Journal of Applied Science and Agriculture, 9(20) Special 2014, Pages: 63-69

AENSI Journals

Journal of Applied Science and Agriculture

ISSN 1816-9112

Journal home page: www.aensiweb.com/JASA

Induction of Multiple Shoot Bud Formation from *Jatropha curcas L*

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ARTICLE INFO

Article history:
Received 30 September 2014
Received in revised form
17 November 2014
Accepted 25 November 2014
Available online 13 December 2014

Keywords:
J. curcas L Hybrid plant (P1 X P3)
Shoot induction Petiole
TDZ (Thidiazuron)

ABSTRACT

Background: This paper described a preliminary study on a simple and efficient protocol for surface sterilization and multiple shoot bud induction of *J. curcas* L using petiole as explants. Multiple shoot bud was induced solely with the application of cytokinin, namely Thidiazuron (TDZ). **Objective:** To develop an efficient sterilization and shoot bud induction protocols of *J.curcas L* plants using petiole for the multiple shoot buds. **Results:** Sterilization technique developed by using 0.1% mercuric chloride ($HgCl_2$) for 5 minutes seems to be very effective in order to diminish the contamination from field sources with 96% survival. Explants culture on Murashige and Skoog's (MS) medium supplemented with TDZ resulted in 34% and 24% shoot induction (2-11 shoot per explant) when supplemented with TDZ at 8mg/L and 10mg/L respectively**.** **Conclusion:** By using 0.1% $HgCl_2$ for 5 minutes contaminations can be diminished effectively and survival rate was recorded until 96%. 8mg/L and 10mg/L TDZ was shown to gives good results for shoot bud induction for this plant.

To Cite This Article: Nasir. N.A.N.M, Anuar, N and Yaakob, Z., Induction of multiple shoot bud formation from *J. curcas L*. ***J. Appl. Sci. & Agric.,*** *9(20): 63-69, 2014*

INTRODUCTION

Jatropha curcas. L belongs to the family of Euphorbiaceae, commonly known as purging nut, physic nut, Barbados nut, Retanjut (Attaya, Geelen and Belal, 2012). The genus Jatropha contains approximately 175 known species (Dehgan, 1984) native to South America and widely distributed in South and Central America and now almost in pantropical (Mukherjee *et al*., 2011). According to Makkar and his colleagues (1997) the most important oil producing part was its seed kernel which contains 40-60% (w/w) of non-edible oil. The oil contains 21% saturated fatty acid and 79% unsaturated fatty acid (Gubitz *et al*., 1999). Moreover, oils derives from Jatropha was highly unsaturated that will not congeal at cool temperature (Gressel, 2008). There are some good properties in Jatropha oil which make it potential as biodiesel which are, low acidity, good oxidation stability as compared to soybean oil, low viscosity as compared to palm oil (Tapanes *et al*., 2007). Viscosity, free fatty acids and density of the oil and the biodiesel are stable within the period of storage (Augustus *et al*., 2002). The oil can be used in special diesel engines (Elsbett engine) and thus vigilant filtration is enough (Bassam, 2010).

Rapid depletion of oil source around the world has brought researchers to find renewable source especially plant-based fuel as an alternative source in order to face energy crisis. The oil of Jatropha is high in cetane value and can be used directly in diesel engines added to diesel fuel as an extender or transesterized to a biodiesel fuel (Mukherje *et al*., 2011). Traditionally, *J. curcas* L can be grown from seed, seedling and cuttings. However, conventional propagation in *J.curcas* L through seeds overwhelmed with unsynchronized flowering, fruit maturity as well as high degree of heterozygosity may possibility pose problem in maintaining their genetic fidelity. Plant tissue culture has been observed by most to be method for rapid cloning in many plant species especially in an important plant such as Jatropha (Kaewpoo and Te-chato, 2009; Kumar and Reddy, 2010; Attaya *et al*., 2012).

Morphological description of J.curcas.L:

Jatropha is monoecious with male and female flowers are on the same plant and the terminal inflorescences contain unisexual flowers (Chang-wei *et al*., 2007). According to Raju and Ezradanam. (2002), the ratio of male

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to female flowers ranges from 29:1. Female flowers are small in size as compare to male flowers. The leaves of *J. curcas* are smooth, cordate, 4-6 lobed and 10-15cm in length and width (Achten *et al*., 2008; Reddy and Pamidimarri, 2010). There are many varieties of Jatropha species, some with yellow, pink or green flowers, red young leaves and green matured leaves. Their fruits kernel commonly has 2, 3 seeds but rarely contained 4 seeds. Ripe fruit change color from green to yellow. The seeds are black 1.5-2cm long and 1cm wide contain 33 percent oil and its chromosomes number is 2n=22 (Heller, 1996). All the characteristics mentioned above were shown in Figure 1.

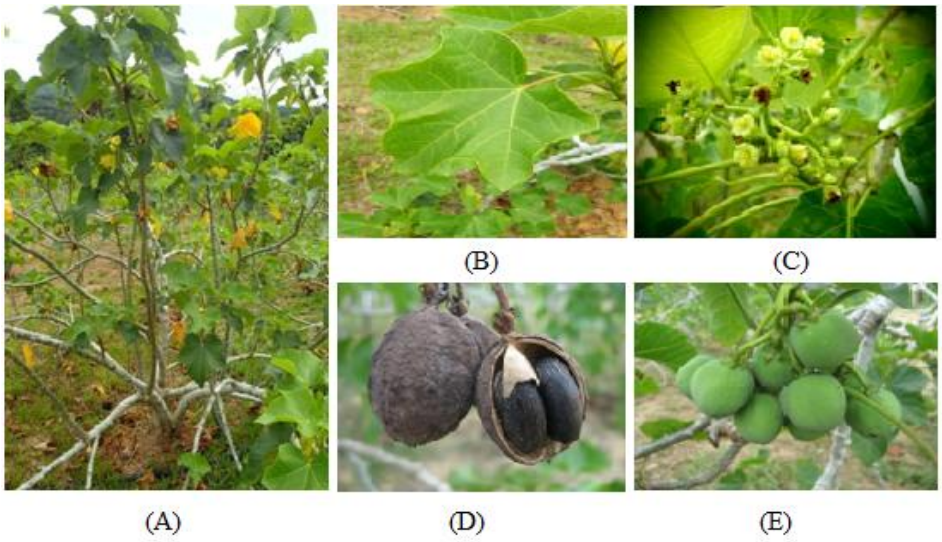

Fig. 1: Phenotypic morphological for *J. curcas* L .A) The whole plant B) Leaves C) Flowers D) Seeds of *J.curcas* E) Fruits of *J.curcas.*

Tissue culture of Jatropha plants:

Plant tissue culture of *J. curcas L* is one of the fast growing, powerful techniques and widely used in this recent years to produce large scale of true to type, high frequency regeneration, disease free, elite and healthy planting material in a rapid rate (Singh *et al*., 2010; Danso *et al*., 2011; Behera *et al*., 2014). Tissue culture technique offer rapid and continuous supply of the planting material (Kaewpoo and Te-chato, 2009). Although traditional propagation method through seeds and stem cutting can be used, they are major limitations exist for each method in order to meet large scale production of true to type of elite plants. Propagation through seeds known to be genetically unstable because seed was naturally pollinated through cross fertilization mating system hence expose to wide amount of heterozygosity, therefore quality traits from mother plants are not fully genetically inheritance to their progeny. Thus, vegetative propagation by stem cutting is not deep rooted, easily uprooted as they do not develop taproot, plants are prone to disease (Heller, 1996) seasonal and insufficient quantity of plant material to meet the demand (Kumar and Reddy, 2012). Therefore, improvements on the micropropagation method for *J.curcas L* are getting spread worldwide recently.

1. Methodology:

Plant material:

Fresh matured petioles were collected from 3 years old of *J. curcas* hybrid (P1 X P3) mother plants, the mother plants were located at experimental plantation, Biodiesel Centre Kuala Pilah, Universiti Kebangsaan Malaysia (UKM), Malaysia. Hybrid plants, P1 X P3 have been selected as the source of explants for shoot induction in culture (Islam, 2009).

Surface sterilization of plant materials:

Surface sterilization was carried out prior to explants preparation to be use in the shoot induction of the culture. A few steps on the sterilization of plant materials were carried out as described in Figure 2. Fresh matured petioles were washed under running tap water for 30 minutes to remove the dust from a surface of plants. Plant material then were washed in Teepol solution (1 drop in 200 ml sterile distilled water) and rinsed 3 times with distilled water. Systemic fungicide, Bavistin (1 ml in 1000ml sterile distilled water) was applied for 1 hour to the plant materials with continuous stirred and rinsed 3 times. Plant materials then were soaked in Tween 20 (1 drop in 200 ml sterile distilled water) for 15 minutes. After this, plants were dipped in 70% ethanol for 1 minute, followed by rinsing twice with sterile distilled water. The plant materials were treated with Mercuric chloride ($HgCl_2$) with concentration of 0.05 - 0.1% w/v for 3, 5 and 7 minutes. Another treatment

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involved sterilization with NaOCl with concentration ranging from 1-3% with exposure period for 10, 15 and 20 minutes. After exposure to all sterilizing agents, explants were rinsed five times with sterile distilled water in order to remove the traces of sterilizing agents.

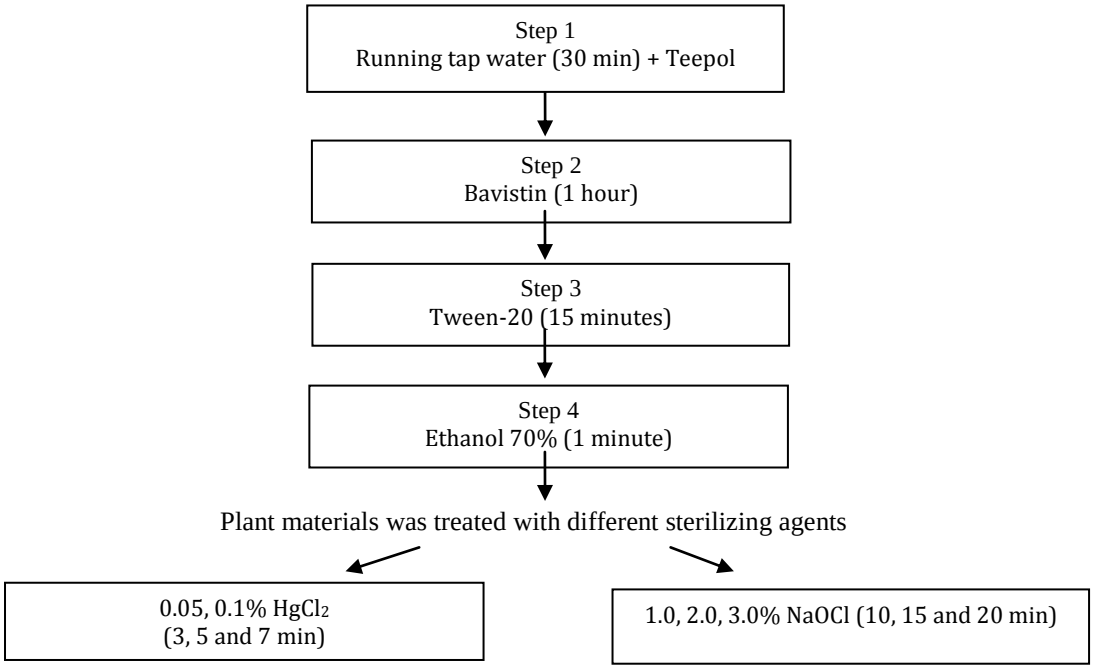

Fig. 2: Surface sterilization of plant material.

Medium and culture condition:

MS medium (Murashige and Skoog, 1962) supplemented with different concentration of TDZ ranging from 0 – 10 mg/L has been applied for the shoot bud induction of *J. curcas L* hybrid. MS medium contains 0.8% w/v agar, 3.0% w/v of sucrose and 0.05% citric acid as a source of antioxidant. The pH for all media was adjusted to 5.8 using 1 N Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) prior to autoclaving at 1.05 kgf/cm^2 pressure at 121˚C at for 20 minutes.

Shoot bud induction:

The sterilized explants were cut in 1.0cm in size, inoculated horizontally in petri dish plate (90mm x 15mm). Explants were cultured on MS agar media with different range of TDZ (0-10mg/L). Each experiment was performed with 5 explants per plate with 5 replicates per treatment. Culture was incubated in a plant growth chamber at 25±2˚C 16 h light/ 8 h dark photoperiod by light intensity of 35-40µmol m^{-2} s^{-1} (cool white fluorescent tubes). The percentage of shoot bud induction and shoot bud per explants were recorded within 5 weeks of culture.

2. Results:

Sterilization protocols:

The survival rates of explants were recorded after 14 days of culture. There were two common sterilizing agents used in this experiment which are NaOCl and $HgCl_2$ and the percentage of sterilization has been recorded in Table 1. By using 0.1% concentration of $HgCl_2$ for 5 minutes, it gave highest percentage of sterilization which indicated as 96% explants survival as compare to the application of NaOCl. In comparison, application of 2% NaOCl (the highest concentration used), for 10 minutes has only achieved 60% sterilization.

Shoot bud induction:

In the present study, 150 explants have been inoculated in MS medium supplemented with different concentration of TDZ. Shoot buds were seen to emerge in all concentration of TDZ ranging from 2-10mg/L within 5-6 weeks of culture. However, the highest percentage of shoot bud induction (34%) and the number of induced shoot buds (11) per explants was achieved in medium supplemented with 8mg/L TDZ within 5 weeks of culture. The lowest percentage of shoot bud induction (8%) was observed in 6mg/L TDZ in 6 weeks of culture. As a negative control explants were cultured in medium without any plant growth regulator (PGR) showed no growth activity as shown in Table 2 and Figure 3 (B).

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Table 1: Percentage of sterilization for *in vivo* hybrid plants *J.curcas*.

Sterilizing Agent	Concentration (%)	Exposure period (min)	% of sterilization
Sodium Hypochlorite		10	0
(NaOCl)	1	15	0
		20	40
		10	60
	2	15	40
		20	40
		10	0
	3	15	60
		20	40
Mercuric Chloride		3	54
($HgCl_2$)	0.05	5	80
		7	76
		3	89
	0.1	5	96
		7	84

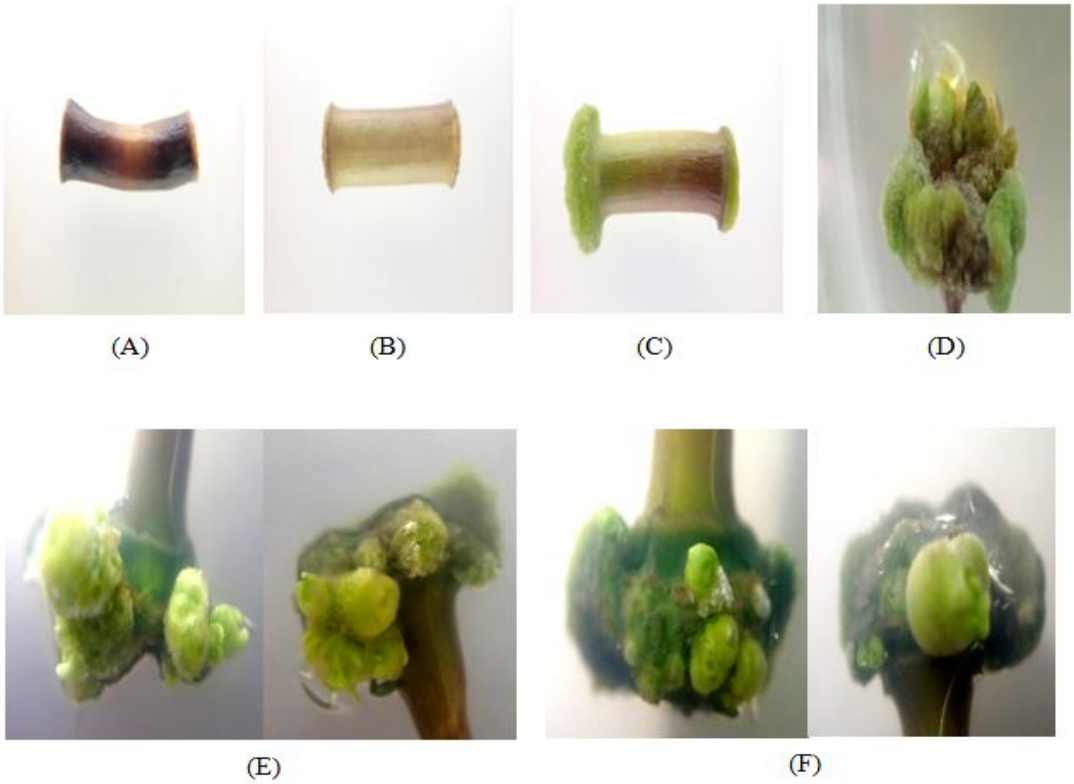

Fig. 3: Shoot bud induction (C - F), A) Explants died due to the long exposure to the EtOH B) Control without plant growth regulator (PGR) C) Swelling at the end cut of petiole explants in 2-3 weeks culture, D) Shoot induced in 4 mg/L of TDZ after 6 weeks, E) 8 mg/L of TDZ after 5 weeks of culture F) 10mg/L of TDZ after 5 weeks of culture.

Table 2: Effect of different concentration of TDZ ranging from 2-10mg/L on shoot bud induction of *J. curcas L.*

Concentration of TDZ (mg/L)	Number of shoot buds per explant			Percentage of shoot bud formation (%)	Period (weeks)
	min	max			
0	0	0		0	0
2	3	6		12	6
4	6	8		16	6
6	1	2		8	6
8	2	11		34	5
10	2	6		24	5

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3. Discussion:

Sterilization of explants material:

The efficiency and successful rate of plant tissue culture relies on several factors such as selection of sterilizing agents, exposure period, types and source of explants (Garg *et al*., 2014). One of the critical parts in this technique is to ensure the cells of explants will not lose their biological activity and any contaminants could be eliminated (Tiwari *et al*., 2012). Here in this experiment, a few steps were performed first in order to get high survival rate of explants. Explants were washed under running tap water for 30 minutes and surface sterilized using Teepol ®. According to Verma *et al*. (2011), application of systemic fungicide such as (Bavistin) will effectively controls different latent fungal contaminations under field conditions. In order to improve the effectiveness of sterilization procedure, ethanol was used prior to treatment with other compound. Ethanols (EtOH) are given short exposure to the explants materials generally only for a few minutes as this powerful sterilizing agent leads to the phytotoxicity. The exposure of explants when treated at 10-15 minutes of EtOH resulted in dying explants indicated by blackish area in the tissue of explants (Tiwari *et al*., 2012) shown in Figure 3(A). According to Rutala and Weber (2008), the application of 70% ethanol leads to the protein denaturation on the bacteria.

In this present work, source of explants (petiole) were collected from field environment. Plants growing on the field environment are invariably contaminated with microorganisms and dust generally confined to the outer surface of the plant. Explants were exposed with different concentration of sterilizing agent which were NaOCl (1%, 2% and 3%) for 10,15 and 20 minutes and $HgCl_2$ (0.05%, 0.1%) for different exposure 3,5 and 7 min to attain the best results for standardization of sterilization procedure of *J.curcas L*. From our results obtained in Table 1, the application of NaOCl for explants of *J.curcas* L was seem not as effective as compare to the used of $HgCl_2$. This was might be due to the source of explants which was collected from field. Eventough NaOCl was a common sterilizing agent, the effectiveness of this sterilizing agent was limited just for explants collected from *in vitro* plants or greenhouse (Kaewpoo and Te-Chato, 2009; Badoni and Chouhan, 2010; Sen *et al*., 2013; Danso *et al*., 2011; Elnour, 2014).

Maximum percentage of sterilization was obtained in treatment with 0.1% $HgCl_2$ for 5 minutes with 96% successful rate. Behera *et al*. (2014) found similar procedure by using 0.1% $HgCl_2$ for 5 minutes was effective for sterilization of explants in *J. curcas L*. Several works on *J. curcas L* also found that similar concentration of 0.1% $HgCl_2$ was suitable for sterilization in *J.curcas L* (Kumar *et al*., 2011; Garg *et al*., 2014). For other plant species *A. malaccensis* taken from natural sources (Daud *et al*., 2012), the same protocols for sterilization were seems to work effectively. The role of mercuric chloride as one of the sterilizing agents was known since 1970, where Gould and Sale found that mercuric chloride work as sterilizing agent by means of inhibiting germination induced by hydrostatic pressure.

Induction of shoot bud:

In the present work, hybrid of *J.curcas* (P1 X P3) was chosen for source of explants by the reason of their good properties of highest positive specific combining ability (SCA) especially in seed yield per plant and their possession of better parent heterosis (Islam, 2009). Micropropagation to produce true-to-type planting material in a large scale for this *J. curcas* hybrid was being carried out and therefore the first step on the shoot bud induction was being highlighted in this paper. Petioles were chosen as the explants materials in this study because there are generous amount of sample that can be obtain from mother plant as compared with other explants such as shoot and nodal explants in Jatropha species (Kaewpoo and Te-Chato, 2009).

TDZ has been applied for shoot bud induction of *J.curcas L*, because TDZ was known as the most active cytokinin-like substance where most woody species positively response to TDZ (Huetteman, 1993). However, the application of TDZ on shoot formation is getting well known as there are also reported in a wide variety of non woody plant species (Qu *et al*., 2002; Gubbuk and Pekmezzi, 2006). Our results have indicated that, 8 mg/L TDZ gives higher regeneration frequency (34%) as compare to other concentration. These results suggest that hybrid plants of *J. curcas L* was suitable to be regenerated through micropropagation technique using the same TDZ concentration. TDZ alone was enough to induce shoot bud in petiole explants of *J.curcas L* (Kumar *et al*., 2010; Kumar *et al*., 2011; Sharma *et al*., 2011; Kumar and Reddy, 2012). Deore and Johnson, (2008) reported that high frequency of multiple adventitious shoot buds was emerged from leaf disc culture from medium supplemented with cytokinin like substance TDZ. Our result further support the role of TDZ in shoot bud induction of *J.curcas L* from petioles explants.

The mechanism on the mode of action of TDZ in shoot bud induction was studied by Jones *et al*. (2007), where the TDZ-induced plants was reported to have increased in concentration of endogenous auxin. This was further supported by Muller and Leyser, (2011) whose claimed that the regulation of both hormone auxin and cytokinin might be upregulating the auxin synthesis during shoot bud activation. Eventough, results obtained in our study showed better shoot bud percentage with high concentration of TDZ (8mg/L) compare to previous study which used lower amount of TDZ (Kumar *et al*., 2011), this may be due to the fact that genotype of explants may influence the results obtained. Genotype is one of the factors that affect regeneration frequency in

J.curcas L (Sharma *et al*., 2011). However, period taken for the shoot bud to induce was seen similar with previous study Kumar and Reddy (2012) whereby shoot bud was emerged within 5-6 weeks of culture.

4. Conclusion:

As the conclusion, one stage of the micropropagation protocol which was the induction of shoot bud of hybrid *J. curcas L* from petioles was established. These procedures also reveal that the establishment of sterilization technique using 0.1% Mercuric chloride for 5 minutes was suitable to be applied for the field source explants from plantation of UKM Biodiesel Kuala Pilah. The average period taken for shoot bud to appear from the explants was 5 to 6 weeks after culture. Eventually, these protocol could be subsequently used in mass propagation of other hybrid plants to produce true to type plantlets through micropropagation.

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