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Antifungal efficacy of three plant extracts in the suppression of panama disease in banana plants

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Abstract

Three plant extracts from *Acacia nilotica, Lawsonia inermis,* and *Ziziphus spina-christi* were evaluated for their antifungal efficacy in the suppression of Panama disease in banana plants. The plants were collected within Niger state environs and were processed following standard procedures. The pathogen was isolated from banana stem showing symptoms of disease on Potato Dextrose Agar. The isolated pathogen was inoculated on two months old healthy banana plantlets obtained from National Centre for Genetic Resources and Biotechnology to ascertain its Pathogenicity. The plant extracts were examined on banana seedlings (*in vivo* experiment) in the screen house for their antifungal efficacy on the pathogen using irrigation method, the seedlings irrigated with sterile distilled water and those irrigated with mancozeb served as the controls (negative and positive) respectively. The results showed a good percentage survival rate of the banana plantlets. Although, the percentage survival of the banana plantlets irrigated with mancozeb had a higher (55 %) survival rate than the three plant extracts; 49 %, 50 % and 20 % (*A. nilotica, L. inermis* and *Z. spina-christi*) extracts respectively, but had no significant differences (P < 0.05) in the plantlets treated with *A. nilotica* (49 %) leaves at 75 % and *L. inermis* (50 and 48 %) leaves at 75 and 50 % concentrations respectively. Field trials of these plant extracts on the control of panama disease are recommended, since they are ecofriendly and could easily be made via a simple process of infusion, they can serve as substitute for conventional fungicides.

Keywords: Banana; Extracts; Mancozeb; Panama disease

1. Introduction

Bananas are monocotyledonous plants in the genus *Musa* belonging to the family Musaceae. They are very large perennial herb with sheaths that form trunks called pseudostems. The fruits appear in about 50-60 days after appearance of flowers [1]. Banana is one of the most popular fruit in the world and ranks among the top 10 food commodities for Africa, Southeast Asia and America [2]. Notably, banana is largely produced by small holder farmers, with around 85 % of global production destined for local markets and only 15 % entering the international trade market [2]. Banana production in Nigeria is found mainly in the Southern States, especially in Cross Rivers State, and a large concentration is grown in parts of Plateau and Benue States, while an isolated concentration is grown in Bida Local Government Area of Niger State [3]. Locally consumed bananas are staple foods or significant additions to diets of those in Africa, Southern Asia, and Tropical America [4 and 5]. Fungi are one of the major disease causing agents on plants and can cause losses up to 90% of agricultural yield [6]. Bananas suffer from various types of fungi diseases, such as Mycosphaerella leaf spots, Black and yellow Sigatoka, Eumusae leaf spot, Mycosphaerella speckle, and Panama disease also known as Fusarium wilt of banana. Panama disease is one of the most destructive, which is considered the most important, because it can reduce banana production in some cases to zero yields [7].

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Panama disease (Fusarium wilt) of banana is the first banana disease to have spread globally [8]. It is a lethal fungal disease caused by the soil-borne Fungus *Fusarium oxysporum f. sp. cubense* (*Foc*). The fungus enters the plant through the roots and invades the vascular tissues (xylem vessels) thereby blocking the flow of water and nutrients, this in essence causes symptoms like progressive yellowing and gradual wilting of plant leaves, eventual collapse at the petiole, and longitudinal splitting of the outer leave sheaths in the pseudostem. Distinguishing internal symptoms like typical discolouration of vascular tissues from light yellow to dark brown is also observed. This appears first in the outer or older leaf sheath, and then extends up to the pseudostem [9]. Once the fungus is established in a field, it persists in the soil for an indefinite period of time. It can survive in the soil for up to 30 years as chlamydospores in infested plant material or in the roots of an alternative host [10]. Panama disease is one of the most destructive diseases of banana plants which has been threatening banana production worldwide. It is not only of a huge concern for the global export sector, but also has a great impact on the domestic production, as many locally preferred cultivars are also endangered, which is threatening the livelihood of millions of small scale farmers [11

Botanical fungicides are presently gaining momentum as they are considered as alternative source for chemicals in the management of soil borne pathogens. The active ingredients in botanicals may either act on the pathogens directly or induce systemic resistance in the host plants resulting in the reduction of disease development [12]. They are considered not only as an alternative to chemicals but also as less expensive, easily available and eco-friendly in the management of various plant diseases including Fusarium wilt [13 and 6]. Plant extracts have opened a new avenue for the control of plant diseases and much attention has been given to the use of secondary metabolites rich plant substances such as phenols, alkaloids, and terpenoids, amongst others, which serve as chemical defense agents against plant diseases [14]. Plant extracts have also been investigated throughout the world for their antifungal activity against a wide range of fungi [15]. The fungicidal and fungistatic potentials of *Acacia nilotica, Lawsonia inermis* and *Ziziphus spina-christi* plant extracts on different plant pathogenic fungi previously reported by various researchers inspired this research. The research was therefore, targeted at investigating the *in vivo* efficacy of the three plant extracts in the control of panama disease on banana plantlets under screen house conditions.

2. Material and methods

2.1. Collection of plant materials, preparation and preservation of plant extracts

Fresh diseased free leaves and barks of *Acacia nilotica* (Egyptian mimosa) and *Ziziphus spina-christi* (Christi thorn) were collected from Tunga, Chanchaga Local Government Area of Niger State while leaves and roots of *Lawsonia inermis* (Henna) were collected from Kutigi, Lavun Local government area of Niger State, Nigeria. The plants were being authenticated by a botanist in the Department of Biological Sciences, Federal University of Technology Minna, Niger State.

Plant samples collected were washed with tap water and disinfected with sodium hypochlorite (0.5 %) for 5 minutes, then rinsed thoroughly with sterile distilled water to remove the remaining sodium hypochlorite, and later dried with whatman No. 1 filter paper to remove excess moisture. The leaves were shade dried under room temperature for two weeks, while the bark and roots were dried for three weeks under same temperature. The plant samples were ground into powder using sterile mortar and pestle. Twenty (20 g) of each ground plant samples were weighed using electric weighing balance (ML-T weighing balance, model ML54T manufactured by METTLER TOLEDO) and poured into sterile No. 1 Whatman filter paper which were placed individually in the extracting flask of the Soxhlet apparatus with water bath heater consisting of six (6) holes of ten cm in diameter (model BST/SXW-6 manufactured by Praxo Instruments and Scientific company), after which 200ml of ethanol was poured into the round bottom flask of the Soxhlet setup, with the extracting chamber attached to the condenser and extracted for 24 hours at a temperature of 55° C. More ground plants were extracted until a substantial amount was obtained. The liquid extracts were concentrated in water bath and then preserved in airtight containers until needed for further analysis [10].

2.2. Preparation of culture media (Potato dextrose agar)

Healthy fresh Irish Potatoes were peeled and washed, out of which 200 grams was weighed and boiled for 20 minutes in 200 ml sterile distilled water. When boiled the liquid was drained into a conical flask 1 litre, 20 g of agar agar and glucose was poured to make a nutrient medium. Sterile distilled water was added to make 1000 ml of the mixture. The flask was corked with cotton wool wrapped in foil paper. The mixture was sterilized at 121° C for 15 minutes in an autoclave and allowed to cool for some minutes (Cheek bearable) after which 0.5 g of chloramphenicol was added and carefully stirred [16].

2.3. Isolation and identification of Fusarium oxysporum f. sp. cubense pathogen of panama disease of banana

Banana stem and leaves showing symptoms of Fusarium wilt disease were collected from banana Farm in Tunga, Chanchaga Local Government Area of Niger State and transported to the Departmental laboratory in paper envelops. The pathogen *F. oxysporum* f. sp. *cubense* was isolated from the infected banana stem collected, by cutting small pieces from diseased stem (about 3-6 mm long) and sterilized in 70% ethanol for one minute and 2% sodium hypochlorite for three minutes. After sterilization, the cut pieces were washed three times with sterile distilled water and then placed on 10 ml Potato Dextrose Agar with an antimicrobial agent (1 ml chloranphenicle) in petri dishes and incubated at 28 ± 2 °C in an incubating chamber. After seven (7) days of incubation, sub-culturing was done to obtain pure culture of *Foc* after which the cultural characteristics like mycelia colour was observed and morphological characteristics such as the shape and size of macro and micro conidia were studied under the microscope [10] and compared with the standard Manuals of Soil Fungi Gillman [17]. The organism was maintained in the media until needed.

2.4. Preparation of inoculum

Inoculum production was performed by inserting little discs of *Fusarium oxysporun* f. sp. *cubense* which was picked from a seven-day old pure culture medium in the centre of the petri dishes with PDA. It was packed in suitable conditions until the fungus filled the plate. After sporulation, conidia were collected adding 10 ml of sterile distilled water, rubbing a brush lightly over the colonies and subjecting the suspension to constant agitation for the spores to liberate, which was to determine the concentration of conidia suspension with the aid Hemocytometer [7]. The macro and microconidia were adjusted to a concentration of 10⁶ conidia/ ml suspension [18].

2.5. Pathogenicity test

The isolated organism was tested on two months old Gold finger tissue culture banana seedlings (three stands) to ascertain the Pathogenicity of the organism. The plants were transferred from polythene bags into plastic buckets and were allowed to acclimatize. After two weeks of acclimatization, the plantlets were then inoculated with 2 mL spore suspension of the *Foc* inoculums with a 2 mL syringe, and were observed for 46 days at 23 days' interval [7] under screen house conditions.

2.6. Experimental design

The experimental pots were laid in Completely Randomized Design with 8 treatments (three leave extracts, two bark extracts, one root extracts, one negative control with distilled water and one positive control with mancozeb), having three replicates each.

2.7. *In vivo* evaluation of three plant extracts in the control panama disease on banana plantlets under screen house condition

The experiment was conducted between June and December, 2017. The three treatments were further evaluated on two months old (Gold finger) tissue culture banana seedlings (Figure 1), which were obtained from National Centre for Genetic Resources and Biotechnology (NACGRAB) Ibadan Oyo State, under screen-house conditions to see their effects on Panama disease in banana.



Figure 1 Two months old tissues culture banana seedlings (collected from NACGRAB Ibadan) in polythene bags

A total of sixty-six (66) samples were collected and allowed to acclimatize for two weeks in the screen house garden of the Department of Biological Sciences, Federal University of Technology, Minna. They were transplanted into seven (7)

litres plastic buckets containing 6.5 kg of soil (Figure 2), cleared of unwanted plants and was treated with 2 % Formalin solution for two weeks' prior the time of transplant [19].



Figure 2 Banana plantlets planted in plastic bucket, inoculated with *Fusarium oxysporum* f. sp. *cubense* and irrigated with the plant extracts under screen house conditions

The seedlings were then inoculated (Figure 3) with 2 mL of *Foc* spore suspension which were injected at five (5) cm above the plants rhizomes with hypodermic syringe and were treated with the extracts at three different concentrations (25, 50 and 75 %) by irrigating with 100 mL of each (6).



Figure 3 Injecting a banana plantlet with *Fusarium oxsporum* f. sp. *cubense* inoculum after two weeks of acclimatization in the Screen House

The control plants were irrigated with sterile distilled water and commercial fungicide (Mancozeb 0.5 g/L) as negative and positive respectively. Observations were taken after 23 days for a period of 6 months' plant growth parameters such as plant height (using metre rule), girth (using tape rule), number of leaves and roots (by physical counting) were measured and recorded, the intensity of the vascular discolouration was also recorded using a scale of 1-6, and the survival rate of the plantlets was estimated [20].

2.8. Data analysis

Data obtained from both *in vitro* and *in vivo* (field) experiments were subjected to statistical analysis of Variance (ANOVA) to determine the significant differences among general significant levels. Duncan multiple range test (DMRT) mean separation was used to separate the means, where there are significant differences. The analysis was carried out using the Statistical Package for Social Sciences, 20th version, at 5 % level of significance.

3. Results and discussion

3.1. Morphological characteristics of Fusarium oxysporum f. sp. cubense

The morphological characteristics of *Foc* observed in this research were similar to those reported by Gnanasekaran *et al.*, [10] who observed that *Foc* produces three types of asexual spores (Macro conidia, micro conida and chlamydospores). Stating that the micro conidia were two celled, macro conidia were three to five celled gradually pointed and curved towards the ends and chlamydospores were round thick walled spores (Figure 4).

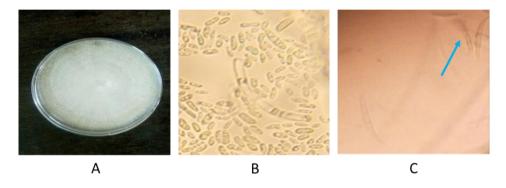


Figure 4 Seven (7) days old culture of *Fusarium oxysporum* f. sp. *cubense* exhibiting the while wooly mycelium. Photomicrograph of *Fusarium oxysporum* f. sp. *cubense* (A); Oval shaped micro conidia in abundance with Macro conidium in the middle having three septate (B); Macro conidia with two to three septate (C)

3.2. Pathogenicity

The results of pathogenicity revealed that, two months after inoculation of the isolated organism into the banana plantlets, the leaves became wilted, progressively yellow which extends from leaf margins towards the midridge, eventual collapse at the petiole, and longitudinal splitting of the outer leaf sheaths in the pseudostem. These symptoms were observed in older leaves (Figure 5).

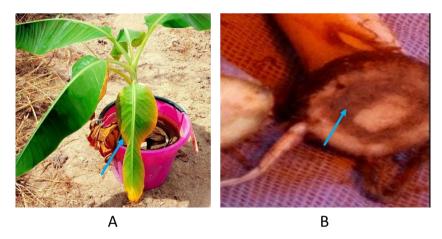


Figure 5 Panama Disease Symptoms. A - External features; leaves yellowing and wilting from the edge towards the mid ridge of the leaves. B-Internal features; brown to blackish discolouration of vascular tissues

This is in conformity with the result reported by Yin *et al.*, [9] who reported that *F. oxysporum* f. sp. *cubense* infects banana plants causing symptoms like gradual wilting, progressive yellowing of the leaves which spreads from the outer

leaf margin to the inner leave margin, eventual collapse at the petiole and longitudinal splitting of the outer leave sheaths. The morphological characteristic symptoms were; white wooly mycelium on PDA, macroconidia were gradually pointed and curved towards the ends. Two celled micro conidia all of which confirms that the causative organism of panama disease to be *F. oxysporum* f. sp. *cubense*.

3.3. *In vivo* evaluation of three plant extracts after six months of pathogen inoculation and treatment in the screen house

It was observed that after six (6) months of pathogen inoculation and treatment with the three plant extracts, the extracts and mancozeb (positive) treated plantlets had close similarities (no significant difference) in the growth parameters, producing a better growth rate than those treated with sterile distilled water which is the negative control. The plant growth parameters of the extracts and mancozeb treated plantlets ranged between 9-12 cm for the girths, the heights recorded was between 63-68 cm, the leave area were between 640-680 cm², while the total number of leaves and roots counted ranged between 8-10 and 21-15 respectively. While the banana plantlets treated with sterile distilled water had 8 cm, 41 cm, 400 cm², 6 and 19 for the girths, the heights, the leave area, the total number of leaves and total number of roots accordingly. This is however, in line with the observations of Khan and Nasreen [6], who reported that *L. inermis* extract at 1 % increase the percentage seed germination of Chikpea (*Cicer aeritinum*) with 30% and protected it from *F. oxysporum in vivo*.

Table 1 Effects of plant extracts on growth parameters of banana plantlets after six months of planting and inoculation with *F. oxysporum* f. sp. *Cubense*

Treatments	Concentrations (%)	Girth (cm)	Height (cm)	Leaf area (cm²)	Total number of leaves	Total number of roots
Plant extracts	75	12 ± 0.58^{b}	68 ± 2.31 ^b	680 ± 28.88 ^b	09 ± 0.58^{b}	22 ± 1.67^{b}
	50	10 ± 0.58^{b}	66 ± 2.08^{b}	660 ± 29.62 ^b	10 ± 0.88^{b}	22 ± 1.15^{b}
	25	09 ± 0.88^{b}	63 ± 2.08^{b}	640 ± 15.23 ^b	09 ± 1.33^{b}	21 ± 1.45^{b}
Mancozeb (Positive control)	0.5 g/L	12 ± 1.00 ^b	67 ± 1.45 ^b	636 ± 03.33 ^b	08 ± 0.33 ^b	25 ± 1.15 ^b
Sterile distilled water (Negative control)	1 L	08 ± 0.58^{a}	41 ± 0.67^{a}	400 ± 14.33 ^a	06 ± 0.33ª	19 ± 0.33ª

Values are means of triplicate ± standard error. Values followed by the same superscript down the column are not significantly different at P < 0.05 Duncan's test.

There was also a disease severity reduction exhibited by the extracts and mancozeb treated plantlets after six (6) months of inoculation and treatment. The intensity of vascular discolouration in the corms of the plantlets is shown in plate A, B C and D. It was observed that, the banana plantlets treated with the extracts all suppressed vascular discolouration in the corms, while the plant treated with mancozeb (positive control) showed corm complete clear discolouration. The plantlets treated with sterile distilled water (negative control) had complete vascular discoulouration in the corm. Fusarium wilt disease severity was calculated using a scale of 1-6. The intensity of vascular discoloration (Table 2) in the banana plantlets treated with A. nilotica leaves extracts at 75, 50 and 25 % concentrations suppressed the disease with a disease score rate of 1.0, 2.0 and 3.0 respectively while those treated with the bark extracts had a score rate of 1.0, 2.0 and 2.0 respectively for the three concentrations. Lawsonia inermis leaves extracts treated plantlets had a score rate of 1.0, 1.0 and 2.0 which were not significantly different accordingly with the positive control treated plantlet, while those treated with L. inermis roots extracts had 2.0 at 75, 50 and 25 % all through. Ziziphus spina-christi leaves extracts treated plantlets had a disease score rate of 3.0 at concentrations 75, 50 and 25 % all through, while the treated plantlets with Z. spina-christi bark extracts had 3.0, 3.0, and 4.0 for 75, 50, and 25 % respectively. The plantlets treated with mancozeb (positive control) recorded a disease score rate of 1.0, and the negative (sterile distilled water) control treated plant had 5.0 severe discolouration. Significant differences at P < 0.05was observed amongst the extracts treated plantlets and the negative control (SDTW) plantlets.

Similarly, Thangavelu and Mustaffa [20], reported that Zimmu leaf extract at five different concentrations (5, 10, 25, 50 and 100 %) were used on banana plantlets inoculated with *Foc* to suppress Fusarium wilt disease. It was observed that at 50 and 100 % concentrations, the disease was completely suppressed and also increases the plant growth parameters such as plant height (up to 98.6 %), girth (up to 71.8 %), total number of leaves (up to 63 %), leaf area (up to 254.5 %),

and total number of roots (up to 88.2 %). The Zimmu leaf extract treatment also enhanced both growth and yield parameters significantly as compared to untreated control plants, 80.8 % increase was observed in bunch weight upon Zimmu leaf extract application and moreover all the plants (100 %), which received Zimmu treatment, yielded salable bunches, whereas, in the case of the control treatment, the amount of salable bunches was only 25 %, which might be due to the effective suppression of Fusarium wilt disease by the Zimmu leaf extract treated plants Gopi and Thangavelu [21].

Table 2 Disease severity score on banana plantlets after six months of inoculation with *F. oxysporum* f. sp. cubense andtreatment with Acacia nilotica, Lawsonia inermis and Ziziphus spina-christi plant extracts

Treatments (extracts)	Plant	Disease severity score (1-6)					
	Parts	SDTW	Mancozeb	Extracts Concentrations (%)			
			(0.5 g/L)	75	50	25	
A. nilotica	L	$5.0 \pm 0.17^{d^*}{}_{a^{**}}$	$1.0 \pm 0.00^{a_{a}}$	1.0 ± 0.15^{a_a}	2.0 ± 0.00 ^b b	$3.0 \pm 0.00^{c_{b}}$	
A. nilotica	В	5.0 ± 0.17 c _a	$1.0 \pm 0.00^{a_{a}}$	$1.0 \pm 0.00^{a_{a}}$	$2.0 \pm 0.17 b_b$	2.0 ± 0.15^{b_a}	
L. inermis	L	5.0 ± 0.17 ^c _a	$1.0 \pm 0.00^{a_{a}}$	$1.0 \pm 0.13^{a_{a}}$	1.0 ± 0.33^{a_a}	2.0 ± 0.67^{b_a}	
L. inermis	R	5.0 ± 0.17 ^c _a	$1.0 \pm 0.00^{a_{a}}$	$2.0 \pm 0.20^{b_{b}}$	2.0 ± 0.00 b	2.0 ± 0.15^{b_a}	
Z. spina-christi	L	5.0 ± 0.17 ^c _c	$1.0 \pm 0.00^{a_{a}}$	$3.0 \pm 0.29^{b_{c}}$	$3.0 \pm 0.13^{b_{c}}$	$3.0 \pm 0.29^{b_{b}}$	
Z. spina-christi	В	$5.0 \pm 0.17 d_a$	$1.0 \pm 0.00^{a}a$	$3.0 \pm 0.67 b_{c}$	$3.0 \pm 0.13^{b}{}_{c}$	4.0 ± 0.20 ^c _c	

Values are means of triplicate ± standard error. *Values followed by the same superscript down the column are not significantly different at P < 0.05 Duncan's test. **Values followed by the same subscript across the rows are not significantly different at p < 0.05 Duncan's multiple range test, (L) = Leaves, (B) = Bark and (R) = Root.

Total percentage survival of the plantlets after six months of inoculation and treatment with the extracts is presented in Table 4.6. In the plants treated with *A. nilotica* (leaves) extracts, 49, 44 and 39% survival was recorded at 75, 50 and 25% concentrations respectively, those treated with the bark extracts of the same plant had 45, 40 and 33% accordingly for 75, 50 and 25%. *L. inermis* (leaves) treated plants at 75, 50 and 25% concentrations had 50, 48 and 40% respectively, while the roots extracts had 45, 42 and 39% at 75, 50 and 25% respectively. Banana plantlets treated with *Z. spina-christi* leaves extracts had a survival rate of 20, 12 and 10% for concentrations 75, 50 and 25% respectively, while the plantlets treated with bark extracts of the same plant had 20, 20 and 15% accordingly for the three concentrations. The control treated plantlets had 55 and 10% positive and negative respectively. However, there were no significant differences (P > 0.05) observed in the percentage survival of the treated plantlets (*A. nilotica* leaves extracts, *L. inermis* leaves extracts at 75 and 50 %) with the mancozeb treated plantlets, but there were differences as compared with those treated with sterile distilled water.

Table 3 Percentage survival of banana plantlets after six months of inoculation with *F. oxysporum* f. sp. *cubense* and treatment with *Acacia nilotica*, *Lawsonia inermis* and *Zizihpus spina-christi* plant extracts

Treatments (extracts)	Plant Parts	Disease severity score (1-6)					
		SDTW	Mancozeb	Extracts Concentrations (%)			
			(0.5 g/L)	75	50	25	
A. nilotica	L	10.0 ^{a*} a**	$55^{cd}a$	49 ^c b	$44^{bc}b$	39 ^b c	
A. nilotica	В	10.0 ^a a	55^{d_a}	45^{c_b}	40^{c_b}	$33b_b$	
L. inermis	L	10.0 ^a a	$55c^{cd}a$	50°c	$48^{bc}c$	$40^{b}c$	
L. inermis	R	10.0 ^a a	55^{d_a}	45^{c_b}	$42^{bc}b$	$39^{b}c$	
Z. spina-christi	L	10.0 ^a a	55^{d_a}	20^{c_a}	12^{b_a}	10^{a_a}	
Z. spina-christi	В	10.0 ^a a	55 ^c a	20 ^b a	10 ^a a	10^{a_a}	

Values are means of triplicate. *Values followed by the same superscript across the rows are not significantly different at P < 0.05 Duncan's test, and **values followed by the same superscript across the rows are not significantly different at P < 0.05 Duncan's test, (L) = Leaves, (B) = Bark and (R) = Root. The Plantlets treated with the extracts from *A. nilotica* (bark), *L. inermis* (Leaves) at 75% concentrations had a disease severity score rate of 1.0 which statistically had no difference (P > 0.05) with the positive control (mancozeb) where 0.1 was recorded, showing that *A. nilotica* and *L. inermis* leaves extracts compete favourabbly with the fungicide (mancob). This is in line with the reports of Fernando *et al.* [6] who stated that, the extracts and essential oils of *C. zeylanicum* and *S. aromaticum* achieved best values in controlling Fusarium wilt disease of banana, obtaining values equal to the fungicide treatment used. The total percentage disease reduction by the three extracts was however best at 75% concentration of *L. inermis* extracts which suppressed panama disease in the banana plantlets by 50, 48, and 40%. This corresponds to the work of Huang *et al.* [22] who studied the effects of Chinese leek (*A. tuberosum*) on Fusarium wilt disease, and reported 58 % reduction of incidence in the banana variety Baxi (AAA) and 79 % in the banana variety Guangfen No. 1 (ABB) under greenhouse conditions.

4. Conclusion

The result from this research showed that, the three plant extracts at 75, 50 and 25 % concentrations suppressed panama disease in banana seedlings (*in vivo*), with a survival rate of 50, 49 and 48 %. *Ziziphus spina-christi* extracts had the lowest effect on the severity of the disease and also the survival rate. The results are promising and the best two plant extracts can go a long way in reducing panama disease on banana fields. The botanical extracts used are hereby, suggested for further field trials to determine their longevity.

Compliance with ethical standards

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Disclosure of conflict of interest

All the three authors of this research article declare that there were no conflicts of interest.

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