Assessing the Efficacy of *Azadirachta indica* Seed Extract on *Fusarium Oxysporum*

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Abstract

Global population pressure has posed great challenge on food security with over 800 million people having no access to adequate food and about two billion faced with hunger and malnutrition. Banana (*Musa spp.)* is the world's fourth most important global food crop after rice, wheat and maize in terms of production. The cultivation of banana is however threatened by pests and diseases and diverse anthropogenic activities which have influenced and changed the climate. Generally, climate change impacts on agriculture and food security across the globe decreasing crop productivity while extreme weather conditions such as flooding, drought, hurricanes etc. increase disease transmission. *Fusarium oxysporum*, a soil borne fungus affects banana production, causing vascular wilts and damage to banana plantations by infecting plant roots, cortex and stele. Several fungicides have been employed to curb these losses. Plant extracts have, however, played significant role in the inhibition of seed-borne pathogenic *F. oxysporum*. Eco-friendly Neem (*Azadirachta indica*) extracts have been reported to gain prominence over inorganic fungicides. This study investigated the effectiveness of Neem (*Azadirachta indica)* seed extract at varying concentrations of 10, 20 and 50% against *F. oxysporum* in Potato Dextrose medium for 120 h. using pour plate and cork boring methods. The results obtained showed inhibited growth of the test fungus with 50% having the greatest percentage inhibition. This indicates that *A. indica* seed extract has fungicidal effect on *F. oxysporum* and has the potential to curb banana losses and hence enhance banana production and thus ensure food security.

Introduction

The world population has posed great challenge on food security. Reports from Food and Agricultural Organization (FAO), (2013) reveal that about 12.5% of the world's population are undernourished. An earlier report (FAO, 2005) showed that of the more than 800 million malnourished world population, 25% were from Sub-Saharan Africa. Globally, banana (*Musa spp.)* is acknowledged as the 4th most important food crop after rice, wheat and corn in terms of gross value production (FAOSTAT, 2003; Babayemi *et al*. 2010; Ravi & Mustapha, 2013). Banana provides basic staple food, supplying up to 25% of food energy requirements for 70–100 million people in Africa (IITA, 2009; Tripathi, 2009; Viljoen, 2010). Banana is cultivated in over 100 countries in the tropics as well as subtropical regions of the globe with about 35% of global production from sub-Saharan Africa (Tripathi, 2005). The cultivation of banana is however threatened by climate change impacts.

Climate Change has been identified as a significant constraint influencing banana yield and vulnerability to diseases (Meadu,

2011). According to the Intergovernmental Panel on Climate Change (IPCC) (2007), Africa is one of the most vulnerable continents to climate change and climate variability. It has been estimated that climate changes in form of extreme weather conditions (rising temperatures, flooding, drought, desertification etc.) are likely to impact on agriculture, seasonal yield losses and global food security in the 21st century especially in the developing world (IPCC, 2009; Byamukama *et al.* 2011). According to Petzoldt & Seaman, (2006) temperature increase has potential impacts on plant diseases especially fungi causing diseases. Specifically, Meadu, (2011) noted that some diseases are affected by very warm weather caused by global warming effect. In addition, rising temperatures affect the diversity of agricultural pests and diseases and are likely to lead to new disease outbreaks (Gregory *et al.* 2009).

The management of pests and diseases (weevils, nematodes, viruses, fungal and bacterial diseases) has played a negative role in food production especially in the last 40 years (Chakrabortya & Newton, 2011; Karamura *et al.* 2012). Research shows that 10–16 % of global harvests are destroyed by pathogens (Chakrabortya & Newton, 2011). According to Oerke, (2006), plant pests and diseases have the potential of depriving humanity of about 82% of the attainable yield and over 50% for other major crops. Specifically, Fusarium wilt, banana bunchy top disease (BBTD), sigatoka, weevils and nematodes were reported as the main constraints in banana plantations (Niyongere *et al.* 2012). Fusarium wilt is one of the mostly widespread and destructive diseases of more than 120 major ornamental and horticultural crops (Hua-Van *et al.* 2001; Davis, 2005). This soil borne fungus causes vascular wilts by infecting plants through the roots and grows internally through the cortex to the stele (Hua-Van *et al*., 2001; Viljoen, 2010). Fusarium wilt of banana (Panama disease) is caused by the soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc) (Rozeita *et al.* 2012). The disease is a major challenge and one of the most damaging banana diseases that adversely affects banana yield worldwide (Stewart *et al.* 2006; Viljoen, 2012). The degree of destruction caused by this disease has led to the development of various management options.

Recently, much attention has been given to biological control of crop pests with the development of safe and economical plant extracts for plant diseases being explored (Verma & Kharwar, 2006; Niaz *et* al. 2008). Plant extracts have played significant role in the inhibition of *F. oxysporum* and in the improvement of seed quality (Nwachukwu & Umechuruba, 2001). Current advances in nematode and *Fusarium* wilt control have been achieved through the application of plant extracts (Ogechi *et al.* 2006). Neem (*Azadirachta indica*) has been identified to be an effective bio-fungicide against over 100 species of different pests with the highest active ingredients present in the seeds (Stroll, 2001). In order to significantly enhance banana production, there is the need to explore the effectiveness of neem extracts to drastically reduce banana yield losses caused by *Fusarium oxysporum.*

This research work was therefore carried out to investigate the efficacy of Neem (*Azadirachta indica*) seed extract incorporated in aseptic Potato Dextrose

Agar (PDA) medium at varying concentrations of 10, 20 and 50% on *Fusarium oxysporum* fungus.

Materials and methods

Sample collection

About 50 g of soil was collected with sterile hand trowel from underneath banana trees in the office complex of the Federal Ministry and Natural Research in Ilorin, Kwara State, Nigeria. The collected soil was thoroughly mixed to obtain representative samples, labeled, transferred into new sterile polythene bag and stored in the refrigerator at - 4°C until needed. Fresh, ripe and healthy neem (*Azadirachta indica*) fruits were collected from Ilorin, Kwara State, Nigeria. The pulp and skin were removed and washed leaving the endocarp (the seed enclosed by the Kernel). These were air dried and de-shelled to obtain the kernels which were blended and stored in air tight plastic containers.

Sterilization technique and media preparation

Clean glass wares were arranged in canisters and oven sterilized at 160° C for an hour. Cork borers were also sterilized by dipping into 70% ethanol and passed over a Bunsen burner before and after use. A wire loop was sterilised by flaming over burning flame until it was red hot, while the media, Potato Dextrose Agar (PDA) was autoclaved at temperature of 121°C for 15 minutes. Fifty (50) g of potatoes were peeled, chopped into small pieces, washed and boiled to softness for about thirty minutes. The liquid content was filtered into a previously sterilized conical flask using clean muslin cloth; 5 g and 3 g of technical Agar and dextrose powder were weighed and added. In addition, distilled water was added to reach the 250 ml mark. The resultant mixture was dissolved on a hot plate with occasional shaking until a homogenous solution was obtained. The mouth of the flask was then corked with cotton wool and wrapped with aluminium foil. The solution was then autoclaved and kept in the refrigerator until needed.

Isolation of test fungi using serial dilution method

One gram of the soil sample was weighed and introduced into already sterilized test tube containing nine (9) ml of sterile distilled water to make a 10-fold dilution. One (1) ml of this dilution was taken with sterile pipette into another sterile test tube already containing nine (9) ml sterile distilled water to make a 10^{-2} dilution. The serial dilution continued up to 10^{-5}. One (1) ml of 10^{-2} to 10^{-5} dilutions was pipetted into different sterile petri dishes after proper shaking. Streptomycin powder, one and half (1.5) g was added to the PDA medium (after autoclaving) to suppress bacterial growth and act as an inhibitory agent against contaminants. The sterile medium was then poured to about two-thirds of the plate at 43° C this was swirled to allow proper and uniform distribution of inoculum. The plates were labeled and incubated at 28° C for 72 hours after which fungal colonies were observed, sub-cultured and pure cultures were obtained. Experiments were carried out on sterilized working surfaces and a sterile environment was maintained by keeping the Bunsen burner on.

Characterisation and identification of fungal isolates

The identification of the fungal isolates was by colonial and microscopic examination. The vegetative and reproductive structures were identified with the use of the microscope. Examination was observed first under low power microscope and later, observations were made under high power microscope in order to identify the spores and other diagnostic structures (Robert *et al.* 1984).

Preparation and sterilization of aqueous neem seed extracts

The previously processed air dried seeds were subjected to water extraction method using distilled water to obtain three concentrations (10, 20 and 50 %) of the extracts. To make the concentrations, 10, 20 and 50 g of blended seeds were dissolved in 100 ml of sterile distilled water respectively. The solutions were allowed to stay for 24 h. to aid proper diffusion of the extract into the water. The solutions were then filtered through a sterile muslin cloth and placed in separate conical flasks. Millipore filter (0.45 µm) was used with a suction pump to filter the extracts under aseptic conditions; the filtrates were then labeled and tested for sterility.

Incorporation of the extracts into PDA medium containing the test fungus

Two methods of extracts incorporation were adopted. These are the Pour Plate and Cork-boring methods.

1. Pour Plate Method

One ml of the seed extract of each of the concentrations was introduced into separate, sterile petri dishes. Sterile PDA medium was then poured into the same plates about half way full aseptically and swirled to allow for uniform distribution of the extract, the medium was then allowed to set at 42° C. The test fungus was then inoculated aseptically using a sterile cork borer of 5 mm diameter. Inoculum plugs were removed from the advancing edge of 5 days old pure culture and transferred in an inverted position with the aid of sterile inoculating needle to the centre of the plates. Two lines were drawn to intersect at the centre to divide the plate into four. The plates were then incubated at room temperature of 28 ± 2° C. A control plate of PDA medium without extract was also inoculated with the test fungus to serve as the control.

2. Cork Boring (Well) Method

About 15 ml of the PDA medium was poured into sterilized petri dishes and allowed to set. Five mm diameter of sterile cork borer was used to remove inoculum plugs from the advancing edge of 5 days old pure culture. This was transferred aseptically onto the centre of the solidified agar medium. The cork borer was re-sterilized and used to create 4 wells equidistant to each other. Three wells were filled with 10, 20, 50% neem seed extracts respectively while one well, was the control containing sterile distilled water, the plates were then incubated at room temperature.

Determination of mycelia growth and percentage inhibition

At 24 h. interval, for 5 days the diameter of the mycelia growths for the plates (including the control) were measured along the perpendicular axes marked at the bottom of the plates. After subtracting the diameter

of the inoculums plug, the average diameters were then recorded respectively. The measurements of each concentration of the anti-fungal agent and the control plates were then compared to determine the rate of growth. The diameter growth of the test organisms, on the control plate was designated as 'a', the diameter of growth on the experimental plate with the extract was designated as 'b'.

The inhibition percentage was calculated as below:

Radial growth

$$(a) = \frac{(y^1 \ldots\ldots y^2)}{d1} + \frac{(x^1 \ldots\ldots x^2)}{d2} - 5 \text{ mm of innoculum} \quad (1)$$

Average diameter – diameter of inoculums

$$(b) = \frac{d^1 + d^2}{2} - 5 \text{ mm of innoculum} \quad (2)$$

Therefore,

$$\text{Percentage inhibition} = \frac{a - b}{a} x\ 100\% \quad (3)$$

a = Radial growth of control plate
b = Radial growth of experimental plate
x_1, x_2 = Vertical growth of organism
y_1, y_2 = Horizontal growth of organism

Results and Discussion

Three (3) fungi were isolated from the banana plant soils these are: *Fusarium oxysporum*, *Aspergillus niger* and *Mucor spp*. However, for this study, only *Fusarium oxysporum* was selected for the anti-fungal study. Neem seed extracts at 10, 20 and 50 g/100 ml concentrations all showed inhibitory effect to *Fusarium oxysporum* growth in ascending order. This fulfils one of the basic criteria of an effective fungicidal formulation which states that it must be lethal to the fungus at very low concentration.

In the pour plate method (Fig, 1), the percentage inhibition of the concentrations of 10, 20 and 50 gm/100 ml were respectively 20, 40 and 53.3% after 24 h. By 72 hours of incubation, the percentage inhibition had increased to 37.5, 50.0 and 65.6%. respectively. For the cork boring method after 24 h. of incubation (Fig. 2), the

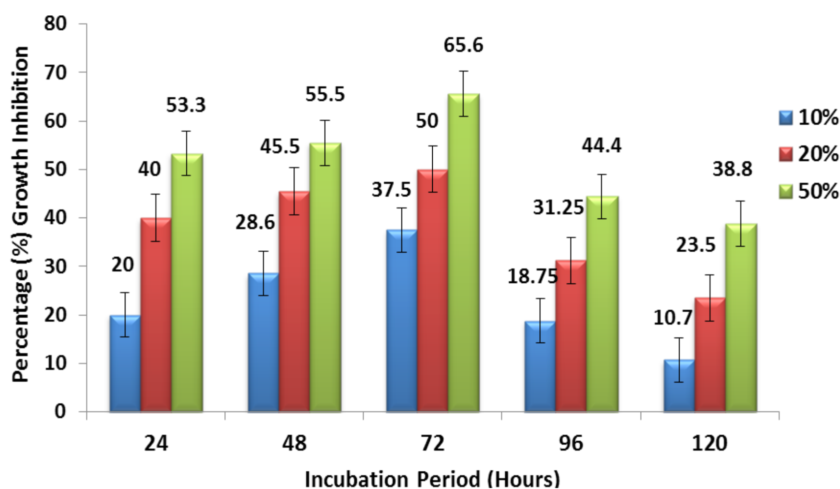

Fig. 1. Effect of *Azadirachta indica* Seed Extract at 10, 20 and 50 % Concentrations on *Fusarium oxysporum* mycelia growth using Pour Plate Method

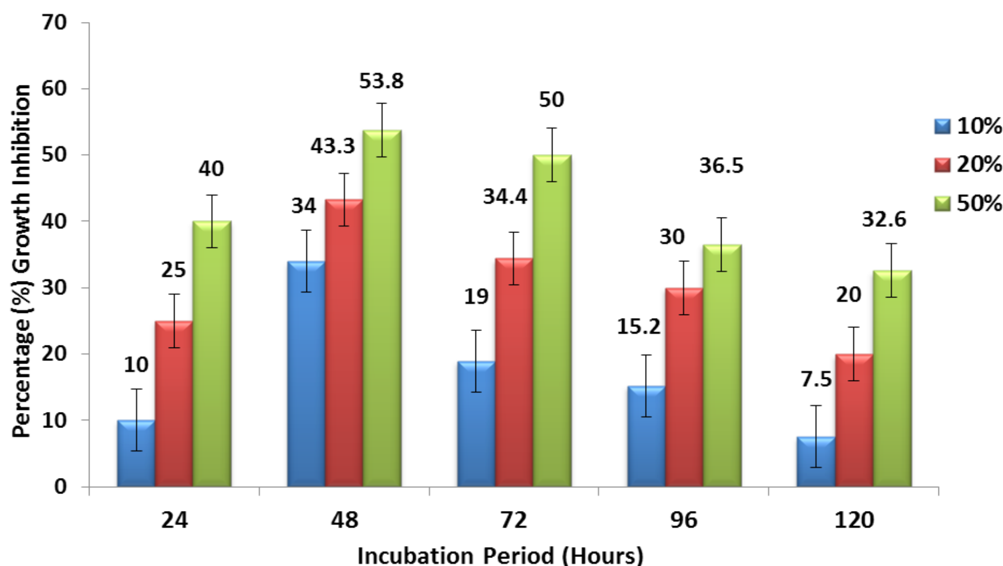

Fig. 2. Effect of *Azadirachta indica* Seed Extract at 10, 20 and 50% Concentrations on *Fusarium oxysporum* mycelia growth using Cork Boring Method

percentage inhibition was 10, 25 and 40%, respectively and by 72 h. of incubation the percentage inhibition had increased to 19.0, 34.4 and 50%, respectively. The control however showed no restriction in fungal growth throughout the incubation period as compared to the rate of growth of *Fusarium oxysporum* in the test samples at varying concentrations of the neem seed extract. This confirms the fungicidal effect of *Azadirachta indica* seed extract on *Fusarium oxysporum*. Results obtained revealed that neem seed extract inhibited the growth of the test fungus at 50 gm/100 ml concentration having the highest and 10 gm/ 100ml having the least percentage inhibitions. This confirms the findings of several researchers, Singh *et al.* (1993), Srivastava *et al.* (1997), Niaz & Kazmi (2005), Agbenin & Marley (2006), Verma & Kharwar (2006) and Hassanein *et al.* (2008) that *Azadirachta indica* plant extract suppresses mycelial growth of *F. oxysporum* and is an effective bio-fungicide with increasing inhibitory potential as its concentration increases.

The percentage inhibitions in the pour plate method were higher compared with those of the cork boring method. This reveals that the pour plate method is a more effective method than the cork boring method. This may be attributed to the uniform distribution of the neem seed extract in the medium and hence increased contact with the test fungus than in the well method where the test fungus had limited contact with the neem seed extract being in the 'wells' alone in the cork boring method. The penultimate and last days (96 and 120 hours) of the incubation showed sharp decrease in percentage inhibition in the two methods. According to Olayinka, (2009) & Oladipo *et al.* (2013) this may be attributed to the exhaustion of nutrients and accumulation of toxic

metabolic wastes into the medium by the fungus. This may therefore reduce the concentration of the neem seed extract and thus its decreased effectiveness.

Conclusion

The outcome of this study establishes the inhibitory effect of *A. indica* seed extract at 10, 20 and 50 g/100 ml concentrations on mycelia growth of *Fusarium oxysporum*. The study also revealed that the percentage inhibition of the test fungus growth increased with increasing concentration of the *A. indica* seed extract. The study concludes that *A. indica* seed extract is an effective bio-fungicide against *Fusarium oxysporum* growth. The neem seed extract at these concentrations can drastically reduce the yield losses of banana plants caused by *Fusarium oxysporum* thus mitigating climate change effect, enhance healthy banana production and hence ensuring food security in Africa and the globe.

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