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Keywords: Adventitious roots; Anatomical study; Histology; Leucospermum; Micropropagation; Proteaceae; Rooting

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Abstract: In vitro rooting of two Leucospermum cultivars (L. cordifolium 'Flame Spike' and L. 'Tango') microcuttings was studied with different indole-3-butyric acid (IBA) treatments: A) including in a solid medium (0, 0.2, 1 and 2 mg L-1), B) by dipping the base of the shoots in 500-2500 mg L-1 for 5 s and C) including in a liquid medium (12.5 and 25 mg L-1) for 24-96h (induction step). After the treatments B and C, shoots were transferred to an auxin-free root elongation medium containing 20 g L-1 sucrose and solidified with 7g L-1 agar. The long-term presence of IBA in the culture-medium inhibited root development. The culture of shoots in a liquid medium with higher IBA concentrations and the application of IBA pulses at the base of the microcuttings proved to be suitable treatments to induce rhizogenesis. Culturing microcuttings in a growth-regulator free solid medium after IBA treatments (B and C) triggered root initiation and differentiation. The results show that the microcuttings cultured in a liquid medium with IBA had the highest percentages of rooting.

The sequence of anatomical changes during rooting was similar in vitro and ex vitro, the origin of the adventitious root in the vascular cambium. In vitro roots with an organized tissue system emerged from the microcutting stems 6 days after the root induction treatments. The acclimatized plantlets showed a suitable root system. Roots were formed ex vitro from the stem vascular cambium and connections were established between the stem vascular bundles and the roots, allowing root elongation. Some modifications were also observed in the basal rooting zone of stems in L. 'Tango'.

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Editors-in-Chief

23th April, 2018

Botany, Ecology and Plant Physiology, Section of Sciences

University of La Laguna, Canary Islands. Spain

Dear Editors-in-Chief

We have completed the revision of our manuscript (Ms. No. HORTI20504) entitled **"Structural and ultrastructural variations in *in vitro* and *ex vitro* rooting of microcuttings from two micropropagated *Leucospermum* (Proteaceae)"** in accordance with the reviewers suggestions. We have eliminated figures (11), modified the tables, added two references and modified text of the manuscript incorporating, among others, the corrections suggested by the reviewers. A Graphical Abstract have added too.

The comments to the Reviewers are listed below in bold lettering, embedded within the corresponding questions.

REVIEWER 1

1. Line 156, trichome is a leafy structure I think the authors mean root hair.

AUTHORS ANSWER: we have changed trichome by root hair.

2. Line 166, not permitted but achieved.

AUTHORS ANSWER: we have changed permitted by achieved.

3. The discussion on the microscopic observations is too long, needs to be shorter.

AUTHORS ANSWER: although discussion of the histological analysis may seem too long, we consider that the results obtained at this level are crucial to understand the ability of acclimatized plantlets to intake water and nutrients and to endure stress under *ex vitro* conditions. Reviewer 2 does not seem to have any problem with this section long.

REVIEWER 2

1. ABSTRACT: I recommend briefly explaining the different protocols and concentration of IBA used in this experiment, and the final result.

AUTHORS ANSWER: we have explained the different protocols and concentrations of IBA used and the final result.

2. MATERIAL AND METHODS: The two protocols used (solid and liquid media) are not well explained, nor are the different concentrations of IBA applied in each one of them.

AUTHORS ANSWER: we have explained the different protocols and the concentrations of IBA applied in each one of them.

3. Table 1: It is not explained in the table if these concentrations have been used in solid or liquid medium.

AUTHORS ANSWER: we added the explanation of the concentrations and media used in table 1.

4. Table 2: The table is not correctly explained in the Material and Method section

AUTHORS ANSWER: we consider that the changes made in Material and Methods explain now the table 2.

5. Figures: there have too many tedious figures/pictures and some are not professional

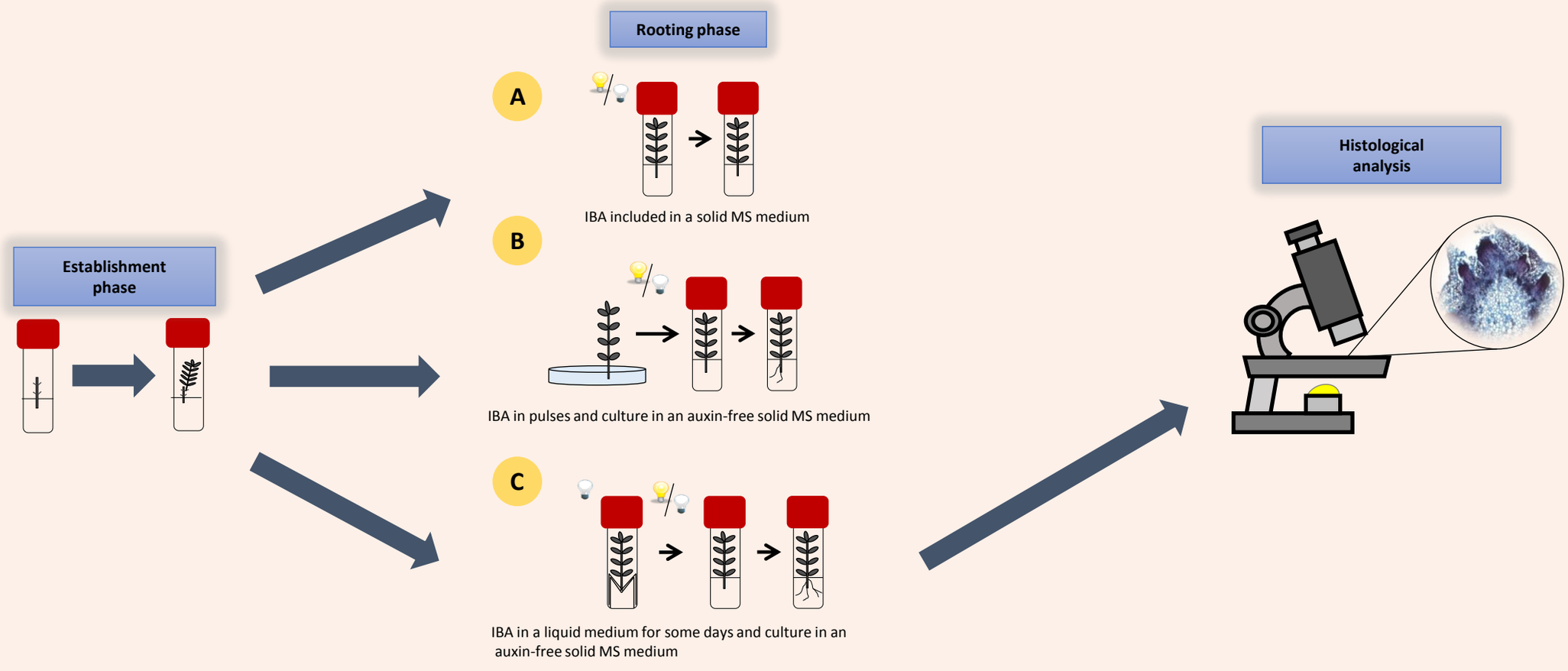
AUTHORS ANSWER: we have eliminated some figures, keeping only the import and informative ones.

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

Sincerely yours,

Emma Suárez

Graphical Abstract



Highlights

- Micropropagation is an alternative to conventional methods of plant propagation.
- Culturing microcuttings in a medium with IBA was necessary to induce rhizogenesis.
- The subculture in a growth-regulator free medium after IBA triggered rooting.
- The origin of the adventitious roots was located in the vascular cambium.

Structural and ultrastructural variations in *in vitro* and *ex vitro* rooting of microcuttings from two micropropagated *Leucospermum* (Proteaceae).

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ABSTRACT

In vitro rooting of two *Leucospermum* cultivars (*L. cordifolium* 'Flame Spike' and *L.* 'Tango') microcuttings was studied with different indole-3-butyric acid (IBA) treatments: A) including in a solid medium (0, 0.2, 1 and 2 mg L^{-1}), B) by dipping the base of the shoots in 500-2500 mg L^{-1} for 5 s and C) including in a liquid medium (12.5 and 25 mg L^{-1}) for 24-96h (induction step). After the treatments B and C, shoots were transferred to an auxin-free root elongation medium containing 20 g L^{-1} sucrose and solidified with 7g L^{-1} agar. The long-term presence of IBA in the culture-medium inhibited root development. The culture of shoots in a liquid medium with higher IBA concentrations and the application of IBA pulses at the base of the microcuttings proved to be suitable treatments to induce rhizogenesis. Culturing microcuttings in a growth-regulator free solid medium after IBA treatments (B and C) triggered root initiation and differentiation. The results show that the microcuttings cultured in a liquid medium with IBA had the highest percentages of rooting.

The sequence of anatomical changes during rooting was similar *in vitro* and *ex vitro*, the origin of the adventitious root in the vascular cambium. *In vitro* roots with an organized tissue system emerged from the microcutting stems 6 days after the root induction treatments. The acclimatized plantlets showed a suitable root system. Roots were formed *ex vitro* from the stem vascular cambium and connections were established between the stem vascular bundles and the roots, allowing root elongation. Some modifications were also observed in the basal rooting zone of stems in *L.* 'Tango'.

Keywords: Adventitious roots; Anatomical study; Histology; *Leucospermum*; Micropropagation; Proteaceae; Rooting.

1. Introduction

Proteaceae comprises about 80 genera with about 1700 species, distributed over the temperate zones of the three southern-hemisphere continents (Australia, Africa and South America) that were connected as Gondwanaland 300 million years ago (Criley, 1998). *Leucospermum* is one of the best known genera of the family. It consists of 48 species of South African shrubs (Rourke, 1972), highly appreciated for their flower heads with long styles, each thickened at the apex to form the pollen presenter. Indeed, their striking appearance makes them commercially important as cut flowers, although Proteaceae also includes some genera with importance as food and the potential of proteas in the pharmaceutical industry has recently been suggested (León et al., 2014).

The cut flower market has expanded in recent decades. Its turnover in the Netherlands was more than € 4 billion (US$ 6.5 billion) in 2010 (Kras, 2011). Due to its importance in the cut flower industry, culture of proteas, especially some *Protea*, *Leucospermum*, *Leucadendron*, *Banksia* (Sedgley, 1998) and *Grevillea* (Joyce et al., 1997) have been distributed over diverse zones like South Africa, Zimbabwe, Mozambique, the USA (southern California and Hawaii), Australia, New Zealand, Israel, Ecuador, Chile, Colombia, Peru, southeast Portugal, Madeira, the Azores, southwest Spain and the Canary Islands. Certainly, the development of this commerce and expansion of its distribution areas have stimulated the search for alternative production systems to conventional cuttings or grafts. In addition, transporting plant material to the different cultivation areas requires plant-health guarantees, which in most cases are not fulfilled, leading to scarce availability. Micropropagation would ensure the health of the material distributed and mass production. *In vitro* culture techniques have been successfully used for micropropagation of many Proteaceae species and cultivars with ornamental interest: *Banksia* (Reuveni et al., 2003), *Grevillea* (Ben-Jaacov & Dax, 1981; Bunn & Dixon, 1992a; Touchell et al., 1992), *Leucadendron* (Pérez-Francés et al., 2001a; Dias Ferreira

et al., 2003; Suárez et al., 2010), *Leucospermum* (Ben-Jaacov & Jacob, 1986; Kunisaki, 1989; Rugge et al., 1989; Tal et al., 1992a, 1992b; Thillerot et al., 2006; Pérez-Francés et al., 2001b), *Protea* (Van Staden et al., 1981; Watad et al., 1992a; Rugge, 1995; Wu & du Toit, 2004; Wu et al., 2007; Wu & du Toit, 2012a, 2012b; Wu & Lin, 2013), *Telopea* (Offord & Campbell, 1992; Offord et al., 1992). Although, as mentioned above, many *Leucospermum* have been successfully propagated *in vitro*, rooting can be difficult and normally microcuttings are rooted in response to a specific plant growth regulator treatment.

From the anatomical point of view, several authors (Vieitez et al., 1981; Moncousin & Gaspar, 1983; Samartin et al., 1986; San-José et al., 1992; Gonçalves et al., 1998; Scaltosyiannes et al., 1998; Ballester et al., 1999; Metaxas et al., 2004; Hatzilazarou et al., 2006; Naija et al., 2008; Millán-Orozco et al., 2011) have recognized three successive stages in the rooting process. These were called induction, initiation and differentiation by Jásik & De Klerk (1997). In *in vitro* plants, analysis of the sequence of histological changes occurring during the rooting process is very useful to understand the ability of these plants to intake water and nutrients, and to endure stress under *ex vitro* conditions.

The present study assesses the effect of different IBA treatments on *in vitro* rooting of *L. cordifolium* 'Flame Spike' and *L.* 'Tango'. Furthermore, the morphological and ultrastuctural studies show the sequence of events occurring in the microcuttings associated with root induction, initiation and differentiation to: 1. identify the tissue which originates the roots, and 2. study the connections between stem and new root tissues on which the plants' survival depends after transplantation.

2. Material and methods

2.1. Plant material

105 Multinodal microcuttings of *Leucospermum cordifolium* 'Flame Spike' (a clone of *L.*
106 *cordifolium*) and *Leucospermum* 'Tango' (*L. glabrum x L. lineare*) were obtained from
107 the experimental fields belonging to the Higher Polytechnic School of Engineering,
108 University of La Laguna, Canary Islands, Spain. Microcuttings were multiplied and
109 maintained on a medium (Murashige & Skoog, 1962) supplemented with 20 g L^{-1}
110 sucrose, 150 g L^{-1} ascorbic acid and solidified with 7g L^{-1} agar (Sigma, plant cell culture
111 tested).

112 2.2. Adventitious rooting and *ex vitro* acclimatization

113 *In vitro* rooting was studied with different indole-3-butyric acid (IBA) treatments:
114 A) including in a solid medium (0, 0.2, 1 and 2 mg L^{-1}), B) by dipping the base of the
115 shoots in 500-2500 mg L^{-1} for 5 s and C) including in a liquid medium (12.5 and 25 mg
116 L^{-1}) for 24-96h (induction step) After the treatments B and C, shoots were transferred to
117 an auxin-free root elongation medium containing 20 g L^{-1} sucrose and solidified with 7g
118 L^{-1} agar. Cultures were incubated in a growth chamber at 24±2°C under Phillips
119 fluorescent daylight tubes (110 µmol m^{-2} s^{-1}) for 16 h.
120 On the 14th day, rooted plants were removed from the culture tube, washed in water and
121 planted in plastic pots filled with a perlite:quartz-sand:peat mixture (3:2:1). The plants
122 were placed in a controlled incubation chamber for 50 days. Relative humidity was
123 gradually reduced from 95% to 60%, while light irradiance was increased from 110
124 µmol m^{-2} s^{-1} to 160 µmol m^{-2} s^{-1} during the acclimatization period.
125 All the experiments were replicated three times with 24 culture tubes each. The rooting
126 experiment results were statistically analysed ($p < 0.05$) individually for each auxin
127 treatment by analysis of variance (ANOVA), followed by Duncan's test (software SPSS
128 for Windows, Version 19.0).

129 2.3. Morphological and ultrastructural studies

During *in vitro* incubation for rooting, the basal 2-4 mm of microcuttings were sampled for histological examination 0, 2, 3, 6, 9 and 12 days after induction treatment with 12.5 mg L^{-1} IBA for 48 h. Samples (2-4 mm) from the acclimatization phase were excised 30 days after transplantation. Sections were processed for light microscopy and transmission electron microscopy using standard protocols. Light microscopy (LM): samples were fixed in FAA solution (formaldehyde 90%, absolute ethanol 5%, acetic acid 5%), for 48 h, transferred to 70% ethanol, dehydrated in an ethanol series and embedded in paraplast-plus. Serial 20 µm thick transverse sections were cut with a rotatory Minot microtome, mounted on slides and stained with safranine and fast green (Johansen, 1940). Semi-thin sections (1 µm) of resin-embedded material were obtained with glass knives and stained with toluidine blue. Observations were made with an LM Leica DM4000B using a Leica QWin computer image apprehension system.

Transmission electron microscopy (TEM): stem segment samples (2-4 mm) were fixed for 2 h in 3% glutaraldehyde and postfixed 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 resin (Spurr, 1969). Semi-thin (0.5-1 µm) and ultra-thin (70 nm) sections were cut using a Reichert-Jung ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were studied using a JEOL JEM-1010 microscope (Electron Microscopy Center, Madrid Complutense University, MCU).

3. Results

3.1. *In vitro* rooting

No root development was observed in either cultivar when IBA was added to the solid medium. However, when the application was via IBA pulses the plants responded presented differently. Thus, in *L. cordifolium* ‘Flame Spike’ microcuttings the highest rooting percentage and number of roots per shoot after application of the IBA pulses

(Table 1) were achieved with 2000 mg L^{-1} IBA (81.2% and 4.7 roots/shoot). Although the rooting percentages were high, the number of roots per shoot was low in all IBA pulses. However, in *L.* 'Tango' (Table 1) both parameters increased with increasing IBA concentration and were optimal, at 2500 mg L^{-1} (50% and 3.23 roots/shoot). No rooting was observed when lower IBA concentrations (less than 1500 mg L^{-1} IBA) were used. In both cultivars, roots showed normal development and appeared frequently over the medium, displaying many root hairs in this zone.

No callus or roots were observed when microcuttings were cultured in liquid medium for 24-96 h (induction medium), before culturing them in a solid medium free of growth regulators. When pulses were applied at the base of the microcuttings, roots began to emerge on day 7 in all treatments. All microcuttings rooted in *L. cordifolium* 'Flame Spike' in the treatments with 25 mg L^{-1} IBA over 48 h (Table 2), although no significant differences were observed when 12.5 mg L^{-1} IBA were used for 48-72 h. The highest number of roots per shoot was achieved when the treatment was 72 h long. In *L.* 'Tango' (Table 2), 100% rooting was achieved over 48 h, independently of the IBA treatment. The highest number of roots per shoot was reached in the same treatment. All the other combinations achieved a minimum of 70% rooting. Figure 1 shows the root development in both cultivars when microcuttings were treated with 12.5 mg L^{-1} IBA for 48 h (Figs. 1a-d) and the new roots obtained during the acclimatization phase (Figs. 1e-h).

3.2. Histological analysis

The processes of initiation and development of adventitious roots were asynchronic.

On day 0: before IBA treatment, transverse shoot sections showed the typical normal organization of *L. cordifolium* 'Flame Spike' and *L.* 'Tango' stem anatomy; simple epidermis, cortex with variable layers of cells, collateral vascular bundles with vascular

cambium visible between xylem and phloem elements, and wide pith (Figs. 2a, 2f). Days 2-3: after IBA treatment, high mitotic activity with periclinal divisions was observed in the cambial zone (Figs. 2b, 2bi). Division increased and small groups of densely-stained dedifferentiated cells appeared, with a prominent central nucleus and small vacuoles (Figs. 2c, 2g). Meristemoids were localized mostly between phloem and cambial cells (Fig. 2c), but also in the interfascicular parenchyma. Meristemoids showed no overall polarity at this stage. Day 6: meristemoid cell division became polarized, differentiated into typically dome-shaped root primordia (Figs. 2d, 2g, 2h). The most differentiated primordia showed parenchyma and vascular bundles with tracheary elements. The first adventitious roots had grown through the cortex and epidermis and were visible at the surface (Fig. 2d). Connection between the vascular system of shoot and root was generally complete before root emergence (Fig. 2d). Days 9-12: most of the primordia emerged at the surface of the basal stem, showing parenchyma and tracheary elements and further divisions at the distal end of the apical meristem gave rise to root caps (Fig. 2e).

Transverse sections of adventitious roots showed a unilayer epidermis and cortical parenchyma made up of isodiametric cells with scarce intercellular spaces (Figs. 3a, 4a). The vascular cylinders consisted of poorly develop vascular bundles surrounding the pith, delimited by the endodermis presenting a weakly defined Casparian strip and a pericycle of uniseriate parenchyma cells (Figs. 3b, 4b). The phloem was located near the periphery of the vascular cylinder and the metaxylem closed to the centre (Figs. 3b, 4b). Parenchyma cells associated with the vascular bundles contained phenolic deposits in the form of droplets in the vacuoles (Figs. 3b, 4b). Some of the adventitious roots had begun to develop secondary roots (4c-d).

Longitudinal section of adventitious roots revealed an apical meristem formed by cells with a euchromatic nucleus and poorly-defined nuclear membrane, large nucleolus and low electron-density cytoplasm with small vacuoles (Figs. 3c-3d). The apices were protected by a cap of large cells with euchromatic nuclei containing nucleolus, mitochondria, rough endoplasmic reticulum and numerous starch deposits (Figs. 3c, 3e, 3f).

After acclimatization, plants of both cultivars showed the same tissue organization in the stem as *in vitro* plants (Fig. 5a). In the case of rooting, they display meristemoid formation in the vascular cambium and radicular primordia breaking through the epidermis and emerging outside (Fig. 5b). However in *L.* 'Tango', other alterations were visible in the structure of the basal zone of the stem (Fig. 6). Firstly, the epidermis and parenchymal cortex were transformed into a peridermis consisting of a variable number of layers of different-sized cells, the outermost densely-dyed cells being mostly detached (Figs. 6a-a1). Absence of perivascular sclerenchyma fibres and collateral vascular bundles that have become notably elongated (Figs. 6b-c). Parenchyma containing different-sized cells and phenolic droplets was situated centripetal to xylem (Fig. 6c). In the innermost layer of this parenchyma there were smaller collateral vascular bundles. The central pith featured large cells, few intercellular spaces and phenol deposits as droplets that in some cases occupied most of the cell lumen (Fig. 6c). Groups of meristemoid-like cells and new groups of xylem cells with circular distribution were also observed in the pith (Fig. 6d).

4. Discussion

4.1 *In vitro* rooting

The size of microcuttings was a very important factor affecting development of the radicular system. Preliminary assays showed that microcuttings under 25-30 mm did not

229 form roots and died within a few weeks, due to tissue necrosis caused by the oxidation
230 of phenolic compounds.
231 This phase was necessary in both cultivars, because no roots appeared in previous
232 phases as occurs in herbaceous species in particular. Many authors report the need for
233 an auxin treatment for rooting different members of the Proteaceae both *in vitro* (Gorst
234 et al., 1978; Ben-Jaacov & Dax, 1981; Williams et al., 1985; Kunisaki, 1989, 1990;
235 Offord et al., 1990; Tal et al., 1992b; Watad et al., 1992b; Leonardi et al., 2001; Pérez-
236 Francés et al., 2001b; Thillerot et al., 2006; Evenor & Reuveni, 2008; Bunn et al., 2010;
237 Olate et al., 2010; Suárez et al., 2010), and *ex vitro* (Offord et al., 1990; Bunn & Dixon,
238 1992a, 1992b; Tal et al., 1992b; Reynoso-Castillo et al., 2001; Croxford et al., 2006).
239 Only Watad et al. (1992b) obtained adventitious roots in *Grevillea* without the
240 application of exogenous auxins *in vitro*, but only in a low percentage (38%).
241 The absence of root development in the media with IBA (0.2, 1 and 2 mg L^{-1}) in both
242 cultivars may be due to exogenous auxin treatment being necessary for the rooting, as in
243 many species. However, in certain cases root development depends on elimination of
244 auxin from the medium. This was observed by Kunisaki (1989) and Olate et al. (2010)
245 in species of *Leucospermum* when they had added IBA to the medium. A reason for this
246 inhibition is the development of a callus at the basal end of microcuttings, caused by
247 continued presence of auxin in the culture medium. This callus normally hinders rooting
248 both *in vitro* and *ex vitro*, although sometimes it is essential for it. Prolonged exposure
249 to auxin can endanger the microcuttings and their radicular development. To resolve
250 this problem a concentrated auxin solution can be applied at the base of the
251 microcuttings for a short time, before culturing them in a medium without growth
252 regulators. This method has proved efficient for the rooting of different members of
253 Proteaceae (Seelye, 1984; Seelye et al., 1986; Kunisaki, 1989; Reynoso-Castillo et al.,

2001; Croxford et al., 2006) and for the rooting of other woody plants as *Quercus lusitanica* (San José et al. 2017). However, in 'Flame Spike' and 'Tango' the highest percentages were obtained when microcuttings were cultivated in a medium with high IBA concentrations for 24-96 h before culturing them in a growth-regulator free medium. This method was used efficiently in *Leucadendron conocarpodendron x L. cuneiforme* 'Hawaii Gold' (Kunisaki, 1989), although similar results were obtained when the auxin exposure period was reduced to 10 min and the concentration increased (Kunisaki, 1990). In other woody species, like *Quercus lusitanica* (San José et al. 2017) and *Quercus ilex* (Martínez et al. 2017) the culture of explants in a medium with IBA for some days before its transferred to an auxin-free medium improved the rooting percentages.

Therefore, the results obtained in 'Flame Spike' and 'Tango' show that exogenous auxin is necessary for root initiation, but for later development of root primordia it must be eliminated from the medium.

On the other hand, the use of a liquid induction medium (Kunisaki, 1989, 1999) can increase the contact between explants and medium, improving auxin and nutrient availability. Later culture on solid medium avoids hyperhydricity symptoms during the initiation and differentiation phases. However, many authors (Kadota & Niimi, 2004; Lucchesini & Mensuali-Soli, 2004; Pati et al., 2006; Pati et al., 2011; Noodezh et al., 2012) have reported that plants rooted easier in liquid medium, with longer roots than those grown on solid medium. This could be due to the relatively poor aeration in the agar-gelled media. Ben-Jaacov & Dax (1981) employed liquid medium with filter paper bridges for *in vitro* rooting of *Grevillea rosmarinifolia* and observed a rooting improvement due to better aeration. Similarly, Offord et al. (1990) used gelled and liquid media sustained with quartz sand and filter paper bridges for *in vitro* rooting of

279 *Telopea speciosissima*. Rooting occurred in all conditions, but plants died during
280 acclimatization. Keeping microcuttings in the dark during the induction phase is
281 generally favourable to rooting (George et al., 2008), since the slower metabolization of
282 endogenous and exogenous auxins in darkness increases peroxidase activity (George et
283 al., 2008). Possibly, when the microcuttings are returned to the light, rapid auxin
284 degradation by the peroxidase improves rooting. It is important to adapt the length of
285 the dark period to the species or cultivar; if too long, leaf yellowing will reduce the
286 survival availability of *ex vitro* plants.

287 4.2. Histological analysis

288 Although the rooting phases proposed by Jásik & De Klerk (1997) are clearly
289 distinguishable in both cultivars, the process was asynchronous, since meristems,
290 radicular primordia and roots were observed in the same cross-section of stem at the
291 same time.

292 Plants treated with IBA 12.5 mg L^{-1} showed the first changes in the basal stem 2-3 days
293 after treatment, consisting of high mitotic activity in the cambial zone. Thus, the
294 cambium, which normally functions as a secondary meristem, acquires the functional
295 characters of a primary meristem: the apical root meristem (Favre & Médard, 1969).
296 This induction phase, identified as such by Jásik & De Klerk (1997), was characterized
297 in apple plants by increased mitotic activity in the cambial cells. However the region of
298 the tissue where cells become activated is thought to depend in part on the presence of
299 competent cells able to respond, and also on the culture medium components (Naija et
300 al., 2008).

301 Later, increased division originates small groups of undifferentiated cells with
302 prominent nuclei and strongly-stained cytoplasm, beginning the initiation phase (Jásik
303 & De Klerk, 1997). These meristemoids appeared six days after the IBA treatment. Cell

304 division then became polarized, originating radicular primordia with a characteristic
305 acuminate shape. The differentiation phase and excrescence began at this point (Jásik &
306 De Klerk, 1997), after which internal organization and emergence ensued. The
307 parenchymal tissue and vascular bundles were developed during primordia formation,
308 while during excrescence the primordia broke through the cortical parenchyma and the
309 epidermis before emerging.
310 In many species, especially in woody plants, a continuous sclerenchyma ring outside the
311 root origin possibly constitutes an anatomical barrier to root emergence (Edwards &
312 Thomas, 1980). In this sense, 'Flame Spike' and 'Tango' stems lacked a continuous
313 sclerenchyma ring since the *in vitro* plants of both cultivars only had small groups of
314 sclerenchyma fibres with poor thickening walls.
315 Although the duration of the root development phases can vary according to species and
316 culture conditions (Moncousin & Gaspar, 1983; Scaltsoyiannes et al., 1998; Metaxas et
317 al., 2004) the sequential anatomical changes observed in 'Flame Spike' and 'Tango'
318 were similar to those described in apple and oak (San-José et al., 1992), chestnut
319 (Vieitez et al., 1981; Gonçalves et al., 1998; Ballester et al., 1999), camellia (Samartin
320 et al., 1986), gardenia (Hatzilazarou et al., 2006), Cuban cedar - *Cedrela odorata*
321 (Millán-Orozco et al., 2011) and apple (Naija et al., 2008).
322 The changes noted in the basal stem of acclimatized *L*. 'Tango' plants could be an effect
323 of the IBA treatment, indeed Govindan & Kathiresan (2014) observed disorganization
324 in the xylem elements and changes in its orientation during the rooting of *Avicennia*
325 *officinalis* shoots treated with auxins.
326 **5. Conclusions**
327 Our study presents a rapid and efficient method for *in vitro* rooting of two
328 *Leucospermum* cultivars and provides evidence, at histological level, about the quality

of the root system. Exogenous IBA was necessary for root initiation, but for later development of root primordia it had to be eliminated from the medium. The origin of adventitious roots in both rooting conditions was located in the cambial zone. These new roots showed functional anatomical traits. The modifications occurred in 'Tango' stem were a consequence of the IBA rooting treatment.

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Table 1. Rooting percentage (%) and number of roots per microshoots obtained in *Leucospermum* 'Flame Spike' and *Leucospermum* 'Tango' after 28 days in culture in a solid medium with IBA (0, 0.2, 1 and 2 mg L^{-1}) or after dipping the base of the shoots in 500-2500 mg L^{-1} IBA for 5 s before its transferred to an auxin-free medium.

		L. cordifolium 'Flame Spike'		*L.* 'Tango'	
IBA (mg L^{-1})	**Pulse**	**% rooting**	**Roots/explant**	**% rooting**	**Roots/explant**
0	--	0	--	0	--
0.2	--	0	--	0	--
1	--	0	--	0	--
2	--	0	--	0	--
500	5 s	60.0^{ab}	3.7^{a}	0	--
1000	5 s	0	--	0	--
1500	5 s	43.8^{ab}	2.1^{a}	14.7^{a}	1.5^{a}
2000	5 s	81.2^{b}	4.7^{a}	20.0^{ab}	1.6^{a}
2500	5 s	75.0^{ab}	4.1^{a}	50.0^{b}	3.2^{a}

Means followed by the same letter in a column are not significantly different at α=0.05

Table 2. Rooting percentage (%) and number of roots per microshoots obtained in *Leucospermum* 'Flame Spike' and *Leucospermum* 'Tango'. Microshoots were cultured in a liquid medium (12.5 and 25 mg L-1) for 24-96 h before its transferred to an auxin-free medium.

	IBA (mg L^{-1})	**24h**		**48h**		**72h**		**96h**	
		%	**Nº**	**%**	**Nº**	**%**	**Nº**	**%**	**Nº**
'Flame Spike'	12.5	50.0^{a}	8.6^{a}	94.0^{a}	10.5^{a}	83.3^{a}	14.6^{a}	50.0^{a}	5.5^{a}
	25	91.6^{b}	15.4^{a}	100.0^{a}	12.9^{a}	100.0^{a}	17.7^{a}	100.0^{b}	13.2^{b}
'Tango'	12.5	81.8^{a}	14.0^{a}	100.0^{a}	14.2^{a}	70.0^{a}	8.2^{a}	91.6^{a}	11.8^{a}
	25	72.0^{a}	11.1^{a}	100.0^{a}	15.2^{a}	87.5^{a}	12.6^{a}	75.0^{a}	10.2^{a}

Means followed by the same letter in a column are significantly different at α=0.05

Figures

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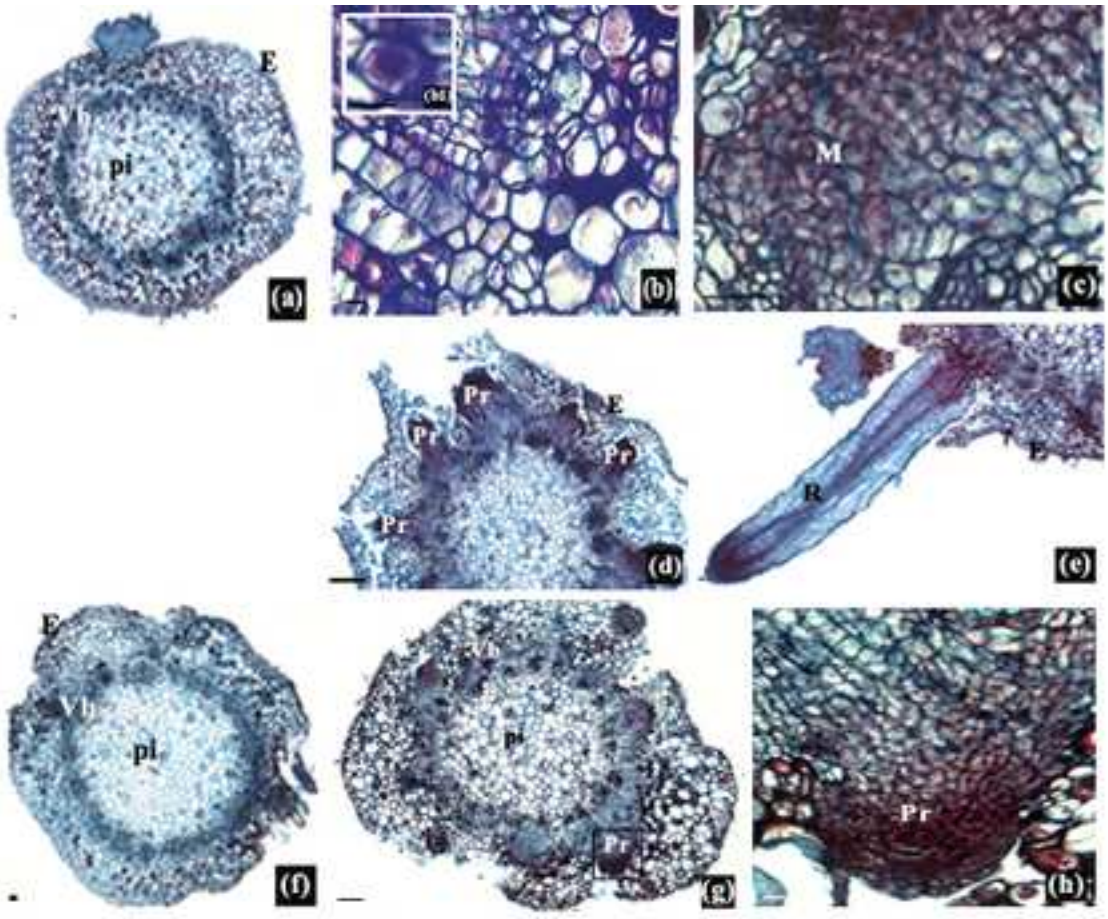

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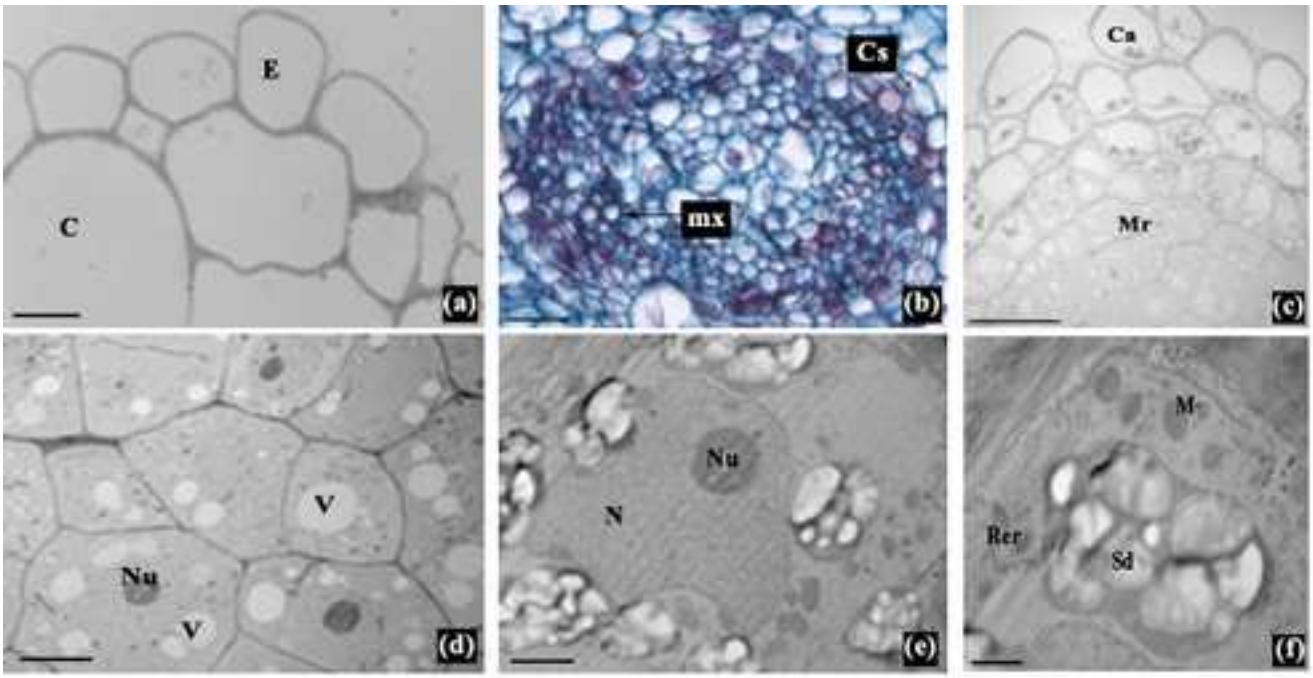

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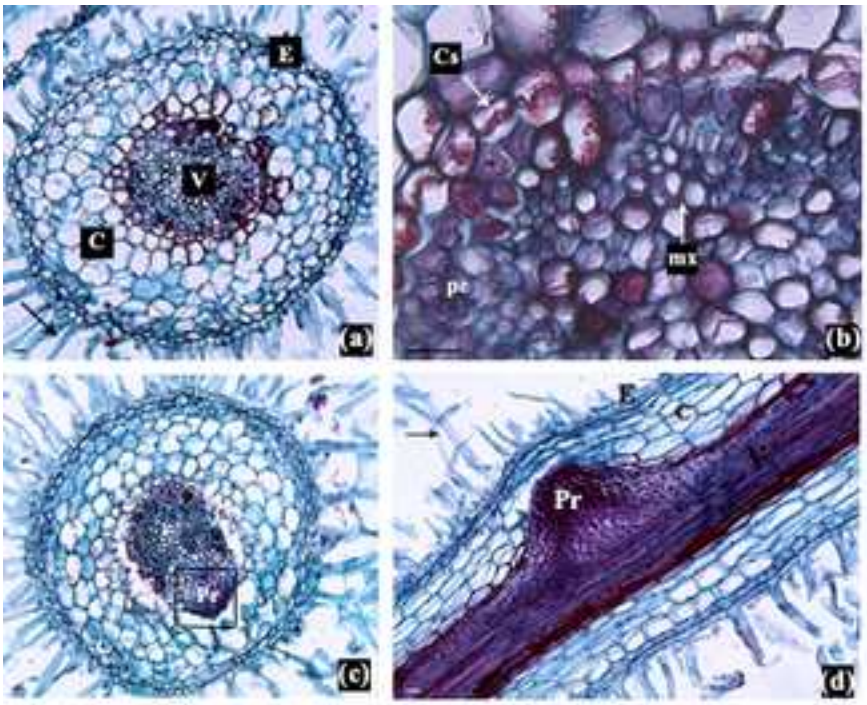

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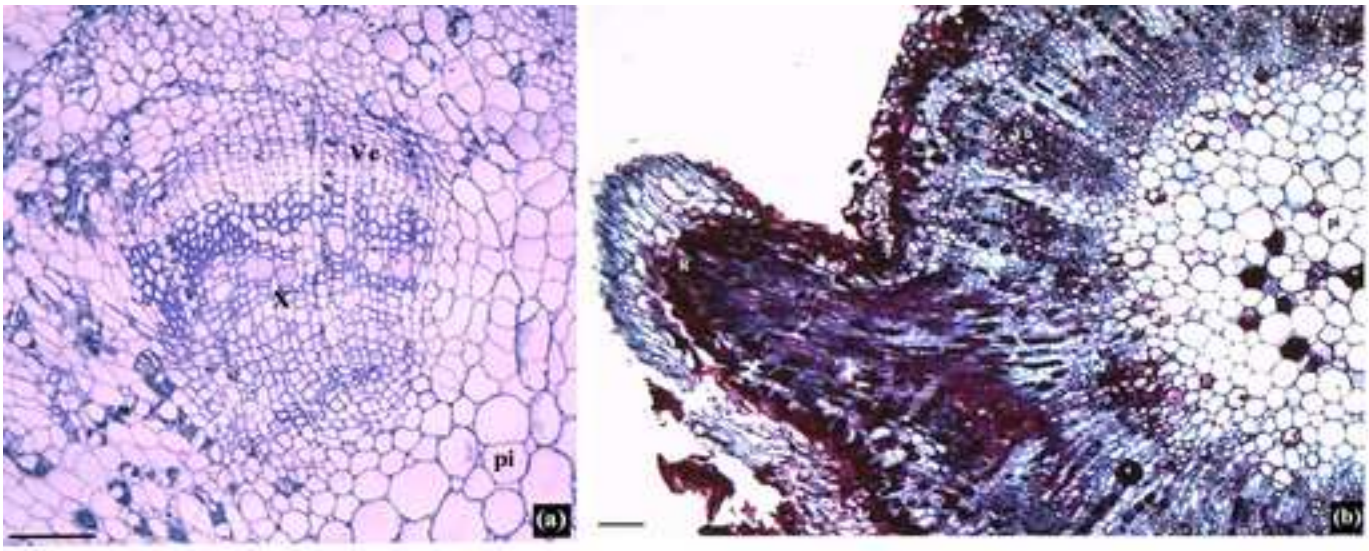

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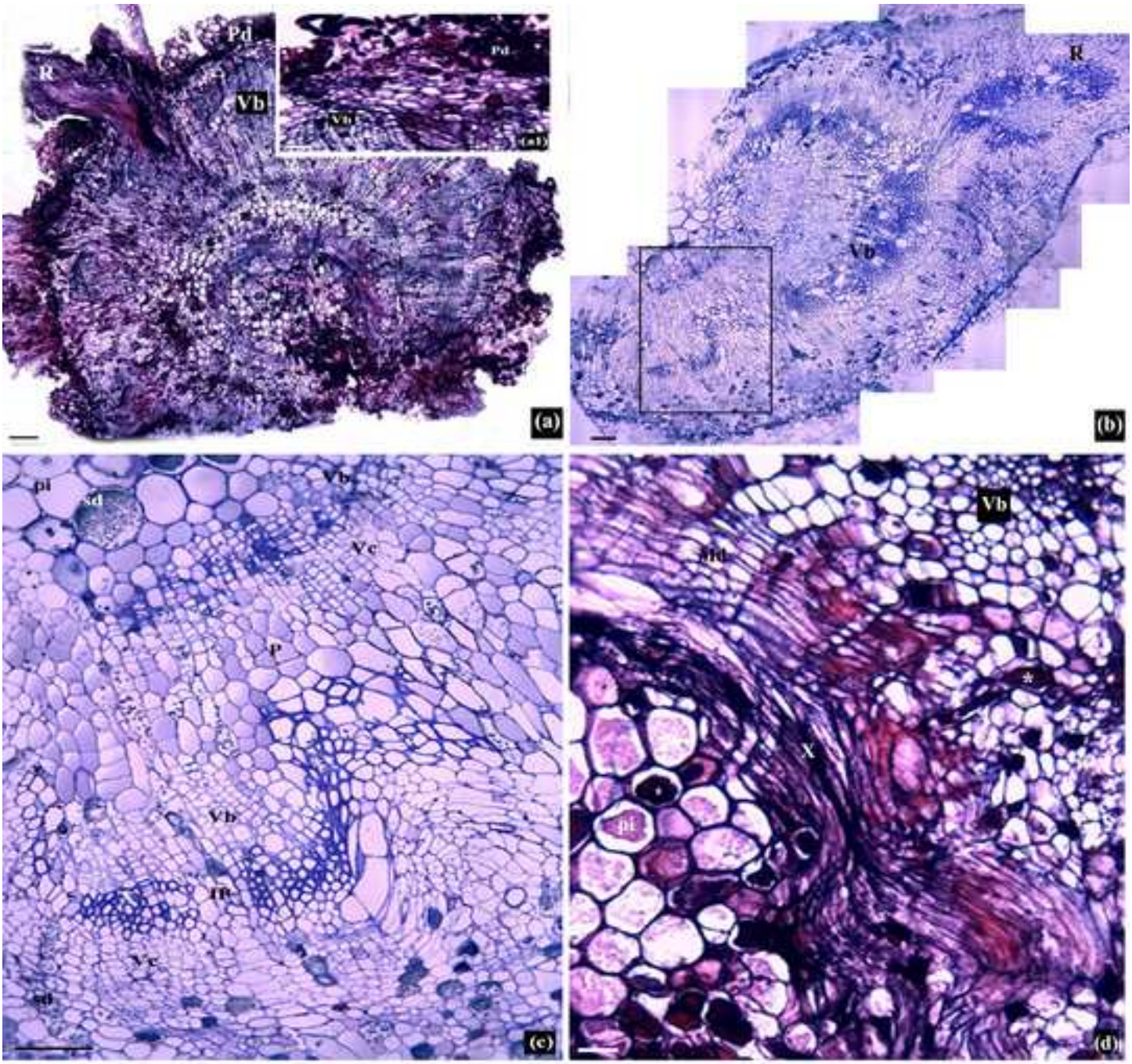

Figure captions

Fig. 1. *In vitro* rooted micropropagated *Leucospermum cordifolium* 'Flame Spike' (a-b) and *L.* 'Tango' (c-d) plants in a ½ MS medium with 12.5 mg L^{-1} IBA for 48h and incubated in darkness before their culture in a ½ MS without growth regulators and plants of *L. cordifolium* 'Flame Spike (e-f) and *L.* 'Tango' plants (g-h) rooted *in vitro*, transplanted in a mixture of perlite:quartz sand:peat and acclimatized during 50 days in a growth chamber.

Fig. 2. Anatomical changes during root induction on *Leucospermum cordifolium* 'Flame Spike' microshoots. (a) Transversal section of control stem. (b-b1) First mitotic divisions in the cambial zone. (c) Meristemoid. (d) Radicular primordia initiates at vascular cambium and crossing the cortical parenchyma to emerge at the surface. (e) Panoramic view of an adventitious root emerging to the surface, and *Leucospermum* 'Tango'. (f) Transversal section of control stem. (g) Many radicular primordia of different sizes initiate at the vascular cambium and crossing the cortical parenchyma. (h) Detail of a radicular primordium breaking the epidermis to emerge at the surface. E: epidermis; Vb: vascular bundles; M: meristemoid; pi: pith; Pr: radicular primordium; R: root. Bars: 25 µm: a, b1-h; 10 µm: b.

Fig. 3. Light and transmission electron microscopy images of *in vitro* rooting of *Leucospermum cordifolium* 'Flame Spike'. (a) Transversal section of *in vitro* rooted stem. Detail of unilayer epidermal and cortex with isodiametric cells. (b) Vascular cylinder surrounding by the endodermis with Caspary strip. (c) Radicular meristem protected by the caliptre. (d) Meristematic cells. (e-f) Details of a caliptre cell. C: cortex; Ca: caliptre cells; Cs: Caspary strip; E: epidermis; M: mitochondria; Mr: radicular meristem; mx; metaxylem; N: nucleus; Nu: nucleolus; Rer: rough endoplasmic reticulum; Sd: starch deposits; V: vacuole. Bars: 25 µm: b; 20 µm: c; 10 µm: a; 5 µm: d; 2 µm: e; 1 µm: f.

Fig. 4. Light microscopy images of *in vitro* roots of *Leucospermum* 'Tango' in transversal sections (a-c) and longitudinal section (d). (a) Unilayer epidermis and cortex with isodiametric cells. (b) Vascular cylinder surrounding by the endodermis with Caspary strip. (c-d) Primary root showed the initiation of a radicular primordium in the pericicle cells and detail of the tracheary elements. C: cortex; Cs: Caspary strip; E: epidermis; en: endodermis; mx: metaxylem; pe: pericicle; Pr: radicular primordium; V: vascular cylinder; arrow: root hairs. Bars: 100 µm: a; 25 µm: b-d.

Fig. 5. Light microscopy images of *ex vitro* rooting of *Leucospermum cordifolium* 'Flame Spike'. (a) Transversal section of a stem vascular bundle in the rooting zone prior to root development. (b) Overview of an adventitious root developed *ex vitro* breaking the epidermis and emerging to the outside. Note the abundance of phenolic content in the root formation zone. pi: pith; R: root; Vb: vascular bundles; Vc: vascular cambium; X: xylem; asterisk: phenolic deposits. Bars: 100 µm: a-b.

Fig. 6. Light microscopy images of *ex vitro* rooting of *Leucospermum* 'Tango'. (a) Transversal section of a rooting zone of a stem stained with safranine-fast green. (a1) Detail of the framed region of Fig. 6a. Severely stained cells of the peridermis. (b) View of the stem with an adventitious root. (c) Detail of the region framed in Fig. 6b. Long vascular bundles and increase in the number of the interphascicular parenchyma layers. (d) Detail of the region framed in Fig. 6a. Group of xylem cells in longitudinal view with circular distribution and presence of meristemoids. Md: meristemoid; IP: interfascicular parenchyma; P: parenchyma; Pd: periderm; pi: pith; R: root; sd: phenolic deposits as scattered droplets; Vb: vascular bundles; Vc: vascular cambium; X: xylem; asterisk: phenolic deposits. Bars: 100 µm: a-a1, b-d.