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Callus Genesis in *Lupinus montanus* HBK from explants cultivated in vitro

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Abstract

Lupinus montanus HBK is a herbaceous species that is widely distributed in Mexico, primarily in the Sierra Madre Occidental and the Transversal Neovolcanic. It is characterized by synthesizing alkaloids whose fungicides, bactericides and hypoglycemic properties have been reported experimentally. However, obtaining these metabolites via extraction from cultivated plants involves investment costs that could turn it into a very profitable activity. In this research the main factors involved in the development of calluses that could be directed toward studying the synthesis and production of secondary metabolites or to micropropagation were evaluated via indirect organogenesis. The research results indicated that the factors evaluated (explant culture medium and incubation) and their interactions show differences in responses; under light conditions callus compact structure is obtained, while in dark friable calluses were obtained. The highest yields were obtained in biomass callus originated from Epicotyl in combinations of 2,4-D, BA and Kinetin in concentrations of the order of (4-6μM; 1-2μM and 2-2μM respectively) under dark conditions.

***Lupinus montanus*, Friable, necrosis, Auxin, cytokinin.**

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Introduction

Several studies point to the importance of in vitro for the initiation of cell suspension cultures for the synthesis of secondary metabolites callogenesis. Similarly, from calluses obtained in vitro can induce the formation of both plants (indirect organogenesis) and somatic embryos (direct embryogenesis) through which could obtain specific clones according own research interests (Martinez et al., 2010).

In woody plants such as *Lupinus montanus* herbaceous- HBK, induction and callus culture is somewhat more complicated compared to other plants. This complication is due primarily to the large amount of metabolic substances tissue released in response to oxidative stress to which it is subjected after being cut (Tang and Newton, 2004). While it is true that the use of some of these metabolites as lupanine alkaloid has been experimentally reported both medical area (Dove et al., 2011) and the agrochemical (Bermudez et al., 2009), it is also true that in the initial explant management stage, the combination of these with phenols and other exudates metabolites is undesirable.

The objective of this research was to evaluate the response to callus formation and their gain in biomass from three different types of explants (hypocotyl and epicotyl Cotyledon) grown in media added with different concentrations of auxin 2,4 dichlorophenoxyacetic acid (2,4-D), cytokinin 6-benzylaminopurine (BA) and 6-furfurylaminopurine (kinetin) and two types of incubation.

Material and methods

The research was conducted on the premises of the Laboratory of plant tissue culture in the Department of Plant Science at the Universidad Autónoma de Chapingo.

Plant material. As seedling explant source *Lupinus montanus* four weeks of age, obtained by germinating seeds in vitro in MS culture media (Murashigue and Skoog, 1962) were used. The seeds were obtained from plants collected in 2011 on the hill of Xipes (19 ° 00 '48' 'N, 97 ° 21' 20 " W), municipality of Libres, Puebla. The plants were identified by taxonomic keys and descriptions of Dunn (1979), subsequently they validated and placed in the National Herbarium of Mexico (MEXU) of the Institute of Biology of the Universidad Nacional Autónoma De México.

Obtaining explant. It was harvested at random a total of 10 seedlings after 4 weeks of growth. The regions used as explant source: hypocotyl, cotyledon and epicotyl, were sectioned in approximate size 3x3x3 mm, and then planted in MS (Murashigue and Skoog, 1962) in petri dishes under six different treatments with 24 replicates each. The treatments were the following T0 = control without plant growth regulators (RCV), T1 = 2,4-D (1.0 **μM**) + BA (0.5 **μM**), T2 = 2,4-D (1.0 **μM**) + kinetin (0.5 M), T3 = 2,4-D (2.0 **μM**) + BA (2.0 M) + kinetin (1.0μM), T4 = 2,4-D (4.0 **μM**) + BA (1.0 M) + kinetin (2.0μM) and T5 = 2,4-D (6.0 **μM**) + BA (2.0 M) + kinetin (2.0μM). In all cases the RCV were supplemented in MS 100%, supplemented with 0.40 mg L-1 thiamine, 100 mg L-1 myo-inositol, 3% sucrose and 7 g L-1 agar, and 100 mg L-1 of activated charcoal at a pH of 5.7 ± 0.1.

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Subsequently, 12 repetitions of each treatment were incubated under conditions of constant darkness at 25 ± 1 ° C, while the remaining 12 for each treatment period were incubated under light / dark (16: 8) at an average temperature of 25 ± 1 ° C, both the incubation period was four weeks to recording variables evaluated.

Callogenic response was evaluated with a qualitative scale of 0 to 1, where 0 = no answer, 1 = callus formation. For the variable level of necrosis, the scale was 0 to 3: where 0 = (<10%), 1 = (> 10 <30%), 2 = (> 30 <60%), 3 (> 60%) . The color and structure of the callus were determined by observations cream or green compact or friable, respectively. Furthermore, for quantitative evaluation variable (biomass) is randomly selected callus six samples of each treatment and the increase in fresh weight was obtained in each case.

Statistical analysis. The results of qualitative variables were analyzed under a completely randomized design (DCA) by Kruskal-Wallis test ($P \leq 0.05$). While results in increased biomass were evaluated in a factorial arrangement DCA, using the model:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + (AB)_{ij} + (AC)_{ik} + (BC)_{jk} + (ABC)_{ijk} + \xi_{ijkl}$$

Where:
Y_{ijkl} = Response biomass increase
μ = General Media
A_i = Effect of the explant (hypocotyl, cotyledon and epicotyl)
B_j = Effect of treatment (2,4-D: BA: Kinetin)
C_k = Effect of incubation (photoperiod: permanent darkness)
$(AB)_{ij}$ = Explant interaction Treatment
$(AC)_{ik}$= Explant interaction: incubation
$(BC)_{jk}$= Interaction treatment: incubation
$(ABC)_{ijk}$= Explant interaction Treatment: incubation
ξijk = Experimental error

The data analysis of variance and Tukey ($P \leq 0.05$) were submitted in all cases the Minitab 17.0 statistical software was employed (2013).

Results and discussion

Callogeneic response. After four weeks it shows that the culture media presenting combinations of plant hormones induced callus formation in three different types of explant under both incubation procedures (photoperiod: permanent darkness), contrasting with that observed in the control treatment (T0) in which there was no response (Figure 1).

No statistical difference ($p = 0.0789$) for callus formation between treatments with combinations of 2,4-D auxin BA with respect to those with combinations of 2,4-D with Kinetin found. Similarly explant level was not statistically different ($p = 0.0876$) because from the three tissues callus formation was presented.

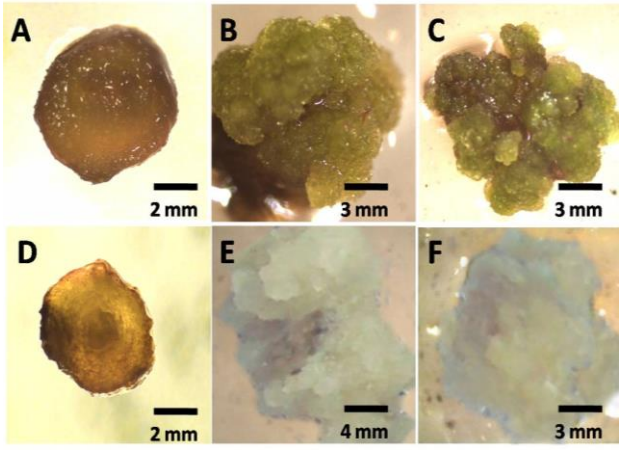

Figure 1 Callus formation under conditions of incubation photoperiod (16: 8). A) Hypocotyl (T0), B) epicotyl (T3), C) Cotyledon (T4); Callus formation under conditions of constant darkness. D) Epicotyl (T0), E) Epicotyl (T5), F) Hypocotyl (T3).

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These results agree with those reported by Montes et al., (2009), who managed to induce callus formation of Lupinus ascherbornii from cotyledon explants of root and hypocotyl, with combinations of 2,4-D and kinetin, in concentrations 1 mg L-1a 2 mg L- 1 (4 to 8 mM).

Degree of necrosis. The Kruskal Wallis noted that statistically there is no difference regarding the degree of necrosis of the explants incubated under conditions of photoperiod and permanent darkness (p-value = 0.105) treatments. In general, under dark conditions, the level of necrosis which had calluses formed from the three types of explant was less than 5% of all occurrences incubated in the different treatments. On the other hand, under conditions of light / dark (16: 8), the level of necrosis was close to 12% of all occurrences; 15 of them were for cotyledon, hypocotyl and 8 to 3 to Epicotyl. It should be noted that the degree of tissue necrosis presenting these corresponded in all cases to the category of level 1 (> 10 <30%).

Callus color and structure. It was observed that both the color and the structure of the callus were intimately associated with the incubation conditions (p-value = 0.000), regardless of the culture medium and the explant source. In terms of light / dark, chlorophyll present in the cells of the callus was expressed generating a uniform green pattern (Figure 2A) while under steady dark, chlorophyll was inhibited, as a result, the fabric the tissue acquired a creamy tonality (Figure 2B).

This fact agrees with the commonly observed in in vitro cultures of various species under both conditions, as said work Parsaeimehr et al., (2010) and Tavakkol et al; (2011), who when evaluating the effect of light and growth regulators on callus induction of herbaceous *Brassica napus* L., Ephedra observed the same tones in corns grown in light and dark.

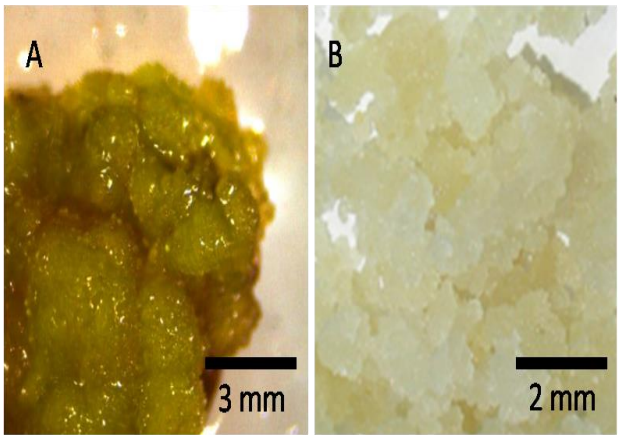

Figure 2 Color and structure calluses. A) Callus formed from hypocotyl explant under conditions of light / dark (T4). B) Callus formed from hypocotyl explant under conditions of permanent darkness (T4).

On the other hand, Figure 2A illustrates the compact structure of callus had grown photoperiod light / dark, while in the second case (Figure 2B), was friable structure type in all instances. This result is in line with those reported by Rajiv and Yadav (2006) on the influence of light on induction of friable calluses incubated at low intensities of light or dark, for various plant species.

Callus biomass. The variance analysis allowed observing the effects that the factors evaluated in increasing biomass. A level explants, there was statistically significant difference (p-value = 0.000) compared to the effect of the explant in biomass response, regardless of the medium or incubation condition which was subjected to (Figure 3A). The explant from which formed callus with the highest biomass was epicótilo (1.3307 ± 0.5151), whereas among the hypocotyl and cotyledon not point Tukey statistical difference (p-value = 0.0637).

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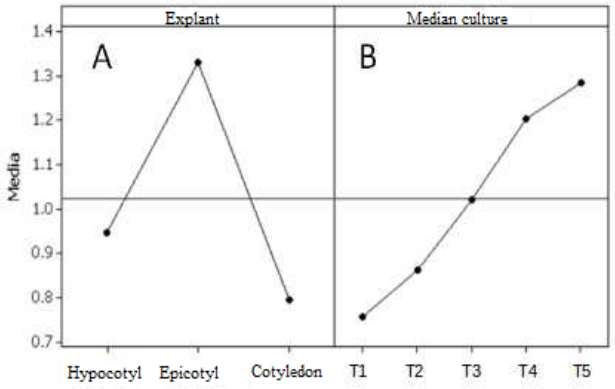

Figure 3 Charts major effects on biomass of corn. A) Effect of explant. B) Effect of culture medium.

A culture media level analysis of variance showed that there was a significantly different response for biomass response (p value = 0.000). In Figure 3B the positive effect of culture media with higher concentrations of 2,4-D, BA and kinetin in the callus biomass increase, regardless of the incubation was observed. Induced responses culture media T4 and T5 were significantly different and greater than those obtained with the other media. Similarly, the effect of incubation factor was statistically different (p-value = 0.000) to the response obtained in permanent darkness photoperiod.

Figure 4 shows the interaction between the various factors discussed in the experiment. The answer callus biomass was higher for the three types of explant cultures incubated under conditions of constant darkness (Figure 4A).

The interaction between culture media and incubation process, significant (p-value = 0.002). The average responses for media with higher content of 1 mM 2,4-D, BA and / or Kinetin (T3, T4, T5) were significantly different from those obtained with the media whose cytokinin concentrations were less than 1 PM (T1, T2).

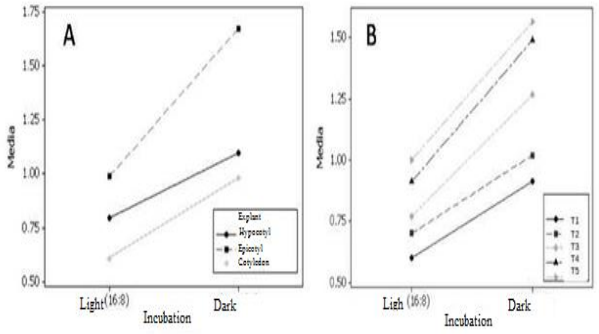

Figure 4 Interaction Charts. E) vs incubation Interaction explant. B) Interaction of culture medium vs incubation.

For any of the explants evaluated under any of the different culture media, the response in biomass is increased, going from a state of incubation in the light, to a phase of permanent darkness (Figure 4A).

The increase in biomass observed in the dark, could be associated to the fact that auxin not undergo degradation by photo-oxidation, allowing a greater extent are present in both the medium (exogenous) and the tissue (endogenous) further promoting cell elongation and consequently the further development of biomass compared to what happens in lighting conditions, as explained some research at the level of plant tissues (Taiz and Zeiger, 2010).

Conclusions

All combinations of different types used RCV induced callus formation from three different explants. Percentages of necrosis of the tissues were very low (5% in darkness photoperiod at 12%) and did not affect the survival of the tissue. Moreover, the calluses formed from any of the explants incubated both photoperiod and dark level differed coloration and structure being green and compact structure under conditions of light / dark, and cream and friable structure under conditions of constant darkness.

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Corns (both compact and friable) generated from epicotyl were those with higher gain in biomass, while cotyledon was the one that produced less biomass. With respect to the culture media, and increasing dose combination of 2,4-D, BA and kinetin potentiate a better response in biomass increase for the three types of explant.

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