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ORIGINAL ARTICLE – Conservation of Nature

Indirect Shoot Regeneration Using 2,4-D Induces Somaclonal Variations in *Cinchona officinalis*

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Abstract

Cinchona officinalis is an important species from the Andean cloud forest that has a low regeneration rate in natural populations. *In vitro* regeneration of *C. officinalis* has been successfully established but somaclonal variation was not evaluated. The regeneration pathway and the number of subcultures on somaclonal variation were evaluated using six ISSR primers that amplified 58 loci of Inter Simple Sequence Repeats (ISSR). A dendrogram based on Jaccard´s genetic distance between the subcultures and the donor plant was produced. The results show that indirect shoot regeneration induces somaclonal variation, in the presence 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with kinetin and 6-Benzylaminopurine (BAP). In combination with 1-Naphthaleneacetic acid (NAA) or with Indole-3-butyric acid (IBA), BAP produces genetically stable explants. The highest proliferation rate was achieved using BAP and IBA. The present research study suggests avoiding the use of 2,4-D when *C. officinalis* is propagated for reintroduction and restoration projects.

Keywords: *In vitro* regeneration, subcultures, auxin, ISSR.

1. INTRODUCTION AND OBJECTIVES

Cascarilla, *Cinchona officinalis* Linneo. (Rubiaceae) is a tree species from the Andean cloud forest of southern Ecuador and northern Peru (Brako & Zarucchi, 1993; Andersson et al., 1998). During the period between the 16th and 19th centuries, this species had enormous importance for the cure of malaria (Jaramillo-Arango, 1947; Cuvi, 2009). The use of tree bark caused a huge decrease in natural populations (Arias, 2017). Besides this, habitat fragmentation due to urban development, agriculture, and livestock establishment resulted in small and dispersed populations of the species (Madsen, 2002; Arias, 2017; Cueva et al., 2019).

Cinchona officinalis presents a high germination rate, ranging from 50 to 87% in assays performed with *in vitro* and *ex vitro* controlled conditions (Caraguay–Yaguana et al., 2016; Armijos-González & Pérez-Ruiz, 2016; Romero-Saritama & Munt, 2017). Despite this fact, the species requires specific conditions to continue its development in nature, resulting in a low regeneration rate in the remnant populations (Acosta, 1947; Garmendia, 2005). In a recent study (Cueva et al., 2019), the genetic diversity of six populations of *C. officinalis* was analyzed. The majority of the populations studied were composed of adult plants located in areas of difficult access in fragmented forest and pasture areas. They suggested that the moderate levels of genetic diversity of these populations have to be conserved, and that it is necessary to develop strategies to protect and conserve the remnant populations.

Ex situ management and conservation techniques are important tools to allow for the recovery and conservation of threatened species. *In vitro* culture is a useful tool for those species that present difficult regeneration under natural conditions, being successfully applied to many rare or threatened species (Slazak et al., 2015; Upadhyay et al., 2015). The species of the *Cinchona* genus have not been the exception. This technique was used in the 1980s and 1990s in order to increase alkaloid productivity through *in vitro* cultivation of various tissues (Koblitz et al., 1983a; Koblitz et al., 1983b; Hay *et al.* 1986, 1987; Hoekstra et al., 1990). The most successful of these techniques is the culture of cells in suspension before callus formation. Some of the cited works had been the base for the establishment of protocols of *in vitro* massive propagation that aim to reintroduce and reinforce the

populations of *C. officinalis* in the natural habitat (Armijos-González & Pérez-Ruiz, 2016).

In vitro culture generates specimens that can be genetically identical to the mother plant; however, in the process, there is a risk of inducing the appearance of variation between the parental cell line and its regenerants (Larkin & Scowcroft, 1981; Sarmast, 2016). The occurrence of these variations is known as somaclonal variation. This is spontaneous and uncontrolled, limiting the usefulness of the mass propagation system because of its unpredictable nature (Singh et al., 2011). Somaclonal variation or genetic fidelity can be analyzed at *different* levels: morphological, cytological, cytochemical, biochemical, and molecular (Chandrika et al., 2008; Ruffoni & Savona, 2013). At the molecular level, the most efficient are molecular markers based on DNA (Tiwari et al., 2013). Among the molecular markers, one of the most used is the Inter Simple Sequence Repeat (ISSR) analysis which allows detecting the variation that occurs in microsatellite regions scattered in the genome (Sharma et al., 2014; Akdemir et al., 2016; Martinez- Estrada et al., 2017; Babu et al., 2018; Rohela et al., 2020).

Even though there are many studies on *in vitro* culture in the *Cinchona* genus, as far as know this is the first work to evaluate somaclonal variation in a *Cinchona* species. In this study, we determine the influence of two factors on the somaclonal variation of *C. officinalis*: the regeneration or propagation pathway using different Plant Growth Regulator (PGR) combinations and the number of subcultures.

2. MATERIALS AND METHODS

2.1. Plant material

The laboratory work was developed in the Plant Physiology Laboratory of the Department of Biological Sciences (3°59'14.2 "S 79°11'52.5 "W) of the Universidad Técnica Particular de Loja, Loja, Ecuador.

Mature seeds of *C. officinalis* were procured from the Aguarango sector in Vilcabamba (Loja-Ecuador). After collection, the seeds were surface-sterilized as described in Armijos-González & Pérez-Ruiz (2016) and germinated on a half strength Gamborg B5 medium. Seeds were incubated at 22.0 °C with 12 h light/12 h dark with a photon flux density of 57 lmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. Seedlings 4 months old and 6-8 cm in height were used to begin cultivation. Each seedling was considered as a donor plant.

2.2. In vitro culture establishment

Nodal segments, with 2 or 3 buds each and 1.0 - 1.5 cm in size, were cultivated on a half strength Gamborg B5 medium containing different combinations of cytokinins (BAP, KIN) and auxins (NAA, IBA, 2,4-D) that resulted in direct shoot or callus formation depending on the type and concentration of the hormone used (Armijos-González & Pérez-Ruiz, 2016). A Gamborg B5 medium was used as control.

2.3. Effect of subculture on shoot multiplication

The effect of subculture cycles on shoot formation was evaluated. New nodal segments derived from the plants of the first culture in the PGR-containing medium were subcultured every three months for 12 months (a total of 4 stages). Beginning with the second subculture, a B5 medium without PGR was used. Both explants and subcultures were exposed to a photoperiod of 12 hours of light and 12 hours with a photon flux density of 57 lmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps, at an average temperature of 23 ± 2°C and an ambient humidity of 65 ± 7%. The multiplication index was calculated as the number of newly formed shoots per each initial shoot and recorded in each subculture stage.

2.4. Analysis of somaclonal variations

Fifty milligrams were taken from each sample, as leaf fragments, and in those where the tissue was not differentiated, callus fragments were taken. The samples were preserved at -80 °C until DNA extraction. DNA extraction was carried out with a DNeasy Plant Mini kit (Qiagen®) following the manufacturer's instructions. The amount and purity of the DNA were quantified in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA integrity was verified by gel electrophoresis in 1% w/v agarose gel staining with Gel Red and visualized in an Enduro TM Gel Documentation System Touch.

2.5. ISSR-PCR analysis

To examine the polymorphism of the genomic DNA in *C. officinalis*, 13 ISSR primers (University of British Columbia Biotechnology Laboratory, Vancouver, Canada) were evaluated. Based on the reproducibility of band pattern and resolution, a total of 6 primers were selected (Table 1).

PCR reactions were performed in a final volume of 20 µL, containing 20 ng of DNA template, 4 µL of 5X buffer containing $MgCl_2$, 0.5 µM of primer, 0.2 mM of dNTPs and 1 U of Taq DNA polymerase (Promega). The PCR product was amplified under the following conditions: one cycle of 4 min at 94 °C, followed by 40 cycles with 30s at 94 °C, 45s at diverse annealing temperatures depending on each specific marker (Table 1) and 2 min at 72 °C, and a final extension of 10 min at 72 °C.

The PCR was carried out in the Applied Biosystems Thermocycler. The visualization of products was carried out in an agarose gel at 2%. The gels were dyed with Gel Red, run at 80 volts/cm and visualized under UV light in an Enduro TM Gel Documentation System Touch. Amplified PCR products that were not clear were repeated and only clear and reproducible bands were considered for the analysis. The sizes of the amplification products were estimated using a 100 bp molecular marker (Invitrogen).

2.6. Experimental design and statistical analysis

All the experiments were repeated twice with a minimum of 36 explants for each subculture with its corresponding control. At random, 2 samples were taken for each subculture and combination for DNA extraction and analysis with ISSR. A total of 84 explants were evaluated.

ISSR fragments were scored as present (1) or absent (0) according to the band amplification resulting in a binary matrix. From the documented gels all the bands were analyzed using the GelAnalyzer (2010) software (Lazar *et al.* 2010). Based on genetic data, the distances between groups were calculated with the Jaccard coefficient. The relationship between the donor plants and the regenerated explants was represented in a dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm. Polymorphism was calculated as the proportion of the total number of polymorphic bands to the total number of bands and expressed as a percentage of each specimen. The analyses were performed in the *R* program, version 3.6.3. (R Development Core Team, 2020).

3. RESULTS AND DISCUSSION

3.1. In vitro culture, shoot development, and multiplication index

Morphogenic responses, such as shoot regeneration, are observed by the application of cytokinins either alone (Razaq et al., 2012; Saha et al., 2014; Sadeghi et al., 2015), or in combination with auxins (Upadhyay et al., 2015; Savitikadi et al., 2020). Both PGRs have a synergistic effect on morphogenic responses, which has been reported in other medicinal species such as *Rhodiolarosea* (Dimitrov et al., 2003). In this work, the combination of 2,4-D with any of the evaluated cytokinins (BAP or Kin) produced indirect shoots (Figures 1 and 2a). The combination of BAP and IBA (5.0 mg L^{-1} and 3.0 mg L^{-1}) produced shoots from compact globular structures, in addition to direct shoot formation (Figure 2c). In *C. robusta* with the use of Kin and 2,4-D (Giroud et al., 1989) and in *C. ledgeriana* with the use of BAP and 2,4-D (Hoekstra et al., 1990), the formation of the same globular structures was reported. The combination of NAA and BAP produced direct shoot formation. The same response was observed in a B5 medium without PGR (Figure 2b).

The highest multiplication index (11.5) was achieved with the use of 5.0 mg L^{-1} BAP and 3.0 mg L^{-1} IBA. Similar responses have been observed in other *Cinchona* species such as *C. ledgeriana* (Hunter, 1979). At the end of the fourth stage of subculture, this PGR combination produced approximately 700 explants from a single plant. In previous work with *C. officinalis*, Armijos-González & Pérez-Ruiz (2016) found the highest proliferation rate with the same combination. The results showed that, when additional subculture is performed, the proliferation rate increases. This effect has been evaluated in several species of bromeliads and is known as habituation to growth regulators (Pierik, 1990).

The lowest multiplication index (1.2) was registered for the explants cultivated in the combination of 5.0 mg L^{-1} BAP + 1.0 mg L^{-1} of 2,4-D (Figure 1).

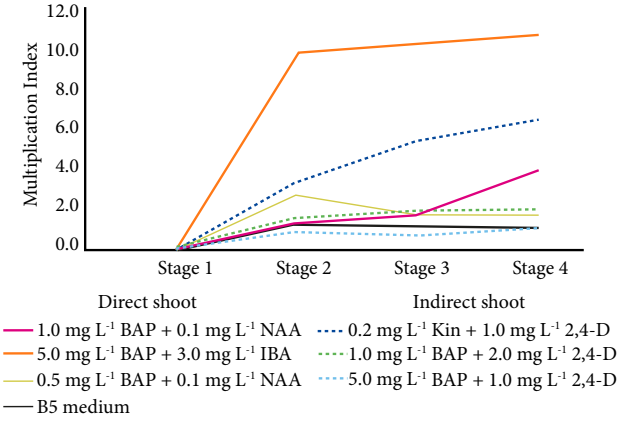

Figure 1. Multiplication index for each Plant Growth Regulator combination evaluated in four developmental stages.

3.2. Marker in formativeness and performance

From a prescreening assay with 13 ISSR primers, six markers were chosen because they generated polymorphic, reproducible, well-differentiated, and bright bands. A total of 58 ISSRs fragments were observed after PCR amplification. The fragment sizes observed were between 200 and 2837 bp using primers with AG and GA dinucleotide repeats (Table 1).

The average fragment number for each primer was 10. A minimum of eight fragments was observed with the

UBC840 marker, and a maximum of 13 bands with the UBC808 primer. The smallest fragment size was 200 bp for the UBC809 marker, and the largest was 2837 bp for the UBC840 marker (Table 1).

The maximum percentage of polymorphism was 39%, observed for the UBC808 marker. This was followed by UBC809, UBC834, and UBC836 (33%). The lowest percentage of polymorphism was observed in the UBC807 marker, with 20%.

Table 1. ISSR primers used for detecting somaclonal variation of *in vitro* regenerated plants of *C. officinalis*.

Primers	Sequence (5´- 3´)	Range (bp)	No. of bands	Monomorphic fragments	Polymorphic fragments	Polymorphism (%)
UBC 807	(AG)8 T	233-1276	10	8	2	20
UBC 808	(AG)8 C	203-1517	13	8	5	39
UBC 809	(AG)8 G	200-1345	9	6	3	33
UBC 834	(AG)8 YT	256-2609	9	6	3	33
UBC 836	(AG)8 YA	233-1544	9	7	2	33
UBC 840	(GA)8 YT	231-2837	8	6	2	25
Total		200-2837	58	40	18	28.7

3.3. Evaluation of the somaclonal variation with ISSR

In the PGR combinations that produced direct shoot formation (Figure 2) or shoot formation from compact globular structures, ISSR markers showed no genetic variation when they were compared to the donor plant. Jaccard's similarity coefficient was 1.0 and there were no groupings. Similar responses had been reported in *Ophiorrhiza mungos,* Rubiaceae (Kaushik et al., 2015).

The UPGMA cluster analysis based on the ISSR analysis revealed genetic differences between the donor plant and the explants cultivated in the PGR combinations that produced indirect shoot formation (Figure 3).

The combination of 0.2 mg L^{-1} Kin - 1.0 mg L^{-1} 2,4-D resulted in the highest genetic distance. The genetic differences were observed from the second stage of culture (Variation coefficient = 0.03) and increased at the third stage (Variation coefficient = 0.06) (Figure 3a; Figure 4).

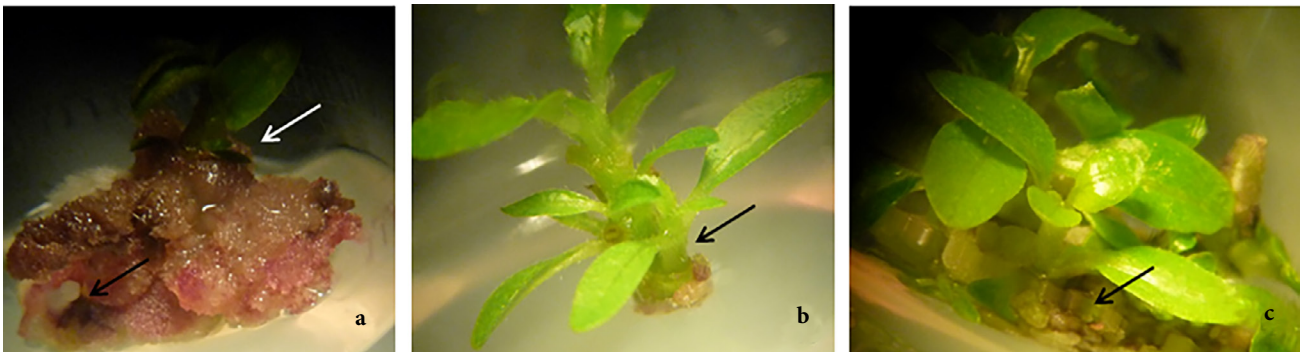

Figure 2. Proliferation of shoots during stage 1: a. Formation of indirect shoots (white arrow) and roots (black arrow) from callus [B5 + 0.2 mg L^{-1} Kin + 1.0 mg L^{-1} 2,4-D]. b. Direct shoot from axillary buds (black arrow) in a B5 medium without regulators. c. Shoot formation from compact globular structures (black arrow) and direct shoot formation [B5 + 5.0 mg L^{-1} BAP + 3.0 mg L^{-1} IBA].

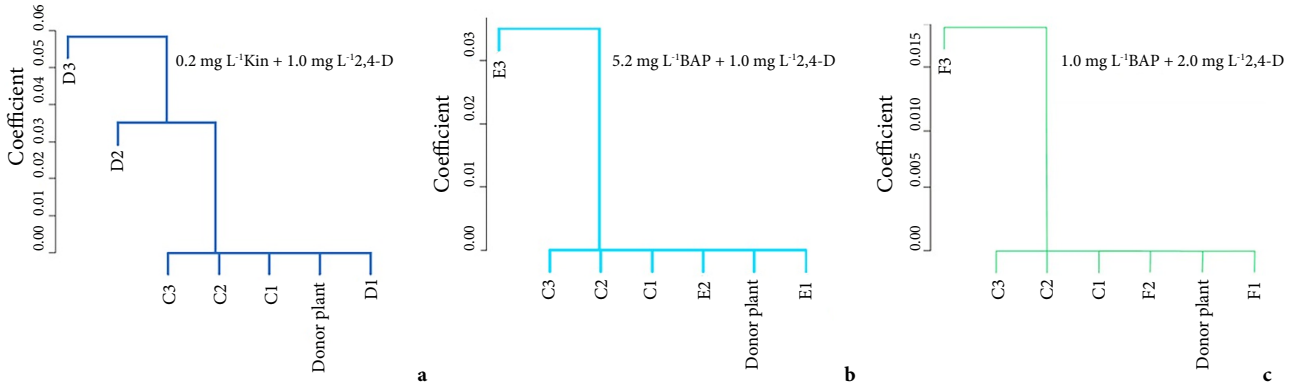

Figure 3. The genetic similarity UPGMA dendrogram is based on Jaccard´s coefficient. The analysis was calculated for each combination of plant growth regulators and subculture with respect to the donor plant. The figure presents the results only for the PGR combinations that resulted in a similarity coefficient different from 1.00. D, E, and F represent the explants which were grown in a medium with PGR: a. B5 medium + 0.2 mg L^{-1} Kin + 1.0 mg L^{-1} 2,4-D; b. B5 medium + 5.0 mg L^{-1} BAP + 1.0 mg L^{-1} 2,4-D; c. B5 medium + 1.0 mg L^{-1} BAP - 2.0 mg L^{-1} 2,4-D. When the letters are followed by numbers, they represent the three different culture stages. Donor plant: represents the control explant used for each PGR combination corresponding to explants from the same donor plant that was grown in the B5 medium.

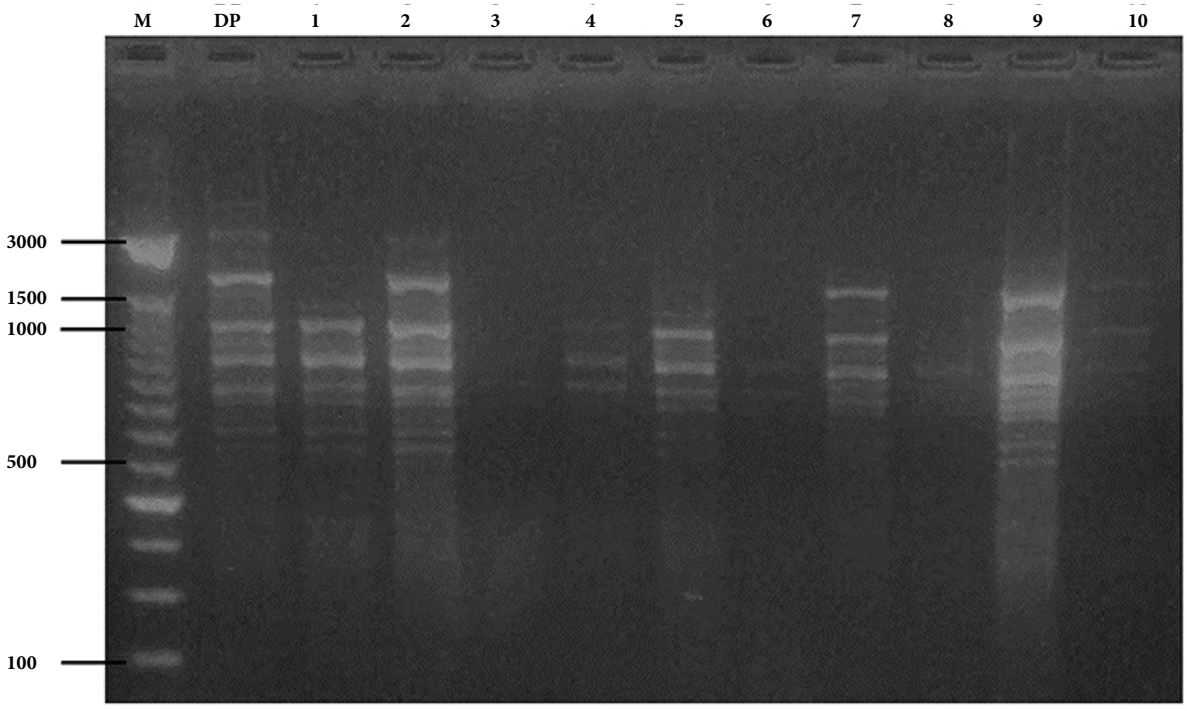

Figure 4. Genetic variation analysis of donor plant and micropropagated, using the ISSR 808 primer. Lane M: 100 bp DNA marker. Lane DP: Donor plant. Lane 1-10: *In vitro* regenerated plant of third of culture in B5 medium + 0.2 mg L^{-1} Kin + 1.0 mg L^{-1} 2,4-D.

The genetic distance observed in the explants cultivated in the other PGR combinations: 5.0 mg L^{-1} BAP - 1.0 mg L^{-1} 2,4-D (Figure 3b) and 1.0 mg L^{-1} BAP - 2.0 mg L^{-1} 2,4-D (Figure 3c), was altered only in the third stage of culture in both combinations when compared to the donor plant, showing variation coefficients of 0.03 and 0.02, respectively.

Several studies have reported that distinct factors influence genetic variation, including the regenerative pathway. However, it is not a rule that direct regeneration has a lower risk of somaclonal variation and those that go through a callous phase have a higher probability of variation (Tang, 2001; Gao et al., 2010; Bairu et al., 2011).

In this work, the compact globular structures that also had been observed in explants of *C. ledgeriana* and *C. robusta* (but in combinations of different PGRs) did not induce genetic differentiation. The observed structures are meristemoids that later differentiate into vascular structures and other tissues (Robins et al., 1986; Hoekstra et al., 1990), organized structures that were also confirmed through histological analysis in *C. officinalis* (Armijos-González & Pérez-Ruiz, 2016). In *Hydrangea macrophylla*, the same combination of regulators did not present the risk of somaclonal variation in the explants (Liu et al., 2011).

The addition of 2,4-D has been reported as a factor associated with genetic abnormalities, stimulating endopolyploidy, amitosis, mutations (Kunakh, 2005; Mohanty et al., 2008), DNA methylation that produces changes in genotype (Chakrabarty et al., 2003; Regalado et al., 2015), and mutation or inactivation of genes by genetic repression in artificial culture conditions (García et al., 2019). In this work, it was observed genetic differences for all the PGR combinations in the presence of 2,4-D. Similar responses have been reported for many species such as in *Aloe vera* (Rathore et al., 2011) and *Bletilla striada* (Wang et al., 2014).

The 2,4-D auxin for *C. officinalis* produced calli, confirming that the type of regeneration through callus (Figure 3) was an important factor that produces genetic variability. The level of somaclonal variation in callus regeneration may be due to the heterogeneity of callus cells and to the accumulation of genomic alterations (Kuznetsova et al., 2006) during long-term culture (Bublyk et al., 2012).

The appearance of variability was not immediate upon exposure to growth regulators, but was evident by the third subculture. Therefore, for *C. officinalis*, the effect of the regulators is also associated with the time of cultivation or with the number of subcultures. Specifically, in the presence of 0.2 mg L^{-1} Kin - 1.0 mg L^{-1} 2,4-D, the variation was detected in the second subculture and increased in the third one. 2,4-D remains in the tissue for a long time and is difficult to remove (García et al., 2019). In general, the greater the number of subcultures, the greater the risk of genetic variation (Devi et al., 2014; Krishna et al., 2016), which may be due to the increased time of exposure to the stress caused by this auxin. This response has been reported for other species. In *Tetrastigma hemsleyanum*, the frequency of variation also tended to increase over time of subculture because of the accumulation of mutations (Peng et al., 2015). In *Pisum sativum*, the variation was observed in the explants obtained from a first subculture and increased in the explants of the second subculture (Kuznetsova et al., 2006) and, for *Musa acuminata*, the variation increased in subcultures through the loss and appearance of new alleles with respect to the mother (Sheidai et al., 2008).

The percentages of genetic variation in *C. officinalis* were between 3 and 6%, although higher genetic differences have been reported for other species with the use of 2,4-D. In explants cultivated from *Lilium orientalis*, the percentage of variation obtained was 8%, and the authors considered this a low percentage of genetic divergence for the regeneration of shoots (Liu & Yang, 2012). In *Lilium* plants with 0.73% polymorphism, the explants obtained were considered genetically and morphologically stable (Yin et al., 2013). Despite the low percentage of genetic variation obtained in the explants of *C. officinalis*, the results suggest that the variation is due to the synergistic effect of the regeneration pathway, the effect of 2,4-D and subcultures. Therefore, the present research study suggests avoiding the use of 2,4-D in order to produce plants with genetic stability.

4. CONCLUSIONS

This is the first study carried out on the genetic stability of the *in vitro* cultivation of *C. officinalis*. The use of IBA and BAP produced a globular compact structure and achieved the highest proliferation index producing 700 plants from a single explant with high genetic fidelity. The presence of 2,4-D induced somaclonal variation in all of the tested combinations. The subculture stages increase somaclonal variation only in the presence of the 2,4-D auxin. This work suggests that direct or indirect propagation pathways without the presence of 2,4-D are good strategies to produce plants used for reintroduction and restoration programs.

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