Jan J. Rybczyński · Michael R. Davey
Anna Mikuła *Editors*

The Gentianaceae - Volume 2: Biotechnology and Applications

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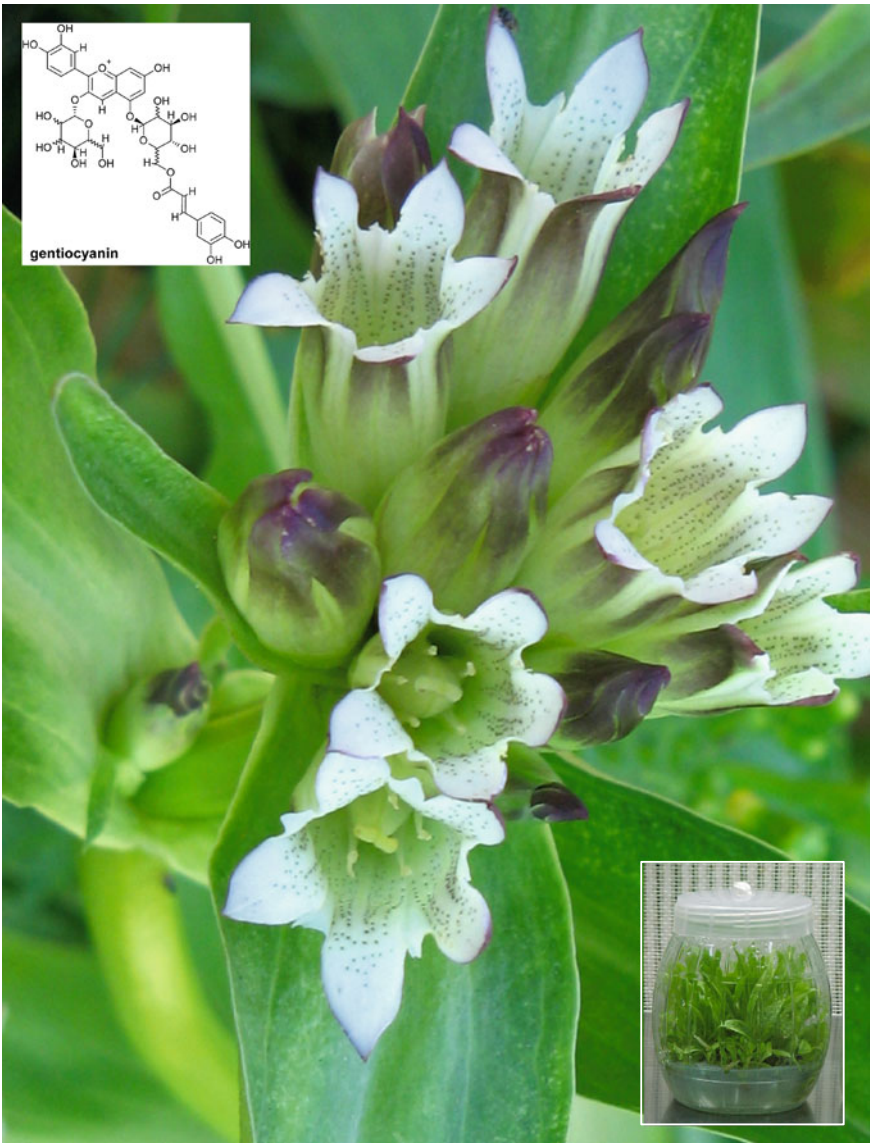

Gentiana tibetica King. (Photograph A. Mikuła)

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This volume is dedicated by the editors to their spouses

Preface

The Gentianaceae, or Gentian family, is worldwide in distribution with approximately 100 genera and about 1800 species that include monocarpic and perennial herbs, shrubs, trees, and lianes, with terrestrial and epiphytic representatives. The plants are diverse in habit, the majority being herbaceous. The tropics are the main source of new species of the Gentianaceae. *Gentiana* (360 species), *Gentianella* (250 species), and *Swertia* (135 species) are the three largest genera; members of the family are protected by law. Several species are important pharmacologically because of their secondary metabolites, as some of the compounds have a broad spectrum of biological activity.

Initial contacts with Gentians often occur during childhood when skin is protected from bacterial infection by *Gencjana* (Polish) or *Violetum Gentianae* (Latin), while children suffering from chicken pox are also painted with *Violetum* to counteract infection by *Herpes virus varicellae*. The importance of Gentians escalated in the 1980s when several studies at the plant level focused on the vegetative propagation of species, such as *Gentiana lutea* and *G. cruciata*, following the recognition of the secondary products synthesized by some members of this genus. Research into Gentians, especially in Poland, was stimulated further by the publication of the "Red Book" of the Polish Flora. This volume included reference to numerous Gentians and Gentianellas, with the need for their multiplication and reintroduction into the wild because of destructive overcollection of wild material for pharmaceutical use, combined with the loss of natural habitats. Some species are now rare and endangered. Variation in plant habit, especially flower morphology and pigmentation, also makes members of the Gentianaceae attractive for outdoor and indoor cultivation. The establishment of the Web site "Gentiana.pl" supplemented the earlier reference site "Gentiana Research Network" established by Dr. Lena Struwe at Rutgers University, New Brunswick, USA. Much deliberation, contacts at the scientific level and discussions with colleagues at Springer resulted in the compilation of these two volumes on Gentians. Volume 1 includes contributions to the characterization of this family of plants, while Volume 2 is devoted to the aspects of biotechnology and their applications.

Volume 1: Characterization and Ecology

Volume 1, comprising 12 chapters, centers upon the characterization and ecology of the Gentianaceae, with some emphasis on the application of molecular and cytological approaches in relation to taxonomy. The first three chapters consider classification of this family of plants, with Chap. 2 reviewing research progress since the earlier revision of the Gentianaceae in 2002. This revision resulted in reclassification of some plants and the naming of new genera. genera. Chapter 3 provides the most comprehensive report to date of the systematics of South American Neotropical woody members of the Gentians, with discussion of the use of cytological and molecular technologies to facilitate classification. Other reviews (Chaps. 4, 5) include details of the Gentianaceae in The Ukraine and Balkan Peninsula, with discussion of the taxonomy of representative species in these regions. Floral pigmentation in members of this family has been a topic of investigation for many years, with the key biochemical steps that result in the diversity of flower colors found in Gentians being summarized in Chap. 6. Other aspects of this diverse, interesting group of plants include the cytology of European species (Chap. 7), and a historical account of the importance of Gentians in herbal medicines, with links to evolution and classification (Chap. 8). Analysis of gene expression in overwintering buds is presented as an approach with which to study several aspects of plant taxonomy, phenotypic characteristics, phylogeography, and pedigree (Chap. 9). Two (Chaps. 10, 11) indicate the importance of Gentians in India in terms of their exploitation as herbal-based medicines, but emphasize the need for conservation to negate the loss of germplasm from natural habitats resulting from random harvesting. Finally, Chap. 12 presents evidence for the importance of fungi from the Phylum Glomeromycota in developing arbuscular mycorrhizal associations with the roots of members of the Gentianaceae. The role of such associations in plant growth and development is also discussed. Volume 1 of the Gentianaceae provides a general, broad-based foundation for more biotechnological approaches that are considered in Volume 2.

Volume 2: Biotechnology and Applications

The Gentianaceae includes species which are popular as ornamentals in the form of cut flowers and pot plants, with market demands necessitating improvement in flower quality, particularly characteristics such as inflorescence longevity. Micropropagation has become a routine procedure for multiplication of horticultural genera, including *Blakstonia*, *Centaurium*, *Genetiana*, *Gentianella*, with seedlings being the most common source of explants for plant propagation in vitro. Although organogenesis is the main route of plant regeneration, somatic embryogenesis is also a pathway in routine use for plant multiplication. These approaches are discussed in detail in Chaps. 1–6. Embryogenic cultures, such as cell

suspensions, are an excellent source of protoplasts for gene transfer by somatic hybridization and cybridization. The relevance of the latter technologies (Chap. 7) is that they generate nuclear and cytoplasmic combinations normally unavailable to plant breeders through conventional sexual hybridization. Techniques presented in Volume 2 also include the generation of haploid and dihaploid plants from cultured anthers, and the genetic variation that may arise from tissue and organ culture (Chaps. 8 and 9). Subsequent chapters discuss the molecular breeding of Gentians, particularly gene transfer by transformation, with associated genetic analyses (Chap. 10). Molecular markers facilitate breeding and cultivar identification. Vegetative propagation to generate genetically uniform populations and, conversely, manipulations to increase genetic variability, often rely upon cryopreservation as a common technology for long-term storage of relevant germplasm (Chap. 11). Other reviews consider the postharvest physiology of Gentian flowers (Chap. 12), and the biosynthesis of secondary metabolites, including antimalarial compounds (Chaps. 13–18). Modification of secondary metabolites has application in human health protection. Interestingly, the beauty of Gentian flowers and the pharmaceutical value of the plants have been the reasons for the special interest in the Gentianaceae since ancient times.

These two volumes should serve as key references for persons from a wide range of disciplines, including students and staff of universities and institutes, as well as professional gardeners and plant hobbyists.

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Abbreviations

(+)	Sign indicates somatic hybrid
μE	MicroE
½ MS medium	Half of original MS medium
1C DNA	Haploid value of nuclear DNA
1*n*	Haploid number of chromosomes
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2C DNA	Diploid value of nuclear DNA
2iP (6-IPA)	isopentenyladenine
2*n*	Diploid number of chromosomes
3C DNA	Triploid value of nuclear DNA
3*n*	Triploid number of chromosomes
4C DNA	Tetraploid value of nuclear DNA
4-CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea
4PU-30	N-(2-chloro-4-pyridyl)-N′-phenylurea
6C DNA	Hexaploid value of nuclear DNA
ABA	Abscisic acid
AC	Active charcoal
ACL	Average chain length
ACPH	Acid phosphatase
ADH	Alcohol dehydrogenase
AGP	Arabinogalactan protein
AP	Acid phosphatase
AS	Adenine sulfate
B5 medium	Medium according to Gamborg et al. (1968)
B5 vitamins	Vitamins according to B5 medium
*Bam*HI	(from Bacillus amyloli) is a type II restriction endonuclease
BAP (BA)	6-benzylaminopurine
Bar	Bialaphos resistance selection marker gene
$CaCl_2$	Calcium dichloride
CaMV	Cauliflower Mosaic Virus 35S promoter
CAPS marker	Cleaved amplified polymorphism sequence marker

CH	Casein hydrolysate
CHS	Chalcone synthase gene
CI	Callus induction
CMS	Cytoplasmic male sterility
Co	Catechol oxidase
cpDNA	Chloroplast DNA
CPI	Carbon preference index
CRES-T	Chimeric Repressor Gene-Silencing Technology
Cx	Cephatoxine
Cy-O	Cytochrome oxidase
DAPI	4′,6-diamidino-2-phenylindole is a fluorescent stain
DC	Direct current
DH	Dihaploid chromosome number
DH plants	Double haploid plants
Dic	Dicamba—3,6-dichloro-2-methoxybenzoic acid
DNA	Deoxyribonucleic Acid
*Eco*RI	Restriction enzyme recognizes G^AATTC
ELISA	Enzyme-linked immunosorbent assay
ELSs	Embryo-like structures
EST	Esterase
EST analysis	Expressed sequence tag analysis
F1	First sexual generation
FDA	Fluorescein diacetate
FID	Flame ionization detector
FITC	Fluorescein isothiocyanate
FLS	Flavonol synthase gene
G2/G1 phase	Phases of nucleus division
GA_3	Gibberellic acid
GC/MS	Gas chromatographic–mass spectrometric analysis
GFP genes	Green fluorescence protein genes
GISH	Genomic *in situ* hybridization
GMOs	Genetically modified organisms
GOT	Glutamate oxalacetate transaminase
H_2O_2	Hydrogen peroxide
*Hind*III	Restriction endonuclease that recognizes the sequence A^AGCT_T
HPLC	High-performance liquid chromatography
HPLC/DAD	High-performance liquid chromatography with diode array detection
HPLC-RP	High-performance liquid chromatography reversed phase
hrs	Hours
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IEF	Isoelectric focusing
IGS region	Intergenic spacer region

Im	Initial callus weight
ISSR	Intersimple sequence repeats
K	Potassium
Kb	Kilobase pairs
KDa	KiloDaltons
Kin	Kinetin—N^6-furfuryladenine
LEA genes	Late embryogenesis abundant genes
LH	Lactalbumin hydrolysate
LN	Liquid nitrogen
LNAs	Long-chain *n*-alkanes
LS	Medium according to Linsmaier and Skoog (1965)
M	Mol
MADS box	is a conserved sequence motif found in genes which comprise the *MADS-box* gene family
MAS	Multi-agent system
MDH	Malate dehydrogenase
MES	Methylethyl sulfide
metAFLP	met amplified fragment length polymorphisms
mg	Milligram
MS medium	Medium according to Murashige and Skoog (1962)
MSD	Network mass selective detector
MspI	Restriction endonuclease that recognizes the sequence C^CG_G
mtDNA	Mitochondrial DNA
n	Number of chromosomes
N	Number of planted explants
NAA	Naphthalene acetic acid
Nc	Number of explants showing callus
NLN medium	Medium according to Takahata and Keller (1991)
NN medium	Medium according to Nitsch and Nitsch (1969)
NOA	Naphthoxyacetic acid
nptII	Kanamycin resistance selection marker gene
P	Phosphate
PCR	Polymerase chain reaction
PCV	Packed cell volume
PEG	Polyethylene glycol
PEM	Proembryogenic mass
PER	Peroxidase
PGRs	Plant growth regulators
PhGLMC	*Petunia hybrida* green leaf mesophyll cells
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
PPF	µmoles m^{-2} s^{-1}
PsGLMC	*Pisum sativum* green leaf mesophyll cells
PVS2	Plant vitrification solution
R	The number of regenerants
RAPD	Randomly amplified polymorphic DNA

rDNA	Ribosomal DNA
RE	Regeneration efficiency
RFLP	Restriction fragment length polymorphism
RNAi	RNA interference
ROS	Reactive oxygen species
RP	Regeneration percentage
rpm	Rotation per minute
rRNA	Ribosomal RNA
RT-PCR	Real-time PCR
s	Second
SA	Adenine sulfate
SDS PAGE	Polyacrylamide electrophoresis
SE	Somatic embryogenesis
SSR markers	Simple sequence repeat markers
STS markers	Sequence tagged site markers
suc	Sucrose
TaqI	Restriction enzyme isolated from the bacterium *Thermus aquaticus*
TCA	Cycle
TDZ	Thidiazuron
TL	Left T-DNA border
TR	Right T-DNA border
TRBC	Trout red blood cells
UV light	Ultraviolet light
VIGS	Virus-induced gene-silencing technology
VSL	Vitrification solution L
w/v	weight/volume
W14 and W15	Proteins accumulated in overwintering buds
WPM medium	Woody plant medium (Lloyd and McCown 1981)
x	Basal chromosome number
Zeat	Zeatin—(*E*)-2-methyl-4-(7*H*-purin-6-ylamino)but-2-en-1-ol
μM	Micromol

Chapter 1
Systems of Plant Regeneration in Gentian In Vitro Cultures

Jan J. Rybczyński, Michael R. Davey, Karolina Tomiczak, Agnieszka Niedziela and Anna Mikuła

Abstract This chapter reviews the development of plant tissue culture and biotechnology of gentians during the last thirty years. The majority of 30 species studied belong to the genus *Gentiana* and those gentians are included into European flora. Biochemical studies aimed secondary metabolites production are not presented in the chapter. Explants from seedling were most frequently used for culture initiation. Differences between particular organs of a few-day-old seedling are significantly different in the presence of MS medium. Leaves from in vitro culture plants were used to describe their morphogenic potential and as a source of the protoplasts for somatic hybridization and transformation. The culture of floral explants helps to get interspecies hybrids and haploids with improving floriculture breeding programs. The shoot and root organogenesis and shoot multiplication play key role in the vegetative propagation of gentians. Embryogenic cultures on semi-solid and in liquid helped to undertake many subjects concerning somatic embryogenesis per se and exploration of embryogenic cell suspension for somatic cell genetic manipulation.

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1.1 Introduction

The first papers summarizing the achievements of gentian plant tissue cultures were published by Barešová (1988) and Miura (1991). Since then, considerable progress has been achieved in the biochemistry and biotechnology of gentian plant tissue cultures. The publication that contains the aim of the studies by Skrzypczak et al. (1993a, b) indicates only six main genera, namely *Blackstonia*, *Centaurium*, *Eustoma*, *Gentiana, Gentianella*, and *Swertia*, with only eleven species. Now, twenty years later, the list of gentians has increased to almost thirty species. Studies which have been carried out on species comprising the family Gentianaceae show considerable evidence for somatic and generative cell manipulation. The study of plant morphogenesis of Gentianaceae species has revealed five patterns of plant differentiation leading, these being.

Explant → cells → direct somatic embryos → plantlets
Explant → callus/cells → indirect somatic embryos → plantlets
Explant → adventitious organ → direct somatic embryos → plantlets
Explant → callus formation → meristem formation →
buds → shoots → roots → plantlets
Explant → apical/axillary buds → multiple shoots → roots → plantlets.

Exploration of these patterns of development has led to the manipulation of cells to generate regenerants with stable or altered chromosome numbers, haploid plants, sexual and somatic hybrids, transformants, and plants with increased production of secondary metabolites.

1.2 Explants Considered for Culture Initiation

1.2.1 Seedling/Plant Explants

Gentian seeds are sexual propagules with a relatively low frequency of germination, and their viability requires special treatment during in vitro and ex vitro experiments. The requirements cover various factors, i.e., chemical treatment with gibberellins (GA_3) and low storage temperature, as an example of a physical treatment. The limits of natural propagation and the pharmaceutical demands for plant material as a source of secondary metabolites are the main reasons for the development of simple and very efficient protocols for in vitro propagation of members of the family Gentianaceae.

1.2.1.1 Shoot and Node Fragments

Regeneration systems of several gentian species have been established using a range of various explants from embryos, seedlings, plantlets, and plants. The majority of papers describe culture initiation taking place with explants from shoot

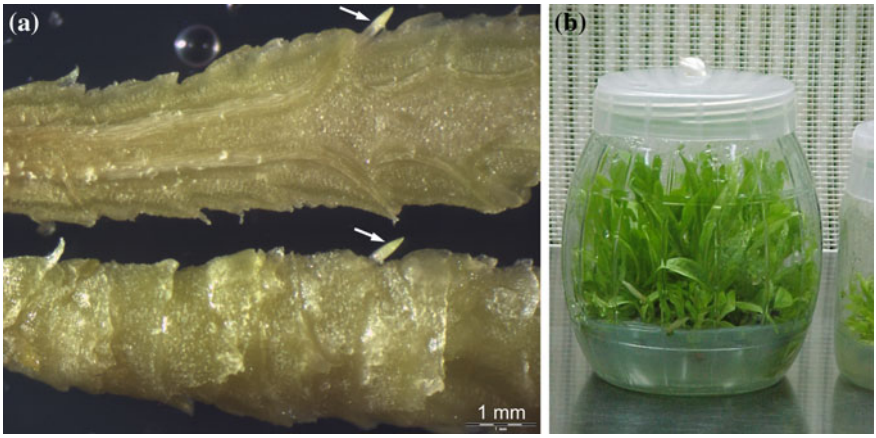

Fig. 1.1 Primary explant and shoot multiplication of *Gentiana* sp. **a** Longitudinal section (*upper*) and general view (*lower*) of a regenerating shoot explant showing axillary buds (*white arrows*) and **b** shoot multiplication culture on 0.5 MS-based medium

tips (apical meristems) and node fragments (axillary buds). Explants originate from seedlings which are a few weeks old, shoot axenic cultures, and plants grown in controlled conditions (Fig. 1.1). Table 1.1 summarizes the species for which multiplication systems have been developed using explants mentioned above. Murashige and Skoog's (1962) medium is very popular for maintenance of plant tissue cultures. In the majority of the studied species, the MS medium was used. The exceptions were as follows: Woody plant medium (WPM) (Lloyd and McCown 1981) for *Gentiana acaulis, Gentiana purpurea* (Momcilović et al. 1997a, b), and *Gentiana triflora* (Zang and Leung 2002), B5 medium (Gamborg et al. 1968) for *Gentiana scabra* (Yamada et al. 1991), and NN medium (Nitsch and Nitsch 1969) for *Gentiana lutea and Gentiana punctata* (Skrzypczak et al. 1993a, b). Murashige and Skoog's medium (1962) was also used at half strength for in vitro propagation of *Swertia bimaculata* and *Swertia chirayita* (Dafadar and Jha 2012; Joshi and Dhawan 2007a, b), while for *G. lutea*, the microelements of MS medium were substituted for microelements of B5 medium (Holobiuc and Blindu 2008). A combination of MS mineral salts with B5 vitamins was sometimes used (Morgan et al. 1997; Cai et al. 2009). It is worth noting that WPM medium applied at double strength greatly stimulated the growth of micropropagated shoots of *G. triflora* var. *axillaryflora* Akita Blue and their flowering in vitro. All of the various media mentioned above were used for 2 years to support the persistence of the regeneration capacity of the systems (Zhang and Leung 2002).

The effects of some factors, such as the application of sucrose, are known to stimulate explant growth and development. For all the gentians studied, sucrose was used as the main energy source. The sucrose concentration varied between 2 and 6 %, with the main application being 3 %. There is only one paper which describes the application of glucose and fructose. However, these two energy sources were