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Effective Sterilization Protocol for Micropropagation of *Musa coccinea* (*Musa SPP*)

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Abstract. In order to reduce contamination on tissue culture of *Musa* family and investigate of different methods of explant sterilization, three methods of aseptic techniques on *Musa coccinea* using male bud and sucker explants were carried out. Using of 100% Clorox for 6 minutes and two times soaking and washing in 70 and 100% ethanol for three and five minutes followed by rinsing via sterilized distilled water was showed 69% survival rate in sucker explants. However, 70% Clorox and ethanol for five and three minutes, respectively followed by rinsing and washing by sterilized distilled water was showed 99% survive of this type explants.

Keywords: In Vitro, Explant, Aseptic Technique, Musa, Sucker, Male Bud

1. Introduction

Micropropagation method is widely used after the discovery of plant growth regulators, auxin and cytokinin. This technique provides opportunities for *in vitro* propagation of higher plants [1], [2]. This method is the *in vitro* technique of growing "sterile" cells, tissues or organs from the intact plant on artificial/synthetic medium. Among the different uses [3], [4], it is a major technique for rapid multiplication of plant materials from tissue or cells of mother plants. However, contamination has been reported as unstoppable problem, which can affect development of all *in vitro* micropropagation technique [5], [6]. Some of the microorganisms include viruses, bacteria, yeast, fungi; have been shown to be harmless to the plant though they introduce fungi, yeast and bacteria into sterile plant culture [7], [4], which are considered harmful to the plant cultures. Although, aseptic conditions are usually applied but many plant cultures do not stay aseptic *in vitro* as they are contaminated. Contamination cannot be eliminated, but it can be managed to reduce both its frequency of occurrence and the seriousness of its consequences. Contaminated plants may have no visible symptoms, reduced multiplication and rooting rates, or may die [8]-[10].

There are some reports of *in vitro* propagation of Musa family via sucker and male bud as explants [11]-[13].The bacterial contamination is one of the major problems in *Musa* (*Musa* spp.) tissue culture [14]. Although, bacterial growth was observed around the explants in the media and plenty number of explants were destroyed in the culture due to endogenous bacteria. The goal of this study is investigate and compare various aseptic techniques on tissue culture of *Musa coccinea* using male bud and sucker explants on MS [15] supplemented with different plant growth regulators.

2. Material and Methods

2.1. Chemicals

Tween 20 (Cat.: 27,434-8) was purchased from Sigma-Aldrich (St. Louis, Mo), 95% ethanol was obtained from System ChemAR® while Clorox was purchased from Jusco hypermarket.

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2.2.Plant materials

The explants of *Musa coccinea* were bought from nursery (*KALIS Nursery and Landscaping*).

All needed glassware, equipment and distilled water were autoclaved under 121℃ for 20 min. The inside surface of laminar flow was wiped by 70% ethanol and was sterilized by UV for 15 min, before explant sterilization.

2.3.General procedure of sucker sterilization

The suckers were washed by soap under tap water and the outer layer was removed. To eliminating soap and soil, they put under tap water and were soaked under running tap water for 30 min. they were rinsed 3 times by distilled water.

2.3.1. Method One

The sucker explants were soaked in 100% Clorox and three drops of Tween-20 for 15 minutes under laminar flow. The explants were rinsed with sterilized distilled water for three times, followed by soaking in 70% ethanol for 5 minutes. As the final step, the suckers were rinsed by sterilized distilled water for three times, and were trimmed, cut and cultured in MS media [13].

2.3.2. Method Two

The sucker explants were soaked in 100% Clorox and three drops of Tween-20 for six minutes under laminar flow. The explants were rinsed with sterilized distilled water for three times, followed by soaking in 70% ethanol and three drops of Tween-20 for five minutes. The suckers were rinsed by sterilized distilled water for three times, and they were immersed in 100% ethanol after the outer layer of pseudostem was removed. They were rinsed by sterilized distilled water for another three times, and after trimming, they were immersed in 100% ethanol for 5 min. They were rinsed by sterilized distilled water for three times and were immersed in 70% ethanol for three min. As the final step, the suckers were rinsed by sterilized distilled water for three times, and they were trimmed, cut and cultured on MS media.

2.3.3. Method Three

The sucker explants were soaked in 100% Clorox and three drops of Tween-20 for six minutes under laminar flow. The explants were rinsed with sterilized distilled water for three times, and were soaked in 70% ethanol and three drops of Tween-20 for ten minutes. The sucker explants were rinsed by sterilized distilled water for three times then were washed by 70% Clorox for ten minutes. They were rinsed by sterilized distilled water for three times and were washed in 70% ethanol for 5 min. The sucker explants were trimmed, cut and cultured on MS media after they were rinsed by sterilized distilled water for three times.

2.4.General procedure of male bud sterilization

The male bud explants were washed by soap under tap water and the outer layers of them were removed up to 5 cm in length and were put into a beaker. To eliminate the soap and other germs from the male bud surface, they were put under running tap water and were soaked for 30 min. The male bud explants were rinsed for 3 times by distilled water.

2.4.1. Method One

The explants were soaked three minutes in 70% ethanol and were rinsed three times with sterilized distilled. The outer layers were removed until reaching the size of 1cm × 0.5cm. Finally, explants were cut into two portions and were cultured in MS media [11].

2.4.2. Method Two

The male buds were immersed in 70% Clorox containing of three drops Tween-20 for five minutes and were rinsed with sterilized distilled water for three times. This procedure was repeated and after rinsing three times by sterilized distilled water, they were immersed for three min in 70% ethanol. The male buds were rinsed with sterilized distilled water for three times and this step was repeated. The male bud explants were cut into two portions and cultured on MS media after rinsing by sterilized distilled water.

2.4.3. Method Three

The male buds were immersed in 70% Clorox containing of three drops Tween-20 for ten minutes and were rinsed with sterilized distilled water for three times. The explants were soaked in 50% Clorox for five min and were rinsed for three times by sterilized distilled water. The male buds were soaked in 10% Clorox for two min, rinsed for three times by sterilized distilled water and were immersed in 70% ethanol for five min. After three times rinsing by sterilized distilled water, they were cultured on MS media.

The culture containers were observed on the two weeks and all data were recorded and were compared. All not clear media, browning or yellowish media or explants were considered as contaminated culture.

3. Statistical Analysis

All experiments were carried out in five blocks. One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version 21. The means were compared with Duncan's multiple comparison test (DMCT) and $p < 1$ was considered to indicate statistical significance.

4. Results

Based on collected data in (Table 1), the survival rate in male bud and sucker explants after sterilization by method one were observed (13 and 34%) respectively. However, this rate was increased to (69 and 78%) in suckers and (99 and 98%) in male buds after aseptic technique was applied via method two and three (Fig. 1 and Fig. 2). According to data analysis, there are significant differences between methods two and three to method one in sucker and male bud explants. Furthermore, method two for both types of explants due to the observed results and easier procedures is suggested.

Table 1. Effect of various aseptic techniques on percentage of aseptic explants, and percentage survival of explants after 14 days of culture.

Sterilization Methods	Mean				
	Male Buds		Sucker		
	Aseptic Explants (%) (± S.E.)	Survival Explants (%) (± S.E.)	Aseptic Explants (%) (± S.E.)	Survival Explants (%) (± S.E.)	
Method 1	34.1 ±5.38	1.97 ±0.84	13.8 ±6.67	1.47 ±1.13	ns
Method 2	47.6 ±7.56 [a]	0.9 ±0.11 [a]	27.3 ±6.42 [a]	1.21 ±1.49 [a]	**
Method 3	43.6 ±6.31 [ab]	1.1 ±0.10 [ab]	18.27 ±10.67 [ac]	0.98 ±0.18 [ab]	*

ns, Non-significant; *, significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at
p<0.05 (DNMRT).

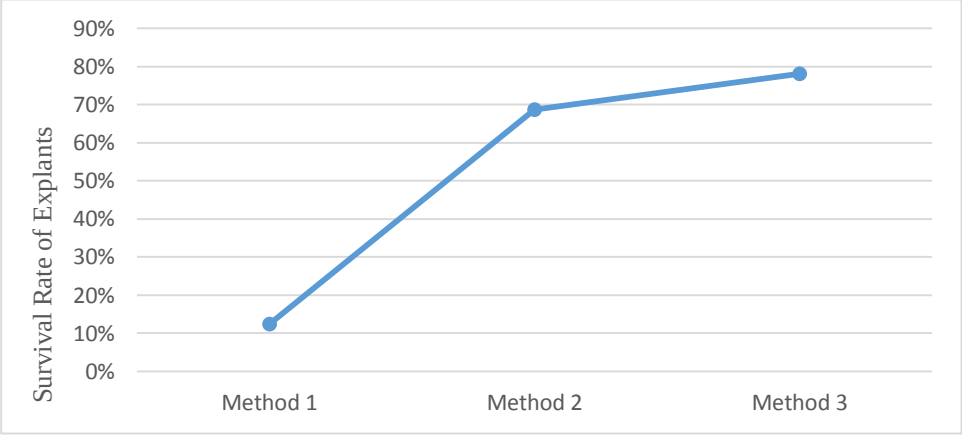

Fig. 1: Survival Rate of Sucker Explants in MS Media after 14 Days of Surface Sterilization

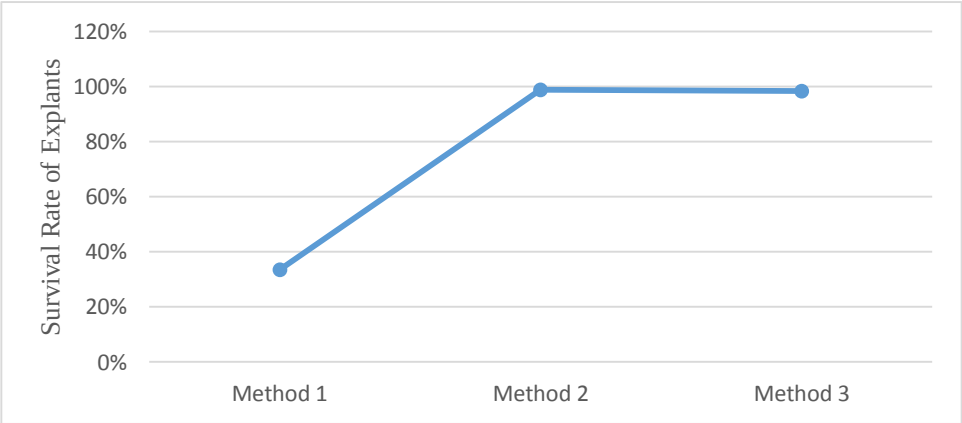

Fig. 2: Survival Rate of Male Bud Explants in MS Media after 14 Days of Surface Sterilization

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