Somatic Embryo Germination of *Jatropha curcas* L in Presence of Sucrose and Poly Ethylene Glycol (PEG)

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Abstract

Jatropha curcas L. is a potential source of a non-edible biofuel. Conventional propagation of *J. curcas* technique has some limitations. Somatic embryo can produce a large number of embryos and obtain a large number of plants all year round. Treatment of sucrose in combination with polyethylene glycol (PEG) was proven to enhance germination of somatic embryos in many plant species. The aim of the study was to investigate the effect of sucrose in combination of PEG on somatic embryo germination in *J. curcas*. Globular somatic embryos at 0.025-0.030 g fresh weight having 0.4-0.5 cm in diameter were grown on MS medium solidified with 3 g/l of Gelzan supplemented with sucrose at 20, 30, 40, and 50 g/l in combination with PEG at 0, 2.5, 5, 10, and 15%. Results showed that the best medium for germination of *J. curcas* somatic embryo cultures was MS medium supplemented with 20 and 30 g/l of sucrose in combination with 5% of PEG. The numbers of germinated embryos per clump had significant enhancement on those medium compared with the control (PEG free treatment) (2.65 to 5.65) and (2.55 to 5.50). In addition, those treatments resulted in the highest percentage of clumps forming germinated embryos (100%), with an average of normal germinated embryos at 94.163 and 96.065%. The addition of 40 and 50 g/l of sucrose in combination with 15% of PEG caused all embryos to fail at germinating.

Keywords: *Jatropha curcas*, germination, somatic embryo, sucrose, polyethylene glycol (PEG)

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Introduction

Jatropha curcas L. is a potential source of a non-edible biofuel. Biofuel extracted from *J. curcas* seeds contains higher oxygen than other biofuels. It has cetane value that can increase the combustion quality. The product is clean, non-toxic and ecologicaly friendly. Biofuel produced by *J. curcas* is efficient due to its low production cost. *J. curcas* is also beneficial for eco-restoration in all types of wasteland as well as useful for medicinal purposes. *J. curcas* kernels have 46-58% oils (iodine value is 93-107) containing mainly oleic (37-63%), linoleic (19-40%) and palmitic (12-17%) acids (Prabakaran & Sujatha 1999; Jha *et al*., 2007; Kalimuthu, 2007). This species originated from South America (Brazil); later it spread to tropical regions in many countries. Conventional propagation of *J. curcas* commonly uses seeds. This technique has some limitations such as poor seed viability, low germination rate, scanty and delayed rooting of seedlings, and vegetative cuttings do not develop a tap-root (Openshaw, 2000).

Somatic embryogenesis is an *in vitro* method to provide a large number of embryos at defined stages of development, and allows alterations of the embryonic environment through manipulations of the culture conditions (Stasolla *et al*., 2004). Plant propagation by somatic embryogenesis not only helps to obtain a large number of plants year round, but also it can act as a powerful tool for genetic improvement of any plant species because of it's single cell origin (Bhansali, 1990; Jha *et al*., 2007).

Several reports have been published on *J. curcas* somatic embryogenesis. Addition of 13.6l M adenine sulphate stimulated the process of development of somatic embryos of *J. curcas*. Mature somatic embryos were converted to plantlets on half strength of MS

basal medium with 90% survival rate (Jha *et al*., 2007). Saxena (2012) stated that maturation and germination of *J. curcas* embryos completed on medium containing 0.2 mg/l of Indole-3-acetic-acid (IAA) in combination with 1.5 mg/l of 6-benzylaminopurine (BAP). The conversion of globular embryos subsequently into cotyledonary embryos took about 40-45 d.

Somatic embryos of *J. curcas* were initiated from the mature embryogenic axis explants cultured on solid Murashige and Skoog (MS) medium containing picloram at 2.5; 3.0; 4.0 and 5 mg/l. Somatic embryogenesis developed directly and indirectly from the end cut of explants. Proliferation of embryogenic callus and maturation of somatic embryos was performed on solid MS medium without plant growth regulators, but limited information for their further growth and embryos germination (Al-Hafiizh *et al*., 2012).

The presence of sucrose in the medium has been reported to have a significant effect in somatic embryogenesis. It increased the frequency of somatic embryo formation in *Carica papaya* L. when they were cultured on the medium supplemented with different concentrations of sucrose. The medium containing 7% of sucrose was able to enhance *C. papaya* L somatic embryogenesis (Fitch, 1993; Mora, 2012). Sucrose at 0.2-0.4% in the culture medium boosts the efficiency of the photosynthetic apparatus of somatic embryos of *Gentiana kurroo* (Rybczyński *et al*., 2007). In addition, Gerdakaneh, (2009), reported that sucrose concentration has been found to play important roles in different stages of the somatic embryogenesis process. Level of sucrose added to the culture medium significantly affects the development and regeneration of cell. In *Dianthus caryophyllus* and *Acacia sinuata*, different concentrations of sucrose influenced the induction of embryonic callus, embryo development and regeneration.

Polyethylene Glycol (PEG), which is a high molecular weight polymer, has been used in cell cultures of several species to stimulate the effects of water stress or to enhance maturation and conversion of somatic embryos into plantlets, similar to those that originate from seeds as observed in *Pinus sylvestris*, *Abies numidica*, *Panax ginseng*, *Cryptomeria japonica*, *Abies cephalonica*, *Aesculus hippocastanum* and *C. papaya* L. (Koehler *et al*. 2013). However there is no report on somatic embryos germination using various concentrations of sucrose and PEG in *J. curcas*. This study was conducted to investigate the effect of sucrose in combination with PEG on the germination of *J. curcas* somatic embryos.

Materials and Methods

In this experiment, globular somatic embryo clumps at 0.025-0.030 g fresh weight having 0.4-0.5 cm in diameter were cultured on MS medium (Murashige & Skoog, 1962) solidified with 3 g/l of Gelzan (TM Caissonlabs) supplemented with sucrose at 20, 30, 40, 50 g/l in combination with PEG 6000 at 0, 2.5, 5, 10, 15 % W/V. Medium pH was adjusted to 5.8 prior to sterilization using autoclave at 15 psi at 121°C for 60 min. Cultures were incubated at 24 ± 2°C, with continuous photoperiod with light intensity of 300-500 lux.

Experiments were set up in a completely randomized design with 25 replicates of each treatment using 5 petri dishes containing 5 clumps embryos in each petri dish. Numbers of germinated embryos per clump were recorded every week from week 1 to week 8 after culture. Diameters of an embryos clump, percentages of normal germinated embryos and percentages of a clump forming germinated embryos were recorded after 8 weeks culture. Growth responses and the development of somatic embryos forming normal and abnormal germinated embryos were also recorded at week 8 after inoculation. Data were analyzed by analysis of variance (ANOVA) (Sokal & Rohlf 1987) to determine the significant levels of differences responses between treatments. Responses differing significantly were compared using Duncan's multiple range test (DMRT) at α 5% probability level using DSAASTAT V.1.1 (open-source software).

Results

At optimal condition, sucrose in combination with PEG stimulated germination of *J. curcas* somatic embryos. Numbers of germinated embryos per clump is presented in

Figure 1. Two weeks after culture, somatic embryos started to germinate on the medium containing 20 g/l of sucrose alone or in combination with PEG at 2.5 and 5% (Figure 1A). On the medium containing 30 g/l of sucrose, the embryos also started to germinate two weeks after culture on the medium without additional PEG and on the medium containing PEG at 2.5%. The embryos started to germinate 3 weeks after culture when they were grown on medium with 30 g/l of sucrose containing 5 and 10% of PEG. The presence of PEG 15% increased numbers of germinated embryos from 0.0 to 0.27 after 5 weeks in culture but lower than PEG at 0, 2.5 and 5% (Figure 1B). On the medium containing sucrose at 40 g/l in combination with 2.5 and 5% of PEG or without PEG, embryos started to germinate at about 3-4 weeks after culture. PEG at 10% supressed germination of embryos, meanwhile, on medium with 15% of PEG no embryo was germinated (Figure 1C). Similarly, on the medium containing sucrose at 50 g/l without addition of PEG or in combination with 2.5 and 5% of PEG, somatic embryos started to germinate 2 weeks after culture. PEG at 10% resulted in only few of number of germinated embryos 4-5 weeks after culture. No germinated embryos were found on the medium containing PEG at 15% (Figure 1.D).

The effect of sucrose in combination with PEG at any concentrations had significant value in variable: diameter of embryo's clump, number of germinated embryo/clump, and percentage of normal germinated embryos of *J. curcas* 8 weeks after culture. Those variables not only significantly affected by individual treatment of sucrose or PEG, but also by interaction of the two factors (sucrose × PEG) and had F value 5.227, 2.722 and 2.691 respectively (Table 1). The correlation value between variable diameter of embryo's clump × number of germinated embryo/ clump: 0.503, diameter of embryo's clump × percentage of normal germinated embryos: 0.477, and number of germinated embryo/ clump × percentage of normal germinated embryos: 0.668 (Table 2).

The highest globular embryos proliferation occurs when embryos grown on the medium containing 20g/l of sucrose without PEG. This treatment achieved diameter of clump embryos highest than others. The growth of globular stage somatic embryo diameter low at the increase of PEG level both in sucrose at 20 g/l and at others concentration of sucrose (Table 3). At medium containing 20 g/l of sucrose without PEG, sucrose at 20 g/l in combination with 5% of PEG, 30 g/l of sucrose alone and in combination with 5% of PEG, 40 g/l of sucrose in combination with 2.5 and 5% of PEG and 50 g/l of sucrose alone and in combination with 5% of PEG achieved normal germinated embryos more than 94% (Table 3 and Figure 3). The highest percentage of clump formed germinated embryos was reached on the medium suplemented 20 and 30 g/l of sucrose in combination with 5% of PEG. All embryos clump cultured on this medium were 100 % germinated (Table 3).

In this experiment, the germination of somatic embryos was depending on the concentration of sucrose and PEG treatments. On the medium containing sucrose at 20, 30, 40 and 50 g/l in combination with 0, 2.5 and 5% of PEG the embryos grew well (Figure 2). Slow growth condition occurs when embryos cultured on the medium containing 10 % of PEG at any concentration of sucrose and 15 % of PEG in combination with 20 and 30 g/l of sucrose (Table 4 and Figure 2). In the medium containing 40 and 50 g/l of sucrose in combination with 15 % of PEG there were no embryos growth and germinated. The size and colours of embryos and germinated embryos also were affected by sucrose and PEG level. In the medium containing sucrose at 20, 30 and 40 g/l without PEG, the colours of embryos were green-yellow and had a big size of germinated embryos (Table 4 and Figure 2). In the medium containing 20, 30 and 40 g/l of sucrose in combination with 2.5 and 5% of PEG and sucrose at 50 g/l in combination with 0, 2.5 and 5% of PEG, the colours of embryos were green-yellow but had a medium size of germinated embryos. In the medium containing 10 % of PEG at any concentration of sucrose and PEG at 15 % in combination with 20 and 30 g/l of sucrose, the colours of embryos were brown-yellow and had a small size of germinated embryos, while at 15% PEG and 40 and 50 g/l of sucrose no embryo germinated (Table 4 and Figure 2).

The distinct figure between normal and abnormal germinated embryo of *J. curcas* were presented in Figure 3. Medium containing 30g/l of sucrose in combination with 5% of PEG gave normal embryos on bipolar features with hypocotyl and epicotyl

grew well (Figure 3A). On the other hand, in medium containing 40 g/l of sucrose in combination with 10% of PEG, several germinated embryos had abnormal shape, hypocotyl did not growth properly (Figure 3B). In the medium containing 30g/l of sucrose in combination 2.5% of PEG also had abnormal germinated embryos, fussion occurs between 2 embryos. In medium containing 20 g/l of sucrose without PEG several germinated embryos transform to callus and continuing proliferation (Figure 3D).

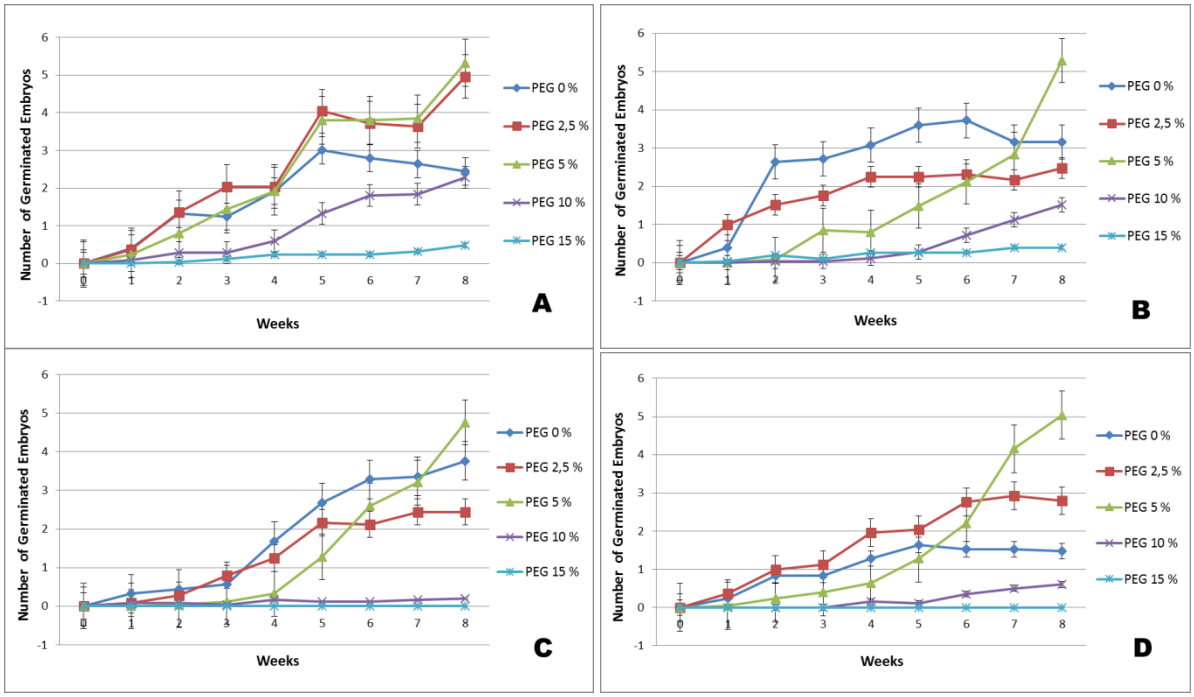

Figure 1. Number of germinated embryos of *J. curcas* grown on medium supplemented with PEG at 0, .5, 5, 10 and 15% in combination with sucrose. A. PEG with sucrose at 20 g/l, B. with sucrose at 30 g/l, C with sucrose at 40 g/l, and D. with sucrose at 50 g/l, respectively

Table 1. Analysis of variance for variable: diameter of embryo's clump, number of germinated embryo, and percentage of normal germinated embryos of *J. curcas* 8 weeks after culture.

No	Variable	F Value			C.V. (%)
		Sucrose	PEG	Sucrose x PEG	
1	Diameter of Embryo's Clump	18.838**	92.387**	5.227**	14.22
2	Number of Germinated Embryo/ Clump	5.826**	51.649**	2.722**	41.63
3	Normal Germinated Embryos (%)	2.944*	26.876**	2.691**	32.20

* : (P < 5%)
** : (P < 1%)

Table 2. Correlation analysis between variable: diameter of embryo's clump, number of germinated embryo, and percentage of normal germinated embryos of *J. curcas* 8 weeks after culture.

No	Variable	Diameter of Embryo's Clump	Number of Germinated Embryo/Clump	Normal Germinated Embryos (%)
1	Diameter of Embryo's Clump	1.000	0.503	0.477
2	Number of Germinated Embryo/ Clump	0.503	1.000	0.668
3	Normal Germinated Embryos (%)	0.477	0.668	1.000

Table 3. Diameter of embryo clump, number of germinated embryo, percentage of normal germinated embryos and percentage of clump formed germinated embryos of *J. curcas* somatic embryos 8 weeks after culture.

Treatments		Diameter of Embryo's Clump (cm)	Number of Germinated Embryo/ Clump	Normal Germinated Embryos (%)	Clump Formed Germinated Embryos (%)
Sucrose (g/l)	PEG (%)				
20	0	**1.870 ± 0.381^{a}**	2.65 ± 0.854^{bc}	**70.139 ± 14.232^{abc}**	80
	2.5	1.178 ± 0.110^{bc}	**5.75 ± 1.879^{a}**	**82.168 ± 12.73^{abc}**	96
	5	1.165 ± 0.070^{bc}	**5.65 ± 1.603^{a}**	**94.163 ± 5.125^{a}**	**100**
	10	0.775 ± 0.262^{ef}	2.75 ± 2.645^{bc}	**84.035 ± 23.111^{abc}**	64
	15	0.558 ± 0.056^{g}	0.60 ± 0.327^{de}	56.667 ± 8.165^{cd}	28
30	0	1.030 ± 0.035^{bcd}	2.55 ± 0.342^{bc}	**96.250 ± 4.383^{a}**	84
	2.5	1.053 ± 0.185^{bcd}	2.65 ± 0.342^{bc}	**76.590 ± 16.146^{abc}**	92
	5	0.965 ± 0.100^{cde}	**5.50 ± 0.775^{a}**	**96.065 ± 4.560^{a}**	**100**
	10	0.673 ± 0.036^{fg}	1.80 ± 1.058^{cde}	**85.714 ± 24.046^{abc}**	56
	15	0.597 ± 0.096^{fg}	0.30 ± 0.383^{e}	31.250 ± 12.500^{de}	16
40	0	1.205 ± 0.109^{b}	**4.20 ± 1.296^{ab}**	**78.310 ± 8.801^{abc}**	84
	2.5	1.050 ± 0.123^{bcd}	2.25 ± 0.755^{cd}	**95.313 ± 5.984^{a}**	76
	5	0.933 ± 0.062^{de}	**4.95 ± 0.619^{a}**	**98.138± 2.162^{a}**	92
	10	0.593 ± 0.034^{fg}	0.25 ± 0.252^{e}	25.000 ± 20.412^{e}	16
	15	0.537 ± 0.012^{g}	0.00 ± 0.000^{e}	0.000 ± 0.000^{e}	0
50	0	1.160 ± 0.099^{bc}	1.80 ± 1.657^{cde}	**91.369 ± 11.800^{ab}**	52
	2.5	0.998 ± 0.043^{bcd}	3.05 ± 1.100^{bc}	**86.881 ±16.149^{abc}**	88
	5	0.973 ± 0.038^{cde}	**5.30 ± 0.902^{a}**	**96.069 ± 3.243^{a}**	92
	10	0.545 ± 0.076^{g}	0.60 ± 0.693^{de}	62.500 ± 47.871^{bc}	24
	15	0.533 ± 0.025^{g}	0.00 ± 0.000^{e}	0.000 ± 0.000^{e}	0

Means followed by the same letter in the same colomn are not significantly different according to the DMRT test ($p<0.05$)

Table 4. Growth response of *J. curcas* somatic embryos 8 weeks after culture

Sucrose (g/l)	PEG (%)	Colour of Embryos	Size of germinated embryo	Growth Condition	Callus Proliferation
20	0	green-yellow	big	grew well	+++++
	2.5	green-yellow	medium	grew well	++++
	5	green-yellow	medium	grew well	++++
	10	brown-yellow	small	slow growth	+++
	15	brown-yellow	small	slow growth	+
30	0	green-yellow	big	grew well	++++
	2.5	green-yellow	medium	grew well	++++
	5	green-yellow	medium	grew well	+++
	10	brown-yellow	small	slow growth	++
	15	brown-yellow	small	slow growth	+
40	0	green-yellow	big	grew well	++++
	2.5	green-yellow	medium	grew well	+++
	5	green-yellow	medium	grew well	+++
	10	brown-yellow	small	slow growth	+
	15	brown-yellow	-	no growth	+
50	0	white-yellow	medium	grew well	++++
	2.5	green-yellow	medium	grew well	+++
	5	green-yellow	medium	grew well	+++
	10	brown-yellow	small	slow growth	+
	15	brown-yellow	-	no growth	+

Discussion

One of the process of expression of cell totipotency in plant cells is somatic embryogenesis. This embryo cell has ability to undergo a complex series of metabolic and morphological coordinated steps to produce a complete and normal plant without sexual process. Jiménez (2005) stated that like their zygotic counterparts, somatic embryos have a single cell origin. Mora (2012) reported that, sucrose has a significant effect in somatic embryogenesis, and found to play important roles in different stages of the somatic embryogenesis process. Heringer *et al*. (2013) reported that the addition of germination promoters, such as PEG is crucial to promote the germination of somatic embryos and conversion into plantlets.

Our results showed that concentration of sucrose in combination with PEG significantly affected somatic embryo germination of *J. curcas* cultures. The growth of somatic embryo diameter low with the increase of PEG levels (Table 3). It indicates that PEG inhibited the proliferation of embryos at globular stage and stimulated embryo germination. In the medium containing sucrose at 20, 30 and 50 g/l without PEG, only few embryos germinated. When embryos were grown on medium supplemented with 10 and 15% of PEG, the number of germinated embryos decreased siqnificantly (Figure 1 and Table 3). These results indicated that the germination of an embryogenic callus in *J. curcas* depended on an optimal concentration of sucrose and PEG. On the medium containing sucrose 40 and 50 g/l with PEG 15% no embryos germinated (Table 3 and Figure. 2).

The optimum concentration of PEG level in somatic embryos germination depend on species and type of embryos. Langhansov *et al*. (2004) reported that in *P. ginseng*, somatic embryos grown on medium MS containing 3.75% of PEG achieved greater structural development, which was demonstrated anatomically. The plantlets obtained from somatic embryos treated with PEG at 3.75% displayed better root formation, facilitating subsequent acclimatisation. PEG also stimulated germination of somatic embryos of *Carica papaya*. Koehler *et al*. (2013) reported that on half strength of MS medium containing 50 g/l PEG, along with 2 g/l activated charcoal and 5 µM ABA, to papaya somatic embryos led to the germination of somatic embryos and the formation of plantlets. This suggested that PEG is an inducer of conversion of somatic embryos to germination. Mishra *et al*. (2010) demonstrated that the supplementation of conversion medium with PEG (4.5%) led to a synchronised germination process of transformed embryos of *C. papaya*.

The scale up production of normal somatic embryos is not always possible for most species, as the development and germination of normal bipolar embryos from embryogenic callus after the globular phase is sometimes problematic. Beyond this stage, embryo development often shows varying degrees of abnormality (Benelli *et al*., 2010). The percentage of normal germinated embryos more than 94% was achieved in the medium containing sucrose at 20 g/l in combination with 5% of PEG, 30 g/l of sucrose without PEG and in combination with 5% of PEG, 40 g/l of sucrose in combination with 2.5 and 5% of PEG and 50 g/l of sucrose without PEG and in combination with 5% of PEG. This indicates that those treatments improve normal germinated embryos in *J. curcas*

Some abnormalities in embryos germination occur when embryos planted on media with low concentration of sucrose. In the medium with sucrose 20 g/l in absent of PEG several germinated embryos of *J. curcas* did not grow normally, some of them turned back to form callus (Figures 2 and 3). Another abnormality occurred when hypocotyl did not grow properly and formed fusion between 2 embryos (Figure 3). This result was similar to Businge *et al*. (2013), they reported that the concentrations of sucrose were also related to the osmotic environment during somatic embryo maturation and germination in Norway spruce. Sucrose is a permeating osmoticum involving apoplastic invertase in the tissue and extracellular invertase in the medium, a different PEG which is a nonpermeating osmoticum (Wind *et al*., 2010). Tereso *et al*. (2007) stated that a maturation medium containing a low sucrose concentration reduced somatic embryo development of *Pinus pinaster*. Benelli *et al*. (2010) reported that the causes various imperfect structures is difficult to establish by anatomical investigation only. It can be hypothesized that growing embryos are affected in their anatomy and histology by

number of negative conditions present in the environment they live in, such as type and concentration of carbon source or plant growth regulators. With respect to sucrose, for example, previous studies reported that this sugar has both a nutritive and an osmotic effect on embryogenesis in several species.

The optimal germination medium for *J. curcas* somatic embryo cultures was determined to be MS medium supplemented with 20 and 30 g/l of sucrose in combination with 5% of PEG. Those treatments exhibited the high value on several variables: number of germinated embryos/ clump, percentage normal germinated embryos and percentage clump germinated embryos (Table 3). This result suggested that the optimum concentration of sucrose in combination with PEG stimulates a shift in the developmental process of the culture, from globular embryos to the production of germination embryos. Therefore, conversion and germination of somatic embryos is dependent on stress that allows embryonic development to progress. As with induction, maturation of somatic embryos also appears to be highly dependent on stress. This stress modifies the DNA methylation pattern and causes the expression of proteins, which is essential to develop somatic embryos, enabling these embryos to correctly mature (Heringer *et al*., 2013). Actually, this studies need to be continued for the response of different plant materials, post maturation treatments, embryo germination using several osmotic agents, optimation of planlet growth and acclimatization to improve the efficiency of plant regeneration system for practical purposes.

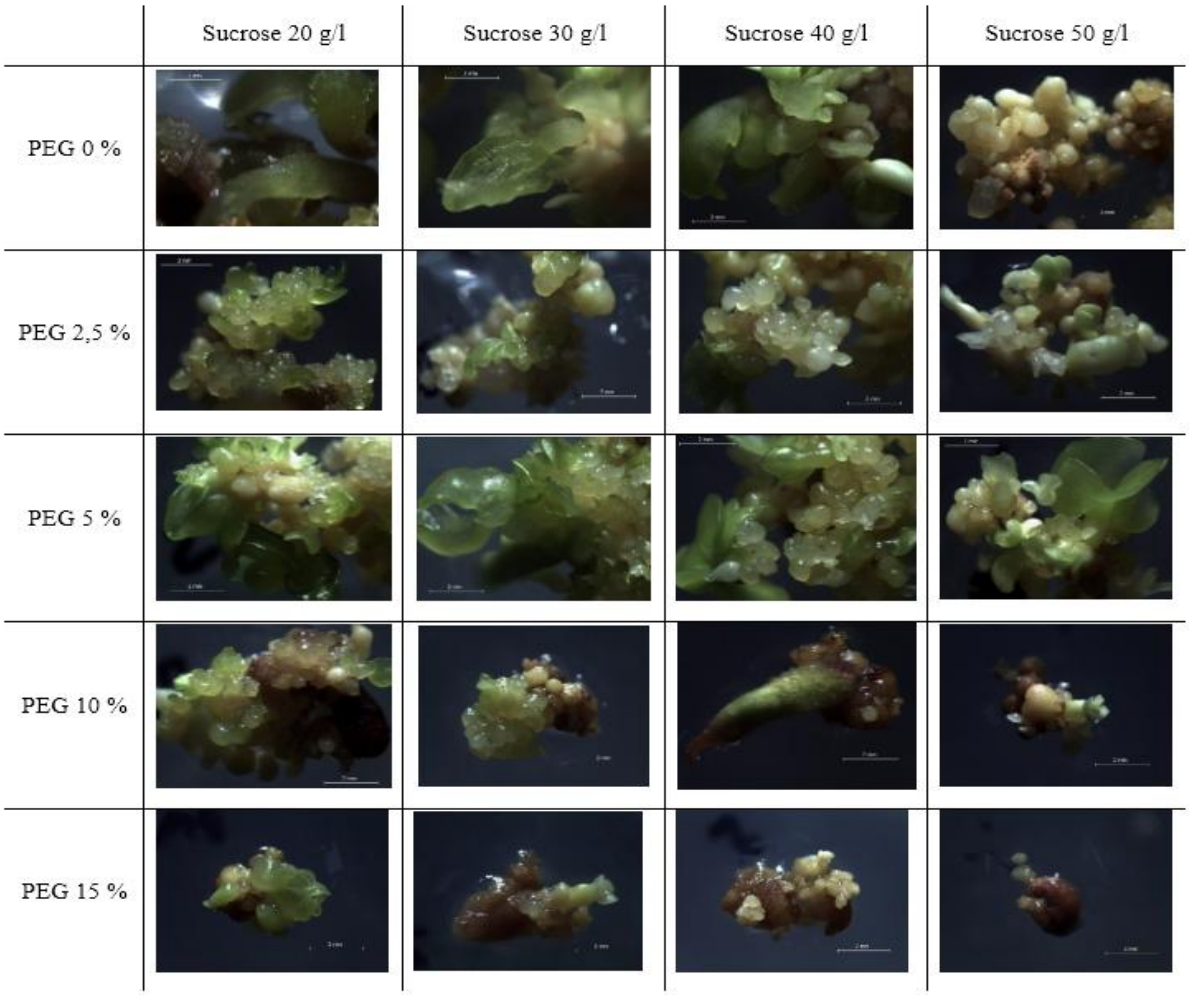

Figure 2. Germination of *J. curcas* somatic embryos 8 weeks after culture. Sucrose at 20, 30, 40 and 50 g/l in combination with PEG at 0, 2.5 and 5% enhanced germination of embryos. PEG at 10 and 15% reduced germination of the embryos.

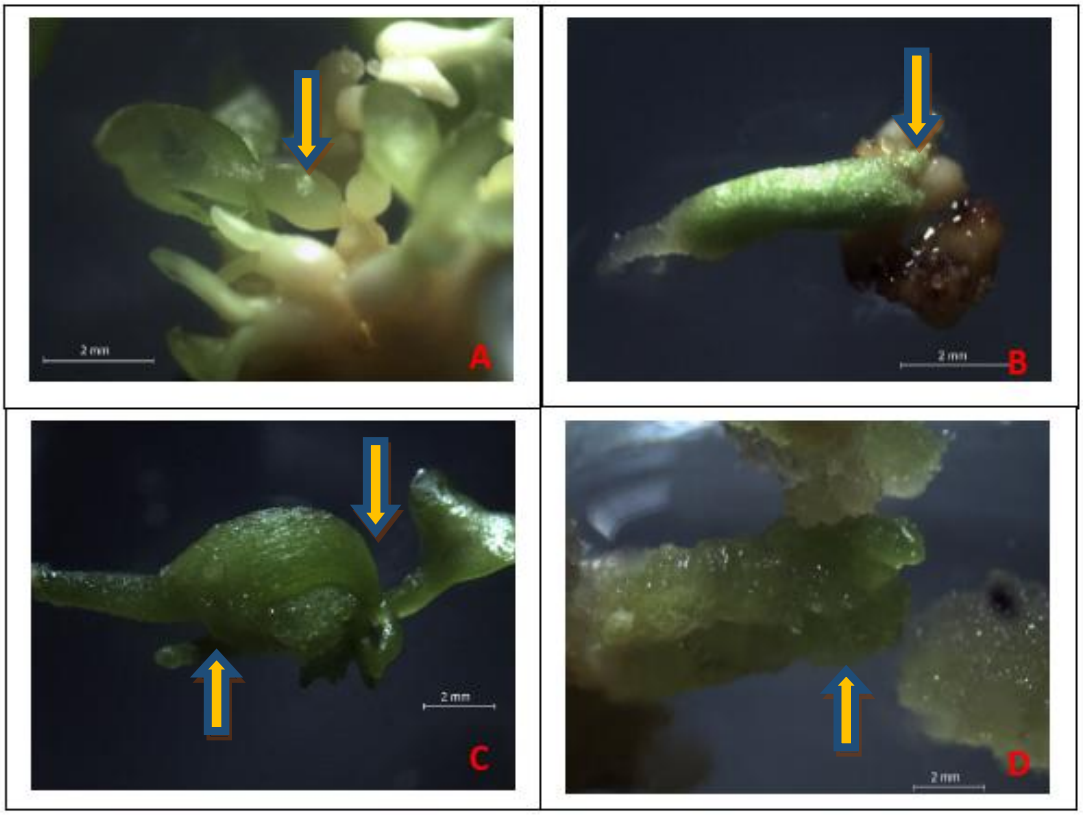

Figure 3. Figure of normal and abnormal germinated embryo of *J. curcas* somatic embryos A. Normal Germinated embryos. B. Abnormal germinated embryo (hypocotyl did not growth properly) C. Abnormal germinated embryos (fusion occurs between 2 embryos) D. Abnormal germinated embryos (germinated embryos transform into callus)

Conclusions

The optimal germination medium for *J. curcas* somatic embryo cultures was MS medium supplemented with 20 and 30 g/l of sucrose in combination with 5% of PEG. Those treatments exhibited the high value on several variables: number of germinated embryos/ clump, percentage of normal germinated embryos and percentage of clump germinated embryos. PEG at 10 and 15% in combination with any sucrose concentrations (20, 30, 40 and 50 g/l) inhibit embryo germination.

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