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Establishment of an *in vitro* culture protocol of *Chuquiraga jussieui* J.F. Gmel. from apical and axillary buds[1]

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Abstract

In vitro establishment of endangered plant species conservation has become a major concern worldwide due to an increase in number of threatened species. Chuquiraga jussieui J.F. Gmel., an Andes mountains shrub is an ideal candidate for the process of conservation due to its state of extinction in Ecuador. Apical and axillary buds of Chuquiraga jussieui were cultivated for 30 days on ½ strength MS (MURASHIGE & SKOOG) [1] medium with different concentrations of BAP (0.1 to 2.0 mg/L), IBA (0.0 - 0.1 mg/L) and BRA (2.0 mg/L) in order to generate shoots. The obtained shoots were transferred to a ½ strength MS multiplication medium supplemented with BAP, IBA and BRA (5 mg/L) for 30 days. The best results for the in vitro multiplication of Chuquiraga jussieui were obtained using ½ strength MS medium supplemented with 2.0 mg/L BAP and 2 mg/L BRA. The rooting process was achieved using IBA (2.0 - 6.0 mg/L) and BAP (0.0 - 0.1 mg/L) added to ½ strength MS medium. The micropropagation of Chuquiragua is critical to conservation efforts in Andean ecosystems due to the ecological role that it plays and our study of the multiplication protocol could overcome this problem.

Key words: apical buds; axillary buds; *Chuquiraga jussieui*; *in vitro*; Ecuador.

1. Introduction

Chuquiragua (*Chuquiraga jussieui* J. F. Gmel.) is an endemic species of the Andean moorlands. Through the years it has been threatened by poor utilization of soils, as well as reforestation with species that erode the land, leading to the reduction of Chuquiragua in its native habitat. This species is known locally as Chuquirahua, Walker flower, and Andes´s flower.

Being a plant of moorland, the possibilities to regenerate and multiply this plant by conventional methods of clonal propagation are scarce due to the nutritional requirements that it has.

The establishment of an *in vitro* culture protocol for Chuquiragua plant species would increase the propagation possibilities, by providing nutrients and conditions which are necessary during shoots induction from apical or axillary buds. This method is beneficial both for the multiplication and rooting phases. Chuquiragua belongs to the family *Asteraceae*, subfamily *Barnadesioideae*, also called a “composed inflorescence”. The *Asteraceae* family is cosmopolitan, and their species inhabit vast areas in the temperate and subtropical regions.

In vitro culture of Chuquiragua is a necessary step for further studies regarding genetic diversity, the functional physiological responses of this species during the establishment of micropropagation protocols and for the production of secondary metabolites with multiple medicinal uses.

This study aimed to establish an *in vitro* culture protocol to obtain Chuquiragua plants starting from apical and axillary buds.

2. Materials and methods

A. Plant samples

Chuquiragua samples were collected from Tungurahua, Ambato province [Al 3535 meters, latitude: 0°18'53,891'' (South) longitude: 78ª26'46,562'' (West)]. The plants specimens selected were those with better phenotypic characteristics such as vigor, greater number of buds, plants free of fungi or other microorganisms and having dense, evergreen leaves.

The plants used as explants sources were maintained in greenhouse conditions, where a phytosanitary control was performed in order to eliminate fungal contamination on the external leaf area of plants, which involved the application of a contact fungicide (Thicarb® SC35-15) with: 2-metoxicarbamil benzimidazol and carbamoyl tetrametil two disulfide in a solution of 0.5% V/V for 15 days with one application every 3 days.

B. Phase I: Explant disinfection and establishment of primary cultures

Chuquiragua´s apical and axillary buds were subjected to a disinfection protocol consisting in washing with water for 30 seconds, then dipping in a solution of 1% W/V detergent with 3% of Tween-20 for 15 minutes in stirring. Immediately 3 rinses of plant material were done using distilled water followed by different concentrations of sodium hypochlorite at appropriated immersion times to prevent the necrosis process of the explants: hypochlorite solutions (0.5 – 1.5% V/V) with 3% of Tween-20 with immersion times (10 – 15 minutes). Three washes of the explants were done with sterile distilled water to remove the disinfectant substances.

Apical and axillary buds were aseptically placed to a half-strength MS culture medium, containing 2% sugar, 0.6% agar and without growth regulators, at pH 5.7. The cultures were incubated at 25 ± 2 °C, in conditions of 40 to 60% relative humidity, 2000 to 2500 lux and photoperiod of 16 hours of light and 8 hours of darkness.

The data were collected after 30 days. The variables tested were: no contamination (NC) and no oxidation (NO). The experimental unit was one apical or axillary bud per culture vessel. A number of 10 repetitions for each treatment were performed during the development of phase I.

C. Phase II: Shoot induction from apical and axillary buds

Apical and axillary buds of an average size of 2 cm were selected for this phase. Buds were disinfected through the protocol described above. After the disinfection process, buds were placed on ½-strength MS culture medium supplemented with six different combinations of 6-benzylaminopurine (BAP), indole butyric acid (IBA) and Brassinolide (BRA). BAP concentrations of 0.1 - 1.0 - 2.0 mg/L, IBA concentrations of 0.0 - 0.1 mg/L and a BRA concentration of 2 mg/L were used to promote the shoot development. Data were recorded after 30 days for the following variables: appearance of shoots (AS) and number of developed shoots (S1). The experimental unit was one apical or axillary bud per culture vessel. During the phase II, 25 replicates per treatment were recorded.

D. Phase III: Shoot multiplication

The shoots obtained in the previous phase were detached and transferred to half-strength MS medium supplemented with 6 different combinations of BAP, IBA and BRA. BAP concentrations of 0.1 - 1.0 - 2.0 mg/L, IBA concentrations of 0.0 - 0.1 mg/L and a BRA concentration of 5 mg/L were used to increase the production of shoots per explant. After 30 days the trials were evaluated for the following variables: number of shoots (S2) and survival of shoots (SS). The experimental unit was one shoot per culture vessel. The number of replicates was 10 per each treatment.

The environmental conditions were the same to those used in the previous phase.

E. Phase IV: Rooting of shoots

After an incubation period the shoots were stabilized, and ready to be transferred and placed individually on culture media with phytoregulators to promote the rooting. For this process a half-strength MS (1962) culture medium was used, containing 4.5% sugar and different concentrations of IBA (2.0 - 4.0 - 6.0 mg/L) and BAP (0.0 - 0.1 mg/L), at pH 5.7. The environmental conditions were the same as the ones previously used, except for the photoperiod which was of 12 hours light and 12 hours dark.

Data were evaluated at 30 days for the variable: presence of roots per shoot (P/S). The experimental unit was one shoot per culture vessel. The number of replicates was 10 per treatment.

F. Data analysis

Analysis of variance and Duncan's multiple comparisons were performed for each variable using InfoStat® (2011I).

3. Results and discussion

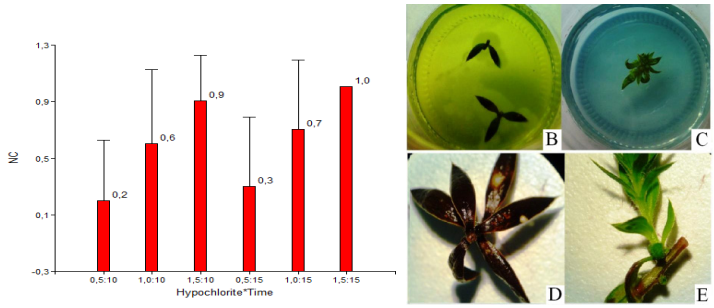
G. Phase I: Introduction and disinfection

High levels of contamination (70 - 80%) were observed in the samples treated with a low concentration of sodium hypochlorite (0.5% V/V), compared to treatments with higher concentrations of sodium hypochlorite (> 1.0% V/V), where only 40% of the samples were contaminated. The samples treated with 1.5% V/V sodium hypochlorite showed very low levels of contamination (<10%) (Table 1).

Treatment	Sodium hypochlorite concentration (% V/V)	Immersion time (min)	Non-contaminated (%)	Non-oxidized (%)
1	0.5	10	20	0
2	1	10	60	0
3	1.5	10	90	0
4	0.5	15	30	20
5	1	15	70	50
6	1.5	15	100	90

Table 1. Percentage of uncontaminated explants (NC) and unoxidized (NO) in disinfection treatments for apical and axillary buds of Chuquiragua (*Chuquiraga jussieui*) in the Phase I.

According to ABDELNOUR & ESCALANT [2] the plant material grown under greenhouse conditions is cleaner than that kept in the field. Therefore, apical and axillary buds that were used in our experiments as explants for culture initiation were collected from plants that were grown in the greenhouse, in controlled environment (Figure 1).



A

Figure 1. Disinfection treatments performed in Phase I for the introduction of Chuquiragua (*Chuquiraga jussieui*) apical and axillary buds. (A) Effect of sodium hypochlorite concentrations and immersion times, (B) Contaminated explants, (C) Non-contaminated explants, (D) Oxidized explants, (E) Non- oxidized explants.

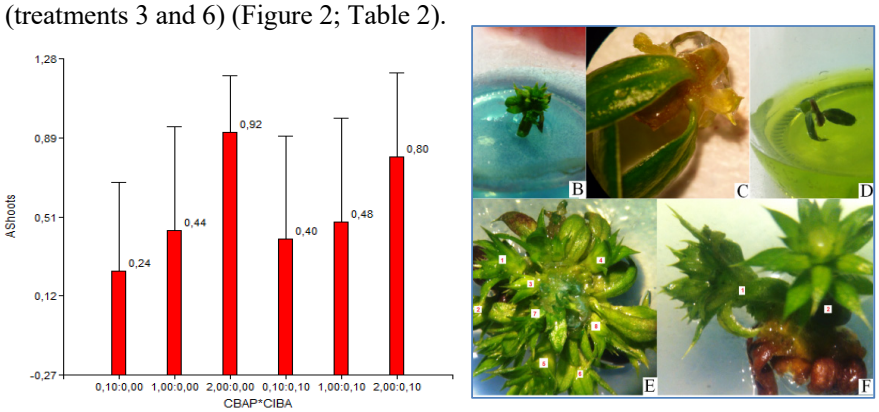
On the other hand, sodium hypochlorite concentrations greater than 1% demonstrated to be quite drastic for 26.67% of explants, causing the necrosis of tissue and lacking of viability (Table 1).

SANCHEZ [3] considered this effect as a result of the potent oxidant characteristic of the sodium hypochlorite. It has been shown to be effective in disinfection process of the explants at 1.5% V/V concentration with an immersion time of 15 minutes. However, explants viability could be drastically affected. In fact, at high concentrations of sodium hypochlorite the immersion time should be lower to avoid oxidation of the plant samples. Out of the three treatments, we observed a high level of disinfection using 1.5% V/V sodium hypochlorite for 10 minutes immersion time, as described in figure 1.

The third treatment was deemed to be appropriate for Chuquiragua disinfection process, the results showing 90% absence of contamination and oxidation of explants.

H. Phase II: Shoots induction from apical and axillary buds

The concentrations of 0.1 mg/L BAP compared to 1.0 mg/L BAP (treatments 1, 2 and 5) were not relevant for the induction of shoots. The concentration of 2.0 mg/L BAP in association or not with 0.1 mg / L IBA proved to be efficient for the shoots induction (treatments 3 and 6) (Figure 2; Table 2).



A

Figure 2. Shoot induction treatments used in Phase II for apical and axillary buds of Chuquiragua (*Chuquiraga jussieui*), (A) Effect of 6-BAP and IBA concentrations, (B) Apical bud with shoot, (C)

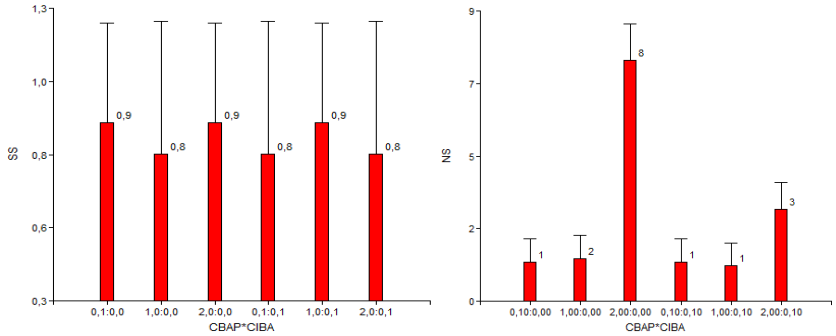
Axillary bud with shoot. (D) Absence of shoot, (E) Apical bud with 8 shoots. (F) Axillary bud with 2 shoots.

Apparently, the offset could be caused by the presence of cytokinin hormone which is present in the culture media (Table 2).

Treatment	6-BAP concentration (mg/L)	IBA concentration (mg/L)	BRA concentration (mg/L)	AS	BS_1
1	0.1	0	2	24	8
2	1	0	2	44	16
3	2	0	2	92	147
4	0.1	0.1	2	40	13
5	1	0.1	2	48	20
6	2	0.1	2	80	66

Table 2. Percentage of shoots appearance (AS) and the number of shoots (S1) in the induction treatments for apical buds and axillary buds of Chuquiragua (*Chuquiraga jussieui*) during the Phase II.

According to JORDAN [4], endogenous cytokinins can induce and fix a type of expression according to the present physiological levels. Plant cells begin their differentiation processes under *in vitro* conditions under the combined effect of these hormones. This explains the responses obtained during the induction and shoots multiplication phases, being recorded that in the presence of the same concentration of BAP (2.0 mg/L) the number of shoots is similar in both phases, while the average starts to differ when the BRA concentration is increased during the multiplication phase (Figure 3).



A B

Figure 3. Shoot multiplication treatments for Chuquiragua (*Chuquiraga jussieui*), (A) Effect of treatments on survival of shoots multiplication (SS_2), (B) Effect of 6 BAP concentrations on the number of sprouts (S_2) during phase III.

According to SALGADO [5], BRA's main physiological effect is to stimulate the growth in a variety of systems such as a complete plant, parts of different organs and vegetative tissues. The increased number of Chuquiragua's shoots in multiplication phase shows that BRA allows a better elongation and development of the new shoots.

Moreover, we also observed that concentrations of 2.0 mg/L BAP and 2 mg/L BRA, in the absence of IBA are more suitable for the establishment of *in vitro* Chuquiragua plants. At these hormone concentrations 92% of the explants produced shoots and also 6.39 shoots per explants were counted (Table 2).

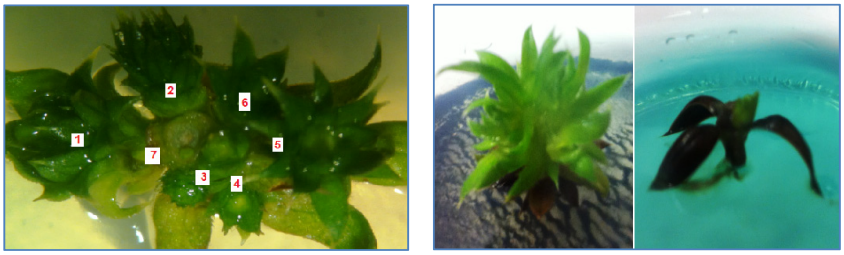
I. Phase III: Shoots multiplication

The increased number of shoots per explants was prominent and it was achieved using a concentration of 2.0 mg/L BAP, in the absence of IBA and an increased concentration of BRA, respectively at 5.0 mg /L, thus obtaining a 7.5 average number of shoots (Table 3) in the treatment 3. It was followed by treatment 6 (Table 3), where the concentration of 0.1 mg/L IBA appears to inhibit the shoot formation, mainly due to the synergism between cytokinin and auxin.

Treatment	6-BAP concentration (mg/L)	IBA concentration (mg/L)	BRA concentration (mg/L)	SS (%)	S_2
1	0.1	0	5	90	14
2	1	0	5	80	15
3	2	0	5	90	75
4	0.1	0.1	5	80	14
5	1	0.1	5	90	13
6	2	0.1	5	80	30

Table 3. Shoots survival rate (SS) and number of buds (S2) in the shoot multiplication treatments for Chuquiragua (*Chuquiraga jussieui*) during Phase III.

According to CRUZ [6] the combination of IBA, kinetin and BAP promote formation and number of shoots per explant at appropriate concentrations for each species, in contrast with the results obtained in our study where in the absence of IBA the appearance and number of shoots increased (Figure 4).



A B C

Figure 4. (A) Countable shoots numbers present in each explant (B) Viable shoot transplanted on shoot multiplication medium. (C) Nonviable shoot transplanted on shoot multiplication medium.

Analysis of variance revealed statistically significant differences (p-value<*0.0001*) between the concentrations of 0.1 mg/L and then lack of IBA during shoots multiplications. This difference indicates an interaction with BAP related to the number of shoots. The result could be affected by the auxin presence because better results were achieved in the absence of IBA (Table 3).

The concentration of cytokinin (BAP) comparing to auxin (IBA) used in this study was 20 to 1. SCHMÜLING [7] indicates that the amount of auxin-cytokinin ratio determines the cellular differentiation in tissue or roots formation and also suggests that higher concentration of cytokinin induce the shoot formation. This is in accordance with the results obtained in the induction and multiplying phase of Chuquiragua, where a BAP concentration of 2.0 mg/L, in the absence of IBA, stimulated efficiently the appearance and the number of shoots.

Analysis of variance revealed no statistically significant differences for the variable survival, which yielded a p-value = 0.9542. This proved that there are no differences between the six treatments used during the shoot multiplication phase. Shoots survival during the stage of shoot multiplication was high and independent to the type of applied

treatment. However, the primary evidence of cell death and necrosis was appearing during the excision of new shoots and cuts from apical and axillary buds (Table 3).

Furthermore, during this phase, the multiplication rate was 7.5 in the number of shoots per explant. This value, according to ROCA & MROGINSKI [8] is important to evaluate the differences between the shoot productions in the *in vitro* systems comparing to vegetative production in the field.

J. Phase IV: Rooting of shoots

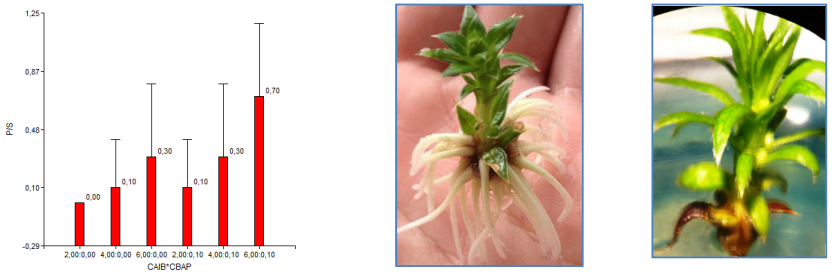
Treatments achieved for the rooting phase are based on the use of the auxine IBA, which according to GOMEZ & ZAMILPA [9] could be effective or not in association with a cytokinin. High concentrations of others auxins, such as α-naphthaleneacetic acid, produce the oxidation of the explant opposite to the use of IAA and IBA, which decrease the oxidation process. Combinations of IBA and 0.1mg/L BAP proved to promote the formation of rhizogenic callus from the shoots.

Analysis of variance revealed that there are statistically significant differences (p=0.0234) between results obtained using various BAP concentrations for inducing the root formation. These results showed that the presence or the lack of this cytokinin infers in the appearance of the roots. 70% of the roots were obtained using the treatment 6, and 30% using treatment 3, which were in the subset B and A, respectively (Table 4).

Treatment	IBA concentration (mg/L)	6-BAP concentration (mg/L)	P/S (%)
1	2	0	0
2	4	0	10
3	6	0	30
4	2	0.1	10
5	4	0.1	30
6	6	0.1	70

Table 4. Percentage of shoot rooting (P/S) in the rooting treatments for Chuquiragua (*Chuquiraga jussieui*), during Phase IV.

The ratio between cytokinins/auxin was less than one (0.0167), which reflected the largest growth of roots (treatment 6). There is a residual effect of BAP hormone used in the induction and shoot multiplication stage, which usually causes an inhibitory effect on the root formation. This effect was evident in the treatments with the concentrations of IBA below 4.0 mg/L, where the presence of roots did not exceed 10%. The application of IBA at high concentrations for the rooting induction did not generate high callus formation. According to PIERIK [10], low concentrations of auxin are enough to generate roots, however does not apply to this species where a higher concentration of IBA was required in the presence or absence of BAP for the development of Chuquiragua's roots (Figure 5).



A B C

Figure 5. Rooting induction treatments used in Phase IV for Chuquiragua shoots (*Chuquiraga jussieui*), (A) Effect of IBA and 6-BAP concentrations, (B) Shoot with the presence of roots, (C) Shoot with no roots.

4. Conclusions

Based on our results we established an adequate treatment to disinfect apical and axillary buds of Chuquiragua - the treatment 3, consisting of 1.5% V/V sodium hypochlorite for 10 min immersion. This treatment reflected the highest average in terms of the absence of contamination (0.90) and oxidation (0.90).

Our results also showed an average of 6.39 shoots per explant and 92% of shoot formation, suggesting that 2.0 mg/L of 6- BAP in the absence of IBA could be an accurate concentration during phase II.

Brassinolide addition in the induction and shoot multiplication steps allows a rapid response of the explants, increasing the yield of shoots per explant as well as their size.

Treatment 3, using 2.0 mg/L 6-BAP in the absence of IBA has the greatest influence on the shoot multiplication with a 7.50 average shoots per explant. The next combination with the good influence on this stage was represented by the treatment 6: 6-BAP vs IBA (6-BAP 2.0 mg/L - IBA 0.1 mg/L) which showed a 3.0 average shoots per explant.

High concentrations of the auxin IBA during the shoot rooting phase allowed rhizogenic callus formation, which subsequently developed roots. The recommended combination of phytohormones for the rooting phase was represented by the treatment 6 consisting on 6.0 mg/L IBA and 0.1 mg/L 6-BAP, which presented the highest average (0.70) in terms of rooting system induction per explant.

The present study established a protocol for the *in vitro* culture of *Chuquiraga jussieui*, allowing the preservation of an important and valuable germplasm and the availability of a viable material for the isolation of secondary metabolites with significant medicinal properties.

Acknowledgments

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