Effect of culture medium on morphogenic processes *in vitro* in *Cinchona officinalis* L.

Efecto del medio de cultivo en los procesos morfogénicos *in vitro* en *Cinchona officinalis* L.

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Abstract

This paper describes the effect of the various plant growth regulators of the culture medium on morphogenic processes *in vitro* in *Cinchona officinalis* L, a highly vulnerable species from southern Ecuador. To do this, different concentrations of NaOCl were used in combination with different immersion times for seed disinfection; for seed germination *in vitro* GA_3 was added to the MS basal culture medium in different concentrations, and for morphogenic processes *in vitro*, different concentrations of auxins and cytokinins were combined. The decrease in the contamination rate was with high concentrations of NaOCl and an increase in the germination rate in 45 days with the addition of 1.0 mg L^{-1} GA_3 to the culture medium the hormonal combination of 0.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP showed a high rate of shoot proliferation and with 1.0 mg L^{-1} NAA a high number of roots was obtained. In the callogenesis phase, the best results were obtained with 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP for callus proliferation. *In vitro* propagation protocols were generated in *Cinchona officinalis* L, for the preservation and conservation of the species.

Keywords
plant growth • germination • organogenesis • callogenesis

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Resumen

Este trabajo describe el efecto de los diversos reguladores de crecimiento del medio de cultivo en los procesos morfogénicos *in vitro* en *Cinchona officinalis* L, una especie altamente vulnerable del sur de Ecuador. Para ello, se utilizó diferentes concentraciones de NaOCl en combinación con distintos tiempos de inmersión para la desinfección de semillas; para la germinación *in vitro* de semillas se agregó AG_3 al medio de cultivo MS basal en diferentes concentraciones, y para los procesos morfogénicos *in vitro* se combinaron diferentes concentraciones de auxinas y citocininas. Se obtuvo la disminución de la tasa de contaminación con altas concentraciones de NaOCl y aumento de la tasa de germinación en 45 días con la adición de 1,0 mg L^{-1} AG_3 al medio de cultivo; la combinación hormonal de 0,5 mg L^{-1} ANA + 2,5 mg L^{-1} BAP mostró una alta tasa de proliferación de brotes y con 1,0 mg L^{-1} ANA se logró obtener un alto número de raíces/explante. En la fase de callogénesis, los mejores resultados se obtuvieron con 1,0 mg L^{-1} 2,4-D + 0,5 mg L^{-1} BAP para proliferación de callo. Se generaron protocolos de propagación *in vitro* en *Cinchona officinalis* L, con fines de preservación y conservación de la especie.

Palabras clave
reguladores del crecimiento • germinación • organogénesis • callogénesis

Introduction

The cascarilla or *Cinchona officinalis* L, is an endemic tree located in the southern Ecuador, which for its medicinal properties has been overexploited, coming to be listed as a endangered species and is considered a priority species of conservation and study in Ecuador (19).

The *in vitro* multiplication of plant species is an alternative for the conservation of this species and arises thanks to the totipotential capacity of plant cells, which allows regenerating a whole plant from small portions of tissues, organs or plant cells under aseptic conditions and controlled (13). The morphogenic response *in vitro* is determined by the interaction of numerous factors: genotype of the donor plant, type of explant and physiological state, chemical composition of the culture medium and physical environment of the incubation of all the factors involved, growing regulators play a fundamental role in the control of morphogenesis (22).

Although the first seedlings of forest species obtained by *in vitro* were obtained in the 1960s, for some species the micro-propagation procedures are still under development, mainly due to the tremendous dependence between the response of the crop and the genotype (8, 14, 17). Species like Cinchona have not been the exception, being used to increase the productivity of alkaloids through the *in vitro* culture of diverse tissues and whole seedlings (Geerlings *et al.*, 1999; Hoekstra *et al.*, 1990; Walton *et al*., 1987); these works developed within the *in vitro* propagation in several species such as the Cinchona have been the basis for the establishment of massive propagation protocols for reintroduction and reinforcement of populations of *C. officinalis* in the natural habitat (5).

As the *in vitro* culture medium is a conservation strategy for threatened species, for allowing the massive multiplication of plants (6) and taking into account that the specimens of *C. officinalis* are sporadic in their natural environment, the rapid loss of viability of the seeds, the recalcitrance in the conservation of the seeds for long periods of time, the scarce natural regeneration, the increasing importance due to its pharmacological properties, among other factors. For this reason, the present study was carried out in order to generate protocols for *in vitro* propagation in *C. officinalis*, analyzing the effect of various plant growing regulators when triggering a satisfactory *in vitro* morphogenic response.

Materials and methods

Identification and selection of trees

The area of influence of the study was located in the southern zone of Ecuador, it shares geographical territories of the Andean, Litoral and Amazon regions; from the extreme north to the south it has a length of 156.8 km and from east to west 156.6 km including the Cantons of Loja, Catamayo and Saraguro, with a total area of 10,793 km^2. The trees of *C. officinalis* were identified based on the description of morphological aspects of the specimens: stem form, filotaxical distribution of the branches along the stem, height of the shaft, DBH (diameter at breast height) and plant health of the tree.

In vitro's seed germination

The seeds of *C. officinalis* were selected considering characteristics such as: shape, size, color, physiological maturity and good plant health conditions. In aseptic conditions, the seeds were subjected to a first disinfection with 70 % ethyl alcohol for 1 min, followed by two five-minute washes with sterile water (5). Then, in groups of 50 seeds, a second disinfection was carried out with sodium hypochlorite (commercial bleach "Ajax Chlorine" 5.25%) 15, 25 and 50 % (v/v) in water, for 5, 10 and 15 min. The culture medium used for sowing of the *in vitro* of the seeds consisted of MS mineral salts (32) supplemented with vitamins (1 mg L^{-1} of thiamine and 100 mg L^{-1} of myo-inositol), sucrose as a source of 2 % carbohydrates, agar (SIGMA®) at 0.6 % as a gelling agent and gibberellic acid (GA_3) in three concentrations (0.0, 0.5 and 1.0 mg L^{-1}). The pH was adjusted to 5.8 ± 0.2 with sodium hydroxide 1N (NaOH) before placing 10 ml parts in 11 x 25 cm glass tubes, then sterilized in an autoclave for 15 minutes at 120 °C and 1.1 kg cm^{-2}. The sowing of 2 seeds/glass tube was established; the seeds were evaluated by direct observation, every 5 days until 45 days after the sowing, the parameters evaluated were: % of contamination and % of germination.

Shoots proliferation

The caulinar apices and nodal segments of *in vitro* seedlings of 5 cm average length were selected with 1-2 nodes. The culture medium used was similar to the seed germination way, supplemented by the combination of plant growth regulators in different concentrations, NAA (naphthaleneacetic acid) 0.5 and 1.5 mg L^{-1} and BAP (6-benzyl-aminopurine) 2.5, 3.0 and 3.5 mg L^{-1}. The pH was adjusted to 5.8 ± 0.2 with sodium hydroxide 1N (NaOH) before placing 30 ml parts in glass vials (Gerber type) of 10 x 5.3 cm, then sterilized in autoclave for 15 minutes at 120 ºC and 1.1 kg cm^{-2}. Two explants/vial were established; 3 sowings were made at constant intervals of 30 days.

The explants were evaluated, every 5 days until 90 days, the parameters evaluated were: number of shoots, shoots length, number of nodes and number of leaves per explant

Rhizogenesis

The nodal and apical segments of 5 cm of average length with 1-2 nodes were selected and planted in a culture medium consisting of MS mineral salts, supplemented separately with three types of auxins: NAA, IAA (indoleacetic acid) and IBA (indolbutyric acid) in 3 different concentrations 0.1, 0.5 and 1.0 mg L^{-1}, without presence of cytokine to stimulate rooting. The pH was adjusted to 5.8 ± 0.2 with NaOH at 1N, then sterilized in an autoclave for 15 minutes at 120 °C and 1.1 kg cm^{-2}, 2 explants/vial was established. During the stages of germination, proliferation and rhizogenesis, the explants were incubated at a temperature of 23 ± 2°C, 70 % relative humidity, a photoperiod of 16/8 hours and an illuminance of 3000 lux at the crop level. The explants were evaluated, every 5 days until 90 days, the evaluated parameters were: number of roots/explant, length of roots/explant. In addition, other parameters were evaluated such as: number of shoot/explant, shoot length and number of leaves/explant.

Callogenesis

From seedlings germinated *in vitro*, aseptically proceeded to dissect in order to obtain nodal segments and caulinar apices with a dimension of approximately 0.5 cm in length and 2 cm in diameter, was seeded in the culture medium consisting of mineral salts MS, supplemented separately and combined with 2,4-D (2,4-dichlorophenoxyacetic acid) in different concentrations, 1.0, 2.0 and 3.0 mg L^{-1}) and BAP at a concentration of 0.5 mg L^{-1}. The pH was adjusted to 5.8 ± 0.2 with NaOH at 1N, then sterilized in an autoclave for 15 minutes at 120 °C and 1.1 kg cm^{-2}. Two explants/vial were established; 3 sowings were made at constant intervals of 30 days. The explants were evaluated, every 5 days up to 90 days, the parameters evaluated were: % callus formation.

Experimental design and statistical analysis

To analyze the conditions of the trial in the phase of disinfection of seeds, a completely randomized design (DCA) with a factorial arrangement of 3 x 3 was used, with 9 treatments and 3 repetitions. The germination phase was evaluated by DCA, with 3 treatments and 3 repetitions. The proliferation phase using a DCA, in a factorial arrangement of 2 x 3, with 6 treatments and three repetitions. The spread phase was evaluated by a DCA, in a factorial arrangement of 3 x 3, with 9 treatments and three repetitions. Finally, the callogenesis phase by DCA, with a factorial arrangement of 3 x 2, with 6 treatments and 3 repetitions. For the statistical analysis of the data obtained in each of the trials, the software InfoStat version 2010 (16) was used, the ANOVA analysis of variance was performed, establishing significant differences with the Duncan test at a level of significance of 0.05.

Results and Discussion

Seeds disinfections

In woody species the contamination rates are higher in many cases, the material comes from trees growing in the field where they have been associated with other organisms, likewise the degree

of contamination is also determined by the climatic conditions of the region, by what is more difficult to obtain clean explants (37). This condition was evident in the present research, since when material from the field was introduced, the high percentage of contamination did not allow its establishment *in vitro*, which forced the use of plant material from seeds germinated *in vitro*, with a sanitary regime more controlled.

The result of the percentage of contamination in *C. officinalis* according to the analysis of variance did not show significant differences between the treatments, a successful reduction of the contamination was obtained using NaClO, benefiting the establishment under *in vitro* conditions of the seeds of *C. officinalis*. It was observed that the higher the concentration of exposure to NaClO, the lower the percentage of contamination. The treatments at 25 % of NaClO during 10 min of immersion and 50 % of NaClO during 5, 10 and 15 min of immersion did not present contamination by fungi and/or bacteria being the best treatments in decrease of the contamination rate, in contrast 15 % NaClO treatments during 5 and 10 min of immersion showed a higher contamination rate of 13.33 %, caused by fungi and/or bacteria (table 1, figure 1A, page 60); filamentous fungi and bacteria are the main contaminants in *in-vitro* seed disinfection tests, they remain attached to seeds under natural conditions (12, 18, 38).

The results of the variable percentage of contamination are related to those obtained by Conde *et al.* (2017) in seeds of *Loxopterygium huasango* spruce ex Egl, using concentrations of 50 % NaOCl, obtained 0 % contamination in the *in vitro* cultivation. Other authors have also successfully used chlorine in crops in which contamination hinders the establishment of seeds and explants at *in vitro* conditions. According to Romero (2000), in disinfection of explants in *Annona muricata*, observed that the higher concentration and time of exposure to NaOCl, the lower percentage of contamination, results similar to those obtained in this research.

Seed germination *in vitro*

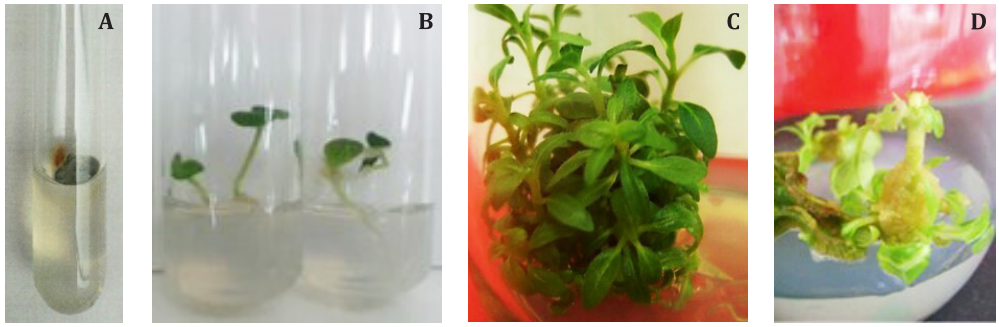
In the present research, the germination of the seeds of *C. officinalis* was considered when the embryo absorbed water and increased its diameter and length, eventually breaking the seed coat. Among the different *in vitro* germination treatments of seeds in *C. officinalis* applying different concentrations of GA_3, significant differences were found ($p = 0.0379$), the maximum germination reached for the species was 74.44 % with the treatment of 1.0 mg L^{-1} GA_3 was presented at 15 days and stabilized 45 days after sowing (table 2, figure 1B, page 60).

In the present research, an acceptable germination of the seeds of *C. officinalis*

Table 1. Effect of sodium hypochlorite (NaOCl) on the disinfection of *C. officinalis* seeds

Tabla 1. Efecto del hipoclorito de sodio (NaOCl) en la desinfección de semillas de *C. officinalis*

Treatments	% contamination
T1 (15 % NaOCl - 15 min immersion)	6.67
T2 (15 % NaOCl - 10 min immersion)	13.33
T3 (15 % NaOCl - 5 min immersion)	13.33
T4 (25 % NaOCl - 15 min immersion)	6.67
T5 (25 % NaOCl - 10 min immersion)	0.00
T6 (25 % NaOCl - 5 min immersion)	6.67
T7 (50 % NaOCl - 15 min immersion)	0.00
T8 (50 % NaOCl - 10 min immersion)	0.00
T9 (50 % NaOCl - 5 min immersion)	0.00



A) seed *in vitro* after the disinfection phase with NaClO in different concentrations (15, 25 and 50%) and different times of immersion (5, 10 and 15 min); B) germination of seeds with 1.0 mg L^{-1} GA_3; C) proliferation of shoots at apices and nodal segments with 0.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP; D) proliferation of calluses in apices and nodal segments.

A) semilla *in vitro* después de la fase de desinfección con NaClO en diferentes concentraciones (15, 25 y 50%) y diferentes tiempos de inmersión (5, 10 y 15 min); B) germinación de semillas con 1.0 mg L^{-1} AG_3; C) proliferación de brotes en ápices y segmentos nodales con 0.5 mg L^{-1} ANA + 2.5 mg L^{-1} BAP; D) proliferación de callos en ápices y segmentos nodales.

Figure 1. *In vitro* germination and proliferation of *C. officinalis* in MS culture medium with different growth regulators.

Figura 1. Germinación y proliferación *in vitro* de *C. officinalis* en medio de cultivo MS con diferentes reguladores de crecimiento.

Table 2. Effect of Gibberellic acid (GA_3) on seed germination of *C. officinalis.*

Tabla 2. Efecto del ácido giberélico (AG_3) en la germinación de semillas de *C. officinalis.*

Treatment	% germination	p - value	E.E
T1 (0.0 mg L^{-1} GA_3)	50.00 AB	0.0379	7.09
T2 (0.5 mg L^{-1} GA_3)	41.11 A		
T3 (1.0 mg L^{-1} GA_3)	74.44 B		

Average ± E.E; the letters in common mean that the values are not statistically different p> 0.05 according to Duncan's test.

Promedio ± E.E; las letras en común significan que los valores no son estadísticamente diferentes p> 0,05 según la prueba de Duncan.

germinated sowed *in vitro* in the MS culture medium was obtained, the germination rate could be influenced by several factors, among which not all the seeds, despite having been selected they were not viable and only those with a viable embryo germinated, also, the seeds of *C. officinalis* are generally non-endospermic, very small and show latency as characteristic of this genus, or also to the ABA concentration of the seeds (4), so that if its concentration is high, germination is low. However, in comparison with what occurs in nature with *C. officinalis*, where the germination of the seeds produced is low and of these only a very small percentage reaches the adult stage (5), the percentage of germination obtained in this work was satisfactory.

It is evident in this work that as the concentration of GA_3 was increased, the percentage of germination increases, which is in concordance with what was mentioned by López-Granados and García-Torres (1996), they pointed out that *in vitro* germination has advantages because it increases the germination rate, reduces the time and homogenizes the germination by the hormonal action (AG_3) stimulating the germination of the seeds.

Other authors have obtained results similar to those obtained in this research, Jäer (2014) showed that seeds of *C. pubescens* germinate between 10 to 40 days using the same amount of 1.0 mg L^{-1} GA_3. Armijos-González and Pérez (2016) showed germination rates in *C. pubescens* of 53.5 % and *C. officinalis* of 56.6 % in germination studies and their relationship with the content of phenols in the seeds, and with the use of photoperiod they increased the germination percentages in *C. pubescens* 90.0 % and *C. officinalis* 86.7 %. Likewise, Koblitz *et al.* (1983) obtained 5.8 % germination in seeds of *C. succirubra* and 15 % for *C. ledgeriana.*

Shoots proliferation

In general, organogenesis is regulated by a cytokinin-auxin relationship, where a high ratio induces caulogenesis and a low ratio induces root. In this way, the cytokinins boost the development of axillary buds by breaking the apical dominance regulated by the auxinic activity of the apex (21). In this investigation, when using BAP in high concentrations with respect to NAA, significant differences were found between *in vitro* caulogenic treatments in apices and nodal segments of *C. officinalis* applying different concentrations of NAA and BAP (p = 0.0016). Treatment with 0.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP obtained the highest proliferation of shoots/explant (6.11), number of leaves (6.18) and nodes/explant (2.93) (table 3; figure 1C, page 60); thus, high concentrations of cytokines (1 to 10 L^{-1}) induce the formation of adventitious buds, but inhibit root formation (21).

As observed in this study, BAP cytokinins were the growth regulator that induced shoots in *C. officinalis*, this exogenous cytokinin supply contributed to obtain this response possibly with synergistic effect on the good physiological condition of the explants. Similar results were described by Córdova (2012), with a hormonal concentration of 0.1 mg L^{-1} NAA + 1 mg L^{-1} BAP was able to obtain 4 shoots/explant in *C. officinalis*.

Table 3. Effect of interaction of the naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) on the proliferation of shoots of *C. officinalis*

Tabla 3. Efecto de la interacción de ácido naftalenoacético (ANA) y 6-bencilaminopurina (BAP) en la proliferación de brotes de *C. officinalis*

Treatments	Nº shoots/ explant	Length of shoots (mm)	Nº leaves/ explant	Nº nodes/ explant
	p-value = < 0.0016 E.E = 0.40	p-value = 0.0029 E.E = 0.58	p-value = 0.0006 E.E = 0.30	p-value = 0.0025 E.E = 0.17
T1 (0.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP)	6.11 A	15.81 AB	6.18 A	2.93 A
T2 (0.5 mg L^{-1} NAA + 3.0 mg L^{-1} BAP)	2.78 BC	12.45 D	3.02 D	1.56 C
T3 (0.5 mg L^{-1} NAA + 3.5 mg L^{-1} BAP)	3.93 B	14.52 BC	4.26 BC	2.04 BC
T4 (1.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP)	2.24 C	13.50 CD	3.81 CD	1.60 C
T5 (1.5 mg L^{-1} NAA + 3.0 mg L^{-1} BAP)	3.10 BC	17.76 A	4.66 B	2.21 B
T6 (1.5 mg L^{-1} NAA + 3.5 mg L^{-1} BAP)	3.09 BC	15.53 BC	3.82 CD	1.79 BC

Average; the letters in common mean that the values are not statistically different p> 0.05 according to Duncan's test.

Promedio ± E.E; las letras en común significan que los valores no son estadísticamente diferentes p> 0,05 según la prueba de Duncan.

Armijos-González and Pérez (2016) in *C. officinalis* with 3.0 L^{-1} IBA + 5.0 L^{-1} BAP obtained 5.3 shoots/explant. Other studies on shoots proliferation in other forest species were reported by Días (2012) in *Cedrela montana* Moritz ex Turcz, with 2.0 mg L^{-1} BAP obtained results of 3 shoots/explant. Daquinta *et al.* (2003) evaluated different concentrations of cytokinins with the aim of stimulating the emission of shoots in *Tectona grandis* and *Swietenia macrophylla*, the best results in *Tectona grandis* were obtained with a MS culture medium + 1.0 mg L^{-1} BAP with an average of 2.6 shoots/explant and 2.3 nodes/explant. In *Swietenia macrophylla* the most effective concentration resulted with the MS + 1.0 mg L^{-1} BAP with an average of 7.2 shoots/explant, among other forest species, in which the use of cytokinins has contributed to its shoots. However, in most of these forest species and in the results observed in this investigation, the concentrations used did not exceed 10 mg L^{-1}. So, in the case of *C. officinalis*, having to use low concentrations of BAP, it is because the plant tissue is efficiently metabolizing the hormone for the proliferation of shoots.

Likewise, similar observations in some studies were described by Daquinta *et al.* (2003), the higher concentration of cytokinin (BAP) leads to greater number of shoots in forest species. Hoekstra *et al.* (1990) showed that when there is a relatively higher level of cytokinins (BAP) against auxins (NAA) in the culture medium, the tissue manifests the formation of new shoots, if on the contrary the levels of the two hormones are reversed so that the proportion of auxins is greater compared to the cytokinins, tissue expression changes and roots originate.

The results also showed, that when decreasing the BAP concentration in the MS culture medium, an increase was observed on the number of nodes and leaves of *C. officinalis* under *in vitro* conditions. Similarly, Ramos *et al.* (2000) in studies on forest species, observed that the number of nodes and leaves in the shoots increased as the levels of cytokinins in the environment decreased. While, inversely it was observed, when increasing the concentration of BAP (1.5 mg L^{-1} NAA + 3.0 mg L^{-1} BAP) in the MS culture medium, shoots with greater length (17.76 mm) were obtained (table 3, page 61), on the other hand, other studies reported that as the concentration of BAP in the culture medium was increased, a decrease in the length of the shoots was observed and with it a reduction in the number of nodes and leaves (33); probably, the low concentrations used of cytokinins in this study lower than 10 mg L^{-1} contributed to not observing this response.

Rhizogenesis

It has been observed that the presence of auxins in the culture medium is essential for root formation *in vitro* and boosts cell lengthening, callus formation and adventitious roots (42).

In the present investigation, significant differences were observed between *in vitro* rooting treatments of caulinar apices and nodal segments adding different concentrations of NAA, IAA and AIB, for root number/explant ($p = <0.0001$) and root length ($p = <0.0001$). It was observed that the treatments with NAA in the MS culture medium were the ones that favored the number and length of roots/explant. The results also showed that, increasing the concentration of NAA to 1.0 mg L^{-1} in the MS culture medium, an increase was observed on the number of roots/explant (5.31); Husen and Pal (2003) also observed that increasing the amounts of auxin in the medium increases the percentages

of rooting. While, inversely, the concentration of NAA at 0.1 mg L^{-1} in the MS culture medium was observed, roots were obtained with a longer length (6.11 mm) (table 4; figure 2B, page 64).

The results obtained show that the number and length of the roots is dependent on the amount of auxin present in the MS culture medium. However, regardless of their size, the roots formed *in vitro* are characterized mostly by being physiologically inefficient and functional due to the easy hydration and nutrition of the tissues from the culture medium. Additionally, it has been observed that, anatomically, the vascular connections between the stem and the roots formed *in vitro* are very weak, which generally favors their rapid deterioration, needing to be quickly replaced by new radical formations during the *ex vitro* conditioning phase (3). Likewise, it was observed that NAA in concentrations of 0.5 mg L^{-1}, boosts the number of shoots/explant (3.74) and shoot length (31.29 mm), and by decreasing NAA to 0.1 mg L^{-1} favored the number of leaves/explant (6.20) (table 4). A positive relationship was observed between NAA and the variables number of roots, length of roots and number and length of shoots, is also a favorable relation for the establishment of the plants in the MS culture medium and will allow a greater survival of *C. officinalis* during establishment in the greenhouse.

Other authors also obtained results similar to those of this study, such as those reported by Lozano (2014) who obtained a 54.16 % roots/explant using concentrations of 5.0 mg L^{-1} NAA with 10.0 g L^{-1} sucrose in *Arabica Coffee*; Quintero (2000) in studies carried out *in vitro* in *Dioscorea*

Table 4. Effect of Naphthalene Acetic Acid (NAA), Indoleacetic Acid (IAA) and Indolebutyric Acid (IBA) in the induction of rooting in apices and nodal segments of *C. officinalis*

Tabla 4. Efecto de ácido naftalenoacético (ANA), ácido indolacético (AIA) y ácido indolbutírico (AIB) en la inducción de enraizamiento en ápices y segmentos nodales de *C. officinalis*

Treatments	Nº roots/ explant	Length of roots (mm)	Nº shoots/ explant	Length shoots/ explant (mm)	Nº leaves/ explant
	p-value = <0.0001 E.E = 0.50	p-value = <0.0001 E.E = 0.40	p-value = 0.0019 E.E = 0.35	p-value = 0.0076 E.E = 2.03	p-value = 0.0011 E.E = 0.40
T1 (0.1 mg L^{-1} NAA)	0.90 B	6.11 A	3.37 AB	29.12 ABC	6.20 A
T2 (0.5 mg L^{-1} NAA)	1.53 B	2.78 B	3.74 A	31.29 A	3.21 C
T3 (1.0 mg L^{-1} NAA)	5.31 A	3.03 B	2.78 ABC	30.76 AB	4.25 BC
T4 (0.1 mg L^{-1} IAA)	0.24 B	2.24 B	3.33 AB	27.40 ABC	3.31 C
T5 (0.5 mg L^{-1} IAA)	0.54 B	3.10 B	1.90 CD	24.10 BCD	4.75 B
T6 (1.0 mg L^{-1} IAA)	0.53 B	3.09 B	1.03 D	22.96 CD	3.73 BC
T7 (0.1 mg L^{-1} IBA)	0.24 B	2.24 B	3.63 A	26.45 ABC	2.99 C
T8 (0.5 mg L^{-1} IBA)	0.54 B	3.10 B	2.46 BC	17.92 D	3.02 C
T9 (1.0 mg L^{-1} IBA)	0.75 B	3.09 B	2.69 ABC	22.63 CD	3.35 C

Average, the letters in common mean that the values are not statistically different p> 0.05 according to Duncan's test.

Promedio ± E.E; las letras en común significan que los valores no son estadísticamente diferentes p> 0,05 según la prueba de Duncan.

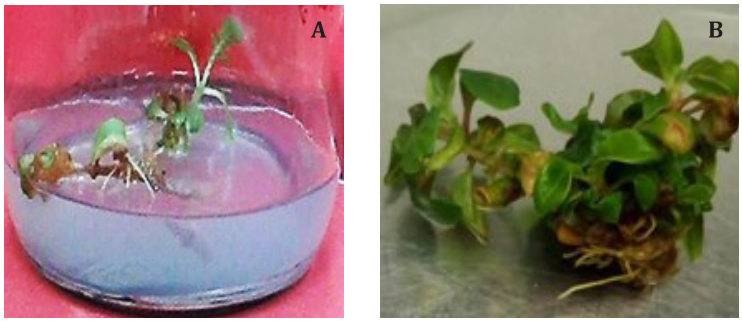
alata obtained an average rooting of 3.6 roots/explant with a concentration of 0.9 mg L^{-1} ANA, and Uribe *et al.* (2012) showed that auxin NAA is an excellent inducer of root growth and callogenic tissue.

Furthermore, with the treatment of 1.0 mg L^{-1} IBA root proliferation was observed by indirect organogenesis with an average of 0.75 roots/explant and root length of 3.09 mm (figure 2A); *C. officinalis*, could spontaneously root through indirect organogenesis depending on the endogenous levels of the growth regulators, even affirming that the presence of high concentrations of IBA in the medium tend to decrease the percentage of rooting and increase the formation of calluses in the base of stems (1).

Similar studies have been carried out in other forest species using IBA to induce rooting in the *in vitro* cultivation. Días (2012) obtained an average *in vitro* rooting of 2.4 roots/explant in *Cedrela montana*, with a concentration of 1.0 mg L^{-1} IBA and a average root length of 30.58 mm; Conde *et al.* (2017) in *Loxopterygium Huasango* with 0.5 mg L^{-1} IBA, and Uribe *et al.* (2012) for the *in vitro* rooting of *Nothofagus glauca* with 1.0 mg L^{-1} of AIB. Thus, Daquinta *et al.* (2003) showed that auxin IBA exerts an inductive rooting effect at the *in vitro* level.

Callogenesis

The results of callus induction *in vitro* showed that when different concentrations of 2,4-D (1.0, 2.0 and 3.0 mg L^{-1}) and BAP (0.5 mg L^{-1}) were applied, significant differences were obtained between treatments ($p = 0.0001$). It was evidenced that the exogenous addition of 2,4-D in combination with BAP to the culture medium, benefited the formation of calluses in the treatments.



A) root proliferation by indirect organogenesis in apices and nodal segments with 1.0 mg L^{-1} IBA; B) root proliferation by direct organogenesis in apices and nodal segments with 1.0 mg L^{-1} NAA.

A) proliferación de raíces por organogénesis indirecta en ápices y segmentos nodales con 1.0 mg L^{-1} AIB; B) proliferación de las raíces por organogénesis directa en ápices y segmentos nodales con 1.0 mg L^{-1} ANA.

Figure 2. *In vitro* root formation in *C. officinalis* in MS culture medium with different growth regulators.

Figura 2. Formación de raíces *in vitro* en *C. officinalis* en medio de cultivo MS con diferentes reguladores de crecimiento.

The highest proliferation of callus *in vitro* (65 %) was achieved with the minimum concentration of 2,4-D (1.0 mg L^{-1}) in combination with BAP (0.5 mg L^{-1}), obtained after 15 days and stabilizing at 65 days (table 5, figure 3A).

Other authors reported similar results in other forest species using BAP and 2,4-D in the MS culture medium (31), demonstrated that BAP + 2,4-D increased the development of calluses with roots and embryogenic potential and that low concentrations of these 2 growth regulators induce a greater development of embryogenic callus. Sharma, *et al.* (2014) showed that the hormonal combination of IBA with BAP generates callus formation, obtained adventitious shoots in *Tylophora indica*, in a culture medium supplemented with 5.0 mg L^{-1} BAP and 3.0 mg L^{-1} IBA. Liu *et al.* (2011) in *Tylophora indica* obtained a high proliferation of callus using a combination of BAP + IBA there are other researches in the type *Cinchona* sp. of the

Table 5. Effect of the interaction of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl-aminopurine (BAP) on the induction of callus of *C. officinalis.*

Tabla 5. Efecto de la interacción de ácido 2,4-diclorofenoxiacético (2,4-D) y 6-bencil- aminopurina (BAP) en la inducción de callos de *C. officinalis.*

Treatments	% of formation of callus	p-value	E.E
T1 (1.0 mg L^{-1} de 2,4-D)	51.67 B	0.0001	8.4
T2 (2.0 mg L^{-1} de 2,4-D)	16.67 A		
T3 (3.0 mg L^{-1} de 2,4-D)	13.33 A		
T4 (1.0 mg L^{-1} de 2.4-D + 0.5 mg L^{-1} BAP)	65.00 B		
T5 (2.0 mg L^{-1} de 2.4-D + 0.5 mg L^{-1} BAP)	48.33 B		
T6 (3.0 mg L^{-1} de 2.4-D + 0.5 mg L^{-1} BAP)	53.33 B		

Average, the letters in common mean that the values are not statistically different p> 0.05 according to Duncan's test.

Promedio ± E.E; las letras en común significan que los valores no son estadísticamente diferentes p> 0,05 según la prueba de Duncan.

A) proliferation of translucent, whitish and spongy callus in explants with 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP; B) change in callus coloration, white to brown and/or brownish; C) differentiation of the compact globular structure (proembryo) with 0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} KIN.

A) proliferación de callo translúcido, blanquecino y esponjoso en explantes con 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP; B) cambio en la coloración del callo, de color blanco a tono marrón y/o carmelita; C) diferenciación de la estructura globular compacta (proembrión) con 0,1 mg L^{-1} 2,4-D + 0,5 mg L^{-1} KIN.

Figure 3. *In vitro* callogenesis of *C. officinalis* in MS culture medium with different combinations of growth regulators.

Figura 3. Callogénesis *in vitro* de *C. officinalis* en medio de cultivo MS con diferentes combinaciones de reguladores de crecimiento.

role of growth regulators in the production of secondary metabolites in cell cultures (7, 36).

The growth regulators are determinants in the embryogenic response, they interact with the levels of endogenous hormones and there is also the possibility that the answers are given by the joint action of two or more regulators (2, 40).

The friable callus presented also small globular structures (figure 3C, page 65), these results are corroborated by Loyola *et al.* (1999) who observed in friable embryogenic tissue the appearance of globular structures characteristic of somatic embryos, refer to these structures as proembryos and indicate they were originated from mesophilic or epidermal cells.

Conclusions

It was possible to stimulate different pathways of morphogenic responses in *C. officinalis*, for which 0 % contamination was achieved by fungi and endogenous bacteria that remain latent in the seeds of the species, applying high concentrations of NaOCl and the germination rate was increased with the addition of 1.0 mg L^{-1} GA_3 to the MS culture medium. A high proliferation rate of shoots, nodes and leaves with 0.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP was also boosted; likewise, with 1.0 mg L^{-1} NAA, a greater number of roots/explant was obtained. Finally, the addition of 1.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP to the MS culture medium benefited the proliferation of callus/explant. Results that contribute to the establishment of *in vitro* plant culture for studies of morphogenesis in endemic forest species.

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