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Artichoke leaf morphology and surface features in different micropropagation stages

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Abstract

Artichoke (*Cynara scolymus* L.) leaf size and shape, glandular and covering trichomes, stomatal density, stomata shape, pore area and epicuticular waxes during micropropagation stages were studied by scanning electron microscopy (SEM) and morphometric analysis with the aim to improve the survival rate after transfer to greenhouse conditions. Leaves from *in vitro* shoots at the proliferation stage showed a spatular shape, ring-shaped stomata, a large number of glandular trichomes and juvenile covering hairs, but failed to show any epicuticular waxes. Leaves from *in vitro* plants at the root elongation stage showed a lanceolated elliptic shape with a serrated border, elliptical stomata, decreased pore area percentage, stomatal density, and mature covering trichomes. One week after transfer to *ex vitro* conditions, epicuticular waxes appeared on the leaf surface and stomata and pore area were smaller as compared to *in vitro* plants. Artichoke acclimatization may be improved by hormonal stimulation of root development, since useful morphological changes on leaves occurred during root elongation.

Additional key words: acclimatization, *Cynara scolymus*, *in vitro* culture, leaf epidermis, stomata, trichomes.

Introduction

Acclimatization of micropropagated plants has a limiting effect on projects involving the production of such plants (Preece and Sutter 1991). *In vitro* plants usually have low survival rates when transferred to soil because they undergo an excess of water loss, attributable to poor stomatal functioning, epicuticular wax formation and water transport (Majada *et al.* 1998).

To establish how micropropagated plants may best be weaned into normal growth conditions, more information is needed regarding structural changes of *in vitro* cultured plantlets (Donnelly and Tisdall 1993). Several authors have carried out research on histological and ultrastructural features of *in vitro* in comparison with *ex vitro* plantlets (Donnelly and Tisdall 1993, Pospíšilová *et al.* 1999, Tichá *et al.* 1999, Apóstolo and Llorente 2000). However, studies on structural characteristics of *in vitro* leaves during *in vitro* stages have been scarcely developed (Donnelly *et al.* 1986, Louro *et al.* 1999, Sha Valli Khan *et al.* 1999).

Several micropropagation protocols for *Cynara scolymus* L. (artichoke) plants with 50 - 80 % survival after *ex vitro* transfer have been reported by Rossi (1992). However, the morphological and anatomical characteristics of the artichoke grown *in vitro* had not been studied. Recently, Brutti *et al.* (2000) described a new *in vitro* propagation protocol for artichoke cv Early French plants with 70 % survival rate. In order to characterize the leaf surface in relation to the different stages of artichoke micropropagation, leaves from *in vitro* culture and *ex vitro* plants were studied by means of morphometric analysis and scanning electron microscopy (SEM).

Materials and methods

Observations were carried out on the third leaf (fully expanded) from the apex. Leaves were collected from different stages of artichoke (*Cynara scolymus* L. cv. Early French) micropropagation by the protocol

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described by Brutti *et al.* (2000): proliferation (M1), pre-rooting (M2), root induction (R1), root elongation (R2), one week after transfer to soil (A1), 4 - 6 weeks after transfer to soil, both persistent leaves (A2) and new leaves (A3) (Fig. 1).

For morphometric studies, leaves were treated by Dizeo de Strittmatter's clearing method (1973). This technique involves: *1*) removal of chlorophyll with boiling ethanol during 30 s; *2*) dissolution of protoplasm using boiling 5 % NaOH and ethanol (1:1) for 1 min and washing three times with distilled water; *3*) removal of brown coloration by bleaching with 50 % sodium hypochlorite and washing three times with water; *4*) clearing by 2.5 g cm^{-3} chloral hydrate 5 to 10 min; and *5*) staining with safranine and mounting with glycerin-gelatin. In abaxial and adaxial leaf surfaces, stomatal density, glandular trichome density, stomatal length and width, as well as pore length and width, were measured on each leaf side ten randomly chosen microscopic fields of known area and repeated three times using a *Wild M20* (*Leica*, Heerbrugg, Switzerland) light microscope. Stomatal length refers to the distance between the ends of guard cells and the width is the transverse distance across them. Besides, percentages of stomata area per leaf area, pore area per leaf area, and pore area per stomata area, as well as total stomata number per leaf area, were calculated.

For SEM, fresh samples were collected from leaves at the end of the different micropropagation stages. Leaves were fixed in FAA (ethanol:formaldehyde:acetic acid:distilled water 10:2:1:7) for at least 2 h, washed in distilled water and dehydrated through acetone series. They were then dried using the critical point technique, mounted on aluminium stubs and coated with platinum. Observations and micrographs of leaf surfaces were carried out using a *Philips 515 SEM* (*CITEFA*, Buenos Aires, Argentina).

Results

Leaves of *in vitro* culture shoots from proliferation and pre-rooting stages (M1, M2) had only 0.5 - 1 % of the mature leaf area of greenhouse grown plants (A3) and were spatular in shape. In the rooting stages (R1, R2), the

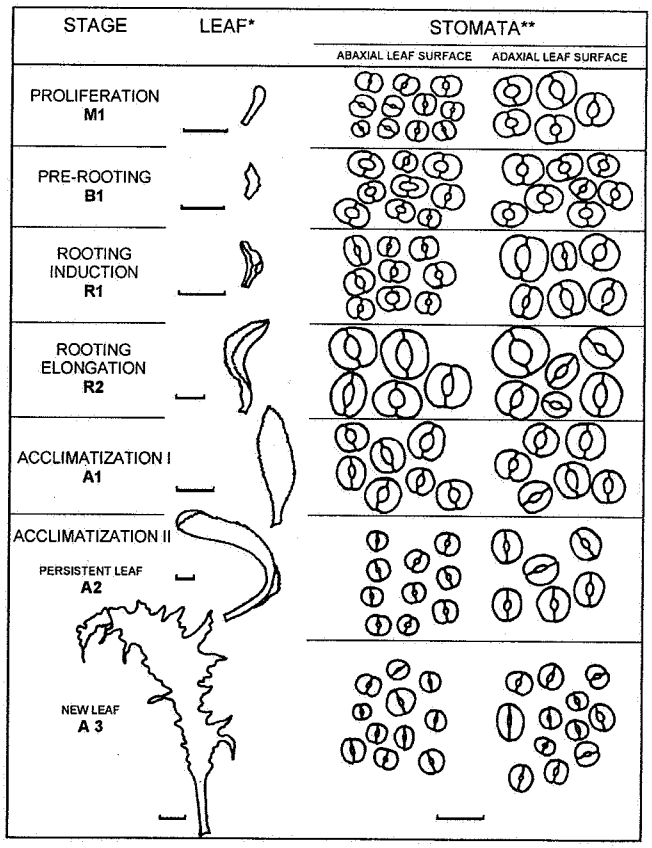

Fig. 1. Leaf and stomata features (adaxial and abaxial leaf surfaces) at different stages of artichoke micropropagation: M1 - proliferation, M2 - pre-rooting, R1 - root induction, R2 - root elongation, A1 - acclimatization I (persistent leaves one week after transfer to soil), A2 and A3 - acclimatization II (A2 - persistent leaves 4 - 6 weeks after transfer to soil, A3 - *ex vitro* new leaves). *Bars*: * - 1 cm; ** - 50 μm.

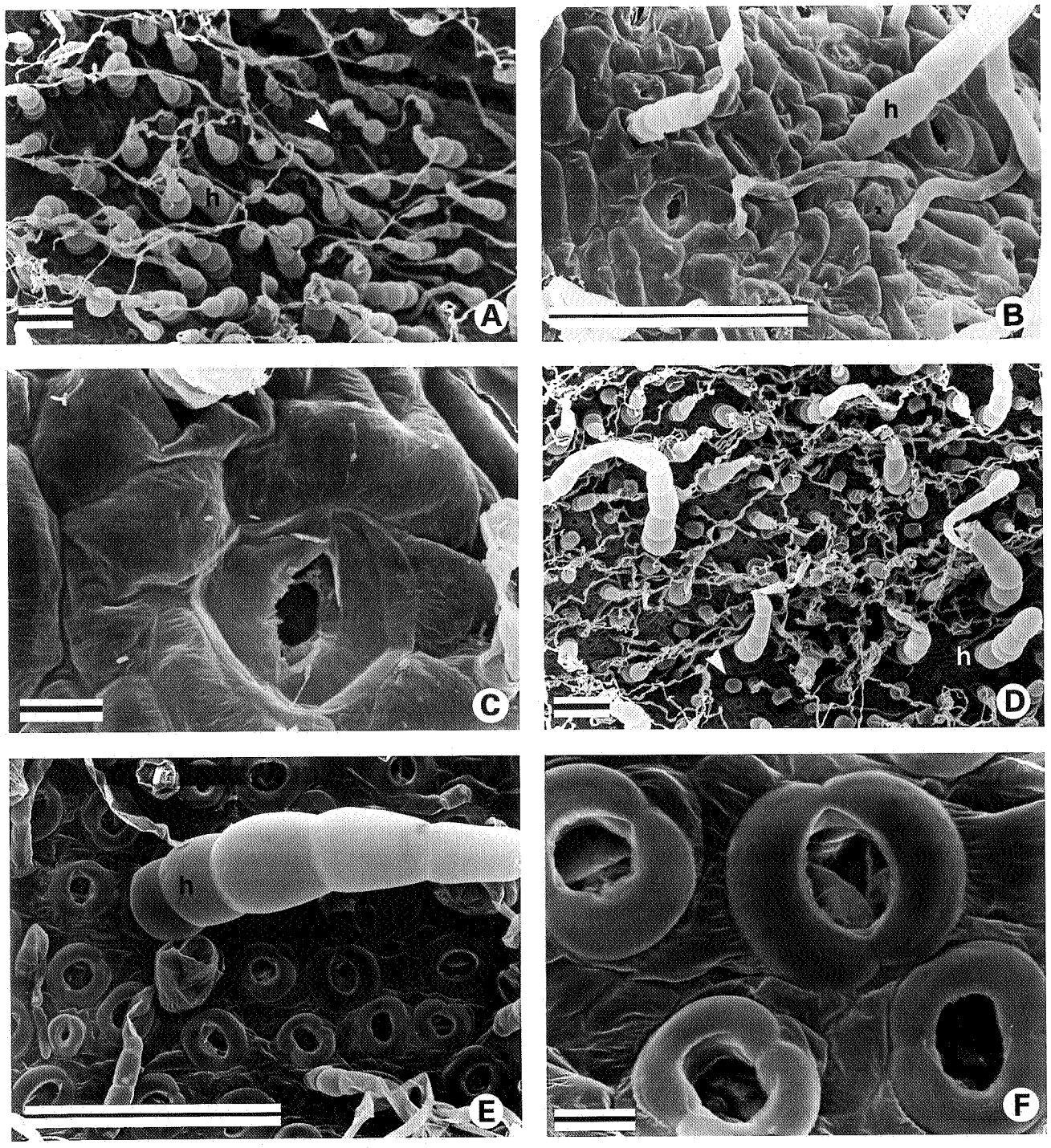

Fig. 2. *A*-C: Leaf surfaces of shoots at proliferation stage (M1): *A* - adaxial surface, *B* - abaxial surface, *C* - stoma and epidermal cells. *D-F*: Leaf surfaces of shoots at pre-rooting stage (M2): *D* - adaxial surface, *E* - abaxial surface; *F* - stomata on abaxial leaf side. *Arrow* - glandular trichome, h - covering hair. *Bars*: *A,B,D,E* 100 μm; *C,F* 10 μm.

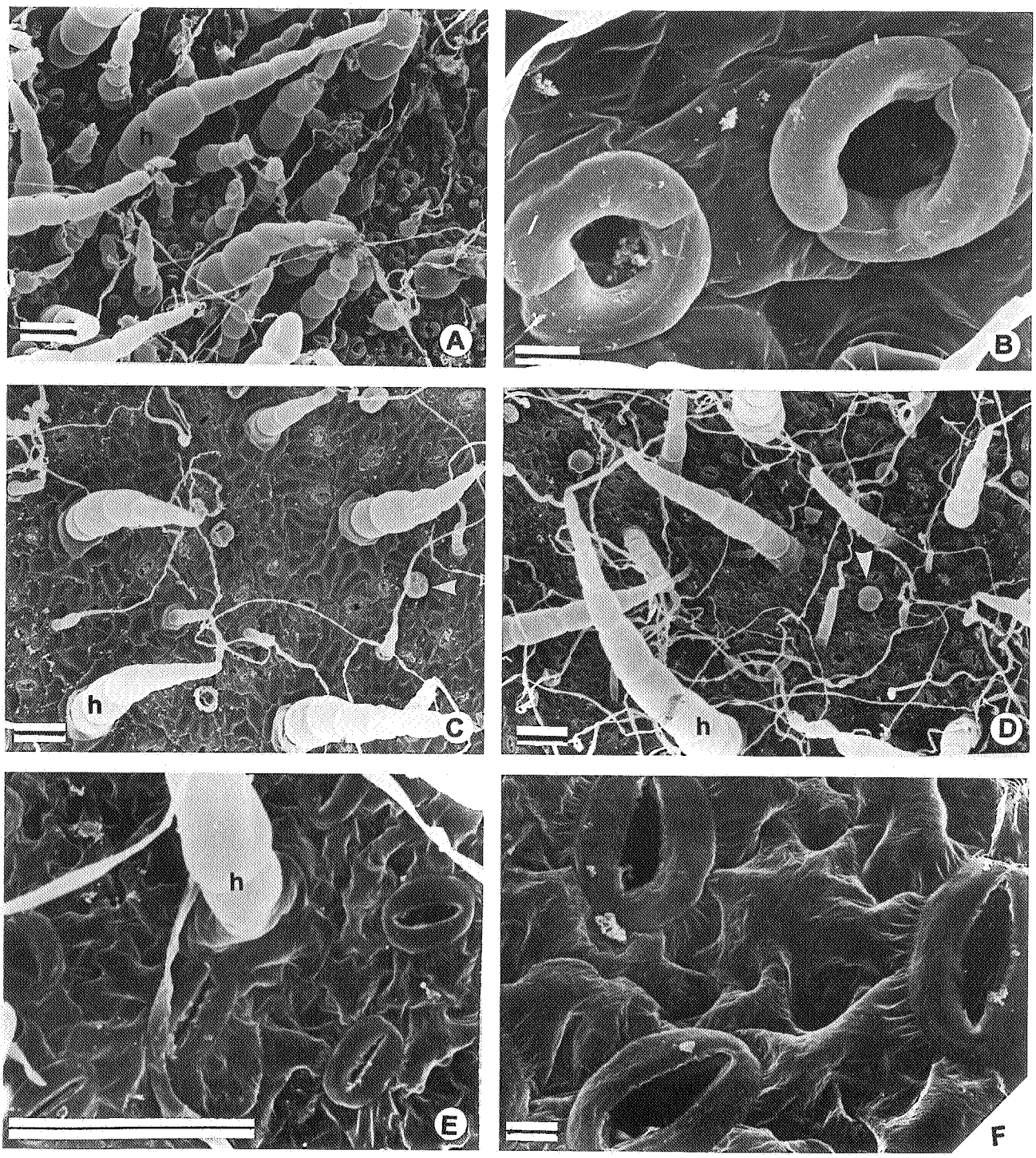

Fig. 3. *A,B*: Leaf surfaces of shoots at root induction stage (R1): *A* - adaxial surface, *B* - stomata. *C-F*: Leaf surfaces of plants at root elongation stage (R2): *C* - adaxial surface, *D* - abaxial surface, *E,F* - stomata and epidermal cells. *Arrow* - glandular trichome, h - covering hair. *Bars*: *A,C,D,E* 100 μm, *B,F* 10 μm.

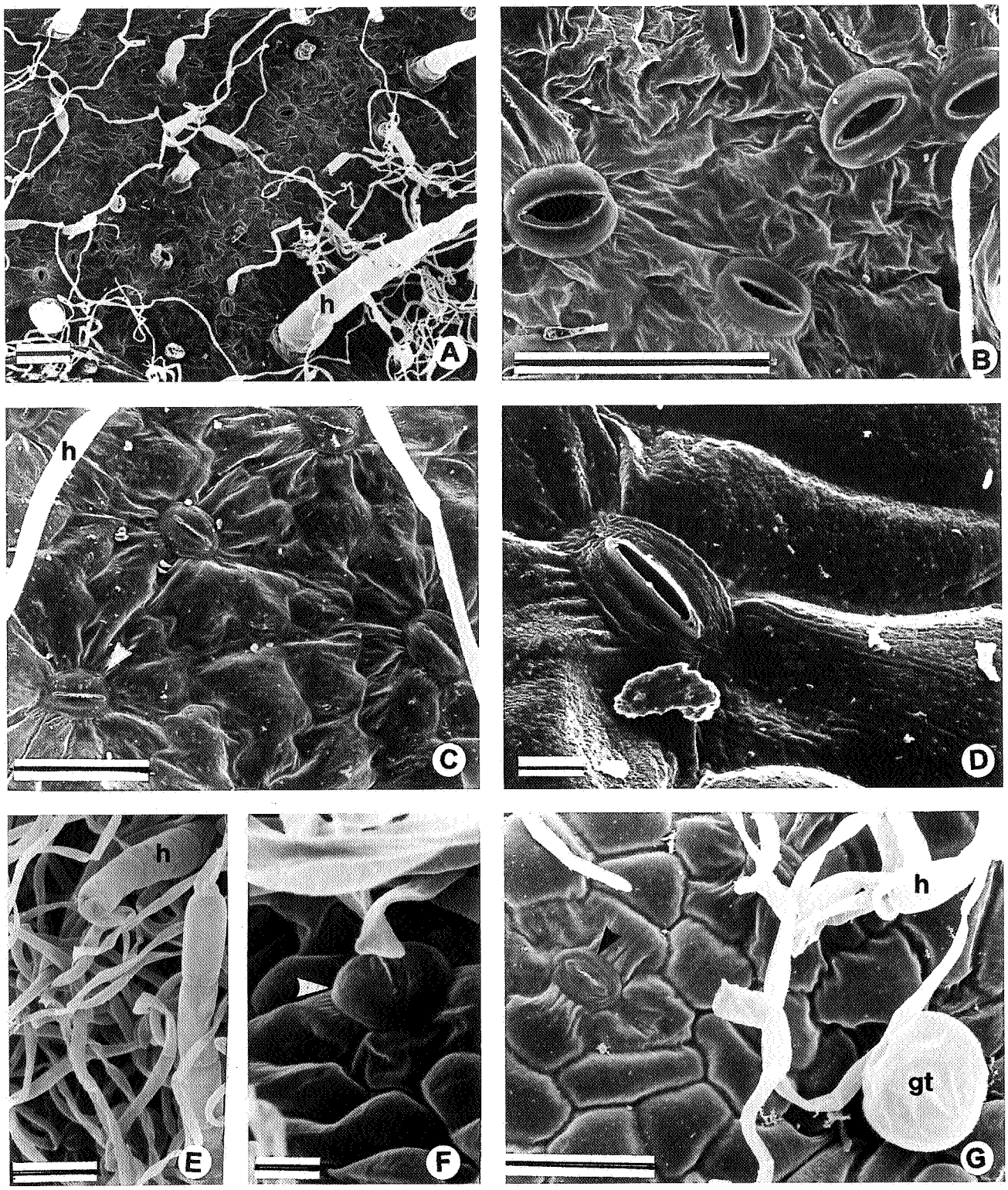

Fig. 4. *A,B*: Persistent leaf surfaces of plants at aclimatization I stage (A1), one week after transfer to soil: *A* - abaxial surface, *B* - stomata. *C,D*: Persistent leaf surfaces of plants at aclimatization II stage (A2), 4 - 6 weeks after transfer to soil: *C* - abaxial surface, *D* - stoma and epicuticular waxes on adaxial leaf side. *E-G*: New leaf surfaces of plants at aclimatization II stage (A3), 4 - 6 weeks after transfer to soil: *E* - abaxial surface, covering hairs, *F* - abaxial surface, stoma and epidermal cells, *G* - adaxial surface. *Arrow* - stoma, gt - glandular trichome, h - covering hair. *Bars*: *A,B* 100 μm, *C,E,G* 50 μm, *D,F* 10 μm.

Table 1. Morphometric analysis of *in vitro* artichoke leaf surfaces at different micropropagation stages. M1, proliferation; M2, pre-rooting; R1, root induction; R2, root elongation; A1, acclimatization I (persistent leaves one week after transfer to soil); A2-A3, acclimatization II: A2, persistent leaves 4-6 weeks after transfer to soil; A3, *ex vitro* new leaves. Means ± SE, $n = 5$ ([a]), 10 ([b]) or 20 ([c]).

Stage	Leaf area[a] [mm²]	Leaf surface	Stomatal density[b] [mm⁻²]	Glandular trichome density[b] [mm⁻²]	Stomata width[c] [µm]	Stomata length[c] [µm]	Stomata number [leaf area⁻¹]	Stomata area [% of leaf area]	Pore width[c] [µm]	Pore length[c] [µm]	Pore area [% of stom. area]	Pore area [% of leaf area]
M1	11.7± 5.7	adaxial	153.1±24.6	20.0± 2.7	39.6±4.7	34.4±5.3	1791	20.2	15.4±5.9	17.4±6.4	19.8	4.1
		abaxial	329.4±40.8	119.4±17.2	23.8±3.9	23.4± 2.6	3855	17.4	4.8±2.2	7.9±2.9	7.0	1.3
M2	24.7± 2.1	adaxial	150.0±27.6	97.3±14.4	38.3±4.8	27.8±3.2	3717	15.4	15.9±1.6	12.2±3.6	18.1	2.9
		abaxial	275.0±43.7	110.4±10.7	32.0±5.5	24.3±2.5	6855	21.8	12.5±3.9	10.5±2.1	16.4	3.6
R1	57.9± 3.1	adaxial	112.1±28.1	17.8± 2.3	36.0±7.5	39.1±6.1	6498	15.7	11.6±5.7	24.2±6.3	19.9	3.1
		abaxial	337.1±41.9	48.6±12.4	28.2±5.9	26.9±4.9	19411	24.5	8.7±4.1	13.9±5.1	16.0	4.1
R2	168.6± 68.8	adaxial	60.4± 6.8	7.2± 1.7	46.6±3.4	52.1±4.6	10191	14.4	15.5±4.1	30.4±5.6	19.5	2.8
		abaxial	106.3±22.1	8.0± 1.1	39.9±7.8	44.0±7.2	17928	18.2	11.9±4.7	27.4±7.2	18.6	3.5
A1	207.3±102.1	adaxial	80.0±10.1	15.6± 4.5	35.3±3.0	38.3±2.8	16578	10.6	11.2±2.5	20.9±3.7	17.4	1.9
		abaxial	120.0±20.0	33.3± 7.6	33.4±2.6	37.9±4.8	24868	15.0	10.1±2.1	23.9±6.1	18.7	2.9
A2	754.7± 21.1	adaxial	33.3± 7.6	6.7± 2.8	28.9±2.1	38.7±1.1	25127	3.5	5.5±0.8	22.0±1.2	10.8	0.4
		abaxial	129.2± 3.6	46.7±11.5	24.5±2.9	26.3±2.1	97470	8.0	4.6±1.1	13.7±3.7	9.8	0.8
A3	1924.8±226.5	adaxial	100.0±10.0	13.3± 5.7	24.2±1.9	28.4±6.7	192481	6.7	6.2±1.7	15.7±3.9	14.1	1.0
		abaxial	133.3±11.4	13.3± 4.9	23.5±4.7	27.5±3.1	256577	8.3	5.5±2.6	14.5±4.0	12.4	1.0

shape of leaves changed to a lanceolate-elliptic with serrated border and leaf area was 14 times greater than at M1. A week after transfer to greenhouse, the shape of leaves formed *in vitro* (A1) remained unchanged although their size increased slightly (1.3 times greater than at R2). These leaves were retained after transplantation for 4 to 6 weeks (A2), their size increasing 6-fold. The first new leaves formed *ex vitro* were lanceolate with serrated border, whereas the following leaves were pinnatilobed (A3) (Table 1, Fig. 1).

Epidermal cells of *in vitro* and *ex vitro* leaves were isodiametric with straight ledges on the adaxial leaf side (Fig. 2*C*) and lobulated ledges on the abaxial leaf side (Fig. 3*F*). *In vitro* leaf epidermis failed to show any epicuticular waxes while *ex vitro* leaf epidermis had scanty granular waxes (Fig. 4*D*).

All leaves had glandular trichomes and covering hairs (Figs. 2 - 4). While mature pluricellular glandular trichomes consisted of two rows of superposed cells and were ovoid in shape (Figs. 3*D*, 4*G*), mature covering trichomes were pluricellular uniseriate with cells of the same kind, but the apical cell differed from the other ones due to its flagelliform shape (Fig. 4*E*).

A large number of glandular trichomes were present on leaves of shoots at M1 to R1 stages. Covering trichome density at these stages was similar on both leaf surfaces. These hairs had juvenile features (shorter and globe-shaped cells with a lengthened terminal cell) (Figs. 2*A,B,D*, 3*A*). The leaves of following stages (M2 to A3) had covering trichomes with mature characteristics, more abundant on the abaxial surface. However, in the leaves at R2 to A3 stages there were fewer glandular trichomes than on leaves from M1 to R1 stages (Figs. 3*C,D*, 4*A,E,G*, Table 1).

The artichoke has amphistomatous leaves. Stomatal density on the abaxial leaf side remained higher than on the adaxial one. Leaves at M1 to R1 stages had higher stomatal density than at following stages. In R2 stomatal density was lower than at M1 to R1 and this feature remained changeless at A1 to A3 (Table 1).

Stomata of adaxial epidermis were larger than those of abaxial epidermis (Table 1, Fig. 1). Leaves from M1 and M2 stages had ring-shaped stomata and pores (Figs. 1, 2*B,C,E,F*), whereas leaves from R1 and R2 stages showed marked heterogeneity in stomatal size and shape, both surfaces displaying increased elliptical stomata (Figs. 1, 3*A-F*). Lastly, in leaves of *ex vitro* transferred artichoke plants (A1 - A3), stomata were elliptical with narrow pores (Figs. 1, 4*B,C,D,F,G*). Leaves from M1 to A1 stages showed greater pore area/stomata area ratio than those from A2 and A3 stages. As regards leaf area, M1 to R1 leaves had the greatest pore area percentage. This value gradually decreased from R2 to A3 leaves probably due to smaller stomatal size and greater leaf area (Table 1).

Discussion

Substantial changes in artichoke leaf morphology and anatomy were observed during *in vitro* stages as well as after transferring plantlets to soil. We observed marked leaf size and shape changes during *in vitro* stages of

artichoke micropropagation. Besides, the shape of leaves formed *in vitro* remained unchanged after transfer to soil although their size increased slightly, as reported by Donnelly and Tisdall (1993) for other species.

In vitro artichoke leaf surfaces failed to show any epicuticular waxes while *ex vitro* leaf surfaces had scarce granular waxes. An improvement in the development of epicuticular waxes after transfer to *ex vitro* conditions has been observed in *Liquidambar styraciflua* and *Brassica*. On the other hand, in *Malus pumila* plantlets, epicuticular waxes were unaffected after transfer, but more plentiful in newly formed leaves (Pospíšilová *et al.* 1999).

Glandular trichome density and covering hair features changed during *in vitro* artichoke stages. Donnelly *et al.* (1986) found fewer trichomes and an altered distribution of glandular and unicellular hairs in Silvan Blackberry *in vitro* leaves compared to greenhouse-grown plants. On the other hand, Apóstolo and Llorente (2000) contended that trichome density was unchanged on *ex vitro* jojoba leaves from micropropagated plants.

In agreement with Louro *et al.* (1999), we found greater stomatal density in the proliferation step than in the root elongation stage of *in vitro* cultures, while Sha Valli Khan *et al.* (1999) failed to observe differences in stomatal density at different *Quercus in vitro* stages. In several species, stomatal density decreases after transplantation (Johansson *et al.* 1992, Noé and Bonini 1996, Tichá *et al.* 1999, Apóstolo and Llorente 2000). However, Donnelly and Tisdall (1993) reported that, depending on species, in *Prunus* spp. and *Rhododendron* (Pospíšilová *et al.* 1999) stomatal density increased after transplantation. On the other hand, in contrast with our results, Pospíšilová *et al.* (1999) showed that, after a short period of acclimatization, stomatal density on both epidermal surfaces of *Nicotiana tabaccum* plants remains unchanged.

Artichoke leaves from *in vitro* culture at the proliferation stage had ring-shaped stomata as observed by other authors in different species (Noé and Bonini 1996, Louro *et al.* 1999, Pospíšilová *et al.* 1999, Sha Valli Khan *et al.* 1999). However, artichoke leaves from cultures at rooting stages showed marked heterogeneity in stomatal size and shape, attributable to the presence of stomata in different development stages, as reported for *Nicotiana tabaccum* (Tichá *et al.* 1999) and *Eucalyptus* (Louro *et al.* 1999). Leaves from *in vitro* artichoke plants at the root elongation (R2) to *ex vitro* stages showed elliptical stomata. This shape is characteristic of *in vivo* stomata endowed with normal function (Willmer 1986, Sha Valli Khan *et al.* 1999). Non-functional ring-shaped stomata of leaves before the R2 stage could be explained by the high cytokinin content in the culture medium which lowers stomatal sensitivity (Radin and Hendrix 1988).

One week after transfer to *ex vitro* conditions, relative humidity was 35 - 40 % and there was no visible wilting. At this time, major anatomical changes on artichoke leaves were observed so the water status of artichoke plants was stabilised quickly. The pore area percentage of such leaves, not exceeding 2 %, agreed with the expected pore area percentage of an *in vivo* leaf (Willmer 1986).

Our results show that acclimatization may be improved by hormonal stimulation of root development since morphological changes on leaves occurred in root elongation medium (R2) that benefit *ex vitro* condition. Similar results have been observed by Van Telgen *et al.* (1992) in various species and Díaz-Pérez *et al.* (1995) in *Malus pumila*. Willmer (1986) reported that long-term application of auxins could exert striking effects upon stomatal functionality. In all likelihood, increased auxin content in the rhizogenesis medium is related to stomatal changes observed during R1 through R2 stages of artichoke micropropagation.

In order to identify further anatomical changes useful to improve plant performance after transfer to soil, several *in vitro* hardening procedures will be carried out. Further studies will show whether *in vitro* hardening helps to improve the survival rate of micropropagated artichoke plants as well as speeding up their acclimatization to greenhouse conditions.

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