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Pathogenicity and cell wall-degrading enzyme activities of some fungal isolates from cowpea (*Vigna unguiculata* [L] Walp)

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Abstract

Nine fungal species isolated from cowpea seeds were used as inocula on four cowpea varieties commonly distributed to farmers in Ilorin, Kwara state, Nigeria by the National Seed Service, a subsidiary of the Federal Ministry of Agriculture and Natural Resources. The effects of fungi on germinability and seedling health were determined using seedling symptom test. Two of the virulent species were screened for the production of cell wall degrading enzymes using viscometric method. All the fungi reduced germination rate in all the cowpea varieties and different types of seedling symptoms were noted for the fungi. The symptoms included seed rot, chlorotic leaf development, stunted growth etc. Production of pectinases and cellulases by *Aspergillus flavus* and *Penicillum* sp. was observed and the virulence of the two organisms could be attributed to the activities of these cell wall degrading enzymes.

Keywords: Cowpea, Seed mycoflora, *Aspergillus* sp, *Penicillium* sp, Pectinases and Cellulases

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INTRODUCTION

Cowpea, *Vigna unguiculata* (L) Walp (Fabaceae), has always been an important grain legume in tropical countries especially Nigeria and a veritable source of dietary protein for the teeming population of human and livestock[1,2]. The dry seed consists of about 25% protein and 67% carbohydrate[3]. In the 1970's, about 94% of the total world crop was produced in Africa[4]. The situation remains much the same today but the optimization of production, which is still grossly at the subsistence level in this region continues to be hampered by pests[5,6,7] and diseases[8,9]. Some of the diseases of cowpea are known to be caused by seed-borne pathogens most of which are fungi[6,8,10,11].

Many phytopathogenic fungi and bacteria have long been known to produce enzymes capable of hydrolyzing the polymeric carbohydrate constituent of higher plants cell wall. This factor might be responsible for the penetration of the fungi into the cowpea seeds[12]. In Nigeria, there is still a dearth of information on cowpea seed-borne fungi and their significance in disease development. Thus, the present study aimed at determining the pathogenicity of some cowpea seed-borne fungi and screening some of the virulent ones for their cell wall degrading enzyme activities.

MATERIALS AND METHODS

Source of organisms

Nine fungal species namely; *Alternaria* sp., *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium* sp., *Fusarium oxysporum*, *Fusarium semitectum*, *Fusarium solani*, *Fusarium* sp. and *Penicillum* sp. isolated on Potato Dextrose Agar (PDA) by the standard procedure of culturing and subculturing, from cowpea seeds were used for the study. The cowpea seeds were obtained from the National Seed Service Ilorin, Kwara state, Nigeria and were stored under refrigeration (4 °C) until needed for use.

Preparation of inocula from the organisms

Spore suspension was prepared from highly sporulating organisms such as *Aspergillus flavus*, and *Aspergillus niger* by washing the spores from agar slants with sterile water. The number of spores was adjusted to $5x10^5$ spores/ml by dilution. Mycelia suspensions of other isolates were prepared by punching several small pieces from 10 day old cultures using 5mm cork borer. The mycelia discs (5mm) were comminuted in 150ml sterile water[13] for 10 seconds in a blender.

Inoculation of seeds

To study the effect of seed borne fungi on germination and seedling growth, the method of Baggett and Fraizer[14] in which seeds were coated with fungal cultures was adopted. Surface-sterilized seeds were mixed with the individual inocula in corked conical flasks for approximately one hour using mechanical shaker. After shaking, the seeds were left to stand in the inoculum for 8-10 hours. The seeds were then dried overnight on sterile paper towel.

Effect of organisms on seed germination and seedling health

Inoculated seeds were sown in small plastic pots filled with steam- sterilized soil. Sterile water was used for wetting the soil while being kept at room temperature for 6-10 days. The pots were later examined for germination and disease symptoms on emerging seedlings.

Cell wall degrading enzymes assay

Two demonstrably virulent fungal isolates of the nine isolates were selected for the assay of cell wall degrading enzyme enzyme activities. The selected organisms were prepared on PDA slants for 3 days at 28 ± 2 °C. The cultures were then separately washed with sterile water to dislodge the spores and the resulting spore suspension was used for preparing enzyme filtrate.

Preparation of enzyme

Seeds of cowpea cultivar (ITA 90-22-2K) susceptible to attack by the virulent fungi were ground in a blender. The ground seeds were sterilized in an autoclave at 15 lb pressure for 15 minutes to get rid of all seed-borne fungi and other contaminants. Two separate 50g of the sterile ground seeds was moistened with 50ml of each inoculum separately and the mixture incubated for 3-5 days at about 28±2°C. Sterile water (150ml) was later added in each case and the mixture agitated for 30 minutes with

mechanical shaker. The resulting mixture was filtered using sterile Whatman No. 1 filter paper. The filtrates were kept under refrigeration until needed.

Enzyme assay

Viscometric method was used in measuring the activity of the enzymes. Viscosity was determined using the method of Endo[15]. The reaction mixture contained 15ml of carboxymethyl cellulose (CMC) (1% w/v) in citrate phosphate buffer (pH 5.5) and 15ml of enzyme filtrate for cellulose assay. For pectinase assay, the mixture contained 15ml pectin (2% w/v) in citrate phosphate buffer (pH 5.5) and 5ml of the enzyme filtrate. Measurements were made in glass viscometer after the mixtures have been incubated for 2 hours at 30°C. The enzymatic activities were expressed in terms of percentage viscosity change. The reducing rate of viscosity was calculated from the following equation:

$$A = \frac{Vo - Vt}{Vo - Vs} \times 100$$

Where, Vo = flow time in seconds of pectin/cellulose + inactivated enzyme. Vt = flow time in seconds of pectin/cellulose + active enzyme. Vs = flow time in seconds of solvent (water) + inactive enzyme. One unit of viscosity reducing activity (VRU) is defined as the quantity of enzyme necessary for 50% viscosity change of 20ml of the reaction mixture at 30°C. Data collected from repeated experiments were pooled and analyzed for variance at 5% level of significance.

RESULTS AND DISCUSSION

Pathogenic effects of seed mycoflora

All the fungi tested reduced percentage seed germination and average height of seedling of the four cowpea varieties compared with the control as shown in Figures 1 and 2.

Among seed borne organisms, fungi cause maximum seed damage, which include reduced germination and vigour[16]. Reduced germinability of seeds may be attributed to damaged embryos from deep seated infection of seeds. All the fungi used in this study induced disease symptoms on germinating seedlings. The symptoms observed ranged from chlorosis to necrotic spots development on the leaves stems and roots while the control remained healthy (Plate 1).

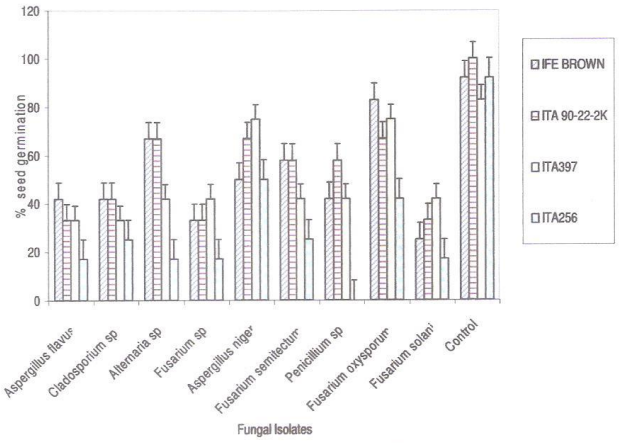


Fig 1: Comparative percentage seed germination in some cowpea cultivarsafter seed inoculation with some seed mycoflora

Error bars are standard errors of the data

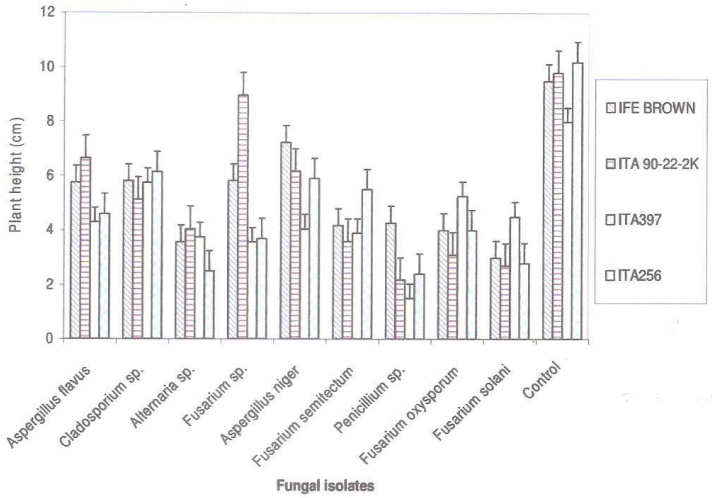


Fig 2: Comparative effect of seed inoculation with some fungal isolates on mean height of cowpea seedlings
Error bars are standard errors of the data

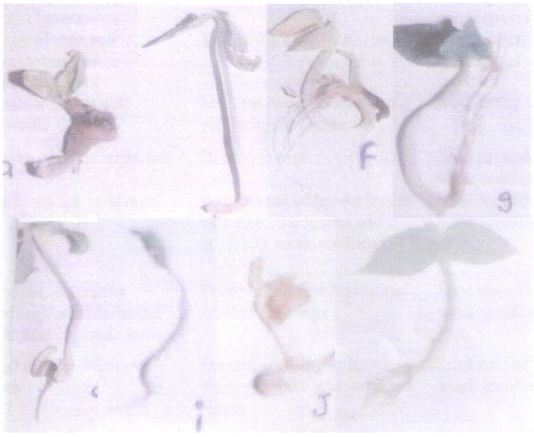


Plate 1: Manifestation of different disease symptoms in germinating cowpea seedlings after infection of the seeds with selected fungal isolates from cowpea seeds.
Top Row from left: *Aspergillus flavus, Cladosporium* sp, *Alternaria* sp. and *Penicillium* sp.
Bottom Row from left: *Fusarium semitectum, F. oxysporium, F. solani* and Sterile Distilled water only (Control).

The impact of fungal inocula on germinating seeds and seedling health which includes seed rot, stunted seedlings and yellowing of leaves is outlined on Table 1.

Many seed borne fungi on cowpea in India have been reported to reduce seed germination and produce symptoms on infected seedlings[17]. It was further observed[17] that fungi such as *Aspergillus flavus* and *Fusarium solani* were associated with damage to plumule, radicle and hypocotyl of germinating seedlings. This kind of observation explains the reducing effects of such fungi, which were also used in this study on seedling development.

Cell wall degrading enzyme activities of some test fungi

The results of the assay for pectolytic and cellulolytic enzyme production by two of the virulent fungi (*Aspergillus flavus* and *Penicillium sp*) are presented in Table 2. A significantly ($P \leq 0.05$) higher volume of enzyme preparation from *A. flavus* than that of *Penicillium sp* was required to bring about 50% viscosity changes in carboxymethyl cellulose (CMC) indicating a higher cellulase activity in the preparation from *Penicillium sp.* Conversely, pectinase activitiy of enzyme preparation from *A. flavus* was significantly higher than that of *Penicillium sp.* Nowithstanding the difference in quantities, the ability of these moulds to produce cellulases and pectinases explains their virulence. The cell wall-degrading enzymes must have facilitated the penetration of the fungi and hence their ability to cause deep seated infection of the seeds and consequent symptom manifestation in the seedlings.

Table 1: Pathogenic effect of fungal isolates on cowpea seedlings

FUNGAL TREATMENT	IFE BROWN	ITA 90-22-2K	ITA 397	ITA 256
Aspergillus flavus	Weak seedling with decayed root and yellowing of first leaves	Root rot and necrotic spots on leaves	Cotyledons wilt quickly with signs of green spores	Seed rot
Cladosporium sp.	Decay of root and development of wrinkled leaves	Yellowing of leaves	Loss of first leaves by seedling	Decay of root system
Alternaria sp.	Death of leaves and chlorosis	Seed rot decay of root and yellowing of leaves	Root decay	Seed rot
Fusarium sp.	Leaves become wrinkled	Damaged root	Seed rot and weak seedlings	Seed rot and weak seedling
Aspergillus niger	Seedlings are weakened	Appearance of brown lesion on roots, first leaves and generally weak seedlings	Seed rot and weak seedlings	Seed rot and weak seedling
Fusarium semitectum	Seed rot, unhealthy seedlings with damaged roots and chlorotic leaves	Dropping of first leaves and stunted seedlings	Seed rot and weak seedlings	Seed rot and stunted seedlings
Penicillium sp.	Seed rot and damaged roots	Stunted seedling wilt damaged roots and wrinkled leaves	Seed rot and weak seedlings	Seed rot
Fusarium oxysporum	Seed rot and stunted growth	Seed rot, stunted seedling loss of first leaves	Seed rot and appearance of brown lesion on roots	Seed rot, root damage and stunted seedling
Fusarium solani	Seed rot and Stunted seedlings	Seed rot, damage to roots, dark spots on cotyledons	Stunted seedlings and seed rot	Seed rot
Control (No fungi)	Healthy seedlings	Healthy seedlings	Healthy seedlings	Healthy seedlings

Table 2: Pectolytic and cellulolytic activities of enzyme preparations from *Aspergillus flavus* and *Penicillium* sp

Fungal Isolate	Enzyme activity (VRU) (ml)	
	Cellulase	Pectinase
Aspergillus flavus	91.20 ± 2.02 b	39.02 ± 1.10 a
Penicillium sp	80.23 ± 2.13 a	51.50 ± 1.22 b

V.R.U (Viscosity Reducing Unit) is defined as quantity of enzyme necessary for 50% viscosity change of 20 ml of the reaction mixture at 30°C. Means ± std. dev. followed by different letter in a column are significantly different at P< 0.05.

In general, the seeds of all the four varieties of cowpea used for this study were to varying extent susceptible to fungal infections and they exhibited the ability to carry over such infections to the seedling stage. The observations made in this study have further underscored the need for regular seed testing and seed treatments with effective protective and systemic fungicidal preparations, by the seed services, before disbursement to growers.

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