Stomatal Response Characteristics as Affected by Long-term Elevated Humidity Levels

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Abstract

Restriction of leaf water loss, by stomatal closure, is decisive for plant survival, especially under conditions of water deficit. This sensitivity of stomata to low water potential is attenuated by high relative air humidity (RH ≥ 85%) during growth, which impedes the plant's ability to survive when subsequently exposed to lower humidities due to a negative water balance. This thesis focuses on the extent of the existing variation and the reasons underlying cultivar differences in their tolerance to high RH, as well as the rate and reversibility of stomatal adaptation to elevated RH in the course of leaf ontogeny. Cut rose was used as a model plant. An experiment on the postharvest water relations of three contrasting cultivars in their sensitivity to high RH showed that the sensitive cultivar (i.e. steepest decrease in the cut flower longevity) underwent a higher increase in the water loss compared to the tolerant cultivars. Preventing vascular occlusion considerably extended the time to wilting in the sensitive cultivar grown at high RH, showing that the high rate of water loss, as a result of plant growth at high RH, can only be detrimental for keeping quality under limiting water uptake conditions. Further investigation showed a large genotypic variation in the regulation of water loss, as a result of leaf development at high RH, and stomatal closing capacity was the key element in this process. The degree to which the stomatal anatomical features were affected and the extent that their functionality was impaired were not correlated. However, higher stomatal density, longer pore length and depth contributed to the higher water loss of high RH-grown leaves (16–30% of the effect depending on the cultivar). Reciprocal change in RH showed that stomatal functioning was no longer affected by the RH level after full leaf expansion. However, expanding leaves were always able to partly adapt to the new RH level. For leaves that started expanding at high RH but completed their expansion after transfer to moderate RH, the earlier this switch took place the better the regulation of leaf water loss. This behaviour of expanding leaves experiencing a shift from high to moderate RH was related with the increasing population of stomata exceeding a critical stomatal length. Contrary to this, leaves initially expanding at moderate RH and transferred to high RH exhibited poor stomatal functioning, even when this transfer occurred very late during leaf expansion. This suggests that stomata at various developmental stages were similarly prone to loss of closing ability, when these had been exposed to high RH prior to full leaf expansion.

Key words: abscisic acid, cuticular permeability, heterogeneity, hydraulic conductivity, pore aperture, relative air humidity, *Rosa hybrida*, stomatal anatomy, stomatal conductance, stomatal growth, stomatal initiation, stomatal malfunctioning, stomatal population, stomatal proximity, vase life.

Στους γονείς μου
(To my parents)

CONTENTS

CHAPTER 1

General introduction

Long keeping quality as well as flower opening to a satisfactory level are very important factors determining consumers' satisfaction and thus choice (Reid and Evans 1986). Furthermore, the increasing number of growers, working with a label for postharvest quality, or selling to buyers who want a vase life guarantee (Rikken 2010), will increase the need to understand the physiological background of keeping quality. Factors determining the keeping quality of cut roses are under intense investigation for several decades (Halevy and Mayak 1981, van Doorn 2011). Currently, most quality manipulation strategies are focused on the postharvest phase, while keeping quality attributes are closely related to the product itself and can only be improved with changing the properties of the product (e.g. stomatal responsiveness, stem hydraulic conductance) during cultivation.

The postharvest behaviour of cut flowers is the compound outcome of many physiological and physical processes occurring in the different cut flower components [flower bud, peduncle, leaves (if any), and stem, Zieslin et al. 1978]. Even though the mechanism underlying each response might be independent (van Doorn 2011), the influence of these factors on vase life is highly interrelated (Spinarova et al. 2007, van Doorn and Vojinovic 1996). Many of the postharvest symptoms observed during vase life are dependent on the water balance of the cut flower, which is determined by the water uptake rate and transpirational water loss (van Doorn 1997, 2011). Negative water balance is one of the most important postharvest quality problems, resulting in shorter vase life of cut flowers, and this is strongly aggravated by high relative air humidity (i.e. RH larger than or equal to 85%) during growth (Mortensen 2001, Mortensen and Fjeld 1998, Torre and Fjeld 2001), but still below the RH levels where infections of *Botrytis cinerea* occur (Morandi et al. 2006, Williamson et al. 1995). Most studies on the effects of high RH on keeping quality of greenhouse ornamentals have been conducted for cut roses and the problem is cultivar specific (Mortensen and Gislerød 1999, 2011).

Elevated relative humidity levels in horticulture

RH is the ratio of the amount of water vapour in the air relative to the amount of water vapour that would be present in the air at saturation, and it is given as a percentage. The air is saturated when it reaches maximum water holding capacity at a given temperature. RH depends on both the amount of moisture available and air temperature. A rise in temperature, while holding the water vapour constant, results in a decrease of RH. This is because the amount of water vapour that could be present at saturation is more at the higher temperature. Vapour pressure deficit (VPD) combines the effects of both RH and temperature, and it is expressed in units of pressure. VPD is the difference between saturation vapor pressure and actual air vapor pressure.

Humidity level as a cultivation factor first drew attention in *in vitro* culture (RH in the culture vessels close to 100%) back in the 1980s, where plantlets produced were found to acquire disturbed water relations after being transferred to conditions of high atmospheric demand for water (Blanke and Belcher 1989, Brainerd and Fuchigami 1981, Capellades et al. 1990). This results in decreased cuttings' success rate and necessitates the hardening procedure (i.e. a gradual VPD increase) (Hazarika 2003, Pospisilova et al. 1999). However, a better control of water loss and a higher success rate has been reported, when vessels were placed at lower humidities during culture (Ghashghaie et al. 1992, Short et al. 1987, Ziv et al. 1987), suggesting that high RH is partly responsible for the deregulation of water loss of the *in vitro* propagated plantlets. A similar problem is induced during rooting of leafy cuttings (elevated humidity levels in the rooting vessels), another widely used method of producing large numbers of genetically identical plants (Fordham et al. 2001a, b). When these plants are transferred to lower ambient humidities, their leaves frequently shrivel and die (Fordham et al. 2001b).

Fifteen years after the large body of data on the water relations of the *in vitro* plants, Mortensen and Fjeld (1995) noticed that high cultivation RH (larger than or equal to 85%) exerts a negative effect on the vase life of cut roses. This observation was later confirmed by a wide range of studies including work in controlled environments (Torre and Fjeld 2001, Torre et al. 2003), as well as experimental (In et al. 2007, Pettersen et al. 2007) and commercial (Marissen and Benninga 2001) greenhouses.

The above mentioned horticultural practices have in common the transfer of plants from a long-term high atmospheric humidity environment (low leaf-air VPD) to conditions of increased evaporative demand (high VPD). Elevated ambient moisture conditions during growth, therefore, induce a number of anatomical and/or functional alterations that impede the plant's ability to survive when exposed to lower humidities.

Stomata formed at high relative humidity are less responsive to closing stimuli

Microscopic stomatal pores in the epidermis of aerial plant organs allow the loss of water vapour to the atmosphere in a process known as transpiration and the entry of carbon dioxide into the plant for photosynthetic carbon fixation (Cowan and Farquhar 1977). Stomatal apertures are rapidly and reversibly regulated by pairs of guard cells that border and define the pores (Schroeder et al. 2001). These cells are kidney-shaped (Fig. 1) in dicots and non-graminaceous monocots and dumbbell-shaped in grasses (Willmer and Fricker 1996). Fine control of stomatal aperture is essential so that the plant neither undergoes excessive water loss and desiccates nor becomes starved for carbon dioxide (Lawson 2009). This fine control is achieved through an exquisite sensitivity of the guard cells to a multitude of environmental and endogenous signals, including light (Shimazaki et al. 2007), temperature (Honour et al. 1995), evaporative demand (i.e. VPD, Peak and Mott 2011), carbon dioxide concentration (Assmann 1999), plant water status (Buckley 2005), and plant hormones, particularly abscisic acid (ABA) (Assmann and Shimazaki 1999). These factors during leaf ontogeny and in a long-term basis determine the speed and the degree of the stomatal response after expansion (Assmann and Wang 2001, Casson and Gray 2008, Hetherington and Woodward 2003).

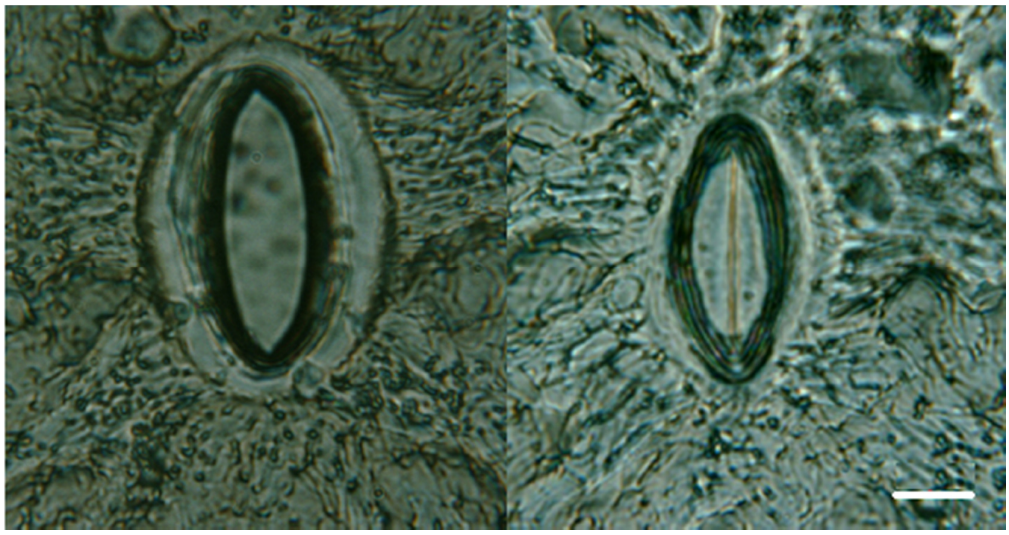

Fig. 1. Open (left) and closed (right) stoma of *Rosa hybrida* L. cv. Prophyta. The white bar is 10 μm.

For instance, stomata of *in vitro* grown plants were insensitive or failed to close fully in response to ABA (Santamaria et al. 1993, Ziv et al. 1987), low leaf water potential (Fordham et al. 2001a), darkness (Wardle and Short 1983, Ziv et al. 1987), mannitol (Brainerd and Fuchigami 1982), and high concentration of carbon dioxide (Brainerd and Fuchigami 1982, Santamaria et al. 1993), treatments that induce closure in functional

stomata. Attenuated stomatal responses were also reported in leafy cuttings (Fordham et al. 2001a, b) and in plants grown under elevated humidity levels (Islam et al. 2010, Kawamitsu 1993, van Meeteren et al. 2009) in a wide range of species. This stomatal malfunctioning is a major cause of the disturbed water relations in plants grown at high RH levels and subsequently transferred to lower humidities (Pettersen et al. 2007, Torre and Fjeld 2001, Torre et al. 2001).

A typical example of disturbed water relations of high RH-grown roses is that they wilt earlier, when placed in a vase, compared to roses grown at moderate RH (Mortensen and Fjeld 1995, Mortensen and Gislerød 1997, 2000). Subsequent studies on the effects of high RH on keeping quality of cut roses revealed that the problem is strongly cultivar dependent (Mortensen and Gislerød 1999, 2005). The decrease in vase life, as a result of plant growth at high RH, varied between as little as 15% (tolerant cultivar) to as much as 75% (sensitive cultivar) (Mortensen and Gislerød 1999). Most studies related to stomatal malfunction after leaf development at high RH are focused on only one genotype (Mortensen 2000, Mortensen et al. 2001, Ottosen et al. 2002), and no in-depth systematic comparison between contrasting genotypes (tolerant vs. sensitive) is available. Although, the striking genotypic differences described for roses in their tolerance to water loss are still poorly understood, they represent a great potential for understanding the underlying processes behind this trait in well-watered plants grown under long-term high RH.

Stomata formed at high relative humidity are longer

Stomata formed under high RH are not only less responsive to closing stimuli, but also have distinct anatomical features (Rezaei Nejad and van Meeteren 2005). For instance, high RH during leaf development results in longer stomata in all examined species so far (Bakker 1991, Fordham et al. 2001b, Gislerød and Nelson 1989, Karbulkova et al. 2008, Torre et al. 2003). Instead, the high RH effect on stomatal density depends on the species. Although no response (Bakker 1991) or a decrease (Rezaei Nejad and van Meeteren 2005) of stomatal density at high RH has been reported, in many species, including rose, high RH tends to increase the stomatal density (Bakker 1991, Gislerød and Nelson 1989, Karbulkova et al. 2008, Torre et al. 2003). Previous studies were focused on stomatal functioning (Rezaei Nejad and van Meeteren 2005, 2007); yet, the relevance of anatomical features on the stomatal functioning in high RH-grown plants has not been addressed. Moreover, studies conducted so far on elevated RH effect on stomatal size have been limited to one genotype. Consequently, its role in the determination of the variability of stomatal functionality among different cultivars has not been addressed.

Santamaria and co-workers (1993) speculated that the bigger stomatal size of plants grown in high RH does not explain *per se* why these are less hydrosensitive. Although there is not as yet experimental evidence against or in favour of this speculation, studies devoted to the smaller stomata, formed under long-term water stress conditions, suggest that stomatal size has an effect on stomatal closure ability. It was demonstrated both mathematically (Spence et al. 1986) and experimentally (Franks and Farquhar 2001) that smaller stomata are mechanically different. A number of subsequent studies confirmed that between species smaller stomata acquire shorter response times (Aasamaa et al. 2001, Franks and Farquhar 2007, Hetherington and Woodward 2003). So, it might be expected that within the stomatal population of a given species, bigger stomata, induced by high RH, have longer response times, and thus contribute more to the higher water loss. The closing ability of stomata with different lengths, has not been previously investigated in plants grown at high RH. Thus, it remains unknown whether the increase in stomatal length, caused by high RH during leaf development, influences their functionality.

Furthermore, the relative contribution of anatomical features to the higher rates of water loss, as a result of plant growth at high RH, has not been investigated previously. Thus, the higher water loss rates have been related to physiological changes mostly. However, at the leaf level stomatal conductance (g_s) is determined by the product of the pore area and the stomatal density (cross-sectional area available for gas fluxes) (Nobel 1991). Since high RH exerts an effect on stomatal density and pore length (Fordham et al. 2001b, Gislerød and Nelson 1989), it is expected to influence g_s independently of pore aperture, representing physiology. Thus, direct (i.e. gravimetric methods, Rezaei Nejad and van Meeteren 2005) or indirect (e.g. infra-red gas analyzer, Ottosen et al. 2002; porometer, Torre et al. 2003; chlorophyll fluorescence, Rezaei Nejad and van Meeteren 2007) measurements of g_s do not only assess treatment effects on stomatal physiology, but also contain the influence of anatomical features. To the best of our knowledge, there are no reports providing the required information at individual pore scale in high RH-grown plants, which would enable to partition differences in g_s among different component variables, such as stomatal density and pore parameters (i.e. aperture, length, and depth).

Abscisic acid (ABA) is strongly implicated as the cause of stomatal malfunction

Although ABA is considered to be a 'stress hormone' (Zeevaart and Creelman 1988), increasing evidence supports that it also plays an important regulatory role in stomatal functioning in the absence of stress (Sharp 2002). It has been suggested that *in vitro* plants are never exposed to water deficits necessary to induce synthesis of ABA (Santamaria et

al. 1993). Since plants must be subjected to a minimum amount of water stress before they can respond to the ABA produced (Raschke 1987), the low ABA levels might be (partly) responsible for the stomatal malfunctioning. In accordance with this hypothesis, it was found that conditions that result in an increase of the endogenous ABA content (e.g. ventilation or ABA addition to the medium) during growth improved the control of water loss (i.e. stomatal responsiveness) of *in vitro* produced plantlets (Aguilar et al. 2000, Pospisilova 1996b, Talavera et al. 2001). In other words, stomatal functionality appears to be related to the ABA accumulation in the leaves, and a minimum ABA level is required for proper stomatal functioning.

Further evidence for such a role of endogenous leaf ABA content comes from ABA-biosynthetic mutants (Koornneef and Jorna 1982, Leon-Kloosterziel et al. 1996) and transgenic plants expressing an anti-ABA antibody (Artsaenko et al. 1995, Wigger et al. 2002), which show a wilty phenotype due to enhanced transpiration related to stomatal malfunctioning. This effect can be entirely reversed by long-term exogenous application of ABA (Koornneef and Jorna 1982, Wigger et al. 2002).

Mortensen and Gislerød (2005) suggested that this is also the case with high RH-grown plants, by demonstrating that severe drought stress (i.e. intensification of root-to-shoot ABA signals, Schachtman and Goodger 2008) during growth at high RH, significantly increased the vase life in five out of six studied cultivars, indicating a stimulation of the stomatal function despite the high RH level. Rezaei Nejad and van Meeteren (2007) went one step further by showing that high RH-grown leaves have reduced foliar ABA concentration, and that stomatal functionality was entirely restored by long-term exogenous application of ABA. Thus, the role of ABA in mediating stomatal malfunctioning under conditions of low evaporative demand is not controversial, though the process this takes place and the reasons for genetic variation in this trait remain elusive.

Rate and degree of stomatal adaptation to contrasting relative humidity levels (possibilities of improvement/deterioration)

Although abnormal stomatal behaviour is a common response in leaves developed at high RH, the dynamics of stomatal adaptation, i.e. the stability of stomatal functionality, when transferring plants from one RH level to a contrasting one seems to be strongly species dependent. For instance, plant transfer after leaf expansion to a new RH level (i.e. moderate to high RH, and vice versa) resulted in a full stomatal adaptation to the new RH environment in *Phaseolus vulgaris* (Pospisilova 1996a), whereas this had no effect on stomatal responsiveness in *Rosa hybrida* (Mortensen and Fjeld 1995, Mortensen and Gislerød 2000). In *Tradescantia virginiana*, plant transfer from moderate to high RH

disrupted the stomatal responses within four days, while moving the plants back to moderate RH did not have any effect (Rezaei Nejad and van Meeteren 2008). Rezaei Nejad and van Meeteren (2008) speculated that a certain ABA level is required not only to induce, but also to sustain stomatal functionality. Since stomatal functioning in rose is independent of post-development conditions, two hypotheses arise: (i) the root-to-shoot ABA signalling is weakened after transfer to high RH, but it is still sufficient to sustain stomatal responsiveness; or (ii) the root-to-shoot ABA signalling after the development of stomatal apparatus is not important in rose. Further research is needed to test these hypotheses.

The leaf expansion period is very critical for its life cycle, since the morphological and physiological attributes established during this phase are key determinants for its behaviour afterwards. This is especially the case with a species, as rose, where stomatal functionality is completely established during leaf ontogeny (Mortensen and Gislerød 2000). However, leaf development under constant RH levels in the growth chambers is quite artificial. In natural environments as well as in protected cultivation, plants are exposed to frequently changing environmental conditions (Körner and Challa 2003). Studies conducted so far were focused on stomatal adaptation in fully expanded leaves, whereas only one study addressed the stomatal adaptation of expanding leaves (Rezaei Nejad and van Meeteren 2008). In that study the growing part of *Tradescantia virginiana* leaves (i.e. near the leaf base) was able to adapt to the new RH level, in contrast with the developed part, which behaved as discussed above for the fully expanded leaves. However, these authors only examined one stage of leaf development (i.e. growing part near leaf base). Thus, it remains unclear, whether the RH level during critical stages, rather than throughout leaf ontogeny, is decisive for stomatal functionality.

In fully expanded leaves, the stomatal anatomy and stomatal population (i.e. absolute number of stomata = density × leaf area) are fixed and, therefore, do not influence the adaptation dynamics' process (Rezaei Nejad and van Meeteren 2008). In contrast, in expanding leaves the stomata are growing (i.e. increase in length), while their population increases till a certain percentage of the final leaf size has been reached (Tichá 1982). This threshold value is species dependent (Rawson and Craven 1975, Ludlow 1991). It remains unknown, whether a moderate RH level is needed only during the first stages of stomatal development (forms close to stomatal initiation), only during the latter stages (forms close to stomatal maturation) or throughout stomatal development to induce stomatal functionality.

Cuticular water loss of leaves developed at high relative humidity

The waxy cuticle of leaves serves to inhibit water loss and thus to decrease the dehydration of the underlying cells (Leon and Bukovac 1978). The waxes vary in thickness and composition, and the inhibition of water loss varies accordingly (O'Toole et al. 1979). The total leaf transpiration results from the water loss via the stomatal and cuticular pathways, representing two resistances connected in parallel (Kerstiens 1995). The relative importance of water loss through the cuticula as compared to the water loss through the open stomata is very variable among species (Kerstiens 1996). This can represent 2 to 29% (Holmgren et al. 1965). This indicates that water loss through the cuticle can be a small or substantia1 fraction of the loss through open stomata. As stomata close, this fraction becomes larger (Boyer et al. 1997). This takes place, for instance, in leaves that are darkened or that dehydrate.

Scant deposition of protective epicuticular waxes on the leaf surface of *in vitro* grown plants, has been regarded as one of the most important factors responsible for excessive water loss, leading to poor transplantation success (Hazarika 2006, Wetzstein and Sommer 1982). Other authors, however, came to the conclusion that cuticular water losses contributed little to the problem (Santamaria and Kerstiens 1994). In case of high RH-grown plants only a limited number of studies have been conducted so far, with antithetical outcomes (Karbulkova et al. 2008, Torre et al. 2001). Thus, the contribution of cuticular water loss to the total leaf water loss under different stomatal closure states has not as yet been investigated in plants grown under high RH conditions, and its significance remains uncertain.

Effect of high relative humidity on the water uptake components

Although the control of water loss is mostly under physiological control (i.e. stomata), water uptake is basically a physical process in cut flowers. From the Ohm's law analogy ($I = V/R$), flow rate (water uptake) is proportional to the driving force (water potential), and to the conductance (inverse of resistance) of the transport path. For a long vase life, the water loss should be replenished by the water uptake (van Doorn 1997, 2011). It is generally accepted that, at least for a large part, the effect of high RH on keeping quality of cut flowers is mediated via water relations (Mortensen et al. 2001, Pettersen et al. 2006). Reduced capacity to control water loss leading to a negative water balance (i.e. water loss > water uptake), as a result of stomatal malfunctioning, has been proposed as the main reason (Mortensen et al. 2007, Pettersen et al. 2007, Rezaei Nejad et al. 2006). However, the effect of high RH on the parameters related to water uptake have received little attention, though a sufficient water uptake would largely compensate the increased water loss and would still result in a positive water balance.

Stem hydraulic conductivity (K_h) is determined by the genotype (van Doorn and Otma 1995) and the cultivation environment (Evans et al. 1996, van Meeteren et al. 2005). K_h decreases during the postharvest phase, due to physical (bacteria or their products, van Doorn 1997) and physiological blockage (wounding reactions, van Doorn 2011), as well as occlusion by emboli (van Doorn 1997, 2011). Air emboli appears in cut open vessels at the moment of harvest and to a certain extent can be reversible when the flower is placed in water (Mensink and van Doorn 2001, van Ieperen et al. 2002). Dry storage increases the severity of embolisms in the cut open vessels, but also induces cavitations (i.e. vapour-filled conduits) in the vessels that are not opened by cutting. K_h depends on xylem conduit anatomy (i.e. length, number and diameter), which also determines its vulnerability to the factors (e.g. air emboli) that decrease it during vase life (Nijsse et al. 2001).

It has been shown that treatment with silver nitrate, an antibacterial compound (expected to improve water uptake), increased the vase life only in high RH-grown roses and not in roses grown in moderate RH (Torre and Fjeld 2001). Moreover, the vase life of roses cultivated at high RH was also shorter than for moderate RH-grown roses even under postharvest elevated RH (81%) conditions (expected to decrease water loss) (Mortensen and Gislerød 2000). Thus, both improving the water uptake and decreasing the water loss during the postharvest phase increased the vase life depending on the preharvest RH level. This difference in the alleviating effect of postharvest treatments might be the result of the higher transpiration stream of high RH-grown roses, or this in combination with higher decrease in K_h. However, there is no data to support these claims.

Aim and outline of the thesis

This project aims at understanding the physiology of stomatal behaviour in response to long-term growth at high RH, the reasons for cultivar differences in their tolerance to high RH and the stomatal adaptation dynamics to contrasting RH levels. Cut rose was used as a model system since: (i) it is one of the most important cut flowers worldwide; (ii) it is by far the plant in which most studies on the present subject have been conducted; and (iii) it is a species in which genetic differences have been observed in terms of tolerance to high RH.

In order to be able to breed for cultivars with more responsive stomata after leaf development at elevated atmospheric humidity, it is important to know the existing variation of this trait. The first part of the thesis (Chapters 2 and 3) focuses on the extent of the existent variation and the reasons underlying genotypic differences in their tolerance to high RH. **Chapter 2** addresses the effects of ambient humidity during

growth on the water balance components of three cut rose cultivars with contrasting sensitivity to high RH. The results presented in this study can be used not only to screen high RH-grown roses, but also to considerably extent their postharvest longevity. **Chapter 3.1** describes the quantitative effects of high RH during leaf development on the stomatal anatomical and response characteristics, as well as on the cuticular permeance of four cut rose genotypes. This study also identifies a previously unrealized effect of anatomical features (i.e. stomatal density, pore length, pore depth) on the higher water loss rates of plants grown in more humid air. **Chapter 3.2** assesses the variation in the stomatal responsiveness of a subset of a segregating tetraploid (K5) cut rose population grown at high RH.

The second part of this thesis (Chapters 4 and 5) deals with the rate and reversibility of stomatal adaptation to elevated RH in the course of leaf ontogeny. **Chapter 4** describes the stomatal adaptation dynamics of expanding leaflets transferred to contrasting RH levels. In **Chapter 5.1**, we examine the stomatal initiation and growth in the studied species. The importance of heterogeneity in stomatal features in relation to the current sampling protocols for determining these features is also discussed. **Chapter 5.2** includes the effect of ambient humidity on the stomatal formation, and gives an anatomical explanation for the observed responses of water loss regulation in expanding leaflets experiencing a shift in RH (Chapter 4).

In the general discussion **(Chapter 6)**, the results of the previous chapters are combined and discussed. Areas that demand further research are also highlighted.

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CHAPTER 2

Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth

Abstract

A constant high relative air humidity (RH) during cultivation can strongly reduce the vase life in some cut rose cultivars. We studied three contrasting cultivars in their tolerance to high RH in order to analyse in detail the water relations during postharvest and better understand this genotypic variation. Plants were grown at moderate (60%) and high (95%) RH, and cut flowers were placed in water immediately after cutting. Flowers of cv. Pink Prophyta grown at high RH did not open throughout vase life, whilst flower opening of cvs. Frisco and Dream was not affected by preharvest RH. Cultivation at high RH resulted in about 80% shorter vase life in Pink Prophyta, whereas in Dream and Frisco the negative effect was considerably smaller (15 and 9% shorter vase life, respectively). The shorter vase life and reduced flower opening of cut roses grown at high RH was due to a higher rate of transpiration both in the light and dark periods. It was found that the leaves of Pink Prophyta grown at high RH could partly close their stomata upon lowering of the water potential or when flower stalks were fed with abscisic acid, but stomata remained far more open than in leaves grown at moderate RH. The RH during cultivation did not affect stem hydraulic conductivity and its recovery after air emboli induction. Preventing vascular occlusion largely alleviated the high-cultivation-RH effect on vase life and flower opening, showing that the effect of high-cultivation-RH becomes only important if water uptake is limited.

Fanourakis D, Carvalho SMP, Almeida DPF, van Kooten O, van Doorn WG, Heuvelink E (2011) Postharvest water relations in cut rose cultivars with contrasting sensitivity to high relative air humidity during growth. Postharvest Biology and Technology (in press)

Introduction

The water relations of cut flowers are dependent on a number of physiological and anatomical traits that regulate the water loss and water uptake rates (reviewed by van Doorn 1997, 2011). These traits are established during the preharvest period, being the result of the complex interaction between genotype and environment during cultivation, and will subsequently determine the potential vase life (i.e. maximum vase life) of a given cut flower. For instance, although the relative air humidity (RH) level during cultivation has no significant effect on crop growth and visual quality (Torre and Fjeld 2001), cut roses grown at high RH (≥ 85%) often have a very short vase life (Mortensen and Fjeld 1998, van Doorn 2011). Nevertheless, this decrease in the life span after cultivation at high RH is strongly dependent on the genotype, varying between as little as 15% (cvs. Dream, Frisco, and Kardinal) to as much as 75% (cv. Amadeus) (Mortensen and Gislerød 1999). Sensitive cultivars grown under elevated RH show precocious senesce symptoms during postharvest, which are typically related to water stress, including a premature flower and leaf wilting as well as pedicel bending (Mortensen and Gislerød 2005, Torre et al. 2001).

The regulation of water loss is mostly under physiological control (i.e. stomata), whereas water uptake is basically a physical process in cut flowers. An adequate stomatal responsiveness to different closing stimuli (e.g. darkness and low water potential) will limit the net loss of water from the cut flower, and will consequently delay early wilting symptoms (Bleeksma and van Doorn 2003, van Doorn et al. 1989). A lower cut flower water loss, can also be induced by addition of antitranspirant compounds in the vase solution (van Doorn 2011). On the other hand, from the Ohm's law analogy, the flow rate (water uptake) is proportional to the driving force (water potential), and to the conductance (inverse of resistance) of the transport path (van Doorn 1997). It has been shown that drought stress results in a reduction of xylem vessel diameter (Lovisolo and Schubert 1998), which in turn leads not only to a lower stem hydraulic conductivity but also to a higher resistance to cavitation (McElrone et al. 2004, Nijsse et al. 2001). Since low water potentials during drought stress can change the xylem anatomy, some opposite changes might be expected when plants are subjected to long-term high water potentials, as a result of elevated RH levels.

A limited capacity to reduce water loss, due to stomatal malfunctioning, is thought to be the main reason for the vase life reduction in plants grown under long-term high RH (Fanourakis et al. 2011, Rezaei Nejad and van Meeteren 2007). Nevertheless, the effect of the RH on stem hydraulic conductivity was not previously investigated. Moreover, the described genotypic variation in the sensitivity to high RH

during preharvest is not yet fully understood. The main objectives of the present work were: (i) to analyse the postharvest water relations of cultivars with contrasting sensitivity to high RH during preharvest, (ii) to assess the cultivation RH effect on the stem hydraulic conductivity and its recovery after artificial induction of air emboli, and (iii) to test if improvement of water uptake, by preventing vascular blockage, can compensate for the higher water loss found after cultivation at high RH. We hypothesized that a shorter vase life, after growth at high RH, results from the combined effect of a higher water loss and a changed xylem anatomy (leading to a higher sensitivity to air emboli). Moreover, we expect that factors which improve water uptake will alleviate this negative effect of high RH on cut flower longevity.

Materials and Methods

Plant material and growth conditions

Rooted cuttings of three cut rose cultivars (*Rosa hybrida* L. cvs. Pink Prophyta, Frisco, and Dream) known to have different decreases in vase life after cultivation at high RH (Mortensen and Gislerød 1999), were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands). The cuttings were planted in 3.6 L pots containing a mixture of cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull, Rhenen, The Netherlands), 3:1, v/v. Cultivar Pink Prophyta (registered cultivar name RUIkuiros) will be called Prophyta in the remainder of this paper.

Five experiments were conducted. In each experiment, plants were grown in four growth chambers as a single shoot (one plant per pot) at a density of 30 plants m^{-2}. In two growth chambers the RH was 60 ± 3% (moderate RH) and in two others it was 95 ± 1% (high RH). The four chambers had a constant day and night temperature (19 ± 1 °C), resulting in vapour pressure deficits (VPDs) of 0.88 ± 0.12 kPa (moderate RH) and 0.11 ± 0.03 kPa (high RH). Climate parameters were recorded automatically every 5 min by data loggers (Fourier Microlog EC650, MicroDAQ.com Ltd, Contoocook, NH).

Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided an 18 h photoperiod and 300 ± 20 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR, determined with a Model LI-250, LI-COR, Lincoln, NE). Radiation levels were measured at 70 cm from the root-shoot interface, which corresponds to the top of fully grown plants. The CO_2 concentration during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Plants were watered automatically with a nutrient solution as described by Fanourakis et al. (2009).

Experiments on postharvest characteristics used second-order shoots, each originating from an individual plant, at the commercial harvest stage [stage 2 according to the Association of Dutch Flower Auctions (VBN 2005); described for experiment 1]. The harvested shoots had a length of approximately 50–60 cm, measured from the primary shoot/secondary shoot junction to the top of the shoot. The night before the experiment, the plants were well irrigated and placed in darkness for 12 h, to ensure maximal turgidity and minimize the presence of natural air emboli (van Doorn and Suiro 1996).

Vase life under non-optimum water uptake conditions (experiment 1)

The flowering stems of the three cultivars were cut in air, left in air for 2–3 min, and their cut end was then washed with sodium hypochlorite solution (2%, v/v; the concentration of commercial bleach solution). All flowers were cut to the same length (49 ± 2 cm), and the same number of five-leaflet leaves per cultivar was left (cvs. Frisco and Prophyta: four leaves, cv. Dream: five leaves). Subsequently, the cut flowering stems were put in flasks (one flower per flask) containing 300 ml of an artificial vase solution (0.7 mM $CaCl_2.2H_2O$, 1.5 mM $NaHCO_3$, 5 μM $CuSO_4.5H_2O$; van Meeteren et al. 2000). The presence of copper sulphate in the vase solution leads to a moderate inhibition of bacterial growth (van Doorn 2011). The top of the flasks was covered with Parafilm, to ensure that water loss could only occur via the flower stalks. These flasks were placed in a climate-controlled room at 20 °C, 50% RH and 10–12 μmol m^{-2} s^{-1} photosynthetic photon flux density at a 12 h on-off cycle, provided by fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands). The height of the vase solution column was held constant over the evaluation period to avoid hydrostatic pressure differences between flowers with different transpiration rates (Mensink and van Doorn 2001).

The termination of vase life was determined based on the occurrence of at least one of the following criteria: (i) bending of the pedicel (bent-neck; i.e. pedicel bends and flower angle becomes larger than 90° from the vertical position); (ii) abscission of more than two petals; (iii) visible wilting of the flower, i.e. loss of petal turgor; and (iv) more than 50% of the number of leaves had abscised, turned yellow, or had desiccated (VBN 2005). Total leaf area was determined at the end of vase life, using a leaf area meter (model 3100 Area Meter, LI-COR, Lincoln, NE).

During the postharvest phase, the flower and flask weights were recorded separately two times a day (time 0 and 12 h after the onset of the light period). The transpiration rate was calculated per unit leaf area. Treatments were compared based on the average transpiration rate over the complete postharvest period. The fresh weight (FW) of each flowering stem was expressed relative to its initial weight. The flower

diameter and opening stage were recorded daily during vase life. The flower diameter was measured by assessing the maximum diameter and the diameter perpendicularly to that one. These two values were averaged. Flower stages were determined using the scale of VBN (2005) (i.e. stage 2: loose pointed bud with cylindrical shape; stage 3: half-open flower; stage 4: open flower; stage 5: maximally opened flower with visible anthers). In this experiment twelve flowers per treatment were assessed.

Vase life under optimum water uptake conditions (experiment 2)

In order to test if improving the water uptake conditions during vase life could compensate the negative effect of high RH during cultivation on keeping quality, vascular blockage (caused by air emboli at the cut surface and bacterial growth in the vase water) was prevented. Flowering shoots of the three cultivars were cut under water to prevent air entrance into the xylem conduits that were opened by cutting. Thus, just prior to cutting, pots were placed in buckets containing degassed sterilized water, whereby the water level was about 5 cm above the primary shoot/secondary shoot junction. Each cut was made using shears that had been sterilized in ethanol and through an internode that had been surface-sterilized by rubbing with a cloth drenched in the same solution (sterile treatment; van Doorn et al. 1991). The flowering stems were cut to the same length, and the same number of five-leaflet leaves per cultivar as described earlier (experiment 1). Subsequently, to further reduce the effect of bacteria on xylem occlusion, the stems were placed in sterilized flasks containing 300 ml of an artificial vase solution (details in experiment 1), which was sterilized (autoclaved at 121 °C for 15 min) and its pH was reduced to 3 with addition of citric acid. This approach was used in place of a vase solution biocide, common in vase life work, to avoid possible effects of chemicals other than on microbes (van Doorn et al. 1990, van Doorn 2011). The flasks were placed under test room conditions and cut flowers were submitted to the same procedures and measurements as described for experiment 1. Additionally, in the cvs. Frisco and Prophyta the flower with the flask was weighed at regular intervals during the light period (time 2, 4, 8, and 10 h after the onset of the light period). In experiment 2, the measurements were carried out in eight flowers per treatment.

Recovery from the decrease in stem hydraulic conductivity (K_h) due to air aspired at the stem cut surface, in cv. Prophyta (experiment 3)

In cv. Prophyta (sensitive) we investigated the effect of high RH on stem hydraulic conductivity (K_h). We also artificially induced the presence of air emboli at the cut surface. All manipulations with plants and stem segments in the laboratory were done under water to prevent the entrance of air into the xylem vessels at the cut surface. Stem

segments of 35 cm length were cut from the plants at 5 cm above the primary shoot/secondary shoot junction with sharp shears. The stems were recut, removing 5 cm from both ends, with a new razor blade. Stem length was then approximately 25 cm. The number of xylem vessels exceeding 20 cm length is very low in rose (less than or equal to 5, van Doorn and Reid 1995). Leaves were removed from the stem segment with a razor blade, leaving 0.2 cm of the petioles on the stem (van Ieperen et al. 2001). Each stem segment contained the same number of nodes, since a nodal structure offers higher resistance to water flow (Salleo et al. 1984), and had similar diameter at both cut ends compared to the other replications. The time between collection from the plant and the start of the measurement was approximately 20 min.

A silicone tube was pushed over the upper cut end of the stem segment (cut end at largest distance from the roots), while the lower end of the stem segment was placed in a container filled with degassed aqueous solution of sodium bicarbonate (1.5 mM), calcium chloride (0.7 mM) and copper sulphate (5 μM) at room temperature (20 ± 2 °C) (van Meeteren et al. 2000). The tube was then connected to a pump (7550-62, Barnant, Barrington, IL) creating a pulling pressure difference of 40 kPa. Actual pressure was measured using a pressure transducer (DVR 5, Vacuubrand, Wertheim, Germany). During these measurements solution flow was always in natural direction, from the lower cut end (closest to the roots) to the upper cut end. Flow through the stem segments was calculated from weight changes of the solution, corrected for evaporation. Flow rates stabilized typically after 5 ± 2 min. Subsequently, the K_h was calculated according to van Meeteren et al. (2000) (Eqn 1), by using the stem segment length (x), the applied pressure difference (ΔP) and the flow rate (q).

$$K_h = \frac{q}{\Delta P / \Delta x} \quad (1)$$

After the K_h-measurement without initial air emboli, and while keeping the stem segments under tension (40 kPa), the segments were lifted out of the solution to allow air entrance for approximately 3 min. The pressure exerted was far lower than the one needed to move the air-water interface (1.5 MPa) through the pores of the pit membranes (van Doorn and Suiro 1996), but higher than the one needed to fill most xylem conduits at the cut surface with air (van Ieperen et al. 2001). After this period of exposure to air, the stem segments were lowered back into the solution and K_h was followed for 2.4 h. In this experiment seven stem segments (one segment per plant) per treatment were used.

Stomatal response to a decrease in leaflet water potential (Ψ_{leaf}), in cv. Prophyta (experiment 4)

The effect of ambient humidity during cultivation on the stomatal responsiveness to a decrease in leaf water potential (Ψ_{leaf}) was investigated in cv. Prophyta (sensitive). The transpiration rate as a function of leaf relative water content (RWC) was evaluated in one set of measurements. Terminal leaflets of the first five-leaflet leaves counting from the apex were detached. Their petioles were immediately recut under degassed water (to prevent cavitation-induced embolism), placed in flasks filled with water, and further incubated for 1 h at about 100% RH (21 °C, VPD close to 0) to establish their saturated FW. Since the leaflets were detached during the light period in the growth chamber, the rehydration process was therefore conducted in the light (15 μmol m^{-2} s^{-1} PAR; following darkness the light-induced stomatal opening requires several min; Mott et al. 1999). Subsequently, the leaflets were removed from water and placed on a table (abaxial surface down) at 21 °C, 50 ± 3% RH, and 50 μmol m^{-2} s^{-1} PAR. Transpiration rate was gravimetrically recorded during 4 h. The leaflets were then dried at 80 °C for 24 h. The RWC was calculated according to Slavik (1974).

In another set of measurements, the Ψ_{leaf} as a function of leaf RWC was determined. Terminal leaflets of the first five-leaflet leaves counting from the apex were detached, their petioles were recut under degassed water and placed in vials filled with water. Subsequently, the leaflets were rehydrated in darkness (to promote stomatal closure). This was done in place of overnight rehydration, common in pressure-volume work, to prevent changes in osmotic potential which can occur within several hours (Auge et al. 1986). Afterwards, the leaflets were covered with a polyethylene sheet of a known mass and weighed to determine their saturated FW. Then the leaflet with the polyethylene sheet still around, was placed in a Scholander-type pressure chamber (Soil Moisture Equipment Corp, Santa Barbara, CA) and the balance pressure ($-\Psi_{leaf}$) was determined. The pressure in the chamber was increased at a rate not higher than 0.02 MPa s^{-1} to avoid cell injury (Kikuta et al. 1985). Leaves were allowed to dry on a table at 21 °C, 50 ± 3% RH, and 50 μmol m^{-2} s^{-1} PAR. Incremental weight loss was gravimetrically determined. The dry weight of the leaflets was obtained as described above. All measurements included 14 leaves (one leaf per plant) per treatment.

Stomatal response to abscisic acid (ABA) feeding during postharvest, in cv. Prophyta (experiment 5)

The efficiency of an antitranspirant compound (abscisic acid, ABA) in decreasing the transpiration rate of cv. Prophyta (sensitive) grown at different moisture ambient conditions was assessed. Care was taken that the vascular blockage was prevented in the tested cut flowers (as described for experiment 2). Flowering stalks were placed in

artificial vase solution with 0 (control) or 100 μM (±)-ABA (Sigma, St. Louis, MO), and kept under test room conditions (as described for experiment 1). The top of the flask was covered with Parafilm, while its sides were wrapped in aluminum foil (to reflect light), since ABA is light sensitive (Davies and Jones 1991). The experiment was stopped when leaf abscission was observed in ABA-fed flowers, and total leaf area was then measured. The transpiration rate was determined as described earlier (experiment 1). In this experiment eight flowers per treatment were used.

Statistical design and analysis

Data were subjected to analysis of variance using Genstat software (10th edition, VSN International Ltd, Hemel Hempstead, Herts, UK). Experiments 1, 2 and 5 had a split-plot design, with RH level as the main factor, and cultivar (experiments 1 and 2) or duration of ABA feeding (experiment 5) as the split factors, respectively. Experiments 3 and 4 were analysed by one-way ANOVA. Treatment effects were tested at 5% probability level and mean separation was carried out using least significant differences based on Student's *t*-test ($P \leq 0.05$).

Results

Vase life, flower opening and flower diameter

The life span of flowering stems cut in air and placed in vase solution with moderate inhibition of bacterial growth (i.e. non-optimum water uptake conditions; experiment 1) was reduced in stalks from plants grown at high RH (95%), compared with those from plants grown at moderate RH (60%). The effect was largest in cv. Prophyta. At moderate RH its vase life was about 19 days, whereas the vase life of flowering stems grown at high RH was only 4 days (Table 1; experiment 1). A small negative effect of high RH during cultivation was found on the vase life of cv. Dream, whilst no significant effect was observed in cv. Frisco (Table 1; experiment 1). Moreover, flower diameter was only significantly inhibited in cv. Prophyta when grown under high RH, resulting in about 30% reduction at the end of vase life (Table 1; experiment 1). A similar trend was observed for flower opening stage, but since these data were not normally distributed no analysis of variance was performed (Table 1; experiment 1). Symptoms that resulted in early termination of vase life after cultivation at high RH were pedicel bending and leaf desiccation in cv. Prophyta, and pedicel bending in cv. Dream. Vase life termination was thus due to early water stress symptoms (Table 2; experiment 1). Such precocious senescence symptoms were not observed in cv. Frisco grown at high RH, which ended its

vase life as a consequence of a natural flower wilting as observed in nearly all flowers of the studied cultivars grown at moderate RH (Table 2; experiment 1).

In experiment 2 the flowers were surface-sterilized, cut from the plant under degassed water, and placed in sterilized vase water. Uptake of air into the cut stems was thereby prevented and microbial effects were drastically reduced (i.e. optimum water uptake conditions). These conditions largely alleviated the negative effect of high RH during cultivation on the length of vase life and on the flower opening and diameter in cv. Prophyta (Table 1; experiment 2). In cv. Dream these conditions completely prevented the effect of high RH during cultivation (Table 1; experiment 2). Contrary to the expectation there was still a negative effect of cultivation RH on the vase life of cv. Frisco (Table 1; experiment 2). In this experiment no water stress symptoms were noted, except leaf desiccation in cv. Dream grown at high RH (Table 2; experiment 2).

Transpiration rate and its diurnal rhythm in the light period

A two days vase life trial, in which the leaves were totally removed on the second day, showed that leaves in cv. Frisco accounted for about 80% of the total transpiration during the light period in flowers grown both at moderate and high RH (data not shown).

Cut roses grown at high RH had higher transpiration rates in the light period, compared with roses grown at moderate RH (Fig. 1A, C, E). In the cvs. Dream and Frisco the effect was only found during the first days of vase life (Fig. 1C, E). Cultivar Prophyta had an average transpiration rate in the light period that was three times higher when grown at high RH, compared to stalks grown at moderate RH (Fig. 1A). The effect was smaller in the cvs. Frisco and Dream (32 and 22% higher transpiration in flowers grown at high RH compared to roses grown at moderate RH, respectively; Fig. 1C, E). These data refer to normal cutting and a moderately large vase water microbial population. When flower stems were surface-sterilized, cut under degassed water, and placed in sterile vase solution, the transpiration was also higher in flowers cultivated at high RH compared to those grown at moderate RH (Fig. 1B, D, F; see also Fig. 7A). There was no clear difference between this experiment (Fig. 1B, D, F) and the one in which the stems had aspired air and the vase water microbial population was much higher (Fig. 1A, C, E).

The diurnal course of transpiration rate was recorded during the vase life of the cvs. Prophyta and Frisco, under water uptake conditions that were close to optimum (stem surface-sterilization followed by cutting stems from the plant under degassed water and placement in sterilized vase solution). A relatively large diurnal oscillation in the transpiration rate was observed in flowers grown at moderate RH, and a much smaller oscillation in flowers grown at high RH (Fig. 2A, B). The transpiration rate during the light period showed a peak during the first hours, while the lowest value was

Table 1. The vase life, flower stage and diameter at the end of vase life of three cut rose cultivars, grown at moderate (60%) or high (95%) relative air humidity (RH). In experiment 1 the stems were cut in air and placed in vase solution with moderate inhibition of bacterial growth (vase life under non-optimum water uptake conditions). In experiment 2 xylem occlusion was largely prevented, as the stems were surface-sterilized, cut under degassed water and placed in sterilized vase solution (vase life under optimum water uptake conditions). The flower stages were according to the scale of VBN, ranging from 2 (loose pointed bud with cylindrical shape) to 5 (maximum opened flower with visible anthers). The flower diameter is the average of the maximum diameter and the diameter perpendicularly to this. Values are the means of 12 (experiment 1) or 8 (experiment 2) replications.

Cultivar	RH (%)	Experiment 1			Experiment 2		
		Vase life (days)	Flower stage[1]	Flower diameter (mm)	Vase life (days)	Flower stage[1]	Flower diameter (mm)
Prophyta	60	19.4 ^{e}	4.3	94.0 ^{bcd}	29.9 ^{a}	4.8	100.0
	95	4.0 ^{a}	3.0	68.4 ^{a}	19.3 ^{c}	4.1	91.0
Frisco	60	18.0 ^{de}	5.0	104.0 ^{e}	26.6 ^{b}	5.0	102.0
	95	16.4 ^{d}	5.0	99.0 ^{de}	20.8 ^{c}	4.6	99.0
Dream	60	13.6 ^{c}	4.3	90.7 ^{bc}	20.3 ^{c}	4.8	97.2
	95	11.5 ^{b}	4.2	88.1 ^{b}	18.1 ^{c}	4.4	93.8
F pr.							
Cv.		<0.001	–	<0.001	<0.001	–	0.019
RH		<0.001	–	<0.001	<0.001	–	0.001
Cv. × RH		<0.001	–	<0.001	<0.001	–	0.250

Means in each column followed by different letters indicate significant differences according to LSD-test.

[1] Flower stage did not show a normal distribution, therefore no F probability is given.

Table 2. Percentage of the incidence of symptoms terminating the vase life of three cut rose cultivars, grown at moderate (60%) or high (95%) relative air humidity (RH). Details of the experiments are given in Table 1. Data refer to 12 (experiment 1) or 8 (experiment 2) replications.

Cultivar	RH (%)	Experiment 1				Experiment 2			
		Symptoms (%)							
		Pedicel bending	Desiccated leaves	Desiccated petals	Wilted flower	Pedicel bending	Desiccated leaves	Desiccated petals	Wilted flower
Prophyta	60	–	–	–	100	–	–	50	50
	95	25	17	–	58	–	–	25	75
Frisco	60	–	–	–	100	–	–	75	25
	95	–	–	–	100	–	–	37	63
Dream	60	8	–	–	92	–	–	88	12
	95	8	–	–	92	–	25	75	–

always observed at the end of the light period (Fig. 2A, B). The amplitudes between the highest and the lowest transpiration rates within the light period are shown in Figures 2C and D. Roses grown under high RH exhibited a considerably smaller amplitude of transpiration during the light period, compared to those grown at moderate RH.

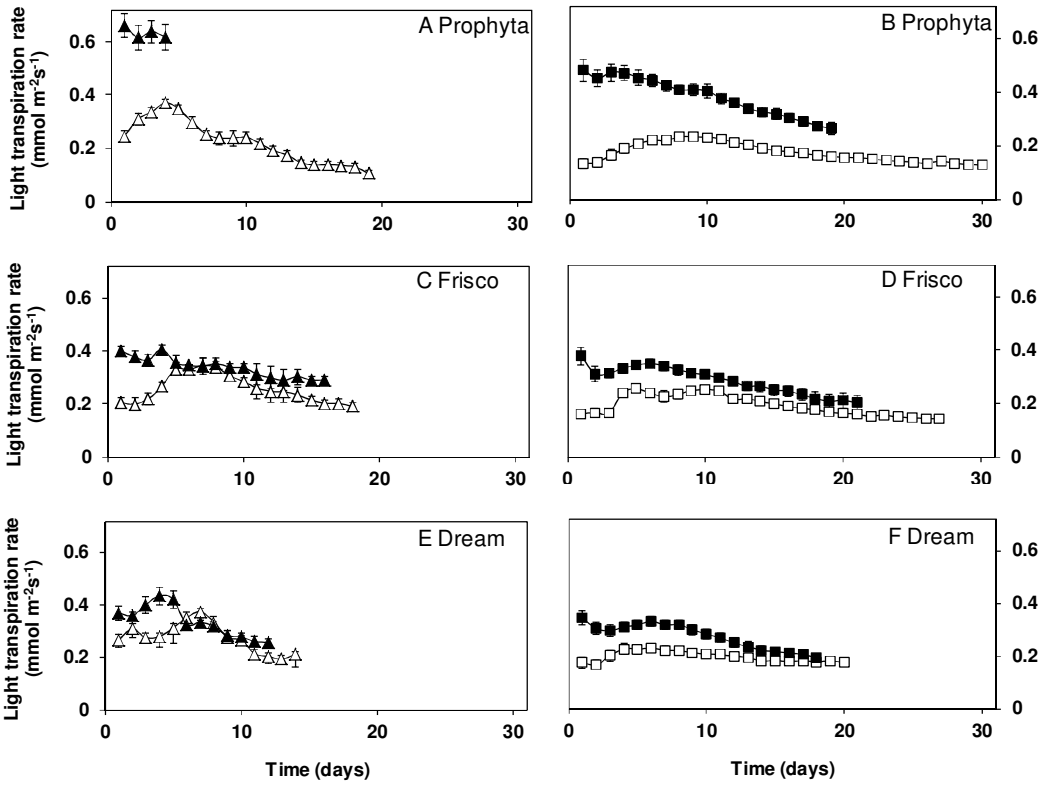

Fig. 1. Transpiration rate in the light period during vase life under non-optimum (A, C, E; experiment 1) and optimum (B, D, F; experiment 2) water uptake conditions of three cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Details of the experiments are given in Table 1. Values are the means of 12 (experiment 1) or 8 (experiment 2) replications ± SEM.

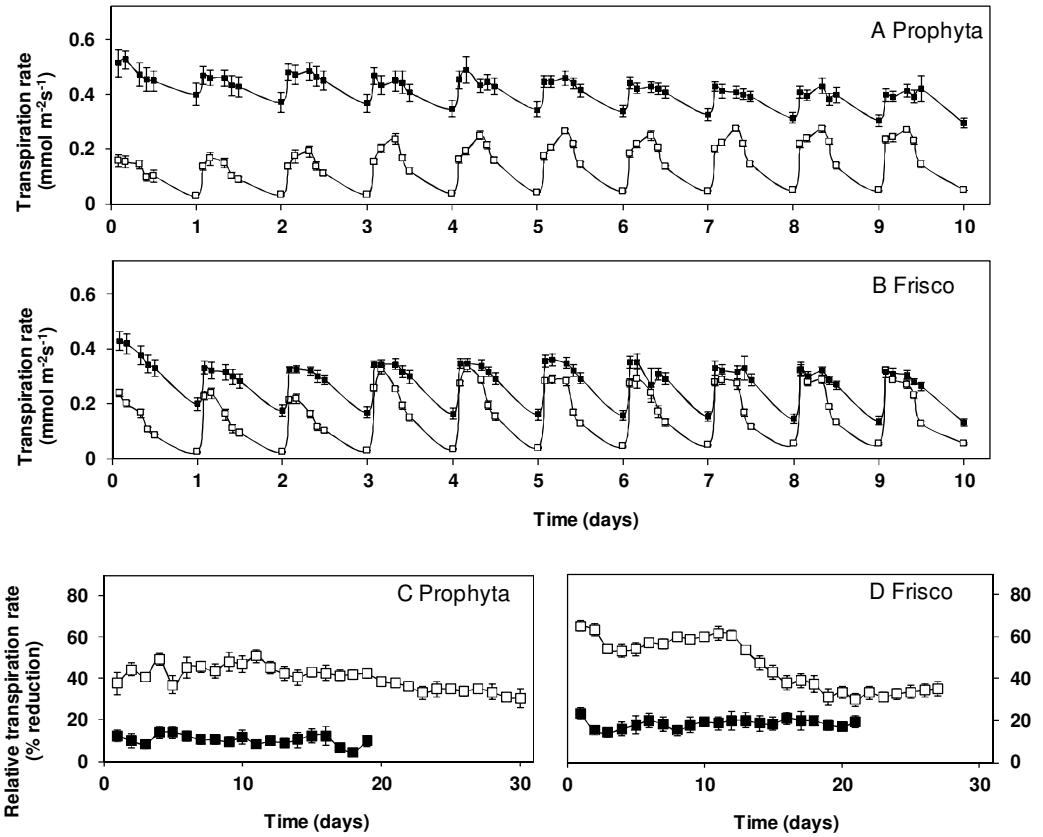

Fig. 2. Transpiration rate (2, 4, 8, 10, 12 h after the onset of the light period, and 12 h after the onset of the dark period) during the first 10 days of vase life under optimum water uptake conditions (experiment 2) of two cut rose cultivars grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. The light and dark periods were 12 h each. Details of the experiment are given in Table 1. Figures C and D depict the relative decrease between the maximum ($T_{L\,max}$) and minimum ($T_{L\,min}$) values of transpiration rate during the light period [i.e. $\frac{(T_{L\,max})-(T_{L\,min})}{(T_{L\,max})}\times 100$]. Values are the means of 8 replications ± SEM.

Transpiration rate in darkness

In all three cultivars tested, a high RH during cultivation significantly increased the transpiration rate in the dark period during vase life (Fig. 3A, C, E). Cultivar Prophyta grown at high RH had on average a five times higher transpiration rate in the dark compared to roses grown at moderate RH. The cvs. Frisco and Dream showed an increase of approximately a factor two. Compared to these data on roses placed under suboptimal water uptake conditions, the effect of cultivation RH on the transpiration rate in darkness was similar in roses that were subjected to optimized water uptake conditions during vase life (Fig. 3B, D, F; see also Fig. 7B).

The difference between the transpiration rate during the light period and the rate of nocturnal transpiration is shown in Figure 4. This difference was large at the beginning of vase life in roses grown under moderate RH, but became lower later on, in roses placed under suboptimal water uptake conditions (Fig. 4A, C, E). This decrease in the difference between transpiration during the light and in darkness, during the course of vase life, was not found (cv. Prophyta; Fig. 4B) or was less pronounced (cvs. Frisco and Dream; Fig. 4D, F) in roses that were subjected to more optimal water uptake conditions.

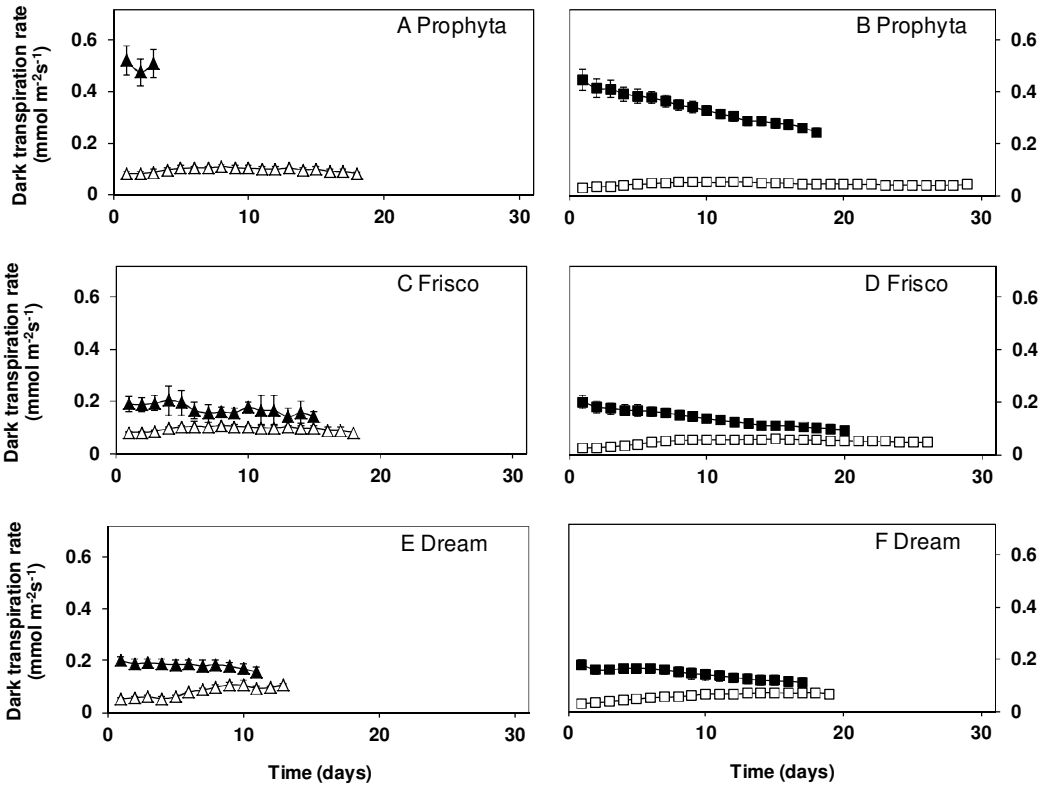

Fig. 3. Transpiration rate in the dark period during the vase life under non-optimum (A, C, E; experiment 1) and optimum (B, D, F; experiment 2) water uptake conditions of three cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Details of the experiments are given in Table 1. Values are the means of 12 (experiment 1) or 8 (experiment 2) replications ± SEM.

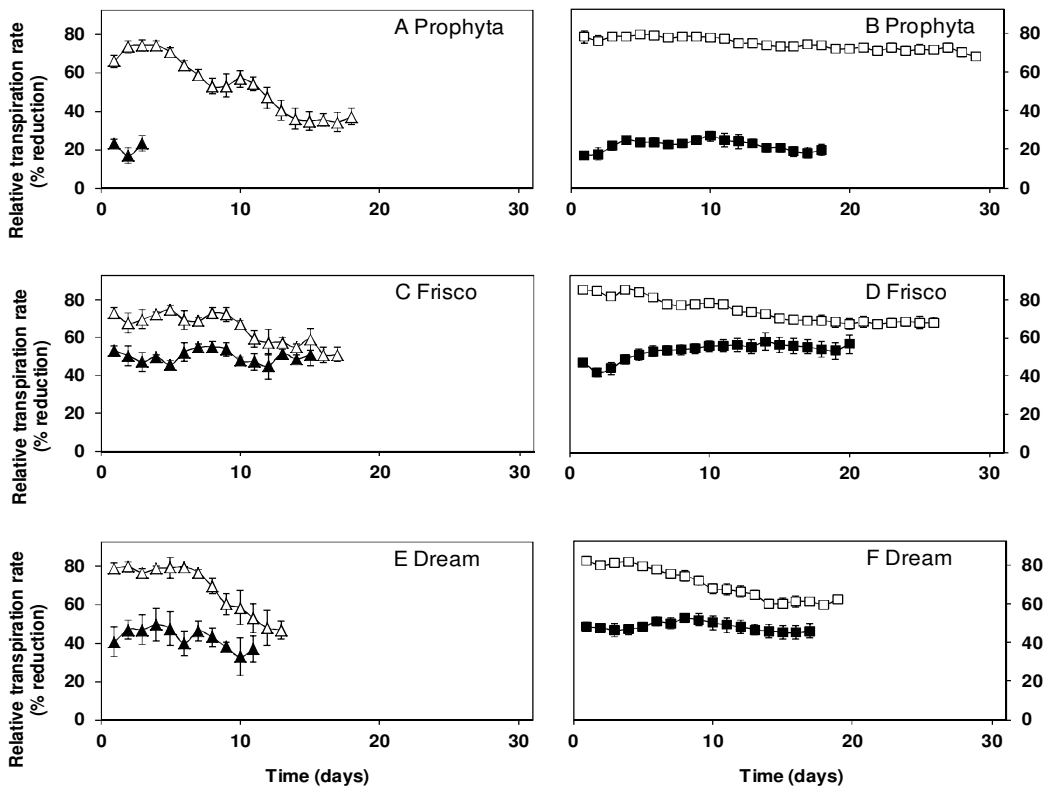

Fig. 4. The relative decrease between the transpiration rate during the light (T_L) and dark (T_D) periods [i.e. $\frac{(T_L)-(T_D)}{(T_L)}\times 100$], during vase life under non-optimum (A, C, E; experiment 1) and optimum (B, D, F; experiment 2) water uptake conditions of three cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Details of the experiments are given in Table 1. The transpiration rate during the light and dark periods is shown in Figures 1 and 3, respectively. Values are the means of 12 (experiment 1) or 8 (experiment 2) replications ± SEM.

Fresh weight (FW)

A large initial increase in FW was observed in roses that were held under rather optimal water uptake conditions during vase life (Fig. 5B, D, F) whilst a smaller FW increase was found in flowers that were exposed to more suboptimal conditions (Fig. 5A, C, E). The RH during cultivation had little effect on the FW in the cvs. Dream (Fig. 5C, D) and Frisco (Fig. 5E, F). Only in cv. Prophyta the FW remained lower, almost throughout vase life, in flowers grown under high RH compared to those grown under moderate RH (Fig. 5A, B).

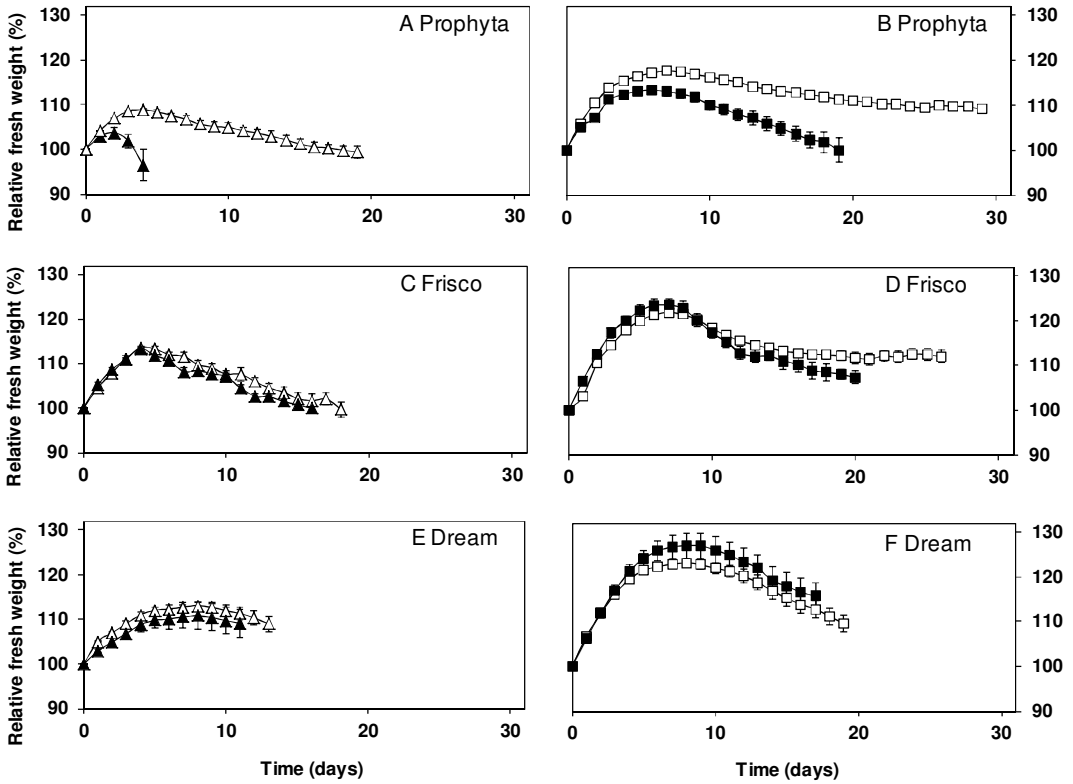

Fig. 5. Relative fresh weight during the vase life under non-optimum (A, C, E; experiment 1) and optimum (B, D, F; experiment 2) water uptake conditions of three cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. The fresh weight of each flowering stem was expressed relative to its initial weight. Details of the experiments are given in Table 1. Values are the means of 12 (experiment 1) or 8 (experiment 2) replications ± SEM.

Recovery from the decrease in stem hydraulic conductivity (K_h) due to air aspired at the stem cut surface, in cv. Prophyta

Stem segments of cv. Prophyta were cut under water. A suction force of 40 kPa was applied to the upper end while the lower end of the stem segment was maintained under water. The absolute K_h values were not affected by the preharvest RH level (P = 0.458; data not shown). The initial K_h was set to 100% (Fig. 6A). After some initial measurements, air was allowed to be aspired at the basal cut surface for 3 min, by lifting the basal end of the segment above the solution surface. This was followed by lowering the segment back into the solution. K_h then showed an initial fast recovery (Fig. 6A). Previous research found that this recovery is due to the partial refilling with solution of xylem conduits in which air had been taken up, resulting in a reconnection between the vase water and the xylem conduits that had not been opened by cutting (van Ieperen et

al. 2002). Later on (from about t = 30 min) a slower increase in K_h was found (Fig. 6A). This has been related to the relatively slow dissolution of the remaining trapped air at the top of the xylem conduits (van Ieperen et al. 2002). After 2.4 h of measurement, the K_h tended to stabilize at 63% of the initial value (before air entrance at the lower stem end). No effects of RH during cultivation were found in the response to air aspiration for 3 min (Fig. 6A).

Stomatal response to a decrease in leaflet water potential (Ψ_{leaf}), in cv. Prophyta

Transpiration rates and Ψ_{leaf} were measured in detached leaflets of cv. Prophyta. The transpiration rate was taken as a measure of stomatal opening. In leaflets from plants that had been cultivated at moderate RH, the stomata showed a rapid closure reaction, starting when the Ψ_{leaf} had dropped to -2.0 MPa. The stomata were almost fully closed when the Ψ_{leaf} was -2.5 MPa (Fig. 6B). The reaction was quite different in leaflets taken from plants that had been cultivated at high RH. The stomata showed a small closing reaction also starting at about -2.0 MPa, but they closed only slightly further at a lower Ψ_{leaf}. When the Ψ_{leaf} had reached -3.0 MPa the stomata were still about half open (Fig. 6B).

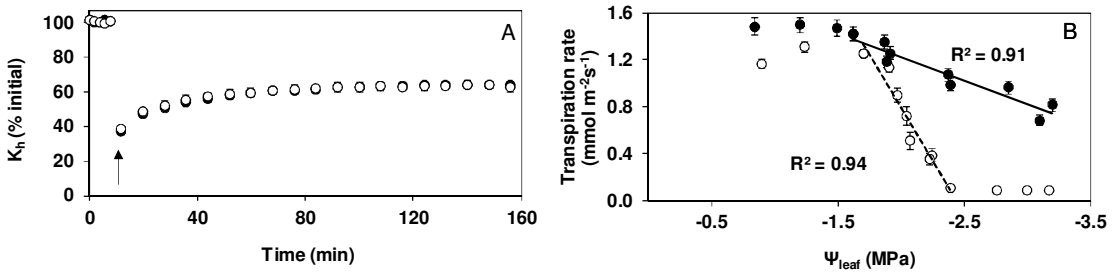

Fig. 6. Stem hydraulic conductivity recovery upon air emboli induction, and transpiration rate as a function of leaflet water potential in cv. Prophyta grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. (A) Hydraulic conductivity (K_h) changes following artificial induction of air emboli at the cut surface of 25 cm stem segments (experiment 3). The arrow depicts the time where the stem segment was allowed to aspire air at the basal cut surface (air aspiration duration was 3 min). Thereafter the end of the stem segment was again placed into the solution. Values are the means of 7 replications ± SEM. (B) Transpiration rate as a function of leaflet water potential (Ψ_{leaf}) during desiccation of detached leaflets (experiment 4). Vertical bars indicate SEM (n = 14).

Stomatal response to abscisic acid (ABA) feeding during postharvest, in cv. Prophyta

The efficacy of adding ABA into the vase solution (antitranspirant compound) on decreasing water loss during postharvest was evaluated in cv. Prophyta roses grown at high RH. Long-term ABA feeding (100 μM) through the stem base resulted in lower transpiration rates during both the light and dark postharvest periods (Fig. 7). However, the long-term ABA feeding via the vase solution was only partly able to counteract the effect of high RH during cultivation on the increased water loss. This is particularly evident during the nocturnal period, where the transpiration rate in ABA-fed stalks from plants grown at high RH is five times higher, as compared to unfed stalks from plants grown at moderate RH (Fig. 7B).

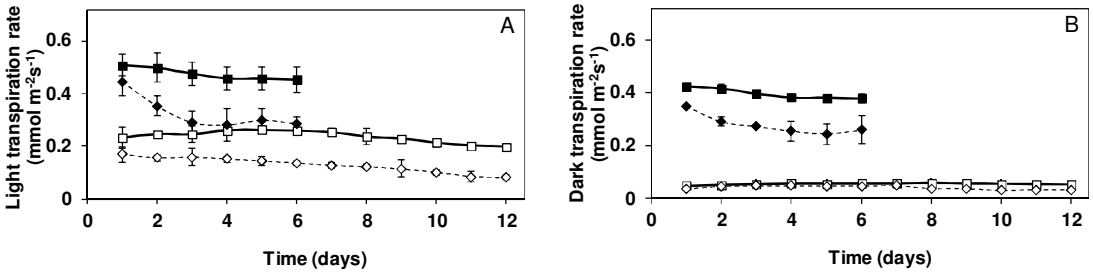

Fig. 7. Transpiration rates in the light (A) and darkness (B) of cv. Prophyta roses placed in a vase solution containing 0 (solid lines) or 100 μM ABA (dashed lines) (experiment 5). Flowers were cultivated at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Xylem occlusion was largely prevented as described for experiment 2 in Table 1. The experiment was terminated when leaf abscission was observed in ABA-fed flowers. Values are the means of 8 replications ± SEM.

Discussion

Cut rose flowers often show water stress symptoms during postharvest. In previous studies it has been found that in some cultivars these water stress symptoms become aggravated by long-term high RH during cultivation (Mortensen and Gislerød 1999, 2005). The present results confirm those findings (Table 1). Moreover, we have studied in detail the water relations of cut flowers grown at moderate and high RH and we addressed the question of how stomatal opening changes in time and reacts to darkness and to a decrease in water potential. In this study we also initiated work to explain the cultivar differences in their sensitivity to long-term high RH.

We observed that in the cultivar that showed a much shorter vase life when grown at high RH (cv. Prophyta, i.e. sensitive cultivar), the rate of transpiration during

both the light and dark periods was, respectively, three- and five-fold higher in cut flowers grown at elevated RH, compared with those grown at moderate RH (Table 1; Fig. 1 and 3). These roses were, therefore, largely unable to recover during darkness (stomatal closing stimulus) from the water stress that naturally occurs in the light period (de Stigter and Broekhuysen 1989). Thus, the rate of water uptake during postharvest period rapidly became insufficient to compensate the enhanced leaf transpiration rate. This resulted in early water stress symptoms in these flowers (Table 2, experiment 1), thus in a decrease of Ψ_{leaf}. Furthermore, it was found that cv. Prophyta roses grown at high RH were much less able to close their stomata in response to this decrease in Ψ_{leaf} (Fig. 6B). The combined effects of a higher initial transpiration rate in the light, a much higher transpiration rate in darkness, and the inhibited response to a decrease in Ψ_{leaf} seem to completely explain why the flowers that had been grown at high RH showed early water stress symptoms and thus had a short vase life. Cultivation at high RH also resulted in inhibition of flower opening in cv. Prophyta (Table 1, experiment 1), which was likely due to the low water potential. In other rose cultivars a decrease in flower water potential also has been shown to reduce flower opening (van Doorn et al. 1991).

When the problems with water uptake were largely prevented (stem surface-sterilization, cutting under degassed water, and placement in sterilised vase solution; experiment 2) the shorter vase life and the inhibition of flower opening in cv. Prophyta were strongly alleviated in high RH-grown plants (Table 1 and 2). Similarly, it has been shown that treatment with silver nitrate, an antibacterial compound, increased the vase life in cut roses cultivated at high RH (Torre and Fjeld 2001). These data show that the water uptake problems, which often occur in most rose cultivars, are the initial cause of the water stress symptoms. However, the poor control of water loss in flowers cultivated at high RH aggravates this problem, whereas flowers grown at moderate RH react to the low Ψ_{leaf} by rapidly closing their stomata (Fig. 6B), which enables a positive water balance during a longer period. Nonetheless, in flowers grown at moderate RH the water uptake still eventually becomes so low that a net water loss occurs, even though the stomata are largely closed. This net water loss is due to the ongoing residual stomatal and cuticular transpiration (Kerstiens 1995, van Doorn 2011). So wilting symptoms will eventually ensue.

The water loss of cv. Prophyta roses that were grown at high RH, and placed in a vase solution, was considerably larger than that in the other two cultivars studied (Fig. 1 and 3). These results are consistent with an earlier study, where stomatal responsiveness to leaf desiccation was significantly lower in cv. Prophyta as compared to cv. Frisco in high RH-grown plants (Fanourakis et al. 2009). Additionally, it was now found that elevated RH during cultivation resulted in a weakened diurnal rhythm during the light

period, as expressed by the amplitude between the highest and the lowest transpiration rate (Fig. 2). Prophyta roses grown at high RH and placed in a vase solution containing ABA had a lower water loss, compared to unfed high RH-grown flower stalks, but still their transpiration rate especially during the darkness was higher than in moderate RH-grown plants (Fig. 7). Recently it has been demonstrated that the role of ABA, in alleviating the negative effects of high RH on stomatal functioning, is restricted to the period of leaf expansion (Fanourakis et al. 2011). Thus, the limited capacity of a long-term ABA feeding to induce stomatal closure when applied via the vase solution (Fig. 7) can be explained by the fact that flower stalks at harvest are totally composed of fully developed leaves. Moreover, in the current study the 100 μM ABA feeding solution was continuously reaching the leaf via the transpiration stream, whereas in Fanourakis et al. (2011) ABA was brushed daily on fully developed leaves at a lower concentration (30 μM). These differences can explain the total absence of stomatal response to ABA in their study and a partial (though limited) response in the current one.

Although stomatal density in cv. Prophyta is not affected by ambient humidity during leaf development, stomata formed under elevated RH levels have longer pore length (Fanourakis et al. 2011), which results in higher water loss rate at the same pore aperture values (Parlange and Waggoner 1970). Higher cuticular water loss, as a result of poor cuticular development, might also contribute to the high rates of water loss in high RH-grown leaves (Karbulkova et al. 2008), though this conclusion has been questioned by other authors (Torre et al. 2001). Thus, part of the higher water loss observed in cv. Prophyta can be possibly related to anatomical features (e.g. bigger stomata and higher cuticular permeability), which might contribute to the increased sensitivity of this cultivar to long-term high RH during growth. Even in the cvs. Frisco and Dream there was a higher water loss as a result of high RH during cultivation, but this did not lead to early visible water stress symptoms (Table 1). Apparently, the increase in water loss in these cultivars was not high enough to reduce the water potential to a level that induced earlier visible symptoms of water stress. Future research is needed to evaluate the relative importance of the physiological and anatomical components in the enhanced water loss among contrasting genotypes grown at elevated RH.

A decrease of the water potential in cut flowers leads to cavitation events (Dixon et al. 1988, Spinarova et al. 2007). When a high number of conduits becomes inoperative, as a result of cavitation, water uptake will become additionally inhibited (van Doorn 1997, 2011). Both air emboli at the cut surface (van Doorn and Jones 1994) and bacterial occlusion (Bleeksma and van Doorn 2003) have been shown to induce cavitation. We have no data on the sensitivity to cavitation in the cvs. Prophyta and Dream, but cv. Frisco was highly resistant to cavitation (which started at a considerably lower water

potential than in cv. Sonia roses; van Doorn and Suiro 1996). It is therefore possible that the genotypic variation in the vase life decrease, as a result of more humid air during cultivation, arises partially from an effect on cavitation, but the role of cavitation in the present differences between cultivars is not yet known.

Unlike our initial hypothesis, no effect of cultivation at high RH was found on the initial values of stem hydraulic conductivity. The absence of such an effect indicates that RH during cultivation had no effect on stem xylem anatomy to an extent that it affected hydraulic conductivity. When the hydraulic conductivity of cv. Prophyta stem segments was reduced because of aspiration of air into the xylem conduits opened by cutting, there was no effect of the RH level during cultivation (Fig. 6A). This suggests that factors such as the wetting angle of the xylem conduits (van Ieperen et al. 2002) were also not considerably affected.

Conclusions

This study clearly demonstrates that the main effect of ambient humidity during preharvest on the water relations during postharvest is closely related to the regulation of water loss, since the stem hydraulic conductivity and its recovery by air emboli were not affected by long-term high RH. Therefore, it is concluded that xylem anatomy does not explain the differential cultivar sensitivity to high RH. Instead, differences between the cultivars could be largely explained by their contrasting capacity to control water loss. For instance, the early water stress symptoms and the inhibition of flower opening in cv. Prophyta (sensitive) grown at high RH were due to a higher rate of water loss compared to those grown at moderate RH, while there was apparently a similar blockage in water uptake. This higher water loss seems to be closely related to stomatal malfunctioning. Cultivar Prophyta that had been cultivated at high RH were found not to close their stomata to the same degree as those cultivated at moderate RH (1) in darkness and (2) when the Ψ_{leaf} decreased. The increase of cut flower water loss, as a result of plant growth at high RH, was much less pronounced in two other cultivars (cvs. Frisco and Dream), compared to cv. Prophyta. It is likely that the higher water loss in these two cultivars grown at high RH results in a water potential, lower than the one needed to induce significant cavitation events. Preventing vascular occlusion, caused by air emboli at the cut surface and bacterial physical blockage, considerably extended the time to wilting and enhanced flower opening in cv. Prophyta roses grown at high RH. This indicates that the high rate of water loss, as a result of plant growth at elevated atmospheric humidity, can only be detrimental for cut flower longevity under limiting water uptake conditions.

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CHAPTER 3

Genotypic variation in the control of water loss as a result of leaf development at high relative air humidity

3.1 A comprehensive analysis of physiological and anatomical components involved in higher water loss rates after leaf development at high humidity

3.2 Breeding cut roses for better keeping quality: first steps

CHAPTER 3.1

A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity

Abstract

Long-term high relative air humidity (RH) during leaf development limits the regulation of leaf water balance. We studied the relative importance of the physiological and anatomical components in the enhanced water loss among contrasting genotypes grown at elevated RH. The stomatal responsiveness to three closing stimuli (desiccation, abscisic acid feeding, light/dark transition), as well as several stomatal features (density, index, size and pore dimensions) and the cuticular permeability (*Pe*) were determined in four rose cultivars, grown under moderate (60%) and high (95%) RH. Moreover, the effects of changes in stomatal density and pore dimensions on the stomatal conductance (g_s) were quantified using a model. Higher water loss, as a result of plant growth at high RH, was primarily caused by an increase in residual g_s, and to a lesser extent due to higher *Pe*. The differential impairment of stomatal closing ability did not associate with differences in stomatal size among genotypes. It was estimated that the higher g_s of high RH-grown plants was mostly due to poor stomatal functionality (70–84% of the effect depending on the cultivar), and to a lesser extent the combined result of higher stomatal density, longer pore length and depth (16–30%). It is concluded that the reduced stomatal closing ability is the primary cause of the large genotypic variation in the control of water loss in high RH-grown plants. Although longer stomata responded similarly to shorter ones, a higher density of stomata with longer pore length contributed significantly to the increased water loss.

Fanourakis D, Heuvelink E, Carvalho SMP (2011) A comprehensive analysis of the physiological and anatomical components involved in higher water loss rates after leaf development at high humidity. (submitted)

Introduction

The regulation of water relations is of utmost importance for the survival of terrestrial plants. Since the leaves represent the main interface between the plant and the atmosphere, restriction of leaf water loss is decisive under conditions of water stress (Burghardt and Riederer 2003). The acquisition of stomata and impervious cuticle were key elements in the evolution of plants, as they work as effective barriers against uncontrolled water loss (Edwards et al. 1996, Hetherington and Woodward 2003). However, several studies have shown that long-term high relative air humidity (RH) during leaf development results in a reduced leaf capacity to control water loss when plants are subsequently subjected to conditions of increased evaporative demand (high leaf to air vapour pressure deficit, VPD). This is the case for plantlets produced in *in vitro* culture (RH in the culture vessels close to 100%), which showed disturbed water relations after transplantation (Aguilar et al. 2000, Brainerd and Fuchigami 1982). A similar problem occurs in protected cultivation when plants are grown under long-term high RH (≥ 85%), as these plants rapidly achieve a negative water balance during postharvest, resulting in a reduced keeping quality (Rezaei Nejad and van Meeteren 2005, Torre and Fjeld 2001, Torre et al. 2001). In cut roses it was found that the impact of high RH during growth on the leaf capacity to control water loss was strongly cultivar dependent (ranging from 11 to 110% water loss increase during leaf desiccation, as compared to leaves from moderate RH-grown plants; Mortensen and Gislerød 1999). Nevertheless, most studies conducted so far have approached this problem by analysing only one genotype (Fordham et al. 2001, Kawamitsu et al. 1993, Ottosen et al. 2002), while very little attention has been paid to the striking genotypic differences described for roses in their tolerance to high RH. This genotypic variation in the control of water loss represents a great potential for understanding the underlying processes behind the tolerance to high RH.

Long-term high RH is known to induce abnormal stomatal functioning, bigger stomata and changed stomatal density in different plant species (Fanourakis et al. 2011, Fordham et al. 2001, Torre et al. 2003). Most studies conducted on this topic have been focused on the lack of proper stomatal closure, which has been pointed out as the main factor involved in the disturbed water relations in high RH-grown plants (Rezaei Nejad and van Meeteren 2005, 2007). Nevertheless, the relevance of the changed anatomical features in the enhanced water loss of high RH-grown plants has been poorly investigated. For instance, it remains unknown if within the stomatal population of a given species an increased stomatal length, caused by high RH, disturbs stomatal functionality contributing indirectly to the higher water loss. This negative relationship

between stomatal size and stomatal functioning has been observed in previous studies when comparing different species, since the ones with larger stomata had slower response times (Aasamaa et al. 2001, Franks and Farquhar 2007). On the other hand, at leaf level the stomatal conductance (g_s) is determined by the product of stomatal density and pore area (i.e. cross-sectional area available for gas fluxes). Since high RH exerts an effect on both stomatal density and pore length (Fordham et al. 2001, Torre et al. 2003), these characteristics are expected to influence *per se* the g_s, having a direct role on the enhanced water loss. Thus, when using direct (i.e. gravimetrically; Fanourakis et al. 2011, Rezaei Nejad and van Meeteren 2005) or indirect methods (e.g. infra-red gas analyzer, porometer, thermal imaging or PSII efficiency under non-photorespiratory conditions; Ottosen et al. 2002, Rezaei Nejad and van Meeteren 2007, Torre et al. 2003) for measuring the g_s those measurements do not only reflect the treatment effect on stomatal physiology (stomatal opening), but also include the influence of the anatomical features, which is often neglected. To the best of our knowledge, there are no reports providing the required information at individual pore scale on high RH-grown plants, which would enable differences in the g_s to be related to its component variables, such as stomatal density and pore dimensions.

Concerning the relative importance of water loss through the cuticle, as compared to the water loss through the open stomata, this can vary between 2 and 29% in non-stressed leaves depending on the species (Holmgren et al. 1965). This fraction becomes higher in leaves subjected to stomatal closing stimuli (Boyer et al. 1997). Many studies on cuticular permeability (*Pe*) in plants grown under prolonged periods of high humidity, have been focused on *in vitro* plants (reviewed by Hazarika 2006, Pospisilova et al. 1999) where a higher *Pe* was frequently observed (Santamaria and Kerstiens 1994, Wetzstein and Sommer 1982). Nevertheless, a comprehensive analysis of the relative contribution of cuticular water loss over the total leaf water loss in non-stressed leaves, or under conditions of maximum stomatal closure in high RH-grown plants, has not yet been performed.

The aim of this research was to (i) assess the genotypic variation in the regulation of water loss in high RH-grown plants, (ii) test whether changes in stomatal size influence their functionality and (iii) separate the relative contribution of the anatomical features (i.e. stomatal density, pore length, pore depth, and *Pe*) from the stomatal opening, on the higher water loss rates in well-watered plants grown under long-term high RH. We tested the hypotheses that the genotypic variation in the control of water loss is primarily driven by differential effects on the stomatal physiology and that changes in stomatal size influence their functionality. *Rosa hybrida* was chosen as a model system, since this is a hypostomatous species (i.e. no stomata were found in the adaxial

leaf surface) facilitating the measurement of *Pe*, and because of the existence of genotypes with contrasting water loss rates when grown under long-term high RH.

Materials and Methods

Plant material and growth conditions

Rooted cuttings of four cut rose cultivars (*R. hybrida* L. cvs. Dream, Frisco, Pink Prophyta and Vendela) were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands) and planted in 3.6 L pots containing a mixture of cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull Rhenen, The Netherlands) (3:1, v/v). Cv. Pink Prophyta will be called Prophyta in the remainder of this paper. These cultivars were selected based on their contrasting water loss rates in response to leaf desiccation after cultivation at high RH: cvs. Dream and Frisco with a lower water loss rate (tolerant cultivars to high RH); cvs. Prophyta and Vendela with a higher water loss rate (sensitive cultivars to high RH) (Mortensen and Gislerød 1999, C Slootweg: personal communication). Twenty four plants per cultivar were randomly distributed over four growth chambers (l × w × h = 1.3 × 0.8 × 1.3 m). Plants were grown as a single shoot (one plant per pot), at a density of 30 plants m^{-2}. In two chambers the RH was 60 ± 3% (moderate RH) and in the other two it was 95 ± 1% (high RH) during the cultivation period. The four chambers had constant day and night temperatures (19 ± 1 °C), resulting in VPDs of 0.88 ± 0.12 kPa (moderate RH) or 0.11 ± 0.03 kPa (high RH). Climate parameters were recorded automatically every 5 min using data loggers (Fourier MicroLog EC650, MicroDAQ.com Ltd, Contoocook, NH). The CO_2 concentration during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided an 18-6 h on-off cycle and 300 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation (Model LI-250, LI-COR, Lincoln, NE). The light intensity was measured at 70 cm from the pot base which corresponds to the top of fully grown plants.

Plants were well-watered automatically with a nutrient solution. Four weeks after planting, when the flower bud became visible, the measurements started on fully expanded leaflets from 12 plants per treatment (one leaflet per plant).

Stomatal responses to closing stimuli

R. hybrida has compound leaves, where the leaflets are in pairs except for the terminal leaflet (having the longest petiole length facilitating evaluations). To study the effect of desiccation, abscisic acid (ABA) feeding and light/dark transition on leaf transpiration

rate, the terminal leaflets of the first, second and third fully grown penta-foliate leaves counting from the apex, were detached. Their petioles were immediately recut under degassed water (to prevent cavitation induced-embolism), placed in flasks filled with water and transferred to the test room. The climate conditions of the test room, where all the closing stimuli were applied, were 21 °C, 50 ± 3% RH (1.24 ± 0.07 kPa VPD) and a photon flux density of 50 μmol m^{-2} s^{-1}.

For the desiccation stimuli, the leaflets were first incubated for 1 h at about 100% RH (21 °C; VPD close to 0) to establish their saturated fresh weight as described by Fanourakis et al. (2011). Subsequently, the leaflets were removed from the water and placed in the test room where the leaflet transpiration rate was gravimetrically measured every 5 to 30 min during 4 h. The leaflet area was then determined using a leaf area meter (model 3100 Area Meter, LI-COR, Lincoln, NE) and the leaflets were dried at 80 °C for 24 h. The relative water content (RWC) was calculated using the following equation (Slavik 1974):

$$\mathrm{RWC} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Saturated Fresh Weight} - \text{Dry Weight}} \times 100 \quad (1)$$

For the ABA stimuli, the leaflets with their petioles in water, were left to stabilize for 1 h in the test room. Subsequently, the transpiration rates were gravimetrically measured for 30 min to guarantee that they had stabilized. Afterwards, the leaflets were kept in the same flasks (control; 0 μM ABA) or transferred to flasks containing an aqueous solution of 100 μM (±)-ABA (Sigma, St. Louis, MO). Both flasks were sealed with parafilm to prevent evaporation. Leaflet transpiration rate was gravimetrically determined by weighing the flasks with the leaflets every 5 to 10 min during 2 h 40 min. The leaflet area was determined as mentioned above and ABA intake was calculated as the product of leaflet transpiration rate multiplied by the ABA concentration of the solution.

A similar procedure to the one used for stomatal responses to ABA was followed to study the effect of light/dark transition stimuli on leaflet transpiration rate. Leaflet transpiration rate was gravimetrically measured for 30 min in light, followed by 2 h 10 min in darkness. The leaflet area was further determined.

Stomatal density, index and dimensions

Stomatal features (i.e. density, index, size, pore length and pore aperture) were measured in four rose cultivars using a leaflet of the first pair of lateral leaflets from the first fully grown penta-foliate leaf (counting from the apex). Since preliminary trials

showed that *R. hybrida* is a hypostomatous species, measurements on stomata were only performed on the abaxial (lower) leaflet surface. These stomatal features were determined using the silicon rubber impression technique (Smith et al. 1989). The details on the procedure for conducting the impression and for image analysis are described by Fanourakis et al. (2011). Five rectangular fields of view (Poole and Kurschner 1999) per leaflet were counted for determining the stomatal density (i.e. number of stomata per unit leaf area) and epidermal cell density (non-stomatal cells) using 12 leaflets (one leaflet per plant), and a magnification of 100 or 250 ×, respectively. Stomatal index [SI = (stomatal density × 100)/(stomatal density + epidermal cell density)] was calculated according to Salisbury (1927).

In 20 randomly selected stomata per leaflet from 12 leaflets (n = 240), the stomatal length, stomatal width, pore length and pore aperture were measured immediately after leaflet detachment and after 35 min of leaflet desiccation using a magnification of 1000 ×. Stoma width was chosen instead of guard cell width, since the latter undergoes changes up to 50% as stomata close (Shope and Mott 2006). To calculate pore area, it was assumed that the pores are elliptical (major axis, pore length; minor axis, pore aperture). For this purpose, the pore length was evaluated separately over the presence of closing stimuli. The pore area per stoma times the stomatal density enabled the calculation of the pore area per unit of leaf area.

Modelling stomatal conductance (g_s)

The sensitivity of g_s to changes in stomatal density, pore length, pore depth and pore aperture of leaflets that have been dehydrated for 35 min was modelled in the four studied cultivars. This information will enable to separate the relative contribution of each stomatal feature to the higher g_s in plants grown under elevated RH. The predicted changes of g_s were estimated by adjusting each of these features within its empirically derived range between moderate and high RH values, while keeping the other features at the average value between the two RH levels (mid-range value). These estimations were conducted as described by Weyers and Lawson (1997) using the following equation (Nobel 1991):

$$g_S = \frac{(\text{mass of air}) \times (\text{diffusion coefficien t}) \times (\text{stomatal density}) \times (\pi \times \text{pore aperture}/2 \times \text{pore length}/2)}{(\text{pore depth}) + (\text{pore aperture}/2 \times \text{pore length}/2)^{0.5}} \quad (2)$$

The mean mass of air used in the calculation of g_s was 41.4 mol m^{-3} and the effective diffusion coefficient for water vapour in air was 2.43×10^{-5} m^2 s^{-1}, both at 21 °C (Jones 1992). The latter value was adjusted to account for molecular collisions with the

pore walls. The weighting used was scaled linearly from 0.67 (at 0 mmol H_20 m^{-2} s^{-1}) to 0.90 (at 300 mmol H_20 m^{-2} s^{-1} and thereafter) (Cowan and Milthorpe 1968). Stomatal pore depth was considered to be equal to the guard cell width (i.e. stomatal width/2), assuming guard cells inflate to a circular cross-section (Franks and Beerling 2009, Franks and Farquhar 2007). The end correction used in the calculation of g_s was according to Nobel (1991). This end correction is added to the pore depth because the concentration patterns of water vapour on both ends of the stomatal pore cause an increase in the effective depth. A number of different formulae for the end correction exists, but the differences in the estimated g_s among them are generally relatively small compared with the pore depth (Nobel 1991).

Cuticular permeability (Pe)

To study the relative contribution of cuticular water loss to the total leaf water loss, the cuticular permeability was evaluated. Young mature leaflets were detached from leaves of the same order (fourth penta-foliate leaves) and double-sealed on the abaxial surface by coating with silicone vacuum grease to which a polyethylene sheet was attached. Leaflets were left to desiccate from the adaxial cuticle (stoma-free) under 1.24 ± 0.07 kPa VPD (21 °C, 50 ± 3% RH) and a light intensity of 2 μmol m^{-2} s^{-1}. This low light intensity was used to prevent leaf heating, since in the absence of stomatal activity the light has no direct role on the cuticular water loss (Boyer et al. 1997). Leaflet temperature was measured with an infrared thermometer (Raytek Raynger ST20, Raytek Corp, Santa Cruz, CA) and remained stable during the evaluation period (≤ 0.4 °C rise). The rate of water loss over time was gravimetrically recorded every 2 or 4 h during the 32 h of desiccation. Finally, the adaxial leaflet surface was sealed as described above and the measurements continued for more 2 h to test the effectiveness of the sealing method. When both surfaces of the leaflet were sealed, weight loss became virtually zero (below the detection limit of 0.015 mmol s^{-1}), indicating that the seal was an effective barrier to water vapour.

To facilitate comparisons between *Pe* and leaf transpiration rate, the former has been expressed in the units used for the latter (the correct unit would be m s^{-1}, Kerstiens 1996). To calculate the residual stomatal transpiration, in evaluations which do not affect *Pe* (i.e. leaflets in water or fed with aqueous ABA solution), the cuticular water loss (initial value, i.e. after 2 h of desiccation) was subtracted from the total leaflet water loss. In this case, *Pe* has been calculated on a total leaflet area basis (i.e. twice the projected leaflet area) assuming that the cuticular properties are identical for both leaf epidermes, though this is not necessarily true (Karbulkova et al. 2008).

Statistical design and analysis

The Genstat software (10th edition, VSN International Ltd, Herts, UK) was used throughout the analysis. The experimental set up was a split-plot design, where RH was the main factor and cultivar was the split factor. Data from the stomatal responses to closing stimuli and *Pe* were subjected to an analysis of variance and treatment effects were tested at 5% probability level using F-test. The Linear Mixed Models method was used to analyze data from the stomatal features (density, index and dimensions), as an unbalanced number of replicates was evaluated (Crawley 2002). Treatment effects were tested at 5% probability level and the mean separation was done using least significant differences based on Student´s *t*-test ($P = 0.05$).

Results

Stomatal responses to closing stimuli

The analysis of pore dimensions after 35 min of leaflet desiccation showed that pore aperture was significantly wider in plants grown at high RH, but the magnitude of this effect was cultivar dependent: ranging from 67% (cv. Dream) up to 359% (cv. Prophyta) (Table 1). Stomatal pore area per unit leaf area after 35 min of desiccation, was significantly higher for plants grown at high RH in all cultivars, but this difference was largest for cvs. Prophyta and Vendela (Table 1).

In all four cultivars, leaflet transpiration rate decreased with time in response to several stomatal closing stimuli, i.e. desiccation (Fig. 1), ABA feeding (Fig. 2) and darkness (Fig. 3). However, stomata were less responsive in high RH-grown plants. Expressing transpiration rate as a function of RWC showed that the RWC, where transpiration rate does not decrease any further, was lower for plants grown at high RH (inserts of Fig. 1). This decrease was more pronounced in cv. Prophyta (49% lower, whereas for the other cultivars this was 13–20%). Leaflets of cv. Prophyta grown at high RH, reached (after 4 h of desiccation) RWC values below 30%, from which a cell will not recover when rehydrated (Lawlor and Cornic 2002). Relationships between transpiration rate and ABA amount fed into the leaflet via the transpiration stream, differed among leaflets from different RH levels (inserts of Fig. 2). The amount of ABA required to minimize the transpiration rate was significantly higher in leaflets grown at high atmospheric humidity calculated both per unit of leaf area and per stoma (Table 2). The relative effect of high RH in all parameters, in response to ABA feeding, was strongest in cv. Prophyta. Stomata of leaflets grown at elevated ambient humidity were also less sensitive to darkness, showing on average 41% lower ability in decreasing the transpiration rate (Fig. 3).

Table 1. Stomatal features of four cut rose cultivars grown at moderate (60%) or high (95%) relative air humidity (RH), just prior to leaflet detachment and 35 min thereafter. Values are the mean of 50 (cv. Prophyta, 95%) or 60 fields of view (stomatal density) and 200 (cv. Prophyta, 95%) or 240 stomata (stomatal anatomical features) measured on fully expanded leaflets. Means followed by different letters indicate significant differences according to LSD-test (comparison in columns).

Cultivar	RH (%)	Stomatal length (µm)	Stomatal width (µm)	Pore length (µm)	Before detachment		After 35 min of desiccation	
					Pore aperture (µm)	Pore area/leaf area (10^3 µm²/mm²)	Pore aperture (µm)	Pore area/leaf area (10^3 µm²/mm²)
Frisco	60	36.5 ^{ab}	26.0 ^{ab}	17.7 ^{ab}	4.47 ^{ab}	3.35 ^{a}	1.08 ^{ab}	1.03 ^{a}
	95	39.2 ^{cd}	27.8 ^{abc}	21.2 ^{c}	5.84 ^{bcd}	5.66 ^{c}	2.12 ^{c}	2.39 ^{c}
Dream	60	37.4 ^{bc}	25.6 ^{ab}	20.0 ^{bc}	3.85 ^{a}	2.57 ^{a}	1.26 ^{b}	0.89 ^{a}
	95	41.8 ^{d}	29.2 ^{bc}	25.3 ^{d}	5.61 ^{bcd}	5.16 ^{bc}	2.11 ^{c}	2.00 ^{b}
Prophyta	60	40.0 ^{cd}	29.9 ^{c}	21.8 ^{c}	5.25 ^{abc}	3.96 ^{ab}	1.13 ^{b}	0.91 ^{a}
	95	47.2 ^{e}	33.8 ^{d}	28.1 ^{d}	7.17 ^{d}	7.40 ^{d}	5.19 ^{e}	5.87 ^{e}
Vendela	60	33.1 ^{a}	28.4 ^{abc}	16.0 ^{a}	5.93 ^{bcd}	3.93 ^{ab}	0.97 ^{a}	0.72 ^{a}
	95	37.8 ^{bc}	30.7 ^{cd}	19.4 ^{bc}	6.79 ^{cd}	6.59 ^{cd}	2.79 ^{d}	2.99 ^{d}
F pr. Cv.×RH		<0.001	<0.001	<0.001	0.001	0.003	<0.001	<0.001

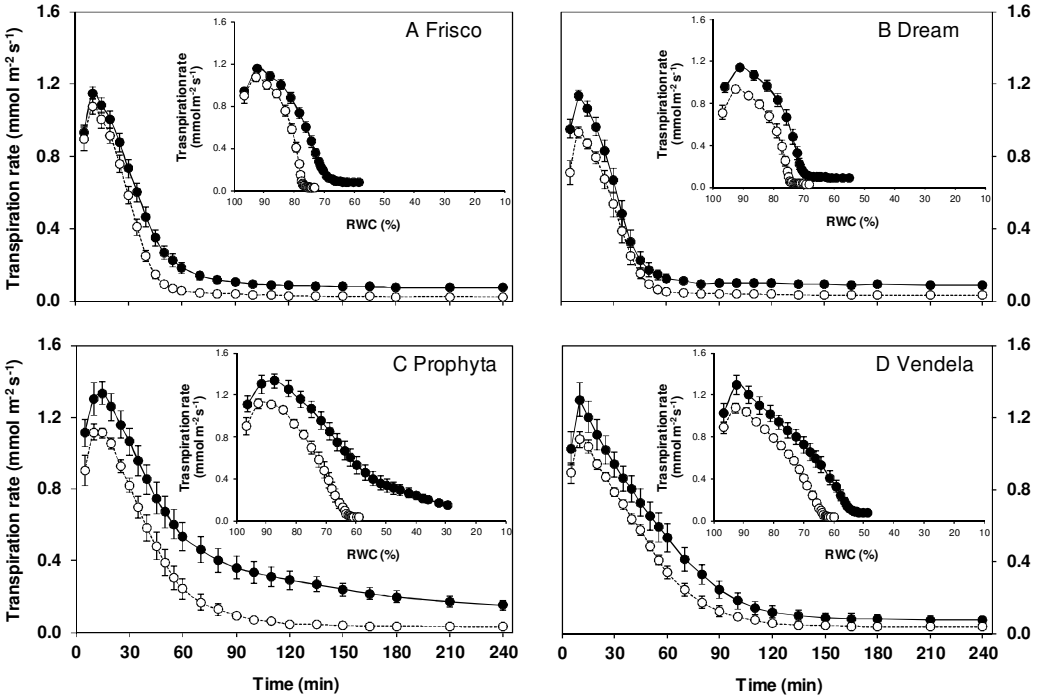

Fig. 1. Dynamics of leaflet transpiration rate during 4 h of desiccation in four cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. The inserts show the relationship between leaflet transpiration rate and leaflet relative water content (RWC). Values are the mean of 12 leaflets ± SEM.

Stomatal density, index and dimensions

Stomatal density, as well as all parameters related to stomatal dimensions, were significantly influenced by the interaction between RH and cultivar (Fig. 4; Table 1). In general, plants grown under long-term high RH, showed 8% (cv. Prophyta) to 22% (cv. Vendela) higher stomatal density (though this effect was not significant for cv. Prophyta; Fig. 4), 16% higher SI (Fig. 5) and bigger stomata (Table 1). Stomatal length increased significantly in all four cultivars but this positive effect of high RH was larger in cvs. Prophyta (18% longer) and Vendela (14% longer), whereas stomatal width was significantly higher only in cv. Prophyta (13% wider). Similarly, both the pore length and pore aperture of non-stressed leaflets were higher in all studied cultivars grown at elevated RH but this effect was stronger in the cvs. Prophyta and Dream. The total pore area per unit of leaf area of non-stressed leaflets was significantly higher in all four cultivars grown at more humid air. On average this was 80% higher, as the combined result of increased pore area per stoma and increased stomatal density.

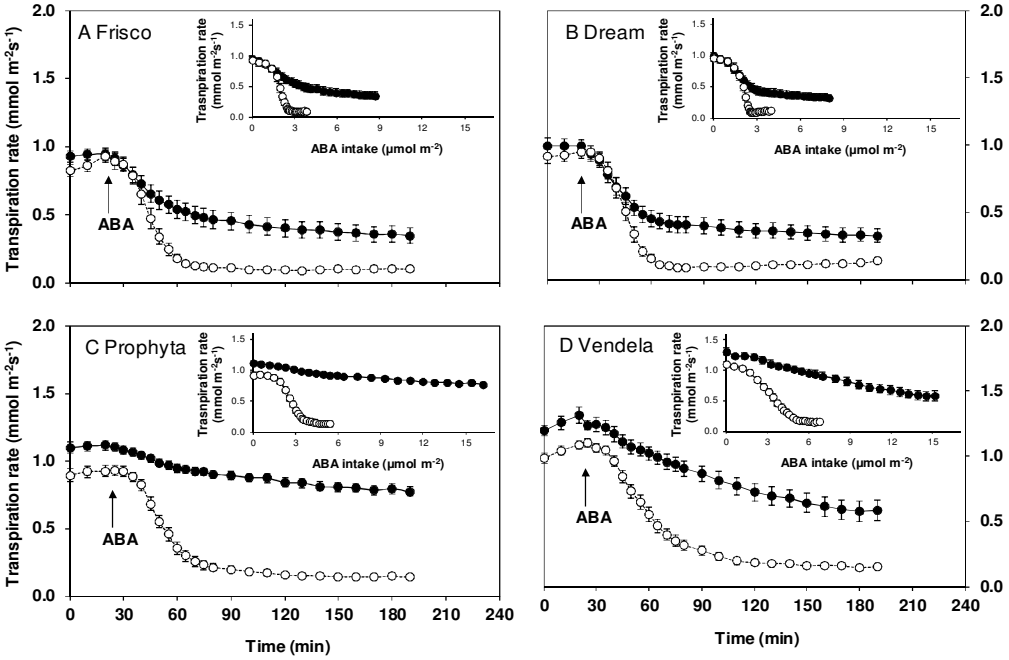

Fig. 2. Dynamics of leaflet transpiration rate during ABA feeding (100 µM) through the transpiration stream in four cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. The inserts show the relationship between leaflet transpiration rate and ABA intake. Black arrows represent the time of ABA feeding (t = 25 min). Values are the mean of 12 leaflets ± SEM.

Table 2. Effect of ABA feeding through the transpiration stream on the stomatal closure in four cut rose cultivars grown at moderate (60%) or high (95%) relative air humidity (RH). Abbreviations: ABA_{LA} = amount of ABA required to stabilize the transpiration rate per leaflet area; ABA_{stoma} = amount of ABA required to stabilize the transpiration rate per stoma. Values are the mean of 12 leaflets ± SEM. Means followed by different letters indicate significant differences according to LSD-test (comparison in columns).

Cultivar	RH (%)	ABA_{LA} (µmol m^{-2})	ABA_{stoma} (pmol $(stoma)^{-1}$)
Frisco	60	2.84 ^{ab}	0.05 ^{a}
	95	7.95 ^{e}	0.13 ^{bc}
Dream	60	2.53 ^{a}	0.06 ^{a}
	95	6.97 ^{de}	0.15 ^{c}
Prophyta	60	4.58 ^{bc}	0.11 ^{b}
	95	14.8 ^{f}	0.32 ^{e}
Vendela	60	5.83 ^{cd}	0.11 ^{b}
	95	14.0 ^{f}	0.22 ^{d}
F pr.			
Cv.×RH		<0.001	<0.001

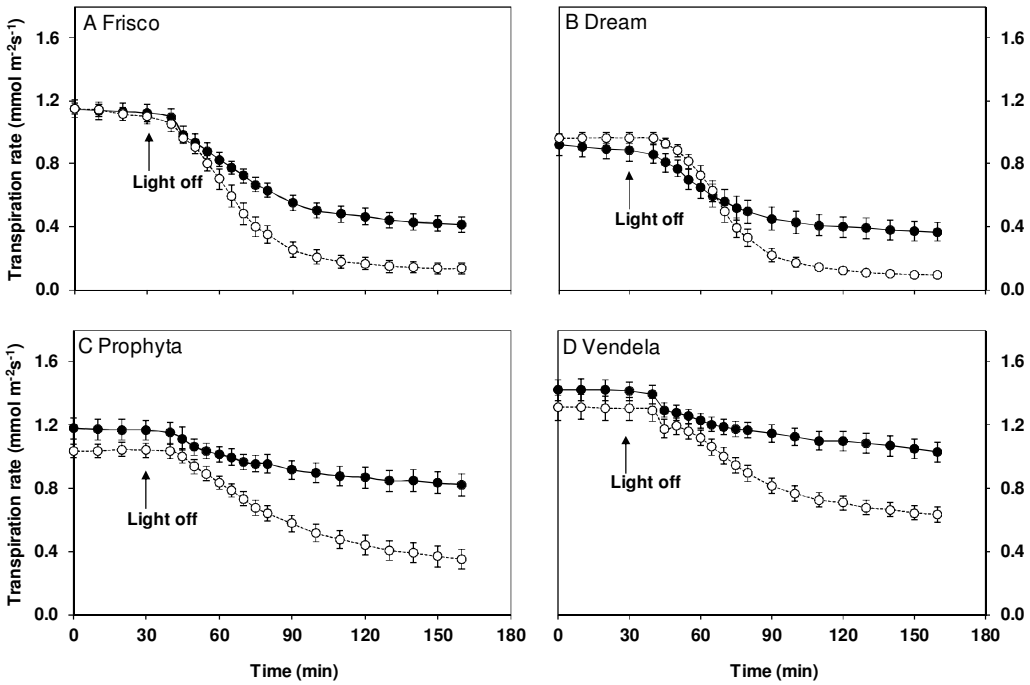

Fig. 3. Dynamics of leaflet transpiration rate after 2 h 10 min of darkness in four cut rose cultivars, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Black arrows represent the time when the light was turned off (t = 30 min). Values are the mean of 12 leaflets ± SEM.

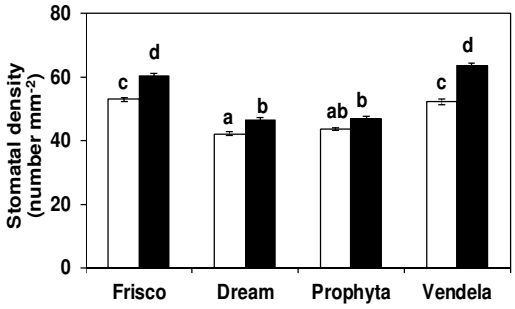

Fig. 4. Stomatal density of four cut rose cultivars, grown at moderate (60%, open columns) or high (95%, closed columns) relative air humidity. Values are the mean of 10 (cv. Prophyta, 95%) or 12 leaflets ± SEM. Different letters indicate significant differences according to LSD-test.

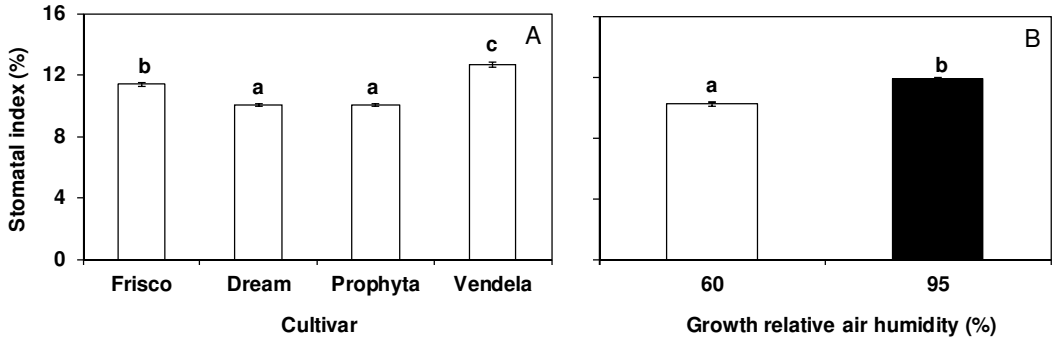

Fig. 5. Effect of the cultivar (A) and relative air humidity [moderate (60%, open columns) and high (95%, closed columns)] (B) on stomatal index. Values are the mean of 22 (cv. Prophyta) or 24 leaflets per cultivar, and 46 (95%) or 48 (60%) leaflets per relative air humidity ± SEM. Different letters indicate significant differences according to LSD-test.

Relationship between stomatal closing ability and stomatal length

In general, under non-stressed conditions, it was found that pore aperture was linearly related to the stomatal length. Thus, the higher the stomatal length, the bigger the pore aperture in both cvs. Frisco and Prophyta (Fig. 6A, B). Nevertheless, when evaluating the stomatal closing ability in response to 35 min desiccation as a function of stomatal length, it was shown that these two traits are not correlated with each other, both in moderate and high RH-grown leaflets (Fig. 6C, D). For instance, within the complete stomatal length range observed in cv. Prophyta grown at high RH (i.e. from 41 to 54 μm), the stomatal aperture was rather constant (5.2 ± 0.2 μm) (Fig. 6D).

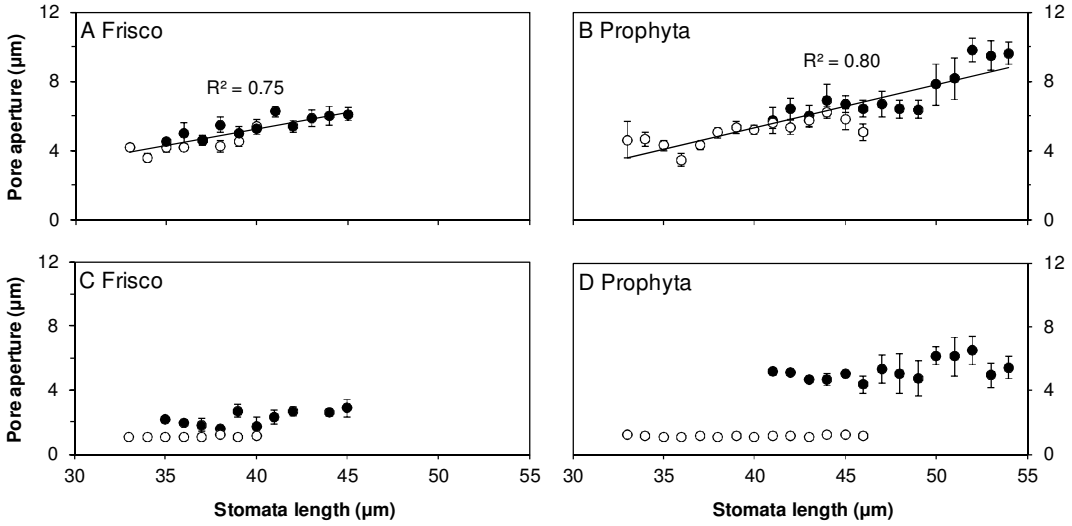

Fig. 6. Relationship between pore aperture and stomatal length under non-stressed conditions (A, B) and after 35 min of desiccation (C, D) in two cut rose cultivars grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Values are the mean of at least 12 stomata ± SEM.

Modelling stomatal conductance (g_s)

A standard formula (Nobel 1991) was used to determine the relative importance of changes in anatomical features and stomatal functionality (stomatal opening) on the higher g_s of high RH-grown leaflets (Fig. 7). Higher pore aperture values were the most important determinant of an increase in g_s (i.e. showing the steepest slope in Fig. 7) caused by high RH during cultivation in all studied cultivars. This was followed by the increased pore length in the cvs. Dream and Prophyta (Fig. 7B, C), while in cvs. Frisco and Vendela increased pore length and stomatal density were of equal importance (Fig. 7A, D). At mid-range values for all other parameters, the relative influence of changes in anatomical features (i.e. higher stomatal density, longer pore length and pore depth) was in the range of 16–30%, with the highest contribution observed in cv. Dream.

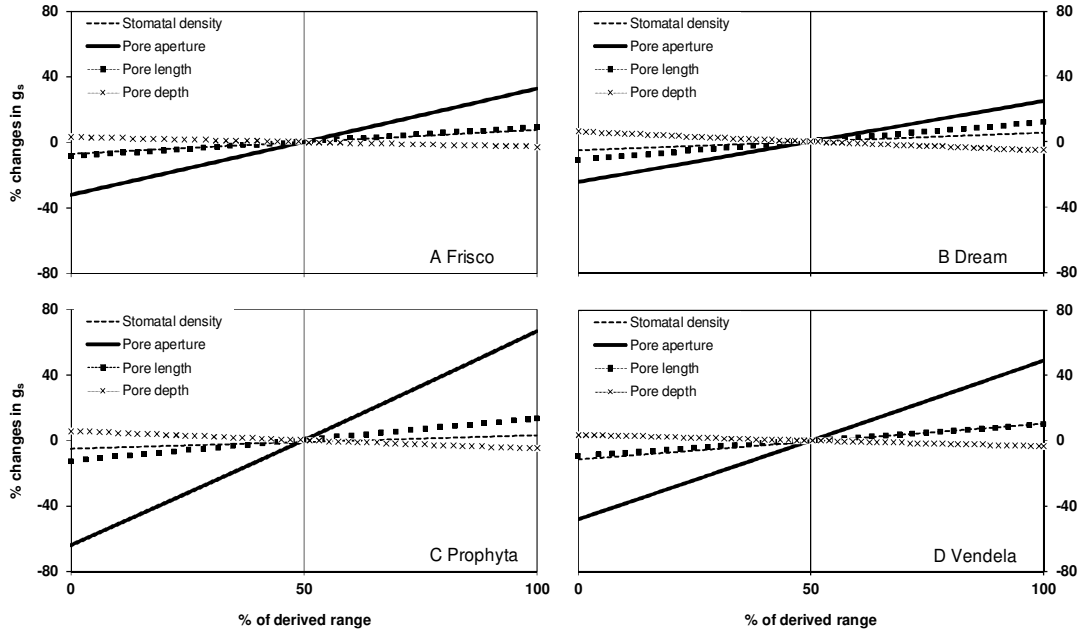

Fig. 7. Predicted sensitivity of four cut rose cultivars' stomatal conductance (g_s) to changes in stomatal density and pore dimensions caused by high relative air humidity (RH) during cultivation within empirically derived ranges under different RH levels (60 and 95%). The range of values employed in the analysis, derived from observations after 35 min of desiccation, is presented in Figure 4 and Table 1. g_s to water vapour was predicted using Eqn 2. The vertical lines represent the g_s obtained using the mid-range value for each variable; this was 65, 60, 111 and 66 mmol H_20 m^{-2} s^{-1} for cvs. Frisco, Dream, Prophyta and Vendela, respectively. Effects of adjusting each anatomical feature within its empirical range were calculated, keeping the other values constant and expressed as a percentage of the mid-range value.

Cuticular permeability (Pe)

The cuticular water loss was determined by allowing leaflet desiccation only from the upper cuticle (stomata free) as the abaxial leaflet surface was sealed. High RH-grown

plants presented a significantly higher (31%) cuticular transpiration rate after 2 h of desiccation, as compared to plants grown at moderate RH ($P = 0.048$; Fig. 8).

The relative contribution of cuticular water loss to the total leaflet water loss was assessed in both non-stressed leaflets (leaflets in water) and under conditions of maximum stomatal closure (leaflets fed with ABA for 2 h 40 min) in leaflets that have been developed at moderate and high RH (data of Fig. 2). In non-stressed leaflets the water loss through the stomata was two orders of magnitude higher than the water loss through the cuticle, irrespective of the moisture ambient conditions during leaf development. Under conditions of maximum stomatal closure, it was found that the residual stomatal transpiration contributed from 57% (cv. Dream) up to 81% (cvs. Frisco, Prophyta and Vendela) to the total leaflet water loss in plants grown under moderate RH and that these values were significantly higher in leaflets developed at high RH. Therefore, the fraction of water loss through the cuticle, compared to the water loss through the stomata, was considerably lower in leaflets developed at high RH than moderate RH-grown leaflets.

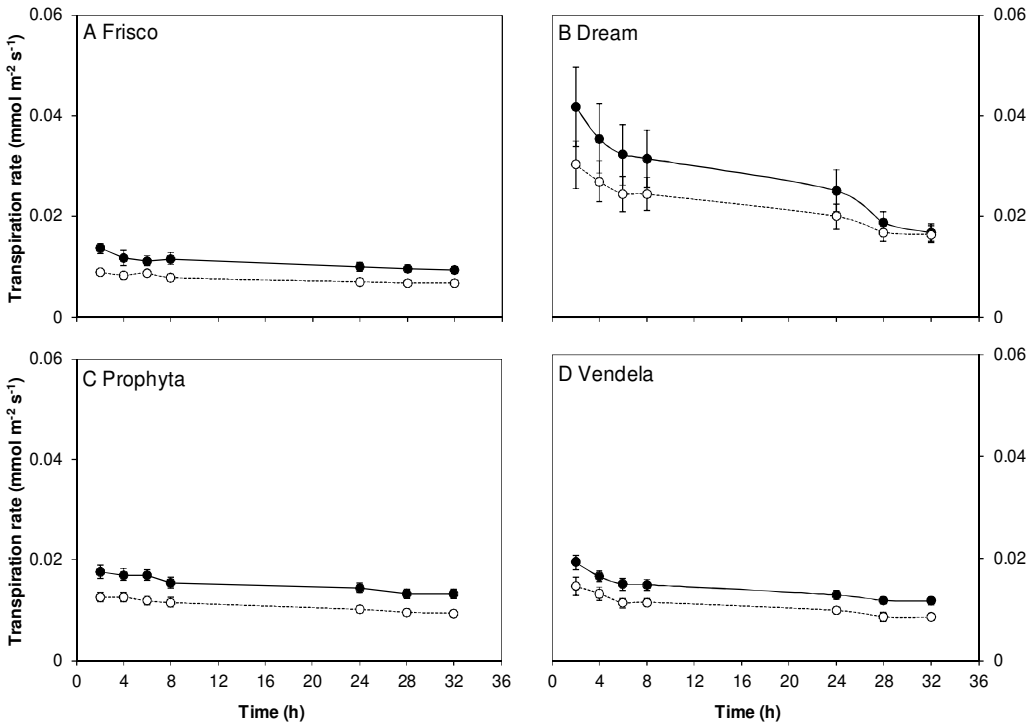

Fig. 8. Dynamics of cuticular transpiration rate during 32 h of desiccation in four cut rose cultivars grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. Values are the mean of 12 leaflets ± SEM.

Discussion

Stomatal closing ability

Long-term high atmospheric humidity (95%) decreased the degree and the speed at which stomata responded to three different closing stimuli (i.e. desiccation, ABA feeding and light/dark transition) resulting in higher transpiration rates in the four studied cultivars (Fig. 1, 2 and 3). These findings are in agreement with previous work on roses (Fanourakis et al. 2011, Torre and Fjeld 2001) and other species (Fordham et al. 2001, Kawamitsu et al. 1993, Rezaei Nejad and van Meeteren 2005). We here clearly demonstrated that there is a strong and consistent genotypic component in the stomatal closing ability as certain cultivars had systematically more responsive stomata after cultivation at high RH (i.e. tolerant cvs. Frisco and Dream; Fig. 1, 2 and 3).

The reasons for a contrasting stomatal sensitivity to high RH are not as yet understood. Recent studies have suggested that short-term lack of ABA was not responsible for non-stomatal closure in high RH-grown leaves (Fanourakis et al. 2011, Rezaei Nejad and van Meeteren 2007). Similarly, in this study we have shown that in the four studied cultivars when grown under high RH a short-term ABA feeding through the leaflet petiole did not induce proper stomatal closure (Fig. 2). Nevertheless, the lower foliar ABA content during leaf development at elevated atmospheric humidity, compared to leaves grown at lower humidities, has been suggested to mediate the reduced stomatal responsiveness of high-RH grown leaves (Rezaei Nejad and van Meeteren 2007, S Torre: personal communication). Thus, the stronger stomatal responsiveness to closing stimuli, observed in the cvs. Frisco and Dream when grown in high RH as compared to cvs. Prophyta and Vendela, might suggest that the former cultivars (i) undergo a lower decrease in the endogenous leaf ABA levels, induced by long-term high RH during growth, (ii) sustain stomatal functionality at lower levels of endogenous ABA or (iii) the same decrease in the bulk leaf ABA takes place, but the ABA content in the ABA action sites, i.e. the symplast and apoplast of guard cells (Finkelstein 2006), is higher. To better understand this process, detailed information on leaf endogenous ABA concentration and compartmentation in contrasting cultivars in relation to their sensitivity to high RH is needed.

Stomatal density, index, and dimensions

Long-term high RH during leaf development besides resulting in higher stomatal density (Fig. 4) and SI (Fig. 5), it also led to bigger stomata in the four studied cultivars (Table 1). Since an increase in the stomatal density *per se* causes a proportional increase in the transpiration rate (Nobel 1991, Parlange and Waggoner 1970), it could be expected that rose genotypes with a higher water loss rate following growth at elevated RH (i.e. sensitive cultivars) would also have a greater increase in the stomatal density. Interestingly, this did not happen as cv. Prophyta (the most sensitive cultivar; Fig. 1, 2

and 3) was the only cultivar where the stomatal density was not significantly enhanced when grown under long-term elevated RH (Fig. 4). Moreover, this cultivar had the lowest stomatal density, revealing that stomatal density is not a key trait for tolerance to high RH.

The higher SI in high RH-grown plants (Fig. 5) was the combined result of increased stomatal initiation and decreased epidermal cell density (data not shown). Therefore, it can be concluded that high RH during leaf expansion affected the cell differentiation in rose. However, no effect of high RH on SI has been reported in other species (Bakker 1991, Rezaei Nejad and van Meeteren 2005).

A positive response of stomatal size to elevated RH levels (Table 1) has been previously described and it appears to be consistent among many different species (Bakker 1991, Gislerød and Nelson 1989, Rezaei Nejad and van Meeteren 2005, Torre et al. 2003). In the current study it was shown that high RH during leaf expansion promoted developmental changes in stomatal size mainly through a significant increase in the stomatal length rather than significantly wider stomata (Table 1). An insensitivity of stomatal width to long-term changes in the moisture ambient conditions during leaf development is in agreement with Aasamma et al. (2001) who also observed that stomatal width was not influenced by the growing conditions (water stress and nitrogen fertilization).

Relationship between stomatal closing ability and stomatal length

To the best of our knowledge the relationship between stomatal closing ability and stomatal length was not previously investigated in plants grown under long-term high RH levels. Unlike our hypothesis that bigger stomata in high RH-grown leaves have a reduced closing ability, the current data show that longer stomata (in dimensions not present at moderate RH) have the same physiological response as shorter stomata (Fig. 6C, D). Thus, it is concluded that longer stomata are not responsible for poor stomatal functioning in high RH-grown leaves.

Modelling stomatal conductance (g_s)

The effects of changes in stomatal density and pore dimensions (aperture, length and depth) on the g_s were estimated in leaflets that had been dehydrated for 35 min (Fig. 7). For all studied cultivars, the main determinant of higher g_s values when grown at elevated RH was pore aperture (stomatal opening). These findings are in agreement with our hypothesis that genotypic variation in the control of water loss, as a result of leaf expansion at high atmospheric humidity, is mainly driven by changes in the stomatal closing ability, rather than anatomical alterations.

This analysis also stresses the importance of the anatomical features (i.e. higher stomatal density, longer pore length and depth) on the enhanced water loss of high RH-grown plants. Assessing the humidity level effect on stomatal closing ability by

measuring the water loss rate and not taking into account the contribution of anatomical features, significantly overestimates this effect.

Cuticular permeability (Pe)

In the four studied cultivars, leaflets developed at high RH had higher *Pe* compared to moderate RH-grown leaflets (Fig. 8, and also described by Karbulkova et al. 2008). In contrast, Torre and co-authors did not find differences in the *Pe* of rose leaflets grown at moderate or high RH, when using a porometer (Torre and Fjeld 2001, Torre et al. 2001).

In non-stressed leaflets, the cuticular contribution to the total leaflet water loss was minimal, which is in agreement with previous studies (Goodwin and Jenks 2005). Moreover, we here show that residual stomatal transpiration is the major determinant of total leaflet transpiration under conditions of maximum stomatal closure in both moderate and high RH-grown plants. Nevertheless, the contribution of the residual stomatal transpiration to the total leaflet transpiration was higher in leaflets developed at high RH. Thus, the higher water loss, as a result of leaf development at elevated ambient humidity, is predominantly caused by the lower stomatal sensitivity to closing stimuli and to a lesser extent due to higher *Pe*.

Conclusions

Plants grown at elevated ambient humidity (95%) showed increased water loss rates when subjected to several stomatal closing stimuli to an extent which was cultivar dependent. This higher water loss resulted from increased residual stomatal transpiration, since the cuticular contribution to the total leaflet water loss was relatively small. Moreover, it was concluded that the stomatal responsiveness to closing stimuli is the basis of the large genotypic variation in the regulation of water loss in high RH-grown plants. The extent that stomatal closing ability was impaired and the degree to which stomatal features (i.e. density and size) were affected were not correlated. Nevertheless, anatomical features (i.e. stomatal density, pore length and depth) have a direct and significant contribution to the higher water loss rates. Hence, these results suggest that increased water loss, as a result of more humid air during leaf expansion, reflects changes in both stomatal closing ability and anatomical features.

Acknowledgements

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CHAPTER 3.2

Breeding cut roses for better keeping quality: first steps

Abstract

Water stress is one of the most common postharvest quality problems, resulting in shorter vase life of cut flowers. Since vase life is a key factor for the consumers' satisfaction, breeding for better control of water loss is an important goal. In this study, we evaluated the stomatal responses to leaflet desiccation in a subset of a segregating tetraploid cut rose population (60 genotypes) grown at high relative air humidity (RH ≥ 85%). Additionally, the vase life was determined in six contrasting genotypes in their stomatal responsiveness. The population screening revealed extreme differences among genotypes, i.e. the relative water content (RWC) after 4 h of leaflet desiccation ranged between 7 and 62% (20 and 51% the RWCs for the two parents). Genotypes with low stomatal responsiveness to desiccation had an average vase life of 8 days, and in two out of three genotypes the flower did not open. These effects are related to high postharvest water loss rates. On the other hand, genotypes with high stomatal responsiveness lasted longer (10−21 days), their flower reached the maximum opening stage, and they showed a better control of water loss. It is concluded that there is a large variation in the stomatal hydrosensitivity, which increases the possibilities for breeding for cultivars with longer vase life (better control of water loss). The selection of genotypes with high stomatal responsiveness, after cultivation at high RH, considerably increases the degree of certainty that the cut flower will last a minimum length of time, and will have an unhampered flower opening.

Fanourakis D, Carvalho DRA, Gitonga VW, van Heusden AW, Almeida DPF, Heuvelink E, Carvalho SMP (2011) Breeding cut roses for better keeping quality: first steps. Acta Horticulturae (in press)

Introduction

Factors determining the keeping quality of cut roses are under intense investigation for several decades (Mayak et al. 1974, van Doorn 2011). Although a significant progress has been made in the understanding of the underlying mechanisms behind this process, the question raised in late 1980s 'why don't roses last?' (Zieslin 1989) has still not been fully answered. For many years research has been focused on minimizing any decrease of the life span after flowers have been cut from the mother plant (i.e. during the postharvest period). This is the case of the large number of studies on the use of different preservative solutions to enhance water uptake (van Doorn 1999). However, the physiological and anatomical traits that determine the potential vase life (i.e. maximum vase life) have already been established during the preharvest phase (Fig. 1). The potential vase life is, therefore, the result of the complex interaction between genotype and environment during cultivation (van Meeteren et al. 2005). On the other hand, the actual vase life will be a function of the potential vase life and of the conditions and duration of the postharvest phase (Fig. 1).

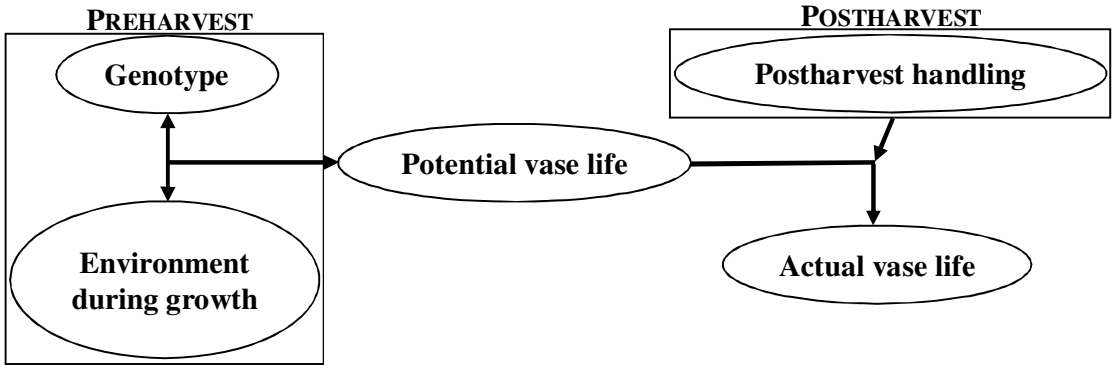

Fig. 1. Schematic representation of the factors involved on the potential and actual vase life.

Many of the postharvest symptoms that lead to vase life termination are dependent on the water balance of the cut flowers, which is determined by the water uptake and transpiration rates (van Doorn 1997, 2011). Water stress (water loss > water uptake) is considered one of the most common postharvest quality problems, leading to precocious senesce of cut flowers (Alaey et al. 2011). Preharvest high relative air humidity (RH ≥ 85%) affects negatively the control of water loss, due to stomatal malfunction, and results in lower potential vase life (Mortensen and Gislerød 1999, Torre and Fjeld 2001). This effect of high RH on stomatal responsiveness is cultivar dependent (Fanourakis et al. 2011b). These authors showed that the relative water content (RWC) after 4 h of desiccation could discriminate between tolerant and sensitive cultivars in a group of four cultivars. Besides being a constraining factor for keeping quality, disturbed water relations, as a result of plant growth at high RH, have been associated with reduced flower opening (Mortensen and Gislerød 2005).

To breed for cultivars with more responsive stomata after cultivation at high RH, it is important to know the existing variation in this trait. The key objectives of this research were: (i) to screen a segregating tetraploid cut rose population grown at high RH for stomatal responses to water loss (i.e. leaflet desiccation), and (ii) to evaluate the vase life and flower opening of contrasting genotypes in their stomatal responsiveness, in order to test the validity of the method employed (i.e. RWC after 4 h of leaflet desiccation) as a reliable screening tool. The knowledge gained in this project is expected to contribute to fasten the selection criteria and procedures for breeding for cultivars with longer vase life (better control of water loss) after cultivation at high RH and to minimize quality problems resulting from increased water loss rates during postharvest phase.

Materials and Methods

Plant material and growth conditions

Plants were grown in a multispan Venlo-type glasshouse (144 m^2) located in central eastern part of The Netherlands (Wageningen, 56^{o}N). Sampling took place from October 2009 to February 2010, using a subset of 60 genotypes from a tetraploid (K5) cut rose population - with a total of 181 individuals, obtained from a cross between two genotypes (P540, P867). This population was created by Yan et al. (2006), for studying resistance to powdery mildew. Cuttings from each of the genotypes were made from mother plants of the same age and rooted in commercial potting soil (Jongkind Grond BV, Aalsmeer, The Netherlands). Each genotype had four replications and several flowering stems per replication when the canopy was closed. The two outer beds, as well as the pots on both ends of each of the remaining beds, acted as borders. Two cuttings were planted per pot on April 2007, using 10 L pots filled with cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) leading to a density of six plants m^{-2}. Four weeks after plantation the plants were pinched, leaving 4–6 leaves on the primary shoots to promote the formation of basal shoots. Plants were structured according to the bending technique, which was applied to low quality shoots (weak, short and blind shoots). These shoots were bent down into the paths weekly, and harvestable shoots formed the upright canopy. Four months after planting, the bent-canopy completely filled the spaces between the rows. Since then, continuous harvesting was practiced. Plants were fertilized using a drip system. The nutrient solution used, is described by Fanourakis et al. (2010). The drainage rate was about 30%. Chemical pest disease control followed commercial practices.

Supplementary light was provided to the crop by high-pressure sodium lamps (SON-T Agro, Philips, Eindhoven, The Netherlands). The lamps were switched on when outside global radiation was lower than 200 W m^{-2}, and switched off when higher than

300 W m^{-2}. The realized RH was 85 ± 0.4 and 89 ± 0.4% for the experiments 1 and 2, respectively. The realized temperature during these periods was 19.1 ± 0.1 and 18.5 ± 0.1 °C respectively, resulting in vapour pressure deficits (VPDs) of 0.32 ± 0.01 kPa (experiment 1) or 0.23 ± 0.01 kPa (experiment 2). The greenhouse was heated by means of heating pipes located below the planting rows. Throughout the experimental period, climate data were automatically recorded each 5 min by a commercial computer system (Hoogendoorn, Vlaardingen, The Netherlands), and average values were calculated per day.

Experiment 1: Population screening

To evaluate the stomatal closing ability in response to desiccation the terminal leaflets of the 1st to 3rd penta-foliate leaves from the top of the plant were detached, their petioles were recut under degassed water (to prevent cavitation induced-embolism), and were placed in flasks filled with water. These leaflets were further incubated in a saturated humidity environment (21 °C, about 100% RH; VPD close to 0) for 1 h to establish their maximum fresh weight. The rehydration process took place under light (15 μmol m^{-2} s^{-1}), since following darkness light-induced stomatal opening requires up to 1 h (Blom-Zandstra et al. 1995). Subsequently, the petioles were removed from water and the leaflets were placed in the test room on a table (abaxial surface down) (21 °C, 50 ± 3% RH, 1.24 ± 0.07 kPa VPD and 50 μmol m^{-2} s^{-1}), where their weight was determined at time 0 and 4 h. Subsequently the leaflets were dried at 105 °C for 24 h. The RWC after 4 h of desiccation was calculated according to Slavik (1974) (Eqn 1).

$$\mathrm{RWC} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Saturated Fresh Weight} - \text{Dry Weight}} \times 100 \qquad (1)$$

These measurements were conducted in 60 genotypes of the K5 tetraploid cut rose population, including the two parents, using fully developed leaflets from stalks with a flower bud at stage 2 (VBN 2005; see section below for detailed description). The leaflet samples were collected between 0800 and 0900 hours (at least 1 h after the onset of the photoperiod). In each genotype, 12 leaflets (one leaflet per stem) were measured.

Experiment 2: Vase life duration and flower opening

Based on the results from Expt. 1, we selected three genotypes with high and three genotypes with low stomatal responsiveness to desiccation. Flower stems were collected at harvest stage [stage 2, VBN (2005)]. Flowering stalks were normalized at the same stem length (60 cm) and number of penta-foliate leaves (four). These were cut in air, left to aspire air for 2–3 min, and placed in flasks (one flower per flask) containing 300 ml of artificial vase solution (0.7 mM $CaCl_2.2H_2O$, 1.5 mM $NaHCO_3$, 0.005 mM $CuSO_4.5H_2O$; van Meeteren et al. 2001). Subsequently, for vase life evaluation, the cut flowering stems

were placed in a climate-controlled room at 21 °C, 50 ± 3% RH and light for 12 subsequent h per day at a photon flux density of 10–12 μmol m^{-2} s^{-1} (provided with fluorescent lamps; TLD 58W/84, Philips, Eindhoven, The Netherlands). The height of the vase solution column was held constant over the evaluation period to avoid hydrostatic pressure differences between genotypes with different transpiration rates (Mensink and van Doorn 2001). The top of the flask was covered with waterproof parafilm, to ensure that water loss could occur via the flower only. The VBN criteria (2005) were used to define the end of vase life [i.e. pedicel bending (bent-neck); more than two fallen petals; flower visibly wilted, i.e. loss of petal turgor; more than 50% of leaves have fallen, turned to yellow or were desiccated]. In this study no *Botrytis cinerea* incidents occurred.

During the postharvest phase, the water loss was recorded daily. Moreover, the symptoms terminating the vase life, the flower stage at that time and the leaf area (model 3100 Area Meter, LI-COR, Lincoln, NE) were determined. For the flower stage, the scale of VBN (2005) was utilized (stage 1: firm pointed bud; stage 2: loose pointed bud with a cylindrical shape; stage 3: half-open flower; stage 4: open flower; and stage 5: maximally opened flower with visible anthers).

Flowering stalks were harvested between 0700 and 0800 hours. Seven flowers per genotype were evaluated.

Results

Population screening

The stomatal responsiveness to leaflet desiccation was evaluated in 60 genotypes of the K5 cut rose population grown at high RH (≥ 85%). The relative water content (RWC) 4 h after the onset of desiccation was used as a measure of stomatal closure. The higher this value, the stronger the stomatal response to desication, and thus the better the control of water loss. The RWC after 4 h of desiccation varied between 7 and 62% (with the parents P867 and P540 having 20 and 51% RWCs, respectively), which reveals a large range of stomatal responses (Fig. 2).

Vase life duration and flower opening

The vase life of genotypes with high stomatal responsiveness after 4 h of desiccation was very variable ranging from 10 to 21 days (Fig. 3A). In contrast, the vase life of genotypes with low stomatal responsiveness did not exceed nine days and had on average 70% higher transpiration rate during the postharvest phase (Fig. 3B). Moreover, unlike the genotypes with high stomatal responsiveness, the latter genotypes did not always reach the stage where anthers are visible, which represents the maximum flower diameter (stage 5; Fig. 4).

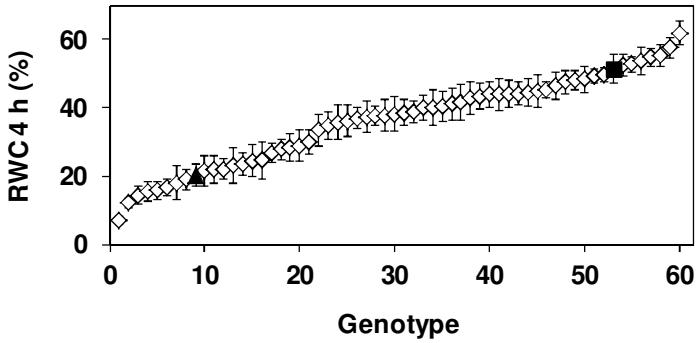

Fig. 2. Relative water content (RWC) after 4 h of leaflet desiccation in 60 genotypes of the K5 cut rose population grown at high relative air humidity (≥ 85%). The filled symbols represent the parents (triangle, P867; square, P540). Vertical bars indicate SEM (n = 12).

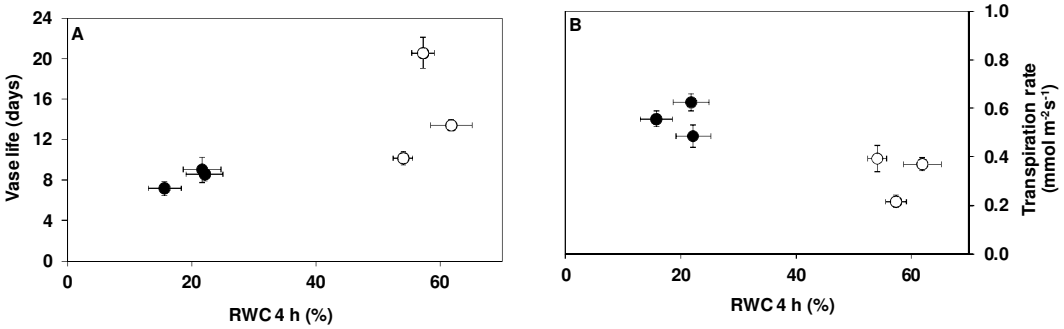

Fig. 3. Length of vase life (A), and average transpiration rate during the postharvest phase (B) as a function of relative water content (RWC) after 4 h of leaflet desiccation in three cut rose genotypes with high (○) and three genotypes with low (●) stomatal responsiveness grown at high relative air humidity (≥ 85%). Vertical (n = 7) and horizontal (n = 12) bars indicate SEM.

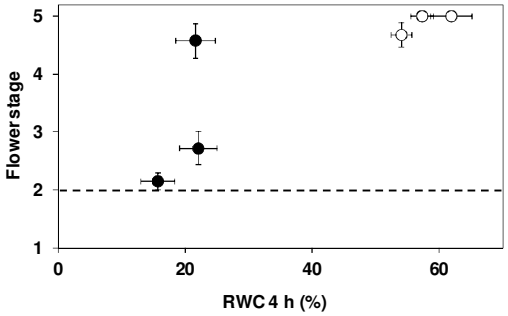

Fig. 4. Flower opening stage at the end of vase life as a function of relative water content (RWC) after 4 h of leaflet desiccation in three cut rose genotypes with high (○) and three genotypes with low (●) stomatal responsiveness grown at high RH (≥ 85%). The flower stages were according to the scale of VBN ranging from 1 to 5. The dashed line depicts the flower stage, at which the flowering stalks were harvested. Vertical (n = 7) and horizontal (n = 12) bars indicate SEM.

Discussion

Due to the increased competition in the ornamental horticultural sector, production of high quality plants is of utmost importance. Keeping quality is a very important factor for the consumers' satisfaction. Furthermore, the increasing number of growers, working with a label for postharvest quality, or selling to buyers who want a vase life guarantee (Rikken 2010), will increase the need to understand the physiological and genetic background of keeping quality.

In this study, a simple method for screening for stomatal responsiveness to water loss is proposed. Using that method a very large variation in the stomatal responses was observed within the 60 studied genotypes of the K5 cut rose population grown at high RH (≥ 85%) (Fig. 2). Cultivar differences in the control of water loss, as a result of plant growth at high RH, has also been recently reported in a group of four commercial cultivars (Fanourakis et al. 2011b). Long-term high RH exerts a negative effect on the stomatal responsiveness to closing stimuli (e.g. desiccation, and light/dark transition, which are conditions that normally take place during the postharvest phase) in several species (Islam et al. 2010, Pospisilova 1996), including rose (Fanourakis et al. 2009, Ottosen et al. 2002). This effect seems to be mediated by a long-term decreased foliar abscisic acid (ABA) content in plants grown at high RH, though the underlying processes behind this phenomenon are not yet fully understood (Fanourakis et al. 2011a, Rezaei Nejad and van Meeteren 2007). The remarkable differences found in the stomatal responsiveness to water loss after cultivation at high RH, may arise from a genotypic variation in the endogenous ABA content or a diferential sensitivity of the guard cells to the same ABA concentration.

The water loss is one of the two components of water balance, which is decisive for the vase life but it is not the only factor (van Doorn 1997, 2011). In genotypes with high stomatal responsiveness the vase life is not precociously limited by the water loss (Fig. 3B), and it will terminate as a result of another limiting factor (e.g. sensitivity to air emboli at the cut surface, and restoration capacity of air emboli). In contrast, the vase life of genotypes with low stomatal responsiveness is always limited by the high water loss. In the current experiment the longest vase life of the latter genotypes was nine days, in contrast with the 21 days achieved in genotypes with high stomatal responsiveness (Fig. 3A). Since in several cases the distances between production sites and the final consumer are still increasing, the vase life must be sufficiently long to meet the needs of the supply chain. In those cases a vase life of nine days might be too short. Besides the keeping quality, flower opening is an important trait. Genotypes with low stomatal responsiveness had a very limited flower opening, often remaining as buds until the end of vase life (Fig. 4). This might be the result of the strong competition for water between the leaves and the flower bud (van Doorn et al. 1991).

Breeding programs for crop improvement in cut roses are mainly focused on productivity, external quality traits (e.g. flower colour or presence of thorns, Gitonga et al. 2009) and improved disease resistance (Yan et al. 2006). Although the vase life of a new cultivar is always tested before they are launched into the market, this is often done in a later phase of the breeding program. Nevertheless, this trait can lead to a rejection of a promising cultivar that has been selected for other purposes. Therefore, an early identification of genotypes with a very poor potential vase life increases the efficiency of the breeding programs.

Currently the map of the K5 population is under construction and QTL analysis will be carried out for the stomatal responsiveness traits. Moreover, the reliability of this screening method should be also tested under moderate RH conditions, which are more common levels in the greenhouse throughout the year (rather than higher or equal to 85%, which is typical for the winter period; Mortensen and Gislerød 2005).

Conclusions

A large genotypic variation has been observed for stomatal responsiveness to leaflet desiccation in a tetraploid cut rose population and a contrasting behaviour between the parents. The RWC after 4 h of leaflet desiccation proved to be a quick and reliable method suitable for large-scale screening of rose genotypes for stomatal responses to water stress. Incorporation of such protocol in the breeding programs will improve the selection procedures and will minimize keeping quality problems throughout the supply chain, since it allows an early detection of genotypes with a short potential vase life.

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CHAPTER 4

Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning

Abstract

Plants of several species, if grown at high relative air humidity (RH ≥ 85%), develop stomata that fail to close fully in case of low leaf water potential. We studied the effect of a reciprocal change in RH, at different stages of leaflet expansion of *Rosa hybrida* L. grown at moderate (60%) or high (95%) RH, on the stomatal closing ability. This was assessed by measuring the leaflet transpiration rate in response to desiccation once the leaflets had fully expanded. For leaflets that started expanding at high RH but completed their expansion after transfer to moderate RH, the earlier this switch took place the better the stomatal functioning. Leaflets initially expanding at moderate RH and transferred to high RH exhibited poor stomatal functioning, even when this transfer occurred very late during leaflet expansion. Applying a daily abscisic acid (ABA) solution to the leaves of plants grown at continuous high RH was effective in inducing stomatal closure at low water potential, if done before full leaflet expansion. After full leaflet expansion, stomatal functioning was no longer affected either by the RH or ABA level. The results indicate that the degree of stomatal adaptation depends on both the timing and duration of exposure to high RH. It is concluded that stomatal functionality is strongly dependent on the humidity at which the leaf completed its expansion**.** The data also show that the effect of ambient RH and the alleviating role of ABA are restricted to the period of leaf expansion.

Fanourakis D, Carvalho SMP, Almeida DPF, Heuvelink E (2011) Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiologia Plantarum 142: 274–286

Introduction

From the time that plants grow on land the proper regulation of water loss is essential to their survival. Stomatal opening is often a compromise between the required uptake of carbon dioxide and the prevention of excessive loss of water. Stomatal movement is regulated by external environmental conditions, such as light, carbon dioxide concentration and vapour pressure deficit (VPD) via a complex signalling network (reviewed by Hetherington and Woodward 2003, Schroeder et al. 2001). These environmental stimuli control not only the opening and closing of stomatal pores on a short-term basis (minutes to hours), but can also permanently alter the stomatal functioning (long-term responses). For example, it is well documented for several plant species that the capacity of the stomata to close in response to water stress is strongly influenced by the relative air humidity (RH) level during growth (Rezaei Nejad and van Meeteren 2005, Torre et al. 2003). Stomata developed under long-term high RH (≥ 85%) show distinct anatomical features and are less sensitive to low leaf water potential compared to those that develop at moderate RH. This phenomenon has been reported in plants grown under high RH and transferred suddenly to conditions of increased evaporative demand, namely leafy cuttings rooted at high RH (Fordham et al. 2001), in vitro propagated plants (Santamaria et al. 1993) and cut flowers grown in greenhouses at high RH (Mortensen and Gislerød 2000). Lack of proper stomatal closure has been identified as the major cause leading to disturbed water relations in high RH-grown plants (Aquilar et al. 2000, Mortensen and Gislerød 2005).

Although stomatal acclimation to RH has been largely described for plants grown continuously at high RH, this acclimation process is not yet fully understood (Rezaei Nejad and van Meeteren 2008). According to Schoch et al. (1980) there is a brief time during which stomatal development is most sensitive to environmental changes. The dynamics of stomatal adaptation to long-term alterations in the RH during cultivation, i.e. the adaption capacity of stomatal apparatus when transferring plants from one RH level to a contrasting one, has been poorly addressed. From the few studies available most were only focused on stomatal adaptation to a contrasting RH after full leaf expansion (e.g. Mortensen and Gislerød 2000, Pospisilova 1996). It was shown that transfer of the plants, after leaf expansion, to a new RH level (i.e. moderate to high RH, or vice versa) resulted in a full stomatal adaptation to the new RH environment in *Phaseolus vulgaris* (Pospisilova 1996), whereas it had no effect on stomatal responsiveness in *Rosa hybrida* (Mortensen and Gislerød 2000). Recently, Rezaei Nejad and van Meeteren (2008) went one step further by comparing the degree of stomatal adaptation to contrasting RH levels in expanding and in fully expanded leaves. It was shown that stomata of *Tradescantia virginiana* were able to adapt to a change in RH but the degree of adaption was higher in expanding leaves. However, these authors only examined one

stage of leaf development. Thus it is still unclear whether stomatal functioning is mostly determined during the first stages of leaf development or during later stages.

The role of abscisic acid (ABA) in the control of stomatal functioning has been well described for several species grown under drought stress conditions (e.g. Zhang and Davies 1990, Zhang and Outlaw 2001). However, the reasons why stomata fail to close fully in response to leaf dehydration in plants subjected to prolonged exposure to high RH are not completely clear. Since the stomata respond to the rate of transpiration rather than to RH itself (Mott and Parkhurst 1991), stomatal malfunctioning could be a consequence of the low transpiration rate. This in turn creates a situation where the plants are subjected to a long-term abnormally high leaf water potential (weaker hydraulic signalling) and to a long-term low leaf ABA concentration (weaker chemical signalling). Since the leaf ABA concentration is influenced by the ABA import via the xylem from the roots (Zhang and Outlaw 2001), it is expected that in plants growing at high RH this import will decrease due to the relatively low rate of transpiration. Increasing evidence supports the hypothesis that long-term low ABA concentration plays an important role in the loss of stomatal functionality in plants grown at high RH (Rezaei Nejad and van Meeteren 2007, 2008). For instance, it was found that a daily exogenous ABA application during leaf development increased stomatal response to desiccation of high RH-grown *Tradescantia virginiana* leaves (Rezaei Nejad and van Meeteren 2007). Furthermore, Mortensen and Gislerød (2005) showed that severe drought stress, which is expected to intensify the ABA signals (Schachtman and Goodger 2008), increased the vase life in five out of six cultivars grown at high RH. Therefore, it is unclear if the aforementioned lack of stomatal adaptation in fully expanded leaves of *Rosa hybrida*, upon transfer to a contrasting RH level, is due to insufficient changes in leaf ABA concentration, or because the stomatal functionality in fully expanded rose leaves is independent of the ABA level. Moreover, to the best of our knowledge there are no studies on the effect of exogenous ABA application on fully expanded leaves, which would allow investigating whether ABA has an active role on stomatal functioning after full leaf expansion.

The aims of the present work were: (i) to investigate the dynamics of stomatal adaptation at various stages of leaf ontogeny (accessed during and after full leaf expansion) in response to long-term high RH; (ii) to test whether long-term exogenous ABA application could improve stomatal functionality during or after complete leaf expansion at high RH; and (iii) to analyze whether root signalling is a prerequisite for sustaining stomatal functionality in leaves fully expanded at moderate RH. We hypothesize that the RH level during critical stages, rather than throughout leaf expansion, is decisive for stomatal functionality, and that drastic changes in the ABA concentration after leaf expansion can influence stomatal functionality.

Materials and Methods

Plant material and growth conditions

Rooted cuttings of the cut rose cv. Pink Prophyta (*Rosa hybrida* L.) were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands), and planted in 3.6 L pots containing a mixture of cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull Rhenen, The Netherlands) (3:1, v/v). The cv. Pink Prophyta was selected due to its sensitivity to high RH (Fanourakis et al. 2009), and will be called Prophyta in the remainder of this paper. A total of five experiments were conducted. In each experiment, plants were grown in four growth chambers (l × w × h = 1.3 × 0.8 × 1.3 m) as a single shoot (one plant per pot) at a density of 30 plants m^{-2}. In two growth chambers the RH was 60 ± 3% (moderate RH) and in two others it was 95 ± 1% (high RH) during the cultivation period. The four chambers had constant day and night temperatures (19 ± 1 °C), resulting in a VPD of 0.88 ± 0.12 kPa (moderate RH) or 0.11 ± 0.03 kPa (high RH). Climate parameters were recorded automatically every 5 min, using data loggers (Fourier MicroLog EC650, MicroDAQ.com Ltd, Contoocook, NH). The CO_2 concentration during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Fluorescent tubes (Philips TLD 58W/84, Eindhoven, The Netherlands) provided an 18 h on-off cycle and 300 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation (Model LI-250, LI-COR, Lincoln, NE). The light intensity was measured at 70 cm from the pot base, which corresponds to the top of the plants at harvest stage. Plants were watered automatically with a nutrient solution, as described by Fanourakis et al. (2009).

Water relations in intact plants during cultivation (experiment 1)

Eight weeks after planting, when the plants were fully developed and had a flower bud with a cylindrical shape and pointed tip, plant transpiration rate was gravimetrically recorded on a daily basis for five days, using nine intact plants at each humidity level. During this period the pots were covered with aluminium foil to prevent evaporation from the substrate, and the amount of solution used for irrigation and that drained from the pots were recorded daily. At the end of the measurements total leaf area per plant was determined using a leaf area meter (model 3100 Area Meter, LI-COR, Lincoln, NE). The plant transpiration rate was normalized per unit leaf area, while the stomatal conductance (g_s) was calculated as a daily average per plant according to the equation of von Caemmerer and Farquhar (1981) (Eqn 1).

$$g_S = \frac{\text{Transpiration rate}}{w_L - w_A} \quad (1)$$

The water vapour gradient between leaf interior and air (W_L - W_A) was calculated using the difference of the saturated mole fraction of water vapour inside the leaf [W_L; 21.7 mmol H_2O mol^{-1} of air at 19 °C, according to Nobel (1991)] and the mole fraction of water vapour in the bulk air outside of the leaf (W_A; 13.0 and 20.6 mmol H_2O mol^{-1} of air at 19 °C, at 60 and 95% RH, respectively). The leaf temperature was assumed to be equal to the air temperature. However, if this assumption would be incorrect, and a leaf with a relatively high rate of transpiration (at moderate RH) would be 1 °C cooler whilst a leaf with a relatively low rate of transpiration (at high RH) would be 1 °C warmer than ambient air, the stomatal conductance would be about 6% higher at moderate RH and 6% lower at high RH than the one quoted here.

Additionally, leaf water potential (Ψ) was measured destructively at regular intervals during the light period (3, 6, and 9 h after the onset of the light period), using plants from both RH levels. The measurements were conducted in three different sets of eight fully expanded leaflets each (using the terminal leaflet of the first penta-foliate leaf counting from the apex of stalks with a flower bud at the stage described above), using a Scholander-type pressure chamber (Soil Moisture Equipment Corp, Santa Barbara, CA).

Stomatal adaptation to a new RH during leaflet expansion (experiment 2)

To determine stomatal adaptation capacity to a long-term alteration in the RH during leaflet expansion, a reciprocal transfer experiment was conducted. Eighteen plants were transferred to the contrasting humidity level (i.e. 60%→95% RH, and 95%→60% RH), while fourteen plants were kept at their original humidity level (controls). Just before transferring the plants, leaves were tagged and the length of the terminal leaflets of the penta-foliate leaves (from first to fifth order counting from the apex) was followed in time. This allowed the calculation of the proportion of the leaflet that expanded at the new humidity level in relation to its final length (whereby final length was defined as the length when the midrib did not elongate significantly for three consequent days). The developmental stage of the leaflets at the moment of transfer was expressed as a percentage of full leaflet expansion by measuring leaflet length (FLE; defined as the proportion of leaflet length at transfer, relative to its final length, Woodall et al. 1998). The moment of transfer corresponded to 90 ± 5% FLE of the terminal leaflet of the fifth penta-foliate leaf counting from the apex, whereas the upper leaflets were at various percentages of their final length. Therefore, after final length had been achieved, leaflets were sorted into eight groups: 21–30, 31–40, 41–50, 51–60, 61–70, 71–80, 81–90, and 91–95% FLE. For example, 20% FLE refers to a leaflet which at the moment of transfer had 20% of its final length, while the remaining 80% developed under the new RH level, after transfer. The stomatal adaptation ability was assessed by determining the transpiration rate of the terminal leaflets in response to desiccation. These leaflets were detached, their petioles were immediately recut under degassed water (to prevent cavitation induced-embolism), placed in flasks filled with water, and further incubated

for 1 h at about 100% RH (21 °C; VPD close to 0) to establish their maximum fresh weight. Since the leaflets were detached during the light period in the growth chamber, the rehydration process was therefore conducted in light (15 μmol m^{-2} s^{-1}), as following darkness the light-induced stomatal opening requires up to 1 h (Blom-Zandstra et al. 1995). Subsequently, the leaflets were removed from water and placed in the test room on a table (abaxial surface down) (50 ± 3% RH, 21 °C, 1.24 kPa VPD and 50 μmol m^{-2} s^{-1}) where the leaflet transpiration rate was measured gravimetrically every 5 to 30 min during 4 h. The leaflet area was then determined and the leaflets were dried at 80 °C for 24 h. The leaflet relative water content (RWC) was calculated according to Slavik (1974) (Eqn 2). This experiment was repeated once.

$$RWC = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Saturated Fresh Weight} - \text{Dry Weight}} \times 100 \qquad (2)$$

Effect of ABA application during leaflet expansion (experiment 3)

To estimate the effect of long-term ABA application on the stomatal functionality during leaflet expansion, the terminal leaflets of the penta-foliate leaves (from first to third order counting from the apex) were gently brushed with an aqueous solution containing 30 μM (±)-ABA (Sigma, St. Louis, MO) on both the abaxial and adaxial surfaces twice a day. In control plants, terminal leaflets were brushed with water in a similar manner. ABA was applied from the start of leaflet unfolding (i.e. when the midrib of the terminal leaflet was visible, corresponding to about 20% of its final length) to the time of full leaflet expansion, in plants grown continuously at moderate and high RH. This ABA application lasted about 14 days and was terminated 48 h before the measurements took place.

After the final length had been achieved, the transpiration rate in response to desiccation was determined as earlier described (experiment 2) using the terminal leaflets of the ABA treated leaves. Additionally, some anatomical features of the stomata were measured on a leaflet of the first pair of lateral leaflets from the first penta-foliate leaf (counting from the apex). The measurements were performed on the abaxial surface, using a part of the leaflet that was midway between the tip and the base and away from the edge. Sites overlying veins were also avoided as they support no stomata. Five fields of view per leaflet were counted for determining the stomatal density using 12 leaflets (one leaflet per plant) and a magnification of 100 ×. In 20 randomly selected stomata per leaflet, the stomatal length, stomatal width, pore length and pore aperture were measured immediately after leaflet detachment using a magnification of 1000 ×. Stoma width was chosen instead of guard cell width, since the latter undergoes changes up to 50% as stomata close (Shope and Mott 2006). These anatomical features were determined using the silicon rubber impression technique (Smith et al. 1989). The leaflet was held horizontally and upside-down until the impression material hardened (< 2 min) to

ensure better replication of pores (Weyers and Meidner 1990). The impression material was then detached and a positive replica was made on clear nail varnish and observed on a microscope slide. Digitized video images were taken using a microscope (Leica, Aristoplan, Bensheim, Germany) which was connected to a digital imaging camera (Nikon DXM-1200, Nikon Corp, Tokyo, Japan). Image processing was done using the freeware UTHSCSA ImageTool program (University of Texas Health Science Centre, San Antonio, TX).

Stomatal adaptation to a new RH in fully expanded leaflets (experiment 4)

To study the effect of a changed RH level on stomatal functionality in young fully expanded leaflets, twelve plants were kept at the humidity level in which they had been grown (controls) while twenty other plants were transferred to the new RH (i.e. 60%→95% RH, and 95%→60% RH). Transfer took place when the flower bud had a cylindrical shape and pointed tip. To follow the time course of stomatal adaptation to the new RH environment, the transpiration rate in response to desiccation was recorded (as described for experiment 2) using terminal leaflets that were detached every two days for a period of 14 days.

Effect of ABA manipulation in fully expanded leaflets (experiment 5)

To investigate the importance of ABA on the dynamics of stomatal functionality in fully expanded leaflets, two treatments were performed: (i) long-term ABA application, twice a day, after complete leaflet expansion under continuous high RH; and (ii) root excision in plants grown continuously at moderate RH.

Both treatments were initiated when stalks had a flower bud with a cylindrical shape and pointed tip. ABA was applied using the same method as described above (experiment 3), and it lasted for 14 days while keeping the plants at high RH. Root removal was carried out in plants grown at moderate RH, while care was taken that the water uptake was undisturbed. The night before the experiment, plants were well irrigated and placed in darkness for 12 h to ensure maximal turgidity and to minimize the presence of natural air emboli (van Doorn and Suiro 1996). Pots with the plants were placed in buckets which contained degassed sterilized water, whereby the water level was about 5 cm above the soil surface. Flowering stems were cut under water to prevent air entrance at the cut surface. Each cut was made using secateurs that had been sterilized in 98% ethanol, and through an internode that had been surface-sterilized by rubbing with a cloth drenched in the same solution (sterile treatment, van Doorn et al. 1991). The flowering stalks were normalized for stem length (47 ± 2 cm) and leaf number (four penta-foliate leaves, resulting in a leaf area of 340 ± 20 cm^2). Subsequently, the shoots were placed in sterilized flasks (one flower per flask) containing sterilized artificial vase solution (0.7 mM $CaCl_2.2H_2O$, 1.5 mM $NaHCO_3$, 5 μM $CuSO_4.5H_2O$; van Meeteren et al. 2000), with a pH adjusted to 3 by addition of citric acid. The cut flowering

stems were placed in a climate-controlled room at 20 °C, 60% RH and light for 12 subsequent h per day at a photon flux density of 10–12 μmol m^{-2} s^{-1}.

In both treatments, the transpiration rate in response to leaflet desiccation was recorded every two days for a period of 14 days.

Statistical design and analysis

Data were subjected to analysis of variance using Genstat software (10th edition, VSN International Ltd, Herts, UK). Experiment 1 was analyzed by one-way ANOVA. Experiment 2 was analyzed using the Linear Mixed Models' method (Crawley 2002), because there were an unbalanced number of replicates. Experiments 3, 4 and 5 were analyzed by two-way ANOVA, where the RH level was the main factor, while ABA application (experiment 3), duration of new RH level (experiment 4), ABA application or root excision (experiment 5) were the split factors, respectively. Treatment effects were tested at 5% probability level and mean separation was done using least significant differences based on Student's *t*-test ($P = 0.05$).

Results

Plant water relations when grown at continuously high or moderate RH

Water relations were measured in intact plants grown continuously at 60 or 95% RH. As expected, because of the lower VPD, plants grown continuously at high RH had a significantly lower transpiration rate during cultivation than plants grown at moderate RH ($P < 0.001$; Fig. 1A). The estimated stomatal conductance (Eqn 1) was about three-fold higher in the plants grown at high RH compared to those from moderate RH (Fig. 1B). The leaf Ψ decreased during the light period, but remained significantly higher in the plants grown at high RH ($P < 0.001$; Fig. 1C).

Concerning the stomatal anatomical features it was found that stomata in plants grown at high RH were bigger than in plants grown at moderate RH, but their density was not affected by the RH level during leaflet development (Table 1). The pore length and aperture, when the lights were on for 2 h, was also greater in plants grown at high RH.

To determine the stomatal response to desiccation, terminal leaflets cut from fully developed leaves were allowed to dehydrate for 4 h. The transpiration rate was taken as a measure of stomatal opening (Fig. 2). After 2 h of desiccation, the transpiration rate in leaflets expanded at high RH was about eight times higher than in those expanded at moderate RH (Fig. 2A). When the leaflets were allowed to desiccate for 4 h, their transpiration rate became similar at both RH levels (Fig. 2A), but the leaflet hydration levels were very different (58 and 11% RWC values for leaflets of plants grown at moderate and high RH, respectively; Fig. 2B).

Table 1. Stomatal characteristics of cut rose cv. Prophyta grown continuously at moderate (60%) or high (95%) relative air humidity (RH) in response to long-term ABA application (0 or 30 μM twice a day throughout leaflet expansion). Values are the mean of 60 fields of view (stomatal density) and 240 stomata (stomatal anatomical features) measured on fully expanded leaflets. Different letters indicate significant differences according to LSD-test (comparison in columns) (experiment 3).

RH	ABA application (μM)	Stomatal density (number mm^{-2})	Stomatal length (μm)	Stomatal width (μm)	Pore length (μm)	Pore aperture (μm)
60%	0	43.1	42.6 ^{c}	31.8 ^{b}	23.7 ^{c}	4.83 ^{b}
	30	43.3	36.0 ^{a}	22.5 ^{a}	19.3 ^{a}	4.10 ^{a}
95%	0	45.3	50.6 ^{d}	36.0 ^{c}	30.7 ^{d}	7.08 ^{c}
	30	44.5	37.6 ^{b}	22.9 ^{a}	21.1 ^{b}	4.35 ^{a}
F pr.						
RH		0.05	<0.001	<0.001	<0.001	<0.001
ABA		0.70	<0.001	<0.001	<0.001	<0.001
RH x ABA		0.57	<0.001	<0.001	<0.001	<0.001

These results show that stomata that developed at high RH were less sensitive to a decrease in leaflet RWC, as they had higher transpiration rates throughout the RWC range (Fig. 2B). Based on these findings, the stomatal closure capacity in response to leaflet desiccation was assessed in the subsequent experiments by measuring both the transpiration rate and the leaflet RWC 2 h after the onset of desiccation, once leaflets were fully expanded.

Stomatal adaptation to a new RH during leaflet expansion

Leaflet elongation growth, expressed as a percentage of full leaflet expansion (FLE) measured in length (i.e. actual leaflet length as a percentage of final length), followed an S-shape pattern in time (Fig. 3A). The percentage of leaflet area increased exponentially with the percentage of FLE (Fig. 3B). Interestingly, leaflet expansion, both in length and in area, was independent of the humidity level during growth (Fig. 3).

To investigate the dynamics of stomatal adaptation throughout leaflet expansion in response to long-term high RH, plants grown at moderate and at high RH were transferred to the other RH level (i.e. 60% to 95% RH and vice versa). At the moment of transfer, the percentage of FLE of individual leaflets varied between 25 and 93% (Fig. 4A, B). This resulted in different durations of exposure to high RH until complete FLE, which reached a maximum of nine days (Fig. 4C, D). To assess the stomatal response to dehydration, the rate of transpiration of cut leaflets and their RWC, measured 2 h after the onset of dehydration, was evaluated. It was found that the leaflets of transferred plants always showed intermediate values between the ones observed for leaflets grown continuously at moderate or at high RH (controls) (Fig. 4). The only exception was found in leaflets that had expanded up to 25–35% FLE under high RH and whereby the plants

had subsequently been transferred to moderate RH. These had a transpiration rate similar to the moderate RH control leaflets. These results revealed that stomata from leaflets initially expanded at a certain RH level and that were attached to plants subsequently transferred to the contrasting RH (at 25 to 93% FLE) always had the capacity to partly adapt to that new RH level. Before 25–35% FLE, a high RH had no effect on stomatal functioning. However, the degree of stomatal adaptation was strongly dependent on the timing and duration of exposure to high RH. In expanding leaflets, the

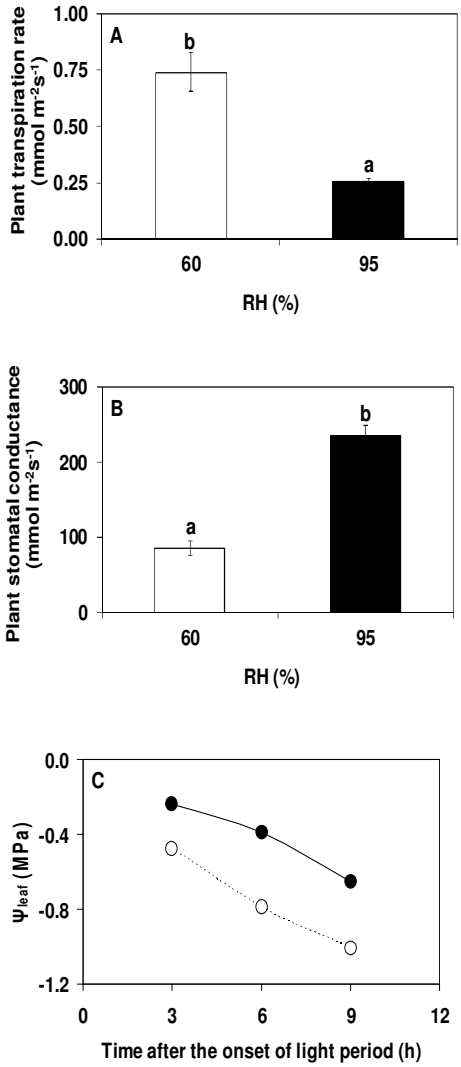

Fig. 1. Plant transpiration rate (A), plant stomatal conductance (B), and leaf water potential (Ψ) at different times after the onset of the light period (C) during cultivation of cut rose cv. Prophyta grown at moderate (open symbols, 60%) and high (closed symbols, 95%) relative air humidity (RH). The measurements were conducted using fully grown plants when the flower bud had a cylindrical shape and pointed tip. Values are the daily mean of nine intact plants (A, B) or the mean of eight leaflets (C) ± SEM. Different letters indicate significant differences according to LSD-test (experiment 1).

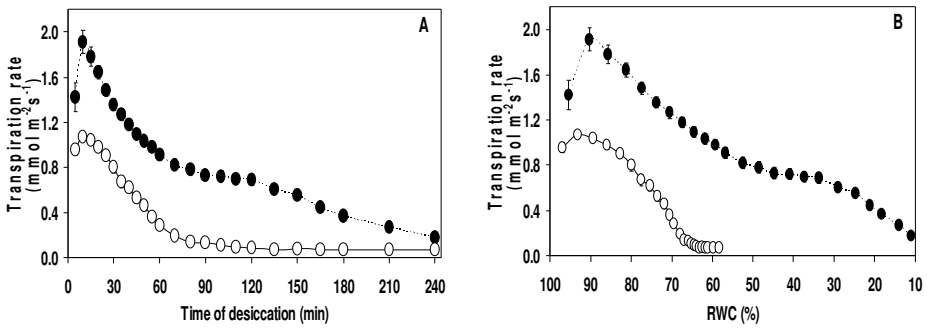

Fig. 2. Transpiration rate as a function of time of desiccation (A) and relative water content (RWC) (B) during four h of leaflet desiccation in cut rose cv. Prophyta, grown continuously at moderate (open symbols, 60%) and high (closed symbols, 95%) relative air humidity. Values are the mean of 14 leaflets ± SEM (experiment 2).

stomatal adaptation capacity was mostly dependent on the RH level at which the last part of leaflet expansion took place. For leaflets that started expanding at high RH but had completed their expansion after transfer to moderate RH (i.e. 95%→60% RH), the moment of transfer had a significant effect on the stomatal capacity to adapt to the new environment (Fig. 4A, B). The younger the leaflets when the shift to moderate RH took place (Fig. 4A, B), i.e. the shorter the period the leaflet was exposed to high RH (Fig. 4C, D), the higher the stomatal capacity to become functional. On the other hand, in leaflets that started expanding at moderate RH and were attached to plants that had been transferred to high RH (i.e. 60%→95% RH), different percentages of FLE at the moment of transfer showed similar stomatal response characteristics, once leaflets were fully expanded (Fig. 4A, B). Surprisingly, the stomatal functionality of a leaflet transferred to high RH at 93% FLE was strongly affected. The same trends as those described here were observed, though the differences were more pronounced when the RWC after 4 h of desiccation was considered (data not shown). These results indicate that the stomatal capacity to respond to desiccation by closing is not completely determined until leaflet expansion has finished.

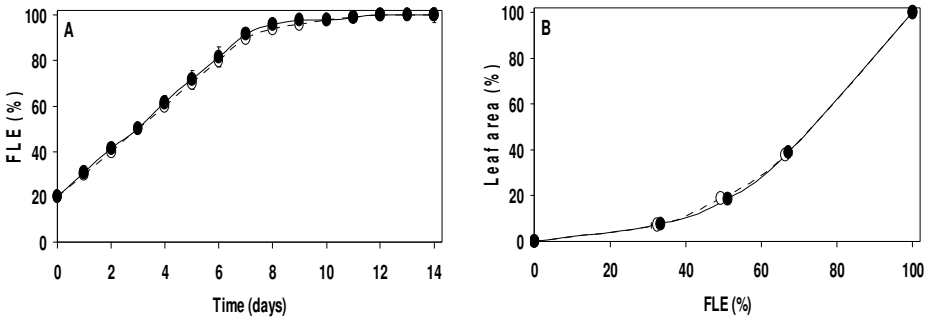

Fig. 3. Percentage of full leaflet expansion (FLE; measured in length) as a function of time (A) and relationship between the percentage of leaflet area and the percentage of FLE (B) in cut rose cv. Prophyta grown continuously at moderate (open symbols, 60%) and high (closed symbols, 95%) relative air humidity, under a constant day and night temperature (19 °C). Time 0 corresponds to the beginning of leaflet unfolding (i.e. when the midrib of the terminal leaflet was visible). Values are the mean of 12 leaflets ± SEM (experiment 2).

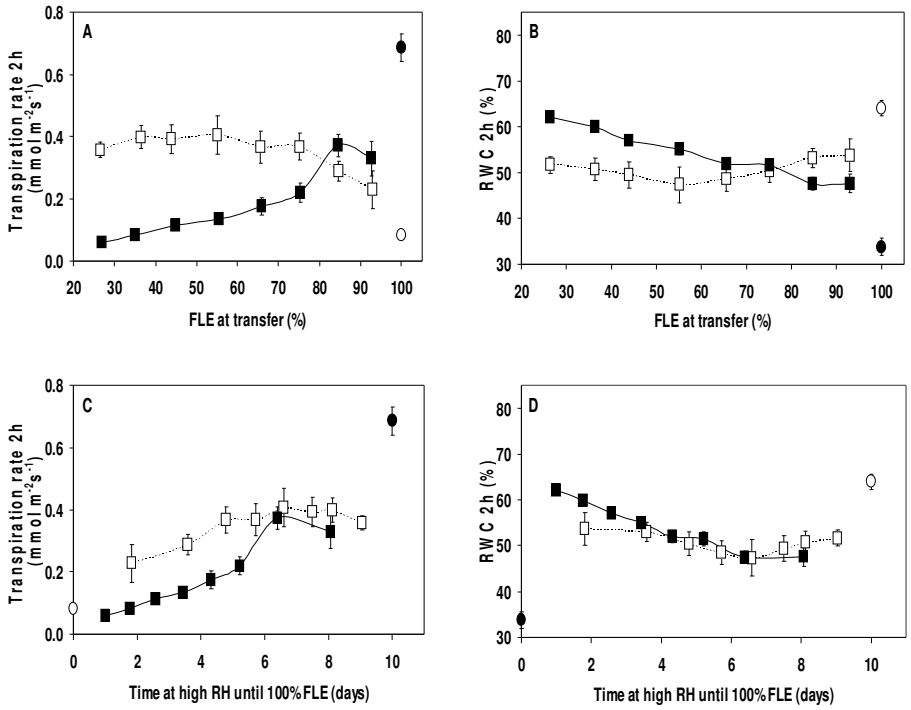

Fig. 4. Transpiration rate (A, C) and relative water content (RWC) (B, D) after two h of leaflet desiccation in cut rose cv. Prophyta grown continuously at moderate (open circles, 60%) and high (closed circles, 95%) relative air humidity (RH) (controls), or transferred to the new humidity environment (open square, 60%→95% RH; closed square, 95%→60% RH) at different percentages of full leaflet expansion (FLE) (A, B), corresponding to different duration of exposure to high RH (C, D). The measurements were conducted after complete leaflet expansion. Values are the mean of at least 18 leaflets ± SEM (experiment 2).

Effect of ABA application during leaflet expansion

In plants that were grown continuously at moderate or high RH, an aqueous ABA solution (0 or 30 μM ABA) was applied on the terminal leaflets twice a day, throughout the period of leaflet expansion. This long-term ABA application did not influence stomatal density (Table 1). In contrast, it resulted in significantly smaller stomata with lower pore dimensions at either RH level (Table 1). There was no significant difference in stomatal width and pore aperture between ABA treated leaflets at the two RH levels (Table 1). Besides the effect of ABA application on stomatal anatomy, stomatal closure during desiccation was largely enhanced in ABA-treated leaflets from plants grown continuously at high RH (Fig. 5). In these leaflets, the transpiration rate after 2 h of desiccation was about 85% lower compared with leaflets that developed at high RH, reaching the same values as those from leaflets grown continuously at moderate RH (Fig. 5A). This resulted in 34% RWC after 2 h of dehydration in untreated leaflets, whereas the RWC was 75% in high RH leaflets treated with ABA (Fig. 5B). Leaflets treated daily with water behaved in a similar way as untreated leaflets, at both RH levels (data not shown).

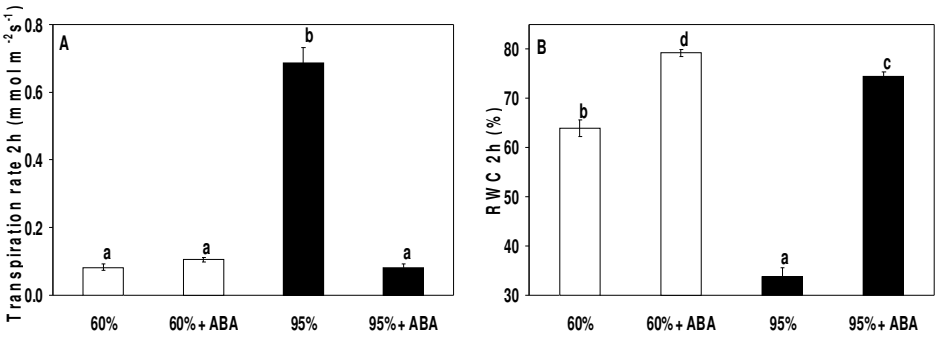

Fig. 5. Transpiration rate (A) and relative water content (RWC) (B) after two h of leaflet desiccation in cut rose cv. Prophyta grown continuously at moderate (open columns, 60%) and high (closed columns, 95%) relative air humidity and treated with long-term ABA application (0 or 30 μM twice a day throughout leaflet expansion). The measurements were conducted after complete leaflet expansion. ABA application ceased 48 h before the measurements. Values are the mean of 10 leaflets ± SEM. Different letters indicate significant differences according to LSD-test (experiment 3).

Stomatal adaptation to a new RH in fully expanded leaflets

To evaluate the dynamics of stomatal adaptation in fully expanded leaves, terminal leaflets were cut every two days from plants that had been transferred to a contrasting RH, for a period between 0 and 14 days. The transpiration rate 2 h after the onset of dehydration did not differ significantly from the controls (leaflets kept at humidity level of expansion), irrespective of the period that the plants had been placed in the new RH environment (Fig. 6A). This was also reflected in the RWC after 2 h of desiccation. The

leaflet RWC had an inverse correlation with the rate of transpiration (Fig. 6B). Thus, leaflets that had expanded under moderate RH showed a normal stomatal closing response to desiccation, even when the plants were subsequently exposed to high RH for as much as 14 days. Similarly, stomata on leaflets fully expanded at high RH were unable to close rapidly during dehydration, even when the plants were further placed at moderate RH for as much as two weeks.

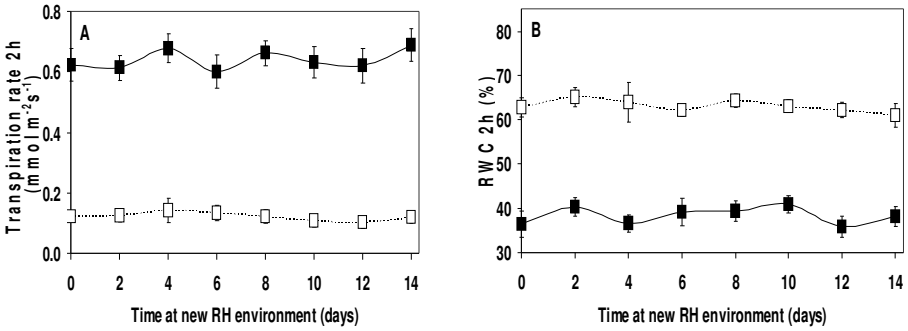

Fig. 6. Transpiration rate (A) and relative water content (RWC) (B) after two h of leaflet desiccation in cut rose cv. Prophyta grown at moderate and high RH and further transferred to a new relative air humidity (RH) environment (open symbols, 60%→95% RH; closed symbols, 95%→60% RH) after complete leaflet expansion. Values are the mean of 14 leaflets ± SEM (experiment 4).

Role of ABA in fully expanded leaflets

To test the hypothesis that drastic changes in ABA concentration after leaflet expansion can influence stomatal functioning, ABA levels were exogenously manipulated both by ABA application in plants grown at high RH and by root excision in plants grown at moderate RH, as described above for experiment 3. During treatments' application, terminal leaflets were sampled every two days for a period of two weeks.

Effect of ABA application on fully expanded leaflets. An ABA solution (30 μM ABA) was applied twice per day on fully expanded leaflets from plants grown continuously at high RH. The stomata of leaflets expanded at high RH did not improve their closing ability after two weeks of exogenous ABA application (Fig. 7).

Effect of root excision. The effect of root excision was evaluated in plants developed at moderate RH. Care was taken that the water uptake in the tested plants was undisturbed. The stomata of leaflets expanded at moderate RH maintained, for at least two weeks, their functionality despite the removal of root signals (Fig. 8).

Taken together the results of these two treatments suggest that after leaflet expansion there were no changes in stomatal responses to desiccation, even when drastic changes in leaf ABA concentration occurred.

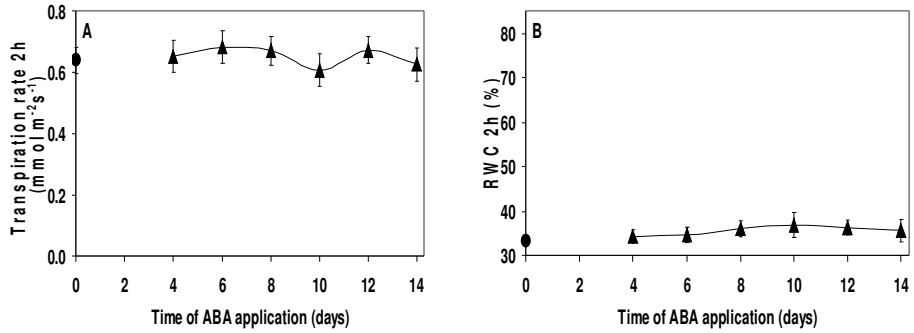

Fig. 7. Transpiration rate (A) and relative water content (RWC) (B) after two h of leaflet desiccation in cut rose cv. Prophyta grown continuously at high relative air humidity (95%) and treated with 30 μM ABA twice a day (triangles) after complete leaflet expansion during a period of 0 days [controls; i.e. non treated leaflets (circle)] until 14 days. ABA application ceased 48 h before the measurements. Values are the mean of 14 leaflets ± SEM (experiment 5).

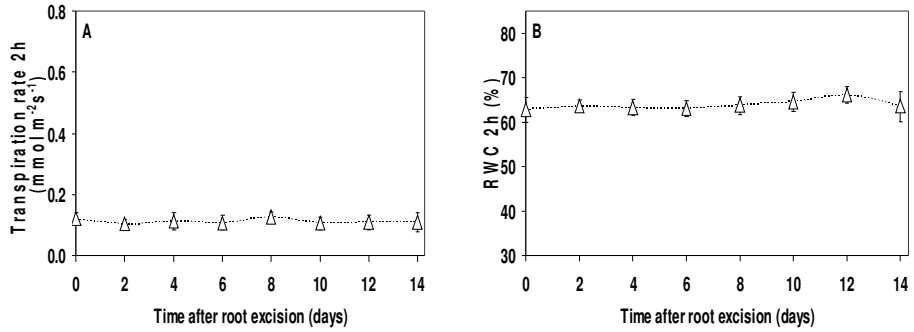

Fig. 8. Transpiration rate (A) and relative water content (RWC) (B) after two h of leaflet desiccation in cut rose cv. Prophyta grown continuously at moderate relative air humidity (60%) and further subjected to root excision after complete leaflet expansion starting at 0 days (controls) until 14 days. Values are the mean of 14 leaflets ± SEM (experiment 5).

Discussion

Stomatal closing behaviour at continuously high or moderate RH

It is well established that long-term high RH negatively affects stomatal regulation of water loss in several species (e.g. *Corylus maxima*, Fordham et al. 2001; *Oryza sativa*, Kawamitsu et al. 1993; *Rosa hybrida*, Torre and Fjeld 2001; *Tradescantia virginiana*, Rezaei Nejad and van Meeteren 2005). The present results confirm those findings in rose, since stomata developed at high RH during cultivation under well-watered conditions remained open in response to desiccation until very low leaflet hydration levels (Fig. 2). By then the leaflet had visibly wilted and is not expected to recover when rehydrated (Lawlor and Cornic 2002).

When leaves are exposed to conditions of a rapid dehydration, due to a fast increase in the transpiration rate, a reduction of the epidermal cell turgor takes place which causes a lower backpressure exerted on the guard cells, leading to a transient increase of stomatal aperture (hydropassive stomatal opening). This temporary response of stomata to rapid changes in leaf hydration, the so-called 'wrong-way' response, is well-described in literature and usually precedes stomatal closure (Buckley 2005, Mott and Franks 2001). The 'wrong-way' response has also been shown in Figure 2 at the onset of leaflet desiccation. This effect was stronger in leaflets grown under high RH due to their higher transpiration rates.

Leaflets subjected to continuously high RH during leaflet expansion also had bigger stomata with longer pores, compared to stomata on moderate RH-grown leaflets (Table 1). Previous studies have shown that when comparing different species, the ones having a higher stomatal size were the ones with the longer response time with respect to water loss (Franks and Farquhar 2007, Hetherington and Woodward 2003). Therefore, it can be hypothesised that within a given species the longer stomata, induced by high RH, are strongly implicated in the inefficient stomatal closure. However, further research is needed to confirm this hypothesis.

Stomatal adaptation to long-term alterations in RH

Although a constant high RH level during leaflet growth clearly results in a poor stomatal closure in response to dehydration, its impact at various stages of leaflet ontogeny was not analyzed previously. In this study it was found that stomatal functionality in rose leaves is completely determined during leaf expansion. Stomata from leaflets that started expanding at a certain RH level and were subsequently transferred to a contrasting one (moderate to high RH and vice versa) between 35 and 93% FLE were always able to partly adapt to the new RH level (Fig. 4). In contrast, out of this range of leaflet developmental stages subjecting the plants to high RH had no effect on stomatal functioning (Fig. 4 and 6). Moreover, this study has shown that in expanding leaflets, the degree of stomatal adaptation was closely related to the timing and duration of exposure to high RH (Fig. 4). It was found that the stomatal adaptation capacity was strongly dependent on the humidity level at which the leaflet completed its expansion and, therefore, high RH should be avoided particularly during the last stages of leaf ontogeny. These findings are in agreement with our hypothesis that the RH level during critical stages, rather than throughout leaf expansion, is decisive for stomatal functionality.

In fully expanded leaves, the stomatal anatomy and stomatal population (i.e. absolute number of stomata = density × leaf area) are fixed. In contrast, in expanding leaves, the stomatal size is either increasing or does not change any more (Tichá 1982). Stomatal initiation often stops before 60 to 80% of leaf expansion has occurred (Rawson and Craven 1975). It might be speculated that developing stomata have a better ability to

adapt to a new environment than fully developed ones (i.e. stomata in which the final length has been achieved). Since rose is a dicotyledonous species, stomatal formation is not synchronous (Larkin et al. 1997). Therefore, during the early phases of leaf expansion, different stomatal developmental stages can be found. This might explain why the sensitivity to high RH is spread over such a large range of leaflet developmental stages (35 to 100% FLE).

When our findings are translated to conditions of changing RH, as in plants grown in natural environments or in protected cultivation, they indicate that only a few days of high RH can induce lack of proper stomatal closure in response to desiccation, at least in the leaves that are in the last phase of elongation. Depending on the RH levels over time, plants grown under such conditions are expected to have some leaves that respond properly to dehydration and others that do not.

ABA manipulation during and after full leaflet expansion

Long-term application of exogenous ABA during leaflet expansion fully counteracted the problem of lack of proper stomatal response to dehydration in rose plants grown at high RH (Fig. 5). The same was shown previously in *Tradescantia virginiana* (Rezaei Nejad and van Meeteren 2007). The fact that ABA application in moderate RH leaflets resulted in a significantly better stomatal closure when the leaflets became dehydrated (Fig. 5B), as compared to ABA treated leaflets expanded in high RH, might be taken to support the hypothesis that the endogenous leaf ABA concentration in rose is lower at high RH-grown leaves. These results provide additional evidence that the weaker hydraulic signal during cultivation at high RH (i.e. low transpiration rates and/or the high leaf water potential; Fig. 1) is not *per se* the dominant factor involved on poor stomatal functioning. It is concluded that a long-term low leaf ABA concentration during stomatal development is the main cause of stomata malfunctioning in well-watered rose plants grown at high RH.

Unlike the results for expanding leaflets (Fig. 5), we show here that daily exogenous ABA application after complete leaflet expansion – keeping the same application conditions (i.e. ABA concentration and treatment duration) – did not have any effect on the stomatal functionality in plants grown continuously at high RH (Fig. 7). Thus, it could be speculated that in fully expanded leaves the cuticula has worked as a barrier for ABA penetration. However, this is not likely since exogenously applied ABA has been shown to penetrate to the leaf interior via the cuticle (Blumenfeld and Bukovac 1972), but also solute uptake takes place through the stomatal pores (Eichert and Burkhardt 2001). Moreover, in the latter study it was found that this uptake is facilitated at elevated humidity due to the increased stomatal opening. Additionally, in our study it was shown a lack of stomatal responses to a change in RH in fully expanded rose leaflets [Fig. 6, and also described by Mortensen and Gislerød (2000)]. Taken together, these results bring us to the conclusion that after full leaflet expansion, stomatal functioning is

no longer affected either by the ABA or RH level. In *Tradescantia virginiana,* the loss of stomatal functionality after transfer to high RH was related to a decrease in the leaf ABA concentration, and the authors proposed that a certain ABA level is required not only to induce, but also to sustain stomatal functionality (Rezaei Nejad and van Meeteren 2008). Since our study shows that stomatal functioning in rose is independent of post-development RH level (Fig. 5), two hypotheses arise: (i) although the root to shoot ABA signalling is weakened after transfer to high RH, this is still sufficient to sustain stomatal responsivity; or (ii) the level of ABA after the development of stomatal apparatus (i.e. in fully developed leaves) is not relevant for a proper stomatal functioning in rose. We tested this hypothesis by excising the root (hormonal and hydraulic) signals for a period of two weeks using plants grown continuously at moderate RH and assessing the response to desiccation in fully expanded leaflets. Similarly to the non excised control plants, stomata on leaflets fully expanded at moderate RH sustained their functionality despite the complete absence of root signals (Fig. 8). Therefore these results suggest that in rose, the functionality of the stomatal apparatus is determined during leaf expansion, and is independent from the ABA level after that period, even when ABA concentration is drastically changed.

Besides the role of ABA on stomatal physiology it was shown that exogenous ABA application resulted in smaller stomatal dimensions (Table 1). Similar results were observed in *Tradescantia virginiana* (leaves treated daily with ABA, Franks and Farquhar 2001) and wheat (ABA was injected regularly in the nutrient solution, Quarrie and Jones 1977). However, exogenous ABA application did not affect stomatal density, while in other studies a higher stomatal density has been reported in ABA treated leaves (Bradford et al. 1983, Franks and Farquhar 2001).

Conclusions

It was demonstrated that the capacity of stomata to close in response to leaf dehydration is completely established during the period of leaf expansion. Moreover, it was concluded that in expanding leaves the degree of stomatal adaptation depends on the duration and timing of exposure to high RH. In general, the longer the exposure to high RH the higher the loss of stomatal functionality in response to desiccation. Furthermore, it was found that stomatal malfunctioning, as a result of plant growth under long-term high RH, is strongly determined during the last part of leaf expansion. A long-term exogenous ABA application (twice a day), throughout leaf expansion, is able to counteract the negative effect of high RH on stomatal functioning. Nevertheless, once the leaves have fully expanded, the stomatal closure capacity is no longer affected either by the RH or the ABA level, even when drastic changes in the leaf ABA concentration take place (i.e. long-term exogenous ABA application in high RH-grown plants and root

removal in moderate RH-grown plants). Thus, these results suggest that the role of ABA on stomatal hydrosensitivity is restricted to the period of leaf expansion.

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CHAPTER 5

Stomatal initiation, development and proximity: role of air humidity

5.1 Large spatial heterogeneity of stomatal features in rose leaflets (*Rosa hybrida* L.) during expansion

5.2 Growing stomata at high relative air humidity exceeding a critical length do not become functional at full leaf expansion

CHAPTER 5.1

Large spatial heterogeneity of stomatal features in rose leaflets (*Rosa hybrida* L.) during expansion

Abstract

Using light microscopy, stomatal initiation, growth and proximity were analysed in *Rosa hybrida* L. Some stomatal features (size, density = number of stomata per unit surface, and index = stomatal number per total cell number) were determined over the entire leaflet surface at 33%, 50%, 67%, and 100% of full leaflet expansion (FLE; proportion of leaflet length relative to its final length). Over 80% of the total stomatal population was initiated between 33% and 67% FLE, and the remainder mainly before 33% FLE. Stomatal growth (based on length increase) mostly took place between 67% and 100% FLE. During leaflet expansion, considerable spatial gradients were found both in stomatal formation and growth. At full expansion, the heterogeneity in stomatal density was only due to variation in the number of stomata, not to features of epidermal cells. Stomatal pore dimensions were measured in non-stressed fully expanded leaflets, and used to estimate stomatal conductance (g_s) over the leaflet. Differences in g_s of 37% were estimated between sampling areas within the leaflet lamina. This variation was due equally to variation in pore aperture and variation in stomatal density. The data indicate that for estimation of leaf transpiration, when using stomatal features, the within-leaf variation needs to be taken into account. The extant sampling protocols for the determination of stomatal features are therefore critically evaluated.

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Introduction

The primary function of stomata is to control the carbon dioxide and water vapour fluxes across the leaf (Cowan and Farquhar 1977, Lawson 2009). Stomatal initiation starts at early stages of leaf expansion (Gay and Hurd 1975, Wild and Wolf 1980). The number of stomata on a leaf usually does not increase further by the time the leaf reaches 10–60% of its final size, depending on the species (Royer 2001). After their initiation, stomata grow considerably in length and width. This growth is also strongly species-dependent. It can take place at an early stage of leaf expansion, or occur only by the time the leaf is already almost fully expanded (Tichá 1982).

Current methods of assessing stomatal features and stomatal pore size often rely on the assumption that these are spatially homogenous over the leaf. Determinations are therefore usually restricted to one sampling area per leaf (Fanourakis et al. 2011, Franks et al. 2009, Haworth et al. 2011). However, some studies have challenged the validity of this assumption. It was observed that stomatal density (i.e. number of stomata per unit area) can vary considerably between areas of the leaf (Lawson et al. 2002, Poole et al. 1996). As far as we know, only one study has shown spatial heterogeneity in stomatal pore aperture, in non-stressed leaves (Smith et al. 1989). Stomatal dimensions and pore sizes are often used to estimate stomatal conductance (g_s) (Lammertsma et al. 2011, Parlange and Waggoner 1970). Given the above critique, one sampling area per leaf might lead to significant errors in g_s, which become magnified when scaling up to the leaf or canopy level (Gupta and Kundu 1965, Lawson 1997). Here, we evaluated the within-leaflet heterogeneity of stomatal features in rose leaflets. In expanding leaflets we measured stomatal size, stomatal density, and stomatal index (the number of stomata per total number of cells), over the entire leaflet surface. Additionally, in fully expanded leaflets we measured stomatal pore dimensions. These data allowed quantification of the errors that are introduced by sampling size or location.

Materials and Methods

Plant material and growth conditions

Rooted cuttings of *Rosa hybrida* L. cv. Pink Prophyta were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands), and planted in 3.6 L pots containing a 3:1 (v/v) mixture of cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull Rhenen, The Netherlands). Plants, one per pot, were grown in growth chambers (l × w × h = 1.3 × 0.8 × 1.3 m) as a single shoot, at a density of 30 plants m^{-2}. The chamber temperature was 19 ± 1 °C and the relative air humidity was 60 ± 3%. Climate parameters were recorded automatically every 5 min, using data loggers (Fourier MicroLog EC650, MicroDAQ.com Ltd, Contoocook, NH). The CO_2

concentration in the air during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided 300 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation (Model LI-250, LI-COR, Lincoln, NE) at 70 cm from the soil level, which corresponds to the top of fully grown plants. An 18 h light - 6 h dark cycle was used. Plants were watered automatically, with a nutrient solution.

Percentage of full leaflet expansion (FLE)

The expanding leaflets were sampled at various stages of elongation growth. Data referred to various stages of leaflet expansion, but were selected later on to represent only 33%, 50%, 67% and 100% of full leaflet expansion (FLE). FLE is defined as the proportion of leaflet length at assessment, relative to final leaflet length (Fanourakis et al. 2011, Woodall et al. 1998). The final leaflet length was determined to be reached when three successive leaflet length data, taken at daily intervals, showed no increase. The 33%, 50%, and 67% of FLE corresponded to 7%, 19%, and 39% of the final leaflet area, respectively.

Stomatal density, index and size

R. hybrida has compound leaves, where leaflets arise on both sides of the rachis (i.e. in pairs) besides the terminal leaflet (odd-pinnate arrangement). Measurements were carried out on one leaflet of the first pair of lateral leaflets from the first order penta-foliate leaf (counting from the apex). One of the lateral leaflets was used for stomatal feature measurements, while the final length was determined in its symmetrical leaflet (paired sampling).

Stomatal characters were evaluated using sampling areas of 1 × 1 cm (Poole and Kürschner 1999). The sampling protocol covered the entire abaxial (lower) leaflet surface (Fig. 1A), as rose is a hypostomatous species (i.e. astomatous adaxial leaflet surface). The number of sampling areas per leaflet varied with the leaflet expansion stage. This was 2, 4, 10, and 28 at 33%, 50%, 67%, and 100% of FLE, respectively. To record the spatial location of the obtained data, these were represented on a three-dimensional Cartesian co-ordinate system. The variables x and y defined the distance of the area to the main vein (i.e. midrib) and to the leaflet base (i.e. petiole-leaflet lamina junction) respectively, while the value of the measured variable was assigned z.

Each sampling area was divided in plots of 1.25 × 1.25 mm (Fig. 1A). The rectangular fields of view were setup in plots (one field of view/plot) located towards the centre of the sampling area. The fields of view within the plot were located in interveinal areas, because veins were devoid of stomata (Weyers and Lawson 1997). Since the areas between the veins were smaller at lower FLE, the area of the field of view was adjusted accordingly. They were 3.8%, 3.8%, 15% and 61% of the total plot area (1.56 mm^2) for

33%, 50%, 67% and 100% FLE, respectively. The fields of view with an area lower than the area of the image taken in this magnification (0.96 mm^2) were taken by selecting part of this image. Using the method of Kubinova (1993) an unbiased decision could be made concerning the inclusion of stomatal and epidermal cells on the edge of a field of view.

Newly formed stomatal initials and epidermal cells can only be distinguished from one another with the use of molecular probes (Boetsch et al. 1995). We studied stomata at a later phase, when they could be distinguished from epidermal cells by light microscopy (i.e. when the stomatal pore length was externally visible). For determining the epidermal cell density (i.e. number of epidermal cells per unit area) and stomatal density, a magnification of 100 × was used, and one representative leaflet or four leaflets per leaflet expansion stage were assessed, respectively. The stomatal index was calculated according to Salisbury (1927) (Eqn 1).

$$\text{Stomatal index} = \frac{\text{Stomatal cells}}{\text{Stomatal cells} + \text{Epidermal cells}} \times 100 \quad (1)$$

To determine length and width of the stomata, a magnification of 1000 × was used. Fifty, 75, and 100 randomly selected stomata in each sampling area were measured in a representative leaflet at 67%, 50%, and 33% FLE, respectively. In six fully expanded leaflets, 40 stomata per sampling area were measured.

The stomatal pore length and pore aperture were also determined in 40 stomata per sampling area of three non-stressed fully expanded leaflets.

All stomatal features were determined using the silicon rubber impression technique (Weyers and Meidner 1990). Details of the impression method and image analysis are described by Fanourakis et al. (2011). For determining epidermal cell density, the microscope and camera were linked to a computer monitor. This was needed as the focus to distinguish epidermal cells was not the same within the same field of view. To ensure that the counts were as accurate as possible, the counted cells were ticked off on a sheet covering the monitor screen.

The contour maps were constructed using MATLAB software (R2008b, The MathWorks, Natick, MA).

Stomatal population

The percentage of stomata present at various leaflet expansion stages was defined as the amount of counted stomata at that stage divided by the final stomatal population (just after complete leaflet expansion). For each lateral leaflet in which stomatal density was determined (a destructive procedure), its symmetrical one was used to count the number of stomata when this leaflet had fully expanded. The absolute number of stomata per leaflet was calculated by multiplying the stomatal density of each sampling area by its surface size and summing these values (Gupta 1961, Tichá 1982) (Eqn 2).

$$\text{Absolute stomatal number} = \sum_{1}^{n} (\text{stomatal density}) \times (\text{sampling area}) \quad (2)$$

Measurements were made in four leaflets per expansion stage. The number of sampling areas per leaflet depended on the expansion stage. They were 2, 4, 10 and 28 sampling areas per leaflet for 33%, 50%, 67% and 100% FLE, respectively.

Modelling stomatal conductance (g_s)

The cross-sectional area available for gas fluxes is the product of the pore area and stomatal density. This product determines stomatal conductance (g_s) for water vapour. In a representative fully expanded leaflet, we predicted g_s (expressed in mmol H_20 m^{-2} s^{-1}) based on a model described by Weyers and Lawson (1997), using the following equation (Nobel 1991) (Eqn 3):

$$g_S = \frac{(\text{mass of air}) \times (\text{diffusion coefficien t}) \times (\text{stomatal density}) \times (\pi \times \text{pore aperture}/2 \times \text{pore length}/2)}{(\text{pore depth}) + (\text{pore aperture}/2 \times \text{pore length}/2)^{0.5}} \quad (3)$$

The density of air and the effective diffusion coefficient for water vapour were taken to be 41.4 mol m^{-3} and 2.43 × 10^{-5} m^2 s^{-1}, respecively, at 21 °C (Jones 1992). According to theoretical considerations that pertain to collisions of water molecules with the pore walls (Cowan and Milthorpe 1968), a weighting factor was introduced. It was taken to be linear, from 0.67 at a flux of 0 mmol H_20 m^{-2} s^{-1} to 0.90 at a flux of 300 mmol H_20 m^{-2} s^{-1} and more (Cowan and Milthorpe 1968). Stomatal pore depth was taken to be equal to guard cell width (i.e. stomatal width/2), based on the assumption that guard cells inflate to a circular cross-section (Franks and Beerling 2009, Franks and Farquhar 2007).

An end correction occurred according to Nobel (1991). This end correction is added to the denominator (i.e. pore depth), because the concentration patterns of water vapour on both ends of the stomatal pore cause an increase of effective pore depth.

Stomatal spacing pattern

The spatial distribution of stomata was analysed by measuring the distance between the nearest neighbour stomata (distances from edge to edge) in three directions, each covering an angle of 120°. The three closest stomata to the reference stoma were first determined, and then it was checked whether these belong spatially in the three directions. If not, the next nearest stoma was included and the same procedure was repeated. We calculated the mean distance between closest neighbour stomata in all three directions (r_A), and tested this against the expected mean distance between closest

neighbours in a spatially randomly distributed population (r_E) (Clark and Evans 1954). The ratio between both distances ($R = r_A/r_E$) indicates whether the type of distribution of the sampled population is random (R = 1), contagious (R < 1; closer than expected by a random pattern) or regular (R > 1; more apart than expected by a random pattern). The R values were calculated from the distances of other stomata to a total of 250 randomly selected stomata per expansion stage. The areas immediately close to the veins were excluded from the analysis. The statistical significance of R was tested by calculating the c value as described by Clark and Evans (1954).

Statistical design and analysis

Data were analysed by one-way ANOVA using Genstat software (10th edition, VSN International Ltd, Hemel Hempstead, Herts, UK). Variance is often indicated by coefficient of variation [(standard deviation/mean) × 100%]. Treatment effects were tested at 5% probability level and mean separation was carried out using least significant differences based on Student's *t*-test ($P \leq 0.05$).

Results

Choice of sampling procedure

In this paper we sampled the entire abaxial leaflet surface, using sampling areas of 1 × 1 cm (Fig. 1A). Sampling areas with a surface less than 1 cm² were present at the base, tip and margins of the leaflet. These were merged, whereby one sampling area was formed at each lateral side of the leaflet, and two at the leaflet tip and leaflet base (Fig. 1A). Towards the centre of each sampling area a number of plots were selected for analysis (Fig. 1A, magnified sampling areas; the plots are shown in black). The microscopic field of view (which was a rectangle) was smaller (39–96%) than these plots. The field of view was placed in the interveinal area of each plot.

In order to decrease the error in the measurements of stomatal and epidermal cell densities (at 100% FLE), graphs were made of the error of the mean versus sample number (sample number is fields of view; Fig. 1B). It was decided that the error had to be limited to 1–2% from the average of ten fields of view. This was realised by counting three fields of view for epidermal cell density and nine fields of view for stomatal density, in each sampling area (Fig. 1B).

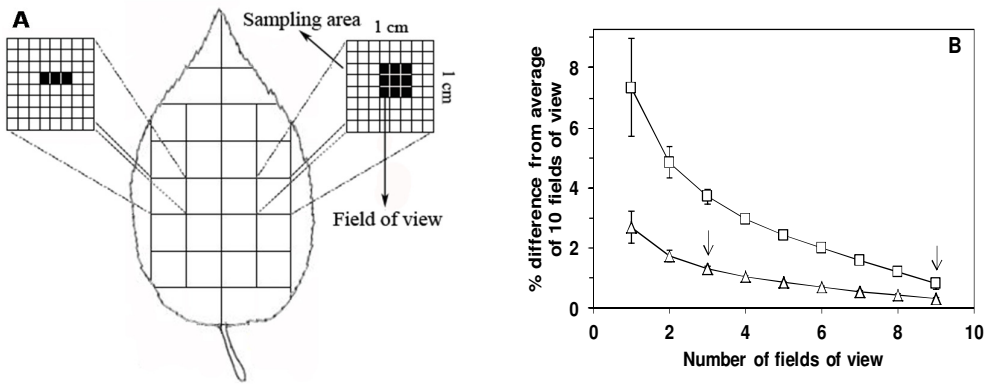

Fig. 1. Sampling protocol for stomatal and epidermal cell features, and the required number of fields of view on leaflets of *R. hybrida* cv. Pink Prophyta. (A) Sampling protocol for the entire lamina of leaflets that had just fully expanded (100% full leaflet expansion, FLE). A frame composed of 1 × 1 cm squares was superimposed upon the leaflet. These 1 x 1 cm squares were called sampling areas. The number of 1 x 1 cm sampling areas shown is an exact copy of the method used. Areas smaller than 1 cm^2 were either included in the laterally adjacent sampling area (at the base and the tip) or were taken together as one sampling area (at the lateral leaflet margins). The sampling areas were divided in 1.25 × 1.25 mm plots. In the central part of each sampling area three plots (for epidermal cell density measurement, left magnification) or nine plots (for stomatal density assessment, right magnification) were selected for fields of view. These plots are indicated as filled boxes. The area of the field of view was 39–96% smaller than the area of the plot, depending on the FLE. Thus the term 'plot' is not a synonym of 'field of view', it only indicates the spatial boundaries of the field of view. (B) Optimizing the number of fields of view for estimating stomatal (squares) and epidermal cell (triangles) densities. The graph shows the decrease in error when taking the average of increasingly more fields of view in a 1 x 1 cm sampling area. The error is expressed as the percentage difference between the average of 10 fields of view and the average of less than 10 fields of view. The arrows depict the number of fields of view per sampling area used in this study. Values are the means of 30 (stomatal density) or 15 (epidermal cell density) sampling areas ± SEM (one sampling area per fully expanded leaflet). When the SE bars are not visible, the SE is smaller than the symbol.

Leaflet size and stomatal population

Leaflet length at full leaflet expansion averaged 7.5 cm, while leaflet width was 4.8 cm on average. The average area of a fully expanded leaflet was 31 cm^2 (Fig. 2A).

The stomatal number over the whole leaflet was calculated by taking the algebraic product of the mean stomatal density in each sampling area and the area size (1 cm^2), and summing these values. Subsequently, the percentage of stomata at various leaflet expansion stages was compared to the total stomatal population at full leaflet expansion, i.e. the time when all stomata had been formed. The number of stomata followed an S-shape pattern during leaflet expansion (Fig. 2B). The increase in the number of stomata took place predominantly between 33% and 67% full leaflet expansion (FLE). During this

period more than 80% of (microscopically recognisable) stomata had been initiated. Thus, at 67% FLE over 90% of the final stomatal population was present (Fig. 2B).

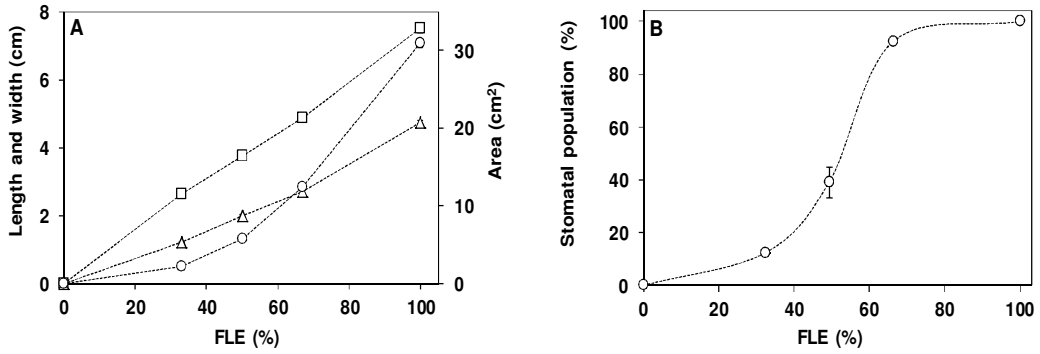

Fig. 2. Leaflet dimensions and the relative number of visible stomata, during elongation growth of leaflets of *R. hybrida* cv. Pink Prophyta. The data on leaflet elongation are expressed as percentage of full leaflet expansion (FLE). (A) Length (squares), width (triangles), and area (circles). (B) The percentage of microscopically visible stomata compared with those when the leaflets had just fully expanded. No more stomata were formed after 100% FLE. Values are the means of 4 (33%, 50% and 67% FLE) or 12 (100% FLE) leaflets ± SEM. The number of 1 x 1 cm sampling areas per leaflet was 2, 4, 10 and 28 for 33%, 50%, 67% and 100% FLE, respectively (nine fields of view/sampling area). When the SE bars are not visible, the SE is smaller than the symbol.

Stomatal density, epidermal cell density, and stomatal index

The mean stomatal and epidermal cell densities, as well as stomatal index were calculated by averaging the values of all sampling areas per leaflet. The stomatal density increased until 67% FLE, followed by a large decrease at complete leaflet expansion (Fig. 3A). In epidermal cells, in contrast, an exponential decrease in density took place throughout the period of leaflet expansion studied (Fig. 3B). The density of epidermal cells on a fully expanded leaflet was 8.8 times less than the one at 33% FLE. The stomatal index was close to 2% at the lowest leaflet expansion stage examined (33% FLE). It increased further during leaflet expansion to 9.4% in the fully expanded leaflet (Fig. 3C).

Stomatal length and width

The mean stomatal length and width were calculated by averaging the values of all sampling areas throughout the leaflet lamina. Stomatal length in leaflets of 33% FLE was less than half of that at full expansion (Fig. 3D). Stomatal length was only slightly higher (14%) than stomatal width at 33% and 50% FLE (Fig. 3D). During further leaflet expansion (67–100% FLE), stomatal length increased more than stomatal width (i.e. stomatal length/stomatal width ratio of 1.32).

The distribution of stomatal length throughout leaflet expansion is shown in Figure 4. It should be noted that in expanding leaflets (33–67% FLE) very short stomata

(about 14 μm) were always observed (Fig. 4A, B, C). The population of stomata slightly longer than 14 μm (the shortest length recorded) considerably increased from 33% through 50% to 67% FLE.

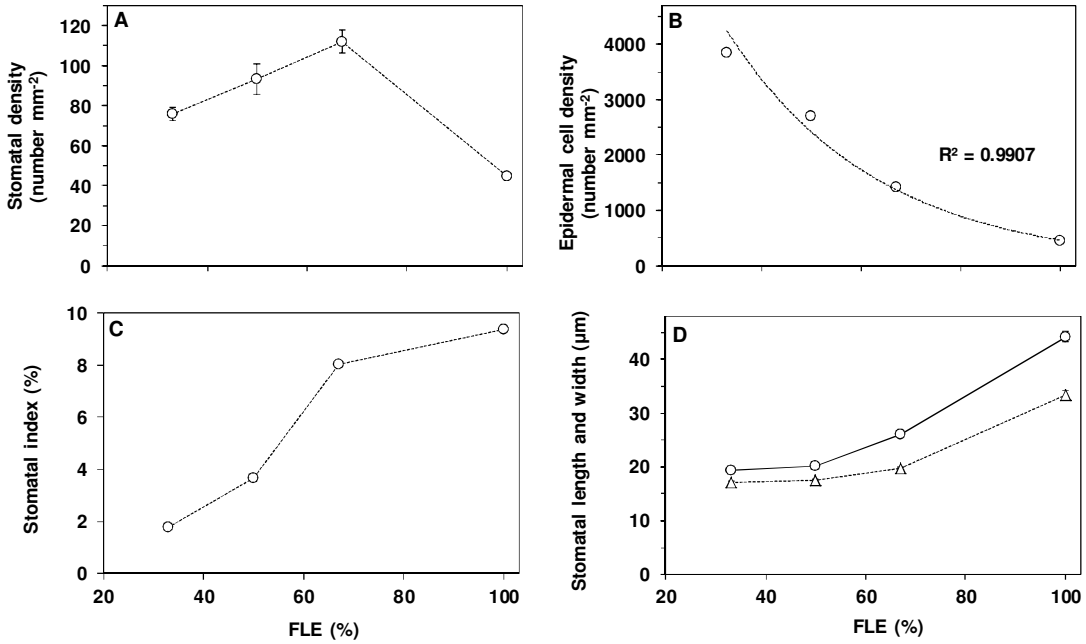

Fig. 3. Stomatal features during leaflet growth in *R. hybrida* cv. Pink Prophyta. (A) Average stomatal density (number of stomata per unit area). (B) Epidermal cell density (number of epidermal cells per unit area). (C) Stomatal index (the number of stomata per total number of cells). (D) Stomatal length (circles) and width (triangles). The data on leaflet elongation are expressed as percentage of full leaflet expansion (FLE). Values are the means of 4 (33%, 50% and 67% FLE, stomatal density) or 6 (100% FLE, stomatal length and width) or 12 (100% FLE, stomatal density) leaflets ± SEM, and refer to one representative leaflet for epidermal cell density, stomatal index, as well as stomatal length and width (33%, 50% and 67% FLE). The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively. Nine or three fields of view/sampling area were assessed from stomatal and epidermal cell densities, respectively. When the SE bars are not visible, the SE is smaller than the symbol.

Gradients in stomatal features

A gradient in stomatal density was observed along the length of expanding and fully expanded leaflets (Fig. 5). At the lowest FLE (33%), stomatal density showed a large increase from base-to-tip (Fig. 5A), but this increase was much less at 50% FLE (Fig. 5B). At 67% FLE, the gradient had become slightly reversed (more at the base than at the tip, Fig. 5C). This gradient was conserved in fully expanded leaflets, but the difference had

become small (insert of Fig. 5D). Close to the leaflet edge, the stomatal density was slightly higher than close to the main vein, at 67% and 100% FLE (Fig. 5E, insert of F).

Epidermal cell density decreased from the leaflet base to the leaflet tip at 33% and 50% FLE (Fig. 6A, B). This gradient had become small by 67% FLE (Fig. 6C), while it was reversed (i.e. slightly higher density of epidermal cells at the leaflet tip compared to leaflet base) in fully grown leaflets (insert of Fig. 6D). No or very weak gradients were found in epidermal cell density along the axis from the main vein to the leaflet edge, at 67% and 100% FLE, respectively (Fig. 6E, F).

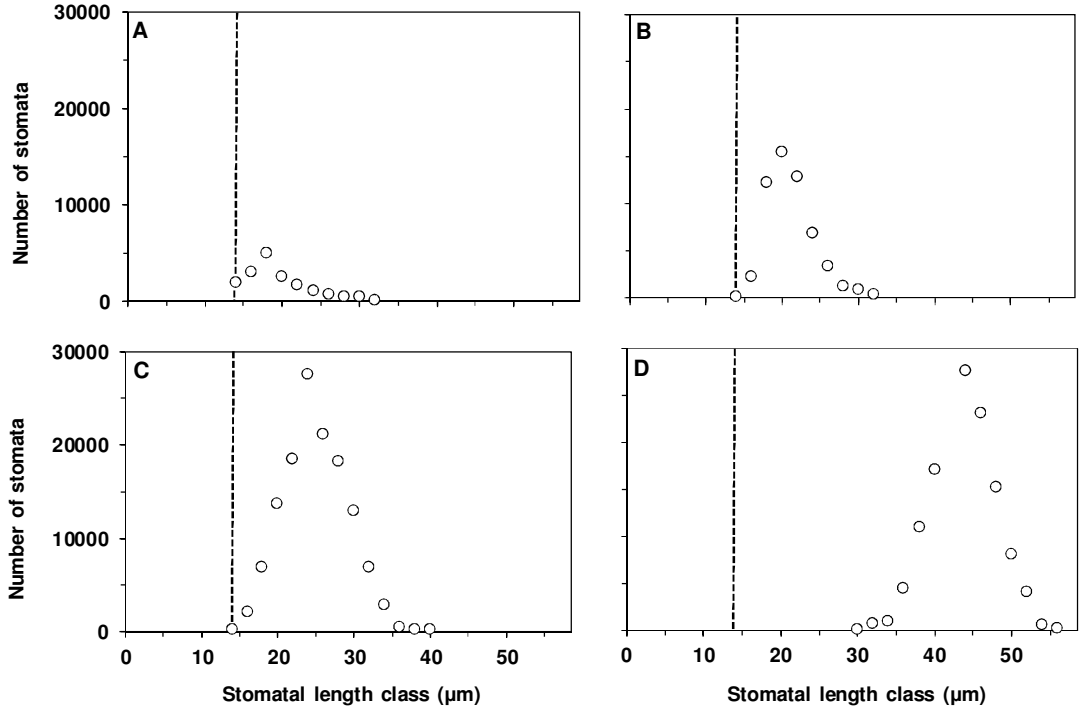

Fig. 4. Frequency distribution of stomata in various stomatal length classes on leaflets of *R. hybrida* cv. Pink Prophyta. The stomatal population was sampled at various percentages of full leaflet expansion (FLE; A: 33%, B: 50%, C: 67%, and D: 100%). The length class intervals were set at 2 µm. The vertical lines depict the 14 µm length class, which represents the shortest length class recorded. Frequency distributions are from representative leaflets. Stomatal length did not change after 100% FLE. The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively.

At 33% FLE, stomatal length was the same throughout the leaflet lamina (Fig. 7A). At later stages of leaflet growth, the stomata close to the leaflet tip were systematically longer (Fig. 7B, C). In fully expanded leaflets the stomatal length (insert of Fig. 7D) and pore length (data not shown) were slightly shorter close to the leaflet base. Instead, lack of gradients was found in stomatal length along the axis from the main vein to the leaflet

edge throughout leaflet expansion (Fig. 7E, F). Gradients were also not observed in pore aperture on non-stressed fully expanded leaflets (data not shown).

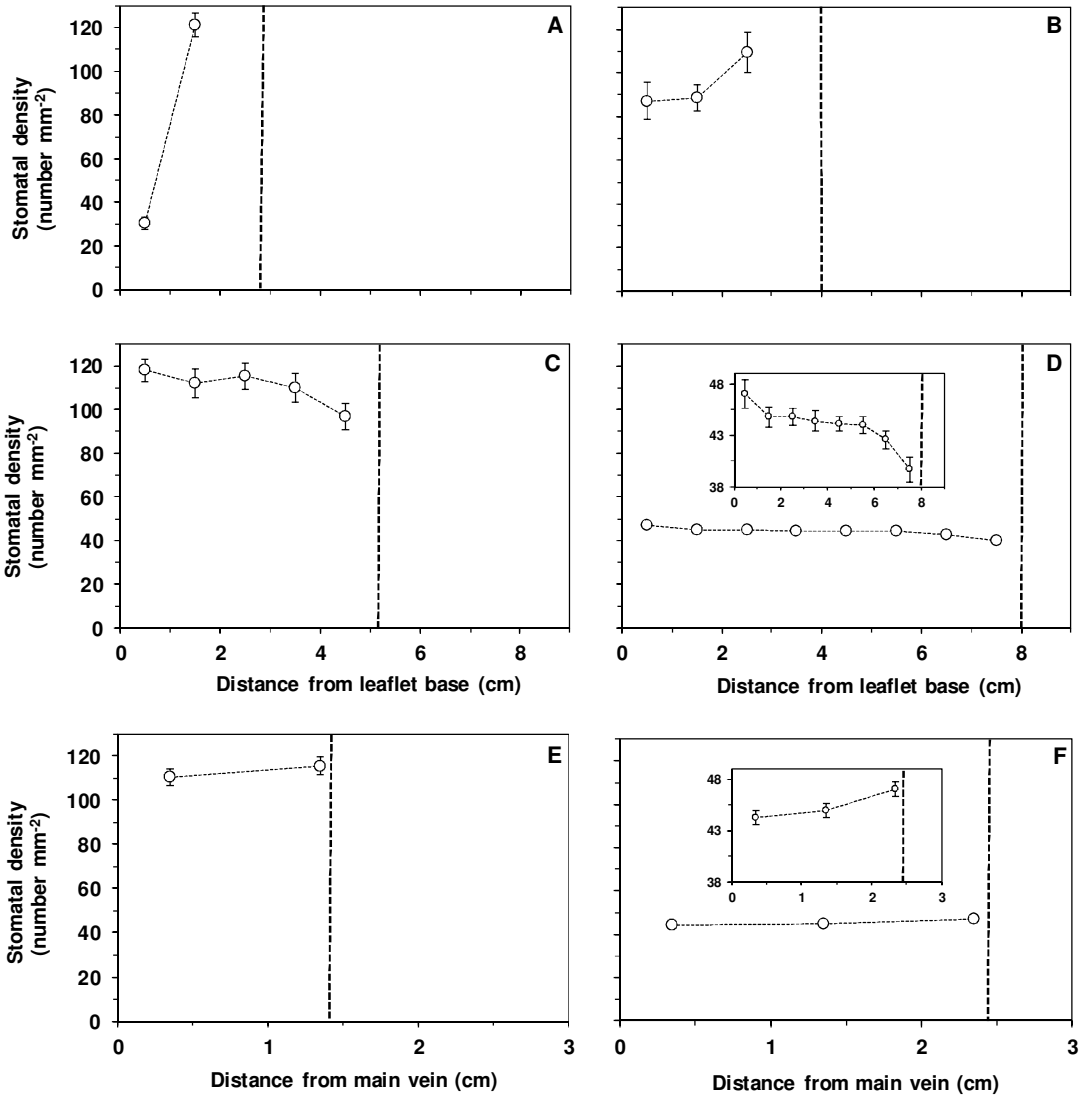

Fig. 5. Heterogeneity of stomatal density over the leaflet of *R. hybrida* cv. Pink Prophyta. Stomatal density is given as a function of the distance from the leaflet base (i.e. close to the petiole-leaflet lamina junction) and the leaflet tip (A, B, C, D) and the distance between the main vein (i.e. midrib) and the leaflet edge (E, F), at various percentages of full leaflet expansion (FLE): A: 33%, B: 50%, C, E: 67%, D, F: 100%. The vertical lines depict the leaflet length (A, B, C, D) or the distance between the leaflet edge and the main vein (E, F). Values are the means of 4 (33%, 50% and 67% FLE) or 12 (100% FLE) leaflets ± SEM. The number of 1 x 1 cm sampling areas per leaflet was 2, 4, 10 and 28 for 33%, 50%, 67% and 100% FLE, respectively (nine fields of view/sampling area). When the SE bars are not visible, the SE is smaller than the symbol.

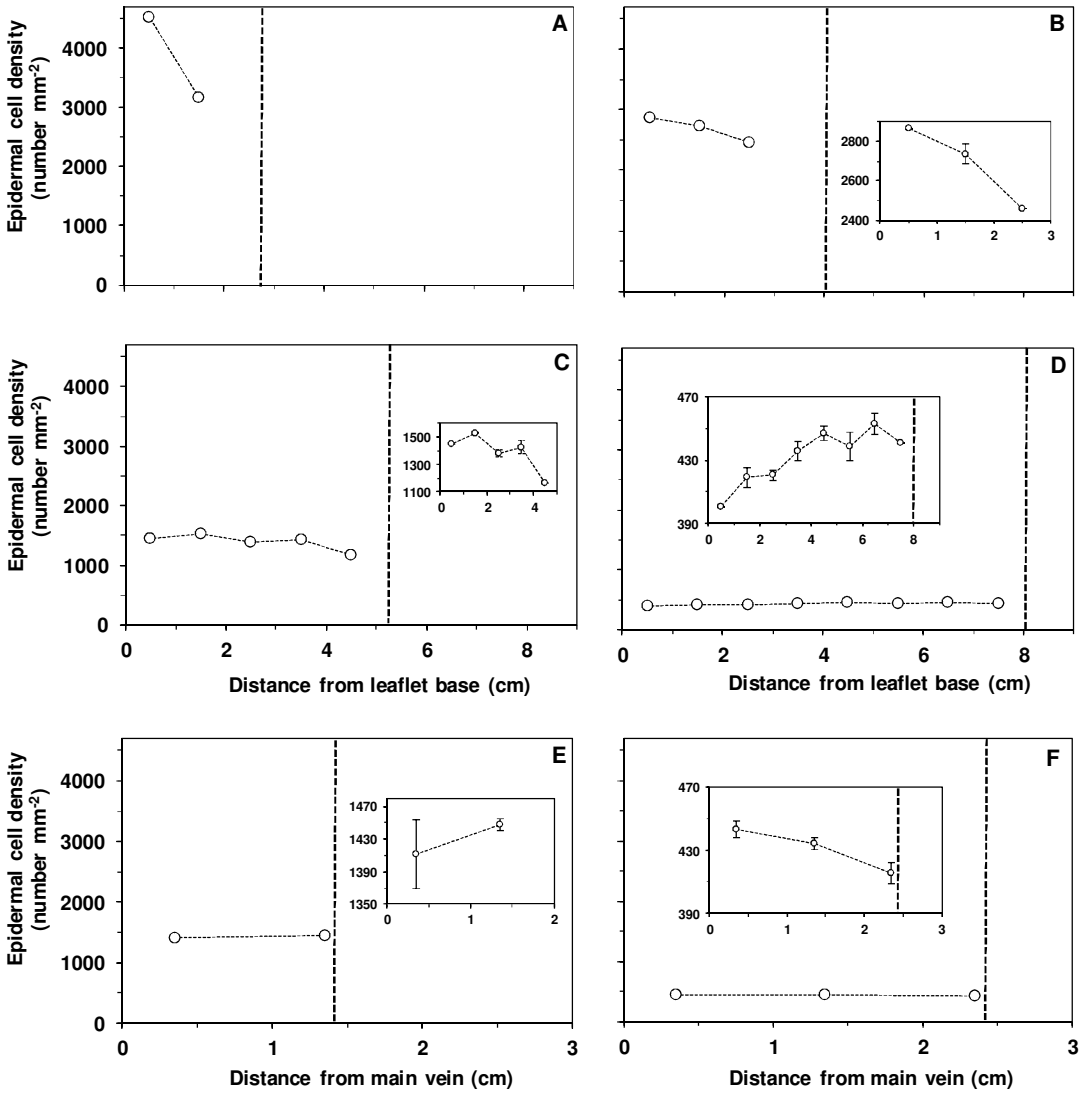

Fig. 6. Heterogeneity of epidermal cell density over the leaflet of *R. hybrida* cv. Pink Prophyta. Epidermal cell density is given as a function of the distance from the leaflet base (i.e. close to the petiole-leaflet lamina junction) and the leaflet tip (A, B, C, D) and the distance between the main vein (i.e. midrib) and the leaflet edge (E, F), at various percentages of full leaflet expansion (FLE): A: 33%, B: 50%, C, E: 67%, D, F: 100%. The vertical lines depict the leaflet length (A, B, C, D) or the distance between the leaflet edge and the main vein (E, F). Values are the means of several sampling areas within one representative leaflet ± SEM. The number of 1 x 1 cm sampling areas per leaflet was 2, 4, 10 and 28 for 33%, 50%, 67% and 100% FLE, respectively (three fields of view/sampling area). When the SE bars are not visible, the SE is smaller than the symbol.

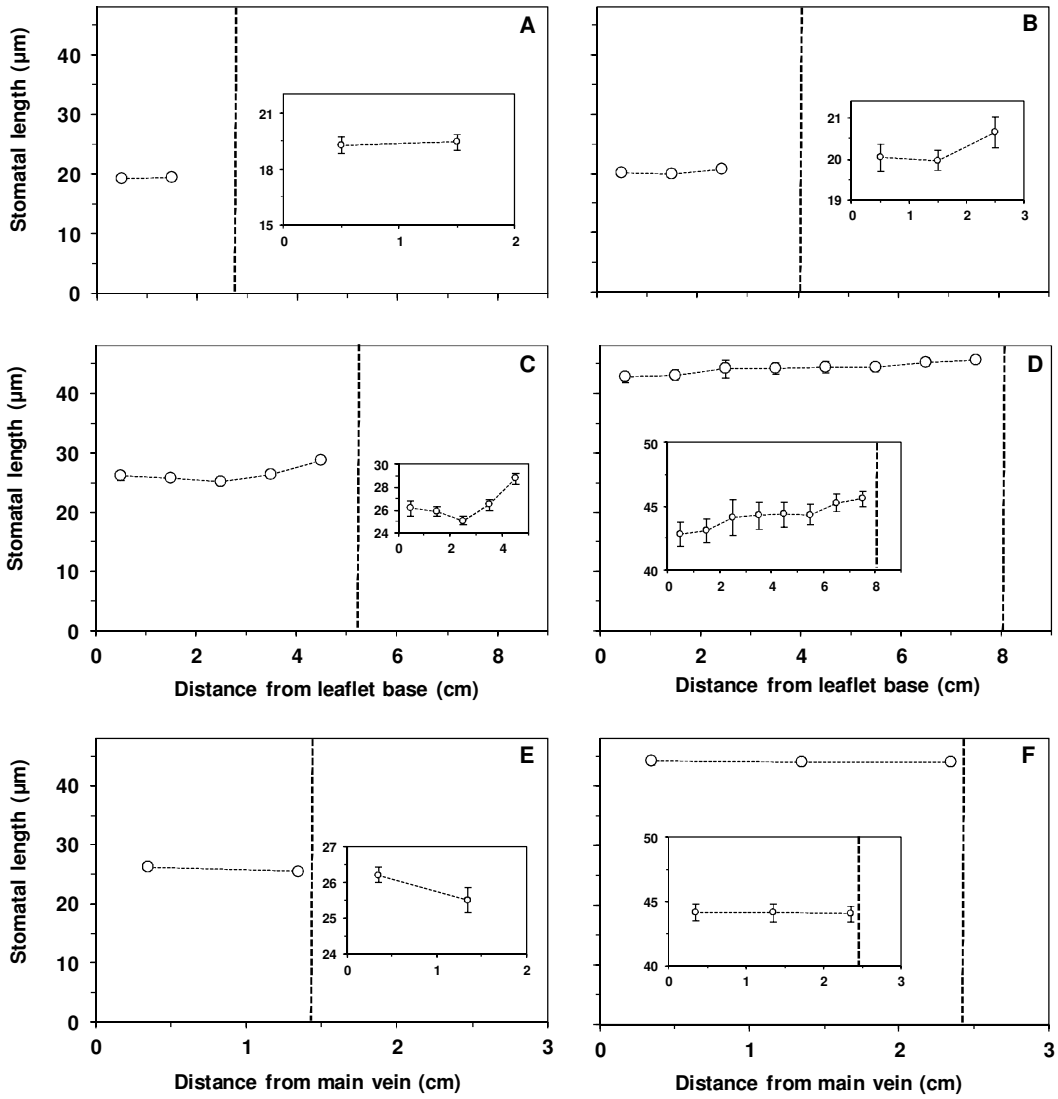

Fig. 7. Heterogeneity of stomatal length over the leaflet of *R. hybrida* cv. Pink Prophyta. Stomatal length is given as a function of the distance from the leaflet base (i.e. close to the petiole-leaflet lamina junction) and the leaflet tip (A, B, C, D) and the distance between the main vein (i.e. midrib) and the leaflet edge (E, F), at various percentages of full leaflet expansion (FLE): A: 33%, B: 50%, C, E: 67%, D, F: 100%. The vertical lines depict the leaflet length (A, B, C, D) or the distance between the leaflet edge and the main vein (E, F). Values are the means of one representative leaflet (33%, 50% and 67% FLE) or 6 leaflets (100% FLE) ± SEM. The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively. When the SE bars are not visible, the SE is smaller than the symbol.

Heterogeneity over the leaflet surface

Contour maps were made of stomatal density, epidermal cell density and stomatal index (Fig. 8). The within-leaflet variation was high at low FLE, but decreased towards 100% FLE. However, differences were still considerable at 100% FLE (Fig. 8; see also Table 1).

We observed symmetry of stomatal and epidermal cell densities at both sides of the main vein, throughout leaflet expansion (Table 1). The data show that the means would be only slightly different (about 2%), if only half of the leaflet lamina had been assessed rather than the whole lamina.

In general, the coefficient of variation of the data on stomatal density, epidermal cell density and stomatal index was higher at the macro scale (i.e. between sampling areas within the leaflet), compared to the micro scale (i.e. between fields of view within the sampling area) (Table 1). This variation was considerably larger during leaflet expansion, compared to fully expanded leaflets. The relative difference of the extremes varied between 18 (epidermal cell density) and 36 (stomatal index) % in fully expanded leaflets.

The coefficient of variation of both stomatal size and pore dimensions was higher at the micro scale, compared to the macro scale (Table 1). Stomatal length and width exhibited low spatial variability in expanding leaflets (33% and 50% FLE), while a larger variability was observed at 67% FLE (Table 1). The relative difference of the extremes in stomatal and pore lengths was about 16% in fully expanded leaflets. An even larger heterogeneity was found in pore aperture: the maximum relative difference across sampling areas within the leaflet was 25%.

Correlations between stomatal index, stomatal density, and epidermal cell density

To evaluate the underlying mechanism(s) behind the local variation in the stomatal characteristics the correlations between stomatal index, stomatal density, and epidermal cell density were examined (Fig. 9). A highly significant correlation was found between stomatal density and stomatal index (Fig. 9A). No correlation was found when epidermal cell density was plotted against stomatal density or index (Fig. 9B, C).

Modelling stomatal conductance (g_s)

The relative importance of stomatal features (i.e. stomatal density, pore aperture, length, and depth) on raising differences in g_s over the leaflet surface was calculated (Fig. 10), using a standard formula (Nobel 1991). Pore aperture and stomatal density had the largest influence on g_s (this resulted in the steepest slopes in Fig. 10). These parameters each contributed about 18% to the heterogeneity of g_s over the leaflet. The contribution of pore length and depth was relatively small (6 and 8%, respectively).

Table 1. Stomatal and epidermal cell features on leaflets of *R. hybrida* cv. Pink Prophyta, at different percentages of full leaflet expansion (FLE). The features are stomatal and epidermal cell densities, stomatal index and stomatal size (i.e. length and width). Minimum and maximum values are given, as well as the coefficient of variation at macro scale (between sampling areas within the leaflet) and micro scale (between fields of view within the sampling area). A comparison is also made between the means of the areas separated by the midrib (symmetry). In fully expanded leaflets the same is done for stomatal pore length and aperture. Values are the means of one representative leaflet (epidermal cell density; stomatal index; stomatal size in 33%, 50% and 67% FLE), 3 leaflets (pore aperture), 4 leaflets (stomatal density in 33%, 50% and 67% FLE), 6 leaflets (stomatal length and width; pore length) or 12 leaflets (stomatal density in 100% FLE), ± SEM. The number of sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively. Nine or three fields of view/sampling area were assessed for stomatal and epidermal cell densities, respectively.

FLE (%)	Min ± SEM	Max ± SEM	Coefficient of variation (%)		Symmetry main vein (%)
			Macro scale	Micro scale	
	Stomatal density (number mm^{-2})				
33	31 ± 3	121 ± 5	85	30	–
50	83 ± 7	109 ± 9	12	9	3
67	95 ± 6	123 ± 5	7	6	4
100	38 ± 1	51 ± 1	7	8	4
	Epidermal cell density (number mm^{-2})				
33	3126	4557	21	8	–
50	2457	2866	6	6	0
67	1116	1536	7	3	1
100	400	470	4	3	0
	Stomatal index (%)				
33	0.9	2.6	69	–	–
50	3.2	4.7	18	–	1
67	7.5	8.8	5	–	1
100	8.4	11.4	7	–	4
	Stomatal length (μm)				
33	19	19	0	22	–
50	20	21	0	15	–
67	25	29	0	16	–
100	41 ± 1	48 ± 1	4	8	–
	Stomatal width (μm)				
33	17	17	0	17	–
50	17	18	0	12	–
67	18	22	0	12	–
100	31 ± 1	36 ± 1	4	8	–
	Pore length (μm)				
100	23 ± 1	27 ± 1	5	10	–
	Pore aperture (μm)				
100	4 ± 1	5 ± 1	8	19	–

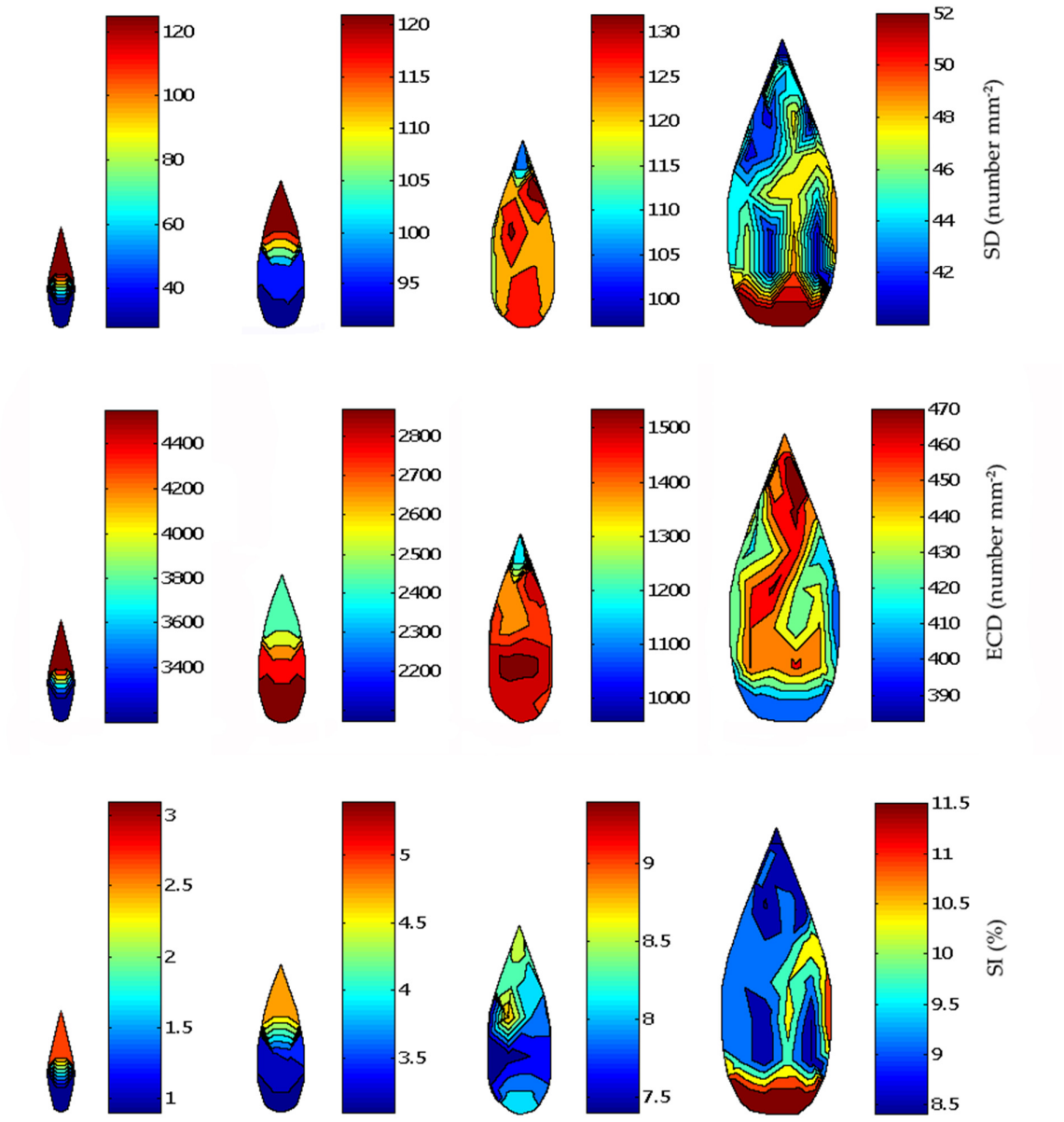

Fig. 8. Contour maps showing spatial heterogeneity of stomatal features over the leaflet of *R. hybrida* cv. Pink Prophyta. These features are stomatal density (SD), epidermal cell density (ECD) and stomatal index (SI), at various percentages of full leaflet expansion (FLE): 33%, 50%, 67% and 100%. Vertical indices (at the right of each FLE) denote the range of values. The data are based on measuring the whole leaflet surface. At full leaf expansion, sampling occurred within 28 areas of 1 cm^2, and nine or three fields of view per sampling area for stomatal and epidermal cell densities, respectively, as indicated in Figure 1. Sampling methods at earlier leaflet growth stages are described under Materials and Methods.

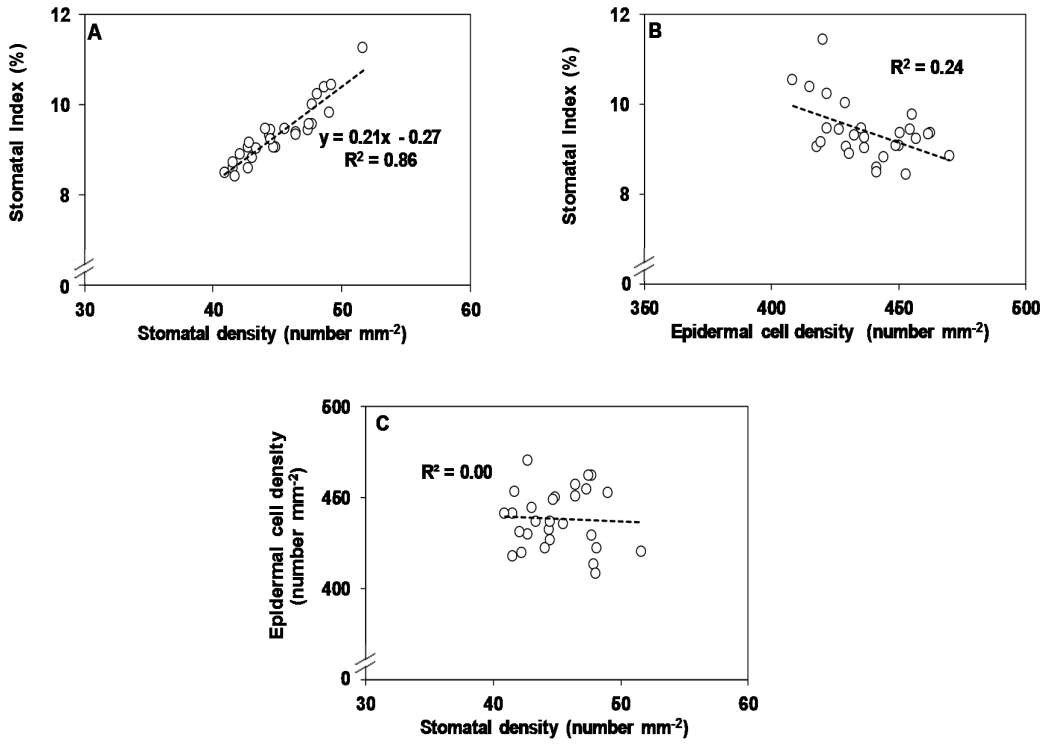

Fig. 9. Correlations between stomatal and epidermal cell features in fully expanded leaflets of *R. hybrida* cv. Pink Prophyta. (A) Correlation between stomatal index and stomatal density. (B) Correlation between stomatal index and epidermal cell density. (C) Correlation between epidermal cell and stomatal densities. Data are based on measuring the whole leaflet surface. Sampling occurred within 28 areas of 1 cm^2, and nine or three fields of view per sampling area for stomatal and epidermal cell densities, respectively, as indicated in Figure 1.

Stomatal spacing pattern

We used the R parameter to quantify stomatal distribution. R is based on measurement of the distances between a reference stoma and its three closest neighbours (Fig. 11). The R value was 0.46 in fully expanded leaflets. This value indicates that the stomata were distributed less than 1/2 of the value they would have (R = 1.0), if they had been randomly spaced. At earlier stages of leaflet expansion (50% and 67% FLE), the stomata were spaced at distances 1/4 and 1/3 of the value expected by a random pattern, respectively. The minimum spacing distance between adjacent stomata was found to be dependent on the leaflet expansion stage (Fig. 11).

Within the leaflet lamina, the stomatal spacing pattern was different in the region close to the veins, as the vein surface contains elongated epidermal cells, without stomata. The reference stomata were, therefore, selected not to be located in the regions close to the veins.

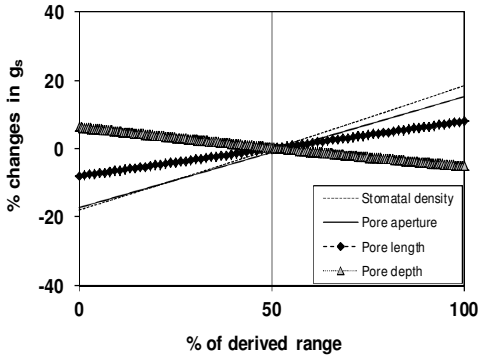

Fig. 10. Predicted sensitivity of the stomatal conductance (g_s) in *R. hybrida* cv. Pink Prophyta to changes in stomatal density and pore dimensions. The analysis employed the following ranges of values derived from observations: pore aperture, 3.9–5.4 μm; stomatal density, 34.2–46.9 stomata mm^{-2}; pore length, 22.3–26.1 μm; pore depth, 15.1–17.2 μm. The g_s to water vapour was predicted using Eqn 3. The vertical line represents the conductance obtained using the mid-range value for each variable; this was 137 mmol H_20 m^{-2} s^{-1}. Effects of adjusting each anatomical characteristic within its empirical range were calculated, keeping the other values constant, and these effects were expressed as a percentage of the mid-range value. The number of sampling areas within the leaflet was 28 (sampling area size of 1 cm^2), and 40 stomata or nine fields of view/sampling area were assessed for stomatal pore dimensions and stomatal density, respectively.

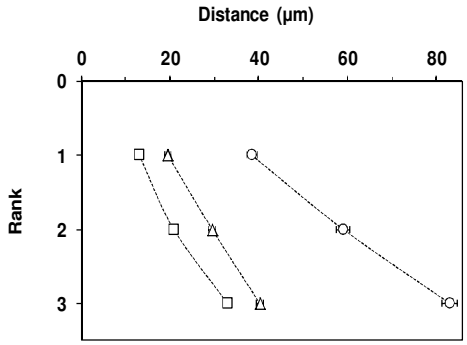

Fig. 11. Stomatal patterning in leaflets of *R. hybrida* cv. Pink Prophyta. Average distances are given from a reference stoma to 3 neighbouring stomata. These have been ranked 1 (nearest stoma) through 3 (most distant stoma). Data refer to various percentages of full leaflet expansion (50%: square; 67%: triangle; 100%: circle). Values are the mean distances of other stomata to 250 reference stomata ± SEM. When the SE bars are not visible, the SE is smaller than the symbol.

Discussion

Stomatal features during leaflet expansion

The contribution of stomata to the total cell number (i.e. stomatal index) has been suggested to remain constant during most of the period of leaf expansion (Kürschner 1997). However, our data (Fig. 3C) and those of Ceulemans et al. (1995) and Fu et al.

(2010) show a marked increase in stomatal index during leaflet growth. This increase in stomatal index was mainly due to a decrease in the epidermal cell density until 67% full leaflet expansion (FLE) (Fig. 3B). During the final expansion phase (67–100% FLE), a considerable decrease was found in both stomatal (which by itself would decrease stomatal index) and epidermal cell densities (Fig. 3A, B) resulting in a further increase of stomatal index (Fig. 3C), whereby the decrease in stomatal density having the largest effect.

The increase in stomatal density from 33% through 50% to 67% FLE was the result of the increasing number of stomata (due to stomatal initiation; Fig. 2B), while the decrease in stomatal density from 67% FLE and onwards was the result of lower stomatal initiation compared to epidermal cell expansion.

At 33% and 50% FLE the stomata had only slightly longer (14%) length than width, giving rise to a rather round stomatal form (Fig. 3D). At later stages of leaflet growth, stomatal length increased more than stomatal width (Fig. 3D), giving stomata their characteristic elliptic shape. Figure 4 shows that stomata of various lengths occurred together at each stage of leaflet growth. This is a characteristic of dicotyledonous plants (Croxdale 2000, Rawson and Craven 1975).

The presence of stomata of very short length (between 14 and about 20 μm), during 33–67% FLE, suggests that stomata were initiated throughout this period. No such small stomata were present at 100% FLE. This indicates that stomatal initiation had finished only by the time of full leaf expansion.

Differences in stomatal density, epidermal cell density and stomatal length over the leaflet

We observed stomatal density gradients during leaflet expansion (Fig. 5A, B, C). Stomata were initially (33–50% FLE) much more dense in the apical leaflet region and subsequently became more frequent towards the base of the leaflet (67% FLE). Similar data have been reported in elongating *Tsuga heterophylla* needles (Kouwenberg et al. 2004) and developing *Prunus persica* leaves (Stavroulaki et al. 2007). In fully expanded rose leaflets, there was only a small difference in stomatal density both between the leaflet base and tip (Fig. 5D) and between the areas close to the main vein and leaflet margins (Fig. 5F). In other species either no clear spatial difference was observed in stomatal density on fully expanded leaves (Lawson et al. 2002, Poole et al. 1996, 2000) or a higher density was observed at the leaf tip and margins (Smith et al. 1989) or in the middle of the lamina (Stavroulaki et al. 2007).

At 50% and 67% FLE, stomata were systematically longer at the leaflet tip compared to the leaflet base (Fig. 7B, C), and this difference was still present at full leaflet expansion (Fig. 7D). Instead, in *Commelina communis* Smith et al. (1989) reported shorter stomata at the leaf tip and leaf margins of fully expanded leaves.

A base-to-tip gradient in the epidermal cell density was observed in expanding leaflets (Fig. 6A, B, C). Lower epidermal cell density at the leaf tip, compared to the leaf

base, has also been found in developing *Ricinus communis* leaves (Heckenberger et al. 1998, Roggatz et al. 1999). Instead, at full rose leaflet expansion the epidermal cell density at the leaflet base was lower than the one at the leaflet tip (Fig. 6D).

The observed within-leaflet differences have implications for sampling. These differences necessitate more than one sampling area per leaflet, especially during leaflet expansion. In expanding leaflets, the location of the sampling areas can now be determined, since the spatial direction and intensity of changes in these features is now known. We observed that the gradients in fully expanded leaflets were smaller (Fig. 5, 6 and 7). Although some gradients occur in a systematic (predictable) pattern (e.g. stomatal density was slightly different at leaflet base and tip), a large part of this heterogeneity is irregular (Fig. 8) which does not allow to determine the location of the sampling area on the leaflet in a manner which would give more accurate estimate of the leaflet real mean. As mentioned in the Introduction, three other studies thus far addressed the within-leaf heterogeneity in stomatal density and index, in fully expanded leaves. They also report considerable heterogeneity (Lawson et al. 2002, Poole et al. 1996, 2000).

It was here demonstrated that heterogeneity in the macro scale, rather than that at the micro scale, was the main source of variability in stomatal and epidermal cell densities of rose leaflets, throughout their expansion (Table 1). In contrast, in individual stomatal features (i.e. stomatal and pore dimensions) the variation at the micro scale was considerably higher than that at the macro scale (Table 1). Consequently, for stomatal and epidermal cell densities a higher number of sampling areas per leaflet (rather than more fields of view per sampling area) will lead to a more accurate estimate of the leaflet real mean. Instead, for stomatal and pore dimensions a higher number of fields of view per sampling area is important for accurate estimates. Specifically for stomatal pore aperture, a higher number of sampling areas per leaflet is also recommended (due to high variability at macro scale, Table 1).

Two forms of macro heterogeneity have been distinguished: (i) trends (i.e. continuous and smooth transition between one area of the leaf to the other), and (ii) patches (i.e. sharp transitions between various areas of the leaf, Weyers and Lawson 1997). It was now found that the heterogeneity in stomatal and epidermal cell densities initially showed the features of trends (parallel contour lines in Fig. 8). This was observed until 50% FLE. During subsequent stages of leaflet expansion (67–100% FLE) these differences were patches, as shown by the mosaic-like pattern over the leaflet surface (Fig. 8).

Origin of within-leaflet variation in stomatal density and index

Variation over the leaf in stomatal density has been attributed to three mechanisms (Beerling and Chanoler 1993, Poole et al. 1996), which are: (i) uneven differentiation of stomatal and/or epidermal cells, resulting in variation in respective cell numbers (the

differentiation hypothesis), (ii) uneven expansion of epidermal cells, causing uneven spacing of stomata (the expansion hypothesis), and (iii) combination of both uneven cell differentiation and uneven epidermal cell expansion (mixed differentiation and expansion hypothesis).

In rose leaflets, a significant correlation was found between stomatal density and index (Fig. 9A). This suggests that the heterogeneity in stomatal density and index resulted from uneven stomatal differentiation (Poole et al. 1996). The lack of correlation between stomatal and epidermal cell densities (Fig. 9C) shows that local variation in epidermal cell expansion did not contribute to the differences in stomatal density and index. Moreover, the lack of correlation between epidermal cell density and stomatal index (Fig. 9B) suggests that uneven epidermal cell differentiation did not contribute to within-leaflet variation in stomatal density and index. The data in the present species therefore agree with the differentiation hypothesis (i): variation in stomatal density and index is due to uneven differentiation of stomatal cells.

Modelling stomatal conductance (g_s)

Between sampling areas on the leaflet we found a difference in g_s of 37%, at 100% FLE (Fig. 10). Variation in g_s was mostly due to differences in stomatal density and pore aperture. These data show that a considerable error can occur when calculating a mean value of g_s for a leaf, when this is based on data from a single sampling area. It is, therefore, evident that neglecting stomatal heterogeneity may give a false picture of the real value of g_s.

Stomatal spacing pattern

We found that stomata were closer to each other than expected by a random pattern, and that this spacing pattern depended on the leaflet expansion stage (Fig. 11). Thus the final stomatal spacing pattern was determined at the end of growth rather than during earlier leaflet expansion. This is in contrast to data reported in *Sansevieria trifasciata* (Kagan and Sachs 1991). The stomatal spatial pattern during leaflet expansion can be implemented by both (i) newly formed stomata being inserted into already spaced stomatal cells, and (ii) expansion of epidermal cells (Croxdale 2000). Given the changes in stomatal initiation (Fig. 2B) and epidermal cell density (Fig. 3B), changes in the stomatal spacing pattern between 50% and 67% FLE were driven by both above-mentioned factors, while changes in the stomatal spacing pattern during the final expansion stage (67–100% FLE) were mostly the result of epidermal cell expansion (since a small fraction of stomata remain to be initiated).

Conclusions

It was demonstrated that in rose leaflets considerable spatial heterogeneity was present in stomatal density and size, as well as in epidermal cell density, especially at early phases of leaflet growth. This heterogeneity cannot be found when using only one 1 x 1 cm sampling area, or even when using a few such areas. In rose, it was adequate to sample only one half of the leaflet, as the other half mirrored the data. Stomatal pore aperture also varied between sampling areas within the lamina of fully expanded leaflets, but also between stomata within these areas. In contrast, stomatal size and stomatal pore length did not vary considerably between sampling areas, but they varied between stomata in each sampling area. This means that measurement of a considerable number of stomata per sampling area is to be recommended. The within-leaflet differences in stomatal features ought to be considered to avoid erroneous conclusions as here illustrated for (i) analysis of stomatal formation and (ii) estimation of leaflet transpiration based on stomatal morphology.

Acknowledgements

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CHAPTER 5.2

Growing stomata at high relative air humidity exceeding a critical length do not become functional at full leaf expansion

Abstract

Cv. Pink Prophyta cut roses that had grown at high relative air humidity (RH, 95%) were previously found to show enhanced transpiration in the light, and their stomata did not properly close in response to darkness or water deficit. This lack of normal stomatal response was increasingly present when the ambient RH had been high at later stages of leaflet growth, before transfer to moderate RH (60%). Here we tested hypotheses relating to the contribution of stomatal features in these phenomena. Stomatal density and size were assessed over the entire leaflet surface at 33%, 50%, 67%, and 100% of full leaflet expansion (FLE; proportion of leaflet length relative to its final length). Stomatal pore dimensions were measured at 100% FLE. High RH during growth did not affect stomatal density, but resulted in longer pore length and wider pore aperture. This explains why cut flowers grown at high RH showed higher rates of water loss in the light. Stomata of fully grown leaflets did not show proper closure after dehydration. This lack of closure was incremental when the period of growth at high RH had been longer (before transferring the plants to moderate RH). A correlation was found between the number of stomata having reached a late stage of development at high RH and their lack of normal closure. When all stomata were shorter than about 32 μm by the time of transfer to moderate RH, their response to dehydration was normal. The more stomata were longer than about 32 μm by the time of transfer to moderate RH, the clearer was the lack of the normal response to dehydration.

Fanourakis D, van Doorn WG, Carvalho SMP, Heuvelink E (2011) Growing stomata at high relative air humidity exceeding a critical length do not become functional at full leaf expansion. (to be submitted)

Introduction

After their initiation, stomata grow considerably in length and width (Gay and Hurd 1975, Wild and Wolf 1980).

Here, we studied stomatal initiation, growth, and pore opening with regard to a problem in the regulation of water loss in cut rose flowers. It was found previously that growth of cv. Pink Prophyta roses at high relative air humidity (RH, 95%) during leaf expansion resulted in a high rate of transpiration (during the light period) in cut flowers, compared with the transpiration of cut flowers grown at moderate RH (60%) (Fanourakis et al. 2011b).

Furthermore, it has been established that the stomata of cut flowers grown at high RH showed only little closure, both as response to darkness and in response to leaf dehydration (Fanourakis et al. 2011b, Torre et al. 2003). Interestingly, the problems with lack of stomatal closure in response to dehydration, measured in fully expanded leaflets, became increasingly greater when the duration of leaflet growth at high RH was longer, before transferring the plants to moderate RH. Thus normal stomatal reaction to dehydration occurred in plants that had been grown at high RH and were transferred to moderate RH by the time the leaflets were still at a very early stage of expansion (25% full leaflet expansion, FLE), but the stomata showed increasing lack of response when the transfer from high to moderate RH took place at later stages of leaflet growth (Fanourakis et al. 2011b).

The present study is meant to find reasons for these observations. It was hypothesised that a) there are more stomata per unit leaflet surface, and these stomata have a longer pore length and a wider pore opening if plants are grown continuously at high RH compared to those grown at moderate RH (this would explain the higher rate of transpiration in the light), and b) there are growth stages where stomatal functionality is more sensitive to the effect of high RH. This would mean that the regulation of water loss is determined by RH dependent on the stage of stomatal growth (development). This might help to explain the increasing effect of high RH during the later stages of leaflet growth.

Materials and Methods

Plant material and growth conditions

Rooted cuttings of the cut rose cv. Pink Prophyta (*Rosa hybrida* L.) were obtained from a commercial propagator (Kordes, De Kwakel, The Netherlands), and planted in 3.6 L pots containing a 3:1 (v/v) mixture of cocopeat (Jongkind Grond BV, Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull Rhenen, The Netherlands). This cultivar was selected because of its increased rate of transpiration when grown at high RH rather

than moderate RH (Fanourakis et al. 2011a, b). Plants, one per pot, were grown in two growth chambers (l × w × h = 1.3 × 0.8 × 1.3 m) as a single shoot, at a density of 30 plants m^{-2}. In one growth chamber the RH was 60 ± 3% and in the other 95 ± 1%. Both chambers had constant day and night temperatures (19 ± 1 °C), resulting in vapour pressure deficits of 0.88 ± 0.12 kPa (moderate RH) or 0.11 ± 0.03 kPa (high RH). Climate parameters were recorded automatically every 5 min, using data loggers (Fourier MicroLog EC650, MicroDAQ.com Ltd, Contoocook, NH). The ambient CO_2 concentration during the light period was 370 ± 50 μmol mol^{-1} (determined using Indoor Air Quality Meter, Model 8760, TSI Incorporated, Shoreview, MN). Fluorescent tubes (TLD 58W/84, Philips, Eindhoven, The Netherlands) provided 300 ± 20 μmol m^{-2} s^{-1} photosynthetically active radiation (Model LI-250, LI-COR, Lincoln, NE) at 70 cm from the soil level, which corresponds to the top of fully grown plants. An 18 h light - 6 h dark cycle was used. Plants were watered automatically with a nutrient solution, as described by Fanourakis et al. (2009).

Percentage of full leaflet expansion (FLE)

The expanding leaflets were sampled at various stages of elongation growth. Data referred to various stages of leaflet expansion, but were selected later on to represent only 33%, 50%, 67% and 100% of full leaflet expansion (FLE). FLE is defined as the proportion of leaflet length at assessment, relative to final leaflet length (Fanourakis et al. 2011a, Woodall et al. 1998). The final leaflet length was determined to be reached when three successive leaflet length data, taken at daily intervals, showed no increase. The 33%, 50%, and 67% of FLE corresponded to 7%, 19%, and 39% of the final leaflet area, respectively.

Stomatal density and size

R. hybrida has compound leaves, where leaflets arise on both sides of the rachis (i.e. in pairs) besides the terminal leaflet (odd-pinnate arrangement). Measurements were carried out on one leaflet of the first pair of lateral leaflets from the first order penta-foliate leaf (counting from the apex). One of the lateral leaflets was used for stomatal feature measurements, while the final length was determined in its symmetrical leaflet (paired sampling).

Stomatal characters were evaluated using sampling areas of 1 × 1 cm (Poole and Kürschner 1999). The sampling protocol covered the entire abaxial (lower) leaflet surface, as rose is a hypostomatous species (i.e. astomatous adaxial leaflet surface). The number of sampling areas per leaflet varied with the leaflet expansion stage. This was 2, 4, 10, and 28 at 33%, 50%, 67%, and 100% of FLE, respectively. To record the spatial location of the obtained data, these were represented on a three-dimensional Cartesian co-ordinate system. The variables x and y defined the distance of the area to the main vein (i.e.

midrib) and to the leaflet base (i.e. petiole-leaflet lamina junction) respectively, while the value of the measured variable was assigned z.

Each sampling area was divided in plots of 1.25 × 1.25 mm. The rectangular fields of view were setup in plots (one field of view/plot) located towards the centre of the sampling area. The fields of view within the plot were located in interveinal areas, because veins were devoid of stomata (Weyers and Lawson 1997). Since the areas between the veins were smaller at lower FLE, the area of the field of view was adjusted accordingly [being 3.8%, 3.8%, 15% and 61% of the total plot area (1.56 mm^2) for 33%, 50%, 67% and 100% FLE respectively]. The fields of view with an area lower than the area of the image taken in this magnification (0.96 mm^2) were taken by selecting part of this image. Using the method of Kubinova (1993) an unbiased decision could be made concerning the inclusion of stomata on the edge of a field of view.

Newly formed stomatal initials and epidermal cells can only be distinguished from one another with the use of molecular probes (Boetsch et al. 1995). We studied stomata at a later phase, when they could be distinguished from epidermal cells by light microscopy (i.e. when the stomatal pore length was externally visible). For determining the stomatal density, a magnification of 100 × was used and four leaflets per expansion stage and humidity level were assessed.

The percentage of stomata present at various leaflet expansion stages was defined as the amount of counted stomata at that stage divided by the final stomatal population (just after complete leaflet expansion). It is to be noted that for each lateral leaflet in which stomatal density was determined (which is a destructive procedure), its symmetrical one was used to count the number of stomata when this leaflet had fully expanded. The absolute number of stomata per leaflet was calculated by multiplying the stomatal density of each sampling area by its surface size and summing these values (Gupta 1961, Tichá 1982) (Eqn 1). Measurements were made in four leaflets per expansion stage and humidity level.

$$\text{Absolute stomatal number} = \sum_{1}^{n} (\text{stomatal density}) \times (\text{sampling area}) \qquad (1)$$

To determine length and width of the stomata, a magnification of 1000 × was used. Fifty, 75, and 100 randomly selected stomata in each sampling area were measured in a representative leaflet per humidity level at 67%, 50%, and 33% FLE, respectively. In six fully expanded leaflets per humidity regime, 40 stomata per sampling area were measured.

The stomatal pore length and pore aperture were also determined in 40 stomata per sampling area of three non-stressed fully expanded leaflets per ambient moisture condition. The pore area was calculated as the area an ellipsis with major and minor axes the pore length and pore aperture, respectively.

All stomatal features were determined using the silicon rubber impression technique (Weyers and Meidner 1990). Details of the impression method and image analysis are described by Fanourakis et al. (2011).

Statistical design and analysis

Data were analysed by one-way ANOVA using Genstat software (10th edition, VSN International Ltd, Hemel Hempstead, Herts, UK). Treatment effects were tested at 5% probability level and mean separation was carried out using least significant differences based on Student's *t*-test ($P \leq 0.05$).

Results

Leaflet dimensions, stomatal density and size

In plants grown at moderate RH (60%), leaflet length at full expansion averaged 7.5 cm, and leaflet width 4.8 cm. Leaflet area increased from 2.2 cm^2 at 33% full leaflet expansion (FLE) to 31 cm^2 at complete expansion (100% FLE), a relative increase from 7 to 100%. In plants grown at high RH (95%), the leaflet area was 12% larger, because the leaflets were slightly longer and wider (data not shown).

The initiation and growth of stomata during leaflet expansion were analysed by determining the stomatal density and size (length and width) at 33%, 50%, 67%, and 100% FLE in the whole abaxial leaflet surface (sampling areas with a size of 1 cm^2).

The mean stomatal density was calculated by averaging the values of all sampling areas (2–28, depending on the FLE), which cover the whole leaflet surface. The stomatal density increased until 67% FLE, followed by a large decrease at complete leaflet expansion (Fig. 1A). Throughout leaflet expansion and at 100% FLE, stomatal density seemed slightly higher on leaflets of plants grown at high RH (Fig. 1A), but this difference was not statistically significant (data not shown).

In fully expanded leaflets stomatal density was slightly higher at the leaflet base than at the leaflet tip (Fig. 2A) and density was also greater at the margins compared to the central areas (Fig. 2B). These gradients were independent of RH during growth (Fig. 2A, B).

The mean stomatal length and width were also calculated by averaging the values of all sampling areas per leaflet (2–28, depending on the FLE). Stomatal length was only 14–22% greater than stomatal width at 33% and 50% FLE (Fig. 1B, C). During further leaflet expansion (67–100% FLE), stomatal length increased more than width, resulting in a stomatal length/width ratio of at least 1.3. Average stomatal length on leaflets at 33% FLE was less than half of that in fully expanded leaflets (Fig. 1B). The length of stomata was not affected by atmospheric humidity, at 33% FLE (Fig. 1B). However, at later stages

of leaflet expansion (50–100% FLE) the average stoma on leaflets expanding at high RH was longer (12–23%, Fig. 1B) and wider (10–17%, Fig. 1C).

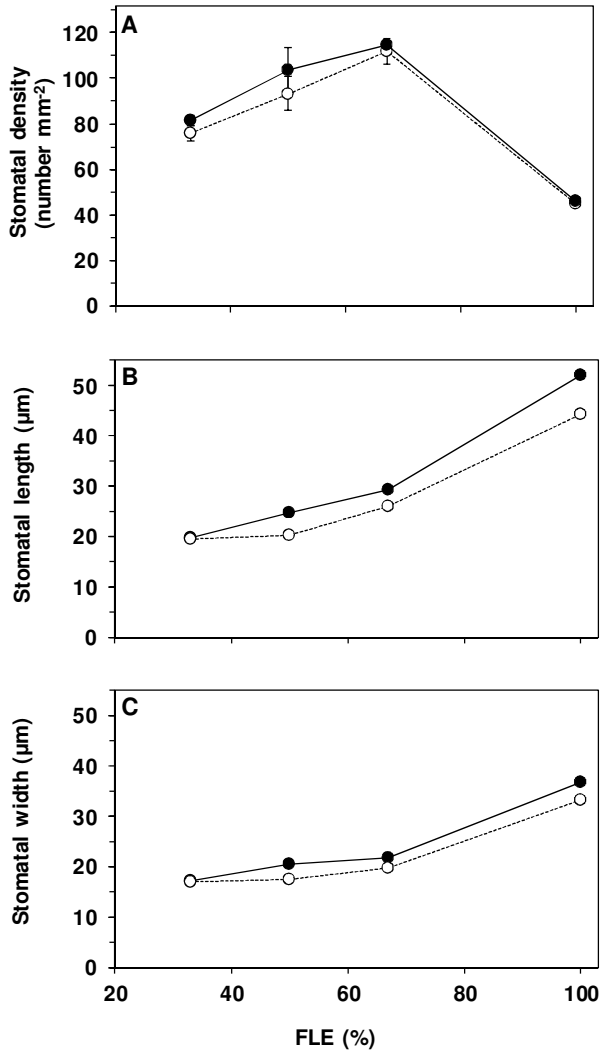

Fig. 1. Stomatal features during leaflet growth in cut rose cv. Pink Prophyta, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity. (A) Average stomatal density. (B) Stomatal length. (C) Stomatal width. The data on leaflet elongation are expressed as percentage of full leaflet expansion (FLE). Values are the means of 4 (33%, 50% and 67% FLE) or 12 (100% FLE) leaflets ± SEM for stomatal density, and refer to one representative (33%, 50% and 67% FLE) leaflet or 6 (100% FLE) leaflets ± SEM for stomatal length and width. The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively. Nine fields of view/sampling area were assessed from stomatal density. When the SE bars are not visible, the SE is smaller than the symbol.

At full expansion, stomatal length was slightly shorter close to the leaflet base (Fig. 2C). This was independent of RH during growth (Fig. 2C). No gradient was found in stomatal length when going from the leaflet axis to the leaflet margins (Fig. 2D).

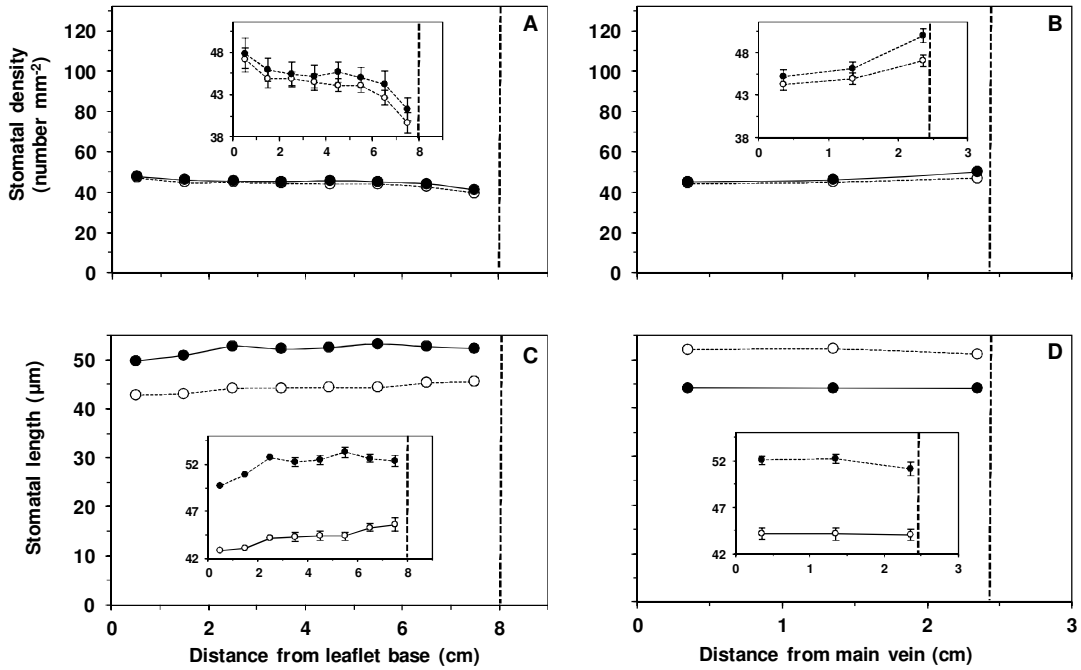

Fig. 2. Heterogeneity of stomatal density and stomatal length over the leaflet surface of cut rose cv. Pink Prophyta, grown at moderate (open symbols, 60%) or high (closed symbols, 95%) relative air humidity. (A, B) Stomatal density. (C, D) Stomatal length. The features are given as a function of the distance from the leaflet base (i.e. close to the petiole-leaflet lamina junction) and the leaflet tip (A, C) and the distance between the main vein (i.e. midrib) and the leaflet edge (B, D) of fully expanded leaflets. The vertical lines depict the leaflet length (A, C) or the distance between the leaflet edge and the main vein (B, D). Values are the means of 6 (stomatal length) or 12 (stomatal density) leaflets ± SEM. Twenty eight 1 x 1 cm sampling areas within the leaflet were assessed (nine field of view and 40 stomata/sampling area for stomatal density and stomatal length, respectively). When the SE bars are not visible, the SE is smaller than the symbol.

Distribution of stomatal length, pore aperture, and pore area

The distribution of stomatal length over the stomatal population throughout leaflet expansion is shown in Figure 3. It should be noted that very short stomata (14 µm) were always present on leaflets that were expanding at both moderate and high RH (Fig. 3A, B, C).

At 33% FLE, no effect of RH was found on the stomatal length distribution (Fig. 3A). However, a clear effect of RH was found at 50% FLE (Fig. 3B) and at later stages of leaflet expansion (Fig. 3C, D). By then, stomatal length had clearly shifted to the right (they were longer) on leaflets from plants grown at high RH. In plants grown at

moderate RH, the majority of the leaflet stomatal population (≥ 92%) was shorter than 32 µm at 67% FLE, while nearly all stomata (99.9%) were longer than 32 µm at 100% FLE (see dashed line in Fig. 3).

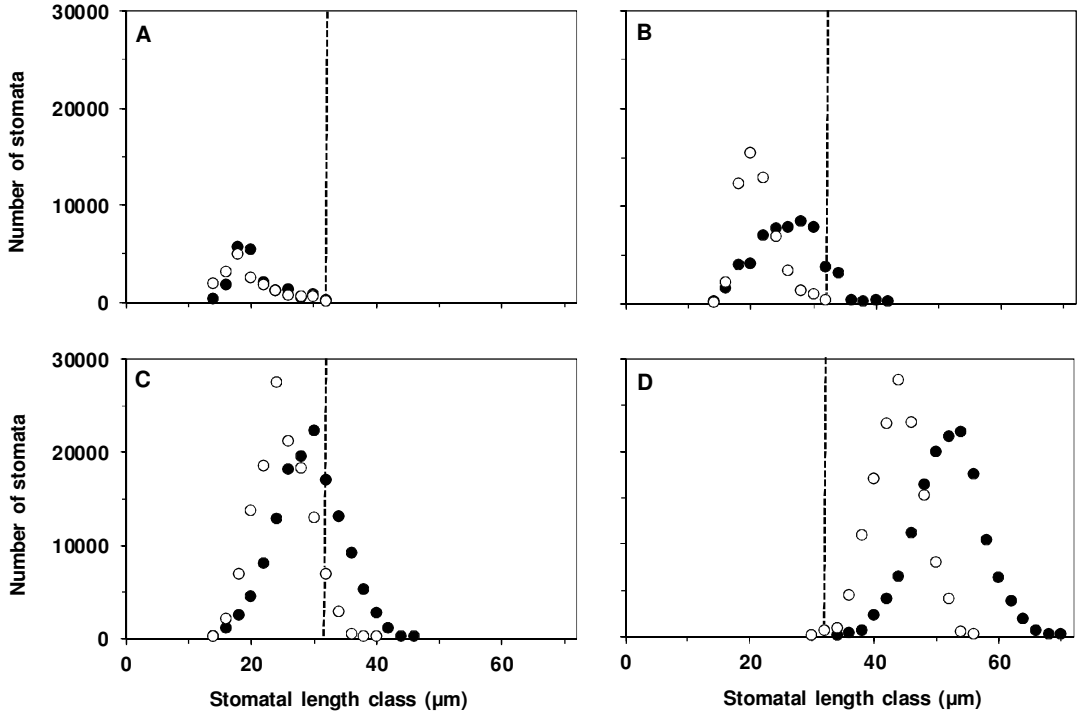

Fig. 3. Frequency distribution of stomata in various stomatal length classes in leaflets of cut rose cv. Pink Prophyta, grown at moderate (open symbols, 60%) or high (closed symbols, 95%) relative air humidity. The stomatal population was sampled at various percentages of full leaflet expansion (FLE; A: 33%, B: 50%, C: 67%, and D: 100%). The length class intervals were set at 2 µm. The vertical lines depict the 32 µm length class. Frequency distributions are from representative leaflets. Stomatal length did not change after 100% FLE. The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively.

Figure 4A shows the response of detached leaflets to 4 h of dehydration. Detached leaflets of plants that had continuously grown at moderate RH (open circle at the Fig. 4A) lost very little water during this period of dehydration. Detached leaflets of plants that had continuously grown at high RH (filled circle at Fig. 4A) did not respond to dehydration by properly closing their stomata, thus lost a considerable amount of water. The triangles in Figure 4A refer to leaflets on plants that had grown at high RH until they had reached the indicated FLE, and were then transferred to moderate RH until the leaflets had fully expanded. The stomatal reaction to dehydration was determined in such fully expanded leaflets, after detachment. Normal stomatal reaction to dehydration only occurred in leaflets of plants that had grown at high RH and were transferred to

moderate RH by the time the leaflets were still at a very early stage of expansion (25% FLE). Problems with stomatal closure, in response to dehydration (measured in detached fully expanded leaves), became increasingly greater when the duration of leaflet growth at high RH was longer, before transferring the plants to moderate RH. Figure 4B shows the percentage of stomata longer than 32 μm at various FLE, in leaflets expanding at high RH. By 33% FLE stomata longer than 32 μm contributed as little as 1% to the total stomatal population.

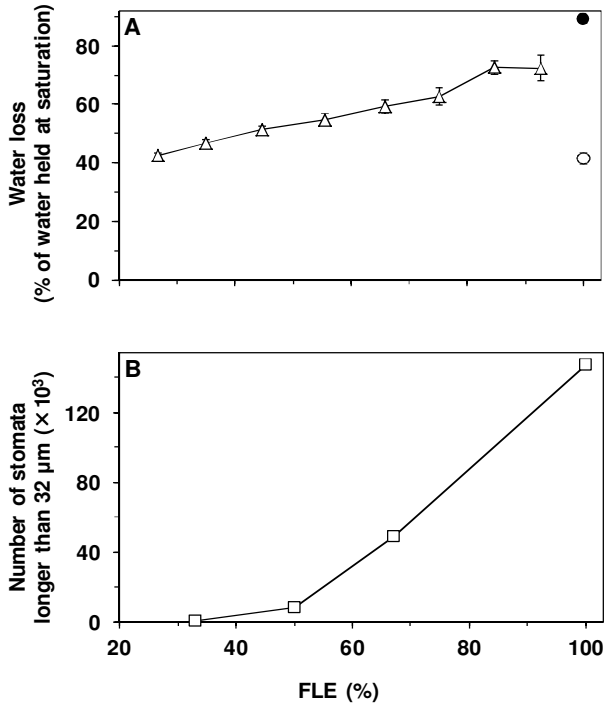

Fig. 4. Relationship between the number of stomata exceeding a certain length (here taken to be 32 μm) at various phases of leaflet growth in cut rose cv. Pink Prophyta plants placed at high relative air humidity (RH, 95%), and stomatal response to leaflet dehydration, measured in fully expanded leaves. The period of leaflet growth is expressed as a percentage of full leaflet expansion (FLE). (A) Number of stomata longer than 32 μm per leaflet, at various stages of leaflet growth. The number of 1 x 1 cm sampling areas per leaflet was 2 (100 stomata/sampling area), 4 (75 stomata/sampling area), 10 (50 stomata/sampling area) and 28 (40 stomata/sampling area) for 33%, 50%, 67% and 100% FLE, respectively. (B) The water loss (expressed as the percentage of the water lost by the leaf at the time of assessment relative to the water contained by the leaf at saturation) after 4 h of dehydration. Triangles: plants grown to the indicated FLE at high RH, but were then transferred to moderate RH (60%) till full leaflet expansion. Closed circle: control continuously grown at high RH. Open circle: control grown continuously at moderate RH. All measurements were conducted after complete leaflet expansion. Values are the means of at least 18 leaflets ± SEM. When the SE bars are not visible, the SE is smaller than the symbol.

This proportion increased to about 35% at 67% FLE, and was 100% in fully expanded leaflets. Comparing the data of Figures 4A and B shows a correlation between the lack of average stomatal closing response upon dehydration, in fully expanded leaves, and the number of stomata that were larger than 32 μm at the time of transfer of the plants from high RH to moderate RH.

The distribution of stomatal pore aperture throughout leaflet lamina on non-stressed fully expanded leaflets grown at moderate or high RH is shown in Figure 5. A higher average pore aperture (dashed line in Fig. 5) and a wider distribution was observed on leaflets from plants grown at high RH, compared to those grown at moderate RH. No spatial gradients over the leaflet lamina were observed for pore aperture on fully expanded leaflets, of plants grown at either atmospheric humidity (data not shown).

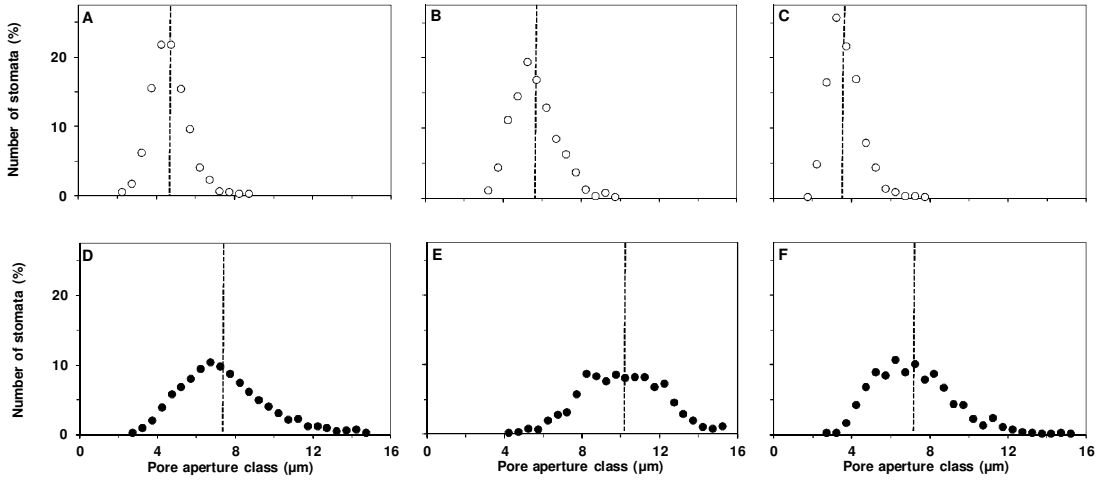

Fig. 5. Frequency distribution of pore aperture in various pore aperture classes of non-stressed fully expanded leaflets of cut rose cv. Pink Prophyta, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity levels. The pore aperture class intervals were set at 0.5 μm. The vertical lines depict the means. Each figure represents one leaflet (twenty eight 1 x 1 cm sampling areas, and 40 stomata/sampling area).

The cross-sectional area per stoma available for gas fluxes is the pore area. The pore area was calculated as the area of an ellipsis, with pore length and pore aperture as major and minor axes. The distribution of the stomatal pore area, on leaflets grown at moderate or high RH, is shown in Figure 6. The pore area distribution shows similarity to the distribution of pore aperture. The variability of pore area of stomata on leaflets that had grown at high RH was considerably larger than that of stomata on leaflets grown at moderate RH.

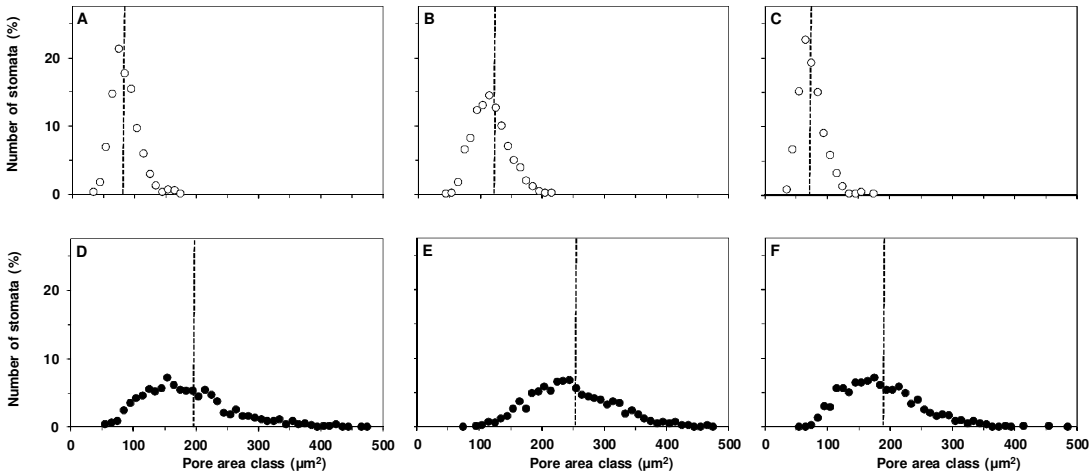

Fig. 6. Frequency distribution of pore area in various pore area classes of non-stressed fully expanded leaflets of cut rose cv. Pink Prophyta, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity levels. The pore area class intervals were set at 10 µm². The vertical lines depict the means. Pore area was taken as the area of an ellipsis with major and minor axes the pore length and pore aperture, respectively. Each figure represents one leaflet (twenty eight 1 x 1 cm sampling areas, and 40 stomata/sampling area). The frequency distribution of pore aperture of these leaflets in sequence is shown in Figure 5.

In fully expanded leaflets (100% FLE), pore length was linearly related to stomatal length (Fig. 7). This was independent of atmospheric humidity during growth.

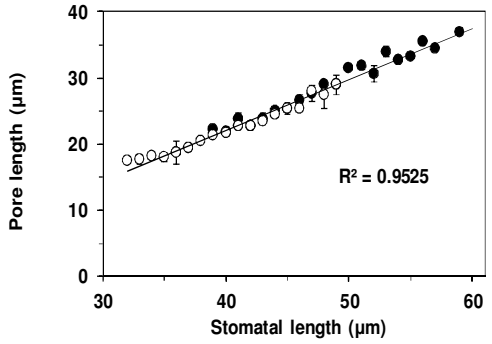

Fig. 7. Relationship between stomatal length and pore length on fully expanded leaflets of cut rose cv. Pink Prophyta, grown at moderate (60%, open symbols) or high (95%, closed symbols) relative air humidity levels. Values are the means of at least 12 stomata ± SEM. When the SE bars are not visible, the SE is smaller than the symbol.

Discussion

Effect of high RH on stomatal density and size

Stomatal density on leaflets of cv. Pink Prophyta roses, measured throughout the leaflet lamina and at various stages of leaflet growth, was not or only little influenced by ambient moisture level during plant growth (60 or 95% RH) (Fig. 1A). In some other rose cultivars, however, a higher atmospheric humidity during plant growth has been shown to result in increased stomatal density on fully expanded leaflets (Torre et al. 2003). The higher transpiration in the light, found in fully expanded leaves that had grown at high RH can therefore in cv. Pink Prophyta roses not be explained by stomatal density, whilst in some other rose cultivars (such as cv. Baroness, Torre et al. 2003) increased stomatal density might be part of the explanation.

The number of stomatal initials (here defined as the shortest microscopically visible stomata, i.e. 14 μm long) was not affected by elevated atmospheric humidity (Fig. 3A, B, C). At 33% FLE the stomatal size was not affected by the RH during growth (Fig. 1B, C; see also Fig. 3A). At later stages of leaflet expansion the stomata in leaflets grown at high RH were longer and wider (Fig. 1B, C). An increase in stomatal length of fully expanded leaves, as a result of high RH during plant growth, has been found in several other species too (Karbulkova et al. 2008, Rezaei Nejad and van Meeteren 2005). This increase in stomatal length and width, during growth at high RH, might allow wider stomatal aperture and larger pore area.

Effect of high RH on pore parameters

Indeed, we found larger stomatal aperture and larger pore area in fully expanded leaflets on plants grown at high RH (Fig. 5 and 6). Pore area is determined by pore length and pore aperture. Pore aperture is actively regulated, and depends on environmental stimuli such as light and evaporative demand (Lawson 2009). In contrast, pore length is a more rigid trait, rather independent of short-term environmental conditions (Lawson et al. 1998).

Wider pore apertures of stomata on leaflets that grew at high RH have been also previously reported (roses: Fanourakis et al. 2011a, Torre et al. 2003; *Tradescantia virginiana*: Rezaei Nejad and van Meeteren 2005). However, previous studies have been limited to one sampling area per leaf. Therefore, possible differences in pore aperture between sampling areas within the leaf have been ignored thus far. We observed that pore aperture was not different within the leaflet in the absence of stomatal closing stimuli, of plants grown at moderate or high RH.

Since larger stomata can achieve wider pore apertures, differences in stomatal size may account for part of the observed variance in pore aperture (Spence 1987). It was now found that the variation in stomatal length of fully expanded leaflets was increased by elevated ambient humidity (Fig. 3D). Consequently, the higher variation among pore

apertures of stomata developed at high RH (Fig. 5), compared to moderate RH-formed stomata, might be partly mediated by the higher variability in stomatal size.

Moreover, we found that longer stomata that developed in high RH had also longer pore length (Fig. 7). Longer stomatal pore length has also been found on *Corylus maxima* leaves grown at elevated RH (Fordham et al. 2001). The increase in pore length in combination with the wider pore aperture, in stomata of rose leaflets grown at high RH, resulted in considerably larger pore areas (Fig. 6). This is apparently the main explanation for the higher rate of transpiration (expressed per unit leaf area) in the light of non-stressed leaves of cv. Pink Prophyta roses that had been grown at high RH, compared to those grown at moderate RH (Fanourakis et al. 2011b): the number of stomata per unit leaf area (i.e. stomatal density) is about the same, but the stomata are longer, have a larger pore length and a wider pore aperture, and thus a larger pore area.

Relationship between stomatal morphology and stomatal closure in response to dehydration

We previously analysed stomatal closure in response to leaf water deficit. This was carried out using fully expanded leaflets continuously grown at moderate (60%) or high (95%) RH. Detached leaflets were held dry for 4 h. This analysis also used fully expanded leaflets of plants that had been transferred from moderate to high RH and vice versa, at various stages of leaflet growth (Fanourakis et al. 2011a). The response to dehydration was determined by measuring the transpiration rate and the relative water content (RWC, expressed as percentage of the water held by the leaf at the time of assessment relative to the water contained by the leaf at saturation). In the previous paper we report the RWC after 2 h of dehydration. Here we show the water loss (= 100 – RWC, i.e. the percentage of the water lost by the leaf at the time of assessment relative to the water contained by the leaf at saturation) after 4 h of dehydration, which magnifies the differences (Fig. 4A).

The stomata of detached fully grown leaflets, from plants that had grown at high RH and that were subsequently transferred to moderate RH exhibited lack of stomatal closure in response to dehydration. However, a full effect (almost no stomatal closure) was only found when the transfer of the plants took place very late during leaflet expansion (i.e. at higher FLE). The earlier the transfer of the plants to moderate RH took place during leaflet expansion, the better was the response of the stomata to dehydration. This was already shown by Fanourakis et al. (2011a). Here we present data after 4 h of leaflet dehydration rather than 2 h dehydration in the previous paper. The relationship between the stomatal closure response to dehydration and the time of transfer of the plants from high to moderate RH was about linear (Fig. 4A). These data might indicate that with increasing time of leaflet growth, a higher percentage of stomata became less responsive to dehydration. This would be understandable if small stomata were not affected by the high RH during growth, but longer stomata did become affected. This idea is represented by the dashed line in Figure 3, whereby the stomata left from the line

are shorter than 32 μm and those at the right from the dashed line are longer. In Figure 4B the number of stomata longer than 32 μm per leaflet, in plants continuously grown at high RH, is shown in time (various stages of leaflet growth). The rather close correlation with the data in Figure 4B suggests that RH during growth does not affect stomata that are shorter than about 30 μm, but does affect stomata that are longer than this size. The data of Figure 4B might well be the explanation of those in Figure 4A.

Additionally, it was previously shown that fully grown leaflets that had initially been expanding on plants growing at moderate RH, but had been transferred to high RH later on during leaflet growth, exhibited a poor stomatal response to dehydration. This poor stomatal response was almost independent of the leaflet expansion stage (FLE ranging between 25% and 67%) at the moment of transfer of the plants from moderate to high RH (Fanourakis et al. 2011a). Thus, stomata at various stomatal growth stages were similarly showing lack of closure. Lack of stomatal closure was even found when the plants had been exposed to high RH for at least 2 days prior to full leaflet expansion. These data suggested that high RH just before full leaflet expansion (and just before the stomata ceased growing) was sufficient to cause full lack of stomatal closure (in response to dehydration). The present results on stomatal morphology do not shed further light on these previous data. The main effect seems therefore physiological rather than morphological.

Conclusions

It is concluded that stomatal density on the whole leaflet surface was not or only little affected by RH during growth. High RH (95%) during leaflet expansion resulted in longer stomata with longer pore length and wider pore apertures throughout the leaflet lamina. This resulted in larger pore area. This seems to explain why cut flowers that had been grown at high RH showed higher rates of water loss in the light, compared to cut flowers grown at moderate RH (60%).

The present results also hint at an explanation of the effect of high RH during growth on the lack of stomatal response to leaflet dehydration (Fanourakis et al. 2011a). The data suggest that there is a critical stomatal length (possibly around 32 μm) where stomatal closing ability is mostly affected by high RH. When stomata shorter than this threshold are exposed to high RH there is no later lack of response to dehydration. In contrast, stomata that are longer than about 32 μm, and are subsequently exposed to moderate RH until full leaflet expansion, did not properly close when the fully grown leaflet was subjected to dehydration.

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CHAPTER 6

General discussion

Genotypic variation in the decrease in vase life after growth at high relative humidity

Several product quality problems originate from adverse growth conditions in the greenhouse, but only become visible in the postharvest phase at supply chain or consumer level (van Doorn 2011). This is especially the case with high atmospheric humidity, a climate parameter that is often ignored, as long as diseases do not appear (Mortensen 2000). However, it has been repeatedly demonstrated that high RH (larger than or equal to 85%) during cultivation decreases the vase life of cut roses (Mortensen and Fjeld 1995, Pettersen et al. 2006, Torre et al. 2001). This effect of high cultivation RH on the vase life strongly depends on the genotype (Mortensen and Gislerød 1999, 2005). Previous studies were focused on the length of vase life, and symptoms ending ornamental value, which were mostly associated with drought stress (In et al. 2007, Marissen and Benninga 2001, Pettersen et al. 2007). Thus, one of the aims of this thesis was to explain the cultivar differences by analysing the water relations of genotypes with contrasting sensitivity to elevated RH (Chapter 2). This study shows that preharvest high RH increases the cut flower water loss during both light and dark periods, with the latter being considerably more affected. The increase of cut flower water loss varied considerably among the studied genotypes. In agreement with the study on the stomatal responses to several closing stimuli on detached leaflets (Chapter 3.1), cv. Pink Prophyta was more affected than cvs. Frisco and Dream.

Besides leaves, sepals and stem are also stomatal bearing organs (Carpenter and Rasmussen 1974, Ganelevin and Zieslin 2002). Leaf removal not only showed that the fluctuation in the transpiration rate between light and dark periods was entirely based on the presence of leaves (also described by Doi et al. 2000, Mayak et al. 1974), but also that the contribution of the leafless stem to the total cut flower water loss was small. On the one hand, sepals acquire functional stomata, though their area is relatively small to induce substantial changes in the cut flower water loss. On the other hand, stomatal density on the rose stem is small, while stomatal activity is absent. Stems grown under high RH had higher water loss, compared to stems grown at moderate RH, also after leaf removal. This indicates higher cuticular water loss, as a result of plant growth at high RH, as found in leaves (Chapter 3.1).

The cv. Pink Prophyta with the highest increase in the water loss showed a very short time to wilting, while in the cvs. Frisco and Dream the higher water loss, as a result of high RH during growth, only slightly accelerated the appearance of visible symptoms of water stress (Chapter 2). It seems likely that the higher water loss of the cvs. Frisco and Dream was lower than the threshold that initiates a strong decline in stem hydraulic conductivity (K_h) (presumably due to cavitation, i.e. vapour-filled conduits) leading to wilting and collapse. In contrast to this, it is our opinion that in cv. Pink Prophyta, the higher water loss causes a xylem tension capable of inducing significant cavitation events. In the absence of roots, which play a crucial role in the embolism repair (Tyree and Sperry 1989), these cavitation incidents progressively accumulate resulting in an escalating decrease of K_h (Dixon and Peterson 1989).

Water loss data is difficult to place in perspective without any information about the cavitation margins (Brodribb and Holbrook 2004). Large differences in the water potential, at which high rates of cavitation take place, between different rose cultivars have been described (van Doorn and Suiro 1996). It is likely that in cultivars with lower xylem vulnerability to cavitation, due to transpiration-induced water tension, the vase life will be less affected by the enhanced water loss, as a result of elevated preharvest RH. Therefore, it would be interesting to investigate the genotypic variation in xylem vulnerability to low water potential and whether this trait is affected by preharvest elevated atmospheric humidity.

The short time to wilting of cv. Pink Prophyta grown at high RH was considerably postponed when the problems with water uptake were largely prevented (by stem surface-sterilization, cutting under degassed water, and placement in sterilised vase solution) (Chapter 2). These data highlight that the higher water loss, as a result of high atmospheric humidity during growth, becomes only important when the water uptake is limiting. The water uptake is basically a physical process in cut flowers. It is determined by the water potential, acting as driving force, and the conductance (inverse of resistance) of the transport path.

Previous studies on pressure-volume curves revealed an effect of RH level on the internal leaf water relations. Luo and Strain (1992) reported a strong increase (0.2 MPa more positive) of leaf water potential in *Abutilon theophrasti* leaves grown at high RH, compared to leaves grown at drier air. A similar effect of elevated humidity on the leaf water potential sensitivity to relative water content has been shown in *Tradescantia virginiana* (Rezaei Nejad et al. 2006). These changes in internal water relations have implications for the cut flower water balance. That is for any given water status the driving force for rehydration is lower in leaves expanded in more humid air. Previous studies on the effect of high RH on the hydrosensitivity of leaf water potential are focused on only one genotype, and it, therefore, remains unknown whether there is genotypic variation in this trait.

The water flow rate through the leaf lamina, from entry at the petiole-shoot junction to the sites of evaporation, besides leaf water potential, depends on leaf hydraulic resistance (R_L). This resistance consists of vascular (venation) and extra-vascular components, while its largest part (50–80%) is located outside the venation system (Cochard et al. 2004). High RH-grown rose leaves have been reported to acquire reduced density of vascular tissue (expected to increase R_L), and increased intercellular air spaces (expected to decrease R_L) (Torre et al. 2003). However, the degree and the relative importance of these responses have not as yet been quantified. Such data will yield a deeper understanding of the consequences of a more humid environment on the leaf water balance.

The anatomical properties of the xylem vessels largely determine the K_h, but also are related to the percentage and rate of recovery after air aspiration at the cut surface (especially vessel diameter distribution, van Ieperen et al. 2002). The K_h and K_h-restoration dynamics of stem segments of the sensitive cv. Pink Prophyta were not affected by the humidity level during growth (Chapter 2), which implies that the xylem structure remained unchanged. This speculation is in agreement with Darlington and Dixon (1991), who reported no significant differences in internal anatomical features of the xylem for rose plants grown at contrasting humidity levels (elevated RH: 77%, versus uncontrolled RH: 30–60%). Thus, stem segments from either RH level are expected to have the same recovery from air emboli (in commercial practice air always enters the vessels that have been opened by cutting as xylem is under negative tension), and the alleviation effect of postharvest treatments involved in embolism repair (such as long vase solution column, low water temperature, and surfactants in: Mensink and van Doorn 2001, van Ieperen et al. 2002) will be similar.

Genotypic variation in the attenuation of stomatal closure after growth at high relative humidity

Stomatal closure in response to different closing stimuli (i.e. decrease in leaflet water content/potential, ABA feeding, and light/dark transition) was attenuated by long-term high RH, resulting in higher transpiration rates compared to leaflets expanded at moderate RH (Chapters 2 and 3.1). This is a common feature of stomata developed at high RH, and it has been shown in a wide range of species (Fordham et al. 2001, Rezaei Nejad and van Meeteren 2005, Torre and Fjeld 2001). However, it was now found that there is a strong and consistent genotypic component in the stomatal responsiveness, since certain cultivars had less responsive stomata after cultivation at high RH.

The stomatal closing in response to leaflet desiccation after leaf development at high RH was evaluated in a subset of a segregating tetraploid cut rose population (60 genotypes) (Chapter 3.2). A large genotypic variation was observed between the individuals, which raises possibilities for breeding cultivars tolerant to high RH. Using sensitive to high RH genotypes is of utmost importance from a fundamental point of view (van Doorn 2011). It helps to understand the physiological processes underlying

stomatal malfunction after cultivation at high RH for example. However by using these, we tend to forget that there is a number of genotypes available, at which the high RH exerts minor effects. The present study highlights that the shorter vase life and reduced flower opening, as a result of plant growth at high RH, might be effectively counteracted via a breeding program that takes into consideration the stomatal responsiveness (Chapter 3.2).

Rezaei Nejad and van Meeteren (2007) suggested that the malfunctioning stomata in leaves developed at high RH, is the result of long-term low leaf ABA concentration. The reasons behind the genotypic variation in the attenuation of stomatal responsiveness after leaf development at high RH are as yet not understood. We were not able to measure the rose leaf ABA concentration with the enzyme linked immunosorbent assay (ELISA) protocol described by Asch (2000), most likely due to a cross-reaction of antibody with other compounds. We hypothesize that the tolerant cultivars (a) undergo a lower decrease in the leaf ABA level, induced by high RH during growth, or (b) the same decrease in the bulk leaf ABA takes place, but either sustain stomatal functionality at lower ABA levels, or the ABA content in the ABA action sites of stomata remains higher. To better understand this, detailed information on leaf ABA concentration and compartmentation should be evaluated for contrasting cultivars in their sensitivity to high RH.

Leaves developed at high RH not only have a lower foliar ABA content (Rezaei Nejad and van Meeteren 2007), but also a lower capacity to produce ABA (personall communication: Louise Elisabeth Arve). Upon water stress, both intraleaf ABA redistribution, by an existing foliar pool of ABA, and ABA synthesis take place resulting in higher ABA accumulation by stomata (Harris and Outlaw 1991, Popova et al. 2000). To evaluate whether the lower pre-existing ABA content as well as the attenuated leaf ability to produce ABA were responsible for the reduced stomatal responsiveness of high RH-grown leaves in short-term, ABA was applied by feeding through the petiole. The sensitivity of stomata to ABA, fed through the petiole, was greatly reduced in high RH-grown leaves, indicating that neither their lower ABA concentration nor their reduced ability to synthesize ABA do account for non-stomatal closure in short-term (also discussed by Rezaei Nejad and van Meeteren 2005).

Besides long-term high RH, environmental (e.g. *in vitro* culture, Aguilar et al. 2000) or genetic factors (e.g. ABA-biosynthetic or transgenic plants expressing an anti-ABA antibody; Artsaenko et al. 1995, Leon-Kloosterziel et al. 1996) that cause a long-term decline in leaf ABA concentration or decreased sensitivity to ABA (e.g. ABA-insensitive mutants, Koornneef et al. 1984), also result in an impaired stomatal ability to respond to closing stimuli. However, in ABA-biosynthetic (Iwai et al. 2003) and ABA-insensitive (Allen et al. 1999) mutants it has been demonstrated that certain signalling cascade steps were impaired or disrupted, and when these were experimentally activated, the stomata reacted similarly to wild type. These results demonstrate that in these mutants long-term

low leaf ABA concentration disrupts specific signal transduction elements, whereas signalling components downstream these regions remain intact. Hence, stomata on leaves with low ABA concentration have the capacity to close, though the signalling events that lead to stomatal closure cannot be induced or are impaired. Further research is needed to confirm this hypothesis at high RH-grown plants.

Consequences of changes in stomatal anatomy caused by high relative humidity

Since species with smaller stomata have faster stomatal responses compared to species with longer stomata (Aasamaa et al. 2001, Franks and Farquhar 2007, Hetherington and Woodward 2003), it might be expected that bigger stomata, induced by high RH, show a lower speed of closure. We tested this approach by examining the closing behaviour of stomata with parallel assessment of their length. Our findings did not support this hypothesis, since stomata had wider aperture after desiccation indepentent of their length (Chapter 3.1). As a consequence, the correlation between the elevated RH effect on stomatal size and closing ability among different genotypes was poor.

High RH during leaf expansion resulted in higher stomatal density (8–22% depending on the cultivar), though this effect was not significant for cv. Pink Prophyta. A higher stomatal density in response to long-term elevated RH has been reported in many other species (Bakker 1991, Gislerød and Nelson 1989, Karbulkova et al. 2008, Kawamitsu et al. 1993) including rose (Torre et al. 2003). This higher stomatal density may be interpreted as an attempt of the species to increase the transpiration rate in an environment with low evaporative demand. An increase in the stomatal density *per se* causes a proportional increase in the transpiration rate (Nobel 1991, Parlange and Waggoner 1970), through its effect on the pore area per leaf area, when keeping the remaining pore features constant. A lower stomatal density as well as a lower increase after cultivation at high RH would, therefore, result in a lower water loss rate upon transfer to high VPD conditions at the same pore aperture values. However when stomatal functionality is differentially affected as in the studied cultivars, stomatal density cannot be used as a selection criterion for lower water loss rates after cultivation at high RH.

Although a higher stomatal density and a longer stomatal size (resulting in longer pore length) are not key traits for sensitivity to high RH, it was now shown that they directly contribute to the higher water loss rates of plants grown at high RH (Chapter 3.1). The contribution of changed anatomy (i.e. higher stomatal density, longer pore length and deeper pore depth), varied between 16 and 30% depending on the genotype. This analysis identifies a previously unrealized effect of anatomical features on the higher postharvest water loss rates of plants grown in more humid air. This also implies that assessing the humidity level effect on the stomatal closing ability by measuring the water loss and not taking into account the contribution of anatomical features overestimates the effect.

Cuticular water loss of leaves developed at high relative humidity

The higher water loss of leaves developed at high RH might be associated with increased stomatal transpiration or this in combination with higher cuticular water loss. In practical terms, evaluating the cuticular contribution to the total leaf water loss has been difficult because of the presence of stomata in both epidermes (i.e. in amphistomatous species). Even when stomata close, leaks may exist that prevent accurate estimation of cuticular properties, since an unknown residual stomatal transpiration may contribute to the cuticular transpiration (Kerstiens 1995, 1996). Poor cuticle development, resulting in high rates of water loss through the epidermis, has been strongly implicated as a major source of higher water loss in *in vitro* plantlets (Hazarika 2006, Pospisilova et al. 1998). A limited number of studies have been conducted in plants grown at high RH (Karbulkova et al. 2008, Torre et al. 2001), with antithetical outcomes. In this study the cuticular water loss was determined by using the adaxial (astomatous) leaf surface. We show that high RH during leaf development resulted in higher water loss through the cuticle (Chapter 3.1). However, the cuticular contribution to the total leaf water loss, compared to the stomatal contribution, was relatively small in all examined cultivars. Although the evaluations reported in the present study were made only on four cultivars, the results nevertheless suggest that the rapid wilting trait of high RH leaves cannot be associated with poor cuticle development.

Adaptation of stomatal closing ability to contrasting relative humidity levels

Determining the possibility of restoring malfunctioning stomata after exposure to long-term high RH is highly important from a practical point of view. However, our results clearly indicate that stomatal functionality of rose leaves is determined during leaf development, and after this period the possibilities to obtain (i.e. by transferring high RH-grown leaves to moderate RH) or to lose (i.e. by transferring moderate RH-grown leaves to high RH) stomatal functionality are absent (Chapter 4), which is in agreement with previous findings (Mortensen and Fjeld 1995, Mortensen and Gislerød 2000). Nevertheless, it has been suggested that a certain foliar ABA content is required to sustain stomatal functionality of moderate RH-grown leaves in both *Phaseolus vulgaris* (Pospisilova 1996) and *Tradescantia virginiana* (Rezaei Nejad and van Meeteren 2008). Thus, two hypotheses arise: (i) although the root-to-shoot ABA signalling is weakened after transfer to high RH, this is still sufficient to sustain stomatal responsiveness; or (ii) the root-to-shoot ABA signalling is not prerequisite for a proper stomatal functioning in fully expanded rose leaves.

Although long-term exogenous ABA application during leaf expansion resulted in proper stomatal functioning (also shown by Rezaei Nejad and van Meeteren 2007), the same ABA application on fully expanded high RH-grown leaves for a period of 2 weeks did not improve their stomatal closing ability (Chapter 4). In agreement with this finding, stomata on leaves developed at high RH and fed with ABA closed less,

compared to ABA-fed stomata formed at moderate RH, despite the continuous supply of ABA for 6 days (Chapter 2). These data highlight that reduced stomatal responsiveness, as a result of leaf expansion at high atmospheric humidity, can no longer be improved after complete leaf expansion by manipulating foliar ABA content in rose.

Moreover, it was shown that root-to-shoot ABA signals are not a prerequisite for normal stomatal functioning of leaves developed at moderate RH, by excising the roots and examining the stomatal responses of detached leaflets for a period of 2 weeks (Chapter 4). Similarly, stomata on attached leaves of moderate RH-grown cut flowers responded by closure in response to a decrease in leaf relative water content and light/dark transition during the whole evaluation period (up to 30 days) despite the complete absence of root signals (Chapter 2). These results support that either the stomatal functionality is independent from the post-development foliar ABA level or the ABA import via the roots is not important for sustaining this level in rose. Further work is needed to investigate these hypotheses.

Unlike fully expanded leaves, stomata from leaflets that started expanding at a certain RH level and were subsequently transferred to a contrasting one (moderate to high RH and vice versa) were always able to partly adapt to the new RH level (Chapter 4). Leaflet expansion stage was expressed as a percentage of full leaflet expansion (FLE, i.e. actual leaflet length as a percentage of final length). The stomatal adaptation dynamics were assessed by measuring the leaflet transpiration rate in response to desiccation once the leaflets were fully expanded. Although this approach was sufficient to illustrate differences in the regulation of leaf water loss, it is necessary to emphasize that this approach reflects the overall behaviour of the stomatal population. Consequently, differences in the closing ability between different stomata within the population might have been masked. Higher water loss might have been the result of (i) equally less closed stomata, or (ii) closed and open stomata within the stomatal population. This heterogeneity in stomatal closure over the leaf surface can be detected by direct measurements of stomatal aperture covering the whole leaf surface (Smith et al. 1989) or by indirect methods (e.g. chlorophyll fluorescence, Rezaei Nejad et al. 2006).

Leaflets initially expanding at moderate RH and transferred to high RH exhibited poor stomatal functioning, nearly independent of the leaflet expansion stage (i.e. FLE) at the moment of transfer (Chapter 4). The stomatal population and growth (increase in length) dynamics in the course of leaflet ontogeny were analysed (Chapter 5.1). Stomatal initiation took place mostly in the first stages of leaflet ontogeny, whilst stomatal growth mostly occurred after this period. Chapters 4 and 5.1 indicate that different stomatal lengths, most probably representing different stages of stomatal development, are similarly prone to loss of closing ability, when these are exposed to high RH. This conclusion supports the general notion that stomatal functionality and size are not built together. Moreover, these results suggest that stomatal closing capacity was rather uniform within the stomatal population (might be used to explain the similar water loss

of fully expanded leaflets which were at different expansion stages at the moment of transfer). Thus, the high RH effect on expanding leaves, that were initially expanding at moderate RH and completed their expansion at high RH, is dominated by its effect on non-fully developed stomata. Since high RH disturbs stomatal functionality in non-fully grown stomata independent of their stage of development, the percentage of FLE (i.e. bearing different stomatal growth stages) at the moment of transfer was not important in determining the leaflet regulation of water loss.

Leaflets initially expanding at high RH and transferred to moderate RH exhibited higher water loss in response to desiccation, when this transfer took place later in leaflet expansion (i.e. higher FLE) (Chapter 4). The water loss of fully expanded leaflets transferred during expansion from high to moderate RH increased linearly with the FLE stage at the moment of transfer. This behaviour could not be explained by stomatal appearance/initiation, which followed an S-shaped pattern (Chapter 5.1), indicating that different stomatal lengths, most probably representing different stages of stomatal development, have a different ability to obtain functionality, when these are exposed to moderate RH. Close examination of stomatal population and growth dynamics indicated that the absolute number of stomata longer than 32 μm increases from 33 through 50 to 67% FLE, while this percentage is expected to further increase from 67 to 100% FLE (Chapter 5.2). It is our opinion that on expanding leaflets transferred from high to moderate RH, a fraction of stomata will be able to close. The stomata that react less to desiccation will be the ones that at the moment of transfer were longer than a threshold length (possibly around 32 μm). Since moderate RH induces functionality in non-fully developed stomata depending on their stage of development, the percentage of FLE (i.e. bearing a stomatal population with different stomatal developmental stages) at the moment of transfer determines the leaflet regulation of water loss.

Conclusion and possibilities for further research

This thesis provides new insights into the effect of elevated ambient humidity on a) the regulation of water loss (stomatal response characteristics) in a range of genotypes, b) the stomatal anatomical characteristics in relation to the enhanced water loss, and c) different stomatal developmental stages in relation to their ability to close at complete development. In the long run, this project contributes to minimize cut flower quality problems due to a negative water balance, resulting from long-term exposure to high RH during growth.

Further research should be directed to identify (i) the underlying mechanism(s) behind genotypic variation in the stomatal responsiveness (by assessing leaf ABA concentration and compartmentation), and (ii) the guard cell signal transduction elements that are disrupted/attenuated (by experimental activation of transduction elements of stomatal closure), as a result of long-term elevated RH.

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SUMMARY

The primary function of stomata is to control dynamically the carbon dioxide and water vapour fluxes across the leaf, two opposite diffusion processes. The rest of the epidermis is covered by a highly impervious barrier, the cuticle, which offers protection against desiccation. Stomatal responses are, therefore, of unquestionable importance to plant water and carbon cycles. These responses in a short-term basis are regulated by internal physiological and external environmental factors. The same factors during leaf development (i.e. long-term basis) determine the stomatal sensitivity to closing stimuli of fully developed leaves. The plant ability to survive an environmental change, therefore, largely depends on its growth history. For instance, high relative air humidity (RH ≥ 85%) during plant growth often leads to a negative water balance and finally wilting, when plants are subsequently moved to lower humidities. Such elevated moisture ambient conditions naturally take place in forests, but also in horticultural industry (e.g. *in vitro* culture, protected cultivation). Stomatal malfunctioning is a major cause for this negative water balance. However, the reasons why stomata fail to close fully during water stress periods in plants subjected to prolonged exposure to high RH and the striking cultivar differences described for cut roses in their tolerance to high RH are still poorly understood. As a result of energy saving practices (e.g. use of energy screens, temperature integration climate control and totally closed greenhouses) it may be expected that high RH levels will occur more frequently in commercial greenhouses. Thus, more keeping quality problems can be foreseen in the near future. This project aims at understanding a) the physiology of stomatal behaviour in response to long-term growth at high RH, b) the reasons for cultivar differences in their tolerance to high RH, and c) the stomatal adaptation dynamics to contrasting RH levels.

In **Chapter 2**, the postharvest water relations of contrasting cut rose cultivars in their sensitivity to high RH were analysed. Plants were grown at moderate (60%) and high (95%) RH, and cut flowers were placed in water directly after cutting. Flowers of cv. Pink Prophyta grown at high RH did not open fully during vase life, though the flower opening of cvs. Frisco and Dream was hardly affected. Compared with moderate RH, cultivation at high RH resulted in about 80% shorter vase life in cv. Pink Prophyta, whilst for cvs. Frisco and Dream this effect was considerably smaller (9 and 15% respectively). The rate of transpiration, both in the light and dark periods, was higher in roses grown at high RH, the difference being larger in cv. Pink Prophyta than in the two other cultivars tested. The growth RH level influenced neither the stem hydraulic conductivity nor its recovery from air emboli in the cut surface. The early water stress symptoms and the inhibition of flower opening in cv. Pink Prophyta flowers grown at high RH were due to

a higher rate of water loss compared to those grown at moderate RH, while there was apparently a similar blockage in water uptake. This higher water loss was due to a larger stomatal opening. Cv. Pink Prophyta roses that had been cultivated at high RH were found not to close their stomata to the same degree as those cultivated at moderate RH in both light/dark transition and when leaf water potential decreased. Preventing vascular occlusion during vase life, caused by bacterial physical blockage, considerably extended the time to wilting and resulted in normal flower opening in cv. Pink Prophyta roses grown at high RH. This indicates that the high rate of water loss, as a result of plant growth at elevated ambient humidity, is only detrimental for cut flower longevity under limiting water uptake conditions.

In **Chapter 3.1**, the relative importance of the physiological and anatomical components in the enhanced water loss among genotypes that differ in their sensitivity to elevated RH was assessed. The stomatal responsiveness to three closing stimuli (desiccation, abscisic acid feeding, light/dark transition), as well as several stomatal features (density, index, size and pore dimensions) and the cuticular permeability (*Pe*) were determined in four cut rose cultivars, grown under moderate (60%) and high (95%) RH. Moreover, the effects of changes in stomatal density and pore dimensions on the stomatal conductance (g_s) were quantified using a model. High RH resulted in lower stomatal sensitivity to all closing stimuli, higher stomatal density, and longer stomata. However, the magnitude of these effects varied considerably with genotype. The degree to which the anatomical features were affected and the extent to which stomatal functionality was impaired were not correlated. Based on the model, it was estimated that higher g_s after 35 min leaf desiccation was mostly due to poor stomatal functionality (70–84% of the effect depending on the cultivar), and to a lesser extent the result of higher stomatal density, longer pore length and depth (16–30%). Additionally, high RH significantly increased *Pe* (31%), but its relative contribution to the total leaf water loss was decreased. In spite of stomatal closing ability being the primary cause of the large genotypic variation in the control of water loss, neglecting the direct contribution of anatomical features to the increased water loss rate would significantly overestimate the effect of long-term high RH on stomatal functioning.

In **Chapter 3.2**, the stomatal responses to desiccation in a subset of a segregating tetraploid cut rose population (60 genotypes) grown at high RH (≥ 85%) were evaluated. Additionally, the postharvest water relations of six contrasting genotypes in their stomatal responsiveness were analysed. A large genotypic variation for stomatal responsiveness to water stress was observed. The relative water content (RWC) after 4 h of leaflet desiccation ranged between 7 and 62%, and the parents showed different behaviour (RWC was 20 and 51%, respectively). The vase life of genotypes with high stomatal responsiveness varied considerably (10–21 days); while in all cases the flower bud reached the maximum opening stage. Instead, in genotypes with low stomatal responsiveness, the length of vase life did not exceed nine days and in two out of three

genotypes the flower did not open fully throughout the vase life. The shorter vase life span and the inhibition of flower opening in genotypes with low stomatal responsiveness, compared to genotypes with enhanced stomatal responses, was attributed to the higher (70%) postharvest water loss rates they exhibited. The results found in this study suggest that there is a large variation in the stomatal closing ability after plant growth in a humid environment, which raises possibilities for breeding tolerant cultivars to high RH. The selection of genotypes with high stomatal responsiveness, after cultivation at high atmospheric humidity, considerably increases the degree of certainty that the cut flower will last a minimum length of time, and will have an unhampered flower opening. Incorporation of such protocols in the breeding programs will improve the selection procedures and will reduce keeping quality problems throughout the chain channel.

In **Chapter 4**, the effect of a reciprocal change in RH, at different stages of leaflet expansion of rose plants grown at moderate (60%) or high (95%) RH, on the stomatal closing ability was studied. The degree of stomatal functioning was assessed by measuring the leaflet transpiration rate in response to desiccation, once the leaves had fully expanded. Intact plants grown at high RH had a significantly higher leaflets water potential throughout the light period than plants grown at moderate RH, despite their much higher stomatal conductance. For leaflets that started expanding at high RH but completed their expansion after transfer to moderate RH, the earlier this switch took place the better the stomatal functioning. Leaves initially expanding at moderate RH and transferred to high RH exhibited poor stomatal functioning, even when this transfer occurred very late during leaflet expansion. Applying a daily abscisic acid (ABA) solution on the leaflets of plants grown at continuous high RH was only effective in inducing stomatal functionality if done before full leaflet expansion. After full leaflet expansion, plants were transferred from one RH to the other, but stomata were functioning as if they were in the previous humidity. Similarly, stomata on leaflets fully expanded at moderate RH followed by the excision of the root (e.g. ABA) signals for a 14 day period did not lose their closing ability. Taken together, these results indicate that stomatal functioning is strongly dependent on the humidity level at which the leaf completed its expansion**.** Once the leaves have fully expanded, stomatal functionality can no longer be affected, even if drastic changes in the leaf ABA concentration take place.

In **Chapter 5.1**, stomatal initiation, growth and proximity were analysed in rose. Stomatal features such as density, index, and size were determined over the entire leaflet surface (sampling areas of 1 cm^2) at 33%, 50%, 67%, and 100% of full leaflet expansion (FLE; proportion of leaf length relative to its final length). Additionally, stomatal pore dimensions were measured in fully expanded leaflets. Over 80% of the total stomatal population was initiated between 33% and 67% FLE. Stomatal growth (based on length increase) mostly took place between 67% and 100% FLE. Incorporating spatial heterogeneity in stomatal density and size was essential to analyse these phenomena.

Considerable within-leaf variation of stomatal density and aperture was also found in fully expanded leaflets. Stomatal conductance (g_s) over the leaf was quantified using a model. Within the leaf lamina, differences in g_s of 37% were estimated. This was equally due to variation in stomatal density and aperture. The data indicate that the within-leaf variation ought to be considered not only for realism but also to avoid erroneous conclusions, as here shown for a) analysis of stomatal formation, and b) estimation of leaf transpiration, when using stomatal features. The extant sampling protocols for the determination of stomatal features are therefore critically evaluated.

In **Chapter 5.2**, the high rate of water loss in the light of cv. Pink Prophyta roses grown at high RH (95%) (Chapter 2) is discussed in relation to the pore features of the entire leaflet surface. Additionally, the decreasing leaf ability to control water loss at full expansion, as the expansion stage at high RH increases, before the subsequent transfer to moderate RH (Chapter 4), is discussed in relation to stomatal initiation and growth. Stomatal density and length were determined over the entire leaflet surface at 33%, 50%, 67%, and 100% of full leaflet expansion (FLE; proportion of leaflet length relative to its final length). Pore length and aperture were also measured at full expansion. Elevated RH did not significantly affect stomatal density, but increased stomatal length. Longer stomata were found to have longer pore length. This longer pore length in combination with wider pore aperture over the entire leaflet surface, compared to stomata formed at moderate RH, seems to explain why high RH-grown cut flowers of this cultivar showed higher rates of water loss in the light. It was also demonstrated that for leaflets grown at elevated ambient humidity the number of stomata longer than 32 μm increased from 1% of the formed stomata at 33% FLE to about 35% at 67% FLE. At 100% FLE all stomata were larger than 32 μm. This increase in the number of stomata longer than 32 μm was positively correlated with less response to dehydration, in stomata on leaflets experiencing a shift from high to moderate RH and cut at 100% FLE.

The experiments and results described in this thesis provide insights into the effects of high RH on the stomatal response and anatomical characteristics in well-watered plants grown at high RH, and into the developmental windows, where the stomatal function can be manipulated by changes in atmospheric humidity. The main achievements and limitations of this thesis are discussed in **Chapter 6**, and suggestions for future research are presented.

SAMENVATTING

De primaire functie van huidmondjes is het op dynamische wijze reguleren van de koolstofdioxide- en waterdampflux in en uit het blad, twee tegenovergestelde diffusieprocessen. De rest van de bladepidermis is bedekt door een moeilijk doordringbare barrière: de cuticula, die bescherming biedt tegen uitdroging. De huidmondjesreactie is daarom van groot belang voor de water- en koolstofbalans van planten. Op korte termijn wordt de huidmondjesreactie bepaald door interne fysiologische factoren en externe omgevingsfactoren. Dezelfde factoren bepalen tijdens de bladontwikkeling de huidmondjesgevoeligheid voor sluitingsimpulsen van volledig ontwikkelde bladeren. De mogelijkheid van de plant om een omgevingsverandering te overleven is grotendeels afhankelijk van de omstandigheden gedurende de groei. Bijvoorbeeld, een hoge relatieve luchtvochtigheid (RV ≥ 85%) tijdens de groei van de plant leidt vaak tot een negatieve waterbalans en uiteindelijk verwelking, wanneer planten vervolgens worden verplaatst naar een lagere luchtvochtigheid. Dergelijke verhoogde omgevingsvochtcondities komen van nature voor in bossen, maar ook in de tuinbouw (bijvoorbeeld *in vitro* cultuur, beschermde teelt). Slecht functionerende huidmondjes zijn een belangrijke oorzaak voor deze negatieve waterbalans. Echter, de redenen waarom de huidmondjes niet volledig sluiten tijdens waterstress in planten, die langdurig zijn blootgesteld aan hoge relatieve luchtvochtigheid, en de opvallende rasverschillen, beschreven voor snijrozen in hun tolerantie voor hoge relatieve luchtvochtigheid, zijn nog steeds slecht begrepen. Als gevolg van energiebesparende maatregelen (bv. gebruik van energieschermen, temperatuur-integratie, air-conditioning en volledig gesloten kassen) kan worden verwacht dat hoge RV niveaus vaker zullen voorkomen in de glastuinbouwpraktijk. Daarom kunnen in de nabije toekomst meer houdbaarheidsproblemen worden verwacht. Dit project is gericht op het begrijpen van a) de fysiologie van het huidmondjesgedrag in reactie op langdurige groei bij hoge RV, b) de redenen voor rasverschillen in tolerantie voor een hoge RV, en c) de dynamiek van de aanpassing van huidmondjes aan contrasterende RV niveaus.

In **hoofdstuk 2** worden de waterrelaties na de oogst van rozenrassen, die verschillen in hun gevoeligheid voor hoge RV, geanalyseerd. Planten werden gekweekt bij een gematigde (60%) en een hoge (95%) RV, en de rozentakken werden direct na het snijden in water geplaatst. Bloemen van het ras 'Pink Prophyta', geteeld bij een hoge RV, gingen niet volledig open tijdens hun vaasleven, terwijl de bloemopening van de rassen 'Frisco' en 'Dream' nauwelijks beïnvloed werd. Vergeleken met een gematigde RV resulteerde de teelt bij hoge RV in een ongeveer 80% korter vaasleven voor het ras 'Pink Prophyta', terwijl voor de rassen 'Frisco' en 'Dream' dit effect aanzienlijk kleiner was

(respectievelijk 9 en 15%). De verdampingssnelheid, zowel in de licht- als donkerperiode, was hoger in rozen geteeld bij hoge RV; het verschil was weliswaar groter bij 'Pink Prophyta' dan bij de andere twee rassen. De RV tijdens de teelt beïnvloedt noch de hydraulische geleidbaarheid van de stengel, noch haar herstel van luchtembolie ontstaan via het snijvlak. De vroege waterstress-symptomen en de remming van de bloemopening bij 'Pink Prophyta' geteeld bij een hoge RV werden veroorzaakt door een hogere waterverliessnelheid in vergelijking met bloemen geteeld bij de gematigde RV, terwijl er blijkbaar een identieke blokkade in wateropname was. Dit hogere waterverlies was te wijten aan een grotere huidmondjesopening. 'Pink Prophyta' rozen, geteeld bij hoge RV, sloten hun huidmondjes niet, in tegenstelling tot rozen gekweekt bij gematigde RV in zowel licht als donker en daarbij nam de waterpotentiaal van het blad af. Wanneer vatverstopping tijdens het vaasleven, veroorzaakt door een fysieke bacteriële blokkade, wordt voorkomen, neemt het vaasleven aanzienlijk toe en is de bloemopening bij 'Pink Prophyta' rozen, geteeld bij een hoge RV normaal. Dit geeft aan dat de hoge waterverliessnelheid, als gevolg van de teelt bij hoge RV, alleen maar nadelig is voor de levensduur van snijrozen als de wateropname limiterend is.

In **hoofdstuk 3.1** wordt het relatieve belang van de fysiologische en anatomische componenten in het toegenomen waterverlies in rassen die verschillen in hun gevoeligheid voor hoge RV beoordeeld. De huidmondjesgevoeligheid voor drie sluitingsstimuli (uitdroging, voeden met abscisinezuur, licht/donker overgang), alsmede diverse huidmondjeskenmerken (dichtheid, index, grootte en afmetingen van de opening) en de cuticulaire permeabiliteit (P*e*) werden bepaald voor vier snijrozenrassen, geteeld onder gematigde (60%) en hoge (95%) RV. Bovendien werden de effecten van veranderingen in de huidmondjesdichtheid en de grootte van de opening op de huidmondjesgeleidbaarheid (g_s) gekwantificeerd met behulp van een model. Hoge RV resulteerde in een lagere gevoeligheid van de huidmondjes voor alle drie sluitingsstimuli, een hogere huidmondjesdichtheid, en langere huidmondjes. Echter, de omvang van deze effecten varieerde aanzienlijk tussen de rassen. De mate waarin de anatomische kenmerken waren beïnvloed en de mate waarin functionaliteit van de huidmondjes werd aangetast waren niet gecorreleerd. Uit modelberekeningen bleek dat de hogere huidmondjesgeleidbaarheid na 35 min bladuitdroging vooral het gevolg was van slechte functionaliteit van de huidmondjes (70–84% van het effect, afhankelijk van het ras), en in mindere mate het gevolg van hogere huidmondjesdichtheid, langere lengte en diepte van de huidmondjesopening (16–30%). Bovendien verhoogde een hoge RV de P*e* aanzienlijk (31%), maar de relatieve bijdrage aan het totale waterverlies via het blad nam juist af. Alhoewel de mogelijkheid van huidmondjes om te sluiten de primaire oorzaak is voor de grote rasverschillen in het reguleren van het vochtverlies, leidt buiten beschouwing laten van de directe bijdrage van anatomische aanpassingen aan het verhoogde vochtverlies tot overschatting van het effect van langdurig hoge RV op het functioneren van de huidmondjes.

In **hoofdstuk 3.2** wordt de huidmondjesreactie op uitdroging van 60 genotypen uit een uitsplitsende tetraploïde rozenpopulatie geteeld bij hoge RV (≥ 85%) geëvalueerd. Bovendien worden de waterrelaties in de na-oogstfase van zes genotypen, die verschillen in huidmondjesrespons, geanalyseerd. Een grote genotypische variatie in de huidmondjesreactie op waterstress werd waargenomen. Het relatieve watergehalte (RWC), na 4 uur van bladuitdroging varieerde tussen de 7 en 62%, en de ouders van de populatie vertoonden verschillend huidmondjes-gedrag (RWC was respectievelijk 20 en 51%). Het vaasleven van genotypen met een sterke respons varieerde aanzienlijk (10–21 dagen), terwijl in alle gevallen maximale bloemknopopening bereikt werd. Het vaasleven van genotypen met geringe huidmondjesrespons was nooit langer dan negen dagen (en in twee van de drie genotypen ging de bloem zelfs niet volledig open tijdens het vaasleven). Het kortere vaasleven en de remming van de bloemopening in genotypen met geringe huidmondjesrespons, in vergelijking met genotypen met een sterke huidmondjesrespons, was het gevolg van het grotere (70%) vochtverlies in de naoogstfase. De resultaten in deze studie suggereren dat er een grote variatie is in de huidmondjesrespons van planten geteeld bij hoge RV, hetgeen mogelijkheden biedt voor de ontwikkeling van cultivars tolerant voor hoge RV. De selectie van genotypen met een sterke huidmondjesrespons, na teelt bij hoge luchtvochtigheid, zal het potentiële vaasleven aanzienlijk verhogen. Integratie van dergelijke protocollen in veredelingsprogramma's zal de selectieprocedures verbeteren en houdbaarheidsproblemen in de gehele keten verminderen.

In **hoofdstuk 4** wordt het effect van een verandering in de relatieve luchtvochtigheid, in verschillende stadia van de strekking van de deelblaadjes van rozen op huidmondjes-functionaliteit bestudeerd. Deze functionaliteit wordt beoordeeld door het meten van de verdampingssnelheid van een deelblaadje in reactie op uitdroging, nadat de deelblaadjes volledig zijn uitgegroeid. Intacte planten, geteeld bij een hoge RV hadden een significant hogere bladwaterpotentiaal tijdens de daglichtperiode, dan planten geteeld bij een gematigde RV, ondanks hun veel hogere huidmondjesgeleidbaarheid. De huidmondjes van deelblaadjes die zich eerst ontwikkelden bij een hoge RV en vervolgens naar een gematigde RV overgeplaatst werden functioneerden beter (hoe eerder tijdens de ontwikkeling dit overplaatsen plaatsvond, hoe beter het functioneren). Huidmondjes van deelblaadjes die van een gematigde RV naar een hoge RV werden verplaatst functioneerden slecht, zelfs als het overplaatsen zeer laat tijdens de uitgroei van het deelblaadje plaatsvond. Het dagelijks toedienen van een abscisinezuur (ABA) oplossing aan de deelblaadjes, geteeld bij continu hoge RV, was alleen effectief voor het verkrijgen van normale huidmondjesfunctionaliteit als het werd gedaan voordat de deelblaadjes volledig uitgegroeid waren. Als planten naar een andere RV werden overgeplaatst nadat het betreffende deelblaadje volledig was uitgegroeid, functioneerden de huidmondjes alsof deze overplaatsing niet had plaatsgevonden. Ook huidmondjes van deelblaadjes die

volledig waren uitgegroeid bij gematigde RV, en vervolgens werden afgesneden van wortelsignalen (bijv. ABA) bleven hun vermogen om te sluiten behouden (gemeten gedurende een periode van 14 dagen). Samengevat geven deze resultaten aan dat het functioneren van huidmondjes sterk afhankelijk is van het luchtvochtigheidsniveau waarbij het blad uitgroeit. Zodra de bladeren volledig zijn uitgegroeid, kan de huidmondjesfunctionaliteit niet meer worden beïnvloed, zelfs niet indien er drastische veranderingen in de ABA concentratie in het blad plaatsvinden.

In **hoofdstuk 5.1** werd de initiatie en de uitgroei van huidmondjes, alsmede de huidmondjesdichtheid geanalyseerd. Huidmondjeseigenschappen zoals dichtheid, index, en grootte werden bepaald over het gehele oppervlak van een deelblaadje (waarnemingsgebieden van 1 cm^2) op 33%, 50%, 67% en 100% van de volledige strekking van het deelblaadje (FLE; lengte van het deelblaadje als percentage van de uiteindelijke lengte). Bovendien werden huidmondjesafmetingen bepaald in volledig uitgegroeide deelblaadjes. Meer dan 80% van het uiteindelijke aantal huidmondjes ontstond tussen 33% en 67% FLE. Huidmondjesgroei (op basis van lengtetoename) vond vooral plaats tussen 67% en 100% FLE. Het rekening houden met ruimtelijke heterogeniteit in huidmondjesdichtheid en -grootte was van essentieel belang bij het analyseren van huidmondjesinitiatie en -groei. Een aanzienlijke variatie binnen een blad in huidmondjesdichtheid en -opening werd ook gevonden in volledig uitgegroeide deelblaadjes. Stomatale geleidbaarheid (g_s) van het blad werd gekwantificeerd met behulp van een model. Het model gaf aan dat binnen een deelblaadje verschillen in de g_s van 37% bestonden. Variaties in huidmondjesdichtheid en variaties in huidmondjesopening droegen daar in gelijke mate aan bij. Het onderzoek laat zien dat het noodzakelijk is met de variatie binnen een blad rekening te houden, om onjuiste conclusies te vermijden, zoals hier aangegeven voor a) de analyse van de vorming van huidmondjes, en b) de schatting van de verdamping van bladeren, op basis van huidmondjeskenmerken. De bestaande steekproefprotocollen voor de bepaling van huidmondjeskenmerken zijn daarom kritisch geëvalueerd.

In **hoofdstuk 5.2**, wordt de hoge snelheid van waterverlies gedurende de lichtperiode van 'Pink Prophyta' rozen geteeld bij een hoge RV (95%) (hoofdstuk 2) geanalyseerd in relatie tot de huidmondjeseigenschappen over het gehele oppervlak van een deelblaadje. Daarnaast wordt de afnemende mogelijkheid die het blad heeft om waterverlies te beperken wanneer deze volledig is uitgegroeid, naarmate het blad een groter deel van de strekkingsgroei bij hoge RV volbrengt, voor de aansluitende overgang naar een gematigde RH (hoofdstuk 4), besproken in relatie tot huidmondjesaanleg en -groei. Huidmondjesdichtheid en lengte werden bepaald over het gehele oppervlak van een deelblaadje op 33%, 50%, 67% en 100% van de volledige uitgroei (FLE; percentage van de lengte van een deelblaadje ten opzichte van de uiteindelijke lengte). Lengte van de huidmondjesopening en de mate van opening werden ook gemeten bij volledig uitgegroeide deelblaadjes. Verhoogde RV had geen significante invloed op de

huidmondjesdichtheid, maar de huidmondjeslengte nam toe. Van langere huidmondjes bleek ook de opening langer te zijn. Deze langere opening in combinatie met een grotere opening over het gehele oppervlak van een deelblaadje, vergeleken met huidmondjes aangelegd en uitgegroeid bij een gematigde RV, leken te verklaren waarom bloemen geteeld bij hoge RV een hogere snelheid van waterverlies in de lichtperiode lieten zien. Ook werd voor deelblaadjes die uitgroeiden bij hoge RV aangetoond dat het aantal huidmondjes langer dan 32 μm steeg van 1% van de gevormde huidmondjes op 33% FLE tot ongeveer 35% bij 67% FLE. Op 100% FLE waren alle huidmondjes langer dan 32 μm. Deze toename van het aantal huidmondjes langer dan 32 μm was positief gecorreleerd aan een geringer worden van de respons op uitdroging, in de huidmondjes van deelblaadjes die later in hun ontwikkeling van hoge naar gematigde RV verplaatst werden en werden afgesneden op 100% FLE.

De experimenten en de resultaten beschreven in dit proefschrift geven inzicht in de huidmondjesrespons en anatomische kenmerken van huidmondjes van planten, die voorzien zijn van voldoende water en geteeld worden bij hoge RV, en in de ontwikkelingsstadia, waar de huidmondjesfunctie kan worden gemanipuleerd door veranderingen in de atmosferische luchtvochtigheid. De belangrijkste resultaten en de beperkingen van het onderzoek beschreven in dit proefschrift worden besproken in **hoofdstuk 6**, en suggesties voor toekomstig onderzoek worden gepresenteerd.

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About the author

Dimitrios Fanourakis was born on 23rd February 1982 in Heraklion, Crete, Greece. After completing high school in his home town in 1999, he undertook his higher education studies at the Agricultural University of Athens (Greece). He received his Diploma degree in plant biotechnology in July 2004 with 7.8/10. After one year of military service, he was awarded a full scholarship from the Alexander S. Onassis public benefit foundation to continue his studies in Greenhouse Horticulture as an MSc student in Wageningen University (The Netherlands). Since the very early stages of his studies, plant water relations never ceased to amaze and surprise him. This may explain why, although a biotechnologist, he chose the area of plant physiology. He received his MSc degree in June 2007 with 8.4/10. In September of that year, he was awarded another scholarship from the same foundation to pursue a PhD in the Horticultural Supply Chains group. This thesis is the outcome of the study.

LIST OF PUBLICATIONS

Papers published in refereed journals

Fanourakis D, Carvalho SMP, Almeida DPF, Heuvelink E (2011) Avoiding high relative air humidity during critical stages of leaf ontogeny is decisive for stomatal functioning. Physiologia Plantarum 142: 274–286

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García NV, van Mourik N, Fanourakis D (2011) Growth responses of two *Anthurium andreanum* genotypes to elevated carbon dioxide concentration. Acta Horticulturae (in press)

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- On the physiological and anatomical responses of stomata to long-term elevated atmospheric humidity (2007)

Writing of project proposal (7.5 ECTS)

- Effects of high relative air humidity during growth on the postharvest quality of cut roses: genotypic variation, analysis and simulation (2007)
- Long-term elevated atmospheric humidity disrupts specific guard cell signal transduction elements leading to impaired stomatal closure (2011)
- Possibilities of light quality manipulation for producing compact horticultural crops (2011)

Post-graduate courses (4.5 ECTS)

- The art of Modelling; PE&RC (2008)
- Advanced Statistics; PE&RC (2009)

Laboratory training and working visits (4.5 ECTS)

- Quantification of endogenous ABA; University of Hohenheim, Stuttgart, Germany (2009)

Competence strengthening/skills courses (1.8 ECTS)

- PhD Competence assessment; WGS (2008)
- Effective behaviour in your professional surroundings; WGS (2011)
- Career assessment; WGS (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (7.1 ECTS)

- Frontier Literature on Plant physiology (FLOP meeting) (2007-2011)
- Several excursions to companies involved in greenhouse production (2007-2011)

Discussion groups/local seminars/other scientific meetings (2.25 ECTS)

- PE&RC Day (2008-2010)
- "Photosynthesis: from femto to Peta and from nano to Global" (2009)
- PE&RC Weekend (2010)
- Library WUR: "How to write a world class paper" (2010)

International symposia, workshops and conferences (10.8 ECTS)

- International Symposium on Postharvest of Ornamental plants; poster presentation; Odense, Denmark (2008)
- 5th International Symposium on Rose Research and Cultivation; 2 posters and an oral presentation; Gifu, Japan (2009)
- 28th International Horticultural Congress; poster and oral presentation; Lisbon, Portugal (2010)

Supervision of 8 MSc students; 80 days

- Response and anatomical characteristics of stomata formed at contrasting humidities
- Postharvest water relations of cut roses grown at high humidity
- Stomatal adaption dynamics to contrasting humidity during leaf expansion
- Stomatal initiation and development : role of air humidity
- Water uptake components in cut rose: role of air humidity
- Does partial root drying mitigate the negative effect of high humidity on stomatal responsiveness?
- Variation of stomatal responses in a cut rose population grown at high humidity
- Vase life and flower opening of a cut rose population grown at high humidity