Plant Cell, Tissue and Organ Culture **30**: 51–57, 1992.

Water relations and growth of rose plants cultured *in vitro* under various relative humidities

Jaleh Ghashghaie, Françoise Brenckmann & Bernard Saugier
Lab. d'Ecologie Végétale, Université de Paris-Sud, F-91405, Orsay, France

Accepted in revised form 28 January 1992

Key words: bottom cooling, porous closure, *Rosa hybrida*, stomatal functioning, water loss

Abstract

Stomatal malfunctioning is one of the main reasons why plants desiccate when transferred from *in vitro* to greenhouse conditions. In order to overcome this problem in *Rosa hybrida* cv. Madame G. Delbard (R) Deladel, two techniques, bottom cooling and water vapour permeable lid, were used. Both methods aimed to increase the vapour pressure gradient between leaf and atmosphere and consequently to improve plant transpiration.

The results showed that these techniques increased leaf resistance to dehydration and improved stomatal regulation. Water relations of treated plantlets were similar to those generally observed in hardened plants: lower leaf water and osmotic potentials, and lower leaf water content than in the control ones. Osmotic adjustment occurred in treated plantlets maintaining turgor pressure. Each technique also induced some effects on growth during the rooting phase: with bottom cooling, roots were shorter, with permeable lids, apices were necrosed.

These results are discussed in terms of physiological causes and in terms of effect during the following acclimatization.

Abbreviations: AWC – absolute water content, DW – dry weight, FW – fresh weight

Introduction

Upon transfer from *in vitro* to greenhouse conditions, rooted plantlets have to undergo changes in trophic status (from heterotrophy to autotrophy) as well as in water status (from high to low relative humidity). Desiccation is one of the main reasons why plants cultured *in vitro* die during this transfer (Brainard & Fuchigami 1982; Maene & Debergh 1987; Short et al. 1987). A poorly developed epicuticular wax layer and malfunctioning stomates lead to an absence in water loss regulation of these plantlets (Sutter & Langhans 1979; Wardle et al. 1979; Brainard et al. 1981; Ziv et al. 1987). These abnormalities are mostly due to a water saturated atmosphere in the culture vessel and thus to a lack of water potential gradient between gel, plant and air. Therefore, it is important to create conditions in the culture vessels that stimulate transpiration so that *in vitro* plants, at rooting stage, behave as normal ones (Grout & Aston 1977).

It has been shown previously that promoting water transport through the plants before transplanting can be achieved by lowering relative humidity in the containers (Brainard & Fuchigami 1981; Ziv et al. 1983; Short et al. 1987; Smith et al. 1990; Preece & Sutter 1991).

Several methods have been used to create low relative humidity in the culture vessel including the use of desiccants (Wardle et al. 1983; Short et al. 1987), covering the culture medium with a layer of lanoline (Wardle et al. 1983), the use of culture vessels with porous closures (Short et al.

1987) or walls (Smith et al. 1990) and the use of saturated salt solutions (Ritchie et al. 1990). But most of these methods are not practical and/or dried the culture medium very quickly. Vanderschaeghe & Debergh (1987) used a 'bottom cooling' technique and reported that condensation of water vapour on the gel surface by cooling the bottom of the vessels not only reduced relative humidity in the container but also avoided desiccation of the culture medium.

The effects of low relative humidity in the culture vessels on stomatal functioning of plantlets have been largely studied (Brainard & Fuchigami 1982; Short et al. 1987; Ziv et al. 1987; Capellades 1989). However, there are only a few data concerning water relations of these plantlets before transplanting (Ziv et al. 1983). In our study, two techniques were used: 'bottom cooling' and 'water vapour permeable lid'. Treated plants were compared to the control ones for various parameters: leaf water potential components (water and osmotic potentials and turgor pressure), leaf absolute water content, root growth (length and number), apex necrosis and stomatal functioning (water loss of excised leaves).

The aim of this work was to examine whether reduced relative humidity in the culture vessel induces modifications in the water relations of rose plants cultured *in vitro*, permitting them to regulate water loss.

Materials and methods

Culture medium

The basic medium contained Murashige & Skoog's (1962) macroelements, microelements and vitamins and 7.5 $g\,l^{-1}$ Touzart and Matignon agar. Plant growth regulators (Sigma) were benzyladenine (7.9 μM) and gibberilic acid (0.3 μM) for multiplication medium, and indole-3-acetic acid (2.9 μM) and indole-3-butyric acid (0.5 μM) for rooting medium. 0.2 $g\,l^{-1}$ charcoal, 40 $g\,l^{-1}$ glucose were added to rooting medium and 30 $g\,l^{-1}$ glucose to multiplication medium. The pH was adjusted to 5.6 before autoclaving. All media were autoclaved at 120°C for 20 min.

Culture conditions

Glass jars (height 13 cm, vol. 850 ml) containing 120 ml of multiplication or rooting medium were used. The containers were closed with screw-on polycarbonate lids and placed in a growth chamber where the conditions were 16 h day with 24°C and 70% relative humidity, and 8 h night with 19°C and 90% relative humidity. Light was provided by white fluorescent tubes (Mazda, Aurora). Photosynthetic photon flux density was about 50 $\mu mol\,m^{-2}\,s^{-1}$ at plant level.

Plant material

A clone of *Rosa hybrida* cv. Madame G. Delbard (R) Deladel was used. At the end of the multiplication stage (day 21), usable shoots ($0.5\ cm < length > 0.3\ cm$) were decapitated and placed on rooting medium for 12 days. Care was taken to have 20 homogeneous shoots for each jar. During multiplication stage, all jars were maintained under controlled conditions as described above. But, during rooting phase, they were subjected to 3 treatments: control, bottom cooling or water vapour permeable lid. All measurements were made on the youngest fully expanded leaf at the end of the rooting phase (day 33). At this stage, leaf number per plant was not significantly different ($p < 0.05$) for three treatments. It was 6.1 ± 0.4 in control, 5.7 ± 0.4 in bottom cooling and 6.0 ± 0.5 in permeable lid treatment. These data are the means $\pm$ confidence intervals ($n = 16$).

'*Bottom cooling*'

A cooling system with an incorporated pump (3.8 $l\,h^{-1}$) was used to force cool water through a system of aluminium pipes (diam. 2.6 cm) with exchange wings. In order to ensure good thermal exchange at the bottom of the jars, an aluminium plate (width 10 cm) was placed on top of each tube. Culture vessels were placed on the aluminium plates. Temperature was adjusted on the cooling system to obtain a gradient of about 4°C between bottom and top of the jar. Temperature of the gel was 20.7°C and temperature of the air in the jar was 24.7°C. Condensation then occurred on the gel surface. Relative

humidity was calculated, as described by Vanderschaeghe & Debergh (1987), by the following formula:

$$R.H. = (e_s(Td))/(e_s(Ta))$$
$$e_s(T) = 6.1078 \exp((17.269 \times T)/(237.3 + T))$$

$e_s(T)$ = saturation vapour pressure at temperature T
Ta = temperature of the atmosphere
Td = dew-point temperature

Thus, in bottom cooled jars, R.H. was about 84% at the top of the jar and 100% at the gel surface.

The temperatures were measured by thermocouples connected to a Wescor microvoltmeter (Model HR-33T).

Permeable lid

To enhance water flux in the culture vessels and thus through the plants, the lids were perforated (diam. 2 cm) and plugged with cellulose fiber which was permeable to water vapour. In this treatment, as in the control, there was no temperature gradient in the jars. Therefore, it was difficult to calculate relative humidity of the air in these cases. However, water loss of the jars was determined by weighing them at the beginning (day 21) and at the end of the rooting phase (day 33). Water loss was 5.3 ± 0.6 ml for jars with permeable lid, 1.3 ± 0.3 ml for control and 0.9 ± 0.2 ml for bottom cooled ones (means $\pm$ confidence intervals, $n = 5$). Water vapour exchange between the jars and the culture room was thus 4 times higher in permeable lid jars than in the control ones indicating that relative humidity of the air in the jars with permeable lids is considerably reduced, thus improving water evaporation from the culture medium and transpiration of plants. In the bottom cooling treatment, the temperature gradient is inversed leading to water condensation on the gel surface. This reduces relative humidity in the jars, but this time water vapour exchange between the jars and the culture room is also reduced, in contrast to the previous treatment.

Plant growth

At the end of the rooting phase (day 33), fresh weight (FW) and dry weight (DW) (dried during 48 h at 80°C) of the leaves, shoot and root lengths and root number were determined. Leaf absolute water content (AWC) was calculated ($AWC = (FW - DW)/DW$).

Relative apex necrosis for each treatment was calculated: (number of 'necrosed apex'/total shoot number per treatment) $\times$ 100. Here 'necrosed apex' takes into account not only the shoots with necrosed apices but also those without a developing or a recently developed young leaf.

Water loss

Excised leaves were allowed to desiccate at room temperature (20 to 25°C) and their water loss was determined during 2 h by weighing them every 30 min. A similar method was used by Capellades (1989). In the present work, four leaves were used for each measurement and four measurements for each treatment. Log_{10} of $(((FW_t - DW)/(FW_0 - DW)) \times 100)$ was calculated: FW_t is the leaf fresh weight during dehydration at time (t) and FW_0 is the initial fresh weight.

Preliminary observations by scanning electron microscopy of the abaxial leaf surface 30 min after leaf excision allowed one to determine stomatal closure ((number of completely closed stomata)/(total number of stomata) $\times$ 100) and stomatal density (number of stomata per mm^2 leaf area). For these observations, the leaves were frozen in liquid nitrogen 30 min after excision. The largest leaf of 3 plants per treatment were examined.

Water potential

A thermocouple psychrometer with a Wescor microvoltmeter (Model HR-33) and C-52 sample chambers were used for leaf water and osmotic potential measurements. One or two leaves were sealed in each chamber for 45–60 min to ensure water vapour equilibrium between the sample and the air in the chamber. All measurements were conducted at room temperature (20 to 25°C). Osmotic potential was measured on previously frozen (−25°C) leaves. Leaf turgor pressure was then obtained from the difference between water and osmotic potentials.

Five jars per treatment were examined and at least 5 plants per jar (15 shoots per treatment) were used for measurement of each parameter. Data were submitted to analysis of variance and LSD was established. The means were compared between 3 treatments and significant differences among them were then tested with $p < 0.05$. The whole experiment was repeated twice and the results are similar to those presented here.

Results and discussion

Plant water relations

Both techniques, bottom cooling and permeable lid, modified significantly water relations of rose plantlets. Leaf water potential decreased from −0.76 MPa in control plants to −0.93 MPa in permeable lid and −1.17 MPa in bottom cooling ones (Table 1). The same trend was observed for leaf osmotic potential. Parallel decrease in leaf water and osmotic potentials maintained turgor pressure in both bottom cooling and permeable lid treatments (Table 1) indicating that osmotic adjustment occurred in treated plants. On the other hand, leaf AWC was significantly lower in bottom cooling (2.7) and permeable lid (3.1) treatments than in control one (3.6). These results suggest that lowering air relative humidity in the culture vessels, during rooting phase, induced some modifications in plant water relations (reduced leaf AWC and leaf water potential, osmotic adjustment and thus turgor maintenance) which are similar to those generally observed in hardened plants. These modifications may be related, as in hardened plants, to structural changes (i.e. smaller cell size, thicker cell walls, . . .) (Cutler et al. 1977; Cutler & Rains 1978) during plant development under water deficit conditions (low relative humidity in the present case).

Water loss

Time course of water loss in excised leaves was illustrated for 3 treatments (Fig. 1). This classical representation permits one to distinguish stomatal closure (Jarvis & Jarvis 1963). Generally, in the first part of this curve (at high water content), stomata are open and thus transpiration is high, but in the second part, stomatal closure occurs and progressively reduces water loss. This was true for plants of the bottom cooling treatment but not for control ones. Permeable lid treatment showed an intermediate behaviour. Water loss was significantly lower in treated plants than in control ones. Control leaves continued to lose water even 90 min after excision, whereas in 'bottom cooling' treatment some resistance to desiccation can be observed and stomatal closure seems to occur 30 min after leaf excision. This was confirmed by stomatal closure calculated from microscopical observations (Table 2). Stomatal control was significantly higher in plants treated by bottom cooling (88%) than in control ones (22%). These results suggested that lowering air relative humidity in the culture vessels improved stomatal functioning in roses cultured *in vitro*. This was in agreement with findings on apple (Brainard & Fuchigami 1981), rose (Capellades et al. 1990) and

Table 1. Leaf water potential components and absolute water content (AWC) measured at the end of the rooting phase on plants cultured under 3 conditions: control, bottom cooling and permeable lid.

Treatment	Control	Bottom cooling	Permeable lid
Water potential (MPa)	−0.76 ± 0.22a	−1.17 ± 0.18b	−0.93 ± 0.14ac
Osmotic potential (MPa)	−1.26 ± 0.19a	−1.74 ± 0.12b	−1.49 ± 0.08c
Turgor pressure (MPa)	+0.50	+0.57	+0.56
AWC	3.6 ± 0.2a	2.7 ± 0.4b	3.1 ± 0.3c

Means ± confidence intervals are presented ($6 < n < 10$ for water potential components and $n = 4$ for AWC).
Data were submitted to analysis of variance and LSD was established. Significant differences among means are then tested, $p < 0.05$.

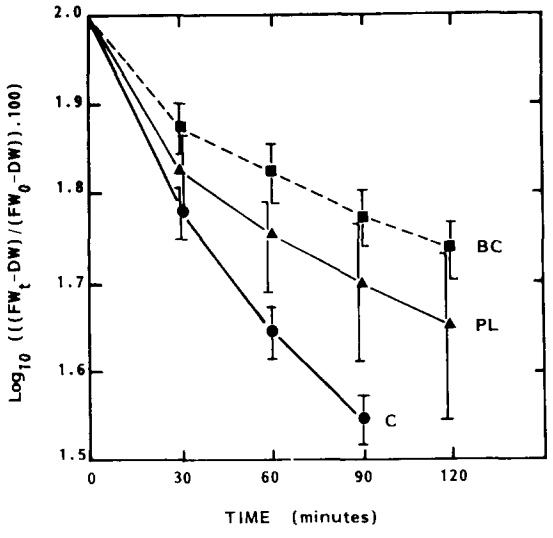

Fig. 1. Kinetics of water loss in excised leaves allowed to dehydrate at room temperature, for control (C), bottom cooling (BC) and permeable lid (PL) treatments. Each point is the mean of 4 measurements and at least 8 leaves were used for each measurement. Bars correspond to confidence intervals. Significant differences among the means are tested: bottom cooling and permeable lid treatments are significantly different from the control with $p < 0.05$. These two treatments are also significantly different but only with $p < 0.1$. This experiment was repeated 10 times and the results are similar to those presented in this figure. FW_0, initial fresh weight; FW_t, fresh weight during dehydration at time (t); DW, dry weight.

chrysanthemum (Short et al. 1987; Smith et al. 1990) plantlets submitted to low relative humidity.

Furthermore, Table 2 shows that stomatal density was about twice greater in leaves of control plants (9.7 mm^{-2}) than in bottom cooling ones (5.0 mm^{-2}). This may also contribute to a lower water loss in treated plants when dehydrated. It has also been reported that stomatal density of *in vitro* plantlets was greater in rose (Capellades et al. 1990) and apple (Brainard et al. 1981) compared to that of plants grown in the greenhouse.

Table 2. Stomatal closure [(number of completely closed stomata)/(total number of stomata) × 100], calculated 30 min after leaf excision, and stomatal density (number of stomata/ mm^{-2} leaf area) determined on 3 leaves per treatment observed by scanning electron microscopy.

Treatment	Control	Bottom cooling
Stomatal closure (%)	22 ± 3a	88 ± 3b
Stomatal density (nb/mm^2)	9.7 ± 1.9a	5.0 ± 2.0b

Means ± confidence intervals are presented (n = 4).
Data were submitted to analysis of variance and LSD was established. Significant differences among means are then tested, $p < 0.05$.

Plant growth

Root growth was affected by bottom cooling but not by permeable lid (Table 3). Bottom cooling remarkably lowered average root length (1.1 ± 0.4 mm) but its effect on root number was not significant. Roots were slightly longer in the permeable lid treatment (5.5 ± 1.8 mm) than in the control one (4.8 ± 1.0 mm). Therefore, the effects of bottom cooling on root growth may be due to low temperature (see Material and methods) rather than to low relative humidity. Shoots were taller in control plants (5.2 ± 1.0 mm) than in bottom cooling (4.3 ± 0.7 mm) and permeable lid ones (4.0 ± 0.8 mm). However, the differences were not significant (Table 3). It has been

Table 3. Average root length and number per plant, shoot height and apex necrosis [(number of necrosed apices/total shoot number per treatment) × 100] determined at the end of the rooting phase on plants cultured under 3 conditions: control, bottom cooling and permeable lid.

Treatment	Control	Bottom cooling	Permeable lid
Root length (mm)	4.8 ± 1.0a	1.1 ± 0.4b	5.5 ± 1.8a
Root number	7.3 ± 1.2a	6.8 ± 1.5a	7.1 ± 1.8a
Shoot height (mm)	5.2 ± 1.0a	4.3 ± 0.7a	4.0 ± 0.8a
Apex necrosis (%)	6	6	14
Survival rate (%)	85	100	90

Means ± confidence intervals are presented (n = 16).
Data were submitted to analysis of variance and LSD was established. Significant differences among means are then tested, $p < 0.05$.

shown in chrysanthemum that growth parameters, including plant height, leaf area and root dry weight, were optimal at 80% RH or when plants were grown initially at 60% RH followed by transfer to 100% RH. The growth was reduced when they were maintained continuously at 100% RH or initially at 100% RH followed by 60% RH (Ritchie et al. 1990).

Apex necrosis was not affected by bottom cooling but it was increased by permeable lid (Table 3). This suggests that permeable lid caused the internal atmosphere in the culture vessel to dry too much, leading to a higher apex necrosis than in the bottom cooling technique. Another explanation for apex necrosis in permeable lid treatment may be salt intoxication due to a high water release from the culture medium. Preliminary observations showed that survival after transplanting was about 100% for bottom cooling treatment, 90% for permeable lid and only 85% for control ones (Table 3). A higher survival in bottom cooling plants, despite their shorter root development, indicates that root number rather than root length is important for transplanting (J.P. Barbe, pers. comm.). Furthermore, the roots developed *in vitro* are breakable and malfunctional.

Conclusion

The two techniques used in this work aimed to reduce RH in the culture vessels and thus to improve plant transpiration. Both techniques had positive effects on leaf resistance to water loss and on stomatal regulation. These effects were accompanied with modifications of leaf water status: they lowered leaf water and osmotic potentials, maintaining turgor pressure. The lower leaf absolute water content and the osmotic adjustment found in plants treated by bottom cooling treatment are characteristics of hardened plants and should allow them to better undergo greenhouse conditions when removed from *in vitro* culture conditions. However, compared to control plants, permeable lid induced a negative effect on growth during rooting phase: apices were more necrosed.

One effect of the treatments is an increased survival rate, more pronounced in the plants submitted to bottom cooling. The plants grown with a permeable lid usually behaved in the middle between the controls and the bottom cooling ones, except for root length that was identical to that of the controls. The effect of bottom cooling appears clear and should be investigated at the industrial level.

Acknowledgements

We are grateful to Prof. Bourdu and Dr. J.P. Barbe for initiating the study, to Prof. P.C. Debergh for interesting discussion on the 'bottom cooling' technique, to Dr. J.Y. Pontailler for technical assistance with 'bottom cooling' system, to Ms C. Perrot for technical assistance with culture media, and to the G. Delbard nurseries for supplying data on the acclimatization stage. This work was funded by Möet-Hennessy Recherche.

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