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A Non Mercuric Shoots Bud Sterilization Technique for *Boesenbergia rotunda* (L.) Mansf. Kulturpfl.

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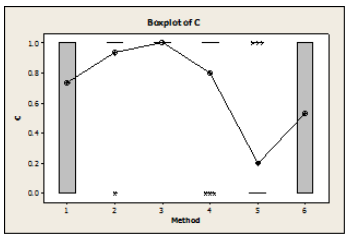
Graphical abstract



Abstract

Boesenbergia rotunda, a medicinal herb under the Zingiberaceae family, has been proven to be the most prominent anticancer remedies. The conventional breeding of this plant is inapplicable as it is susceptible to rhizome soft rot and leaf spot diseases. The yellow rhizome also produces limited buds. Therefore it is necessary to propagate this plant through *in vitro* propagation to obtain abundant uniform planting materials. Unfortunately, high cost is incurred due to the high shoot bud explants contamination level. Hence, it is addressed in the present study to solve the contamination problem. Mercuric Chloride is well known to solve this problem, but it is not advisable to use, because of its poisoning and other hazardous effects. Moreover, explant sterilization technique should also accelerate the shoot response. To find an alternative, 6 different surface sterilization methods (SSM) were designed and evaluated on the explants where different combinations of sodium hypochlorite and ethanol (instead of mercuric chloride) were applied. The sterilized shoot bud explants were then cultured on Murashige & Skoog (MS) media with no additional vitamins or plant growth regulator under the light below 25ºC. The contamination was recorded for 3 consecutive weeks along with visible shoot responses. SSM 5 showed minimum contamination and maximum visible shoot response, compared to other SSMs. Therefore, it is suggested that SSM5 could be used to conduct surface sterilization to avoid contamination problem.

Keywords: *Boesenbergia rotunda*; surface sterilization; shoot buds explants; mercury chloride; contamination

Abstrak

Boesenbergia rotunda, sejenis tumbuhan ubatan berasal daripada keluarga Zingiberaceae, telah terbukti sebagai remedi antikanser yang terkenal. Pembiakan konvensional tumbuhan ini tidak boleh diguna pakai lagi kerana ia mudah terdedah kepada penyakit reput lembut rizom dan bintik daun. Selain itu, rizom tumbuhan ini juga menghasilkan tunas yang terhad. Oleh itu, adalah perlu untuk membiak tumbuhan ini melalui pembiakan *in vitro* bagi mendapatkan bahan tanaman yang seragam dan dalam kuantiti yang besar. Malangnya, kos yang tinggi akan ditanggung akibat daripada tahap pencemaran eksplan tunas pucuk yang tinggi. Maka, usaha untuk menyelesaikan permasalahan ini adalah amat disyorkan. Penggunaan merkurik klorida dalam pensterilan eksplan telah dikenalpasti dapat membantu menyelesaikan masalah ini, tetapi atas faktor merkuri dan lain-lain kejadian yang berbahaya, ia tidak digalakkan. Selain itu, teknik pensterilan eksplan juga seharusnya mempercepatkan kadar percambahan tunas. Untuk mencari alternatif kepada penggunaan merkurik klorida ini, 6 kaedah pensterilan permukaan eksplan (SSM) yang berbeza telah dibentuk dan dinilai ke atas eksplan di mana kombinasi berlainan di antara natrium hipoklorit dan etanol (selain merkurik klorida) telah diguna pakai. Eksplan tunas pucuk yang steril telah dikultur pada media Murashige & Skoog (MS) tanpa sebarang penambahan kandungan seperti vitamin atau pengawal seliaan pertumbuhan tumbuhan, di bawah cahaya pada suhu tidak melebihi 25ºC. Tahap pencemaran kultur telah direkod selama 3 minggu berturut-turut bersama-sama tindak balas percambahan tunas. SSM 5 telah menunjukkan pencemaran minimum dan percambahan tunas yang maksimum berbanding SSMs yang lain. Oleh itu, SSM 5 digalakkan untuk diguna pakai sebagai teknik pensterilan permukaan bagi mengelakkan permasalahan pencemaran yang ketara.

Kata kunci: *Boesenbergia rotunda*; pensterilan permukaan; eksplan tunas pucuk; merkurik klorida; pencemaran

1.0 INTRODUCTION

World Health Organisation (WHO) estimates that 84 million people will die of cancer between 2005 and 2015 without intervention and cancer itself is known as the second leading cause of death. While *B. rotunda* has been proven as the most prominent promising anticancer remedy, it has attracted more than one specialist in recent years (Jing *et al.*, 2011). Large-scale multiplication was done conventionally but the lack of seed set in its family makes conventional breeding methods inapplicable (Yusuf *et al.*, 2011).

Additionally, conventional propagation allows transmission of soil borne pathogens while many of the species are susceptible to rhizome soft rot disease and leaf spot (Balachandran *et al.*, 1990; Chan, 2004). The small central rhizomes produced limited buds, making it impossible to keep relying on conventional breeding. Therefore it is necessary to develop new, simple and cost-effective method to obtain abundant uniform planting materials within relative short time (Çirak *et al.*, 2007).

To date, there are two reports on the rapid and efficient micropropagation of the *B. rotunda* through shoot derived callus and shoot bud explants (Yusuf *et al.*, 2010; Yusuf *et al.*, 2011). In many stages involved in *In-vitro* propagation, the sterilization of explants for aseptic culture establishment is the most challenging one. It is a process of making explants free from contamination before culturing, in which the contamination is caused mostly by fungal, yeast and bacterial contaminant. These microbes compete unfavorably with cultured tissue for nutrients. Consequently it leads to waste of time, effort and material which if not mitigated can have serious economic problems (Srivastava *et al*, 2010). Unfortunately, both of the above mentioned reports are aided with the usage of toxic Mercuric Chloride as its surface sterilizing agent which can lead to mercury poisoning. The aim of this study therefore was to find alternative, non-Mercuric Chloride, surface sterilization method for *In vitro* propagation of *B. rotunda* shoot bud explants.

2.0 EXPERIMENTAL

2.1 Plant Materials

B. rotunda shoots were selected as explants as it is proven the most efficient explant for its rapid propagation (Yusuf *et al.*, 2010; Yusuf *et al.*, 2011). It is also known that shoot tips and meristem tips are the most popular source of explants to initiate tissue cultures.

2.2 Experimental Procedure

Rhizome was cleaned, rinsed and placed in open container to allow shoot sprouting to 2-4 cm length. Sprouted shoots were then collected and washed under water for 30 minutes and surface sterilized with 6 different planned methods. Contact of sterilants is improved by vigorous agitation to dislodge air bubbles on explants surfaces. Ethanol and sodium hypochlorite were used for surface sterilization. An additional source of sodium hypochlorite was also used from the locally produced bleach solution containing 2.5% sodium hypochlorite in the study. The outer explants tissue layer was then removed carefully before the treated explants were placed vertically in vials containing 25 ml culture medium. MS culture media was used in the present investigation (Murashige and Skoog ,1962). The vials were then placed under the light below room temperature of 25ºC. Five replications were done on each treatment and results were observed for 3 weeks.

2.3 Surface Sterilization Methods (SSMs)

2.3.1 SSM 1

Shoot buds were initially washed with distilled water in washing vial. The buds were then soaked in 20% sodium hypochlorite for 30 seconds and 80% sodium hypochlorite solution for another 1 minute with a drop of Tween 20. The buds were rinsed with sterile distilled water after each treatment (Table 1).

2.3.2 SSM 2

Shoot buds were initially washed with distilled water. The buds were then soaked in 20% sodium hypochlorite for 30 seconds with a drop of Tween 20. Then, it was treated with 70% and 95% ethanol solution for 30 seconds and 2 minutes consecutively. The buds were rinsed with sterile distilled water after each treatment (Table 1).

2.3.3 SSM 3

Shoot buds were initially washed with sterile distilled water. The buds were then soaked in 20% sodium hypochlorite solution for 5 minutes with a drop of Tween 20. Then it is treated with 70% ethanol solution and 100% ethanol for 30 seconds and 10 minutes consecutively. The buds were rinsed with sterile distilled water after each treatment (Table 1).

2.3.4 SSM 4

Shoot buds were soaked in 20% sodium hypochlorite with a drop of Tween 20 for 20 minutes, 95% ethanol solution for 15 seconds and back again into the first treatment. Shoot buds were rinsed with sterile distilled water thrice after each treatment (Table 1).

2.3.5 SSM 5

Shoot buds were dipped into a 95% ethanol solution for 2-3 seconds and immersed in 20% and 80% sodium hypochlorite solutions with a drop of Tween 20 for 20 minutes and 10 minutes consecutively, respectively. Shoot buds were dipped into 70% ethanol solution for 2-3 seconds between each treatment. Finally, the treated buds were rinsed four times with sterile distilled water for duration of 1 minute per rinse (Table 1).

2.3.6 SSM 6

Shoot buds were immersed in 70% ethanol solution for 1 minute and 20% sodium hypochlorite for 10 minutes. Then, it was rinsed thrice with sterile distilled water before the outer layer of the shoot buds were peeled off (Table 1).

3.0 RESULTS AND DISCUSSION

Whilst medicinal herbs are moving to mainstream uses especially *B. rotunda*, large scale propagation will be necessary. As conventional method lacks in producing disease-free seedlings and seed set due to limited buds it seems impossible to rely on as described and cited by Yusuf *et al.* (2011). A micropropagation method has been developed but the possibility of cost-inefficiency and hazardous incident might occur through several contaminations and mercury toxicity. Surface sterilization seems to be the most important treatment prior to any culture initiation as ineffective sterilization will hinder

progress of the study and indirectly incur great loss. It is relatively important to have a much safer working environment if possible. Recent successful and reported culture activities of *B. rotunda* was surface sterilized by Mercuric Chloride, the finding of this study hopes for the solution of minimizing both the contamination and reducing mercury toxicity usage by using other convenient sterilants.

Previously, 46.8% clean cultures was obtained through disinfection with Sodium Hypochlorite in experiment conducted by Moutia and Dookun (1998) and later 74.3% explants survived the culture even with the aid of ethanol, Mercuric Chloride and Sodium Hypochlorite (Rai and Misra, 2003). Present study has combined several sterilizing agents' concentration which was considered effective to develop a surface sterilization method for *B. rotunda* shoot bud explants. It was understood that the role of ethanol was to kill bacteria and fungi through dehydration and often given a brief wash and longer duration would not be any harm as the tissue that will develop is within the sterilized outer surface. In the other hand, Sodium Hypochlorite main role was to kill microbes through the process of oxidizing cell. The combination of these two sterilizing agents were recommended for efficient surface sterilization by Oyebanji *et al*. (2009)

The contamination of explants were assessed by the presence of fungi, mycelium and bacterial infections symptoms in the studied vials while the percentage of contaminations were acquired through calculating the number of contaminated vials in each SSM group during the 3 weeks study periods. According to Table 2 and Figure 1, 100% contamination from Week 1 thus no longer suitable for surface sterilizing whereas SSM 2 started off with 80% contamination but reached 100% contamination by Week 2. Meanwhile, SSM 1, 4 and 6 gave 80% contamination by Week 3, while SSM 5 yielded only 20% contamination from Week 1 till Week 3 constantly. Our study has given an increment in the survival rate from surface sterilization SSM 5 of 80% compared to the previous study mentioned. Ethanol dipping given at the end of each treatment was theoretically to dehydrate as much as possible bacteria and fungi present and it is proven to be efficient in the present study.

To further explain the significance of the result obtained, both Figure 2 and 3 shows the distribution of the data set. Figure 2 illustrates SSM 3 having the highest maximum mean of 1.0 contamination number while SSM 5 has the lowest mean of 0.2 contamination number. As proven, for the best of avoiding the contamination of *B. rotunda* shoot bud explants, SSM 3 is not applicable between all of the studied methods. While Figure 3 illustrates SSM 5 as having the largest mean of about 0.6 responding, uncontaminated number and SSM 6 at the weakest, has the smaller valid mean of 0.2 responding and uncontaminated number while SSM 1, 2, 3 and 4 shows the zero possibility of neither responding nor uncontaminated results. Thus, to acquire the best and successful micropropagation of *B. rotunda* shoot bud explants, SSM 5 is applicable between all of the studied methods.

The detailed review of earlier studies reveals insufficient data on micropropagation of the fairly beneficial herb, B. *rotunda* let alone its surface sterilization which is vital where minute error leads to great loss (Srivastava *et al*, 2010). It is also reviewed that most of the sterilization process was aided by the use of rather toxic substance of Mercuric Chloride. Results of the study reveal that the method developed hold the potential to be reproduced and utilized for the efficient and safe surface sterilization of *B. rotunda* shoot bud explants and simultaneously conserving this promising anticancer remedy economically.

Table 1 Tabulated procedures of different Surface Sterilization Methods (SSMs)

STEP / METHODS	SSM1	SSM2	SSM3	SSM4	SSM5	SSM6
1	Initial rinsing with dH_2O					
2	20% NaClO (*30 seconds*)	20% NaClO + A drop of Tween 20 (*30 seconds*)	20% NaClO + A drop of Tween 20 (*5 minutes*)	20% NaClO + A drop of Tween 20 (*20 minutes*)	95% Ethanol (*2-3 seconds*)	70% Ethanol (*1 minute*)
3	dH_2O rinsing once			dH_2O rinsing 3 times	70% Ethanol (*2-3 seconds*)	20% NaClO (*10 minutes*)
4	80% NaClO (1 minute)	70% Ethanol (*30 seconds*)		95% Ethanol (*15 seconds*)	20% NaClO + A drop of Tween 20 (*20 minutes*)	dH_2O rinsing 3 times
5	dH_2O rinsing once rinse			dH_2O rinsing 3 times	70% Ethanol (*2-3 seconds*)	
6		95% Ethanol (*2 minutes*)	100% Ethanol (*10 minutes*)	20% NaClO + A drop of Tween 20 (*20 minutes*)	80% NaClO + A drop of Tween 20 (*10 minutes*)	
7		dH_2O rinsing once rinse		dH_2O rinsing 3 times	dH_2O rinsing 4 times (*1 minute per rinse*)	

Table 2 The effects of different methods on surface sterilization and shoot response

Observation period	SSM 1		SSM 2		SSM 3		SSM 4		SSM 5		SSM 6	
	C	R	C	R	C	R	C	R	C	R	C	R
Week 1	60%	0%	80%	0%	100%	0%	80%	0%	20%	0%	20%	0%
Week 2	80%	0%	100%	0%	100%	0%	80%	0%	20%	80%	60%	40%
Week 3	80%	0%	100%	0%	100%	0%	80%	0%	20%	80%	80%	20%

(C: Contaminated, R: No contamination with visible shoot response)

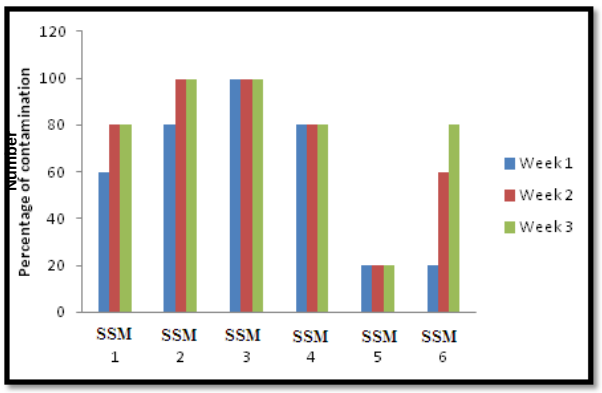


Figure 1 Percentage of contamination observed for 3 consecutive weeks

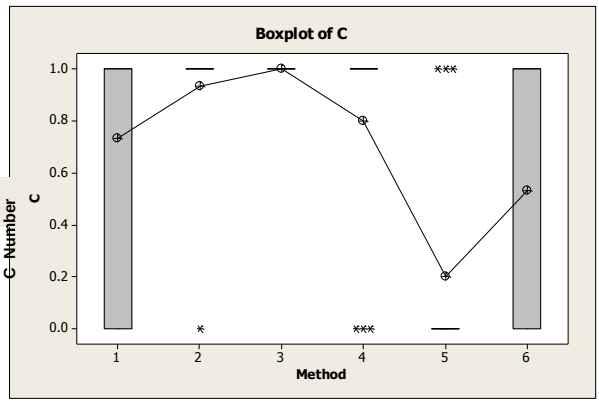


Figure 2 Boxplot of contaminated explants (C) data

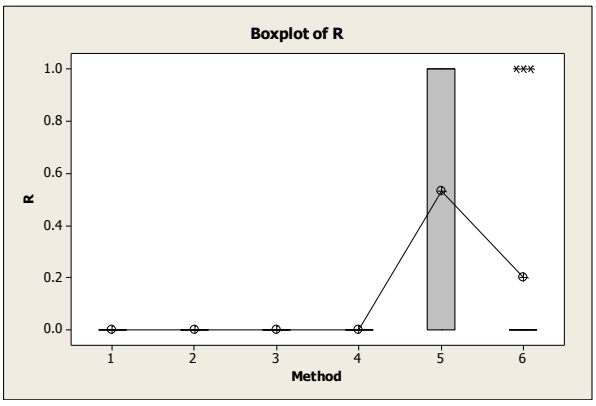


Figure 3 Boxplot of uncontaminated explants with visible shoot responses (R)

4.0 CONCLUSION

The efficacy of any explants sterilization method includes minimum contamination and maximum shoot response in plant tissue culture experiments. Optimization of a suitable surface sterilization for *B. rotunda* shoot bud explants is conducted and it is revealed that if the explants is treated with 95% and 70 % ethanol (2-3 seconds and 5-6 seconds, respectively) as well as with 20% and 80 % sodium hypochlorite solutions with a drop of Tween 20 (20 minutes and 10 minutes, respectively), minimum contamination and maximum shoot response is reported. It is therefore suggested to use this surface sterilization, instead of Mercuric Chloride, would be better in plant tissue culture.

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