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Original Research Article

Determination of polyunsaturated fatty acids in *Jatropha curcas* somatic embryos and the effect of abiotic sources

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The present study reports the accumulation of polyunsaturated fatty acids and effect of media on *Jatropha curcas* somatic embryos. Plant cells were cultivated in three different growth media supplemented with various carbon, growth regulators and polyethylene glycol in triplicates. In addition The PUFA composition was determined using GC-MS and spectrophotometer. The maximum relative growth capacity of 0.621 and specific growth rate of 0.242 gm day^{-1} was found from cells cultivated in Murashige-Skoog media. The biomass productivity was found to be 4.531gmL^{-1} day^{-1}. The fatty acid content of suspension culture and callus found to be 8±0.23% and 9±0.16% respectively extracted using chloroform: methanol (2:1). The GC-MS method for rapid determination of fatty acid profile is reliable and reproducible for further bioprocess and biotechnological studies of *Jatropha* tissue culture system.

Key words: Fatty acids, *Jatropha curcas* L., lipids, somatic embryos, suspension culture, callus.

INTRODUCTION

Plant cell culture is a suitable *in vitro* manipulation technique in plant cell biotechnology particularly somatic embryogenesis. The interplay between fundamental medium components and basic plant biology gives models to investigate the process of induction and development of somatic embryogenesis. Several plant somatic embryo regulation strategies for cultivation of plant cells *in vitro* have been reported by several research groups for various applications (Jayasankar et al. 2000). *In vitro* selection, embryo rescue, preservation of germplasm and other *in vitro* technologies to both applied and basic plant research are some of advantages in plant biotechnology through somatic embryogenesis as a tool.

Likewise plant can be cultivated *in vitro* and/or *ex vitro* for the production of industrially important fatty acids for various applications. Plant fats and oils are known to be the major sources of polyunsaturated fatty acids (PUFA). However, problems exist such as the instability of the PUFA content brought about by changes in certain intrinsic/extrinsic biotic and abiotic constraints in conventional cultivation systems. The average temperature and the local climatic conditions have strong influence on fatty acid composition during plant growth and development (Lajara et al., 1990). However, in an in vitro condition these factors could be controlled in a plant system. The various biotic and abiotic constrains cannot affect the stable production of various form of fatty acid components through plant tissue culture techniques. Hence, the important of plant biotechnology through tissue culture is remarkable to explore the potential of the plant.

Lipid biosynthesis and its regulation through *B. napus* somatic embryogenesis have been reported elsewhere (Pomeroy et al., 1991). Their accumulation is developmentally regulated as in Zygotic Embryogenesis. The PUFA can be elongated or further unsaturated to physiologically active substances, such as prostaglandins or leukotrienes via the various fatty acid metabolic pathways has also been reported (Bjerve et al. 1987).

The major fatty acids found in the oil samples of *Jatropha curcas* are oleic (41.5– 48.8%), linoleic (34.6–44.4%), palmitic (10.5–13.0%), stearic (2.3–2.8%) acids, cis-11-eicosenoic acid (C20:1) and cis-11, 14-eicosadienoic acid (C20:2) in the oil (Martınez-Herrera et al., 2006). Oil contents, physicochemical properties, fatty acid

composition and energy values of seed samples of four species of *Jatropha* (*J. curcas, J. glandulifera, J. gossypifolia* and *J. multifida*) have been reported (Banerji et al. 1985).

Biotechnological approaches for crop improvement, somatic embryogenesis and micro propagation of *J. curcas* have been studied by several authors for various applications (Jha et al., 2007). None of these reports show the accumulation of fatty acids in *Jatropha curcas in vitro* so far. In this study, we reported accumulation of total lipid and determination various form of fatty acid from *Jatropha curcas* tissue culture biomass. This work can be helpful for further plant growth regulations, bioprocess and biotechnological studies of fatty acid metabolism to further understand and exploit the potential of *Jatropha curcas* for various industrial applications.

MATERIALS AND METHODS

Plant material and its preparation

The seed of *Jatropha curcas* was procured from Suntan, Tamil Nadu, India. The *in vitro* culture biomass was maintain in MS media supplemented with 3% sucrose, 3mg/l BAP, 2 mg/l TDZ and 0.5mg/l IBA for germination. 0.35% bacteriological agar, 0.12% gelrite (Gelrite® Gelan Gum, sigma) was used for solid phase cultures. The pH of the media was adjusted to 5.8±0.2 before autoclaving and incubated in 16/8 hrs photoperiods for one month. Cell suspension cultures were initiated from callus grown in a solid agar media for 4weeks of same medium composition. The cultures were kept in orbital shaker maintained at 100rpm and 16/8 light/dark photoperiods. Henceforth, the stock suspension was subcultured every 10days to freshly prepared medium in 1:1 ratio. The rest fifty percent of the culture was used for various analyses like estimation of fatty acid. The plant materials were dried overnight at 60°C in oven and then sieved and then taken for further the study. All chemicals were procured from Hi media, India unless and otherwise mentioned.

Estimation of carbon sources in cell suspension culture.

Sucrose content of the cell suspension culture was estimated using anthrone method. Samples from the cell suspension culture were withdrawn at an interval of every 7 days for 45 days and estimated for the changes in the sucrose concentration as follows. A diluted sample in the range of 20-100µl were used each and made the total volume to 200µl with sterile water. Thereafter 100µl of 30% KOH were added to each test tube and incubated in a boiling water bath for 10mins. The solution was cooled for 5min. At an interval of 2mins, 3ml of anthrone prepared in 70% H_2SO_4 were added while keeping the solution at 45°C and incubated for 20 min. The optical density of different sugars was read at 540 nm using Hitachi UV/Vis spectrophotometer. The standard curve was plotted with concentration against absorbance using sucrose as standard.

Lipid extraction and fatty acid determination

Extraction of lipids from the plant biomass was performed according to the modified procedure (Lee et al., 1996). Lipids were exhaustively extracted with chloroform/methanol (2:1, v/v), filtered and concentrated by rotary evaporator (Buchi, Swiss) at 50°C. For phase separation 20ml 0.5% NaCl solution was added by gently shaken and kept until a clear separations observed. The chloroform layer was successfully taken and concentrated using a rotary evaporator. Thereafter the sample was analysed by GC-MS using linoelic acid as a standard, and UV/Vis spectrophotometer using triolein as a standard.

Extraction and spectrophotometer quantification of triglyceride

The callus tissue grown in the aforementioned media composition was harvested in after three weeks. 100mg of the tissue was extracted and purified using a modified procedure (Feirer et al., 1989). Thereafter, 800 µ1 of purified supernatant was added to a test tube with a snap cap and 200 µl of 1 N KOH was added with the tubes held in a 60°C water bath for 5 min. After cooling to room temperature, 200 µl of m-sodium periodate solution in 2M acetic acid was added to each tube. After exactly 10 min., 1.2 ml of 2M ammonium acetate, isopropanol and actylacetone mixture (250:500:1) was added and the tubes returned to the 60°C water bath for ½ h. The optical density of the sample was read at 410 nm using Hitachi UV/Vis spectrophotometer.

Sample preparation for GC-MS analysis

Approximately 2 mg of extracted fatty acid was dissolved in 0.2 ml of chloroform. 0.2 ml of 20 mM? cupric acetate monohydrate in methanol and 1 ml of 0.5 N HC1 in methanol were added, and the mixture was left for 10-60 min at 37°C. The reaction mixture was extracted three times with 2 ml of chloroform after addition of 2 ml of water. The pooled extracts were washed with water and then evaporated to dryness under a flow of nitrogen (Hoshi et al., 1973). The analysis of fatty acid was performed with 450-GC varian 220-MS IT mass spectrometer (Column: Perkin-Elmer HP 5-MS=60m×250µm; oven: initial temp. 160°C for 3 min, ramp 7 °C/min to 220°C for 10min; inj=270°C; volume=1µl; split ratio=100:1; carrier gas=He; solvent delay=5min; transfer temp=250°C; source temp=180°C). The individual constituents showed by GC were identified by comparing their MS with standard compounds of NIST and Willey libraries.

Table 1. GC-MS profile of the fatty acid compassions of *Jatropha* cell

Molecular formula	IUPAC name	Systematic name	Percentage	
			Seed reported	Tissue culture
18:3n-3	9,12,15-octadecatrienoic	Linolenic acid	-	10.911
18:2 (n-6)	Cis-9,11-octadecadienoic	Conjugated linoleic acid	-	26.778
18:2n-6	Cis-9,12-octadecadienoic	linoleic acid	42.3	-
18:1n-9	Cis -9-octadenoic	Oleic	36.5	23.421
16:0	Hexadecanoic	palmatic acid	13.4	33.129.

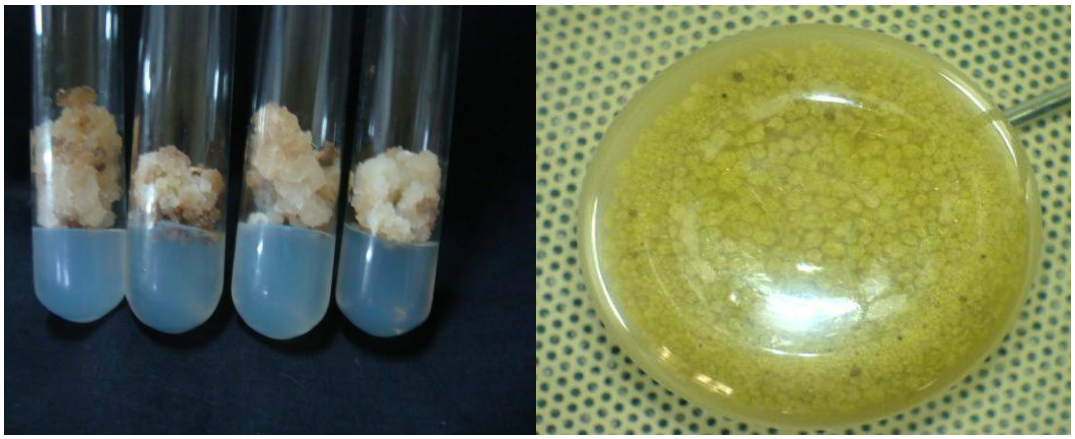

Figure1: *In vitro* grown cell biomass: a) callus after 21 days b) somatic embryos in liquid state

Data analysis

The results were analyzed statistically using SPSS version 10 (SPSS Inc., Chicago, IL, USA). One-Way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) for mean comparison between values of the treatment was used with 5% level of significance. Peak identification of the fatty acids in the analyzed margarine samples was carried out by the comparison with retention times and mass spectra of known standards. Standard methyl esters of palmitic, stearic, oleic and linoleic acids were used for the confirmation of GC-MS libraries result.

RESULTS AND DISCUSSION

Cell cultivation and culture maintenance

The cultivation of *J. curcas* in both liquid state tissue culture systems using different growth media components, growth regulators, carbon sources and nitrogen source was carried out. From media components, MS media supplemented with various combinations of auxin/cytokinin, carbon sources and nitrogen sources showed significant callus induction, development, and proliferation. BAP in combination of with IBA and TDZ showed significant induction of callus in this study. Slower with induction was observed in BAP and 2, 4-D combinations. Of all the carbohydrates used 3% sucrose showed improved callus formation in MS media supplemented with 2mg/l BAP, 0.15mg IBA and 0.2mg/l TDZ. Significant proliferation was achieved when 100mg/l of Glutamine in the aforementioned compositions was added (Table 1). The optimum media was found to be MS media supplemented with 3% sucrose, 2mg/l BAP, 0.15mg IBA and 0.2mg/l TDZ for further experiments. The callus formed in the aforementioned conditions was taken to liquid system. The *in vitro* grown cell biomass is shown in Figure 1 as above.

The effect of Media on the biomass

Three media components MS, B5 and WPM were used and the percentage of induction and the number of somatic embryos per explants were recorded every 21 days after induction. Biomass productivity of the cells was determined by ratio of the change in cell weight increase to maximum cell weight per the cultivation time during which the maximum cell weight is obtained. The Higher biomass productivity was obtained in MS medium at the 16^{th} day of the culture time. The maximum biomass productivity of the cells in B5 and WPM were obtained at 12^{th} day of the culture time. The kinetics of the biomass productivity on the basis of dry cell weight is shown below (Figure 2). The ratio of maximum dry cell weight to preceding cell dry

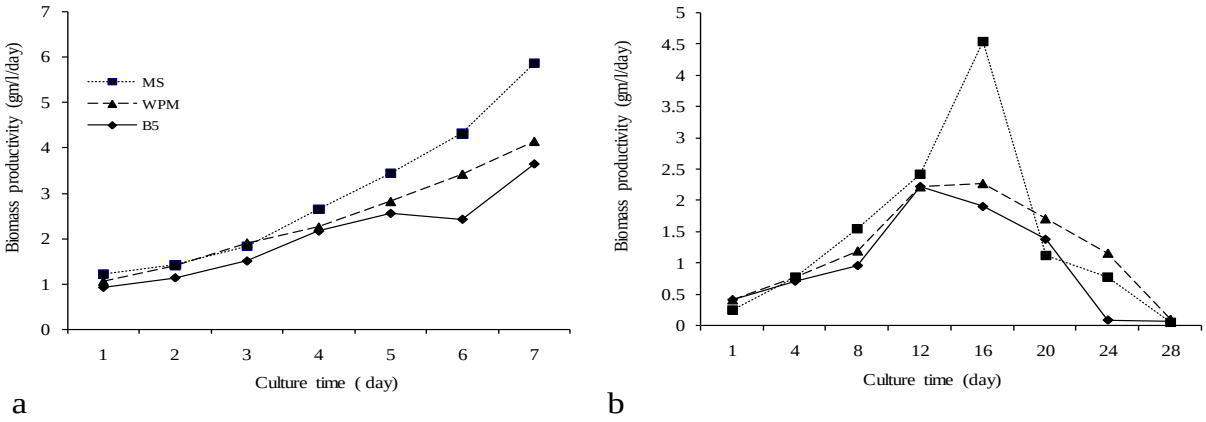

Figure 2: Biomass productivity on the basis of dry cell mass Biomass: data taken a. every day, b. every four days

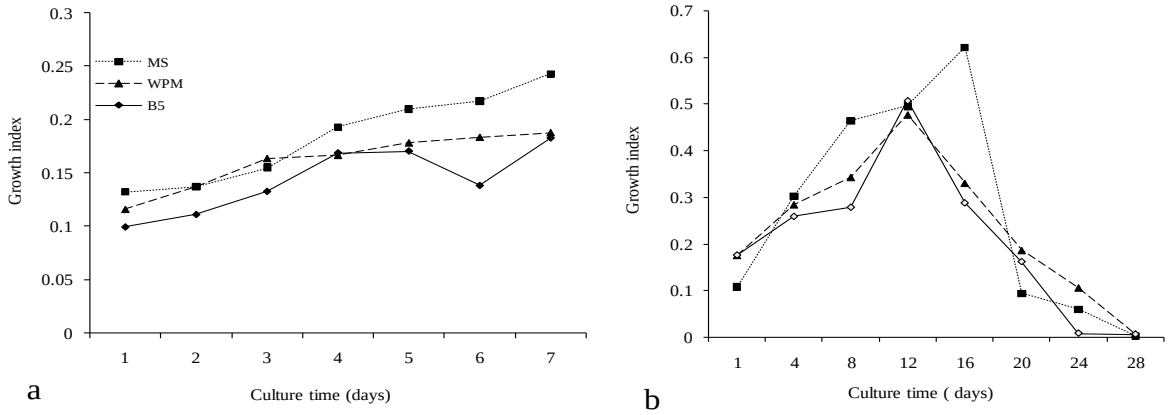

Figure 3: The growth index calculated on the basis of dry cell weight; a. every day; b. every four days

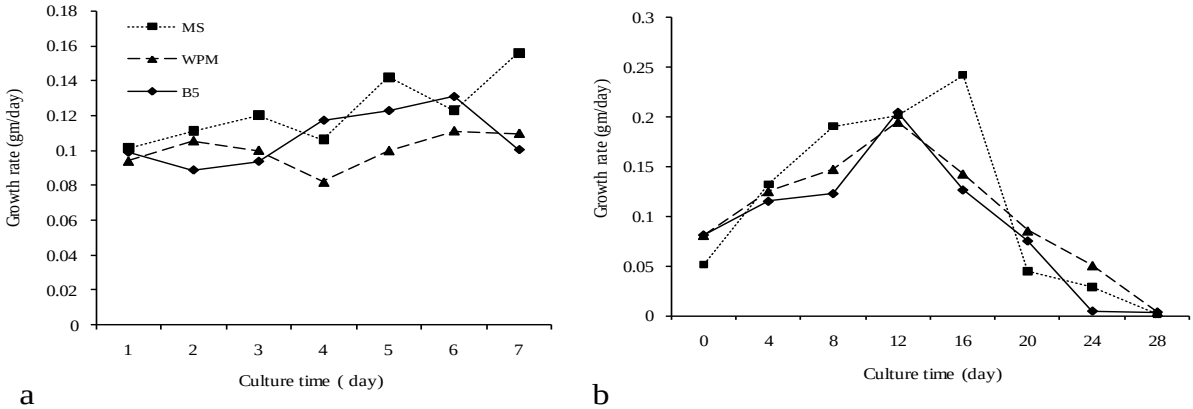

Figure 4: The specific growth rate on the basis of dry cell: a. everyday b. every four days

weight was expressed as Growth Index. The growth index increases at the first 7th days of the culture time in all the three growth media. The maximum increase in growth index was observed at the 16th days of the culture time in MS medium which is higher than the other media (Figure 3).The specific growth rate was measured in terms of the biomass gain at specific cultivation time as described (Figure 4). Like the other growth kinetics measurements the maximum growth rate was obtained at the 16th days of the culture time in M S medium. The culture in MS medium

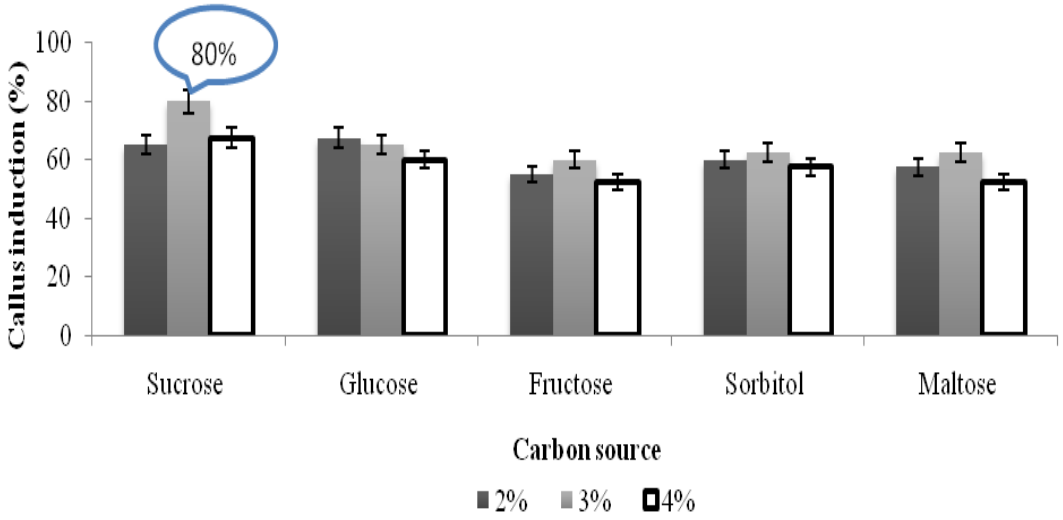

Figure 5: Effect of carbon sources on callus proliferation

was observed sharply increased especially the first 8 days unlike B5 and WPM. Based on this growth rate, the corresponding doubling time of the plant cells in a liquid system was calculated.

The effect of carbohydrate source on callus induction

Various plant species have different ability to utilize carbohydrate. Such carbohydrates as a carbon source are indispensable for the *in vitro* manipulation of plant cells. The quality and/or quantity of the outcome are also depending on the type and optimum concentration of these carbohydrates. Low carbon dioxide and light supplies *in vitro* condition is the main reason that plant requires sugar for growth especially in liquid phase culture system. They are not able to grow properly without an exogenous supply of carbohydrates (Ticha et al., 1998). Hence, the effect of various carbon sources which includes fructose; glucose, maltose, sucrose and sorbitol (sugar alcohol) were evaluated to improve callus induction, proliferation and formation of somatic embryo.

The effect of fructose, maltose and sorbitol on calli proliferation and development was not significant at optimal concentration (Figure 5). It is found that 3% sucrose gave the maximum proliferation of callus followed by 2% glucose. Although, glucose was found to be less suitable than sucrose, reported as an important additive for callus induction and embryogenic callus formation of *Miscanthus* sp. (Petersen et al., 1999).

Sucrose is used in plant cell culture for various purposes either as a carbon source or as a regulator of cell osmosis. The effect of exogenous supply of sucrose on plant cells have been reported elsewhere (Ticha et al., 1998). High sucrose levels might also cause cell dehydration according to other reports (Meurs et al., 1992), and affect cell proliferation. In this experiment, we also observed this effect. 3% sucrose has improve the callus proliferation by 80% in MS_3 media supplemented with 2.46 µM /L IBA, 9.08 µM /L TDZ and 13.3 BAP µM /L (Figure 5). This optimal concentration was considered for further somatic embryo formation and maturation of callus studies.

The other carbon source, maltose has significant influence on somatic embryogenesis of *Petunia* anthers (Raquin, 1983). In support of the effect of maltose, an increase in callus induction and plantlet regeneration has also been reported from winter triticale and wheat *in vitro* androgenesis (Karsai et al. 1994). Improved callus induction in rice microspore culture has been reported elsewhere (Finnie et al., 1989). Sometime, maltose equally or surpass sucrose in supporting embryogenesis in a number of species, including carrot (Kinnersley and Henderson 1988), alfalfa (Strickland et al., 1987). Similarly, maltose led to a much higher germination rate from asparagus somatic embryos than sucrose (Kunitake et al., 1997).

Mostly, maltose has been used both as a carbon source and as an osmoticum. Compared to sucrose there is a slower rate of extracellular hydrolysis, it is taken up more slowly, and hydrolysed intracellularly more slowly according to the reports. However, the effect of maltose in our study is less significant either for callus induction, proliferation or somatic embryo formation (Figure 5).

Like other carbohydrate, fructose has also been reported to be effective in preventing hyperhydricity (Rugini et al. 1987). It has been also reported that fructose supports callus induction and proliferation of *Bougainvillea* sp. over sucrose or glucose (Steffen et al., 1988).. Fructose has reported the best sugar for the production of adventitious shoots from *Glycine max* cotyledonary nodes (Wright et al. 1986). Shoot and leaf growth; and axillary shoot formation

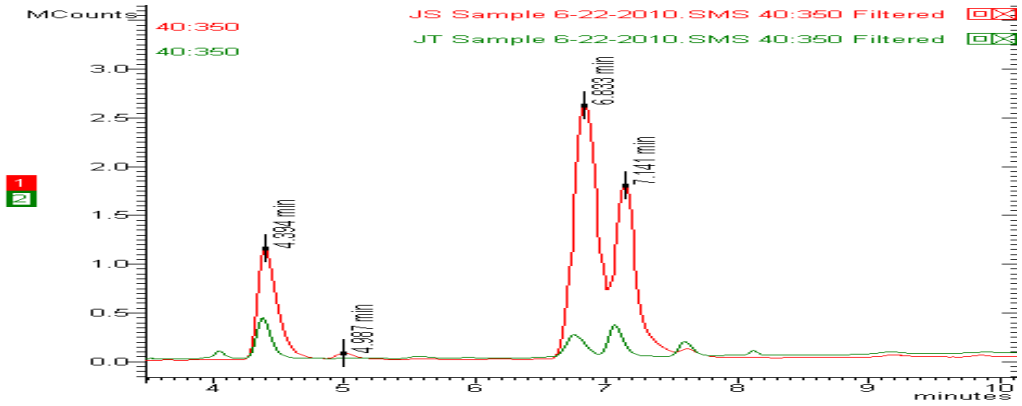

Figure 6: GC-MS spectra of fatty acid extracted from seed and tissue culture

in *Castanea* shoot cultures were stimulated when sucrose was replaced by fructose. The growth of basal callus was reduced and it was possible to propagate from mature explants of *C. crenata*, although this was not possible on the same medium supplemented with sucrose (Chauvin and Salesses,1988). However, fructose was reported to be toxic to carrot tissue. On other aspect, when filter sterilized, fructose supported the growth of callus cultures which had a final weight 70% of those grown on sucrose. Furthermore, it have been reported that fructose improve somatic embryos per cultured explant to 4 fold higher than sucrose (Loiseau et al., 1995). However, such strong role for the aforementioned species was not observed in this study.

The other carbon sources used was sugar alcohols for its effect as osmoticum for callus maturation. The ability of Rosaceae to use sorbitol as a carbon source is reported to be variety dependent. The apple rootstock produced abnormal shoots on sorbitol. The significant effect of sorbitol on shoot induction in apple has been reported elsewhere (Karhu, 1997). However, in this study callus induction was found to been inhibited in MS_3 media supplemented with sorbitol, Figure 5.

The fatty acid composition from the biomass was determined using GC-MS. The fatty acid content of suspension culture and callus found to be 8±0.23% and 9±0.16% respectively. The fatty acid composition in cell cultures differed remarkably from those in the tissues of the mother plant reported elsewhere (Becker and Makkar, 2008). The result showed that the palmatic acid is found to be higher *in vitro* grown biomass (33.12%). Unusual fatty acid like conjugated linoleic acid (CLA) was also found in higher composition (26.77%). However, CLA have not been reported so far from *Jatropha* seed oil. Palmitic acid is the most abundant fatty acid in most of the cultures. Linolenic acid which was not reported to be present in *Jatropha* seed also found in tissue culture lesser amount (10.91%) (Gemmrich and Shraudolf, 1980). However, linoleic acid was not observed in this study. The composition of the fatty acid in the tissue culture biomass and seed is shown Figure 6.

The various PUFA composition of the seed and the *in vitro* grown cell mass is shown Table 1 above. The fatty acid composition of the oils in the different *J. curcas* seeds, compared with the reported values, is recorded in Table 1. The fatty acids found common in all the oil samples were oleic, linoleic, palmitic and stearic. The major fatty acid in the Veracruz samples was oleic acid, whereas in the Morelos sample it was linoleic acid. This variation is possibly due to soil and climatic conditions. The results showed that the oil is composed mainly of unsaturated fatty acids (oleic and linoleic acid). The results obtained are very similar to those reported for *J. curcas* seed provenances from different countries (Banerji et al. 1985). The present study shows that both cis-11-eicosenoic acid (C20:1) and cis-11,14-eicos- adienoic acid (C20:2), which have not been reported by earlier workers. It is very important to mention that the phorbol esters were found in high levels only in the sample from Coatzacoalcos (3.85 mg/g). In the oils from Castillo de Teayo, Pueblillo, and Yautepec seed samples, phorbol esters were not detected. These seeds could thus be recommended for utilization by the food/feed industry after treatment for removing the less toxic anti-nutrients. It is, however, necessary to carry out in vivo studies to confirm their harmlessness. Looking to the fatty acid composition various additive, like PEG_{1500}, PEG_{4000}, glutamine, aspartic acid, jasmonic acid, salicylic acid under aseptic condition were tried. In all the case the composition of palmatic was found to be higher (Figure 7).

CONCLUSION AND RECOMMENDATION

The biosynthesis of long chain fatty acid through cell culture yielded 8+0.23% and 9+0.16% in liquid and solid state culture system. The present investigation suggests the biosynthesis and determination of polyunsaturated fatty acids in *Jatropha curcas* somatic embryos. The advantage of such biosynthesis of PUFA using this protocol over other

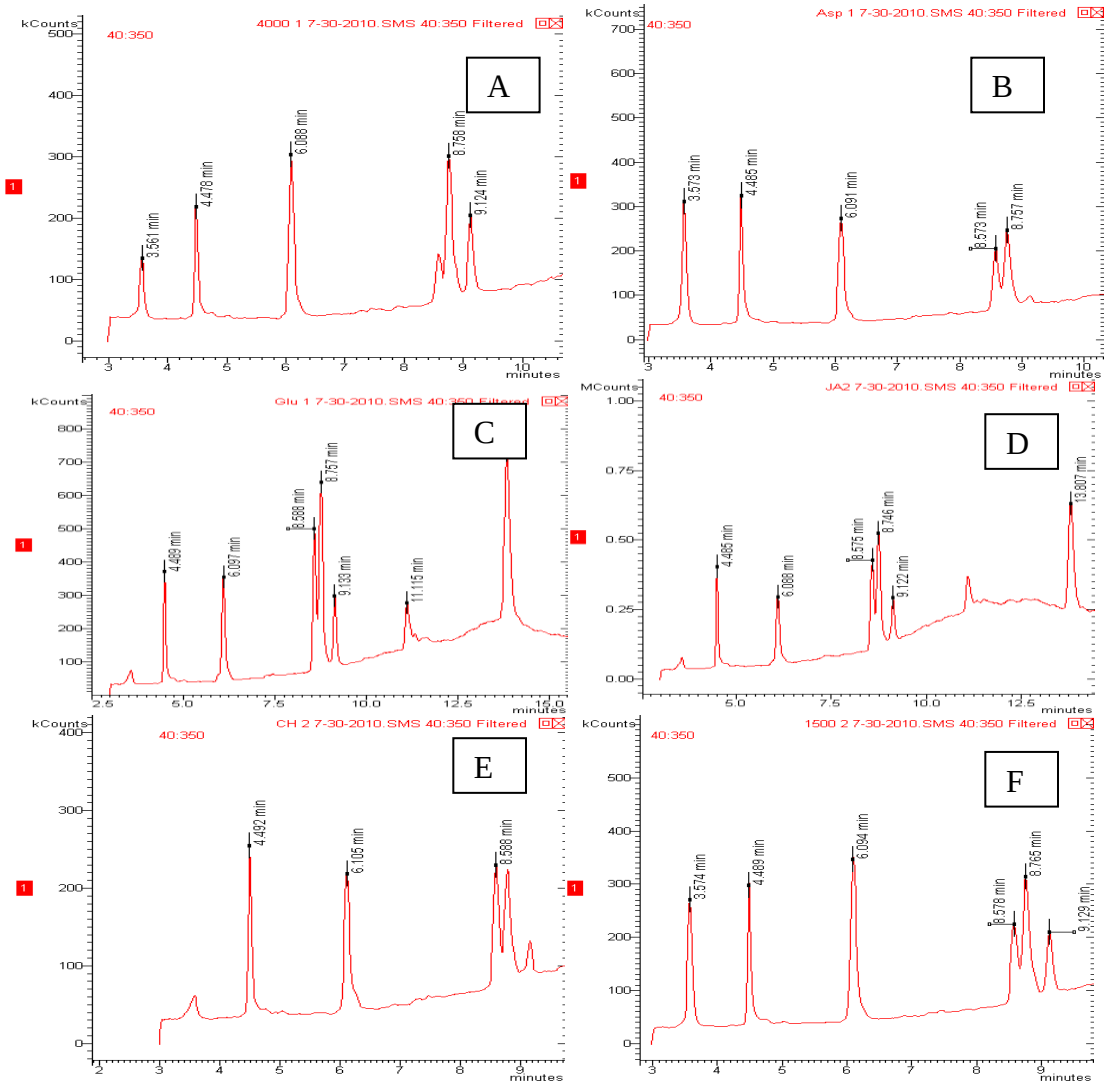

Figure 7: The effect of A) PEG_{4000}, B) Aspartic acid, C) Glutamine, D) Methyl jasmonate, E) Casein hydrolysate, F) PEG_{1500} on fatty acid composition

methods currently in use is that the PUFA are quite consistent. This study may therefore lead to the development of a simple bioprocess for the biosynthesis of important PUFA for various applications. However, different media components, various biological and physiochemical parameters were the major to make a strong conclusion. It could be considered from the present work that the significant associations of the culture conditions and the various elicitors are vital points in laboratory and/or large scale production of PUFA.

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