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Full Length Research Paper

Effects of *Fusarium verticilloides*, its metabolites and neem leaf extract on germination and vigour indices of maize (*Zea mays* L.)

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Maize (*Zea mays* L.) seed samples were collected from three markets in Minna, Niger State to investigate the effect of *Fusarium verticilloides*, its metabolites and neem (*Azadirachta indica*) leaf ethanolic extract on germination of the seeds and seedling vigour. Among the fungi genera isolated from the seeds samples were *Aspergillus* spp. (41.18%), *Fusarium* spp. (29.41%) and *Rhizopus* spp. (23.53%). *F. verticilloides* metabolite was extracted using dichloromethane and phosphoric acid (10:1) while powdered neem leaf was extracted with ethanol for 72 h. The experiment, which was made up of eight treatments and three replicates, was set up in a germination chamber. At 9 days after sowing (9 DAS), the seeds applied with combined *F. verticilloides* mycelia and their metabolites had the least germination percentage (30%), the highest rot-index (3.5) and the least seedling vigour (0.93). The seeds applied with neem extract only had the highest germination percentage of 100%, the least rot index (0.5) and seedling vigour index (5.4). Though neem leaf extract proved to be fungitoxic against *F. verticilloides,* further investigation on the pathogenic mode of action of fusariotoxin on germination and growth of cereal crops on the field is necessary.

Key words: *Fusarium verticilloides*, metabolites, neem leaf extract, germination, vigour indices, maize.

INTRODUCTION

Maize (*Zea mays* L.) is ranked second to wheat among the world cereal crops and the most important cereal crop in sub-saharan Africa (FAO, 1996). It has been a major source of energy for humans and farm animals; and it is used for producing several non-food products (IITA, 2006). Seed-borne pathogenic fungi have been reported to cause field and market losses of maize (Onifade, 2000). Gbodi (1986) found that the most important field fungi of maize in Africa are *Fusarium* spp. and found to be one of the limiting factors in pre- and postharvest stages of maize production. *Fusarium verticilloides* (Sacc.) Nirenberg was previously known as *Fusarium moniliforme* Sheldon. It is known to be mycotoxigenic and characterized by microconidia being produced in chains. Specifically reported is the cob-rot of maize caused by *F. verticilloides* and *F. gramineum* which often led to reduction in quality of the infected seeds (Singh, 2005). Christensen and Kaufnaiann (1988) reported estrogenic fusariotoxins such as trichothecens, zearalenone, and zearalenol that could adversely affect seeds, human and animal health.

Sowing of fungi-free seeds might be the best management option but most farmers in Nigeria and other developing countries find it difficult to obtain certified pathogenic fungi-free or resistant seeds. Those seeds that look healthy externally might be internally infected with pathogenic fungi. Though application of synthetic seeds dressing fungicides were reported to be effective against these seed-borne fungi, they are unaffordable by most resource-poor farmers (Salako, 2002). Additionally, synthetic fungicides such as methyl bromide or benomyl were reported to produce toxic residue on the environment (Anastasiah et al., 2001) and their continuous appli-

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Table 1. Incidence of fungi in maize seeds samples collected from three markets in Minna, Niger State.

Fungus	Incidence			Total Incidence	Rel. frequency (species)	Rel. frequency (genera)
	Bosso market	Central market	Tunga market			
Aspergillus flavus	2/6	-	1/4	3/17	17.65	41.18
Aspergillus niger	1/6	2/7	-	3/17	17.65	
Aspergillus glacus	-	1/7	-	1/17	5.88	
Fusarium verticilloides	1/6	1/7	-	2/17	11.76	29.41
Fusarium nivale	-	1/7	2/4	3/17	17.65	
Neurospora sitophillus	1/6	-	-	1/17	5.88	5.88
Rhizopus nigricans	1/6	2/7	1/4	4/17	23.53	23.53
Total incidence (%)	6 (35.29)	7 (41.18)	4 (23.53)	17	100	100

cation could lead to the development of resistant strains (Mehrota and Aggrawal, 2005).

There are several reports on animal and human mycotoxicoses but reports are scanty on the effects of botanicals on pathogenesis of seed moulds and mycotoxin with respect to seeds viability and seedlings vigour. This study was conducted to confirm the fungi genera associated with maize seeds in Minna and determine the effect of neem leaf extracts on *F. verticilloides,* its metabolites on germination and vigour indices of maize seeds.

MATERIALS AND METHODS

Maize seed samples were obtained from Bosso, Tunga and central markets in Minna, Niger state (Lat. 9° 37' N of the equator and Long. 6° 33' E of the Greenwich Meridian) during the wet of August, 2007. The labeled samples were stored in a refrigerator 4°C.

Seed-borne fungi isolation and identification

The seeds samples from each market were aseptically placed in Petri dishes containing an autoclaved Sabouraud Dextrose Agar (SDA), incubated in lamina hood at 28°C and examined from 5 - 9 days. To identify the fungi, mycelia speck from each colony were aseptically placed on a slide, stained with lactophenol blue, covered with slips and viewed under microscope (x 40). The identification was accomplished using fungi catalogue in the Microbiology Department in F.U.T., Minna and also by comparing with the confirmed strains by CABI Biosciences Identification Services (IMI 392668) maintained on maize seeds kept in Biochemistry Laboratory of the University. *F. verticilloides* isolates were aseptically subcultured in other to obtain its pure culture.

Neem leaf extraction

Healthy *Azadirachta indica* leaves were collected from Bosso campus of Federal University Technology (FUT), Minna. The shade-dried neem leaves were crushed with mortar and pestle and blended into powder using an electric blender (National Mx 319 M). Three hundred grams of the powder were cold extracted with 900 ml ethanol for 72 h in a conical flask. The extracts was sieved into beaker using a cheesecloth and evaporated to dryness using Heildulph rotary evaporator at 55°C. The stock extract was kept in a refrigerator at 4°C until used.

Extraction of the fungus metabolite

Five hundred grams of confirmed fungi-free maize seeds (Swan-1-SR) obtained from National Cereal Research Institute, Badeggi, Niger State was measured into eight Buchuner flasks and added with 200 ml of distilled water, mixed thoroughly and left overnight for moisture equilibration (Gbodi, 1986). They were sterilized by autoclaving at 121°C for 15 min and then cooled. Fifteen grams of dissolved biomass of pure culture of *F. verticilloides* were added to the maize samples by under aseptic condition and left in the inoculation hood for 21 days to allow for massive growth of the fungus and the synthesis of the secondary metabolites.

The extraction of mycotoxin was by adding 500 ml dichloromethane (84.93 g/mol) and 50 ml of 1 M H_2SO_4 to 500 g of maize seeds in the Buchuner flask. It was left to stand for 30 min and blended thoroughly. The homogenate was then separated using suction pump filtering process. The extracted metabolite was concentrated in a beaker on a heating water bath at 55°C. As the solvent distilled off, the residue was placed in vial and kept in a refrigerator at 25°C until used for the maize seeds treatment.

Seed germination procedure, data collection and analysis

Certified fungi-free maize seeds were used for the experiment. This was through screening of the seeds and disinfestations with sodium hypochlorate. Ten treated seeds were sown per plate. The eight treatments (Table 2) were placed in a completely randomized design (CRD) with three replicates in a germination chamber in the laboratory of the Crop Production Department. Germination % at 5, 7 and 9 days after sowing (DAS) was recorded. Also recorded were the length of hypocotyls, length of roots, root indices and seedling vigour index. The vigour index was calculated by modified Randahawa et al. (1985) method given by the formula:

$$V.I. = (HL + RL) \times \%G$$

Where V.I. = Vigour index, HL = hypocotyl length, RL = root length, and %G = % germinated. The seedling vigour index was subjected to analysis of variance and means separated using Duncan Multiple Range Test (DMRT) at 5% level of probability.

RESULTS

Table 1 shows the incidence and percentage occurrence of fungal colonies isolated from maize seed samples from three markets in Minna. Seed samples from central markets had the highest incidence of fungi species (41.18%)

Table 2. Effects of *F. verticilloides,* its metabolites and neem leaf extract on germination of maize seeds, their hypocotyl and root length.

Treatment	% Germination			Hypocotyl length (cm)			Root length (cm)		
	5 DAS	7 DAS	9 DAS	5 DAS	7 DAS	9 DAS	5 DAS	7DAS	9 DAS
*Fus** mycelia	30	30	40	2.0	2.3	2.4	0.6	0.9	1.2
Fus. met**	20	30	30	1.8	1.9	2.1	0.6	1.0	1.1
Fus. mycelia +*Fus.* met.	10	20	30	1.2	1.4	1.6	0.5	0.8	1.0
*Fus.*mycelia+ neem extract	40	60	70	2.1	2.4	2.5	0.7	1.0	1.3
Fus. met. + neem extract	30	50	60	2.0	2.5	2.7	0.7	1.1	1.3
Fus. mycelia+*Fus.* met.+ neem extract	30	50	60	1.9	2.4	2.6	0.6	1.0	1.2
Neem extract	40	80	100	2.8	3.1	3.9	0.7	1.2	1.4
Control (distilled water)	50	70	90	2.9	3.3	3.8	0.8	1.3	1.5

F. verticilloides; * metabolite.

Table 3. Effects of *F. verticilloides,* its metabolites and neem leaf extraction % seedling vigour, rot and vigour indices.

Treatment	% Seedling mortality		Rot-index			Vigour index (x 100)		
	7 DAS	9 DAS	5 DAS	7 DAS	9 DAS	5 DAS	7 DAS	9 DAS
*Fus.** mycelia	10	30	2.5	3.0	3.0	0.78^{d} *	0.87^{d}	1.44^{c}
Fus. met.**	10	20	2.0	2.0	2.0	0.48^{e}	0.87^{d}	0.99^{cd}
Fus. mycelia +*Fus.* met.	30	30	2.0	2.5	3.0	0.17^{f}	0.44^{e}	0.93^{d}
*Fus.*mycelia+ neem extract	10	10	0.5	1.0	1.5	1.12^{c}	2.10^{b}	2.66^{b}
Fus. met. + neem extract	10	20	0.5	1.0	1.5	0.81^{d}	1.80^{bc}	2.40^{b}
*Fus.*mycelia+*Fus.* met.+ neem extract	10	20	1.0	1.0	1.5	0.75^{d}	1.70^{c}	2.28^{b}
Neem extract	0	0	0	0	1.0	1.40^{b}	3.44^{a}	5.30^{a}
Control (distilled water)	0	10	0	0	0	1.85^{a}	3.22^{a}	4.77^{a}

*Column means followed by common letter(s) are not significantly different (P ≥ 0.05) by DMRT.
***F. verticilloides*; *** metabolite.

while the least was found at Tunga market (23.53%). *Aspergillus* spp. had the highest occurrence (30.18%), while *Neurospora* spp. had the least (5.88%).

Table 2 shows the effects of the various treatments on germination, hypocotyls and root length. It was observed that the adverse effect of *Fusarium* mycelia was less than that of its metabolite contamination. At 9 DAS, the germination percentage of the *Fusarium* infected-seeds applied with neem extracts increased by 20%. Seeds applied with neem extract alone had 100% germination which was 10% higher than that of the seeds in the control plates. Also the highest hypocotyl and root lengths (4.9 and 1.4 cm) were observed under this treatment. The germination of the seeds applied with the metabolites combined with neem extracts also increased by 20%. On the 9 DAS, seeds applied with fungi metabolites and combined *Fusarium* and its metabolites had the highest seedling mortality (30%) while the seeds applied with neem extract recorded no dead seedling. At 5 and 7 DAS, the rot-index on the seeds and hypocotyl under various treatments followed the same pattern.

The seeds that received *F.* mycelia only had the highest rot-indices. This was closely followed by the seeds that were applied with the combined *Fusarium* and its metabolites. This indicated that more rottenness might result from mycelia infection than from its metabolites.

The rot-index of seeds applied with neem extract or distilled water was zero. On the 9 DAS, the seeds applied with *F.* mycelia and combined *Fusarium* and its metabolites had the highest and the same rot index of 3.0. This might be due to synergist interaction of the pathogenic *F.* mycelia and toxic metabolite. Table 3 shows the seedling vigour index of seeds applied with various treatment of *Fusarium*, its metabolites and neem extracts.

At 5 DAS, the highest vigour index (1.85) was observed from the seeds in the control plate and was significantly higher than the seeds applied with neem extract (1.40). On the 7 DAS and 9 DAS, those seeds applied with neem leaf extracts, showed the highest vigour index. The least vigour index was from those applied with *F. verticilloides* mycelia and its metabolites.

DISCUSSION

The identified moulds on Maize seeds was in line with the report of Moss and Smith (1995) that *Fusarium, Aspergillus and Penicillium* species are considered to be

the most significant toxigenic moulds of cereal crops. The factors influencing the development of seed-borne fungi include the moisture content of the seeds or grains, prevailing temperature, storage period and degree of seed invasion with the pathogen. Others are level of host genetic resistance, activities of insects and mites and amount of foreign materials in the seeds lot (Miller and Trenholen, 1994).

It has been found in this study that *F. verticilloides* singly or in combination with its metabolites reduces germination percentage and seedling vigour of maize seeds. Mehrotra and Aggarwal (2003) reported that most pathogenic seed-borne hyphae progressively ramify through the protoplast cells as the cell membranes are disrupted. *Fusarium* metabolites could bring about rapid softening and necrosis of tissues. This might be due to presence of trichothecenes, deoxynivalenol and fuminisins B_1, B_2 and B_3 present in the metabolites (Marassas et al., 1995). *Fusarium* spp. and their metabolites have been implicated in seed viability, wilting of plants, stem flaccidity and leave necrosis (Mehrotra and Aggarwal, 2003).

The increase in germination of the mycelia-inoculated seeds applied with the neem extracts might be due to fungitoxicity of neem extract or their stimulatory effects that enhance germination of seeds. Neem extracts might be antidotal to *Fusarium* metabolites thus reducing its toxicity. The report of Enikuomehin and Peters (2001) confirmed that the cold water extract from the leaves of *A. indica,* sprayed fortnightly have the ability to reduce the incidence and severity of the *Cercospora* leaf spot and leaf blight disease of three sesame varieties. Their results indicated the prospect of these affordable natural fungicides in the control of field disease of sesame. Yaradua (2007) reported *Fusarium* spp. to be among the pathogenic fungi that neem extract is fungitoxic to. The author attributed the fungitoxic efficacy of neem to several biologically active chemical neem components such as tritepernoids, salanin, nimbolinin and azadirachtin groups. Srivastava and Kalman (1997) tested neem seed cake, seed and fruit extract, seed kernel powder and seed oil and confirmed their utility to control a wide spectrum of fungal pathogens like *Pyricularia grisea* causing blast disease of rice.

Conclusion

Most of the maize seeds sold in Minna markets were internally borne with moulds. Seeds or grains sold in such markets should be inspected periodically and certified to be mould free by an appropriate agency. The presence of *F. verticilloides* singly or in combination with its metabolites in maize seeds reduced germination percentage and seedling vigour. The adverse effect of *F. verticilloides* mycelia was less severe on germination than its metabolites. It was further observed that neem ethanolic extract applied to maize seeds infected with the fungus or its metabolites witnessed improved germination percentage and seedling vigour. Neem leaf is easily renewable than other parts and than synthetic fungicides and their extracts proved to be fungitoxic to *F. verticilloides.* Through interdisciplinary efforts and provision of modern tools and facilities, bioactive ingredients in the crude extracts could be isolated and purified so that standard commercial fungicide could be formulated from it. A well-prepared neem extract formulation will be easier for the resource-poor farmers to afford and renew than synthetic seed-dressing fungicides. Further investigation on the mode of action of the extract on the fungus and the pathogenic action of purified fusariotoxin on germination and growth of cereal crops in the laboratory and on the field are imperative.

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