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

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ABSTRACT

To better understand the poor regulation of water loss after leaf development at high relative air humidity (RH), the relative importance of the physiological and anatomical components was analyzed focusing on cultivars with a contrasting sensitivity to 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 transpiration rate (CTR) 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 modified version of the Brown and Escombe equation. 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 CTR. It was estimated that in leaflets subjected to desiccation the enhanced g_s in high RH- as compared to moderate RH-grown plants was mostly due to poor stomatal functionality and to a lesser extent the combined result of higher stomatal density and longer pore length. It is concluded that the reduced degree and, specially, the reduced rate of stomatal closure are the primary causes of the large genotypic variation in the control of water loss in high RH-grown plants. Furthermore, it was found that although changes in stomatal length have no influence on stomatal functionality, changed anatomical features *per se* represent a significant and direct contribution to the increased water loss.

Introduction

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. 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 (Brainerd and Fuchigami, 1982; Aguilar et al., 2000). 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 (reviewed by Fanourakis et al., 2013). In rose, a large genotypic variation in the sensitivity to high RH was found (ranging from 11 to 110% water loss increase during leaf desiccation, as compared to leaves from moderate RH-grown plants; Mortensen and Gislerød, 1999). Despite this genotypic variation in the leaf capacity to control water loss, the processes behind the tolerance to high RH are not yet fully understood.

Long-term high RH is known to induce abnormal stomatal functioning, bigger stomata and changed stomatal density in different plant species (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 suggested as the main factor involved in the disturbed water relations in high RH-grown plants (Aguilar et al., 2000; Rezaei Nejad and van Meeteren, 2007). Nevertheless, the relevance of the changed anatomical features in the enhanced water loss of high RH-grown plants has hardly been studied. For instance, a negative relationship between stomatal size and stomatal functioning has been observed when comparing different species (Aasamaa et al., 2001; Franks and Farquhar, 2007; Drake

Abbreviations: ABA, abscisic acid; CTR, cuticular transpiration rate; g_s, stomatalconductance; PAR, photosynthetically active radiation; RH, relative air humidity; RWC, relative water content; VPD, vapour pressure deficit.

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et al., 2013), but such relationship has not been investigated within a given species. At leaf level the stomatal conductance (g_s) is not only determined by stomatal pore aperture, but it also depends on stomatal density, pore length and pore depth (Franks and Beerling, 2009; Roth-Nebelsick et al., 2012). Since it has been demonstrated that high RH exerts an effect on both stomatal density and pore length (Fordham et al., 2001; Torre et al., 2003), changes in these characteristics might influence *per se* the g_s, having a direct role on the enhanced water loss. Thus, when using direct (*i.e.* gravimetry, Rezaei Nejad and van Meeteren, 2005; Fanourakis et al., 2011) or indirect (*e.g.* infra-red gas analyzer, porometer, thermal or chlorophyll fluorescence imaging; Ottosen et al., 2002; Torre et al., 2003; Rezaei Nejad and van Meeteren, 2007) methods for measuring g_s, those measurements do not only reflect the RH effect on stomatal physiology (stomatal opening), but also include the influence of the anatomical features (*i.e.* stomatal density, pore length and depth), which is often neglected. To the best of our knowledge, there are no studies 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 each of its component variables, such as stomatal density and pore dimensions. This analysis can only be performed by estimating the sensitivity of g_s to high RH-induced changes in each stomatal feature (*e.g.* based on the equation of Brown and Escombe (1900), or modified versions of this equation; Nobel, 1991; Taylor et al., 2012).

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 transpiration rate (CTR) in plants grown under prolonged periods of high humidity, have been focused on *in vitro* plants, where a higher CTR was frequently observed (reviewed by Pospisilova et al., 1999; Hazarika, 2006). Nevertheless, a comprehensive analysis of the relative contribution of CTR 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 and understand 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 and quantify the relative contribution of each anatomical feature (*i.e.* stomatal density, pore length, pore depth, and CTR) and of stomatal functionality (pore aperture), on the higher water loss rates in well-watered plants grown under long-term high RH. The hypotheses that the genotypic variation in the control of water loss is primarily driven by differential effects on the stomatal physiology and that the changes in stomatal size influence their functionality were tested.

Materials and methods

Plant material and growth conditions

Rooted cuttings of four cut rose cultivars (*Rosa 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 B.V., Aalsmeer, The Netherlands) and perlite (Agraperlite nr. 3, Pull Rhenen, The Netherlands) (3:1, v/v). *R. hybrida* was chosen as a model system, since this is a hypostomatous species (*i.e.* no stomata were found on the adaxial leaf surface) facilitating the assessment of the CTR, and because of the existence of cultivars with contrasting water loss rates when grown under long-term high RH. 'Dream' and 'Frisco' were selected due to their lower water loss rate during vase life (tolerant cultivars to high RH), whereas 'Pink Prophyta' (called 'Prophyta' in the remainder of this article) showed a higher water loss rate (sensitive cultivar to high RH) (Fanourakis et al., 2012). 'Vendela' has also been indicated as a cultivar belonging to the latter group (C. Slootweg, personal communication). Twenty-four plants per cultivar were randomly distributed over four growth chambers ($l \times w \times h = 1.3 \times 0.8 \times 1.3$ 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 $60 \pm 3\%$ (moderate RH), and in the other two it was $95 \pm 1\%$ (high RH) during the cultivation period. The four chambers had constant day and night temperatures ($19 \pm 1\,^{\circ}C$), resulting in vapour pressure deficits (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, USA). 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, USA). 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 (PAR; Model LI-250, LI-COR, Lincoln, NE, USA). The irradiance was measured at 70 cm from the root-shoot interface, which corresponds to the top of fully grown plants.

Plants were well-watered automatically with a nutrient solution (Fanourakis et al., 2009). 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 the evaluations mentioned below). 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, $50 \pm 3\%$ RH (1.24 ± 0.07 kPa VPD) and a photon flux density of 50 μmol m^{-2} s^{-1} PAR (TLD 58W/84, Philips, Eindhoven, The Netherlands).

For the desiccation stimulus, 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 5–30 min during 4 h. The leaflet area was then determined using a leaf area meter (model 3100 Area Meter, LI-COR, Lincoln, NE, USA) 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 stimulus, 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 20 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, USA). Both flasks were sealed with parafilm to prevent evaporation. Leaflet transpiration rate was gravimetrically determined by weighing the flasks with the leaflets every 5–10 min