Surface sterilization of *Ocimum* seeds and tissues with biosynthesized nanosilver and its effects on callus induction

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Abstract

Plant tissue culture is a basic and fundamental component of plant biotechnology. Nowadays, nanomaterials especially nanosilver (NS) are being used as an antimicrobial agents for surface sterilization of explants in tissue culture. In this study, biosynthesized nanosilver (BNS) was used for the surface sterilization of *Ocimum* seeds and tissues and its effects on callus induction were evaluated. The seeds and tissues were exposed to different concentrations of BNS (10, 50 and 100 mg/l) as well as 5% Clorox for five exposure times (5, 10, 20, 30 and 60 min) and effects on germination, callus induction and surface sterilization were determined. The BNS was found very effective on surface sterilization as 100% decontamination was achieved with no adverse effect on explant viability and callus formation but rather had stimulating effect on formation of callus. The study concluded that BNS can be used as an antimicrobial agent in surface disinfection of explants therefore extending the frontiers of the potential application of biosynthesized nanosilver in tissue culture.

Keywords: Tissue culture, *Ocimum*, callus, nanosilver, disinfection, plant biotechnology

1. Introduction

Nanotechnology is the study of nano-objects having sizes generally between 1-100 nm [1]. "Nanobiotechnology" is a term that blends nanotechnology and biotechnology terms and is the crossing point of nanotechnology, materials science, molecular biology and biomedical sciences. In both nanobiotechnology and nanotechnology, the principal zone of interest is nanoparticles [2]. Nowadays, plant extracts and microorganisms are presently utilized as bioreductors in nanobiotechnological studies so as to synthesize nanoparticles from metal ions. This technique is termed as “bio-synthesis” or “green synthesis” of nanoparticles [3]. It is an emerging alternative
technology as it is ecofriendly, compared to conventional physical and chemical synthesis methods, which are energy demanding, expensive, and often generate toxic chemicals and vapours [4].

Silver nanoparticles are the most generally utilized among the other metal nanoparticles. They have antiseptic, antibacterial, antifungal and antiviral properties relying upon their capacity to attack a wide range of organic processes in microorganisms prompting the disruption of cell membrane structure and the breakdown of plasma membrane potential. This subsequently leads to depletion in the intracellular ATP and in this way guaranteeing the cell death. In addition, they are eco-friendly and biocompatible with low lethality towards the earth and human [2].

Presently, plant tissue culture has direct commercial importance, and its applications in basic research such as genetics, biochemistry, cell biology, and biotechnology are evidences for its usefulness. Plant tissue culture does not only provide a method for mass propagation of tissues but also makes possible the production of disease-free and genetically modified plants. It also provides a way for secondary metabolite production [5]. Despite all the advantages of this technique, some methodological obstacles, mainly the contamination of explants, hinders its exploitation as an efficient technique for biotechnological research. Both external and internal contaminations of plant tissues during tissue culture process turn out to be a prevailing problem. This is because microorganisms, mostly fungi, and bacteria which are major contaminants, grow much faster than plant cells in growth media and take up all the nutrients, thus preventing the plants from growing [6].

Surface sterilization is the most important step in plant tissue culture as it is a pre-requisite for tissue culture techniques to be carried out under sterile conditions. Microbial growth rates far exceed plant cell division in culture. Thus, surface sterilization endeavours to remove all microorganisms that will thrive under *in vitro* conditions whilst still maintaining explant viability [7]. Mostly utilized agents for surface sterilization in tissue culture are sodium hypochlorite, ethanol, calcium hypochlorite, mercuric chloride, surfactants, and antibiotics. They are not ecofriendly and are toxic to living organisms. They also have very toxic effects on plant cells and tissues that are cultured. Their effects range from browning of the tissues to complete inhibition of growth in plant cells and tissues under culture conditions [8]. Therefore, an appropriate surface sterilizing agent is very important as the key to successful procedures for plant tissue culture.
Although, there are a variety of techniques to reduce the possibility of fungal and bacterial contaminations during in vitro propagation, examples of such include repetitive sub-culturing and meristem culture (the pathogen-free part of the plant). However, designing a more effective approach to sterilize plant tissues still seems inevitable to eliminate the labour-intensive trial and errors and the time-consuming decontamination approaches in existence. In order to eliminate the tenacious bacterial and fungal contaminations in plant tissue culture, treatment with antibiotics and antifungal agents have been used which greatly lessen the contaminations. However, it has been reported that antibiotics are usually phytotoxic, and have an inhibitory effect on cell multiplication, regeneration, callus induction, and explants’ survival [9]. Moreover, lack of an optimal protocol for sterilization of field, greenhouse-derived (ex vitro) or orchard explants may result in a scarcity of samples for further tissue culture research [9].

The genus *Ocimum* (family-Lamiaceae), is a versatile aromatic genus well known for its economically important essential oils of enormous medicinal properties [10]. Therefore, in this study, silver nanoparticles synthesized via biological procedure and 5% Clorox were used as surface sterilization agents on the seeds of five *Ocimum* species and tissues of three *Ocimum* species. This was with a view to evaluate the efficiency of BNS as a surface sterilization agent in tissue culture processes compared to 5% Clorox as a standard.

2. Materials and Methods

This research was carried out at the Tissue Culture Laboratory, National Centre for Genetic Resources and Biotechnology (NACGRAB), Moor Plantation, Ibadan, Nigeria. Seeds of five *Ocimum* species (*O. basilicum*, *O. gratissimum*, *O. africanum*, *O. canum* and *O. sanctum*) and tissues (leaves, stems and inflorescence) of three *Ocimum* species (*O. basilicum*, *O. canum* and *O. sanctum*) collected from screen house grown plants were used. The nanosilver (BNS) used was Kola pod (*Cola nitida*) mediated silver nanoparticles [11] obtained from the Nanotechnology Research Group (*NANO*), Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria. The properties of this nanosilver are shown in Table1.
Table 1. Properties of Kola pod (*Cola nitida*) mediated biogenic synthesized silver nanoparticles used [11]

| Properties                  | Mode of synthesis                          | Max absorbance | Size     | Stability                  | Dispersity         | Shape         | Phase                        | Colour         |
|------------------------------|---------------------------------------------|----------------|----------|----------------------------|-------------------|---------------|-------------------------------|----------------|
|                              | By kolanut (*Cola nitida*) pod extract       | 431.5 nm       | 12-80 nm | Stable, without aggregation| Polydisperse       | Fairly spherical | Face-centered cubic crystalline| Dark brown     |

2.1 *Ocimum* Seeds Sterilization

Prior to the sterilization processes, the seeds were poured in a test tube and filled with water. The floated seeds were regarded as non-viable seeds and were sieved. Prior to the BNS applications, 70% ethanol was first applied for 3 min to the seeds and they were rinsed with sterile distilled water twice. The seeds were later exposed to 100, 50 and 10 mg/l BNS with five exposure times (5, 10, 20, 30 and 60 min). Two control groups were used; negative control group where sterile distilled water was used and the positive control group where 5% commercial Clorox bleach (8.25% active sodium hypochlorite) was used with five exposure times (5, 10, 20, 30 and 60 min) for surface sterilization. They were rinsed in sterile distilled water five times and inoculated on semi solid MS medium (MS basal salts supplemented with 3% sucrose (30 g/L) and 0.1% myoinositol (0.1 g/L), with pH 5.8 ± 0.1) [12]. The cultures were maintained in a growth chamber at 25± 2°C with light intensity of 10-20 μmol m⁻² s⁻¹ provided by white fluorescent bulbs for photoperiod of 16 h of light and 8 h of darkness. The cultures were observed daily for the commencement of seed germination and at the second week, percentage of sterile and germinated seeds were determined by observations of seeds in culture condition. All the surface sterilization processes were conducted under the laminar flow hood to maintain a sterile environment.
2.2 Plant Tissues Sterilization

Plants parts with trichomes (especially with glandular trichomes) such as the leaf, the stem and the inflorescence, were collected from screen house (at flowering stage). These parts were collected in a clean beaker and washed under running tap water for 30 min to wash off adhering dirt coming along with them from the screen house. They were surface sterilized by dipping in concentrations of BNS (100 m, 50 and 10 mg/l) with five exposure times (5, 10, 20, 30 and 60 min). Two control groups were used; negative control group where sterile distilled water was used and the positive control group where 5% commercial Clorox bleach (8.25% active sodium hypochlorite) was used with five exposure times (5, 10, 20, 30 and 60 min) for surface sterilization. They were later rinsed in sterile distilled water five times.

Glandular trichomes were excised from the sterilized plants part (the leaf, the stem and the flower) using a sterile scalpel and forceps with the aid of a dissecting microscope. The glandular trichomes were carefully excised from the epidermal wall without contamination by the epidermal cells and were then inoculated on callus induction media (MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BAP for O. basilicum and O.sanctum and MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP for O. canum). The cultures were maintained in a growth chamber at 25± 2 °C with light intensity of 10-20 μmol m$^{-2}$ s$^{-1}$ provided by white fluorescent bulbs for photoperiod of 16 h of light and 8 h of darkness. The cultures were observed daily for the commencement of callus formation and at the 10th day of culture, percentage of disinfected glandular trichome explants and percentage of explants that induced callus were observed. The intensity of the callus formed was observed after 3 weeks of culture. All the surface sterilization and the culture processes were conducted under the laminar flow hood to maintain a sterile environment.

2.3 Statistical Analysis

Each treatment of 10 samples were replicated thrice, totaling 30 explants per treatment ($n \geq 30$) in order to have an approximately normal distribution of the sample mean [13]. Data were statistically analyzed using one-way Analysis of Variance (ANOVA) with significant means separated using Duncan’s Multiple Range Test (DMRT) at $P = 0.05$, on IBM Statistical Package for Social Science (SPSS) statistics version 25 software. Results were presented as mean ± standard deviation (SD).
3. Results and Discussion

3.1 Effects of Different Concentrations and Exposures of BNS on *Ocimum* Seeds

The percentages of disinfected seeds were observed after 10 days of culture and the control bottles with full contaminated seeds were discarded. Daily observations were made on the remaining cultures to check for any contamination again and no contaminations were recorded after the 10th day in all the treatments. The percentage germinated seeds were observed after 14 days of culture (Table 2).

The seeds of *O. canum* and *O. basilicum* started sprouting after 4 days of culture, while *O. sanctum*, *O. africanum* and *O. gratissimum* started sprouting after the 6th day of culture. No adverse effect was observed on sterilized plantlets compared to the positive control groups. They all started to germinate within the same days with approximately the same height as the controls.

In the negative control group where sterile distilled water was used as disinfectant, the cultures were heavily contaminated and no growth was observed. The contaminants were observed on the seeds after 2 days of culture and grew larger rapidly till they cover the whole surface of the medium as shown in Plate 1C. But in the BNS treated group where full contaminations were not noticed, the contaminants were observed on the seeds after 5 days of culture which was 3 days later than the negative control group. The rate of growth of the contaminants were not as rapid as in the negative control group as shown in the 10 days old culture in plates 1A, D and E. The contaminants were seen mounted on the seeds, thus preventing their growth as seen in Figures 1A, B, D and E where some of the contaminated seeds showed expression of root but no shoot.

The results showed that all used concentrations of BNS were efficient in decontaminating the *Ocimum* plant seeds with varying exposure times and even at higher concentration of 100 mg/l with 60 min exposure time, the BNS seems not to have any harmful effect on the seeds germination.

Nartop [14] also reported successful decontamination (100%) of Lamiaceae seeds with green synthesized silver nanoparticles with no lethal effect on the seeds viability. Although not all disinfected seed showed 100% germination, this does not seem to be associated with the effect of the BNS as there were no significant differences in the percentage germinated where 100% decontaminations were achieved. In some instances, 100% seed germinations were recorded at some high concentration and longer exposure time, as seen in *O. canum* seed. The disparity in percentage germinations could be ascribed to the seeds viability as low seed viability of *Ocimum*
species had been reported [15, 16]. Also it was reported that Ocimum species germination rate varies [15].

When the seeds were treated with very low concentration of BNS (10 mg/l) for 5 min, 100% decontamination was not achieved. However 96% was achieved in O. canum being the highest percentage decontamination followed by 83, 66, 63 and 56% achieved in O. bacilicum, O. sanctum, O. gratissimum and O. africanum respectively. Notwithstanding 100% decontamination was achieved when they were treated with the same concentration for 10 min in O. bacilicum and O. canum followed by 96% in O. africanum and 83% in O. gratissimum and O. sanctum seeds. At 20, 30 and 60 min exposure time with the same concentration of BNS, 100% decontamination of the seeds were achieved in all the species. This shows that the BNS possessed great antimicrobial properties at a very low concentration which is similar to the report of Matsumura et al. [17] that the toxicity of nanosilver to microbial cells is evident even at very low concentration. Although in a related research conducted by Mahna et al. [18] on Arabidopsis seeds, 10 mg/l was not effective in decontaminating the seeds. It was reported that plant-derived silver nanoparticles demonstrated better antimicrobial activity than others synthesized through physical and chemical methods [19], which is in consonance with the result obtained in this study.

When the seeds were treated with 100 mg/l BNS for 5 min, 100% decontamination was achieved in all the species with the exception of O. africanum (93% decontamination). However, with an increase in the exposure time to 10, 20, 30 and 60 min, 100% decontaminations were achieved in all the species without affecting the seed viability. This differs to the result reported by Mahna et al. [18], where treatment of the Arabidopsis seeds with 100 mg/l of non-biosynthesized nanosilver (Nanocid L-2000) for 30 and 60 min resulted in 100% decontamination but none of the seed survived. This shows that BNS is more plant-friendly than other silver nanoparticles synthesized by other methods. Nartop [14] also reported that green synthesized nanosilver showed no negative effect on both germination and morphology of plantlets when used to sterilize Lamiaceae seeds.

In this study, the exposure time was a critical parameter in decontamination of the explants. When BNS was used at higher concentrations of 100 mg/l for 5 min, 100% decontamination was not achieved but as the exposure time increases, full decontaminations were achieved even at the lowest concentration of 10 mg/l. This corroborates the work of Abdi et al. [20], which is the first
time NS was used in tissue culture, where 89% decontamination was achieved. Mahna et al. [18] also achieved 100% decontamination with treatment of Arabidopsis seeds in 100 mg/l AgNPs for 1 to 5 min.

In the positive control group where 5% Clorox was used, 100% decontamination was achieved in all the exposure times used in all the species. However, the exposure time of the seeds to the treatment was a critical parameter. When the 5% Clorox was used with short exposure time (5 and 10 min) for the seeds, it functioned as an antimicrobial agent leaving no harm on the seeds and their germination. High germination percentages of up to 86% in *O. africanum*, 96% in *O. basilicum* and *O. canum* and 90% in *O. gratissimum* and *O. sanctum* were observed. However with an increase in the exposure time to 20, 30 and 60 min, there was drastic reduction in the germination percentages (Table 2). Seeds germination of only 10% in *O. africanum*, 6.67% in *O. basilicum*, 3.33% in *O. gratissimum* and 0% in *O. canum* and *O. sanctum* were observed when exposed to the Clorox solution for 60 min. These low germination percentages could be as a result of the prolonged exposure of the explants to sodium hypochlorite (NaOCl). Yildiz and Ekiz [21] opined that direct contact of explant with NaOCl during the sterilization process can have a severe effect on regeneration capacity.
Table 2. Seed germination percentage of surface sterilized *Ocimum* seeds with biosynthesized nanosilver

| Sterilization Methods | *O. africum* seed | *O. basilicum* seed | *O. canum* seed | *O. gratissimum* seed | *O. sanctum* seed |
|-----------------------|------------------|------------------|----------------|----------------------|------------------|
| BNS (mg/l)            | (%) D ± SD       | (%) G ± SE       | (%) D ± SE     | (%) G ± SE           | (%) D ± SE       |
| 10                    | 56.67±5.77b      | 50.00±10.00c     | 83.33±5.77b    | 66.67±5.77b          | 66.67±5.77b     |
| 10                    | 93.33±5.77a      | 83.33±5.77b     | 100.00±0.00a   | 96.67±5.77a          | 73.33±5.77b     |
| 20                    | 100.00±0.00a     | 96.67±5.77a     | 100.00±0.00a   | 96.67±5.77a          | 93.33±5.77a     |
| 30                    | 100.00±0.00a     | 90.00±0.00ab    | 100.00±0.00a   | 96.67±5.77a          | 96.67±5.77a     |
| 60                    | 100.00±0.00a     | 93.33±5.77a     | 100.00±0.00a   | 96.67±5.77a          | 93.33±5.77a     |
| 50                    | 63.33±5.77b      | 50.00±10.00c     | 100.00±0.00a   | 96.67±5.77b          | 83.33±5.77b     |
| 10                    | 100.00±0.00a     | 86.67±5.77b     | 100.00±0.00a   | 93.33±5.77a          | 100.00±0.00a    |
| 20                    | 100.00±0.00a     | 90.00±0.00ab    | 100.00±0.00a   | 96.67±5.77a          | 93.33±5.77a     |
| 30                    | 100.00±0.00a     | 90.00±0.00ab    | 100.00±0.00a   | 96.67±5.77a          | 96.67±5.77a     |
| 60                    | 100.00±0.00a     | 96.67±5.77a     | 100.00±0.00a   | 96.67±5.77a          | 93.33±5.77a     |
| 100                   | 93.33±5.77b      | 86.67±5.77a     | 100.00±0.00a   | 90.00±10.00a          | 90.00±10.00a    |
| 10                    | 100.00±0.00a     | 100.00±0.00a    | 100.00±0.00a   | 93.33±5.77a          | 100.00±0.00a    |
| 20                    | 100.00±0.00a     | 90.00±10.00a    | 100.00±0.00a   | 96.67±5.77a          | 93.33±5.77a     |
| 30                    | 93.33±5.77a      | 93.33±11.55a    | 100.00±0.00a   | 96.67±5.77a          | 96.67±5.77a     |
| 60                    | 93.33±5.77a      | 96.67±5.77a     | 100.00±0.00a   | 96.67±5.77a          | 96.67±5.77a     |

Values within column followed by the same letter are not significantly different at P = 0.05; (%) D ± SD is the percentage decontaminated seeds ± standard deviation and, (%) G ± SD is the percentage of germinated seed ± standard deviation.
Figure 1. *Ocimum basilicum* culture showing contamination mounted on the seed surface sterilized with 5 mg/l BNS for 5 min (red arrow) (A), *O. canum* contaminated seed showing root but no shoot when treated with 5 mg/l BNS for 5 min (red arrow=contaminant) (green arrow=root) (B), *O. africanum* negative control (seed treated with sterile distilled water) culture showing full contamination (C), *O. gratissimum* culture showing contaminated, non-germinated seeds (red arrows); non-contaminated, non-germinated seeds (blue arrows); non-contaminated, germinated seed (yellow arrow) (D), and *O. sanctum* culture showing contaminated, non-germinated seed (red arrow); non-contaminated, non-germinated seed (blue arrow); non-contaminated, germinated seeds (yellow arrows) (E)
3.2 Effects of Different Concentrations and Exposures to BNS on Glandular Trichome Explants and Callus Culture of Ocimum species

The percentage of disinfected glandular trichome explants were calculated after 10 days of culture when all the calli have been formed in all the treatments. The bottles with contaminations were discarded while daily observations were made on the uncontaminated calli for any contamination, and no contaminations were recorded after the 10th day in all the treatments and species. The percentages of explants that induced callus were observed after the 10th day of culture. The intensity of the callus formed was determined after 3 weeks of culture. The data were presented in Table 3 for O. basilicum, Table 4 for O. canum and Table 5 for O. sanctum.

In the negative control where sterile distilled water was used to disinfect the explants, the cultures were heavily contaminated and no growth was observed as the contaminations have covered the surfaces of the media (Figures 2A and 2D). Treatments with BNS for the species where 100% decontaminations were not achieved manifested delay in the onset of contaminations. Contamination was noticed on the 6th day of culture compared with the negative control group which started to show at the 3rd day. In addition, the rate of growth of the contaminations in the treatments with BNS was not as rapid as in the negative control group. This delay observed show that even at very low concentration with little exposure time, the toxicity of BNS to the microbial cells is evident which is in agreement with the report by Matsumura et al. [17].

In the positive control group where 5% Clorox was used, 100% decontamination was recorded in all the species with varying exposure time. The highest percentage callus induction was 86% in O. basilicum (Table 3) and O. canum (Table 4), and 83% in O. sanctum (Table 5). The exposure time had significant effect on the callus induction as the degree of callus formation reduces with an increase in the exposure time till no callus was induced at higher exposure time. This showed that prolonged exposure of plant tissues to Clorox even at low concentration is detrimental to them [21].

BNS was effective in decontaminating the explants in all the species with 100% decontamination achieved in all the concentrations cum varying exposure time. Exposure time had significant effect on the explants decontamination as higher decontamination was achieved with an increase in the exposure time. No significant effect was observed for exposure time on the callus induction in treatments where 100% decontamination was achieved. Almost all the disinfected explants induced callus, suggesting that high concentration of BNS (100 mg/l) with longer exposure time (60 min) has no detrimental effect on the explants. This result corroborates that of Mahna et al. [18], where
nanosilver was used on tomato cotyledon explants and another report [22], where up to 97% decontamination was achieved. In the later, all surface sterilized *Rosmarinus officinalis* stem explants using *Rubia tinctorum* L. cell culture extracts mediated biosynthesized silver nanoparticles induced callus.

The calluses were observed starting to form on the surface of the medium after 8 days of culture in *O. basilicum* and 6 days of culture in *O. canum* and *O. sanctum* in all treatments with BNS. There was evident growth in treatments with higher concentrations of BNS with longer exposure time. This was 2 days earlier than in the positive control where 5% Clorox was used which showed no growth until the 10th day of culture in *O. basilicum* and 8th day of culture in *O. canum* and *O. sanctum*. Moreover the number of explants that induced calluses were higher in treatment with BNS, where 100% decontaminations were achieved compared with the positive control group as shown in Tables 3, 4 and 5 for *O. basilicum*, *O. canum* and *O. sanctum*, respectively. This result revealed that BNS stimulated the explant to form callus 2 days earlier than Clorox treated explants, which indicates that BNS possess stimulating effect towards callus induction.

In explants exposed to high concentrations of BNS with long exposure times, the degree of callus formation increased in *O. basilicum* and *O. sanctum* as shown in Tables 3 and 5, respectively, while in *O. canum* both the degree of callus formation and root initiation increased as shown in Table 4, Figures 2B and 2C. These results pointed to the possibility of BNS producing a stimulating effect on the induction and intensity of callus formation, which could be as a result of the absorption of the BNS by the explants as observed during the sterilization processes. After rinsing of the plant tissues (explants) three times with sterile distilled water, traces of the BNS colour (brown) could still be seen on them. Stimulating effects of camel milk mediated AgNPs in culture media for callus growth from leaf explants of *Solanum nigrum* (L.) have been reported by Ewais et al. [23]. They reported that the frequency of callus formation (89%) and the fresh weight of the callus (4.67 g per leaf explant) were increased on MS medium augmented with 5 mg/l BA, 3 mg/l NAA and 8 mg/l AgNPs. Aghdaei et al. [24] and Fazal et al. [25] also reported the stimulating effect of AgNPs in culture media.
Table 3. The effect of different concentrations and exposures of BNS on callus of *Ocimum basilicum* glandular trichome explants cultured on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BAP

| Sterilization Methods | Decontaminated callus induction (%) ± SD | Explants | Intensity of callus formation | Colour | Texture |
|-----------------------|----------------------------------------|----------|-----------------------------|--------|---------|
| **Sterilization Methods** | **BNS (mg/l)** | **Exposure time (min)** | | | |
| | 10 | 5 | 0.00±0.00c | 0.00±0.00c | - | - | - |
| | 10 | 86.67±5.77b | 83.33±5.77b | + | creamy | Friable and soft |
| | 20 | 100.00±0.00a | 96.67±5.77a | + | creamy | Friable and soft |
| | 30 | 100.00±0.00a | 93.33±5.77a | + | creamy | Friable and soft |
| | 60 | 100.00±0.00a | 93.33±5.77a | + | creamy | Friable and soft |
| | 50 | 5 | 93.33±5.77b | 90.00±10.00a | + | creamy | Friable and soft |
| | 10 | 100.00±0.00a | 93.33±5.77a | + | creamy | Friable and soft |
| | 20 | 100.00±0.00a | 90.00±0.00a | + | creamy | Friable and soft |
| | 30 | 100.00±0.00a | 93.33±5.77a | + | creamy | Friable and soft |
| | 60 | 100.00±0.00a | 100.00±0.00a | ++ | creamy | Friable and soft |
| | 100 | 5 | 100.00±0.00a | 90.00±0.00c | + | creamy | Friable and soft |
| | 10 | 100.00±0.00a | 93.33±5.77ab | + | creamy | Friable and soft |
| | 20 | 100.00±0.00a | 96.67±5.77ab | + | creamy | Friable and soft |
| | 30 | 100.00±0.00a | 100.00±0.00a | + | creamy | Friable and soft |
| | 60 | 100.00±0.00a | 93.33±5.77ab | ++ | creamy | Friable and soft |
| +ve Control | 100.00±0.00a | 83.33±5.77a | + | creamy | Friable and soft |
| | 10 | 100.00±0.00a | 86.67±5.77a | + | creamy | Friable and soft |
| | 20 | 100.00±0.00a | 83.33±5.77a | + | creamy | Friable and soft |
| | 30 | 100.00±0.00a | 0.00±0.00b | - | - | - |
| | 60 | 100.00±0.00a | 0.00±0.00b | - | - | - |
| -ve Control | 0.00±0.00 | 0.00±0.00 | - | - | - |

Values within column followed by the same letter are not significantly different at P = 0.05; ++ = Profuse callus mean; + = Moderate callus mean; - = Absent
### Table 4. The effect of different concentrations and exposures of BNS on glandular trichome explants and callus culture of *Ocimum canum* cultured on MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l BAP

| Sterilization Methods | BNS (mg/l) | Exposure time | Decontaminated callus induction (% ± SD) | callus induction (%) ± SD | Intensity of callus formation | Colour | Texture | Intensity of root formation |
|-----------------------|------------|---------------|----------------------------------------|--------------------------|-----------------------------|--------|---------|--------------------------|
| +ve Control           | 100        | 5             | 0.00±0.00c                             | 0.00±0.00c               | -                           | -      | -       | -                         |
|                       | 10         | 5             | 50.00±0.00b                            | 46.67±5.77b              | +                           | Creamy and brownish          | Hard, Compact and rooty    | +                         |
|                       | 20         | 100.00±0.00a  | 93.33±5.77a                           | +                        | Creamy and brownish         | Hard, Compact and rooty      | +                         |
|                       | 30         | 100.00±0.00a  | 93.33±5.77a                           | +                        | Creamy and brownish         | Hard, Compact and rooty      | +                         |
|                       | 50         | 5             | 56.67±5.77b                            | 50.00±10.00b             | +                           | Creamy and brownish          | Hard, Compact and rooty    | +                         |
|                       | 10         | 100.00±0.00a  | 86.67±5.77a                           | +                        | Creamy and brownish         | Hard, Compact and rooty      | +                         |
|                       | 20         | 100.00±0.00a  | 90.00±0.00a                           | +                        | Creamy and brownish         | Hard, Compact and rooty      | +                         |
|                       | 30         | 100.00±0.00a  | 96.67±5.77a                           | ++                       | Creamy and brownish         | Hard, Compact and rooty      | ++                        |
|                       | 60         | 100.00±0.00a  | 96.67±5.77a                           | ++                       | Creamy and brownish         | Hard, Compact and rooty      | ++                        |
| -ve Control           |            | 0.00±0.00d    | 0.00±0.00d                             | -                        | -                           | -      | -       | -                         |

Values within column followed by the same letter are not significantly different at P = 0.05; Where, ++ = Profuse callus mean; + = moderate callus mean; - = Absent; * = Dominant.
Table 5. The effect of different concentrations and exposures of BNS on callus of *Ocimum sanctum* glandular trichome explants cultured on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BAP

| Sterilization Methods | Decontaminated (%) ± SD | Explants callus induction (%) ± SD | Intensity of callus formation | Colour | Texture |
|-----------------------|-------------------------|----------------------------------|-----------------------------|--------|---------|
| **BNS (mg/l)**        | **Exposure time**       | **(%) ± SD**                     | **(%) ± SD**                |        |         |
| 10                    | 5                       | 0.00±0.00d                       | 0.00±0.00d                  | -      | -       |
|                       | 10                      | 26.67±5.77c                     | 23.33±5.77c                | +      | Creamy and Greenish** Hard and Compact |
|                       | 20                      | 70.00±0.00b                     | 70.00±0.00b                | +      | Creamy and Greenish** Hard and Compact |
|                       | 30                      | 100.00±0.00a                    | 93.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 60                      | 100.00±0.00a                    | 93.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
| 50                    | 5                       | 46.67±5.77b                     | 40.00±0.00c                | +      | Creamy and Greenish** Hard and Compact |
|                       | 10                      | 90.00±10.00a                    | 83.33±5.77b                | +      | Creamy and Greenish** Hard and Compact |
|                       | 20                      | 100.00±0.00a                    | 90.00±0.00ab               | +      | Creamy and Greenish** Hard and Compact |
|                       | 30                      | 100.00±0.00a                    | 93.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 60                      | 100.00±0.00a                    | 96.67±5.77a                | ++     | Creamy and Greenish** Hard and Compact |
| 100                   | 5                       | 100.00±0.00a                    | 90.00±0.00a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 10                      | 100.00±0.00a                    | 93.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 20                      | 100.00±0.00a                    | 93.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 30                      | 100.00±0.00a                    | 96.67±5.77a                | ++     | Creamy and Greenish** Hard and Compact |
|                       | 60                      | 100.00±0.00a                    | 93.33±5.77a                | ++     | Creamy and Greenish** Hard and Compact |
| +ve                   | 5                       | 73.33±5.77b                     | 70.00±0.00b                | +      | Creamy and Greenish** Hard and Compact |
| Control               | 10                      | 100.00±0.00a                    | 83.33±5.77a                | +      | Creamy and Greenish** Hard and Compact |
|                       | 20                      | 100.00±0.00a                    | 76.67±5.77b                | +      | Creamy and Greenish** Hard and Compact |
|                       | 30                      | 100.00±0.00a                    | 26.67±11.55c               | +      | Creamy and Greenish** Hard and Compact |
|                       | 60                      | 100.00±0.00a                    | 0.00±0.00d                 | -      | -       |
| -ve                   | Control                 | 0.00±0.00                       | 0.00±0.00                  | -      | -       |

Values within column followed by the same letter are not significantly different at P = 0.05; Where, ++ = Profuse callus mean; + =Moderate callus mean; - = Absent; **= More dominant
Figure 2. *Ocimum africanum* culture showing full contaminated explants when sterilized with sterile distilled water (A), Formation of moderate rooting callus from glandular trichome explant of *Ocimum canum* surface sterilized with 10 mg/l BNS for 60 min (B), Formation of profuse rooting callus from glandular trichome explant of *Ocimum canum* surface sterilized with 100 mg/l BNS for 60 min (C), and *Ocimum gratissimum* culture showing full contaminated explant when sterilized with sterile distilled water (D).

4. Conclusion

The results obtained from this study showed that all used concentrations of BNS were efficient in decontaminating the *Ocimum* plant seeds and tissues with 20 min exposure time. At higher concentration of 100 mg/l with longer expose time (60 min), the BNS posed no harmful effect on the seeds, tissues and callus induction but rather possess stimulating effect on callus formation. This
ascertained that biosynthesized nanosilver possess great antimicrobial properties just like chemically synthesized nanosilver and other sterilization agents used in tissue culture. It can be employed as an efficient tool for removing contaminants from plant tissues, at right dose and exposure time. Although, it has not yet become a universal decontamination agent, therefore, to extend its use in in vitro culture of other plants, further investigations should be conducted on diverse plant species and different explants.

Acknowledgements
The authors would like to thank the Director, National Centre for Genetic Resources and Biotechnology (NACGRAB), Moor Plantation, Ibadan, Nigeria, for his permission to make use of the Tissue Culture Laboratory and Tissue Culture unit staffs are appreciated for their technical assistance. We are also grateful to Nanotechnology Research Group (NANO+), Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria, for providing the biosynthesized nanosilver used.

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