Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2010 8(3), 790-796
ISSN: 1695-971-X
eISSN: 2171-9292

Use of multinodal explants for micropropagation of *Leucadendron* 'Safari Sunset'

E. Suárez[1]*, J. F. Pérez-Francés[1] and J. A. Rodríguez-Pérez[2]

[1] *Departamento de Biología Vegetal. Facultad de Farmacia. Universidad de La Laguna (ULL). 38071 La Laguna (Tenerife). Spain*

[2] *Departamento de Ingeniería, Producción y Economía Agraria. Escuela Técnica Superior de Ingeniería Agraria. Universidad de La Laguna (ULL). 38071 La Laguna (Tenerife). Spain*

Abstract

Leucadendron 'Safari Sunset' is one of the most valuable *Proteaceae* cultivar, due to its economic importance as cutflower. *In vitro* tissue culture of this cultivar and others may offer a massive cloned plant production that permit to satisfy the market demand. Multinodal explants of *Leucadendron* 'Safari Sunset' were established *in vitro* on a half strength Murashige and Skoog medium supplemented with 2.2 µM benzylaminopurine (BAP). Various antioxidant treatments were used to reduce the presence of phenolic compounds in culture. No important differences in growth and multiplication between these treatments were detected during *in vitro* establishment and multiplication, but explants cultured on a medium with 1 g L^{-1} of polyvinylpyrrolidone (PVP) developed a higher number of buds in the establishment phase. Proliferation of axillary shoots was achieved with 0.88 µM BAP, being slightly higher on a medium with 2.2 µM BAP although the buds were deformed, showing abnormal leaves. The use of phytagel in this phase increased proliferation rate and shoot growth. Low percentages rooting were obtained when microcuttings were cultured on the same basal medium supplemented with several indole-3-butyric acid (IBA) concentrations. The best rooting percentage (37.5%) was obtained with 44.1 µM IBA. This work shows that micropropagation of *L.* 'Safari Sunset' is possible with multinodal explants, although is necessary to optimize the protocol for commercial applications.

Additional key words: antioxidant treatment, *in vitro* tissue culture, ornamental plants, phenolic compounds, Proteaceae.

Resumen

Empleo de explantos multinodales para la micropropagación de *Leucadendron* 'Safari Sunset'

Leucadendron 'Safari Sunset' es uno de los cultivares más importantes de *Proteaceae* debido a su importancia económica como flor cortada. El cultivo *in vitro* de este cultivar y otros, puede ofrecer una producción masiva de clones que permita satisfacer la demanda del mercado. Se establecieron *in vitro* explantos multinodales de *L.* 'Safari Sunset' en un medio Murashige y Skoog, con los macronutrientes a la mitad de su concentración, y bencilaminopurina (BAP) 2,2 µM. Para reducir la presencia de compuestos fenólicos en los cultivos, se emplearon diferentes tratamientos antioxidantes. No se observaron diferencias importantes en cuanto al crecimiento y multiplicación de las yemas, entre los antioxidantes empleados durante el establecimiento y multiplicación *in vitro*, sin embargo, se obtuvo un mayor número de yemas (25 ± 2,82) en los explantos sembrados en un medio con 1 g L^{-1} de polivinilpirrolidona (PVP). La multiplicación de las yemas se logró con BAP 0,88 µM. La tasa de proliferación fue ligeramente superior al adicionar al medio de cultivo BAP 2,2 µM, pero en este medio se desarrollaron yemas deformes, con hojas anormales. El empleo de fitagel incrementó la tasa de multiplicación y crecimiento de los vástagos. Se obtuvieron bajos porcentajes de enraizamiento al cultivar los microesquejes en el medio basal suplementado con diferentes concentraciones de ácido indol-3-butírico (IBA). El mejor porcentaje de enraizamiento (37,5%) se obtuvo con IBA 44,1 µM. Este trabajo muestra que es posible la micropropagación de *L.* 'Safari Sunset' a partir de explantos multinodales, aunque es necesario optimizar el protocolo con fines comerciales.

Palabras clave adicionales: compuestos fenólicos, cultivo *in vitro* de tejidos, plantas ornamentales, *Proteaceae*, tratamiento antioxidante.

* Corresponding author: ensuarez@ull.es
Received: 27-04-09; Accepted: 05-07-10.

Abbreviations used: BAP (benzylaminopurine), GA_3 (giberellic acid), IBA (indole-3-butyric acid), NAA (naphthaleneacetic acid), PVP (polyvinylpyrrolidone), 1/2 MS (half-strength macronutrient salts).

Introduction

'Safari Sunset' (*L. salignum* × *L. laureolum*), is an ornamental plant widely cultivated for cut flower. It is the most cultivated *Leucadendron* on a worldwide basis. First bred in New Zealand, it expanded rapidly to other zones. It is present in all the protea producing regions: New Zealand, Israel, Australia, Hawaii, California, Chile, Equador, South Africa, Zimbabwe, Azores and Madeira among others. Presently, millions of floral stems are sold at flower auctions in Holland, being extensively used by florists for floral arrangements (baskets, centrepieces, etc.) and bouquets.

It has dark green leaves and its terminal bracts present a luminous red during summer, darkening with the course of the season to adopt a dark wine-red colour. Owing to its fast growth and early entrance in production it has become a highly profitable cultivar. Its propagation by stem cuttings is easy, but it is frequently difficult to satisfy the demand for plants using conventional methods of vegetative propagation. Micropropagation could be a valid alternative to solve this problem, as well as to provide the indispensable plant health guarantee for vegetative material exchange at an international level. The need to establish protocols for the micropropagation of different types of proteas has been supported by the IPA (International Protea Association) at the International Protea Conference held in 2006 in San Diego, California.

Although diverse authors have studied the micropropagation of some proteas (Hu and Wang, 1983; Ben-Jaacov and Jacobs, 1986; Seelye *et al*., 1986; Rugge *et al*., 1989; Kunisaki, 1989, 1990; Bunn and Dixon, 1992a,b; Watad *et al*., 1992a,b; Pérez-Francés *et al*., 1995; Rugge, 1995; Dias Ferreira *et al*., 2003; Wu and du Toit, 2004), results have not been totally satisfactory, as at present there is still no firm that specialises in marketing *in vitro* produced protea plants. A previous report by Pérez-Francés *et al*. (1995) showed the effect of some factors on *in vitro* multiplication of this cultivar. In this study, the use of benzylaminopurine (BAP) improved bud proliferation rates. The addition of naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) or gibberellic acid (GA_3) to the proliferation medium containing BAP did not significantly affect the formation of new buds per explant or their length. The presence of activated charcoal in the medium clearly inhibited bud proliferation and an MS medium (Murashige and Skoog, 1962) at half strength produced the best response. Dias Ferreira *et al*. (2003) worked with the same cultivar, obtaining the best results on a modified MS medium containing ascorbic acid and a combination of BAP and GA_3 when shoot tips were used for establishment. They obtained only 20% elongated plants with explants from the tender parts of the shoots.

In the present paper, we report a method for micropropagating *Leucadendron* 'Safari Sunset' from multinodal (2-3 node) explants. These are a good option for this technique, since they provide 2-3 buds per explant.

Material and methods

Establishment

Explants were removed from actively growing terminal shoots of plants growing in a greenhouse and used to initiate *in vitro* culture. Shoots 20 cm in length were dipped into a 2 g L^{-1} Benlate solution (50% Benomyl) for 1 hour. The clean materials were given a quick (5 s) rinse in 70% ethanol, followed by washing in distilled water. These were then surface-sterilized in a 5% sodium hypochlorite solution with one drop L^{-1} of Tween 80 (surfactant) under vacuum for 15 min, followed by three washes in sterilized distilled water. Before planting segments in the culture medium, they were dipped into different antioxidant solutions to diminish excessive oxidation.

Multinodal segments (3-5 cm length with 2-3 nodes) were placed in basal MS medium (Murashige and Skoog, 1962) containing half-strength macronutrient salts (1/2 MS), 3% sucrose, 7 g L^{-1} agar, and 100 mg L^{-1} myo-inositol. The 1/2 MS medium was supplemented with plant growth regulators and the antioxidant substances and adjusted to pH 5.7 before autoclaving. Antioxidant treatments were used in the establishment phase to reduce explant oxidation: a combination of citric (1,500 mg L^{-1}) and ascorbic acid (100 mg L^{-1}) (Pérez-Francés *et al.,* 1995) or polyvinylpyrrolidone (PVP) (1 g L^{-1}), employed in different ways: T1 (ascorbic and citric acid solution shaken for 30 min before culture), T2 (T1 followed by darkness for three days after culture), T3 (T1 then subculture every three days during the first week of culture), T4 (ascorbic and citric acid included in the medium), P1 (PVP solution shaken as in T1), P2 (P1, then treated as in T2), P3 (P1 then subcultured as in T3), P4 (as T4 but with PVP), O (as T2 without antioxidants), SF (subcultured as in T3 without antioxidants). The cultures were maintained for 8 weeks during which they were transferred once

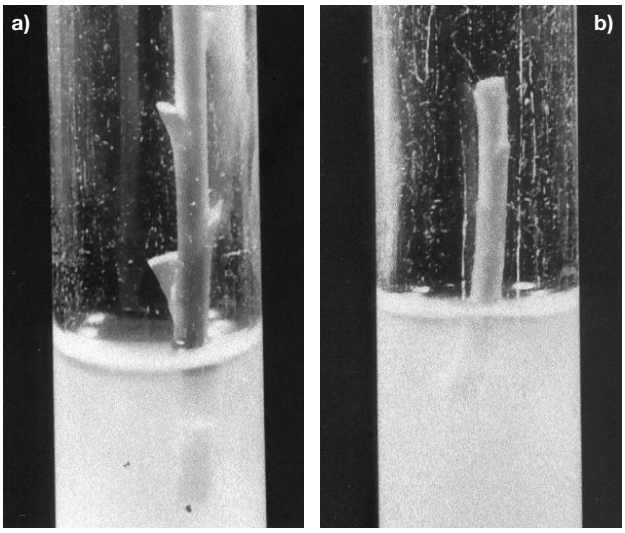

Figure 1. Multinodal explants. a) Explant A: explants where petioles were cut off leaving 1-2 mm petioles. b) Explant B: explants where petioles were totally removed.

after 4 weeks of incubation. Twenty millilitres of medium was dispensed into each (20 × 150 mm) glass tube and autoclaved at 103.5 kPa and 121°C for 20 min. Explants were divided in two groups (Fig. 1). Leaves of group A explants were cut off leaving 1-2 mm petioles, whereas leaves of explants B were totally eliminated. During establishment, explants were inserted into the basal medium supplemented with 2.2 µM BAP. Cultures were maintained at 25 ± 2°C, in a culture room illuminated with cool white fluorescent tubes (110 µmol m^{-2} s^{-1}) on shelves during the 16 h photoperiod. Within 2 weeks, axillary buds sprouted and 2 weeks later, buds were excised and subcultured in a multiplication medium.

At least 24 tubes were raised for each treatment and the experiment was repeated two times.

Multiplication

Multiplication was carried out using BAP at two concentrations (0.88 µM and 2.2 µM) and two gelling agents: 0.7% agar (agar-agar/gum-agar, Sigma Chemical, St. Louis, Mo. A1296) and 0.25% phytagel (Sigma). Multiplication rates were recorded during the first four subcultures.

Rooting

For rooting, microcuttings (1.5-2.5 cm length) from the multiplication phase were cultured on i) 1/2 MS medium supplemented with NAA (37.8, 43.2, 48.6, 54, 59.4 µM) or IBA (34.3, 39.2, 44.1, 49, 53.9 µM) or ii) dipped in a solution of IBA (0.73, 2.45, 4.90 mM) or NAA (0.81, 2.70, 5.40 mM) for 5 s and then cultured on a 1/2 MS medium without growth regulators.

At least 24 tubes were raised for each treatment and all the experiments were repeated three times. Number of bud-breaks were recorded at weekly intervals.

Results were analysed statistically by analysis of variance, using the SPSS statistical software package (version 17.0). Standard error of the mean was calculated and is indicated by ± sign.

Results

Establishment

When multinodal segments from green shoots were used, 66.7% of explants sprouted buds on a medium with PVP and BAP 2.2 µM, producing approximately two buds per explant. Table 1 shows the mean values of buds developed 4 weeks after culture for each treatment. Explants A developed a higher number of buds after all treatments assayed, and a mean of 25 buds sprouted when PVP was included in the medium (P4), in comparison with the 7 buds developed by explants B during the same treatment. The highest number of buds (10) produced by explants B was obtained when explants remained

Table 1. Buds mean developed four weeks after culture with each antioxidant treatment in each explant type C (control, without antioxidant treatment), T1 (ascorbic and citric acid solution shaken for 30 min before culture), T2 (T1 followed by darkness for three days after culture), T4 (ascorbic and citric acid included in the medium), P1 (PVP solution shaken as in T1), P4 (as T4 but with PVP), O (as T2 without antioxidants), SF (subcultured every three days during the first week of culture without antioxidants). Explants A (leaves were cut off leaving 1-2 mm petioles), Explants B (leaves were totally eliminated)

Treatment	Explant A	Explant B
C	21 ± 5.65^{ab}	4 ± 4.24^{ab}
T1	18 ± 2.82^{ab}	4.5 ± 2.12^{ab}
T2	20 ± 5.65^{ab}	7.5 ± 3.5^{ab}
T4	17 ± 5.65^{ab}	8 ± 0.0^{ab}
P1	22.5 ± 13.43^{b}	10 ± 1.14^{b}
P2	25 ± 2.82^{ab}	6 ± 4.24^{ab}
O	11.5 ± 3.53^{a}	3 ± 3.82^{a}

Values in each column followed by different letter are significant different at $P = 0.05\%$.

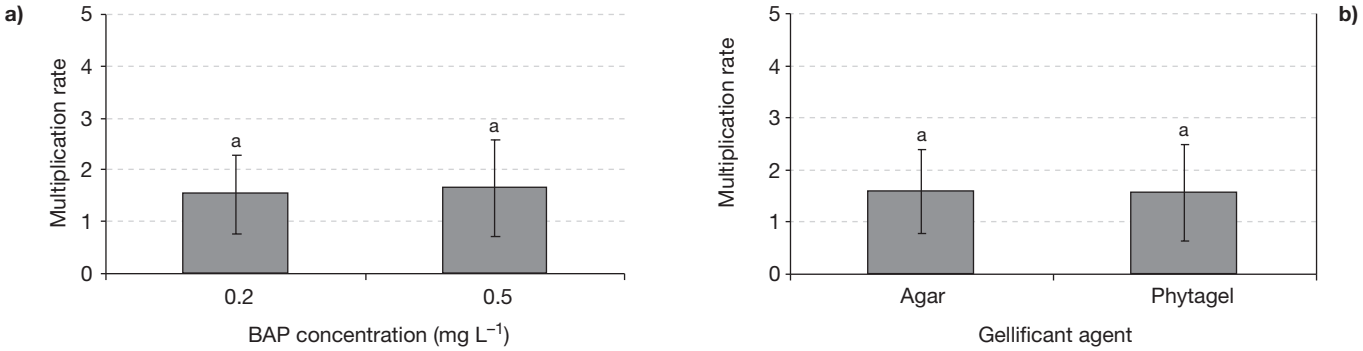

Figure 2. Effect of a) different BAP concentrations and b) gellificant agent on the multiplication rate of *Leucadendron* 'Safari Sunset'. Means and standard deviations for independent variables and ANOVA tests. The same letters indicate no significant differences at $P = 0.05\%$.

30 min in a PVP solution before being cultured in the medium (P1), although it was very low in comparison with the buds produced by explants A (23 buds).

All antioxidant treatments used were effective for explant oxidation and necrosis control, but no important differences between them were detected. Nevertheless, some observations suggested that explants smaller than 30 mm in length presented greater browning symptoms, but statistical data are insufficient to verify this.

Multiplication

The buds with greatest development during the establishment phase were isolated from the explants and subcultured on a multiplication medium consisting of 1/2 MS medium and two different BAP concentrations (2.2 µM and 0.88 µM). Multiplication rates obtained were low in both cases and no significant differences were identified between them (Fig. 2a). However, shoot cultures growth with BAP 2.2 µM showed stunted growth with small and abnormal leaves. Moreover these buds presented more symptoms of necrosis than buds obtained on a 1/2 MS medium supplemented with 0.88 µM BAP, which showed normal development (Fig. 3a-b).

Though the multiplication rates did not alter for the different antioxidant treatments used (Table 2), they did depend on the subculture number, showing a significantly decline in the third subculture (Table 2). With the use of phytagel as a gelling agent, multiplication rates continued to be low (Fig. 2b), and no significant differences were observed in the multiplication rates in comparison with the buds multiplied in a medium gelled with agar. However, buds obtained with phytagel showed stronger growth in both BAP concentrations without hyperhydricity symptoms and presented less symptoms of browning (Fig. 3c-d).

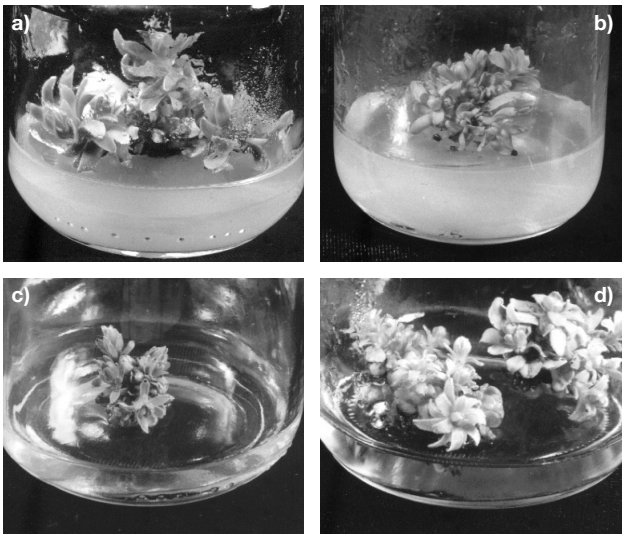

Figure 3. Multiplication of *Leucadendron* 'Safari Sunset': a) and b) in a medium gelled with agar, a) 0.8 µM BA and b) 2.2 µM BA; c) and d) in a medium gelled with phytagel, c) 0.8 µM BA, d) 2.2 µM BA.

Table 2. Effect of different treatments and subcultures on the multiplication rates of *L.* 'Safari Sunset'. Multiplication rates means obtained by Duncan's test

Treatment	Multiplication rate	Subcultures	Multiplication rate
C	1.45[a]	1	1.77[a]
P1	1.61[a]	2	1.58[ab]
O	1.65[a]	3	1.35[b]
		4	1.67[a]

Same letters within a column indicate no significantly differences at $P = 0.05\%$. C: control. P1: PVP solution for 30 min on sharking before culture. O: darkness during three days after culture.

Table 3. Rooting percentages obtained with different indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) concentrations

Growth regulator	Concentration (µM)	Rooting (%)
IBA	34.3	25
	39.2	25
	44.1	37.5
	49.0	12.5
	53.9	12.5
NAA	37.8	0
	43.2	25
	48.6	0
	54.0	0
	59.4	0

Rooting

When microshoots (1.5-3 cm length) were cultivated in a 1/2 MS rooting medium containing NAA or IBA, after 4 weeks in the establishment phase and 16 weeks in a multiplication phase (4 subcultures), IBA gave better results than NAA (Table 3).

Root induction was preceded by callusing at the base of the shoot on IBA concentrations higher than 34.3 µM. On a 1/2 MS medium with 49 µM IBA, which proved to be the best, 37.5% of shoots formed roots. Roots appeared after 4 weeks and the maximum response was observed after 7 weeks, reaching between 25-40 mm in length (Fig. 4).

Roots obtained with IBA concentrations above 49 µM showed very little growth (1-2.5 mm in length). No rooting was seen when microshoots were dipped in an IBA solution and then cultured on a 1/2 MS medium without growth regulators. The addition of activated charcoal to the medium did not improve the rooting percentage or root length.

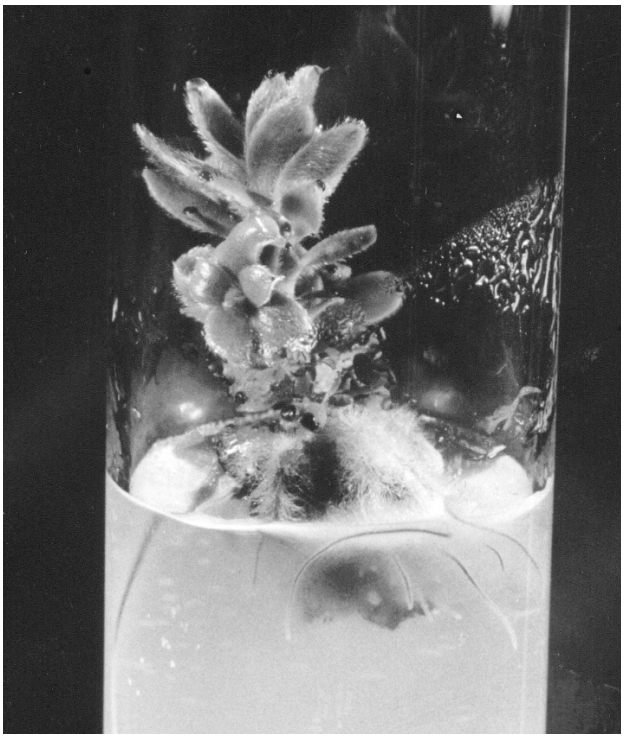

Figure 4. A rooted microcutting of *Leucadendron* 'Safari Sunset' on a 1/2 MS medium with 44.1 µM IBA.

Discussion

Use of multinodal explants provided more than one bud per explant and reduced browning. Dias Ferreira *et al*. (2003) studied three types of 'Safari Sunset' explants: shoot tips (5-10 mm), explants from the green part of the shoots and explants from the remaining lignified shoots. They achieved a 65% bud elongation from shoot tips but only 20% from explants from the green part of the shoots, in an MS medium including a combination of growth regulators. They did not obtain positive results from the oldest explants. Similar results were found by Rugge *et al*. (1989), who reached a higher percentage of sprouted buds using multinodal green explants than with semi-lignified and lignified explants.

Our results were better when the explant leaves were cut off leaving 1-2 mm petioles, than with explants without petioles. This could be due to excessive phenols production in the zone that produced damage on the axillary meristems. In previous studies with members of the *Proteaceae*, the utilization of explants with and without leaf remnants gave rise to differences. In many of them, authors used explants with 1-5 mm of leaf (Ben-Jaacob and Jacobs, 1986; Watad *et al.,* 1992b; Rugge, 1995), though others used explants without remains of leaf (Seelye *et al.,* 1986; Kunisaki, 1989), nevertheless only Pérez-Francés *et al*. (2001b) compared the development of axillary buds in these two types of explants with *Leucadendron discolor*, with an inferior response in explants with 1-3 mm of petiole than with the other type.

The need for a study on the action of antioxidant treatments on the *in vitro* culture of this plant arises from the *Proteaceae* being rich in phenolic compounds (Malan, 1992; Watad *et al*., 1992b; Rugge, 1995; Wu

and du Toit, 2004). After the dissection of explants, these compounds may be oxidized by different enzymes (polyphenoloxidases and tyrosinases) which are synthesized and/or liberated due to injury during excision and sterilization of tissues (Torres, 1989). Oxidation products are known to inhibit the enzymatic activity, browning the tissues and darkening the medium. Darkening can be reduced by limiting the production of phenolic compounds, modifying the redox potential, inactivating the phenolases, and reducing the activity of the phenolase and/or the availability of substrate (Hu and Wang, 1983).

In several reports concerning *Proteaceae*, the use of an antioxidant solution is necessary in some phases of culture: citric and/or ascorbic acid (Pérez-Francés *et al*., 1995; Dias Ferreira *et al*., 2003; Wu and du Toit, 2004), PVP (Pérez-Francés *et al*., 2001a,b), or BAP applied to the entire mother plant at 200 mg L^{-1}. This latter significantly reduced browning and promoted bud sprouting *in vitro* (Rugge, 1995; Pérez-Francés *et al*., 2001b). It is also possible to apply an etiolation treatment to a new actively growing shoot on the mother plant (Watad *et al*., 1992b). In our present study, we evaluate the effect of different antioxidant treatments on explant oxidation, using PVP and an ascorbic and citric acids solution combined with darkness and frequent subculture. The PVP absorbs the phenolic substances so they cannot be oxidized by the polyphenoloxidases, whereas the citric and ascorbic acid prevent oxidation of the phenolic compounds. Explant incubation in darkness in the initial period and the frequent transfer of explants into fresh medium were the other treatments assayed. No differences in tissue oxidation were observed between the different treatments. Results during the multiplication phase were similar to those reported by Pérez-Francés *et al*. (1995); nevertheless Dias Ferreira *et al*. (2003) obtained higher multiplication rates, although they used a combination of different growth regulators. Buds obtained in a medium with 2.2 μM BAP browned rapidly due to their poor development. There are cases in which the effect of the BAP at certain concentrations inhibits bud elongation, causing browning. To diminish this, the BAP concentration can be reduced in this phase, or be combined with an auxin or gibberellin to eliminate the inhibitory effect of BAP on bud growth (Orellana Pérez, 1998). In our work, using a lower BAP concentration was sufficient to promote bud development and reduce the risk of browning.

Multiplication rates obtained were slightly higher but not significantly different when phytagel was used as agar substitute, moreover these buds were longer. Similar results in 'Safari Sunset' using a gelling agent were obtained by Pérez-Francés *et al*. (1995). Any differences observed may be a result of the higher purity of phytagel and unidentified impurities in the agar (Sharma and Ramamurthy, 2000), which could contribute to inhibiting shoot growth (Hu and Wang, 1983). There are some reports of phytagel addition improving the multiplication phase in different plants (Saadat and Hennerty, 2002; Thimmappaiah and Sadhana, 2002).

The major problem in our study was the low rooting percentage obtained. Rooting *in vitro* may be reduced due to a long period in culture (Norton and Norton, 1986), moreover, the presence of a cytokinin in the medium during the establishment and multiplication phases (Torres, 1989; Peddaboina *et al*., 2006) can also negatively affect rooting. This can be increased using high cytokinin concentrations (Figueiredo *et al*., 2001). In our case, presence of BAP during the establishment and multiplication phases could have reduced the rooting rates.

In conclusion, the present work shows that micropropagation of *Leucadendron* 'Safari Sunset' is possible with multinodal explants, although for commercial applications it will be necessary to optimize the protocol and study the factors that affect rooting in this cultivar after a relatively long period in culture.

Acknowledgement

The authors wish to thank Dra. María Teresa Ramos Domínguez (Dpto. Estadística, Investigación Operativa y Computación, Universidad de La Laguna) for her excellent assistence with the statistical analysis.

References

BEN-JAACOV J., JACOBS G., 1986. Establishing *Protea, Leucospermum* and *Serruria in vitro*. Acta Hort 185, 39-52.

BUNN E., DIXON K.W., 1992a. *In vitro* propagation of the rare and endangered *Grevillea scapigera* (*Proteaceae*). HortScience 24(3), 261-262

BUNN E., DIXON K.W., 1992b. Micropropagation of *Stirlingia latifolia* (*Proteaceae*), an important cut flower from Western Australia. Hort Science 27(4), 368.

DIAS FERREIRA C., DIAS J.D., CANHOTO J.M., 2003. *In vitro* propagation of *Leucadendron laureolum* × *L. salignum* cv. 'Safari Sunset': ultrastructural and ana-

tomical studies of regenerated plantlets. Acta Hort 602, 29-38.

FIGUEIREDO S.F.L., ALBARELLO N., VIANA V.R.C., 2001. Micropropagation of *Rollinia mucosa* (Jacq.) Baill. *In Vitro* Cell Dev Biol Plant 37, 471-475.

HU C.V., WANG J.P., 1983. Meristem shoot tip and bud culture. In: Handbook of plant cell (Evans D.A., Sharp W.R., Ammirato P.V, Vamada V., eds). MacMillan Publ, NY. Vol. l, pp. 177-227.

KUNISAKI J.T., 1989. *In vitro* propagation of *Leucospermum* Hybrid, 'Hawaii Gold'. HortScience 24(4), 686-687.

KUNISAKI J.T., 1990. Micropropagation of *Leucospermum*. Acta Hort 264, 45-48.

MALAN D.G. 1992. Propagation of Proteaceae. Acta Hort 316, 27-34.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15, 473-497.

NORTON M.E., NORTON C.R., 1986. Change in shoot proliferation with repeated *in vitro* subculture of shoot of woody species of *Rosaceae*. Plant Cell Tiss Org Cult 5(3), 187-197.

ORELLANA PÉREZ P.A., 1998. Propagación vía organogénesis. In: Propagación y mejora genética de plantas por biotecnología (Pérez Ponce J.N., ed). Instituto de Biotecnología de Plantas, Santa Clara, Cuba. pp. 151-178. [In Spanish].

PEDDABOINA V., THAMIDALA C., KARAMPURI S., 2006. *In vitro* multiplication and plant regeneration in four *Capsicum* species using thidiazuron. Sci Hortic 107, 117-122.

PÉREZ-FRANCÉS J.F., EXPÓSITO A.J., RODRÍGUEZ-PÉREZ J.A., 1995. Effect of different factors on *in vitro* multiplication of *Leucadendron* 'Safari Sunset' (*Proteaceae*). Acta Hort 387, 115-120.

PÉREZ-FRANCÉS J.F., RAYA-TAMAYO V., RODRÍGUEZ-PÉREZ J.A., 2001a. Micropropagation of *Leucospermum* 'Sunrise' (*Proteaceae*). Acta Hort 545, 161-165.

PÉREZ-FRANCÉS J.F., RAVELO B.J., RODRÍGUEZ-PÉREZ J.A., 2001b. *In vitro* establishment and proliferation of axillary bud cultures of *Leucadendron discolor* (*Proteaceae*). Acta Hort 545, 179-185.

RUGGE B.A., 1995. Micropropagation of *Protea repens*. Acta Hort 387, 121-127.

RUGGE B.A., JACOBS G., THERON K.I., 1989. Factors affecting bud sprouting in multinodal stem segments of *Leucospermum cv.* Red Sunset *in vitro*. J Hort Sci 65(1), 55-58.

SAADAT Y.A., HENNERTY M.J., 2002. Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.). Sci Hortic 95, 251-260.

SHARMA S.K., RAMAMURTHY V., 2000. Micropropagation of 4-years-old elite *Eucalyptus tereticornis* trees. Plant Cell Reports 19, 511-518.

SEELYE J.F., BUTCHER S.M., DENNIS D.J., 1986. Micropropagation of *Telopea speciosissima*. Acta Hort 185, 281-285.

THIMMAPPAIAH SHIRLY R.A., SADHANA P.H., 2002. *In vitro* propagation of crashew from young trees. In Vitro Cell Dev Biol Plant 38(2), 152-156.

TORRES K.C., 1989. Tissue culture techniques for horticultural crops. Van Nostrand Reinhold. 224 pp.

WATAD A.A., BEN-JAACOV J., TAL E., SOLOMON H., 1992a. *In vitro* propagation of *Grevillea* Species. Acta Hort 316, 51-53.

WATAD A.A., BEN-JAACOV J., COHEN C., TAL E., SOLOMON H., 1992b. *In vitro* establishment of *Protea obtusifolia*. Acta Hort 316, 59-62.

WU H.C., DU TOIT E.S., 2004. Reducing oxidative browning during *in vitro* establishment of *Protea cynaroides*. Sci Hortic 100, 355-358.