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Full Length Research Paper

Simple, effective and economical explant-surface sterilization protocol for *c*owpea, rice and sorghum seeds

O. B. Oyebanji, O. Nweke, O. Odebunmi, N. B. Galadima, M. S. Idris, U. N. Nnodi, A. S. Afolabi* and G. H. Ogbadu

Biotechnology Advanced Laboratory, Sheda Science and Technology Complex, P. M. B. 186, Garki, Abuja FCT, Nigeria.

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Three different surface sterilization methods were evaluated using seeds and excised embryos of cowpea, rice and sorghum as explants: Method 1: Ethanol alone in concentrations of 95, 90, 85 and 70% at different time intervals and observed at different days. Method 2: Locally produced bleaching solution (JIK® -Reckitt and Benckiser (Nig) Ltd) containing 3.5% Sodium hypochlorite) at different time intervals, observed at different days. Method 3 (The control): The routinely used two step sterilization procedure using 90% ethanol for 3 min followed by sodium hypochlorite 3.5% for 30 min. This is commonly used in most laboratories. However, neat concentration of the locally produced bleaching solution of JIK® (-Reckitt and Benckiser (Nig) Ltd) containing 3.5% sodium hypochlorite was used instead of the pure sodium hypochlorite solution. Our results showed that Method 2 produced the highest reduction in bacterial and fungal contamination (0%) at time intervals between 20 - 45 min. The search for a simple, rapid and economical method of sterilizing explants for tissues culture, instead of the orthodox two-step -two reagent - technique, necessitated these experiments; we would, therefore recommend this technique due to its simplicity and economy.

Key words: Explant, surface sterilization, cowpea, rice, sorghum, JIK®

INTRODUCTION

Contamination with microorganisms is considered to be the single most important reason for losses during *in vitro* culture of plants. Such microorganisms include viruses, bacteria, yeast, fungi, etc (Omamor et al., 2007). These microbes compete adversely with plant tissue cultures for nutrients. The presence of these microbes usually result in increased culture mortality but can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003).

Despite the best timing and selection efforts, it is almost impossible to eliminate contamination from *in vitro* grown plants and losses due to contamination *in vitro* average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories (Leifert et al., 1994). The cumulative result is an abundant waste of time, effort and materials which if not mitigated can have severe economic consequences (Webster et al., 2003).

During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period. The disinfectants widely used are sodium hypochlorite (which dates back to the the mid 18th century- Miche' and Balandreau, 2001), calcium hypochlorite, ethanol (or isopropyl alchohol), mercuric chloride, hydrogen peroxide, silver nitrate and bromine water (Rai university lab-manual, 2003). Hypochlorite is known to be a very effective killer of bacteria, even micromolar concentrations are enough to reduce bacterial populations significantly. However, little is known about the exact mechanisms of its bacteriocidal activity. When diluted in water the hypochlorite salts

*Corresponding author. E -mail: abolade.afolabi@wagner.edu. Tel: +234 (0) 803 488 1092.

($NaOCl$, $Ca(OCl)_2$) lead to the formation of HoCl whose concentration is correlated with bactericidal activity (Nakagarwara et al., 1998). Sodium hypochlorite, usually purchased as laundry bleach is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explant because of phytotoxicity. Calcium hypochlorite is used mostly in Europe and the concentration generally used is 3.25%, it may be less injurious to plant tissues than sodium hypochlorite (https://www.msu.edu/course/css/451/).

Ethanol is a powerful sterilizing agent but also extremely phyto-toxic. Therefore, the explant is typically exposed to it for only a few seconds or minutes. Explants such as seeds or dormant buds can be treated for longer periods of time since the tissue that will develop is actually within the structure that is being surface sterilized.

To enhance effectiveness in sterilization procedure, a surfactant like Tween 20® is freqently added to the sterilizing solution (and in some laboratories a mild vacuum is applied during the procedure); in general, the sterilizing solutions containing the explants are continously stirred during the sterilization period (https://www.msu.edu/course/css/451/).

Ethanol, in general, is used prior to treatment with other compounds. The use of a two-step (two-source) sterilization procedure has proven beneficial with certain species. Ethanol is usually combined with hypochlorite for effectiveness, e.g. the use of 90 or 70% ethanol for 3 min and sodium hypochlorite(3.5%) for about 30 min.

Prior to this study, the standard surface sterilization technique used in our laboratory is the use of 90% ethanol for 3 min, followed by the local household bleach commonly called JIK® (Reckitt and Benckiser (Nig) Ltd), which contains 3.5% sodium hypochlorite. The aim of this study, therefore, was to determine the simplest and least expensive method of sterilizing explants for *in vitro* plant tissue cultures.

MATERIALS AND METHODS

Plant material

Rice seeds (NCRI Line DA 29 and FARO 29) for these experiments were collected from the National Cereal Research Institute (NCRI), Badeggi, Cowpea (IT960-610 and IT97K-568-18) from the International Institute of Tropical Agriculture (IITA), Ibadan and Sorghum (Bigero) from the Institute of Agricultural Research (IAR), Samaru-Zaria. All Institutes are located in Nigeria.

Chemicals

Ethanol was obtained from the Sigma-Aldrich Lab Chemicals, Germany. The locally produced bleach solution JIK® (Reckitt and Benckiser (Nig) Ltd) containing. 3.5% sodium hypochlorite was used instead of the expensive Sigma- Aldrich® Sodium hypochlorite solution.

Experimental technique

Briefly, the seeds (explants) in the container were washed and rinsed 3 times in sterile distilled water in the laminar flow/biosafety cabinet. The sterilizing agents (depending on the method used) with a drop or two of Tween 20 were then added to cover the explants and stirred continously for the different time intervals according to the methods described below. The sterilizing agents were then decanted and the explants were rinsed thoroughly three times with sterile distilled water. Seeds were placed on sterile tissue to dry and were cultured on water agar media. From some of the sterilized cowpea seeds, embryos were excised using a sterile knife and forceps. In the two step sterilization procedure (Method 3), 90% ethanol was added first for 3 min and decanted, rinsed 3 times with sterile distilled water before the addition of the neat JIK® (containing 3.5% sodium hypochlorite) for 30 min. Sunsequently, the seeds were rinsed thoroughly with sterile distilled water 3 times before culturing on the media. All experiments were repeated twice.

Method 1

The effect of ethanol alone on the cowpea seeds and detached embryos (from sterilized seeds) and on dehusked whole rice seeds (as explants) at different concentrations of 95, 90, 85 and 70% at different time intervals of 5, 10, 15 and 30 min was studied. Observation for contamination was carried out on days 3, 6 and 9.

Method 2

The effect of neat JIK® (containing 3.5% Sodium hypochlorite) on similar explants as in method 1, at different time intervals of 5, 10, 15, 20, 30 and 45 min was studied.

Method 3

The effect of 90% ethanol for 3 min and 3.5% Sodium hypochlorite for 30 min which is routinely used and also very effective (but not economical) was also investigated and used as the control.

RESULTS

Method 1: Surface sterilization of explants with different concentration of ethanol with variable sterilization time

Cowpea seeds

Table 1 and Figure 1 (A & B) shows the results obtained when ethanol alone was used in surface sterilization of cowpea seeds. The percent contamination observed on all days showed that it is not suitable for sterilization.

Cowpea embryos

Cowpea seeds were surface sterilized as above and whole embryos asceptically excised under laminar flow

Table 1. Sterilization of cowpea seeds using various concentration of ethanol at various intervals.

Ethanol conc. (%)	CS (3 days - 10 min)	CS (6 days - 10 min)	CS (9 days - 10min)	CS (3 days - 15 min)	CS (6 days - 15 min)	CS (9 days - 15 min)	CS (3 days - 30 min)	CS (6 days - 30 min)	CS (9 days - 30 min)
T1 (95)	100%*	100%	100%	100%	100%	100%	100%	100%	100%
T2 (90)	100%	100%	100%	100%	100%	100%	100%	100%	100%
T3 (85)	100%	100%	100%	100%	100%	100%	100%	100%	100%
T4 (70)	100%	100%	100%	100%	80%	80%	20%	20%	40%

*Contamination percentages after days 3, 6 and 9.
CS = Cowpea seeds.

1A 1B

Figure 1 (A&B): Cowpea seeds sterilized with 95% ethanol for 10mins had 100% contamination after 9 days of culture. B. Cowpea seeds sterilized with 3.5% sodium hypochlorite (Jik) for 30 minutes had 0% contamination after 9 days of culture.

Table 2. Sterilization of cowpea seeds using various concentration of ethanol at various intervals.

Ethanol conc. (%)	CE (3 days - 10 min)	CE (6 days - 10 min)	CE (9 days - 10 min)	CE (3 days - 15 min)	CE (6 days - 15 min)	CE (9 days - 15 min)	CE (3 days - 30 min)	CE (6 days - 30 min)	CE (9 days - 30 min)
T1 (95)	60%*	60%	80%	0%	60%	0%	0%	0%	0%
T2 (90)	0%	0%	0%	0%	0%	0%	0%	0%	0%
T3 (85)	0%	0%	0%	0%	0%	0%	0%	0%	0%
T4 (70)	0%	0%	0%	10%	0%	10%	0%	0%	0%

*Contamination percentages after days 3, 6 and 9.
CE = Cowpea embryos.

Table 3. Sterilization of rice seeds using various concentrations of ethanol at various intervals

Ethanol conc. (%)	Rice (3 days - 10 min)	Rice (6 days - 10 min)	Rice (9 days - 10 min)	Rice (3 days - 15 min)	Rice (6 days - 15 min)	Rice (9 days -15 min)	Rice (3 days - 30 min)	Rice (6 days - 30 min)	Rice (9 days - 30 min)
T1 (95)	0%*	10%	10%	0%	10%	10%	0%	30%	30%
T2 (90)	0%	30%	30%	10%	10%	10%	0%	10%	10%
T3 (85)	0%	20%	20%	0%	0%	0%	0%	0%	0%
T4 (70)	10%	30%	70%	0%	0%	40%	0%	0%	30%

*Contamination percentages after days 3, 6 and 9.

Table 4. Sterilization of sorghum seeds using various concentration of ethanol at various intervals.

Ethanol conc. (%)	SS (3 days - 10 min)	SS (6 days - 10 min)	SS (9 days - 10 min)	SS (3 days - 15 min)	SS (6 days - 15 min	SS (9 days - 15 min)	SS (3 days - 30 min)	SS (6 days - 30 min)	SS (9 days - 30 min)
Conc.(95)	0%*	10%	10%	0%	0%	0%	0%	0%	0%
Conc.(90)	0%	0%	10%	0%	0%	0%	0%	0%	0%
Conc. (85)	0%	0%	10%	0%	0%	0%	0%	0%	0%
Conc. (70)	0%	0%	0%	0%	0%	0%	0%	0%	0%

SS = Sorghum seeds.
*Contamination percentages after days 3, 6 and 9.

hood after thorough rinsing of the seeds with sterile distilled water. Table 2 showed the results of this experiment.

Rice seeds

Table 3 also show the results obtained when ethanol alone was used in surface sterilization of rice seeds. The percent contamination observed on all days showed that it is not suitable for sterilization.

Sorghum seeds

Table 4 shows the results obtained when ethanol alone was used in surface sterilization of sorghum seeds.

Method 2: Surface sterilization of explants with neat solution of locally produced bleach JIK® containing 3.5% sodium hypochlorite Seeds of rice, sorghum and cowpea were sterilized with JIK containing 3.5% sodium hypochlorite at different time intervals of 5, 10, 15, 20, 30 and 45 min. Embryos of cowpea were also excised from sterilized cowpea seeds. Cultures were then observed for contamination after 3, 6 and 9 days of culture. Table 5 shows the results obtained.

Method 3: The effect of 90% ethanol for 3 min and 3.5% Sodium hypochlorite for 30 min which is routinely used and also very effective (but not economical) was also investigated and used as the control.

DISCUSSION

Reducing contamination through two-step two-reagent procedure, as described by Mathews and Duncan (1993) is a laborious and drawn-out process. The use of ethanol or the combination of ethanol and other disinfectants is very expensive. In a typical tissue culture laboratory, ethanol, sodium hypochlorite and sucrose are a few of the major investments and any means of reducing this cost would be significantly useful especially in the developing countries. Additionally simplifying existing technique will have benefit in timesaving.

In this study, cowpea seeds with varied ethanol treatments had the highest rate of contamination at the different time intervals with 80-100% contamination after

Table 5. Sterilization of different seeds with JIK containing 3.5% sodium hypochlorite.

JIK conc. (%)	CS (3 days)	CS (6 days)	CS (9 days)	CE (3 days)	CE (6 days)	CE (9 days)	Rice (3 days)	Rice (6 days)	Rice (9 days)	SS (3 days)	SS (6 days)	SS (9 days)
JI-5 min	100%*	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%	10%
J2-10 min	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%	10%
J3-15 min	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%	10%
J4-20 min	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
J5-30 min	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
J6-45 min	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

CS = Cowpea seeds; CE = Cowpea embryos; SS = Sorghum seeds.
*Contamination percentages after days 3, 6 and 9.

Table 6. Results obtained with the routine and well established standard method of explant surface sterilization.

Conc. (%)	CS (3 days)	CS (6 days)	CS (9 days)	CE (3 days)	CE (6 days)	CE (9 days)	Rice (3 days)	Rice (6 days)	Rice (9 days)	SS (3 days)	SS (6 days)	SS (9 days)
90% Alc. and 3.5% NaOCl	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

CS = cowpea seeds, CE = cowpea embryos, SS = sorghum seeds.

9 days. This is probably due to the fact that cowpea are usually heavily infested with pests resulting in heavy load of various organisms. As expected, embryos are “cleaner” since seeds are internally sterile. However, there are seeds with heavy systemic bacterial infections and other methods of sterilization are required (Ksenija and Dragana, 2005). Cowpea seeds also had high contamination rates on exposure to 3.5% sodium hypo-chlorite at 5 -15 min, but no contamination at 20 - 45 min after 9 days. In this experiment, 85% ethanol was also effective but is more expensive than the locally produced bleach JIK.

Webster et al. (2003) suggested that prudent selection of explants from the healthy parent plants coupled with an effective surface sterilization method should be the goal in avoiding culture contamination.

In conclusion, results of this study have demonstrated that the use of locally produced bleach (JIK®) containing 3.5% hypochlorite (JIK®) for 30 min is as effective as the regular 2-step 2-reagent technique. Consequently, we would recommend its usage because of its simplicity and economy.

REFERENCES

CSS 451 (2009) Biotechnology Applications for Plant Breeding and Genetic. January 13, Course and laboratory orientation; Sterile techniques and tissue culture ...https://www.msu.edu/course/css/451/

Kane M (2003). Bacterial and fungal indexing of tissue cultures http://www.hos.ufl.edu/moreweb/TissueCulture/class1/Bacterial%20and%20fungal%20indexing%20of20tissue%20cultures.doc.

Ksenija JT, Dragana MV (2005). Different sterilization methods for overcoming Internal Bacterial Infection in sunflower seeds. *Matica Srpska Novi Sad*, Proc. Nat. Sci. 109: 59-64

Leifert C, Morrris EC, Waites MW (1994). Ecology of microbial saprophytes and pathogens in tissue culture and field grown plants: reasons for contamination problems *in vitro*. Crit. Rev. Plant Sci. 13(2): 139-183.

Mathews R, Duncan EJ (1993). A method to reduce microbial contamination *in vitro* culture. Third Caribbean Biotechnology Conference, pp. 160-166.

Miche L, Balandreau J (2001). Effects of rice seeds sterilization with Hypochlorite on inoculated Burkholderia vietnamiensis. Appl. Environ. Microbiol. 67(7): 3046-3052.

Nakagarwara S, Goto T, Nara M, Ozawa Y, Hotta K, Arata Y (1998). Spectroscopic characterisation and the pH dependence of bacterial activity of the aqueous chlorine solution. Anal. Sci. 14: 691-698.

Omamor IB, Asemota AO, Eke CR, Ezia EI (2007). Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR) Afr. J. Agric. Res. 2(10): 534-537.

Webster SK, Seymour JA, Mitchell SA, Ahmad MH (2003). A novel surface sterilization method for reducing microbial contamination of field grown medicinal explants intended for *in vitro* culture. Biotechnology Centre, U.W.I, Mona, Kingston 7, Jamaica, West Indies.