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Zearalenone Production by Naturally Occurring *Fusarium Species* on Maize, Wheat and Soybeans from Nigeria

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ABSTRACT

The occurrence of *Fusarium* species on maize, wheat and soybean in store in Southwestern Nigeria was determined followed by the study of the Zearalenone producing capacity of the isolates on soybean using wheat as standard under tested conditions. One hundred and seventy-three isolates representing 12 *Fusarium* species were isolated and identified as *F. acuminatum, F. compactum, F. culmorum, F. equiseti, F. graminearum, F. lateritium, F. poae, F. proliferatum, F. semitectum, F. sporotrichioides, F. subglutinans* and *F. verticillioides*. *F. sporotrichioides* recorded the highest isolation frequency (96%) on maize, *F. graminearum* (100%) on wheat and *F. semitectum* (80%) on soybeans. Zearalenone determination using the Veratox® ELISA quantitative test kit showed that *F. equiseti* (Corda) Sacc. IMI 393764 ($S_{os}1$) produced the highest zearalenone on soybean (27.2µg kg^{-1}) under the tested conditions while no zearalenone was detected for *F. poae* ($S_{ak}1$) and *F. subglutinans* ($S_{ek}2$). All tested isolates produced ZEA on wheat from day 12. Kinetic study of zearalenone production showed that this toxin was not detected at day 6. The levels of zearalenone produced between day 12 and 18 at 30°C tripled on an average while at 25°C and between the 24th and 30th days, levels almost doubled. This is the first report of *F. lateritium* occurrence on wheat in Nigeria and zearalenone production by *F. equiseti* (Corda) Sacc. IMI 393764 on soybean.

Key Words: Zearalenone, *Fusarium*, maize, wheat, soybean.

INTRODUCTION

The occurrences of *Fusarium* species on maize, wheat and soybean have been reported (Ishii et al 1974; El-Kady and El-Maraghy 1982; Vaamonde et al 1987; Langseth 1998; Park et al 1999; Fandohan et al 2003; Adejumo et al 2007; Broggi et al 2007). These phytopathogenic field-to-store fungi contaminate the food materials on field, indeed accompany them to their storage points and may express the capacity to produce associated mycotoxins thus leading to a progressive deterioration in food quality (Aziz et al 1998). The resulting alarming economic loss reflected as reduced productivity, direct commodity loss, livestock losses due to death and lower growth rates, and human illness has sustained research interests in the investigation of toxin producing capabilities of *Fusarium* species on food materials (Kommedahl et al 1979; Sydenham et al 1988; ApSimon et al 1990; Smith et al 1997; Geraldo et al 2006; Seeling et al 2006).

In Nigeria, *Zea mays* L. (maize) ranks third as the main staple food and industrial cash crop while *Triticum aestivum* L. (wheat), which is not widely grown, and *Glycine max* L. Merill (soybean) serve as additional food and feed supplements for humans and livestock. Due to the grossly inadequate storage facilities, storage duration, temperature and humidity levels as well as other climatic factors surrounding food production in Nigeria several losses have been recorded and health of consumers affected.

Zearalenone (ZEA), also referred to as F-2 toxin is a potent resorcyclic acid lactone metabolite that exhibits stability at high temperatures and estrogenic activity on mammals (Mirocha and Christensen 1974; Ryu et al 1999). The application of maize and wheat as substrates for ZEA production is on record and *F. acuminatum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. sporotrichioides*, *F. semitectum*, etc have been implicated (O'Neill et al 1983; Jiménez et al 1996; Martins and Martins 2002; Geraldo et al 2006). However, there is paucity of information on ZEA production on soybeans though it has served as substrate for growth of some fusaria (Richardson et al 1985; Vaamonde et al 1987).

El-Kady and El-Maraghy (1982) recorded that strains of *F. equiseti*, *F. oxysporum* and *F. moniliforme* from Egypt produced ZEA on optimized medium while sorghum-based traditional malt wort and beer in Botswana have been shown to contain some levels of ZEA (Nkwe et al 2005). In Nigeria, Adejumo et al (2007) reported the occurrence of high ZEA levels in maize samples from the Southwest valuing 779µg kg^{-1} with a mean of 49µg kg^{-1}.

Though contamination of food materials by *Fusarium* species does not necessarily denote toxin production, it is on record that toxin production is influenced by physicochemical factors such as the incubation temperatures, available moisture content and storage duration (Milano and Lopez 1991; Jiménez

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et al 1996; Bresler et al 1998; Martins and Martins 2002). This study which has both agricultural and medical significance was therefore aimed at investigating the occurrence of *Fusarium* species contaminating maize, wheat and soybean in store in Southwestern Nigeria, a tropical country, and determining the ZEA-production capacity of tested isolates on soybean using wheat as standard under tested conditions.

MATERIALS AND METHODS

Samples

Fifty samples each of stored maize, wheat and soybean grains were bought from various retailers in local markets situated in 5 states within Southwestern Nigeria namely Lagos, Ogun, Ondo, Osun and Ekiti states. These samples which weighed 200g each were kept in clean labeled polyethylene bags at 4^{o}C until further analyses were to be carried out.

Isolation and Identification

The dilution plating method of Dubey and Maheshwari (2005) with modifications was used in the isolation of fungi. Random batches of 5g were taken in duplicates from each sample lot of seeds, surface sterilized with 1% NaOCl for 2 minutes, rinsed thrice in sterile distilled water and blended to fine particles in a high-speed blender with 195ml of sterile 0.1% peptone water. Serial dilutions were carried out in the same diluents and 0.1ml of each dilution was spread (duplicate) on acidified potato dextrose agar (PDA) fortified with 50mg chloramphenicol, (Difco Laboratories, Detroit, MI) in Petri dishes. Incubation was at 25^{o}C for 5days.

Pure cultures of *Fusarium* isolates were established using the single spore technique of Nelson et al. (1983). Single-spored cultures were subsequently placed on PDA and carnation leaf agar media and incubated at 25^{o}C under fluorescent and UV lamps (uv-c G15T8) for 12 h per day to promote fungal growth and sporulation (Bosch and Mirocha 1992). Species identifications were determined following the descriptions in the manual of Nelson et al (1983) and Leslie and Summerell (2006). Stock cultures were stored in sterilized soil (Park et al 1999) and recovered on PDA as needed. An isolate with peculiar characteristics was deposited at CABI Bioscience, UK, and confirmed as *Fusarium equiseti* (Corda) Sacc. IMI 393764. The isolation frequency (*fr*) and relative density (*Rd*) of isolates were calculated as follows (Broggi et al 2007):

$$fr(\%) = (n / N) \times 100 \qquad Rd(\%) = (n_i / N_i) \times 100$$

Where, n is the number of samples where a species of *Fusarium* occurred; N is the total number of samples; n_i is the number of isolates of a species; N_i is the total number of fusaria isolated. The asymptotic tests for equality of proportions and Fischer exact test were used to compare the *Rd* and analyze possible differences in the *fr* of fungal species, respectively, using the Statistix 4.1 package.

One randomly selected isolate of each species that occurred on soybean were used for the study of ZEA production kinetics and they were; *F. acuminatum* ($S_{ab}3$), *F. culmorum* ($S_{lg}1$), *F. equiseti* (Corda) Sacc. IMI 393764 ($S_{os}1$), *F. poae* ($S_{ak}1$), *F. semitectum* ($S_{lg}3$), and *F. subglutinans* ($S_{ek}2$).

Substrate preparation and inoculation for "In Vitro" ZEA production

Substrates for ZEA production contained no preformed ZEA and were prepared by modifying the procedures of Martins and Martins (2002) and Smith et al. (2004). Fifty grams each of whole wheat and soybeans were weighed into separate 250ml conical flasks and 20ml of distilled water was added to each flask. Water activities were adjusted to 0.95 followed by substrate sterilization by autoclaving. After an overnight cooling period, each flask was inoculated with 5mm disc of 7day old cultures. Flasks were shaken daily for the first 3days.

Inoculated flasks were incubated at temperatures of 25 and 30^{o}C for 30days. Cultures were examined for pH changes and ZEA at selected incubation times (6, 12, 18, 24 and 30days). All experiments were duplicated.

ZEA determination

Procedures in 48-well commercial Veratox® Enzyme-linked Immunosorbent Assay (ELISA) quantitative test kits for ZEA (Product number 8110, Lot number 4127) manufactured by Neogen Corporation, Lansing MI, USA, were employed for the extraction, screening and quantitation of ZEA in samples. Samples were in triplicates and screening and quantitation were carried out immediately on each sample after extraction using

a Neogen microwell reader (9302) at absorbance and differential filters of 450nm and 650nm, respectively. Results were automatically calculated and interpreted. Limit and range of quantitations were 5µg kg^{-1} and 100µg kg^{-1}, respectively.

RESULTS AND DISCUSSION

A total of 173 isolates representing 12 *F.* species were isolated and identified as *F. acuminatum* (8 isolates), *F. compactum* (4 isolates), *F. culmorum* (8 isolates), *F. equiseti* (11 isolates), *F. graminearum* (51 isolates), *F. lateritium* (2 isolates), *F. poae* (4 isolates), *F. proliferatum* (6 isolates), *F. semitectum* (13 isolates), *F. sporotrichioides* (47 isolates), *F. subglutinans* (4 isolates) and *F. verticillioides* (15 isolates). The most predominant species on maize from the sampled locations was *F. sporotrichioides* with higher *fr* and *Rd* (96% and 23.12%, respectively) than other species ($p < 0.01$). *F. graminearum* predominated on wheat with only significant higher *Rd* (23.69%) than other species ($p < 0.01$). On soybeans, *F. semitectum* predominated with higher *fr* and *Rd* (80% and 5.78%, respectively) than other species ($p < 0.01$). Their isolation frequencies and relative densities of species in the grains are shown in Table 1.

Table 1. Isolation frequency (*fr*) and relative density (*Rd*) of fusaria isolated from maize, wheat and soybean seeds in Southwestern Nigeria

S/N	Species	maize		wheat		soybeans	
		fr	*Rd*	*fr*	*Rd*	*fr*	*Rd*
1.	*F. acuminatum*	-	-	30	2.89	26	1.73
2.	*F. compactum*	20	1.16	24	1.16	-	-
3.	*F. culmorum*	28	2.89	22	0.58	24	1.16
4.	*F. equiseti*	34	3.47	26	2.31	10	0.58
5.	*F. graminaerum*	50	5.78	100	23.69	-	-
6.	*F. lateritium*	-	-	20	1.16	-	-
7.	*F. poae*	18	1.16	14	0.58	10	0.58
8.	*F. proliferatum*	40	1.73	30	0.58	-	-
9.	*F. semitectum*	-	-	22	1.73	80	5.78
10.	*F. subglutinans*	-	-	30	1.16	10	1.16
11.	*F. sporotrichioides*	96	23.12	76	4.05	-	-
12.	*F. verticillioides*	82	5.78	50	2.89	-	-

fr: isolation frequency (%); *Rd*: relative density (%)

The occurrences of *F. compactum*, *F. culmorum*, *F. equiseti*, *F. graminearum, F. poae*, *F. proliferatum, F. sporotrichioides*, *F. subglutinans* and *F. verticillioides* on maize and wheat from this experiment have been supported by the reports of Jiménez et al (1996) and Sohn et al (1999) that isolated these fusaria from temperate grains. Adejumo et al (2007) had also isolated a larger number of *Fusarium* species from tropical grain (Southwestern Nigerian maize) although we observed a wider numerical difference from the percentage frequencies reported by them. The differences in isolate kind and numerical frequency of isolation can be attributed to the prevailing storage and climatic conditions such as storage duration, temperature and humidity levels, etc, during the time prior to sample collection (April - August) and sample size.

All 12 species isolated were present on the wheat samples indicating that Nigerian wheat harbored the most fusaria than maize and soybean. The occurrence of all the isolates on wheat except *F. lateritium* is in accordance with the reports of (Broggi et al 2007; Uoti 2008). Afanide et al (1976) reported the presence of

the perfect state of *F. lateritium*, *Gibberella bacatta*, on diseased leaves of *Celosia argentea* L. from Southwestern Nigeria but not on wheat. Also, the presence of *F. lateritium* on wheat grains is noteworthy because Leslie and Summerell (2006) described this species as one capable of surviving and colonizing wood trees and shrubs. Therefore, there could be a relationship between agronomic practices and species regional dominance, and the presence of *F. lateritium*.

Our data show that the only 6 species isolated from soybeans are *F. acuminatum* (3 isolates), *F. culmorum* (2 isolates), *F. equiseti* (Corda) Sacc. IMI 393764 (1 isolate), *F. poae* (1 isolate), *F. semitectum* (10 isolates) and *F. subglutinans* (2 isolates). Though soybeans have been noted not to extensively support fusaria growth and toxin production, *F. equiseti* (Corda) Sacc. and *F. semitectum* have been reported by Neergaard (1977) and Vaamonde et al. (1987) to occur on soybean seed lots.

All tested isolates produced ZEA on wheat from day 12 though at varying levels with lowest levels of 7µg kg^{-1} and highest of 98µg kg^{-1} (Table 2). Eugenio et al (1970) and Vaamonde et al (1987) reported that they did not detect ZEA on soybean inoculated with *F. equiseti* and *F. semitectum* and suggested that biosynthesis of this toxin is quite restricted in certain legumes (soybeans, beans and peanuts) but Richardson et al. (1985) reported that *Fusarium* isolates were able to produce ZEA and T-2 toxin on soybean and soybean meal. Our data in Table 2 show that *F. poae* ($S_{ak}1$), and *F. subglutinans* ($S_{ek}2$) lack the ability to biosynthesize ZEA on soybean while *F. equiseti* (Corda) Sacc. IMI 393764 ($S_{os}1$) produced the highest ZEA on soybean (27.2µg kg^{-1}) under the stated conditions than other tested isolates. At both incubation temperatures, it could produce appreciable amount of ZEA capable of causing estrogenic activity in mammals. It is further revealed that there is a relationship between incubation temperatures, duration and ZEA production on soybean which agrees with the report of Jiménez et al. (1996). Kinetic study of ZEA production showed that no ZEA was detected at day 6 (Table 2). Between day 12 and 18 at 30°C, the level of ZEA produced tripled significantly ($p<0.05$) on an average while at 25°C and between the 24th and 30th days, levels almost doubled. This implies that on a long term storage of this grain, some strains of *Fusarium* may biosynthesize ZEA and in increasing quantities, depending on other factors such as temperature and water activity. No significant pH changes were observed after day 12 for all isolates on wheat and soybean substrates (data not shown).

Table 2. Amount of ZEA (µg kg^{-1}) produced on wheat and soybean under varied conditions

Code		Incubation time (days) and temperature (°C)									
		6		12		18		24		30 (days)	
		A	B	A	B	A	B	A	B	A	B (°C)
$S_{ab}3$	S	-	-	-	-	-	7.0±0.05	9.0±0.01	8.5±0.09	15.1±0.02	9.1±0.01
	W	-	-	7.9±0.00	7.0±0.55	9.6±0.11	11.5±0.01	21.0±0.00	17.5±0.04	30.1±0.01	19.2±0.00
$S_{lg}1$	S	-	-	-	-	-	5.0±0.01	5.1±0.03	5.8±0.00	6.0±0.01	5.9±0.09
	W	-	-	9.9±0.11	7.8±0.00	23.5±0.03	25.8±0.10	65.0±0.00	43.0±0.02	98.0±0.10	55.7±0.00
$S_{os}1$	S	-	-	-	5.0±0.07	5.4±0.00	17.5±0.02	7.0±0.12	25.0±0.03	16.0±0.00	27.2±0.05
	W	-	-	10.1±0.00	11.7±0.10	21.6±0.00	22.7±0.01	31.1±0.07	30.0±0.03	46.0±0.02	34.0±0.10
$S_{ak}1$	S	-	-	-	-	-	-	-	-	-	-
	W	-	-	7.2±0.06	-	11.1±0.10	5.0±0.07	13.8±0.00	8.1±0.08	15.5±0.00	10.4±0.01
$S_{lg}3$	S	-	-	-	5.0±0.02	-	14.0±0.04	9.0±0.01	16.4±0.06	17.5±0.10	17.5±0.00
	W	-	-	6.5±0.10	5.1±0.00	11.9±0.03	9.7±0.03	20.0±0.00	15.1±0.11	26.5±0.07	21.0±0.04
$S_{ek}2$	S	-	-	-	-	-	-	-	-	-	-
	W	-	-	5.4±0.01	5.1±0.06	13.9±0.07	5.9±0.00	16.0±0.11	6.8±0.03	17.0±0.01	8.4±0.08

A: 25(°C) B: 30(°C) W: wheat substrate S: soybean substrate

Results are means of triplicates with standard deviations

It can be concluded that many fusaria colonize stored maize, wheat and soybean from Southwestern Nigeria, and suggested that ZEA production on soybean is highly dependent on the strain of *Fusarium* among many other factors. Also that the occurrence of fusaria and their subsequent capacity to produce ZEA on the tested substrates could pose serious damaging health concerns to the human and livestock consumers of these food materials thereby threatening life continuity. Therefore a quick intervention strategy in reducing the invasion of agricultural produce by toxigenic molds will be carefully studied and selected. This is the first

report of *F. lateritium* occurrence on wheat in Nigeria and zearalenone production by *F. equiseti* (Corda) Sacc. IMI 393764 on soybean.

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