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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 24–96 h (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 7 g 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'.

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 and Dax, 1981; Bunn and Dixon, 1992a; Touchell et al., 1992), *Leucadendron* (Pérez-Francés et al., 2001a; Dias Ferreira et al., 2003;

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Suárez et al., 2010), *Leucospermum* (Ben-Jaacov and Jacobs, 1986; Kunisaki, 1989; Rugge et al., 1989; Tal et al., 1992a,b; Thillerot et al., 2006; Pérez-Francés et al., 2001b), *Protea* (Van Staden et al., 1981; Watad et al., 1992a; Rugge, 1995; Wu and du Toit, 2004; Wu et al., 2007; Wu and du Toit, 2012a,b; Wu and Lin, 2013), *Telopea* (Offord and 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 and Gaspar, 1983; Samartin et al., 1986; San-José et al., 1992; Gonçalves et al., 1998; Scalsoyiannes et al., 1998; Ballester et al., 1999; Metaxas et al., 2004; Hatzilazarou et al., 2006; Naija et al., 2008; Millan-Orozco et al., 2011) have recognized three successive stages in the rooting process. These were called induction, initiation and differentiation by Jásik and 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

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

2.2. Adventitious rooting and ex vitro acclimatization

In vitro rooting 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 24–96 h (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 7 g L^{-1} agar. Cultures were incubated in a growth chamber at 24 ± 2 °C under Phillips fluorescent daylight tubes (110 µmol m^{-2} s^{-1}) for 16 h.

On the 14th day, rooted plants were removed from the culture tube, washed in water and planted in plastic pots filled with a perlite:quartz-sand:peat mixture (3:2:1). The plants were placed in a controlled incubation chamber for 50 days. Relative humidity was gradually reduced from 95% to 60%, while light irradiance was increased from 110 µmol m^{-2} s^{-1} to 160 µmol m^{-2} s^{-1} during the acclimatization period.

All the experiments were replicated three times with 24 culture tubes each. The rooting experiment results were statistically analysed ($p < 0.05$) individually for each auxin treatment by analysis of variance (ANOVA), followed by Duncan's test (software SPSS for Windows, Version 19.0).

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

Table 1
Rooting percentage (%) and number of roots per microshoots obtained in *Leucospermum cordifolium* '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 $\alpha = 0.05$.