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Surface Sterilization Protocol for *In Vitro* Propagation of *Artocarpus heterophyllus* Lam.

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Abstract The present study investigated various surface sterilization techniques for *in vitro* propagation using different explants of *Artocarpus heterophyllus* Lam. grown in West Garo Hills, Meghalaya, India under open and natural environmental conditions. Sterilizing agents viz. ethanol, mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) were tested for sterilization at different concentrations and time of exposure. Five explants viz. auxiliary bud, shoot tips, nodal segments, internodal segments and leaf discs were taken for the study. Observation on contamination, browning and survivability were recorded daily till 30 days after inoculation. Highest survivability of auxiliary bud (50%) and shoot tips (66.6%) were achieved when sterilization was done with 70% ethanol for 1 minute, followed by 0.1% $HgCl_2$ for 5 minutes. Sterilization with 70% ethanol for 1 minute followed by 0.2% $HgCl_2$ for 3 minutes achieved 50% green and alive explants for both nodal segments and internodal segments. In case of leaf explants, treatment with 70% ethanol for 30 seconds followed by 10% NaOCl for 5 min produced 83.3% of green and alive explants. Treatment with either extreme high concentration of $HgCl_2$ or extreme low concentration of NaOCl was not found to be suitable in controlling contamination of fungal, bacterial and browning of the explants.

Keywords *Artocarpus heterophyllus,* Jackfruit, Surface sterilization, *In vitro* propagation.

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Introduction

Jackfruit (*Artocarpus heterophyllus* Lam.) grows well in areas receiving annual rainfall more than 1, 200 mm and is widely grown in eastern and southern regions of India. The agro-climatic conditions prevailing in low hills and plain areas of India as a whole offer splendid scope for successful cultivation of jackfruit. Garo Hills of Meghalaya falls under tropical and sub-tropical climatic zone and hence, is bestowed with biodiversity and natural resources of various flora and fauna. Jackfruit trees are abundantly grown in almost every household of Garo Hills. Scientific cultivation of jackfruits and scientific food processing and preservation center have enormous potential in this region. However, more than 80% of jackfruits are wasted or rotting under the trees of Garo Hills region due to lack of awareness with regard to value added products and unorganized marketing channel. In spite of having several jackfruit trees growing in the region, the pro-

duction and quality is not assured. Non-availability of quality fruit with uniform characters at one collection point discourages the traders and hence most of the produce is wasted. The heterogeneity of the jackfruit trees is primarily due the fact that the jackfruit trees of Garo Hills are mostly seedling progeny and hence they are not true-to-type. This issue can be addressed by mass propagation through plant tissue culture technique of elite jackfruit germplasm by selecting somatic or vegetative explants. The selection and production may be made according to the demand from the processing units and market to ensure steady marketing and/or consumption. Hence, this may have positive impact on improvement of livelihood opportunities of rural communities of Garo Hills. But the most important, critical and challenging step for successful plant tissue culture technique is to make the explants free from microbial contamination. An average contamination rates ranging between 3-5% at every subculture under *in vitro* conditions results in the wastage of time, effort and materials, which have serious economic impact on *in vitro* propagation (Srivastava et al. 2010). Successful work using 0.1% of $HgCl_2$ for shoot tips and nodal segments of *A. heterophyllus* has been reported by Azam et al. (2009) Ashrafuzzaman et al. (2012) and Harb et al. (2015). However, standard sterlization protocol of many other explants of *A. heterophyllus* is yet to be studied. Therefore, a study was carried out to evaluate the efficiency of different surface sterilants on various explants of jackfruit.

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Materials and Methods

The explants used for the present study i.e., fresh auxiliary buds, shoots tips, nodal segments, internodal segments and young leaves were collected from 10 years old mature field grown *Artocarpus heterophyllus* tree growing in Tura, Meghalaya, India 25.5257°N longitude and 90.2106° Elatitude).

The collected explant materials were brought to the laboratory and washed thoroughly with running tap water for 20-30 minutes. These were then brushed with soft brush to remove adhering dirt followed by washing with 0.1% liquid detergent (Teepol, qualigens, India) for 1-2 minutes followed by several wash with double distilled water. The explant material were then excised with the help of sterilized scalpel and forceps and transferred to the laminar air flow cabinet for further aseptic sterilization. These were then surface sterilized with different concentrations of sterilizing agents as follows.

Treatment with ethanol

The excized explants were dipped in 70% ethanol (v/v) in different timings (1 minute for auxiliary buds, shoot tips, nodal segments and internodal segments and 30 seconds for leaf explants). After pre-treatment with ethanol, the explants were rinsed with double distilled water 4-5 times to lower the toxic effect of ethanol.

Treatment with mercuric chloride ($HgCl_2$)

Each explants like auxiliary buds, shoots tips, nodal segments, internodal segments and leaf discs were sterilized with $HgCl_2$ (w/v) solution at a concentrations of 0.05%, 0.1%, 0.2% and 0.3%, to identify the best results for survival. The explants immerzed in the $HgCl_2$ solution were sterilized maintaining three different timings i. e., 3, 5 and 10 minutes. These were then washed in double distilled water 3-4 times to remove the sterilant. Thereafter, the basal ends of sterilized explants were trimmed before inoculating in the MS medium. The percentage of contamination, browning and viability were recorded.

Treatment with sodium hypochlorite (NaOCl)

Similarly, different explant materials were sterilized with NaOCl (v/v) solution at four different concentrations i.e. , 5%, 6%,8% and 10% and four different timing i.e., 5, 10, 15 and 20 minutes to find the best results of the two sterilizing agents. These were

then washed in double distilled water 3-4 times and the basal ends of explants were trimmed before inoculating in the MS medium for contamination, browning and viability studies.

Medium and culture conditions

Murashigs and Skoog (MS) basal medium (1962) was used for all the culture experiments. The required culture nutrients like macronutrients, micronutrients, vitamins and sucrose were first dissolved iin double distilled water to prepare the culture media. Thereafter, Agar (15g/l) gelling agent was added for solidifying the medium. The pH was then adjusted to 5.8 by adding 1 N HCl or 1 N NaOH and the medium was autoclaved at 121°C and 15 psi for 15 minutes. On cooling of media, the explants were cultured in tube (15× 2.5 cm) containing 10 ml and 100 ml flasks having 40 ml of MS medium. The cultures were incubated in air conditioned culture room at 25°±2°C temperature, 60% relative humidity (RH) under a photo periodic period of 16 h light and 8 h dark cycles.

Statistical analysis

Observations of cultures were recorded daily for contamination, browning and survival expressed in percentage. The experiment was laid down incompletely randomized design (CRD) with the five explants per treatment repeated six times. The data of the contaminated, browned and surviving explants were assessed at 7-30 days of culture period. Recorded data were analyzed statistically using analysis of variance technique (ANOVA).

Results and Discussion

The data Table 1 revealed that the highest percentage of survivablity (50%) of auxiliary bud of jackfruit was achieved when explants were sterilized using 70% ethanol for 1 minute followed by 0.1 $HgCl_2$ solution for 5 minutes. It was observed that sterilization with NaOCl (5 to 10%) did not show any survivability in case of auxiliary bud. Shoot tip also exhibited highest survivability (66.66%) when sterilization with 70 ethanol for 1 minute followed by

Table 1. Effect of various concentrations and exposure duration of $HgCl_2$ and NaOCl on contamination, browning and survivability of different explants of *Artocarpus heterophyllus*. CNT- Contamination, BRN-Browning, SRV-Survival.

		Exposure	Auxiliary buds			Shoot tips		
Sterilizing agents	Conc (%)	duration (in mins)	CNT (%)	BRN (%)	SRV (%)	CNT (%)	BRN (%)	SRV (%)
Control	0.0	0.0	83.3	0.00	16.6	66.6	16.6	16.6
		3	16.6	50.0	33.3	33.3	16.6	50.0
	0.05	5	33.3	33.3	33.3	16.6	50.0	33.3
		10	50.0	16.6	33.3	16.6	50.0	33.3
		3	50.0	50.0	0.00	16.6	33.3	50.0
$HgCl_2$	0.1	5	0.00	50.0	50.0	16.6	16.6	66.6
		10	16.6	66.6	16.6	16.6	50.0	33.3
		3	16.6	66.6	16.6	33.3	50.0	16.6
	0.2	5	16.6	66.6	16.6	33.3	16.6	50.0
		3	66.6	16.6	16.6	33.3	66.6	0.00
	0.3	5	16.6	66.6	16.6	33.3	66.6	0.00
		10	16.6	83.3	0.00	66.6	33.3	0.00
	5	15	66.6	33.3	0.00	83.3	16.6	0.00
		20	50.0	50.0	0.00	33.3	66.6	0.00
		10	33.3	66.6	0.00	50.0	50.0	0.00
	6	15	50.0	50.0	0.00	50.0	50.0	0.00
NaOCl		8	50.0	50.0	0.00	83.3	16.6	0.00
	8	10	66.6	33.3	0.00	33.3	66.6	0.00
		5	83.3	16.6	0.00	33.3	66.6	0.00
	10	8	33.3	66.6	0.00	66.6	33.3	0.00
		10	100.0	0.00	0.00	50.0	50.0	0.00
$CD_{0.01}$			48.7	34.5	11.7	14.2	28.1	42.8

Table 1. Continued.

Sterilizing agents	Conc (%)	Exposure duration (in mins)	Nodal segments CNT (%)	BRN (%)	SRV (%)	Internodal segments CNT (%)	BRN (%)	SRV (%)	Leaf discs CNT (%)	BRN (%)	SRV (%)
Control	0.0	0.0	83.3	0.00	16.6	100.0	0.00	0.00	100.0	0.00	0.00
		3	50.0	50.0	0.00	50.0	50.0	0.00	33.3	66.6	0.00
	0.05	5	50.0	50.0	0.00	50.0	50.0	0.00	33.3	66.6	0.00
		10	50.0	50.0	0.00	50.0	50.0	0.00	0.00	50.0	50.0
		3	83.3	0.00	16.6	33.3	50.0	16.6	33.3	50.0	16.6
$HgCl_2$	0.1	5	66.6	33.3	0.00	66.6	33.3	0.00	33.3	66.6	0.00
		10	66.6	33.3	0.00	66.6	33.3	0.00	0.00	50.0	50.0
		3	16.6	33.3	50.0	0.00	50.0	50.0	33.3	66.6	0.00
	0.2	5	50.0	50.0	0.00	0.00	66.6	33.3	66.6	33.3	0.00
		3	33.3	33.3	33.3	33.3	33.3	33.3	50.0	50.0	0.00
	0.3	5	16.6	83.3	0.00	16.6	50.0	33.3	50.0	50.0	0.00
		10	83.3	16.6	0.00	83.3	16.6	0.00	100.0	0.00	0.00
	5	15	66.6	33.3	0.00	33.3	66.6	0.00	83.3	0.00	16.6
		20	100.0	0.00	0.00	50.0	50.0	0.00	100.0	0.00	0.00
		10	50.0	16.6	33.3	50.0	33.3	16.6	100.0	0.00	0.00
	6	15	66.6	16.6	16.6	66.6	16.6	16.6	100.0	0.00	0.00
NaOCl		8	66.6	33.3	0.00	83.3	16.6	0.00	66.6	0.00	33.3
	8	10	66.6	33.3	0.00	100.0	0.00	0.00	100.0	0.00	0.00
		5	66.6	33.3	0.00	100.0	0.00	0.00	16.6	0.00	83.3
	10	8	33.3	66.6	0.00	66.6	33.3	0.00	66.6	0.00	33.3
		10	50.0	50.0	0.00	100.0	0.00	0.00	50.0	0.00	50.0
$CD_{0.01}$			35.3	20.2	13.6	18.8	17.3	11.8	21.8	12.3	27.8

0.1 $HgCl_2$ solution for 5 minutes. NaOCl showed no survivability incase of shoot tip (Table 1). Sterilization method using 70% ethanol for 2 minutes in combination with 0.2% of $HgCl_2$ for 5 minutes was reported for auxiliary buds, shoot tips, apical bud and cotyledons of *A. heterophyllus* (Zaher and Abd 2008). In case *Citrus megaloxycarpa*, sterilization of shoot tips with 70% ethanol for 4 minutes followed by 0.2% $HgCl_2$ for 7 minutes was carried out by Haripyaree et al. (2011). In the present study, we have exposed the explants in ethanol for very less time as well as in low concentration of $HgCl_2$ to prevent the explants from undesirable toxicity of tissues. With the increase in concentration and exposure time of $HgCl_2$, browning of the explants tissue was observed. Similar studies using ethanol and $HgCl_2$ for other field grown plant species have been reported in case of auxiliary buds of *Aegle marmelos* (L.) (Puhan and Rath 2012) and shoot tips of *Vitis vinifera* L. (Khan et al. 2015).

Nodal segments and intermodal segments exhibited highest survivability (50%) when sterilized with70% ethanol for 1 minute followed by 0.2% $HgCl_2$ for 3 minutes. Study on internodal segments in other species like treatment with $HgCl_2$ in *Solanum surattense* (Rahman et al. 2011) and treatment with NaOCl in *R. damascene* (Tabesh et al. 2013) have been reported. Azam et al. (2009) studied the effect 0.1% $HgCl_2$ for 5-15 minutes treatment in nodal segments of *A. heterophyllus*. Although, we have obtained in 0.2%$HgCl_2$, but the exposure time was less. Similar use of $HgCl_2$ in other plant species have been reported in *Myrica esculenta* (Sreedevi and Damodharam 2015).

The surface sterilization for leaf explants treated with 70% ethanol for 30 seconds followed by 10% NaOCl for 5 minutes resulted 83.3% of green leaf discs forthe establishment of cultures and induction for callus of *A. heterophyllus* (Table 1). In case of leaf explants, for exposure timing of ethanol was less which is desirable for young leaf explants, as they are very delicate to with stand a long exposure of treatment of sterilants and may die due to toxicity and internal tissue injury. Ndakidemi et al. (2013)

also reported that more exposure of ethanol as sterilizing agent is extremely phototoxic to tissues of leaf explants.

Treatment of $HgCl_2$ showed an adverse browning of leaf explants resulting into necrosis, whereas treatment of NaOCl has established culture of explants for leaf discs which remained alive and green after 30 days, which also initiated callus. Guma et al. (2015) studied 5% NaOCl for 10 minutes sterilization of leaf explants of *Coccinia a byssinica*. Isikala et al. (2010) obtained 10% NaOCl for 10 minutes to be the best for *Amygda luscommuni* leaf explants.

Due to cross pollination of *Artocarpus heterophyllus*, it exhibits a wide genetic variation and huge diversity within its population. Therefore, propagation of this species through seed germination never bears the fruits true-to-type of the mother plant. Moreover, the seeds of *A. heterophyllus* are re-calcitrant in nature and difficult to germinate after a short period of storage. *In vitro* propagation may be an alternative method for propagation of *A. heterophyllus* giving a homogenous type of fruits. But, *in vitro* propagation for such a woody species is a challenging task as it realizes high toxic phenolic substances resulting in browning and death of the explants. Hence, utmost care is required regarding the various factors of age, health and seasons in selecting the explants for *in vitro* propagation of *A. heterophyllus*.

In a nut shell, the current research revealed that among the different sterilants studied for various types of explants, the highest survivability of auxiliary bud (50%) and shoot tips (66.6%) were achieved when sterilization was done with 70% ethanol for 1 minute, followed by 0.1% $HgCl_2$ for 5 minutes. Sterilization with 70% ethanol for 1 minute followed by 0.2% $HgCl_2$ for 3 minutes achieved 50% green and alive explants for both nodal segments and internodal segments. In case of leaf explants, treatment with 70% ethanol for 30 seconds followed by 10% NaOCl for 5 mins produced 83.3% of green and alive explants. Treatment with either extreme high concentration of $HgCl_2$ or extreme low concentration of NaOCl was not found to be suitable in controlling contamination of fungal, bacterial and browning of the explants.

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