Nig. J. Biotech. Vol. 38 (2) : 47-55 (Dec 2021)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: https://dx.doi.org/10.4314/njb.v38i2.5

NIGERIAN JOURNAL OF BIOTECHNOLOGY

In vitro antifungal activities of mancozeb/phytosynthesized zinc oxide nanoparticles against *Eurotium* sp. isolated from diseased cassava plant (*Manihot esculenta* Crantz)

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Abstract

Nanoparticles are substances ranging from 1 – 100 nm in size and they have improved property such as increased surface area to volume ratio. In this study, *in vitro* antifungal activities of mancozeb/phytosynthesized zinc oxide nanoparticles against *Eurotium* sp. isolated from diseased cassava plants (*Manihot esculenta* crantz) were determined. Zinc oxide (ZnO) nanoparticles was synthesized using *Moringa* leaf extract and characterization of the biosynthesized nanoparticles was done using UV-Vis spectrophotometer. Different combinations of zinc oxide nanoparticles and mancozeb were prepared corresponding to 25/75, 50/50 and 75/25 ZnO nanoparticles/mancozeb respectively. Antifungal testing using the test nanoparticles/mancozeb combinations was carried out using the food poisoning method. The results obtained from this study indicate that zinc oxide nanoparticles/mancozeb combinations significantly inhibited the growth of the test pathogen with varying rates of inhibition. One hundred percent (100 %) inhibition of *Eurotium* sp. was obtained by 25/75, 75/25 and 100 % nanoparticles treatments. Future direction to this study is to investigate how ZnO nanoparticles/mancozeb combinations could be used for crop protection against phytopathogens. The mode of action of the test nanoparticles should be further investigated.

Keywords: Fungicide, Plant, Pathogens, Synergism, ZnO.

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Introduction

There is great concern over global food security, which is facing severe challenges across the world. It is estimated that by 2050, an additional 70% food production is needed to fulfill the demand of the growing human population (Godfray et al., 2010). The effect of pathogens on food crops cannot be over emphasized as damages caused by phytopathogens continue to draw attention worldwide (Zhen-Xing and Bin-Feng, 2014). Plant pathogens such as bacteria, viruses and fungi cause lots of havoc when they attack crop plants, thereby resulting to low yield of the plants (Savary et al., 2012). The protection of important crop plants such as cassava against pathogens has a critical role to play in meeting the high food demand of the ever-growing human population.

Cassava originated from Latin America, and it has been grown by the indigenous Indian population for at least 4000 years (Akinpelu et al., 2011). It is among the major food crops grown in Africa. It is very common, affordable and widely used globally (Alves, 2002). It is highly rich in starch (carbohydrate) as well as other nutrients such as proteins, vitamins, potassium, sodium and magnesium (Desse and Taye, 2001). It is cultivated as food (for humans and animals) and as industrial raw material (FAO, 2012). Some foods derived from cassava are beverages, tapioca, cassava flour, starch, and cassava chips. It also plays an important dietary role in the diets of almost one billion people worldwide (Prochnik et al., 2012). Farmers are faced with several challenges such as pest and diseases in the cultivation of cassava, which leads to low yield and losses. Various methods have been adopted to manage diseases of cassava and they have several

limitations, including health hazard associated with the use of chemicals. Currently, the use of nanoparticles and nanotechnology is gaining importance in the control of plant pathogens.

A nanoparticle is a material comprising of particles ranging from 1 – 100 nm in size. It exhibits unusual physical, chemical and biological activity due to its reduced small sizes (Rao and Paria, 2013; Mariselvan et al., 2014). Nanoparticles are synthesized based on two approaches namely: top-down (breakdown) and bottom-up (buildup). Based on this, different strategies have been developed (Kandasamy and Sorna, 2015). Phytosynthesis of nanoparticles is considered to be cost-effective and environment friendly, hence, it can easily be scaled-up for large scale production (Ahmed et al., 2016). In this study, *Moringa oleifera* leaf extract was used for the synthesis of zinc oxide (ZnO) nanoparticles. There seems to be limited studies on synergistic activities of nanoparticles and commercially available fungicides such as mancozeb, $C_4H_6N_2S_4Mn$. $C_4H_6N_2S_4Zn$ (at lower concentrations). Therefore, the current study investigated the antifungal activities of phytosynthesized zinc oxide (ZnO) nanoparticles/mancozeb against *Eurotium* sp. isolated from diseased cassava.

Materials and Methods

Collection of Sample

The diseased cassava plants used for this study were obtained from a cassava farm (GPS location: N6^{0}23'45.40344", E5^{0}37'12.65088") located at Ovia North-East Local Government Area, Benin, City, Edo State, Nigeria. The fresh *Moringa oleifera* leaves were got from a tree growing in an open place (GPS location: N6^{0}23'37.42548", E5^{0}37'13.2474") around junior staff quarters, University of Benin, Benin city, Edo State, Nigeria.

Preparation of Potato Dextrose Agar (PDA)

The medium, PDA used for this study was prepared following the manufacturer's instruction. The PDA was prepared by dissolving 39 g of powdered PDA in 1 liter of sterile distilled water. The sterilization of the medium was done by autoclaving at 121^{o}C for 15 minutes under pressure. After sterilization, it was cooled to 45-50^{o}C and aseptically dispensed into sterile Petri dishes. An antibiotic (250 ml chloramphenicol) was added to 250 ml of PDA to inhibit bacteria growth.

Isolation of fungal pathogen from diseased cassava

The cassava samples were prepared by teasing the plant part used (leaves) into smaller pieces, which was then followed by surface sterilization with alcohol to remove any surface contaminants. Direct plating method of fungal isolation was used. The prepared samples were aseptically inoculated on the already dispensed medium using sterile forceps. The culture was incubated at room temperature (28±2^{o}C) for 72 hours and observed for fungal growth. Pureculture of the isolate was obtained by picking single isolated mycelia of the fungi with the help of sterilized wire loop and placing on fresh PDA medium. The incubation of the culture was done at room temperature (28±2^{o}C) for 72 hours.

Identification of fungal Isolate

The fungal isolate was identified through macroscopy and microscopy. The morphological characteristics were observed and described. The isolate was also observed on the microscope after staining with lactophenol blue according to Obiazikwor and Shittu (2018).

Preparation of Moringa oleifera leaf extract

This was prepared as follows: The leaves were removed from the stalk and washed with sterile distilled water. This was then surface sterilized using 70% ethanol to remove contaminants. Twenty (20) grams of the washed leaves was weighed using weighing balance. This was then blended with mortar and pistil. The blended leaves were suspended in 100 ml of sterile distilled water, mix thoroughly, followed by boiling for 2 minutes. Whatman filter paper was used to filter the extract.

Phytosynthesis of zinc oxide nanoparticles

This was carried out following the adapted method of Alavi et al. (2019). The precursor used for the synthesis of zinc oxide nanoparticles was zinc nitrate hexahydrate ($ZnO(NO_3)2.6H_2O$. A concentration of 0.1 M of the precursor solution was prepared. An aliquot of 10 ml of the prepared plant extract was added to 50 ml of the precursor. The resulted solution was stirred vigorously for an hour. Reduction of ($ZnO(NO_3)2.6H_2O$ with *Moringa oleifera* leaf extract resulted in yellow–black

colour (Plate 1).

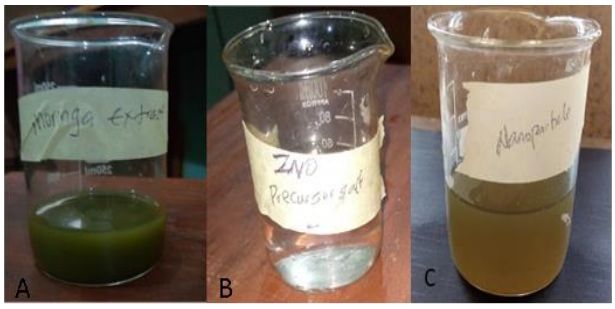

Plate 1: Picture of *Moringa* leaf extract (A), ZnO precursor solution (B) and ZnO Nanoparticles (C)

Characterization of zinc oxide nanoparticle using spectrophotometer

The absorbance of phytosynthesized nanoparticles was examined using UV–vis spectrophotometer. This was carried out by measuring the absorbance at regular intervals (1, 24 and 48 hours after synthesis) within 300-800 nm wavelengths.

Preparation of Mancozeb solution

This was prepared as follows: 16 g of the mancozeb solution was dissolved in 8 L of distilled water according to the manufacturer instruction. This was used to form the zinc oxide nanoparticles/mancozeb combinations. The combinations include 25/75, 50/50, 75/25 zinc oxide nanoparticles/mancozeb respectively.

Antifungal testing

The antifungal activities of zinc oxide nanoparticles and mancozeb combinations against the test pathogen were carried out using the food poisoning method. Five different concentrations corresponding to 100, 75-25, 50-50, 25-75 and 0% (control) of zinc oxide nanoparticles and mancozeb was prepared. The 100% concentration was taken to be the stock solution for both nanoparticles and mancozeb, the 75-25% was prepared by dispensing 75 ml of nanoparticles into 25 ml of the mancozeb. In addition, the 50-50% was prepared by dispensing 50 ml of nanoparticles into 50 ml of the mancozeb, while the 25-75% was prepared by dispensing 25 ml of nanoparticles into 75 ml of mancozeb. The 0 % was taken as the control. An aliquot of 2 ml of the nanoparticles and mancozeb combination for the different concentrations was added to 20 ml of PDA after pouring under sterile conditions. This was shaken carefully and allowed to solidify. After solidifying, the test pathogen was inoculated by picking a culture plug of the fungal culture and placing it at the center of the solidified medium. This was then incubated at room temperature and fungal mycelia growth was measured for a period of 7 days.

The rate of inhibition of fungal growth by the nanoparticles and mancozeb combinations treatments was calculated using the following formula:

Inhibition rate = Average mycelia growth of control – average mycelia growth of r ×100 / average mycelia growth of control.

(Where r = average mycelia growth of other treatments apart from control).

Statistical analysis

Each treatment was repeated in triplicates and results were presented as mean ± standard error. The data obtained from this study were subjected to descriptive statistics (using Microsoft Excel) and parametric statistics using the Statistical Package for the Social Sciences (SPSS), version 20 software. An alpha value of 0.05 was taken as the level of significance.

Results

The fungal pathogen isolated in this study was identified as *Eurotium* sp. Table 1 shows the morphological description of the isolate. The absorbance values of the biologically synthesized zinc oxide nanoparticles taken after 2, 24, 48 hours of synthesis is shown in Figure 1. The peak of the absorbance was recorded at 400 nm.

Table 1: Morphological description of fungal isolate associated with disease cassava plant

Morphology	Isolate 1
Form	Filamentous
Elevation	Flat
Margin	Filamentous
Texture	Hairy colony
Pigmentation	White
Optical property	Opaque
Size	Small
Identified organism	***Eurotium* sp.**

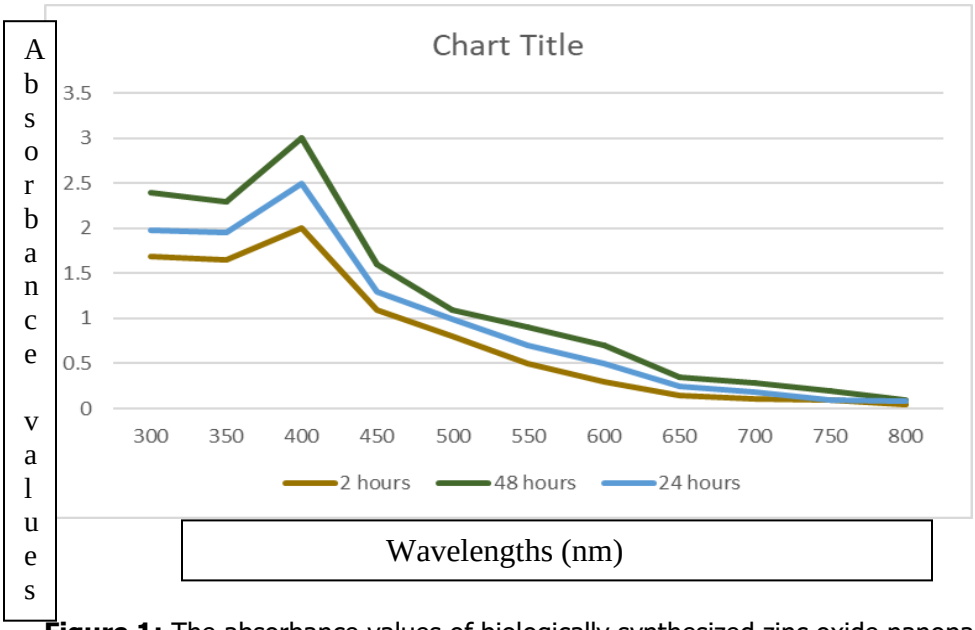

Figure 1: The absorbance values of biologically synthesized zinc oxide nanoparticles after2, 24 and 48 hours of synthesis

Table 2 shows the effect of phytosynthesized ZnO nanoparticles/mancozeb combinations and mancozeb on the mycelia growth of *Eurotium* sp. Total inhibition of the test organism was recorded in all the days observed for 25/75, 75/25 treatments. The highest mycelia growth was recorded at day 7 forthe control treatment. The effect of zinc oxide nanoparticles/mancozeb combinations and zinc oxide nanoparticles on the mycelia growth of *Eurotium* sp. is shown in Table 3. Total inhibition of the test pathogen wasobtained in all the days observed for 25/75, 75/25 and 100 % nanoparticles treatments (Plate 2). The highest mycelia growth (0.43) at day 7 was recorded for the control treatment.

Table 2: Effect of zinc oxide nanoparticles/mancozeb combinations and mancozeb on themycelia growth of *Eurotium* sp.

Treatments	Mycelia growth measurements (cm)					
	Day2	Day3	Day4	Day5	Day6	Day7
Control (0%)	0.17^{b}±0.02	0.02^{ab}±0.04	0.30^{b}±0.05	0.38^{b}±0.04	0.43^{b}±0.07	0.43^{b}±0.07
25/75	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00
50/50	0.00^{a}±0.00	0.07^{ab}±0.04	0.11^{ab}±0.06	0.13^{ab}±0.07	0.18^{ab}±0.09	0.27^{ab}±0.14
75/25	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{b}±0.00
100 % Mnz	0.00^{a}±0.00	0.18^{b}±0.03	0.33^{b}±0.17	0.33^{b}±0.17	0.33^{b}±0.18	0.33^{ab}±0.18

Values are presented as mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.
Legend: Mnz = Mancozeb; 25/75 = 25 ml zinc oxide nanoparticles, 75 ml mancozeb;50/50 = 50 ml zinc oxide nanoparticles, 50 ml mancozeb;75/25 = 75 ml zinc oxide nanoparticles, 25 ml mancozeb.

Table 3: Effect of zinc oxide nanoparticles/mancozeb combinations and zinc oxide nanoparticleson the mycelia growth of *Eurotium* sp.

Treatments	Mycelia growth measurement (cm)					
	Day2	Day3	Day4	Day5	Day6	Day7
Control (0%)	0.17^{b}±0.02	0.23^{b}±0.04	0.30^{b}±0.05	0.38^{c}±0.04	0.43^{c}±0.07	0.43^{b}±0.07
25/75	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00
50/50	0.00^{a}±0.00	0.07^{a}±0.04	0.12^{a}±0.07	0.13^{b}±0.07	0.18^{b}±0.09	0.27^{b}±0.14
75/25	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00
100 % NPs	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00

Values are presented as mean ± standard error; figures bearing similar superscripts within columns are not significantly different using Duncan's Multiple Range (DMR) test at 0.05 level of significance.
Legend: 100 % NPs = 100 % nanoparticles; 25/75 = 25 ml zinc oxide nanoparticles, 75 ml mancozeb;50/50 = 50 ml zinc oxide nanoparticles, 50 ml mancozeb;75/25 = 75 ml zinc oxide nanoparticles, 25 ml mancozeb.

Plate 2: Effect of zinc oxide nanoparticles/mancozeb combinations and zinc oxide nanoparticles on the mycelia growth of *Eurotium* sp. after 7 days of incubation
Legend: A = 0% (control); B = 25/75 = 25 ml zinc oxide nanoparticles, 75 ml mancozeb; C = 50/50 = 50 ml zinc oxide nanoparticles, 50 ml mancozeb; D = 75/25 = 75 ml zinc oxide nanoparticles, 25 ml mancozeb; E = 100 % ZnO nanoparticles

Figure 2 shows the inhibition rates of zinc oxide nanoparticles/mancozeb combinations, zinc oxide nanoparticles and mancozeb against *Eurotium* sp. One hundred percent (100 %) inhibition of *Eurotium* sp. (a) was obtained by 25/75, 75/25 and 100 % nanoparticles treatment.

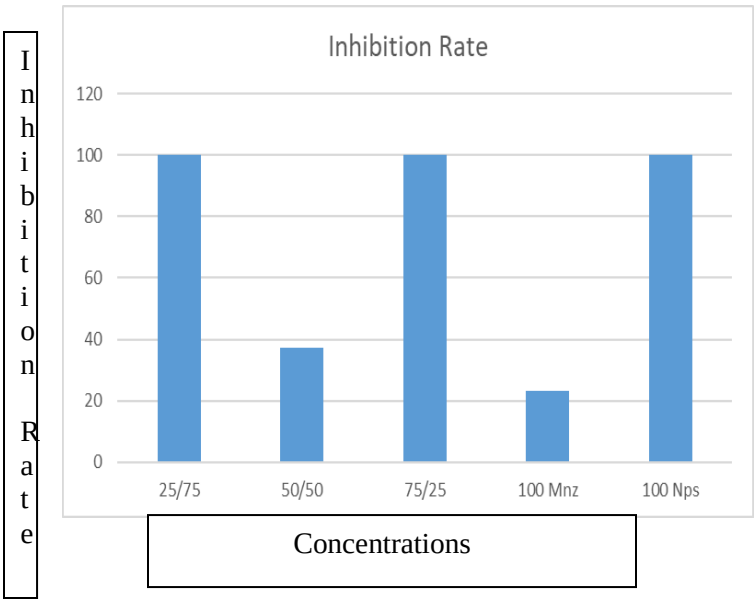

Figure 2: Rates of inhibition of zinc oxide nanoparticles/mancozeb combinations, zinc oxide nanoparticles and mancozeb against *Eurotium* sp.
Legend: 25/75 = 25 ml zinc oxide nanoparticles, 75 ml mancozeb; 50/50 = 50 ml zinc oxide nanoparticles, 50 ml mancozeb; 75/25 = 75 ml zinc oxide nanoparticles, 25 ml mancozeb; 100 % Mnz = Mancozeb; 100 % NPs = zinc oxide nanoparticles

Discussion

The fungal pathogen isolated from diseased cassava leaves in this study was identified as *Eurotium* sp. It has been documented that diverse *Eurotium* spp. have been sometimes isolated from fairly saline soils and water (Grishkan et al., 2003).

The absorbance values of the phytosynthesized zinc oxide nanoparticles after 2, 24, 48 hours of synthesis were recorded (Figure 1). The maximum absorbance peak was obtained at 400 nm wavelength. The results obtained from this study agree with previous study carried out by Khorsand et al. (2011), who reported that zinc oxide nanoparticles have its absorbance peak at 400 nm wavelength.

The antimicrobial activities of ZnO nanoparticles/mancozeb combinations and mancozeb on the mycelia growth of *Eurotium* sp. was presented (Table 1). One hundred percent (100 %) inhibition against the test pathogen was obtained by 25/75 and 75/25 treatments after 7 days of incubation. Mycelia growth of 0.33 cm was given by 100 % Mancozeb treatment compared to the control which gave 0.43 cm after seven days of incubation. These results indicate that there was synergistic effect in the antifungal activity of the combined antimicrobial agents. Some of the proposed antimicrobial mechanisms of ZnO nanoparticles include production of reactive oxygen species which elevates lipid peroxidation (Tiwari et al., 2018). However, this should be further investigated, most especially in fungal organisms. The effect of zinc oxide nanoparticles/mancozeb combinations and zinc oxide nanoparticles on the mycelia growth of *Eurotium* sp. shows that 100 % inhibition was obtained by zinc oxide nanoparticle treatment (Table 2) after 7 days of incubation. This suggests that the synergistic effect observed in Table 1 could be attributed to the antimicrobial activity of ZnO nanoparticles. However, the mechanisms underlying the synergism observed in this study should be investigated. The antimicrobial activities of ZnO nanoparticles in this research agree with earlier works done on the subject. Basavaraju et al. (2020) reported that zinc oxide nanoparticles have anticancer and antimicrobial attributes. Antibacterial efficacies of ZnO nanoparticles against the multi-drug resistant *Acinetobacter Baumanni* was reported by Tiwari et al. (2018).

The rates of inhibition of zinc oxide nanoparticles/mancozeb combinations, zinc oxide nanoparticles and mancozeb against the mycelia growth of *Eurotium* sp. is shown in Figure 2. One hundred percent (100 %)

inhibition against the growth of *Eurotium* sp. was obtained by 25/75, 75/25 and 100% nanoparticles treatments. The mechanism of the inhibitory effect of zinc oxide nanoparticles on fungi is not fully understood, however, several authors have reported the inhibitory action of zinc oxide nanoparticles. He et al. (2011) reported that zinc nanoparticles inhibit fungal growth by disrupting cellular activities thus leading to distortion in fungal hyphae. At high concentrations, zinc oxide nanoparticles can lead to complete inhibition by preventing the development of conidiophores and conidia. Arciniegas-Grijalba et al. (2017) suggested that the actions of ROS and/or Zn^{2+} are responsible for the inhibitory property of zinc oxide nanoparticles.

Conclusion

Green synthesis of zinc oxide nanoparticles using *Moringa* leaf extract was achieved in this study. Zinc oxide nanoparticles were effective against the mycelia growth of *Eurotium* sp. The different combinations of zinc oxide nanoparticles/mancozeb significantly inhibited the growth of the test pathogen with varying rates of inhibition obtained. Future direction to this study is to investigate how ZnO nanoparticles/mancozeb combinations could be used for crop protection against phytopathogens. The mode of action of the test nanoparticles should be further investigated.

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