Journal of Oleo Science
Copyright ©2015 by Japan Oil Chemists' Society
doi : 10.5650/jos.ess14206
J. Oleo Sci. **64**, *(3) 325-330 (2015)*

JOURNAL OF OLEO SCIENCE
Jos

NOTE

Effect of Plant Growth Regulators on Fatty Acids Composition in *Jatropha curcas* L. Callus Culture

Ludwi Rodríguez Hernandez[1], Martha A. Rodríguez Mendiola[1], Carlos Arias Castro[1] and Federico A. Gutiérrez-Miceli[2]*

[1] *Area de Posgrado en Ciencias en Agrobiotecnología. Instituto Tecnológico de Tlajomulco, Jalisco, México.*
[2] *Department of Plant Biotechnology, Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, México.*

Abstract: The influence of Naphtaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) on callus formation, its morphology and fatty acids profile were examined from *Jatropha curcas* L. Embryo from seeds of *J. curcas* L. were sown in Murashige and skoog (MS) medium with NAA and BAP. All treatments induced callus formation, however callus morphology was different in most of the treatments. Higher callus biomass was presented with 1.0 NAA + 0.5 BAP mg/L. Plant growth regulators modifies the fatty acids profile in callus of *J. curcas* L. BAP was induced linoleic and linolenic acids.

Key words: naftalen acetic acid, 6-benzylaminopurine, callus culture, fatty acid profile

1 INTRODUCTION

Jatropha curcas L. is a plant that belongs to the Euphorbiaceae family, it is widely spread in the tropical and subtropical areas[1)]. Historically this plant has been associated to multiple uses in ground recovery, and processing of feed, food, pesticides, cosmetics and anticancer medicine[2–5)]. Recently the oil of the seed of *J. curcas* L. has been studied, due to its potential as raw material for the biodiesel production[6)]. Varieties with little or no antinutrients compounds such as phorbolesters, trypsin inhibitors, phytates, saponins and lectins have been found in Mexico, furthermore they contain a significant amount of oil in their seeds (8.020% to 54.28%)[7–9)]. Currently, has been developing *in vitro* propagation protocols for *J. curcas* L., using different explants at contrasting concentrations and combinations of plant growth regulators[10)]; With leaves, Thidiazuron (TDZ; 0.90 μM) in combination with indol-3-butíric acid (AIB, 0.98 μM) produce adventitious shoots. In apical meristems, the best result was obtained with Benzylaminoupurine (BAP; 2.5 μM). The purpose of all these protocols was spreading this plant in a massive way[11)]. Other studies with *J. curcas* L., induce the formation of callus tissue in MS medium containing 1.0 mg/L, IBA and 0.5 mg/L BA,[12)].

The callus are cell clusters in an undifferentiated state, which can be induced to a cell re-differentiation process through the addition of vegetal growth regulators, as well as to produce secondary metabolites of industrial interest. The requirements of plant growth regulators and its concentrations can influence in the formation of more viable callus, callus growth and could be induce changes in metabolism in the callogenesis phase[12)].

With somatic embryos of jojoba (*Simmondsia chinensis* (Link) Schneider) have been found that the plant growth regulators induced changes in fatty acids profile[13)]. With *J. curcas* L, the cell viability, fatty acid content, and characteristics were used to select the most promising cell line according to its fatty acid profile and ability to grow and develop under *in vitro* conditions[14)]. Therefore in the present investigation the influence of NAA and BAP on the callus formation, its morphology and the fatty acids profile were examined in *Jatropha curcas* L.

2 EXPERIMENTAL PROCEDURES

2.1 Obtaining, disinfestations, and *in vitro* establishment of plant material

Mature fruits were collected from Suchiapa, Chiapas; Mexico (16° 37′ 06″ iN, 93° 05′ 28″ iW). Fruit outer fleshy

*Correspondence to: Federico A. Gutiérrez-Miceli, Department of Plant Biotechnology, Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, México.
E-mail: fgmiceli@gmail.com
Accepted November 27, 2014 (received for review September 13, 2014)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

coat was removed and the seeds were superficially disinfected with 10% of sodium hypochlorite, subsequently, the inner hard seed coat was take out, and were submerged in an Agrimicin 500 (0.5%) + Metalaxil (0.25%) solution with agitation for 10 min. Seeds were rinsed with sterile distilled water five times, and they were submerging again in a 0.1% mercury chloride solution ($HgCl_2$) for 5 min. The seeds were rinsing with sterile distilled water, and placed on a sterile filter paper. Zygotic embryo with cotyledone leaf were obtained from disinfected seeds and were sown in Murashige and Skoog medium[15] supplemented with 0.5, 1.0 and 3.0 mg/L of Naphtaleaneacetic acid (NAA) and 0.5, 1.0 y 3.0 mg/L of Benzilaminopurine (BAP) (Table 1). The medium pH was adjusted to 5.8 and it was sterilized in autoclave at 1.5 kg/cm^2 of pressure and 121℃ for 20 min. Cultures were incubated at 25℃, with a 24/0 photoperiod. The observations of the callus, root, stem, and leaves formation were made after 35 and 40 days of culture in 10 flasks containing 3 embryos. Calluses were replanted every 25 days, using the same composition of the medium for every treatment described in Table 1. Data is present as the percentage of each parameter. The callus fresh weight was taken at the 40 day of culture.

2.2 Callus viability estimation

The callus viability was estimated by the use of fluoresceine diacetate (FDA) according to the method described in ref.16, a 5 mg/mL FDA stock solution was prepared in acetone and it was kept at 4℃, a 0.1 mL aliquot from the stock solution was added to 5 mL of distilled water, the calluses samples were placed in containers, and two drops of FDA solution were added, after two min the viable cells fluoresced as a result of the lighting with ultraviolet light, using a microscope of fluorescence (Leica DMLS model DC100). The viability percentage was estimated considering the amount of cells that fluoresce in each field.

2.3 Oil extraction and preparation of fatty acid methyl esters (FAMEs)

The fresh viable calluses were dehydrated in a Labconco lyophilizer at constant temperature of −40℃ and 1.33×10^{-3} mBar, for 48 h. For the extraction of oil was used 0.5 g of lyophilisate calluses (Soxhlet method) using a BUCHI extraction equipment (B-811), 20 cycles for 2 h, using petroleum ether as solvent. The methyl esters were prepared according to the method described in ref. 17. Were taken 100 μL of oil and 1 mL of 2 M NaOH in methanol was added, it was heated in water bath at 80℃ for 20 min with

Table 1 The effect of Naphtaleaneacetic acid (NAA) and Benzylaminopurine (BAP) on callus, root, stem and leaves formation in *J. curcas* L.

Treatment	Concentration (mg/L)	Response (%)			
		Root	Leaves	Stem	Callus
Control	0	80 a	40 dc	100 a	0 c
BAP	0.5	0 b	0 e	0 e	100 a
BAP	1.0	0 b	0 e	0 e	100 a
BAP	3.0	0 b	0 e	0 e	100 a
NAA	0.5	0 b	60 cb	60 cb	100 a
NAA	1.0	0 b	80 ba	80 ba	100 a
NAA	3.0	0 b	0 e	0 e	80 ba
NAA + BAP	0.5 + 3.0	0 b	100 a	80 ba	100 a
NAA + BAP	1.0 + 0.5	0 b	100 a	20 ed	100 a
NAA + BAP	1.0 + 1.0	0 b	0 e	40 dc	100 a
NAA + BAP	1.0 + 3.0	0 b	0 e	0 e	100 a
NAA + BAP	3.0 + 0.5	0 b	0 e	0 e	60 b
NAA +BAP	3.0 + 1.0	0 b	0 e	0 e	60 b
NAA + BAP	3.0 + 3.0	0 b	0 e	0 e	60 b
Mean		5.71	27.14	27.14	82.85
SE		0.053	0.119	0.447	0.1253
LSD (95%)		0.1514	0.3386	0.3709	0.3551
CV (%)		409.13	192.86	165.01	45.81

Mean values with the same letter in the column are not statistically different ($p < 0.05$).

constant agitation. The sample was cooled and mixed with 1mL of BF_3 14% in methanol, and it was heated again at 80℃ for 20 min with constant agitation, later the methyl esters were extracted using 1mL Hexane HPLC grade.

2.4 Determination of fatty acid composition

The fatty acids obtained from extracts of *J. curcas* L. callus were analyzed by gas chromatography coupled with mass spectrometry (GC-MS, Agilent Technologies 7890A, United States). The column was (DB-WAXter, J & W Scientific 122-7362 de 60m × 250 μm × 0.25 μm). Helium as carrier gas (1 mL/min). 1 μL of FAMEs extract was injected in mode split. The temperatures of injector and detector were 250℃ and 230℃ respectively. The oven was programmed with temperature flux at 150℃ for 5 min, to 30℃ min^{-1} to 210℃, to 1℃/min to 213℃, and finally 20℃/min to 225℃ for 12 min. The detection was made through a mass selective detector (Agilent Technologies 5975C). Compounds in each sample were identified by comparing with mass spectra of National Institute of Standards and Technology Library (NIST05).

2.5 Statistical analysis

The statistical significant differences of the growth regulators effect on *Jatropha curcas* L. callus for biomass, and fatty acids profile, were determined by a variance analysis (ANOVA), applying the minimum significant difference criterion (LDS) α 0.05.

3 RESULTS

3.1 Effect of plant growth regulator (PGRs) on embryos and callus induction of *J. curcas* L

Roots, leaves, stems were observed in embryos culture. Roots were observed in treatment without added PGRs. Leaves and stems were encountered in the treatments 0.5 and 1.0 mg/L NAA, NAA + BAP (0.5 mg/L + 3.0 mg/L), NAA + BAP (1.0 mg/L + 0.5 mg/L), stems only was detected in the treatment NAA + BAP (1.0 mg/L + 1.0 mg/L) (Table 1). All the treatments with PGRs, individually and/or combined with the different concentrations induced the callus formation. Additions of BAP and NAA to different concentrations induced the callus formation with values from 80 to 100%. When higher NAA concentration was mixed with BAP at the three different concentrations, a decrease in callus formation was observed, reporting values of 60% (Table 1).

Table 2 The effect of Naphtaleaneacetic acid (NAA) and Benzylaminopurine (BAP) on biomass and morphology of *J. curcas* L. callus.

Treatment	Concentration (mg/ L)	Callus biomass (g)	Callus Morphology
Control	0	0	–
BAP	0.5	2.81 dcba	Compact, green-yellow
BAP	1.0	3.55 ba	Compact, green-yellow
BAP	3.0	2.31 edcb	compact coffee
NAA	0.5	2.04 edcb	Fluffy soft, coffee-clear
NAA	1.0	1.46 edcb	Compact, light-coffee
NAA	3.0	0.83 ed	Granular, Green-Light
NAA + BAP	0.5 + 3.0	3.39 cba	Fluffy White, green
NAA + BAP	1.0 + 0.5	4.67 a	Soft fluffy, green-light
NAA + BAP	1.0 + 1.0	1.64 edcb	Compact, light brown, green
NAA + BAP	1.0 + 3.0	2.37 edcba	Soft spongy, green-light
NAA + BAP	3.0 + 0.5	0.43 e	Granular fluffy, green-light
NAA +BAP	3.0 + 1.0	0.83 ed	Granular fluffy, green-light
NAA + BAP	3.0 + 3.0	1.06 edc	Friable, light coffee, yellowish-green
Mean		1.93	
SE		0.26	
LSD		2.10	
CV (%)		78.67	

Mean values with the same letter in the column are not statistically different ($p < 0.05$).

Table 3 The effect of Naphtaleaneacetic acid (NAA) and Benzylaminopurine (BAP) on fatty acid profile in *J.curcas* L. callus culture.

fatty acids	Treatments (mg/L)						LSD (95%)
	NAA			BAP			
	0.5	1.0	3.0	0.5	1.0	3.0	
Pentanoic acid m.e (C5:0)	*nd	*nd	*nd	*nd	0.72 a	*nd	0.042
Nonanedioico acid d.e (C11:0)	*nd	*nd	*nd	*nd	1.33 a	*nd	0.098
Tetradecanoic acid m.e (C14:0)	*nd	*nd	*nd	*nd	1.35 b	1.57 a	0.118
12-tetradecenoic acid m.e (C14:1)	*nd	*nd	*nd	*nd	*nd	0.41 a	0.034
Hexadecanoic acid m.e (C16:0)	*nd	*nd	17.16 b	*nd	26.28 a	24.93 a	3.066
7-hexadecenoic acid m.e (C16:1)	*nd	*nd	*nd	*nd	*nd	0.25 a	0.177
16-heptadecenoic acid m.e (C16:1)	*nd	*nd	7.43 a	*nd	*nd	*nd	0.623
Octadecanoic acid m.e (C18:0)	*nd	*nd	*nd	*nd	8.65 b	12.21 a	0.609
9-octadecenoic acid m.e (C18:1)	*nd	*nd	*nd	*nd	*nd	23.78 a	0.520
8-octadecenoic acid m.e (C18:1)	*nd	*nd	*nd	*nd	12.63 a	*nd	0.427
10-octadecenoic acid m.e (C18:1)	*nd	*nd	55.44 a	*nd	*nd	*nd	7.426
8,11-octadecadienoic m.e (C18:2)	*nd	*nd	*nd	*nd	11.53 b	17.44 a	0.535
9,12,15-octadecatrienoic acid m.e (C18:3)	*nd	*nd	*nd	*nd	7.19 b	14.10 a	0.563

Mean values with the same letter in the row are not statistically different ($p < 0.05$). * nd: not detected

3.2 Effects of the PGRs, on callus biomass fresh weight and microscopic morphology of the callus

Calli were detected after 35 days of culture. The highest value of callus biomass fresh weight (4.67 g) was registered in the combined treatment NAA + BAP (1.0 + 0.5) mg/L (Table 2). PGRs induced callus with different color and consistency. The callus color was different ranging from light brown, light green, yellowish green to green (Table 2). Callus consistency (compact, soft, and friable) was also different. In the treatment NAA + BAP (3.0 + 3.0 mg/L) callus morphology was friable and light Brown in contrast with the rest of the treatments that had a compact and soft consistence.

3.3 Fatty acids profile (FAP) in the seed and callus of *Jatropha*. curcas L

The oil content in the seeds of *J. curcas* L. was of 46.4 ± 4.2% (W/W) whereas fatty acids composition was: Hexadecanoic acid m.e (16:0), Octadecanoic acid m.e (18:0), 9-octadecenoic acid m.e (18:1), and 8,11-octadecadienoic m.e (18:2) (Table 3). In the quantitative analysis of FAP in callus obtained from different treatments was found Pentanoic acid m.e (5:0), Nonanedioico acid d.e (11:0), Tetradecanoic acid m.e (14:0), 12-tetradecenoic acid m.e (14:1), Hexadecanoic acid m.e (16:0), 7-hexadecenoic acid m.e (16:1), 16-heptadecenoic acid m.e (16:1), Octadecanoic acid m.e (18:0), 9-octadecenoic acid m.e (18:1), 8-octadecenoic acid m.e (18:1), 10-octadecenoic acid m.e (18:1), 8,11-octadecadienoic m.e (18:2), and 9,12,15-octadecatrienoic acid m.e (18:3) acids (Table 3). In treatments 0.5 and 1.0 mg/L of NAA and 0.5 mg/L BAP, no fatty acids was detected. In the BAP treatments 1.0 and 3.0 mg/L, were determined eight different types of fatty acids, among them is highlighted polyunsaturated acids such as: 8,11-octadecadienoic m.e (18:2) (Omega 6) and 9,12,15-octadecatrienoic acid m.e (18:3) (Omega 3), also in these treatments was found a greater number and proportion of fatty acids: Hexadecanoic acid m.e (16:0), Octadecanoic acid m.e (18:0), 9-octadecenoic acid m.e (18:1), 8,11-octadecadienoic m.e (18:2) and 9,12,15-octadecatrienoic acid m.e (18:3). In the combined treatments NAA + BAP at least one of the fatty acids from the polyunsaturated type was determined (Table 4).

4 DISCUSSION

NAA and BAP played an important role in the formation and microscopic morphology of the callus obtained from the embryos of *Jatropha curcas* L. Friable callus was obtained with high concentrations of both regulators. These results coincide with[18, 19] who report that the combined additions of NAA and BAP yielded higher callus formation. The non-accumulation and detection of fatty acid in the calluses obtained in this study in treatments 0.5 and 1.0 mg/L of NAA and 0.5 mg/L BAP (Table 3) can be because some hormones may be degraded or conjugated with sugar and/or amino acid to form biologically inert compounds, and lose their functionality, and not being able to be translocated to the meristematic zone[19], however, with 3.0 of

Table 4 The effect of Naphtaleaneacetic acid (NAA) and Benzylaminopurine (BAP), at different concentrations, on the fatty acids obtained from callus *J.curcas* L.

Fatty acids	Treatments (NAA + BAP) mg/L						LSD (95%)
	0.5 3.0	1.0 0.5	1.0 1.0	3.0 0.5	3.0 1.0	3.0 3.0	
Pentanoic acid m.e (C5:0)	*nd	*nd	*nd	0.74 a	*nd	*nd	0.030
Pentanoic acid 14- methyl, m.e (C6:0)	*nd	9.91 b	30.15 a	*nd	*nd	*nd	5.151
Nonanedioic acid d.e (C11:0)	*nd	*nd	*nd	1.50 a	*nd	*nd	0.137
Tetradecanoic acid m.e (C14:0)	*nd	*nd	*nd	1.38 a	*nd	1.53 a	0.269
Hexadecanoic acid m.e (C16:0)	*nd	16.03 c	*nd	22.29 b	32.65 a	28.48 a	4.331
Heptadecanoic acid 16-methyl m.e (C16:0)	*nd	3.93 b	*nd	*nd	8.12 a	*nd	1.153
Octadecanoic acid m.e (C18:0)	*nd	*nd	*nd	7.98 c	10.40 b	12.37 a	1.322
9-octadecenoic acid m.e (C18:1)	*nd	*nd	*nd	15.34 b	23.34 a	23.95 a	1.848
10-octadecenoic acid m.e (C18:1)	*nd	10.03 b	33.58 a	*nd	*nd	*nd	6.977
8,11-octadecadienoic m.e (C18:2)	*nd	*nd	*nd	5.22 c	14.68 a	7.63 b	0.963
9,12-octadecadienoic acid m.e (C18:2)	*nd	4.78 b	*nd	*nd	6.19 a	*nd	0.775
9,12,15-octadecatrienoic acid m.e (C18:3)	*nd	*nd	*nd	2.71 b	*nd	3.56 a	0.670

Mean values with the same letter in the row are not statistically different ($p < 0.05$). * nd: not detected

NAA, three fatty acids were detected in significant proportions: Hexadecanoic acid (17.16%), 16-heptadecenoic acid m.e (7.43%), and 10-octadecenoic acid m.e (55.44%). The methodology used to callus obtain from the zygotic embryos of *J. curcas* L. using NAA and BAP to different concentrations, yielded positive results on the fatty acids profile, finding more oil content in callus obtained *in vitro* in comparison with the oil contained naturally from the seeds collected in field. This results is important and demonstrating that the PGRS can modify the fatty acids profile in plants, and promote the biosynthesis of fatty acids in callus culture[13].

The results show that the treatments with BAP (1.0 and 3.0 mg/L), influenced the fatty acids biosynthesis because induced a greater number and proportions of FA. The results obtained in this investigation confirm what has already been shown with somatic embryos of jojoba that the PGR's modify the fatty acids profile, benefiting the synthesis of fatty acids from the polyunsaturated types[13]. A possible explication is related with BAP action on the biosynthesis of fatty acids[20], it could be a factor that influence in the synthesis of enzymatic proteins such as piruvate kinases related with the formation of carbonated chains in the biosynthesis of fatty acids in plants[21]. Another possibility could be related to citokinins activity in the translocation to the meristematic zone and the metabolic processes in different genes[19]. The BAP effect in lowest concentrations (0.5 mg/L) can be explained by their differential uptake, varied translocation rates to meristematic regions and metabolic processes. However, It has been reported that the concentration of cytokinins influence the proliferation of shoots, better response to higher concentration in *J. curcas* L.[8], similar data were found in the present study, where the lowest concentration of cytokinin did not influence the fatty acid biosynthesis in *J. curcas* L. callus, where the presence of fatty acids was not detected (Table 3), contrary to the treatments with 1.0 and 3.0 mg/L.

Has been reported that the combined addition of 2,4-D and BA, has induced to the formation of the essential polyunsaturated fatty acid C18:2 (omega-6) linoleic acid, (omega-3) alpha-linolenic acid (ALA), with higher values of long chain saturated fatty acids C16:0 palmitic acid and monounsaturated fatty acid C18:1 oleic acid[13], these results agree with those found in this work, where the addition combined of NAA 3.0 mg/L BAP at 0.5, 1.0 and 3.0 mg/L, favored the biosynthesis of FAs with long chains, saturated and unsaturated, found Hexadecanoic acid m.e (C16:0), Octadecanoic acid m.e (C18:0), 9-octadecenoic acid m.e (C18:1), 8,11-octadecadienoic m.e (C18:2), 9,12-octadecadienoic acid m.e (C18:2), and 9,12,15-octadecatrienoic acid m.e (C18:3) (Table 4). Majority appearance of long chain fatty acids, could be due to the action of plant growth regulators has been shown that compounds of C18: 2 and/or C18: 3 tends to increase its size when there is the presence of 2, 4-D and BA[13]. Therefore the above-described effect may explain the low appearance of short-chain fatty acids in the treatment added with NAA 3.0 mg/L and BAP 0.5, 1.0 and 3.0 mg/L, in this work.

The results show that callus culture of *Jatropha* curcas L. was able of fatty acids synthesize and NAA and BAP addition were influenced on qualitative and quantitative FA profile. This result is of great relevance for future investigations for obtaining oil with polyunsaturated FA for com-

mercial purposes obtained in the callus stage and for FA induction in suspension cell culture. This experiment also opens the possibility to elicit and promote fatty acids biosynthesis in callus culture.

5 CONCLUSION

The callus morphology was related to concentration and combination of NAA and BAP, the exposure of embryos of *Jatropha curcas* L., to vegetal growth regulators induced the synthesis of fatty acids in the callogenic stage. Callus culture was able to synthesize polyunsaturated fatty acids resulting in high quality oil. Fatty acids production in callus culture can be the beginning of production on a large scale, using bioreactors.

ACKNOWLEDGMENTS

Rodríguez Hernandez thanked PRONABES the scholarship program. Maria Celina Lujan Hidalgo of ITTG, Chiapas, México, for her assistance in the chromatographic analysis.

REFERENCES

1) Schmook, B,: Serralta-Peraza, L. *J. curcas*: distribution and uses in the Yucatan Peninsula of Mexico. In: Gubitz GM, Mittelbach M, Trabi M(eds)Biofuels and industrial products from *Jatropha curcas*. Dbv-Verlag, Graz, pp 53-57(1997).
2) Staubmann, R.; Ncube, I.: Gubitz, G. M.; Steiner, W.; Read, J. S. Esterase and lipase activity in *Jatropha curcas* L. *J. Biotechnol.* **75**, 117-126(1999).
3) Openshaw, K. A review of *Jatropha curcas*: an oil plant of unfulfilled promise. *Biomass Bioenergy* **19**, 1-15(2000).
4) Lin, J.; Zhou, X. W.; Tang, K. X.; Chen, F. A survey of the studies on the resources of *Jatropha curcas. J. Trop. Subtrop. Bot.* **12**, 285-290(2004).
5) Mampane, K. J.; Joubert, P. H.; Hay, I. T. *Jatropha curcas*: use as a traditional Tswana medicine and its role as a cause of acute poisoning. *Phytother. Res.* **1**, 50-51(2006).
6) Moser, B. R. Biodiesel production, properties, and feedstocks. *In Vitro Cell. Dev. Biol.-Plant* **45**, 229-266(2009).
7) Makkar, H. P. S.; Martínez-Herrera. J.; Becker, K. Variations in seed number per fruit, seed physical parameters and contents of oil, protein and phorbol esters in toxic and non-toxic genotypes of *Jatropha curcas. J. Plant. Sci.* **3**, 260-265(2008).
8) Nitish, K.; Anand, V. K.; Muppala, R. Plant regeneration of non-toxic *Jatropha curcas*-impacts of plant growth regulators, source and type of explants. *J. Plant Biochem. Biotechnol.* **20**, 125-133(2011).
9) Ovando-Medina, I.; Espinosa-Garcia, F. J.; Núñez-Farfán, J.; Salvador-Figueroa, M. Genetic Variation in Mexican *Jatropha curcas* L. Estimated with Seed Oil Fatty Acids. *J. Oleo Sci.* **60**, 301-311(2011).
10) Khurana-Kaul, V.; Kachhwaha, S.; Kothari, S. L. Direct shoot regeneration from leaf explants of *Jatropha* curcas in response to thidiazuron and high copper contents in the medium. *Biologia Plantarum* **54**, 369-372(2010).
11) Purkayastha, J.; Sugla, T.; Paul, A.; Solleti, S. K.; Mazumdar, P.; Basu, A.; Mohommad, A.; Ahmed, Z.; Sahoo, L. Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas. Biologia Plantarum* **54**, 13-20(2010).
12) He, Y.; Guo, X.; Lu, R.; Niu, B.; Pasapula, V.; Hou, P.; Cai, F.; Xu, Y.; Chen, F. Changes in morphology and biochemical indices in browning callus derived from *Jatropha curcas* hypocotyls. *Plant Cell Tiss. Organ Cult.* **98**, 11-17(2009).
13) Aly, M. A. M.; Amer, E. A.; Al-Zayadneh, W. A.; Eldin, A. E. N. Growth regulators influence the fatty acid profiles of in vitro induced jojoba somatic embryos. *Plant Cell Tiss. Organ Cult.* **93**, 107-114(2008).
14) Correa, S. M.; Atehortúa, L. Lipid Profile of In Vitro Oil Produced Through Cell Culture of *Jatropha curcas. J. AOAC Int.* **95**, 1161-1169(2012).
15) Murashige T.; Skoog, F. A revised medium for rapid bioassays with tobacco tissue cultures. *Physiol. Plant* **15**, 473-497(1962).
16) Wildholm, J. The use of fluoresceindiacetate and phenosafranine for determining viability of culture plant cells. *Stain Tech.* **47**, 189-194(1972).
17) Egan, H.; Kirk, R. S.; Sawywer. R. Analisis de Alimentos de Pearson. CECSA. México(1981).
18) Koroch, A.; Juliani, H. R.; Kapteyn, J.; Simon, J. E. In vitro regeneration of Echinacea purpurea from leaf explants. *Plant Cell Tiss. Organ Cult.* **69**, 79-83(2002).
19) Kaminek, M. Progress in cytokinin research. *Trends Biotech.* **10**, 159-162(1992).
20) Redig, P.; Shaul, O.; Inze, D.; Van, Montagu, M.; Van, Onckelen, H. Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. *FEBS Lett.* **391**, 175-180(1996).
21) Ireland, R. J.; DeLuca, V.; Dennis, D. T. Isozymes of pyruvate kinase in etioplasts and chloroplasts. *Plant Physiol.* **63**, 903-907(1979).