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In Vitro Sterilization Protocol for Micropropagation of Musa (AAA group) 'Amritsagar' Musa (AAB group) 'Malbhog' and Musa (AAB group) 'Chenichampa' Banana

KEYWORDS: Sterilant, Micropropagation, contamination, surface sterilization and explants

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ABSTRACT *Sterilization procedure was standardized for three popular banana cultivars viz., Musa (AAA group) 'Amritsagar'; Musa (AAB group) 'Malbhog'; and Musa (AAB group) 'Chenichampa'. Two sterilants, sodium hypochlorite (0.5 % and 1.0 %) and mercuric chloride (0.1 %) were used both singly and in combination for surface sterilization of explants under different exposure time ranging from 5 to 15 minutes. Sterilized explants were inoculated on four different MS media viz., MS Basal, MS+BAP 0.2 mg L^{-1}, MS +BAP 0.3 mg L^{-1} and MS +BAP 0.5 mg L^{-1} to evaluate the response of different chemicals. The observations were recorded regularly up to 30 days with respect to the dead cultures, infected cultures and healthy cultures. Result showed that a treatment combination of Sodium hypochlorite (1.0%) for 15 minutes followed by $HgCl_2$ (0.1%) for 7 minutes gives the highest percentage of aseptic culture establishment in in vitro condition.*

INTRODUCTION

In vitro propagation technique for banana involves various steps i.e. selection of explants (suckers), its sterilization, initiation and establishment, shoot proliferation and rooting of microshoots. The first condition for the success of in vitro propagation is the getting aseptic culture. The maintenance of aseptic or sterile conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explant itself must be sterilized. The importance is to keep the air, surface and floor free of dust and it is require carrying out all the operation in laminar airflow sterile cabinet.

The use of field grown plants as a direct source of explant material for the production of 'clean' in vitro plantlets, presents a major challenge. Microbial contaminations are the major hurdle to the initiation and maintenance of viable in vitro cultures. Explant contamination occurs due to several plant and environmental related factors such as plant species, age of the plant, explant source and prevailing weather condition. Despite the best timing and selection efforts it is difficult to eliminate contamination from in vitro grown plants. Losses due to contamination in in vitro condition average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Boxus & Terzi, 1987, 1988 ; Leifert et al., 1990), the majority of which is caused by fungal, yeast and bacterial contaminants (Leifert, et al., 1994).

For in vitro culture initiation, explants are normally collected from field grown plants, so the plant material is liable to be contaminated by microorganisms which must be disinfected before explants are transferred to in vitro conditions. Variations in sterilization procedure have been proposed by several researchers. Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of banana explants (Cronauer and Krikorian 1984; Mendes et al., 1996; Muhammad et at., 2004). Some other investigators have replaced sodium hypochlorite with low concentration of mercuric chloride (Banerjee & Sharma 1988; Habiba et al., 2002; Molla et al., 2004, Titov et al., 2006).

Sterilization is the process of making explants contamination free before in vitro establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants have to be standardized to minimize the injury to the explant for achieving better survival rate. Two different chemicals i.e. sodium hypochlorite (0.5 % and 1.0 %) and Mercuric chloride (0.1%) were used for the study to standardize the best sterilization protocol for in vitro propagation of Musa cv. Amritsagar (AAA), Malbhog (AAB) and Chenichampa (AAB).

MATERIAL AND METHOD

The present study was carried out at Department of Biotechnology, Gauhati University, Assam and at The Energy and Resources Institute, Guwahati, Assam with the objective to evaluate the effect of different sterilants at different concentrations and different exposure time on banana explants for in vitro propagation.

Three banana cultivars viz., Amritsagar (AAA), Malbhog (AAB) and Chenichampa (AAB) were considered for the research programme. These cultivars have economic importance to the people of the state. Amritsagar (AAA) is good table banana cultivars and fairly resembles the internationally reputed banana Gros Michel, which once occupied 63% of the world market. Plants are medium sized, fruit size good, rind medium thick and the ripe banana develops a bright yellow colour. Malbhog (AAB) is one of the most popular table banana cultivar indigenous to Assam and has a high demand on market due to its sweet aroma, taste and higher post harvest life. It is a medium tall cultivar flowers in 18 months. Cheni Champa (AAB) is one of the hardiest medium tall banana cultivar. The plant is resistant to Fusarium wilt and fairly resistant to bunchy top disease. Fruits are small in size with thin peel, creamy pulp and sub-acid taste.

The pre treated suckers of all the three cultivars were washed under running tap water for 30 minutes to 1 hour to remove the dirt (Plate 1). Thereafter suckers were trimmed into 3-4 inched sizes (Plate 2). The trimmed explants were further treated with Savlon (Johnsons & Johnsons) for 15 minutes. Thereafter, explants containing meristem and rhizomatous base were treated with a mixture of 2 % Sodium Hypochlorite + Captan or Dithane M-45 1 g/L of water and Rifampicin (0.1%) for 45 minutes (Plate 3). Tween-20 was added as wetting agent to enhance maximum penetration of sterilizing agents. After that explants were rinsed with clean water for 4 times and a quick dip (15 sec) in 70 % alcohol was given before transferring the explants in sterile environment (Laminar Air Flow Cabinet). Explants were then dipped in double distilled water, Ascorbic Acid, Citric Acid and a solution of Ascorbic acid and citric acid 100 mg/L for 1 hour before surface sterilization and 50 mg/L Ascorbic Acid, 50 mg/L Citric

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Acid and a solution of Ascorbic acid and citric acid 100 mg/L (Table 1) for 10 minutes after sterilization and trimming further in sterile environment. After that explants were taken out from the solution and washed with sterile water and then treated with Sodium Hypochlorite solution (0.5-1.0 %) for different period of exposure (7, 10, and 15 minutes) followed by 4 times washing with sterile water (Table 2) . The treated suckers were further pealed up by removing one more scale and treated with 0.1 % $HgCl_2$ for different period of exposure (5 and 7 minutes) and washed with sterile water for 4 times. Finally the final trimming was done to a size of 2.0 cm and dipped in a sterile solution of FL Cystine (15 mg/L) for 30 minutes and then explants were directly inoculated in four different MS media viz., Bi1: MS Basal, Bi2: MS+BAP 0.2 mg L^{-1}, Bi3: MS +BAP 0.3 mg L^{-1} and Bi4: MS +BAP 0.5 mg without washing and incubated in the Plant Growth Room (PGR) at $25^0C \pm 2^0$ C with 16 hours illuminations and 8 hours dark phases. Contamination percentage at weekly interval and microshoots production at 2 weeks intervals were recorded and the contaminated cultures were discarded immediately and autoclaved at 121^0 C and 15 lbs p.s.i pressure for 1 hour.

Table 1: Different pre-treatment tested to prevent browning of explants

Pre-treatment	Before surface sterilization		After sterilization	
	Treatment	Time	Treatment	Time
PT1	dH_2O	1 hr	dH_2O	10 min
PT2	Ascorbic acid (100 mg/l)	1 hr	Ascorbic acid (50 mg/l)	10 min
PT3	Citric acid (100 mg/l)	1 hr	Citric acid (50 mg/l)	10 min
PT4	Ascorbic acid (100 mg/l) + Citric acid (100 mg/l)	1 hr	Ascorbic acid (100 mg/l) + Citric acid (100 mg/l)	10 min

Table 2: Sterilants, concentration and exposure time for sterilization of banana explant

Treatment	Sterilants	Concentration (%)	Exposure time (Min.)
T1	Sodium hypochlorite	0.5	7
T2	Sodium hypochlorite	0.5	10
T3	Sodium hypochlorite	0.5	15
T4	Sodium hypochlorite	1.0	7
T5	Sodium hypochlorite	1.0	10
T6	Sodium hypochlorite	1.0	15
T7	$HgCl_2$	0.1	5
T8	$HgCl_2$	0.1	7
T9	Sodium hypochlorite	1.0	10
	$HgCl_2$	0.1	7
T10	Sodium hypochlorite	1.0	15
	HgCl2	0.1	7

Plate 1: Pre-treatment of suckers

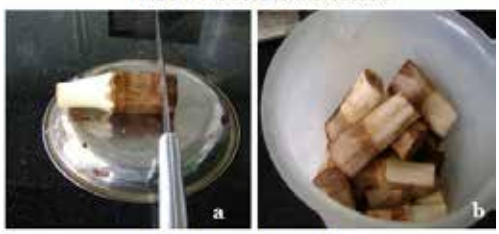

Plate 2: Preparation of explants-Trimming of explants

RESULTS

The present study was conducted to standardize the best sterilization protocol for in vitro propagation of Musa cv. Amritsagar, Malbhog and Chenichampa. Different pre-treatment methods were tested to prevent browning of explants before surface sterilization and after sterilization. Treatment combination Ascorbic acid (100 mg/l) + Citric acid (100 mg/l) for 1 hour before surface sterilization and 10 minutes after sterilization (PT4) gave the best results with regard to number of days for initial browning of explants and days required for first subculture (Table 3). For sterilization of explants two different chemicals i.e. sodium hypochlorite (0.5 % and 1.0 %) and Mercuric chloride (0.1%) were used for the present study with treatment duration of 7, 10, and 15 minutes for sodium hypochlorite and 5 and 7 minutes for Mercuric chloride respectively.

In this study, the treatment combination T10, T9, T6 and T8 for both Amritsagar and Malbhog were found to be the best combination with regard to achieving highest percentage of contamination free healthy culture, whereas in case of Chenichampa treatment combination T10, T9, T6 and T5 gave the best result following the disinfection procedure described in the materials and methods section. T10 gave the best results with regard to per cent of health culture establishment with 85, 75, and 90 per cent for Amritsagar, Malbhog and Chenichampa respectively (Table 5). Axillary buds showed cent percent viability along with the emergence of 2-3 shoots per node within four weeks of initiation (Plate 3).

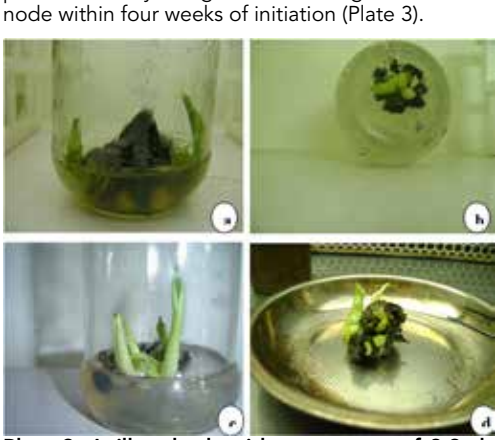

Plate 3: Axillary buds with emergence of 2-3 shoots per node within four weeks of initiation

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Effect on infection of cultures:
Result showed that with increase in exposure time the infection was decreases in both the chemicals. The infection was notably much lower in the NaOCl (1.0 %) and $HgCl_2$ (0.1%) with 15 and 7 minutes duration (T10) respectively. The single treatment either with NaOCl or $HgCl_2$ showed higher infection (Table 5, Figure 1)

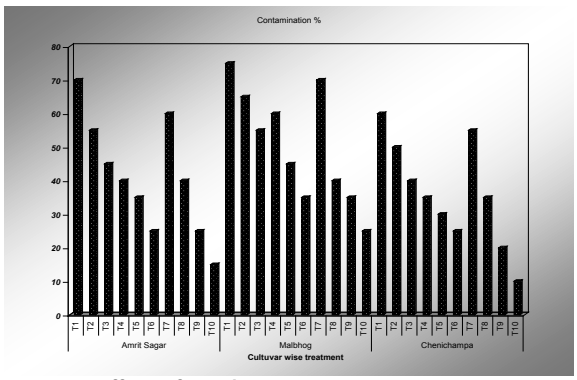

Figure 1: Effect of sterilants on contamination per cent

Effect on healthy cultures (overall survivals):
The data depicted in the table 5 indicate that with the increase in concentration and exposure duration of both the chemicals the survival rate was also increased (Figure 2). The survival obtained with 15 minute of NaOCl followed by 7 minutes of $HgCl_2$ (T10) treatment was significantly higher than all other concentration and exposure duration of both the chemicals.

Suitable sterilization chemical combination:
While comparing the effect of $HgCl_2$ and NaOCl, comparatively the NaOCl was found better than $HgCl_2$. A treatment combination of Sodium hypochlorite (1.0%) for 15 minutes followed and $HgCl_2$ (0.1%) for 7 minutes resulted the highest percentage of aseptic culture establishment in in vitro condition followed by Sodium hypochlorite (1.0%) for 10 minutes and $HgCl_2$ (0.1 %) for 7 minutes and Sodium hypochlorite (1.0%) for 15 minutes alone.

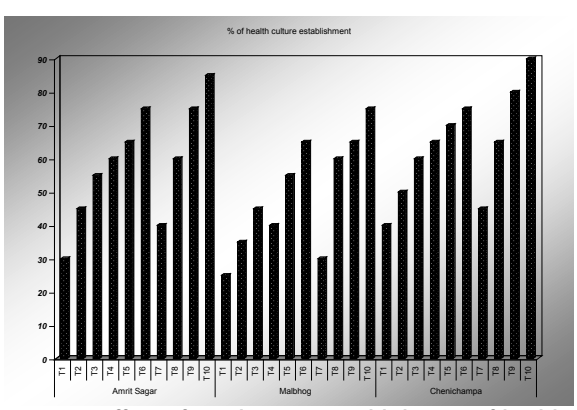

Figure 2: Effect of sterilants on establishment of healthy cultures

Table 3: Response of different antioxidant to prevent the browning of banana explants and media

Cultivar	Media	Initial browning of explants (days)	Days required for first subculture
Amritsagar	PT-1	1.67_c	3.25_d
	PT-2	2.25_c	5.58_c
	PT-3	4.92_b	6.67_b
	PT-4	6.67_a	13.08_a
	S.Ed.±	0.29	0.33
	$CD_{0.05}$	0.59	0.67
Malbhog	PT-1	2.50_d	2.67_d
	PT-2	4.08_c	4.33_c
	PT-3	5.83_b	6.25_b
	PT-4	8.25_a	12.08_a
	S.Ed.±	0.31	0.29
	$CD_{0.05}$	0.63	0.59
Chenichampa	PT-1	2.75_d	4.17_d
	PT-2	3.75_c	5.92_c
	PT-3	6.17_b	7.17_b
	PT-4	8.00_a	12.67_a
	S.Ed.±	0.28	0.35
	$CD_{0.05}$	0.56	0.71

Table 4: Effect of initiation media on initiation of banana cultivars (Amritsagar, Malbhog and Chenichampa)

Media	Amritsagar	Malbhog	Chenichampa
Bi1	23.33	35.00	40.00
Bi2	86.67	88.33	78.33
Bi3	76.67	75.00	68.33
Bi4	46.67	58.33	53.33

Table 5: Effect of surface disinfectants on per cent contamination and number of healthy cultured established in banana cultivars (Amrit Sagar, Malbhog and Chenichampa) explants

Cultivar	Treatment	No. of explants inoculated	No. of explants contaminated	No. of healthy cultures established	Contamination %	% of health culture establishment
Amrit Sagar	T1	20	14	6	70	30
	T2	20	11	9	55	45
	T3	20	9	11	45	55
	T4	20	8	12	40	60
	T5	20	7	13	35	65
	T6	20	5	15	25	75
	T7	20	12	8	60	40
	T8	20	8	12	40	60
	T9	20	5	15	25	75
	T10	20	3	17	15	85
Malbhog	T1	20	15	5	75	25
	T2	20	13	7	65	35
	T3	20	11	9	55	45
	T4	20	12	11	60	40
	T5	20	9	11	45	55
	T6	20	7	13	35	65
	T7	20	14	6	70	30
	T8	20	8	12	40	60
	T9	20	7	13	35	65
	T10	20	5	15	25	75

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Cultivar	Treatment	No. of explants inoculated	No. of explants contaminated	No. of healthy cultures established	Contamination %	% of health culture establishment
Chenichampa	T1	20	12	8	60	40
	T2	20	10	10	50	50
	T3	20	8	12	40	60
	T4	20	7	13	35	65
	T5	20	6	14	30	70
	T6	20	5	15	25	75
	T7	20	11	9	55	45
	T8	20	7	13	35	65
	T9	20	4	16	20	80
	T10	20	2	18	10	90

DISCUSSION:

The use of field grown plants as a direct source of explants for the production of 'clean' in vitro plantlets, presents a major challenge with regard to microbial contaminations during the process of initiation and maintenance of viable in vitro cultures. Losses due to contamination in in vitro condition average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Boxus & Terzi, 1987, 1988 ; Leifert et al., 1990), the majority of which is caused by fungal, yeast and bacterial contaminants (Leifert, et al., 1994).

Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of banana explants (Cronauer and Krikorian 1984; Mendes et al., 1996; Muhammad et at., 2004). Some other investigators have replaced sodium hypochlorite with low concentration of mercuric chloride (Banerjee and Sharma 1988; Habiba et al., 2002; Molla et al., 2004, Titov et al., 2006). Double disinfection method has also been applied by some researchers, where first large size explants are disinfected, followed by shoot tip excision and finally disinfection by some other chemical agents (Silva et al., 1998; Nandwani et al., 2000; Rahman et al., 2002; Madhulatha et al., 2004). Sometimes explants are treated with fungicides and antibiotics to minimize the contamination load in in vitro cultures (Van den Houwe 1998; Nandwani et al., 2000). Ethanol has also been used by a number of research workers for disinfection purposes (Silva et al., 1998; Rahman et al., 2002; Jalil et al., 2003).

Onuoha et al., (2011) achieved the contamination free Plantain culture (100%) in the explants treated with $HgCL_2$ for 6 min. Houwe et al., (1998) reported that treatment of shoot tips with Rifampicin at 100 mg. l^{-1} during 1 month resulted in 100 % bacteria free explants without any phytotoxity. Amongst the two sterilants i.e. NaOCl and $HgCl_2$, NaOCl was found better for controlling the infection and it had not any adverse effect on explant even in long duration (15 minutes). Sodium hypochlorite at higher concentration (1.0 %) has turned out to be a better sterilant than mercuric chlorite alone at 0.1 % for 5 minutes treatment time. However, a treatment combination of Sodium hypochlorite (1.0%) for 15 minutes followed and $HgCl_2$ (0.1%) for 7 minutes resulted the highest percentage (85, 75 and 90 %) of aseptic culture establishment in banana cultivars Amritsagar, Malbhog and Chenichampa respectively in in vitro condition followed by Sodium hypochlorite (1.0%) for 10 minutes and $HgCl_2$ (0.1 %) for 7 minutes and Sodium hypochlorite (1.0%) for 15 minutes alone.

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