Rev. FCA UNCUYO. 2012. 44(2): 119-130. ISSN impreso 0370-4661. ISSN (en línea) 1853-8665.

In vitro propagation of *Glandularia peruviana* (L.) Small, an ornamental native plant from South America

Propagación *in vitro* de *Glandularia peruviana* (L.) Small, una especie ornamental nativa de América del Sur

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Originales: Recepción: 12/09/2011 - Aceptación: 24/08/2012

ABSTRACT

The flower market is characterized by being both eager for novelties and highly competitive. The exploration of native species with ornamental potential represents a remarkable area of research, since it entails the introduction and development of novel promising ornamental crops. The genus *Glandularia*, widely distributed in Argentina, holds an enormous ornamental potential, due to the variety of colors of its inflorescences (red, violet, white, rose and lily), and extended flowering period. There is little information on tissue culture of *Glandularia*, thus highlighting the relevance of this research. In this work, the conditions for *in vitro* multiplication of *G. peruviana* were optimized. It was concluded that WPM supplemented with TDZ, in concentrations ranging from 1.1 to 9.0 µM, was the most adequate treatment, rendering a multiplication rate of approximately 10 *de novo* shoots per explant. This paper presents a protocol for the *in vitro* propagation of this species and introduces interesting prospects in the application of biotechnological tools to breed *Glandularia*.

Keywords

plant tissue culture • micropropagation • native germplasm • Verbenaceae • callus

Palabras clave

cultivo de tejidos • micropropagación • germoplasma nativo • Verbenaceae • callo

RESUMEN

El mercado de plantas ornamentales se caracteriza por ser altamente competitivo y ávido de novedades. La exploración de especies nativas con potencial ornamental representa una interesante área de investigación, ya que permite la introducción y desarrollo de nuevos cultivos ornamentales a partir de ese germoplasma. El género *Glandularia*, ampliamente distribuido en Argentina, se caracteriza por la variedad de colores de sus inflorescencias (rojo, violeta, blanco, rosa y fucsia), además de un prolongado período de floración. La falta de comunicaciones publicadas sobre el cultivo de tejidos de *Glandularia* le confiere relevancia a la investigación llevada a cabo en el presente reporte. En este trabajo se probaron sobre medio WPM diferentes reguladores del crecimiento, solos y combinados entre sí y fue posible optimizar las condiciones para la multiplicación *in vitro* de *G. peruviana*. Los resultados obtenidos muestran que de las citocininas probadas, TDZ, en concentraciones que van desde 1,1 hasta 9,0 µM, fue el regulador de crecimiento más adecuado, con una tasa de multiplicación de aproximadamente 10 brotes *de novo* por explanto. Este trabajo presenta un protocolo para la propagación masiva de esta especie y muestra una interesante perspectiva en la aplicación de herramientas biotecnológicas para el mejoramiento del género *Glandularia*.

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INTRODUCTION

Floriculture is a very competitive industry worldwide and the whole sale volume is approximately 50 billion EURO (5). The flower market is characterized by being both eager for novelties and highly competitive (16). In order to provide for the markets high demand, millions of ornamental plants are routinely produced *in vitro* annually, and around 156 genera are propagated using this biotechnological technique in different laboratories around the world (16). The exploration of wild species that might have ornamental potentials represents an interesting area of research to develop novel promising ornamental crops (9). In time, this will allow obtaining a portion of the market through the rational use of native genetic resources.

South America has historically contributed actively to the development of this market; but this contribution has been carried out by the incorporation of its native ornamental germplasm to breeding programs of trans-national companies. The fact that numerous species can be found in international catalogues evidences this. Unfortunately, the countries in this region have not benefited from this contribution and the local producers are compelled to pay royalties for the right to commercialize these ornamental varieties. One of the most outstanding examples is *Alstroemeria*. This situation is the natural result of the lack of local interest in investigation and development in this particular field of study (5).

Among the native species with ornamental potential, the genus *Glandularia* stands out. This genus was described by Gmelin in 1796 and belongs to the *Verbenaceae* family, which comprises around 50 species. These are found in template and subtropical regions of North and South America. In Argentina, it is found in the provinces of Buenos Aires, Entre Ríos, Corrientes, Formosa and Santa Fe (2). The plants that belong to this genus are perennial and herbaceous and they owe their commercial popularity to the attractive color of their flowers, which are white, red, pink, lilac or violet; their prolonged summer flowering period and also, their rusticity. Some of them can be erect while others have creeping growth habits (2).

In vitro plant tissue culture, which is based on the totipotency of plant cells, is perhaps one of the most basic of the many applications that Biotechnology has to offer. However, it is a very powerful tool in the breeding and massive propagation of plants (9). In fact, micropropagation using nodal segments is a method used extensively to multiply plants. Its main advantages lie in the perpetuation of selected genotypic characters, a high propagation rate, the optimization of the space for its exploitation, the improvement in the sanitary conditions of the plants and the possibility of facilitating the international exchange of germplasm (16). An additional benefit is that, in the context of a breeding program, genetic variability can be promoted *in vitro*, either inducing cellular dedifferentiation followed by plant regeneration (4, 6) or using mutagenic agents, for example the alkaloid colchicine (1).

The ornamental value of the multiple species belonging to this genus can be largely augmented if their architecture is modified, obtaining more compact plants. As has been mentioned above, biotechnology has a number of powerful tools that can

be successfully employed to improve species, such as transformation, somaclonal variation or *in vitro* polyploidization, among others (7). The advantage of these approaches is that they can increase genetic variability without "diluting" the expression of the rest of the genes for which the specimen was selected, a frequent problem when classic breeding methods are involved (5). In light of this, it is of utmost importance to adjust a protocol allowing the *in vitro* propagation of *Glandularia*, since this is an essential requirement both for the application of biotechniques to breed the species and for the massive propagation of the developed products.

In this context, the fact that there are few reports on tissue culture of *Glandularia* emphasizes the relevance of this research. In fact, the only references found regarding the micropropagation of this genus were the investigations carried out by Marino *et al.* (11) and Ponce *et al.* (14), in addition to previous work developed in the Institute of Floriculture (INTA-Castelar, Argentina) (10). In the present work, the adjustment of a simple and effective protocol for the *in vitro* culture of *G. peruviana* is proposed.

MATERIALS AND METHODS

Disinfection of explants and establishment *in vitro* of *G. peruviana*

The explants (nodal segments) employed in this assay were obtained from a *G. peruviana* mother plant that was grown in standard greenhouse conditions. Prior to explanting, the mother plant was sprayed three times every two days with 2.5 ml/L of Kasumin® (Kasugamycine: fungicide and bactericide). To disinfect, 1.0 cm long nodal segments of 30 explants were washed with tap water during 10 minutes, and then sonicated during 20 min. They were then submerged in ethanol 70 % (1 min), followed by 5.5% active chloride (20 min) and 0.01% Tween 80.

Finally, in a laminar flow cabinet, they were washed 5 times with sterile distilled water and the oxidized sectors were removed. The explants were cultured in Woody Plant Medium (WPM) (12) without plant growth regulators (PGR), supplemented with 20 g/L sucrose and solidified with 0.7 % agar (Sigma®)**.** The pH was adjusted to 5.6 with KOH (0.1 N and 0.5 N). In this and all the assays reported in this paper, the physical culture conditions were 16h photoperiod using (52 μmol.m^{-2}.seg^{-1}) and 23 ± 2°C. In all the cases involved, the subcultures were repeated every 30 days. The results were statistically analyzed using ANOVA and the Tukey test ($p<0.05$), through the program Statistica (Statsoft. 6.1, Start Soft. Inc., USA).

In vitro response of nodal segments of *G. peruviana* to different PGRs

For these experiments, the source of explants was the *in vitro* shoots cultured on the same basal medium (WPM) used for *in vitro* establishment. In all cases, 0.75 - 1.0 cm long nodal segments were transferred to basal medium supplemented with the PGR indicated in each case. The number of explants (n) for each treatment was 10 and the assays were repeated twice. The explants were cultured on WPM supplemented with 6-benzylaminopurine (BAP) (μM): 0.0; 2.0 and 4.0 and 1-naphthaleneacetic acid (NAA) (μM): 0.0; 2.5 and 5.0; in all possible combinations.

Response to different cytokinins

The *in vitro* response of *G. peruviana* to the following cytokinins was evaluated: 6-benzylaminopurine (BAP); 6-furfurylaminopurine (KIN) and thidiazuron (TDZ) in the following concentrations (µM): 0.0; 9.0; 13.0 and 18.0.

Assay with TDZ

The basal medium was supplemented with the following concentrations of TDZ (µM): 0.0; 0.4; 1.1; 2.2; 4.5; 6.6 and 9.0. The explants were cultured on Petri dishes (10 cm diameter). The calli with shoots were subcultured to 15.0x2.5 cm tubes containing PGR-free WPM. For the rooting step the shoots were isolated from the original explant and transferred to the same medium.

Acclimatization

Acclimatization was carried out according to Escandón *et al* (6): the *G. peruviana* plantlets were carefully washed in order to eliminate the remaining agar in the roots. They were then transferred to 10 cm diameter pots containing peat-based potting substrate (Growing Mix®) and maintained in a humid chamber, which consisted of transparent polyethylene bags, and kept in the acclimatization greenhouse. The bags were gently perforated periodically to allow gaseous exchange and thus the gradual adaptation of the explant to the external atmosphere. After eight days no condensation water was detected in the polyethylene bags and they were removed.

The plantlets were watered periodically in the acclimatization greenhouse and remained there 21 days. Finally they were transferred to a standard greenhouse.

RESULTS

The combination of pre-treatment of the donor plant with Kasugamycine plus the disinfection protocol (sonication/ethanol/chloride+detergent), allowed a recovery of 70% healthy *G. peruviana in vitro* explants.

Table 1. Average number of shoots per explant obtained with the different combinations NAA/BAP tested cultured on WPM.
Tabla 1. Promedio de yemas por explanto obtenido con diferentes combinaciones de ANA/BAP.

PGR (µM)	BAP		
NAA	0.0	2.0	4.0
0.0	3.6^a	2.2^b	1.8^b
2.5	1.6^b	2.0^b	2.0^b
5.0	1.6^b	2.0^b	2.0^b

Different letters indicate differences between treatments (Tukey test, $p < 0.05$).
Letras distintas indican diferencias significativas entre tratamientos (Tukey test, p < 0,05).

Table 1 (page 122) indicates the average number of shoots per explant obtained with the nodal segments of *G. peruviana* cultured in WPM using different NAA/BAP combinations. There were significant differences between the control treatment (PGR-free) and the other treatments.

Nodal segments produced normal shoots (photo 1a and 1b, page 124) and other responses that depended on the presence, type and relations of the PGRs. When NAA was used as the sole PGR, the induction of roots was observed (photo 1c, page 124); while BAP alone induced the development of "bud-like" malformed structures (photos 1d and 1e, page 124).

Oxidation was a common feature in all the treatments, including the controls. An example of this process is exhibited in photo 1f (page 124). In addition, in those treatments where both PGRs were combined, almost all the explants presented hyperplasic characteristics (photo 1g, page 124), root development (photo 1h, page 124) and abnormal structures (bud-similar structures) (photo 1i, page 124). The normal plant development can be appreciated in the control treatment as shown in photo 1j (page 124).

In table 2, it can be observed that in all the tested treatments (with and without cytokinins), there was shoot development. However, the treatment with 9.0 µM TDZ induced multiplication rates that were significantly higher in relationship to the other PGRs tested. On the other hand, it is also significant that both KIN and TDZ presented better results than BAP in terms of multiplication rate. In fact, BAP showed no differences from the control (table 2).

Table 2. Average number of shoots per explant obtained with the different cytokinins used cultured on WPM. Cyt.: cytokinin.
Tabla 2. Promedio de yemas por explanto obtenido con las diferentes citocininas usadas. (Cyt.: citocinina).

Cyt.	µM	0.0	9.0	13.0	18.0
BAP		2.0bc	1.7bc	1.9 cd	1.2ab
KIN		1.0ab	2.6 e	1.1 a	1.0 a
TDZ		1.0ab	3.9 f	2.4 de	1.6ab

Different letters indicate differences between treatments (Tukey test, $p < 0.05$).
Letras distintas indican diferencias significativas entre tratamientos (Tukey test, $p < 0,05$).

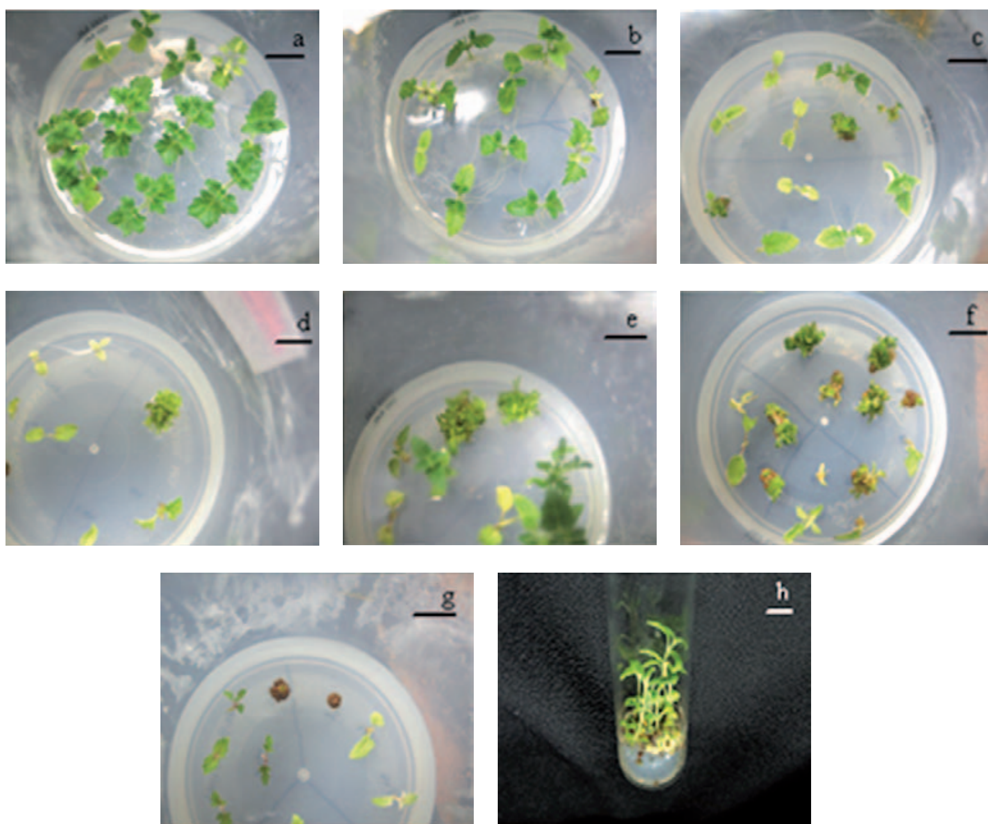
Photo 2 (page 125) shows the response of nodal segments of *G. peruviana* to different concentrations of TDZ. Explants cultured with and without the lowest concentration of TDZ (0.4 µM) showed root development, internodal elongation and a slight hyperplasia on its base (in contact with the medium) but without callus production (photos 2a and 2b, page 125). When the concentration of TDZ was gradually increased, the appearance of roots diminished and there was an increase in callus tissue (photos 2c and 2d, page 125), as well as symptoms of vitrification and tissue oxidation (photos 2f and 2g, page 125). The development of *de novo* shoots once the calli with shoots were transferred to PGR-free medium as shown in photo 2h (page 125).

a) Buds developed (arrowed) onto a callus mass cultured in 4.0 μM BAP treatment; the black arrow indicates the malformed structures showing oxidation signs. Bar: 2.5 mm. b) Isolated callus showing bud development in the treatment with 4.0 μM BAP. Bar: 5 mm. c) Nodal segment cultured in 5.0 μM NAA. Bar: 5 mm. d) "Bud-like" structures at the base of an explant cultured in 4.0 μM BAP. Bar: 2.5 mm. e) Fully oxidized "bud-like" structures (arrowed) (2.0 μM BAP). Bar: 5 mm. f) Oxidation process in an explant cultured on 4.0 μM BAP. Bar: 2.5 mm. g) Hyperplasia at the base of the explants and death at the top, intermediate relations of BAP and NAA treatments. Bar: 5 mm. h) Root development from the callus growing at 2.0 μM BAP / 2.5 μM NAA. Bar: 2.5 mm. i) "Bud-like" structures (arrowed) in an explant cultivated in 2.0 μM BAP / 2.5 μM NAA. Bar: 2.5 mm. j) Control with root development (arrowed). Bar: 5 mm.

a) La flecha blanca indica las yemas desarrolladas sobre el callo en 0,4 μM BAP. La flecha negra muestra las estructuras mal formadas y oxidadas. Escala: 2,5 mm. b) Desarrollo de yemas sobre callos aislados 4,0 μM BAP. Escala: 5 mm. c) Segmento nodal cultivado en 5 μM ANA. Escala: 5 mm. d) Estructura tipo yema en la base del explanto en medio adicionado con 4,0 μM BAP. Escala: 2,5 mm. e) La flecha indica una estructura malformada y oxidada. Escala: 5 mm. f) Explanto oxidado (4,0 μM BAP). Escala: 5 mm. g) Hiperplasia en la base del explanto y muerte en la parte superior (combinaciones intermedias de ANA/BAP. Escala: 5 mm. h) Callo generando raíz (2.0 μM BAP / 2.5 μM de ANA. Escala: 2.5 mm). i) Estructura tipo yema en el mismo tratamiento que H) 2.0 μM BAP / 2.5 μM NAA. Escala: 2.5 mm. j) Control con desarrollo de raíces. Escala: 5 mm.

Photo 1. Nodal segments of *G. peruviana* after 30 days of culture on different NAA/BAP combinations.

Foto 1. Segmentos nodales de *G. peruviana* luego de 30 días de cultivo en diferentes combinaciones de ANA/BAP.



a) Control without cytokinin. b) 0.4 µM. c) 1.1 µM. d) 2.2 µM. e) 4.5 µM. f) 6.6 µM. g) 9.0 µM. h) Evolution of the culture with 1.1 µM, once transferred to fresh medium (PGR free), after 60 days of initiation of culture. Bar: 1cm.

a) Control. b) 0,4 µM. c) 1,1 µM. d) 2,2 µM. e) 4,5 µM. f) 6,6 µM. g) 9,0 µM. h) Evolución del cultivo en 1,1 µM, una vez transferido a medio libre de reguladores. Escala: 1 cm.

Photo 2. Response of nodal segments of *G. peruviana* towards different treatments containing TDZ after 30 days of culture.
Foto 2. Respuesta de los segmentos nodales de *G. peruviana* a los 30 días de cultivo en diferentes concentraciones de TDZ (µM).

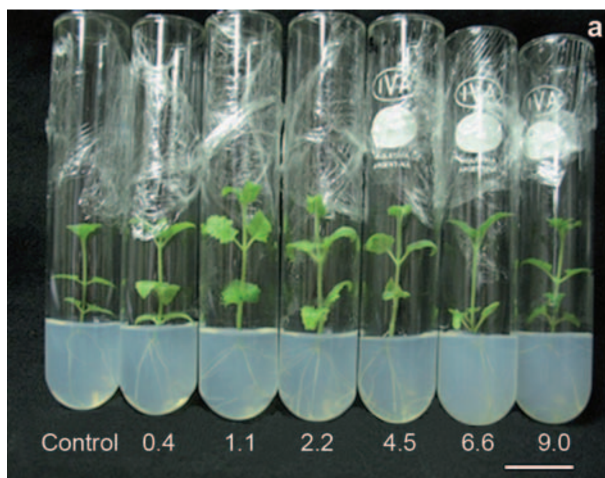
After 30 days, the corresponding multiplication rates were calculated (table 3, page 126). All the treatments tested yielded *de novo* shoots. ANOVA analysis showed significant differences between the shoot number produced by the control treatment and treatments with TDZ in concentrations of 1.1 µM. There were no significant differences among treatments using TDZ in concentrations above 1.1 µM, and the multiplication rates observed fluctuated between 11 buds per explant when 1.1 µM TDZ was used and 7 for 2.2 µM TDZ (table 3, page 126). It is important to highlight the fact that in all the treatments with concentrations of TDZ above 1.1 µM, multiple shoots were observed in the callus tissue formed at the base of the explants (photo 2).

Table 3. Average number of shoots per explant obtained with the different TDZ concentrations tested cultured on WPM.S/E: shoots per explant.
Tabla 3. Promedio de yemas por explanto obtenido con las diferentes concentraciones de TDZ aplicadas.

TDZ (μM)	S/E
0.0	1[a]
0.4	1[a]
1.1	11[b]
2.2	7[b]
4.5	8[b]
6.6	10[b]
9.0	8[b]

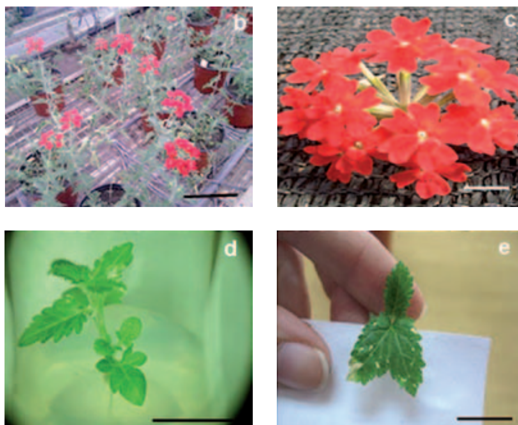
Different letters indicate differences between treatments (Tukey test, $p < 0.05$).
Letras distintas indican diferencias significativas entre tratamientos (Tukey test, $p < 0,05$).

Once the shoots were isolated, individually transferred and cultured in WPM, all of them rooted, independently of the original treatment. Furthermore, all the plantlets were acclimatized and they were finally grown under standard greenhouse conditions. Photo 3a shows details of the shoots, derived from the different TDZ treatments, in late rooting stage. Photo 3b (page 127) exhibits *ex vitro* plants grown under standard greenhouse conditions and in photo 3c (page 127) the details of an inflorescence from one of the recovered *ex vitro* plants can be appreciated. Photos 3d and 3e (page 127) display the malformations detected in leaves in some *G. peruviana* plants. Generally, these aberrations consisted in changes in the shape of the leaf (figure 3d, page 127) and/or fused leaves (photo 3e, page 127). Single-leaf verticiles were also observed.



a) Rooted plantlets from *in vitro* treatments of TDZ. Bar: 2.5 cm.
a) Plántulas enraizadas provenientes de los diferentes tratamientos con TDZ. Escala: 2,5 cm.

Photo 3. Rooting and acclimatization step of *G. peruviana*.
Foto 3. Enraizamiento y aclimatación de *G. peruviana*.



b) Flowering *ex vitro* plants under standard greenhouse conditions. Bar: 5 cm. c) Detail of the inflorescence. Bar: 1 cm. *In vitro* (d) and *ex vitro* (e) leaves showing morphological changes. Bar: 1 cm.

b) *Ex vitro* plantas florecidas bajo condiciones de invernáculo. Escala: 5 cm. c) Detalle de la inflorescencia. Escala: 1 cm. Aspecto de hojas *in vitro* (d) y *ex vitro* (e) con cambios morfológicos. Escala: 1 cm.

Photo 3. Rooting and acclimatization step of *G. peruviana*.
Foto 3. Enraizamiento y aclimatación de *G. peruviana*.

DISCUSSION

The genus *Glandularia* possesses enormous ornamental potential, due to, among other factors, the great variety in the colors of its flowers and its crop hardiness. However, there are few references regarding tissue culture in this genus (10, 11, 14). In fact, with the exception of the papers mentioned above, we have not found other reports in the literature surveyed. This is common when the goal is the study of native species.

From an ornamental point of view, one of the main features that need improvement in *G. peruviana* is its architecture. If it can be introduced *in vitro*, the use of other biotechniques such as mutagenesis, transgenesis, etc., could be applied in order to address this issue and obtain more compact plants.

Concerning the *in vitro* establishment of *G. peruviana*, the results show that the disinfection protocol applied was adequate, since 70% of the treated plants were recovered. The quantity and quality of the material obtained was suitable to perform all the tests proposed.

The *in vitro* tissue culture conditions for *G. peruviana* appear to very demanding in terms of nutritional requirements. In a previous report (10) it was determined that WPM was the most adequate medium to establish the culture of nodal segments of *G. peruviana* and other *Glandularia* species, such as *G. tenera* and *Glandularia* sp. (10).

This finding is consistent with the fact that poor soils are the natural habitat of these species. Taking this characteristic into account, WPM was chosen as basal medium, since its nitrogen concentration is 4 times lower than MS (13).

When these experiments were initiated, the only report found on tissue culture of *Glandularia* genus was reported by Marino *et al.* (11). These authors tested combinations of BAP and indole-3-butyric acid (IBA) in *G. perakii*. Their results showed a low multiplication rate. For the present work, due to the fact that NAA and BAP combinations are often used successfully in other plant species (8, 15, 17, 18), it was decided to start our tests using combinations of these growth regulators.The concentrations and the ratio of NAA and BAP used in this experiment were not adequate for the multiplication of *G. peruviana*. In fact, photo 1 (page 124) shows that few shoots and bud-like structures were obtained only when BAP was used as the sole PGR, but after 30 days of culture the main response was oxidation. Furthermore, the maximum multiplication rate observed was 3 shoots per explant.

In reference to the differences observed with the control responses registered in the first experiment (table 1, page 122), it would be possible to speculate that this fact could be due to topophysis phenomena. In some species, these strongly condition the response of the explant (3).

In an effort to improve the multiplication rate per explant that would allow, for instance, subsequent *in vitro* mutagenesis assays, attention was turned to the effect of different cytokinins on single nodal segments. The results obtained showed that *G. peruviana* responded better to low concentrations of all the PGRs tested, and the best multiplication rate was observed with 9.0 µM TDZ (table 2, page 123). Based on this data, it was concluded that TDZ was the most adequate PGR for this particular purpose, and an experiment was designed to adjust a protocol in order to promote the generation of multiple shoots. Table 3 (page 126) shows that given the conditions of this experiment, the amount of TDZ needed to obtain the highest multiplication rate would lie within the range of the concentrations tested. In fact, in concentrations below 1.1 µM the average of shoots per explant was approximately 7 times lower, and in the upper concentrations no significant differences were detected among the number of shoots per explant recovered, at least up to 9.0 µM TDZ (table 3, page 126).

Working on other species of *Glandularia*, Iannicelli *et al*. (10) found that *G. tenera* required a combination of KIN/NAA for its propagation. In the case of *Glandularia* sp *in vitro* multiplication needed less amounts of TDZ (0.5 µM).

It is important to emphasize that in both reports the oxidation process was very important since it can strongly condition future biotechnical procedures. In order to breed *Glandularia in vitro* a method to prevent or minimize the occurrence of this process would be essential. For example, the addition of antioxidants to culture medium could be standardized (10).

Comparing the results obtained here with those reported by Ponce *et al.* (14), the most relevant difference lies in the multiplication rates. This fact could be explained by several reasons, and one of them could be, hypothetically, genotypic variability. It could also be hypothesized that the basal medium used by these authors, MS, contributed to the low performance reported. Another difference found is that Ponce *et al*. (14) only reported a low percentage of single nodal segments as shoot generators, while in the present report most of the explants generated *de novo* shoots (photo 2, page 125). Except for donor plant conditions, we cannot speculate on other explanations for this discrepancy. Moreover, the report of Ponce *et al.* (14) does not indicate if the recovered shoots rose from pre-existing meristems or appeared as *de novo* shoots.

In the present report, as it is shown in photo 1a (page 124) and 2h (page 125), the *de novo* shoot regeneration occurred from the structures formed at the base of the explant and not from the pre-existing meristems. This passage through a callus phase may explain the appearance of the variants shown in photos 3d and 3e (page 127). Indeed, the process of the tissues dedifferentiation and the possible release of polyphenols (evidenced by the oxidation produced) are known to generate levels of stress high enough to cause the phenomenon of somaclonal variation (4), such as that detected in this report.This observation is of invaluable importance, since it could become the basis of a protocol to obtain a source of genetic variability *in vitro*.

CONCLUSIONS

This report shows a consistent and simple protocol for *in vitro* multiplication of *G. peruviana*. Under the culture conditions applied, WPM and TDZ appear to be decisive for the adequate multiplication rate obtained. In contrast, with the other PGRs used (KIN and BAP with or without NAA) the multiplication rates obtained were significantly lower. The oxidation process is very important since it conditions the progress of *in vitro* propagation of *G. peruviana*. These results open a very interesting scenario for the implementation of other biotechnologicaltoolsfor the breeding of this genus.

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Acknowledgements

We thank Pablo Alejandro Marinangelli for his critical revision of the manuscript and suggestions.

Also we also thank Carlos Díaz and Eduardo García Lager for their technical support.