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Structural and ultrastructural differences between field, micropropagated and acclimated leaves and stems of two Leucospermum cultivars (Proteaceae)
--Manuscript Draft--

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Abstract:	The anatomy of field, in vitro and acclimatized shoots (leaves and stems) of two cultivars of Leucospermum (L. cordifolium 'Flame Spike' and L. 'Tango') was compared using light, scanning and transmission electron microscopy. Field plants showed several scleromorphic anatomical structures related to excess solar radiation such as: cuticle thickness, subepidermal collenchyma and sclerenchyma. Furthermore, a large quantity of phenolic deposits present in the cell lumen of various tissues is also a scleromorphic feature. The special conditions during in vitro culture result in plantlets with abnormal morphology and anatomy. These disorders are associated with the gaseous environment in the culture vessels, low irradiance in the incubation chamber and the addition of sucrose, nutrients and growth regulators to the culture medium. After transfer from in vitro to ex vitro conditions, substantial changes in leaf and stem anatomy were observed, above all in cuticle thickness, epidermal characteristics (stomatal and trichome index, and stomatal and pore size), differentiation of leaf mesophyll, chloroplast structure, and amount and localization of phenolic deposits. These changes allowed the plants to adapt to the new environmental conditions. The study of anatomical features of in vitro shoots facilitated adapting the acclimation protocol to predict which plantlet would survive the critical acclimation stage.

Response to Reviewers:	Emma Suárez Toste Facultad de Farmacia. Universidad de La Laguna Avenida Astrofísico Francisco Sánchez 38071 San Cristóbal de La Laguna Tenerife, Islas Canarias España ensuarez@ull.es

Editors-in-Chief
Plant Cell, Tissue and Organ Culture

August 23, 2018

Dear Editors-in-Chief

Thank you for considering our research article entitled "Structural and ultrastructural differences between field, micropropagated and acclimated leaves and stems of two Leucospermum (Proteaceae)" by Carmen Alfayate, Juan Felipe Pérez-Francés, Juan Alberto Rodríguez-Pérez and Emma Suárez for publication in Plant Cell, Tissue and Organ Culture.

In view of your comments and the Associate Editor's, it is important to explain that the article titled "Structural and ultrastructural variations in in vitro and ex vitro rooting of microcuttings from two micropropagated Leucospermum (Proteaceae). Scientia Horticulturae (2018), Vol. 239:300-307" was not yet cited in the present paper because it was not published at the time of submitting this manuscript. Of course, we planned to cite it once you decided it was appropriate for publication in Plant Cell, Tissue and Organ Culture.

In addition, this manuscript and the article cited above are part of the same research, but in that previous paper we only described the anatomical changes occurring in the rooting zone during their root development. While it is true that the stem anatomy before rooting is described, it was provided add the citation on this point. Furthermore, all the leaf and stem anatomy descriptions described in the manuscript are original (except description of stems of in vitro plants, but citations have been added) as well as the microscopy images, which have not been published previously.

We hope that these explanations clarify any doubt you had regarding the originality of this research article.

Finally, all the changes suggested by the editors and reviewers have been made. The comments to the Reviewers are listed below in bold lettering, embedded within the corresponding questions.

REVIEWER 1

1. Line 55 perhaps should read.-'Proteas have been successfully grown in the Canary Islands for 20 years.'
AUTHOR ANSWER: we have changed it.

2. Line 66 perhaps could read as- '…however the results showed low survival rates…'
AUTHOR ANSWER: we have changed it.

3. Line 91- what was the age of leaves collected from the field?
AUTHOR ANSWER: we have added some more information about the field plants (line 94).

4. Line 91- can you clarify 'in vitro cultures were 70 days old' i.e. were they initiated from field plants and cultured for 70d, if so what was the initiation protocol.
Alternatively, were the plants subcultured at day 1 and grown for 70 days, if so were they subcultured to fresh media every 21 days.
AUTHOR ANSWER: we have added the explanation in the manuscript (line 105).

REVIEWER 2

1. Line 60: vegetative propagation is easy…it would be interesting to specify how the vegetative propagation is done, by cuttings, or.
AUTHOR ANSWER: we have added the method of vegetative propagation

2. 50 days-old acclimated plants: why 50 day was chosen? Any parameters were formerly measured to conclude that at this moment the plants were acclimate?
AUTHOR ANSWER: we have added in the manuscript the explanation (Line 120).

3. The tissue culture protocol mentioned here is based on a formerly published reference? If so, please include the reference.
AUTHOR ANSWER: the reference have been added.

4. Philips instead of Phillips
AUTHOR ANSWER: we have changed it.

5. Roots were induced by culture shoots in darkness…for how many days.
AUTHOR ANSWER: we have added in the manuscript the explanation (Line 107).

6. After induction the shoots were transferred to an auxin-free medium… for how long?
AUTHOR ANSWER: we have added in the manuscript the explanation (Line 114).

7. No information is given on the division between persistent and newly formed leaves

in the acclimated plants in the material and methods section.

AUTHOR ANSWER: we have added the division between persistent and newly formed leaves in material and methods section (Lines 125 and Lines 154-160).

8. 5x10 repeats in abaxial and 5x10 repeats in adaxial leaf surfaces were done? How many repeats were done for the stainings and how many were observed before conclusions were made?

AUTHOR ANSWER: yes, 5x10 repeats in abaxial and 5x10 repeats in adaxial leaf surfaces were done. We cannot specify how many stains were made before conclusions but many of them were made and observed both for optical and transmission microscopy.

9. No information is given on the growth conditions of field plants and how these plants were obtained. Radiation levels?

AUTHOR ANSWER: we have added information about the growth conditions of field plants and how these plants were obtained in the manuscript (line 94).

10. Please use consistently the abbreviations on AP, FP, IP.

AUTHOR ANSWER: we have done it.

11. Often in the results section one refers to the sections. F. exp. Line 152 'The cuticle was thicker', line 165 'most of the stomata are single spaced,…: is it not possible to clarify some of these things by measurements done on the sections? Other measurements are given (f.exp. line 217-227) and I wonder if these measurements cannot be statistically analysed?

AUTHOR ANSWER: we have added to table 2 the measurements and statistical analysis of the cuticle thickness in leaves and stems. The explanation about the 'single spaced' have been added in line 189.

12. Line 286: strange sentence…water and nutrients in the medium and growth chamber…

AUTHOR ANSWER: we have changed the sentence (line 305).

13. Line 289: during micropropagation and/or…IP and/or AP or add respectively

AUTHOR ANSWER: we have added respectively (line 312)

14. Line 304: clarify what is meant by absence of cytokinins.

AUTHOR ANSWER: no cytokinins were added to the culture medium.

15. Line Conner and Conner, specify in which plant

AUTHOR ANSWER: we have added the name of the plant.

In light of the changes and justifications mentioned above we hope this revised version is suitable for publication in Plant Cell Tissue and Organ Culture. We are looking forward to hearing from you soon.

Sincerely yours,

Emma Suárez Toste Ph.D.
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1 **Structural and ultrastructural differences between field, micropropagated and**

2 **acclimated leaves and stems of two *Leucospermum* cultivars (Proteaceae)**

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Abstract

The anatomy of field, *in vitro* and acclimatized shoots (leaves and stems) of two cultivars of *Leucospermum* (*L. cordifolium* 'Flame Spike' and *L.* 'Tango') was compared using light, scanning and transmission electron microscopy. Field plants showed several scleromorphic anatomical structures related to excess solar radiation such as: cuticle thickness, subepidermal collenchyma and sclerenchyma. Furthermore, a large quantity of phenolic deposits present in the cell lumen of various tissues is also a scleromorphic feature. The special conditions during *in vitr*o culture result in plantlets with abnormal morphology and anatomy. These disorders are associated with the gaseous environment in the culture vessels, low irradiance in the incubation chamber and the addition of sucrose, nutrients and growth regulators to the culture medium. After transfer from *in vitro* to *ex vitro* conditions, substantial changes in leaf and stem anatomy were observed, above all in cuticle thickness, epidermal characteristics (stomatal and trichome index, and stomatal and pore size), differentiation of leaf mesophyll, chloroplast structure, and amount and localization of phenolic deposits. These changes allowed the plants to adapt to the new environmental conditions.

The study of anatomical features of *in vitro* shoots facilitated adapting the acclimation protocol to predict which plantlet would survive the critical acclimation stage.

Keywords: Adaptation; *In vitro* tissue culture; *Leucospermum*; Plant anatomy; Proteaceae; Tissue features.

Introduction

Leucospermum (Proteaceae) consists of 48 species confined to South Africa (Rourke 1972). Most of the known species (92%) are distributed over the Cape province, between Port Elizabeth and the Oliphants River mouth. Due to their striking inflorescences, *L. cordifolium* 'Flame Spike' and *L.* 'Tango' are two of the *Leucospermum* cultivars most

appreciated by the cut flower industry. 'Flame Spike' is a very appealing cultivar of *Leucospermum cordifolium* and presents long flower stems carrying striking light orange-red flowers. *Leucospermum* 'Tango' is a cultivar of *L. glabrum* x *L. lineare* parentage that displays a vividly colored bloom head, with bright red perianth ribbons contrasting strikingly with the orange styles (Matthews 2002). Proteas have been successfully grown in the Canary Islands for 20 years ago in the Canary Islands, and the results were good. At present, their cultivation is widespread on La Palma, Tenerife and Gran Canaria and around 1 million protea blooms per year are produced for export to Europe, the USA, Japan, etc.

Although vegetative propagation by cuttings is easy for these plants (Jacobs and Steenkamp 1976; Rodríguez-Pérez *et al.* 2009, 2011; Suárez *et al.* 2010; Oliveira *et al.* 2012; Rodríguez-Pérez *et al.* 2014; Vera Batista 2016), micropropagation could be an alternative to satisfy demand as well as to provide plant health guarantees for international trade. Furthermore, *in vitro* tissue culture is a fast disease-free and energy-saving method to maintain production steady throughout the year. Diverse authors have studied the micropropagation of *Leucospermum* (Ben-Jaacov and Jacobs 1986; Kunisaki 1989, 1990; Pérez-Francés *et al.* 2001; Rugge *et al.* 1989; Tal *et al.* 1992; Thillerot *et al.* 2006; van Staden and Borman 1976) however the results showed low survival rates in the acclimation phase. In most cases, the survival percentage of *in vitro* plants after transplanting to *ex vitro* conditions is a consequence of the adaptive morphological and functional changes that leaves and stems undergo in the particular *in vitro* conditions. In particular, these are high relative humidity, low light, no supplementary CO_2 and high sucrose and nutrient concentration in the medium. Knowledge of these modifications permits adapting acclimation protocols to the *in vitro* plant characteristics, which achieves greater success in this phase. Features acquired during *in vitro* culture are thinning of the

cuticle and a lower amount of phenolic deposits etc. These should be modified gradually

during the acclimation phase, especially in plants such as proteas that have numerous

scleromorphic structures attributable to direct exposure to sunlight in their leaves and

stems. These structures protect the mesophyll from excess solar radiation. Other defence

and protection mechanisms are secondary metabolites and phenolic compounds with

antioxidant properties. Plant phenols constitute a group of natural products of great

structural diversity and wide phylogenetic distribution (Zucker 1983). The presence of

phenolic compounds is well known in Proteaceae. Although they are not widely studied,

their potential antioxidant activity has recently been suggested, making them interesting

for medical applications and the development of "natural food preservatives" (León *et al*.

2014).

In the present paper, we report a comparative structural and ultrastructural study of the

leaves and stems of both cultivars, *L.* 'Flame Spike' and *L.* 'Tango' from field,

micropropagated and acclimated plants. The knowledge of these characteristics is useful

to adapt the micropropagation and acclimation protocols to obtain better survival results.

Materials and methods

Plant material

Leaves and stems were collected from the field, (FP), *in vitro* 70 days-old plants (IP) and

50 days-old acclimated plants (AP). FP were 10 year-old plants cultured by cuttings in

the experimental fields belonging to the Higher Polytechnic School of Engineering,

University of La Laguna, Canary Islands, Spain (28°28´42.65´´ N, 16°19´08.68´´ W;

altitude 564 m above sea level). Irradiance was approximately 250 W/m^2. Scarcely

lignified young shoots were cut, keeping them in water in order to avoid breaking their

inner water-column. Micropropagated plants were obtained from the cultivation of

multinodal segments from FP, *in vitro* on half-strength macronutrient MS medium

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101 (Murashige and Skoog 1962) supplemented with 20 g/l sucrose, 150 mg/l ascorbic acid

102 and solidified with 7 g/l agar (agar-agar/gum-agar, Sigma Chemical, St. Louis, Mo.

103 A1296). The pH was adjusted to 5.8 prior to autoclaving at 121º C for 24 minutes.

104 Cultures were incubated in a growth chamber at 24±2º C under Philips fluorescent

105 daylight tubes (110 µmol m^{-2}s^{-1}) during the 16-h photoperiod. Subcultures into fresh

106 medium were carried out for 21 days, and the plantlets remained in these conditions for

107 70 days. Roots were induced by culture of the shoots in darkness for 2 days, on liquid

108 basal medium containing 12.5 mg/l IBA (root induction medium), as described previously

109 (Suárez *et al*., 2018).

110 After induction treatment, the shoots were transferred to an auxin-free medium containing

111 20 g/l sucrose and solidified with 7g/l agar (root elongation medium). The medium pH

112 was adjusted to 5.8 prior to autoclaving at 121º C for 24 minutes. Cultures were incubated

113 in a growth chamber at 24±2°C under Philips fluorescent daylight tubes (110 µmol m^{-2} s^{-}

114 ^{1}) during the 16 h photoperiod. Plantlets remained in the elongation medium for 26 days,

115 until they reached a suitable size for transplant.

116 Rooted plantlets were transplanted into plastic pots filled with a perlite:quartz-sand:peat

117 mixture (3:2:1). Plants were acclimated for 7 weeks in a controlled incubation chamber.

118 The relative humidity was gradually reduced from 95% to 60% while the light irradiance

119 was increased from 110 µmol m^{-2} s^{-1} to 160 µmol m^{-2} s^{-1} during the acclimation period.

120 We considered that plants were acclimated when new leaves developed and they reached

121 an adequate size. This was accompanied by a strong development of the root system.

122 Morphological and ultrastructural studies

123 Leaves and stems from FP, IP and AP were excised and processed for light microscopy

124 (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

125 using standard protocols. Samples of persistent and new leaves were taken from the AP.

Light microscopy (LM): samples were fixed in FAA solution (formaldehyde 90%, absolute ethanol 5%, acetic acid 5%) for 48h, transferred to 70% ethanol, dehydrated in an ethanol series (Johansen 1940) and embedded in paraplast plus. Transverse sections (20 µm) were cut with a rotatory Minot microtome, mounted on slides and stained with safranin and fast green (Johansen 1940). Semi-thin sections (1 µm) of resin-embedded material were cut with glass knives and stained with toluidine blue.

To study leaf trichomes and stomata, epidermal peels were taken from abaxial and adaxial leaf surfaces in the middle of branches and plantlets, and mounted in glycerin. Observations and photomicrographs were carried out using a Leica Mod. D4000B light microscope.

Transmission electron microscopy (TEM): small leaves and stem pieces (2-4 mm) were fixed for 2h in 3% glutaraldehyde, post-fixed in 1% OsO_4, both in phosphate buffer (PB) 0.1 M. They were then dehydrated in an ethanol gradient series, transferred to acetone, and embedded in Spurr's resin (Spurr 1969). Semi-thin (0.5-1 µm) and ultra-thin sections (70 nm) were cut using a Reicher-Jung ultramicrotome. Ultra-thin sections were stained with uranyl acetate and lead citrate. The sections were photographed in a JEOL JEM-1010 transmission electron microscope (Electron Microscopy Centre, Madrid Complutense University, MCU).

Scanning electron microscopy (SEM): leaf pieces (3 mm^2) were fixed in 2.5% glutaraldehyde in PB 0.05 M (pH=7). They were dehydrated in ethanol gradient series, dried using 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and coated with silver. Observations and photomicrographs of the foliar surface were made using a JEOL JSM-6300 scanning electron microscope (General Services for Supporting Research, University of La Laguna).

Statistical analysis

151   Stomatal index parameters (SI) (Salisbury 1929), stomata and pore width, and length and

152   trichome index (TI) were measured ==on both surfaces==, in five randomly selected

153   microscopic fields in each slide (ten slides per sample).

154   ==Cuticle thickness was measured ten times on both surfaces of five leaves of each type. In==

155   ==AP, new leaves developed during the acclimation and persistent leaves were considered==

156   ==those developed *in vitro* that remained on the plant during acclimation.==

157   ==Cuticle thickness of the stem was measured in five plants of each type and ten==

158   ==measurements were made per plant. In AP, measurements were taken in the zone of the==

159   ==stem developed during acclimation (new stem) and the zone developed *in vitro* (persistent==

160   ==stem).==

161   Data were analysed using analysis of variance (ANOVA) with Duncan´s multiple range

162   test (α=0.05%). All data were evaluated using SPSS v.19.0 (SPSS Inc., Chicago, IL,

163   USA).

164   **Results**

165   The histological pattern of leaves and stems in *L. cordifolium* 'Flame Spike' and *L.*

166   'Tango' were similar in the three types of plants (FP, IP and AP). Some of the

167   scleromorphic characteristics lost during the *in vitro* phases were recovered in the *ex vitro*

168   acclimation.

169   Leaf anatomy

170   Transverse sections of 'Flame Spike' and 'Tango' leaves from FP, IP and AP presented

171   a unilayered epidermis with isodiametric cells covered by a smooth cuticle (Figs. 1a, f, k,

172   p and 2a, g, l, q). The cuticle was thicker in FP and in persistent and new leaves of AP,

173   ==on both epidermises (Table 2). There were no significant differences in cuticle thickness==

174   ==between leaves from IP or AP in either cultivar or surface, except for the AP-P leaves of==

175   =='Tango', which showed significant differences with FP and AP (Table 2).==

176 Epidermal cells with thick and electron-dense internal and external tangential cell walls

177 had phenolic deposits as scattered droplets and fine grain in field and persistent 'Flame

178 Spike' leaves (Fig. 1a1), while they were absent in 'Tango' (Fig. 2a1) and in

179 micropropagated and new acclimated leaves in both cultivars (Figs 1f1, p and 2g, q).

180 'Flame Spike' and 'Tango' leaves presented simple trichomes (Figs. 1a-b, f-g, k-l, p-q

181 and 2a-b, h, l-m, q-r) with striate cuticles and a thickened basal cell (Figs. 1c, h, m, r and

182 2c, i, n, s). Leaves are amphistomatic (Figs. 1a, f, k, p and 2a, g, m, s), with open or closed

183 kidney-shaped guard cells with an elliptical aperture, situated at the level of the epidermal

184 cells (Figs. 1d, h, n, s and 2d, j, o, s). The guard cells had a thick cuticle and the

185 chloroplasts had poorly developed inner membranes and a large amount of starch deposits

186 in the stroma. Both epidermis in paradermal view had brachyparacytic stomata. In micro-

187 propagated 'Flame Spike' and 'Tango' leaves (Figs. 1g and 2h), and in persistent and new

188 leaves from 'Tango' AP (Figs. 2m, r), most of the stomata are singly spaced on the leaf

189 epidermis, separated by at least one epidermal cell, but a minority of them are arranged

190 adjacently without a stomata-free region surrounding each stoma (Figs. 1i and 2j, o, t). In

191 persistent leaves from both cultivars, some open stomata showed extrusion of

192 parenchymal tissue through the pore (Figs. 1k1, o and 2l1-2l2). Nevertheless this was not

193 observed in new leaves (Figs. 1p1 and 2q1). The mesophyll had a distinct palisade and

194 spongy parenchyma on both surfaces in field (Figs. 1a and 2a) and persistent acclimated

195 leaves (Figs. 1k and 2l). These leaves presented an organized palisade parenchyma

196 formed of two layers of cells, abundant chloroplasts surrounding the central vacuole with

197 an organized thylakoid system, many lipid inclusions and a few starch grains in the

198 stroma. The cell lumen was filled with phenolic deposits as fine granular material in

199 'Flame Spike' (Figs. 2a1), and as scattered droplets in IP in both cultivars (Figs. 1g and

200 2g1) and persistent leaves of 'Tango' AP (Fig. 2q1). Spongy round parenchyma cells had

201 chloroplasts surrounding the central vacuole and phenolic deposits as scattered droplets

202 in the lumen of 'Flame Spike' FP, IP and in the persistent leaves of AP (Figs. 1a, f, k and

203 2.g, l). *In vitro* leaves showed a poorly developed mesophyll with a significant amount of

204 air spaces in both cultivars (Figs. 1f and 2g). Mesophyll cells presented abundant

205 chloroplasts with a disorganized thylakoid membrane system and few phenolic deposits

206 as scattered droplets in both cultivars. The margin of the leaf blade of FP 'Flame Spike'

207 leaves had 2 layers of sub-epidermal collenchyma (Fig. 1a1). Collenchyma cells had

208 thickened corners, spherical chloroplasts surrounding the vacuole with lipid inclusions in

209 the stroma and phenolic deposits as scattered droplets in the cell lumen. The primary and

210 secondary vascular bundles were surrounded by sclerenchyma in a cap-shaped manner.

211 Sclerenchyma cells exhibited marked thickening in FP (Figs. 1e and 2e) and persistent

212 leaves of AP (Figs. 1k and 2p) in both cultivars, but was scarce in IP (Figs. 1k and 2k)

213 and new leaves of AP (Figs. 1t and 2u) in both cultivars. Vascular bundles showed

214 collateral arrangement, with adaxial phloem (Figs. 1e, j1, k, t and 2e, k, p, u). Xylem

215 elements had thickened secondary cell wall in all types of plants (Fig. 1l).

216 Parenchyma cells associated with vascular bundles exhibited chloroplasts with an

217 organized thylakoid membrane system, many lipid inclusions in the stroma and phenolic

218 deposits as scattered droplets in the cell lumen (Fig. 2f). Phenolic deposits were scarce in

219 parenchyma cells of IP. Vascular cambium consisted of 2-3 rows of cuboidal thin-walled

220 cells in FP and IP for both cultivars. Persistent and new leaves of both AP cultivars had a

221 slight increase in the number of vascular cambium rows (3-4 in persistent leaves and 4-5

222 in new leaves). Phloem cells showed thin-walls and absence of phenolic deposits in the

223 cell lumen.

224 Epidermal parameters

In 'Flame Spike', the stomatal index (SI) was significantly greater in FP leaves on both surfaces, while 'Tango' had the greater SI in AP-N (acclimated plantas-new leaves) leaves, independently of which surface (Table 1). Data analysis confirmed significant differences in leaf stomata, pore length and width, with larger stomata on IP leaves (Table 1). Trichome index (TI) was higher in IP leaves on both surfaces, although there were non-significant differences on the abaxial side. In 'Tango' cultivar, there were no substantial differences in SI between FP and IP on either surface but TI was higher in AP (Table 1). The low percentage of 'Flame Spike' acclimated plants did not permit study of their epidermal parameters.

Stem anatomy

In cross-section, the stem exhibited concentric tissue arrangement (Figs. 3a, e, h, k and 4a, f, i, l), with a unilayered epidermis of papillose cells in FP from 'Flame Spike' (Fig. 3b) and isodiametric cells in 'Tango' FP (Fig. 4b), IP (Figs. 3f and 4f) and AP from both cultivars (Figs. 3h, l and 4., 13). Epidermis was covered by a smooth cuticle, its thickness depending on the type of cultivar and plant.

Cuticle of FP from 'Flame Spike' had a thickness of 6.9 µm (Table 2), showing by two zones (Fig. 3.1i). The inner reticulate zone had electron-opaque fibrils coming from the underlying cell walls. The outer zone was thinner and electron-clear. 'Tango' FP cuticle thickness was 3.7 µm (Table 2) and presented only one electron-dense layer. In-vitro 'Flame Spike' cuticle was 1.1 µm thick (Table 2) and also showed 2 zones (Figs. 3f-f1). The inner one was in direct contact with the external epidermal cell walls and showed a reticulate structure and the outer zone was thinner with a poorly-defined amorphous structure. The 'Tango' IP cuticle was 1.4 µm wide (Fig. 4f). In AP cuticle, the thickness depends on the type of stem and cultivar (Table 2) (Figs. 3i, l and 4j1, m).

249 Phenolic deposits as scattered droplets were present in the epidermal cell lumen of new

250 stems in both cultivars AP (Figs. 3l and 4m) but absent in the other type of stems (Figs.

251 3b, f, i and 4b, f, j1). Both cultivars presented simple trichomes (Figs. 3a, f, i, k and 4a, f,

252 i, l). Both FP cultivars (Figs. 3b and 4b) and the new stem of 'Tango' AP (Fig. 4m)

253 showed a sub-epidermal collenchyma, consisting of 2-3 layers of thick-walled cells at the

254 corners. Their cytoplasm contained phenolic deposits as scattered droplets in 'Flame

255 Spike' and in 'Tango' AP, and as a fine grain filling the cell lumen in 'Tango' FP. The

256 chloroplasts were spherical and located around the vacuole, presenting lipid inclusions in

257 the stroma. The parenchymatous cortex consisted of a variable number of cell layers, FP:

258 8-10 in 'Flame Spike' (Fig. 3a) and 6-9 in 'Tango' (Fig. 4a); IP: 8-10 in 'Flame Spike'

259 (Fig. 3g) and 5-10 in 'Tango' (Fig. 4f); persistent AP: 6-8 in both cultivars (Figs. 3h and

260 4i), new AP: 8-10 in both cultivars (Figs. 3k and 4l), with scarce intercellular spaces.

261 Parenchyma cells showed phenolic deposits as scattered droplets in 'Flame Spike' FP

262 (Fig. 3b), in 'Tango' IP (Fig. 4a) and in both cultivars AP (Figs. 3i, l and 4j1, m) and as

263 fine grain in 'Tango' FP (Fig. 4b). Vascular bundles were surrounded by a cap-shaped

264 sclerenchyma. Sclerenchyma cells were numerous in 'Tango' FP (Figs. 3c and 4c) and

265 persistent stem (Figs. 3g and 4c1), in Tango they exhibited markedly thickened walls with

266 a narrow central lumen, which was absent in IP stems (Figs. 4g). There were

267 sclerenchyma cells with thick walls in persistent stem of AP in both cultivars (Figs. 3j

268 and 4j-k) and in the new stem of 'Tango' (Fig. 4n), while they were scarce in 'Flame

269 Spike' (Fig. 3m). Collateral vascular bundles (Figs. 3d, j, m and 4c, g, k, n) with

270 secondary growth surround extensive parenchyma pith. The centrifugal phloem was

271 formed of different layers of irregular cells located above the vascular cambium. Xylem

272 elements showed various sizes and secondary growth in both cultivars (Figs. 3d, g1, j, m

273 and 4d, h, k, n), but in *in vitro* stem xylem cells were smaller and had thickened walls.

274 The central pith contained spherical cells with scarce intercellular spaces and phenolic

275 deposits in 'Tango' FP (Fig. 3a), in the new stems of 'Tango' AP, and in persistent AP

276 stems of both cultivars (Figs. 3j and 4j).

277 **Discussion**

278 The main differences at the histological level between leaves and stems of FP, IP and AP

279 of *L. cordifolium* 'Flame Spike' and *L.* 'Tango' are related to the nutritional and

280 environmental conditions. The histological adaptations by the two cultivars to the

281 different environments were similar, showing considerable differences in both leaves and

282 stems from the plants grown under natural conditions (FP).

283 Leaf anatomy

284 In leaves, the reduced thickness of the IP cuticle could be caused by the closed vessels,

285 since they produce an atmosphere with high relative humidity and scarce gas exchange

286 inside the vessels (George *et al*. 2008). During acclimation, although humidity was

287 reduced, the scarce gas exchange inside the growth chamber prevented normal cuticle

288 development. Furthermore, the non-vented vessels and high nutrient concentration in the

289 medium caused poor development of the secondary walls of epidermal cells (Majada *et*

290 *al*. 2001) and negative effects in the epidermal foliar structure (Dickinson 2000). The

291 reduction in the tangential external epidermal wall cells of AP in both cultivars may also

292 cause limited plant survival, which along with the thin cuticle contributed to

293 impoverishment of one of the main plant defence mechanisms against excessive water

294 loss (Louro *et al*. 2003) and radiation (Pospíšilová *et al*. 1999).

295 FP epidermal cells in both cultivars presented phenolic deposits as scattered droplets

296 ('Flame Spike') and fine grain ('Flame Spike' and 'Tango'). The accumulation of phenols

297 in tissues, especially the epidermis and peripheral cells, has been related with the function

298 of protecting inner tissues against excess visible and ultraviolet irradiation (Salatino *et al*.

299   1988; Jordan *et al*. 2005; León *et al*. 2014). Therefore, their absence in IP epidermal cells

300   could be due to low irradiance in the growth chambers in addition to the use of closed

301   containers. In both cultivars, PA persistent leaves showed high adaptation to the stronger

302   light intensity in terms of the phenolic deposits visible in the epidermal cells, whereas in

303   new leaves they were absent. These plants were grown in chambers with a low light

304   intensity compared to the radiation levels FP was exposed to, which would explain the

305   scarce presence of phenolic deposits in their epidermal cells. Furthermore, Theunissen

306   and Jordan (1990) reported that the high availability of water and nutrients in the medium

307   and the relative humidity in the growth chamber could be another reason for the low

308   presence of phenols in these leaves. These authors related the fluctuation of phenolic

309   content in leaves with plant development in nutritional and environmental unfavorable

310   conditions.

311   The use of closed vessels during micropropagation and a closed growth chamber during

312   acclimation could cause the reduced cuticle size in IP and AP respectively. In these

313   conditions, gaseous exchange is limited and an atmosphere with high relative humidity is

314   formed. (George *et al*. 2008).

315   This feature is one of those responsible for the excessive evapotranspiration losses after

316   transplant. This is the main factor related to plant death and low survival rates (Hazarika

317   2006). Although we did not analyse the epicuticular waxes, Sutter (1984) reported that

318   their composition can differ according to environmental conditions.

319   IP stomata showed some morphological and physiological features that point to a certain

320   functionality. First, elliptical guard cells have been related with normal stomatal

321   functioning by Jausoro *et al*. (2010). Micropropagated plants usually had rounded guard

322   cells (George *et al*. 2008), suggesting reduced functionality. Secondly, the presence of

323   open and closed stomata also points to functionality, since the stomata of

324   micropropagated plants normally remain open due to the presence of sucrose in the

325   medium.

326   This IP feature could also be related to the absence of cytokinins in the culture medium

327   (Apóstolo *et al*. 2005). Finally, the presence of plastids rich in starch and large vacuoles

328   in the guard cells indicate a similar metabolic activity to FP guard cells. The increase in

329   stomatal length and width on both foliar surfaces of IP and AP in comparison with the FP

330   stomata of both cultivars may be related with water-loss after transplant (Zobayed *et al*.

331   2001).

332   The differences observed between the SI of FP, IP and AP leaves on both surfaces are in

333   contrast to those reported by Conner and Conner (1984) ==in *Solanum laciniatum*==, according

334   to which this index normally shows no significant differences among *in vitro*, acclimated

335   and green-house grown plants. The differences suggest variations at cellular and

336   developmental level, since stomatal differentiation is determined at the early stages of

337   epidermal morphogenesis.

338   In both cultivars and in the three types of plants, the stomata were located scattered over

339   the leaf epidermis, separated from one another by at least one intervening epidermal cell,

340   forming the denominated "stomata free region" (Tang *et al*. 2002; Bergmann 2004).

341   However, IP leaves of both cultivars and AP persistent and new leaves of 'Tango'

342   presented two stomata located in direct contact via their guard or subsidiary cells,

343   originating stomatal clusters (Serna and Fenoll 2000, 2002). Clusters were also observed

344   in micropropagated camphor-tree and rhubarb plants, and Zhao *et al.* (2006a, b) suggested

345   they are due to variations at cellular and developmental level. Clusters were formed when

346   during the formation of one stoma the guard mother-cell undergoes two symmetrical

347   divisions instead of one, originating two stomata. Clusters have been described by Gan *et*

348   *al*. (2010) as a modification of the stomatal patterning due to a gaseous signal such as

349 ethylene or CO_2 concentration changes, which are common features inside the culture

350 vessels (Serna and Fenoll 1997).

351 In persistent leaves of AP in both cultivars there were open stomata with parenchyma

352 cells or other content protruding outwards through the pore. They are likely due to the

353 poor control that stomata developed *in vitro* have over gradual humidity changes during

354 the acclimation phase. No references to these structures were found in the literature.

355 Leaf trichomes of both cultivars are typical of the Proteaceae family (simple and

356 elongated). As in *Leucospermum conocarpodendron* (Skelton *et al*. 2012), *Adenanthos*

357 *cuneatus* and *Bellendena montana* (Carpenter *et al*. 2005) they showed a widened basal

358 cell. This type of pubescence, described by Rourke (1972) in almost 22 of the 47 species

359 of *Leucospermum* has traditionally been attributed to the water economy of plants. This

360 is consistent with the TI obtained in both cultivars, since plants grown under lower

361 relative humidity and higher light intensity (FP) presented a higher TI than those under

362 higher relative humidity and low light intensity (IP). Although it may also be related to

363 radiation protection, as Skelton *et al.* reported (2012) for *Leucospermum*

364 *conocarpodendron* and Jordan *et al*. (2005) for *Leucospermum coniferum*.

365 The poor mesophyll differentiation in 'Tango' especially, and the large amount of

366 intercellular spaces are some of the main features of IP. These abnormalities are

367 consequences of the microenvironment in the culture vessels and appear to be related to

368 different ventilation levels, as reported by Majada *et al*. (2001, 2002) in *Dianthus*

369 *caryophyllus*. Moreover, in these plants photosynthetic activity is reduced due to the

370 addition of sucrose and nutrients to the culture medium, low irradiance in the growth

371 chamber and the high CO_2 concentration in the culture vessels (Hazarika 2006). Similar

372 features were observed in the mesophyll of new leaves in 'Flame Spike' AP and indicate

373 poor adaptation of these plants to the acclimation conditions.

374 Although *in vitro* culture conditions can affect chloroplast development negatively, in

375 both cultivars chloroplasts showed a similar morphology to those in FP, and an

376 appropriate organization of the plastid membrane system. Stoyanova-Koleva *et al*. (2012)

377 and Stefanova *et al*. (2013) attributed this to the absence of growth regulators in the

378 establishment medium, since adding cytokinins to the medium can induce changes in

379 thylakoid orientation and their ability to become organized into a granum. The presence

380 of electron-dense granules in the chloroplast stroma of the IP mesophyll cells could be

381 due to the stress induced by irradiance and the nutritional composition of the culture

382 medium (Ladygin and Semenova 1993; Ladygin *et al*. 2008; Lucchesini *et al*. 2006; Serret

383 and Trillas 2000).

384 Among the functions of parenchyma cells is the development/production and storage of

385 substances such as phenolic compounds. Salatino *et al*. (1988) ascribe to phenols the

386 function of protecting inner tissues against excess visible and ultraviolet irradiation.

387 Another function seems to be antipredator protection (Jordaan and Theunissen 1992). In

388 'Flame Spike' and 'Tango', phenols contribute to protection from radiation, so they are

389 present mainly in the epidermis, collenchyma and mesophyll in the leaves of both FP

390 cultivars, and in reduced quantities in IP exposed to low radiation levels. The increased

391 phenolic deposits in persistent leaves of AP could be an adaptation to the raised light

392 intensity in the growth chamber, in contrast to the incubation chamber. The similar

393 location and ultrastructure of phenolic deposits observed in FP and in AP persistent leaves

394 were recently described by León *et al.* (2014) in *Protea* 'Susara'. These authors studied

395 the antioxidant activity of these compounds, suggesting their potential use in the

396 pharmaceutical industry.

397 The IP of both cultivars showed small groups of sclerenchyma fibres with poorly

398 thickened secondary walls. Similar features were observed by Dias Ferreira *et al*. (2003)

399 in *Leucadendron* 'Safari Sunset' micropropagated plants. Poorly thickened walls have

400 been linked with the absence of auxins in the culture medium, since these hormones

401 regulate lignin deposition in the cell walls (Bisbis *et al.* 2003; Hatzilazarou *et al.* 2006;

402 Ďurkovič and Mišalová 2009).

403 The FP, IP and AP had collateral vascular bundles with abaxial phloem; there were

404 thinner xylem and phloem cell walls in IP. However, the presence of plasmodesmata

405 confirmed the transport of substances by symplastic pathways between sieve elements

406 and companion cells. The parenchyma associated with the vascular bundles in FP showed

407 chloroplasts with a well-developed endomembrane system and large lipid droplets in the

408 stroma. Different-sized electron-dense plastoglobules were detected in IP, owing to the

409 nutritional and incubation conditions.

410 Stem anatomy

411 The stems of both cultivars showed thinner internal and external tangential cells walls in

412 IP. As in leaves, this could be related with the low light intensity in the growth chambers,

413 during *in vitro* rooting. In addition, the reduction in cuticle thickness, especially in 'Flame

414 Spike' was significant. In FP and IP, the stem cuticle showed two distinct layers as

415 proposed by Jeffree (2006): the inner reticulated layer (cuticular layer) and the outer layer

416 (cuticle proper). The cuticle proper was thicker in FP than IP. Since the cuticle has the

417 same protective function, differences in its thickness should be due to the environmental

418 conditions. It was thicker in the persistent and new stems than in IP of both cultivars and

419 as noted above, the composition may vary depending on the environment (Sutter, 1974).

420 This indicates an adaptation to the acclimation conditions. Cuticle in stems has the same

421 protective function as in leaves and the differences observed between the different plants

422 may indeed be due to the environmental conditions in which they developed. The FP and

423 IP stems showed scattered stomata with the guard cells at the same level as subsidiary

424 cells and the same type of simple and elongated leaf trichomes that Skelton *et al.* (2012)

425 and Carpenter *et al.* (2005) described in other members of Proteaceae.

426 Subepidermal collenchyma was only observed in FP and AP new stems. The location and

427 presence of thick primary cell walls provide extra structural support. León *et al.* (2014)

428 described similar subepidermal collenchyma in the stem of the *Protea* 'Susara'.

429 The main differences between FP, IP and AP cortex were the amount and morphology of

430 the phenolic deposits. Phenols were especially abundant in FP, appearing as scattered

431 droplets ('Flame Spike') or as fine grain occupying the cell lumen ('Tango'). However,

432 no phenolic deposits were observed in IP cortex (Suárez *et al.* 2018). The absence of these

433 deposits in the cortical cells of the IP would support the hypothesis of these substances

434 acting as an adaptation to environmental conditions, in particular protection against UV

435 radiation.

436 The stem of 'Flame Spike' and 'Tango' FP and AP presented perivascular sclerenchyma

437 formed by highly thickened walls, with the lumen obliterated or not. In 'Flame Spike' IP

438 stem, sclerenchyma was formed by small scattered groups of thin-fibre walls and was

439 absent in 'Tango'. The organization of vascular bundles showed more thickened cell walls

440 in FP than in IP, due to the absence of growth regulators in the culture medium. The

441 chloroplasts in parenchyma cells contained electron-dense granules in the stroma, which

442 as discussed above could be related to the nutritional and incubation conditions during IP

443 development. The reduction in the number of vascular cambium layers indicates less

444 activity in this tissue.

445 The large pith observed in FP stems of both cultivars could be another xeromorphic

446 feature of these plants (Evert 2006), due to its storage function. Therefore, the smaller

447 amount of this tissue in IP (Suárez *et al.* 2018) and in AP could result from an increased

448 availability of water and nutrients in the culture medium.

449 *In vitro* plantlets did not show the appropriate structure to survive transfer to soil, whereas

450 most acclimated plants did acquire the necessary protective characteristics. In some cases

451 these were not achieved, causing death of the plant.

452 In view of the histological study of the FP, IP and AP, we consider it necessary to design

453 an acclimation protocol that combines very gradual changes in environmental conditions

454 during the first phases of the *ex vitro* culture, since these plants have a slow growth

455 process. The main factors to take into account would be relative humidity and irradiance.

456 A reduction in relative humidity in the growth chamber would improve the survival of

457 the plants by reducing water loss by transpiration. In addition, the increase in irradiance

458 would allow a greater development of the cuticle, as well as an increase in the phenols

459 content.

460 **Author Contributions** ES and CA planned and designed the research, JA Rodríguez-

461 Pérez contributed plant material, ES performed the experiments and collected data. ES,

462 CA, JF Pérez-Francés and JA Rodríguez-Pérez analysed the data, ES wrote the

463 manuscript and CA supervised the writing. All the co-authors reviewed the manuscript

464 before submission.

465 **Compliance with ethical standards**

466 **Conflict of Interest** The authors declare that they have no conflict of interest.

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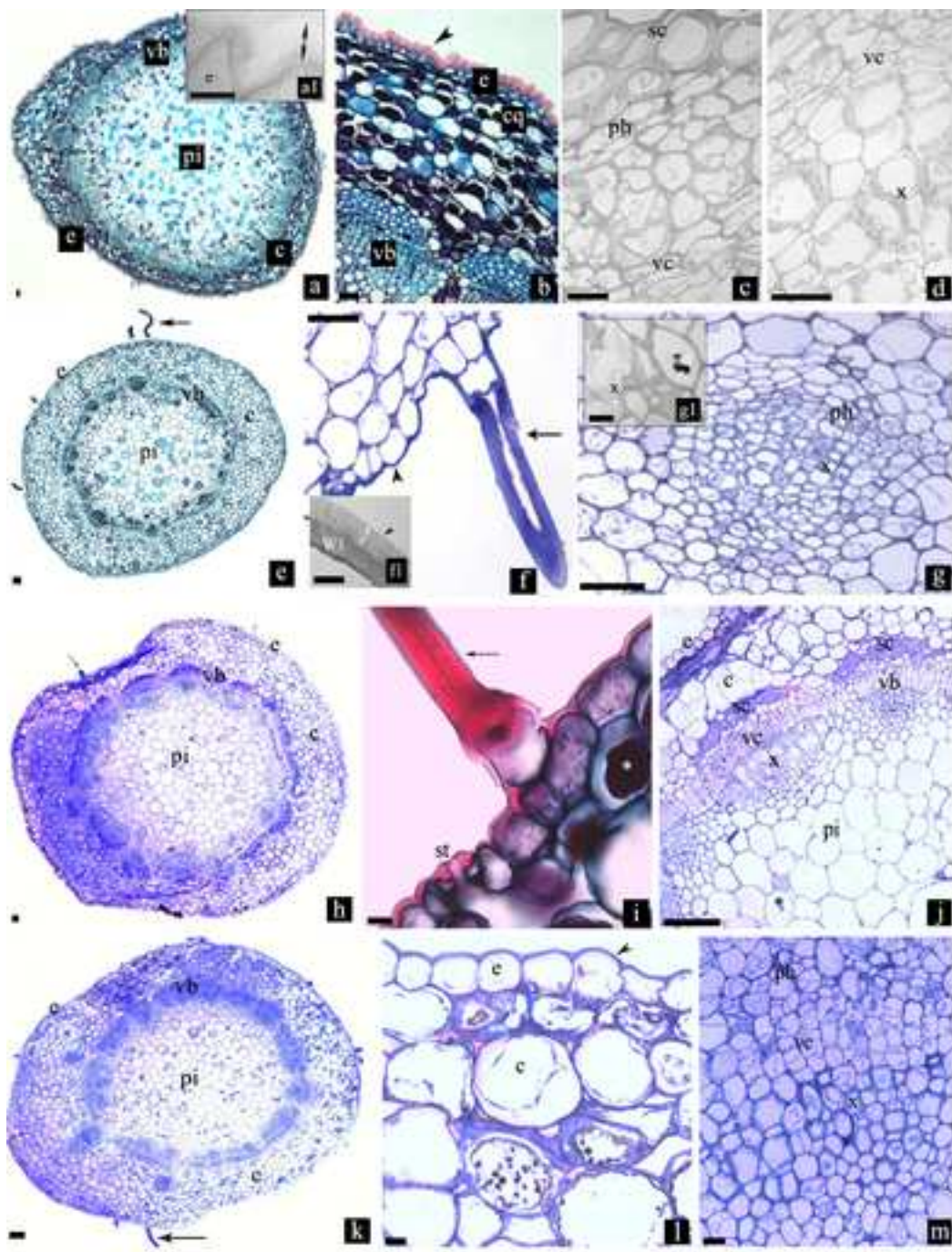
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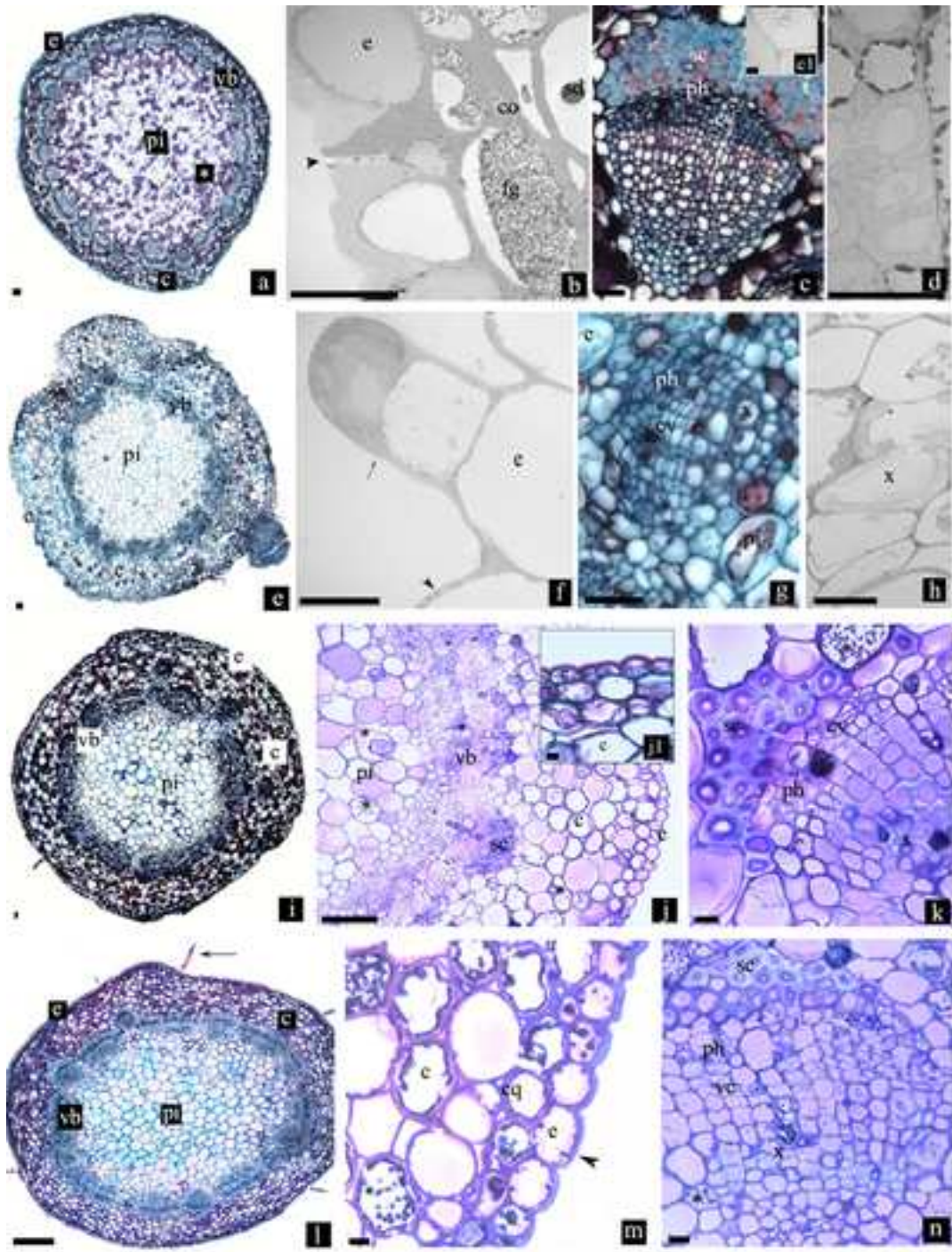
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<u>Key Message</u>

The study of the structural and ultrastructural features of leaves and stems allowed to determinate the anatomical changes occurred in plants during the micropropagation and acclimation.
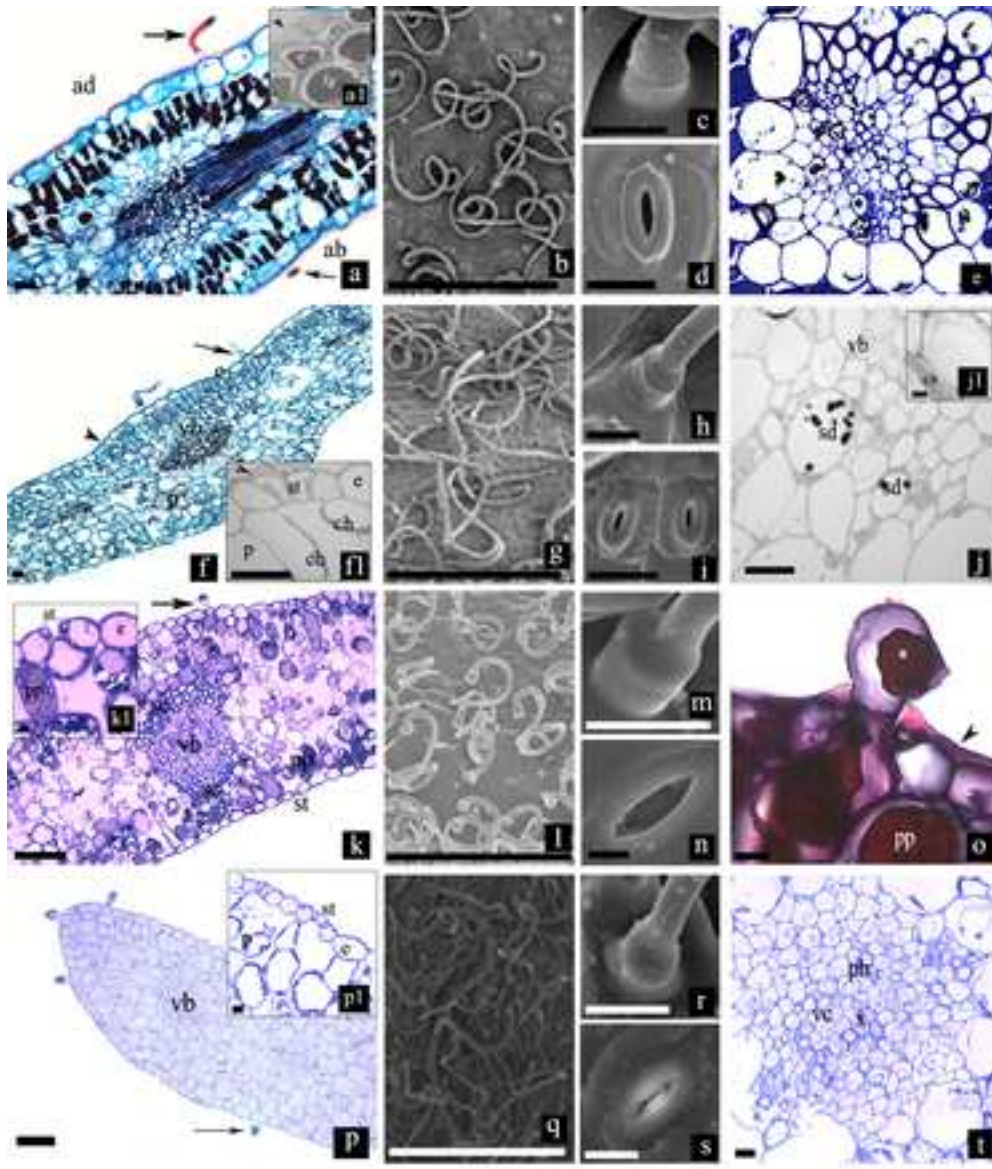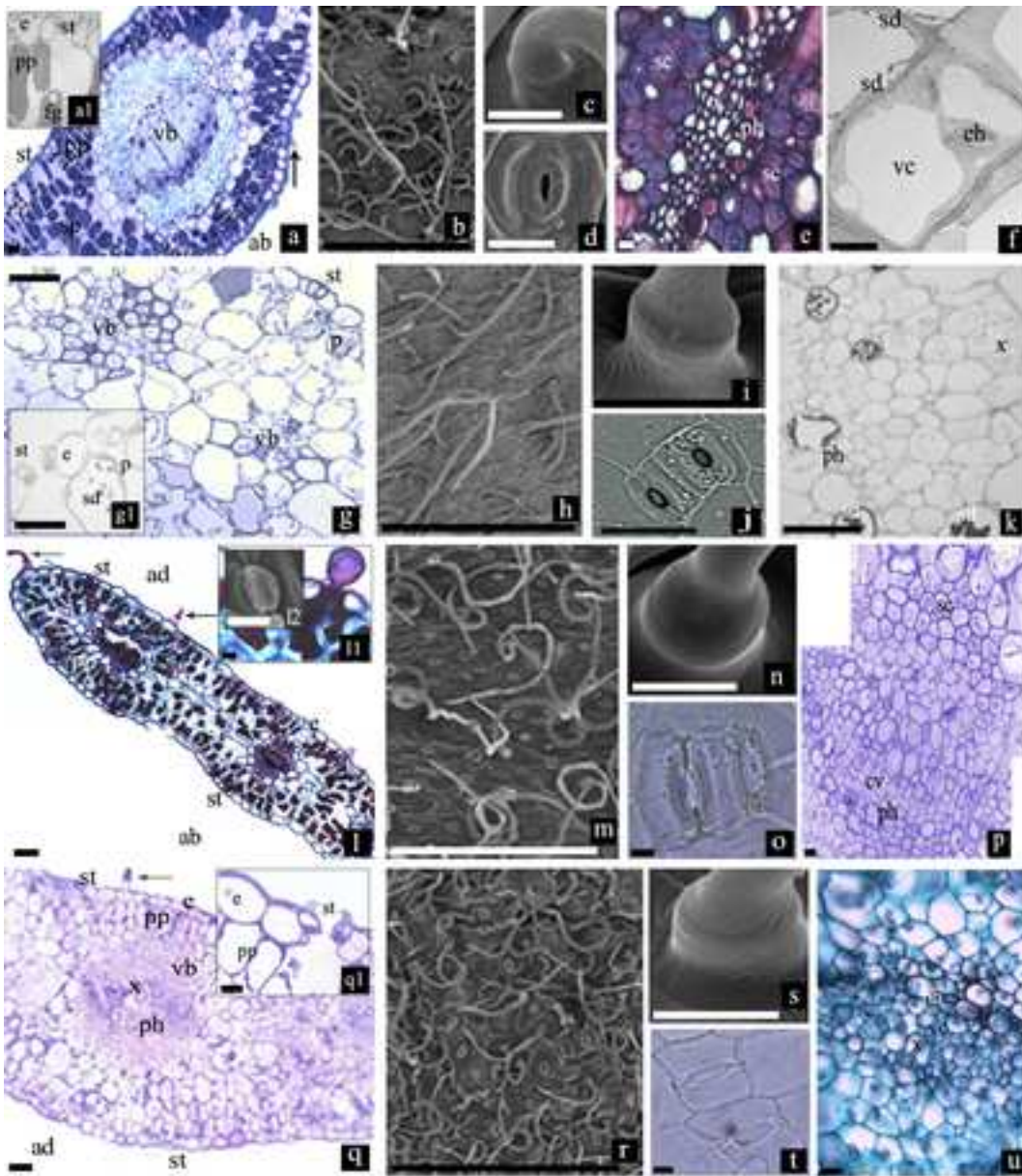
1   Fig. 1 Light, transmission and scanning electron microscopy images of *Leucospermum*

2   *cordifolium* 'Flame Spike' leaves from field (a-e), *in vitro* (f-j) and acclimated plants:

3   persistent leaves (k-o) and new leaves (p-t). **a** Unilayered epidermis with wide cuticle and

4   stomata on the adaxial and abaxial sides, mesophyll with palisade and spongy

5   parenchyma, and vascular bundles with sclerenchyma. **a1** Subepidermal angular

6   collenchyma with phenolic deposits in the cellular lumen. **b** Epidermal cells in frontal

7   view, adaxial (o abaxial) surface with stomata and trichomes. **c** Basal cell and reticulate

8   epidermis of a trichome. **d** Paracytic stoma. **e** Collateral vascular bundle, with

9   perivascular sclerenchyma fibres. **f** Unilayered epidermis with a thin cuticle and stomata

10 on adaxial and abaxial sides, poor mesophyll. **f1** Palisade parenchyma with significant air

11 spaces and chloroplasts. **g** Epidermal cells with frontal view, adaxial (or abaxial) surface

12 with stomata and trichomes. **h** Basal cell and reticulate epidermis of a trichome. **i** Stomatal

13 cluster of two stomata. **j** Collateral vascular bundle, with scarce development and

14 phenolic compounds as scattered droplets in the vascular parenchyma. **j1** Detail of a

15 xylem element with thickened walls and xylem parenchyma with chloroplast. **k**

16 Unilayered epidermis with a thin cuticle and stomata on adaxial and abaxial sides,

17 mesophyll with palisade and spongy parenchyma and vascular bundles with

18 sclerenchyma. **k1** Open stoma with parenchyma cell content protruding through the

19 stomata. **l** Epidermal cells with frontal view, adaxial (or abaxial) surface with stomata and

20 trichomes. **m** Basal cell and striated epidermis of a trichome. **n** Paracytic stoma. **o** Open

21 stoma with parenchyma cell content protruding through the pore. **p** Uni-layered epidermis

22 with thin cuticle and stomata on adaxial and abaxial sides. Mesophyll poorly structured.

23 p1 Epidermis and poorly organized palisade parenchyma without phenol deposits. **q**

24 Epidermal cells in frontal view, adaxial (or abaxial) surface with stomata and trichomes.

25 **r** Basal cell and reticulate epidermis of a trichome. **s** Paracytic stomata. **t** Collateral

vascular bundle without perivascular sclerenchyma fibres. ab: abaxial; ad: adaxial; co: collenchyma; ch: chloroplast; e: epidermis; fg: phenolic deposits as fine granular; p: parenchyma; pp: palisade parenchyma; sd: phenolic deposits as scattered drops; sp: sponge parenchyma; st: stoma; vb: vascular bundle; arrow: trichome; arrow head: cuticle; star: stomatal cluster. Bars: 600 μm: b, g; 500 μm: l, n, q; 100 μm: k, p; 50 μm: i; 40 μm: d, m; 30 μm: r; 25 μm: a, a1, e, f, f1; 20 μm: s; 10 μm: c, h, j, k1, o, p1, t; 1 μm: j1

Fig. 2 Light, transmission and scanning electron microscopy images of *Leucospermum* 'Tango' leaves from field (a-f), *in vitro* (g-k) and acclimated plants; persistent leaves (l-p) and new leaves (q-u). **a** Unilayered epidermis with thick cuticle and stomata on adaxial and abaxial sides, differentiated mesophyll and vascular bundles with sclerenchyma. **a1** Palisade parenchyma with phenolic deposits occupying the entire vacuolar volume. **b** Epidermal cells in the frontal view, adaxial (abaxial) surface with stomata and trichomes. **c** Basal cell and reticulate epidermis of a simple trichome. **d** Open brachyparacytic stoma. **e** Vascular bundle with perivascular sclerenchyma fibres. **f** Xylem parenchyma with phenolic deposits surrounding the vacuole membrane. **g** Unilayered epidermis with a thin cuticle and stomata on adaxial and abaxial surfaces, poor mesophyll, with a poorly developed palisade layer. **g1** Poorly developed mesophyll, with spherical cells and a few phenolic deposits. **h** Epidermal cells in frontal view, adaxial (abaxial) surface with stomata and trichomes. **i** Basal cell and reticulate epidermis of a simple trichome. **j** Cluster of two stomata. **k** Collateral vascular bundle, with limited development and phenolic compounds in the vascular parenchyma. **l** Unilayered epidermis with a thick cuticle and stomata on adaxial and abaxial sides, differentiated mesophyll and vascular bundles with sclerenchyma. **l1-l2** Light and SEM photomicrograph of an open stoma with parenchyma cell content extruded through the pore. **m** Epidermal cells in frontal view, adaxial

(abaxial) surface with stomata and trichomes. **n** Basal cell and reticulate epidermis of a simple trichome. **o** Cluster of two stomata. **p** Collateral vascular bundle with perivascular sclerenchyma. **q** Unilayered epidermis with a thin cuticle and stomata in adaxial and abaxial sides, differentiated mesophyll and vascular bundles without sclerenchyma. **q1** Poorly developed mesophyll without phenolic deposits. **r** Epidermal cells in frontal view, adaxial (abaxial) surface with stomata and trichomes. **s** Basal cell and striated epidermis of a simple trichome. **t** Cluster of two stomata. **u** Collateral vascular bundle, with limited development and without perivascular sclerenchyma fibres. ab: abaxial; ad: adaxial; ch: chloroplast; e: epidermis; m. mitochondria; p: parenchyma; ph: phloem; pp: palisade parenchyma; sc: sclerenchyma; sd: phenolic deposits as scattered drops; sp: sponge parenchyma; st: stoma; v: vacuole; vb: vascular bundle; x: xylem; arrow: trichome; arrow head: cuticle. Bars: 500 µm: h, m, r; 400 µm: b; 100 µm: l, q; 40 µm: l2; 30 µm: d, i, j; 25 µm: a, a1, g; 20 µm: c, k, n, s; 10 µm: e, o, l1, p, t, q1, u; 8 µm: g1; 3 µm: f

Fig. 3 Light and transmission electron microscopy images of *Leucospermum cordifolium* 'Flame Spike' stem from field (a-d), *in vitro* (e-g) and acclimated plants; persistent stem (h-j) and new stem (k-m). Stem cross-section. **a** Concentric tissue organization. **a1** Detail of cell wall with cuticle. **b** Unilayered epidermis covered by a thick cuticle, subepidermal angular collenchyma. **c** Vascular cambium and phloem elements. **d** Xylem elements and xylem parenchyma. **e** Concentric tissue organization. **f** Unilayered epidermis covered by a thin cuticle, simple trichome. **f1** Detail of cell wall and cuticle with two different zones, the inner shows a reticulate structure and the outer is thinner and amorphous. **g** Vascular bundle without perivascular sclerenchyma fibers. **g1** Detail of xylem elements and xylem parenchyma with phenolic deposits as scattered droplets. **h** Concentric tissue organization. **i** Unilayered epidermis covered by a thick cuticle. Simple trichome and

stoma. **j** Vascular bundles with perivascular sclerenchyma fibers. **k** Concentric tissue organization. **l** Unilayered epidermis covered by a cuticle. Cortex with phenolic deposits as scattered droplets. **m** Vascular bundle without perivascular sclerenchyma fibers. c: cortical parenchyma; ch: chloroplast; co: collenchyma; e: epidermis; ip: interfascicular parenchyma; m: mitochondria; ph: phloem; pi: pith; sc: sclerenchyma; sd: phenolic deposits as scattered drops; v: vacuole; vb: vascular bundle; vc: vascular cambium; x: xylem; arrow: trichome; arrow head: cuticle; asterisk: phenolic deposits as scattered droplets; double arrowhead: cuticle thickness. Bars: 100 μm: i, l, m; 25 μm: a, b, e, f, g, h, k; 20 μm: a1, d, g1; 10 μm: c, j; 1 μm: f1

Fig. 4 Light and transmission electron microscopy images of *Leucospermum* 'Tango' stem from field (a-d), *in vitro* (e-h) and acclimated plants; persistent stem (i-k) and new stem (l-n). Stem cross-section. **a** Concentric tissue organization. **b** Unilayered epidermis covered by a wide cuticle. Subepidermal angular collenchyma. **c** Collateral vascular bundle with perivascular sclerenchyma fibers. **c1** Detail of sclerenchyma fibers with thickened walls. **d** Xylem and pith ray showing phenolic deposits in the lumen. **e** Concentric tissue organization. Unilayered epidermis covered by a thin cuticle. **f** Cuticle, epidermis and simple trichome. **g** Collateral vascular bundle. **h** Xylem with thickened wall. **i** Concentric tissue organization. **j** Vascular bundles. Cortex and pith with phenol deposits as scattered droplets. **j1** Unilayered epidermis covered by a wide cuticle. **k** Collateral vascular bundle with perivascular sclerenchyma fibres. **l** Concentric tissue organization. **m** Unilayered epidermis covered by a wide cuticle and subepidermal collenchyma. **n** Collateral vascular bundle with perivascular sclerenchyma fibres. c: cortical parenchyma; co: collenchyma; e: epidermis; fg: phenolic deposits as fine granules; ph: phloem; pi: pith; sc: sclerenchyma; sd: phenolic deposits as scattered drops;

101   vb: vascular bundle; vc: vascular cambium; x: xylem; arrow: trichome; arrow head:

102   cuticle; asterisk: phenol deposits. Bars: 100 µm: j, l, m, n; 25 µm: a, b, f, g, h; 20 µm: c,

103   e, i; 10 µm: d, j1, k; 5 µm: c1

Table 1 Some abaxial and adaxial leaf-surface characteristics of field *Leucospermum cordifolium* 'Flame Spike' and *Leucospermum* 'Tango' plants, as compared to micro-propagated and acclimated plants. FP: plants from field, IP: *in vitro* plants, AP-P: acclimated plants-persistent leaves; AP-N: acclimated plants-new leaves, PL: pore length; PW: pore width; SI: stomatal index, SL: stomatal length; SW: stomatal width; TI: trichome index.

| | | *L. cordifolium* 'Flame Spike' | | | | | | *L.* 'Tango' | | | | | |
		SI	SL (μm)	SW (μm)	PL (μm)	PW (μm)	TI	SI	SL (μm)	SW (μm)	PL (μm)	PW (μm)	TI
Adaxial	FP	18.7[a]	24.6[a]	16.7[a]	14.7[a]	7.9[a]	8.9[a]	17.2[a]	21.2[a]	13.2[a]	10.8[a]	6.3[a]	8.1[a]
	IP	12.9[b]	34.1[b]	24.5[b]	17.9[b]	11.3[b]	9.0[b]	17.8[a]	32.2[b]	22.2[b]	12.3[b]	4.6[b]	6.2[b]
	AP-P	*	*	*	*	*	*	17.5[a]	26.0[d]	15.7[d]	13.8[c]	7.6[d]	9.8[d]
	AP-N	*	*	*	*	*	*	22.1[b]	22.4[d]	14.5[d]	12.3[b]	6.7[d]	14.2[d]
Abaxial	FP	16.5[a]	24.2[a]	16.9[a]	13.8[a]	8.2[a]	7.1[a]	16.3[a]	22.0[a]	14.1[a]	11.2[a]	6.7[a]	6.7[a]
	IP	13.7[b]	36.8[b]	24.1[b]	19.7[b]	10.8[b]	9.4[b]	17.5[a]	36.2[b]	21.8[b]	14.5[b]	4.5[b]	6.4[a]
	AP-P	*	*	*	*	*	*	16.4[a]	26.5[c]	16.5[c]	14.1[bc]	8.1[c]	7.7[a]
	AP-N	*	*	*	*	*	*	21.2[b]	23.1[a]	14.6[a]	13.2[c]	6.7[a]	11.7[b]

Values in each column followed by different letter are significantly different at α=0.05%.

* The low percentage of acclimated plants did not permit a study of their epidermal parameters.

Table 2. Leaves and stems cuticle thickness of field *Leucospermum cordifolium* 'Flame Spike' y *Leucospermum* 'Tango' plants, as compared to micro-propagated and acclimated plants. FP: plants from field, IP: *in vitro* plants, AP-Ps: acclimated plants-persistent stem; AP-Ns: acclimated plants-new stem.

			L. cordifolium 'Flame Spike'	*Leucospermum* 'Tango'
Leaves	Adaxial	FP	6.66^a	7,01^a
		IP	1.10^b	1.07^b
		AP-P	1.84^b	2.27^c
		AP-N	1.70^b	1.73^b
	Abaxial	FP	7.48^a	7.08^a
		IP	1.02^b	0.96^b
		AP-P	1.53^b	1.74^b
		AP-N	1.63^b	1.67^b
Stems		FP	6.90^a	3.07^a
		IP	1.10^b	1.40^b
		AP-P	2.27^c	2.47^c
		AP-N	2.03^c	3.28^d

Values in each column followed by different letter are significantly different at α=0.05%.