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***In vitro* Propagation of *Oreocallis grandiflora* (Lam.) R. Br., A Medicinal Threatened Plant**

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Authors' contributions

This work was carried out in collaboration between all authors. Authors POG and CTA conceived and designed the study. Authors EMY and EMM executed and performed the experimental work. Author POG wrote the first draft of the manuscript. Author CTA reviewed the first manuscript and wrote the final manuscript. All authors read the draft and gave consent for its publication.

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ABSTRACT

Aims: To promote the conservation and biotechnology approach of *Oreocallis grandiflora*, a promising wild, medicinal and agroforestry Peruvian plant, a methodology for its *in vitro* propagation was performed.

Study Design: Disinfection and germination of seed, followed by shoot multiplication and rooting using *in vitro* techniques.

Place and Duration of Study: Follicles were collected in Yupa - Huaraz (9°30'26.76"S, 77°28'21.33"W) and the experimental procedure was done between January 2013 and December 2016.

Methodology: Follicles with different colors were disinfected with ethanol 70% at different times,

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and seeds were placed on basic culture medium composed by $\frac{1}{2}$ Murashige and Skoog, sucrose ($30 \text{ g}\cdot\text{L}^{-1}$) and phytigel ($3 \text{ g}\cdot\text{L}^{-1}$), at pH 5.7. Shoot induction was performed using N^6 -benzylaminopurine (BAP) at 0.5, 1, 2, 3 and $4 \text{ mg}\cdot\text{L}^{-1}$. For root induction, α -naphthalenacetic acid (NAA) or gibberellic acid (GA_3) at 0.25, 0.50, 0.75 and $1.0 \text{ mg}\cdot\text{L}^{-1}$ were used.

Results: Disinfection of follicles at 15 and 20 min. of 70% ethanol were successful at 100%, and all seed germinated without addition of any growth regulator. The best shoot induction treatments were 3.0 and $4.0 \text{ mg}\cdot\text{L}^{-1}$ of BAP at 60 days of PGR exposition. For the root development PGR supplement was not necessary, but GA_3 improved rooting percentage and explants growth while NAA induced callusing.

Conclusion: This is the first report of *in vitro* propagation of *O. grandiflora*, and provides important results to promote its domestication, *ex situ* conservation, and biotechnological applications.

Keywords: α -naphthalenacetic acid; gibberellic acid; N^6 -benzylaminopurine; *Oreocallis grandiflora*; Peruvian plant; Proteaceae.

1. INTRODUCTION

Oreocallis grandiflora (Lam.) R. Br. is a shrub known as "chacpá", "cucharilla", "llama llama", "saltaperico", or "atash". It grows between 1500-4000 m.a.s.l. in the Peruvian Andean valleys from Cusco to San Martín [1,2,3], but there are also reports of populations in southern Ecuador [4]. It is used as hedgerows, and its wood is used as firewood and basketry [3]. In traditional medicine, its cooked leaves are used for ulcers, and the infusion for uterus ailments and colds [1,3,4]; the decoction of its flowers is used for vaginal bleeding, ovary and uterus inflammation [5]. Decoction of its stems is drunk for liver problems, and the whole plant is mixed with other plants to be used in baths for cold [6]. Pharmacological studies have shown that ethanolic extract from its leaves have hypoglycemic activity by inhibiting α -amylase and β -glucosidase, also show antioxidant activity, with IC_{50} of $15 \mu\text{g}\cdot\text{mL}^{-1}$ compared to $5 \mu\text{g}\cdot\text{mL}^{-1}$ of α -tocopherol, providing evidence that *O. grandiflora* is a promising plant for the treatment of diabetes [7]. In spite of the ethnobotanical usefulness of *O. grandiflora*, in Peru, this species is endangered due to irrational use and absence of initiatives for its propagation [1], this problem is worsened by agricultural and forestry expansion with exotic species, that reduces the possibilities of regeneration and conservation in its natural habitats. Besides, *O. grandiflora* shows a poor capacity of vegetative propagation when live stakes are used [1]. Reynel and Marcello [2] mention that its seeds have high percentage of germination on soil with mycorrhizal fungi and greenhouse conditions, but also these seeds can be eaten by rodents. None reports about its pathogens was found, but some *O. grandiflora* plants, in its natural habit, show thin dark layer on leaves and stems similarly to

smut fungi which seems to be a pathogen of this species.

On the other hand, the use of biotechnology through plant micropropagation techniques allows the rapid production of plants at low cost, and in limited spaces [8]. These techniques are based on somatic cell culture, or tissue and organs culture under controlled conditions, to produce a high number of plantlets free of microorganisms, and in short times compared with the conventional propagation [9,10]. Thus, micropropagation is becoming an alternative for multiplication and preservation of endangered species, especially wild medicinal plants; and it can get high quality seedlings free for viral, fungal and bacterial pathogen [8].

According to these considerations, the *in vitro* tissue culture of *O. grandiflora* was conducted in order to propose an alternative for its preservation. In this sense, hypocotyls of *O. grandiflora* were evaluated about their ability to multiply by shoots, and rooting process using common plant growth regulators (PGRs) and PGR-free culture medium.

2. MATERIALS AND METHODS

2.1 Plant Material and Disinfection of Follicles

Follicles were collected in Yupa - Huaraz ($9^{\circ}30'26.76'' \text{ S}$, $77^{\circ}28'21.33'' \text{ W}$, 3554 m.a.s.l.). They were transported in sterile bags, and stored at 4°C for about five days. Branches with flowers were deposited in the David Smith Herbarium – UNASAM (Fig. 4A).

The basic medium used in all micropropagation stages was $\frac{1}{2}$ Murashige and Skoog ($\text{MS}\frac{1}{2}$)

supplemented with 30 g·L⁻¹ sucrose and 3 g·L⁻¹ phytigel, and with pH adjusted to 5.7. The environmental conditions were darkness at 24 ± 2°C for seed germination; while for establishment, shooting and rooting, the seedlings were grown using a photoperiod of 16 hours of light at 22.4 photons, and temperature of 20 ± 2°C.

To perform the disinfection of *O. grandiflora* seeds; red follicles, completely closed, healthy and undamaged were immersed in 70% ethanol at 0, 5, 10, 15 and 20 minutes. For each treatment 10 follicles were used, each one was considered as a replicate. These treated follicles were opened with a sterile small saw; and 10 seeds per follicle were grown on basic medium in polycarbonate Magenta (V8505 - Magenta™ vessel, Sigma). Disinfection efficiencies [%] were evaluated after 7 days. Ten treated seeds of each disinfected follicle were considered 100%.

2.2 *In vitro* Germination and Seedling Establishment

To evaluate germination success, five stages of seed maturation were considered based on follicle color: green, yellow, reddish-yellow, red, and brown (Fig. 4B). These were obtained following the best disinfection treatment identified in the previous step. Ten follicles per stage were used, and 10 seeds of each follicle were grown on the basic medium in polycarbonate Magenta. Germination percentage was evaluated at 15 days. The total number of seeds by follicle was considered 100%. Germinated seedlings followed growing for 35 more days to complete their *in vitro* establishment.

2.3 *In vitro* Shoot Multiplication

Under sterile conditions, seedlings were cut at their stem base to obtain hypocotyls of 3.0 cm long, they were transferred to 55 mL glass culture tubes, with 10 mL of basic medium supplemented with 0.5, 1, 2, 3 and 4 mg·L⁻¹ BAP (Sigma, B3408), and control without BAP. The samples were grown under conditions described in section 2.1. All treatments were evaluated using 2 groups, and each group had 10 hypocotyls. The percentage of shooting, and number of shoots per explant were evaluated at 15, 30, 45 and 60 days.

2.4 *In vitro* Rooting

Before rooting, shoots - containing their apical bud - from the multiplication step were

transferred to PGR-free culture medium to grow for 30 days. Shoots of similar size and thickness were selected and transferred to basic medium, with PGR supplement. NAA (Sigma, N0640) or GA₃ (Sigma, G7645) were used separately as PGR supplements at 0.25, 0.50, 0.75 and 1.0 mg·L⁻¹. Control without PGR supplement was also used. All treatments had two groups, each group had 10 shoots. Culture conditions are described in section 2.1. The parameters evaluated were: roots and callus percentage, number of roots per plantlet, main root length, and the aerial plant part length (stem with leaves) at 30, 60 and 90 days.

2.5 Statistical Analysis

The statistical design used to evaluate the percentage of disinfection and germination was the Completely Randomized Block Design (CRBD) using ten follicles (blocks) with ten seeds (replicates) for each one. The CRBD was also used to evaluate shooting, rooting and callusing; where they were design using two blocks with ten replicates each one. Number of shoots, number of roots, length of the main roots, and length of aerial part of the explants were evaluated using the Completely Randomized Design (CRD), and for which it was considered only the samples that showed positive effect represented in the percentage of shooting or rooting. Averages and standard deviations were calculated, ANOVA and the Duncan's test (*P* < 0.05) were used for mean comparison in all cases.

3. RESULTS AND DISCUSSION

3.1 Plant Material, Disinfection of Follicles, Germination, and Explant *In vitro* Establishment

Although the mature follicles of *O. grandiflora* are deshiscent, immature follicles were used to ensure that the seeds would be mostly aseptic, requiring only follicle surface disinfection. Disinfection at 100% was reached at 15 and 20 minutes exposure to 70% ethanol (Fig. 1A), which shows that its contaminant microorganisms are sensitive to ethanol. In comparison, Lema-Rumińska and Kulus [11] obtained an 83.3% disinfection efficiency of *Astrophytum asterias* seeds after treating them with 70% ethanol and 1.58% NaOCl for 15 minutes.

Seed germination is an important process in the plant life cycle and it is influenced by

environmental and physiological dormancy factors [12]. *O. grandiflora* has shown that the breaking of dormancy in its seeds did not have special requirements; furthermore, these had 100% germination without the stimulus of exogenous plant growth regulators supplied (Fig. 1B). In comparison with *A. asterias* a 70% germination rate on the same medium was recorded [11]. Also, normal growth was observed without exogenous plant growth regulators supplied in the *in vitro* establishment process. This features described before could be advantageous for *O. grandiflora* seeds, because that allows disinfected seeds to germinate and establish without PGR supplements or other special conditions.

3.2 *In vitro* Shoot Multiplication

No shooting process was observed on basic PGRs-free medium; however, a positive response was observed with BAP-supplemented in medium, which is an important cytokinin of low cost used in many biotechnological procedures because it stimulates cell division, affecting all stages of plant development and growth [13]. The development of axillar shoot needs to break the apical dominance which is controlled by auxin [14]; this breaking could be attained with the addition of cytokinin, because cytokinin

influences the distribution of auxin within the plant, also regulates the sensitivity to auxin via regulation of the Aux/IAA protein SHY2 [15], also Wang et al. [16] have found that cytokinin signaling precedes *WUS* expression in leaf axils, and activates *WUS* expression *de novo* in the leaf axil to promote axillary meristem initiation. But also, it has been described others molecular and biochemical mechanisms of diverse functions in regulating developmental and physiological processes that respond to cytokinin [15,17,18].

Since shoot formation is controlled mainly by cytokinin [9,10,15,16], and cytokinin is critical for cell division and shoot formation during *in vitro* plant regeneration [15]; shoot multiplication of *O. grandiflora* was assayed using BAP, a cytokinin initially produced synthetically but also occur at low levels naturally in some species [15]. Accordingly, it was found that shoot formation and number of shoots per explant had a direct relation with the concentration of BAP, where it was obtained up to 100% shooting in treatments with 2, 3 and 4 mg·L⁻¹ BAP at 15 days of PGR exposition (Fig. 2A). Likewise, the highest number of shoots was achieved using 3 and 4 mg·L⁻¹ BAP at 60 days with an average around 9 shoots per explants (Figs. 2B and 4C).

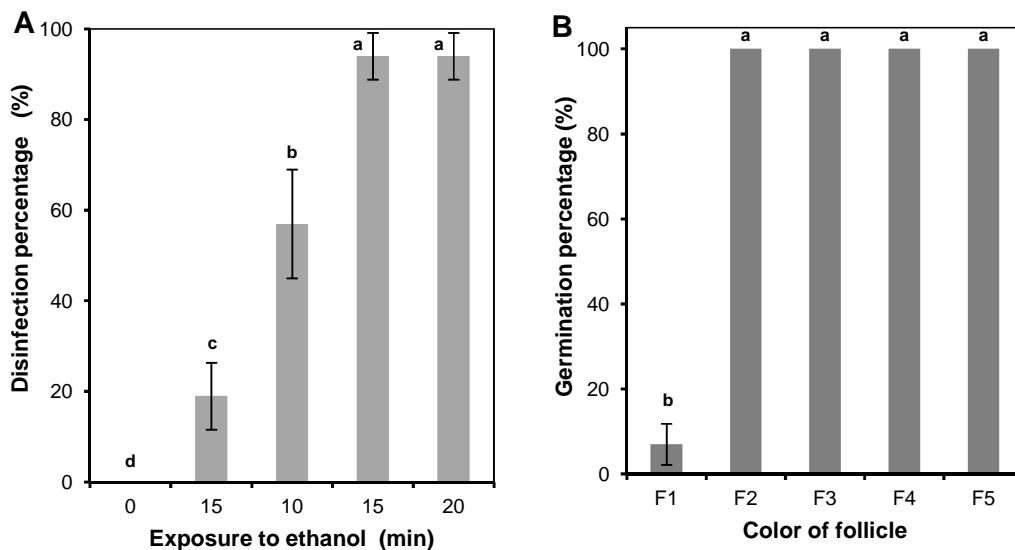


Fig. 1. Follicle disinfection and seed germination of *Oreocallis grandiflora*. A. Disinfection percentage with 70% ethanol at 0, 5, 10, 15, and 20 min.; B. Germination percentage of seed in different maturation stage based on follicle color: green (F1), yellow (F2), reddish-yellow (F3), red (F4) and brown (F5)

Values represent means of 10 block with 10 replicates each one \pm SD; and letters indicate the groups with significant differences according Duncan's statistical test ($P < 0.05$)

Similar results have been reported in some shrubs and trees, for example *Feronia limonia* produced 11.3 shoot per explant using 1.13 mg·L⁻¹ BAP [19]. *Anona squamosa* hypocotyls produced up to 23 shoot per explant with 5 mg·L BAP [20]; *Morus alba* var. *shidareguwa* showed the best shoot multiplication treatment using 1.0 mg·L⁻¹ BAP at 30 days [8]. The new shoots were cultured on PGR-free after 30 days, and did not show alterations in their leaf and stem morphology rather they had similar feature to *O. grandiflora* plants in their natural environment.

3.3 In vitro Rooting

Auxin plays an important role in the rooting process [21], but this complex process dependent of the interaction of auxin-cytokinin [22,23]. Molecular studies show that auxin-cytokinin interaction controls the root meristem development, mutually regulate their pathways and metabolisms controlling the cell division and cell differentiation of the root meristem [22]. Beside, auxins are used in tissue culture to induce callus formation, Kareem et al. [23], in their review of the molecular and biochemical mechanisms and pathways during *de novo* plant regeneration, mention that auxin play an

important role activating the development of callus, and organogenesis of root from callus.

In this sense, NAA is an artificial auxin which is used in many cases as rooting inducer, for example in *Feronia limonia*, 0.19 mg·L⁻¹ NAA induced 83% of rooting [19]. Then, the effect of NAA on rooting of *O. grandiflora* was evaluated. It was found that NAA at different doses (0.25, 0.50, 0.75 and 1.0 mg·L⁻¹) have induced callusing in shoots of *O. grandiflora*, followed by wilting and death of shoots up to 60 days (Table 1, and Fig. 4E). Kareem et al. [23] refer that the presence of callus is an effect of auxin rich culture medium; further, the formation of callus at the base of the shoots of *O. grandiflora* would be in concordance with some results reported for others woody species treated with NAA at 0.2 to 3 mg·L⁻¹ [20,24]. The wilting and death of shoots were reported by Olmos et al. [25], who explained that the callus around the cutting area of shoot disrupts the vascular connections, affecting water and nutrient assimilation. Furthermore, Porfirio et al. [21] in the review of adventitious root formation of *Olea europaea* mention that the auxin accumulated in the cutting shoots difficult rooting, suggesting that the presence of auxin required for rooting induction

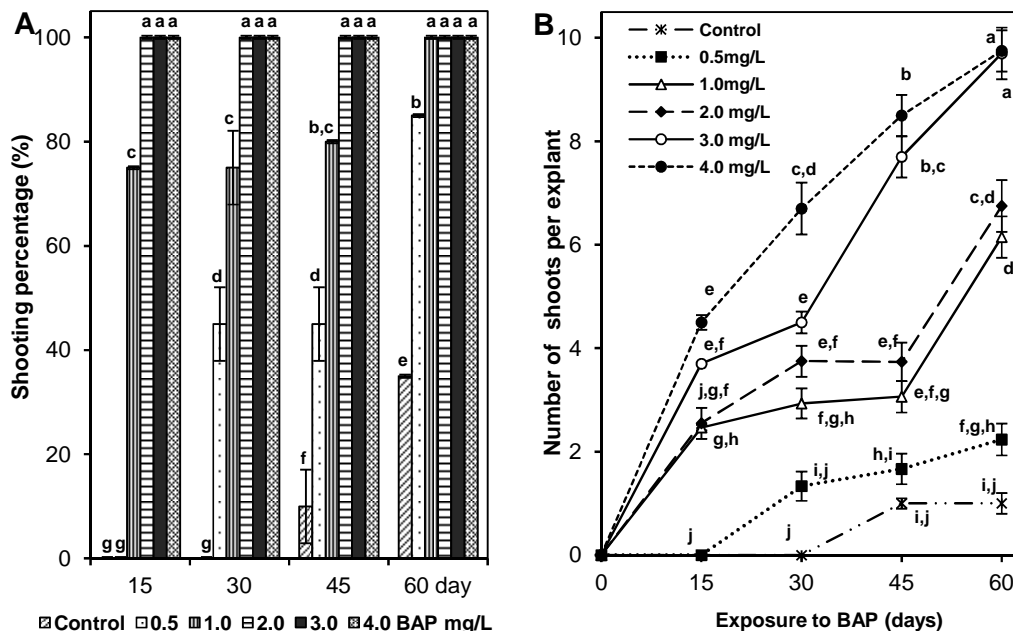


Fig. 2. In vitro shooting of *Oreocallis grandiflora* using BAP. A. Shooting percentage; and B. Number of shoots per explant

Values of 2A represent means of two blocks ± SD (each block had 10 hypocotyls); and values of 2B represents means of replicates with shoots ± SD. Letters indicate the groups with significant differences according Duncan's statistical test (P<0.05)

Table 1. Effects of NAA on *Oreocallis grandiflora* morphogenesis during rooting stage

Parameters	Exposure (days)	NAA (mg·L ⁻¹)				
		Control	0.25	0.50	0.75	1.0
Callusing (%)	30	0.0	65.0 ± 7.1	80.0 ± 0.0	100 ± 0.0	100 ± 0.0
	60	0.0	95.0 ± 7.1	100 ± 0.0	100 ± 0.0	100 ± 0.0
	90	0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Rooting (%)	30	30.0 ± 0.0	0.0	0.0	0.0	0.0
	60	40.0 ± 0.0	0.0	0.0	0.0	0.0
	90	45.0 ± 7.1	0.0	0.0	0.0	0.0
Aerial plant length ± SD (mm)	30	28.9 ± 1.9	31.3 ± 3.2	31,6 ± 2.4	27.9 ± 2.1	26.8 ± 2.5
	60	33.5 ± 2.7	32.0 ± 3.7	30,3 ± 2.5	26.4 ± 2.3	23.6 ± 2.6
	90	34.9 ± 4.3	*	*	*	*

Percentages of callusing and rooting are means of two blocks with 10 samples each one ± SD. Values of aerial plant length are means of 20 replicates ± SD. *, the plants died

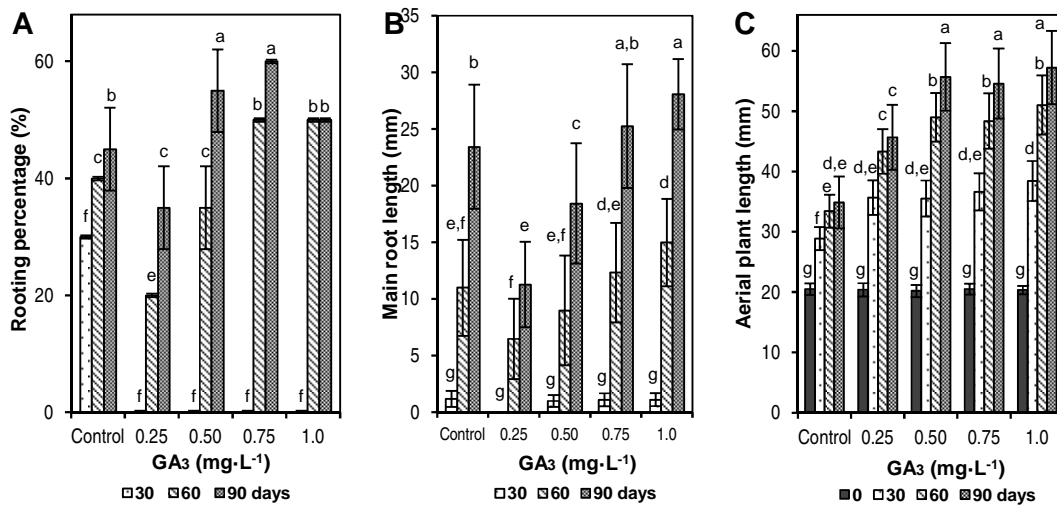


Fig. 3. *In vitro* rooting of *Oreocallis grandiflora* using gibberellic acid (GA₃). A. Rooting percentages; B. Main root length; and C. Aerial plant length (stem with leaf)

Values represent means of two blocks with 10 samples ± SD (3A); and means of samples that had roots ± SD (3B, 3C). Letters indicate the groups with significant differences according Duncan's statistical test (P < 0.05)

would have an opposite effect upon rooting expression. For this reason, some rooting methodologies include two steps: rooting induction with short times of NAA, followed by rooting expression on culture medium without NAA [9,21].

Aroonpong and Chang [8] also found that auxins were unnecessary for *in vitro* rooting of 'Shidareguwa' mulberry. Similar result was found in the PGR-free rooting medium of *O. grandiflora* (Figs. 3A, 3B, 4E); and it could be due to the presence of enough endogenous auxin in the plantlets that stimulates root formation without exogenous auxin supplement [26]. However, the culture medium supplemented with GA₃ increased rooting percentages, in this sense,

0.5 mg·L⁻¹ and 0.75 mg·L⁻¹ at 90 days induced 60% of rooting compared with 45% of PGRs-free culture medium (Figs. 3A and 4E). Likewise, similar results were found for the length of main roots, where 0.75 and 1.0 mg·L⁻¹ GA₃ at 90 days showed the best results with 25-28 mm, followed by PGRs-free culture medium with 18 mm (Fig. 3B). Furthermore, the use of GA₃ has shown positive influence on the aerial plant length, where 0.50, 0.75 and 1.0 mg·L⁻¹ GA₃ at 90 days were the best treatments with more vigorous plants, of 54 - 57 mm (Figs. 3C and 4E). Zang et al. [27] found that GA₃ improved the root system of rabbiteye blueberry (*Vaccinium ashei*), probably because gibberellins promote plant growth by stimulating photosynthesis, increasing levels of photosynthetic pigments, area and

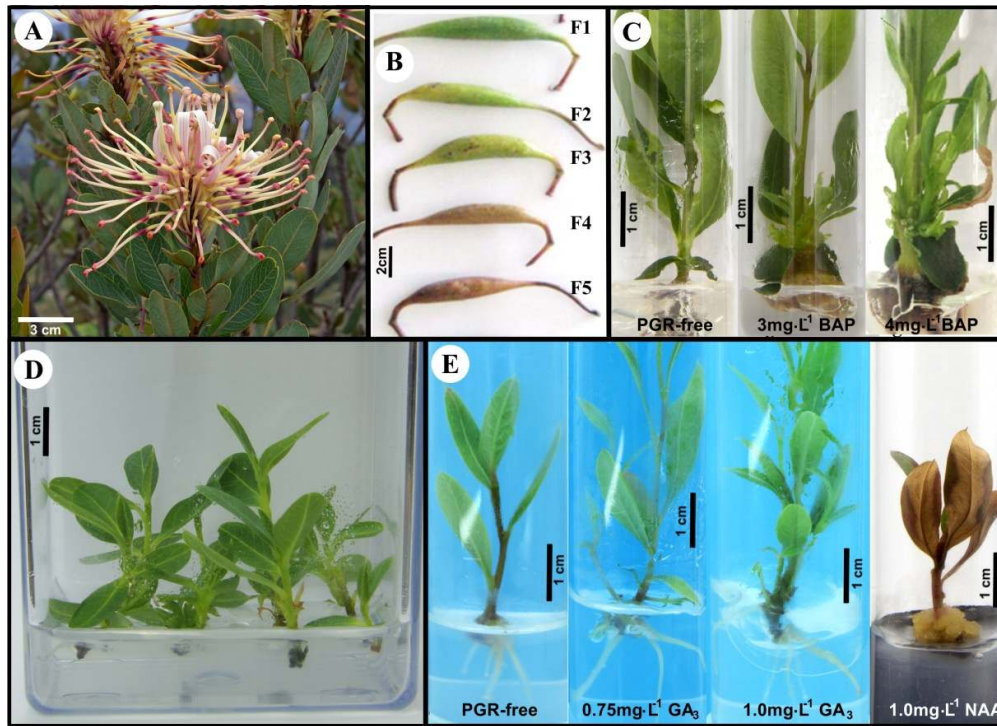


Fig.4. Plant material and micropropagation stages of *Oreocallis grandiflora* (Lam.) R. Br. A. Plant in its natural environment; B. Follicles used to evaluate seed germination: green (F1), yellow (F2), reddish-yellow (F3), red (F4) and brown (F5); C. Shooting at 60 days; D. Shoots establishment on PGR-free MS $\frac{1}{2}$ culture medium; E. Rooting at 90 days

weight of fresh and dry leaf [27,28,29]. Similarly, GA₃ plays a role on the interlayer meristem near the base of internode, inducing plant elongation [26,30].

Thus, the use of exogenous GA₃ in the rooting culture medium of *O. grandiflora* increased shoot size, rooting percentage, and root length. Then, our results have improved rooting and microshoot quality, that are important properties for acclimatization and survival of plantlets from *in vitro* tissue culture [31]. In this sense, preliminary acclimatization of *in vitro* seedlings of *O. grandiflora* was performed using natural and low-cost substrates (sterile sand, peat and moss, alone or in combinations), and it was found that probably the best substrate for the survival and growth of *O. grandiflora* in greenhouse conditions was peat-sand (1:1), during the first 30 days in chamber humidity the survival percentage was 100%, but when moisture decreased this percentage decreased down to 46% at 90 days, these results suggest that acclimatization needs more studies considering the investigation of possible soil microbial association such as mention Reynel and Marcelo [2].

The main stages of the *in vitro* micropropagation of *O. grandiflora* are represented in the Fig. 4.

4. CONCLUSION

Currently, biotechnological techniques contribute to the conservation, domestication and rational use of medicinal plant species. In that sense, micropropagation of *O. grandiflora* has shown us that 3.0 and 4.0 mg·L⁻¹ BAP were suitable for shoot formation; auxin promote callusing; and rooting is possible without supplement of exogenous plant growth regulators. However, the addition of GA₃ improves rooting percentage, root and aerial plant length. Finally, the results obtained here provide important information for *in vitro* propagation of *O. grandiflora*, but the methodology is susceptible to improvements.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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