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Effect of Various Surface Sterilant on Contamination and Callus Regeneration of Ashoka (*Saraca asoca* Roxb. De Wilde) from Leaf Segment Explant

Sandeep Rout* and Neelam Khare

College of Forestry, Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad-211007, Uttar Pradesh, India

**Corresponding Author*

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A B S T R A C T

The present experiment was conducted to find out the most suitable surface sterilant and timing for controlling contamination in the leaf segment explants of Ashoka (*Saraca asoca* Roxb. De Wilde) using MS as basal medium with BAP (2.0 mg/l) and NAA (0.5mg/l). Among the various sterilants and timing the explants sterilized with 0.1% $HgCl_2$ for 15 min + 1% NaOCl for 2 min significantly reduced the percentage of fungal contamination (6.67%), maximum aseptic culture (93.33%). Callus percentage maximum was recorded in T_{19} (0.1% $HgCl_2$ for 15 min + 1% NaOCl for 2 min) (93.33%) and callus spread (2.33×1.00 cm) was recorded in similar treatment. *In vitro* propagation of this high valued medicinal tree is of great importance for the mass supply of disease free planting materials.

Introduction

Trees constitute an important component of forests. It is an admitted fact that tree is the invariable resources for providing food, fodder, timber and medicines items of daily life. So there is natural and anthropogenic pressure on these natural resources. Tree has been the treasure house of a wide range of valuable medicinal and aromatic plants on account of vast diversity in climatic condition. Most of them used in Ayurvedic, Unani, Siddha, Homeopathic, Allopathic and other alternative medicinal practices such as folk medicine, household remedies, naturopathy. The WHO has estimated the present demand for medicinal plants is approximately US $14 billion per year (Sharma, 2004). With time deforestation for cultivation of food, fodder, shelter and pasture diversion for the developmental purpose and removal of valuable trees affected the forest resources adversely at a greater rate making them threatened.

Ashoka (*Saraca asoca* Roxb.De Wilde) syn. *Saraca indica* belongs to family Fabaceae is a medium sized evergreen tree growing up to 9m high, with numerous spreading and drooping glabrous branches. Leaves are

pinnate, 30-60cm long having 2-3 pairs of lanceolate leaflets. Flowers are orange or orange yellow, arranged in dense corymbs and very fragrant. Fruits are flat black pods, leathery and compressed with 4-8 seed/pod. Seeds are ellipsoid oblong and compressed, The bark is dark brown to grey or black with a warty surface. The thickness varies from 5mm to 10mm. The tree is found almost throughout India, except North-Western India, Up to 750m. It is also found in the Andaman Islands.

Saraca asoca posses varied medicinal uses. The bark is useful in dyspepsia, fever, burning sensation, ulcers, leucorrhoea and pimples. The leaf juice mixed with cumin seeds are used for treating stomachalagia. The flowers are considered to be uterine tonic and dysentery. The well known Ayurvedic preparations are "Ashokarishta" is prescribed in leucorrhoea and other diseases of female (Nudrat, *et al*. 2005). Producing commercially valuable Ashoka with high levels of medicinal properties requires *Saraca asoca* trees to be a minimum of 15 years old the age at which they will be harvested *Saraca asoca* is expensive compared to other types of woods, therefore to maximize the profit of bark is harvested by removing from the tree.

In vitro propagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm. The technique has great potential for rapid and large-scale multiplication of true to type planting material (Pierik, 1989). The desire of every researcher in tissue culture studies is to eliminate or prevent contamination, but unfortunately contamination cannot be eliminated totally but can be managed to reduce both frequency and occurrence, this can be achieved by surface sterilization (Barpanda *et al.* 2017). Surface sterilization is effective and cheap for getting contamination free culture. Hence, the present investigation was undertaken to find out the effect of various surface sterilant on contamination and callus regeneration of Ashoka (*Saraca asoca* Roxb. De Wilde) from Leaf segment explants.

Materials and Methods

The investigation was carried out at the Biotechnology-cum- Tissue Culture Center, OUAT, Bhubaneswar. The chemicals used for the present study were analytical reagents of excel R grade of Merck (India), Qualigen fine Chemicals, and Himedia Laboratories Ltd. (India). Auxins, Cytokinins, Myo-inositol and Fe-EDTA were from Sigma (USA) and Agar from Himedia Lab Ltd (India). For the preparation of MS culture medium (Murashige and Skoog, 1962) required quantities of macronutrients, micronutrients, Fe-EDTA, vitamins and plant bio regulators were taken from the stock solution and required quantity of sucrose dissolved in water was added fresh to the medium. The pH of the solution was adjusted to 5.7$\pm$ 0.1 using 0.1N NaOH or 0.1 N HCl. The volume was made up to 1 liter with distilled water. Agar (0.8% w/v) was added to the medium boiled and poured to the culture tubes and plugged with non-absorbent cotton. Plugged culture tubes containing culture medium were autoclaved for the 20 minutes at 121^0C and 15 Psi pressure. The autoclaved medium was kept in a laminar air flow bench for cooling. All the glassware were dipped in the detergent solution overnight and washed under running tap water. They were rinsed with distilled water and then dried in an oven for 2hrs at 150^0C. Forceps, petridishes and scaples were thoroughly cleaned with isopropanol and wrapped with paper and kept in a clean sterilized in the autoclave at 15 psi and 121^0C for 20 minutes. The working chamber of laminar air flow cabinet was wiped with isopropanol. Filtered air (80-100 cft/min) to ensure that particles do not settle in working area was blown for 5min. The sterilized materials to be used (except living

tissue) were kept made the chamber and exposed to UV light for 30 minutes. The sterilized explants were inoculated in culture tubes containing the media. Cut ends of explants will be kept in such a way so as to have maximum contact with the medium. All the aseptic manipulations such as surface disinfection of explants, preparation and inoculation of explants and subsequent culturing were carried out in the laminar air flow cabinet. The working table of laminar airflow cabinet and spirit lamp was sterilized by swabbing with absolute alcohol. All the required materials like media, spirit lamp, matchbox, glassware etc., were transferred on to the clean laminar airflow. The UV light will switch on for half an hour to achieve aseptic environment inside the cabinet.

Explant was collected from the plus tree identified in Bhubaneswar (Khurda) Odisha. The explants were washed thoroughly in running tap water for 30 minutes, followed by tween 20 for 15 min. Further aseptic surface sterilization was carried out with 2% bavistin with constant stirring which was then rinsed out after 30 min with sterile distilled water for three times. The sterilized leaf explants were then prepared by into individuals, subjected to further surface sterilant solution as per different treatment for different timing and a control in the laminar airflow. These sterilized explants were then cultured on Murashige and Skoog (1962) medium supplemented with plant bioregulators BAP (2.0 mg/1) and NAA (0.5mg/1) with 8%(w/v) agar, 30% (w/v) sucrose replicated thrice. The observation of the following parameters was recorded after 60 days after inoculation (DAI) on Fungal %, Bacterial % contamination, death %, survival % and aseptic %. For callusing study, following observation on days to callusing, % of callusing, callus spread, colour of the callus and nature of callus recorded at 60 DAI. After inoculation, the cultures were kept, at 25 ± 2^0 C in an air conditioned room with a 16 hours photo period (3000-3200 lux) light intensity and 80% relatively humidity. All of the experiments were replicated thrice and 10 culture tubes per replication in each treatment were taken. The raw data obtained during the experimental observations were subjected to completely randomized design (Gomez and Gomez, 1984). The significance and non-significance of the treatment effect were judged with the help of 'F' variance ratio test. Calculated 'F' value was compared with the table value of 'F' at 5% level of significance. The data were transferred from where ever required before suitability of Analysis of Variance (ANOVA) analyzed in statistical package SAS version 7.0.

Results and Discussion

The results of the experiment on timing of sterilant in leaf segment explants(Table.1) revealed that the leaf segment explants surface sterilised by 0.1% $HgCl_2$ for 15 minutes+ 1% NaOCl for 2 Min. significantly reduced the percentage of fungal infection(6.67 %) and the data stood at par with the treatment T_{12} (0.1% $HgCl_2$ for 15 minutes. Significantly maximum fungal infection 100% was recorded in control i.e. T_1 and T_{13} (1% NaOCl for 5 Min).

The result of bacterial infection showed non-significant results. In treatment T_1 (control) led condition there is no bacterial infection as all the explants have contaminated due to fungal infection. However lower percentage of bacterial infection (3.33) was recorded in treatment T_9 (0.1% $HgCl_2$ for 8 minutes), T_{10} (0.1% $HgCl_2$ for 9 minutes).

Aseptic culture was significantly higher (93.33%) with T_{19} (0.1% $HgCl_2$ for 15 minutes + 1% NaOCl for 2 Min) and the data stood par with T_{12} (0.1% $HgCl_2$ for 15minutes). There was no aseptic culture as all the explants have contamination due to fungal infection in treatment T_1 (control) and T_{13}.

Table.1 Effect of surface sterilant and duration of time on leaf segment explants [MS+2.0 mg/l BAP+0.5mg/l NAA (60DAI)]

Treatment	Fungal %	Bacterial%	Death %	Aseptic %	Survival %
T_1 (Tap water) (Control)	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_2 (0.1% $HgCl_2$ 1 min)	90.00 (71.56)	0.00 (0.00)	0.00 (0.00)	10.00 (18.44)	10.00 (18.44)
T_3 (0.1% $HgCl_2$ 2 min)	80.00 (63.44)	0.00 (0.00)	0.00 (0.00)	20.00 (26.56)	20.00 (26.56)
T_4 (0.1% $HgCl_2$ 3 min)	76.67 (61.10)	0.00 (0.00)	0.00 (0.00)	23.33 (28.86)	23.33 (28.86)
T_5 (0.1% $HgCl_2$ 4 min)	76.67 (61.10)	0.00 (0.00)	0.00 (0.00)	23.33 (28.86)	23.33 (28.86)
T_6 (0.1% $HgCl_2$ 5 min)	76.67 (61.10)	0.00 (0.00)	0.00 (0.00)	23.3328.86)	23.3328.86)
T_7 (0.1% $HgCl_2$ 6 min)	66.67 (54.78)	0.00 (0.00)	0.00 (0.00)	33.33 (35.24)	33.33 (35.24)
T_8 (0.1% $HgCl_2$ 7min)	63.33 (52.71)	0.00 (0.00)	0.00 (0.00)	36.67 (37.26)	36.67 (37.26)
T_9 (0.1% $HgCl_2$ 8 min)	36.67 (37.26)	3.33 (10.47)	0.00 (0.00)	60.00 (50.77)	60.00 (50.77)
T_{10} (0.1% $HgCl_2$ 9 min)	33.33 (35.24)	3.33 (10.47)	0.00 (0.00)	63.33 (52.71)	63.33 (52.71)
T_{11} (0.1% $HgCl_2$ 10 min)	30.00 (33.21)	10.00 (18.44)	0.00 (0.00)	70.00 (56.79)	70.00 (56.79)
T_{12} (0.1% $HgCl_2$ 15 min)	10.00 (18.44)	10.00 (18.44)	0.00 (0.00)	80.00 (63.44)	80.00 (63.44)
T_{13} (1% NaOCl 5 min)	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_{14} (1% NaOCl 10 min)	90.00 (71.56)	0.00 (0.00)	0.00 (0.00)	10.00 (18.44)	10.00 (18.44)
T_{15} (1% NaOCl 15 min)	80.00 (63.44)	0.00 (0.00)	0.00 (0.00)	20.00 (26.56)	20.00 (26.56)
T_{16} (70% Propanol 5 min)	90.00 (71.56)	0.00 (0.00)	0.00 (0.00)	10.00 (18.44)	10.00 (18.44)
T_{17} (70% Propanol 10 min)	90.00 (71.56)	0.00 (0.00)	0.00 (0.00)	10.00 (18.44)	10.00 (18.44)
T_{18} (70% Propanol 15 min)	86.67 (68.57)	0.00 (0.00)	0.00 (0.00)	13.33 (21.39)	13.33 (21.39)
T_{19} (0.1 % $HgCl_2$ 15 min + 1% NaOCl 2 min)	6.67 (14.94)	0.00 (0.00)	0.00 (0.00)	93.33 (75..00)	93.33 (75..00)
SE(m)±	**2.61**	-	-	**7.12**	**7.12**
CD at 5%	**7.39**	-	-	**20.00**	**20.00**

**Value in parenthesis is arc sine value

Table.2 Impact of timing of sterilant on callus initiation and development of the leaf segments explants [MS+2.0 mg/l BAP+0.5mg /l NAA (60DAI)]

Treatments	Days to Callusing	Colour of Callus	Nature of the Callus	Callusing %	Callus Spread (cm)	
					L	**B**
T_1 (Tap water)(Control)	0.00	-	-	0.00 (0.00)	0.00	0.00
T_2 (0.1% $HgCl_2$ 1 min)	37.67	Off white	Fragile	10.00 (18.44)	0.25	0.47
T_3 (0.1% $HgCl_2$ 2 min)	38.33	Off white	Fragile	20.00 (26.56)	0.25	0.53
T_4 (0.1% $HgCl_2$ 3 min)	38.00	Off white	Fragile	23.33 (28.86)	0.33	0.50
T_5 (0.1% $HgCl_2$ 4 min)	38.00	Off white	Fragile	23.33 (28.86)	0.50	0.50
T_6 (0.1% $HgCl_2$ 5 min)	38.00	Off white	Fragile	23.3328.86)	0.57	0.47
T_7 (0.1% $HgCl_2$ 6 min)	38.00	Off white	Fragile	33.33 (35.24)	0.68	0.55
T_8 (0.1% $HgCl_2$ 7min)	42.67	Off white	Fragile	36.67 (37.26)	0.58	0.70
T_9 (0.1% $HgCl_2$ 8 min)	43.00	Off white	Fragile	60.00 (50.77)	0.72	0.85
T_{10} (0.1% $HgCl_2$ 9 min)	46.00	Off white	Fragile	63.33 (52.71)	2.92	1.00
T_{11} (0.1% $HgCl_2$ 10 min)	46.00	Off white	Fragile	70.00 (56.79)	1.65	0.95
T_{12} (0.1% $HgCl_2$ 15 min)	47.00	Off white	Slightly fragile	80.00 (63.44)	1.83	1.00
T_{13} (1% NaOCl 5 min)	0.00	Off white	Fragile	0.00 (0.00)	0.00	0.00
T_{14} (1% NaOCl 10 min)	48.67	Off white	Fragile	10.00 (18.44)	0.32	0.48
T_{15} (1% NaOCl 15 min)	49.33	Off white	Fragile	20.00 (26.56)	0.27	0.50
T_{16} (70% Propanol 5 min)	40.00	Off white	Fragile	10.00 (18.44)	0.37	0.48
T_{17} (70% Propanol 10 min)	41.33	Off white	Fragile	10.00 (18.44)	1.45	0.70
T_{18} (70% Propanol 15 min)	42.00	Off white	Fragile	13.33 (21.39)	1.47	0.75
T_{19} (0.1 % $HgCl_2$ 15 min + 1% NaOCl 2 min)	47.00	Off white	Slightly fragile	93.33 (75..00)	2.33	1.00
SE(m)±	**0.87**	**-**	**-**	**7.12**	-	-
CD at 5%	**2.48**	**-**	**-**	**20.00**	-	-

**Value in parenthesis is arc sine value

Percentage of the death of the explants was significantly low (0.00) in all treatments. There is no death of the explants due to surface sterilants in treatments. The explants surface sterilised with by 0.1% $HgCl_2$ for 15 minutes + 1% NaOCl for 2 Min recorded significantly higher percentage of survival (93.33%) followed by T_{12} (0.1% $HgCl_2$ for 15 minutes) (80.00%). The results are in close conformity with those of Beura *et al.* (2016), Ghochhayat and Beura *et al.* (2017). However mercuric chloride gave better results or in combination when compared to NaOCl and 70% Propanol. $NaOCl_2$ and 70% Propanol alone did not found acceptable sterilants even on increasing time similar findings are also reported in *Asparagus densiflorus* (Amutha *et al.* 2008). The sterilizing agent should be used for an appropriate duration to control contamination. However $HgCl_2$ which has mainly antibacterial action was more efficient and should be used for decontamination percentage (Rihan , 2012)

The data presented in table 2. revealed that explants treated with 0.1% $HgCl_2$ for 1 minute significantly reduced the days to callus initiation(37.67) remaining at par with T_4 (0.1% $HgCl_2$ for 3 minutes),T_5 (0.1% $HgCl_2$ for 4 minutes), T_6 (0.1% $HgCl_2$ for 5 minutes) and T_7 (0.1% $HgCl_2$ for 6 minutes) and callusing percentage were significantly higher (93.33 %) in T_{19} (0.1% $HgCl_2$ for 15 minutes +1% NaOCl for 2 Min) remaining at par with T_{12} (0.1% $HgCl_2$ for 15 minutes). The callus spread was higher (2.33 × 1.00) in T_{19} (0.1% $HgCl_2$ for 15 minutes +1% $NaOCl_2$ for 2 Min). Considering all the characters of the impact of sterilant, leaf segments surface sterilized with 0.1% $HgCl_2$ for 15 minutes +1% $NaOCl_2$ for 2 Min was found to be best for Ashoka (*Saraca asoca* Roxb. De Wilde). The results are in alignment with the findings of Patnaik and Beura, (2008); Gochhayat *et al.* (2017).

It was concluded that for *in vitro* propagation of Ashoka (*Saraca asoca* Roxb. De Wilde) leaf segment explants sterilized with 0.1% $HgCl_2$ for 15 min + 1% NaOCl for 2 min is more effective for getting maximum aseptic culture, survival of explants along with maximum callus growth and found to be most effective for the purpose of mass multiplication with-in short period of time.

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