Somatic Embryogenesis and Transformation Studies in *Schlumbergera* and *Rhipsalidopsis*

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Zusammenfassung

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Somatische Embryogenese und Untersuchungen zur Transformation bei *Schlumbergera* und *Rhipsalidopsis*

Schlagwörter: Genetische Transformation - Osterkaktus - Transgen - Weihnachtskaktus

Die In-vitro-Kultur bietet für Kakteen Möglichkeiten zur Vermehrung, somatischen Embryogenese und genetischen Transformation. In der vorliegenden Arbeit wurde somatische Embryogenese an Phyllokladien-Explantaten von *Schlumbergera truncata* cv. Russian Dancer induziert. Kallus, der sich an Phyllokladien-Explantaten gebildet hatte, wurde über einen Zeitraum von 16 Monaten auf MS-Medium mit Cytokininen kultiviert. Im Hinblick auf die Induktion somatischer Embryonen war diese Behandlung einer kürzeren Etablierungsphase überlegen. Somatische Embryonen differenzierten nach drei bis fünf Monaten, wenn Kallus, der in flüssigem Medium auf SH- oder MS-Basis mit 7 µM Kinetin kultiviert worden war, auf festes MS-Medium entweder mit 0,45 µM 2,4-D oder hormonfrei überführt wurde. Die Medienzusammensetzung, Wachstumsregulatoren sowie die Dauer der Kultur auf cytokininhaltigen Medien hatten einen Einfluss auf die Embryogenese. Die höchste durchschnittliche Anzahl von Embryonen in unterschiedlichen Stadien wurde erzielt, wenn die erste Kulturphase von 30 Tagen in flüssigem SH-Medium, und die zweite auf hormonfreiem MS-Medium erfolgte. Ungefähr 70 % der somatischen Embryonen keimten auf G-Medium. Anschließend wurden die auf somatische Embryonen zurückgehenden Pflanzen erfolgreich in Erde überführt. Sie zeigten genetische Stabilität im Vergleich zu den Ausgangspflanzen mit Ausnahme der Pflanzen, die sich aus somatischen Embryonen mit mehr als vier Keimblättern entwickelt hatten. Zusätzlich wurde ein sehr effizientes Regenerationssystem über Adventivsprossbildung für *Schlumbergera* cv. Alex und *Rhipsalidopsis* cv. CB5 entwickelt. Darüber hinaus wurden diese effizienten Regenerationssysteme weiterentwickelt und erfolgreich eingesetzt, um erstmals transgene Pflanzen von *Rhipsalidopsis* cv. CB5 zu erzeugen, die das *uidA*-Gen und das selektierbare Markergen *nptII* enthielten. Einige Faktoren, die die Transformation von *Rhipsalidopsis*-Kallus beeinflussen, wurden untersucht. Transformierte *Rhipsalidopsis*-Kallusse wurden nach längerer Kultur auf Medien mit 600 mg/l Kanamycin erhalten. Eine Vor-Inkubation von *Agrobacterium tumefaciens* in SIM-Medium mit Acetosyringon steigerte die Häufigkeit, mit der transgene Kallusse erzielt wurden. Desweiteren war ein zusätzlicher Waschschritt der Kallus-Explantate mit Cefotaxim nach der Ko-Kultur notwendig, um *Agrobacterium tumefaciens* zu unterdrücken. Der Verzicht auf Kanamycin im Medium für den letzten Kulturschritt sowie die Kultur der transformierten Kallusse unter Ernährungsstress führte zur Bildung transgener Adventivsprosse. Mit diesem Ansatz wurde eine Transformationsrate von 22,7 % erreicht. Die in dieser Arbeit erzielten Ergebnisse zeigen, dass der *Agrobacterium*-vermittelte Gentransfer ein vielversprechender Ansatz für die Erzeugung neuer Genotypen bei diesen beiden Kakteenarten ist.

Abstract

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Somatic Embryogenesis and Transformation Studies in *Schlumbergera* and *Rhipsalidopsis*

Keywords: Christmas cactus - Easter cactus - genetic transformation - transgene

Tissue culture has emerged as a tool for cacti micropropagation, somatic embryogenesis and genetic manipulation. Somatic embryogenesis was induced from phylloclade explants of *Schlumbergera truncata* cv. Russian Dancer. Callus that developed on phylloclade explants and sub-cultured over a period of 16 months on MS medium containing cytokinins was superior for the induction of somatic embryos compared to callus grown for a shorter time in the establishment phase. Somatic embryos were induced after three to five months when callus grown in SH- or MS-based liquid media supplemented with 7.0 μM kinetin was transferred onto solid MS-based medium with either 0.45 μM 2,4-D or without hormones. Embryogenesis was affected by the type of medium, plant growth regulators and duration of callus exposure to cytokinins. The highest average numbers of embryos in the different stages were achieved using SH liquid medium for the first culture and MS medium without hormones after 30 days. Approximately 70% of somatic embryos germinated on G medium. Furthermore, plants derived from somatic embryos were successfully potted in soil, and they showed, except those derived from somatic embryos with more than four cotyledons, genetic stability compared to mother plants. A highly efficient regeneration system through adventitious shoot formation was also developed in *Schlumbergera* cv. Alex and *Rhipsalidopsis* cv. CB5. Moreover, the development of these efficient regeneration systems was further exploited and success was demonstrated as the first report in obtaining *Rhipsalidopsis* cv. CB5 shoots transgenic for the *uidA* gene and the selectable marker *nptII* gene. Some of the factors influencing transformation of *Rhipsalidopsis* callus explants were evaluated. Transformed *Rhipsalidopsis* calli were obtained by extended culture on media containing 600 mg/l kanamycin. The pre-incubation of *Agrobacterium tumefaciens* in SIM medium containing acetosyringone raised the frequency of transgenic calli. Furthermore, a washing step with cefotaxime for the callus explants after co-culture was necessary to remove the excess *Agrobacterium*. The removal of kanamycin from the final medium together with the culture of the transformed calli under nutritional stress led to the formation of transgenic adventitious shoots. With this approach, a transformation efficiency of 22.7% was achieved. The results obtained in this study suggested that *Agrobacterium*-mediated transformation is a promising approach for generating new genotypes of these cacti.

Dedication

To my
Family
and
Friends

With Love

Acknowledgements

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Abbreviations

1-MCP	1-methylcyclopropene
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ,γ-dimethylallylamino) purine
2n	The characteristic number of chromosomes in a somatic cell (diploid number)
ABA	Abscisic acid
AVG	Aminoethoxyvinylglycine
B_5	Gamborg et al., (1968) medium
BA	6-benzyladenine
BME	β-mercaptoethanol
BM3	Gupta and Durzan (1986) medium
BSA	Bovine serum albumine
CaMV 35S	Cauliflower mosaic virus promoter
cm	Centimeter
cSUT	Carrot sucrose transporter gene
cv(s)	Cultivar(s)
dH_2O	Deionized H_2O
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ESEM	Environmental scanning electron microscope
etr1-1	Ethylene mutant receptor gene
fbp1	Flower specific promoter
G medium	¾ MS salts, 0.1 g l^{-1} myo inositol, 50 g l^{-1} sucrose, Staba vitamins and 3 g l^{-1} gelrite
g l^{-1}	Gram per liter
GA_3	Gibberellic acid
GLM	General linear model
GUS	β-Glucuronidase
gusA	β-Glucuronidase gene from *Escherichia coli*
gusAint	β-Glucuronidase gene containing an intron in the coding region
h	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
in	Inch
kin	Kinetin
L2	Phillips and Collins (1979) medium
LB	Luria Broth medium
MES	2-(*N*-Morpholino) ethane-sulfonic acid
min	Minute
µM	Micro molar
Mpa	Megapascal
MS	Murashige and Skoog (1962) medium
N	Normality
Na2HPO4	Disodium hydrogen phosphate
NAA	α-naphthaleneacetic acid

NaH2PO4	Sodium dihydrogen phosphate
nptII	Neomycin phosphotransferase gene
OD_{600}	Optical density at 600 nm
$_P$-CPA	*p*-chlorophenoxyacetic acid
PEDCs	Pre-embryogenic determined cells
PEG	Polyethylene glycol
PVPP	Polyvinylpolypyrrolidone
rpm	Revolution per minute
SAS	Statistical analysis system
SDS	Sodium Dodecyl Sulfate
SH	Schenk and Hildebrandt (1972) medium
SIM medium	10 g l^{-1} bacto peptone, 5 g l^{-1} NaCl, 10 g l^{-1} yeast extract, 0.5 *mM* acetosyringone and 20 *mM* tri sodium citrate
SSC	Sodium chloride sodium citric acid
STS	Silver thiosulphate
T-DNA	Transferred DNA
TDZ	Thidiazuron
TE	Transformation efficiency
Ti	Tumour-inducing
TIBA	2,3,5-triiodobenzoic acid
uidA	Coding region of the *β*-glucuronidase gene
v/v	Volume/ volume (Percentage by volume)
vir	Virulence gene
w/v	Weight/ volume

1.0 General Introduction

1.1 Overview

Cacti are dicotyledonous perennial plants with specialised features adapted for survival in arid and other climatic conditions (Hubstenberger *et al.*, 1992).

Common and scientific names: The holiday cactus group generally consists of those plants derived from the two species: (1) Thanksgiving cactus (*Shlumbergera truncata*), (2) Christmas cactus (*Schlumbergera x buckleyi*); synonym *S. bridgesii* (Ramirez and Lang, 1997; Dole and Wilkins, 1999). Plants cultivated as Easter cactus are a clonally propagated selection of *Rhipsalidopsis gaertneri* (Regel) Moran, *R. rosea* (Lagerheim) Britton & Rose, and their interspecific hybrids [*R. x graeseri* (Werdermann) Moran] (Liberty Hyde Bailey Hortorium, 1976; Barthlott, 1979). Moreover, *Hatiora gaertneri* has been used as a synonym for *Rhipsalidopsis gaertneri* (Barthlott, 1987; Dole and Wilkins, 1999).

Two species, *S. truncata* and *S. russelliana*, have been hybridized to produce the well-known Christmas cacti that are widely cultivated as flowering potted plants (McMillan and Horobin, 1995).

Much confusion exists over the identity of the species used in the research. *Schlumbergera truncata* was formerly named *Epiphyllum truncatum* and *Zygocactus truncatus* (Hammer, 1992). These names are used in the literature with the common name Christmas cactus, but it would appear from photographs and descriptions that most of the work was done with Thanksgiving cactus. Most of the new hybrids that are called Christmas cactus in the trade also appear to be Thanksgiving cactus, *S. truncata* (Hammer, 1992).

Origin: *Schlumbergera*, popularly known as 'Christmas cactus' or 'Thanksgiving cactus', and *Rhipsalidopsis*, also known as 'Easter cactus', are epiphytes native to forests in Brazil (Hammer, 1992; Sriskandarajah and Serek, 2004).

General Introduction

Family and related taxa: These cacti belong to the Cactaceae family. The three cacti are confusing because of frequent taxonomic name changes, interspecific hybrids and similar appearance. All three species consist of modified stems called phylloclades which are flat-like structures that photosynthesize. *Hatiora* differ from *Schlumbergera* in that the *Hatiora* flower perianth tube is shorter, the stamens are separated and the stigmatic lobes are spreading. Further, *Hatiora* phylloclades have shallow marginal indentations with obvious bristle-like spines, while the phylloclades of *Schlumbergera* do not have obvious "spines" (Dole and Wilkins, 1999). The main difference between the Thanksgiving cactus (*S. truncata*) and the Christmas cactus (*S. x buckleyi*) is that the former has strongly toothed or pointed edges along the margins of the phylloclades; the latter has smooth-edged margins (Heins *et al.*, 1981; Rünger and Poole, 1985; Wilkins and Rünger, 1985; Hammer, 1992).

Uses and economic importance: The main economic importance of these cactus species lies in world-wide horticultural trade as ornamental plants. The major countries for production of ornamental cacti include Denmark, USA, Japan, Great Britain, Germany and Holland (Sriskandarajah and Serek, 2004). A total of 8 and 2.8 million plants of *Schlumbergera* and *Rhipsalidopsis*, respectively, were produced in Denmark in 2005. This constitutes about 70-80% of the production of the European market of these cacti.

Christmas and Easter cactus are popular plants sold during the spring and fall holidays. Holiday cactus are suitable for hanging baskets (pendulos types), 4-in. (10 cm) pots (semipendulous types), or 3-in. (7.5 cm) pots (erect types) (Dole and Wilkins, 1999). These cacti are grown as flowering potted plants (Boyle, 1997) and they make colourful potted plants which adapt well in the home and re-flower with modest care (Dole and Wilkins, 1999).

Schlumbergera species of holiday cactus have become very popular ornamental plants for their beautiful flowers, wide flower-colour range, and unusual plant form (Lang and Cushman, 1993). Their short-day photoperiodic flowering response and easy care make them a favourite plant of consumers during the Christmas season (Ramirez and Lang, 1997).

1.2 Tissue culture of the Cactaceae family

In their review, Fay and Gratton (1992) have summarized the *in vitro* culture work of cactus. Early studies focused on callus formation and proliferation. King (1957) reported the induction of callus in several species and later Sachar and Iyer (1959) investigated the effects of auxins, cytokinins and gibberellins on callus formation from placental tissue of *Opuntia dillenii*. Minocha and Mehra (1974) found that callus induction on a range of tissues of *Mammillaria prolifera* was dependent on the presence of 2,4-D in the medium. Kolár *et al*. (1976) were the first to obtain cactus shoots from *in vitro* callus cultures of *Mammillaria woodsii*. Shoots were subsequently recovered from callus of *Mammillaria elongata* (Johnson and Emino, 1979).

Johnson *et al*. (1976) found that shoot tips of Christmas cactus and Easter cactus grown on rotating liquid medium, produced an average of 9 shoots per explant in 8 weeks. The best results were obtained using liquid Linsmaier-Skoog media with 30 g/l sucrose and 10 mg/l kinetin.

Mauseth (1979) successfully demonstrated the micropropagation of cacti by axillary shoot proliferation. Following these pioneering efforts, numerous kinds of cactus have been propagated by multiplication of axillary shoots. Depending on the genus, explants consisted of the shoot apex of seedlings, lateral or vertical sections of plantlets or cladodes, single areoles or single tubercles (George, 1996).

Clayton *et al*. (1990) have investigated the micropropagation of 11 rare or endangered cacti species belonging to the subtribe Cactinae, using shoot tips as explants. Their study showed that low or no auxin but moderate to high cytokinin concentrations were required for axillary shoot production. Furthermore, all species rooted spontaneously on hormone-free media. However, several species rooted better on media containing auxin.

Boyle and Marcotrigiano (1997) reported that organogenesis in Easter cactus (*Hatiora gaertneri*) can be controlled by varying the concentration of BA and GA_3 in the culture medium. The number of flower buds and new phylloclades, from phylloclades

cultured *in vitro*, increased linearly as BA concentration increased from 0 to 444.1 μM. GA_3 increased the number of new phylloclades when present in moderate concentrations (2.9 or 28.9 μM), but inhibited flower bud formation when present in concentrations as low as 0.3 μM.

Recently, García-Saucedo *et al*. (2005) have developed a protocol for *in vitro* micropropagation of *Opuntia* cactus plants. Their method consisted of growing cladode explants on MS medium (Murashige and Skoog, 1962) containing BA and GA_3. The shoots produced were used as secondary explants and grown again on medium containing BA for bud formation. Rooting occurred when IBA was added to the medium.

The formation of somatic embryos in cacti was also reported. Somatic embryos were induced in *Ariocarpus retusus*, from callus produced from seedlings germinated earlier on solid MS medium containing 20 g/l sucrose and 20% (v/v) coconut water (Stuppy and Nagl, 1992). After 3-4 months of culture, the first somatic embryo appeared. For further growth, somatic embryos were placed on hormone-free MS medium.

Torrez-Muñoz and Rodríguez-Garay (1996) reported plant regeneration in *Turbinicarpus pseudomacrochele* through indirect somatic embryogenesis on solid MS medium supplemented with L_2 vitamins, 3 mg/l 2,4-D, 2 mg/l NAA, 2 mg/l kinetin, 500 mg/l L-glutamine, 250 mg/l casein hydrolizate and solidified with 8 g/l agar.

Recently, Marín-Hernández *et al* (1998) reported the *in vitro* somatic embryogenesis in the severely endangered cactus *Mammillaria san-angelensis*. Somatic embryo structures of both unicellular and multicellular origin were formed from ovule integuments cultured in a modified B_5 medium (Gamborg *et al*., 1968) supplemented with 2,4-D (4 mg/l) plus kinetin (2 mg/l). However, plant regeneration was not obtained.

1.3 Plant transformation

Genetic engineering has opened up new avenues to modify crops and provided new solutions to solve specific needs. The powerful combination of genetic engineering and conventional breeding programs permit useful traits encoded by transgenes to be introduced into commercial crops within an economically viable time frame (Hansen and Wright, 1999). There is a great potential for genetic manipulation of crops to enhance productivity through increasing resistance to pests (Cho *et al*., 2001), diseases (Chen and Punja, 2002) and environmental stress (Uchimiya *et al*., 2002; Lim *et al*., 2005).

The development of methods for introducing genes into plants is an essential step in metabolic manipulation of plant secondary compounds. This provides a potential strategy to improve the production of high-value secondary metabolites (Bae *et al*., 2005).

Genetic engineering in floriculture is providing a valuable means of expanding the floriculture gene pool and so, promoting the generation of new commercial varieties. Commercialisation of genetically engineered flowers is currently confined to novel coloured carnations (Tanaka *et al*., 2005).

The production of novel flower colour has been the first success story in floriculture genetic engineering (Meyer *et al*., 1987). Other traits that have received attention include floral scent, floral and plant morphology, senescence of flowers both on the plant and post-harvest and disease resistance (Tanaka *et al*., 2005).

Three major techniques were mainly used for transforming plants. Those included *Agrobacterium*-mediated transformation (Toldi *et al*., 2002; Gruchala *et al*., 2004), protoplast transformation (Tsugawa *et al*., 1998; Wang *et al*., 2000) and biolistics or microprojectile bombardment (Zhang *et al*., 2000).

For transformation, antibiotics or herbicides have been widely used as selection agents to identify transformants. Hygromycin (Arencibia *et al*., 1998; Cho *et al*., 2001; Bae *et al*., 2005), kanamycin (Aida and Shibata, 1996; Nebauer *et al*., 2000;

Araújo *et al*., 2004; Sriskandarajah *et al*., 2004), bialaphos (Zhang *et al*., 2000), and phosphinothricin (Chen and Punja, 2002; Kang *et al*., 2005) have been used as selection agents to stop non-transformed tissues or cells from growing.

1.4 Statement of the problem

The main post-greenhouse problem of *Schlumbergera* and *Rhipsalidopsis* is flower bud abscission (Hammer, 1992). Furthermore, these cacti have been considered as ethylene sensitive (Serek and Reid, 1993), which leads to abscission of flowers and buds. This bud abscission can occur in cactus during long distance shipment of plants (Cameron and Reid, 1981) and thus can cause economic loss. However, chemical treatments utilizing ethylene inhibitors, like silver thiosulfate (STS), to improve the display life of cactus have caused environmental concerns (Serek and Reid, 1993).

Theoretically, hybrids between *Hatiora* and *Schlumbergera* may serve as a bridge for introgressing desirable traits from one genus to the other. Numerous interspecific and intergeneric hybrids have been documented for the Cactaceae, some of which originated in the wild while others arose from deliberate crosses (Hawkes, 1982, 1983; Rowley, 1994).

Interspecific hybrids have been produced by crossing *H. gaertneri* with *H. rosea* (Moran, 1953) and *S. truncata* with *S. opuntioides* (Löfgren & Dusén) D. Hunt, *S. orssichiana* Barthlott & McMillan, and *S. russelliana* (Barthlott and Rauh, 1977; McMillan and Horobin, 1995).

In their study, Boyle and Idnurm (2003) have pointed out that there are barriers to intergeneric hybridization that must be overcome to increase the success rate in crossing Easter cactus with holiday cactus. However, only one intergeneric hybrid, that has proven to be male sterile, has flowered (Boyle and Idnurm, 2003). Hence, improving these cacti through classical breeding techniques is limited and alternative, more efficient technologies will be needed to expand the gene pool in these plants.

Recently, through the introduction of new tools of biotechnology, crossing barriers have been overcome, and genes from unrelated sources have become available to be

introduced asexually into plants (Repellin *et al*., 2001). A transgenic approach to genetic improvement would allow modification of various traits without altering the essential characteristics of the plant cultivar (Iocco *et al*., 2001).

The application of gene technology to create new cultivars with desirable characteristics, such as ethylene resistance, has been highlighted for plants like carnation (Bovy *et al*., 1999), petunia (Gubrium *et al*., 2000) and *Campanula carpatica* (Sriskandarajah *et al*., 2004). In these studies, a mutant *etr1-1* allele that confers insensitivity to ethylene was used. Because of the bud drop problem of cacti due to ethylene sensitivity, molecular approaches through genetic engineering can have a high potential for increasing the display life of flowering cactus potted plants. Micropropagation and efficient plant regeneration from tissue cultures are required steps for the development of protocols for plant improvement through genetic engineering (Pozueta-Romero *et al*., 2001).

Tissue culture has emerged as a tool for cacti micropropagation and has been used in different genera like *Cereus*, *Equinocereus*, *Ferocactus*, *Mammillaria*, *Opuntia* and others (Escobar *et al*., 1986; Machado and Prioli, 1996; Pérez-Molphe *et al*., 1998; Juárez and Passera, 2002). There have been a few studies on *in vitro* regeneration of *Schlumbergera* and *Rhipsalidopsis*. In these studies, regeneration occurred via axillary and adventitious shoots but the rate at which these shoots were produced was slow and unsatisfactory (Johnson *et al*., 1976; Sriskandarajah and Serek, 2004).

Another route for *in vitro* plant regeneration, which has not been reported in either *Schlumbergera* or *Rhipsalidopsis*, is through somatic embryogenesis. Somatic embryos reportedly originate from single cells, so one could avoid the problem of producing plants with chimeric tissues. However, this problem could occur when regeneration originates from shoots (Nagmani *et al*., 1987; Nuti Ronchi and Giorgetti, 1995; Kuksova *et al*., 1997; Mandal and Gupta, 2003; Mithila *et al*., 2003). The other important aspect of somatic embryos is their practical application in large scale vegetative propagation (Fki *et al*., 2003; Langhansová *et al*., 2004; Pinto *et al*, 2004; Zouine *et al*., 2005). Using this method, large number of embryos of uniform physiological and growth characteristics can be produced (Von Aderkas *et al*., 2001).

Furthermore, somatic embryos could be used for cloning of somaclonal variants and synthetic seed production (Moghaddam *et al*., 2000).

Agrobacterium-mediated transformation via somatic embryogenesis has been a common approach for genetic improvement in many plant types like geranium (KrishnaRaj *et al*., 1997), grapevine (Iocco *et al*., 2001), carrot (Chen and Punja, 2002), *Lilium* (Mercuri *et al*., 2003), pearl millet (Goldman *et al*., 2003) and rose (Condliffe *et al*., 2003). When somatic embryogenesis occurs indirectly, embryogenic callus provide a large population of embryogenic competent cells that are extremely amenable for transformation by *Agrobacterium*. Each transformed cell represents an independent transgenic line, thus tremendously increasing the number of transformation events in a regenerable tissue and improving transformation frequencies (Leelavathi *et al*., 2004).

1.5 Objectives

Overall goal

To explain the factors influencing morphogenesis in *Schlumbergera* and *Rhipsalidopsis* in order to improve the regeneration systems in these plants. This can be the basis for genetic improvement work in *Schlumbergera* and *Rhipsalidopsis*.

Specific goals

(1) To improve the *in vitro* regeneration systems in *Schlumbergera* and *Rhipsalidopsis* through:

 I. Screening a number of cultivars from both genera for somatic embryogenesis potential.

 II. Induction, maturation and plant regeneration via somatic embryos in the responsive cultivars.

 III. Improving protocols for adventitious shoot regeneration.

(2) To provide histological evidence of the discrete vascular connections and the key developmental stages of the somatic embryos.

(3) To study the genetic stability of the plants derived from somatic embryos.

(4) To develop methods for introducing foreign genes into *Schlumbergera* and *Rhipsalidopsis* through genetic transformation:

 I. Developing a general transformation protocol for the reporter *uidA* gene and the selectable marker *nptII* gene.

 II. Developing a transformation protocol for the gene *etr1-1* that confers insensitivity to ethylene.

2.0 Plant regeneration via somatic embryogenesis in *Schlumbergera*

Abstract

Keywords: Christmas cactus - multiple cotyledons - secondary embryos – organogenesis - adventitious shoots

Somatic embryogenesis was induced from phylloclade explants of *Schlumbergera* cv. Russian Dancer. Callus developed on phylloclade explants and sub-cultured over a period of 16 months on MS medium containing mainly cytokinins was superior for the induction of somatic embryos compared to callus grown for a shorter time in the establishment phase. Subculture of callus grown in SH- or MS-based liquid media supplemented with 7.0 μM kinetin and transferred onto solid MS-based medium with either 0.45 μM 2,4-D or without hormones resulted in the differentiation into somatic embryos. SH-based medium proved better than MS-based medium when used as the first medium for the induction of somatic embryogenesis. However, somatic embryogenesis, contrary to adventitious shoot formation, was reduced when 2,4-D was included in the MS-based medium used for final transfer. This is contrary compared to the medium without growth regulators, indicating a critical hormonal balance was reached. Somatic embryos developed root and shoot poles when grown on G medium. Furthermore, plants from somatic embryos were successfully potted in soil and showed an increase in height and formation of a second set of phylloclades (secondary phylloclades). With the exception of plants derived from somatic embryos with more than four cotyledons, plants derived from somatic embryos showed genetic stability compared to mother plants. Histological studies showed that somatic embryos had no detectable connection with the mother explants and that advanced stages of somatic embryos had a contained vascular system. Medium, plant growth regulators, duration of callus exposure to cytokinins and presence or absence of gauze were shown to affect morphogenesis in *Schlumbergera* and *Rhipsalidopsis*. A highly efficient regeneration system in cultivars Alex and CB5 was described. The occurrence of anomalous and secondary embryos was also recorded in this study.

2.1 Introduction

2.1.1 Overview

Easter cactus [*Rhipsalidopsis* spp and interspecific hybrids] and Holiday cactus [*Schlumbergera* spp and interspecific hybrids] are popular, flowering, potted plants in North America and northern Europe (Scott *et al*., 1994).

While axillary and adventitious shoots in *Schlumbergera* and *Rhipsalidopsis* have been produced *in vitro* from phylloclade explants and callus cultures (Sriskandarajah and Serek, 2004) and from shoot tips (Johnson *et al*., 1976), the frequency at which these shoots are produced is still slow and unsatisfactory. However, somatic embryogenesis is another pathway that could play a role in the current improvement programmes of tissue culture in these two crops.

Induction of somatic embryogenesis has been reported in some cactus plants, including *Turbinicarpus pseudomacrochele* (Torres-Muñoz and Rodríguez-Garay, 1996), *Mammillaria san-angelensis* (Marín-Hernández *et al*., 1998), *Opuntia ficus-indica* (Da Costa *et al*., 2001) and in other succulent plants such as *Agave victoria-reginae* (Rodríguez-Garay *et al*., 1996). However, up to now somatic embryogenesis has not been reported in *Schlumbergera* or *Rhipsalidopsis*.

2.1.2 Morphogenesis and regeneration

The inherent potentiality of a plant cell to give rise to a whole plant, a capacity which is often retained even after a cell has undergone final differentiation in the plant body, is described as ´cellular totipotency`. For a differentiated cell to express its totipotency it first undergoes dedifferentiation followed by redifferentiation (Bhojwani and Razdan, 1996; Nishiwaki *et al*., 2000). This potentiality has been exploited through the culture of protoplasts, cells, tissues and organs *in vitro*. In cultured material it has been possible to study such processes as cytodifferentiation, and organ and somatic embryo formation. Morphogenesis, or the origin of form, can

be examined through manipulation approaches, descriptively, physiologically, biochemically or at the molecular level (Thorpe, 1998).

2.1.3 Organogenesis

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissue (Thorpe, 1998; Victor *et al*., 1999).

2.1.4 Somatic embryogenesis

Somatic embryogenesis is defined as a process in which a bipolar structure, containing a root/shoot axis, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue. This process occurs through the same key stages of embryo development as zygotic embryogenesis (i.e. globular, heart and torpedo stages) (Sharma and Millam, 2004). Somatic embryogenesis is a multi-step regeneration process starting with formation of proembryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration (Von Arnold *et al*., 2002).

Sometimes, somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors (Mavituna and Buyukalaca, 1996). Often, the somatic embryos or the embryogenic cultures can be cryopreserved (Mathur *et al*., 2003; Winkelmann *et al*., 2004), which makes it possible to establish gene banks (Von Arnold *et al*., 2002). Furthermore, *in vitro* somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement (Santacruz-Ruvalcaba *et al*., 1998). In this regard, somatic embryos play a key role in current genetic transformation methods.

The first observation of *in vitro* somatic embryogenesis was made in *Daucus carota* (Reinert, 1958, 1959; Steward *et al*., 1958). Since then, the potential for somatic embryogenesis has been shown in many plant species. Plant regeneration through

somatic embryos has been induced using ovules (Winkelmann *et al*., 1998), epicotyls (Little *et al*., 2000), ray florets (Tanaka *et al*., 2000), stem internodes (Seabrook and Douglass, 2001), cotyledons (Mandal and Gupta, 2003), leaves (Fki *et al*., 2003) and zygotic embryos (Pinto *et al*., 2004) as explants.

However, somatic embryos in some species are formed directly from specific explants cultured on a medium without growth regulators. This occurred in nucellus of citrus, which is thought to compromise pre-embryogenic determined cells (PEDCs) (Sharp *et al*., 1980).

Common embryogenesis-related gene expression has been postulated to exist in plants (Reinbothe *et al*., 1992). DNA methylation plays a role in gene activity and cell differentiation (Munksgaard *et al*., 1995). Somatic embryogenesis could be induced by stressful conditions manifested through methylation changes (Leljak-Levanić *et al*., 2004). Some of the factors that can influence the induction of somatic embryos and subsequent plant development are the following:

2.1.4.1 Growth regulators

Hormones are the most likely candidates in the regulation of developmental switches. Auxins and cytokinins are the main plant growth regulators concerning cell division and differentiation related to induction and development of somatic embryogenesis (Fehér *et al*., 2003). However, embryo development in somatic embryos has been reported in the absence of growth regulators (Choi *et al*., 1998). Non-hormonal inducers can also be used to promote the somatic embryogenic transition. Such inducers include high sucrose concentration or osmotic stress (Kamada *et al*., 1993), heavy metal ions (Pasternak *et al*., 2002) and high temperature (Kamada *et al*., 1989).

A) Auxins

The well-studied somatic embryogenic systems, such as alfalfa, carrot, celery, coffee, orchardgrass, and most of the cereals, require a synthetic auxin for the induction of somatic embryogenesis followed by transfer to an auxin-free medium for embryo differentiation (Bhojwani and Razdan, 1996). 2, 4-D alone, or in combination with

other plant growth regulators, has been the most commonly used auxin for the induction of somatic embryogenesis in plants like pearl millet (Goldman *et al*., 2003), barley (Sahrawat and Chand, 2004) and elm (Conde *et al*., 2004).

Fehér *et al*. (2001, 2002) suggested that 2, 4-D above a certain concentration, has a dual effect, as an auxin (directly or through the interference with endogenous IAA) and as a stressor.

In the last few years a large body of experimental observation has accumulated, suggesting that temporal and spatial changes in endogenous auxin, particularly indoleacetic acid (IAA), and abscisic acid (ABA) levels are important factors controlling the embryogenic fate (Fehér *et al*., 2003).

The type and concentration of auxin can affect somatic embryo development and morphology. Mandal and Gupta (2003) found that NAA at 10.74 μM was optimum for high frequency of safflower somatic embryos, whereas IAA provided the maximum number of somatic embryos per culture. On the contrary, 2, 4-D failed to elicit any embryogenic response. Furthermore, they found that in the p-CPA-supplemented medium, somatic embryos did not mature, and their development ceased at the globular or heart-shaped stage. Maximum numbers of well-developed somatic embryos at the cotyledonary stage were obtained with 5.37 μM NAA plus 2.22 μM BA. However, auxins were found to be inhibitory for somatic embryogenesis in oncidium (Chen and Chang, 2004).

B) Cytokinins

In some plants, embryo formation was promoted by cytokinins and reduced or inhibited by auxins. Chen and Chang (2001), found that embryo formation was retarded on leaf explants of Oncidium ´Grower Ramsey` by the four auxins tested (IAA, IBA, NAA and 2, 4-D), whereas the highest percentage of embryo formation was recorded using media containing TDZ, 2iP and kinetin. Moreover, in the genus *Abies*, it was reported that cytokinin alone is effective in induction of somatic embryos from cultured zygotic embryos (Guevin and Kirby, 1997). However, in

chrysanthemum, kinetin was reported to induce somatic embryogenesis in combination with an auxin, particularly indoleacetic acid (IAA) (Tanaka *et al*., 2000).

Thidiazuron, known as TDZ, either alone or in combination with other growth regulators, has been found to induce somatic embryogenesis in many plant species (Guevin and kirby, 1997; Victor *et al*., 1999; Mithila *et al*., 2003; Zhang *et al*., 2005). TDZ also induces shoot organogenesis in a wide variety of plant species (Malik and Saxena, 1992; Saito and Suzuki, 1999; Mithila *et al*., 2003). Moreover, TDZ, a synthetic phenylurea-type cytokinin, is believed to modulate the metabolism of endogenous auxins and cytokinins (Murch and Saxena, 2001). However, the precise mode of action of TDZ remains undetermined. Studies in geranium (*Pelargonium x hortorum* Bailey) have indicated that exposure of hypocotyl sections to TDZ enhances the accumulation and translocation of auxin within the tissues (Murch and Saxena, 2001).

C) Abscisic acid (ABA)

In some species, the use of ABA was found to improve the process of somatic embryogenesis. Carrot seedlings formed somatic embryos when cultured on medium containing ABA as the sole source of growth regulator (Nishiwaki *et al*., 2000). Langhansová *et al.* (2004) reported that ABA treatment promoted regeneration and rooting of *Panax ginseng* somatic embryos.

ABA application alone or combined with other substances like PEG (polyethylene glycol) or activated carbon, has become a routine method for stimulation of somatic embryo maturation in plants like *Hevea brasiliensis* (Linossier *et al*., 1997), cork oak (García-Martín *et al*., 2005), date palm (Zouine *et al*., 2005) and norway spruce (Pullman *et al*., 2005).

D) Daminozide

The growth retardant daminozide is used to reduce the stem length of *in vitro*-propagated plantlets. Daminozide basically results in plantlets with shorter internodes, with darker green leaves and stems, and shorter, more uniformly distributed roots

(Sipos *et al*., 1988). Daminozide also improved survival of transplants of potato (Sipos *et al*., 1988). Furthermore, when added to the rooting medium of *in vitro* nodal cuttings of potato, daminozide led to a significantly higher leaf area of the plantlets soon after transfer to the greenhouse (Tadesse *et al*., 2000).

Many growth retardants exert their influence by inhibiting cell division in the subapical zones of the shoot apex, causing subsequent cell enlargement, resulting in reduced stem elongation (Jarret, 1997).

Few reports are dealing with the effect of daminozide on *in vitro* organogenesis or somatic embryogenesis. Gmitter and Moore (1986) reported that sweet orange ovules cultured on media supplemented with 2,4-D and daminozide in the light were the most responsive in terms of total embryo production. Daminozide at certain concentrations was also reported to severely reduce rooting (Ferreira and Janick, 1996).

2.1.4.2 Sugars

In plants, sugars are not only the source of a carbon skeleton and energy, but are also regulators in many aspects of life's activities. These include metabolism, assimilating partitioning and transporting, growth and development, stress responses and others.

Sugar regulates these activities by enhancing or repressing expression of relevant genes (Koch, 1996; Smeekens, 2000; Rolland *et al*., 2002). Sucrose has been the most tested carbon source for induction of somatic embryogenesis. It has been used mostly at 3% of the culture medium in a wide range of plants including peanut (Little *et al*., 2000), *Helianthus maximiliani* (Vasic *et al*., 2001) *Eucalyptus globulus* (Pinto *et al*., 2002) and African violet (Mithila *et al*., 2003).

In some plants, reduced levels of sucrose (1-2%) in the maturation media were reported to ease the conversion of somatic embryos (Conde *et al*, 2004). The sugar type and concentration influence the expression of somatic embryogenesis. Biahoua and Bonneau (1999) reported for spindle tree (*Euonymus europaeus* L.), that the frequency of somatic embryogenesis increased steadily with increasing sucrose

concentration. They concluded that elevated concentrations of sucrose mainly act osmotically, stimulating the emergence of numerous somatic embyos. An opposite response was observed with increasing glucose concentration, and it was found that elevated concentrations of glucose have an inhibitory effect, independent of their osmotic effect.

Recent studies have drawn attention to the signal transduction system as a major regulatory function of sugars. Yang *et al* (2004) have studied the effect of sucrose on the germination and radicle elongation of carrot somatic embryos. They concluded that there is a sucrose-specific effect regulating elongation of carrot somatic embryo radicles, and that this effect is correlated to signal transduction. Northern hybridization revealed that there is a marked increase in the expression of a carrot sucrose transporter gene (*cSUT*) at the beginning of somatic embryo germination, and this is attributed to regulation on the level of transcription. However, this study excluded osmotic stress as the regulating mechanism.

2.1.4.3 Medium

The demand by plants for nutrients is diverse and different plant species require specific nutrient elements at specific stages. In fact, each of the tissue culture media that have been published in the literature was developed on the bases of a specific genotype of a particular plant species (Lin and Zhang, 2005).

MS medium is a universal medium used in plant tissue culture. It has been used widely for the induction of somatic embryogenesis (Tanaka *et al*., 2000; Vasic *et al*., 2001; Pinto *et al*., 2002; Conde *et al*., 2004). In some studies, MS medium at half salts strength was also reported to be used for somatic embryo induction (Winkelmann *et al*., 1998; Kim *et al*., 2003) and conversion into plantlets (Langhansová *et al*., 2004). In other cases, MS salts were used with B5 vitamins instead of MS vitamins (Victor *et al*., 1999; Little *et al*., 2000).

There are many examples in literature of the different media and variability in individual components of the media used for somatic embryogenesis, in particular, and plant tissue culture in general. In *Cucurbita pepo* L., the embryo development

was affected by the nitrogen source or by manipulating the availability of auxin (presence or absence) (Leljak-Levanić *et al*., 2004). Medium BM3 (Gupta and Durzan, 1986) was used for maintenance and multiplication of developing embryo suspension masses of *Picea abies* (Pullman *et al*., 2005). Moghaddam *et al*. (2000) reported the promotion of somatic embryos in sugar beet when proline was used together with TIBA.

Rodríguez-Garay *et al*. (1996) described the induction of direct somatic embryogenesis in *Agave victoria-reginae* on agar-solidified MS medium supplemented with L2 vitamins (Phillips and Collins, 1979). Germination of somatic embryos was achieved on half-strength MS medium or on half-strength SH medium (Schenk and Hildebrandt, 1972), both of which lacked growth regulators.

2.1.4.4 Age of plant material and culture conditions.

In some plant species, the *in vitro* morphogenesis response is influenced considerably by the age and the physiological state of the donor plant. Seedlings of carrot with hypocotyls longer than 3.0 cm were not able to form somatic embryos when treated with ABA. This indicated that ABA sensitivity to embryogenesis decreases with age (Nishiwaki *et al*., 2000). In another study, seed coats picked from 14- and 18- day old carrot seedlings exhibited a relatively high ability to develop somatic embryo formation compared to those from 10-day old seedlings (Ogata *et al*., 2005).

Treatments, like exposure to plant growth regulators, and the duration to which the plant material is exposed early in the *in vitro* culture can affect the final observed morphogenic response (Victor *et al*., 1999; Von Arnold *et al*., 2002). In African violet, increasing the length of exposure to TDZ from 3 to 9 or even 35 days appeared to have a positive effect on *de novo* shoot organogenesis at lower concentrations (up to 1.5 *μM*) (Mithila *et al*., 2003). In European spindle tree, the stimulation of somatic embryos from zygotic embryos dissected from seeds stored at 4 ºC for 2-4 months was important for the expression frequency of somatic embryos than a cold storage for 10-12 months at -1.30 MPa osmotic potential (Biahoua and Bonneau, 1999).

2.1.5 Pathways of somatic embryogenesis

Somatic embryogenesis generally occurs through two different pathways; namely, directly without forming the callus phase (Chen and Chang, 2004; Zhang *et al*., 2005) and indirectly following callus formation from explants (Griga, 2002; Kim *et al*., 2003; Conde *et al*., 2004). Direct embryogenesis, without the callus phase, is likely to be more suitable for mass propagation because genetic rearrangement is limited compared with the embryogenesis via calli, which often showed aberrant chromosome numbers during culture (Tanaka *et al*., 2000).

2.1.6 Cotyledonary morphology of somatic embryos

Generally, zygotic embryos of dicotyledonous plants have two discrete cotyledons lateral to the embryo axis, but in somatic embryos the cotyledon number shows great diversity (Soh, 1996). The production of embryos with more than two cotyledons (multiple, poly) (Choi *et al*., 1997; Griga 2002) is well documented. Moreover, embryos with other forms of cotyledons such as jar-shaped (Choi *et al*., 1997), were also described.

The objective of this study was to induce somatic embryogenesis and plant regeneration in *Schlumbergera* and *Rhipsalidopsis*. Furthermore, the effect of medium type, growth duration of callus on culture medium and plant growth regulators on the induction of somatic embryos were evaluated.

2.2 Materials and Methods

2.2.1 Plant material

Mother plants of *S. truncata* (cvs. Russian Dancer, Exotic Dancer, Carribean Dancer, Malindi and Alex) and *R. gaertneri* (cvs.CB1, CB4, CB5 and CB6) were grown in 0.51 (10 cm square) pots in a greenhouse with 16 h light (provided by SON-T sodium lamps during winter months, 600 μmol m^{-2} s^{-1} at plant surface) at 25-28 ºC. The plants were watered, without fertilizer, at the base without wetting the foliage, in order to reduce problems during surface sterilisation. When the experiments started, the plants were about 1-year-old with 3-5 tiers of mature phylloclades.

2.2.2 Establishment phase

Phylloclade explants were surface sterilised in 1.5% active chlorine from sodium hypochlorite with 0.02% Tween 20 for 15 min. The explants were then thoroughly washed five times with sterile water. After the removal of chlorine-damaged areas, each phylloclade was cut through the midrib into 2-3 pieces and grown in a medium consisting of MS salts, Staba vitamins (Staba, 1969), 22.7 μM TDZ and 1.3 μM NAA, 3% w/v sucrose and gelled with 3 g l^{-1} gelrite (maintenance medium). Growth regulators were dissolved in DMSO to make stock solutions and were added to the media after autoclaving. The pH of all media was adjusted to 5.7 before autoclaving.

Callus developed on explants was used for the next experiments. All of the cultures, unless otherwise stated, were incubated in light in a growth room (17-h photoperiod of 66 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes) at 25-28 ºC.

2.2.3 Preliminary experiment

From each of the nine cultivars of *Schlumbergera* and *Rhipsalidopsis*, small callus pieces were grown in liquid MS-based medium supplemented with 7.0 μM kinetin. After 20, 30, 40 and 50 days, callus was transferred onto solid MS medium supplemented with 0.45 μM 2,4-D (MSD), 0.57 μM IAA (MSIA), 0.54 μM 4-CPA

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(MSCPA) or 0.62 μM Daminozide (MSDM). Callus was either placed directly on the surface of the medium or on a piece of gauze on the medium.

The formation of somatic embryos, roots and shoots was recorded. Based on the results, the following experiments using selected cultivars (sections 2.2.4 and 2.2.5) were conducted.

2.2.4 Somatic embryogenesis in cv. Russian Dancer

Two types of callus were defined to see the effect of the growth duration of callus on the induction biology of somatic embryogenesis. The first type of callus was grown for a relatively short period of time on the establishment medium (designated as H callus). The second type of callus was grown for a longer time during the establishment phase (Ho callus). H callus was obtained by sub-culturing the regenerated callus from the explants twice over a period of three months on maintenance medium. Ho callus developed on explants was sub-cultured onto fresh maintenance medium approximately every two months over a period of nine to twelve months. Then it was grown 4.5 months on MS medium containing 26.6 μM BA, 27.2 μM TDZ, 27.4 μM zeatin, 3% w/v sucrose and 3 g l^{-1} gelrite, before the start of the embryogenesis experiment.

Induction of somatic embryos

Eight callus pieces (approximately 100 mg each) were transferred to 30 ml of liquid medium in Erlenmeyer flasks (100 ml). Two media were employed; the first medium was based on MS salts and the second medium was SH-based medium. Both media were supplemented with 0.1 g l^{-1}myo-inositol, 7.0 μM kinetin and 3% w/v sucrose. There were 6 flasks for each medium and the cultures were shaken at 120 rpm using a rotary shaker and incubated at 27-29 ℃ under a light intensity of 4 μmol m^{-2} s^{-1} with 12 h-photoperiod.

Callus was grown for 30 days in SH medium, whereas in MS-based medium, it was grown for 20 and 40 days. Callus was then filtered through sieves (200 μm), and 0.5 g callus was placed on gauze (TZMO SA, Poland) which was placed on MSD, or on

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MS-based medium without hormones (MSWH). The medium contained 3% w/v sucrose and was gelled with 3 g l^{-1} gelrite. Each petri dish containing 0.5 g of callus was considered as a replicate, and the petri dishes were placed randomly on shelves in the growth room.

To study the effects of gauze and type of auxin used on the induction of somatic embryos, callus transferred after 20 days from MS-based medium was grown on MSD and MSIA. In this case, callus was placed directly on the surface of the medium (- gauze), or on a piece of gauze on the medium (+ gauze).

When somatic embryos began appearing, they were proliferated by transferring them to the respective media, MSD and MSWH, after six weeks. Embryos consisting of 2 cotyledons were classified as normal embryos, and those consisting of 1 or more than 2 cotyledons or other forms such as vase-like, were classified as abnormal.

Embryo maturation and subsequent plant regeneration

After culturing on the proliferation medium for two months, somatic embryos were separated and transferred to a medium consisting of ¾ MS salts, 0.1 g l^{-1} myo inositol, 50 g l^{-1} sucrose, Staba vitamins and 3 g l^{-1} gelrite (G medium) for germination. Ten embryos (from the normal and abnormal types) were placed on the surface of G medium in plastic containers (99 mm x 91 mm, Danefeld, Breeding Station, Denmark) and grown further.

To accelerate the growth of germinated embryos, the embryos were taken from G medium after about 3 months and transferred to B medium consisting of 2.2 μM BA, ½ MS salts, 7.5 g l^{-1} sucrose and 5 g l^{-1} glucose. Five germinated embryos were placed on the surface of the medium in each plastic container.

Transfer to greenhouse conditions

Plantlets from somatic embryos were transferred after 3 weeks from B medium to plastic pots (5.5 cm, OS Plastic A/S Denmark) containing a mixture of autoclaved peat and perlite (2:1) and were grown under greenhouse conditions. Acclimatization

of the plants was carried out by wrapping them with a plastic cover. After one week, the cover was removed gradually and small holes were made in it to enable an exchange of air and humidity.

Morphology and histology of somatic embryos

Embryos in the globular and torpedo stages were harvested and observed using an environmental scanning electron microscope (ESEM) (Quanta 200 SEM, FEI Europe B.V., Eindhoven, Netherlands).

Somatic embryos at the different developmental stages were fixed in histochoice overnight at room temperature and then washed 3 times at 20 min intervals using distilled water. The samples were dehydrated in isopropanol solutions (10 %, 30 %, 50 %, 70 %, 85 %, 95 %, twice 100 %) for 2 hrs in each step and embedded in paraffin. Sections, of 10 μm, were cut using a rotary microtome (Microm HM340E). The sections were mounted on glass slides and placed on a warm tray (40 ºC) overnight. For deparaffinizing, sections were passed through 100% ultraclear (3 times each for 15, 10 and 5 min) and then a graded series of isopropanol solutions each at 3 min intervals (100, 95, 85, 70, 50, 30, 20 and 10%). Samples were then rinsed with distilled water (5-15 min) and stained with 0.1 % Toludine blue in citrate buffer (pH 4.4). Samples were photographed using Leica DMR fluorescence microscope (Fotoequipment: Leica DC 300F Leica, Germany).

Morphology of zygotic embryos/somatic embryos

Seeds of *Schlumbergera* cv. Thor-Ritt were sterilized in 1.5 % sodium hypochlorite solution for 8 min and rinsed three times with sterile water. Seeds were then germinated in petri dishes on MS-based medium containing Staba vitamins, 3% w/v sucrose and 3 g l^{-1} gelrite.

The stages observed from seed germination of the cv. Thor-Ritt were compared to the main stages of somatic embryogenesis in the cv. Russian Dancer.

Cytological analysis of the regenerated plants

Chromosome counting in root tips was done as described by Parks and Boyle (2003). Root tips (1-2 cm) were collected from *Schlumbergera* mother plants and embryos which had 2, 4, or more cotyledons and pretreated in a saturated solution of *Para*-dichlorobenzene for 5 h at room temperature. *Para*-dichlorobenzene was dissolved in methanol to make a saturated solution. Root tips were rinsed in deionized H_2O (d H_2O), fixed and stored at room temperature for 24 h. The fixation solution consisted of 3 parts ethanol 95% to one part glacial acetic acid (by volume). Rinsed root tips were hydrolyzed for 11 min in 1 *N* HCl at 60 C° and then rinsed in dH_2O. Apices were stained in a drop of Feulgen reagent (0.5% (w/v) *Para*-rosaniline) for 1 hr in the dark at room temperature. Feulgen reagent was prepared by dissolving 0.5 g of *Para*-rosaniline in 100 ml ethanol. Meristems were squashed in a drop of acetocarmine. Acetocarmine was prepared by dissolving 0.5 g carmine in 45% glacial acetic acid (45 ml glacial acetic acid combined with 55 ml distilled water). Slides were viewed with a Leica DMR fluorescence microscope. At least five root tip cells were counted for each sample.

2.2.5 Somatic embryogenesis in cvs. Alex and CB5

Callus developed on explants from cv. CB5 was sub-cultured onto fresh maintenance medium approximately every two months over a period of nine to twelve months. The callus from cv. Alex was sub-cultured over a period of seven months.

Induction of somatic embryos

The procedure is similar to that reported in section 2.2.4, but with modifications. Small callus pieces (approximately 5.5 g) were transferred to 40 ml of liquid medium in Erlenmeyer flasks (250 ml). The medium used was based on MS salts supplemented with 0.1 g l^{-1} myo-inositol, kinetin at 7.0 μM for cv. Alex and 4.7 μM for cv. CB5, 3% w/v sucrose. There were 8 flasks for each medium and the cultures were shaken at 120 rpm using a rotary shaker and incubated at 27-29 ℃ under a light intensity of 4 μmol m^{-2} s^{-1} with 12 h-photoperiod.

Callus was grown for 30 and 50 days in this medium. Then callus was filtered through sieves (200 μm), and callus (0.5 g for cv. CB5, 1.0 g for cv. Alex) was placed on gauze which was placed on the surface of one of the following: MSD, MSIA, MSDM, or MSWH media. The medium contained 3% w/v sucrose and was gelled with 3 g l^{-1} gelrite. Each petri dish (9 cm in diameter) containing 0.5 or 1.0 g of callus was considered as a replicate and seven replicates were used for each medium. Petri dishes were placed randomly on shelves in the growth room.

2.2.6 Data collection and statistical analysis

Each experiment was repeated twice over a 2 year period. In addition to the number of embryos in the defined developmental stages, the number of adventitious shoots was also counted. The dry weight of shoots was also recorded. Shoots were dried in an oven at 70 ºC for two weeks. Embryos were considered germinated if they formed at least one 2 mm-long root and had a clear shoot apex with at least one phylloclade. The frequency of secondary embryogenesis on the germinated embryos was also recorded.

The survival percentage of plants in soil (number of surviving plants / total number of plantlets transferred to soil x 100) was recorded 45 days after transferring the embryos to soil. At the same time, growth of plants derived from somatic embryos (plant height, number of primary and secondary phylloclades) was recorded. Chromosomes were counted in root tips of plants derived from somatic embryos of *Schlumbergera* cv. Russian dancer that had 2, 4 or more cotyledons, and in the mother plants. For the experiment with the cv. Russian Dancer, data were presented as mean values and their standard errors calculated. Treatments consisted of unequal replicates (replicate numbers designated as n) according to the availability of plant material.

A factorial randomized experimental design was used for the data of the adventitious shoot regeneration in the cultivars Alex and CB5. Statistical procedures were performed using SAS software (SAS-Institute Inc, 1996)-general linear model (GLM) procedure according to the model Y= time treat and time*treat. Differences between means were examined using t test at P level < 0.05.

2.3 Results

2.3.1 Preliminary experiment

Differences were observed in the organogenic and embryogenic potential between cultivars from both *Schlumbergera* and *Rhipsalidopsis* in response to the different media tested. After about three to five months, cv. CB1 did not exhibit any growth response when grown on any of the media in the final transfer, regardless of the duration of culture in the first liquid medium. Cultivar Exotic Dancer formed only roots, but no incidence of shoots or somatic embryos was recorded. Cultivars CB4 and CB6 showed adventitious shoot formation with low frequency when grown on MSD (< 5 shoots per petri dish). However, a good regeneration potential through the formation of adventitious shoots was observed with the cvs. CB5 and Alex in almost all media tested.

The induction of somatic embryogenesis was observed only in *Schlumbergera* cvs. Russian Dancer, Alex, Carribean Dancer and Malindi. Somatic embryos were induced 3-6 months after transfer from the liquid medium onto MSDM, MSCPA, MSD and MSIA.

The results indicated that duration of callus growth in the first liquid medium, presence or absence of gauze may influence the regeneration potential. For example, somatic embryos of the cv. Carribean Dancer were induced from callus grown for 50 days in the first liquid medium and then transferred onto MSDM without using gauze. In contrast, no somatic embryos were seen on MSDM medium from callus grown only for 20 days in the first medium, or when MSDM was used with gauze. However, this effect was genotype dependent (Table 1).

Table 1. Treatment combinations that resulted in the induction of somatic embryos in the preliminary experiment

Cultivar	Transfer (Days)	Medium	Gauze
Carribean Dancer	50	MSDM	-
	20	MSCPA	+
	50	MSIA	+
Malindi	20	MSDM	-
Russian Dancer	40	MSD	+
Alex	30	MSDM	+

2.3.2 Somatic embryogenesis

A) Cultivar Russian Dancer

Induction of somatic embryos

Somatic embryos in cv. Russian Dancer were induced after three and a half months from the start of the embryogenesis experiment when callus was transferred from the liquid SH-based medium (containing 7.0 μM kinetin) after 30 days to the MSWH or after five months when callus was grown in the liquid MS medium (containing 7.0 μM kinetin) and transferred after 40 days to the MSWH or MSD.

When the embryogenesis experiment began, the colour of the callus grown in the liquid media was greenish-brown. Callus texture ranged from medium to hard. After the transfer to the second medium, a yellowish, soft, friable callus began forming on the older brownish callus, from which somatic embryos later began differentiating (Figure 1).
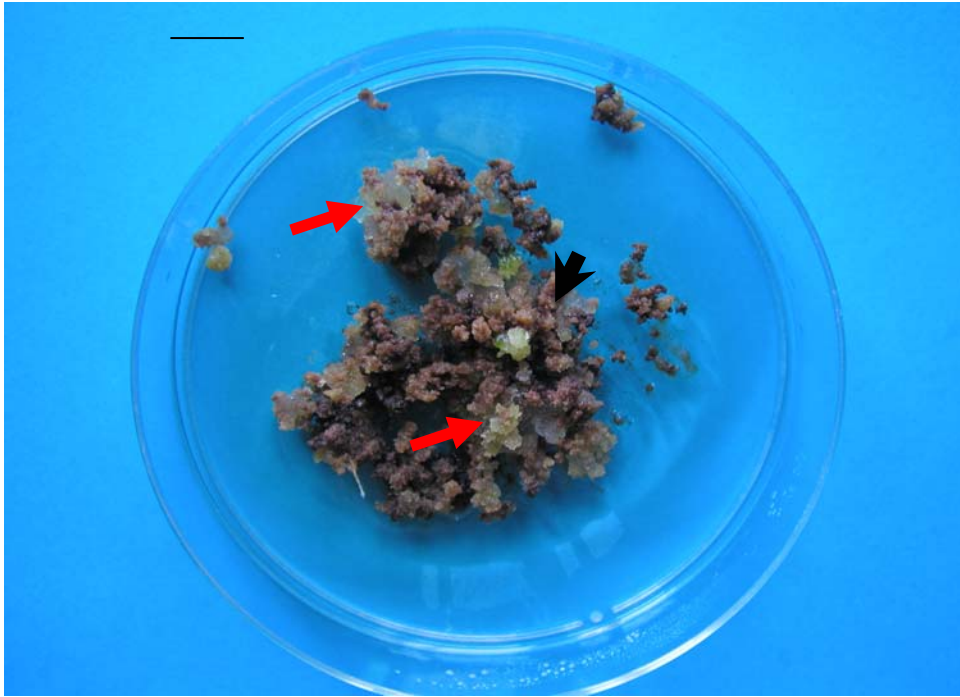
Figure 1. The development of friable, soft, yellowish callus (*arrow; red*) on the older brownish callus (*arrowhead; black*) on MSWH medium after the transfer from SH-based liquid medium. *Bar*: 1cm.

The somatic embryos began to form as whitish proembryogenic mass (Figure 2 a), from which globular embryos (Figure 2 b) formed and began elongating (Figure 2 c). They then successively developed into the torpedo stage (Figure 2 d & e) and cotyledonary stage (Figure 2 f). The embryos were easily detachable from the callus inside the cultures and showed clear root and shoot poles indicating no vascular connection with the original tissue. Induction of somatic embryogenesis was favoured by Ho-type callus. The highest average numbers of embryos in the globular (14.7), elongation (43.7), torpedo (26.2) and the two-cotyledon (47) stages were achieved using SH medium for the first culture and MSWH medium after 30 days. When MS medium was used for the first culture, about twice the number of embryos in the two-cotyledon stage was formed on the second medium MSWH when compared to that on MSD (Table 2). However, no somatic embryogenesis was recorded on MSD, when callus was transferred 20 days from liquid MS medium or 30 days from liquid SH medium.
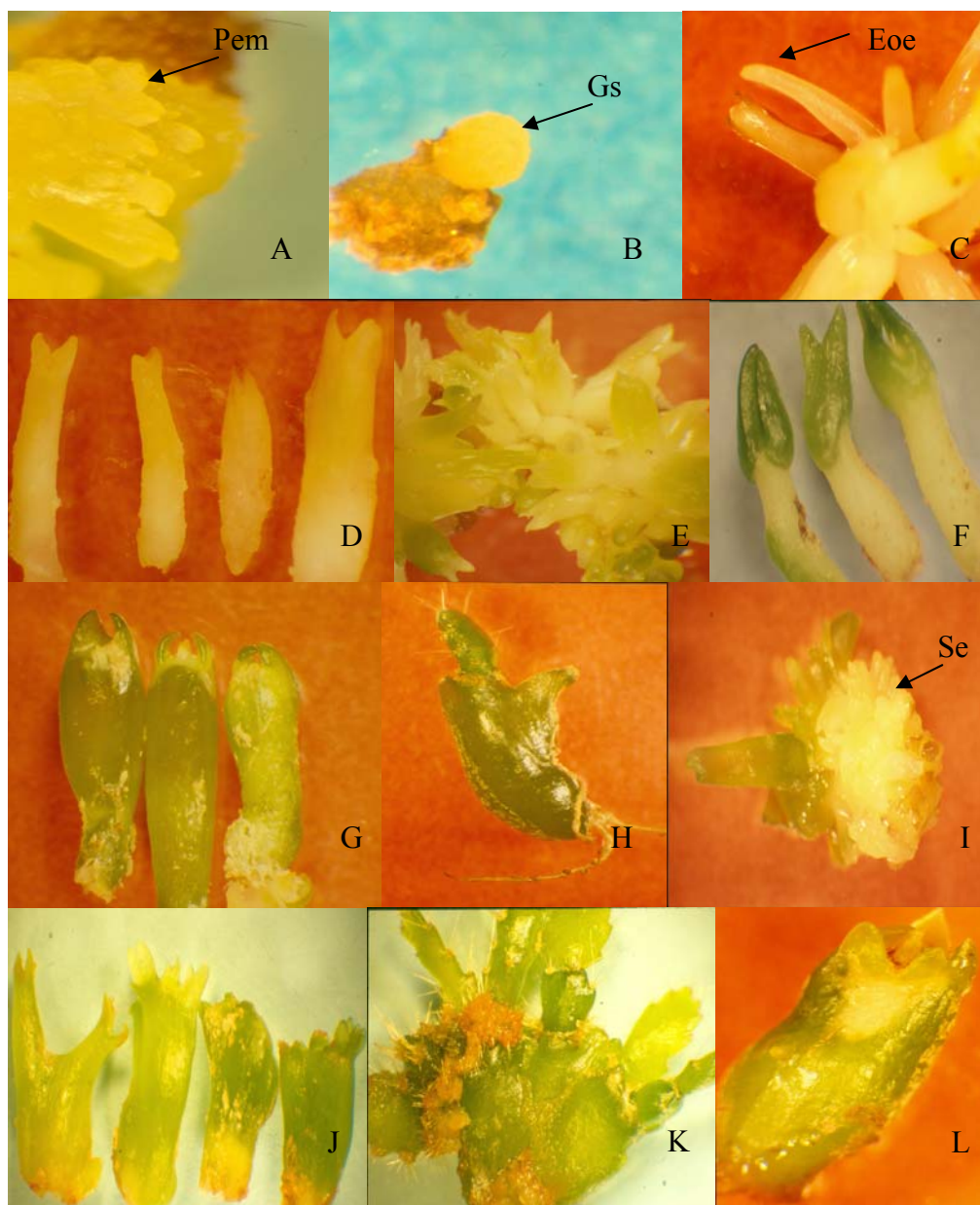
Figure.2 Plant regeneration via somatic embryogenesis in *S. truncata* cv. Russian Dancer. **(A)** Proemryogenic mass (Pem) (x18). **(B)** Globular stage (Gs) (x18). **(C)** Elongation of embryos (Eoe) (x18). **(D)** Torpedo stage (x18). **(E)** Mixed stage of elongating and torpedo embryos (x6). **(F)** Embryos with 2 cotyledons (x18). **(G)** Embryos with clear shoot apex (x9). **(H)** Complete plantlet with new phylloclade forming areoles with spines (x9). **(I)** Secondary embryos (Se) (x8). **(J)** Embryos show multiple finger-like cotyledons (x6) **(K)** which formed later the new phylloclades (x6). **(L)** Vase-like embryos with cotyledons arranged in a circle at the embryo apex, with the new growth primordia located in the centre (x18).

Table 2.Effects of media and transfer dates on the average number of somatic embryos in the different developmental stages in *S. truncata* cv. Russian Dancer.

Callus	First medium	Transfer (days)	Second medium	No. of embryos/ 0.5 g callus			
H0				Globular	Elongation	Torpedo	2 cotyledon
	SH,7.0 μM kin	30	MSWH	14.7 ± 4.6^1	43.7 ± 10.2^1	26.2 ± 5.9^1	47.0 ± 12.9^1
	MS,7.0 μM kin	40	MSD	3.0 ± 0.9^2	5.7 ± 1.3^2	5.9 ± 1.7^2	10.4 ± 2.9^2
			MSWH	10.6 ± 1.1^3	13.8 ± 4.6^3	18.8 ± 4.9^3	21.4 ± 6.6^3
H	MS,7.0 μM kin	40	MSD	0.1 ± 0.1^4	0	0	0.3 ± 0.1^4

The results are the mean ± standard error of [1] (n:6), [2] (n:7), [3] (n:5) and [4] (n:8)

During the course of somatic embryo development, different patterns of cotyledon morphology and shoot apex structure of embryos were observed. In addition to the normal dicotyledonous embryos, anomalous embryos with multiple cotyledons developed (Figure 2 j & k). Vase-like embryos with cotyledons arranged in a circle at the embryo apex, with the new growth primordia located in the centre (Figure 2 l), were noticed.

All of these embryogenic structures were seen simultaneously in all the media tested. When callus was grown in liquid SH-based medium and transferred after 30 days to MSWH medium, the highest average number of abnormal embryos were those with 3 and 4 cotyledons (25), (16), respectively (Figure 3 I). Using this combination of media and transfer date, a total of 77 abnormal embryos (Figure 3 II), a total of 124 normal and abnormal embryos and a total of 208 embryos were formed (Figure 3 III). The response was higher than when MS-based medium was used for the first culture. 62-74% of the total number of embryos in the cotyledonary stage was of the anomalous type (Figure 3 IV).
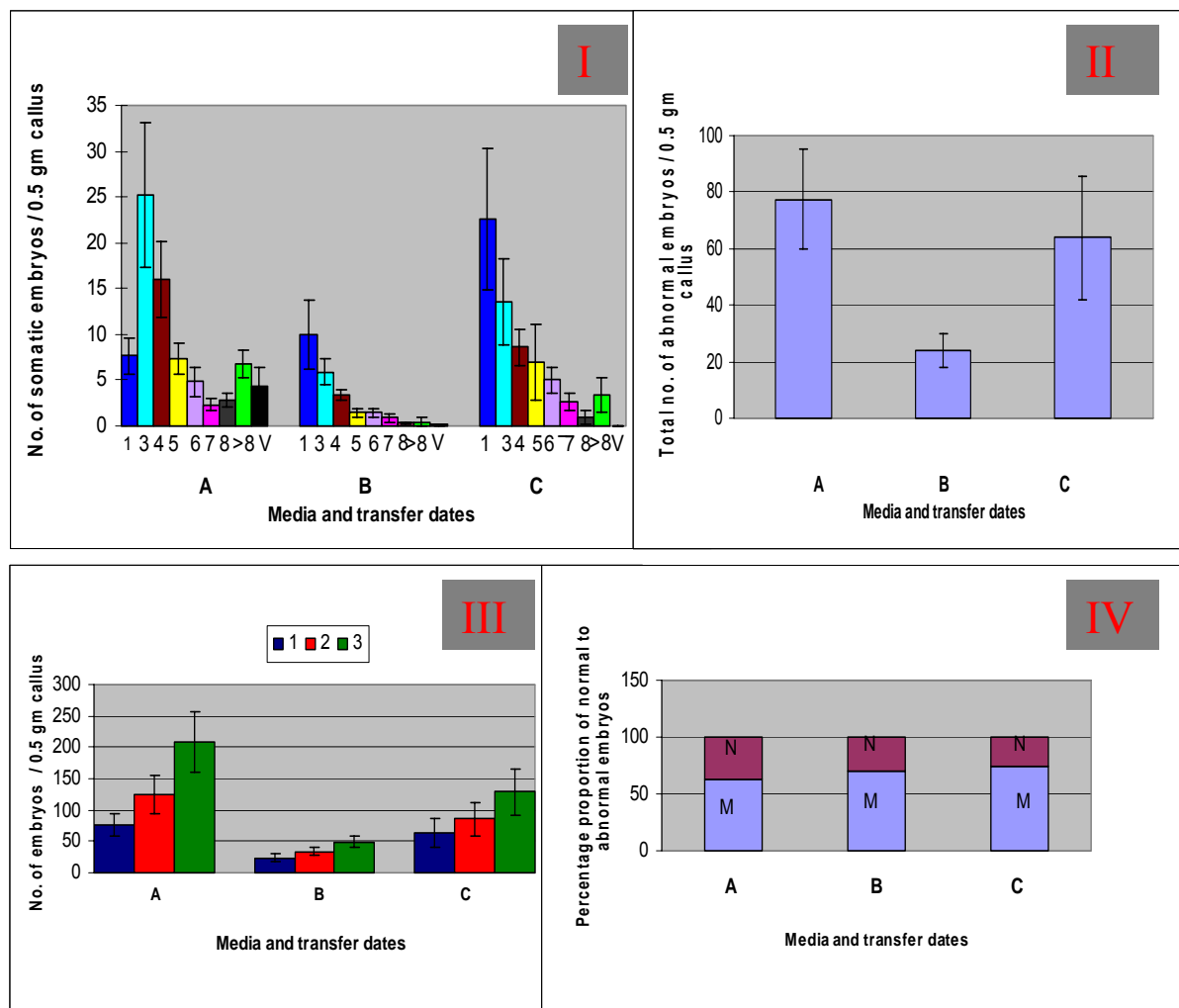
Figure 3. Effects of media and transfer dates on the (**I**) Number of somatic embryos, with 1, 3, 4, 5, 6, 7, 8 and more than 8 (>8)cotyledons and those defined as vase-like embryos(V). (**II**) Total number of abnormal embryos. (**III**): Total number of the abnormal embryos **(1)**, The sum of the normal and the abnormal embryos **(2),** Total number of the embryos formed including normal and the abnormal embryos plus those in the globular, elongation and torpedo stages **(3). (IV)** Proportion of normal (N) to abnormal embryos (M), from cv. Russian Dancer. Ho-type of callus was transferred from **(A)** SH liquid medium supplemented with 7.0 *μM* kinetin after 30 days onto (MSWH) **(B)** MS liquid medium supplemented with 7.0 *μM* kinetin after 40 days onto (MSD) or **(C)** MS liquid medium supplemented with 7.0 *μM* kinetin after 40 days onto (MSWH). The results are the mean ± standard error of A (n:6), B (n:7) and C (n:5). The description for the media and transfer date's combinations designated by **(A)**, **(B)** and **(C)** will be the same in figures 4, 5 and 7.

Using H callus, only a limited somatic embryogenesis was recorded on MSD-grown callus that was transferred after 40 days from liquid MS-based medium supplemented with 7.0 μM kinetin (Table 2).

Somatic embryo development in *S. truncata*, as shown in this study, was asynchronous since all phases up to cotyledon phase were observed in the same embryogenic callus at the same time.

Embryo maturation and subsequent plant regeneration

Embryos began rooting after 2-3 weeks on G medium (Figure 2 g & h, Figure 4 V). After three months on this medium, approximately 70% germination was recorded in dicotyledonous embryos. These embryos were differentiated earlier from Ho callus grown on either SH-based medium or MS-based medium supplemented with 7.0 μM kinetin, and then transferred after 30 days (from SH medium) onto MSWH medium or after 40 days (from MS medium) onto MSD medium (Figure 4 I). However, 72-90% of embryos formed roots, regardless of shoot formation (Figure 4 II). This occurred whether SH- or MS-based medium was used for the first culture.

The germination of abnormal embryos was also recorded. Abnormal embryos with 3, 4, or more than 4 cotyledons showed significantly higher germination percentages (53-78%) than embryos with 1 cotyledon (23-44%) in all media and combinations of transfer dates (Figure 4 III). Embryos with one cotyledon also showed slightly less root formation than the rest of the abnormal embryos (Figure 4 IV).

However, by this time many new secondary embryos had developed on old embryos (Figure 2 i). Similar to the trend obtained with the average number of somatic embryos in the two-cotyledonary stage (Table 2), the frequency of secondary somatic embryos was about two-fold when embryos were grown on MS-based liquid medium as the first medium and transferred after 40 days onto MSWH compared to those transferred to MSD as the last media (Figure 5 I). Moreover, the average number of secondary somatic embryos per primary embryo was three to four times more on MSWH medium than on MSD, regardless of the type of medium used for the first step (Figure 5 II).
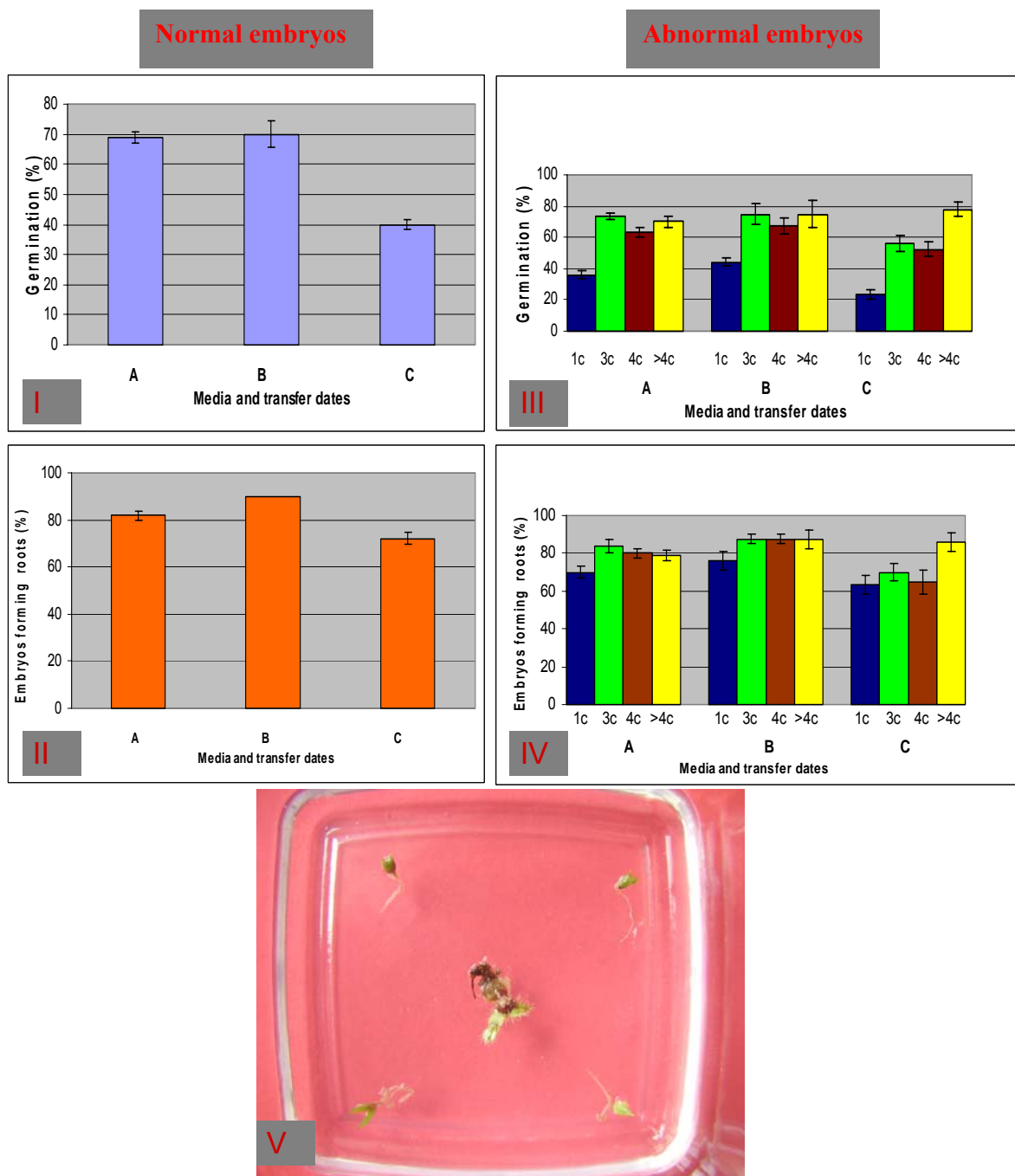
Figure 4. Germination of somatic embryos of *S. truncata*.cv Russian Dancer. The results are the mean ± standard error of **I.** and **II.:** A(n:17), B(n:5) and C (n:10); **III.** and **IV.**: A(1c, 3c, 4c, >4c; n: 5, 8, 6, 9, respectively), B(1c, 3c, 4c, >4c; n: 5, 4, 4, 4, respectively) and C (1c, 3c, 4c, >4c; n: 6, 5, 4, 5, respectively). **V.** Germinated somatic embryos after one month on G medium.

Secondary somatic embryos formed on abnormal primary embryos showed the same trend. The frequency of secondary somatic embryos and the average number of secondary somatic embryos per primary abnormal embryo were less when MSD was used as the last medium compared to MSWH medium (Figure 5 III & IV, respectively).
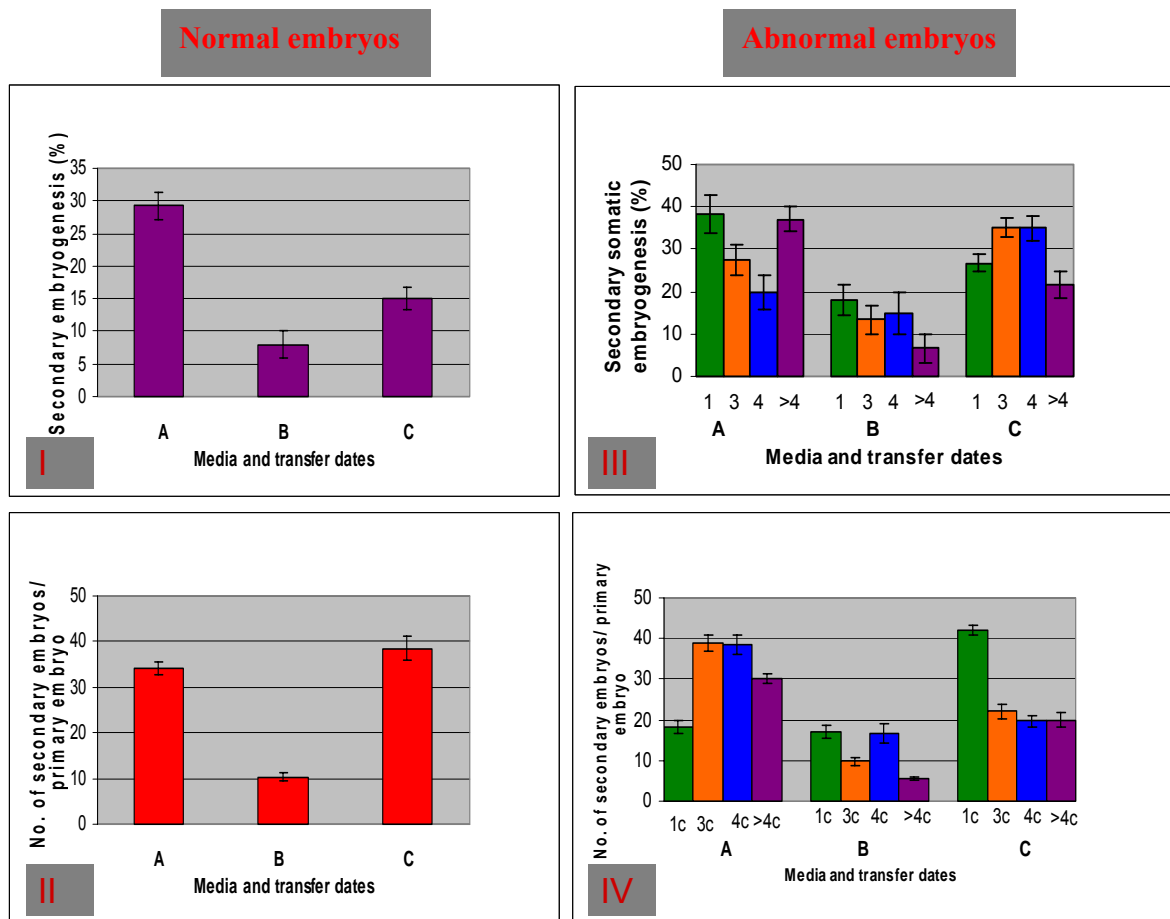
Figure 5. Secondary embryogenesis of *S. truncata* cv Russian Dancer. The results are the mean ± standard error of **I.** A (n:13), B (n:5) and C (n:10), **II.** A (n:38), B (n:4) and C (n:15) , **III.** A (1c, 3c, 4c, >4c; n: 4, 8, 4, 7, respectively), B (1c, 3c, 4c, >4c; n: 5, 3, 2, 3, respectively) and C (1c, 3c, 4c, >4c; n: 6, 6, 4, 6, respectively), **IV.** A (1c, 3c, 4c, >4c; n:14, 22, 8, 25, respectively), B (1c, 3c, 4c, >4c; n: 9, 4, 3, 2, respectively) and C (1c, 3c, 4c, >4c; n: 16, 20, 14, 13, respectively).

Transfer to greenhouse conditions

A total number of 439 germinated embryos from all combinations of media and different transfer dates that originated from Ho-type callus, were transferred to soil to evaluate their survival rates. When somatic embryos were left more than 3 weeks on B medium, they began callusing at the bases. However, plants from somatic embryos with 1, 2, 3, 4, or more than 4 cotyledons showed excellent acclimatisation and survival rates of (70-80%), (65-81%), (84-94%), (75-88%) and (70-87.5%), respectively (Table 3). They also showed an increase in height and formation of the second set of phylloclades (secondary phylloclades) (Figure 6, Figure 7I, II and III). A maximum average height of 2.6 cm was recorded for those plants developed from somatic embryos with more than 4 cotyledons. Those resulted from callus transferred after 40 days from MS-based medium onto MSWH (Figure 7 I). Moreover, plants developed from somatic embryos with more than 4 cotyledons, and to a lesser extent those plants developed from somatic embryos with 4 cotyledons, had the highest average number of primary and secondary phylloclades per plant (Figure 7 II & III).

Morphology and histology of the somatic embryos

In this investigation, the main stages of somatic embryogenesis from globular to cotyledonary stages were observed. The development of the embryo began with small, densely cytoplasmic cells that underwent a series of organized divisions (Figure 8 a, *arrowhead*). Such meristematic centres led to the formation of globular-stage somatic embryos (Figure 8 b). The appearance of the globular structures was coupled with the development of the protoderm, which is the outermost layer of a developing embryo (Figure 8 a, *arrow*).

The next developmental stage was the oblong embryo (Figure 8 c). The oblong stage is considered as a transition stage between the globular and the elongated embryos. When embryos began elongating, they were easily separated from the mother tissue and showed clear, distinct root and shoot poles (Figure 8 d, *arrowhead*). They also developed a clear vascular system (Figure 8 d, *arrow*). The torpedo stage marked the beginning of the two cotyledons (Figure 8 e, *arrowhead*). At this stage, the first signs

of the two cotyledons (*arrowhead*) were seen around the top middle notch (*arrow*) in the apical meristem (Figure 8 e & f).

Somatic embryos further progressed through the early (Figure 8 g) and middle cotyledonary stages (Figure 8 h) until they attained well-developed and mature cotyledons (Figure 8 i).

Table 3. Effects of media and transfer dates on the percentage of survival of *S. truncata* cv. Russian Dancer somatic embryos from Ho-type callus.

First medium	Transfer (days)	Second medium	Somatic embryo survival (%)				
			1ct	2ct	3ct	4ct	>4ct
SH,7.0 μM kin	30	MSWH	$73_{(11)*}$	$81_{(70)}$	$84_{(49)}$	$75_{(32)}$	$70_{(50)}$
MS,7.0 μM kin	40	MSD	$80_{(15)}$	$65_{(31)}$	$94_{(17)}$	$77_{(13)}$	$87.5_{(16)}$
		MSWH	$70_{(20)}$	$70_{(27)}$	$89_{(36)}$	$88_{(17)}$	$74_{(35)}$

* Numbers between brackets in the lowercase represent the number of embryos evaluated.

Figure 6. Regenerated plants from somatic embryos of *S. truncata* cv. Russian Dancer grown in soil for two months.
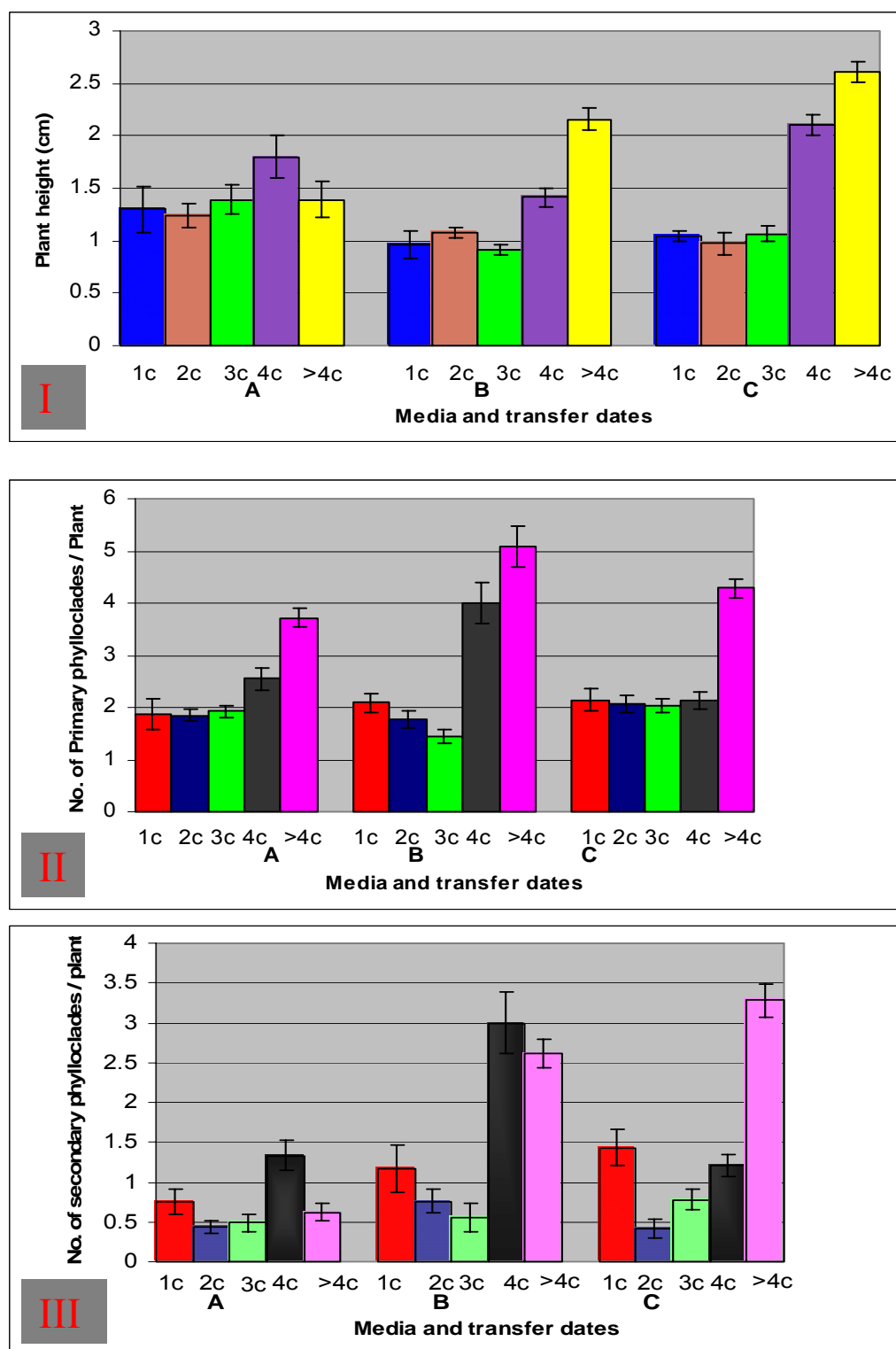
Figure 7. Growth of plants derived from somatic embryos of *S. truncata* cv. Russian Dancer. **I., II.** and **III.:** A (1c, 2c, 3c, 4c, >4c; n: 8, 50, 41, 24, 34, respectively), B (1c, 2c, 3c, 4c, >4c; n: 12, 21, 16, 9, 14, respectively) and C (1c, 2c, 3c, 4c, >4c; n: 14, 17, 30, 14, 24, respectively).
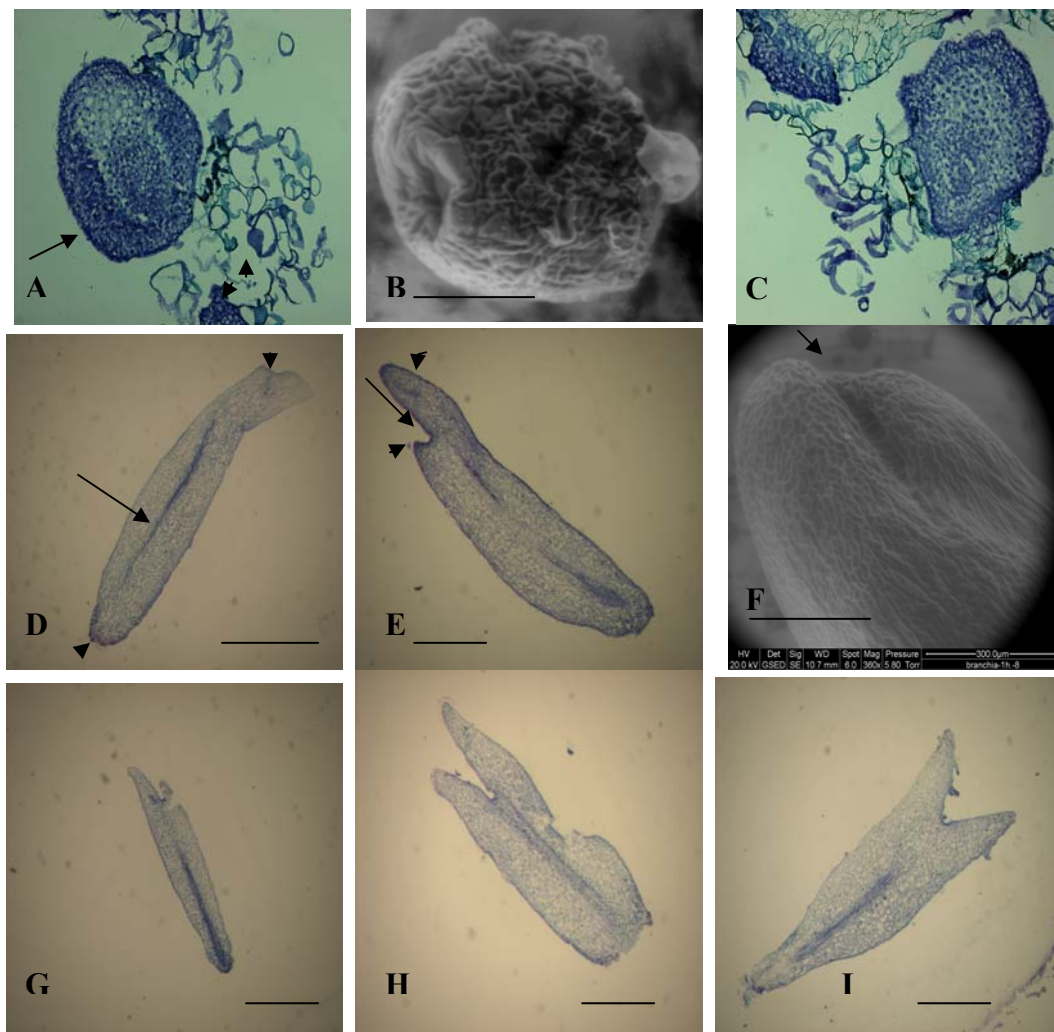
Figure 8. Histological analysis revealing the key developmental stages in *S. truncata* cv. Russian Dancer somatic embryogenesis. (**A**) Globular embryo with visible protoderm (*arrow*) and a nearby section of callus cells preparing for somatic embryogenesis with apparent meristematic zones (*arrowhead*) characterized by dense cytoplasm and intensely stained nuclei (x10). (**B**) Isolated somatic embryo in globular phase photographed by (ESEM) (*Bar*: 300 μm). (**C**) Oblong-shaped somatic embryo (x10). (**D**) Elongated embryo, with a closed basal end (*arrowhead: bottom*) and contained vascular system (*arrow*) (*Bar*: 350 μm). (**E**) Early torpedo-shaped somatic embryo, with a notch (*arrow*) in the middle of the shoot apex, giving rise to the first signs of the 2 cotyledons (*arrowhead*) (*Bar*: 240 μm). (**F**) The shoot apex of a somatic embryo in the torpedo stage photographed by (ESEM), showing the notch (*arrow*) and the very beginning of the 2 cotyledons (*Bar*: 300μm). (**G**) (**H**) and (**I**) Early, middle and late-cotyledonary stage showing the gradual development of the 2 cotyledons (*Bars*: 300 μm (G); 240 μm (H); 240 μm (I))

Organogenesis

Both organogenesis and embryogenesis occurred at the same time. Roots were seen after 3-4 weeks when callus was transferred from the liquid medium to the second solid medium. Adventitious shoots began forming after 4 weeks on MSWH when Ho callus was transferred from SH-based medium. Adventitious shoots formed in almost all types of callus with different types of media and combinations of transfer dates. Similar to the tendency with somatic embryos, adventitious shoots formed at higher frequency when the older callus was used. However, unlike somatic embryos, when 2,4-D was included in the second medium, formation of shoots was clearly enhanced (Table 4).

Table 4. Effects of media and transfer dates on the average number of adventitious shoots of *S. truncata* cv.Russian Dancer from Ho-type callus.

First medium	Transfer (days)	Second medium	Average number of shoots/ 0.5gm callus			
			Scale 1[1]	Scale 2[2]	Scale 3[3]	∑ shoots
MS, 7.0 μM kin	20	MSD	$9.0\pm0.7^{*,x}$	$2.6\pm0.4^{*,x}$	$0.4\pm0.4^{*,x}$	$12.0\pm0.9^{*,x}$
	40	MSD	6.3 ± 0.5^{y}	3.3 ± 0.5^{y}	0.4 ± 0.2^{y}	9.9 ± 0.7^{y}
		MSWH	0.3 ± 0.2^{y}	0	0	0.3 ± 0.2^{y}
SH, 7.0 μM kin	30	MSD	6.5 ± 0.6^{y}	2.9 ± 0.4^{y}	0.1 ± 0.1^{y}	9.5 ± 0.6^{y}
		MSWH	1.8 ± 0.4^{y}	1.6 ± 0.3^{y}	0	3.4 ± 0.6^{y}

[1] shoots that are less than 1 cm long

[2] shoots that are between 1 and 2 cm long

[3] shoots that are more than 2 cm long

* Only data from the first combination (media and transfer date) represent the average number of shoots per 1 gm callus. The results are the mean ± standard error of [x] (n:5) and [y] (n:8).

Effects of auxin type and gauze on induction of somatic embryos

Adventitious shoots, however not somatic embryos, were formed when callus was transferred after 20 days from liquid MS-based medium onto MSD or MSIA, regardless of whether gauze was used or not. More shoots regenerated on MSIA than

on MSD. In this regard, a total of 20 shoots were produced on MSIA compared to 4 shoots regenerated on MSD when gauze was not used (Figure 9).

The addition of gauze on the surface of MSD enhanced the regeneration of shoots (Figure 9). A total of 12 shoots regenerated when gauze was used compared to only 4 shoots formed in the absence of gauze. However, this effect was not clear with MSIA. Approximately the same number of shoots was produced on MSIA, in the presence and absence of gauze (Figure 9).
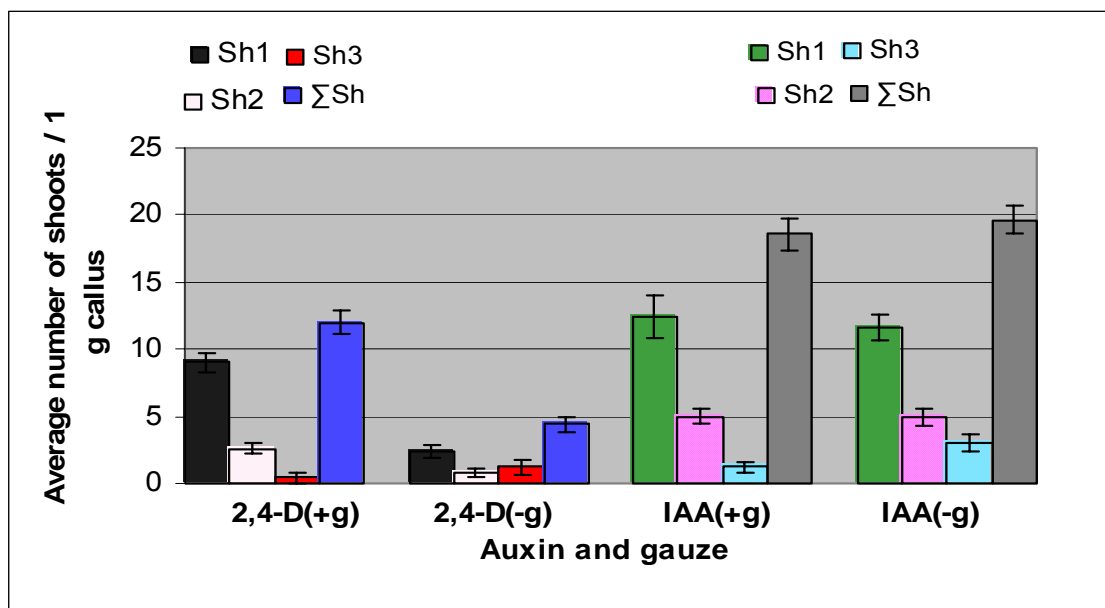
Figure 9. Effects of auxin type and gauze on the regeneration of adventitious shoots in *S. truncata* cv. Russian Dancer. Gauze was either placed on the surface of the medium (+g) or it was not used (-g). Sh1: Shoots that are less than 1 cm long, Sh2: Shoots that are between 1 and 2 cm long, Sh3: Shoots that are more than 2 cm long, ∑ Sh: total number of shoots.

Morphology of zygotic embryos\ somatic embryos

After the rupture of seed coat, developing *Schlumbergera* zygotic embryos exhibited the torpedo, early, middle and late cotyledonary stages (Figure 10 a). The corresponding stages of somatic embryos showed a good resemblance (Figure 10 b).

Typical heart-shaped somatic embryos were not observed in *Schlumbergera*. Based on the germination of zygotic embryos, this stage was also absent (Figure 10 a).

However, differences were noticed between zygotic and somatic embryos. Zygotic embryos normally exhibited an increase in the gradient of green colour, from light to dark green, with the development from torpedo to late cotyledonary stages (Figure 10 a). Somatic embryos were white at the globular and elongation stages (Figure 10 c). Once the torpedo stage began, a change in the colour of somatic embryos was observed. Somatic embryos at torpedo and sometimes cotyledonary stages displayed a colour gradient (Figure 10 c & e). Sometimes the lower part of the embryo was green and the upper part with the cotyledons was white (Figure 10 c). In other cases, the opposite was noticed. Cotyledons were green and the lower part of the embryo was white (figure 10 e).

In addition to the formation of embryos with multiple cotyledons, somatic embryos exhibited a broad spectrum of morphological abnormalities in the dicotyledonous stage. Compared to zygotic embryos, which exhibited a clear V shape of the 2 cotyledons (Figure 10 d), somatic embryos with fused cotyledons and a narrow angle between the two cotyledons, were observed (Figure 10 e).

Dicotyledonous somatic embryos with various sizes of cotyledons were also evident. For example, embryos that were wide in the middle with short cotyledons were observed (Figure 10 f).

Cytological analysis of the regenerated plants

Plants derived from somatic embryos with 4, or more than 4, cotyledons had the highest average plant height (Figure 7 I) and showed more primary and secondary phylloclade formation than embryos with other types of cotyledons (Figure 7 II & III). This finding, coupled with the observed large size of embryos bearing 4 or more than 4 cotyledons (Figure 11), led to the study of the genetic stability of somatic embryo-derived plants. The chromosome number of mother plants was diploid ($2n=2x=22$) (Figure 12 I). In plants derived from somatic embryos with either 2 or 4 cotyledons, the ploidy level was maintained ($2n=2x=22$) (Figure 12 II & III). However, plants

derived from somatic embryos with more than 4 cotyledons were triploid (Figure 12 IV).
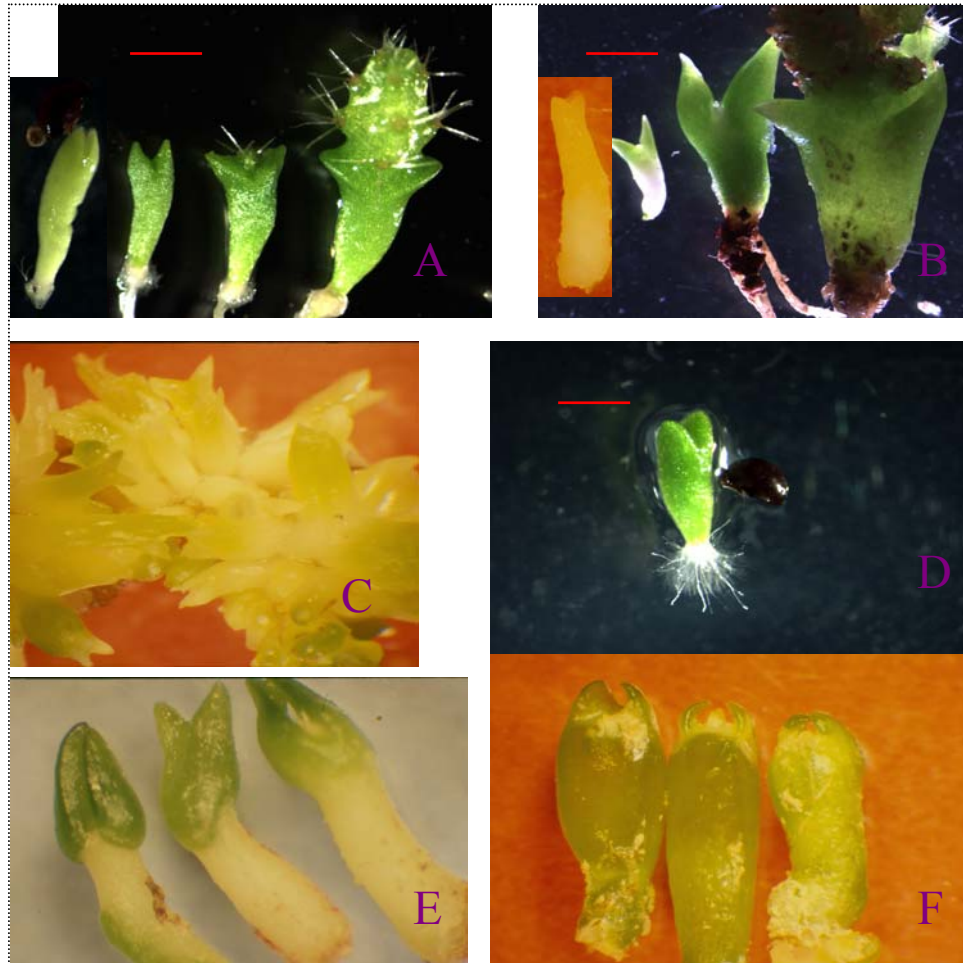
Figure 10. Morphology of zygotic and somatic embryos. (**A**) Gradual development of zygotic embryos (*Bar*: 1.50 mm) (**B**) Gradual development of somatic embryos. (*Bar*: 1.70 mm) (**C**) Mixed stage of elongating and torpedo embryos (x6). Morphology of the two cotyledons from zygotic embryos (*Bar*: 1.50 mm) (**D**) and somatic embryos (**E & F;** x18 & x9, respectively).

Figure 11. Plants derived from somatic embryos with 2, 3, 4 or more cotyledons (from left to right, respectively).
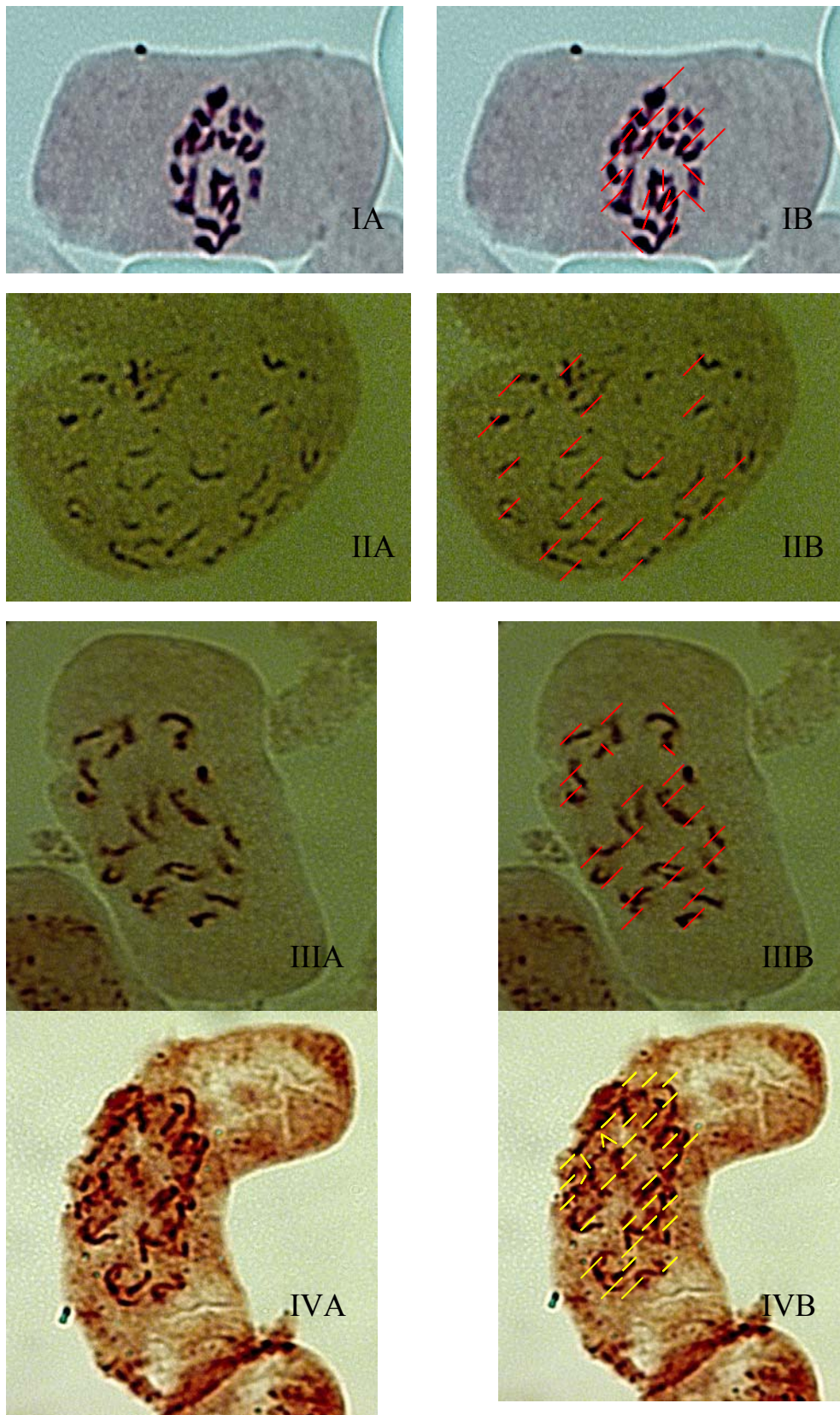
Figure 12. Chromosome numbers in *S. truncata* cv. Russian Dancer somatic embryos.
(I) Diploid chromosome set (2n=2x=22) of the mother plant, (II) Diploid chromosome

set (2n=2x=22) of dicotyledonous embryos, (III) Diploid chromosome set (2n=2x=22) of somatic embryos with 4 cotyledons, (IV) Triploid chromosome set (2n=3x=33) of somatic embryos with more than 4 cotyledons. (A) Original slides (B) Individual chromosomes are marked to indicate their number. Objects were photographed at x1000.

B) Cultivars Alex and CB5

Morphogenesis

Both cultivars produced adventitious roots and shoots. Somatic embryogenesis was induced only in cv. Alex.

Induction of somatic embryos

The incidence of somatic embryos was recorded in cv. Alex from callus that was grown for 30 days in liquid MS-based medium (containing 7.0 μM kinetin), and then transferred on either MSWH or MSDM. Somatic embryos were induced at low frequency. Only callus in one replicate out of seven replicates for each medium produced friable, white, reddish callus with 3-6 embryos.

Organogenesis

Differences were observed in the colour and texture of the callus from both cultivars that were grown in the first MS liquid media. Callus texture from cultivar Alex was medium to hard after 30 days of growth in the liquid medium. Its colour was greenish-yellow. However, the callus from cv. CB5 was characterized by its hard texture and greenish-red colour.

Adventitious roots and shoots were seen after about three to five weeks when callus was transferred from the liquid media to the various solid media. For cv. CB5, in addition to the adventitious roots and shoots, white transparent structures had formed by that time on callus gown on MSD. Those structures later produced roots.

Effects of the media and transfer dates on the adventitious root and shoot formation

Different media were used in the final transfer phase to test the effect of auxins and daminozide on morphogenesis in *Schlumbergera* and *Rhipsalidopsis*. Two to three months after the callus of cv. Alex was transferred to the different media, new soft, watery callus had developed on the older callus in the different media (Figure 13). This new callus was rhizogenic, giving rise only to roots.

The interaction between the transfer dates and the media was significant for the average number of adventitious shoots for both cultivars. The results from cv. Alex showed that the longer the callus is grown in the liquid medium, the more the regeneration of adventitious shoots in terms of both the average number and dry weight of shoots (Table 5).

The callus that was grown for 50 days in the first MS liquid medium, and then grown for 5 months on either MSWH or MSDM, significantly produced the highest average number of total shoots (40 and 45, respectively) (Figure 14 a). This combination of transfer date and medium also resulted in the highest average shoot weight of 0.26 (MSWH) and 0.31 g (MSDM), respectively (Table 5). In contrast, callus grown for 30 or 50 days in the first liquid medium and then transferred onto MSD, significantly produced the least average number of shoots (1 and 4, respectively). The average dry weight of shoots produced from this treatment combination was also poor (Table 5). Roots formed in almost all of the combinations of medium and transfer dates (Table 5). The adventitious shoots formed on the callus had formed a second set of phylloclades, with the least response recorded for the callus grown on MSD. Cultivar CB5 showed the opposite trend than that of cv. Alex for adventitious shoot formation. Callus grown for 30 days in the first liquid medium showed significantly higher response in almost all the media in the final transfer than callus grown for 50 days in the liquid medium (Table 6).
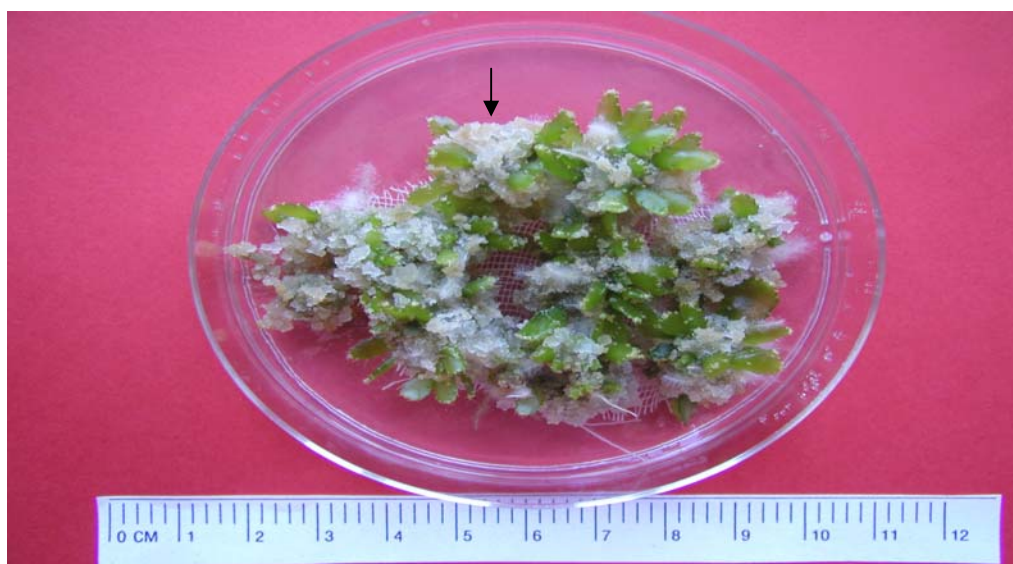
Figure 13. Callus culture of *S. truncata* cv. Alex grown for four months on MSIA medium, showing shoot regeneration and the development of new, soft, watery, whitish callus (arrow) on the top.

Table 5. Effects of media and transfer dates on the average number of adventitious shoots of *S. truncata* cv. Alex.

Medium	Transfer (days)	Average number of shoots/ 1.0 g callus				Shoots Dry weight (g)	roots
		Scale 1[(1)]	Scale 2[(2)]	Scale 3[(3)]	∑ shoots		
MSD	30	0.57 f	0.57 e	0.00	1.14 d	0.004±0.004	-
	50	1.14 ef	3.14 ed	0.00	4.29 d	0.02±0.002	+
MSWH	30	3.14 de	8.00 cb	2.57 b	13.71 c	0.14±0.03	+
	50	7.71 b	22.14 a	9.71 a	39.57 a	0.26±0.05	+++
MSDM	30	5.71 bc	12.57 b	3.57 b	21.86 b	0.18±0.03	+
	50	10.29 a	24.71 a	9.57 a	44.57 a	0.31±0.02	++
MSIA	30	3.86 cd	6.29 dc	2.29 b	12.43 c	0.10±0.02	+
	50	7.00 b	11.29 b	8.14 a	26.43 b	0.28±0.04	+++

[(1)] shoots that are less than 1 cm long
[(2)] shoots that are between 1 and 2 cm long
[(3)] shoots that are more than 2 cm long
Numbers within each column having the same letter are not significantly different at $P=0.05$ according to t –test. The results of shoot dry weight are the means ± standard errors. Qualitative characterisation of root production: -, absence; +, few; ++, good; +++, very good.
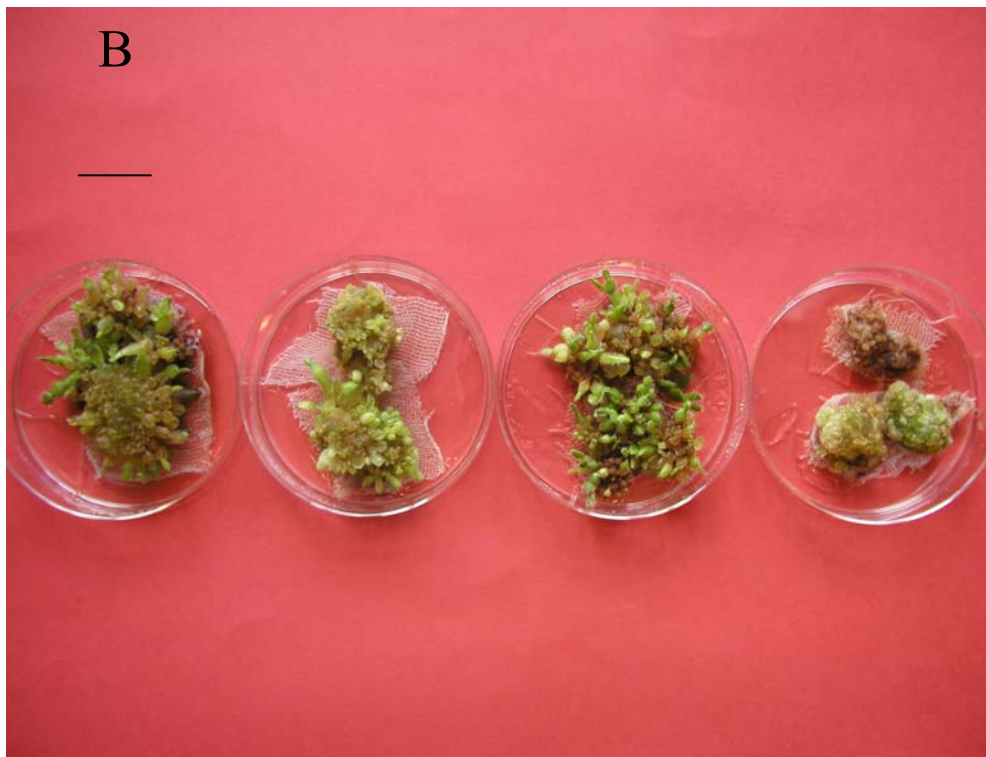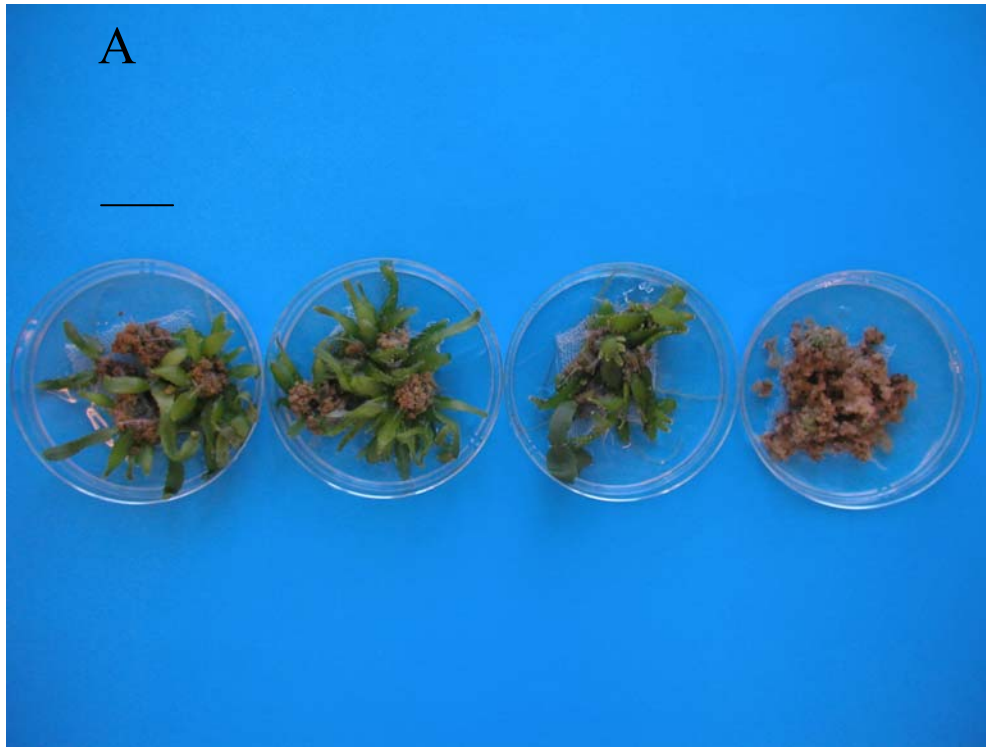
Figure 14. Adventitious shoot organogenesis (A) Alex callus grown for 50 days in liquid MS-based medium, then transferred to media MSWH, MSDM, MSIA and MSD (from left to right, respectively). *Bar*: 2.6 cm (B) CB5 callus grown for 30 days in liquid MS-based medium, then transferred to media MSWH, MSIA, MSDM and MSD (from left to right, respectively) *Bar*: 2.6 cm.

The highest significant average of total shoots was obtained with callus grown for 30 days in liquid medium, and then transferred to MSWH (495) or MSDM (447) (Figure 14 b). The response from MSWH was significantly higher than that from MSDM. On both media, approximately the same average dry weight of shoots was recorded (0.66 g) (Table 6).

Similar to cultivar Alex, callus grown on MSD showed poor response for both average number of shoots produced and their average dry weight. On this medium, most of the shoots produced were small and less than 3 mm long (Table 6). These shoots were pale green compared to the darker shoots produced from the other treatments. In contrast to other treatments, shoots produced on MSD did not form secondary phylloclades. Adventitious roots were formed in all combinations of media and transfer dates (Table 6).

Table 6. Effects of media and transfer dates on the average number of adventitious shoots of *R. gaertneri* cv. CB5.

Medium	Transfer (days)	Average number of shoots/ 0.5 g callus				Shoots Dry weight (g)	roots
		Scale 1[1]	Scale 2[2]	Scale 3[3]	$\sum$ shoots		
MSD	30	101.00 ^{b}	0.00	0.00	101.00 ^{e}	0.03±0.008	++
	50	41.86 ^{d}	4.57 ^{e}	0.00	46.43 ^{f}	0.009±0.004	++
MSWH	30	131.14 ^{a}	304.57 ^{a}	59.14 ^{a}	494.86 ^{a}	0.66±0.04	++
	50	69.57 ^{c}	74.29 ^{c}	22.57 ^{b}	166.43 ^{c}	0.15±0.03	++
MSDM	30	112.86 ^{ab}	277.14 ^{b}	57.14 ^{a}	447.14 ^{b}	0.67±0.02	+
	50	72.71 ^{c}	62.29 ^{cd}	13.00 ^{b}	148.00 ^{cd}	0.13±0.03	++
MSIA	30	67.14 ^{c}	70.57 ^{c}	13.86 ^{b}	151.57 ^{cd}	0.17±0.03	+
	50	52.43 ^{cd}	46.43 ^{d}	15.14 ^{b}	114.00 ^{de}	0.12±0.04	+

[1] shoots that are less than 3 mm long

[2] shoots that are between 3 and 7 mm long

[3] shoots that are more than 7 mm long

Numbers within each column having the same letter are not significantly different at P=0.05 according to t–test. The results of shoot dry weight are the means ± standard errors. Qualitative characterisation of root production: +, few (< 10); ++, good (> 10).

2.4 Discussion

Adventitious root and shoot organogenesis were observed in both *Schlumbergera* and *Rhipsalidopsis*. However, somatic embryogenesis was induced only in *Schlumbergera* cultivars. Differences were observed between cultivars in both plant types for morphogenesis response. For example, *Rhipsalidopsis* cv. CB5 and *Schlumbergera* cv. Alex showed a good potential for adventitious shoot formation compared to the other cultivars tested.

In *Schlumbergera*, differences between cultivars were also evident in terms of the time required for the induction of somatic embryos. This response was affected by the treatment combinations of media, callus transfer dates, plant growth regulators and presence or absence of gauze.

Similarly, genotypic differences for the induction of somatic embryogenesis have been observed in *Citrus* (Gmitter and Moore, 1986), potato (Seabrook and Douglass, 2001) and cassava (Hankoua *et al*., 2005). Furthermore, Seabrook and Douglass (2001) reported that differences existed between cultivars of potato for the time required to form embryos.

Somatic embryogenesis occurred indirectly through the callus phase in *S. truncata* from phylloclade explants. Somatic embryos differentiated from friable, yellowish callus that developed on older callus.

It is likely that a hormonal balance, represented by the ratio of cytokinins to auxins, controls the morphogenic response observed in *Schlumbergera* and *Rhipsalidopsis*.

Callus of the cv. Russian Dancer, which was sub-cultured for a long time in the establishment phase on medium containing mainly cytokinins, proved superior in both somatic embryogenesis induction and organogenesis compared to callus grown for a shorter time on the establishment media. Apparently, embryogenesis and adventitious shoot organogenesis were promoted by an increase in cytokinins through the long incubation of callus on media with high cytokinin to auxin ratio until a critical balance

was reached that, in turn, controlled the fate of the somatic cells. This view is further supported by the lack of somatic embryogenesis on MS media containing 2,4-D, when callus of cv. Russian Dancer was transferred after 20 days on liquid MS medium or after 30 days on liquid SH medium. Therefore, it seems that the period starting from the 20th day of callus growth in the liquid culture in the induction phase is critical for the regulation of the developmental switches in cv. Russian Dancer. Thus, it was clear that the presence or absence of auxins in the last solid media used was the decisive factor for determination of the morphogenic response. In this regard, MSWH favoured somatic embryo induction, whereas MSD and MSIA favoured adventitious shoot regeneration (organogenesis). The same conclusion can be reached for the other cultivars of *Schlumbergera* and for *Rhipsalidopsis*. However, the length of callus exposure to cytokinins and the duration of callus growth in the liquid media, which is required to reach the critical hormonal balance, were different in the cultivars of *Schlumbergera*, other than Russian Dancer, and in *Rhipsalidopsis*. For example, somatic embryos were induced after 4 months on MSDM, when callus of cv. Malindi was subcultured for seven months in the establishment phase on medium containing mainly cytokinins, and then grown for 20 days in the liquid medium.

It seems that the embryogenic callus was transformed from a heterotrophic to an autotrophic state and thus became habituated for both auxins and cytokinins. This was demonstrated by the continuous production of repetitive somatic embryos when this embryogenic callus was transferred monthly onto MS-based medium without hormones. Furthermore, the primary embryos or even the cotyledons derived from the primary embryos, when grown on MS medium without hormones, were able to form new secondary embryos. This indicated indefinite autotrophic growth capacity similar to that exhibited by the habituated embryogenic callus. Habituation was earlier reported for the *in vitro* cultures of *Nicotiana tabacum* (Meins and Lutz, 1980), *Nicotiana bigelovil* (Bennici, 1983), sugarbeet (van Geyt and Jacobs, 1985) and soybean (Christou, 1988).

In *Rhipsalidopsis*, once the hormonal balance was reached adventitious shoots began forming. However, it could be that the white, transparent, round structures formed on the callus of CB5 on MSD medium were intermediate structures between somatic embryos, roots and possibly shoots. Furthermore, under the conditions of the

experiment and the physiological state of the callus, it seemed that the hormonal balance was in favour of root formation. Provided that the callus in *Rhipsalidopsis* is hard compared to the soft fine callus observed in most of the *Schlumbergera* cultivars, it might be possible that further attempts by manipulation of the plant growth regulators and changing callus texture could lead to somatic embryogenesis induction in *Rhipsalidopsis*.

Generally, cactus is known to produce high levels of auxins (Hubstenberger *et al*., 1992). Srikandarajah and Serek (2004) reported that accumulation of cytokinin-like substances due to repeated culture in media containing cytokinin may have contributed to balancing out the high endogenous levels of auxins, thus improving adventitious shoot formation in phylloclade explants of cv. CB4 of *Rhipsalidopsis*. Chen and Chang (2001) found that embryo formation was retarded on leaf explants of *Oncidium* ´Grower Ramsey` by the four auxins tested (IAA, IBA, NAA and 2, 4-D), whereas the highest percentage of embryo formation was recorded using media containing TDZ, 2iP and kinetin.

From the present study, TDZ may have a role in the regulation of morphogenesis. It could be that TDZ, a cytokinin-like substance, contributed to the enhanced adventitious shoots and somatic embryogenesis induction. Furthermore, TDZ may modulate the metabolism of endogenous auxins and cytokinins. TDZ, either alone or in combination with other growth regulators, has been found to induce somatic embryogenesis in many plant species (Victor *et al*., 1999; Mithila *et al*., 2003; Zhang *et al*., 2005). TDZ also induces shoot organogenesis in a wide variety of plant species (Malik and Saxena, 1992; Saito and Suzuki, 1999; Mithila *et al*., 2003). However, although the precise mode of action of TDZ remains undetermined, Murthy *et al*., (1995) states that TDZ-induced morphogenesis is related to the levels of endogenous growth regulators.

The usage of gauze on the surface of MSD, but not MSIA, enhanced the regeneration of adventitious shoots of Russian Dancer derived from callus that was grown for 20 days in liquid MS-based medium. Therefore, we partially concluded, at least for MSD, that the gauze has modified the environment where callus was grown by

creating a favourable contact with the medium. This enabled better utilization of nutrients from the medium, as well as the removal of metabolites by callus.

Takezawa (2003) highlighted the concept of organ engineering in animals. This included development of ideal cellular scaffolds for maintaining the activity of functional cells, for regulating cell behaviour and for reconstructing three-dimensional multicellular masses. Gauze made of natural cotton fibers, therefore, was used because each cotton fiber is sufficiently soft enough to be included in three-dimensional multicellular masses and possess a high capacity of absorbing water by capillary phenomenon. Therefore, such cellular scaffolds sustain capillary networks that can provide fresh culture medium to all cells and remove metabolites from the cells.

Despite the effect of gauze, more cv. Russian Dancer shoots regenerated on MSIA compared to MSD, from callus that was grown for 20 days in liquid MS-based medium. The different response observed between these two auxins reflects the differences in their uptake, transport and metabolism (De Klerk *et al*., 1997), modulated possibly by the levels of endogenous auxins.

The superiority of SH-based medium over MS-based medium in the induction experiments of cv. Russian Dancer, in terms of the highest average number of embryos obtained, indicated that differences in the concentrations or combinations of nutrients contributed to the optimal expression of embryogenic potential.

The experiments with cvs. Alex and CB5 provided more insights with respect to morphogenesis in *Schlumbergera* and *Rhipsalidopsis*.

Unlike cv. Russian Dancer, the highest numbers of total shoots in both cvs. Alex and CB5 were significantly produced on MSWH and MSDM media. The least response was observed on MSD medium. However, reflecting genotypic differences, the two cultivars showed the opposite trend for the time required in the liquid medium used in the first culture. Whereas callus from cv. CB5 required only 30 days, the callus from cv. Alex needed 50 days of growth in the liquid medium to produce the highest average number of total shoots.

Those findings, together with the observation that somatic embryos were induced in cv. Alex on either MSWH or MSDM, indicated that absence or reduction of auxin is necessary after long exposure to cytokinins for adventitious shoot regeneration in both plant types and for somatic embryo induction in *Schlumbergera*.

Similar to these results, daminozide was found to enhance the production of somatic embryos derived from sweet orange ovules when used together with 2,4-D compared to treatment with only 2,4-D (Gmitter and Moore, 1986). Thus, it is possible that daminozide reduces the concentration of endogenous auxins or negates its effect (George, 1996).

Somatic embryos in cv. Russian Dancer showed variation in cotyledon morphology. In addition to the normal embryos, abnormal somatic embryos showing 1, 3, 4 or even more than 4 cotyledons were recorded. The production of abnormal somatic embryos with multiple cotyledons (Choi *et al*., 1997; Griga, 2002; Kim *et al*., 2003) or other forms of cotyledons like jar-shaped cotyledons (Choi *et al*., 1997), is well documented. The production of somatic embryos with multiple cotyledons was considerable for a continuous culture on a medium containing cytokinins (Lee and Soh, 1993), on 2,4-D-containing medium (Cho *et al*., 1998) or on ABA-containing medium (Lee and Soh, 1994).

In agreement with the present study, Guevin and Kirby (1997) reported the formation of cotyledonary-stage somatic embryos with stunted cotyledons and reduced embryo axes.

The formation of secondary somatic embryos in cv. Russian Dancer noticed in this study did not hamper the maturation and eventual acclimatization of plants under greenhouse condition. In the literature there have been many reports on secondary somatic embryogenesis induced on embryos in several plants, such as *Arachis hypogaea* L. (Little *et al*., 2000), *Helianthus maximiliani* (Vasic *et al*., 2001), *Manihot esculenta* Crantz (Woodward and Puonti-Kaerlas, 2001) and *Quercus ilex* L (Mauri and Manzanera, 2003).

The histological investigation showed that the somatic embryos had no detectable connection with the mother explants and that advanced stages of somatic embryos had a contained vascular system. Histological studies in other plants confirm the same results (Kärkönen, 2000; Quiroz-Figueroa *et al*., 2002; Sharma and Millam, 2004).

The chromosome numbers reported here for *Schlumbergera* cv. Russian Dancer agree with the base number x = 11 for the Cactaceae (Turner, 1994; Pinkava *et al*., 1998; Parks and Boyle, 2003).

With the exception of plants derived from somatic embryos with more than 4 cotyledons, *Schlumbergera* (cv. Russian Dancer) somatic embryo-derived plants showed genetic stability when compared to the mother plants. However, plants derived from somatic embryos with more than 4 cotyledons were morphologically different than plants derived from other types of somatic embryos. Those plants were triploid, showing the highest average plant height and more phylloclade formation compared to plants derived from somatic embryos with fewer cotyledons.

Although in this study, not all the regenerated plants derived from somatic embryos were analysed, it was possible to find a relationship between the manifested morphological characteristics and the ploidy level at an early stage of development. Furthermore, the production of such polyploidy plants from somatic embryos could be exploited further through somaclonal variation (Larkin and Scowcroft, 1988) for the production of new plant genotypes for breeding. In this respect, polyploids often have desirable horticultural features such as larger flowers and stems and a more upright habit (Blakeslee, 1941; Sparnaaij, 1979; Levin, 1983; Karle, 1996).

Normally, plant regeneration through somatic embryogenesis considerably decreases the possibility of chimeral plant production. Genetic stability using this method was reported to be maintained in *Cyclamen persicum* Mill. (Winkelmann *et al*., 1998), *Phoenix dactylifera* L. (Fki *et al*., 2003) and *Ulmus minor* Mill. (Conde *et al*., 2004).

2.5 Conclusion

This study describes for the first time a protocol for the production of somatic embryogenesis in *S. truncata* cv. Russian Dancer. Using SH-based medium supplemented with 7.0 *μM* kinetin for the first culture, then MSWH medium after 30 days gave the highest average number of embryos in the two-cotyledon stage. Significant regeneration efficiency of primary embryos coupled with the continuous production of secondary embryogenesis is highly beneficial. Phenotypic variability was not found between somatic embryo-derived plants and mother plants, except for those plants derived from somatic embryos with more than 4 cotyledons. The production of polyploidy plants from somatic embryos could be exploited further through somaclonal variation for the production of new plant genotypes for breeding purposes.

Regeneration through adventitious shoots in *Schlumbergera* cv. Alex and *Rhipsalidopsis* cv. CB5 has a high potential. The highest average numbers of total shoots in cvs. Alex and CB5 were obtained on MSWH or MSDM. This occurred when callus was grown for 50 days for cv. Alex, or 30 days for cv. CB5, in liquid MS-based medium containing 7.0 *μM* kinetin, for cv. Alex, or 4.7 *μM* kinetin, for cv. CB5 in the first culture.

The protocols established in the present study for the formation of somatic embryos and adventitious shoots open the window for plant genetic improvement through transformation techniques.

3.0 *Agrobacterium tumefaciens*-mediated transformation of *Rhipsalidopsis* and *Schlumbergera*

Abstract

Keywords: *Agrobacterium tumefaciens* – cactus - genetic transformation - nutritional stress - *Rhipsalidopsis gaertneri*

A protocol for *Agrobacterium tumefaciens*-mediated genetic transformation of *Rhipsalidopsis* cv. CB5 was developed. This is the first report on the transformation of *Rhipsalidopsis*. Calli derived from phylloclade explants and sub-cultured onto fresh maintenance medium over a period of nine to twelve months, were co-cultivated with *A. tumefaciens* LBA4404. Plasmid constructs carrying the *nptII* gene, as a selectable marker, and the reporter *uidA* gene were used. Some of the factors influencing T-DNA transfer to *Rhipsalidopsis* callus explants were assessed. Transformed *Rhipsalidopsis* calli with a vigorous growth phenotype were obtained by extended culture on media containing 600 mg/l kanamycin. The pre-incubation of *A. tumefaciens* in SIM medium containing acetosyringone raised the frequency of transgenic calli. However, after 9 months of stringent selection pressure, the removal of kanamycin from the final medium together with the culture of the transformed calli under nutritional stress led to the formation of several transgenic adventitious shoots. Transformation was confirmed by GUS staining (for *uidA* gene), ELISA analysis and Southern blot hybridization (for the *nptII* gene). With this approach, a transformation efficiency of 22.7% was achieved. In a separate set of experiments, the *Agrobacterium* strain AGL0, harbouring the plasmid pBEO210 that contains both *etr1-1* and *nptII* genes, was inefficient when used to transform callus explants from *Rhipsalidopsis* cv. CB5 and *Schlumbergera* cv. Alex. The loss of morphogenic potential in callus explants from cv. Alex may explain the lack of success in transforming this genotype. However, key variables in *Agrobacterium*-mediated gene transfer can be further optimised when the bacterial strain AGL0 is used. Overall results described in this study demonstrate that *Agrobacterium*-mediated transformation is a promising approach for these cacti.

3.1 Introduction

3.1.1 Overview

For potted plants with numerous flowers, flower longevity partially defines the duration that plants remain attractive; therefore, it is a major component of plant quality. Flower longevity ranged from 7 to 12 days and from 4 to 6 days for *Rhipsalidopsis* and *Schlumbergera*, respectively (Scott *et al.*, 1994). The display life of many flowering potted plants is limited by the loss of flowers, buds or leaves, caused by ethylene in the air or by stresses that induce ethylene responses (Fjeld and Moe, 1985; Reid, 1985; Reid and Wu, 1992). Stress can occur during packing, transit and even retail display. The drop of flowers and buds can be caused by exogenously applied ethylene. High temperature and low light intensity condition, which can easily occur when plants are transported, also induces flower abscission (Cameron and Reid, 1981). Flowers also produce endogenous ethylene when exposed to stress or during the natural senescence process (Cameron and Reid, 1981; Serek and Reid, 1993).

Christmas cactus has been shown to be sensitive to ethylene (Cameron and Reid, 1981; Serek and Reid, 1993). Bud drop is actually a major problem that can reduce the display life in *S. truncata* (Serek and Reid, 1993). Flower and bud drop of Christmas cactus could reach 30% during long distance transit (Cameron and Reid, 1981). Efforts had been made to reduce the undesirable effects of ethylene on flowers and buds of *Schlumbergera*. Foliar application of STS, an inhibitor of ethylene action (Veen and van de Geijn, 1978), significantly reduced flower and bud abscission of *Schlumbergera* plants stressed by exposure to ethylene even 4 weeks after application (Cameron and Reid, 1981). However, because STS is environmentally hazardous (Serek and Reid, 1993) and costly, researchers have been seeking alternative tools to control the effects of ethylene in the display life of potted plants.

Inhibitors of ethylene biosynthesis have been shown to be ineffective in the presence of exogenous ethylene (Serek and Reid 1993). Therefore, it was necessary to find environmentally-safe methods for blocking ethylene responses at the receptor level.

Transformation studies

In the last decade, a volatile ethylene binding inhibitor 1-methylcyclopropene (1-MCP), a non-toxic compound, has been discovered (Serek *et al*., 1994). This compound inhibited bud abscission and wilting of flowers of potted plants of *Schlumbergera* cv. Dark Marie in the presence of exogenous ethylene (Serek and Sisler, 2001). 1-MCP, blocks ethylene responses by binding irreversibly to ethylene receptors (Serek *et al*., 1994; Sisler and Serek, 1997; Kebenei *et al*., 2003), and after pre-treatment most of the receptors are blocked. However, during further plant development new sites are synthesized (Müller *et al*., 2000) and such de novo receptors are not protected by 1-MCP. Consequently, the exposure of plant material to exogenous ethylene can cause senescence symptoms.

When compared to 1-MCP, STS has proven to be more efficient in preventing bud drop in *S. truncata* caused by ethylene (Serek and Sisler, 2001). It appears that silver ion remains in the plant tissue for a longer time and after synthesis of new sites, continuously inactivates ethylene responses (Serek and Sisler, 2001)

The display life of flowering *Rhipsalidopsis* potted plants, for the purpose of increasing flower sales, was also addressed through scientific research. Flower longevity for *R. gaertneri* cvs. 'Evita', 'Purple Pride' and 'Red Pride' increased as postproduction temperature was decreased (Hartley *et al*., 1995). The number of days required for the last flower to open also increased as the temperature was decreased. No efforts have been made in increasing the postproduction longevity of *R. gaertneri* other than through development of improved cultivars.

In summary, the efficacy of 1-MCP to prevent the adverse effects of ethylene is limited by its short term residual activity in some plants (Macnish *et al*., 2004) and consequently its modest effect in overcoming the ethylene-mediated flower senescence (Reid *et al*., 2002). This, together with the environmental concern regarding the phytotoxicity of STS, necessitates the need to seek alternative tools, most likely through molecular approaches to control the effects of ethylene in the display life of potted *Schlumbergera* and *Rhipsalidopsis* plants.

Strategies have been used to engineer prolonged vase-life in carnations (Bovy *et al*., 1999), Petunia (Gubrium *et al*., 2000) and Campanula (Sriskandarajah *et al*., 2004)

without the need for chemical treatments. For this purpose, a mutant *etr1-1* allele that confers insensitivity to ethylene was introduced into those plants. The results from those studies showed that genetic transformation is a promising route to obtain ornamental crops with greatly enhanced shelf life.

Development of an efficient system for transformation in *Schlumbergera* and *Rhipsalidopsis* for introducing foreign genes into plants would be useful in modifying ornamental characteristics such as ethylene sensitivity, flower colour and flower shape, and disease resistance.

An efficient system for the induction of somatic embryogenesis in *Schlumbergera* and regeneration through adventitious shoot formation in both *Schlumbergera* and *Rhipsalidopsis* was achieved in the first part of this study. These improved systems of regeneration in both plant types were the basis for further work aimed at genetic improvement of these plants through transformation techniques.

3.1.2 *Agrobacterium*-mediated transformation

The natural ability of the soil microorganism *Agrobacterium* to transform plants is exploited in the *Agrobacterium*-mediated method. During the process of transformation, a specific segment of the vector, T-DNA, which can be engineered to contain a selectable marker and/ or genes of interest, is transferred from the *Agrobacterium* to the host plant cells and inserted into the nuclear genome. These functions are mediated by a set of virulence genes with optimal expression occurring in the presence of phenolic inducers, such as acetosyringone, that are released by wounded plant cells (Gelvin, 2003).

Agrobacterium-mediated transformation has been the tool for the transformation of many dicotyledonous crops. It will most likely be the method of choice in plant species in which efficient T-DNA transfer is possible. This is because of the relative ease and precision of gene transfer to intact, regenerable explants (Hinchee *et al.*, 1998).

Generally, *Agrobacterium*-mediated transformation offers some advantages in comparison with direct transformation methods as: (a) transfer of relatively large segments of DNA with little rearrangement; (b) integration of low copy numbers of the gene into the plant chromosomes; (c) being a simple and economical procedure; (d) stable gene expression with more genes intact and (e) production of transgenic plants with better fertility (Arencibia *et al*., 1998; Dai *et al*., 2001).

Beside its natural plant hosts, *Agrobacterium tumefaciens* has also been demonstrated to have the ability to transform yeast (Bundock *et al*., 1995), cultivated mushrooms (Chen *et al*., 2000), human cells (Kunik *et al*., 2001) and fungi (Degefu and Hanif, 2003). The versatility of *Agrobacterium* in its ability to transform is one of the unique advantages of this technique over other conventional transformation methods.

However, the low efficiency of transformation and adaptability to monocotyledonous plants are major disadvantages of *Agrobacterium*-mediated transformation (Dai *et al*., 2001).

The development of binary Ti vectors has revolutionized the use of *Agrobacterium* to introduce genes into plants. The two main components for successful *Agrobacterium*-mediated gene transfer, the T-DNA and the *vir* region, can reside on separate plasmids. These form the basis of modern Ti plasmid vectors, termed binary Ti vectors (Hoekema *et al*., 1983).

The flexibility of modern binary Ti vectors will permit the choice of plant selectable marker genes and the promoters that initiate and drive their transcription. This will permit any combination of selectable marker genes in order to explore such options as double and single selection protocols, and alternative selectable marker genes (Hellens *et al*, 2000).

Factors affecting *Agrobacterium*-mediated transformation

Efficiency of plant transformation depends on many factors. The genotype, applied techniques and modification of plant regeneration are the most important among

them. Therefore, the following are some of the variables that should be tested to guarantee a successful outcome.

Genotype

Different genotypes or cultivars of the same plant species have been reported to show different transformation rates (Cao *et al*., 1998). Sigareva *et al*. (2004), noticed variation in transformation efficiency between elite lines of tomato, with SG048 and 00-5223-1 lines producing more transgenic plants at a higher transformation rate than from 00-0498-B.

Starting explants

Typically, *Agrobacterium*-mediated transformation is performed using different explants. Leaves (Toldi *et al*., 2002; Kang *et al*., 2005), seeds (Lim *et al*., 2005), pollens (Li *et al*., 2004), shoot internodes (Horlemann *et al*., 2003), cotyledons (Zaragozá *et al*., 2004), shoot apex (Gould *et al*., 2002), and embryogenic calli (Chai *et al*., 2004; Leelavathi *et al*., 2004) have been used as explants for *Agrobacterium*-mediated transformation.

Explant age was found to influence plant transformation efficiency. Cao *et al* (1998) reported that for some blueberry cultivars, explant age influenced the number of GUS-expressing leaf zones and calli.

Bacterial strain

One of the most important factors influencing frequency of *Agrobacterium*-mediated transformation is susceptibility of the host genotype to the specific *Agrobacterium* strain. Nadolska-Orczyk and Orczyk (2000) have compared the virulence of three *Agrobacterium* strains (LBA4404, C58C1 and EHA105) and reported that transformation efficiency was the highest (8.2 transgenic plants per 100 explants tested) when the hypervirulent EHA105 strain was used. Cao *et al*. (1998) have reported similar results when comparing EHA105 and LBA4404 strains.

Strains EHA101 and EHA105 are more effective than strain LBA4404 since both are derived from supervirulent wild-type strain A281 (Hood *et al.* 1986, 1993), whereas strain LBA4404 was derived from less virulent strain Ach5 (Hoekema *et al.*, 1983).

Optical density

Bacterial suspensions are grown to the optimal optical density (OD_{600}), which allows for efficient plant transformation. Normally, OD_{600} between 0.5-1.0 is used for inoculating plant material (Dai *et al.*, 2001; Kim *et al.*, 2004; Bae *et al.*, 2005). However, in some studies an OD_{600} of more than one was used. Sriskandarajah *et al* (2004) used *Agrobacterium* suspension without dilution (OD_{600}=2-2.5) for inoculating cotyledon and hypocotyl explants of *Campanula carpatica*. They pointed out the need to have ample bacteria during explant co-cultivation. However, bacterial suspensions grown to an OD_{600} less than 0.5 have also been reported (Chen and Punja, 2002).

***vir* genes inducers**

Many proteins encoded by *vir* genes play essential roles in the *Agrobacterium*-mediated transformation process. Acetosyringone, a phenolic compound secreted by wounded plant cells, is known as a factor inducing the *vir* genes of *Agrobacterium* (Stachel *et al.*, 1985). Acetosyringone, at a temperature below 28 ºC, sucrose and acidic pH were found to influence the expression of *virG* and *virD* in the *vir* region of Ti plasmids (Alt-Mörbe *et al.*, 1989).

Gruchala *et al.* (2004) reported an increased number of transgenic shoots of strawberry plants when *A. tumefaciens* was incubated in LB or MS medium with acetosyringone and IAA. However, some studies showed inhibitory (Orlikowska *et al.*, 1995) or no effect (Miguel and Oliveira, 1999) of acetosyringone on plant transformation efficiency. Nadolska-Orczyk and Orczyk (2000) reported that acetosyringone had no apparent influence on the transformation efficiency from pea cotyledons with the hypervirulent strain EHA. It did, however, affect transformation efficiency when the moderately virulent strain C58C1 was used.

Recently, it has been demonstrated that *Agrobacterium* strains engineered with additional *virE/ virG* genes could be used to improve the transformation efficiency in rose (Kim *et al*., 2004). Similar results were obtained in rice, where the addition of *vir* gene(s) significantly improved transformation, and in particular, the addition of *virGwt* was the most beneficial, doubling the overall performance of the pGreen/ pSoup vector system (Vain *et al*., 2004).

Co-cultivation duration

After inoculation, explants are incubated on the appropriate solid medium for 48-72 hours to allow for DNA transfer and integration. However, the duration for co-cultivation that results in efficient transformation varies between different plant species.

Explants are normally co-cultivated with *Agrobacterium* for periods ranging from 2 (Cho *et al*., 2001) to 7 days (Jia *et al*., 1989). In blueberry, 2 days of co-cultivating explants with *Agrobacterium* resulted in almost no visible GUS expression. At least 3 or 4 days of co-cultivation was required for efficient transformation of *gusAint* gene into leaf explants (Cao *et al*., 1998).

Preconditioning of explants

Preconditioning the explants before exposure to bacteria helps in inhibiting damage (necrosis) and increases the transformation efficiency. Improvement in transformation frequency upon reconditioning of the explants has been reported in sugarbeet (Jacq *et al*., 1993); *Populus nigra* (Confalonieri *et al*., 1994); tobacco (Sunilkumar *et al*., 1999); *Brassica napus* (Cardoza and Stewart, 2003) and *Campanula carpatica* (Sriskandarajah *et al*., 2004).

This improvement in transformation efficiency as a result of preconditioning can be attributed to the active cell division (Sangwan *et al*., 1992), which contributed to the preparation of cells for active transfer of genes and also for the subsequent regeneration (Sriskandarajah *et al*., 2004).

Preconditioning is done by pre-treatment of the explants for certain durations prior to inoculation and co-cultivation. This is done by growing explants on media under environmental conditions that are genotype-dependent. Cardoza and Stewart (2003) defined those conditions for *Brassica napus* as preconditioning of hypocotyls for 72 h on MS medium supplemented with 1 mg/l 2,4-D and 30 g/l sucrose. Under a different set of conditions, preconditioning in *Campanula carpatica* was done by growing seedlings in the dark (Sriskandarajah *et al*., 2004). Those seedlings were etiolated and pale, and served as the source of explants that resulted in increased transformation efficiency.

In this study, success was demonstrated as the first report in obtaining transgenic *Rhipsalidopsis* shoots for the reporter *uidA* gene and the selectable marker *nptII* gene.

3.2 Materials and Methods

3.2.1 Plant material

Mother plants of *S. truncata* cv. Alex and *R. gaertneri* cv. CB5 were grown in 0.51 (10 cm square) pots in a greenhouse with 16 h light (provided by SON-T sodium lamps during winter months, 600 μmol m^{-2} s^{-1} at plant surface) at 25-28 ºC. The plants were watered without fertilizer, at the base without wetting the foliage, which reduced problems during surface sterilisation. When the experiments started, the plants were about 1-year-old with 3-5 tiers of mature phylloclades.

3.2.2 Establishment phase

Phylloclade explants were surface-sterilised in 1.5% active chlorine from sodium hypochlorite with 0.02% Tween 20 for 15 min. The explants were then thoroughly washed five times with sterile water. After the removal of chlorine-damaged areas, each phylloclade was cut through the midrib into 2-3 pieces and grown in a medium consisting of MS salts, Staba vitamins, 22.7 μM TDZ and 1.3 μM NAA, 3% w/v sucrose and gelled with 3 g l^{-1} gelrite (Maintenance medium). All the plant growth regulators were dissolved in DMSO to make stock solutions and then added to the medium after autoclaving.

Callus developed on explants from cv. CB5 was sub-cultured onto fresh maintenance medium approximately every two months over a period of nine to twelve months. The callus from cv. Alex was sub-cultured over a period of seven months. All of the cultures, unless otherwise stated, were incubated in light in a growth room (17-h photoperiod of 66 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes) at 25-28 ºC.

3.2.3 Transformation studies

Tolerance of *Schlumbergera* and *Rhipsalidopsis* to kanamycin

The kanamycin sensitivity of *Schlumbergera* and *Rhipsalidopsis* was tested using callus and phylloclade explants. Non-transformed callus explants from *Schlumbergera* cv. Alex and *Rhipsalidopsis* cv. CB5 were cultured on maintenance medium containing varying levels of kanamycin (0, 50, 100, 200 and 500 mg/l). Each petri dish was divided into two sections and five callus explants from each cultivar were placed on the medium in each section. Similarly, non-transformed *in vitro* phylloclades from *Rhipsalidopsis* cv. CB5 were cut into small pieces and six phylloclade explants were grown on MS medium containing 26.6 μM BA, 27.2 μM TDZ, 27.4 μM zeatin, 3% w/v sucrose and 3 g l^{-1} gelrite (B6T6Z6). This medium contained various levels of kanamycin (0, 50, 100, 200, and 500 mg/l). Both callus explants and phylloclade explants were marked at the bottom of each petri dish to monitor growth as formation of new callus or adventitious shoots. Five replicates were used for each experiment.

Bacterial strains and plasmids

Two *A. tumefaciens* strains were used in this study: strain AGL0, harbouring the plasmid pBEO210, which contains the *etr1-1* gene under the control of the flower specific promoter *fbp1*, and the *nptII* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter. This plasmid was obtained from the University of Wageningen. Strain LBA4404, harbouring both a helper plasmid pBBRlMCS containing a mutant *VirG* gene (van der Fits *et al.*, 2000), and plasmid pBI121 containing the genes *nptII* and *uidA* (without intron), both under the control of the CaMV 35S promoter, was obtained from Leiden University, The Netherlands.

Inoculation

Bacterial suspensions were prepared using single colonies of bacteria in YEP medium (10 g/l bacto peptone, 5 g/l Nacl and 10 g/l yeast extract) and cultured overnight at 28 ºC on a rotary shaker at 120 rpm. For cv. CB5, these suspensions were used for

inoculation at different OD_{600} (0.4, 0.5-0.6, and without dilution). For the inoculation with the bacterial strain AGL0, suspensions were tested further at lower OD_{600} (0.1-0.3). However, for cv. Alex, bacterial suspensions were used at OD_{600} (0.5-0.6) with the bacterial strain AGL0 and at OD_{600} (0.6-0.7) with the bacterial strain LBA4404. Twenty to twenty-five callus explants were added to the flasks containing the bacterial suspension. The flasks were then gently shaken at 25 rpm for 20 min at 28 ºC. In a separate experiment the pre-incubation of bacterial cells with acetosyringone was studied. Single colonies of bacteria were cultured overnight in YEP medium to OD_{600} (0.8-1.0), then the bacterial suspension was diluted using SIM medium (10 g/l bacto peptone, 5 g/l Nacl, 10 g/l yeast extract, 0.5 mM acetosyringone, 20 mM tri sodium citrate, pH 5.5) to OD_{600} (0.6). Acetosyringone was dissolved in DMSO to make a stock solution. The bacterial suspension was grown once again overnight to OD_{600} (1.0) then diluted with SIM solution to OD_{600} (0.6). Explants were then planted on co-cultivation medium.

Co-cultivation

Inoculated callus explants were transferred to MS-based medium supplemented with 0.1 g l^{-1} myo-inositol, kinetin at 4.7 μM for cv. CB5 or 7.0 μM for cv. Alex, 3% w/v sucrose and 3 g l^{-1} gelrite (MSCo medium). The medium had acetosyringone added at 15 mg/l. Six to eight explants were placed on the surface of the medium in petri dishes, and incubated for 24 hours (under light and temperature conditions described in the growth room).

Post co-cultivation treatment

After co-cultivation, the explants were washed thoroughly four times with distilled water and two times (five minutes each) with 500 mg/l cefotaxime. Twenty to twenty-five callus pieces were transferred to 40 ml of selection liquid medium in Erlenmeyer flasks (250 ml). The medium used was based on MS salts supplemented with 0.1 g l^{-1} myo-inositol, kinetin at 4.7 μM for cv. CB5 or 7.0 μM for cv. Alex and 3% w/v sucrose, 600 mg/l kanamycine and 300 or 500 mg/l cefotaxime (MSI medium). Control medium lacked kanamycin but contained cefotaxime (10 explants per flask). Flasks were shaken at 120 rpm using a rotary shaker and incubated at 27-29 ºC under

a light intensity of 4 μmol m^{-2} s^{-1} with 12 h-photoperiod. Kanamycin and cefotaxime were dissolved in water to make the needed stock solutions.

After 30 days (for cv. CB5) or 50 days (for cv.Alex), callus explants were transferred to MS-based medium supplemented with 0.62 μM Daminozide, 3% w/v sucrose, 3 g l^{-1} gelrite, (250, 400 or 600 mg/l) kanamycin and 300 mg/l cefotaxime (MSII medium) (6-8 explants per petri dish). Control medium lacked kanamycin. While still on selective medium, some of the callus explants of cv. CB5 formed new yellow-reddish callus. The newly formed callus was tested for *Gus* expression, and some of the putative transgenic callus lines were transferred to the same fresh selective medium (MSII). The remainder of the transgenic calli was sub-cultured on maintenance medium supplemented with 600 mg/l kanamycin and 300 mg/l cefotaxime. This aimed to multiply the putative transgenic calli to be utilized in the following experiment to recover transformed shoots.

Recovery of transformed shoots

This last study was done only in the cv. CB5, to encourage regeneration and the production of transgenic shoots from the transgenic callus. For this purpose, the previously described system utilising MSI, and later MSII media was modified.

Explants from two independent putative transgenic callus lines were used. Four to six callus pieces from each putative callus source were transferred to 40 ml of MSIII liquid medium in Erlenmeyer flasks (250 ml). MSIII medium was based on MS salts supplemented with 0.1 g l^{-1}myo-inositol, 4.7 μM kinetin, 3% w/v sucrose, 500 mg/l cefotaxime and (0 or 500 mg/l kanamycin). Flasks were shaken at 120 rpm using a rotary shaker and incubated at 27-29 ºC under a light intensity of 4 μmol m^{-2} s^{-1} with 12 h-photoperiod.

After 30 days, callus explants were transferred from MSIII medium to MSIV medium. This medium was MS-based medium supplemented with 0.62 μM daminozide, 3% w/v sucrose, 3 g l^{-1} gelrite, (0, 100, 300 or 500 mg/l) kanamycin and 300 mg/l cefotaxime. Four callus pieces from each treatment were placed on the surface of the

different media in plastic containers and grown further. These callus explants were left on MSIV medium for 4.5 months without being transferred to fresh media.

β-Glucuronidase assay

GUS activity was assessed histochemically, using calli and adventitious shoots from both control and transformed cultures. Plant tissues were placed under vacuum in fixative (0.3% formaldehyde, 10 mM MES pH 5.6, 0.3 M mannitol) for 2 minutes. After samples were left 4-5 minutes at room temperature, they were washed three times in phosphate buffer. Phosphate buffer was prepared by adding 11.54 ml of 0.5 M Na2HPO4 combined with 21.15 ml of 0.2 M NaH2PO4. Then water was added to a final volume of 200 ml at pH 7.0. Shoots and calli were incubated in 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronid$e$ (X-Gluc) in phosphate buffer at 37 ºC for two to four hours. Stained tissues were washed with 70% ethanol before being examined under the microscope.

NPTII-ELISA assay

Tissues from a total of 9 independent putative transformed callus lines and 3 control non-transformed calli (two replicates each) were evaluated by ELISA for the expression of the *nptII* gene (NPTII-ELISA kit from Agdia, Elkhart, Indiana, USA). Positive controls included those provided with the kit at three dilutions (1:3, 1:10 and 1:30) along with bacterial strain AGL0. Tissues were homogenised in 100 μl of the buffer provided with the ELISA kit and the supernatant was used for the NPTII-ELISA assay according to the manufacturer's protocol. The plate containing samples was read at 450 nm. The protein content in some of the samples was measured according to the method of Bradford (1976).

Genomic DNA extraction and Southern analysis

Total genomic DNA was isolated from control and kanamycin-resistant calli using the DNeasy Plant Mini kit (Qiagen, UK) with modifications. From 800 to 1000 mg of plant material were weighed for each sample. About 115 mg of PVPP was added while grinding plant material under liquid nitrogen to a fine powder. In the first step

of DNA preparation, the amounts of AP1 buffer and RNase A stock solution were increased six-fold and 180 μl 50% w/v PEG 20,000 was added to the mixture before vortexing. Similarly, the amount of AP2 in the later steps was increased six-fold. Other steps of the protocol were followed as mentioned in the handbook of the kit.

Ten μg of the genomic DNA from the control and one of the putative transgenic callus lines was digested at 37 ºC overnight with *Hind*III, (*Hind*III and *Eco*R1), and (*Hind*III and *Xba*1). One putative transgenic callus line was digested only with *Hind*III. These enzymes cut once in the T-DNA region, but outside the *nptII* region (Figure 15). This allowed estimating the number of *nptII* gene copies. DNA fragments were then separated by agarose gel electrophoresis (0.8% Seakem GTG) at 30 volts overnight and transferred to a Hybond N membrane (Amersham, UK) using 10x SSC (3M NaCl, 0.3M Na$_3$Citrate) according to standard procedures (Sambrook *et al*., 1989).

Filters were pre-hybridized at 60 ºC in hybridization buffer (Church buffer; 0.25 *M* sodium phosphate buffer pH 7.2, 1m*M* EDTA, 1% BSA and 7% SDS) for 2 h. The DNA probe used for hybridization was a 0.7 kb fragment of the *nptII* gene amplified by PCR from the plasmid pBEO210 described above. The DNA fragment for the *nptII* gene was labelled with ^{32}P-dCTP using the Megaprime kit (Amersham, UK) and hybridization was performed overnight at 65 ºC. The filter was then washed in 2xSSC, 0.1% SDS, 1xSSC, 0.1% SDS and finally 0.1xSSC, 0.1% SDS, for 2 h in each solution. The membrane was exposed to X-ray film at −70 ºC for 1 week before developing.

With the exception of NPTII-ELISA assay which was done only once, each experiment or analysis was repeated at least twice.

(A)

SalI *Bam*H I *Eco*R I   *Eco*RI ClA1

| LB | Pfbp 1 | | *etr1-1* | Tnos | RB |

(B)

RB   LB

Sph I NOS P *nptII* NOS T CaMV 35S P B-glucuronidase NOS T

Pst I *Sph* I   *Eco*R1

Hind III *Pst* I *Xba* I *Sma* I

Sph I   *Bam*H I
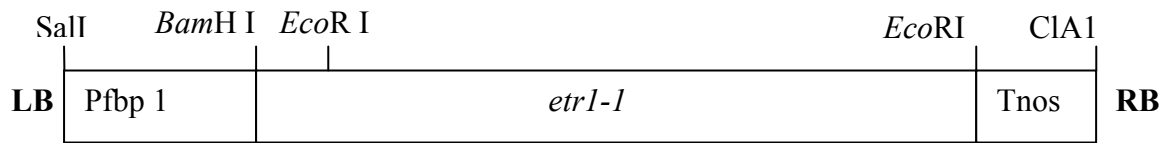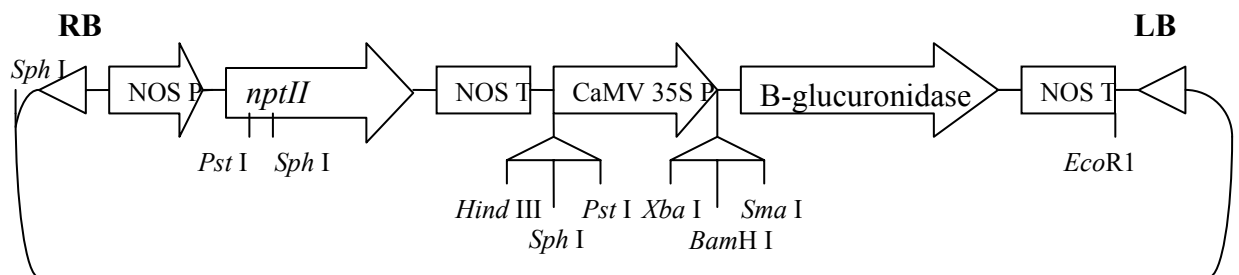
Figure 15. T-DNA constructs used for *Schlumbergera* and *Rhipsalidopsis* transformation. (A) *Sal*I / *Cla*1 insert of plasmid pBEO210. (B) Plasmid construct pBI121. LB, T-DNA left border; RB, T-DNA right border; Pfbp 1, *Petunia hybrida FBP1* promoter; *etr1-1*, *Arabidopsis thaliana etr1-1* allele; Tnos, polyadenylation region of the *A. tumefaciens* nopaline synthase gene; NOS P, Nopaline synthase gene promoter; *nptII*, neomycin phosphotransferase gene; NOS T, nopaline synthase gene terminator; CaMV 35S P, cauliflower mosaic virus 35S promoter; *β*-glucuronidase, coding region of the *β*-glucuronidase gene.

3.3 Results

Tolerance of *Schlumbergera* and *Rhipsalidopsis* to kanamycin

The sensitivity of *Schlumbergera and Rhipsalidopsis* callus and phylloclade explants to kanamycin was established prior to transformation experiments in order to determine the effective concentration for selecting transformed cells.

After one month, callus explants increased in size on the medium with no selection. The presence of kanamycin at 50 and 100 mg/l resulted in increased callus growth. However, the colour of the callus was pale green compared to the darker green callus grown with no selection. Callus growth decreased when kanamycin concentration increased to 200 mg/l. Callus from both cultivars stopped growing on medium containing kanamycin at 500 mg/l (Figure 16 a).

Similarly, non-transformed phylloclade explants had overgrown, forming new callus on wound edges and showed axillary bud growth on media containing 0, 50 and 100 mg/l kanamycin (Figure 16 b). This response was much reduced with 200 mg/l kanamycin, and in the presence of 500 mg/l kanamycin, phylloclade explants showed no growth signs. Two weeks later, phylloclade and callus explants showed necrosis and browning on media containing 500 mg/l kanamycin. After three months from the start of the experiment, both callus and phylloclade explants turned pale yellow on media containing 500 mg/l kanamycin.

From the results of this experiment, kanamycin at 600 mg/l was chosen to select transformed cells.

Transformation

One-hundred sixty callus explants from cv. Alex inoculated with either bacterial strain (80 callus explants inoculated with each strain) failed to produce any transformed calli or shoots.
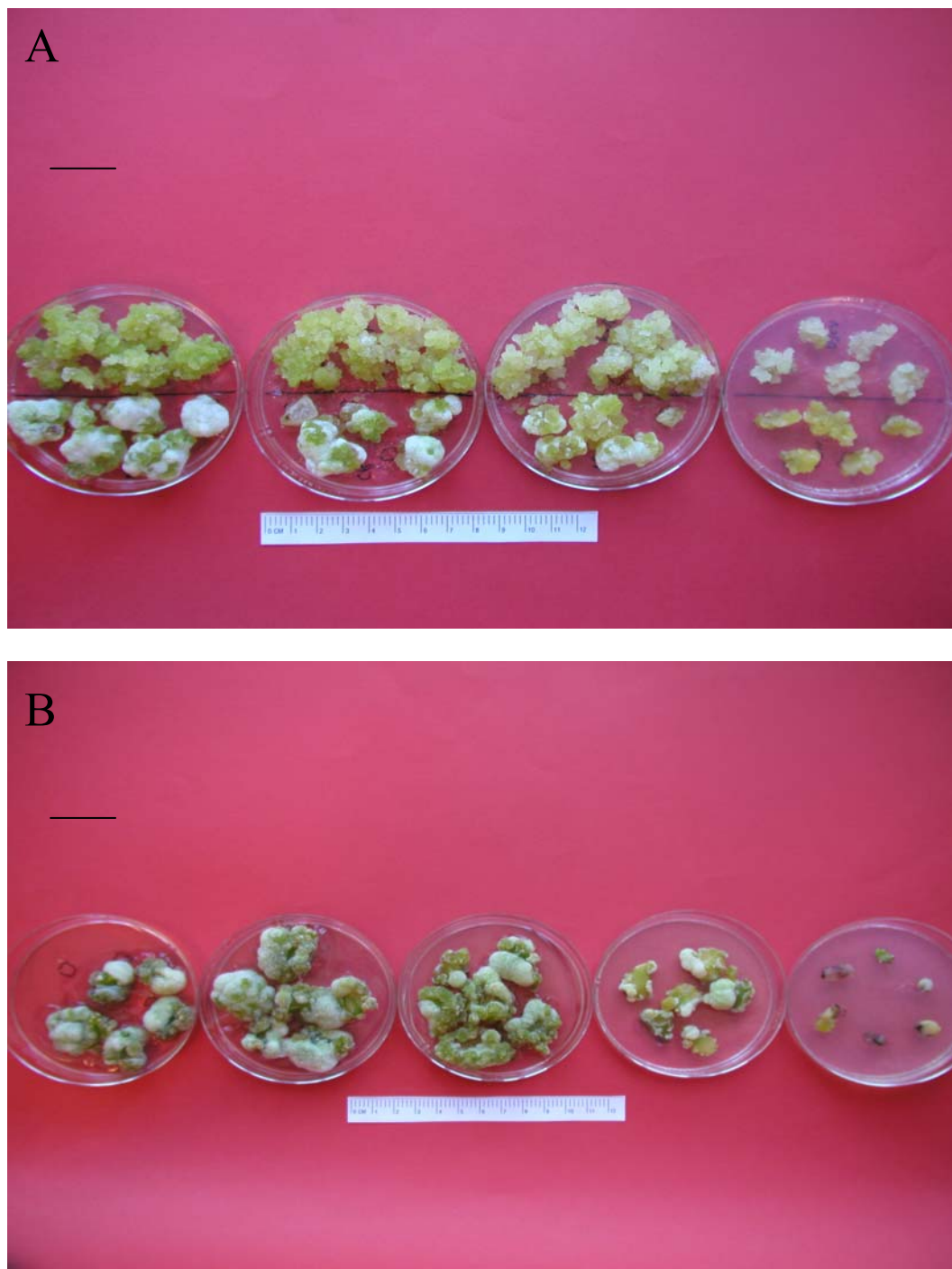
Figure 16. Tolerance of *Schlumbergera* and *Rhipsalidopsis* to kanamycin. **(A)** Non-transformed callus from cultivars Alex (petri dish; upper half portion) and CB5 (petri dish; lower half portion) grown on maintenance medium containing kanamycin (50, 100, 200, 500 mg/l; from left to right, respectively) *Bar*: 2.6 cm. **(B)** *in vitro* phylloclades from cultivar CB5 grown on B6T6Z6 containing kanamycin (0, 50, 100, 200, and 500 mg/l; from left to right, respectively). *Bar*: 3.2 cm.

By the time those calli were transferred from MSI medium with or without selection, they were brown and did not survive later on the following media. The *Agrobacterium* strain AGL0, containing the *etr1-1* gene was ineffective in producing transformed calli or shoots when used to inoculate callus explants from the cv. CB5 under the conditions employed in these experiments. A total of 100 callus pieces inoculated with this strain at OD_{600} (0.1-0.4) and 50 callus pieces inoculated at OD_{600} (0.6-0.9), only produced one new callus piece under selection. However, this callus piece did not grow further when transferred to maintenance medium containing 600mg/l kanamycin. On the contrary, it deteriorated and turned brown. In control treatments, without kanamycin, callus explants were overgrown after 3 months on MSII medium, with many shoot already formed.

However, transformed calli and shoots were later achieved when callus explants of cv. CB5 were inoculated with the bacterial strain LBA4404. Therefore, the next results will be dealing with the aspects for this transformation success.

From preliminary experiments, it was found that it is necessary after the co-cultivation stage to wash callus explants four times with sterilized distilled water and two times (5 minutes for each) with 500 mg/l cefotaxime. This is to eliminate bacterial re-growth in subsequent steps. The maintenance of selection pressure at 600 mg/l of kanamycin in MSI and MSII media, after co-cultivation, was also necessary to produce transgenic calli (Table 7).

Histochemical GUS assay

Twenty callus pieces and 17 shoots collected from different transformation events and tested for GUS expression, were all found to be positive (Table 7 & 8). Those transgenic tissues showed blue staining, indicating the presence of GUS activity (Figure 17 I & II). No GUS activity was detected in non-transformed control calli and shoots (Figure 17 I & II).

Table 7. Transformation of *Rhipsalidopsis* cv. CB5 calli infected with *A. tumefaciens* strain LBA4404.

OD [a]	Total No. of callus explants inoculated	First medium	Co-cultivation Time (days)	Second medium	Culture time (days)	Third medium	No. of regenerated calli	No. of GUS positive calli	Frequency of GUS positive calli (%) [b]
0.6-0.7 [c]	120	MSCo	1	MSI (500 mg/l cef [d])	30	MSII (600 mg/l kan [e])	13	10	76.9
0.5-0.6	44	MSCo	1	MSI (500 mg/l cef)	30	MSII (600 mg/l kan)	3	2	66.7
	6	MSCo	1	MSI (500 mg/l cef)	30	MSII (250 mg/l kan)	0	0	0
0.5	20	MSCo	1	MSI (300 mg/l cef)	30	MSII (600 mg/l kan)	0	0	0
0.4	86	MSCo	1	MSI (300 mg/l cef)	30	MSII (600 mg/l kan)	11	8	72.7
	24	MSCo	1	MSI (300 mg/l cef)	30	MSII (400 mg/l kan)	2	0	0

[a] Optical density

[b] Number of GUS-positive calli per total number of regenerated calli x 100

[c] Experiment with SIM medium

[d] Cef-cefotaxime

[e] Kan-kanamycin

Table 8. Transformation efficiency of *Rhipsalidopsis* cv. CB5. Callus explants were co-cultivated with *A. tumefaciens* strain LBA4404.

First stage of transformation		Recovery of transformed shoots										
OD [a]	Total No. of callus explants inoculated	Independent putative transgenic callus lines	First medium	Second medium	Total No. of transgenic callus explants	No. of callus explants forming shoots	Frequency of callus explants forming shoots [b] (%)	Total No. of shoots produced	No. of GUS positive shoots	TE1 (%) [c]	TE2 (%) [d]
0.5-0.6	44	1	MSIII (+500 mg/l kan)	MSIV (- kan [e])	52	20	38.5	192	10	22.7	19.2
				MSIV (100 mg/l kan)	40	12	30	38	0	0	0
				MSIV (300 mg/l kan)	44	7	15.9	42	2	4.5	4.5
				MSIV (500 mg/l kan)	32	2	6.3	5	2	4.5	6.3
			MSIII (-kan)	MSIV (- kan)	56	29	51.8	212	3	6.8	5.4
0.5-0.6	44	2	MSIII (-kan)	MSIV (- kan)	16	4	25	46	0	0	0

(a) OD-Optical density

(b) Number of transgenic callus explants forming shoots as a percentage of the total number of transgenic callus explants x 100

(c) TE1-Transformation efficiency1: Number of GUS-positive shoots per total number of callus explants inoculated x 100

(d) TE2-Transformation efficiency2: Number of GUS-positive shoots per total number of transgenic callus explants x 100

(e) – Kan- Absence of kanamycin
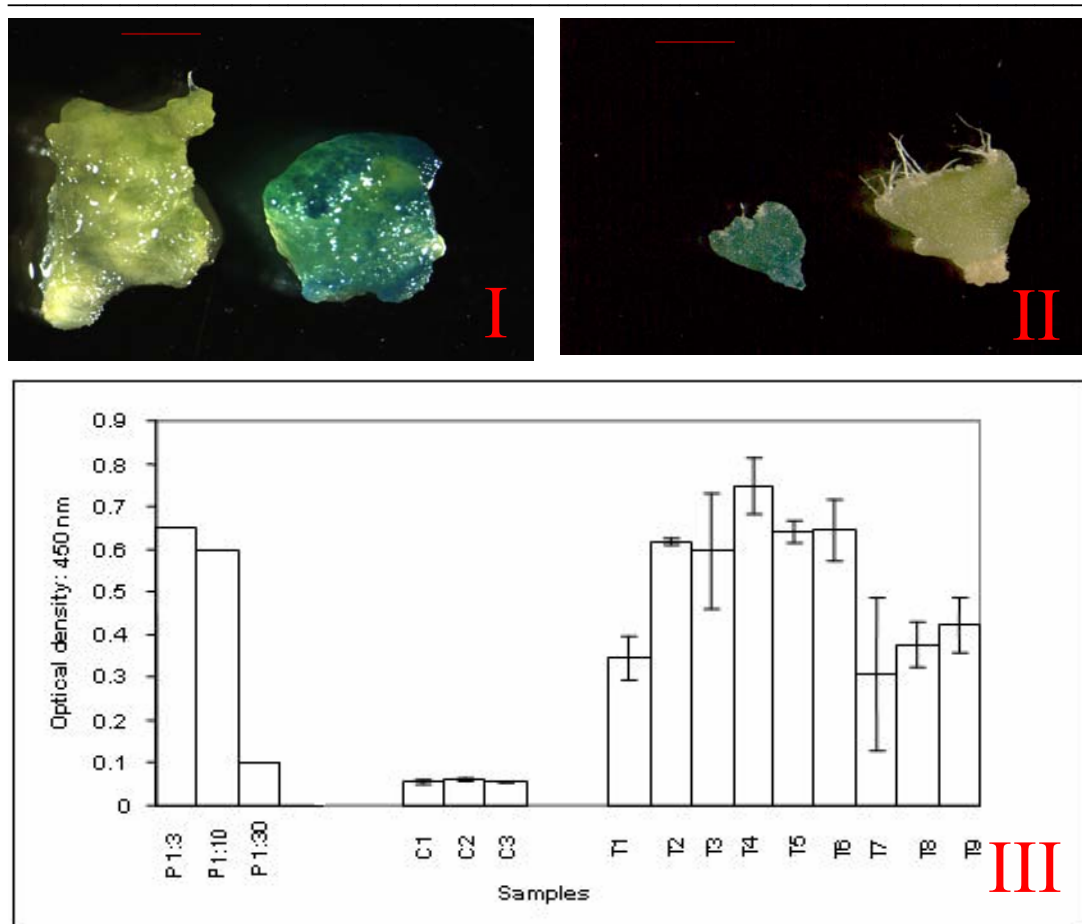
Figure. 17 Transformation of *Rhipsalidopsis* cv. CB5 (**I**) Histochemical GUS assay: transgenic (blue staining; right) and non-transgenic (no activity; left) calli. (*Bar*: 1.4 mm) (**II**) Transgenic (left) and non-transgenic (right) shoots (*Bar*: 1.4 mm) (**III**) OD values obtained by ELISA reader at 450 nm for the immuno-detection of NPTII transgene expression of *Rhipsalidopsis* callus lines. *T1-T9* Independent transformed callus lines; *C1-C3* Non-transformed callus lines; *P* Positive controls provided with the kit at three dilutions (1:3, 1:10, 1:30).

Effect of bacterial cell density (OD$_{600}$) and acetosyringone on transformation

Eighty callus explants inoculated with *A. tumefaciens* suspension (without dilution) failed to produce shoots or any callus growth. However, when a total of 150 callus explants were inoculated in a bacterial suspension with OD$_{600}$ (0.4-0.6), the frequency of GUS positive calli (%) ranged from 67-73%. This occurred with selection pressure maintained at 600 mg/l of kanamycin (Table 7). The frequency of GUS positive calli

rose to 77% at OD_{600} (0.6-0.7), when bacterial cells were pre-incubated with acetosyringone (Table 7).

In those experiments, after one month of growth in liquid MSI medium supplemented with kanamycin at 600 mg/l, callus explants were completely dark or dark-reddish. In control medium, lacking kanamycin, explants were reddish-green and overgrown. After 3 months, on MSII medium with selection, some of the callus explants formed new yellow-reddish callus. However, when these putative transgenic callus lines were transferred to the same fresh selective medium, they failed to produce shoots. At the same time, callus explants grown without selection were overgrown with many shoots.

Recovery of transgenic shoots

Callus explants multiplied from two independent putative transgenic callus lines were maintained under selection pressure for five months. To regenerate transgenic shoots, it was necessary to compromise between regeneration potential and selection pressure. For this purpose, the presence or absence of kanamycin in MSIII and MSIV media, together with testing the increase in kanamycin concentration (100, 300 and 500 mg/l) in MSIV on regeneration potential were investigated.

For the explants from the first putative transgenic callus line, it could be seen that the absence of kanamycin from both MSIII and MSIV media produced the highest frequency of callus explants that formed shoots (52%). These treatment combinations also produced the highest number of shoots (212) (Table 8). When kanamycin was present in MSIII medium, followed by an increase in kanamycin in MSIV (0, 100, 300 and 500 mg/l) regeneration of adventitious shoots decreased as presented by the decrease in the frequency of callus explants that formed shoots (38.5, 30, 15.9 and 6.3%, respectively) (Table 8). It appeared that the presence of kanamycin in MSIII had balanced out its absence in MSIV, which in turn, resulted in the highest TE1 and TE2 (22.7 and 19.2%) (Table 8). This view can be further understood in that the complete absence of kanamycin in both MSIII and MSIV media, although increased regeneration potential (52 and 25%; frequency of callus explants that formed shoots

for callus lines 1 and 2, respectively), resulted in low transformation efficiencies (TE1) (6.8 and 0%, respectively) (Table 8).

Enzyme-linked immunosorbent assay

The ability to detect NPTII by ELISA is based on the expression of the inserted *nptII* gene. Therefore, the detection of NPTII by ELISA confirmed the presence of the corresponding gene product inserted in the transformed calli. The nine putatively transgenic callus lines tested showed high expression of *nptII* (Figure 17 III). OD values from transgenic calli were 5-13 times higher than that of the control calli (Figure 17 III). Moreover, the calculated NPTII for some of the transgenic calli was 0.33-3.34, on the basis of nano-grams per milligram total protein, which is 3-30 times higher than that of control calli.

Southern blot analysis

Southern blot analysis of the genomic DNA from two randomly selected putative transgenic callus lines (both kanamycin resistant and GUS positive calli), confirmed the presence of the *nptII* gene in the plant genome. These calli, unlike the control, had the sequences that hybridized to the DNA fragment of the *nptII* gene (kan1 probe) (Figure 18). The DNA of the putative transgenic callus digested with *Hind*III alone or in combination with *Eco*R1 or *xba*1 showed multiple hybridization bands suggesting multiple integrations or multiple transformation events. Only one hybridization band was detected from the DNA from the other callus piece digested only with *Hind*III (Figure 18).
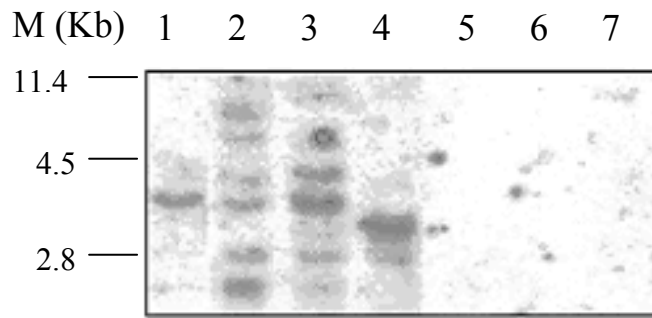
Figure 18. Southern analysis of genomic DNA of transgenic *Rhipsalidopsis* calli with kan1 probe M: *Pst*I digested λ DNA ladder. Lane 1: DNA from first putative transgenic callus digested only with *Hind*III. Lanes 2, 3 and 4: DNA from the second putative transgenic callus digested with *Hind*III, (*Hind*III and *Eco*R1), and (*Hind*III and *Xba*1), respectively. Lanes 5, 6 and 7: DNA from non-transformed control callus digested with *Hind*III, (*Hind*III and *Eco*R1), and (*Hind*III and *Xba*1), respectively.

3.4 Discussion

3.4.1 Methods

Like all the species in the cactus family, the absence of straightforward protocols in *Schlumbergera* and *Rhipsalidopsis* for molecular testing, has strongly hampered the progress in those plants. Isolation of nucleic acids for molecular gene expression analysis is extremely difficult due to the very high content of polysaccharides and polyphenols (De la Cruz *et al*., 1997). Available protocols are laborious and time consuming. Furthermore, those methods are based on repeated phenol/chloroform extractions and multiple ultra-centrifugations (Valderrama-cháirez *et al*., 2002).

As part of the development of a transformation protocol for *Schlumbergera* and *Rhipsalidopsis*, an easy and rapid method for the isolation of genomic DNA has been established. This method is based on commercially-available DNeasy Plant Mini Kit, and enables the rapid isolation of DNA for southern blot analysis from callus explants.

The protocol does not necessitate the addition of toxic β-mercaptoethanol (BME), although increased levels of BME have previously been suggested as a way of avoiding poly-phenol oxidation (De la Cruz *et al*., 1997; Lal *et al*., 2001).

When extracting DNA from phylloclades and calli, in preliminary experiments, the extract was highly viscous and gluelike because of the considerable amount of gummy polysaccharides. Such thick extracts were noticed in *Cinnamomum tenuipilum* (Zeng and Yang, 2002).

To avoid the precipitation of insoluble complexes, resulting in very poor yield, the volumes of AP1 and AP2 buffers were increased up to six-fold. This diluted the gummy polysaccharides and thus reduced the viscosity of the extract. The yield could be further increased by scaling up the extraction protocol and applying the increased volume to the same column in subsequent steps.

The combined application of PVPP and high molecular weight PEG (HMW PEG) seemed to be advantageous to increase the yield and quality of genomic DNA. HMW PEG was reported to bind phenolic and other compounds that may interfere with nucleic acids, thus allowing the increased yield of RNA (Gehrig *et al.*, 2000). Moreover, Salzman *et al.* (1999) observed a moderate effect of PVPP in preventing quantitative loss of RNA.

Applying the modification described herein, it was possible to extract sufficient genomic DNA for southern blotting from both *Schlumbergera* and *Rhipsalidopsis* using DNeasy Plant Mini Kit from Giagen.

Regarding ELISA assay, the background obtained in the test was a small negative value. Therefore, this value was taken as zero and the OD readings from ELISA reader were not changed for further calculations.

3.4.2 Results

This is the first successful report on the transformation of *R. gaertneri*. Transformed shoots were achieved when callus explants were inoculated with the bacterial strain LBA4404.

The results obtained by GUS expression, ELISA assay and Southern hybridization confirmed the presence of the *uidA* gene and *nptII* gene in the selected transgenic callus lines. Hybridization of the digested DNA of one of the transgenic callus lines to the DNA fragment of the *nptII* gene yielded multiple hybridization bands suggesting multiple integrations or multiple transformation events. Moreover, the formation of transgenic CB5 shoots was confirmed by GUS assay as shown by the dark blue histochemical stain in the shoots tested.

Some of the factors that had effects on the transformation efficiency and led to the formation of transformed shoots were optimised. It seems that *Agrobacterium* when used without dilution was deleterious for the callus explants and thus prevented any desirable response. On the contrast, using the bacterial suspension with an OD_{600}

between 0.4 and 0.7 resulted in the formation of transformed calli at a high frequency, and contributed to the eventual recovery of transformed shoots.

Similar to the methodology used in this study, a washing step of the explants after co-cultivation with cefotaxime for the elimination of *A. tumefaciens* was included in the transformation of sugarcane (Arencibia *et al*., 1998), rice (Dai *et al*., 2001), colonial bentgrass (Chai *et al*., 2004) and tomato (Sigareva *et al*., 2004).

The pre-incubation of bacterial cells in SIM medium containing acetosyringone raised the frequency of transgenic GUS positive *Rhipsalidopsis* calli. Acetosyringone is known as a factor inducing the *vir* genes of *Agrobacterium* (Stachel *et al*., 1985) and was reported to improve transformation efficiency in *Cucumis melo* (Bordas *et al*., 1997), *Glycine max* (Trick and Finer, 1997), *Craterostigma plantagineum* Hochst (Toldi *et al*., 2002), *Fragaria x ananassa* Duch (Gruchala *et al*., 2004).

The recovery of transformed shoots in this study was achieved through the manipulation of two factors. The first factor was the selection pressure applied. It was found that kanamycin at 500 mg/l was effective in allowing the growth of only transformed cells. However, the working concentration of kanamycin was raised a little higher to 600 mg/l to reduce the chance for escapes. Keeping a stringent selection pressure was necessary for the formation of transgenic calli.

An effective selection system should establish a good compromise between the regeneration capacity of the explants and the effectiveness of the selection applied (Araújo *et al*., 2004).

After a total of 9 months on media with high selection pressure using 600 mg/l kanamycin, it was necessary to determine the effect of reducing kanamycin level on the possible regeneration of shoots from the transgenic callus. Therefore, the adventitious shoot regeneration system composed of two steps (MSI and MSII media) established for cv. CB5 was repeated with modifications that included testing different levels of kanamycin (MSIII and MSIV media) on shoot regeneration potential. It was found that the presence of kanamycin at this high level was still necessary when transgenic callus was grown in MSIII. However, it seemed that this

prolonged incubation on media with high selection pressure allowed the complete removal of kanamycin in the final medium (MSIV), thus resulting in the highest transformation efficiency of 22.7%. It can be stated that a procedure based on a stringent selection pressure is required for recovery of transformed plants and thus reducing the possibility of escapes (Arencibia *et al*., 1998; Zhang *et al*., 2000; Araújo *et al*., 2004; Chai *et al*., 2004).

The second factor affecting the recovery of transformed shoots was the nutritional stress. The callus explants were grown on MSIV final medium for 4.5 months without changing or transfer to fresh new media. By this time, the media were depleted inside the containers and the callus pieces were overgrown and had formed shoots. This situation led to a nutritional and dehydration stress. This nutritional stress together with the removal of kanamycin after a prolonged incubation of the callus on media with a stringent selection pressure led to the stimulation of adventitious shoot formation.

Leelavathi *et al*. (2004) also reported that nutritional as well as dehydration stress applied at the selection stage for embryogenic cotton calli, induces direct development of transformed somatic embryos. This resulted in a high frequency of transformation.

As indicated by this study, transformation normally reduces regeneration efficiency and more time is needed for adventitious shoot formation. Many shoots formed after 3 months from callus grown on MSII medium without selection. By this time, however, only transgenic callus began forming in the presence of kanamycin selection. Formation of transgenic shoots occurred 6 months later when further modifications in the regeneration system were made. These results were confirmed in transformation studies on strawberry (Gruchala *et al*., 2004) and *Medicago truncatula* (Araújo *et al*., 2004). In these studies, regeneration of shoots (from strawberry) or somatic embryos (from *M. truncatula*) was faster and more prolific using the normal regeneration system without selection compared to *Agrobacterium*-mediated transformation.

In the present study, transformation efficiency showed a good consistency when calculated on the basis of the total number of the callus explants inoculated (TE1) or on the basis of the number of transgenic callus used in the final experiment (TE2).

Although transformed shoots were produced when callus explants of cv. CB5 were inoculated with the bacterial strain LBA4404, no success was obtained with the strain AGL0. Bae *et al.* (2005) reported that bacterial strains can vary in efficiency when used for transformation. In their study, *Agrobacterium* strain EHA105 harbouring pCAMBIA vector was more effective for the transformation of dandelion than the GV3101 strain harbouring pBI121.

In the present study, the control callus explants inoculated with bacterial strain AGL0, and then grown in MSI and MSII media without selection, were overgrown and formed shoots after about 3-4 months. This indicated that key variables in *Agrobacterium*-mediated gene transfer can be further optimised when using the bacterial strain AGL0. Those may include pH and the salt concentration in the infection medium, sugar type, use of acetsyringone, type and age of explant (using old or young phylloclades), and age of callus cultures used as explants.

However, for the purpose of introducing *etr1-1* gene into the genome of these cacti, other bacterial strains such as EHA101 and EHA105, which are supervirulent type strains (Hood *et al.* 1986, 1993), could be used. New vectors could also be constructed with more options for optimisation.

The morphogenic potential of callus from cv. Alex was considerably changed over time. Green callus was dominated by white, watery callus when maintained by sub-culturing on maintenance medium. This can explain the lack of response, even without selection, when such callus was used as explants for the *Agrobacterium*-mediated transformation. It could be concluded that this callus became less competent for transformation with a decreased number of actively dividing cells when the transformation experiments began.

The choice of suitable starting explants is important for genetic transformation (Toldi *et al.*, 2002; Chai *et al.*, 2004). Therefore, only vigorously growing and actively dividing cells, that are competent for agroinfection and are capable of regenerating into a whole plant, shall be used for transformation experiments (Arencibia *et al.*, 1998; Leelavathi *et al.*, 2004).

3.5 Conclusion

A transformation system mediated by *Agrobacterium* was established in *Rhipsalidopsis* cv. CB5 using *uidA* as a reporter gene and *nptII* gene as a selectable marker. The stringent selection from the beginning with 600 mg/l kanamycin, together with pre-incubation of bacteria in a SIM medium supplemented with acetosyringone were necessary to produce transgenic calli at a high frequency. The inclusion of a washing step with cefotaxime for the callus explants after co-culture aimed at removing the excess of *Agrobacterium* in subsequent steps. Both the nutritional stress with the removal of kanamycin in the final MSIV medium, had contributed to the eventual recovery of transformed shoots.

In this report, relatively high transformation efficiency (maximum 22.7%) was demonstrated. Therefore, it can be concluded that *Agrobacterium*-mediated transformation is a promising approach for these cacti.

Results from the present investigation confirm that creation of genetically-modified plants requires very precise studies on each genotype, step and factor influencing regeneration and transformation processes.

Summary

A protocol was developed for the production of somatic embryos in *Schlumbergera* cv. Russian Dancer (Figure 19). A highly efficient regeneration system through adventitious shoots in *Schlumbergera* cv. Alex and Rhipsalidopsis cv. CB5 was also demonstrated (Figure 19). Medium, plant growth regulators, duration of callus exposure to cytokinins and presence or absence of gauze were shown to affect morphogenesis in *Schlumbergera* and *Rhipsalidopsis*. The production of polyploidy plants from somatic embryos could be exploited further through somaclonal variation for the production of new plant genotypes for breeding purposes. These improved systems of regeneration in both plant types formed the basis for the subsequent transformation studies.

A transformation system mediated by *Agrobacterium* was established in *Rhipsalidopsis* cv. CB5 using *uidA* as a reporter gene and *nptII* gene as a selectable marker. Some of the factors that had effects on the transformation of *Rhipsalidopsis* and *Schlumbergera* were studied. The starting explants, selection system, bacterial strain, induction of *vir* genes by acetosyringone, the inclusion of a washing step with cefotaxime for callus explants after co-culture, and nutritional stress were found to affect transformation efficiency in these plants.

Summary

Embryogenesis
(cv. Russian Dancer)
Protocol

Organogenesis
(cv. Alex)
Protocol

Organogenesis
(cv. CB5)
Protocol

Callus induction
Establishment phase
Explant (Phylloclades)
1) Maintenance medium
(9-12 months)

Callus induction
Establishment phase
Explant (Phylloclades)
1) Maintenance medium
(7 months)

Callus induction
Establishment phase
Explant (Phylloclades)
1) Maintenance medium
(9-12 months)

2) MS+26.6 μM BA
+27.2 μM TDZ
+27.4 μM zeatin

(4.5 months)

Adventitious shoots
experiment
a) MS+7.0 μM kinetin

(50 days)

b) MSWH or MSDM

(5 months)

Adventitious shoots
(highest average number)

Adventitious shoots
experiment
a) MS+4.7 μM kinetin

(30 days)

b) MSWH or MSDM

(4 months)

Adventitious shoots
(highest average number)

Embryogenesis experiment
1) Induction of somatic embryos
a) SH+7.0 μM kinetin
(1 month)

b) MSWH
(2.5-3 months)

Appearance of
somatic embryos

(3.5 months)
Somatic embryos
Proliferation

2) Germination of embryos
G medium
3 months

3) Development of plantlets
B medium
3 weeks

4) Establishment of plants in

soil

Figure 19. Flow chart showing the protocols developed for the *in vitro* regeneration of *Schlumbergera* and *Rhipsalidopsis*

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Appendices

I) Media composition

Appendix 1. MS medium stocks and final recipe

A) Stocks

Macro Elements, 10x	g/l
KNO_3	19.00
NH_4NO_3	16.50
KH_2PO_4	1.70
$CaCl_2$	3.32
$MgSO_4.7H_2O$	3.70
Micro Elements, 1000x	g/l
$MnSO_4.1H_2O$	16.90
KI	0.83
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins, 1000x	g/100ml
Glycin	0.20
Thiamine HCl	0.01
Pyridoxine HCl	0.05
Nicotinic acid	0.05
Iron, 100x	g/100ml
$FeSO_4.7H_2O$	0.278
$Na_2EDTA.2H_2O$	0.372

B) MS medium Final recipe

MS medium	1 liter
Macro	100 ml
Micro	1 ml
Iron	10 ml
Vitamins	1 ml*
Sucrose	30 g
pH	5.7
Gelrite	3 g

* When Staba vitamins (100x stock solution) are used, 10 ml is added for 1 liter of MS medium

Appendices

Appendix 2. Staba vitamins (100x stock solution)

Staba vitamins	mg/l
Cyanocobalamin	0.15
Folic Acid	50.00
p-Aminobenzoic Acid	50.00
Riboflavin	50.00
Biotin	100.00
Choline Chloride	100.00
Thiamine HCl	100.00
Nicotinamide	200.00
Pyridoxine HCl	200.00

Appendix 3. SH medium recipe

Micro Elements	mg/l
$CoCl_2.6H_2O$	0.10
$CuSO_4.5H_2O$	0.20
FeNaEDTA	19.80
H_3BO_3	5.00
KI	1.00
$MnSO_4.H_2O$	10.00
$Na_2MoO_4.2H_2O$	0.10
$ZnSO_4.7H_2O$	1.00
Macro Elements	mg/l
$CaCl_2$	151.00
KNO_3	2500.00
$MgSO_4$	195.05
$(NH_4)H_2PO_4$	300.00
Vitamins	mg/l
myo-Inositol	1000.00
Nicotinic acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	5.00
Sucrose	30 g/l
pH	5.7
Gelrite	3 g/l

Appendices

II) Chemicals

Appendix 4. List of the main chemicals and their manufacturers used in this study.

Chemical	Company	Chemical	Company
Acetosyringone	Sigma/USA	Sodium citrate	Sigma/USA
BA	Duchefa/Netherlands	Sodium hypochlorite	Sigma/USA
Bacto peptone	Sigma/USA	Sucrose	Duchefa/Netherlands
Biotin	Duchefa/Netherlands	TDZ	Duchefa/Netherlands
Bradford reagent	Sigma/USA	Thiamine HCL	Duchefa/Netherlands
BSA	Sigma/USA	Toludine blue	Sigma/USA
Carmine	Sigma/USA	Tween 20	Duchefa/Netherlands
Cefotaxime	Sigma/USA	Ultraclear	Baker/Netherlands
Choline Chloride	Duchefa/Netherlands	X-Gluc	Sigma/USA
Daminozide	Sigma/USA	Yeast extract	Duchefa/Netherlands
DMSO	Duchefa/Netherlands	Zeatin	Duchefa/Netherlands
EDTA	Sigma/USA	2,4-D	Duchefa/Netherlands
Ethanol	Sigma/USA	4-CPA	Sigma/USA
Folic acid	Duchefa/Netherlands		
Formaldehyde	Sigma/USA		
Gelrite	Sigma/USA		
Glacial acetic acid	Sigma/USA		
Glucose	Duchefa/Netherlands		
Histochoice	Sigma/USA		
IAA	Duchefa/Netherlands		
Isopropanol	Sigma/USA		
Kanamycin	Sigma/USA		
Kinetin	Duchefa/Netherlands		
Mannitol	Duchefa/Netherlands		
MES	Duchefa/Netherlands		
Methanol	Sigma/USA		
Myo inositol	Duchefa/Netherlands		
NAA	Duchefa/Netherlands		
NaCl	Duchefa/Netherlands		
NaH2PO4	Sigma/USA		
Nicotinic acid	Duchefa/Netherlands		
p-Aminobenzoic acid	Sigma/USA		
p-Aminobenzoic acid	Sigma/USA		
Paraffin	Sigma/USA		
PEG 20 000	Sigma/USA		
PVPP	Sigma/USA		
Pyridoxol HCl	Duchefa/Netherlands		
Riboflavin	Duchefa/Netherlands		
SDS	Duchefa/Netherlands		

III) NPTII-ELISA assay

Appendix 5. Absorbance values for the standard solutions of BSA

Standard solutions of BSA (µg/ml)	Absorbance (595 nm)
0.00	0.000
0.10	0.358
0.25	0.359
0.50	0.363
1.00	0.365
2.50	0.407
5.00	0.482
10.00	0.590
20.00	0.904
40.00	1.199
60.00	1.363
80.00	1.471
100.00	1.534
120.00	1.591
140.00	1.631
160.00	1.676
180.00	1.698
200.00	1.726
220.00	1.718
240.00	1.762
260.00	1.788
280.00	1.81
300.00	1.836
360.00	1.879
380.00	1.922
400.00	1.904
440.00	1.942
460.00	1.962
480.00	1.979
500.00	1.981

Standard curve(total protein)

$$y = 0.001x + 1.4939$$
$$R^2 = 0.9658$$
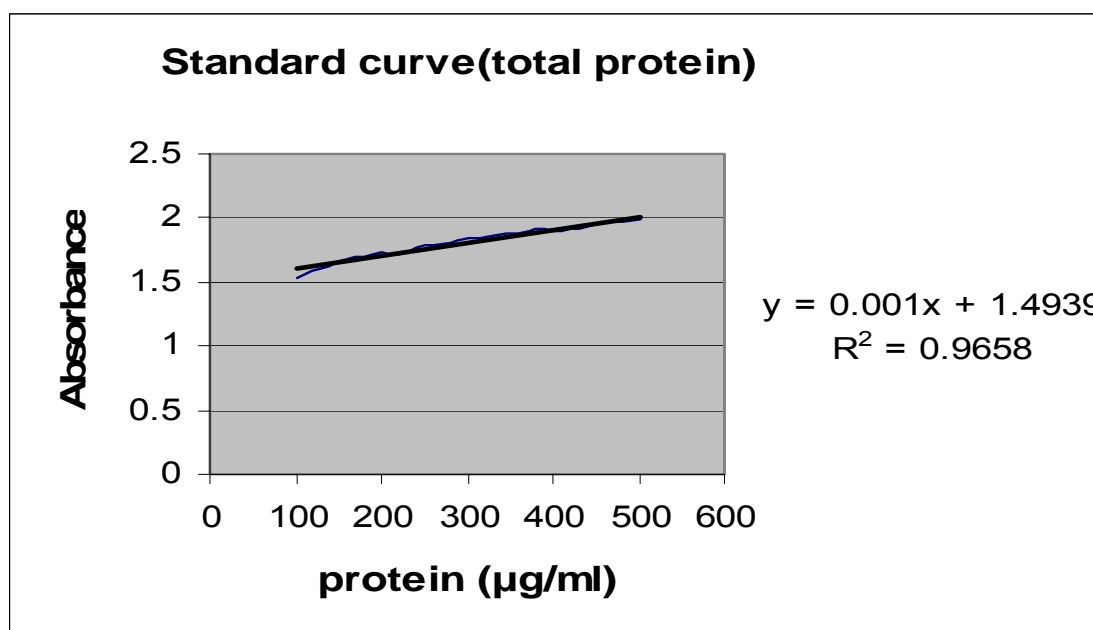
Appendix 6. Standard curve used to calculate total amount of protein in the transgenic and control calli.

Appendix 7. Total amount of protein in transformed and non-transformed calli measured according to the method of Bradford

Callus number	Absorbance (595 nm)	Total protein (µg/ml)	Total protein (mg/ml)
C1	1.717	223.1	0.2231
C2	1.942	448.1	0.4481
T2	1.873	379.1	0.3791
T3	1.941	447.1	0.4471
T4	1.874	380.1	0.3801
T6	1.871	377.1	0.3771
T7	1.909	415.1	0.4151
T9	1.885	391.1	0.3911

C: CB5 non-transgenic callus. T: CB5 transgenic callus; total protein was not calculated for T1, T5 and T8. Each value is the average of two replicates.
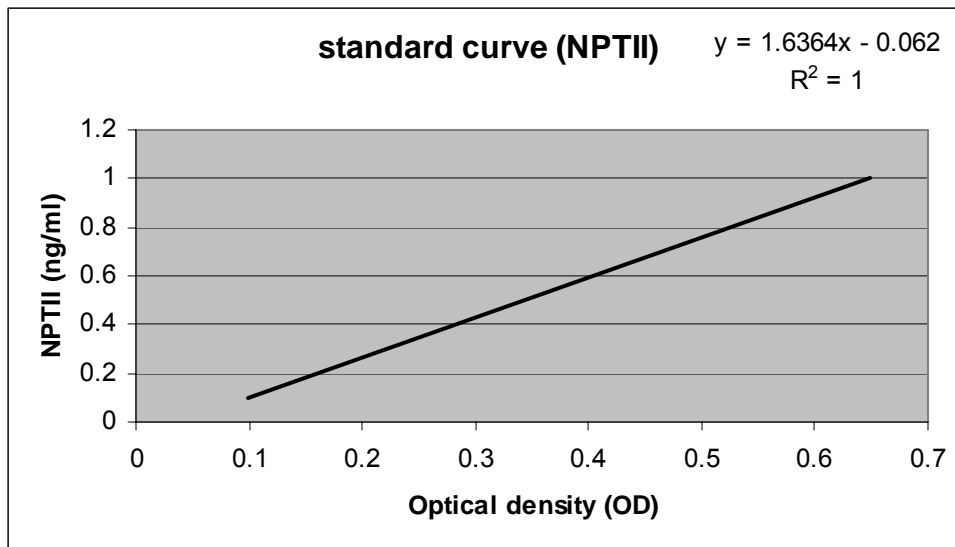
Appendix 8. Standard curve for the calculation of NPTII concentration (ng/ml) based on a known positive control concentration (3 ng/ml). OD values from diluted positive controls (1:3) and (1:30) were plotted versus the corresponding known concentration of 1 and 0.1 ng/ml, respectively.

Appendices

Appendix 9. Immunodetection of transgene expression by enzyme-linked immunosorbent assay (ELISA). Values in last column represent nanograms NPTII per milligram total protein in callus explants. Callus numbers: C1-C3 controls; T1-T9 independent transformants. P: positive controls (1:3 and 1:30). Nd: not determined

Callus number	OD (450 nm)	NPTII (ng/ml)	Total protein (mg /ml)	(ng NPTII)/ (mg total protein)
c11	0.051	0.02	0.2231	0.09
c12	0.06	0.04	0.2231	0.18
c21	0.058	0.03	0.4481	0.07
c22	0.063	0.04	0.4481	0.09
c31	0.054	0..03	Nd	Nd
c32	0.058	0.03	Nd	Nd
P 1:3	0.649	1	Nd	Nd
P 1:30	0.099	0.1	Nd	Nd
T1a	0.295	0.42	Nd	Nd
T1b	0.395	0.58	Nd	Nd
T2a	0.608	0.93	0.3791	2.45
T2b	0.627	0.96	0.3791	2.53
T3a	0.459	0.69	0.4471	1.54
T3b	0.729	1.13	0.4471	2.53
T4a	0.68	1.05	0.3801	2.76
T4b	0.813	1.27	0.3801	3.34
T5a	0.667	1.03	Nd	Nd
T5b	0.613	0.94	Nd	Nd
T6a	0.571	0.87	0.3771	2.31
T6b	0.716	1.11	0.3771	2.94
T7a	0.126	0.14	0.4151	0.33
T7b	0.485	0.73	Nd	Nd
T8a	0.428	0.64	Nd	Nd
T8b	0.325	0.47	Nd	Nd
T9a	0.484	0.73	Nd	Nd
T9b	0.358	0.52	0.3911	1.33

Appendices

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IV) Summary of ANOVA tables

Appendix 10. Effects of media and transfer dates on the average number of adventitious shoots of *S. truncata* cv. Alex

1. Shoot Scale 1

Source of variation	DF[1]	MS[2]	F value	Significance
Time	1	144.64	36.43	***
Treat	3	123.71	31.16	***
Treat*Time	3	12.45	3.14	*
Error	48	3.97		

2. Shoot Scale 2

Source of variation	DF	MS	F value	Significance
Time	1	1003.02	51.80	***
Treat	3	762.78	39.39	***
Treat*Time	3	107.92	5.57	**
Error	48	19.36		

3. Shoot Scale 3

Source of variation	DF	MS	F value	Significance
Time	1	315.88	57.25	***
Treat	3	129.49	23.47	***
Treat*Time	3	36.26	6.57	***
Error	48	5.52		

4. Total no. Shoots

Source of variation	DF	MS	F value	Significance
Time	1	3778.57	79.22	***
Treat	3	2412.05	50.57	***
Treat*Time	3	362.62	7.60	***
Error	48	47.70		

[1] DF: Degree of freedom

[2] MS: Mean square

*, **, *** Significant at $P \leq 0.05$, 0.01, or 0.001, respectively

Appendices

Appendix 11. Effects of media and transfer dates on the average number of adventitious shoots of *R. gaertneri* cv. CB5

1. Shoot Scale 1

Source of variation	DF[1]	MS[2]	F value	Significance
Time	1	26972.16	66.29	***
Treat	3	4924.40	12.10	***
Treat*Time	3	1645.64	4.04	*
Error	48	406.88		

2. Shoot Scale 2

Source of variation	DF	MS	F value	Significance
Time	1	188964.45	383.12	***
Treat	3	112133.45	227.35	***
Treat*Time	3	53443.88	108.36	***
Error	48	493.22		

3. Shoot Scale 3

Source of variation	DF	MS	F value	Significance
Time	1	5520.29	50.33	***
Treat	3	4971.07	45.32	***
Treat*Time	3	1995.57	18.19	***
Error	48	109.69		

4. Total shoot number

Source of variation	DF	MS	F value	Significance
Time	1	453240.07	343.23	***
Treat	3	218177.45	165.22	***
Treat*Time	3	84284.98	63.83	***
Error	48	1320.52		

[1] DF: Degree of freedom

[2] MS: Mean square

*, **, *** Significant at P ≤ 0.05, 0.01, or 0.001, respectively

Publications

Journals

Al-Ramamneh, E. A., Sriskandarajah, S. and Serek, M. 2006. Plant regeneration via somatic embryogenesis in *Schlumbergera truncata*. Plant Cell Tiss. Organ Cult., 84:333-342.

Al-Ramamneh, E. A. and Sander, L. 2005. Easy and rapid isolation of RNA from small samples of fresh cactus. Plant Molecular Biology Reporter (submitted).

Al-Ramamneh, E. A, Sriskandarajah, S. and Serek, M. 2005. *Agrobacterium tumefaciens*-mediated transformation of *Rhipsalidopsis* and *Schlumbergera*. Plant Cell Rep. (Submitted)

Abstracts

Golombek, S. and Al-Ramamneh, E. A. 2002. Drought tolerance mechanisms of pearl millet. Deutscher Tropentag, Witzenhausen, Germany.

Sriskandarajah, S., Al-ramamneh, E. and Serek, M. 2004. Enhanced regenerative capacity in *Rhipsalidopsis* and *Schlumbergera* explants by *in vitro* manipulation. 5th *in vitro* culture and Horticultural Breeding (IVCHB), Hungary, Debrecen, Acta Horticulture (in press).

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe sowie dass diese Arbeit noch nicht als Dissertation oder andere Prüfungsarbeit vorgelegt worden ist.

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