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Environment Influences Anatomy of Stomata and Epidermal Cells in Tissue-cultured *Rosa multiflora*

M. Capellades[1], R. Fontarnau[2], C. Carulla[3], and P. Debergh[4]

Laboratory for Horticulture and I.W.O.N.L., State University Gent, Coupure links 653, B-9000 Gent, Belgium

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Abstract. **The surface structure of rose (*Rosa multiflora* L. cv. Montse) leaves formed in vitro under several environmental conditions (light level, relative humidity) and with various growth regulator treatments was studied by light and scanning electron microscopy. The epidermis from leaves developed in cultures grown under a higher light level and a lower relative humidity (80 µmol·s^{-1}·m^{-2} and 75% RH) than the conditions used in commercial laboratories (25 µmol·s^{-1}·m^{-2} and 100% RH) showed anatomical modifications of the epicuticular wax, stomata, and epidermal cells similar to that of greenhouse-grown plant leaves. These results indicate that cultured plantlets can resemble greenhouse-grown plants under modified environmental conditions. In vitro pretreatment will reduce transplant losses and shorten the acclimatization period in the greenhouse.**

The survival and growth of in vitro plantlets is still problematic for some crops. Reduced cuticle development, abnormal stomatal function (Brainerd and Fuchigami, 1981; Fuchigami et al,, 1981) and poorly developed vascular systems (Fabbri and Bartolini, 1985; Grout and Aston, 1977; Leshem, 1983; Ziv et al., 1981) are suggested causes for the susceptibility of plantlets to water stress during acclimatization. Gradual hardening-off with periods of decreasing humidity are necessary for plantlets to survive the transition from culture to the greenhouse or the field. Despite elaborate and time-consuming acclimatization protocols using humidity tents and/or intermittent mist, significant losses are often incurred, particularly in dicotyledonous woody species (Wardle et al., 1983) and herbaceous plants susceptible to vitrification; e.g., *Dianthus* (Ziv et al., 1981) and *Cynara scolymus* (unpublished data).

Hardening-off plantlets in vitro, by reducing the humidity (Maene, 1985; Maene and Debergh, 1987; Vanderschaeghe and Debergh, 1987) and increasing the irradiance, might reduce losses because the plantlets would be less fragile when removed from culture. The present study was undertaken to determine if environmental changes in the culture container could improve the quality of in vitro plantlets before transplanting to greenhouse conditions.

Materials and Methods

One-year-old cultures of 'Montse' rose grown on a Hasegawa (1980) multiplication medium were used for the experiments. Stage II cultures (Debergh and Maene, 1981) were grown in 320-ml jars with opaque polypropylene screw-on lids and containing 80 ml of medium. The cultures were incubated in a cabinet with controlled atmosphere at 23 ± 2C, under 25 µmol·s^{-1}·m^{-2} PAR provided by Philips TLD n° 83 fluorescent lamps, and a photoperiod of 16 hr. Ambient relative humidity was not controlled. Shoots multiplied readily and were transferred to fresh medium every 5 weeks.

To study possible variations of the leaf surface induced by manipulation of light intensity and relative humidity, shoots from 5-week-old Stage II cultures were excised and transferred to Stage IIIa solid culture medium (Hasegawa, 1980) in which 3 mg of benzyladenine/liter were replaced by 1.5 mg·liter^{-1}, and maintained at 25 µmol·s^{-1}·m^{-2} and 100% RH for 2 weeks; they were then placed under 80 µmol·s^{-1}·m^{-2} at either 100% RH (Stage IIIa-100% RH) or 75% RH (Stage IIIa-75% RH) for 3 weeks. Five jars were cultured for each treatment. The relative humidity in the containers was lowered via bottom-cooling at shelf level (Maene, 1985; Maene and Debergh, 1987; Vandershaeghe and Debergh, 1987). Temperature was 23 ± lC at leaf level in the container. Six-month-old greenhouse-acclimatized roses were used for anatomical comparisons. Plants were placed on benches at temperatures varying between 20 and 24C, under a light intensity level of 120 µmol·s^{-1}·m^{-2} and 75% RH. Plants were fertilized every 2 weeks with 2 g of Alkrisal Special/liter (N.V. Schering, Berlin, F.R.G.).

For scanning electron microscopy (SEM) and light microscopy (LM), fresh leaf samples were collected from 10 expanded leaves of 5-week-old Stage II, Stage IIIa (75% and 100% RH), and control plants. Leaf sections of 1 x 4 mm were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) for at least 2 hr and washed in the same buffer. For SEM, samples were dehydrated through an ethanol and amylacetate series and

Table 1. Some abaxial and adaxial leaf-surface characteristics of micropropagated rose as compared to greenhouse-grown (control) plants[z].

Stage of development[y]	Stomata per mm²	Hydathodes per mm²	Length of abaxial epidermal cells (µm)	Length of adaxial epidermal cells (µm)
Stage II	193 a	0	33.1 a	26.0 a
Stage IIIa–75% RH	136 b	0	50.5 b	50.5 b
Stage IIIa–100% RH	95 c	175	33.3 a	45.4 b
Control	112 b	0	72.0 c	70.0 c

[z]Significant differences among means in a column by Duncan's multiple range test, $P = 0.05$.
[y]Stage IIIa plantlets were held at 100% RH for 2 weeks, then at either 75% or 100% RH for 3 weeks.

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[1]On leave from the biology faculty of the Univ. of Barcelona, Spain. Supported by a grant from the CIRIT, Generalitat de Catalunya, Barcelona, Spain.
[2]Head, Scanning Microscopy Unit, Servei de Microscopic Electronics, Univ. of Barcelona, Marti Franqués s/n, 08028 Barcelona, Spain.
[3]Assistant, Scanning Microscopy Unit, Servei de Microscopic Electronica, Univ. of Barcelona.
[4]To whom reprint requests should be addressed.

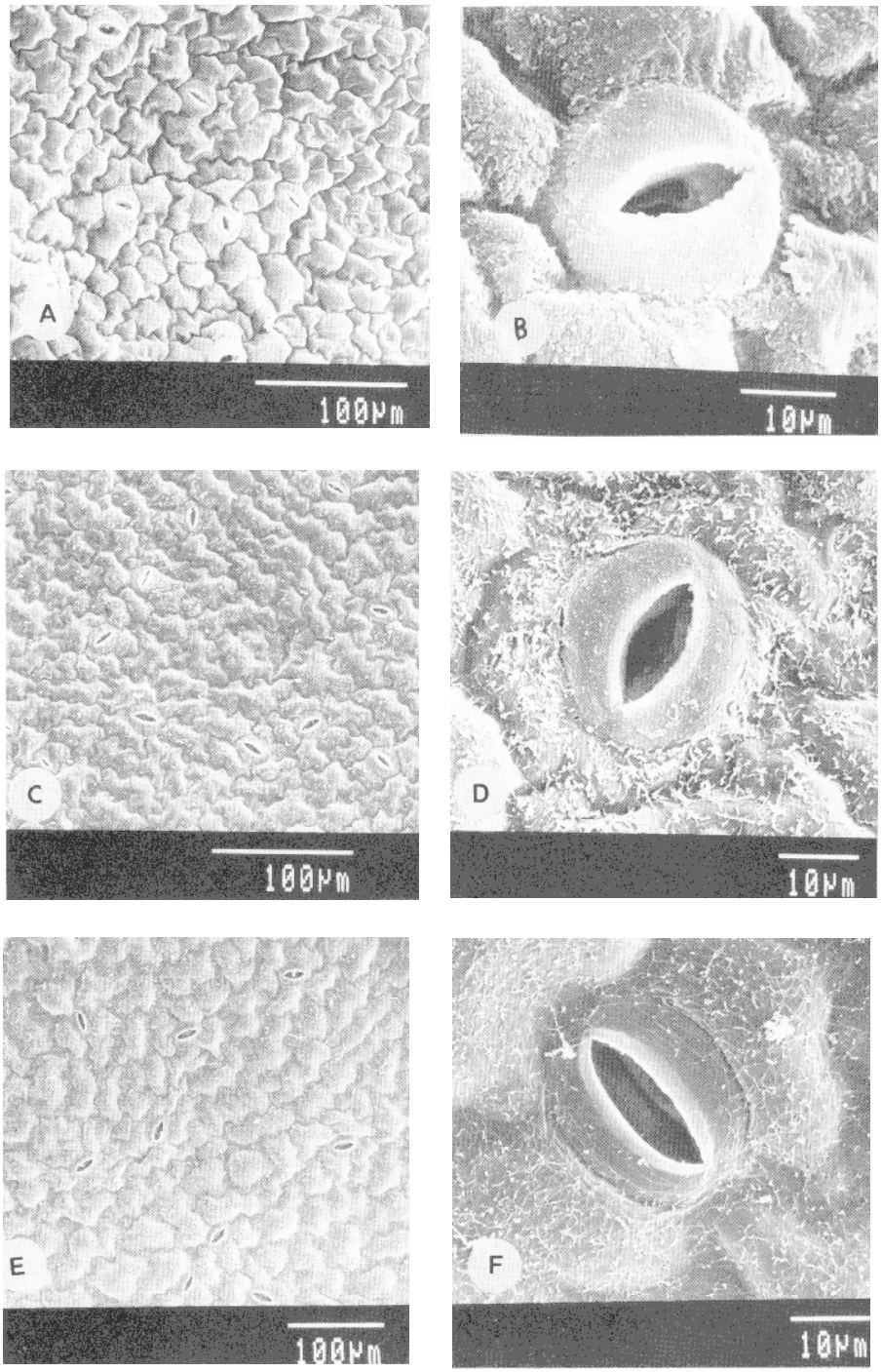

Fig. 1. SEM photomicrographs of the abaxial surface of micropropagated and field-grown 'Montse' roses. (A) Stage II (25 µmol·s^{-1}·m^{-2} and 100% RH), showing numerous raised and spherical stomata. (B) Detail of part A showing much crystalline epicuticular wax. (C) Stage IIIa–75% RH (80 µmol·s^{-1}·m^{-2} and 75% RH) showing fewer and more elliptical stomata. (D) Detail of part C showing epicuticular crystalline wax deposition. (E) Greenhouse-grown plant. (F) Detail of part E showing less crystalline epicuticular wax deposition than in parts B or D.

critical-point-dried with CO_2 in a Polaron E 3000. The samples were mounted on copper stubs with colloidal silver, sputter-coated with 50 nm gold (coating unit Polaron E 5000), and viewed under a JEOL JMS 840 SEM at 15 kV. Three samples of each treatment were examined. For LM, samples were embedded in Spurr's resin (Spurr, 1969), sectioned 0.5 to 1.0 mm thick, stained with 0.05% toluidine blue, and observed and photographed in a Zeiss Standard universal LM.

Lower and upper epidermal cell lengths and stomatal lengths and frequency were determined by microscopic examination of five leaf surface imprints in the LM. Epidermal impressions were made by applying a thin pellicle of transparent fingernail polish on the leaf surface and letting it dry for 10 min. The imprints were removed from the leaf with clear adhesive tape and glued on a microscope slide. The length of 25 stomata and the length (greatest length of the cell) of 25 epidermal cells was

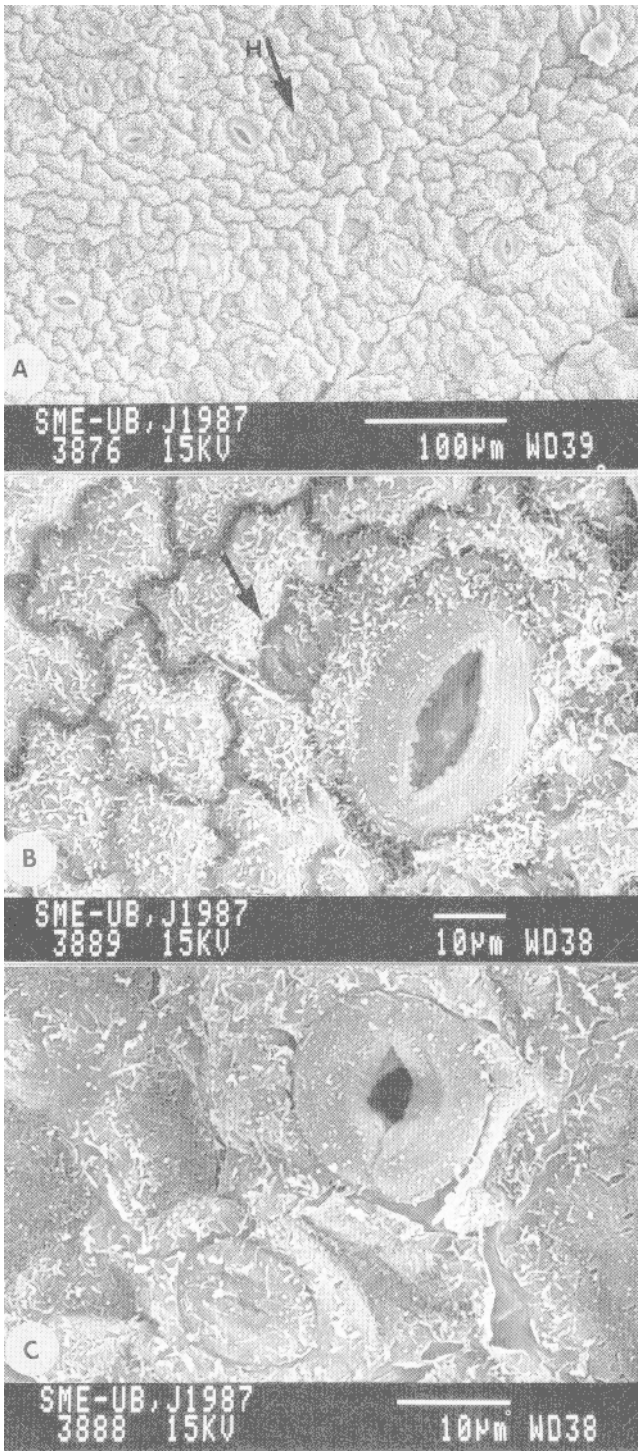

Fig. 2. SEM photomicrographs of Stage IIIa-100% RH 'Montse' leaves. **(A)** Hydathodes (H) can be seen between the normal-sized elliptical stomata. **(B)** Detail of an elliptical, normal-sized stomata with a hydathode in its proximity (arrow). **(C)** Detail of a hydathode photographed at higher magnification.

measured. The number of stomata of 25 microscopic fields was sampled. Data were submitted to analysis of variance, and the LSD was established according to Duncan's multiple range test.

To study the capability of stomata to close, imprints of isolated leaves kept at 40% RH for 15 min were made.

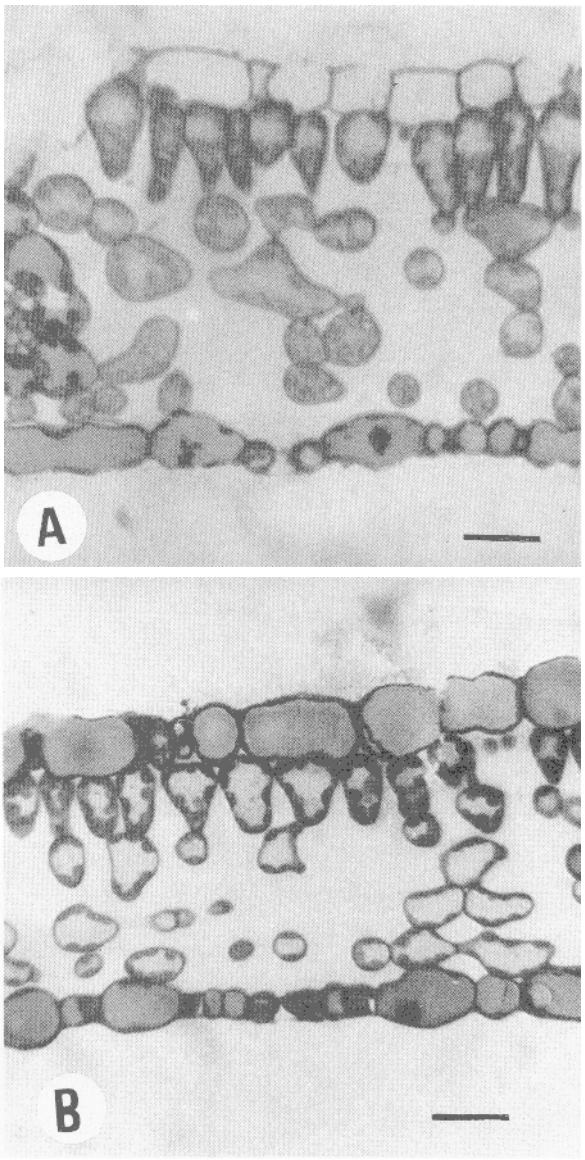

Fig. 3, Light photomicrographs of sections of Stage IIIa 'Montse' leaves. **(A)** Stage IIIa-100% RH showing stomata with guard cells protruding above the epidermal surface. **(B)** Stage IIIa-75% RH showing guard cells at the same level as the epidermal surface. Scale bar = 21 µm.

Results

Stomata were confined to the abaxial leaf surface. Stomatal density was significantly greater in Stage II plantlets than in Stage IIIa–75% RH, Stage IIIa–100% RH, or greenhouse-grown (control) plants. Presence of hydathodes was only observed in Stage IIIa–l00% RH plantlets. Nonsignificant differences were found between stomatal densities in leaves of Stage IIIa-75% RH and control plants (Table 1). Furthermore, stomatal length was similar for all treatments (between 27 and 28 µm) as was the stomatal index, (S + H)/(S + E + H), which ranged from 0.111 to 0.152 and where S = stomata/mm^2, F = epidermal cells/mm^2 and H = hydathodes/mm^2.

Variations in stomatal and epidermal cell shape were observed among treatments. Stomata of Stage II plantlets (Fig. 1 A and B) were more spherical than those of Stage IIIa-75% RH

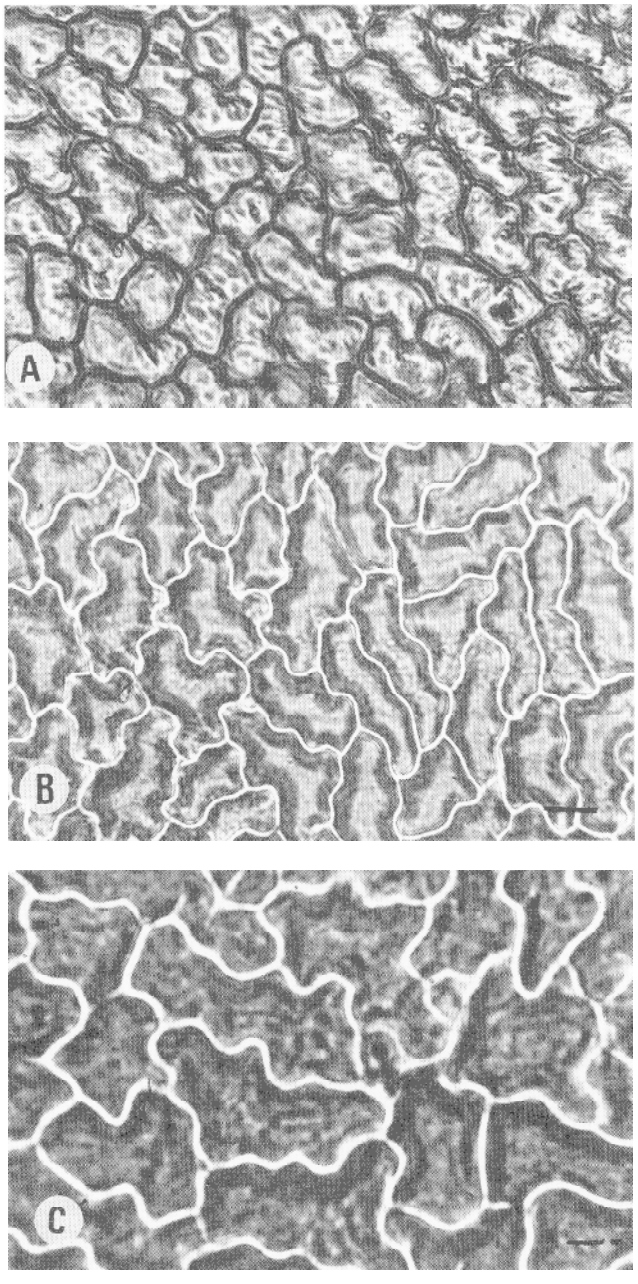

Fig. 4. Light photomicrographs of adaxial surface imprints of leaves of 'Montse' roses from Stage II, Stage IIIa, and greenhouse-grown plants. **(A)** Small and irregularly shaped Stage II epidermal cells showing few sinuous undulations of the anticlinal cell walls. **(B)** Epidermal cells of Stage IIIa-75% leaves are longer and have more sinuous undulations than those of Stage II. **(C)** Control plant epidermis showing longer and more undulated epidermal cells than Stage II or Stage IIIa plantlets. Scale bar = 12 μm.

(Fig. 1 C and D), Stage IIIa-100% RH (Fig. 2 A and B), and control leaves (Fig. 1 E and F), which had more elliptical stomata.

In Stage IIIa–100% RH plantlet leaves, small and spherical stomata (Fig. 2A) occurred among the normal-sized stomata. They resembled the anatomy of water pores of hydathodes (Fig. 2 A-C) and were never seen on leaves of plantlets developed at 75% RH or on control plants.

Stomata of Stage II and Stage IIIa-100% RH leaves (Fig. 3A) were raised above the surface of epidermal cells, whereas in Stage IIIa-75% RH leaves (Fig. 3B) or control plant leaves, stomata were sunken below the surface of epidermal cells.

The epidermal cells from leaves of Stage II cultures varied in shape and size, having few sinuous undulations in anticlinal walls (Fig. 4A). Epidermal cells of Stage IIIa–75% RH leaves (Fig. 4B) were more regularly shaped and closely resembled those of leaves grown ex vitro (Fig. 4C). Epidermal cells of Stage IIIa-100% RH leaves had similar morphology as these of Stage IIIa-75% RH.

Leaf impressions showed that epidermal cells and guard cells of Stage II plantlets collapsed within 15 min after they were separated from the plantlet and placed in 40% RH, whereas the stomata of Stage IIIa–75% RH leaves closed and the epidermal cells did not collapse. In Stage IIIa–100% RH leaves, stomata closed, hydathodes remained open, and the epidermal cells did not collapse.

From the SEM observations of the leaf surface, we have the impression that the crystalline structure of the epicuticular wax differs between treatments (Figs 1 A-F, 2 A and B).

Discussion

By increasing the light intensity and reducing the RH in the culture container environment, it was possible to induce in vitro the same transformations found by numerous authors during the ex vitro acclimatization period in the greenhouse (Donnelly et al., 1987; Fabbri et al., 1986; Wetzstein and Sommer, 1983).

Wetzstein and Sommer (1983) found stomatal densities that were significantly greater in cultured than in field-grown or acclimated plantlet leaves. However, we had nonsignificant differences in stomatal frequency between Stage IIIa–75% RH leaves and control leaves. Wetzstein and Sommer (1982) observed collapse of epidermal and guard cells of tissue-cultured leaves when plantlets were removed from culture. They attributed this phenomenon to the beam and vacuum effects of SEM observations. We believe that this phenomenon maybe due to the leaf desiccation during manipulations. We have also observed this kind of collapse in LM impressions of Stage II leaves, but not in Stage IIIa–75% RH or Stage IIIa–100% RH leaves.

Superficial, circular, and more numerous stomata were observed on plantlets of *Liquidambar styraciflua* grown in vitro than in those that had been removed from culture (Wardle et al., 1983). During acclimatization of *L. styraciflua* plants, stomata became ellipsoid, depressed, and less numerous (characteristics similar to nonmicropropagated field plants). We observed comparable changes in Stage IIIa plantlets grown at 75% RH.

Very small, spherical stomata were seen among the normal-sized stomata on Stage IIIa–100% RH leaves (Fig. 2A), but never on Stage IIIa–7590 RH leaves (Fig. 1C). One hypothesis to explain these findings could be that these structures are hydathodes, which occur in many plant families, including the *Rosaceae* (Fahn, 1979). The significant differences in the ion content observed with an energy-dispersive X-ray analyzer (EDAX) between the stomata and the supposed hydathodes, and the very high frequency of hydathodes when compared to the stomata in the Stage IIIa–100% RH leaves, would support this hypothesis (Capellades, 1989). Hydathodes are thought to be involved in the regulation of water uptake, maintaining an upward flow of water and minerals when the transpiration stream is suppressed because of the humid conditions of the environment (Devlin and Witham, 1983; Juniper, 1960). Hydathodes

were seen on leaf apices and serrations of tented ex vitro *Rubus* transplants in the greenhouse (Donnelly et al., 1987).

Differences in shape and size of epidermal cells of the adaxial surface were found between treatments, as also noted by Wetzstein and Sommer (1982). Irregularly shaped epidermal cells, without or with few undulations, were seen in leaves of Stage II plantlets (Fig. 4A), while, in Stage IIIa–75% RH and in control leaves (Fig. 4 B and C), sinuous undulations were evident. Epidermal cells of similar morphology as those in Fig. 4B were observed in leaves of Stage IIIa–100% RH plantlets.

We achieved, with plants still in culture, anatomical and morphological characteristics similar to those reported by others during acclimatization. Work correlating survival with morphological and anatomical aspects would be the next step in demonstrating that in vitro hardening would be a way to reduce losses incurred during removal of plantlets from culture to ex vitro conditions.

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