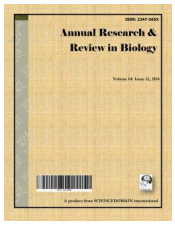

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In vitro Control of Microbial Contamination of Sweet Potatoes Cultured with Nodal Explants

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Authors' contributions

This work was carried in collaboration between all authors. Authors RA and HYS designed the study and wrote the protocol. Authors SA, PAC and HYS conducted the experiments and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The use of nodal explants for *in vitro* propagation promotes direct regeneration of cultures, but may cause high levels of microbial contamination due to large size of the explants. We evaluated different surface sterilization protocols, and inclusion of antibiotics and fungicides in growth medium to control microbial contamination of sweet potatoes (*Ipomoea batatas*) propagated *in vitro* with nodal explants. Three surface sterilization methods (low- 70% ethanol for 1 min, followed by 10% NaOCl for 15 mins; moderate- 70% ethanol for 3 mins, followed by 20% NaOCl for 10 mins; and high- 90% ethanol for 3 mins, followed by 30% NaOCl for 10 mins), plus a control where only distilled water was used to rinse the explants, were assessed. In addition, hormone-free Murashige and Skoog (MS) basal medium was amended with different rates of Chloramphenicol and Benomyl.

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The results showed high levels of direct regeneration of plantlets, but microbial contamination was also high, which emanated from both endogenous and exogenous sources. Surface sterilization without medium amendments controlled mainly the exogenous contaminants, but endogenous contaminants were still problematic, leading to the contamination of cultures even 4 wks after inoculating. Amending the culture medium with Chloramphenicol and Benomyl, reduced microbial contamination but it inhibited the growth of plantlets. In general, the moderate surface sterilization plus amending the culture medium with 0.5 g l^{-1} Chloramphenicol and 0.1 g l^{-1} Benomyl was the most effective in reducing contamination and causing minimal inhibition to plantlet growth.

Keywords: Sweet potato; germplasm conservation; tissue culture; microbial contamination; nodal explants.

1. INTRODUCTION

Sweet potato, believed to have originated from tropical America, is a dicotyledonous plant of the *Convulaceae* family [1,2]. Sweet potato is very nutritious and an important source of complex carbohydrates, dietary fiber, vitamin A and C, proteins, iron, calcium, and beta carotene [3-5]. Studies show that regular consumption of sweet potatoes can improve the body's immune system and resistance to infection [6]. In addition, sweet potato is a low input crop, and can successfully be cultivated in diverse agro-ecological conditions [7]. These nutrition and agronomic features make sweet potato a potential crop to combat food shortage, malnutrition, and poverty [7-9].

Continual genetic modifications and increasing reliance on modern crop species suggest the importance of germplasm conservation. However, field conservation of vegetative propagated crops poses major problems to curators of germplasm, especially in developing countries [10]. This is because of losses from pests and diseases, drought stress, wild fires, and thefts. Alternative method of germplasm conservation is the use of tissue culture techniques in medium formulated for maintenance under slow growth. Usually, embryogenic calli are first produced from shoot, leaf, or petiole explants of sweet potatoes before regenerating in medium amended with growth hormones [1,5,11]. Callus induction and subsequent regeneration of sweet potato plantlets is reported to cause somaclonal variation [12], which is undesirable for germplasm conservation that requires maintenance of genetic fidelity to the donor plant.

More importantly, studies indicate that *in vitro* cultures of sweet potatoes are prone to microbial contamination emanating from both endogenous and exogenous sources, which leads to culture mortality [5,13]. Isolation of apical shoot meristems for *in vitro* culturing has been successfully used to eliminate pathogens [14,15]. Nevertheless, since meristem tips are zone of undifferentiated cells, they usually form calli before regeneration, even when the initial culturing media are amended with growth hormones [14]. The use of nodal explants may promote direct regeneration of plantlets [16]. However, microbial contamination would be very high due to large size of the explant. We a) evaluated different surface sterilization protocols, and b) the addition of antibiotic and fungicide to culture medium to control microbial contamination of sweet potatoes propagated *in vitro* with nodal explants.

2. MATERIALS AND METHODS

2.1 Experimental Materials

Vine cuttings of two sweet potato genotypes (MOHC 1 and 199062.1) were obtained from Crop Research Institute, Council for Scientific and Industrial Research, Fumesua, Ghana. The vines were cut into 4 to 6-node sections and planted in approximately 1.5-litre pails filled with loamy soil. The pails were kept in lath house to avoid direct contact of the sprouts with rain which could serve as a source of contaminant. The propagated vines were maintained for 6 wks before excising nodes segments for tissue culturing.

2.2 Surface Sterilization Studies

2.2.1 Surface sterilization

One node per explants, approximately 2 cm long, were excised with a scalpel from the propagated sweet potato vines and transferred to a clean GA_7 vessels containing distilled water. The explants were rinsed four times with sterile distilled water for five to ten minutes and

transferred to a laminar flow hood. Three surface sterilization treatments were applied, plus a control where only distilled water was used to rinse the explants (Table 1). After disinfection with ethanol, the explants were rinsed with three to five changes of sterile distilled water depending on the treatment. Four drops of Tween-20 (Polyoxyethylene 20 sorbitan monolaurate) was added to the sodium hypochlorite (NaOCl) solution to provide good contact of the sterilant with all surfaces of the tissue. The explants were then rinsed with several changes of sterile distilled water.

Table 1. Surface sterilization treatments with ethanol and sodium hypochlorite (NaOCl)

Surface sterilization	Description
Control	No ethanol or NaOCl applied
Low	70% ethanol for 1 min, followed by 10% NaOCl for 15 mins
Moderate	70% ethanol for 3 mins, followed by 20% NaOCl for 10 mins
High	90% ethanol for 3 mins, followed by 30% NaOCl for 10 mins

2.2.2 Culture medium preparation and inoculation

Hormone-free MS basal medium [17] of 1 liter with 30 g sucrose was prepared, and 3.5 g phytagel (Sigma-Aldrich Co. LLC, Saint Louis, MO, USA) was added while the medium was placed on a hot magnetic stirrer which provided heat to dissolve the phytagel. MS medium supplemented with 30 g l^{-1} sucrose is the conventional culture medium for *in vitro* propagation of sweet potato [18]. The pH of the medium was adjusted to 5.8 and dispensed into test tubes in aliquots of 10 ml each while they were hot. The test tubes were capped and autoclaved at 121°C and at a pressure of 103.4 kPa for 15 minutes. The medium was allowed to cool after autoclaving and then stored in a refrigerator at 4°C. The sterilized explants were held with a sterilized pairs of forceps, while a surgical blade was used to trim the explants to about 3 mm below the node, and 6 mm above the node. The explants were immediately removed and inoculated onto the culture medium, using one explant per test tube. The entire process was carried out under the laminar flow hood to minimize contamination. Also, the surgical blade and the pair of forceps were sterilized after every explant was cultured to avoid cross contamination.

Inoculated cultures were transferred to a growth chamber and were exposed daily to 16 hours of light, with light illuminance of 47.3 µmol. m^{-2}. s^{-1} for root and shoot development. The growth chamber was maintained at 24±2°C temperature and relative humidity of 70%.

2.2.3 Experimental management and statistical analysis

The experiment was a two factor factorial arrangement of the two genotypes and four sterilization treatments in a completely randomized design with three replications. Each replication was an average of five sub-cultures. Culture contamination and general growth of plantlets were assessed 1, 2, and 4 wks after culture. Validity of normality, equal variance, and independence assumptions on the error terms were checked by assessing the residuals, and appropriate data transformation performed when applicable [19]. Analysis of variance test was performed using the PROC GLM procedure in SAS 9.4 [20]. Significant levels were determined at $P = .05$, and mean separation conducted with the least squares means (LSMEANS) and adjusted Tukey multiple comparison procedure. Based on the level of contamination and general health of plantlets, the best surface sterilization method was selected and used in the medium amendment study.

2.3 Medium Amendment Studies

The same hormone-free MS basal growth medium used for the surface sterilization studies was prepared. However, the medium was amended with different rates of antibiotics (Chloramphenicol; $C_{11}H_{12}Cl_2N_2O_5$) and systemic fungicide (Benomyl; $C_{14}H_{18}N_4O_3$) prior to autoclaving. The amendment treatments included a) control; no Chloramphenicol and Benomyl, b) moderate amendment; 0.5 g l^{-1} Chloramphenicol and 0.1 g l^{-1} Benomyl, and c) high amendment; 1 g l^{-1} Chloramphenicol and 0.2 g l^{-1} Benomyl. The moderate surface sterilization protocol was used for the medium amendment studies. In addition, excision and inoculation procedures, as well as growth chamber conditions were the same as described previously.

The experiment was a two factor factorial arrangement of the two genotypes and three amendment strategies in a completely randomized design with three replications. Every replicate constituted five sub-cultures. Data was

collected after 8 wks by counting the number of cultures that were contaminated, regenerated directly, showed malformed growth, and also showed no growth response before senescing. Explants that regenerated directly but got contaminated later were still considered to have directly regenerated. The data was analyzed with the PROC GLM procedure in SAS 9.4 [20], and significant levels determined at *P =.05*.

3. RESULTS AND DISCUSSION

3.1 Surface Sterilization Effects on Microbial Contamination

There were no significant genotype and genotype × surface sterilization interaction effects on all measured parameters in the studies. Compared to the control, the surface sterilization treatments significantly reduced the number of contaminated cultures observed after 1 wk of inoculation (Fig. 1). However, the level of microbial contamination increased rapidly with time regardless of the surface sterilization treatment. Averaged over the moderate and high surface sterilization methods, microbial contamination at wk 1 was 4.36%, which increased to 76.1% and 94.6% after 2 and 4 wks, respectively. There was 100% contamination in the control and low sterilization methods after culturing for 2 and 4 wks (Fig. 1), respectively. Although, the moderate and high sterilization methods exhibited similar efficiency in controlling microbial contamination, plantlet growth was significantly impaired in the high sterilization, making the moderate sterilization the preferred method.

The results of this work support previous studies that indicated *in vitro* propagation of sweet potatoes are prone to microbial contamination [5,13]. Epiphytic and endophytic organisms can cause losses of explants at every growth stage. Exogenous contaminants are found on the surface of the explant and hence show fast growth rates, usually occurring within 2-3 days or sometimes up to 1 wk after culture [21]. Conversely, endogenous microbial contaminants reside in the tissues and are usually symbiotic and beneficial to most plants, but become pathogenic when the plants are stressed such as weakening of their cell walls [22,23]. They can cause contamination of cultures even after several weeks of inoculation [5,21]. Although, surface sterilization can adequately eliminate exogenous contaminants, it is not efficient in controlling endogenous contaminants [5,22]. This was confirmed in the current study as increased contamination of cultures that were surface sterilized even after 4 wks of inoculation.

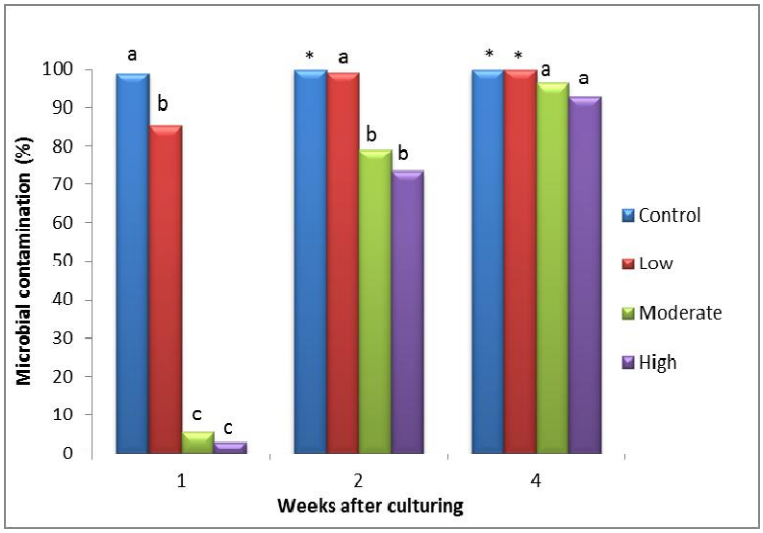

Fig. 1. Surface sterilization effects on microbial contamination of *in vitro* cultures of sweet potato. Within growth stage and surface sterilization methods, means followed by same letter are not significantly different using the least squares means (LSMEANS) and adjusted Tukey multiple comparison procedure *(P=.05)*, *Not included in statistical analysis due to lack of variance

We observed that all contaminated explants died even if initially they showed some growth. This could be due to microbial contaminants utilizing the nutrient rich medium for growth and eventually out-competing the explants for nutrients and oxygen. Studies show that some contaminants also cause death to explants by secreting phytotoxins, which have deleterious effects on explants [22]. The concentration and time of exposure of the explants to the surface disinfectants also adversely affected the growth of the explants. Growth inhibition of cultures was greatest in the high sterilization treatment.

3.2 Performance of Explants in Medium Amended with Chloramphenicol and Benomyl

Amending the growth medium with various rates of Chloramphenicol and Benomyl showed different effectiveness in reducing microbial contamination of cultures (Fig. 2). The high medium amendment was the most effective, followed by moderate amendment, and the control treatment showed the greatest microbial contamination. 100% explant mortality was observed in all cultures that were contaminated, even though they showed initial signs of growth (Fig. 3A). Culture growth response was observed as greening of explants before direct shoot initiation (Fig. 3B), which occurred within 1 wk after inoculation. Although the high medium amendment was the most effective in controlling microbial contamination, direct regeneration of explants was, however, significantly reduced (Table 2). This was 70 and 75% smaller compared to the moderate medium amendment and the control treatment, respectively. The high medium amendment also significantly increased the number of explants that showed malformed growth (Fig. 3C). More importantly, most of the explants cultured in the medium amended with high rates of Chloramphenicol and Benomyl did not respond to growth at all, which eventually died. As high as 59.3% of the explants responded that way, compared to 18.4% and 9.33% in the moderate medium amendment and control treatment, respectively.

The significant reduction in microbial contamination upon amending the growth medium with Chloramphenicol and Benomyl, and after surface sterilization, indicates that most of the contaminants were bacteria and fungi, consistent with previous studies [5,13,22,24]. Benomyl is a systemic fungicide which acts as multiplication inhibitor by binding to microtubules and interfering with cell functions, such as meiosis and intracellular transportation. Chloramphenicol prevents protein chain elongation by inhibiting peptidyl transferase activity of bacterial ribosome. Although effective in controlling microbial contaminants, the amendment with antibiotic and fungicide adversely altered the growth and development of the plantlets. This was observed as malformed growth or the lack of growth which led to browning of explants and subsequent death. In addition, none of the explants cultured in the present study showed root initiation after 8 wks of culturing. However, root formation began 12 wks after culturing (Fig. 4). Root initiation is a high energy formation process that requires metabolic substrate, usually carbohydrate [10]. Previous studies have reported root formation and healthy sweet potato plantlets in hormone free MS medium supplemented with 30 g l^{-1} sucrose as used in this study [18,25]. Root formation in sweet potato plantlets after culturing for 6 wks have also been reported [18]. As such, the lack of root formation within the 8 wk period in this study could be due to injuries sustained by the explants [14].

Table 2. The effects of growth medium amendment with Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$) and Benomyl ($C_{14}H_{18}N_4O_3$) on direct regeneration, malformed growth, and the proportion of plantlets that showed no sign of growth after culturing for 8 wks

Medium amendment	Directly regenerated	Malformed growth	No growth response
	◄	%	►
Control	87.3a	3.33a	9.33c
Moderate amendment	72.0b	9.56b	18.4b
High amendment	21.6c	19.1a	59.3a

Within growth stage and surface sterilization, means followed by same letter(s) are not significantly different using the least squares means (LSMEANS) and adjusted Tukey multiple comparison procedure (P=.05)

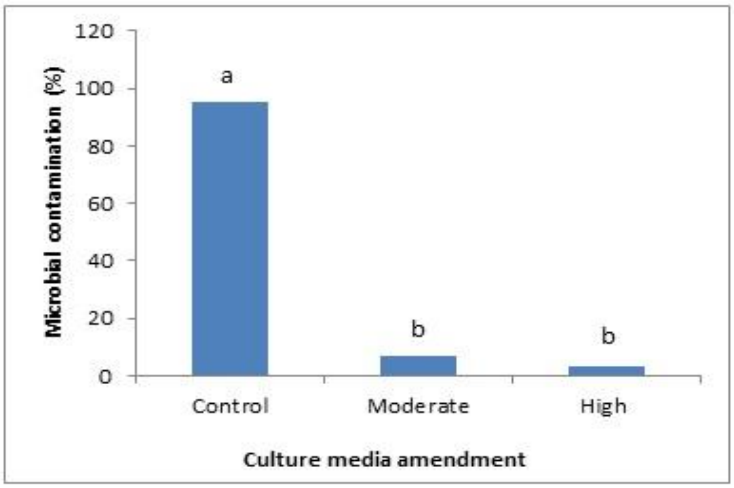

Fig. 2. Microbial contamination as affected by medium amendment with different rates of Chloramphenicol and Benomyl after culturing for 8 wks. Means followed by same letters are not significantly different using the least squares means (LSMEANS) and adjusted Tukey multiple comparison procedure *(P=.05)*; Control constituted no amendment, moderate was amendment with 0.5 g l^{-1} Chloramphenicol and 0.1 g l^{-1} Benomyl; and high was 1 g l^{-1} Chloramphenicol and 0.2 g l^{-1} Benomyl

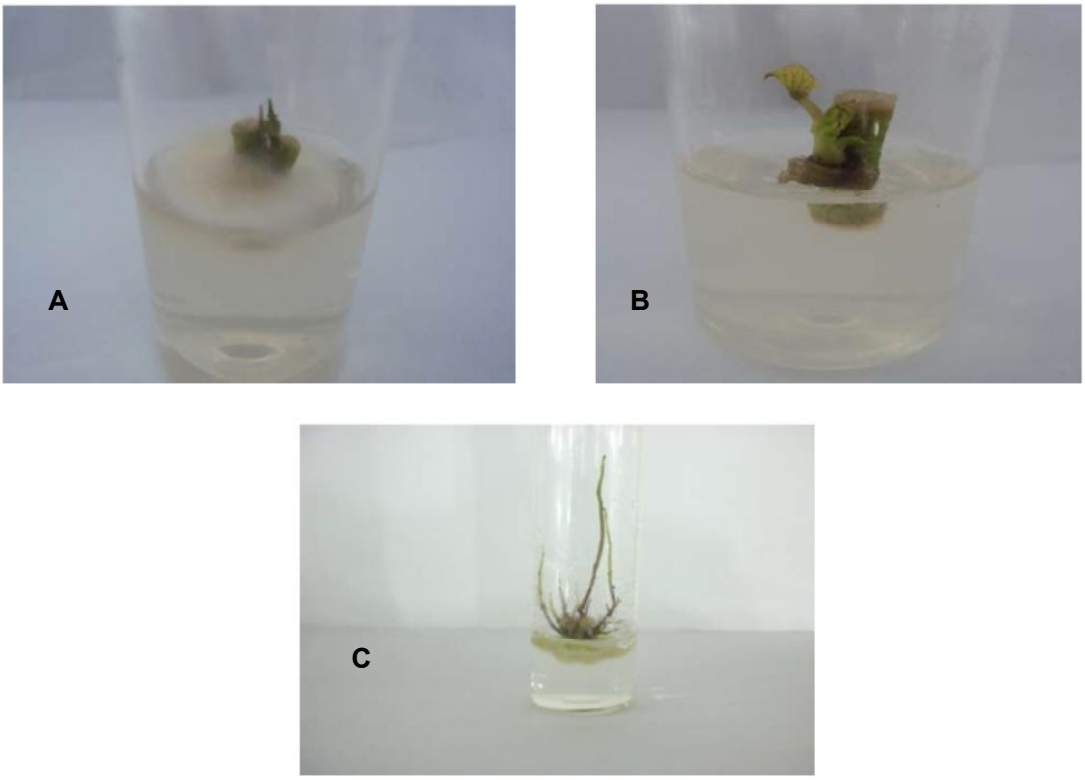

Fig. 3. *In vitro* sweet potato plantlets showing a) microbial contamination 2 wks after culture, b) direct regeneration 4 wks after culture, and c) malformed growth 8 wks after culture in MOHC 1 sweet potato genotype

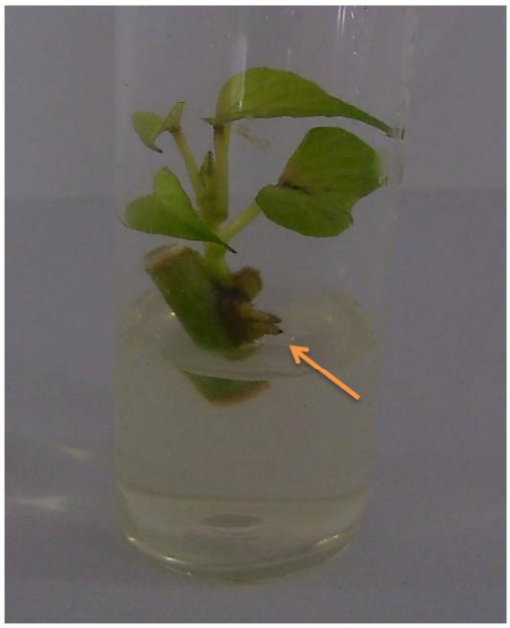

Fig. 4. *In vitro* sweet potato plantlet (199062.1 genotype) showing root formation 12 wks after culturing

4. CONCLUSIONS

In vitro propagation of sweet potatoes with nodal explants enhanced direct regeneration of plantlets but it suffered from high microbial contamination. The microbial contaminants emanated from both endogenous and exogenous sources. Although surface sterilization controlled exogenous contaminants, endogenous contaminants were still problematic, leading to culture contamination even 4 wks after inoculating. Amending the culture medium with Chloramphenicol and Benomyl, reduced microbial contamination but inhibited the growth of plantlets.

In general, the moderate surface sterilization plus amending the culture medium with 0.5 g l^{-1} Chloramphenicol and 0.1 g l^{-1} Benomyl was the most effective in reducing contamination and causing minimal inhibition of plantlet growth. Further studies that would evaluate different antibiotics and fungicides at various rates, in order to reduce contamination, while maintaining the growth of plantlets, would be important in promoting *in vitro* conservation of sweet potato. There is the need also to apply molecular techniques to assess whether regenerated plantlets from nodal explants are true to type, especially because of the use of chemicals which injured the explants.

CONSENT

It is not applicable.

ETHICAL APPROVAL

There were no ethical concerns pertaining to this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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