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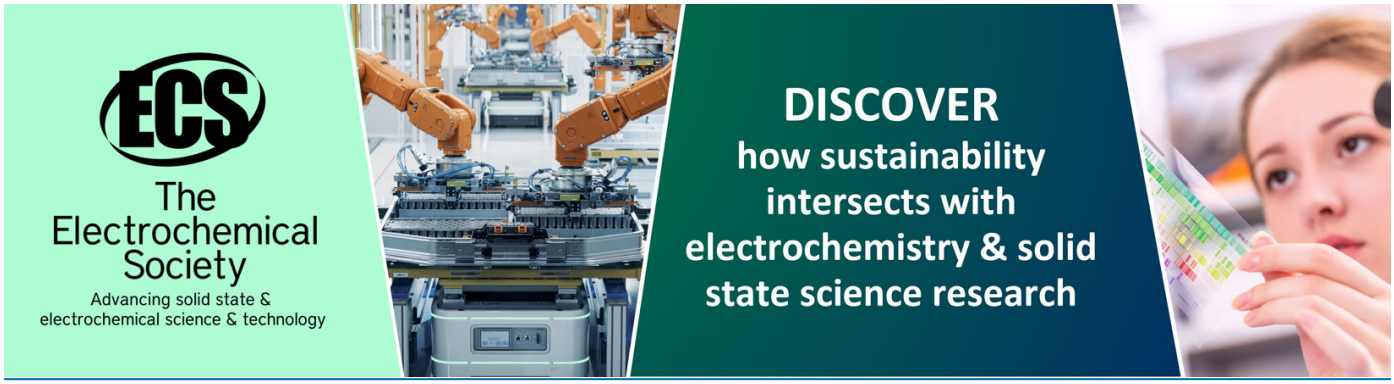

Establishing surface sterilization protocol for nodal culture of *Solanecio biafrae*.

[1]Bello Oluwakemi A, [2]Esan Edward. B and [1]Obembe Olawole. O.

[1]Department of Biological Sciences, College of Science and Technology, Covenant University, P. M. B 1023 Canaanland Ota, Ogun State, Nigeria.
[2]Department of Basic Sciences, Babcock University, Ilishan-Remo, Ogun State Nigeria.

Correspondence email: olawole.obembe@covenantuniversity.edu.ng

ABSTRACT
Surface sterilization of plant materials is a very problematic step in establishing plant tissue culture protocol. Prior to introduction into sterile Murashige and Skoog (MS) media, nodal explants obtained from potted plants of *Solanecio biafrae* grown in the screenhouse were given various surface sterilization treatments. A total of 16 treatments with varying time of immersion in two sterilant successions, 70% (v/v) ethanol (20 s, 1 min, 3 min and 5 min) and 10% (w/v) calcium hypochlorite ($Ca(ClO)_2$) granules (5, 10, 15 and 20 min) at varying times and then rinsed three times with sterile distilled water. 100% clean culture was obtained from treatments containing 70% ethanol (20 s)+10% $Ca(ClO)_2$ (15 min) and 70% ethanol (3 min)+10% $Ca(ClO)_2$ (20 min) after 4 weeks in culture. 70% ethanol (20 s)+10% $Ca(ClO)_2$ (15 min) is proposed as the best surface sterilization protocol as it gave 90% growth compared to 60% growth recorded in 70% ethanol (3 min)+10% $Ca(ClO)_2$ (20 min). This is a preliminary study as more research is ongoing to establish an efficient, effective and reproducible protocol for the surface sterilization of different explants used for the tissue culture of *S. biafrae* obtained from open field.
Keywords: Murashige & Skoog, calcium hypochlorite ($Ca(ClO)_2$, surface sterilization, nodal explant, *Solanecio biafrae*.

1. INTRODUCTION
Vegetative shoot serve as a good starting material/explant for *in vitro* culture of plants (Obembe, 2000). However, the heavy microbial contamination loads they harbour, especially the axillary nodes, make *in vitro* culture establishment problematic. This problem of microbial contamination is usually overcome through effective surface sterilization of explants among other aseptic techniques. Surface sterilization of explant is a process which involves the immersion of explants into appropriate concentration of chemical sterilant(s) or disinfectant(s) for a specified time resulting in the establishment of a contamination-free culture. Literature showed that various types of disinfectants are used, they include ethanol (or isopropyl alcohol), hypochlorite-sodium (NaOCl)/calcium ($Ca(ClO)_2$), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$) and bromine water (Abrham, 2011; Singh *et al*., 2011; Teixeira da Silva *et al*., 2016). However, the required type, concentration and time of exposure of the disinfectant(s) differ for different plant and plant parts (Srivastava *et al*., 2010). Ethanol is a sterilizing agent that is so powerful but is also phytotoxic. It is generally used at the concentration of 70% (v/v) (Abbasi *et al*., 2016) for only a few seconds or minutes, followed by treatment with other disinfectant(s). Hypochlorite, on the other hand, is a very effective bacteria killer which reduces bacterial populations significantly, even with concentrations at micromolar level. However, sodium hypochlorite is quite aggressive for explant disinfection which makes the use of calcium hypochlorite, being a mild sterilant preferable (Abbasi *et al*., 2016; Badoni and Chauhan, 2009; Cruz-Martínez *et al*., 2017). Among treatments with three sterilizing agents ($Ca(ClO)_2$, NaOCl and $HgCl_2$) for sterilization of *Ziziphus spina-christi* (L.) Desf., Assareh and Sardabi

(2005) reported that $Ca(ClO)_2$ was satisfactory. The objective of this study is to develop a surface sterilization protocol for *in vitro* establishment of *Solanecio biafrae* nodal explants using available industrial grade ethanol and calcium hypochlorite granules.

2. MATERIALS AND METHODS

Nodal explants of about 2-3 cm were taken from new growth flushes from potted plants of *Solanecio biafrae* grown in the screenhouse.. They were thoroughly washed under running tap water with liquid detergent and immersed in 70% ethanol for varying exposure time (20 seconds, 1 minute, 3 minutes and 5 minutes) followed by surface sterilization with 10% $Ca(ClO)_2$ for different exposure time (5, 10, 15 and 20 minutes) and then rinsed with three consecutive changes of sterile distilled water. The sterilized single node explants had their bleached ends trimmed and were cultured on Murashige and Skoog (MS) basal (Murashige and Skoog, 1962) supplemented with 3% sucrose, pH 5.8, 0.8% agar and autoclaved at 121°C for 15 min. The sterilization and transfer procedures were carried out in the Laminar flow hood. The cultures were kept in the growth room with 16 hr photoperiod under 3000 lux light intensity at 25±2°C. The experiment had 10 replicates in each treatment and arranged in Completely Randomized Design. They were maintained for 4 weeks and observations on percentage clean cultures, germination frequency and tissue survival were recorded.

3. RESULTS AND DISCUSSION

All type of explants including seeds, spadices or spathes and leaves have been frequently surface sterilized with calcium hypochlorite, either alone or in combination with ethanol (70%) (Teixeira da Silva *et al*., 2015). The result of this study show that increasing exposure time for 70% ethanol was not effective in removing contaminants from the nodal explants of *Solanecio biafrae*. This may be due to the decontaminant toxicity as the concentration increased (Rodrigues *et al*., 2013). The combinatorial effect of 70% ethanol and 10% calcium hypochlorite, however, could be used to obtain high contamination-free culture.

Figure 1a showed that at the exposure time of 20 sec, T3 gave the best result (100% clean culture) followed by T4 (79%). T1 gave the least percentage clean culture (54%). The percentage clean culture increased as the time of exposure increased from 5 to 15 min but a further increase in exposure time to 20 min resulted in decline of percentage clean culture. This agrees with Obembe (2000) which reported that step-wise treatment of 70% ethanol for 20 seconds and 10% (w/v) $Ca(ClO)_2$ for 10 minutes resulted in the best sterilization procedure for *Cola nitida*; and Slusarkiewicz-Jarzina *et al*. (2005) when surface sterilization of seeds of *Cannabis sativa* in $Ca(ClO)_2$ solution for 15 minutes gave the best result in comparison to exposure time of 6 and 8 minutes. At one minute exposure time, T6 gave the best result (88% clean culture) while other treatments resulted in the same percentage clean cultures (54%) (Figure 1b). This disagrees with dos Santos *et al*. (2015) that reported immersion of leaf explants in70% ethanol for 1 minutes and soaked in 10% $Ca(ClO)_2$ for 30 minutes being the most effective and resulting in 90% disinfection. When the exposure time was further increased to 3 minutes (Figure 1c), the percentage clean culture increased with increasing exposure time. T12 gave the best result (100% clean culture) while T9 gave the least percentage clean culture (20%). A further increase in the exposure time for 5 minutes showed that the percentage clean culture decreased as the time of exposure increased. T13 gave the best result (100% clean culture) followed by T14 (77%) while T16 gave the least percentage clean culture (40%). Pretreatment of *Launaea taraxacifolia* with 70% ethanol for 5 min, followed by step-wise double disinfection in 10% and 15% sodium hypochlorite was also reported to give satisfactory result (Obembe *et al*., 2017).

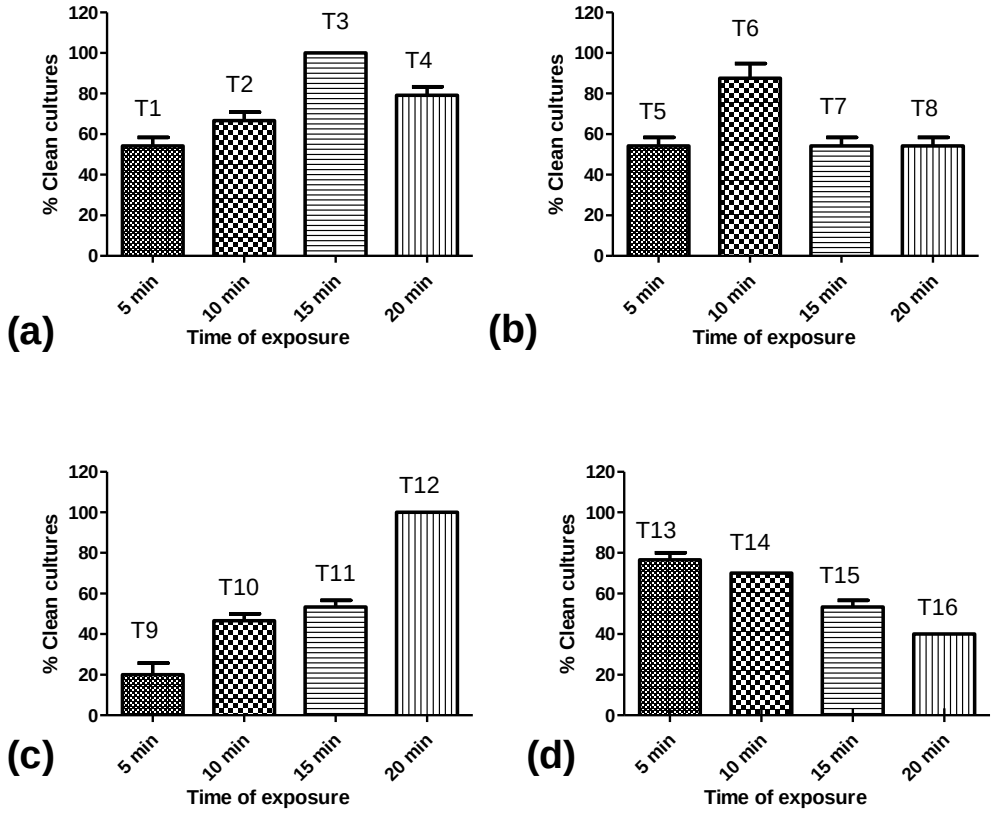

Figure 1: The percentage of clean culture of S. biafrae with 70% ethanol at varying exposure time of (a) 20 seconds, (b) 1 minute, (c) 3 minutes, (d) 5 minutes and 10% Ca(ClO)2 at varied exposure time (5, 10, 15 and 20 minutes).

The overview of the result showed that the most effective treatments for disinfection were T3 (70% ethanol for 20 seconds followed by 10% calcium hypochlorite for 15 minutes and T12 (70% for 3 minutes followed by 10% calcium hypochlorite for 20 minutes), which resulted in 100% of the explants without contamination after four weeks in culture. However, with the increase in exposure time of the sterilants, survival rate was hampered. The survival obtained with T3 (70% ethanol for 20 seconds+10% calcium hypochlorite for 15 minutes) had 90% survival and growth compared to 60% recorded in T12 (70% ethanol for 3 minutes+10% calcium hypochlorite for 15 minutes) (Figure 2a&b).

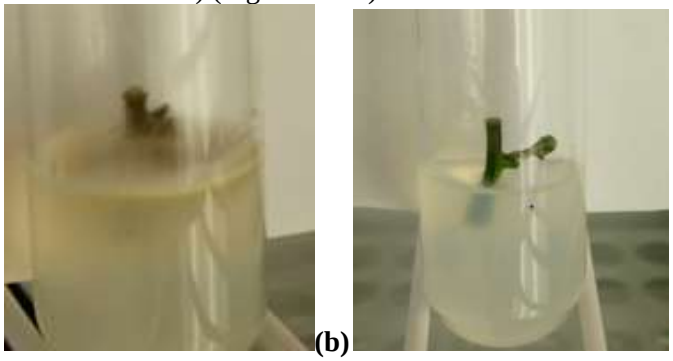

Figure 2(a) contaminated culture (b) clean culture with sprout

4. CONCLUSION

Disinfection of *S. biafrae* nodal explants collected from screen house can be achieved at a level of 90% with the immersion of the explants in 70% ethanol (20 s)+10% Ca(ClO)2 (15 min) and 70% ethanol (3 min)+10% Ca(ClO)2 (20 min). Meanwhile for survival and growth, 70% ethanol (20 s)+10% Ca(ClO)2 (15 min) is to be adopted.

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