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Development and Optimization of a Surface Sterilization Protocol for the Tissue Culture of *Pleurotus tuber-regium (Fr) Sing.* and *Auricularia auricula-judae*

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ABSTRACT: *This study was carried out to develop and optimize a surface sterilization protocol for the sterilization of explants in the tissue culture of the edible mushrooms Pleurotus tuber-regium (Fr) Sing. and Auricularia auricula-judae. The explants were exposed to two sterilization agents; 70% ethanol (EtOH) and JIK® a locally produced bleaching solution containing 3.5% (w/v) sodium hypochlorite at concentrations of 2.5% (v/v) and 5.0% (v/v) with varying time intervals of one, two, five, ten and fifteen minutes. The results showed that treatment of explants with 5.0% (v/v) sodium hypochlorite solution for ten minutes preceded by 70% ethanol pre-treatment for two minutes gave the least explants contamination (0%) and highest explants survival (100%) for Pleurotus tuber-regium, while treatment of explants with 70% ethanol for one minute followed by 5.0% (v/v) sodium hypochlorite solution for five minutes, gave the highest survival rate (100%) and least explants contamination (0%) for Auricularia auricula-judae.*

KEYWORDS: ethanol, explants, mushrooms, sterilization agents, tissue culture

INTRODUCTION

Mushrooms are an economically important type of fungi which have long been used as a valuable food source and as traditional medicines around the world. Presently, in many parts of sub-Saharan Africa, mushrooms present a low- cost alternative source of high quality protein [1].

Pleurotus tuber-regium is one of a class of mushrooms generally referred to as 'oyster mushrooms'. They are fungi which belong to the phylum basidiomycota and order agaricales [2]. Oyster mushrooms have a very good organoleptic acceptability and as such are an excellent delicacy in many parts of the world because of their naturally occurring flavour and pleasant taste [3], [4]. Pleurotus tuber-regium is the most common and widely consumed oyster mushroom in Nigeria and many tribes in Sub-Saharan Africa [5], [6]. Unlike most other mushrooms, Pleurotus tuber-regium has a sclerotia stage in its lifecycle which can withstand extreme harsh ecological conditions

[7]. Fruit bodies and sclerotia of the mushroom are usually collected from the wild and used as food and medicine [8]. The sclerotia can be stored quite easily for many months and even years before being used as food. They can also be used to produce sporophores or fruitbodies which can be stored for use later or eaten fresh. The ability to store the sclerotia for later use can be exploited to provide a possible solution to the challenge of protein food shortage and malnutrition. *Auricularia auricula-judae* commonly called 'wood ear' is also an edible mushroom consumed in Nigeria. The genus *Auricularia* is presently the fourth most important cultivated mushrooms in the world after *Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus* [9]. This rapid increase in the domestication of the mushroom from the wild is attributed to its nutritional and medicinal properties which includes antitumor, hypocholesterolemic, antiviral and antibacterial effects [10], [11]. The tissue culture of mushrooms has become necessary and important as a result of the gradual extinction of these fungi due to climate change and the depletion of forest habitats. Tissue culture techniques offer a rapid method of mass propagation as well as availability of the mushroom at any time of the year. Microbial contamination results in losses during in vitro cultivation and the success of the tissue culture process depends largely on having an effective surface sterilization protocol for the sterilization of explants to eliminate these microbes and prevent culture contamination and mortality. The sterilization process should preserve the biological activity of the explants and effectively eliminate the contaminating microorganisms [12]. In literature, ethanol, sodium hypochlorite and the detergent Tween 20 have been used as disinfectants in explants- surface sterilization of different plants. However, there is no documented use of these sterilizing agents for the sterilization of mushrooms. This study was therefore carried out to determine the most effective type, concentration and duration of disinfectants and hence develop an effective explants surface sterilization protocol for *Pleurotus tuber-regium* and *Auricularia auricula-judae*.

MATERIALS AND METHODS

This study was conducted at the Biotechnology Advanced Research Centre of Sheda science and Technology complex, Abuja, Nigeria.

Sterilization of equipment and laminar flow hood

The glass wares and other apparatus used for the experiment were autoclaved at 121^{o}C for fifteen minutes to eliminate any bacteria or fungal deposits and then kept under aseptic conditions until use. The laminar flow hood was swabbed with 70% ethanol and then the autoclaved beakers, conical flasks, PDA plates, cutting pads, sterile distilled water, forceps and scalpel were transferred into the laminar flow hood and the UV light turned on for fifteen minutes.

Chemicals

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

Source of explants

Explants for *Pleurotus tuber-regium* were obtained from the sclerotia purchased from 'Ntezi Nkwo' market in Ishielu LGA of Ebonyi State, and from healthy sporophores of *Auricularia auricula* growing in the botanical garden of Sheda Science and Technology Complex, Abuja.

Experimental protocol

The mushrooms were cut into small sizes and washed under running tap water for three to five minutes to get rid of sand particles and dirt on the surface. This was followed by washing with liquid soap and rinsing three to four times with distilled water. The explants were then put in sterile beakers and transferred into the laminar flow hood. Here the outer tissues of the sporophore and sclerotium was removed on sterile cutting pads exposing the internal tissues which were cut into smaller sizes and then rinsed again with sterile distilled water. Five different sterilization treatments were then carried out as shown in Table 1. In the first treatment (A_{1-5}), the explants were treated with 70% ethanol alone for one, two, five, ten and fifteen minutes after which they were rinsed four to five times with sterile distilled water and then put out on sterile tissue pads to dry. In Experiments (B_{1-5}) and (C_{1-5}), the explants were treated with 2.5% and 5.0% (V/V) sodium hypochlorite solution respectively for one, two, five, ten and fifteen minutes after which they were rinsed four to five times with sterile distilled water and put on sterile tissue pads to dry. While in experiments (D_{1-5}) and (E_{1-5}), the *Pleurotus tuber-regium* and *Auricularia auricula-judae* explants were first treated with 70% ethanol before being treated with 2.5% and 5.0% (V/V) sodium hypochlorite solution. Three drops of the detergent Tween 20 per 20 ml of solution was added to the sodium hypochlorite solution. This process was followed by washing four to five times with sterile distilled water and then drying on sterile tissue pads.

Table 1: Sterilization agents used in the protocol optimization

Treatment code	Sterilization agent
A_{1-5}	70% ethanol
B_{1-5}	2.5% (v/v) sodium hypochlorite
C_{1-5}	5.0% (v/v) sodium hypochlorite
D_{1-5}	2.5% (v/v) sodium hypochlorite + 70% ethanol
E_{1-5}	5.0% (v/v) sodium hypochlorite + 70% ethanol

For each treatment, the explants were agitated vigorously in the sterilizing solution. After drying, the explants were then inoculated on potato dextrose agar (PDA) in Petri dishes. The cultures were observed after five days of culturing and data was recorded on the mortality and number of contaminated and survived (clean) cultures. The data recorded was converted into percentage.

RESULTS AND DISCUSSION

Contamination in in-vitro culture results in considerable economical losses [13].Sterilization is therefore an important step which determines the success or failure of the tissue culture process as inadequate sterilization protocols compromises in-vitro culture techniques. The efficacy of sterilization is dependent on the type, concentration and time of exposure to the sterilant(s) used.

Sterilization with 70% ethanol: a common sterilization agent routinely used for sterilization in the laboratory, is ethanol at a concentration of 70% (v/v). Despite being a powerful sterilization agent, it is an ineffective sterilant when used alone. This is because explants culture contamination and high mortality is recorded with 70% ethanol sterilization treatment of the explants as seen in the explants cultures in Plate 1(A & B).

A B

C **D**

Plate 1 (A & B) contaminated explants cultures with 70% ethanol sterilization; (C) clean *Pleurotus tuber-regium* explants culture (D) clean *Auricularia auricula-judae* explants culture

Furthermore the cyto-toxicity of ethanol is evident from the results in Table 2. Explants exposure to ethanol for more than two minutes resulted in 100% mortality in *P. tuber-regium* while for *Auricularia auricula*, mortality increased following treatment of explants with 70% ethanol beyond one min. The unsuitability of 70% ethanol treatment alone as a sterilization method has also been recorded by other authors [12], [14].

	Mortality (%)	
Time (Mins)	***Pleurotus tuber-regium***	***Auricularia auricula***
1	0	10
2	10	95
5	100	100
10	100	100
15	100	100

Table 2 Effect of sterilization with 70% ethanol alone on explants mortality

Sodium hypochorite sterilization with 70% ethanol pre-treatments: Hypochlorite is a well-known and widely used anti-bacterial agent so much so that even micromolar concentrations show antibacterial activity [14]. Sodium hypochlorite is the most frequent choice for surface sterilization and has been used as a sterilant by many researchers [12], [14], [15-17]. Various researchers have used a combination of sterilants in different concentrations to achieve desired results and have developed surface sterilization protocols for various plants. Ethanol is sometimes used prior to explants treatment with other sterilants [12]. But the duration of pre-treatment with ethanol has to be determined empirically for each explants type. In this study, ethanol pre-treatment time was found to be one minute for *Auricularia auricula* and two minutes for *Pleurotus tuber-regium*. Following the ethanol treatments, the mushroom explants were treated with 2.5% and 5.0% sodium hypochorite solutions at varied time intervals of one, two, five, ten and fifteen minutes. For *Auricularia auricula* (Figure 2), the highest percentage contamination was recorded with treatment of the explants with 2.5% sodium hypochlorite alone. A reduction in percentage contamination was observed with an increase of the sodium hypochlorite solution concentration to 5.0% (v/v), ethanol pre-treatment of the explants and increase in the time of exposure of the explants to the sterilants. To determine the most effective sterilization method, a balance must be determined between explants level of contamination obtained by a sterilization method and the level of survival of the explants after such treatments. Thus comparing the percentage contamination in Figure 2 and survival from the data in Figure 3, the least contamination and highest survival was recorded with ethanol pre-treatment of the *Auricularia auricula-judae* explants for one minute followed by 5.0% sodium hypochlorite solution for five minutes. This treatment resulted in clean cultures shown in Plate 1(D).

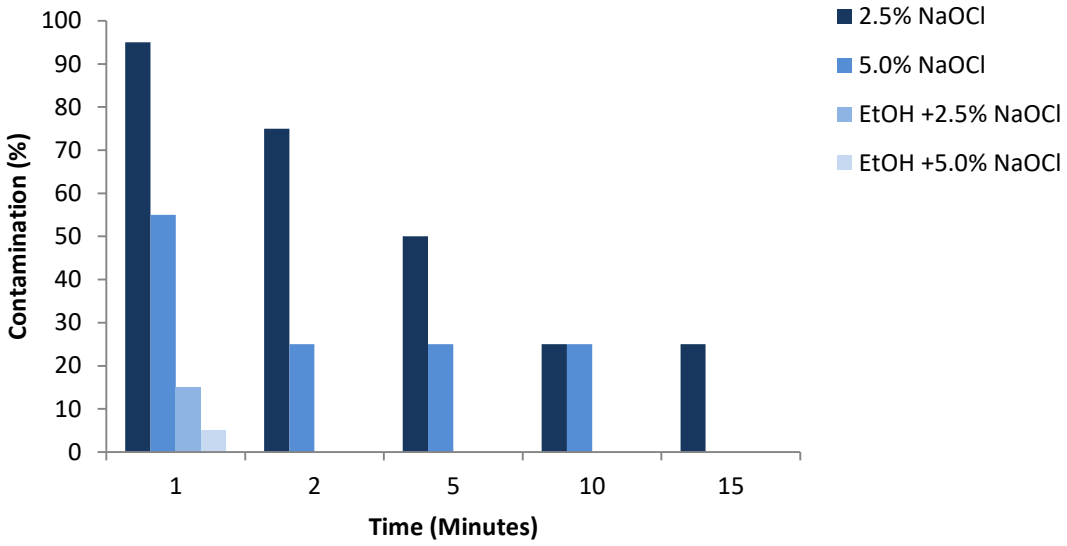

Figure 2 Contamination (%) of Auricularia auricula-judae explants culture sterilized with ethanol and sodium hypochlorite solution

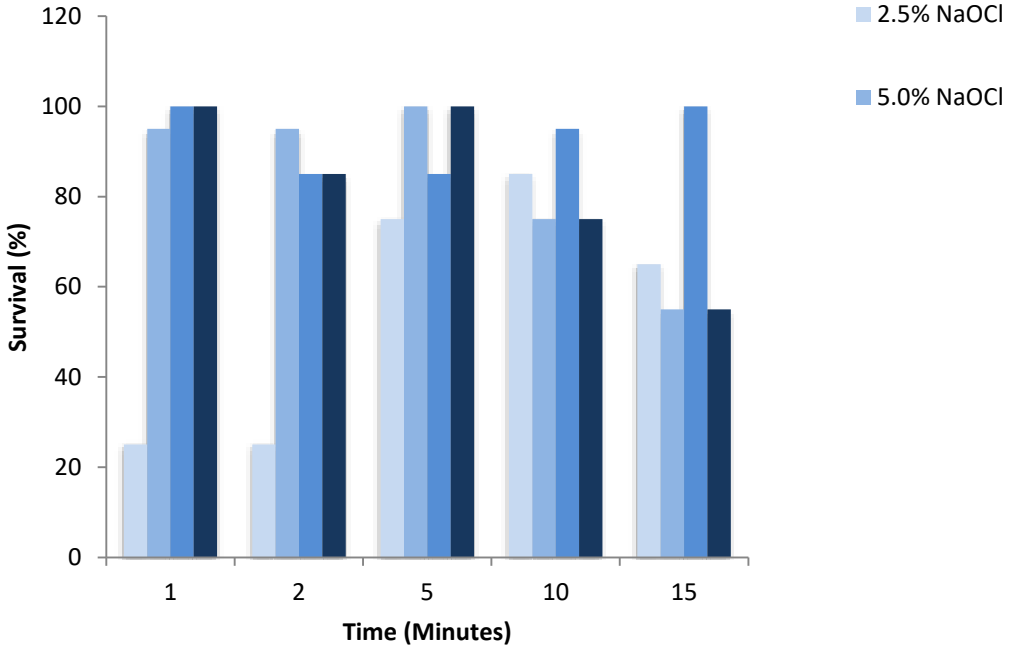

Figure 3 Survival (%) of Auricularia auricula-judae explants culture sterilized with ethanol and sodium hypochlorite solution

For Pleurotus tuber-regium, a comparison of the percentage contamination (Figure 4) and percentage survival (Figure 5) shows that pre-treatment of the explants with ethanol for two minutes followed by 5.0% sodium hypochlorite solution for two to ten minutes gave the highest explants survival and least contamination resulting in clean cultures

shown in Plate1(C). Generally *Pleurotus tuber-regium* explants had a higher survival rate than those of *Auricularia auricula*. This could be correlated with their physical properties as "Osu" which is the *Pleurotus tuber-regium* sclerotium used in this study is made up of hardened mycelia while *Auricularia auricula* has soft gelatinous tissues. For both mushrooms, increasing the exposure time of the explants to the sterilants increases the mortality of the explants. This has also been observed for peach plant, Juncus effuses L., sweet potato and oil palm [14], [18-20].

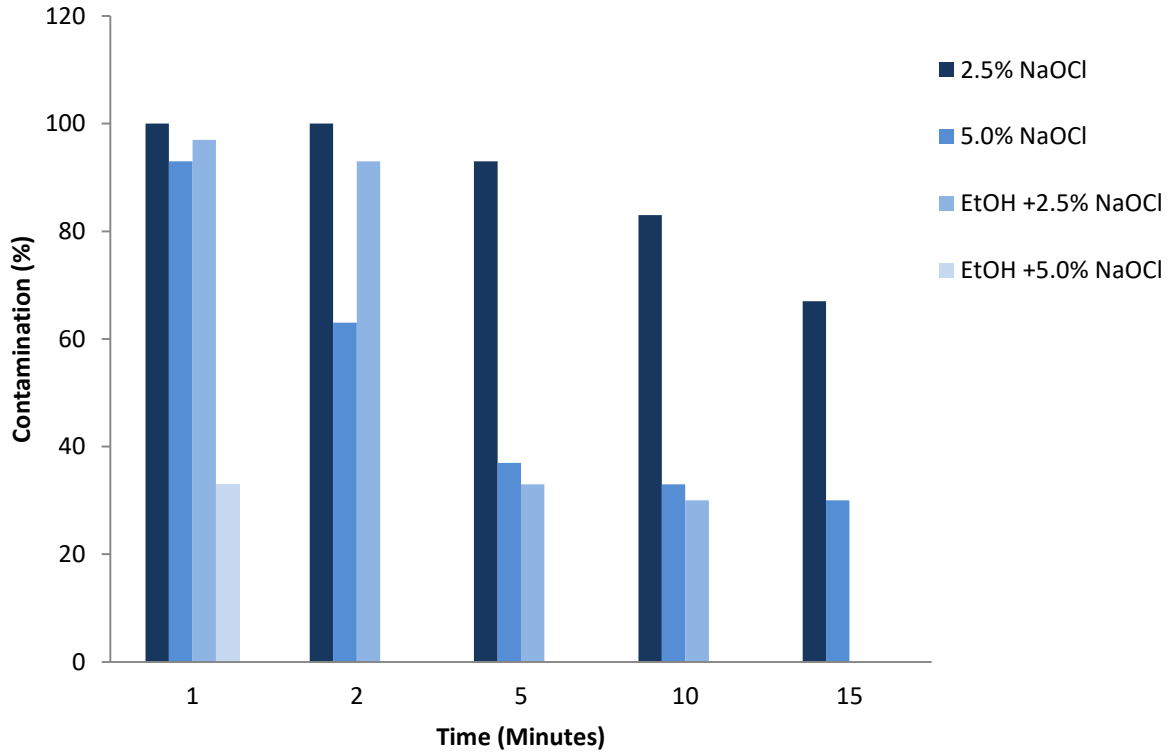

Figure 4 Contamination (%) of Pleurotus tuber-regium explants culture sterilized with ethanol and sodium hypochlorite solution

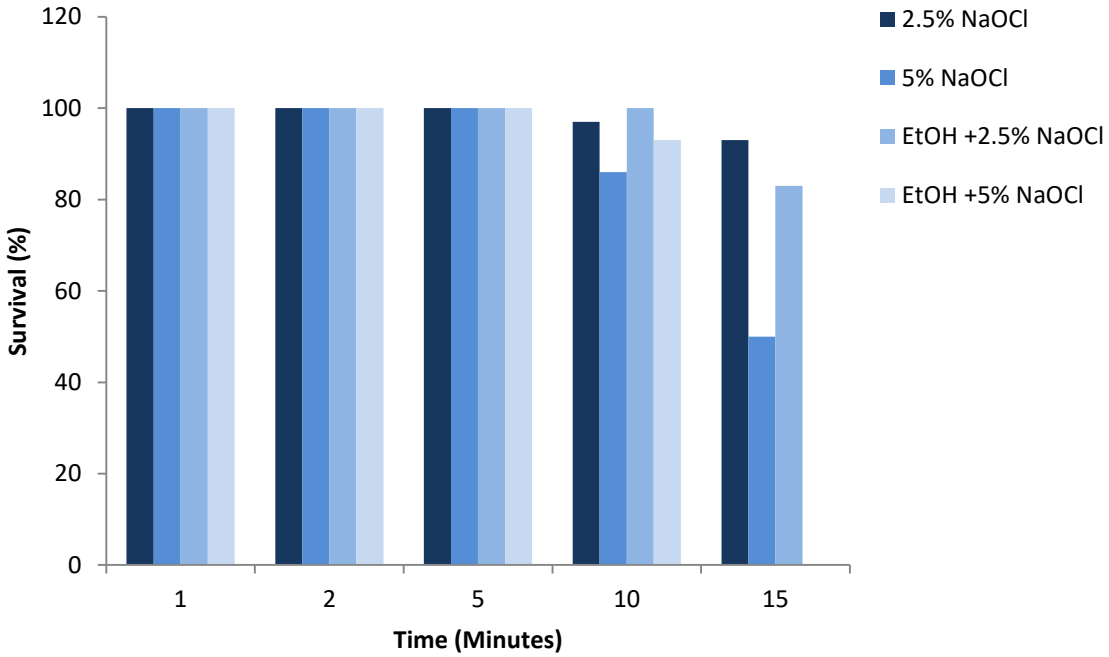

Figure 5 Survival (%) of Pleurotus tuber-regium explants culture sterilized with ethanol and sodium hypochlorite solution

CONCLUSION

This study shows that a combination of 70% ethanol pre-treatment for one minute and 5.0 % sodium hypochlorite treatment of *Auricularia auricula-judae* explants for five minutes is the most effective surface sterilization method for the mushroom. Similarly, 70% ethanol pre-treatment for two minutes followed by 5.0% sodium hypochlorite treatment for ten minutes is the most effective sterilization method for *Pleurotus tuber-regium* sclerotium.

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