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IN VITRO CHARACTERIZATION OF FIXED OIL CONTENTS FROM CALLUS CULTURES OF JOJOBA (*SIMMONDSIA CHINENSIS* L.)

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ABSTRACT

Objective of the present study was to investigate and determine fixed oil (lipids) contents from *in vitro* induced calluses of leaf, cotyledon and intermodal explants of jojoba (*Simmondsia chinensis* L.). The leaf and internodal explants were from micropropagated *in vitro* plants whereas mature seeds were used for the acquisition of cotyledons. Sterilized explants were cultured on different concentrations (1, 2, 4, 6, 8 or 10 µM) of either 2, 4-Dichlorophenoxyacetic acid (2, 4-D) or α-Naphthaleneacetic acid (NAA) alone or in combination (1+1, 2+1, 4+1, 6+2, 8+2 or 10+2 µM) with 6-Benzylaminopurine (BAP) or Kinetin (Kin) for callus induction. Highly proliferating callus lines (CLs) growing on 8 µM 2, 4 - D (CL-1), 10 µM 2, 4-D (CL-2) and 2, 4-D (10 µM) + 2 µM BAP (CL-3) were selected for the determination of fixed oil contents by distillation method. Highest rate of callus induction (100%) as well as 6.87 gm fresh weight was obtained from leaf explant on CL-3 after 99 days of culture. Amount of fixed oil content (290mg/6gm FW), acid value (0.320), free fatty acid (0.160) and saponification number (91) were highest from calluses of cotyledons on CL-1 as compared to other callus lines CL-2 or CL-3. The present investigation demonstrated an efficient method for determination of the fixed oil contents and related biochemical parameters from calluses of jojoba.

Keywords: *Callus, fixed oil, free fatty acid, jojoba, liquid wax.*

INTRODUCTION

Simmondsia chinensis L. is commonly known as 'jojoba' belongs to the family Simmondsiaceae, deer nut or wild hazel (Steven, 2000). This is a small semi-hardwood 2-20 feet high shrub. Jojoba is wide spread over 18,500 hectares and the demand of its oil is 6400-200,000 tons per year throughout the world (Hussain *et al.* 2011). Jojoba is also known as desert shrub, drought resistant crop cultivated in waste and dry places in different regions of Pakistan of Bahawalpur, D.I. Khan, Karachi and Quetta (Aftab *et al*., 2008).

Secondary metabolites primarily serve as reserve stored food material in plants. Such chemicals provide protection to plants as well as contribute to the preparation of life-saving medicines, confectionaries, cosmetic and health promoting activities (Tietela *et al.,* 2021). Fixed oil is an important metabolite of jojoba (Aftab *et al*. 2008) contained 50% lipids of its dry seed weight. Oil quantity in jojoba seed is twofold the amount of other oil yielding crops (Kumar *et al.,* 2012). Jojoba oil is golden in color "unsaturated liquid wax with odorless greasy feel" (Kumar *et al.,* 2012). Jojoba lipids are straight chain monoesters of alcohol and acids (Wisniak, 1987). It has different uses for synthesis of high-pressure lubricants, renewable energy source, preparation of cosmetics, fire retardants, food, transformer oils, electrical insulators and pharmaceuticals (Wisniak, 1987).

Oil yield may be enhanced by introducing improved verities and disease-free plant production. For this purpose, plant tissue culture technology is usually employed for the improvement of plant health, vigor and rapid multiplication. Cells of callus tissue are totipotent that may have ability to regenerate into complete plant. It is therefore prerequisite to harvest whole callus tissue acquainted with its oil yielding capacity for subsequent commercial application. Thus, aim of the present work was to induce callus and selection of suitable callus lines having improved growth for the determination of fixed oil contents of jojoba.

MATERIALS AND METHODS

Plant material and culture conditions

Jojoba seeds were procured from PU Seed Centre, University of the Punjab, Lahore, Pakistan. Seeds were thoroughly washed under running water followed by 0.1% $HgCl_2$ for 10 min, and finally decontaminated with 6% (v/v) active chlorine in the form of NaOCl (Robin Bleach, Reckitt Benckiser, Pakistan) for another 15 min. Thoroughly rinsed seeds with autoclaved distilled water under sterile conditions were cultured on MS (Murashige & Skoog 1962) medium + 0.5 µM 6-Benzylaminopurine (BAP) for 25 days for the establishment of axenic seedlings at 25 ± 2 °C and 16h photoperiod (35 µmol $m^{-2}s^{-1}$).

Callus induction and growth

There were 36 treatments to test callus induction from cotyledon, internode and leaf explants. Axenic seedlings were cut to prepare cotyledons, internode and leaf explants (0.5 – 1.0 cm^2) and cultured on MS medium supplemented with different concentrations (1, 2, 4, 6, 8 or 10 µM) of either 2, 4-Dichlorophenoxyacetic acid (2, 4-D) or α-Naphthaleneacetic acid (NAA) alone or in combination (1+1, 2+1, 4+1, 6+2, 8+2 or 10+2 µM) with 6-Benzylaminopurine (BAP) or Kinetin (Kin) for callus induction under complete dark conditions at the temperature mentioned above. The data for callus induction were taken after 49 days of initial culture.

Selection of callus lines and fresh weight (FW)

Cultures with rapid and healthy callus growth were categorized as callus lines (CL). Such lines were growing on 2,4-D 8 µM (CL-1), 2,4-D 10 µM (CL-2), 2,4-D 10 µM + 2 µM BAP (CL-3). Calluses of these lines were further cultured on the

respective fresh medium. Fresh weights (FW) of the calluses were then taken after 14, 63 and 99 days of re-culture.

Extraction of total lipids from CL

Total lipids were extracted by immersing 6g FW callus tissue from selected CL in distillation flask fitted with condenser containing 100 ml n-hexane. Flask was placed on the heating bath to heat the solvent at 60 °C. The same process was used for lipids extraction for all other experimental tissues. After 60 min, condenser was removed and n-hexane containing lipid contents were evaporated at 50 °C by agitating. Weight of the remaining evaporated material was recorded and calculated total lipids contents by the following formula.

$$\text{Lipid contents (\%)} = \frac{\text{Wt. of extracted lipids}}{\text{Wt. of callus tissue}} \times 100$$

Biochemistry

For biochemical investigation, the modified method of Akubugwo *et al*. (2008) was followed.

Acid value

Acid value was determined by dissolving 50 mg oil (W) in equal proportion (1:1 v/v) of 1.5 ml ethyl alcohol: diethyl ether solvent. This was then titrated by stirring against 0.1N NaOH (V) and phenolphthalein was used as indicator. Acid value was determined by using the following formula.

Acid value = $\frac{56.1 \times N \times V}{W}$

Free fatty acid (%FFA) was determined by multiplying acid value with factor 0.503 as follows: %FFA = 0.503 × acid value

Saponification number:

Saponification number was determined by taking 50 mg oil in conical flask and dissolved in 1 ml of 0.5% ethanolic KOH and refluxed on water bath for 30 min. Then few drops of phenolphthalein indicator were added and the hot mixture was titrated against 0.5 N HCl taken in the burette. A blank reading was recorded and determined the saponification number by using the following formula.

Saponification number = $\frac{56.1N\,(V1-V2)}{W}$

N= Normality of HCl

V_1= Volume of HCl used in test

V_2= Volume of HCl used in blank

W= Weight of oil used

Data analyses

Statistically data were analyzed by ANOVA using the function Duncan's multiple range test (DMRT) for comparison of means. Mean values of fresh weight of calluses were compared using the function box plot. All analyses were carried out at $p<0.05$ probability level as determined by SPSS v. 16.0.

RESULTS AND DISCUSSION

Callus induction

The rate of callus induction was significantly improved by increasing the concentration of 2,4-D alone from cotyledon explants (Table 1). Highest (100%) callus induction was obtained at the both concentrations of 8µM or 10µM 2,4-D from cotyledon explants after 49 days of initial culture (Table 1). Similarly, 100% callus induction was also observed at 10 µM 2,4-D + 2 µM BAP from leaf explants. Callus induction at lower concentrations of NAA alone was good enough while higher levels seemed detrimental, when BAP or Kin was used along with NAA; the harmful effect vanished.

Authors observed a little callus from internode on all media types that may be due to the hard texture of an explant. Fig. 1 showed the trend of callus induction frequency from different explants on different media formulation. Cotyledon was most responsive explant as compared to leaf or internode.

Table 1. Effect of various treatments involving MS medium supplemented with various growth regulators on callus induction from different explants of jojoba after 49 days

Tr. No.	PGRs (µM)				Callus induction (%)		
	2,4-D	NAA	BAP	Kin	Cotyledon	Internode	Leaf
1	1				90.22 ± 6.55^{abc}	50.55 ± 4.51^{fgh}	20.21 ± 2.60^{ijk}
2	2				92.38 ± 4.33^{abc}	66.66 ± 3.55^{ef}	60.82 ± 3.21^{ef}
3	4				93.21 ± 4.02^{ab}	50.22 ± 4.22^{fgh}	66.63 ± 6.51^{def}
4	6				93.82 ± 5.01^{ab}	51.25 ± 4.45^{fgh}	70.25 ± 7.33^{cde}
5	8				100^{a}	81.33 ± 5.55^{cd}	100^{a}
6	10				100^{a}	95.21 ± 5.21^{ab}	100^{a}
7		1			81.55 ± 6.66^{cd}	25.21 ± 2.52^{ij}	0
8		2			75.33 ± 3.31^{cde}	25.21 ± 4.52^{ij}	88.33 ± 3.21^{bc}
9		4			66.61 ± 4.51^{def}	33.33 ± 3.21^{hij}	66.66 ± 6.32^{def}
10		6			66.61 ± 6.65^{def}	0	25.42 ± 4.24^{ijk}
11		8			40.01 ± 4.32^{d}	40.41 ± 4.25^{hi}	40.05 ±4.25^{ij}
12		10			41.22 ± 3.32^{d}	40.36 ± 2.15^{hi}	41.33 ± 1.25^{ij}
13	1		1		92.25 ± 7.33^{abc}	50.21 ± 3.33^{fgh}	75.55 ± 5.21^{cd}
14	2		1		93.15 ± 6.21^{ab}	53.63 ± 5.55^{fg}	76.51 ± 4.37^{cd}
15	4		1		97.25 ± 3.20^{ab}	55.68 ± 5.61^{fg}	75.75 ± 4.25^{cd}
16	6		2		97.15± 7.33^{ab}	80.82 ± 6.45^{cd}	81.37 ± 3.40^{bc}
17	8		2		98.01 ± 6.33^{ab}	89.37 ± 7.55^{b}	88.25 ± 4.44^{b}
18	10		2		100^{a}	98.45 ± 6.45^{a}	100^{a}
19	1			1	66.25 ± 3.33^{def}	40.44 ± 4.25^{hi}	50.51 ± 5.55^{fij}
20	2			1	66.37 ± 4.21^{def}	42.25 ± 3.21^{hi}	60.02 ± 3.33^{ef}
21	4			1	71.91 ± 4.25^{de}	48.37 ± 3.00^{gh}	62.55 ± 4.22^{ef}
22	6			2	75.55 ± 5.21^{cde}	50.51 ± 4.21^{fgh}	68.25 ± 4.01^{def}
23	8			2	76.51 ± 6.66^{cde}	55.33 ± 3.33^{fg}	68.37 ± 7.33^{def}
24	10			2	80.25 ± 5.51^{cd}	60.21 ± 2.11^{efg}	71.25 ± 3.21^{cde}
25		1	1		21.22 ± 2.01^{hi}	15.99 ± 3.21^{ij}	27.13 ± 4.10^{ijk}
26		2	1		25.33 ± 3.21^{hi}	19.21 ± 3.25^{ij}	30.21 ± 2.11^{ijk}
27		4	1		26.25 ± 2.33^{hi}	21.27 ± 4.02^{ij}	30.25 ± 2.11^{ijk}
28		6	2		38.19 ± 4.17^{gh}	35.02 ± 4.12^{hij}	35.29 ± 3.33^{ijk}
29		8	2		41.21 ± 2.11^{gh}	40.25 ± 2.21^{hi}	45.21 ± 2.22^{ij}
30		10	2		49.23 ± 4.27^{fg}	41.20 ± 4.21^{hi}	47.22 ± 5.55^{ij}

31	1	1	60.21 ± 5.77^{efg}	94.88 ± 6.45^{ab}	66.66 ± 4.32^{def}
32	2	1	62.71 ± 3.11^{efg}	83.45 ± 4.51^{cd}	41.24 ± 2.24^{ij}
33	4	1	62.61 ± 4.21^{efg}	75.45 ± 6.51^{bc}	53.45 ± 5.21^{fij}
34	6	2	64.66 ± 5.63^{def}	41.21 ± 4.25^{hi}	60.66 ± 6.32^{ef}
35	8	2	66.33 ± 5.63^{def}	35.33 ± 2.41^{hij}	65.23 ± 6.32^{def}
36	10	2	79.14 ± 5.63^{cd}	35.42 ± 3.63^{hij}	71.46 ± 6.32^{cde}

The observed values given in the columns are the mean of three replicates of three independent experiments. Mean values (±SE) indicated with small alphabetical different letters are significantly different as per DMRT (Duncan's multiple range test $p \leq 0.05$).

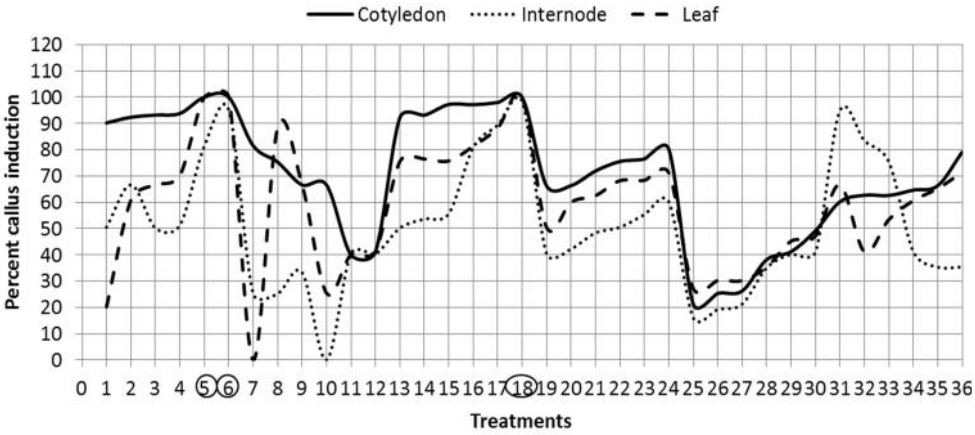

Fig. 1. Comparison of values and trend percent callus induction of each treatment from different explants of 49-day old cultures of jojoba. Encircled treatments indicate CL-1, CL-2 and CL-3, respectively

Selection of callus lines (CL)

There were three treatments (2, 4-D 8 µM or 10 µM and 2, 4-D 10 µM + 2 µM BAP) that formed 100% callus on both cotyledon and leaf explants. Color of calluses was different of white and milky appearance observed at the periphery of the excised cotyledon at 8 µM 2, 4-D after 14 days of initial culture (Fig. 2a). Such calluses proliferated further and formed huge amount of translucent friable mass with leathery and grayish appearance after 21 days (Fig. 2b). Calluses obtained with 10 µM 2, 4-D + 2 µM BAP from leaf explants were soft textured observed after 63 days of culture (Fig. 2c). Friable calluses were observed with 2 µM NAA + 2 µM BAP (Fig. 2d). It was therefore such calluses formed on these treatments selected and designated as CL. The FW of CL was significantly increased with the passage of time after 14, 63 and 99 days of culture (Fig. 3). Highest FW (6.87gm) was obtained from leaf explants followed by 6.65 gm from cotyledon with CL-3 after 99 days.

Quantity and characteristics of total lipid contents

Highest amount of total lipids (290 mg) was obtained from cotyledons cultured on CL-1 followed by CL-3 (250 mg). In case of leaf explants, amount of lipid was higher (120 mg) with CL-3 as compared to 110 mg of other callus lines (Table 2).

Callus tissue from internodal explants produced 110 mg lipids with CL-3 followed by CL-2 (90 mg) and CL-1 (100 mg).

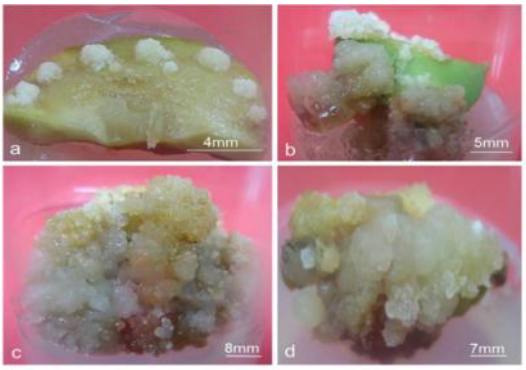

Fig. 2. Callus induction and proliferation in jojoba. a) Callus initiation at the periphery of cotyledon at 8 µM 2, 4 -D after 14 days. b) Translucent and grayish callus proliferation on cotyledonary explant at 10 µM 2, 4 -D after 21 days. c) 63-day old multicolored mass of callus induced with 2, 4-D 10 µM + BAP (2 µM). d) Friable callus induction at 2 µM NAA + 2 µM BAP after 63 days

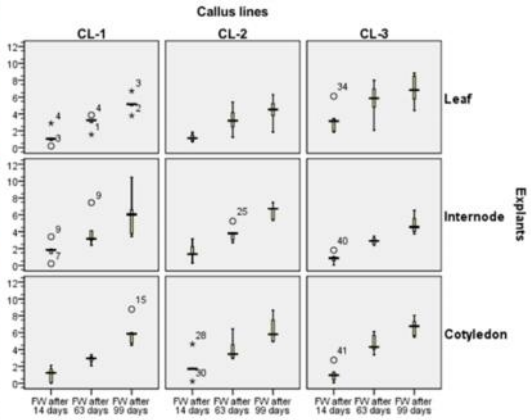

Fig. 3. Fresh weight (FW) of callus lines (CL-1: 2, 4-D 8 µM, CL-2: 2, 4-D 10 µM, CL-3: 2, 4-D 10 µM + 2 µM BAP) of different explants of jojoba. Box plot indicates upper and lower quartile range of fresh weight.

Table 2. Total lipid contents from callus lines of different explants of jojoba

Callus lines	Total lipid contents (mg/6gm FW)		
	Cotyledon	**Internode**	**Leaf**
CL-1	290 ± 0.01^{a} (4.66)	100± 0.01^{a} (0.50)	110± 0.01^{b} (1.69)
CL-2	210 ± 0.02^{c} (3.23)	90 ± 0.01^{b} (0.45)	100± 0.01^{b} (1.69)
CL-3	250 ± 0.02^{b} (3.84)	110± 0.02^{a} (0.46)	120 ± 0.02^{a} (1.84)

Oil contents (%) = weight of oil / weight of callus used for extraction × 100, Values in parenthesis are the percentages of oil contents, CL-1: 2, 4-D 8 µM, CL-2: 2, 4-D 10 µM, CL-3: 2, 4-D 10 µM + 2 µM BAP

Mean values (±SE) indicated with small alphabetical different letters are significantly different as per DMRT (Duncan's multiple range test $p \leq 0.05$).

The present study demonstrated that medium composition of CL-3 was most effective for callus induction and callus FW production from leaf explants. Whereas, CL-1 produced highest amount of lipid contents (290 mg/6gm FW of callus) from cotyledon explants. Chemical characteristics of lipids in terms of acid value (0.320), percent free fatty acids (0.160) and saponification number (91) were highest from cotyledonary explant grown at CL-1 followed by CL-2 and CL-3 (Table 3). The amount of acid value was ranged from 0.320-0.201, FFA 0.160-0.10 and saponification number 91 to 88 in the present study. We observed that callus induction from internodal explant had smallest amount of all parameters that decreased from CL-1 to CL-3. In the contemporary literature, information vis-à-vis indigenous determination of oil in callus cultures is completely lacking in jojoba.

Table 3. Chemical properties of callus oil from different explants of jojoba

Callus source		Acid value (Meq Kg^{-1})	%Free Fatty Acid (FFA)	Saponification number
Cotyledon	CL-1	0.320 ± 0.110^{a}	0.166 ± 0.045^{a}	91 ± 4.25^{a}
	CL-2	0.290 ± 0.013^{b}	0.150 ± 0.041^{ab}	91 ± 3.25^{a}
	CL-3	0.250 ± 0.045^{c}	0.131 ± 0.025^{ab}	89 ± 6.33^{ab}
Internode	CL-1	0.210 ± 0.045^{cd}	0.112 ± 0.036^{abc}	89 ± 4.25^{ab}
	CL-2	0.201 ± 0.033^{d}	0.101 ± 0.045^{bc}	89 ± 6.55^{ab}
	CL-3	0.201 ± 0.021^{d}	0.101 ± 0.025^{bc}	88 ± 7.88^{ab}
Leaf	CL-1	0.220 ± 0.041^{cd}	0.115 ± 0.074^{abc}	89 ± 6.56^{ab}
	CL-2	0.215 ± 0.036^{cd}	0.110 ± 0.042^{abc}	89 ± 6.22^{ab}
	CL-3	0.217 ± 0.085^{cd}	0.112 ± 0.036^{abc}	88 ± 4.66^{ab}

Mean values (±SE) indicated with small alphabetical different letters are significantly different as per DMRT (Duncan's multiple range test $p \leq 0.05$).

Secondary metabolites are reserve food materials stored within the plant organs such as leaves, stems, roots or seeds. Parenchymatous tissues are the key components where these metabolites accumulate in the form of various chemical constituents of lipids or carbohydrates. A callus is an amorphous parenchymatous mass of cells gathers metabolites as predisposition until regenerated. Growth regulators, specifically auxins control the cell division activity and intricate metabolites production. Whereas gamma radiations have also been reported for increased wax oil contents in calluses of jojoba (El-Shabrawi *et al.* 2019). We obtained highest callus growth on 2, 4-D (8 or 10 µM) supplemented media. Farhadi *et al*. (2017) also achieved similar results by using 2, 4-D in *Allium hirtifolium*. On the other hand, El-Shabrawi *et al.* (2019) obtained highest callus induction of jojoba with 0.5 mg/l kinetin + 6% sucrose. Similar to our results, Kumar *et al*. (2012) reported 97.3% callus induction from field collected leaf explants of jojoba on MS medium supplemented with 2 mg/L 2,4-D + 0.5 mg/L BAP + 100 mg/L casein hydrolysate after 22 days of initial culture. In the present study, all concentrations of NAA induced callus albeit the higher levels 8 µM or 10 µM seemed detrimental. The negative effect of NAA on callus growth has been reported in *Zingiber officinale* (El-Nabarawy *et al*. 2015). Leaf explants produced proliferating calluses similar to Arce and Jordan (1988) with 5 mg/L NAA + 0.1 mg/L BAP. The explants containing one node and a shoot tip seemed vital for invigorating the axillary shoots of jojoba cultured on MS medium supplemented with BA or Kin alone (10 mg/L) or in combination with 0.2 mg/L IBA (Rossi 1996). Similarly, 2 mg/L BAP induced callus formation after 14 days and sub-cultured on B_5 + 1 mg/L 2, 4-D + 0.1 mg/L Kin as compared to the callus induction from leaf explants on B_5 + 1mg/L 2, 4-D + 0.1 mg/L Kin (Jabeen 1999). These reports showed explant's response similar to our findings albeit we used MS medium + 8 µM 2, 4-D or 2 µM BAP.

Authors demonstrated that cotyledon was good enough for callus induction whereas nodal explant seemed recalcitrant. Generally, calluses obtained on the combination of both auxin and cytokinin has much more potential for metabolite production (Verma *et al*. 2016) and hard tissues are less responsive for callus induction under in vitro conditions (Asakuraa and Hoshino 2017).

Cotyledonary structures of somatic embryos have been reported for the determination of liquid wax likely that of jojoba seeds (Lee and Thomas 1985). We extracted lipids from calluses of seedling tissues and greenish cotyledons of germinating seeds, whereas Aftab *et al*. (2008) used 6 to 1-year-old plant tissues and mature cotyledons of jojoba. They reported higher fixed oil contents (1502 mg) from calluses of cotyledons as compared to us. This may be due to the difference of satiated plant material at the time of collection. Palmer *et al*. (1994) support this phenomenon that older calluses retained maximum amount of oil than fresh ones. Explant collection from different sources plays fundamental role on the production of lipid contents (Palmer *et al*., 1994). In contrast, traces of lipid contents from the older callus tissues have been reported in the present study. It seems that the proliferating calluses from different explants usually keep the capacity to produce maximum amount of lipids to that of intact plants.

FFA and acid value are important indicators for the quality of the jojoba oil. We obtained higher value of the biochemical parameters of jojoba callus cultures. High acid value demonstrated that oil has more carbon residue resulted more combustion potential in the diesel engines. FFA composition, acid value, palmitic acid and linoleic acid have been reported from callus cultures of *Ajuga* (Sahakyan *et al.*, 2010). Identification of high amount of FFA and other parameters in the present study demonstrated that jojoba oil may become a renewable energy source and substitute of conventional diesel (Durrett *et al*., 2008; Correa and Atehortúa, 2012).

CONCLUSION

It was observed that 2, 4-D was the best auxin for high yielding callus production from green cotyledons of jojoba. Moreover, such calluses may also provide the basis for subsequent regeneration for improved and high yielding crop production. High amount of FFA in the oil of jojoba provides the evidence for an alternative source of energy. This is first report demonstrating the determination of biochemical parameters from callus tissues of jojoba. A very simple and reproducible method for high amount of callus production for sustainable agricultural practices.

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