Factors affecting flower development and quality in *Rhododendron simsii*

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Factoren die de bloemontwikkeling en bloeikwaliteit van azalea (*Rhododendron simsii*) beïnvloeden

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Summary

As part of the genus *Rhododendron*, azalea is well known for its luxuriant flowering. Each year, 30 million plants are produced with a production value of 48.2 million euro in East Flanders (Belgium). High-quality flowering is essential and is considered to be a homogeneous bud burst in the forcing greenhouse and a continuous development to fully open flowers at indoor conditions. Growers continuously strive to improve the quality of their products and a simple question arose: "Why do azalea flowers not always open at indoor conditions?" We tried to answer this question by an in-depth physiological research of the complex flowering process.

Analysing the production methods, we see drastic changes over the last decade. The complete production process is forced to a higher productivity, but as a result it becomes more challenging to keep flowering quality up to standards. A year-round production of flowering plants is established through a shorter and different timing of production, an increased use of plant growth regulators, the use of supplemental light during forcing and a new range of cultivars. These cultivars can be divided into three groups according to their natural flowering time: early flowering, semi-early flowering and late flowering.

In a first part of this research, we focus on flower differentiation and flower bud dormancy.

First, we investigated the effects of the altered production processes on flower initiation and differentiation. As we expected large genotypic differences between cultivars of different flowering groups, the experiments are performed using early-, semi-early- and late-flowering cultivars.
We hypothesized that the increased use of the plant growth regulators (chlormequat and paclobutrazol; inhibitors of the gibberellin biosynthesis pathway) to control the vegetative growth and initiate flowering would affect flower differentiation rate. Our results show that although flower initiation is triggered by chlormequat, subsequent flower differentiation is not influenced

by the application of plant growth regulators (PGR). Nevertheless, plants treated with PGR, have an evenly distributed flower bud formation at plant level compared to control plants.

A different timing of the production cycle means that climatic conditions (temperature, light intensity, day length) are different during flower differentiation. Flower differentiation could be hastened by higher temperatures and light intensities or by shorter days. However, under the tested circumstances, no significant differences in differentiation rate are observed.

There are however, large genotypic variations in differentiation rate. Semi-early-flowering cultivars develop faster than early- and late-flowering cultivars. And even among the late-flowering cultivars, two distinct groups are characterised based on their differentiation rate.

Second, after the initiation and differentiation of the flower buds, flower bud dormancy is installed. A period at low temperatures is required to break dormancy and to continue flower development to anthesis. Up to now, there is no easy parameter to determine when dormancy is broken. Therefore, we investigated the possibility to link the end of the dormancy-period with endogenous ABA content in flower buds and/or sensitivity of the flower buds to exogenous ABA. ABA levels decrease during a cold period for semi-early- and late-flowering cultivars, while for early-flowering cultivars levels remain stable, which makes a correlation between ABA levels and dormancy duration difficult. Sensitivity of the flower buds to ABA is more correlated with flower earliness than with flower homogeneity, which makes it not possible to link this with whole-plant dormancy release. Our results of endogenous ABA content and sensitivity to ABA of azalea flower buds could thus not be linked to dormancy release in a straightforward manner. There are cultivar differences in endogenous ABA content and influences of climatic factors during flower differentiation on ABA sensitivity.

The use of paclobutrazol, which is also in inhibitor of ABA catabolism, can increase the ABA content in buds. As this higher ABA content might lead to an increase in flower bud dormancy, we tested if high concentrations of paclobutrazol affected the need for cold to break dormancy. Flower buds of a late-flowering cultivar increase their ABA content after treatment with paclobutrazol, but ABA levels are unaffected for the early-flowering cultivar. However, for both cultivars, paclobutrazol increases the need for chilling to obtain an equal quality of flowering.

It has been suggested that for azalea, flowering time was related to the amount of low temperatures (chilling units) needed to break dormancy. Our results confirm this suggestion, as early-flowering cultivars require less chilling units to flower compared to semi-early- and late-flowering cultivars. Unfulfilled chilling requirements result in late and non-homogenous flowering.

In a second part of this research, source-sink relationships and carbohydrate metabolism are investigated.

First, we characterized the photosynthetic capacities of an early-flowering and late-flowering cultivar during the forcing period, when plants are placed in heated greenhouses to force them to flowering. Whole-plant photosynthesis measurements show that the light requirements differ among cultivars. Early-flowering cultivars need more light to balance photosynthesis and respiration. This minimum light requirement is not influenced by the flower bud opening (closed to colour-showing), but increases with increasing temperatures.

As global warming leads to increased irregular and unexpected warm spells, natural chilling requirements to break dormancy are at risk. Controlled cold treatment can provide an answer to this problem, but the dark and cold conditions will have consequences on carbohydrate metabolism. Ideal dormancy-breaking temperatures do not block metabolism to the same extend as storage temperatures (2°C), therefore, we investigate the effect of a 7°C cold treatment on carbohydrate metabolism. This cold treatment increases the amount of glucose and fructose when initial leaf starch content is high. When leaf starch content is low at the start of cold treatment, plants will be heavily depleted in carbon at the end. Again, genotypic variations were present; a semi-early-flowering cultivar is able to keep its carbon reserves longer than early-flowering cultivars. Dark respiration decreases during cold treatment, but increased again afterwards and stabilised after three days. Also photosynthesis stabilised after three days in favourable conditions, making it unnecessary to include a low-light acclimation phase.

After dormancy is broken, plants are placed in a heated greenhouse with supplemental light to force the plants to flower. Anthesis requires high amounts of carbohydrates for petal growth. We investigated the effect of supplemental light on carbohydrate metabolism. We hypothesized that the low light levels during the post-production phase are insufficient to provide enough photoassimilates to ensure further development of flowers and a certain amount of carbon reserves must be present at the end of the production phase. Results show that supplemental light during forcing will provide enough photoassimilates to partially recover the lost carbohydrates and starch after cold storage. These regained carbon reserves are rapidly used in the post-production phase, indicating that a sufficient amount must be present at the end of the production. Invertase activity increases in the petals, resulting in increased levels of glucose and fructose, needed for petal expansion.

In conclusion, the reasons for the two main problems with flower quality (non-homogeneous flowering and the arrest of flower development at indoor conditions) have been identified in this research and can be prevented by correct production techniques. First, flower homogeneity is influenced by the amount of chilling units which plants receive during the dormancy period. Unfulfilled chilling requirements will lead to non-homogeneous flowering. Second, the arrest of flower opening at indoor conditions is due to insufficient leaf starch reserves. Forcing plants to flower with the appropriate amount of supplemental light will prevent this problem by building-up reserves before sale.

Samenvatting

Azalea is een belangrijke sierplant voor Oost-Vlaanderen (België). Elk jaar worden 30 miljoen planten geproduceerd met een productiewaarde van 48,2 miljoen euro. Een goede bloeikwaliteit is essentieel en betekent enerzijds een homogene opening van de bloemknoppen in de forcerie (verwarmde serre waar de planten in bloei worden getrokken) en anderzijds een verdere ontwikkeling tot open bloemen in de huiskamer. Telers proberen continu de kwaliteit van hun product te verbeteren, en de eenvoudige vraag "Waarom komen bloemen niet altijd open in de huiskamer?" werd een hot-topic. In dit onderzoek bekijken we het complexe bloeiproces om een antwoord te geven op deze vraag.

De productiemethodes worden onder de loep genomen en tonen grote wijzigingen over de jaren heen. De productiviteit verhoogt en tegelijkertijd komt de uitdaging om de bloeikwaliteit optimaal te houden. De jaarrond productie van bloeiende planten wordt mogelijk door het gebruik van een kortere en variërende productiecyclus, het gebruik van plantengroeiregulatoren, assimilatie-belichting in de forcerie en een reeks nieuwe cultivars. Deze cultivars worden ingedeeld volgens bloeitijdstip: vroeg bloeiende, middelvroeg bloeiende en laat bloeiende cultivars.

In een eerste deel van dit onderzoek, wordt gefocust op bloemknopontwikkeling en -dormantie.

Allereerst wordt de invloed van de gewijzigde productiemethodes op de bloeminitiatie en bloemdifferentiatie onderzocht. Aangezien er een variatie tussen genotypen verwacht wordt, worden de experimenten uitgevoerd met vroeg bloeiende, middelvroeg bloeiende en laat bloeiende cultivars.

We veronderstellen dat een stijging in het gebruik van plantgroeiregulatoren (chloormequat en paclobutrazol, inhibitoren van de gibberelline biosynthese) om de vegetatieve groei onder controle te houden en de generatieve groei te stimuleren, ook de snelheid van bloem-differentiatie kan wijzigen. Uit de resultaten blijkt dat hoewel bloeminitiatie bevorderd wordt

door chloormequat, de bloemdifferentiatie niet wordt beïnvloed door het gebruik van de plantgroeiregulatoren.

De productiecyclus kan op verschillende tijdstippen in het jaar starten. Dit zorgt voor verschillende klimatologische factoren (temperatuur, lichtintensiteit, daglengte) tijdens de bloemdifferentiatie die zo versnelt of vertraagd kan worden. Significante verschillen in snelheid van bloemdifferentiatie worden echter niet gevonden onder de verschillende omstandigheden in de uitgevoerde experimenten. De differentiatie snelheid wordt voornamelijk beïnvloed door het genotype. Middelvroege cultivars hebben een snellere ontwikkeling dan vroege en late cultivars. Zelfs binnen de groep van de late cultivars, kunnen nog twee groepen worden onderscheiden op basis van de snelheid in bloemdifferentiatie.

Ten tweede wordt de bloemknop dormantie bestudeerd die volgt op de bloemdifferentiatie. Een koude periode is nodig om deze bloemknop dormantie te doorbreken en anthese mogelijk te maken. Een methode om het doorbreken van de bloemknop dormantie eenduidig te bepalen is er momenteel nog niet. We onderzoeken daarom een mogelijke link tussen het einde van dormantie en de endogene ABA concentraties in de bloemknop en/of de gevoeligheid van de bloemknop voor exogeen toegediend ABA. De endogene concentraties ABA dalen gedurende de koude periode voor middelvroege en late cultivars, terwijl de concentratie stabiel blijft in bloemknoppen van vroege cultivars. Een duidelijke correlatie tussen endogene ABA concentratie en dormantie is daarom moeilijk te bepalen. De gevoeligheid van bloemknoppen op exogeen toegediend ABA blijkt meer gecorreleerd te zijn met bloeivroegheid dan met bloeihomogeniteit, waardoor het niet mogelijk is om een link te leggen met het doorbreken van dormantie op plantniveau. Onze resultaten van de endogene ABA-concentraties en gevoeligheid voor ABA van azalea bloemknoppen kunnen dus niet gelinkt worden aan het breken van de bloemknop dormantie. Er zijn zowel verschillen in endogene ABA-concentraties tussen cultivars als effecten van omgevingsparameters tijdens de bloemknopontwikkeling op de gevoeligheid aan ABA.

Het gebruik van paclobutrazol, dat ook een inhibitor van ABA katabolisme is, kan de inhoud van ABA in de knoppen verhogen. Deze hogere ABA concentraties kunnen op hun beurt de koudebehoefte om dormantie te doorbreken verhogen. Na een behandeling met paclobutrazol hebben de bloemknoppen van de late cultivar inderdaad een verhoging in endogeen ABA, maar dit is niet zo voor de vroege cultivar. Toch blijkt voor beide cultivars de koudebehoefte voor het doorbreken van dormatie groter te zijn. Er wordt ook gesuggereerd dat het bloeitijdstip van azalea gerelateerd is aan de koudebehoefte van de betreffende cultivar. Dit onderzoek bevestigt dat het aantal koude-eenheden, nodig om dormantie te doorbreken, verschilt van cultivar tot

cultivar. Vroeg bloeiende cultivars hebben minder koude nodig dan laat bloeiende cultivars. Onvoldoende koude leidt tot een late en niet-homogene bloei.

In het tweede deel van dit onderzoek, worden de source-sink relaties en het koolhydraatmetabolisme onderzocht.

We karakteriseren eerst de fotosynthese capaciteiten van een vroege en late cultivar tijdens de forceerperiode, dit is wanneer planten in een verwarmde serre in bloei worden getrokken. Fotosynthesemetingen op plantniveau tonen aan dat vroeg bloeiende cultivars meer licht nodig hebben dan late cultivars om fotosynthese en respiratie in balans te houden. Deze minimale lichtbehoefte wordt niet beïnvloedt door de opening van de bloemknopop (van kleurtonend tot kaarsvlam), maar stijgt wel met stijgende temperaturen.

De opwarming van de aarde leidt tot onregelmatige en onverwachte warme perioden, hierdoor komt de natuurlijke koude periode om dormantie te doorbreken onder druk te staan. Een gecontroleerde koudebehandeling kan hier een antwoord bieden op dit probleem, maar de donkere en koude omstandigheden zullen gevolgen hebben voor het koolhydraatmetabolisme. Ideale temperaturen om dormantie te doorbreken, blokkeren het metabolisme van de plant niet in dezelfde mate als bij bewaartemperaturen (2 °C), daarom wordt het effect van een 7 °C koudebehandeling op het koolhydraatmetabolisme onderzocht. Door deze koudebehandeling stijgt de concentratie glucose en fructose als de zetmeelgehaltes in het blad hoog genoeg zijn. Wanneer het zetmeelgehalte in de bladeren bij de start van de koudebehandeling laag zijn, wordt de koolstofvoorraad uitgeput op het einde van de koudebehandeling. Er zijn opnieuw variaties tussen de verschillende genotypes: middelvroeg bloeiende cultivars zijn beter in staat hun koolhydraatreserves te bewaren dan vroeg bloeiende cultivars. Verder daalt de donkerrespiratie tijdens de koudebheandeling, maar stijgt opnieuw nadien en stabiliseert na drie dagen. Ook de fotosynthese stabiliseert na drie dagen in gunstige omstandigheden, hierdoor is het niet nodig de planten te acclimatiseren aan de lichtomstandigheden in de forcerie.

Na het doorbreken van de dormantie, worden de planten in een verwarmde serre met extra licht geplaatst, om de planten in bloei te trekken. De anthese (openkomen van de bloemknop) vereist een hoge hoeveelheid suikers voor de groei van de kroonbladen. We onderzochten het effect van extra licht op het koolhydraatmetabolisme. Hierbij veronderstelden we dat de lage lichtniveaus tijdens de post-productie fase onvoldoende zijn om voor genoeg suikers te zorgen voor de verdere ontwikkeling van de bloemen en dat een bepaalde hoeveelheid reservekoolstof aanwezig moet zijn op het einde van de productiefase. Onze resultaten tonen aan dat de extra belichting zorgt voor voldoende aanmaak van koolhydraten en zetmeel om een deel van de koolhydraten

verloren tijdens de koudebewaring terug aan te vullen. Deze koolstof reserves (voornamelijk zetmeel in het blad) worden snel terug gebruikt in de post-productie fase, wat aangeeft dat een voldoende hoeveelheid aanwezig moet zijn aan het einde van de productie. De activiteit van invertase verhoogt in de kroonbladen, wat resulteert in een stijging van glucose en fructose, nodig voor de expansie van de kroonbladen.

Samenvattend kunnen we zeggen dat de oorzaken van de twee grote problemen rond bloeikwaliteit (niet-homogene bloei en het niet verder ontwikkelen van de bloem in de huiskamer) in dit onderzoek geïdentificeerd zijn en voorkomen kunnen worden door gebruik te maken van de juiste productiemethodes. De bloeihomogeniteit wordt beïnvloed door de koudesom die planten krijgen tijdens de dormantie. Onvoldoende koude, of koude op het verkeerde moment, leidt tot niet uniform openen van de knoppen. Het niet open komen van bloemen in de huiskamer is te wijten aan onvoldoende zetmeelreserves in de bladeren van de plant. De planten moeten in bloei getrokken worden met voldoende licht om zetmeelreserves te kunnen opbouwen en een goede bloeikwaliteit te garanderen.

Abbreviations

α_c	quantum efficiency
A	leaf area
ABA	abscisic acid
AG	AGAMOUS
AI	acid invertase
AP1	APETALA1
AP3	APETALA3
CA	flower buds in candle stage
CO	CONSTANS
CS	colour-showing buds
CU	chilling unit
CWAI	cell wall-bound acid invertase
DIF	difference between day and night temperatures
DLI	daily light integral
DPI	daily photosynthesis integral
DTT	dithiothreitol
F	forcing at natural light intensities
FA	forcing with supplemental light
FAL	forcing with supplemental light until buds show colour, followed by indoor conditions
FI	flowering index
FLC	FLOWERING LOCUS C
FLM	FLOWERING LOCUS M
FT	FLOWERING LOCUS T

FW	fresh weight
G	green buds
GA	gibberellin
HPAE-PAD	high performance anion-exchange chromatography with pulsed amperometric detection
I_c	light compensation point
LAI	leaf area index
LFY	LEAFY
MDT	mean daily temperature
NI	neutral invertase
OF	open flowers
P_N	net photosynthesis
P_{max}	maximum net photosynthesis
PAK	Project Azalea Kwaliteit
PAR	photosynthetically active radiation
PGI	protected geographical indication
PGR	plant growth regulator
PI	PISTILLATA
PVPP	polyvinylpyrrolidon
R_D	dark respiration
ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SLA	specific leaf area
SOC1	SUPPRESSOR OF CONSTANTS 1
SPS	sucrose phosphate synthase
SUS	sucrose synthase
SVP	SHORT VEGETATIVE PHASE
TFL1	TERMINAL FLOWER1

Chapter 1

General introduction

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1.1   Taxonomy, morphology and origin

The genus *Rhododendron* of the Ericaceae family includes both rhododendrons and azaleas. The genus contains evergreen and deciduous shrubs and occasionally small to large trees or epiphytes. Leaves alternate and have usually smooth edges. In most species, flower buds are terminal; in some cases there are flower buds in the upper leaf axils. Exceptionally, the apex is vegetative and all inflorescence are found in leaf axils. The calyx is 5-lobed; the corolla is composed of 5 petals and can have a funnel or campanulate form. The flower has five to ten stamens and the pollen is released from apical pores in the anthers. The pistil contains the ovary, which stands on top of the receptacle, and a terminal style with a round stigma. Fruits are capsules and seeds are numerous and very small (Hume, 1956).

Rhododendrons are used as ornamental plants in landscaping and as flowering pot plants. Taxonomists have made several classifications over the years, mainly based on morphological characteristics. The most popular classification is the one from Chamberlain et al. (1996) where the genus is divided into eight subgenera: *Azaleastrum*, *Candidastrum*, *Hymenanthes*, *Mumeazalea*, *Pentanthera*, *Rhododendron*, *Therorhodion* and *Tsutsusi*. More recently, Goetsch et al. (2005) proposed a classification in 5 subgenera (Table 1.1), based on a phylogeny study. For this purpose, a large part of the *RPB2-1* gene, encoding a major RNA polymerase II subunit, from 87 species was analysed. The identified monophyletic groups, together with morphological information resulted in a revision of the 8 subgenera from Chamberlain et al. (1996) into 5 subgenera: *Rhododendron*, *Hymenanthes*, *Azaleastrum*, *Choniastrum* and *Therorhodion*.

The evergreen garden rhododendron can be found in subgenus *Hymenanthes*, section *Pontica*. Deciduous azaleas belong to subgenus *Hymenanthes*, section *Pentanthera* and evergreen azaleas,

including the modern pot azalea, to subgenus *Azaleastrum*, section *Tsutsusi*. 'Tsutsusi' is the Japanese word for 'evergreen azalea' and this section includes 66 species.

Table 1.1 Classification of the genus *Rhododendron* in five subgenera according to Goetsch et al. (2005)

Subgenus	Section
Rhododendron	*Pogonanthum*
	Rhododendron
	Vireya
Hymenanthes	*Pontica*
	Pentanthera
Azaleastrum	*Tsutsusi*
	Sciadorhodion
Choniastrum	
Therorhodion	

The main ancestor of the modern pot azalea (in this thesis referred to as 'azalea') is accepted to be *Rhododendron simsii* Planch (Figure 1.1). Hence, pot azaleas are also known as *R. simsii* hybrids. *R. simsii* is native to East Asia, where it grows in the hilly areas of China (Chang Jiang valley, in Sichuan and Yunnan), Thailand, Laos and Myanmar at altitudes of 1,000-2,600 m and was brought to Europe early in the nineteenth century. The shrubs, from 1 to 2.5 m high, have red (varying from pink to deep-red), funnel-shaped flowers. The economically very important traits: early flowering and ability to produce sports (see 1.2) are features coming from *R. simsii*.

Figure 1.1 The first colour drawing of *Rhododendron simsii* from Curtis's Botanical Magazine (1812)

Three other species might also have contributed to the origin of the azalea: *R. indicum*, *R. mucronatum* and *R. scabrum*. *R. indicum* is native to Japan, where it grows along river benches as very compact shrubs with small, leathery leaves. The broad, funnel-shaped carmine-red flowers

bloom very late (May). *R. indicum* introduced the globular plant architecture and carmine-red flower colour. *R. scabrum* occurs on the Ryukyu Islands in the Pacific. This subtropical species is not winter-hardy, has very long, hairy leaves, is very vigorous and produces the biggest flowers of all azaleas in purple, carmine-red and red. *R. mucronatum* has not been found as wild plant in nature, but was described based on a white-flowering garden plant in China and Japan, which can produce progeny with purple flowers. The large and purple flowers in azalea are features coming from *R. mucronatum* and *R. scabrum* (Hume, 1956; Heursel, 1999).

Nowadays, the modern pot azalea comes in different plant shapes, flower colours and flower shapes. In total, over 150 cultivars are commercially available. Azalea typically has a globular plant architecture, which can range from very small (baby azalea) to large plants grown over several years. Next to this globular form, there are also speciality forms such as pyramids and single-stem topiary. Single or double flowers can range in colour from red over purple to pink and white. Further, cultivars are divided into flowering groups according to their natural flowering time: (very) early-flowering cultivars (August), semi-early-flowering cultivars (November) and late-flowering cultivars (February). The natural flowering time here, refers to the earliest time when flowering will occur in a traditional production scheme, when plants are forced to flower but without extra cultural techniques to advance or delay flowering (see 1.4). Table 1.2 lists the cultivars used in this thesis with their natural flowering time and flower characteristics.

Table 1.2 Overview of the cultivars (*R. simsii* hybrids) used in this thesis with their natural flowering time and flower characteristics (Heursel, 1999)

Flowering group	Cultivar	Flowering time	Flower characteristics
Early	'Nordlicht'	August	Double; red
	'Lunterra'	August	Double; salmon
	'Sima'	August	Double; white with pink edge
	'Ilona'	August	Double; pinks with white edge
	'Mw. Ed. Troch'	August	Double; white
	'Inka'	August	Double; pink
Semi-early	'Otto'	November	Double; red
	'M. Marie'	December	Double; pink
	'Mont Blanc'	December	Double; white
Late	'Thesla'	January	Double; salmon
	'Sachsenstern'	January	Single; white with red edge
	'Tamira'	February	Double; red
	'Mw. G. Kint'	February	Single; white or pink with white edge

1.2 Azalea breeding

Historically, German breeders were the most successful by introducing 47% of the cultivars on the Belgian market, whereas Belgian breeders only introduced 38% (Heursel, 1999). Nowadays, Germany counts only two important breeders (Rannacher and Stahnke-Dettmer). In Belgium, a few individual growers, a major company Hortinno® and ILVO (Institute for Agricultural and Fisheries Research) are active in azalea breeding. The cooperative AZANOVA is a group of 21 nurserymen that invests in the ILVO breeding program.

Breeding in azalea focuses on flower colour and shape, leaf shape, growth vigour, plant architecture, earliness of flowering, post-production quality (longevity of flowers, absence of brown bud scales), and disease and pest resistance.

The first commercial hybrid was bred in 1867, but the most important event in breeding was in 1967 with the development of the cultivar 'Helmut Vogel'. The introduction of this very early-flowering cultivar led to the almost all-year-round production (August – May) of flowering azalea. Nowadays, 'H. Vogel' and its sports are the most important cultivars in commercial production with a market share of 65% (van Trier, 2012).

Breeding methods to find novelties include the screening of sports, conventional breeding and new techniques, such as in vitro-assisted breeding. A sport has been defined as "an individual exhibiting in whole or in part a sudden spontaneous deviation beyond the normal limits of individual variation usually as a result of mutation, especially of somatic tissue" (Pratt, 1980). Flower colour sport formation is caused by a random point mutation in flower buds causing one flower on a plant to have a different colour from the rest. Flower bud sports are propagated vegetatively to form new cultivars that retain the characteristics of the new morphology (new colour), but have the same vegetative characteristics of the parent. The new cultivar is often referred to as 'a sport' from the original cultivar. 'H. Vogel' is known for its easy formation of sports and the bud sporting series consists of over 20 different cultivars with different flower colours. Of all cultivars in pot azaleas, 52% are a result of sports (Horn, 2002).

Conventional breeding is not always straight forward in azalea. Making crosses is not a simple task as flowering times differ between cultivars and flowers often appear sterile, especially the doubled-flowers which mostly lack stamens or have deformed carpels. Azalea is a cross-pollinator; self-pollination is possible but will result in inbreeding depression (lower seed yield and growth vigour). Since azalea is vegetatively propagated, the progeny of a cross between two cultivars or species can be cloned and further vegetatively propagated.

In vitro-assisted breeding has been shown to be a useful tool to produce sports and polyploid plants (Eeckhaut et al., 2009; Eeckhaut et al., 2013).

In the future, molecular-assisted breeding can become a method to select for flower colours. The first important step was made by Heursel (1999) who identified the inheritance of colour pigments. Nowadays, the responsible genes for the biosynthesis of anthocyanins have been identified and the first steps have been taken in molecular-assisted breeding and in further unravelling the complete azalea genome (De Keyser, 2010). Together with *Agrobacterium*-mediated or microprojectile bombardment transformation of azalea (Deroles et al., 2002), new flower colours might be introduced.

1.3 Economic importance of azalea for the Ghent region

The modern pot azalea is a typical product from Belgium, and more specifically from the Ghent region. The production area of azalea is located for 96.2% in East Flanders and with 318 ha accounts for 37.1% of total ornamental industry (nursery stock exclusive). With a production of 30 million azaleas each year and a production value of 48.2 million euro in 2012, almost 80% of European azaleas are produced in East Flanders. The export value is 28.5 million euro (inclusive rhododendron) and has a positive trade balance of 28.1 million euro. Plants are exported to France, Italy, The Netherlands, Germany and other European countries (EROV, 2012).

In 2010, the 'Ghent azalea' was the first ornamental plant to be recognised as a regional product and designated with a European Protected Geographical Indication (PGI). Only plants with 80% colour-showing buds, cultivated in East Flanders and that comply with strict quality standards are allowed to be sold as 'Ghent azalea'. These quality criteria are drawn up and controlled by 'Project Azalea Kwaliteit' (PAK). Nurserymen, who are PAK-members, are subject to independent monitoring and have to take part in flowering quality tests in order to control the pre-defined quality requirements. (www.gentseazalea.be)

1.4 Cultivation of azalea

Almost 100% of the azalea assortment is vegetatively propagated by taking cuttings; only older cultivars are still grafted onto a rootstock, but are commercially less interesting because of the longer production cycle.

In this paragraph, the description of a common production scheme for azalea in Belgium is given. One to five cuttings are placed immediately in the final pot with a mixture of 9:1 peat: coconut fibres (v/v) and a pH of 4.5 and are covered with plastic. The high humidity and a soil temperature

of 23-25°C will stimulate root formation without the use of rooting hormones. After 8 to 10 weeks, plastic foil is removed and plants are decapitated a first time. Depending on the desired plant diameter, plants are successively decapitated. With every decapitation the number of branches increases as the removal of the apical meristem lifts the apical dominance, enabling the outgrowth of axillary buds. The major part of the vegetative growth, after the second decapitation, takes place on outdoor container fields in summer. During the vegetative growth phase, plants are watered manually (by most growers) or automatically based on irradiation sum (10 to 20 MJ m^{-2}) with 6-8 L m^{-2} (in our experiments). Fertilization consists of 0.5 kg/WM m^3 Osmocote Exact Lo.Start (NPK 15-8-11 + 2MgO + TE, Everris) mixed with the substrate and extra fertigation (NPK 20-7-10, pH=4.5, EC=0.8-1.5 mS/cm). Further in the thesis, this is referred to as standard horticultural conditions.

To control the vegetative growth and initiate the generative phase, plants are treated with plant growth regulators (PGR) (Keever, 1990; Marosz and Matysiak, 2005; Meijón et al., 2009). The most common used PGR are chlormequat (Cycocel®) and paclobutrazol (Bonzi®). Both PGR are allowed for use on ornamentals in Belgium as one or two successive applications, to inhibit growth and initiate flower formation (www.fytoweb.be). They are applied as a foliar spray until run-off. In practice, multiple applications of PGR on a weekly basis creates the desired globular form; as smaller shoots can still continue to grow after a first application, larger shoots are immediately retarded in their growth. When flower buds are fully formed, plants are treated again with paclobutrazol to prevent the outgrowth of axillary shoots (Keever and Foster, 1991) and multiple buds can be formed at the end of each branch.

Before anthesis, flower buds require a period of cold to break flower bud dormancy. The amount of chilling units necessary to break dormancy depends on the cultivar (early vs late flowering) and the production methods (treatment with plant growth regulators; Pettersen, 1976). The optimum dormancy-breaking temperatures for azalea have been subject to some research. Temperatures from 2 to 24°C have been tested. Optimum temperature to break dormancy, proved to be around 6-8°C. Higher temperatures can be used, but extra light during the cold period should be given to ensure plant quality. Early-flowering cultivars flowered satisfactory after a cold treatment of 6 weeks at 14°C, or even 18°C (Pettersen, 1971, Bodson, 1989). However, Kromwijk (2001) showed that lower temperatures (8°C) increased flower homogeneity. These higher temperatures did not result in satisfactory flowering of late-flowering cultivars, even when prolonging the period at 12°C to 8 weeks (Pettersen, 1971). Lower temperatures (2-5°C) always resulted in a less homogenous flowering (Kromwijk, 2001). A period of cold can be replaced by a GA-treatment on

fully developed buds (Bodson, 1986; Chang and Sung, 2000; Larson and Sydnor, 1971). However, cold treatment did provide better flowering results according to Black et al. (1990).

When flower bud dormancy is broken, plants can be forced to flower. This is a critical period of only two to three weeks in a 12 to 18 month production cycle. Forcing takes place in heated greenhouses (20-21°C) with supplemental light, when natural light levels are low. Commercial forcing starts in August and continues until May.

Two examples of a production schedule for an early and late production are shown in Table 1.3. These production schedules can be adjusted, depending on the desired flowering date. Therefore, rooting date, the number and timing of decapitations and the timing of the successive PGR treatment can be adapted. The use of cold storage makes it possible to advance flowering (breaking dormancy in a controlled way) or to delay flowering (storage of plants at 2°C).

Table 1.3 Examples of an early and late production schedule; for plants grown as 4 cuttings per pot and decapitated twice

	Early production	Late production
Planting of cuttings	July	December
First decapitation	September	February
Second decapitation	January	June
First PGR treatment	May	August
Forcing	August (early-flowering cultivar)	December (early-flowering cultivar) February (late-flowering cultivar)

1.5   Flowering: a complex process

Flowering is a crucial process in the reproductive success of species. Annual plants grow, reproduce, and senesce within a growing season. In woody perennial plants, on the other hand, 3 phases in plant development can be distinguished: a juvenile phase, adult vegetative phase and adult reproductive phase. Plants need to progress from the juvenile phase to the adult phase to be able to flower. The length of the juvenile phase varies greatly among species, lasting less than a year, to 45 years or longer (Kozlowski and Pallardy, 1997; Tan and Swain, 2006). In this juvenile phase, the vegetative meristems will not react to any floral stimulus to initiate flowering.

Despite this difference, the extensive knowledge of flowering control in *Arabidopsis* can be used to help understand the flowering process in woody plants. There are several distinct stages in the flowering process: floral induction, flower initiation and flower formation and development. In most woody plants of the temperate zone, flower buds are initiated in summer or autumn, they remain dormant over winter and anthesis occurs in spring.

1.5.1 Floral induction

Figure 1.2 shows the different pathways of floral induction in *Arabidopsis*. The process of floral induction can be regulated by environmental cues (day length, temperature, abiotic stress) or by internal factors (plant development, hormones). We discuss these factors based on the knowledge of *Arabidopsis* and provide information on pathways in woody plants and if possible in *Rhododendron* and azalea.
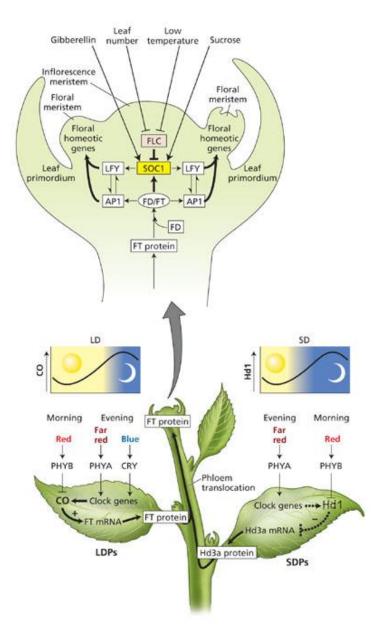
Figure 1.2 Developmental pathways for flowering in *Arabidopsis*: the photoperiodic pathway is located in the leaves and involves the expression of *CONSTANS* (*CO*) and the production of FLOWERING LOCUS T (FT) protein; the autonomous and vernalization pathway negatively regulate *FLOWERING LOCUS C* (*FLC*). The genes that initiate the developmental cascade towards flowering are *APETALA1* (*AP1*) and *LEAFY* (*LFY*). AP1 is directly induced by *FT*, and *LFY* is directly induced by *SUPPRESSOR OF CONSTANS 1 (SOC1)*. Both *FT* and *SOC1* are positively regulated by *CO* and negatively regulated by *FLC* (Taiz and Zeiger, 2010).

- Day length

Photoperiodism or the length of day and night can trigger floral induction. The perception of day length is regulated by the circadian clock and makes it possible to have a seasonal response of flowering. There are two main categories of photoperiodic responses (1) short-day plants will only flower in short days or their flowering is accelerated by short days and (2) long-day plants will only flower in long days or their flowering is accelerated by long days. Flowering in long-day plants is promoted when the day length exceeds the critical day length and the other way around for short-day plants. Species that flower under any photoperiodic condition are day-neutral plants and are insensitive to day length (Hayama et al., 2004).

The perception of the photoperiodic signal happens in the leaves, where *CONSTANS* (*CO*) integrates circadian rhythm and light signal input (Figure 1.2). The CO protein in *Arabidopsis* accumulates in long days and induces transcription of the *FLOWERING LOCUS T (FT)* gene in the companion cells of the leaf phloem. The FT protein is then exported to the phloem sieve elements from where it is transported through the phloem to the shoot apex, where FT induces the expression of *APETALA1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) to initiate the formation of flowers (Amasino and Michaels, 2010).

In woody perennials, photoperiod is more widely recognized in the control of growth promotion and cessation, than as direct flower promoting environmental key. Böhlenius et al. (2006) showed that there are parallels between the pathways controlling flowering time in *Arabidopsis* and the SD-induced growth cessation in *Populus*. In *Arabidopsis*, transcript levels of *CO* show a diurnal expression pattern, peaking close to the end of the day. Under short days, *CO* expression peaks in the dark, when the *CO* protein is unstable. Consequently, *FT* expression remains low in short days and flowering is not induced. Similarly, in *Populus*, the *CO* transcript peaks in the dark during short days (below the critical day length), and *FT* expression is not induced in the leaves and growth cessation is triggered. Overexpression of *CO* poplar homologs showed that not the amount of mRNA levels is critical, but the diurnal expression pattern of *CO* (Hsu, 2012).

Even though the similar *CO/FT* mechanism plays a role, the down-stream regulation is different. In *Arabidopsis,* FT is transferred to the apex where it induces flowering; in *Populus*, the *FT* expression in the leaves results in growth.

The parallels with floral induction are less obvious. Böhlenius et al. (2006) stated that the *CO/FT* regulatory module might also play a role in photoperiodic induction of flowering. As there was a gradual increase in *FT* expression as the tree grew older, they suggested that a critical level of *FT* expression is needed to initiate flowering. It was argued that each annual cycle of growth and

dormancy will lead to a gradual release of the chromatin structure-based *FT* repression, making *FT* more and more susceptible to transcriptional activation by *CO*.

Further research by Hsu et al. (2011) showed that two paralogs, *FT1* and *FT2*, homologous to the *Arabidopsis FT* gene, might differ in their regulation and function. Whereas *FT2* is expressed in the leaves during the vegetative growth in response to warm temperatures and long days; *FT1* determines the onset of the reproductive phase and is expressed in preformed leaves enclosed in vegetative buds in response to winter temperatures.

A homologous *FT* gene has also been identified during floral initiation in *Rhododendron* (Cheon et al., 2013).

Even though the molecular basis of photoperiodic control in floral induction in woody plants remains to be investigated, *Rhododendron* and azalea show several photoperiodic responses regarding to floral induction. *Rhododendron* has been reported to be a short-day plant (Criley, 1969), but also long-day varieties exist (Sharp et al., 2010). Also in azalea there are cultivar differences in photoperiodic floral induction (Table 1.4). The effect of day length on floral induction was also dependent on temperature; at a temperature of 15°C plants were day neutral, while at temperatures of 20°C plants acted as short-day plants (Pettersen, 1972; Bodson, 1983). Recently, Meijón et al. (2011b) showed that the change of long-day to short-day photoperiod can be responsible for floral induction in azalea.

Table 1.4 Different azalea cultivars (*R. simsii* hybrids) show different floral induction responses to photoperiodic control

Cultivar	Origin	Photoperiodic control	References
'Red Wing'	American	facultative short day	Pettersen and Kristoffersen (1969)
'Reinhold Ambrosius'	European	facultative long day	Pettersen and Kristoffersen (1969), Bodson (1983)
'Ambrosiana'	European	facultative short day	Pettersen (1972), Bodson (1989)
'Whitewater'	American	short day	Pettersen (1972)
'Skylark'	American	short day	Pettersen (1972)
'Kingfisher'	American	short day	Pettersen (1972)
'Knut Erwén'	European	facultative intermediate	Bodson (1983)
'Hexe'	European	short day	Criley (1969)
'H. Vogel'	European	day neutral	Bodson (1989)
'Blaauw's Pink'	European	short day	Meijón et al. (2011b)

- Cold temperatures

Cold temperatures are in some cases required for floral initiation. In the vernalization pathway of *Arabidopsis,* cold temperatures repress the expression of *FLOWERING LOCUS C* (*FLC*), a gene that acts as a repressor of flowering. Cold temperatures result in epigenetical changes which involves chromatin remodelling. The chromatin of *FLC* is converted from euchromatin to heterochromatin and effectively silences the gene, enabling flowering (Amasino and Michaels, 2010).

Low temperatures have also been shown to play a role in floral induction in subtropical and tropical trees; however, the response varies greatly between species (Kozlowski and Pallardy, 1997). The major difference between vernalization in *Arabidopsis* and low temperature induction of flowering in woody plants is the required temperature. Whereas vernalization requires temperatures between -1°C and 10°C; subtropical and tropical trees often require temperatures around 15-20°C. It is not clear if temperature provides an inductive stimulus in temperate deciduous trees, however, temperature can affect the intensity of floral initiation (Wilkie et al., 2008). The molecular background on cold induced flowering in woody plants is not clear yet (Tan and Swain, 2006), however, Meijón (2011b) showed changes in DNA methylation at the onset of flower initiation after cold treatment in azalea.

- Ambient temperature

The flowering response to ambient temperatures during the vegetative growth varies among species. Higher temperatures (25 or 27°C instead of 23°C) will promote flowering in *Arabidopsis*. It was suggested that *FLOWERING LOCUS M* (*FLM*) participates in the temperature pathway. And another major regulator of flowering in response to ambient temperatures is *SHORT VEGETATIVE PHASE* (*SVP*), which acts as a floral repressor downstream of the autonomous pathway and has a *FLC*-like manner in regulating *FT* and *SOC1* (Srikanth and Schmid, 2011).

- Abiotic stress

Abiotic stress (drought, flooding, salt, nutrients, heat …) can enhance flower initiation as a survival mechanism. It is shown in *Arabidopsis* that there is a crucial relationship between the epigenetic modifications associated with floral initiation and development and the modifications associated with stress tolerance (Yaish et al., 2011). Stress situation, such as water or nutrient deficiency and heat, can also trigger flowering in woody perennials. It has been demonstrated for *Rhododendron* that water deficit promotes flowering (Sharp et al., 2009).

- Developmental stage of the plant

The developmental stage of plants or the number of leaves can control flowering in *Arabidopsis*. This can happen in two ways. (1) Plants must reach a minimum size before they become sensitive to other floral induction factors, e.g. low temperatures for vernalization. (2) Leaf number can trigger an autonomous pathway which in itself represses *FLC*. This autonomous pathway is also sugar-mediated. Sucrose produced by photosynthesis moves from the leaves to the shoot apex, where it promotes floral induction (Corbesier et al., 1998).

Also in woody plants carbohydrates can play a role in flower initiation, as high sucrose concentrations at the apex inducing flowering (Wilkie et al., 2008).

- Gibberellin

Gibberellins (GA) can have a strong influence on flowering. In *Arabidopsis,* gibberellins will enhance flowering as GAs stimulate the expression of *FT*, *LEAFY* (*LFY*) and *SOC1* (Srikanth and Schmid, 2011). However, in many angiosperm woody species, GAs inhibit flowering and a GA-biosynthesis inhibitor stimulates flowering by a decrease in GA levels (Kozlowski and Pallardy, 1997; Wilkie et al., 2008). In rose, it was shown that exogenous applied GA_3 resulted in the accumulation of *RoKSN* (Randoux et al., 2012), a homologue of *TFL1* (*TERMINAL FLOWER1*) which acts opposite to *FT* (Hanano and Goto, 2011). This prevents flower initiation, which was also seen in *Citrus* where the expression of *FT* decreases after a GA_3 treatment (Goldberg-Moeller et al., 2013).

1.5.2   *Flower initiation and flower development*

Once floral induction took place, flower initiation and further differentiation will take place. There are two major groups of genes that regulate floral development: meristem identity genes which encode transcription factors that are necessary for the initial induction of the second group of genes, the organ identity genes. These floral organ identity genes directly control floral identity (Irish, 2010).

- Floral meristem identity genes

In *Arabidopsis,* the critical meristem identity genes that must be activated are: *SOC1*, *AP1* and *LFY*. *SOC1* and *LFY* play a central role in flower initiation by integrating the signals from different pathways of environmental and endogenous regulators of floral induction (Figure 1.2). If *SOC1* is activated, it turns on the expression of *LFY*, which triggers the expression of *AP1*. *AP1* then stimulates the expression of *LFY*, which creates a positive feedback loop. In *Rhododendron,* homologues of *LFY* and *SOC1* have been isolated (Cheon et al., 2012).

- Floral organ identity genes

The floral organs arise from proliferation of the floral meristem. The genetic pathways controlling the specification of different organ identities are well studied. The floral organ identity genes fall into three classes: types A, B and C and the control of organ identity relies on these three types of genes and is referred to as the ABC model (Figure 1.3). Type A activity, encoded by *AP1* and *AP2*, controls the organ identity in the first and second whorls. Type B genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) control the second and third whorls. Type C gene *AGAMOUS* (*AG*) controls the third and fourth whorls. The activity of type A genes alone specifies the sepals. For petal formation, both A and B genes are required. Type B and C genes form stamens and the activity of C alone defines carpels. Of these floral organ identity genes, only a homologue to *AP3* has been isolated in *Rhododendron* (Cheon et al., 2011).

Loss of function of one of the ABC-genes will lead to flower malformation. Woody plants have a variety of floral modifications. In *Rhododendron,* sepals, stamens and carpels can become petaloïd, which results in beautiful doubled-flowers but leaving them infertile.
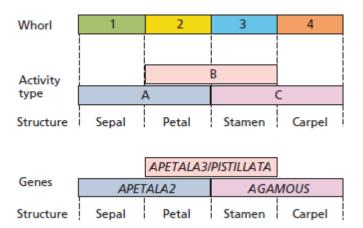
Figure 1.3 ABC model in *Arabidopsis* to determine the floral organ identity based on the interactions of type A, B and C genes (Taiz and Zeiger, 2010)

After the activation of the floral organ identity genes, the formation of floral parts occurs sequentially and can be followed microscopically in azalea. Bodson (1983) defined 9 stages in the development of the flower bud (Figure 1.4). Stage 0: vegetative bud; stage 1: initiation of bud scales, stage 2: initiation of flower primordia, stage 3: sepal initiation, stage 4: petal initiation, stage 5: stamen initiation, stage 6: carpel initiation, stage 7: initiation of style elongation, stage 8: the ovary contains ovules. The initiation phase comprises stages 1-2. Differentiation starts from stage 3.
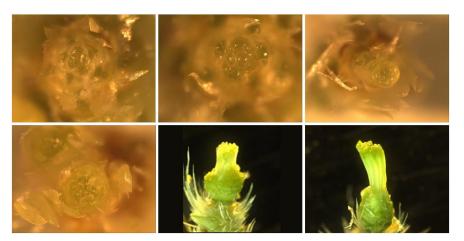
Figure 1.4 Flower development of azalea. Top: stage 1, 2 and 4; bottom: stage 5, 7 and 8

1.5.3   Dormancy

Before further development of the flower buds to anthesis, there is a period of rest, i.e. dormancy. Dormancy is a common mechanism in woody plants of the temperate zone to prevent buds from resuming growth in unfavourable conditions. To break dormancy and to be able to continue further development, buds require a prolonged exposure to low temperatures. This chilling requirement is different among species and among cultivars (Cooke et al., 2012).

Three types of dormancy have been defined by Lang (1987) depending on which factors play a role. During ecodormancy, environmental factors prevent growth, while for paradormancy and endodormancy the growth inhibition arises from the plant itself. Paradormancy refers to factors from other plant parts inhibiting growth (e.g. apical dominance); endodormancy refers to factors inside the dormant structure itself which prevent initiation of growth from meristems (with the capacity to resume growth), under favourable environmental conditions (Rohde and Bhalerao, 2007). The regulation and mechanisms of dormancy are complex and still not fully understood. The induction of endodormancy can be regulated by the environmental factors shortening day length and lower temperatures. This makes it difficult to distinguish physiological and molecular changes associated with dormancy regulation from those of cold acclimation. Bud dormancy has been extensively studied and a number of reviews are present dealing with physiological and molecular aspects of bud dormancy in trees (Arora et al., 2003; Horvath et al., 2003; Chao et al., 2007; Rohde and Bhalerao, 2007; Ruttink et al., 2007). Research on flower bud dormancy focused mainly on temperate fruit trees and it is believed that similar processes as for vegetative buds play a role. However, the depth of dormancy can be different between vegetative and generative buds (Horvath, 2009). Flower buds seem to be less dormant than vegetative buds, but still require an equal amount of chilling units (Gariglio et al., 2006). Research on *Rhododendron* mainly

focusses on cold acclimation and cold hardiness of flower buds, but not on the process of flower bud dormancy (Arora and Rowland, 2011).

1.5.4 Anthesis

The last step in the flowering process is anthesis, i.e. the opening of the flower. The opening of flowers is characterised by 2 steps: petal growth by cell division, and unfolding of the petals by cell expansion. In these processes sugar supply is essential as flower development is associated with increased soluble carbohydrates in the petal cells. These carbohydrates provide energy for growth respiration and also lower the osmotic potential which promotes the influx of water (Reid, 2003). The research on flower opening has mainly been done on ornamental plants such as roses (Kumar et al., 2007), lily (van Doorn and Han, 2011), tulip and *Alstroemeria* (Collier 1997)*;* and focussed mainly on source-sink relations and sucrose metabolism, as they prove to be essential for petal growth and expansion (Kumar et al., 2008b). For cut flowers, post-harvest quality has been thoroughly investigated with attention to sucrose metabolism, and ethylene induced senescence (van Doorn and Han, 2011).

During flowering of azalea, different stages in flower opening can be distinguished which are used to determine the quality of flowering. Buds develop from a closed (green) bud, to a colour-showing bud (CS), candle stage bud (CA) and ultimately an open flower (OF) (Figure 1.5). The commercial forcing period ends when plants have buds in the colour-showing stage or in the candle stage.

Figure 1.5 Four stages during flowering, from left to right: green bud (G), colour-showing bud (CS), candle-stage bud (CA), open flower (OF)

1.6 Research objectives and thesis outline

This research addresses a central question of azalea growers: "Why do azalea flowers not always open at indoor conditions?" This question could be dissected in 2 sub-questions: (1) what is the basis of the non-uniform opening of buds in the forcing greenhouse and (2) why is flower development held back at the candle stage at indoor conditions? Simple questions, however, finding the answers was far more complex.

Indeed, over the last decade production methods have evolved drastically. A year-round production of azalea (from August till May) is established with a shorter production time (more cuttings per pot and a reduced number of decapitations), an increased frequency of PGR applications and the use of supplemental light during forcing, in short the complete production process has been forced to a higher productivity, but with consequences for flowering quality.

To advance the selling period, production schedules of early-flowering cultivars were altered, to obtain flowering plants in August and September. However, this means a production under different climatic conditions, especially day length and temperature could have negative effects on flower bud differentiation and flower bud dormancy induction and release. Additionally, a range of new cultivars was introduced since the research on Belgian pot azalea by Bodson (1989). Further, analysing the applied cultural techniques, it became obvious that the number of PGR applications increased since 1994, though from 2007 on, the number of applications stabilized around 6 (Figure 1.6A). However, there is still a steady increase to combine different products during one treatment (Figure 1.6B). As a result, the use of the more persistent PGR, paclobutrazol, has also increased over the years. This might have its effect on flower bud differentiation, flower bud dormancy and anthesis.

The main objective of this thesis was therefore to understand the different factors that influence the quality of flowering in azalea through a step by step in depth approach of the physiological processes that play a role from flower differentiation, up to flowering.
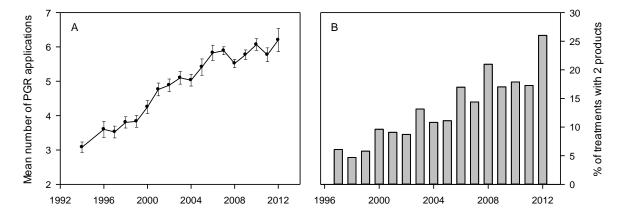
Figure 1.6 (A) Increased use of plant growth regulators over the years; (B) Percentage of treatments with a combination of 2 plant growth regulators (data PAK)

Since this research started based on questions of azalea growers, the experiments were performed on a semi-practical scale under greenhouse conditions and on outdoor container fields where the climate is less controlled compared to growth chambers. This approach ensures a rapid implementation of the results by the growers.

Unravelling the processes involved in flowering starts in **chapter 2** by examining flower bud initiation and differentiation. The effect of increased doses of plant growth regulators on flower differentiation is tested for 2 cultivars belonging to a different flowering group. It is known that the use of PGR hasten flower initiation (Meilan, 1997), however, information on flower differentiation is much less clear. It is hypothesized, that by using higher amounts of growth regulators the differentiation rate of the flower bud could be hastened. Further, flower differentiation is compared among 13 genotypes of different natural flowering periods. As all plants were treated with PGR to start flower initiation at the same moment we can look at the intrinsic developmental rate of the different genotypes. We investigate if the hypothesis of Bodson (1983) that earliness of flowering could be partly related to flower differentiation rate is valid for these 13 commercial cultivars. Last, we investigate the seasonal effects on flower differentiation for an early-flowering cultivar. The early-flowering cultivars have a variable production schedule, depending on the planned flowering period (August – December). Both light and temperature might affect the rate of flower bud development.

Chapter 3 addresses the period of flower bud dormancy. Since natural flowering times between cultivars differ a lot, it is assumed that their cold requirements are different (Andreini et al., 2014): late-flowering cultivars requiring a higher amount of chilling units to break dormancy compared to early-flowering cultivars. Up to now, there is still no easy parameter to determine when dormancy is broken. As abscisic acid (ABA) might play a role in dormancy induction and maintenance, we looked at the possibility if endogenous concentration in the flower bud, or the sensitivity to exogenous ABA in a bio-assay, could be used as a marker for dormancy release. Paclobutrazol, being one of the frequent used PGR, is an inhibitor of ABA catabolism (Saito et al., 2006) and increases the ABA content in buds which can increase the dormancy period and need for cold to break dormancy. The effect of a high dose of this PGR, also applied at a late stage in flower bud development (near the onset of dormancy), is evaluated. Endogenous ABA content, sensitivity to exogenous ABA and chilling requirements are measured. Subsequently, the effect of an early and late production cycle of an early-flowering cultivar on dormancy is discussed. Flower bud development under different day lengths, might differently affect dormancy induction. Further, to optimise the artificial cold treatment (7°C) to break dormancy, it was tested in which flower bud stage plants are most responsive to cold treatment. This was tested on the same 13 cultivars used in **chapter 2**.

The last three experimental chapters focus on carbohydrate metabolism. Anthesis requires high amounts of carbohydrates for petal growth, hence source-sink metabolism will play a crucial role in high quality flowering. First, we determined the minimum light requirements of an early- and

late-flowering cultivar in **chapter 4**, by measuring photosynthesis at a whole plant level on plants where flower bud dormancy is released. Before entering the forcing greenhouse, source-sink balance alters (**chapter 5**). First, an alteration in the physiological state of the plant takes place. Dormant flower buds have a low sink capacity which demands a low input of carbohydrates; once dormancy is broken, flower buds become highly active sinks requiring high amounts of sugar for their development. Second, during artificial cold treatment, the level of carbohydrates will decrease, to what extent this decrease occurs, is followed for three cultivars with different flowering times. Next, the transfer of plants from cold, dark conditions, to warm and supplemental light conditions might require an adaption phase for photosynthesis, or might even induce photoinhibition. Therefore, leaf photosynthesis measurements are done during the first days, immediately after cold storage. Finally, in **chapter 6** we look more into detail at the sucrose metabolism during flowering. At indoor conditions, light levels are low and will not provide enough photoassimilates for flower opening; on the contrary, respiration might lead to a decrease in available carbohydrates in the plant. To ensure good flowering, a certain amount of reserve carbohydrates have to be present when plants are sold. We examine how we can manipulate these reserves prior and during the forcing period. It is investigated to what extend supplemental light can compensate for a loss in carbohydrates during a previous dark period (**chapter 5**). Investigating the sucrose metabolism in the petals, must provide us information on the sink strength during different light conditions.

General findings and future perspectives are concluded in **chapter 7**.

CHAPTER 2

Flower differentiation of azalea depends on genotype and not on

the use of growth regulators.

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Chapter 2

Flower differentiation of azalea depends on genotype and not on the use of growth regulators

Flowering is a complex process which starts with the induction and development of the flower buds. For azalea (*Rhododendron simsii* hybrids), flower induction was hastened by the application of chlormequat and took place within 11 days after treatment. Subsequent flower bud differentiation was not altered by the application rate of the plant growth regulators (PGR) chlormequat and paclobutrazol, nor by temperature or light sum. There were, however, large genotypic variations in flower bud differentiation rate. For all cultivars, a linear phase until flower primordia were fully differentiated and the style started to enlarge (flower bud stage 7), was followed by a slower final development (to stage 8). The linear phase was fastest for the semi-early-flowering cultivars ('Mont Blanc', 'M. Marie' and 'Otto'), requiring only 46 to 48 days reaching flower bud stage 7 after the first PGR treatment. Two late-flowering cultivars ('Thesla' and 'Sachsenstern'), had the slowest differentiation, requiring 64 days to reach stage 7. The early-flowering cultivars ('H. Vogel' sports) and two late-flowering cultivars ('Mw. G. Kint' and 'Tamira') required 54 and 52 days, respectively, after the first PGR treatment until stage 7. To reach flower bud stage 8, a similar trend in velocity was seen, the semi-early-flowering cultivars requiring the least amount of days (17 to 18 days), the late-flowering cultivars 'Thesla' and 'Sachsenstern' requiring the highest amount of days (24) and the early-flowering cultivars and the late-flowering cultivars 'Mw. G. Kint' and 'Tamira' requiring an intermediate number of days (20 to 22 days).

2.1 Introduction

The genus *Rhododendron* of the Ericaceae family includes both rhododendrons and azaleas. The evergreen garden rhododendron can be found in subgenus *Hymenanthes*, section *Pontica*.

Deciduous azaleas belong to subgenus *Hymenanthes*, section *Pentanthera* and evergreen azaleas, including the modern florist azalea (*Rhododendron simsii* hybrids), to subgenus *Azaleastrum*, section *Tsutsusi* (Goetsch et al., 2005).

Rhododendrons are characterized by a rhythmic vegetative growth. This periodic growth is controlled by day length and influenced by temperature and light intensity (Väinölä and Junttila, 1998). Flower initiation of *Rhododendron* 'Roseum Elegans' occurs under natural long days, when the vegetative growth flush is at 1/3 of its total length (Adams and Roberts, 1968). To promote flower bud set and enhance flowering, plants can be treated with plant growth regulators (Ranney et al., 1994; Gent, 1995). After flower bud differentiation, a period of flower bud dormancy precedes anthesis. The whole period, from initiation to anthesis, lasts almost one year.

Florist azaleas on the other hand, exhibit a continuous vegetative growth which allows a year-round production. Over 150 commercial cultivars are available and are divided into groups according to their natural flowering period: early- (August), semi-early- (November) and late- (February) flowering cultivars. Flower initiation depends on environmental factors such as day length, irradiance and temperature (Criley 1969; Larson and Biamonte, 1972; Pettersen, 1972; Pettersen, 1973; Bodson, 1983), but also the genetic background of the cultivars will exert an effect (Bodson, 1983). To assure a year-round production, plant growth regulators are used to start flower initiation independent of these factors (Bodson, 1989; Marosz and Matysiak, 2005; Meijón et al., 2009; Meijón et al., 2011a). The commonly used plant growth regulators chlormequat and paclobutrazol block the gibberellin biosynthesis pathway (Rademacher, 2000). Chlormequat interferes in the early steps of GA (gibberellin) biosynthesis by blocking the cyclases copalyl-diphosphate synthase and *ent*-kaurene synthase. Paclobutrazol inhibits the oxidation of *ent*-kaurene into *ent*-kaurenoic acid by blocking cytochrome P450-dependent monooxygenases. The result of both growth regulators is a decrease in endogenous gibberellins which results in flower initiation. Next, flower differentiation is influenced by the same factors as initiation (Bodson, 1989). However, since the research of Bodson (1989), breeding efforts resulted in an important change of cultivars, although the earlier described flowering groups remain valid. Also, current production schedules apply higher rates of plant growth regulators to strictly control vegetative growth, though effects on generative development are not documented. This paper studies if increased doses of plant growth regulators might influence the developmental flower pattern for 2 cultivars belonging to a different flowering group. Further, flower differentiation is compared among 13 *R. simsii* hybrid genotypes and for 1 cultivar seasonal effects are also documented. This fundamental information can be used to schedule production.

2.2   Materials and methods

2.2.1   Plant material

Azalea cuttings were rooted in a mixture of 9:1 peat:coconut fibres (v/v) in 12 cm pots (4 cuttings/pot). Plants were grown under standard horticultural conditions and were decapitated twice to stimulate branching. To initiate the generative phase and to suppress the outgrowth of axillary buds, plants for experiment 2 and 3 were treated six times with 2.25 g L^{-1} chlormequat and two times with 0.012 g L^{-1} paclobutrazol (foliar spray until run-off).

2.2.2   Experiment 1 Effect of PGR application

The first experiment is performed with 2 cultivars: 'Nordlicht' (sport of 'H. Vogel'), characterised by a natural early-flowering period and 'Mw. G. Kint', characterised by a natural late-flowering period. Cuttings were rooted in July 2008 and in December 2008 for 'Nordlicht' and in July 2008 for 'Mw. G. Kint'. 'Nordlicht' plants potted in July 2008 were decapitated for a second time on 23 January 2009 and remained in the greenhouse (early production), while those potted in December 2008 were transferred outside after the second decapitation on 25 May 2009 (late production). For 'Mw. G. Kint' the second decapitation was on 11 June 2009 after which plants were transferred outside.

Three different treatments with plant growth regulators were applied: a control treatment without PGR, a standard and a high dose PGR treatment. For the standard treatment, plants were sprayed weekly, 6 times with 2.25 g L^{-1} chlormequat of which the last two treatments were combined with 0.012 g L^{-1} paclobutrazol. The high dose PGR treatment consisted also of 6 applications with 2.25 g L^{-1} chlormequat, but the last three applications were combined with 0.04 g L^{-1} paclobutrazol. Treatment started on 4 May 2009, 5 August 2009 and 18 August 2009 for the early and late production cycle of 'Nordlicht' and 'Mw. G. Kint', respectively.

2.2.3   Experiment 2 Seasonal variations

Flower initiation and differentiation of 'Nordlicht' was followed for 3 production cycles: the spring (early production) of 2009 and the summer/autumn (late production) of 2009 and 2010. Cuttings were rooted in July 2008, December 2008 and December 2009. Plants potted in July 2008 were decapitated for a second time on 23 January 2009 and remained in the greenhouse. Plants potted in December 2008 and 2009 were transferred outside after the second decapitation on 25 May 2009 and 3 June 2010, respectively. Weekly treatment with plant growth regulators started on 4 May 2009, 4 August 2009 and 3 August 2010 for plants potted in July 2008, December 2008 and 2009, respectively.

2.2.4   Experiment 3 Genotypic variation in flower development

Cultivars belonging to different groups according to their natural flowering time were used; six sports of the early-flowering cultivar 'H. Vogel': 'Nordlicht', 'Lunterra', 'Sima', 'Ilona', 'Mw. Ed. Troch' and 'Inka'; 'M. Marie', 'Otto' and 'Mont Blanc' as semi-early-flowering cultivars and four late-flowering cultivars, 'Mw. G. Kint', 'Tamira', 'Thesla' and 'Sachsenstern'. Cuttings of all cultivars were rooted at the end of 2009. After the second decapitation (3 June 2010) plants were transferred from the greenhouse to an outside container field. Weekly treatment with plant growth regulators started on 3 August 2010.

2.2.5   Assessment of flower bud development

Three to five plants per cultivar were randomly selected once or twice a week. Flower bud differentiation was observed on three buds per plant (OLYMPUS SZX9 stereo microscope). Since each bud contained several primordia, a mean stage was determined per bud, based on the floral scale of Bodson (1983) which comprises nine stages; stage 0: vegetative bud, stage 1: initiation of bud scales, stage 2: initiation of flower primordia, stage 3: sepal initiation, stage 4: petal initiation, stage 5: stamen initiation, stage 6: carpel initiation, stage 7: initiation of style elongation, stage 8: the ovary contains ovules. The initiation phase comprises stages 1-2. Differentiation starts from stage 3. The rate of differentiation ρ was calculated as the reciprocal of days.

2.2.6   Climatic registration

Temperature and irradiation were measured by the weather station on the greenhouses of the PCS Ornamental Plant Research (51.058°N, 3.88°E), and 20 minute averages were recorded. A mean daily temperature, a mean daily light integral (DLI) and total irradiation sum were calculated and used to evaluate results.

2.2.7   Statistical analysis

Regression analysis of flower development was done in SigmaPlot Version 11.0 (Systat Software Inc., San Jose, CA, USA). Linear regression was used to describe flower bud development till stage 7 for comparison of PGR treatments and seasonal effects. The start of flower initiation (stage 1) was calculated based on the obtained regressions. Flower bud development till stage 8 was described by a sigmoid logistic regression for genotypic comparison. A reduced model, invariant of cultivars, was fitted through the data and the change in residual sums of squares was used to compute the F-statistic to find significant differences between cultivars of a same flowering time.

2.3 Results

2.3.1 Effect of PGR on flower development

Flower differentiation was followed for 'Nordlicht' and 'Mw. G. Kint' (Figure 2.1). Differentiation progressed linear and was not significantly different between control plants and PGR-treated plants for either of the cultivars (Table 2.1; early production of 'Nordlicht' p=0.53; late production of 'Nordlicht' p=0.97; 'Mw. G. Kint' p=0.18). In PGR-treated plants, initiation was enhanced by 11 days during the early production cycle of 'Nordlicht', but was unaffected for 'Mw. G. Kint'. As flower initiation during the late production cycle of 'Nordlicht' started already before the first PGR application, no effect on initiation could be measured.

Table 2.1 Regression parameters (a, b) and coefficient of determination (R^2) describing the linear flower bud differentiation in function of days after the first PGR application (flower bud stage = a + b x days after first PGR treatment). The regression was calculated for the early-flowering cultivar 'Nordlicht' in an early and late production cycle and the late-flowering cultivar 'Mw. G. Kint'.

Cultivar	PGR application[y]	a ± SE	b[z] ± SE	R^2
'Nordlicht'	Control	-1.737 ± 0.371	0.125 ± 0.008	0.875
early production	Standard	-0.419 ± 0.133	0.132 ± 0.004	0.970
	High	-0.527 ± 0.140	0.133 ± 0.004	0.968
'Nordlicht'	Control	1.734 ± 0.375	0.136 ± 0.013	0.848
late production	Standard	2.205 ± 0.313	0.137 ± 0.011	0.890
	High	2.169 ± 0.324	0.133 ± 0.011	0.877
'Mw. G. Kint'	Control	-0.312 ± 0.208	0.137 ± 0.006	0.946
	Standard	-0.605 ± 0.194	0.130 ± 0.005	0.948
	High	-0.290 ± 0.194	0.144 ± 0.005	0.957

[y] Control plants without PGR; standard PGR application: 6 x chlormequat (2.25 g L^{-1}) + 2 x 0.012 g L^{-1} paclobutrazol; high PGR application: 6 x chlormequat (2.25 g L^{-1}) + 3 x 0.04 g L^{-1} paclobutrazol

[z] slopes are not significantly different between different PGR applications (p=0.05, comparison of linear regressions)
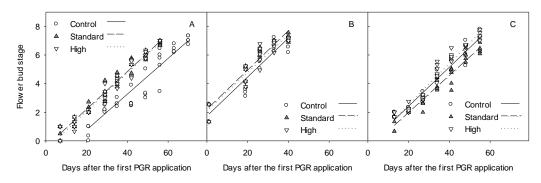
Figure 2.1 Flower bud differentiation in function of days after the first PGR application for the early-flowering cultivar 'Nordlicht' in early (A) and late (B) production and the late-flowering cultivar 'Mw. G. Kint' (C). Control plants without PGR; standard PGR application: 6 x chlormequat (2.25 g L^{-1}) + 2 x 0.012 g L^{-1} paclobutrazol; high PGR application: 6 x chlormequat (2.25 g L^{-1}) + 3 x 0.04 g L^{-1} paclobutrazol

2.3.2   Seasonal effects

As mentioned above, 'Nordlicht' was initiated (stage 1) 11 days after the first PGR application in the spring of 2009, and already initiated (stage 2) at the start of PGR application in the late production of 2009. In contrast, initiation for the late production of 2010 took place 10 days (calculated after extrapolation of the linear regression, Figure 2.2) after the first PGR application. For all three production cycles, flower differentiation progressed linear to stage 7 (Figure 2.2A) in function of the number of days after the first PGR application. The differentiation rates did not differ significantly between the three production cycles (linear regression analysis, p=0.64) despite the different climatic conditions (Table 2.2). The early and late production cycle in 2009 received an almost equal average temperature of 18.2 and 17.9 °C, between stage 3 and stage 7, but the irradiation sum for the late production cycle (343 MJ m^{-2}) was higher than for the early production cycle in the greenhouse (245 MJ m^{-2}). The following year, the average temperature outside (14.3 °C) was lower while the irradiation sum (239 MJ m^{-2}) was comparable with the early production cycle of 2009.

Table 2.2 Flower bud rate of development (ρ) between stage 3 and stage 7 with climatologic conditions for three production cycles of the early-flowering cultivar 'Nordlicht'

	Stage 3 $\rightarrow$ 7		Average temperature (°C)	Irradiation sum (MJ m^{-2})	Mean DLI (mol m^{-2} d^{-1})
	days	ρ (d^{-1})			
Early production (2009)	30	0.033	18.2	245	17.3
Late production (2009)	29	0.034	17.9	343	24.2
Late production (2010)	29	0.034	14.3	239	17.0
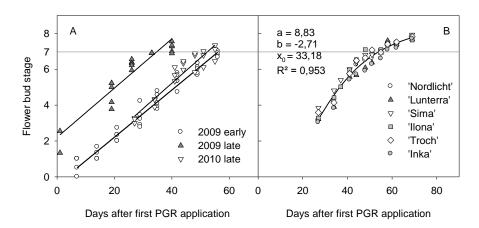
Figure 2.2 (A) Linear flower bud differentiation in function of days after the first PGR application for the early-flowering cultivar 'Nordlicht': early production in 2009 (y = -0.419 + 0.132 x; R² = 0.970), late production in 2009 (y = 2.205 + 0.137 x; R² = 0.890) and in 2010 (y = -0.435 + 0.141 x; R² = 0.898); (B) Flower bud differentiation rate decreases towards stage 8 for all sports of 'H. Vogel' in 2010 (y=a*(x/x$_0$)^abs(b)/(1+(x/x$_0$)^abs(b)))

2.3.3 Genotypic variation in flower development

In general, flower differentiation initially followed a linear pattern, towards the beginning of stage 7 differentiation slowed down to reach stage 8. The six sports of the early-flowering cultivar 'H. Vogel' developed similar (Figure 2.2B). Stage 7 and 8 were reached 54 and 76 days after the first PGR application. When comparing the three semi-early-flowering cultivars (Figure 2.3), 'Mont Blanc' differed significantly (p=0.003) from 'M. Marie' and 'Otto'. To reach stage 7 and 8 'Mont Blanc' required 46 and 63 days after the first PGR application, whereas 'M. Marie' and 'Otto' reached these stages 2 and 3 days later, respectively. The rate of development for all three semi-early-flowering cultivars was faster than for 'H. Vogel' sports (Table 2.3).
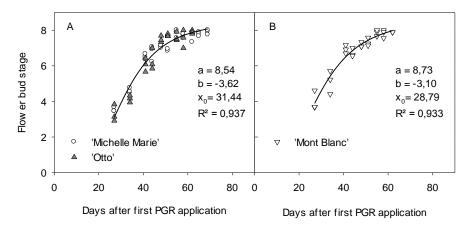
Figure 2.3 Flower bud differentiation in function of days after the first PGR application for the semi-early-flowering cultivars (A) 'M. Marie', 'Otto' and (B) 'Mont Blanc'
(y=a*(x/x0)^abs(b)/(1+(x/x0)^abs(b)))

Table 2.3 Rate of flower bud development (ρ) between stage 3 and stage 7, and stage 7 and 8 with climatologic conditions for 13 cultivars grouped according to their differentiation rate. The six bud sports of 'H. Vogel' are grouped together as no significant difference was found between these cultivars.

Cultivar	Stage 3 → 7		Stage 7 → 8		Average temperature (°C)	Irradiation sum (MJ m^{-2})	Mean DLI (mol m^{-2} d^{-1})
	days	ρ (d^{-1})	days	ρ (d^{-1})			
'H. Vogel' sports	28	0.035	22	0.045	15.3	624	17.8
'M. Marie' + 'Otto'	21	0.047	18	0.055	16.2	563	18.5
'Mont Blanc'	22	0.045	17	0.058	16.3	549	18.9
'Mw. G. Kint' + 'Tamira'	24	0.041	20	0.049	15.8	605	18.0
'Thesla' + 'Sachsenstern'	35	0.029	24	0.042	14.5	672	16.7

The late-flowering cultivars (Figure 2.4) differed significantly among each other (p<0.001). Two groups could be distinguished, 'Mw. G. Kint' and 'Tamira' developed clearly faster than 'Thesla' and 'Sachsenstern' (Table 2.3). 'Mw. G. Kint' and 'Tamira' reached stage 7 and 8 after 52 and 73 days, which is 2-3 days faster than 'H. Vogel' sports. 'Thesla' and 'Sachsenstern' were the slowest developing cultivars. It took 64 and 88 days to reach stage 7 and 8, respectively.
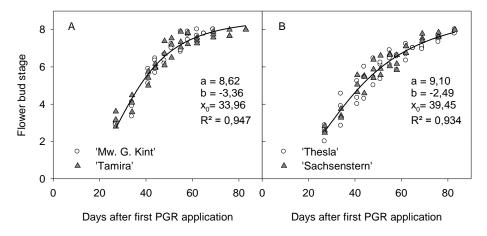
Figure 2.4 Flower bud differentiation in function of days after the first PGR application for the late cultivars (A) 'Mw. G. Kint', 'Tamira', (B) 'Thesla' and 'Sachsenstern'
(y=a*(x/x0)^abs(b)/(1+(x/x0)^abs(b)))

2.4 Discussion

2.4.1 Effect of PGR on flower initiation and differentiation

Plant growth regulators can be used to control vegetative growth and to promote flowering in woody plants (Meilan, 1997). Also in *Rhododendron* and azalea the use of plant growth regulators interfering in the GA biosynthesis pathway, such as paclobutrazol, chlormequat and daminozide improve plant architecture and flowering (Bodson, 1989; Keever and Foster, 1991; Marosz and Matysiak, 2005; Meijón et al., 2009). The blocking of the GA biosynthesis pathway will lead to a reduced level of GAs, which leads to floral induction in certain woody plant species. The role of the GA pathway in floral induction in woody plants still needs a lot of research, however, recently progress has been made. In rose, it was shown that exogenous applied GA_3 resulted in the accumulation of *RoKSN* (Randoux et al., 2012), a homologue of *TFL1* (*TERMINAL FLOWER1*) which acts opposite to *FT (FLOWERING LOCUS T)* (Hanano and Goto, 2011). This prevents flower initiation, which was also seen in *Citrus* where the expression of *FT* decreases after a GA_3 treatment (Goldberg-Moeller et al., 2013). Homologous genes were also isolated in evergreen azalea where an increase in *LFY (LEAFY)* and a decrease in *TFL1* were found during the initiation of flowering (Cheon et al., 2011). And a PGR treatment increased the expression of *LFY* and a *FT*-like

gene (De Keyser et al., 2013a). When initiating flowering with PGR, there will be other pathways affected as well, as is seen in azalea where a PGR treatment did not only alter GA levels, but also other phytohormones, such as cytokinins, and DNA methylation levels, which can be associated with the floral signal according to Meijón et al. (2011b).

The use of PGR not only hastened flower bud initiation, but also led to a more uniform flower bud development on plants in our experiments. In non-treated plants, flower bud stages had a higher variability per plant resulting in a lower R^2 value for the linear regression of flower bud differentiation (Table 2.1). Also, the plants which initiated floral meristems before the first PGR treatment had a lower R^2 value. Nevertheless, the use of PGR (standard and enhanced doses) did not alter the rate of flower bud differentiation, indicating a continuous process. Katz et al. (2003) suggests that by repressing the vegetative growth with PGR, more photo-assimilates are available for the inflorescence meristems, leading to a faster flower development. In our case, we saw no such effect on differentiation rate. This might be due to the fact that the difference in vegetative growth between control plants and PGR-treated plants was not strong enough as initiation in control plants was only delayed by 2 weeks.

2.4.2 Seasonal effects on flower bud development

Effects of photoperiod and temperature on flower initiation of azalea are well documented (Bodson, 1989; Criley, 1969; Pettersen, 1972); but the phase of floral organ differentiation is less studied. Therefore, differentiation was followed in an early and late production cycle, over two successive years for 'Nordlicht'. In general, the rate of development in plants increases linearly with temperature up to some optimum, beyond which development rate is slowed. We found that differentiation rates in function of days after the first PGR application, were constant and the rate of development (ρ) from stage 3 to 7 was 0.033 - 0.034 d^{-1} (Table 2.2). A clearly lower temperature in 2010 did not slow down the rate of flower differentiation. This is in accordance with the findings of Bodson (1989) in controlled conditions where the differentiation rate at 15°C did not differ from that at 20°C for the early-flowering cultivar 'H. Vogel'. This suggests that the floral differentiation of the sports of 'H. Vogel' has a low sensitivity in this temperature range. A higher light sum (late production cycle in 2009) did not affect the differentiation rate of 'Nordlicht' either. Bodson (1983) described that there was a higher developmental rate for late-flowering cultivars with higher light intensities. However, the tested DLIs in that paper were ranging between 2.3 and 9.2 mol m^{-2} d^{-1}, which are much lower values than in our study (17.0 and 24.3 mol m^{-2} d^{-1}). The light intensities in our field study were therefore probably saturating, resulting in no influence on development. Oh et al. (2009) showed also in cyclamen that above a critical DLI of 12 mol m^{-2} d^{-1} no further effect of a higher DLI on time to flower was observed.

2.4.3 Genotypic variation

Bodson (1983) postulated that earliness of flowering between cultivars does not result from earliness of flower initiation, but may be partly related to their different irradiance requirements during flower differentiation. In this experiment mean DLIs were higher than 16.7 mol m^{-2} d^{-1} and therefore not limiting for development (Table 2.3). Furthermore, the floral initiation started at the same date for all cultivars by applying PGR. We did not find a pattern between earliness of flowering and differentiation rate. Our results show that semi-early cultivars have a faster developmental rate, both from stage 3 to 7 (ρ= 0.046 d^{-1}) as from stage 7 to 8 (ρ= 0.056 d^{-1}) than the early 'H. Vogel' sports (ρ= 0.035 d^{-1} from stage 3 to 7 and ρ= 0.045 d^{-1} from stage 7 to 8). The late cultivars, however, develop slower than the semi-early cultivars, and show an important variation. 'Thesla' and 'Sachsenstern' had a very slow flower bud developmental rate (ρ= 0.029 d^{-1} from stage 3 to 7 and ρ= 0.042 d^{-1} from stage 7 to 8) though flower bud development of 'Mw G Kint' and 'Tamira' was superior to the 'H Vogel' sports. The six sports of 'H. Vogel' did not differ in their flower bud development, indicating that the occurrence of bud mutations resulting in a different flower colour had no influence on flower bud differentiation. It is clear that cultivars differ in flower development, not only between cultivars of a same natural flowering time, but also in these groups. Pettersen and Kristoffersen (1969) could also indicate differences in differentiation between azalea cultivars. Differences in flower development between cultivars are also observed in other woody plants like apple (Hoover et al., 2004; Koutinas et al., 2010), pear (Marafon et al., 2010) blackberry (Takeda et al., 2002) and black currant (Sønsteby and Heide, 2013).

2.5 Conclusions

Flower bud differentiation until stage 7 is linear; towards stage 8, differentiation slows down. The rate of differentiation is not influenced by a PGR treatment, only initiation is enhanced by an application with chlormequat. Large genotypic variations in flower bud differentiation rate were seen. Flower bud development was completed fastest for the semi-early-flowering cultivars ('Mont Blanc', 'M. Marie' and 'Otto'), requiring only 63 to 66 days after the first PGR treatment. Two of the late-flowering cultivars ('Thesla' and 'Sachsenstern') had the slowest development, requiring 88 days while the early-flowering cultivars and the late-flowering cultivars 'Mw. G. Kint' and 'Tamira' completed development in 73 to 76 days.

CHAPTER 3

Flower bud dormancy of *Rhododendron simsii* is influenced by genotype, plant growth regulators and flower bud developmental stage

Chapter 3

Flower bud dormancy of azalea is influenced by genotype, plant growth regulators and flower bud developmental stage

Before anthesis, the flower buds of azalea (*Rhododendron simsii* hybrids) are characterised by a period of dormancy. Prolonged exposure to cold is needed to fulfil the chilling requirements and synchronize flowering. Three different cultivars with different natural flowering times were followed during cold treatment at 7°C to break dormancy. The changes in ABA levels during release of dormancy differed between the cultivars and no simple relationship between ABA levels and dormancy duration could be established. The early-flowering cultivar 'Nordlicht' maintained a basal ABA level, while the semi-early-flowering cultivar 'M. Marie' and the late-flowering cultivar 'Mw. G. Kint' had twice the amount of ABA at the start of cold treatment, which decreased with increasing cold. Sensitivity to ABA was more correlated to flower earliness than to flower homogeneity, but this response was also cultivar dependent. A clear correlation with dormancy release could not be made. However, the natural flowering time of the three cultivars was also reflected in the chilling requirements, the early-flowering cultivar requiring less chilling than the semi-early- and late-flowering cultivars.

During the production of azalea, plant growth regulators such as chlormequat and paclobutrazol, are frequently used. This PGR treatment did affect the chilling requirements of the plants. A delay in flowering time and a more heterogeneous flowering of PGR-treated plants was observed compared to control plants. The late-flowering cultivar 'Mw. G. Kint' responded to the PGR treatment by increasing its endogenous ABA content.

Further, to have an optimal response to cold and ensure good flowering, flower buds have to be sufficiently developed (stage 7 or 8) at the start of cold treatment.

3.1 Introduction

Woody plants of the temperate zone exhibit growth-dormancy cycles to synchronize with seasonal variations, in which bud dormancy protects the plant from resuming growth in unfavourable conditions. Lang (1987) defined three types of dormancy: ecodormancy, endodormancy and paradormancy. During ecodormancy environmental factors prevent growth, while for paradormancy and endodormancy the growth inhibition arises from the plant itself. Paradormancy refers to factors from other plant parts inhibiting growth; endodormancy refers to factors inside the dormant structure itself which prevent initiation of growth from meristems (with the capacity to resume growth) under favourable environmental conditions (Rohde and Bhalerao, 2007).

Bud dormancy is a complex process with different factors influencing induction, maintenance and release. Dormancy induction is preceded by growth cessation followed by bud set, which are controlled by the environmental cues photoperiod (short days) and temperature (cold), but can be controlled autonomously in some species. Once dormancy is established, prolonged cold temperatures are the main factor to break dormancy. The amount of cold necessary to resume growth (bud burst) is the chilling requirement and differs among species and among cultivars (Cooke et al., 2012).

In most woody perennials, short days induce growth cessation and bud development by the formation of bud scales. After upregulation of the photoperiodic pathway, ethylene biosynthesis and signal transduction pathways are transiently activated, after which the first signs of bud formation are visible. Following this peak in ethylene biosynthesis, most metabolites are downregulated, indicating a progressive inactivation of growth, except for an upregulation of abscisic acid (ABA) biosynthetic enzymes which leads to a peak in ABA concentration (Ruttink et al., 2007). The induction of ABA is generally observed at the onset of endodormancy, while ABA levels drop and ABA biosynthesis is downregulated as buds are released from endodormancy (Xu et al., 2006; Horvath et al., 2008; Karlberg et al., 2010; Bai et al., 2013). Also in dormancy maintenance, ABA could have a role as shown in birch (Rinne et al., 1994). However, ABA levels don't always correlate with dormancy patterns, as was shown in willow by Barros and Neill (1986). In this study, dormancy was correlated to the sensitivity of the buds to exogenously applied ABA. Nevertheless, ABA does not need to be linearly correlated with dormancy to have an effect on its development (Faust, 1997).

Next to a downregulation of ABA biosynthesis, an upregulation of gibberellin (GA) biosynthesis is seen during dormancy release (Karlberg et al., 2010; Gai et al., 2013). An application of GA was also able to release dormancy in buds of *Populus* (Rinne et al., 2011), as well as in flower buds of

Japanese apricot (Zhuang et al., 2013), azalea (Bodson, 1986) and *Rhododendron* (Chang and Sung, 2000). However, treatment with GA$_3$ could not completely substitute for chilling requirements to break dormancy in azalea flower buds (Larson and Sydnor, 1971). Cold temperatures remain the main factor to release buds from endodormancy. Up to now, there is no physiological or molecular marker that easily indicates the fulfilment of chilling requirements. Recent gene expression analysis could overcome this problem as Leida et al. (2012) proposed for flower bud dormancy in peach. However, a clear break point will not be easy to find as dormancy induction and release is a gradual process (Faust et al., 1997; Junttila and Hänninen, 2012).

R. simsii hybrids have similar adaptive mechanisms as woody plants of the temperate zone, namely, flower bud dormancy and acclimation to lower, though not freezing temperatures. Flower bud dormancy gives the plant the ability to synchronise flowering. When chilling requirements are satisfied, buds will resume growth within 2–3 weeks of favourable growing conditions (Arora et al., 2003). Unfulfilled chilling requirements will lead to erratic bud break and heterogeneous flowering. Temperatures to break dormancy are generally between 2.5 and 9°C (Richardson et al., 1974) and the optimum for *R. simsii* hybrids is around 7°C (Pettersen, 1971; Bodson, 1989; Kromwijk, 2001).

During the production cycle of azalea, plants are treated with plant growth regulators (paclobutrazol, chlormequat) to control the vegetative growth and to initiate flower bud formation. Paclobutrazol is an inhibitor of ABA catabolism, by inhibiting ABA 8'-hydroxylase, which catalyses the first step in the oxidative degradation of ABA (Saito et al., 2006). Thus, treatment with paclobutrazol can increase ABA content, as shown by Upreti et al. (2013) in flower buds of mango. This increase in ABA content might lead to a deeper dormancy. There is evidence in potato tubers (Tekalign and Hammes, 2004) and azalea (Pettersen, 1976) that the use of paclobutrazol increased the dormancy period, reflected by a higher need for cold.

Over 150 commercial azalea cultivars exist, which differ in their natural flowering time. We investigated if cultivars belonging to different flowering groups differ in their requirements for chilling units. As ABA can be both inducing and maintaining dormancy we investigated whether flower bud dormancy in azalea was linked to ABA levels in the bud or if sensitivity to ABA maintained buds dormant. Next, we investigated if the use of plant growth regulators (chlormequat and paclobutrazol) might enhance dormancy by determining endogenous ABA levels, sensitivity to exogenously applied ABA and chilling requirements for dormancy release. Finally, using 13 cultivars, we tried to define a developmental stage of the flower bud where dormancy is installed and the accumulation of chilling units starts.

3.2 Materials and methods

3.2.1 Experiment 1 Effect of dormancy-breaking cold treatment on endogenous ABA content, ABA sensitivity and quality of flowering

Azalea (*R. simsii* hybrids) cultivars belonging to three different natural flowering groups were used. Cuttings of the early-flowering cultivar 'Nordlicht', the semi-early-flowering cultivar 'M. Marie' and the late-flowering cultivar 'Mw. G. Kint' were rooted in 12 cm pots (4/pot). Plants were grown under standard horticultural conditions, decapitated twice and treated six times with 2.25 g L^{-1} chlormequat and two times with 0.012 g L^{-1} paclobutrazol. Plants were subjected to a dormancy-breaking cold treatment (dark room at 7°C), one week after the flower primordia were fully differentiated and the style started to enlarge (stage 7; Bodson 1983). Temperature was recorded every 15 minutes and chilling units were calculated according to the Utah model (Richardson et al., 1974). Control plants were transferred to a greenhouse at non-dormancy-breaking temperatures. For 'Nordlicht', cold treatment started at 29 June 2009 and control plants received an average temperature of 21.1 ± 4.5 °C. 'M. Marie' started its cold treatment at 12 October 2009 and control plants received an average temperature of 13.6 ± 2.8 °C. Control plants of 'Mw. G. Kint' received an average temperature of 20.0 ± 1.3°C starting from 1 December 2011. From the start of the cold treatment, samples were taken weekly from flower buds of control plants and cold-treated plants, to determine the endogenous ABA concentrations and to test the responsiveness to exogenous ABA in a bio-assay.

This experiment was repeated in 2011 ('Nordlicht', 'M. Marie') to evaluate the quality of flowering. Forcing conditions were a constant temperature of 21°C, an average relative humidity of 60 % and a photoperiod of 16 h with a supplementary light level of 75-80 µmol m^{-2} s^{-1} (SON-T PHILIPS 600W) at plant canopy level. Quality of flowering was determined on 10 plants after 0 to 8 weeks of cold treatment (7°C).

3.2.2 Experiment 2 Effect of plant growth regulators (PGR) on endogenous ABA content, ABA sensitivity and quality of flowering

Azalea cuttings of the early-flowering cultivar 'Nordlicht' and the late-flowering cultivar 'Mw. G. Kint' were rooted in 12cm pots (4/pot). 'Nordlicht' was grown in an early production cycle (rooting in July 2008) and a late production cycle (rooting in December 2008). Plants were grown under standard horticultural conditions and decapitated twice to stimulate branching. PGR applications started on 4/5/2009, 5/8/2009 and 18/8/2009 for the early production of 'Nordlicht', the late production of 'Nordlicht' and 'Mw. G. Kint', respectively. A control treatment, without

plant growth regulators, was compared to a high dose PGR treatment with 6 applications of 2.25 g L^{-1} chlormequat, the last three were combined with 0.04 g L^{-1} paclobutrazol followed by an extra treatment with 0.04 g L^{-1} paclobutrazol when the flower buds were in stage 7, this one day prior to the cold treatment at 7°C. Every 2 or 3 weeks samples were taken from flower buds to determine the endogenous ABA concentrations and to test the responsiveness to exogenous ABA in a bio-assay. Subsequently, plants were forced after 0 to 8 weeks of cold to assess quality of flowering on 10 plants. Forcing conditions were as described above.

3.2.3 Experiment 3 Optimum flower bud stage to respond to cold treatment

Thirteen azalea cultivars with three different natural flowering times were used; six sports of the early-flowering cultivar 'H. Vogel': 'Nordlicht', 'Lunterra', 'Sima', 'Ilona', 'Mw. Ed. Troch' and 'Inka'; 'M. Marie', 'Otto' and 'Mont Blanc' as semi-early-flowering cultivars and four late-flowering cultivars, 'Mw. G. Kint', 'Tamira', 'Thesla' and 'Sachsenstern'. Cuttings of all cultivars were rooted in 12 cm pots (4/pot) at the end of 2009. After the second decapitation (3/06/2010) plants were transferred from the greenhouse to an outside container field. To initiate the generative phase, vegetative plants were treated with 2.25 g L^{-1} chlormequat for the first time on 3/08/2010. Six more applications of which one combined with 0.012 g L^{-1} paclobutrazol followed weekly after the first treatment. Flower bud development was followed by dissections twice a week. To break dormancy, the early-cultivar 'H. Vogel' was placed at 7 °C for 5 weeks when flower buds were differentiated up to, respectively, flower bud stage 5, 6 and 7. Semi-early- and late-flowering cultivars were at the start of flower bud stage 6, 7 and 8 when kept at 7°C for 7 weeks. After cold treatment, plants were forced as described above and after 5 weeks, the quality of flowering was determined on 7 plants per cultivar.

3.2.4 Assessment of flower bud development

Flower bud differentiation was observed on three buds per plant for three to five randomly chosen plants (OLYMPUS SZX9 stereo microscope). Since each bud contained several primordia, a mean stage was determined per bud using the floral scale of Bodson (1983) which comprises nine stages; stage 0: vegetative bud, stage 1: initiation of bud scales, stage 2: initiation of floral primordia, stage 3: sepal initiation, stage 4: petal initiation, stage 5: stamen initiation, stage 6: carpel initiation, stage 7: initiation of style elongation, stage 8: the ovary contains ovules.

3.2.5 ABA bio-assay

Flower buds were disinfected with 1 % mercury (I) chloride and 10 % sodium hypochlorite. The outer bud scale was removed and buds were transferred onto solid medium (7 g L^{-1} agar) in test

tubes. The McCown Woody Plant Medium including vitamins (Duchefa Biochemie bv) was used with or without (control) 1 µM (±)-ABA. Buds remained close by exogenous ABA when buds are dormant (Barros and Neill, 1986). Bud burst was considered to occur when the scales unfurl. Every week the number of opened buds was registered, for a maximum of 84 days. The data were expressed as Σ-84 values, if a bud opened on day 1 then its Σ-84 value is 83, if it opened on day 2 then its Σ-84 value is 82, if it opened on day 84 or did not open its Σ-84 value is 0. Twenty four buds were used per treatment and after 12 weeks the mean Σ-84 values were calculated.

3.2.6 Endogenous ABA concentrations

Extraction and purification of ABA was done as described by Chen et al. (1997). Grounded samples were extracted in cold 80 % aqueous methanol (5 ml g^{-1} FW) overnight at 4°C with 10 mg L^{-1} butylated hydroxytoluene to prevent oxidation. After centrifugation at 10,000 g (4°C, 20 min), the supernatant was passed through a C18 Sep-Pak cartridge (Waters). The efflux was collected and dried in a stream of N$_2$. The residue was dissolved in 1.5 ml phosphate-buffered saline (0.01 M, pH 9.2) and adjusted to pH 8.5, then partitioned three times with an equal volume of ethyl acetate. The remaining water phase was adjusted to pH 2.5 and again extracted three times with ethyl acetate. The ethyl acetate phases were pooled and dried in a stream of N$_2$. The residue was dissolved in 200 µl of 100 % methanol and diluted with tris-buffered saline (25 mM, pH 7.5). Free ABA levels were quantified by ELISA with a phytodetek-ABA kit (Agdia-Biofords, France).

3.2.7 Quality of flowering

For experiments 1 and 2, quality of flowering was followed by weekly counting the number of opening buds. The flowering percentage was calculated and used to determine the earliness of flowering as days between start of forcing and 10 % flowering, and the uniformity of flowering as days between 10 and 90 % flowering.

Quality of flowering was assessed for the third experiment after 5 weeks of forcing by counting the number of opened flower buds on 7 plants to determine a total flowering percentage per plant. Further, the opened buds were categorized into three groups according to their developmental stage: colour-showing (CS), candle stage (CA) and open flowers (OF). To compare the flower development between the different objects, a flowering index (FI) was calculated based on the percentages of the developmental stage of the flower: FI = 1 * %CS + 2 * %CA + 3 * %OF.

3.2.8 Statistical analysis

Results were analysed using SPSS statistical software Version 19.0 (SPSS Inc., Chicago, USA). Analyses were carried out by ANOVA and means were separated with Tukey's HSD test or by Student's *t*-test (p=0.05). If normality or equality of variances was not met, the non-parametric Mann-Whitney U Test was used.

3.3 Results

3.3.1 Experiment 1 Effect of the dormancy-breaking cold treatment on endogenous ABA content, ABA sensitivity and quality of flowering

The bio-assay for 'Nordlicht' (Figure 3.1A) indicated that at least 4 weeks at 7°C were required for flower buds to lose sensitivity to exogenous ABA. Endogenous ABA content in the flower buds of cold treated plants remained at a low average level of 0.768 nmol g^{-1} FW (Figure 3.2A). Flower buds of control plants, on the other hand, increased their ABA content during the first two weeks until 1.446 nmol g^{-1} FW, followed by a continuous decrease. The earliness of flowering (Table 3.1) improved significantly with increasing cold period, reaching 23.2 days after 5 weeks at 7°C. Flowering homogeneity was good, reaching 17.3 days after 3 weeks at 7°C, but further improved by three days after 4 and 5 weeks at 7°C.
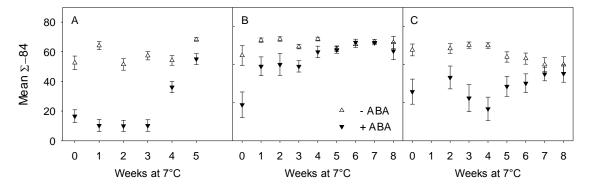
Figure 3.1 Flower bud sensitivity (Σ-84) to 1 μM ABA in response to increasing weeks of cold treatment at 7°C for 'Nordlicht' (A), 'M. Marie' (B) and 'Mw. G. Kint' (C) on medium with (▼) and without (△) ABA (mean ± SE, n=24)

The semi-early-flowering cultivar 'M. Marie' was less sensitive to exogenous ABA (Figure 3.1B), so only small differences were seen between buds on medium with or without ABA and after 5 weeks at 7°C, there were no differences at all. The endogenous ABA content of the flower buds at the start of the cold period was more than double (1.738 nmol g^{-1} FW) compared to 'Nordlicht' (Figure 3.2B). After a strong decrease in ABA concentration the first week, levels steadily

decreased further to reach 0.600 nmol g^{-1} FW after 8 weeks at 7°C. Flowering results (Table 3.1) showed that earliness of flowering was only slightly affected by the cold period in a non-consistent way. Flowering homogeneity on the other hand, improved up to 11.3 days after 6 weeks at 7°C, having no further benefit of extra cold.
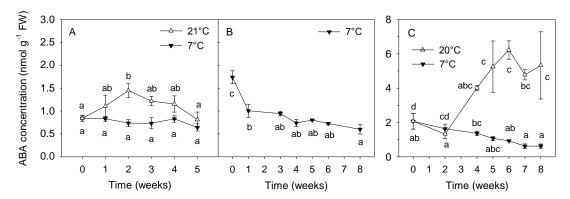
Figure 3.2 Variation of endogenous ABA concentrations in flower buds of 'Nordlicht' (A), 'M. Marie' (B) and 'Mw. G. Kint' (C) under non-dormancy-breaking temperatures (△) and after increasing weeks at 7°C (▼). For 'M. Marie', the control has been omitted as temperatures were not strictly non-dormancy-breaking temperatures. (mean ± SD, n=3, different letters indicate significant differences according to Tukey's HSD test for each temperature treatment, p=0.05)

Table 3.1 Effect of increasing chilling units on the earliness of flowering (days between the start of forcing and 10 % flowering) and homogeneity of flowering (days between 10 and 90 % flowering) for 'Nordlicht', 'M. Marie' and 'Mw. G. Kint'

Weeks at 7°C	Chilling Units	'Nordlicht' [z]		'M. Marie' [y]		'Mw. G. Kint' [y]	
		Earliness (days)	Homogeneity (days)	Earliness (days)	Homogeneity (days)	Earliness (days)	Homogeneity (days)
0	0	52.0 e	26.8 c			38.8 e	- [x]
1	168	44.8 d	21.0 cb	13.4 c	24.0 b		
2	336	39.6 d	21.3 cb	12.0 bc	20.0 b	26.4 cd	35.0 c
3	504	31.8 c	17.3 ac	12.9 bc	17.9 b	24.7 d	13.8 a
4	672	28.6 b	14.0 a	13.1 c	16.3 ab	20.6 c	17.1 ab
5	840	23.2 a	14.8 ab	6.8 a	18.2 b	13.4 a	22.0 b
6	1008			12.7 c	11.3 a	15.3 b	11.7 a
7	1176			10.5 b	11.0 a	15.4 b	11.2 a
8	1344			7.5 a	11.5 a	15.3 b	15.4 ab

[z] Means (n=10) followed by different letters are significantly different (p=0.0033, Mann-Whitney U Test)

[y] Means (n=10) followed by different letters are significantly different (p=0.0018, Mann-Whitney U Test)

[x] 90 % flowering not reached

Flower buds of the late-flowering cultivar 'Mw. G. Kint' showed sensitivity to exogenous ABA up to 6 weeks at 7°C (Figure 3.1C). For 7 and 8 weeks at 7°C there were no significant differences with buds on the control medium. The endogenous ABA level at the start of the cold treatment (2.070 nmol g^{-1} FW) was similar to the concentration found in 'M. Marie' (Figure 3.2C). A significant decrease towards 1.072 nmol g^{-1} FW after 5 weeks of cold treatment was seen, followed by a non-significant decrease until levels reached 0.620 nmol g^{-1} FW after 7 and 8 weeks at 7°C. In flower buds of control plants, ABA concentrations increased strongly. Earliness of flowering (Table 3.1) improved until 5 weeks at 7°C to 13.4 days, extra cold did not advance flowering further. Flowering homogeneity reached the best results after 6 weeks at 7°C, reaching 11.7 days.

3.3.2 Experiment 2 Effect of plant growth regulators on endogenous ABA content, ABA sensitivity and quality of flowering

The effect of a high dose of plant growth regulators was investigated for the early-flowering cultivar 'Nordlicht' during an early and late production cycle. The flower buds of the early production showed a clear response to exogenously applied ABA and control plants (not treated with PGR) lost sensitivity more rapidly than treated plants (Figure 3.3A), indicating that control plants were less dormant. In contrast, the flower buds of the late production showed less sensitivity to ABA, and the difference between the control and treated plants was not significant (Figure 3.3B).
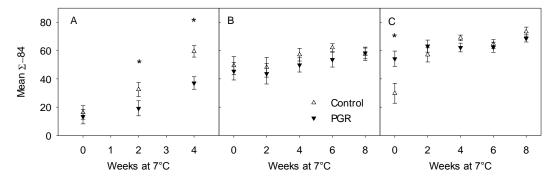
Figure 3.3 Flower bud sensitivity (Σ-84) to 1 μM ABA in response to PGR treatment and this for increasing weeks of cold treatment at 7°C for 'Nordlicht' in early (A) and late (B) production and 'Mw. G. Kint' (C). Control (△) plants without PGR; PGR-treated (▼) plants received 6 x chlormequat (2.25 g L^{-1}) + 4 x 0.04 g L^{-1} paclobutrazol (mean ± SE, n=24)
* indicate a significant difference (Mann-Whitney U, p=0.05) between the control and PGR-treated plants.

Endogenous flower bud ABA concentrations for 'Nordlicht' showed very little difference between control plants and plants treated with PGR (Figure 3.4A-B). For the early production, ABA levels showed a clear decrease between 2 and 5 weeks at 7°C, for control plants to 0.494 nmol g^{-1} FW and for PGR-treated plants to 0.774 nmol g^{-1} FW, a significant difference after 5 weeks at 7°C. During the late production, ABA levels deceased significantly during cold treatment, but no significant difference was seen between control and PGR-treated plants, both reaching an average level of 0.569 nmol g^{-1} FW after 5 weeks at 7°C.
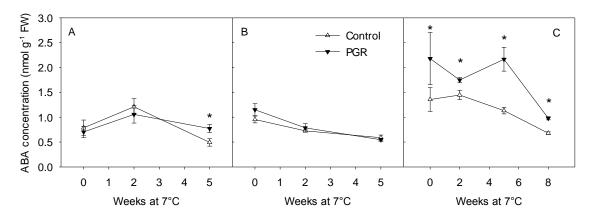
Figure 3.4 Effect of PGR treatment on endogenous ABA concentrations in flower buds of 'Nordlicht' in early (A) and late (B) production and 'Mw. G. Kint' (C). Control (△) plants without PGR; PGR-treated (▼) plants received 6 x chlormequat (2.25 g L^{-1}) + 4 x 0.04 g L^{-1} paclobutrazol.
* indicates a significant difference (T-test, p=0.05) between the control and PGR-treated plants.

Quality of flowering (Table 3.2) on the other hand, showed clearly the effect of the PGR application. Flowering was delayed with 23.1 and 14.6 days for the early and late production, respectively, when plants were treated with PGR. With increasing cold treatment, the difference was reduced to 10 days of delayed flowering, after 5 weeks of cold for the early production. Prolonging the cold treatment eliminated the delay in earliness of flowering as seen from 7 weeks at 7°C on, for the late production cycle. For the early production of 'Nordlicht', flowering was very heterogeneous (homogeneity of flowering > 20 days) when plants were treated with PGR. For the late production, the differences were less pronounced, PGR-treated plants showed by average, from 3 weeks cold on, a homogeneity score which was only 2.5 days longer than control plants.

Control plants of the late-flowering cultivar 'Mw. G. Kint' showed a higher response to exogenously applied ABA at the start of cold treatment (Figure 3.3C). During the cold treatment, the bio-assay did not show any differences between control and PGR-treated plants. In contrast with the early-flowering cultivar 'Nordlicht', 'Mw. G. Kint' showed a clear significant difference in endogenous ABA levels between control and PGR-treated plants (Figure 3.4C). After 8 weeks at 7°C the flower buds of PGR-treated plants contained still 0.980 nmol g^{-1} FW, while flower buds of

Table 3.2 Effect of PGR treatment on earliness of flowering (days between the start of forcing and 10% flowering) and homogeneity of flowering (days between 10 and 90% flowering) for 'Nordlicht' in early and late production and 'Mw. G. Kint' after increasing weeks at 7°C. Control plants without PGR; PGR treated-plants received 6 applications with chlormequat (2.25 g L^{-1}), and four applications with 0.04 g L^{-1} paclobutrazol.

		Weeks at 7°C (chilling units)							
		0	2 (336)	3 (504)	4 (672)	5 (840)	6 (1008)	7 (1176)	8 (1344)
'Nordlicht' early production	Earliness (days) Control	18.5 a	15.1 a	11.6 a	13.0 a	10.0 a			
	Earliness (days) PGR	41.6 b	33.1 b	28.3 b	25.0 b	20.0 b			
	Homogeneity (days) Control	-*	-	39.9	30.0 a	21.8 a			
	Homogeneity (days) PGR	-	-	-	55.9 b	42.1 b			
'Nordlicht' late production	Earliness (days) Control	28.4 a	24.6 a	22.0 a	19.6 a	18.1 a	13.3 a	14.0 a	13.0 a
	Earliness (days) PGR	43.0 b	30.2 b	29.7 b	22.6 b	20.5 b	20.0 b	13.6 a	14.0 b
	Homogeneity (days) Control	-	16.1 a	13.4 a	8.8 a	8.7 a	10.2 b	6.4 a	6.0 a
	Homogeneity (days) PGR	-	22.7 b	16.0 a	12.8 b	12.1 b	7.3 a	10.3 b	9.8 b
'Mw. G. Kint'	Earliness (days) Control	66.3 a			38.6 a	33.7 a	29.8 a	25.9 a	21.0 a
	Earliness (days) PGR	77.0 b			48.7 b	41.3 b	35.0 b	32.1 b	31.4 b
	Homogeneity (days) Control	-			15.3 a	13.7 a	11.7 a	8.6 a	14.1 b
	Homogeneity (days) PGR	-			23.9 b	25.0 b	21.0 b	17.1 b	11.5 a

Means (n=10) followed by different letters are significantly different (p=0.05, Mann-Whitney U Test)

*: 90 % flowering not reached

control plants decreased the ABA level to 0.697 nmol g^{-1} FW. For 'Mw. G. Kint' flowering was delayed regardless of cold treatment with an average of 8 days when plants are treated with PGR (Table 3.2). Also flowering homogeneity is affected by PGR application, control plants flowered more homogeneous, and had a lower homogeneity score with an average of 9 days compared to PGR-treated plants. However, after 8 weeks at 7°C, flowering was slightly more homogeneous in PGR-treated plants (11.5 days) compared to control plants (14.1 days).

3.3.3 Experiment 3 Optimum flower bud stage to respond to cold treatment

Since all sports of the early-flowering cultivar 'H. Vogel' gave the same flowering results, a mean value is shown in Table 3.3. It is clear that the optimal response to cold treatment is seen when applied at flower bud stage 7, with a high total flowering percentage of 94.5 % and flowering index of 167.5. Transfer of plants to 7°C with flower buds at stage 5 and 6 resulted in a lower flowering percentage but also a less uniform and later flowering indicated by a smaller flowering index of 66.5 and 86.8, respectively.

For the semi-early-flowering cultivars (Table 3.3), 'M. Marie' showed clearly an optimal response to the cold treatment at stage 8. 'Otto' and 'Mont Blanc' have a higher flowering percentage of 69.0 % and 69.3 % and a higher FI of 99.8 and 112.7 at stage 7 compared to 'M. Marie' (23.4 %, FI = 27.7). Nevertheless, an improvement was seen in both flowering percentage (to 87.6 % and 75.7 %) and flowering index (181.7 and 150.5), even though not significant for 'Mont Blanc', when cold treatment started at flower bud stage 8.

Table 3.3 Percentage of flowering (%) and flowering index (FI) after 5 weeks of forcing for early- and semi-early-flowering cultivars when cooled for 5 (840 CU, early-flowering cultivars) and 7 (1176 CU, semi-early-flowering cultivars) weeks at different floral developmental stages

	'H. Vogel' sports		'M. Marie'		'Otto'		'Mont Blanc'	
	%	FI	%	FI	%	FI	%	FI
Stage 5	53.6 b	66.5 b						
Stage 6	70.8 b	86.8 b	0.7 c	0.7 c	34.7 c	36.4 c	67.6 a	102.2 b
Stage 7	94.5 a	167.5 a	23.4 b	27.7 b	69.0 b	99.8 b	69.3 a	112.7 ab
Stage 8			87.4 a	161.6 a	87.6 a	181.7 a	75.7 a	150.5 a

Means (n=7) followed by different letters are significantly different (p=0.0167, Mann-Whitney U Test)

The four late-flowering cultivars can be divided in two groups (Table 3.4); 'Mw. G. Kint' and 'Tamira' show both poor flowering regardless of the flower bud stage at the start of cold treatment. The flowering index for these cultivars is extremely low: 9.8 and 47.0, respectively. The second group with 'Thesla' and 'Sachsenstern' had poor flowering at stage 6 and improved flowering at stage 7 and stage 8. Starting cold treatment at stage 8 resulted in a flowering percentage of 79.0 % and 77.6 %; and a flowering index of 111.4 and 151.4 for 'Thesla' and 'Sachsenstern', respectively.

Table 3.4 Percentage of flowering (%) and flowering index (FI) after 5 weeks of forcing for four late-flowering cultivars when cooled for 7 weeks (1176 CU) at different floral developmental stages.

	'Mw. G. Kint'		'Tamira'		'Thesla'		'Sachsenstern'	
	%	FI	%	FI	%	FI	%	FI
Stage 6	0.0 b	0.0 b	0.0 b	0.0 b	3.4 c	3.4 c	15.1 b	15.1 c
Stage 7	6.1 ab	6.1 a	0.0 b	0.0 b	66.3 b	66.4 b	69.5 a	75.0 b
Stage 8	9.8 a	9.8 a	41.3 a	47.0 a	79.0 a	111.4 a	77.6 a	151.4 a

Means (n=7) followed by different letters are significantly different (p=0.0167, Mann-Whitney U Test)

3.4 Discussion

3.4.1 Effect of dormancy-breaking cold treatment on endogenous ABA content, ABA sensitivity and quality of flowering

The early-flowering cultivar 'Nordlicht' shows a basal ABA content in the flower buds. This level is not altered by the cold treatment. In the bioassay, dormant buds should remain closed on medium with ABA. A high sensitivity to ABA is seen until 3 weeks of cold treatment, though after four to five weeks of cold, the buds completely lose sensitivity to ABA. Barros and Neill (1986) also showed a strong responsiveness to exogenous ABA in willow buds, while the endogenous ABA concentrations were low at that time. Earliness of flowering of 'Nordlicht' keeps improving with increased cold treatment, while flower homogeneity improves up to 4 weeks of cold treatment. 'Nordlicht' will need at least 672 CU for high quality flowering, and not the absolute ABA content, but the sensitivity to ABA is indicative for optimal flowering in this cultivar during an early production cycle.

The semi-early-flowering cultivar 'M. Marie' has more than double the amount of ABA at the start of cold treatment. A sharp decrease in ABA levels during the first week at 7 °C is followed by a slow decrease towards basal levels. The responsiveness to ABA shows a similar pattern. At the start of cold treatment, sensitivity is very high, and a sharp decrease in sensitivity occurs during the first week at 7°C. Earliness of flowering is not affected by increasing cold treatment, however,

the variation between buds decreases up to 6 weeks of cold treatment (flower homogeneity < 12 days). ABA levels and responsiveness to ABA are less indicative for dormancy release in 'M. Marie', where 1008 CU or higher gave the best flowering results.

'Mw. G. Kint' has even a slightly higher ABA content at the start of cold treatment compared to 'M. Marie'. The concentration in ABA levels decreases continuously until a basal level was reached after 5 weeks at 7°C. The continuous decrease of endogenous ABA levels with increasing cold has also been observed in different plant species as *Rhododendron* 'Prize' (Pemberton et al., 1985), lily bulbs (Xu et al., 2006) and birch (Rinne et al., 1994). The sensitivity to ABA is present the first four weeks of cold treatment, concomitant, the earliness of flowering improves the first four weeks at 7°C, while homogeneity of flowering is hardly affected by minimum 2 weeks of cold. So for 'Mw. G. Kint', there is a correlation between high ABA concentration, sensitivity to ABA and earliness of flowering, which all improved up to 5 weeks of cold. The optimal flowering homogeneity is reached one week later, after 1008 CU.

Table 3.5 Mean (minimum – maximum) daily temperature and day length of the week preceding the cold treatment for experiment 1 and 2

	'Nordlicht' early production	'Nordlicht' late production	'M. Marie'	'Mw. G. Kint'
Start 7°C treatment	29/06	22/09	12/10	1/12
Temperature (°C) (min – max)	20.6 (11.8 – 31.0)	16.7 (9.9 – 26.3)	14.1 (7.6 – 20.3)	11.4 (5.7 – 18.7)
Day length	long day	day neutral	short days	short days

ABA biosynthesis peaks at the onset of bud dormancy after a short-day induction of growth cessation and bud formation in poplar (Ruttink et al., 2007). Also Karlberg et al. (2010) showed that short days induce higher ABA levels. For 'M. Marie' and 'Mw. G. Kint' bud formation took place under decreasing day length and short days (Table 3.5), which resulted in a high endogenous ABA content. For 'Nordlicht' on the other hand, bud formation and dormancy induction took place under long days. However, for the late production of 'Nordlicht' (Figure 3.4) low ABA levels were also observed when bud formation and dormancy induction took place under decreasing photoperiod. 'Nordlicht' might be a genotype that doesn't really enter endodormancy. Forcing plants early (in August) without cold treatment is possible, however, flowering is heterogeneous and 100% bud break cannot be achieved. With a cold treatment before forcing, flowering is improved drastically: homogenous and near 90% bud break. Sung and Chang (2001) showed that *R. mucronatum* is able to flower when plants are shifted from relatively cold (15/13°C), but non-dormancy-breaking temperatures to higher temperatures. So in this case temperatures are not low enough to break dormancy, but improve flowering nonetheless.

Looking at the flowering results in Table 3.2, flower homogeneity is markedly better for the late production even with only 2 weeks of cold treatment. During the late production, plants received already cooler temperatures (16.7°C compared to 20.6°C) before cold treatment, which clearly improve flowering. So it can be assumed that true endodormancy is not induced in 'Nordlicht'.

We also followed ABA concentration when plants stayed at high temperatures ('Nordlicht') or were put from low temperatures to high temperatures when dormancy was installed ('Mw. G. Kint'). For both cultivars, there is an increase in ABA content under these conditions. It was also observed in willow buds that during summer (warmer temperatures) ABA levels increased (Alvim et al., 1976; Barros and Neill, 1986). This indicates that cold treatment is necessary to decrease or keep ABA at a basal level to allow further development of the flower.

Determining the exact amount of chilling units necessary to break dormancy is not evident, as there are large genotypic differences. Also in peach flower bud chilling units can range between 300 and 1220 CU (Leida et al., 2012; Zhebentyayeva et al., 2013). To predict dormancy release in apricot flower buds, Andreini (2014) divided the cultivars into three groups: early, intermediate and late cultivars. These three groups showed differences in chilling requirements and different responses to temperature during dormancy. However, the order of dormancy release is equivalent to the natural flowering times. Therefore, we would expect the late-flowering cultivar 'Mw. G. Kint' to have the highest chilling requirements, however, CU were equal to 'M. Marie'. Yet, we cannot exclude with our experiment that 'Mw. G. Kint' might have a higher cold requirement, as temperatures before the start of the cold treatments were already reaching dormancy-breaking levels. Two weeks before the start of cold treatment, 'M. Marie' did not accumulated any CU, while 'Mw. G. Kint' already encountered 155 CU, so chilling accumulation had already started for this cultivar and the total cold requirements will be slightly higher than for 'M. Marie'.

3.4.2 Effect of plant growth regulators (PGR) on endogenous ABA content, ABA sensitivity and quality of flowering

As paclobutrazol is an inhibitor of ABA catabolism (Saito et al., 2006), we expected to find effects of the applied plant growth regulators on ABA content and ABA sensitivity. Our results, however, show that genotypes react differently. The buds of the early-flowering cultivar 'Nordlicht' have no increase in endogenous ABA content. The inhibition of ABA catabolism by paclobutrazol must be limited in this cultivar. On the other hand, the buds of the late-flowering cultivar 'Mw. G. Kint' have the expected increase in ABA content in PGR-treated plants. The levels decrease with increasing weeks of cold, but remain higher than in the untreated control plants. Only slight

differences in sensitivity to exogenous ABA are seen, except for the early production cycle of 'Nordlicht' where flower bud development took place under long days. The increased sensitivity to exogenous ABA in the early production cycle of 'Nordlicht' by PGR also reflects in the flowering results, earliness of flowering after 4 weeks of cold is still delayed by 12 days. For the late production cycle, no increase in ABA levels or sensitivity to exogenous ABA are seen, still flowering is delayed by 3 days. If the cold period of plants from the late production cycle of 'Nordlicht' is prolonged, quality of flowering becomes equal between control plants and PGR-treated plants. This was also shown by Pettersen (1976). Also for 'Mw. G. Kint' earliness of flowering was delayed in PGR-treated plants, after 8 weeks at 7°C there was still a 10 days difference. Keever and Foster (1991) showed that the effect of paclobutrazol on dormancy is cultivar dependent and found a delayed flowering of 2 to 4 days.

The prolonged dormancy period of PGR-treated plants can still be a result of the blocking of the GA biosynthesis pathway by chlormequat and paclobutrazol. Gai et al. (2013) showed an increased activation of GA biosynthesis during dormancy release, this by an increase in an ent-kaurene synthase gene which functions in the first steps of GA biosynthesis. This step in the GA-biosynthesis pathway is a target of chlormequat. Paclobutrazol blocks the formation of ent kaurenic acid from ent-kaurene. As paclobutrazol has a long persistence in woody plants (Gent, 1997; Meilan, 1997) this can still have an effect on dormancy release. So our results also indicate that the up regulation of GA is necessary for dormancy release of flower buds and a previous inhibition of the GA-biosynthesis pathway may delay this process.

3.4.3 Floral developmental stage determines the start of dormancy-breaking treatments

As discussed above, 'Mw. G. Kint' might have already started with the accumulation of CU at the start of our experiment, which makes it difficult to know when dormancy-breaking temperatures have an effect. Further, dormancy develops gradually after growth cessation, and floral organ differentiation continues during dormancy establishment and the chilling period (Reinoso et al., 2002; Jones et al., 2009; Yamane et al., 2011). It is therefore impossible to define exactly at which flower bud stage dormancy is installed. Nevertheless, a clear effect of flower bud stage on the starting point of an artificial dormancy-breaking cold treatment was observed. For early-flowering cultivars chilling accumulation can start at stage 7, while for semi-early- and late-flowering cultivars stage 8 is preferred for the start of the cold treatment. The poor flowering of two of the late-flowering cultivars ('Mw. G. Kint' and 'Tamira') will be a result of insufficient cold. 1176 CU were not sufficient to break dormancy and to guarantee good flowering. In 'Mw. G. Kint' chilling accumulation in natural conditions can start at/before stage 7, as discussed above, but for

artificial cold treatment stage 8 is preferred. These results are of great practical use, as also Larson and Sydnor (1971) suggested that the stage of bud development is much more meaningful than the number of weeks from the final decapitation, in determining the optimum time to subject plants to dormancy-breaking treatments.
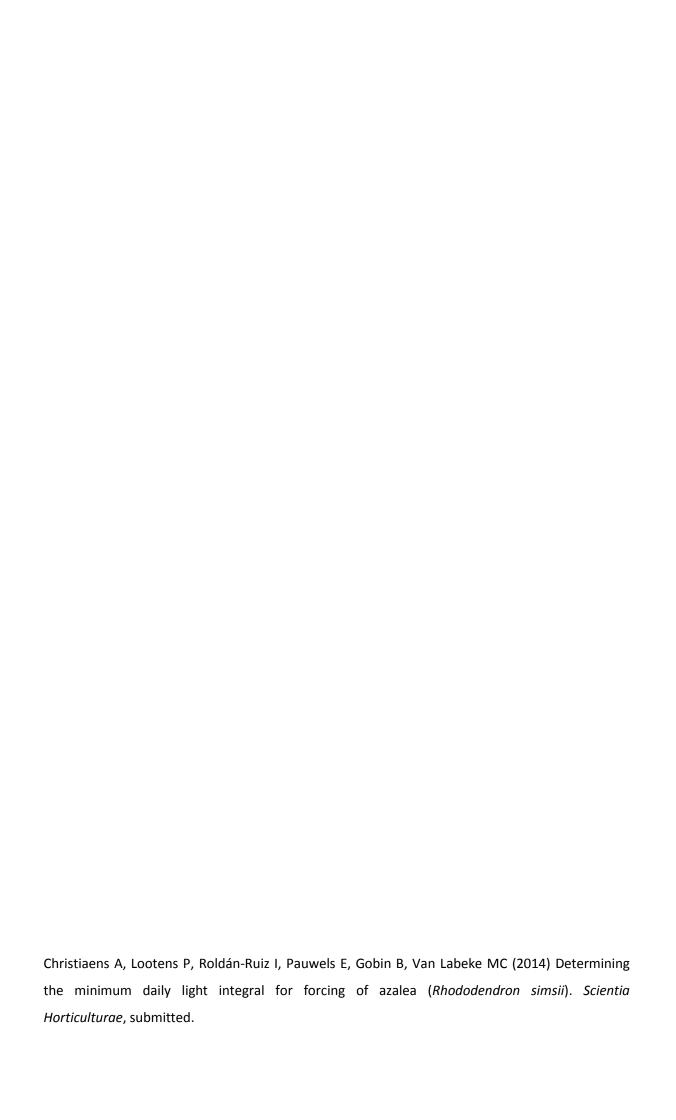
3.5   Conclusions

We found a great variability in chilling requirements among the tested azalea cultivars. In this study the changes in ABA levels during release of dormancy differed between the cultivars and no simple relationship between ABA levels and dormancy duration could be established. Overall, it seems that the sensitivity to ABA was more correlated with flower earliness than with flower homogeneity and so linking the results of the bio-assay to whole-plant dormancy release is not possible for every genotype. PGR treatment might enhance dormancy and increase the need for chilling in certain conditions. When applying artificial cold treatment at 7°C, the flower bud must be at an advance stage of development to result in a high quality of flowering.

CHAPTER 4

Determining the minimum daily light integral for forcing of azalea

(*Rhododendron simsii*)

Christiaens A, Lootens P, Roldán-Ruiz I, Pauwels E, Gobin B, Van Labeke MC (2014) Determining the minimum daily light integral for forcing of azalea (*Rhododendron simsii*). *Scientia Horticulturae*, submitted.

Chapter 4

Determining the minimum daily light integral for forcing of azalea (*Rhododendron simsii*)

Forcing of florist azalea is a relative short, though important step in the production cycle of this ornamental. This phase determines the flowering quality which is important for the economic value of the plant. Forcing is performed inside heated greenhouses (21°C), and application of supplemental light is increasing in popularity among growers. Optimizing the use of supplemental light is essential to minimize energy costs. Here, the minimum daily light integral (DLI) needed to balance photosynthesis and respiration for two *Rhododendron simsii* cultivars was determined. Whole-plant net CO_2 exchange measurements resulted in a minimum DLI for photosynthesis of 2.1 mol $m^{-2}d^{-1}$ for the early-flowering cultivar 'Nordlicht' and of 1.7 mol $m^{-2}d^{-1}$ for the late-flowering cultivar 'Thesla'. Increasing the forcing temperature by 6°C resulted in a 72% increase of the minimum DLI for photosynthesis of 'Thesla'. The developmental stage of the flower buds had no effect on the minimum DLI. Further, forcing experiments showed that to obtain a high-quality flowering plant, this minimum DLI for photosynthesis must be raised by at least 57%.

4.1   Introduction

In ornamentals, light is a crucial factor in plant development and final plant quality. The daily light integral (DLI) can be defined as the amount of photosynthetically active radiation (PAR) received on a daily basis. It is expressed as mol $m^{-2}\,d^{-1}$, and is affected both by light intensity and photoperiod. Average monthly DLI outdoors ranges between 5 and 60 mol $m^{-2}\,d^{-1}$ depending on time of year and latitudes between 30° and 50° (Korczynski et al., 2002). It has been demonstrated that DLI can have a profound effect on root and shoot growth of seedlings and cuttings. An increased DLI increases sucrose levels in leaves, which are positively correlated with

improved rooting (Rapaka et al., 2005; Zerche and Druege, 2009). A higher dry weight of shoots and roots, root:shoot dry weight ratio and root number under higher DLI has been found in several ornamentals, including *Impatiens*, *Petunia*, *Tecomastans* and *Angelonia angustifolia*. (Lopez and Runkle, 2008; Torres and Lopez, 2011; Currey et al., 2012; Hutchinson et al., 2012). Final plant quality (total dry weight, total number of flowers and the rate of flowering) improves in *Kalanchoe* (Currey and Erwin, 2011), *Limonium* (Chen et al., 2010), and different annual bedding plants (Faust et al., 2005) by an increased net photosynthetic rate under higher DLI (Oh et al., 2009). In cut roses, a complete opening of flower buds is only achieved at high light levels (300 μmol m^{-2} s^{-1}) so that carbohydrate content increases in the petals (Kumar et al., 2008b; Kuiper et al., 1996), ensuring energy supply and osmotic potential. Adding sucrose to the water of cut roses under low light levels in post-harvest conditions does not have the same effect as providing high light levels (Kuiper et al., 1996). Therefore, the use of supplemental light to increase DLI is commonly used to improve plant quality in ornamentals. The time of the day when extra light is provided does not affect the dry matter accumulation, as this is linked to the total light integral (Markvart et al., 2009; Kjaer et al., 2011).

Temperature has also an important influence on plant quality, by determining the demand of carbohydrates needed for biological processes and growth (Gent and Seginer, 2012). If temperatures are low, carbohydrate demand will be low. Under these circumstances the supply of carbohydrates under high DLI might exceed the demand, resulting in a net accumulation of starch and sugars. Correspondingly, increasing the mean daily temperature (MDT) leads to an earlier flowering, but a decreasing number of open flowers, as demonstrated in *Petunia*, *Salvia*, *Tagetes*, *Celosia*, *Impatiens* and *Dahlia* (Pramuk and Runkle, 2005; Moccaldi and Runkle, 2007; Blanchard et al., 2011; Blanchard and Runkle, 2011). For these bedding plants carbohydrate demand is higher at higher temperatures. Higher temperatures reduce the time to flower which limits the time to harvest sunlight, which in turn results in a lower carbon accumulation before flowering and a reduced number of flowers at the end of the production cycle. Similar to flowering time, the flower bud opening is hastened by increasing the MDT as seen in roses and chrysanthemum (Kofranek and Reid, 1983).

Rhododendron is an important horticultural crop with both deciduous and evergreen species and cultivars. In greenhouse production the most important cultivars are derived from the evergreen species *Rhododendron simsii* and *Rhododendron obtusum* (common name azalea). In these cultivars floral initiation and development of the flower bud is separated from the actual flower bud opening by a period of dormancy. Once flower bud dormancy is broken, the final phase in the production of azaleas, the forcing, can start. At this step marketable plants with colour showing flower buds are produced. The time of year at which forcing starts depends on the cultivar. For

very early-flowering cultivars forcing starts in August and continues during autumn and winter. Forcing of very late-flowering cultivars starts in February until April/May. The availability of natural light varies considerably, depending on the period of the year at which the forcing cycle starts. In the northern hemisphere, the natural daily light integral decrease towards December/January and increase again from then on. During the forcing months November, December and January natural light is a limiting factor. Extra light during these months has been shown to hasten flowering (forcing can be shortened with an average of 5 days in November to 3 days in February), improve homogeneity of flowering and intensify flower colour (own observations).

To optimize the light strategy during forcing of azaleas, information is needed on the minimum DLI necessary to produce quality plants. First, we quantified the minimum daily light integral required to balance daily respiration and photosynthesis for an early-flowering and late-flowering azalea cultivar at different temperatures and for flower buds at different developmental stages. To avoid the difficult extrapolation of results generated at the leaf level, CO_2 exchange was measured on a whole-plant level (Jarvis, 1995; Anderson et al., 2000). Next, plants were forced to flower under several light conditions; the minimum DLI for photosynthesis as well as higher DLIs were compared to determine the optimum DLI for high-quality flowering plants.

4.2 Materials and methods

4.2.1 Plant material

Cuttings of two *Rhododendron simsii* hybrids, the early-flowering cultivar 'Nordlicht' and the late-flowering cultivar 'Thesla', were rooted in 12 cm pots (4/pot) in September 2010. The substrate contained a mixture of 9:1 peat:coconut fibres (v/v). Plants were grown under standard cultural conditions and were decapitated twice to stimulate branching. To initiate the generative phase and to suppress the outgrowth of axillary buds, plants were treated six times with 2.25 g L^{-1} chlormequat and two times with 0.012 g L^{-1} paclobutrazol (foliar spray until run-off) in a time span of 8 weeks during July-August 2011. Apical buds were dissected at regular time intervals to monitor flower bud differentiation. Cold storage (7°C, 5 weeks) to release flower bud dormancy of 'Nordlicht' started on 12 September 2011, when the floral primordia were fully differentiated and the style started to enlarge (stage 7; Bodson, 1983). Cold treatment of 'Thesla' started after differentiation of the egg-cells (stage 8) on 1 December 2011 and was maintained for 7 weeks. Subsequently, plants were transferred to a greenhouse maintained at a constant day/night temperature of 21°C with a ventilation set point of 24°C. Screens closed during the day at 200 W m^{-2} and supplemental light (SON-T high pressure lamps) was used during the night and/or

day to ensure a 16h day length and sufficient light intensity. 'Nordlicht' was transferred to the forcing greenhouse on 17 October 2011 and 'Thesla' was transferred on 18 January 2012 for 4 days prior to the photosynthesis measurements of plants at the closed buds stage. Light levels during those first days of acclimation for 'Nordlicht' were as follows: a natural day length of 10.5 hours with an average light intensity of 134 ± 39 µmol m^{-2} s^{-1} and 5.5 hours day length extension with an average light intensity of 74 ± 1 µmol m^{-2} s^{-1}. For 'Thesla' light intensities during the natural day length of 8.5 hours were 36 ± 13 µmol m^{-2} s^{-1} of natural light and 72 ± 2 µmol m^{-2} s^{-1} of supplemental light; and 7.5 hours of day length extension with an average light intensity of 73 ± 1 µmol m^{-2} s^{-1}.

Plants were kept in forcing conditions until buds showed colour for a second round of photosynthesis measurements. For 'Nordlicht' the light intensity during the prolonged period in the forcing greenhouse decreased slightly as the natural light intensity reached an average of 121 ± 59 µmol m^{-2} s^{-1}. For 'Thesla' the mean daily natural light intensity varied from 24 µmol m^{-2} s^{-1} to 172 µmol m^{-2} s^{-1} resulting in an average light level of 63 ± 45 µmol m^{-2} s^{-1}.

4.2.2   Whole-plant gas exchange measuring technique

Photosynthesis was measured at whole-plant level in an open CO_2 gas-exchange system (Figure 4.1). Six Plexiglas cuvettes (internal dimensions: 22.3 x 35 x 50 cm) were installed in a growth chamber (Johnson Controls, Brussels, Belgium). A software controlled program (Labview, National Instruments, Austin, TX, USA) was used to regulate temperature, relative humidity and light intensities (provided by fluorescent lamps) inside the growth chamber. CO_2 concentrations inside the growth chamber were set at 450 µmol mol^{-1} at the beginning of measurements. Air from inside the growth chamber was pumped into each cuvette at a rate of 27.08 ± 1.31 L min^{-1}. Air was extracted alternately from the growth chamber ($CO_{2,in}$) or from a cuvette ($CO_{2,out}$), controlled by magnet valves and analysed with a LI-7000 CO_2/H_2O gas analyser (LI-COR, Lincoln, USA). This resulted in measurements every 8 minutes for each plant. The measurement signal became stable within one minute and the average value of the last two seconds was recorded. Simultaneously, temperature and relative humidity inside the growth chamber and in the measuring cuvette at plant level were recorded. Data were automatically stored in a data file (Labview, National Instruments, Austin, TX, USA). Light intensities were measured and recorded separately every minute (DL2, Delta-T, Cambridge, England) on top of the cuvettes with quantum sensors (LICOR, Lincoln, NE, USA) and recalculated to plant level using a conversion factor related to the transmission of each Plexiglas cuvette.

- —— Air of the growth chamber
- ------ Air of the measuring cuvette
- ☐ Ventilator
- ☐⋈ Magnetic valve
- ◯ Pump
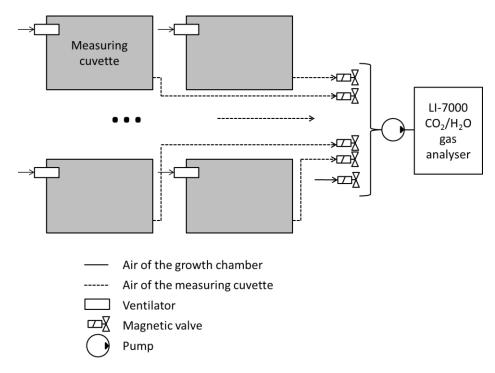
Figure 4.1 Schematic overview of the open CO$_2$ gas exchange measuring system. Six plastic cuvettes were placed in a growth chamber; air from the growth chamber entered the measuring cuvettes by means of a ventilator. Air was extracted from each measuring cuvette and from the growth chamber and sequentially analysed by the LI-700 CO$_2$/H$_2$O gas analyser.

Net photosynthesis was calculated as follows:

$$P_N = \frac{\Delta CO_2 \times F}{A} \frac{273.15}{273.15 + T_c} \frac{1}{22.4}$$

With ΔCO_2 $CO_{2,in} - CO_{2,out}$ (µL L^{-1})

   F Flow rate (L s^{-1})

   A Leaf area (m^2)

   273.15/(273.15 + T_c)   Correction factor for the temperature inside the cuvette T_c (°C)

   22.4   Molar volume of CO$_2$ (µL µmol^{-1})

Since plants were grown from 4 cuttings, it was impossible to seal off the root system and substrate before gas exchange measurements. Net photosynthesis included thus root respiration and microbial or fungal respiration due to organic matter breakdown. Root respiration is considered as a part of the whole-plant carbon exchange. As at the time of the measurements a high root density was present and only a negligible amount of substrate, we considered the total pot respiration as root respiration.

Plants were watered up to field capacity before measurements started to avoid water stress.

After gas exchange measurements plant area and total leaf area were determined by image analysis (WiT 8.3, Teledyne Dalsa, Canada) (Lootens et al., 2000). Leaf area index (LAI) was calculated and was for both cultivars $4.2 \pm 0.1 \, m^2 \, m^{-2}$. Dry weight of leaves and branches was determined separately by drying them at 70°C for 24 h. The specific leaf area (SLA) for 'Nordlicht' was $109.5 \pm 3.3 \, cm^2 \, g^{-1}DW$ and for 'Thesla' $122.2 \pm 1.5 \, cm^2 \, g^{-1}DW$.

4.2.3   Determining the minimum DLI

In a first step the minimum DLI was calculated using daily carbon assimilation data. Net photosynthesis was measured on 6 plants for 'Nordlicht' and 12 plants for 'Thesla' during a 24 h cycle, simulating a natural day: dark period – photoperiod – dark period. Four different photoperiods were tested: 6, 7, 9 and 10 hours. Light intensity during the photoperiod was set to reach $80 \, \mu mol \, m^{-2} \, s^{-1}$ and ranged from 65.3 ± 1.0 to $80.8 \pm 1.1 \, \mu mol \, m^{-2} \, s^{-1}$, depending on the position of the cuvette in the growth chamber. Temperature was $20.3 \pm 0.3 \, °C$ and relative humidity was $74.0 \pm 2.2 \, \%$ at plant level. CO_2 concentration in the growth chamber during measurements was $421.7 \pm 19.3 \, \mu mol \, mol^{-1}$ for 'Nordlicht' and $430.0 \pm 13.5 \, \mu mol \, mol^{-1}$ for 'Thesla'. For each plant and simulated day, the DLI was calculated and plotted against the calculated daily photosynthesis integral (DPI). The minimum DLI at which the DPI = 0 was determined using linear regression.

Whole-plant light response measurements offered a second way to determine the minimum DLI. Net photosynthesis was measured at increasing irradiances from 0 to $800 \, \mu mol \, m^{-2} \, s^{-1}$ in 12 replicates for both cultivars. Measurements at each light intensity were repeated until the net photosynthesis reached a stable level (after approximately 30 to 40 minutes). At plant level, temperature was $20.1 \pm 0.9°C$ and relative humidity was $71.8 \pm 2.5 \, \%$. During measurements, CO_2 concentration in the growth chamber was $438.5 \pm 12.9 \, \mu mol \, mol^{-1}$ for 'Nordlicht' and $438.4 \pm 10.1 \, \mu mol \, mol^{-1}$ for 'Thesla'. Maximum net photosynthesis (P_{max}), quantum efficiency (α_c), and light compensation point (I_c) were calculated by fitting the curve $P_N = P_{max} [1–\exp(–\alpha_c(I–I_c)/P_{max})]$, with P_N the net photosynthesis ($\mu mol \, CO_2 \, m^{-2} \, s^{-1}$) and I the light intensity ($\mu mol \, m^{-2} \, s^{-1}$). Dark respiration ($R_D$) is the positive value of net photosynthesis calculated when $I = 0$ (Lootens et al., 2004). The minimum DLI was determined as follows: $[(P_N(0) \times 86,400 \, s \, d^{-1})/(P_N(0)-P_N(74))] \times 74 \, \mu mol \, m^{-2} \, s^{-1} \times 10^{-6}$. With $P_N(0)$ and $P_N(74)$ the net photosynthesis rate in dark conditions and at $74 \, \mu mol \, m^{-2} \, s^{-1}$ (the mean light intensity during the 24 h cycle measurements), respectively, and 86,400 the number of seconds in one day.

4.2.4 Effect of temperature on minimum DLI

For 'Thesla' the light response curves were measured and fitted (as described above) at different temperatures, 19, 21, 23 and 25°C, this for 12 replicates. CO_2 concentration in the growth chamber during measurements was 431.7 ± 10.0 µmol mol^{-1}. The photosynthetic parameters P_{max}, α_c and I_c were plotted against temperature measured at plant level. The response of measured dark respiration rates to temperature was described by the Arrhenius equation: $R_D = A\ e^{-Ea/RT}$. Where A is a constant; E_a (J mol^{-1}) is the apparent activation energy of the dark respiration reaction; R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T (K) is the absolute temperature. The minimum DLI was calculated as above, with the dark respiration determined by the Arrhenius equation.

4.2.5 Effect of developmental stage on minimum DLI

During the forcing period closed flower buds develop to the colour showing stage. Whole-plant light response curves were determined as described above after forcing the plants for 28 days ('Nordlicht') and 26 days ('Thesla'), when buds showed colour (n = 12). Minimum DLI was calculated as described in section 2.3.

4.2.6 Effect of developmental stage on carbohydrate content

For the late-flowering cultivar 'Thesla', which was forced during the period of the year with very low light conditions, the difference in carbon allocation to leaves and flower buds between the two developmental stages was evaluated. Leaves or flower buds of two plants were pooled and grinded in liquid nitrogen (n = 3). Soluble carbohydrates were extracted from 200 mg fresh weight in 6 mL 80% ethanol for 3 h at 45°C. After centrifugation at 7500 g (5 min), the supernatant was purified with 50 mg mL^{-1} polyvinylpyrrolidon (PVPP). The concentrations of glucose, fructose and sucrose in filtered (0.45 µm, Millipore) and diluted samples were quantified by means of high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) using a Dionex series chromatograph, equipped with a CarboPack PA10 column.

Starch in the leaves was determined by acid hydrolysis of the remaining pellet (1 M HCl for 1 h at 95°C). The pH of the supernatant was adjusted to 7.6 and the sample was diluted to 10 mL. Starch content, expressed as glucose equivalents, was determined enzymatically by the reduction of NADP$^+$ (measured at 340 nm, UV/VIS 916, GBC Scientific Equipment, Australia) with a hexokinase/glucose-6-phosphate dehydrogenase assay.

4.2.7   Forcing experiments

The early-flowering cultivar 'Nordlicht' was forced to flower under four different DLIs. The minimum DLI for photosynthesis of 2.1 mol m^{-2} d^{-1}; a control DLI of 5.4 mol m^{-2} d^{-1} based on measurements of practical forcing conditions; and two DLIs in between: 3.2 and 4.3 mol m^{-2} d^{-1} were chosen as set points. The minimum DLI and the control DLI were tested in a first experiment in 2011 (forcing started on 24 October 2011), the higher DLIs together with the control were tested in a second experiment in 2012 (forcing started on 26 November 2012) with plants grown and treated equally as in 2011. Forcing took place at 21°C and a relative humidity of 65%. Light intensity was recorded every minute during forcing with quantum sensors (LICOR, Lincoln, NE, USA). DLIs were reached by increasing the natural light with supplementary light (high-pressure sodium lamps, 75-80 µmol m^{-2} s^{-1} at canopy level during the first experiment, 110-115 µmol m^{-2} s^{-1} at canopy level during the second experiment). When flower buds showed colour, plants were placed in a climate chamber mimicking living room conditions (20°C, 11 µmol m^{-2} s^{-1}, 14h photoperiod) to assess post-production quality. From the start of forcing, flowering was followed on 5 plants by counting the number of opening flower buds. Time to flower was calculated as the number of days between the start of forcing and 10% flowering. Post-production quality was assessed 38 days after the start of forcing by calculating a flowering index (FI). Therefore opened buds were categorized in three groups according to their developmental stage: colour showing (CS), candle stage (CA) and open flowers (OF). The flowering index was calculated based on the percentages of the developmental stage of the flower: FI = 1 * %CS + 2 * %CA + 3 * %OB.

4.2.8   Statistical analysis

Data in the text are presented as means ± standard error (SE). Results were analysed using SPSS statistical software Version 19.0 (SPSS Inc., Chicago, IL, USA) and means were compared by Student's *t*-test (p=0.05). Comparison of means of flowering parameters was done by the non-parametric Kruskal Wallis and Mann-Whitney U test. Light response curves and the Arrhenius equation were fitted and linear regression was performed using SigmaPlot Version 11.0 (Systat Software Inc., San Jose, CA, USA).

4.3 Results

4.3.1 Daily carbon assimilation

In Figure 4.2, the measured net photosynthesis is shown for 6 plants of 'Thesla' during a simulated day with a photoperiod of 10 h. Ten minutes after the light was switched on, maximum photosynthesis rate was reached. Net photosynthesis remained stable until the end of the photoperiod, at a rate of 2.79 ± 0.06 µmol CO_2 m^{-2} s^{-1} at the highest light intensity and 1.72 ± 0.06 µmol CO_2 m^{-2} s^{-1} at the lowest light intensity. Dark respiration rates remained stable at 0.62 ± 0.04 µmol CO_2 m^{-2} s^{-1} before and after the photoperiod. A similar trend was found for 'Nordlicht' with the net photosynthesis rate ranging between 1.78 ± 0.05 µmol CO_2 m^{-2} s^{-1} and 1.16 ± 0.04 µmol CO_2 m^{-2} s^{-1} and dark respiration rate at 0.76 ± 0.03 µmol CO_2 m^{-2} s^{-1}.
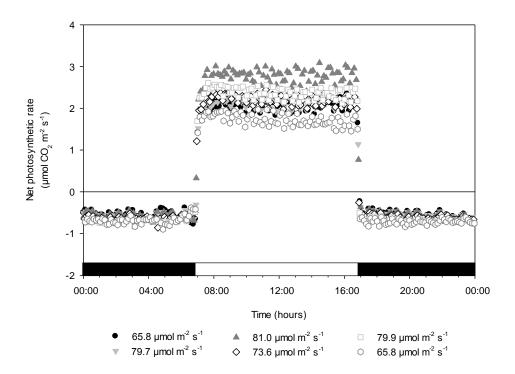
Figure 4.2 Whole-plant net photosynthetic rate for 6 plants ('Thesla') during a 24 h period; measurements were performed simultaneously in 6 cuvettes with slightly different light intensities at plant level. Black bars: dark conditions – white bar: light conditions (10 h)

4.3.2 Determining the minimum DLI

Based on the data obtained during the 24 h cycle measurements, DPI was calculated and plotted against the measured DLI (Figure 4.3). The linear regression fit was high ($R^2 > 0.806$). For 'Nordlicht' DPI increased significantly slower with increasing DLI than for 'Thesla' (Table 4.1). Based on the regressions, the minimum DLI (at DPI = 0) for 'Nordlicht' was 2.10 mol m^{-2} d^{-1} and 1.66 mol m^{-2} d^{-1} for 'Thesla'. A second approach to estimate the minimum DLI was based on the response of net photosynthesis to irradiation. Light response curves for 'Nordlicht' and 'Thesla' are shown in Figure 4.4 (for closed flower buds). Using the photosynthetic parameters from Figure 4.4 and Table 4.2 a minimum DLI of 2.50 mol m^{-2} d^{-1} was determined for 'Nordlicht' and 1.75 mol m^{-2} d^{-1} for 'Thesla'.

Table 4.1 Regression parameters (a, b) and coefficient of determination (R^2) describing the linear regression analysis of the daily photosynthesis integral (DPI, mmol CO_2 m^{-2} s^{-1}) in relation to the daily light integral (DLI, mol m^{-2} s^{-1}) for 'Nordlicht' and 'Thesla'. DPI = a + b x DLI

Cultivar	a ± SE	b ^{z} ± SE	R^2
'Nordlicht'	-64.02 ± 6.87	30.44 ± 3.18 b	0.806
'Thesla'	-71.43 ± 4.63	43.15 ± 2.13 a	0.899

^{z} slopes are significantly different between cultivars (p=0.05, comparison of linear regressions)
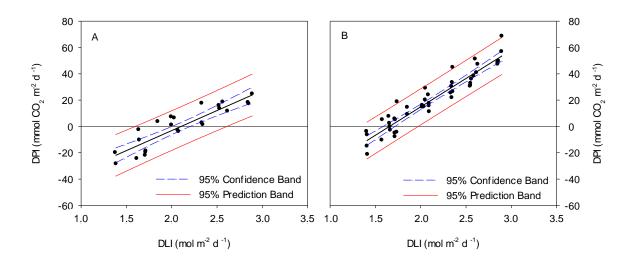
Figure 4.3 Daily photosynthesis integral (DPI) in function of the daily light integral (DLI) for 'Nordlicht' (A) and 'Thesla' (B)

Table 4.2 Parameters of the light response curve, the maximum net photosynthesis (P_{max}), the quantum efficiency (α_c), the light compensation point (I_c) and the dark respiration (R_D) are shown for 'Thesla' at two developmental stages

Flower bud	P_{max} (μmol CO_2 m^{-2} s^{-1})	α_c (μmol CO_2 μmol^{-1})	I_c (μmol m^{-2} s^{-1})	R_D (μmol CO_2 m^{-2} s^{-1})
Closed	8.91 a	0.039 b	18.09 a	0.73 a
Colour showing	9.16 a	0.042 a	18.90 a	0.82 a

Means (n=12) followed by different letters are significantly different (p=0.05, *t*-test)
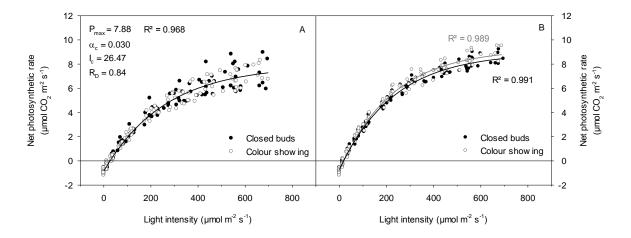
Figure 4.4 Light response curves of whole-plant net photosynthesis (P_N) for 'Nordlicht' (A) and 'Thesla' (B) and effect of developmental stage, comparing plants with closed buds (n=12) and colour showing buds (n=12)

4.3.3   Effect of temperature on photosynthesis and DLI

The effect of temperature on the photosynthetic parameters was investigated for 'Thesla' (Figure 4.5). Temperature did not influence maximum net photosynthesis (P_{max}), which stayed at 9.35 μmol CO_2 m^{-2} s^{-1}. Quantum efficiency decreased slightly from 0.040 to 0.037 μmol CO_2 μmol^{-1} with increasing temperature from 19°C to 25°C (R² = 0.195). Temperature affected strongly the light compensation point and the dark respiration; both increasing at higher temperatures, from 16.37 μmol m^{-2} s^{-1} and 0.75 μmol CO_2 m^{-2} s^{-1} at 19°C to 29.09 μmol m^{-2} s^{-1} and 1.30 μmol CO_2 m^{-2} s^{-1} at 25°C. The activation energy for dark respiration was 65.62 kJ mol^{-1}. Minimum DLI calculated at a constant temperature of 19, 21, 23 and 25°C was 1.72, 2.09, 2.50 and 2.96 mol m^{-2} d^{-1}, respectively; increasing the temperature by 6°C increased the minimum DLI by 72%.
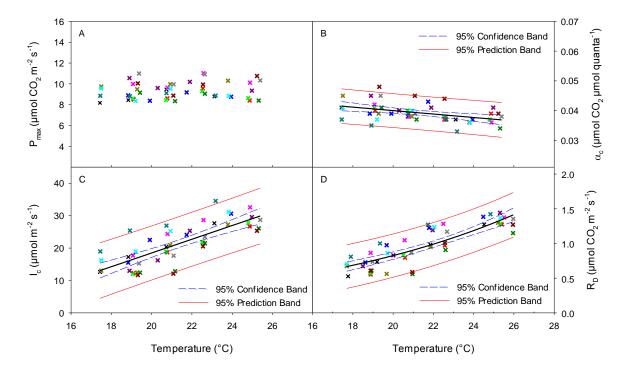
Figure 4.5 Effect of temperature on the calculated photosynthetic parameters P_{max} (A), α_c (B), I_c (C) and measured R_D (D) for 'Thesla'. Regressions: (B) y = 0.0516 - 0.0006 x (R^2 = 0.195); (C) y = -23.91 + 2.12 x (R^2 = 0.600); (D) R_D = 0.41e+12 exp(-65,618/RT) (R^2 = 0.720)

4.3.4 Effect of developmental stage on DLI

To estimate the effect of the developing flower bud on the minimum DLI, light response curves for plants with colour showing buds were recorded (Figure 4.4). For 'Nordlicht' no significant differences were found in light response parameters, thus minimum DLI did not change during the forcing period. For 'Thesla' a minor, but statistically significant, difference in the quantum efficiency was observed. During the forcing period α_c increased from 0.039 µmol CO_2 µmol^{-1} for plants with closed buds to 0.042 µmol CO_2 µmol^{-1} later in the forcing period. This resulted in a minimum DLI of 1.83 mol m^{-2} d^{-1}. This DLI was not significantly different (p = 0.59) from the 1.77 mol m^{-2} d^{-1} for plants with closed buds.

4.3.5 Effect of developmental stage on carbohydrate content

Flower opening affects the source-sink relationship (Table 4.3). In the leaves of 'Thesla' the concentration of glucose and starch remained stable at an average of 2.59 mg g^{-1}FW and 50.59 mg g^{-1}FW respectively. The concentration of fructose was higher in leaves of plants with closed flower buds (2.97 mg g^{-1}FW), compared to plants with colour showing flower buds

(1.91 mg g^{-1}FW). The same was found for sucrose content, which decreased from 14.39 mg g^{-1}FW to 10.90 mg g^{-1}FW during the forcing period.

Table 4.3 Effect of flower developmental stage on carbohydrate content of source (leaves) and sink (flower buds) for 'Thesla'

Sample tissue	Flower bud stage	Glucose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	Sucrose (mg g^{-1} FW)	Starch (mg g^{-1} FW)
Leaves	Closed	2.78 a	2.97 a	14.39 a	50.39 a
	Colour showing	2.40 a	1.91 b	10.90 b	50.78 a
Flower buds	Closed	3.08 b	1.55 b	7.42 a	
	Colour showing	8.45 a	6.05 a	5.29 b	

Means (n=3) followed by different letters are significantly different (p=0.05, *t*-test)

The major change between the developmental stages was found in the flower buds. Closed flower buds had low glucose and fructose concentrations of 3.08 and 1.55 mg g^{-1} FW, respectively. These values increased significantly in colour showing buds to 8.45 and 6.05 mg g^{-1} FW. Sucrose decreased from 7.42 mg g^{-1} FW in closed flower buds to 5.29 mg g^{-1} FW in colour showing buds.

4.3.6   Flowering quality under different DLIs

The obtained DLIs were slightly higher than the set points (Table 4.4). The control DLI of 6.2 and 5.6 mol m^{-2} d^{-1} in experiment 1 and 2 respectively, resulted in a fast opening of flower buds (average 10.1 days) and a high percentage of fully opened flowers (average 72.9 %), with an average FI of 264.7 indicating that all buds showed colour (Table 4.4). The minimum DLI reached during forcing was 2.4 mol m^{-2} d^{-1} and this was insufficient to ensure a good quality of flowering. Time to flower was delayed by almost 6 days compared to the control DLI. Even though the flowering index of 200 indicates that a high percentage of buds opened, a maximum of only 39.9 % flowers were fully open. The two intermediate DLIs 3.3 and 4.5 mol m^{-2} d^{-1}, resulted in a flowering quality with 65.9 and 64.9 % fully opened flowers and a FI of 253.3 and 251.3 that was not significantly different from the highest DLI. Flowering under these conditions started, however, two days later compared to the control.

Table 4.4 Flowering quality under different DLIs for the early-flowering cultivar 'Nordlicht': time to flower (days to reach 10% flowering), maximum % open flowers and flowering index 38 days after the start of forcing

	DLI (mol m-2 d-1)	Time to flower (days)	Max % open flowers	Flowering Index
Experiment 1	2.4 ± 0.1	15.5 c	39.9 b	209.2 b
	6.2 ± 0.1	10.4 a	74.6 a	268.1 a
Experiment 2	3.3 ± 0.1	12.0 b	65.9 a	253.3 a
	4.5 ± 0.1	12.0 b	64.9 a	251.3 a
	5.6 ± 0.1	9.8 a	71.1 a	261.2 a

Means (n=5) followed by different letters are significantly different (p=0.01, Mann-Whitney U test)

4.4 Discussion

4.4.1 Method to determine the minimum DLI

Minimum DLI to balance photosynthesis and respiration can be calculated in two ways. The labour intensive method of measuring net photosynthesis in several 24 h cycles with different photoperiods has the advantage that it takes into consideration the actual plant photosynthesis and respiration over a complete 24 h cycle. Changes in respiration during the night or photosynthesis during the day and transition between dark and light are included in the calculations. Measurement of a light response curve on the other hand requires less effort, but when calculating the minimum DLI fluctuations during the night, the day and the transitions, are not taken into account. Here, a higher minimum DLI was found using light response curves for both 'Nordlicht' and 'Thesla', although the differences were small and within the 95% prediction band of the linear regression of DPI in function of DLI (Figure 4.3). The 24 h measurements showed that dark respiration and net photosynthesis stayed constant and that the transitions from dark to light and vice versa were rapid, explaining the minor differences in minimum DLI between both methods. So both approaches to determine the minimum DLI render reliable results. However, for fast determination of the minimum DLI, light response curves can be advised.

Nonetheless, the measuring conditions must be monitored closely. During the measurements of the light response curves a ΔCO_2 (difference in CO_2 between growth chamber and the inside of the cuvette) of maximum 100 was recorded in our measurements, whereas during a simulated day ΔCO_2 was maximum 30, consistent with values reported elsewhere (Takahashi et al., 2008). The high CO_2 differential during measurements of the light response curves was observed at high PAR, when the relative contribution of CO_2 level to the net carbon exchange rate is also high (Jiao

et al., 1991). During the measurements at high PAR there might have been a reduction in photosynthesis rate due to CO_2 depletion at plant level (inside the cuvette). But, this will be a minor reduction, as Ceulemans et al. (1984) showed in azalea that the net CO_2 gas exchange rate decreased only with 5% when the external CO_2 concentration decreased from 400 to 300 ppm at 700 µmol m^{-2} s^{-1}. Furthermore, we recalculated the minimum DLI using only the measurements with a light intensity below 120 µmol m^{-2} s^{-1} (where the ΔCO_2 does not exceed 30) and had the same results.

Furthermore, there are some considerations to be made using photosynthesis measurements of 24h cycles to determine the minimum DLI. Carbohydrate availability in the plants can influence respiration rates, so light conditions before measurements will influence the minimum DLI by altering the carbohydrate status of the plant. Therefore, light conditions before and during measurements must be similar. In addition to this, if light conditions before measurements are very high, the severe reduction in light intensity will lead to the need of an acclimation period in which the plant regains their initial carbon use efficiency. This acclimation period is species dependent and can take up to 12 days (Frantz and Bugbee, 2005). So the minimum DLI determined will only be of practical use when similar light levels are used during measurements as in practical conditions (acclimation conditions before measurements).

In our case, the light intensities during the 4 days acclimation for 'Thesla' (91 ± 5 µmol m^{-2} s^{-1}) were not substantially higher than during measurements (74 µmol m^{-2} s^{-1}), but the total DLI was higher during the acclimation phase (5.2 ± 0.7 mol m^{-2} d^{-1}). This could lead to an increase in soluble carbohydrates and therefore increase the respiration rate. However, since plants have been in a dark cold room before entering the forcing greenhouse, carbohydrates will be low at the start of acclimation (see chapter 5). During the first days in the forcing greenhouse, photosynthesis recovers and carbohydrates are produced. As a result, a significant increase in starch is seen, which is stored in the plastids, but the level of soluble carbohydrates in the leaves remains relatively stable (chapter 5), and therefore, probably not affecting the respiration rate. This combined with the fact that dark respiration of the plant will be predominantly maintenance respiration, as there is no substantial growth (flower buds are fully formed and there is no vegetative growth); we can assume that the carbohydrate availability had no major influence on respiration (Amthor, 1989). It is also shown in our results of the 24 h cycle measurements that respiration rates remain stable. Moreover, the altered carbohydrate availability in the leaves seen during further acclimation and flower development to colour showing buds, and the higher light intensities during this longer acclimation period did not alter the minimum DLI significantly.

For 'Nordlicht' on the other hand, acclimation started already in higher light intensities (113 ± 5 μmol m^{-2} s^{-1}). This might have led to the higher DLI found for 'Nordlicht' as photosynthesis was less efficient compared to 'Thesla'.

4.4.2   Minimum DLI is cultivar dependent

Apart from the possible effect of the different light acclimation conditions, minimum DLI will also be influenced by genotype. DPI increases more rapidly at increasing DLI for the late-flowering cultivar 'Thesla' than for the early-flowering cultivar 'Nordlicht'. 'Thesla' has a more efficient photosynthesis, as indicated by a higher quantum efficiency, higher maximum net photosynthesis and lower light compensation point. Among growers, 'Thesla' is considered a cultivar with high growth vigour, which is confirmed by its high specific leaf area (see 4.2.2), a typical characteristic of fast growing cultivars (Poorter and Remkes, 1990). Poorter et al. (1990) stated that the relative growth rate of a plant correlates with photosynthesis expressed per unit leaf dry weight. In our experiments, the higher growth vigour of 'Thesla' correlated also with a higher net photosynthetic rate per unit leaf dry weight. Using the specific leaf area, P_N was recalculated and at 74 μmol m^{-2} s^{-1} P_N was 2.30e-2 μmol CO_2 g^{-1} s^{-1} for 'Thesla' and 1.44e-2 μmol CO_2 g^{-1} s^{-1} for 'Nordlicht'. In addition, the higher growth vigour of 'Thesla' positively correlated with a higher whole-plant net photosynthetic rate expressed per unit leaf area. For *Rhododendron simsii*, Ceulemans et al. (1980, 1984) showed also a positive correlation between growth rate and whole-plant photosynthesis.

4.4.3   Effect of temperature on DLI

The minimum DLI of 'Thesla' decreased significantly with decreasing temperature. The combination of a lower dark respiration with an increased net photosynthesis during the light period opens ways for energy saving. For dark respiration a Q_{10} (temperature range 19-25°C) of 2.3 was estimated, meaning that an increase of 10°C will lead to a more than doubled respiration rate. Similar values, a Q_{10} of 2.32 for intermediate canopy leaves and 2.84 for lower canopy leaves, were reported by Bolstad et al. (1999) for *Rhododendron maximum*. So decreasing the night temperature from 21°C to 19°C will result in a decrease of the dark respiration and lead to a lower minimum DLI of 1.8 mol m^{-2} d^{-1} compared to 2.1 mol m^{-2} d^{-1} at constant 21°C (recalculated based on the measurements at different temperatures). A lower dark respiration probably affected the light compensation point, which also decreased with declining temperature. Maximum net photosynthesis was not affected by temperature, meaning that the photosynthesis was measured in the temperature optimum of the plant (Ranney et al., 1995). This temperature optimum lays between 19°C and 25°C, in accordance with previous growth experiments on

Rhododendron simsii by Pettersen (1972). So the increase in net photosynthesis at 74 µmol m^{-2} s^{-1} during the light period with decreasing temperature is a result of a lower dark respiration and almost unchanged quantum efficiency. For photosynthesis, both reduction of the day and the night temperature are advantageous. The minimum DLI of 2.1 mol m^{-2} d^{-1} at constant 21°C can be decreased to 1.7 mol m^{-2} d^{-1} by forcing at a constant temperature of 19°C.

As temperature affects respiration, it will also affect growth. During the forcing of azalea growth is limited to the opening of the flower buds. Nevertheless, decreasing the MDT to 19°C will delay flowering (Pramuk and Runkle, 2005; Blanchard et al., 2011; Gent and Seginer, 2012). Since the forcing cycle should be fast, an alternative strategy to reduce heating costs might be to use a positive DIF (difference between day temperature and night temperature) by maintaining the MDT over 24 h through a lower night temperature and higher day temperature. It has been demonstrated that this strategy does not affect the flowering time in *Lilium longiflorum* (Fisher et al., 1996) and several bedding plants (Blanchard and Runkle, 2011). According to our calculations a day/night temperature of 23/19°C would give a DLI of 2.0 mol m^{-2} d^{-1}, which is slightly lower than the DLI at constant 21°C. This small positive DIF might even have a positive effect on bud opening by increasing cell expansion (Erwin et al., 1994; Bachman and McMahon, 2006). Indeed, the opening of a flower is a process driven by cell division, but mostly by cell expansion (van Doorn and van Meeteren, 2003). Cell expansion is a process which can be enhanced by a positive DIF, but for which a high import of photo-assimilates is necessary to maintain the osmotic potential. The import of photoassimilates can be influenced by a low night temperature; however, the small decrease in night temperature from 21 to 19°C will probably have limited effect on carbon translocation as it was shown for *Rhododendron* that day temperature was more detrimental (Rowe et al., 1994). Consequently, a positive DIF can decrease the minimum DLI, with probably negligible effects on flower opening. In this way the effect is double: saving energy for heating and for assimilation light.

4.4.4   Effect of developmental stage on DLI

Developing flowers require high amounts of soluble carbohydrates for growth and to maintain a high vacuolar osmotic potential to drive water uptake for cell expansion. Developing flower buds are strong sinks which accumulate high concentrations of glucose and fructose, which are provided by sucrose transported from the leaves. This strong sink activity and the high demand of photo-assimilates will enhance net photosynthesis of leaves (Paul and Foyer, 2001). On the other hand, the developing flower will have an increased respiration rate, as demonstrated for azalea, grapefruit, tulip and *Alstroemeria* (Ballantyne, 1963; Collier, 1997; Bustan and Goldschmidt, 1998). Measurements of photosynthesis at the whole-plant level integrate the effect of a higher

net photosynthesis of leaves and higher respiration of colour showing flower buds. If both processes balance each other the overall plant net photosynthesis will remain unchanged in light conditions, as is observed here. As respiration in the leaves will be predominantly maintenance respiration, this respiration is likely to be consistent during the forcing period. Dark respiration might change because of the increased flower bud respiration, but a significant difference between the two developmental stages investigated was not detected here. This indicates that during the early stages of flower bud development, respiration does not increase strongly. This is in agreement with the results of Lay-Yee et al. (1992), who found that during the early stages of flower development in *Hemerocallis* respiration did not alter, but it increased dramatically when petals were fully expanded. Therefore during the forcing phase, up to colour showing buds, DLI should not be adapted due to alterations in source/sink activity.

4.4.5 Minimum DLI and plant quality

Using the data of a typical reference year in Belgium (Dogniaux et al., 1978), DLI was calculated inside a greenhouse with 70% light transmission (Figure 4.6). During the forcing months November, December and January natural light levels result in DLIs often lower than the estimated minimum DLIs for photosynthesis. This will result in a net loss of carbon; therefore supplementary light during the forcing period is necessary. The estimated DLI values to keep a steady-state between photosynthesis and respiration proved insufficient for high quality flowering (Table 4.4). A DLI of $3.3\,mol\,m^{-2}\,d^{-1}$ is at least required for good post-production flowering quality as determined by the forcing experiment, this is 57% higher than the minimum DLI for photosynthesis. Higher light integrals will not contribute to a cost-effective forcing. Comparing this DLI to the daily light integrals of a reference year in Belgium, supplementary lighting from November through mid-February will be necessary to force azalea. Still, azalea is a crop with low light requirements, compared to pot plants such as *Maranta*, *Phalaenopsis* or *Spathiphyllum* which have medium light requirements (between 6 and $12\,mol\,m^{-2}\,d^{-1}$). Crops with high light requirements such as the cut flowers *Alstroemeria*, *Chrysanthemum*, *Gladiolus* and rose need a DLI higher than $18\,mol\,m^{-2}\,d^{-1}$ to meet high-quality standards (Torres and Lopez, 2010).
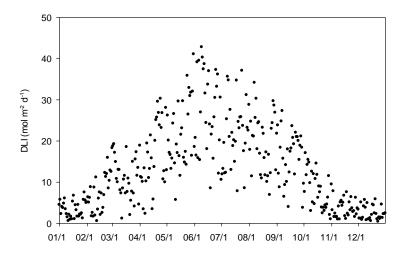
Figure 4.6 Daily light integral inside a greenhouse (70% transmission) for a typical reference year in Belgium

4.5 Conclusions

A minimum daily light integral for photosynthesis of 2.1 mol m^{-2} d^{-1} was estimated for the early-flowering cultivar 'Nordlicht' and 1.7 mol m^{-2} d^{-1} for the late-flowering cultivar 'Thesla'. This minimum DLI was affected by the forcing temperature and increased by 72% when temperature increased by 6°C. The development from closed to colour showing buds had no effect on the minimum DLI requirements of the cultivars investigated. To obtain high-quality flowering plants, a DLI must be given which is at least 57% higher than the estimated DLIs for photosynthesis.

CHAPTER 5

Cold storage to overcome dormancy affects the carbohydrate status and photosynthetic capacity of *Rhododendron simsii*

Chapter 5

Cold storage to overcome dormancy affects the carbohydrate status and photosynthetic capacity of *Rhododendron simsii*

Global warming leads to increasing irregular and unexpected warm spells during autumn, and therefore natural chilling requirements to break dormancy are at risk. Controlled cold treatment can provide an answer to this problem. Nevertheless, artificial cold treatment will have consequences for carbon reserves and photosynthesis. In this paper, the effect of dark cold storage at 7°C to break flower bud dormancy in the evergreen *Rhododendron simsii* was quantified. Soluble carbohydrates and starch content in leaves and flower buds of an early- ('Nordlicht'), semi-early- ('M. Marie') and late- ('Mw. G. Kint') flowering cultivar showed that carbon loss due to respiration was lowest for 'M. Marie', while 'Mw. G. Kint' was completely depleted of starch reserves at the end of cold treatment. Gene isolation resulted in a candidate gene for sucrose synthase (*SUS) RsSUS* which appears to be homologous to *AtSUS3* and had a clear increase in expression in leaves during cold treatment. Photosynthesis measurements on 'Nordlicht' and the late-flowering cultivar 'Thesla' showed that during cold treatment, dark respiration decreased by 58% and 63%, respectively. Immediately after cold treatment, dark respiration increased and stabilized after 3 days. The light compensation point followed the same trend as dark respiration. Quantum efficiency showed no significant changes during the first days after cold treatment, but was significantly higher than in plants with dormant flower buds at the start of cold treatment. In conclusion, photosynthesis stabilized 3 days after cold treatment and was improved compared to the level before cold treatment.

5.1 Introduction

Rhododendron simsii Planch. (Ericaceae) is a semi-evergreen shrub found at elevations of 1,000–2,600 m and widely distributed in subtropical China (Li et al., 2012). The species is submitted to

annual temperature changes between summer and winter that increase with altitude. Therefore, *R. simsii* and its hybrids have developed similar adaptive mechanisms as woody plants of the temperate zone, namely, flower bud dormancy and acclimation to lower but not freezing temperatures. Lower temperatures during winter are necessary to release the flowers from their dormant state. Unfulfilled chilling requirements lead to erratic bud break and a low quality of flowering. When chilling requirements are satisfied, buds will resume growth within 2–3 weeks of favourable growing conditions (Arora et al., 2003). Temperatures for dormancy breaking are generally between 2.5 and 9°C (Richardson et al., 1974) and the optimum for *R. simsii* hybrids is around 7°C (Pettersen, 1971; Bodson, 1989; Kromwijk, 2001). These lower temperatures will, however, affect different plant processes. The rate of biochemical reactions decreases as temperature falls due to limitations in enzyme capacity, and so metabolic and developmental processes slow down (Reid, 1991), although development can continue during the chilling period of dormant buds (Jones et al., 2009).

We use *R. simsii* hybrids as a model for evergreen plants with flower bud dormancy, such as *Camellia*, *Helleborus* and other *Rhododendron* species. The application of controlled cold storage will increase in view of global warming. As cold periods decrease and irregular and unexpected warm spells during autumn increase, chilling requirements will be more difficult to fulfil in a natural way (Luedeling et al., 2011). However, the prevailing dark conditions during controlled cold treatment prevent photosynthesis and thus carbohydrate production. In these conditions carbon and energy will be supplied mainly through degradation of stored starch reserves. Carbon deprivation subsequently leads to reduced enzymatic activities related to carbohydrate metabolism and respiration. In the extreme case of total depletion of carbon reserves, proteins, amino acids and fatty acids are used as a substrate for maintenance respiration (Ishizaki et al., 2005).

During dark and cold conditions, photosynthetic integrity is affected by blocking the oxygen evolution through a loss of manganese; although oxygen-evolving activity is restored within 30 minutes after returning to 18-20°C and moderate light conditions (Shen et al., 1990, Sonoike, 1998). A more severe impact on the photosynthetic apparatus is possible, as carbon starvation leads to proteolysis and loss of chlorophyll and mitochondria (Keech et al., 2007). Bringing plants to higher temperatures and natural light conditions after several weeks of cold treatment will require a period in which photosynthesis must recover to its maximum capacity, in which light-induced enzymes must be transcribed and activated. If the light harvesting complexes are damaged after an artificial cold treatment, then a sudden change to high light intensities might lead to photooxidative damage as the absorption of excess excitation energy leads to the

production of excess reactive oxygen species (ROS), and thus lower light conditions are necessary for the photosynthetic apparatus to recover (Hou et al., 2010).

To date, studies on carbon metabolism during dark storage of woody cuttings and nursery seedlings have been performed at temperatures around 0°C, which almost completely blocks metabolism (L'Hirondelle et al., 2007; Martens et al., 2007). However, effects at dormancy-breaking temperatures are not well documented. In this research we focus on the changes in carbohydrate pools (leaves and flower buds) and expression of genes related to carbohydrate metabolism of evergreen *Rhododendron* during dark cold storage at 7°C. Next, we study the photosynthetic behaviour during acclimation to light and ambient temperatures and the effects on carbohydrate pools. *R. simsii* cultivars belonging to 3 different groups with respect to earliness of flowering and chilling requirements were chosen to obtain insight in the genotypic variation.

5.2   Materials and methods

5.2.1   *Plant material and experimental set-up*

Four *R. simsii* hybrid cultivars were used in the experiments: the early-flowering cultivar 'Nordlicht', the semi-early-flowering cultivar 'M. Marie' and the late-flowering cultivars 'Mw. G. Kint' and 'Thesla'. The four cultivars are characterized by different chilling requirements to break flower bud dormancy and subsequently a longer period at 7°C: 'Nordlicht' requires 672 chilling units (CU), 'M. Marie' 1008 CU, 'Thesla' 1176 CU and 'Mw. G. Kint' 1344 CU, calculated according to the Utah model (Richardson et al., 1974). Plants were grown from four cuttings in 12 cm pots under standard horticultural conditions. To stimulate ramification, plants were decapitated twice. Plants were treated with growth regulators (six applications with 2.25 g L^{-1} chlormequat and 2 applications with 0.012 g L^{-1} paclobutrazol) to initiate the generative phase and supress outgrowth of axillary buds. Apical buds were dissected at regular time intervals to monitor flower bud differentiation. The dormancy-breaking treatment at 7°C started for the early-flowering 'Nordlicht' when the floral primordia were fully differentiated and the style started to enlarge (stage 7; Bodson, 1983); for the other cultivars, cold treatment started after differentiation of the egg cells (stage 8). Detailed dates of the experimental set-up are shown in Table 5.1. Simultaneously, control plants were transferred to a greenhouse at non-dormancy-breaking temperatures (average of 21.1 ± 4.5°C for 'Nordlicht', 13.6 ± 2.8°C for 'M. Marie' and 20.0 ± 1.3°C for 'Mw G. Kint') for gene expression analysis.

In a first experiment, soluble carbohydrates and starch content was followed weekly, both in leaves and flower buds, for a period of 5 weeks of cold storage (7°C) for 'Nordlicht' (from 29/06/2009 until 3/08/2009) and 8 weeks for 'M. Marie' (from 12/10/2009 until 7/12/2009) and

'Mw. G. Kint' (from 1/12/2011 until 26/01/2012). For gene expression analysis, plants were sampled each week during cold storage and control plants (ambient temperature) were sampled simultaneously.

In a second experiment, photosynthesis was measured for 'Nordlicht' and 'Thesla' prior to cold storage (when flower buds were still dormant). After 5 weeks ('Nordlicht'; from12/09/2011 until 17/10/2011) or 7 weeks ('Thesla'; from 8/12/2011 until 26/01/2012) cold storage, plants were transferred to the greenhouse at 21°C and 16 h supplementary light (SON-T, 75-80 µmol m^{-2} s^{-1} at plant canopy level). Photosynthesis was measured daily to follow recovery. On each day of photosynthesis measurements leaf samples were taken to analyse soluble carbohydrates and starch content.

Table 5.1 Experimental set-up: date of planting, last (2nd) decapitation, first PGR application, start of cold treatment and the mean temperature and mean irradiation sum 1 week prior to cold treatment

	'Nordlicht' [z]	'Nordlicht' [y]	'M. Marie' [z]	'Mw. G. Kint' [z]	'Thesla' [y]
Rooting of cuttings	07/2008	11/2010	10/2009	11/2010	11/2010
Last decapitation	23/01/2009	7/06/2011	25/06/2009	26/6/2011	27/06/2011
First PGR application	4/05/2009	27/07/2011	13/08/2009	22/08/2011	25/08/2011
Start cold treatment	29/06/2009	12/09/2011	12/10/2009	1/12/2011	8/12/2011
Temperature	21.9°C	16.8°C	16.7°C	11.5°C	18.1°C
Irradiation sum	13.6 MJ m^{-2}	6.0 MJ m^{-2}	3.5 MJ m^{-2}	1.6 MJ m^{-2}	1.7 MJ m^{-2}

z Plants for determination of carbohydrate content during cold treatment
y Plants for photosynthesis measurements

5.2.2   Determination of soluble sugars and starch

Three replicates of pooled leaves or flower buds from two plants were ground in liquid nitrogen and stored at -80°C until analysis. Ground tissue (200 mg) was extracted in 6 mL 80 % ethanol for 3 h at 45°C. After centrifugation at 7500 g (5 min), the supernatant was purified with 50 mg mL^{-1} polyvinylpyrrolidone (PVPP). The concentrations of glucose, fructose, sucrose, raffinose and stachyose in filtered (0.45 µm; Millipore) diluted samples were quantified by means of high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) using a Dionex series chromatograph, equipped with a CarboPack PA10 column, a pulsed amperometric detector and a gold electrode (Dionex, Sunnyvale, CA, USA). The concentration of starch was determined after extraction of soluble carbohydrates, through acid hydrolysis of the remaining pellet with 1 M HCl for 1 h at 95°C. The pH of the supernatant was adjusted to 7.6 and the sample was diluted to 10 mL. Starch content, expressed as glucose equivalents, was determined enzymatically by the reduction of NADP^{+} (measured at 340 nm, UV/VIS 916; GBC Scientific Equipment, Australia) with a hexokinase/glucose-6-phosphate dehydrogenase assay.

The total carbon content of leaves and flower buds was calculated as the sum of all soluble carbohydrates and the starch content.

5.2.3 Gene expression analysis of carbohydrate metabolism genes

Degenerate primers were developed for the isolation of candidate genes for sucrose synthases (*SUS*), neutral invertase and α-amylase. cDNA amplicons were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced according to the protocol of the Big Dye Terminator v1.1 Cycle Sequencing Kit on an ABI Prism 3130*xl* Genetic analyser (Applied Biosystems). BlastX (Altschul et al., 1997) was used to confirm fragment identity and Clustal Omega (Sievers et al., 2011) was used for the phylogenetic analysis.

Leaf disks of two mature leaves per plant were harvested directly in Eppendorf tubes; samples of 3 plants were bulked in this way. All sampling was done at the end of the light period and samples were immediately frozen in liquid nitrogen. Plant material was stored at -80°C prior to analysis. Grinding was done as described in De Keyser et al. (2013b). A CTAB-based protocol was used for RNA extraction (Wang and Stegemann, 2010). RNA was treated with the DNA-free kit (Ambion; rigorous treatment for high concentrated samples) and subsequently purified using 7.5M LiCl (Ambion) according to the manufacturer's protocol. Absorbance ratios were measured to determine RNA quality, and a SPUD assay (Nolan et al., 2006) was used to check for PCR inhibition. cDNA synthesis (including noRT samples) and RT-qPCR (including reference gene validation) were performed as described in De Keyser et al. (2013b). RT-qPCR primers for *SUS* (forward: 5'-GGAAATCTTGTTGCGTCTTTGTT-3'; reverse: 5'-GTCCGAGTCAGGATATTTCGTTTT-3') were developed with Primer3Plus software. All RT-qPCR data analysis was done by means of the qBase[+] software (Biogazelle; Hellemans et al., 2007) with gene-specific amplification efficiencies and multiple validated reference genes.

5.2.4 Photosynthesis measurements

Light response curves of full-grown sun adapted leaves were measured on at least 6 plants at a constant CO_2 concentration of 400 µmol CO_2 mol^{-1}, 60% relative humidity and a leaf temperature of 21°C with an open gas exchange system (LI-6400, LI-COR, Lincoln, USA). Air flow rate was set at 250 µmol s^{-1}. The following irradiances were used: 1000, 750, 500, 250, 100, 50, 30, 10 and 0 µmol m^{-2} s^{-1}. The photosynthetic parameters - maximum net photosynthesis (P_{max}), quantum efficiency (α_c), and light compensation point (I_c) - were calculated by fitting the curve $P_N = P_{max} [1-\exp(-\alpha_c(I-I_c)/P_{max})]$. Dark respiration ($R_D$) is the positive value of net photosynthesis calculated when $I = 0$ (Lootens et al., 2004).

5.2.5 *Statistical analysis*

Physiological data and photosynthetic parameters were analysed using SPSS statistical software Version 19.0 (SPSS Inc., Chicago, USA) and means were separated by Student's *t*-test or ANOVA followed by Tukey HSD test (p=0.05). Light response curves were fitted using SigmaPlot version 11.0 (Systat Software, San Jose, CA, USA).

5.3 Results

5.3.1 *Carbohydrate metabolism during cold storage*

In leaf tissue, glucose and fructose levels increased during cold treatment for 'Nordlicht' (Figure 5.1A-B) and 'M. Marie' (Figure 5.2A-B). Initial levels were comparable for both cultivars: glucose 1.85 and 1.27 mg g^{-1}FW, respectively, fructose 1.55 and 1.39 mg g^{-1}FW, respectively. In contrast, the initial glucose (3.17 mg g^{-1}FW) and fructose (2.61 mg g^{-1}FW) concentrations were higher in the leaves of 'Mw. G. Kint', but decreased continuously with increasing weeks at 7°C to 2.06 and 1.42 mg g^{-1}FW, respectively (Figure 5.3A-B). The oligosaccharides raffinose and stachyose accumulated in leaves of 'Nordlicht' (Figure 5.1C-D) and 'M. Marie' (Figure 5.2C-D). At the end of cold treatment raffinose levels were higher for 'Nordlicht' (1.16 mg g^{-1}FW) compared to 'M. Marie' (0.83 mg g^{-1}FW), while stachyose levels were higher for 'M. Marie' (0.47 mg g^{-1}FW) than 'Nordlicht' (0.25 mg g^{-1}FW). Raffinose content in the leaves of 'Mw. G. Kint' started with a high initial concentration of 1.14 mg g^{-1}FW, comparable with the concentration at the end of cold treatment for 'Nordlicht', but decreased rapidly the first week and continued a slow decrease to 0 at the end of 8 weeks (Figure 5.3C). No stachyose was found in the leaves (Figure 5.3D). Leaf sucrose concentrations remained stable at 12.78 mg g^{-1}FW for 'Nordlicht' and 19.12 mg g^{-1}FW for 'M. Marie' during 5 weeks of cold treatment (Figure 5.1E and Figure 5.2E). Prolonged cold for 'M. Marie' did cause a decrease in sucrose thereafter: to 14.32 mg g^{-1}FW after 8 weeks. Sucrose levels decreased slowly from the start for 'Mw. G. Kint', from 11.15 to 6.70 mg g^{-1}FW (Figure 5.3E). Starch content in 'M. Marie' showed the same trend as sucrose levels: a stable concentration for 5 weeks (64.74 mg g^{-1}FW) followed by a decrease to 43.60 mg g^{-1}FW after 8 weeks at 7°C (Figure 5.2F). On the other hand, starch concentrations dropped rapidly during the first weeks of cold treatment in the leaves of 'Nordlicht' and 'Mw. G. Kint', leaving only 15.83 mg g^{-1}FW of the initial 64.74 mg g^{-1}FW in 'Nordlicht' (Figure 5.1F) and 0.68 mg g^{-1}FW from the low initial start concentration of 22.54 mg g^{-1}FW in 'Mw. G. Kint' (Figure 5.3F).

The total carbon content in the leaves decreased during cold treatment for all three cultivars. For 'Nordlicht', the decrease from 2700 to 1287 μmol C g^{-1}FW was continuous, while in 'M. Marie' carbon content started to decrease only after 5 weeks of cold, from a higher initial concentration

of 3043 µmol C g^{-1}FW to 2563 µmol C g^{-1}FW at the end of 8 weeks at 7°C. The total initial carbon content in the leaves of 'Mw. G. Kint' was 1376 µmol C g^{-1}FW, which is remarkably lower compared to the other cultivars, and decreased continuously to only 373 µmol C g^{-1}FW at the end of cold treatment.
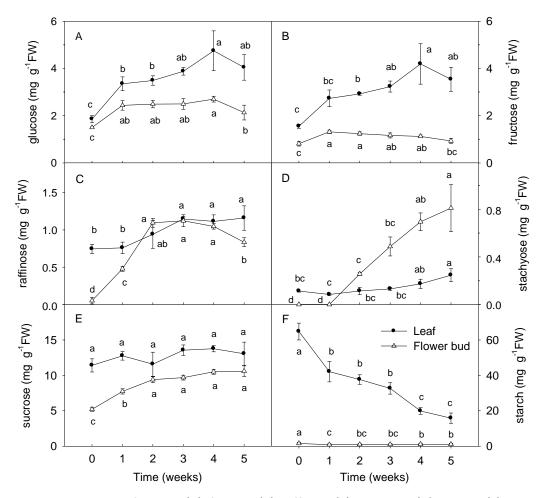
Figure 5.1 Concentrations of glucose (A), fructose (B), raffinose (C), stachyose (D), sucrose (E) and starch (F) in leaves (●) and flower buds (△) of 'Nordlicht' during increasing weeks at 7°C. (mean ± SD; n=3; different letters in time are significantly different according to Tukey HSD (p=0.05))

In the flower buds, only 'Nordlicht' showed a significant increase in glucose and fructose levels during the first week of cold treatment: from 1.50 and 0.81 mg g^{-1}FW to 2.44 and 1.31 mg g^{-1}FW, respectively (Figure 5.1A-B). From the second week onwards, concentrations stabilized and declined towards the end of cold treatment. For 'M. Marie' (Figure 5.2A-B) and 'Mw. G. Kint' (Figure 5.3A-B) glucose and fructose concentrations decreased steadily from the start. Raffinose and stachyose accumulated far more in flower buds than in leaves for 'Nordlicht' (Figure 5.1C-D). Raffinose increased from 0.05 to 1.10 mg g^{-1}FW in the first three weeks after which the concentration stabilized; after 5 weeks, the concentration decreased to 0.84 mg g^{-1}FW. Stachyose levels increased constantly from 0 to 0.81 mg g^{-1}FW. The increase in raffinose and stachyose for

'M. Marie' was less pronounced compared to 'Nordlicht' and concentrations started to decrease towards the end of the cold treatment (Figure 5.2C-D). Raffinose levels decreased from week 3 onwards, to an initial concentration of 0.18 mg g^{-1}FW. Stachyose levels continued to increase until week 6 to 0.50 mg g^{-1}FW and fell back to 0.38 mg g^{-1}FW after 8 weeks. In flower buds of 'Mw. G. Kint' raffinose (0.53 mg g^{-1}FW) and stachyose (0.31 mg g^{-1}FW) levels remained relatively stable during the 8 weeks of cold treatment (Figure 5.3C-D). Sucrose accumulated in flower buds of 'Nordlicht' during the first 2 weeks, after which the concentration stabilized at 10.05 mg g^{-1}FW (Figure 5.1E). Also for 'M. Marie', an increase was seen the first week from 7.70 to 10.15 mg g^{-1}FW, but levels slowly decreased thereafter to reach the initial concentration again after 8 weeks at 7°C (Figure 5.2E). The decrease in sucrose started immediately for 'Mw. G. Kint', from 7.74 to 5.62 mg g^{-1}FW (Figure 5.3E). Starch content in flower buds was negligible compared to that in leaves (Figure 5.1F, Figure 5.2F and Figure 5.3F) for all cultivars.
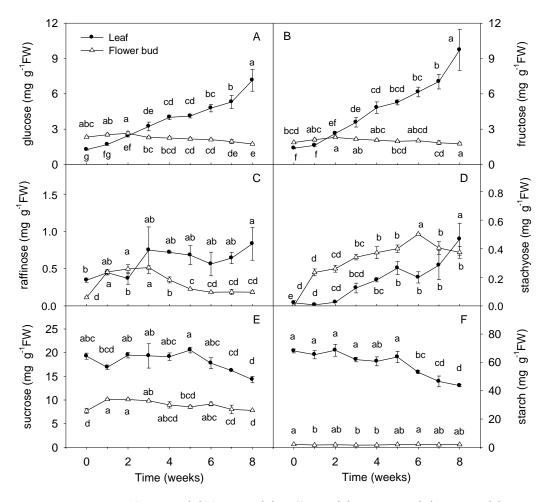
Figure 5.2 Concentrations of glucose (A), fructose (B), raffinose (C), stachyose (D), sucrose (E) and starch (F) in leaves (●) and flower buds (△) of 'M. Marie' during increasing weeks at 7°C. (mean ± SD; n=3; different letters in time are significantly different according to Tukey HSD (p=0.05))

At the start of cold treatment, the total carbon content in flower buds was lowest in 'Nordlicht' (314 µmol C g^{-1}FW) compared to 484 and 432 µmol C g^{-1}FW for 'M. Marie' and 'Mw. G. Kint', respectively. During the first weeks of cold treatment, carbon content increased in flower buds of 'Nordlicht' (596 µmol C g^{-1}FW) and 'M. Marie' (606 µmol C g^{-1}FW) after which the concentration decreased to 568 µmol C g^{-1}FW and 476 µmol C g^{-1}FW, respectively. For 'Mw .G. Kint', there was a continuous decrease, reaching 285 µmol C g^{-1}FW at the end of cold treatment.
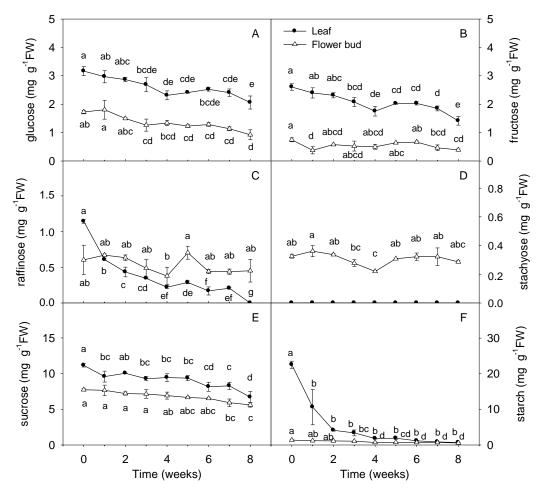

Figure 5.3 Concentrations of glucose (A), fructose (B), raffinose (C), stachyose (D), sucrose (E) and starch (F) in leaves (●) and flower buds (△) of 'Mw. G. Kint' during increasing weeks at 7°C. (mean ± SD; n=3; different letters in time are significantly different according to Tukey HSD (p=0.05))

5.3.2 Gene expression profiling during cold treatment

A candidate gene for *SUS* (*RsSUS*; Acc. N° HG969196) was successfully isolated. The fragment length was 346bp; BlastX confirmed homology with orthologous genes from other species (Table 5.2). Phylogenetic analysis classified the azalea *SUS* gene in the Sus II group (Chen et al., 2012) together with *AtSUS3*, *OsSUS4* and *GaSUS6* (Figure 5.4). The last gene is one of the few members of the cotton *SUS* gene family that is expressed in leaves (Chen et al., 2012). For other enzymes of the carbohydrate metabolism, α-amylase and neutral invertase, sequence homology

was not optimal (data not shown) so these genes were not retained for further gene expression analysis.

Table 5.2 Sequence homology (Blastx; swissprot database) of the *SUS* gene isolated in azalea (*RsSUS*; Acc. N° HG969196) with orthologs in other species

Species	Gene	Acc. N°	E-value	Homology
Arabidopsis thaliana	*AtSUS3*	sp|Q9M111.1	3E-69	88%
Oryza sativa	*OsSUS4*	sp|Q10LP5.1	3E-67	86%
Arabidopsis thaliana	*AtSUS2*	sp|Q00917.3	1E-65	85%

The absorption ratios of RNA ($A_{260/230}$) were suboptimal (Additional data 1), but no PCR inhibition was present in these samples according to the results of the SPUD assay (Additional data 2). Hence, all RNA was considered of sufficient quality for RT-qPCR. In qbase+, standard-curve derived gene and run specific amplification efficiencies (Additional data 3) were used for calculation of gene expression values (calibrated normalised relative quantity: CNRQ). Reference gene validation resulted in the use of 3 reference genes for gene expression normalization (GAPDH, HK5 and HK47). Normalization factor stability (M-value) within the specific assays was 0.783 (CV = 0.321), 0.532 (CV = 0.211) and 0.976 (CV = 0.397) for 'Nordlicht', 'M. Marie' and 'Mw. G. Kint', respectively. There were no DNA contamination problems from the noRT samples, and no primer dimers were detected in the melting profiles.
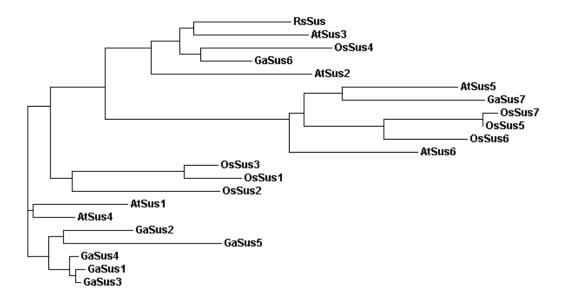
Figure 5.4 Phylogenetic analysis of the azalea *SUS* gene (*RsSUS*) with other plant homologs. Isozymes in corresponding plant species are according to Chen et al. (2012): cotton, *GaSUS* 1 to 7; *A. thaliana*, *AtSUS* 1 to 6 and rice *OsSUS* 1 to 7

When we examined the expression profile of *SUS* during cold treatment, it was significantly up-regulated in leaves of all 3 cultivars grown at 7°C compared to plants kept in the greenhouse (Figure 5.5). In 'Nordlicht', *SUS* expression continuously increases to 4 weeks of cold treatment, then decreased slightly but remained 7-fold higher compared to plants in the greenhouse. In 'M. Marie', expression immediately peaks after 1 week of cold treatment, with a 7-fold difference, but then decreased to only 2-fold difference at the end of treatment. A similar pattern was seen in the expression profile in 'Mw. G. Kint': an immediate increase of 10 and 20-fold differences after 1 and 2 weeks, respectively. But the expression then dropped rapidly to a steady-state level with only a 2 to 3-fold difference compared to the greenhouse plants. Expression in flower buds was negligible compared to leaves and therefore not discussed in this paper.
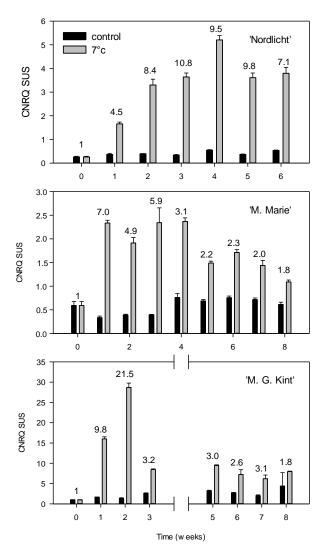
Figure 5.5 Gene expression profiles (CNRQ, not log-transformed) of *SUS* in leaves of 'Nordlicht', 'M. Marie' and 'Mw. G. Kint' during dormancy breaking at 7°C (grey bars) and control in the greenhouse (black bars). Error bars indicate SE. Numbers above the bars correspond to fold differences between 7°C and control.

5.3.3 Effect of cold treatment on photosynthesis and carbohydrate metabolism

In terms of photosynthetic parameters during forcing conditions after cold treatment (Table 5.3), there were no significant differences in maximum net photosynthesis (6.11 µmol CO_2 m^{-2} s^{-1}) for 'Nordlicht', while P_{max} significantly increased from 7.06 to 11.23 µmol CO_2 m^{-2} s^{-1} after 3 days in forcing conditions for 'Thesla'. Quantum efficiency remained stable for both cultivars, at 0.054 µmol CO_2 µmol^{-1} ('Nordlicht') and 0.052 µmol CO_2 µmol^{-1} ('Thesla'). The light compensation point increased during the first two days in forcing conditions, from 9.22 and 6.58 µmol m^{-2} s^{-1} on day 0 to 12.01 and 10.17 µmol m^{-2} s^{-1} on day 2 for 'Nordlicht' and 'Thesla', respectively; after which I_c returned towards its initial values. Dark respiration increased significantly for 'Nordlicht': from 0.48 µmol CO_2 m^{-2} s^{-1} immediately after cold treatment to 0.69 µmol CO_2 m^{-2} s^{-1} after one day in forcing conditions. During the following days in forcing conditions, dark respiration remained stable, and pair-wise comparisons showed significant differences between all days and day 0 (p<0.02). Dark respiration remained relatively stable for 'Thesla', and there was only a slight increase from 0.35 to 0.50 µmol CO_2 m^{-2} s^{-1} after 1 day of forcing.

Table 5.3 Photosynthetic parameters maximum net photosynthesis (P_{max}), light compensation point (I_c) and dark respiration (R_D), soluble sugars and starch content for 'Nordlicht' and 'Thesla' before and after cold storage (7°C).

Cultivar	Days after cold	P_{max} (µmol CO_2 m^{-2} s^{-1})	I_c (µmol m^{-2} s^{-1})	R_D (µmol CO_2 m^{-2} s^{-1})	Soluble sugars (mg g^{-1}FW)	Starch (mg g^{-1}FW)
'Nordlicht'	-35	4.88 b	25.96 b	1.14 c	18.4 d	68.1 a
	0	5.86 a	9.22 a	0.48 a	24.2 a	33.1 b
	1	5.67 a	11.68 a	0.69 b		
	2	6.44 a	12.01 a	0.66 ab	23.5 ab	40.0 b
	3	6.24 a	10.43 a	0.59 ab	20.9 c	39.8 b
	4	6.33 a	10.15 a	0.61 ab	22.9 b	43.2 b
'Thesla'	-49	11.01 a	18.47 c	0.95 b	23.7 bc	63.8 a
	0	7.06 b	6.58 a	0.35 a	25.1 b	16.2 e
	1	9.16 ab	9.77 b	0.50 a	27.0 a	25.2 d
	2	9.60 ab	10.17 b	0.52 a	24.1 bc	42.2 c
	3	11.23 a	8.98 ab	0.47 a	23.4 bc	46.2 c
	7	10.97 a	8.15 ab	0.47 a	22.4 c	58.7 b

Means (n=6-8 for photosynthetic parameters, n=3 for soluble carbohydrates and starch) followed by different letters are significantly different (p=0.05, Tukey HSD test), cultivars were analysed separately.

For both of the above cultivars, photosynthesis stabilized after 3 days in forcing conditions. Comparing with photosynthesis at the start of cold treatment, there were differences in both cultivars. 'Nordlicht' plants with dormant flower buds had a significantly (t-test, p=0.08) lower maximum net photosynthesis of 4.88 µmol CO_2 m^{-2} s^{-1} compared to 6.24 µmol CO_2 m^{-2} s^{-1} after 3

days of stabilization. Also quantum efficiency in plants with dormant flower buds (0.040 µmol CO_2 µmol^{-1}) was significantly lower (p<0.001) compared to the 0.054 µmol CO_2 µmol^{-1} in plants that are ready to flower. 'Thesla', on the other hand, showed no significant differences in P_{max} and α_c. Light compensation point was for 'Nordlicht' (25.96 µmol m^{-2} s^{-1}) and 'Thesla' (18.47 µmol m^{-2} s^{-1}) significantly higher in plants with dormant flower buds compared to plants ready to flower: 10.43 and 8.98 µmol m^{-2} s^{-1}, respectively. The same is seen for dark respiration where 1.14 and 0.95 µmol CO_2 m^{-2} s^{-1} before cold treatment decreased to 0.59 and 0.47 µmol CO_2 m^{-2} s^{-1} after cold treatment for 'Nordlicht' and 'Thesla', respectively.

Parallel to the photosynthesis measurements, carbohydrates were measured in the leaves (Table 5.3). At the start of cold treatment, soluble sugar content for 'Nordlicht' (18.3 mg g^{-1}FW) and 'Thesla' (23.6 mg g^{-1}FW) was lower compared to the content after cold treatment (24.1 and 25.1 mg g^{-1}FW, respectively). In forcing conditions, the concentration increased the first day for 'Thesla' to 27.0 mg g^{-1}FW, after 2 days concentrations decreased in both cultivars. On the other hand, starch levels were significantly higher before cold treatment in both cultivars. Returning plants to light conditions led to a continuous increase in starch content: from 33.1 to 43.2 mg g^{-1}FW after 4 days for 'Nordlicht' and from 16.2 to 58.7 mg g^{-1}FW after 7 days for 'Thesla'.

5.4 Discussion

5.4.1 Carbohydrate metabolism during cold storage

Cold storage at 7°C induce metabolic changes that also occur under cold acclimation. Cold acclimation starts when plants are exposed to low, non-freezing temperatures for days or weeks. These low temperatures enhance the activity of the starch degrading enzymes (α-amylase and β-amylase; Kaplan et al., 2006) and induce a substantial increase in soluble carbohydrates in plant cells. Recently, Schulze et al. (2012) showed that the concentrations of glucose and fructose increase considerably in the vacuole, making it the major area for sugars during cold treatment. These carbohydrates function as cryoprotectants by increasing solute concentration and thus depressing the freezing point of cells and through stabilizing membranes. Wright and Aung (1975) found that in mid-winter the predominant sugars in *Rhododendron* 'Sweetheart Supreme' and 'Hexe' are glucose, fructose and sucrose. Also our results show an increase in glucose and fructose content and high levels of sucrose in 'Nordlicht' and 'M. Marie' during cold treatment. Sucrose synthase (SUS) is a key enzyme in sucrose catabolism. The increase in *SUS* gene expression at 7°C in both cultivars explains the observed increase in glucose and fructose. The phylogenetic study of our putative *RsSUS* gene showed a close relationship with *AtSUS3* (Table 5.2). In *Arabidopsis,* the expression level of *AtSUS3* in rosette leaves increased after 48 h at 4°C (Baud et al., 2004).

Further, raffinose and stachyose are formed in response to cold, this mainly in the flower buds. These oligosaccharides increase also in flower buds of *Forsythia* (Flinn and Ashworth, 1995) during cold acclimation. As raffinose and stachyose interact with membranes, they can increase membrane stability (Hincha et al., 2003).

In contrast, the soluble carbohydrate content in the leaves of 'Mw. G. Kint' shows a steady decline despite the up-regulation of *RsSUS* one and two weeks after the start of the cold treatment. Further investigation is needed to understand why *RsSUS* is only up regulated in the first 2 weeks of the cold treatment. This is probably not due to a decrease in sugar content, as sugar levels do not regulate the expression of *AtSUS3* (Baud et al., 2004). The lack of maintenance of the initial sugar content in 'Mw. G. Kint' is likely due to the low leaf starch storage pool at the start of treatment and its depletion after 2 weeks at 7°C. Furthermore, higher levels of soluble sugars were already present in the leaves of 'Mw. G. Kint' at the start of cold treatment, as compared to the 2 other cultivars. These differences between the cultivars at the start of treatment can be explained by the environmental conditions. Cold treatment for 'Nordlicht' started in early summer, with sufficient light (Table 5.1) and photosynthesis resulting in a high level of starch. Also, for 'M. Marie' light was still sufficient for maintenance of starch levels in autumn, while the cold treatment for 'Mw. G. Kint' started at the end of autumn, when light levels outside were low and temperatures had already reached dormancy-breaking levels (Pettersen, 1971). These temperatures resulted in an increase of soluble carbohydrates (hence the higher initial concentrations) and, due to the low photosynthesis, a decrease in starch levels. Apparently, the decreased starch levels seem to induce *SUS* expression at the start of cold treatment in 'Mw. G. Kint'. Once starch was depleted after 3 weeks, *SUS* expression dropped, suggesting that starch content tends to have an impact on *SUS* gene expression. However, more experiments are needed to confirm this.

There are large differences between cultivars in ability to maintain carbon levels during cold treatment. In particular, 'M. Marie' is able to maintain carbon reserves for a long period at 7°C. This cultivar has a higher demand for cold than 'Nordlicht', and therefore seems better adapted to longer periods at low temperatures. However, a sufficient carbohydrate reserve is needed at the start of the storage period. The low carbohydrate reserves in 'Mw. G. Kint' at the beginning of cold treatment result in plants heavily depleted in carbon at the end of cold treatment, which will have repercussions on flowering.

5.4.2   *Photosynthetic recovery after cold treatment*

Recovery of photosynthesis occurs rapidly: after 3 days, the photosynthetic parameters stabilize. This indicates that there is no severe damage to the photosynthetic apparatus, which would have

led to a longer recovery time. When cold-acclimated *Rhododendron catawbiense* was brought into favourable temperature conditions, the capacity of carbon assimilation fully recovered over a period of approximately 14 days, with a linear and rapid recovery for the first 4 days (Harris et al., 2006). It was suggested that chronic photoinhibition might cause this slow recovery, although plants were kept at light intensities of only 140 µmol m^{-2} s^{-1}.

When plants are forced in August or September, natural light intensities are high. It might be necessary after cold treatment to acclimate *R. simsii* plants under moderate light conditions. Immediate exposure of dark stored, white spruce seedlings to high light intensities after 6 months of storage, resulted in a decline in maximum net photosynthesis, as compared to plants exposed to low light levels, which started to increase maximum net photosynthesis after cold storage (Camm et al., 1993). The authors argued that after 6 months of dark, cold storage the protective capability of the membranes was probably near saturation at the low light levels, so the membranes possessed limited ability to undergo further changes permitting dissipation of extra energy, resulting in chronic photoinhibition at high light levels. Hou et al. (2010) suggested a gradual increase in light intensities after dark shipping of *Phalaenopsis* was necessary to achieve better photosynthetic recovery. Even though we assume that enzymatic activity is rapidly restored within the first hours of light exposure, it is possible that immediately after cold storage high light intensities might cause chronic photoinhibition. However, in our experiment a peak of 289 µmol m^{-2} s^{-1} during the first days of forcing resulted in no indication of chronic photoinhibition.

5.4.3 Effect of dormancy status of the flower buds on photosynthesis

Photosynthesis is less efficient in plants with dormant flower buds at the start of cold treatment, compared to plants ready to flower after cold treatment. Increases in maximum net photosynthesis and quantum efficiency and decreases in light compensation point and dark respiration are observed. These effects on photosynthesis are also found in dormant seedlings of Norway spruce (Helenius et al., 2005); where the maximum efficiency of photosystem II was lower than in growing seedlings. The differences in maximum net photosynthesis and quantum efficiency between plants with dormant flower buds and plants ready to flower are the result of altered source-sink relationships in the plant. When flower buds become dormant, the sink demand decreases (Richardson et al., 2010) and the unloading of the phloem is decreased, leading to high sucrose levels in the source, which in turn results in a lower photosynthetic rate due to a decreased expression of photosynthetic genes (Paul and Foyer, 2001). In our plants, we did not see an accumulation of sucrose, but there were high levels of starch, which can also lead to feed-back inhibition of photosynthesis (Paul and Foyer, 2001). When dormancy is broken,

flower buds become highly active sinks with a continuously increasing demand for sucrose to support the flower opening, which thus requires high photosynthetic activity.

5.4.4   Effect of cultivar on photosynthesis

Maximum net photosynthesis was much higher and the light compensation point was lower for the late-flowering cultivar 'Thesla' compared to the early-flowering cultivar 'Nordlicht'. Possible explanations include higher growth vigour of 'Thesla' leading to a higher photosynthesis rate (Ceulemans et al., 1984) or the fact that 'Thesla' had already acclimated to lower light levels (Ehleringer and Sandquist, 2010), since the first measurements on 'Thesla' started at the end of November. These plants already spent two months inside the greenhouse at lower light levels (Table 5.1), while for 'Nordlicht' on the other hand, plants were grown outside until the first measurements in September.

5.5   Conclusions

During dark, cold treatment (7°C) of *Rhododendron simsii* there is an increase in glucose and fructose when initial leaf starch content is high. When leaf starch content is low at the start of cold treatment, plants will be heavily depleted in carbon at the end of cold treatment. The expression of the successfully isolated candidate gene for *SUS* (*RsSUS*) increased during cold treatment, resulting in an increase of glucose and fructose. This putative *RsSUS* gene showed a close relationship to *AtSUS3*. After the dormancy-breaking cold treatment, photosynthesis stabilized in 3 days and was enhanced compared to plants with dormant flower buds, before the cold treatment.

CHAPTER 6

Source-sink metabolism during flowering of azalea is influenced by

light conditions

Chapter 6

Source-sink metabolism during flowering of azalea is influenced by light conditions

Flower opening relies on the import of carbohydrates. In this paper, we investigated in which stage of flower development the quality of flowering is most susceptible for reduced photosynthates. The light conditions prior to cold treatment to break dormancy played only a minor role. Light conditions after cold treatment, during forcing, proved to be most determining for quality of flowering. During cold treatment, leaf carbohydrates decreased significantly. To increase the leaf soluble carbohydrates and starch content during forcing, supplemental lighting is needed. Continued flower development at indoor conditions, rely on these leaf carbon reserves. Developing flowers strongly increase their glucose and fructose content, which is accompanied by an increased invertase activity. Carbohydrate metabolism and quality of flowering was also followed for plants treated with a high dose of paclobutrazol. Foliar soluble carbohydrates and starch levels decreased slightly less during cold treatment for paclobutrazol-treated plants. However, in forcing conditions, the carbohydrate content in the petals during flower opening was not affected by the high dose of paclobutrazol. Flower opening rate was not affected by the high dose of paclobutrazol, only flower diameter proved to be smaller.

6.1   Introduction

As hybrid within the genus *Rhododendron*, azalea is well known for its beautiful flowering. For complete flower opening, petal growth is essential. This is a process of cell division and cell expansion. Whereas cell division in petals is the primary mechanism during the first stages of petal growth, cell expansion becomes the dominant process to fully open flowers (Reale et al., 2002). Cell expansion is a combined process of cell wall weakening, carbohydrate partitioning and water uptake. During flower opening, an increase in the expression of an expansin gene was seen

by Ma et al. (2012). These expansins are extracellular proteins involved in cell wall modifications, which contribute to cell expansion. Further, large amounts of soluble carbohydrates accumulate in the petals, especially in vacuoles (Yamada et al., 2009). The import of carbohydrates is necessary for biosynthesis and maintenance, but serves also as osmoticum. As a consequence of carbohydrate import, the osmotic water potential in petal cells is lowered, promoting water influx which drives cell expansion (Tarpley and Sassenrath, 2006).

To provide soluble carbohydrates to the developing flower (sink), sucrose is the main sugar transported from the source leaves to the sink, by phloem transport. This transport is driven by a pressure difference between sources and sinks (Münch, 1930). Hence, sucrose metabolism is a key factor in flowering, as sucrose must be hydrolysed to continue phloem unloading in the sinks and maintain phloem transport from source to sink. Key enzymes in sucrose metabolism are sucrose phosphate synthase (SPS), sucrose synthase (SUS) and invertases. *SPS* genes are mostly expressed in photosynthetic tissues, but SPS activity has also been shown to play a role in flower tissue of orchids and rose where it was associated with increased sucrose content in the petals (Li et al., 2003; Kumar et al., 2007). SUS catalyses the reversible reaction that splits sucrose into fructose and UPD-glucose. SUS activity in sink organs has been correlated with sucrose unloading and sink strength in tomato fruit (Sun et al., 1992; D'Aoust et al., 1999). The non-reversible cleavage of sucrose into fructose and glucose is catalysed by invertases. Invertases are present in three different isoforms, which differ in their biochemical properties and subcellular localisation. Soluble neutral invertase (NI) is located in the cytosol, soluble acid invertase (AI) in the vacuole and the insoluble acid invertase is bound to the cell wall (CWAI). Invertases have been shown to play a major role in flowering by determining floral sink strength (Bihmidine et al., 2013). AI mainly plays a role in cell osmoregulation and cell expansion (Balk and de Boer, 1999; Ranwala and Miller, 2008; Kutschera and Niklas, 2013). CWAI plays a key role in phloem unloading by converting sucrose into hexoses after sucrose is translocated from the phloem to the apoplast (Roitsch and González, 2004) and this enables petals to increase their sink strength.

Translocation of photoassimilates depends on source supply and sink demand. During high sink activity, there is a high use of photoassimilates and the phloem unloading rate is enhanced, thus lowering the turgor of sink phloem and thereby increasing mass flow. This will stimulate phloem loading in the source, lowering the carbohydrate levels there (Ainsworth and Bush, 2011). These lower carbohydrate levels will stimulate photosynthetic activity by releasing feed-back inhibition and up-regulation of genes for photosynthesis (Koch, 1996). On the other hand, when sink demand is low, carbohydrates accumulate in the source leaves, down-regulating photosynthesis by suppressing photosynthetic gene expression (Paul and Foyer, 2001). The amount of sucrose available in the source leaves for transport to the sinks depends highly on photosynthetic activity

(Lemoine et al., 2013). Limited light not only creates a shortage of photoassimilate supply from the leaves, but also decreases the ability to transport photoassimilates as the expression of a sucrose transporter can be downregulated (Ishibashi et al., 2014).

In azalea (*Rhododendron simsii* hybrids), quality is highly dependent on the continuous development of closed flower buds to open blooms in indoor conditions where light levels are very low (10-12 µmol m^{-2} s^{-1}). During these low light conditions, photosynthesis will be insufficient to provide carbohydrates to sustain the developing flower and stored carbohydrates will be necessary for further development. As a shortage of carbohydrates often leads to the arrest of flower bud development, we aim to quantify if a threshold level of carbohydrate reserve is necessary for high-quality post-production performance of azalea. Therefore, we influenced the photosynthate level and thus the carbohydrate pools. In our approach, we investigated at which stage of flower development the quality of flowering is most susceptible to reduced photosynthate. We therefore applied two light levels at 3 different time intervals (1) before the start of the dormancy-breaking treatment, by shading the plants (2) immediately after cold (and dark) treatment, during the forcing up to open flowers and (3) from colour-showing buds up to open flowers, by using supplemental light. As there are differences in minimum light requirements between early- and late-flowering cultivars, we quantified soluble carbohydrates and starch levels in leaves and flowers of an early- and late-flowering cultivar. To determine sink strength, we quantified the activity of enzymes (neutral invertase, acid invertase, cell wall-bound invertase, sucrose synthase) involved in the sucrose metabolism. During azalea production, high doses of the persistent growth regulator paclobutrazol are used. Since paclobutrazol inhibits the GA-biosynthesis and gibberellins play a major part in flower development, especially in petal elongation (Goto and Pharis, 1999), we investigated the influence of this PGR on carbohydrate pools, sink strength and flowering.

6.2 Materials and methods

6.2.1 Plant material

Two cultivars differing in their natural flowering time and chilling requirements to break dormancy were used in this experiment. Four cuttings of the early-flowering cultivar 'Nordlicht' and the late-flowering cultivar 'Sachsenstern' were rooted and grown in a 12 cm pot according to standard horticultural practice. Plants were decapitated twice and transferred to an outdoor container field at the beginning of July 2010. At 3 August 2010 plants were weekly treated with plant growth regulators (six applications with 2.25 g L^{-1} chlormequat) to initiate the generative phase and to supress the outgrowth of axillary buds.

6.2.2   Experimental set-up 1: manipulating photosynthate level

The general overview of the experimental set-up is given in Figure 6.1, the experiment started with 180 plants for 'Nordlicht' and 240 plants for 'Sachsenstern', each treatment consisted of minimal 30 plants to follow flowering and to take samples during the different stages of flowering.
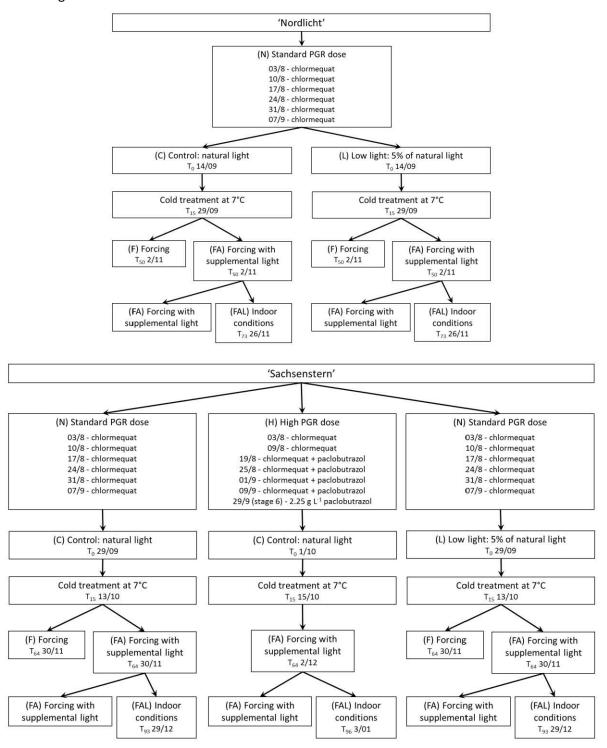
Figure 6.1: Schematic overview of the experimental set-up for 'Nordlicht' (top) and 'Sachsenstern' (bottom)

To alter the carbohydrate content before cold treatment, plants were subjected to two different light intensities for 15 days (Table 6.1) (=pre-treatment). When stamen initiated (flower bud stage 5; Bodson, 1983) in flower buds of 'Nordlicht' and when carpels initiated (flower bud stage 6) in flower buds of 'Sachsenstern', plants were brought inside the greenhouse at 18°C and divided into two groups. One control group received natural light conditions and one group received very low light levels (less than 5 % of the control group). After two weeks flower bud stage 7 for 'Nordlicht' and 7-8 for 'Sachsenstern' was reached and plants were stored in a dark cold room (7 °C) to break flower bud dormancy for 5 and 7 weeks, respectively. Soluble carbohydrates and starch content was followed in leaves and flower buds (3 replicates, 1 replicate is a bulk sample derived of 2 random plants) at 3 time points: the start of the low light treatment, the start of the cold treatment and the start of forcing.

Table 6.1 Start date and mean daily light integral (DLI) during the different phases in the experiment for 'Nordlicht' and 'Sachsenstern'

	'Nordlicht'		'Sachsenstern'	
	Start date	DLI (mol m^{-2} d^{-1})	Start date	DLI (mol m^{-2} d^{-1})
First PGR treatment	3/08/2010	21.0 ± 7.5	3/08/2010	19.6 ± 7.2
(C) Control light	T_0 14/09/2010	6.6 ± 0.9	T_0 29/09/2010 (1/10)[z]	4.7 ± 1.1
(L) Low light	T_0 14/09/2010	0.2 ± 0.2	T_0 29/09/2010, T_0	0.1 ± 0.1
Cold treatment	T_{15} 29/09/2010	0	T_{15} 13/10/2010 (15/10)	0
(F) Forcing	T_{50} 2/11/2010	1.3 ± 0.8	T_{64} 30/11/2010	1.4 ± 1.1
first week of forcing		2.5 ± 0.7		0.9 ± 0.4
(FA) Forcing with supplemental light	T_{50} 2/11/2010	5.2 ± 0.8	T_{64} 30/11/2010 (2/12)	5.2 ± 0.8
(FAL) Indoor conditions	T_{73} 26/11/2010	0.7	T_{93} 29/12/2010 (3/01)	0.7

[z] In parentheses the dates of plants treated with a high dose of PGR

Before forcing, plants were again split in two groups. One group was forced under natural light conditions and a second group with 16 h supplementary light (SON-T, 75-80 μmol m^{-2} s^{-1} at plant canopy level). Forcing temperature was a continuous 21.2 ± 0.3 °C and relative humidity was 57.8 ± 5.5%. During forcing, samples were taken for analysis of soluble carbohydrates, starch and enzyme activity. Leaves and flower buds (3 replicates, 1 replicate is a bulk sample derived of 2 random plants) were taken at 4 developmental stages: after 1 week of forcing when buds were still closed (G), colour-showing buds (CS), buds in the candle stage (CA) and fully open flowers (OF).

To further assess the quality of flowering at indoor conditions, plants from the forcing greenhouse with supplemental light were split again at the colour-showing stage and one half was placed in a growth chamber under controlled conditions mimicking indoor conditions. Temperature was 19.8 ± 0.4 °C, relative humidity 73.7 ± 9.5% and light intensity at plant canopy level was 11 µmol m^{-2} s^{-1} for 18 hours. Sampling continued also in the climate chamber to determine soluble carbohydrates, starch and enzyme activities on plants with buds in the candle stage and fully open flowers.

During the experiment photosynthetic active radiation (QS, Delta-T Devices, Cambridge, UK) was measured at canopy level and data were recorded every 5 minutes by a data logger (34970A, Agilent Technologies).

6.2.3 Experimental set-up 2: influence of PGR

For the late-flowering cultivar 'Sachsenstern' the effect of paclobutrazol on flowering was examined. Plants were treated with a standard dose of PGR, as mentioned above, a weekly application with 2.25 g L^{-1} chlormequat starting at 3 August 2010 and with a high dose of PGR, also containing the 6 applications with chlormequat, of which the last four were combined with paclobutrazol (0.04 g L^{-1}) and an extra application with paclobutrazol was done at flower bud stage 6, one day prior to the start of the shading experiment. Plants with a high dose of PGR followed the same path as the control light plants in experimental set-up 1, but with a two day delay (Figure 6.1, Table 6.1).

6.2.4 Soluble carbohydrates and starch content

Grounded tissue (200 mg) of leaves and flower buds was extracted in 6 ml 80 % ethanol for 3 h at 45 °C. After centrifugation at 7500g (5 min), the supernatant was purified with 50 mg ml^{-1} PVPP. The concentrations of glucose, fructose and sucrose in filtered (0.45 µm, Millipore) diluted samples were quantified by means of high performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) using a Dionex series chromatograph, equipped with a CarboPack PA10 column, a pulsed amperometric detector and a gold electrode.

The concentration of starch is determined by the acid hydrolysis of the remaining pellet after extraction of the soluble carbohydrates. The dried pellet was treated with 1 M HCl for 1 h at 95 °C for starch hydrolysis. The pH of the supernatant was adjusted to 7.6 and the sample was diluted to 10 ml. Starch content, expressed as glucose equivalents, was determined enzymatically by the reduction of NADP$^+$ (measured at 340 nm, UV/VIS 916, GBC Scientific Equipment, Australia) with a hexokinase/glucose-6-phosphate dehydrogenase assay.

6.2.5   Enzyme activities

Three enzymes were analysed in the petals: soluble acid invertase, soluble neutral invertase and cell wall-bound acid invertase. Activity of sucrose synthase in the petals was not found. For the extraction of the soluble invertases, 2 g plant material was homogenized in 6 ml ice cold extraction buffer (50 mM Hepes-NaOH, 20 mM $MgCl_2.6H_2O$, 5 mM dithiothreitol (DTT), 10 mM iso-ascorbic acid, 1 mM Na_2EDTA, 0.1 % (v/v) Triton X-100, pH 7.5) and filtered over Miracloth. The filtrate, containing the soluble invertases, was centrifuged at 10,000 g and 4 °C for 10 minutes. The residue with the cell wall-bound acid invertase was washed three times with extraction buffer without DTT and Triton X-100 and incubated for 24 h in 3 ml incubation buffer (20 mM MES-KOH, 1 M NaCl, 5 mM DTT, pH 6). After incubation, samples were centrifuged at 10,000 g and 4 °C for 10 minutes. To determine the activity of acid invertase (soluble and cell wall-bound), 50 µl of the extract is incubated with 200 µl reaction buffer (100 mM acetate buffer, 100 mM sucrose, pH 5) for 45 minutes at 30 °C (3 replications for each extract). The reaction was stopped in boiling water, while a blank was put immediately in boiling water. The samples were cooled on ice and the formed glucose was determined by a LabAssay Glucose kit (Wako Chemicals GmbH, Neuss, Germany). The same procedure was followed to determine the activity of neutral invertase, but the reaction buffer contained 50 mM Hepes-NaOH and 50 mM sucrose at pH 7. Enzyme activities are expressed as µmol (glucose) $min^{-1} g^{-1}$ (protein). Therefore, protein content of the extracts was determined according to the method of Bradford (Bradford, 1976).

6.2.6   Assessment of the quality of flowering

During the forcing period, flowering was followed on 10 plants by counting weekly the number of flower buds in different developmental stages: closed, green buds (G), colour-showing buds (CS), candle stage buds (CA) and fully open flowers (OF). Earliness of flowering was determined as the number of days between start of forcing and 10 % colour-showing buds. The total flowering (CS + CA + OF) percentage was calculated and used to determine the homogeneity of flowering as days between 10 and 90 % flowering.

6.2.7   Statistical analysis

Data were analysed using SPSS statistical software Version 19.0 (SPSS Inc., Chicago, USA) and means were separated by Tukey's HSD test (soluble carbohydrates, starch) or by the non-parametric Mann-Whitney U test (flowering quality).

6.3 Results

6.3.1 Carbohydrate content before forcing

Soluble carbohydrate and starch content was followed in leaves and flower buds at the start and end of the pre-treatment, and at the end of the cold treatment (Table 6.2 - Table 6.3).

For 'Nordlicht' there was a significant effect of the 2 weeks of low light conditions (T_{15}) on glucose, sucrose and starch content in the leaves. The low light conditions decreased glucose levels significantly from 1.9 to 1.3 mg g^{-1} FW, while under control conditions levels stayed constant. The same was seen for fructose; however, the decrease in concentration from 1.7 to 1.4 mg g^{-1} FW was not significant. Sucrose levels decreased both in control as well as in low light conditions, but the decrease in control conditions from 16.1 to 9.6 mg g^{-1} FW was less than to 5.6 mg g^{-1} FW in low light conditions. The same trend is seen for starch: in control conditions there was a decrease from 63.3 to 45.3 mg g^{-1} FW while in low light conditions starch decreased to 27.2 mg g^{-1} FW. A cold treatment at 7°C for five weeks (T_{50}, Table 6.2) increased the concentrations of glucose and fructose in the leaves. The increase was highest for the plants from the control light conditions before cold treatment. Glucose increased to 4.5 and 2.8 mg g^{-1} FW, fructose increased to 3.5 and 2.6 mg g^{-1} FW for control and low light-treated plants, respectively. Sucrose and starch showed the opposite trend and decreased significantly during cold treatment. Sucrose decreased to 7.0 and 5.0 mg g^{-1} FW and starch to 8.1 and 0.8 mg g^{-1} FW for control and low light-treated plants, respectively.

Table 6.2 Effect of light levels applied before a dormancy-breaking treatment on carbohydrate levels at 3 time intervals (T_0: start of the pre-treatment, T_{15}: after 2 weeks of pre-treatment and just before the start of the cold treatment, T_{50}: after the cold treatment) in the leaves and flower buds of 'Nordlicht'

	Glucose (mg g^{-1} FW)			Fructose (mg g^{-1} FW)			Sucrose (mg g^{-1} FW)			Starch (mg g^{-1} FW)		
	Control		Low light	Control		Low light	Control		Low light	Control		Low light
Leaves												
T_0	1.94 b		1.94 b	1.65 b		1.65 b	16.12 a		16.12 a	63.27 a		63.27 a
T_{15}	2.11 b	*	1.32 c	1.68 b		1.36 b	9.62 b	*	5.64 b	45.26 b	*	27.20 b
T_{50}	4.51 a	*	2.84 a	3.54 a	*	2.61 a	6.96 c	*	4.98 b	8.06 c	*	0.77 c
Flower buds												
T_0	0.63 b		0.63 b	0.36 b		0.36 b	4.04 b		4.04 a	1.34 a		1.34 a
T_{15}	0.64 b	*	0.53 b	0.37 b		0.35 b	4.17 b		4.17 a	0.88 b	*	0.55 b
T_{50}	1.97 a	*	1.34 a	0.92 a	*	0.76 a	6.97 a	*	4.82 a	1.02 b	*	0.71 b

Means (n=3) for leaves or flower buds followed by different letters are significantly different (p=0.05, Tukey's HSD test)
* indicates a significant difference (p=0.05, Student's t-test) between control and low light pre-treatment.

Concentrations of soluble carbohydrates in the flower buds remained constant at an average level of 0.6, 0.4 and 4.1 mg g^{-1} FW for glucose, fructose and sucrose, respectively, during the 2 weeks of pre-treatment (T_0 to T_{15}). Starch concentrations on the other hand decreased, form 1.3 to 0.9 mg g^{-1} FW in control plants and to 0.5 mg g^{-1} FW in low light-treated plants. Cold storage (T_{50}) did increase the soluble carbohydrates in the flower buds, more in control plants than low light-treated plants. Glucose increased to 2.0 and 1.3 mg g^{-1} FW, fructose increased to 0.9 and 0.8 mg g^{-1} FW in control and low light-treated plants, respectively. Sucrose only increased in control plants from 4.2 to 7.0 mg g^{-1} FW, while in low light-treated plants the concentration stayed around 4.3 mg g^{-1} FW. Starch levels in the flower buds did not differ significantly before (T_{15}) and after cold treatment (T_{50}).

At the end of cold treatment (T_{50}, start of forcing), the difference in concentration of soluble carbohydrates and starch in the leaves and flower buds between control and low light-treated plants was significant.

Table 6.3 Effect of light levels applied before a dormancy-breaking treatment on carbohydrate levels at 3 time intervals (T_0: start of the pre-treatment, T_{15}: after 2 weeks of pre-treatment and just before the start of the cold treatment, T_{64}: after the cold treatment) in the leaves and flower buds of 'Sachsenstern'

	Glucose (mg g^{-1} FW)		Fructose (mg g^{-1} FW)		Sucrose (mg g^{-1} FW)		Starch (mg g^{-1} FW)	
	Control	Low light	Control	Low light	Control	Low light	Control	Low light
Leaves								
T_0	2.04 a	2.04 a	1.38 a	1.38 a	13.38 a	13.38 a	61.14 a	61.14 a
T_{15}	1.75 ab *	1.22 b	1.15 a	0.92 b	6.85 b *	4.72 b	26.84 b *	16.94 b
T_{64}	1.35 b	1.30 b	1.28 a	1.29 a	4.50 c	3.97 b	2.41 c	1.06 c
Flower								
T_0	0.99 a	0.99 a	0.46 a	0.46 ab	4.38 ab	4.38 a	1.43 a	1.43 a
T_{15}	1.06 a *	0.88 a	0.40 a	0.40 b	3.82 b *	3.19 b	0.69 b *	0.57 b
T_{64}	0.91 a	1.00 a	0.50 a	0.53 a	4.76 a	4.89 a	0.63 b	0.63 b

Means (n=3) for leaves or flower buds followed by different letters are significantly different (p=0.05, Tukey's HSD test)
* indicates a significant difference (p=0.05, t-test) between control and low light pre-treatment.

Also in leaves of 'Sachsenstern' there was a significant effect of the low light conditions before cold treatment in glucose, fructose, sucrose and starch levels (Table 6.3). In control plants glucose and fructose stayed constant, but under low light conditions, concentrations decreased from 2.0 to 1.2 mg g^{-1} FW and from 1.4 to 0.9 mg g^{-1} FW, respectively. Both control and low light-treated plants had a decrease in sucrose and starch. Sucrose levels decreased from (T_0) 13.4 to (T_{15}) 6.9 and 4.7 mg g^{-1} FW and starch levels from (T_0) 61.1 to (T_{15}) 26.8 and 16.9 mg g^{-1} FW, for control and

low light-treated plants, respectively. Cold treatment (7 weeks at 7°C, T_{64}) did not change glucose and fructose concentrations. Sucrose decreased significantly for control plants to 4.5 mg g^{-1} FW, while for low light-treated plants the decrease to 4.0 mg g^{-1} FW was not significant. Starch content decreased to 2.4 and 1.1 mg g^{-1} FW for control and low light-treated plants, respectively.

Flower buds of 'Sachsenstern' showed no difference in glucose (1.0 mg g^{-1} FW) and fructose (0.5 mg g^{-1} FW) content, neither during the pre-treatment nor during the cold treatment. Sucrose concentration slightly decreased at the end of pre-treatment (T_{15}), but increased again to reach the initial level of 4.6 mg g^{-1} FW after cold treatment (T_{64}). Starch concentration in flower buds decreased during the pre-treatment from 1.4 to 0.7 and 0.6 mg g^{-1} FW for control and low light-treated plants, respectively. During cold treatment starch concentrations stayed low.

At the end of cold treatment (T_{64}, start of forcing), there were no significant differences in carbon content in leaves and flower buds between control and low light-treated plants for 'Sachsenstern'.

6.3.2   Carbohydrate content during forcing

Even though there were significant differences at the start of forcing (T_{50}) between control plants and low light-treated plants in leaf soluble carbohydrates and leaf starch content for 'Nordlicht', this difference disappeared almost immediately during forcing (Figure 6.2). The significant differences during forcing were to be found between the two forcing conditions: forcing under natural light (F) and forcing with supplemental light (FA). Concentrations of glucose and fructose in the leaves decreased immediately during F and stayed at a basal level of 1.3mg g^{-1} FW from the colour-showing stage onwards. During FA, on the other hand, concentrations of glucose and fructose increased after 1 week of forcing and then decreased towards this basal level which was only reached at full bloom. A same trend was seen for leaf sucrose content, a continuous decrease until 3.4 mg g^{-1}FW during F. For FA, an increase was seen until 12.3 mg g^{-1}FW at the colour-showing stage after which levels decreased towards 5.8 mg g^{-1}FW. Starch levels stayed very low (< 9.2 mg g^{-1}FW) during F; on the other hand, a strong increase was seen during FA, where levels reached a plateau (27.7 mg g^{-1}FW) after 1 week of forcing until the candle stage. When flowers were fully open, starch content decreased again strongly.
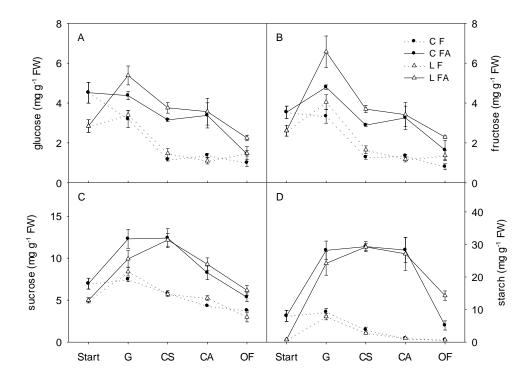
Figure 6.2: Effect of light intensity prior to the cold storage and during forcing on concentrations of glucose (A), fructose (B), sucrose (C) and starch (D) in leaves of 'Nordlicht'. C: control light level (6.6 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.2 mol m^{-2} d^{-1}) before cold treatment; F: forcing under natural light conditions (1.3 mol m^{-2} d^{-1}); FA: forcing with supplemental light (5.2 mol m^{-2} d^{-1}).
x-axis: Start: start of forcing (T_{50}), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers.

During the opening of the flower buds, glucose and fructose concentrations increased strongly in the petals when buds started showing colour, while sucrose concentrations decreased after the colour-showing stage (Figure 6.3). Differences in carbohydrate levels at the start of forcing disappeared also immediately in the first week. The differences are to be found between the forcing conditions; however, these differences were small compared to those in the leaves. Glucose concentrations increased less rapid during F compared to FA, in the colour-showing stage concentrations were 7.1 and 11.1 mg g^{-1}FW, respectively. Fructose concentrations showed no clear differences between the objects. Sucrose decreased during flowering, and a difference between F and FA is seen when flowers are fully open. During F concentrations continue to decrease strongly until 0.2 mg g^{-1}FW, while during FA concentrations stabilize at 1.8 mg g^{-1}FW.
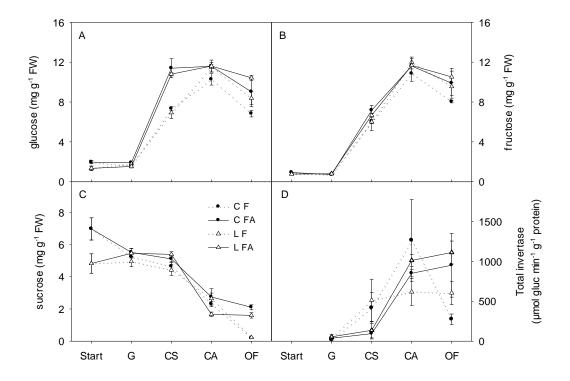
Figure 6.3: Effect of light intensity prior to the cold storage and during forcing on concentrations of glucose (A), fructose (B), sucrose (C) and total invertase activity (D) in flower buds of 'Nordlicht'. C: control light level (6.6 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.2 mol m^{-2} d^{-1}) before cold treatment; F: forcing under natural light conditions (1.3 mol m^{-2} d^{-1}); FA: forcing with supplemental light (5.2 mol m^{-2} d^{-1}).

x-axis: Start: start of forcing (T$_{50}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers.

There were no significant differences in leaf carbon content for 'Sachsenstern' at the start of forcing (T_{64}) between control plants and low light-treated plants and also during forcing, no significant differences were seen. The effect of supplemental light during forcing was, however, even more clear than for 'Nordlicht' (Figure 6.4). Samples during F were only taken until the colour-showing stage, as due to very poor flowering, buds hardly made further progress in opening. During FA, glucose, fructose, sucrose and starch concentrations increased the first week to 2.6, 2.7, 9.7, 15.2 mg g^{-1}FW, respectively, after which a constant decrease is seen until the end of flowering.
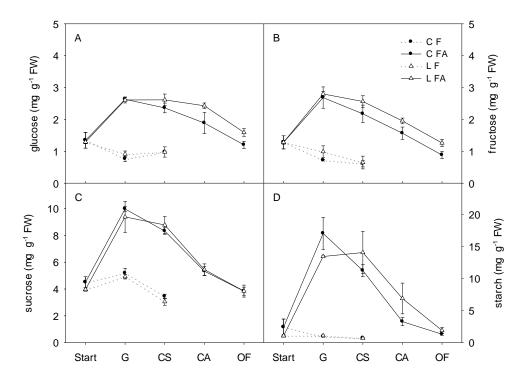

Figure 6.4: Effect of light intensity prior to the cold storage and during forcing on concentrations of glucose (A), fructose (B), sucrose (C) and starch (D) in leaves of 'Sachsenstern'. C: control light level (4.7 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.1 mol m^{-2} d^{-1}) before cold treatment; F: forcing under natural light conditions (1.4 mol m^{-2} d^{-1}); FA: forcing with supplemental light (5.2 mol m^{-2} d^{-1}).
x-axis: Start: start of forcing (T_{64}), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers.

The glucose and fructose concentrations in flower buds increased rapidly during FA when buds showed colour, while the sucrose concentration decreased with further opening of the flowers (Figure 6.5). Light conditions before cold treatment, did not result in significant differences at the start of cold treatment, and also during the first stages of forcing no differences were found. Only when flowers were fully open, the concentrations of glucose, fructose and sucrose were slightly lower in plants that received a low light treatment before cold treatment. For control plants, glucose, fructose and sucrose concentrations were 13.4, 17.8 and 3.3 mg g^{-1} FW, respectively, while in low light-treated plants concentrations were 11.0, 10.0 and 2.4 mg g^{-1} FW, respectively.
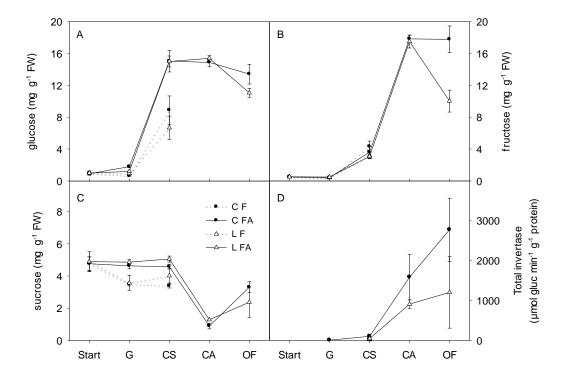

Figure 6.5: Effect of light intensity prior to the cold storage and during forcing on concentrations of glucose (A), fructose (B), sucrose (C) and total invertase activity (D) in flower buds of 'Sachsenstern'. C: control light level (4.7 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.1 mol m^{-2} d^{-1}) before cold treatment; F: forcing under natural light conditions (1.4 mol m^{-2} d^{-1}); FA: forcing with supplemental light (5.2 mol m^{-2} d^{-1}).
x-axis: Start: start of forcing (T$_{64}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers.

6.3.3 Enzymatic activity during forcing

Invertase (acid, neutral and cell wall-bound acid) activity was measured in the flower buds during forcing. In green buds of 'Nordlicht', after 1 week of forcing, total invertase activity was very low (Figure 6.3D). During forcing, activity increased until the candle stage and remained more or less constant towards the end of flowering. As also seen with the soluble carbohydrate content, the difference in total invertase activity was most clear at the colour-showing stage between plants forced under natural light and supplemental light. The highest activity was seen in plants under natural light with 465 µmol glucose min^{-1} g^{-1} protein, compared to 116 µmol glucose min^{-1} g^{-1} protein with supplemental light. Overall, total invertase activity in 'Nordlicht' was predominantly acid invertase activity, while neutral invertase showed the lowest activity (Table 6.4).

Table 6.4 Protein concentration, acid invertase (AI), neutral invertase (NI) and cell wall-bound acid invertase (CWAI) in developing flowers of 'Nordlicht' at the colour-showing stage (CS), candle stage (CA) and open flowers (OF) during forcing with (5.2 mol m^{-2} d^{-1}) and without (1.3 mol m^{-2} d^{-1}) supplemental light and during flowering at indoor conditions. (means ± SE, n=3)

Flower stage	light levels before cold treatment	supplemental light during forcing		Protein (µg g^{-1} FW)	AI	NI	CWAI
					(µmol glucose min^{-1} g^{-1} protein)		
CS	control	-	C F	145 ± 30	202 ± 54	37 ± 37	178 ± 176
		+	C FA	178 ± 39	51 ± 27	11 ± 11	33 ± 32
	low light	-	L F	106 ± 21	292 ± 125	71 ± 49	150 ± 92
		+	L FA	203 ± 25	12 ± 6	26 ± 25	98 ± 77
CA	control	-	C F	40 ± 8	807 ± 364	406 ± 225	51 ± 37
		+	C FA	102 ± 4	701 ± 141	150 ± 150	0
		+	C FAL	76 ± 16	643 ± 216	208 ± 123	209 ± 155
	low light	-	L F	106 ± 11	377 ± 27	234 ± 153	7 ± 7
		+	L FA	113 ± 30	521 ± 53	158 ± 94	334 ± 141
		+	L FAL	158 ± 17	363 ± 64	75 ± 50	72 ± 52
OF	control	-	C F	156 ± 12	189 ± 29	18 ± 18	70 ± 36
		+	C FA	85 ± 9	524 ± 169	212 ± 212	216 ± 117
		+	C FAL	143 ± 11	292 ± 18	50 ± 36	160 ± 80
	low light	-	L F	176 ± 26	289 ± 35	0	315 ± 112
		+	L FA	103 ± 6	629 ± 136	261 ± 121	223 ± 119
		+	C FAL	261 ± 10	125 ± 29	0	22 ± 21

Total invertase activity in the flower buds of 'Sachsenstern' during forcing was only measured for plants under supplemental light and gradually increased in the candle stage and at fully open flowers (Figure 6.5D). Here we did see a lower activity in low light-treated plants compared to control plants; however, the difference was not significant. In the candle stage, activity was 1586 and 911 µmol glucose min^{-1} g^{-1} protein for control and low light-treated plants, respectively. In fully open flowers, activity was 2778 and 1204 µmol glucose min^{-1} g^{-1} protein for control and low light-treated plants, respectively. Total invertase activity in 'Sachsenstern' during the candle stage was predominantly neutral invertase (Table 6.5). When flowers were fully open the cell wall-bound acid invertase was the predominant invertase for low light-treated plants, while for control plants neutral invertase was the main invertase.

Table 6.5 Protein concentration, acid invertase (AI), neutral invertase (NI) and cell wall-bound acid invertase (CWAI) in developing flowers of 'Sachsenstern' at the colour-showing stage (CS), candle stage (CA) and open flowers (OF) during forcing with (5.2 mol m^{-2} d^{-1}) and without (1.4 mol m^{-2} d^{-1}) supplemental light and during flowering at indoor conditions. (means ± SE, n=3)

	Flower stage	light levels before cold treatment	supplemental light during forcing	Protein (µg g^{-1} FW)	AI	NI	CWAI
					(µmol glucose min^{-1} g^{-1} protein)		
C FA	CS	control	+	479 ± 60	19 ± 18	2 ± 1	82 ± 35
L FA	CS	low light	+	335 ± 64	47 ± 46	0	0
C FA	CA	control	+	72 ± 13	284 ± 34	867 ± 552	435 ± 63
C FAL	CA	control	+	40 ± 4	866 ± 457	923 ± 479	0
L FA	CA	low light	+	77 ± 9	62 ± 62	811 ± 148	38 ± 37
L FAL	CA	low light	+	79 ± 9	427 ± 178	283 ± 130	102 ± 102
C FA	OF	control	+	44 ± 3	577 ± 401	1604 ± 345	597 ± 47
C FAL	OF	control	+	46 ± 4	504 ± 259	613 ± 253	216 ± 215
L FA	OF	low light	+	54 ± 5	169 ± 88	358 ± 358	677 ± 474
L FAL	OF	low light	+	45 ± 14	564 ± 264	402 ± 101	1315 ± 666

6.3.4   Post-production carbohydrate content

To determine post-production quality, only plants forced with supplemental light (FA) were examined, as forcing under natural light conditions (F) resulted already in poor flowering in greenhouse circumstances (see further). When buds showed colour, plants were placed at indoor conditions. Looking at the soluble carbohydrates and starch content in leaves of 'Nordlicht', there is a clear effect from the transfer from the greenhouse to indoor conditions (FAL) (Figure 6.6). Differences between control plants and low light-treated plants were not present at the colour-showing stage (CS) and also during further flowering at indoor conditions, no differences were noted. Glucose, fructose and sucrose decreased immediately during the post-production phase. Also leaf starch content decreased drastically at indoor conditions to very low levels: 5.9 and 0.7 mg g^{-1} FW at candle stage and open flowers, respectively.
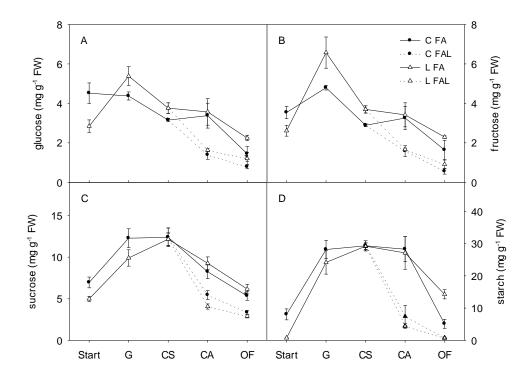
Figure 6.6: Concentrations of glucose (A), fructose (B), sucrose (C) and starch (D) in leaves of 'Nordlicht' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). C: control light level (6.6 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.2 mol m^{-2} d^{-1}) before cold treatment.
x-axis: Start: start of forcing (T$_{50}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

Also in the flower buds of 'Nordlicht', the effect of the low light intensities during post-production was clearly seen in glucose and fructose content, which decreased during candle stage and fully open flowers and became significantly lower than during continuous FA (Figure 6.7). When flowers were fully open, glucose and fructose concentrations in the greenhouse were 9.7 and 10.2 mg g^{-1} FW compared to 5.7 and 6.9 mg g^{-1} FW at indoor conditions, respectively. Sucrose concentrations, however, decreased during flowering and no effect of the indoor conditions was seen.
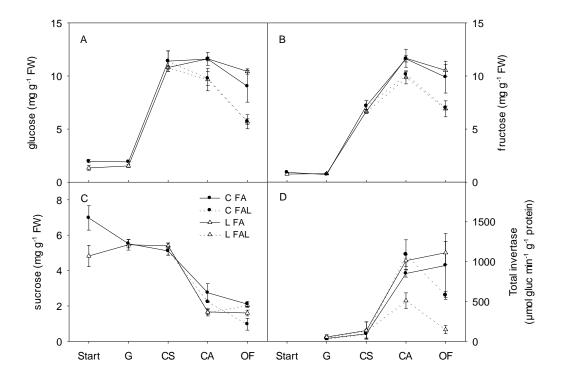
Figure 6.7: Concentrations of glucose (A), fructose (B), sucrose (C) and total invertase activity (D) in flower buds of 'Nordlicht' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). C: control light level (6.6 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.2 mol m^{-2} d^{-1}) before cold treatment.
x-axis: Start: start of forcing, G (T$_{50}$): 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

Post-production leaf carbohydrate content in 'Sachsenstern' followed the same trend as in 'Nordlicht' (Figure 6.8). Differences between control plants and low light-treated plants were minor. The largest difference was seen immediately after transfer to indoor conditions, in the candle stage. Concentrations at indoor conditions were lower and reached a basal level for glucose, fructose, sucrose and starch of 1.3, 0.9, 2.4 and 0.6 mg g^{-1} FW, respectively. When flowers were fully open, concentrations in the leaves of plants in the greenhouse (FA) reached the same basal levels.
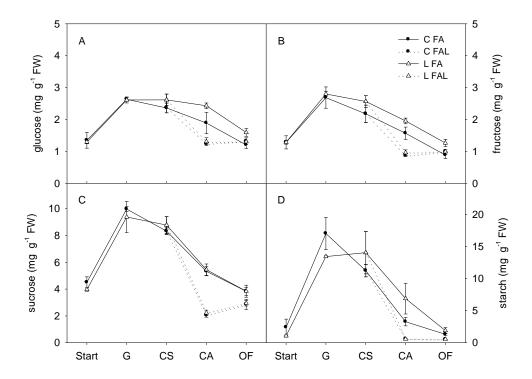

Figure 6.8: Concentrations of glucose (A), fructose (B), sucrose (C) and starch (D) in leaves of 'Sachsenstern' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). C: control light level (4.7 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.1 mol m^{-2} d^{-1}) before cold treatment.
x-axis: Start: start of forcing (T$_{64}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

In the flower buds of 'Sachsenstern', there was also a decrease in glucose in the candle stage (Figure 6.9); while fructose concentrations increased, but not to the levels as in forcing conditions. Candle-stage buds of plants in FA had glucose and fructose concentrations of 15.1 and 17.7 mg g^{-1} FW, compared to 13.0 and 12.9 in FAL, respectively. When flowers were fully open, differences were no longer present, except for the fructose concentration in FA for control plants, which were higher compared to the other objects. Sucrose concentrations did not differ much, when the flowers were fully open, there was a trend that FA plants had a higher concentration than FAL plants.
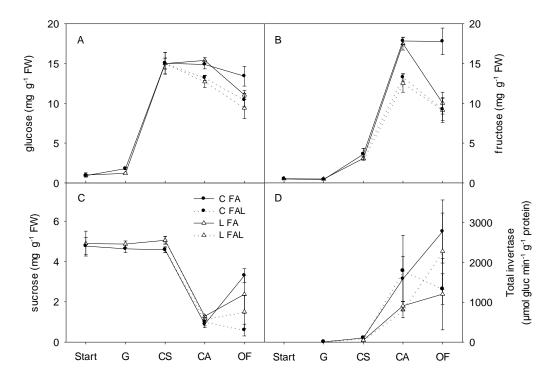

Figure 6.9: Concentrations of glucose (A), fructose (B), sucrose (C) and total invertase activity (D) in flower buds of 'Sachsenstern' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). C: control light level (4.7 mol m^{-2} d^{-1}) before cold treatment; L: low light level (0.1 mol m^{-2} d^{-1}) before cold treatment.
x-axis: Start: start of forcing (T$_{64}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

6.3.5   Enzymatic activity during post-production

For 'Nordlicht' total invertase activity during post-production conditions further increased until the candle stage, but dropped when flowers were fully open; this in contrast with plants in the forcing greenhouse, which maintained their invertase activity when flowers were fully open (Figure 6.7D). There was also a difference during post-production conditions between control plants and low light-treated plants. Low light-treated plants did not increase their total activity as much as the other plants: 510 compared to 975 µmol glucose $min^{-1} g^{-1}$ protein in low light-treated and control plants, respectively.

During post-production conditions in 'Sachsenstern' (Figure 6.9D), no differences of invertase activity were seen in the candle stage between greenhouse and indoor conditions and also when flowers were fully open total invertase activity was not significantly different. The total invertase activity during the candle stage and open flowers in post-production conditions was predominantly neutral and acid invertase (Table 6.4 and Table 6.5).

6.3.6   Quality of flowering

The effect of supplemental light compared to natural light conditions on earliness of flowering, flower homogeneity and maximum percentage of open flowers was determined; this for plants with a different light pre-treatment prior to cold (Table 6.6). Control plants and low light-treated plants did not differ in their quality of flowering under the same forcing conditions. Forcing with supplemental light improved the quality of flowering significantly. Earliness of flowering was improved by 5 days for 'Nordlicht' and by 14 days for 'Sachsenstern'. Since 90 % flowering was not reached during forcing under natural light conditions, flower homogeneity could not be calculated. The homogeneity for plants forced with supplemental light, was 13 and 22 days for 'Nordlicht' and 'Sachsenstern', respectively. The maximum percentage of open flowers was very low without supplemental light: only 49 and 18 % compared to 80 and 68 % with supplemental light, for 'Nordlicht' and 'Sachsenstern', respectively.

Table 6.6 Earliness of flowering, flower homogeneity and maximum percentage of open flowers (OF) for 'Nordlicht' and 'Sachsenstern'. C: control light level before cold treatment; L: low light level before cold treatment; F: forcing under natural light conditions; FA: forcing with supplemental light.

	light levels before cold treatment	supplemental light during forcing	'Nordlicht'			'Sachsenstern'		
			Earliness (days)	Uniformity (days)	% OF	Earliness (days)	Uniformity (days)	% OF
C F	Control	-	22.7 b	-	49.8 b	38.1 b	-	14.5 b
C FA		+	17.9 a	12.4	81.98 a	25.6 a	20.4	62.8 a
L F	Low light	-	23.8 b	-	48.92 b	39.4 b	-	21.1 b
L FA		+	18.6 a	13.9	77.74 a	24.5 a	24.0	73.8 a

Means (n=10) followed by different letters are significantly different (p=0.05, Mann-Whitney U Test) between forcing with (+) and without (-) supplemental light.

Post-production quality was assessed for plants forced with supplemental light (Table 6.7). There was no significant difference in flower homogeneity between plants under forcing conditions and indoor conditions. There was however, a significant difference in % OF, for 'Nordlicht' and 'Sachsenstern' only 50 and 46% flowers opened completely at indoor conditions, respectively.

Table 6.7 Earliness of flowering, flower homogeneity and maximum percentage of open flowers for 'Nordlicht' and 'Sachsenstern'. C: control light level before cold treatment; L: low light level before cold treatment; FA: forcing with supplemental light; FAL: forcing with supplemental light followed by indoor conditions at the colour-showing stage.

	light levels before cold treatment	Flowering after colour-showing stage	'Nordlicht'		'Sachsenstern'	
			Uniformity (days)	% OF	Uniformity (days)	% OF
C FA	Control	greenhouse	12.4 a	82.0 a	20.4 a	62.8 a
C FAL		indoor	12.4 a	54.4 b	26.5 a	43.9 b
L FA	Low light	greenhouse	13.9 a	77.7 a	24.0 a	73.8 a
L FAL		indoor	14.0 a	46.0 b	26.6 a	48.3 b

Means (n=10) followed by different letters are significantly different (p=0.05, Mann-Whitney U Test) between greenhouse and indoor conditions.

6.3.7   Effect of a high dose of paclobutrazol

In Table 6.8 the soluble carbohydrates and starch content is shown in leaves and flower buds of 'Sachsenstern' treated with a standard and high dose of PGR. At the start of cold treatment (T_{15}), there are significant differences in the leaves; a high dose of PGR resulted in a higher amount of fructose (1.7 compared to 1.1 mg g^{-1} FW), sucrose (8.4 compared to 4.5 mg g^{-1} FW) and starch (31.1 compared to 26.8 mg g^{-1} FW). Also in the flower buds, sucrose content was higher (4.2 mg g^{-1} FW) in plants with a high dose of PGR. These differences increased during cold treatment, at the end of cold treatment (T_{64}) leaves of plants with a high dose of PGR contained 2.5, 2.7, 8.4, 6.8 mg g^{-1} FW glucose, fructose, sucrose and starch, respectively, while standard PGR-treated plants contained 1.2, 1.3, 4.5, 2.4 mg g^{-1} FW, respectively. Also in flower buds, significant higher concentrations in plants treated with a high dose of PGR were seen: 1.5, 0.7, 7.2 mg g^{-1} FW glucose, fructose and sucrose, respectively, compared to 0.9, 0.5, 4.8 mg g^{-1} FW in plants treated with a standard dose PGR, respectively.

Table 6.8 Effect of a PGR application (standard PGR dose: 6 x 2.25 g L^{-1} chlormequat; high PGR dose: 6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol) applied before the dormancy-breaking treatment on carbohydrate levels at 3 time intervals (T_0: start of the treatment, T_{15}: after 15 days of pre-treatment and just before the start of the cold treatment, T_{64}: after the cold treatment) in the leaves and flower buds of 'Sachsenstern'

	Glucose (mg g^{-1} FW)			Fructose (mg g^{-1} FW)			Sucrose (mg g^{-1} FW)			Starch (mg g^{-1} FW)		
	Standard		High	Standard		High	Standard		High	Standard		High
Leaves												
T_0	2.04 a	*	1.72 c	1.38 a		1.18 c	13.38 a		13.32 a	61.14 a		67.00 a
T_{15}	1.75 ab		1.99 b	1.15 a	*	1.71 b	6.85 b	*	8.62 b	26.84 b	*	31.12 b
T_{64}	1.35 b	*	2.48 a	1.28 a	*	2.68 a	4.50 c	*	8.41 b	2.41 c	*	6.78 c
Flower												
T_0	0.99 a		1.34 a	0.46 ab		0.68 a	4.38 ab		4.70 b	1.43 a	*	0.77 a
T_{15}	1.06 a		1.33 a	0.40 b		0.55 a	3.82 b	*	4.27 b	0.69 b		0.68 b
T_{64}	0.91 a	*	1.48 a	0.50 a	*	0.66 a	4.76 a	*	7.18 a	0.63 b		0.74 ab

Means (n=3) for leaves or flower buds followed by different letters are significantly different (p=0.05, Tukey's HSD test)
* indicates a significant difference (p=0.05, t-test) between control treatment and low light pre-treatment.

During forcing with supplemental light (Figure 6.10), carbohydrate levels in the leaves showed differences between standard- and high-PGR-treated plants, while at indoor conditions these differences disappeared. Glucose and fructose levels in plants treated with of high dose of PGR were higher during the first week of forcing until the colour-showing (CS) stage, where levels were almost equal. During further flower opening, hexose levels in plants treated with a high dose of PGR were still slightly higher. Sucrose content in the leaves showed no differences during forcing between plants with a different dose of PGR, the difference at the start of forcing (T_{64}) disappeared after 1 week of forcing. Starch content was higher at the start of forcing as well as in the colour-showing and candle stage for plants treated with a high dose of PGR. Starch depleted, regardless of the PGR dose, when plants were brought to indoor conditions.
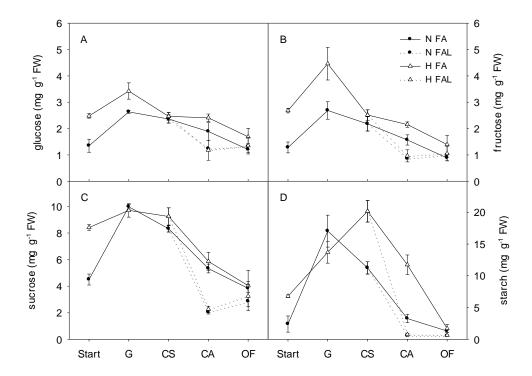
Figure 6.10 Effect of PGR dose on concentrations of glucose (A), fructose (B), sucrose (C) and starch (D) in leaves of 'Sachsenstern' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). N: standard dose PGR (6 x 2.25 g L^{-1} chlormequat); H: high dose PGR. (6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol).
x-axis: Start: start of forcing (T_{64}), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

In flower buds (Figure 6.11), differences between plants treated with a standard and a high dose of PGR were less obvious. Sucrose levels decreased very rapidly from the start of forcing (7.2 mg g^{-1} FW) until a basal level (1.4 mg g^{-1} FW) in the colour-showing stage, if a high dose of PGR was applied. Plants treated with a standard dose maintained their sucrose levels (4.7 mg g^{-1} FW) until the colour-showing stage and decreased thereafter. Total invertase activity was not significantly different between PGR-treated plants (Figure 6.11D). NI was the predominant invertase during opening in the forcing greenhouse and at indoor conditions. However, in plants treated with a high dose of PGR, open flowers contained a higher activity of AI; this was seen in forcing conditions (Table 6.9) and at indoor conditions (Table 6.10).

Quality of flowering for plants treated with a standard and high dose of PGR were not highly different (Table 6.11). There was no delay in flowering in plants treated with the high dose of PGR (earliness of 25 days); also flower homogeneity was not significantly different in forcing conditions and indoor conditions; but slightly better for plants treated with a high dose of PGR. In forcing conditions the percentage of open flowers was significantly higher (74.3%) in plants treated with a high dose of PGR, compared to 62.8% for plants treated with a low dose of PGR. Nevertheless, at indoor conditions, there was no difference detected in percentage of open flowers (42.8%).
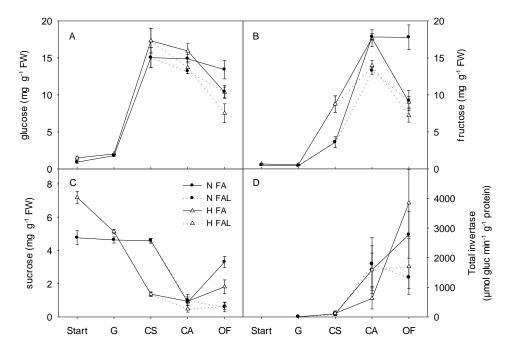
Figure 6.11 Effect of PGR dose on concentrations of glucose (A), fructose (B), sucrose (C) and total invertase activity (D) in flower buds of 'Sachsenstern' during flowering in the greenhouse with supplemental light (FA; 5.2 mol m^{-2} d^{-1}) and flowering at indoor conditions from the colour-showing stage (FAL; 0.7 mol m^{-2} d^{-1}). N: standard dose PGR (6 x 2.25 g L^{-1} chlormequat); H: high dose PGR (6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol).
x-axis: Start: start of forcing (T$_{64}$), G: 1 week forcing, CS: colour-showing stage, CA: candle stage, OF: open flowers

Table 6.9 Effect of PGR dose on protein concentration, acid invertase (AI), neutral invertase (NI) and cell wall-bound acid invertase (CWAI) in developing flowers of 'Sachsenstern' at the colour-showing stage (CS), candle stage (CA) and open flowers (OF) during forcing with supplemental light. Standard PGR dose: 6 x 2.25 g L^{-1} chlormequat; high PGR dose: 6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol. (means ± SE, n=3)

Flower stage	PGR treatment	Protein (µg g^{-1} FW)	AI	NI	CWAI
			(µmol glucose min^{-1} g^{-1} protein)		
CS	standard	479 ± 60	19 ± 18	2 ± 1	82 ± 35
CS	high	519 ± 50	51 ± 21	38 ± 21	34 ± 33
CA	standard	72 ± 13	284 ± 34	867 ± 552	435 ± 63
CA	high	171 ± 6	135 ± 32	143 ± 108	347 ± 214
OF	standard	44 ± 3	577 ± 401	1604 ± 345	597 ± 47
OF	high	21 ± 3	1210 ± 401	1814 ± 344	837 ± 379

Table 6.10 Effect of PGR dose on protein concentration, acid invertase (AI), neutral invertase (NI) and cell wall-bound acid invertase (CWAI) in developing flowers of 'Sachsenstern' at the candle stage (CA) and open flowers (OF) during flowering at indoor conditions after forcing with supplemental light. Standard PGR dose: 6 x 2.25 g L^{-1} chlormequat; high PGR dose: 6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol. (means ± SE, n=3)

Flower stage	PGR treatment	Protein (µg g^{-1} FW)	AI	NI	CWAI
			(µmol glucose min^{-1} g^{-1} protein)		
CA	standard	40 ± 4	866 ± 457	923 ± 479	0
CA	high	68 ± 3	833 ± 452	608 ± 299	152 ± 58
OF	standard	46 ± 4	504 ± 259	613 ± 253	216 ± 215
OF	high	48 ± 16	1272 ± 579	305 ± 263	125 ± 99

Table 6.11 Effect of PGR dose on earliness of flowering, flower homogeneity and maximum percentage of open flowers (OF) for 'Sachsenstern'. C: control light level before cold treatment; FA: forcing with supplemental light; FAL: forcing with supplemental light followed by indoor conditions at the colour-showing stage. Standard PGR dose: 6 x 2.25 g L^{-1} chlormequat; high PGR dose: 6 x 2.25 g L^{-1} chlormequat + 5 x 0.04 g L^{-1} paclobutrazol.

	light levels before cold treatment	PGR treatment	'Sachsenstern'		
			Earliness (days)	Homogeneity (days)	% OF
C FA	greenhouse	standard	25.6 a	20.4 a	62.8 b
C FA		high	25.9 a	15.8 a	74.3 a
C FAL	indoor	standard		26.5 a	43.9 a
C FAL		high		18.6 a	41.7 a

Means (n=10) followed by different letters are significantly different (p=0.05, Mann-Whitney U Test) between plants treated with normal and high dose of PGR.

6.4 Discussion

6.4.1 Sucrose metabolism during flowering under optimal conditions

During flower development in the greenhouse with assimilation light, we see the expected increase in glucose and fructose in the petals, and a decrease in sucrose. This has been observed in many other species such as petunia, *Ranunculus*, rose, lily, tulips (Hachiya and Noguchi, 2008; Shahri and Tahir, 2011), were glucose and fructose serve as osmoticum to promote water influx and petal expansion. To supply the necessary carbohydrates for flower opening, carbohydrates are reallocated from the leaves to the flowers. Especially towards the end of flowering, a marked decrease in foliar starch is seen. As fully open flowers cover the leaves, photosynthesis is strongly reduced and the starch reserves are depleted. In azalea petals, starch is not present, in contrast with other flowers as *Alstroemeria* (Collier, 1997), rose (Sood et al., 2006; Kumar et al., 2008b) and *Dendrobium* (Yap et al., 2008). In these flowers, the petal starch content increases during the first stages of flower opening, and is used again towards a fully open flower to further increase the glucose and fructose content.

Sucrose degradation in the petals of azalea is predominantly done by the acid invertases; also neutral invertase activity is present, but no SUS activity is measured (data not shown). The activity of the enzymes increased during flower opening. Also in rose petals acid invertase (soluble and cell wall-bound) activities are higher than neutral invertase activities and increase during flower development. But also increasing SUS activity was detected, though its activity was weak compared to the invertase activity (Yamada et al., 2007; Kumar et al., 2008a; Horibe et al., 2013).

Invertase cleavage of sucrose predominates in tissues where carbohydrates are catabolized for respiration and has been associated with cell expansion (Winter and Huber, 2000). SUS activity, on the other hand, is the dominant activity in accumulating sinks, e.g. fruit, when the products of sucrose cleavage are used for biosynthesis of carbohydrate polymers like starch (Sun et al., 1992; Winter and Huber, 2000; Koch, 2004; Moscatello et al., 2011). The absence of SUS activity in azalea petals might explain the lack of starch in the flower. In contrast with roses, where SUS activity was detected and a substantial increase in starch is seen during the first stages of flower opening (Kumar et al., 2007).

The invertase activity in azalea increases most after the colour-showing stage. This point corresponds with a drop in sucrose and increase in fructose content in the flowers. However, a clear increase in glucose and fructose was also observed in colour-showing buds. The high increase in invertase activity later in development might be related to a higher sink activity, due to increased size and a higher respiration rate when petals start to expand (candle stage) (Lay-Yee et al., 1992).

6.4.2   *Effect of forcing conditions on sucrose metabolism*

Two weeks before the cold treatment to break flower bud dormancy, plants were subjected to a low light treatment to decrease the carbon reserves in the leaves. However, the control group also decreased their starch content significantly, as control plants were transferred from the outside container field to the greenhouse, light levels decreased resulting in decreased starch content. The following cold treatment decreased carbon content further and resulted in plants with very low starch reserves. Especially the low light-treated plants were almost completely depleted in starch and in the case of 'Sachsenstern' were unable to increase their soluble carbohydrates as cryoprotectants during cold treatment.

Despite the differences in starch content between control plants and low light-treated plants at the start of forcing, this difference did not result in a major difference when plants were forced under the same light conditions. Forcing under natural light conditions resulted for both cultivars in very poor flowering, for 'Sachsenstern' only a few buds opened and no further analyses after the colour-showing stage could be done. In contrast, 'Nordlicht' did have more buds that fully opened, but still the percentage was very low. This difference between both cultivars might be found in the different light conditions during the first week of forcing. For 'Nordlicht' a DLI of 2.5 mol m^{-2} d^{-1} was measured, which is slightly higher than the minimum DLI of 2.1 mol m^{-2} d^{-1} determined in chapter 4, while for 'Sachsenstern' the 0.9 mol m^{-2} d^{-1} was already lower than the minimum DLI of 1.7 mol m^{-2} d^{-1} of the late-flowering cultivar. During the whole forcing period under natural light, DLIs were for both cultivars lower than their respective minimum DLIs, which makes the supply of photoassimilates too limited to increase starch reserves and assure proper flowering. Also in rose, low light levels limit photosynthesis and the amount of photoassimilates is insufficient to meet the flower demands and mobilization of carbohydrates from the base of the plant is important for further development (Mattson et al., 2008). Plants forced with assimilation light are able to build up some of their starch reserves during the first week of forcing, however, the obtained levels were still a lot lower than before cold treatment. Clear differences between forcing under natural light and with assimilation light were seen in the carbohydrate status of the leaves. Soluble carbohydrates and starch were markedly lower due to inadequate photosynthesis. However, the differences seen in the flowers were a lot smaller in 'Nordlicht'. At the colour-showing stage there was a lower glucose concentration in natural light conditions, but fructose and sucrose levels were equal. Since not all flower buds on the plants under natural light developed, samples were taken from developing flowers. Probably the limited leaf carbohydrate pool was used for those buds that opened. In cut flowers longevity of open flowers could be increased by removing other floral buds which compete for the inadequate amount of

carbohydrates (van Doorn and Han, 2011). A similar effect in azalea is possible as the non-developing flower buds are aborted and so excluded from carbohydrate supply, as a result, enough sugars are provided to flowers that do develop. Differences in total invertase activity were not significant, but at the colour-showing stage, plants under normal light conditions had a slightly higher activity. This is also seen during shading of Japanese pear, where the activities of acid invertase in the bud increased to enhance the sink strength (Ito et al., 2003). This increased sink strength might have stimulated the loading of sucrose at the source leaves providing still high levels of carbohydrates to opening flowers, provided that source leaves contained enough photoassimilates.

6.4.3 Post-production sucrose metabolism and flowering quality

The quality of flowering after production will be strongly influenced by the available carbohydrates. There is an immediate drop in soluble carbohydrates and starch in leaves when plants are placed at indoor conditions. Differences in the flower buds are much smaller, however, a lower glucose and fructose content is observed at indoor conditions. Post-harvest quality in cut roses shows that starch from petals is consumed to provide glucose and fructose for continued opening (Kumar et al., 2007). The total invertase activity in flowers for 'Nordlicht' and control plants of 'Sachsenstern' decreased at indoor conditions. Again in rose, the same is observed, in detached flowers, enzyme activities decrease (Horibe et al., 2013).

Quality of flowering at indoor conditions is also markedly lower, as the total percentage of fully opened flowers is lower. Probably the low starch reserves at the start of cold treatment and consequently at the end of cold treatment, resulting in a concentration of 29.3 mg g^{-1} FW for 'Nordlicht' and 12.6 mg g^{-1} FW for 'Sachsenstern' at the transfer to indoor conditions, is not high enough to ensure a higher percentage of fully open flowers at indoor conditions.

6.4.4 Effect of a high dose of paclobutrazol on sucrose metabolism and flowering

A first remarkable observation is the higher starch and soluble carbohydrate content at the end of cold treatment in plants treated with a high dose of PGR. It was shown that paclobutrazol decreased the activity of α-amylase (Pagano et al., 1997), which can explain the higher starch content in the leaves at the end of cold treatment.

Gibberellins can increase sink demand, which is related to increased activities of cell wall-bound invertase and neutral invertases in Japanese pear fruit, (Zhang et al., 2007), while in *Phalaenopsis* GA$_3$ stimulates sink activity by increasing sucrose synthase activity and not the invertase activity (Chen et al., 1994). A treatment with the GA-biosynthesis inhibitor paclobutrazol can decrease the amount of soluble invertase (González and Cejudo, 2007). In our case, we did not see a clear

significant effect of the paclobutrazol treatment on invertase activity. Although GA levels are presumably still lower in paclobutrazol-treated plants, it does not affect invertase activity. Paclobutrazol is a persistent plant growth regulator, nonetheless, 7 weeks of cold storage might have some effect on the breakdown of paclobutrazol in the plant. Furthermore, the flower bud is a highly expanding organ, so there might be a dilution effect during development (Gent, 1997), negating the possible negative effect on invertase activity. Therefore, the effect of paclobutrazol on flowering is minimal, even resulting in a slightly higher percentage of fully opened flowers. However, there is still a significant effect on flower diameter. Paclobutrazol-treated plants had a smaller diameter of 6.8 cm compared to 7.5 cm for standard-treated plants, which is a regularly observed effect (Ranney et al., 1994).

6.5   Conclusions

As there was no starch detected in the developing petals of azalea, increasing the glucose and fructose content relies on the transport of sucrose from the leaves. Therefore, flowering in azalea is a source-limiting process and needs high light levels to provide enough photoassimilates. Supplying higher light intensities should preferentially be done during the forcing itself. Prior to indoor conditions, it will be important to increase the carbohydrate reserve of the plants. Especially starch content in the leaves will be the determining factor for quality of flowering in the post-production phase, as these levels drop immediately at indoor conditions. A high dose of paclobutrazol applied during flower bud differentiation, did not affect the flowering response of the plants, but resulted in smaller flowers.

CHAPTER 7

Conclusions and future perspectives

Chapter 7

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Azalea production in East Flanders accounts for approximately 30 million plants each year. Since plants are sold as flowering pot plants it goes without saying that quality of flowering must be optimal. High quality flowering in azalea equals a homogenous bud burst and a continuous development to fully open flowers at indoor conditions. Growers continuously strive to improve the quality of their products and a central question arose: "Why do azalea flowers not always open at indoor conditions?" It was clear from the beginning that a single clear cut answer would not be easy to find, as flowering in azalea is a complex process starting with flower bud initiation and development, followed by flower bud dormancy and then anthesis. Since the extensive research of Bodson (1989) on flowering of Belgian pot azalea, the production techniques have changed. First of all, breeding efforts lead to constant renewal of cultivars, there are over 150 commercial cultivars differing in their natural flowering time. Second, to ensure a year-round production of flowering plants, extra cultivation techniques (multiple cuttings per pot, plant growth regulators, cold storage, and supplemental light) are used to 'force' plants into the right developmental stage at the right time. These changes have led to many new and unanswered questions, which we address in this thesis. First, we discuss the genotypic variations and production timing of flowering azalea. Flower bud differentiation rate is different among cultivars (chapter 2), and also flower bud dormancy is cultivar dependent (chapter 3). Early and late flowering of 'H. Vogel' sports are controlled by production schedule. Differences in flower bud development and dormancy were not found. Further, the photosynthetic capacities and metabolic rate of cultivars will be different leading to different light requirements (chapter 4) and different responses in carbohydrate metabolism during cold treatment (chapter 5) and forcing (chapter 6).

Second, the increased use of plant growth regulators is addressed. Plant growth regulators are used to initiate the generative phase, but have, in contrast with what was first assumed, no

influence on differentiation rate in our experimental set-up (chapter 2). The PGR did however; influence the dormancy status of flower buds, requiring higher amounts of chilling units to result in a similar flowering quality (chapter 3). The effect of PGR on anthesis was minor (chapter 6)

Third, cold storage at low temperatures (2-3°C) has always been used to store cuttings and plants before sale. But as results showed a clear improvement of flowering when plants received a 7°C cold treatment, this extra step is included for early-flowering cultivars. This 7°C will break dormancy in a controlled and efficient manner (chapter 3), however, not without consequences towards soluble carbohydrates and starch reserves and photosynthesis as shown in chapter 5.

Fourth, over the years preliminary trials with supplemental light already indicated that flowering could be enhanced and be more uniform. Extra light means extra photo-assimilates which will be beneficial for anthesis as petal growth requires high amounts of sugars. However, the forcing period is short (2-3 weeks). Therefore, we investigated which light levels were sufficient to replenish starch and soluble carbohydrates depletion due to cold treatment to ensure flowering at indoor conditions (chapter 6), taking into account the minimum light requirements of the plants (chapter 4).

To conclude we sum up some guidelines to ensure good quality flowering.

Genotypic variations

There is a constant renewal of cultivars with different characteristics. We followed 13 different commercial cultivars during flower differentiation (chapter 2) and flower bud dormancy (chapter 3). The 13 cultivars belong to three different groups according to their natural flowering period: early-flowering, semi-early-flowering and late-flowering cultivars. Flower differentiation rate showed clear differences among the cultivars. Next, the flower bud stage that responded best to the cold treatment was also different between early-flowering cultivars and later flowering cultivars. The six early flowering sports of 'H. Vogel' all had the same differentiation rate and response to cold treatment. Fifty four days after the first PGR treatment of vegetative plants, flower bud stage 7 was reached and proved to be the optimal stage to start cold treatment. Flower bud development was completed fastest for the semi-early-flowering cultivars: 'Mont Blanc' requiring 63 days and 'M. Marie' and 'Otto' 66 days to reach flower bud stage 8 after the first PGR treatment. Flower bud stage 8 proved to give the best flowering results after cold treatment. The four late-flowering cultivars could be divided in two groups with highly different rates of differentiation. Flower bud stage 8 proved also to be optimal to start cold treatment and was reached 88 days after the first PGR treatment for 'Thesla' and 'Sachsenstern'; and after 73 days for 'Mw. G. Kint' and 'Tamira'. Interestingly, we see that the faster developing cultivars have the poorest flowering results. Furthermore, we observed that accumulation of chilling units might

begin before flower bud stage 8 is reached. The flower development between stages 7 and 8 takes a few weeks, while our artificial dormancy-breaking treatment started at the beginning of stage 7 and 8 respectively. So if further optimisation of CU is needed, climate room experiments should be developed where the flower development can take place at a constant temperature and where at several time points between stage 7 and 8 the cold treatment starts.

Bodson (1983) found that earliness of flowering could be linked partly to flower differentiation rate and partly to flower bud dormancy. This was partially valid for the cultivars we tested. We found no clear order in flower differentiation rate between early-, semi-early- and late-flowering cultivars so this differentiation rate per se will not affect earliness of flowering. However, an indirect relation might be present if environmental conditions during differentiation rate proof to influence flower bud dormancy. In vegetative poplar buds, warmer temperatures during the autumn hasten short-day induced growth cessation and result in a deeper dormancy (Kalcsits et al., 2009). Nonetheless, we found a clear difference in chilling requirements between the cultivars, early-flowering cultivars requiring less chilling units than semi-early-flowering cultivars and late-flowering cultivars.

The obtained results in this thesis provide growers already with an easy tool to determine the ideal time for cold treatment. As they can count the days from the first PGR treatment (on vegetative plants) to the ideal flower bud stage for a 7°C treatment, regardless of the dose of PGR and regardless of the cultivation period for 'H. Vogel' (chapter 2).

A more in-depth study of dormancy and cold treatment was done on three cultivars of the three different flowering groups. As discussed in chapter 3, the role of abscisic acid will probably be limited to the first stages of dormancy as levels decrease rapidly during cold treatment and this only in the semi-early- and late-flowering cultivars. The early-flowering cultivar had basal ABA concentrations during cold treatment. The high loss of carbon reserves during cold treatment in the early-flowering cultivar might be due to the fact that this cultivar did not enter true endodormancy and respiration rates were not down regulated as much as in completely dormant plants. Monitoring respiration rates at bud level during development form stage 7 to stage 8 at different temperatures could clarify this.

Light requirements also differed between early- and late-flowering cultivars (chapter 4). Late-flowering cultivars require less light to balance respiration and photosynthesis and also had a lower dark respiration and higher maximum net photosynthesis. In forcing conditions this would mean that the late-flowering cultivar requires less light to obtain a similar accumulation of soluble carbohydrates and starch as early-flowering cultivars. However, in chapter 6 we did not see higher carbohydrate concentrations in the leaves of the late-flowering cultivar, only in the petals there

were higher concentrations of soluble carbohydrates. Perhaps the different late-flowering cultivars used in chapter 4 and 6, have different responses. To better identify the variability of light requirements among cultivars, extra measurements should be performed on different cultivars of a same flowering group.

Production schedules

The early flowering sports of 'H. Vogel' have different production schedules depending on the intended flowering period (August – December). This different timing will also result in different climatic factors during flower initiation, differentiation and putative dormancy. We tested this effect on an early and late production cycle for 'Nordlicht'. First, differentiation rate was not affected by the production cycle, and even in two successive years there was no different differentiation rate due to temperature or light differences. Differences in ABA sensitivity of the buds during late and early production were not reflected in a different earliness of flowering, but different flower homogeneity.

As semi-early- and late-flowering cultivars have a broader variety in flower colours and leaf shapes, it might be interesting to force them to flower earlier than normal. This will require additional research. First, the issue of flower initiation seems less important as PGR regulates the start of the flower initiation. Second, the differentiation rate of these cultivars might be more susceptible to environmental parameters than the tested early cultivars in our experiments, temperature especially, as was shown for certain late-flowering cultivars by Bodson (1989). Also the different day lengths might have a role, although the total daily light sum (which is correlated with day length) will probably exert a much higher effect on differentiation (Bodson, 1989). Third, the different day lengths, when advancing the production, might play a role on dormancy initiation and the depth of the dormancy status of the flower buds. A molecular approach as in poplar might be the correct way to explore this further; using both early-flowering and late-flowering cultivars.

Our experiments always took place under natural climatic conditions and under standard cultivation practice. Even though for the early-flowering cultivar we did not see any difference in flower differentiation and chilling requirements, the total number of opened buds for the early production plants was significantly less than during the late production cycle. Furthermore, during different cultivation years we saw also different flowering responses of the plants, even though we treated the plants exactly the same at the same moment: flower earliness could differ a few days, flower homogeneity was not affected. This might be due to the depth of dormancy that can be influenced by both day and night temperatures (Kalcsits et al., 2009). This makes it more

difficult to provide the exact number of CU necessary for cultivars to break dormancy. Further research should focus on the effect of these environmental factors, especially in respect with climate change. To control these factors, experiments should be carried out in growth chambers where the conditions can be maintained constant.

Increased use of plant growth regulators

Over the last decade the use of plant growth regulators to control vegetative growth and stimulate generative growth has increased a lot. Next to chlormequat, the more persistent growth regulator paclobutrazol increased in popularity because of its fast mode of action to control vegetative growth. Paclobutrazol is an inhibitor of GA-biosynthesis (Rademacher, 2000) and also a putative inhibitor of ABA-catabolism (Saito et al., 2006), resulting in decreased levels of GA and increased levels of ABA. Since GA plays a major role in flower initiation, end of dormancy and petal growth at anthesis and ABA plays a major role in flower bud dormancy we investigated the implications of increased plant growth regulator applications on azalea flowering.

We tested the effect of PGR on flower initiation and differentiation and found, as expected (Meilan, 1997), that flower initiation could be enhanced when vegetative plants were treated with PGR. The use of PGR also led to a more uniform flower bud development at plant level. Control plants had a higher variability in flower bud stage, this was also clearly seen for 'H. Vogel' by Bodson (1989). As natural flower initiation might also depend on the availability of carbohydrates, buds on smaller shoots with fewer leaves, might be delayed in initiation resulting in a plant with flower buds at different developmental stages. This high variability can have its consequences when plants must receive a cold treatment at 7°C to break dormancy as flower bud stage is highly important in the response to this cold treatment (chapter 3). Plants with a uniform developmental flower bud stage are preferred; if plants have buds in different stages at the start of cold treatment a non-uniform flowering afterwards is expected.

The rate of flower differentiation was not affected by a PGR treatment in the three cultivars ('Nordlicht', 'M. Marie', 'Mw. G. Kint') with different natural flowering times. Also Bodson (1989) found this effect for the early-flowering cultivar 'H. Vogel'. In contrast, for the semi-early- and late-flowering cultivars Bodson (1989) observed an increased developmental rate during the early stages of development. In our experimental set-up, we initiated flowering with chlormequat and applied paclobutrazol when buds were already in stage 3. No effect on flower differentiation rate during the early stages was observed for late-flowering cultivars and neither did the supplemental paclobutrazol application accelerate differentiation. However, it might still be interesting to initiate flowering in vegetative plants directly with paclobutrazol. Since a treatment of 'H. Vogel'

with paclobutrazol can increase the expression of *LFY* (De Keyser et al., 2013a), which precedes that of other meristem-identity genes with flower-specific expression, flower differentiation might be enhanced by this specific PGR. It is certainly worthwhile to research if this up-regulation is valid for cultivars belonging to different flowering groups and verify also development. The dose of paclobutrazol will probably be of minor importance in flower bud differentiation as differentiation rate was not affected by the dose of paclobutrazol in the research of Bodson (1989) and in our experiment. This is highly relevant to a potential cold treatment at 7°C, as the amount of days from the start of PGR treatment, regardless of the dose, is an easy indicator of the flower developmental stage, which is an important criterion to start the cold treatment to break dormancy.

The cold requirements of plants are affected by PGR (chapter 3). As paclobutrazol might increase the ABA level (Saito et al., 2006) and hence the dormancy status of the plant, we verified the effect of four successive late applications where the last treatment was done one day prior to cold treatment. The early-flowering cultivar 'Nordlicht' did not respond to this late paclobutrazol treatments with an increase in ABA, but the late-flowering cultivar 'Mw. G. Kint' had significant higher concentrations of endogenous ABA in the flower buds. This resulted in an increased need for chilling units to have a similar optimal flowering response compared to control plants. As ABA did not increase in the early-flowering cultivar, we hypothesize that reduced GA levels might be more important to explain the prolonged dormancy period. As GA biosynthesis increases during dormancy release (Karlberg et al., 2010), the use of paclobutrazol (which blocks the GA biosynthesis pathway) might delay dormancy release. Measuring the endogenous GA levels might increase our understanding for these higher chilling requirements. Indeed only knowledge of gene regulation might be insufficient (Zhao et al., 2007). It is known that GAs control their own homeostasis with feedback and feed-forward regulation of active GA levels in the cytosol. Yet paclobutrazol blocks earlier in the biosynthesis and thus the precursors for the active GA-biosynthesis might only be present at very low levels in the cells.

Interestingly, during cold treatment plants treated with paclobutrazol did show less decrease in carbohydrates compared to plants treated with only chlormequat (chapter 6). So even if the chilling requirements are higher in paclobutrazol treated plants, the effect on carbohydrate reserves at the end of the cold period might be minor. Further research might reveal the reasons why paclobutrazol slowdowns the decrease in reserves. First, it might be interesting to investigate if the ABA concentration also increases in leaves after a paclobutrazol treatment and if this might affect the carbohydrate status of the plant as ABA can repress the expression of *α-amylase* genes (Bethke et al., 1997). Second, measuring the activity of α-amylase and invertase might be useful,

as both activities decrease by paclobutrazol treatment (Pagano et al., 1997; González and Cejudo, 2007). Third, respiration measurements of control plants and plants treated with paclobutrazol might also give extra information. As vegetative growth is strongly inhibited by paclobutrazol, respiration rates could also be decreased, which then leads to a smaller loss of reserves during cold storage.

In chapter 6 the subsequent effects of PGR on anthesis are discussed. Plants treated with chlormequat are compared with plants treated with supplemental high doses of paclobutrazol. Even though paclobutrazol is a more persistent PGR and it might be assumed to still have an effect on the levels of GA, effects on carbohydrates, invertases and flowering were marginal. As discussed in chapter 6, the minor effects of paclobutrazol on flower sucrose metabolism might be explained by the dilution factor in these rapid growing petal tissues. To confirm this, endogenous concentrations of paclobutrazol in the flowers should be measured. It would also be interesting to measure paclobutrazol in both leaves and flower buds throughout the production cycle to have an idea of the persistence of this substance in azalea. Although flower opening was not impaired, the flower diameter and thus petal cell elongation was significantly smaller.

Cold storage

Cold storage at 2-3°C has always been used to store cuttings and plants; storage at 7°C is a recent practice. The stay at 7°C must break flower bud dormancy in an efficient and controlled way. However, this period must be as short as possible, since plants at those temperatures are more likely to suffer from drought stress and are at increased risk of *Botrytis* compared to plants stored at 2-3°C. To keep this period as short as possible, but still effective, an indicator for dormancy release might come in handy. In chapter 3 we determined the endogenous ABA concentrations and the sensitivity to exogenous ABA during cold storage of 3 cultivars with different flowering times. We could not find an overall correlation between the ABA concentrations, sensitivity to ABA and dormancy release for all three cultivars. We showed that a basal level of ABA might be a prerequisite for dormancy release, but it will not be the ultimate factor releasing flower buds from dormancy. For a quick and efficient indicator of dormancy release more research is needed. Up till now the best way to determine dormancy release is to observe flowering after different chilling units. The expression patterns of genes seem more and more promising to be this easy indicator (Leida et al., 2012). A commercial test of a Dutch company (NSure) that claims to predict dormancy release in *Viburnum* is an example of such a molecular test.

During storage at 7°C metabolism is slowed down, however, this will be to a lesser extent than at 2 °C. This enables the flower buds to further develop to an equal flower bud stage of 8 and

subsequently very homogenous flowering. Linked to this 'high' 7°C temperature, respiration rates during dark cold storage are higher than at 2°C, resulting in a decrease of carbohydrate reserves as shown in chapter 5. After cold treatment, photosynthesis recovery was fast and an adaptation period at low light levels before forcing was not necessary. Next to photosynthesis recovery, also recovery of carbon reserves was fast. Foliar soluble carbohydrates remained relatively stable during the first days of acclimation, while starch content increased significantly (chapter 5). One week of acclimation, stabilized or slightly decreased leaf carbohydrate levels (chapter 6). So recovery of the plants is very fast, both for photosynthesis and carbohydrate content. However, in practice early-flowering cultivars can be stored at 7°C for four weeks after which they are kept for several days or weeks at 2°C until market demands. It should be investigated if these extra weeks at 2°C will result in (severe) damage of the light harvesting complexes if light conditions are high at the moment of forcing. If so, then an adaptation period under low light intensity might be necessary.

Light: before, during and after forcing

In chapter 5 we determined the loss of soluble carbohydrates and starch in plants stored at 7°C. However, not only plants that are stored in a cold room will lose carbon reserves. As discussed in chapter 4, DLIs during November, December and January in the greenhouse are often lower than the estimated minimum DLIs for photosynthesis. Consequently, plants that receive a natural cold period in the greenhouse will also lose carbon content, though probably to a lesser extent. To make up for the losses made during this dormancy period, supplemental light in the forcing greenhouse is necessary. As shown in chapter 6, only plants which receive supplemental light increase their carbohydrate content again. These carbohydrates are crucial for flower opening, as we see a strong increase in glucose and fructose in the petals during opening. However, we did not see strong significant differences between plants with and without supplemental light in carbohydrate content and invertase activity in the petals. This might be due to the fact that we always sampled buds that actually developed to opening flowers. Buds that did open had enough sink strength to accumulate soluble carbohydrates, while buds that remain closed are assumed to have insufficient sink strength. As sink strength is sometimes correlated with the size of the sink (Marcelis, 1996) it was seen that especially the smaller buds did not open.

To test post-production quality, plants were removed from the forcing greenhouse with supplemental light at the early stages of colour-showing buds and placed in a climate room mimicking indoor conditions. The lack of light immediately decreased the starch content in the leaves of plants. This makes leaf starch reserves the main storage of carbohydrates in azalea; this

in contrast with for example cut roses where starch is accumulated in the petals (Kumar et al., 2008b). In short, our results show clearly that light during the forcing period will be determinative for flowering quality at indoor conditions. However, as energy costs increase, the use of supplemental light is costly. Therefore, the minimum light requirements determined in chapter 4 can be used to optimize the use of supplemental light. In Denmark an alternative light control system (DynaLight desktop) exists that automatically defines the most cost-efficient use of supplemental light (Kjaer et al., 2012). The program uses set points for daily photosynthesis integral, forecasted solar irradiance and the electricity price. As we determined our minimum DLI based on photosynthetic measurements, we can provide a daily photosynthesis integral (DPI) as well. This DPI might be a more appropriate tool to control supplemental lighting compared to DLI, as it will also take into account the levelling of photosynthetic rates (P_{max}) at periods of high light intensity.

To further decrease energy costs, a different night/day temperature regime might be useful, as respiration rates are lower during a cooler night (chapter 4). Nevertheless, further experiments should confirm if the developmental rate of the flowers is not affected by these colder night temperatures.

Guidelines and recommendations for high quality flowering

The reasons for the two main problems with flower quality (heterogeneous flowering and the arrest of development at indoor conditions) have been identified in this research and some guidelines for correct production techniques are formulated below.

First, flower homogeneity is influenced by the amount of chilling that plants receive during the dormancy period. Unfulfilled chilling requirements will lead non uniform flowering. However, an easy indicator for flower bud release could not be found in endogenous ABA concentrations or the easy bio-assay. Also the proper use of plant growth regulators is relevant. It will ensure an even development of the flower buds, which is essential to have an optimal result from the artificial cold treatment at 7°C. But high doses of plant growth regulators will increase the chilling requirements, compared to untreated plants.

Second, the arrest of flower opening at indoor conditions is due to insufficient leaf starch reserves. Forcing plants to flower with the appropriate amount of supplemental light will prevent this problem by building-up reserves before sale. In light limiting conditions these starch reserves are broken down to provide soluble carbohydrates to the developing flower. Care must be taken with the storage (2-3°C) of plants when they leave the forcing greenhouse, the build-up starch reserves will be used again before flowers develop further at indoor conditions.

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Additional data to chapter 5

Additional data 1: RNA concentration and purity.

Description: RNA quantity and purity (A_{260}/A_{280} and A_{260}/A_{230}) was measured of each sample using a NanoDrop spectrophotometer.

Sample	Genotype	Concentration (ng μL^{-1})	A_{260}/A_{280}	A_{260}/A_{230}
1	'Nordlicht'	441.48	1.80	1.02
5	'Nordlicht'	272.69	1.92	1.37
9	'Nordlicht'	338.49	1.86	1.13
13	'Nordlicht'	336.99	1.98	1.59
17	'Nordlicht'	412.05	1.84	1.18
21	'Nordlicht'	304.65	1.89	1.08
25	'Nordlicht'	410.02	1.78	2.06
29	'Nordlicht'	228.12	1.88	1.23
33	'Nordlicht'	341.01	1.89	1.42
37	'Nordlicht'	334.35	1.98	1.67
41	'Nordlicht'	309.34	1.79	0.99
45	'Nordlicht'	1029.17	1.88	1.25
49	'Nordlicht'	449.23	1.85	2.12
1	'M. Marie'	839.38	2.03	1.44
5	'M. Marie'	882.93	2.04	1.62
9	'M. Marie'	946.70	2.01	1.48
13	'M. Marie'	848.66	1.99	1.43
17	'M. Marie'	1145.58	2.02	1.52
21	'M. Marie'	947.36	2.09	1.81
26	'M. Marie'	1223.22	1.98	1.59
29	'M. Marie'	1099.75	2.10	1.74
33	'M. Marie'	1090.79	2.07	1.67
37	'M. Marie'	858.38	2.02	1.59
41	'M. Marie'	1200.85	2.00	1.66
45	'M. Marie'	1123.15	2.01	1.57
49	'M. Marie'	892.64	2.07	1.94
53	'M. Marie'	1330.63	2.11	1.98
57	'M. Marie'	1009.69	2.07	1.68
61	'M. Marie'	1290.76	2.07	1.71
65	'M. Marie'	998.04	1.97	1.55

Additional data 1 (continued)

Sample	Genotype	Concentration (ng μL^{-1})	A_{260}/A_{280}	A_{260}/A_{230}
1	'Mw. G. Kint'	295.9	2.04	1.97
5	'Mw. G. Kint'	295.53	2.05	1.83
9	'Mw. G. Kint'	163.2	2.08	1.81
13	'Mw. G. Kint'	347.18	2.04	1.94
17	'Mw. G. Kint'	201.25	2.05	1.67
21	'Mw. G. Kint'	213.34	2.04	1.65
25	'Mw. G. Kint'	136.18	2.04	1.57
37	'Mw. G. Kint'	192.00	2.05	1.66
41	'Mw. G. Kint'	214.09	2.07	1.73
45	'Mw. G. Kint'	184.46	2.07	1.63
49	'Mw. G. Kint'	313.21	2.05	2.07
53	'Mw. G. Kint'	117.69	2.04	1.35
57	'Mw. G. Kint'	231.57	2.03	1.46
69	'Mw. G. Kint'	242.32	2.06	1.77
73	'Mw. G. Kint'	174.47	2.04	1.56

Additional data 2: Results of the SPUD assay.

Description: Mean Cq values obtained after the analysis of 3 sample types (RNA, cDNA and control) in a SPUD assay. Both RNA and cDNA samples were diluted and Cq values of the dilution series were measured to demonstrate no inhibitors could be diluted out. Hence, samples were free of PCR inhibitors.

Sample	Mean Cq	Stdev Cq	Min Cq	Max Cq
Control	24.38	0.06	24.30	24.47
RNA	24.59	0.06	24.51	24.68
cDNA	24.55	0.04	24.49	24.60

Dilution	RNA (± 2*stdev)	cDNA (± 2*stdev)
1	24.59 (± 0.12)	24.55 (± 0.08)
1/5	24.51 (± 0.22)	24.61 (± 0.06)
1/25	24.60 (± 0.08)	24.66 (± 0.12)
1/125	24.57 (± 0.03)	24.65 (± 0.08)

Additional data 3: PCR efficiencies of the standard curves.

Description: Summary of slopes, intercepts and R² of the standard curves made of a dilution of 5/6 standards for each gene under analysis. The PCR efficiency (E) and the standard deviation on E (SD(E)) were calculated according to the formulas described in Hellemans et al. (2007). Data were calculated for every experiment.

Experiment	Gene	Slope	Intercept	R²	# of standards	E	SD (E)
'Nordlicht	SUS	-3.684	40.232	0.999	6	1.868	0.013
	HK5	-3.558	33.464	1	6	1.910	0.005
	HK47	-4.056	45.523	0.999	5	1.764	0.013
	GAPDH	-3.848	40.175	0.999	6	1.819	0.010
'M. Marie'	SUS	-3.616	41.920	0.999	6	1.890	0.010
	HK5	-3.535	35.741	0.999	6	1.918	0.012
	HK47	-4.007	47.933	0.998	5	1.776	0.015
	GAPDH	-3.765	42.707	0.999	5	1.843	0.009
'Mw. G. Kint'	SUS	-3.637	39.365	1	6	1.884	0.008
	HK5	-3.501	34.563	0.999	6	1.930	0.014
	HK47	-3.951	37.334	1	6	1.791	0.004
	GAPDH	-3.776	38.365	0.999	6	1.840	0.009

Curriculum vitae

Personal data

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Education

2005-2007 **Master in Bioscience Engineering: Cell and Gene Biotechnology**

Ghent University, Faculty of Bioscience Engineering, Ghent.

Master thesis: "Transformatie van katoen (*Gossypium hirstum* L.): oriënterend onderzoek voor de ontwikkeling van een meristeem-gebaseerde transformatie-methode"

Promotor: Prof. Dr. Ir. Godelieve Gheysen, Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University.

2001-2005 **Master in Industrial Science: Biochemistry**

University College West Flanders (howest), Department PIH, Kortrijk.

Master thesis: "Onderzoek naar het effect van geselecteerde bacteriën op de reproductiesnelheid van verschillende rotiferenklonen"

Promotor: Lic. Christophe Wille, Department PIH, University Collage West Flanders.

1995-2001 Secondary education: Science-Mathematics

Sint-Jozefscollege, Izegem.

Professional career

2012 – now   **Scientific associate**

Lab of In Vitro Biology and Horticulture, Department of Plant Production, Faculty of Bioscience Engineering, Ghent University (50%)
PCS Ornamental Plant Research (50%)
IWT-agricultural research project: "Kennisgedreven sturing van plantfysiologische processen in de sierteelt ter bevordering van plantkwaliteit".

2008 – 2012   **Scientific associate**

Lab of In Vitro Biology and Horticulture, Department of Plant Production, Faculty of Bioscience Engineering, Ghent University (50%)
PCS Ornamental Plant Research (50%)
IWT-agricultural research project: "Bloeiregulatie en –kwaliteit bij azalea: interactie tussen genetische, fysiologische en teeltgebonden factoren".

2007 – 2008   **Scientific associate**

Inagro (research & consultancy in agriculture and horticulture)
IWT-agricultural research project: "Fytomonitoring bij glasgroenten voor 'on-line' diagnose van groei en ontwikkeling en als hulpmiddel voor kwaliteitsbewaking en duurzame teeltstrategieën".

Research related activities

Tutor of bachelor and master students

- Veerle Vandaele (2008-2009) Invloed van teelttechnieken op de bloeikwaliteit bij azalea. Master thesis, Hogeschool Gent.
- Maarten Van de Vijver (2011-2012) Invloed van het koolstofmetabolisme op de bloeikwaliteit bij azalea. Master thesis, Hogeschool Gent.
- Bart Van Calenberge (2011-2012) Karakterisering van bloemknopdormantie en invloed van bewaring op bloeikwaliteit bij azaela. Master thesis, Hogeschool Gent.
- Barbara De Smet (2011-2012) Fysiologische processen en stress responsen in houtachtige planten. Research project MA1 Biochemistry and Biotechnology, Faculty of Sciences, Ghent University.
- Dorien Van Wesemael (2012-2013) Koolhydraatreserves bepalen bloeikwaliteit bij azalea. Master thesis, Faculty of Bioscience Engineering, Ghent University.

International conference contributions

- 28th International Horticultural Congress
  22-27 August 2010, Lisbon, Portugal, poster presentation

- 9th International Plant Cold Hardiness Seminar
  17-22 July 2011, Luxembourg, Luxembourg, poster presentation

- 2nd International Symposium on Woody Ornamentals of the Temperate Zone

1-4 July 2012, Ghent, Belgium, oral presentation

- 7th International Symposium on Light in Horticultural Systems
  15-18 October 2012, Wageningen, The Netherlands, poster presentation

National conference contributions

- National meeting on Secondary Metabolites and Molecular Farming (BPBA)
  November 7, 2008, Ghent, Belgium, poster presentation

- National meeting on Plant Hormones: New Insights for Biotechnology (BPBA)
  November 13, 2009, Gembloux, Belgium, poster presentation

- 18[de] studiedag azalea
  February 25, 2010, Destelbergen, Belgium, oral presentation

- 19[de] studiedag azalea
  February 24, 2011, Destelbergen, Belgium, oral presentation and practical demonstration

- 20ste studiedag azalea
  March 1, 2012, Destelbergen, Belgium, oral presentation

- Plant research@FBW
  May 4, 2012, Ghent, Belgium, oral presentation

- Inspiration days at floreac: L'Azalée, une tradition pour l'avenir!
  August 21, 2012, Lochristi, Belgium, oral presentation

- 21ste studiedag azalea
  February 21, 2013, Destelbergen, Belgium, oral presentation and moderator workshop "To flower or not to flower"

- Workshop 'Post-harvest kwaliteit in de tuinbouw'
  April 15, 2013, Melle, Belgium, oral presentation

- Workshop 'Beworteling bij jongplanten'
  October 10, 2013, Destelbergen, Belgium, oral presentation

- Plant research@FBW
  February 5, 2014, Ghent, Belgium, oral presentation

- 19th national symposium on applied biological sciences
  February 7, 2014, Gembloux, Belgium, oral presentation

A1-publications

Christiaens A, Pauwels E, Gobin B, Van Labeke MC (2014) Flower differentiation of azalea depends on genotype and not on the use of plant growth regulators. *Plant Growth Regulation*, doi:10.1007/s10725-014-9948-2.

Christiaens A, De Keyser E, Lootens P, Pauwels E, Roldán-Ruiz I, De Riek J, Gobin B, Van Labeke MC (2014) Cold storage to overcome dormancy affects the carbohydrate status and photosynthetic capacity of *Rhododendron simsii*. *Plant Biology*, doi:10.1111/plb.12195.

Christiaens A, Dhooghe E, Pinxteren D, Van Labeke MC (2012) Flower development and effects of a cold treatment and a supplemental gibberellic acid application on flowering of *Helleborus niger* and *Helleborus* x *ericsmithii*. *Scientia Horticulturae* 136:145-151. IP 1.396

P1-publications

Christiaens A, Van Labeke MC, Pauwels E, Gobin B, De Keyser E, De Riek J (2013) Flowering response of an early and late flowering azalea cultivar (*Rhododendron simsii*) to cold treatment. *Acta Horticulturae* 990:83-89.

Berruti A, Scariot V, Borriello R, Bianciotto V, Della Beffa MT, Christiaens A, Van Labeke MC, De Keyser E (2013) Rationalization of *Camellia japonica* L. pot cultivation: a multidisciplinary approach. *Acta Horticulturae* 990:159-166.

De Keyser E, De Riek J, Christiaens A, Van Labeke MC, Pauwels E, Gobin B (2013) Gene expression profiling of candidate genes for flowering initiation and dormancy breaking in *Rhododendron simsii* hybrids. *Acta Horticulturae* 990:187-192.

Christiaens A, Van Labeke MC, Pauwels E, Gobin B, De Keyser E, De Riek J (2012) Flowering quality of azalea (*Rhododendron simsii*.) following treatments with plant growth regulators. *Acta Horticulturae* 937:219-224.

Berruti A, Scariot V, Christiaens A, Van Labeke MC (2012) Changes in ABA levels in vegetative and flower buds during dormancy in *Camellia*. *Acta Horticulturae* 953:247-254.

Vermeulen K, De Swaef T, Steppe K, Christiaens A, Bleyaert P, Dekock J, Aerts JM, Berckmans D (2011) Quantifying plant responses of a tomato crop under a thermal screen. *Acta Horticulturae* 893:831-838.

Submitted for peer review

Christiaens A, Lootens P, Roldán-Ruiz I, Pauwels E, Gobin B, Van Labeke MC (2014) Determining the minimum daily light integral for forcing of azalea (*Rhododendron simsii*). *Scientia Horticulturae*, submitted.

Other publications

Christiaens A, Van Labeke MC (2014) Beworteling van stekken onder LED-belichting. *Sierteelt & Groenvoorziening* 1:27-28.

Christiaens A, Van Labeke MC (2013) Fysiologische processen tijdens transport en bewaring. *Sierteelt & Groenvoorziening* 13:28-30.

Christiaens A (2013) Onderzoeksproject plantenfysiologie. *Azalea informatief*.

Christiaens A (2012) Bloeikwaliteit bij azalea: 4 jaar onderzoek in een notendop. *Sierteelt & Groenvoorziening* 16:21-23.

Christiaens A, Lootens P (2012) Belichting bij het forceren van azalea, een noodzaak! *Sierteelt & Groenvoorziening* 7:32-34.

Christiaens A (2011) Een gekoelde azalea heeft licht nodig. *Verbondsnieuws* 16:15-16.

Christiaens A (2011) Niet iedere Gentse azalea kan in stadium 7 naar de koelcel! *Verbondsnieuws* 16:15-16.

Christiaens A (2010) Wat gebeurt er met de Gentse azalea bij 7°C? *Verbondsnieuws* 15:28-29.

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Christiaens A (2009) Bloei 'Sachsenstern' in forcerie en huiskamer. *Azalea informatief*.

Christiaens A, Vandaele V (2009) Bloei 'Sachsenstern' in forcerie en huiskamer. *Verbondsnieuws* 13:29-30.

Christiaens A (2008) Bloeiregulatie en –kwaliteit bij azalea: interactie tussen genetische, fysiologische en teeltgebonden factoren. *Sierteeltonderzoek in Vlaanderen* 2008, p23.

Bleyaert P, Christiaens A, Verbruggen B, Vermeulen K (2008) Beweegbaar belommeringsdoek bij sla beloftevol bij correct gebruik. *Proeftuinnieuws* 20:36-38.

Christiaens A, Bleyaert P, Vermeulen K, Steppe K, Lemeur R, Dekock J, Aerts JM, Berckmans D (2008) Plantsensoren bepalen wateropname en waarschuwen voor droogte. *Proeftuinnieuws* 17:32-34.

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