Research highlight

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Preliminary investigations on the control of yam (*Dioscorea rotundata* Poir) tuber rot through nanoscience

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Abstract: *In vitro* and *in vivo* experiments were conducted to evaluate the bioactivity of nanoparticles of silver and neem (AgNeemnano) solution on some organisms that cause yam rot. In the *in vitro* study, organisms were isolated from a decaying yam tuber and identified as *Fusarium moniliforme* and *Lasiodiplodia theobromae*. The organisms were cultured in Sabouraud dextrose agar. With a cork borer of diameter 8 mm, cups were made at the centre of each of the agar plates, and then 0.05 ml AgNeemnano solution in varying concentrations (28.40, 56.81, 113.63, and 227.27 mg/ml) was aseptically introduced into the cups starting from the lowest to the highest concentration. The plates were incubated at 35°C for 48 h, and the zones of inhibition were determined. In the *in vivo* study, sterile yam slices, each weighing approximately 12.6 g, were dipped into each level of AgNeemnano solution for 3 min and thereafter inserted into the Petri dishes containing each of the organisms. The minimum inhibition concentration (MIC) of the AgNeemnano that inhibited the mycelial growth of *F. moniliforme* was 0.8 mg/ml, while the MIC of the AgNeemnano for *L. theobromae* was 0.5 mg/ml. The sterile yam slices dipped in the AgNeemnano solution and later inserted into cultured organisms were not attacked by the rot-causing organisms.

Keywords: nanotechnology; neem (*Azadirachta indica*); silver nitrate; yam storage.

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1 Introduction

Yam, a starchy tuber of the genus *Dioscorea*, is a staple food in the tropics and some parts of Europe and America. The southern part of Nigeria is known for high-volume production of yam where a high premium is attached to yam-based foods [1]. Over the last decade, yam became an export crop in most parts of the yam belt of West Africa, following high demand from other countries of the world. The post-harvest losses of yam, which occur shortly after harvest, have been a major challenge to the production and marketing of yams [2]. These losses are usually associated with microbial attack by rot-causing micro-organisms whose activities were exacerbated by mechanical injuries inflicted on yam tubers, which probably occurred during harvesting [3]. Research has shown that most of the post-harvest losses in yams result majorly from microbial attacks [4]. In south-eastern Nigeria, yams are stored in bans, carefully separated from each other under the shade until the next planting season. Storage or preservation of fresh yam tuber in low-temperature facilities such as refrigerators has been shown to keep the yam but causes dehydration, which induces deterioration when the yam is returned to room temperature [4]. Application of other preservation methods such as irradiation [4] has been reported to influence the physiological activities such as dormancy extension and slow rate of sprouting, which causes serious weight loss of stored yam tubers. Losses through microbial attack or pathogenic organisms are not readily controlled by the aforementioned technologies.

Besides, a number of micro-organisms, such as *Botryodiplodia theobromae*, *Fusarium moniliforme*, *Penicillim sclerotigenum*, *Rosellina bunnode*, *Aspergillus niger*, *Hendersonula forulvidae*, *Macrophomina phaseoli*, and *Rhizopus nodosus*, have been implicated as the major causes of yam rot in storage [5]. The use of synthetic chemicals of fungicide and bactericide in the control of microbial attacks of crops and crop products has been reported; however, these chemicals have been shown to have toxic

effects on humans and the environment, in addition to declining potency of the chemicals following resistance by the target organisms [5]. Recently, botanical extracts (organic chemicals) with anti-fungal and anti-bacterial properties were reported to be effective in the control of some pathogenic organisms especially in the post-harvest management of crop and crop products [6]. These organic chemicals are non-toxic, readily available, cost-effective, and highly degradable [6]. The notable plants from where the organic chemicals are extracted include but are not limited to *Azadirachta indica*, *Elaeis guineensis*, *Cassia alata*, and *Moringa oleifera* [7]. Application of chemical treatments such as gibberellic acid, thiabendazole, and deltamethine on stored yam has been reported to be effective; however, they are imported products and may not even be affordable for the rural farmers that produce the yam [8]. There is, therefore, the need to develop a technology that is non-toxic to the environment, cost-effective, and could easily be adopted by rural farmers.

The application of low-cost nanobiotechnology – a combination of silver nitrate and neem *Azadirachta indica* leaf extract, otherwise termed AgNeemnano – might provide a viable solution to the post-harvest problems of yam. Each of the constituent elements has variously been shown to have anti-bacterial and anti-fungal properties [9, 10]. Nanobiotechnology is a combination of organic and metallic nanoparticles whose chemical property is far different from each constituent element [11]. Nanoparticles hold great potentials in various contexts. This study, therefore, sets to investigate the potential of nanobiotechnology (AgNeemnano) in the control of rots of fresh yam tubers in storage.

The objectives of this study were as follows:

1. To test the bioactivities of neem (*Azadirachta indica*) leaf extracts and silver nitrate (AgNeemnano) solution on rot-causing organisms of yam (*Dioscorea* spp.); and
2. To ascertain the minimum inhibitory concentration (MIC) of the AgNeemnano solution that inhibits the growth of rot-causing organisms in stored yam.

2 Materials and methods

Two experiments were conducted in the Department of Crop Science, University of Nigeria, Nsukka. Nsukka is located on a plateau by latitude 6°52′N and longitude 7°23′E on an altitude of 400 m above sea level. The experiments involved *in vitro* and *in vivo* trials conducted with a decaying yam tuber suspected to have been infested with rot-causing micro-organisms. The tuber had soft rot that covered about 25% of the entire yam tuber. Prior to the trials, the yam was kept in a humid environment so as to create a conducive ground for micro-organism infestation. Other materials were silver nitrate salt and neem (*Azadirachta indica*) leaf extracts. The fresh leaves of neem *(A. indica*) were harvested from the botanical garden of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

2.1 Nanoparticle preparation and size characterisation

2.1.1 Neem particle preparation

The neem (*Azadirachta indica*) leaves were air dried on the laboratory bench for 4 days. The air-dried leaves were ground to powder with the aid of a hammer mill, model 4 (Thomas Miller, USA). The ground leaves weighing 50 g were added to 1 l deionised water in a conical flask. The solution was boiled on an electric heater for 25 min. After boiling, it was stirred with a stirrer continuously at 28–48°C for 15 min. The solution in the conical flask was put on a magnetic stirrer for another 5 min to ensure that all the sediment nutrients were extracted and contained in the clear liquid. This solution was subjected to particle size characterisation and distribution using a transmission electron microscope (TEM) and viewed in the Moticam machine (Motic Image, China). The particle size was determined by dropping 1 ml aliquot of the solution on a slide and then viewing under a TEM. Four different particle sizes of the neem broth were read from the Moticam machine for four consecutive times, and the mean of the values (58 μm) was taken as the particle size.

2.1.2 Silver nanoparticle preparation

Silver nano was prepared by adding 1.7 g of silver nitrate ($AgNO_3$) to 1 l deionised water. The particle size was determined using the same procedure as described above. The particle size obtained was constantly 10 μm for the four times the value was read.

2.1.3 AgNeemnano preparation and particle size characterisation

The silver nitrate solution was added to 500 ml neem solution, and the setup was allowed to stay for 24 h. The

resultant solution was termed silverneem nanoparticle (AgNeemnano). Particle size characterisation for the AgNeemnano solution was done three times using the same procedure as described above. The particle sizes obtained were summed up, and the mean value (20 µm) was taken as the particle size of AgNeemnano.

The AgNeemnano with particle size of 20 µm was used as the treatment at different levels of concentration for the *in vitro* and *in vivo* studies of yam tuber in storage.

2.2 *In vitro* experiment

This was done in May 2015 when yam tubers had stayed up to 5 months in storage and evidences of yam rot were very prominent. The rot-causing organisms were isolated, identified, and characterised following the procedure outlined by Pitt and Hocking [12]. The morphological structures of the organisms in lactophenol blue stain were captured and filmed with Moticam machine for ease of identification.

2.3 Determination of the inhibition zone diameter (IZD) of AgNeemnano

With the aid of a flame-sterilised wire loop, the soft/wet part of the tuber was scooped into a prepared growth medium, Sabouraud dextrose agar, and allowed to stay for 48 h to enable the rot-causing organisms to grow. The agar plates were divided into four sections (tubes) using a marker and labelled 1, 2, 3, and 4 for the different concentrations of 227.27, 113.63, 56.81, and 28.40 mg/ml of AgNeemnano, respectively. Using a cork borer of diameter 8 mm, cups were made at the centre of each of the four sections. Then, 0.05 ml each of the dilution of the AgNeemnano solution was aseptically introduced into the cups starting from the lowest to the highest concentration. The plates were labelled and incubated for 48 h at the temperature of 35°C, and the IZDs were determined using a metre rule. The concentration use efficiency (CUE) was calculated as the additional increase in IZD following an upward change in concentration of AgNeemnano.

2.4 *In vivo* experiment

A healthy yam tuber was washed clean with 75% dilution of sodium hypochlorite and rinsed with sterile water. Sterile Petri dishes were aseptically seeded with 0.1 ml of freshly prepared suspension of *Fusarium moniliforme* and the same procedure was done for *Lasiodiplodia theobromae* using a sterile pipette. Three pieces of yam each weighing 12.6 g were cut from the tuber and dipped in the prepared AgNeemnano solution and then placed in sterile Petri dishes containing the test micro-organisms. Other pieces of yams were cut from the same source but were not treated with AgNeemnano solution. Each of the cut piece of yam was placed in the prepared suspension of *F. moniliforme* as well as in the suspension of *L. theobromae* and allowed to stay for 24 h.

3 Results

The particle size distributions of the neem broth, silver nitrate, and the combination of neem and silver nitrate are shown in Figures 1–3, respectively. Neem has variable particle sizes, which were larger than the silver nitrate particles. A combination of the two substances (AgNeemnano) resulted in a colour change different from either the neem or silver nitrate, and the particle sizes were in between the sizes of neem and silver nitrate.

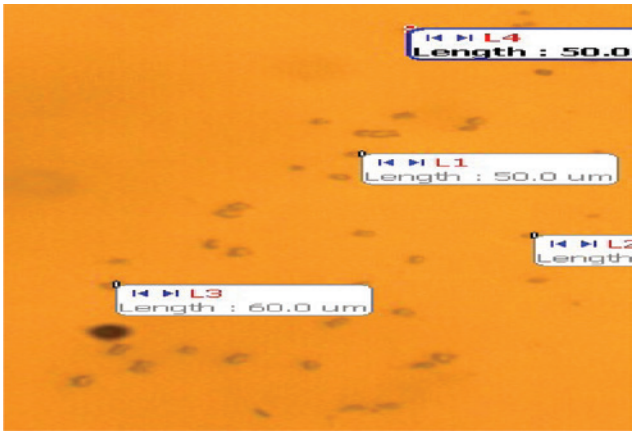

Figure 1: Particle size distribution of neem broth.

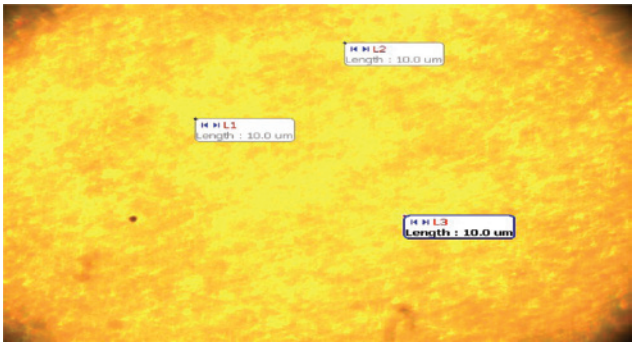

Figure 2: Particle size distribution of silver nitrate.

Figure 3: Particle size distribution of AgNeemnano.

The rot-causing organisms found in yam tuber in the study area at the time of this study were *Fusarium moniliforme* and *Lasiodiplodia theobromae*. The bioactivities of AgNeemnano on *F. moniliforme* varied with the varying concentration of the solution (Table 1). At the concentration of 28.40 mg/ml, the IZD was 13.0 mm but increasing the concentration to 227.27 mg/ml resulted in an IZD of 20.0 mm. Similarly, varying the concentration of AgNeemnano affected its activities on *L. theobromae* (Table 2). The MIC of the AgNeemnano that inhibited the mycelial growth of *F. moniliforme* was about 0.9 mg/ml as shown in Figure 4, while the MIC of AgNeemnano for *L. theobromae* was <0.5 mg/ml (Figure 5). Doubling the concentration did not show a corresponding increase in the IZD. When the concentration was increased from 56.81 to 113.63 mg/ml for *Fusarium moniliforme*, the CUE was only 3 mm. When this concentration was doubled or increased to 227.27 mg/ml, the IZD increased in a decreasing trend with

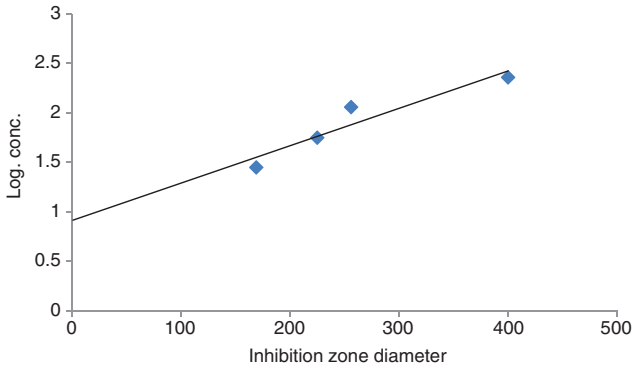

Figure 4: Graph of the log concentration and IZD showing the point of MIC (in mg/ml) of AgNeemnano solution against *Fusarium moniliforme*.

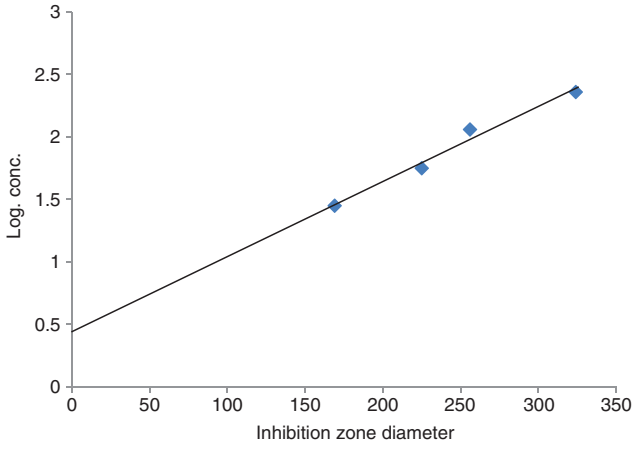

Figure 5: Graph of the log concentration and IZD showing the point of MIC (in mg/ml) of AgNeemnano solution against *Lasiodiplodia theobromae*.

Table 1: Effects of varying concentrations and log concentration on IZD, CUE, and IZD² of silverneem on *Fusarium moniliforme*.

Concentration (mg/ml)	Log. of conc.	IZD (mm)	CUE (mm)	IZD² (mm)
227.27	2.36	20.0	2	400
113.63	2.06	18.0	3	324
56.81	1.75	15.0	2	225
28.40	1.45	13.0	–	169

Table 2: Effects of varying concentrations and log concentration on IZD, CUE, and IZD² of silverneem on *Lasiodiplodia theobromae*.

Silverneem conc. (mg/ml)	Log. of conc.	IZD (mm)	CUE (mm)	IZD² (mm)
227.27	2.36	18.0	2.0	324.0
113.63	2.06	16.0	1.0	256.0
56.81	1.75	15.0	2.0	225.0
28.40	1.45	13.0	–	169.0

reduction in CUE. The cut surfaces of the untreated yam tuber appeared white or colourless, while the cut surface of tuber treated with AgNeemnano appeared dark brown (Figure 6). The isolated micro-organisms that were found in the yam tubers as captured by the Moticam camera had large particle sizes (Figure 7).

4 Discussion

The individual substances (neem broth and silver nitrate) used in this study had a peculiar colour and different particle sizes; however, when they were mixed together to generate AgNeemnano, a different colour and a different particle size were obtained. This is evidently a complete chemical reaction with a new product that has properties different from the individual component reactants.

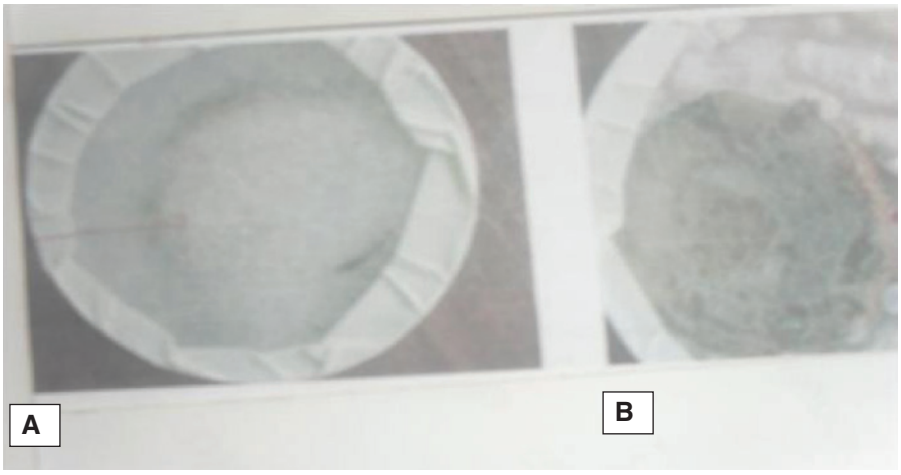

Figure 6: Photograph of untreated tuber (A) and tuber treated with AgNeemnano solution (B) against *Fusarium moniliforme* and *Lasiodiplodia theobromae*.

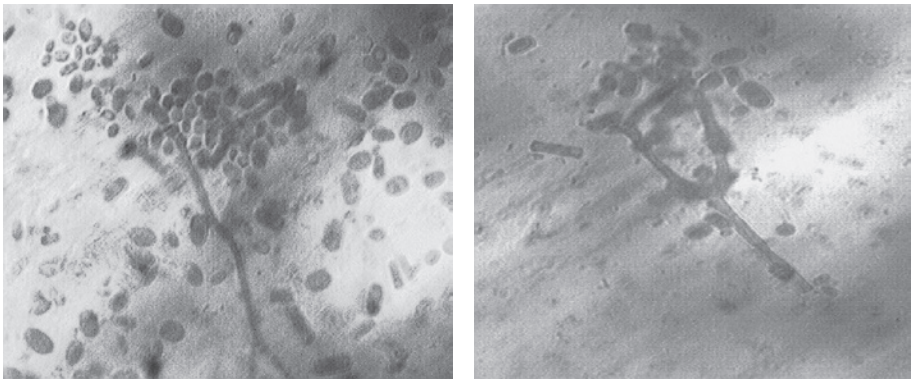

Figure 7: Microphotographs of the organisms associated with soft rot in yam tuber (*Dioscorea rotundata*).

This preliminary investigation of nanobiotechnology for its effectiveness as an anti-fungal agent of soft rot in yam gave encouraging and interesting results. The MIC of AgNeemnano that inhibited the mycelial growth of *Fusarium moniliforme* was about 0.9 mg/ml, while the MIC of AgNeemnano for *Lasiodiplodia theobromae* was <0.45 mg/ml. These micro-organisms were sensitive to the AgNeemnano solutions at very low concentrations compared to the MIC of *Cassia alata* leaf extracts (48.78 and 87.50 mg/ml) on *Rhodotorula mucilaginosa* and *Scopulariopsis brevicaulis*, respectively, as reported by Eze and Eze [8].

The MIC of any chemical is as important as the optimum inhibition concentration (OIC) for social, environmental, and economic reasons. It is evident in this study that while the MIC of AgNeemnano for *Fusarium moniliforme* is 24.40 mg/ml, the OIC is 113.63 mg/ml, beyond which the IZD decreased. Given that silver is non-toxic to humans and the environment, it is not economical to increase the concentration beyond this point as additional increases in concentration resulted in a very low increase in IZD, and therefore, poor CUE of the silverneem. The MIC of AgNeemnano for *Lasiodiplodia theobromae* was also the same with that of *F. moniliforme* but the OIC varied. The CUE of AgNeemnano decreased at the concentration of 113.63 mg/ml and increased again at the concentration of 227.27 mg/ml. Although it is not clear what caused the fall and rise in the value of the CUE of the silverneem on the *L. theobromae*, it is likely that the organism resisted the bioactivity of this chemical at that concentration but became susceptible when the concentration was further increased. The optimum inhibition concentration of silverneem for *L. theobromae* was not attained in this study; therefore, further research is recommended.

Interestingly, silver is metallic in nature; therefore, the combination of silver and neem (AgNeemnano) solution in this study is not likely to be degradable as in the

case of *Cassia alata* leaf extracts or neem leaf extracts alone. This means that AgNeemnano could represent a lead source of novel antimicrobial agents capable of controlling rots of yam in storage if properly utilised. In rational drug therapy, the concurrent administration of two or more antimicrobial agents is often essential and sometimes mandatory in order to achieve the desired therapeutic effects [13].

The mycelial growth of the pathogenic organisms was noticeable even with unaided eyes. The dark brown colour of the treated tuber could be due to the drying up of yam exudates that sealed the cut surface and changed in colour, while the colourless or white surface of the untreated tuber suggests well-covered mycelial growth of the pathogenic organisms. The morphological features of the micro-organisms as captured by the Moticam camera suggest that these two organisms that cause yam rot in the study area have big particle sizes and might be virulent to other micro-organisms. Again, few numbers of pathogenic organisms of yam identified in this study could be attributed to the prevalent weather conditions, which probably were not conducive to other organisms. It has been reported that micro-organisms thrive better in a high-humidity environment with relative humidity of 70–90% than in low-humidity regions [14].

In this study, the sensitivity of the micro-organisms to AgNeemnano varied with the concentration, suggesting that this new metallic nanoparticle has multiple anti-microbial properties that could be applied to other fields of study, and not only in food crop preservation. This result also corroborates the report by Furno et al. [10] who had stated earlier that nanoparticles hold great potential in various contexts. A combination of silver nitrate and neem leaf extracts is a reaction of metal and a non-metallic substance resulting in a product that could maintain the durable property of metal with antimicrobial (antibacterial and antifungal) spectrum that deals with two or more organisms with different sensitivity patterns [14]. The most interesting aspect of this study is that the green metallic nanoparticles have no toxic chemicals and no effects on food [10].

5 Conclusion

This new method of biosynthesis of green metallic nanoparticles has different levels of bioactivities on the rot causing micro-organisms. Again, many micro-organisms were not found in this study. It is likely that location, type of yam, and time of storage could be responsible for the non-abundance of the micro-organisms in the study area. However, it is evident in this study that the use of metallic nanoparticles for storage of yam has the potential to keep yam free from all manners of microbial attack, and will enable yam to be stored for a period longer than the traditional 2–4 months after harvest. Further research is recommended to substantiate our findings, especially on the optimum concentration of the solution and different application methods. The technology is cheap, easy to apply, and with non-toxic effects on humans and the environment, and can be applied correctly in the farm field.

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Bionotes

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