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Effect of forcing solutions used to break the seasonal influence on in vitro axillary bud sprouting of two Leucospermum (R. Br.) cultivars

--Manuscript Draft--

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Abstract:	In woody plants, the availability of plant material for in vitro use is usually limited to the short active growth period of the mother plants. To overcome this limitation, an effective protocol is described to break the seasonal effect on in vitro axillary bud sprouting in Leucospermum cordifolium ‘Flame Spike’ and Leucospermum ‘Tango’. This enables homogenous production throughout the year. Shoots were harvested at four different times of the year, sterilized and cultured in forcing solutions containing a quarter-strength of Murashige and Skoog medium salts (MS, 1962), supplemented with benzyladenine (BA) (0-100 mg L-1) and gibberellic acid (GA3) (0 or 10 mg L-1). Ascorbic acid at 150 mg/l was added to avoid explant oxidation. After 10 days, multinodal explants were cultured on half-strength MS solid growth regulator-free medium or with GA3. Three weeks later, explants were subcultured in fresh medium. The effect of pre-treatment is very clear in the case of L. cordifolium ‘Flame Spike,’ where the percentages without pre-treatment reached 20% in the August 2014 harvest and the maximum was 53.81% in November 2013 in those with pre-treatment. Although the effect of forcing solutions in L. ‘Tango’ was not evident, supplementation with 50 mg L-1 BA turned out to be optimal in February, May and August 2014. Its use also reduced the time required for in vitro establishment of explants in both cultivars.
Response to Reviewers:	PLANT CELL TISSUE AND ORGAN CULTURE Editors-in-Chief 12th March, 2021 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 (PCTOC-D-20-00533) entitled “Use of forcing solutions to break the stationary influence on in vitro axillary bud sprouting of two Leucospermum (R. Br.) cultivars.” in accordance with the reviewers suggestions. We have enhanced the statistical analysis and modified text of the manuscript incorporating among others, the corrections suggested by the Associate Editor and Reviewers. In view of the statistical analysis we decide to change the manuscript title to “Effect of forcing solutions used to break the seasonal influence on in vitro axillary bud sprouting of two Leucospermum (R. Br.) cultivars”. Finally the English language have been reviewed again.
The comments to the Reviewers are listed below in bold lettering, embedded within the corresponding questions.

Associate Editor
1. Why have the authors chosen these two cultivars?
AUTHOR ANSWER: Leucospermum genus is one of the Proteaceae genera that has adapted better to the climatic and edaphic conditions of the Canary Islands, being one of the most cultivated. The two cultivars chosen are two of the most economically important cultivars for the Canary Islands due to their extensive cultivation mainly for the export of cut flowers to Europe. However, its propagation by stem cuttings does not always offer good results and it is also slow, so micropropagation would allow increasing the production, as well as obtaining healthy plant material for export.
3. Have they evidenced a different reactivity concerning the in vitro inoculation at different times of the year?
AUTHOR ANSWER: Previous experiences on micropropagation of these cultivars (unpublished) and another cultivar of Leucospermum (L. ‘High Gold’; Pérez-Francés et al. 1992) seemed to evidence a different behavior of explants depending of the time of collection, related it to the phenological state of the mother plants.
4. It is unclear to me the experimental set up and I found it too confusing. How many repetitions? Different years?
AUTHOR ANSWER: All experiments were repeated three times in the same year.
5. Why did they perform a different pre-treatments for the two varieties? Why were different substrates used for the two varieties after the “forcing solutions” treatments?
AUTHOR ANSWER: In our laboratory, there were already previous experiences (unpublished) about the micropropagation of these two cultivars. Media were selected based on this previous experience.

6. In the Material and Methods at line 98, the authors should better identify the climate conditions for the different months selected to judge the influence of inoculation period on in vitro success.
AUTHOR ANSWER: A paragraph has been added including information about the flowering and growing periods of these plants in Tenerife, as well as a table with the climatic conditions at the times of collection the plant material (temperature (ºC), rainfall (mm), relative humidity (%) and sunshine hours (h).
7. Results, line 143: although the authors are saying that they can’t show the statistical analyses for the shoot length, they can say to us something about the further development
AUTHOR ANSWER: Buds did not grow any further. When they were isolated from the primary explant and cultured in the same culture medium they became necrotic due to its small size. A sentence about this has been added in the manuscript.
It seems to me that the control is lacking were the authors are evaluating the effect of the forcing solution (table 3). Did the authors try to put the buds only in a water solution as pre-treatment?
AUTHOR ANSWER: We previously tried a treatment with only a water solution, but no results were obtained so we decided not to include them in this research.
REVIEWER 1
Changes proposed by reviewer 1 are highlighted in yellow in the manuscript.
1. Lines 29-30: Please write the complete genus (Leucospermum)
AUTHORS ANSWER: we have changed “L.” by “Leucospermum”
2. Line 38: The word research is confusing here. Do you mean reached?
AUTHORS ANSWER: we have changed “research” by “reached”

	3. Line 39: Of which year? AUTHORS ANSWER: we have added “August 2014” and “November 2013” 4. Line 39: was AUTHORS ANSWER: we have changed “is” by “was” 5. Line 42: Of which year? AUTHORS ANSWER: we have added “August 2014” 6. Line 47: family AUTHORS ANSWER: we have added “family” 7. Line 62: add a comma AUTHORS ANSWER: we have added a comma 8. Line 77: “and pre-treatment to the mother plants or to isolated branches can be applied (consider revision) AUTHORS ANSWER: we have changed “and pre-treatment to the mother plants or to isolated branches can be applied” by “and pre-treatments to the mother plants or to isolated branches can be applied” 9. Line 91: “preparation of explants” or “explants preparation” AUTHORS ANSWER: we have changed “preparation explants” by “preparation of explants” 10. Line 98: Which year? AUTHORS ANSWER: we have changed “November, February, May and August” by “November (2013), February, May and August (2014)” 11. Line 126: Bud-breaks were recorded after 21 days and microshoot length after 70 days? If not, the word respectively is not needed. AUTHORS ANSWER: Bud-breaks were recorded after 21 days and microshoots length after 70, so the word respectively is needed 12. Line 142: How is that? AUTHORS ANSWER: Because of the number of buds obtained was so low it was not possible to do a statistical analysis of length. 13. Line 149, 261, 264, 279, 290: Add the specie AUTHORS ANSWER: Leucospermum ‘Tango’ is a hybrid between L. lineare x L. glabrum, as is cited in line 54. We consider that is correct to cited it at the beginning of the manuscript and then name it with the short form of the cultivar. With L. cordifolium ‘Flame Spike’ is different because it is a clone of L. cordifolium and the correct form to name it is L. cordifolium ‘Flame Spike’. 14. Line 205: Collected AUTHORS ANSWER: we have changed “recollected” by “collected” 15. Line 282: The most suitable explants AUTHORS ANSWER: we have changed “the explants most suitable” by “the most suitable explants” REVIEWER 2 Changes proposed by reviewer 2 are highlighted in green in the manuscript. 1. Abstract: concentrations of BA and GA, please check these concentrations, there are not identical with those mentioned in M&M and Tables AUTHORS ANSWER: description of concentrations of BA and GA3 have been changed in Material and Methods (highlighted in green in the manuscript). 2. Line 37-40: strange sentence (verb) AUTHORS ANSWER: 3. Line 54: interspecific cross x (not italic) AUTHORS ANSWER: we have changed “x” by “x” 4. Line 80: typo micro nutrientes AUTHORS ANSWER: we have changed “micronutrients” by “micro nutrients” 5. Change subtitles of 2.2 and 2.3 to a more informative subtitle (line 104 and 110) and idem dito in results section AUTHORS ANSWER: -we have changed “2.2. Without forcing solutions” by “2.2. Development of axillary buds in vitro in absence of a pre-treatment with forcing solutions” -we have changed “2.3. With forcing solutions” by “2.3. Development of axillary buds in vitro after a pre-treatment with forcing solutions” -Idem in the results section 6. Line 114: BA (0-1 mg/l) typo also explain which concentrations were specifically tested (not only range) AUTHORS ANSWER: we have changed “BA (0-100 mg L-1 and GA3 (0-10 mg L-1)” by “BA (0, 25, 50, 75 and 100 mg L-1 and GA3 (0 or 10 mg L-1) 7. Pictures are not informative, maybe better to select 1 or 2

	AUTHORS ANSWER: We think that the pictures are informative, as they show the malformed buds obtained in the media with a combination of BA and GA3 and the absence of these anomalies with the use of forcing solutions. They also allow to visualize the different sizes between treatments. We have grouped together the two figures in one. 8. Statistical tests can be done better. Check if a multifactorial test (effect of season, cultivar and effect of medium composition) can be done. a, b in statistical tests, give 'a' to the highest value. AUTHORS ANSWER: Results were analysed too by two way analysis of variance (ANOVA) (effect of season and effect of media). Results were added to the tables and new sentences have been added in Results section (highlighted in green in the manuscript). 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. Departamento de Botánica, Ecología y Fisiología Vegetal Universidad de La Laguna

Effect of forcing solutions used to break the seasonal influence on *in vitro* axillary bud sprouting of two *Leucospermum* (R. Br.) cultivars.

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ABSTRACT

In woody plants, the availability of plant material for *in vitro* use is usually limited to the short active growth period of the mother plants. To overcome this limitation, an effective protocol is described to break the seasonal effect on *in vitro* axillary bud sprouting in *Leucospermum cordifolium* 'Flame Spike' and *Leucospermum* 'Tango'. This enables homogenous production throughout the year. Shoots were harvested at four different times of the year, sterilized and cultured in forcing solutions containing a quarter-strength of Murashige and Skoog medium salts (MS, 1962), supplemented with benzyladenine (BA) (0-100 mg L^{-1}) and gibberellic acid (GA_3) (0 or 10 mg L^{-1}). Ascorbic acid at 150 mg/l was added to avoid explant oxidation. After 10 days, multinodal explants were cultured on half-strength MS solid growth regulator-free medium or with GA_3. Three weeks later, explants were subcultured in fresh medium. The effect of pre-treatment is very clear in the case of *L. cordifolium* 'Flame Spike,' where the percentages without pre-treatment reached 20% in the August 2014 harvest and the maximum was 53.81% in November 2013 in those with pre-treatment. Although the effect of forcing solutions in *L.* 'Tango' was not evident, supplementation with 50 mg L^{-1} BA turned out to be optimal in February, May and August 2014. Its use also reduced the time required for *in vitro* establishment of explants in both cultivars.

Keywords: Axillary buds, Micropropagation, Multinodal explants, *In vitro* tissue culture, Pre-treatment, Proteaceae.

1. Introduction

The Proteaceae family comprises about 80 genera of trees and shrubs; the genus *Leucospermum* R. Br. is particularly outstanding, with 48 shrub species distributed across South Africa. The members of this genus are distinguished by their striking terminal inflorescences. *Leucospermum* plays an essential role in ornamental plant

production due to its commercialization as cut flowers. *Leucospermum cordifolium* 'Flame Spike' and *L.* 'Tango' are two cultivars selected in South Africa. 'Flame Spike' is a clone of *L. cordifolium* and is characterized by bright red terminal inflorescences, while *L.* 'Tango' is a hybrid of *L. lineare* x *L. glabrum*, with red-orange terminal inflorescences.

In the first decades of the twentieth century, cultivation and development of new cultivars of the species began in diverse parts of the world due to the small geographical range of the *Leucospermum* genus. South Africa, Australia and New Zealand stand out as initial producing areas. The United States, Ecuador, Chile, Southeast Portugal, Madeira, the Azores, Southwest Spain, the Canary Islands, Zimbabwe and Mozambique joined later.

The success of protea cultivation in the northern hemisphere solved the problems in obtaining certain cultivars throughout the year, and to satisfy the demand, new alternative techniques for conventional vegetative propagation were promoted. Among these new techniques, *in vitro* tissue culture produces plants in aseptic conditions to facilitate worldwide exchange and large yields of plants throughout the year, thus promoting the commercialization of these plants or flowers worldwide.

In many species, an optimal phytosanitary and physiological state of the mother plants at the time of harvest is important before initiating any micropropagation protocol. Occasionally, the percentages of buds obtained *in vitro* from material collected at different times of the year can differ due to the physiological state of the mother plants. Many authors refer to this as a seasonal effect, which occurs mainly in woody and semi-woody species (Thakar and Bhargava 1999; Chitra and Padmaja 2002; Romano et al. 2002; Chaturvedi et al. 2004; Schoene and Yeager 2005; Kartsonas and Papafotiou 2007; Mishra et al. 2008; Arora et al. 2010; Shekhawat and Manokari 2016) and even in

proteas (Pérez-Francés et al. 2001a,b).

Among the different techniques used to modify the physiological state of the mother plants, growth regulators can be added to the culture media and ~~the application of~~ pre-treatments to the mother plants or to isolated branches can be applied. This breaks the seasonal effect, so as to obtain steady homogeneous production throughout the year. Pre-treatments consist mainly of solutions of growth regulators with or without macro- and micro nutrients. The regulators used are cytokinins and gibberellins, which induce bud sprouting and increase the number of new shoots. Pre-treatments can be applied as a foliar spray to the mother plants or as forcing solutions to isolated branches (Yang and Read 1997; Read and Preece 2003; Nas et al. 2012; Grabkowska et al. 2014; Bukhari et al. 2016; Kumari et al. 2017). Application of gibberellic acid (GA_3) to the mother plants of *Telopea speciosissima* (Offord et al. 1992) or benzyladenine (BA) to *Protea repens* (Rugge 1995), *Leucospermum* 'Sunrise' (Pérez-Francés et al. 2001b) and *Banksia coccinea* (Olate et al. 2010) activated axillary bud development in mother plants. These solutions are especially important because, depending on their content, they can break bud dormancy or at least stimulate sprouting.

Leucospermum cordifolium, parent of the clone *Leucospermum cordifolium* 'Flame Spike', and their clones and hybrids are the most studied members of the *Leucospermum* genus. Van Staden and Bornman (1976) initiated *in vitro* tissue culture studies of *L. cordifolium*. Although they did not develop a complete micropropagation protocol, they established the starting point for future researches on propagation of this species and some of its clones and hybrids. Additionally, Ben-Jaacov and Jacobs (1986) did a brief preliminary trial using a natural hybrid between *L. cordifolium* and *L. lineare*, *L.* 'Red Sunset', using an AND medium with benzyladenine (BA). Later, the most used medium for axillary bud sprouting of members of *Leucospermum* has been

that of Murashige and Skoog (MS, 1962) (Rugge et al. 1989; Tal et al. 1992 a,b; Pérez-Francés et al. 2001b; Thillerot et al. 2006). Furthermore, bud production has been enhanced using a combination of BA and gibberellic acid (GA_3) in the medium (Tal et al. 1992a, Thillerot et al 2006). These researchers employed uni- or multinodal explants to develop axillary buds, although Rugge et al. (1989) also studied the influence of explant position on the shoot (terminal, middle and basal section) and also in solidified medium with *L*. 'Red Sunset'. They obtained the best results using non-lignified terminal explants placed with the feeder leaf immersed in the solidified medium. Pérez-Francés et al. (2001b) studied the influence of a pre-treatment of the mother plants of *Leucospermum* 'Sunrise' with BA before culturing the nodal explants *in vitro*. They showed that the response of the explants depended on time of harvest.

While these research studies allowed plants of the *Leucospermum* genus to be developed *in vitro*, sprouting bud counts were insufficient for this technique to be adopted for commercial purposes. With this in mind, we describe a comparative study of *in vitro* development of axillary buds obtained after the application of forcing solutions to isolated branches of *Leucospermum cordifolium* 'Flame Spike' and *L*. 'Tango', without similarly treating the mother plants.

2. Material and methods

2.1. Plant material and preparation of explants

Shoots (10-15 cm) of *L. cordifolium* 'Flame Spike' and *L*. 'Tango' were collected from 10 year-old plants cultured in the experimental fields belonging to the Higher Polytechnic School of Engineering, University of La Laguna, Canary Islands, Spain (28º28′42.65′′N, 16º19′08.68′′W; altitude 564 m above sea level). The temperature in the growing area varied between 12.4º C mean in February 2014 and 20.9º C in August 2014. This latter was the driest month with 5 mm rainfall and 79% relative humidity.

These *L. cordifolium* 'Flame Spike' plants bloom in September and October, and the buds begin to sprout between February and May. 'Tango' begins to flower in November and its buds sprout between May and August (Table 1). These mother plants were irrigated, fertilized and periodically treated with fungicides and insecticides, so they presented an appropriate nutritional and phytosanitary status at the time of collection.

Plant material was harvested at four different times of year, November 2013, February, May and August (2014). These months were selected to study the development of plants *in vitro* and the effect of forcing solutions on plant material in different phenological stages of the mother plants.

After removing leaves, the shoots were washed in water and Tween 20 for 1h, disinfected by immersion in a fungicide solution (thiophanate-methyl 45% w/v and propamocarb 60.5% w/v) for 1h, dipped into 70% ethanol for 5s and rinsed with distilled water. Shoots were then surface-sterilized in a 4% sodium hypochlorite solution containing 10 drops L^{-1} of Tween 20 under vacuum for 30 min, and rinsed 3 times in distilled water.

2.2. Development of axillary buds *in vitro* in the absence of a pre-treatment with forcing solutions

Sterilized shoots were cut into multinodal segments (2-3 cm length). Multinodal explants were cultured in test tubes containing half-strength (½) MS, 20 g L^{-1} sucrose, 150 mg L^{-1} ascorbic acid and 7 g L^{-1} agar. Media for *L. cordifolium* 'Flame Spike' were supplemented with benzyladenine (BA) (0, 0.5 and 1 mg L^{-1}) and gibberellic acid (GA_3) (0, 0.5 and 1 mg L^{-1}) and for *L.* 'Tango', media were supplemented with BA (0, 1 and 2 mg L-1) and GA_3 (0, 1 and 2 mg L^{-1}). The pH was adjusted to 5.8 before autoclaving.

2.3. Development of axillary buds *in vitro* after pre-treatment with forcing solutions

Sterilized shoots were cultured in test tubes containing 3 ml of quarter-strength (¼ MS) liquid medium, 20 g L^{-1} sucrose, 150 mg L^{-1} ascorbic acid. No growth regulators were added to the solutions for *L. cordifolium* 'Flame Spike' culture. Solutions for *L.* 'Tango' culture were supplemented with BA (0, 25, 50, 75 and 100 mg L^{-1}) and GA_3 (0 or 10 mg L^{-1}). The pH was adjusted to 5.8 before autoclaving. After 10 days in the solutions, shoots were cut into multinodal explants (2-3 cm length) and cultured on a solid ½ MS medium without growth regulators (*L. cordifolium* 'Flame Spike' and *L.* 'Tango') or with 1 mg L^{-1} GA_3 (*L. cordifolium* 'Flame Spike').

2.4. Growth conditions

All cultures were incubated in a growth chamber at 25±2 °C under Philips fluorescent daylight tubes (120 µmol $m^{-2}s^{-1}$) for 16 h. Explants were subcultured every 3 weeks on the same culture medium. At least 48 tubes were raised for each treatment and the experiments were repeated three times.

2.5. Statistical analysis

At least 24 tubes of each treatment were cultured and all the experiments were repeated three times. Percentage of bud-breaks and mean microshoot length were recorded after 21 and 70 days in culture, respectively. Results were analysed statistically by one or two way analysis of variance (ANOVA). The means of each treatment were compared by Duncan's multiple range test (α=0.05), using the SPSS statistical software package (version 19.0) (SPSS Inc. Chicago, IL, USA).

3. Results

3.1. Development of axillary buds *in vitro* in the absence of a pre-treatment with forcing solutions

The percentage of buds obtained in *L. cordifolium* 'Flame Spike' and *L.* 'Tango' was affected by both medium composition and time of collection (Tables 2, 3). In general,

175 the bud percentage in *L. cordifolium* 'Flame Spike' was very low when growth
176 regulators were added to the medium without pre-treatment, independently of the type
177 of growth regulator, its concentration and the period when the plant material was
178 collected.
179 Absence of growth regulators in the medium inhibited the development of axillary buds
180 in explants of *L. cordifolium* 'Flame Spike' collected in November and February (Table
181 2), although the budding percentages of obtained in May and August 2014 (5.66 and
182 8.33 % respectively) were very low. Addition of BA to the medium yielded very low
183 percentages in the four collection periods. The highest (20%) was achieved in the
184 explants collected in August and cultured in a medium supplemented with 0.5 mg L^{-1}
185 BA and 0.5 mg L^{-1} GA_3 (Table 2). All bud lengths were measured, but the low bud
186 count obtained in all media and collection periods prevented statistical analysis and data
187 interpretation. These buds showed only limited development, becoming necrotic 7-10
188 days after their isolation.
189 Regarding micro-shoot morphology, those developed in media without growth
190 regulators (Fig. 1a), or with only BA (Fig. 1b) or GA_3 (Figs. 1f, g) showed normal
191 growth. In contrast, those developed in media with combined BA and GA_3 showed
192 abnormalities, thickened stems and deformed, excessively broad or thin leaves (Figs.
193 1c-e), independently of the collection period.
194 In *L.* 'Tango', bud percentages were in all cases higher than those obtained in *L.*
195 *cordifolium* 'Flame Spike' (Table 2). The highest were reached when the plant material
196 was collected in May (Table 3). Addition of BA as the only growth regulator increased
197 the percentages only in November, as the BA concentration increased. However, in
198 February, May and August an increase in BA concentration led to a lower budding
199 percentage, although it was only significant in February. The highest percentage of

buds, over the four months, was obtained when explants were cultured on media supplemented with combinations of BA and GA_3, especially with 2 mg L^{-1} BA and 1 mg L^{-1} GA_3. Only in February were higher percentages obtained in the media without growth regulators (Table 3). In general, presence of GA_3 in the culture medium as the only growth regulator decreased bud sprouting significantly in all months.

After 70 days of culture, the longest micro-shoots were obtained in the medium with 1 mg L^{-1} GA_3 in all four months (Table 3). Normally, their length decreased on increasing the concentration of BA. The BA+GA_3 combination only improved on the results obtained in the absence of regulators or with BA only, in particular in the months at concentrations of 1 mg L^{-1} BA and 1 mg L^{-1} GA_3 (Table 3).

As happened in *L. cordifolium* 'Flame Spike', shoots developed in media without growth regulators (Fig. 1h), or with BA (Figs. 1i-j), or GA_3 (Figs. 1n-o) presented normal development. In contrast, those obtained in media with combined BA+GA_3 showed thickened and deformed stems with excessively broad or thin leaves (Figs. 1k-m), independently of the collection period.

3.2. Development of axillary buds *in vitro* after a pre-treatment with forcing solutions

The percentage of buds obtained in *L. cordifolium* 'Flame Spike' and *L.* 'Tango' was again affected by both composition of the medium and time of collection (Tables 4, 5).

In *L. cordifolium* 'Flame Spike', the use of forcing solutions comprising a ¼ MS medium increased the percentages of buds (Table 4), compared with those obtained without it (Table 2). The highest percentages were obtained by adding GA_3 to the establishment medium, although differences were significant only in May. The highest of all was obtained in November (53.81%) and the lowest in February and August, regardless of the addition or absence of GA_3. The presence of GA_3 in the establishment medium also increased shoot length significantly in all months (Table 4).

225 The use of forcing solutions increased the *L*. 'Tango' bud percentage (Table 5) in
226 comparison with those obtained without this pre-treatment (Table 3).
227 The presence of BA as the only regulator in the forcing solutions increases the
228 percentage of buds in all the harvests (Table 5) at all dates, especially in February, May
229 and August when BA was added at 50 mg L^{-1} and in November with 100 mg L^{-1} BA.
230 In all cases, the combination of BA and GA_3 produced a lower bud percentage (Table
231 5), especially in November. However, in May no significant differences were observed
232 between any of the treatments.
233 Shoot length was very similar in all treatments (Table 5). No differences were observed,
234 whether or not GA_3 was added to the forcing solution. In most cases, an increase in BA
235 concentration reduced shoot length, but addition of GA_3 enhanced it only in May and
236 August. In November and February the longest shoots were obtained in solutions
237 without growth regulators, although without significant differences from the shoots
238 developed after pre-treatments with BA.
239 In both cultivars shoot morphology was similar in all media, without anomalies in
240 leaves and stems (Figs. 2a-b; 2c-k). The only differences observed were in shoot length,
241 those developed in the medium supplemented with 1 mg L^{-1} GA_3 being longer in *L.*
242 *cordifolium* 'Flame Spike' (Fig. 2b), and those supplemented with combined BA+GA_3
243 (Figs. 2h-k) in *L.* 'Tango'.

244 **4. Discussion**

245 According to the literature, the method of axillary buds is the most widely used for
246 micropropagation of protea in general and *Leucospermum* species and cultivars in
247 particular (Ben-Jaacov and Jacob 1986; Kunisaki 1989, 1990; Olate et al. 2010; Rugge
248 et al. 1989; Tal et al. 1992; Thillerot et al. 2006).

In spite of the poor percentages of buds obtained in *L. cordifolium* 'Flame Spike' without applying a pre-treatment, the best results were in May and August, corresponding to the period of active growth of the mother plants on Tenerife. Consequently, bud development was inferior when plant material was collected during the flowering period. Pérez-Francés et al. (2001) reported similar results in *Leucadendron discolor*, obtaining the highest percentages of budding in May (period of active growth on Tenerife) and the lowest in January, just before flowering. The same results were observed in *L.* 'Tango' explants.

In the absence of pre-treatments, addition of BA to the establishment medium was not a determinant factor in development of axillary buds in *L.* 'Tango', since no significant differences were observed when it was not present in the medium. Concentrations of BA included in the culture media were between 0 and 2 mg L^{-1}. This is because the optimal concentrations of BA used are normally lower than 2 mg L^{-1} for members of the Proteaceae family, since higher concentrations reduce the percentages of buds (Mulwa and Bhalla 2000; Gitonga 2008).

Axillary bud sprouting is mainly regulated by cytokinins. However, it has been seen that in many woody plants, such as *Prunus avium* (Elfving et al. 2011), *Populus tremula x P. tremuloides* (Rinne et al. 2016), *Jatropha curcas* (Ni et al. 2015, 2017) and *Magnolia sirindhornieae* (Cui et al. 2019), their effect increases with the presence of GA_3 in the culture medium. This has been observed in members of the Proteaceae family (Seelye 1984; Offord et al. 1992; Watad et al. 1992; Rugge et al. 1995; Dias Ferreira et al. 2003; Wu and du Toit 2012) and *Leucospermum* genus (Tal et al. 1992a; Pérez-Francés et al. 2001b). In *L. cordifolium* 'Flame Spike' and *L.* 'Tango' this effect was also found when both hormones were added to the medium at certain concentrations, in accordance with

273 the results obtained by Tal et al. (1992a) in *Leucospermum cordifolium* with an optimal
274 concentration of both regulators of 1mg L^{-1}.
275 Nevertheless, these authors did not report notable morphological differences between
276 buds sprouting in different media, as we observed in *L. cordifolium* 'Flame Spike' and
277 *L.* 'Tango'. Such modifications have not been described previously in other members of
278 the Proteaceae family. However, in *Annona emarginata* (Annonaceae; Freitas et al.
279 2016), combining BA and GA_3 in the culture medium also induced irregular malformed
280 plants. On the other hand, in *Camelia sinensis* (Gonbad et al. 2014), buds were
281 morphologically abnormal when GA_3 was combined with thidiazuron (TDZ) instead of
282 BA. This shows that GA_3 can induce different responses in other species and even in
283 different genotypes of the same species (Cui et al. 2019).
284 Adding GA_3 as the only regulator in the culture medium decreased the percentage of
285 buds sprouting in all media, although the shoots were longer. This negative effect could
286 be due to using an inappropriate concentration of GA_3 (Cui et al. 2019).
287 In both cultivars, the low percentage of buds obtained in the absence of pre-treatments
288 could be a consequence of many factors. Age of the mother plant, its physiological and
289 phytosanitary state, the conditions in which it has developed, and the harvesting season
290 can all significantly affect the later behaviour of the explants *in vitro*. In our case, it
291 seems that time of collection influences the success of the establishment phase. This is
292 because the mother plants of both cultivars were grown in the field, not in a greenhouse
293 where growing conditions can be modified. In other woody and semi-woody species
294 (Romano and Martins-Loução 1992; Thakar and Bhargava 1999; Kartsonas and
295 Papafotiou 2007), a marked seasonal effect has been seen in the *in vitro* plant response.
296 Nevertheless, in our case differences are due to both collection time and culture medium
297 composition. In those plants, this seasonal phenomenon influences the success or failure

298 of cultivation in terms of the plant material collected. In both cultivars, this factor
299 probably increased the percentage of buds sprouting when plant material was collected
300 during the growth period of the mother plants. Moreover, the differences observed in
301 budding according to the concentration of growth regulators added to the medium could
302 be due to the need for an exogenous cytokinin, to break the latency of the axillary buds
303 collected in the flowering period. Axillary bud sprouting induced in solutions with BA
304 and GA_3 stimulates protein expression, thus increasing tissue growth and elongation
305 (Yang and Read 2004).
306 On the other hand, the shoots of *L. cordifolium* and *L.* 'Tango' collected were younger
307 and less lignified in the growing period and therefore had greater regenerative capacity.
308 Moreover, in plants with high phenols content, such as *L. cordifolium* 'Flame Spike'
309 and *L.* 'Tango' (Suárez et al. 2018, 2019), young tissues frequently produce less
310 phenolic compounds, so differences observed in the development of axillary buds in the
311 two cultivars may also be due to fluctuations in the exudation of phenols through the
312 year, as observed in *Quercus suber* (Romano and Martins-Loucao 1992), *Bauhinia*
313 *vahlii* (Dhar and Upreti 1999), *Eucalyptus tereticornis* (Sharma and Ramamurthy 2000),
314 *Ceratonia siliqua* (Romano et al. 2002) and *Smilax campestris* (Rugna et al. 2008).
315 The use of mother plants grown in greenhouses or growth-chambers with controlled
316 conditions was not possible in our case, so applying nutrient solutions with or without
317 growth regulators may be a good alternative to improve the low budding percentage
318 obtained without these pre-treatments. As mentioned above, pre-treatment need not
319 always to be applied to the whole plant, but also to isolated stems before sterilization
320 (Yang and Read 1993; Read and Qiguang 1987; Ercisli et al. 2001) or afterwards (Yang
321 and Read 1997; Moura and Silva 2009). In our case we used juvenile stems, so we

decided to carry out the pre-treatments in aseptic conditions, dividing the stems into multinodal explants later.

In *L. cordifolium* 'Flame Spike' and *L.* 'Tango', results showed that nutritional improvement in the isolated branches achieved by pre-treatment also improved bud development, even without adding growth regulators to the solution. As noted by Read and Preece (2003), this is because the most suitable explants for micropropagation were taken from plants provided with appropriate mineral nutrition.

5. Conclusions

Our study presents an efficient method for the development of axillary buds *in vitro* from two cultivars of the *Leucospermum* genus. The application of a pre-treatment to isolated branches from both cultivars induced high percentages of axillary buds throughout the year. It was necessary to improve the nutritional status of the isolated branches for adequate development of axillary buds in both cultivars and addition of BA caused higher bud sprouting in *L.* 'Tango'. However, for commercial applications it may be necessary to optimize the protocols.

Author Contributions ES and JFP-F planned and designed the research, JAR-P contributed plant material, ES performed the experiments and collected data. ES, JFP-F and JAR-P analysed the data, ES wrote the manuscript and ES, JFP-F, JAR-P and CA supervised the writing. All the co-authors reviewed the manuscript before submission.

Compliance with ethical standards

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

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Key Message

The used of nutritive solutions with and without growth regulators allowed to reduce the seasonal effect on *in vitro* axillary buds sprouting and increased the percentage of new shoots.

Figure 1

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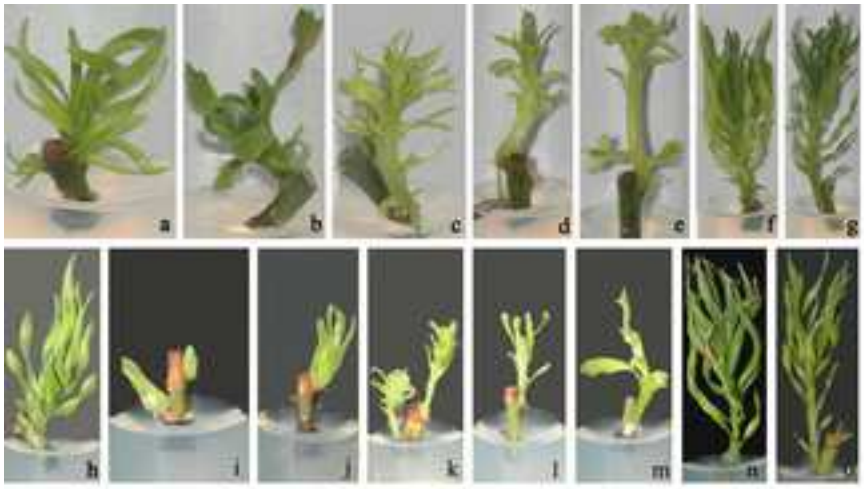

Figure 2

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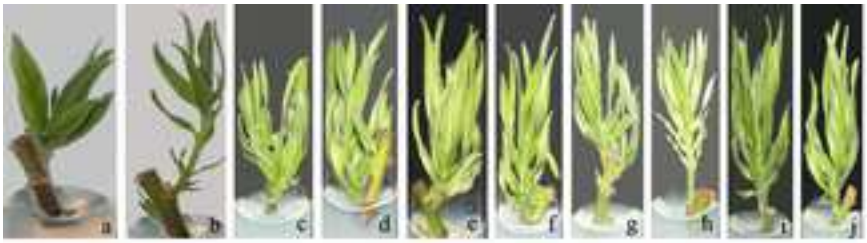

Fig. 1. a-g: Buds of *Leucospermum cordifolium* 'Flame Spike' after 70 days in culture on a ½ MS medium supplemented with BA and GA_3 (a: without growth regulators, b: 0.5 mg L^{-1} BA, c: 0.5 mg L^{-1} BA + 0.5 mg L^{-1} GA_3, d: 0.5 mg L^{-1} BA + 1 mg L^{-1} GA_3, e: 1 mg L^{-1} BA + 1 mg L^{-1} GA_3, f: 0.5 mg L^{-1} GA_3, g: 1mg L^{-1} GA_3). h-o: *Leucospermum* 'Tango' after 9 weeks in culture on a ½ MS medium supplemented with BA and GA_3 (h: without growth regulators, i: 1 mg L^{-1} BA, j: 2 mg L^{-1} BA, k: 1 mg L^{-1} BA + 1 mg L^{-1} GA_3, l: 2 mg L^{-1} BA + 1 mg L^{-1} GA_3, m: 2 mg L^{-1} BA + 2 mg L^{-1} GA_3, n: 1 mg L^{-1} GA_3, o: 2 mg L^{-1} GA_3).

Fig. 2. a-b: Buds of *Leucospermum cordifolium* 'Flame Spike' obtained after use of forcing solutions composed of ¼ MS without growth regulators, and subsequent culture of multinodal explants on a solid ½ MS medium supplemented with GA_3 (a: without growth regulators, b: 1 mg L^{-1} GA_3). c-k: *Leucospermum* 'Tango' obtained after the use of forcing solutions composed of ¼ MS supplemented with BA and GA_3 (c: without growth regulators, d: 25 mg L^{-1} BA, e: 50 mg L^{-1} BA, f: 75 mg L^{-1} BA, g: 100 mg L^{-1} BA, h: 25 mg L^{-1} BA + 10 mg L^{-1} GA_3, i: 50 mg L^{-1} BA + 10 mg L^{-1} GA_3, j: 75 mg L^{-1} BA + 10 mg L^{-1} GA_3, k: 100 mg L^{-1} BA + 10 mg L^{-1} GA_3) and subsequent culture on a ½ MS medium without growth regulators.

Table 1 Climatic values in the growing area of mother plants of *Leucospermum cordifolium* 'Flame Spike' and *Leucospermum* 'Tango' (Agencia Estatal de Meteorología, 2021).

Month-Year	Temperature (ºC)	Precipitation (mm)	RH (%)	Hours of sun
November-2013	16.3	45	85	123
February-2014	12.4	118	85	140
May-2014	16.5	12	80	170
August-2014	20.9	7	79	263

Table 2 Effect of different concentrations of BA and GA_3 on budding (21 days) of *Leucospermum cordifolium* 'Flame Spike' in a ½ MS medium, at four different times of the year, November (Nov), February (Feb), May (May) and August (Aug).

Growth regulators (mg L^{-1})		**Buds (%)**			
BA	**GA_3**	**Nov**	**Feb**	**May**	**Aug**
0	**0**	0^{a}	0^{a}	$5,66^{a}$	$8,33^{ab}$
0,5	**0**	$1,35^{a}$	$1,06^{ab}$	$0,90^{b}$	$6,11^{ab}$
0,5	**0,5**	$1,87^{a}$	$1,35^{ab}$	$3,24^{ab}$	20^{b}
0,5	**1**	0^{a}	$1,35^{ab}$	0^{b}	$8,64^{ab}$
1	**1**	$2,53^{a}$	$3,33^{ab}$	0^{b}	$8,77^{ab}$
0	**0,5**	0^{a}	0^{a}	0^{b}	0^{a}
0	**1**	$2,77^{a}$	$4,76^{b}$	0^{b}	$11,36^{ab}$

Significance of two way ANOVA	Buds (%)
Medium	*
Time of the year	*
Medium x Time of the year	NS

Means in each columns by different letter are significant different at α=0.05.
*Significant at α=0.05, NS: no significant at α=0.05.

Table 3 Effect of different concentrations of BA and GA_3 on budding (21 days) of *Leucospermum* 'Tango' in a ½ MS medium, at four different times of the year, November (Nov), February (Feb), May (May) and August (Aug). Shoot length was recorded after 70 days in culture.

		Buds (%)				Length (mm)			
BA (mgL^{-1})	**GA_3 (mgL^{-1})**	**Nov**	**Feb**	**May**	**Aug**	**Nov**	**Feb**	**May**	**Aug**
0	**0**	36.95^{bc}	30.27^{a}	50.56^{a}	29.59^{a}	$16,53^{ab}$	$18,29^{a}$	$21,04^{a}$	$18,69^{ac}$
1	**0**	42.83^{abc}	16.26^{bc}	49.00^{a}	26.11^{a}	$11,50^{cd}$	$6,87^{c}$	$14,10^{bc}$	$12,99^{a}$
2	**0**	54.60^{a}	13.29^{bc}	47.50^{a}	17.83^{a}	$8,86^{de}$	$6,66^{c}$	$10,96^{c}$	$7,10^{b}$
1	**1**	38.70^{bc}	13.10^{bc}	55.16^{a}	18.99^{a}	$16,52^{ab}$	$18,61^{a}$	$26,00^{d}$	$16,42^{a}$
2	**1**	49.01^{ab}	20.17^{ab}	56.55^{a}	31.27^{a}	$13,41^{ac}$	$14,97^{ab}$	$17,29^{ab}$	$16,03^{a}$
2	**2**	47.05^{abc}	10.85^{bc}	44.96^{a}	24.74^{a}	$14,49^{abc}$	$9,97^{bc}$	$15,00^{bc}$	$18,35^{ac}$
0	**1**	32.15^{c}	4.48^{c}	29.76^{b}	28.38^{a}	$16,80^{b}$	$33,24^{d}$	$33,91^{e}$	$27,71^{c}$
0	**2**	11.16^{d}	17.39^{bc}	30.28^{b}	15.35^{a}	$7,03^{e}$	$19,25^{a}$	$28,56^{d}$	$17,20^{a}$
Significance of two way ANOVA				Buds (%)				Length (mm)	
Medium				*				*	
Time of the year				*				*	
Medium x Time of the year				*				*	

Means in each columns by different letter are significant different at α=0.05
*Significant at α=0.05, NS: no significant at α=0.05.

Table 4 Effect of a forcing solution without growth regulators on subsequent budding of *Leucospermum cordifolium* 'Flame Spike' multinodal explants in a ½ MS medium with or without GA_3. Bud percentage was recorded after 21 days in culture and shoot length after 70 days in culture.

	Nov		Feb		May		Aug	
GA_3 ($mg\ L^{-1}$)	%	mm	%	mm	%	mm	%	mm
0	49.89^{a}	17.39^{a}	2.70^{a}	**	12.01^{a}	$20,27^{a}$	9.21^{a}	$8,70^{a}$
1	53.81^{a}	25.83^{b}	2.85^{a}	**	41.89^{b}	$30,44^{b}$	10.41^{a}	$26,50^{b}$
Significance of two way ANOVA			Buds (%)				Length (mm)	
Medium			*				*	
Time of the year			*				NS	
Medium x Time of the year			*				NS	

** The low percentages of buds did not allow to study its length.
Means in each columns by different letter are significant different at α=0.05
*Significant at α=0.05, NS: no significant at α=0.05.

Table 5 Effect of different BA and GA_3 concentrations added to the forcing solution on subsequent budding of *Leucospermum* 'Tango' multinodal explants cultured in a ½ MS0. Bud percentage was recorded after 21 days in culture and shoot length after 70 days in culture.

		Buds (%)				Length (mm)			
BA (mgL^{-1})	**GA_3 (mgL^{-1})**	**Nov**	**Feb**	**May**	**Aug**	**Nov**	**Feb**	**May**	**Aug**
0	**0**	52.34^{a}	41.42^{ac}	40.50^{a}	52.66^{a}	22.56^{a}	22.61^{a}	21.80^{ab}	20.27^{ad}
25	**0**	61.43^{a}	59.36^{c}	35.74^{a}	72.44^{bc}	20.07^{abc}	22.66^{a}	17.84^{a}	19.09^{a}
50	**0**	61.46^{a}	59.92^{c}	50.26^{a}	77.42^{c}	20.33^{abc}	18.88^{a}	18.44^{a}	18.03^{a}
75	**0**	58.63^{a}	43.41^{ac}	42.98^{a}	42.12^{a}	18.07^{bc}	21.40^{a}	20.13^{ab}	6.00^{b}
100	**0**	63.07^{a}	57.64^{c}	44.93^{a}	70.03^{bc}	20.16^{abc}	18.14^{a}	25.48^{b}	12.65^{c}
25	**10**	38.82^{b}	22.02^{b}	36.98^{a}	53.37^{a}	22.17^{ab}	19.95^{a}	22.14^{ab}	22.82^{de}
50	**10**	27.46^{b}	20.57^{b}	35.38^{a}	44.32^{a}	16.73^{c}	20.37^{a}	25.36^{b}	24.09^{e}
75	**10**	38.32^{b}	44.13^{ac}	35.11^{a}	42.55^{a}	18.23^{bc}	20.05^{a}	23.37^{ab}	22.87^{de}
100	**10**	28.79^{b}	28.60^{ab}	40.92^{a}	57.46^{ab}	18.59^{abc}	16.68^{a}	23.26^{ab}	22.99^{de}

Significance of two way ANOVA	Buds (%)	Length (mm)
Medium	*	*
Time of the year	*	*
Medium x Time of the year	*	*

Means in each columns by different letter are significant different at α=0.05

*Significant at α=0.05, NS: no significant at α=0.05.

Letter

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