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STANDARDIZATION OF SURFACE STERILIZATION PROTOCOL OF FIELD GROWN *Stevia rebaudiana* PRIOR TO *IN VITRO* CLONAL PROPAGATION

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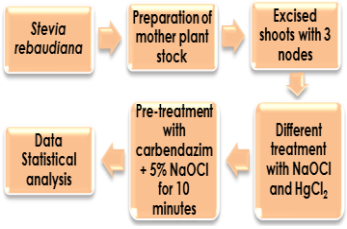

Abstract

The high demand of *Stevia rebaudiana*, commonly known as Stevia encourages people for commercial-scale cultivation. *In vitro* clonal propagation is a potential alternative for conventional propagation due to its advantages. However, microbial contamination is a constant problem for *in vitro* clonal propagation of field grown plant. Therefore, the aim of this study was to establish an effective surface sterilization protocol for Stevia prior to *in vitro* propagation. Two disinfecting agents, NaOCl and $HgCl_2$ with different concentrations and various exposure times were tested for surface sterilization of shoot tips and nodal explants. Results show that treatment of explants with NaOCl was more effective compared to $HgCl_2$ and 5% NaOCl for 10 minutes with 0.2% carbendazim (fungicide) pre-treatment showed least contamination and highest survival rate. Fungal contamination was observed to be a main problem in this study and it occurred within 2-3 days of inoculation, leading to the death of the explants.

Keywords: *In vitro* propagation, Surface sterilization, *Stevia rebaudiana*, NaOCl, $HgCl_2$

1.0 INTRODUCTION

Since dawn of civilization, medicinal plants have been considered as an integral part of human life to combat diseases. Currently, plants are highly investigated worldwide with the aim to find new pharmaceuticals as alternative remedies, that are free from side effects caused by synthetic chemicals [1]. About 75-80% of population worldwide depends on medicinal plants for the primary health care system. It is estimated that more than 80,000 of total plant species have been used for medicinal purposes, of which about 1300 plant species used traditionally in Malaysia [2]. The increasing global market demand of medicinal plants and its related products was US$ 60 billion in 2003 and is expected to be around US$ 5 trillion by 2050 [3; 4]. Therefore, there has been accelerated demand of commercially important medicinal plants and nowadays, commercial-scale production of medicinal plants is the foremost issue to meet the commercial demand.

Stevia rebaudiana Bertoni is one of the commercially important medicinal plants, commonly known as Stevia. This plant is in high demand due to its high non-caloric sweetening value with many potential medicinal properties. Stevia belongs to the Asteraceae family and is a perennial herbaceous shrub originated from Paraguay and part of Argentina and Brazil [5]. This plant, especially the leaves, produces glycosides, primarily stevioside and rebaudioside which are estimated to be 300 times sweeter than cane sugar with zero calories [6]. Stevia has been widely used as substitute for sugar in Japan for decades and also as food additives and supplements. However, in USA Stevia leaf and its extract were banned in the early 1990s, but some specific glycoside extracts (such as rebaudioside-A) were approved for use as food additives in 2008. The European Union also approved Stevia extracts as

additives in 2011 [7; 8]. Moreover, stevioside and rebaudioside A have no toxicity effects on human health, and safe for human consumption [9; 10]. Stevia leaves have superiority over other high-potency sweeteners because of its functional and sensory properties; therefore it would be a potential alternative source of high-potency sweetener for the growing natural food market in the future. For these reasons, many countries including Malaysia have shown interest in its commercial cultivation and research activities for the last two decades to fill up the shortage of sugar and to curb obesity and diabetes. Malaysians consume a lot of sugar in their daily food intake, which is harmful for human and to satisfy local demand Malaysia depends largely on imported sugar. This unhealthy diet habit may be partially responsible for the alarming rise in diabetic and obesity cases. The prevalence of diabetes increased in Malaysia to 20.8% among Malaysian adults (≥ 30 years) in 2011 [11] and the prevalence might be reduced by using Stevia instead of commercial sugar. Stevia does not affect blood glucose level [12]. Moreover, stevioside and rebaudioside A potentially stimulate the secretion of insulin and normalized blood sugar levels in experimental rats; hence Stevia may serve a potential role as treatment in type 2 diabetes mellitus [13; 14].

Considering the high medicinal and commercial value, it is necessary to grow Stevia commercially. However, Stevia is usually grown via seed but the seed germination percentage is very low and conventional propagation is limited to vegetative means which is slow and difficult in meeting the commercial quantities required. So, alternative techniques like *in vitro* clonal propagation holds potential for producing large number of plantlets within a short time. However, microbial contamination is a constant problem for *in vitro* clonal propagation. There are several factors that involved either directly or indirectly for the contamination for example the nutrient medium itself is a good growth condition for microbial growth and explant taken from field grown plant is also a major source of microorganisms. Microbial contamination adversely affects plant tissue culture leading to culture mortality, variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting [15]. Sometimes it is also impossible to remove internal contamination if the explants were taken from the field grown plants. Thus, it is crucial to establish a suitable and optimum surface sterilization technique for field grown explants of Stevia. In this study, we compared the effects of using different type of disinfectants with different concentrations and variation on exposure time with the objective to find a suitable surface sterilization technique for shoot tips and nodes of field grown *S. rebaudiana* plants.

2.0 EXPERIMENTAL

2.1 Explants Source

Healthy and juvenile shoot tips and nodes were collected from 2-3 months old field grown *Stevia rebaudiana* accession MS007. The mother plants were grown at Kulliyyah of Science, International Islamic University, Kuantan campus, Malaysia.

2.2 Surface Sterilization of Explants

Two sterilizing agents or disinfectants namely NaOCl and HgCl2, with different concentrations and various exposure time were used to surface sterilize the explants collected from the field. A total of seven different treatments (T1-T7) were chosen and the details of all treatment are shown in Table 1. For each treatment, 10 shoot tips and 30 nodes were used. All glassware, instruments and distilled water were autoclaved prior to surface sterilization of the explants. Firstly, the explants were washed with running tap water for 30 minutes and Tween 20 wash for 10 minutes (2 drops/100 ml). Next, the explants were rinsed with sterile distilled water for 6 minutes and these steps were repeated 5 times. Prior to treatments, explants were exposed to 70% ethanol for 30 seconds. The explants were then treated by the seven different treatments inside laminar air flow hood. At the end of each treatment, the explants were rinsed with sterile distilled water for 5 minutes and repeated for 5 to 6 times. Finally, the explants were dried by using sterile blotting paper and trimmed to approximately 1-1.5 cm. The surface sterilized explants were then inoculated in basal Murashige and Skoog (MS) medium [16] supplemented with 30 g/l sucrose and 6.5% agar to evaluate the response of explants to different treatments. All cultures were placed in the growth room with 25±2°C temperature, 60-70% humidity and 16 hours photoperiod (2500 lx). The explants were incubated in a growth room for 10 days and were observed every day for any contamination and morphological appearances.

Table 1 Experimental design of consisting seven different treatments for surface sterilization of Stevia shoot tips and nodes

Treatment	NaOCl		0.1% $HgCl_2$ (w/v) (min)	Source
	% (v/v)	Time (min)		
T1	1	30	-	[17]
T2	1.5	20	-	[18]
T3	5	10	-	[19]
T4	-	-	5	[20]
T5	-	-	8	[21]
T6	5	5	3	[22]
T7	10	2	2	[23]

T1= Treatment 1; T2= Treatment 2; T3= Treatment 3; T4= Treatment 4; T5= Treatment 5; T6= Treatment 6; T7= Treatment 7.

2.3 Standardization of Sterilization Protocol

Based on the observation and results of initial experiments, fungal contamination was very high in every treatment and bacterial contamination was less. Since treatment 3 (T3) gave the highest rate of survival for both explants, further experiment was conducted using T3. Explants were pretreated with 0.2% carbendazim (fungicide) for 30 minutes prior to the use of Tween 20 and followed by the surface sterilization process. This new treatment was termed T3F, (F for fungicide). The rest of the procedure and culture and growth conditions were the same as the initial experiments.

2.4 Statistical Analysis

Each treatment was repeated for 3 times. After 10 days of observation, percentage of contaminated explants, percentage of survival and death of explants were counted. The experimental design was completely randomized. The recorded data was subjected to one-way analysis of variance (ANOVA) and the means were compared by Tukey HSD multiple range tests ($P \leq 0.05$).

3.0 RESULTS AND DISCUSSION

The *in vitro* culture of any cell, organ and tissue can be contaminated basically from 4 types of sources. These sources are the plant (internal as well as external), the nutrient medium (insufficiently sterilized), the air, and the research worker (improper sterile techniques) [24]. In this study, the first possible source of contamination ie plant source was critically evaluated.

From the results obtained, it shows that the aseptic establishment of field grown Stevia was affected by fungi as well as bacteria (Figure 1). The loss of explants due to fungal contamination was more serious compared to bacterial contamination where it can be observed in all treatments. In this study, fungal contamination occurred within 2-3 days of inoculation and leading to the death of the explants

Figure 1 A representative photo of effect of different surface sterilization treatments on shoot tips of *Stevia rebaudiana*

Effect of NaOCl in disinfecting shoot tips and nodes of Stevia is time and concentration dependent (Table 2, Figure 2(a) and 2(b)). The effectiveness of the treatments (T1, T2 and T3) increases with the increase in concentration but less exposure of time. NaOCl at the lowest concentration (1%) in T1, was less effective to inhibit the growth of microorganisms with 30% and 25.56% contamination for shoot tips and nodes, respectively. The treatment with 1.5% NaOCl for 20 minutes (T2) helped to reduce contamination with 23.33% in the case of shoot tips but for nodal explants the percentage of contamination increased (32.22%). Contamination percentage of both explants was tremendously reduced to 16.67% (shoot tips) and 18.89% (nodes) when the concentration of NaOCl was increased to 5%. At the same time, percentage of survival for both explants increased with the highest concentration of NaOCl (76.67% for both explants). A high level of survival rate was reported for tomato seeds where different concentrations of NaOCl (3, 4, 5 and 6%) were tested with different time durations (15 and 20 mins). Among the treatments, 5% NaOCl produced 77.07% healthy, uniform and contamination free explants in tomato seed [25]. When dissolved in water, the hypochlorite salts leads to the formation of HOCl, which is responsible to eliminate fungal or bacterial contamination [26], which makes NaOCl as an effective and widely used disinfectant against microorganisms.

Table 2 Effect of different type of disinfectants with different concentrations and variation of exposure of time on inoculated shoot tips and node explants of *S. rebaudiana*. Different letters indicate significant differences between the values of pairs of treatments within column at $P \leq 0.05$. SEM= Standard Error of Mean; T1= Treatment 1, T2= Treatment 2, T3=Treatment 3, T4= Treatment 4, T5= Treatment 5, T6= Treatment 6, T7= Treatment 7, T3F= Treatment 3 with Fungicide (Carbendazim)

Treatment	Shoot tips (Mean±SEM)			Node (Mean±SEM)		
	Contamination (%)	Death (%)	Survival (%)	Contamination (%)	Death (%)	Survival (%)
T1	30.00 ± 10.00^{a}	13.33 ± 5.09^{m}	56.67 ± 15.03^{xy}	25.56 ± 5.01^{ab}	5.56 ± 1.70^{m}	68.89 ± 3.39^{xu}
T2	23.33 ± 5.09^{ac}	16.67 ± 5.09^{mn}	60.00 ± 10.00^{xy}	32.22 ± 5.01^{a}	4.44 ± 1.70^{m}	63.33 ± 3.33^{yu}
T3	16.67 ± 5.09^{ac}	6.67 ± 5.09^{m}	76.67 ± 1.92^{xz}	18.89 ± 5.01^{bd}	4.44 ± 1.70^{m}	76.67 ± 3.33^{x}
T4	16.67 ± 5.09^{ac}	20.00 ± 5.77^{mn}	63.33 ± 10.18^{xy}	18.89 ± 2.31^{bd}	27.78 ± 4.21^{n}	53.33 ± 1.92^{z}
T5	16.67 ± 1.92^{ac}	46.67 ± 5.09^{op}	36.67 ± 5.09^{y}	15.56 ± 1.70^{bd}	31.11 ± 0.64^{n}	53.33 ± 1.92^{z}
T6	13.33 ± 5.09^{ac}	36.67 ± 15.03^{np}	50.00 ± 10.00^{xy}	21.11 ± 1.70^{ad}	23.33 ± 1.92^{n}	55.56 ± 3.57^{yz}
T7	50.00 ± 10.00^{b}	10.00 ± 1.39^{m}	40.00 ± 10.00^{y}	44.44 ± 1.70^{c}	8.89 ± 1.70^{m}	46.67 ± 3.33^{z}
T3F	**6.67 ± 5.09^{c}**	**0.00 ± 0.00^{m}**	**93.33 ± 5.09^{z}**	**10.00 ± 3.33^{d}**	**2.22 ± 1.70^{m}**	**87.78 ± 1.70^{v}**

Since $HgCl_2$ is a highly toxic agent for plants, 0.1% $HgCl_2$ was used with different duration of exposures, 5 minutes (T4) and 8 minutes (T5). In both treatments, they were more effective than NaOCl in removing contaminants with 16.67% in both treatments for shoot tips and 18.89% (T4) and 15.56% (T5) for nodes (Table 2, Figure 2(a) and 2(b)). However the death percentage increased proportionally with concentration and exposure time, where exposure time of 8 minutes resulted in maximum death percentage both for shoot tips and nodes with 46.67% and 31.11%, respectively. It was reported that disinfection with 0.1% $HgCl_2$ proved to be the most phytotoxic, resulting in necrosis and death of *Solanum tuberosum* sprouts [27]. Increasing the exposure time of explants to 0.1% $HgCl_2$ did not give significant difference in percentage of contamination.

The combination of NaOCl and $HgCl_2$ showed quite satisfactory results. Although the percentage of contamination was the lowest for shoot tips with 13.33% when treated with treatment T6 (5% NaOCl for 5 minutes and 0.1% $HgCl_2$ for 3 minutes) compared to other treatments the survival rate was only 50% and 55.56% for shoot tips and nodes, respectively (Table 2, Figure 2(a) and 2(b)). For treatment T7 where 10% NaOCl for 2 minutes in combination with 0.1% $HgCl_2$ for 2 minutes was used, less percentage of death for both shoot tips and nodes could be observed with 10% and 8.89%, respectively. Treatment T7 did not eliminate all the surface contaminants with percentage of contamination of 50% and 44.44% for shoot tips and nodes, respectively. From this result, it can be deduced that when two disinfecting agents are being used in combination, there should be a balance between the concentration used and the exposure time. This is to ensure that all microorganisms are successfully removed without compensating the survival of explants. The use of two sterilizing agents has been shown in sugarcane in which 0.1% $HgCl_2$ for 5 minutes in combination with 90% ethanol for 10 minutes gave satisfactory result [28].

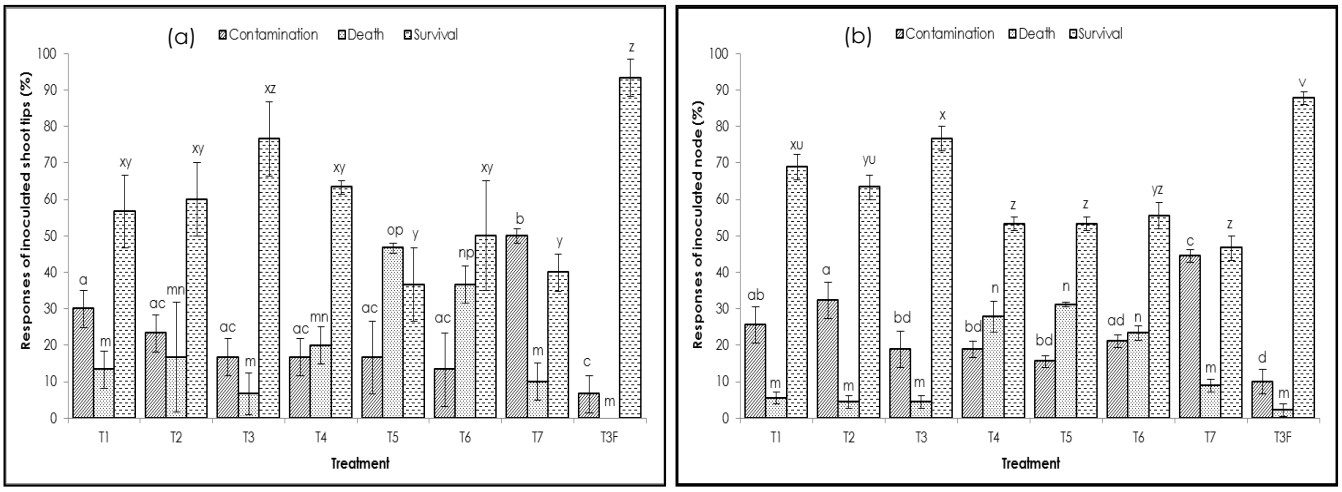

Figure 2 Effects of disinfectants with different concentrations and variation of exposure of time on inoculated (a) shoot tips and (b) nodes of *Stevia rebaudiana*. Different letters indicate significant differences among treatments at $P \leq 0.05$

Although from all treatments (T1-T7), T3 showed maximum survival rate with least contamination and death for both explants, the fungal contamination was still unsatisfactory level, which is main impediment for *in vitro* clonal propagation of Stevia. Therefore, carbendazim (fungicide) was used to overcome this problem. Pre-treatment of explants with 0.2% carbendazim followed by treatment T3 (named as T3F) was evaluated. This treatment gave highest survival rate with 93.33% for shoot tips and 87.78% for node explants and minimal contamination (6.67% for shoot tips and 10% for nodal explants) compared to other treatments (see Table 2, Figure 2). Carbendazim was effective to control fungal contamination in *in vitro* culture of *Momordica dioica* [29] and *Gymnema sylvestre* [30]. Similar findings were reported in *Andrographis paniculata*, where the plants were sprayed with fungicide prior to collection plant materials [31].

4.0 CONCLUSION

The use of suitable disinfectant with optimum concentration and exposure time is an important factor for the establishment of Stevia aseptic culture from field grown plant. This report describes a suitable protocol for surface sterilization of shoot tips and nodes from field grown Stevia, which could be feasible to use for *in vitro* propagation of Stevia. To reduce fungal contamination and to increase survival rate, pretreatment with carbendazim (fungicide) is highly effective.

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