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ADVANCED BREEDING TECHNIQUES TO INDUCE VARIATION IN WOODY ORNAMENTALS

Thesis submitted in fulfilment of the requirements for the degree of
doctor (PhD) in Applied Biological Sciences.

Dutch translation of the title:

GEAVANCEERDE VEREDELINGSTECHNIEKEN VOOR HET INDUCEREN VAN VARIATIE IN HOUTACHTIGE SIERGEWASSEN

To refer to this thesis:

Denaeghel H. (2018) Advanced breeding techniques to induce variation in woody ornamentals. PhD. Thesis, Faculty of Bioscience Engineering, Ghent University.

ISBN: 978-94-6357-107-4

TABLE OF CONTENTS

SAMENVATTING

Constante productinnovaties zijn commercieel erg belangrijk voor siertelers, vooral in het marktsegment van de visueel aantrekkelijke planten. Gewenste nieuwigheden zijn trendgevoelig; belangrijke kenmerken zijn winterhardheid, biotische en abiotische stress tolerantie, bloemkleur en -vorm, enz. Naarmate de tuinen steeds kleiner worden, neemt ook de vraag naar compacte sierplanten toe. In deze studie werden daarom geavanceerde veredelingstechnieken geïmplementeerd voor houtachtige planten en geëvalueerd op hun vermogen om nieuwe variatie in het algemeen, en meer specifiek compactheid, te induceren. *Escallonia* en *Sarcococca* werden door BestSelect CVBA, een samenwerkingsverband van Vlaamse siertelers, geselecteerd als zijnde commercieel interessant. Deze geslachten zijn vrij onbekend en weinig of geen veredeling werd reeds toegepast. In dit proefschrift werden deze twee genera gebruikt als casestudies voor houtachtige siergewassen. Als eerste techniek werd interspecifieke hybridisatie toegepast op het genus *Sarcococca*. Hierbij werden moleculaire en cytogenetische methoden gebruikt om de compatibiliteit van ouderlijke soorten te evalueren en de hybride status van het verkregen nageslacht te bepalen. Ten tweede werden, als meer geavanceerde veredelingstechnieken, polyploïdisatie en co-cultivatie met rhizogene *Agrobacterium*-stammen toegepast om nieuwe genetische variatie te induceren. Tot nu toe zijn deze technieken onbenut voor houtachtige siergewassen, maar ze bieden vele mogelijkheden om nieuwe, interessante variatie te creëren voor de ontwikkeling van visueel aantrekkelijke en/of gezondere planten. Aangezien bij *Sarcococca* een trage *in vitro* vermeerdering voor een gebrek aan plantmateriaal zorgde, werden maar een beperkt aantal experimenten ingezet voor co-cultivatie met rhizogene *Agrobacterium* stammen. *Escallonia* werd gebruikt zowel voor polyploïdisatie als voor co-cultivatie met rhizogene *Agrobacterium* stammen.

Een collectie van 23 *Escallonia*-genotypen en 18 *Sarcococca*-genotypen werd morfologisch, cytogenetisch en fylogenetisch geanalyseerd. Eén *Escallonia*-genotype was tetraploïd, *E. pendula*, terwijl alle andere *Escallonia*-genotypen diploïd bleken. Veel natuurlijke *Escallonia*-hybriden zijn al beschreven, zelfs tussen ver verwante soorten. Het *Sarcococca*-geslacht omvatte zowel diploïden als tetraploïden, met een grote variatie in genoomgroottes. Gebaseerd op cytogenetische, fylogenetische en morfologische informatie, konden de *Sarcococca*-genotypen worden onderverdeeld in vijf clusters. Deze informatie was belangrijk om met succes een interspecifiek hybridisatieprogramma op te zetten voor *Sarcococca*. Hierbij waren er geen ploïdiebarrières, hoewel de efficiëntie van interploïdiekruisingen lager was dan voor kruisingen

tussen ouders met een zelfde ploïdieniveau. Ook unilaterale incongruentie kwam voor. De hybride status van de nakomelingen werd geverifieerd door AFLP-analyse, en het bepalen van unieke vadermerkers in de nakomelingen. Vele echte hybriden werden teruggevonden, zelfs resulterend uit kruisingen tussen verschillende ploïdieniveaus. F1-zaailingen van *Sarcococca* worden nu op het veld verder geëvalueerd als pre-breeding materiaal en potentiële nieuwe cultivars.

Voor *Escallonia* werd een efficiënt protocol voor chromosoomverdubbeling ontwikkeld voor drie geselecteerde soorten, *E. illinita, E. rosea* en *E. rubra*. Het protocol omvatte een continue toediening van 5 µM trifluraline gedurende 10 weken in een vast *in vitro* medium. De verkregen tetraploïden werden geacclimatiseerd en fenotypisch geanalyseerd. Een strategie voor beeldanalyse van bovenaanzicht en zijaanzicht werd uitgewerkt, waarmee de groei, vertakking, circulariteit en visuele densiteit van de planten kon worden gemeten. Er werd veel variatie waargenomen tussen tetraploïden van de verschillende soorten, variërend van kleine en compacte tot minder vertakte en spichtige fenotypen. Tetraploïden in het veld hadden grotere bloemen dan hun diploïde tegenhangers en hun koude tolerantie nam toe (*E. rubra*) of bleef hetzelfde (*E. rosea*). Uit de resultaten is het duidelijk dat polyploïdisatie een variabel effect op het fenotype van *Escallonia* heeft en in staat is om interessante eigenschappen voor houtachtige siergewassen te creëren.

Rhizogene *Agrobacterium*-stammen zijn in staat hun *rol*-genen over te brengen in het planten-DNA. Introductie van deze *rol*-genen veroorzaakt de groei van “hairy roots” op de plaats van infectie. Theoretisch bevatten scheuten die zijn geregenereerd op de hairy roots deze *rol*-genen, met als gevolg veranderingen in fenotype en fysiologie. In deze studie werden verschillende bacteriële stammen getest (Arqua1, NCPPB 2659, LMG 63 en MAFF02-01266) en interacties tussen gastheer en stam werden waargenomen. Een co-cultivatieprotocol werd ontwikkeld en hairy roots werden geoogst. Voor *Escallonia* had de Arqua1-stam de meeste potentie om hairy roots te induceren, gevolgd door LMG 63 en MAFF02-10266. De stam NCPPB 2659 was niet in staat om hairy roots in *Escallonia* te induceren. De verkregen hairy roots bevatten verschillende combinaties van *rol*-genen, aangetoond met PCR. Er werden echter nog geen scheuten geregenereerd, dus het effect van de *rol*-genen op *Escallonia* kon nog niet worden vastgesteld.

De resultaten van dit doctoraatsonderzoek tonen aan dat interspecifieke hybridisatie en polyploïdisatie zeker waardevol zijn om nieuwe variatie in *Sarcococca* en *Escallonia* te induceren. De verkregen hybriden en tetraploïden zullen in samenwerking met de telers van BestSelect CVBA worden geëvalueerd op hun waarde als cultivar of veredelingsmateriaal. Bovendien werd

een proof-of-concept geleverd waaruit blijkt dat *Escallonia* met succes kan worden geïnfecteerd door rhizogene *Agrobacterium*-stammen en dat hairy roots, waarin *rol*-genen werden overgebracht, werden geproduceerd. Er is echter meer onderzoek nodig om scheuten op de hairy roots te regenereren en de impact van de *rol*-genen op het fenotype van *Escallonia* te evalueren. Een efficiënte fenotyperingsmethode werd ontwikkeld op basis van beeldanalyse, die de visuele evaluatie van veel planten op een objectieve manier mogelijk maakt. *Escallonia* en *Sarcococca* werden gebruikt als case-studies van houtachtige siergewassen. Het succesvol toepassen van de gebruikte geavanceerde veredelingstechnieken biedt echter verdere veredelingsopportuniteiten in andere houtachtige siergewassen.

SUMMARY

Constant product innovations are commercially very important for ornamental growers, especially in the market segment of visually attractive plants. Desired novelties are trend-sensitive; interesting characteristics are winter hardiness, resistance against variable biotic and abiotic stresses, flower color and shape, etc. Nowadays, as gardens keep getting smaller, also the demand for compact ornamentals increases. In this study, advanced breeding techniques were implemented for woody plants and evaluated for their ability to induce new variation in general, and compactness specifically. *Escallonia* and *Sarcococca* were selected as being commercially interesting by BestSelect CVBA, a cooperation of Flemish ornamental growers. These genera are rather unknown and no breeding programs have been set-up before. In this dissertation, these 2 genera were used as case-studies for woody ornamentals. As a first technique, interspecific hybridization was attempted on the genus *Sarcococca*. Hereby, the use of molecular and cytogenetic tools was evaluated to determine cross compatibility of parental species and to verify the hybrid status of the obtained progeny. Secondly, as more advanced breeding techniques, polyploidization and co-cultivation with rhizogenic *Agrobacterium* strains were implemented to induce new genetic variation. Up till now, these techniques are unexploited for woody ornamentals, yet they offer countless opportunities to create novel, interesting variation for the development of visually attractive and/or healthier plants. Few experiments for co-cultivation with rhizogenic *Agrobacterium* strains could be set-up with *Sarcococca,* due to low availability of plant material caused by a slow *in vitro* growth. The genus *Escallonia* was used in both polyploidization experiments as in co-cultivation with rhizogenic *Agrobacterium* strains.

A collection of 23 *Escallonia* genotypes and 18 *Sarcococca* genotypes was analyzed morphologically, cytogenetically and phylogenetically. One *Escallonia* genotype was tetraploid*, E. pendula*, while all other *Escallonia* genotypes were diploid. Natural hybridization within *Escallonia* is described, even between distantly related species. The *Sarcococca* genus included both diploids and tetraploids, with a large variation in genome sizes. Based on cytogenetic, phylogenetic and morphological information, the *Sarcococca* genotypes could be divided in five clusters. This information was used to successfully set-up an interspecific hybridization program for *Sarcococca*. No ploidy barriers seemed to occur, although the efficiency of interploidy crosses was lower than for intraploidy crosses. In some cases, unilateral incongruity occurred. The hybrid status of the progeny was verified by AFLP analysis, by determining the presence of paternal markers in the progeny. Many true hybrids were recovered, even from interploidy crosses. F1

seedlings from *Sarcococca* are now planted on the field for further evaluation as pre-breeding material and potential new cultivars.

For *Escallonia*, an efficient polyploidization protocol was developed on three selected species, *E. illinita, E. rosea* and *E. rubra*. The best method consisted of a continuous application of 5 µM trifluralin during 10 weeks in a solid growth medium. The obtained tetraploids were acclimatized and phenotypically analyzed. A strategy for image analysis of top view and side view pictures was elaborated, enabling to measure the growth, branching, circularity and visual fullness of the plants. Much variation was observed between tetraploids of the different species used, ranging from small and compact to less branched and spindly phenotypes. Acclimatized tetraploids in the field had larger flowers than their diploid counterparts, and their cold tolerance either increased (*E. rubra*) or remained the same (*E. rosea*). From the results it is clear that polyploidization has a variable effect on the phenotype of *Escallonia*, and is capable of creating interesting traits for woody ornamentals.

Rhizogenic *Agrobacterium* strains are capable of transferring their *rol*-genes into the plant DNA. Introduction of these *rol*-genes causes the growth of hairy roots on the site of infection. Theoretically, shoots regenerated on the hairy roots contain these *rol*-genes, with alterations in phenotype and physiology as a consequence. In this study, several bacterial strains were tested (Arqua1, NCPPB 2659, LMG 63 and MAFF02-01266), and host-strain interactions were observed. A co-cultivation protocol was developed and hairy roots were harvested. For *Escallonia*, the Arqua1 strain had most potential to induce hairy roots, followed by LMG 63 and MAFF02-10266. The NCPPB 2659 strain was not capable of inducing hairy roots in *Escallonia*. The obtained hairy roots contained different combinations or *rol*-genes, as proven with PCR. However, no shoots were regenerated on the hairy roots yet, so the effect of the *rol*-genes on *Escallonia* could not be determined.

This PhD investigation proves that the interspecific hybridization and polyploidization are for sure valuable tools to induce novel variation in *Sarcococca* and *Escallonia*. The performance of obtained hybrids and tetraploids will be evaluated in cooperation with the growers of BestSelect CVBA for their value as cultivar or pre-breeding material. Furthermore, a proof-of-concept is delivered showing that *Escallonia* can be successfully infected by rhizogenic *Agrobacterium* strains and can produce hairy roots containing *rol*-genes from the bacteria. However, more investigation is needed to regenerate shoots on hairy roots and to evaluate the impact of the *rol*-genes on *Escallonia* phenotypes. An efficient phenotyping approach was developed based on

image analysis, which allows the visual evaluation of many plants in an objective way. *Escallonia* and *Sarcococca* were used as case-studies of woody ornamentals. But the successful application of the advanced breeding techniques implemented, also offers further breeding opportunities in other woody ornamental genera.

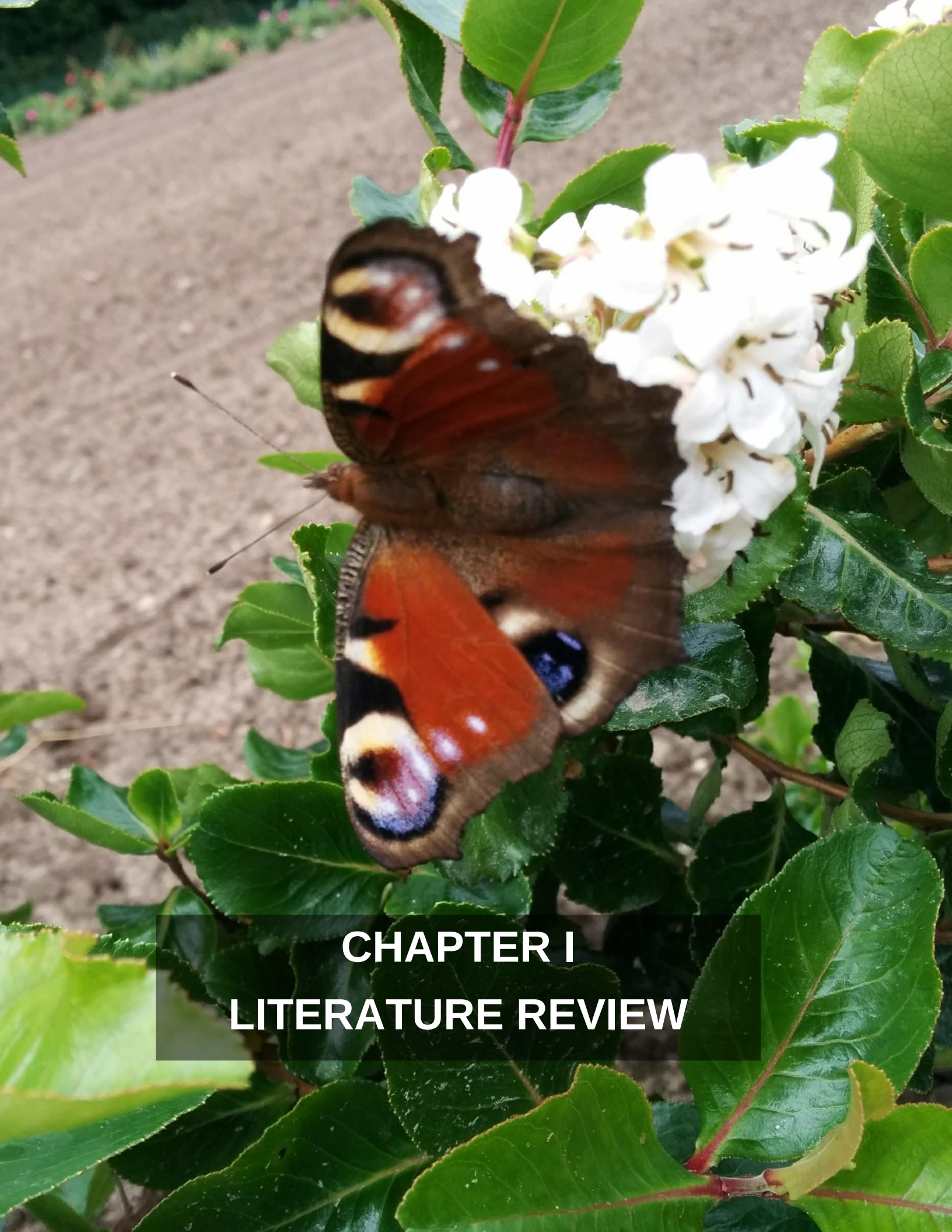
ABBREVIATIONS

2-IP	2-isopentenlyadenine
ABA	abscisic acid
AFLP	amplification fragment length polymorphism
ags	agropine synthase gene
ANOVA	analysis of variance
AU	approximately unbiased p-values
aux	auxin synthesis gene
BAP	6-benzylaminopurine
BB	axillary budburst
BCCM	Belgian co-ordinated collection of micro-organisms
BIL	branch internode length
BL	branch length
BP	bootstrap probability value
bp	base pairs
BR	bounding rectangle
CaMV	cauliflower mosaic virus
CDPK	calcium-dependent protein kinase
CHROM	chromosome number
CoHu	convex hull
COL	colchicine
Coll.	collection
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
CTAB	cetyltrimethylammonium bromide
cus	cucumopine synthase gene
CVBA	cooperative company with limited liability
D	diploid
DAPI	4',6-diaminidino-2-phenylindole
DEGs	differentially expressed genes
DNA	deoxyribonucleic acid
EC	electrical conductivity
EC_{ref}	electrical conductivity reference value before autoclaving
$EC_{ref,aut}$	electrical conductivity reference value after autoclaving
EC_{samp}	electrical conductivity sample value before autoclaving
$EC_{samp,aut}$	electrical conductivity sample value after autoclaving
EtOH	ethanol
FISH	Fluorescence *in situ* hybridization

FLCOL	flower color
FRCOL	fruit color
GEN	genome size
GFP	green fluorescent protein
GISH	genomic in situ hybridization
GMO	genetically modified organism
GUS	glucuronidase
HA	shoot hairiness
HCl	hydrogen chloride
HPLC	high-performance liquid chromatography
I(t)	index of injury at a given temperature t
ITS	internal transcribed spacer
kDa	kiloDalton
KIN	kinetin
KOH	potassium hydroxide
KWT	Kruskal-Wallis test
LMG	lab for microbiology Ghent
LT50	lethal temperature where 50% of plant cells were injured
LW	leaf length/width ratio
MAFF	ministry of agriculture, forestry and fisheries of Japan
mas	mannopine synthase gene
MBC	minimal bounding circle
miRNA	microRNA
mis	mikimopine synthase gene
MSM/MS	Murashige and Skoog medium
mT	meta topolin
MWUT	Mann-Whitney U test
MYA	malt yeast agar
NAA	1-naphthaleneacetic acid
NaOCl	sodium hypochlorite
NARO	national agriculture and food research organization of Japan
NBGM	National Botanical Garden in Meise (Belgium)
NCPPB	national collection of plant pathogenic bacteria
NPA	naphthylphtalamic acid
NSL	new shoot length
NSIL	new shoot internode length

OD	optical density
ORF	open reading frame
ORY	oryzalin
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PI	propidium iodide
PLRV	potato leaf roll virus
PS	plant size
PVP	polyvinylpyrrolidone
qPCR	quantitative polymerase chain reaction
RcApx1	ascorbate peroxidase gene
RcCSD1	Cu/Zn superoxide dismutase gene
RH	relative humidity
Ri-plasmid	root-inducing plasmid
RNA	ribonucleic acid
rol	root oncogenic loci gene
ROS	reactive oxygen species
RS	regenerated shoots
SDR	second division restitution
ST	number of styles
SV	side view
SV_fill	% of the area of the bounding rectangle was filled by the plant in side view
SV_he	plant height in side view
SV_pl_ar	plant area in side view
SV_wi	plant width in side view
T	tetraploid
T4SS	type IV secretion system
T-DNA	transfer-DNA
TDZ	thidiazuron
TIBA	2,3,5-triiodobenzoic acid
Ti-plasmid	Tumor inducing plasmid
TL-DNA	left transfer DNA
TMV	tobacco mosaic virus
TR-DNA	right transfer DNA
TRI	trifluralin
TV	top view
TV_circ	plant circularity in top view
TV_fill	% of the area of the minimal bounding circle that was filled by the plant in top view

TV_pl_ar	plant area in top view
T-yield	tetraploid yield
UPGMA	unweighted pair group method with arithmetic mean
USDA	United States department of agriculture
VIB	Flemish institute for biotechnology
vir	virulence genes
WPM	woody plant medium
YEG	yeast extract glucose



CHAPTER I
LITERATURE REVIEW

1. LITERATURE REVIEW

To keep ornamental growers competitive on the national and international market, product innovation and improvement of crop performance are very important. Yet, product innovation is trend-sensitive. Nowadays, gardens keep getting smaller and the demand for potted and compact attractive cultivars grows. Specific and/or compact growth forms are therefore an important selection criteria in the breeding of woody ornamentals.

Escallonia and *Sarcococca* are two woody ornamental genera within the section of attractive, evergreen container grown plants. The introduction of new cultivars with a more compact growth habit or other novel aesthetically relevant traits, could broaden the assortment. For *Escallonia* and *Sarcococca*, modern breeding techniques are not exploited. However, they can introduce new genetic variation and can enable faster selection. Therefore, in this thesis, the genera were characterized, and three advanced breeding techniques were applied: interspecific hybridization, polyploidization, and introduction of *rol*-genes from rhizogenic *Agrobacterium* species into the plant genome via co-cultivation.

In this chapter, a concise overview of the genera *Escallonia* and *Sarcococca* is given, followed by the state of art of interspecific hybridization, polyploidization, and the introduction of *rol*-genes from rhizogenic *Agrobacterium*.

1.1. ESCALLONIA

1.1.1 Phylogenetics, taxonomy and occurrence

The genus *Escallonia* Mutis ex L. f. contains about 40 species and 20 botanical varieties, distributed along the Andean mountains and in different ecosystems in southern Brazil and central Argentina (Figure 1.1) (Morello and Sede, 2016, Sede et al., 2013). The genus was named by the Spanish botanist Mutis, in honor of his friend and teacher Escallon (Bean and Murray, 1989a). The genus is classified as follows:

Kingdom: Plantae
Division: Magnoliophyta (Angiospermae)
Clade: Asterids
Order: Escalloniales
Family: Escalloniaceae
Genus: *Escallonia*

Other genera in the family of the Escalloniaceae are *Anopterus, Tribeles, Forgesia, Eremosyne, Polyosma*, and *Valdivia* (Angiosperm Phylogeny Group, 2016). Recent studies show that *Escallonia* is member of a monophyletic group with *Forgesia* and *Valdivia* as sister clades, instead of a paraphyletic groups with *Forgesia* and *Valdivia* having separate ancestors (Sede et al., 2013, Zapata, 2013).

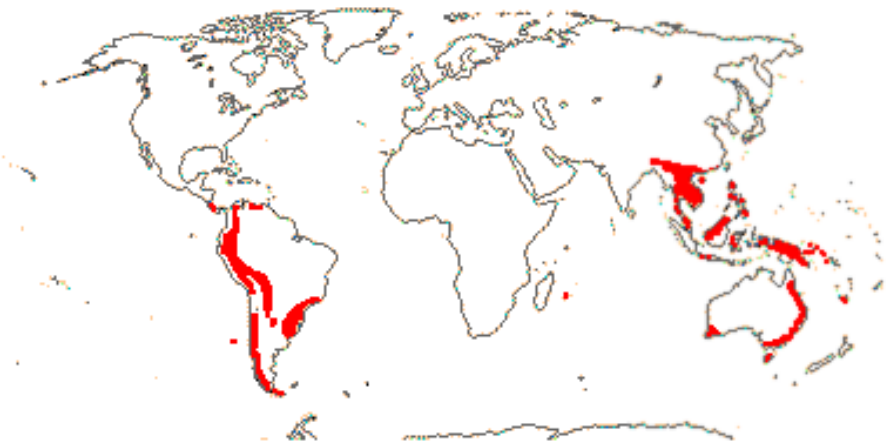


Figure 1.1: Distribution area of the Escalloniaceae. The genera *Anopterus* and *Eremosyne* originate from Australia, *Polyosma* from South-East Asia and East Australia, *Forgesia* from La Réunion, and *Escallonia, Tribeles and Valdivia* from South America (Modified from the Angiosperm Phylogeny Website, Stevens (2001 onwards)).

Several complications arise in the systematics of the species within the *Escallonia* genus. Many species have the same chromosome morphology and number with a high potential for hybridization. Zielinski (1955) analyzed 21 different *Escallonia* genotypes, and reported them all being diploid with 2n = 2x = 24 chromosomes. These results were confirmed for *E. macrantha,* and *E. thyrsoidea* by Darlington and Wylie (1955) and for *E. rubra* by Darlington and Wylie (1955) and Hanson and Leitch (2002). Furthermore, the species boundaries can be unclear, due to large variations in morphology, and some species seem to be intermediate between two sympatric species (Morello and Sede, 2016, Zapata, 2013). Numerous hybrids and cultivars have been described, and many of them have an *E. rubra* and *E. virgata* background, such as *E.* 'Langleyensis' and *E.* 'Edinburgh' (=*E.* 'Edinensis'). *E.* 'Donard Seedling', *E.* 'Apple Blossom', and *E.* 'Slieve Donard' resulted from backcrosses of *E. virgata* x *E.* 'Langleyensis', while *E. rubra* 'C.F. Ball' originated from a backcross of (*E. rubra* var. *macrantha* x (*E. rubra* x *E. rubra* var. *macrantha*)). The genotype *E.* 'Red Elf' is a seedling from a hybridization between *E. rubra* 'C.F. Ball' and *E.* 'William Watson', with the latter being a seedling of *E.* 'Langleyensis' (Bean and Murray, 1989a, Hilliers Garden, 1991, Krüssmann, 1960).

The biogeographic history of *Escallonia* species is likely associated with Andean orogeny. Both Zapata (2013) and Sede et al. (2013) divided the *Escallonia* species in clades concurring with early geographic separation, with a multilocus phylogenetic analysis and plastid DNA sequence data respectively. Two possibilities arise to account for the diversification, either environmental gradients along steep elevation in the mountainous areas give rise to development of new, separate species, or the species themselves evolved broader environmental tolerances.

1.1.2 Morphological characteristics and use

The plant morphology displays a high variation between the species, and intraspecific variability is shown in e.g., shape and size of flower organs or the presence of hairs and glands. *Escallonia* species are evergreen, except for *E. virgata* (Bean and Murray, 1989a, Hilliers Garden, 1991, Krüssmann, 1960), and have white to red hermaphroditic flowers, with a honey fragrance and nectar production (Anderson et al., 2001, Bean and Murray, 1989a). The flowers are arranged in terminal panicles or racemes, with five petals, sometimes forming a tube (Figure 1.2) (Bean and Murray, 1989a, Krüssmann, 1960). Pollination has rarely been studied, and most likely occurs both by self-pollination as by cross-pollination, by a variation of birds and insects (Anderson et al., 2001, Bean and Murray, 1989a, Díaz-Forestier et al., 2016, Valdivia and Niemeyer, 2006). The ovary is semi-inferior and enclosed in a top- or bell-shaped receptacle or hypanthium. The ovary

is surmounted by a disk, of which the shape and depth can be used for identification (Bean and Murray, 1989a). The fruit is a capsule, with many small seeds (Bean and Murray, 1989a, Krüssmann, 1960).

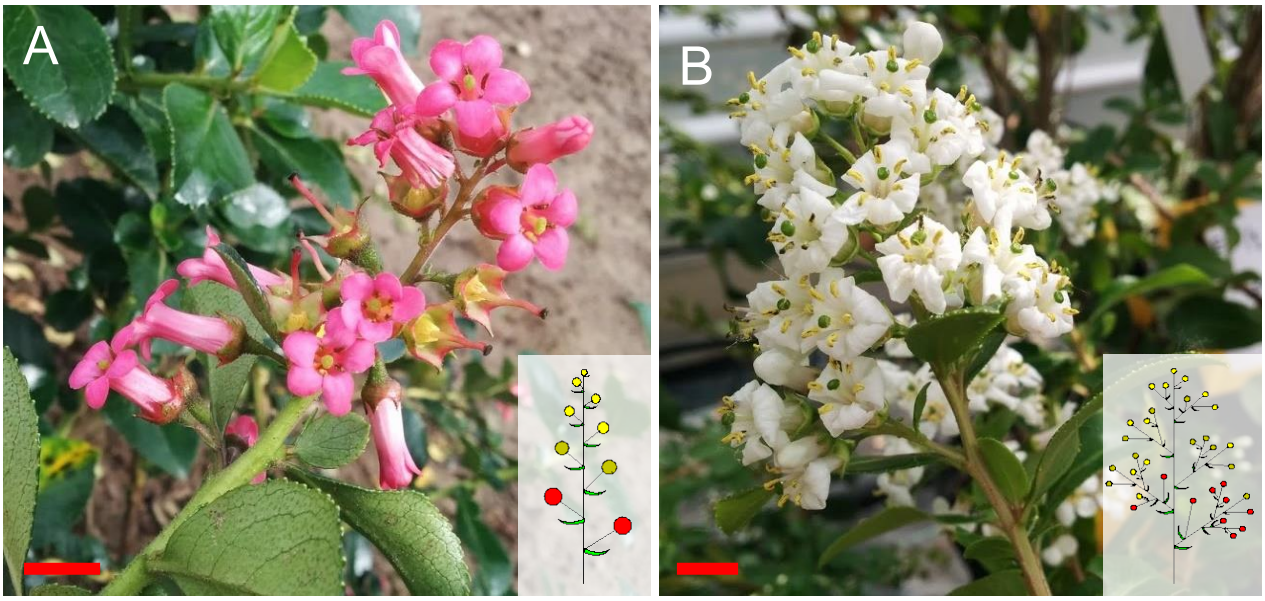


Figure 1.2: Flowers with five petals forming a tube, arranged in terminal racemes (A) in *Escallonia rubra* var. *macrantha* or in terminal panicles (B) in *E.* 'Iveyi'. Red bar = 1 cm.

The leaves are arranged alternate, without stipules, often in the axil of a larger leaf, actually representing a short branch (Bean and Murray, 1989a, Krüssmann, 1960) (Figure 1.3). *Escallonia* species have resin glands on leaves and branches, but this is more abundant in the wild species than in the cultivated ones (Bean and Murray, 1989a). Sizes range from shrubs (1.5-2.5 m) to small trees (< 3m) (Hilliers Garden, 1991, Krüssmann, 1960). *Escallonia* is used as a hedging plant, especially in coastal regions, since they display a moderate salt tolerance (for example *E. rubra,* Cassaniti et al. (2009)). However, they need some protection from wind and cold. Depending on the species, *Escallonia* can thrive in USDA zones from 10 to 7b, which corresponds to mean minimum temperatures of -1.1°C to -14.9°C (Hoffman and Ravesloot, 1998).

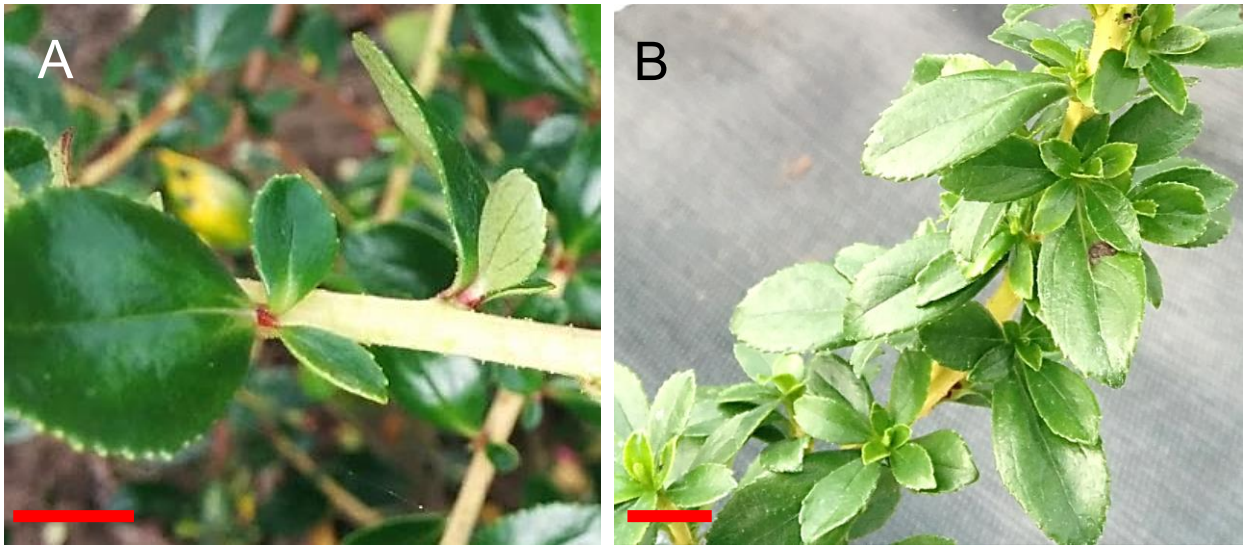


Figure 1.3: Clusters of leaves in the axil of a larger leaf of *Escallonia* 'Red Elf' (A) and *E.* 'Apple Blossom' (B), actually representing a short branch. Red bar = 1 cm.

Tea of leaves and stems of *E. illinita* is used in traditional medicine to treat liver diseases, rheumatism and kidney pain. HPLC analysis of an infusion of dried areal parts of *E. illinita* showed the presence of 16 phenolic compounds and high amounts of flavonoids with a good antioxidant activity (Simirgiotis et al., 2012). *E. pulverulenta* is used to produce high quality honey (Díaz-Forestier et al., 2016, Montenegro et al., 2009).

1.2 SARCOCOCCA

1.2.1 Phylogenetics, taxonomy and occurrence

The *Sarcococca* Lindley genus ("sweet box" or "Christmas box") contains ± 20 species, native to the south of China, the Himalaya Mountains, Afghanistan, Pakistan, Vietnam, Sri Lanka and the northeast of India. According to Hoffman and Ravesloot (1998) and Dirr (2011), the genus is classified as follows:

Kingdom: Plantae
Division: Magnoliophyta (Angiospermae)
Clade: Eudicots
Order: Buxales
Family: Buxaceae
Genus: *Sarcococca*

The Buxaceae family comprises 7 genera, namely *Sarcococca, Buxus, Notobuxus, Pachysandra, Haptanthus, Didymeles* and *Styloceras* (the Angiosperm Phylogeny Website, Stevens (2001 onwards)). Studies on the relationships between *Sarcococca* species are rare. Taxonomic studies based on floral morphology were performed on *S. confusa*, *S. ruscifolia,* and *S. hookeriana* var. *humilis* (Endress and Igersheim, 1999, Von Balthazar and Endress, 2002). The basic chromosome number is x = 14. Chromosome counts of 3 species (*S. saligna*: 2*n* = 2*x* = 28; *S. humilis:* 2*n* = 4*x* = 56; *S. ruscifolia*: 2*n* = 4*x* = 56) were published (Darlington and Wylie, 1955).

The species *S. hookeriana* and *S. ruscifolia* and their cultivars are commonly cultivated in Europe (Sealy, 1986). Until now, new introductions are either spontaneous mutations or lucky findings, such as *Sarcococca ruscifolia* 'Dragon Gate', which was discovered in 1980 at the Dragon Gate temple in Yunnan, China (Dirr, 2011).

1.2.2 Morphological characteristics and use

Sarcococca species are evergreen flowering shrubs (Flora of China, 2008, Jarvis, 1989, Köhler, 2007, Bean and Murray, 1989b, Krüssmann, 1960). They slowly reach 1.2-1.5 m high (Flora of China, 2008). The shrubs have dark, glossy foliage, with alternate leaves (Bean and Murray, 1989b, Krüssmann, 1960). In winter they bear green-white to light pink flowers that are sweetly fragrant. The plants are monoecious, with unisexual flowers in small, axillary clusters or racemes (Endress and Igersheim, 1999, Flora of China, 2008, Krüssmann, 1960, Sealy, 1986) (Figure 1.4).

Several female flowers occur at the base of the inflorescence, while the male flowers occupy the upper part (Von Balthazar and Endress, 2002).The male flowers have four tepals and four stamens, the female flowers four to six tepals (Bean and Murray, 1989b, Krüssmann, 1960). Both do not have petals. The female flower can have two or three styles, which is used for identification (Flora of China, 2008, Krüssmann, 1960) (Figure 1.5). The fruit is an ovoid to globose fleshy drupe (Flora of China, 2008, Bean and Murray, 1989b, Krüssmann, 1960) (Figure 1.6). The drupes are green when unripe, turning bright red, purple or black when matured (Bean and Murray, 1989b, Krüssmann, 1960), and contain two or three seeds, depending on the number of styles (Flora of China, 2008). The plants have an ornamental value in gardens, as they thrive in the shade (Bean and Murray, 1989b, Flora of China, 2008) and are winter-flowering fragrant shrubs. *Sarcococca* species can survive in USDA zones 9 to 6, depending on the species, concurring with minimum mean temperatures of -6.6°C up to -23.3°C.

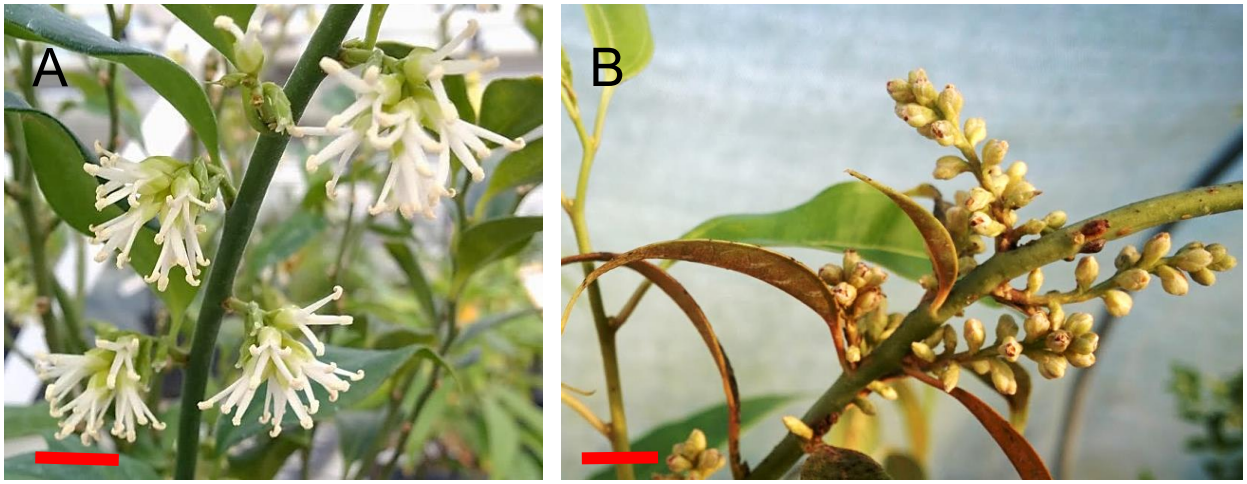


Figure 1.4: Flowers in axillary clusters (A) on *Sarcococca confusa* and in axillary racemes (B) on *S. coriacea*. Red bar = 1 cm.

The leaves and shoots of several *Sarcococca* sp. have been used in traditional medicine for stomach disorders, blood disorders, muscle aches (Ahmad et al., 2015), malaria, rheumatism and skin infections (Moghaddam et al., 2010). Several studies show the presence of pharmacologically active substances with different biological activities in *S. saligna* (Jan et al., 2017, Moghaddam et al., 2010, Musharraf et al., 2012, Jiang et al., 2016), *S. hookeriana* (Zhang et al., 2013, Jiang et al., 2016), *S. wallichii* (Adhikari et al., 2015), and *S. ruscifolia* (Zhang et al., 2015b). Their steroidal alkaloids have immunomodulatory and hepatoprotective agents and a high antioxidant activity (Jan et al., 2017, Iqbal et al., 2015), and can potentially be used to treat prostate cancer (Brossard

et al., 2013), and Alzheimer's disease (Konrath et al., 2013). An ethanol extract shows antifungal activity against *Aspergillus* sp. (Moghaddam et al., 2010).

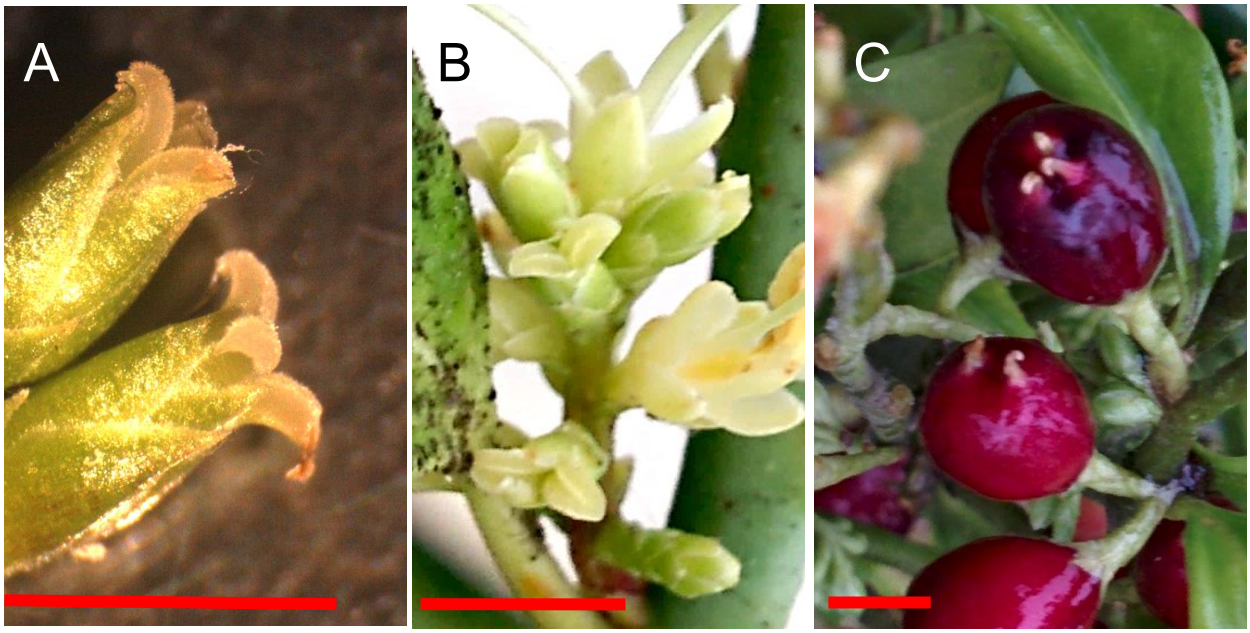


Figure 1.5: The number of styles in the female flower is a characteristic used for determination of the *Sarcococca* species. (A) *S. ruscifolia* (S06) has 3 styles; (B) *S. coriacea* (S02) has 2 styles; (C) *S. confusa* has both 2 and 3 styles in the female flower on the same plant, as seen here on the drupes. Red bar = 0.5 cm.

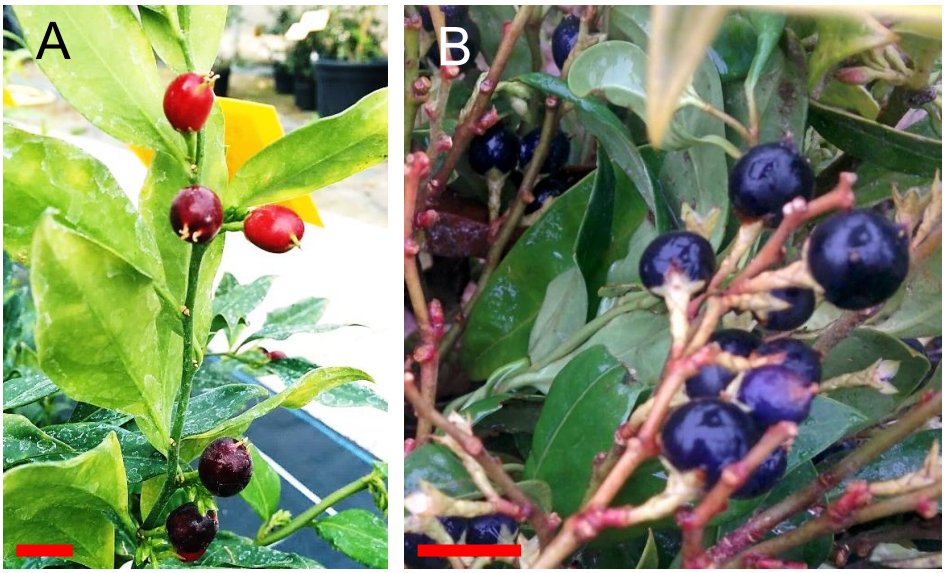


Figure 1.6: (A) Red immature drupes on *Sarcococca confusa* (S01), which turn black at maturity, and (B) black drupes on *S. hookeriana* var. *humilis* (S09). Red bar = 1 cm.

1.3 BREEDING METHODS

1.3.1 Interspecific hybridization

Sexual hybridization is considered as a significant process in plant evolution and speciation (Kuligowska et al., 2016b, Abbott et al., 2013). As a consequence, interspecific hybridization is one of the most used breeding tools that contributed to the development of many plant cultivars in the food, feed and ornamental sector. Hybrids can display an intermediary phenotype of the parental species, but also novel characteristics can be observed. However, several pre- and post-zygotic barriers can hamper the fertilization and embryo development of hybrids (reviewed by Kuligowska et al. (2016b) and Tonosaki et al. (2016)).

The success of interspecific breeding has been augmented by technological advances, such as *in vitro* embryo rescue, polyploidization, pistil manipulation, etc. to overcome aforementioned barriers. These advances were followed by molecular techniques such as the screening of the ploidy level, the analysis of the chromosome numbers, and molecular markers to analyze phylogenetic distances between species, enabling to assess the efficiency of hybridization between potential parental species (Granados Mendoza et al., 2013, Kubota et al., 2012). Molecular marker technology allows to confirm the hybrid status of the progeny (Elliott et al., 2004) (reviewed by Kuligowska et al. (2016b) and Tonosaki et al. (2016)).

Many woody ornamental interspecific hybrids were developed, e.g., *Acacia* (Kato et al., 2014), *Betula* (Czernicka et al., 2014), *Calluna* (Behrend et al., 2015), *Hibiscus* (Kuligowska et al., 2016a, Van Laere et al., 2007), *Hydrangea* (Granados Mendoza et al., 2013) *Jasminum* (Deng et al., 2017) and *Viburnum* (Xie et al., 2017) among many others. In the genera *Escallonia* and *Sarcococca*, interspecific hybridization breeding programs are not reported. But in the, to *Sarcococca* related, genus *Buxus* interspecific hybridization was reported to be successful (Van Laere et al., 2015). The genetic distance and cytogenetic characteristics were determined on several European and Asiatic *Buxus* species (Van Laere et al., 2011a), and more than 7000 inter- and intraspecific crosses made (Van Laere et al., 2015). It could be concluded that genetic distance, geographical distribution and ploidy differences did cause a decline in crossing efficiency, but they were not absolute crossing barriers. Interploidy crosses were more efficient if the female parent had the highest ploidy level. This clearly shows that the efficiency of interspecific crosses could increase with prior knowledge of (cyto)genetic characteristics.

1.3.2 Polyploidy

1.3.2.1 Importance of polyploidy

Polyploidy is the presence of more than two complete sets of chromosomes per cell nucleus, which can either originate from different parents (allopolyploidy), or can be the result of a whole-genome doubling and thus contains copies of the same genome (autopolyploidy). Several studies tried to estimate the incidence of polyploidy worldwide, but the results vary widely, extending from 30% to 70% for Angiosperms (Sattler et al., 2016, Ramsey and Schemske, 1998). Polyploids are generally more abundant in areas with unstable environmental conditions or more extreme climates, such as permafrost areas and mountainous zones (Madlung, 2013, Brochmann et al., 2004). Harsher climatic events lead to a higher frequency of polyploid offspring, for example glaciation and warming periods, such as the Cretaceaous-Tertiary extinction period 65 million years ago. These were key moments for polyploidization events and their subsequent establishment (Bretagnolle and Thompson, 1995, Fawcett et al., 2009). Due to the redundancy of genetic material in polyploids, mutations and genetic drift in gene copies can occur without compromising essential functions. This gives cause to neo- or sub-functionalization of genes, phenotypic diversification, heterosis, changes in reproduction, and a higher flexibility to adapt to changing environmental conditions, which ultimately leads to a new species (Bretagnolle and Thompson, 1995, Sattler et al., 2016, Ramsey and Schemske, 1998, Comai, 2005). A second advantage is the heterosis effect, which is stable in polyploids. In hybrids, the heterosis decays in subsequent generations (Madlung, 2013). Taken all together, polyploidization, followed by gene loss and/or diploidization is important for species evolution and diversification (Jiao et al., 2011).

1.3.2.2 Naturally occurring systems of polyploid induction

Two types of polyploids are described: allopolyploids and autopolyploids (Comai, 2005). Allopolyploids can be subdivided into two classes, namely true and segmental allopolyploids. True allopolyploids result from the hybridization between distantly related species. The orthologous chromosomes from the different parents are too differentiated and form bivalents instead of multivalents, resulting in disomic inheritance. On the contrary, in segmental allopolyploids where the parental species are more closely related with only partially differentiated chromosomes, a mix of univalent, bivalent, and/or multivalent pairing of chromosomes during meiosis is possible, resulting in aneuploid progeny, but the frequency varies according to species (Comai, 2005, Dolyle, 1986). This multivalent pairing and subsequent aneuploid progeny is also very common in neopolyploids (Comai, 2005). Well known examples of allopolyploids are upland cotton and bread

wheat. Cotton, *Gossypium hirsutum* (2x = 4x = 52), combines the genome of *G. herbaceum* and of *G. raimondii* or *G. gossypioides* (Sybenga, 1992, Wendel et al., 1995). Allohexaploid bread wheat has a genome composition of AABBDD, with the A genome from *Triticum monococcum, T. boeoticum* or another *Triticum* sp. The B genome most likely comes from an *Aegilops* species, while the D genome is derived from *Aegilops squarrosa* (Sybenga, 1992). Ornamental examples of allopolyploids are found in the genus *Hibiscus* (Contreras et al., 2009) and *Rubus* (Wang et al., 2015).

Autopolyploids contain copies of the same genome. Many important crops are autopolyploids, such as the solanaceous crops tomato and potato, bananas, kiwifruit, leek, some ryegrasses, sugar beet, watermelon, some apple cultivars (reviewed by Sattler et al. (2016)) and many ornamentals, such as *Buddleja* (Van Laere et al., 2008), *Buxus* (Van Laere et al., 2011a) *Chrysanthemum* (Won et al., 2017), *Hibiscus* (Contreras et al., 2009) and roses (Yokoya et al., 2000). Sometimes ploidy chimeras occur, such as the differently colored tetraploid edges of petals in otherwise diploid Belgian azalea (Eeckhaut et al., 2006, De Schepper et al., 2004).

Several pathways for the formation of auto- and allopolyploids have been studied. The two main systems are endopolyploidization and the formation and fusion of unreduced gametes (Sattler et al., 2016). Endopolyploidization is somatic doubling in sporophytic tissues. This endopolyploid tissue could then grow into a fertile shoot, produce 2n gametes and subsequently polyploid offspring (Bretagnolle and Thompson, 1995, Ramsey and Schemske, 1998, Yant and Bomblies, 2016). However, endopolyploid tissue is discernable from polyploid tissue by the arrangement of the chromosomes around the centromere, as shown in Figure 1.7. Somatic doubling does not increase the heterozygosity, which is an evolutionary disadvantage (Bretagnolle and Thompson, 1995).

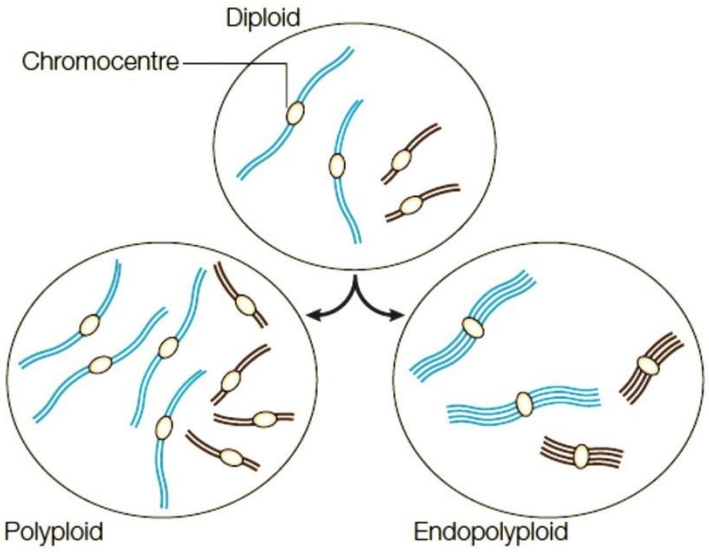


Figure 1.7: Endoploidy involves side-by-side replication of chromosomes, resulting in sister chromosomes on a single chromocentre, while true polyploids have distinct chromocentres for all chromosomes (Modified from Comai (2005)).

In nature, most polyploids have arisen by sexual polyploidization via unreduced gametes (Horn, 2002a, Sattler et al., 2016, Yant and Bomblies, 2016). The frequency by which unreduced gametes are formed depends on the plant species, the genotype and even on the specific flower within one plant. Interspecific hybrids are known to produce 50 times more unreduced gametes than non-hybrids, due to meiotic irregularities with poor chromosome pairing (Ramsey and Schemske, 1998). Allopolyploids can result from the fusion of two unreduced gametes, so-called bilateral polyploidization. Unilateral polyploidization is a fusion between an unreduced and a normal, reduced gamete, forming a 'triploid bridge' (Horn, 2002a, Yant and Bomblies, 2016, Sattler et al., 2016, Bretagnolle and Thompson, 1995, Ramsey and Schemske, 1998). While triploids produce very little functional gametes because of their unbalanced number of chromosomes, small number of euploid gametes (x or 2x) do develop. Also hybrid triploids can occur, resulting from a cross between a diploid and a tetraploid parent. This triploid produces allotetraploids after a subsequent backcrossing with the tetraploid parent, with an unreduced gamete from the diploid parent, or via self-fertilization. Chromosome doubling through unreduced gametes increases the heterozygosity, which is advantages in determining the success in the establishment of the newly formed polyploid, both for plant breeding as in wild populations (Bretagnolle and Thompson, 1995, Ramsey and Schemske, 1998).

Plant breeders have elicited the production of unreduced gametes by treatments with heat, cold, antimitotic agents, etc, to use 2n gametes for their breeding strategies (Younis et al., 2014), e.g., in *Populus* (Guo et al., 2016, Wang et al., 2017a), cassava (Lai et al., 2015), *Eucommia* (Li et al., 2016) and *Begonia* (Dewitte et al., 2010).

1.3.2.3 Synthetic autopolyploidization and the consequences for breeding

Breeding programs based on synthetic autopolyploidy induction, are introduced in the 1930s (Hancock, 1997). From the 90's and onward, *in vitro* chromosome doubling became more popular because it provides a stable and standardized environment (Dhooghe et al., 2011). In autopolyploidization experiments, many parameters are species specific, such as the explant type and the mitotic inhibitor, its mode of application and the most efficient combination of concentration and exposure time (Dhooghe et al., 2011). Ergo, no standard protocol for somatic autopolyploidization exists.

Mitotic inhibitors, more specific the metaphase inhibitors, such as colchicine, oryzalin and trifluralin, disturb the formation of the spindle, which is essential for polar migration of the homologous chromosomes to the daughter cells (Bartels and Hilton, 1973, Dewitte and Murray, 2003). Colchicine inhibits the addition of tubulin-dimers to the microtubuli (Bartels and Hilton, 1973). It has carcinogenic effects on humans and a low affinity for plant tubulins, while oryzalin and trifluralin have a higher affinity for plant tubulins. The latter can therefore be used at mere micromolar concentrations, reducing the amount of antimitotic agent needed and the risk to human health (Hansen and Andersen, 1996, Planchais et al., 2000). Oryzalin and trifluralin disturb the formation of the tubulin-dimers, the building blocks for the microtubuli (Bartels and Hilton, 1973). However, colchicine is still the most popular metaphase inhibitor for woody species, e.g., in *Hebe* (Gallone et al., 2014), *Lavandula* (Urwin, 2014), *Thymus* (Tavan et al., 2015), *Ziziphus* (Shi et al., 2015), *Eriobotrya* (Blasco et al., 2015), *Rosa* (Feng et al., 2017), *Ligustrum* (Fetouh et al., 2016), *Populus* (Xu et al., 2015) and *Malus* (Hias et al., 2017). Oryzalin and/or trifluralin were only scanty used, e.g., in *Cercis* (Nadler et al., 2012), *Hebe* (Gallone et al., 2014) and *Rosa* (Feng et al., 2017), but are usually more efficient (Dhooghe et al., 2011).

The main breeding goals to obtain by polyploidization are production factors and yield for food and feed crops, and visual attributes for ornamental plants (Leus et al., 2012). Polyploidized genotypes are found to have large morphological and physiological differences compared to their counterparts. Although changes in gene expression and epigenetics are thoroughly investigated

in paleopolyploids (reviewed by Adams and Wendel (2005), Comai (2005), Parisod et al. (2010), Madlung (2013), Gallagher et al. (2016)), the immediate effect of chromosome doubling in first generation neopolyploids is not well understood, due to the complexity of polyploid genomes and its epigenetics. Genetic changes such as genome rearrangements and loss of repeated elements are reported, but also changes in epigenetics and in gene expression (Osborn et al., 2003). In a maize ploidy series (1x, 2x, 3x and 4x), the RNA content of leaf tissue was analyzed. For most genes tested, the transcript level was proportional to the ploidy level, and only exceptionally differentially expressed genes (DEGs) were noticed (Guo et al., 1996). Also in *Citrus limonia*, the amount of DEGs was less than 1% (Allario et al., 2011). Fasano et al. (2016) analyzed the transcriptome of four tetraploid lines of two *Solanum* species. For the four *S. bulbocastanum* tetraploids, the transcription level was proportionate to the gene dosage. This gene-dosage effect was not present in the four *S. commersonii* tetraploids. A second difference between the two species was found in the DEGs, which occurred preferentially in pericentromeric regions for *S. commersonii* tetraploids, while no preferential region was observed for *S. bulbocastanum.* The DEGs were different for each tetraploid line and varied between 1800 and 3800. They concluded that changes in gene expression after autopolyploidization are genotype-specific, and subsequently, so are the morphological and physiological changes.

The basic consequence of polyploidy is an increased cell size, caused by the larger number of gene copies (gigas effect) (Sattler et al., 2016, Allario et al., 2011), and an increased chloroplast number and chlorophyll content (Hias et al., 2017, Feng et al., 2017, Allario et al., 2011). Therefore, polyploids may have larger organs than their diploid counterparts, such as larger and thicker leaves, flowers, and fruits. For example, tetraploid leaves were larger in *Citrus* (Guerra et al., 2014), *Spathiphyllum* (Van Laere et al., 2010), *Paulownia* (Tang et al., 2010), *Platanus (Liu et al., 2007)*, *Buddleja* (Van Laere, 2008) and *Petunia* (Regalado et al., 2017). But also smaller organs are reported, e.g., tetraploid *Malus* x *domesticus* displayed a decreased leaf size (Hias et al., 2017), and some tetraploid *Rosa* genotypes had less leaflets or smaller leaves (Feng et al., 2017) (Figure 1.8).

An increased cell size does not implicate an increased plant size, since the number of cell divisions can be reduced in polyploids (Hias et al., 2017, Horn, 2002a, Sattler et al., 2016). Several studies on polyploidization reported a decreased growth, e.g., in *Buddleja* (Rose et al., 2000a), *Citrus* (Allario et al., 2011, Guerra et al., 2014), *Platanus* (Liu et al., 2007), *Petunia* (Regalado et al., 2017), *Hibiscus* (Contreras et al., 2009) and *Spathiphyllum* (Van Laere et al., 2010). In *Rosa* tetraploids, some genotypes displayed a significant decrease in plant size, and other genotypes

not (Figure 1.8) (Feng et al., 2017). Also the opposite is possible, an increased vigor can be caused by an increased chromosome number, e.g., in triploid apples (Sedov, 2014, Sedov et al., 2014) and hexaploid *Hibiscus* (Van Laere et al., 2006).

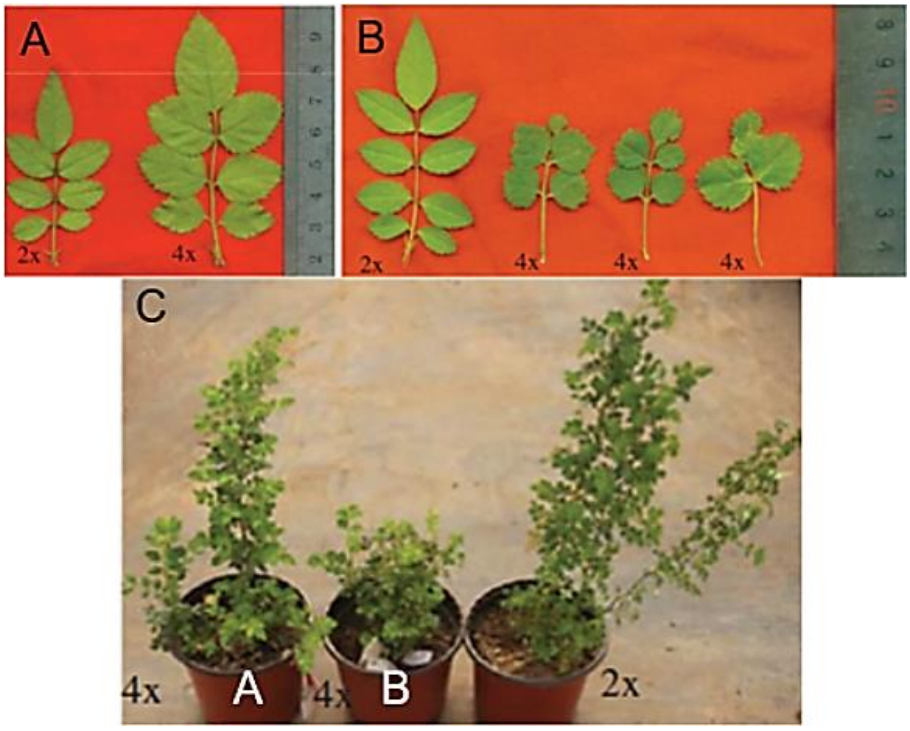


Figure 1.8: Both (A) and (B) compare the leaves of a diploid (2x) and tetraploid (4x) *Rosa multiflora*, but large differences were present between two different polyploidization events. This difference was also visible in plant height (C), between the tetraploids (4x, A and B) and the diploid (2x). (Source: (Feng et al., 2017))

Besides morphological changes, physiological changes in for example stress resistance and flowering are reported (Regalado et al., 2017, Van Laere et al., 2010, Levin, 2002). A better drought tolerance was found in tetraploid *Spathiphyllum* (Van Laere et al., 2010), and in pentaploid *Betula* (Li et al., 1996). An increase in salt tolerance was found in tetraploid *Malus* (Zhang et al., 2015a). In tetraploid *Lonicera*, an increase in both heat tolerance and drought was observed (Li et al., 2009, Li et al., 2011). Tetraploid *Malus, Citrus,* and *Rosa* leaves displayed a darker green color, due to a higher chlorophyll content (Hias et al., 2017, Feng et al., 2017, Allario et al., 2011, Guerra et al., 2014). Triploid *Malus* showed an increased scab immunity, larger fruits and an increased autogamy (Sedov, 2014, Sedov et al., 2014). *Citrus* (*Poncirus trifoliata, C. sinensis* x *P. trifoliata, and C. reshni*) 4x seedlings were more tolerant to salt stress when insufficiently watered (Saleh et al., 2008). However, when sufficiently watered, salt stress was higher for 4x seedlings than for 2x seedlings (*P. trifoliata* and *C. deliciosa*), as they accumulated more toxic ions due to a

higher transpiration and uptake rate (Mouhaya et al., 2010). According to Ruiz et al. (2016), differences in root architecture and anatomy occurred between diploid and tetraploid *Citrus sinensis* seedlings, which can cause differential uptake of ions.

Polyploidy can cause a decrease in fertility, because of failures in pairing of multiple homologous chromosomes during cell division. Ergo, breeding programs with autopolyploidy induction, usually focus on crops cultivated for their vegetative organs or on vegetatively propagated species (Paterson, 2005). However, a decrease in fertility can be advantageous in the development of seedless fruits, such as triploid mandarins (Aleza et al., 2009) or to prevent the spreading of seedlings and seed increase in an otherwise invasive plant species (Horn, 2002), such as *Buddleja* (Leus et al., 2012).

Polyploidization can also be used as a tool to circumvent ploidy barriers in interspecific hybridization. In *Lilium* breeding, tetraploids were made to improve the fertility of an F1 interspecific hybrid to enable backcrossing (Zhang et al., 2017). Also in *Rosa,* tetraploids were created of two diploid cultivars, to enable the creation of fertile tetraploid hybrids with another tetraploid *Rosa* genotype (Feng et al., 2017). A hybrid of two wild *Arachis* species was polyploidized to provide resistance genes for the cultivated allotetraploids peanut varieties (de Paula et al., 2017).

The cases above demonstrate the capricious effect of polyploidization. In an attempt to understand this fickle reaction, Riddle et al. (2006) studied haploid, diploid, triploid and tetraploid *Zea mays*, all derived from the same diploid cultivar (Figure 1.9). They concluded that the ploidy level has a large effect on the observed phenotype, but also that the genetic background and the ploidy level interact, meaning that polyploidization can have a different effect for each species/cultivar used. In addition, evidence suggests that plant species possess an optimal ploidy degree for maximum growth, and ploidy levels above or below this optimum show a growth reduction (Hias et al., 2017). In *Malus,* triploids showed increased vigor over diploids, while tetraploids decreased in vigor (Hias et al., 2017, Sedov et al., 2014). Research on *Hibiscus syriacus* showed that hexaploids are increased in vigor compared to tetraploids (Van Laere, 2008), while Belgian azalea shows a decrease in vigor in hexaploids, and in increase in tetraploids, compared to diploids (Eeckhaut, 2003) (Figure 1.10).The hexaploid *Hibiscus* 'Azurri', resulting from a cross between the tetraploid *H.* 'Oiseau Blue' and its doubled octaploid, was much more vigorous than the tetraploid. A hexaploid azalea was discovered in a seedling population of *Rhododendron* 'Starlight' (4x) x *R.* 'Casablanca tetra' (4x) after an unilateral polyploidization. On the contrary to the *Hibiscus* hexaploid, this azalea hexaploid showed a stunted growth compared to the diploids and tetraploids

from which it originated. In the fruit tree *Ziziphus jujuba,* tetraploids exhibited the same growth rate as diploids, but were markedly more sturdy. Obtained *Ziziphus* octaploids were slow growing and not able to survive winter conditions (Shi et al., 2015).

Figure 1.9: The effect of different ploidy levels on the *Zea mays* inbred line B73. Adult haploid (1x), diploid (2x), triploid (3x), and tetraploid (4x) plants are displayed. A meter stick is included for reference. (Source: (Riddle et al., 2006))

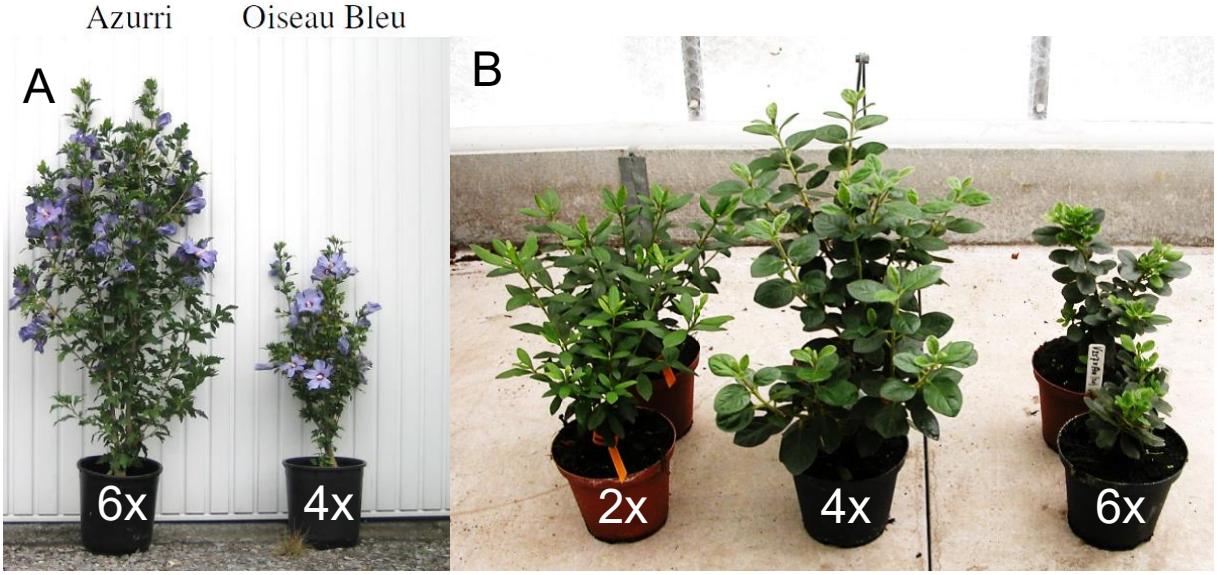


Figure 1.10: The effect of different ploidy levels on (A) *Hibiscus syriacus* and (B) Belgian azalea (*Rhododendron simsii* hybrids). (A) The tetraploid (4x) *Hibiscus* cultivar 'Oiseau Bleu' was polyploidized into an octaploid (8x). The hexaploid (6x) 'Azurri' is the result of a cross between 'Oiseau Bleu' (4x) and 'Oiseau Bleu' (8x). (B) A tetraploid azalea (4x) was created from the diploid 'Starlight'. The hexaploid (6x) was discovered in a 4x x 4x seedling population, a result of the tetraploid 'Starlight' x 'Casablanca tetra', an unilateral polyploidization by unreduced gametes. (Source: (A) (Van Laere, 2008); (B) (Eeckhaut et al., 2006)).

1.3.3 rol-gene introduction with rhizogenic *Agrobacterium* strains

1.3.3.1 Rhizogenic Agrobacterium strains and their Ri-plasmid

Agrobacterium rhizogenes was first described as a pathogen of economic importance by Riker et al. (1930) in a study on hairy root disease on apple. It was officially named and placed within the *Agrobacterium* genus in 1942 by Conn (1942) and was later changed to *Rhizobium rhizogenes.* It is a gram-negative, soil-dwelling pathogen with flagella, attacking roots. It has been studied as the cause of the 'hairy root' or 'crazy root' syndrome on several dicotyledonous crops, such as tomato, melon, cucumber and aubergine (Figure 1.11). Infected plants typically display extensive root proliferations, and strong vegetative growth, which causes a decline in fruit production in crops such as tomatoes (Bosmans et al., 2017).

Figure 1.11: Hairy root disease on 4-month-old hydroponically grown tomato plants. Left: healthy roots; right: extensive root proliferation caused by rhizogenic *Agrobacterium* biovar 1 (Source: Bosmans et al., 2017)

Virulent bacterial strains contain the Ri-plasmid, so-called for its root-inducing capacity. Wild type Ri-plasmids vary in size around 10-30 kbp, depending on the type (Chandra, 2012). Since bacteria can exchange plasmids, sometimes *A. tumefaciens* or *A. radiobacter* can contain the Ri-plasmid and also cause the 'hairy root' disease (Lacroix and Citovsky, 2016). Therefore, the virulent bacterial strains possessing the Ri-plasmid are referred to as 'rhizogenic *Agrobacterium* strains' (Bosmans et al., 2017). Biovar 1 is a complex of several species, including *A. tumefaciens* and *A. radiobacter*, but containing a Ri-plasmid instead of a Ti-plasmid, while biovar 2 strains have an *Rhizobium rhizogenes* background with a Ri-plasmid (Portier et al., 2006). Rhizogenic *Agrobacterium* strains can transfer the T-DNA (transfer-DNA) on the plasmid to plant cells during a natural infection process (Chen and Otten, 2017). Plant-derived signal molecules trigger the

virulence genes (vir) on the Ri-plasmid, and the resulting Vir-proteins mediate the transfer of the T-DNA into the host (Lacroix and Citovsky, 2016). The transfer process and the involved proteins are shown in Figure 1.12. VirD2 makes a single-stranded DNA molecule of the T-DNA, and stays connected to the 5' terminus. The coupling factor VirD4 transports the protein-T-DNA complex to the type IV secretion system (T4SS), a channel through the cell membrane composed of VirD4/VirB proteins, which transfers the T-DNA and accompanying proteins to the host cell cytoplasm. The VirE2 protein packages the T-DNA in a helical nucleoprotein complex and facilitates its passage into the nucleus. Subsequently, the DNA fragment is unwrapped, converted to a double-stranded form and inserted in the host-DNA by the hosts own DNA repair machinery (Lacroix and Citovsky, 2016). Thus, the gene transfer is a complex system, involving gene functions from the Ri-plasmid, the bacterial genome and the plant genome. The place of incorporation into the host-DNA is not specific, and can occur several times within one plant cell. This naturally occurring type of genetic engineering is called horizontal gene transfer (Lacroix and Citovsky, 2016, Kyndt et al., 2015).

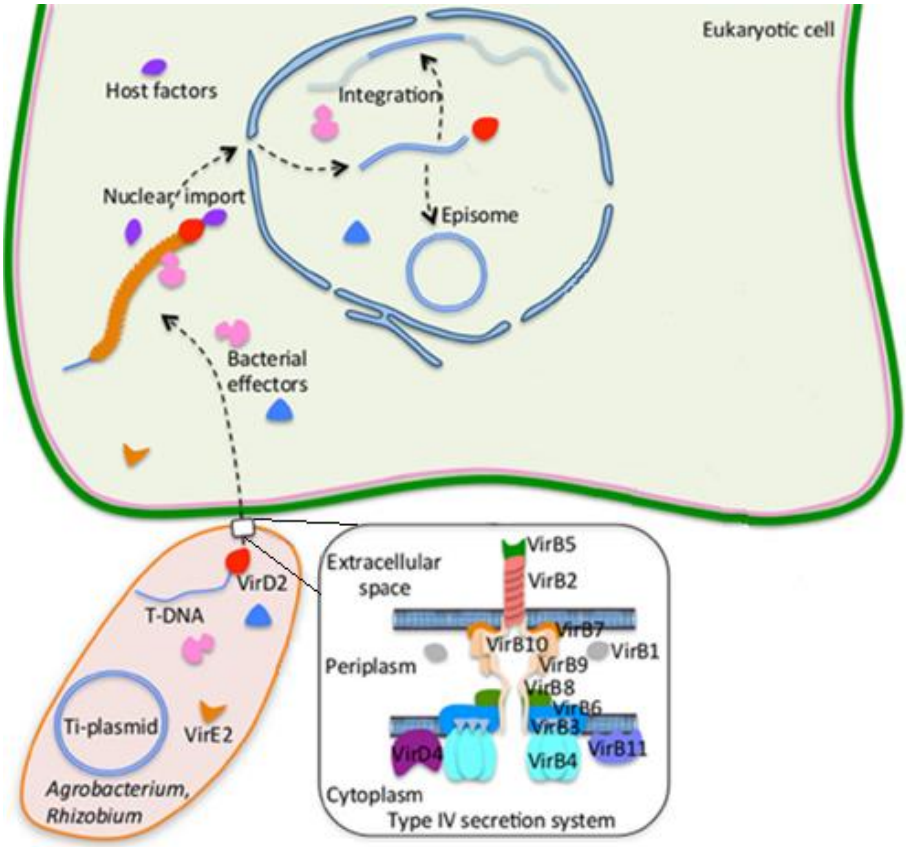


Figure 1.12: Schematic summary of the T-DNA transfer from bacteria to an eukaryotic cell by way of the type IV secretion system (T4SS). The VirD2 protein guides the single-stranded T-DNA to the eukaryotic cytoplasm by way of the T4SS, consisting of VirD4 and VirB proteins. The VirE2 proteins envelop the T-DNA for easier access to the nucleus. Once there, host factors take over the T-DNA and incorporate it into the host DNA. (Modified from Lacroix and Citovsky (2016))

The genes on the T-DNA can be divided in two functional groups, namely the root oncogenes, which cause neoplastic or tumorous growth of the host cells, and the opine biosynthesis genes, which cause the neoplastic growth on the host to produce certain opines and secrete them in the rhizosphere (Vladimirov et al., 2015). Opines are the product of condensed amino acids and sugars or ketoacid. They serve as source of energy and food for the rhizogenic *Agrobacterium* strains and are not present in non-transfected plant roots (Chilton et al., 1982, Vladimirov et al., 2015). By thus shaping their own niche with their own specific food, rhizogenic *Agrobacterium* can outcompete other rhizosphere-dwelling bacteria (Mauro et al., 2017). In nature, the gene-transfer of rhizogenic *Agrobacterium* strains to a plant host could be a survival strategy of the bacteria to make the plants produce opines in the rhizosphere. But it could also be possible that the gene-transfer increases the survival of the host plant by improving the plant fitness, indirectly assuring the opine production continues (Mauro et al., 2017, Arshad et al., 2014, Bettini et al., 2016a). The latter hypothesis is supported by the observation that some *Linaria* and *Nicotiana* species contain homologous sequences to rhizogenic *Agrobacterium* T-DNA genes fixed in their DNA after an infection somewhere during their evolution (Matveeva et al., 2012, Aoki et al., 1994, Matveeva and Sokornova, 2017, Suzuki et al., 2002).

Virulent rhizogenic *Agrobacterium* strains can be classified by their opine type, which are agropine, mannopine, cucumopine, and mikimopine strains (Vladimirov et al., 2015, Christensen and Müller, 2009b). The mikimopine and cucumopine (Figure 1.13) strains possess the *mis* gene (mikimopine synthase) and the *cus* gene (cucumopine synthase) respectively (Vladimirov et al., 2015).

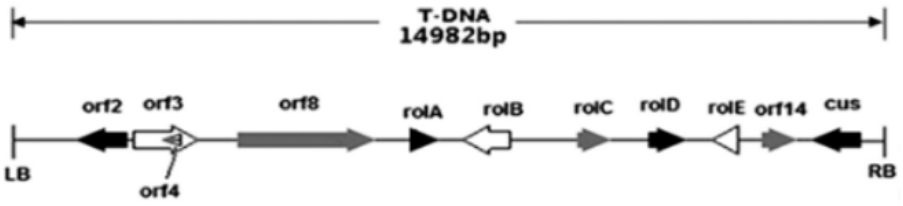


Figure 1.13: Schematic representation of the ORFs (open reading frames) on the transfer DNA (T-DNA) on the Ri-Plasmid pRi2659 of the cucumopine type NCPPB 2659. (Source: (Xiang et al., 2016)).

On the contrary, mannopine and agropine type strains have a multistage biosynthesis pathway to produce their opines. Mannopine plasmids possess the *mas1* and *mas2* genes (mannopine synthase), forming mannopine from glucose and glutamine. The agropine strains have both *mas1* and *mas2* genes, and first produce mannopine, which is then converted to agropine by the *ags*

gene (agropine synthase). Agropine strains have a TL- (left) and TR-(right) DNA (Figure 1.14), all other opine type strains have only one single T-DNA. The TR-DNA shows many similarities to the T-DNA found in Ti-plasmids of *A. tumefaciens*, while the TL-DNA displays no homologues, except the 25-bp terminal sequences at the left and right border of the TL (Nilsson and Olsson, 1997, Slightom et al., 1986). The TR-DNA of agropine strains possess the *mas1*, *mas2,* and *ags* genes. The most commonly used rhizogenic *Agrobacterium* strains are classified by type in Table 1.1 (Christensen and Müller, 2009b).

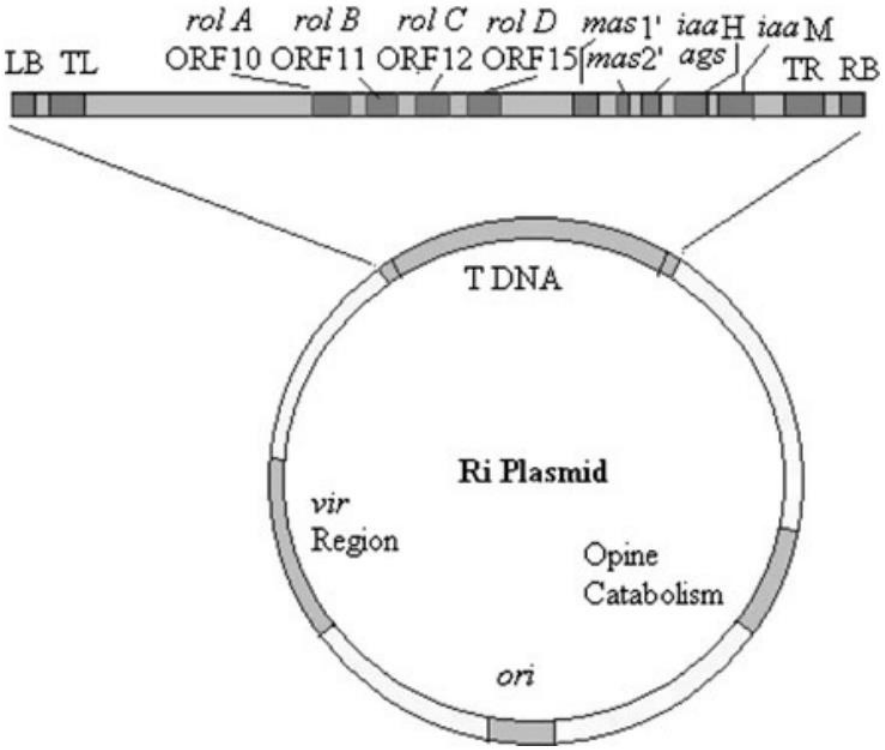


Figure 1.14: Schematic representation of the Ri-plasmid (root-inducing) of an agropine type strain. LB: left border; TL: left T-DNA; TR: right T-DNA; RB: right border. (Source: (Chandra, 2012))

Besides genes for opine synthesis, the T-DNA has four *rol*-genes (root oncogenic loci), *rolA, rolB, rolC,* and *rolD*. They correspond with ORFs 10, 11, 12, and 15 respectively. ORFs 3, 8, 13 and 14 have also been identified as containing oncogenes, but their effect is less studied (Lutken et al., 2012, Slightom et al., 1986, Lemcke and Schmülling, 2002, Wang et al., 2016, Kodahl et al., 2016). The *rol*-genes are not active when in the Ri-plasmid (except for *rolA,* see further), but are transcriptionally activated after integration in the plant genome. Shoots that are regenerated on roots with the T-DNA integrated, also contain and express that T-DNA. The *rol*-genes cause an effect on the phenotype of the regenerated plants, indicated as Ri-phenotype.

Table 1.1: The most commonly used rhizogenic *Agrobacterium* strains classified according to opine-type [z].

Opine-type	Ri-Plasmid	Strain [y]	Biovar	Reference
Agropine	pRiA4	A4, ATCC 43057	2	(Petit et al., 1983)
	pRi15834	ATCC 15834	2	(Costantino et al., 1981, Petit et al., 1983)
	pRi1855	NCPPB 1855	2	(Costantino et al., 1981, Filetici et al., 1987)
Mannopine	pRi8196	NCIB 8196	2	(Costantino et al., 1981)
Cucumopine	pRi2659	NCPPB 2659	1	(Combard et al., 1987, Xiang et al., 2016)
Mikimopine	pRi1724	MAFF03-01724	1	(Isogai et al., 1988, Moriguchi et al., 2000)
		MAFF02-10266	1	(Isogai et al., 1988, Moriguchi et al., 2000)

z) Modified from Christensen and Müller (2009b)

y) Strains used in this thesis are underlined

1.3.3.2 The pleiotropic effects of rol-genes on the plant

The changes caused by introduction of *rol*-genes can be phenotypic, e.g., changes in growth and branching, leaf and flower characteristics and rooting ability, but also physiological, e.g., changes in secondary metabolite production (reviewed by Georgiev et al. (2012)), phytoremediation (Malandrino et al., 2017), disease resistance and tolerance to biotic and abiotic stresses. In several studies, plants were infected with rhizogenic *Agrobacterium* strains with a single *rol*-gene or different combinations of *rol*-genes, to investigate the contribution of each gene to the hairy root phenotype and their interactions. Each individual *rol*-gene causes different effects by themselves, but different *rol*-genes also have complicated interactions with each other, either intensifying the effect or counteracting.

The ***rolA***-gene has no clear identified role yet. *rolA* encodes for a small (11.4 kDa) basic protein with no homology to known proteins (Vilaine et al., 1998, Rigden and Carneiro, 1999). *RolA* folds comparable to the bovine papillomavirus-1 E2 DNA binding domain, and has been observed to bind to DNA (Rigden and Carneiro, 1999). A *RolA*-GUS construct showed the presence of the RolA protein in the cell membrane fraction, proposing that it could be a non-integral, cell membrane-associated protein (Vilaine et al., 1998). Probably, it has a role in protecting the cells from proteolysis by interfering with the protein degradation pathway regulated by auxin, resulting in an increase in protein stability (Barros et al., 2003). RolA also causes changes in gibberellin content (Schmülling et al., 1993), auxin content (Schmülling et al., 1993, Bettini et al., 2016b) and abscisic acid content (Bettini et al., 2016b).

The *rolA* promotor contains 3 domains, A, B and C. The level of accumulation of *rolA* mRNA in each organ is determined by the cooperation of distinct domains of the *rolA* promotor (Carneiro and Vilaine, 1993). The three domains work cooperatively for *rolA* expression in leaves. But in stems and roots, domain A has an inhibitory effect on domain B and C (Carneiro and Vilaine, 1993).

The *rolA*-gene is the only *rol*-gene that is transcribed in the rhizogenic *Agrobacterium* strains themselves, driven by a spliceosomal intron that contains a prokaryotic promotor (Magrelli et al., 1994, Pandolfini et al., 2000). Spliceosomal introns are removed during RNA splicing by eukaryotes, but not by prokaryotes. Its expression in the bacteria is highest during the stationary growth phase and might play a role during conditions of high cell density (Pandolfini et al., 2000). The *rolA*-gene causes phenotypic changes, such as wrinkled leaves and a stunted growth with shortened internodes, e.g., in rice (Lee et al., 2001), tomato (Bettini et al., 2016b), apple rootstock (Xue et al., 2008, Holefors et al., 1998) and tobacco (Carneiro and Vilaine, 1993). Wrinkled leaves in tobacco could be assigned to *rolA,* since the leaves of regenerated tobacco plants with *rolA* were wrinkled, and this did not occur in any transformant without *rolA* introgression (Figure 1.15) (Sinkar et al., 1988a, Schmulling et al., 1988). Furthermore, a delayed flowering, small flowers and a reduction of male fertility could be observed, e.g., in tomato, which resulted in small fruits (Bettini et al., 2016b), and in tobacco (MartinTanguy et al., 1996).

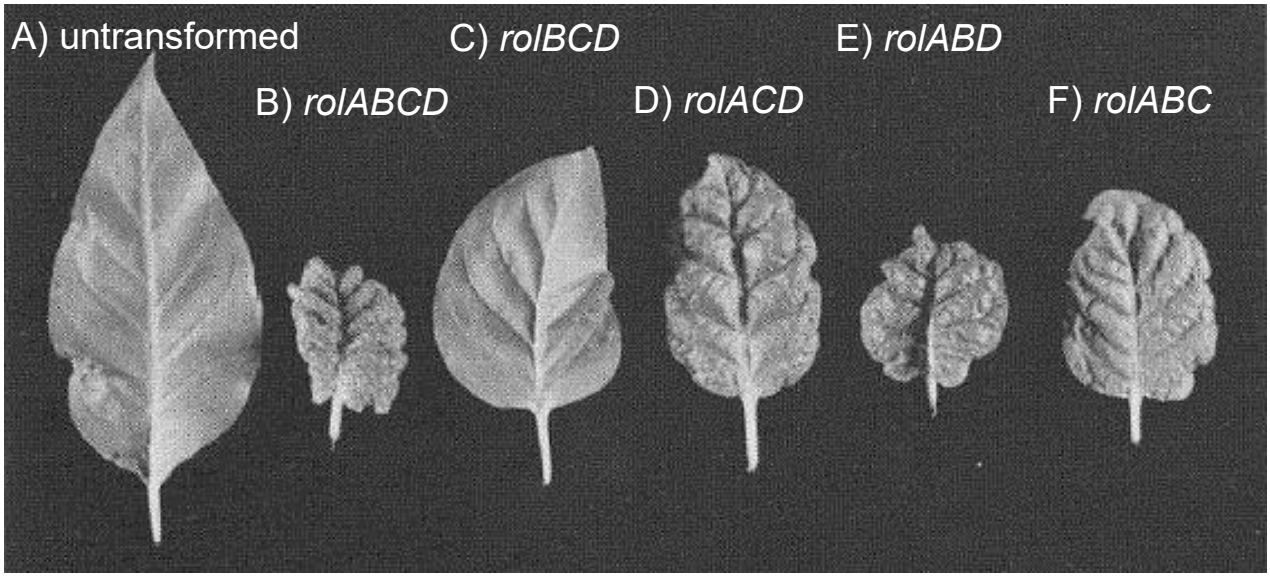


Figure 1.15: Comparison of leaves of *Nicotiana tabacum* transformed with different rol-combinations. A) untransformed, B) transformed with wild type rhizogenic *Agrobacterium* strain A4 with *rolABCD*, C) *rolBCD*, D) *rolACD*, E) *rolABD*, F) *rolABC*. The wrinkled leaf phenotype is only visible in transformants containing rolA. (Modified from Sinkar et al. (1988a)).

Changes in the level of secondary metabolites and disease resistance have been observed. Tomatoes with *rolA* showed an increased tolerance to *Fusarium* (Bettini et al., 2016b). A higher level of the secondary metabolites anthraquinones was observed in *Rubia cordifolia* calli (Shkryl et al., 2008).

rolA is also capable of inducing roots on species, but to a lesser extent than *rolB* (Spena et al., 1987a, Vilaine et al., 1998) (see further). For example, root induction on *in vitro* tissues was possible in tobacco (Carneiro and Vilaine, 1993, Spena et al., 1987b) and not in *Kalanchoë* (Spena et al., 1987b).

The ***rolB*** gene is the most studied of the four *rol*-genes. It is the crucial gene in promoting hairy root initiation and elongation. The roots are characterized by fast growth, high branching and plagiotropism. The RolB protein has a tyrosine phosphatase activity (Baumann et al., 1999, Filippini et al., 1996). It removes phosphate groups from phosphorylated tyrosine residues on proteins. This has an influence on the auxin signal transduction pathway (Shankar et al., 2015). Consequently the RolB protein causes a 10^3 to 10^4-fold increase in auxin sensitivity, which is likely the trigger for organogenesis of meristems (Baumann et al., 1999). According to Altamura et al. (1994), the effect of *rolB* is not the induction of rooting specifically, but an increased and earlier meristem formation, resulting in an enhanced flowering and root formation in tobacco. The *rolB* gene does not impose root regeneration upon a cell, but rather enhances the regeneration process in already differentiated cells (Di Cola et al., 1996, Altamura, 2004).

Little is known about the actual transcriptional response of the plant after transformation with *rolB.* A study of differentially expressed genes (DEGs) in *rolB*-tomato revealed an overexpression of chloroplast functional genes, resulting in a more efficient protection of the photosynthetic apparatus from excess energy, and a more efficient use of low light for photosynthesis (Bettini et al., 2016a). This could be a strategy to counteract the negative effect of the increased root mass and opine production to help the plant to survive after infection, and subsequently to provide opines for the bacteria.

It has recently been shown that many of the traits generated by *rolB* can be attributed to RNA-silencing with microRNA (miRNA) (Bulgakov et al., 2015). The overexpression of miRNAs in plants has often been mentioned in literature, and this is usually associated with the occurrence of biotic or abiotic stresses (reviewed by Noman et al. (2017) and Wang et al. (2017b)). *rolB* increases the expression of several genes encoding for components that are essential to the miRNA processing machinery (Bulgakov et al., 2015).

The *rolB*-promotor is developmentally regulated, as its activity correlates with the state of differentiation of the plant cells. Altamura et al. (1991) visualized the *rolB* activity with a GUS reporter gene in tobacco tissues, and noticed its activity only in initial cells in all types of meristems. They found five domains (A to E) on the promotor, each responsible for the expression in a different tissue (phloem, root, pericycle, shoot and flower).

Typical phenotypic changes for *rolB*-plants are an increased rooting capacity, earlier flowering with more flowers, an early necrosis of leaves, and a reduced growth due to reductions in internode length and apical dominance, e.g., in *Arabidopsis* (Kodahl et al., 2016), tomato (Arshad et al., 2014), tobacco (Schmülling et al., 1988), pear rootstock (Zhu et al., 2003), apple rootstock (Zhu et al., 2001), and kiwi trees (Rugini et al., 1991). However, no increase in rooting ability was present in *rolB-Rosa hybrida* (van der Salm et al., 1997). Tomato plants with *rolB* carried the same number of flowers as the control, but due to a loss of pollen viability, less fruits were produced. Fruits from a *rolB*-tomato plant were smaller but ripened faster (Figure 1.16) (Arshad et al., 2014).

Figure 1.16: Phenotypical changes of 2 *rolB* transformant tomato plants cv. 'Rio Grande' showing A) a growth reduction in mature plants containing *rolB*, and B) a size reduction of fruits. Modified from (Arshad et al., 2014).

From all four *rol*-genes, *rolB* influences secondary metabolism strongest (Bulgakov, 2008). A decrease in ginsenoside production was observed in ginseng callus cell lines (Bulgakov et al., 1998). A 15-fold increased anthraquinones production occurred in *rolB*-transfomed *Rubia cordifolia* calli (Shkryl et al., 2008) and a 100-fold increase of resveratrol in *Vitis amurensis* (Kiselev et al., 2007). In *rolB* tomato, an increase in lycopene, ascorbic acid, phenolics and free radical

scavenging activity was observed, resulting amongst others in an increased defense to fungi (Arshad et al., 2014). Other studies also indicated that *rolB* plays a role in the tolerance of transformed plants to biotic and abiotic stresses. The *rolB*-gene caused an increase in expression of antioxidant genes which decreased the level of reactive oxygen species (ROS) in several plant species transformed with *rolB* (*Rubia cordifolia, Panax ginseng* and *Arabidopsis thaliana*) (Bulgakov et al., 2012, Veremeichik et al., 2012).

It has been proposed that the ***rolC***-gene codes for a β-glucosidase, which hydrolyses inactive cytokinin glucosides, and subsequently releases free cytokinins (Estruch et al., 1991). However, Schmülling et al. (1993) did not confirm this on tobacco and potato transformants. Although *rolC* overexpression indeed led to a small increase of free cytokinins in tobacco and potato transformants, only a few of the phenotypic changes that an increase in cytokinins would cause, were visible on the *rolC*-plants. Furthermore, the rooting characteristics and reduction of chlorophyll content in the leaves that are typical for *rolC*-transformants, could not be induced by application of exogenous cytokinins nor by the expression of cytokinin synthesizing genes. Overexpression of *rolC* also led to an altered response to plant hormones, such as auxins, cytokinins, abscisic acid (ABA), gibberellic acid and the ethylene precursor 1-aminocyclopropanecarboxylic acid, e.g., in tobacco (Schmülling et al., 1993), tomato (Bettini et al., 2010) and *Chrysanthemum* (Mitiouchkina and Dolgov, 2000). The increased rooting ability of *rolC*-plants also indicates an influence of RolC on the auxin sensitivity or pathway (Kaneyoshi and Kobayashi, 1999, Zuker et al., 2001, Koshita et al., 2002).

The *rolC* promotor is highly active during seed germination and embryotic development, and is expressed in phloem tissue, bundle sheath cells and vascular parenchyma (Graham et al., 1997, Sugaya and Uchimiya, 1992, Fujii et al., 1994). Several control elements were found that regulate the *rolC* promotor activity in phloem, leaves, roots and seedlings (Sugaya and Uchimiya, 1992). A sucrose responsive region was found in the *rolC* promotor region for expression in phloem, indicating that sucrose can act here as a signal molecule (Yokoyama et al., 1994).

rolC expression causes dwarfism by shortening internodes, reducing apical dominance and increasing the formation of axillary shoots, e.g., in tobacco (Schmülling et al., 1988), Japanese persimmon trees (Koshita et al., 2002), carnation (Zuker et al., 2001), *Chrysanthemum* (Mitiouchkina and Dolgov, 2000) and orange trees (Kaneyoshi and Kobayashi, 1999). Furthermore, a reduction of flower size and male fertility was observed in *rolC*-tobacco (Schmülling et al., 1988). For *rolC*-carnations, the flowers were smaller in some transformants, but not in all. However, more flowering stems were produced, which is a desirable trait in a flowering ornamental

(Zuker et al., 2001). Also in *Chrysanthemum,* a reduction in size of the floral head, but an increase in the number of floral heads was observed (Mitiouchkina and Dolgov, 2000).
rolC affects the secondary metabolite production, e.g., a threefold increase in ginsenosides was observed in *rolC-Panax ginseng* hairy root cultures (Bulgakov et al., 1998), a rise in phenolic and flavonoid content in *Lactuca* (Ismail et al., 2016) and a resveratrol increase in *Vitis* callus cultures (Dubrovina et al., 2010).
rolC-transformed potatoes had an increased resistance to the potato leaf roll virus (PLRV), which is a phloem-limited virus (Graham et al., 1997). No increased resistance was observed in *rolC*-tobacco with the tobacco mosaic virus (TMV) (Reimann-Philipp and Beachy, 1993), which does not replicate in the vascular tissue, were *rolC* is very active.
Although *rolC* in itself was not capable of inducing roots on *in vitro* leaf discs, hairy roots of *rolBC*-tobacco was much more branched and had more vigorous growing roots compared to hairy roots of *rolB*-tobacco (Schmülling et al., 1988). Cuttings from *rolC*-transformed plants also showed increased rooting ability in *ex vitro* conditions e.g., in fruit trees (Koshita et al., 2002, Kaneyoshi and Kobayashi, 1999) and carnations (Zuker et al., 2001).

The ***rolD***-gene encodes for an ornithine cyclo-deaminase, which catalyzes the conversion of ornithine to proline (Trovato et al., 2001). Proline has been shown to play an important role in the transition from vegetative to reproductive state, for inducing inflorescence formation (Mattioli et al., 2008).
rolD expression exhibits a strict developmental control, it is highly expressed in differentiated tissues in each organ, its activity is highest in mature tissues, decreasing slowly with age until switched of at senescence (Mauro et al., 2017, Trovato et al., 1997). The *rolD*-promotor is often used in gene-constructs to regulate the expression of genes (Wan et al., 2012, Kang et al., 2011). The *rolD*-gene causes earlier flowering and an increase in flower number, due to a rise in proline level (Altamura, 2004). This was confirmed in *rolD*-tomatoes, they developed a significantly higher number of flowers per inflorescence, of flowers transitioning into fruits and of fruits per plant with the same fruit weight. Furthermore, *rolD*-tomatoes also showed an increased tolerance towards *Fusarium* (Bettini et al., 2003). The same phenotypic changes were observed in *rolD-Arabidopsis* (Falasca et al., 2010).

All *rol*-genes alter the expression of CDPK (calcium-dependent protein kinase) in plants. CDPKs have an impact on biotic and abiotic stress signaling (Harmon et al., 2000, Harper et al., 2004, Romeis et al., 2001). The alteration of the *CDPK*-gene expression by *rolC* in *Panax ginseng* contributed to the formation of somatic embryos (Kiselev et al., 2009, Kiselev et al., 2008), and an

increased salt tolerance in cell cultures (Kiselev et al., 2010). Also in pRiA4-transformed calli of *Rubia cordifolia* a considerable change in *CDPK* expression profiles was observed. It appeared that *CDPK* genes who were involved in ROS (reactive oxygen species) production were downregulated, while *CDPK*-genes involved in ROS-detoxification were upregulated, enhancing stress tolerance (Veremeichik et al., 2014). Plant pathogens try to decrease ROS levels in plants, which increases their chances of survival (Bretz et al., 2003, Underwood et al., 2007). In addition, this decrease in ROS levels also enhances the stress tolerance of the host plant itself. As previously mentioned, *rolB* decreased the ROS level in several plant species (*Rubia cordifolia, Panax ginseng* and *Arabidopsis thaliana*) (Bulgakov et al., 2012, Veremeichik et al., 2012). Several other antioxidant genes were also upregulated in *Rubia cordifolia* through the *rol*-genes, such as the ascorbate peroxidase gene *RcApx1* and the Cu/Zn superoxide dismutase gene *RcCSD1* (Shkryl et al., 2010). Several researchers used *rol*-gene transformations to increase the production of a secondary metabolite, e.g., for medicinal purposes in *Rubia tinctorum* (Perassolo et al., 2017), *Lactuca sativa* (Ismail et al., 2017, Ismail et al., 2016), *Daucus carota* (Rachamallu, 2016) *Withania somnifera* (Thilip et al., 2015), *Berberis aristata* (Brijwal and Tamta, 2015), *Agastache foeniculum* (Nourozi et al., 2014), *Gentiana scabra* (Huang et al., 2014), *Arnica montana* (Petrova et al., 2013), and *Rhinacanthus nasutus* (Cheruvathur et al., 2015), for the production of essential oils in *Lavandula* (Tsuro and Ikedo, 2011), or for the improvement of nutritional quality in tomato (Arshad et al., 2014).

1.3.3.3 Introduction of T-DNA from wild type rhizogenic Agrobacterium strains

The introduction of *rol*-genes has been used by ornamental breeders to increase the product range and to improve production and post-harvest performance, to meet the demands of both consumers and producers (Christensen and Müller, 2009b). The intensity of the effect of the *rol*-genes on the plant can vary between and within plant species, ranging from non-changed phenotype to a strong Ri-phenotype, due to several reasons (Christensen and Müller, 2009b, Christensen et al., 2008, Baumann et al., 1999, Carneiro and Vilaine, 1993). The degree of alterations is correlated with the gene copy number that was inserted into the host DNA, the place of insertion, and whether the T-DNA is inserted completely, or only fragments. For example, a higher copy number of *rolA,* caused a higher level of *rolA* expression in *rolA*-tomatoes, which reduced the fitness severely and caused a high susceptibility for *Fusarium* infection. Tomatoes with less *rolA*-copies had a lower level of expression which caused an increased tolerance for *Fusarium* (Bettini et al., 2016b). Also

the rhizogenic *Agrobacterium* strains itself can cause different effects, e.g., strains with the pRiA4 agropine plasmid also contain auxin genes (*aux1* and *aux2*), which are not located on the mannopine, mikimopine and cucumopine type plasmids. This variation in Ri-phenotype allows for further selection for desired characteristics. The Ri-phenotype is dominantly inherited in a Mendelian fashion (Lütken et al., 2012, Durand-Tardif et al., 1985), and is stable via vegetative propagation (Pellegrineschi et al., 1994). However, on plants with the Ri-phenotype, lateral shoots with a non-Ri-phenotype have occasionally been observed, due to a transcriptional inactivation of the T-DNA genes (Sinkar et al., 1988b, Tepfer, 1984).

The phenotype of many different plant species transformed with the pRi-plasmid of natural occurring rhizogenic *Agrobacterium* strains is reviewed by Christensen and Müller (2009b) and Casanova et al. (2005). The introduction of *rol*-genes in *Kalanchoë blossfeldiana*, a potted, flowering ornamental, resulted in 6 Ri-lines with a reduced height due to a decrease in internode length. The number of lateral shoots was either increased, decreased, or stayed the same. Leaves were wrinkled, but the Ri-lines differed in degree of wrinkling. A delay of flowering was observed in some Ri-lines, but not in all. Inflorescences were more dense, smaller, and contained less flowers, which were also smaller (Christensen et al., 2008). They also displayed a reduced dry weight of the shoots, leaves and flowers, due to a reduced plant height, leaf area and number of flowers. A change in biomass distribution was observed, a part of the biomass shifted from flowers to leaves, and an increase in vegetative lateral shoots was seen, while reproductive lateral shoots decreased (Christensen et al., 2009). Although the number of flowers decreased, the longevity of detached flowers increased with several days and were less responsive to ethylene (Christensen and Müller, 2009a). The inheritance of the *rol*-genes into the F2 progeny was studied by crossing a transformed cultivar ‘Molly’ with a non-transformed cultivar ‘Sarah’ (Figure 1.17). The F2 progeny containing *rol*-genes showed a wider variation in compactness than the F1 generation, which is a very interesting feature for breeders to select for plants with the desired Ri-phenotype (Lütken et al., 2012).

Rehmannia elata, a herbaceous ornamental known as Chinese foxglove, with *rol*-genes, displayed a reduced plant height due to an increased number of lateral shoots and a decrease in internode length, slightly wrinkled leaves with a reduced size, and a higher number of flowers. These changes in phenotype varied between three transformants (Kim et al., 2012). *In vitro* Ri-lines of kiwi (*Actinidia deliciosa*) displayed a slow initial growth, a high rooting ability and darker, wrinkled leaves. However, after acclimation and potting, the characteristics diminished (Rugini et al., 1991).

Regenerated plants from *Plumbago rosea* showed extremely shortened internodes resulting in a rosette appearance (Satheeshkumar et al., 2009). Furthermore, wrinkled leaves and an abundant root formation was present. *Limonium* is a genus of perennial herbs species with a woody rhizome, used as fresh and dried cut flower. Transformation resulted in early flowering, a higher flower density, a compact plant habitus, reduced leaf area, and an increased rooting system. Flowers were smaller, but more numerous (Mercuri et al., 2001). A super-compact, compact, and semi-compact type were recovered. The degree of dwarfness could not be explained by the number of T-DNA copies, since each type contained two (Mercuri et al., 2003). Several other studies on herbaceous species indicated regeneration on hairy roots, but without further phenotyping of the regenerants, e.g., in several *Campanula* species (Hegelund et al., 2017), *Centaurium erythraea* (Subotic et al., 2003) and *Amaranthus* (Swain et al., 2010).

Although many woody species are known for their recalcitrance *in vitro* (Rastogi et al., 2008, Rugini et al., 1991), several woody species were also successfully transformed and regenerated. *Lavandula* x *intermedia*, an ornamental shrub valued for its scent, was transformed by Tsuro and Ikedo (2011) and 85 regenerated plants were recovered. Of these, 49 died in the field at the beginning of the summer, probably due to an increased sensitivity to the seasonal changes. The 26 surviving plants showed dwarfism and extensive lateral branching. Only 9 plants flowered, with a 1 month delay compared to non-transformed plants, and with very short flower stalks. Ri-lines of silver birch (*Betula pendula*), an important tree species for wood production, displayed a shorter, slower, and more bushy growth, with smaller leaves and a larger root system in some regenerants without the TR-DNA, and a more vigorous, but delayed growth in plants with TR-DNA and thus containing both *rol*-genes and auxin genes (Figure 1.18) (Piispanen et al., 2003).

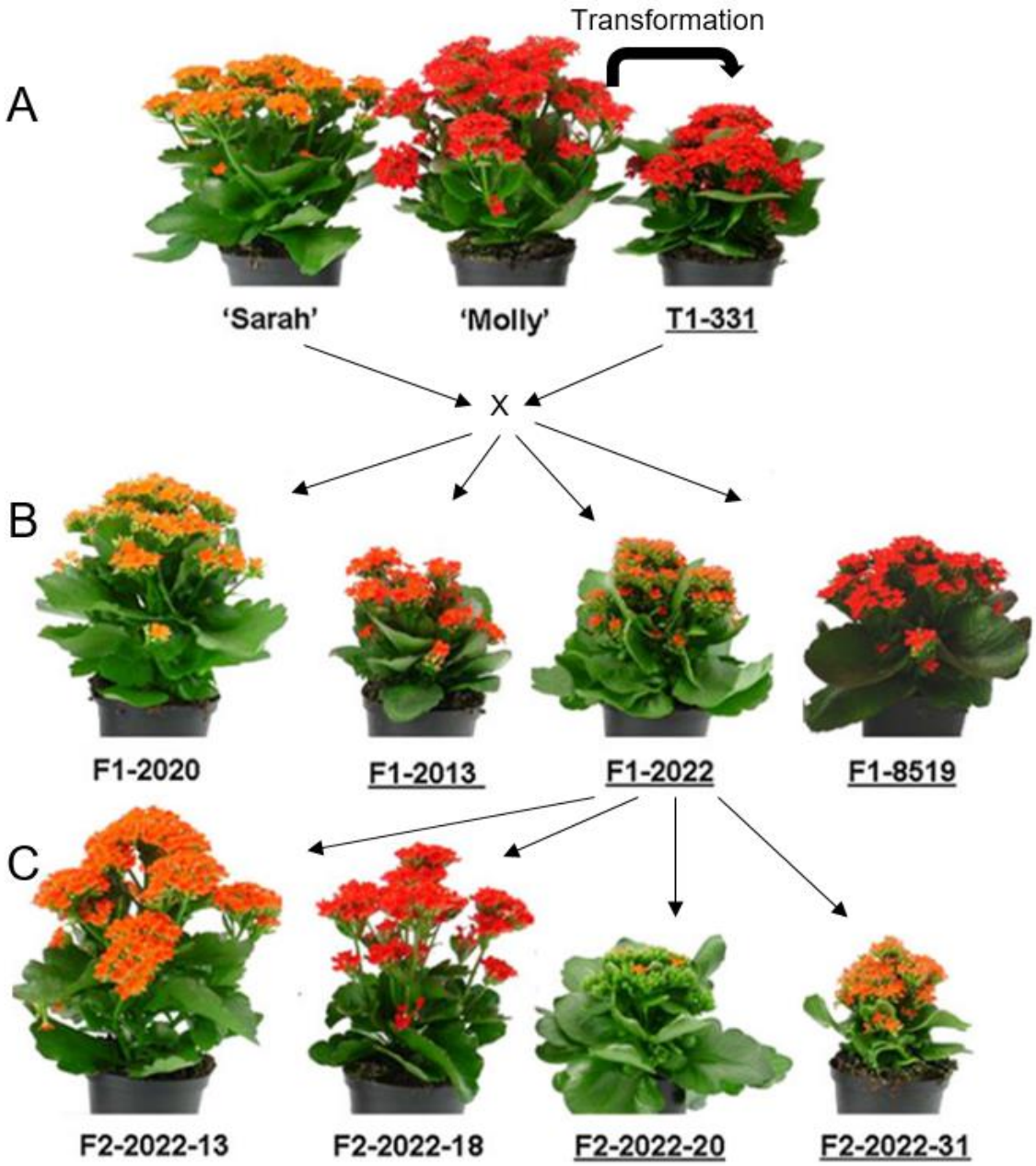


Figure 1.17: Overview of different *Kalanchoë blossfeldiana* lines at an age of 102 days. A) The commercial cultivar 'Molly' (Knud Jepsen A/S, Denmark) was transformed with the wild type rhizogenic *Agrobacterium* strain ATCC15834 (Christensen et al., 2008). The transformant T1-331 was crossed with the commercial cultivar 'Sarah' (Knud Jepsen A/S, Denmark), resulting in B) the F1 plants. The plant F1-2022 was selfed, resulting in C) the F2 generation. Plants were *rol*-genes are present are underlined. (Modified from (Lütken et al., 2012)).

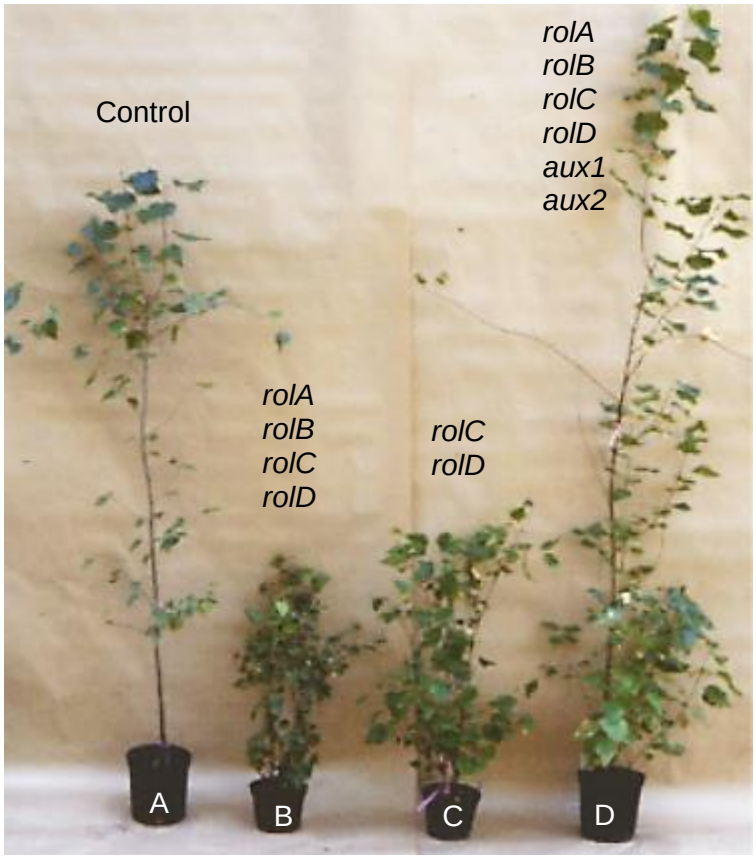


Figure 1.18: Several phenotypes of *Betula pendula*, transformed with a rhizogenic *Agrobacterium* strain containing pRiA4, retaining different genes, indicated in the figure (Modified from Piispanen et al. (2003)).

Regenerants of *Malus baccata*, an apple rootstock, showed a decrease in plant height, etiolated, wrinkled and/or clustered leaves and a vigorous rooting. The Ri-lines also displayed an increase in survival rate during acclimation in the greenhouse (Wu et al., 2012). *Aralia elata,* a woody shrub which produces secondary metabolites with medical properties, was also successfully transformed with *rol*-genes. The regenerated shoots from Ri-lines displayed an increased survival rate during acclimation, wrinkled leaves with shorter petioles and prolific rooting (Kang et al., 2006). In *Catharanthus roseus,* regenerated shoots showed prolific rooting with extensive lateral branching, wrinkled leaves and shortened internodes (Choi et al., 2004). However, the regenerants did not always exhibit all three of these characteristics. Ri-lines were derived from *Aesculus hippocastanum*, an ornamental tree. The regenerated plants showed the typical Ri-phenotype, but in variating degree, including a decreased plant height and more narrow and darker green leaves with a shortened petiole. However, no wrinkled leaves were observed (Zdravkovic-Korac et al., 2004). The induction of *rol*-genes into woody species with wild type

rhizogenic *Agrobacterium* strains have also been conducted on *Hibiscus rosa-sinensis* (Christensen et al., 2015), but no successful regeneration was reported as yet.

1.3.3.4 rol-gene introduction and regeneration

Different techniques are used for the introduction of the *rol*-genes into plant material such as injection of the rhizogenic *Agrobacterium* strains suspension into the plant tissue (Majumdar et al., 2011) or wounding the plant tissue with a scalpel dipped in a bacterial culture (Perassolo et al., 2017, Nourozi et al., 2014, Alpizar et al., 2006). A commonly used transformation procedure is co-cultivation of the host-plant material with a culture of actively growing rhizogenic bacteria (Christensen and Müller, 2009b, Christey and Braun, 2005). This technique has been used frequently and with varying success in several plant species, such as *Campanula* (Hegelund et al., 2017), *Rhinacanthus* (Cheruvathur et al., 2015), *Gentiana* (Huang et al., 2014), *Arnica* (Petrova et al., 2013), and *Rehmannia* (Kim et al., 2012), and even in woody species, such as *Berberis* (Brijwal and Tamta, 2015), *Lavandula* (Tsuro and Ikedo, 2011), *Aralia* (Kang et al., 2006), and *Juglans* (Falasca et al., 2000).

Firstly, a suspension of rhizogenic *Agrobacterium* strains and plant explants are prepared. The explants are immersed into the bacterial suspension and placed on a co-cultivation medium containing acetosyringone or other wound signaling molecules. After the co-cultivation period, the explants are submerged in a liquid medium containing antibiotics and placed on a solid medium with antibiotics, to eliminate the bacteria.

Co-cultivation of a plant species with a rhizogenic *Agrobacterium* strains can result in the production of hairy roots, easily distinguished by their vigorous, branched, plagiotropic growth and their hairy appearance. The morphology of hairy roots at the inoculation site can differ considerably between plant species and bacterial strains in thickness, branching, hairiness, age and growth rate. Several factors can influence the efficiency of the hairy root induction, as indicated in Figure 1.12. Firstly, the bacterial factors need to be considered. Acetosyringone or other molecules indicating wounded plant material are added to increase *vir* activity (Ismail et al., 2017). Secondly, plant factors influence the efficiency of hairy root formation. Many explant types have been shown to be susceptible for infection by rhizogenic *Agrobacterium* strains, such as leaves, stems, petioles, shoots, roots, hypocotyls, embryos, etc. The most important characteristic for the explant is the presence of a cut or wounded surface (Christey and Braun, 2005). However, explants differ in susceptibility within one plant species. Thirdly, the host-pathogen relationship needs to be considered, as every bacterial strain has its own host plant spectrum.

When hairy roots are harvested, shoots need to be regenerated. Herbaceous species are generally easier to propagate and regenerate *in vitro.* For *Rehmannia elata,* shoot regeneration from hairy roots even occurred spontaneously, starting 2 weeks after excising the roots, and 2-3 shoots were produced per hairy root explant (Kim et al., 2012). However, most other species need addition of plant growth hormones in the medium to promote shoot regeneration. The hormone balance and concentrations required are dependent on the plant species of interest. Sometimes direct regeneration is not possible, and a callus phase with somatic embryogenesis is required. And even then, some species are recalcitrant and did not regenerate shoots on the hairy root cultures (Christensen and Müller, 2009b). For *Kalanchoë*, shoots developed after 4 weeks on regeneration medium with N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) or thidiazuron (TDZ) (Christensen et al., 2008). For *Campanula,* only 1 out of 3 species successfully regenerated shoots on the hairy roots, although they were all tested on the same media (Hegelund et al., 2017). The efficiency of the regeneration in *Catharanthus roseus* was highly dependent on the genotype used (Choi et al., 2004). This shows that the variation in response to exogenous plant hormones for shoot regeneration can be high, and the regeneration procedure can be different for every species. For transformations in new genera, several species or genotypes should be included to maximize the chance on successful regeneration (Hegelund et al., 2017, Choi et al., 2004). Before shoots can be acclimatized in the greenhouse, the plantlets are checked for the absence of rhizogenic *Agrobacterium* strains, by conducting a PCR analysis for the *vir*-genes (Christey and Braun, 2005). Therefore, the choice and concentration of antibiotics added to the medium is paramount.

1.3.3.5 Regulation of products resulting from Agrobacterium transformation

A genetically modified organism (GMO) is defined by the European Union (EU) in Directive 2001/18, Article 2(2) as "an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination". Annex I A, part 2 lists several techniques that are not considered to result in genetic modification, which includes natural processes such as conjugation, transduction and transformation. However, these processes may not involve the use of recombinant DNA or RNA molecules or GMOs as defined in Annex I A, part 1. This means that the introduction of the T-DNA from a wild type rhizogenic *Agrobacterium* strain into the plant genome is seen as a naturally occurring process, and therefore is not considered as a GMO. Regenerated shoots on the resulting hairy roots contain this T-DNA or parts of it, and can thus be used for further breeding. Cross-overs during meiosis can result in progeny containing only one *rol*-gene or a combination

of *rol*-genes. These plants are also considered as the result from a natural process and thus not a GMOs. In this study, only wild type strains of rhizogenic *Agrobacterium* species are used, and the resulting plants are not GMOs. However, if the Ri-plasmid of the rhizogenic *Agrobacterium* strain has been altered, e.g., by adding GUS (glucuronidase) (Barros et al., 2003) for detection, by changing the promotors of the *rol*-genes with the CaMV 35S promotor (Cauliflower Mosaic Virus) (Arshad et al., 2014, Lee et al., 2001), or when the Ri-plasmid was exchanged for an artificially created vector containing only pieces of the T-DNA (Kiselev et al., 2010), then the resulting regenerated shoots are considered as GMOs. These techniques can be valuable to do research on the effect of each individual gene and its promotor on the plants (Bettini et al., 2016a, Bettini et al., 2016b), but can never be brought outside of the lab in the EU.

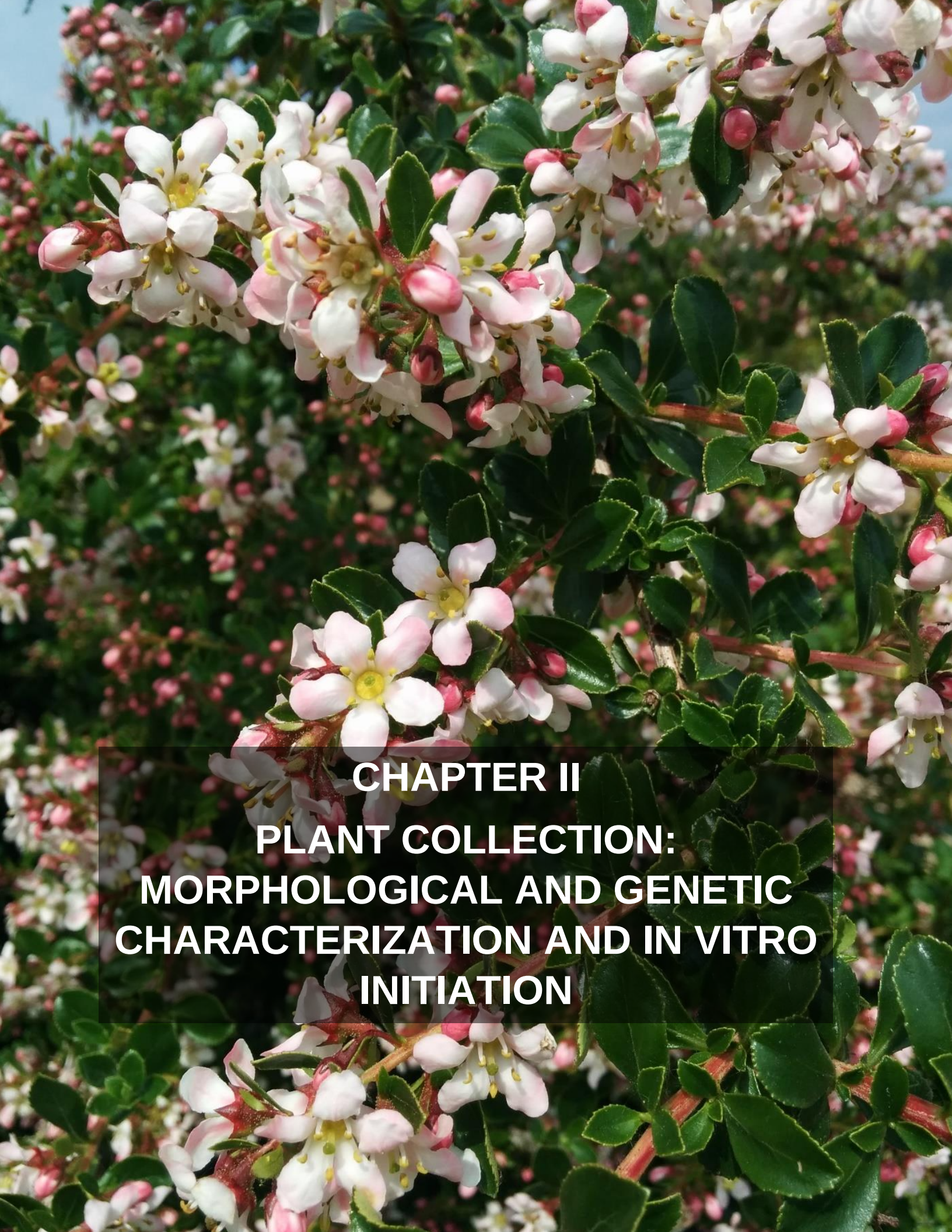
1.4 OBJECTIVES

The main goal of this project was to generate interesting new phenotypic and genotypic variation in the genera *Escallonia* and *Sarcococca*, two genera in the segment of visually attractive potted woody ornamentals. To do so, advanced breeding techniques, interspecific hybridization, polyploidization and introduction of *rol*-genes of rhizogenic bacteria, were implemented. The generated plant material was then valued either as potential cultivar or as pre-breeding material. Both genera were suggested by BestSelect CVBA, a Flemish cooperation of ornamental growers.

More specific the following objectives were set:

1. To build a breeders collection of both *Sarcococca* and *Escallonia* and evaluate the variation among genotypes by morphological characterization. (Chapter II)
2. To gather (cyto)genetic and phylogenetic knowledge of the collected genotype in order to assess their breeding possibilities and values. (Chapter II)
3. To set-up suitable *in vitro* initiation and propagation media for the genotypes of both *Escallonia* and *Sarcococca*. (Chapter II)
4. To evaluate the possibilities for interspecific hybridization within *Sarcococca* using an integrated approach of molecular and cytogenetic techniques to characterize the hybrid nature of the progeny. (Chapter III)
5. To optimize a protocol for efficient *in vitro* mitotic polyploidization to create new variation within *Escallonia* and *Sarcococca,* depending on the available *in vitro* stock (Chapter IV)
6. To develop a protocol for efficient introgression of *rol*-genes from wild type rhizogenic *Agrobacterium* strains in *Escallonia* and *Sarcococca* (Chapter V), depending on the available *in vitro* stock (objective 3).
7. To implement an efficient and robust phenotyping tool for evaluating the variation in resulting plant material based on image analysis. (Chapter IV)

The final conclusions on the obtained knowledge, protocols and plant materials, and the future perspectives of the used techniques in *Escallonia* and *Sarcococca,* and in woody ornamentals in general, are then discussed in the final Chapter VI.



CHAPTER II

PLANT COLLECTION: MORPHOLOGICAL AND GENETIC CHARACTERIZATION AND IN VITRO INITIATION

Parts of Chapter II are published in:

- Denaeghel H, Van Laere K, Leus L, Van Huylenbroeck J, Van Labeke MC (2017) Interspecific hybridization in *Sarcococca* supported by analysis of ploidy level, genome size and genetic relationships. Euphytica 213: 149.
- Denaeghel H, Van Laere K, Leus L, Lootens P, Van Huylenbroeck J, Van Labeke MC (2018) The variable effect of polyploidization on the phenotype in *Escallonia*. Frontiers in Plant Science 9: 354.

2. PLANT COLLECTION: MORPHOLOGICAL AND GENETIC CHARACTERIZATION AND IN VITRO INITIATION

2.1 INTRODUCTION

A plant collection was constructed for *Escallonia* and *Sarcococca*. Botanical species, varieties and cultivars were gathered from botanical gardens, arboreta and growers. Morphological characteristics of the collected genotypes were compared to descriptions in botanical guides, redetermined using a determination key (*Sarcococca*, see addendum) and compared to living plants and herbarium specimens in the National Botanical Garden of Meise (Belgium) to confirm the identity of the acquired plants. An analysis of the morphological characteristics was used to assess the visual attractiveness of the genotypes for further use in breeding programs.

Knowledge of ploidy level differences, genome size and genetic relationships between species facilitates interspecific hybridization in ornamentals. Such (cyto)genetic information can be used to improve the efficiency of a breeding program, as was done in *Hydrangea* (Granados Mendoza et al., 2013). By combining the knowledge of phylogenetic relatedness, genetic distances, chromosome numbers, ploidy levels, and the success rate of previous hybridization attempts, Granados Mendoza et al. (2013) determined the average genetic distance were direct crosses between two species are successful. With this information, they proposed candidates for bridge-crosses to combine attractive traits from two species in previously unsuccessful hybridizations. A similar approach has also been proven successful in e.g., *Brassica* (Mohanty et al., 2009), *Asparagus* (Kubota et al., 2012), and *Solanum* (Jansky and Hamernik, 2009).

For *Sarcococca* and *Escallonia* only limited (cyto)genetic information is available. For *Sarcococca,* the basic chromosome number is x = 14; chromosome counts of 3 species (*S. saligna*: 2*n* = 2*x* = 28; *S. humilis:* 2*n* = 4*x* = 56; *S. ruscifolia*: 2*n* = 4*x* = 56) are published (Darlington and Wylie, 1955). Sequences of nuclear internal transcribed spacers (ITS) and plastid DNA were used to unravel the phylogenetic relationship of the Buxaceae family, but relationships within the *Sarcococca* genus were only partially resolved (Von Balthazar et al., 2000). All analyzed *Escallonia* genotypes are reported to be diploid with 24 chromosomes (Zielinski 1955, Darlington and Wylie 1955, Hanson et al. 2002). A multi-locus phylogenetic analysis from two nuclear loci and one chloroplast locus has been conducted (Zapata, 2013). Furthermore, an analysis of plastid DNA sequence data of 2 intergenic spacers and one gene, showed that the evolutionary history of the genus *Escallonia* can be linked to historical processes, such as Andean orogeny (Sede et al., 2013).

In this chapter, a morphological analysis of the acquired plants was conducted, to increase our knowledge about the morphological variation present in the collection and the value of individual genotypes for breeding purposes. Furthermore, chromosome counts were performed for 21 *Escallonia* genotypes and 17 *Sarcococca* genotypes. Also the genome size of the genotypes and their genetic relationships were unraveled using flow cytometry and AFLP marker analysis. With this information, the efficiency of an interspecific breeding program can be enhanced (Chapter III). Furthermore, this (cyto)genetic information will also be useful to set-up experiments for polyploidization (Chapter IV) and *rol*-gene introduction (Chapter V), for example to see whether closely related genotypes show the same sensitivity towards bacteria or chemicals used.

Making use of *in vitro* plant material provides a stable and controllable environment and thus enhances success of several breeding techniques, such as polyploidization and co-cultivation with rhizogenic agrobacteria (Dhooghe et al., 2011, McCown, 2000). For example, tetraploid induction in *Morus alba* was 80% more efficient using an *in vitro* method compared to *ex vitro* (Chakraborti et al., 1998). Therefore, *in vitro* shoot culture was initiated for *Escallonia* and *Sarcococca* genotypes. Preformed buds were collected, decontaminated, and initiated *in vitro* to have a uniform and continuous shoot growth. Hereby the composition of the culture medium and environment (temperature, lighting, etc.) was optimized and a rooting and acclimation protocol was developed. Shoots from the *in vitro* stocks were used for polyploidization (Chapter IV) and introduction of *rol*-genes from rhizogenic *Agrobacterium* (Chapter V).

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Eighteen *Sarcococca* genotypes and 25 *Escallonia* genotypes (species and cultivars) were collected from breeders and botanical gardens, during the spring and summer of 2014. The genotypes *Sarcococca humilis* (S16), *S. vagans* (S17) and *S. wallichii* (S18) were added to the collection in 2016. The acquisition origin and reported winter hardiness are presented in Table 2.1 (*Sarcococca*) and Table 2.2 (*Escallonia)*. For *Sarcococca*, also the geographic region and habitat where the genotypes are found in nature is given (Table 2.1). From each genotype, one plant was planted in the field (51°0'N, 3°48'E, Melle, Belgium), and one or two plants were grown as a container plant (peat based substrate with 1.5 kg/m³ fertilizer: 12N:14P:24K + trace elements, pH 5.0-6.5, EC 450 µS/cm) and kept in a frost free greenhouse. Unfortunately, cuttings from *Escallonia alpina* (E11) and *E. resinosa* (E13) did not survive, and could not be included in further research. Each acquisition was given a unique code for identification (S01 – S18 for *Sarcococca* in Table 2.1*,* and E01- E25 for *Escallonia* in Table 2.2).

2.2.2. Characterization of the collection

2.2.2.1 Morphological characterization and verification of the collection

The *Sarcococca* genotypes were redetermined with an identification key based on vegetative properties, developed by Jan De Langhe, dendrologist at the Ghent University Botanical Garden (Ghent, Belgium) in collaboration with Arboretum Wespelaar (Haacht, Belgium) (See Addendum for the vegetative key of *Sarcococca*) (http://www.arboretumwespelaar.be/EN /Identification_keys_and_illustrations/?). The hairiness of the shoots was observed with a 10x magnifying lens.

For both *Sarcococca* and *Escallonia*, morphological descriptions from botanical guides and both the living and herbarium collection of the National Botanical Garden in Meise (Belgium) were used to verify the botanical species or cultivars names under which the plants were acquired (Bean and Murray, 1989b, Dirr, 2011, Flora of China, 2008, Hilliers Garden, 1991, Krüssmann, 1960, Sealy, 1986, Schneider and van de Laar, 1970). Vouchers were made of each genotype and added to the collection at the National Botanical Garden in Meise. Morphological characteristics, such as flower color, leaf lamina size and shape, and the plant size and appearance, were determined. The occurring leaf lamina shapes are depicted in Figure 2.1. Leaf lamina lengths and widths were

measured on minimum 10 fully developed leaves randomly selected on at least 2 plants, preferably on one field-grown plant and on a greenhouse-grown plant. However, not all field-grown plants survived the winter, in which case the leaves were all sampled on plants in the greenhouse. The leaves were scanned and measured using ImageJ (Abramoff et al., 2004). The length of the leaf lamina was measured from the tip to the start of the petiole. The width was measured at the widest position of the leaf lamina perpendicular to the length. For *Sarcococca,* the plant size was measured on one plant per genotype that had been grown outdoors for 3 years (51°0'N, 3°48'E, Melle Belgium). If no field-grown plants were available, a value was assigned by combining information from botanical descriptions and plant appearance in the greenhouse. Finally, for *Sarcococca*, also the number of styles in the female flower and the hairiness of the shoots were determined, since these are used for determination of the species by the botanical publications used.

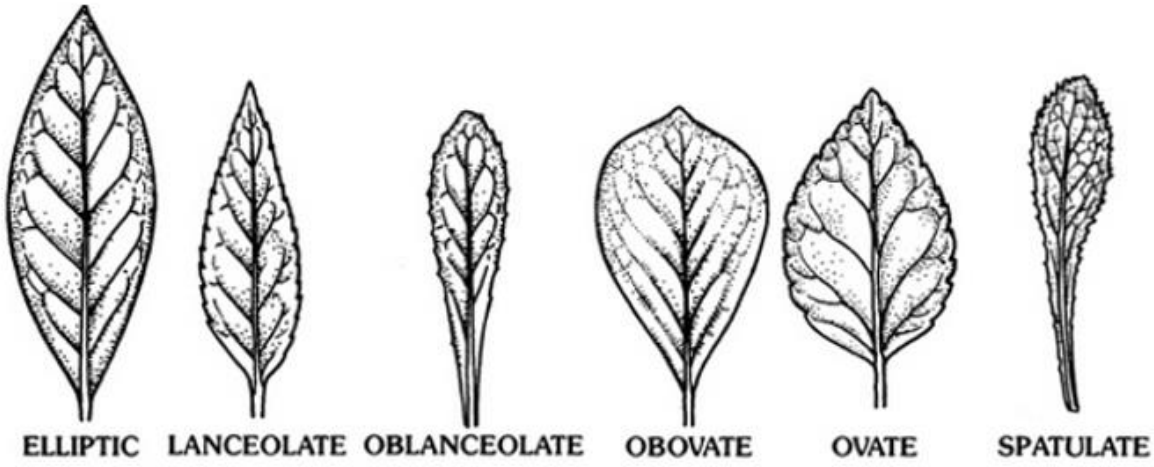


Figure 2.1: Leaf lamina shape terminology used for the morphological analysis of *Escallonia* and *Sarcococca* genotypes. (Modified from Swink and Wilhelm (1994))

Table 2.1: Overview of the collected *Sarcococca* genotypes, their code and place of acquisition, with the documented winter hardiness and geographic origin.

Accession name	Code	Place of acquisition[z]	winter hardiness (°C)[y]	Geographic Origin[x] altitude	environment	Region
S. confusa Sealy	S01	DN	-14.9	-	-	-
S. confusa Sealy	S12	PE	-14.9	-	-	-
S. coriacea (Hooker) Sweet	S02	AW	-	600-2500 *	forests, hills *	Nepal, India, Burma, China (Yunnan), Vietnam, Thailand, Indonesia *
S. hook. 'Purple Stem'	S15	BRMB	-17.7	-	-	-
S. hook. var. *digyna* Franch.	S08	PE	-17.7	1000-3500m	forests	C. China
S. hook. var. *humilis* Rehd. & Wils.	S09	PE	-17.7	-	-	China [Hupeh, E. Szechuan] *
S. humilis Stapf	S16	DN	-17.7	-	-	China [Hupeh, E. Szechuan] *
S. orientalis C.Y. Wu	S03	BRMB	-17.7	200-1000m	forests, streamsides	SE China
S. orientalis C.Y. Wu	S11	PE		200-1000m	forests, streamsides	SE China
S. rusc. var. *chinensis* (Franch.) Rehd. & Wils.	S06	PE	-12.2	200-2600m	Forests on mountain slopes , streamsides	W. China
S. rusc. 'Dragon Gate'	S07	PE	-12.2	200-2600m	Forests on mountain slopes , streamsides	W. China
S. saligna (D. Don) Muell.-Arg.	S04	BRMB	-6.6	1200-2300m	evergreen forests	Taiwan, Tibet, Afghanistan, India, Nepal, Pakistan
S. saligna (D. Don) Muell.-Arg.	S10	PE	-6.6	1200-2300m	evergreen forests	Taiwan, Tibet, Afghanistan, India, Nepal, Pakistan
S. saligna (D. Don) Muell.-Arg.	S13	RBGE (nr: 19851807)	-6.6	1200-2300m	evergreen forests	Taiwan, Tibet, Afghanistan, India, Nepal, Pakistan
S. vagans Stapf	S17	PE	-	500-800m	forests, mountain valleys	China (Hainan, S. and W. Yunnan), Myanmar, Vietnam
S. wallichii Stapf	S05	PE	-12,2*	1300-2700m	forests on mountain slopes or in valleys	Tibet, China (S. and W. Yunnan), Bhutan, NE India, Myanmar, Nepal
S. wallichii Stapf	S18	PE	-12,2*	1300-2700m	forests on mountain slopes or in valleys	Tibet, China (S. and W. Yunnan), Bhutan, NE India, Myanmar, Nepal

z) AW: Arboretum Waasland, Beveren, Belgium; PE: Plantentuin Esveld, Boskoop, The Netherlands; BRMB: Boomkwekerij Rein & Mark Bulk, Boskoop, The Netherlands; DN: tree nursery De Neve, Oosterzele, Belgium; RBGE: Royal Botanic Garden Edinburgh, Edinburgh, Scotland.
y) Hoffman and Ravesloot (1998), unless indicated with *, then Dirr (2011)
x) Data from Flora of China (2008); unless indicated with *, then Sealy (1986)
-: no data found

Table 2.2: Overview of the collected *Escallonia* genotypes, their code and place of acquisition, with the documented winter hardiness

Accession name	Code	Acquisition		Winter hardiness (°C) [y]
		Grower/botanical garden [z]	Accession number	
E. alpina DC.	E12	HG	-	-6.6
E. alpina DC.	E19	BRMB	-	-6.6
E. 'Apple Blossom'	E22	PE	-	-14.9
E. bifida Link & Otto	E08	BGM	19084138	-6.6
E. 'Donard Seedling'	E06	DN	-	-14.9
E. 'Edinburgh'	E24	RBGE	20050334 A	-12.2
E. illinita Presl.	E01	DN	-	-12.2
E. illinita Presl.	E17	BGUW	XX-0-WU-ESC000001	-12.2
E. 'Iveyi' (Veith)	E07	DN	-	-14.9
E. iveyi (x) Veith	E09	BGM	19391832	
E. laevis 'Gold Ellen'	E02	DN	-	-12.2
E. 'Langleyensis' (Veith)	E21	TS	1347	-12.2
E. myrtoidea Bertero ex DC.	E23	RBGE	20130304	-
E. pendula Ruiz & Pav.	E10	BGM	19843175	-
E. 'Red Dream'	E05	DN	-	-12.2
E. 'Red Elf'	E03	DN	-	-12.2
E. rosea Griseb.	E14	HG	-	-
E. rubra (Ruiz & Pav.) Pers.	E16	RBGE	19924317*B	-12.2
E. rubra 'C.F. Ball'	E20	BGM	19810040	-12.2
E. rubra var. *macrantha* (Hook. & Arn.) Reiche	E04	DN	-	-12.2
E. rubra var. *rubra*	E18	HB	C0834	-12.2
E. 'Slieve Donard'	E25	RBGE	19653608 A	-14.9
E. stricta (x) Remy.	E15	HG	-	-

z) DN: tree nursery De Neve, Oosterzele, Belgium; BGM: Botanical Garden Meise, Meise, Belgium; RBGE: Royal Botanical Garden Edinburgh, Edinburgh, Scotland; HG: Hillier Gardens, Ampfield, Romsey, UK; BGUW: Botanischer Garten Universität Wien, Vienna, Austria; HB: Hortus Botanicus, Amsterdam, The Netherlands; BRMB: Boomkwekerij Rein & Mark Bulk, Boskoop, The Netherlands; TS: Ter Saksen, Beveren, Belgium; PE: Plantentuin Esveld, Boskoop, The Netherlands

y) Hoffman and Ravesloot, 1998

2.2.2.2 Genome size measurements and chromosome counts

Genome sizes (pg/2C) of the plants in the collection were determined with a Partec PAS III flow cytometer equipped with a 488 nm laser (20 mW solid state laser, Sapphire 488-20) (Partec, Münster, Germany). The samples were chopped according to Galbraith et al. (1983). Staining with propidium iodide (PI) was performed using the PI Cystain kit (Partec). The samples were incubated in the dark at 5°C for at least 30 min before measuring. As internal standard maize (*Zea mays* 'CE-777'; 2C = 5.43 pg) (Lysak and Dolezel, 1998) or pea (*Pisum sativum* 'Ctirad'; 2C = 9.09 pg) (Dolezel et al., 1998) were used for *Sarcococca.* For *Escallonia*, tomato *Solanum esculentum* 'Stupicke' (2C = 1.96 pg) (Dolezel et al., 1992) was used as internal standard, except for *E*. *pendula* where *Zea mays* 'CE-777' was used as internal standard. At least 5 measurements were made, on 3 different plants on 5 different days. Histograms were analyzed using FloMax software (Partec). Terminology on 2C values was used as defined by Greilhuber et al. (2005).

Chromosome numbers were determined from actively growing root meristems. Young root tips (± 1 cm) were incubated for 3h in an antimitotic mixture (2.5 µM colchicine + 1 mM 8-hydroxyquinoline) and fixated in 3:1 EtOH:acetic acid for at least 30 min at room temperature. Digestion of *Sarcococca* was performed at 37°C for 75 min using a 1% enzyme suspension (1% cellulase, 1% pectolyase and 1% cytohelicase in 10mM citric acid buffer), for *Escallonia* for 60 to 100 min (depending on the genotype) using 1% enzyme suspension (1% cellulase and 1% pectolyase in 10mM citric acid buffer). Cell suspensions and chromosome slides were made according to the SteamDrop protocol (Kirov et al., 2014) with 2:1 and 1:1 EtOH:acetic acid as the first and second fixative (for both *Sarcococca* and *Escallonia*), and stained with DAPI. Slides were examined with fluorescence microscopy (AxioImager M2, 1000x, Zeiss) equipped with an Axiocam camera and ZEN software (Zeiss). Chromosome analyses and size measurements were carried out on 10 well-spread metaphases of each genotype by DRAWID software version 0.26 (http://drawid.xyz/; (Kirov et al., 2017)).

2.2.2.3 Principal component analysis on morphological traits, genome sizes and chromosome numbers of Sarcococca

The Principal Component Analysis (PCA) was executed on 18 *Sarcococca* genotypes, based on number of styles in the female flower (ST), the leaf lamina L/W ratio (LW), flower color (FLCOL), fruit color (FRCOL), shoot hairiness (HA), chromosome number (CHROM), 2C value (GEN), and plant size (PS). The plant characteristics were converted to ordinal values (Table 2.3). Clustering was performed using the princomp function and plotted with ggplot, with ellipses drawn with a probability of 0.95 (R Core Team, 2015).

Table 2.3: Assignment of values to the used morphological characteristics in the Principal Component Analysis (PCA) analysis

Characteristic	Value	Characteristic	Value
Flower color	**FLCOL**	**Shoot hairiness**	**HA**
greenish white	1	glabrous	1
white	2	puberulous	2
cream	2	**Plant Size**	**PS**
white to rose	3	x<50	1
cream to rose	3	50<x<80	2
Fruit color	**FRCOL**	80<x<110	3
red	1	110<x	4
red to black	2	**Number of styles**	**ST**
dark (black/blue/purple)	3	2	1
		2 or 3	2
		3	3

2.2.2.4 AFLP analysis and phylogenetic tree

A phylogenetic tree based on an AFLP analysis was created for 15 *Sarcococca* genotypes and 22 *Escallonia* genotypes. The *Sarcococca* genotypes *S. humilis* (S16), *S. vagans* (S17), and *S. wallichii* (S18) were at the moment of sampling not yet acquired, and therefore not included. The DNA of *Escallonia* genotype *E. rubra* var. *rubra* was not of sufficient quality and thus omitted from the AFLP analysis. For DNA extraction, the modified CTAB DNA isolation protocol of Doyle and Doyle (1987) was used with 20 mg of lyophilized young leaf material as starting material. AFLP reactions were executed according to the protocol described for *Buxus* in Van Laere et al. (2011a). AFLP reactions were run on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with the GeneScan 500Rox kit for labeling (Applied Biosystems). Four fluorescent-labeled EcoRI and MseI primer combinations with 6 selective bases were used for the selective amplification. The primers used for *Sarcococca* were E-AAG+M-CAG, E-ACA+M-CAT, E-AAC+M-CTG and E-ACT+M-CTA, and for *Escallonia* E-AAC+M-CTG, E-ACA+M-CAT, E-ACT+M-CTA and E-AGG+M-CTT. Analysis was done using the ABI Prism Genemapper software version 4.1 (Applied Biosystems). Peaks lower than 50 were removed from the analysis, together with all markers occurring in more than 95% or less than 5% of the population. A phylogenetic tree (dendrogram) was constructed using a UPGMA clustering method with Jaccard indices and bootstrap values, executed in R (R version 3.2.0) (R Core Team, 2015).

2.2.3 *In vitro* initiations

Young, non-woody shoots were collected on greenhouse grown plants, between March and October of 2014 and 2015. After rinsing the shoots with demineralized water, the leaves were

excised and the shoot cut into nodal sections of ± 1 cm. The nodal sections were sterilized in 70% EtOH for 1 min, rinsed with sterile demineralized water, submerged in 1% NaOCl for 20 min, and then rinsed again with sterile demineralized water. After 30 min drying on sterile paper, the cut surfaces of the nodal sections were cut off with a sterile scalpel, since these were damaged by the EtOH and NaOCl treatments and would delay or inhibit the regrowth on *in vitro* medium. The cut nodal sections were transferred into a tube (Ø 2 cm) with ± 10 mL solid growth medium. The tubes were placed in a growth chamber (ambient temperature: 23 ± 1°C, photoperiod: 16 h, light intensity: 35 µmol/m²s, bottom cooling: 18 ± 1°C). The used media are described in Table 2.4. All media contained 30 g/L sucrose, 7 g/L agar for plant tissue culture (Lab M Limited, Heywood, Lancashire, UK) and had a pH between 5.8 and 6, adjusted with KOH or HCl. Two different types of medium were used: Murashige and Skoog medium (MSM including vitamins, Duchefa Farma, Haarlem, The Netherlands) (Murashige and Skoog, 1962) and Woody Plant medium (WPM, Duchefa Farma) (Lloyd and McCown, 1980), at full or half strength. Several plant hormones were tested: the cytokinins 6-benzylaminopurine (BAP), zeatin, 2-isopentenlyadenine (2-IP), meta topolin (mT), and the auxin 1-naphthaleneacetic acid (NAA). Non-temperature-sensitive plant hormones were added before autoclaving (15 min, 121°C, 0.5 MPa) and temperature sensitive hormones after autoclaving by filter sterilization when the media cooled down below 60°C. Contaminated nodes were removed. These nodes were not included to determine the success rate of the *in vitro* initiations. Shoots that developed on the nodes were transferred after 2 to 15 weeks (depending on the genotype) to a solid growth medium for further propagation and placed in the growth chamber (conditions see above) in a 500 mL jar containing 100 mL medium. This *in vitro* stock was sub-cultured on fresh medium every 3-4 months.

Shoots of *E. rubra* and *E. rosea* were, before the start of this project (2013), successfully initiated and propagated on medium 3 (MSM with 2 mg/L BAP and 0.1 mg/L NAA). They were subcultured on medium 26 (MSM with 0.15 mg/L BAP and 0.05 mg/L NAA) for further propagation. For these genotypes, no further *in vitro* initiations and medium optimizations were made.

Table 2.4: Media used for *in vitro* shoot induction and multiplication of *Sarcococca* and *Escallonia* nodal sections

Use	Medium number	Macro-nutrients [z]	Hormones [y] (mg/L)				
			BAP	Zeatin	2-IP	mT	NAA
Shoot induction	1	MSM	1.00	-	-	-	0.10
	2	MSM	1.00	-	-	-	0.50
	3	MSM	2.00	-	-	-	0.10
	4	MSM	2.00	-	-	-	0.50
	5	MSM	0.50	-	-	-	1.00
	6	MSM	2.00	-	-	-	-
	7	MSM	0.20	-	-	-	-
	8	MSM	0.50	-	-	-	-
	9	MSM	1.00	-	-	-	-
	10	MSM	-	-	-	-	0.50
	11	WPM	0.20	-	-	-	-
	12	WPM	0.50	-	-	-	-
	13	WPM	1.00	-	-	-	-
	14	WPM	2.00	-	-	-	-
	15	WPM	-	-	-	-	-
	16	WPM	0.10	-	-	-	-
	17	1/2 WPM	-	0.10	-	-	-
	18	1/2 WPM	-	1.00	-	-	-
	19	1/2 WPM	-	2.50	-	-	-
	20	1/2 WPM	-	5.00	-	-	-
	21	1/2 WPM	-	-	0.50	-	-
	22	1/2 WPM	-	-	2.50	-	-
	23	1/2 WPM	-	-	5.00	-	-
	24	1/2 WPM	-	-	10.00	-	-
Multipli-cation	25	WPM	-	-	-	0.25	-
	26	MSM	0.15	-	-	-	0.05

z) Murashige and Skoog Medium including vitamins (MSM, Duchefa Farma); Woody Plant Medium (WPM, Duchefa Farma).
y) cytokinins: 6-benzylaminopurine (BAP); 2-isopentenyladenine (2-IP); metatopolin (mT); auxin: 1-naphthaleneacetic acid (NAA).

2.3 RESULTS

2.3.1 Characterization of the *Sarcococca* collection

2.3.1.1 Morphological characterization and verification

Based on the vegetative identification key (addendum), the genotype *S. hookeriana* 'Purple Stem' (S15) could not be determined further than *S. hookeriana,* since the variant *S. hookeriana* 'Purple Stem' was not included in the vegetative key. However, as this genotype clearly showed purple stems, the name *S. hookeriana* 'Purple Stem' was not changed. The correct botanical name of *S. humilis* is *S. hookeriana* var. *humilis,* and is adapted as such (Table 2.5). Both genotypes *S. ruscifolia* 'Dragon Gate' and *S. ruscifolia* var. *chinensis* could not be determined further than *S. ruscifolia,* and were renamed as such. The genotypes *S. saligna* S04 and S10 were both redetermined as *S. hookeriana* 'Ghorepani' and renamed (Table 2.5). The redetermined names will be used as shown in Table 2.5 further on.

Most of the morphological characteristics of the collected *Sarcococca* genotypes confirmed the characteristics found in literature in case a taxonomic description was available (Table 2.6). However, some minor deviations were present. *S. vagans* differed from the descriptions as it had 2 and 3 styles in the female flowers on the same plant instead of 2 styles as mentioned in literature (Flora of China, 2008, Sealy, 1986). For *S. hookeriana* var. *humilis* S09 and S16, the L/W ratio was not in agreement with earlier published ratios in literature. The morphological descriptions confirm the redetermination of the (previously named *S. saligna*) genotypes S04 and S10 to *S. hookeriana* 'Ghorepani' by the identification key. The leaf lamina L/W ratio distinguished *S. saligna* (S13) from *S. hookeriana* 'Ghorepani' (S04) and (S10). Both literature references who describe *S. saligna* agree on the description of leaf lamina shape to be narrow lanceolate (Bean and Murray, 1989b, Krüssmann, 1960) and the leaves of *S. saligna* (S13) best match this description (Figure 2.2). The other two genotypes S04 and S10 have more narrow-elliptic to narrow-oblanceolate shapes. The leaf lamina L/W ratio also differed between *S. saligna* (S13) and *S. hookeriana* 'Ghorepani' (S04) and (S10), respectively 4.7 ± 0.6, 5.2 ± 0.4 and 5.3 ± 1.0, due to much longer leaf laminas of S13 (9.8 ± 1.0 cm) compared to the leaf lamina lengths of S04 (6.5 ± 1.1 cm) and S10 (6.2 ± 1.7 cm).

Table 2.5: Redetermination of the botanical names of the acquired *Sarcococca* genotypes with a vegetative key[z], verification of the botanical name with the living collection and herbarium[y], and the assigned voucher numbers.

Accession name	Determined name with vegetative key[z]	code	verified with[y] living collection	verified with[y] herbarium	voucher number[x]
S. confusa	*S. confusa*	S01	19392121	-	BR0000025666496V
S. confusa	*S. confusa*	S12	19392121	-	BR0000025666472V
S. coriacea	*S. coriacea*	S02	-	*S. coriacea*	BR0000025666397V
S. hookeriana 'Purple Stem'	*S. hookeriana*	S15	-	-	BR0000025666267V
S. hookeriana var. *digyna*	*S. hookeriana* var. *digyna*	S08	-	935872	BR0000025666311V
S. hookeriana var. *humilis*	*S. hookeriana* var. *humilis*	S09	19840106	-	BR0000025666571V
S. humilis	*S. hookeriana* var. *humilis*	S16	19840106	-	BR0000025666465V
S. orientalis	*S. orientalis*	S03	-	-	BR0000025666243V
S. orientalis	*S. orientalis*	S11	-	-	BR0000025666274V
S. ruscifolia var. chinensis	*S. ruscifolia*	S06	-	-	BR0000025666564V
S. ruscifolia 'Dragon Gate'	*S. ruscifolia*	S07	-	-	BR0000025666281V
S. saligna	*S. hookeriana* 'Ghorepani'	S04	-	-	BR0000025666519V
S. saligna	*S. hookeriana* 'Ghorepani'	S10	-	-	BR0000025666557V
S. saligna	*S. saligna*	S13	-	*S. saligna*	BR0000025666427V
S. vagans	*S. vagans*	S17	-	-	BR0000025666526V
S. wallichii	*S. wallichii*	S05	-	931590	BR0000025666540V
S. wallichii	*S. wallichii*	S18	-	931590	BR0000025666359V

z: Changes in names are underlined. The vegetative key was developed by Jan De Langhe, dendrologist at the Ghent University Botanical Garden (Ghent, Belgium) in collaboration with Arboretum Wespelaar (Haacht, Belgium) (See Addendum for the vegetative key of *Sarcococca*) (http://www.arboretumwespelaar.be/EN)/Identification_keys_and_illustrations/?)
y: Herbarium vouchers without cataloged number are mentioned with species name. Herbarium and living collection consulted at the National Botanical Garden in Meise (Belgium). -: no specimen available.

x: voucher numbers assigned by the National Botanical Garden in Meise (Belgium) for addition to the herbarium.

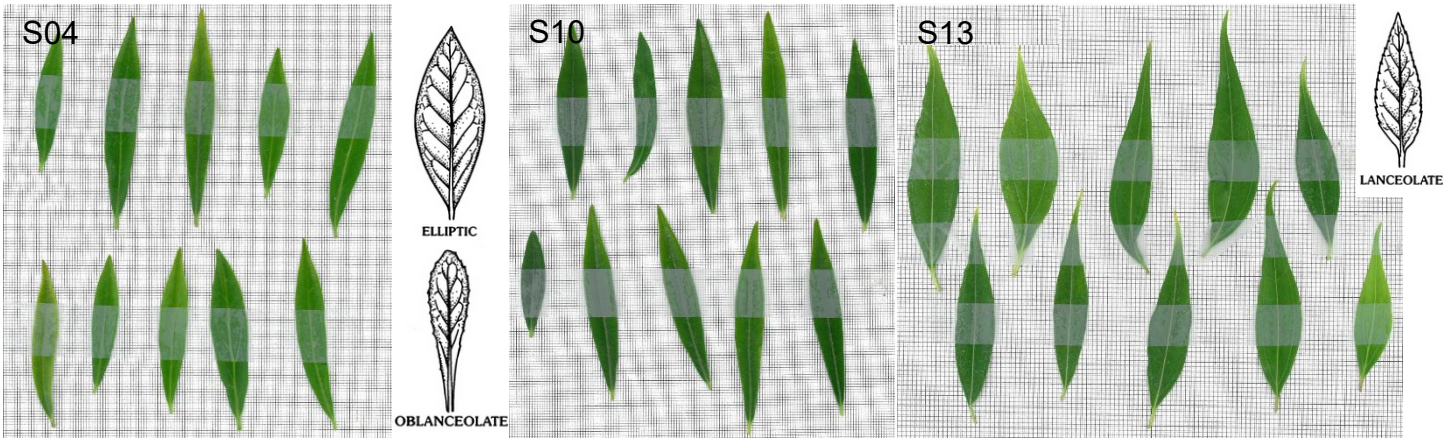


Figure 2.2: The leaves of the genotypes S04 and S10, who are more narrow-elliptic to oblanceolate. The leaves of *S. saligna* S13, who are shaped lanceolate. Leaf lamina shape drawing modified from Swink and Wilhelm (1994).

Table 2.6: Most distinguishable morphological characteristics of the parental Sarcococca genotypes found in literaturea (Lit.) compared to the acquired plants (A.p.). Leaf length/width ratio (L/W) were calculated on minimum 10 leaves per genotype. Flower color varied from greenish white (gr. white) to white, cream or white with rose (wh/ro). Shoots are either puberulous (pub.) or glabrous (glab.). For mature fruit color, leaf shape and general appearance of the plant, no differences between literature and the acquired plant were found.

Sarcococca genotype	code	L/W ratio		Number of styles		Flower color		Shoot hairiness		Plant height[b] (cm)		Mature fruit color	Leaf shape	General appearance
		A.p.	Lit.	A.p.	Lit.	A.p.	Lit.	A.p.	Lit.	A.p.	Lit.			
S. confusa	S01	2.7 ± 0.0	2.5 - 3	2 or 3	2 or 3	cream	cream	pub	pub	50<x<80	90<x<200	red to black	elliptic to elliptic ovate	densely branched, dark green
S. confusa	S12	2.7 ± 0.2	2.5 - 3	2 or 3	2 or 3	cream	cream	pub	pub	50<x<80	90<x<200	red to black	elliptic to elliptic ovate	densely branched, dark green
S. coriacea	S02	3.0 ± 0.2	2.3 - 3.8	2	2	gr. white	-	glab	glab	100<x	x<250	blue/black	elliptic to ovate	sprawling habit, ribbed branches
S. hookeriana 'Purple Stem'	S15	3.8 ± 0.4	-	2	2	white	white	pub	glab	50<x<80	-	black	narrow elliptic	erect growing, midribs and branches flushed purple
S. hookeriana var. *digyna*	S08	4.5 ± 0.2	3.7 - 5.3	2	2	wh/ro	wh/ro	pub	pub	50<x<80	30<x<100	black	narrow elliptic	suckers, green or purplish stems
S. hookeriana var. *humilis*	S09	3.2 ± 0.2	3.5 - 4	2	2	wh/ro	wh/ro	pub	pub	x<50	20<x<60	blue/black	elliptic to narrow elliptic	dwarf, densely branched, suckers
S. hookeriana var. *humilis*	S16	3.2 ± 0.1	3.5 - 4	2	2	wh/ro	wh/ro	pub	pub	x<50	20<x<60	blue/black	elliptic to narrow elliptic	dwarf, densely branched, suckers
S. orientalis	S03	2.8 ± 0.1	3	2	2	wh/ro	wh/ro	pub	pub	50<x<80	60<x<300	black	ovate-lanceolate to obovate-oblanceolate	strong growing upright shrub
S. orientalis	S11	2.7 ± 0.2	3	2	2	wh/ro	wh/ro	pub	pub	50<x<80	60<x<300	black	ovate-lanceolate to obovate-oblanceolate	strong growing upright shrub
S. ruscifolia	S06	2.3 ± 0.1	-	3	3	cream	-	pub	-	80<x<100	60<x<90	red	narrow lanceolate to narrow elliptic	dwarf
S. ruscifolia	S07	2.4 ± 0.1	2 - 3.2	3	3	white	white	pub	pub	80<x<100	60<x<200	red	lanceolate to elliptic	branched towards the top, rounded
S. hookeriana 'Ghorepani'	S04	5.2 ± 0.4	4 - 7	3	3	gr. white	gr. white	pub	pub	50<x<80	60<x<120	purple	narrow lanceolate	small shrub with erect green stems
S. hookeriana 'Ghorepani'	S10	5.3 ± 1.0	4 - 7	3	3	gr. white	gr. white	pub	pub	100<x	60<x<120	purple	narrow lanceolate	small shrub with erect green stems
S. saligna	S13	4.7 ± 0.6	4 - 7	3	3	gr. white	gr. white	glab	glab	100<x	60<x<120	purple	narrow lanceolate	small shrub with erect green stems
S. vagans	S17	4.0 ± 0.3	2 - 4	2 or 3	2	gr. white	-	glab	glab	100<x	100<x<300	-	elliptic-ovate-lanceolate	long branches, recurved
S. wallichii	S05	-	1.8 - 3.6	3	3	white	-	glab	glab	100<x	60<x<300	purple/black	(ob)lanceolate	long branches, recurved
S. wallichii	S18	3.0 ± 0.0	1.8 - 3.6	3	3	white	gr. white	glab	glab	100<x	60<x<300	purple/black	(ob)lanceolate	long branches, recurved

a: literature cited: Krüsmann (1960); Sealy (1986); Bean and Murray (1989); Hilliers Garden (1991); Flora of China (2008); Dirr (2011)
-: no information
b: measured after 3 years on the field

The genotypes *S. coriacea* (S02), *S. wallichii* (S05) and *S. wallichii* (S18) were not sufficiently developed for use of the determination key. Yet, thanks to the morphological descriptions in botanical guides (Bean and Murray, 1989b, Dirr, 2011, Flora of China, 2008, Hilliers Garden, 1991, Krüssmann, 1960, Sealy, 1986, Schneider and van de Laar, 1970), the *S. coriacea* (S02) could be distinguished as such, due to the presence of 2 styles in the female flower, while both *S. wallichii* genotypes S05 and S18 possessed 3 styles.

2.3.1.2 Cytogenetic analysis of the Sarcococca collection

Chromosome counts showed that the *Sarcococca* genotypes can be divided into a diploid (2*n* = 2*x* = 28) and a tetraploid (2*n* = 4*x* = 56) group (Table 2.7). The diploid *S. hookeriana* genotypes (S04, S08, S09, S10, S15 and S16) had a 2C value between 4.11 and 4.20 pg/2C. The other diploid genotypes *S. coriacea, S. saligna*, *S. vagans*, and *S. wallichii* (S05 and S18), had a 2C value between 7.25 - 9.63 pg/2C, twice as high as the previous mentioned diploids. The tetraploid genotypes (*S. confusa, S. orientalis* and *S. ruscifolia* (S06 and S07)) had a 2C value between 7.91 - 8.33 pg/2C. The chromosome lengths of the diploids with a small genome size (± 4 pg/2C) and the tetraploids varied between 1.13 pm and 6.97 pm. The diploid group with a large genome size (± 8 pg/2C) had much longer chromosomes, ranging from 1.28 pm to 12.39 pm (Figure 2.3).

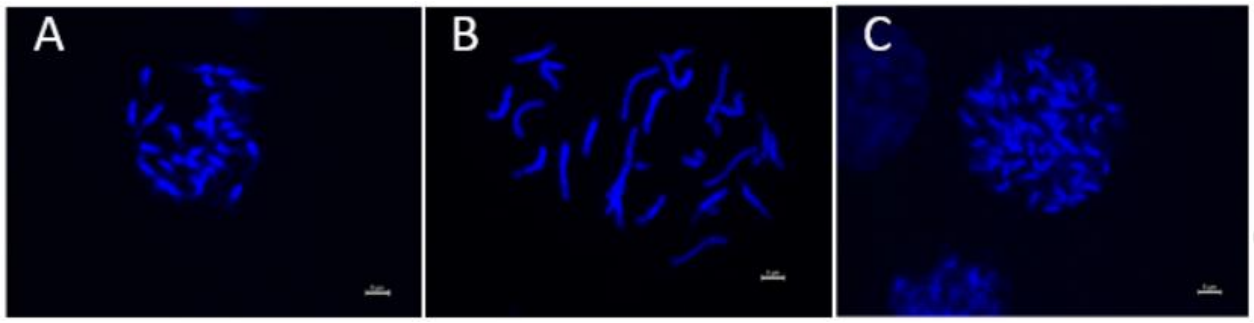


Figure 2.3: A) 28 chromosomes of the diploid *Sarcococca saligna* (S04). B) 28 chromosomes of the diploid *S. vagans* (S17), and C) 56 chromosomes of the tetraploid *S. ruscifolia* (S06).

Table 2.7: Genome sizes of the *Sarcococca genotypes* with the internal standard used, measured chromosome numbers on minimum 10 chromosome spreads and the length of the shortest and longest chromosome.

Sarcococca genotype	Code	Internal standard[z]	Genome size (pg/2C)[y]	Chromosome number (n=10)	Shortest chromosome (pm) [x]	Longest chromosome (pm) [x]
S. hook. 'Purple Stem'	S15	Pea	4.20 ± 0.10	28	2.18 ± 0.09	6.11 ± 1.33
S. hook. var. *digyna*	S08	Pea	4.12 ± 0.07	28	1.64 ± 0.36	5.55 ± 1.03
S. hook. var. *humilis*	S09	Pea	4.11 ± 0.15	28	1.97 ± 0.26	6.06 ± 0.06
S. hook. var. *humilis*	S16	Pea	4.16 ± 0.17	28	2.28 ± 0.32	4.17 ± 0.31
S. hook. 'Ghorepani'	S04	Pea	4.16 ± 0.11	28	2.34 ± 0.31	6.08 ± 1.07
S. hook. 'Ghorepani'	S10	Pea	4.14 ± 0.06	28	2.27 ± 0.26	6.47 ± 0.79
S. saligna	S13	Maize	9.63 ± 0.28	28	2.22 ± 0.32	10.83 ± 1.17
S. vagans	S17	Maize	9.53 ± 0.18	28	2.27 ± 0.18	12.05 ± 1.01
S. wallichii	S05	Maize	7.25 ± 0.15	28	1.28 ± 0.20	9.98 ± 0.15
S. wallichii	S18	Pea	7.34 ± 0.16	28	1.51 ± 0.22	12.39 ± 2.09
S. coriacea	S02	Maize	7.33 ± 0.15	28	1.70 ± 0.31	9.60 ± 3.52
S. confusa	S01	Maize	8.18 ± 0.24	56	1.62 ± 0.26	4.24 ± 0.85
S. confusa	S12	Maize	8.17 ± 0.13	56	2.41 ± 0.23	6.23 ± 0.67
S. orientalis	S03	Maize	8.25 ± 0.31	56	-	-
S. orientalis	S11	Maize	8.33 ± 0.08	56	1.63 ± 0.12	4.70 ± 0.19
S. ruscifolia	S06	Maize	8.00 ± 0.09	56	1.13 ± 0.29	4.80 ± 0.75
S. ruscifolia	S07	Maize	7.91 ± 0.22	56	1.71 ± 0.34	6.97 ± 1.94

z) Genome sizes of internal standards: Maize (*Zea mays* 'CE-777') 2C=5.43pg; Pea (*Pisum sativum* 'Ctirad') 2C=9.09pg

y) Genome size with standard deviation. At least 5 measurements have been made, on 3 different plants on 5 different days.

x) Chromosome lengths measured on at least 3 metaphases.

The PCA (Figure 2.4) had two components with an eigenvalue larger than 1, comprising 46.8% and 32.2% of the variance. The most important characteristics for component 1 are the positively correlated shoot hairiness (HA, 0.970) and flower color (FLCOL, 0.902) and the negatively correlated plant size (PS, -0.794). The second component is mostly correlated negatively by the genome size (GEN, -0.800) and positively by fruit color (FRCOL, 0.748).

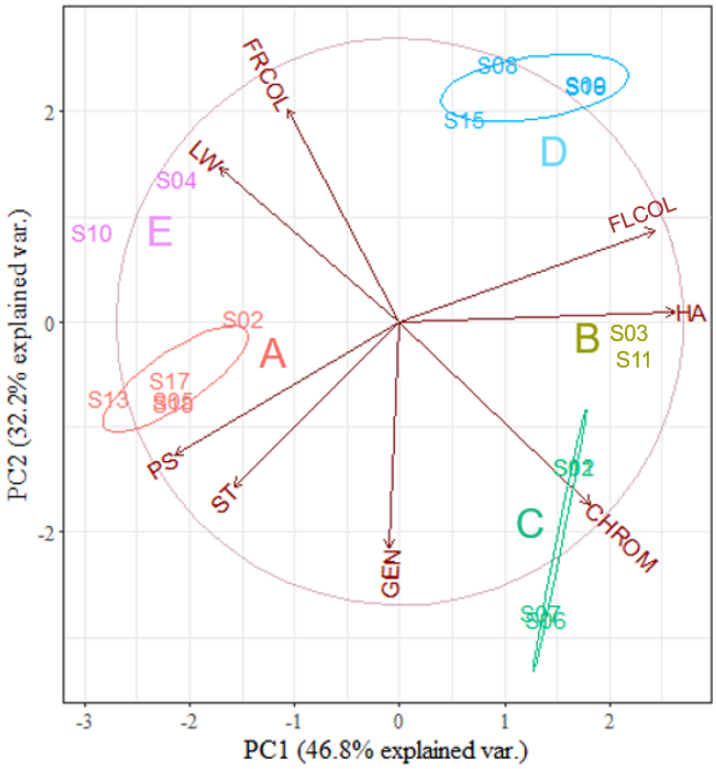


Figure 2.4: Principal Component Analysis (PCA) of the morphological and cytogenetic traits of the collected *Sarcococca* genotypes. A-E: see phylogenetic tree in Figure 2.5. PS: plant size; ST: number of styles; GEN: genome size; CHROM: chromosome number; HA: shoot hairiness; FLCOL: flower color; FRCOL: fruit color; LW: leaf lamina length/width ratio.

2.3.1.3 Genetic relationships revealed by AFLP analysis

The AFLP analysis of 15 genotypes resulted in 753 polymorphic bands. The phylogenetic tree (Figure 2.5) clusters the genotypes in 5 groups. The first cluster (A) comprises *S. coriacea* (S02) and *S. wallichii* (S05) in the phylogenetic tree, two diploids with a 2C value around 8 pg. Cluster B contains both *S. orientalis* genotypes (S03 and S11), tetraploids with a 2C value around 8 pg. Cluster C comprises the *S. ruscifolia* genotypes (S06 and S07) and both *S. confusa* (S01 and S12) genotypes, which are all tetraploid. The *S. hookeriana* genotypes (S08, S09, S15 and S16), all diploid with a small 2C value around 4 pg, are grouped together in Cluster D. Cluster E contains two *S. hookeriana* 'Ghorepani' genotypes, which are also diploid with a small 2C value around 4 pg.

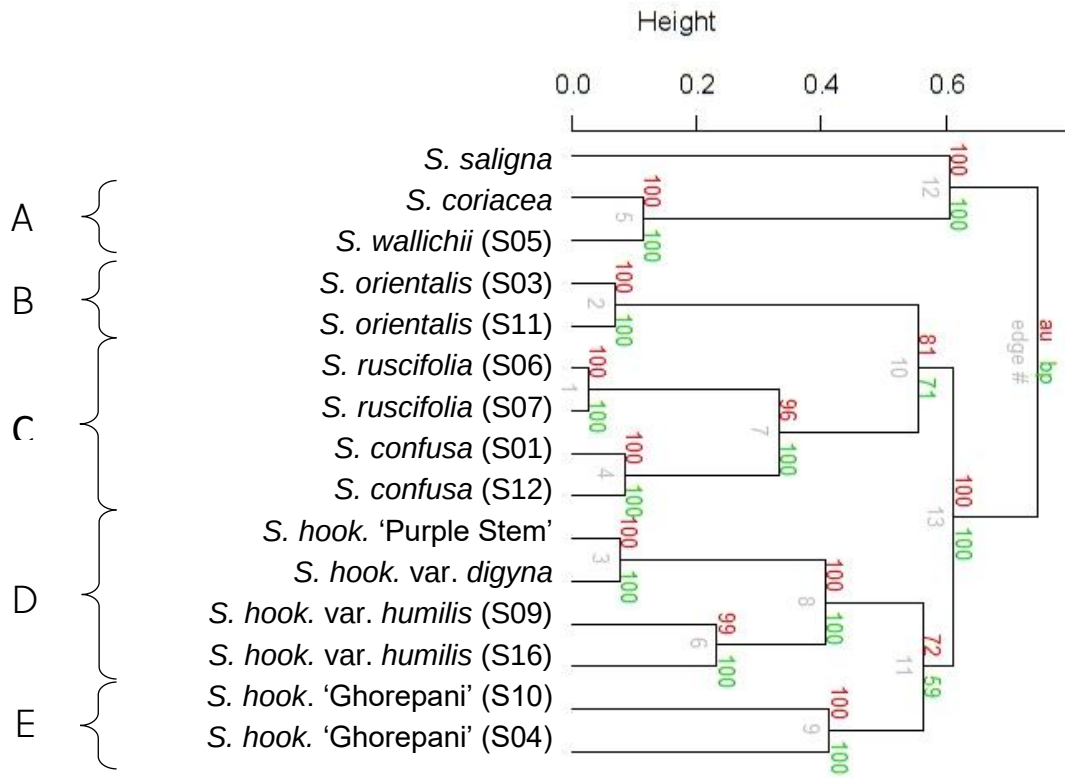


Figure 2.5: Phylogenetic tree of 15 *Sarcococca* genotypes divided in 5 clusters with an UPGMA clustering method. *S. saligna* (S13) is not included in any cluster. AU: Approximately Unbiased p-values computed by multiscale bootstrap resampling. BP: Bootstrap Probability value.

2.3.2 Characterization of the *Escallonia* collection

2.3.2.1 Morphological characterization and verification

The names of the genotypes *E.* 'Iveyi', *E. iveyi* (x), *E.* 'Langleyensis', *E. pendula* and *E. rubra* 'C.F. Ball' could be confirmed with living specimens, and *E. alpina* (E19), *E. bifida, E. illinita* (E17), *E. pendula, E. rubra* and *E. rubra* var. *macrantha* could be confirmed with dried herbarium specimens (Table 2.8). In the living collection of the NBGM, a *E.* 'Red Elf' was present, but it was not similar to *E.* 'Red Elf' (E03) in our collection. Furthermore, the genotypes *E. alpina* (E12) and *E. illinita* (E01) were not alike the specimens in the herbarium collection. However, as none of the genotypes could be unambiguously redetermined, the accession names were held.

Table 2.8: Verification of the botanical names of the accessions in our *Escallonia* collection with the living collection and herbarium[z] and the assigned voucher numbers[y].

		verified with[z]		
Accession name	code	living collection	herbarium	voucher number[y]
E. alpina	E12	-	~~873539~~	BR0000025666441V
E. alpina	E19	-	873539	-
E. 'Apple Blossom'	E22	-	-	BR0000025666366V
E. bifida	E08	-	873440	BR0000025666298V
E. 'Donard Seedling'	E06	-	-	BR0000025666373V
E. 'Edinburgh'	E24	-	-	-
E. illinita	E01	-	~~873556~~	BR0000025666403V
E. illinita	E17	-	873556	BR0000025666182V
E. 'Iveyi'	E07	19391832	-	BR0000025666328V
E. iveyi (x)	E09	19391832	-	BR0000025666380V
E. laevis 'Gold Ellen'	E02	-	-	BR0000025666250V
E. 'Langleyensis'	E21	19810040	-	BR0000025666489V
E. myrtoidea	E23	-	-	BR0000025666458V
E. pendula	E10	19843175	870060	BR0000025666304V
E. 'Red Dream'	E05	-	-	BR0000025666342V
E. 'Red Elf'	E03	~~19940006-07~~	-	BR0000025666199V
E. rosea	E14	-	-	-
E. rubra	E16	-	502790	BR0000025666236V
E. rubra 'C.F. Ball'	E20	19826497	-	-
E. rubra var. *macrantha*	E04	-	999314	BR0000025666205V
E. 'Slieve Donard'	E25	-	-	-
E. stricta (x)	E15	-	-	-

z: Herbarium vouchers without cataloged number are mentioned with species name. Herbarium and living collection consulted at the National Botanical Garden in Meise (Belgium). -: no specimen available. If the number is crossed out, our specimen did not concur with the specimen in Meise.
y: voucher numbers assigned by the National Botanical Garden in Meise (Belgium) for addition to the herbarium. -: no voucher made, since the genotypes were no longer present in our collection.

The morphology of the *Escallonia* genotypes in the collection was compared with descriptions in botanical guides (Bean and Murray, 1989a, Hilliers Garden, 1991, Krüssmann, 1960) (Table 2.9). Many differences between the collected genotypes and literature were noted. Both *E. alpina*

genotypes (E12 and E19) had deviating characteristics. E12 was much larger and had panicles instead of racemes, and E19 had red flowers instead of white. They also showed large differences in leaf lamina sizes, however, both fell within the values in literature (Figure 2.9). However, with the herbarium specimen of the NBGM, the genotype *E. alpina* (E19) appeared to be correctly named, while *E. alpina* (E12) was not. *E.* 'Donard Seedling' (E06) had larger leaf laminas than described in literature. The two *E. illinita* genotypes (E01 and E17) showed differences in leaf lamina shapes, with the leaves of E17 being more slender. However, for both genotypes the leaf lamina length and width values are within the values described by literature. Yet *E. illinita* (E01) carries white with rose flowers instead of white, and the shrub size is smaller than described. Both genotypes show a vigorous, arching growth form, have sticky leaves with the same, strong odor, which is not observed in any of the other genotypes. A herbarium specimen at the NBGM confirmed that *E. illinita* (E17) is most likely aptly named, while *E. illinita* (E01) could be a hybrid of *E. illinita. E.* 'Iveyi' (E07) and *E.* (x) *iveyi* (E09) are phenotypically very alike, and concur with the descriptions in literature and the specimens at the NBGM. The genotype of *E. rosea* is very deviating from the descriptions, the leaves are much larger, flowers are white with rose panicles instead of white racemes, and the shrub is much larger. The flowers of *E. rubra* 'C.F. Ball' (E20) deviate from descriptions, both in color – dark rose instead of crimson red - and shape (Figure 2.10) (Schneider and van de Laar, 1970). However, *E. rubra* 'C.F. Ball' (E20) appeared very similar to the living specimen at the NBGM (Table 2.8). Therefore, their accession name and identifying code will be used further on.

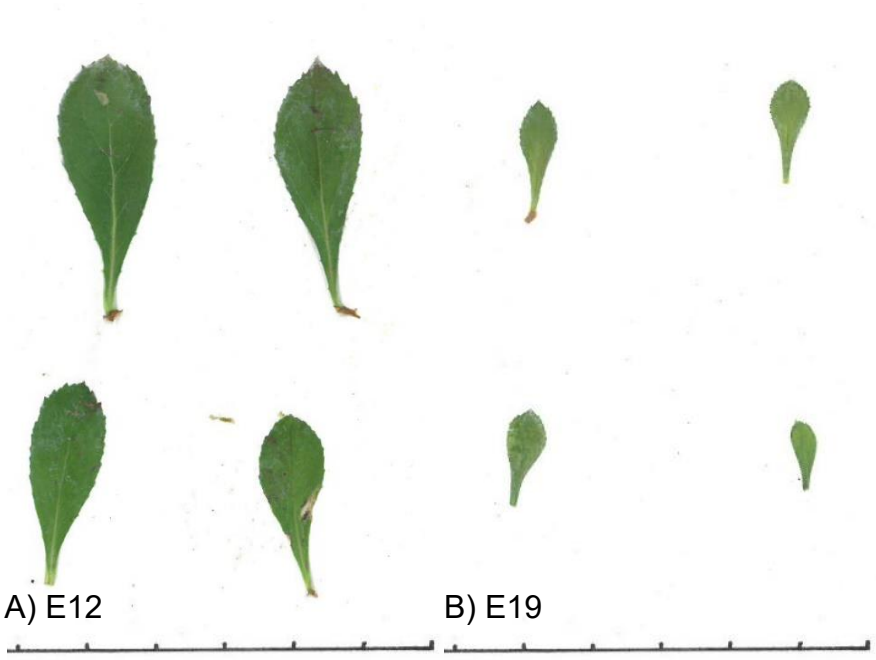


Figure 2.6: (A) leaves of *E. alpina* (E12); (B) leaves of *E. alpina* (E19). Bar: ticks on 1 cm.

Table 2.9: Most distinguishable morphological characteristics of the *Escallonia* genotypes found in literature[z] (Lit.) compared to the acquired plants (A.p.). Leaf length and width were calculated on minimum 10 leaves per genotype. Flower color varied from white (white) to white with rose (wh/ro) and dark red.

Escallonia genotype	code	hybrid	Leaf length		Leaf width		Flower color		Plant height (cm)		Inflorescence		Leaf shape	General appearance
			Coll.	Lit.	Coll.	Lit.	Coll.	Lit.	Coll.[y]	Lit.	Coll.	Lit.		
E. alpina	E12	-	3.2 ± 0.6	1.3 - 3.3	1.2 ± 0.3	0.3 - 1.3	white	white	150 - 200	60 - 150	panicles	raceme	obovate to spatulate-obovate, cuneate base	dense shrub, wider than high
E. alpina	E19		1.4 ± 0.3		0.5 ± 0.1		red		< 100		-			
E. 'Apple Blossom'	E22	*E.* 'Langleyensis' x *E. virgata*	3.4 ± 0.6	-	1.4 ± 0.2	-	wh/ro	wh/ro	< 100	150	panicles	-	elliptic	about as wide as it is high, slow growing
E. bifida	E08	-	6.3 ± 1.0	1.3-7.6	2.1 ± 0.3	-	white	white	200 - 250	up to 300	dense panicles	rounded terminal panicle	narrowly oval to obovate or spatulate, rounded base	large shrub to small tree
E. 'Donard Seedling'	E06	*E.* 'Langleyensis' x *E. virgata*	3.2 ± 0.6	1.3-2.5	1.5 ± 0.3	-	wh/ro	wh/ro	150 - 200	up to 300	racemes	-	obovate	arching branches, vigorous
E. 'Edinburgh'	E24	*E. rubra* x *E. virgata*	2.0 ± 0.4	small	0.8 ± 0.1	small	rosy carmine	rosy carmine	100 - 150	200 - 250		-	-	less pendulous than *E.* 'Langleyensis', long, arching branches
E. illinita	E01	-	4.9 ± 0.7	1.9-6.4	2.0 ± 0.3	0.9-3.2	wh/ro	white	150 - 200	up to 300	panicles	cylindrical panicles	obovate or oval, tapered base	open, loose-habited, strong smell
E. illinita	E17		5.5 ± 1.0		1.8 ± 0.3		white		250 - 300		panicles			
E. 'Iveyi'	E07	(*E. rosea* x *E. rubra*) x *E. bifida*	5.8 ± 1.2	2.5-7.0	3.0 ± 0.6	1.9-3.8	white	white	200 - 250	up to 300	large panicles	large pyramidal panicles	oval, tapered base	-
E. iveyi (x)	E09		5.4 ± 1.1		2.7 ± 0.6		white		250 - 300		large panicles			
E. laevis 'Gold Ellen'	E02		6.5 ± 1.1	-	2.2 ± 0.4	-	dark rose	dark rose	< 100	120 - 180	short, dense panicles	short, dense panicles	narrowly obovate to oval, tapered base	robust habit
E. 'Langleyensis'	E21	*E. rubra* x *E. virgata*	1.8 ± 0.3	1.3-2.5	0.6 ± 0.1	0.7-1.3	rosy carmine	rosy carmine	-	up to 250		short racemes	obovate to narrowly oval	long, slender, arching shoots, vigorous

Table 2.9 (ctd.): Most distinguishable morphological characteristics of the *Escallonia* genotypes found in literaturea (Lit.) compared to the acquired plants (A.p.). Leaf length and width were calculated on minimum 10 leaves per genotype. Flower color varied from white (white) to white with rose (wh/ro) and dark red.

Escallonia genotype	code	hybrid	Leaf length		Leaf width		Flower color		Plant height (cm)		Inflorescence		Leaf shape	General appearance
			Coll.	Lit.	Coll.	Lit.	Coll.	Lit.	Coll.[y]	Lit.	Coll.	Lit.		
E. myrtoidea	E23		3.6 ± 1.0	-	1.8 ± 0.5	-	-	-	100 - 150	-	-	-	-	
E. pendula	E10		14.8 ± 3.4	-	3.6 ± 1.0	-	white	-	-	small tree	-	-	-	-
E. 'Red Dream'	E05		2.8 ± 0.5	-	1.3 ± 0.2	-	red	red	-	-	-	-	-	
E. 'Red Elf'	E03	*E.* 'William Watson' x *E.* 'C.F. Ball'	3.2 ± 1.0	-	1.5 ± 0.6	-	red	red	150 - 200	120 - 240	racemes	-	-	vigorous, free flowering
E. rosea	E14	-	3.5 ± 1.0	0.8 - 2.5	1.1 ± 0.3	0.3-0.6	wh/ro	white	200 - 250	120 - 240	panicles	slender racemes	narrowly obovate, tapered base	bushy
E. rubra	E16	-	3.8 ± 0.6	2.5-5	1.4 ± 0.2	1.6-2.5	red	crimson	100 - 150	up to 400	loose panicles	loose panicles	obovate to ovate to lanceolate (very variable)	peeling bark, vigorous
E. rubra 'C.F. Ball'	E20	(*E. rubra* var. *macrantha* x *E. punctata)* x *E. rubra* var. *macrantha*	2.1 ± 0.5	large	1.1 ± 0.3	large	dark rose	crimson	-	210 - 240	racemes	-	-	very vigorous
E. rubra var. *macrantha*	E04		5.7 ± 0.9	2.5-7.6	3.0 ± 0.6	1.3-4.4	crimson	red	100 - 150	180 - 400	racemes	racemes / panicle	broadly oval to obovate	dense bush of luxuriant habit, vigorous
E. 'Slieve Donard'	E25	*E.* 'Langleyensis' x *E. virgata*	1.8 ± 0.3	small	0.6 ± 0.1	small	wh/ro	rose to carmine	150 - 200	150-210	racemes	-	-	arching habit, compact
E. stricta (x)	E15	*e. leucantha* x *E. virgata*	4.6 ± 0.9	-	2.2 ± 0.5	-	red	red	150 - 200	-	loose panicles	-	-	

z: literature cited: Krüsmann (1960); Sealy (1986); Bean and Murray (1989a); Hilliers Garden (1991);
-: no information
y: measured after 3 years on the field

2.3.2.2 Cytogenetic characterization

All but one genotype of the collected plants were diploid with 24 chromosomes. Only *E. pendula* was tetraploid with 48 chromosomes (Table 2.10). Diploid genome sizes ranged from 1.06 ± 0.02 pg/2C for *E.* 'Langleyensis' to 1.43 ± 0.03 pg/2C for *E. bifida*. The chromosome lengths of the diploids vary between 1.12 ± 0.28 µm and 4.04 ± 1.06 µm, with the shortest chromosome for *E.* 'Red Elf' and the longest for *E. laevis* 'Gold Ellen'. The tetraploid *E. pendula* had even larger chromosomes, up to 5.56 ± 0.94 µm.

Table 2.10: Genome sizes of the *Escallonia* genotypes, measured chromosome numbers and the length of the shortest and longest chromosome.

Escallonia genotype	code	2C value (pg/2C)[z]	Chromosome number	Shortest chromosome (µm)[y]	Longest chromosome (µm)[y]
E. alpina	E12	1.16 ± 0.07	24	1.67 ± 0,33	2.82 ± 0.42
E. alpina	E19	1.13 ± 0.04	24	1.35 ± 0,13	2.58 ± 0.14
E. 'Apple Blossom'	E22	1.13 ± 0.03	24	1.35 ± 0.23(2)	2.87 ± 0.58(2)
E. bifida	E08	1.43 ± 0.03	24	1.75 ± 0.38	3.96 ± 0.65
E. 'Donard Seedling'	E06	1.07 ± 0.06	24	1.70 ± 0.13	3.55 ± 0.29
E. 'Edinburgh'	E24	-	24	1.84 ± 0.25	3.40 ± 0.44
E. illinita	E01	1.18 ± 0.04	24	1.53 ± 0.09	3.62 ± 0.53
E. illinita	E17	1.31 ± 0.08	24	1.79 ± 0.12	3.99 ± 0.59
E. 'Iveyi'	E07	1.32 ± 0.16	24	1.48 ± 0.11	3.68 ± 0.72
E. iveyi (x)	E09	1.25 ± 0.03	24	-	-
E. laevis 'Gold Ellen'	E02	1.21 ± 0.12	24	1.46 ± 0.25	4.04 ± 1.06
E. 'Langleyensis'	E21	1.06 ± 0.02	24	1.37 ± 0.01	2.50 ± 0.23
E. myrtoidea	E23	1.20 ± 0.03	24	1.75 ± 0.11(1)	3.24 ± 0.21(1)
E. pendula	E10	2.17 ± 0.16	48	1.63 ± 0.20	5.56 ± 0.94
E. 'Red Dream'	E05	1.20 ± 0.12	24	1.61 ± 0.16(1)	2.97 ± 0.16(1)
E. 'Red Elf'	E03	1.11 ± 0.06	24	1.12 ± 0.28	3.28 ± 1.22
E. rosea	E14	1.11 ± 0.06	24	1.37 ± 0.07	3.27 ± 0.26
E. rubra	E16	1.07 ± 0.06	24	1.43 ± 0.22	3.53 ± 0.56
E. rubra 'C.F. Ball'	E20	1.07 ± 0.03	24	1.37 ± 0.04	2.96 ± 0.06
E. rubra var. *macrantha*	E04	1.12 ± 0.03	24	1.43 ± 0.26	3.27 ± 1.07
E. rubra var. *rubra*	E18	1.12 ± 0.02	24	-	-
E. 'Slieve Donard'	E25	-	24	1.66 ± 0.17	3.29 ± 0.46
E. stricta (x)	E15	1.15 ± 0.04	24	1.55 ± 0.11	2.67 ± 0.22

z) Genome size with standard deviation. At least 5 measurements have been made, on 3 different plants on 5 different days.

y) Chromosome lengths measured on at least 3 metaphases, unless indicated by (1): on 1 metaphase or (2): on 2 metaphases.

2.3.2.3 AFLP analysis

The phylogenetic tree resulting from the AFLP analysis (Figure 2.7) shows that the two *E. illinita* genotypes (E01 and E17) and the two *E. alpina* (E12 and E19) genotypes differed genetically. *E. pendula* was most distantly related to the other *Escallonia* genotypes. The genotypes *E.* 'Apple Blossom', *E.* 'Donard Seedling', *E. rubra* 'C.F. Ball', *E.* 'Slieve Donard', *E.* 'Langleyensis' and *E.* 'Edinburgh' were clustered together. All these genotypes are hybrids of *E. rubra* and *E. virgata,* except for *E. rubra* 'C.F. Ball', which is a hybrid of two *E. rubra* varieties.

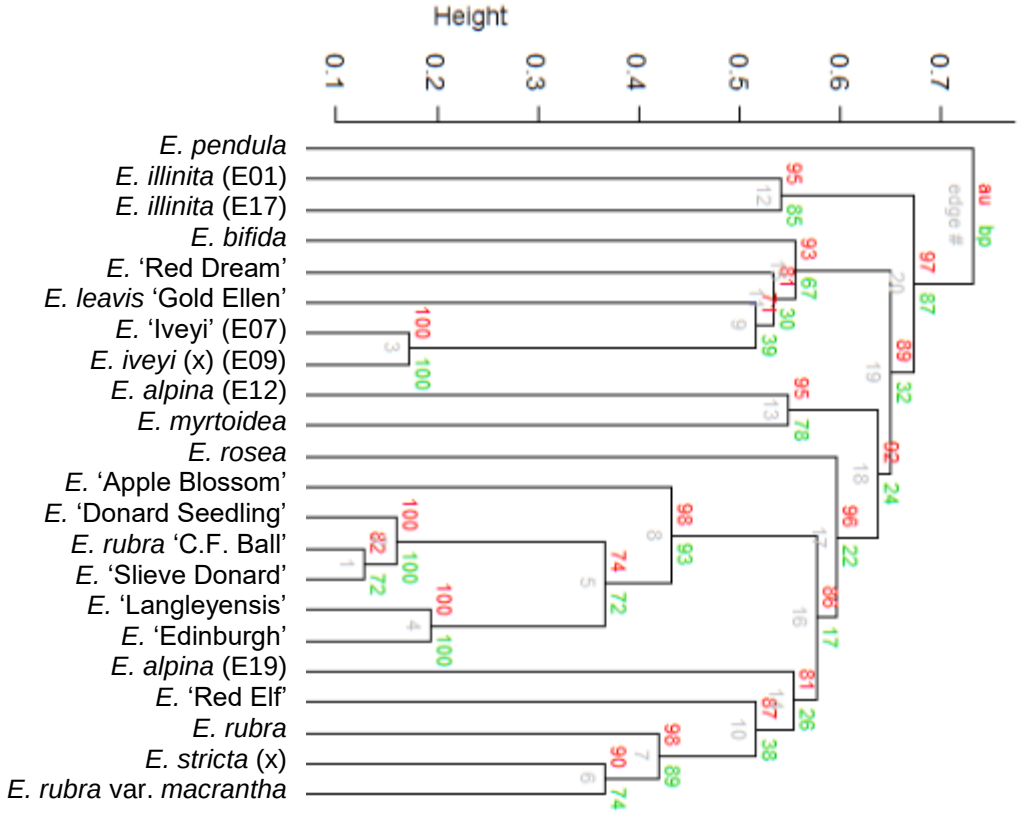


Figure 2.7: Phylogenetic tree of the collection of *Escallonia* species. AU (red): Approximately Unbiased p-values. computed by multiscale bootstrap resampling. BP (green): Bootstrap Probability value.

2.3.3 *In vitro* initiations of *Escallonia* and *Sarcococca* genotypes

Nodal sections of the other *Escallonia* genotypes and *Sarcococca* genotypes were placed on different media. The total number of nodal sections that were initiated on the different media and the number of successful initiations after 15 weeks are displayed in Table 2.11 (*Escallonia*) and Table 2.12 (*Sarcococca*). Both for *Escallonia* and *Sarcococca*, *in vitro* initiation of nodal sections was very difficult and genotype dependent. It was not possible to assign one medium as being the most optimal for initiation of all genotypes.

For *Escallonia*, medium 1 to 5, (MSM with BAP and NAA) were for none of the genotypes the most successful medium. Media 17 to 20 (1/2 WPM with zeatin) were only tested for *E. pendula,* and were not successful for shoot induction for that genotype. Media 21 to 24 (1/2 WPM with 2-IP) were tested on three *Escallonia* genotypes, and quite successful for *E.* 'Edinburgh' and *E. pendula,* but not for *E. rubra* var. *macrantha.* For the majority of the *Escallonia* genotypes tested, the most successful initiation medium for shoot induction are either the media 6 to 9 (MSM with BAP), or the media 11 to 14 (WPM with BAP). For *in vitro* initiations of *Escallonia* genotypes, medium 14 scored best for 10 out of the 22 genotypes initiated. However, for *E.* 'Apple Blossom' and *E.* 'Slieve Donard', no successful initiations happened on medium 14.

For further multiplication, the induced shoots were placed on medium 26, (MS with small concentrations of BAP and NAA). This medium appeared to have the best results in propagating the in vitro stock of *E. illinita* (E01 and E17), *E. rubra, E. rubra* 'C.F. Ball', *E.* 'Edinburgh' and *E. myrtoidea*. Several other genotypes, i.e. *E. laevis* 'Gold Ellen', *E.* 'Red Elf', *E. pendula*, *E.* 'Langleyensis' and *E. rosea,* showed less vigorous growth on medium 26, and the growth of the shoots in the *in vitro* stock declined. However, no further optimization for the shoot multiplication medium was done.

For *Sarcococca,* media 2, 4, 8, 20, 23 and 24 were least successful. Media 9 and 12 (MSM and WPM respectively with 0.5 mg/L BAP) were most successful for 3 and 4 genotypes out of 15. Further multiplication of successfully initiated *Sarcococca* genotypes was performed on medium 25, which contained WPM as medium composition and mT as growth hormone. However, *Sarcococca in vitro* multiplication was difficult, due to the slow growth of the genotypes.

In vitro stocks of the *Escallonia* genotypes *E.* 'Apple Blossom' (E22), *E.* 'Edinburgh' (E24), *E. illinita* (E01), *E. illinita* (E17), *E. laevis* 'Gold Ellen' (E02), *E.* 'Langleyensis' (E21), *E. myrtoidea* (E23), *E. pendula* (E10), *E.* 'Red Elf' (E03), *E. rosea* (E14), *E. rubra* (E16) and *E. rubra* 'C.F. Ball' (E20) and of the *Sarcococca* genotypes *S. coriacea* (S02), *S. orientalis* (S03), *S. hookeriana* 'Ghorepani' (S04), *S. wallichii* (S05) and *S. hookeriana* var. *digyna* (S08) were maintained for further breeding experiments in chapter 3, 4 and 5.

Table 2.11: Number of nodal sections successfully initiated after 15 weeks and the total number of nodal sections (contaminated nodes not included) for a range of media with different hormonal compositions. The most successful media for each *Escallonia* genotype are underlined[z].

		Medium used																				
Escallonia genotype	code	1	2	3	4	5	6	7	8	9	11	12	13	14	17	18	19	20	21	22	23	24
E. alpina	E12	-	-	-	-	-	-	-	-	-	6 / 6	5 / 5	5 / 6	6 / 6	-	-	-	-	-	-	-	-
E. alpina	E19	-	-	-	-	-	-	-	-	-	6 / 6	6 / 6	6 / 6	6 / 6	-	-	-	-	-	-	-	-
E. 'Apple Blossom'	E22	-	-	-	-	-	5 / 6	5 / 6	3 / 6	5 / 5	1 / 6	0 / 6	0 / 6	0 / 6	-	-	-	-	-	-	-	-
E. bifida	E08	-	-	-	-	-	0 / 7	0 / 7	1 / 5	1 / 3	10 / 11	7 / 9	9 / 11	10 / 10	-	-	-	-	-	-	-	-
E. 'Donard Seedling'	E06	2 / 12	-	3 / 12	-	4 / 12	10 / 10	10 / 11	10 / 10	10 / 10	13 / 17	13 / 17	13 / 17	11 / 14	-	-	-	-	-	-	-	-
E. 'Edinburgh'	E24	-	-	-	-	-	-	-	-	-	2 / 4	3 / 4	1 / 3	4 / 4	-	-	-	-	6 / 6	6 / 6	5 / 5	5 / 5
E. illinita	E01	2 / 6	3 / 6	3 / 6	1 / 6	3 / 6	5 / 5	6 / 6	6 / 6	4 / 4	-	-	-	-	-	-	-	-	-	-	-	-
E. illinita	E17	-	-	-	-	-	5 / 5	5 / 5	4 / 4	2 / 2	6 / 6	6 / 6	6 / 6	6 / 6	-	-	-	-	-	-	-	-
E. 'Iveyi'	E07	1 / 6	1 / 6	2 / 6	2 / 6	1 / 5	2 / 6	2 / 6	3 / 5	2 / 6	-	-	-	-	-	-	-	-	-	-	-	-
E. iveyi (x)	E09	-	-	-	-	-	4 / 8	0 / 10	2 / 8	3 / 9	5 / 7	6 / 6	6 / 6	6 / 6	-	-	-	-	-	-	-	-
E. laevis 'Gold Ellen'	E02	0 / 6	1 / 6	1 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	5 / 6	6 / 7	4 / 7	5 / 6	-	-	-	-	-	-	-	-
E. 'Langleyensis'	E21	-	-	-	-	-	0 / 3	1 / 5	1 / 4	0 / 1	12 / 24	13 / 26	11 / 24	9 / 24	-	-	-	-	-	-	-	-
E. myrtoidea	E23	-	-	-	-	-	-	-	-	-	8 / 14	8 / 13	7 / 12	6 / 10	-	-	-	-	-	-	-	-
E. pendula	E10	-	-	-	-	-	6 / 6	6 / 6	4 / 4	5 / 5	11 / 13	13 / 14	13 / 14	8 / 14	3 / 12	2 / 12	1 / 12	3 / 11	4 / 5	6 / 6	6 / 6	6 / 6
E. 'Red Dream'	E05	2 / 12	-	2 / 12	-	2 / 12	2 / 7	3 / 8	3 / 10	3 / 10	2 / 6	2 / 6	4 / 6	3 / 6	-	-	-	-	-	-	-	-
E. 'Red Elf'	E03	1 / 12	-	0 / 12	-	2 / 12	2 / 12	2 / 10	2 / 10	3 / 11	6 / 9	6 / 10	4 / 9	6 / 8	-	-	-	-	-	-	-	-
E. rubra 'C.F. Ball'	E20	-	-	-	-	-	4 / 15	5 / 13	5 / 15	5 / 16	9 / 17	10 / 18	11 / 18	10 / 17	-	-	-	-	-	-	-	-
E. rubra var. *macrantha*	E04	-	-	-	-	-	1 / 4	2 / 3	1 / 3	3 / 6	4 / 11	4 / 10	5 / 11	4 / 11	-	-	-	-	0 / 6	0 / 6	0 / 6	0 / 6
E. 'Slieve Donard'	E25	-	-	-	-	-	-	-	-	-	0 / 4	0 / 4	0 / 4	0 / 4	-	-	-	-	-	-	-	-
E. stricta (x)	E15	-	-	-	-	-	-	-	-	-	3 / 7	1 / 4	3 / 4	3 / 7	-	-	-	-	-	-	-	-

z) Data for *E. rosea* and *E. rubra* was not available. The genotypes were already *in vitro* available at the start of this project, and no further initiations were made.

Table 2.12: Number of nodal sections successfully initiated after 15 weeks and the total number of nodal sections (contaminated nodes not included) for a range of media with different hormonal compositions. The most successful media for each Escallonia genotype are underlined[z].

Sarcococca genotype		Medium used																				
	code	1	2	3	4	5	6	7	8	9	11	12	13	14	17	18	19	20	21	22	23	24
S. confusa	S01	-	-	-	-	-	1 / 6	3 / 8	1 / 6	2 / 6	1 / 6	4 / 6	1 / 5	1 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	2 / 5	2 / 5	0 / 6
S. confusa	S12	3 / 12	-	1 / 12	0 / 12	0 / 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. coriacea	S02	5 / 12	-	1 / 12	-	0 / 12	6 / 8	7 / 7	7 / 8	5 / 29	6 / 6	6 / 6	6 / 6	5 / 5	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 5
S. hook. 'Purple Stem'	S15	-	-	-	-	-	0 / 5	0 / 7	0 / 9	0 / 8	-	-	-	-	5 / 6	6 / 6	5 / 6	4 / 6	-	-	-	-
S. hook. var. *digyna*	S08	0 / 6	0 / 6	2 / 6	0 / 6	0 / 6	0 / 2	0 / 2	0 / 1	0 / 26	5 / 17	5 / 18	1 / 7	1 / 6	1 / 6	2 / 6	2 / 6	3 / 6	2 / 6	1 / 6	0 / 6	0 / 6
S. hook. 'Ghorepani'	S04	2 / 12	-	1 / 12	-	2 / 12	0 / 5	0 / 6	0 / 6	0 / 29	4 / 22	9 / 23	2 / 8	1 / 10	0 / 6	0 / 6	2 / 6	0 / 6	2 / 6	2 / 6	1 / 6	0 / 6
S. hook. 'Ghorepani'	S10	-	-	-	-	-	-	-	-	-	-	-	-	-	0 / 6	0 / 6	0 / 6	0 / 6	-	-	-	-
S. hook. var. *humilis*	S09	1 / 12	-	0 / 12	-	4 / 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. hook. var. *humilis*	S16	-	-	-	-	-	-	-	-	1 / 23	-	-	-	-	-	-	-	-	-	-	-	-
S. orientalis	S03	-	-	-	-	-	2 / 3	3 / 5	3 / 4	3 / 3	1 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	0 / 6	-	-	-	-
S. orientalis	S11	-	-	-	-	-	4 / 5	2 / 5	2 / 5	16 / 24	-	-	-	-	0 / 6	2 / 6	0 / 6	1 / 6	-	-	-	-
s. ruscifolia	S07	5 / 12	-	10 / 12	-	0 / 12	3 / 6	2 / 6	1 / 4	6 / 8	-	-	-	-	0 / 6	0 / 6	0 / 6	0 / 6	-	-	-	-
s. ruscifolia	S06	2 / 12	-	0 / 12	-	0 / 12	-	-	-	23 / 23	-	-	-	-	0 / 11	1 / 12	0 / 12	0 / 10	2 / 6	1 / 6	1 / 6	0 / 6
S. saligna	S13	0 / 12	-	2 / 12	-	0 / 12	3 / 11	1 / 8	3 / 11	4 / 10	-	-	-	-	2 / 23	4 / 22	3 / 23	4 / 23	2 / 5	3 / 5	1 / 5	1 / 5
S. wallichii	S05	2 / 12	-	1 / 12	-	1 / 12	47 / 56	4 / 5	5 / 6	2 / 4	5 / 5	6 / 6	5 / 5	4 / 5	-	-	-	-	-	-	-	-

2.4 DISCUSSION

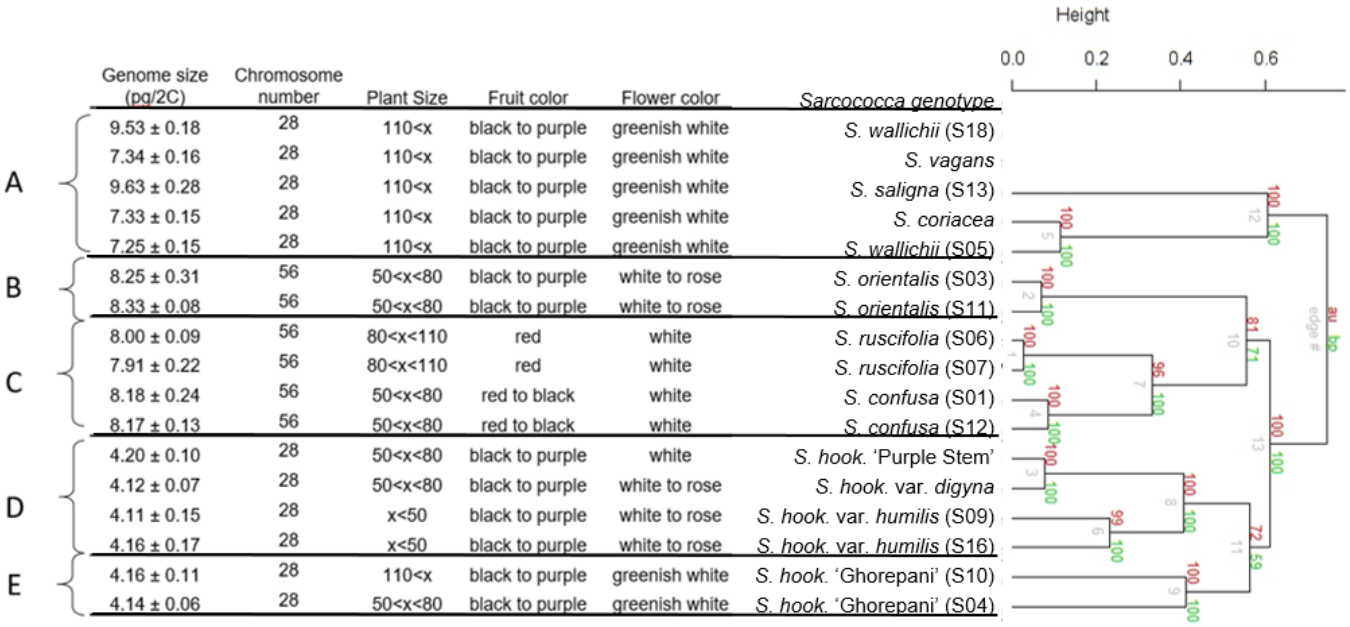
2.4.1 Classification of *Sarcococca* genotypes into 5 clusters based on their ploidy level, genome size, and genetic relatedness

Based on the brief taxonomic study using morphological characteristics and 2C values, most genotypes appeared to be named correctly, except for the genotypes S04 and S10, which were redetermined as *S. hookeriana* 'Ghorepani', instead of *S. saligna.* The female flowers of *S. vagans* in our collection have 2 or 3 styles on the same plant, although the Flora of China (2008) and Sealy (1986) stated that this species should only have 2 styles. No reference mentioned a *Sarcococca* species with 2 or 3 styles on the same plant other than *S. confusa,* but the *S. vagans* in our collection does not resemble *S. confusa* in any other way. All other easily distinguishable characteristics described by the Flora of China (2008) and Sealy (1986) for *S. vagans* matched the plant named *S. vagans* in our collection. An anomalous number of styles is also reported previously for *S. hookeriana* var. *digyna* (Sealy, 1986) - "very rarely an anomalous flower may be found with 3 styles" - which might support our hypothesis that the genotype in our collection is a *S. vagans* but with an anomalous number of styles.

To our information, this is the first study on 2C values and chromosome counts for *Sarcococca*. Most genotypes in our collection are diploid. They can be divided in two groups according to their genome size, one group has a small genome (~4 pg/2C), the other group has a genome size twice as large (~8 pg/2C). The *S. confusa, S. orientalis* and *S. ruscifolia* genotypes are tetraploids with a genome size double the smallest diploid 2C value. No tetraploids with a 2C value twice that of the diploids with the largest 2C value have been found. For several *Sarcococca* genotypes chromosome numbers have been documented (Darlington and Wylie, 1955). For *S. hookeriana* var. *humilis* a chromosome number of 56 is reported, but this does not concur with our observations of 28 chromosomes. However, little information on the plant material and on the methods used for this observation is available, thus the reason for this discrepancy between the documented chromosome number and our results remains unclear. Other documented chromosome numbers are 28 for *S. saligna* and 56 for *S. ruscifolia,* which is confirmed by our results.

In Figure 2.8, the data from the three analyses, namely the AFLP analysis, the cytogenetic data and the most discerning traits from the PCA, are combined. All three analysis methods correlate in the clustering of the genotypes The first cluster (A) comprises *S. coriacea* (S02), *S. wallichii*

(S05), and *S. saligna* (S13) in the phylogenetic tree, with *S. vagans* (S17) and *S. wallichii* (S18) included based on the PCA. All species in this cluster are diploid with a 2C value around 8 pg, have greenish white flowers, and are the plants with the largest plant size in the collection. Cluster B contains both *S. orientalis* genotypes (S03 and S11), both tetraploid with a 2C value around 8 pg. Cluster C comprises the *S. ruscifolia* genotypes (S06 and S07) and both *S. confusa* (S01 and S12) genotypes, which are tetraploids. The tetraploid genotypes of cluster C are furthermore visually discernable by the combination of a red fruit color and pure white flowers. The *S. hookeriana* genotypes (S08, S09, S15 and S16) are grouped together in Cluster D, which are all diploid with a small 2C value around 4 pg. Plant sizes in cluster D are rather small and they carry white or rosy white flowers with dark fruits. Cluster B, C and D comprise the same genotypes in both the phylogenetic tree and the PCA. Cluster E comprises the remaining *S. hookeriana* genotypes (S04 and S10) diploids with a small genome size, greenish flowers and black fruits. The AFLP shows that these genotypes are not the same species, which is also shown by their deviating plant size.



	Genome size (pg/2C)	Chromosome number	Plant Size	Fruit color	Flower color	*Sarcococca* genotype
A	9.53 ± 0.18	28	110<x	black to purple	greenish white	*S. wallichii* (S18)
	7.34 ± 0.16	28	110<x	black to purple	greenish white	*S. vagans*
	9.63 ± 0.28	28	110<x	black to purple	greenish white	*S. saligna* (S13)
	7.33 ± 0.15	28	110<x	black to purple	greenish white	*S. coriacea*
	7.25 ± 0.15	28	110<x	black to purple	greenish white	*S. wallichii* (S05)
B	8.25 ± 0.31	56	50<x<80	black to purple	white to rose	*S. orientalis* (S03)
	8.33 ± 0.08	56	50<x<80	black to purple	white to rose	*S. orientalis* (S11)
C	8.00 ± 0.09	56	80<x<110	red	white	*S. ruscifolia* (S06)
	7.91 ± 0.22	56	80<x<110	red	white	*S. ruscifolia* (S07)
	8.18 ± 0.24	56	50<x<80	red to black	white	*S. confusa* (S01)
	8.17 ± 0.13	56	50<x<80	red to black	white	*S. confusa* (S12)
D	4.20 ± 0.10	28	50<x<80	black to purple	white	*S. hook.* 'Purple Stem'
	4.12 ± 0.07	28	50<x<80	black to purple	white to rose	*S. hook.* var. *digyna*
	4.11 ± 0.15	28	x<50	black to purple	white to rose	*S. hook.* var. *humilis* (S09)
	4.16 ± 0.17	28	x<50	black to purple	white to rose	*S. hook.* var. *humilis* (S16)
E	4.16 ± 0.11	28	110<x	black to purple	greenish white	*S. hook.* 'Ghorepani' (S10)
	4.14 ± 0.06	28	50<x<80	black to purple	greenish white	*S. hook.* 'Ghorepani' (S04)

Figure 2.8: Most important characteristics of the *Sarcococca* genotypes to discern the clusters made in the AFLP and PCA analysis.

For *S. saligna,* all botanical guides agree on the description of leaf lamina shape to be narrow lanceolate (Bean and Murray, 1989b, Krüssmann, 1960) and the leaves of *S. saligna* (S13) best match this description (Figure 2.2). The two genotypes S04 and S10, originally named *S. saligna,* have more narrow-elliptic to narrow-oblanceolate shapes. The leaf lamina L/W ratio of *S. saligna* (S13) was also smaller than of S04 and S10, due to longer leaves. *S. saligna* (S13) best resembles

S. wallichii (S18) and *S. coriacea* in general appearance and frondescence, while both S04 and S10 resemble the *S. hookeriana* genotypes and are therefore redetermined *S. hookeriana* 'Ghorepani' with the help of the vegetative key. The clustering of these genotypes in the phylogenetic tree and the PCA backs up this observation (Figure 2.8).

Introgression of genes from one genotype into another can be directed more efficiently by knowledge of the relationships among species and cultivars, as shown in *Hydrangea* (Granados Mendoza et al., 2013) and *Asparagus* (Kubota et al., 2012). The information gathered about the studied *Sarcococca* species allows us to gain insight in crossing efficiencies and possible incompatibilities. Crosses within clusters of the phylogenetic tree are most likely to have a higher crossing efficiency, primarily because of their closer relationship. For example, it could be expected that the genotypes in cluster A will probably cross quite well with cluster B, and cluster D with cluster E, as they have the same 2C value and ploidy level. Other crosses between clusters are expected to be more difficult due to differences in 2C value and ploidy level, which can cause a ploidy barrier (Lin, 1984; Sutherland and Galloway, 2017; Xie et al., 2017). To increase the information that can be extracted from this analysis, more genotypes need to be analyzed. Some additional species, e.g., *S. longipetiolata, S. longifolia, S. zeylanica* and *S. confertiflora* (Flora of China, 2008, Sealy, 1986) were not present in our collection. In addition, the knowledge gained in our study can also be used to increase the effectiveness of other breeding techniques, such as polyploidization.

2.4.2 Easy hybridization within *Escallonia* is expected

Many discrepancies in morphology between the genotypes in our breeders' collection and the descriptions in botanical guides are present. By comparing our accessions to specimens in the NGBM, we could verify the botanical name of nine genotypes. Correct identification is very difficult due to inadequate morphological descriptions, the variability of traits on the plants themselves such as leaf and plant size, and an easy natural interspecific hybridization leading to many genotypes with intermediary characteristics. *E. illinita* (E01) is an example of an intermediary genotype. It has several characteristics that are typical for *E. illinita,* such as long arching branches, sticky leaves and a strong odor, but it has a deviating flower color, leaf color and plant size from *E. illinita* (E17). It is possible that *E. illinita* (E01) is the result of a natural hybridization or a mutation. *E. rosea* (E14) differed from descriptions for many traits, such as flower color, leaf size, type of inflorescence and plant size (Figure 2.9). By comparing this genotype to botanical

descriptions, it could be *E.* x *exoniensis* or its cultivar 'Balfourii', which are hybrids between *E. rosea* and *E. rubra*, reaching up to 6 meter and 3 meter respectively. Their flowers are rose-tinted and organized in panicles (Bean and Murray, 1989a, Krüssmann, 1960). Another possibility is *E.* 'Newreyensis', a hybrid of *E.* 'Langleyensis' and *E. rosea. E.* 'Newreyensis' is a upright shrub reaching 4 meters with large aromatic leaves and white rose-tinged flowers (Hilliers Garden, 1991). It could also be an as yet undescribed intermediary phenotype as a result of an interspecific hybridization. However, none of these hypotheses were verified with a genetic analysis or with specimen vouchers, as the proposed cultivars were not present in the NBGM. Since both *E. illinita* E01 and *E. rosea* E14 could not be identified with certainty, we continued to use the name and code throughout this work.

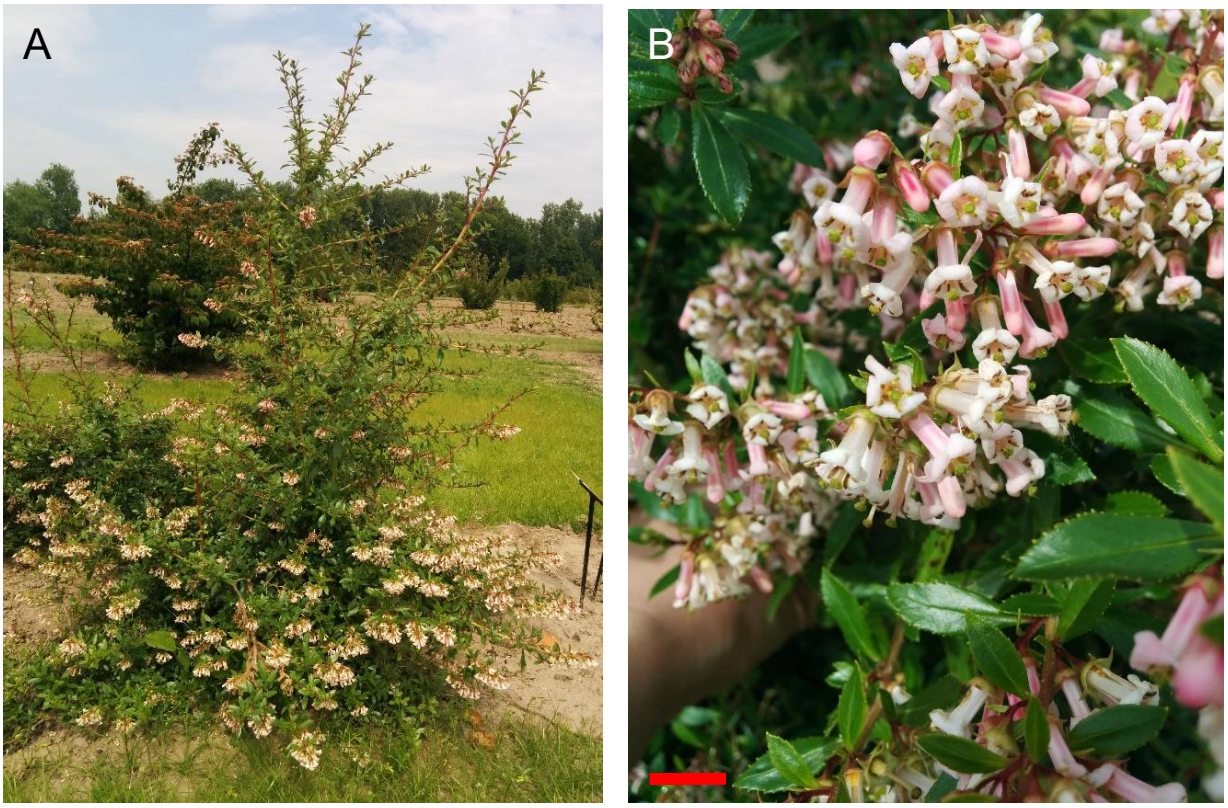


Figure 2.9: A) Shrub of *E. rosea* in our collection with long upright branches and B) its flowers arranged in panicles. Red bar = 1 cm.

Several genotypes with the same name from different accession sites displayed differences in morphology. Based on morphological descriptions and herbarium specimens, *E. alpina* (E19) from the Tree Nursury of Rein and Mark Bulk (BRMB, Boskoop, The Netherlands) was closest to a true

E. alpina (Bean and Murray, 1989a, Hilliers Garden, 1991, Krüssmann, 1960), while *E. alpina* (E12) from Hillier Garden (HG, Ampfield, United Kingdom) had a deviating flower color (white instead of red). The genotypes *E.* 'Iveyi' (E07) and *E. iveyi* (x) (E09) (=(*E. rosea* x *E. rubra*) x *E. bifida*) (Bean and Murray, 1989a, Hilliers Garden, 1991, Krüssmann, 1960) could be the same genotype from a different acquisition site, from Tree Nursery De Neve (DN, Oosterzele, Belgium) and the NBGM respectively, since they clustered very closely together in the phylogenetic tree (Figure 2.7).

The phylogenetic tree (Figure 2.7) showed that *E. pendula* was the most distantly related species. This concurs with the results of (Sede et al., 2013), who created a phylogenetic tree of *Escallonia* spp. using plastid DNA. *E. pendula* was also the only tetraploid species. Except for the groups *E.* 'Langleyensis' and *E.* 'Edinburgh', *E.* 'Iveyi' (E07) and *E. iveyi* (x) (E09), and *E.* 'Donard Seedling', *E. rubra* 'C.F. Ball' and *E.* 'Slieve Donard', all other *Escallonia* genotypes were quite genetically distant. Although *E.* 'Donard Seedling', *E. rubra* 'C.F. Ball' and *E.* 'Slieve Donard' were closely related, they were visually distinct by flower color and leaf sizes (Table 2.5, Figure 2.10). However, as *E. rubra* 'C.F. Ball' should be a hybrid of two *E. rubra* varieties, namely *E. rubra* var. *macrantha* and *E. rubra* var. *rubra,* this genotype should not be clustered with *E.* 'Donard Seedling' and *E.* 'Slieve Donard', but with *E. rubra* and *E. rubra* var. *macrantha* (Figure 2.7). Also the flower phenotype of *E. rubra* 'C.F. Ball' in our collection does not concur with literature, but rather with the flower phenotype of *E.* 'Donard Seedling' and *E.* 'Slieve Donard' (Figure 2.10) (Schneider and van de Laar, 1970). It is possible that this genotype is *E.* 'Victory' rather than *E. rubra* 'C.F. Ball', the previous being described as having spatulate leaves of 2-2.5 cm long and flat, carmine rose flowers, which concurs with the phenotype of the genotype E20. Also, *E.* 'Victory' is a hybrid of *E. rubra* var. *rubra* and *E. virgate,* which would cluster together with *E.* 'Donard Seedling' and *E.* 'Slieve Donard', as was the case.

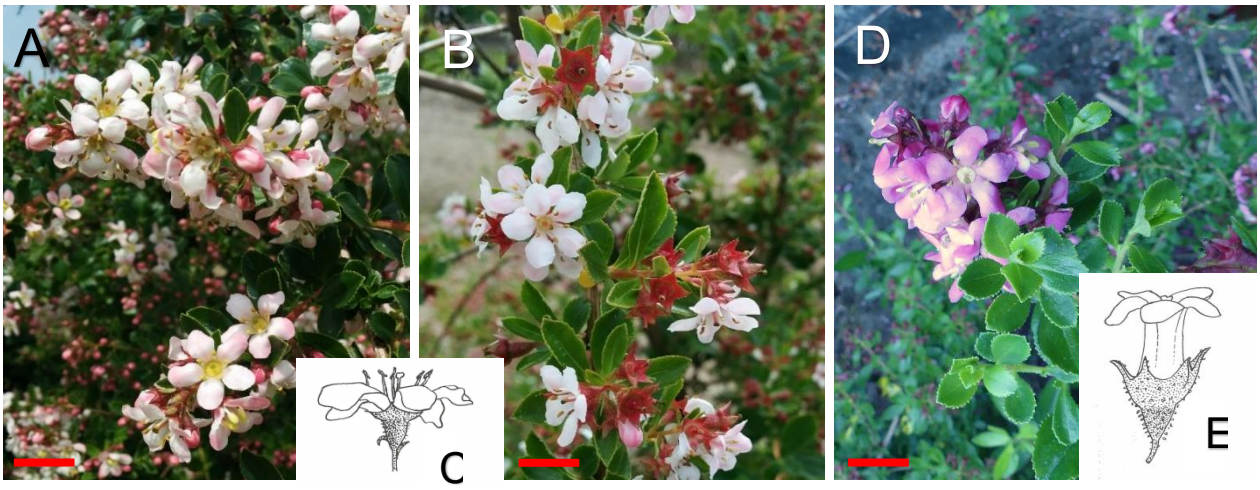


Figure 2.10: A) *Escallonia* 'Donard Seedling', B) *E.* 'Slieve Donard', C) and their flower phenotype according to Schneider and van de Laar (1970). D) *E. rubra* 'C.F. Ball', E) and the flower phenotype for *E. rubra* var. *macrantha* and its hybrid *E. rubra* 'C.F. Ball' according to Schneider and van de Laar (1970). Red bar = 1 cm.

The Kew C-Values database contains information on the genome size of *E. rubra,* 0.85 pg/2C measured with Feulgen densitometry and of *E. pulverulanta,* 1.13 pg/2C measured with flow cytometry with PI (Kew Botanical Garden, 2001). *E. rubra* in our collection had a 2C value of 1.07 ± 0.06 pg, measured with flow cytometry with PI. The difference in analyzing method could account for the difference in 2C value (Moscone et al., 2003). The 2C value of *E. pulverulenta* with PI flow cytometry as published by (Kew Botanical Garden, 2001) is within the range of the values that resulted from our analysis.

Many natural *Escallonia* hybrid cultivars are described, indicating an easy interspecific hybridization. Data of chromosome counts showed that all but one species have 24 chromosomes. Diploid genome sizes varied between 1.06 ± 0.02 pg/2C for *E.* 'Langleyensis' and 1.43 ±0.03 pg/2C for *E. bifida.* No information was found about crosses between these latter two species. However, *E.* 'Iveyi' (or *E. iveyi* (x)) is a hybrid of (=(*E. rosea* x *E. rubra*) x *E. bifida*), and the genome sizes of *E. rosea* and *E. rubra* were also rather small (1.11 ± 0.06 pg/2C and 1.07 ± 0.06 pg/2C respectively), while the genome size of *E. bifida* is about 30% bigger. The hybrid *E.* 'Iveyi' and *E. iveyi* (x) had 2C values of 1.32 ± 0.16 and 1.25 ± 0.03 pg/2C, respectively, in between the parental genome sizes. This indicates that the variation in genome size is not always a barrier for interspecific hybridization. Furthermore, the genotypes *E. rubra* and *E. bifida* are very distantly related as shown by the AFLP analysis (Figure 2.7), indicating that hybridization within the diploid

genotypes of our collection is possible. Yet, hybridizations with *E. pendula* seem likely to be difficult, this species is genetically most distant from all other genotypes in our collection and has a different ploidy level. Differences in ploidy level can cause a post-fertilization block, due to a change in the maternal:paternal genome ratio in the endosperm (Lin, 1984; Sutherland and Galloway, 2017; Xie et al., 2017). Not surprisingly, no data of crosses between the tetraploid *E. pendula* and any of the diploid genotypes is available in literature.

2.4.3 *In vitro* initiation of *Escallonia* and *Sarcococca* is genotype dependent

For *Escallonia* genotypes, media containing the auxin NAA were not successful for *in vitro* initiation of shoots. Other media only contained cytokinins (BAP, Zeatin, 2-iP), but none of the tested initiation media was potent for shoot initiation in all genotypes tested. Although medium 14 (WPM with 2.0 mg/L BAP) was the best medium for 10 out of 22 *Escallonia* genotypes, no shoot development was seen for *E.* 'Apple Blossom' and *E.* 'Slieve Donard. Therefore, medium 14 could not be used as universal initiation medium for *Escallonia*. However, most of the genotypes were either successfully initiated on the MS media (6 to 9) or WPM media (11 to 14), containing the cytokinin BAP. Thus, if new genotypes of *Escallonia* should be initiated *in vitro,* the media to start with should be an MS or WPM based medium enriched with the cytokinin BAP and without any auxin.

No such conclusions could be made for *Sarcococca*, since none of the tested media was successful for more than 4 of the 15 genotypes. Furthermore, initiated genotypes displayed a very slow growth. To develop an efficient initiation and propagation protocol, further testing with more nodal explants and other medium compositions is necessary.

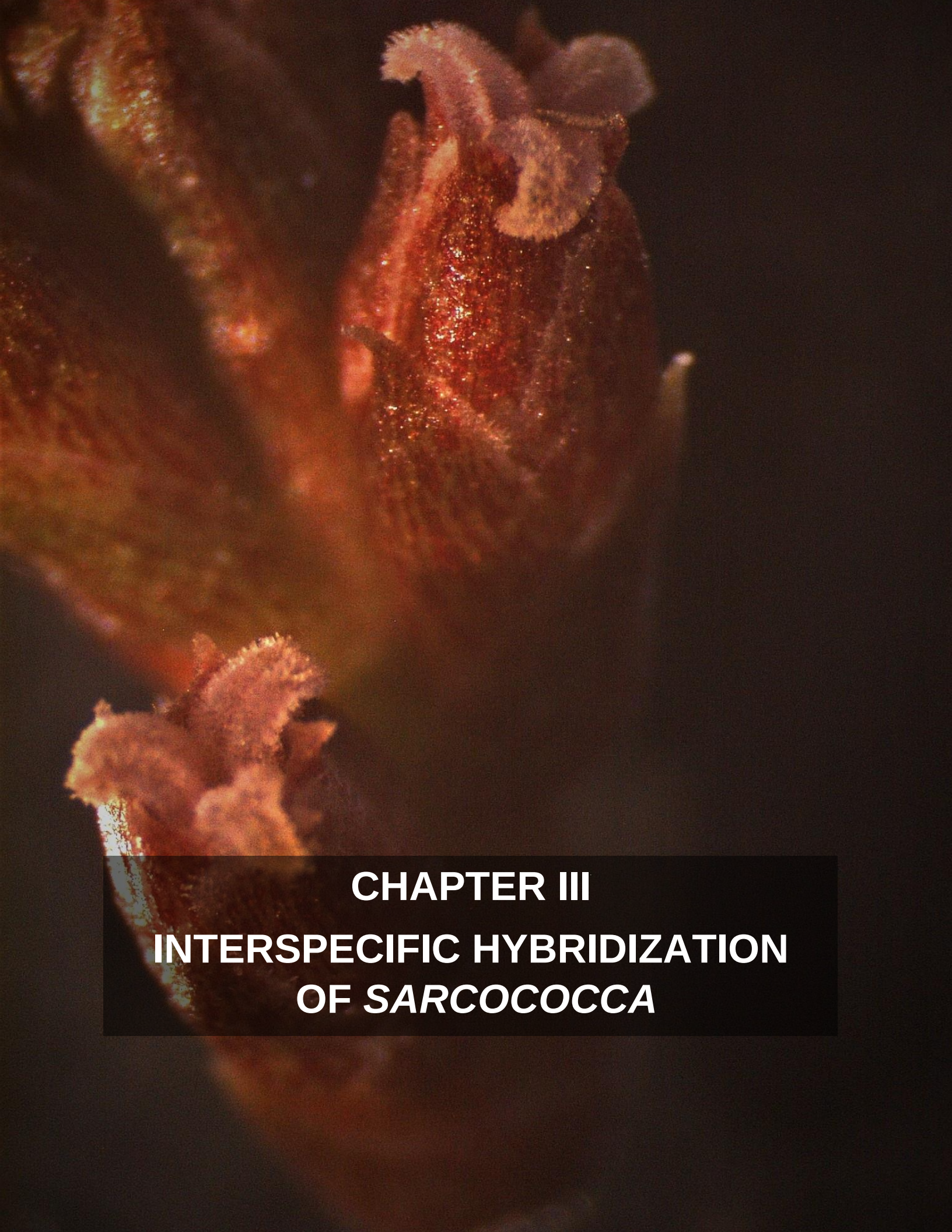
In general, four steps need to be followed to obtain an efficient *in vitro* initiation and propagation: 1) isolation of preformed buds, 2) stabilization of the shoot culture, 3) optimization and 4) acclimation and rooting. For perennials and woody species, the problems usually arise in the second step (McCown, 2000). The efficiency of the shoot regeneration from vegetative plant material can change during the season. Woody species are often characterized by episodic growth flushes during the growing season. The growth potential of these flushes are predetermined in the bud that was formed in the previous growing season. If they maintain this episodic growth-habit *in vitro,* random flushes of shoots are produced for a short duration (McCown, 2000). A possibility to circumvent this episodic growth is by using very juvenile tissues, preferably embryonic, which are

more responsive. However, for many vegetative propagated woody ornamentals, seedlings are genetically different from the parent genotype of interest for further biotechnological improvements. Furthermore, *in vitro* cultures of perennials and woody species often display a slow growth (Mantovani et al., 2013), which causes an inadvertent selection for fast growing genotypes. Another problem is the high production of secondary metabolites, usually phenolic compounds, which can interfere with active substances in the media (McCown, 2000). All these complications can be seen as reason for the slow growing *Sarcococca* genotypes *in vitro*.

2.5 CONCLUSIONS

Both genera *Sarcococca* and *Escallonia* were characterized morphologically, cytogenetic and phylogenetic. This showed some errors in the names of the genotypes in both genera, but especially in *Escallonia*. Comparing the acquired genotypes to voucher specimens and living specimens in the National Botanic Garden in Meise (Belgium) helped to identify some of the acquired genotypes. Furthermore, a vegetative identification key for *Sarcococca* also ameliorated the determination of the genotypes. In the genus *Escallonia,* many intermediate genotypes are present due to a frequent natural interspecific hybridization and a variable phenotype within one species. These intermediate genotypes are difficult to identify. With the collected information on *Escallonia*, little difficulties for further interspecific hybridization to combine wanted traits are foreseen. Within the *Sarcococca* genus, differences in chromosome number and genome size were found, and the genotypes were clustered in five groups according to phylogenetic, genetic and morphological characteristics. These data were used to choose parents for an efficient hybridization program (Chapter III), and to choose candidate genotypes for chromosome doubling (Chapter IV) and *rol*-gene introgression (Chapter V).

Several genotypes of both *Sarcococca* and *Escallonia* have been initiated *in vitro* and stocks were made. For *Escallonia,* an efficient initiation and multiplication protocol could be developed for several genotypes, but not all. The well performing genotypes were used in further experiments (Chapter IV and Chapter V). For *Sarcococca,* the *in vitro* stocks were slow growing; further optimization of the propagation protocol is needed



CHAPTER III

INTERSPECIFIC HYBRIDIZATION OF *SARCOCOCCA*

Parts of Chapter III are published in:

DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J, VAN LABEKE MC (2017) Interspecific hybridization in *Sarcococca* by analysis of ploidy level, genome size and genetic relationships. Euphytica, 213: 149

3 INTERSPECIFIC HYBRIDIZATION OF *SARCOCOCCA*

3.1 INTRODUCTION

Little breeding research has been done for *Sarcococca*. New introductions were either spontaneous mutations or lucky finds. However, well-designed interspecific hybridization can be useful for increasing genetic variation, as some hybrids display hybrid vigor and combine traits from different species (reviewed by Kuligowska et al. (2016b) and Lutken et al. (2012)). Interspecific hybrids are reported for *Buxus*, the largest genus within the Buxacecae (Van Laere et al., 2015, Van Trier and Hermans, 2005). A collection of *Buxus* species was analyzed for chromosome number and ploidy level, phylogenetic relationships and genome sizes (Van Laere et al., 2011a). Based on this information, interspecific and interploidy hybridization were successfully performed, followed by a confirmation of the hybrid status of the progeny with AFLP (Van Laere et al., 2015). It was shown that geographical distribution and genetic distance did not hamper the hybridization. The same observation was also made by Honda et al. (2003).

In this study, the genetic and cytogenetic information on *Sarcococca* gathered in Chapter II was used to set-up cross breeding experiments to evaluate species compatibility and possibility to obtain interspecific hybrids.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

The *Sarcococca* parent collection is described in Chapter II (2.2.1 Plant Material).

3.2.2. Hybridization

Flowering occurred from December until March. The male flowers, when still closed, were removed with tweezers from the seed parent. When the female flower was fully opened and receptive, hand pollination was performed by rubbing anthers with releasing pollen from the male parent over the stigma. Whenever possible, reciprocal crosses were made. As a control, also some intraspecific crosses were done. An overview of the different parental combinations is shown in Table 3.1. Fruits were harvested when mature (about 6 months after pollination). Seeds were sown in trays with moistened sand, stratified for 3 months at 7°C and then placed in the greenhouse at ± 20°C. Successful seedlings (Figure 3.1) were transplanted in P9 pots (9 cm x 9 cm x 9.5 cm) with peat mixture (1.5 kg/m fertilizer: 12N:14P:24K + trace elements, pH 5.0-6.5, EC 450 µS/cm) and kept in a frost free greenhouse, usually between 4-6 months after stratification.

Figure 3.1: A hybrid *Sarcococca* seedling after transplanting to a P9-pot (9 x 9 x 9.5 cm), showing several developed leaves.

3.2.3. Chromosome counts and 2C value measurements of F1 seedlings

The 2C values of the F1 seedlings and their parents were measured using 4',6-diaminidino-2-phenylindole (DAPI) instead of propidium iodide (PI), because obtained peaks when using DAPI were more narrow and resulted in less deviation. Analysis of the DAPI stained samples was performed on a Partec Cyflow Space equipped with a UV LED (365 nm) (Partec). Samples were prepared as described in Chapter 2 with the following modifications: buffer 1 (500 µL 0.1M citric acid monohydrate and 0.5% Tween 20) and buffer 2 (750 µl 0.4 M $Na_2HPO_4.12H_2O$, 2mg/L DAPI, 0.1% polyvinylpyrrolidone (PVP)) (modified from Otto (1990)) were used instead of the PI Cystain kit (Partec). Tomato (*Solanum esculentum* 'Stupicke'; 2C = 1.96 pg) (Dolezel et al., 1992) was used as internal standard. Progenies containing less than 20 F1 seedlings were completely tested; for larger F1 populations, 20 F1 seedlings were randomly selected for analysis. All histograms were analyzed using FloMax software (Partec). Terminology on 2C values was used as defined by Greilhuber et al. (2005).

3.2.4. AFLP analysis

AFLP on the F1 seedlings was performed as described in chapter 2 (2.2.4) for the parental genotypes. The hybrid nature of the F1 seedlings was verified by selecting the unique markers for both female and male parents for each cross combination. The number of unique markers of the male parent inherited by the seedlings was determined. Seedlings possessing more than 25% unique male markers were recorded as an F1 hybrid. Seedlings with a number of unique male markers between 10% and 25% were recorded as a "partial hybrid". At rates below 10% unique male markers, the seedling was not considered to be progeny of the indicated female and male parent. For cross combinations with less than 35 progeny plants, all seedlings were tested; for larger populations, 35 seedlings were randomly selected for analysis.

3.3. RESULTS

3.3.1. Interspecific hybridization

A total of 1228 interspecific crosses were made, yielding 133 mature fruits (11% overall pollination efficiency) and 109 seedlings (Table 3.1). A total of 403 intraspecific crosses were made, resulting in 242 mature fruits (60% overall pollination efficiency) and 215 seedlings. Of 21 interspecific cross combinations made, only 7 yielded fruits, of which 5 produced seedlings. Crossing efficiency (= amount of resulting fruits / amount of flowers pollinated) of the interspecific cross combinations resulting in seedlings was between 18.2% and 68.0%, with *S. ruscifolia* (S06) x *S. wallichii* (S05) and *S. ruscifolia* (S07) x *S. confusa* (S01) reaching the highest crossing efficiencies. Intraspecific cross combinations resulting in seedlings had crossing efficiencies between 28.6% and 71.0%, with *S. hookeriana* var. *digyna* x *S. hookeriana* 'Ghorepani' (S04) and *S. hookeriana* 'Ghorepani' (S10) x *S. hookeriana* var. *digyna* reaching the highest crossing efficiencies.

Crosses between two tetraploids (intraploidy crosses) had a higher crossing efficiency than interploidy crosses. No interspecific crosses between two diploids were made. Two out of four crosses between two tetraploids bore fruits and seedlings, namely *S. ruscifolia* (S06) x *S. confusa* (S01) and *S. ruscifolia* (S07) x *S. confusa* (S01), with crossing efficiencies of 29.3% and 60.0%, respectively. In contrast, only 5 out of 15 cross combinations between a diploid and a tetraploid parent resulted in fruits, and only 3 of those 5 resulted in seedlings. Crosses between a tetraploid female and a diploid male with a 2C value of 4 pg were not successful, no fruits were produced. For crosses between a tetraploid female and a diploid male with the same genome size (8 pg/2C), the only cross made was successful in yielding fruits and seedlings, with a high efficiency of 68.0%, namely *S. ruscifolia* (S06) x *S. wallichii* (S05). When the female parent was a diploid with a 2C value around 4 pg and the male a tetraploid (2C = 8 pg), smaller crossing efficiencies were reached compared to crosses between two diploids or two tetraploids, i.e. between 2.3% and 18.2%.

Unilateral incongruity occurred in the cross combinations *S. hookeriana* var. *digyna* x *S. hookeriana* 'Ghorepani' (S04), *S. hookeriana* var. *digyna* x *S. confusa* (S01), *S. ruscifolia* (S06) x *S. ruscifolia* (S07), and *S. ruscifolia* (S07) x *S. confusa* (S01); these cross combinations generated between 18.2% and 71.0% fruits, while all the reciprocal crosses yielded no fruits.

Table 3.1: Overview of the intra- and interspecific crosses between Sarcococca genotypes grouped according to 2C-value and chromosome count, with the number of hand pollinations made, number of fruits obtained, the crossing efficiency and the number of seedlings.

Parentage ♀	Parentage ♂	Hand pollinations	Fruits	Crossing efficiency [z]	Seedlings
Intraspecific crosses					
♀ (2n = 2x = 28, 2C = 4 pg) x ♂ (2n = 2x = 28, 2C = 4 pg)					
S. hook. var. *humilis* (S09)	*S. hook.* var. *digyna*	42	12	28,6	12
S. hook. var. *digyna*	*S. hook.* 'Ghorepani' (S04)	31	22	71	12
S. hook. 'Ghorepani' (S04)	*S. hook.* var. *digyna*	21	0	0	-
S. hook. 'Ghorepani' (S10)	*S. hook.* var. *digyna*	285	202	70,9	188
♀ (2n = 4x = 56, 2C = 8 pg) x ♂ (2n = 4x = 56, 2C = 8 pg)					
S. ruscifolia (S06)	*S. ruscifolia* (S07)	10	6	60	3
S. ruscifolia (S07)	*S. ruscifolia* (S06)	14	0	0	-
TOTAL intraspecific crosses		*403*	*242*		*215*
Interspecific interploidy crosses					
♀ (2n = 2x = 28, 2C = 4 pg) x ♂ (2n = 4x = 56, 2C = 8 pg)					
S. hook. var. *digyna*	*S. ruscifolia* (S07)	51	0	0	-
S. hook. var. *digyna*	*S. confusa* (S01)	66	12	18,2	4
S. hook. var. *digyna*	*S. ruscifolia* (S06)	*	*	*	3
S. hook. var. *digyna*	*S. confusa* (S12)	62	0	0	-
S. hook. 'Ghorepani' (S04)	*S. confusa* (S01)	25	0	0	-
S. hook. 'Ghorepani' (S04)	*S. ruscifolia* (S07)	36	0	0	-
S. hook. 'Ghorepani' (S10)	*S. confusa* (S12)	174	5	2,9	0
S. hook. 'Ghorepani' (S10)	*S. ruscifolia* (S06)	86	2	2,3	0
S. hook. 'Ghorepani' (S10)	*S. ruscifolia* (S07)	42	0	0	-
S. hook. var. *humilis* (S09)	*S. ruscifolia* (S07)	93	0	0	-
♀ (2n = 4x = 56, 2C = 8 pg) x ♂ (2n = 2x = 28, 2C = 4 pg)					
S. ruscifolia (S07)	*S. hook.* var. *digyna*	40	0	0	-
S. confusa (S01)	*S. hook.* var. *digyna*	22	0	0	-
S. ruscifolia (S06)	*S. hook.* var. *digyna*	34	0	0	-
S. ruscifolia (S06)	*S. hook.* 'Ghorepani' (S04)	14	0	0	-
♀ (2n = 4x = 56, 2C = 8 pg) x ♂ (2n = 2x = 28, 2C = 8 pg)					
S. ruscifolia (S06)	*S. wallichii* (S05)	25	17	68	13
S. ruscifolia (S06)	*S. orientalis* (S03)	44	0	0	-
S. ruscifolia (S07)	*S. orientalis* (S03)	73	0	0	-
Interspecific intraploidy crosses					
♀ (2n = 4x = 56, 2C = 8 pg) x ♂ (2n = 4x = 56, 2C = 8 pg)					
S. ruscifolia (S06)	*S. confusa* (S01)	290	85	29,3	78
S. ruscifolia (S06)	*S. confusa* (S12)	16	0	0	-
S. ruscifolia (S07)	*S. confusa* (S01)	20	12	60	11
S. confusa (S01)	*S. ruscifolia* (S07)	15	0	0	-
TOTAL interspecific crosses		*1228*	*133*		*109*

z) Crossing efficiency: number of fruits obtained / number of hand pollinations

*: data missing

3.3.2. Analysis of the F1 progeny

Progeny of crosses between diploids with comparable 2C values have a similar 2C value as their parents (Table 3.2). The analyzed progeny of an interploidy cross (*S. hookeriana* var. *digyna* x *S. ruscifolia* (S06)), resulted in triploid hybrids, with an intermediate 2C value (mean 5.56 pg/2C between both parental 2C values (3.74 pg/2C and 6.81 pg/2C). The triploid status of the progeny obtained from *S. ruscifolia* (S06) and *S. wallichii* (S05) was harder to confirm by flow cytometry because the parents have an almost equal 2C value, namely 6.81 pg/2C and 7.43 pg/2C for the parents, and a mean of 6.94 pg/2C for the progeny.

The AFLP analysis on the F1 seedlings resulted in 325 polymorphic markers (Table 3.3). The tested seedlings were either full F1 hybrids (>25% of paternal markers present) or partial hybrids (between 10% and 25% of paternal markers). No seedlings with less than 10% of paternal markers occurred. All tested seedlings of diploid cross combinations (41) were full hybrids. For the 3 tetraploid cross combinations, 24 out of 27 measured progeny were full hybrids, while 3 were partial hybrids. The diploid (2C = 4 pg) x tetraploid (2C = 8 pg) cross combinations resulted in 4 partial hybrids and no full hybrids. The 13 plantlets tested from a tetraploid (2C = 8 pg) x diploid (2C = 8 pg) cross combinations with equal 2C values gave 9 partial hybrids and 4 full hybrids.

Table 3.2: Detailed overview of the inter- and intraspecific crosses of *Sarcococca* which yielded seedlings, with an indication of the parental 2C-values and chromosome numbers, the number of the progeny analyzed and their 2C-values.

Parentage		2C-value parents (pg)[z]		Chromosome number parents		progeny analyzed	2C-value progeny (pg)[z]		
♀	♂	♀	♂	♀	♂		mean	min	max
S. hook. var. *digyna*	*S. hook.* 'Ghorepani' (S04)	3.74	3.62	28	28	10	3.69	3.62	3.80
S. hook. var. *humilis*	*S. hook.* var. *digyna*	3.65	3.74	28	28	9	3.69	3.62	3.84
S. hook. 'Ghorepani' (S10)	*S. hook.* var. *digyna*	3.69	3.74	28	28	20	3.65	3.54	3.73
S. hook. var. *digyna*	*S. ruscifolia* (S06)	3.74	6.81	28	56	3	5.56	5.33	5.84
S. ruscifolia (S06)	*S. wallichii* (S05)	6.81	7.43	56	28	13	6.94	6.79	7.36
S. ruscifolia (S07)	*S. confusa* (S01)	7.43	7.05	56	56	10	6.88	6.74	7.21
S. ruscifolia (S06)	*S. confusa* (S01)	6.81	7.05	56	56	20	6.99	6.38	7.40
S. ruscifolia (S06)	*S. ruscifolia* (S07)	6.81	6.90	56	56	3	7.04	6.86	7.18

z) measured with DAPI and tomato *Solanum esculentum* 'Stupicke' (2C=1.96 pg) as internal standard, measured once.

Table 3.3: Overview of the AFLP marker analysis of the *Sarcococca* progeny. The parental genotypes are shown with their 2C-values and chromosome numbers (# chrom.), the number of unique paternal markers (♂ mark.), the number of true F1 hybrids (>25% of paternal markers) and the number of partial hybrids (25%>x>10% of paternal markers).

Parentage		2C-value (pg/2C)[z]		# Chrom.		progeny analyzed	♂ mark.	True F1	Partial
♀	♂	♀	♂	♀	♂				
S. hook. var. *digyna*	*S. hook.* 'Ghorepani' (S04)	4.12 ± 0.07	4.16 ± 0.11	28	28	6	56	6	0
S. hook. 'Ghorepani' (S10)	*S. hook.* var. *digyna*	4.16 ± 0.11	4.12 ± 0.07	28	28	35	67	35	0
S. hook. var. *digyna*	*S. confusa* (S01)	4.12 ± 0.07	8.18 ± 0.24	28	56	4	75	0	4
S. ruscifolia (S06)	*S. wallichii* (S05)	8.00 ± 0.09	7.25 ± 0.15	56	28	13	41	4	9
S. ruscifolia (S07)	*S. confusa* (S01)	7.91 ± 0.22	8.18 ± 0.24	56	56	7	48	7	0
S. ruscifolia (S06)	*S. confusa* (S01)	8.00 ± 0.09	8.18 ± 0.24	56	56	20	45	17	3
S. ruscifolia (S06)	*S. ruscifolia* (S07)	8.00 ± 0.09	7.91 ± 0.22	56	56	3	18	3	0

z) 2C-values measured with flow cytometry with PI, described in Chapter II (2.2.2.2).

3.4. DISCUSSION

The morphological, molecular and cytogenetic information gathered about the studied *Sarcococca* species allowed us to gain insight in crossing efficiencies and (in)compatibilities (Chapter II). Crosses within the same phylogenetic cluster are most likely to have a higher crossing efficiency, primarily because of their closer relationship, but also their similar 2C value and ploidy level. Three cross combinations within cluster C were made. Two out of these three crosses resulted in seedlings, namely *S. ruscifolia* (S06) x *S. confusa* (S01) and *S. ruscifolia* (S07) x *S. confusa* (S01) (Table 3.1). It could be expected that crosses between genotypes with a larger genetic distance are likely to have a lower crossing efficiency or not to be possible at all (Granados Mendoza et al., 2013). However, after performing crosses between species belonging to different clusters, it could be concluded that distant genetic relationships did not always interfere with hybridization, although the wider crosses showed lower crossing efficiencies in some cases. For example, the cross between *S. ruscifolia* (S06) and *S. wallichii* (S05), situated in clusters C and A respectively, did show a high crossing efficiency (68.0%) and 13 seedlings from 17 drupes. On the other hand, only 2 out of 6 crosses between species from clusters C and E resulted in drupes (*S. hookeriana* 'Ghorepani' (S10) x *S. confusa* (S12) and *S. hookeriana* 'Ghorepani' (S10) x *S. ruscifolia* (S06) with crossing efficiencies of 2.9% an 2.3% respectively), and none of these cross combinations resulted in seedlings. Few crosses with genotypes from the clusters A (*S. coriacea, S. saligna* (S13), *S. vagans* and *S. wallichii* (S05 and S18)) and B (*S. orientalis* (S03 and S11)) were performed, due to slight differences in flowering time. The *S. orientalis* genotypes (S03 and S11) flowered rather early, in November, the genotypes from cluster A in December, while the other genotypes flowered in January-February. To overcome this time-barrier, the use of frozen pollen can be investigated, as was done in *Viburnum* (Xie et al., 2017).

In many other genera, interploidy crosses are hampered by ploidy barriers (Lin, 1984, Sutherland and Galloway, 2017, Xie et al., 2017). This can include difficult embryo development and sterility of the triploid progeny (Aleza et al., 2009, Van Laere et al., 2008). Polyploidization is therefore considered a major speciation mechanism (Köhler et al., 2010, Sutherland and Galloway, 2017). This is shown by Lin (1984) in maize, who concluded that a 2:1 maternal:paternal genomic ratio in the endosperm is needed for an optimal embryo development. Several mechanisms exist to overcome this difficulty, such as formation of unreduced gametes, autogamy or apomixis (Bicknell and Koltunow, 2004, Köhler et al., 2010, Sutherland and Galloway, 2017). In our interploidy crosses in *Sarcococca*, the creation of triploid progeny was possible. This is in agreement with earlier observations in the related genus *Buxus* (Van Laere et al., 2015). However, the pollination

efficiency is generally lower in these interploidy crosses, in *Buxus* (Van Laere et al., 2015) and *Sarcococca.* One exception for this was the interploidy cross between *S. ruscifolia* (S06) and *S. wallichii* (S05), where the crossing efficiency was 68% and 13 seedlings were obtained after 25 hand pollinations.

The cross efficiency observed depended on the specific cross combination and the direction of the cross, which was also the case for the related genus *Buxus* (Van Laere et al., 2015). Unilateral incongruity was observed, although more crosses in both directions are needed to prove this clearly. This unilateral incongruity can be caused by physical barriers, such as differences in style lengths (Kuligowska et al., 2015), but also by the presence of proteins for self-incompatibility (Chalivendra et al., 2013). Considering the previously mentioned effects on cross efficiency (phylogenetic distance, ploidy number, genome size, unilateral incongruence), the number of drupes obtained per pollination could possibly be augmented. Pollen fertility of *Sarcococca* is reported to be reduced (70-90%), due to meiotic abnormalities (Saggoo et al., 2011), which can be evaluated by staining with acetocarmine solution and *in vitro* germination of the pollen (Kuligowska et al., 2015). Aniline blue staining of pollinated styles could reveal information about the pollen tube growth and the stage and conditions in which the style is most receptive, as shown in *Viburnum* (Xie et al., 2017). Furthermore, treatment of the style with plant hormones (e.g., gibberellic acid, auxins) or germination medium could increase the fruit set (Xie et al., 2017). Although no information on the number of viable seeds per drupe was collected – depending on the number of styles in the female flower, drupes can contain 2 or 3 seeds – the germination efficiency was rather low. This can be caused by the differences in genome sizes between the parental species. The 19 harvested drupes from these crosses yielded only four seedlings (21%), while intraspecific and intraploidy crosses both yielded around 90% seedlings/drupes. Also interploidy crosses between genotypes with an equal genome size yielded 76% seedlings/drupes. The viability of these seeds could be determined with a tetrazolium test (Xie et al., 2017), if the embryos are viable, *in vitro* embryo rescue could increase the number of hybrid seedlings obtained. The overall germination efficiency can be possibly increased by modifying the germination conditions. The stratification conditions can be optimized by altering the temperature and period of stratification (Balestri and Graves, 2016), or a period of warm stratification could be added (Santiago et al., 2014). Scarring or removing of the seed pericarp, e.g., mechanical (Karlsson et al., 2005) or by sulphuric acid (Mattana et al., 2018), could also increase the germination efficiency. Other seed pretreatments to increase the germination rate are soaking in water or adding hormones such as gibberellic acid (Fogliani et al., 2017).

Furthermore, in a small genus such as *Sarcococca,* it is highly likely that two desired characteristics are present in plant species with a different 2C value or ploidy level, for example when the red drupes of *S. ruscifolia* (tetraploid, ± 8 pg/2C) are to be combined with the compact growing phenotype and slender leaves of the *S. hookeriana* 'Ghorepani' or *S. hookeriana* var. *humilis* (diploid, ± 4pg/2C). If a specific cross between a tetraploid and a diploid is desired, the efficiency could be augmented by doubling the chromosome number of the diploid in a polyploidization event, yielding a tetraploid, which can be crossed probably more efficiently with the original tetraploid. This technique has been used successfully in woody ornamentals such as *Citrus* (Aleza et al., 2009), *Buddleja* (Van Laere et al., 2008) and roses (Allum et al., 2007), and can be a future perspective in the interspecific breeding program of *Sarcococca*.

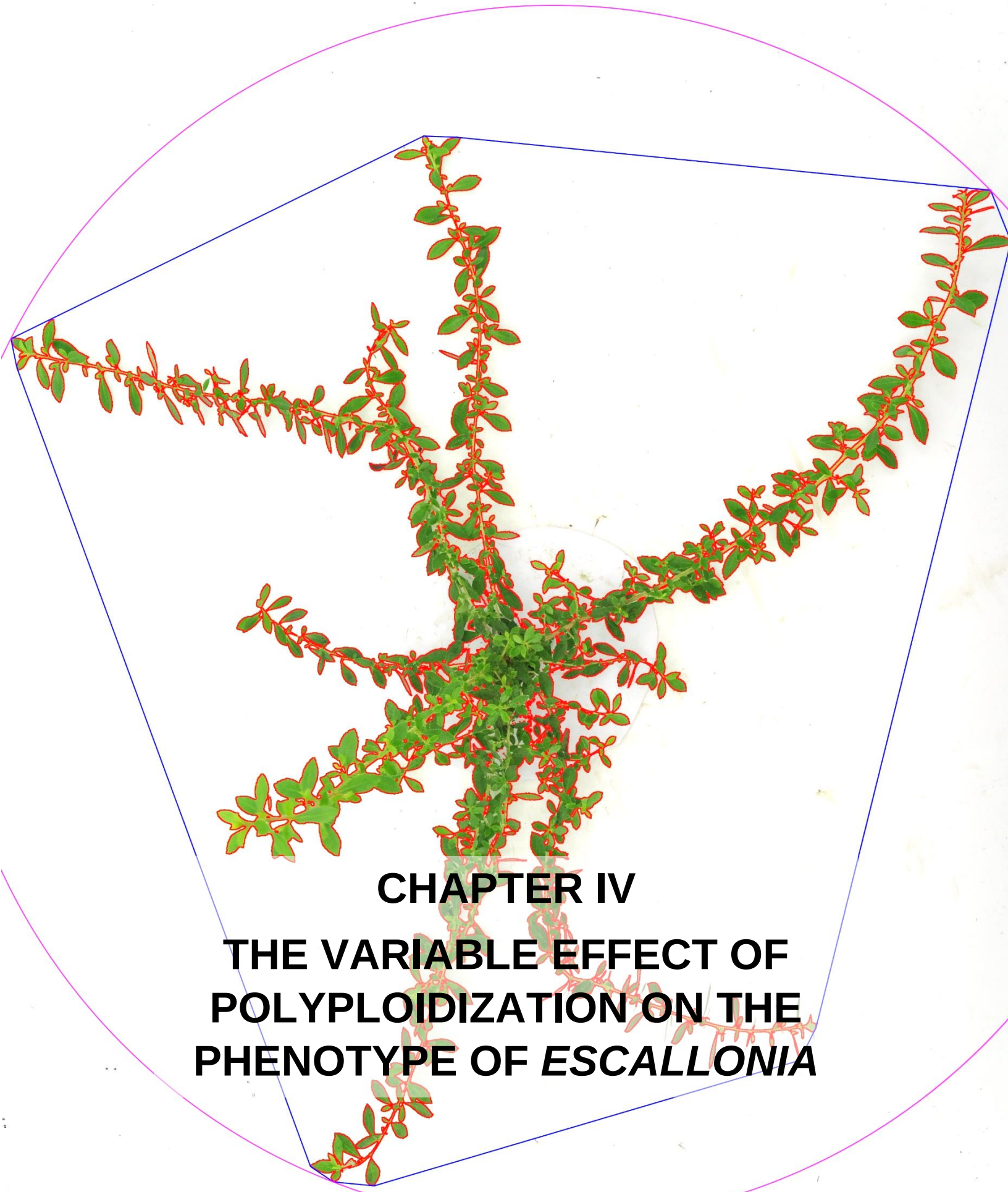
In progeny resulting from interspecific crosses, AFLP analysis is valuable for assessing their hybrid nature, as shown in *Buxus* (Van Laere et al., 2015). No prior sequence information is needed for AFLP (Meudt and Clarke, 2007), which is beneficial in the study for hybridity in taxa where little is known about the genomic composition. In this study, the majority (82%) of the progeny proved to be true hybrids (Table 3.3). Only 16 out of 88 (18%) of the progeny tested contained between 25% and 10% of the unique male parental markers, indicated as partial hybrids. Of these, 13 are resulting from a cross between parents with a different ploidy level. Hypotheses for the creation of partial hybrids are either substitution lines, where only a few male chromosomes substitute the female chromosomes, or addition lines, where a few male chromosomes are added. Because an interspecific cross combines two genomes, the new genomic constitution can lead to genetic and epigenetic reorganization and loss of chromosomes due to intergenic conflicts which can be seen as early as in the F1 hybrid (Wu et al., 2015, Groszmann et al., 2013, He et al., 2013). Furthermore, these hypotheses of partial hybrids are supported by the results of meiotic studies in *S. pruniformis* and *S. saligna* by Saggoo et al. (2011). The meiotic course of these species was abnormal, showing transfer of chromatin material through a channel between two pollen mother cells and the presence of laggards during anaphase I and II, and telophase I and II. This results in pollen grains with either extra or too little chromosomes. The parental species who produced partial hybrids could be studied to see whether these also show meiotic abnormalities. GISH and chromosome counts could further elucidate the chromosomal composition in the partial hybrids. In several other studies, partial hybrids were detected after interspecific hybridization, amongst others in *Buxus* (Van Laere et al., 2015), *Hydrangea* (Van Laere, 2008) and Ranunculaceae (Dhooghe, 2009). No seedlings with less than 10% of male parental markers were found, indicating that no self-pollinations occurred and the emasculation procedure was adequate.

Currently, the obtained interspecific hybrids are grown on the field (Figure 3.2) for further evaluation in cooperation with the growers from BestSelect CVBA. All the hybrids are viable, however, as they are still too young, their (commercial) value could not be determined as yet.

Figure 3.2: A) Intraspecific hybrids of *S. hookeriana* 'Ghorepani' (S10) x *S. hookeriana* var. *digyna* (S08). B) Interspecific hybrids of *S. ruscifolia* (S06) x *S. confusa* (S01). Red bar = 20 cm.

3.5. CONCLUSIONS

This is the first study to explore the possibilities of interspecific hybridization in *Sarcococca*. Crossing efficiency was mostly dependent on the ploidy level and 2C value of the parents. Existing ploidy level and 2C value differences among the *Sarcococca* genotypes did not completely hinder the creation of hybrid progeny (partial or true hybrids) but such crosses had a lower crossing efficiency. The hybrid status of the F1 progeny was confirmed for almost all seedlings. The seedlings have been planted under field conditions for further phenotypical evaluation and selection.



CHAPTER IV

THE VARIABLE EFFECT OF POLYPLOIDIZATION ON THE PHENOTYPE OF *ESCALLONIA*

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Parts of this chapter are published in:

- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J & VAN LABEKE MC (2015). Induction of tetraploids in *Escallonia* spp. Acta Horticulturae, 1087, 453-458.
- DENAEGHEL H, VAN LAERE K, LEUS L, LOOTENS P, VAN HUYLENBROECK J, VAN LABEKE MC (2018) The variable effect of polyploidization on the phenotype in *Escallonia*. Frontiers in Plant Science, 9

4. THE VARIABLE EFFECT OF POLYPLOIDIZATION ON THE PHENOTYPE OF *ESCALLONIA*

4.1 INTRODUCTION

Polyploidization, or the manipulation of the number of basal chromosome sets (x), is one of the main breeding tools in ornamentals to create variation in phenotype and physiology of plants (Dhooghe et al., 2011, Sattler et al., 2016, Horn, 2002b). The basic consequence of polyploidization is an increase in cell size caused by the larger number of gene copies (gigas effect) (Sattler et al., 2016). Therefore, polyploids may have larger organs than their diploid counterparts, such as larger and thicker leaves, flowers, and fruits (Feng et al., 2017, Tang et al., 2010). However, an increased cell size does not implicate an increased plant size, as the number of cell divisions can be reduced in polyploids and thus result in more compact growing genotypes (Sattler et al., 2016, Horn, 2002b, Hias et al., 2017). More compact growing polyploids have been found in *Malus* (Hias et al., 2017), *Buddleja* (Rose et al., 2000b), *Petunia* (Regalado et al., 2017), *Rosa* (Feng et al., 2017), *Platanus* (Liu et al., 2007) and *Eriobotrya* (Blasco et al., 2015) among others. Other changes can include darker green leaves, stronger and thicker stems, a higher petal number and deeper color in flowers and a delay in flowering time. However, also albinism, malformations and brittle wood have been reported (Dhooghe et al., 2011). Besides morphological changes, physiological changes, e.g., stress resistance and flowering period, are reported for polyploid plants (Regalado et al., 2017, Van Laere et al., 2010, Levin, 2002) due to an increase in genome flexibility (Levin, 2002). A change in drought tolerance was found in tetraploid *Spathiphyllum* (Van Laere et al., 2010), in autotetraploid *Malus* (Zhang et al., 2015a) and in pentaploid *Betula* (Li et al., 1996). An increase in drought tolerance was found in *Citrus* (Ruiz et al., 2016). In tetraploid *Lonicera*, an increase in both heat tolerance and drought was observed (Li et al., 2011, Li et al., 2009).

Nowadays, development of compact growing plants is a major goal in many breeding programs for woody ornamentals. Currently, compactness is obtained by frequent pruning (Mutlu and Kurtulan, 2015, Meijon et al., 2009), by changing environmental factors such as light and temperature regimes for indoor plants (Clifford et al., 2004, Löfkvist, 2010, Bergstrand and Schussler, 2013), or by using plant growth regulators such as chlormequat, paclobutrazol, trinexapac-ethyl, and daminozide (PPDB, 2017, Löfkvist, 2010, Lutken et al., 2012, Mutlu and Kurtulan, 2015, Meijon et al., 2009). However, the chemical approach is currently under debate for environmental reasons (Lutken et al., 2012, Wang et al., 2011). Therefore, as a more durable

approach, in this chapter the use of polyploidization to generate more compact woody ornamental plants is evaluated.

In vivo and *in vitro* polyploidy induction has been widely applied in ornamental breeding. However, each species responds differently to polyploidization and tailored protocols need to be developed. Most protocols are developed *in vitro*, but some species are very difficult to initiate and propagate *in vitro*, and *in vivo* chromosome doubling protocols are needed, e.g., for *Ziziphus* (Shi et al., 2015). Once polyploids are created, traits need to be evaluated. Most phenotyping studies on polyploid ornamentals measure the characteristics of interest, e.g., internode length, leaf length and width, flower diameter, number and length of branches, etc., manually on a limited number of plants (Liu et al., 2007, Stanys et al., 2006, Tang et al., 2010, Van Laere et al., 2010, Regalado et al., 2017, Ari et al., 2015). However, in contrast, image analysis enables a more in-depth analysis of plant characteristics (Fahlgren et al., 2015), as performed on leaf area to compare diploid and tetraploid apple trees (Hias et al., 2017). Image analysis of whole shoots augmented the information on plant architecture and the number of plants that could be analyzed, e.g., in rose (Li-Marchetti et al., 2015) and pea (Humplik et al., 2015a).

In order to create new variation in general and to create more compact plants specifically, polyploids were generated of a selected number of *Escallonia* genotypes. The slow *in vitro* growth of the *Sarcococca* stock did not allow for polyploidization experiments in this genus. The generated *Escallonia* tetraploids were characterized for compactness and plant shape using an image analysis approach. As *Escallonia* is only moderately hardy (-1.1°C to -14.9°C) (Hoffman and Ravesloot, 1998), therefore new (compact) cultivars with an improved cold tolerance would be a commercial success. Ergo, a cold tolerance test is performed on the resulting tetraploids. In addition, horticultural traits of interest, such as rooting capacity and flower characteristics were quantified.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

The collection and *in vitro* initiation of the plant material is described in Chapter II. Three genotypes, *E. illinita* (E01)*, E. rosea* (E14) and *E. rubra* (E16) were chosen for further experiments and will be referred to as *E. illinita, E. rosea* and *E. rubra* further in this chapter. They were plentiful available *in vitro,* and displayed morphological differences.

4.2.2 Chromosome doubling

Preliminary experiment
Nodal explants (0.2-0.5 cm) of *E. rosea* and *E. rubra* were submerged in 100 mL liquid Murashige and Skoog medium (Murashige and Skoog, 1962) (MSM, MS including vitamins, Duchefa) with 30 g/L sucrose, pH = 5.9 ± 0.1, with addition of 1000 µM colchicine (COL, dissolved in 5 mL of demineralized water), 150 µM oryzalin (ORY, dissolved in 1 mL 99% EtOH) or 150 µM trifluralin (TRI, dissolved in 1mL acetone). The nodes were incubated for 2 and 3 days on a gyratory shaker (75 rpm) in a growth chamber (ambient temperature: 23 ± 1°C, photoperiod: 16 h, light intensity: 35 µmol/m²s, bottom cooling: 18 ± 1°C). Each treatment was performed on 30 nodes. The nodes in the control treatment were submerged in liquid MSM without antimitotic agents. After exposure, the nodes were rinsed in sterile water and placed on solid MS growth medium with 30 g/L sucrose, 0.15 mg/L BAP, 0.05 mg/L NAA, 7 g/L agar (Lab M), and pH = 5.9 ± 0.1 (6 nodes per jar) without mitotic inhibitors and placed in the growth chamber.

4.2.2.1 Shock treatments

Nodal explants (0.2-0.5 mm) of *E. illinita, E. rosea,* and *E. rubra* were submerged in 100 mL liquid MS medium with 30 g/L sucrose, pH = 5.9 ± 0.1, with addition of 0, 50, 150, or 250 µM of oryzalin (ORY; dissolved in 1 mL 99% EtOH) or trifluralin (TRI; dissolved in 1 mL acetone). For *E. rosea*, also 2000 µM of COL was used. The nodes were incubated for 2, 3 or 4 days on a gyratory shaker (60 rpm) in a growth chamber (ambient temperature: 23 ± 1°C, photoperiod: 16 h, light intensity: 35 µmol/m²s, bottom cooling: 18 ± 1°C). Each treatment was performed on 30 nodes (2 jars with 15 nodes). The control treatment (0 µM; only addition of 1 mL EtOH or acetone in analogy with the treatments) contained 18 nodal explants (2 jars with 9 nodes). When the allotted exposure time was reached (2, 3 or 4 days) the nodal explants were rinsed with sterile demineralized water and transferred to solid MS growth medium with 30 g/L sucrose, 0.15 mg/L BAP, 0.05 mg/L NAA, 7 g/L agar and pH = 5.9 ± 0.1 (6 nodes per jar), and placed in the growth chamber.

4.2.2.2 Continuous treatments

Nodal explants (0.2-0.5 mm) of *E. illinita, E. rosea* and *E. rubra* were placed on 100 mL solid MS growth medium containing either 0, 1, 5 or 10 µM ORY (dissolved in 40 µL EtOH) or TRI (dissolved in 40 µL acetone) and grown in the growth chamber (for conditions see above) for 6, 8 or 10 weeks. Each treatment was performed on 30 nodes (6 nodes per jar). The control treatment (0 µM; only addition of 40 µL EtOH or acetone in analogy with the treatments) contained 18 nodal explants (6 nodes per jar). After the allotted exposure time (6, 8 or 10 weeks), the nodal explants were rinsed with sterile distilled water, transferred to 100 ml solid MS growth medium with 30 g/L sucrose, 0.15 mg/L BAP, 0.05 mg/L NAA, 7 g/L agar and pH = 5.9 ± 0.1 (6 nodes per jar), and placed in the growth chamber.

4.2.2.3 Analysis of ploidy level with flow cytometry

The mortality (%) and tetraploid yield (T-yield, %) of the preliminary experiment, the shock and continuous treatments were determined 12 weeks after the start of the experiment. Contaminated nodes were excluded from the calculations, the mortality and the T-yield were calculated from the total number of non-contaminated nodes. Non-surviving shoots are included in the T-yield. From each surviving nodal explant, a single developing axillary shoot was selected, indicating one possible polyploidization event. To determine the ploidy level of this event, a young leaf was sampled (10 à 20 mm^2). Samples were chopped according to Galbraith et al. (1983), stained with 4',6-diaminidino-2-phenylindole (DAPI) using a citrate buffer (500 µL 0.1M citric acid monohydrate and 0.5% Tween 20) and a phosphate buffer (750 µL 0.4 M $Na_2HPO_4.12H_2O$, 2mg/L DAPI, 0.1% polyvinylpyrrolidone (PVP) (modified from Otto (1990), and analyzed with a flow cytometer equipped with an UV LED (365 nm) (Cyflow Space, Partec). Histograms were analyzed using FloMax software (Partec). An *in vitro* sample of a non-treated diploid plant of the same species was used as external standard.

Each nodal explant that resulted in a tetraploid was identified by a unique code (T01, T02,…) for each species. Diploid plants from the control treatment were similarly named (D01, D02,…). The diploid controls and tetraploid events are therefore referred to by these codes below.

4.2.3 Characterization of the tetraploids

4.2.3.1 Acclimatization of plant material

All tetraploid shoots form the preliminary experiment, the shock and continuous treatments, were transferred to a solid MS rooting medium (30 g/l sucrose, 0.5 mg/l NAA, 7 g/l agar, pH = 5.9 ± 0.1) for approximately six weeks. An equal number of diploid shoots from the control treatments were also transferred to this MS rooting medium. When the first roots emerged, plantlets were acclimated in the greenhouse (photoperiod of 16 h; mean temperature day: 22.9°C and night: 19.9°C; fertilization: N-P-K+MgO 20-5-10+2 at EC = 1 mS/cm and pH = 5) in a peat based substrate (Saniflor: 1.5 kg/m³ fertilizer: 12N:14P:24K + trace elements, pH 5.0-6.5, EC 450 µS/cm). These plants were used as mother plants for cutting production. Cuttings were rooted in peat substrate (Saniflor: 1.5 kg/m³ fertilizer, 12N:14P:24K + trace elements, pH 5.0-6.5, EC 450 µS/cm) without auxin treatment in the greenhouse under a tunnel covered with white plastic to maintain humidity at 100% RH. Rooted cuttings were used for further experiments.

4.2.3.2 Rooting capacity

For determining the rooting capacity, the diploid and tetraploid numbers were grouped in homoploid groups per *Escallonia* genotype. For *E. rosea* and *E. rubra,* 4 replicates of ± 50 cuttings (± 5 cm) were made from the diploid control group and from the tetraploid group. For *E. illinita,* 2 replicates of ± 60 cuttings (± 5 cm) were made per ploidy group. The cuttings were taken from randomly chosen numbers in the homoploid groups. After five weeks, the rooted cuttings were rinsed with tap water, photographed, the roots were excised and the fresh weight of the roots was determined on an analytical balance. Subsequently, the roots were dried for 48h at 70°C and weighed again to determine the dry matter content.

4.2.3.3 Morphological traits

4.2.3.3.1 Growth, branching and leaf characteristics

Apical cuttings (± 5 cm) were made for each number from 6-month-old mother plants for all three genotypes (*E. illinita, E. rosea* and *E. rubra*). Five weeks after cutting, measurements were made on at least four rooted cuttings per number. The length of the new apical shoot (NSL) and its internode length (NSIL) were determined. Subsequently, the cuttings were pinched, leaving four

nodes of the new grown shoot. Seven weeks after pinching, the axillary budburst (BB, % of nodes on the main stem that sprouted), axillary branch length (BL) and the branch internode length (BIL) were measured on the plantlets. Ten full-grown leaves were collected on each of five randomly chosen plantlets of each tetraploid and diploid number, and photographed. Leaf length, width, and surface area were measured in ImageJ. The length was measured from the leaf base to the tip. The width was measured on the widest point of the leaf, perpendicular to the height.

4.2.3.3.2 Flower characteristics

Tetraploid and diploid mother plants were planted in the field (51°0'N, 3°48'E, Melle, Belgium) in October 2016 for *E. rosea* and *E. rubra* and in October 2017 for *E. illinita* . After one year, flowering has occurred only on *E*. *rubra* diploids and tetraploids and on *E. rosea* diploids. The flowers of the diploid controls and tetraploid genotypes of *E*. *rubra* were grouped. For the diploid group, 96 flowers were collected vs. 93 flowers for the tetraploid group. In addition, 76 flowers were collected from the original plant in our breeders' collection in the field. Top and side view photographs were taken of the flowers then analyzed for flower length, tube width and corolla surface area using ImageJ (Abramoff et al., 2004). The flower length was measured from the base of the receptacle to the corolla.

4.2.3.4 Plant architecture

After pinching the plantlets, pictures, were taken of the diploid and tetraploid numbers. For *E. rosea* and *E. rubra*, pictures were taken 11 weeks after pinching from all diploid controls and all tetraploid events. For *E. illinita* pictures were taken five weeks after pinching, due to a more vigorous growth, but only from five diploid controls and five tetraploid events, randomly selected.. Top view pictures were taken from ten clones of the selected diploid and tetraploids. Five clones were photographed from the side. For *E. rosea* and *E. rubra*, the clones were photographed from the side three times, each time turning the plant 120°. For *E. illinita,* the clones were photographed from the side two times, turning 90° between two pictures. For the top view, convex hull (CoHu) area and perimeter, and the minimal bounding circle (MBC) were determined in ImageJ (Abramoff et al., 2004) (Figure 4.1 A). The convex hull was created with the Hull and Circle plugin provided by ImageJ (Abramoff et al., 2004). With the convex hull area and perimeter, the circularity (Circ) was determined. This formula returns a value between 0 and 1, with 1 being a perfect circle.

$$Circ = \frac{4 * \pi * CoHu\ area}{(CoHu\ perimeter)^2}$$

For the side view, the plant surface, width and height were determined in ImageJ (Abramoff et al., 2004) (Figure 4.1 B), by using the Measure function (bounding rectangle) on the selected plant surface.

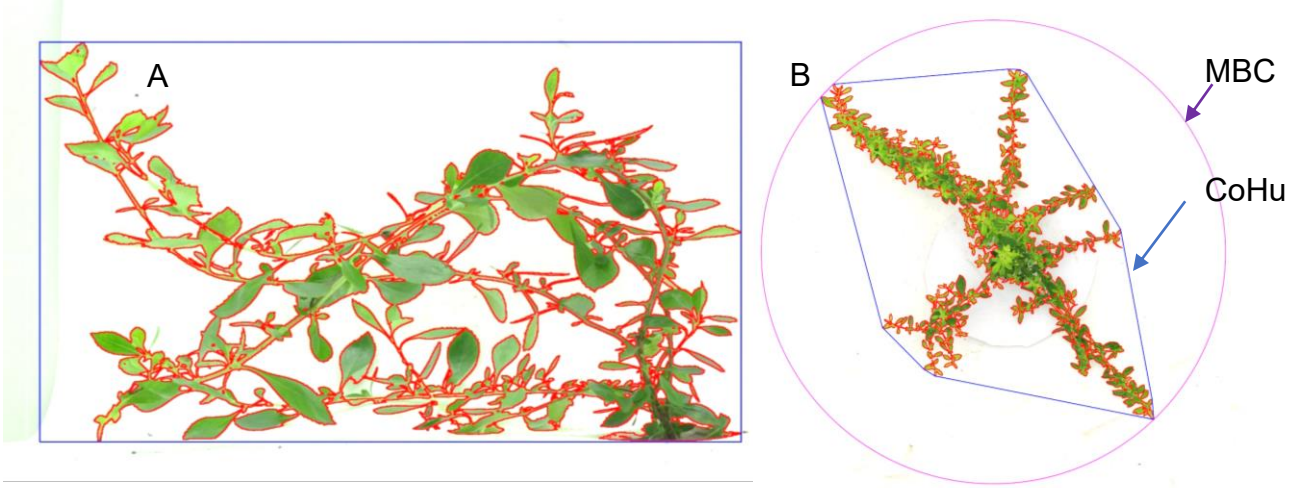

Figure 4.1: A) Side view photograph of the tetraploid *Escallonia rubra* T04 with the bounding rectangle (blue) and analyzed plant area (red) indicated. B) Top view photograph of the tetraploid *E. rosea* T04 with the convex hull (CoHu, blue), the minimal bounding circle (MBC, purple), and the analyzed plant area (red) indicated. Photographs taken eleven weeks after the pinching of the cuttings.

4.2.3.5 Controlled freezing test

Shoots of at least one diploid and one tetraploid number of *E. rosea* and *E. rubra* were collected in January 2017 after a cold period (13 days in November and December 2016 with minimum temperatures < 0°C) on field-grown plants. The analyzed numbers were chosen randomly. No cold tolerance test could be performed on *E. illinita,* as they had not yet been cold-acclimated. The shoots were dissected in stem pieces of ± 1 cm, each containing one axillary bud. Ten randomly chosen stem pieces were weighed on an analytical balance, dried for 48h at 70°C, then weighed again to determine the dry matter content. Fifty stem pieces per number were placed in Eppendorf tubes (2 mL) with 0.5 mL distilled water and a few clean grains of sand. The stem tissue was frozen in a cryostat (Polystat 37, Fisher Scientific, Waltham, MA, USA) from 0°C to -35°C at a rate of -6°C/h. This was done for five replicates per cultivar and per freezing temperature. The positive controls were kept at a reference temperature of 4°C, the negative controls at -80°C. After the target temperatures were reached, the samples were transferred to vials containing 10 mL of incubation medium with 0.002% Triton-X and 10 mM boric acid (Ögren et al., 1997). The vials were shaken (200 rpm) for 20 h. To determine the degree of injury, the electrical conductivity (EC) of the samples was determined before (EC_{samp}) and after autoclaving ($EC_{samp,aut}$) (15 min, 121°C,

0.5 MPa). The positive control (4°C) was used as reference (EC_{ref} and $EC_{ref,aut}$). The index of injury (Flint et al., 1967) was determined for each sample as follows:

$$I(t) = \frac{\left(\frac{\text{EC samp}}{\text{EC samp, aut}} - \frac{\text{EC ref}}{\text{EC ref, aut}}\right)}{1 - \frac{\text{EC ref}}{\text{EC ref, aut}}} * 100$$

The formula to determine the index of injury I(t) is based on the principle that when dying plant cells burst, they release their content, and thus the EC value of the surrounding solution rises, which was measured after the cold treatment and the shaking. Subsequently, the surviving cells were killed by autoclaving and the total EC value was determined. The EC values of the reference samples at 4°C take the damage caused by sampling and the experiment itself into account. To calculate the LT50, the % of injury at -80°C was interpreted as 100%, and the other injury values were compared with this value. LT50-values (the temperature where 50% of plant cells were injured) were determined via sigmoidal regression on the adjusted injury values as determined by Lim and Arora (1998).

4.2.3.6 Statistical analysis

Statistical analysis was done in R, version 3.2.0 (R Core Team, 2015). All data were first analyzed for normality using the Shapiro-Wilk Test (p=0.05). For data that were not normally distributed, group comparison was done with the Kruskal-Wallis Test (KWT) and pairwise comparison with the Mann-Whitney U Test (MWUT). This was done for the data on rooting capacity and the data on root dry weight. MWUT and KWT were also used for part of the data concerning growth and branching, namely BL, BIL and axillary budburst of *E. illinita,* all the characteristics of *E. rosea,* and the BL, BIL and axillary budburst of *E. rubra*. The data of the leaf sizes of *E. rosea* and *E. rubra* were not normally distributed, as were the data from the top view and side view images of *E. rosea, E. rubra* and *E. illinita,* and the length and width of the flower tube of *E. rubra*. All data were analyzed using p = 0.05 unless stated otherwise. Boxplots for the figures in Supplementary Data were plotted in R, version 3.2.0 (R Core Team, 2015) using the boxplot function. The bottom and top of the box are the lower and upper quartiles; the band in the middle displays the median. The upper/lower whisker extends to the highest/lowest value, up to a maximum length of 1.5 times the box length. Higher/lower values are indicated as a dot. The diploid and tetraploid numbers were sorted by means.

For normally distributed data, group comparisons were performed with ANOVA and pairwise comparison was done using the t-test. This was done for the data on growth and branching, namely NSL and NSIL of *E. illinita,* and the NSL and NSIL of *E. rubra*. The data of the leaves of *E. illinita* and the area of the flower top view were analyzed using ANOVA and the t-test.

The phylogenetic trees were plotted with the pvclust package, using the UPGMA clustering method with Jaccard indices in R (version 3.2.0) (R Core Team 2015).

A principal component analysis (PCA) was executed for the diploids and tetraploids of all three *Escallonia* genotypes. The PCA contained the analyzed morphological traits, namely the length of the new apical shoot (NSL) and its internode length (NSIL), the axillary budburst (BB), axillary branch length (BL) and the branch internode length (BIL). Furthermore, the PCA included the plant architecture traits from the pictures in top view (TV), namely the plant area (TV_pl_ar), the circularity (TV_circ) and the % of the area of the minimal bounding circle that was filled by the plant (TV_fill). The architecture traits from the pictures in side view (SV) used were the plant area (SV_pl_ar), the plant height and width (SV_he and SV_wi, respectively) and the % of the area of the bounding rectangle that was filled by the plant (SV_fill).The graphs were made in R (version 3.2.0) using the prcomp function and plotted with the ggbiplot function, with ellipses drawn with a probability of 0.95 (R Core Team 2015).

4.3 RESULTS

4.3.1 Chromosome doubling

4.3.1.1 Preliminary experiment

An overview of the tetraploid yield (T-yield) and mortality of the preliminary experiment are displayed in Table 4.1. The detailed results (including mixoploids and contaminations) are displayed in table 1 in the Addendum. For COL experiments on *E. rosea*, only the 3 day treatments with 2000 µM COL yielded tetraploids, but only 3.3%. Both treatments with ORY yielded much more tetraploids than COL, namely 29.2% and 33.3% for 2 days and 3 days respectively. TRI treatments had a T-yield of 0% and 12.5% for 2 and 3 days respectively. For *E. rosea,* 3 day treatments yielded more tetraploids on average than 2 day treatments, namely 12.3% and 7.2% respectively.

For *E. rubra,* a 2 day COL treatment yielded 10.0%, which was 3 times lower than a 2 day ORY or TRI treatment with T-yields of 32.1% and 35.1% respectively. For the 3 day treatment, ORY yielded most tetraploids (22.0%), followed by COL (16.7%) and then by TRI with only 8.3% tetraploids. On average for *E. rubra,* 3 day treatments were more effective in inducing tetraploids than 2 day treatments, with T-yields of 25.7% and 15.6% respectively. On average for both *Escallonia* genotypes, treatments with 150 µM ORY yielded the most tetraploids, namely 30.7% after 2 days and 27.7% after 3 days.

The mortality in this experiment was quite fluctuating. For *E. rosea,* the mortality in the 2 day treatment was much higher than for the 3 day experiment, except for ORY. For *E. rubra*, In the 2-day treatments, COL caused a higher mortality than both ORY and TRI. In the 3 day treatment, COL caused no mortality, while the mortalities of ORY and TRI were very high (45.8% and 77.8% respectively). The mortality of the 3-day control treatment was much higher (31.8%) than for the 2-day control treatment (0.0%).

For *E. rosea,* in total 240 nodes were treated, of which 34 tetraploids were recovered. For *E. rubra,* a total of 180 nodes were treated with mitotic inhibitors, which yielded 38 tetraploids (Table 1 in Addendum).

Table 4.1: The tetraploid yield (T-yield, %) and the mortality (%) of the preliminary experiment on *E. rosea* and *E. rubra* with different mitotic inhibitors, exposure times and concentrations.

Treatment			*E. rosea*		*E. rubra*	
Time (days)	Mitotic inhibitor	Conc. (µM)	T-yield (%)	Mortality (%)	T-yield (%)	Mortality (%)
2	COL	2000	0.0	13.3	-	-
2	COL	1000	0.0	36.7	10.0	20.0
2	TRI	150	0.0	58.3	35.1	5.3
2	ORY	150	29.2	12.5	32.1	7.1
2	Control	0	0.0	0.0	0.0	0.0
3	COL	2000	3.3	3.3	-	-
3	COL	1000	0.0	0.0	16.7	0.0
3	TRI	150	12.5	0.0	8.3	77.8
3	ORY	150	33.3	20.0	22.0	45.8
3	Control	0	0.0	4.2	0.0	31.8

With these results, further experiments were designed. Since COL did not yield more tetraploids than ORY and TRI and due to its high toxicity to humans, COL was not used in further experiments. The shock experiment was expanded, with more concentrations of ORY and TRI, and with a longer exposure time of 4 days. The erratic mortality in this shock experiment led to the design of a less stressful, continuous experiment with lower concentrations of ORY and TRI.

4.3.1.2 Shock experiments

An overview of the T-yield and the mortality of the shock treatments are shown in Figure 4.2. The detailed results (including mixoploids, octaploids and contaminations) are displayed in Table 2 (*E. illinita*), Table 3 (*E. rosea*) and Table 4 (*E. rosea*) in the Addendum. In ORY treatments, a positive correlation between T-yield and ORY concentration was found for all three genotypes, with an average from 5.5% up to 24.9% for 50 µM and 250 µM, respectively. In TRI treatments T-yield reached a plateau at 50 µM for *E. rosea* and *E. rubra* and at 150 µM for *E. illinita .* An increase in exposure time caused a decrease in T-yield (on average from 21.4 to 6.8% for 2 days to 4 days, respectively) and an increase in mortality (on average from 6.8 to 30.2% for 2 days to 4 days, respectively) in ORY treatments. Only for *E. illinita,* the effect on the mortality was reversed, with a small decrease in mortality from 14.4 to 8.9% (Figure 4.2). In TRI treatments, the exposure time did not cause changes in average T-yield and mortality.

The ORY treatment that yielded the best results for all three *Escallonia* genotypes was 2 days of 250 µM ORY, resulting in a high T-yield (22.0 to 56.7%) and low corresponding mortality (0.0 to 4.9%). No best treatment for TRI could be identified, as all concentrations yielded the approximate

same number of tetraploids. In the control treatment of *E. rosea* a limited number of tetraploids and mixoploids were found, due to the high stress induced by the treatment.

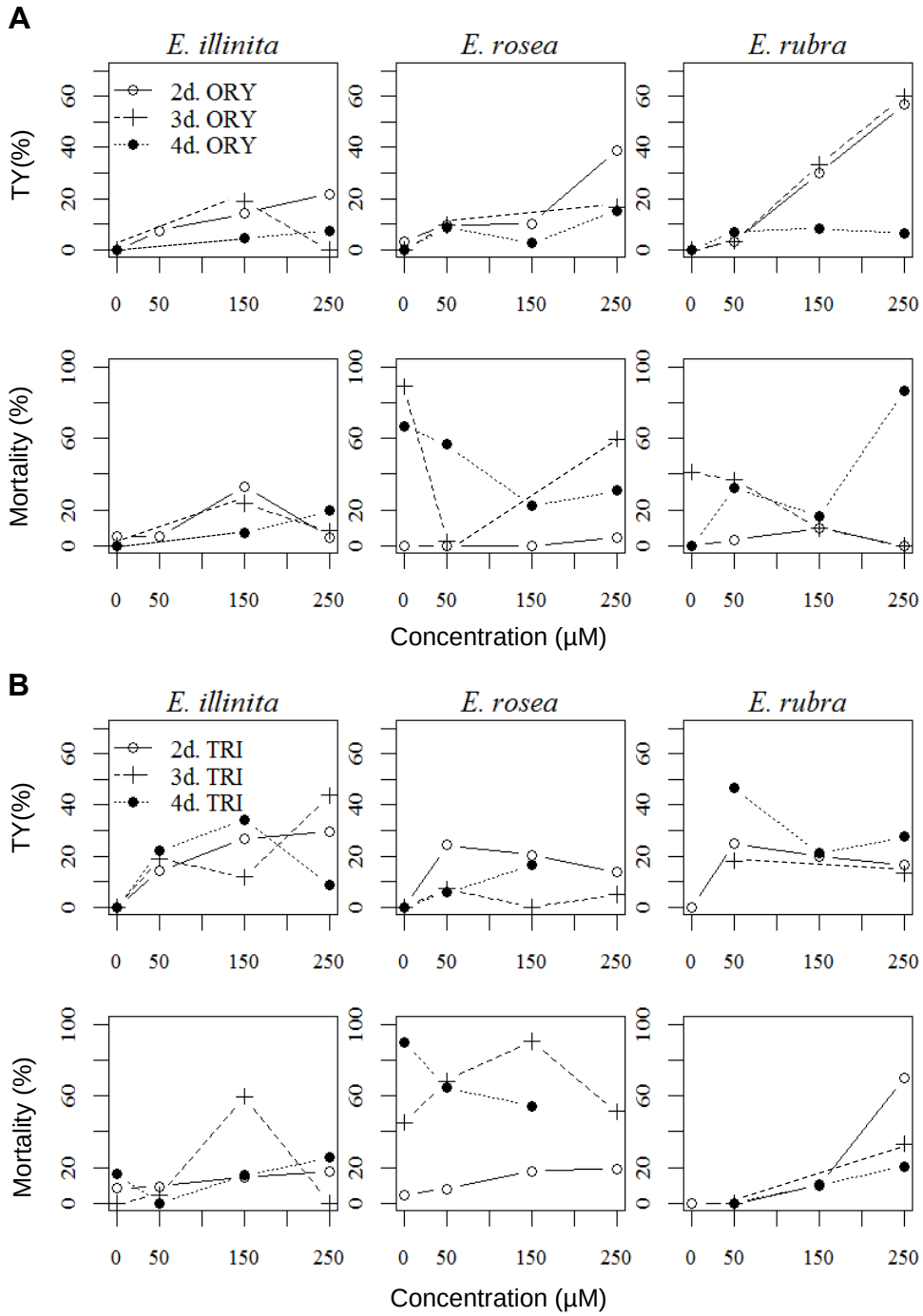

Figure 4.2: Tetraploid yield (T-yield, %) and mortality for *Escallonia illinita, E. rosea*, and *E. rubra* in a shock experiment of 2, 3, and 4 days with 0 (control), 50, 150, and 250 µM of A) oryzalin (ORY) or B) trifluralin (TRI). Graphs plotted in R (version 3.2.0) (R Core Team 2015)

For *E. illinita,* of the 571 non-contaminated nodes, 31 tetraploids were created with ORY and 67 with TRI (Table 2 in Addendum). For *E. rosea,* 585 nodes were treated, of which 42 tetraploids were recovered with ORY and 36 with TRI (Table 3 in Addendum). For *E. rubra,* 470 treated nodes yielded 61 tetraploids with ORY and 57 with TRI (Table 4 in Addendum). Only for *E. illinita* a large difference in T-yield was present between ORY and TRI, with TRI yielding a double amount of tetraploids.

Seven octaploids were generated for *E. illinita* in the treatments with 150 µM TRI for 2, 3, and 4 days, and with 150 µM ORY for 2 and 4 days (Table 2 in Addendum). One octaploid *E. rosea* was recovered in the 4 days treatment of 50 µM TRI (Table 3 in Addendum). Five octaploid *E. rubra* were recovered from 4 days of 150 µM TRI, 3 days with 50 µM TRI, 2 days with 150µM ORY and 3 days with 250 µM ORY (Table 4 in Addendum). The percentage mixoploids for the shock experiments with ORY reached up to 33.3%, and with TRI up to 31.7% (Table 2, Table 3 and Table 4 in Addendum). No mixoploids or octaploids were retained for further in-depth evaluation.

4.3.1.3 Continuous experiments

An overview of the T-yield and mortality after the continuous treatments are shown in Figure 4.3. The detailed results (including mixoploids, octaploids and contaminations) are displayed in Table 5 (*E. illinita*), Table 6 (*E. rosea*) and Table 7 (*E. rubra*) in Addendum. Differences between TRI and ORY were observed. For all three species, TRI induced more tetraploids compared to ORY. This effect was most substantial in *E. illinita* (on average 5.5% T-yield with ORY, 32.3% with TRI). On average, ORY led to higher mortalities than TRI.

The T-yield reached a plateau at 5 µM ORY for *E. rosea,* and at 1µM ORY for *E. rubra.* For *E. illinita,* the T-yield was positively correlated with the concentration. The mortality of all three species increased with ORY concentrations (on average from 11.6 to 38.7%). For TRI treatments, similar observations were made for both T-yield and mortality. For *E. rosea* and *E. rubra* the plateau in T-yield was reached at 5 µM TRI, while the T-yield of *E. illinita* increased with the concentration. No clear effect of the exposure time on the T-yield and mortality was found for both ORY and TRI. *E. rosea* and *E. rubra* showed a small decline in T-yield with increasing exposure and a small increase in mortality.

In this continuous experiment, a 10 week exposure of 5 µM of TRI was the overall best treatment, despite of species-dependent sensitivity towards the antimitotic agents used. For *E. rosea* and *E. rubra,* this resulted in respectively 43.5% and 54.2% tetraploids. For *E. illinita,* 10 weeks on 5 µM

of TRI resulted in a 63.4% T-yield, which was a close second to 8 weeks of 10 µM with a T-yield of 65.5%. The mortality of 10 weeks of 5 µM TRI of all three species was 12.5% or lower. For ORY, the best yielding treatment, or close second best for *E. rosea,* was 8 weeks with 1 µM of ORY. However, the T-yield of the best ORY treatment was much lower or similar to the T-yield in the best TRI treatment. Some continuous treatments yielded octaploids. One *E. illinita* octaploid was recovered after 10 weeks with 10 µM TRI. For *E. rosea,* all 10 week and 6 week TRI treatments yielded a sum of 10 octaploids. For *E. rubra,* the following treatments yielded 14 octaploids together: 6 weeks of 1 µM and 5 µM ORY, 6 weeks of 1 µM and 10 µM TRI, 8 weeks of 5 µM and 10 µM TRI, and 10 weeks of 5 µM TRI. The percentage mixoploids for the continuous experiments with ORY reached up to 31.0%, and with TRI up to 82.9%. No mixoploids or octaploids were retained for further evaluation.

Of the 688 non-contaminated nodes of *E. illinita*, ORY treatments yielded 20 tetraploids and TRI 100 (Table 5 in Addendum). For *E. rosea,* 625 nodes were treated, of which 57 tetraploids were recovered with ORY and 60 with TRI (Table 6 in Addendum). For *E. rubra,* 462 treated nodes yielded 35 tetraploids with ORY and 72 with TRI (Table 7 in Addendum). For both *E. illinita* and *E. rubra,* T-yields with TRI were at least twice as high compared with ORY, while for *E. rosea* there was no difference.

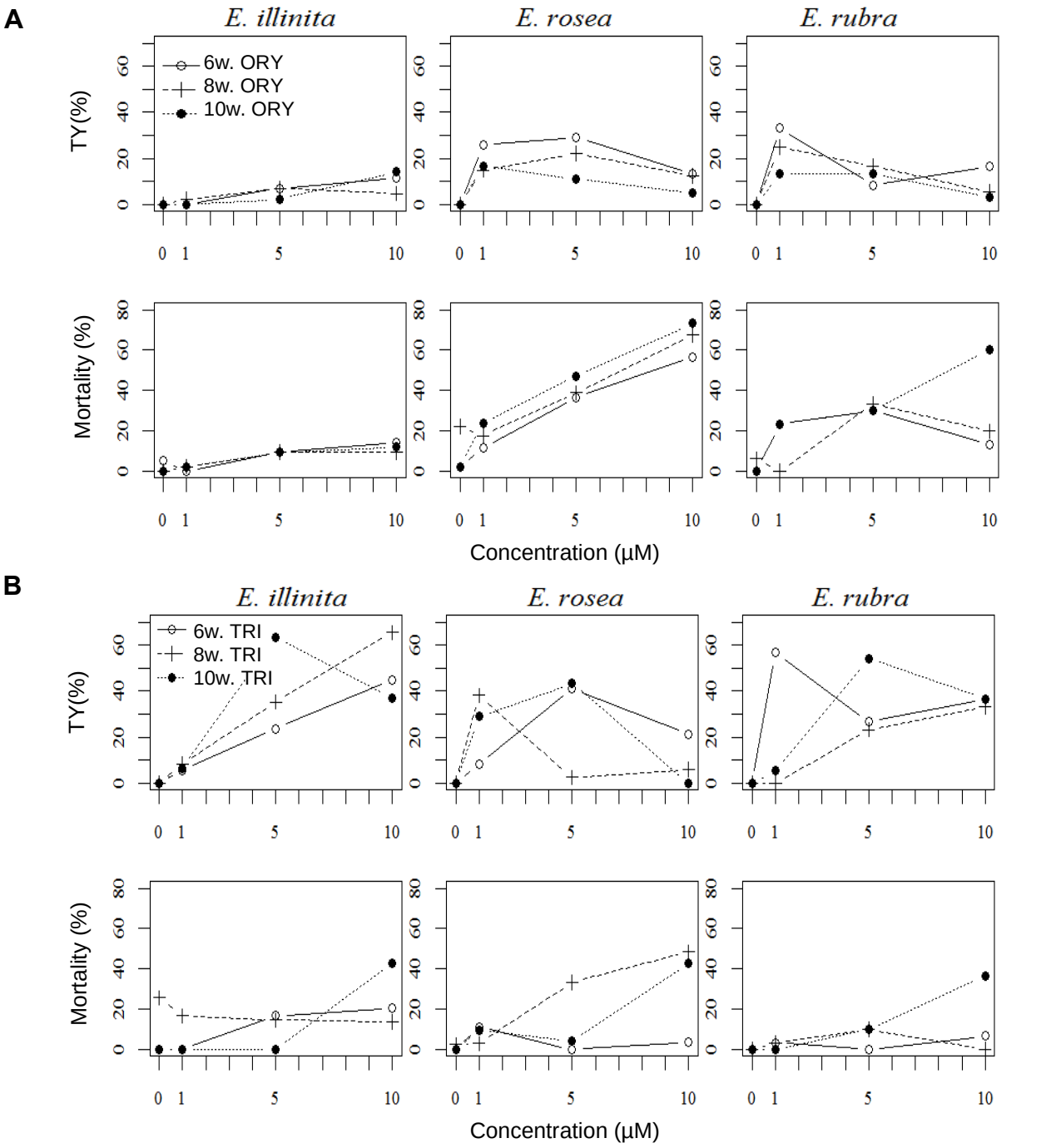

Figure 4.3: Tetraploid yield (T-yield, %) and mortality for *Escallonia illinita, E. rosea*, and *E. rubra* in a continuous experiment of 6, 8, and 10 weeks with 0 (control), 1, 5, and 10 µM of oryzalin (ORY) (C) and trifluralin (TRI) (D). Graphs plotted in R (version 3.2.0) (R Core Team 2015)

4.3.2 Characterization of the tetraploids

For *E. rosea* and *E. rubra,* the tetraploids and diploid controls used for phenotyping resulted from the preliminary experiment. For *E. rosea,* six tetraploids and four diploid controls survived the acclimation in the greenhouse. For *E. rubra* 23 tetraploids and 16 diploid controls were recovered.

For *E. illinita,* the plants used for phenotyping resulted from the continuous treatments. After acclimatization in the greenhouse, 10 diploid controls and 23 tetraploid numbers of *E. illinita* were randomly chosen for further phenotyping.

4.3.2.1 Rooting capacity

The rooting capacity of cuttings after five weeks did not differ between the diploid and tetraploid group for any of the three genotypes (Table 4.2). In addition, the root dry weight did not significantly differ between diploids and tetraploids.

Table 4.2: Rooting capacity and dry weight of cuttings of diploid (D) and tetraploid (T) *E. illinita, E. rosea,* and *E. rubra.*

Genotype	Ploidy group	Number of cuttings	Repetitions	Rooting (%)	Root dry weight (g)
E. illinita	D	136	2	94.9^{NS}	1.11 ± 0.96^{NS}
	T	126	2	96.1	1.06 ± 0.74
E. rosea	D	194	4	78.9^{NS}	0.56 ± 0.44^{NS}
	T	252	4	78.2	0.49 ± 0.31
E. rubra	D	188	4	80.3^{NS}	1.66 ± 2.71^{NS}
	T	192	4	79.2	1.84 ± 2.29

Statistical differences per genotype between diploids and tetraploids (MWUT, NS: not significant; S: significant at p = 0.05)

4.3.2.2 Morphological traits

4.3.2.2.1 Growth and branching

For all three species, much phenotypic variation for each trait was present within both the diploid control group and the tetraploid group. Diploids and tetraploids were not clearly separated for each trait, many intermediate phenotypes were present. Yet when analyzing the average results, trends could be observed (Table 4.3).

Table 4.3: New apical shoot length (NSL) and its internode length (NSIL), axillary branch length (BL) and its internode length (BIL), and the axillary budburst (% of buds on the main stem that sprouted) of *E. illinita, E. rosea,* and *E. rubra* diploid controls (D) and tetraploid numbers (T), measured on at least 4 plantlets per plant number (mean ± standard deviation).

Genotype	Ploidy group	Analyzed plant numbers	NSL (cm)	NSIL (cm)	BL (cm)	BIL (cm)	Axillary budburst (%)
E. illinita	D	6	11.0 ± 4.5 NS	1.0 ± 0.2 ***	23.6 ± 2.6 *	1.4 ± 0.1 *	15.6 ± 2.0 **
	T	23	11.4 ± 2.4	1.1 ± 0.2	20.1 ± 2.9	1.5 ± 0.1	18.0 ± 2.3
E. rosea	D	4	3.8 ± 2.8 ***	0.3 ± 0.1 ***	6.8 ± 2.2 ***	0.4 ± 0.1 ***	93.1 ± 10.3 **
	T	6	1.3 ± 1.2	0.2 ± 0.2	3.4 ± 2.0	0.3 ± 0.1	85.1 ± 15.2
E. rubra	D	16	4.9 ± 4.3 *	0.4 ± 0.3 ***	8.2 ± 2.1 ***	0.6 ± 0.1 ***	81.5 ± 17.0 **
	T	23	5.5 ± 3.9	0.5 ± 0.3	8.6 ± 2.0	0.7 ± 0.1	76.2 ± 16.7

Statistical analysis within the genotype between diploids and tetraploids with MWUT for pairwise comparison, except for NSL and NSIL of *E. illinita* and *E. rubra*, a T-test for pairwise comparison was used. NS: not significant; *: significant for p = 0.05; **: significant for p = 0.01; ***: significant for p = 0.001.

For *E. illinita,* the average length of the new apical shoot (NSL) did not differ significantly between diploids and tetraploids, yet its mean internode length (NSIL) increased significantly. In a further developed state (7 weeks later), tetraploids had a significantly higher axillary budburst (+ 2.4 %) on the main stem than diploids. The presence of more branches in tetraploids caused a significant decrease in average branch length (BL). The internode length of the axillary branches (BIL) was also significantly increased with ± 0.1 cm in tetraploids, as was the case for the NSIL. Thus for *E. illinita,* polyploidization created a tendency for shorter branches but longer internodes (Figure 4.4).

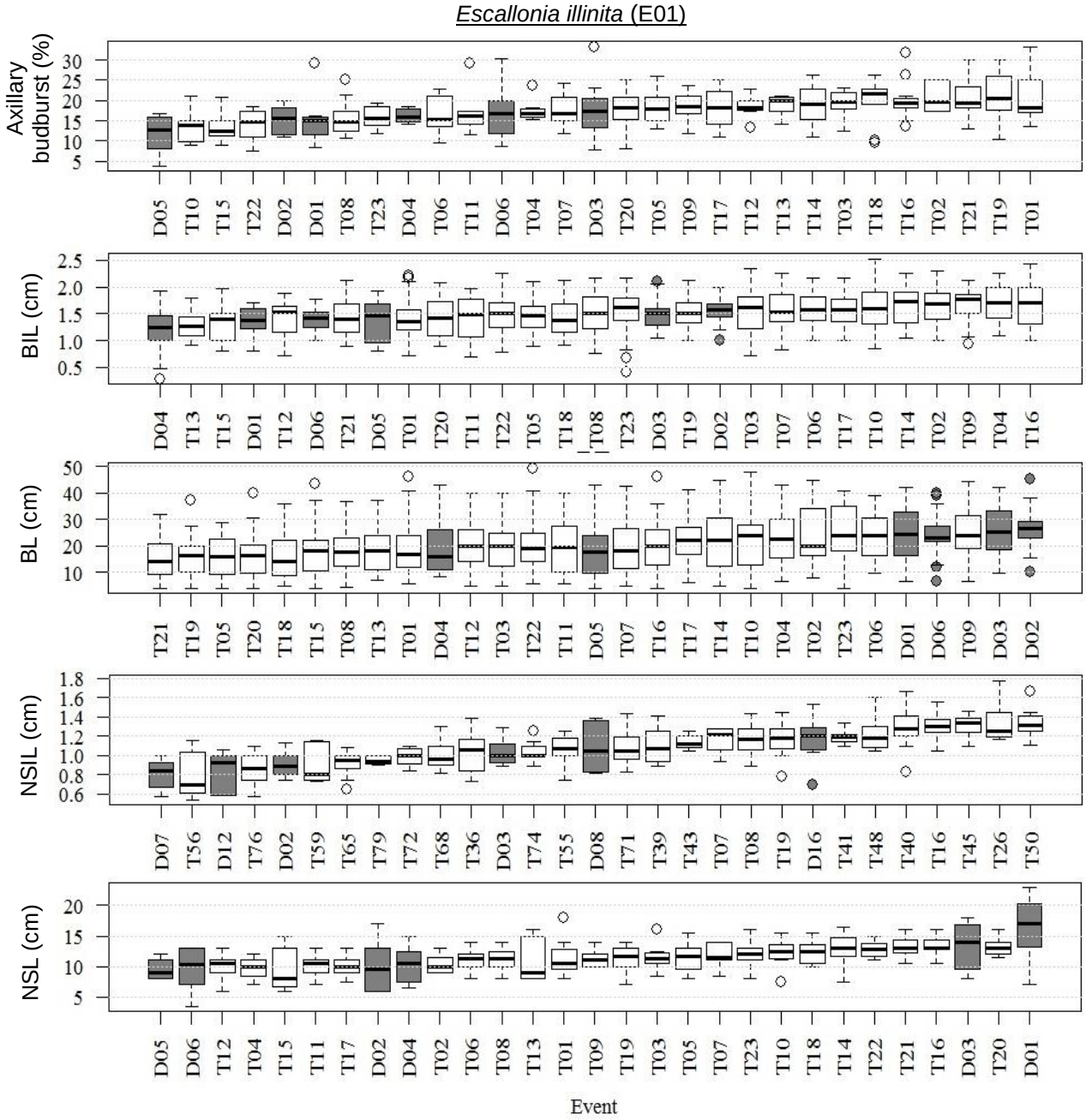

Figure 4.4: Morphological characteristics of diploid (D) and tetraploid (T) numbers of *Escallonia illinita* . The length of the new apical shoot (NSL) and its internode length (NSIL) of the 5-week-old rooted cuttings. The length of the branches (BL), their internode lengths (BIL), and the axillary budburst (%) of the pinched plantlet 17 weeks after cutting. (Diploid = grey, Tetraploid = white).

For *E. rosea*, on average the tetraploids reached about half the length of diploids. Both the NSL and the BL of tetraploids were significantly shorter. In addition, the internode length was significantly shorter in tetraploids than in diploids for both the NSIL and the BIL. The diploid axillary budburst decreased significantly by ± 8% due to polyploidization. Polyploidization resulted in a one-directional change towards smaller and slower growing plants for *E.* rosea (Figure 4.5).

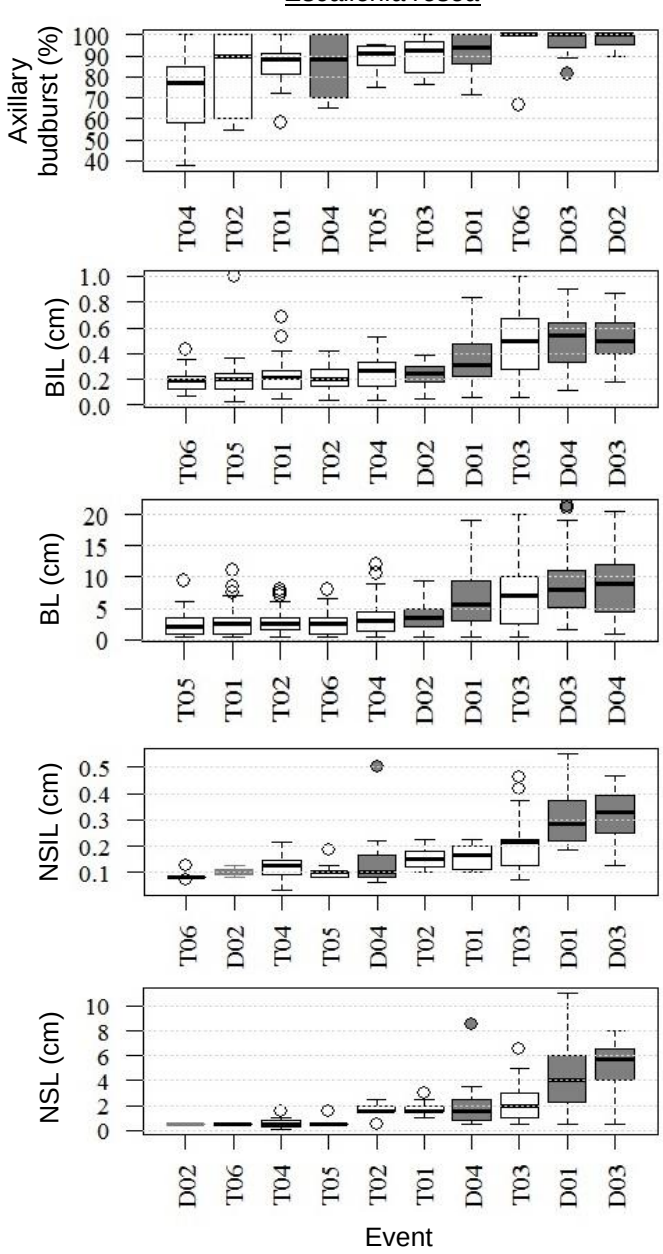

Figure 4.5: Morphological characteristics of diploid (D) and tetraploid (T) numbers of *Escallonia rosea.* The length of the new apical shoot (NSL) and its internode length (NSIL) of the 5-week-old rooted cuttings. The length of the branches (BL), their internode lengths (BIL), and the axillary budburst (%) of the pinched plantlet 17 weeks after cutting. (Diploid = grey, Tetraploid = white).

Tetraploids of *E. rubra* were larger and faster growing than the diploid controls. The NSL increased significantly with 0.6 cm. The increase was smaller for BL (only ± 0.4 cm) but highly significant. NSIL and BIL increased significantly. The axillary budburst of the diploid controls was significantly higher (5%) than of the tetraploid numbers. Thus it could be concluded that polyploidization resulted in a one-sided broadening or the variation present in the diploid group towards faster growing and taller plants (Figure 4.6).

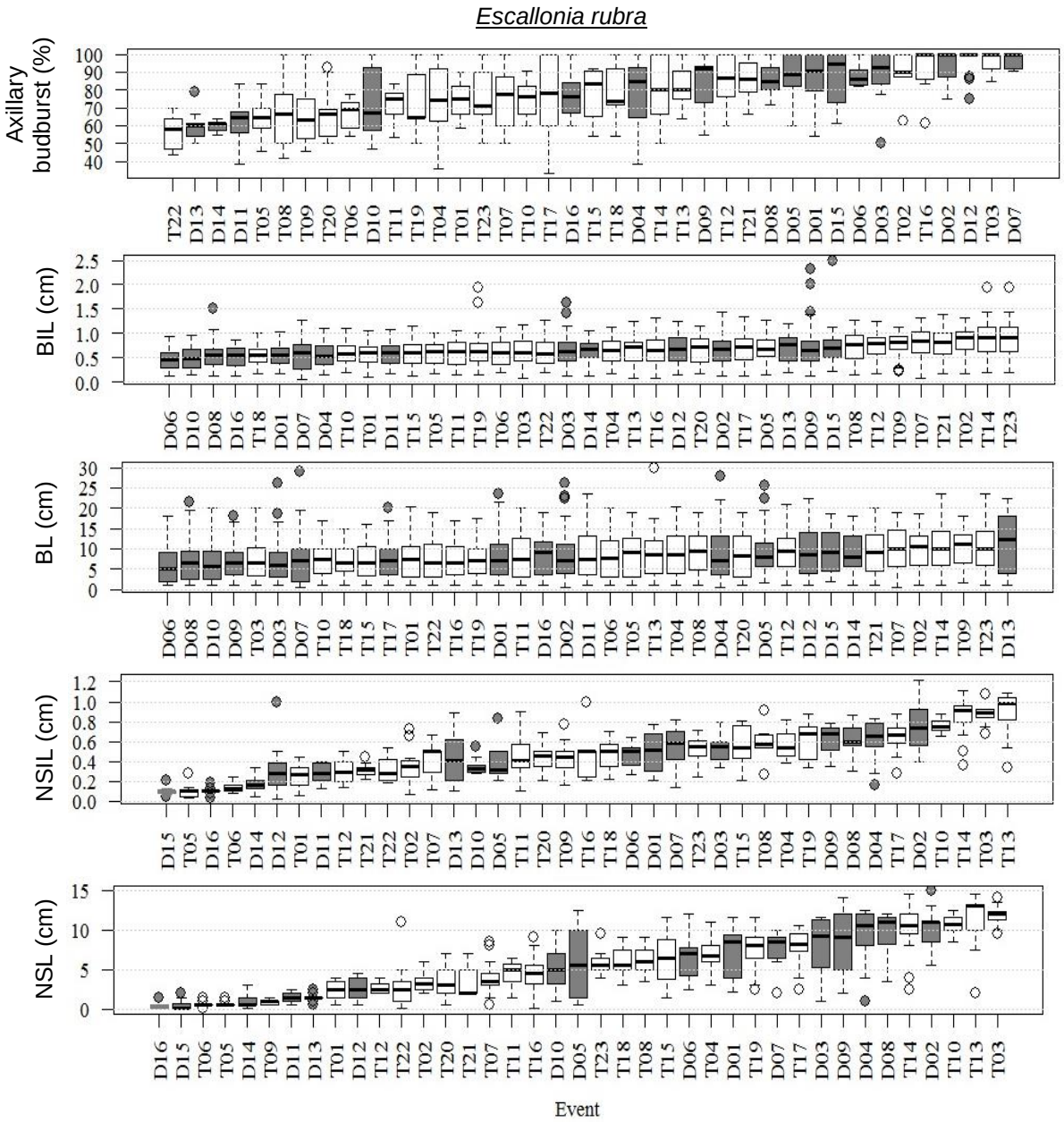

Figure 4.6: Morphological characteristics of diploid (D) and tetraploid (T) numbers of *Escallonia rubra.* The length of the new apical shoot (NSL) and its internode length (NSIL) of the 5-week-old rooted cuttings. The length of the branches (BL), their internode lengths (BIL), and the axillary budburst (%) of the pinched plantlet 17 weeks after cutting. (Diploid = grey, Tetraploid = white).

4.3.2.2.2 Leaf morphology

For both *E. illinita* and *E. rubra*, chromosome doubling caused wider leaves and a larger leaf surface (Table 4.4). The leaves from tetraploid *E. illinita* numbers were significantly wider, but not longer than the leaves of diploid controls. This resulted in a significant decrease in length/width ratio (L/W) and in a significant increase in leaf surface of ± 0.71 cm². Tetraploid *E. rubra* leaves were significantly wider (± 0.51 cm), but not longer than the leaves of diploids. This resulted in a significant decrease in L/W and a significant increase in leaf surface of ± 1.14 cm². In contrast, *E. rosea* tetraploid leaves were significantly reduced in both leaf length and width, resulting in a significant decrease of ± 0.27 cm² in leaf surface. The decrease was proportional for length and width, so no significant changes in L/W ratio were observed.

Table 4.4: Leaf length, width, length/width ratio (L/W), and leaf surface of diploid (D) and tetraploid (T) *E. illinita, E. rosea,* and *E. rubra,* collected on at least 4 plantlets per plant number (means ± standard deviation).

Genotype	Ploidy group	Analyzed plant numbers	N° of leaves	Length (cm)	Width (cm)	L/W ratio	Leaf surface (cm²)
E. illinita	D	6	390	4.17 ± 0.70 NS	1.75 ± 0.31 ***	2.40 ± 0.25 ***	4.49 ± 1.32 ***
	T	23	1270	4.12 ± 0.83	2.10 ± 0.46	1.98 ± 0.24	5.20 ± 1.99
E. rosea	D	4	180	2.55 ± 0.73 **	0.92 ± 0.32 **	2.92 ± 0.64 NS	1.63 ± 0.88 ***
	T	6	320	2.33 ± 0.66	0.85 ± 0.33	2.87 ± 0.60	1.36 ± 0.82
E. rubra	D	16	450	4.27 ± 0.83 NS	1.70 ± 0.35 ***	2.53 ± 0.36 ***	4.80 ± 1.80 ***
	T	23	1259	4.26 ± 0.81	2.21 ± 0.47	1.96 ± 0.25	5.94 ± 2.14

Statistical analysis within the genotype between diploids and tetraploids. (NS: not significant; *: significant for p = 0.05; **: significant for p = 0.01; ***: significant for p = 0.001). Pairwise comparison with the Mann-Whitney U Test for *E. rosea* and *E. rubra*, T-Test for *E. illinita* .

4.3.2.2.3 Flower characteristics

The diploid controls (D), the plants in our collection (Coll) and the tetraploid numbers (T) of *E. rubra* started flowering by mid-June (Figure 4.7). Flowers from the diploid control plants were significantly shorter (D: 0.96 ± 0.06 cm; Coll: 0.99 ± 0.06 cm) and wider (D: 0.28 ± 0.03 cm; Coll: 0.27 ± 0.03 cm) than flowers from the genotype of *E. rubra* in the collection (p = 0.001 and 0.01 respectively) (Figure 4.7). The corolla surface (D: 0.31 ± 0.06 cm²; Coll: 0.30 ± 0.04 cm²) was not significantly different between diploid controls and the collection genotype. As shown in Figure 4.7, the flowers of the tetraploid group were significantly larger and wider than both the collection and diploid flowers (T: 1.18 ± 0.11 cm long and 0.35 ± 0.03 cm wide) (p = 0.001). The corolla surface of the tetraploid flowers (0.47 ± 0.08 cm²) was significantly larger than the corolla surface of both the collection and diploid flowers (p = 0.001).

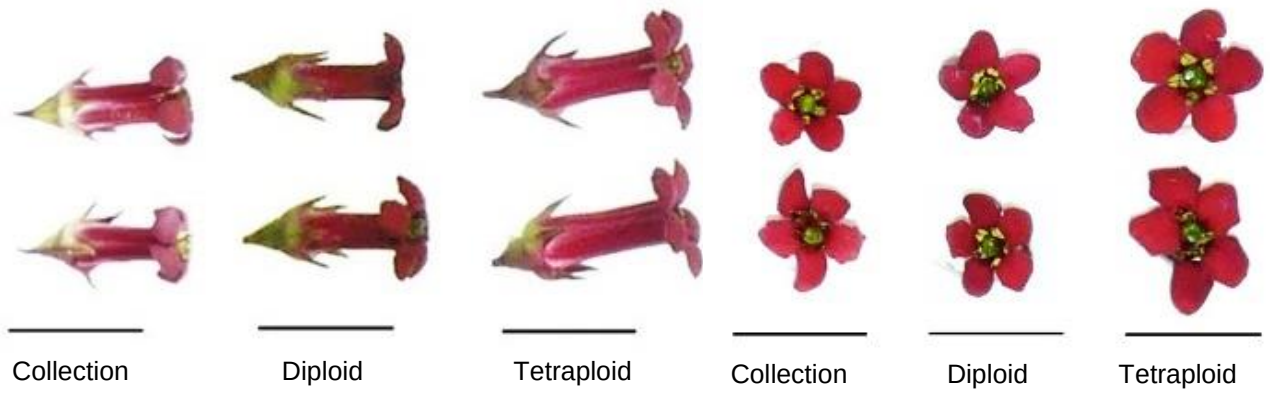

Figure 4.7: Side view and top view of flowers of *Escallonia rubra* from the plant in our collection, from the diploid controls, and the tetraploid numbers in the field. (bar = 1 cm)

4.3.2.3 Plant architecture

Results of the image analysis of *E. illinita, E. rosea* and *E. rubra* are shown in Table 4.5 for the top view images and in Table 4.6 for the side view images. For *E. illinita,* images of only five out of ten diploids and five out of 23 tetraploids were analyzed as a subsample. The plant area in both top and side view decreased significantly, on average with 32% and 52% respectively. This was due to a large decrease in plant width of 47% on average. The decrease of the plant area in side view, was also due to a decrease in plant height of 13% on average. In top view, the circularity and the % of the MBC filled by the plant area increased significantly in tetraploids, however this was not the case in side view. The variation within and between the diploids and tetraploids of *E. illinita* is shown in Figure 4.8. It is clear that diploids and tetraploids are not clearly separated for the analyzed traits. The range of variation is rather shifted, with many intermediate genotypes. Polyploidization of *E. illinita* resulted in slightly smaller, fuller and more circular plant types.

Table 4.5: Plant area, circularity of the convex hull and the % filled by the plant in the minimal bounding circle (MBC) from the images in top view, measured on at least 4 plantlets per number (mean ± standard deviation).

Escallonia species	Ploidy group	Analyzed plant numbers	Top view		
			Plant Area (cm²)	Circularity [z]	% of MBC filled
E. illinita	D	5	188.9 ± 96.7**	0.78 ± 0.08*	18.9 ± 7.1***
	T	5	128.4 ± 77.7	0.81 ± 0.08	29.5 ± 10.1
E. rosea	D	4	319.1 ± 141.4 ***	0.76 ± 0.08 **	12.6 ± 4.7 ***
	T	6	129.7 ± 102.1	0.81 ± 0.07	24.5 ± 8.7
E. rubra	D	16	394.1 ± 110.7^{NS}	0.75 ± 0.09***	12.1 ± 3.3***
	T	23	371.5 ± 86.8	0.46 ± 0.08	15.0 ± 4.8

Statistical analysis within the genotype between diploids and tetraploids with MWUT for pairwise comparison. NS: not significant; *: significant for p = 0.05; **: significant for p = 0.01; ***: significant for p = 0.001.

z) Circularity= 4 * π * area of convex hull / (perimeter of convex hull)²

Table 4.6: Plant area, width, height, and the % of the bounding rectangle (BR) filled by the plant from the side view images, measured on at least 4 plantlets per number (mean ± standard deviation).

Escallonia species	Ploidy group	Analyzed plant numbers	Side view			
			Plant Area (cm²)	Plant width (cm)	Plant height (cm)	% of BR filled
E. illinita	D	5	122.0 ± 51.9***	34.8 ± 12.5***	18.2 ± 5.2**	20.4 ± 6.1^{NS}
	T	5	58.4 ± 35.7	18.3 ± 7.9	15.3 ± 3.7	20.7 ± 5.3
E. rosea	D	4	136.5 ± 59.0 ***	47.7 ± 15.2 ***	16.6 ± 8.5 **	18.6 ± 4.4 ***
	T	6	91.7 ± 48.7	29.0 ± 12.1	12.5 ± 5.2	26.3 ± 6.2
E. rubra	D	16	191.0 ± 54.6*	58.2 ± 14.5***	21.0 ± 8.8^{NS}	17.9 ± 6.0*
	T	23	180.4 ± 49.2	50.1 ± 13.3	20.8 ± 7.8	19.5 ± 6.5

Statistical analysis within the genotype between diploids and tetraploids with MWUT for pairwise comparison. NS: not significant; *: significant for p = 0.05; **: significant for p = 0.01; ***: significant for p = 0.001.

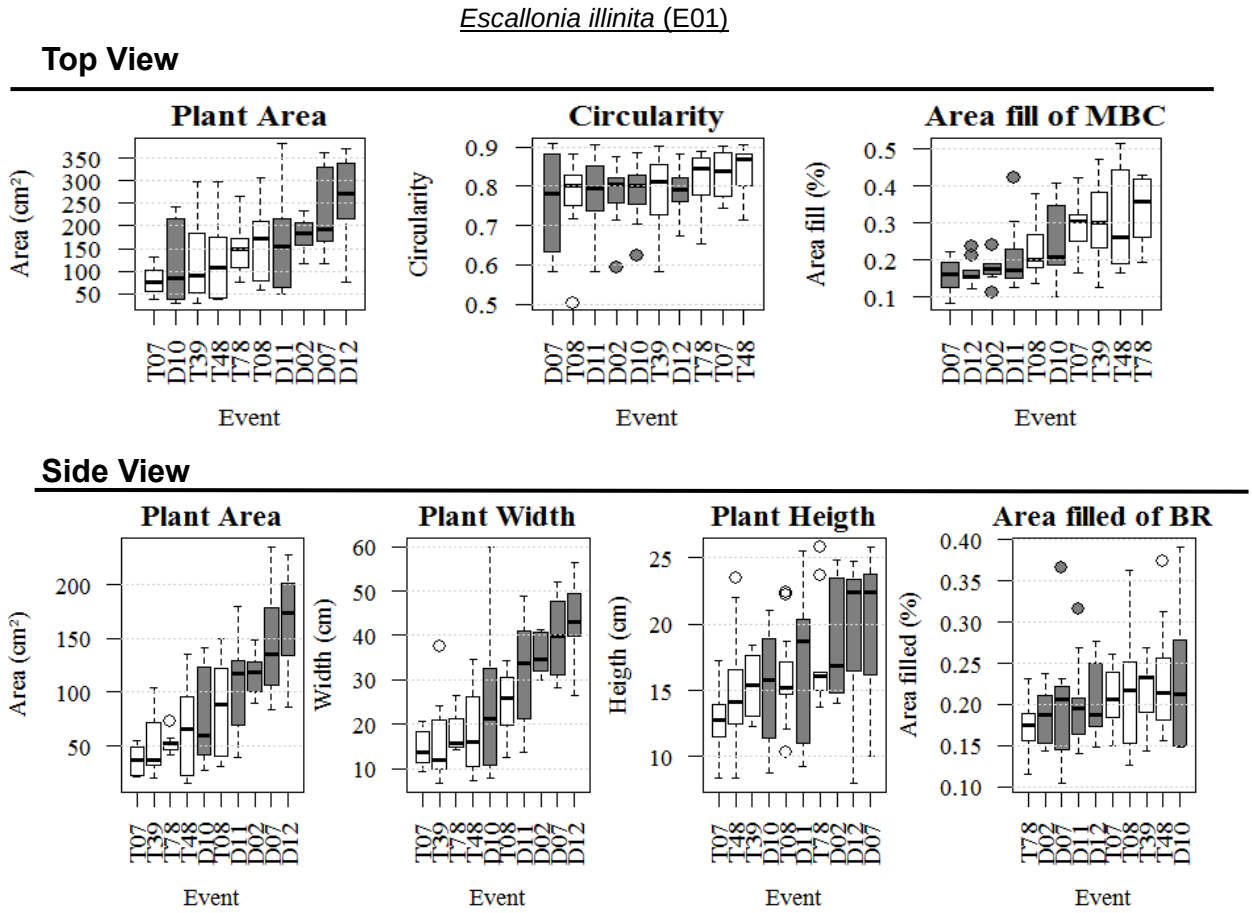

Figure 4.8: Analysis of a selected number of *Escallonia illinita* diploid (D) and tetraploid (T) numbers. Top view was analyzed for plant area (cm²), the circularity, and the % of the area of the minimal bounding circle (MBC) filled with the plant. Side view was analyzed for plant area (cm²), plant width (cm) and height (cm), and the % of the area of the bounding rectangle (BR) filled with the plant (Diploid = grey, Tetraploid = white).

The tetraploids of *E. rosea* were much smaller than the diploids. The average plant area decreased significantly by ± 60% in top view and with ± 33% in side view on average and the plant width and height of tetraploids decreased by ± 39% and ± 25% respectively. The percentage of the area the plant occupies in the bounding rectangle (BR) was significantly larger in tetraploids than in diploids. This was also significant for the percentage of the minimal bounding circle (MBC) filled by the plants. In addition, the circularity of the tetraploids was significantly larger than the diploids. The variation within and between individual diploids and tetraploids is shown in Figure 4.9. Diploids and tetraploids are not clearly separated for the analyzed traits. The range of variation is rather shifted, with intermediate genotypes. Tetraploid *E. rosea* were not only smaller in area, height and width, but they were much denser and less spindly than the diploids.

Escallonia rosea

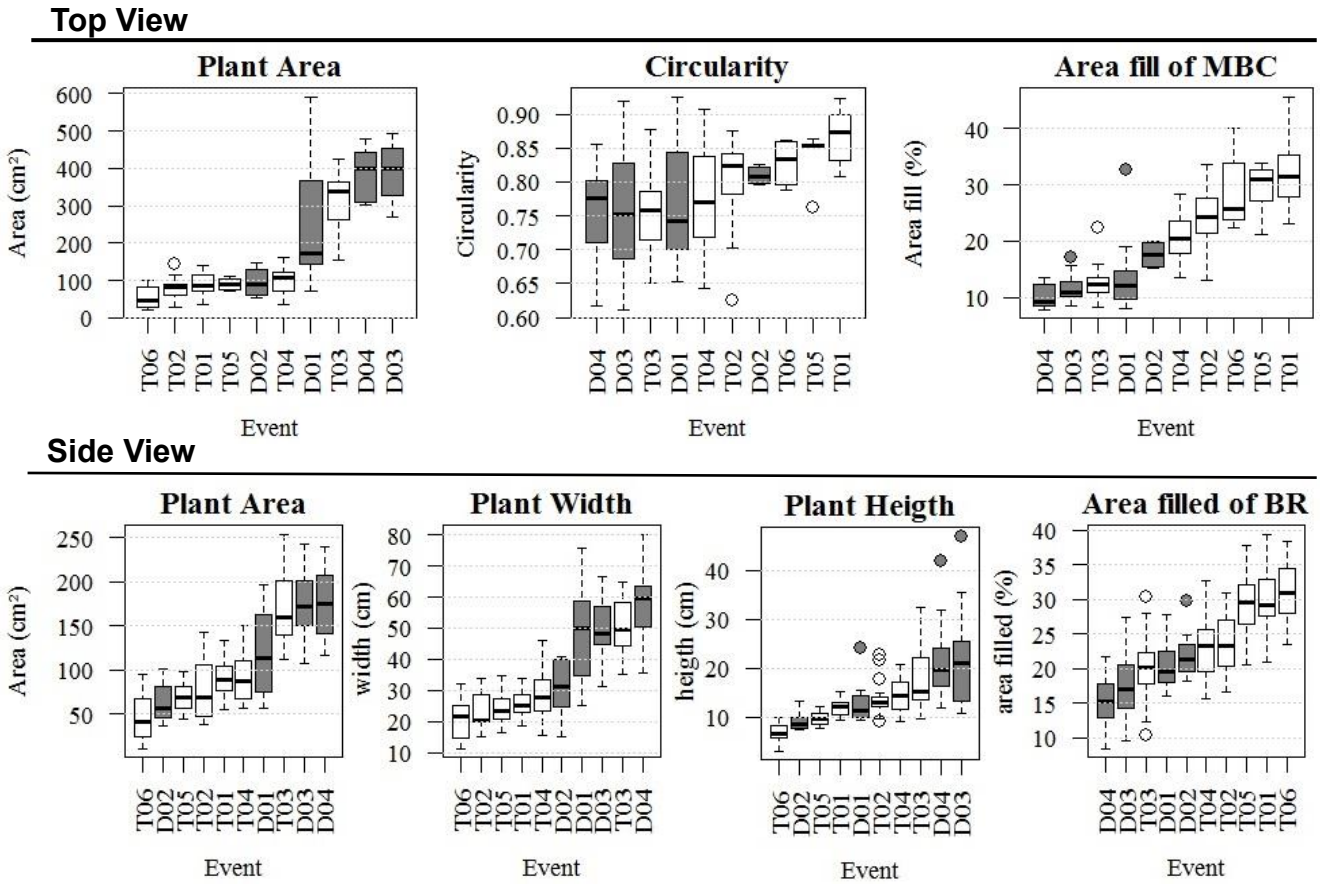

Figure 4.9: Analysis of *Escallonia rosea* diploid (D) and tetraploid (T) numbers. Top view was analyzed for plant area (cm²), the circularity, and the % of the area of the minimal bounding circle (MBC) filled with the plant. Side view was analyzed for plant area (cm²), plant width (cm) and height (cm), and the % of the area of the bounding rectangle (BR) filled with the plant (Diploid = grey, Tetraploid = white).

For *E. rubra,* the average plant area viewed from the side decreased significantly (± 6%) in tetraploids compared to diploids (Table 4.6), due to a reduction in plant width of ± 14% on average. The plant height and plant area in top view were not significantly changed. The circularity of tetraploids was significantly lower than in diploids, with a decrease of ± 39%. The percentage in which the area of the MBC and the BR are filled by the plants both increase significantly with ± 24% and ± 9% respectively. The variation within and between individual diploids and tetraploids of *E. rubra* is shown in Figure 4.10. Polyploidization causes a shift in the range of the traits analyzed. Tetraploid *E. rubra* are larger and less circular than their diploid counterparts.

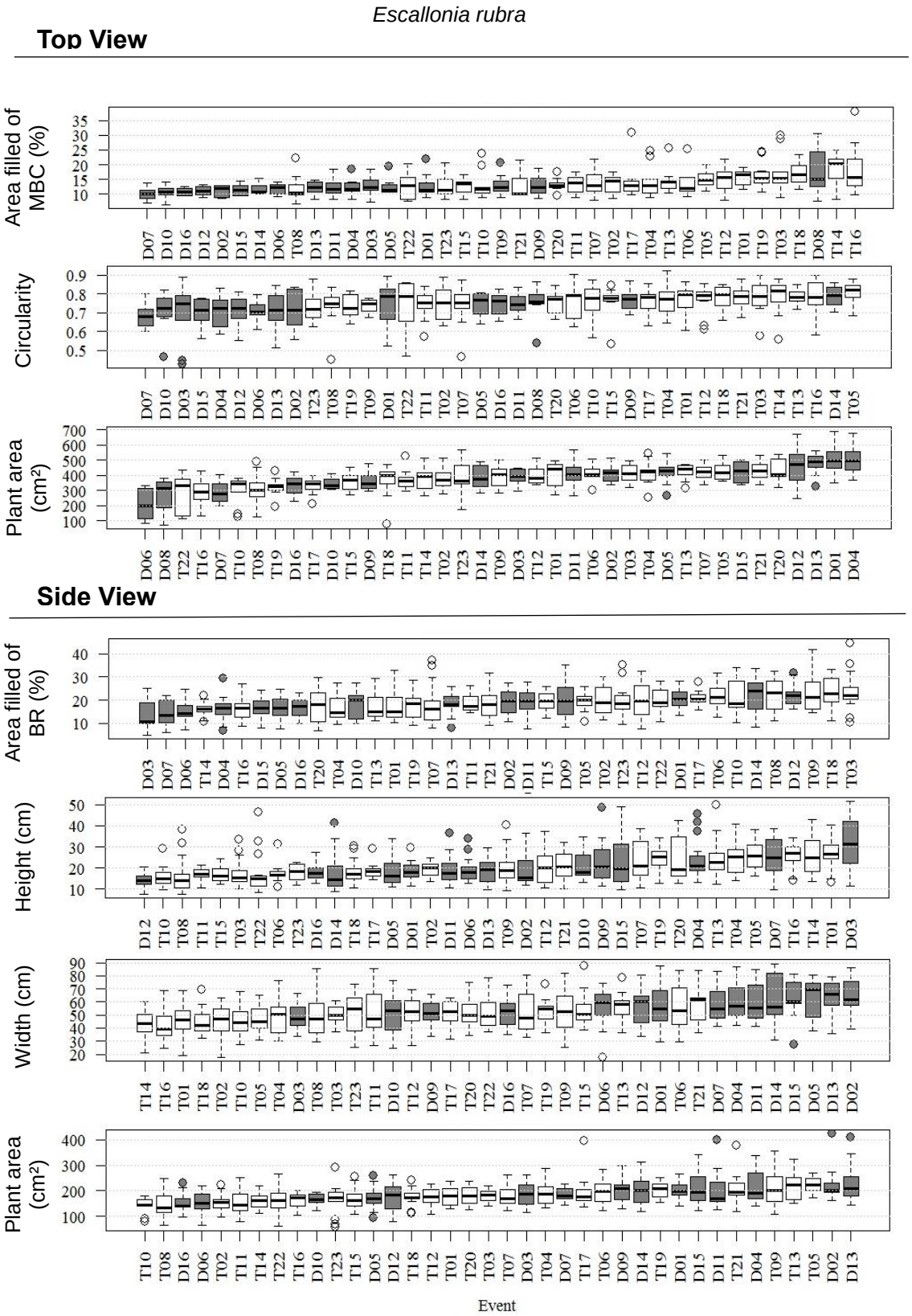

Figure 4.10: Analysis of *Escallonia rubra* diploid (D) and tetraploid (T) numbers. Top view was analyzed for plant area (cm²), the circularity, and the % of the area of the minimal bounding circle (MBC) filled with the plant. Side view was analyzed for plant area (cm²), plant width (cm) and height (cm), and the % of the area of the bounding rectangle (BR) filled with the plant (Diploid = grey, Tetraploid = white).

4.3.2.4 PCA of the phenotyping traits

The different morphological and architectural traits obtained by image analysis for selected diploids and tetraploids of *E. illinita* were analyzed by PCA (Figure 4.11). Three principal components (PCs) had an eigenvalue larger than 1, explaining 84.0% of the variance. PC1 was mainly determined negatively by NSL, NSIL, TV_circ and TV_fill (-0.853, -0.890, -0.748 and -0.925 respectively) and positively by TV_pl_ar, SV_pl_ar, SV_he en SV_wi (0.907, 0.955, 0.973 and 0.930 respectively). PC2 is mainly determined by SV_fill (-0.757), and PC3 by BL and BIL (0.656 and 0.676 respectively). While comparing PC1 to PC2, a clear group separation is present (Figure 4.11 A) by all the traits composing PC1, namely NSL, NSIL, TV_circ, TV_pl_ar, SV_pl_ar, SV_he, SV_wi and SV_fill. Group separation when comparing PC1 to PC3 (Figure 4.11 B) was also caused by the same traits.

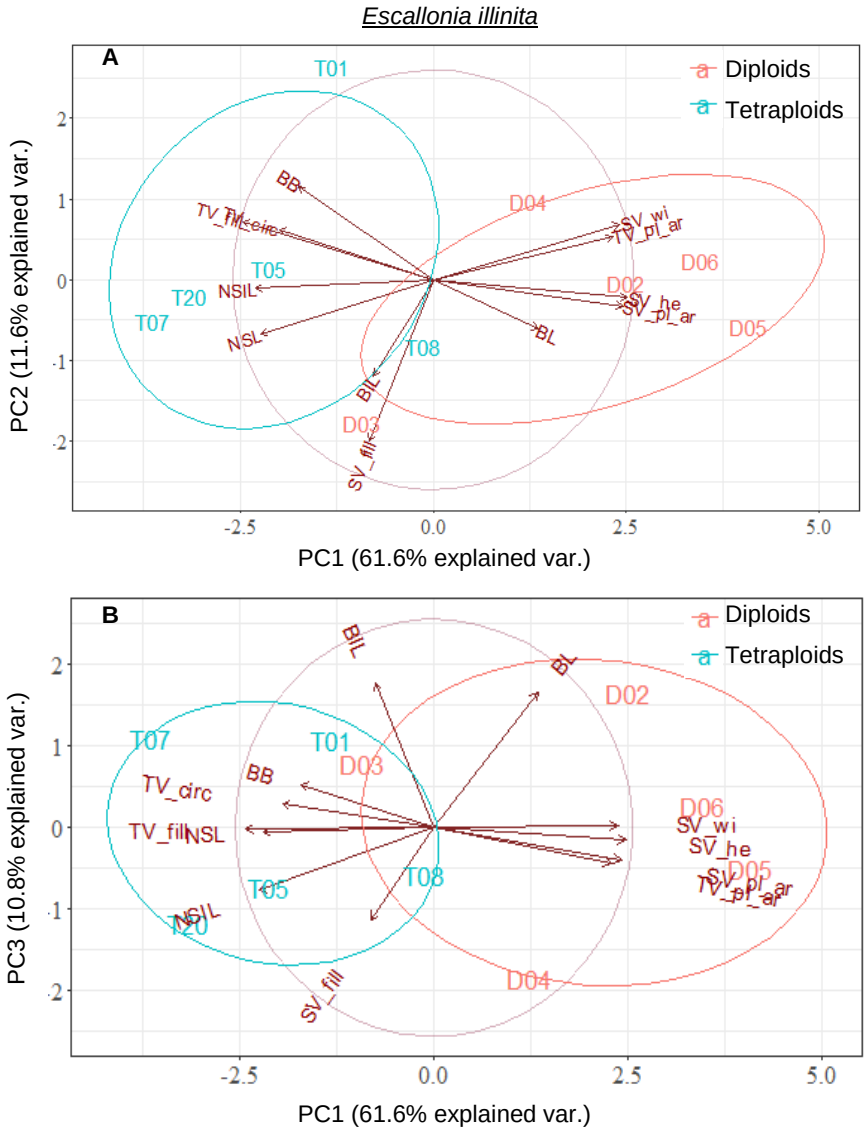

Figure 4.11: Principal Component Analysis (PCA) of the selected 5 diploids and 5 tetraploids of *Escallonia illinita.* 3 PCs with an eigenvalue larger than one, explained 84.0% of the variation. (A) PC1 vs. PC2, with 73.2% of the variance. (B) PC1 vs. PC3, with 72.4% of the variance. NSL: new shoot length; NSIL: new shoot internode length; BB: budburst; BL: branch length; BIL: branch internode length, TV_pl_ar: plant area in top view, TV_circ: circularity; TV_fill: % filled of the minimal bounding circle in top view; SV_pl_ar: plant area in side view; SV_he: plant height in side view, SV_wi: plant width in side view; SV_fill: % filled of the bounding rectangle in side view. Ellipses have a probability of 0.95.

PCA of *E. rosea* on 12 traits could be reduced to 2 PCs, explaining 85.0% of the variation (Figure 12). PC1 was mostly determined by the positively correlated SV_fill and TV_circ (0.897 and 0.856) and by the negatively correlated NSL, NSIL, BL, BIL, TV_pl_ar, SV_pl_ar, SV_he, and SV_wi (-0.822, -0.783, -0.979, -0.968, -0.980, -0.945, -0.885, and -0.962 respectively). The diploid and tetraploid group were mainly separated by BB and TV_fill. However, if we do not take the deviating phenotypes of T03 and D02 into account, any of the used traits could be used as group separators.

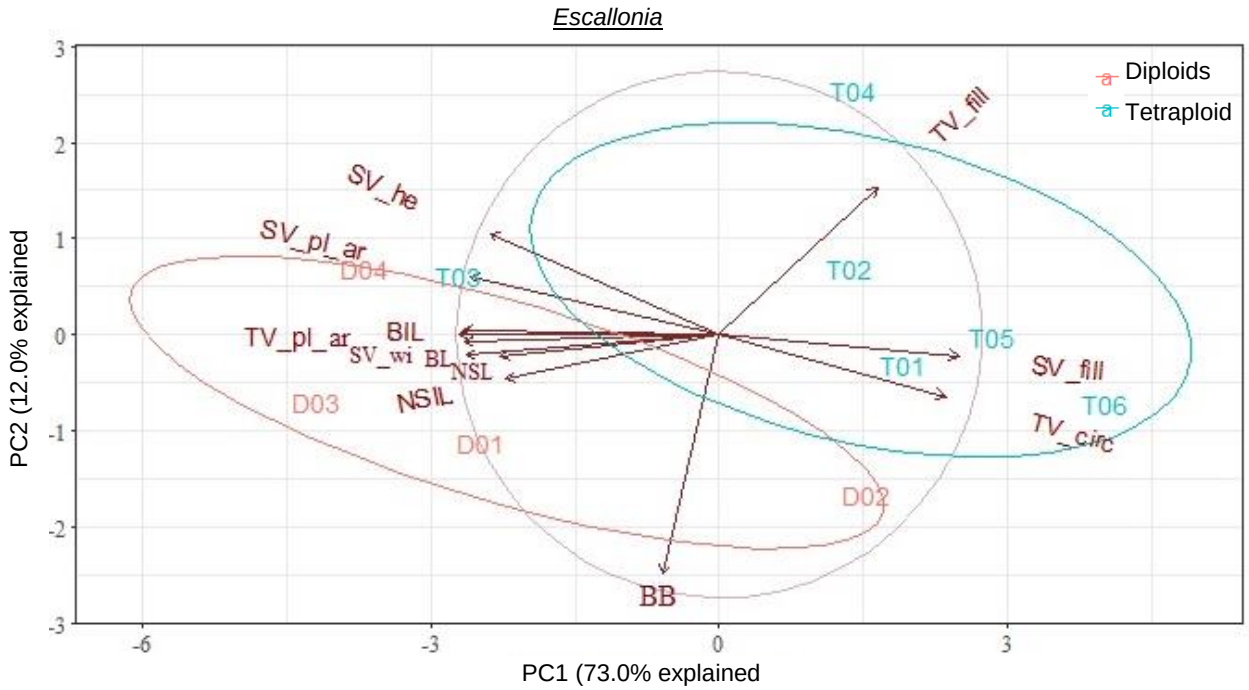

Figure 4.12: Principal Component Analysis (PCA) of the diploids and tetraploids of *Escallonia rosea*. 2 PCs with an eigenvalue higher than one, explained 85.0% of the variation. NSL: new shoot length; NSIL: new shoot internode length; BB: budburst; BL: branch length; BIL: branch internode length, TV_pl_ar: plant area in top view, TV_circ: circularity; TV_fill: % filled of the minimal bounding circle in top view; SV_pl_ar: plant area in side view; SV_he: plant height in side view, SV_wi: plant width in side view; SV_fill: % filled of the bounding rectangle in side view. Ellipses have a probability of 0.95.

For *E. rubra,* five PCs had an eigenvalue higher than 1, explaining 84.5% of the variance between all the homoploid groups. PC1 was positively correlated with SV_wi (0.730) and negatively correlated with NSL, NSIL, and TV_fill (-0.673, -0.678 and -0.617 respectively). PC2 was mostly determined by the positively correlated traits BL, BIL, TV_circ and TV_fill (0.636, 0.692, 0.667, and 0.628 respectively). In Figure 4.13 A, a clear separation between diploids and tetraploids was made by TV_fill, TV_circ and SV_wi. PC3 was determined positively by SV_he (0.686) and negatively by SV_fill (-0.653). Comparing PC1 to PC3 (Figure 4.13 B), group separation was mainly caused by SV_pl_ar and TV_pl_ar, and to a minor extent by SV_wi and BL. The most distinguishing trait to determine PC4 was the positively correlated SV_he (0.621) and for PC5 BIL (-0.525) and SV_pl_ar (0.563). Although PC4 and PC5 explained 11.9% and 10.5% of the variance, respectively, no separation was made between the diploid and the tetraploid group with these components.

Escallonia rubra

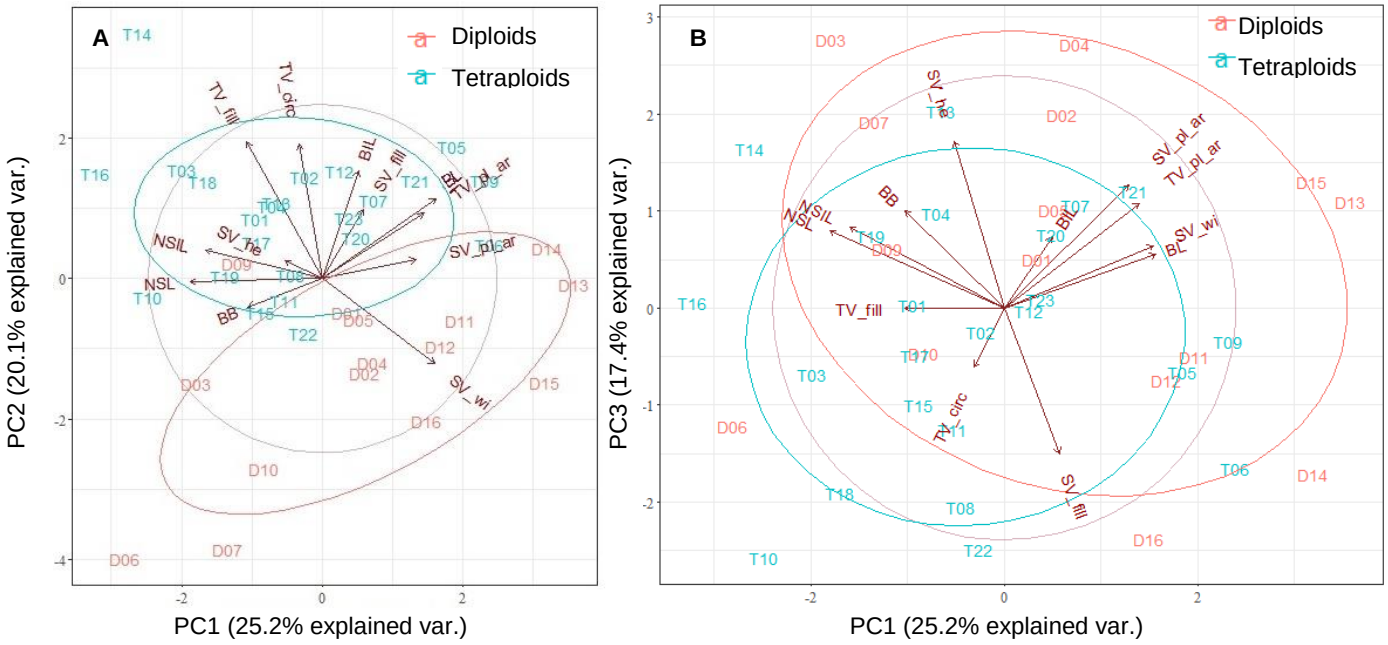

Figure 4.13: Principal Component Analysis (PCA) of the diploids and tetraploids of *Escallonia rubra.* 5 PCs with an eigenvalue larger than one, explained 84.5% of the variation. (A) PC1 vs. PC2, with 45.3% of the variance. (B) PC1 vs. PC3, with 42.6% of the variance. PC4 and 5 are not plotted, these components did not separate the diploid and tetraploid group. NSL: new shoot length; NSIL: new shoot internode length; BB: budburst; BL: branch length; BIL: branch internode length, TV_pl_ar: plant area in top view, TV_circ: circularity; TV_fill: % filled of the minimal bounding circle in top view; SV_pl_ar: plant area in side view; SV_he: plant height in side view, SV_wi: plant width in side view; SV_fill: % filled of the bounding rectangle in side view. Ellipses have a probability of 0.95.

4.3.2.5 Cold tolerance

The index of injury for *E. rosea* and *E. rubra* for the analyzed temperatures are shown in Figure 4.14. For *E. rosea*, 1 diploid (D02) and 2 tetraploids (T03 and T05) were analyzed in the controlled freezing test (Figure 4.14 A). Polyploidization had no effect on the calculated LT50: -15.1°C and -13.8°C for T03 and T05 respectively compared to -14.6°C for the diploid D02.

In contrast, a positive effect of polyploidization on cold tolerance was observed in *E. rubra*. The LT50 for T05 of *E. rubra* was significantly lower compared to D02, namely -11.8°C versus -7.7°C respectively (Figure 4.14 B).

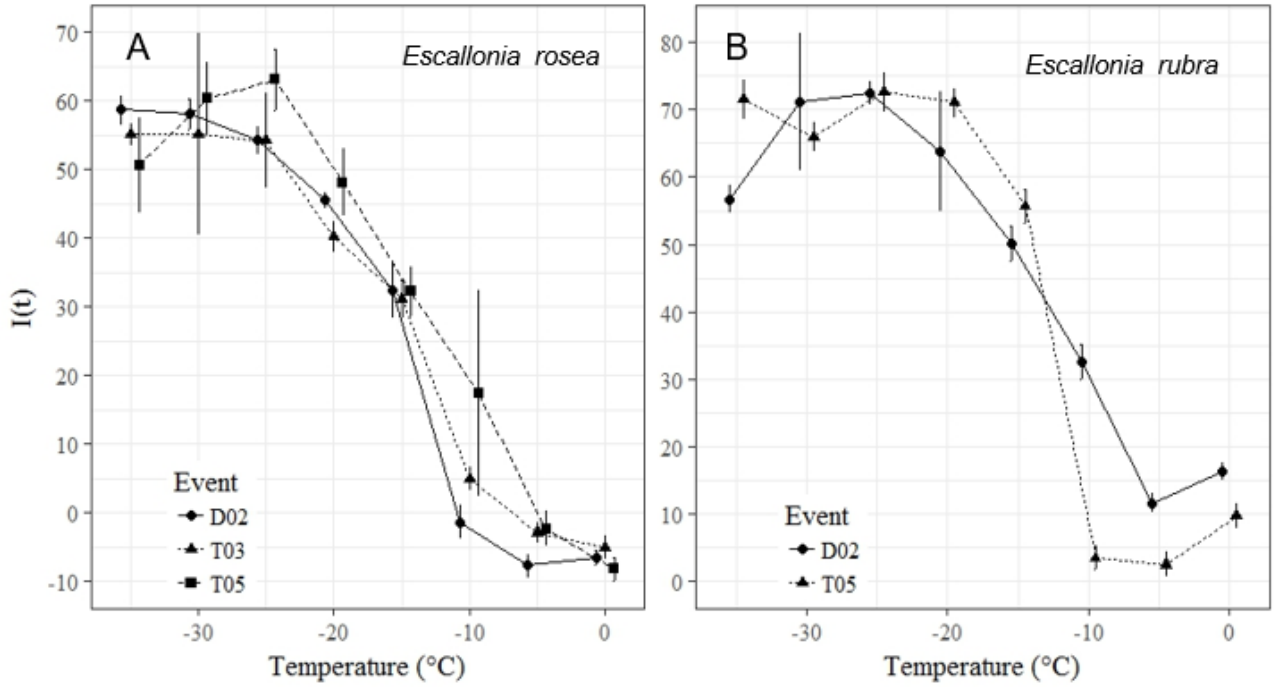

Figure 4.14: The Index of injury (I(t)) of the controlled freezing test for A) 1 diploid (D02) and 2 tetraploids (T03 and T05) of *E. rosea*. and B) 1 diploid (D02) and 1 tetraploid (T05) of *E. rubra*.

4.4 DISCUSSION

4.4.1 In vitro continuous treatment with trifluralin is most efficient to induce tetraploids in *Escallonia*

From the preliminary experiment could be concluded that colchicine (COL) was not efficient to induce tetraploids in *Escallonia*. The differences in T-yield and mortality that were observed between COL, oryzalin (ORY) and trifluralin (TRI) might be due to differences between the mitotic inhibitors in mode of action, solubility, penetration or transportation in the plant tissue, but also in sensitivity among the *Escallonia* genotypes. The mode of action differs between COL and ORY/TRI. COL inhibits the addition of tubulin-dimers to the microtubuli, while ORY and TRI disturb the formation of the tubulin-dimers themselves (Bartels and Hilton, 1973). Furthermore, COL is more carcinogenic for humans and has a lower affinity for plant tubulin dimers compared to ORY and TRI (Hansen and Andersen, 1996, Planchais et al., 2000). Ergo, COL was not further used in experiments. Both antimitotic agents ORY and TRI showed to be effective, and were maintained for further experiments. More concentrations and exposure times were applied to investigate the dose and exposure-effect on the different *Escallonia* genotypes. Since an fluctuating mortality was witnessed in the preliminary experiment, most likely due to the stress during the treatment, a less stressful treatment was set-up, namely a continuous treatment with long duration times and lower doses of mitotic inhibitors added to the growth medium.

Overall results of all experiments showed that the best treatments, yielding the most tetraploids, were 2 days of 250 µM ORY (T-yield of 39.2%) and 10 weeks of 5 µM TRI (T-yield of 53.7%). Moreover, these treatments also displayed low mortalities. Compared to similar studies, our polyploidization treatments were highly efficient with low mortalities. E.g., in *Rosa* 'Thérèse Bugnet', a high tetraploid yield of 67% was reached, but also 80-100% mortality (Kermani et al., 2003). Also for *Thymus persicus,* a high T-yield was reached, but with a mortality of 83% (Tavan et al., 2015). In *Hebe,* similar results as on our *Escallonia* were obtained, with T-yields of 46% and a mortality of only 10% (Gallone et al., 2014). In mandarins, only some mixoploids were recovered, no tetraploids, with mortalities up to 100% (Aleza et al., 2009). In *Buddleja davidii,* no tetraploids or mixoploids could be achieved with a similar treatment (Van Laere et al., 2011b). Therefore we can conclude that the developed protocol for *in vitro* polyploidization in *Escallonia* was very efficient.

As mentioned before, in all shock treatments the mortality was erratic and quite high, even in control treatments, indicating a high stress level caused by the shaking treatment and the addition

of EtOH or acetone as solvents for ORY and TRI. As a consequence of that stress, several tetraploids and mixoploids were recovered in the control treatment of *E. rosea*. High stress levels can cause endopolyploidy or endoreduplication (Barrow and Jovtchev, 2007). Spontaneous induction of polyploid plants during *in vitro* regeneration has also been reported in *Phalaenopsis* (Chen et al., 2009) and in *Gentiana* (Tomiczak et al., 2015). Given that the stress induced by the shock treatment caused great variation in mortality, we could conclude that the continuous treatment was most stable.

Octaploid *Escallonia* were recovered in all three species and for both mitotic agents in several treatments. Trifluralin induced more octaploids compared to oryzalin (1.7% versus 0.5%), for all three species across all experiments. Long exposure times can lead to a redoubling to produce octaploids (Dhooghe et al., 2011, Allum et al., 2007). However, no octaploids could be acclimatized due to poor viability and lack of growth vigor. This unfavorable effect of higher ploidy levels has also been observed in octaploid *Rosa rugosa* hybrids (Allum et al., 2007). As discussed in Chapter I (0) there is an optimal ploidy level for maximum growth, and ploidy levels above or below this optimum show a growth reduction (Hias et al., 2017). Furthermore, this effect is different for each plant species (Riddle et al., 2006). For the used *Escallonia* genotypes, octaploidy is not an optimal ploidy level, resulting in a loss of viability.

4.4.2 Changes in plant morphology and physiology after polyploidization

Three *Escallonia* species were polyploidized, and genotype dependent effects on the phenotype of the resulting tetraploids was observed (Table 4.7, Figure 4.15). Our results indicate that no phenotypic predictions on the outcome of a polyploidization experiment can be made, as every species has to be evaluated separately. This interaction between genetic background and ploidy level was also demonstrated by Riddle et al. (2006) who studied the effect of polyploidization on the phenotype of 1x, 2x, and 4x plants of four *Zea mays* cultivars.

Table 4.7: Average effect of polyploidization on the rooting capacity, morphological traits, leaves, the plant architecture and flowers of the *Escallonia* genotypes.

Genotype	Rooting capacity	Morphologic traits					Leaves			
		New Shoot Length	Branch Length	New Shoot Internode Length	Branch Internode Length	Axillary Budburst	Length	Width	L/W ratio	Surface
E. illinita	=	=	↘	↗	↗	↗	=	↗	↘	↗
E. rosea	=	↘	↘	↘	↘	↘	↘	↘	=	↘
E. rubra	=	↗	↗	↗	↗	↘	=	↗	↘	↗

Table 4.7: ctd.

Genotype	Top View			Side View				Flowers		
	Plant Area	Circularity	Area Filled	Plant Area	Width	Height	Area Filled	Length	Width	Corolla surface
E. illinita	↘	↗	↗	↘	↘	↘	=	-	-	-
E. rosea	↘	↗	↗	↘	↘	↘	↗	-	-	-
E. rubra	=	↘	↗	↘	↘	=	↗	↗	↗	↗

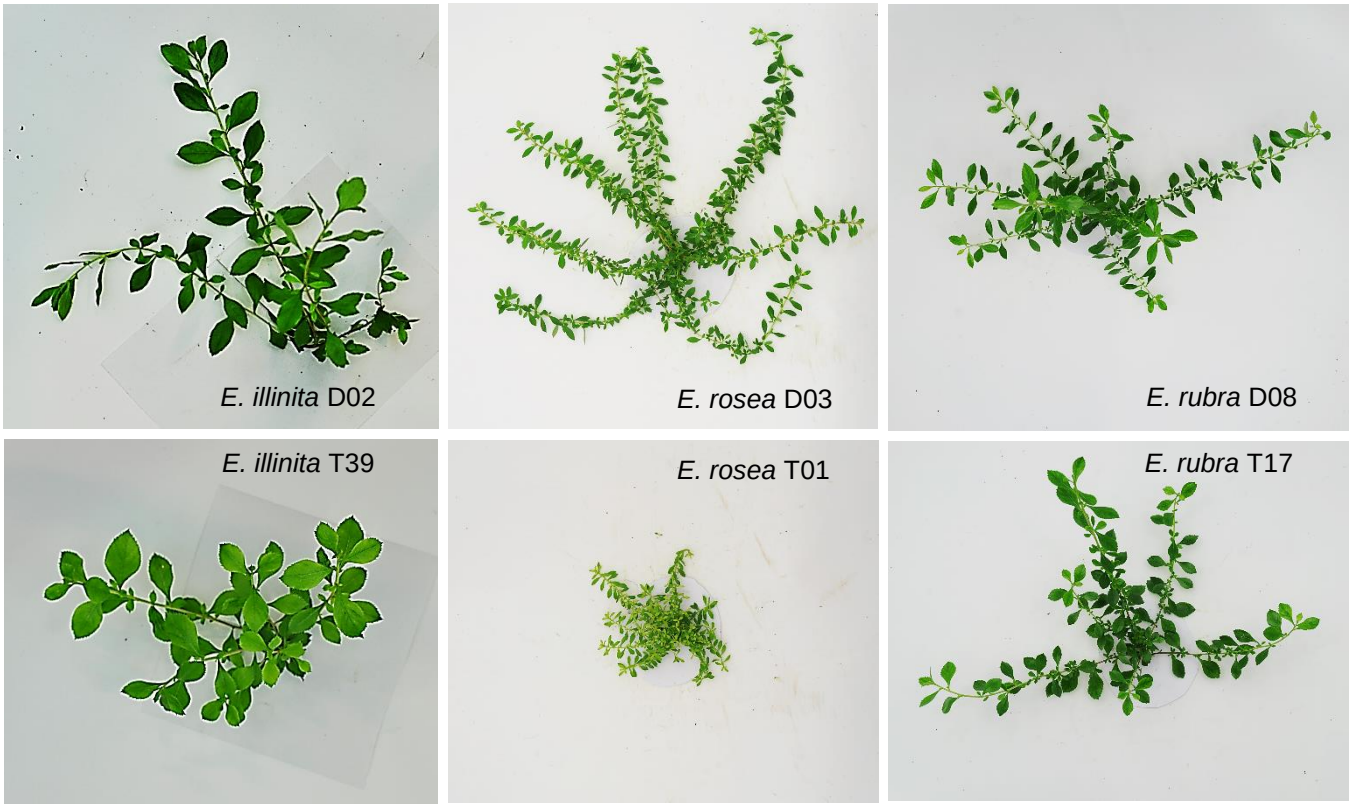

Figure 4.15: Top view from *Escallonia illinita, E. rosea* and *E. rubra*, from a diploid (D, top) and a tetraploid event (T, bottom).

Plant architecture determines the visual attractiveness, an important criterion for the commercial success of ornamental plants (Li-Marchetti et al., 2015). The architecture of a plant consists of the relative arrangement of each of its parts. Four important characteristics can be distinguished: (1) branching process, (2) growth process, (3) the morphological differentiation of axes, and (4) the position of reproductive structures (Barthelemy and Caraglio, 2007). In the present study the branching and growth process were analyzed for tetraploid and diploid *Escallonia* genotypes.

An increased budburst or axillary branching on the main stem was obtained in tetraploid *E. illinita,* while both *E. rubra* and *E. rosea* displayed a decrease in axillary budburst in tetraploids. Branching is controlled by apical dominance, the shoot apex controls the outgrowth of axillary buds (Cline, 1994). Several hypotheses attempt to explain branching control by apical dominance, and a common factor appears to be the levels of auxin and cytokinin, or the sensitivity of the plant tissues towards these hormones (reviewed by Dun et al. (2006)). If polyploidization influences the production, transport or sensitivity towards auxin or cytokinin, changes in apical dominance can occur. A lower apical dominance leads to a higher axillary budburst and more branches, i.e., a higher number of nutrient sinks. The same amount of nutrients that normally would go to the outgrowth of the apical shoot is divided among the branches, leading to a slower growth of each of the branches compared to the apical shoot (Dun et al., 2006). This process is applied artificially by growers of ornamentals by frequent mechanical or chemical pruning, or by applying plant hormones exogenously (Meijon et al., 2009, Mutlu and Kurtulan, 2015). To analyze the growth process, the primary growth of the rooted cutting and the outgrowth of axillary branches after pinching were measured and the internodal distance was determined. Clearly, both results of branching and internode length were necessary to interpret the effect on the overall plant size. The increase in axillary budburst for tetraploid *E. illinita* could potentially cause more, shorter branches to grow. However, an increased internode length reduced the effect of the increased axillary budburst on the compactness, resulting in only slightly shorter branches. The combination of a decrease in both axillary budburst and internode length led to a large decrease in size and plant area in tetraploid *E. rosea*. A decrease in plant height by reducing internode length after polyploidization was also observed in garden petunia (Regalado et al., 2017), in *Rosa multiflora* (Feng et al., 2017), in *Platanus* (Liu et al., 2007), in *Thymus* (Tavan et al., 2015), and in *Eriobotrya* (Blasco et al., 2015). In tetraploid *E. rubra,* chromosome doubling caused both a significant increase in internode length and a decrease in axillary budburst. The overall effect was an increased plant size and a looser plant habitus, as shown by the decrease in circularity. A similar

increase in plant size was found in *Vitex* (Ari et al., 2015) and in some *Rosa* genotypes (Kermani et al., 2003).

Image analysis of *E. rosea, E. rubra* and *E. illinita* added information of visual attractiveness to the information of growth and branching such as circularity and the bushiness. In *E. rosea,* where a significantly lower axillary budburst in tetraploids could indicate less dense plants, the circularity and the % filled by the plant of the MBC and the BR clearly showed more circular and dense plants. For *E. rubra,* the image analysis showed no increase in size, except for plant width. The increase in the % of area of the MBC or BR that was filled by the plant, was likely caused by an increase in leaf area. The large decrease in circularity was caused by a polar outgrowth of branches on the main axis in tetraploids. This is not a desired characteristic by growers or consumers. It is clear that the image analysis adds valuable information about the plant architecture that could not be derived from the measurements of plant height, internode length and axillary budburst. Traits such as plant area and circularity would have been very difficult to determine correctly by means of visual scoring. This type of 2D image analysis has several advantages. It is non-invasive and could be repeated to analyze the growth dynamic over time (Fiorani and Schurr, 2013). Free, open-source software, such as ImageJ, is available to create a customized program for analysis. The correct analysis of the plant area, however, can be biased by overlapping or curling leaves when the image is taken from only one view (e.g., top view) (Humplik et al., 2015b). This was a problem with tetraploid *E. rubra*, where a decrease in plant width and axillary budburst should have resulted in a decreased plant area in top view, but was counteracted by an increased leaf area. Therefore, side view images from different angles were included. The efficiency of this image analysis could be optimized by determining the number of pictures in side view necessary to obtain the required information. A high-throughput phenotyping study on cereals and pea only included two side view images, rotated 90° vertically, (Golzarian et al., 2011, Humplik et al., 2015a), which could reduce the time needed to take the images. For *E. illinita,* only two images in side view were made, by rotating the plant 90°, of a randomly selected number of diploids and tetraploids. The analysis of the morphological traits alone showed slightly shorter branches due to an interaction between a higher axillary budburst on the main stem and longer internodes. This information was confirmed by the image analysis, showing a significant decrease in plant dimensions, but also an increase in circularity. In top view images, an increased fill of the MBC by the plant was found, but not an increased fill of the BR in the side view images. This increase of the MBC fill could be due to an increase in leaf size, as was also found for *E. rubra.* The image analysis of a subset of tetraploids is sufficient to determine the general effect of

polyploidization on the phenotype, yet it does not identify the single genotype that has the most wanted characteristics, for example the most compact genotype. Yet, image analysis on a subset of genotypes can determine the general trend quickly and easily, after which one can decide if an in-depth analysis is useful.

A PCA was conducted on the traits resulting from the growth and branching analysis and the image analysis in order to find the traits that are most important in discerning diploids from tetraploids. For *E. rosea,* the homoploid groups could be separated by axillary budburst (BB) and the percentage the plant filled the minimal bounding circle in top view (TV_fill). However, two highly deviant phenotypes were present, namely D05 and T03. If these numbers were not present in the PCA, all traits analyzed could be used to discern the homoploid groups. This indicates that for *E. rosea,* polyploidization causes changes in all morphological and architectural traits analyzed. For *E. rubra,* the most discerning traits were the plant area in top and side view (TV_pl_ar and SV_pl_ar), the plant width (SV_wi), the circularity (TV_circ) and the fullness in top view (TV_fill). For *E. illinita,* the PCA of the randomly selected diploids and tetraploids showed the importance of the new shoot length and its internode length (NSL and NSIL), the circularity (TV_circ), the plant area in top and side view (TV_pl_ar and SV_pl_ar), the plant width and height (SV_wi and SV_he) and the fullness in side view (SV_fill). None of the traits measured were common group separators for all three genotypes. Again, this indicated the very variable effect of polyploidization on the phenotype of tetraploid *Escallonia* genotypes.

In all analyzed genotypes, the tetraploids broadened the phenotypic variation that was already present in the original diploid phenotype. A genome-dosage effect is considered as a major contributor to the added variation by polyploidization. However, this usually leads to more intermediate phenotypes, and only for some alleles to extreme phenotypes (Osborn et al., 2003). This effect has mostly been studied on an evolutionary scale for natural auto- or allo-polyploids, and only occasionally in the first generation of synthetic autopolyploids.

Beside plant architecture, foliage and flower morphology are important characteristics for visual attractiveness of an ornamental. An increase in organ size is quite common after polyploidization (Dhooghe et al., 2011). The observed leaf morphology changes after polyploidization differed for the three *Escallonia* genotypes. Tetraploid *E. illinita* and *E. rubra* showed wider leaves than diploids, thus a decrease in L/W ratio and increase in leaf surface. An increase in leaf size due to *in vitro* chromosome doubling has been observed in *Rosa* (Allum et al., 2007, Feng et al., 2017) and in *Vitex* (Ari et al., 2015), and after *in vivo* chromosome doubling in *Ziziphus* (Shi et al., 2015).

A decrease in L/W ratio was also observed in *Spathiphyllum* (Van Laere et al., 2010). A decrease in leaf area, as was the case for *E. rosea,* was similarly present in tetraploid apple (Hias et al., 2017) and in *Buddleja globosa* (Van Laere et al., 2011b). Flowers of *E. rubra* tetraploids were larger than flowers of the diploid counterparts. An increase in flower size was reported for *Rosa* (Allum et al., 2007), *Vitex* (Ari et al., 2015), and *Paulownia* (Tang et al., 2010).

Rooting capacity, which is important for commercial propagation, was not affected by polyploidization. A delay in rooting and a decreased root length has been reported previously in tetraploid *Thymus* (Tavan et al., 2015), but for *Escallonia,* this was not the case. Changes in stress resistance are often a consequence of chromosome doubling (Regalado et al., 2017, Van Laere et al., 2010, Levin, 2002). Since winter hardiness is an issue for *Escallonia* breeders, a cold tolerance test was conducted. Polyploidization did not have a negative effect on the cold tolerance of the tetraploids, and even an increase in cold tolerance was measured for tetraploid *E. rubra*. According to Hoffman and Ravesloot (1998) *E. rubra* can survive up to -12.2°C. However, this manual on nursery plants does not indicate how this value is achieved, and no further literature has been found. For *E. rosea,* no data on winter hardiness could be found, therefore our data could not be compared to literature.

4.5 GENERAL CONCLUSIONS

In this study, an efficient polyploidization protocol for the studied *Escallonia* species was set up, and tetraploids were characterized for their morphological traits and plant architecture. For both *E. illinita* and *E. rosea*, more compact phenotypes were obtained, but further field evaluations are needed to evaluate older plants. For *E. rubra,* tetraploid flowers were larger and the cold tolerance of tetraploids was increased. In addition, rooting of the tetraploids scored at least as good as the original diploid genotypes. Therefore, we conclude that polyploidization is an efficient breeding tool to induce useful variation in *Escallonia*. The results of the image analysis added valuable information on the compactness and visual attractiveness of the plants, which would be hard to quantify with the standard morphological measurements such as shoot length and number of branches.

CHAPTER V
INTRODUCTION OF *ROL*-GENES FROM RHIZOGENIC *AGROBACTERIUM* STRAINS

5 INTRODUCTION OF ROL-GENES FROM RHIZOGENIC *AGROBACTERIUM* STRAINS

5.1 INTRODUCTION

The introgression of *rol*-genes (root oncogenic loci) from a rhizogenic *Agrobacterium* strain may introduce new phenotypic and physiological characteristics in plants, accomplished by the integration of transfer-DNA (T-DNA), which contains four *rol*-genes (*rolA, rolB, rolC* and *rolD*), in the host plant DNA. Each *rol*-gene has its own effect on the plant. Due to differences in the copy number of the inserted *rol*-genes, combination of *rol*-genes inserted, or the location where the (fragments of) T-DNA is (are) inserted in the plant DNA, variable effects on the phenotype and physiology can be observed in different regenerated shoots (Chapter I). Typical changes in plants with *rol*-genes are compactness due to a loss of apical dominance and a decrease in internode length, wrinkled leaves, changes in flowering time, an increased rooting ability and changes in secondary metabolite concentrations (reviewed by Christensen and Müller (2009b)). Different rhizogenic *Agrobacterium* strains can contain different combinations of *rol*-genes or other genes, such as *aux*-genes (auxin synthesis), which has an impact on the resulting plant phenotype (Chapter I). In this study, the wild type rhizogenic *Agrobacterium* strains Arqua1 (Chandra, 2012, Jouanin et al., 1987, White et al., 1985), LMG 63 (Young et al., 2004, Trypsteen et al., 1991), NCPPB 2659 (synonym K599) (Mankin et al., 2007, Serino et al., 1995, Xiang et al., 2016) and MAFF02-10266 (synonym A13) (Tsuro and Ikedo, 2011, Tsuro et al., 2005) are used. The Arqua1 strain is an agropine type containing the pRiA4 plasmid with a split T-DNA (White et al., 1985, Jouanin et al., 1987). This split T-DNA contains two separate T-DNA strings, named right T-DNA (TR) and left T-DNA (TL), which can be transferred to the host cell and introduced in the host DNA separately (Jouanin et al., 1987). The TR-region carries the auxin synthesis genes *aux1* and *aux2* (Camilleri and Jouanin, 1991) and the opine synthesis genes, while the TL-region carries the four *rol*-genes (Chandra, 2012). LMG 63 is a mannopine type, the plasmid is not known (Trypsteen et al., 1991). NCPPB 2659 contains the pRi2659 plasmid of the cucumopine type (Mankin et al., 2007, Serino et al., 1995), with all four *rol*-genes (Xiang et al., 2016). For NCPPB 2659, also the gene at ORF3 has been shown to induce compactness in tobacco and *Arabidopsis* (Wang et al., 2016). The specific Ri-plasmid in the mikimopine MAFF02-10266 strain is unknown. Arqua1 and MAFF02-10266 are both biovar 1 strains, meaning they have an *Agrobacterium tumefaciens* genetic background, but with a Ri-plasmid and no Ti-plasmid (Christensen and Müller, 2009b). NCPPB 2659 is a biovar 2 strain, with a *Rhizobium rhizogenes* genetic background and a Ri-

plasmid (Christensen and Müller, 2009b). All these bacterial strains, except for LMG 63, are proven to be effective in woody species. Arqua1 has successfully transformed *Prunus* (Bosselut et al., 2011), apple rootstock (Pawlicki-Jullian et al., 2002), *Vitis* (Jittayasothorn et al., 2011), avocado (Prabhu et al., 2017), *Coffea arabica* (Alpizar et al., 2006), *Hibiscus* (Christensen et al., 2015), and *Semecarpus* (Panda et al., 2017). NCPPB 2659 has induced hairy roots in *Populus* (Neb et al., 2017) and in *Coffea arabica* (Alpizar et al., 2006), and MAFF02-10266 in *Lavandula* (Tsuro and Ikedo, 2011).

The aim is to induce hairy root production upon co-cultivation with the 4 rhizogenic *Agrobacterium* strains. For this, a protocol for *rol*-gene introduction was developed for several *Escallonia* and *Sarcococca* genotypes for which an *in vitro* stock was present. Furthermore, the efficiency of the rhizogenic *Agrobacterium* strains Arqua1, LMG 63, NCPPB 2659, and MAFF02-10266 was evaluated. Secondly, it was tried to regenerate shoots from these hairy roots, also containing rol-genes. Shoots can regenerate spontaneously (Kim et al., 2012, Subotic et al., 2003, Christensen and Müller, 2009b, Mehrotra et al., 2013), or after the addition of exogenous phytohormones (Gunjan et al., 2013, Hegelund et al., 2017, He-Ping et al., 2011, Swain et al., 2010, Tsuro and Ikedo, 2011). The need for exogenous hormones can vary between genotypes within a single species (Momcilovic et al., 1997). For some species, regeneration has not been achieved (Christensen and Müller, 2009b, Christensen et al., 2015). Finally, in this study, the presence of *rol*-genes in the hairy roots and regenerated shoots were evaluated, together with the absence of bacteria (absence of *virD2*-genes). For this, a robust PCR-based protocol was optimized.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

The collection of plants (2.2.1) and the *in vitro* initiation and propagation (2.2.3) are described in Chapter II. For the introduction of *rol*-genes, several *Escallonia* genotypes (*E.* 'Edinburgh' (E24), *E. illinita* (E01), *E. myrtoidea* (E23)*, E. pendula* (E10)*, E. rosea* (E14)*, E. rubra* (E16) and *E. rubra* 'C.F. Ball' (E20)) and *Sarcococca* genotypes (*S. coriacea* (S02)*, S. wallichii* (S05) and *S. hookeriana* var*. digyna* (S08)) were used. For these genotypes, a sufficient *in vitro* stock was built-up.

5.2.2 Storage and maintenance of rhizogenic *Agrobacterium* strains

The Arqua1 strain was donated by the Laboratory for Applied *In Vitro* Plant Biotechnology from the Department Plants and Crops of Ghent University. The LMG 63 strain was received from the Ghent University collection BCCM/LMG (Belgian Co-ordinated Collection of Micro-organisms). The NCPPB 2659 strain was obtained from VIB (Flemish Institute for Biotechnology), and the MAFF02-10266 strain was provided by the Genebank Project from NARO (National Agriculture and Food Research Organization). The bacteria were freeze-dried and stored at -196°C in liquid N_2. For use, the bacteria were grown in the dark at 28°C for 48h and then kept at 4°C in the dark. MAFF02-10266 was grown on a solid YEG medium (Yeast Extract Glucose broth: 10 g/L yeast, 10 g/L D-glucose, 1 g/L $(NH_4)_2SO_4$, 0.25 g/L KH_2PO_4, 15 g/L Bacto agar) while Arqua1, NCPPB 2659 and LMG63 were grown on a solid MYA medium (Malt Yeast Agar: 5 g/L yeast extract, 0.5 g/L casein hydrolysate, 8 g/L mannitol, 4.1 g/L $MgSO_4$ x $7H_2O$, 5 g/L NaCl, 15 g/L Bacto Agar, pH 6.6) (Wang, 2006). Every 4 weeks, a new culture of the four strains was started on YEG or MYA.
Protocol for *rol*-gene introduction

The *rol*-gene introduction protocol, and subsequent sub-culturing and data collection is schematically showed in Figure 5.1.

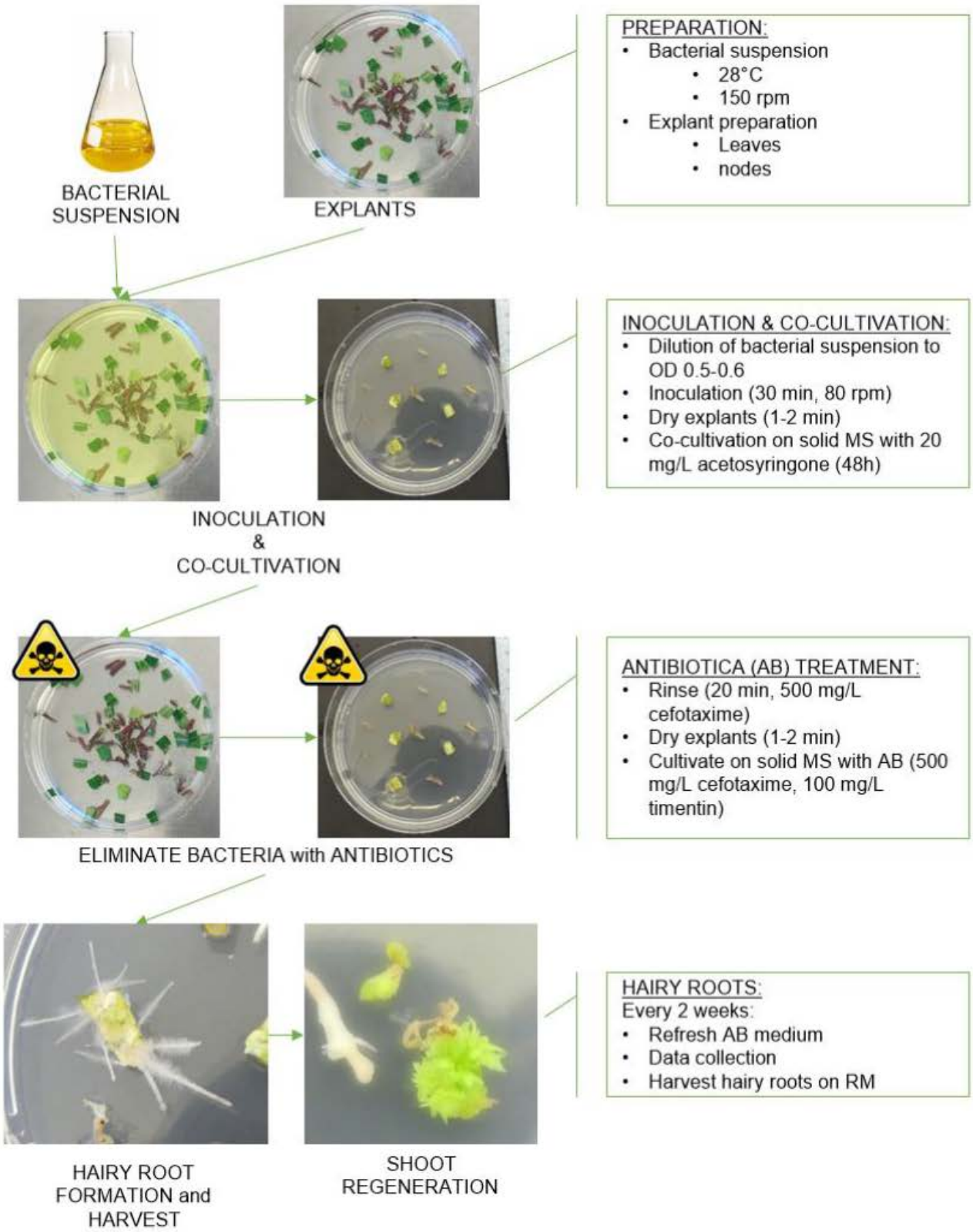

Figure 5.1: Schematic overview of the protocol for co-cultivation with rhizogenic *Agrobacterium* strains.

5.2.3 Protocol for co-cultivation with rhizogenic *Agrobacterium* strains

5.2.3.1 Bacteria and explant preparation

One bacterial colony was transferred to 50 mL liquid MYA (for LMG63, NCPPB 2659 and Arqua1) or 50 mL liquid YEG (for MAFF02-10266), and incubated in the dark on a rotary shaker (150 rpm) for 24h at 28°C.

For both *Escallonia* and *Sarcococca,* the explants (leaf or nodal sections) were prepared from *in vitro* stock material. Leaf explants were cut from a young leaf, always containing a midrib and a cut edge all around. Nodal explants were 0.3-0.6 cm long (Figure 5.2). Leaf and nodal explants were collected in a petri dish (Ø 9 cm) with ± 15 mL liquid MSM containing 30 g/L sucrose and a pH of 5.9 ± 0.1. Per experiment, 4 Petri dishes were filled with a total of 80 leaf and 80 nodal explants; 60 explants of each type were to be co-cultivated, and 20 explants of each type were to be used in a control treatment. The explants were placed in the dark in a growth chamber (ambient temperature: 23 ± 1°C).

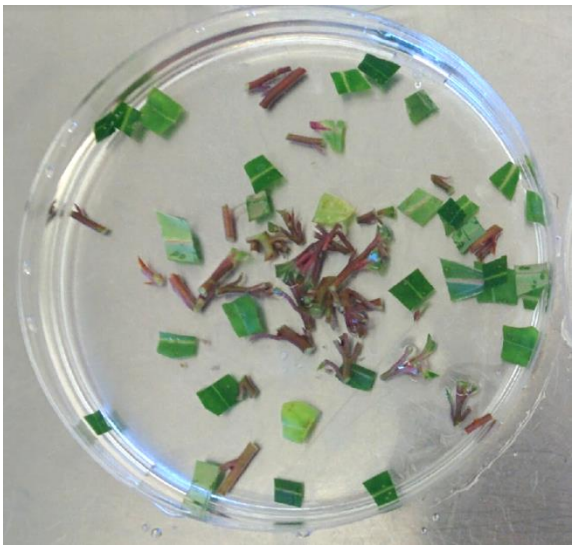

Figure 5.2: Leaf and nodal explant preparation of *Sarcococca hookeriana* var. *digyna* (S08) in liquid MSM. Leaf explants were cut on all four sides and contained a midrib. (Petri dish Ø 9 cm)

Table 5.1 lists the number of experiments that were conducted for each *Escallonia* or *Sarcococca* genotype – rhizogenic *Agrobacterium* strain combination.

Table 5.1: The number of experiments for each genotype - strain combination. Each treatment contained 60 explants.

Escallonia	Arqua1	LMG63	NCPPB 2659	MAFF02-10266
E. illinita	3	2	1	1
E. rosea	3	1	1	1
E. rubra	2	1	1	1
E. myrtoidea	1	1	1	-
E. 'Edinburgh'	-	1	-	1
E. rubra 'C.F. Ball'	1	-	-	-
Sarcococca				
S. coriacea	1	-	-	-
S. wallichii (S05)	1	-	-	-
S. hookeriana var. *digyna* (S08)	1	-	-	-

5.2.3.2 Inoculation and co-cultivation

The density of the liquid bacterial culture was analyzed with a spectrophotometer (iMark Microplate Reader, Bio-Rad Laboratories, Hercules, California, USA). Therefore, the bacterial culture was diluted with liquid MYA or YEG, depending on the strain (see 5.2.2), to obtain 1/3, 1/2 and 2/3 dilutions. The dilution with an optical density between 0.5 and 0.6 OD (measured at 600 nm) was applied on the prepared explants, by replacing the liquid MSM in the petri dishes or tubes with 15 mL of the liquid bacterial cultures. In the control treatments, the same amount of MYA or YEG was added but without bacteria. Cultures were incubated in the dark on a rotary shaker (80 rpm) for 30 min. Subsequently, the explants were air dried on sterile paper for 1-2 min to remove an excess of bacteria, placed on fresh solid MSM (30 g/L sucrose, MS including vitamins (Duchefa), 6 g/L agar (LabM), pH 5.9 ± 0.1) with 20 mg/L acetosyringone, and incubated in the dark in the growth chamber (23 ± 1°C) for 48h.

5.2.3.3 Antibiotics treatment

After the co-cultivation, all explants were transferred to a Petri dish with ± 20 mL liquid MSM with 500 mg/L cefotaxime for 20 min and stirred regularly. Subsequently, the explants were air dried for 1-2 min on sterile filter paper, placed on solid MSM (30 g/L sucrose, MS including vitamins (Duchefa), 6 g/L agar, pH 5.9 ± 0.1) with 500 mg/L cefotaxime and 100 mg/L timentin, and incubated in the dark at 23 ± 1°C.

Every two weeks, the MSM medium containing antibiotics (500 mg/L cefotaxime and 100 mg/L ticarcillin) was refreshed, since these antibiotics decrease in activity over time. When hairy roots appeared, they were photographed, the hairy roots (>1 cm) excised and firmly pressed into the regeneration medium (see 5.2.4). Based on the photographs, information on the explant type which rooted, the number of hairy roots per explant and the time of rooting (weeks after infection) was collected. All experiments were kept at least 12 weeks, even if no roots were produced in that period.

5.2.4 Regeneration protocols

Several media for shoot regeneration were applied. The hormonal composition of the regeneration media (RM) are shown in Table 5.2. All regeneration media were based on MS salts and vitamins (Duchefa), 30 g/L sucrose, 6 g/L agar (LabM) and pH 5.9 ± 0.1. The first four weeks after harvesting the hairy roots, they were placed on regeneration medium containing 250 mg/L cefotaxime and 50 mg/L timentin. After four weeks, no antibiotics were added anymore. Three different approaches for shoot regeneration were applied: (1) continuous exposure to a medium without exogenous plant hormones (RM00); (2) continuous exposure to media supplemented with 0.1 mg/L to 0.5 mg/L of either thidiazuron (TDZ), kinetin (KIN), or 6-benzylaminopurine (BAP), the latter cytokinin was complemented with the auxin 1-naphthaleneacetic acid (NAA) (RM01-RM06), and (3) a shock treatment during 2-4 weeks on a medium with a relative high concentration (1 mg/L to 7 mg/L) of cytokinins (RM07, RM08, RM10 and RM11), followed by a medium containing a 10-fold lower cytokinin concentration (RM01, RM09, RM12 and RM13 respectively). The hairy roots were placed in the growth chamber (ambient temperature: 23 ± 1°C, photoperiod: 16 h, light intensity: 35 µmol/m²s, bottom cooling: 18 ± 1°C), and the regeneration medium was refreshed every four weeks.

Table 5.2: Hormonal composition of the regeneration media (RM) for shoot formation on the hairy roots.

Medium name	BAP (mg/L)	TDZ (mg/L)	KIN (mg/L)	NAA (mg/L)
RM00	-	-	-	-
RM01	-	0.1	-	-
RM02	-	0.5	-	-
RM03	-	-	0.1	-
RM04	-	-	0.5	-
RM05	0.1	-	-	0.1
RM06	0.5	-	-	0.1
RM07	-	1.0	-	-
RM08	3.0	-	-	-
RM09	0.3	-	-	-
RM10	7.0	-	-	-
RM11	-	3.0	-	-
RM12	1.0	-	-	-
RM13	-	0.3	-	-

All media contained 30 g/L sucrose, 4.4 g/L MS including vitamins (Duchefa), 6 g/L agar (LabM) and pH 5.9 ± 0.1.

5.2.5 *rol*-gene and bacteria detection in shoots and hairy roots

The DNA extraction was performed according to the modified CTAB (cetyltrimethylammonium bromide) DNA isolation protocol (Doyle and Doyle, 1987). For the hairy roots, 100 mg of young root tips from hairy roots of maximum 4 weeks old and with vigorous growth was used (Figure 5.3), and for the regenerated shoots, 100 mg of young leaf material was used. A sample of the rhizogenic *Agrobacterium* strains was added as a positive control, and leaf tissue of the *in vitro* stock plants as a negative control. All samples were tested for the presence of ITS (Internal Transcribed Spacer) markers. PCR for ITS included the following steps: 95°C for 10 min, 35 cycles at 95°C for 30 sec for denaturation, annealing at 58°C for 40 sec and 72°C for 1 min for elongation. The cycles were followed by 10 min at 72°C for final elongation. The PCR mix contained 10 µM of the primers and 5 U/µL of AmpliTag Gold (Applied Biosystems, Foster City, CA, USA). The ITS region is evolutionarily conservative and useful for phylogenetic reconstruction in plants (Baldwin, 1992). By confirming the presence of the ITS genes in the DNA samples, it is proven that the DNA of the hairy root and shoot samples and the plant controls is of sufficient quality.

Regenerated shoots must be free of rhizogenic *Agrobacterium* strains before transfer to the greenhouse for acclimation, since these bacteria cause problematic hairy root diseases in greenhouses (Bosmans et al., 2017) and according to the biosafety protocols. Furthermore, if rhizogenic *Agrobacterium* strains are still present on the root or plant samples, the *rol*-genes in the bacteria are detected and no exclusive proof on the presence of the *rol*-genes in the samples can be given. Therefore, qPCR detection of the *virD2*-gene, located on the Ri-plasmid was

performed with a LightCycler 480 (Roche, Bazel, Switserland) (primers are confidential). The same DNA samples were used in a qPCR analysis to detect the individual *rol*-genes (primers are confidential). The lengths of *rolA, rolB, rolC* and *rolD* are respectively 282 bp, 233 bp, 310 bp and 320 bp. The qPCR included following steps: pre-incubation at 95°C for 2 min, 40 amplification cycles of 95°C for 5 sec, 55°C for 10 sec and 72°C for 20 sec. Melting occurred at 95°C for 5 sec, 60°C for 1 min and back to 97°C. The qPCR mix contained 10 µM of the primers and 5 u/µL SensiFAST mix (Bioline Reagents Ltd, London, UK).

Figure 5.3: Hairy roots from *Escallonia illinita* (E01) after co-cultivation with LMG 63. The root tips harvested for qPCR analysis are encircled in red. Red bar: 1 cm.

5.2.6 Statistical analysis of the results

Statistical analysis was done in R, version 3.2.0 (R Core Team, 2015). Pairwise comparison was done using the t-test on a significance level of $p = 0.05$.

5.3 RESULTS

5.3.1 Hairy root formation on *Escallonia* and *Sarcococca*

Arqua1 infects all tested *Escallonia* genotypes, and NCPPB 2659 none. None of the *Sarcococca* genotypes could be infected by Arqua1, and no further experiments could be initiated, due to the limited explant availability caused by the very slow *in vitro* multiplication of *Sarcococca*.

The cumulative percentage of rooted explants in the different *Escallonia* experiments is presented in Figure 5.4. The phenotypic difference between hairy roots produced on explants inoculated with a rhizogenic *Agrobacterium* strain and on control explants was clearly visible for all genotypes used (Figure 5.5). The hairy roots were thicker, more branched and hairier than the roots on control explants. Furthermore, the abundance of hairy roots per explant (Table 5.3) was higher for successfully inoculated explants with hairy roots, than for explants in the control treatments with regular roots. In the control treatments, regular roots were found on leaves and nodes of *E. illinita,* respectively 1.6 ± 0.7 roots/leaf explant in week 8 and 1.4 ± 0.7 roots/nodal explant in week 6. Two nodes produced one regular root for *E. rosea,* and one node produced two regular roots for *E. rubra.* Due to this easy visual distinction, only roots with the hairy root phenotype were harvested on inoculated explants.

Table 5.3: The highest abundance of hairy roots per explant type and the week after infection in which this occurred.

Genotype	Bacterial strain	Explant type and week number			
		Leaves	Week	Nodes	Week
E. illinita (E01)	Arqua1	5.0 ± 3.3	6	3.4 ± 1.6	6
	LMG 63	3.7 ± 3.0	4	3.2 ± 3.6	6
	MAFF 02-10266	3.6 ± 3.0	6	2.0 ± 1.9	6
	NCPPB 2659	0	-	0	-
E. rosea	Arqua1	3.3 ± 0.9	8	2.4 ± 0.9	6
	LMG 63	0	-	3.3 ± 3.5	4
	MAFF 02-10266	0	-	0	-
	NCPPB 2659	0	-	0	-
E. rubra	Arqua1	3.3 ± 1.5	6	3.0 ± 1.7	8
	LMG 63	4.2 ± 1.6	6	2.9 ± 1.0	6
	MAFF 02-10266	0	-	0	-
	NCPPB 2659	0	-	0	-
E. myrtoidea	Arqua1	4.4 ± 2.5	6	4.6 ± 2.5	6
	LMG 63	0	-	0	-
	NCPPB 2659	0	-	0	-
E. 'Edinburgh'	LMG 63	1.5 ± 0.5	6	0	-
	MAFF 02-10266	0	-	0	-

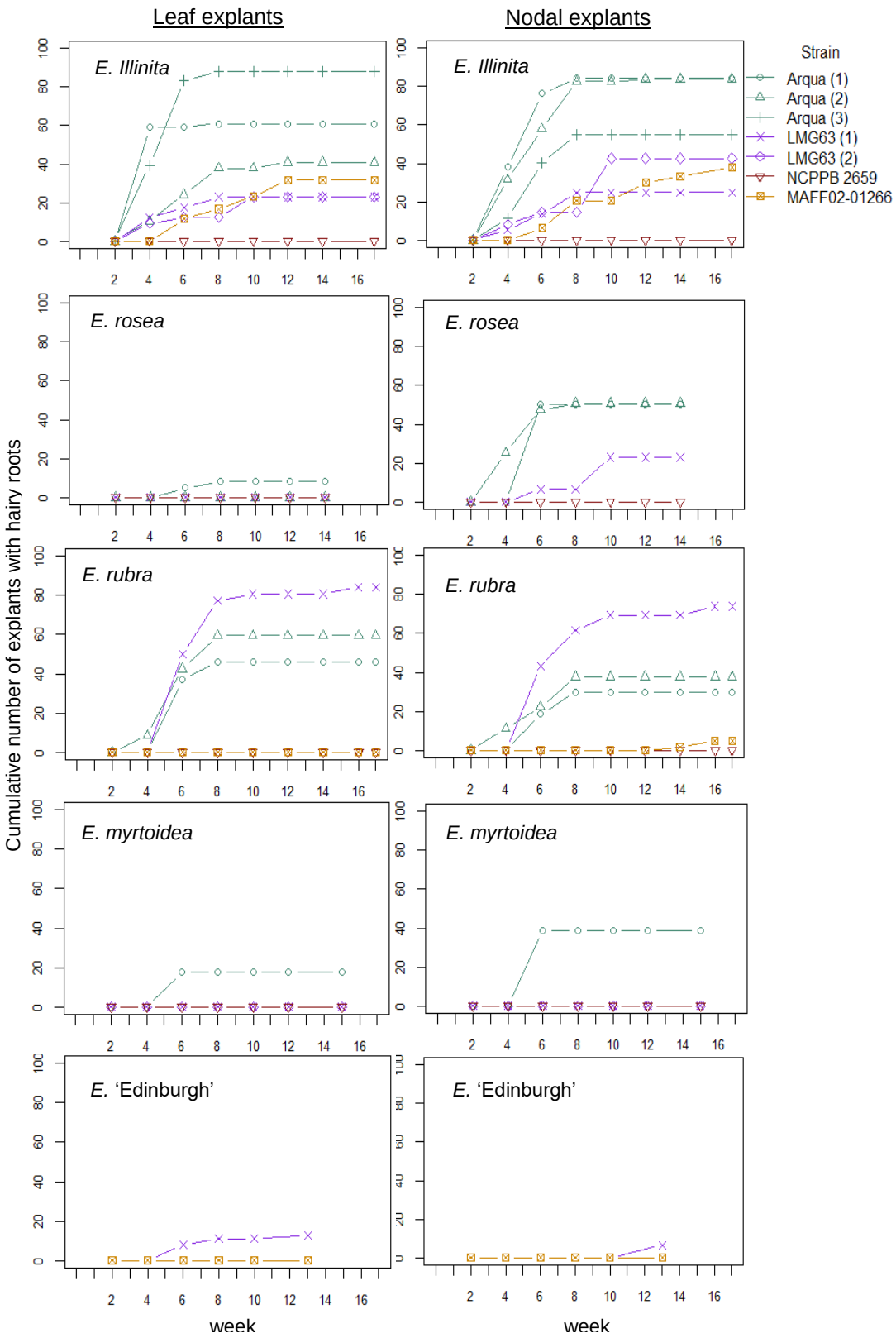

Figure 5.4: The cumulative percentage of rooted leaf and nodal explants for the different combinations of rhizogenic Agrobacterium strains (Arqua1, LMG63, NCPPB 2659, and MAFF02-10266) and the *Escallonia* genotypes (*E. illinita*, *E. rosea*, *E. rubra*, *E. myrtoidea* and *E.* 'Edinburgh'). Repeated experiments are indicated between brackets in the figure legend.

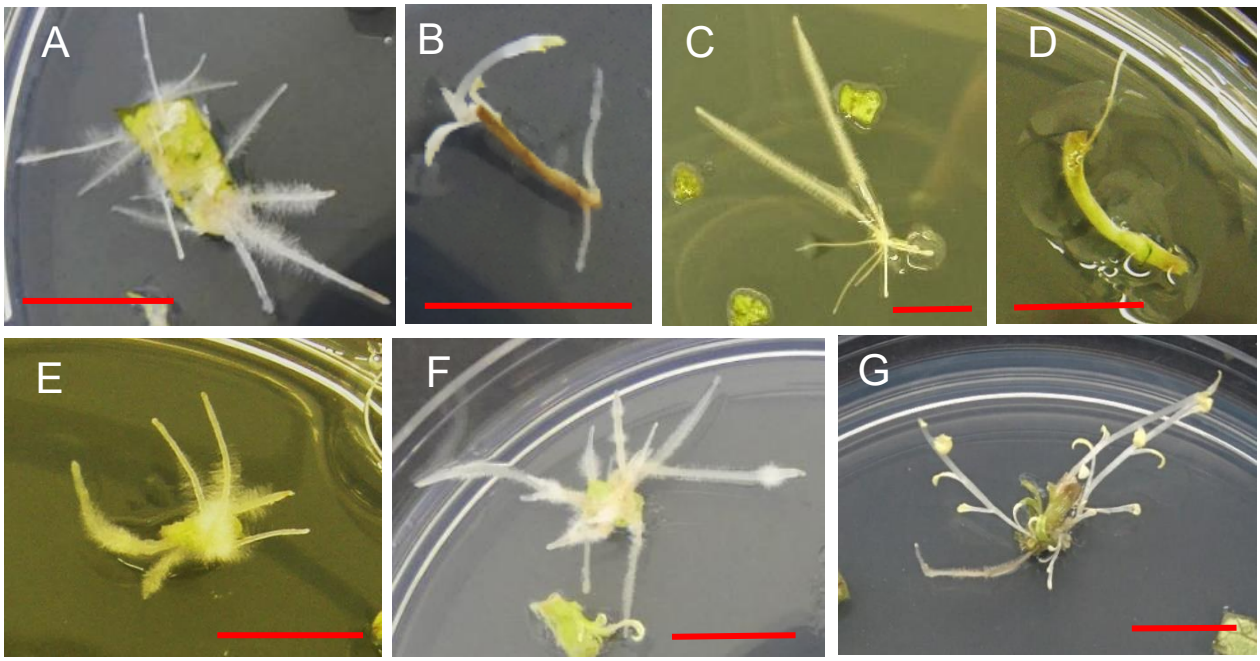

Figure 5.5: Differences in root phenotype produced on different *Escallonia* genotypes after 6 weeks. (A) an *E. illinita* leaf explant exposed to Arqua1 and (B) an *E. illinita* nodal explant in a control treatment. (C) An *E. rosea* nodal explant after inoculation with Arqua1 and (D) an *E. rosea* nodal explant in a control treatment after 8 weeks. (E) A *E. rubra* leaf explant after inoculation with Arqua1, (F) a *E. rubra* leaf explant after inoculation with LMG 63 and (G) a *E. rubra* nodal explant in a control treatment. Red bar = 1cm.

With *E. illinita*, three experiments with Arqua1 were conducted, yielding hairy roots on average on 63.2 ± 19.3% of the leaves and 74.3 ± 13.8% of the nodes. Two experiments with LMG 63 yielded on average 23.0 ± 0.1% and 33.8 ± 8.8% hairy roots on leaves and nodes respectively, and the one experiment with MAFF02-10266 yielded 31.7% and 38.1% hairy roots on leaves and nodes respectively. On average over all experiments with *E. illinita*, the percentage of explants yielding hairy roots was not significant between leaves and nodes. A difference in virulence between the strains was present. The Arqua1 strain yielded significantly more hairy roots (average for both nodes and leaves is 71.5 ± 18.1%) than the LMG 63 strain (average for both nodes and leaves is 28.4 ± 8.2%). Explants co-cultivated with Arqua1 showed a steeper increase in the number of explants with hairy roots in the first 6 weeks after the co-cultivation, compared to both LMG 63 and MAFF02-10266 (Table 5.3, Figure 5.4). A total number of 549 hairy roots (Arqua1), 123 hairy roots (LMG63) and 74 hairy roots (MAFF02-10266) were harvested on *E. illinita* explants.

With *E. rosea,* two experiments with Arqua1 resulted in on average 4.1 ± 4.1% and 50.4 ± 0.4% of leaf and nodal explants respectively with hairy roots, and inoculation with LMG 63 in 0% and 23.0% on leaves and nodes respectively. Overall, *E. rosea* yielded significantly ($p = 0.04$) more hairy roots on nodal explants (41.3 ± 13.0% on average for all co-cultivations), than on leaf

explants (2.7 ± 3.9% on average for all co-cultivations). Arqua1 resulted in twice as much hairy roots on nodal explants than LMG 63, but its statistical significance could not be proven due to a lack of repeats of LMG 63. Explants inoculated with Arqua1 showed a steeper increase in hairy root production the first 6 weeks after the co-cultivation compared to explants inoculated with LMG 63 (Table 5.3, Figure 5.4). Respectively 100 (Arqua1) and 14 (LMG63) hairy roots were harvested after co-cultivation of *E. rosea* explants.

With *E. rubra,* two experiments with Arqua1 were conducted, the mean percentage of leaves and nodes resulting in hairy roots was respectively 52.9 ± 6.7% and 33.9 ± 3.9%. Leaves and nodes inoculated with LMG 63 yielded 83.9% and 73.8% respectively. Nodal explants inoculated with MAFF02-10266 showed roots only after 14 weeks, but these were not displaying the hairy root phenotype and were thus not considered as such. The number of leaf- and nodal explants producing hairy roots was not significantly different. Explants inoculated with either Arqua1 or LMG 63 showed a steep increase in hairy root production between 4 and 8 weeks after the co-cultivation (Table 5.3, Figure 5.4). A total number of 196 (Arqua1) and 166 (LMG63) hairy roots were harvested on *E. rubra* explants.

For *E*. *myrtoidea*, only Arqua1 induced hairy roots on leaf and nodal explants, 17.5% and 38.9% respectively. The hairy roots appeared between 4 and 6 weeks after the co-cultivation, with the highest number of hairy roots/explant at 6 weeks (Table 5.3). No hairy roots appeared after inoculation with LMG 63. Co-cultivation with Arqua1 yielded 45 hairy roots on *E. myrtoidea* explants.

A total of 12.7% of the *E.* 'Edinburgh' leaf explants inoculated with LMG 63 produced hairy roots, with the highest increase in hairy root production between 4 and 8 weeks after inoculation. Only 6.5% of the inoculated nodal explants formed roots at 13 weeks. Furthermore, many of the roots on nodal explants did not show a distinct hairy root phenotype, ergo they were not considered as hairy roots. A total of 11 hairy roots were harvested after co-cultivation with LMG 63. The experiment with MAFF02-10266 did not result in hairy root formation and no hairy roots grew on control explants.

5.3.2 Shoot regeneration

A limited amount of regenerated shoots (RS) appeared on the harvested hairy roots. Hereby, none of the followed approaches proved to be more efficient. For *E. illinita*, six shoots were regenerated,

five after Arqua1 co-cultivation and one after LMG 63 co-cultivation. Two Arqua1-shoots (E01-RS01 and E01-RS02) were harvested on RM11 (3.0 mg/L TDZ), after four and six weeks. One Arqua1 shoot (E01-RS03) was harvested after six weeks on RM10 (7.0 mg/L BAP). The fourth shoot (E01-RS04) was harvested after four weeks on RM10 (7.0 mg/L BAP) and 16 weeks on RM12 (1.0 mg/l BAP). The fifth Arqua1-shoot (E01-RS05) was obtained after five weeks on RM08 (3.0 mg/L BAP) and 11 weeks on RM09 (0.3 mg/L BAP). The shoot of *E. illinita* that regenerated on a LMG 63-hairy root (E01-RS06), appeared after five weeks on RM07 (1.0 mg/L TDZ).

For *E. rubra,* a shoot regenerated on a LMG 63 root (E16-RS01), one after 2 weeks on RM07 (1.0 mg/L TDZ) and one after 4 weeks on RM01 (0.1 mg/L TDZ).

For *E. rosea,* one shoot was regenerated after LMG 63 co-cultivation (E14-RS01), after 2 weeks on RM07 (1.0 mg/L TDZ), followed by 4 weeks on RM01 (0.1 mg/L TDZ).

5.3.3 Confirmation of the absence of rhizogenic *Agrobacterium* and the presence of the *rol*-genes

A qPCR analysis with the *virD2*-gene was performed on 112 hairy root samples, from 6 different *Escallonia* genotypes (*E.* 'Edinburgh', *E. illinita, E. myrtoidea, E. rosea, E. rubra* and *E. rubra* 'C.F.Ball'), on the regenerated shoots that had enough leaves (E01-RS03, E01-RS06 and E16-RS01) and on 2 different rhizogenic *Agrobacterium* strains (Arqua1 and LMG 63). All hairy root samples and control plant samples tested positive for the ITS markers, indicating a sufficient DNA quality for further PCR analyses. None of the tested hairy roots, and shoots contained the *virD2*-gene, while it was present in the samples of the tested bacteria (Figure 5.6). Three stock plants (*E. illinita, E. rubra* and *E. rubra* 'C.F. Ball') also contained the *virD2*-gene, but further sequencing of the DNA sample and a BLAST (Basic Local Alignment Search Tool)(NCBI, 2018) analysis showed that this *virD2*-gene originated from other bacteria, namely *Methylobacterium*, *Shewanella* and an unidentified species respectively and not from rhizogenic *Agrobacterium* strains.

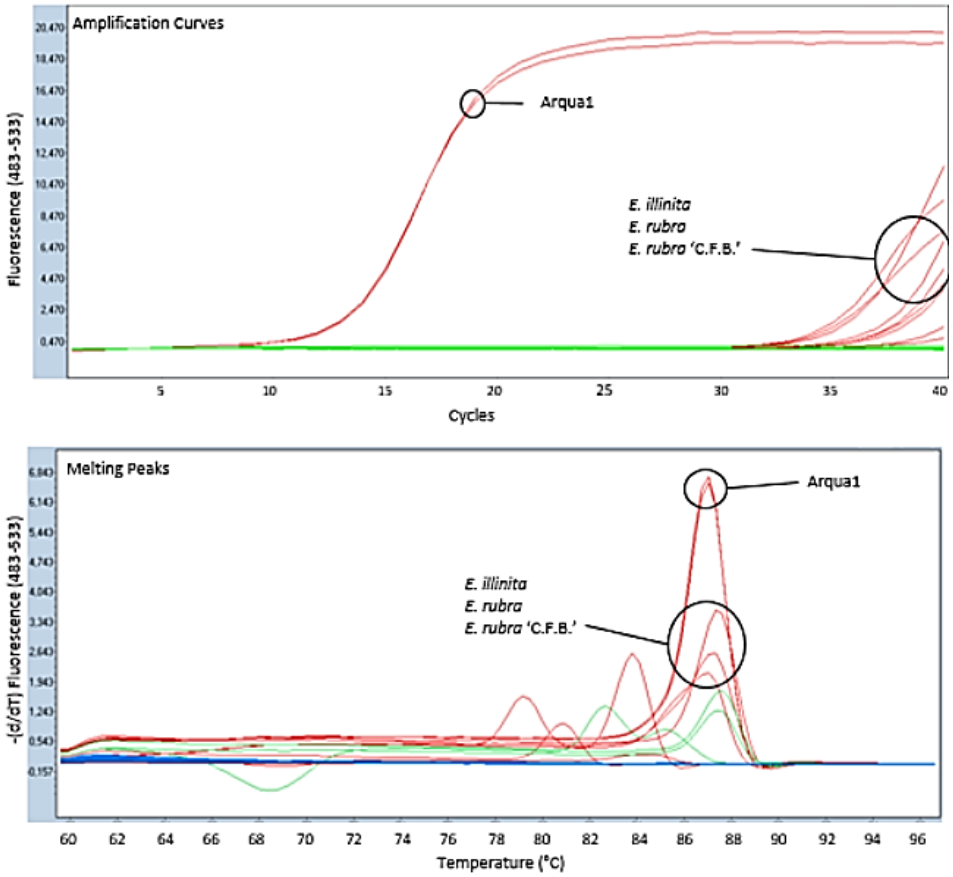

Figure 5.6: Amplification curves and melting peaks of the qPCR analysis to detect the *virD2*-gene in the hairy root samples inoculated with Arqua1, with the *in vitro* stock plants as negative control and Arqua1 as positive control.

The different combinations of *rol*-genes that were present in the hairy root samples are shown in Table 5.4. Out of 112 tested hairy root samples, only 3 did not contain any *rol*-genes, namely 2 roots of *E. rosea* and one of *E. rubra,* both harvested after inoculation with Arqua1. However, these roots did show a hairy root phenotype, with plagiotropic growth and extensive branching. Most hairy roots resulting from an Arqua1 infection contained all four *rol*-genes (75 of 79 hairy roots). Only 1 hairy root of *E. illinita* contained only *rolD.* For hairy roots resulting from a LMG 63 infection, none of the hairy roots contained all four *rol*-genes, although the LMG 63 bacteria added as positive control did contain all four *rol*-genes. All 24 tested LMG 63-hairy roots of *E. illinita* and one LMG 63-hairy root of *E. rubra* contained *rolABC,* eight LMG-63-hairy roots of *E. rubra* contained *rolAD.*

None of the regenerated shoots tested contained any of the *rol*-genes. The roots on which these shoots originated were not tested for *rol*-genes, since they did not grow sufficiently.

Table 5.4: Presence (+) or absence (-) of the *rol*-genes in the analyzed hairy roots of different *Escallonia* genotypes, infected with either Arqua1 or LMG 63

Bacterial strain	*Escallonia* genotype	# Hairy roots	*rolA*	*rolB*	*rolC*	*rolD*
LMG 63	*E. illinita*	24	+	+	+	-
	E. rubra	8	+	-	-	+
		1	+	+	+	-
	Total	**33**	**33**	**25**	**25**	**8**
Arqua1	*E. illinita*	21	+	+	+	+
		1	-	-	-	+
	E. rosea	16	+	+	+	+
		2	-	-	-	-
	E. rubra	22	+	+	+	+
		1	-	-	-	-
	E. myrtoidea	2	+	+	+	+
	E. 'Edinburgh'	9	+	+	+	+
	E. rubra 'C.F. Ball'	5	+	+	+	+
	Total	**79**	**76**	**75**	**75**	**76**

5.4 DISCUSSION

5.4.1 Differences in host-strain interactions

The T-DNA transfer into the host plant cell requires a complex interaction of proteins of both the rhizogenic *Agrobacterium* strain and the host plant (Lacroix and Citovsky, 2016). Therefore, it is not surprising that various strains of rhizogenic *Agrobacteria* display different virulence towards different plant species. In our study, we could conclude that the rhizogenic *Agrobacterium* strains showed clear differences in efficacy to induce hairy roots on the different *Escallonia* genotypes (Table 5.3, Figure 5.4). The most infectious strain for *Escallonia* species was obviously Arqua1, which was capable of inducing hairy roots with confirmed presence of *rol*-genes in all five *Escallonia* genotypes that were inoculated with Arqua1. Arqua1 was followed by LMG 63, capable of infecting four out of six inoculated genotypes, and MAFF02-10266, infecting only one out of four *Escallonia* genotypes. The introgression of the *rol*-genes of LMG 63 in *E. illinita* and *E. rubra* was confirmed with qPCR. The least efficient strain for inducing hairy roots in *Escallonia* was NCPPB 2659, which transferred *rol*-genes in none of the four tested genotypes. Although NCPPB 2659 is capable of infecting woody plant species, e.g., *Coffea* (Alpizar et al., 2006), *Elaeagnus* (Berg et al., 1992) and poplar (Neb et al., 2017), *Escallonia* seemed not to be a suitable host. Indeed most reports of successful *rol*-gene introduction by NCPPB 2659 are on leguminous crops such as soybean (Xiang et al., 2016), *Phaseolus* (Estrada-Navarrete et al., 2007) and peanut (Liu et al., 2016, Pilaisangsuree et al., 2018, Guimaraes et al., 2017). It is clear that this capacity for hairy root induction is genotype specific, since Arqua1 induced more hairy roots than LMG 63 in *E. illinita* and *E. rosea,* while this was the other way around in *E. rubra.* Different host-strain reactions were also found in poplar (Neb et al., 2017), in *Hyoscyamus*, (Akramian et al., 2008), and in *Coffea arabica* var. Caturra, (Alpizar et al., 2006).

The efficiency of hairy root induction on the explants varies for the different bacterial strains and *Escallonia* genotypes. Overall, both Arqua1 and LMG 63 were able to induce hairy roots in ± 85% of the explants, depending on the genotype and the explant type. The strain MAFF02-10266 only reached a maximum of 38% explants with hairy roots and NCPPB 2659 none. These results concurred with hairy root induction efficiencies found in literature for other woody species, for example, an optimum of 61% was reached in *Semecarpus* (Panda et al., 2017), up to 95% hair root induction in *Rauwolfia* (Mehrotra et al., 2013) and up to 60% in *Hibiscus* (Christensen et al., 2015). The combination of *E. illinita* with Arqua1, and *E. rubra* with LMG 63 yielded around 80% explants with hairy roots, which is within the range of the efficiencies found in literature. For the

other *Escallonia* genotypes, other bacterial strains could be investigated to increase the infection efficiency.

5.4.2 Shoot regeneration is the bottle-neck of *rol*-gene induction in *Escallonia*

Spontaneous shoot regeneration on hairy roots or directly on the inoculated explants has been reported (Kim et al., 2012, Subotic et al., 2003, Christensen and Müller, 2009b, Mehrotra et al., 2013). For *Escallonia* however, only a few shoots were obtained by adding phytohormones to the growth media. Altogether, eight shoots regenerated on 1278 hairy roots on several regeneration media; 3 shoots could already be tested but did not contain *rol*-genes. An efficient method for shoot regeneration on *Escallonia* hairy roots still needs to be developed.

The rhizogenic *Agrobacterium* strain Arqua1 contains several auxin genes in its TR-DNA, which are also transferred into the plant DNA (White et al., 1985, Jouanin et al., 1987). These auxin genes influence the auxin biosynthesis in the transformed plant cells (Camilleri and Jouanin, 1991) and thus interact with root morphology, and create hairy root-like roots without the actual insertion of *rol*-genes, which decreases the relative yield of regenerants containing *rol*-genes. Also, it is possible that the auxin genes in Arqua1-hairy roots influence potential shoot regeneration by changing the auxin/cytokinin ratio. Therefore, it would be interesting to evaluate the presence of *aux*-genes in the hairy roots and regenerants by qPCR. In *Campanula*, shoots only regenerated on roots that did not contain the auxin genes (Hegelund et al., 2017). The addition of auxin transport inhibitors such as naphthylphtalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) in the *in vitro* media could counteract the effect of the auxin genes and increase shoot regeneration. This has been shown in *Beta vulgaris* hairy roots (Ninkovic et al., 2010). However, the MAFF02-10266 and LMG 63 strains used in our study do not contain auxin genes and their hairy roots are equally incapable of producing shoots for *Escallonia*. Also the *rol*-genes themselves play a role in shoot regeneration, as they can influence hormone sensitivity and the hormone pathways, which in turn influences the capacity for shoot regeneration on the hairy roots. For example, the RolA protein can influence the auxin content (Bettini et al., 2016b, Schmülling et al., 1993), *rolB* influences the auxin signal transduction pathway (Baumann et al., 1999, Shankar et al., 2015), and the RolC protein changes the auxin sensitivity or pathway (Kaneyoshi and Kobayashi, 1999, Koshita et al., 2002, Zuker et al., 2001). To circumvent the bottle-neck of shoot regeneration, it could be considered to include a callus-inducing step in the regeneration process. This was useful for shoot regeneration in *Campanula* (Hegelund et al., 2017). A medium with an intermediate auxin/cytokinin ratio could first induce callus on the hairy roots. This callus should then be transferred to a medium with a low auxin/cytokinin ratio for shoot induction.

5.4.3 The *rol*-genes are successfully introduced in the hairy roots

The *virD*2 gene of the rhizogenic *Agrobacterium* strains was not present in any of the samples. This means that the applied treatment with antibiotics was sufficient to remove all bacteria. Cefotaxime and timentin both are shown to have a inhibiting effect on the growth of *Agrobacterium* strains at the concentrations used (Nauerby et al., 1997) and are used frequently in similar studies (Akramian et al., 2008, Alpizar et al., 2006, Brijwal and Tamta, 2015, Cheruvathur et al., 2015, Hegelund et al., 2017, Huang et al., 2014, Nourozi et al., 2014, Petrova et al., 2013). Timentin is a mixture of ticarcillin and clavulanic acid (Nauerby et al., 1997). Both ticarcillin and cefotaxime are β-lactam antibiotics, and interrupt the synthesis of peptidoglycan causing the rupture of the cell wall. Some gram-negative bacteria however contain β-lactamase activity, which inactivates β-lactam antibiotics. This is counteracted by the addition of clavulanic acid, which is an inhibitor of β-lactamase. Timentin is even shown to have a positive effect on shoot regeneration on tobacco leaves (Nauerby et al., 1997).

The successful infection and introduction of all four *rol*-genes by both Arqua1 and LMG 63 were proven. Most of the hairy roots tested for Arqua1 showed the introgression of all four *rol*-genes. Hairy roots induced by LMG 63 showed either the presence of *rolA, rolB* and *rolC,* or the presence of *rolA* and *rolD.* This could indicate that the T-DNA is not always transferred to the plant DNA in one piece, but can be inserted in fragments. However only few studies in literature have tested the presence of all four *rol*-genes, so the frequency of fragmentation of the T-DNA is difficult to ascertain. Fragmentation of the T-DNA has been reported for the pRiA4 plasmid in *Chrysanthemum cinerariaefolium,* where hairy root lines contained either *rolABC* or *rolBC* (Khan et al., 2017), in *Dracocephalum forestii,* where either *rolB* or *rolC* was introduced (Weremczuk-Jezyna et al., 2016), and in *Bacopa monnieri,* where three regenerants contained either *rolAB, rolABC* or only *rolC* (Majumdar et al., 2011). For the agropine plasmid pRi15834, the insertion of fragments of the T-DNA was observed, e.g., in *Arnica montana* (Petrova et al., 2013) and in apple rootstock (Pawlicki-Jullian et al., 2002). Hairy root lines of *Tetrastigma hemsleyanum* differed greatly in the presence of 11 tested genes (Du et al., 2015). However, the insertion of the whole T-DNA fragment is much more common according to literature (Petrova et al., 2013, Kim et al., 2012, He-Ping et al., 2011, Kang et al., 2006, Choi et al., 2004).

Although none of the roots on which shoots regenerated could be tested for the presence of *rol*-genes, the assumption that they did not contain any can be made. These roots did not grow vigorously, while vigorous growth is a typical phenotype for roots with *rol*-genes. This difference

between vigorously growing, branching roots and slow-growing, non-branched and less hairy roots has also been seen in *Campanula* hairy root lines (Hegelund et al., 2017).

Each *rol*-gene has its own function and its own effect on the phenotype and physiology of the transformed plant (reviewed by Mauro et al. (2017) and Bulgakov (2008)). Shoots regenerated on hairy roots with different *rol*-gene combinations will probably show different phenotypes. As *rolA* is associated with wrinkled leaves and a delay in flowering time, regenerants with only *rolA* will probably not be desirable as an ornamental (Bettini et al., 2016b, MartinTanguy et al., 1996, Schmülling et al., 1988, Sinkar et al., 1988a). However, plants with only *rolD* are likely to show an increased flower number and earlier flowering (Altamura, 2004, Bettini et al., 2003). Also *rolB* is associated with earlier flowering, but its main effect on the phenotype is a decrease in apical dominance, resulting in more branched, fuller and compact plants (Arshad et al., 2014, Rugini et al., 1991, Zhu et al., 2001, Zhu et al., 2003, Kodahl et al., 2016). The *rolC*-gene causes dwarfism by reducing internode length and apical dominance (Kaneyoshi and Kobayashi, 1999, Koshita et al., 2002, Mitiouchkina and Dolgov, 2000, Schmülling et al., 1988). Also the copy number of the inserted T-DNA and the place of insertion in the plant genome can influence the resulting phenotype. The place of insertion and the copy number can be visualized by FISH (fluorescence *in situ* hybridization) (Kirov et al., 2014). The copy number can also be determined by Southern blot (Wagenknecht and Meinhardt, 2011, Collier et al., 2005), digital droplet PCR or inverse PCR (Nakagaki et al., 2018). Although the function and effect of each *rol*-gene separately has been studied extensively, their interactions with each other and with the plant species in which they are inserted are difficult to unravel, and the effect of the *rol*-genes on the phenotype of *Escallonia* species is not predictable.

5.5 CONCLUSIONS

A protocol for hairy root induction in several *Escallonia* species has been obtained. Also, the introduction of *rol*-genes into particular *Escallonia* hairy roots has been proven. Shoot regeneration from hairy roots remains a bottleneck and the search for an optimal shoot regeneration protocol continues. For *Sarcococca,* the *in vitro* stock grew too slowly to do many experiments. In future, more rhizogenic *Agrobacterium* strains need to be tested, and the protocol optimized.

CHAPTER VI
CONCLUSIONS, FINAL REMARKS AND FUTURE PERSPECTIVES

6 CONCLUSIONS, FINAL REMARKS AND FUTURE PERSPECTIVES

The main goal of this thesis was to create new variation within the genera *Escallonia* and *Sarcococca.* In urban and peri-urban environments gardens keep getting smaller or people have only a small balcony. This results in an increase in the market segment of potted compact woody ornamentals. Both *Escallonia* and *Sarcococca* are rather unknown in the assortment of flowering shrubs. For both genera, only a limited number of species or cultivars is commercially available. To our knowledge, no breeding activities are reported nor for *Escallonia*, nor for *Sarcococca*. These genera were suggested by BestSelect CVBA, a Flemish cooperation of ornamental growers, as having potential as ornamental shrub, especially when novelties such as more compact growing cultivars or cultivars with a higher number of inflorescences could be created. To meet this main objective, a breeders collection was made and analyzed for several traits, to increase the efficiency of the subsequently applied advanced breeding techniques such as interspecific hybridization, chromosome doubling and the introduction of *rol*-genes from rhizogenic *Agrobacterium* strains were studied.

IN DEPTH CHARACTERIZING OF THE PARENTAL PLANT COLLECTIONS TO INCREASE BREEDING EFFICIENCY

A breeders collection of 23 *Escallonia* genotypes and 18 *Sarcococca* genotypes was created, and analyzed morphologically. The collection of *Escallonia* contained 9 species, 2 varieties and 12 cultivars. Yet, the *Escallonia* genus consists of about 40 species and 20 varieties (Morello and Sede, 2016, Sede et al., 2013). In future, it might be interesting to enlarge the *Escallonia* collection with more species to increase the genetic diversity. The collection of *Sarcococca* genotypes contained 12 species, 4 varieties and 2 cultivars. As the genus has about 20 species (Bean and Murray, 1989b, Flora of China, 2008, Hilliers Garden, 1991), this collection is more complete.

The morphological traits of the collected genotypes were compared to literature, and the genotypes were verified with specimens in the living collection and herbarium at the National Botanical Garden in Meise (Belgium). For *Sarcococca,* a vegetative identification key uncovered some errors, e.g. our accessions *S. saligna* (S04) and (S10) were actually *S. hookeriana* ‘Ghorepani’. For *Escallonia*, many differences were found. This can have various causes. Firstly, there is a lack of knowledge on the genus *Escallonia.* The taxonomical name of several species has changed over time, and morphological descriptions in literature are not very elaborate. Secondly, the genus *Escallonia* itself is very loose with species-boundaries. Many genotypes have intermediary characteristics and a lot of variation is displayed within one species, such as plant

size and leaf size. The *Escallonia* genus has very little variation in genome size and ploidy level, with only one tetraploid out of the 23 collected genotypes. In our collection of *Escallonia*, 12 cultivars resulting from an interspecific hybridization were present. These hybrids and the small variation in ploidy level and genome sizes, could indicate that interspecific hybridization holds possibilities for breeding in *Escallonia.*

INTERSPECIFIC HYBRIDIZATION IS A VALUABLE TOOL TO INDUCE VARIATION IN *ESCALLONIA* AND *SARCOCOCCA*

Interspecific hybridization is a breeding technique that has resulted in new cultivars of many crops and ornamentals (Kuligowska et al., 2016b). Also for *Escallonia* and *Sarcococca* many possibilities for improvement are expected by the use interspecific hybridization. To have more insight into the breeders' value of our collected genotypes, they were analyzed for genome size, ploidy level and chromosome number, and a phylogenetic tree was composed with AFLP markers (Chapter II). Large differences in genome sizes and ploidy levels were observed between *Sarcococca* genotypes. The genetic variation measured by AFLP markers showed a grouping within the *Sarcococca* genus that concurred with the cytogenetic information and several morphological characteristics such as flower and fruit color, and plant size. Within the genus *Escallonia*, cytogenetic differences were minor. The AFLP analysis showed a large genetic variation, and a clustering of the cultivars according to their genetic background. These data improved the knowledge about the possible presence of crossing barriers, e.g., due to ploidy differences, can provide insight on the cross compatibility of potential parent species and predict the success rate of crosses (Van Laere, 2008, Honda et al., 2003).

For *Escallonia*, no severe difficulties are expected for interspecific hybridization in *Escallonia.* Natural hybrid cultivars are already described in literature, even between diploids with a difference in genome sizes (1.06 pg/2C – 1.43 pg/2C), e.g., the hybrid *E.* 'Iveyi' (or *E. iveyi* (x)) (=(*E. rosea* x *E. rubra*) x *E. bifida*). The genome sizes of *E. rosea* and *E. rubra* were one of the smallest measured while the genome size of *E. bifida* is the largest diploid measured. Furthermore, these parental genotypes were rather distantly related. A preliminary hybridization experiment for *Escallonia* was started, ± 300 flowers have been pollinated for 12 different interspecific crosses, of which ± 30% produced seeds (data not showed in this PhD thesis). Among the progeny planted on the field, many interesting compact growth forms are found (Figure 6.1).

For *Sarcococca*, more interspecific barriers are expected based on the results of ploidy analysis, genome size analysis and phylogenetic relationships. Our results from inter- and intraspecific crosses provided information on the real crossing ability of species with differences in ploidy level,

genome size and genetic distance (Chapter III). It could be concluded that distant genetic relationships did not always interfere with hybridization, although the wider crosses showed lower crossing efficiencies in some cases. Interploidy crosses yielded less fruits than intraploidy crosses, but no complete ploidy block was present. Within the progeny of an intraploidy cross, true hybrids could be found. The progeny of the interploidy crosses was analyzed for genome size and number of paternal markers (AFLP) to determine their hybrid status.

The efficiency of the interploidy crosses probably can be augmented by doubling the chromosome number of the diploid in a polyploidization event, yielding a tetraploid, which can then be crossed with the other (tetraploid) parent. This technique has been used successfully in *Citrus* (Aleza et al., 2009), *Buddleja* (Van Laere et al., 2011b), and roses (Allum et al., 2007). The crossing efficiency could also be increased by gathering information about pollen fertility (Kuligowska et al., 2015) and the optimal time and conditions for style receptivity (Xie et al., 2017). The fruit set can be enhanced further by hormone treatments (Xie et al., 2017). Furthermore, the germination efficiency could be increased by altering the stratification conditions (Balestri and Graves, 2016) or pretreatment of the seeds, with hormones or by scarring the pericarp (Karlsson et al., 2005, Mattana et al., 2018).

Further evaluation of interesting *Sarcococca* and *Escallonia* hybrids in consultation with the growers of BestSelect CVBA is necessary. The hybrids are currently planted on the fields at ILVO to evaluate flowering characteristics, winter hardiness in field conditions, growth vigor, etc.. which is necessary to assure that qualitative and visually attractive cultivars can be selected and commercialized.

Figure 6.1: Progeny of interspecific crosses within *Escallonia* with interesting characteristics. A) *E. laevis* 'Gold Ellen' (E02) x *E. illinita* (E01), B) *E.* 'Iveyi' (E07) x *E. illinita* (E01) and C) flowers of *E. illinita* (E01) x *E.* 'Donard Seedling' (E06).

IN VITRO INITIATION AND MULTIPLICATION TO PROVIDE CLONAL MATERIAL

To be able to perform *in vitro* breeding techniques, such as polyploidization and *rol*-gene introduction, under controlled conditions, the collected genotypes were initiated *in vitro* to provide clonal propagated material. For both genera, 23 different medium compositions were tested, with 2 different types of macro-nutrients (Murashige and Skoog medium and Woody Plant Medium) and different concentrations and combinations of cytokinins (BAP, zeatin, 2-IP, mT) and auxin (NAA). Twelve *Escallonia* genotype and five *Sarcococca* genotypes were successfully initiated and multiplicated *in vitro*. Several *Escallonia* genotypes grew rather vigorously *in vitro*, and provided sufficient plant material for further *in vitro* experiments. Others showed initially a vigorous growth, but this declined after 2 years of multiplication, so further medium optimization is needed. All *Sarcococca* genotypes showed a slow growth *in vitro*, and could therefore only be used to a limited extend for *in vitro* breeding techniques. Further optimization of the initiation and multiplication media are needed to promote growth and reduce the stress on the *in vitro* plantlets.

DEVELOPMENT OF AN EFFICIENT POLYPLOIDIZATION PROTOCOL FOR *ESCALLONIA*

Three *Escallonia* genotype were selected for mitotic polyploidization (*E. illinita* (E01)*, E. rosea* and *E. rubra*). To develop an efficient protocol for chromosome doubling, several mitotic inhibitors and techniques were applied. A preliminary experiment showed that oryzalin and trifluralin were more effective than colchicine, which was thus omitted from following experiments. This inefficiency of colchicine compared to other mitotic inhibitors was also observed in other plant species (Ari et al., 2015, Gallone et al., 2014, Nonaka et al., 2011, Yoon et al., 2017). Successive experiments included two types of treatments: i) shock treatments with high concentrations of oryzalin and trifluralin (50-250 µM) and short exposure times (2-4 days), and ii) continuous treatments with low concentrations (1-10 µM) and long exposure times (6-10 weeks). Both shock and continuous treatments yielded a high percentage of tetraploids (on average 39.2% and 53.7% respectively). In the shock treatment a fluctuating mortality and spontaneous chromosome doubling in the control explants was observed. This was most likely due to the stress induced by high concentrations of mitotic inhibitors and/or their solvents, and the physical treatment. Sometimes spontaneous chromosome doubling is reported as a side effect of *in vitro* multiplication (Barow and Jovtchev 2007; Chen et al. 2009; Jelenic et al. 2001; Meyer et al. 2009). Overall, 10 weeks with 5 µM of trifluralin in solid growth medium was chosen as the most effective protocol for chromosome doubling in *Escallonia.* In similar studies, equally high tetraploid yields could be reached, but this was accompanied by very high mortality rates of 80% or higher (Kermani et al.,

2003, Tavan et al., 2015). Sometimes no tetraploids (Van Laere et al, 2011b) or only mixoploids (Aleza et al., 2009) could be obtained. So compared to literature, our protocol is highly efficient and can probably be used for the polyploidization of other *Escallonia* genotypes as well. Ploidy induction in more genotypes could provide insights whether closely related genotypes show the same response towards mitotic inhibitors and the same changes in the obtained tetraploids.
For *Sarcococca*, no polyploidization was attempted, due to a lack of *in vitro* plant material. Possible phenotypes of interest of synthetic *Sarcococca* polyploids could be a more compact and fuller growth form, with larger fruits and flowers. The natural occurring tetraploids, namely the *S. ruscifolia* genotypes and *S. confusa,* are quite compact, but form occasional long erect branches and require frequent pruning to keep in shape. To develop a similar polyploidization protocol for *Sarcococca,* firstly the *in vitro* multiplication medium needs to be optimized. Due to their very slow growth on the current medium, the uptake of nutrients, and consequently the uptake of the mitotic inhibitors from the media is very slow and the polyploidization experiment would probably have to last a much longer time to create full tetraploids and not mixoploids, than was necessary for *Escallonia.* The treatment of germinating seedlings could be an alternative way to induce polyploidy in *Sarcococca.* However, seedlings possess more genetic variation compared to clonal propagated material. Ergo, the observed variation in obtained polyploids cannot solely be attributed to the polyploidization itself.

IMAGE ANALYSIS PROVED TO BE A ROBUST AND EFFICIENT PHENOTYPING APPROACH

The obtained tetraploids were assessed for several characteristics. The image analysis of the top and side view was a valuable tool to efficiently evaluate several characteristics on the plantlets. Naturally, taking the images of the plantlets in a standardized photo booth takes time, but certainly less than measuring all characteristics manually on the many clones themselves. If additional data are needed, e.g., a color analysis of the foliage, or the angle of the secondary branches, this can be generated from the same images, instead of having to make new cuttings for analysis. Another advantage of image analysis is that some characteristics can be quantified, while they would otherwise have been scored visually, e.g., the circularity and the fullness of the plant. This removes the subjectivity of the observer(s). For the image analysis of *E. illinita* (E01) tetraploids, only a subset of five diploids and five tetraploids were photographed. The analysis of this subset could indicate the trend of the phenotypical changes. With only a limited amount of time to take the images of a subset of plants, a selection can already be made. If the results from the subset are promising, further images can be made for a full analysis. Our developed image analysis approach provides a robust and efficient phenotyping of the generated plants.

The different effects of polyploidization on phenotype and physiology described in literature are plethora. This abundance of changes was also observed in the tetraploids of the different *Escallonia* species. The largest differences in size and compactness could be attributed to changes in internode length and to the differences in budburst on the main stem and their interaction. For tetraploid *E. rubra,* an increased internode length and decreased budburst increased the plant size, and decreased the bushiness of the plant. Likewise, *E. rosea* tetraploids had a decreased budburst, but combined with a large decrease in internode size, plants were much smaller and denser than the diploids. *E. illinita* (E01) tetraploids showed an increased budburst, and the increase of the number of branches could potentially cause a decreased growth rate. However, this was compensated with an increased internode length, and this combination resulted in only slightly smaller plants, but visually denser.

Moreover, leaf and flower characteristics, rooting ability of cuttings and the cold tolerance were determined. For *E. illinita* (E01) and *E. rubra,* the leaf width and surface increased due to polyploidization, while for *E. rosea,* leaf sizes decreased remarkably. Flower characteristics could only be determined for *E. rubra* tetraploids. Tetraploid flowers were significantly larger than diploids. The rooting ability of the tetraploid cuttings was as high as of the diploids cuttings, which is an important commercial characteristic for vegetatively propagated woody ornamentals. The cold tolerance of tetraploids either stayed the same (*E.* rosea) as their diploid progenitor or increased (*E. rubra*). Overall, it was clear that mitotic polyploidization enlarged the variation, and that this can lead to visually attractive compact plants and enlarged flowers. All tetraploids are planted in the field for further evaluation and selection. If no ploidy barrier exist within *Escallonia,* the development of sterile triploids could be interesting. Triploid cultivars are usually sterile, which can lead to a prolonged flowering time (Van Laere, 2008), and prevent the spreading of seedlings of an invasive plant species (Leus et al., 2012).

DEVELOPMENT OF A PROTOCOL FOR EFFICIENT INTROGRESSION OF *ROL*-GENES

The introgression of *rol*-genes into the plant genome is known to induce compactness. However, limited data with this technique is available in woody ornamentals. This study has proven the successful introduction of *rol*-genes into the plant genome of *Escallonia*, and hairy roots cultures were obtained, but no shoots with *rol*-genes were regenerated yet from the hairy roots. The shoots that were obtained, originated from small, feeble roots, that most likely did not contain *rol*-genes. Therefore, a stringent selection of hairy roots is important to avoid useless effort in regeneration of non-transformed roots. Although spontaneous shoot regeneration has been reported in different species (Kim et al., 2012, Subotic et al., 2003, Christensen and Müller, 2009b, Mehrotra et al.,

2013, Christensen et al., 2008, Hegelund et al., 2017), this was not observed for *Escallonia*. Woody species in general are known for their recalcitrance *in vitro* (Rastogi et al., 2008, Rugini et al., 1991). Furthermore, differences between species from the same genus in regeneration potential are reported (Choi et al., 2004, Hegelund et al., 2017). We already observed differences in *in vitro* initiation and multiplication media between *Escallonia* species, so most likely also the shoot regeneration medium will have to be optimized for each species. Several cytokinins have not yet been tested for shoot regeneration, such as mT, 2-iP and CPPU (1-(2-chloropyridin-4-yl)-3-phenylurea = forchlorfenuron). Furthermore, a callus-inducing step with an intermediate cytokinin/auxin balance could ameliorate shoot regeneration (Hegelund et al., 2017). Hairy roots originating from agropine type strains can contain the TR-DNA with its auxin genes. These genes cause changes in the auxin/cytokinin content in the roots, which can hamper shoot regeneration. A solution could be to add auxin transport blockers such as TIBA or NPA in the regeneration medium (Ninkovic et al., 2010). The presence of the *aux*-genes can be detected with (q)PCR, roots with the *aux*-genes can easily be selected.

Introduction of *rol*-genes in *Sarcococca* has been attempted – unsuccessfully – with the Arqua1 strain. However, many differences in efficacy between bacterial strain - species combinations can occur (Hegelund et al., 2017, Setamam et al., 2014), as was also the case for *Escallonia.* It is recommended to test the other bacterial strains for *Sarcococca*. Several other strains that have not been used in this study are also available. Furthermore, the slow growth of the *in vitro* stock of *Sarcococca* is a limiting factor and optimization of the *in vitro* growth medium is needed. Another possibility for the induction of hairy roots could be using *in vivo* plant material, by wounding stems or leaves and inoculating the bacteria on the wounded surface (Alpizar et al., 2006, Majumdar et al., 2011, Nourozi et al., 2014, Perassolo et al., 2017). Also inoculating *in vitro* germinating seedlings could be tried, but the genetic variation of seedlings has to be taken into account during further evaluation of shoots.

FINAL CONCLUSIONS AND PERSPECTIVES

In conclusion, for *Escallonia*, interspecific hybridization is very feasible, and interesting hybrids were obtained. Furthermore, in this thesis also polyploidization in this genus has proven valuable for induction of interesting traits, e.g., compactness, increased cold tolerance and larger flowers. An efficient protocol for chromosome doubling is developed which can deliver *in vitro* tetraploids in 12 weeks. Provided that *in vitro* initiation and multiplication is optimized further, more genotypes can successfully be polyploidized and analyzed phenotypically with the elaborate image analysis. These results lead to the conclusion that further breeding in *Escallonia* with interspecific

hybridization and polyploidization will be quick, efficient, and leading to aesthetically pleasing and qualitative cultivars. Although the introgression of *rol*-genes has proven its worth in other ornamentals and offers many possibilities for breeding, the development of shoots with *rol*-genes has not been achieved for *Escallonia*. Shoot regeneration will most likely require much effort in the optimization of the regeneration medium, and that medium will probably be different for each genotype, or even different for each genotype-bacterial strain combination.

For breeding within *Sarcococca,* it can be concluded that interspecific hybridization was most successful. The knowledge of the ploidy levels, genome sizes and genetic distances greatly improved the understanding of the genus and the potential efficiency of the breeding program. The bottleneck for the application of the *in vitro* breeding techniques is clearly the *in vitro* multiplication. Further optimization of the multiplication and regeneration media are advised, as the *in vitro* breeding techniques certainly can add valuable traits to the genus. For example, polyploidy can cause larger fruits, which are the eye-catcher of *Sarcococca* in spring and summer. Besides, these polyploids could be interesting for interspecific hybridization, as natural tetraploids are already present in the collection and can then be crossed with artificially made tetraploids. The introgression of *rol*-genes could increase the flowering time or increase the tolerance of the photosynthetic apparatus to excess energy, so they can be grown in the sun. Although no *in vitro* breeding techniques were successful yet, the possibilities justify further effort. Alternatively, both polyploidization and the co-cultivation with rhizogenic *Agrobacterium* strains could be attempted on germinating seedlings. This juvenile tissue could be more susceptible to the applied treatments.

In this study, we used *Escallonia* and *Sarcococca* as case studies to investigate the possibilities of several techniques for breeding purposes, and the obtained results can be extrapolated for woody ornamentals in general. When starting a breeding program with a rather unknown genus, it is advisable to collect morphological, cytogenetic and phylogenetic information of the potential parental species. With this information, interspecific hybridization can lead to a straightforward development of interesting hybrids. Although in this thesis it was not examined for *Escallonia* and *Sarcococca,* the efficiency of interspecific hybridization can be further augmented with information on e.g., pollen quality, the period of optimal receptivity of the style, embryo rescue, etc. For inducing new variation within the genus, both polyploidization and *rol*-gene introgression are valuable techniques. However, for each new genus, optimization of the *in vitro* initiation and multiplication, and protocol development for both polyploidization and *rol*-gene introgression, are required. As the *in vitro* stock of *Sarcococca* is increasing, both techniques can be optimized for this genus in future research, and better conclusions for woody ornamentals in general will be

made. Finally, an efficient and robust image analysis approach was developed. This approach can easily be adapted for the phenotyping of other (woody) ornamental plants and to other types of research with effects on the morphology.

ADDENDUM

Vegetative Key to the genus *Sarcococca*

01 a) Shoot pubescent (10x LENS) ……02
b) Shoot glabrous (10x LENS) ……07

02 a) Lamina narrowly elliptic with largest width in the middle AND L/W > 3/1…*S. hookeriana*
- Lamina apex acuminate to caudate, margin at least in part of the leaves undulate.
S. hookeriana var. *digyna*
- Lamina apex acute to (sub-)acuminate, margin predominantly flat.
- suckering shrug, 25-60 cm, often shoots with leaves (sub-)opposite…….*S. hookeriana* var. *humilis*
- shrub ± erect, 60-120 cm, leaves alternate……..*S. hookeriana* var. *hookeriana*

b) Lamina (broadly) elliptic, L/W ≤ 3/1, OR at least in part of the foliage with largest width below middle ……03

03 a) Lamina rigid AND midvein length predominantly 6-9 cm ……*S. orientalis*
b) Lamina supple, OR midvein length predominantly ≤ 6 cm ……04

04 a) Current year shoot initially green but quickly becoming brown…….. *S. bleddynii*
b) Current and secondary shoot green ……..05

05 a) Lamina elliptic, with largest width in the middle (fruit black) ……*S. confusa*
b) Lamina ovate to lanceolate, with largest with below the middle (fruit red) ……06

06 a) Lamina ovate: L/W ≤ 3/1, margin often ± undulate ……...*S. ruscifolia*
b) Lamina narrowly ovate to lanceolate: L/W > 3/1, margin flat .*S. ruscifolia* ‘Dragon Gate’

07 a) Lamina narrowly elliptic, L/W 3/1 - 5/1 ……08
b) Lamina elliptic, L/W ≤ 3/1 ……..09

08 a) Lamina predominantly 7-15 cm x 10-20 mm. Apex acuminate to caudate …...*S. saligna*
b) Lamina predominantly ≤ 8cm x 5-120 mm. Apex ± acute ……..*S. hookeriana* ‘Ghorepani’

09 a) Lamina midvein length predominantly 10-20 cm ……10
b) Lamina midvein length predominantly ≤ 10 cm ……..11

10 a) Lamina apex 2-3 cm, gradually long acuminate to caudate ……..*S. coriacea*
b) Lamina apex 1-2 cm, rather abruptly to short acuminate ……*S. vagans*

11 a) Lamina width predominantly ≥ 3.5 cm ……12
b) Lamina width predominantly < 3 cm ……...13

12 a) Lamina broadly elliptic L/W < 2/1, base attenuate ……*S. brevifolia*
b) Lamina elliptic L/W > 2/1, base cuneate ……..*S. trinervia*

13 a) Lamina apex acuminate, 1 cm ……*S. zeylanica*
b) Lamina apex acuminate to caudate, 1-2 cm ……*S. wallichii*

Table 1: Results[z] from the preliminary ploidization experiment on *Escallonia rosea* (E14) and *E. rubra* (E16) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) colchicine (COL), trifluralin (TRI) and oryzalin (ORY).

Genotype	Mit. inh.	Exp.T. (days)	Conc. (µM)	Treated nodes	Contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Non-surviving nodes
E. rosea	COL	2	2000	30	0 (0.0%)	23 (76.7%)	0 (0.0%)	3 (10.0%)	4 (13.3%)
	COL	2	1000	30	0 (0.0%)	19 (63.3%)	0 (0.0%)	0 (0.0%)	11 (36.7%)
	TRI	2	150	30	18 (60.0%)	2 (16.7%)	0 (0.0%)	3 (25.0%)	7 (58.3%)
	ORY	2	150	30	6 (20.0%)	10 (41.7%)	7 (29.2%)	4 (16.7%)	3 (12.5%)
	control	2	0	6	0 (0.0%)	6 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	COL	3	2000	30	0 (0.0%)	21 (70.0%)	1 (3.3%)	7 (23.3%)	1 (3.3%)
	COL	3	1000	30	12 (40.0%)	14 (11.8%)	0 (0.0%)	4 (22.2%)	0 (0.0%)
	TRI	3	150	30	6 (20.0%)	11 (45.8%)	3 (12.5%)	10 (41.7%)	0 (0.0%)
	ORY	3	150	30	0 (0.0%)	11 (36.7%)	10 (33.3%)	3 (10.0%)	6 (20.0%)
	control	3	0	24	0 (0.0%)	23 (95.8%)	0 (0.0%)	0 (0.0%)	1 (4.2%)
E. rubra	COL	2	1000	30	0 (0.0%)	13 (43.3%)	3 (10.0%)	8 (26.7%)	6 (20.0%)
	TRI	2	150	30	0 (0.0%)	9 (30.0%)	14 (46.7%)	5 (16.7%)	2 (6.7%)
	ORY	2	150	30	0 (0.0%)	2 (6.7%)	5 (16.7%)	5 (16.7%)	18 (60.0%)
	control	2	0	5	0 (0.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	COL	3	1000	30	0 (0.0%)	13 (43.0%)	5 (16.7%)	12 (40.0%)	0 (0.0%)
	TRI	3	150	30	0 (0.0%)	0 (0.0%)	1 (3.3%)	1 (3.3%)	28 (93.3%)
	ORY	3	150	30	0 (0.0%)	1 (3.3%)	3 (10.0%)	5 (16.7%)	21 (70.0%)
	control	3	0	3	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 2: Results[z] from the shock experiment on *Escallonia illinita* (E01) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (days)	conc. (µM)	Treated nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	2	0	18	0 (0.0%)	18	17 (94.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.6%)
ORY	2	50	40	0 (0.0%)	40	33 (82.5%)	2 (5.0%)	3 (7.5%)	0 (0.0%)	2 (5.0%)
ORY	2	150	42	0 (0.0%)	42	15 (35.7%)	6 (14.3%)	6 (14.3%)	1 (2.4%)	14 (33.3%)
ORY	2	250	41	0 (0.0%)	41	22 (53.7%)	8 (19.5%)	9 (22.0%)	0 (0.0%)	2 (4.9%)
ORY	3	0	18	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	3	50	42	42 (100.0%)	0	- (-%)	- (-%)	- (-%)	- (-%)	- (-%)
ORY	3	150	42	0 (0.0%)	42	20 (47.6%)	4 (9.5%)	8 (19.0%)	0 (0.0%)	10 (23.8%)
ORY	3	250	42	30 (71.4%)	12	7 (58.3%)	4 (33.3%)	0 (0.0%)	0 (0.0%)	1 (8.3%)
ORY	4	0	18	0 (0.0%)	18	18 (100.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	4	50	42	42 (100.0%)	0	- (-%)	- (-%)	- (-%)	- (-%)	- (-%)
ORY	4	150	42	0 (0.0%)	42	33 (78.6%)	3 (7.1%)	2 (4.8%)	1 (2.4%)	3 (7.1%)
ORY	4	250	41	0 (0.0%)	41	20 (48.8%)	10 (24.4%)	3 (7.3%)	0 (0.0%)	8 (19.5%)
TRI	2	0	42	0 (0.0%)	36	22 (61.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (5.6%)
TRI	2	50	35	0 (0.0%)	35	22 (62.9%)	10 (28.6%)	6 (17.1%)	0 (0.0%)	4 (11.4%)
TRI	2	150	42	1 (2.4%)	42	9 (21.4%)	13 (31.0%)	11 (26.2%)	2 (4.8%)	6 (14.3%)
TRI	2	250	41	7 (17.1%)	29	8 (27.6%)	10 (34.5%)	10 (34.5%)	0 (0.0%)	6 (20.7%)
TRI	3	0	39	0 (0.0%)	39	17 (43.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	3	50	36	0 (0.0%)	36	25 (69.4%)	7 (19.4%)	8 (22.2%)	0 (0.0%)	2 (5.6%)
TRI	3	150	41	0 (60.0%)	34	8 (23.5%)	3 (8.8%)	5 (14.7%)	1 (2.9%)	25 (73.5%)
TRI	3	250	41	24 (60.0%)	29	8 (27.6%)	1 (3.4%)	7 (24.1%)	0 (0.0%)	0 (0.0%)
TRI	4	0	42	0 (0.0%)	42	15 (35.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (7.1%)
TRI	4	50	42	24 (57.1%)	30	10 (33.3%)	4 (13.3%)	4 (13.3%)	0 (0.0%)	0 (0.0%)
TRI	4	150	41	0 (0.0%)	41	9 (22.0%)	8 (19.5%)	13 (31.7%)	2 (4.9%)	6 (14.6%)
TRI	4	250	41	6 (14.6%)	35	16 (45.7%)	7 (20.0%)	3 (8.6%)	0 (0.0%)	9 (25.7%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 3: Results[z] from the shock experiment on *Escallonia rosea*(E14) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (days)	conc. (µM)	Treated nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	2	0	29	0 (0.0%)	29	25 (86.2%)	3 (10.3%)	1 (3.4%)	0 (0.0%)	0 (0.0%)
ORY	2	50	41	0 (0.0%)	41	28 (68.3%)	9 (22.0%)	4 (9.8%)	0 (0.0%)	0 (0.0%)
ORY	2	150	40	0 (0.0%)	40	24 (60.0%)	12 (30.0%)	4 (10.0%)	0 (0.0%)	0 (0.0%)
ORY	2	250	41	0 (0.0%)	41	13 (31.7%)	10 (24.4%)	16 (39.0%)	0 (0.0%)	2 (4.9%)
ORY	3	0	42	24 (57.1%)	18	2 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	16 (88.9%)
ORY	3	50	41	0 (0.0%)	41	31 (75.6%)	5 (12.2%)	4 (9.8%)	0 (0.0%)	1 (2.4%)
ORY	3	150	42	42 (100.0%)	0	- (-%)	- (-%)	- (-%)	- (-%)	- (-%)
ORY	3	250	42	0 (0.0%)	42	8 (19.0%)	2 (4.8%)	7 (16.7%)	0 (0.0%)	25 (59.5%)
ORY	4	0	42	6 (14.3%)	36	10 (27.8%)	2 (5.6%)	0 (0.0%)	0 (0.0%)	24 (66.7%)
ORY	4	50	41	18 (43.9%)	23	6 (26.1%)	2 (8.7%)	2 (8.7%)	0 (0.0%)	13 (56.5%)
ORY	4	150	42	6 (14.3%)	36	22 (61.1%)	5 (13.9%)	1 (2.8%)	0 (0.0%)	8 (22.2%)
ORY	4	250	38	12 (31.6%)	26	13 (50.0%)	1 (3.8%)	4 (15.4%)	0 (0.0%)	8 (30.8%)
TRI	2	0	42	0 (0.0%)	42	40 (95.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (4.8%)
TRI	2	50	35	0 (0.0%)	37	13 (35.1%)	12 (32.4%)	9 (24.3%)	0 (0.0%)	3 (8.1%)
TRI	2	150	42	0 (0.0%)	39	17 (43.6%)	7 (17.9%)	8 (20.5%)	0 (0.0%)	7 (17.9%)
TRI	2	250	41	6 (14.3%)	36	21 (58.3%)	3 (8.3%)	5 (13.9%)	0 (0.0%)	7 (19.4%)
TRI	3	0	39	0 (0.0%)	42	23 (54.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	19 (45.2%)
TRI	3	50	36	0 (0.0%)	41	5 (12.2%)	5 (12.2%)	3 (7.3%)	0 (0.0%)	28 (68.3%)
TRI	3	150	41	0 (0.0%)	42	4 (9.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	38 (90.5%)
TRI	3	250	41	0 (0.0%)	39	13 (33.3%)	4 (10.3%)	2 (5.1%)	0 (0.0%)	20 (51.3%)
TRI	4	0	42	0 (0.0%)	40	3 (7.5%)	1 (2.5%)	0 (0.0%)	0 (0.0%)	36 (90.0%)
TRI	4	50	42	6 (15.0%)	34	5 (14.7%)	4 (11.8%)	2 (5.9%)	1 (2.9%)	22 (64.7%)
TRI	4	150	41	16 (40.0%)	24	4 (16.7%)	3 (12.5%)	4 (16.7%)	0 (0.0%)	13 (54.2%)
TRI	4	250	41	36 (92.3%)	3	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 4: Results[z] from the shock experiment on *Escallonia rubra* (E16) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (days)	conc. (µM)	Treated nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	2	0	18	1 (5.6%)	17	17 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	2	50	30	0 (0.0%)	30	27 (90.0%)	1 (3.3%)	1 (3.3%)	0 (0.0%)	1 (3.3%)
ORY	2	150	30	0 (0.0%)	30	7 (23.3%)	10 (33.3%)	9 (30.0%)	1 (3.3%)	3 (10.0%)
ORY	2	250	30	0 (0.0%)	30	3 (10.0%)	10 (33.3%)	17 (56.7%)	0 (0.0%)	0 (0.0%)
ORY	3	0	17	0 (0.0%)	17	10 (58.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	7 (41.2%)
ORY	3	50	30	0 (0.0%)	30	16 (53.3%)	2 (6.7%)	1 (3.3%)	0 (0.0%)	11 (36.7%)
ORY	3	150	30	0 (0.0%)	30	16 (53.3%)	1 (3.3%)	10 (33.3%)	0 (0.0%)	3 (10.0%)
ORY	3	250	30	0 (0.0%)	30	7 (23.3%)	3 (10.0%)	18 (60.0%)	2 (6.7%)	0 (0.0%)
ORY	4	0	18	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	4	50	30	2 (6.7%)	28	14 (50.0%)	3 (10.7%)	2 (7.1%)	0 (0.0%)	9 (32.1%)
ORY	4	150	30	18 (60.0%)	12	9 (75.0%)	0 (0.0%)	1 (8.3%)	0 (0.0%)	2 (16.7%)
ORY	4	250	30	0 (0.0%)	30	1 (3.3%)	1 (3.3%)	2 (6.7%)	0 (0.0%)	26 (86.7%)
TRI	2	0	42	0 (0.0%)	17	17 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	2	50	35	6 (20.0%)	24	16 (66.7%)	2 (8.3%)	6 (25.0%)	0 (0.0%)	0 (0.0%)
TRI	2	150	42	0 (0.0%)	30	9 (30.0%)	12 (40.0%)	6 (20.0%)	0 (0.0%)	3 (10.0%)
TRI	2	250	41	0 (0.0%)	30	4 (13.3%)	0 (0.0%)	5 (16.7%)	0 (0.0%)	21 (70.0%)
TRI	3	0	39	12 (6.7%)	6	3 (50.0%)	0 (0.0%)	3 (50.0%)	0 (0.0%)	0 (0.0%)
TRI	3	50	36	8 (26.7%)	22	12 (54.5%)	5 (22.7%)	4 (18.2%)	1 (4.5%)	0 (0.0%)
TRI	3	150	41	24 (80.0%)	6	3 (50.0%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	0 (0.0%)
TRI	3	250	41	0 (0.0%)	30	12 (40.0%)	4 (13.3%)	4 (13.3%)	0 (0.0%)	10 (33.3%)
TRI	4	0	42	7 (41.2%)	10	6 (60.0%)	1 (10.0%)	1 (10.0%)	0 (0.0%)	2 (20.0%)
TRI	4	50	42	0 (0.0%)	30	11 (36.7%)	5 (16.7%)	14 (46.7%)	0 (0.0%)	0 (0.0%)
TRI	4	150	41	6 (24.0%)	19	9 (47.4%)	3 (15.8%)	4 (21.1%)	1 (5.3%)	2 (10.5%)
TRI	4	250	41	0 (0.0%)	29	10 (34.5%)	5 (17.2%)	8 (27.6%)	0 (0.0%)	6 (20.7%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 5: Results[z] from the continuous experiment on *Escallonia illinita* (E01) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (weeks)	conc. (µM)	# Nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	6	0	19	0 (0.0%)	19	18 (94.7%)	0 (0.0.%)	0 (0.0%)	0 (0.0%)	1 (5.3%)
ORY	6	1	42	0 (0.0%)	42	40 (95.2%)	2 (4.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	6	5	42	0 (0.0%)	42	22 (52.4%)	13 (31.0%)	3 (7.1%)	0 (0.0%)	4 (9.5%)
ORY	6	10	47	6 (12.8%)	41	19 (46.3%)	7 (17.1%)	4 (9.8%)	0 (0.0%)	5 (12.2%)
ORY	8	0	24	6 (25.0%)	18	12 (66.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	8	1	42	0 (0.0%)	42	38 (90.5%)	2 (4.8%)	1 (2.4%)	0 (0.0%)	1 (2.4%)
ORY	8	5	42	0 (0.0%)	42	24 (57.1%)	11 (26.2%)	3 (7.1%)	0 (0.0%)	4 (9.5%)
ORY	8	10	42	0 (0.0%)	42	27 (64.3%)	9 (21.4%)	2 (4.8%)	0 (0.0%)	4 (9.5%)
ORY	10	0	18	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	10	1	42	0 (0.0%)	42	40 (95.2%)	1 (2.4%)	0 (0.0%)	0 (0.0%)	1 (2.4%)
ORY	10	5	42	0 (0.0%)	42	35 (83.3%)	2 (4.8%)	1 (2.4%)	0 (0.0%)	4 (9.5%)
ORY	10	10	42	0 (0.0%)	42	23 (54.8%)	8 (19.0%)	6 (14.3%)	0 (0.0%)	5 (11.9%)
TRI	6	0	42	6 (14.3%)	36	36 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	6	1	35	0 (0.0%)	35	32 (91.4%)	1 (2.9%)	2 (5.7%)	0 (0.0%)	0 (0.0%)
TRI	6	5	42	0 (0.0%)	42	20 (47.6%)	5 (11.9%)	10 (23.8%)	0 (0.0%)	7 (16.7%)
TRI	6	10	41	12 (29.3%)	29	6 (20.7%)	4 (13.8%)	13 (44.8%)	0 (0.0%)	6 (20.7%)
TRI	8	0	39	0 (0.0%)	39	29 (74.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (25.6%)
TRI	8	1	36	0 (0.0%)	36	23 (63.9%)	4 (11.1%)	3 (8.3%)	0 (0.0%)	6 (16.7%)
TRI	8	5	41	7 (17.1%)	34	11 (32.4%)	6 (17.6%)	12 (35.3%)	0 (0.0%)	5 (14.7%)
TRI	8	10	41	12 (29.3%)	29	3 (10.3%)	3 (10.3%)	19 (65.5%)	0 (0.0%)	4 (13.8%)
TRI	10	0	42	0 (0.0%)	42	42 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	10	1	42	12 (28.6%)	30	25 (83.3%)	3 (10.0%)	2 (6.7%)	0 (0.0%)	0 (0.0%)
TRI	10	5	41	0 (0.0%)	41	7 (17.1%)	8 (19.5%)	26 (63.4%)	0 (0.0%)	0 (0.0%)
TRI	10	10	41	6 (14.6%)	35	2 (5.7%)	4 (11.4%)	13 (37.1%)	1 (2.9%)	15 (42.9%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 6: Results[z] from the continuous experiment on *Escallonia rosea*(E14) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (weeks)	conc. (µM)	Treated nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	6	0	42	0 (0.0%)	42	41 (97.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.4%)
ORY	6	1	41	6 (14.6%)	35	16 (45.7%)	6 (17.1%)	9 (25.7%)	0 (0.0%)	4 (11.4%)
ORY	6	5	42	1 (2.4%)	41	5 (12.2%)	8 (19.5%)	12 (29.3%)	1 (2.4%)	15 (36.6%)
ORY	6	10	42	12 (28.6%)	30	6 (20.0%)	3 (10.0%)	4 (13.3%)	0 (0.0%)	17 (56.7%)
ORY	8	0	42	6 (14.3%)	36	28 (77.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (22.2%)
ORY	8	1	40	0 (0.0%)	40	23 (57.5%)	4 (10.0%)	6 (15.0%)	0 (0.0%)	7 (17.5%)
ORY	8	5	42	6 (14.3%)	36	10 (27.8%)	4 (11.1%)	8 (22.2%)	0 (0.0%)	14 (38.9%)
ORY	8	10	40	0 (0.0%)	40	5 (12.5%)	3 (7.5%)	5 (12.5%)	0 (0.0%)	27 (67.5%)
ORY	10	0	42	0 (0.0%)	42	41 (97.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.4%)
ORY	10	1	42	0 (0.0%)	42	18 (42.9%)	7 (16.7%)	7 (16.7%)	0 (0.0%)	10 (23.8%)
ORY	10	5	42	6 (14.3%)	36	5 (13.9%)	10 (27.8%)	4 (11.1%)	0 (0.0%)	17 (47.2%)
ORY	10	10	41	0 (0.0%)	41	2 (4.9%)	7 (17.1%)	2 (4.9%)	0 (0.0%)	30 (73.2%)
TRI	6	0	42	1 (2.4%)	41	41 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	6	1	35	6 (16.2%)	31	9 (29.0%)	8 (25.8%)	9 (29.0%)	2 (6.5%)	3 (9.7%)
TRI	6	5	42	12 (34.3%)	23	7 (30.4%)	4 (17.4%)	10 (43.5%)	1 (4.3%)	1 (4.3%)
TRI	6	10	41	12 (36.4%)	21	5 (23.8%)	6 (28.6%)	0 (0.0%)	1 (4.8%)	9 (42.9%)
TRI	8	0	39	0 (0.0%)	39	38 (97.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.6%)
TRI	8	1	36	0 (0.0%)	34	8 (23.5%)	12 (35.3%)	13 (38.2%)	0 (0.0%)	1 (2.9%)
TRI	8	5	41	0 (.0.0%)	39	10 (25.6%)	15 (38.5%)	1 (2.6%)	0 (0.0%)	13 (33.3%)
TRI	8	10	41	0 (0.0%)	33	6 (18.2%)	9 (27.3%)	2 (6.1%)	0 (0.0%)	16 (48.5%)
TRI	10	0	42	0 (0.0%)	41	41 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	10	1	42	6 (14.3%)	36	21 (58.3%)	8 (22.2%)	3 (8.3%)	0 (0.0%)	4 (11.1%)
TRI	10	5	41	0 (0.0%)	39	16 (41.0%)	6 (15.4%)	16 (41.0%)	1 (2.6%)	0 (0.0%)
TRI	10	10	41	0 (0.0%)	28	3 (10.7%)	14 (50.0%)	6 (21.4%)	4 (14.,3%)	1 (3.6%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

Table 7: Results[z] from the continuous experiment on *Escallonia rubra* (E16) with different concentrations (conc.) and exposure times (Exp.T.) of the mitotic inhibitors (Mit. Inh.) trifluralin (TRI) and oryzalin (ORY).

Mit. inh.	Exp.T. (weeks)	conc. (µM)	Treated nodes	Contaminated nodes	Non-contaminated nodes	Diploid nodes	Mixoploid nodes	Tetraploid nodes	Octaploid nodes	Non-surviving nodes
ORY	6	0	18	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	6	1	30	0 (0.0%)	30	10 (33.3%)	2 (6.7%)	10 (33.3%)	1 (3.3%)	7 (23.3%)
ORY	6	5	30	6 (20.0%)	24	4 (16.7%)	8 (33.3%)	2 (8.3%)	1 (4.2%)	9 (37.5%)
ORY	6	10	30	12 (40.0%)	18	4 (22.2%)	7 (38.9%)	3 (16.7%)	0 (0.0%)	4 (22.2%)
ORY	8	0	16	0 (0.0%)	16	14 (87.5%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	1 (6.3%)
ORY	8	1	30	6 (20.0%)	24	13 (54.2%)	5 (20.8%)	6 (25.0%)	0 (0.0%)	0 (0.0%)
ORY	8	5	30	6 (20.0%)	24	2 (8.3%)	8 (33.3%)	4 (16.6%)	0 (0.0%)	10 (41.7%)
ORY	8	10	30	12 (40.0%)	18	6 (33.3%)	5 (27.8%)	1 (5.6%)	0 (0.0%)	6 (33.3%)
ORY	10	0	18	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ORY	10	1	30	0 (0.0%)	30	12 (40.0%)	7 (23.3%)	4 (13.3%)	0 (0.0%)	7 (23.3%)
ORY	10	5	30	0 (0.0%)	30	3 (10.0%)	13 (43.3%)	4 (13.3%)	1 (3.3%)	9 (30.0%)
ORY	10	10	30	0 (0.0%)	30	5 (16.7%)	6 (20.0%)	1 (3.3%)	0 (0.0%)	18 (60.0%)
TRI	6	0	42	0 (0.0%)	18	18 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	6	1	35	0 (0.0%)	30	3 (10.0%)	6 (20.0%)	17 (56.7%)	3 (10.0%)	1 (3.33%)
TRI	6	5	42	0 (0.0%)	30	17 (56.7%)	5 (16.7%)	8 (26.7%)	0 (0.0%)	0 (0.0%)
TRI	6	10	41	0 (0.0%)	30	4 (13.3%)	11 (36.7%)	11 (36.7%)	2 (6.7%)	2 (6.7%)
TRI	8	0	39	6 (33.3%)	12	12 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	8	1	36	0 (0.0%)	30	26 (86.7%)	3 (10.0%)	0 (0.0%)	0 (0.0%)	1 (3.3%)
TRI	8	5	41	0 (0.0%)	30	6 (20.0%)	13 (43.3%)	7 (23.3%)	1 (3.3%)	3 (10.0%)
TRI	8	10	41	18 (60.0%)	12	2 (16.7%)	5 (41.7%)	4 (33.3%)	1 (8.3%)	0 (0.0%)
TRI	10	0	42	6 (33.3%)	12	12 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TRI	10	1	42	12 (40.0%)	18	16 (88.9%)	1 (5.6%)	1 (5.6%)	0 (0.0%)	0 (0.0%)
TRI	10	5	41	6 (20.0%)	24	5 (20.8%)	3 (12.5%)	13 (54.2%)	0 (0.0%)	3 (12.5%)
TRI	10	10	41	0 (0.0%)	30	0 (0.0%)	4 (13.3%)	11 (36.7%)	4 (13.3%)	11 (36.7%)

z) The percentage of contaminated nodes is calculated on the total amount of treated nodes. All other percentages are calculated on the amount of non-contaminated nodes.

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DANKWOORD

“Life is like a box of chocolates, you never know what you’re gonna get.” Deze gevleugelde woorden van Forrest Gump uit de gelijknamige film beschrijven het leven in het algemeen, maar zijn ook specifiek van toepassing op het verloop van een doctoraatsonderzoek, zijnde de doos chocoladepralines. Ik werd op de hoogte gebracht van deze specifieke doos chocolade door prof. Dirk Reheul, en als onverbeterlijke chocoladesnoeper ben ik hem daarvoor heel dankbaar!

Het assortiment pralines was samengesteld door het ILVO en BestSelect CVBA, een samenwerkingsverband van Vlaamse siertelers, en ik ben hen zeer erkentelijk dat ze mij geschikt vonden om van die pralines te snoepen. Eerst volgde de voorbereidende literatuurstudie om mezelf bekend te maken met de verschillende plantengewassen en technieken en het opstellen van een planning, dus ik zette een theetje klaar, een muziekje op en de knop van mijn broek al open. Ik had graag alle leden van de doctoraatscommissie bedankt voor hun tijd en het delen van hun ervaring om mijn planning en werk bij te sturen waar nodig, zodat ik niet alle chocolaatjes in één keer in mijn mond probeerde te proppen en mij te verslikken. Bedankt aan Dr. Ir. Tom Eeckhaut, Dr. Ir. Johan Van Huylenbroeck, Dr. Ir. Leen Leus, Dr. Ir. Emmy Dhooghe en prof. Dr. Ir. Stefaan Werbrouck, en in het bijzonder aan mijn promotoren Dr. Ir. Katrijn Van Laere en prof. Dr. Ir. Marie-Christine Van Labeke. Hun positieve aanpak en het aanbrengen van talloze oplossingen hielpen mij door bottlenecks en uit doodlopende straatjes. Bedankt voor jullie steun en inzet in deze 4 jaar, jullie optimisme hielp mij om door te zetten en toch het volgende chocolaatje te nemen wanneer ik er eventjes mijn buik van vol had.

De examencommissie, bestaande uit prof. Dr. Ir. Kris Verheyen, prof. Dr. Ir. Stefaan Werbrouck, prof. Dr. Renate Müller, prof. Dr. Paul Goetghebeur en dr. Ir. Emmy Dhooghe, hebben deze scriptie kundig geëvalueerd, hun opmerkingen droegen bij tot de kwaliteit van dit werk. Deze master chocolatiers had ik daarom graag bedankt voor hun tijd en expertise.

Ik wil graag ook mijn collega’s bedanken, die mij met veel plezier hielpen bij het uitpakken van de chocolaatjes uit hun papiertje en het analyseren van de samenstelling. Bedankt Dr. Ir. Ellen De Keyser voor de moleculaire expertise, Dr. Ir. Jan De Riek voor het verhelderen van de verschillende statistische analyses en Dr. Ir. Peter Lootens voor de snelle cursus in fotografie en image analysis. Mijn planten waren altijd kundig verzorgd in de serre en op het veld, waarvoor dank aan Roger Dobbelaere, Frederik Delbeke, Jo Degroote en Dieter Coppens. Voor de moleculaire analyses werd ik vakkundig begeleid door Laurence Desmet, Magali Losschaert en

Veerle Buysens, en voor de ploïdiemetingen door Dr. Ir. Leen Leus en Hilde Carlier. Voor het *in vitro* gedeelte wil ik Ronald Van den Oord, Pepijn De Raeymacker en Michiel Carron bedanken, en zeker ook Kristien Janssens en Sophie Carbonelle. Zij maakten het *in vitro* werk onvergetelijk door hun gezellige babbels, luisterende oren en bekwame handen! De boog kan ook niet altijd gespannen staan, en dus had ik graag ook Johan Snoeck en Geert Lejeune bedankt voor hun luchtige babbels over onze gemeenschappelijke hobby's, honden, moto's en musiceren.

Mijn mede-doctoraatsstudenten in de bureau, die elk aan hun eigen doos chocolade bezig waren, wil ik ook heel graag bedanken. Ik zag hen worstelen met dezelfde bottlenecks en doodlopende straatjes, maar ook juichen wanneer de praline de perfecte samenstelling had. Dit samen doorleven van deze pieken en dalen maakte ons een hechte groep met veel steun aan elkaar. In het bijzonder wil ik Silvia Bruznican en Jeroen Van Der Veken bedanken, zij brachten het grootste deel van de 4 jaar met mij door, samen snoepend aan onze chocolaatjes, en stonden altijd klaar voor een lach of met een luisterend oor.

Ik wil ook graag mijn vriendengroep en familie bedanken, die altijd achter mij staan. Zij zijn mijn witte pralines met pistachevulling, mijn favoriet! Dankzij hen was er tijd voor ontspanning, musiceren, en de mogelijkheid tot uiting van frustraties, zoals de squash met mijn beste vriendin Sien. Zij zorgden ook voor de nodige relativering, want ondanks mijn verwoede pogingen zijn de meeste onder hen na 4 jaar nog steeds blijven steken op een zeer rudimentaire kennis van plantenveredeling. Zo blijf ik mooi met mijn twee voeten op de grond. Mijn zus Jannie wil ik bedanken voor het opstellen van de afkortingenlijst. Ze heeft dit zeer kundig gedaan, hoewel ze er geen idee van had hoeveel pagina's ze zou moeten doornemen! In het bijzonder wil ik ook mijn vriend Nikos bedanken, die mij helpt om problemen vanuit een ander oogpunt te bekijken, om creatieve oplossingen te bedenken, om mij op te beuren in de downs en mee te vieren met de ups. Ik wil ook mijn hond en kat bedanken, voor hun onvermoeibare aanwezigheid tijdens het schrijfproces, als respectievelijke voeten- en schootwarmer.

Na 4 jaar is de doos chocolaatjes nog lang niet leeg, en ik draag deze met pijn in het hart over aan m'n collega's van het ILVO, die door mijn resultaten gericht verder kunnen snoepen. Ik hoop dat dit dankwoordje ook een vraag kan oplossen die velen zich stelden tijdens die 4 jaar, namelijk waarom ik toch zoveel ga gaan lopen? Omdat je dan een chocolaatje meer kan snoepen!

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TRAINING AND EDUCATION

2014-2018 PhD in Applied Biological Sciences
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PROFESSIONAL EXPERIENCE

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Research project: Advanced breeding techniques in woody ornamentals (in cooperation with BestSelect CVBA)

May 2013-March 2014 Greenhouse Technician – Bayer CropScience – Astene
Organisation and execution of different breeding projects via crossing and backcrossing in European winter oil seed rape

January 2013 – April 2013: Greenhouse Worker – Bayer CropScience – Astene
Support in the execution of breeding projects with crosses or doubled haploids in European winter oil seed rape

PUBLICATIONS

Articles in journals included in Science Citation Index

- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J, VAN LABEKE MC (2017) Interspecific hybridization in *Sarcococca* by analysis of ploidy level, genome size and genetic relationships. Euphytica, 213: 149
- DENAEGHEL H, VAN LAERE K, LEUS L, LOOTENS P, VAN HUYLENBROECK J, VAN LABEKE MC (2018) The variable effect of polyploidization on the phenotype in *Escallonia*. Accepted for publication in Frontiers in Plant Science

Artikels in proceedings of scientific conferences

- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J & VAN LABEKE MC 2015. Induction of tetraploids in *Escallonia* spp. Acta Horticulturae, 1087, 453-458.

Vulgarizing articles

- Veredeling in Escallonia (2015) Sierteelt en groenvoorziening (20): 24

CONFERENCES, SYMPOSIA AND SEMINARS

International conferences and symposia

- DENAEGHEL H, VAN LAERE K, LEUS L, EECKHAUT T, VAN HUYLENBROECK J & VAN LABEKE MC. Alternative *in vitro* breeding techniques to induce compactness in woody ornamentals (poster). BPBA 8th Symposium: “New Breeding Technologies”. Brussel, België (14 November 2014).
- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J & VAN LABEKE MC. Induction of tetraploids in *Escallonia* spp. (poster) Eucarpia 25th international Symposium, section ornamentals: ‘Crossing Borders’. Melle, België (28 juni - 2 juli 2015).
- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J & VAN LABEKE MC. Polyploidization as a tool to introgress compact growth in *Escallonia.* (voordracht) VISCEA Plant Genetics & Breeding Technologies II. Wenen, Oostenrijk (1-2 februari 2016).
- DENAEGHEL H, VAN LAERE K, LEUS L, VAN HUYLENBROECK J & VAN LABEKE MC. Breeding for compactness in *Escallonia* (poster). WOTZ, 3rd international Symposium on Woody Ornamentals of the Temperate Zone. Minneapolis, Minnesota, USA. (2-5 august 2016).

Seminars

- 8-12 September 2014: 2nd PhD Summer School Plant Breeding and Sexual Reproduction. Ghent University, Faculty of Bioscience Engineering. Organized by prof. Danny Geelen.
- 8-9 November 2017: Study trip plant phenotyping to the Plant Phenotyping Centre in Jülich and Bayer Crop Science in Monheim (Duitsland Ghent University, Faculty of Bioscience Engineering. Organised by AgroFBW, prof. Marie-Christine Van Labeke
- Seminar for the tree nursery sector at PCS (Centre for ornamental plant research), Destelbergen. 4/02/2016 and 23/02/2018

STUDENT SUPERVISION

Internships

- Polyploidization of woody ornamentals (*Escallonia* sp.). Stijn Van de Sompele, Master of Science in Bioscience Engineering: Cell and gene biotechnology. Ghent University, Faculty of Bioscience Engineering. (3 – 31 July 2015)
- Induction of *rol*-genes in woody ornamentals via co-cultivation with *Rhizobium rhizogenes.* Willem Desmedt, Master of Science in Bioscience Engineering: Cell and gene biotechnology. Ghent University, Faculty of Bioscience Engineering. (17 July – 14 August 2015)
- Polyploidization and co-cultivation with *Rhizobium rhizogenes* in *Escallonia* spp. Sofie Denaeghel, Master of Science in Bioscience Engineering: Cell and gene biotechnology. Ghent University, Faculty of Bioscience Engineering. (4-29 July 2016)

Thesis students

- Ploidy breeding in *Escallonia*. Siel Desmet. Master of Science in Bioscience Engineering: agriculture and horticulture, Ghent University, Faculty of Bioscience Engineering. (2014-2015)
- Rhizogenic *Agrobacterium* strains: a potential technique to induce new traits in the woody ornamental shrub *Escallonia*. Evelyne Stevens. Master of Science in the Industrial Sciences: biochemistry, Ghent University, Faculty of Bioscience Engineering. (September 2016 - January 2018)

SCIENTIFIC SERVICES

- 15/10/2015 and 19/01/2017: workshop on polyploidization and flow cytometry for the Sietinet community.
- 16 and 30/01/2018: workshop on *rol*-gene introgression with rhizogenic *Agrobacterium* strains for the VLAIO-project ‘Compact plants without growth inhibitors’, by ILVO, UGent,AgroCampus and supported by the Flemish Government and the European Union.

RELEVANT COURSES

- Advanced Academic Skills: English Writing (3 March – 12 May 2015)
- Speed Reading. (February 17 and 24, 2016)