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Full Length Research Paper

The effect of organic soil amendments on stalk rot of maize caused by *Fusarium verticillioides*

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The effect of 3 organic soil amendments viz., cassava peel (*Manihot esculenta*, Cranz), sawdust (*Gmelina arborea*, Roxb) and leaves of (*Cedrela odorata*, L) on the stalk rot of maize (*Zea mays* L.) caused by *Fusarium verticillioides* was investigated. Fourteen treatments made up of single or combined treatments with pathogenic or non-pathogenic inoculation of concentrations 3:1, 2:2 and 1:3 were added to 15.8 g sterilized soil. Growth parameters data on leaf numbers, stem girth, plant height and leaf area were collected biweekly. All treatments had significant effects on plant heights, number of leaves, leaves areas, stems girths and on disease indices and disease severities of the treated plants compared to controls. Concentration 2 (2:2) had the highest effect on all the growth parameters considered and gave the lowest disease index (P= 0.05, R^2= 0.98) and disease severity (P= 0.05, R^2= 0.92) in the treated plants. Plants treated with cassava peels combined with *C odorata* had significantly lowest disease index and severity thus, competing favourably with *F verticillioides*. Severity of stalk rot of maize can reduce significantly in amended soils compared to unamended soils.

Key word: Organic amendment, stalk rot, *F verticillioides,* toothpick inoculation, maize stem, *Gmelina arborea*, *Manihot esculenta*, *Cederela odorata*.

INTRODUCTION

Maize is one of the most important cereal crops cultivated in Nigeria (Iken and Amusa, 2004). Maize is the most important cereal crop and staple food for about 1.2 billion people (Macauley, 2015). It belongs to the grass family Poaceae. Maize constitutes a major part of diet in Nigeria. It started as a subsistence crop and gradually became a major important crop in the commercial sector on which many agro based industries depend on for raw materials (Iken and Amusa, 2004). In the last decade it registered as the highest growth rate among all food grain. The total production of maize in 2020 was estimated to be about 12 million metric tons (Doris Dokua Sasu, 2022).

However, the production of maize in the whole world

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including Nigeria is threatened by various constraints including its susceptibility to both pest and diseases that cause pre- and post-harvest losses in Nigeria. Pathogenic infections are considered as one of the most prevalence limiting factors that contribute to reduction in the cultivation of maize. Among the variety of pathogens, *Fusarium* is considered a devastating fungal menace in maize cultivation (Agoda et al., 2011).

Stalk rot of maize is the most destructive disease worldwide and reduced yield by 10.0% (Li et al., 2010) and may increase to 30.0 to 50.0% in serious cases (Yu et al., 2017) caused by *F. verticilliodes*. The pathogen has been documented to be a major cause of stalk rot of maize (Akinbode et al., 2014; Christensen and Wilcoxson, 1996; Drepper and Renfro, 1990). *F. verticillioides* infect maize across the world, posing one of the greatest threats of toxin contamination especially fumonisin. The stalk rot caused on maize results in stalk breakage and lodging thereby making harvesting difficult and consequently reduces yield. The fungus *F. verticillioides,* penetrate stalk and root directly or spread systemically in the plant after infection that originated from seed borne inoculum (Akinbode et al., 2014; Olawuyi et al., 2011; Munkvold and Desjardins, 1997).

Organic soil amendments made from plant and animal remains are of more importance than inorganic fertilizer because it consists of relatively stable decomposed materials resulting from accelerated biological degradation of organic matter under controlled aerobic conditions (Epstein, 1997). Several studies have shown that organic amendments can be very effective in controlling diseases caused by pathogens such as *F* spp. (Lewis and Papavizas, 1977; Akanmu et al., 2013), since most subsistence farmers do not have the means to purchase synthetic fungicides.

Several methods have been employed in controlling various plant pathogens including: physical, chemical, biological and cultural methods. Synthetic pesticides are known to be highly efficient and effective in the management of crop diseases (Akanmu et al., 2013); however, the indiscriminate use of fungicides has posed a serious threat to human health and to the ozone layer. There is therefore a need for a more environmentally and human friendly approach of controlling plant diseases.

Application of organic amendments to the soil is emerging as an economically and environmentally acceptable alternative to disposal through landfill because of its agronomic benefits and ability to reduce predisposal to soil borne diseases. The use of organic amendments for the managements of plant disease has been documented to achieve several successes and the level of disease control has been documented to be consistent or predictable to an extent (Akamu et al., 2013). The aim of the study was to determine the effect of selected organic soil amendment on the survival of *F. verticillioides* in maize stalk and maize production, with regard to combination of amendments.

MATERIALS AND METHODS

Experimental site

The pot experiment was conducted in the screen house of the Department of Botany, University of Ibadan, Oyo state, Nigeria.

Source of materials used

Maize seeds, (genotype DMR-LSR-Y) were obtained from the Institute of Agricultural Research and Training (IAR and T), Ibadan.

Source of botanical samples

Fresh cassava peels were collected in bags from a cassava processing center at the outskirt of University of Ibadan in Agbowo area of Ibadan. Sawdust of *Gmelina arborea* were collected from a major sawmill at Bodija in the area of Ibadan, while fresh leaves of *Cedrela odorata* were collected from the botanical garden of the University. All materials were dried at room temperature for 2 to 3 weeks, and then milled to fine particles, with a 2 mm sieve. The infected stalks of maize were collected from the International Institute of Tropical Agriculture, Moniya, Ibadan, Oyo state Nigeria.

Isolation and identification of pathogen

The infected stalk samples were cut into small pieces up to about 1.5 to 2 cm and surface sterilized in a mixture of 1% sodium hypochlorite and placed on potato dextrose agar (PDA) media containing anti-bacterial (lactic acid) drops for 3 to 5 days in an incubator at a temperature of 30 ± 1°C.The colonies of observed fungal growth were sub-cultured on agar media till pure cultures of suspected *F. verticillioides* isolates were obtained. The identification and taxonomic classification was aided by the use of Fusarium identification manuals “Fuskey” Seifert (1996) and pictorial atlas for identification of fungi by Watanabe (2002) for decription of colony morphology.

Preparation of fungal spores’ count of *F verticillioides*

The mycelia growth of 5 days old culture of *F. verticillioides* were harvested by rinsing with sterile distilled water into a sterile bottle and the solution was sieved using a double folded cheese cloth to allow the passage of fungal spores which were later counted using the haemocytometer(BLAUBBRAND: BRT17810-1EA) to approximately 3×10^{5} spores/ml.

Screen house experiment

Top soil collected from the nursery of the Botany Department, University of Ibadan was sieved and sterilized using an electric soil sterilizer and left to cool. Six kilogram of soil were placed in several 15×11 cm polypots and 15.8 g of various treatment were added, two weeks prior planting in other to decompose, before the test crop (DMR-LSR-Y) was planted.

Experimental design

The pot experiment was arranged in a Completely Randomized Design (CRD) with three replications. The individual and combined effects of treatments were observed. The treatments consisted of:

Figure 1a. Inoculation of treated toothpick into the internode of the maize stalk.
Source: Authors

Treatment 1= cassava peels + *F.verticillioides*
Treatment 2 = sawdust + *F.verticillioides*
Treatment 3 = *Cedrela odorata* + *F.verticillioides*
Treatment 4 = cassava peels + sawdust + *F.verticillioides*
Treatment 5= cassava peels + *Cedrela odorata* + *F.verticillioides*
Treatment 6= sawdust + *Cedrela odorata* + *F.verticillioides*
Treatment 7 (Control 1) = *F.verticillioides*
Treatment 8 (Control 2) = Control.
Each of the sawdust and botanical treatments were combined in the ratio 3:1 (75/25), 2:2 (50/50) and 1:3 (25/75).

Planting and inoculation of maize

Maize seeds were planted in organic amended soil, three seeds per pot. Adequate management practices such as wetting, thinning and weeding were carried out. Maize plants were inoculated at tasseling stage (7 weeks after planting) with *F. verticillioides* using toothpick method (Figure 1a) as modified by Drepper and Renfro (1990); Hameed et al., (1997); Sobowale (2011).

Disease incidence rating and analysis

Fourteen weeks after planting (7weeks after inoculation) maize stems were observed for rot formation. The incidence and severity of disease were determined around the inserted toothpicks at harvest (7weeks after inoculation) as shown in Figure 1b. This was achieved by centrally splitting the stalk lengthwise and recording the extent of spread of rot on a modified form of the Hooker's scale (Iken and Amusa, 2004) which indicates the percentage of infection in the inoculated internode, using the scale below:

1 = 0 to 4% of internode rotten.
2 = 5 to 25% of internode rotten.
3= 26 to 50% of internode rotten.
4 = 51 to 75% of internode rotten.
5 = 76 to 100% of internode rotten as modified by (Sobowale, 2011).

Grain yield was also recorded seven weeks after inoculation (14WAP); yield was estimated based on weight of grain at 15%moisture. Disease data on 0 to 5 scale were transformed to percentages for statistical analysis as follows (Hameed et al., 1997):

$$\text{Disease index} = \frac{\sum \text{Disease rating in plants examined}}{\text{Total number}}$$

$$\text{Disease severity\%} = \frac{\text{Disease index}}{5} \times 100$$

Data collection and analysis

Data on leaf number, plant height (cm), stem girth (mm) and leaf area (cm^2) were obtained from two weeks after planting on a weekly basis till twelve weeks after planting. The results obtained were subjected to Analysis of Variance (ANOVA) using SAS package (1993) version and means were separated with Duncan Multiple Range Test (DMRT) at p=0.05

RESULTS

The mean comparisons of the treatments on growth parameters of the treated maize plants gave significant

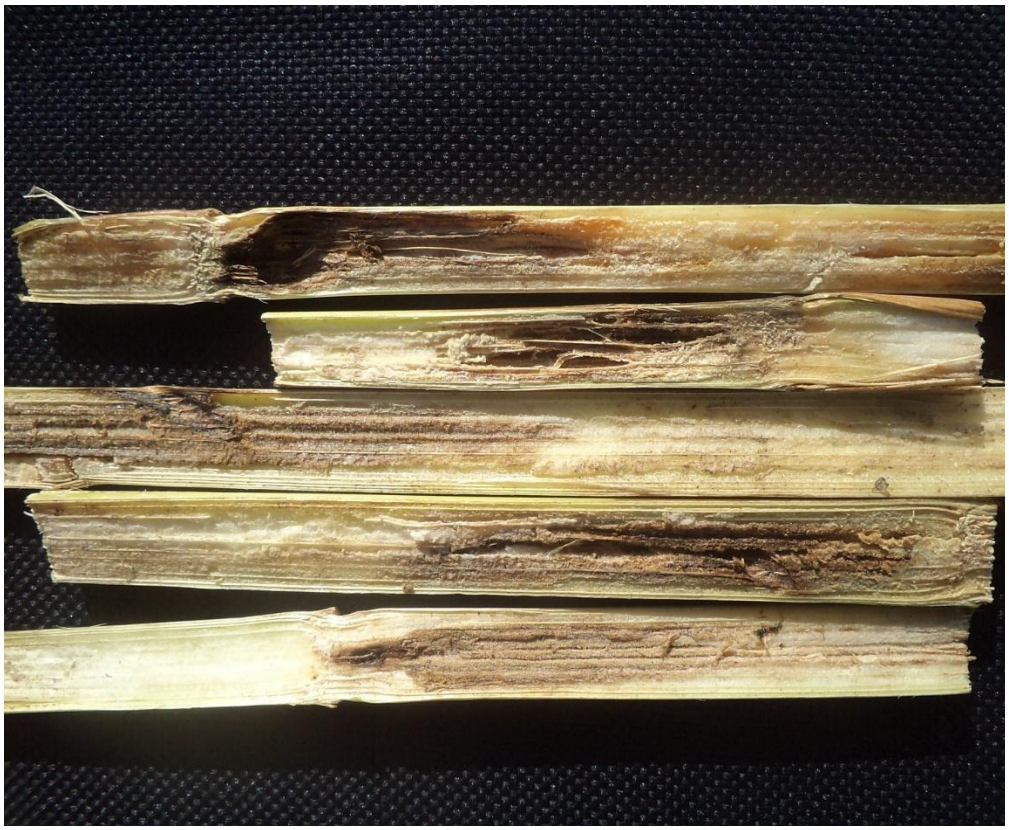

Figure 1b. Split treated maize stem (internode) with severe rot formation.
Source: Authors

differences at *P*=0.05 (Table 1). The number of leaves of the maize plants treated with T5 (cassava peel+*C. odorata+F. verticillioides*) and T6 (sawdust+leaf+ *F. verticillioides*) were significantly different (p=0.05, R^2= 0.54) from Control 1 (T7) maize + *F. verticillioides*. The number of leaves of the maize plants treated with T1 (cassava peels + *F. verticillioides*) was significantly different from Control 2 (T8). However, the number of leaves of maize that received other treatments were not significantly different from controls 1 and 2 (*P*=0.05). The stem girth and plant height of the maize treated with T1, T2 (sawdust + *F verticillioides*), T3 (*C.odorata* + *F.verticillioides*), T4 (cassava peels + sawdust + *F verticillioides*), T5 and T6 were less than controls (1 and 2) (*P*=0.05, $R^{2\ =}$ 0.85) compared to other treatments. The leaf area of the maize treated with T1 to T4 were significantly different from the controls 1 and 2 (*P*=0.05, R^2=0.77). However, the leaf area of maize that received treatments T5 and T6 were not significantly different from control (*P*= 0.05).

Table 2 show the effect of different concentrations of treatment on some growth parameters of the maize after treatment. The number of leaves of maize affected were significantly higher and similar (*P*=0.05, R^2=0.54) in groups treated with concentration 1 (3:1) and 2 (2:2) than those that received concentration 3 (1:3). The plant height of maize treated with concentration 2 were significantly higher (*P*=0.05, R^2= 0.95) than those that received concentrations 1 and 3. The leaf area of maize treated with concentration 2 was significantly higher (*P*=0.05, R^2= 0.77) than those that received concentrations 1 and 3.

The summary of mean (*P*=0.05, R^2= 0.95) rot formation and rot rating for maize stem that received different treatments are presented in Table 3. Treatments 1 to 8 had moderate rot formation within the inoculated internode ranging from as low as 27% in reatment 3 has no ratio, treatment 4 (2:2), treatment 5 (1:3 and 3:1), treatment 6 (1:3 and 2:2) to slighty higher rot formation of 47% in treatment 1:40% treatment 2:33% in treatment 4 (1:3 and 3:1) and treatment 6 (3:1). However, severe rot formation was recorded within inoculated internodes of control 1 (T7) 93% and control 2 (T8) with 67%.

Table 4 gives means comparison of disease index and disease severity of maize plants after receiving different treatments. The disease indices and disease severity recorded in plants that received T1 to T6 were significantly lower than Controls 1 and 2 (*P*= 0.05, R^2= 0.98).

The effect of different treatment combinations on shoot and root weight of the treated maize plants are presented

Table 1. Means comparison of various treatments on growth parameters of maize plants.

Treatment	No. of leaves	Stem girth (mm)	Plant height (cm)	Leaf area (cm^2)
T1	7.15^{ba}	3.97^{cb}	102.45^{a}	221.34^{a}
T2	7.09^{bc}	3.89^{cb}	101.25^{ab}	206.21^{b}
T3	6.70^{cd}	3.92^{cb}	95.86^{cde}	200.22^{cb}
T4	6.93^{bc}	3.98^{cb}	94.26^{cde}	192.43^{cd}
T5	6.52^{d}	3.89^{cb}	84.09^{f}	171.26^{f}
T6	6.54^{d}	3.62^{d}	84.42^{f}	145.12^{f}
T7	7.00^{bc}	2.75^{e}	75.49^{g}	152.61^{f}
T8	6.67^{cd}	2.86^{e}	78.46^{g}	148.18^{f}
$LSD_{0.05}$	0.10	0.05	1.59	3.95
R^2	0.54	0.85	0.95	0.77

Means followed by the same letter(s) in each column are not significantly different at ($p \geq 0.05$).
Source: Authors

Table 2. Effect of treatment concentrations on some growth parameters of treated maize plants.

Concentration	Number of leaves	Stem girth (mm)	Plant height (cm)	Leaf area (cm^2)
1 (3:1)	7.02^{a}	3.47^{a}	87.03^{b}	175.47^{b}
2 (2:2)	6.92^{a}	3.50^{a}	89.69^{a}	184.43^{a}
3 (1:3)	6.71^{b}	3.48^{a}	87.17^{b}	174.07^{b}
$LSD_{0.05}$	0.12	0.05	1.95	4.84
R^2	0.54	0.85	0.95	0.77

Means followed by same letter(s) in the same column are not significantly different ($p \geq 0.05$)
Source: Authors

Table 3. Mean percentage rot formation and rating within inoculated internode of maize stem after different treatments.

Treatment	Scale	Mean rot formation (%)
T1= CP + SD + *F. verticillioides*	3	47
T2= CP + Leaf + *F. verticillioides*	3	40
T3= SD +Leaf + *F. verticillioides*	3	27
T4C1= CP + SD + *F. verticillioides*	3	33
T4C2= CP + SD + *F. verticillioides*	3	27
T4C3= CP + SD + *F. verticillioides*	3	33
T5C1= CP + Leaf + *F. verticillioides*	3	27
T5C2= Maize + CP + Leaf + *F. verticillioides*	2	20
T5C5= Maize + CP + Leaf + *F. verticillioides*	3	27
T6C1= Maize + SD +Leaf + *F. verticillioides*	3	27
T6C2= Maize + SD +Leaf + *F. verticillioides*	3	27
T16C3= Maize + SD +Leaf + *F. verticillioides*	3	33
T7= Maize + *F. Verticillioides*	5	93
T8= Maize only	4	67

CP= Cassava peels, SD= Saw dust, Leaf= *Cedrela odorata,* C1= concentration 1(1:3) C2= concentration 2(2:2) C3= concentration 3(3:1).
Source: Authors

in Table 5. The shoot weights of plants that received treatment combinations 1 and 2 were significantly different

Table 4. Means comparison of disease severity and disease Index of the maize plants after receiving different treatments.

Treatment	Disease index	Disease severity
1	2.33[c]	3.00[c]
2	2.00[d]	3.00[c]
3	1.33[g]	3.00[c]
4	1.55[e]	3.00[c]
5	1.22[h]	2.67[d]
6	1.44[f]	3.00[c]
7 (Control 1)	4.67[a]	5.00[a]
8 (Control 2)	3.33[b]	4.00[b]
R^2	0.98	0.92

Means followed by same letter(s) in the same column are not significantly different (p≥0.05).1=cassava peels +*F. verticillioides*, 2 = sawdust +*F. verticillioides*, 3 = leaf +*F. verticillioides*, 4=cassava peels + sawdust + *F. verticillioides*,5=cassava peels+*Cederela odorata* + *F .verticillioides*, 6=sawdust+*Cederela odorata*+ *F. verticillioides*, 7(Control1) = *F. verticillioides*, 8(Control 2) = control.
Source: Authors

Table 5. Effect of different treatment combinations on shoot and root weight of infected maize plants.

Treatment combination	Shoot weight (g)	Root weight (g)
1	20.02[b]	3.52[a]
2	21.71[a]	4.08[a]
$LSD_{0.05}$	1.65	0.76
R^2	0.79	0.48

Means followed by same letter(s) in the same column are not significantly different (p≥0.05). 1= single treatment + *Fusarium verticillioides,* 2= combined + *Fusarium verticillioides.*
Source: Authors

from each other (*P*=0.05, R^2= 0.79) while root weights were similar (*P*=0.05, R^2= 0.48).

Table 6 shows the effect of different concentrations of treatments on Disease severity, Disease index and cob yield after treatments. The disease index and disease severity of plants treated with concentration 2 (2:2) were significantly lower (*P*= 0.05, R^2= 0.98) than those that received concentrations 1(3:1) and 3 (1:3). The cob yield of plants treated with concentration 2(2:2) were significantly higher (*P*= 0.05, R^2= 0.98) than those that received concentrations 1 (3:1) and 3 (1:3).

DISCUSSION

The observed changes (increase) in height and stem girth of maize plants that received all the treatments compared to control indicates that these amendments stimulated plant growth even in the presence of a pathogen. This agrees with the reports of Chilimba (2002) and Ajanga et al. (2003) who opined that plant diameter and shoot length was significantly higher in amended soil as compared to controls; an indication that the amendments stimulated plant growth.

The higher plant growth as a result of organic amendment could be associated with the release of nitrogen for plant use which support the result of Akanbi (2002) while Adebayo et al. (2012) who stated that the increase in plant growth was as a result of nitrogen released which is essential for chlorophyll and protoplasm formation.

The rot formation in the stem of maize plants on unamended soil was significantly higher than those on amended soils; this showed the impact of the amendments on rot formation within the maize stem. It is possible that the amendments adversely affected the rot forming fungi within the soil. This supports the report of Ajanga et al. (2003) who reported high recovery of *F. moniliforme* in unamended soil. The significant reduction in disease severity (stalk rot) of plants that received (*C.*

Table 6. Effect of different treatment concentrations on Disease Index, Severity and cob yield.

Concentration	Disease index	Disease severity	Cob yield
1 (3:1)	2.41[a]	3.40[a]	23.04[a]
2 (2:2)	2.18[b]	3.10[b]	23.69[b]
3 (1:3)	2.43[a]	3.45[a]	23.03[a]
R^2	0.98	0.92	0.98

Means followed by same letter(s) in the same column are not significantly different ($p \geq 0.05$).
Source: Authors

odorata at ratio 2:2**)** could be due to the biological activity of the treatment against the rot forming pathogen (*F. verticillioides*). However, the suppression of the pathogen by the soil amendment cannot be said to be absolute or consistent. The effective result generally obtained with *C. odorata* (2:2) and combined treatments of cassava peel and *C. odorata* (2:2) supports the result of (Akamu et al., 2013). In their work, Akanmu et al. (2013) concluded that 5 g/ml of cassava peels extracts had a significant effect on *F. anthophilum* and that the efficacy of peels was an effective biocontrol agent for the management of Fusarium disease in millet seedlings. Falade et al. (2006) affirmed the efficacy of cassava seed extracts in the control of *Scelerotium rolfsii* and *F. oxysporium* in Southwestern Nigeria. Akanmu et al. (2020) in their studies revealed that organic amendments are effective in controlling diseases caused by pathogens such as *F.* sp.

The observed disease severities of plants that received different treatments showed significant impacts of different treatments on the rot forming fungus (*F. verticillioides*). Treatments with cassava peels + *C. odorata*, had the highest effect on disease severity compared to other treatments, and can thus be said to be preferred above others. The results obtained in the study could be attributed to differences in mode of action of all treatments considered. This agrees with the findings of Elad et al. (2010) who reported that the differences in effect of organic amendments on *Fusarium verticillioides* at two recovery intervals from ear rot of maize could be attributed to the rate and mode of disease depression such as antagonism, antibiosis and lysis.

The general reduction in disease severity of maize by *C. odorata* (leaves) and cassava peels compared to control could also be due to increased population of other more competitive fungi and bacteria in amended soil leading to decline in disease severity. This agrees with the reports of Saravanan et al. (2008) and Ajanga et al. (2003). They reported that soils high in organic matter support huge populations of diverse mirco-organisms and because of this plant diseases may be suppressed by the activities of plant-associated micro-organisms. *C odorata* has been reported by Asekun et al. (2013) to contain flavanoids.

The variations recorded in organic amendments used could be as a result of variation in the materials used. This agrees with the findings of Flores et al. (2006) who concluded that not only do amendments from different materials vary in disease suppression, but those from different batches of the same material are also variable. The high rot formation recorded with saw dust could be because of the greater surface area found in wood materials limiting the availability of nitrogen needed by soil microorganisms to break down the material.

The combined treatments were more effective than single treatments against disease severity supports findings (Akanmu et al., 2020), which stated that organic amendments can improve physical and chemical properties of soil.

Generally, this study revealed that *C. odorata*, cassava peels and sawdust have the potential to suppress the causal pathogen (*F. verticillioides*) with a resultant enhanced growth and yield of maize. Different soil amendments used are of benefits to maize production but may be effective in different proportions. *C odorata* and cassava peels are appropriate as soil amendments for maize cultivation. Severity of stalk rot of maize can be said to reduce significantly in amended soils compared to unamended soils. The soil amendments may also have adverse effect on some soil borne pathogens such as *F verticillioides* serving as an alternative treatment (Kenganal et al., 2017). Conclusively, the use of combined treatments such as cassava peels and *C odorata* which competed favourably with *F verticillioides* can thus be suggested as a good soil amendment against such fungi.in the control of stalk rot of maize.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbiology spectrum and antibiotic susceptibility of bacterial pathogen from ready-to-eat sliced pineapple and water melon

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This study was conducted to examine microbiological quality and antibiotic susceptibility of bacterial pathogen of ready to eat sliced pineapple and water melon. Pineapples and water melon were purchased from three different vendors in Sango, University of Ibadan and Apete markets. Isolation and identification of pathogens were carried out using culture dependent method, morphological and biochemical characterization, respectively. Bacterial isolates were tested for antibiotic susceptibility. Results showed that all samples were contaminated. *Lactobacillus* species had the highest percentage frequency of occurrence (35%), while *Proteus*, *Pseudomonas, Shigella* and *Salmonella* species had the least (5%). Multiple drug resistances were observed among the isolates when subjected to Ampicillin, Ceftazidine, Cefuroxine, and Augumentin. Despite the dominance of *Lactobacillus* spp. in this study, the presence of *Escherichia coli, Shigella* spp., and *Salmonella* spp., *Staphylococcus aureus, Klebsiella pneumonia* and *Enterobacter* species together with their multiple drug resistance call for public health attention. Quality of vended fruits will be better assured if there are improved hygienic practices and awareness among fruit vendors as well as the consumers. There is need to monitor the spread of multiple drug resistant antimicrobial pathogens in vended fruits to avoid outbreaks.

Key words: Microbiological quality, antibiotic susceptibility, multiple drug resistance ready to eat, pineapple, water melon.

INTRODUCTION

Balanced diets enriched by fruits and vegetables are of utmost importance due to their nutritional values and ability to reduce risk of illnesses (Mahmoud et al., 2019). Fruits could be eaten wholly or sliced (Erhirhie et al., 2020). Ready-to-eat sliced fruits are fruits that have been cut open, sliced into bits, but remain in the fresh state and sold from retail outlets. They are usually eaten immediately, easily accessible, convenient and most especially cheaper than the whole fruit (Nwachukwu et al., 2008).

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In Nigeria where street fruit vending is very common probably due to modern lifestyle, industrialization, economic downturn, materialism and lack of time to prepare proper meal (Fowoyo, 2012), there is little information on the incidence of food borne diseases as related to the street vending fruits. However, microbial studies on each fruits in American, Asian, European and some African countries have revealed increased bacteria pathogen in ready to eat fruits (Mahale et al., 2008). Several bacteria such as *Salmonella* species, *Shigella* species*, Listeria monocytogens, Campylobacter* species, *Aeromonas* species, *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas* species have been associated with contaminated sliced fruits (Eni et al., 2010; Fowoyo, 2012; Asmaru and Sahile, 2013; Nwachukwu and Osuocha, 2014; Shenge et al., 2015).

Additionally, increase in the incidence of antimicrobial resistance bacteria in humans, animals and the environment is a major concern in both human and veterinary medicine and Infectious Diseases Report noted that drug resistant organisms are prevalent worldwide (WHO, 2000). Fruits can be infected with antibiotic resistance contaminants though several sources. Manure use in enhancing soil fertility harbours bacteria that can be carrying the Antibiotic Resistant Genes (ARG), which can be horizontally transferred to soil bacteria. This in turn, can contaminate fruits and vegetables. This is also applicable when there is faecal contamination of soil or spray of soil with antimicrobial agents. Also, during plant tissue culture, inclusion of antimicrobial agents in the culture medium can prevent or often treat microbial contamination but increase the chance of build-up of antibiotic resistance (Rashmi et al., 2017).

This work therefore aimed to determine microbial spectrum of ready-to-eat sliced pineapple and water melon fruits together with the assessment of bacteria susceptibility to antibiotics.

MATERIALS AND METHODS

Sample collection

On the 2nd of January, 2020, samples of sliced pineapple (*Ananas comosus)* and watermelon (*Citrullus lanatus*) were purchase randomly at three different sellers in three markets (Apete, Sango and University of Ibadan), Oyo State, Nigeria. All samples were collected in sterile plastic bags and transported to laboratory for analysis.

Enumeration and identification of microorganisms

Isolation of bacteria from the fruit samples

From each fruit sample, 2 g was cut and mashed in a mortal using a pestle and 1 g of sample was weighed into 9 ml of sterile distilled water in McCartney bottle. A 10-fold serial dilution of the solution was done in sterile distilled water and 0.1 ml of dilutions 10^{-3}, 10^{-5} and 10^{-6} were plated using pour plate technique on eosin methylene blue agar (EMB), *Salmonella-Shigella* agar (SSA) for bacterial isolation and identification. All samples were duplicated and incubated at 37°C for 24 h for bacteria and 72 h at 30°C (Cheesbrough, 2011). After incubation, the cultured plates were observed for growth and distinct colonies were selected and streaked on their respective medium, the pure cultures were inoculated on slant and stored in the refrigerator for further studies. Phenotypic identification of the bacteria isolates were carried out according to Boone et al. (2005).

Antibiotic sensitivity test

The susceptibility of each isolate to commonly available antibiotics was carried out using disc diffusion method. Mueller Hilton agar was prepared according to manufacturer's specification and sterilized in an autoclave at 121°C for 15 min. The isolated organisms were inoculated on the agar using a swab stick. Each antibiotic disc (10 µg) was aseptically placed on the surface of the plate with sterile forceps, to ensure complete contact with the agar surface. The plates were incubated for 24 h at 37°C, clear zone of incubation around the antibiotic disc on the plate indicated susceptibility of the isolates. Inhibition clear zones were measured in millimetre and grouped into antibiotics sensitive and resistant isolates (Cheesbrough, 2011).

RESULTS AND DISCUSSION

All the pineapple samples were contaminated. Pineapple bought from Apete pineapple (APP) had average viable count of 2.3×10^{-4} - 4.5×10^{-5} cfu/ml bacteria cells, Sango pineapple (SPP) had a viable count of 1.8×10^{-3} - 4.5×10^{-5} cfu/ml and University of Ibadan pineapple had a viable count of 4.7×10^{-3}- 4.0×10^{-5} cfu/ml. Water melon bought from Apete had total viable count of 3.7×10^{-4} - 1.1×10^{-7}, Sango 1.2×10^{-4} - 1.7×10^{-4} and University of Ibadan 2.5×10^{-4} - 2.5×10^{-7} (Table 1).

Apete pineapple (APP) had three major groups of bacteria isolated and identified as *E. coli, Lactobacillus* species and *S. aureus.* Sango pineapple samples (SPP) had a wider spread of organisms such as *S. aureus*, *E. coli, Lactobacillus* spp., *Salmonella* spp, *Proteus* and *Bacillus subtilis* while *Shigella* spp*., Pseudomonas aeruginosa, S. aureus* and *B. subtilis* were isolated from University of Ibadan pineapple samples (UPP) sample. The water melon samples had seven similar bacteria isolates, identified as *S. aureus, Enterobacter* species, *P. aeruginosa, Lactobacillus* spp*., E. coli, Klebsiella pneumonia* and *Shigella* spp*.*

Among all the bacterial isolates from sliced pineapples (Figure 1a), *Lactobacillus* spp. had the higher percentage occurrence of 35%, followed by *S. aureus* at 18%, *E. coli* and *B. subtilis* at 12% while *Proteus*, *Pseudomonas* spp*., Shigella* spp. and *Salmomella* spp. had the occurrence rate of 5%. With the exception of *Pseudomonas* and *Klebsiella* spp*.* which are the least (8%), all other bacteria isolates had same occurrence level (16.8%) in water melon (Figure 1b).

According to Wadamori et al. (2016), various pathogenic microbes can contaminate fresh fruits and

Table 1. Total viable count of microorganisms from ready-to-eat sliced pineapple and water melon.

Lab. Code	Bacteria count
APP	2.3×10^{-4} - 4.5×10^{-5}
AWM	32.7×10^{-4} - 1.1×10^{-7}
SPP	1.8×10^{-3} - 4.5×10^{-5}
SWM	1.2×10^{-4} - 1.7×10^{-4}
UPP	4.7×10^{-3} - 4.0×10^{-5}
UWM	2.5×10^{-4} - 2.5×10^{-7}

APP- Apete pineapple, AWM- Apete water melon, SWM- Sango water melon, SPP- Sango pineapple, UPP -U.I. pineapple, UWM- U.I. water melon.
Source: Author

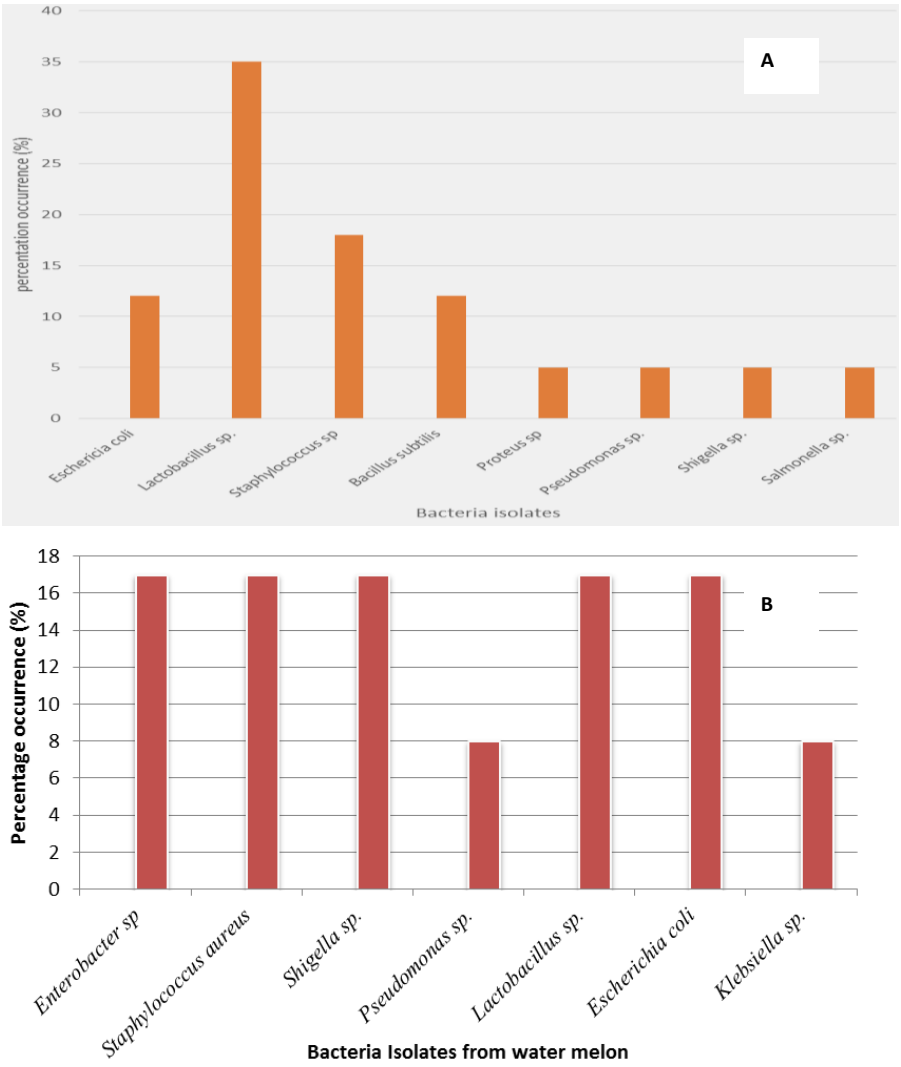

Figure 1. Percentage occurrence (%) of the bacterial isolates on the ready-to-eat sliced (a) Pineapple fruit and (b) water melon fruit
Source: Author

Table 2. Antimicrobial activities of bacteria isolated from sliced pineapple.

Bacterial isolate	Antibiotics							
	Gen 10 µg	CPR 10 µg	AMP 10 µg	NIT 10 µg	OFL 10 µg	CAZ 10 µg	CRX 10 µg	AUG 10 µg
Escherichia coli	+	++	-	+	++	-	-	-
Lactobacillus spp.	++	++	-	++	+	-	-	-
Staphylococcus aureus	+++	+++	-	+++	+++	-	-	-
Bacillus subtilis	+	++	-	++	++	-	-	-
Proteus spp.	-	++	-	-	++	-	-	-
Pseudomonas aeroginosa	+	++	-	+	++	-	-	-
Shigella spp.	+++	++	-	-	++	-	-	-
Salmonella spp.	++	+	-	++	++	-	-	-

+Weak inhibition (diameter <8 mm), ++Moderate inhibition (diameter>8-12 mm), +++ Strong inhibition (diameter >12-above), -No inhibition, GEN-Gentamycin, CPR-Ciprofloxacin, OFL-Ofloxacin, NIT, AMP-Ampicillin, CAZ-Ceftazidine, CEX-Cefuroxine, AUG-Augumentine
Source: Author

vegetables at any point in the chain. The presence of these bacteria contaminants indicated that, microbial contaminants of fruits and vegetables occur mainly because of resident microflora in the soil, application of non-resident microflora via animal manures, sewage or irrigation water, transportation and handling (Ray and Bhunia, 2007; Ofor et al., 2009). Similar report had been previously shown though not in Oyo State (Adebolu and Ifesan, 2001; Tambekar and Mundhada, 2006; Uzeh et al., 2009; Eni et al., 2010). Although it is worthy to note that *Lactobacillus* spp. are normal flora of the fruit. Their presence could also be due to the fact that they are obiquitious occurring naturally in a variety of niches, including the gastrointestinal tract and plants (Mohania et al., 2008). Their dominance could be traced to their ability to inhibit the growth of pathogenic bacteria by producing antimicrobial substances (such as lactic acid) (Olateru et al., 2020).

The presence of *Staphylococcus* indicates unhygienic handling of fruits. The samples obtained from Sango pineapples had variety of organisms; this might be attributed to poor handling and storage condition (Bello et al., 2016)

The prevalence of pathogenic organism in all samples can be as a result of improper washing and decontamination of these produce. In support, Eni et al. (2010) isolated *S. aureus* with percentage occurrence rate of 29.2%, followed by *Klebsiella* spp. (12.5%), *Salmonella* spp. (12.5%), *E. coli* (4.2%) and *Actinomycetes* (4.2%) from ready to eat fruits and vegetable in Sango-Ota, Ogun State Nigeria. As noted by Buck et al. (2003), enteric pathogens such as *E. coli* and *Salmonella* spp. are among the greatest concerns during food-related outbreaks. Several cases of typhoid fever outbreak have been associated with eating contaminated vegetables grown in or fertilized with contaminated soil or sewage (Beuchat, 2002). Besides, contamination observed in this study may be attributed to storage conditions or cross contamination between fruits as a result re-washing with used water or water recycled by the vendor or processor (Topalcengiz et al., 2017).

Tables 2 and 3 present the distribution of isolates susceptibility and resistance to specific antibiotic agents. The result showed that susceptibility of the isolates to different antibiotic varied. Ofloxacin, Gentamycin, and Ciprofloxacin, have the highest inhibitory properties against all isolates while Nitrofurantoin has the lowest inhibitory properties. All isolates showed high resistance to Ampicillin, Ceftazidine, Cefuroxine, and Augumentin. *E. coli, B. subtilis, P. aeruginosa* and *Salmonella* spp., were partially susceptible to Gentamycin, Ciprofloxacin, Nitrofurantoin and Ofloxacin. *S. aureus* was sensitive to Gentamycin, Ciprofloxacin, Nitrofurantoin and Ofloxacin. *Shigella* spp. was sensitive to Gentamycin and partially sensitive to Ciprofloxacin, Nitrofurantoin and Ofloxacin. *Proteus* species was resistant to Gentamycin and Nitrofurantoin and partially sensitive to Ciprofloxacin and Ofloxacin. However, *Pseudomonas* spp. isolated from ready to eat water melon was resistant to Gentamycin and *Klebisella* to Ampicillin. The presence of resistant bacteria pathogen may be attributed to continuous indiscrete use of antibiotics as therapy and as an additive. In support, Meher et al. (2011) reported that emergence of drug resistance is one of the most serious health problems in developing countries. Abuse of antibiotics have resulted in increase in prevalence of antibiotic resistant bacteria strains, vice-verse increase bacterial infections (Nuermberger and Bishai, 2004; Kummerer, 2004; Rashmi et al., 2017).

Conclusion

It is very important to note that the outward appearance

Table 3. Antimicrobial activities of bacteria isolated from sliced water melon.

Bacteria isolate	Antibiotics							
	GEN 10 µg	CPR 10 µg	OFL 10 µg	NIT 10 µg	AMP 10 µg	CAZ 10 µg	CEX 10 µg	AUG 10 µg
Enterobacter spp.	+	++	++	+	-	-	-	-
Staphylococcus aureus	++	+++	++	++	-	-	-	-
Pseudomonas spp.	-	+++	+++	+++	-	-	-	-
Lactobacillus spp.	+	+++	++	++	-	-	-	-
Escherichia coli	++	++	++	+	-	-	-	-
Klebsiella spp.	++	++	++	-	-	-	-	-
Shigella spp.	+++	++	++	++	-	-	-	-

+Weak inhibition (diameter <8 mm), ++Moderate inhibition (diameter>8-12 mm), +++Strong inhibition (diameter >12-above), -No inhibition, GEN-Gentamycin, CPR-Ciprofloxacin, OFL-Ofloxacin, NIT, AMP-Ampicillin, CAZ-Ceftazidine, CEX-Cefuroxine, AUG-Augumentine.
Source: Author

of fruits gave a wrong assessment of the microbial quality. Hence, awareness is to be created both for the consumers and the vendors. There is also need to increase surveillance of antibiotic resistant pathogen in ready to eat foods.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Regulation of the *bE* and *bW* genes in *Sporisorium scitamineum* using silver nanoparticles synthesized with *Carissa spinarum* extract

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Sugarcane smut is caused by the fungus *Sporisorium scitamineum.* It is a disease of economic importance in the sugarcane industry because it can cause losses that can lead to total crop failure. Bio-synthesized silver nanoparticles have been found to possess antimicrobial properties, yet they have not been explored against *S. scitamineum.* Optimization of the mixtures using ultraviolet-visual spectroscopy (UV-Vis) showed peaks in the range of 340 to 400 nm. The Fourier transform infrared spectroscopy (FTIR) analysis identified proteins as essential capping agents, and reducing sugars were responsible for reducing the silver nitrate to nanoparticles and stabilizing the nanoparticles. They have the highest antifungal activity at 5 mg/ml, while the minimum inhibitory and fungicidal concentrations were 0.078 mg/ml. The *in-vivo* assays showed a significant (P<0.05) reduction of the pathogen biomass in plants treated with the nanoparticles compared to the control plants. The application of 0.0585 mg/ml of the nanoparticles to the *S. scitamineum* resulted in a significant (P<0.05) increase in the expression of the *bE* and *bW* genes. Silver nanoparticles that were synthesized using *C. spinarum* crude extract inhibited the growth of *S. scitamineum* both *in-vitro* and *in-vivo* and had a regulatory effect on the expression of the pathogenicity genes in the fungus.

Key words: *Sporisorium scitamineum*, sugarcane smut, *Carissa spinarum*, biosynthesized silver nanoparticles, antifungal activity.

INTRODUCTION

Sugarcane**,** *Saccharum officinarum*, is a perennial grass that belongs to the Poaceae family. It is primarily grown to produce sugar, but also has other by-products which include biofuel, ethyl alcohol (ethanol), molasses, rum,

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straw and bagasse (Yamane, 2018). Sugarcane production is challenged by several insect pests and diseases. Among these is the sugarcane smut, which is caused by the fungus *Sporisorium scitamineum* (Syd) M. Piepenbr., M. Stoll & Oberw (Comstock, 2000).

Sugarcane smut is a major challenge to sugarcane production because of its potential to spread quickly and the considerable losses that it causes (Cui et al., 2020; Jacques-Edouard et al., 2020). The disease has been reported to cause yield losses that range from 30 to 100%, and sometimes even leading to the elimination of some varieties (Rajput et al., 2022).

Sugarcane smut is primarily managed by the use of resistant varieties. This usually poses challenges to maintain because the *S. scitamineum* evolves to produce new strains that are not inhibited by the host resistance mechanism of these varieties (Jacques-Edouard et al., 2020). Other management practices include hot water treatment of seed cane, the use of fungicides and also rouging of infected sugarcane stalks or stools. The use of fungicides usually has very limited efficacy since they are unable to penetrate the waxy coat of the sugarcane, and they also have deleterious impacts on the environment (Cui et al., 2020).

Silver nanoparticles have been traditionally known for their antimicrobial activities. They are produced by physio-chemical methods that include ion sputtering or pulsed laser ablation, reduction, solvo-thermal synthesis, hydrothermal and sol-gel methods. The nanoparticle synthesis methods can be broadly categorised as chemical, physical, photochemical and biological. Chemical and physical methods are generally expensive, harmful and inflammable, yet biosynthesis is cost-effective, energy-saving and environmentally benign as it uses microorganisms and plant extracts. Phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides, and enzymes present in plants are used as reducing, capping and stabilizing agents (Chouhan, 2018).

Bio-synthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal and antiviral properties, with no environmental concerns and development of microbial resistance (Velu et al., 2017; Ahmadi et al., 2021). These characteristics have ignited an increasing interest in the biosynthesis of silver nanoparticles. The b-AgNPs can be produced by either using microorganisms (fungi or bacteria) or plant materials. The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018).

Conventionally, silver has been known and used for its antimicrobial activity (Jamiu and Bello, 2018). When reduced to their nano-form, silver nanoparticles (AgNPs) possess novel and more efficient antimicrobial properties, owing to their large surface-to-volume ratio, size, shape and structure (Ahmadi et al., 2020). Bio-synthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal and antiviral properties, with no environmental concerns or development of microbial resistance. These characteristics have ignited increasing interest in the synthesis of silver nanoparticles using the green method or biosynthesis (Ahmadi et al., 2020). The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018).

Sporisorium scitamineum, formerly known as *Ustilago scitamineum,* is genetically similar to the maize pathogen *Ustilago maydis* which has been extensively studied with attributes from the availability of the complete genome sequence. The reproduction process of the pathogen is mainly regulated by two loci, locus *a* and locus *b* (Yan et al., 2016).

The *a*-locus has been studied and found to regulate the pheromone and pheromone receptor system or cell-cell recognition system, while the *b*-locus encodes for homeodomain proteins that function as transcription factors (Yan et al., 2016).

The *b* locus is formed by the *bE* and *bW* genes which are responsible for the differentiation of self and non-self, a heterodimeric transcription factor which will only be active when these have been contributed by different alleles. These genes encode for the homeodomain proteins *bE* (HD1) and *bW* (HD2) which are present and conserved in the genomes of Ustilaginaceae which includes both the genera *Ustilago* and *Sporisorium* (Peters et al., 2020; Que et al., 2014).

The *bE* and *bW* genes are involved in several pathways that include the pathogenic development of the fungi, the cell cycle regulation, mitosis as well as DNA replication (Yan et al., 2016).

This study aims to investigate the efficacy of silver nanoparticles (AgNPs) that have been synthesized with *Carissa spinarum* against the fungus *S. scitamineum in-vivo* and to investigate the gene-regulatory effects of the b-AgNPs on the *bE* and *bW* genes of the fungus. Considering the known antimicrobial applications of b-AgNPs and the availability of the medicinal plant *Carissa spinarum* in Kenya, we decided to evaluate the antifungal efficacy of the b-AgNPs on *S. scitamineum* as well as their regulatory effects on the *bE* and *bW* genes which are responsible for the pathogenicity of the fungus.

MATERIALS AND METHODS

Collection and identification of the fungus

This study was conducted in Kenya during the period from 2021 to 2022. The smut-infected plants were identified at the Sugarcane Research Institute in Kisumu, Kenya. The sori were cut from the infected sugarcane plants and bagged to prevent any spread to healthy plants. The spores were maintained at the molecular biology laboratory (Pan African University). These spores were rinsed three times with distilled water, by centrifugation at 10 000 rcf for 1 min, and cultured in potato dextrose agar (PDA) by spreading the suspended spores using a sterile swab. The plates were

incubated in darkness at 28°C (Singh et al., 2005; Cui et al., 2020). To purify the cultures, the fungal isolates were transferred onto new plates and incubated in darkness at 28°C (Que et al., 2014).

The fungal genomic deoxyribonucleic acid (DNA) was extracted from mycelia using a Zymo Fungal and

Bacterial Genomic DNA Extraction Kit (Inqaba Biotech, South Africa), following the instructions of the manufacturer. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and a nanodrop spectrophotometer. To verify the identity of the fungus, the DNA that was extracted from the samples was amplified on conventional PCR using the *bE*4 (5'-CGCTCTGGTTCATCAACG - 3') and *bE*8 (5'-TGCTGTCGATGGAAGGTGT - 3') primers that are specific for *S. scitamineum* (Izadi and Moosawi-jorf, 2007; Zhang et al., 2015).

Conventional PCR amplification was carried out in a 25 μL volume containing 1 µl of 0.1 ng/µl gDNA, 12.5 μL of 2x OneTaq master mix, 0.5 μL of each of the upstream and downstream primers and 10.5 μL of water. The PCR amplification was performed following a thermal cycling programme of 95°C for 5 min; 35 cycles of 95°C for 30s, 52°C for 30s, and 68°C for 40 s; and a final extension at 72°C for 5 min. The PCR amplicons were checked for quality in a 1% agarose gel electrophoresis and then documented.

Plant extract preparation

The *Carissa spinarum* leaves were sourced from the Jomo Kenyatta University of Agriculture and Technology's (JKUAT) botanical garden, in Kenya.

The leaf extract preparation was done by rinsing the leaves with sterile water, drying them and cutting them into small pieces using a blender. 50 g of the leaf sample was heated at 80°C in 250 ml of sterile water in a 500 ml Erlenmeyer flask for 30 min. The crude leaf extract was then filtered using Whatman No. 1 and stored at 4°C (Velu et al., 2017).

Biosynthesis of silver nanoparticles using *C. spinarum*

To synthesize the b-AgNPs, 1 mM of silver nitrate was formulated by adding 0.167 g of silver nitrate into 1 L of distilled water. The mixture of the silver nitrate and the plant's crude extract was kept for 24 h in darkness at 28°C in a 150 rpm shaking incubator (Velu et al., 2017).

Optimizing the nanoparticles

The b-AgNPs were optimised under different reaction conditions which included leaf extract reaction volume (2, 3, 4, 5, 6, 7, 8 and 9 ml) and the duration of incubation of the AgNPs in darkness which was varied at 0, 2, 4, 12, 24, 48 and 72 h (Houllou et al., 2019). While optimizing each parameter, the other parameter was kept constant.

The b-AgNPs were isolated from the optimized mixture by centrifugation at 12000 rcf for 20 min. The pellet was then purified using distilled water and washed twice to ensure better separation of free entities from the AgNPs. The b-AgNPs were kept at -20°C for 24 h, moved to -80°C to be kept for 48 h, and then they were lyophilized and used for further characterization (Velu et al., 2017).

Characterization of the b-AgNPs

UV-Vis spectra analysis

The sample (1 ml) of the suspension was collected periodically to monitor the completion of bio-reduction of Ag^+ in an aqueous solution. The UV-Vis spectrum of the solution was measured between wavelengths 200 and 800 nm using the Jenway Model 6800 Spectrophotometer Flight Deck with a resolution of 1nm (Sanchooli et al., 2018).

FTIR analysis

The nanoparticle characterization included ascertaining the active biomolecules responsible for the reduction; capping and stabilising by FTIR Spectrometer model 8400, Shimadzu. For the FTIR analysis, the dried b-AgNPs were added to FTIR-grade potassium bromide (KBr) in 1: 100 ratios and observed in the range of 4000 to 400 cm^{-1} (Qais et al., 2019).

Transmission electron microscopy (TEM) analysis

The analysis to determine the morphology, size and shape of the nanoparticles was done using the JEM-2100 Electron Microscopy. The TEM sample grid with a continuous silicon oxide film was prepared, as well as the glassware and apparatus. The sample grid was then derivatized by exposing the silicon oxide to 10µl of aminopropyldimethylethoxysilane solution. The b-AgNPs were then citrate-stabilized for them to have a negative charge to attract to the positively charged TEM surface grid (Bonevich and Haller, 2010; Qais et al., 2019).

Antifungal activity of the b-AgNPs

The antifungal activity of the synthesized AgNPs was assayed using the disc diffusion assay method. The *S. scitamineum* was cultured in PDA media and the treatments were replicated three times. The fungal suspension was calibrated using phosphate-buffered saline (PBS) solution to match the McFarland turbidity standard. This standard was made by mixing 1% Barium Chloride (0.05ml) and 1% Sulphuric acid (9.95 ml) to produce a 0.5 McFarland standard which is equivalent to 1.5×10^8 colony forming units (CFU). The plates were incubated in darkness at 28°C for 48 h. The b-AgNPs were dissolved in distilled water to make a stock solution of 20 mg/ml. The stock solution was then serially diluted to make various concentrations (0.62, 1.25, 2.5, 5 and 10 mg/ml) that were placed on the surface of inoculated agar plates using the disc diffusion method. The positive control was the standard fungicide nystatin and the negative control was distilled water. The antifungal activity was measured by the diameter of the inhibition zone in millimetres (Sanchooli et al., 2018; Ahmadi et al., 2021).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the b-AgNPs

This assay aimed to determine the least amount of the b-AgNPs that inhibited the growth of the fungi completely. The fungus was cultured on nutrient media broth and 200 µl was transferred into different wells on a 96 well plate. The fungal suspension was calibrated to match the McFarland turbidity standard. Then 200 µl of serially diluted b-AgNPs (0.0097, 0.0195, 0.039, 0.078, 0.165, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 mg/ml) was added onto the wells and incubated for 24 h. The negative control was the fungus without the b-AgNPs but water and the positive control was the b-AgNPs without the fungus. To aid with the observation of the MIC in broth culture, a fluorescent blue dye called resazurin was used. Resazurin is reduced by metabolic activity to a pink colour (resorufin), and where there is no metabolic activity, it remains blue (Kowalska-Krochmal and Dudek-Wicher, 2021). After the 24 hours incubation, 10 µl of resazurin was added and incubated in darkness

Table 1. The list of primers that were designed and optimized for the relative RT-qPCR assays.

Gene ID	Primer	Forward primer	Product size	Position
JQ290342	*bE - F* *bE*- R	TGGATCAGATATGGCGTCAA GCTCTCTGCTTAGCCCTCCT	179bp	237-415
MZ773250	*bW* - F *bW - R*	GCTTTCACTCCTTGGAGCAC TTCCGATGGTGAGATTAGGC	172bp	1042-1213
KJ194461	*ITSa* - F *ITSa* - R	TGAGGGTTTTGCCATTTACC GCTTCTTGCTCATCCTCACC	150bp	456-605
DQ352817	*GAPDHa* - F *GAPDHa* - R	TTTCCGTCGTTGACCTTACC AAGATGGACGAGTGCGAGTT	166bp	692-857

Source: Authors

for 2 h, to determine the metabolic state of the fungi in the wells by colour changes. The lowest b-AgNP concentration without changing its colour to pink (remaining blue) was recorded as the minimum inhibitory concentration (Sanchooli et al., 2018). The lowest concentration of the b-AgNPs that killed 100% of the fungi is known as the minimum fungicidal concentration (MFC). From the MIC assays, the wells with the least concentration that had no colour change (recorded MIC) and the subsequent well (slight colour change) were then cultured on PDA to observe the least AgNP concentration that inhibited fungal growth (Qais et al., 2019).

Antifungal activity of the b-AgNPs in-vivo

The efficacy of the b-AgNPs to control the pathogen *in-vivo* was evaluated by measuring the pathogen biomass (copy number) using qPCR. Healthy seed cane of the smut-susceptible variety CO421 was obtained from the Sugarcane Research Institute (SRI) in Kisumu, Kenya, and grown in the greenhouse.

The *S. scitamineum* teliospores that were provided by the SRI were suspended in distilled water cultured by spreading using a sterile swab on PDA and incubated at 28°C for five days. The mycelium was then transferred to a yeast extract liquid medium and incubated at 28^{o}C while shaking at 150 rpm for two days before it was used for inoculation. The inoculum suspension was calibrated using phosphate-buffered saline (PBS) solution to match the McFarland turbidity standard. The fungi were then harvested by centrifuging at 4000 rcf for 5 min. The cells were rinsed with distilled water twice before re-suspending them on 1 ml distilled water. Inoculation was done three weeks after germination following the injure and paste method (Olweny et al., 2008). The negative control was inoculated with distilled water (Yan et al., 2016; Sun et al., 2019). The success of inoculation was confirmed by conventional PCR using the *bE4* and *bE8* primers.

After two weeks, post-inoculation, the plants were treated with the b-AgNPs (MIC and 0.5 MIC). The extraction of DNA was done three weeks after inoculation. To treat the infected plants, the plants were completely cut off at a height of 5 cm to mimic harvesting before the treatments were applied to the vascular bundle. The treatments included; plants that were treated with water (positive control), plants that were treated with 0.078 mg/ml of b-AgNPs (MIC), plants that were treated with 0.039 mg/ml of b-AgNPs (1/2 MIC) and healthy plants as a negative control. The fungal genomic DNA was extracted and the pathogen biomass after the treatment with the b-AgNPs was quantified on a RT-qPCR using the *bE* mating- type gene-specific primers (F-CCAACGACGAAAGCGCGACG and R-GACTCTCTGCGAGCGGGCAT). The cycle conditions were: 95°C for 5 min, 95°C for 30 s and 60°C for 30 s (Nayaleni et al., 2021).

Gene expression analysis of the bE and bW genes in S. scitamineum

The fungus was cultured in nutrient broth media for 48 h. The culture (2 ml) was then transferred into tubes and was mixed with an equal amount of b-AgNPs. The three treatments were; fungi treated with 0.0585 mg/ml of b-AgNPs (75%MIC), fungi treated with 0.039 mg/ml of b-AgNPs (50%MIC), and fungi treated with water as a positive control. Total RNA was extracted from these samples at 3, 6, 9 and 12 h (Thornton and Basu, 2011) and the quality and concentration of RNA were analysed by 1% agarose gel electrophoresis and using a nanodrop spectrophotometer.

The RT-qPCR quantification (SYBR Green) treatments included the fungi with b-AgNP treatments (0.059 and 0.039 mg/ml) and the cDNA from fungi that was treated with distilled water as a positive control. The qPCR cycle conditions were: 95°C for 12 min, 95°C for 15 s and 60°C for 20 s.

The primers (Table 1) that were used for the RT-qPCR were designed based on the *bE* gene (JQ290342.1), *bW* gene (MZ773250.1) and the GAPDH gene (DQ352817.1) as a housekeeping gene (Thornton and Basu, 2011).

Data analysis

For the antifungal activity of the b-AgNPs statistical analysis was performed using a one-way analysis of variance, which was used to compare the differences among samples using their inhibition zones. P values ≤ 0.05 will be considered significant and all antifungal assays will be performed with 3 replications (Sanchooli et al., 2018). The MIC and MFC analyses were evaluated qualitatively.

RESULTS

Identification of the fungus

To confirm the identity of the fungus, the collected isolates were screened by conventional PCR using the *S. scitamineum-specific primers bE4 and bE8. The three*

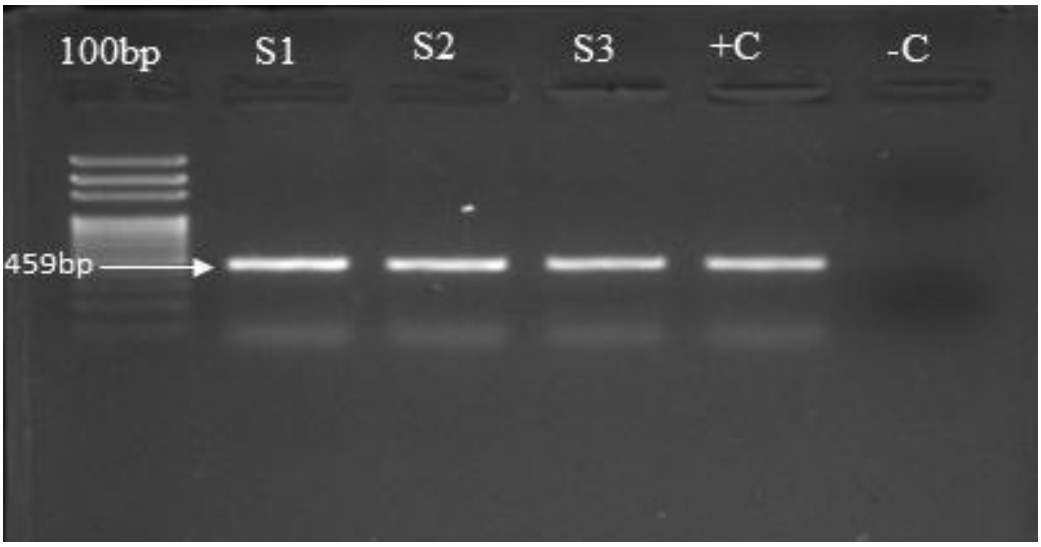

Figure 1. Gel documentation indicates the amplification of the fungal genomic DNA using the *S. scitamineum* specific primers, bE4 and bE8, that were used to confirm the identity of the pathogen. The primers had a positive amplification on the three isolates (S1, S2 and S3) and the positive control (+C) with a 459 bp amplicon size.
Source: Authors

isolates (S1, S2 and S3) were extracted and amplified along with a known positive sample (+C). All the samples had a positive amplification of a 459 bp fragment (Figure 1), which was consistent with the findings by Izadi and Moosawi-jorf (2007).

Biosynthesis and characterizing of the biosynthesized nanoparticles

Mixing the silver nitrate (100 ml) with the crude extract of *C. spinarum* (3 ml) resulted in a colour change of the mixture from light pale to dark brown.

UV-Vis spectroscopy analysis

The analysis showed absorption peaks for the b-AgNPs at 385 nm (Figure 2).

Fourier transform infrared (FTIR) analysis

The FTIR analysis results of the b-AgNPs (Figure 3) shows the band at 3364 cm^{-1}which corresponds to the N-H stretching of proteins' secondary amide. The peak at 1589 cm^{-1} indicates stretch vibrations for the -C=C- bond, while the benzene rings C=C and C-C are shown by the peak at 1404 cm^{-1}. The C-H bond in the pyridine ring appears at 1335 cm^{-1} and the C-OH phenols appear at 1003 cm^{-1}, while the peaks at 500 to 709 cm^{-1} show the presence of AgNPs. The TEM analysis was able to provide the study with the sizes, shapes and texture of the b-AgNPs. The nanoparticles that were produced ranged between 3 and 33 nm in size (Figure 4). They were spherical in shape and smooth in texture.

Antifungal activity of the b-AgNPs

The different b-AgNP amounts had a significant ($P < 0.05$) difference in their ability to inhibit fungal growth. Among the treatments, 5 mg/ml had the largest inhibition zone, followed by 10, 2.5, 1.25, and 0.62 mg/ml and then followed the crude extract (Figure 5). There was a significant ($P < 0.05$) difference between the b-AgNP treatment that had the highest inhibition zone (5 mg/ml) and the standard antifungal nystatin, while the negative treatment showed no inhibition. The treatment with 5 mg/ml also had a significantly ($P<0.05$) higher inhibition zone when compared to the treatment with the crude extract.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the AgNPs

The determination of the minimum inhibitory concentration of the b-AgNPs was observed with the use of resazurin dye. The dye turned pink in wells where there was metabolic activity (lower b-AgNP dosages) and remained blue where there was no metabolic activity (Sanchooli et al., 2018). The minimum concentration that had an

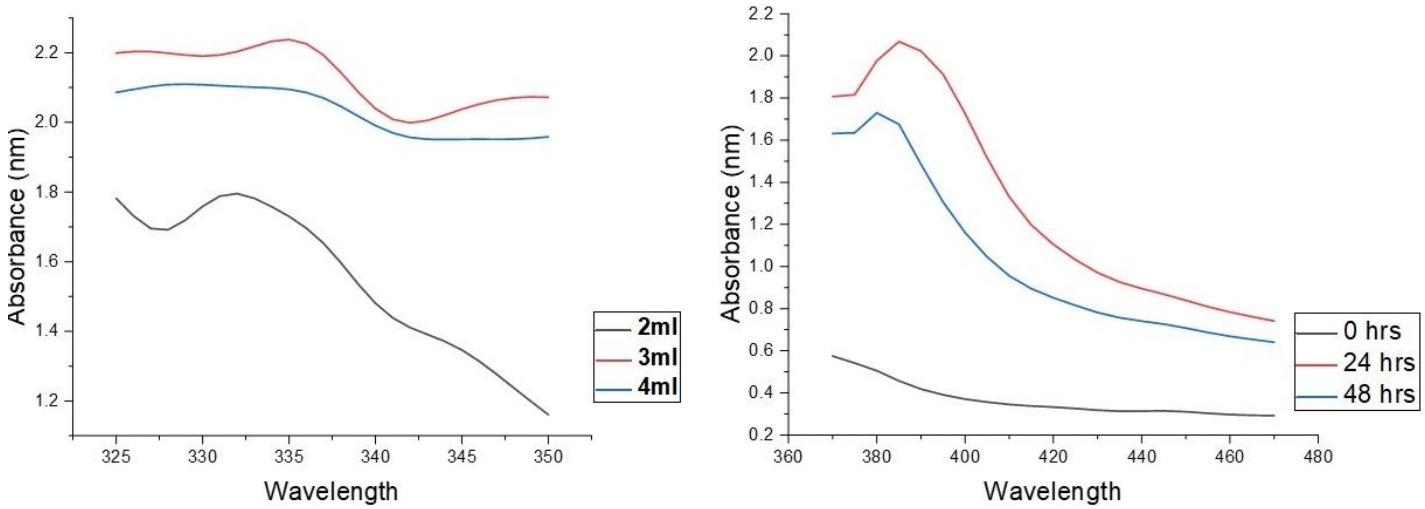

Figure 2. The optimization of the b-AgNPs by varying the incubation period, observing the absorbance at a wavelength from 200 to 800 using UV-Vis spectroscopy.
Source: Authors

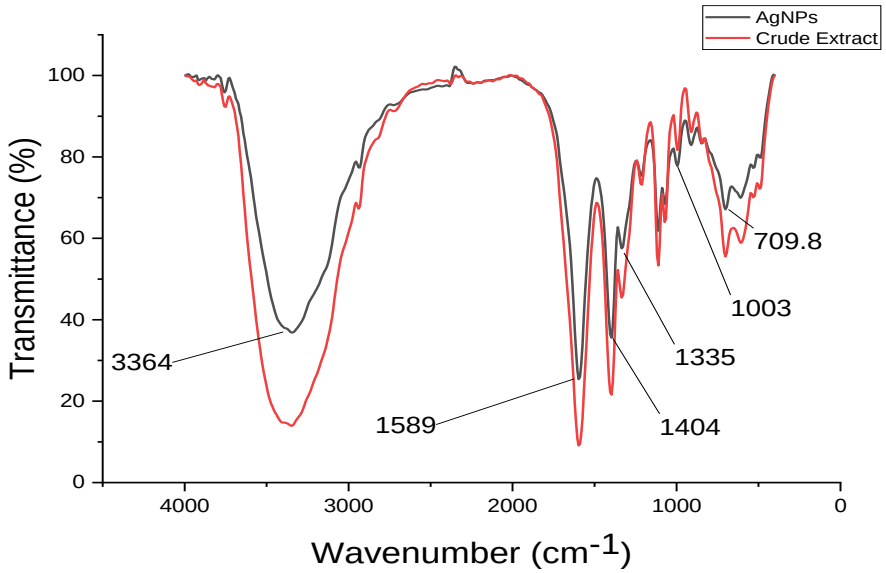

Figure 3. The FTIR results indicate the presence and sites (bands) of the biomolecules that are responsible for reducing the $AgNO_3$ to b-AgNPs well as those responsible for capping and stabilising the AgNPs.
Source: Authors

absence of metabolic activity was observed to be 0.078 mg/ml.

On the MIC assay, the contents of the wells that were observed to be the minimum inhibitory concentration (0.078 mg/ml) along with the first concentration to have a colour change to pink (0.039 mg/ml) were cultured to observe the concentration that had no fungal growth. The minimum fungicidal concentration was observed to be 0.078 mg/ml, which is similar to the minimum inhibitory concentration.

The effect of the b-AgNPs on pathogen colonization *in-vivo*

The b-AgNP treatment was able to significantly (at $P<0.05$) reduce the pathogen titres in the plants that were treated with the nanoparticles seven days after treatment. The total genomic DNA of the treatment plants was extracted and the presence of the fungal DNA was confirmed by the amplification of the *bE* primers that produced a 179 bp amplicon.

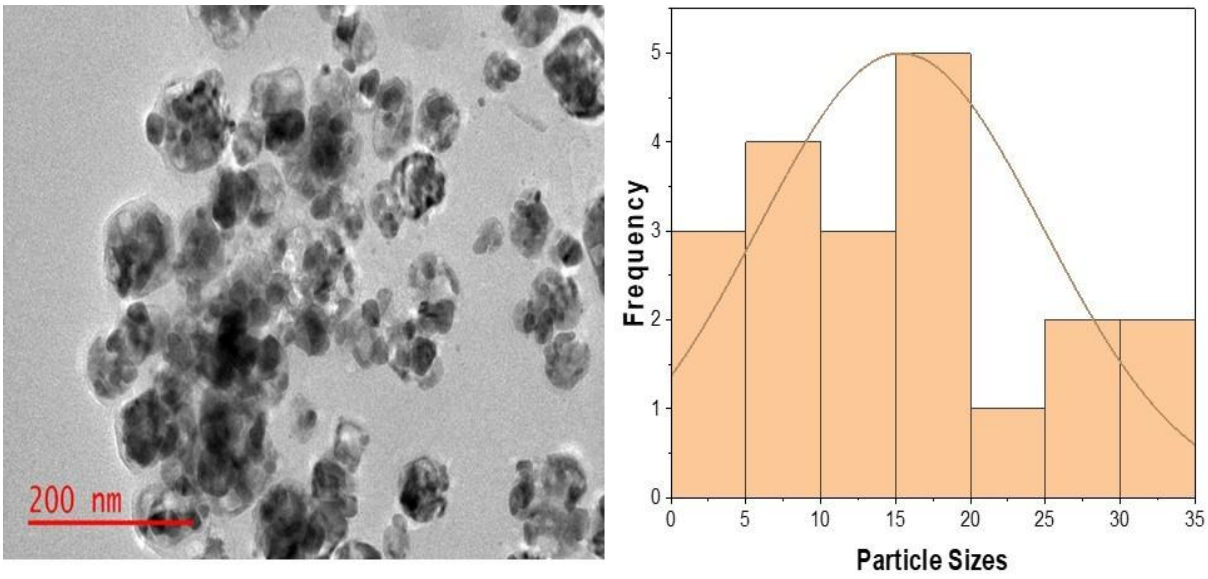

Figure 4. The TEM results indicating the sizes and shapes of the b-AgNPs that were produced by using *C. spinarum.*
Source: Authors

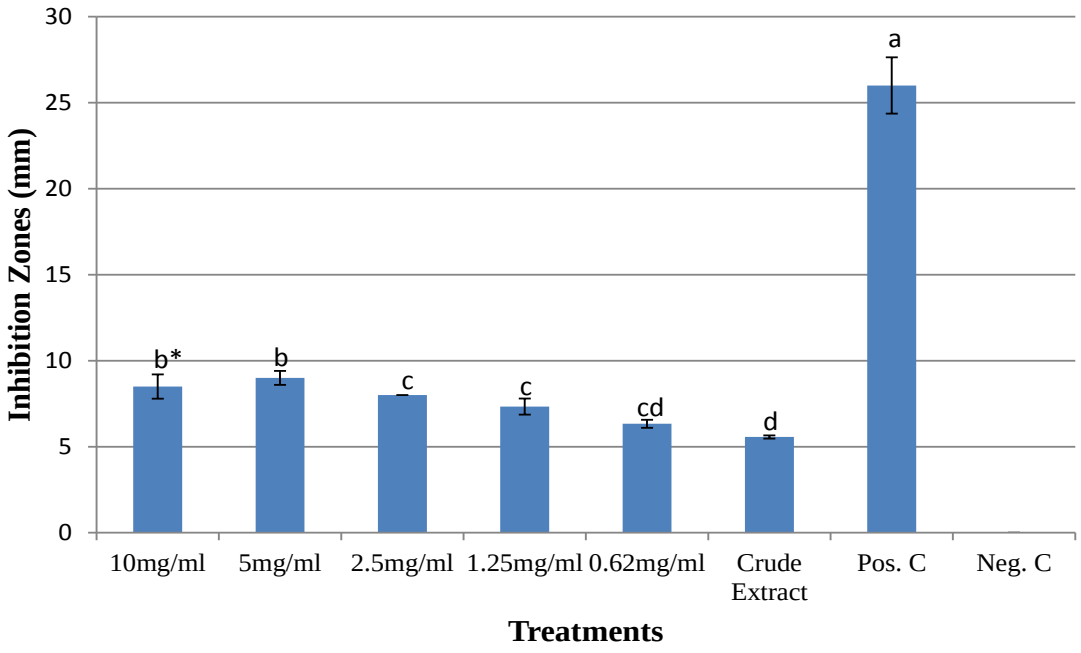

Figure 5. The inhibition zones of b-AgNPs tested *in-vitro* on *Sporisorium scitamineum* at 0.62, 1.25, 2.5, 5 and 10 mg/ml. Nystatin and distilled water were included as positive and negative control, respectively. The error bars indicate the standard deviation of the treatment means. *Means followed by the same letter (per treatment) are not significantly different at P<0.05.
Source: Authors

The treatment with the MIC concentration of the b-AgNPs (0.078 mg/ml) had the least fungal DNA quantity with 0.00038 ng/ml, whilst the treatment with 0.039 mg/ml of b-AgNPs had 0.005 ng/ml of the fungal DNA (Figure 6). The positive control had 0.1017 ng/ml of fungal DNA.

Gene expression analysis of the *bE* and *bW* genes in *S. scitamineum*

The optimization of the *GAPDHa, bE* and *bW* primers produced a positive amplification of fragments that were

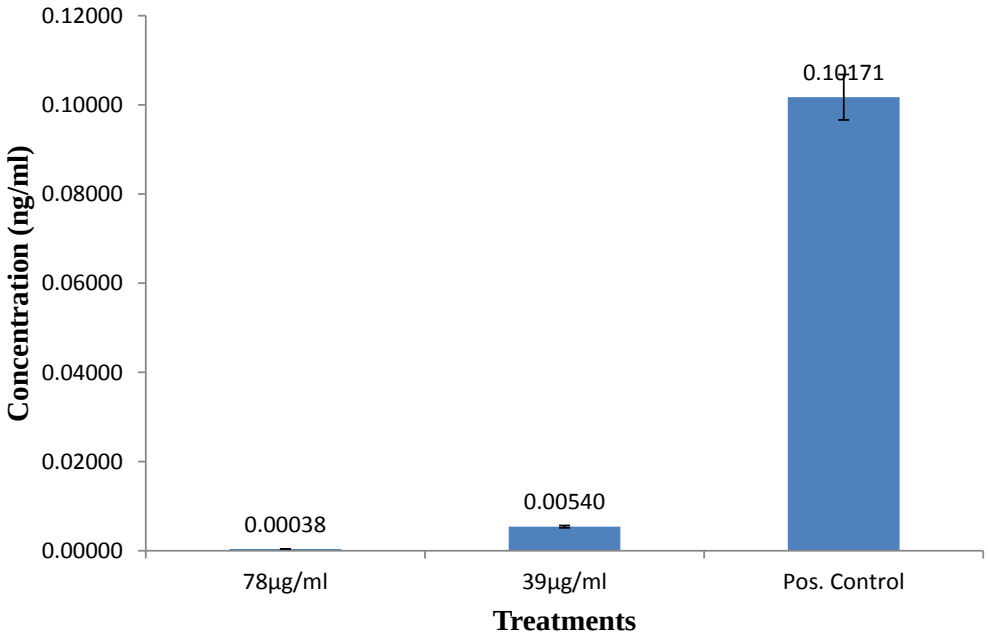

Figure 6. The pathogen biomass quantification in the plants at 7 days after treatment with varying b-AgNP concentrations compared to the positive control.
Source: Authors

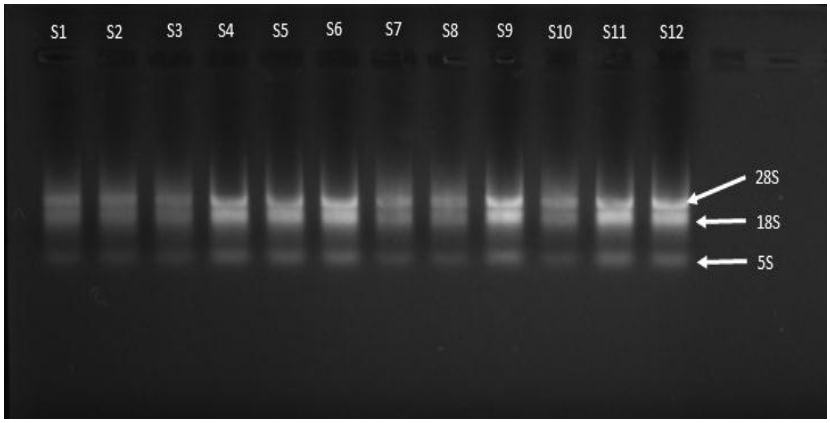

Figure 7. Integrity of the RNA that was extracted from the 12 samples showing the e28S, 18S and 5 S rRNAs.
Source: Authors

166, 179 and 172 bp, respectively. All the primers were annealed at 52°C.

The *ITSa* primers were used to verify the success of the conversion of the extracted total RNA to cDNA and a 150 bp fragment was observed in all the 12 samples (Figures 7 and 8). The amplification of all the samples warranted for the downstream RT-qPCR assays.

The application of 0.0585 mg/ml (3/4 MIC) of b-AgNPs to the *S. scitamineum* resulted in increased expression of the *bE* gene when compared to the regular expression of the gene. The expression increased significantly ($P<0.05$) from three hours to reaching an 8-fold peak after nine hours of exposure to the b-AgNPs. At 12 h, the expression levels of the gene had normalized to the regular expression levels (Figure 9).

An increased expression level was also observed on the *bE* gene when the dosage of the b-AgNPs was reduced to 0.039 mg/ml. A significant ($P<0.05$) increase to an 8-fold peak level was reached only after three hours, and then the expression levels of the gene were reduced back to their regular expression level after 6 to 12 h (Figure 9).

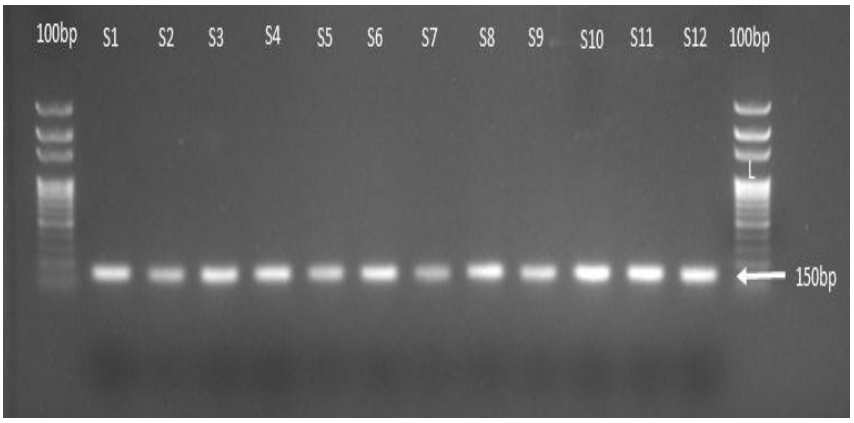

Figure 8. Amplification of the *ITSa* primer to confirm the synthesis of cDNA from the extracted RNA. The amplification produced a 150 bp fragment in all 12 samples.
Source: Authors

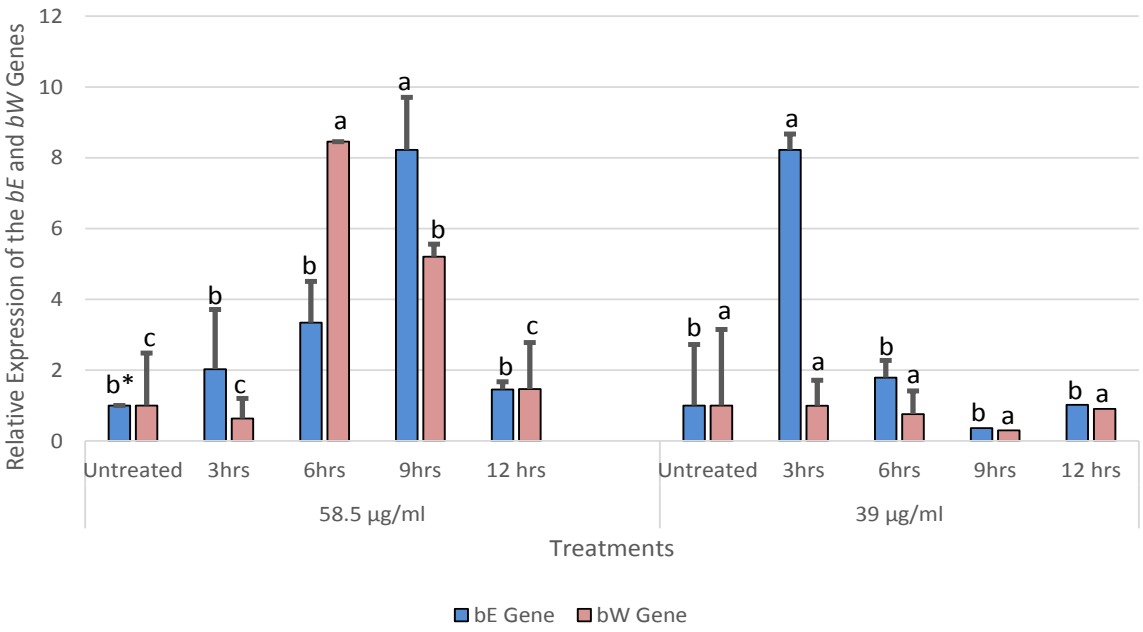

Figure 9. The relative expression levels of the *bE* and *bW* genes after treatment with 58.5 and 39 µg/ml of b-AgNPs. Total RNA was extracted from these treatments at 3, 6, 9 and 12 h after treatment. The level of significance was P=0.05. *Means followed by the same letter (per treatment) are not significantly different at P<0.05.
Source: Authors

The expression levels of the *bW* gene were also observed to increase significantly (P<0.05) upon exposure to the b-AgNPs to reach an 8-fold peak level, after six hours, when compared to its regular expression. The level of expression of the gene was then gradually reduced to almost the regular expression after 12 h of exposure to the b-AgNPs (Figure 9). When the fungus was treated with a dosage of 0.039 mg/ml b-AgNP, the expression of the *bW* gene had no significant difference throughout the observed period of 12 h.

The change in expression levels of the target genes was restored to normal expression levels about 12 h after treatment with the b-AgNPs.

DISCUSSION

The colour change upon mixing the silver nitrate with the

crude extract is due to the occurrence of the surface plasmon resonance (SPR) phenomenon which is caused by the interaction of the conduction electrons of the AgNPs (Sharma et al., 2014). The phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides and enzymes that are present in plants are used as reducing, capping and stabilising agents (Chouhan, 2018). During the optimization of the b-AgNPs, the peaks were observed with the absorbance spectra of AgNP solutions which range between 300 and 600 nm (Qais et al., 2019).

The FTIR analysis of b-AGNPs validates the activity of biomolecules that are in charge of the reduction and stabilization of the b-AgNPs (Khatoon et al., 2017). The synthesised nanoparticles were surrounded by proteins and other functional groups such as terpenoids. These results indicate the strength of the carbonyl groups from the proteins and amino acids to bind with metal, thereby capping the AgNPs. The presence of the reducing sugars could indicate their responsibility in reducing the $AgNO_3$ to AgNPs and stabilizing the AgNPs (Khatoon et al., 2017; Inam et al., 2021). Inam et al. (2021) confirmed that the biomolecules that are identified by the FTIR are the ones responsible for the stability of the b-AgNPs. The biosynthesis method produces the most stable AgNPs, especially when compared to chemical synthesis (deMelo et al., 2020; Inam et al., 2021; Vala et al., 2021).

The TEM analysis was able to provide the study with the sizes, shapes and texture of the b-AgNPs. By their definition, the size of nanoparticles should range between 1 and 100 nm. Their nano-scale size, morphological substructure and shape are of great importance as they give the AgNPs the physicochemical properties that suit them for their multiple applications (Khatoon et al., 2017; Sanchooli et al., 2018). The synthesis and characterisation of AgNPs made from *C. spinarum* have not been documented before this study.

The antifungal activity of b-AgNPs was also observed by Velu et al. (2017) against the plant pathogenic fungi; *Colletotrichum acutatum, Phytophthora capsici, Phytophthora drechsleri* and *Cladosporium fulvum.* Resistance to the b-AgNPs that were synthesized from sugarcane leaves was observed on *Didymella bryoniae* (Al-zubaidi et al., 2019). The standard antifungal nystatin may have displayed significantly (at $P<0.05$) higher inhibition, but the limitation of using fungicides is the failure to penetrate the waxy coat of sugarcane (Cui et al., 2020).

The resazurin assay qualitatively observes and differentiates dead and live cells by turning pink when it is exposed to metabolic activity. Resazurin is reduced by metabolic activity to a pink colour (resorufin), and where there is no metabolic activity, it remains blue (Kowalska-Krochmal and Dudek-Wicher, 2021). The colour change is caused by the reduction of the resazurin (blue) by the mitochondrial reductase into resorufin, a fluorescent pink colour. The minimum inhibitory concentration is the lowest value of the b-AgNPs that resulted in the complete death of the fungus, while the minimum fungicidal concentration is the lowest concentration of the b-AgNPs that kills 99.9% of the fungus, and verified by zero growth when cultured on media. This study observed the MIC and MFC of the b-AgNPs of *Carissa spinarum* to be 0.078 mg/ml.

The antimicrobial mechanism of growth inhibition by AgNPs is still not yet fully understood. Some authors have reported damage to the cell wall and cell membrane, while some have reported AgNPs penetrate the cells and cause damage to the cell organelles and thereby resulting in apoptosis (Al-zubaidi et al., 2019). Other reports attribute the inhibition mechanism to be caused by the effect on the ability of microbial DNA to replicate once it comes to contact with the AgNPs, inactivation of the ribosomes which ultimately fails to express proteins. The increase in the inhibition effect was directly proportional to the increase in the concentration of the AgNPs. This could be caused by the increased number of b-AgNPs that attach to the fungus until a saturation point is reached. This result corroborates the findings by Al-zubaidi et al. (2019).

The pathogenicity of the smut pathogen is a required mechanism for it to colonize the plant and continuously draw nutrients to complete its life cycle. Su et al. (2016) observed a slower rate of colonization in smut-resistant sugarcane varieties when compared to the susceptible varieties. Alternative to the use of resistant genotypes for the management of sugarcane smut, fungicides could be a better alternative. The fungicides are only effective on pathogens that they will be in direct contact with on the plants' surfaces at the time of spraying; otherwise, they are ineffective in treating systemic pathogens because they are not able to penetrate the waxy coat of the sugarcane plant (Cui et al., 2020). This study has demonstrated the ability of b-AgNPs to control the smut pathogen post-colonization of the sugarcane plant. It is important to further determine the cytotoxicity of the b-AgNPs on the plant cells as well as their effects on the agronomic properties of the sugarcane.

Peters et al. (2020) also observed an upregulation of the *sod1*, *sod2* and *katG* genes in *S. scitamineum* in the presence of hydrogen peroxide (H_2O_2) *in-vitro*, which then returned to normal expression levels after 180 min of exposure. Hydrogen perioxides was found to be produced by smut-resistant sugarcane varieties, especially upon being colonized by *S. scitamineum*. The up-regulation of the *bE* and *bW* genes is observed as an increase in the pathogenicity of the fungus in the presence of the b-AgNPs, especially at the dosage that is below the observed minimum inhibitory concentration. The significant upregulation of the gene expression is returned to its regular expression level after 12 h of treatment, which could be a signal of overcoming the inhibitory effect of the b-AgNPs. This shows that the growth inhibition of the *S. scitamineum* upon exposure to

the b-AgNPs is caused by their effect on the genes that are responsible for the pathogenicity of the fungi.

Conclusion

The AgNPs that were synthesized by using *C. spinarum* had antifungal activity against *S. scitamineum*. The b-AgNPs were found to have the highest antifungal activity at 5 mg/ml, whilst the MIC and MFC were found to be 0.078 mg/ml. When the b-AgNPs were tested on plants that were challenged by the fungal pathogen, these plants were found to have low tithers when compared with the plants that were not treated. The application of b-AgNPs was found to have a regulatory effect on the *bE* and *bW* genes in *S. scitamineum.* The application of 0.0585 mg/ml of the b-AgNPs to the *S. scitamineum* resulted in a significant ($P<0.05$) increase in expression of the *bE* and *bW* genes, while the treatment with 0.039 mg/ml significantly ($P<0.05$) increased the expression of the *bE* gene, but there was not significant ($P>0.05$) change in the expression of the *bW* gene. The *bE* and *bW* genes are responsible for the pathogenicity in *S. scitamineum,* and the regulatory effect of the b-AgNPs on these genes could be part of the basis of the antifungal activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antifungal activity of essential oil-based formulations used in corn preservation in Burkina Faso

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Fungi and their toxins have an impact on the hygienic standards and market value of corn (*Zea Mayas* L.) in Burkina Faso. Though effective, other pesticides have been shown to be harmful. This study aims to aid in the management of corn diseases by assessing the effectiveness of bio-fungicide formulations. In this study, the blotter method was used to assess the antifungal activity at doses of 0.5% (5 g/kg) and 1% (10 g/kg) of powdered formulations of *Cymbopogon giganteus* (F1) and *Eucalyptus camaldulensis* (F2) essential oils on the fungal flora of a corn sample. Untreated controls were also observed. The results of this study demonstrated that *Aspergillus flavus* and *Aspergillus parasiticus*, which could produce aflatoxin, were more susceptible to the 0.5% dose at F1 than at F2. Though *Aspergillus niger* and *Rhizopus* sp. were resistant to 0.5% of F1 and 0.5% of F2, while *Fusarium* sp. was resistant to 0.5% of F1. All of the above fungi were vulnerable to 1% of F1 and F2. Therefore, these two formulations could be utilized successfully to combat the decline in the hygienic, nutritional, and market value of corn in Burkina Faso caused by aflatoxin contamination and other toxins produced by these fungi. However, it would be important to evaluate the synergistic effect of these biofungicide formulations *in situ* and on a large scale.

Key words: Antifungal, *Cymbopogon giganteus, Eucalyptus camaldulensis*, essential oil, biofungal formulation, corn.

INTRODUCTION

In Burkina Faso, corn is the second most cultivated cereal after sorghum. Its production increased from 1,133,480 tonnes in 2011 to 1,170,898 tonnes in 2020 (FAO/OMS, 2020). Despite this production, food insecurity, amplified by the risks of uncontrolled food poisoning, remains a real problem in Burkina Faso.

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Indeed, cereal production is particularly affected by numerous ecological constraints (Waongo et al., 2013). In addition, the impact of rudimentary production methods and poor storage conditions increases the risk of contamination by toxins. Also, stored corn is subject to daily attacks by toxin-producing moulds. To protect crops and ensure their good phytosanitary quality, various crop management methods are used. Among them, those using synthetic chemicals have proved to be effective and have shown convincing results. For example, peroxydan induces a total inhibition (100%) of the mycelial growth of two fungi, *Botrytis cinerea* and *Penicillium digitatum* at a dose of 2.5% (Elbouchtaoui et al., 2015). Unfortunately, the use of these products has disastrous environmental, health, and economic repercussions. Environmental pollution leads to the contamination of surface water and groundwater, the contamination of food products from treated areas or surrounding areas, the contamination of certain herbaceous or medicinal plants, the gradual disappearance of certain species of useful herbs in animal feed, and some aquatic animal species (Tarnagda et al., 2017). This pollution also leads to a decrease in soil fertility, as well as a loss of biodiversity and the selection of strains of pests resistant to pesticides (Sala et al., 2000; Lehmann et al., 2018). Cases of acute poisoning by pesticides constitute a public health problem. Indeed, more than 385 million cases of unintentional acute poisoning worldwide each year (Boedeker et al., 2020) and more than 29% in the two major university hospitals, between the years 2006 and 2007, linked to the intensification of the use of pesticides by agriculture (Ouédraogo et al., 2011). Costs resulting from pesticide poisonings now exceed the total annual amount of development assistance given to the sub-Saharan African region for basic health care. The total cost of pesticide-related illnesses and injuries could reach $90 billion (USD) by 2020 (Heindel et al., 2013). In Burkina Faso, in particular, the cost of economic losses linked to the mismanagement of chemicals in the agricultural sector is estimated at around 4.2 billion CFA francs (Lankoande and Maradan, 2013). These synthetic products, of their exorbitant costs and their toxicity, also constitute an economic burden for farmers. Indeed, these pesticides are one of the ways to the impoverishment of the peasants, because the high residues of pesticides in agricultural products also raise food safety products and constitute a serious obstacle to exports. These ecological problems and their consequences on the environment, human and animal health have prompted the development of innovative post-harvest management strategies based in particular on the search for new substances that are effective, less toxic, less expensive, and respectful of the environment. In this context, several studies have already proven the effectiveness of natural substances in the fight against fungi. The essential oils of *Cymbopogon schoenanthus*, *Lippia multifliora* and *Ocimum americanum*, tested on cow pea seed-borne fungi, were found to reduce the contamination rates of *Colletotrichum dematium* and *Fusarium* spp., *Cladosporium* sp. and *Macrophomina phaseolina*, respectively (Toé et al., 2022). In view of the antifungal potential of these essential oils on various pathogenic fungal species, they could be a promising alternative strategy for combating moulds. Given the results obtained, the use of these bio-pesticides could be an interesting alternative to the use of conventional pesticides, particularly chemical pesticides. This alternative based on the use of local natural resources at a lower cost and ecologically sustainable could contribute to reducing farmers' expenses, preserving the environment, sustainably managing soil fertility, guaranteeing the quality of crops, and respecting sanitary and phyto-sanitary standards for food products. All these aspects will contribute to developing the maize sector to sustainably improve its contribution to food security, quality and nutritional aspects, access to markets, poverty reduction, and accelerated growth of the national economy. It is in this context that the present study falls under the general objective of which is to evaluate the antifungal activities of two essential oil-based formulations with a view to developing a fungicide for the management of toxigenic moulds in corn in Burkina Faso.

MATERIALS AND METHODS

Plant material

For the study, the corn variety Kamboinsé Extra Precoce Jaune (KEJ) was collected in Dédougou (12°28′N 3°28′W) located in Burkina Faso using a sterile transparent bag during the period of September 2021 and kept at 5°C in the SAMSUNG RL4352LBASP refrigerator at the Laboratory of Phytopathology (CNRST/INERA), CREAF Kamboinsé for less than 24 h before analysis.

Biopesticides based on essential oils

Two ready-to-use formulations based on essential oils of *C. giganteus* (F1) and *E. camaldulensis* (F2) fixed on powdered starch were used.

The control pesticide

Calthio MIX 485 WS (CM) (Imidacloprid 350 g/kg, thiram 100 g/kg, Metalaxyl 35 g/kg), a systemic insecticide or fungicide used as a seed treatment for corn against soil-borne pests (CSP, 2020), was used in this study as a reference control. The dose used in the study was the manufacturer's recommended dose of 5 g of product per 1 kg of grain (or 0.5%).

Processing of corn grains

The treatment of corn grains with the fungicides F1, F2 and Calthio MIX 485 WS was done by coating. For each formulation, doses of 5 g fungicide per kg of corn (or 0.5% concentration) and 10 g fungicide per kg of corn (or 1% concentration) were tested. The

tests were carried out in the presence of an untreated control (negative control) and a treatment with a reference pesticide, Calthio MIX 485 WS (positive control). Thus, 4 treatments per formulation (that is, 8 samples in total) were tested:

1. 2 samples of untreated corn (NT) ;
2. 2 samples of corn treated with Calthio MIX 485 WS at a 0.5% dose (CM);
3. 2 samples of corn treated respectively with the bio-fungicide formulations based on *C. giganteus* and *E. camaldulensis* essential oil at a dose of 0.5% (F1-1 and F2-1);
4. 2 corn samples treated with the essential oil formulations of *C. giganteus* and *E. camaldulensis* at a 1% dose (F1-2 and F2-2).

Seed health analysis

The health analysis of corn was carried out using the blotting paper method (ISTA, 1999). Corn grains were placed equidistantly in 9 cm diameter Petri dishes (10 grains per Petri dish) containing 3 layers of blotting paper soaked in sterile distilled water. For each treatment, 200 seeds were used in 4 replicates of 50, following a Fischer block design. The seeded dishes were kept in incubation at 22 ± 3°C and illuminated by an alternating cycle of 12 h per day of darkness and 12 h of near-ultraviolet light, for 7 days following the method described by Mathur and Kongsdal (2003). The incubated grains were removed and inspected individually with a Leica M210 stereomicroscope (zoom range 7.5 - 60x) for identification of the fungi by macroscopic characters (colour of mycelium, pycnidia, acervuli and conidiophores). A sample of the observed fungus was then mounted between slide and coverslip and observed with a Motic BA210 light microscope (N-WF 10 x/20 mm wide field eyepieces with dioptre adjustment on both tubes, 360° binocular rotating head, 2 CCIS EF-N PLANS 4x, 10x, 40x, and 100x immersion objectives). The identification of the fungus was confirmed by examining the microscopic characters (shape and size of the conidia, structure of the mycelium) with reference to the identification manual for fungi by Mathur and Kongsdal (2003). The species of fungi present on each grain were noted, and the percentage of grains infected by a given fungus was calculated for each seed treatment. The effect of each treatment in controlling the fungi was evaluated in comparison with the fungal infection indices obtained with untreated corn and corn treated with the reference fungicide.

Statistical analysis

The data obtained per treatment and per replication, on grain mould infection rates were subjected to an analysis of variance (ANOVA) with the statistical software XLSTAT-Pro 7.5.2 version 2016 and the means were compared using the Student Newman Keuls (SNK) test at the probability threshold of $p = 0.05$.

RESULTS

The fungal species detected in the sanitary analysis of treated and untreated seeds were *Aspergillus niger*, *Aspergillus flavus*, *Fusarium* sp., *Rhizopus* sp. and *Aspergillus parasiticus*. The results of the analysis of variance (Table 1) revealed that the seed treatments had a significant effect on the reduction of all these fungi. The lowest infection rates were noted on seeds treated with Calthio MIX 485 WS, while the highest infection rates were obtained with untreated seeds (NT). Thus, Calthio MIX 485 WS showed high fungicidal activity compared to the untreated control. *Aspergillus niger, Aspergillus flavus, Fusarium* sp., *Rhizopus* sp. and *Aspergillus parasiticus* were reduced to 8.75±3.75, 21.75±9.25, 13.75±4.75, 3.25±3.37 and 3.12±1.41%, respectively, for Calthio MIX 485 WS compared to 83±8, 82.5±8, 59.5±10, 38.5±19 and 11.5±1.37% for untreated seeds.

Effects of F1 biopesticide on seed-borne fungi in corn

The results of the analysis of variance (Table 1) revealed that the corn treatment with the essential oil formulation of *C. giganteus* (F1) at dose 2 resulted in a significant reduction of the infection rates of all the exceptional fungi. Indeed, the contamination rates of the grains in *A. niger, A. flavus*, *Fusarium* sp., *Rhizopus* sp. and *A. parasiticus* decreased from 83 ± 8 to 44.25 ± 10.75%, from 82.5 ± 8 to 35.75 ± 7.37%, from 59.5 ± 10 to 23.75 ± 5.25%, from 38.50 ± 19 to 7 ± 8.37% and from 11.5 ± 1.37 to 5.5 ± 1.25% respectively. On the other hand, the contamination rate of the grains by *Fusarium* sp. was 59.5 ± 10 to 69 ± 8% with F1-1. Calthio MIX 485 WS also resulted in significant decreases in fungal infection levels compared to the untreated seeds treatment, although with slightly greater reductions than F1-2.

Effects of the biopesticide F2 on seed-borne fungi in corn

The results of the analysis of variance (Table 1) revealed that corn treatment with the essential oil formulation of *Eucalyptus camaldulensis* (F2) resulted in a significant reduction of fungi except for *Aspergillus niger* and *Rhyzopus* sp. for which the low dose biopesticide of 0.5% (F2-1) was ineffective. Indeed, the contamination rates of grains by *A. niger, A. flavus*, *Fusarium* sp., *Rhizopus* sp. and *A. parasiticus* went from 83 ± 8 to 73 ± 9 %, from 82.5 ± 8 to 52.5 ± 5.5 %, from 59.5 ± 10 to 33.5 ± 4.25 %, from 38.50 ± 19 to 34 ± 11 % and from 11.5 ± 1.37 to 7.75 ± 0.87 % respectively with F2. On the other hand, the contamination rate of the grains by *Aspergillus niger* and *Rhyzopus* sp. increased from 83 ± 8 to 88.5 ± 5.5 % and from 38.50 ± 19 to 49 ± 4 % respectively with F2-1.

Correlation of treatments and mycological profile

The principal component analysis (PCA) (Figures 1 and 2) showed a distribution of the data around two axes (*F*1 and *F*2: 91.37%). Thus, the 1st and 2nd dimensions of the PCA (F1 and F2) explained 91.37% of the information contained in the dataset. This means that if we summarize the 5 variables studied (*A. niger, A. flavus, Fusarium* sp.,

Table 1. Analysis of variance of the data from the sanitary analysis of treated and untreated corn grains.

Sample	*A. niger* (%)	*A. flavus* (%)	*Fusarium sp.* (%)	*Rhizopus sp.* (%)	*A. parasiticus* (%)
NT	83 ± 8^{a}	82.5 ± 8^{a}	59.5 ± 10^{ab}	38.50 ± 19^{a}	11.5 ± 1.37^{a}
F2-1	88.5 ± 5.5^{a}	74 ± 7^{ab}	47.5 ± 6.75^{bc}	49 ± 4^{a}	11 ± 1^{a}
F2-2	73 ± 9^{a}	52.5 ± 5.5^{bc}	33.5 ± 4.25^{cd}	34 ± 11^{ab}	7.75 ± 0.87^{ab}
F1-1	76 ± 3^{a}	42.5 ± 9.5^{c}	69 ± 8^{a}	29.75 ± 5^{abc}	6.25 ± 1.25^{bc}
F1-2	44.25 ± 10.75^{b}	35.75 ± 7.37^{cd}	23.75 ± 5.25$^{d}{}_{e}$	7 ± 8.37^{bc}	5.5 ± 1.25^{bc}
CM	8.75 ± 3.75^{c}	21.75 ± 9.25^{d}	13.75 ± 4.75$_{e}$	3.25 ± 3.37^{c}	3.12 ± 1.41^{c}
Pr > F	0.0000	0.0000	0.0000	0.0000	0.0000
Significant	Yes	Yes	Yes	Yes	Yes

In the same column, the means assigned to the same alphabetical letter are not significantly different at the 5% threshold, according to the Student-Newman-Keuls multiple classification test; NT = Not treated; F1-1: *Cymbopogon* at the 0.5 % dose; F1-2: *Cymbopogon* at the 1 % dose; F2-1: *Eucalyptus* at the 0.5 % dose; F2-2: *Eucalyptus at* the 1 % dose; CM: Calthio MIX.
Source : Authors

Rhizopus sp. and *A. parasiticus*) by the two dimensions, then we recover 98% of the information contained in this dataset. In other words, we have an excellent summary that synthesizes these 5 variable almost perfectly. The first dimension of the PCA (*F*1) allowed the different treatments to be ranked in decreasing order of their antifungal activity (Figures 1 and 2). From left to right, the points furthest from untreated seeds correspond to the most fungicidal treatments. Thus, the biopesticides F1 and F2 were less effective than the synthetic pesticide Calthio MIX 485 WS, but F1 was more fungicidal than F2. The PCA shows that the fungus rate decreases with increasing treatment dose. The *F*1 dimension of the PCA (Figures 1 and 2) also informs us of the relationship between these fungi. Thus, there is a close correlation between these different fungi, especially between *A. flavus* and *A. parasiticus*, due to the proximity of their eigenvector values. The *F*2 dimension of the PCA (Figures 1 and 2) provides information on the level of resistance of these fungi to the different treatments. *Fusarium* sp. is most resistant to the F1 biopesticide, while *Rhizopus* sp. is most resistant to the F2 biopesticide.

DISCUSSION

In the present study, the fungal analysis of maize grains of the Kamboinsé Extra Précoce Jaune (KEJ) variety made it possible to identify a diversity of fungi such as *A. niger*, *A. flavus*, *Fusarium* sp., *Rhizopus* sp. and *A. parasiticus*. Previous studies conducted outside of Burkina Faso have found similar results. Indeed, *A. flavus*, *A. niger*, *Fusarium oxysporum*, *Penicillium* sp. and *Rhizopus* sp. were detected on corn with yellow grains, not disinfected, with an average temperature and relative humidity of 25°C and 40% respectively, harvested in Côte d'Ivoire (Ky and Diomandé, 2017). *A. flavus*, *A. nige*r, *R. stolonifer* and *Penicillium* sp. have been identified in corn-based samples produced in Benin (Agassounon et al., 2020). The presence of these moulds in corn implies that the contamination likely occurs either before field harvest or during improper drying, storage and processing of corn grains into finished products. The presence of these moulds in corn implies that the contamination likely occurs either before field harvest or during improper drying, and storage of corn grains (Czerwiecki et al., 2010). Corn exposed to the air in large basins and without adequate protection during sale is likely to be contaminated with fungal and bacterial spores (Toffa et al., 2013). Mould growth in corn could also be favoured by inadequate physico-chemical parameters, including the amount of free water (A_w), temperature, presence of oxygen, nature of the substrate and pH (Siramon et al., 2013).

The results of the analysis of variance revealed that the seed treatments with the biopesticides F1, F2 and the synthetic pesticide Calthio MIX 485 WS had a significant effect on the reduction of all these fungi compared to the untreated control.

Calthio MIX 485 WS showed the highest fungicidal activity. In fact, the lowest infection rates were noted on seeds treated with Calthio MIX 485 WS. *A. niger, A. flavus, Fusarium* sp., *Rhizopus* sp. and *A. parasiticus* were strongly reduced by CM (3.12-1.75%) compared to NT (11.50-83%). Similar effects of Calthio MIX 485 WS have been described in several previous studies applied to corn (Szwejkowska et al., 2008), cow peas and groundnut seeds (Toé et al., 2022). The efficacy of Calthio MIX 485 WS against these fungi is likely to be due to the fungicidal activity of one of its active molecules (thiram), which acts through direct contact with the seed against numerous seed-borne pathogenic fungi, reduces the incidence of seed-borne rots and damping-off on seedlings (Séguy, 1999).

Corn treatment with the essential oil formulation of *C. giganteus* (F1) was also effective at both low and high doses in controlling the fungi identified in corn, resulting

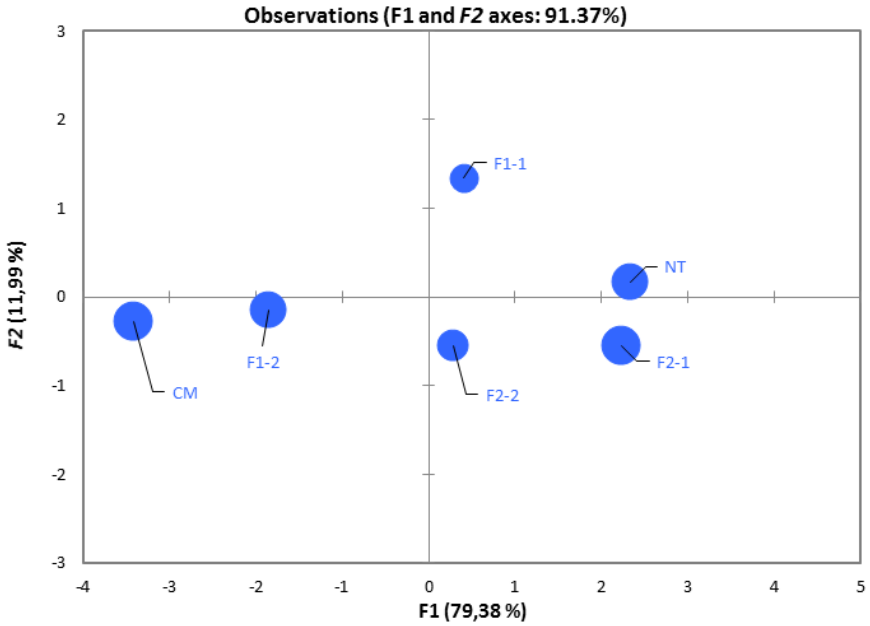

Figure 1. Classification of treatments in descending order of effectiveness.
Source : Authors

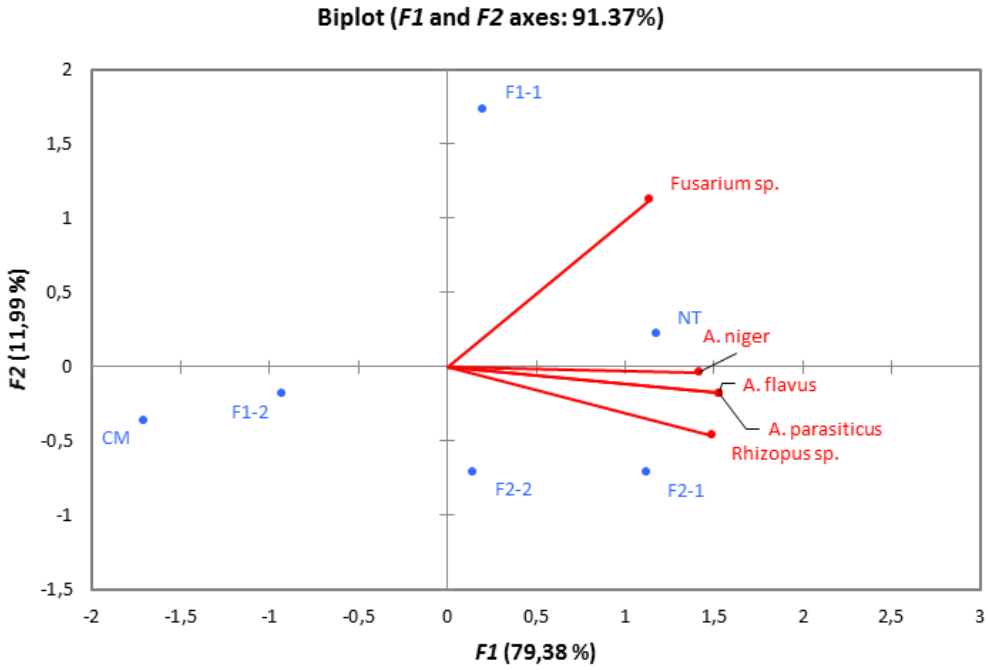

Figure 2. Relationships between treatment and frequency of contamination of corn.
Source: Authors

in a significant reduction in these fungi. Indeed, with F1, the level of contamination of treated corn was significantly reduced for all fungi except for *Fusarium* sp. when the biopesticide was used at the low dose of 0.5 % (F1-1). The rate of grain contamination by *Fusarium* sp. increased from 59.5 ± 10 to 69 ± 8 % with F1-1. This effect was similarly observed in earlier studies where *C. citratus* essential oil was also only effective at high doses (1%). Similarly, *A. indica* seed oil accelerated the growth of *F. verticillioides* on stored corn (Fandohan et al.,

2004). The fungicidal effect of F1 would then increase with the dose of the treatment applied and more precisely with the dose of its constituents such as limonene, Z-Mentha-1(7),8-dien-2-ol, E-Mentha-1(7),8-dien-2-ol, E-*p*-Mentha-2,8-diene-ol and Z-*p*-Mentha-2,8-diene-1-ol (Bossou et al., 2020). This hypothesis corroborates the results of another study where it was shown that 1.0 µL/mL of *C. citratus* essential oil with 79% citral was more fungicidal than that with 68.4% citral (Martinazzo et al., 2019).

Seed treatment with the high-dose formulation of *E. camaldulensis* (F2) essential oil was also effective in controlling the fungi identified in corn, resulting in a significant reduction of these fungi, albeit with lower reduction rates. In addition, some fungi such as *A. niger* and *Rhyzopus* sp. were resistant to the low dose (0.5 %) of this biopesticide; the rate of contamination of grain by these fungi increased from 83 ± 8 to 88.5 ± 5.5% and from 38.50 ± 19 to 49 ± 4% respectively. Other studies also proved the effectiveness of *E. camaldulensis* essential oil against *A. flavus* (Abo Elgat et al., 2020) A. *parasiticus* NRRL2999 *in-vitro* (Rasooli et al., 2009) *A. niger* (Siramon et al., 2013) against five species of *Fusarium* at 10 ml/ml (Gakuubi et al., 2017) and *Rhizopus stolonifer* (Nasr et al., 2018). However, application of essential oil from *E. camaldulensis* leaves to tree wood showed no inhibition of *A. niger* at low concentrations (156.25 ppm) (Salem et al., 2016). The efficacy of F2 would therefore increase with the treatment dose and more specifically in synergy with the high oxygen content (at least 60%) of the essential oil (Barra et al., 2010) namely *α*-phellandrene, 1,8-cineole, *p*-cymene, α-pinene and *γ*-terpinene (Samaté et al., 2011).

The Principal Component Analysis (PCA) allowed us to draw a partial conclusion to our analysis by classifying in decreasing order of their effectiveness, (i) the type of pesticide (ii) the dose of the pesticide (iii) the type of relationship existing between the pesticide and these fungi and (iv) the level of resistance of these fungi to different treatments. Thus, the synthetic pesticide (Calcio MIX 485 WS), used as a reference control, was the most effective, followed by the formulation based *C. giganteus* essential oil (F1), and *E. camaldulenssis* (F2) respectively. The fungicidal efficacy of these two formulations increased with the prescribed dose. The aflatoxigenic pathogens *A. flavus* and *A. parasiticus are* thought to share close links at the ecological and sensitivity levels to the different pesticides. Specific resistance of *Fusarium* sp. and *Rhizopus* sp. to low doses of F1 and F2, respectively, was reported. However, the resistance of these fungi was attributed to the low content of oxygenated compounds present in essential oils (Barra et al., 2010). Although the resistance of these fungi was attributed to the low content of oxygenated compounds in the essential oils, this phenomenon of resistance could be further clarified by in-depth toxicological studies. These studies will allow us to have a better knowledge of the mechanism of action of these essential oils towards fungi.

Conclusion

From the study of the effect of bio-fungicides based on essential oils of *C. giganteus* and *E. camaldulensis*, it appears that these formulations are effective against the mycelial growth of potentially aflatoxinogenic pathogens, *A. flavus* and *A. parasiticus*, of *A. niger*, of the genera *Fusarium* and *Rhizopus*. This study, therefore, shows that these two formulations could effectively replace synthetic pesticides in the fight against phytopathogenic and toxigenic fungi. On the one hand, this would contribute by improving the health of plants, animals, consumers and the environment. On the other hand, this would make it possible to ensure that corn produced complies with international standards, guarantee its competitiveness and consequently promote its speculation on national and international markets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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