Original Research Article

Effect of Some Fungal and Bacterial Organisms on the Growth of Cowpea (V*igna unguiculata* (L.) Walp) Seedlings

ABSTRACT

Aims: Cowpea [*Vigna unguiculata* (L.) Walp] is a legume widely consumed in Africa. The effect of eight organisms viz: *Fusarium oxysporum*, *Aspergillus niger*, *Botryodiplodia theobromae*, *Rhizopus stolonifer*, *Pseudomonas* sp., *Corynebacterium* sp., *Micrococcus* sp. and *Xanthomonas* sp. on the growth of *Vigna unguiculata* seedlings was determined.
Methodology: Spore suspension of each fungal organism was prepared from pure cultures grown on Potato Dextrose Agar (PDA) plates and the bacteria used were obtained from slants. Fungal spores were standardized with the help of a haemocytometer slide and gelatine (0.1%) was used as a sticker. Using serial dilution method, 0.1ml of each of the isolated bacteria was collected from the 10^{-3} dilution and sprayed on the young seedlings using the run-off method. The cowpea seedlings were separately inoculated with each organism at the three leaf stage, three weeks after planting. Seedlings were artificially inoculated by spraying the adaxial surface of the leaves until water-soaked spots were obtained. The experiment was allowed to stand for 2 months and the leaf number, root length, shoot length and total seedling height of the cowpea seedlings were determined.
Results: Symptoms observed on seedlings were: stunted growth, drying of leaves, few fibrous roots, yellowing of leaves, wilting, necrotic lesions, leaf spot, darkening of leaf veins and blight. Most of the test organisms were pathogenic to *V. unguiculata* causing varying degrees of damage. *Fusarium oxysporum* caused the most deterioration on cowpea seedlings when compared to the other treatments.
Conclusion: This study has demonstrated the ability of culture filtrates of pathogenic microorganisms to express symptoms in seedlings and transmit diseases to healthy seedlings.

Keywords: Bacteria, Fungi, Inoculation, Pathogens, Symptoms, Vigna unguiculata

1. INTRODUCTION

Vigna unguiculata (L.) Walp. (cowpea) is an annual herbaceous legume cultivated for its edible seeds or for animal feed. The crop serves as both vegetable and pulse crop. It is a major source of proteins, essential vitamins, minerals and amino acids in most tropical third world countries [1,2]. They are mostly grown for grain but a small proportion (about 10%) are grown as fresh pods eastern Asia or as green leafy vegetables, fodder or fresh pods in Africa [3]. Cowpeas are annual herbaceous crops that are erect, climbing or prostrate with a strong principal root and many spreading lateral roots in surface soil [4]. *V. unguiculata* has a well-developed root system and can grow up to 80 cm for the erect varieties, and up to 2 m for the climbing cultivars. Germination in cowpea is epigeal. The first pair of true leaves are simple and opposite while subsequent leaves are trifoliate with oval leaflets. Pods occur in

pairs, mostly vertical and pending, but they can also be erect. They contain 8-20 seeds and are cylindrical in shape, 2-6 cm long and 3-12 mm broad. Seeds can be black, white or pink brown [5].

Cowpeas are adapted to warm season and are grown in the tropical and subtropical zones, in sub-Saharan Africa and in Asia, the Caribbean, Central America, South America, around the Mediterranean Sea and the United States of America. Temperatures are suitable for cowpea all year round in the tropical zones while in the subtropical zones, temperatures are just suitable in the summer. Over 95% of the world's cowpea production takes place in Sub-Saharan Africa, with about 12.5 million hectares of land under cowpea cultivation globally in 2014 [6]. The Sudan savannah zone of Nigeria is the centre of maximum diversity of cultivated cowpeas. Nigeria (4 million ha) has the largest area of cowpea cultivation according to FAOSTAT [6]. The second largest producing continent is Asia and it represents less than 3% of cowpea production worldwide from 1993 to 2014. Over this period of time, most of the cultivation in Asia was done in Myanmar [6]. In Africa, *V. unguiculata* is mostly grown on lowlands but can be cultivated at an altitude of up to 1800 m.

Cowpea is prone to its natural enemies. Although cowpea is used as bio-fertilizer in agriculture and it's a good source of amino acids for humans, however, commercial production of the legume is highly affected by pests (especially arthropods) and pathogenic organisms [7]. Insects of various types cause devastating losses on cowpea, but nematodes, bacterial, fungal and viral diseases also cause losses. Several fungal and bacterial pathogens have been reported to infect crop plants which lead to decrease in their yield and consequently reduce profit. Therefore, the aim of this study is to ascertain the effect of some fungal and bacterial organisms on the growth of *V. unguiculata* seedlings and also to determine the symptoms caused by these organisms on cowpea.

2. MATERIAL AND METHODS

2.1 Source of Fungi

Botryodiplodia theobromae, *Fusarium oxysporum*, *Rhizopus stolonifer*, *Aspergillus niger*, *Pseudomonas* sp*., Corynebacterium* sp*., Micrococcus* sp*.* and *Xanthomonas* sp. were isolated from diseased *Vigna unguiculata* seeds [8] using Standard Blotter Method [9] and Agar Method described by Klement and Voros [10]. Whatman's filter papers were soaked in sterile distilled water and placed on Petri dishes. Sterilized cowpea seeds were plated on the filter papers and then incubated for 7days at room temperature (28^{o}C). Fungal growth was observed on the filter papers and the resulting fungi were sub-cultured on Potato Dextrose Agar (PDA) medium. Isolated fungi were identified according to the guidelines issued by Umechuruba and Elenwo [11] and Ataga *et al*. [12]. Pure culture of each fungus was used as inoculum.

2.2 Inoculation of Healthy Cowpea Seedlings with Culture Filtrates of Test Fungi and Bacteria

Five healthy cowpea seeds were planted in polythene bags containing fine grained sterile sandy loam soil obtained from the back of Faculty of Science (Ofrima Hall), University of Port Harcourt, Rivers State. Nine treatments were used and each treatment was replicated five times. A total of 45bags were used in this experiment for the eight test organisms (fungi and bacteria) and the control. Completely Randomized Design (CRD) was used as the experimental layout. The plants were irrigated at 24hours interval.

The test organisms used in this study were: *Fusarium oxysporum*, *Aspergillus niger, Botryodiplodia theobromae, Rhizopus stolonifer, Pseudomonas* sp*., Corynebacterium* sp*., Micrococcus* sp*.* and *Xanthomonas* sp. Spore suspension of each fungus was prepared from culture in Petri-dishes and the bacteria from the slants. For the bacterial organisms, serial dilution method was used from the 10^{-3} dilution where 0.1ml of each of the isolated bacteria was sprayed using the run-off method. Mycelia suspensions ofthe fungal isolates were prepared by punching 7 day old fungal cultures in agar plates using5mm cork borer. The agar pieces of each test fungus were separately lodged into 10ml of distilled water and then sieved through double layer muslin cloth to remove the hyphae, agar lumps and other impurities. The spore suspension of each fungus was standardized at 10,000 spores per ml, in gelatine water. The spore suspension was standardized with the help of a haemocytometer slide. Gelatine (0.1%) was used as a sticker.

After three weeks, the cowpea seedlings were separately inoculated with each test organism at the three leaf stage. Test seedlings were artificially inoculated by spraying the abaxial surface of the leaves until water-soaked spots were obtained using a hand operated sprayer. After spraying, each plant was covered with a sterile polythene bag for 24hours to maintain about 100% relative humidity. The control plants were sprayed to run-off with sterile distilled water only and covered with polythene bags for 24hours. The plants were water sprinkled at 24hours interval and examined for signs of infection. Where symptoms occurred; isolation was carried out again to confirm the identity of the isolates.

2.3 Data Collection and Data Analysis

The experiment was studied for two months. The leaf number, root length, shoot length and total seedling height of the cowpea seedlings were determined at the end of the experiment. The number of emerged leaves was recorded daily for two months. The length of the shoot was measured from the root-collar to the terminal bud with a meter rule. The length of the longest root was measured with a meter rule. Total seedling height was obtained by measuring from the tip of the longest root to the shoot terminal bud with a meter rule.

Analysis of variance (ANOVA) was carried out on all data collected. Duncan's Multiple Range Test (DMRT) was used to determine whether three or more means differ significantly. Bar graphs were plotted and the standard error bars noted at 95% confidence limit.

3. RESULTS AND DISCUSSION

3.1 Effect of Microorganisms on Cowpea Seedlings

The morphological characteristics of the fungal organisms used in the study are presented in Table 1.

Table 1: Morphological characteristics of the fungal organisms used in the study

Morphological Characteristics	Fungal organism
Black colony with powdery surface	*Aspergillus niger*

Dark grey cottony colonies	*Botryodiplodia theobromae*
Copious cottony colony with black globules	*Rhizopus stolonifer*
Snow white colony with dry surface	*Fusarium oxysporum*

**Source: Iyanyi and Ataga (2014)*

The effect of *Fusarium oxysporum*, *Aspergillus niger*, *Botryodiplodia theobromae*, *Rhizopus stolonifer*, *Pseudomonas* sp., *Corynebacterium* sp., *Micrococcus* sp*. and Xanthomonas* sp*.* on the seedling growth of cowpea are presented in Figures 1 to 3. The test organisms reduced the seedling growth of cowpea ($P< 0.05$) infected three weeks after planting.

The mean number of infected leaves (disease incidence) 2months after planting showed that *Fusarium oxysporum* (7.1) had the highest mean number of infected leaves followed by *Aspergillus niger* (6.8), *Pseudomonas* sp. (6.2), *Xanthomonas* sp. (6.1), *Rhizopus stolonifer* (5.1), *Botryodiplodia theobromae* (4.6), *Corynebacterium* sp. (3.3) and *Micrococcus* sp. (3.1) respectively.The control had no infected leaves (Figure 1).

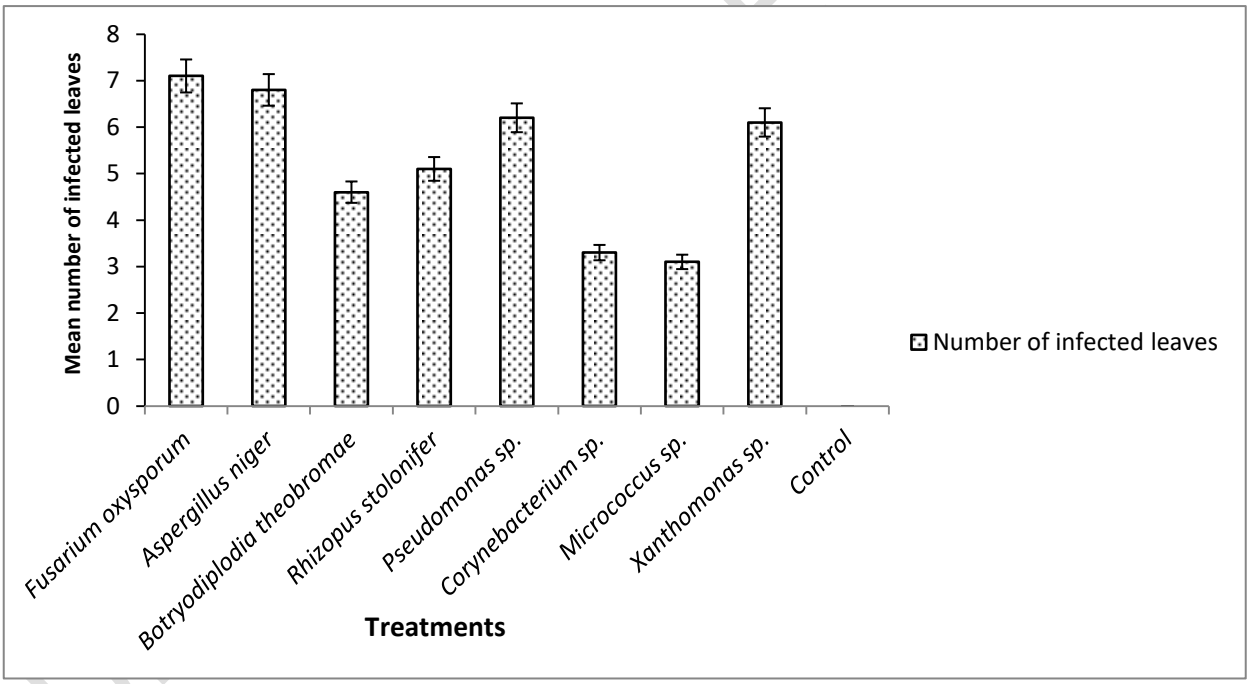

Figure 1: Effect of microorganisms on number of infected leaves (disease incidence) of *Vigna unguiculata* seedlings two months after planting
**I= Standard error ($P\leq0.05$)*

Fusarium oxysporum (34.3%) caused the highest reduction in leaf mean number of cowpea seedlings followed by *Aspergillus niger* (27.1%), *Botryodiplodia theobromae* (21.0%), *Pseudomonas* sp. (18.6%), *Xanthomonas* sp. (17.6%), *Rhizopus stolonifer* (16.7%), *Corynebacterium* sp. (14.3%) and *Micrococcus* sp. (11.0%) when compared with the control (Figure 2).

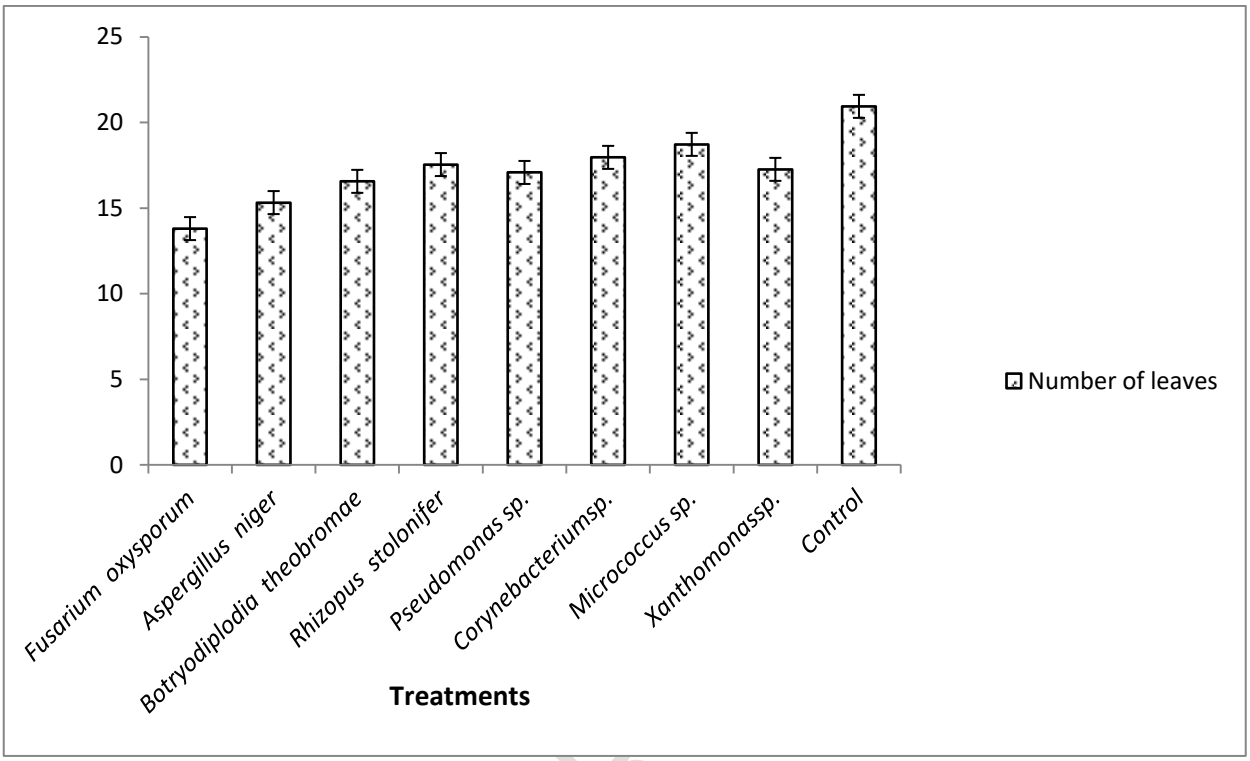

Figure 2: Effect of microorganisms on leaf number of *Vigna unguiculata* seedlings two months after planting.
**I= Standard error (P≤0.05)*

Fusarium oxysporum (31.6%) caused the highest reduction in the mean root length of cowpea seedlings followed by *Aspergillus niger* (28.7%) *Rhizopus stolonifer* (24.3%) and *Pseudomonas* sp. (24.3%), *Botryodiplodia theobromae* (22.8%), *Corynebacterium* sp. (18.4%) and *Micrococcus* sp. (16.2%) when compared with the control (Figure 3). *Fusarium oxysporum* (45.7%) caused the highest reduction in mean shoot length of cowpea seedlings followed by *Aspergillus niger* (38.2%), *Botryodiplodia theobromae* (34.7%), *Rhizopus stolonifer* (30.7%*), Pseudomonas* sp. (23.6%), *Corynebacterium* sp. (8.5%), *Xanthomonas* sp. (5.1%) and *Micrococcus* sp. (5.0%) when compared with the control (Figure 3).

Fusarium oxysporum (40%) caused the highest reduction in the total seedling height of cowpea followed by *Aspergillus niger* (34.6%), *Botryodiplodia theobromae* (29.9%), *Rhizopus stolonifer* (27.8%), *Pseudomonas* sp. (23.9%), *Xanthomonas* sp. (17.9%) *Corynebacterium* sp. (12.5%) and *Microccus* sp. (9.3%) when compared with the control (Figure 3).

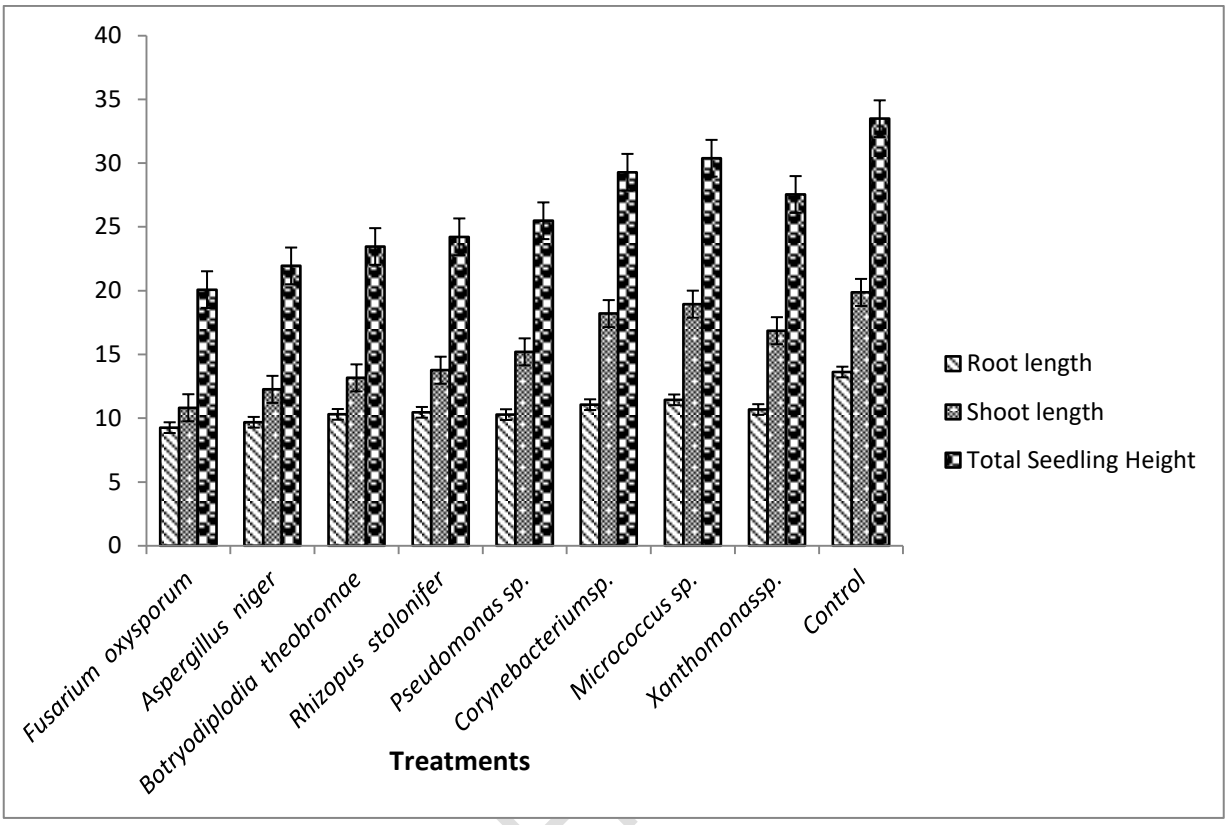

Figure 3: Effect of microorganisms on root length, shoot length and total seedling height of *Vigna unguiculata* seedlings two months after planting.
**I= Standard error ($P \leq 0.05$)*

Fungal culture filtrate of *Fusarium oxysporum*, *Aspergillus niger*, *Botyodiplodia theobromae* and *Rhizopus stolonifer* reduced the seedling growth of cowpea seedlings ($P<0.05$). The results showed that *Fusarium oxysporum* caused the highest reduction in both shoot and root length of cowpea seedlings. Reduction in seedling growth may be due to the amount of metabolites induced by this fungus which interferes with the normal metabolic and physiological processes of the seedlings. *Fusarium* wilt caused by *Fusarium oxysporum* [13]. *F. oxysporum* is responsible for limitation in cowpea production in different parts of the world.

Bacterial culture filtrates of *Pseudomonas* sp., *Xanthomonas* sp., *Corynebacterium* sp. and *Micrococcus* sp. significantly reduced the growth of cowpea seedlings ($P<0.05$). Adebayo *et al*. [14] reported *Pseudomonas* sp., *Xanthomonas* sp. and *Corynebacterium* sp. infestation on cowpea and maize grains. *Pseudomonas* sp. has been reported as a growth-promoting and antagonistic microrganism by many authors [15,16]. Venkatachalam *et al*. [17] reported the reduction in shoot length and root length in maize and raddish repectively caused by culture filtrates of *Streptomyces viridochrogenes* and *Streptomyces clavifer*. Pectinases, cellulases and hydrolytic enzymes, are involved in the mechanisms used by bacteria to penetrate into and persist in host plants. The cell wall of plants consists of cellulose, while the middle lamella between cell walls is made up of mainly pectin. Hydrolysis of methyl-ester groups of cell wall pectins is catalyzed by pectinases. The ability of endophytes to degrade pectate could be an important factor in the colonization of the interspatial region between plant cells [18].

3.2 Disease Symptoms Observed on Inoculated Cowpea Seedlings

Cowpea seedlings infected with the different test organisms were observed for two months. Different symptoms manifested as a result of the effect of the organisms on the seedlings.

Nine types of disease symptoms were observed to be associated with the seedlings. Seedlings stunted (S_1), few fibrous roots (S_2), yellowing of leaves (S_3), drying of leaves/defoliation (S_4), wilting (S_5), blight (S_6), leaf spot (S_7), necrotic lesions (S_8) and darkening of leaf veins (S_9). Cowpea seedlings infected with *Aspergillus niger* showed stunted growth, yellowing and drying of leaves. Those infected with *Fusarium oxysporum* all showed stunted growth, yellowing, wilting and drying of leaves. Seedlings infected with *Rhizopus stolonifer* showed darkening of veins, necrotic lesions and wilting. *Pseudomonas* infected seedlings resulted in wilting and yellow to light brown spots on leaves. *Xanthomonas* infected seedlings were subjected to defoliation and leaf blight. Defoliation also occurred with *Corynebacterium* infected seedlings with yellow necrotic leaf lesions and small dark brown lesions. Seedlings inoculated with *Micrococcus* sp. had dried leaves. The control plants showed no symptoms. The symptoms are represented in Table 2.

Table 2: Disease symptoms associated with cowpea seedlings.

S/N	Microorganism	Disease symptoms types	% of diseased seedlings of cowpea
1	*Fusarium oxysporum*	S_1	19
		S_2	17
		S_3	20
		S_4	17
		S_5	22
2	*Aspergillus niger*	S_1	9
		S_3	10
		S_4	13
3	*Rhizopus stolonifer*	S_5	7
		S_8	5
		S_9	6
4	*Botryodiplodia*	S_4	6
	theobromae	S_7	4
5	*Pseudomonas* sp.	S_5	14
		S_7	18
6	*Corynebacterium* sp.	S_4	6
		S_8	4
7	*Micrococcus* sp.	S_4	4
8	*Xanthomonas* sp.	S_4	10
		S_6	15
9	Control	No disease symptoms	

Oluyemisi *et al*. [19] reported that seed inoculation with some seed mycoflora induced disease symptoms in cowpea seedlings. The organisms caused chlorosis and necrotic spots

on the leaves, stems and roots. The cell wall-degrading enzymes (pectinases and cellulases) produced by these organisms must have facilitated the penetration of the fungi. Kritzinger *et al*. [20] observed the effect of fumonisin (a mycotoxin produced by *Fusarium* sp.) on cowpea. Some of the contents of the cytoplasm passed through the plasma membrane as it separated from the cell wall. Irregular sized vacuoles were formed due to the contraction of the protoplasm. The destructive effects seen in the ultra structure of the cell might play a role in the significant reduction in germination, root and shoot. *Fusarium oxysporum* is one of the important and diverse plant pathogenic fungi infecting nearly 150 plant species. The pathogen of each plant is specific and referred as formae specials [21]. One of the important cowpea diseases is *Fusarium oxysporum* and this species possess risk to production of wheat, banana, tomato, beans, peas, palm, onions, sorghum, cowpea, potatoes, garlic and maize etc [22]. The genus *Fusarium* consists of several species that produce mycotoxins responsible for various animal diseases.

The genus *Aspergillus* is widely distributed in various habitats and can grow on a wide range of substrates. *Aspergillus niger* is usually found as a saprophyte growing on stored grain, dead leaves, compost piles and other decaying vegetation. The conidiophores have smooth walls and are hyaline or dark near the vesicle. Hussain *et al*. [23] reported the pathogenecity of species belonging to the genera, *Fusarium* and *Aspergillus* to be highly infective by producing mycotoxins that are involved in retarding seedlings growth of maize. *Botyodiplodia theobromae* has been reported to be able to colonize many plants as both a pathogen and an endophyte [24,25]. *B*. *theobromae* is a common rot fungus that causes great economic losses in the cultivation of various crops such as banana, cocoa, yam and mango [26]. *Botryodiplodia theobromae* has also been reported to cause bark canker and die-back of pear trees in India [27].

Rhizopus stolonifer is usually considered as the most important species of the genus *Rhizopus*. *R. stolonifer* is a plant pathogen and in most plant hosts, it is a weak parasite. Plant disease symptoms associated with *R. stolonifer* are watery areas that are rapidly covered by coarse, copious gray cottony colony with black globules (sporangia) at the tips [28].

Fungal organisms contain cell wall degrading enzyme (CWDE) which consists of laccases and peroxidases. These enzymes are used for the degradation of glycoside hydrolases and lignin. Fungi secrete pectinases, cellulases and hemicellulases for the degradation of pectin, cellulose and hemicellulose respectively [29]. Plant immune responses are deactivated by effector proteins and this facilitates the colonization of the plant host by the pathogen [30]. Fungal effector proteins are of two types: those secreted in between the plant cells (apoplastic) and those accumulated inside the plant cells where the membranes are situated (cytoplasmic) [31].

Reddish leaf spots on cowpea seedlings infected by *Xanthomonas phaseoli* was earlier reported by Manyangarirwa *et al*. [32]. Claudius-Cole *et al*. [33] reported bacteria blight caused by *Xanthomonas axonopodis p*v. *vignicola* on cowpea.*Xanthomonas campestris* pv. *vignicola* was reported by Okechukwu *et al*. [34] to be responsible for bacterial blight disease and post-emergence seedling mortality in cowpea. *Xanthomonas* is a well-known genus of bacterial plant pathogens whose members cause a variety of diseases in economically important crops worldwide [35]. Many strains of *Xanthomonas* produce the extracellular polysaccharide, xanthan which is used in the pharmaceutical and food industries. This polymer is also believed to be involved in a number of phases involved in the bacterial disease cycle.

4. CONCLUSION

Plant pathogens cause diseases which reduce both quality and quantity of plant products obtained by farmers after harvest. The main objective of plant pathology is to prevent diseases and widespread outbreak of destructive diseases. The test organisms caused various degrees of deterioration in the inoculated *Vigna unguiculata* seedlings with *Fusarium oxysporum* causing the highest damage. This means that all the organisms were pathogenic on *V. unguiculata*. Most of the organisms used in this study are seed-borne pathogens. Seed-borne microorganisms can be transmitted into healthy young seedlings through inoculation or through dispersal of spores and other ways. Dormant fungal spores that over-wintered from one farming season to another can spread to healthy plants in the field thereby causing destruction. Poor storage of seeds may create a favourable environment for the growth of pathogens and infected seeds from a previous harvest may lead to disease spread. Therefore, adequate seed testing and regular seed treatment is of utmost importance so as to ensure that growers produce healthy plant products and also to help maintain sustainable production of cowpea for the teaming population of Africa and the world at large since this species is a very good and cheap source of plant protein. Though most of the chemicals and fungicides used in the treatment of seeds before planting and in the control of these organisms are so expensive and not affordable by most subsistence farmers, use of plant ash (potash) in dusting these seeds before planting and use of pepper (*Capsicum annum*) in storing seeds goes a long way in protecting them from pathogens infestation. Farmers are advised to employ different strategies such as cultural practices, application of bio-control agents, sowing pathogen-free seeds and planting of cowpea genotypes with resistance to the pathogen among others in order to control these diseases.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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