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

In this project, shoots of apical and axillary buds were induced on MS 50% medium essaying different concentrations of BAP, IBA and BRA. Then, generated shoots were transferred to a MS 50% multiplication medium, using BAP, IBA and BRA. Subsequently, obtained shoots were induced for rooting in MS 50% medium with IBA and BAP. The results reflected that MS 50% medium with: BAP 2.0 mgL^{-1} + BRA 2.0 mgL^{-1}, allows the shoot induction of apical and axillary buds in a 92%, and also MS 50% medium with: BAP 2.0 mgL^{-1} + BRA 5.0 mgL^{-1} during multiplication phase, permited to obtain more number of shoots per buds. To finish, 70% of rooted shoots were obtained with MS 50% medium with: IBA 6.0 mgL^{-1} BAP + 0.1 mgL^{-1}.

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ABSTRACT

The establishment of native and vulnerable plant species using *in vitro* culture has become a major research objective. In this project, shoots of apical and axillary buds were induced on MS 50% medium (1962) essaying different concentrations of BAP (0.1 to 2.0 mgL^{-1}), IBA (0.0 - 0.1 mgL^{-1}) and BRA (2.0 mgL^{-1}). Then, generated shoots were transferred to a MS 50% multiplication medium, using BAP (0.1 to 2.0 mgL^{-1}), IBA (0.0 - 0.1 mgL^{-1}) and BRA (5.0 mgL^{-1}). Subsequently, obtained shoots were induced for rooting in MS 50% medium in the presence of IBA (2.0 - 6.0 mgL^{-1}) and BAP (0.0 - 0.1 mgL^{-1}). The results reflected that MS 50% medium supplemented with: BAP 2.0 mgL^{-1} + BRA 2.0 mgL^{-1}, allows the shoot induction of apical and axillary buds in a 92%, and also MS 50% medium supplemented with: BAP 2.0 mgL^{-1} + BRA 5.0 mgL^{-1} during multiplication phase, permited to obtain more number of shoots per buds. To finish, 70% of rooted shoots were obtained with MS 50% medium supplemented with: IBA 6.0 mgL^{-1} + BAP 0,1 mgL^{-1}. The micropropagation of *Chuquiraga jussieui* is critical to conservation efforts in Andean ecosystems due to the important ecological role that it plays.

Key words: *Chuquiraga jussieui*, apical buds, axillary buds, *in vitro*, BAP, IBA, Ecuador.

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 locally known as Chuquirahua, Walker flower, and Andes´s flower.

Being a plant of moorland, the possibilities to regenerate and multiply plants 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, providing nutrients and conditions, which are necessary during shoots induction from apical or axillary buds. This method also will benefit in multiplication and rooting phases, respectively. Chuquiragua belongs to the family *Asteraceae*, subfamily *Barnadesioideae*, also called an "inflorescence composed". 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 and functional physiological responses of this species during the establishment of protocols for micropropagation, also production of secondary metabolites, which are involved in multiple medicinal uses. This study aimed to establish an *in vitro* culture protocol to obtain Chuquiragua plants from apical and axillary buds.

METHODS

A. Plant samples

Chuquiragua samples were collected from Tungurahua, Ambato province (Al 3535 m.s.n.m., location: 01°16, 2325, 078°45232O). The plants specimens selected were those with better phenotypic characteristics such as greater number of buds, fungus-free plants or microorganisms, vigor, with dense evergreen.

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 benzimidazole and carbamyl tetrametiltio disulfide in a solution of 0.5% v/v for 15 days with one application every 3 days.

B. Phase I: Introduction and disinfection

Chuquiragua´s apical and axillary buds were subjected in a disinfection protocol, which consisted in a washing with water for 30 seconds, then they are dipped in a solution of

1% w/v of 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 and immersion times appropriated to prevent necrosis process of the explants: hypochlorite solutions (0,5 – 1,5% v/v) with 3% of Tween-20 with immersion times (10 – 15 minutes). To finish, 3 washes of explants were done with sterile distilled water.

Apical and axillary buds were established in Murashige & Skoog (1962) 50% culture medium, 2% sugar, pH 5.7, 0.6% agar and no growth regulators. The cultures were incubated at 25 ± 2 °C, 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 an apical or axillary bud per bottle. A number of 10 repetitions for each treatment was performed for the development of phase I.

C. Phase II: Induction of shoots 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 disinfection, buds were planted in MS 50% culture medium supplemented with 6 different combinations of 6-benzylaminopurine (BAP), indole butyric acid (IBA) and Brassinolide (BRA). Concentrations of BAP 0.1 - 1.0 - 2.0 mgL^{-1}, IBA concentrations: 0.0 - 0.1 mgL^{-1} and concentration BRA: 2 mgL^{-1} were used to promote shoot formation. Data were recorded at 30 days for the following variables: appearance of shoots (AS) and number of shoots produced (S1). The experimental unit was an apical or axillary bud per bottle. During the phase II, 25 replicates per treatment were recorded.

D. Phase III: Shoots multiplication

The shoots obtained in the previous phase were dissected and transferred to MS 50% medium (1962) supplemented with 6 different combinations: BAP, IBA and BRA. BAP concentrations 0.1 - 1.0 - 2.0 mgL^{-1} IBA concentrations: 0.0 - 0.1 mgL^{-1} and BRA concentration: 5.0 mgL^{-1} were used to increase the production of shoots per explant. After 30 days trials were evaluated for the following variables: number of shoots (S2) and survival of shoots (SS). The experimental unit was a shoot per bottle. The number of replicates were 10 per each treatment.

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

E. Phase IV: Rooting of shoots

After incubation period shoots are stabilized, and ready to be sectioned and placed individually in culture media with phytoregulators that promoted root development. MS 50% medium with 4.5% sugar, pH 5.7, were used, supplemented with IBA and BAP. IBA concentrations: 2.0 - 4.0 -6.0 mgL^{-1}, BAP concentrations: 0.0 - 0.1 mgL^{-1}. The environmental conditions were the same as used above. Photoperiod was 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 a shoot per bottle. The number of replicates were 10 per treatment.

F. Data analysis

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

RESULTS

A. Phase I: Introduction and disinfection

High levels of contamination, 70 - 80%, were observed in the samples treated with low concentration of sodium hypochlorite (0,5% v/v), while for treatments with higher concentrations of sodium hypochlorite (> 1.0% v/v) 40% of the samples showed contamination. The samples treated with 1,5% v/v sodium hypochlorite showed very low levels of contamination (<10%) (Table 1). According to Abdelnour Ana, Jean Escalant, (1994), plant material grown under greenhouse conditions are cleaner than those kept in the field. Apical and axillary buds that were used as plant samples for introduction were collected from plants that were grown in controlled in greenhouse environment (Figure 1).

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 viability (Table 1). According to Sánchez, M., (2004), this effect can be a result of the potent oxidant characteristic of the sodium hypochlorite. It has been shown to be effective in disinfection of the explants by immersion for 15 minutes at 1.5% v/v concentrations. 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 plant samples. Out of the three treatments, we observed the highest level of disinfection at 1.5% v/v and 10 minutes immersion time, as described figure 1. After sodium hypochlorite and immersion time experiments, the third treatment was deemed to be appropriate for Chuquiragua disinfection process. Results showed 90% absence of contamination and oxidation.

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

Treatment	Sodium hypochlorite Concentration (% v/v)	Inmersion time (min)	Uncontaminated (%)	Unoxidized (%)
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
		Rate	61,67	26,67

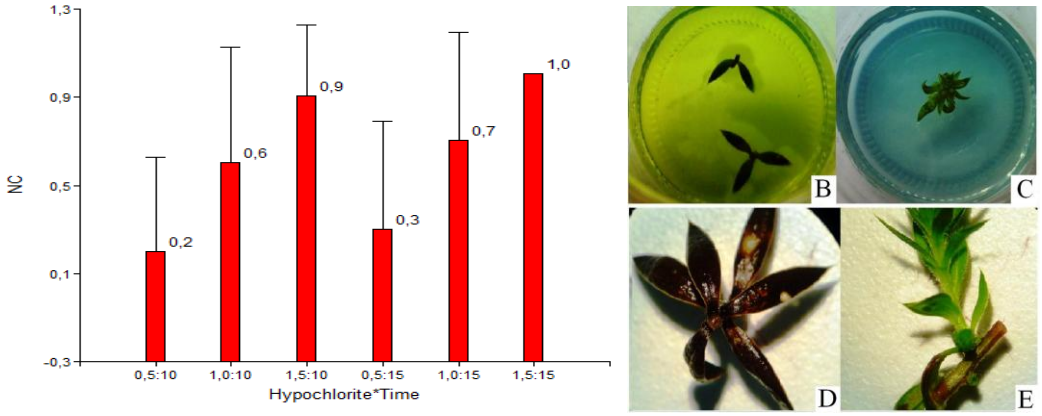

Figure 1. Disinfection treatments performed in Phase I for introduction of apical and axillary buds of Chuquiragua (*Chuquiraga jussieui*), (A) Effect of sodium hypochlorite concentration and immersion time, (B) Contaminated explants, (C) No contaminated explants, (D) Oxidized explant, (E) No oxidized explant.

B. Phase II: Induction of shoots from apical and axillary buds

Concentrations of BAP 0.1 mgL^{-1} in regards to obtaining shoots presented no relevance in contrast to the concentration of BAP 1.0 mgL^{-1} (treatment 2 and 5) which induces formation of shoots, while the concentration of BAP 2.0 mgL^{-1} in combination or not with IBA (0.1 mgL^{-1}) induce shoot formation efficiently (treatment 3 and 6) (Figure 2). Apparently, the offset could be caused by the presence of cytokinin hormone, hormone which

was presence in the culture media (Table 2). According with Jordan, M. (2006), endogenous cytokinins can induce and fix a type of expression according to physiological levels to be present. Plant cells begin their differentiation processes under *in vitro* conditions in the presence of the combination of these hormones, which justifies the responses obtained during the induction and shoot multiplication phase, where it was reflected that in the presence of the same concentration of BAP (2.0 mgL^{-1}) the number of shoots is approximate in both phases, while the average difference is due to the increase of BRA concentration during multiplication phase (Figure 5).

According to Salgado, R. (2008), BRA's main physiological effect is to stimulate the growth in a variety of systems; complete plant, parts of different segments and vegetative tissues. The increased number of Chuquiragua's shoots in multiplication phase shows that BRA allows better elongation and development of new shoots.

Moreover, we also observed that concentrations of BAP 2.0 mgL^{-1}, IBA 0.0 mgL^{-1} and BRA 2.0 mgL^{-1} are more suitable for the establishment of *in vitro* Chuquiragua´s plants. At this hormone concentrations 92% of the explants produced shoots, and also 6.39 shoots per explants were counted (Table 2).

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

Treatment	6-BAP concentration (mgL^{-1})	IBA concentration (mgL^{-1})	BRA concentration (mgL^{-1})	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
			Rate	54,67	45

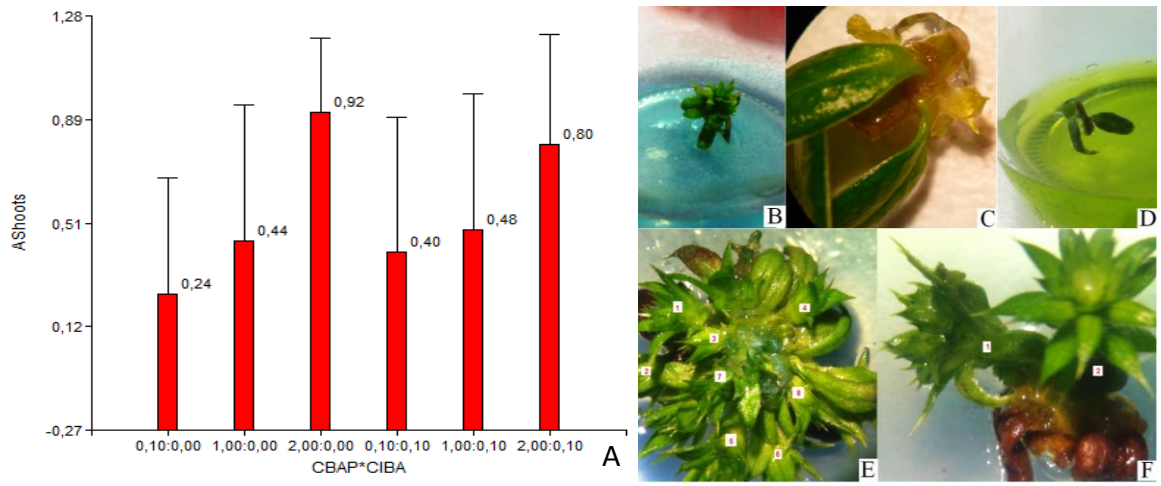

Figure 2. Shoots induction treatments used in Phase II for apical and axillary buds of Chuquiragua (*Chuquiraga jussieui*), (A) Effect of 6-BAP y IBA concentrations, (B) Apical bud with shoot, (C) Axillary bud with shoot. (D) Absence of shoot, (E) Apical bud with 8 shoots. (F) Axillary bud with 2 shoots.

C. Phase III: Shoots multiplication

The increased number of shoots per explants was prominent, and it was obtained by concentration of BAP (2.0 mgL^{-1}) in absence of IBA and increasing concentration of BRA (5.0 mg / L), obtaining a 7.5 average number of shoots (Table 3) in treatment 3. It was then followed by treatment 6 with an average of 3.00 (Table 3), which refers to the concentrations employing regulators, being 0.1 mgL^{-1} IBA the element that appears to inhibit shoot formation. This is mainly due to the synergism between cytokinin and auxin. According to Cruz, M. (2004) the combination of IBA, kinetin and BAP promote formation and number of shoots per explant at appropriate concentrations for each species, which is contrasted with this study, because in the absence of IBA the appearance and number of shoots increased (Figure 3).

Analysis of variance revealed statistically significant differences (p- value *<0,0001*) between the concentrations of 0.1 mgL^{-1} and 0.0 mgL^{-1} of IBA during shoots multiplications.

This difference indicates an interaction with BAP related with the number of shoots. These numbers could have been affected by auxin presence, because better results were founded in the absence of IBA (Table 3).

The concentration of cytokinin (BAP) in relation to auxin (IBA) used in this study was 20 to 1, Schmülling, T. (2004) indicates that the amount of auxin-cytokinin ratio determines the cellular differentiation in tissue or roots formation, also suggests that the higher concentration with respect to auxin promotes shoot formation, which is consistent with results obtained in the induction and multiplying phase of Chuquiragua, where at concentrations of BAP 2.0 mgL^{-1} and 0.0 IBA mgL^{-1} the appearance and number of shoots was stimulated efficiently.

Analysis of variance revealed no statistically significant differences for the variable survival, which yielded a p-value = *0.9542*, which means, there are no differences between the six treatments used during the shoot multiplication phase. Shoots survival during shoot multiplication stage was high and not dependent on the type of treatment employed, however, the primary source of cell death and necrosis was presented during extraction of new shoots and cuts of 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 with Roca W, Mroginski L. (1993), is an important variable needed to be evaluated. It allows the production of shoots differences between *in vitro* systems with vegetative production in field.

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

Treatment	6-BAP concentration (mgL^{-1})	IBA concentration (mgL^{-1})	BRA concentration (mgL^{-1})	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
			Rate	85,71	26,83

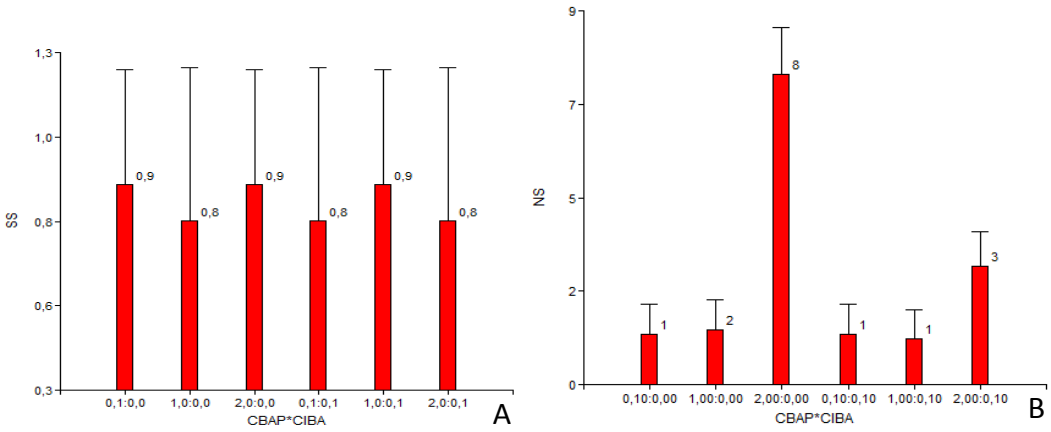

Figure 3. Shoot multiplication treatments for Chuquiragua (*Chuquiraga jussieui*), (A) Effect of treatments on survival of shoots multiplication (SS_2), (B) Effect of concentration of 6-BAP in the number of sprouts (S_2) during phase III.

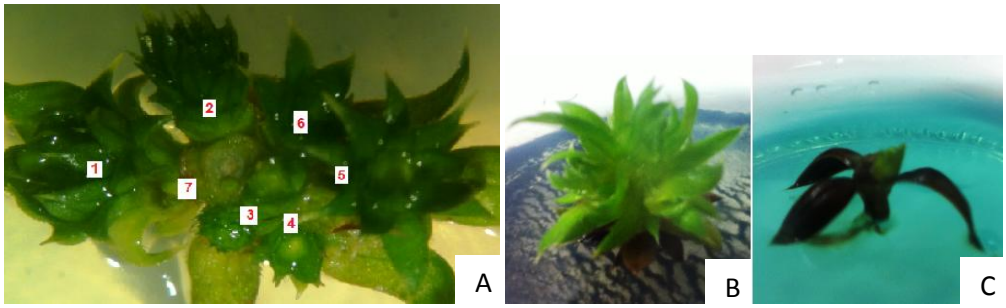

Figure 4. (A) Countable shoots numbers present in each explant (B) Shoot multiplication medium transplanted viable. (C) Shoot multiplication medium transplanted nonviable.

D. Phase IV: Rooting of shoots

Treatments for the rooting phase are based on the use of indole butyric acid, which according to Gomez, Y., Zamilpa, A. (2009), using as IBA rooting promoter could be effective or not in combination with a cytokinin. High concentrations of others auxins, such as α-naphthaleneacetic acid, produce the oxidation of the explant, as opposed to the use of IAA and IBA, which decrease the oxidation of the explant. Combinations with IBA and BAP at 0.1 mgL^{-1} showed to promote the formation of rhizogenic callus from shoots, with good root sizes.

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

The relationship between cytokinin/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 during the induction and shoot multiplication, which usually causes an inhibitory effect on the roots formation. This effect was evident in the treatments with concentrations of IBA below 4.0 mgL^{-1}, in which the presence of roots did not exceed 10%. The application of IBA at high concentrations for shoot rooting induction did not generate high callus formation. According with Pierik (1990), low concentrations of auxin are enough to generate roots. However, does not apply in this species, because for the development of Chuquiragua's root higher concentrations of IBA were required in the presence or absence of BAP (Figura 4).

Table 4. Percentage shoots rooting presence (P/S) in shoot rooting treatments for Chuquiragua (*Chuquiraga jussieui*) in Phase IV.

Treatment	IBA concentration (mgL^{-1})	6-BAP concentration (mgL^{-1})	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
		Rate	25

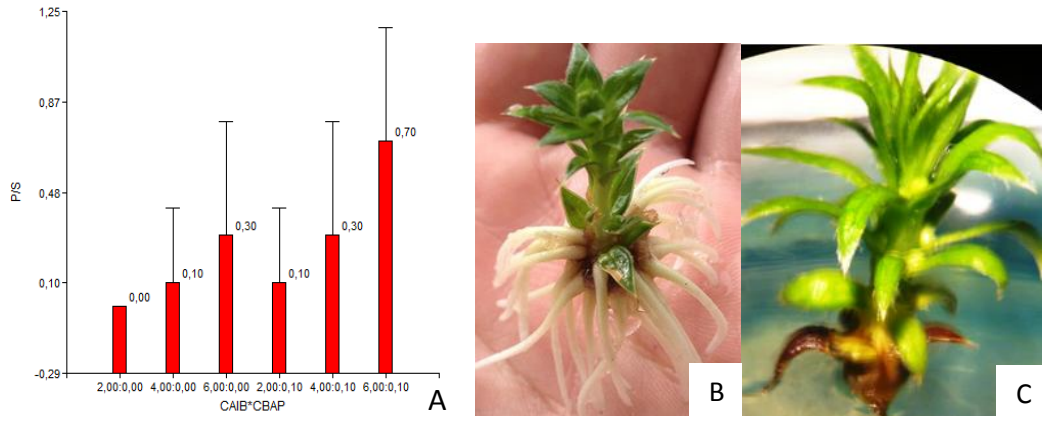

Figure 5. Rooting induction treatments used in Phase IV for Chuquiragua shoots (*Chuquiraga jussieui*), (A) Effect of IBA and 6-BAP concentrations, (B) Shoot with the presence of roots, (C) Shoot with no roots.

DISCUSSION

From our results an adequate treatment to disinfect apical and axillary buds of Chuquiragua is treatment 3, which consists of 1.5% v/v of sodium hypochlorite with 10 min of immersion time. 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 mgL^{-1} of 6- BAP in the absence of IBA could be an accurate concentration during phase II.

Brassinolide addition in the induction step and shoot multiplication allows a rapid response of the explants, yielding a number of shoots per explant, as well as a good size thereof.

Treatment 3: 6-BAP vs IBA (6-BAP 2.0 mgL^{-1} - IBA 0.0 mgL^{-1}) suggests to be an adequate combination for shoot multiplication with 7.50 average shoots per explant. The next best combination was treatment 6: 6-BAP vs IBA (6-BAP 2.0 mgL^{-1} - IBA 0.1 mgL^{-1}) which showed 3.0 average shoots per explant.

The best combination for shoot multiplication was treatment 3: 6-BAP vs IBA (6-BAP 2.0 mgL^{-1} - IBA 0.0 mgL^{-1}), with an average of 7.50 shoots per explant, compared to treatment 6: 6-BAP vs IBA (6-BAP 2.0 mgL^{-1} - IBA 0.1 mgL^{-1}) in which was obtained an average of 3.0 shoots per explant.

High concentration of auxin indole butyric acid (IBA) during shoot rooting phase allowed rhizogenic callus formation, which subsequently developed roots.

The recommended combination for rooting phase was the treatment 6: IBA vs 6-BAP (IBA 6.0 mgL^{-1} – 6-BAP 0.1 mgL^{-1}), which presented the highest average (0.70) in terms of presence of rooting system per explant.

The present study established a protocol for *in vitro* culture of *Chuquiraga jussieui*, allowing to obtain viable plants significantly.

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