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Carbon Source Requirement For *In Vitro* Plantlet Regeneration From Zygotic Embryos Of *Jatropha Curcas* L. (A Biodiesel Plant)

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Abstract

Jatropha curcas L. is an important non-edible biodiesel plant. The nature of the seed makes conventional propagation difficult since it has a hard seed coat that delays germination. In this study, embryos were freed from the seed nutrients and cultured in MS basal media in which seven carbohydrates (glucose, fructose, galactose, sucrose, maltose, lactose and raffinose) were tested at 1.0 to 5.0% concentration for the most efficient carbon source for the propagation of *Jatropha curcas* through zygotic embryo culture. In the first experiment, only glucose, fructose and sucrose supported embryo growth (germination) substantially with a maximum percentage germination of 92.2±6.7, 37.8±6.9 and 29.3±4.8; and germination rates of 0.25, 0.11, 0.10 respectively, showing sucrose (at 3 and 5% concentration) as the most effective in this respect. In the second experiment in which only sucrose was employed, germination rates under 1%, 3% and 5% sucrose levels were 0.14, 0.25 and 0.33 respectively. Percentage germination after 10 days for 1%, 3% and 5% were 60±5.2, 94.2±9.2, and 94.8± 4.5 respectively. The mean fresh weight (mg) of the plantlets from 3% sucrose concentration had the highest value of 71.28±11.42 with the least weight occurring under 1% sucrose concentration (18.78 ±3.74). The mean numbers of leaves were 1.2±0.2, 1.9±0.3 and 0.9±0.2 for 5%, 3% and 1% concentrations respectively. This study showed that although a number of monosaccharides (glucose and fructose) and the disaccharide (sucrose) supported embryo growth in this study, sucrose at 3% concentration was shown to be the most effective carbon source for the propagation of *J. curcas* zygotic embryos. Also, high concentrations of sucrose seem to inhibit shoot growth and development while lower concentrations of sucrose have been shown to be less effective for adventitious shoot formation.

Keywords: *Jatropha curcas*, Zygote, Embryo, Culture, Carbon source.

1.0 Introduction

Jatropha curcas (Euphorbiaceae) is a shrub that grows to a height of about 6 m and it is worldwide in distribution. In recent years, this plant has received extensive attention in view of its great economic importance, as the seed has been shown to be a source of biodiesel. The use of this plant for large scale bio-diesel production will be of great importance in view of its potential in addressing the impending energy crises, reducing carbon emission and increasing the income of farmers (Gubitz *et al*., 1999; Openshaw, 2000; Zhou *et al*., 2006).

Conventionally, the plant is propagated either by direct seeding or the use of stem cuttings. However, conventional propagation is limited by problems associated with poor seed viability, low germination, scanty and delayed rooting of seedlings and vegetative cuttings (Openshaw, 2000). To overcome these constraints, tissue culture techniques involving the use of various explants (meristems, hypocotyl, stem/leaf segments, embryos) in micropropagation will offer rapid and continuous supply of the planting materials (Chawla, 2002). Previously, shoot regeneration from various explants of *Jatropha* have been reported (Sujatha and Mukta, 1996). However the studies on tissue culture of *Jatropha curcas* have been confined to leaflet, petiole, cotyledon and hypocotyls (Sujatha and Mukta, 1996; Lu *et al*., 2003). Somatic embryogenesis has been reported in *J. Curcas* (Jha *et al*., 2007) which requires 16 weeks for plant regeneration. This study was conducted to mass propagate, and to obtain high frequency regeneration of *Jatropha curcas* using zygotic embryo explants in modified MS basal media employing seven carbohydrates at 1.0-5.0 per cent as carbon sources.

2.0 Materials and Methods

2.1 Source of Explants

The explants employed in this study consisted of zygotic embryos derived from mature seeds (see Plate 1) of *Jatropha curcas* plants harvested from the gardens of the National Centre for Energy Research and Development, University of Nigeria, Nsukka. Prior to culturing the embryos (plate 2), the seeds were immersed for 12h in a beaker containing sterile distilled water. The seeds were then surface –sterilized by complete immersion for 20 min in 1.17% (w/v) sodium hypochlorite (NaOCL) which was obtained by dissolving one part of jik (a laundry bleach containing 5.25% NaOCL) in four parts of distilled water. This step was necessary in order to eliminate possible surface contaminants (usually bacteria and fungi) from the surface of the seeds. The seeds were then rinsed in three changes of sterile distilled water to wash off the bleach and left to dry on sterile absorbent paper for 10 mins. Thereafter, each one was picked up with sterile forceps, and immersed for 5 seconds in 70 per cent ethanol and drained on sterile absorbent paper. The embryo was excised from the seed using a pair of forceps and scalpels under a dissecting binocular microscope prior to inoculation on the appropriate growth media. Embryo excision and culture were done in the laminar air flow chamber previously exposed to ultraviolet radiation for 30 minutes in order to avoid contamination.

2.2 Establishment of Cultures / Sprouting of Embryos.

In the first experiment, modified medium of Murashige and Skoog (1962) was employed as the basal medium and seven carbohydrates, namely; glucose, fructose, galactose, sucrose, maltose, lactose and raffinose were tested at 0-5% levels as carbon source for the growth of the embryos. In the second experiment, only sucrose was employed as carbon source and also applied at concentrations ranging from 0.0 to 5.0 per cent. was supplemented with 0-5 per cent. The pH of the medium was adjusted to 5.8 with 1M NaOH or HCL, and each medium was solidified using Fluka agar at 7.0 gl^{-1} prior to autoclaving by steam sterilization at 103 KN M^{-2} pressure and 121^0C for 15 mins. The embryos were cultured singly in Pyrex test tubes at 27 ± 2^0C under 16-h light/ 8-h dark photoperiod at a photon flux density of 60 µmol $m^{-2}S^{-1}$ provided by cool white fluorescent tubes. All operations starting from the preparation of explants to establishment of cultures were carried out in a Laminar flow hood previously kept sterile by exposure to ultraviolet light for 30 min. The cultured embryos were left to grow for 10 days after which they were scored for the requisite growth parameters. Ten replicate tubes containing an embryo each were randomly selected under each treatment and scored for the following: time course in sprouting (%), germination rate, mean length of shoots and roots, mean fresh weight of sprouts produced, mean number and area of leaves produced in culture.

2.3 Statistical Analysis

All the experiments involving the treatments (0, 1, 3 and 5 per cent carbon sources) consisted of 20 replicate explants per treatment. Treatment means were tested for significance using Fishers Least Significance Difference (F-LSD) at 5 per cent level.

3.0 Results

In the first experiment in which seven carbohydrates were screened for the most effective carbon source(s) for the culture of the embryos, only glucose, fructose and glucose supported embryo sprouting to any meaningful extent within ten days in culture while embryo germination was also possible but to a much lower extent with galactose, maltose, lactose and raffinose at 1.0-5.0 concentration. Embryos failed to sprout in media devoid of carbon source (control) (see Table 1).

In the second experiment in which only sucrose was employed as the carbon source and used at 0.0-5.0 per cent, sprouting commenced within four days among embryo explants grown in media containing 5% sucrose, whereas it did not commence until four days in media containing 0%(control), 1% and 3% sucrose concentration in which percentage sprouting was 20.0, 52.5 and 0.0 per cent respectively (Figure 1). By the end of the 10^{th} day when sprouting had levelled off in most of the treatments, the highest percentage sprouting (94.%) was recorded for those containing 3% and 5%sucrose while 1% sucrose supported only 60% germination (Figure 1). Germination rate of the embryos (determined as the number of days to 50% sprouting) was dependent

Table 1: Maximum germination (%) attained by excised zygotic embryos of *Jatropha curcas* after 10 days of growth in Murashige and skoog media containing 0-5% concentration of the various Carbohydrates as Carbon Source.

Type of Carbon Source	Level at which carbon source was tested				
	0% (control)	1%	3%	5%	10%
Glucose	0±0	16.7±6.2	37.8±6.9	18.8±6.5	8.3±3.1
Fructose	0±0	11.9±3.0	29.3±4.8	10.4±2.9	4.9±0.7
Galactose	0±0	8.9±2.7	26.0±3.8	11.6±2.7	4.2±0.9
Sucrose	0±0	37.8±5.3	92.2±6.2	83.4±7.9	18.3±5.7
Maltose	0±0	22.0±4.8	27.6±6.1	14.9±3.7	10.7±2.9
Lactose	0±0	16.7±4.7	19.8±3.9	9.9±2.3	4.9±0.7
Raffinose	0±0	3.9±1.3	5.6±1.7	2.7±0.8	1.4±0.5

Table 2: Per Cent Germination and Germination Rate of Excised *Jatropha curcas* Embryos as Affected by the Various Carbon Sources at 3 Per Cent Concentration.

Type of Carbon Source	Per cent Germination	Germination Rate
Glucose	37.8±6.9	0.11
Fructose	29.3±4.8	0.10
Galactose	26.0±3.8	0.08
Sucrose	92.2±6.2	0.25
Maltose	27.6±6.1	0.07
Lactose	19.8±3.9	-
Raffinose	5.6±1.7	-
Control	0±0	-

Germination rate was obtained as the reciprocal of the number of days to 25 per cent seed germination under the various treatments.

the concentrations of carbon source employed. For example, the highest rate of sprouting (0.33) was recorded for those supplemented with 5%, while 1% and 5% sucrose had 0.14 and 0.25 respectively. There was no sprouting (germination) among untreated (control) embryos. However, the mean length of shoot and root for 5% and 3% sucrose levels were 0.81±0.2, 0.59±0.14 and 1.42±0.40, 0.70±0.20 respectively while there was significant difference in the length of root and shoot produced there was no significance difference (p=0.05) between their fresh weights (Table 3). Plates 3, 4 and 5 show the nature of plantletets produced in media containing 1%, 3% and 5% sucrose respectively. Also, embryos in 1% sucrose level had the least weight, length of shoot and root when compared to the ones in 5% and 3% sucrose levels respectively. The number of leaves for (1, 3 and 5) per cent sucrose level was 0.7±0.2, 1.9±0.3 and 1.2±0.2 respectively (See Table 3).

Table 3: Mean number, length and fresh weight of sprouts produced by *Jatropha curcas* Embryo after 10 days of growth in MS media containing 0,1,3 and 5% Sucrose concentrations.

Sucrose conc.	Length of shoot (cm)	Length of roots (cm)	Fresh weight (mg)	No. of leaves
5% sucrose	0.81±0.2	0.59±0.14	71.28±11.42	1.2±0.2
3% sucrose	1.42±0.40	0.70±0.20	108.66 ±9.96	1.9±0.3
1% sucrose	0.38±0.09	0.43±0.11	18.78 ±3.74	0.7 ±0.2
0% sucrose	0±0	0±0	0±0	0±0

Plate 1: *Jatropha Curcas* Seed

Plate 2: *Jatropha Curcas* embrayo

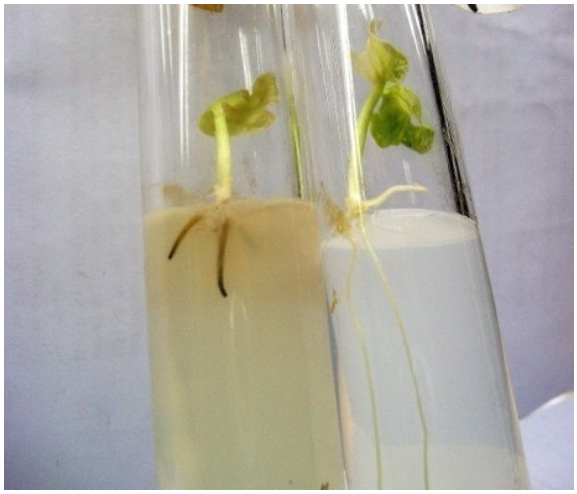

Plate 3: Ten day old *J. curcas* plantlet in 1% sucrose

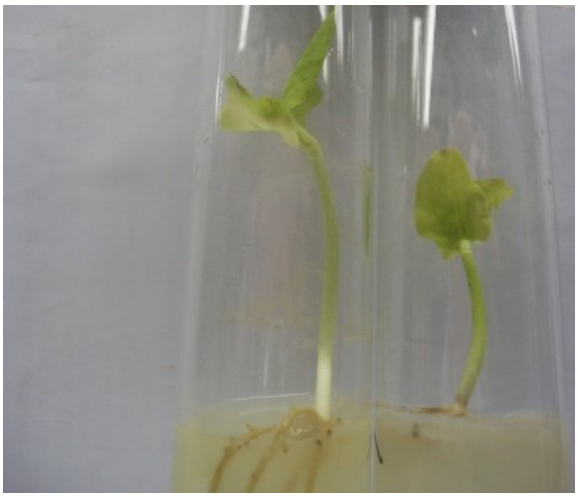

Plate 4: Ten day old *J. curcas* plantlet in 3% sucrose

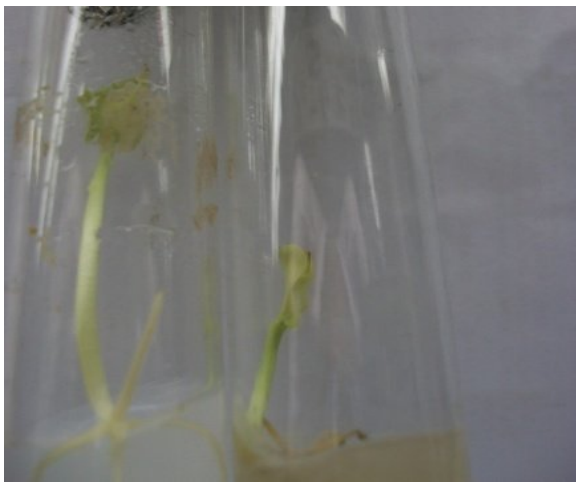

Plate 5: Ten day old *J. curcas* plantlet in 5% sucrose

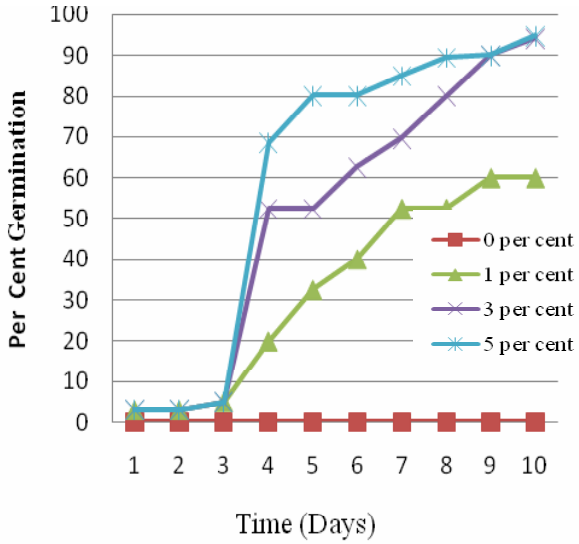

Figure 1: Time Course in Percentage Sprouting of *J. curcas* Embryos under 0, 1, 3 and 5 Per Cent Sucrose Concentrations.

4.0 Discussion

Plant cells and tissues in a culture medium lack autotrophic ability and therefore need external carbon for energy. Excised *Jatropha curcas* embryos, as is the case in this study, are devoid of the nutrients that otherwise confer autotrophy to the developing embryo *in vivo* and hence the inability of the embryos to grow in the absence of a carbon source (control). The superiority of sucrose over the other sugars employed as carbon source in this study, is consistent with earlier reports for other plants such as *Striga senegalensis* (Okonkwo, 1966) and *Dioscorea rotundata* (Okezie, *et al.*, 1994). The addition of an external carbon source to the medium serves to provide the energy required to drive the metabolic processes that lead to growth, thus enhancing the proliferation of cells and regeneration of green shoots (Nowak *et al*., 2004). The optimal sucrose concentration in a medium should be sufficient to satisfy the basic energy requirements for cell division/ differentiation and not impose any negative osmotic effects on shoot formation. This indicates that sucrose acts not only as a carbon energy source in a medium but also as an osmotic substance and that different concentrations of sucrose control the induction and growth of shoots (Gibson, 2000).The optimum sucrose concentration as an efficient carbon source has been examined in tissue cultures of some plant species such as *Paederia foetida* and *Elaeocarpus robustus*, in which 3% sucrose enhanced shoot growth and development. Shoot elongation of alfalfa leaflets regenerated from a callus was promoted when the sucrose concentration in the medium was 1% to 3%(Ramage and Williams, 2002). Lower concentrations of sucrose have been shown to be less effective for adventitious shoot formation. On the other hand, the detrimental effect of a high sucrose concentrations on shoot formation demonstrated in this study, implies that the osmotic level in the medium may be inhibitory to further shoot development. Thus, high concentrations of sucrose seem to inhibit shoot growth and development (Nowak *et al*., 2004). The embryos of *Jatropha curcas* L. in this study showed different responses to sucrose concentrations. Thirty grams per litre sucrose (i.e. 3% concentration) was noted to favour the growth of the embryos *in vitro* by increasing the shoot to root ratio unlike 5% that decreased the shoot to root ratio. Thus, this might have imposed a negative osmotic effect on shoot formation. One per cent sucrose had the least values of the growth parameters studied. At this point, sucrose concentration seemed to be limiting, hence a decline in shoot growth.

5.0 Conclusion

Methods described in this study have the potential for mass producing *J. curcas* plantlets which, after hardening, would be raised *ex vitro* for ensuring a steady supply of diesel oil from the seeds. Somaclonal variants arising from this propagation method (Bhojwani and Razdan, 1996) would include those with desirable attributes that would enhance steady availability of elite cultivars of this plant for purposes of providing a cleaner and more environmentally friendly substitute for fossil fuels.

References

Bhojwani, S.S. and Razdan, M.K. 1996, "Plant Tissue Culture: Theory and Practice", Elsevier Publisher, Amsterdam, pp 166.

Chawla, H.S. 2002, "Introduction to Plant Biotechnology" (2nd ed.), Oxford and IBH Publishers, New York, pp 562.

Gibson, S. 2000, "Plant Sugar-Response Pathways, Part of a Complex Regulatory Pathway", *Plant Physiology*, **124**, 1532-1539.

Gubitz, G. M., Mittelbach, M. and Trabi, M. 1999, "Exploitation of the tropical oil seed plant '*Jatropha curcas* L.,' *Bioresources and Technology*, **67**, 73-82.

Jha, T.B., Mukherjee, P. and Datta, M.M.2007, "Somatic Embryogenesis in *Jatropha curcas* L.: An Important Biofuel Plant. *Plant Biotechnology,* **1**, 135–140.

Lu, W. D., Wei, Q., Tang, L., Yan, F. and Chen, F. 2003, "Induction of callus from *Jatropha curcas* and Rapid Propagation," *Chinese Journal of Applied Environmental Biology*, **9(2)**, 127-130.

Murashige, T. and Skoog, F. 1962, "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissues Cultures", *Plant Physiology,* **15**(3), 473-497.

Nowak, B., Miczynski, L. and Hudy, L. 2004, "Sugar Uptake and Utilization during Adventitious Bud Differentiation on *In Vitro* Leaf Explant of *Prunus domestica*. *Plant Cell Tissue and Organ*

Culture, 76, 255-260.

Okezie, C.E.A., Okonkwo, S.N.C. and Nwoke, F.I.O. 1994, "Carbon Source Requirement for the Culture of White yam (*Diosorea rotundata*) Embryos *in vitro"*, Proceedings of the 9th Sympo-sium of the International Society for Tropical Root Crops 20-26 October, 1991, Accra, Ghana (F. Ofori and SK. Hahn, eds) pp 329-334.

Okonkwo, S.N.C. 1966, "Studies on *Striga senegalensis.* IV. *In vitro* Culture of Seedlings", Effect of various Sugars and Glutamine, *American Journal of Botany* 53, 687-693.

Openshaw, K. 2000, "A Review of *Jatropha curcas*: an Oil Plant of Unfulfilled Promise", *Biomass and Bioenergy*, **19**, 1-15.

Ramage, C.M. and Williams, R.R. 2002, "Mineral Nutrition and Plant Morphogenesis" *In Vitro Cellular and Developmental Biology-Plant*, 38, 116-124

Sujatha, M. and Mukta, N. 1996, "Morphogenesis and Plant Regeneration from Tissue Culture of *Jatropha curcas"*, *Plant Cell, Tissue and Organ Culture* **44**, 135-141

Zhou, H., Lu, H. and Liang, B. 2006, "Solubility of Multicomponent System in the Biodiesel Production by Transesterification of *Jatropha curcas* L. oil with methanol", *Journal of Chemical Engineering Data*, **51,**1130-1135.