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Study on ploidy level of micropropagated *Jatropha curcas* L. via flow cytometry

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Jatropha curcas L. is considered as one of potential source for a non-edible biofuel-producing energy crop throughout the world. The seed oil content varies from 4 to 40%, and thus the seed viability and rate of germination are low. Various explant types as epicotyls and hypocotyls of *J. curcas* were cultured on MS medium supplemented with different combinations of PGRs. The results showed that MS medium supplemented with 0.5 mg/l kinetin (KN) in combination with 0.25 mg/l thidiazuron (TDZ) provided average number of shoots at 15 shoots per responding explant from epicotyl explants. Hypocotyl explants gave average number of shoots at 22.76 shoots per responding explant in 0.5 mg/l KN and 0.25 mg/l IBA containing medium after 30 days of culture. In all PGRs tested, shoots formation from callus culture was small and short which could not be elongate into normal plantlets. After that shoot explants were cut and transferred to MS medium supplemented with 0.5 mg/l BA in combination with 0.25 mg/l IBA. They were green in color and elongate. Flow cytometry was used to analyse the DNA content of nuclei isolated from intact plant tissue and from leaves stems and callus cultures *in vitro*. They showed diploid status with the same 2C DNA content. The results from this experiment suggested that this protocol could produce uniform plantlets.

Key words: *Jatropha curcas* L., micropropagation, PGRs, diploid, flow cytometry

Introduction

Jatropha curcas L. (Euphobiaceae) or physicnut is an multi-purpose, zero-waste perennial plant (Datta *et al.,* 2007). It is a hardy perennial shrub of Latin American origin that widespread throughout the tropical regions of the world (Deore and Johnson, 2008). Physicnut is considered as one of potential source for a non-edible biofuel-producing energy crop throughout the world. *J. curcas* biofuel contains more oxygen, with a higher cetane value increasing the combustion quality, is cleaned, non toxic, eco-friendly and economic due to its

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low production cost (Jha *et al.,* 2007). Kernels yield 46-58% of a semi-dry oil (iodine value 93-107) and contain mainly oleic (37-63%), linoleic (19-40%) and palmitic (12-17%) acids as constituent fatty acids (Sujatha and Mukta, 1996). The development of techniques for *in vitro* culture is necessary to generate material for germplasm preservation, rapid propagation and crop improvement to increase field productivity and profitability leading to full supply of the demand for physic nuts, and future development (Thepsmorn *et al.,* 2006b). *In vitro* techniques have been used for propagation of many plant species (Thepsmorn *et al.,* 2006a). Tissue culture techniques offer rapid and continuous supply of the planting material. These techniques were undertaken to circumvent the problems associated with large scale multiplication of *J. curcas* (Sujatha and Mukta, 1996). Evaluation of tissue culture propagated plants of *J. curcas* revealed that they have advantage over seed propagated plants in terms of yield and yield related traits (Sujatha *et al.,* 2005).

Flow cytometry enables DNA amounts to estimate rapidly from all parts of tissue even callus. It is therefore a means by which to determine ploidy level of these tissue rapidly where basic information about chromosome numbers and DNA amount of a plant group is known (Blakesley *et al.,* 2002). Leaf tissue is a particularly convenient source of material for the study of ploidy in mature trees from which rooted cuttings, as a source of material for chromosome counts, are not easily obtained. The fluorochrome 4´, 6-diamidino-2-phenylindole (DAPI) has a preference for AT bases (Dolezel *et al.,* 1992) and is, therefore, not suitable for the precise measurement of DNA amount. However, samples for flow cytometry using DAPI are easily prepared and thus this fluorochrome is wildly used in ploidy studies. Propidium Iodide (PI) is another fluorochrome that is wildly used in flow cytometry. As it shows no base preference, it is suitable for the determination of absolute DNA amounts [4]. Eventhough tissue culture of *J. curcas* was successfully established evaluation of somaclonal variation but the variation that was not reported.

In the present study, we put attempts in evaluation of uniformity of plantlets obtained from various sources of explant using flow cytometry.

Materials and methods

Plant material

Seeds of *J. curcas* were surface sterilized in 20% (v/v) and 10% (v/v) Clorox with 0.02% (v/v) Tween 20 for 20 min and 10 min respectively, followed by three times rinse in sterile distilled water. Zygotic embryos were cultured on plant growth regulator (PGR)-free MS medium (Murashige and Skoog, 1962), then various sources of explants were taken from 15 day-old

seedling. Epicotyl and hypocotyl explants were separately excised and cultured on MS with a series of PGRs.

Culture conditions

All culture media were supplemented with sucrose 30 g/l and solidified with 7.5 g agar. The pH of the culture media was adjusted to 5.6 ± 0.2 before autoclaving. The cultures were incubated at 25°C ± 2°C under 14 h photoperiod using cool, white fluorescent light at 20 µmol/m^2/sec.

Induction of adventitious shoots

The epicotyl and hypocotyl explants taken from 15-day-old seedlings were separated and transferred to MS medium supplemented with BA (0.5 mg/l) in combination with IBA (0.05, 0.1 and 0.25 mg/l) or KN (0.5 mg/l) in combination with IBA (0.05, 0.1 and 0.25 mg/l) or KN (0.5 mg/l) in combination with 2, 4-D (0.05, 0.1 and 0.25 mg/l) or TDZ (0.5 mg/l) in combination with 2, 4-D (0.05, 0.1 and 0.25 mg/l) or KN (0.5 mg/l) in combination with TDZ (0.05, 0.1 and 0.25 mg/l). Each treatment consisted of 20 explants with 2 replications. After culture for 6 weeks percentage multiple shoot formation and number of shoot per responding explants were compared using completely randomized design (CRD) and mean among treatments separated by Duncant's multiple range test (DMRT).

Flow cytometry analysis

Young leaf sample from field grown diploid, leaves and stem derived from culturing epicotyl and callus (approximately 1 cm^2) were chopped with a shape razor blade in a Petri dish containing 5 ml buffer (LB01 lysis buffer; DoleŽel *et al*., 1992). This buffer consisted of 15 mM Tris, 2 mM Na EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM **ß**-mercaptoethanol and 1 ml/l Triton X-100, pH 7.5. The nuclei suspension was filtered through 50 µm nylon mesh and centrifuged at 3000 rpm for 2 min to remove debris. After removing debris, pellet was dissolved with 500 µl of extraction buffer and mix thoroughly. Fifty µl of PI was added to the nuclei suspension and incubated for at least 5 min at room temperature to stain nuclei completely. The fluorescent intensities of each nuclei suspension were measured by Epics XL, equipped with a 488 nm argon laser and long path filter (Becton Dickinson FACSCalibur™). The data of DNA content from each plant material were compared.

Results

Shoots induction from epicotyl

The best response on multiple shoot formation and from epicotyl explants was obtained on culture medium supplemented with KN in combination with TDZ. Those PGR containing medium gave the best result in direct shoot bud formation. The number of shoots obtained from 0.5 mg/l KN and 0.25 mg/l TDZ containing medium was the highest at 15 shoots per responding explants, significant difference ($P \leq 0.05$) to other combinations. (Table 1). However, shoot bud formation from each PGRs were small and short and could not be excised to induce root. Thus, complete plantlets were not obtained. Induced shoot buds were elongated by transferring a cluster of tiny shoot to the MS medium supplemented with 0.5 mg/l BA in combination with 0.25 mg/l IBA. Shoots formation from these PGRs gave the best shoots characteristic. The leaves were green in color and stems and shoots were elongated (Fig. 1A). In other PGRs tested, shoot formation was smaller and shorter, especially the combination of TDZ and 2, 4-D (Fig. 1D). IBA in combination with KN at all concentrations gave single elongated shoot without callus formation (Fig. 1E).

Table 1. Mean number of explants, which produced shoots induction of cultured on MS medium supplemented with various concentrations of plant growth regulators from epicotyl explants of *J. curcas* L.

Conc. of PGRs (mg/l)				No. of shoots/explants from epicotyls
Control				1.00±0.00^{e}
BA	0.5	IBA	0.05	3.86±0.23^{de}
	0.5		0.1	4.29±0.16^{cd}
	0.5		0.25	5.19±0.24^{cd}
KN	0.5	IBA	0.05	2.88±0.12^{d}
	0.5		0.1	2.93±0.18^{d}
	0.5		0.25	2.67±0.12^{de}
TDZ	0.5	2, 4-D	0.05	3.61±0.11^{de}
	0.5		0.1	2.80±0.13^{d}
	0.5		0.25	3.64±0.15^{de}
KN	0.5	2, 4-D	0.05	3.18±0.21^{d}
	0.5		0.1	2.45±0.31^{de}
	0.5		0.25	2.60±0.30^{de}
KN	0.5	TDZ	0.05	13.00±1.14^{b}
	0.5		0.1	12.56±1.08^{b}
	0.5		0.25	15.00±1.40^{a}
F-test				*
C.V. (%)				41.76

*Significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Mean sharing letter in common within column is not significant difference by DMRT.

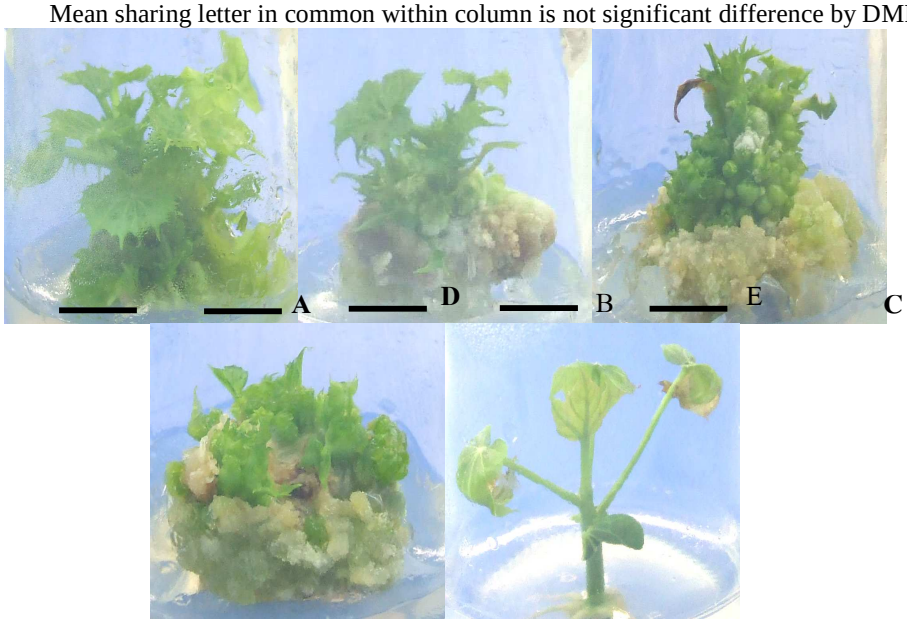

Fig. 1. Characters of shoots induced from epicotyl explants of *Jatropha carcas* L. (bar = 1 cm).

(A) MS + 0.5 mg/l BA + 0.05, 0.1 and 0.25 mg/l IBA alone

(B) MS+ 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l 2, 4-D

(C) MS+ 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l TDZ

(D) MS+ 0.5 mg/l TDZ + 0.05, 0.1 and 0.25 mg/l 2, 4-D

(E) MS + 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l IBA

Shoots induction from hypocotyl

The best response on multiple shoot formation from hypocotyl explants was obtained on MS medium supplemented with 0.5 mg/l KN in combination with 0.25 mg/l IBA at 22.76 shoots/responding explant, followed by 0.5 mg/l KN in combination with 0.25 mg/l 2, 4-D (21.47) significant difference ($P \leq 0.05$) to other combinations (Table 2). Shoot formation from all PGRs was green in color and small in size. Callus formation from this explant could not be regenerated into plantlet (Fig. 2A-C). Induced shoot buds were multiplied and elongated into healthy shoots following transfer to the MS medium supplemented with 0.5 mg/l BA in combination with 0.25 mg/l IBA.

Table 2. Mean number of shoots, which produced on MS medium supplemented with various concentrations of plant growth regulators from hypocotyl explants of *J. curcas* L.

Conc. of PGRs (mg/l)				Average no. of shoots/responding epicotyl
Control				1.00±0.00^{g}
BA	0.5	IBA	0.05	5.76±0.53^{ef}
	0.5		0.1	5.64±0.37^{ef}
	0.		0.25	5.5±0.39^{ef}
KN	0.5	IBA	0.05	13.13±0.65^{b}
	0.5		0.1	8.64±0.60^{c}
	0.5		0.25	22.76±1.27^{a}
TDZ	0.5	2, 4-D	0.05	12.33±0.65^{b}
	0.5		0.1	9.12±0.70^{c}
	0.5		0.25	21.47±1.09^{a}
KN	0.5	2, 4-D	0.05	5.91±0.22^{def}
	0.5		0.1	4.6±0.28^{ef}
	0.5		0.25	4.5±0.38^{f}
KN	0.5	TDZ	0.05	7.73±0.39^{d}
	0.5		0.1	6.53±0.31^{de}
	0.5		0.25	6.42±0.40^{def}
F-test				*
C.V. (%)				26.51

* Significant difference at $P \leq 0.05$ according to Duncan's multiple range test.
Mean followed by a common letter in column were not significant difference by DMRT.

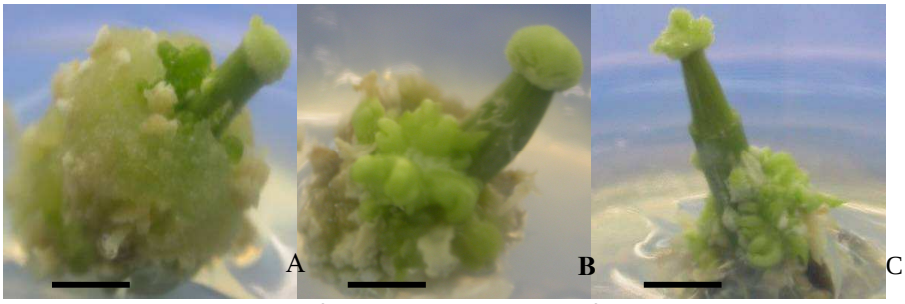

Fig. 2. Callus and shoots induction from hypocotyl explants of *Jatropha curcas* L. (bar = 1 cm), (A – B) Callus and shoots induction from hypocotyls cultured on MS + 0.5 mg/l BA + 0.05, 0.1 and 0.25 mg/l IBA, 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l 2, 4-D, 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l TDZ and 0.5 mg/l TDZ + 0.05, 0.1 and 0.25 mg/l 2, 4-D, (C) Shoots induction from hypocotyls cultured on MS + 0.5 mg/l KN + 0.05, 0.1 and 0.25 mg/l IBA.

Flow cytometry analysis

Young leaf, callus and stem from explants cultured on MS medium supplemented with the best result PGRs were used for flow cytometric measurement. Figure 3 showed the result of flow cytometric measurements with three types of histograms. Diploid plants containing 2C DNA showed G1 peak at the position (channel 100) that had been determined by analyzing the diploid plants. This suggested that plantlets obtained directly from stem explants or callus have the same ploidy level. It might be no variation occur by this procedure.

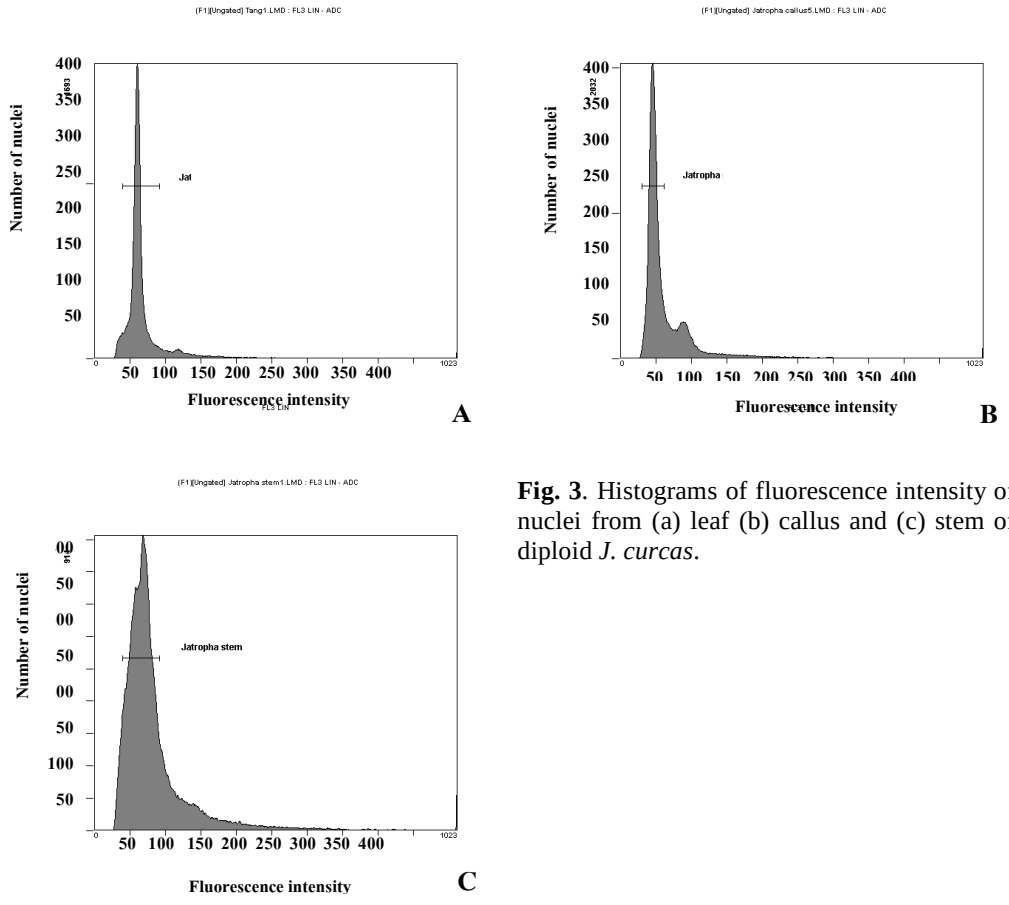

Fig. 3. Histograms of fluorescence intensity of nuclei from (a) leaf (b) callus and (c) stem of diploid *J. curcas*.

Discussion

This study demonstrated the different explant responded to the different PGRs. Epicotyl explant gave the best result in adventitious shoots in 0.5 mg/l KN in combination with 0.25 mg/l TDZ containing medium whereas hypocotyls explant gave the best result in 0.5 mg/l KN together with 0.25 mg/l IBA containing medium. Average number of shoots obtained from the former explant were lowere than those obtained from the latter explant. The different response might be due to meristematic activity of the cells contained in those explants. Hypocotyl explants consisted of appropriate balance of endogenous growth regulators lead to the higher development of meristematic cells to be meristemoid structure. Similar result was obtained in *J. integerrima* (Sujatha and Mukta, 1993; Sujatha *et al.,* 2000). However, Qin *et al.* (2004) and Sujatha and Mukta (1996) found that epicotyl of *J. curcas* gave the best result in average number of shoot bud formation. Moreover, they reported that BA was the potent cytokinin for maximum number of shoot bud formation whereas KN was proved to be the best in this present study. The different response to different cytokinin might be tissue specific, physiological stage and geographical plantation. Those factors affected growth and development of the explant and endogenous growth regulators as well. Beyond these two explants leaf disc, petiole, cotyledon and axillary bud were also reported to give a good result in shoot bud regeneration (Datta *et al.,* 2007; Deore and Johnson, 2008; Jha *et al.,* 2007; Sujatha *et al.,* 2005). Unfortunately, our preliminary results in culturing those explants revealed only callus formation without regeneration. Therefore, hypoctyl and epicotyl explants of *J. curcas* should be the best explants in our present study at this time.

Flow cytometry is frequently used in plant cells to date to determine ploidy level of tissue and plants following their manipulation *in vitro* or their treatment with mitogenic substances (Ochatt, 2008). The analysis of nuclear DNA content in *J. curcas* from leaf, stem and callus showed no changes among those three different explants. Our results confirmed that there is no variation in ploidy level of micropropagated plants derived from both epicotyl and hypocotyl culture. Plantlets obtained through this protocol were uniform. Meesawat *et al.* (2008) also showed that nuclear content of adult pigeon orchid grown in greenhouse and *in vitro* obtaining plantlets was the same (2C DNA). In case of *Astragalus chrysochlorus*, the ploidy of somatic embryos (25 day-old cell suspension) was 81% true-to-type (2C DNA) revealed by flow cytometric analysis (Kara and Ari, 2008). Rival *et al.* (1997) also used flow cytometry as a tool for determination of variation in oil palm tissue culture. Each sources of plant material gave the same ploidy level and showed no variation. However,

fast growing callus (FGC) gave slightly lower content of DNA. Regardingly, they suggested that plantlets regenerated from FGC were off-type.

Conclusion

This study revealed that epicotyl and hypocotyl were the best explants for micropropagation through direct induction of adventitious shoots when cultured on MS medium supplemented with KN (0.5 mg/l) in combination with TDZ (0.25 mg/l) (15.00) and KN (0.5 mg/l) in combination with IBA (0.25 mg/l) (22.76), repectively. Plants could be regenerated after transferred and cultured on MS medium supplemented with 0.5 mg/l BA in combination with 0.25 mg/l IBA. The regenerants obtained from this protocol were normal under the evaluation by flow cytometry.

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