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Standardization of surface sterilization protocols for culture establishment in banana cv. Karpurachakkarakeli(AAB) male flower buds*in vitro*

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ABSTRACT

Plant tissue culture is a proven technique for producing banana seeds in large quantities, uniformly and in a short time to support good quality banana seeds. The banana flower meristem can be a potential explant. The banana flower meristem offers the opportunity to regenerate plants with agronomic characteristics. This study aimed to regenerate banana flowers *in vitro* with different sucrose and BA (Benzyladenine) concentrations after standardized surface sterilization protocols. The study used a Completely Randomized Design (CRD), two factorial designwith surface sterilents and gelling agents. The results showed that the treatmentT_{15} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)) in G_1 (0.25% gelrite) recorded the lowest fungal and bacterial contamination (0.00, 0.00) & (0.73, 0.53) respectively, in i*n vitro* cultures of male flower buds of banana cultivar KarpuraChakkarakeli (AAB). While, the combination of BA (4 mgL^{-1}) and sucrose (30 mgL^{-1}) concentration had directly induced organogenesis in banana male flower explants.

Keywords: Male flower buds, Surface sterilents, gelling agents, BA

INTRODUCTION

The banana (*Musa* spp.) is a member of the Musaceae family. Bananas are high in carbohydrate, minerals, phosphorus, calcium, potassium, and vitamin C, and are popular because of their year-round availability, prolific production, and high customer acceptance.Latin America and Asia are the leading producers of banana.Many biotic and abiotic variables limit its production and, as a result, its export value is effected (Aquil*et al.,* 2012). Following the discovery of plant growth regulators, auxin and cytokinin, the micropropagation method has become frequently employed. The *in vitro* technique of growing "sterile" cells, tissues, or organs from an intact plant on artificial/synthetic medium is known as this approach (Pierik, 1995;Farzinebrahimi, 2012).For large-scale generation of homogeneous and aggressively developing propagules for field establishment, tissue culture techniques utilizing shoot or meristem tips are ideal. A combination of mutant breeding and *in vitro* culture has been proposed as a potential banana enhancement strategy(Novak *et al.,* 1990). Flower meristems have the potential to be used as explants. This technique is cost-effective, has fewer latent contaminants than shoots put in the soil, and allows for the regeneration of plants with specified agronomic traits and yields (Darvari*et al.,* 2010).

Contamination, on the other hand, has been described as an unstoppable issue that can stymie the development of any *in vitro*micropropagation technology (Enjalric*et al.,* 1998; Odutayo*et al.,* 2002). Viruses, bacteria, yeast, and fungi are examples of microorganisms that have been proven to be safe to plants despite introducing fungi, yeast, and bacteria into sterile plant cultures (Cassels, 1996; Omamor*et al.,* 2002) which are believed detrimental to plant cultures.Obtaining aseptic culture is the first requirement for successful *in vitro* growth. For successful tissue culture methods, aseptic or sterile conditions must be maintained. All culture jars, media, and devices used in treating tissues, as well as the explant itself must be sanitized to ensure an aseptic environment. It is critical to keep the air, surface, and floor free of dust, and this necessitates performing all operations in a sterile cabinet with laminar airflow.

The specific objectives of the present study are:

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(i) To find-out the specific concentration of surface sterilization agent for the control of the contamination during the initiation of the culture

(ii) (ii) To find-out the specific gelling agent to optimize the initiation rate along with the reduction in the contamination.

MATERIAL AND METHODS

Plant materials: The explants (male flower buds)of *Musa accuminata* cv. KarpuraChakkarakeli(AAB) were bought from healthy field. The inside surface of laminar flow was wiped by 70% ethanol and was sterilized by UV for 15 min, before explant sterilization. The outer layers of the male bud explants were excised up to 5 cm in length and placed in a beaker after being cleaned with soap and tap water. The male bud surface was bathed in flowing tap water for 30 minutes to remove the soap and other bacteria. Distilled water was used to rinse the male bud explants three times.

Medium preparation: All needed glassware, equipment and distilled water were autoclaved under 121°C for 20 min. Following the preparation of MS and plant growth regulators (PGRs) stock solutions and proper mixing, the pH of the medium was adjusted to 5.8 and autoclaved for 20 minutes at 121°C (Murashige and Skoog, 1962). For all male flower explants, MS basal medium was enriched with 30 gL^{-1} Sucrose, 1.0 gL^{-1} Active Charcoal, 7.5 gL^{-1}agar, 0.25% gelritegL^{-1}, and 500 mgL^{-1} filter sterilizedcefotaxime, and supplemented with 1.5 mgL^{-1} BAP in combination with 0.2 mgL^{-1}NAAfor culture establishment. The culture medium was autoclaved and then kept in a clean, dust-free chamber for 4-5 days before use to check for contamination.

Sterilization treatment: For sterilization, processed male flower buds are treated with different concentration of mercuric chloride and sodium hypochlorite for the different interval of time. Different combination of treatments was recorded in table 1.

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Treatment	Sterilant	Exposure time (min.)
T_1	Sodium hypochlorite (0.5%)	7
T_2	Sodium hypochlorite (0.5%)	10
T_3	Sodium hypochlorite (0.5%)	15
T_4	Sodium hypochlorite (1%)	7
T_5	Sodium hypochlorite (1%)	10
T_6	Sodium hypochlorite (1%)	15
T_7	$HgCl_2$ (0.1%)	5
T_8	$HgCl_2$ (0.1%)	7
T_9	$HgCl_2$ (0.1%)	10
T_{10}	Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%)	7
T_{11}	Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%)	10
T_{12}	Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%)	13
T_{13}	Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)	7
T_{14}	Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)	10
T_{15}	Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)	13
T_{16}	Control	no treatment

Experimental design and data analysis: A Factorial Completely Randomized Design was used to perform the trials (F-CRD). Data on the proportion of contamination-free explants that survived sterilization were analyzed using ANOVA, and significant differences across treatments were evaluated using Duncan's multiple range tests at a 5% level of significance using SAS software.

RESULT

The quantity of NaOCl, $HgCl_2$ and its interaction with gelling agents showed a significant difference ($P<0.05$) in overcoming contamination and enhancing the survival of shoot tip explants, according to an analysis of variance (ANOVA). When compared to the control, using NaOCl to sterilize explants resulted in considerably lower contamination levels and a higher

explant survival rate in culture.The goal of this study was to standardize the optimum sterilizing procedure for *Musa* cv. KarpuraChakkarakeli(AAB) *in vitro* propagation in male flower bud (Fig.1 & Fig. 2). To avoid browning of explants before and after surface sterilization, various pre-treatment techniques were explored.Maximum contamination per cent of explants exhibited i.e. fungal contamination after 10 days (3.33%) was noted under the treatment of sodium hypochlorite (1%) with exposure time of 7 when initiated on G_1(gelrite 0.25%) on the other hand maximum contamination (3.33%) on G_2(0.7% agar) was observed when the explants were treated with sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%) for 10 minutes. Interaction effect for fungal contamination (2.50%) was maximum when the explants were treated with 1% sodium hypochlorite(Table 1 @ Table 2). Maximum contamination per cent of explants displayed bacterial contamination after 10 days (3.33%) when explants treated with sodium hypochlorite (1%) + $HgCl_2$ (0.1%)for 13 minutes and initiated on G_1 (gelrite 0.25%), while maximum contamination (3.33%) wasseen when the explants does not treated with any sterilant and initiated on G_2 (0.7%)

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Fig. 1 Direct regeneration of male flowers of KarpuraChakkarakeli (AAB).**A.**Explants undergo rupture to form CLB **B**.Explants undergo swelling after 15 days. **C**Explants forming callus with small green size in G_1(0.25% gelrite)

When the explants were treated with Sodium hypochlorite (1%) + $HgCl_2$ (0.1%) and does not treated with any sterilant, the interaction effect for bacterial contamination (2.50%) was at its peak. There were no significant differences between the treatments or their interactions, according to the findingsas shown in the table 2.

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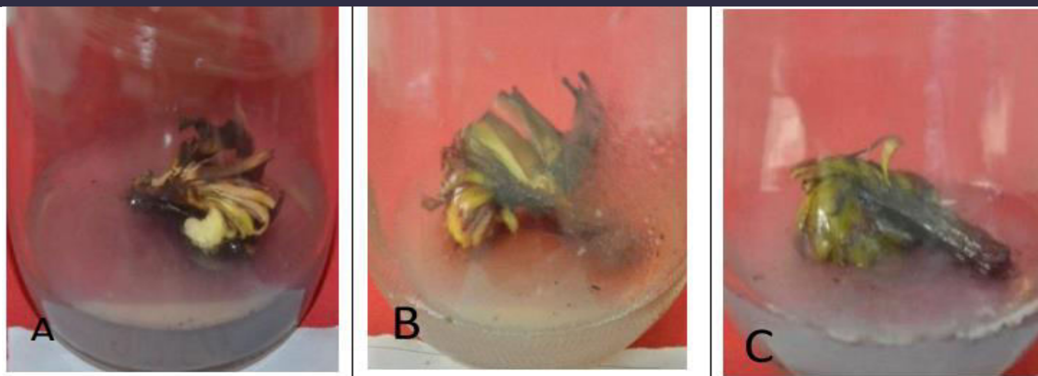

Fig. 2A. Explant death T_3(Sodium hypochlorite (0.5%) 15min **B**. T_{14} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)) 10min **C**. T_{15} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)) 15minin G_1(0.25% gelrite)in G_2(0.7% agar)

DISCUSSION

The utilization of field grown plants as a direct source of explants for the generation of 'clean' *in vitro* plantlets poses a significant microbial contamination problem during the start and maintenance of viable *in vitro* cultures (Fig.1 & Fig. 2). In the majority of commercial and scientific plant tissue culture laboratories, losses due to contamination in *in vitro* conditions average between 3 and 15% at every subculture (BoxusandTerzi, 1988; Leifert*et al*., 1990), with the majority of losses caused by fungal, yeast, and bacterial contaminants (Leifert*et al*., 19)

For surface sterilization of banana explants, sodium hypochlorite is the most widely employed disinfectant (Muhammad *et al.,* 2004). Other researchers have used a low concentration of mercuric chloride to substitute sodium hypochlorite (Habiba*et al.,* 2002; Molla*et al.,* 2004, Titov*et al.,* 2006).Some researchers have used a double disinfection approach, in which large size explants are disinfected first, followed by excision, and then disinfection with additional chemical agents (Nandwani*et al*., 2000; Rahman*et al*., 2002; Madhulatha*et al*., 2004). To reduce contamination in *in vitro* cultures, explants are sometimes treated with fungicides and antibiotics (Nandwani*et al*., 2000). Ethanol has also been utilized for disinfection by a number of researchers (Rahman*et al*., 2002; Jalil*et al*., 2003).In explants treated with $HgCl_2$ for 6 minutes, Onuoha*et al*. (2011) obtained contamination-free culture (100%).

Growth of explants during the culture period requires a relatively long time

forming callus and in some parts of the explant shows symptoms of browning. Browning symptoms in culture, if left too long it will cause explant death (Marlin and Hermansya, 2012). Organogenesis of explants in vitro occurs in two different ways, namely directly and indirectly (Titov*et al*., 2006). This research suggested that BA and sucrose's addition would regenerate explants through organogenesis directly, inducing bud formation, but explants could only grow to form cauliflower-like bodies (CLB)(Fig.1 & Fig. 2).These organs are referred to as cauliflower-like bodies. The organ turned green but had not been able to form buds. CLB formed in this study was 15% of all samples observed. Concentration of 4 mg/L BA does not induce male flowers and 98% of male flower hands turn brown in a month (Prabhath& Silva, 2014). Comparable results have been reported by Hernandez and Garcia (2008) for varieties cultivated by Musa AAA, Cavendish inflorescences that have not shown organogenesis or proliferation responses in basal media MS cytokinin deficiency. All cells or plants may not be manipulated in vitro due to differences in the ability to grow or regenerate from each cell type and plant genotype.

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Table 2. Effect of different combinations and concentrations of sterilization agent and treatment time on nature of contamination during culture establishment from immature male flower buds of banana cv. KarpuraChakkarakeli (AAB)

Treatment combinations	Exposure time (Min.)	Nature of contamination					
		Per cent of explants exhibited fungal contamination			Per cent of explants exhibited bacterial contamination		
		Gelling agents			Gelling agents		
		G_1 (gelrite 0.25%)	G_2 (0.7% agar)	MEAN	G_1 (gelrite 0.25%)	G_2 (0.7% agar)	MEAN
T_1 (Sodium hypochlorite (0.5%))	7	0.00	1.67	**0.83**	1.67	0.00	**0.83**
T_2 (Sodium hypochlorite (0.5%))	10	1.67	0.00	**0.83**	0.00	0.00	**0.00**
T_3 (Sodium hypochlorite (0.5%))	15	0.00	1.67	**0.83**	0.00	1.67	**0.83**
T_4 (Sodium hypochlorite (1%))	7	3.33	1.67	**2.50**	0.00	1.67	**0.83**
T_5 (Sodium hypochlorite (1%))	10	1.67	0.00	**1.67**	0.00	0.00	**0.00**
T_6 (Sodium hypochlorite (1%))	15	1.67	0.00	**0.83**	0.00	0.00	**0.00**
T_7 ($HgCl_2$ (0.1%)	5	0.00	1.67	**0.83**	0.00	1.67	**0.83**
T_8 ($HgCl_2$ (0.1%)	7	1.67	1.67	**1.67**	0.00	0.00	**0.00**
T_9 ($HgCl_2$ (0.1%)	10	0.00	1.67	**0.83**	1.67	0.00	**0.83**
T_{10} (Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%))	7	1.67	0.00	**1.67**	0.00	1.67	**0.83**
T_{11} (Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%))	10	0.00	3.33	**1.67**	0.00	0.00	**0.00**
T_{12} (Sodium hypochlorite (0.5%) + $HgCl_2$ (0.1%)	13	0.00	0.00	**0.00**	0.00	0.00	**0.00**
T_{13} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%))	7	1.67	0.00	**0.83**	0.00	0.00	**0.00**
T_{14} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%))	10	1.67	0.00	**1.67**	0.00	0.00	**0.00**
T_{15} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%))	13	0.00	1.67	**0.83**	3.33	1.67	**2.50**
T_{16} (Control)	no treatment	1.67	1.67	**1.67**	1.67	3.33	**2.50**
Mean		**0.73**	**1.04**		**0.52**	**0.73**	
		SE.m±	CD at 5%		SE.m±	CD at 5%	
T		0.161	NS		0.132	NS	
G		0.057	NS		0.047	NS	
T×G		0.228	NS		0.186	NS	

CONCLUSION

The results showed that the treatment T_{15} (Sodium hypochlorite (1%) + $HgCl_2$ (0.1%)) in G_1 (0.25% gelrite) recorded the lowest fungal and bacterial contamination (0.00, 0.00) & (0.73, 0.53) respectively,in i*n vitro* cultures of male flower buds of banana cultivar KarpuraChakkarakeli (AAB).While, the combination of BA (4 mgL^{-1}) and sucrose (30 mgL^{-1}) concentration had directly induced organogenesis in banana male flower explants. Growth and development of banana flower explants maximally form Cauliflower-Like Bodies.Growth and development of banana flower explants maximally form Cauliflower-Like-Bodies resembling cauliflower.. There is a need to develop climate-resilient crops to face global warming in the near future with this current innovative technology.

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