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ORIGINAL PAPER

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

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Abstract 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 or 48 days to reach 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 to reach 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 (17–18 days), the late-flowering cultivars 'Thesla' and 'Sachsenstern' requiring the highest amount of days (24 days) and the early-flowering cultivars and the late-flowering cultivars 'Mw. G. Kint' and 'Tamira' requiring an intermediate number of days (20–22 days).

Keywords *Rhododendron simsii* hybrids · Flower initiation · Paclobutrazol · Chlormequat · Flowering

Abbreviations

GA	Gibberellin
PGR	Plant growth regulator
DLI	Daily light integral

Introduction

The genus *Rhododendron* of the Ericaceae family includes both rhododendrons and azaleas. The evergreen garden rhododendron can be found in the subgenus *Hymenanthes*, section *Pontica*. Deciduous azaleas belong to the subgenus *Hymenanthes*, section *Pentanthera* and evergreen azaleas to the subgenus *Azaleastrum*, section *Tsutsusi* including the modern florist azalea, *Rhododendron simsii* hybrids (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,

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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 (PGR) (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 in groups according to their natural flowering period: early (from August), semi-early (from November) and late (from February) flowering cultivars. Flower initiation depends on environmental factors such as day length, irradiance and temperature (Criley 1969; Larson and Biamonte 1972; Pettersen 1972, 1973; Bodson 1983) but also the genetic background of the cultivars will exert an effect (Bodson 1983). To assure a year-round production, PGR are used to start flower initiation independent of these factors (Bodson 1989; Marosz and Matysiak 2005; Meijón et al. 2009, 2011a). The commonly used PGR chlormequat and paclobutrazol block the gibberellin biosynthesis pathway at two different steps (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 PGR to strictly control vegetative growth though effects on generative development are not documented. This paper studies if increased doses of PGR might influence the developmental flower pattern for two cultivars belonging to a different flowering group. Further, flower differentiation is compared among 13 *R. simsii* genotypes and for one cultivar seasonal effects are also documented. This fundamental information can be used to schedule production.

Materials and methods

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 cultural conditions and were pinched 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).

Experiment 1: Effect of PGR applications

This experiment is performed with two 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 pinched 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 pinch on 25 May 2009 (late production). For 'Mw. G. Kint', the second pinch was on 11 June 2009.

Three different treatments with PGR were applied: a control treatment without PGR, a standard and a high dose PGR treatment. For the standard treatment, plants were treated weekly, six 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 six 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.

Experiment 2: Seasonal variations

Flower initiation and differentiation of 'Nordlicht' was followed for three 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 pinched 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 pinch on 25 May 2009 and 3 June 2010, respectively. Weekly treatment with PGR started on 4 May 2009, 4 August 2009 and 3 August 2010 for plants potted in July 2008, December 2008 and 2009 respectively.

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'; 'Michelle 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 pinch (3 June 2010) plants were transferred outside. Weekly treatment with PGR started on 3 August 2010.

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.

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 min averages were recorded. A mean daily temperature, a mean daily light integral (DLI) and total irradiation sum were calculated and used to evaluate results.

Statistical analysis

Regression analysis of flower development was done in Sigma Plot 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.

Results

Effect of PGR on flower development

Flower differentiation was followed for 'Nordlicht' and 'Mw. G. Kint' (Fig. 1). Differentiation progressed linear and was not significantly different between control plants and PGR-treated plants for either of the cultivars (Table 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.

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, Fig. 2a) after the first PGR application. For all three production cycles flower differentiation progressed linear to stage 7 (Fig. 2a) in function of the number of days after the first PGR application. The differentiation rates did not significantly differ between the three production cycles (linear regression analysis, $p = 0.64$) despite the different climatic conditions (Table 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 (14.3 °C) was lower while the irradiation sum (239 MJ m^{-2}) was comparable with the early production cycle of 2009.

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 similarly (Fig. 2b). Stage 7 and 8 were reached 54 and 76 days after the first PGR application. When comparing the three semi-early-flowering cultivars (Fig. 3), 'Mont Blanc' differed significantly ($p = 0.003$) from 'Michelle Marie' and 'Otto'. To reach stage 7 and 8 'Mont Blanc' required 46 and 63 days after the first PGR application whereas 'Michelle Marie' and 'Otto' reached these stages respectively 2 and 3 days later. The rate of development for all three semi-early-flowering cultivars was faster than for 'H. Vogel' sports (Table 3). The late-flowering cultivars (Fig. 4) differed significantly among each other ($p < 0.001$). Two groups could be distinguished, 'Mw.

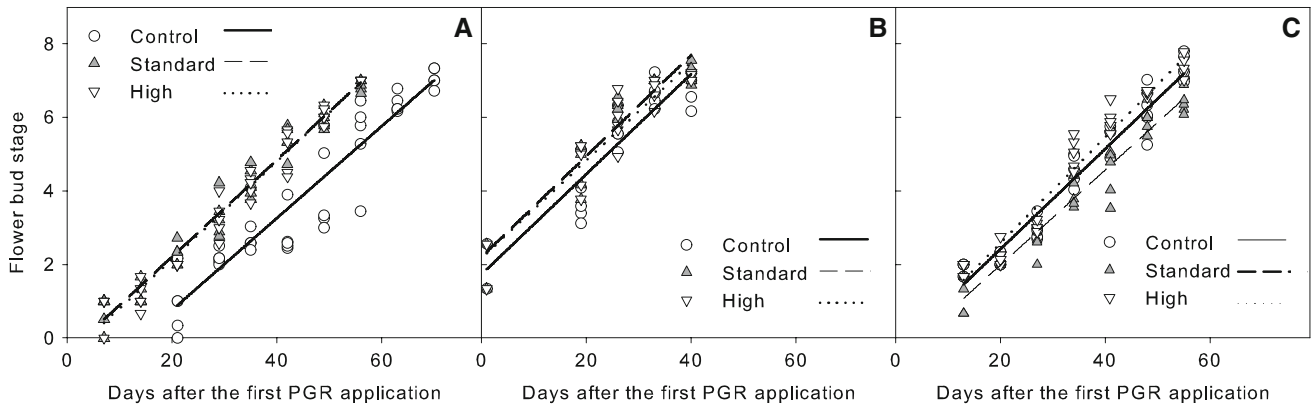

Fig. 1 Flower bud differentiation in function of days after the first PGR application for the early-flowering cultivar 'Nordlicht' in early (**a**) and late (**b**) production and the late-flowering cultivar 'Mw. G. Kint' (**c**). Control plants without PGR; standard PGR application: six times chlormequat (2.25 g L^{-1}) supplemented on the last two applications with 0.012 g L^{-1} paclobutrazol; high PGR application: six times chlormequat (2.25 g L^{-1}) supplemented on the last three applications with 0.04 g L^{-1} paclobutrazol

Table 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)

Cultivar	PGR application[y]	a ± SE	b[z] ± SE	R^2
'Nordlicht' early production	Control	−1.737 ± 0.371	0.125 ± 0.008	0.875
	Standard	−0.419 ± 0.133	0.132 ± 0.004	0.970
	High	−0.527 ± 0.140	0.133 ± 0.004	0.968
'Nordlicht' late prodcution	Control	1.734 ± 0.375	0.136 ± 0.013	0.848
	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

The regression was calculated for the early-flowering cultivar 'Nordlicht' in early and late production cycle and the late-flowering cultivar 'Mw. G. Kint'

[y] Control plants without PGR; standard PGR application: six times chlormequat (2.25 g L^{-1}) supplemented on the last two applications with 0.012 g L^{-1} paclobutrazol; high PGR application: six times chlormequat (2.25 g L^{-1}) supplemented on the last three applications with 0.04 g L^{-1} paclobutrazol

[z] Slopes are not significantly different between different PGR applications ($p = 0.05$, comparison of linear regressions)

G. Kint' and 'Tamira' developed clearly faster than 'Thesla' and 'Sachsenstern' (Table 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.

Discussion

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 PGR interfering in the GA biosynthesis pathway such as paclobutrazol, chlormequat, 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* (Randous 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

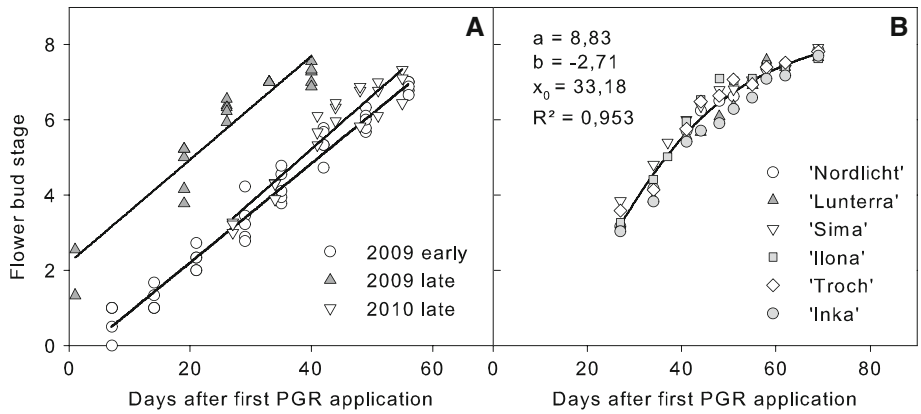

Fig. 2 **a** Linear flower bud differentiation in function of days after the first PGR application for the early-flowering cultivar 'Nordlicht': early production in 2009 ($y = -0.419 + 0.132$ x; $R^2 = 0.970$), late production in 2009 ($y = 2.205 + 0.137$ x; $R^2 = 0.890$) and in 2010 ($y = -0.435 + 0.141$ x; $R^2 = 0.898$); **b** flower bud differentiation rate decreases towards stage 8 for all sports of 'H. Vogel' in 2010. ($y = a * (x/x_0)^{abs(b)}/(1 + (x/x_0)^{abs(b)})$)

Table 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 → 7		Average temperature (°C)	Irradiation sum (MJ m^{-2})	Mean DLI (mol m^{-2} day^{-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

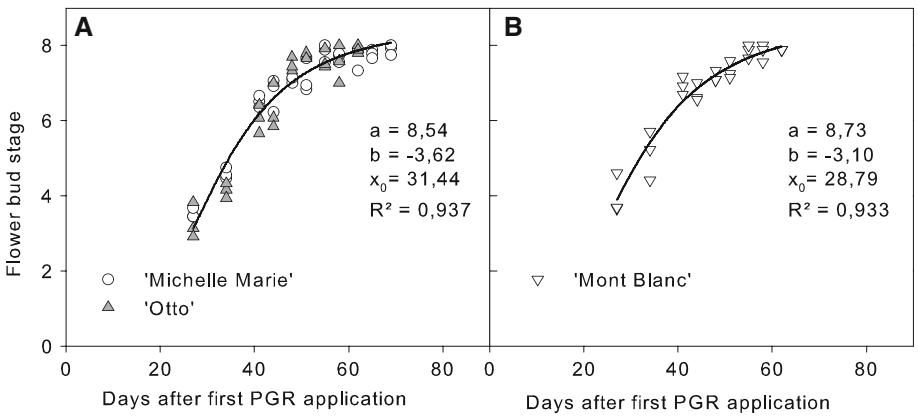

Fig. 3 Flower bud differentiation in function of days after the first PGR application for the semi-early-flowering cultivars **a** 'Michelle Marie', 'Otto' and **b** 'Mont Blanc'. ($y = a * (x/x_0)^{abs(b)}/(1 + (x/x_0)^{abs(b)})$)

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. 2013). 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 cytokinis, 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 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

Table 3 Flower bud rate of 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

Cultivar	Stage 3 → 7		Stage 7 → 8		Average temperature (°C)	Irradiation sum (MJ m^{-2})	Mean DLI (mol m^{-2} day^{-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 six bud sports of 'H. Vogel' are grouped together as no significant difference was found between these cultivars

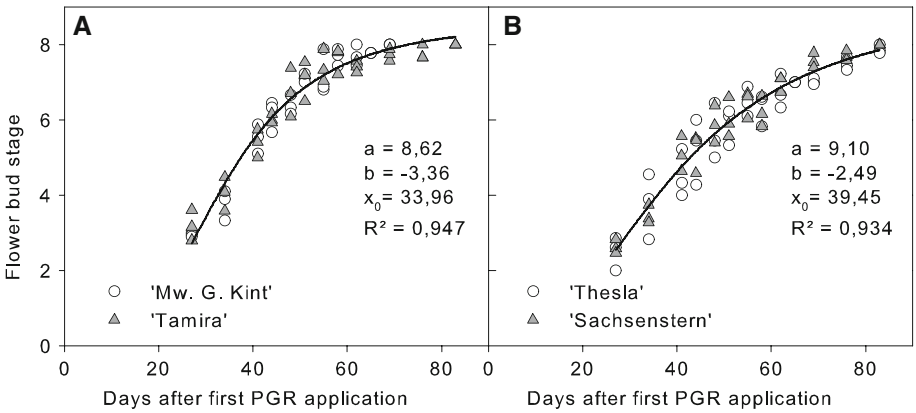

Fig. 4 Flower bud differentiation in function of days after the first PGR application for the late-cultivars **a** 'Mw. G. Kint', 'Tamira', **b** 'Thesla' and 'Sachsenstern'. (y = a * (x/x_0)^abs(b)/(1 + (x/x_0)^abs(b)))

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.

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', a sport of H. Vogel. 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 day^{-1} (Table 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} day^{-1}, which are much lower values than the 17.0 and 24.3 mol $m^{-2}day^{-1}$ in our study. 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 $m^{-2}day^{-1}$ no further effect on time to flower was observed.

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 our experiment, mean DLIs were higher than 16.7 mol m^{-2} day^{-1} and therefore not limiting for development (Table 3). Furthermore, the floral initiation started at the same date for all cultivars by applying PGR. Therefore, we did not find a pattern between earliness of flowering and a faster differentiation rate. Our results show that semi-early cultivars have a faster developmental rate, both from stage 3 to 7 ($\rho = 0.046$ day^{-1}) as from stage 7 to 8 ($\rho = 0.056$ day^{-1}) than the early 'H. Vogel' sports ($\rho = 0.035$ day^{-1} from stage 3 to 7 and $\rho = 0.045$ day^{-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 ($\rho = 0.029$ day^{-1} from stage 3 to 7 and $\rho = 0.042$ day^{-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).

Conclusions

Flower bud differentiation until stage 7 is linear; towards stage 8 differentiation slows down. This 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 63–66 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 73–76 days.

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