbuch 1985: 85-92.

PREIL W, ENGELHARDT M (1977). Meristem culture of azaleas (*Rhododendron simsii*). Acta Horticulturae, 78: 203-208.

PRYOR R (1973). Hybridization between evergreen and deciduous azaleas. Quarterly Bulletin of the American Rhododendron Society, 27: 212-214.

PRYOR R, FRAZIER L (1968). Colchicine-induced tetraploid azaleas. HortScience, 3: 283-286.

PRYOR R, FRAZIER L (1970). Triploid azaleas of the Belgian-Indian series. HortScience, 5: 114-115.

PRZYWARA L, PIROG H, KONIECZNY R, JACH M (1996). The use of tissue culture methods in interspecific hybridization within the genus *Trifolium* L. Acta Biologica Cracoviensia, 38: 53-65.

PRZYWARA L, WHITE D, SANDERS P, MAHER D (1989). Interspecific hybridization of *Trifolium repens* with *Trifolium hybridum* using in ovulo embryo and embryo culture. Annals of Botany, 64: 613-624.

QUAZI M (1988). Interspecific hybrids between *Brassica napus* L and *B. oleracea* L. developed by embryo culture. Theoretical and Applied Genetics, 75: 309-318.

RADOJEVIC L (1995). Somatic embryogenesis in *Aesculus hippocastanum* L. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht: 409-444.

RAGHAVAN V (1980). Embryo culture. International Reviews in Cytology, 11: 209-240.

RAGHAVAN V (1997). Molecular embryology of flowering plants. Cambridge University Press, New York, 690 pp.

RANGAN T (1984). Culture of ovules. In: Vasil, I (ed) Cell culture and somatic cell genetics of plants: laboratory procedures and their applications. Orlando, Academic Press, vol 1: 227-231.

RAO P, SUPRASANA P (1996). Methods to double haploid chromosome numbers. In: Mohan S, Sopory S, Veilleux R (eds) Current plant science and biotechnology in agriculture: *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, vol 1: 317-339.

RAQUIN C (1985). Induction of haploid plants by *in vitro* culture of *Petunia* ovaries pollinated with irradiated pollen. Zeitschrift fur Pflanzenzuchtung, 94: 166-169.

RAYSSIGUIER C, THALER D, RADMAN M (1989). The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. Nature, 342: 396-401.

REED S, COLLINS G (1978). Interspecific hybrids in *Nicotiana* through *in vitro* culture of fertilized ovules. Journal of Heredity, 69: 311-315.

REESE G (1950). Beiträge zur Wirkung des Colchicins bei der Samenbehandlung. Planta, 38: 324-376.

REGNER F, STADLHUBER A, ROMANN H (1996). Somatic embryogenesis in grapevine (*Vitis vinifera*). Mitteilungen von Klosterneuburg, -Rebe und -Wein, Obstbau und Fruchteverwertung, 46, 105-113.

REINERT J (1959). Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen in Gewebekulturen aus Karotten. Planta, 53: 318-333.

RIERA-LIZARAZU D, RINES H, PHILLIPS R (1996). Cytological and molecular characteristics of oat x maize partial hybrids. Theoretical and Applied Genetics, 93: 123-135.

RIHOVA L, TUPY J (1996). Influence of 2,4-D and lactose on pollen embryogenesis in anther culture of potato. Plant Cell, Tissue and Organ Culture, 45: 269-272.

ROBERTS A, LLOYD D, SHORT K (1990). *In vitro* procedures for the induction of tetraploidy in a diploid rose. Euphytica, 49: 33-38.

RODE J, DUMAS DE VAULX R (1987). Obtention de plantes haploïdes de carotte (*Daucus carota* L.) issues de parthénogenèse induite in situ par du pollen irradié et culture *in vitro* des graines immatures. Comptes Rendus de l' Academie de Sciences Paris, t305, série III: 225-229.

ROLDAN-RUIZ I, DE RIEK J, DENDAUW J, VAN BOCKSTAELE E, DE LOOSE M (1997). Fluorescent detection of AFLP fragments. EMBO-course 1997, Protocol book.

ROOSE M, GOTTLIEB L (1976). Genetic and biochemical consequences of polyploidy in *Tragopogon*. Evolution, 30: 818-830.

ROSE J, KUBBA J, TOBUTT K (2000a). Chromosome doubling in sterile *Syringa vulgaris* x *S. pinnatifolia* hybrids by *in vitro* culture of nodal explants. Plant Cell Tissue and Organ Culture, 63: 127-132.

ROSE J, KUBBA J, TOBUTT K (2000b). Induction of tetraploidy in *Buddleia globosa*. Plant Cell Tissue and Organ Culture, 63: 121-125.

ROUSE J (1993). Inter- and intraspecific pollinations involving *Rhododendron* species. Journal of the American Rhododendron Society, 47: 23-45.

ROUSE J, WILLIAMS E, KNOX R (1988). A vireya azaleodendron in flower. Journal of the American Rhododendron Society, 42, 133-135.

ROWE P (1974). Methods of producing haploids; parthenogenesis following interspecific hybridization. In: Kasha K (ed) Haploids in higher plants. University of Guelph: 43-52.

ROY A, LEGGETT G, KOUTOULIS A (2001). *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Reports, 20: 489-495.

ROYAL HORTICULTURAL SOCIETY (1966). Royal Horticultural Society Colour Chart. Royal Horticultural Society, London.

RYBERG H, RYBERG M, SUNDQVIST C (1993). Plastid ultrastructure and development. In: Sundqvist C, Ryberg M (eds) Pigment-protein complexes in plastid: synthesis and assembly. Academic Press, San Francisco: 25-62.

SALLEY H, GREER H (1992). Rhododendron hybrids, 2nd edition. Timber Press, Portland, 344pp.

SAMYN G, DE SCHEPPER S, VAN BOCKSTAELE E (2002). Adventitious shoot regeneration and appearance of sports in several azalea cultivars. Plant Cell Tissue and Organ Culture, 70: 223-227.

SANCHEZ A, MARIANI C (2002). Expression of the ACC synthase and ACC oxidase coding genes after self-pollination and incongruous pollination of tobacco pistils. Plant Molecular Biology, 48: 351-359.

SANTAMOUR F, DUMUTH P (1978). Carotenoid flower pigments in *Rhododendron*. HortScience, 13: 461-462.

SANTAMOUR F, PRYOR R (1973). Yellow flower pigments in *Rhododendron*: a review for breeders. Quarterly Bulletin of the American Rhododendron Society, 27: 214-216.

SATO S, KATOH N, YOSHIDA H, IWAI S, HAGIMORI M (2000). Production of doubled haploid plants of carnation (*Dianthus caryophyllus* L.) by pseudofertilized ovule culture. Scientia Horticulturae, 83: 301-310.

SAUTON A, DUMAS DE VAULX R (1987). Obtention de plantes haploids de carotte (*Daucus carota* L) issue de parthénogenèse in situ par du pollen irradié. Agronomie, 7: 141-148.

SAX K (1930). Chromosomal stability in the genus Rhododendron. American Journal of Botany, 17: 247-251.

SCHMIDT E, GUZZO F, TOONEN M, DE VRIES S (1997). A leucin-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development, 124: 2049-2062.

SCHUM A, MATTIESCH I, TIMMANN E, HOFMANN (1993). Regeneration of diploids via gynogenesis in *Allium porrum* L. Gartenbauwissenschaft, 45: 242-251.

SHAKED H, KASHKUSH K, OZKAN H, FELDMAN M, LEVY A (2001). Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome in wide hybridization and allopolyploidy in wheat. Plant Cell, 13: 1749-1759.

SHALABY T (1996). Producing double haploid plants, through ovule and anther culture technique in *Cucurbita pepo* L. Master of Science Thesis, Faculty of Agriculture, Tanta University, Egypt.

SHAMINA N, DOROGOVA N, GONCHAROV N, ORLOVA A, TRUNOVA S (1999). Abnormalities of spindle and cytokinine behavior leading to the formation of meiotic restitution nuclei in intergeneric cereal hybrids. Cell Biol Int, 23: 863-870.

SHARMA D, KAUR R, KUMAR K (1996). Embryo rescue in plants - a review. Euphytica, 89: 325-337.

SHARMA H (1995). How wide can a wide cross be? Euphytica, 82: 43-64.

SHEPHERD K (1999). Cytogenetics of the genus Musa. International Network for the Improvement of Banana and Plantain. Montpellier, France. IPGRI, Rome: 1-157.

SHIM K, KIM K, HA Y (1993). Characteristics of triploid cultivars Diana and Helene in *Hibiscus syriacus L*. Journal of the Korean Society for Horticultural Science, 34: 54-67.

SIBI M, KOBAISSI A, SHEKAFANDEH A (2001). Green haploid plants from unpollinated ovary culture in tetraploid wheat (*Triticum durum* Defs.). Euphytica, 122: 351-359.

SILVA J, DEBERGH P. (2001). Somatic embryogenesis from flower explants of Cocoa (*Theobroma cacao* L.). Mededelingen van de Faculteit Landbouwwetenschappen, Universiteit Gent, 66: 31-34.

SKOVSTED A (1941). Chromosome numbers in the Malvaceae II. Comptes rendus des travaux du laboratoire Carlberg, Série Physiologique, 23: 195-242.

SLOCOMBE S, MAITZ M, HUEROS G, BECKER H, YAN G, MÜLLER M, VAROTTO S, SANTANDREA G, THOMPSON R (1999). Genetic control of endosperm development. In: Russo V, Cove D, Edgar L, Jaenisch R, Salamini F (eds). Development: genetics, epigenetics and environmental regulation. Springer, Berlin: 185-197.

SMITH-WHITE S (1955). The life history and genetic system of *Leucopogon juniperus*. Heredity, 9 : 79-91.

SNEATH P, SOKAL R (1973). Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman, San Francisco, 573 pp.

SOLOV'EVA L (1990). Number of chloroplasts in guard cells of stomata as an indicator of the ploidy level of apple seedlings. Cytology and Genetics, 24: 1-4.

SOLTIS D, SOLTIS P (1993). Molecular data and the dynamic nature of polyploidy. Critical Reviews in Plant Sciences, 12: 243-273.

SOLTIS P, SOLTIS D (2000). The role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences, 97: 7051-7057.

SONG K, LU P, TANG K, OSBORN T (1995). Rapid genome change in synthetic polyploids of Brassica and its implications for polyploid evolution. Proceedings of the National Academy of Sciences, 92: 7719-7723.

SONG K, PARK H (1998). High frequency plant regeneration through suspension culture in *Hibiscus syriacus*. Journal of the Korean Society for Horticultural Science, 39: 843-848.

SONG K, PARK H (1999). Plant regeneration through protoplast culture in *Hibiscus syriacus* L. Journal of the Korean Society for Horticultural-Science, 40: 93-98.

SONG K, PARK H, KIM K (1998). Embryo abortion in intraspecific crosses in *Hibiscus syriacus*. Journal of the Korean Society for Horticultural Science, 39: 635-640.

SOPORY S, MUNSHI M (1996). Anther culture. In: Mohan S, Sopory S, Veilleux R (eds) Current plant science and biotechnology in agriculture: *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, vol 1: 145-176.

SPETHMANN W (1979). Untersuchungen über das Zustandekommen der Blütenfarbe bei *Rhododendron*. Mitteilungen der Deutschen Dendrologischen Gesellschaft, 71: 125-143.

SPETHMANN W (1987). A new infrageneric classification and phylogenetic trends in the genus *Rhododendron* (Ericaceae). Plant Systematics and Evolution, 157: 9-31.

STEBBINS G (1958). The inviability, weakness, and sterility of interspecific hybrids. Advance in Genetics, 9: 147-215.

STEBBINS G (1971). Chromosomal evolution of higher plants. Edward Arnold Ltd, London, 216 pp.

STETTLER R (1968). Irradiated mentor pollen: its use in remote hybridization of black cottonwood. Nature, 219: 746-747.

SULLIVAN N (1989). Succesful pollination. Journal of the Australian Rhododendron Society, 28:47-49.

TÄCKHOLM G (1920). On the cytology of the genus Rosa. Svensk Botanik Tidskrift, 14: 300-311.

TAJI A, KUMAR P, LAKSHMANAN P (2002). *In vitro* plant breeding. Haworth Press, New York, 167 pp.

TAKAMURA T, MIYAJIMA I (1996). Colchicine induced tetraploids in yellow-flowered cyclamens and their characteristics. Scientia Horticulturae, 65: 305-312.

TAL M (1980). Physiology of polyploids. In: Lewis W (ed) Polyploidy: biological relevance. Plenum Press, New York: 61-75.

TAYLOR N, ANDERSON M, WUESENBERRY K, WATSON L (1976). Doubling the chromosome number of *Trifolium* species using nitrous oxide. Crop Science, 16: 516-518.

THIERFELDER A, LUHS W, FRIEDT W (1992). Breeding of industrial oil crops with the aid of biotechnology: a review. Industrial crops and products, 1: 261-271.

TILNEY-BASSETT R (1986). Plant chimeras. Edward Arnold Publishers, London, 199 pp.

TOBUTT K (1992). Breeding woody ornamentals at east Malling, with particular reference to *Sambucus nigra*. Acta Horticulturae, 320: 63-68.

TODOROVA M, IVANOV P, SHINDROVA P, CHRISTOV M, IVANOVA I (1997). Doubled haploid production of sunflower (*Helianthus annuus* L.) through irradiated pollen-induced parthenogenesis. Euphytica, 97: 249-254.

TOURAEV A, ILHAM A, VICENTE O, HEBERLE-BORS E (1996). Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Reports, 15: 561-565.

TULECKE W (1964). A haploid tissue culture from the female gametophyte of *Ginkgo biloba*. Nature, 203: 94-95.

UCHIMIYA H, KAMEYA T, TAKAHASHI N (1971). *In vitro* culture of unfertilized ovules in Solanum melongena and ovaries in *Zea mays*. Japanese Journal of Breeding, 21: 247-250.

UHL C (1978). Chromosomes of Mexican *Sedum*, II Section Pachysedum. Rhodora, 80: 491-512.

UHLINGER R (1982). Wide crosses in herbaceous perennials. HortScience, 17: 570-574.

URESHINO K, KAWAI M, MIYAJIMA I (2000). Factors of unilateral cross incompatibility between several evergreen azalea species and *Rhododendron japonicum flavum*. Journal of the Japanese Society for Horticultural Science, 69: 261-265.

URESHINO K, MIYAJIMA I, AKABANE M (1998). Effectiveness of three-way crossing for the breeding of yellow-flowered evergreen azalea. Euphytica, 104: 113-118.

URESHINO K, MIYAJIMA I, OZAKI Y, KOBAYASHI N, MICHISHITA A, AKABANE M (1999). Appearance of albino seedlings and ptDNA inheritance in interspecific hybrids of azalea. Euphytica, 110: 61-66.

VÄINÖLÄ A (2000). Polyploidization and early screening of *Rhododendron* hybrids. Euphytica, 112: 239-244.

VAN BOCKSTAELE E (2001). Toegepaste plantenveredeling. Opleidingsonderdeel, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Gent, 223 pp.

VAN CREIJ M (1997). Interspecific hybridization in the genus *Tulipa*. Thesis Wageningen, Landbouwfaculteit, Nederland, 163 pp.

VAN CREIJ M, KERCKHOFFS D, VAN TUYL J (1997). Application of three pollination techniques and of hormone treatments for overcoming interspecific crossing barriers in *Tulipa*. Acta Horticulturae, 430: 547-557.

VAN DE LAAR H (1997) *Hibiscus syriacus*. Dendroflora, 34: 43-60.

VAN DE WEYER W (1920). Hybrid Buddleias. The Gardeners' Chronicle, 68: 181.

VAN DEN BULK R, DE VRIES – VAN HULTEN H, CUSTERS J, DONS J (1994). Induction of embryogenesis in isolated microspores of tulip. Plant Science, 104: 101-111.

VAN DUREN M, MORPURGO R, DOLEZEL J, AFZA R (1996). Induction and verificaton of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica, 88: 25-34.

VAN GEYT J, SPECHMANN G, D'HALLIUN K, JACOBS M (1987). *In vitro* induction of haploid plants from unpollinated ovules and ovaries of the sugar beet (*Beta vulgaris* L.). Theoretical and Applied Genetics, 73: 920-925.

VAN HUYLENBROECK J, DE RIEK J, DE LOOSE M (2000). Genetic relationships among *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis* revealed by AFLP, morphology and ploidy analysis. Genetic Resources and Crop Evolution, 47: 335-343.

VANOVERSCHELDE M (1989). Essais de polyploïdisation dans le cadre de la sterilité dans le genre *Rhododendron*. Mémoire en vue de l'obtention du grade d'ingenieur agronome, UCL, 89 pp.

VAN SUMERE C, VANDECASTEELE K, DE LOOSE R, HEURSEL J (1985). Reversed-phase – HPLC analysis of flavonoids and the biochemical identification of cultivars of evergreen azalea. Biochemistry of Plant Phenolics, Annual Proceedings of the Phytochemical Society of Europe, 25: 17-43.

VAN TUYL J, DE JEU M (1997). Methods for overcoming interspecific crossing barriers. In: Shivanna K, Sawhney V (eds). Pollen biotechnology for crop production and improvement. University Press, Cambridge: 273-292.

VAN TUYL J, DE VRIES J, BINO R, KWAKKENBOS A (1989). Identification of 2n-pollen producing interspecific hybrids of Lilium using flow cytometry. Cytologia, 54: 737-745.

VAN TUYL J, MEIJER H, VAN DIËN M (1992). The use of oryzalin as an alternative for colchicine *in vitro* chromosome doubling of *Lilium* and *Nerine*. Acta Horticulturae, 325: 625-630.

VAN TUYL J, VAN DIËN M, VAN CREIJ M, VAN KLEINWEE T, FRANKEN J, BINO R (1991). Application of *in vitro* pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Science, 74: 115-116.

VAN TUYL J, VAN HOLSTEYN H (1996). Lily breeding research in the Netherlands. Acta Horticulturae, 414: 35-45.

VAUGHAN M, VAUGHN K (1988). Carrot microtubules are dinitroaniline resistant. I. Cytological and cross-resistance studies. Weed Research, 2: 73-83.

VAUGHN K (2000). Anticytoskeletal herbicides. In: Nick P (ed) Plant microtubules, potential for biotechnology. Springer, Berlin: 193-205.

VAUGHN K, LEHNEN L (1991). Mitotic disrupter herbicides. Weed Science, 39: 450-457.

VAUGHN K, VAUGHAN M (1988). Mitotic disrupters from plant cells, effects on plants. In: Cutler H (ed) Biologically active natural products: potential uses in agriculture. American Chemical Society, Washington DC: 273-293.

VAUGHN K, VAUGHAN M (1990). Structural and biochemical characterization of dinitroanilineresistant *Eleusine*. In: Green M, Le Baron H, Moberg W (eds) Managing resistance to agrochemicals: from fundamental research to practical strategies. American Chemical Society, Washington DC: 364-375.

VEILLEUX R (1985). Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breeding Reviews, 3: 253-288.

VERHOEVEN H, RAMULU K, DIJKHUIS P (1990). A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginifolia*. Planta, 182: 408-414.

VERVAEKE I (2002). Functional anatomical study of the fertilization in Bromeliaceae. Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences, KUL, 209 pp.

VERVAEKE I, PARTON E, MAENE L, DEROOSE R, DE PROFT MP (2001). Prefertilization barriers between different Bromeliaceae. Euphytica, 118: 91-97.

VILLAR M, GAGET-FAUROBERT M (1997). Mentor effects in pistil-mediated pollen-pollen interactions. In: Shivanna K, Sawhney V (eds). Pollen biotechnology for crop production and improvement. University Press, Cambridge: 315-332.

VOS P, HOGERS R, BLEEKER M, REIJANS M, VAN DE LEE T, HORNES M, FIJTERS A, POT J, PELEMAN J, KUIPER M, ZABEAU M (1995). AFLP™: a new technique for DNA fingerprinting. Nucleic Acids Research, 21: 4407-4414.

VOSS D (2001). What is an azalea? Journal of the American Rhododendron Society, 55: 188-192.

VROEMEN C, DE VRIES S (1999). Flowering plant embryogenesis. In: Russo V, Cove D, Edgar L, Jaenisch R, Salamini F (eds). Development: genetics, epigenetics and environmental regulation. Springer, Berlin: 121-132.

WADA K (1974). Hybridizing myths of evergreen azaleas. Quarterly Bulletin of the American Rhododendron Society, 28: 101-105.

WAGNER G (1979). Content and vacuole/extravacuole distribution of neutral sugars, free amino acids and anthocyanen in protoplasts. Plant Physiology, 64: 88-93.

WALTERS S, BRADY A, BRICKELL C, CULLEN J, GREEN P, LEWIS J, MATTHEWS V, WEBB D, YEO P, ALEXANDER J (1996). Araceae. In: The European Garden Flora Vol II: monocotyledons. Cambridge University Press, Cambridge: 75-112.

WANG C, KUANG B (1981). Induction of haploid plants from the female gametophyte of *Hordeum vulgare* L. Acta Botanica Sinica, 23: 329-330.

WANG P, CHEN Y (1986). A study on the application of C17 medium for anther culture. Acta Botanica Sinica, 28: 38-45.

WENDEL J (2000). Genome evolution in polyploids. Plant Molecular Biology, 42: 225-249.

WENSLAFF T, LYRENE P (2000). The use of mentor pollination to facilitate wide hybridization in blueberry. HortScience, 35: 114-115.

WERBROUCK S (1987). Bestuiving en zaadzetting bij *Rhododendron simsii* Planch. Werk einde studiën, Rijksuniversiteit Gent, Faculteit van de Landbouwwetenschappen, 95 p.

WERBROUCK S (1997). Studiecentrum voor *in vitro* veredeling van sierplanten, overeenkomst nr. D 1/4 6185/5707A, verslag van de werkzaamheden van 01/02/96 tot 30/11/97, 32 pp.

WERBROUCK S, DEBERGH P (1995). Imazalil enhances the shoot inducing effect of benzyladenine in *Spathiphyllum floribundum* Schott. Journal of Plant Growth Regulation, 14: 105-107.

WERBROUCK S, DEBERGH P (1996). Imidazole fungicides and paclobutrazol enhance cytokinin-induced adventitious shoot proliferation in Araceae. Journal of Plant Growth Regulation, 15: 81-85.

WERBROUCK S, DHUYVETTER H, SAIBO N, VANDERSTRAETEN D, DE SCHEPPER S, DEBERGH P (2003). Physiological and morphological evidence of brassinosteroid-biosynthesis inhibition by the fungicide imazalil. Physiologia Plantarum, accepted for publication.

WERBROUCK S, REDIG P, VAN ONCKELEN H, DEBERGH P (1996). Gibberellins play a role in the interaction between imidazole fungicides and cytokinins in Araceae. Journal of Plant Growth Regulation, 15: 87-93.

WERBROUCK S, EECKHAUT T, DEBERGH P (2000). Induction and conversion of somatic embryogenesis on the anther filament of *Spathiphyllum* Schott. Acta Horticulturae,

520: 263-269.

WHEELER M, FRANKLIN-TONG V, FRANKLIN F (2001). The molecular and genetic basis of pollen-pistil interactions. New Phytologist, 151: 565-584.

WHITECROSS M, WILLING R (1975). Hybridization of incompatible poplars following solvent treatment of stigmas. Experientia, 31: 651-653.

WILHELMI L, PREUSS D (1997). Blazing new trails: pollen tube guidance in flowering plants. Plant Physiology, 113: 307-312.

WILLIAMS E, KAUL V, ROUSE J, KNOX R (1984). Apparent self-incompatibility in *Rhododendron ellipticum, R. championae* and *R. amamiense*: a post-zygotic mechanism. Plant Cell Incompatibility Newsletter, 16: 10-11.

WILLIAMS E, KNOX R, ROUSE J (1982). Pollination sub-systems distinguished by pollen tube arrest after incompatible interspecific crosses in *Rhododendron* (Ericaceae). Journal of Cellular Science, 53: 255-277.

WILLIAMS E, WEBB M (1987). Enclosure of pollinated flowers weakens the gametophytic self-incompatibility response in *Lycopersicon peruvianum*. Journal of Experimental Botany, 38: 1756-1764.

WILLIAMS E, ROUSE J (1988). Disparate style lengths contribute to isolation of species in *Rhododendron*. Australian Journal of Botany, 36: 183-191.

WILLIAMS E, ROUSE J (1990). Relationships of pollen size, pistil length and pollen tube growth rates in *Rhododendron* and their influence on hybridization. Sexual Plant Reproduction, 3: 7-17.

WILLIAMS E, ROUSE J, PALSER B, KNOX R (1990). Reproductive biology of *Rhododendron*. Horticultural Reviews, 12: 1-67.

WU A, ZHU B, LI Q (1996). Studies on somatic embryogenesis of *Pinellia pedatisecta* Schott. Acta Agronomica Sinica, 22: 66-71.

YAMAGUCHI S (1986). *In vitro* culture of remote hybrid seedlings aiming to breed new yellow flowered evergreen azalea. Plant and Cell Incompatibility Newsletter, 18: 50-51.

YAO J, COHEN D (1996). Production of triploid *Zantedeschia* hybrids using embryo rescue. New Zealand Journal of Crop and Horticultural Science, 24: 297-301.

YAO J, COHEN D (2000). Multiple gene control of plastome-genome incompatibility and plastid DNA inheritance in interspecific hybrids of *Zantedeschia*. Theoretical and Applied Genetics, 101: 400-406.

YAO J, COHEN D, ROWLAND R (1994). Plastid DNA inheritance and plastome-genome incompatibility in interspecific hybrids of *Zantedeschia* (Araceae). Theoretical and Applied Genetics, 88: 255-260.

YAO J, COHEN D, ROWLAND R (1995). Interspecific albino and variegated hybrids in the genus *Zantedeschia*. Plant Science, 109: 199-206.

YU T, YEAM D (1972). A survey on flower types and colors in *Hibiscus syriacus* L. Journal of the Korean Society for Horticultural Science, 11: 55-61.

YU T, YEAM D, KIM Y (1976). A study on the breeding of *Hibiscus syriacus* L. on hybridization among introduced, tetraploids and *H. rosa-sinensis*. Journal of the Korean Society for Horticultural Science, 17: 107-112.

ZENKTELER M (1990). *In vitro* fertilization and wide hybridization in higher plants. Critical Reviews in Plant Sciences, 9: 267-279.

ZHANG Y, LESPINASSE Y (1991). Pollination with gamma-irradiated pollen and development of fruits, seeds and parthenogenetic plants in apple. Euphytica, 54: 101-110.

ZHENG Q, DESSAI, A, PRAKASH C (1996). Rapid and repetitive plant regeneration in sweetpotato via somatic embryogenesis. Plant Cell Reports, 15: 381-385.

ZHU Z, WU H (1979). *In vitro* induction of haploid plantlets from unpollinated ovaries of *Triticum aestivum* and *Nicotiana tabacum*. Acta Genetica Sinica, 23: 499-501.

Curriculum vitae

Personalia

Naam en voornamen: Tom Gerard Roger Eeckhaut
Adres: Poststraat 28, 9860 Oosterzele (Balegem)
Tel 0485/86 93 95
E-mail: t.eeckhaut@clo.fgov.be
Geboorteplaats en -datum: Gent, 14 juni 1974
Nationaliteit: Belg
Burgerlijke staat: ongehuwd

Studies

Middelbaar onderwijs (1986-1992)
Algemeen Secundair Onderwijs
richting Latijn-Wiskunde
College O.L.V. Van Deinsbeke (Zottegem)
afgestudeerd met onderscheiding

Hoger Onderwijs (1992-1997)
Bio-ingenieur in de cel- en genbiotechnologie
Universiteit Gent
afgestudeerd met onderscheiding

Scriptie: "Screenen van fytohormonale effecten van fungiciden" (15/20).

Beroepservaring

01/02/98 – 30/11/99: Wetenschappelijk medewerker (Laboratorium voor Tuinbouwplantenteelt, Vakgroep Plantaardige Productie, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent)
Titel onderzoeksproject: "*In vitro* veredelingstechnieken voor Araceae en azalea" (Ministerie van Middenstand en Landbouw).

01/12/99 – 30/09/00: Wetenschappelijk medewerker (Departement voor Plantenveredeling, Centrum voor Landbouwkundig Onderzoek, Melle)
Titel onderzoeksproject: “Veredelingsstrategieën met behulp van moleculair biologische technieken en productvernieuwing van vegetatief vermeerderde sierplanten” (DWTC).

01/10/00 – 30/09/02: Wetenschappelijk medewerker (Departement voor Plantenveredeling, Centrum voor Landbouwkundig Onderzoek, Melle)
Titel onderzoeksproject: “Creatie van nieuwe bloemkleuren bij azalea en *Hibiscus*: mogelijkheden van embryo rescue en in vitro bevruchting” (DWTC).

01/10/02 – 31/12/02: Wetenschappelijk medewerker (Departement voor Plantenveredeling, Centrum voor Landbouwkundig Onderzoek, Melle)
Titel onderzoeksproject: "Monitoring" (AventisCropScience)

01/01/03 – 31/01/03: Wetenschappelijk medewerker (Departement voor Plantenveredeling, Centrum voor Landbouwkundig Onderzoek, Melle)
Titel onderzoeksproject: "Creatie van bloemkleurvariatie in azalea" (O&O)

01/02/03-nu: Wetenschappelijk medewerker (Departement voor Plantenveredeling, Centrum voor Landbouwkundig Onderzoek, Melle)
Titel onderzoeksproject: "Evaluatie van het genetisch potentieel van inlandse rozenspecies met het oog op behoud van genetische diversiteit en gebruik in de tuinbouw" (IWT)

Begeleiding van scripties

- VANDENHOECK PATRICK (1999). A1 Farmaceutische & Biologische Technieken, Erasmushogeschool Jette.
- VAN MOER NELE (2000). A1 Landbouwkunde, optie Tuinbouw, Tuinbouwschool Sint-Niklaas.
- TAILLEUX WOUTER (2002). Ind. Ing. Tuinbouw, CTL Gent (2001-2002).
- OP DE BEECK STIJN (2003). Ind. Ing. Tuinbouw, H.I. Kempen (2002-2003).

Bijgewoonde congressen, symposia en studiedagen

- 02/08/98 - 08/08/98: XXV International Horticultural Congress, Brussels. Poster: Somatic embryogenesis on anther filaments of *Spathiphyllum*
- 16/10/98: Phytohormones, Croissance et development. Liège, Institut de Botanique B22, BPTCG
- 19/11/98 - 21/11/98: 5th Plant Embryogenesis Workshop - 2nd European Plant Embryogenesis Network Meeting. Barcelona. Poster: Somatic embryogenesis on anther filaments of *Spathiphyllum*: effect of developmental stage
- 10/12/98: Symposium on Plant Regeneration, organisation: Cost 8.22. DVP, Merelbeke.
- 08/01/99: FWO Research Community on "Plant Growth Regulators: metabolism, function and mode of action": 1st international meeting, Antwerpen
- 13/10/99: 5th PhD Symposium. Faculty of Agricultural and Applied Biological Sciences, Gent. Poster: Ploidy manipulation in *Spathiphyllum wallisii.*
- 23/11/00 – 26/11/00: COST 843 meeting, Blankenberge
- 03/07/2001 - 06/07/2001: 20th International Symposium Eucarpia – Section Ornamentals. Posters: Creation of tetraploid *Rhododendron simsii* hybrids / Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis.
- 24/09/01 – 25/09/01: 15th Forum for Applied Biotechnology, Gent. Poster: Creation of tetraploid *Rhododendron simsii* hybrids
- 05/11/01 – 06/11/01: COST 851 Brussel: 1st Management Committee Meeting
- 09/05/02- 12/05/02: COST 851, Working Group I, Boedapest. Oral presentation: Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis.

- 16/05/02- 21/05/02: Rhododendrons in Horticulture and Science, Royal Botanical Gardens, Edinburgh. Poster: Creation of tetraploid *Rhododendron simsii* hybrids.
- 24/08/03 – 29/08/03: 21st International Symposium Eucarpia – Section Ornamentals. Oral Presentation: Interspecific crosses in the *Rhododendron* genus involving *R. simsii* hybrids. Posters: The DvP azalea collection as a tool for classical and molecular breeding / Interspecific hybridization in flowering shrubs.

Publicaties

Wetenschappelijk

EECKHAUT T, DEBERGH P (1999). Ploidy manipulation in *Spathiphyllum wallisii*. Mededelingen van de Faculteit Landbouwwetenschappen, Universiteit Gent, 64/5: 111-115.

EECKHAUT T, SAMIJN G, VAN BOCKSTAELE E (2001). *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. Mededelingen van de Faculteit Landbouwwetenschappen, Universiteit Gent, 66: 451-454.

EECKHAUT T, SAMIJN G, VAN BOCKSTAELE E (2002). *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. In: Van Huylenbroeck J, Van Bockstaele E, Debergh P (eds) Acta Horticulturae, 572: 43-49.

EECKHAUT T, SAMIJN G, VAN BOCKSTAELE E (2003). Interspecific breeding in the *Rhododendron* genus involving *R. simsii* hybrids. In: Forkmann G, Hauser B, Michaelis S (eds) Acta Horticulturae, 612: 165-170.

EECKHAUT T, WERBROUCK S, DENDAUW J, VAN BOCKSTAELE E, DEBERGH P (2001). Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell Tissue and Organ Culture, 67: 181-189.

WERBROUCK S, EECKHAUT T, DEBERGH P (2000). Induction and conversion of somatic embryogenesis on the anther filament of *Spathiphyllum* Schott. In: Van der Plas L, De Klerk G (eds) Acta Horticulturae, 520: 263-270.

WERBROUCK S, JUMLI, EECKHAUT T, DEBERGH P (1997). Screening hormonal effects of fungicides with a *Ficus benjamina* L. chimera. Mededelingen van de Faculteit Landbouwwetenschappen, Universiteit Gent, 62/3b.

In voorbereiding

EECKHAUT T, WERBROUCK S, LEUS L, VAN BOCKSTAELE E, DEBERGH P. Polyploidization of *Spathiphyllum wallisii* Regel through somatic embryogenesis.

EECKHAUT T, LEUS L, DE RAEDT A, VAN BOCKSTAELE E. Detection of polyploidy in *Rhododendron luteum* Sweet, 'Hardy Ghent'and 'Rustica' hybrids.

Vulgariserend

EECKHAUT T (1998). Inductie van embryo's op antherenfilamenten van *Spathiphyllum*. Sierteeltonderzoek in België 1998, 27.

EECKHAUT T, SAMIJN G (2000). Polyploïdisatie van azalea's. Sierteeltonderzoek in België, 63.

EECKHAUT T, SAMIJN G (2000). Studie van 'embryo rescue' als hulpmiddel bij incompatibele kruisingen binnen Rhododendron. Sierteeltonderzoek in België, 64.

EECKHAUT T, SAMIJN G (2001). Studie van prefertilisatiebarrieres bij incompatibele kruisingen binnen Rhododendron. Sierteeltonderzoek in België, 57.