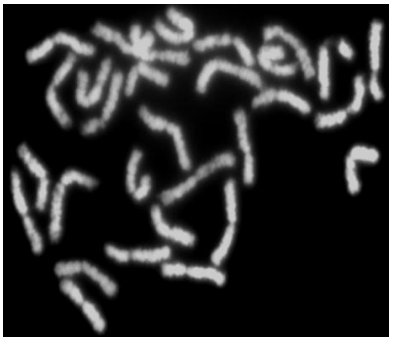

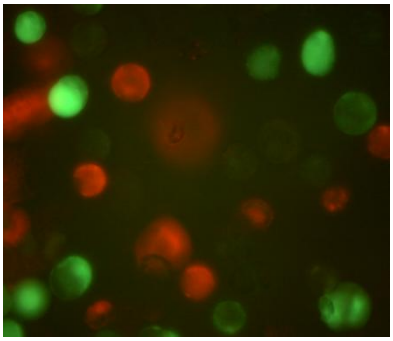

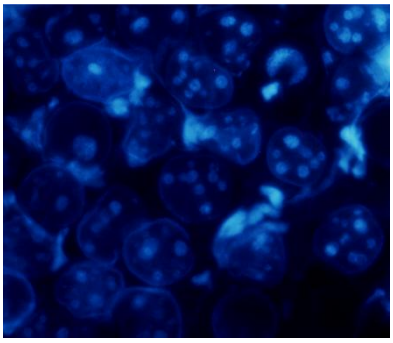

Molecular cytogenetic studies and technology development for creating aroid (Araceae) asymmetric somatic hybrids

Prabhu Shankar Lakshmanan

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Know the self to be sitting in the chariot,
the body to be the chariot,
the intellect the charioteer,
and the mind the reins.

(Katha Upanishad, ~ 1400 BC)

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Molecular cytogenetic studies and technology development for creating aroid (Araceae) asymmetric somatic hybrids

Prabhu Shankar Lakshmanan

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

The title in Dutch:

Moleculair cytogenetische studies en technologieontwikkeling voor het creëren van asymmetrische somatische hybriden binnen de aronskelkfamilie (Araceae)

Cover pictures:

A *Zantedeschia aethiopica* 'spadix' covered by colorless 'spathe' as an example for aroid flower morphology, a DAPI stained chromosome spread of *Spathiphyllum wallisii*, a mix of FDA stained *Z. rehmanii* (green) and RITC stained *S. wallisii* (red) protoplasts prior to fusion and DAPI stained micronucleated *S. wallisii* microspores. All the microscopic pictures were captured under a fluorescence microscope using UV irradiation.

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It was dusk on the Graslei. I saw people enjoying their talks, drinks and puffs. Watching that scene made me happy. I strolled through the city streets and ate a warm waffle... So began my first day in Ghent. I loved it then and have loved it ever since.

In October 2009, under the magical influence of those enchanting moments in Ghent, I officially started my joint PhD between UGent and Moscow State Agrarian University. I joined ILVO's 'protoplast team' with Tom Eeckhaut, Dieter Deryckere and Ronald van den Oord. Ronald was patient when teaching me *in vitro* techniques. Technical discussion with Dieter and Ronald about improving protocols helped me work more efficiently. To my surprise, even Pepijn De Raeymaecker (who seemed to be cold at first) started to warm up to me. Joking and laughing with Dieter and Pepijn was fun.

I went to Moscow in 2010 for cytogenetic experiments as a part of this PhD program. There I worked with a Russian version of Dieter and Pepijn - Michel Bodelin and Dimitry Romanov. (When I came here for a PhD, I sure didn't expect my life to be filled with this kind of people! A Mediterranean version of these guys would be Panagiotis Theocharis and Luca Pipino; the Indian version would be Hemant Pratap Singh and Joji John.) I thank you all for the fun we had! The city of Moscow was pleasant to explore and our other colleagues were very warm-hearted. During the winter of 2011, I was "cold-treated" and sent back to Belgium. Now when I miss Moscow, I just go to the big freezer at ILVO and stay there for a while, full of nostalgia.

I am very lucky to have marvelous teachers, tuition masters, mentors, guides, yoga masters and supporters. I have always wondered how they could help a person without having any expectations and with such great patience. I remember every one of you and thank you all. In particular, I thank Prof. Van Bockstaele for his supervision. I thank Prof. Khrustaleva for her guidance and her efforts to start this collaboration in spite of all the tough administrative procedures. I also thank the board of examiners: Prof. Van Damme, Prof. Werbrouck, Prof. Van Labeke and Dr. Geerts for their valuable comments on this doctoral thesis.

My most heartfelt thanks go to Tom for everything he has taught me and his fine mentorship throughout my stay at ILVO. Special thanks go to Johan Van Huylenbroeck for his training on critical writing and guidance on publishing and presenting the results. I would like to thank Katrijn Van Laere and Leen Leus for their help in cytogenetic and cytological analysis, Jorien Oomen for her technical assistance in cytogenetic experiments and Miriam Levenson for her proof reading efforts.

I am also grateful to Kristiaan Van Laecke, Emmy Dhooghe, Prof. Elena Kalashnikova, Prof. Alexander Soloviev, Irina Lapitskaya, Ilya Kirov, Laurence Desmet, Anne-Marie Van Exter, Kristien Janssens and all of my other colleagues from ILVO and Moscow State Agrarian University.

Fortunately or unfortunately, I also have to thank the UZ hospital emergency units, orthopedics and general practitioners for treating me after the multiple accidents and illnesses I suffered during my stay in Belgium. My friends (Mary, Arthi, Linda, Vasia, Vidhya, Fenila, Jacob, Karthik, Giacomo,…) were always there for me whenever I was in need. Thanks for the food you cooked for me when I was sick, even when your food made me sicker ☺. It was the thought that counted…

I give thanks to my mother for teaching me feelings and emotions. I am grateful to my father for showing me how to be straight-forward and honest. My three sisters have cared for me to the extreme, which has spoilt me rotten. I thank them for their continued love and care. I love my mom, I like my dad and I adore my sisters. My nephews, nieces, cousins and my brothers-in-law love me the most and I love them too.

Some wise old men have told me that I am always surrounded by the best, especially when it comes to people. They were right. I give thanks and respect to these wise elders who have shown up out of nowhere to give me the taste to appreciate life, my own being and my connection to nature.

The journey has been wonderful so far and will continue to be!

25·11·2013, Melle, Belgium.

The aim of this dissertation was to produce Araceae (aroid) asymmetric somatic intergeneric (between two different genera) hybrids and to use molecular cytogenetic tools to select fusion products. Aroids are monocots that are mostly known for their ornamental values as cut flowers, pot and landscape plants. Intergeneric or interspecific hybridization is a method to introduce valuable traits desired by ornamental industry. Due to sexual breeding barriers, distant breeding is sometimes impossible. In Araceae, there is no report yet on successful intergeneric hybrid production. One of the ways to overcome the barriers is asexual hybridization through protoplast fusion. Protoplasts are plant cells without cell wall. Therefore, they can be easily fused. If the fusion is performed between two different parent protoplasts with complete genomes, it is referred to as symmetric; if a parent's genome (donor) is fragmented and fused, the fusion is asymmetric. As only a small part of the genome is inserted, less undesired genes are incorporated and less gene conflict occurs in the asymmetric hybrid cell; thus there are less restrictions for complete regeneration. There are reports on unsuccessful attempts to produce intergeneric symmetric fusion between *Anthurium* and *Spathiphyllum.* Our aim was to develop a model system to create asymmetric hybrids, which could lead to incorporate economically important traits such as disease resistance, flower color or leaf shape, etc., in Araceae. We studied *Anthurium andreanum*, *Spathiphyllum wallisii* and *Zantedeschia spp.*

Cytological and cytogenetic information such as genome size, chromosome morphology and size differences could distinguish hybrids from parent plants. Hybridity can further be confirmed using molecular cytogenetic analysis through *in situ* hybridization (ISH) using repetitive DNA sequences (FISH) and/or genomic DNA of parents (GISH). To create a general protocol for molecular cytogenetics that could be useful for future breeding programs in a large sense, besides the three above mentioned genera, we also included *Monstera deliciosa, Philodendron scandens* and *Syngonium auritum.* More specifically, the following goals were set in this study: (i) cytological and cytogenetic analysis in Araceae model plants as a basis for implementing molecular cytogenetic characterization in putative fusion products, (ii) protoplast and tissue culture studies including the establishment of an explant system, isolation, fusion and regeneration techniques, and (iii) the development of genome fragmentation technology using microprotoplasts for asymmetric hybrid production (Fig. 1).

A detailed view of intergeneric hybridization techniques, problems, solutions, and recent advances in somatic hybridization techniques is presented in chapter 1. That chapter contains a brief outline of the Araceae plant family, its breeding history and recent research activities. It also lists the techniques used in this study.

With the aim of creating a general protocol for aroids, we performed cytological and karyotypical studies on six genera using *Anthurium andreanum*, *Monstera deliciosa, Philodendron scandens,* S*pathiphyllum wallisii*, *Syngonium auritum* and *Zantedeschia elliottiana.* Physical mapping of 45S and 5S rDNA repeats using fluorescence *in situ* hybridization

(FISH) techniques were performed as a basis for implementing molecular cytogenetic studies in putative hybrid selection (Chapter 2).

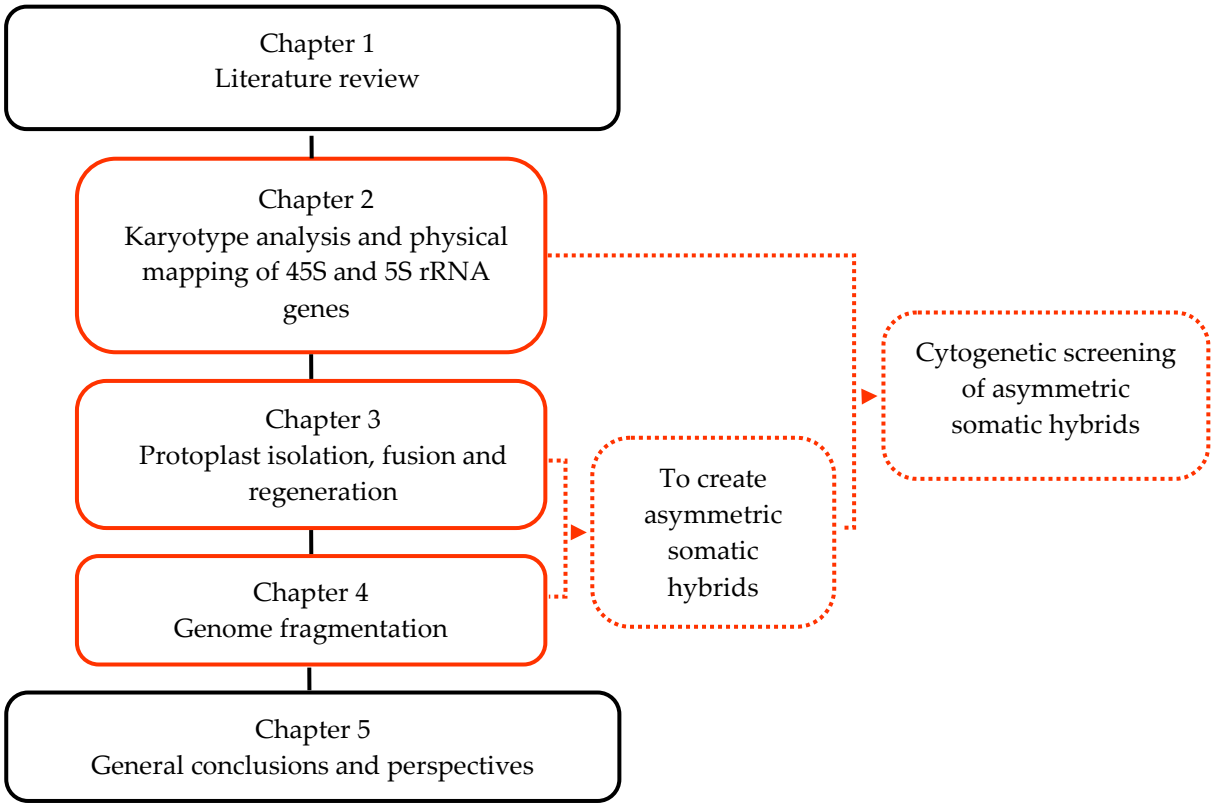

Fig. 1 The outline of the doctoral thesis entitled 'Molecular cytogenetic studies and technology development for creating aroid (Araceae) asymmetric somatic hybrids'. The colored part of the flow chart represents the overall research; the dotted lines show the final aim; and the continuous lines represent the specific research goals.

Chapter 3 summarizes our efforts to study protoplast isolation from various sources, including chemical and electrical fusion methods and regeneration methods. It also describes the establishment of culture systems for protoplast donor tissues. We used *Anthurium, Spathiphyllum* and *Zantedeschia* as model plants for tissue culture and protoplast related studies.

As a start for microprotoplast mediated chromosome transfer, we established a genome fragmentation system through micronucleation using *S. wallisii* developing microspores (Chapter 4). We tested the micronucleation efficiency of various mitotic inhibitors and genotypes; and evaluated multiple parameters.

The last chapter presents the results and findings from this study and a short general outlook for future research (Chapter 5).

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ, γ-dimethylallylamino) purine
AC	alternate current
AFLP	amplification fragment length polymorphism
AgNORs	silver stained nucleolus organizing region
ANOVA	analysis of variance
Antidig-POD	anti-digoxigenin peroxidase
APM	amiprophos-methyl
A-T	adenine-thymine
B5	Gamborg B5 salts
BA	6-benzyladenine
BAP	6-benzylaminopurine
BUT	butamiphos
CAPS	cleaved amplified polymorphic sequence
CIPC	chlorpropham
CMAn	*Anthurium* stock calli culture media
CMSp	*Spathiphyllum* stock calli culture media
CMZn	*Zantedeschia* stock calli culture media
COL	colchicine
cpDNA	chloroplast DNA
CY3	cyanine dye 3
DAPI	4', 6-diamidino-2-phenylindole
DC	direct current
Dig	digoxigenin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCM	flowcytometry
FDA	fluorescein diacetate
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FW	fresh weight
G-C	guanine-cytosine
GC-MS	gas chromatography-mass spectrometry
GFP	green fluorescent protein
GISH	genomic *in situ* hybridization
HCl	hydrochloric acid
hPa	hectoPascal
HPLC	high-performance liquid chromatography
HRM	high resolution melting

HRP	horse radish peroxidase
ILVO	Institute for agricultural and fisheries research
IOA	iodoacetamide
IRAP	interretroelement amplified polymorphism
ISH	*in situ* hybridization
ISSR	intersimple sequence repeat
ITS	internal transcribed spacers
KIN	kinetin
KM-OA	Kao and Michayluk-organic acids
LMPA	low melting point agarose
LS	Linsmaier and Skoog salts
m	metacentric
MES	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
MMCT	microprotoplast mediated chromosome transfer
MNi	micronuclei
MS	Murashige and Skoog salts
MSAP	methylation-sensitive amplified polymorphism
mtDNA	mitochondrial DNA
N6	Chu N6 salts
NAA	naphthaleneacetic acid
NOR	nucleolar organizing region
NTS	non-transcribed spacer
ORY	oryzalin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
PMAn	*Anthurium* stock plant culture media
PMC	pollen mother cell
PMSp	*Spathiphyllum* stock plant culture media
PMZn	*Zantedeschia* stock plant culture media
PP	protoplast
PP/g FW	protoplast per gram fresh weight
PRO	propyzamide
RAPD	random amplification of polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
REMAP	retroelement-microsatellite amplified polymorphism
RFLP	restriction fragment length polymorphism
Rho/RITC	rhodamine β isothiocyanate

RNAse	ribonuclease
rRNA	ribosomal ribonucleic acid
SCGE	single cell gel electrophoresis
SE	standard error
sm	submetacentric
SNP	single nucleotide polymorphism
SQ RT-PCR	semi-quantitative real-time PCR
SSC	saline sodium citrate buffer
SSR	simple sequence repeats
st	subtelocentric
t	telocentric
TDZ	thidiazuron
TN buffer	(tris-HCl, NaCl) buffer
TNB	tris-NaCl-blocking buffer
TNT buffer	tris-NaCl-tween buffer
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloric acid
Tyr-FISH	tyramide-fluorescence *in situ* hybridization
Tyr-FITC	tyramide-fluorescein isothiocyanate
UV-C	ultraviolet C irradiation
X	X-ray irradiation
γ	gamma-ray irradiation

De Vries, 1900 Sleper and Poehlman, 1995 Von Hanstein, 1880 Klercker, 1892

Cocking, 1960 Kao *et al.* 1974 Stebbins, 1958 Hogenboom, 1975

Küster, 1909 Carlson *et al.* 1972 Dudits *et al.* 1980 Saito and Nakano, 2002

Dobzhansky, 1951 Morejohn *et al.* 1987 Verhoeven *et al.* 1990 Ramulu *et al.* 1995

Matthews *et al.* 1999 Fournier and Ruddle, 1977 Lakshmanan *et al.* 2013

Eriksson and Jonasson, 1969 Townsend, 1897 Kao and Michayluk, 1975

Chapter 1
Literature review

Murashige and Skoog, 1962 Zimmermann and Scheurich, 1981 Gleba *et al.* 1982

De Jong, 2003 Galbraith *et al.* 1983 Pardue and Gall, 1969 Eeckhaut *et al.* 2013

Appels and Honeycutt, 1986 Croat, 1982 Leitch and Heslop-Harrison, 1992

Schwarzacher *et al.* 1989 Jiang and Gill, 1993 Khrustaleva and Kik, 1998

Werbrouck *et al.* 1998 Snijder *et al.* 2007 Kuehnle *et al.* 1991 Pongchawee *et al.* 2006

Duquenne *et al.* 2007 Mayo *et al.* 1997 Shibuya, 1956 Henny and Chen, 2010

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Eeckhaut, T., Lakshmanan, P. S., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Progress in plant protoplast research. Planta, 238: 991-1003. doi: 10.1007/s00425-013-1936-7.

1.1 Introduction

The agricultural development in the Neolithic period was probably the stepping stone for modern civilization (Wessel, 1984). Early humans domesticated wild plants and selected better plant materials for cultivation; this is the basic plant breeding technique. The selected plant materials were introduced in new areas and hybridized without human intervention. Later, seeds of the better performing plants were collected and stored for the next cultivation. Early farmers slowly learned the basic plant breeding principles and laid the foundation for modern plant breeding, which remains essential to agricultural development. Modern plant breeding is based on genetic principles. It flourished after the rediscovery of Mendel's work in 1900 by De Vries. Plant breeding has been defined as: « the art and the science of improving the heredity (the genetic characters so transmitted) of plants for the benefit of mankind » (Sleper and Poehlman, 1995). Plant breeding results in new cultivars with useful traits for commercial growers, farmers and gardeners.

In ornamental plant industry, new varieties are desired for their morphological characteristics such as flower or foliage differences, or their physiological properties such as biotic or abiotic stress resistance. The introduction of new plants selected from the wild still remains one of the primary introduction methods. These newly collected plants are subjected to evaluation, prior to release, such as taxonomic identification, propagation methods, cultivation and assessment of their horticultural values. However, international commercialization of new valuable wild plants falls under the limitations of the Convention on Biological Diversity, United Nations, 1992. Another source of genetic variation is spontaneously occurring mutations (Debergh, 1992). These somatic mutations might be due to changes in chromosome number, loss of genes or transposon activities. As the most ornamentals are vegetatively propagated, these mutations may result in stable mutant clones. They may be used as starting material for further breeding purposes or directly released as new cultivars (Van Harten, 2002).

In ornamentals, introgression of valuable traits can also be achieved through traditional breeding between distantly related species or genera (Van Tuyl and De Jeu, 1997; Liu *et al.* 2005). Depending on crossing parents, *intraspecific* (within a single species), *interspecific* (between different species) and *intergeneric* (between different genera) hybridization can be distinguished. Numerous examples of hybrids from distantly related species and genera are available in many plants (Knobloch, 1972; Sharma and Gill, 1983; Sharma, 1995).

Interspecific or intergeneric crosses are not possible between some species and/or genera due to sexual incongruity barriers (Hogenboom, 1975). Incongruity is not related to (self-) incompatibility, a system typically occurring after crosses between closely related genotypes that have evolved to encourage outbreeding and heterozygosity in flowering plants (de

Nettancourt, 1977, Franklin-Tong and Franklin, 2000). As opposed to self-incompatibility, interspecific incompatibility or incongruity is the result of barriers determined by evolutionary divergence of physiology or morphology between species (Hogenboom, 1973). Incongruity occurs as a result of the lack of genetic information in both parent plants that is necessary to complete pre- and post- fertilization processes. Unlike self-incompatibility, which is mainly a prefertilization barrier, incongruity can cause both pre- and post-fertilization barriers (Raghavan, 1997). Dobzhansky (1951) and Stebbins (1958) classified the factors affecting easy gene flow through sexual breeding into pre- or post-fertilization barriers. Prezygotic incongruity can easily be identified as failure of pollen germination, pollen tube growth or fertilization (De Verna *et al.* 1987). Postzygotic barriers comprise inhibition of seed formation and germination, albino hybrids, hybrid breakdown and hybrid sterility (Sharma, 1995; Van Tuyl *et al.* 1991).

Knowledge about the sexual barriers in the attainment of desired hybrids, between distantly related species or genera, inspired plant breeders to develop new techniques. Techniques to overcome pre- and post- fertilization barriers are reviewed by Van Tuyl and De Jeu (1997) and Jansky (2006). For pre-fertilization barriers, reciprocal crosses, pollination on immature or aged stigma, chemical applications such as hormones, organic solvents, mentor pollination, heat or irradiation treatment of style, the cut-style method, and *in vitro* pollination can be applied. Post fertilization barriers can be overcome by *in vitro* culture of ovaries, ovules or embryo. But the above techniques are not applicable to every species. Bridge crosses are a possible method when two species/genera are incompatible but both are compatible with an intermediate plant. In this case, an intermediate crossing is first performed with a compatible plant and the resulting interspecific hybrid is subsequently crossed with a desired parent plant; in this way, genomes or segments are indirectly combined between incompatible species. Bridge crosses are time-consuming, however (Jansky, 2006).

Modern techniques of tissue culture and genetic engineering allow us to move from traditional plant breeding methods to asexual methods. One of these methods is somatic hybridization or protoplast fusion, which has yielded distant hybrids between plants in several genera (Waara and Glimelius, 1995; Grosser *et al.* 2000; Johnson and Veilleux, 2001; Orczyk *et al.* 2003; Guo *et al.* 2004; Liu *et al.* 2005; Puite, 2006).

Somatic hybridization has three main advantages over transgenic approaches: (i) it broadens the germplasm base, (ii) allows the transfer of uncloned multiple genes, and (iii) generates products that are not subjected to the same legal regulations as transgenic lines (Grosser and Gmitter, 2005; 2011). However, according to Belgian Biosafety Directive 2001/18/EC which states 'cell fusion (including protoplast fusion) or hybridization techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or

more cells by means of methods that do not occur naturally can be considered as genetic modification'. Considering the definition, one might claim protoplast fusion as transgenic approach because parent plants cannot be crossed sexually and asymmetric fusion as fragmentation treatment doesn't happen naturally. In case of asymmetric fusion, no new genes are introduced; on the contrary, the number of genes is reduced. Furthermore, protoplast reviews report asymmetric hybrids spontaneously obtained after symmetric fusion (Eeckhaut *et al*. 2013). Meaning symmetric fusion, which is not considered as transgenic approach, could also yield asymmetric hybrids. Also in sexual crossing, genes or chromosomes of one partner can often be eliminated after crossing. The irradiation could be considered as mutagenesis and micronucleation toxin treatments as polyploidization.

Somatic hybridization is one of many breeding tools available to create various new genomic combinations, and can be used to transfer both mono- and polygenic traits (Thieme *et al.* 2004). In recent years, it has been used frequently as an alternative for incompatible sexual crossing, although (with the exception of polyploidization) other genomic effects like chromosome rearrangements are more typically observed in somatic hybrids than in their sexual counterparts (Chevre *et al.* 1994).

Articles on protoplast fusions in different plant families and species are listed in earlier reviews (Melchers and Labib, 1974; Davey and Kumar, 1983; Gleba and Sytnik, 1984; Bravo and Evans, 1985; Davey and Power, 1988; Waara and Glimelius, 1995; Davey *et al.* 2005 and Liu *et al.* 2005). A typical somatic breeding protocol can be subdivided into protoplast isolation, fragmentation (in case of asymmetric hybridization), fusion, regeneration and selection (Johnson and Veilleux, 2001; Razdan, 2003). Reviews by Davey *et al.* (2010) and Grosser *et al.* (2010) describe numerous protocols for isolation, culture, fragmentation and fusion.

1.2 Protoplast isolation

Protoplasts are cells without a cell wall; the term 'protoplast' was first defined by Von Hanstein in 1880, meaning 'first formed' (Greek: protos-first; plastos-formed). The cell wall can be removed either mechanically (Klercker, 1892) or enzymatically (Cocking, 1960). Enzymatic isolation of a protoplast is preferred over mechanical isolation, because larger number of protoplasts can be isolated with less labor (Davey and Kumar, 1983). Commonly known enzyme types are pectinase and cellulase. Pectinase digests the pectin-rich matrix, while cellulase is used to digest the cellulose-rich cell wall. The enzymatic incubation is typically performed under dark conditions. Protoplast isolation efficiency depends on many factors such as enzyme combination and concentration, osmotic pressure, incubation time, explant source and plant species. Preincubation of explant in an osmotically corrected solution helps to obtain less damaged and more viable protoplasts (Frearson *et al.* 1973;

Ortin-Parraga and Burgos, 2003). Several agents can create an osmotic equilibrium between the protoplasts and their environment: metabolically inactive sugar alcohols such as mannitol and sorbitol are most frequently applied but also glucose, sucrose or salts can be used (Fleck *et al.* 1982; Smith *et al.* 1984; Razdan, 2003).

Some well-known protoplast sources are: mesophyll cells (Tavazza *et al.* 1986; Cardi *et al.* 1990; Zhou *et al.* 2005; Eeckhaut and van Huylenbroeck, 2011; Deryckere *et al.* 2012); suspension cells (Abdullah *et al.* 1986; Tang *et al.* 2001; Inoue *et al.* 2004; Shiba and Mii, 2005); calli (Luo *et al.* 1998; Kim *et al.* 2005; Chabane *et al.* 2007; Kanwar *et al.* 2009); hypocotyls (Rakosy-Tican *et al.* 2007; Grzebelus *et al.* 2012a); somatic embryos (Sun *et al.* 2005b; Duquenne *et al.* 2007); cotyledons (Sutiojono *et al.* 2002; Pati *et al.* 2005) and guard cells (Hall *et al.* 1996 and Pandey *et al.* 2002). Furthermore, the physiological condition and the age of the source tissue also affect protoplast release and viability. After cell wall removal, the protoplasts are filtered through a 50-100 μm mesh size filter and purified by flotation and/or sedimentation. In *Rosa*, sucrose flotation and enzyme treatment are integrated, eliminating one step from the protocol (Pati *et al.* 2008). For heterogeneous protoplasts, such as *Helianthus maximiliani*, Ficoll gradient isolation (flotation) is efficient (Taski-Ajdukovic *et al.* 2006). The viability of the isolated protoplasts can be assessed by fluorescein diacetate (FDA) dye which enters the live cells cytoplasm where esterase hydrolyzes the acetate residues and leaves fluorescein. This can be observed under fluorescence microscopy under UV irradiation (Duquenne *et al.* 2007).

1.3 Protoplast fusion

The first somatic hybridization (protoplast fusion) was described more than a century ago by Küster (1909) and many new techniques have been introduced afterwards. Protoplast fusion can be either symmetric or asymmetric, depending on the nature of genetic contribution of fusion partners (Fig. 1.1).

1.3.1 Symmetric fusion

In symmetric fusion, the complete genomes of both parent protoplasts are fused (Fig. 1.1). The first successful somatic symmetric interspecific hybrid reported was in tobacco (Carlson *et al.* 1972). Since then, many improvements and somatic hybrids have been made in a diversity of species. Complete regeneration of plants was accomplished after many fusions (reviewed in Johnson and Veilleux *et al.* 2001; Davey *et al.* 2005; Liu *et al.* 2005). However, as two complete genomes fuse, a phenomenon called 'gene conflict' may arise as certain chromosomes repel one another. Moreover, the technique introduces a significant amount of unwanted genetic material. These limitations result in: abnormal growth (Sherraf *et al.* 1994), regeneration of hybrids with low fertility (Spangenberg *et al.* 1994; Kisaka *et al.* 1998; Hu *et al.* 2002), non rooted shoots (Wakita *et al.* 2005; Sonntag *et al.* 2009), slow hybrid growth (Zhou *et*

al. 2006; Wang *et al.* 2011c) and recalcitrant calli (Liu *et al.* 2007; Pati *et al.* 2008; Han *et al.* 2009) or microcalli (Duquenne *et al.* 2007; Geerts *et al.* 2008).

1.3.2 Asymmetric fusion

In asymmetric fusion, the fragmented genome of partner (the 'donor') is fused with the complete genome of the other partner (the 'acceptor') (Fig. 1.1). Asymmetric fusion is widely applied to circumvent the above barriers in symmetric fusion. For example, symmetric hybrids between B*rassica napus* and *Lesquerella fendleri* are self-sterile, but asymmetric hybrids of the same fusion partners were self-fertile (Skarzhinskaya *et al.* 1996). Similiarly, symmetric

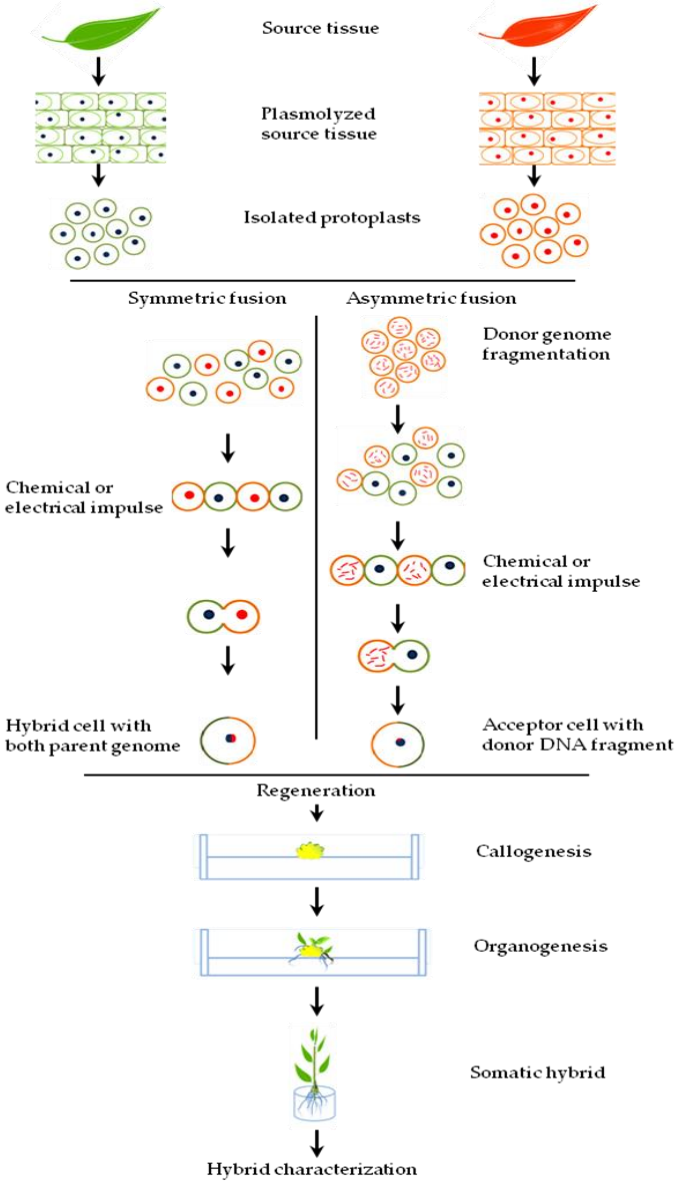

Fig. 1.1 Schematic representation of symmetric and asymmetric somatic hybridization techniques (Lakshmanan *et al.* 2013). Plasmolyzed explants are treated with enzyme mixture to obtain protoplasts. Protoplasts are fused either chemically or electrically. Fused cells are regenerated into plantlets and screened for selecting somatic hybrids.

fusion between *Orychophragmus violaceus* and *B. napus* yielded sterile hybrids, whereas asymmetric hybrids were fertile (Hu *et al.* 2002). By introgressing fewer genes than after sexual crossing or symmetric somatic fusion, the number of backcrosses can be significantly reduced. Also, cytoplasmic genomes can be recombined with nuclear genomes for application like cytoplasmic male sterility introduction (Liu *et al.* 2005).

Asymmetric fusion also encourages the elimination of much of the donor's redundant genetic material in the somatic hybrid (Waara and Glimelius, 1995; Liu *et al.* 2005). Moreover, in asymmetric fusions, most karyotype instability-causing donor genes are eliminated during the first post-fusion mitoses, as opposed to symmetrical fusions, after which eliminations can occur up to the first sexually derived generation (Cui *et al.* 2009). In other words, not only does asymmetric fusion introduce fewer genes in a recipient genome after fragmenting the donor genome, but elimination of disadvantageous genes or chromosomes also proceeds faster.

Table 1.1 outlines and highlights the last decade of research on asymmetric protoplast fusion. The most studied families were Brassicacae and Poaceae, followed by Rutaceae. Biotic resistance introduction, genetic variation, agronomic traits such as seedless fruits, hybrid analysis, fragmentation technology development and secondary metabolite production were recently the most important aims for asymmetric hybridization. Abiotic resistance introduction, hybridization, genome mapping and the establishment of chromosome addition lines were rarely-studied objectives. Other motives were plastome and/or cytoplasmic male sterility transfer (Sun *et al.* 2005a).

Many successful asymmetric hybrids were reported for the first time. Using UV irradiated asymmetric hybrids a radiation hybrid panel was established for *Lolium multiflorum* (Cheng *et al.* 2006). Taski-Adjukovic *et al.* (2006) regenerated an asymmetric hybrid between sunflower and *Helianthus maximiliani* for the first time.

Acceptor protoplast sources for asymmetric hybridization existed mainly of suspension cell cultures, mesophyll, callus and hypocotyls. The donor protoplast source differed in about 30% of the cases from the one for the acceptor. Brassicaceae and Asteraceae hypocotyl acceptor protoplasts were combined with mesophyll donor protoplasts (Taski-Ajdukovic *et al.* 2006; Scholze *et al.* 2010; Wang *et al.* 2011a). The number of asymmetric hybridization realized through PEG fusion was four times the number of fusions generated by electrical fusion.

Apart from morphological characterization, the majority of the publications reporting on complete plant regeneration describe the use of molecular tools to unravel the genomic constitution of the alleged hybrids. DNA markers were sometimes complemented with isozyme analysis, sodium dodecyl sulphate polyacrylamide gel electrophoresis or sequence

analysis. The most frequently employed molecular markers were Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Amplification Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and Cleaved Amplified Polymorphic Sequence (CAPS). PCR-RFLP and CAPS analysis using mitochondrial or chloroplast universal primer pairs were efficient and reliable methods for characterizing the cytoplasmic genome. This technique was applied for both chloroplast and mitochondria screening, whereas SSR was only used once for chloroplast evaluation. Southern blotting for chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) was employed to screen cytoplasmic DNA, whereas Northern blotting was used once for chloroplast evaluation. Besides molecular markers, cytogenetic tools as chromosome counting, flow cytometry and genomic *in situ* hybridization (GISH) were mentioned to distinguish asymmetric hybrids.

Table 1.1 Asymmetric protoplast fusion in different plant families and species during 2004-2013 (Lakshmanan *et al*. 2013)

Plant family and species (acceptor + donor)[s]	Aim	Protoplast source[t]	Fragmentation [u]	Fusion Method[v]	Best Result[w]	Characterization			Reference
						Cytogenetic[x]	DNA-markers	Other methods	
Apiaceae + Gentianaceae									
Bupleurum scorzonerifolium + *Swertia mussottii**	Secondary metabolites	C	UV	PEG	P	GISH	RAPD, SQ RT-PCR	Isozyme analysis, mitochondrial and chloroplast DNA specific probes on Southern blots, HPLC	Wang *et al.* 2011b
Bupleurum scorzonerifolium + *Swertia tetraptera**	Secondary metabolites	SC + C	UV	PEG	P	CC, GISH	RAPD, SSR(C)	Isozyme analysis, HPLC	Jiang *et al.* 2012
Bupleurum scorzonerifolium + *Gentianopsis paludosa**	Secondary metabolites	SC	UV	PEG	P	CC	RAPD, RFLP	Isozyme analysis, HPLC, GC-MS, 5S rDNA spacer sequence analysis	Yu *et al.* 2012
Apiaceae + Taxaceae									
Bupleurum schorzonerifolium + *Taxus chinensis mairei*	Secondary metabolites	SC	UV	PEG	C	CC	RAPD, SQ RT-PCR	Isozyme analysis, HPLC	Zhang *et al.* 2011
Asteraceae									
Helianthus annuus + *H. maximiliani**	Biotic resistance	H + M	UV	EF	P		RAPD	Isozyme analysis	Taski-Ajdukovic *et al*. 2006
Brassicaceae									
Brassica napus + *Isatis indigotica**	Genetic variation, Secondary	M	IOA(A) + UV	PEG	P	CC, GISH	AFLP, CAPS(C)[y]	Pollen fertility	Du *et al.* 2009
Brassica napus + *Orychophragmus violaceus*	Chromosome addition lines	M	IOA(A) + UV		P	CC, GISH			Zhao *et al.* 2008
Brassica oleracea + *B. nigra*	Genetic variation, Biotic resistance	H + M	UV	PEG	P	CC, FCM, GISH	AFLP, CAPS(C), CAPS(M)[z]	Mitochondrial DNA specific probes on Southern blots, resistance screening	Wang *et al.* 2011a

Brassica oleracea botrytis + *B. carinata* + *B. juncea* + *B.nigra* + *Sinapis alba*	Biotic resistance	H + M	X	PEG	P		RAPD	Resistance screening	Scholze *et al.* 2010
Brassica oleracea capitata + *Barbarea vulgaris* + *Capsella bursapastoris* + *Diplotaxis tenuifolia* + *Hesperis matronalis* + *Matthiola incana* + *Raphanus sativus*	Biotic resistance	H + M	X	PEG	P		RAPD	Resistance screening	Scholze *et al.* 2010
Orychophragmus violaceus + *Lesquerella fendleri* (GFP) *	Plastome transfer	M + C	γ	PEG	P		ITS, CAPS(M)	Isozyme analysis, GFP	Ovcharenko *et al.* 2011
Brassicaceae + Apiaceae									
Arabidopsis thaliana + *Bupleurum schorzonerifolium**	Secondary metabolites	C	IOA(A) + UV	PEG	P	CC, GISH,	RAPD	5S rDNA spacer sequence analysis	Wang *et al.* 2005a
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Fragmentation tool		UV	PEG	P	CC,GISH	RAPD, SSR		Wang *et al.* 2011d
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Hybrid analysis, fragmentation tool	C + SC	γ	PEG	P	CC	RAPD, SSR	Histology	Wang *et al.* 2012
Brassicaceae + Poaceae									
Arabidopsis thaliana + *Triticum aestivum**	Hybrid analysis	SC + C	UV	PEG	P	CC, GISH	RAPD, SSR, CAPS(C)	Isozyme analysis	Deng *et al.* 2007
Malvaceae									
Gossypium hirsutum + *G. klozschianum*	Alternative for symmetric somatic hybridization	SC	UV	EF	P	CC	RAPD, SSR, CAPS(C)		Yang *et al.* 2007b
Musaceae									
Musa 'Guoshanxiang' + *M. acuminate**	Biotic resistance	SC	IOA(A) + UV	PEG	P	CC	RAPD, ISSR		Xiao *et al.* 2009

Poaceae									
Festuca arundinacea + *Triticum aestivum**	Hybrid analysis	SC	UV	PEG	P	CC, GISH	RAPD, SSR(C), MSAP	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Cai *et al.* 2007
Oryza sativa japonica + *O. meyeriana**	Biotic resistance	SC	IOA(A) + X	PEG	P	CC	RAPD	Resistance screening	Yan *et al.* 2004
Triticum aestivum + *Avena sativa*	Hybrid analysis	SC	UV		P	ISH	SSR		Xiang *et al.* 2010
Triticum aestivum + *Haynaldia villosa*	Biotic resistance, protein content	SC + C	γ	PEG	P	CC, GISH	RFLP(C)	Isozyme analysis, 5S rDNA spacer sequence analysis	Zhou and Xia, 2005
Triticum aestivum + *Lolium multiflorum**	Biotic resistance	SC	UV		P	CC	RAPD, SSR	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Cheng and Xia, 2004
Triticum aestivum + *Lolium multiflorum*	Agronomic traits, biotic resistance	SC	IOA(A) + X	EF	P	CC	RFLP, AFLP	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Ge *et al.* 2006
Triticum aestivum + *Lolium multiflorum*	Radiation hybrid panel / genome mapping	-	UV	-	P	GISH	RFLP, SSR	Sequencing	Cheng *et al.* 2006
Triticum aestivum + *Setaria italic**	Abiotic resistance	(SC+C) +C	UV	PEG	P	GISH, CC	RAPD, RFLP(C), RFLP(M)	Isozyme analysis, 5S rDNA spacer sequence analysis	Xiang *et al.* 2004
Poaceae + Apiaceae									
Festuca arundinacea + *Bupleurum schorzonerifolium**	Hybrid analysis	SC	UV	PEG	P	CC	RAPD	Isozyme analysis, 5S rDNA spacer sequence analysis	Wang *et al.* 2011c
Triticum aestivum + *Bupleurum schorzonerifolium**	Genetic variation, genome mapping	C + SC	UV	PEG	P	GISH	CAPS, RAPD, SSR	Isozyme analysis	Zhou *et al.* 2006
Rutaceae									
Citrus paradisi + *C. sinensis*	Genetic variation	SC	IOA(A) + γ		P	FCM	AFLP		De Bona *et al.* 2009a
Citrus paradisi + *Swinglea glutinosa*	Fragmentation tool	SC	γ and UC	PEG	C		AFLP		De Bona *et al.* 2009b
(Citrus reticulata x C. sinensis) + *C.sinensis*	Genetic variation	SC	IOA(A) + γ		P	FCM,	AFLP		De Bona *et al.* 2009a

(Citrus reticulata x C. sinensis) + *C. unshiu*	Seedless fruits	SC	UC	EF	C	FCM	SSR, CAPS(C)		Xu *et al.* 2006
(Citrus reticulata x C. sinensis) + *Swinglea glutinosa*	Fragmentation tool	SC	γ and UC	PEG	C		AFLP		De Bona *et al.* 2009b
Citrus sinensis + *C. unshiu*	Fragmentation tool	SC	UV	EF	P	CC, FCM	RAPD, AFLP, CAPS(C)		Xu *et al.* 2007
Solanaceae									
Nicotiana tabacum + *N. repanda*	Cytoplasmic male sterility	M	Rhodamine 6G (A)	PEG	P	CC	RAPD, CAPS(M)	Isozyme analysis	Sun *et al.* 2005a
Petunia hybrid +*Nicotiana tabacum*	Plastome transfer	M	UV	PEG	P		RAPD, CAPS(C)	Mitochondrial DNA specific probes on Southern blots, chloroplast RNA specific probes on Northern blot	Sigeno *et al.* 2009

[s] Species labeled with * were fused for the first time

[t] C: callus; CO: cotyledon; H: *in vitro* hypocotyls; M: mesophyll cells from *in vitro* leaves; SC: suspension cells

[u] (A): for acceptor; IOA: iodoacetamide; UC: ultracentrifugation; UV: UV-ray irradiation; X: X-ray irradiation; γ: gamma-ray irradiation

[v] EF: electrofusion; PEG: chemical fusion with polyethylene glycol; when not mentioned the fusion method is described in earlier publications

[w] C: callus; P: hybrid plants

[x] CC: chromosome counting; FCM: flow cytometry; (G)ISH: (genomic) *in situ* hybridization

[y] (C) on chloroplast DNA

[z] (M) on mitochondrial DNA

1.3.2.1 Irradiation on donor genome

To obtain asymmetric hybrids, Dudits *et al.* (1980) introduced irradiation. UV rays (Hall *et al.* 1992), X-rays (Scholze *et al.* 2010) and γ-rays (Ovcharenko *et al.* 2011) can be used. In the past, X- or gamma-rays were more frequently used for donor protoplast fragmentation, but now UV-irradiation is more widely applied (Table 1.1). One persistent problem is the quantification of DNA damage after an irradiation treatment. Abas *et al.* (2007) presented Comet assay single cell gel electrophoresis (SCGE) as a reliable tool to observe single and double strand breaks in mesophyll protoplasts of *Nicotiana plumbaginifolia*. In some cases, asymmetric fusions were accomplished without fragmentation treatment, decreasing possible long term irradiation effects on hybrid growth and development (Li *et al.* 2004).

1.3.2.2 Microprotoplast mediated chromosome transfer (MMCT)

Genome irradiation often induces random chromosome breakage, deletion, rearrangements of genes and sterility of hybrid cells (Famelaer *et al.* 1989; Gleba *et al.* 1988; Puite and Schaart, 1993; Wijbrandi *et al.* 1990). In cucumber, the negative effect of UV-C irradiation on cell wall regeneration, protoplast viability and the intensity of the nuclei after DAPI staining is also evident (Navratilova *et al.* 2008). Therefore, the use of micronuclei and microprotoplasts mediated chromosome transfer (MMCT), which was originally developed for mammalian cells by Fournier and Ruddle (1977), has been considered as an alternative method for partial genome transfer (Doherty and Fisher, 2003).

Mass induction of micronucleation and efficient isolation of the microcells are key aspects in any microprotoplast mediated chromosome transfer for successful transfer of a partial genome (Ramulu *et al.* 1993). Microtubules are involved in several processes such as migration of chromosomes, cell structure, guidance and arrangement of the cellulose micro fibrils, the cell wall formation, the intracellular movement and cell differentiation (Morejohn, 1991; Jordan and Wilson, 1998). Toxic substances such as herbicides or colchicine prevent their normal polymerization (Hansen *et al.* 1998). These spindle toxins generally block fast growing suspension cells, or synchronized cells, in their metaphase and scatter chromosomes which later decondense to form micronuclei in the cytoplasm. After cell wall removal and ultracentrifugation (30000-100000g for 20-30 min), these micronucleated cells form microprotoplasts, some containing nuclei and others lacking them (cytoplasts) (Ramulu *et al.* 1995; Wallin *et al.* 1977). These microprotoplasts can be further filtered through sequential filters of smaller pore width. Cell suspensions are used as source material in *Nicotiana plumbaginifolia* (Verhoeven *et al.* 1990), *Haemanthus katherinae* Bak. (Morejohn *et al.* 1987; Binsfeld *et al.* 2000), *Helianthus giganteus* (Binsfeld *et al.* 2000), *Citrus unshiu* (Zhang *et al.* 2006) and *Beta vulgaris* (Famelaer *et al.* 2007).

For recalcitrant species that cannot yield a fast growing suspension culture, developing microspores (Fig. 1.2) can be alternatively used (Matthews *et al.* 1999; Saito and Nakano, 2002). Furthermore, the risk of mutation accumulation in suspension cells can be avoided

(Guo and Grosser, 2005). Additionally, when using microspores instead of suspension cultures, there is no need for a synchronizing treatment, as meiotic cycles are usually synchronous within an anther (McCormick, 1993). Matthews *et al.* (1999) induced microcells in dissected potato microspores treating with spindle toxins. However, they could not efficiently isolate microprotoplasts in large numbers. Later, Saito and Nakano (2002) successfully isolated gametic microprotoplasts using sequential filtering after flowcytometry sorting of microcells.

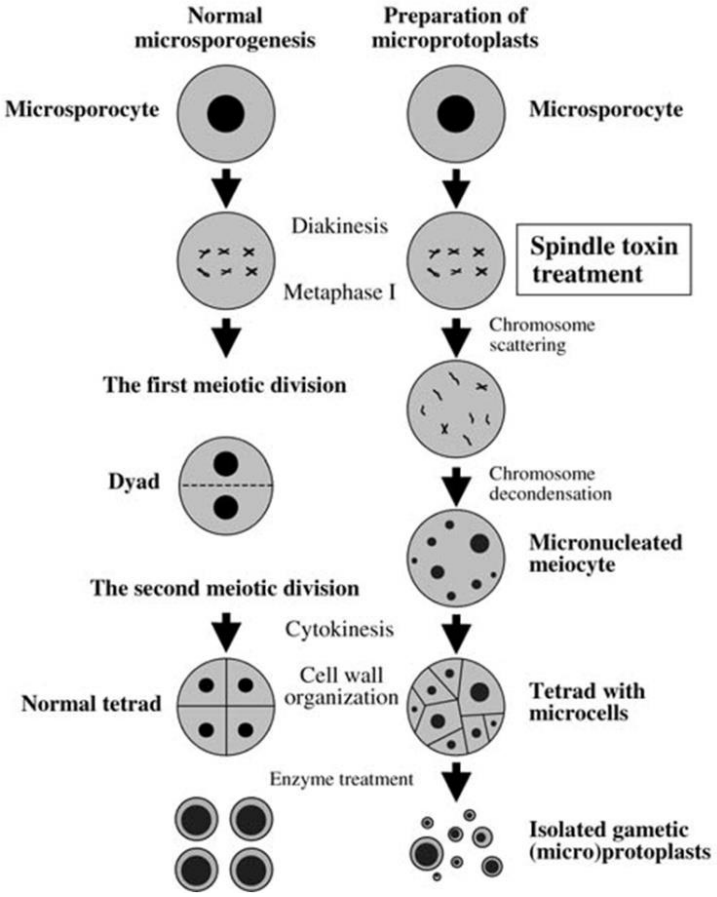

Fig. 1.2 Schematic representation of gametic microprotoplast isolation technique (Saito and Nakano, 2002a). Spindle toxin blocks spindle fiber formation and induces subsequent chromosome scattering in microsporocytes. Scattered chromosomes later decondense into micronucleated meiocytes and microcells. By treating the microcells with enzymes mixture microprotoplasts are directly obtained.

The effect of spindle toxins depends on their type, dose, and the incubation period and the plant genotype. Oryzalin (ORY) was an effective micronucleator in suspension cells as well as in developing microspores (Binsfeld *et al.* 2000; Matthews *et al.* 1999; Morejohn *et al.* 1987; Verhoeven *et al.* 1990). The same authors also reported a detrimental or reduced micronucleation effect when the toxins' dose is higher (more than 25μM). Conversely, Saito and Nakano (2001) mentioned ORY induced less micronucleation than other toxins such as amiprophos-methyl, butamiphos, propyzamide and chlorpropham. Compared with irradiation, genome fragmentation in microprotoplasts can be better quantified, using flow

cytometry or fluorescence microscopy (Ramulu *et al.* 1988; Famelaer *et al.* 2007). Furthermore, microprotoplasts might further be fragmentized using irradiation techniques and used either for asymmetric hybrid production or radiation mapping (de Bona *et al.* 2009b).

1.3.2.3 Cytoplasmic inactivation

Metabolic inhibitors, such as iodoacetamide (IOA) and rhodamine 6G can be used to obtain asymmetric fusions. The exact mode of action of IOA has not yet been described but it is known that the compound inhibits protoplast division by irreversibly inactivating the cytoplasm. Fusion of IOA-treated recipient parental protoplasts with irradiated donor protoplasts could produce cybrids. In *Cichorium*, successful asymmetric protoplast fusion has been performed between γ-ray-irradiated sunflower protoplasts and iodoacetate-treated red chicory protoplasts (Varotto *et al.* 2001). Sun *et al.* (2005) reported cybridization between *Nicotiana tabacum* and *N. repanda* using rhodamine 6G. Metabolic inhibitor treatments prevent cell division, but fusion with non treated protoplasts restores cell division ability, thus opening pathways to heterokaryon or cybrid selection.

1.4 Protoplast fusion methods

Because their cell wall is removed, protoplasts can be fused. Tobacco was the first crop in which successful interspecific somatic hybridization was reported (Carlson *et al.* 1972). This fusion was spontaneous, but fusions can also be induced through mechanical pushing, $NaNO_3$ treatment, or high pH/Ca^{++} treatment (Razdan, 2003). Currently, fusion is nearly exclusively performed through polyethylene glycol (PEG) (Kao *et al.* 1974) or electrofusion (Zimmermann and Scheurich, 1981). Chemical fusogens cause the isolated protoplasts to adhere to each other, to agglutinate and to facilitate fusion. Chemofusion is non-specific and inexpensive but can be cytotoxic. For electrofusion, protoplasts are first aligned in a low strength electric field generated by alternating current, upon which fusion is induced by application of one or a few high-voltage direct current pulses. The electroporation induced by these pulses enables cell fusion. Electrofusion is less lethal to the cells than chemical fusion but expensive. After fusion, different types of homokaryons or heterokaryons can be created, as well as alloplasmic hybrids (cybrids) (Liu *et al.* 2005).

The efficiency of electrical and chemical fusion, the two main somatic hybridization tools, was compared for banana. With regard to frequency of binary fusion, the chemical PEG induced protoplast fusion was best. However, with respect to mitotic activities, somatic embryogenesis and plant regeneration, electrical fusion was better (Assani *et al.* 2005). Also, a novel protoplast fusion method was developed (electrochemical protoplast fusion), which combines the advantages of the two classical methods. This new procedure is based on chemically induced protoplast aggregation and DC pulse-promoted membrane fusion. The necessity of PEG removal is one of the main drawbacks of chemical fusion. Classical chemical fusion protocols require two to three washes. Removal of PEG in the novel technique was simpler because of its low concentration. After the DC pulse-promoted

membrane fusion, high numbers of symmetric somatic hybrids and cybrids were obtained (Olivares-Fuster *et al.* 2005).

Fusion events can be monitored with fluorescent markers, or by observing cell organelles. Chen (2005) evaluated natural pigmentation in red cabbage (+ *B. juncea*) fusions to distinguish hybrids. Khan and Grosser (2004) observed both gray starch bodies of sweet orange and chloroplasts of *Citrus micrantha* in fusion products between both. The use of both leaf and hypocotyls derived protoplasts could be especially useful for autofluorescence based heterokaryon selection: for instance, red autofluorescence of mesophyll protoplasts can be combined with fluorescein diacetate (FDA) staining of embryo protoplasts (Duquenne *et al.* 2007; Przetakiewicz *et al.* 2007). Durieu and Ochatt, (2000) and Pati *et al.* (2008) identified heterokaryons by labeling parents with fluorescent dyes [fluorescein isothiocyanate (FITC) and rhodamine-B-isothiocyanate (RITC)]. Thus labeled heterokayons on a grid or coverglass were subsequently selected after microcalli formation for further culture (Pati *et al.* 2008). Biotinilation of a *Solanum* donor protoplast line can be applied directly to protoplasts without affecting their viability and cell wall regeneration. In addition, the use of green and colorless protoplasts was helpful for the identifcation of heterokaryons during the fusion process and for monitoring the efficiency of selection after magnetic cell sorter column separation of protoplasts (Borgato *et al.* 2007a). Green fluorescent protein (GFP) transgenic citrus lines have frequently been used as tools for fusion monitoring. GFP allows screening after the first days (after a few days, chloroplast lose their green color and autofluorescence) and before any morphological traits are present (Guo and Grosser, 2005).

1.5 Protoplast regeneration

Theoretically, protoplasts are totipotent, meaning that after their isolation and subsequent culture they have the capability to dedifferentiate, re-enter the cell cycle, go through repeated mitotic divisions and then proliferate or regenerate into various organs. In other words, applying the correct physical and chemical stimuli would suffice to regenerate fertile plants through tissue culture practices. Factors such as culture media, protoplast density, protoplast source and culture methods influence successful regeneration.

1.5.1 Culture system

Generally, cell wall regeneration is the first step in any protoplast regeneration process. On occasion, protoplasts go through nuclear division before cell wall regeneration has taken place (Eriksson and Jonasson, 1969). Townsend (1897) first reported cell wall regeneration by plant protoplasts; Nagata and Takabe (1970) used Calcofluor for cell wall formation observation under UV light. Though protoplasts form a cell wall after enzyme removal, still they need to be protected in an osmotically corrected solution until they can withstand the turgor pressure in the culture system. The gradual reduction of osmoticum is preferred over continuous or sudden osmotic changes, as this facilitates cell growth (Kao and Michayluk, 1980; Davey *et al.* 2004). Osmotic stabilizers such as sugar alcohols, mannitol or sorbitol and

carbon sources sucrose or glucose are added to the basal medium. Kao and Michayluk (1975) enabled *Vicia* protoplast culture at low densities after media enrichment with organic acids, sugars, sugar alcohols and casamino acids. After this publication, protoplast culture at low densities became possible for more species. Other media have been derived from MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al.* 1968) formulations. Sometimes a complex undefined medium is composed by adding coconut milk, albumin serum or conditioned media obtained from cells (Kao and Michayluk, 1975; Gleba *et al.* 1982; Chabane *et al.* 2007; Rizkalla *et al.* 2007). Novel approaches such as electrical stimulation, addition of surfactants, antibiotics, polyamines, artificial oxygen carriers and physical gaseous exchange have been detailed by Davey *et al.* (2005).

Chemical modifications have contributed to regeneration of some recalcitrant species or materials. In *Beta vulgaris*, protoplast regeneration recalcitrance is a main problem. The plating efficiency of mesophyll cells drastically increases, however, after adding 100 nM phytosulfokine, a peptide growth factor that has antioxidant properties but possibly also generates a nurse cell effect (Grzebelus *et al.* 2012b). The supply of exogenous arabinogalactan protein-rich extracts significantly improved the protoplast-derived callus organogenesis (Wisniewska and Majewska-Sawka, 2007; Wisniewska and Majewska-Sawka, 2008). Galactoglucomannan-derived oligosaccharides in very low concentrations act as regulatory/signaling molecules in plant cells elongation, differentiation and development. Combined with NAA, they positively influenced not only the viability, but also the protoplast regeneration and division. They influenced both quality and quantity of extracellular proteins in regenerating protoplasts. They probably fulfill a protective role in the spruce protoplast regeneration (Kakoniova *et al.* 2010).

Plant hormones evidently remain one of the most important parameters for protoplast regeneration (Nagata and Takebe, 1970; Böhmer *et al.* 1995). Auxin 1-NAA additions to protoplasts isolated from leaves of 6-day-old wheat seedlings induced an increase in the cytosolic calcium concentration within 5-10 seconds, while the physiologically non-active analogue, 2-NAA, did not (Shishova *et al.* 2004). The amplitude of calcium increase depended on the concentration of 1-NAA. A complicated mechanism of auxin-induced rise in cytosolic Ca is suggested. Within the first few seconds of hormone-plant cell interaction, the hormone directly activates Ca- channels in the plasma membrane and induces a Ca influx from the external medium. Later (within 300-400 seconds), a second, more intensive, rise in Ca can be obtained by activation of intracellular calcium stores. A 2,4-D shock was found to be indispensable for *Helianthus* protoplast regeneration (Taski-Adjukovic *et al.* 2006). The cytokinin TDZ induces morphogenesis, probably through modulation of auxin levels (Böhmer *et al.* 1995; Xiao *et al.* 2007; Thomas, 2009); for shoot induction, the cytokinin type may be decisive (Borgato *et al.* 2007b). Interactions of gibberellic acid (Yang *et al.* 2007a; An *et al.* 2008) as well as ethylene inhibitors (Guo *et al.* 2007b) with regeneration were studied. Endogenous hormones can interact with exogenously applied plant growth

regulators, as demonstrated by the different reaction of multiple explants types on phytohormone treatments (Sun *et al.* 2005c).

Polyamines are known to be involved in a variety of growth and developmental processes in higher plants, as well as in adaptation to stresses (Smith, 1985; Kusano *et al.* 2008). The isolation process contributes to increased putrescine levels, which are higher in non-totipotent tobacco protoplasts than in totipotent tobacco protoplasts (Papadakis *et al.* 2005). During culture, putrescin predominates over other polyamines, and the highest accumulation is found in totipotent protoplasts. The authors suggest that the levels and metabolism of the intracellular polyamines could be related to the expression of totipotency of plant protoplasts. Polyamines probably play a role in the antioxidant machinery that is induced after the generation of reactive oxygen species. Rakosy-Tican *et al.* (2007) found that a combination of spermidine and hemoglobin increase plating efficiency but was unable to provoke full regeneration. They propose spermidine to stimulate mitosis and to reduce stress impacts.

In liquid cultures at high density, the protoplasts try to form a middle lamella with adjacent protoplasts. This results in cell aggregation (Cocking, 1970; Pojnar *et al.* 1968). This problem can be overcome by immobilizing protoplasts in agarose culture media, leading to subsequent regeneration of whole plants from single protoplasts (Nagata and Takebe, 1970; Takebe *et al.* 1971). Liquid medium rarely yields better protoplast division (Castelbanque *et al.* 2010). The lower colony formation in liquid medium is assumed to be caused by a shortage of aeration and light (Azad *et al.* 2006) or a release of toxic components (Duquenne *et al.* 2007).

A general finding was the better performance of protoplasts when embedded in alginate or agarose. In the genus *Cichorium*, regeneration of a wide variety of species and genotypes could be accomplished by agarose bead culture (Deryckere *et al.* 2012). In some cases, beads, discs, layers, thin layers or extra thin films are also used. A major advantage of embedding systems is the easier handling of the cultures which permits replacement of the culture media without disturbing the development of the microcolonies and may prevent microbial contamination. When discs are used, protoplasts divide at a higher rate at the edge (Rakosy-Tican *et al.* 2007). Pati *et al.* (2005) observed in *Nicotiana tabacum* and *Lotus corniculatus,* that the thinner the matrix, the higher plating efficiencies were. For carrot protoplast culture, layer thickness was minimized by circular rotation of the protoplast/alginate suspension during application and before polymerization (Grzebelus *et al.* 2012a). Also the embedding agent type affects the final outcome, possibly by interacting with genotype, osmolarity, temperature, culture system or aeration (Prange *et al.* 2010a; Kielkowska and Adamus, 2012). This is in accordance with earlier postulations on the positive effect of embedding by membrane stabilization through lipid peroxidase inhibition, the prevention of leakage of cell wall precursors or other metabolites, and lower ethylene levels (Bajaj, 1989). Moreover, protoplast aggregation leading up to poor oxygen supply and browning is avoided (Pati *et al.*

2008; Lian *et al.* 2011). Also, the osmotic pressure changes steadily instead of stepwise (Kanwar *et al.* 2009).

Shrestha *et al.* (2007) propose that the reasons for efficient division of cell suspension of *Phalaenopsis* protoplasts are the better dilution of inhibitory substances and the better distribution of nutrients. Niedz (2006) regenerated somatic embryos through culture of citrus protoplasts on semi-permeable membranes, which enabled a better oxygen supply to the cells. *Nicotiana tabacum* protoplasts were cultured in microfluidic polydimethylsiloxane channels with microtubes for continuous medium supply; these successfully developed into a microcolony within four weeks. This illustrates the benefits of the microdevice-based method (Ko *et al.* 2006).

Actively-dividing cells can sometimes promote protoplast cultures by releasing growth factors such as amino acids. These cells are called 'nurse cells'. For example, division of protoplasts from embryogenic rice cell suspensions was effectively stimulated by nurse cells of *Lolium multiflorum* (Jain *et al.* 1995). Protoplasts/spheroplasts or cells that have been X-irradiated to inhibit division can also exert a similar nurse effect. As in Brassicaceae, IOA is added to prevent division of unfused protoplasts, who subsequently could nurse the fused cells (Tu *et al.* 2008). This approach was successful in *Brassica juncea* and *B. oleracea* fusions (Chen *et al.* 2005). A nurse layer of tuber mustard cells significantly increases regeneration of cauliflower (Sheng *et al.* 2011) and red cabbage protoplasts (Chen *et al.* 2004a). This was the first time that a red cabbage protoplast culture was successful; without a nurse layer, microcalli did not form. The sustained division of banana protoplasts also occurs exclusively when a feeder system is implemented (Xiao *et al.* 2007), and not in liquid medium; possibly the feeder layer has a signaling function in addition to providing nutrients. Cell suspensions are often used as feeder layers (Kyozuka *et al.* 1987; Petersen *et al.* 1992; He *et al.* 2006), and their efficiency is determined by their culture time, possibly because a more vigorous growth coincides with the release of more stimulatory substances in the medium that can initiate divisions in otherwise recalcitrant protoplasts. Not all cell suspensions are a suitable source for a feeder layer. Genotype, pretreatment and medium of the original callus can all affect the final efficiency of the protocol.

1.5.2 Source material

Protoplasts isolation and regeneration has always been difficult for monocotyledonous species. Protoplasts isolated from monocot mesophyll tissue rarely undergo sustained mitotic division (Potrykus and Shillito, 1986; Vasil, 1988; Potrykus, 1990) and are thus recalcitrant to regeneration. Also, callus and embryogenic cell suspensions have been extensively used as the source of protoplasts in *Lolium* species (Wang *et al.* 1993) and other monocotyledons (Nielsen *et al.* 1993; Taylor *et al.* 1992). Embryogenic suspension cells in particular were used as protoplast source in monocot species (Chabane *et al.* 2007). Like callus and unlike mesophyll cells, suspension cells contain more mitochondria, suggesting a

better energy supply to dividing protoplasts (Moreira *et al.* 2000). Although cell suspensions theoretically are the best starting material they are often hard to accomplish in cereals (Li *et al.* 2004). Another drawback is the possible introduction of cytological aberrations or mutations (Grosser *et al.* 2007).

1.5.3 Complementary effects in fusion products

After fusion, lack of regeneration capacity of one or both fusion partners can be used as a selection tool to eradicate nonfused protoplast regeneration of the recalcitrant parent (Guangmin and Huimin, 1996; Liu *et al.* 2007; Sheng *et al.* 2008; Wang *et al.* 2008b; Patel *et al.* 2011; Wang *et al.* 2012). Non regenerative tissues like citrus mesophyll cells are routineously applied for the same reason (Fu *et al.* 2004). Citrus somatic hybrid cells are usually more vigorous and have higher capacity for embryogenesis than unfused cells and homo-fused protoplasts from suspension parent (Guo and Grosser 2005). Especially when the suspension parent has lost the ability to regenerate and even the capacity for embryogenesis, the regenerated plantlets are always of hybrid origin (Wu *et al.* 2005; Cai *et al.* 2006). When recalcitrance of donor protoplasts is not total, they can still be outcompeted if hybrids develop more vigorously (Cappelle *et al.* 2007). Guo and Grosser (2005) demonstrated that *Citrus sinensis* (GFP transgene mesophyll parent) + *C. reticulata* (callus) hybrids grew more vigorously than *C. reticulata* regenerants; the mesophyll parent did not regenerate. The same phenomenon was observed after *C. unshiu* + *C. microcarpa* fusions (Cai *et al.* 2006). In contrast, slower division rates of heterokaryons (compared to homokaryons or unfused protoplasts) can cause them to disappear from the cell mix after fusion. Flow sorting might solve this problem by sorting out hybrid cells (Sheng *et al.* 2008). However, when mesophyll cells are used to create somatic hybrids, their lack of calli differentiation capacity might decrease hybrid regeneration capacity (Szcerbakowa *et al.* 2005).

Metabolic complementation can be used to distinguish somatic hybrid products i.e. auxotrophic (the inability to synthesize a particular organic compound required for growth) cells may regain the ability to synthesize the required compound after fusion with other cell lines. For example, two mutant cell lines of *Nicotiana tabacum* L. 'Gatersleben', which lacked nitrate reductase and thus auxotrophic for reduced nitrogen, were fused and the hybrids were found to grow on a nitrate containing medium (Glimelius *et al.* 1978). Similarly, amino acid auxotrophs of *N. plumbaginifolia* mutants were selected based on the complementation effects after fusion (Negrutiu *et al.* 1992). However, auxotrophic mutants in plants are not widely available and are less use in hybrid selection.

1.6 Selection and characterization of somatic hybrids

A set of tools is used to monitor or direct the fusion event and thus to optimize the entire process. Although fluorescing agents can be used to label hybrid fusion products during fusion, regeneration can be disabled due to cytotoxic side-effects. Screening is therefore usually performed during or after *in vitro* regeneration. Apart from morphological markers,

many tools have been developed (Liu *et al.* 2005; Eeckhaut *et al.* 2013): flow cytometry, *in situ* hybridization, isoenzymes and molecular markers. A thorough screening can be complicated by genotype instability such as chromosome loss or by hybrid growth vigor, as in sexual hybrids (Eeckhaut *et al.* 2006).

1.6.1 Morphological markers

The morphology of somatic hybrids can either be intermediate, which is common in symmetric hybrids, or similar to one parent. Asymmetric hybrids can also possess intermediate morphology (Liu *et al.* 2005). However, morphological characters alone are insufficient for confirming hybridism.

1.6.2 Molecular and isozyme markers

Molecular markers have been used for identification of many somatic hybrids, such as randomly amplified polymorphism DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), cleaved amplification polymorphic sequences (CAPS), intersimple sequence repeat (ISSR) and 5S rDNA spacer sequence (Sakomoto and Taguchi, 1991; Bauer-Weston *et al.* 1993; Hansen and Earle, 1997; Zubko *et al.* 2002; Xia *et al.* 2003).

DNA markers are sometimes complemented by isoenzyme analysis. Isozymes are coded by genes and therefore, variation in isozyme pattern through gel banding can reveal hybrid nature. Isozymes can be isolated from a variety of tissues by relatively simple, rapid and inexpensive procedures. For example, Zhou *et al. (*2006) distinguished asymmetric hybrids between *B. scozonerifolium* and *T. aestivum* by observing novel bands in esterase profiles that were not present in the both parents. Kostenyuk *et al.* (1991) confirmed the stability of intergeneric hybrids *R.serpentina* + *V. minor* using transferase patterns. However, molecular markers are better performing than isozyme markers. Somewhat less conventional techniques have been employed, like InterRetroelement Amplified Polymorphism (IRAP) and Retroelement-Microsatellite Amplified Polymorphism (REMAP) (Patel *et al.* 2011) or microsatellite anchored fragment polymorphism (Thieme *et al.* 2010).

Spectrometrical methods constitute another group of screening tools. As isozymes, proteins also genetically coded, and these proteins can be identified or quantified using mass spectrometers. Roddick and Melchers (1985) used spectrometry techniques to quantify steroidal glycoalkaloid content. The differentially expressed proteins can reveal hybrid nature in somatic hybridization (Gancle *et al.* 2006; Wang *et al.* 2010). In addition, tools have been developed to determine the practical value of the screening products, such as biotests for abiotic or biotic tolerance or resistance, pollen fertility testing, fatty acid analysis in Brassicaceae or determination of diverse agronomical traits. In *Solanum*, they are combined with enzyme-linked immunosorbent assay (ELISA) (Nouri-Ellouz *et al.* 2006, Thieme *et al.* 2008, Tiwari *et al.* 2010).

Sometimes complementary resistance and metabolic gene introgression were used to select hybrids. Thieme *et al.*, (2010) obtained and selected *Solanum tuberosum* hybrids resistant to the PVY virus, Colorado beetle and late blight (*Phytophtora*). Multiple resistances were also found, along with high morphological and agronomic variation. Scholze *et al.* (2010) produced the first resistant raphano-brassica symmetric and asymmetric hybrids. These showed new resistance types along with multiple resistances, including turnip mosaic virus. Jiang *et al.* (2009) obtained *Brassica* + *Camelina* hybrids with an increased linolenic acid content compared to *B. napus*. This indicates introgression of genes coding for elongases and hydroxylases. Similar results were reported by Du *et al.* (2008) who crossed *B. napus* with *Lesquerella fendleri* and observed a modified synthesis of linoleic, linolenic, eicosanoic and erucic acids, suggesting the transfer of polygenic traits. In *Brassica* + *Sinapis* hybrids, CAPS analysis was performed to verify the presence of the FAE1 gene, responsible for elongation of C18:1 to C22:1 (erucic acid). It is assumed that differences in erucic acid content are caused by this gene (Wang *et al.*, 2005b).

PCR-RFLP and CAPS using mitochondrial or chloroplast universal primer pairs have proven to be efficient and reliable methods for characterizing the cytoplasmic genome. They replace southern blot analysis with mitochondrial or plastidial probes. Compared to RFLP with labeled probes, CAPS is simpler, more rapid and less expensive (Guo *et al.* 2004). Chloroplast SSR is even more convenient and efficient as it eliminates the need for enzyme cutting following PCR reaction (Cheng *et al.* 2005). Also **s**equencing of common bands and searching for restriction endonuclease sites could be cheaper and more convenient than actual CAPS analysis (though after sequencing CAPS could be used to confirm the results). High resolution melting analysis, a screening technique based upon insertions, deletions or single nucleotide polymorphism (SNP)- induced altered dissociation behavior of double stranded DNA has become a highly sensitive method for genotyping (Wu *et al.* 2008). Deryckere *et al.* (2013) applied CAPS to unravel the constitution of mitochondria and chloroplast in *Cichorium* somatic hybrids. High resolution melting can become a standard for mtDNA and cpDNA screening, as, through combination with a PCR reaction, it can outcompete laborious and costly sequencing analysis. Promising as it may be, it has its shortcomings in establishing recombination events. For that purpose, it is best combined with sequencing.

1.6.3 Cytological and cytogenetic markers

Information on ploidy level is obtained directly, through chromosome counting and/or indirectly, through flow cytometry (Backhall *et al.* 1994; Hu *et al.* 2002a; Ge *et al.* 2006). Flow cytometry has become a very important tool in plant breeding. It provides a fast and accurate determination of nuclear DNA content, which is indirectly related to the ploidy level (Galbraith *et al.* 1983; Dolezel, 1991; Dolezel and Bartos, 2005). Flow cytometry is mainly used for 3 purposes: (i) characterization of available plant material, including screening of possible parent plants for breeding programs, (ii) ploidy level determination after polyploidization experiments and (iii) offspring screening after interspecific crosses (Eeckhaut *et al.* 2005).

Ploidy measurements are performed on DAPI (an AT base specific dye) stained nuclei. Estimates of the genome sizes are obtained by flow cytometry using intercalating fluorochrome propidium iodide (PI) stained nuclei. In interspecific and intergeneric crosses, the resulting hybrids will normally exhibit an intermediate ploidy level and/or genome size compared with the parent plants. Genome size analysis by flow cytometry proved its use for the determination of interspecific hybrids in many plant genera, e.g., *Allium* (Van der Valk *et al.* 1991), *Coffea* (Barre *et al.* 1998), *Cucurbita* (Sisko *et al.* 2003), weedy *Hieracium* (Morgan-Richards *et al.* 2004; Suda *et al.* 2007) and weedy *Amaranthus* spp. (Rayburn *et al.* 2005). The flow cytometric confirmation of the hybrid origin of regenerants seems to be superior to other available methods since it is relatively cheap and fast. However, the screening capacity of hybrids based on genome size is limited because parental genome sizes need to differ significantly (Van Tuyl and Boon, 1997).

Chromosome morphology and size differences between parent genomes and hybrids, i.e. karyotype analysis, can be used for a more precise characterization of hybrids (listed in Liu *et al.* 2005; Wang *et al.* 2011b).

1.6.3.1 Karyotyping and fluorescence *in situ* hybridization (FISH)

In eukaryotes, chromosomes follow mitotic and meiotic cycles of DNA replication, condensation, division, and decondensation. The morphology of metaphase chromosomes is relatively conserved and the size, centromere (primary constriction) position, and presence and location of the nucleolar organizers (secondary constriction) are characteristic for individual chromosome types. The analysis of metaphase chromosomes is known as karyotyping. The karyotypes generally display chromosomes in ordered sequence of decreasing length. Identification of individual chromosomes is based on morphological characteristics like arm length, and primary and secondary constrictions.

In fluorescent chromosome banding, fluorescent dyes bind directly to DNA, either uniformly or basepair specifically, and certain fractions of chromosomes or different types of heterochromatin can be differentiated and some information about the DNA sequence behind the chromosome elucidated (Schwarzacher, 2003). When DNA differences between species are too large (very different karyotype, large sequence divergence), this can be an indication that interspecific crosses might not be successful.

Buongiorno-Nardelli and Amaldi (1969), John *et al.* (1969) and Pardue and Gall (1969) claimed the first successful *in situ* hybridizations of cell spreads and tissue preparations. They assumed that chromosomal DNA could be made single stranded and allowed to hybridize *in situ* with labeled probe DNA molecules. They used radioactively labeled satellite DNA and showed that sequences with particular base pair compositions were concentrated in particular chromosome regions, including the nucleolar organizing region (NOR). Later, non-radioactive, fluorescent labeling was developed and opened the possibility to address chromatin regions (defined repetitive and single-copy sequences of

nucleic acids) of individual chromosomes on the basis of DNA sequence information in addition to morphological features (Shubert *et al.* 2001). The method is based on the site specific hybridization of single-stranded DNA probes labeled with hapten (e.g. biotin or digoxigenin) or directly with fluorophore (e.g. FITC) to denatured, complementary target sequences on cytological preparation like metaphase chromosomes or interphase nuclei (Fig. 1.3). Fluorescence visualizes the probe sequences at the hybridization site.

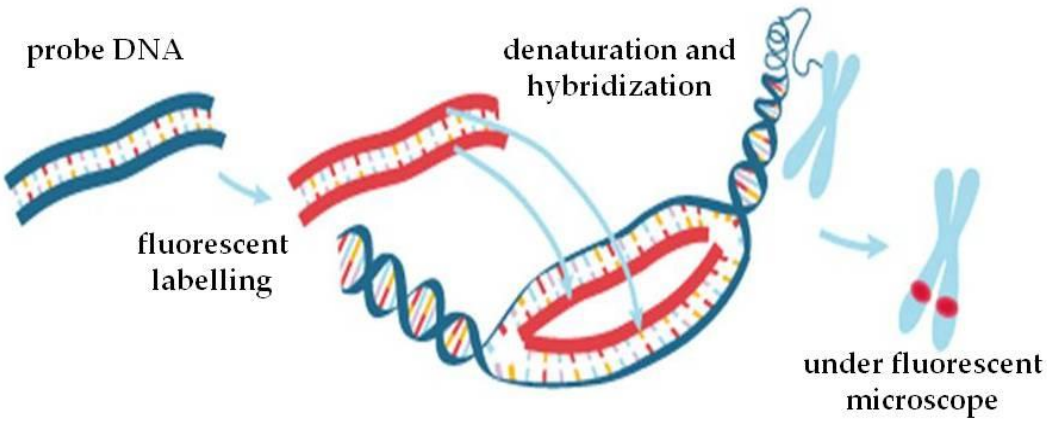

Fig. 1.3 Fluorescence *in situ* hybridization (FISH) principle. Probe DNA is labeled with fluorescent dyes, hybridized with the chromosomal DNA and visualized under a fluorescent microscope to detect the location of stable site hybridization of probe on the chromosome (Picture courtesy: Abnova).

FISH of single-copy DNA sequences including disease-related and other economically important genes in plant species has become indispensable in map-based cloning and other physical mapping strategies. FISH is also valuable for identifying the sites of highly repetitive genes, e.g., ribosomal genes (rRNA genes), which are difficult to map by other methods (Leitch and Heslop-Harrison, 1992; De Jong, 2003). The localization of this ribosomal DNA using FISH plays a major role in chromosome identification and karyotype analysis. rRNA genes have been isolated from many plant species and used as probes for FISH (Schwarzacher, 2003). In eukaryotes, rRNA genes are organized as families of tandem repeated units located at one or a few chromosomal sites (Appels and Honeycutt, 1986; Flavell, 1986). 45S rDNA sequences are on the NOR of satellite chromosomes (Gerlach and Dyer, 1980; Leitch and Heslop-Harrison, 1992; Roa and Guerra, 2012). On metaphase chromosomes, NORs can be identified as secondary constrictions and, owing to the abundance of argyrophylic proteins, they can be visualized by silver staining (Goodpasture and Bloom, 1975). However, during metaphase, only NORs that are transcriptionally active in the previous interphase form secondary constrictions and can be silver stained (AgNORs) (Sumner, 1982). Results from different laboratories indicate that nucleoli are also composed of silent NORs (Akhmanova *et al.* 2000; Sullivan *et al.* 2001; Strohner *et al.* 2001). 45S FISH detects both the active and the silent NORs. The 45S rDNA repeat unit consists of highly conserved coding regions (18S, 5.8S and 25S rDNA), internal transcribed spacers and non-transcribed spacers. In flowering plants, it ranges from 7.5 kb in *Oryza sativa* to 18.5 kb in *Trillium tschonoskii* (Appels and Honeycutt, 1986). Variation in non-transcribed spacer (NTS)

length and sequence causes the length heterogeneity of rDNA units within and between species. Because the 5S and 45S units are highly conserved, probes isolated originally from wheat can be used to localize the 45S and 5S genes in most eukaryotic species. rDNAs linked with the nucleolar bearing chromosomes have been frequently detected in the karyotype of various crops (Jiang and Gill, 1993; 1994).

1.6.3.2 Genomic *in situ* hybridization (GISH)

Among all specific adaptations of FISH, the use of total genomic DNA has become most useful in the discrimination of parental species in plant hybrids. This genome painting technique or GISH (Schwarzacher *et al.* 1989) is based on hybridization with genomic DNA of one of the parental species as probe, labeled with biotin or digoxigenin, for example. The addition of an excess amount of unlabeled DNA from the other parent (blocking DNA) substantially increases the specificity of probing and enables more closely related species to be distinguished.

Though molecular markers are also widely used for monitoring alien genetic material in a plant genome (Garcia *et al.* 1995, Fedak, 1999, Yamagishi *et al.* 2002), a large number of markers that represent different chromosome regions should be used to analyze a complete chromosome. Moreover, a molecular marker approach often does not provide an answer whether one or multiple copies of a particular gene/chromosome are present in a plant (Dong *et al.* 2001). In this respect, GISH is a powerful tool to clearly differentiate chromosomes of different parental genomes in hybrids and their backcross progenies as well as to trace intergenomic chromosome rearrangements (Schwarzacher *et al.* 1989, Parokonny *et al.* 1997 and Takahashi *et al.*1997). The review by Xia (2009) contains many examples of the successful application of GISH on asymmetric hybrids.

The ultimate correlation of DNA sequence and molecular data to the structure and organization of chromosomes and nuclei is achieved through DNA *in situ* hybridization (Gall and Pardue, 1969; John *et al.* 1969). It also assists in identification and characterization of chromosomes and chromosome segments, by visualizing recent or evolutionary chromosome arrangements and changes in sequence abundance during evolution (Schwarzacher, 2003).

GISH was successfully used to identify the component genomes of interspecific hybrids of closely related species *Hordeum vulgare, H. chilense, H. bulbosum* and between *Secale cereale* and *S. africanum* (Anamthawat-Jonsson K *et al.* 1990 and Leitch, 1991). The technique has also been employed in many plant species and their interspecific and intergeneric hybrids such as *Allium* (Hizume, 1994, Keller *et al.* 1996, Khrustaleva and Kik, 1998; 2000), *Gasteria* x *Aloe* (Takahashi *et al.* 1997) and tomato (Ji *et al.* 2004). GISH has successfully identified intergenomic recombinant chromosomes regenerated from asymmetric hybrids between *Nicotiana plumbaginifolia* (acceptor) and *N. sylvestris* (donor). The fate of the recombinant

chromosomes was monitored by GISH (Parokonny *et al.* 1994). Other, more recent reports on asymmetric hybrid analysis using GISH are listed in Table 1.1.

GISH has also successfully discriminated genomes in other somatic hybrids between tomato and wild eggplant (Escalante *et al.*1998), rice and its wild relatives (Shishido *et al.* 1998), tobacco and its wild relatives (Kenton *et al.* 1993, Kitamura *et al.* 1997), potato and tomato (Jacobsen *et al.* 1995) and intergeneric somatic hybrids in rice (Jelodar *et al.* 1999). Recently, GISH has become as a standard screening tool for somatic hybrids (Eeckhaut *et al.* 2013).

More precise identification of chromosome rearrangements can be obtained through combined FISH/GISH (Moscone *et al.* 1996; Lim *et al.* 2000) which uses *in situ* hybridization with total genomic DNA as a probe to distinguish genomes and with chromosome-specific DNA probes to identify pairs of mitotic chromosomes or visualization of pairing of homoeologous chromosomes at meiosis (Abbasi *et al.* 1999; Cao *et al.* 2000). Sequential FISH and GISH methods were useful to precisely identify *Avena sativa* L. chromosome segments introgressed with *Triticum aestivum* L. in an asymmetric hybrid (Xiang *et al.* 2010). Recent achievements in the analysis of hybrids using GISH, such as higher probe concentration and a long hybridizing period on plants with extremely small sized genome and chromosomes can be useful (Ali *et al.* 2004; Van Laere *et al.* 2010). Tyr- and/or high sensitive FISH can visualize even very small chromosomal targets, as small as 500 bp on metaphase spreads (Khrustaleva and Kik, 2001; De Jong, 2003).

1.7 The family Araceae

Kingdom: Plantae (Plants)
Subkingdom: Tracheobionta (Vascular plants)
Super division: Spermatophyta (Seed plants)
Division: Magnoliophyta (Flowering plants)
Class: Liliopsida (Monocotyledons)
Subclass: Arecidae
Order: Arales
Family: Araceae (Arum family)

The Araceae (commonly known as aroids) is a very widely distributed monocotyledonous family. While some members are found in temperate regions, most are found in tropical and subtropical species. There are about 105 genera and 3300 species globally (Mayo *et al.* 1997). The leaves are often found with broad netted venation. The inflorescence is very consistent in having a dense mass of apetalous (petal less) flowers on a central 'spadix'. The flowers are often covered in a leaf like 'spathe', which can be colored or colorless. Because of this attractive nature, aroids are commonly appreciated as ornamentals. Known ornamental aroids are *Aglaonema, Alocasia, Anthurium, Colocasia, Dieffenbachia, Epipremnum, Gonatanthus, Homalomena, Monstera, Philodendron, Scindapsus, Spathiphyllum, Syngonium* and *Zantedeschia* (Henny, 1988; Chan, 2003; Henny and Chen, 2010). Ornamental aroids are used as cut

flowers, cut foliage, pot plants, and landscape plants. According to the Dutch Central Bureau of Statistics, in the Netherlands, the total auction turnover in 2011 of *Anthurium*, *Spathiphyllum* and *Zantedeschia* was 95 x 10^6€, including cut flowers and pot plants. Some ornamental aroids, such as *Aglonema, Dieffenbachia, Epipremnum, Philodendron, Scindapsus,* and *Spathiphyllum,* remove indoor volatile organic compounds produced from paints and electronic devices (Wolverton *et al.* 1989, Yang *et al.* 2009). Other than their use as ornamentals, the family also offers many other benefits. Plowman (1969) classified the economic and commercial uses of aroids such as production of essential oil from *Acorus*, leaf extracts of *Caladium* for treatment of cancerous ulcers, dyes from *Dieffenbachia seguine*, and a powder used as an antidote for snakebite from *Dracontium asperum*. *Colocasia esculenta* and *Xanthosoma sagittifolium* are cultivated as food crops. Volatile oil obtained from *Acorus calamus* is a repellent against *Callosobruchus chinensis*, a beetle destroying legumes in storage conditions (Su, 1977).

1.7.1 Cultivation and breeding history

Croat has collected and revised thousands of aroids which are currently in use in foliage plant industry (Croat, 1982, 1983, 1986, 1997 and 1998). New cultivars were also introduced after mutant clone or somaclonal variant selection such as *Dieffenbachia maculata* 'Perfection 007' which is a sport with bright striped petioles (Henny, 1977); also *Syngonium* produces excellent somaclonal variants (Henny and Chen, 2010). The Royal Horticultural Society produced two *Dieffenbachia* hybrids, namely, 'Bausei' (*D. maculate* x *D. weirii)* in 1870 and 'Memoria-corsii' (*D. maculata* x *D. wallisii*) in 1881. Those are the oldest *Dieffenbachia* hybrids which are still in industrial cultivation (Chen *et al.* 2005). Interspecific hybridization produced many hybrids in *Aglaonema* and *Alocasia* through conventional breeding (reviewed in Henny and Chen, 2010). Furthermore, interspecific hybridization is well investigated in *Anthurium* (Kamemoto and Kuehnle, 1996; Henny, 1999). Transgenic *Anthurium* has been produced by Kuehnle *et al.* (1991; 2001). In *Philodendron,* interspecific hybridization was referred back to 1887 with the production of *P. corsinianum,* a hybrid between *P. lucidum* and *P cariaceum* (Wilfret and Sheehan, 1981). McColly and Miller (1965) reported successful *Philodendron* interspecific hybrids. In *Zantedeschia,* bridge crosses were also performed by Yao *et al.* (1995) and only albino hybrids were obtained because of plastome incompatibility.

In Belgium, around 1965, Achiel Cornelis started crossings between *Spathiphyllum wallisii* seedlings and the cultivar 'Mauna Loa'. After approximately 20 years of selection, 'Daniël' was patented and marketed by Deroose plants. During the period 1993-2000, 60000 to 80000 'Daniël' plantlets per month were sold. Additionally, the cultivar was used for further breeding by Daniël Cornelis. This yielded the well-known cultivars, 'Alfa', 'Galaxy', 'Frederick', 'Lima', 'Mara', 'Marcay', 'Max' and 'Stephanie'. These have been established a place in the European market as well as in the US. Currently, 25000 to 30000 'Lima' plantlets and 25000 to 80000 'Alfata' are being sold per month. Among consumers, plants with darker leaves, white spathe, less fertilizer requirements and less pollen are desired. The most

important pathogens are *Cylindrocladium* and *Phytophthora*. Other than *Spathiphyllum* breeding, Cornelis also bred *Anthurium* but they were not marketed. According to our knowledge, Daniël Cornelis is the only aroid breeder in Belgium (Daniël Cornelis, personal communication).

Other efforts were done to improve adventitious shoot regeneration for transformation or mutant selection in *Anthurium, Dieffenbachia* and *Spathiphyllum* (Orlikowska *et al.* 1995). Eeckhaut *et al.* (2001) induced homozygous *Spathiphyllum* through gynogenesis, which can be used to produce inbred lines. In *Zantedeschia,* crosses were made using *Zantedeschia rehmanii*, with pink flowers, *Z. albomaculata*, with white spotted flowers, and *Z. elliottiana,* with yellow flowers (Shibuya, 1956). Polyploidization in *Spathiphyllum* was performed by Eeckhaut *et al.* (2004) and Vanstechelman *et al.* (2010); these polyploids were more resistant to drought (Van Laere *et al.* 2011). Imidazole fungicides induce adventitious shoots in *S. wallisii* and *A. andreanum* and they can be useful for micropropagation or mutagenesis (Werbrouck and Debergh, 1995; Werbrouck *et al.* 1996a, b). Imazalil in combination with benzyladenine (BA) induced shoots dramatically in *S. floribundum*. Similar effects were observed in *A. andreanum* when prochloraz was added in combination with BA. This shoot inducing effect of imidazole fungicides in Araceae could partially be based on their inhibition of gibberellic acid and alteration in endogenous cytokinins metabolism (Werbrouck *et al.* 1996a). Somatic embryogenesis and regeneration of plants were obtained from *Spathiphyllum* anther filaments (Werbrouck *et al.* 1998). Studies on somatic embryogenesis and mass micropropagation of *Anthurium* are reported by Hamidah *et al.* (1994; 1995 a, b; 1997 a,b).

Selection of varieties for resistant breeding is also prominent in aroids (Ivancic, A. 1995; Anaïs *et al.* 1998; Snijder *et al.* 2004a, b; Goktepe *et al.* 2007; Seijo, 2010). Bacterial blight disease caused by *Xanthomonas campestris* pv. *dieffenbachiae* affects a broad range of ornamental and edible aroids including *Anthurium*, *Colocasia* (taro), *Aglaonema, Syngonium, Xanthosoma, Dieffenbachia*, *Epipremnum*, *Dracaena, Alocasia, Spathiphyllum, Rhaphidophora*, *Caladium,* and *Philodendron* (Hayward, 1972; Nishijima, 1985). Cultivar resistance to bacterial blight disease can be used to produce commercial resistant varieties (Anaïs *et al.* 1998). *Spathiphyllum* production is mainly affected by *Cylindrocladium spathiphylli* and *Phytophthora parasitica;* however, *Myrothecium roridum*, *Rhizoctonia* spp., *Sclerotium rolfsii* and *Xanthomonas campestris* pv. *Dieffenbachiae* can also cause extensive damage under appropriate conditions. Also *Rhodanobacter spathiphylli* spp. been isolated from *Spathiphyllum* roots (Simone, 1994; De Clerq *et al.* 2006). Bacterial soft rot caused by *Erwinia carotovora* subsp. *carotovora* is a major disease in *Zantedeschia* spp., particularly in cultivars from the section *Aestivae*. *Z. aethiopica* is more resistant to soft rot than *Aestivae* species. To date, interspecific hybrids between genotypes of the *Aethiopica* and the *Aestivae* section obtained after embryo rescue have only delivered albino plants (Snijder *et al.* 2007).

Protoplast regeneration of *Pinellia* was reported by He *et al.* (1996). Kuehnle (1997) isolated and cultured protoplasts from axenic tissues of etiolated *Anthurium*, but only microcolonies

were reported. Murakami *et al.* (1995; 1998) mentioned protoplasts regeneration from *Colocasia esculenta* var. *antiquorum* and intraspecific autotetraploid somatic hybrids of taro (*Colocasia esculenta* Schott). However, the frequency of protoplast regeneration was very low and the procedure was time consuming. In other reports, no successful protoplast regeneration was mentioned for different species (Pongchawee *et al.* 2006; Duquenne *et al.* 2007; Pongchawee *et al.* 2007). Furthermore, intergeneric hybrids in Araceae have not yet been reported (Duquenne *et al.* 2007).

antigen antibodies genome size peroxidase

flow cytometry hybridization G-C interstitial 5S rDNA

B chromosomes chromosome markers A-T cloning FISH

phase contrast microscope DAPI buffer RNAse stringency

telocentric PI pepsin 45S rDNA metacentric

Nick translation satellite tandem repeats pTa71 short arm

Chapter 2
Karyotype analysis and physical mapping of 45S and 5S rRNA genes

NOR paraformaldehyde 2C α-bromonaphthalin

distal SSC long arm chromosome number renaturation

digoxigenin denaturation subtelocentric ethanol arm ratio

citrate buffer Tyr-FITC tyramide mbp metaphase

karyotype asymmetry AI 8-hydroxyquinoline centromeric index

fluorescence microscope karyotype idiogram simultaneous FISH

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2.1 Introduction

In breeding programs, cytogenetic information of parent plants can be useful to trace parental markers in putative hybrids as well as to select suitable parents. Therefore, we applied cytogenetic studies on six economically important species from aroid genera to gather information for future breeding and to analyze somatic hybrid products. In Araceae, Petersen (1989; 1993) has reviewed the works on aroid chromosome numbers. Karyotype studies have been performed on *Anthurium* (Kaneko and Kamemoto, 1979), *Sauromatum* (Fu-Hua *et al.* 2001), *Dieffenbachia (*Chen *et al.* 2007), *Colocasia* (Begum *et al.* 2009) and *Zantedeschia* (Ghimire *et al.* 2012). Cabrera *et al.* (2008) published an extensive phylogenetic study on Araceae familial, subfamilial and tribal monophyly level using plastid DNA. A karyotype is an organised profile of the chromosomes of a eukaryotic cell that provides information about the chromosome number, size and morphology. In a karyotype, chromosomes are arranged and numbered by size from the largest to the smallest. In addition, we can use fluorescence *in situ* hybridization (FISH) techniques to physically localize repetitive sequences (e.g. 45S or 5S rDNA) as chromosome markers. Physical mapping of gene markers and repetitive sequences such as 45S or 5S rRNA using FISH has not yet been reported in literature for Araceae.

In *Anthurium,* B-chromosomes have been reported (Sharma and Bhattacharyya, 1961, Kaneko and Kamemoto, 1979, Marutani *et al.* 1993). B-chromosomes are considered to be unnecessary components in the karyotypes of some plants, fungi and animal species. They are present in some individuals of a population and absent in others. They do not pair or recombine with any members of the standard diploid (or polyploid) set of chromosomes (A chromosomes) at meiosis and their inheritance is non-mendelian and irregular (Jones and Houben, 2003).

In this study, flow cytometric analysis for genome size measurements, karyotype construction, and FISH mapping for repetitive sequences were performed. We also screened the occurrence of B-chromosomes-like structures. In order to establish a general FISH protocol for Araceae, we performed FISH using 45S and 5S rDNA repetitive sequences on *Anthurium andreanum*, *Monstera deliciosa, Philodendron scandens, Spathiphyllum wallisii*, *Syngonium auritum*, and *Zantedeschia elliottiana.* Tyramide-FISH (Tyr-FISH) was used to locate 5S rDNA when conventional FISH was not sensitive enough. These model plants are distantly related and among them *Philodendron*, *Syngonium* and *Zantedeschia* belong to sub-family aroideae; *Monstera* and *Spathiphyllum* to monsteroideae, and *Anthurium* to pothoideae (Cabrera *et al.* 2008).

2.2 Materials and methods

2.2.1 Plant materials

A. andreanum '061' and *S. wallisii* 'Domino' were present in the ILVO collection; *M. deliciosa* 'Variegata', *P. scandens* and *S. auritum* were obtained from the greenhouse of Tsitsin RAS

Botanical Garden, Moscow, Russia; *Z. elliottiana* '068' was provided by Sandegroup, the Netherlands.

2.2.2 Acclimatization

A. andreanum '061', *S. wallisii* 'Domino' and *Z. elliottiana* '068' in vitro plants were transferred to hormone free medium (Chapter 3; Table 3.1) for four weeks. After removing agar, the plantlets were treated with 8.5 mM benzimidazole and planted in plastic trays filled with potting soil (Saniflor®, NV Van Israel) and covered with a glass plate for three days. The plantlets were gradually exposed to greenhouse conditions (20±2°C; 16h/day at 30 μmol m^{-2} s^{-1} photosynthetic period, 60±3% relative humidity) by opening the glass cover partially after three days. All plants were placed in ceramic pots, filled with potting soil and watered two days before collecting the root tips.

2.2.3 Genome size measurements

Approximately 25 mm^2 young leaf material was co-chopped with the same amount of leaf material of a reference plant in 400 μL extraction buffer (Partec, Cystain PI absolute P, according to the manufacturers' protocol). The chopped material was then filtered through a nylon filter with 50 μm mesh size (Celltrics®, Partec) and incubated for at least 60 minutes in 2 mL staining buffer at 4°C in darkness. The staining buffer contained (per sample) 12 μL propidium iodide stock solution and 6 μL RNase solution (Partec, Cystain PI absolute P). At least 5000 nuclei were analysed per sample. Obtained data were analysed using Flomax software on a CyFlow space of PASIII (Partec).

The following reference plants were used: *Pisum sativum* L. 'Ctirad' (9.09 pg/2C; Doležel *et al.* 1998) for *S. wallisii* 'Domino'; *Solanum lycopersicum* L. 'Stupické Polní Rané' (1.96 pg/2C ; Doležel *et al.* 1992) for *P. scandens, S. auritum* and *Z. elliottiana* '068'; and *Glycine max* 'Polanka' Merr. (2.5 pg/2C; Doležel *et al.* 1994) for *A. andreanum* '061' and *M. deliciosa* 'Variegata. At least three repeats were analyzed. The genome size was calculated based upon peak position ratios of the sample plants and the reference plants. The influence of plant cytosolic compounds on fluorochrome accessibility of nuclear DNA was tested. To this end, we tested the stability of the peak positions of the reference plants, either with or without sample plants, in all tests.

2.2.4 Chromosome spread preparation

Actively growing root tips were collected. The root tips of *S. wallisii* 'Domino' were pretreated in ice-cold (4°C) water overnight. *A. andreanum* '061', *M. deliciosa* 'Variegata', *P. scandens, S. auritum*, and *Z. elliottiana* '068' root tips were pretreated in a α-bromonaphthalin solution (10 μL in 10 mL water) overnight at 4°C. After the pretreatment, the root tips were fixed in Carnoy solution (3:1 absolute ethanol-acetic acid) at least 1 h at room temperature. They were either used immediately or stored at -20°C until use. The Carnoy solution was removed by washing the root tips three times in tap water for 20 min. The root tips were

digested in a pectolytic enzyme mixture [0.1% (w/v) pectolyase Y23 (Duchefa, Haarlem, the Netherlands), 0.1% (w/v) cellulase onozuka RS (Duchefa, Haarlem, the Netherlands) and 0.1% (w/v) cytohelicase (Sigma-Aldrich, Steinheim, Germany)] in 10 mM citrate buffer (10 mM tri sodium citrate + 10 mM citrate, pH 4.5) at 37°C for 1h.

Chromosome preparations were made according to the spreading method of Pijnacker and Ferwerda (1984). One meristem was transferred to a clean slide using a Pasteur pipette, the excess buffer was removed using Whatman filter paper and a drop of 45% acetic acid was added. The cells were spread with needles on a heating plate (42°C) for 2 min and slightly tilted for 1 min. Seven drops of ice-cold Carnoy solution was added to the slides and they were rinsed briefly in 98% ethanol. The slides were air-dried for 1 h and stored at 4°C until further use. The best slides were selected under a phase contrast microscope (Leica DM IRB) and used for karyotype and FISH analysis.

2.2.5 *In situ* hybridization

Plasmid clone pTa71 containing a 9 kb *Eco*RI fragment of the 45S rDNA from *Triticum aestivum* L. (Gerlach and Bedbrook, 1979), and plasmid clone pScT7 containing a 462 bp fragment of 5S rDNA from rye (Lawrence and Appels, 1986) were used. Isolated pTa71 and pScT7 plasmids were labelled with a Biotin-Nick Translation Kit (Roche Diagnostics Gmbh, Mannheim, Germany) and Digoxigenin-Nick Translation Mix (Roche Diagnostics Gmbh, Mannheim, Germany), respectively, according to manufacturer's instructions. They were subsequently checked using gel electrophoresis.

Slides were pretreated with 4% (w/v) paraformaldehyde for 10 min at room temperature and air dried after sequential washes in 70% (-20°C), 90% and 100% ethanol for 3 min each (Leitch and Heslop-Harrison, 1994). DNA denaturation and *in situ* hybridisation were done according to Schwarzacher and Leitch (1993) and Schwarzacher and Heslop-Harrison (1994). The hybridization mixture was made of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2x SSC (Saline Sodium Citrate buffer), 0.25% (w/v) sodium dodecyl sulphate and 2 ng/μL labelled DNA. The hybridization mixture was denatured at 80°C for 5 min and placed on ice for 5 min. After the hybridisation mixture (40 μL) was added to the slides, a 5 min denaturation process was carried out at 80°C. Then the slides were incubated overnight in a humid chamber at 37°C to hybridize. The slides were washed in 2x SSC at room temperature for 15 min, then transferred to 0.1x SSC at 48°C for exactly 30 min to give a stringent wash (78%). Finally, they were washed again in 2x SSC for 15 min at room temperature. To reduce non-specific binding of antibodies and thus to lower the background fluorescence, 100 μL of 1% TNB [Boeringer blocking reagent in TN buffer (0.1 M Tris-HCl, 0.15 M NaCl, and pH 7.5)] was added to the slides and incubated for 10 min at 37°C in a humid chamber. Biotin-labelled DNA was detected with 5 μL CY3-conjugated streptavidin and amplified with 1 μL biotinylated goat-antistreptavidin (Vector Laboratories, Burlingame, CA, USA) followed by addition of CY3-conjugated streptavidin. Digoxigenin-labelled probes were detected using

FITC conjugated anti-Dig antibody (0.01% FITC in TNB; Roche Diagnostics Gmbh, Mannheim, Germany) from sheep and 1 μL anti-sheep FITC from rabbit diluted in TNB. These detection steps were performed at 37°C in a humid chamber for 1 h.

2.2.6 Tyramide-FISH

For Tyr-FISH, the plasmid carrying the 5S rRNA gene of rye (pScT7, Lawrence and Appels, 1986) was labeled using Digoxigenin Nick Translation Mix (Roche). Probe hybridization and detection were performed according to Khrustaleva and Kik (2001) with minor modifications. Slides were fixed in 4% buffered paraformaldehyde in 1xPBS (1xPBS: 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.5) for 8 min before the RNAse treatment and 10 min before denaturation. Inactivation of endogenous peroxidase was done by incubating the slides in 0.01M HCl for 8 min (Liu *et al.* 2006). Pepsin treatment was performed for 30 sec at room temperature. The hybridization mixture contained 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 2xSSC, 0.25% sodium dodecyl sulphate and 2.75 ng/μL labelled DNA. The hybridization mix was denatured at 75°C for 5 min and subsequently placed on ice for 5 min and added to the chromosome preparations. Slides were then denatured for 10 min at 80°C. A 82% stringency washing was attained by washing the slides twice in 2xSSC for 37°C, twice in 25% (v/v) formamide in 0.4xSSC for 10 min at 42°C and finally in 2xSSC for 3 min at 37°C. 100 μL Horseradish Peroxidase (HRP) conjugated antibodies [antiDig-POD (Roche, Diagnostics Gmbh, Mannheim, Germany) diluted 1:100 in TNB] were applied). After washing three times in TNT buffer, the tyramide solution containing 200 μL Tyr-FITC and 10% dextran sulphate in 1x Amplification Diluent (Perkin Elmer, Inc.,Waltham, Massachusetts, USA) was applied.

2.2.7 Microscopy and karyotyping

The slides were counterstained with 1 μg/mL 4',6-Diamidino-2-phenylindole (DAPI) and mounted with Vectashield® (Vector Laboratories, Burlingame, CA, USA). Slides were examined under a Zeiss Axio Imager microscope (Carl Zeiss MicroImaging, Jena, Germany). Images were captured by AxioCam and Axiovision 4.6 software, Zeiss. Chromosome analysis was done on at least five well-spread metaphases of each genotype using MicroMeasure (Reeves, 2001) for Windows, version 3.3. Relative chromosome length, arm length, centromeric index and the position of the hybridization signal were measured. Characterization of chromosome morphology was done based on Levan *et al.* (1964). Chromosomes were arranged in order of decreasing length.

The asymmetry of the karyotype was evaluated according to Paszko (2006). A theoretical symmetrical karyotype could be defined as a karyotype in which all chromosome arm ratio is 1.0 and all chromosomes are equal in length. The interchromosomal asymmetry coefficient of variation for chromosome length (CV_{CL}) was calculated as the ratio between the standard deviation (S_{CL}) and mean chromosome length (X_{CL}): $CV_{CL} = S_{CL} / X_{CL} \times 100$. The intrachromosomal asymmetry coefficient of variation in centromere position (CV_{CI}) was

evaluated as the ratio between the standard deviation (S_{CI}) and the mean centromeric index (X_{CI}): $CV_{CI} = S_{CI} / X_{CI} \times 100$. The asymmetry index (AI) was evaluated as $CV_{CL} \times CV_{CI} / 100$. The degrees of chromosome compaction [Genome size 1C (Mbp) / mean total chromosome length (μm)] were calculated assuming that they are uniform along the entire chromosome.

2.3 Results

The obtained data from the results are summarized in Table 2.1 and 2.2. The pictures including idiograms with the FISH signal positions and B-chromosome like structures are presented in Fig. 2.1, 2.2 and 2.3.

2.3.1 Flow cytometry

Flow cytometric analysis showed small genome sizes for *P. scandens, S. auritum* and *Z. elliottiana* while *S. wallisii* had the biggest genome size (7.39 ± 0.04 pg/1C). The results are summarized in Table 2.2. No cytosolic compound effects on fluorochrome accessibility on nuclear DNA were observed.

2.3.2 Karyotype analysis

2.3.2.1 *Anthurium andreanum* (2n=2x=30)

Three metacentric and 12 sub-metacentric chromosomes were observed (Table 2.1 A and Fig. 2.2 A). *A. andreanum* chromosomes were the biggest among all the plants tested ranging from 15.77 ± 1.72 μm and 6.20 ± 0.10 μm (Table 2.2). The first two pairs of metacentric chromosomes are distinctive in that they were markedly larger than all the remaining chromosomes. The centromeric indexes of submetacentric chromosomes ranged from 0.25 ± 0.01 μm to 0.34 ± 0.05 μm. Karyotype asymmetry index was 5.49. Even though *A. andreanum* possessed bigger chromosomes, the degree of chromosome compaction was not much different from the other plants except *S. wallisii* (Table 2.2).

We observed B-chromosome-like structures in *A. andreanum* metaphase spreads. Approximately 19.75% cells possessed two B-chromosomes-like structures, while 34.57% spreads showed one and 45.68% showed none (Fig. 2.3). The size of B-chromosome-like structures was 3.32 ± 0.12 μm, which is about 1.87 times less than the size of the shortest chromosome of the complement (6.20 ± 0.10 μm).

2.3.2.2 *Monstera deliciosa* (2n=2x=60)

Monstera deliciosa 'Variegata' is the diploid species with the highest basic chromosome number (x=30) and the longest total chromosome complement among all analyzed genera. The chromosome sizes ranged from 7.76 ± 0.99 μm to 3.35 ± 0.40 μm. The complement was rather difficult for karyotyping due to the uniformity of chromosome morphology, i.e., a slight difference between several chromosome pairs in size of length and centromere position (Table 2.1 B). Most of chromosomes are metacentric (26 pairs) and only four pairs

were submetacentric. The chromosomes contain on average of 41.98 ± 0.29 million base pairs per 1 μm.

2.3.2.3 *Philodendron scandens* (2n=2x=32)

P. scandens is a diploid species with basic chromosome number x=16. Its chromosomes were the smallest of all genera tested (Table 2.2). The centromeric index ranged from 0.10 ± 0.02 μm to 0.45 ± 0.01 μm. The karyotype contained 10 pairs of metacentric, 2 pairs of submetacentric, 3 pair of subtelocentric and 1 pair of telocentric chromosomes (Table 2.1 C and Fig. 2.2 C). The karyotype asymmetry (6.58) was the highest of the all the six genera (Table 2.2). The chromosome compaction was 37.74 ± 0.39 Mbp/μm.

2.3.2.4 *Spathiphyllum wallisii* (2n=2x=30)

The idiogram of the karyotype is shown in Fig. 2.2 D. *S. wallisii* is diploid species and has a basic chromosome number of x=15. Its karyotype contained 15 metacentric chromosomes. Chromosome size ranged from 8.58 ± 0.02 μm to 5.64 ± 1.35 μm. The karyotype asymmetry (0.70) was the lowest and chromosome compaction was the highest (66.49 ± 0.37 Mbp/μm) of all the genera tested (Table 2.2).

2.3.2.5 *Syngonium auritum* (2n=2x=24)

S. auritum is a diploid species and has the lowest basic chromosome number (x=12) (Fig. 2.2 E). The size of chromosomes ranged from 8.71 ± 1.91 μm to 3.07 ± 0.57 μm. The first pair of metacentric chromosomes can be easy identified due to distinctively large size and morphology. The karyotype of this species consisted of eight pairs of metacentric chromosomes and four pairs of submetacentric chromosomes (Table 2.1 E). The degree of chromosome compaction was 38.85 ± 2.02 Mbp/μm.

2.3.2.6 *Zantedeschia elliotiana* (2n=2x=32)

Z. elliottiana is a diploid species with a basic chromosome number of x=16. All chromosomes in the complement were metacentric, and form a continuous group of chromosomes that decrease slightly in length (Table 2.1 F). The karyotype showed a highly symmetrical karyotype with an index of 0.90. The chromosome sizes ranged from 6.51 ± 2.10 μm to 3.01 ± 0.85 μm. The degree of chromosome compaction was the lower than the other Araceae genera tested (17.84 ± 0.59 Mbp/μm).

2.3.3 *In situ* hybridization of 45S and 5S rDNA repeats

Secondary constrictions or satellites that indicate the position of 45S rDNA were not detected on the monochrome-stained chromosomes in all analyzed species. Therefore we carried out FISH experiments probing with 45S rDNA for visualization of the position of the ribosomal genes. The results are shown in Fig. 2.1 and summarized in Table 2.2. In all plants, two 45S rDNA sites were visualized except in *Z. elliottiana* which had four 45S rDNA sites. 45S rDNA

sites were seen in a distal position of *A. andreanum* and *Z. elliottiana* short arms. The 45S rDNA signals were observed on the proximal position of the short arms in other species except *P. scandens,* whose telocentric chromosome and delivered signal at the terminal position (Fig 2.1 C). Using Tyr-FISH, 5S rDNA was also mapped. *S. wallisii* showed the signal in the interstitial position of the chromosome 13 short arm while *Z. elliottiana* showed it in the proximal position of the long arm of chromosome 10.

Table 2.1 Karyotype data of *Anthurium andreanum*, *Monstera deliciosa*, *Philodendron scandens*, *Spathiphyllum wallisii*, *Syngonium auritum* and *Zantedeschia elliottiana*. Data are means ± SE. At least five well-spread metaphases were analyzed. m-metacentric; sm-sub metacentric; t-telocentric; st-sub telocentric.

A) *Anthurium andreanum* '061'

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	15.77±1.72	11.89±0.72	0.44±0.02	m
2	14.28±0.88	10.76±0.39	0.43±0.02	m
3	9.71±0.53	7.31±0.18	0.26±0.01	sm
4	9.31±0.71	7.01±0.24	0.26±0.04	sm
5	9.07±0.28	6.83±0.07	0.30±0.04	sm
6	8.89±0.56	6.70±0.20	0.29±0.00	sm
7	8.40±0.63	6.33±0.20	0.31±0.02	sm
8	7.84±0.59	5.90±0.23	0.25±0.01	sm
9	7.83±0.47	5.89±0.18	0.39±0.05	m
10	7.42±0.46	5.58±0.14	0.30±0.03	sm
11	7.25±0.59	5.46±0.19	0.31±0.03	sm
12	7.19±0.69	5.41±0.23	0.30±0.02	sm
13	6.74±0.16	5.07±0.03	0.34±0.05	sm
14	6.67±0.49	5.02±0.18	0.30±0.03	sm
15	6.20±0.10	4.83±0.03	0.29±0.04	sm

B) *Monstera deliciosa* 'Variegata'

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	7.76±0.99	5.40±0.23	0.45±0.02	m
2	7.04±0.70	4.90±0.08	0.46±0.01	m
3	6.65±0.85	4.62±0.11	0.41±0.02	m
4	6.44±0.73	4.33±0.12	0.38±0.00	m
5	6.22±0.68	4.24±0.14	0.48±0.02	m
6	6.08±0.76	3.81±0.08	0.37±0.02	sm
7	5.49±0.78	3.73±0.11	0.40±0.04	m
8	5.38±0.61	3.60±0.06	0.38±0.03	m
9	5.18±0.61	3.53±0.03	0.41±0.02	m
10	5.12±0.60	3.47±0.03	0.46±0.02	m
11	5.08±1.73	3.39±0.03	0.38±0.02	m
12	5.00±0.63	3.34±0.05	0.35±0.02	sm
13	4.82±0.59	3.31±0.02	0.45±0.03	m
14	4.77±0.62	3.23±0.03	0.42±0.03	m
15	4.66±0.60	3.16±0.02	0.40±0.03	m
16	4.56±0.62	3.11±0.03	0.42±0.02	m
17	4.50±0.63	3.07±0.03	0.44±0.01	m
18	4.44±0.65	3.05±0.05	0.44±0.03	m
19	4.40±0.65	3.01±0.07	0.42±0.01	m
20	4.35±0.64	2.98±0.05	0.44±0.02	m
21	4.30±0.61	2.92±0.05	0.41±0.02	m
22	4.22±0.58	2.89±0.03	0.46±0.03	m
23	4.18±0.55	2.86±0.05	0.35±0.03	sm
24	4.12±0.59	2.82±0.06	0.38±0.02	m
25	4.07±0.58	2.70±0.04	0.37±0.01	sm
26	3.90±0.62	2.63±0.06	0.47±0.02	m
27	3.80±1.49	2.67±0.03	0.41±0.00	m
28	3.65±0.60	2.52±0.06	0.44±0.02	m
29	3.59±0.56	2.48±0.04	0.47±0.01	m
30	3.35±0.40	2.32±0.04	0.44±0.01	m

C) *Philodendron scandens*

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	3.81±0.75	8.49±0.22	0.41±0.02	m
2	3.65±0.79	8.13±0.22	0.40±0.01	m
3	3.28±0.43	7.34±0.13	0.20±0.01	st
4	3.19±0.40	7.14±0.17	0.38±0.03	m
5	3.09±0.34	6.92±0.13	0.43±0.01	m
6	2.97±0.47	6.65±0.19	0.10±0.02	t
7	2.92±0.37	6.54±0.11	0.26±0.01	sm
8	2.80±0.31	6.30±0.11	0.14±0.02	st
9	2.78±0.33	6.25±0.14	0.22±0.04	st
10	2.69±0.36	6.01±0.16	0.40±0.01	m
11	2.54±0.48	5.70±0.25	0.43±0.01	m
12	2.41±0.26	5.42±0.11	0.40±0.02	m
13	2.30±0.39	5.15±0.20	0.42±0.01	m
14	2.26±0.17	5.07±0.08	0.36±0.02	sm
15	2.12±0.29	4.75±0.15	0.45±0.01	m
16	1.87±0.33	4.20±0.17	0.44±0.01	m

D) *Spathiphyllum wallisii* 'Domino'

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	8.58±0.02	7.94±0.01	0.45±0.01	m
2	8.32±0.41	7.71±0.07	0.44±0.03	m
3	7.99±0.08	7.40±0.00	0.44±0.00	m
4	7.85±0.10	7.27±0.00	0.45±0.00	m
5	7.62±0.75	7.05±0.15	0.47±0.01	m
6	7.26±0.43	6.72±0.11	0.44±0.01	m
7	7.19±0.23	6.66±0.07	0.43±0.00	m
8	7.06±0.32	6.54±0.09	0.41±0.00	m
9	6.98±0.30	6.46±0.08	0.42±0.04	m
10	6.93±0.29	6.42±0.08	0.47±0.00	m
11	6.86±0.08	6.35±0.03	0.41±0.05	m
12	6.73±0.27	6.24±0.07	0.40±0.00	m
13	6.51±0.19	6.03±0.03	0.47±0.00	m
14	6.45±0.08	5.98±0.00	0.40±0.01	m
15	5.64±1.35	5.23±0.29	0.39±0.02	m

E) *Syngonium auritum*

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	8.71±1.91	13.36±0.43	0.41±0.01	m
2	7.83±1.32	12.11±0.16	0.44±0.01	m
3	7.20±1.81	11.05±0.61	0.33±0.03	sm
4	6.27±1.29	9.66±0.39	0.39±0.02	m
5	5.97±1.34	9.13±0.38	0.37±0.02	sm
6	5.20±0.98	8.03±0.31	0.35±0.02	sm
7	4.99±0.94	7.72±0.40	0.31±0.03	sm
8	4.31±1.10	6.63±0.46	0.42±0.02	m
9	4.14±0.85	6.37±0.16	0.46±0.01	m
10	3.77±0.70	5.84±0.20	0.47±0.01	m
11	3.44±0.58	5.34±0.16	0.43±0.02	m
12	3.07±0.57	4.75±0.19	0.41±0.01	m

F) *Zantedeschia elliottiana* '068'

Chromosome number	Absolute length (μm)	Relative length (%)	Centromeric index	Chromosome type
1	6.51±2.10	8.83±0.28	0.42±0.02	m
2	5.83±1.61	7.96±0.19	0.41±0.02	m
3	5.53±1.58	7.54±0.12	0.38±0.02	m
4	5.37±1.58	7.32±0.11	0.41±0.01	m
5	5.17±1.63	7.02±0.15	0.40±0.02	m
6	4.94±1.45	6.72±0.10	0.44±0.01	m
7	4.77±1.39	6.51±0.10	0.40±0.01	m
8	4.61±1.39	6.28±0.15	0.42±0.02	m
9	4.46±1.24	6.09±0.05	0.39±0.02	m
10	4.25±1.23	5.80±0.14	0.43±0.01	m
11	4.12±1.15	5.64±0.09	0.43±0.02	m
12	4.00±1.11	5.49±0.12	0.42±0.02	m
13	3.77±0.96	5.19±0.11	0.40±0.01	m
14	3.48±0.90	4.80±0.09	0.38±0.01	m
15	3.36±0.94	4.63±0.14	0.40±0.02	m
16	3.01±0.85	4.17±0.21	0.44±0.01	m

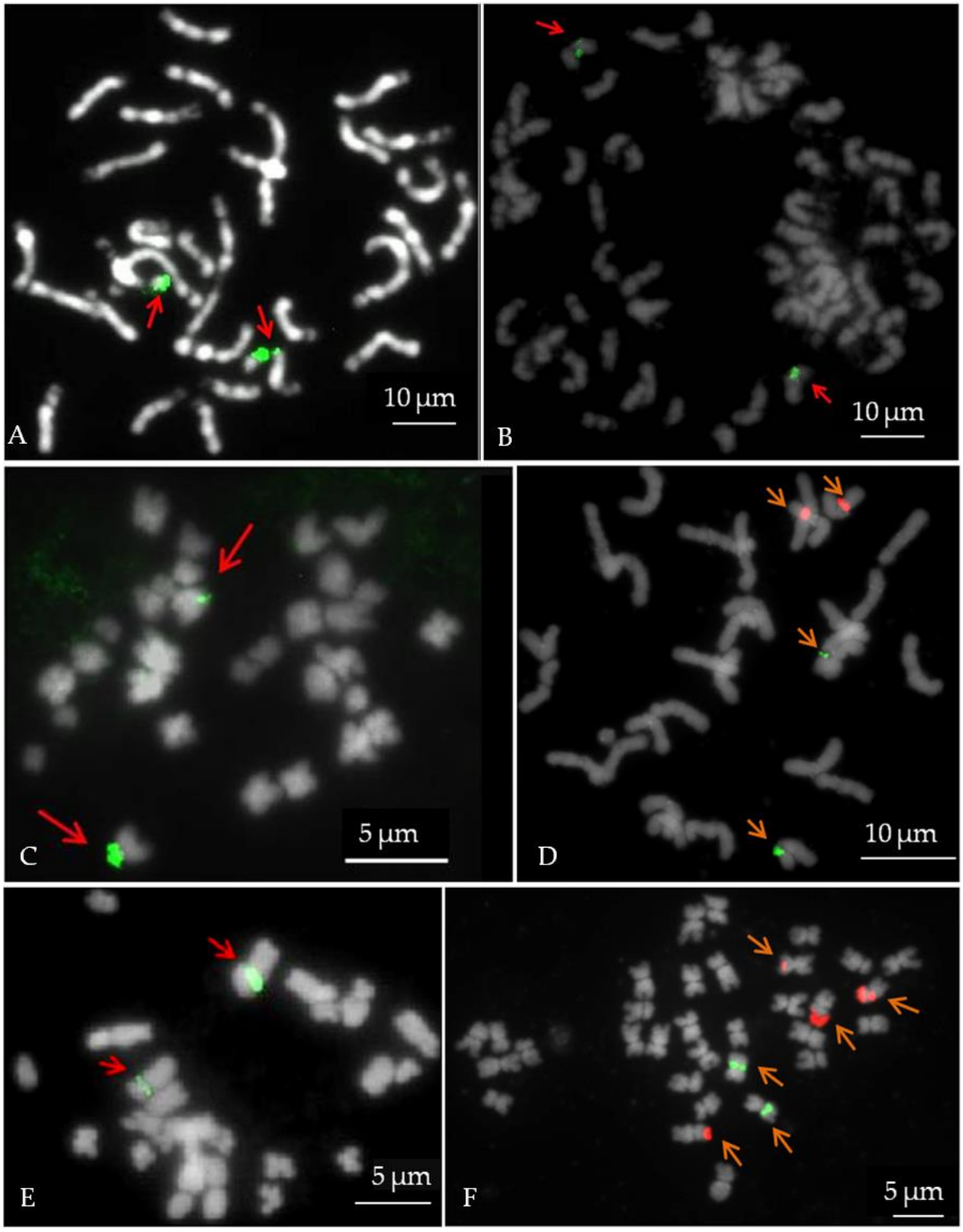

Fig. 2.1 DAPI stained mitotic metaphases with FISH signal: (A) *Anthurium andreanum* '061'; (B) *Monstera deliciosa* 'Variegata'; (C) *Philodendron scandens*; (D) *Spathiphyllum wallisii* 'Domino'; (E) *Syngonium auritum*; and (F) *Zantedeschia elliotiana* '068'. A-E: 45S rDNA sites were observed by FITC (green) by conventional FISH. D: 5S visualized by CY3 (red signal) by conventional FISH. F: 45S sites were visualized by CY3 (red signal) and FITC 5S (green signal) by Tyr-FISH. The arrows indicate the hybridization sites.

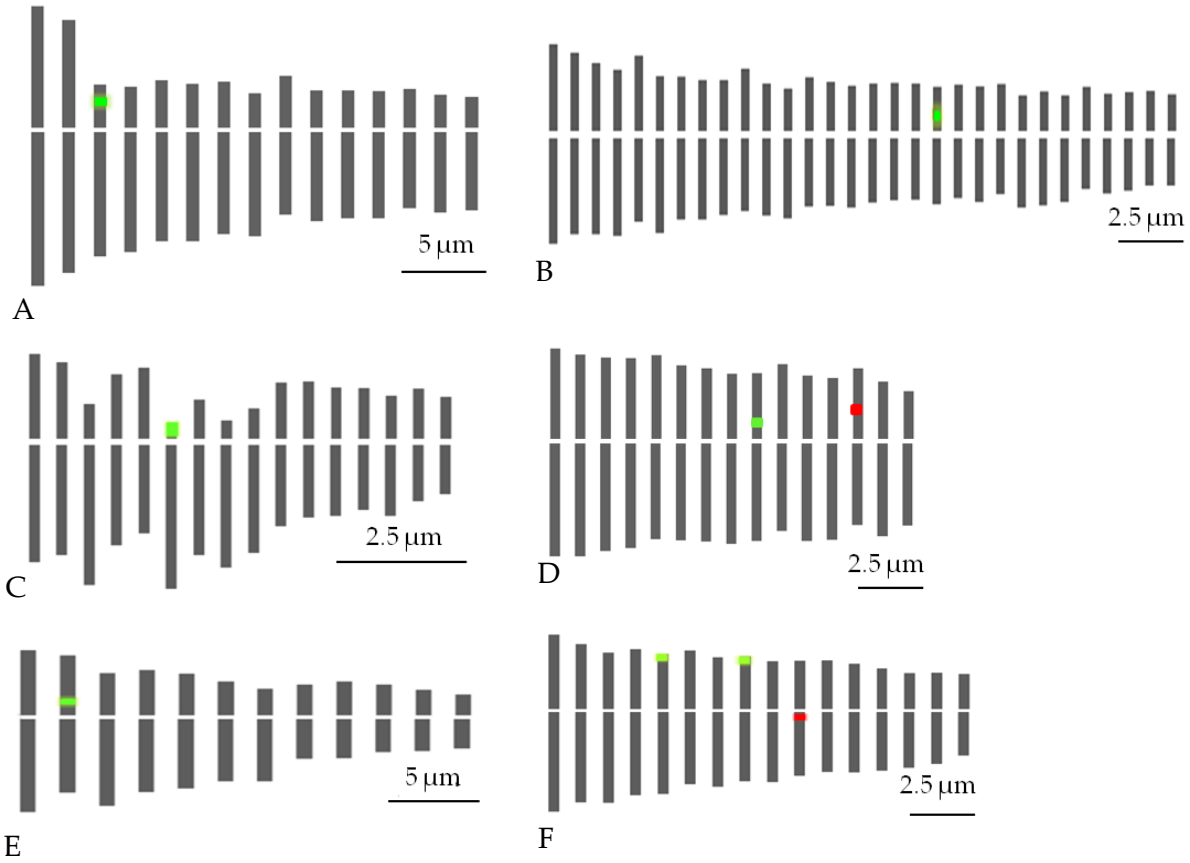

Fig. 2.2 Idiograms with indication of 45S (green) and 5S rDNA (red) FISH signal: (A) *Anthurium andreanum* '061'; (B) *Monstera deliciosa* 'Variegata'; (C) *Philodendron scandens*; (D) *Spathiphyllum wallisii* Regel 'Domino'; (E) *Syngonium auritum*; and (F) *Zantedeschia elliotiana* '068').

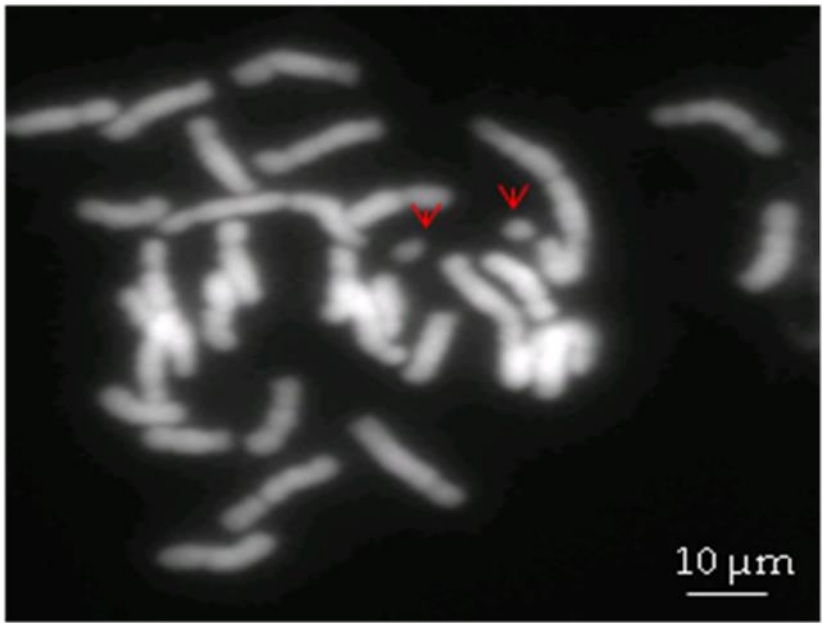

Fig. 2.3 DAPI stained chromosome spreads of *Anthurium andreanum* '061' with presumable B-chromosomes (indicated by arrows).

Table 2.2 Summary of genome size and karyotypic data for *Anthurium andreanum* '061', *Monstera deliciosa* 'Variegata', *Philodendron scandens*, *Spathiphyllum wallisii* 'Domino', *Syngonium auritum* and *Zantedeschia elliottiana* '068'.

	Anthurium andreanum	*Monstera deliciosa*	*Philodendron scandens*	*Spathiphyllum wallisii*	*Syngonium auritum*	*Zantedeschia elliottiana*
Genome size (pg/1C)	5.27 ± 0.08	6.36 ± 0.22	1.74 ± 0.01	7.39 ± 0.04	2.60 ± 0.04	1.35 ± 0.01
Chromosome number	2n=2x=30	2n=2x=60	2n=2x=32	2n=2x=30	2n=2x=24	2n=2x=32
Chromosome formula	3m+12sm	26m+4sm	10m+2sm+3st+1t	15 m	8m+4sm	16m
Total chromosome complement (μm)[1]	132.72 ± 1.39	147.14 ± 0.39	44.64 ± 0.52	107.97 ± 0.58	64.88± 1.25	73.19 ± 0.99
Length of the longest chromosome (μm)	15.77 ± 1.72	7.76 ± 0.99	3.81 ± 0.75	8.58 ± 0.02	8.71 ± 1.91	6.51 ± 2.10
Length of the shortest chromosome (μm)	6.20 ± 0.10	3.35 ± 0.40	1.87 ± 0.33	5.64 ± 1.35	3.07 ± 0.57	3.01 ± 0.85
Asymmetry index (AI)	5.49	1.90	6.58	0.70	4.31	0.90
Degree of compaction (Mbp/μm)	38.52 ± 0.19	41.98 ± 0.29	37.74 ± 0.39	66.49 ± 0.37	38.85 ± 2.02	17.84 ± 0.59
rRNA gene(s) : chromosome number(s)	45S : 3	45S : 19	45S : 6	45S : 9 and 5S : 13	45S : 2	45S : 5, 8 and 5S : 10
Number of spreads analyzed after FISH	3	5	5	2	4	6

Data are averages ± SE.

[1]Total chromosome length at haploid level

m- metacentric; sm- submetacentric; st- subtelocentric; t- telocentric

2.4 Discussion

The success of interspecific or intergeneric crosses using traditional breeding mainly depends on how closely the parental species are (cyto) genetically related. Differences between parent plants concerning genome size, chromosome number and centromeric position of pairing chromosomes decide the fate of hybrid chromosome pairing during meiosis. Somatic hybridization as an asexual method allows overcoming genetically determined pre- and post-hybridization barriers. However, heterokaryons division and subsequent regeneration remains partly dependent on the genomic and (cyto)genetic structure of parental species. Moreover, screening of somatic hybrids and monitoring of genetic material introgression from donor species to recipient species are based on the knowledge of parental chromosome complements and genomes. In this study, the genomes and chromosome complements of six distantly related Araceae plants (Cabrera *et al.* 2008) were characterized using flow cytometry, karyotype analysis and FISH with rRNA genes as probes in such detail that these data can be used for genome constitution analysis of their somatic hybrids. The aim of this chapter is to gather information that is useful for breeding programs.

Among all six Araceae genera, genomic DNA content was the highest in *A. andreanum*, *M. deliciosa* (5.27 pg/1C) and *S. wallisii* (7.39 pg/1C). *P. scandens* (1.74 pg/1C) and *Z. elliottiana* (1.35 pg/1C) had smaller genome sizes. In literature, Araceae genome size varies between 0.33 and 15.83 pg/1C. Small genomes of 0.60 pg/1C and 2.3 pg/1C for *Lemna* and *Xanthosoma* respectively and a big genome size of 15.00 pg/1C for *Orontium* were reported (Soltis *et al.* 2003). Genome sizes reported for *A. andreanum* Linden (4.49 pg/1C) by Bliss et al (2012), and *Spathiphyllum* (7.11 pg/1C) by Zhao *et al.* (2012) are in agreement with our results. However, for *Z. elliottiana* the total genomic content calculated in our study (1.35 pg/1C) was higher than reported by Ghimire *et al.* (2012; 0.59 pg/1C). Therefore, we also performed analysis using *Pisum sativum* L. 'Ctirad' as the reference plant for *Z. elliottiana* flow cytometric analysis and calculated the genome size as 1.30 pg/1C.

In our study, the genome size variability among Araceae genera did not correlate with karyotype asymmetry as it was demonstrated for Liliaceae (Peruzzi *et al.* 2009). *S. wallisii* had the highest genome size (7.39pg/1C) and the lowest karyotype asymmetry (0.70) while *P. scandens* had a relatively small genome (1.74 pg/1C) and the highest karyotype asymmetry (6.58). Based on this observation it might be suggested that there is a negative correlation between genome size and karyotype asymmetry in Araceae. However, for Z. *elliottiana* both a smallest genome size (1.35 pg/1C) and a low karyotype asymmetry (0.90) were observed.

Araceae vary in chromosome size, structure and basic number. The commonly known chromosome number for *A. andreanum* is 2n=30 (Marutani *et al.* 1993) which is in agreement with our results. However, there is a report of 2n=32 for *A. andreanum* (Sheffer and Croat, 1983). The karyotype of *A. andreanum* from our study and of *A. warocqueanum* in the study of

Kaneko and Kamemoto (1979) was similar, i.e. 2 pairs of large chromosomes, 1 pair of satellite chromosomes and 12 pairs of small chromosomes. However, the size of the chromosomes differed because they are different species. Additionally, the choice of the pretreatment, fixating agents and chromosome preparation techniques considerably influences the structure of chromosomes (Sharma and Bhattacharyya, 1961). We also observed one or two B-chromosome-like structures in *A. andreanum* in different spreads. It is possible that B-chromosomes are absent in some cells of the same species (Jones and Houben, 2003). The size of the B-chromosomes in *A. ochranthum* was smaller than the smallest chromosome while in *A. garagaranum* the B-chromosome had the same size as the smallest regular chromosome (Marutani *et al.* 1993). Further analyzes at meiotic stage are needed to confirm their presence and to exclude that they are broken chromosome arms or satellites.

In literature, different *M. deliciosa* chromosome numbers (2n=56, 58, 60 and 70) have been reported by different authors (Petersen 1989). Our counts for *M. deliciosa* 'Variegata' (2n=60) are in agreement with Marchant (1970). The varying chromosome numbers mentioned by different authors might be due to aneuploid derivations. The aneuploid chromosome number changes may result from chromosome losses or gains after meiotic irregularities (Petersen 1989). The higher chromosome numbers in *M. deliciosa* compared to other Araceae plants, might indicate either an ancient (allo)polyploidy origin of the genus or a high basic chromosome number.

A chromosome number of 30 was reported for *Spathiphyllum* (Marchant, 1973) which is the same as in our study. Our *P. scandens* results are in agreement with Marchant (1971b). For *Syngonium*, *Syngonium wendlandii* has a same chromosome number as *S. auretum* in our study, while other *Syngonium* species have varying chromosome numbers (Marchant, 1971b). In *Zantedeschia,* Marchant (1971a) reported a chromosome number of 32(2n). Karyotypic data obtained on *Z. elliottiana* by Ghimire *et al.* (2012) are not in agreement with our results. This could be mainly due to a poor quality of slide preparation, pictures, aceto-orcein staining and less repeats in their study. Moreover, DAPI staining (fluorescent) is preferred over other staining methods for species with small chromosomes as it can provide a stronger signal (Maluszynska, 2003, Van Laere *et al.* 2008).

The most common type of chromosome morphology was metacentric, followed by sub metacentric. Subtelocentric (3st) and telocentric (1t) chromosomes were observed in *P. scandens*. *M. deliciosa* (2n=60) showed the longest total chromosome length (147.14 ± 0.39 μm) while *P. scandens* (2n=32) showed the shortest length (44.64 ± 0.52 μm). *P. scandens* showed the highest asymmetry index as it contained subtelocentric and telocentric chromosomes. Karyotypic symmetry varies according to the presence of different chromosome types. A symmetrical karyotype mainly possesses metacentric and submetacentric chromosomes of approximately equal size whereas asymmetric karyotypes arise by shifts in centromeric

position towards the telomere, and/or by addition or deletion of chromatin in some chromosomes, which gives rise to size differences (Stebbins, 1971).

S. wallisii 'Domino' had the biggest genome size. However, the total chromosome complement was lower than in *A. andreanum* and *M. deliciosa*. *Z. elliottiana* had a higher chromosome length (73.19 ± 0.99 μm) than *P. scandens* and *S. auritum* although its DNA content was lower. A direct correlation between total chromosome complement and genomic content is reported (Cerbah *et al.* 2001, Zonneveld, 2004). However, negative correlation also has been reported (Van Laere *et al.* 2008).

DNA condensation varies among plants. For instance, in onion condensation was six times higher than in tomato (Khrustaleva and Kik, 2001). Van Laere *et al.* (2008) and Lysak *et al.* (1999) even reported varying genomic condensation differences among genera and subspecies as well as among accessions. They also proposed the geographical origin of the plants, even within species, as a probable cause for the differences. In our studies, *Z. elliottiana,* bearing chromosomes which are less condensed than other genera and is the only plant from South Africa, whereas all the other genera in this study originated in tropical America. *S. wallisii* chromosomes were quite compact compared to other genera tested. However, there is no clear proof yet that geographical origin plays a major role in DNA condensation.

No secondary constriction could be distinguished in our DAPI stained spreads. DAPI binds to A-T rich heterochromatic regions, whereas the nucleolar organizing region (NOR) is composed of G-C rich tandem repeats (Lima-de-Faria, 1976). Generally, 45S rDNA is associated with a NOR in eukaryotes and NOR is often positioned with a secondary constriction such as satellites (Roa and Guerra, 2012). Sometimes, these secondary constrictions are lost during slide preparation. There are few reports of 45S rDNA signal presence without satellites (Ricroch *et al.* 1992, Van Laere *et al.* 2008). In our study, 45S rDNA signals were observed in the short chromosome arms. This is in agreement with Lima-de-Faria (1976). 45S signals were localized near the centromere, except for *Z. elliottiana* signals that were present at the distal end of the short arms.

5S rDNA was not visualized through conventional FISH, except in *S. wallisii*. However, high sensitive Tyr-FISH was useful to visualize 5S rDNA in *Z. elliottiana.* Khrustaleva and Kik (2001) demonstrated that the signal sensitivity can be increased 100 times using Tyr-FISH which could be an explanation for the results obtained in our study with *Z. elliottiana*. This might be due to low copy number when used conventional FISH (Dagne *et al.* 2000) and needed signal amplification. Macas *et al.* (2002) mentioned satellite repeats could also undergo rapid changes in copy numbers. The signal intensity of the rDNA FISH depends on the number of repetitive units in the cluster, the chromosome compaction, the number of detection layers applied for signal amplification, and the type of hapten used for labeling (Chevalier *et al.* 1997). As it was reported earlier, the signal strength can also be related to

low copy number of rDNA (Dagne *et al.* 2000). According to Lapitan (1991), in plants, the copy number of the rDNA is rather high, between 600 and 8500 in a haploid genome. Although FISH is not a completely quantitative technique, the intensity of the signal can be related to the copy number (Leitch and Heslop-Harrison, 1992). Applying Tyr-FISH on other genera is still a perspective. The differences in 45S and 5S rDNA position can contribute to the distinction of somatic hybrids from parent genomes using FISH.

2.5 Conclusion

We have established a general protocol for karyotyping and FISH in Araceae genera *A. andreanum*, *M. deliciosa, P. scandens, S. wallisii*, *S. auritum* and *Z. elliottiana* that are potential parental plants in an intergeneric somatic hybridization breeding program. Moreover, a high sensitive Tyr-FISH protocol to locate 5S rDNA genes has also been established for *Z. elliottiana*. Cytological analyses have been performed to measure genome sizes. Our results give a clear view on the cytogenetic differences among genera within Araceae. Our optimized protocols can be a basis for GISH analysis in the characterization of regenerated protoplast fusion products.

explants UV-rays PP/mL scalpel/pinsets centrifugation

rhodamine FDA PP concentraion MS salts LMPA

2,4-D friable calli organic acids conditioned media NAA

driselase alginate osmotic solution amino acids +/- NH_4^+

mannitol KM salts AC/DC TDZ cell wall

macerase culture mthods PEG Pluronic® F-68 sterile

Chapter 3
Protoplast isolation, fusion and regeneration

nurse cells high hormone pulse laminar air flow cellulase

light high Ca/pH microcolony compact calli shaker

Tween 20 bead culture meristematic PP sorbitol PP fusion

two cell stage mesophyll PP PP alignment lamella donor PP

liquid over agar regenerative calli coconut water bovine serum

plasmolysed explant acceptor swing-out rotor millicell culture

Parts of this chapter have been published in:

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3.1 Introduction

Protoplasts are plant cells whose cell wall has been removed. They can theoretically be regenerated into whole plants under suitable physical and chemical conditions. One of the well-known manipulations of plant protoplasts is producing somatic hybrids between plants, mostly between two species or genera, which cannot be crossed sexually. In interspecific or intergeneric somatic hybridization, asymmetric fusion is preferred over symmetric fusion, as it generates less genome conflict between the two parent genomes and less unwanted genetic material is introduced into the acceptor genome.

Successful protoplast regeneration depends on physical factors such as protoplast source, density and culture method, and chemical factors such as composition of the medium. These factors vary according to plants as well as genotypes and are empirically optimized. Protoplast fusion, to obtain somatic hybrids, is often performed through chemical or electrical methods. To save time and efforts protoplast regeneration is generally optimized before fusion. However, complementary effects of fusion products are sometimes reported in literature. In order to optimize protoplast regeneration and asymmetric hybrid production protocol, in this study we aimed establishing calli and cell suspension system, isolated protoplasts from different explant sources, attempted asymmetric fusion through electrical and chemical fusion, various protoplast culture methods and chemical manipulations were tested.

Previously, Duquenne *et al.* (2007) attempted electrical fusion between protoplasts of *Spathiphyllum wallisii* leaves and *Anthurium scherzerianum* embryos. Microcalli formed, but did not regenerate further. Therefore, regeneration of protoplasts isolated from various *Anthurium, Spathiphyllum* and *Zantedeschia* explants, such as leaves, petioles, etiolated calli, anther calli, petiole calli, etc., was attempted. To this end, various callus types were established, including anther filament, petiole and etiolated internode calli from *Spathiphyllum* cultivars, and friable calli from *Zantedeschia* leaves, petioles and tubers as well as cell suspensions. Regeneration of protoplasts through various culture methods and systems was examined. Furthermore, asymmetric protoplast fusion was attempted.

3.2 Materials and methods

All *A. andreanum* '061' was present in the ILVO collection. *S. wallisii* genotypes '6054', '6332', '6341', '6409', '6526' were provided and coded by Deroose Plants; 'Daniël' and 'Domino' were present in the ILVO collection. *Zantedeschia aethiopica* 'Green goddess', *Z. elliotiana* '068' and *Z. rehmanii* 'Universe' were provided by Sandegroup, the Netherlands.

The basal media for stock plants and calli [*Anthurium* stock plant culture media (PMAn) and *Anthurium* stock calli culture media (CMAn), *Spathiphyllum* stock plant culture media (PMSp) and *Spathiphyllum* stock calli culture media (CMSp), *Zantedeschia* stock plant culture media (PMZn) and *Zantedeschia* stock calli culture media (CMZn)] are presented in Table 3.1.

Table 3.1 Basal media for stock plants and calli of *Anthurium andreanum, Spathiphyllum wallisii* and *Zantedeschia spp.*

Components	Stock plants			Stock calli		
	PMAn	PMSp	PMZn	CMAn	CMSp	CMZn
MS macro and micro elements	1x	1x	1x	1x	1x	1x
MS vitamins	1x	-	1x	1x	1x	1x
MES monohydrate (mg/L)	-	-	-	-	1065	-
Casein hydrolysate (mg/L)	-	-	-	-	100	-
Sucrose (g/L)	20	30	30	20	30	30
BA (mg/L)	-	2.5	2.5	1	0.2	0.1
2iP (mg/L)	1	-	-	-	-	-
NAA (mg/L)	-	-	-	1	-	-
2, 4-D (mg/L)	-	-	-	-	4	3

All tissue culture media, used for stock plant material maintenance, were supplemented with 0.7% plant tissue culture agar (Lab M Limited, UK) unless mentioned otherwise; the pH was adjusted to 5.8. They were sterilized by autoclaving at 121°C, for 30 min, at 500hPa pressure. All the stock solutions and sterilized media were stored in the refrigerator (4°C). The stock plant cultures were maintained in Meli jars containing 100 mL solidified medium in a culture room of 23 ± 2°C under a 16 h photoperiod at 54 μ mol m^{-2} s^{-1} photosynthetic active radiation, supplied by cool white fluorescent lamps (Philips 35W/840). All the calli initiations and cultures were performed in 90x15 mm Petri dishes (unless mentioned otherwise) in dark in cuture room conditions. Etiolated plants in Meli jars were maintained in the culture room in darkness. The proliferated shoots and calli were transferred to new medium every 6 weeks. All Petri dishes were sealed with Parafilm. One way-analysis of variance (ANOVA) was performed using Statistica 11.0. Mean separation was accomplished using the Duncan test (p=0.05).

3.2.1 Preparation of material for isolation

All explant materials, species and genotypes used for protoplast isolation and regeneration are shown in Table 3.2.

Table 3.2 Cultivars and explants used for protoplast isolation and regeneration

	Anthurium andreanum	*Spathiphyllum wallisii*	*Zantedeschia*
Plant organs	-	Leaves[3,4]	Leaves[5,6,7]
	-	Petioles[3]	Petioles[5]
	-	Meristematic shoots[3,4]	Tuber[6]
Calli material	Leaf calli[8]	Petiole calli[3,4]	Leaf calli[5]
	-	Etiolated internode calli[3,4]	Tuber calli[6]
	-	Anther filament calli[1,2]	-

[1] '6526'; [2] '6527'; [3] 'Daniël'; [4] 'Domino'; [5] *Z. rehmanii* 'Universe'; [6] *Z. elliotiana* '068',
[7] *Z. aethiopica* 'Green Goddess' and [8] '061'

For the induction of *A. andreanum* leaf calli and *S. wallisii* anther filament calli, protocols described by Hamidah *et al.* (1997b) and Werbrouck *et al.* (2000), respectively, were used (described in 3.2.1.1 and 3.2.1.2). For calli induction from other explant types, we started several experiments. Hundred explants (5 explants/90x15 mm Petri dish x 5 repeats x 4 experiments) were tested for each cultivar in all tests.

3.2.1.1 Callus induction from *A. andreanum* leaf explants

A. andreanum in vitro leaves were cut across the midrib and placed on CMAn and cultured in a culture room. Calli formed were placed on new media after every 6 weeks (Hamidah *et al.* 1997b).

3.2.1.2 Callus induction from *S. wallisii* explants

Filament calli (Werbrouck *et al.* 2000)

Inflorescences of '6526' and '6527' were cut when the spathum was still tightly closed. They were rinsed in 70% ethanol and sterilized for 15 min in a 10% NaOCl solution with two drops of Tween 20 and finally rinsed three times in sterile distilled water. The spadix was excised and the dissected anthers with filaments were cultured on media containing Murashige and Skoog (1962) macroelements, Nitsch (1969) microelements, 1.2 μM thiamine HCl, 230 μM NaFeEDDHA, 555 μM myo-inositol, supplemented with 4% sucrose, 0.25% gelrite, 2.5 μM TDZ and 10 μM 2, 4-D in 60x15 mm Petri dishes. After 6 weeks, the protrusions were transferred to the same medium but with 0.2 μM TDZ and 10 μM 2, 4-D in 90x15 mm Petri dishes.

Petiole and etiolated internode calli

Petioles and etiolated internodes of *in vitro S. wallisii* cultivars 'Daniël' and 'Domino' were cut into 0.5 cm length explants and cultured on half strength MS medium with 3% sucrose and different compositions of TDZ, 2iP and 2, 4-D (Table 3.3).

3.2.1.3 Friable callus induction from *Zantedeschia* explants

Z. elliotiana '068' and *Z. rehmanii* 'Universe' tubers from *in vitro* plants were cut into 10 x 10 mm pieces and cultured on CMZn with various TDZ and 2, 4-D combinations as shown in Table 3.4. *Z. rehmanii* 'Universe' leaf blades were cut with a sterile scalpel along with the vein while the midrib was still attached. These wounded explants were cultured on different media same as used for *S. wallisii* petioles (Table 3.3). Friability was evaluated by crushing the calli and cell separation using a sterile pincet. The color was compared to white paper.

3.2.1.4 Establishment of the *Zantedeschia* suspension culture

One gram of friable *Z. rehmanii* 'Universe' leaf calli and *Z. elliotiana* '068' tuber calli were separately chopped into small pieces and suspended in 10 mL of liquid CMZn containing 2

mg/L TDZ and 1 mg/L 2, 4-D in a sterile Erlenmeyer flask. The cell cultures were shaken on a rotary shaker at 120 rpm in darkness at 28°C. After 5 days, the suspension was filtered through a 400 μm pore width SEFAR nylon sterile filter to remove cell clumps. Ten milliliters of 3 x 10^5 cells/mL suspension was added to 40 mL fresh media in sterile Erlenmeyer flasks. The suspensions were kept on a rotary shaker at 60 rpm in darkness at 28°C for further use. The culture was refreshed each week by repeating the last step.

3.2.2 Protoplast isolation

3.2.2.1 Differentiated tissue

Plant organs (4 weeks old) mentioned in Table 3.2 above were chopped and pre-incubated for 1 h in 0.5 M mannitol solution in a 90 x 15 mm Petri dish. After the pre-incubation, the mannitol solution was replaced by 15 mL mixture of 1.5% Cellulase Onozuka R-10 (Duchefa Biochemie BV, the Netherlands), 0.5% Macerase R-10 (Duchefa Biochemie BV, The Netherlands) and 0.5% Driselase (Sigma-Aldrich, Belgium) dissolved in 0.5 M mannitol solution (pH = 5.5) (Duquenne *et al.* 2007). The enzyme solution was sterilized through a nylon filter of 0.22 μm pore size. The suspension was incubated at 23°C on a rotary shaker at a constant speed of 50 rpm in darkness. After 16h, the digest was passed through a sterile nylon filter with 100 μm sized pores and collected in a test tube. The filtrate was centrifuged in a swingout rotor (100 g, 10 min). The pellet was dissolved in 10 mL flotation medium (0.6 M sucrose and 3mM MES, pH 6). The protoplasts were separated from debris using density gradient centrifugation (100 g, 10 min) after gently layering 1 mL rinse medium (0.5 M sorbitol, 10 mM $CaCl_2$, 10 mM MES, pH 6) onto this flotation medium. The floated protoplasts were transferred into a fresh tube and washed with rinse medium to remove the remaining debris and enzymes by centrifuging again (100 g, 10 min). The protoplast pellet was resuspended in 0.5 M mannitol solution. The protoplasts were counted using a Burker counting chamber. At least four repeats were performed for each test.

3.2.2.2 Calli

Calli explants of about 5-6 weeks of age were used. Protoplasts were isolated using 0.5% cellulase, 0.3% macerase and 0.5% driselase. The rest of the procedure was similar to the above mentioned procedure (3.2.2.1) except that the digest filtration was done using a sterile nylon filter with a 50 μm pore size.

For *S. wallisii* filament calli, 1 g fresh weight (FW) calli was suspended in 5 mL enzyme mix in 60x15 mm Petri dishes and the protoplasts yield after 16, 18, 20 and 22 h incubation was tested. A minimum of four repeats were performed for each test.

3.2.3 Protoplast fusion

Protoplast fusion, both electrical and chemical, was attempted using *S. wallisii* 'Daniël' and *Z. rehmanii* 'Universe' protoplasts. Protoplasts were stained either with fluorescein diacetate

(FDA) or rhodamine β isothiocyanate (Rho) (Sigma-Aldrich). Stock solutions of 5 mg FDA/mL acetone and 30 mg Rho/mL acetone were prepared. To stain protoplasts, 100 µL FDA and Rho stock solutions were added separately to 5 mL of 10^5 PP/mL solutions. After 1 min incubation, excess stain was removed by washing three times in 0.5 M mannitol medium. Donor protoplasts (*Z. rehmanii* 'Universe') were irradiated under UV light (254 nm, 5 min) generated by a 13.4 W G30T8/OF, Sylvania lamp and fused with *S. wallisii* 'Daniël' (Table 3.5).

3.2.3.1 Electrical fusion

Fusion was performed using an Eppendorf Multiporator®. Protoplasts of *S. wallisii* 'Daniël' and *Z. rehmanii* 'Universe' were mixed in a 1:1 (10^5 PP/mL) ratio in 0.5 M mannitol solution containing 1 mM $CaCl_2.2H_2O$ and MES (pH 5.8). Fifty microliters of the mixture were pipetted between the electrodes of the micro-fusion chamber. A 70, 80, 90 and 100 V/cm alternating current (AC) was applied for a 30, 40, 60 and 90 s for protoplast alignment. Two direct current (DC) pulses of 3000, 4000 or 5000 V/cm for 40 µs were applied to induce membrane fusion. The fusion process was microscopically observed to analyze the alignment process and the fusion. Fused protoplasts were regenerated.

3.2.3.2 Chemical fusion

Equal volumes of two protoplast populations (10^5 PP/mL), stained differently as described above (3.2.3), were mixed together and allowed for 5 min to settle down at the bottom of a Petri dish (60 x 15 mm). Fifty microliters of PEG solution (89.6 mM PEG 3350 + 10.2 mM $CaCl_2.2H_2O$ + 735 µM KH_2PO_4 + 7.8 mM DMSO and 0.5 M mannitol, pH 5.5) were first added around the protoplast mix droplet and subsequently in the center; then the whole was gently mixed. The mix was incubated for 1 min to stimulate protoplast agglutination (Fig. 3.2 A,B). A high pH solution (0.5 M mannitol + 100 mM glycine; pH 10.5) and a calcium chloride solution (0.5 M mannitol + 100 mM $CaCl_2.2H_2O$) were prepared on beforehand and mixed freshly (1:1). This mix (50 µL) was added to the protoplast mix droplet to induce fusion. The fusion events were monitored under a fluorescence microscope (Leica DM IRB, DFC320 Leica Camera System). FDA stained protoplasts were green while rhodamine stained protoplasts fluoresced red. After 5 min incubation, the protoplasts were suspended for 15 min in liquid culture medium and washed 3 times by centrifuging (100 g, 10 min) and subsequently suspended in liquid culture medium.

3.2.4 Protoplast regeneration

The standard protoplast culture medium was based on Kao and Michayluk (1975) and supplemented with 0.09 M sucrose, 0.31 M mannitol, 0.45 µM 2, 4-D, and 2.22 µM BAP and 2.69 µM NAA. Different methods and media compositions were used to test protoplast regeneration capacity (Table 3.5). Vitamin solutions were prepared separately, sterilized using 0.22 µm pore width sterile filters and added to media at room temperature. The pH of

the media was corrected to 5.6. The standard protoplast concentration was 10^5 PP/mL irrespective of the culture method. In the beginning of each experiment, 0.31 M mannitol and 0.09 M sucrose were present in the culture medium. After 7 days, the mannitol concentration was reduced to 0.16 M. After seven more days, all mannitol was removed from the media. The cultures were refreshed with new medium on a weekly basis. All regeneration experiments were classified according to culture types, chemical manipulations and explant sources. All protoplasts were cultured in 60 x 15 mm Petri dishes (unless mentioned otherwise). The Petri dishes were sealed with Parafilm and incubated either in darkness or light at 23°C. More information on regeneration system attempted is given in Table 3.5. Each test had a minimum of 5 repeats.

3.2.4.1 Culture types

Liquid culture

Protoplasts were adjusted to the desired concentration and suspended in 5 mL liquid culture media in Petri dishes (Fig. 3.1 A). At the end of each week, the protoplasts were centrifuged (100 g, 10 min) or sedimented in a 50 mL falcon tube bottom and the media were replaced.

Liquid over agarose

Four mL protoplast culture media solidified with 0.7% agar was dispersed in Petri dishes. Subsequently, 3 mL protoplast suspension in liquid media was pipetted onto the solidified media (Fig. 3.1 B).

Agarose beads in liquid culture

For making immobilized agarose beads, the basal medium was supplemented with 0.6% low melting point agarose (LMPA), autoclaved and cooled to room temperature. The protoplasts were mixed with LMPA medium (1:1), so that the mixture contained the desired protoplast concentration. Droplets of 50 or 100 µL containing protoplasts were dispensed into the Petri dishes. The solidified droplets were covered with 5 mL liquid protoplast culture medium (Fig. 3.1 C).

Calcium alginate beads culture

The protoplast suspension was mixed (1:1) with alginate solution (40 g/L Na-alginate + 4.26 g/L MES + 0.25 M mannitol, pH 5.6). The mixture was dropped from a height of 5 cm into 5 mL of $CaCl_2$ solution (5.88 g/L $CaCl_2.2H_2O$ + 4.26 g/L MES + 0.25 M mannitol, pH 5.6) into the Petri dishes. After the drops had solidified, the $CaCl_2$ solution was removed and replaced by culture medium (Fig. 3.1 D).

Other tests

Light conditions were tested by culturing the Petri dishes in dark, partial light (28 µ mol m^{-2} s^{-1}; Philips 35W/840 lamp) (covered with white paper) and normal light conditions during two days or the complete culture period. Slicing LMPA beads and culture in glass Petri dishes (35 x 10 mm, Greiner bio-one) was also attempted.

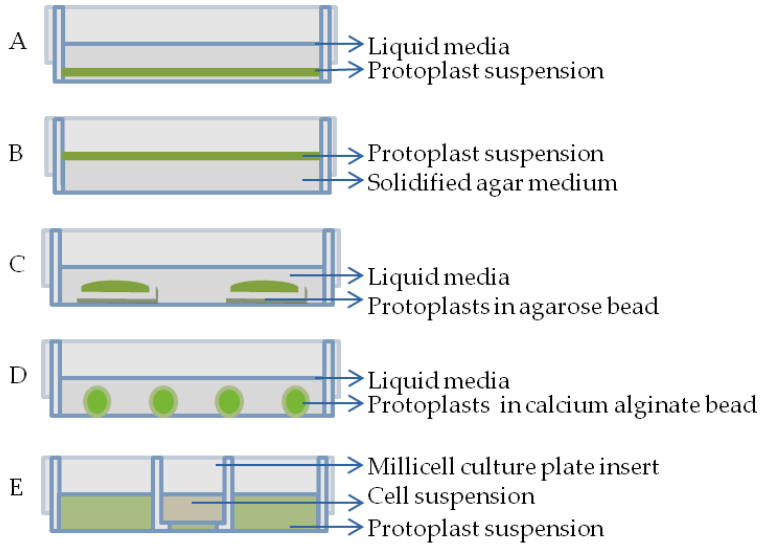

Fig. 3.1 Attempted protoplast culture methods (A) Liquid; (B) Liquid over agar; (C) Agarose bead; (D) Ca-alginate bead and (E) Nurse culture

3.2.4.2 Chemical manipulations

Conditioned media

Feeder solution experiments were performed with 5-day-old *Z. rehmanii* 'Universe' cell suspensions (3.2.4.1). Most cells were removed by centrifugation (100 g, 10 min) and the supernatant was filter-sterilized through a 0.22 µm pore width sterile filter. Conditioned media were mixed with standard culture media. The mix contained 0, 17, 33, 50, 67, 83 or 100% conditioned medium. Protoplasts (10^5 PP/mL) embedded in agarose beads were covered with 6 mL media mix.

Nurse culture

For the nurse cell culture, 5 mL protoplast suspension was poured into a Petri dish. A Millicell® culture plate insert (0.45 µm pore width cellulose filter, 30 mm diameter) was soaked in protoplast culture medium for 30 min and approximately 3, 5, or 10 x 10^3 cells/mL of suspension cells (3 mL) were poured onto the filter inside the insert (Fig. 3.3 E). Also in other tests, 10^3 and 10^5 PP/mL *Z. rehmanii* 'Universe' protoplasts were used as nurse cells and suspended in liquid media around agarose beads containing *S. wallisii* protoplasts.

Other tests

The following carbon sources were tested: sucrose (0.06 and 0.09 M) mannitol (0.08, 0.17, 0.25 and 0.40 M) and glucose (0.08 and 0.17 M). In other test, the protoplasts were cultured in 0.09

M sucrose until the formation of four cell stage. After the four cell stage was observed, 0.08 M glucose was provided (Eeckhaut *et al.* 2008). The following parameters were tested: protoplast density (0.2, 0.3, 0.5, 1, 5 x 10^5 PP/mL), different basal media, phytohormones, complex compounds (organic acids, bovine serum and coconut milk), oxygen carriers and surfactants (0.01, 0.1 and 1% Pluronic® F-68; 0.05, 0.1 and 1% Tween 20) and ammonium concentration (Table 3.5).

3.2.4.3 Explant sources

Different explant sources and genotypes were tested (Table 3.2). Plants were precultured for 24h in liquid basal medium (PMSp) containing 13, 26, or 39 µM NAA, 11.3, 22.6 or 33.9 µM 2,4-D or 11.1, 22.2 or 33.3 µM BAP (Table 3.1) under culture room conditions (see 3.2) and used for protoplast isolation. As an alternative to chopping, protoplasts were isolated from leaves sliced in the direction of the veins while the midrib was still attached and incubated in enzyme solution for protoplast isolation.

3.3 Results

3.3.1 Preparation of isolation tissue

The results from callus induction and suspension culture establishment in *A. andreanum*, *S. wallisii*, *Z. elliotiana* and *Z. rehmanii* are summarized in Fig. 3.2.

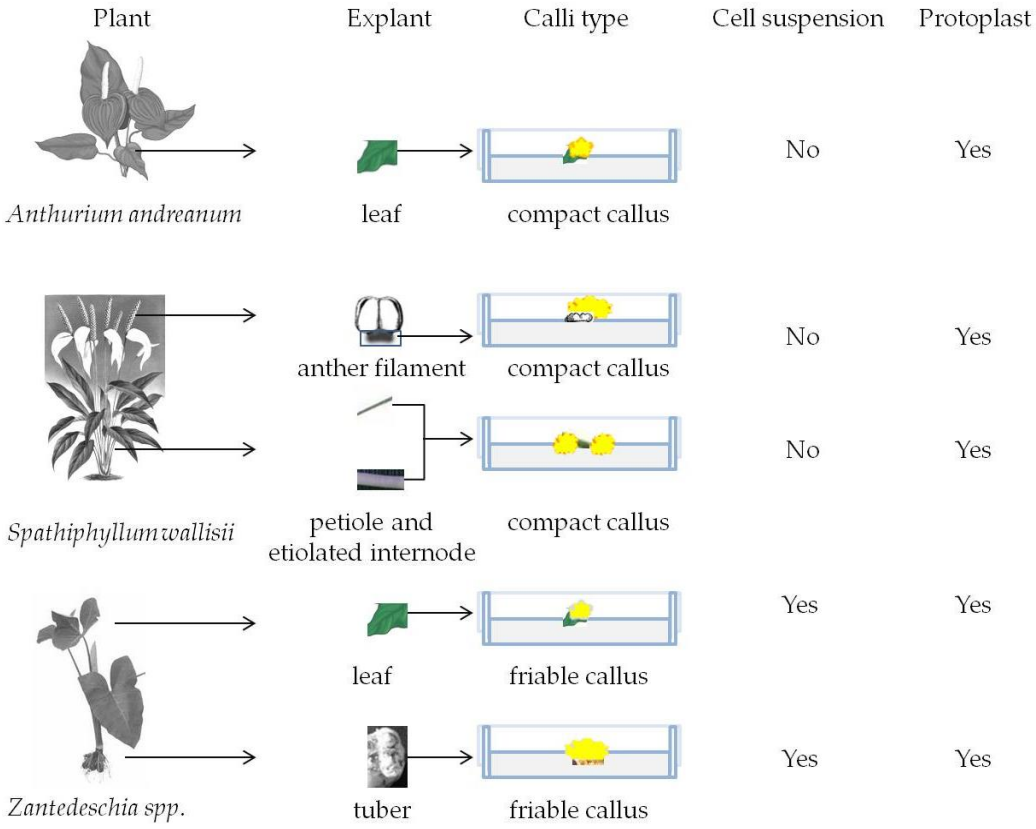

Fig. 3.2 The schematic summarizes the results from the establishment of callus and suspension system for protoplast isolation from *Anthurium andreanum*, *Spathiphyllum wallisii* and *Zantedeschia elliotiana* and *Z. rehmanii*.

Calli induced from *A. andreanum* and *S. wallisii* were used for protoplast isolation but did not yield cell suspension because of their high compact nature. *Zantedeschia spp.* produced non regenerative friable calli and suspension cells. However, suspension culture were not useful for protoplast isolation as they were not vigourously dividing. Furthermore, protoplasts were directly isolated form *Zantedeschia* friable calli. All the results are further detailed in the below sections.

3.3.1.1 Callus induction from *A. andreanum* leaf explants

Compact, yellow and regenerative calli were obtained (Fig. 3.3 A). These could only be used for protoplast isolation but not for suspension culture establishment.

3.3.1.2 Callus induction from *S. wallisii* explants

Filament calli

Anther filaments produced compact, creamy white and regenerative calli (Fig. 3.3 B). Because of the highly compact nature, these calli were not suitable to obtain suspension cells, hence petioles and etiolated internodes were used as explants for calli induction.

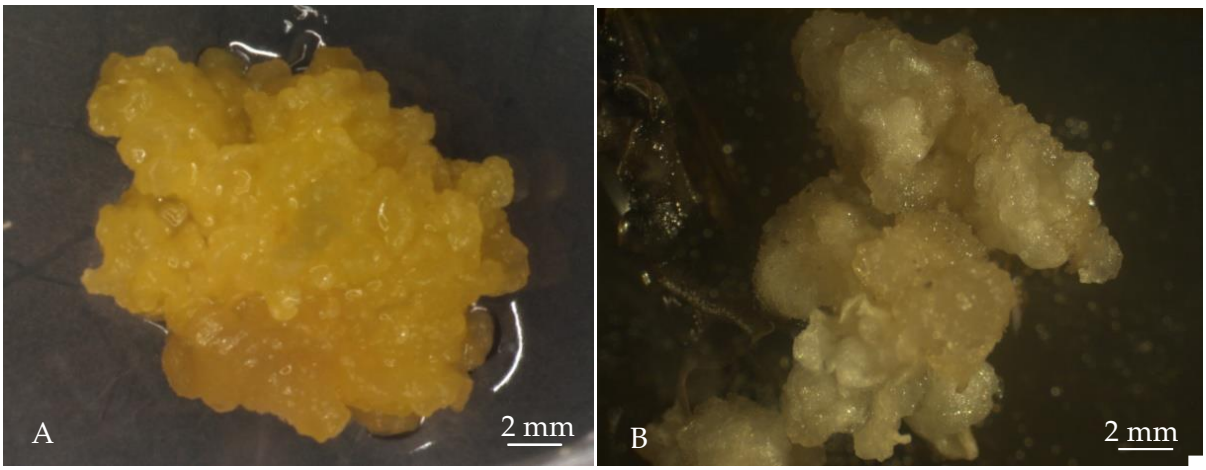

Fig. 3.3 Calli from (A) *Anthurium andreanum* '061' leaf and (B) *Spathiphyllum wallisii* '6527' anther filament

Petiole and etiolated internode calli

Between 16-68% of the 'Daniël' petioles treated with TDZ and 2, 4-D yielded calli. Only 16-40% of the 'Domino' petioles produced calli. 'Daniël' petioles regenerate best (68%) after a combination of 1mg/l TDZ and 0.2 mg/l 2, 4-D whereas 'Domino' petioles (40%) preferred 2 mg/l TDZ and 1mg 2, 4-D (Table 3.3). Shoots and roots formed directly on 2iP + 2, 4-D treated petioles without a callus phase, but 'Domino' etiolated internodes produced calli. The mean weight of the calli is was 0.25-0.77 g and 0.65-0.74 g for 'Daniël' and 'Domino' petioles respectively (Table 3.3). Internodes from the etiolated plants of both cultivars treated with TDZ and 2, 4-D efficiently transformed into calli (Table 3.3). The biggest calli were obtained from etiolated internodes from both cultivars, rather than from the petioles. The optimal

phytohormone concentration for calli weight differs for both cultivars. All calli are compact, creamy white colored and regenerative (Fig. 3.4 A, B). Calli induced from both explants were used for protoplast isolation.

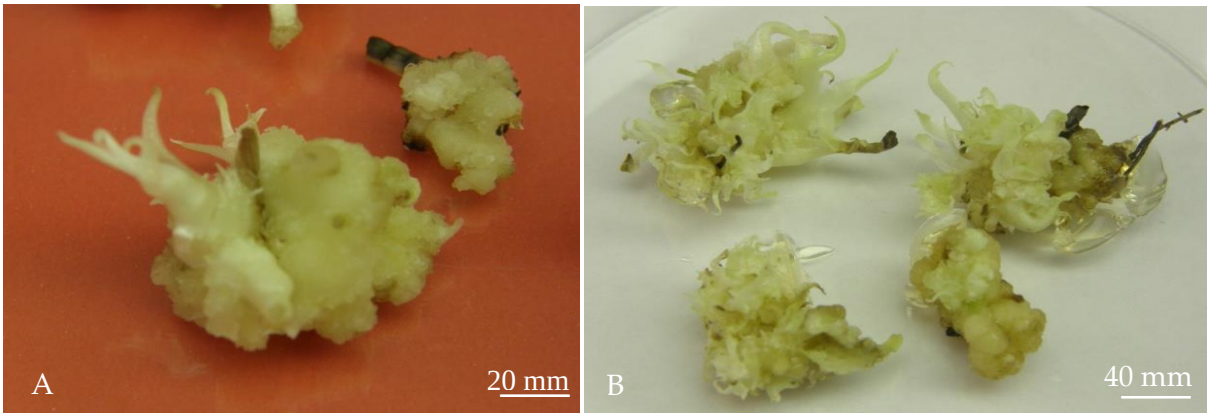

Fig. 3.4 Calli from *Spathiphyllum wallisii* 'Daniël' petioles (A) and 'Domino' etiolated internodes (B)

Table 3.3 Callus induction from *Spathiphyllum wallisii* petioles and etiolated internodes: mean explant weight and calli formation percentage. Significantly different means (n=10) are labeled per column (Duncan, $p<0.05$). Means followed by the same letter are not significantly different.

Phyto-hormonal combination	Petiole calli				Etiolated internode calli			
	'Daniël'		'Domino'		'Daniël'		'Domino'	
	Mean weight (g)	Calli %	Mean weight (g)	Calli %	Mean weight (g)	Calli %	Mean weight (g)	Calli %
0.2 mg TDZ + 0.1 mg 2,4-D	0.77±0.41 a	40	0.74±0.37 a	36	0.59±0.30 b	98	0.76±0.52 a	97
1 mg TDZ + 0.2 mg 2,4-D	0.70±0.31 a	68	-	16	0.58±0.25 b	97	1.02±0.45 a	98
2 mg TDZ + 1 mg 2,4-D	0.25±0.05 b	16	0.65±0.45 a	40	1.09±0.32 a	96	0.90±0.30 a	97
2 mg 2iP + 2 mg 2,4-D	0 b	0	0 b	0	0 c	0	-	30

- not available; calli dried;
Calli % - calli formation percentage
Data are means ± SE.

3.3.1.3 Callus induction from *Zantedeschia* explants

In Table 3.4, Fig. 3.5 A, B and Fig. 3.6 A, the calli types formed from *Z. rehmanii* 'Universe' tubers are presented. *Z. elliotiana* '068' tuber explants formed 40% highly friable calli on 2 mg/L TDZ + 1 mg/L 2, 4-D enriched medium; the other combinations did not induce any calli. Friable calli were further used for protoplast isolation and suspension culture.

Table 3.4 Callus induction from *Z. rehmanii* 'Universe' tubers

CMZn +		Callus type
TDZ mg/l	2,4-D mg/l	
0.25	1	Yellow; friable
0.5	1	Yellow turned brown
1	1	Yellow; friable
2	1	Brown
0.25	2	Brown
0.5	2	Brown
1	2	Yellow; slightly friable
2	2	Yellow; slightly friable
0.2	0.1	Compact calli
1	0.2	No calli; direct shoot formation

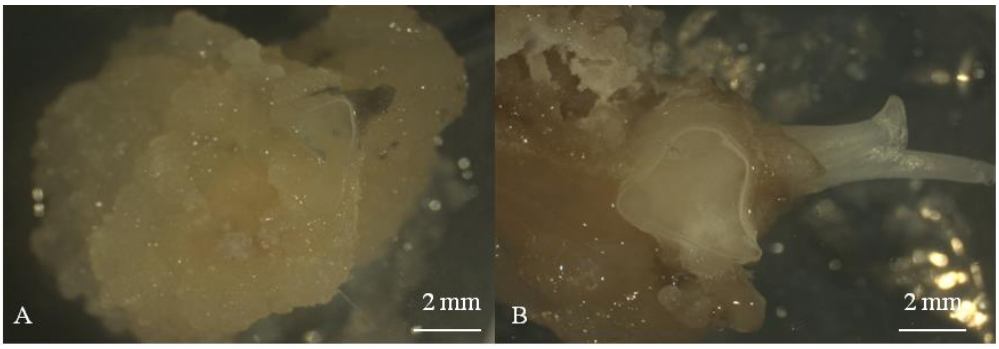

Fig. 3.5 Friable calli induced from *Zantedeschia rehmanii* 'Universe' tubers (A); direct shoot formation (B)

3.3.1.4 Establishment of the *Zantedeschia* suspension culture

Cells were separated in suspension media (Fig. 3.6 B). Tests with both leaf calli and tuber calli failed to yield continuously dividing cells. These cell cultures were used as nurse cells or for conditioned media for protoplast regeneration experiments, rather than as protoplast sources.

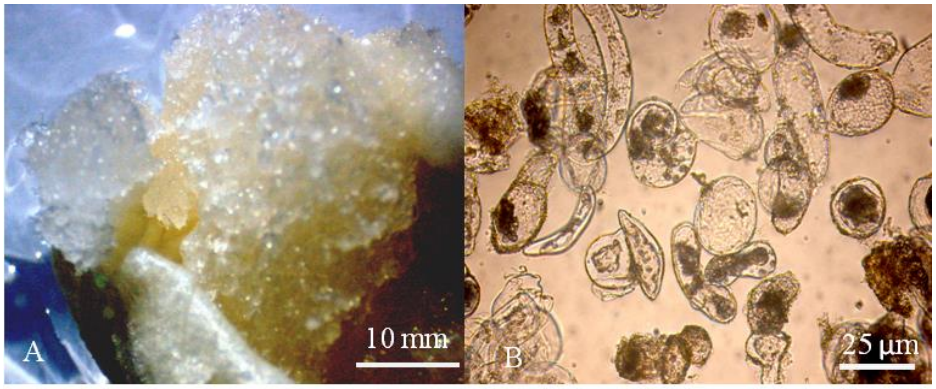

Fig. 3.6 *Zantedeschia rehmanii* 'Universe' leaves friable callus (A) and the derived cell suspension culture (B)

3.3.2 Protoplast isolation

3.3.2.1 Differentiated tissue

From both *S. wallisii* 'Daniël' and *Z. rehmanii* 'Universe' leaves, the obtained protoplast yield was about 10^6 PP/g FW and was not significantly different (Fig. 3.7). For both species, significantly fewer protoplasts were isolated from petioles. *S. wallisii* meristematic shoots yielded 0.9 x 10^6 PP/g FW, whereas the protoplast yield of *Z. elliotiana* '068' tubers was 0.42 x 10^6 PP/g FW.

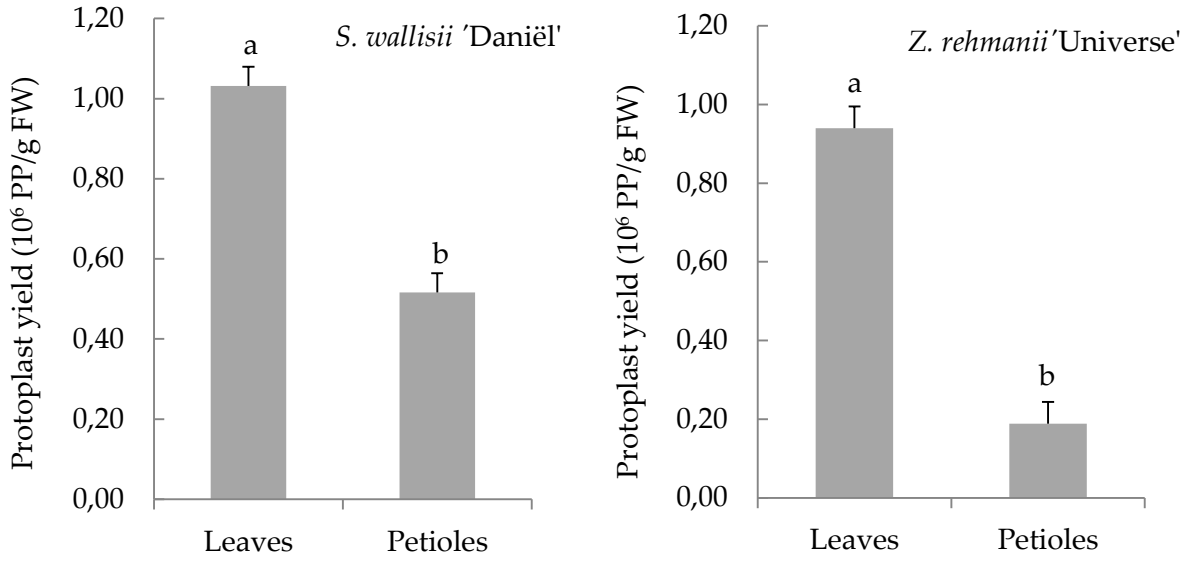

Fig. 3.7 Protoplast yield from *Spathiphyllum wallisii* 'Daniël' and *Zantedeschia rehmanii* 'Universe' leaves and petioles. (Data are means ± SE; n=5). Means indicated by the same letter are not significantly different (Duncan, $p<0.05$).

3.3.2.2 Calli

After 16 h incubation of *S. wallisii* '6527' embryogenic anther filaments, undigested cell clumps could be observed. Incubation was thus extended to 18, 20 and 22 hours. The highest yield of 1.3 x 10^5 PP/g FW was obtained after 18 h treatment. As the incubation times increased further, protoplast counts significantly decreased (Fig. 3.8). When etiolated internode calli were used, a maximum yield of 5 x 10^5 PP/g FW and 3.2 x 10^5 PP/g FW was obtained for 'Daniël' and 'Domino', respectively. Petiole calli resulted in significantly smaller yields for both genotypes (Fig. 3.9).

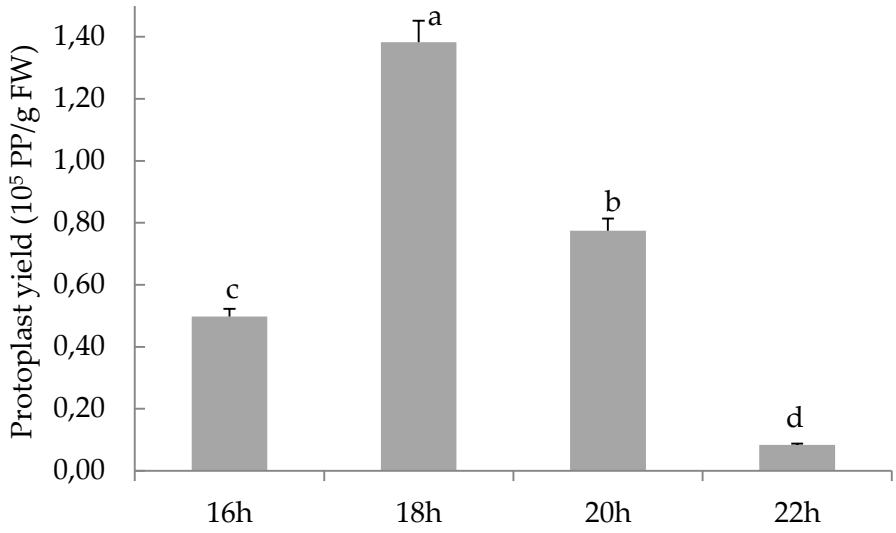

Fig. 3.8 Protoplast yield and enzyme duration effect in *Spathiphyllum wallisii* '6527' filament calli (Data are means ± SE; n=4). Means indicated by the same letter are not significantly different (Duncan, $p<0.05$).

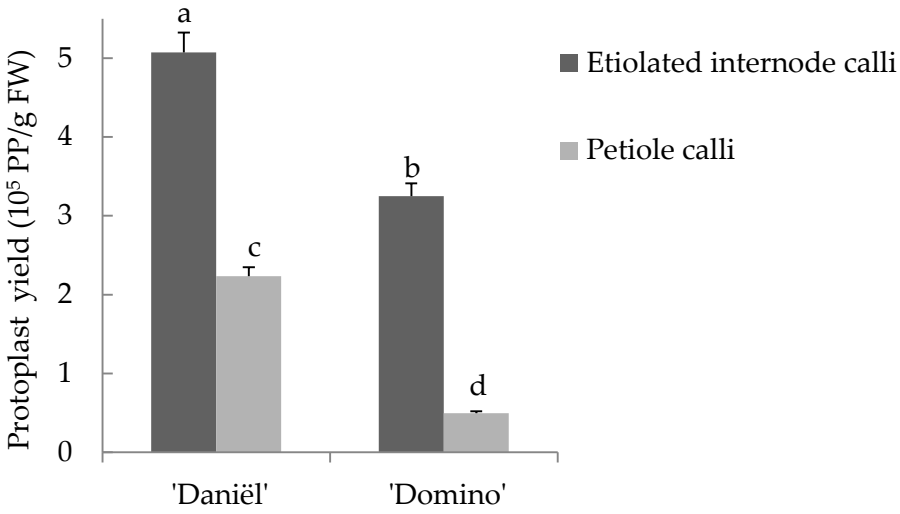

Fig. 3.9 Protoplast yield from etiolated internode and petiole calli of S*pathiphyllum wallisii* 'Daniël' and 'Domino' (Data are means ± SE; n=5). Means indicated by the same letter are not significantly different (Duncan, $p<0.05$).

A. andreanum '061' leaf calli protoplast isolation yielded about 10^5 PP/g FW. *Z. rehmanii* 'Universe' leaf calli and *Z. elliotiana* '068' tuber explants yielded the lowest protoplast amounts, 7.7 x 10^3 and 9.1 x 10^3 PP/g FW, respectively.

In all the species used in our experiments, higher amounts of protoplasts can be isolated from leaves, as compared to petioles and calli explants. *S. wallisii* meristematic shoots yielded approximately the same protoplast number as leaves. Filament calli required a higher incubation period. The most efficient callus type for protoplast regeneration was derived from *S. wallisii* etiolated internodes. *Z. elliotiana* '068' tubers yielded more protoplasts than the calli induced from them.

3.3.3 Protoplast fusion

3.3.3.1 Electrical fusion

A 80-90 V/cm alternate current (AC) 60 sec pulse aligned the protoplasts (Fig. 3.10 A). Two 3000 V/cm direct current (DC) pulses for 40 μs were applied to induce fusion (Fig. 3.10 B).

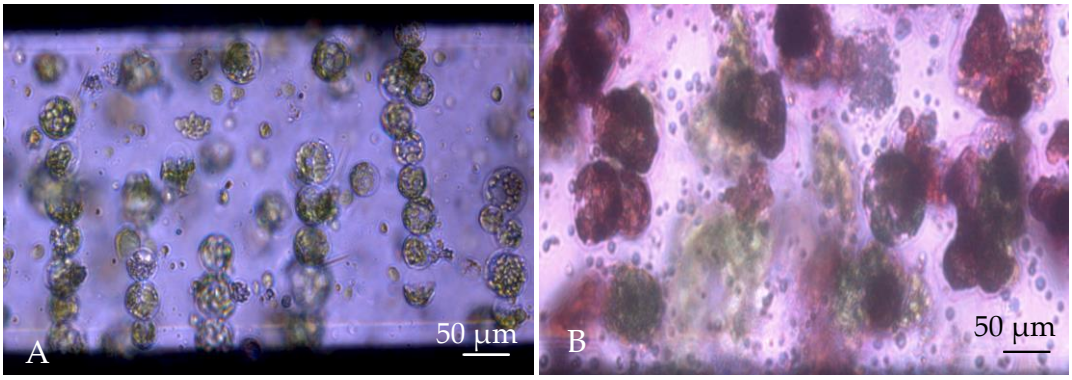

Fig. 3.10 Electrical fusion (A) Alignment of protoplasts after alternate current (AC); (B) fused protoplast after direct (DC) pulse. Green: *Zantedeschia rehmanii* 'Universe' leaf protoplasts, Red: *Spathiphyllum wallisii* 'Daniël' leaf protoplasts.

3.3.3.2 Chemical fusion

Protoplast agglutination occurred after 1 min incubation in PEG solution (Fig. 3.11 A,B). After 5 min incubation in high pH and calcium chloride solution mix the complete fusion was observed (Fig. 3.11 C).

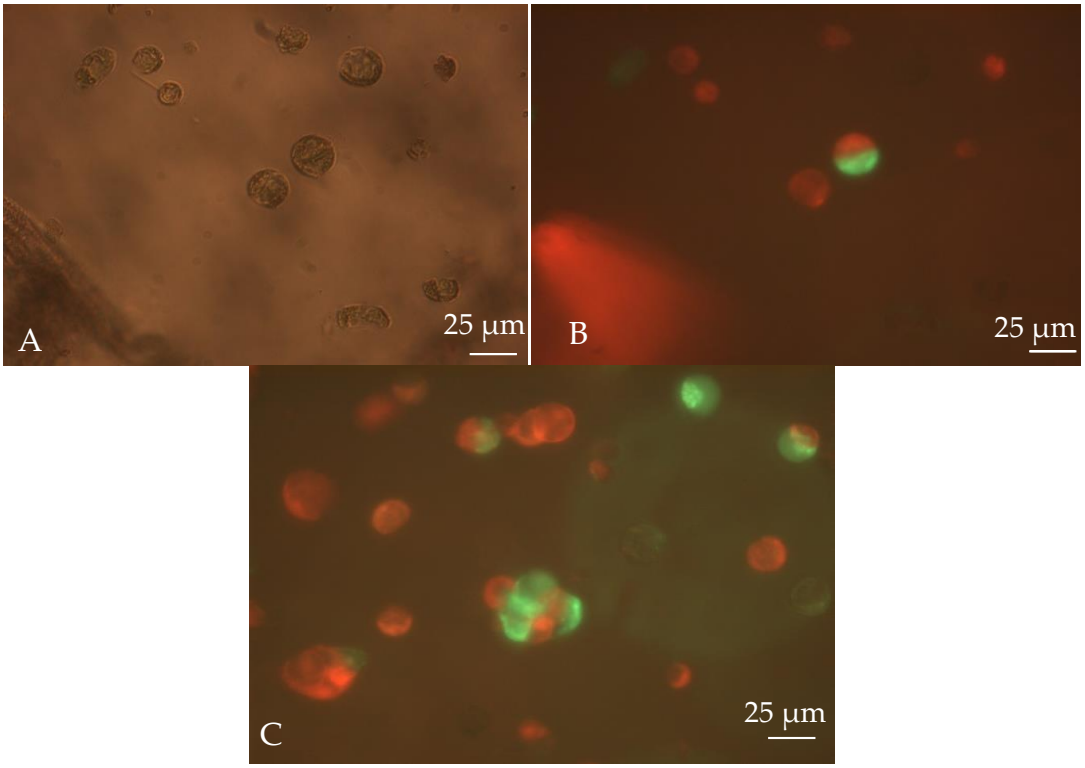

Fig. 3.11 Chemical fusion: (A) and (B) association of protoplasts; (C) fusion after adding high pH solution. Green: *Zantedeschia rehmanii* 'Universe' leaf protoplasts, Red: *Spathiphyllum wallisii* 'Daniël' leaf protoplasts.

3.3.4 Protoplast regeneration

Protoplast regeneration results are summarized in Table 3.5. The first cell division appeared 6-7 days after culturing. Four cell stage and microcolonies were formed after 4 and 6 weeks of culture, respectively (Fig. 3.12). No further division was observed. Both 0.09 M sucrose and 0.08 M glucose are suitable carbon sources. A protoplast density of 10^5 PP/mL was the most suitable to obtain microcolonies. KM salts supplemented with 0.45 μM 2, 4-D, and 2.22 μM BAP and 2.69 μM NAA favored microcolony formation. However, if Tween 20 or Pluronic® F-68 were added, microcolonies were observed within 2 weeks. Moreover, less protoplast agglutination and subsequent damage were observed in the liquid culture system in the presence of Tween 20 or Pluronic® F-68.

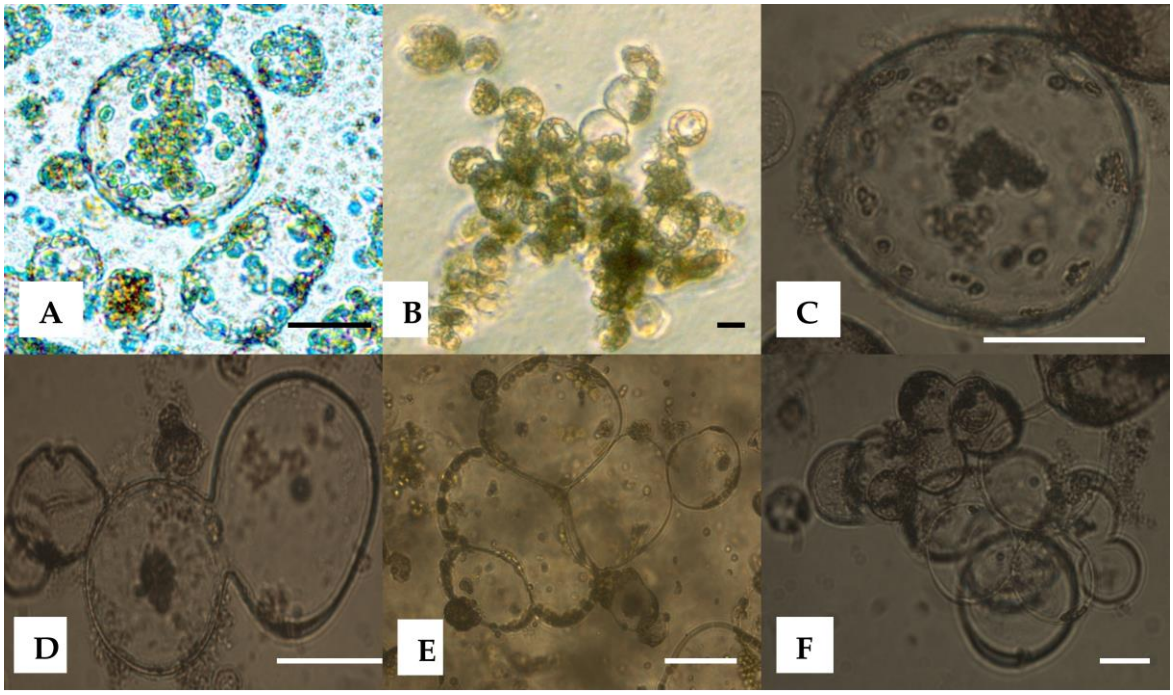

Fig. 3.12 Protoplast culture of *Zantedeschia rehmanii* 'Universe' leaf protoplasts in liquid medium: two cell stage (A) and microcolony (B); *Spathiphyllum wallisii* 'Daniël' petiole calli protoplast culture in medium enriched with 0.01% Pluronic® F-68: single protoplast (C), first division (D), second division (E) and microcolony (F). The bars represent 10 μm.

Conditioned media obtained from *Zantedeschia* suspension cell cultures could induce the second divisions but no further divisions were observed in *S. wallisii* calli protoplasts. First divisions were obtained when amino acids, coconut milk and calf serum used. No further growth was observed, however. Presence of NH_4NO_3 and KM organic acids is vital to obtain microcolonies as no protoplast divisions were observed in their absence. No other medium salts yielded microcolonies than KM salts for *S. wallisii* and *A. andreanum*. However, *Zantedeschia* protoplasts divided until the microcolony stage in either KM or MS salts enriched with KM organic acids. *A. andreanum* and *S. wallisii* cultivars yielded microcolonies in agarose beads culture in the dark, while *Zantedeschia* protoplasts divided in both liquid

and agarose beads in dark or light conditions. Among all the explants tested, *Zantedeschia* tubers or friable calli were not able to divide. Petioles from *S. wallisii* and *Zantedeschia* could only yield up to the four-cell stage. Asymmetric protoplast fusion products, obtained either through chemical or electrical fusion, did not divide.

Table 3.5 Protoplast regeneration experiments and their results in *Anthurium andreanum*, *Spathiphyllum wallisii* and *Zantedeschia spp.*

Genotype	Explant	Salts	Medium adaptations	Method	Light (h)
MICROCOLONY FORMATION					
Spathiphyllum wallisii 'Daniël'	Leaves	KM		Agarose beads	0
	Meristematic shoots	KM	Nurse culture; 0.1% Pluronic® F-68; 0.05% Tween 20	Agarose beads	0
	Petiole calli/Etiolated internode calli	KM	0.1% Pluronic® F-68; 0.05% Tween 20	Agarose beads	0
	Meristems/Etiolated internode calli/Leaves	KM	High hormone pulse pretreatment on explants	Agarose beads	0
Spathiphyllum wallisii 'Domino'	Leaves	KM		Agarose beads	0
	Meristematic shoots	KM	Nurse culture; 0.1% Pluronic® F-68; 0.05% Tween 20	Agarose beads	0
	Petiole calli/Etiolated internode calli	KM	0.1% Pluronic® F-68; 0.05% Tween 20	Agarose beads	0
Spathiphyllum wallisii '6526'	Filament calli	KM		Agarose beads	0
Spathiphyllum wallisii '6527'	Filament calli	KM		Agarose beads	0
Anthurium andreanum '061'	Leaf calli	KM	Nurse culture	Agarose beads	0
Zantedeschia elliotiana '068'	Leaves	KM/MS + KM OA		Agarose beads/ Liquid	0/16
Zantedeschia aethiopica 'Green Goddess'	Leaves	KM/MS + KM OA		Agarose beads/ Liquid	0/16

FOUR CELL STAGE					
Spathiphyllum wallisii 'Daniël'	Petiole calli/Etiolated internode calli/Leaves	KM	Conditioned media; nurse culture	Agarose beads	0
	Petioles	KM		Agarose beads	0
Spathiphyllum wallisii 'Domino'	Petiole calli/Etiolated internode calli/Leaves	KM	Conditioned media; nurse culture	Agarose beads	0
Zantedeschia rehmanii 'Universe'	Petioles	KM		Agarose beads	0
TWO CELL STAGE					
Spathiphyllum wallisii 'Daniël'	Leaves	KM	2.7 mM glutamine, 1.7 mM proline, 1% casein hydrolysate, 0.2% coconut milk, 0.4% calf serum	Agarose beads	0
Zantedeschia rehmanii 'Universe'	Leaves	KM	2.7 mM glutamine, 1.7 mM proline, 1% casein hydrolysate, 0.2% coconut milk, 0.4% calf serum	Agarose beads	0
	Leaves	KM		Liquid/Liquid over agarose	0
NO DIVISION					
Anthurium andreanum '061'	Leaf calli	KM	Explants treated on hormone free media	Agarose beads	0
Spathiphyllum wallisii 'Daniël'	Leaves/Etiolated internode calli	KM	1.36 or 4.52 μM 2,4-D or 0.54,1.61, 5.4, 10.74 or 16.13 NAA combined with 0.49, 1.48 or 4.92 μM 2iP or 0.44, 1.33, 4.44 or 13.32 μM BAP or 2.23, 4.46 or 6.69 μM KIN or 0.46, 1.37 or 4.56 μM zeatin	Agarose beads	0
	Leaves	KM	without NH_4NO_3	Agarose beads	0

	Leaves	KM	without KM OA	Agarose beads	0
	Leaves	MS/N6/B5 /LS/MS + KM OA/ MS macro + Heller micro		Agarose beads	0/16
	Leaves/Etiolated internode calli	KM/MS + KM OA		Liquid/Liquid over agarose/ Ca-alginate beads	0/16
Zantedeschia rehmanii 'Universe'	Leaves	KM	1.36 or 4.52 μM 2,4-D or 0.54,1.61, 5.4, 10.74 or 16.13 NAA combined with 0.49, 1.48 or 4.92 μM 2iP or 0.44, 1.33, 4.44 or 13.32 μM BAP or 2.23, 4.46 or 6.69 μM KIN or 0.46, 1.37 or 4.56 μM zeatin	Agarose beads	0
	Leaves	KM	without NH_4NO_3	Agarose beads	0
	Leaves	KM	without KM OA	Agarose beads	0
Zantedeschia rehmanii 'Universe'	Leaves	MS/N6/B5 /LS/MS macro + Heller micro		Agarose beads	0/16
	Leaf calli	KM		Agarose beads	0/16
	Leaves	KM/MS + KM OA		Ca-alginate beads	0/16

	Tubers	KM	Agarose beads/ Liquid	0/16
	Tuber calli	KM	Agarose beads/ Liquid	0/16
Zantedeschia elliotiana '068'	Leaf calli	KM	Agarose beads	0/ 16
	Tubers	KM	Agarose beads/ Liquid	0/16
	Tuber calli	KM	Agarose beads/ Liquid	0/16
Spathiphyllum wallisii 'Daniël' (A) + *Zantedeschia rehmanii* 'Universe' chemical fusion products (D) *	Leaves + leaves	KM	Agarose beads/ Liquid	0/16
Spathiphyllum wallisii 'Daniël' (A) + *Zantedeschia rehmanii* 'Universe' electrical fusion products (D) *	Etiolated internode calli + leaves	KM	Agarose beads/ Liquid	0/16

LS: Linsmaier and Skoog (1965); MS: Murashige and Skoog (1962); N6: Chu *et al.* (1975); B5: Gamborg *et al.* (1968)
KM OA: Organic acids Kao and Michayluk (1975)
*-asymmetric fusion products
A-acceptor parent; D-donor parent

3.4 Discussion

Somatic hybridization is an alternate method for overcoming sexual barriers in conventional breeding between two distant genera. Compared to symmetric fusion, asymmetric fusion introduces less undesired genes and thus less genome conflict in hybrid products. Somatic hybridization starts with protoplast source selection, isolation of protoplasts, fusion and complete regeneration. The main aim of this project is to produce aroid intergeneric asymmetric somatic hybrids. To reach the goal, we studied the influence of source material and isolated quality protoplasts from various sources. Subsequently, we performed asymmetric heterofusion and attempted regeneration in three aroid genera *Anthurium*, *Spathiphyllum* and *Zantedeschia.*

Calli were induced on *S. wallisii* petioles, etiolated internodes and anther filament calli. The calli type affected the protoplast yield. If highly compact calli such as filament calli were treated with enzyme solution for 18 h, the highest PP yield was obtained. When the duration was reduced, cells clumps were observed whereas long incubated cells had bursted. Fewer protoplasts were obtained from calli (10^5 PP/g FW) than from leaf explants (10^6 PP/g FW). Similar results were reported by Duquenne *et al.* (2007). Additionally, etiolated *S. wallisii* 'Daniel' and 'Domino' internode calli yielded more protoplasts (Fig. 3.8) than the filament and petiole calli obtained by Duquenne *et al.* (2007).

Aroid protoplast regeneration has been very difficult to attain. Murakami *et al.* (1995) reported low frequency and slow regeneration in taro. *A. andreanum* protoplasts did not regenerate after the first few divisions (Kuehnle, 1997), and *S. wallisii* and *A. scherzerianum* protoplasts did not divide beyond the microcolony stage (Duquenne *et al.* 2007). He *et al.* (1996) and Murakami *et al.* (1995) reported plant regeneration from suspension cell protoplasts of *Pinellia ternata* and taro, respectively. The higher protoplast regeneration ability might be due to the presence of more dedifferentiated cells and mitochondria than in the callus (Moreira *et al.* 2000). Therefore, as a first step towards suspension culture, a system to obtain regenerative and friable calli was attempted. However, *S. wallisii* etiolated stems or internode calli did not yield friable calli for suspension cells. Only *Z. rehmanii* and *Z. elliotiana* leaf and tuber explants yielded soft, friable, non regenerative calli. These calli yielded neither fast growing suspension cells nor regenerating protoplasts. The regenerative ability of callus or suspension cells is important to obtain plantlets from protoplasts (Murakami *et al.* 1995). But establishing a regenerative suspension culture is difficult and time consuming (Li *et al.* 2004). Mesophyll protoplasts are unsuitable for protoplast regeneration in *Cryptocoryne wendtii* De Wit (Pongchawee *et al.* 2007) and other monocots (Potrykus and Shillito, 1986; Vasil, 1988; Potrykus, 1990). A possible reason is the lack of callus differentiation capacity of mesophyll cells (Szcerbakowa *et al.* 2005). Embryogenic suspension cells are preferred over mesophyll cells as protoplast source in monocot species (Taylor *et al.* 1992; Nielsen *et al.* 1993; Wang *et al.* 1993; Chabane *et al.* 2007).

Actively dividing 'nurse' cells can sometimes promote protoplast regeneration by releasing growth factors such as amino acids. For example, the division of embryogenic rice cell suspensions protoplasts was most effectively stimulated by nurse cells of *Lolium multiflorum* (Jain *et al.* 1995). A nurse layer of tuber mustard cells significantly increases regeneration of cauliflower (Sheng *et al.* 2011) and red cabbage protoplasts (Chen *et al.* 2004a). Also the sustained division of banana protoplasts occurs exclusively when a feeder system is implemented (Xiao *et al.* 2007). The feeder layer may possibly have a signaling function in addition to providing nutrients. Similarly, a feeder solution or conditioned media increased protoplast division of *Chrysanthemum indicum* (Zhou *et al.* 2005), but only microcolonies were reported. Moreover, vigorously growing cell suspensions are often used as feeder layers and their efficiency is determined by their culture period and the release of more stimulatory substances in the medium that can initiate divisions in the more recalcitrant protoplasts (He *et al.* 2006). In our studies, only four cell stage or microcolonies were observed (Table 3.5). The slow growth of the suspension cells might make them unsuitable for nurse culture or conditioned media.

When the protoplasts were continuously cultured in a high concentration of osmotica, we observed flaccid and plasmolysed cells. As in Davey *et al.* (2004), the gradual reduction of osmoticum facilitated cell divisions.

Protoplast culture at low densities became possible using the Kao and Michayluk (KM, 1975) medium composition. Other media have also been derived from MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al.* 1968) formulations. Our results showed that KM encourages division up to the microcolony stage in all the plants tested. However, *Zantedeschia* could also divide in MS media enriched with KM organic acids. Yamada *et al.* (1986) reported addition of organic acids was necessary for microcolony formation which is in agreement with our results. Innovative approaches such as the addition of surfactants and artificial oxygen carriers have been detailed by Davey *et al.* (2005). When we used Tween 20 and Pluronic® F-68, microcolonies were observed within 2 weeks of culture instead of 4 to 6 weeks. Possibly these surfactants stimulate cell growth by better uptake of nutrients, hormones and oxygen. However, they did not enable sustained cell division.

Complex undefined medium compiled by adding coconut milk, albumin serum or conditioned media obtained from cells were reported in literature (Kao and Michayluk, 1975; Gleba *et al.*, 1982; Chabane *et al.* 2007; Rizkalla *et al.* 2007). Yamada *et al.* (1986) reported that addition of calf serum improved division frequency and subsequent regeneration of rice protoplasts. Beneficiary effects of adding casein hydrolysate, coconut water as well alanine, proline and glutamine in maize protoplast culture media have been reported (Imbrie-Milligan *et al.* 1987). In our crops, addition of amino acids, calf serum and coconut milk did not improve microcolony formation.

Inhibitory effects of NH_4^+ on protoplast culture were reported and reduction or complete removal had been applied (Kao and Michayluk, 1975; Toriyama and Hinata, 1985, Yamada *et al.* 1986; Imbrie-Milligan *et al.* 1987). Yin *et al.* (1993) found NH_4^+ to be necessary for protoplast division and callus formation in *Oryza sativa* L. Likewise, our results indicate that NH_4^+ removal inhibits protoplast division.

Plant hormones are important parameters for protoplast regeneration. Typically, auxin and cytokinins are necessary for sustained divisions (Davey *et al.* 2005). A 2, 4-D shock was essential for plant regeneration of sunflower protoplasts (Taski-Adjukovic *et al.* 2006). Endogenous hormones can interact with exogenously applied plant growth regulators, as demonstrated by the different reaction of multiple explants types on various phytohormone treatments (Sun *et al.* 2005c). High pulse hormone shock treatments applied to Araceae explants to increase endogenous hormonal level did not promote sustained division after microcolony formation. Furthermore, protoplasts isolated from different explants did not divide continuously.

Protoplast clumps were formed in liquid protoplast culture. This might be due to the formation of middle lamella with adjacent protoplasts while forming the first cell wall, which could result in cell aggregation (Pojnar *et al.* 1968; Cocking, 1970). Addition of surfactants such as Tween 20 or Pluronic® F-68 to liquid media highly reduced protoplast aggregation and increased cell division (Lowe *et al.* 2001). However, liquid culture was suitable for obtaining microcalli from *Zantedeschia* spp. but not from *A. andreanum* or *S. wallisii.* Castelblanque *et al.* (2010) also reported that liquid medium rarely yields better protoplast division. A shortage of aeration and light (Azad *et al.* 2006) or a release of toxic components (Duquenne *et al.* 2007) might cause lower colony formation in liquid media. Immobilizing protoplasts on agarose culture media was suitable for microcalli formation in all the species used in this study. Moreover, clumping protoplasts in the culture could be avoided. Additionally, embedding systems ease the handling of the cultures while replacing the culture media without disturbing the development of the microcolonies and may prevent microbial contamination. Furthermore, the osmotic pressure changes steadily instead of stepwise (Kanwar *et al.* 2009). Nevertheless, *Z. aethiopica, Z. elliotiana* and *Z. rehmanii* protoplasts could divide, until microcolony stage, in both liquid and agarose bead culture.

S. wallisii and *A. scherzerianum* protoplasts only formed microcolonies when they were embedded in agarose beads. In liquid culture or Ca-alginate they underwent only the first or second division. This might be due to the accumulation of the toxic substances released by dying protoplasts, the poor oxygen supply at the bottom of liquid culture, or a toxicity in calcium alginate beads as reported by Duquenne *et al.* (2007). Also, the embedding agent type may affect the final outcome, possibly by interacting with genotype, osmolarity, temperature, culture system or aeration (Prange *et al.* 2010a; Kielkowska and Adamus, 2012). Bajaj (1989) also mentioned membrane stabilization through lipid peroxidase inhibition, the

prevention of leakage of cell wall precursors or other metabolites, and lower ethylene levels, as the consequence of a particular embedding type.

As reported by Rakosy-Tican *et al.* (2007), we also observed increased microcolony formation at the edges of the agarose beads, which explains the need for thinner matrices. Therefore, we reduced final LMPA from 0.6% to 0.3% and the bead volume from 100 to 50 μL. Despite this we observed no further growth after the microcalli formed.

Protoplast concentration as well as carbon source is crucial for protoplast regeneration. High protoplast densities (>10^6 PP/mL) consume nutrients and release toxic products rapidly, which then inhibit sustained protoplast growth (Davey *et al.* 2005). In this study, a concentration of 10^5 PP/mL was suitable to promote microcolony formation all the genera studied. Sucrose promoted four-cell-stage formation and glucose stimulated more microcalli formation from the four cell stage (Eeckhaut *et al.* 2008).

We attempted asymmetric fusion between *S. wallisii* 'Daniël' and *Z. rehmanii* 'Universe' protoplasts by irradiating donor protoplasts before chemical or electrical fusion. It is believed that fragmentation of donor genome, prior to fusion, encourages the elimination of much of its redundant genetic material in the somatic hybrids. Moreover, in asymmetric fusions, most karyotype instability causing donor genes are eliminated during the first post-fusion mitoses, as opposed to symmetrical fusions after which eliminations can occur up to the first sexually derived generation (Cui *et al.* 2009).

Currently, protoplast fusion is almost performed either through PEG (Kao *et al.* 1974) or electrofusion (Zimmerman and Scheurich, 1981). Chemical fusion is inexpensive but can be cytotoxic. Electrofusion is less cytotoxic than chemical fusion, but expensive. In our case, both fusion techniques did not yield any divisions (Table 3.5). A possible reason might be that irradiation on donor protoplast genomes might have induced negative effects such as random chromosome breakage, deletion, rearrangements of genes and sterility of hybrid cells (Famelaer *et al.* 1989; Gleba *et al.* 1988; Puite and Schaart, 1993; Wijbrandi *et al.* 1990).

3.5 Conclusion

We have established an embryogenic calli culture system for *S. wallisii* 'Daniël' and 'Domino' from their petioles and etiolated internodes. These calli yielded more protoplasts than filament calli. Though friable calli could be induced from *Z. rehmanii* and *Z. elliotiana,* a fast growing cell suspension culture was not attained. Asymmetric fusion was attempted through chemical and electrical fusion. Different regeneration methods were tested and microcolony formation from *Zantedeschia, Spathiphyllum* and *Anthurium* protoplast was established. No regeneration beyond microcolony formation was accomplished in any species tested.

DNA fragments alternative to UV MMCT asymmetry fusion mitosis

spindle toxin amide herbicide carbamate microspores microcells

microtubules anthers PMC DAPI stain ball metaphases

fluorescence microscope dyads ball metaphases chromosome bridge

irradiation alternative CIPC mass micronucleation inflorescenes

microPP MNI indices suspension cells microspores genotypes

Chapter 4
Genome fragmentation

antimicrotubular propyzamide MNi ORY incubation period

spadix stage anther dissection tetrads chromosome mass centromeres

butamiphos DMSO random gene breaks sterility gene deletion

fragmentation cultivars pollen sporopollenin enzyme

flow cytometry microspore observation late tetrad early tetrad

average MNi Ca^{2+} deregulation shrunken cells chromosome clumps

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4.1 Introduction

For asymmetric fusion, the genome of the donor protoplast is fragmented. Subsequently, these fragments are integrated in the acceptor genome (Forsberg *et al.* 1998). Because only a fragment of the donor genome is integrated, gene conflicts are expected to be smaller. Therefore, asymmetrically fused protoplasts theoretically divide more efficiently, regenerate more easily, and are more fertile. Various fragmentation strategies have been published (in Chapter 1; section 1.3.2). Irradiation often induces random chromosome breakage, deletion, rearrangement of genes and sterility of hybrid cells. Duquenne *et al.* (2007) attempted asymmetric somatic hybridization between *Spathiphyllum wallisii* protoplasts and UV-irradiated *Anthurium scherzeranium* protoplasts and obtained hybrid cells that could not be regenerated. Moreover, there is no suitable method to quantify fragmentation done by UV irradiation. MMCT (Microprotoplast Mediated Chromosome Transfer) was chosen as an alternative to overcome these barriers (Ramulu *et al.* 1995). No fast growing *S. wallisii* cell suspension cultures are currently available.

Our objective was to induce micronucleation starting from developing microspores of *S. wallisii*. The micronucleation leads to direct microcell formation in microspores that possess one or few chromosomes. These cells could help to isolate microprotoplasts by enzyme treatment and can be fused with protoplasts of other aroid parent to produce asymmetric hybrids. Therefore, parameters such as flower stage, mitotic inhibitor type and dose, and treatment duration through application of mitotic inhibitors were optimized. Additionally, the best protocol was tested on different cultivars.

4.2 Materials and methods

4.2.1 Plant materials

Six diploid *S. wallisii* Regel commercial genotypes ('6054', '6332', '6341', '6409', '6526') (provided and coded by Deroose Plants, Evergem, Belgium) and 'Daniël' (ILVO, Merelbeke, Belgium) were used. All the plants were grown in standard greenhouses under natural light conditions. The day/night temperature was maintained at 20 ± 2°C and the relative humidity at 70%.

4.2.2 Spindle toxins

In the experiments, 5 spindle toxins were used: amiprophos-methyl PESTANAL® [APM, *O*-methyl-*O*-*O*-(4-methyl-6-nitrophenyl)-*N*-isopropyl phosphorothioamidate, a phosphoric amide herbicide], chlorpropham PESTANAL® [CIPC, isopropyl *N*-(3-chlorophenyl)carbamate, a carbamate] and propyzamide VETRANAL™ [PRO, 3,5-dichloro-*N*-(1,1- dimethylpropyl)benzamide, an amide herbicide] were purchased from Sigma-Aldrich, Seelze, Germany; oryzalin [ORY, 4-(dipropylamino)-3,5-dinitro-benzenesulfonamide, a dinitroaniline herbicide] from Duchefa Biochemie, Haarlem, the Netherlands; and butamiphos [BUT, O-ethyl O-6-nitro-m-tolyl sec-

butylphosphoramidothioate, a phosphoric amide herbicide] from Wako Pure Chemical Industries, Osaka, Japan. All stock solutions (5 mM) were prepared in 100% dimethylsulfoxide and stored at -20°C. APM, BUT, ORY and PRO inhibit microtubule assembly while CIPC inhibits mitosis and microtubule organization.

4.2.3 Sterilization of plant material

Spadices (see 1.7) were collected from the greenhouse, washed in 70% ethanol for 3 minutes, disinfected in a 1% NaOCl solution with 2 drops of Tween 20 for 10 minutes and thoroughly rinsed 3 times in sterile distilled water.

4.2.4 Tested parameters

4.2.4.1 Spadix stage

An introductory experiment was conducted for identifying a suitable spadix stage by harvesting '6526' flowers at 6, 8 and 10 days after their first appearance at the very bottom of the plant, inside the leaf sheath (Fig. 4.1). Anthers were dissected and suspended in micronucleation medium containing half-strength MS salts (Murashige and Skoog, 1962), double strength MS vitamins, 1 g/L casamino acids, and 100 g/L sucrose (pH 5.8). The medium was autoclaved at 121°C for 30 min at a pressure of 500 hPa and supplemented with 50 μM ORY afterwards. All treatments were done in Petri dishes (60 x 15 mm) containing 5 mL medium in 4 repetitions. Petri dishes were placed in a culture room at 23 ± 2°C, 16 h photoperiod and 40 $\mu molm^{-2}s^{-1}$ photosynthetic active radiations, supplied by cool white fluorescent lamps (OSRAML36W/31). Observations were made on the fourth day.

Fig. 4.1 Different developmental stages of *Spathiphyllum wallisii* Regel spadices. The stages were determined based on the first appearance of the spadix at the very bottom of the plant, inside the leaf sheath. **A:** 6 days old. **B:** 8 days old. **C:** 10 days old.

4.2.4.2 Flower position, plant material type and exposure time

In experiment 1, the effects of the flower position on the spadix, the type of plant material and exposure time to the spindle toxin were tested. To do so, both isolated anthers and whole spadices were collected from the upper, middle and lower part of a *S. wallisii* '6526' inflorescence. The collected material was suspended in the micronucleation medium supplemented with 50 μM ORY. All treatments were performed as above. Observations were made on day 2, 4 and 8.

4.2.4.3 Spindle toxin type and concentration

In experiment 2, the efficiency of five spindle toxins (APM, BUT, CIPC, ORY, PRO) on micronucleation was examined. Based on the results of experiment 1, we used 5 complete '6526' spadices and dissected all anthers. To minimize developmental stage effects, we collected all anthers in a single Petri dish in 20 mL of basic micronucleation medium. Subsequently, anthers were transferred with a spatula to micronucleation medium supplemented with the various toxins at a concentration of 0, 10, 20, 50 or 100 μM. The anthers were analyzed after 24h, 48h and 72h exposure. The rest of the procedure was as described for introductory experiment. After determination of the spindle toxins that resulted in the highest micronucleation indices, the exact number of MNi induced by these compounds was monitored. For this screening a distinction was made between pollen mother cells (PMCs), dyads and tetrads.

4.2.4.4 Plant genotype

In experiment 3, five other cultivars ('6054', '6332', '6341', '6409' and 'Daniël') were used to control the applicability of the protocol for a range of genotypes. Isolated anthers of these 5 genotypes were suspended in micronucleation medium supplemented with 10 μM ORY during 72h, based on the micronucleation indices that were determined in experiment 2. Depending on the genotype, we used 3 spadices ('6409') or 1 spadix (all other genotypes). The rest of the procedure was performed as described for experiment 1.

4.2.5 Microscopic analysis and micronucleation index

Cells were studied under a fluorescence microscope (Leica DM IRB). To this goal, they were stained with a mixture of 20 μL Vectashield and 1 μg/mL 4', 6-diamidino-2-phenylindole (DAPI). After squashing under a glass cover slip, samples were visualized by UV irradiation; the DAPI fluorescent cell nuclei were detected through a blue filter, whereas the cell walls were visualized under white light. Pictures were captured using a Leica DFC 320 camera with accompanying software (Leica Application Suite). The formation of abnormalities such as ball metaphases (complete destruction of spindle fibers followed by chromosome 'clumping') and chromosome bridges (chromatin bridges between centromeres) was also observed and registered.

The micronucleation index was defined as the ratio between the number of micronucleated cells and the total cell number and used to quantify the effects of the parameters. To calculate the index, pollen mother cells, dyads and tetrads were taken into account. Mature pollen was ignored because its exine layer was easily DAPI stained; this inhibited fluorescence observation of micronuclei inside the cell.

4.2.6 Statistical analysis

For each treatment, 400 cells (4 repetitions of 100 cells per test) were analyzed. Analysis of variance (ANOVA) was performed using Statistica 10.0. A multifactorial analysis was done for experiment 1 and 2, while the introductory experiment and experiment 3 were treated as a one-factor experiment. Mean separation was accomplished with Duncan test (p=0.05).

4.3 Results

Micronuclei were seen in PMCs, dyads and tetrads (Fig. 4.2 A-C and I). Though mature pollen could not be used to calculate micronucleation indices, micronuclei were occasionally observed inside (Fig. 4.2 J).

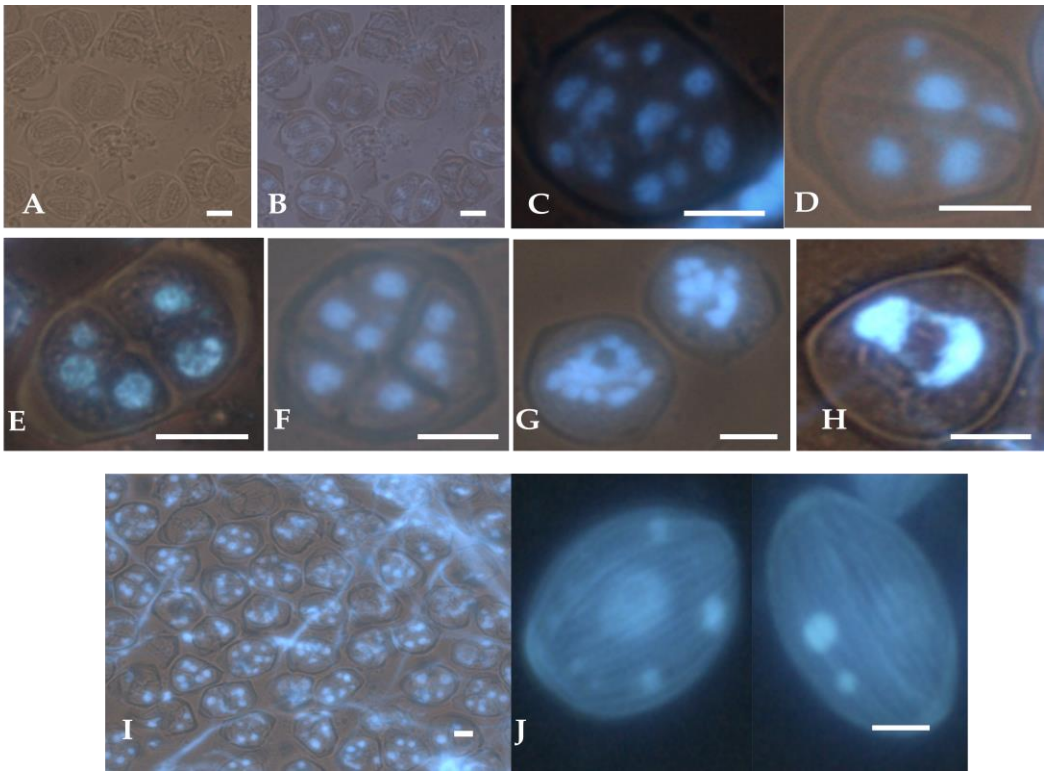

Fig. 4.2 Micronucleation in microspores of *Spathiphyllum wallisii* Regel '6526' after treatment with various antimitotic toxins and visualization through DAPI staining. **A-B:** Control treatment after 72h of incubation, **A:** under bright field; **B:** under UV filter. **C-F:** Micronuclei (MNi) in different pollen developmental stages, **C:** pollen mother cell with MNi (CIPC, 20 μM, 48h); **D:** dyad with MNi (ORY, 10 μM, 72h); **E:** tetrad with MNi (ORY, 10 μM, 72h); **F:** microcell formation at late tetrad stage (CIPC, 50 μM, 72h). **G-H:** Chromosomal abnormalities, **G:** ball metaphases (CIPC, 50 μM, 72h); **H:** chromosome bridges (ORY, 50 μM, 72h). **I:** cluster of microspores with MNi (ORY, 10 μM, 72h). **J:** MNi in pollen (ORY, 10 μM, 72h). The bars represent 10 μm.

By observing cell wall formation, we distinguished micronuclei at dyad and early tetrad stage (Fig. 4.2 B-C) and microcell formation at late tetrad stage (Fig. 4.2 D). Other abnormalities were also seen, such as chromosome bridges and ball metaphases (Fig. 4.2 E-H).

4.3.1 Spadix stage

In the introductory experiment, the micronucleation percentages were 6.25 ± 0.85, 75.25 ± 3.20 and 50.00 ± 0.91 for 6, 8 and 10-day-old inflorescences, respectively; they were all significantly different from one another. We chose 8-day-old inflorescences for further experiments.

4.3.2 Flower position, plant material type and exposure time

Statistical analysis of data obtained in experiment 1 showed that the position of the anthers on the spadix and the plant material type do not cause significantly different micronuclei indices. However, significant interactions between exposure time and plant material or position on the spadix were observed (Table 4.1).

Table 4.1 ANOVA of the effect of the flower position on the spadix, the exposure time and the plant material used on the micronucleation in *Spathiphyllum wallisii* Regel '6526' microspores treated by 50 μM oryzalin.

Parameter	p-value and significance level
Position on spadix	0.548 NS
Exposure time	0.000 ***
Plant material	0.252 NS
Position on spadix x exposure time	0.013 *
Position on spadix x plant material	0.107 NS
Exposure time x plant material	0.011 *
Position on spadix x exposure time x plant material	0.284 NS

***: significant, $p<0.001$; *: significant, $0.01<p<0.05$; NS: not significant

Therefore, these interactions were analyzed separately (Fig. 4.3). No significant differences between micronucleation indices were seen between 2 and 4 days of exposure to 50 μM ORY. This result was not influenced by spadix position nor by the plant material type. After 8 days of exposure, a significant decrease was observed for samples at the lower and upper spadix (Fig. 4.3 A) and in dissected anthers (Fig. 4.3 B).

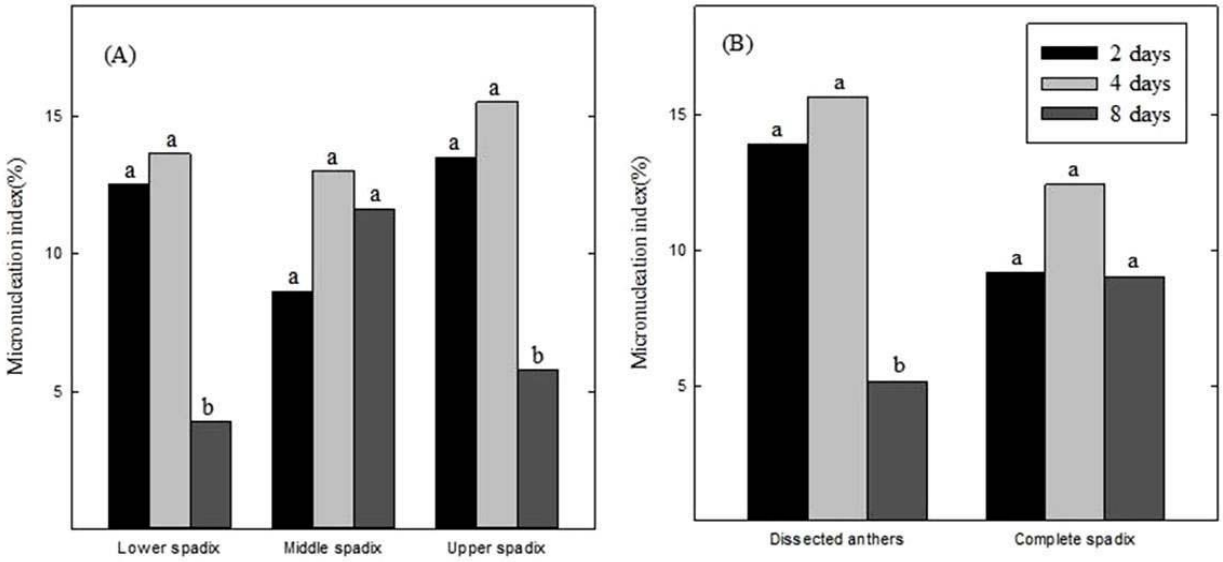

Fig. 4.3 Interactions between exposure time and flower position on the spadix (A) and exposure time and plant material (B) on the micronucleation index in *Spathiphyllum wallisii* Regel '6526' microspores after treatment with 50 µM ORY. Significantly different means (Duncan, $p < 0.05$) are marked by different symbols. Means indicated by the same letter are not significantly different (n=4).

4.3.3 Spindle toxin type and concentration

Experiment 2 showed significant interactions between all tested parameters (spindle toxin, concentration and exposure time) (Table 4.2). Therefore, results were presented separately for each spindle toxin (Fig. 4.4). Significantly higher micronucleation indices were obtained after treatment with ORY or CIPC compared to APM, BUT and PRO. The optimal concentration for all the products tested was situated between 10 to 50 µM. At a higher concentration, the efficiency decreased. In general, exposure time had a smaller influence.

Table 4.2 ANOVA of the effect of the mitosis inhibitor, the dose applied and the exposure time on the micronucleation in *Spathiphyllum wallisii* Regel '6526' microspores.

Parameter	p-value and significance level
Mitosis inhibitor	0.000 ***
Dose	0.000 ***
Exposure time	0.866 NS
Mitosis inhibitor x dose	0.000 ***
Mitosis inhibitor x exposure time	0.019 *
Dose x exposure time	0.002 **
Mitosis inhibitor x dose x exposure time	0.018 *

***: significant, $p<0.001$; **: significant, $0.001<p<0.01$; *: significant, $0.01<p<0.05$; NS: not significant

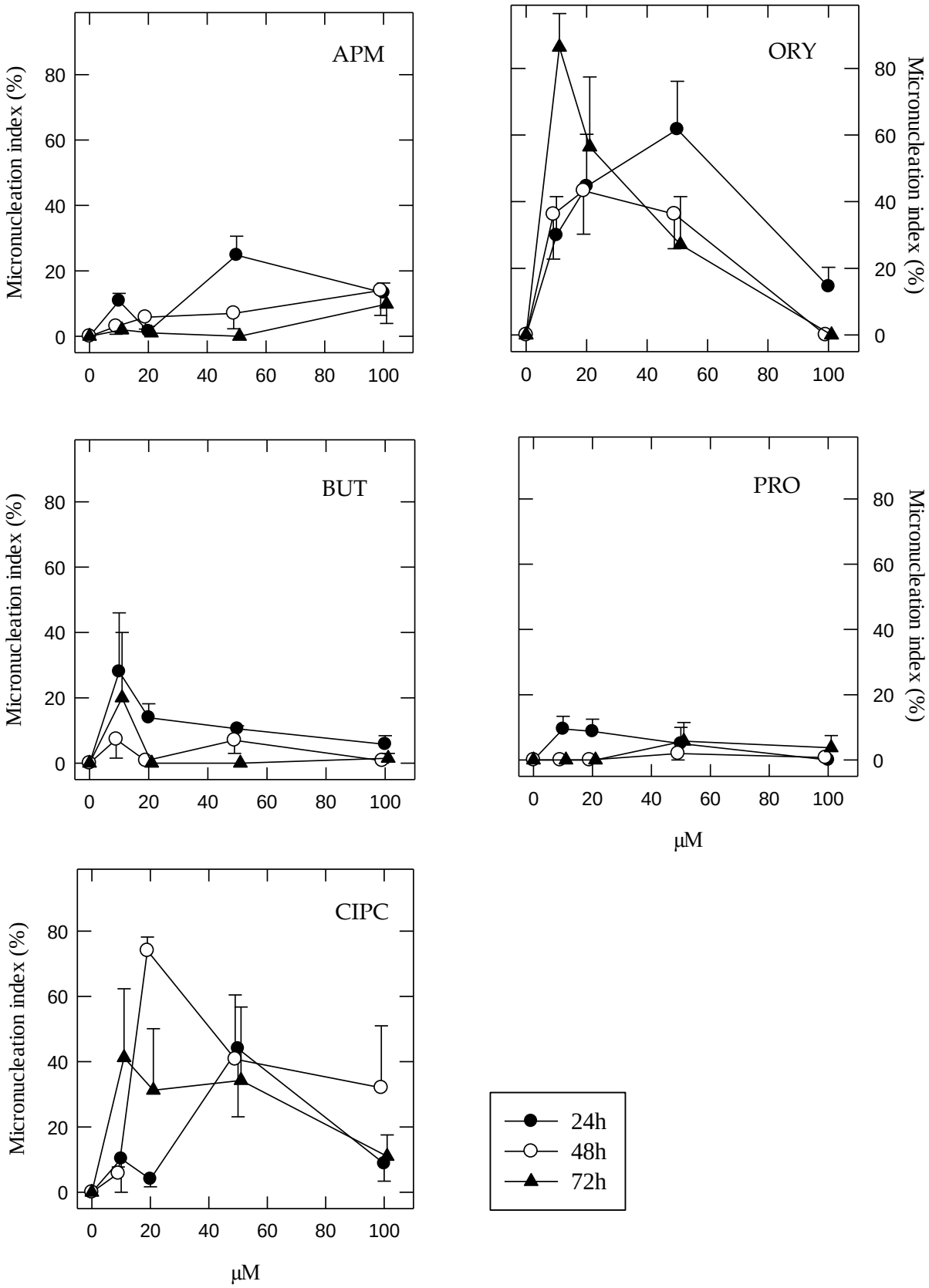

Fig. 4.4 Effect of mitosis inhibiting compounds APM, BUT, CIPC, ORY and PRO, concentration used, and exposure time on the micronucleation index in *Spathiphyllum wallisii* '6526' microspores (data are means ± SE, n=4).

More detailed information on the exact number of micronuclei per micronucleated cell generated by ORY and CIPC treatments is presented in Table 4.3. When CIPC was used, maximal micronucleation was already observed in PMC. After the reduction division, in dyads or tetrads, the MNi number was reduced by half, parallel to the chromosome number per cell. On the other hand, ORY induced relatively more MNi in dyads and tetrads. The average number of MNi (i.e., extra nuclei) found in micronucleated cells varied between 6.44 and 1.67 for CIPC and 5.50 and 0.83 for ORY. The maximal number of MNi observed was 12 for CIPC and 9 for ORY. In other words, CIPC induces MNi sooner than ORY.

Table 4.3 Effect of CIPC and ORY on *Spathiphyllum wallisii* '6526' microspores and micronuclei counts. The data presented here are averages of 24h, 48h and 72h treatments. (Data are means ± SE).

Toxin (μM)	Cell Type	Average cell number with MNi (n=12)		Average MNi number in micronucleated cells [x]		Maximal MNi number [x]	
		CIPC	ORY	CIPC	ORY	CIPC	ORY
10	PMC	7.75±2.74	30.25±8.25	5.03±0.32	5.20±0.36	9	9
	Dyad	2.50±1.41	6.08±2.66	3.67±0.44	3.03±1.63	5	8
	Tetrad	8.83±6.74	5.92±3.32	6.00±0.76	4.00±2.02	9	8
20	PMC	25.83±10.28	24.50±9.01	3.72±2.17	3.67±0.60	12	7
	Dyad	5.75±4.20	7.83±3.39	2.67±1.33	3.83±0.44	5	6
	Tetrad	4.83±4.83	3.08±1.66	1.83±1.83	3.50±1.76	6	6
50	PMC	27.58±10.41	22.50±5.46	6.44±0.51	4.17±0.33	11	7
	Dyad	2.67±1.81	11.00±5.57	2.25±1.15	3.06±1.65	6	9
	Tetrad	2.50±2.50	4.75±1.76	1.67±1.67	5.50±0.29	5	7
100	PMC	15.00±7.18	0.50±0.50	5.83±1.09	0.83±0.83	12	3
	Dyad	0.92±0.92	1.00±0.56	1.67±1.67	1.00±1.00	6	3
	Tetrad	1.33±1.09	2.67±1.39	1.67±1.67	1.83±1.83	5	6

[x] $2<n<12$; cells without micronuclei were not included

4.3.4 Plant genotype

All genotypes tested in experiment 3 yielded micronucleated cells. Among the different genotypes, micronucleation indices varied between 53% for '6332' and 2% for '6409'. Because of high variations, genotype effects were not statistically significant (Table 4.4).

Table 4.4 Average micronucleation index in microspores of 5 *Spathiphyllum wallisii* cultivars after 10 μM ORY treatment during 72h (Data are means ± SE, n=4).

Genotype	Micronucleation index (%)
6054	44 ± 18
6332	53 ± 25
6341	21 ± 15
6409	2 ± 2
'Daniël'	40 ± 20

4.4 Discussion

MMCT is an alternative donor genome fragmentation method for producing asymmetric somatic hybrids. Mass micronucleation in donor source cells is the first step in isolating microprotoplasts. As an alternative for suspension cells, developing microspores can also be treated with spindle toxins and microcells can be directly obtained. As a first step in establishing MMCT, in this study we report the optimal conditions and mitotic inhibitors type for mass micronucleation and microcells induction. These microcells, containing one or a few chromosomes, can be a source for isolating microprotoplasts.

Micronucleation (Fig. 4.2) was observed when treating microspores of *S. wallisii* with several antimitotic agents. These agents are believed to block the growth of microtubules and to stop the movement of chromosomes to the poles, creating chromosome masses. Additionally, Hertel *et al.* (1980) suggest that lower concentrations (5-15 μM) of ORY and APM deregulate and inhibit Ca^{2+} level uptake by plant mitochondria which subsequently lead to micronucleation by forming nuclear membranes around the fragmented genome.

In our experiments, micronuclei were present throughout all developmental phases from PMC to mature pollen. Although the micronucleation efficiency could not be quantified in the latter phase, we expect that micronuclei are abundantly present in mature pollen, based on the efficient micronucleation in tetrads and the observation of micronuclei in pollen with poorly stained cell walls. For younger cells, the micronucleation frequency and quality observed depended mainly on the chemical used, the concentration and the exposure time.

When 6-day-old inflorescences were treated with toxins, many PMC with intact nuclei were observed on the third day. When the treatment was prolonged to 4 and 5 days, the toxins

induced detrimental effects such as shrinking or decaying of cells. At this stage, inflorescences were not sufficiently susceptible to the mitotic inhibitors. On the other hand, 10-day-old spadices produced more mature pollen than 8-day-old inflorescences. At that time, cell exposure to the toxins was no longer optimal. Henny and Chen (2010) state that all flowers on a *Spathiphyllum* spadix mature simultaneously. However, minor differences in developmental stages of cell divisions exist within each anther, which explains why we observed dyads and tetrads along with mature pollen.

In experiment 1, the optimal exposure period was 4 days, but after 2 days the micronucleation index was not significantly lower. After 8 days of ORY exposure, both flower positions on the spadix and plant material used had a significant effect on micronucleation (Table 4.1, Fig. 4.3). One possible explanation might be the difference in developmental stage of the anthers from the bottom to the top of the inflorescence. Cells exposed at an ORY concentration as high as 50 μM can be assumed to have a higher risk of being completely arrested after longer incubation times, thus decreasing micronucleation indices. An interaction with the pollen developmental stage, as shown in Fig. 4.3, is probable in this case. Likewise, in dissected anthers the developmental stage might be arrested compared to undetached ones. Significant effects of developmental stage were also reported in potato, where successful micronucleation was only achieved by applying toxins on separated anthers but not on buds or inflorescences (Matthews *et al.* 1999).

After 4 days of incubation, many shrunken cells appeared; this severely reduced the number of cells suitable for MNi counting. Furthermore, we can expect cell shrinking to be negatively correlated with chromosome scattering. For this reason, in following experiments we exposed the cells to mitosis inhibitors for a maximal time frame of 72h. Moreover, the results obtained by this experiment demonstrate that developmental stage effects are not statistically relevant after a short exposure time. We decided to use dissected anthers from the whole spadix as plant material source in subsequent testing.

Among the antimicrotubular toxins, significant differences were observed in experiment 2. The most efficient toxins were CIPC and ORY, while APM, BUT and PRO had only a limited or almost no effect. Not surprisingly, interactions with dose and exposure time were significant (Table 4.2). Similarly, Ramulu *et al.* (1991) found an increased percentage of micronucleated cells as well as chromosome scattering, along with the increase of duration of the treatment in potato suspension cells, when comparing APM and ORY with colchicine. They also reported that 30 μM ORY and 32 μM APM induced a higher frequency of micronucleated cells at 30h and 48h, respectively.

After treatment with 10 μM ORY for 72h, the most cells with micronuclei were produced; the micronucleation index was 86.4% (Fig. 4.4). Likewise, in *L. longiflorum,* about 90% of microspores produced more than 4 nuclei when treated with CIPC of 10 μM, while PRO (5 or 10 μM) and colchicines (COL) (120 or 240 μM) induced less than 10% micronucleated cells, after an exposure time of 72-96h (Saito and Nakano 2002a). An efficiency of 5-9% of

micronucleated cells was obtained using ORY at lower concentrations (15 and 30 μM) in *Nicotiana plumbaginifolia* suspension cells (Verhoeven *et al.* 1990); 25 μM ORY also induces about 10% microcell formation in microspores of *Solanum tuberosum* L (Matthews *et al.* 1999). Also, both articles reported that APM had promising effects. However, this was not confirmed in our study. Although APM, an amide herbicide, has approximately the same mode of action as ORY, its efficiency was significantly lower. ORY was also found to be effective in inducing micronucleation on *Haemanthus katherinae* Bak. and *Helianthus giganteus* suspension cells (Binsfeld *et al.* 2000; Morejohn *et al.* 1987). Conversely, in *Hemerocallis hybrida* 'Stella d'Oro' suspension cultured cells, ORY induced less micronucleation when compared to other toxins such as APM, BUT, CIPC and PRO (Saito and Nakano 2001). Also in other publications, APM, BUT or PRO were relatively more successfully applied for micronucleation (Ramulu *et al.* 1994; Saito and Nakano 2001; Saito and Nakano 2002b). This disagreement with our results can be explained by differences in plant material, not only genotypic, but also physiological, such as a different division activity. For instance, in the latter articles cell suspensions were used as donor material and mitosis inhibitors were often combined with synchronizing agents such as hydroxyl urea and microfilament disrupting agents such as cytochalasin B.

A comparison of the results from experiments 1 and 2 shows that after treatment with 50 μM ORY, 27-62% and 14-16% micronucleated cells were formed in experiments 2 (1-3 days incubation) and 1 (2-4 days incubation), respectively (Fig. 4.3 and 4.4). We assume that this is caused by developmental stage differences, as different spadices were used for both experiments.

From a qualitative point of view, smaller micronuclei are more interesting for possible future applications such as microprotoplast isolation. We expect that the average DNA amount and the chromosome number per micronucleus are negatively correlated with chromosome scattering and the number of micronuclei per cell. For that reason we checked the number of micronuclei generated by the 2 antimitotic compounds that yielded most cells with micronuclei, being ORY and CIPC (Table 4.3). Because exposure time had no significant effect on micronucleation in the previous experiment (Table 4.2), the mean result of the 24, 48 and 72h treatments was evaluated. Compared to ORY, CIPC resulted in a higher number of MNi per cell. This suggests a better fragmentation, although the micronucleation index was lower. Also in meiocytes of *Lilium longiflorum,* CIPC was found to be most efficient. The mean number of micronuclei induced was 7.5 with a maximum of 20 MNi which was higher than after treatment with APM, colchicine or PRO (Saito and Nakano 2002a). CIPC, a carbamate, alters the orientation of spindle microtubules which leads to multiple spindle formation. Thus chromosomes move to many poles, resulting in more scattering than ORY (Vaughn and Lehnen 1991). Micronuclei are more often found in PMCs than in dyads or tetrads, which can be explained by the application of the antitubular toxins in an early developmental stage.

Besides micronucleation, we observed abnormalities such as chromosome clumping, known as ball metaphase, and chromosome bridges. The same ball metaphases were observed by Damon (1957), Ramulu *et al.* (1987) and Verhoeven *et al.* (1990). However, in *Citrus unshiu* cultured cells, no such ball metaphases were observed (Zhang *et al.* 2006). Chromosome bridges were reported by Peng *et al.* (2003) in APM treated root tips of *Triticum durum*. They also occur during 2n pollen development in *Begonia* that could be induced by the dinitroaniline trifluralin (Dewitte *et al.* 2010a; 2010b). In our experiment, ball metaphase was seen in all toxin treatments, in varying frequency (up to 0.42% in PRO treated cells and 1.81% in ORY treated cells), while chromosome bridges were only observed in ORY treated cells in a very low frequency of 0.17%.

We tested the protocol that yielded the maximal micronucleation index in '6526' (10 μM ORY during 72h) on 5 other *S. wallisii* genotypes in experiment 3. It provoked micronucleation in all genotypes, and in all but 1 at relatively high frequencies (Table 4.4). Only cultivar '6409' produced only 2% micronucleated cells. The lower micronucleation indices compared to 86% obtained in Experiment 2 can be explained by the optimization of the protocol for '6526'; probably antimicrotubular toxin concentration and exposure are not optimal for other cultivars. However, experiment 3 succeeded in demonstrating that micronuclei were formed in developing microspores of a range of genotypes. For future applications, we recommend that micronuclei inducing protocols are optimized per genotype.

Significant differences between cultivars were not recorded because the variation within a single cultivar was generally large (Table 4.4). Comparisons between different genotypes are also complicated because the developmental stages of different spadices are not exactly equal. Furthermore, in '6409', low amounts of immature pollen were present, accounting for the need to harvest material from 3 different spadices, even increasing variation. Also in *Lilium* almost no genotypic effects were seen and in all cultivars tested efficient induction of micronucleation was possible (Saito and Nakano 2002a).

4.5 Conclusion

From our study, we conclude that mass micronucleation is possible in microspores of various *S. wallisii* Regel genotypes using either CIPC or ORY. The optimal treatment is probably genotype specific; moreover, the most efficient treatment for induction of micronucleated cells does not necessarily bring forth the largest number of micronuclei. We assume that minor developmental stage differences, which are not easy to control, may affect micronucleation efficiency.

aroid breeding intergeneric barriers asymmetry hybrids cytogenetic

advantages of FISH 1st step to GISH parents selection gene FISHing

additional knowledge of B's explant studies embryos for synthetic seed

need embryogenic suspension electrical fusion < chemical only microcolonies

suspension PP trial based study < rationalised irradiation vs MMCT microPP

use of meiotic microPP useful for monosomic additional lines applying comet FISH

Chapter 5
General conclusions and perspectives

laser technology irradiation on microPP chemical fusion for microPP

useful for hybrid analysis micronuclei transfer need for microPP isolation

easy to quantify fragmentation avoids random gene breaks no gene deletion

calla lily for tissue and cell culture studies more study on suspension cells

flow cytometry for microPP monocot PPs recalcitrant need friable calli

limitation of ISH insights for PP source selection regenerative suspension cells

5.1 Introduction

Araceae is a species-rich monocot family. Due to their attractive nature, aroids are mostly appreciated as ornamental plants. Intergeneric or interspecific hybridization is the main tool for innovation in ornamentals. However, sexual barriers are hampering successful intergeneric crosses between aroids as well as between many other genera. Therefore, the potential of somatic fusion was evaluated. Symmetric protoplast fusions are often correlated with regeneration problems. For this reason, in this project, we aimed for asymmetric fusion in Araceae model plants *Anthurium andreanum*, *Spathiphyllum wallisii* and *Zantedeschia spp.* and tried to establish some steps towards our final goal to create asymmetric intergeneric somatic hybrids. To analyze the parent plants in the putative asymmetric hybrids, we used molecular cytogenetic technique for physical mapping of 45S and 5S rRNA genes in the parent plants as a starting step towards optimizing GISH protocol. The specific goals of this PhD were: i) karyotype construction and mapping of rRNA genes in Araceae species, ii) establishment of protoplast isolation, fusion and regeneration protocols and iii) development of a donor genome fragmentation system to create asymmetric somatic hybrids.

5.2 Karyotype analysis and physical mapping of 45S and 5S rRNA genes

Karyotypes and a general protocol for FISH using rRNA genes have been established for the first time in Araceae genera *A. andreanum*, *Monstera deliciosa, Philodendron scandens, S. wallisii*, and *Syngonium auritum.* Additionally, a tyramide-FISH protocol for locating 5S rRNA genes has also been established for *Z. elliottiana*. Furthermore, cytological analysis has been performed to measure genome sizes (Chapter 2). These results will assist to a large extent in identifying putative hybrids and form a solid basis to implement GISH analysis in the characterization of regenerated protoplast fusion products.

As discussed in chapter 2, studies on chromosome numbers have already been extensively used for aroid plant systematics; our study provides additional useful data for breeding programs or evolutionary studies. The karyotype analysis and the chromosome counts can be used to study taxonomic relationships, evolutionary events, chromosomal aberrations and cellular functions. The similarities and differences in chromosome morphology, chromosome length, centromeric position and karyotype asymmetry can be potential indicators for successful interspecific crosses. Therefore, our results may be used by breeders to select suitable parents for interspecific or intergeneric crosses in aroids. Besides chromosome morphology and size differences, karyotype analysis can reveal parental chromosomes in intergeneric crosses or somatic hybrids. Somatic hybrids have been characterized using chromosomes numbers and nuclear DNA content analysis (Fahleson *et al.* 1988; Sundberg *et al.* 1991; Smyda *et al.* 2013). However, it is not always sufficient as there can be fewer chromosomes in hybrids than the parental chromosome sum (Liu *et al.* 2005). Therefore, a more solid and specific chromosomal marker is appreciated. ISH is a very powerful tool for accurate distinction of asymmetric hybrids and parents and has been increasingly used in

recent years (Chapter 1, Table 1.1). In literature, there was no report on *Monstera, Philodendron, Spathiphyllum* and *Syngonium* karyotype or chromosome morphology. This is also the first report of FISH mapping of rRNA genes in Araceae. The general FISH protocol we established for Araceae species with rDNA repeats will also provide a basis for phylogenetic and evolutionary studies as achieved in Asteraceae and Siberian Larix species (Goryachkina *et al.* 2013; Pellicer *et al.* 2013). However, when conventional FISH with 45S and 5S rRNA genes was applied, either separately or simultaneously, only 45S rRNA genes were observed in all genera. Only in *Spathiphyllum*, it was able to identify 5S rRNA genes using conventional FISH. Therefore, Tyr-FISH was applied for *Zantedeschia spp*. and signals for 5S rDNA were observed. The Tyr-FISH method developed in our study will take us a step further than conventional FISH that seems less sensitive for species with small chromosomes that possess fewer 5S rDNA copies such as *Zantedeschia*.

Using FISH, we can also physically map single-copy DNA sequences of interesting genes i.e. disease susceptibility or other economically important genes relevant for breeding programs. Using GISH, we can track chromosome fragments or complete chromosomes present in asymmetric hybrids as well as intra or intergenomic translocation and chromosome rearrangements (Liu *et al.* 2005). As mentioned in chapter 1, GISH can be used for monitoring genomic stabilization; if chromosome elimination occurs preferentially rather than randomly, it would be a convenient tool to evaluate the effects of fusion and regeneration related parameters, and thus to optimize these parameters in an effort to stimulate or impede regeneration of particular genome types (Lakshmanan *et al*. 2013). Our optimized FISH protocols will be the first step for establishing GISH in Araceae and can be used in future aroid breeding programs. However, to apply GISH successfully in putative hybrids, optimization of the probe/block DNA ratio and the best labelling and detection system for the probe should be studied. If the chromosomes are small, they may be hard to visualize and well-trained skills are important because the procedure can fail during many key steps. The preparation of well-dispersed chromosomes is the most important factor in ISH. In addition, the optimal ratio of blocking DNA to probe DNA is critical, and this ratio depends on the phylogenic relationship of the species tested-the closer the phylogenic relationship, the higher the ratio. Another limitation of ISH is that we cannot evaluate mitochondrial or chloroplast genome recombination. To this aim, molecular markers such as AFLP, CAPS, SSR or HRM can be applied to screen the plastome (Chapter 1).

In literature, B-chromosomes have been reported for *Anthurium spp.* and we also observed B-chromosome like structures in some spreads. However, more studies are needed to confirm B-chromosomes during meiosis, as B-chromosomes are not involved in regular chromosome (A-chromosome) pairing (Jones 2003). There are also B-specific sequences which can be used to confirm the presence and distinguish B chromosomes from A-chromosomes through FISH techniques (Houben *et al.* 2013). The presence of B-chromosomes, in rye, correlates to the condensation of both rDNA regions during interphase and satellite repeat located on A-

chromosomes (Houben *et al.* 2013). It might be interesting to extend our FISH study to apply on B-chromosomes in *Anthurium* and add knowledge to existing B-chromosome studies and condensation of satellite regions. B-chromosomes were used to study homologous pairing in maize (Jones 1995). B-chromosomes may also be used as vectors for transgenesis (Houben *et al.* 2011). However, B chromosomes have little effect on an individual's phenotype and applications are yet unexploited.

5.3 Protoplast isolation, fusion and regeneration

Successful protoplast regeneration depends on parameters such as protoplast source, protoplast density in culture, chemical media composition, physical culture method, refreshment rates and plant hormones. To test these parameters, an extensive study has been performed as detailed in chapter 3. We established for the first time an embryogenic calli system from *S. wallisii* petioles and etiolated internodes. This latter method gives the possibility to produce large numbers of embryos in bioreactors. These embryos can then be incorporated in synthetic seeds. These embryos (or explants) can also be used for genetic transformation and cryopreservation studies. It might be a powerful tool for *in vitro* ploidy manipulation or mutation breeding in *Spathiphyllum*.

As already mentioned in chapter 1, in monocots or other recalcitrant species, suspension cell protoplasts usually are the most efficient material with regard to regeneration. In our experiments, *S. wallisii* did not yield friable calli that are required to initiate a cell culture. Though *Zantedeschia spp.* yielded friable calli, the cells did not divide vigorously during suspension subculture. A possible future study could lead deeper into the use of various sugar types which might lead to successful, fast growing embryogenic cells. When highly friable calli are not induced, adding a low concentration of pectolytic enzymes (for eg. 0.001% pectinase) to chopped calli material in suspension culture may help to separate cells in the initial culture. Although establishing a suspension cell culture is time consuming and labor intensive, it can be used, not only as a protoplast source, but also for transformation studies, as single cells can be ample targets for maximizing transformant-agent exposure and may facilitate unicellular approach.

Previously, Araceae protoplasts were isolated from *Anthurium* and *Spathiphyllum* spp. (Kuehnle, 1997; Duquenne *et al.* 2007). We also obtained protoplasts from *Zantedeschia. Zantedeschia* tuber calli and tubers yielded an insufficient number of protoplasts; probably the higher accumulation of calcium oxalate crystals, compared to leaves and petioles, damaged protoplasts during isolation. For protoplast isolation from species with oxalate crystal accumulation, the floating layer method used in our study might prevent centrifuging and pelleting damage. However, it depends on the level of crystal accumulation.

Electrical fusion was performed based on Duquenne *et al.* (2007) using *Spathiphyllum* and *Zantedeschia spp.* protoplasts. We also established chemical fusion between protoplasts of *Spathiphyllum* and *Zantedeschia spp.* However, the fusion products did not divide.

For protoplast regeneration, we tested different parameters (Table 3.5). However, we could only obtain microcolonies in our model crops. In literature, except for *Pinellia* (He *et al.* 1996), no successful regeneration has been achieved in aroids. In general, protoplast regeneration is often problematic in monocots. In our study as well as in other studies, monocot mesophyll cells do not divide in a sustained way and are recalcitrant. Like suspension cells, calli also contain more mitochondria which can provide a better energy supply to dividing protoplasts. However, also the embryogenic calli protoplasts we used for protoplast regeneration did not go through sustained division. Possibly, suspension cells may be more suitable for protoplasts regeneration. As explained before, cell suspensions are often hard to accomplish, especially in monocots and there is the possibility of cytological aberrations or mutations (Li *et al.* 2004; Grosser *et al.* 2007). Therefore, a future research on cell suspension culture establishment should be necessary.

Our tests revealed that culture in agarose beads and in the dark yielded the best results for all three model plants. Light conditions may have unfavored division due to high polyphenol oxidase activities that could lead to oxidation of the phenolic compounds that are abundant in *Spathiphyllum,* and subsequent cell browning (Zhao *et al.* 2012). Liquid culture and culture in Ca-alginate beads were not suitable for any species tested. Toxic substances in alginate beads might prevent division as supposed by Duquenne *et al.* (2007). *Zantedeschia spp.* protoplasts could also form microcolonies in liquid culture either in dark or light conditions. However, agglutination of cells was unavoidable. Addition of surfactants avoided clump formation and was less laborious than culture in beads. Also, adding surfactants such as Pluronic® F-68 and Tween 20 speeded up microcolony formation from 4-6 weeks to 2 weeks after isolation. This method can be applied for slowly dividing protoplast of other species as Pluronic® F-68 is non toxic. Although, we obtained microcalli, further regeneration was not achieved.

The KM mineral composition was suitable for *Anthurium*, *Spathiphyllum* and *Zantedeschia spp.* However, for *Zantedeschia spp.*, both KM and MS supplemented with KM organic acid could yield microcolonies. Also organic acids and NH_4NO_3 were necessary for microcolony formation. Addition of amino acids, coconut milk and calf serum did promote division beyond two cell stage. When we used nurse culture or conditioned media methods, either we obtained only microcolonies or only four cell stage. In other words, a nurse effect was not demonstrated. Further studies using vigorously dividing cells as nurse cells or for conditioned medium preparation might increase the chances of success. Administering high hormone pulses on explants to increase endogenous hormone levels that can interact with exogenously applied plant growth regulators is another option to introduce explants variability. Calli induced from embryos, hypocotyls or seedlings provide an alternative cell source. Special treatments such as electric stimulation, micofluidic channels for continuous media supplementation or polyamine addition to reduce stress might be helpful. Also a wider range of hormonal combinations and addition of antioxidants can be explored with

regard to protoplast regeneration. However, in this type of research, we expect it will be necessary to switch from trial and error based studies towards more rationalized approaches such as detailed studies on antioxidant machinery, the search for genes controlling the plant-regeneration abilities, proteome studies and phytohormonal monitoring (Eeckhaut *et al*. 2013).

5.4 Genome fragmentation

For asymmetric fusion, micronucleation on developing microspores of *S. wallisii* has been performed. These micronucleated cells can be the first step to isolate microprotoplasts for producing asymmetric somatic hybrids with other aroids (Chapter 4).

We established an efficient micronucleation system on microspores of *S. wallisii* 'Daniël' for genome fragmentation. Mitotic inhibitors CIPC and ORY induce mass micronucleation in *S. wallisii* microspores. However, from our results we could conclude that the optimal treatments are genotype specific and minor adaptations to the protocol should be considered for other cultivars. Other toxins APM, BUT, and PRO were not as efficient as CIPC and ORY. The optimal concentration of all toxins was between 10 to 50 μM. Also the flower stage matters as flower developmental stages can differ between spadices, and within a single spadix. The exposure time was of less importance for micronucleation. When CIPC was used, the number of micronuclei observed in PMC was maximal. After the reduction division, in dyads or tetrads, the MNi number was reduced by half, parallel to the chromosome number per cell. In other words, CIPC induced MNi sooner than ORY. ORY induced relatively more MNi. In dyads and tetrads, it induced more MNi per average cell than CIPC. Moreover, the ORY micronucleation index was higher. Therefore, ORY was preferred over CIPC. However, CIPC and ORY can be suitable toxins for MNi induction in microspores.

The flower stage selection and anther collection methods established may be applied in other aroids as they share the unique flower structure of Araceae (spadices). Microprotoplasts can be isolated by enzyme incubation of microcells induced from the toxin treated microspores directly. However, complementary to a study on optimization of enzyme application, sequential filtering and sorting is still needed. Quantification of fragmentation through microscopic observation and flow cytometric sorting of microprotoplasts has already been reported in literature. Despite all the advantages over somatic microprotoplast technology, microspore micronucleation has not been exploited for microprotoplast induction except in *Solanum tuberosum* L. and *Lilium longiflorum* (Matthews *et al.* 1999; Saito and Nakano, 2002). Our study could be the basis for meiotic microprotoplast isolation in other economically important crops.

Successful production of monosomic additional lines through somatic microprotoplast-protoplast fusion between transgenic potato and tomato, subsequent successful GISH

characterization has been reported (Ramulu *et al.* 1996). Therefore, theoretically, *Spathiphyllum* microprotoplasts containing one or a few chromosomes can be isolated using our technique and fused with protoplasts to create asymmetric somatic hybrids between *Zantedeschia* and other aroids. However, electrical fusion might be impeded as diameter variations between protoplasts and microprotoplasts might prevent alignment and membrane breakdown (Zimmermann *et al.* 1982). Therefore, the chemical fusion protocol developed in chapter 3 is probably the most suitable to produce asymmetric hybrids using microprotoplast and protoplast fusion (Binsfield *et al.* 2000; de Bona *et al.* 2009 b). In the near future, laser assisted microdissection, optical trapping and even fusion can significantly contribute to the implementation of this innotive technology. So far, laser induced fusion has not been applied in any protoplast-microprotoplast combinations. Additionally, the micronuclei may also be collected in glass needles and microinjected in protoplasts, as has been done previously for organel transfer (Knoblauch *et al.* 1999).

Combining microprotoplast and irradiation might further fragmentize the donor chromosomes, facilitate the introgression of few donor genes in hybrids and be a start for radiation mapping. However, our first goal remains successful asymmetric hybrid creation. A particularly useful tool would be single cell gel electrophoresis (SCGE) also known as comet assay. For establishing comet assay, there are certain limitations. In plants, UV irradiated cells can possess self repair mechanisms; therefore, the nuclei have to be carefully extracted in dark and cold conditions. However, it is time consuming, labour intensive and limited number of samples can be analyzed in an experiment. Combining comet with FISH (comet-FISH) may help identifying UV damage induced on specific genome regions as demonstrated in *Crepis capillaris* using 5S and 25S rDNA sequences (Kwasniewska *et al.* 2012).

5.5 General outlook

We have successfully established a general protocol for FISH technique using 6 aroids. We also extended high sensitive Tyr-FISH to 5S rRNA genes in *Zantedeschia.* This technique will be the basis for aroid hybrid plants analysis, either somatic or sexual, through ISH. However, further protocol optimization for GISH is still needed while analyzing hybrids using the parent plants. Additionally, to confirm B-chromosomes, a study at meiotic level is required.

We have established different explants system in *Spathiphyllum* and *Zantedeschia* and used for protoplasts regeneration studies. Various protoplast regeneration methods were attempted and useful information about protoplasts source selection in aroids was retained. These attempts will provide useful insights towards more efficient protoplast source selection. Nevertheless, additional research is necessary to optimize protocols. For instance, the potential of suspension cells as protoplast source still needs to be evaluated. Various regeneration methods are so far unexploited. Moreover, there is a need to conduct more rationalized studies on protoplast regeneration such as profound study of phytohormones,

chromosomal condensation and reactive oxygen species (ROS), rather than to perform trial and error based experimentation.

For fragmenting the donor genome, we have established an efficient micronucleation system in *S. wallisii* microspores. However, there is a further need to optimize microprotoplast isolation and to quantify genome fragmentation. Once microprotoplasts are obtained, they can be fused with protoplasts based on the chemical fusion method optimized in our study. This study can be useful to quantify fragmentation and partial genome transfer in economically important crops.

A general observation was that *Spathiphyllum* and *Anthurium* cells divided slowly which hampered chromosome preparations for FISH. The same was already observed in *Colocasia esculanta* (Murakami *et al.* 1995). However, *Zantedeschia* grows faster than the other two model plants and its chromosome preparation and molecular cytogenetics were easier than in other model crops. Moreover, we demonstrated that it is possible to induce friable calli and suspension cells from *Zantedeschia spp.* However, inducing regenerative friable calli from *Zantedeschia spp.* is also required for a successful suspension cell cultures. Additionally, after fast growing suspension cells are established, micronucleation techniques may be applied on somatic cells implementing the technology developed in chapter 4. However, a synchronizing treatment as well as ultracentrifugation is required and the risk of mutation is higher than after microspore micronucleation. Based on the knowledge gathered in this study, *Zantedeschia* might be an interesting model for cytogenetic studies and tissue culture manipulations. Moreover, interspecific hybrids in *Zantedeschia* have not yet been successfully produced, except albinos. Disease resistant traits such as resistance to bacterial soft rot in *Z. aethiopica* may be transferred, to the section *Aestivae* which is prone to the disease, or to other sections through somatic hybridization.

Araceae is a monocot family that comprises many species with ornamental values. Intergeneric or interspecific hybridization is a means to introduce genetic variations into economically important crops, especially in ornamentals. However, no reports have been published about successful Araceae intergeneric hybrid production. Such crosses are difficult to achieve due to various sexual barriers. Somatic hybridization is a known asexual method for overcoming the sexual barriers related to intergeneric or interspecific crosses. Asymmetric fusion limits the introgression of unwanted genes and the genome repelling effect, phenomena that are typical in symmetric fusion products.

Genome fragmentation can be achieved through micronucleation, an alternative for irradiation. Irradiation often induces dimer formation, random gene deletion and rearrangements. Moreover, micronucleation caused genome fragmentation can be quantified microscopically and flow cytometrically. Developing microspores can be used for microprotoplast mediated chromosome transfer (MMCT) more quickly than suspension cells, as batch cell cultures are time consuming to establish.

Molecular cytogenetic techniques, such as *in situ* hybridization, provide accurate information like parent gene copy or chromosome numbers in the hybrids. It is a reliable method for many asymmetric somatic hybrids. Therefore, we performed studies to support the production of Araceae asymmetric somatic intergeneric hybrids and Araceae molecular cytogenetic characterization. In this dissertation, we compiled information which could be useful for the production and analysis of asymmetric hybrids in Araceae as well as in other crops.

Genome sizes were measured, karyotypes were constructed, and a general protocol for FISH has been established for the first time for six Araceae genera: *Anthurium, Monstera, Philodendron, Spathiphyllum, Syngonium* and *Zantedeschia*. 45S rDNA repeats were visualized by conventional FISH in all the genera tested. In *S. wallisii*, 5S rDNA repeats were visualized by conventional FISH. A highly sensitive Tyr-FISH protocol was established to visualize 5S rRNA genes in *Z. elliottiana*. We also collected information about B-chromosome-like structures in *A. andreanum*.

One of the parameters for successful protoplast regeneration is the type of source plant material. Therefore, we induced calli that could be used as a protoplast source as well as for the establishment of suspension cell cultures. We induced compact calli from *S. wallisii* petioles and etiolated internodes; and friable calli from *Zantedeschia spp.* Using friable calli, suspension cell cultures were established, but they did not divide vigorously. Protoplasts were isolated from various plant organs and tissues including leaves, petioles, meristematic cells and tubers as well as the abovementioned calli. Chemical and electrical fusion protocols were optimized using *S. wallisii* and *Z. elliottiana* protoplasts.

Protoplast regeneration into microcolonies was attained in *Anthurium, Spathiphyllum* and *Zantedeschia*. Agarose bead culture and dark conditions favored protoplast division. A KM

mineral composition supplemented with 0.09 M sucrose or 0.08 M glucose, 0.45 μM 2, 4-D, and 2.22 μM BAP and 2.69 μM NAA was suitable. A gradual, weekly reduction of mannitol concentration from 0.31 M to 0 M stimulated microcolony formation. A density of 10^5 PP/mL was optimized for protoplast division. Addition of 0.1% Pluronic® F-68 or 0.05% Tween 20 induced microcolonies within two weeks.

As a preliminary step towards MMCT starting from developing microspores of *S. wallisii*, we optimized parameters such as flower age and mitotic inhibitor type, concentration and incubation period. We selected the highest micronucleation inducing mitotic inhibitors and tested the toxin efficiency in various genotypes.

Data from our karyotype studies, including chromosome morphology and asymmetry differences, can be useful to select suitable parent plants for future Araceae intergeneric/interspecific breeding programs. This data can also be useful to differentiate hybrids from parent plants. FISH results could identify putative hybrids and form a solid basis to implement GISH analysis in the characterization of regenerated protoplast fusion products. Additional research is needed to confirm the presence of B-chromosome in *A. andreanum*.

The embryogenic callus formation system established in this study can be useful in artificial seed and commercial plant production. For protoplast studies, the potential of suspension cells as explant source requires further study, and various regeneration methods are as yet unexploited. First, a fast growing cell suspension culture has to be established. In general, a more rational approach to protoplast regeneration instead of trial and error based studies is necessary.

The micronucleation system established from our research can be used to isolate meiotic microprotoplasts in large numbers. A further study should quantify the genome fragmentation through flow cytometry. This study can also be the onset for the production of mitotic microprotoplasts after a fast growing suspension culture has been established. Overall, this study provides useful insights towards producing asymmetric somatic hybrids and subsequent hybrid selection within Araceae as well as in other economically important crops.

De monocotyle plantenfamilie Araceae omvat tal van soorten met sierwaarde. Intergenerische of interspecifieke hybridisatie is een middel om genetische variaties te induceren in economisch belangrijke teelten, vooral bij sierplanten. Binnen Araceae werden intergenerische hybriden nog niet beschreven; bovendien compliceren uiteenlopende kruisingsbarrières dergelijke seksuele kruisingen. Somatische hybridisatie is een beproefde methode om deze voor intergenerische of interspecifieke kruisingen typische barrières te omzeilen. Asymmetrische fusie beperkt de introgressie van ongewenste genen en het afstoten van complete genomen, die beide typisch voorkomen in symmetrische fusieproducten.

Zowel micronucleatie als bestraling zijn geschikte technieken voor genoomfragmentatie. Bestraling is vaak de oorzaak van dimeervorming, random gen deletie en genoomherschikkingen. Bovendien kunnen de effecten van genoomfragmentatie via micronucleatie microscopisch en flowcytometrisch gekwantificeerd worden. Ontwikkelende microsporen zijn een mogelijke bron van microprotoplasten voor chromosoom transfer via microprotoplasten (MMCT) en zijn sneller beschikbaar dan suspensiecellen, waarvan continu delende batch culturen moeten aanwezig zijn, wat veel tijd vraagt.

Moleculair cytogenetische technieken zoals *in situ* hybridisatie onthullen het precieze kopieaantal van ouderlijke genen of het exacte chromosoomaantal in de hybriden. Het is een betrouwbare methode gebleken voor de screening van verschillende asymmetrische somatische hybriden. Om die reden verrichtten we naast onderzoek gericht op de productie van asymmetrische producten binnen Araceae ook onderzoek naar moleculair cytogenetische karakterisering binnen deze familie. De in deze scriptie verzamelde informatie kan een belangrijke hulp betekenen bij zowel de productie als de analyse van asymmetrische somatische hybriden in Araceae of in andere gewassen.

Genoomgroottes warden gemeten, karyotypes warden opgesteld, en een algemeen FISH protocol werd voor het eerst verwezenlijkt in 6 Araceae geslachten: *Anthurium, Monstera, Philodendron, Spathiphyllum, Syngonium* en *Zantedeschia*. 45S rDNA repeats werden gevisualiseerd m.b.v. conventionele FISH in al deze genera. In *S. wallisii* konden op deze manier ook 5S rDNA repeats worden aanschouwelijk gemaakt. We stelden een heel gevoelig Tyr-FISH protocol op dat in staat was 5S rRNA genen in *Z. elliottiana* te visualiseren. Ook verzamelden we informatie over op B-chromosoom lijkende structuren *A. andreanum*.

Eén van de belangrijkste parameters voor succesvolle protoplast regeneratie is het type donormateriaal. We induceerden de vorming van calli, die konden gebruikt worden als protoplastbron en als uitgangsmateriaal voor celsuspensies. Zowel compacte calli, op *S. wallisii* bladstelen en afgebleekte internodiën als losse calli, van *Zantedeschia spp,* werden hiervoor aangewend. Het laatste type callus kon gebruikt worden om celsuspensies op te starten, maar deze deelden niet snel genoeg. Protoplasten warden geïsoleerd uit uiteenlopende plantenorganen en -weefsels zoals bladeren, bladstelen, meristemen, knollen

en de hierboven vermelde calli. *S. wallisii* en *Z. elliottiana* protoplasten waren het uitgangsmateriaal om chemische en elektrische fusie te optimaliseren.

We verwezenlijkten protoplast regeneratie tot microkolonies in *Anthurium, Spathiphyllum* en *Zantedeschia*. Agarose bead cultuur en groei in het donker stimuleerden de deling van protoplasten. Een minerale samenstelling gebaseerd op KM zouten en verrijkt met 0.09 M sucrose of 0.08 M glucose, 0.45 μM 2, 4-D, 2.22 μM BAP en 2.69 μM NAA was geschikt. Een stapsgewijze, wekelijkse vermindering van de mannitol concentratie van 0.31 M tot 0 M bevorderde de vorming van microkolonies. De optimale dichtheid voor protoplast deling was 10^5 PP/mL. Het toevoegen van 0.1% Pluronic® F-68 of 0.05% Tween 20 induceerde kolonievorming binnen 2 weken.

In een eerste stap naar MMCT uitgaand van ontwikkelende microsporen van *S. wallisii*, optimaliseerden we parameters zoals bloemleeftijd en type, blootstellingduur en concentratie van de mitotische inhibitor. We bepaalden welke inhibitors de meeste microkernen induceerden en testten de efficiëntie van het protocol in verschillende genotypes.

Data uit onze karyotype studies, zoals chromosoommorfologie and asymmetrieverschillen, kunnen nuttig zijn om geschikte ouderplanten voor toekomstige interspecifieke of intergenerische kweekprogramma's binnen Araceae te selecteren, en om mogelijke hybriden van hun ouders te onderscheiden. FISH kan hybriden identificeren en vormt een basis voor de toepassing van GISH om de exacte chromosomale samenstelling van deze fusieproducten te analyseren. Bijkomend onderzoek zal definitief uitsluitsel moeten geven over de aanwezigheid van B-chromosomen in *A. andreanum*.

De ontwikkeling van een systeem voor inductie van embryogeen callus is mogelijk nuttig voor productie van artificieel zaad en commerciële plantproductie. Wat betreft protoplastregeneratie, dient het potentieel van suspensiecellen als uitgangsmateriaal nog steeds getest te worden, net als verschillende regeneratiemethoden. Allereerst moet een vlot delende celsuspensiecultuur worden verwezenlijkt. Een meer doordachte aanpak van protoplastregeneratie, in plaats van uitsluitend trial en error gebaseerd onderzoek, is onontbeerlijk.

Ons micronucleatiesysteem kan gebruikt worden om grote hoeveelheden meïotische microprotoplasten te isoleren. Verder flowcytometrisch onderzoek moet een techniek vinden om de genoomfragmentatie te kwantificeren. Deze scriptie kan ook een aanzet geven voor de productie van mitotische protoplasten, na de verwezenlijking van vlot delende celsuspensieculturen. Samengevat biedt dit onderzoekswerk nuttige inzichten met het oog op de productie van asymmetrische somatische hybriden binnen Araceae en andere economisch interessante gewassen.

Abas, Y., Touil, N., Kirsch-Volders, M., Angenon, G., Jacobs, M. and Famelaer, I. (2007). Evaluation of UV damage at DNA level in *Nicotiana plumbaginifolia* protoplasts using single cell gel electrophoresis. Plant Cell Tiss Organ Cult., 91:145–154.

Abbasi, F.M., Brar, D.S., Carpena, A.L., Fukui, K., and Khush, G.S. (1999). Detection of autosyndetic and allosyndetic pairing among A and E genomes of Oryza through genomic *in situ* hybridization. RGN, 16:24-25.

Abdullah, R., Cocking, E. C., and Thompson, J. A. (1986). Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Biotechnol., 4:1087-1090.

Acebey, A., Krömer, T., Maass, B L., and Kessler, M. (2010). Ecoregional distribution of potentially useful species of Araceae and Bromeliaceae as non-timber forest products in Bolivia. Biodiversity and Conservation, 19:2553-2564.

Akhmanova, A., Verkerk, T., Langeveld, A., Grosveld, F., and Galjart, N. (2000). Characterisation of transcriptionally active and inactive chromatin domains in neurons. J. Cell. Sci., 24:4463-4474.

Ali, H. B. M., M. A. Lysak, and I. Shubert (2004). Genomic *in situ* hybridisation in plants with small genomes is feasible and elucidated the chromosomal parentage in interspecific *Arabidopsis* hybrids. Genome, 47:954-960.

An, H., Jin, S., Kang, B., and Park, H. (2008). Production of somatic hybrids between satsuma mandarin (*Citrus unshiu*) and navel orange (*Citrus sinensis*) by protoplast fusion. Journal of Plant Biology, 51:186-191.

Anaïs, G., Darrasse, A., and Prior, P. (1998). Breeding anthuriums (*Anthurium andreanum* L.) for resistance to bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae*. In XIX International Symposium on Improvement of Ornamental Plants, 508:135-140.

Anamthawat-Jónsson, K., Schwarzacher, T., Leitch, A. R., Bennett, M. D., and Heslop-Harrison, J. S. (1990). Discrimination between closely related Triticeae species using genomic DNA as a probe. Theoretical and Applied Genetics, 79:721-728.

Appels, R., and Honeycutt, R. L. (1986). rDNA: evolution over a billion years. In: S. K. Dutta (ed.), DNA systematics II. CRC Press Inc., Boca Raton. Plant DNA, 81-135.

Asano, Y., and Sugiura, K. (1990). Plant regeneration from suspension culture-derived protoplasts of *Agrostis alba* L. (redtop). Plant Science, 72:267-273.

Assani, A., Chabane, D., Haicour, R., Bakry, F., Wenzel, G., and Foroughi-Wehr, B. (2005). Protoplast fusion in banana (*Musa* spp.): Comparison of chemical (PEG:Polyethylene glycol) and electrical procedure. Plant Cell Tissue and Organ Culture, 83:145-151.

Assani, A., Haïcour, R., Wenzel. G., Foroughi-Wehr, B., Bakry, F., Côte, F.X., Ducreux, G., Ambroise, A., and Grapin, A. (2002). Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (*Musa* spp.). Plant science, 162:355-362.

Azad, M., Yokota, S., Ishiguri, F., and Yoshizawa, N. (2006). Plant regeneration from mesophyll protoplasts of a medicinal plant, *Phellodendron amurense* Rupr. In Vitro Cellular and Developmental Biology-Plant, 42:502-507.

Azqueta, A., and Collins, A. R. (2013). The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Archives of Toxicology, 87:1-20.

Bajaj Y (1989). Plant protoplasts and genetic engineering. Biotechnology in Agriculture and Forestry Vol 9. Springer-Verlag, Heidelberg, New York.

Barre, P., Layssac, M., D'Hont, A., Louarn, J., Charrier, A., Hamon, S., and Noirot, M. (1998). Relationship between parental chromosomic contribution and nuclear DNA content in the coffee interspecific hybrid *C. pseudozanguebariae* x *C. liberica* var 'dewevrei'. Theor. Appl. Genet., 96:301-305.

Bauer-Weston, B., Keller, W., Webb, J., and Gleddie, S. (1993). Production and characterization of asymmetric somatic hybrids between *Arabidopsis thaliana* and *Brassica napus*. Theor. Appl. Genet., 86:150-158.

Begum, K. N., and Alam, S. S. (2009). Karyotype analysis in three morphological forms of *Colocasia fallax* Schott. Cytologia, 74:209-214.

Belgian Biosafety Server: Directive 2001/18/EC:
http://www.biosafety.be/GB/Dir.Eur.GB/Del.Rel./2001_18/2001_18_A1A.html

Binsfeld, P., Wingender, R., and Schnabl, H. (2000). Characterization and molecular analysis of transgenic plants obtained by microprotoplast fusion in sunflower. Theor. Appl. Genet., 101:1250-1258.

Bliss, B. J., and Suzuki, J. Y. (2012). Genome size in *Anthurium* evaluated in the context of karyotypes and phenotypes. AoB plants, 2012, pls006.

Böhmer, P., Meyer, B., and Jacobsen, H. J. (1995). Thidiazuron-induced high frequency of shoot induction and plant regeneration in protoplast derived pea callus. Plant Cell Reports, 15:26-29.

Borgato, L., Conicella, C., Pisani, F., and Furini, A. (2007a). Production and characterization of arboreous and fertile *Solanum melongena* plus *Solanum marginatum* somatic hybrid plants. Planta, 226:961-969.

Borgato, L., Pisani, F., and Furini, A. (2007b). Plant regeneration from leaf protoplasts of *Solanum virginianum* L. (Solanaceae). Plant Cell Tissue and Organ Culture, 88:247-252.

Bravo, J., and Evans, D. (1985). Protoplast fusion for crop improvement. Plant Breeding Reviews, 3:193-218.

Buongiorno-Nardelli, M., and Amaldi, F. (1969). Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. Nature, 225:946-947.

Cappelle, C., Morchen, M., Hilbert, J., and Rambaud, C. (2007). Regeneration and molecular characterization of a male sterile interspecific somatic hybrid between *Cichorium intybus* and *C. endivia*. Plant Science, 172:596-603.

Cai, X., Liu, H. and Guo, W. (2006). GFP expression as an indicator of somatic hybrids between transgenic Satsuma mandarin and calamondin at embryoid stage. Plant Cell Tissue and Organ Culture, 87:245-253.

Cai, Y., Xiang, F., Zhi, D., Liu, H., and Xia, G. (2007). Genotyping of somatic hybrids between *Festuca arundinacea* Schreb. and *Triticum aestivum* L. Plant Cell Reports, 26:1809-1819.

Cao, M., Sleper, D. A., Dong, F., and Jiang, J. (2000). Genomic *in situ* hybridization (GISH) reveals high chromosome pairing affinity between *Lolium perenne* and *Festuca mairei*. Genome, 43:398-403.

Cardi, T., Puite, K. J., and Ramulu, S. K. (1990). Plant regeneration from mesophyll protoplasts of *Solanum commersonii* dun. Plant Science, 70:215-221.

Carlson, P. S., Smith, H.H., and Dearing, R. (1972). Parasexual interspecific plant hybridization. Proceedings of the National Academy of Sciences of the United States of America, 69:2292-2294.

Centraal Bureau voor de Statistiek, Land en tuinbouwcijfers (2012). LEI Wageningen Universiteit en Researchcentrum,'s-Gravenhage. (http://www.cbs.nl).

Cerbah, M., Mortreau, E., Brown, S., Siljak-Yakovlev, S., Bertrand, H., and Lambert, C. (2001). Genome size variation and species relationships in the genus *Hydrangea*. Theor. Appl. Genet., 103:45-51.

Castelblanque, L., Garcia-Sogo, B., Pineda, B., and Moreno, V. (2010). Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis. Plant Cell, Tissue and Organ Culture, 100:107-112.

Chan, L.K., Tan, C. M., and Chew, G.S. (2003). Micropropagation of the Araceae ornamental plants. Acta Hort. (ISHS), 616:383-390.

Chabane, D., Assani, A., Bouguedoura, N., Haicour, R., and Ducreux, G. (2007). Induction of callus formation from difficile date palm protoplasts by means of nurse culture. Comptes Rendus Biologies, 330:392-401.

Chen, L., Zhang, M., Xiao, Q., Wu, J., and Hirata, Y. (2004a). Plant regeneration from hypocotyl protoplasts of red cabbage (*Brassica oleracea*) by using nurse cultures. Plant Cell Tissue and Organ Culture, 77:133-138.

Chen, S., Xia, G., Quan, T., Xiang, F., Jin, Y., and Chen, H. (2004b). Introgression of salt-tolerance from somatic hybrids between common wheat and *Thinopyrum ponticum*. Plant Sci., 167:773–779.

Chen, A., Zhuang, M., and Huang, M. (2007). Karyotype of the two species of genus *Dieffenbachia* (Araceae). Acta Botanica Yunnanica, 29:441-441.

Chen, J., McConnell, D. B., Henny, R. J., and Norman, D. J. (2010). The foliage plant industry. Horticultural Reviews, 31: 45-110.

Chen. J., McConnell, D. B., and Henny, R. J. (2005). The world foliage plant industry. Chronica Hort, 45:9-15.

Cheng, A., and Xia, G. (2004). Somatic hybridisation between common wheat and Italian ryegrass. Plant Science, 166:1219-1226.

Cheng, A., Cui, H., and Xia, G. (2006). Construction of a primary RH panel of Italian ryegrass genome via UV-induced protoplast fusion. Plant Biology, 8:673-679.

Chevalier, J., Yi, J., Michel, O., and Tang, X. M. (1997). Biotin and digoxigenin as labels for light and electron microscopy *in situ* hybridization probes: Where do we stand? Journal of Histochemistry and Cytochemistry, 45:481-491.

Chevre, A., Eber, F., Margale, E., Kerlan, M. C., Primard, C., Vedel, F., Delseny, M., and Pelletier, G. (1994). Comparison of somatic and sexual *Brassica napus - Sinapis alba* hybrids and their progeny by cytogenetic studies and molecular characterization. Genome, 37:367-374.

Cho, M. S., and Zapata, F. J. (1988). Callus formation and plant regeneration in isolated pollen culture of rice *Oryza sativa* L. cv. Taipei 309. Plant Science, 58:239-244.

Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Yin, K. C., Chu, C. Y., and Bi, F. Y. (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. (Peking), 18:659-668.

Cocking, E. C. (1960). A method for the isolation of plant protoplasts and vacuoles. Nature, 187:927-929.

Cocking, E. C. (1970). Virus uptake, cell wall regeneration and virus multiplication in isolated plant protoplasts. Int. Rev. Cytol., 28:89-124.

Cocking, E. C. (1972). Plant cell protoplasts-isolation and development. Ann. Rev. Plant Physiol., 23:29-50.

Convention on Biological Diversity. (1992). Secretariat, C. B. D. Convention on Biological Diversity. (http://www.cbd.int/convention)

Cornelis, D. (1997a). U.S. Patent No. PP9,944. Washington, DC: U.S. Patent and Trademark Office.

Cornelis, D. (1997b). U.S. Patent No. PP09,943. Washington, DC: U.S. Patent and Trademark Office.

Cove, D. J. (1979). The uses of isolated protoplasts in plant genetics. Heredity, 43(3):295-314.

Croat, T. B. (1982). A revision of *Syngonium* (Araceae). Ann. Missouri Bot. Gard., 68:565-651.

Croat, T. B. (1983). A reversion of the genus *Anthurium* (Araceae) of Mexico and Central America. Part I. Mexico and Middle America. Ann. Missouri Bot. Gard., 70:211-420.

Croat, T. B. (1986). A reversion of the genus *Anthurium* (Araceae) of Mexico and Central America. Part II. Panama, Nonogr. Syst. Bot. Mo. Bot Gard., 14:1-204.

Croat, T. B. (1992). Species diversity of Araceae in Colombia: preliminary survey. Ann. Mo. Bot. Gard., 79:17-28.

Croat, T. B. (1997). A revision of *Philodendron* subgenus *Philodendron* (Araceae) of Central America. Annals of the Missouri Botanical Garden Press, 84:311-704.

Croat, T. B. (1998). History and current status of systematic research with Araceae. Aroideana, 21:26-145.

Cui, H., Yu, Z., Deng, J., Gao, X., Sun, Y., and Xia, G. (2009). Introgression of bread wheat chromatin into tall wheatgrass via somatic hybridization. Planta, 229:323-330.

Dagne, K., Cheng, B., and Heneen, W. K. (2000). Number and sites of rDNA loci of *Guizotia abyssinica* (L. f.) Cass. as determined by fluorescence *in situ* hybridization. Hereditas, 132:63-65.

Damon, E. (1957). Investigations into the cyto-morphogenetic effects of colchicine on varieties of *Sorghum vulgare*. Proc Okla Acad Sci., 38:9-13.

Davey, M. R., and Kumar, A. (1983). Higher plant protoplasts - retrospect and prospect. Int. Rev. Cytol. Suppl., 16:219-299.

Davey, M. R., Anthony, P., Power, J. B., and Lowe K. C. (2005). Plant protoplasts: status and biotechnological perspectives. Biotechnology Advances, 23:131-171.

Davey, M., and Power, J. (1988). Aspects of protoplast culture and plant regeneration. Plant Cell, Tissue and Organ Culture, 12:115-125.

Davey, M., Anthony, P., Patel, D., and Power, J. (2010). Plant protoplasts: isolation, culture and plant regeneration. In: Davey, M., and Anthony, P. (Eds) Plant cell culture: essential methods, John Wiley and Sons, New York, USA, Chapter 9:153-173.

De Bona, C., Gould, J., Miller, J., Stelly, D., and Louzada, E. (2009a). *Citrus* asymmetric somatic hybrids produced via fusion of gamma-irradiated and iodoacetamide-treated protoplasts. Pesquisa Agropecuaria Brasileira, 44:454-462.

De Bona, C., Stelly, D., Miller, J., and Louzada, E. (2009b). Fusion of protoplasts with irradiated microprotoplasts as a tool for radiation hybrid panel in *Citrus*. Pesq. Agropec. Bras., 44:1616-1623.

De Clercq, D., Van Trappen, S., Cleenwerck, I., Ceustermans, A., Swings, J., Coosemans, J., and Ryckeboer, J. (2006). *Rhodanobacter spathiphylli* sp. nov., a gammaproteobacterium isolated from the roots of *Spathiphyllum* plants grown in a compost-amended potting mix. International journal of systematic and evolutionary microbiology, 56:1755-1759.

De Jong, H. (2003). Visualising DNA domains and sequences by microscopy: a fifty-year history of molecular cytogenetics. Genome, 46:943-946.

De Nettancourt, D. (1977). Incompatibility in angiosperms. Sexual plant reproduction, 10:185-199.

De Verna, J. W., Myers, J. R., and Collins, G. B. (1987). Bypassing prefertilisation barriers to hybridization in Nicotiana using *In vitro* pollination and fertilisation. Theoretical and Applied Genetics, 73:665-671.

De Vries, H. (1900). Sur la loi de disjonction des hybrides. Comptes Rendus de l'Academie des Sciences (Paris), 130:845-847.

Debergh, P. C. (1992). The *In vitro* techniques: their contribution to breeding and multiplication of ornamentals. In International Workshop on Floriculture and Nursery Industries and Environment, 353:122-133.

Deng, J., Cui, H., Zhi, D., Zhou, C., and Xia, G. (2007). Analysis of remote asymmetric somatic hybrids between common wheat and *Arabidopsis thaliana*. Plant Cell Reports, 26:1233-1241.

Deo, P. C., Tyagi, A. P., Taylor, M., Becker, D. K., and Harding, R. M. (2009). Improving taro (*Colocasia esculenta* var. esculenta) production using biotechnological approaches. The South Pacific Journal of Natural and Applied Sciences, 27:6-13.

Deryckere, D., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2012). Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the *Cichorium* genus. Plant cell reports, 31:2261-2269.

Deryckere, D., De Keyser, E., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2013). High-resolution melting analysis as a rapid and highly sensitive method for *Cichorium* plasmotype characterization. Plant Mol Biol Rep. 31:731-740.

Devanand, P. S., Chen, J., Henny, R. J., and Chao, C. C. T. (2004). Assessment of Genetic Relationships among *Philodendron* cultivars using AFLP markers. Journal of the American Society for Horticultural Science, 129:690-697.

Dewitte, A., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2010a). Induction of 2n pollen formation in *Begonia* by trifluralin and N_2O treatments. Euphytica, 171:283-293.

Dewitte, A., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2010b). Meiotic aberrations during 2n pollen formation in *Begonia*. Heredity, 104:215-223.

Dobzhansky, T. (1951). Genetics and the origin of species, 3rd edn., Columbia Univ. Press, New York.

Doherty, A. M. O., and Fisher, E. M. C. (2003). Microcell-mediated chromosome transfer (MMCT): small cells with huge potential. Mammalian Genome, 14:583-592.

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

Dolezel, J., and Bartos, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot., 95:99-110.

Doležel, J., Doleželová, M., and Novák, F. J. (1994). Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminate* and *M. balbisiana*). Biol. Plantarum, 36:351-357.

Doležel, J., Greilhuber, J., Lucretti, S., Meister, A., Lysak, M. A., Nardi, L., and Obermayer, R. (1998). Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann. Bot., 82:17-26.

Doležel, J., Sgorbati, S., and Lucretti, S. (1992). Comparison of three DNA fluorochromes for flow-cytometric estimation of nuclear DNA content in plants. Physiol Plantarum, 85:625-631.

Dong, F., McGrath, J. M., Helgeson, J. P., and Jiang, J. (2001). The genetic identity of alien chromosomes in potato breeding lines revealed by sequential GISH and FISH analyses using chromosome-specific cytogenetic DNA markers. Genome, 44:729-734.

Du, X., Ge, X., Zhao, Z., and Li, Z. (2008). Chromosome elimination and fragment introgression and recombination producing intertribal partial hybrids from *Brassica napus* x *Lesquerella fendleri* crosses. Plant Cell Reports 27: 361-271.

Du, X., Ge, X., Yao, X., Zhao, Z., and Li, Z. (2009). Production and cytogenetic characterization of intertribal somatic hybrids between *Brassica napus* and *Isatis indigotica* and backcross progenies. Plant Cell Reports, 28:1105-1113.

Duquenne, B., Eeckhaut, T., Werbrouck, S., and Van Huylenbroeck, J. (2007). Effect of enzyme concentrations on protoplast isolation and protoplast culture of *Spathiphyllum* and *Anthurium*. Plant Cell Tissue Organ Cult., 91:165-173.

Duquenne, B., Eeckhaut, T., Werbrouck, S., and Van Huylenbroeck, J. (2006). *In vitro* somatic embryogenesis and plant regeneration in *Zantedeschia* hybrids. Plant Cell, Tissue And Organ Culture, 87:329-331.

Durieu, P., and Ochatt, S. J. (2000). Efficient intergeneric fusion of pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplasts. Journal of Experimental Botany, 51:1237-1242.

Eeckhaut, T., Lakshmanan, P. S., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Progress in plant protoplast research. Planta, Online:1-13.

Eeckhaut, T., Lakshmanan, P., Van Huylenbroeck, J., and Duquenne, B. (2008). Development of microcolonies in protoplast culture of *Spathiphyllum wallisii*. In VI International Symposium on *In vitro* Culture and Horticultural Breeding, 829:51-54.

Eeckhaut, T., Leus, L., and Van Huylenbroeck, J. (2005). Exploitation of flow cytometry for plant breeding. Acta Phys. Plant., 27:743-750.

Eeckhaut, T., Van Laere, K., De Riek, J., and Van Huylenbroeck, J. (2006). Overcoming interspecific barriers in ornamental plant breeding. In: Floriculture, ornamental and plant biotechnology. Floriculture, ornamental and plant biotechnology, 540-551.

Eeckhaut, T., Werbrouck, S., Dendauw, J., Van Bockstaele, E., and Debergh, P. (2001). Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell Tissue Organ Cult., 67:181-189.

Eeckhaut, T., Werbrouck, S., Leus, L., Van Bockstaele, E., and Debergh, P. (2004). Chemically induced polyploidization of *Spathiphyllum wallisii* Regel through somatic embryogenesis. Plant Cell Tissue Organ Cult., 78:241-246.

Eriksson, T., and Jonasson, K. (1969). Nuclear division in isolated protoplasts from cells of higher plants grown *in vitro*. Planta, 89:85-89.

Escalante, A., Imanishi, S., Hossain, M., Ohmido, N., and Fufui, K. (1998). RFLP analysis and genomic *in situ* hybridisation (GISH) in somatic hybrids and their progeny between *Lycopersicon esculentum* and *Solanum lycopersicoides*. Theor. Appl. Genet., 96:719-726.

Fahleson, J., Dixelius, J., Sundberg, E., and Glimelius, K. (1988). Correlation between flow cytometric determination of nuclear DNA content and chromosome number in somatic hybrids within Brassicaceae. Plant cell reports, 7:74-77.

Fahleson, J., Eriksson, I., Landgren, M., Stymme, S., and Glimelius, K. (1994). Intertribal somatic hybrids between *Brassica napus* and *Thlaspi perfoliatum* with high content of the *T. perfoliatum*-specific nervonic acid. Theor. Appl. Genet., 87:795-804.

Famelaer, I., Gleba, Y., Sidorov, V., Kaleda, V., Parokonny, A., Boryshuk, N., Cherep, N., Negrutiu, I., and Jacobs, M. (1989). Intrageneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Nicotiana sylvestris* obtained by gamma-fusion. Plant Sci., 61:105-117.

Famelaer, I., Verhoeven, H. A., Dijkhuis, P., and Ramulu, K. S. (2007). A study of the process of synchronisation and micronucleation in *Beta vulgaris* and the monitoring of an isolation procedure for micro-nuclei and micro-protoplasts by confocal laser scanning microscopy and flow cytometry. Plant Cell Tissue and Organ Culture, 90:169-179.

Fedak, G. (1999). Molecular aids for integration of alien chromatin through wide crosses. Genome, 42: 584-591.

Flavell, R. B. (1986). The structure and control of expression of ribosomal RNA genes. Oxf. Surv. Plant. Mol. Cell. Biol., 3:252-274.

Fleck, J., Durr, A., Fritsch, C., Vernet, T., and Hirth, L. (1982). Osmotic-shock 'stress proteins' in protoplasts of *Nicotiana sylvestris*. Plant Science Letters, 26:159-165.

Forsberg, J., Dixelius, C., Lagercrantz, U., and Glimelius, K. (1998). UV dose-dependent DNA elimination in asymmetric somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*. Plant Sci., 131:65-76.

Fournier, R.E., and Ruddle, F.H. (1977). Stable association of the human transgenome and host murine chromosomes demonstrated with trispecific microcell hybrids. Proc Natl Acad. Sci., 74:3937-3941.

Franklin-Tong, V. E., and Franklin, F. C. H. (2000). Self-incompatibility in *Brassica*: the elusive pollen S gene is identified. Plant Cell, 12:305-308.

Frearson, E. M., Power, J.B., and Cocking, E. C. (1973). The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol., 33:130-137.

Fu-Hua, B. I. A. N., Zhong-Lang, W. A. N. G., Heng, L. I., and Kai-Yun, G. U. A. N. (2001). Karyotypic studies on two species in the genus *Sauromatum* (Araceae)[J]. Acta Botanica Yunnanica, 4: 011.

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

Gall, J. G., and Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proceedings of the National Academy of Sciences, 63:378-383.

Galston, A. W., and Sawhney, R. K. (1990). Polyamines in plant physiology. Plant physiology, 94:406-410.

Gamborg, O. L., Miller, R. A., and Ojima, O. (1968). Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell. Res., 50:151-158.

Gancel, A. L., Grimplet, J., Sauvage, F. X., Ollitrault, P., and Brillouet, J. M. (2006). Predominant expression of diploid mandarin leaf proteome in two citrus mandarin-derived somatic allotetraploid hybrids. Journal of agricultural and food chemistry, 54(17):6212-6218.

Garcia, G. M., Stalker, H. T., and Kochert, G. (1995). Introgression analysis of an interspecific hybrid population in peatus (*Archis hypogaea* L.) using RFLP and RAPD markers. Genome, 38:166-176.

Ge, T., Lin, X., Qin, F., Yu, S., and Yu, Y. (2006). Protoplast electrofusion between common wheat (*Triticum aestivum* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) and regeneration of mature cybrids. *In vitro* Cellular and Developmental Biology-Plant, 42:179-187.

Geerts, P., Druart, P., Ochatt, S., and Baudoin, J. (2008). Protoplast fusion technology for somatic hybridisation in *Phaseolus*. Biotechnologie Agronomie Societe et Environnement, 12:41-46.

Gerlach, W. L., and Bedbrook, J. R. (1979). Cloning and characterisation of ribosomal RNA genes from wheat and barley. Nucleic Acids Res., 7:1869-1885.

Gerlach, W. L., and Dyer, T. A. (1980). Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Research, 8:4851-4865.

Ghimire, B. K., Yu, C. Y., Kim, H. J., and Chung, I. M. (2012). Karyotype and nucleic acid content in *Zantedeschia aethiopica* Spr. and *Zantedeschia elliottiana* Engl. African Journal of Biotechnology, 11:11604-11609.

Gleba, Y. Y., Momot, V. P., Cherep, N. N., and Skarzynskaya, M. V. (1982). Intertribal hybrid cell lines of *Atropa belladonna* (X) *Nicotiana chinensis* obtained by cloning individual protoplast fusion products. TAG Theoretical and Applied Genetics, 62:75-79.

Gleba, Y., and Sytnik, K. (1984). Protoplast fusion: genetic engineering in higher plants. In: Shoeman, R. (Ed) Monographs on theoretical and applied genetics. Springer-Verlag, Berlin, Germany, 115-161.

Gleba, Y., Hinnisdaels, S., Sidorov, V., Kaleda, V.., Parokonny, A., Boryshuk, N., Cherep, N., Negrutiu, I., and Jacobs, M. (1988). Intergeneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Atropa belladonna* obtained by gamma-fusion. Theor. Appl. Genet., 76:760-766.

Glimelius, K., Eriksson, T., Grafe, R., and Müller, A. J. (1978). Somatic hybridization of nitrate reductase deficient mutants of *Nicotiana tabacum* by protoplast fusion. Physiologia Plantarum, 44:273-277.

Goktepe, F., Seijo, T., Deng, Z., Harbaugh, B. K., Peres, N. A., and McGovern, R. J. (2007). Toward breeding for resistance to *Fusarium* tuber rot in *Caladium:* inoculation technique and sources of resistance. Hort. Science, 42:1135-1139.

Goodpasture, C., and Bloom, S. E. (1975). Visualisation of nucleolar organiser regions in mammalian chromosomes using silver staining. Chromosoma, 53:37-50.

Goryachkina, O. V., Badaeva, E. D., Muratova, E. N., and Zelenin, A. V. (2013). Molecular cytogenetic analysis of Siberian *Larix* species by fluorescence *in situ* hybridization. Plant Systematics and Evolution, 299:471-479.

Grosser, J. W., Ollitrault, P., and Olivares-Fuster, O. (2000). Somatic hybridization in *Citrus*: an effective tool to facilitate variety improvement. *In Vitro* Cellular and Developmental Biology-Plant, 36:434-449.

Grosser, J., and Gmitter, F. (2005). 2004 SIVB Congress Symposium Proceedings Thinking Outside the Cell-Applications of somatic hybridization and cybridization in crop improvement, with *Citrus* as a model. *In vitro* Cellular and Developmental Biology Plant, 41:220-225.

Grosser, J., Chandler, J., and Duncan, L. (2007). Production of mandarin plus pummelo somatic hybrid citrus rootstocks with potential for improved tolerance/resistance to sting nematode. Scientia Horticulturae, 113:33-36.

Grosser, J., and Gmitter, F. (2011). Protoplast fusion for production of tetraploids and triploids: applications for scion and rootstock breeding in *Citrus*. Plant Cell Tiss. Organ Cult., 104:343-357.

Grosser, J., Calovic, M., and Louzada, E. (2010). Protoplast fusion technology-somatic hybridization and cybridization. In: Davey, M., and Anthony, P. (Eds) Plant cell culture: essential methods. John Wiley and Sons, New York, USA, 175-198.

Grzebelus, E., Szklarczyk, M., and Baranski, R. (2012a). An improved protocol for plant regeneration from leaf and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss. Organ Cult., 109:101-109.

Grzebelus, E., Szklarczyk, M., Gren, J., Sniegowska, K., Jopek, M., Kacinska, I., and Mrozek, K. (2012b). Phytosulfokine stimulates cell divisions in sugar beet (*Beta vulgaris* L.) mesophyll protoplast cultures. Plant Growth Regul., 67:93-100.

Guangmin, X., and Huimin, C. (1996). Plant regeneration from intergeneric somatic hybridization between *Triticum aestivum* L. and *Leymus chinensis* (Trin.) Tzvel. Plant Science, 120:197-203.

Guo, W. W., Cai, X. D., and Grosser, J. W. (2004). Somatic cell cybrids and hybrids in plant improvement. Molecular Biology and Biotechnology of Plant Organelles, Chapter 3:635-659.

Guo, W., Wu, R., Cheng, Y., and Deng, X. (2007a). Production and molecular characterization of *Citrus* intergeneric somatic hybrids between red tangerine and citrange. Plant Breeding, 126:72-76.

Guo, Y., Bai, J., and Zhang, Z. (2007b). Plant regeneration from embryogenic suspension-derived protoplasts of ginger (*Zingiber officinale* Rosc.). Plant Cell Tissue and Organ Culture, 89:151-157.

Guri, A., Dunbar, L. J., and Sink, K. C. (1991). Somatic hybridization between selected *Lycopersicon* and *Solanum* species. Plant Cell Rep., 10:76-80.

Guo, W. and Grosser, J. (2005). Somatic hylbrid vigor in *Citrus*: Direct evidence from protoplast fusion of an embryogenic callus line with a transgenic mesophyll parent expressing the GFP gene. Plant Science, 168:1541-1545.

Hall, R. D., Krens, F. A., and Rouwendal, G. J. A. (1992). DNA radiation-damage and asymmetric somatic hybridization - is UV a potential substitute or supplement to ionizing-radiation in fusion experiments. Physiologia Plantarum, 85:319-324.

Hall, R. D., Riksen-Bruinsma, T., Weyens, G., Lefèbvre, M., Dunwell, J. M., and Krens, F. A. (1996). Stomatal guard cells are totipotent. Plant physiology, 112:889-892.

Hamidah, M., Debergh, P. C., and Abdul Karim, A. G. (1994). Mass propagation of *Anthurium andreanum* L. through meristemoid clusters and retarded shoots. Mededelingen-Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen. Universiteit Gent, 59.

Hamidah, M., Debergh, P. C., and Abdul Karim, A. G. (1995a). Nodule culture of *Anthurium scherzerianum* Schott. Mededelingen-Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent, 60.

Hamidah, M., Debergh, P. C., and Abdul Karim, A. G. (1995b). Somatic embryogenesis of *Anthurium scherzerianum* Schott. Mededelingen-Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent, 60.

Hamidah, M., Debergh, P., and Karim, A. G. A. (1997a). Cyclic somatic embryogenesis of *Anthurium scherzerianum* Schott. Acta Horticulturae, 447:123-124.

Hamidah, M., Karim, A. G. A., and Debergh, P. (1997b). Somatic embryogenesis and plant regeneration in *Anthurium scherzerianum*. Plant Cell, Tissue and Organ Culture, 48:189-193.

Han, L., Zhou, C., Shi, J., Zhi, D., and Xia, G. (2009). Ginsenoside Rb(1) in asymmetric somatic hybrid calli of *Daucus carota* with *Panax quinquefolius*. Plant Cell Reports, 28:627-638.

Hansen, L. N., and Earle, E. D. (1997). Somatic hybrids between *Brassica oleracea* L. and *Sinapis alba* L. with resistance to *Alternaria brassicae* (Berk) Sacc. Theor. Appl. Genet., 94:1078-1085.

Hansen, N. J. P., and Andersen, S. B. (1998). *In vitro* chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). Euphytica, 102:101-108.

Harms, C. T. (1983). Somatic incompatibility in the development of higher plant somatic hybrids. Quarterly Review of Biology, 58:325-353.

Hayward, A. C. (1972). A bacterial disease of *Anthurium* in Hawaii. Plant Dis. Rep., 56:904-908.

He, Y., Zhu, C., He, M., and Hao, S. (1996). Protoplast culture and plant regeneration of *Pinellia ternata.* Plant Cell Reports, 16:92-96.

Henny, R. J. (1977). Breeding, growing, and observing *Dieffenbachia* species and seedlings. In Proc. Fla. State Hort. Soc., 90:94-96.

Henny, R. J. (1988). Ornamental aroids: culture and breeding, in horticultural reviews, Volume 10 (ed J. Janick), John Wiley and Sons, Inc., NJ, USA, 10:1-33.

Henny, R. J. (1999). 'Red Hot' Anthurium. Hort. Science, 34:153-154.

Henny, R. J. and Chen, J. (2010). Cultivar development of ornamental foliage plants, Plant breeding reviews, Volume 23 (ed J. Janick), John Wiley and Sons, Inc., Oxford, UK, 23:245-290.

Hertel, C., Quader, H., Robinson, D., Marme, D. (1980). Anti-Microtubular herbicides and fungicides affect Ca^{2+} transport in plant-mitochondria. Planta, 149:336-340.

Hizume, M. (1994). Allodiploid nature of *Allium wakegi* Araki revealed by genomic *in situ* hy bridization and localization of 5S and 18S rDNAs. 遺伝学雑誌, 69:407-415.

Hoffmann, F., and Adachi, T. (1981). 'Arabidobrassica': chromosomal recombination and morphogenesis in asymmetric intergeneric hybrids. Planta, 153:586-893.

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

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

Houben, A., Banaei-Moghaddam, A. M., and Klemme, S. (2013). Biology and evolution of B chromosomes. Springer Vienna. In Plant Genome Diversity, 2:149-165.

Houben, A., Nasuda, S., and Endo, T. R. (2011). Plant B chromosomes. Humana Press. In Plant chromosome engineering, 701: 97-111.

Hu, Q., Hansen, L., Laursen, J., Dixelius, C., and Andersen, S. (2002). Intergeneric hybrids between *Brassica napus* and *Orychophragmus violaceus* containing traits of agronomic importance for oilseed rape breeding. Theor. and Appl. Gen., 105:834-840.

Imbrie-Milligan, C., Kamo, K. K., and Hodges, T. K. (1987). Microcallus growth from maize protoplasts. Planta, 171:58-64.

Inoue, K., Kato, T., Kunitake, H., and Yabuya, T. (2004). Efficient Production of Polyploid Plants via Protoplast Culture of *Iris fulva*. Cytologia, 69:327-333.

Ivancic, A. (1995). Abnormal and unusual inflorescences of taro, *Colocasia esculenta* (Araceae). Australian Journal of Botany, 43:475-489.

Jacobsen, E., De Jong, H., Kamstra, S.A., Van den Berg, P. M. M. M., and Ramanna, M. S. (1995). Genomic *in situ* hybridisation (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity, 74:250-257.

Jain, R. K., Davey, M. R., Cocking, E. C., and Wu, R. (1997). Carbohydrate and osmotic requirements for high-frequency plant regeneration from protoplast-derived colonies of Indica and Japonica rice varieties. Journal of exper. botany, 48:751-758.

Jain, R. K., Khehra, G. S., Lee, S-H., Blackball, N. W., Marchant, R., Davey, M. R., Power, J. B., Cocking, E. C. ,and Gosal, S. S. (1995). An improved procedure for plant regeneration from Indica and Japonica rice protoplasts. Plant Cell Reports 14:515-519.

Jansky, S. (2006). Overcoming hybridization barriers in potato. Plant brdng., 125:1-12.

Jelodar, N. B., Blackhall, N. W., Hartman, T. P. V., Brar, D. S., Khush, G., Davey, M. R., Cocking, E. C., and Power, J. B. (1999). Intergeneric somatic hybrids of rice (*Oryza sativa* L. (+) *Porteresia coarctata* (Roxb.) Tateoka). Theor. Appl. Genet., 99:570-577.

Ji, Y., Pertuzé, R., and Chetelat, R. T. (2004). Genome differentiation by GISH in interspecific and intergeneric hybrids of tomato and related nightshades. Chromosome Res., 12:107-116.

Jiang, J. M., and Gill, B.S. (1993). Sequential chromosome banding and *in situ* hybridisation analysis. Genome, 36:792-795.

Jiang, J., and B. S. Gill, (1994). Non-isotropic *in situ* hybridization and plant genome mapping: the first 10 years. Genome, 37:717-725.

Jiang, J., Zhao, X., Tian, W., Li, T., and Wang, Y. (2009). Intertribal somatic hybrids between *Brassica napus* and *Camelina sativa* with high linolenic acid content. Plant Cell Tissue and Organ Culture, 99:91-95.

Jiang, L., Cai, Y., Xia, G., and Xiang, F. (2012). Introgression of the heterologous nuclear DNAs and efficacious compositions from *Swertia tetraptera* Maxim. into *Bupleurum scorzonerifolium* Willd. via somatic hybridization. Protoplasma, 249:737-745.

John, H. L., Birnstiel, M. L., and Jones, K. W. (1969). RNA-DNA hybrids at the cytological level. Nature, 223: 912-913.

Johnson, A. A., and Veilleux, R. E. (2001). Somatic hybridization and applications in plant breeding. Plant Breeding Reviews, 20:167-226.

Jones, N., and Houben, A. (2003). B chromosomes in plants: escapees from the A chromosome genome?. Trends in plant science, 8:417-423.

Jones, R. N. (1995). Tansley review no. 85. B chromosomes in plants. New Phytologist, 131:411-434.

Jordan, M. A., and Wilson, L. (1998). Use of drugs to study role of microtubule assembly dynamics in living cells. Methods in enzymology, 298:252-276.

Kakoniova, D., Hlinkova, E., Liskova, D., Kollarova, K. (2010). Oligosaccharides induce changes in protein patterns of regenerating spruce protoplasts. Centr Eur J Biol, 5: 353-363.

Kamemoto, H., and Kuehnle, A. R. (1996). Breeding *Anthurium* in Hawaii. University of Hawaii press.

Kaneko, K., and Kamemoto, H. (1979). Karyotype and B chromosomes of *Anthurium warocqueanum.* Journal of Heredity, 70:271-272.

Kanwar, K., Bhardwaj, A., and Deepika, R. (2009). Efficient regeneration of plantlets from callus and mesophyll derived protoplasts of *Robinia pseudoacacia* L. Plant Cell Tissue and Organ Culture, 96:95-103.

Kao, K. N., and Michayluk, M. R. (1980). Plant regeneration from mesophyll protoplasts of alfalfa. Zeitschrift für Pflanzenphysiologie, 96:135-141.

Kao, K. N., Constabel, F., Michayluk, M. R., and Gamborg, O. L. (1974). Plant protoplast fusion and growth of intergeneric hybrid cells. Planta, 120:215-227.

Kao, K., and Michayluk, M. (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta, 126:105-110.

Keller, E. R. J., Shubert, I., Fuchs, J., and Meister. A. (1996). Interspecific crosses of onion with distant *Allium* species and characterisation of the presumed hybrids by means of flow cytometry, karyotype analysis and genomic *in situ* hybridisation. Theor. Appl. Genet., 92:417-424.

Kenton, A., Parokonny, A. S., Gleba, Y. Y., and Bennett, M. D. (1993). Characterisation of the *Nicotiana tabacum* L. genome by molecular cytogenetics. Mol. Gen. Genet., 240:159-169.

Khrustaleva, L. I., and Kik, C. (1998). Cytogenetical studies in the bridge cross *Allium cepa* x (*A. fistulosum* x *A. roylei*). Theoretical and Applied Genetics, 96(1):8-14.

Khrustaleva, L. I., and Kik, C. (2000). Introgression of *Allium fistulosum* into *A. cepa* mediated by *A. roylei.* Theoretical and Applied Genetics, 100(1): 17-26.

Khrustaleva, L. I., and Kik, C. (2001). Localization of single-copy T-DNA insertion in transgenic shallots (*Allium cepa*) by using ultra-sensitive FISH with tyramide signal amplification. The Plant Journal, 25:699-707.

Kielkowska, A., and Adamus, A. (2012). An alginate-layer technique for culture of *Brassica oleracea* L. protoplasts. In Vitro Cell.Dev.Biol. Plant, 48:265–273.

Kim, J., Bergervoet, J., Raemakers, C., Jacobsen, E., and Visser, R. (2005). Isolation of protoplasts, and culture and regeneration into plants in *Alstroemeria*. *In Vitro* Cellular and Developmental Biology – Plant, 41:505-510.

Kisaka, H., Kisaka, M., Kanno, A., and Kameya, T. (1998). Intergeneric somatic hybridization of rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.) by protoplast fusion. Plant Cell Reports, 17:362-367.

Kitamura, S., Inoua, M., Ohmido, N., and Fufui, K. (1997). Identification of parental chromosomes in the interspecific hybrids of *Nicotiana rustica* L., *N. tabacum* L. and *N. gossei* Domin, *N. tabacum* L. using genomic *in situ* hybridisation. Japan J. Breed., 47:67-70.

Klercker, J. A. F. (1892). Eine methode zur isolierung lebender protoplasten. Ofvers Vetensk-Akad Forh Stokh, 9:463-475.

Knoblauch, M., Hibberd, J. M., Gray, J. C., and Van Bel, A. J. E. (1999). A galinstan expansion femtosyringe for microinjection of eukaryotic organelles and prokaryotes. Nat. Biotechnol., 17:906-909.

Knobloch, I. W. (1972). Intergeneric hybridization in flowering plants. Taxon, 21:97-103.

Ko, J. M., Ju, J., Lee, S. H., and Cha, H. C. (2006). Tobacco protoplast culture in a polydimethylsiloxane-based microfuidic channel. Protoplasma, 227:237-240.

Kostenyuk, I., Lubaretz, O., Borisyuk, N., Voronin, V., Stöckigt, J., andGleba, Y. (1991). Isolation and characterization of intergeneric somatic hybrids in the Apocynaceae family. Theoretical and Applied Genetics, 82(6):713-716.

Kuehnle, A. (1997). Progress in protoplast isolation and culture from axenic tissues of hybrid *Anthurium*. Aroideana, 20:147-154.

Kuehnle, A. R., and N. C. Sugii, (1991). Introduction of tumors in *Anthurium andraeanum* by *Agrobacterium tumefaciens*. Hort. Science, 26:1325-1328.

Kuehnle, A. R., Chen, F. C., and Sugii, N. C. (2001). Transgenic *Anthurium*. In: Bajaj, Y. P. S. (ed.), Biotechnology in agriculture and forestry. Vol. 48. Transgenic crop III. Springer-Verlag, Berlin-Heidelberg, 48:4–15.

Küster, E. (1909). Über die Verschmelzung nachter protoplasten. Ber. Dtsch. Bot. Ges., 27:589-598.

Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. Planta, 228:367-381.

Kwasniewska, J., Grabowska, M., Kwasniewski, M., and Kolano, B. (2012). Comet-FISH with rDNA probes for the analysis of mutagen-induced DNA damage in plant cells. Environ. Mol. Mutagen., 53:369-75.

Kyozuka, J., Hayashi, Y., and Shimamoto, K. (1987). High frequency plant regeneration from rice protoplasts by novel nurse culture methods. Molecular and General Genetics MGG, 206:408-413.

Lakshmanan, P. S., Eeckhaut, T., Deryckere, D., Van Bockstaele, E. and Van Huylenbroeck, J. (2013). Asymmetric somatic plant hybridization: status and applications. American Journal of Plant Sciences, 4:1-10.

Lapitan, N. L. V. (1991). Organization and evolution of higher plant nuclear genomes. Genome, 35:171-181.

Lawrence, G. J., and Appels, R. (1986). Mapping the nucleolus organizer region, seed protein loci and isozyme loci on chromosome 1 R in rye. Theor. Appl. Genet., 71:742-749.

Leitch, I. J., and Heslop-Harrison, J. S. (1992). Physical mapping of the 18S-5.8S-26S rRNA genes in barley by *in situ* hybridization. Genome, 35:1013-1018.

Leitch, I. J., and Heslop-Harrison, J. S. (1994). Detection of digoxigenin-labelled DNA probes hybridising to plant chromosomes in situ. In: Isaac PG (ed.) Protocols for nucleic acid analysis by non-radioactive probes, Methods in molecular biology, volume 28, Humana Press, Totowa, NJ, 177-186.

Leitch, I. J., Leitch, A. R., and Heslop-Harrison, J. S. (1991). Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. Genome, 34:329-333.

Levan, A., Fregda, K., and Sandberg, A. A. (1964). Nomenclature for centromeric position on chromosomes. Hereditas, 52:201-220.

Li, Y., Guan, L., Lou, L., Cui, G., Yao, Y., Wang, H., and Chen, X. (1999). Laser-induced tobacco protoplast fusion. Science in China Series C:Life Sciences, 42:122-127.

Li, C., Xia, G., Xiang, F., Zhou, C., and Cheng, A. (2004). Regeneration of asymmetric somatic hybrid plants from the fusion of two types of wheat with Russian wildrye. Plant Cell Reports, 23:461-467.

Lian, Y., Lin, G., Zhao, X., and Lim, H. (2011). Production and genetic characterization of somatic hybrids between leaf mustard (*Brassica juncea*) and broccoli (*Brassica oleracea*). *In Vitro* Cellular and Developmental Biology-Plant, 47:289-296.

Lim, K. Y., Matyasek, R., Lichtenstein, C. P., and Leitch, A. R. (2000). Molecular cytogenetic analyses and phylogenetic studies in the *Nicotiana* section Tomentosae. Chromosoma, 109:245-258.

Lima-de-Faria, A. (1976). The chromosome field. I. Prediction of the location of ribosomal cistrons. Hereditas, 83:1-22.

Linsmaier, E. M., and Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. Physiologia. Plantarum, 18:100-127.

Liu, B., Tao, W. J., Zhang, Z. H., Xing, M., Xie, H., He, M. Y., and Hao, S. (1995). Intergeneric protoplast fusion between *N. rustica* L. and *L. barbarum* L. and hybrid shoot formation. Acta Biol. Exp. Sin., 28:319-327.

Liu, G., Amin, S., Okuhama, N. N., Liao, G., and Mingle, L. A. (2006). A quantitative evaluation of peroxidase inhibitors for tyramide signal amplification mediated cytochemistry and histochemistry. Histochemistry and cell biology, 126:283-291.

Liu, J. H., Xu, X. Y., and Deng, X. X. (2005). Intergeneric somatic hybridization and its application to crop genetic improvement. Plant Cell Tiss. and Org. Cult., 82:19-44.

Long, C. L., and Liu, and K. M. (2001). *Colocasia lihengiae* (Araceae: Colocasieae), a new species from Yunnan, China. Botanical Bulletin of Academia Sinica, 42:313-317.

Lowe, K. C., Anthony, P., Davey, M. R., and Power, J. B. (2001). Beneficial effects of Pluronic F-68 and artificial oxygen carriers on the post-thaw recovery of cryopreserved plant cells. Artificial Cells, Blood Substitutes and Biotech., 29:297-316.

Luo, J. P., and Jia, J. F. (1998). Plant regeneration from callus protoplasts of the forage legume *Astragalus adsurgens* Pall. Plant Cell Reports, 17:313-317.

Lysak, M. A., Dolezelova, M., Horry, J. P., Swennen, R., and Dolezel, J. (1999). Flow cytometric analysis of nuclear DNA content in *Musa*. Theor. Appl. Genet., 98:1344-1350.

Macas, J., T. Me´szaros, and M. Nouzova, (2002). PlantSat: a specialized database for plant satellite repeats. Bioinformatics, 18:28-35.

Maluszynska, J. (2003). Cytogenetic tests for ploidy level analysis - chromosome counting. In: M. Maluszynska, K. J. Kasha, B. P. Forster, and I. Szarejko, (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands. Doubled haploid production in crop plants: a Manual, Chapter 5:391-395.

Marchant, C. J. (1970). Chromosome variation in Araceae: I: Pothoeae to Stylochitoneae. Kew Bulletin, 24:315-322.

Marchant, C. J. (1971a). Chromosome variation in Araceae: II: Richardieae to Colocasieae. Kew Bulletin, 25:47-56.

Marchant, C. J. (1971b). Chromosome Variation in Araceae: III: Philodendreae to Pythoniëae. Kew Bulletin, 25:323-329.

Marchant, C. J. (1972). Chromosome Variation in Araceae: IV: Areae. Kew Bulletin, 26:395-404.

Marchant, C. J. (1973). Chromosome variation in Araceae: V. Acoreae to Lasieae. Kew Bulletin, 28:199-210.

Marutani, M., Sheffer, R. D., and Kamemoto, H. (1993). Cytological analysis of *Anthurium andraeanum* (Araceae), its related taxa and their hybrids. American journal of botany, 80:93-103.

Matthews, D., Millam, S., and Wilkinson, M. (1999). Factors influencing the utility of gametic microprotoplasts for partial genome transfer in potato. Plant Cell Rep., 18:786-790.

Mayo, S. J., Bogner, J., and Boyce, P. C. (1997). The genera of Araceae. Kew: Royal Botanic Gardens, Kew 370:1.

McColley, R. H., and Miller, H. N. (1965). *Philodendron* improvement through hybridization. Florida State Hort. Soc., 78:409-415.

McCormick, S. (1993). Male gametophyte development. Plant Cell, 5:1265-1275.

Melchers, G., and Labib, G. (1974). Somatic hybridization of plants by fusion of protoplasts. Molec. gen. Genet., 135:277-294.

Millam, S., Payne, L. A., and Mackay, G. R. (1995). The integration of protoplast fusion-derived material into a potato breeding programme - a review of progress and problems. Euphytica, 85:451-455.

Moreira, C., Chase, C., Gmitter, F., and Grosser, J. (2000). Inheritance of organelle genomes in citrus somatic hybrids. Mol Breeding, 6: 401-405.

Morejohn, L. C., and Fosket, D. E. (1991). The biochemistry of compounds with anti-microtubule activity in plant cells. Pharmacology and therapeutics, 51:217-230.

Morejohn, L., Bureau, T., Molebajer, J., Bajer, A., and Fosket, D. (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. Planta, 172:252-264.

Morgan-Richards, M., Trewick, S. A., Chapman, H. M., and Krahulcova, A. (2004). Interspecific hybridisation among *Hieracium* species in New Zealand: evidence from flow cytometry. Heredity, 93:34-42.

Moscone, E. A., Matzke, M. A., and Matzke, A. J. M. (1996). The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgene inserts in amphidiploid tobacco. Chromosoma, 105:231-236.

Murakami, K., Kimura, M., and Matsubara, S. (1995). Plant-regeneration from protoplasts isolated from callus of taro (*Colocasia esculenta* Schott). Journal of the Japanese Society for Horticultural Science, 63:773-778.

Murakami, K., Nishioka, J., and Matsubara, S. (1998). Production of somatic hybrids in taro (*Colocasia esculenta* Schott). Journal of the Japanese Society for Horticultural Science, 67:607-612.

Murakami, K., Yokoyama, H., and Matsubara, S. (1992). Callus formation and plant regeneration from etiolated stem of taro (*Colocasia esculenta* Schott). Journal of the Japanese Society for Horticultural Science, 61:367-374.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15:473-497.

Nagata, T., and Takebe, I. (1970). Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta, 92:301-308.

Navrátilová, B. (2004). Protoplast cultures and protoplast fusion focused on Brassicaceae - a review. Hort. Sci. (Prague), 31:140-157.

Navratilova, B., Luhova, L., and Petrivalsky, M. (2008). Effect of UV-C irradiation on mesophyll protoplasts of *Cucumis sativus*. Plant Cell Tiss. and Org. Cult., 94:313-318.

Negrutiu, I., Hinnisdaels, S., Cammaerts, D., Cherdshewasart, W., Gharti-Chhetri, G., and Jacobs, M. (1992). Plant protoplasts as genetic tool: selectable markers for developmental studies. The International journal of developmental biology, 36:73-84.

New, E. H., and Paris, C. D. (1967). Virescence in *Zantedeschia*. In Proceedings of the American Society for Horticultural Science, 92:685-695.

Niedz, R. (2006) . Regeneration of somatic embryos from sweet orange (*C. sinensis*) protoplasts using semi-permeable membranes. Plant Cell Tissue and Organ Culture, 84:353-357.

Nielsen, K. A., and Knudsen, E. (1993). Regeneration of green plants from embryogenic suspension on cultures of kentucky blue grass (*Poa pratensis* L.). Journal of plant physiology, 141:589-595.

Nishijima, W. T., and Fujiyama, D. K. (1985). Bacterial blight of *Anthurium*. Hawaii Cooperative Extension Service, Hawaii Institute of Tropical Agriculture and Human Resources.

Nitsch, J.P., and Nitsch, C. (1969). Haploid plants from pollen grains. Science, 163:85-87.

Nouri-Ellouz, O., Gargouri-Bouzid, R., Sihachakr, D., Triki, M., Ducreux, G., Drira, N., and Lakhoua, L. (2006). Production of potato intraspecific somatic hybrids with improved tolerance to PVY and *Pythium aphanidermatum*. Journal of Plant Physiology, 163:1321-1332.

Olivares-Fuster, O., Duran-Vila, N., and Navarro, L. (2005). Electrochemical protoplast fusion in *Citrus*. Plant Cell Reports, 24:112-119.

Ooka, J. J. (1990). Taro diseases. In, taking taro into the 1990s (Eds Hollyer, J. R., and Sato, D. M.). University of Hawaii, 51-59.

Orczyk, W., Przetakiewicz, J., and Nadolska-Orczyk, A. (2003). Somatic hybrids of *Solanum tuberosum* - application to genetics and breeding. Plant cell, tissue and organ culture, 74:1-13.

Orlikowska, T., Sabala, I., and Nowak, E. (1995). Adventitious shoot regeneration on explants of *Anthurium, Codiaeum, Dieffenbachia, Gerbera, Rosa* and *Spathiphyllum* for breeding purposes. Ornamental Plant Improvement: Classical and Molecular, 420:115-117.

Ortin-Parraga, and F., and Burgos, L. (2003). Isolation and culture of mesophyll protoplasts from apricot. J. Hortic. Sci. Biotechnol., 78:624-628.

Ovcharenko, O., Momot, V., Cherep, N., Sheludko, Y., Komarnitsky, I., Rudas, V., and Kuchuk, N. (2011). Transfer of transformed *Lesquerella fendleri* (Gray) Wats. chloroplasts into *Orychophragmus violaceus* (L.) OE Schulz by protoplast fusion. Plant Cell Tissue and Organ Culture, 105:21-27.

Pandey, S., Wang, X. Q., Coursol, S. A., and Assmann, S. M. (2002). Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. New Phytol., 153:517-526.

Pijnacker, L. P., and Ferwerda, M. A. (1984). Giemsa C-banding of potato chromosomes. Canadian journal of genetics and cytology, 26(4):415-419.

Papadakis, A, Paschalidis, K, and Roubelakis-Angelakis, K. (2005). Biosynthesis profile and endogenous titers of polyamines differ in totipotent and recalcitrant plant protoplasts. Physiologia Plantarum, 125:10-20.

Parokonny, A. S., Kenton, A. Y., Gleba, Y. Y., and Bennett, M. D. (1992). Genome reorganization in *Nicotiana* asymmetric somatic hybrids analysed by *in situ* hybridization. The Plant Journal, 2:863-874.

Parokonny, A. S., Kenton, A., Gleba, Y. Y., and Bennett, M. D. (1994). The fate of recombinant chromosomes and genome interaction in *Nicotiana* asymmetric somatic hybrids and their sexual progeny. Theoretical and Applied Genetics, 89:488-497.

Parokonny, A. S., Marshall, J. A., Bennett, M. D., Cocking, E. C., Davey, M. R., and Brian Power, J. (1997). Homoeologous pairing and recombination in backcross derivatives of tomato somatic hybrids [*Lycopersicon esculentum* (+) *L. peruvianum*]. Theor. Appl. Genet., 94:713-723.

Paszko, B. (2006). A critical review and a new proposal of karyotype asymmetry indices. Plant Systematics and Evolution, 258:39-48.

Patel, D., Power, J., Anthony, P., Badakshi, F., Heslop-Harrison, J., and Davey, M. (2011). Somatic hybrid plants of *Nicotiana* x *sanderae* (+) *N. debneyi* with fungal resistance to *Peronospora tabacina*. Annals of Botany, 108:809-819.

Pati, P., Sharma, M., and Ahuja, P. (2005). Extra thin alginate film: an efficient technique for protoplast culture. Protoplasma, 226:217-221.

Pati, P., Sharma, M., and Ahuja, P. (2008). Rose protoplast isolation and culture and heterokaryon selection by immobilization in extra thin alginate film. Protoplasma, 233:165-171.

Pellicer, J., Garcia, S., Vallès, J., Kondo, K., and Garnatje, T. (2013). FISH mapping of 35S and 5S rRNA genes in Artemisia subgenus *Dracunculus* (Asteraceae): changes in number of loci during polyploid evolution and their systematic implications. Botanical Journal of the Linnean Society. 171:655-666.

Peng, Y., Wang, Z., Cheng, L., and Chen, H. (2003). Effect of phosphoric amide herbicide APM on the structure and protein composition of chromosome in *Triticum durum*. Plant Prod. Sci., 6:134-138.

Peruzzi, L., Leitch, I. J., and Caparelli K. F. (2009). Chromosome diversity and evolution in Liliaceae. Annals of Botany, 103:459-475.

Petersen, G. (1989). Cytology and systematics of Araceae. Nord. J. Bot., 9:119-158.

Petersen, G. (1993). New chromosome numbers in Araceae. Willdenowia, 239-244.

Petersen, W. L., Sulc, S., and Armstrong, C. L. (1992). Effect of nurse cultures on the production of macro-calli and fertile plants from maize embryogenic suspension culture protoplasts. Plant Cell Reports, 10:591-594.

Pijnacker, L. P., and Ferwerda, M. A. (1984). Giemsa C-banding of potato chromosomes. Canadian journal of genetics and cytology, 26(4), 415-419.

Plowman, T. (1969). Folk uses of new world aroids. Economic Botany, 23:97-122.

Pojnar, E., and Cocking, E. C. (1968). Formation of cell aggregates by regenerating isolated tomato fruit protoplasts. Nature, 218:289-289.

Pongchawee, K., Nanakorn, U., Lamseejan, S., and Poompuang, S. (2007). Protoplast isolation and culture of aquatic plant *Cryptocoryne wendtii* De Wit. Kasetsart J, 41: 300-310.

Pongchawee, K., Nanakorn, U., Lamseejan, S., Poompuang, S., and Phansiri, S. (2006). Factors affecting the protoplast isolation and culture of *Anubias nana* Engler. International Journal of Botany, 2: 193-200.

Potrykus, I. (1980). The old problem of protoplast culture: cereals. In: Ferenczy L, Farkas GL (eds) Advances in protoplast research. Hungarian Academiy of Science Press, 243–254.

Potrykus, I., and Shillito, R. D. (1986). Protoplasts: isolation, culture, plant regeneration. Methods Enzymol, 118:549-678.

Prange,A., Bartsch,M., Serek,M., and Winkelmann,T. (2010a). Regeneration of different *Cyclamen* species via somatic embryogenesis from callus, suspension cultures and protoplasts. Scientia Horticulturae, 125:442-450.

Prange,A., Serek,M., Bartsch,M., and Winkelmann,T. (2010b). Efficient and stable regeneration from protoplasts of *Cyclamen coum* Miller via somatic embryogenesis. Plant Cell, Tissue and Organ Culture, 101:171-182.

Puite, K. J. (2006). Progress in plant protoplast research. Physiol. Plant., 85:403-410.

Puite, K., and Schaart, J. (1993) Nuclear genomic composition of asymmetric fusion products between irradiated transgenic *Solanum brevidens* and *Solanum tuberosum* - Limited elimination of donor chromosomes and polyploidization of the recipient genome. Theor. Appl. Genet., 86:237-244.

Raghavan, V. (1997). Molecular embryology of flowering plants. University Press, Cambridge.

Rakosy-Tican, E., Aurori, A., Vesa, S., and Kovacs, K. M. (2007). *In vitro* morphogenesis of sunflower (*Helianthus annuus*) hypocotyl protoplasts: the effects of protoplast density, haemoglobin and spermidine. Plant Cell Tissue and Organ Culture, 90:55-62.

Ramulu, K. S., Dijkhuis, P., Rutgers, E. , Blaas, J., Krens, F. A., Verbeek, W. H. J., Colijn-Hooymans, C. M., and Verhoeven, H. A. (1996). Intergeneric transfer of a partial genome and direct production of monosomic addition plants by microprotoplast fusion. Theoretical and Applied Genetics, 92:316-325.

Ramulu, K. S., Dijkhuis, P., Famelaer, I., Cardi, T., and Verhoeven, H. A. (1993). Isolation of sub-diploid microprotoplasts for partial genome transfer in plants - enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes. Planta, 190:190-198.

Ramulu, K., Dijkhuis, P., Famelaer, I., Cardi, T., and Verhoeven, H. (1994). Cremart: a new chemical for efficient induction of micronuclei in cells and protoplasts for partial genome transfer. Plant Cell Rep., 13:687-691.

Ramulu, K., Dijkhuis, P., Rutgers, E., Blaas, J., Verbeek, W., Verhoeven, H., and Colijnhooymans, C. (1995). Microprotoplast fusion technique - A new tool for gene-transfer between sexually-incongruent plant-species. Euphytica, 85:255-268.

Ramulu, K., Verhoeven, H., and Dijkhuis, P. (1987). Mitotic dynamics of induced micronuclei and prospects for chromosome-mediated gene transfer in plants. Theor. Appl. Genet., 75:575-584.

Ramulu, K., Verhoeven, H., and Dijkhuis, P. (1991). Mitotic blocking, micronucleation, and chromosome doubling by oryzalin, amiprophos-methyl, and colchicine in potato. Protoplasma, 160:65-71.

Rayburn, A. L., McCloskey, R., Tatum, T. C., Bollero, G. A., Jeschke, M. R., and Tranel, P. J. (2005). Genome size analysis of weedy *Amaranthus* species. Crop Science, 45:2557-2562.

Razdan, M. K. (2003). Introduction to Plant Tissue Culture, 2 edn., Enfield, NH, USA: Science Publishers, Inc.

Reeves, A. (2001). MicroMeasure: a new computer program for the collection and analysis of cytogenetic data. Genome, 44:439-443.

Rick, C. (1982). Isozymes in plant breeding. California Agriculture, 36:28-28.

Ricroch, A., Peffley, E. B., and Baker, R. J., (1992). Chromosomal location of rDNA in *Allium*: *in situ* hybridization using biotin-labeled and fluorescein-labeled probe. Theoretical and Applied Genetics, 83:413-418.

Rizkalla, A., Badr-Elden, A., and Nower, A. (2007). Protoplast isolation, salt stress and callus formation of two Date Palm genotypes. Journal of Applied Sciences Research, 3:1186-1194.

Roa, F., and Guerra, M. (2012). Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evolutionary Biology, 12:225.

Roddick, J. G., and Melchers, G. (1985). Steroidal glycoalkaloid content of potato, tomato and their somatic hybrids. Theoretical and Applied Genetics, 70(6):655-660.

Saito, F., and Nakano, M. (2001). Partial synchronization of cell division and micronucleation in suspension-cultured cells of *Hemerocallis hybrida*: the effects of hydroxyurea and various spindle toxins. Breeding Sci., 51:285-291.

Saito, H., and Nakano, M. (2002a). Isolation and characterization of gametic microprotoplasts from developing microspores of *Lilium longiflorum* for partial genome transfer in the Liliaceous ornamentals. Sex. Plant Reprod., 15:179-185.

Saito, H., and Nakano, M. (2002b). Isolation and characterization of microprotoplasts from propyzamide-treated cell suspension cultures of *Hemerocallis hybrida*. Breeding Sci., 52:51-56.

Sakomoto, K., and Taguchi, T. (1991). Regeneration of intergeneric somatic hybrid plants between *Lycopersicon esculentum* and *Solanum muricatum*. Theor. Appl. Genet., 81:509-513.

Scholze, P., Kramer, R., Ryschka, U., Klocke, E., and Schumann, G. (2010). Somatic hybrids of vegetable *Brassicas* as source for new resistances to fungal and virus diseases. Euphytica, 176:1-14.

Schubert, I., P. F. Fransz, J. Fuchs, and J. H. De Jong, (2001). Chromosome painting in plants. Methods. Cell Sci., 23:57-69.

Schwarzacher, T. (2003). DNA, chromosomes, and *in situ* hybridisation. Genome, 46:953-962.

Schwarzacher, T., and Heslop-Harrison, J. S. (1994). Direct fluorochrome-labelled DNA probes for direct fluorescent *in situ* hybridisation to chromosomes. In: Isaac PG (ed.) Protocols for nucleic acid analysis by non-radioactive probes, Methods in molecular biology, volume 28, Humana Press, Totowa, NJ, 167-176.

Schwarzacher, T., and Leitch, A. R. (1993). Enzymatic treatment of plant material to spread chromosomes for *in situ* hybridisation. In: Isaac PG (ed.) Protocols for nucleic acid analysis by non-radioactive probes, Methods in molecular biology, volume 28, Humana Press, Totowa, NJ, 153-160.

Schwarzacher, T., Leitch, A. R., Bennett, M. D., and Heslop-Harrison, J. S. (1989) *In situ* hybridisation of parental genomes in a wide hybrid. Ann. Bot., 64:315-324.

Seijo, T. E., Peres, N. A., and Deng, Z. (2010). Characterization of Strains of *Xanthomonas axonopodis* pv. dieffenbachiae from Bacterial Blight of Caladium and Identification of Sources of Resistance for Breeding Improved Cultivars. Hort. Science, 45:220-224.

Sharma, A. K., and Bhattacharyya, U. C. (1961). Structure and behavior of chromosomes in species of *Anthurium* with special reference to the accessory chromosomes. Proceedings of the National Institute of India, Part B, Biological Science, 27:317-328.

Sharma, H. (1995). How wide can a wide cross be? Euphytica, 82:43-64.

Sharma, H. C., and Gill, B. S. (1983). Current status of wide hybridization in wheat. Euphytica, 32:17-31.

Sheffer, R. D., and Croat, T. B. (1983). Chromosome numbers in the genus *Anthurium* (Araceae) II. American journal of botany, 858-871.

Sheng, X., Zhao, Z., Yu, H., Wang, J., Xiaohui, Z., and Gu, H. (2011). Protoplast isolation and plant regeneration of different doubled haploid lines of cauliflower (*Brassica oleracea* var. botrytis). Plant Cell Tiss Organ Cult, 107:513–520.

Sherraf, I., S. Tizroutine, M. H. Chaput, M. Allot, I. Mussio, D. Sihachakr, L. Rossignol, and G. Ducreux. (1994). Production and characterization of intergeneric somatic hybrids through protoplast electrofusion between potato (*Solanum tuberosum*) and *Lycopersicon pennellii*. Plant cell, tissue and organ culture, 37:137-144.

Shiba, T. and Mii, M. (2005). Plant regeneration from mesophyll and cell suspension-derived protoplasts of *Dianthus acicularis* and characterization of regenerated plants. *In Vitro* Cellular & Developmental Biology - Plant, 41:794-800.

Shibuya, R. (1956). Intercrossing among pink calla, white-spotted calla and yellow calla. Intercrossing among pink calla, white-spotted calla and yellow calla. Kasai, Tokyo.

Shishido, R., Apisitwanish, S., Ohmido, N., Okinaka, Y., Mori, K., and Fukui, K. (1998). Detection of specific chromosome reduction in rice somatic hybrids with the A, B and C genomes by multi-color genomic *in situ* hybridisation. Theor. Appl. Genet., 97:1013-1018.

Shishova, M., Lindberg, S. (2004). Auxin induces an increase of Ca2þ concentration in the cytosol of wheat leaf protoplasts. Journal of Plant Physiology, 161:937–945.

Shrestha, B., Tokuhara, K., and Mii, M. (2007). Plant regeneration from cell suspension-derived protoplasts of *Phalaenopsis*. Plant Cell Reports, 26:719-725.

Sigeno, A., Hayashi, S., Terachi, T., and Yamagishi, H. (2009). Introduction of transformed chloroplasts from tobacco into petunia by asymmetric cell fusion. Plant Cell Reports, 28:1633-1640.

Simone, G., Elliott, M., and Mullin, R. (1994). Florida Plant Disease Control Guide, Volume 1. Plant Pathology Dept., University of Florida, Gainesville FL. 362.

Sisko, M., Ivancic, A., and Bohanec, B. (2003). Genome size analysis in the genus *Cucurbita* and its use for determination of interspecific hybrids obtained using embryo-rescue technique. Plant Science, 165:663-669.

Skarzhinskaya, M., Landgren, M., and Glimelius, K. (1996). Production of intertribal somatic hybrids between *Brassica napus* L. and *Lesquerella gendleri* (Gray) Wats. Theor. Appl. Genet., 93:1242-1250.

Sleper, D.A. and Poehlman, J.M. (1995). Breeding Field Crops, 3.

Smith, M. A. L., Palta, A. P., and McCown, B. H. (1984). The measurement of isotonicity and maintenance of osmotic balance in plant protoplast manipulations. Plant Science Letters, 33:249-258.

Smith, T. A. (1985). Polyamines. Annual Review of Plant Physiology, 36:117-143.

Smyda, P., Jakuczun, H., Dębski, K., Śliwka, J., Thieme, R., Nachtigall, M., and Zimnoch-Guzowska, E. (2013). Development of somatic hybrids *Solanum× michoacanum* Bitter.(Rydb.)(+) *S. tuberosum* L. and autofused 4x *S.× michoacanum* plants as potential sources of late blight resistance for potato breeding. Plant cell reports, 32:1231-1241.

Snijder, R. C., Brown, F. S., and Van Tuyl, J. M. (2007). The role of plastome-genome incompatibility and biparental plastid inheritance in interspecific hybridization in the genus *Zantedeschia* (Araceae). Floriculture and Ornamental Biotechnology, 1:150-157.

Snijder, R. C., Cho, H. R., Hendriks, M. M., Lindhout, P., and Van Tuyl, J. M. (2004a). Genetic variation in *Zantedeschia* spp.(Araceae) for resistance to soft rot caused by *Erwinia carotovora* subsp. carotovora. Euphytica, 135:119-128.

Snijder, R. C., Lindhout, P., and Van Tuyl, J. M. (2004b). Genetic control of resistance to soft rot caused by *Erwinia carotovora* subsp. carotovora in *Zantedeschia* spp.(Araceae), section *Aestivae*. Euphytica, 136:319-325.

Soltis, D. E., Soltis, P. S., Bennett, M. D., and Leitch, I. J. (2003). Evolution of genome size in the angiosperms. American Journal of Botany, 90:1596-1603.

Sonntag, K., Ruge-Wehling, B., and Wehling, P. (2009). Protoplast isolation and culture for somatic hybridization of *Lupinus angustifolius* and *L. subcarnosus*. Plant Cell Tissue and Organ Culture, 96:297-305.

Spangenberg, G., Valles, M. P., Wang, Z. Y., Montavon, P., Nagel, J., and Potrykus, I. (1994). Asymmetric somatic hybridization between tall fescue (*Festuca arundinacea* Schreb.) and irradiated Italian ryegrass (*Lolium multiflorum* Lam.) protoplasts. Theoretical and applied genetics, 88:509-519.

Stebbins, G. (1958). The inviability, weakness, and sterility of interspecific hybrids. Advances in Genetics, 9:147-215.

Stebbins, G. (1971). Chromosomal evolution of higher plants. Edward Arnold Ltd., London.

Strohner, R., Nemeth, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., and Längst, G. (2001). NORC - a novel member of mammalian ISWI-containing chromatin remodeling machines. EMBO J., 20:4892-4900.

Stuart, D. A., and Strickland, S. G. (1984). Somatic embryogenesis from cell cultures of *Medicago sativa* L the role of amino acid additions to the regeneration medium. Plant science letters, 34:165-174.

Su, H.C.F. (1977). Insecticidal properties of black pepper to rice weevils and cowpea weevils. Journal of Economic Entomology, 70:18-2.

Suda, J., Krahulcova, A., Travnicek, P., Rosenbaumova, R., Peckert, T., and Krahulec, F. (2007). Genome size variation and species relationships in *Hieracium* sub-genus *Pilosella* (Asteraceae) as inferred by flow cytometry. Ann. Bot., 100:1323-1335.

Sullivan, G. J., Bridger, J. M., Cuthbert, A. P., Newbold, R. F., Bickmore, W. A., and McStay, B. (2001). Human acrocentric chromosomes with transcriptionally silent nucleolar organizer regions associate with nucleoli. The EMBO Journal, 20:2867-2877.

Sumner, A. T. (1982). The nature and mechanisms of chromosome banding. Cancer Genet. Cytogenet., 6:59-87.

Sun, Y., Xue, Q., Ding, C., Zhang, X., Zhang, L., Wang, W., and Ali, S. (2005a). Somatic cybridization between *Nicotiana tabacum* and *N. repanda* based on a single inactivation procedure of nuclear donor parental protoplasts. Plant Sc., 168:303-308.

Sun, Y., Zhang, X., Huang, C., Nie, Y., and Guo, X. (2005b). Factors influencing *in vitro* regeneration from protoplasts of wild cotton (*G. klotzschianum* A) and RAPD analysis of regenerated plantlets. Plant Growth Regulation, 46:79-86.

Sun, Y., Zhang, X., Huang, C., Nie, Y., and Guo, X. (2005c). Plant regeneration via somatic embryogenesis from protoplasts of six explants in Coker 201 (*Gossypium hirsutum*). Plant Cell Tissue and Organ Culture, 82:309-315.

Sundberg, E., and Glimelius, K. (1991). Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within Brassicaceae. Theoretical and Applied Genetics, 83:81-88.

Sutiojono, E., Nonhebel, H. M., and Kantharajah, A. S. (1998). Short communication factors affecting protoplast culture of *Cucumis melo* 'Green Delica'. Ann. of Bot., 81:775-777.

Szczerbakowa, A., Boltowicz, D., Lebecka, R., Radomski, P., and Wielgat, B. (2005). Characteristics of the interspecific somatic hybrids *Solanum pinnatisectum* (+) *S. tuberosum* H-8105. Acta Physiol Plant, 3:265–273.

Takahashi, C., Leitch, I. J., Ryan, A., Bennett, M. D., and Brandham, P. E. (1997). The use of genomic *in situ* hybridization (GISH) to show transmission of recombinant chromosomes by a partially fertile bigeneric hybrid, *Gasteria lutzii* x *Aloe aristata* (Aloaceae), to its progeny. Chromosoma, 105:342-348.

Takebe, I., Labib, G., and Melchers, G. (1971). Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften, 58:318-320.

Tang, K., Sun, X., An, D., Power, J. B., Cocking, E. C., and Davey, M. R. (2001). A simple and rapid procedure to establish embryogenic cell suspensions as a source of protoplasts for efficient plant regeneration from two Chinese commercial rice cultivars. Plant cell, tissue and organ culture, 66:149-153.

Taski-Ajdukovic, K., Vasic, D., and Nagl, N. (2006). Regeneration of interspecific somatic hybrids between *Helianthus annuus* L. and *Helianthus maximiliani* (Schrader) via protoplast electrofusion. Plant Cell Reports, 25:698-704.

Tavazza, R., and Ancora, G. (1986). Plant regeneration from mesophyll protoplasts in commercial potato cultivars (Primura, Kennebec, Spunta, Desirée). Plant Cell Reports, 5:243-246.

Taylor, P. W., Ko, H. L., Adkins, S. W., Rathus, C., and Birch, R. G. (1992). Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids). Plant Cell, Tiss. Org. Cult., 28:69-78.

Thieme, R., Darsow, U., Rakosy-Tican, L., Kang , Z., Gavrilenko, T., Antonova, O., Heimbach, U., and Thieme, T. (2004). Use of somatic hybridization to transfer resistance to late blight and potato virus Y (PVY) into cultivated potato. Plant Breed Seed Sci., 50:113–118.

Thieme, R., Rakosy-Tican, E., Gavrilenko, T., Antonova, O., Schubert, J., Nachtigall, M., Heimbach, U., and Thieme, T. (2008). Novel somatic hybrids (*Solanum tuberosum* L. plus *Solanum tarnii*) and their fertile BC1 progenies express extreme resistance to potato virus Y and late blight. Theoretical and Applied Genetics, 116:691-700.

Thieme, R., Rakosy-Tican, E., Nachtigall, M., Schubert, J., Hammann, T., Antonova, O., Gavrilenko, T., Heimbach, U., and Thieme, T. (2010). Characterization of the multiple resistance traits of somatic hybrids between *Solanum cardiophyllum* Lindl. and two commercial potato cultivars. Plant Cell Reports, 29:1187-1201.

Thomas, T. (2009). Isolation, callus formation and plantlet regeneration from mesophyll protoplasts of *Tylophora indica* (Burm. f.) Merrill: an important medicinal plant. In Vitro Cellular and Developmental Biology-Plant, 45:591-598.

Tiburcio, A. F., Masdéu, M. A., Dumortier, F. M., and Galston, A. W. (1986). Polyamine metabolism and osmotic stress I. Relation to protoplast viability. Plant physiology, 82:369-374.

Toriyama, K., and Hinata, K. (1985). Cell suspension and protoplast culture in rice. Plant Science, 41:179-183.

Townsend, (1897). Der Einfluss des Zellkernes auf die Bildung der Zellhaut. Jahrbuch. Wiss. Bot., 30: 484-510.

Tu, Y., Sun, J., Liu, Y., Ge, X., Zhao, Z., Yao, X., and Li, Z. (2008). Production and characterization of intertribal somatic hybrids of *Raphanus sativus* and *Brassica rapa* with dye and medicinal plant *Isatis indigotica*. Plant Cell Reports, 27:873-883.

Van der Valk, P., Kik, C., Verstappen, F., Everink, J. Y., and De Vries, J. N. (1991). Independent segregation of 2 isozyme markers and inter-plant differences in nuclear-DNA content in the interspecific backcross (*Allium fistulosum* L. x *A. cepa* L.) x *A. cepa* L. Euphytica, 55:151-156.

Van Harten, A. M. (2002). Mutation breeding of vegetatively propagated ornamentals. Breeding for ornamentals: Classical and molecular approaches. Kluwer Academic Publishers, Dordrecht, 105-128.

Van Huylenbroeck, J. (2010). Status of floriculture in Europe. In: protocols for *in vitro* propagation of ornamental plants. Jain, S. M., Ochatt, S. J., Humana Press, 365-376.

Van Laere, K., França, S. C., Vansteenkiste, H., Van Huylenbroeck, J., Steppe, K., and Van Labeke, M. C. (2011). Influence of ploidy level on morphology, growth and drought susceptibility in *Spathiphyllum wallisii*. Acta Physiologiae Plantarum, 33:1149-1156.

Van Laere, K., L. Khrusteleva, J. Van Huylenbroeck and E. Van Bockstaele, (2010). Application of GISH to characterize woody ornamental hybrids with small genomes and chromosomes. Plant Breeding, 129:442-447.

Van Laere, K., Van Huylenbroeck, J., and Van Bockstaele, E. (2008). Karyotype analysis and physical mapping of 45S rRNA genes in *Hydrangea* species by fluorescence *in situ* hybridization. Plant Breeding, 127:301-307.

Van Tuyl, J. M., and De Jeu, M. J. (1997). Methods for overcoming interspecific crossing barriers. Pollen Biotech. for Crop Production and Improvement, 273-292.

Van Tuyl, J. M., Van Dien, M., Van Creij, M., Van Kleinwee, T., Franken, J., and Bino, R. (1991). Application of *in vitro* pollination, ovary culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sc., 74:115-116.

Vanstechelman, I., Eeckhaut, T., Van Huylenbroeck, J., and Van Labeke, M. C. (2010). Histogenic analysis of chemically induced mixoploids in *Spathiphyllum wallisii*. Euphytica, 174:61-72.

Varotto, S., Nenz, E., Lucchin, M., and Parrini, P. (2001). Production of asymmetric somatic hybrid plants between *Cichorium intybus* L. and *Helianthus annuus* L. Theoretical and Applied Genetics, 102:950-956.

Vasil, V., Perl, R. J., and Vasil, J. K. (1988). Somatic hybridization in the Gramineae: *Triticum monococcum* + *Pennisetum americanum* (L.) K. Schum. (pearl millet). J. Plant Physiol., 123:160-163.

Vaughn, K., and Lehnen, L. (1991). Mitotic disrupter herbicides. Weed Sci. Soc. Am., 39:450-457.

Ventura, L., A. Giovannini, M. Savio, M. Donà, A. Macovei, A. Buttafava, D. Carbonera, and A. Balestrazzi. (2013). Single Cell Gel Electrophoresis (Comet) assay with plants: Research on DNA repair and ecogenotoxicity testing. Chemos., 92:1-9.

Verhoeven, H., Ramulu, K., and 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.

Von Hanstein, J. (1880). Einige Züge aus der Biologie des Protoplasmas. Bot. Abhandlungen, 4:1-56.

Waara, S., and Glimelius, K. (1995). The potential of somatic hybridization in crop breeding. Euphytica, 85:217-233.

Wakita, Y., Yokota, S., Yoshizawa, N., Katsuki, T., Nishiyama, Y., Yokoyama, T., Fukui, M., and Sasamoto, H. (2005). Interfamilial cell fusion among leaf protoplasts of *Populus alba*, *Betula platyphylla* and *Alnus firma*: assessment of electric treatment and in vitro culture conditions. Plant Cell Tissue and Organ Culture, 83:319-326.

Wallin, A., K. Glimelius, and T. Eriksson. (1977). Enucleation of plant protoplasts by Cytochalasin B. Z. Pflanzenphysiol., 87:333-340.

Wang, G., Tang, Y., Yan, H., Sheng, X., Hao, W., Zhang, L., Lu, K., and Liu, F. (2011a). Production and characterization of interspecific somatic hybrids between *Brassica oleracea* var. botrytis and *B. nigra* and their progenies for the selection of advanced pre-breeding materials. Plant Cell Reports, 30:1811-1821.

Wang, J., Zhao, C., Liu, C., Xia, G., and Xiang, F. (2011b). Introgression of *Swertia mussotii* gene into *Bupleurum scorzonerifolium* via somatic hybridization. BMC Plant Biology, 11:71-71.

Wang,L., Pan,Z., and Guo,W. (2010). Proteomic analysis of leaves from a diploid cybrid produced by protoplast fusion between Satsuma mandarin and pummelo. Plant Cell Tissue and Organ Culture, 103:165-174.

Wang, M., Peng, Z., Hong, S., Zhi, D., and Xia, G. (2012). Hybrid inflorescences derived from gamma-fusion of *Arabidopsis thaliana* with *Bupleurum scorzonerifolium*. Protoplasma, 249:197-205.

Wang, M., Peng, Z., Wang, L., Zhao, J., Che, J., and Xia, G. (2011c). Different rates of chromosome elimination in symmetric and asymmetric somatic hybridization between *Festuca arundinacea* and *Bupleurum scorzonerifolium*. Russian Journal of Plant Physiology, 58:133-141.

Wang, M., Peng, Z., Wang, M., Guo, W., Zhao, J., Zhi, D., and Xia, G. (2011d). The behavior of *Bupleurum scorzonerifolium* as a parent of somatic hybrid introgressed lines is associated with UV resistance of its chromosomes. Russian Journal of Plant Physiology, 58:615-621.

Wang, M., Xia, G., and Peng, Z. (2005a). High UV-tolerance with introgression hybrid formation of *Bupleurum scorzonerifolium* Willd. Plant Science, 168:593-600.

Wang, Y., Sonntag, K., Rudloff, E., and Chen, J. (2005b). Intergeneric somatic hybridization between *Brassica napus* L. and *Sinapis alba* L. Journal of Integrative Plant Biology 47:84-91.

Wang, Z. Y., Nagel, J., Potrykus, I., and Spangenberg, G. (1993). Plants from cell suspension-derived protoplasts in *Lolium* species. Plant Science, 94:179-193.

Werbrouck, S. P. O., Eeckhaut, T. G. R., and Debergh, P. C. (1998). Induction and conversion of somatic embryogenesis on the anther filament of *Spathiphyllum* Schott. In XXV International Horticultural Congress, Part 10: Application of Biotechnology and Molecular Biology and Breeding-*In vitro* 520:263-270.

Werbrouck, S. P. O., and Debergh, P. C. (1995). Imazalil enhances the shoot-inducing effect of benzyladenine in Spathiphyllum floribundum Schott. Journal of plant growth regulation, 14(2), 105-107.

Werbrouck, S. P., and Debergh, P. C. (1996b). Imidazole fungicides and paclobutrazol enhance cytokinin-induced adventitious shoot proliferation in Araceae. Journal of plant growth regulation, 15:81-85.

Werbrouck, S. P., Redig, P., Van Onckelen, H. A., and Debergh, P. C. (1996a). Gibberellins play a role in the interaction between imidazole fungicides and cytokinins in Araceae. Journal of plant growth regulation, 15:87-93.

Werbrouck, S. P., van der Jeugt, B., Dewitte, W., Prinsen, E., Van Onckelen, H. A., and Debergh, P. C. (1995). The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. Plant Cell Reports, 14:662-665.

Werbrouck, S., Eeckhaut, T., and Debergh, P. (2000). Induction and conversion of somatic embryogenesis on the anther filament of *Spathiphyllum* Schott. Acta Hort., 520:263-269.

Werbrouck, S., Goethals, K., Van Montagu, M., and Debergh, P. (1999). Surprising micropropagation tools. Plant Biotechnology and *In Vitro* Biology in the 21st Century. Springer Netherlands, 667-672.

Wessel, T. (1984). The agricultural foundations of civilization. Agriculture and Human Values, 1:9-12.

Wijbrandi, J., Zabel, P., and Koornneef, M. (1990). Restriction fragment length polymorphism analysis of somatic hybrids between *Lycopersicon esculentum* and irradiated *L. peruvianum*: Evidence for limited donor genome elimination and extensive chromosome rearrangements. Mol. Gen. Genet., 222:270-277.

Wilfret, G. J., and Sheenhan, T. J. (1981). Development of new foliage plant cultivars. In: J. N. Joiner (ed.), Foliage plant production. Prentice-Hall, NJ, 126-136.

Wilson, L., and Jordan, M. A. (1995). Microtubule dynamics: taking aim at a moving target. Chemistry and biology, 2:569-573.

Wisniewska, E., and Majewska-Sawka, A. (2007). Arabinogalactan-proteins stimulate the organogenesis of guard cell protoplasts-derived callus in sugar beet. Plant Cell Reports, 26:1457-1467.

Wisniewska, E., and Majewska-Sawka, A. (2008). The differences in cell wall composition in leaves and regenerating protoplasts of *Beta vulgaris* and *Nicotiana tabacum*. Biologia Plantarum, 52:634-641.

Wolverton, B. C., Johnson, A. and Bounds, K. (1989). Interior landscape plants for indoor air pollution abatement. Final Rept. NASA, Stennis Space Center, Mississippi.

Wu, S., Wirthensohn, M., Hunt, P., Gibson, J., and Sedgley, M. (2008). High resolution melting analysis of almond SNPs derived from ESTs. Theor. Appl. Genet., 118:1-14.

Xia, G. M., Xiang, F. N., Zhou, A. F., Wang, H., and Chen, H. M. (2003). Asymmetric somatic hybridization between wheat (*Triticum aestivum* L.) and *Agropyron elongatum* (Host) Nevishi. Theor. Appl. Genet., 107:299-305.

Xiang, F., Wang, J., Xu, C., and Xia, G. (2010). The chromosome content and genotype of two wheat cell lines and of their somatic fusion product with oat. Planta, 231:1201-1210.

Xiang, F., Xia, G., Zhi, D., Wang, J., Nie, H., and Chen, M. (2004). Regeneration of somatic hybrids in relation to the nuclear and cytoplasmic genomes of wheat and *Setaria italica*. Genome, 47:680-688.

Xiao, W., Huang, X., Huang, X., Chen, Y., Dai, X., and Zhao, J. (2007). Plant regeneration from protoplasts of *Musa acuminata* cv. Mas (AA) via somatic embryogenesis. Plant Cell Tissue and Organ Culture, 90:191-200.

Xiao, W., Huang, X., Gong, Q., Dai, X., Zhao, J., Wei, Y., and Huang, X. (2009). Somatic hybrids obtained by asymmetric protoplast fusion between *Musa silk* cv. Guoshanxiang (AAB) and *Musa acuminata* cv. Mas (AA). Plant Cell Tissue and Organ Culture, 97:313-321.

Xu, X., Hu, Z., Li, J., Liu, J., and Deng, X. (2007). Asymmetric somatic hybridization between UV-irradiated *Citrus unshiu* and *C. sinensis*: regeneration and characterization of hybrid shoots. Plant Cell Reports, 26:1263-1273.

Xu, X., Liu, J., and Deng, X. (2006). Isolation of cytoplasts from Satsuma mandarin (*Citrus unshiu* Marc.) and production of alloplasmic hybrid calluses via cytoplast-protoplast fusion. Plant Cell Reports, 25:533-539.

Yamada, Y., Yang, Z. Q., and Tang, D. T. (1986). Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Reports, 5:85-88.

Yamagishi, M., Abe, H., Nakano, M. and Nakatsuka, A. (2002). PCR-based molecular markers in Asiatic hybrid lily. Scientia Horticulturae, 96:225-234.

Yan, C., Qian, K., Yan, Q., Zhang, X., Xue, G., Huangfu, W., Wu, Y., Zhao, Y., Xue, Z., Huang, J., Xu, G., and Wu, P. (2004). Use of asymmetric somatic hybridization for transfer of the bacterial blight resistance trait from *Oryza meyeriana* L. to *O. sativa* L. ssp japonica. Plant Cell Reports, 22:569-575.

Yang, D. S., Pennisi, S. V., Son, K. C., and Kays, S. J. (2009). Screening indoor plants for volatile organic pollutant removal efficiency. HortScience, 44:1377-1381.

Yang, X., Guo, X., Zhang, X., Nie, Y., and Jin, S. (2007a). Plant regeneration from *Gossypium davidsonii* protoplasts via somatic embryogenesis. Biologia Plantarum, 51:533-537.

Yang, X., Zhang, X., Jin, S., Fu, L., and Wang, L. (2007b). Production and characterization of asymmetric hybrids between upland cotton Coker 201 (*Gossypium hirsutum*) and wild cotton (*G. klozschianum* Anderss). Plant Cell Tissue and Organ Culture, 89:225-235.

Yao, J. L., Cohen, D., and Rowland, R. E. (1995). Interspecific albino and variegated hybrids in the genus *Zantedeschia* Plant science, 109:199-206.

Yin, Y., Li, S., Chen, Y., Guo, H., Tian, W., Chen, Y., and Li, L. (1993). Fertile plants regenerated from suspension culture-derived protoplasts of an Indica type rice (*Oryza sativa* L.). Plant cell, tissue and organ culture, 32:61-68.

Yu, Y., Li, Z., Wang, P., and Xiang, F. (2012). Genetic and biochemical characterization of somatic hybrids between *Bupleurum scorzonerifolium* and *Gentianopsis paludosa*. Protoplasma, 249:1029-1035.

Zhang, F., Wang, P., Ji, D., Kang, G., and Xiang, F. (2011). Asymmetric somatic hybridization between *Bupleurum scorzonerifolium* Willd. and *Taxus chinensis* var. mairei. Plant Cell Reports, 30:1857-1864.

Zhang, Q., Liu, J., and Deng, X. (2006). Isolation of microprotoplasts from a partially synchronized suspension culture of *Citrus unshiu*. J. Plant Physiol., 163:1185-1192.

Zhao, J., Cui, J., Liu, J., Liao, F., Henny, R. J., and Chen, J. (2012). Direct somatic embryogenesis from leaf and petiole explants of *Spathiphyllum* 'Supreme'and analysis of regenerants using flow cytometry. Plant Cell, Tissue and Organ Culture (PCTOC), 110:239-249.

Zhao, Z., Hu, T., Ge, X., Du, X., Ding, L., and Li, Z. (2008). Production and characterization of intergeneric somatic hybrids between *Brassica napus* and *Orychophragmus violaceus* and their backcrossing progenies. Plant Cell Reports, 27:1611-1621.

Zhou, A., and Xia, G. (2005). Introgression of the *Haynaldia villosa* genome into gamma-ray-induced asymmetric somatic hybrids of wheat. Plant Cell Reports, 24:289-296.

Zhou, C., Xia, G., Zhi, D., and Chen, Y. (2006). Genetic characterization of asymmetric somatic hybrids between *Bupleurum scorzonerifolium* Willd and *Triticum aestivum* L.: potential application to the study of the wheat genome. Planta, 223:714-724.

Zhou, J., Wang, B., and Zhu, L. (2005). Conditioned culture for protoplasts isolated from chrysanthemum: an efficient approach. Colloids Surf B Biointerfaces, 45:113-119.

Zimmermann, U., and Scheurich, P. (1981). High frequency fusion of plant protoplasts by electric fields, Planta, 151:26-32.

Zimmermann, U., and Vienken, J. (1982). Electric field-induced cell-to-cell fusion. Journal of Membrane Biology, 67:165-182.

Zonneveld, B. J. M. (2004). Genome size in Hydrangea. In: Van Gelderen, J., Van Gelderen, D. M., (eds.) Encyclopedia of Hydrangeas. Timber Press, Portland, Ore, USA, 245-251.

Zonneveld, B. J. M., Leitch, I. J., and Bennett, M. D. (2005). First nuclear DNA amounts in more than 300 angiosperms. Annals of Botany.

Zubko, M. K., Zubko, E. I., and Gleba, Y. Y. (2002). Self-fertile cybrids *Nicotiana tabacum* (+ *Hyoscyamus aureus*) with a nucleo– plastome incompatibility. Theor. Appl. Genet., 105:822-828.

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Higher Secondary School Leaving Certificate (HSSC)	Govt. Higher Secondary School, Panaimarathupatty, Salem, Tamil Nadu, India	2002
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Research projects

- Molecular cytogenetic studies and technology development for creating aroid (Araceae) asymmetric somatic hybrids. Joint doctorate between University of Ghent, Gent, Belgium and Russian State Agrarian University, Moscow, Russia (Promoter: Prof. Erik Van Bockstaele, Co-promoter: Prof. Ludmila Khrustaleva, From October-2009 to September-2012)
- Induction of microcalli and microprotoplast in *Spathiphyllum spp.*, ILVO, Melle, Belgium (Guide: Dr. Tom Eeckhaut, 2008)
- Protoplast isolation and regeneration of *Spathiphyllum wallisii,* ILVO, Melle, Belgium (Guide: Dr. Tom Eeckhaut, 2007)
- Studies on micropropagation and molecular techniques. Loyola College, Chennai, India (Tutor: Dr. Ravindran, 2006)
- Isolation and purification of plasmid DNA and analysis using PCR techniques. Golden Jubilee Biotech Park, Chennai, India (Tutor: Mrs. Radhika, 2006)

Industrial projects

- Documentation, raw material management and professional risk management, La Lègumerie de la Cote d'Opale, Longuenesse, France (Tutor: Mr.S.Pecqueraux, 2007)
- Germination, nutritional analysis, preservation and marketing of seeds. Abbaye de Woestyne, France (Tutor: Mr. Bertrand Gabelle, 2007)

Achievements and awards

- Young scientist grant awarded in 24th EUCARPIA symposium (2012)
- Awarded Special Research Fund (BOF) scholarship for joint doctorate between UGent and Moscow State Agrarian University (2009-2012)
- Awarded French government's ÉGIDE scholarship for M.Sc. (2007-08)
- Outstanding Leadership Award, Loyola College (2005-06)
- Elected president of Students' Union, Loyola College (2005-06)
- Received meritorious scholarship from University of Madras (2003-06)
- School first rank holder (92.3%) in the state level HSSC examination (2002)
- School first rank holder (92.2%) in the state level SSLC examination (2000)

Conferences and contributions

- 02.06.2013 - 07.06.2013: 8th International symposium on *in vitro* culture and horticultural breeding, Coimbra, Portugal. Lecture: "Micronucleation in developing *Spathiphyllum wallisii* Regel microspores for microprotoplast preparation"
- 23.11.2012: 6th BPBA Symposium: Biotechnology targeting roots and rooting, ILVO, Melle, Belgium. Poster: "Micronucleation in developing *Spathiphyllum wallisii* Regel microspores for microprotoplast preparation"
- 02.09.2012 - 07.09.2012: 24th EUCARPIA international symposium on ornamental breeding, Warsaw, Poland. Lecture: "Micronucleation in developing *Spathiphyllum wallisii* Regel microspores for microprotoplast preparation"
- 18.06.2012 - 30.06.2012: International summer school of young scientists, TIMACAD, Moscow, Russia. Lecture: "Somatic hybridization for plant breeding"
- 18.09.2011 - 22.09.2011: 7th International symposium on *in vitro* culture and horticultural breeding, Ghent, Belgium. Poster: "Embryogenic callus formation from the petioles of *Spathiphyllum wallisii*"
- 13.11.2009: Plant hormones: new insights for biotechnology, Gembloux, Belgium
- 31.08.2009 - 04.09.2009: 23rd International EUCARPIA symposium, Section ornamentals "Colourful breeding and genetics", Leiden, The Netherlands
- 06.03.2006: National Conference on "Students rights and responsibilities under democracy" Loyola College, Chennai, India. Prime organizer as the president of Students' Union

Scientific publications

Peer reviewed

- Lakshmanan, P. S., Van Laere, K., Eeckhaut, T., Van Huylenbroeck, J., Van Bockstaele, E. and Khrustaleva, L. I. Karyotype analysis and visualization of 45S

rRNA genes using fluorescence *in situ* hybridization in aroids (Araceae). (Manuscript submitted in Genome)

- Eeckhaut, T., Lakshmanan, P. S., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Progress in plant protoplast research. Planta, 238: 991-1003
- Lakshmanan, P. S., Eeckhaut, T., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Asymmetric somatic plant hybridization: status and applications. American Journal of Plant Sciences, 4: 1-10
- Lakshmanan, P. S., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2013). Micronucleation by mitosis inhibitors in developing microspores of *Spathiphyllum wallisii* Regel. Plant cell reports, 32: 369-377

Proceedings

- Lakshmanan, P. S., Eeckhaut, T., Van Huylenbroeck, J., Van Bockstaele, E., and Khrustaleva, L. I. (2012, September). Micronucleation in developing *Spathiphyllum wallisii* Regel microspores for microprotoplast preparation. Acta Hort., 953:129-133
- Lakshmanan, P. S., Eeckhaut, T., Van Huylenbroeck, J., and Van Bockstaele, E. (2011, September). Embryogenic callus formation from the petioles of *Spathiphyllum wallisii*. Acta Hort., 961:231-234
- Eeckhaut, T., Duqenne, B., Lakshmanan, P. S., and Van Huylenbroeck, J. (2008, August). Development of microcolonies in protoplast culture of *Spathiphyllum wallisii*. Acta Hort., 829:51-54