CrossRef DOI of original article:

In Vitro Multiplication of Hemp (Cannabis sp.) in Cotopaxi-Ecuador

Received: 1 January 1970 Accepted: 1 January 1970 Published: 1 January 1970

Abstract

Index terms—

1 I. INTRODUCTION

In the industry of Cannabis spp. There are many hundreds of psychoactive and non-psychoactive varieties whose uses are mainly destinated to the medicinal, cultural, and recreational field; the cannabis industry has high potential of growing due to the increase in number of countries focused in its medicinal use. The production of Cannabis in the world has increased since 2011 to 2017 to 406.1 tons (t), and it is expected that licit global market change from 7% in 2018 to 44% in 2025, moving 214 billion of dollars (166 licit and 48 illicit) (Ramírez, Naranjo and Torres 2018).

Among the advantages found to make cultivation of these species viable in countries close to the equator, it is mentioned an adequate legal framework, lower production costs (lighting, labor, inputs), good productive infrastructure, availability, and a favorable productive fabric (pharmacy and floriculture). It is important to promote Cannabis industry due to its potential for creation of job, and export (Ramírez, Naranjo and Torres 2018).

The Cannabis plant has existed for about 10000 years since the discovery of agriculture in the Old World; it is one of the oldest crops of man, it is a source of hemp fiber, oil, achenes (food seeds), narcotic properties used in medicine and pharmacology in the treatment of diseases, and accepted in London Journal of Research in Science: Natural and Formal many world religions (Schultes et al. 2000). Botanically, the Cannabis is part of the Cannabaceae family, which contains two genres, Cannabis and Humulus; and three species for the current crop, C. indica, C. sativa, and C. ruderalis, different by their form of growth, achenes, and mainly by their fiber (Schultes et al. 2000;Thomas 2012). In recent years, the active principle cannabidiol (CBD), an indirect antagonist of tetrahydrocannabinol (THC), has received more importance due to its pharmacological and non-addictive effects.

In Ecuador, given the event of legalization of Cannabis on September 17 in 2019, approved by the national assembly, there is a legal regulation for medicinal or therapeutic and industrial purposes. The content must be less than 1% THC and between 10-15% CBD. In addition, there are seven licenses: 1) Import and commercialization of seeds; 2) Sowing and production of seeds;). Therefore, it is described an in vitro shoot multiplication method using achenes as initial explant.

2 II. MATERIALS AND METHODS

The). In all stages, cultures remained in growth chamber at temperature of 24 ± 2 °C, and photoperiod of 16 hours of light and 8 hours of darkness.

3 Introduction and germination (Stage 1)

A disinfection treatment was applied prior to in vitro introduction by soaking the achenes in running water for 20 minutes (Chaohua et al. 2016), and superficially sterilizing with alcohol 75% (v/v) for 2 minutes and 30 seconds, followed by immersion in sodium hypochlorite (NaClO) 3% (v/v) with a surfactant agent for 25 minutes (Galán-Ávila et al. 2020), and several washes with sterile distilled water after each disinfectant agent. The disinfection treatment combined investigations with achenes as initial explant. The variables were germination percentage, and contamination. London Journal of Research in Science: Natural and Formal

4 Germinated achenes (Stage 2)

Germinated achenes CRNTIO (in vitro), M.B. (in vitro), and P.V. (peat), were subcultured in previously autoclaved peat, containing 2.0 mg?L -1 of 6-BAP (Galán-Ávila et al. 2020; Villezcas 2020), macro and micronutrients in sealed plastic vessels to avoid explant dehydration. The variables were number of leaves, explant length (cm), and number of nodes.

5 In vitro shoot multiplication (Stage 3)

Nodal segments of germinated achenes were subcultured in GENNBIO medium with different concentrations of plant growth regulators (Table 2). The data were arranged in 2 x 2 factorial design, two varieties, two hormonal treatments, and n replicates; and through analysis of variance (ANOVA) means were compared using Tukey's test. Additionally, exploratory cultures were carried out with explants from a mother plant. The variables were number of shoots or leaves, and explant length (mm). Statistical packages InfoStat 2016, and Minitab 16 were used.

6 III. RESULTS AND DISCUSSION

The material consisted of achenes product of mass selection process, adapted to the equatorial line. The method was developed for cultivars of Cannabis sativa L.; landraces are heterozygous populations due to cross-pollination; and represent an important gene pool in plant breeding programs.

7 Introduction and germination (Stage 1)

In this research, an achene is considered germinated (Figure 1), when the radicle exceeds 1 mm in length outside its cover (Prohens, Soler and Nuez 1999). The use of antibiotics is recommended after disinfection with alcohol or chlorine (Smith 2000), to avoid contamination once the achenes have germinated.

8 Percentage of germination

The highest percentage of in vitro germination (n = 50) was obtained with M.B. = 32%, followed by CNRTIO = 16%, and in peat with P.V. = 8%. Previous studies with Cannabis mention an in vitro germination percentage (5%) similar to that obtained with P.V., however, the concentration of agar-agar and landraces are key factors to increase these percentages and reduce germination time and emergence of cotyledons (< 7 days), due to the fact that by traditional methods germination and emergence of cotyledons occurs after the fourth day with imported varieties of hemp (Simbaña 2005). Previously, higher germination percentage was obtained in peat (n = 16) with M.B. = 93.75% (Figure 2). By using these methods, in vitro and in peat, controlled nutritive microenvironments are generated (Ioannidis, Tomprou, and Mitsis 2022), space is reduced, and times are optimized. London Journal of Research in Science: Natural and Formal

9 Germinated achenes (Stage 2)

As the explants developed, those called off type were discarded.

10 Number of leaves

The number of leaves (mean) was higher in P.V. = 14 (n = 2) compared to M.B. = 10.67 (n = 3); assuming that the variances are the same, and applying T test for independent samples, these means are significantly different, with p-value = 0.0099.

11 Length of explant

While, the explant length (mean) did not present significant differences between P.V. = 11.7 cm and M.B. = 9.0 cm, with p-value = 0.3067.

12 Number of nodes

On the other hand, the number of nodes was statistically similar between P.V. = 6.5 and M.B. = 5.0, with p-value = 0.2048 (Figure 3).

13 In vitro multiplication of shoots (Stage 3)

In vitro shoot multiplication in Cannabis sp. was executed by adding antibiotic solutions to the protocol, mainly fungicides and bactericides in standardized concentrations during the process.

14 Number of shoots or leaves

In a completely randomized design (DCA), the landrace*regulator interaction did not present significant differences, since p-value = 0.2213; therefore, the analysis was continued with the main effects landrace and regulator, sending the landrace*regulator effect to the error; thus, the number of shoots or leaves (mean ± S.E.)

was statistically different between P.V. = 1.70 ± 0.51 and M.B. = 0.00 ± 0.51, with p-value = 0.0304 (Table **??**; Figure **??**). In previous studies with Cannabis variety Changtu, it is mentioned the obtention of 1.83, 2.00, and 1.74 auxiliary shoots using KT in proliferation stage, fourteen days after subculture (Wang 2009), similar to the number of shoots or leaves obtained in this research with P.V. using young explants, since they response better to in vitro conditions compared to differentiated tissues, due to the high rate of mitosis (Zwenger 2014).

15 Explant length

In a completely randomized design (CRD), the landrace*regulator interaction did not present significant differences, since p-value = 0.2213; however, applying Tukey's test, different groups were observed, being the experimental point P.V.:Cit = 3.20 mm ± 0.77 the largest explant (Figure 5), which means that the best result was obtained with application of KIN in P.V. London Journal of Research in Science: Natural and Formal The analysis was continued with the main effects landrace and regulator, sending the landrace*regulator effect to the error; thus, explant length (mean ± S.E.) was statistically different between P.V. = 2.00 mm ± 0.57 and M.B. = 0.00 mm ± 0.57, with p-value = 0.0234 (Table 4; Figure **??**). Normality of errors was checked using Ryan-Joiner's test (similar to Shapiro-Wilk), with p-value = 0.061.

16 IV. CONCLUSIONS

In stage 1, the cannabis landrace with the highest in vitro germination and using peat was M.B. = 32% and 93.75%, respectively; followed by in vitro germination in CNRTIO = 16%, and P.V = 8%.

In stage 2, the number of leaves (mean) was statistically different between P.V. = 14 and M.B. = 10.67. The explant length (cm) and the number of nodes were similar between P.V. and M.B.; however, the latter are essential for shoot multiplication process at industrial scale.

In stage 3, it was significantly demonstrated the highest number of shoots or leaves (mean ± S.E.) in P.V. = 1.70 ± 0.51, also the largest explant length in P.V. = 2.00 mm ± 0.57 in presence of cytokinin fourteen days after subculture, respectively. Finally, in CRNTIO2 was observed callogenesis response in presence of kinetin in the selected culture medium.

The impact of this project is the development of an efficient protocol for shoot multiplication, and regeneration of hemp plantlets to be genetically transformed, and conserved in germplasm banks for industrial uses in Cotopaxi-Ecuador.

17 This page is intentionally left blank

London Journal of Research in Science: Natural and Formal 10 [1] [2]

Figure 1:

1

Figure 2: Figure 1 :

Figure 3: Figure 2 :

Figure 4: Figure 3 :

34

Figure 5: Table 3 :Figure 4 :

5

Figure 6: Figure 5 :

2

Explant	Growth regulators			References
Stage 2 explant (M.B. and P.V.)	0.25 mg?L -1	AIA and 1.0 mg?L -1	BAP,	(Galán-Ávila et al. 2020; Villezcas 2020)
Stage 2 explant (M.B. and P.V.)	2.0 mg?L -1		KIN	(Wang et al. 2009)
Mother plant (CRNTIO)	0.25 mg?L -1	AIA and 1.0 mg?L -1	BAP,	(Galán-Ávila et al. 2020; Villezcas 2020; Wang et al. 2009)
	2.0 mg?L -1		KIN	

Figure 7: Table 2 :

4

Landrace	Mean ± S.E.	N	Group
P.V.	2.00 ± 0.57	10	B
M.B.	0.00 ± 0.57	10	A

Figure 6: Explant length (mm) in P.V. landrace at multiplication stage in presence of kinetin. Source: GENNBIO. London Journal of Research in Science: Natural and Formal 6

Figure 8: Table 4 :

[1] © 2023 Great] Britain Journals PressIn Vitro Multiplication of Hemp (Cannabis sp.) in Cotopaxi-Ecuador
[2] London Journal of Research in Science: Natural and Formal

17 THIS PAGE IS INTENTIONALLY LEFT BLANK

.1 ACKNOWLEDGMENTS

To London Journals Press (LJP).

Conflicts of interest: the authors declare no conflict of interest.

[London Journal of Research in Science: Natural and Formal] , *London Journal of Research in Science: Natural and Formal*

[Terranova ()] , Terranova . 1998. Bogotá, Colombia. (Enciclopedia agropecuaria Terranova. Producción agrícola 2. Tomo 3)

[Wang ()] 'A micropropagation system for cloning of hemp (Cannabis sativa L.) by shoot tip culture'. R Wang . *Pak. J. Bot* 2009. 41 p. .

[Chaohua ()] 'A rapid shoot regeneration protocol from the cotyledons of hemp (Cannabis sativa L.)'. C Chaohua . *Industrial Crops and Products* 2016. 83 p. .

[Acuerdo Ministerial No. 109. 1, 48. Available online ()] https://www.agricultura.gob.ec/wp-content/uploads/2020/10/109-2020-1.pdf *Acuerdo Ministerial No. 109. 1, 48. Available online*, 2020. Ministerio de Agricultura y Ganadería.

[Ioannidis et al. ()] 'An alternative in vitro propagation protocol of Cannabis sativa L. (Cannabaceae) presenting efficient rooting, for commercial production'. K Ioannidis , I Tomprou , V Mitsis . 10.3390/plants11101333. https://doi.org/10.3390/plants11101333 *Plants* 2022. 11 p. .

[Changoluisa and Peñafiel ()] *Análisis del potencial productivo del cannabis no psicoactivo (Cannabis sativa) con fines investigativos para la industrialización en la provincia de Cotopaxi*, V Changoluisa , D Peñafiel . 2021. Ecuador. Universidad Técnica de Cotopaxi (Graduate thesis)

[Wahby ()] *Aproximaciones biotecnológicas tendentes a la mejora del cáñamo (Cannabis sativa L.): Obtención y cultivo de raíces transformadas, transformación genética y regeneración in vitro*, I Wahby . 2007. España: Universidad de Granada. (Doctoral thesis)

[Morales and Andrade ()] 'Biotechnological plant breeding applied to purple blackberries'. J Morales , P Andrade . 10.21931/RB/2023.08.01.7. http://dx.doi.org/10.21931/RB/2023.08.01.7 *Revis Bionatura* 2023. 8 (1) p. 7.

[Thomas ()] 'Cannabis cultivation'. M Thomas . *A complete grower´s guide. 3rd edition*, (San Francisco, CA, USA) 2012. Green Candy Press.

[Doemer ()] 'Cell division regulation'. P Doemer . *Biochemistry and molecular biology of plants*, B En , W Buchanan , R Gruissem , Jones (eds.) (USA) 2000. American Society of Plant Physiologists. p. .

[Galán-Ávila ()] 'Development of a direct in vitro plant regeneration protocol from Cannabis sativa L. seedling explants: developmental morphology of shoot regeneration and ploidy level of regenerated plants'. A Galán-Ávila . *Frontiers in Plant Science* 2020. 11 p. .

[Pilalumbo and Unaucho ()] *Efecto de dos medios de cultivo y tres niveles de ácido giberélico en el crecimiento de cannabis (Cannabis sativa) obtenidas por germinación in vitro en el laboratorio de la facultad CAREN*, H Pilalumbo , M Unaucho . 2022. Ecuador. Universidad Técnica de Cotopaxi (Graduate thesis)

[Wain ()] 'El control químico del crecimiento de las plantas'. R L Wain . *Los reguladores de las plantas y los insectos*, N R En , Ondarza (eds.) (México) 1980. CONACYT. p. .

[Alvarado and Benalcázar ()] *El negocio del cannabis florece en Pedro Moncayo*, P Alvarado , W Benalcázar . https://www.elcomercio.com/actualidad/negocio-cannabis-pedro-moncayo-cannandes 2021. (html?utm_source=facebook&utm_medium=social&utm_campaign=photopost&fbclid=IwAR30TtM4zHzDNwnDenn8eivroGTSqRXNisTKgBWWFooJDOkHaeu1Cg5FhD0)

[Océano ()] *Enciclopedia práctica de la agricultura y ganadería*, Océano . 1999. Barcelona, España.

[Hernández ()] *Ensayo de variedades de cáñamo en La Vega Baja del Segura*, U M Hernández . 2016. España. Universidad Miguel Hernández (Graduate thesis)

[Simbaña ()] *Evaluación de tres variedades de cáñamo (Cannabis sativa var. sativa) bajo tres densidades de siembra para la obtención de fibra (Graduate thesis)*, A Simbaña . 2005. Ibarra, Ecuador: Pontificia Universidad Católica.

[Ramírez et al. (2018)] 'La industria del cannabis medicinal en Colombia. Una ventana de oportunidad para la transformación productiva de Colombia'. J Ramírez , J Naranjo , A Torres . *FEDEDESARROLLO* 2018. 18 sep.

[La legalización de la siembra, cultivo y cosecha de cannabis en Ecuador entró en vigor ()] *La legalización de la siembra, cultivo y cosecha de cannabis en Ecuador entró en vigor*, https://www.eluniverso.com/noticias/2020/06/26/nota/7886121/legalizacion-siembra-cultivo-cosecha-cannabis-ecuador-entro 2020. El Universo

[Malpica ()] K Malpica . *Marihuana. Available online: https://Marihuana -El cáñamo de las Indias*, 2004. (mindsurf.net)

17 THIS PAGE IS INTENTIONALLY LEFT BLANK

[Morales and Chiluisa-Utreras ()] J Morales , V Chiluisa-Utreras . *Mejoramiento biotecnológico de plantas y modificación genética. Editorial Grupo Compás*, (Guayaquil, Ecuador) 2022.

[Inec ()] *Módulo ambiental de la encuesta de superficie y producción agropecuaria continua*, Inec . https://www.ecuadorencifras.gob.ec/documentos/web-inec/Encuestas_Ambientales/Informacion_am-biental_en_la_agricultura/2016/informe_ejecutivo_ESPAC_2016.pdf 2016. 2016. Instituto Nacional de Estadística y Censos.

[Villezcas ()] *Obtención de células de Cannabis sativa L. in vitro, por citocinina no convencional, metatopolina*, G Villezcas . 2020. México. Universidad Autónoma de Chihuahua (Postgraduate thesis)

[Saad and Elshahed ()] 'Plant tissue culture media'. A I Saad , A M Elshahed . *Recent advances in plant in vitro culture*, En A Leva , L Rinaldi (eds.) 2012. InTech. p. .

[Smith ()] *Plant tissue culture: Techniques and experiments*, R H Smith . 2000. College Station, Texas; USA. Department of Soil and Crop Sciences, Texas A&M University

[Bhojwani and Razdan] *Plant tissue culture: Theory and practice*, S S Bhojwani , M K Razdan . Delhi, India: ELSEVIER.

[Schultes ()] *Plantas de los dioses. Las fuerzas mágicas de las plantas alucinógenas*, R Schultes . 2000. México: Fondo de Cultura Económica.

[Salgado ()] *Proyecto Startup: Green In Vitro*, A Salgado . 2020. Ecuador. Universidad San Francisco de Quito (Graduate thesis)

[Ratikanta ()] M Ratikanta . *Fibras vegetales en el mundo: aspectos botánicos: calidad y utilidad. Trillas*, (México D. F., México) 1995.

[Zwenger ()] *The biotechnology of Cannabis sativa. 2nd Edition*, S Zwenger . 2014. New York, USA: Extreme Publications, Inc.

[Prohens et al. ()] 'The effects of thermotherapy and sodium hypochlorite treatments on pepino seed germination, a crucial step in breeding programmes'. J Prohens , S Soler , F Nuez . *Annals of Applied Biology* 1999. 134 p. .