

Leaf Anatomy and Stomatal Morphology of Greenhouse Roses Grown at Moderate or High Air Humidity

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ADDITIONAL INDEX WORDS. epicuticular wax, postharvest life, stomatal aperture, *Rosa*, transpiration rate, water loss

ABSTRACT. Single node cuttings with one mature leaf were taken from *Rosa ×hybrida* 'Baroness' and rooted in water culture. The plants were subjected to either 90% (high) or 70% (moderate) relative humidity (RH) in climate chambers. Single stem roses with intact roots were transferred to 40% (low) RH to investigate the stomatal response to water stress. Moderate RH plants showed decreasing leaf conductance from day 1 to day 3 during both light and dark phases, in contrast to high RH roses, which showed almost similar leaf conductances during the 3 days. Leaf samples were studied with a light microscope (LM) and a scanning electron microscope (SEM) to quantify morphological and structural changes. Epidermal imprints showed a significantly higher number of stomata and longer stomata, as well as a wider stomatal apertures on roses grown at high RH. The high RH leaves showed a reduced density of vascular tissue and thinner leaves when compared to moderate RH leaves. Enlarged intercellular air-space (ICA) was found due to a reduced number of spongy and palisade mesophyll cells. No obvious difference in shape, size, undulation or the structure of the epicuticular wax was observed in SEM between high and moderate RH grown leaves. In conclusion, roses subjected to high RH showed differences in leaf anatomy, stomatal morphology and stomatal function, which may explain the loss of water control of these plants. Stomatal ontogenesis should occur at RH conditions below 85% to secure roses with a high postharvest quality potential.

Postharvest life of cut roses is often limited by water stress. Roses do not undergo a natural senescence process, but end their vase life because of premature wilting of flowers and leaves as well as a bent-neck (Burdett, 1970; Torre and Fjeld, 2001; Zieslin et al., 1978). Transpiration rate and the capacity of the flower tissue to retain water, as well as the water uptake rate and transport determine the water balance of cut flowers in general (Reviewed by Halevy and Mayak, 1981; van Doorn, 1997). Preharvest conditions are known to influence the vase life of roses by affecting their ability to control postharvest water loss. For instance, increasing the daylength in the greenhouse by artificial light, results in an increase in the duration of stomatal opening, higher water loss from the flowering shoot and hence early wilting after cutting (Mortensen and Fjeld, 1998; Sloopweg and van Meeteren, 1991). Also humid air (>85% to 90% RH) during growth is a critical environmental factor reducing the postharvest life of cut roses, mainly due to uncontrolled water loss from the cut shoot (Mortensen and Fjeld, 1995; 1998; Mortensen and Gislerød, 1999; Torre and Fjeld, 2001; Torre et al., 2001). Plants cultured in vitro are widely known to display desiccation when transferred to soil and placed in greenhouses. The problem is thought to be due to the high RH of the culture vessels, and plants have shown considerable modifications from what is normally seen in tissue structure and functioning (Brainerd et al., 1981; Sciutti and Morini, 1995; Wardle and Short, 1983). In particular, alterations in leaf anatomy, such as reduced cuticle development and reduced stomatal closure, appear. One objective of this study was to elucidate the extent to which plants grown at high RH undergo similar anatomical and

morphological changes as described for plants regenerated in vitro. In addition, the study was undertaken to evaluate if differences in leaf morphology between high and moderate RH could explain the distinct postharvest behavior of these roses.

Materials and Methods

PLANT MATERIAL AND GROWING CONDITIONS. Rooted stems of *Rosa ×hybrida* 'Baroness' were grown in growth chambers from single-node segments with a mature leaf in water culture (Torre and Fjeld, 2001). During the rooting period, RH was kept above 90%. After rooting, RH was adjusted to either high: 90% ± 5% (−14.2 MPa) or moderate: 70% ± 5% (−48.2 MPa) RH. Temperature was kept at 20 ± 1 °C and the CO₂ at 700 ± 50 μL⁻¹L⁻¹ during the entire experimental period. All experiments were carried out during the darkest period of the year (January to April and September to December) with a minimal influence of natural sunlight. Artificial light provided by high-pressure-sodium-lamps (SON-T) was supplied 20 h every 24 h period with a 4 h dark period given in between. The average light intensity was 75 ± 10 μmol·m⁻²·s⁻¹ (measured with a LI-185; LI-COR). Plants were harvested at commercial stage, A (Itzhaki et al., 1990).

POROMETER MEASUREMENTS. Leaf conductance (mmol·m⁻²·s⁻¹) was measured on the same plant for three days to study the stomatal response to water stress. Roses with intact roots were transferred from the chambers directly to a test room, placed in 0.5-L vases with double distilled water and acclimated for 8 h before the first measurement with an AP4 porometer (Delta-T Devices LTD, Cambridge, U.K.). The temperature in the test room was 20 °C, RH was 40% (−127.4 MPa) and an irradiation level of 14 μmol·m⁻²·s⁻¹ was provided by fluorescent tubes for 12 h per day. Measurements were taken on the abaxial side of the leaves, twice daily, once in the light period and once in the dark period (3 h after light off), corresponding to a time when plants had light or

Received for publication 16 Oct. 2002. Accepted for publication 28 Jan. 2003. The authors thank Vigdis Revhaug for technical assistance. Thanks also to Elisabeth Eng and Trygve Krekling (Department of Chemistry and Biotechnology) and Nina Nagy (Norwegian Forest Research Institute) for assistance during the microscopy work. The work was financially supported by the Norwegian Research Council, project number 107008/111.



Fig. 1. Leaves from 'Baroness' roses grown at high (90%) and moderate (70%) RH, 35 minutes after detachment. The detached leaves were placed in 20 °C, RH was 40% (-127.4 Mpa) and an irradiation level of 14 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was provided by fluorescent tubes.

Table 1. Relative changes in leaf conductance during light and darkness measured on roses with intact roots developed at high (90%) and moderate (70%) RH in response to continuous placement in a test room [RH was 40% (-127.4 Mpa)] and an irradiation level of 14 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was provided by fluorescent tubes 12 h daily. (n = 12).

Day	Leaf conductance ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)			
	90% RH		70% RH	
	Light	Darkness	Light	Darkness
1	100	100	100	100
2	91	96	74	58
3	110	100	69	58

dark during cultivation. Samples were taken from two independent experiments, each with six plants per treatment.

STOMATAL MORPHOLOGY. Roses with intact roots were transferred to a test room (described above) and acclimated for at least one hour prior to sample collection. Epidermal impressions were made on fresh intact upper leaves by applying transparent glue, which covered ≈ 2 to 3 cm^2 of the abaxial surface. After drying (2 to 3 min) the imprints were removed from the leaf with clear adhesive tape and glued on a microscope slide. All samples were taken interveinal near to the midrib. Stomatal length, aperture and frequency were measured with use of video microscopy and digital image processing (see below). Samples were taken from three independent experiments, each with five plants per RH treatment.

LIGHT MICROSCOPY AND SEM PROCEDURE. For SEM (JEOL JSM 840 SEM) and LM, fresh fully developed upper leaves were collected and interveinal samples were taken close to the midrib. Sample sections were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 50 $\text{mmol}\cdot\text{L}^{-1}$ L-piperazine-N-N'-bis(2-ethane sulfonic) acid buffer (pH 7.2) for 24 h at room temperature.

Fixed tissue was rinsed with the same buffer, and dehydrated in an ethanol series (70% to 80% to 90% to 96% to 4 \times 100%). For SEM, fixed and dehydrated samples were critical point dried with CO_2 , mounted on aluminum stubs and sputter coated with palladium-platinum. Samples for LM were infiltrated with L.R. White acrylic resin (TAAB Laboratories, U.K), and polymerized at 60 °C for 24 h. For the LM study, semithin (1 to 2 μm) sections were cut with a glass knife. The sections were dried on silanated slides, stained with Stevenel's blue (del Cerro et al., 1980), mounted with immersion oil, and imaged and analysed in a Leitz Aristoplan light microscope with use of video microscopy and digital image processing (Image Pro Plus, ver. 3.0). For carbohydrate staining, the periodic acid/Schiffs (PAS) method was used. LM sections were oxidized with periodic acid (1%) for 30 min, then washed with distilled water, and treated with Schiff's reagent (Sigma Chemical Co.) for 2 h in darkness. Control slides were incubated in distilled water prior to treatment with Schiff's reagent. After several washes, the sections were dried, mounted, and imaged as above. PAS is selective for carbohydrate residues with vicinal-hydroxyl groups, which in the first step is oxidized to aldehyde groups by periodic acid, and then reacts with Schiff's reagent to give a pink-colored complex (Hotchiss, 1948; McManus, 1948). Samples were taken from two independent experiments, each with 5 plants per RH treatment for both SEM and LM analysis.

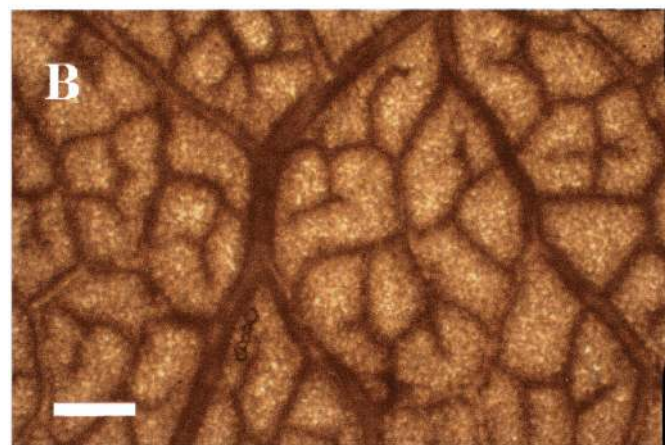
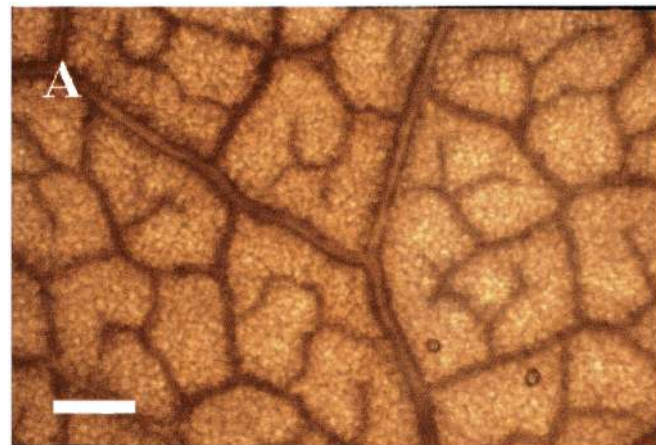


Fig. 2. Detached upper leaves from 'Baroness' roses grown at (A) high (90%) and (B) moderate (70%) RH, bleached by placement in ethanol (100%) for 24 h. Photographs were taken interveinal close to the mid-rib. Scale bars = 0.5 mm.

Table 2. Leaf morphology characteristics^z of roses grown at high (90%) and moderate (70%) RH.

Leaf morphology	90% RH	70% RH
Leaf thickness (μm)	139.8 a ^y	167.4 b
Thickness of upper epidermis cells (μm)	16.3 a	18.9 b
Thickness of lower epidermis cells (μm)	13.5 a	17.3 b
ICA (%)	49.1 a	39.9 b
Spongy mesophyll cell number/mm ²	628 a	797 b
Palisade mesophyll cell number/mm ²	911 a	1003 b

^zStatistical difference was calculated by a two-way ANOVA with two independent replications of the experiment. Data was sampled from five plants in each replication.

^yDifferent letters in the same line indicate significant difference ($P \leq 0.05$).

Table 3. Stomatal characteristics^z of roses grown at high (90%) and moderate (70%) RH.

Stomatal characteristics	90% RH	70%RH
Stomatal number / mm ²	52.0 a ^y	39.0 b
Stomatal aperture (μm)	12.6 a	9.0 b
Stomatal length (μm)	36.0 a	32.4 b

^zStatistical difference was calculated by a two-way ANOVA with three independent replications of the experiment. Data was sampled from five plants in each replication.

^yDifferent letters in the same line indicate significant difference ($P \leq 0.05$).

Results

LEAF CHARACTERISTICS. Leaves of high and moderate RH roses showed similar general appearance during development and at the stage of harvest. However, the transpiration rate was much higher in high RH roses than in moderate RH roses. This was clearly demonstrated by detached leaves. Fully developed leaves from roses grown at high RH showed wilting signs 30 min after detachment (Fig. 1). The conductivity of high RH roses with intact roots did not show any tendency to decrease during time: at day 3 the leaves still showed the same relative conductance as the first day of measurement, both in light and darkness. In contrast, the stomata of moderate RH roses responded when placed in dry air with decrease in relative leaf conductance over time being observed in light and darkness (Table 1). The leaves of roses have reticulately branched veins, which are pinnate (with one central vein, the midrib, and smaller branching veins). For studying the vascular bundle pattern between two branches, it was clear that the density of the vasculature was higher in roses grown at moderate RH than high RH (Fig. 2). In addition, significantly thinner leaves were found in high RH plants (Table 2).

STOMATAL AND EPIDERMAL CHARACTERISTICS. Stomatal morphology confined to the abaxial surface of leaves is presented in Table 3. A significantly higher number of stomata and longer stomata were found in plants grown at high RH compared to plants grown at moderate RH. The variability in stomatal size was higher in the high RH plants (data not shown),

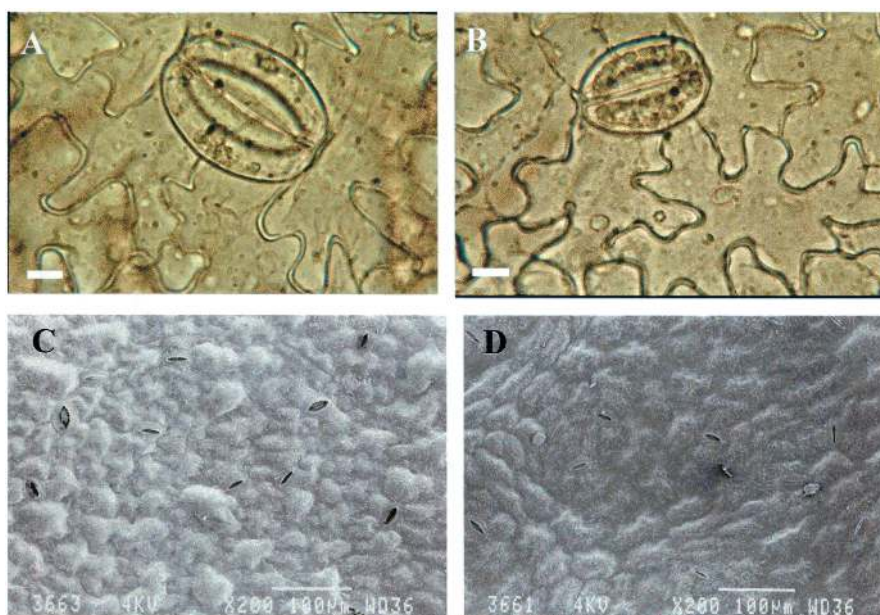
and several of the stomata were remarkably larger than those of moderate RH plants. Figure 3A shows a giant stomata from high RH leaves and a normal one from moderate RH leaves (Fig. 3B). Stomatal aperture was found to be $\approx 25\%$ wider on the imprints taken from high RH plants compared to the moderate RH plants. The epidermis and stomata of high and moderate RH leaves were studied with the SEM (Fig. 3C and 3D). The lower epidermal surface on the high and moderate RH leaves looked somewhat similar. No obvious variation in shape, size or undulation was observed in the SEM between high and moderate RH. Furthermore, no apparent differences in the structure of the epicuticular wax were observed between the two treatments. No hydathodes were observed on either high or moderate RH leaves.

LEAF MORPHOLOGY. Cross-sections of leaves showed that elevated RH induced structural modifications (Fig. 4, Table 2). Significantly more intercellular air spaces and a reduced number of both palisade and spongy mesophyll parenchyma cells were measured on the high RH leaves. The thickness of epidermal cells on both sides was lower in high than in moderate RH leaves (Table 2). Moderate RH leaves showed a stronger tendency for developing two layers of palisade parenchyma. The PAS reagent stained cell walls and starch grains of the tissue. In general, the moderate RH leaves tended to show more starch grains and a stronger coloration compared to the high RH tissue (Fig. 4).

Discussion

Stomatal behavior can be influenced by environmental factors in a direct or indirect manner. When a plant is exposed to water stress, tissue water potential will normally decrease. This condition is transmitted to the guard cells via hydroactive and/or hydropassive mechanisms and stomata close (Willmer and Fricker, 1996). Despite the much higher water loss from high RH roses they continued to lose just as much water during the three days in light and darkness. This means that the stomata of the high

Fig. 3. Epidermal cells of a 'Baroness' rose leaf. Abaxial epidermis peel of upper leaves grown in (A) high (90%) and (B) moderate (70%) RH. Note that the guard cells of the high RH leaves contain less starch grains. Scale bars = 8μm. Scanning electron micrographs of the abaxial epidermal surface of leaves grown in (C) high and (D) moderate RH showing wax cover and numerous stomata.



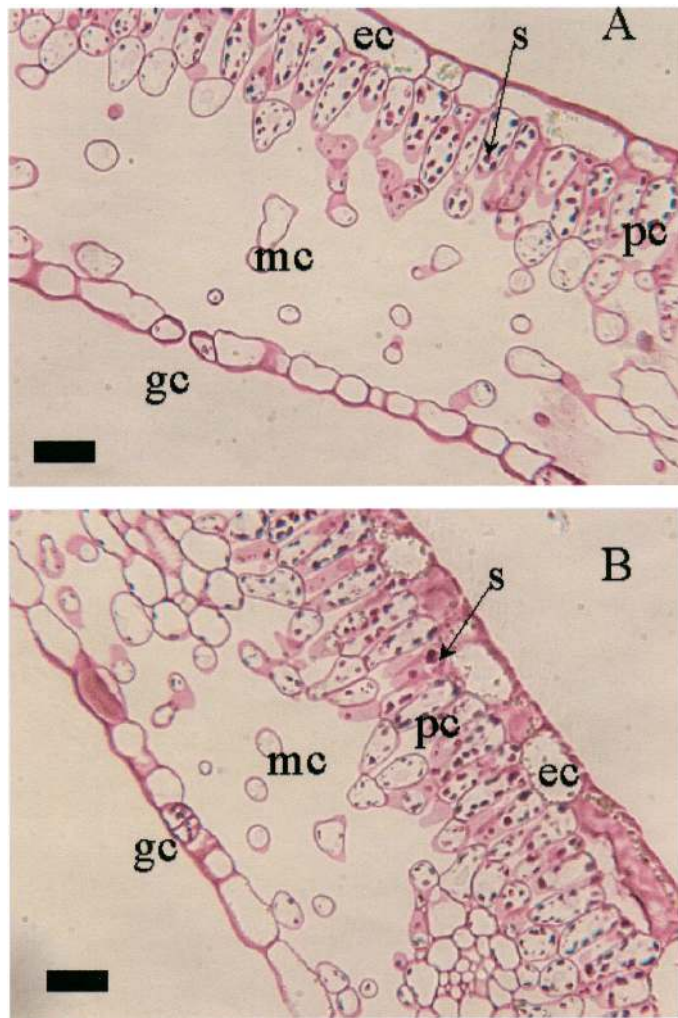


Fig. 4. Light photomicrograph cross-sections of 'Baroness' rose leaves grown at (A) high (90%) and (B) moderate (70%) RH, stained with carbohydrate staining (PAS). Note the strong coloration of the moderate RH upper epidermis. ec = epidermal cell, gc = guard cell, mc = mesophyll cell, pc = palisade cell, s = starch. Scale bars = 12.5 μ m.

RH roses are insensitive to water stress and that the situation is somehow irreversible. In contrast, moderate RH roses responded to dehydration by stomatal closure, indicating that they are more competent to adapt when transferred to an interior climate than high RH roses.

The microscopy analysis showed that RH affected the morphological development of the leaf tissue during growth. Alterations in stomatal morphology and enlarged ICA due to reduced palisade and spongy mesophyll parenchyma cells were observed in leaves of plants grown at high RH. Microscopic examinations of leaves produced *in vitro* have shown, in many cases, that they either possess a very thin palisade tissue or lack it all together. Most of the unorganized mesophyll consists of spongy parenchyma that is rich in ICA (Paques and Boxus, 1987). Alterations in stomatal morphology in micropropagated plants have been reported in several species, including *Malus* (Blanke and Belcher, 1989), *Delphinium* (Santamaria et al., 1993), *Prunus* (Sciutti and Morini, 1995) and in *Rosa* (Cappellades et al., 1990; Ghashghaie et al., 1992). Exceptionally large stomata, many of which have developed abnormally with pores 'locked' open are commonly found in *in vitro* plants (Willmer and Fricker, 1996).

In the present study, occasionally large stomata could be observed on the high RH leaves.

The fact that stomata are large does not explain why high RH leaves dehydrate rapidly after detachment. In some studies, discontinuity in the epidermis and epidermal pores in association with stomata have been found in *in vitro* plants, and suggested these are the main cause of desiccation *in vivo* as was the case for *Gypsophila* (Gribble et al., 1996). Discontinuity was not observed on the epidermis of the high RH rose leaves when studied with the SEM in the present study. Neither did we observe any obvious differences in shape, size nor undulations of the epidermal layer as described previously for *in vitro* rose plants (Cappellades et al., 1990). Recent suggestions have indicated reduced cuticle development and lack of cuticular waxes to be one of the major causes for desiccation of *in vitro* plants (Fabbri et al., 1986; Sutter and Langhans, 1982). However, no obvious difference in epicuticular wax was observed with the SEM between high and moderate RH plants. Finally, it may be concluded that the excessive water loss of the high RH plants cannot be explained by a poorly developed epidermis, either on the adaxial or abaxial side. The former was previously confirmed by porometer measurements (Torre and Fjeld, 2001).

A lower density of vascular tissue was observed on high RH leaves. The differentiation and development of the vascular bundles are known to be affected when plants are grown under very humid conditions (Smith et al., 1986). Enlarged ICA and guard cells protruding above the epidermal surface are structural adaptations that may assist in increasing the water loss from high RH plants. However, the main reason for the high water loss of high RH plants seems to be a higher number of stomata with a reduced function. There is currently little information on the molecular control of stomatal initiation and spacing patterns. It is however well known that the stomatal index depends on the environment in which the leaves develop (Schoch et al., 1980). Lack of stomatal closure has been observed in several micropropagated plants, including roses (Cappellades et al., 1990; Sallanon et al., 1993; Short et al., 1981). In roses, the lack of closure was attributed to high levels of K, which the guard cells accumulated from the salt rich culture medium (Short et al., 1981). A lower Ca level is currently found in leaves of high RH roses. However, by manipulating the K/Ca ratio, only slight or inconspicuous differences in the day/night transpiration rate ratio were observed in rose leaves (Torre et al., 2001). Hence, it is most unlikely that nutrient status of the high RH plants can explain the reduced stomatal response to water stress.

Several authors have suggested that failure of stomatal closing lies mainly in the guard cell wall (Ziv et al., 1987) and that stomata from leaves raised *in vitro* cannot be fully repaired or become functional (Sallanon et al., 1993). The fact that the high RH roses did not respond fully to darkness (Torre and Fjeld, 2001; Torre et al., 2001) or to water stress over time, indicated a similar structural and/or physiological malfunctioning of the stomata. However, more research is needed to define the limitation on closing more in detail.

In conclusion, the excessive water loss from the high RH leaves is due to a higher number of stomata with a wider aperture that are unable to close fully. High RH tends to affect the anatomical and morphological characteristics of the leaves in a similar, however in a less apparent, way as reported in micropropagated plants. Earlier work has shown that RH during growth is decisive for the postharvest life. Cut roses grown at high RH (85% to 90%) show premature wilting of flowers and leaves and bent necks.

The structural and functional characteristics of leaves developed at high RH described in the present study may explain why these roses show such a poor postharvest longevity. Thus, stomatal ontogenesis should occur at RH conditions below 85% to secure roses with a high postharvest quality potential.

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