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IN VITRO CULTURE ESTABLISHMENT AND SHOOT PROLIFERATION OF *Jatropha curcas* L

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ABSTRACT

***Jatropha curcas* seeds (immature, mature, fully mature) were collected and surface sterilized using different concentrations of Clorox (5.25% sodium hypochlorite –NaOCl) with different exposure times followed by dipping in 70% and 100% ethanol for two minutes and flaming. Surface sterilized seeds were inoculated on Murashige and Skoog, McCown's Woody Plant and B5 media with and without incorporating activated charcoal at 1 gL^{-1}. Shoot tips were excised from *in vitro* germinated seedlings and cultured on B5 medium containing different combinations of benzylaminopurine, kinetin and naphthaleneacetic acid. Experiments were designed according to a Factorial Completely Randomized Design and replicated 20 times. Data were analyzed using SAS computer software. Surface sterilization of seeds in 100% Clorox for 30 minutes followed by dipping in 100% ethanol for two minutes and flaming over the blue flame of a Bunsen burner recorded 100% aseptic cultures. Mature seeds in B5 medium showed early seed germination and best seedling growth than immature and fully mature seeds in B5, MS and WPM media. Shoots proliferated on B5 medium supplemented with 1 mgL^{-1} benzylaminopurine, 1 mgL^{-1} Kinetin and 2 mgL^{-1} naphthaleneacetic acid resulted the highest number of shoots (6.6 shoots per explant). Supplementing B5 medium with activated charcoal at 1 gL^{-1} did not have a significant effect on seed germination and seedling growth.**

***Key words**:* Biofuel, Establishment medium, Micropropagation, Proliferation

INTRODUCTION

There is a growing interest on biofuel as an alternative for fossil fuel due to ever increasing price. Biofuel production using food crops has now been shifted to non-food crops due to the world-wide food crisis. Jatropha is an economically viable, non -food biofuel crop, which can be grown in a wide range of climatic and soil conditions, and can easily be intercropped with a wide range of crop/tree species. With the scarcity of seeds and limitations in vegetative cuttings, there is a demand for large-scale production of high-health planting material having high yield and high oil content. This study was aimed at developing a micropropagation protocol for jatropha. The specific objectives of the study were to identify the correct maturity stage of seeds for culture establishment, and to optimise surface sterilization procedure and *in vitro* establishment medium to achieve high germination percentage followed by *in vitro* shoot proliferation of *J. curcas*.

METHODOLOGY

The research was conducted at the tissue culture laboratory, Department of Crop Science, Faculty of Agriculture, University of Ruhuna during the period of August 2008 to March 2009.

Surface sterilization

Fruits belonging to three maturity stages (immature - green/yellow, mature - yellow/brown and fully mature- brown) of *Jatropha curcas* were collected from trees growing in natural habitats for all the experiments. For surface sterilization, seeds were separated from fully mature fruits. Whole fruits were used for mature and immature stages of fruit as it was difficult to remove seeds. The seeds/fruits were washed under running tap water for half an hour, then dipped in 25%, 50%, 75% or 100% Clorox (5.25% sodium hypochlorite –NaOCl) with different exposure times (10, 20 and 30 min) followed by dipping in 70% and 100% ethanol for two minutes and subsequent flaming over a Bunsen burner. After dipping in 100% ethanol fully mature seeds, immature and mature fruits were burnt over a flame inside the lamina flow cabinet. Number of contaminations was counted once a week up to one month and the experiment was repeated five times. Results were based on average of five means.

Culture establishment

After surface sterilization, immature seeds were cultured without removing the seed coat. The seed coat of mature and fully mature seeds were removed and cultured on MS (Murashige and Skoog 1962), WPM: McCown's Woody Plant (Lloyd and McCown 1980) and B5 (Gamberg *et al.*1968) media with and without 1 gL^{-1} activated charcoal. Germination and survival counts were recorded once a

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week up to a month. The experiment was repeated twice and results were based on treatment means of two experiments.

***In vitro* shoot proliferation**

Shoots were excised from *in vitro* germinated seedlings and cultured on B5 media supplemented with combinations of benzylaminopurine (BA - 1, 2 and 3 mgL^{-1}), Kinetin (Kin - 0 and 1 mgL^{-1}) and naphthaleneacetic acid (NAA - 1 and 2 mgL^{-1}). Number of shoots generated per each initial shoot culture was recorded periodically for three months period.

Statistical Analysis

All experiments were designed according to Factorial Completely Randomized Design (CRD) and each treatment was replicated 20 times. Analysis of Variance was performed using SAS computer software.

RESULTS AND DISCUSSION

Surface sterilization

When immature and mature fruits and fully mature seeds were treated for 30 min with 100% Clorox followed by 100% ethanol for 2 minutes and flaming, there was no contamination detected. Contamination percentages increased with decreasing levels of Clorox (25%, 50% and 75%) for all the maturity stages (Fig. 1). Treatments which were not flamed after dipping in 100% ethanol were all contaminated (data not presented).

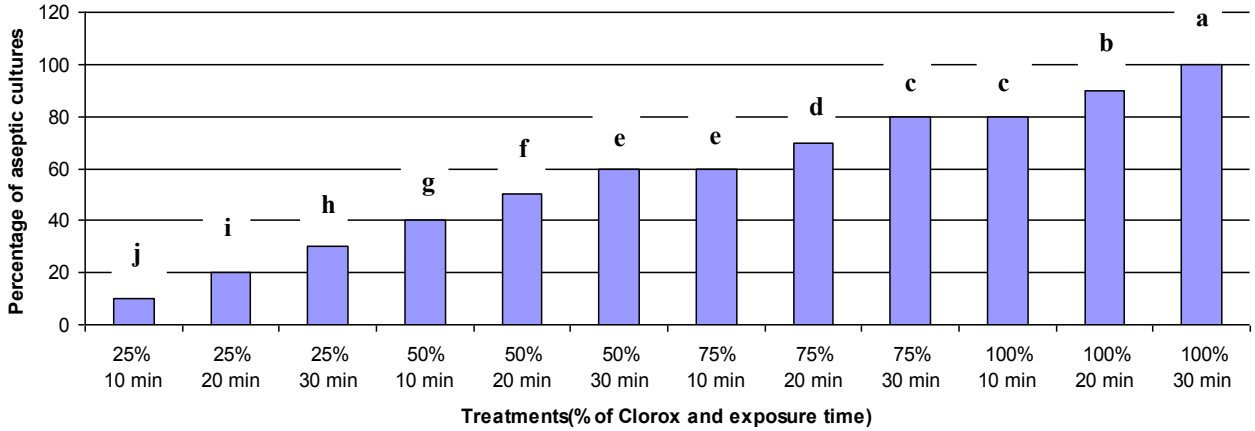
Immature pods, mature pods and fully mature seeds showed same pattern when considering the percentages of aseptic cultures resulted due to different concentrations of Clorox and exposure times used for surface sterilization (Fig. 2). Therefore 100% Clorox with 30 minutes exposure time followed by dipping in 100% ethanol for 2 minutes followed by flaming over a burner was the best surface sterilization procedure for *J. curcas*.

When surface sterilizing plant parts it is necessary to disinfect tissues with a minimum amount of cellular damage to the host tissue (Conger 1987). NaOCl is the most common chemical agent used to sterilize plant tissues which is more effective and less expensive (Prasad 1999). Some woody tissues such as buds, twigs and seeds were cleaned by immersing them briefly in a 70 % ethanol solution (Conger 1987). The results of the current study proved the necessity of both Clorox and ethanol for surface sterilization to avoid contaminations during *in vitro* culture establishment of *J. curcas*.

For surface sterilization of jatropha seeds, Qin *et al*. 2004 used 70% ethanol for 1 minute after removing seed coats, followed by 0.15% $HgCl_2$ for 25 minutes. In another study seeds were surface sterilized using 70% ethanol for 3 minutes followed by 0.12% $HgCl_2$ for 10 minutes (Kalimuthu *et al*. 2007). However, in the current study $HgCl_2$ was not used due to high toxicity of it for humans.

Culture establishment

Maturity stage of the seeds and the basal media had a significant effect on seed germination and subsequent growth of seedlings ($P \leq 0.05$). Mature seeds showed 100% germination and they grew well on B5 medium while immature seeds showed abnormal growth with subsequent seedling death. Some seedlings derived from fully mature seeds were contaminated due to fungal infections after 2 weeks from culture establishment (Table 1). The success in cell, tissue and organ culture technology is related to the selection or development of the culture medium and the decision on using type of media for the metabolic needs of the cultured cells and tissue (Kumar 2001). In some studies embryos were excised from surface sterilized seeds to establish cultures on MS medium (Qin *et al*. 2004 and Kali-



CV= 8.68 LSD= 5. 79

Figure 1: Percentages of aseptic cultures as affected by different Clorox concentrations and exposure time followed by flaming over a burner (after 4 weeks)

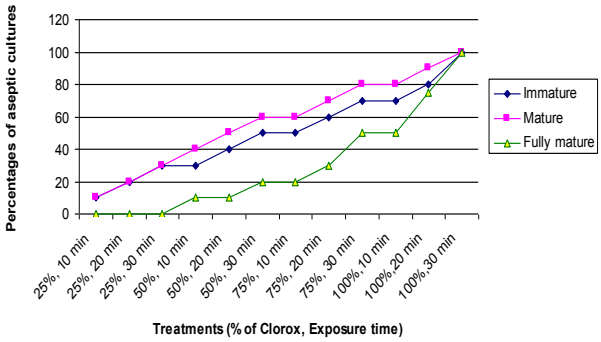


Figure 2: Effect of surface sterilization methods on aseptic culture establishment of *J. curcas* seeds (fully mature stage) and fruits (mature and immature stages) after 4 weeks

muthu *et al*. 2007). However, the maturity stage of the seeds and the germination percentages achieved with MS medium were not reported.

In the present study, among the three media tested, B5 was the best for seed germination of *J. curcas* and better results were achieved using mature seeds compared to immature and fully mature stages.

Activated charcoal acts as an absorbent in tissue culture media (Razdan 2003). However, in the present study, there was no significant effect of supplementing culture media with activated charcoal on seed germination and subsequent growth of jatropha (Table 1).

Table 1: Effect of maturity stage and culture medium on seed germination and seedling growth

Maturity stage of seeds	Medium	Activated charcoal	Germination percentage ± SD	Survival percentage ± SD
Immature	MS	Present	77.5^{d}±5.00	37.5^{f}±5.0
Immature	MS	Absent	77.5^{d}±5.00	37.5^{f}±5.0
Immature	WPM	Present	70.0^{e}±0.0	35.0^{f}±5.7
Immature	WPM	Absent	70.0^{e}±0.0	40^{e}±0.0
Immature	B5	Present	95.0^{b}±5.7	50.0^{d}±0.0
Immature	B5	Absent	92.0^{b}±5.0	50.0^{d}±0.0
Mature	MS	Present	70.0^{e}±0.0	70.0^{b}±0.0
Mature	MS	Absent	70.0^{e}±0.0	70.0^{b}±0.0
Mature	WPM	Present	60.0^{f}±0.0	60.0^{c}±0.0
Mature	WPM	Absent	60.0^{f}±0.0	60.0^{c}±0.0
Mature	B5	Present	100.00^{a}±0.0	100.0^{a}±0.0
Mature	B5	Absent	100.00^{a}±0.0	100.0^{a}±0.0
Fully mature	MS	Present	57.5^{g}±5.0	45.0^{d}±5.7
Fully mature	MS	Absent	57.5^{g}±5.0	45.0^{d}±5.7
Fully mature	WPM	Present	50.0^{h}±0.0	47.5^{d}±0.0
Fully mature	WPM	Absent	50.0^{h}±0.0	47.5^{d}±0.0
Fully mature	B5	Present	80.0^{c}±0.0	60.0^{c}±0.0
Fully mature	B5	Absent	80.0^{c}±0.0	60.0^{c}±0.0
			CV= 3.79	**CV= 5.58**

Means in the columns with the same superscript are not significantly different at P≤ 0.05

In vitro shoot proliferation

Growth regulators must be added selectively to culture media. The type of growth regulators and concentrations used vary according to the culture purpose. There are several types of plant growth regulators, each having a well defined effect on growth and development (Kumar 2001). Qin *et al*. (2004) reported that 0.1 mgL^{-1} IBA and 0.5 mgL^{-1} BA in MS medium can achieve highest shoot regeneration frequency from epicotyl explants of jatropha. Kalimuthu *et al*. (2007) reported that 1.5 mgL^{-1} BA, 0.5 mgL^{-1} Kin and 0.1mgL^{-1} IAA in MS medium can achieve highest proliferation (30- 40 shoots per explant) rates within 30- 40 days when nodal explants of jatropha were used. Therefore, during the current study, the effect of BA, Kin and NAA in B5 medium on shoot proliferation was evaluated.

Significantly (P≤ 0.05) higher mean number of shoots was achieved using 1mgL^{-1} each of BA and Kin with NAA (1 or 2 mgL^{-1}) and 1 mgL^{-1} each of Kin and NAA with 2 mgL^{-1} of BA in B5 media (Table 2). Presence of Kin is important to enhance shoot proliferation while increased levels (2 and 3mgL^{-1}) of BA inhibited shoot proliferation (Table 2). To further optimize the shoot proliferation, we suggest testing other combinations of Kin and BA.

There was no significant effect of NAA level on shoot proliferation (Table 02). Therefore, the effect of other auxins (IAA and IBA) and lower concentrations of NAA on shoot proliferation need to be further examined. It is important to incorporate above growth regulators in other basal media

Table 2: Effect of BA, Kin and NAA concentrations on shoot proliferation of *J. curcas* in B5 medium

BAP concentration (mgL^{-1})	Kinetin concentration (mgL^{-1})	NAA concentration (mgL^{-1})	No of Shoots ± SD
1	1	1	6.5^{a}±0.5
1	1	2	6.6^{a}±0.8
1	0	1	4.4^{d}±0.5
1	0	2	4.8^{c}±0.8
2	1	1	6.4^{a}±0.5
2	1	2	4.3^{d}±0.4
2	0	1	3.9^{d}±0.6
2	0	2	4.1^{d}±0.8
3	1	1	5.6^{b}±0.4
3	1	2	5.0^{c}±0.0
3	0	1	3.4^{e}±0.5
3	0	2	3.4^{e}±0.5
			CV= 12.38

Means ± standard error in the column with the same superscript are not significantly different at P≤ 0.05

(MS and WPM) to study the effect of medium on shoot multiplication as well. Above will help to increase the shoot proliferation rate of *in vitro* cultures, as the rates recorded in the current study are lower than those reported by Kalimuthu *et al*. (2007).

CONCLUSION

Mature seeds of *Jatropha curcas* can be successfully surface sterilized using 100% Clorox with an exposure time of 30 minutes followed by dipping in 100% ethanol for 2 minutes and flaming. They can be germinated successfully *in vitro* in B5 medium solidified with agar. Incorporation of activated charcoal at 1 gL^{-1} into the B5 medium did not have a significant effect on seed germination and seedling growth. Shoots excised from *in vitro* grown seedlings can be proliferated in B5 medium with 1 mgL^{-1} BA, 1 mgL^{-1} Kin and 2 mgL^{-1} NAA to generate 6.6 shoots per explant. Future experiments should be focused on shoot proliferation ability in few sub cultures, rooting of shoots and hardening before commercialization of the technology.

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