ANTIFUNGAL ACTIVITY OF LEAF EXTRACTS OF SOME PLANT SPECIES ON *Mycosphaerella fijiensis* MORELET, THE CAUSAL ORGANISIM OF BLACK SIGATOKA DISEASE IN BANANA (*Musa acuminata*)

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ABSTRACT

The effect of leaf extracts of *Vernonia amygdalina* Del., *Azadiracta indica* A. Juss and *Ocimum gratissimum* L. on conidiospore germination, mycelia extension and lesion development of *Mycosphaerella fijiensis* Morelet, the causal agent of black Sigatoka disease of banana was investigated. An increased antifungal activity was noticed with a corresponding increase in concentration of aqueous extracts from all the plants. A 100% concentrated extract of *O. gratissum* completely inhibited conidial germination ($P< 0.05$). There was a general decrease in the incidence of lesion occurrence on all the banana plants sprayed with plant extracts in the field in a descending order of *O. gratissimum*, *A. indica* and *V. amygdalina*. Extracts of *O. gratissimum* compared favourably with the disease control achieved with benomyl application. These findings are discussed in relation to plant chemical means of disease control on banana.

Keywords: *Mycosphaerella fijiensis*, black Sigatoka, banana, plant extracts, plant disease control, antifungal activity.

1. INTRODUCTION

Banana and plantain (*Musa* spp.) are important food crops in East and West Africa [1]. Annually sub-saharan Africa produces approximately 35% of the world's 68 million tons of banana and plantain. These food crops provide more than 25% of the carbohydrates consumed by the approximately 350 million people in the region [2]. Plantain and banana are cultivated mostly in the moist tropics and, in Nigeria, they are found mostly in the south of the country.

Damage caused by pests and diseases on plantain and banana has increased over the years. One of the most important constraints to plantain and banana production is a leaf spot disease called black Sigatoka caused by the fungus *Mycosphaerella fijiensis* Morelet. Black Sigatoka is also considered as one of the most destructive diseases of *Musa* sp. in the world. It causes losses by reducing the functional leaf surface of plants, resulting in small, unevenly ripened bananas that may fall and fail to reach maturity [3-4]. The disease has been introduced into Africa recently, and has spreaded rapidly throughout all plantain and banana producing areas in the area [5]. In Nigeria and elsewhere in Africa, where plantain and banana are produced, the disease results in annual yield loss of 30-50% [6-7].

Great advances are being made in controlling black Sigatoka diseases. Control strategies which have been adopted include inspection and quarantine, cultural methods, fungicides and petroleum oil application, and breeding for varietal resistance [7, 2, 4]. Biological control of plant pathogens by employing antagonistic microorganisms has proved to be a reliable alternative to the use of chemicals in the effective management of some diseases [8 -11]. Studies on the use of plant extracts for control of diseases have shown the importance of natural chemicals as possible sources of non-phytotoxic and easily biodegradable alternative fungicides [12]. However, this growing and promising method of diseases control has not been attempted on the black Sigatoka disease.

There is an urgent need to look for a method of control of black Sigatoka that will be affordable, durable and free of chemicals that can pollute the environment. This work, therefore, examines the anti-microbial effects of bitter leaf (*Vernonia amygdalina* Del.), neem leaf (*Azadirachta indica* A. Juss) and scent leaf (*Ocimum gratissimum* Linn.) extracts on *Mycosphaerella fijiensis* Morelet, the causal organism of black Sigatoka disease of banana (*Musa acuminata*).

2. MATERIALS AND METHODS

2.1 Collection of *Mycosphaerella* samples

Leaves from Cavendish banana cultivars "dwarf Cavendish" with Sigatoka lesion were collected from an evaluation trial farm at the University of Benin, Nigeria. At the laboratory leaf portions containing disease lesion were cut into segments with dimensions of about 4 x 4 cm and put into McCartney bottles containing 10 ml of sterile water. The McCartney bottles were sealed and shaken vigorously for about 45 seconds. This vigorous shaking with fresh sterile water was repeated twenty five times. The rinsed leaf segments were then surface sterilized in 70% alcohol for 30 seconds. Thereafter, the leaf segments were rinsed twice with sterile water, dried between sterile tissue paper and plated out on potato dextrose agar (PDA) containing antibiotics mixture (streptomycin and penicillin at 0.6 mg / ml) to discourage bacterial growth. There were four replicate leaf segments per Petri-dish and four replicate plates for each of the individual banana leaves portions. Plates were incubated at room temperature (28 ± 2^0 C) in a laboratory bench and kept 30 cm under a 1.5 m fluorescent daylight tube for 10-h photo–period daily for five days when fungal outgrowths were isolated and identified using the methods of Emoghene *et al.* [4]. Pure cultures of the fungal isolates were obtained and preserved on PDA slants in the refrigerator at 4^0C with sub-culturing at monthly intervals.

2.2 Pathogenicity test

A pathogenicity test was carried out by the leaf segment method [4]. The youngest unfurled leaves of banana in the multi-locational trial farm in the University of Benin were used for this test. Portions (segments) of the leaves measuring 7cm by 7cm and cut under sterile distilled water were washed in four changes of sterile distilled water and then surface sterilized in 70% alcohol for 30 seconds and rinsed twice in sterile water. The middle of the adaxial (upper) surface of each leaf segment was wounded by lightly pricking an area of approximately 5 mm in diameter with sterile needle. Each leaf surface was placed, adaxial surface up, into a 9 – 12 cm sterile petri - dish lined with sterile moistened tissue paper to maintain high relative humidity. Three sterile glass rods were placed beneath the leaf segments to keep them away from contact with the moistened tissue paper. The wounded area of a leaf segment was then inoculated with a 5 mm agar disc from a 5-day old culture of *M. fijiensis* isolates, with the mycelial surface in contact with the leaf. Wounded but uninoculated leaf segments served as control. There were three replicate leaf segments per isolate of *M. fijiensis*. The plates were incubated for seven days at room temperature ($28 \pm 2\ ^{0}C$) at 10-hour photoperiods using a 1.5 m fluorescent daylight tube. Observations were made for development of disease symptoms every 24 hours. *M. fijiensis* was reisolated as described above from the affected leaf sections after the incubation period.

2.3 Preparation of leaf extracts and leaf extracts agar

Fresh leaves of *V. amygdalina*, *A. indica* and *O. gratissimum* were thoroughly washed under running tap water then sterile distilled water, and air dried ($28^{0}C$) for 2 h, then homogenized into a paste of 1 kg for each leaf with a blender (mixer model 830 L, Hong Kong).

Hot water extracts were obtained by infusing the paste from each plant separately with 100 ml sterile distilled water using 250 ml conical flasks in water bath at 90 ^{0}C for one hour. Thereafter, the suspension was filtered through sterile muslin cloth. Different concentration of 10,

25, 50 and 100 % were prepared by dilution with water. Five ml from each of the dilutions was dispensed into 9-cm diameter Petri dishes after which 20 ml of melted PDA were poured into the plate, shaken together and allowed to solidify, forming potato dextrose leaf extract agar.

2.4 Effect of leaf extracts of *Vernonia amygdalina*, *Azadiracta indica* and *Ocimum gratissimum* on germination of *M. fijiensis* spores

Conidiospores suspension was obtained from 15 - day old PDA culture plates of *M. fijiensis* and were separately suspended in 100, 50, 25 and 10 % dilutions of leaf extracts of *V. amygdalina*, *A. indica* and *O. gratissimum*. The concentration of the spores was 10,000 spores/ml. Controls were prepared using sterile distilled water. Four replicate drops of the suspension were incubated on sterile microscope slides placed inside petri dish moisture chambers and incubated at room temperature ($28 \pm 2\ ^{0}C$). Conidial germination was examined every hour for up to 9 hours.

2.5 Mycelia extension growth rate of *Mycosphaerella fijiensis* on leaf extract agar

Inoculum discs of 5 mm in diameter obtained from the edge of a six day old culture of *M. fijiensis* on PDA were inoculated face downwards at the center of each of the different leaf extract agar plates. PDA without leaf extract served as control. Three replicate plates of leaf extract agar per isolate were incubated at room temperature (28 ± 2) under a 1.5m fluorescent daylight tube for ten hours photoperiod for up to seven days. Daily measurements of the mycelial extension of the cultures were determined by measuring culture size along two diameters. Mycelial growth inhibition is taken as growth of the fungus on the leaf extract agar expressed as percentage of growth on the PDA.

2.6 Field evaluation of tissue extracts and benomyl

Two month old seedlings of *Musa acuminata* cv dwarf Cavendish from the multi-locational evaluation trial farm in the University of Benin were raised in plastic pots each containing 10 kg of sterilized field soil. Potted plants were arranged in groups of 30 in an open field. Each group was subdivided into three groups of 10 seedlings and given the following treatments. (a) Spraying plants with 500 ml of plant extract (50 % concentration) and another 5 with benomyl (3 g/l) two days before inoculation of *Mycosphaerella fijiensis* (b) Simultaneous inoculation of *M. fijiensis* and spraying of plant extracts or benomyl on 5 banana each and (c) Inoculation of the pathogen 14 days after spraying 5 banana with the plant extracts.

Spraying with sterile distilled water served as control. The percentage disease incidence was taken as the number of plants with lesion expressed as the percentage of the whole set. The data obtained were subjected to ANOVA and mean comparisons were according to Duncan's Multiple Range Test [13].

3. RESULTS

Grayish colonial growth was observed 2 – 3 days after the strips of infected leaves were inoculated on plates. The light yellow margin become deeper in color and better defined by the sixth day. Also, the center brown became dry and turned brown. *M. fijiensis* was reisolated from the infected leaf segments, thus confirming that the organism is the causal agent of black leaves Sigatoka disease. The control did not show lesion development.

A general trend of increased anti-fungal activity was noticed with a corresponding increase in concentration of the aqueous extracts of all the plants (Tables 1, 2). At highest concentration of 100%, spore germination and mycelial growth of *M. fijiensis* was entirely inhibited by the leaf

extract of *O. gratissimum*. *O. gratissimum leaf extract* showed significantly higher antifungal activity than *A. indica* and *V. amydalina* at all concentrations of leaf extract studied (Tables 1, 2).

Table 1. Percentage inhibition of conidisopore production by leaf extracts of *Azadiracta indica*, *Vernonia amygdalina* and *Ocimum gratissimum*.

Concentration of extract	*Vernonia amygdalina* (%)	*Azadiracta Indica* (%)	*Ocimum gratissimum* (%)
100	64. 8 e	98. 5 a	100 a
50	25. 0 f	60. 1 b	70.4 h
25	8. 1 g	32. 1 c	20.3 j
10	6.2 d	7. 6 d	14.3 k

Values followed by the same letters are not significantly different (P>0.05) according to Duncan's multiple range test.

Table 2. Percentage inhibition of mycelial growth of *Mycosphaerella fijiensis* on agar plates by plant extracts of *Vernonia amygdalina*, *Azadiracta indica* and *Ocimum gratissimum* after 7 days of inoculation.

Concentration of extracts	*Vernonia amygdalina* (%)	*Azadiracta indica* (%)	*Ocimum gratissimium* (%)
100	64.8 a	100 e	100 e
50	28.3 b	68.3 a	75.1 h
25	13.3 c	16.4 f	32.1 j
10	3.8 d	8.2 g	18.1 f

Values followed by the same letters are not significantly different (P> 0.05) according to Duncan's multiple range test.

Lesion on whole plant reduced by the introduction of the extracts (Table 3). Results show greatest inhibition when leaf extracts were applied at the same time as the pathogen was inoculated. The inhibition of the lesion was higher in the treatments on which the extracts were sprayed two days the pathogen had been inoculated (Table 3). There was a general decrease in the incidence of the occurrence of lesions on banana plants sprayed with the extracts in the field (Table 3). Disease incidence was lowest with *O. gratissimum* followed by *A. indica* and *V. amygdalina* in a descending order (Table 3). There was no significant difference between banana sprayed with benomyl and *Ocimum gratissimum* 14 days after inoculation (Table 3). Both *in vitro* or *in vivo*, *Vernonia amygdalina* extract had the lowest effect on the inhibition of mycelia, spore germination or lesion development of *M. fijiensis* (Table 3).

Table 3. Percentage incidence of occurrence of *Mycosphaerella fijiensis* lesion on banana leaf sprayed with plant extracts of *Vernoria amygdalina*, *Azadiracta indica* and *Ocimum gratissimum* in the field.

Sprayed	**Inoculum Regime**		
	Two days before inoculation (%)	**Simultaneous inoculation (%)**	**14 days after inoculation (%)**
Vernonia	43.8 a	38.4 a	42.7 a
Azadiracta	20.2 b	14.1 f	18.6 b
Ocimum	15.4 c	3.1d	5.4d
Benomyl	5.6d	0 h	4.3d
Control	61.1e	68.1 e	59.3e

Values in the same column followed by a similar letter are not significantly different (P> 0.05) according to Duncan's multiple range test.

4. DISCUSSION

Several workers have emphasized the advantage of using higher plants in controlling plant diseases [12, 14, 15] and use of microorganism [16, 17, 8 - 11] but very little work has been done on use of natural plant products as pesticide for control of black Sigatoka disease of banana.

The present study has shown that *Vernonia amygdalina, Ocimum gratissimum* and *Azadirachta indica* have some antimicrobial substances that affect the germination and growth of *M. fijiensis*. However, the effect of *V. amygadalina* was less inhibitory. It has been suggested [18] that the growing pathogen metabolises the *V. amygdalina* extracts and therefore this is not an effective antimicrobial agent for *M. fijiensis*. Active principles present in plants are influenced by many factors which include the age of plant, extracting solvent, method of extraction and time of harvesting plant materials [19, 20]. Water extract of neem leaves was found to be more effective in reducing the spore germination and growth of *Colletotrichum* than organic solvent extraction [15, 21]. The possible explanation for the superiority of water extract over organic solvent is that organic solvent may have reacted with the active ingredients in the neem leaf, forming inactive compound [15]. However, this is in contrast with others workers [12, 22-23] who reported that organic extractions were more effective than water extractions in controlling *Rhizoctonia solani* in cowpea, Piper bitter leaf extracts against *Pyricularia oryzae* and *Cajanus cajan* on some pathogens of man. The reason they advanced was that the organic solvent extracted the fungicidal principle more effectively than water. The use of aqueous extract in this study could be the reason for low level of control obtained with some of the plant extracts used.

The result of this study, suggested *Ocimum gratissimum* and *Azadiracta indica* could be useful in controlling black Sigatoka disease of banana. The plant extract from *O. gratissimum* compared favourably well with benomyl which has been used for control of the fungus.

The outcome of this study showed that the use of plant extracts from *Ocimum gratissimum* and *Azadiracta indica* has potential as a substitute for chemical pesticide for control of Sigatoka disease of banana. This approach to plant disease management is economically and environmentally viable. The results look promising and should therefore be tried out as an appropriate repeat spraying regime through the life cycle of banana plants.

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