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Leaf anatomy of highbush blueberry grown *in vitro* and during acclimatization to *ex vitro* conditions

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Abstract

Leaves of micropropagated highbush blueberry (*Vaccinium corymbosum*) cv. 'Bluetta' have been observed during the acclimatization phase. *In vitro*-developed leaf cells were circular and small, the spongy parenchyma was discontinuous and disorganized and formed by 1 - 2 layers of cells with large intercellular spaces and the palisade to spongy mesophyll thickness ratio was 1:1.5. After rooting *ex vitro*, the first leaves formed under natural conditions showed substantial changes in the anatomical characteristics. After 6 months, the plants produced leaves similar to those in field-grown plants. The palisade cells were rectangular, the spongy parenchyma was formed by 3 - 4 layers of cells and the intercellulars were around the stomata. Leaves from field-grown plants lost 24 % of water during 150 min after excision while leaves from *in vitro* shoots lost about 50 % of water in the same time. Leaves from *in vitro* shoots showed a higher number of smaller stomata (361 per mm^2), with the guard cells forming a circular ring; the stomata frequency in field-grown leaves was 241 per mm^2 and the guard-cells were elliptical.

Additional key words: leaf histology, stomata, *Vaccinium corymbosum*, water loss.

Introduction

Acclimatization of *in vitro*-produced propagules is critical in the micropropagation of many woody species. Anatomical and physiological abnormalities developed *in vitro* do not enable the propagules to regulate transpiration at the time of transplanting *ex vitro*, causing severe water loss and eventually death (Romano and Martins-Lonçao 1994). Low amount or lack of epicuticular waxes causes excessive plantlet transpiration and consequent desiccation (Gribaudo *et al.* 1994). Also density,

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abnormal structure and physiology of stomata have been implicated in their water imbalance (Preece and Sutter 1991). The stomatal density of *in vitro*-grown plantlets versus field-grown plants is higher in some species (Wetzstein and Sommer 1983) and lower in others (Brainerd and Fuchigami 1981, Gribaudo *et al.* 1994).

Aim of the research was to study the anatomical and morphological characteristics of *in vitro*-formed leaves in comparison with field-grown plants in highbush blueberry (*Vaccinium corymbosum*).

Materials and methods

Research was carried out at the Institute of Arboriculture of Milan in the tissue culture laboratory and nearby greenhouse. Propagules of *Vaccinium corymbosum* cultivar 'Bluetta' were grown *in vitro* on PMN medium (Eccher *et al.* 1986) with 7.5 mg dm^{-3} of N6(2-isopentenyl)adenine (2iP), subcultured every 45 d, in growth-chamber at temperature of 24 ± 1 °C, irradiance (PAR) at shelter level of 50 µmol m^{-2} s^{-1} and photoperiod of 16 h. For rooting, 30 mm long shoots were treated by quick-dipping in a 1 g dm^{-3} solution of indolebutyric acid (IBA) in 10 % ethanol and planted in peat into small plastic boxes (micro-greenhouses - 50 × 40 × 30 cm, with transparent cover) and kept for 60 d, until well rooted (Noé and Eccher 1994). They were then transferred into the greenhouse to complete acclimatization and cultured in 7 × 7 cm square pots in a mixture of peat and perlite 4:1 (v/v).

The observations have been carried out on 6 leaf samples collected from different plant growth stages and environmental conditions: *1*) leaves from proliferating *in vitro* shoots, *2*) leaves from *ex vitro*-rooted plantlets, cultured in growth chamber (Fig. 1); *3*) *in vitro*-developed persisting leaves, from acclimatized plantlets after 1 month in greenhouse (Fig. 1); *4*) the youngest leaves developed in greenhouse (Fig. 1); *5*) greenhouse-developed mature leaves from 6-month-old acclimatized plantlets; 6) leaves from 2-year-old micropropagated plants.

Anatomy of leaves: Observations were carried out on thin sections with an optical microscope; 240 and 480 × magnifications were used to observe single cells of epidermis, mesophyll and stomata.

The tissues were excised from the central portion of the leaf and were rapidly killed and fixed overnight in FAA. The samples were dehydrated through tertiary butanol series at incremental concentrations and embedded in paraplast. Cross sections (15 µm thick) were glued on glass wells. After paraplast elimination by immersions in xylol and washings in ethanol, the samples were coloured with saphrine and bright green. The samples were then re-dehydrated with ethanol first and xylol afterwards. Right before the microscope observation, the samples were covered with Canadian balm to avoid light dispersion when passing through glass-air-glass.

Water loss: Comparative water loss analyses were made following the method described by Romano and Martins-Loçao (1994). Detached leaves were immersed in

distilled water and kept for 3 h at low cool white light (irradiance 10 μmol m^{-2} s^{-1}) and temperature of 24 °C to enable full water saturation. They were then placed in Petri dishes with abaxial surface facing up and kept at temperature of 38 °C and relative humidity of 50 %. Water loss was measured by weighting the leaves every 10 min for the first hour and every 20 min thereafter. The leaves were finally dried at 80 °C for 24 h to measure the dry mass. The experiments have been repeated 3 times.

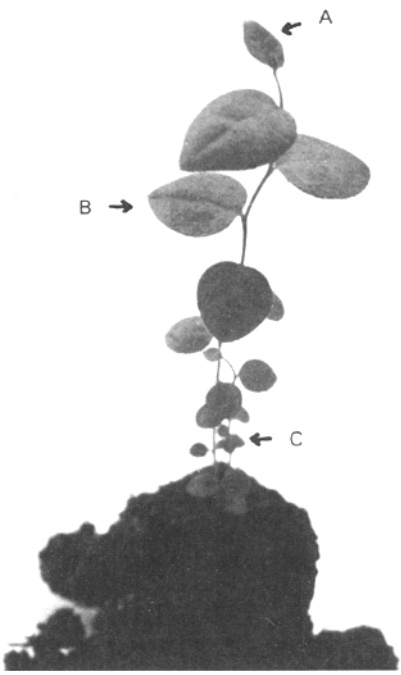

Fig. 1. *In vivo* rooted plantlets: *in vitro*-developed persisting leaves (*C*), disclosing leaf at apex (*A*) and leaves underneath (*B*).

Stomatal observations: Leaves were collected and placed abaxial side up in Petri dishes with high relative humidity to preserve dessication. Stomatal frequency and shape were observed by optical microscope on non-fixed leaves using 40 × magnifications. Stomatal frequency, expressed as number of stomata per mm^2 of leaf surface, was measured in 4 randomly chosen microscope fields (0.25 mm length and 0.175 mm width) and repeated 4 times.

Results and discussion

In vitro-developed leaves from shoots in proliferation phase were smaller, thinner and of different shape in comparison with field-grown ones (Table 1), also leaf cells

Table 1. Characteristics of leaves.

Type of leaf	Length [mm]	Width [mm]	Thickness [μm]	Palisade parenchyma [μm]	Spongy-parenchyma [μm]	Stomata [mm^{-2}]
Growth chamber						
1) leaves from *in vitro* shoots, in proliferation	4.3 d	3.5 d	98 d	27 c	40 d	361
2) leaves from *ex vitro*-rooted plantlets	11.0 b	8.8 b	101 c	28 c	42 d	170
Greenhouse						
3) *in vitro*-developed persisting leaves	4.6 d	3.9 d	94 d	29 c	40 d	195
4) newly *ex vitro*-formed leaves	7.6 c	5.1 cd	111 b	34 b	45 c	386
5) from 6-month-old acclimatized plantlets	10.3 b	8.3 b	112 b	33 b	51 b	178
Field						
6) leaves from 2-year-old micropropagated plants	22.3 a	16.2 a	140 a	44 a	65 a	241

Different letters correspond to statistical significant difference in means ($P \leq 0.05$, according to Duncan's test).

were circular and smaller (Fig. 2). Palisade and spongy parenchyma was formed only by 1 - 2 layers of cells with large intercellular spaces. The palisade and spongy mesophyll thickness ratio was 1:1.5 (Table 1). After transplanting and rooting *ex vitro* - but still under artificial light in the growth chamber - the new leaves were larger (Fig. 1), but showed the same anatomic characteristics as *in vitro*-formed ones (Table 1). When the cuttings were transferred in greenhouse, the already developed leaves did not change. Irradiance and spectral composition appeared to affect leaf development, regardless if *in vitro* or *ex vitro* (Table 1): leaves developed under cool-white lamps showed thinner palisade (27 - 29 μm) and spongy (40 - 42 μm) mesophyll than those developed under natural light. The first leaves formed under natural light showed substantial increase in leaf thickness (111 μm) and thickness of palisade (34 μm) and spongy (45 μm) mesophyll (Table 1). It took quite a long time before the plants started to produce leaves similar to those from field-grown plants. The palisade cells were typically rectangular and showed regular and continuous distribution (Fig. 3); the spongy parenchyma was formed by 3 - 4 layers of cells and the only empty spaces were around the stomata.

Leaves from *in vitro*-growing shoots absorbed much more water during the saturation than other leaves; leaf dry matter was almost the same in the 3 leaf types, ranging from 20.8 to 21.6 % (Fig. 4).

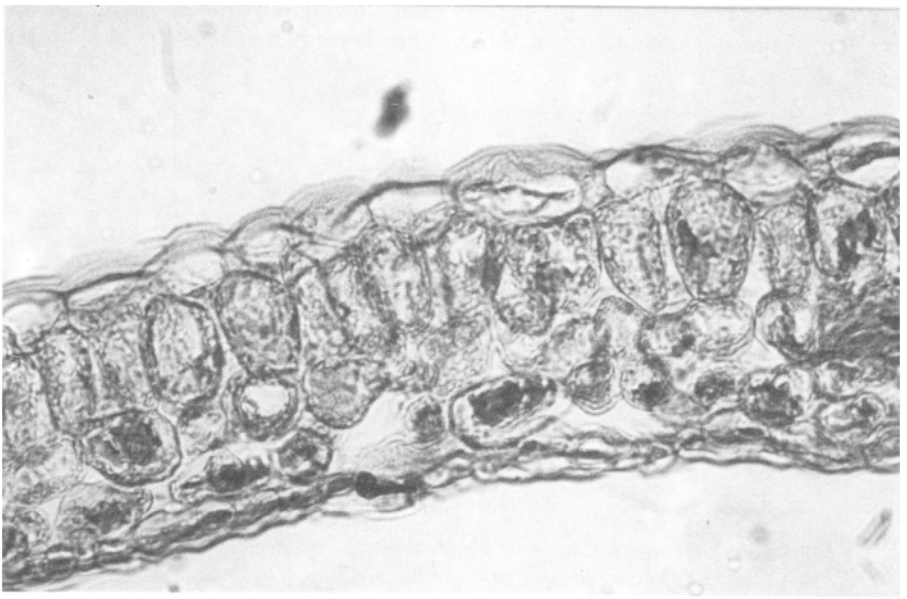

Fig. 2. Section (480 × enlargements) of leaves grown *in vitro*.

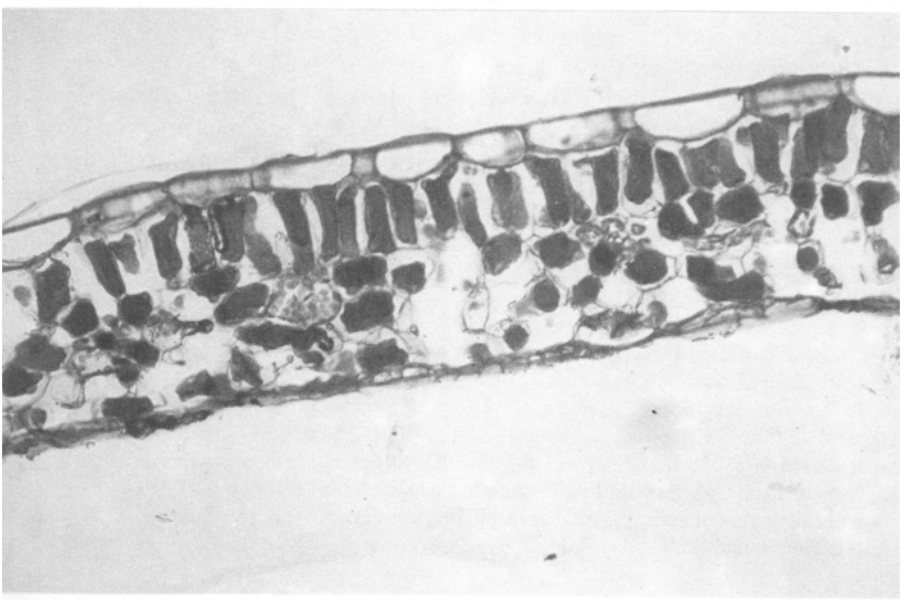

Fig. 3. Section (240 × enlargements) of leaves from field-grown plants.

Leaves from fully acclimated plants lost 24 % of their initial mass within 2.5 h; the water loss rate was higher in the first 30 min, slowing down afterwards. Leaves from *in vitro* shoots lost about 50 % and leaves newly formed *ex vitro* 60 % of water within 2.5 h (Fig. 4).

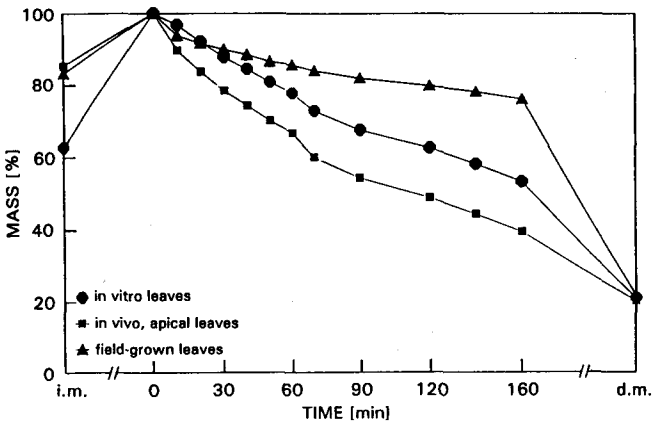

Fig. 4. Water loss of leaves taken from *in vitro* shoots, *in vivo* plantlets and field-grown plants, measured as mass percentage in comparison to mass at full turgidity. (i.m. - initial mass at excision; d.m. - dry mass).

Stomata were present only on the abaxial surface, except for the *in vitro* cultured leaves which had stomata also on the adaxial surface.

The number of stomata in leaves from *in vitro* propagules was high (361 per mm^2) and their guard cells collapsed and formed a circular ring. In leaves formed *in vitro* and then exposed to natural conditions, the number of stomata was lower (195 mm^{-2}) and stomata size increased although leaf size remained similar. In leaves from *ex vitro* rooted cuttings in growth chamber, the stomata remained circular, but the stomata frequency decreased to 170 mm^{-2} (Table 1) and the stomata size increased. The stomata density measured in field-grown leaves was 241 mm^{-2} and the guard-cells were elliptical.

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