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EFFECTS OF TEMPERATURE ON THE SIMULTANEOUS PRODUCTION OF ZEARALENONE AND DEOXYNIVALENOL BY *FUSARIUM GRAMINEARUM* ON MAIZE

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ABSTRACT

Fusarium graminearum **(Schwabe), a fungus commonly encountered on maize (*Zea mays* L) in Zambia, was analyzed for toxin production under temperature conditions prevalent during periods of high infestations. Ground maize samples were adjusted to a moisture content of 40% and inoculated with an isolate of *F. graminearum*. The samples were incubated at 26°C or 16°C for 10 weeks. Samples were analyzed for the toxins, zearalenone, deoxynivalenol, and nivalenol every two weeks. Nivalenol was not detected in any of the samples. Approximately five times more zearalenone than deoxynivalenol was produced at all temperatures tested. More zearalenone was produced at 16°C (5-1300 mg kg^{-1}) than at 26°C (5-750 mg kg^{-1}). When the fungus was grown at 16°C for five weeks and transfered to 26°C, production of zearalenone was stimulated. Although more deoxynivalenol was produced by the fungus at 26°C (1–62 mg kg^{-1}) than at 16°C (1–50 mg kg^{-1}), most deoxynivalenol was produced by the fungus grown at 16°C for five weeks and then transferred to 26°C for the next five weeks (1-137 mg kg^{-1}). Growth of the fungus at 26°C for 5 weeks before transfer to 16°C did not result in any significant rise in either zearalenone or deoxynivalenol production.**

Key Words: **Toxins, Zambia, *Zea mays***

RÉSUMÉ

Fusarium graminearum **(Schwabe), un champignon communément trouvé sur le maïs (*Zea mays* L.) en Zambie a été analysé pour production de toxine dans des conditions prévalant pendant les périodes de hautes infestations. Les échantillons de maïs broyés ont été ajustés à 40% d'humidité et inoculés avec un isolat de *F. graminearum*. Les échantillons ont été incubés à 26 ou 16°C pendant 10 semaines. Ils ont été analysés toutes les deux semaines pour les toxines zearalenone, deoxynivalenol, et nivalenol. Nivalenol n'a été détectée dans aucun des échantillons. Zearalenone été approximativement 5 fois plus que deoxynivalenol à toutes les températures d'expérience. Plus de zearalenone était produite à 16°C (5-11300 mg kg^{-1}) qu' à 26°C (5-750 mg kg^{-1}). Quand le champignon était cultivé à 16°C pendant 5 semaines puis transferé à 26°C, la production de zearalenone était stimulée. Bien que le champignon produisait plus de deoxynivalenol à 26°C (1-62 mg kg^{-1}) qu ' à 16°C(1-50 mg kg^{-1}), la plus grande quantité de deoxynivalenol était produite lorsque le champignon était cultivé à 16°C pour 5 semaines et transferé à 26°C pour les 5 semaines suivantes. La culture du champignon à 26°C pendant 5 semaines et son transfer à 16°C n'a pas réalisé une augmentation significative de production que ce soit de zearalenone on de deoxynivalenol.**

Mots Clés: **Toxine, Zambie, *Zea mays***

INTRODUCTION

Cob rot is the most serious disease of maize in Zambia and *Fusarium* and *Diplodium* species are the major causative agents. Grain invaded by certain species of *Fusarium* may be toxic to animals and humans due to toxins produced by the fungi. Toxin-producing *Fusarium* species are widespread in nature and can be found on cereals, feeds, and vegetables (Marasas *et al.*, 1984). Some of the important species are *F. equiset* (Corda) Sacc, *F. nivale* (Fr) Ces, *F. sporotrichioides* (Sherb), *F. moniliforme* (Sheldon), and *F. graminearum* (Schwabe). Many of these toxigenic species of *Fusarium* produce more than one toxin (Marasas *et al.*, 1984).

Among the *Fusarium* species, *F. graminearum* and *F. moniliforme* are the most common (MacDonald and Raemaeker, 1974; Marasas *et al.*, 1984). These fungi will grow only when the moisture content of the grain is fairly high, and by the time the grain is dry enough to harvest no further damage occurs. In the past, the National Marketing Board (NAMBOARD) bought moulded maize from farmers during seasons of every high infestations and attempted to recover the good grain (Anonymous, 1974). However, because of the high cost of the cleaning process, NAMBOARD no longer buys grain if more than 2% of seeds are infested by mould. Farmers who acumulate moulded grain are therefore now forced to use it as animal feed supplement or to brew local beer. This moulded maize contains high levels of zearalenone and deoxynivalenol (Lovelace and Nyathi, 1977; Marasas *et al.*, 1977; Siame and Lovelace, 1989).

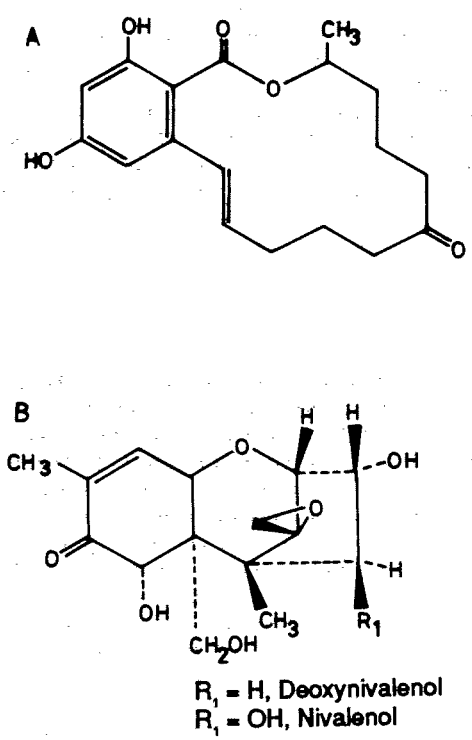

Figure 1.The structure of (A) zearalenone, (B) Deoxynivalenone, and nivalenol

Isolates of *F. graminearum* produce several toxins including zearalenone, deoxynivalenol, and nivalenol (Fig. 1). *F. graminearum* produces either zearalenone and deoxynivalenol or zearalenone and nivalenol (Ichinoe *et al.*, 1983). One isolate of *F. graminearum* which produces both deoxynivalenol and nivalenol on rice was recently isolated (Sigiura *et al.*, 1990). Apart from single fungal species producing more than one toxin, several toxigenic fungi can occur on the same sample (Abbas *et al.*, 1988).

Zearalenone causes hyperestrogenism and infertility in swine and was found in feeds associated with infertility and hyperestrogenism in diary catle (Shotwell, 1977). Deoxynivalenol causes emesis, feed refusal, and decreased weight gains in swine (Forsyth *et al.*, 1977). Deoxynivalenol was also isolated from maize consumed by humans in Transkei, a region of high esophageal cancer incidence (Thiel *et al.*, 1982). Little information is available on the toxicity of nivalenol on livestock and humans. However, nivalenol was shown to be more toxic than deoxynivalenol in experimental animals (Ueno and Ishii, 1985).

One environmental factor which influnces the type and amount of toxins produced is temperature. Zearalenone production on rice and maize by *Fusarium* species is favoured by temperatures between 12°C and 16°C (Eugenio *et al.*, 1970; Greenhalgh *et al.*, 1983) while production of deoxynivalenol and nivalenol are favoured by higher temperatures ranging between 25 and 28°C (Greenhalgh *et al.*, 1983). This is the higher range of temperatures commonly found in the maize growing regions of Zambia.

In this study, *F. graminearum* isolated from Zambian maize was analyzed for the types and amounts of toxins produced under Zambian temperature conditions prevalent during periods of high *Fusarium* infestation (average maximum and minimum temperatures of 26°C and 16°C, respectively).

MATERIALS AND METHODS

Source of standards. Zearalenone and deoxynivalenol used as standards were obtained from Sigma Chemical Co, UK, and Nivalenol from Wako Chemicals, Germany. All other reagents used were analytical products from commercial sources in Zambia.

Isolation of *F. graminearum*. A few lightly infected maize kernels obtained locally were washed in running tap water. The kernels were soaked in 5% sodium hypochlorite for 10 minutes, rinsed with sterile water, and then soaked for 10 minutes in penicillin solution (0.3 g l^{-1}). Treated kernels were transfered to moistened filter papers in petri-dishes and incubated for one week at room temperature (23–25°C). Single conidia isolates of the fungus were subcultured on Potato Dextrose Agar and incubated at 25°C for one week. The pink fungal growth was identified as *F. graminearum* (Schwabe) at Mount Makulu Government Agricultural Research Station, Chilanga, Zambia. Fungal spores were suspended in sterrile water for inoculation.

Inoculation of maize samples. A sample of healthy maize kernels (with no detectable zearalenone, deoxynivalenol, and nivalenol) was ground in a coffee grinder (Moulinex, Type 241.2.00, France) after which 300 g were transferred to 1 liter glass flasks and autoclaved at 120°C for one hour. The moisture content was adjusted to 37% (wet-weight basis) with sterile water. *F. graminearum* suspended in 10 ml sterile water was added to each flask, and the moisture content was adjusted to 40%. The inoculated samples were incubated at the following temperatures: (i) 16°C for 10 weeks, (ii) 26°C for 10 weeks, (iii) 16°C for 5 weeks followed by 5 weeks at 26°C, and (iv) 26°C for 5 weeks followed by 5 weeks at 16°C.

Extraction and determination of toxins. A 50 g sample was removed from each flask every two weeks and divided into two 25 g samples. One portion was analyzed for zearalenone while the other portion was analyzed for deoxynivalenol and nivalenol.

The method by Bennett *et al.* (1985) with modifications described by Siame and Lovelace (1989) was used for zearalenone. Zearalenone was extracted from the samples with chloroform and partitioned with sodium hydroxide. The pH of the aqueous layer was lowered with citric acid and zearalenone was taken up in dichloromethane. The dichloromethane was evaporated to dryness, and the residue was taken up in 200 µl of chloroform for thin layer chromatography (TLC) on 0.2 mm silica gel plates (E Merck, Germany). Quantification was by visual comparison with standards.

Deoxynivalenol and nivalenol were extracted as described by Scott *et al.* (1986) with modifications so that TLC could be used in the determination step (Siame and Lovelace, 1989). Samples were extracted with methanol-water (1:1, v/v). The extracts were eluted with ethyl acetate and further cleaned and taken up in 200 µl dichloromethane-methanol (9:1, v/v) for TLC. Quantification was also by visual comparison with standards.

Gas chromatographic. A Hitachi model 263–50 gas chromatograph equipped with a flame ionization detector was used to confirm the identity of the toxins as well as to determine the concentration of selected sample toxins. Trimethyl silane (TMS) derivatives of the toxins were prepared as described by Kamimura *et al.* (1981), the derivatizing reagent being N-trimethylsilylimidazole-trimethylchlorosilane-ethyl acetate (1.0:0.2:9.0, v/v/v). The TMS derivatized toxins were seperated on a glass column, 2 m x 5 mm (i.d), packed with 2% OV-17 on 80–100 mesh Uniport.

RESULTS

Fungal growth was vigorous at 16 and 26°C. By the end of the first week, the whole substrate surface was covered by white mycelial growth which later turned pink. Fungal invasion of the

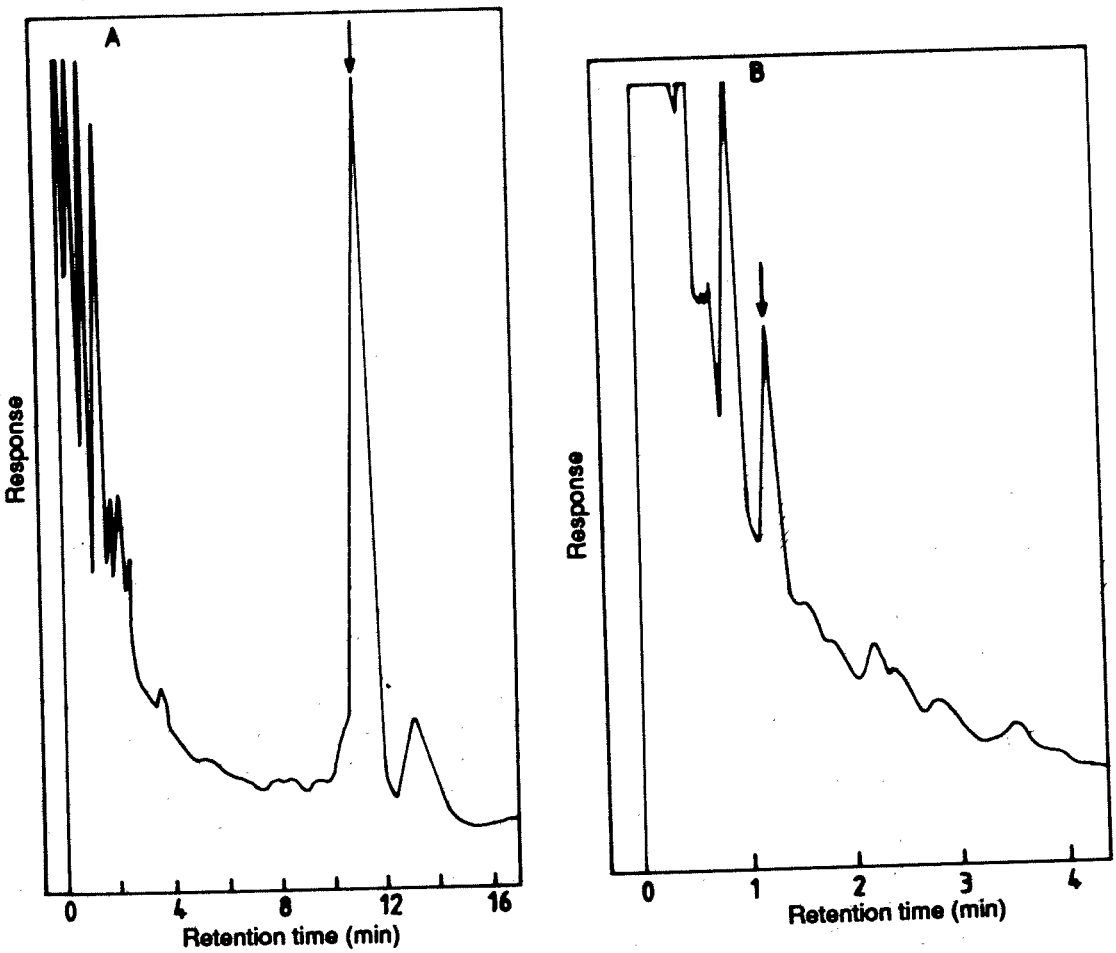

Figure 2. Gas chromatograms of zealenone and deoxynivalenol produced by *Fusarium graminearum*. The toxin peaks had retention times of 11.5 and 1.2 minutes (arrowed) and concentrations were calculated to be 240 mg kg^{-1} and 137 mg kg^{-1}, respectively. The concentrations of the toxins were estimated at 260 and 120 mg kg^{-1}, respectively, using TLC. A 2 m glass column packed with 2% OV-17 on Uniport and a flame ionization detector were used.

substrate in all flasks was complete after eight weks.

The zearalenone method with TLC quantitation recovered 80% of added toxin with a detection limit of 50 mg kg^{-1}. This was comparable to the original method by Bennett *et al.* (1985) in which HPLC was used for quantitation. One-dimensional TLC gave very good resolution of zearalenone. The recoveries for deoxynivalenol and nivalenol were 80% and 86% of added toxins, respectively. The detection limits on TLC were 80 mg kg^{-1} for both deoxynivalenol and nivalenol, comparable to that obtained by Scott *et al.* (1986) in which gas chromatographic quantitation was used. Two-dimensional TLC provided better quantitation. Gas chromatographic quantitation gave consistently lower toxin concentrations than those obtained by TLC quantitation. In the gas chromatography, the retention time for zearalenone was 11.5 min, while that for deoxynivalenol was 1.2 min (Fig. 2). No nivalenol was detected in the extracts. Zearalenone and deoxynivalenol were produced in all flasks regardless of incubation temperature. Approximately five times more zearalenone than deoxynivalenol was produced in all flasks.

More zearalenone was produced by the fungus incubated continuously at 16°C than at 26°C (Fig. 3). Most of the zearalenone was produced after 6 weeks incubation, but production continued to

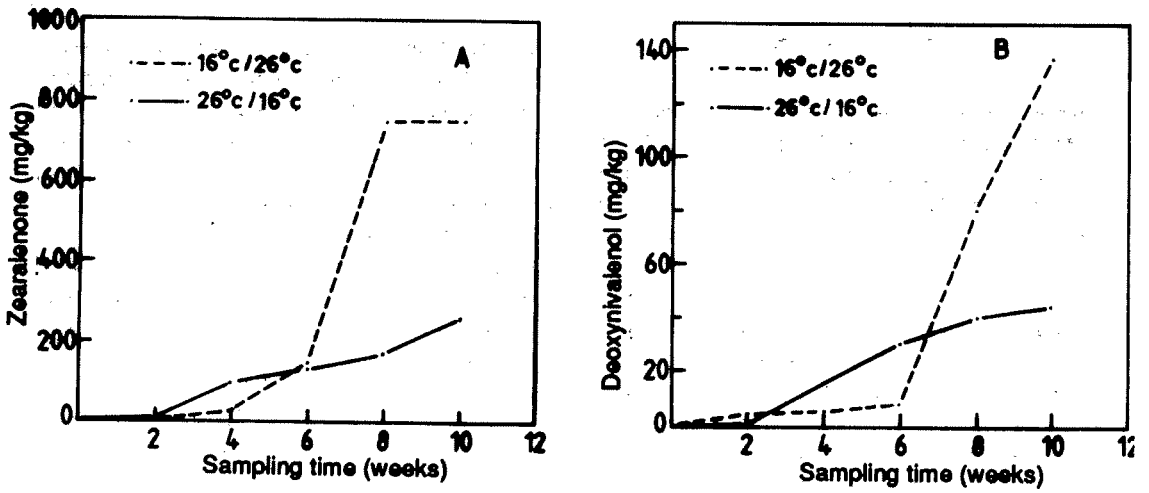

Figure 3. Zearalenone and Deoxynivalenol production by *Fusarium graminearum* at 16°C and 26°C. Samples were assayed for the toxins every two weeks. TLC quantitation was by visual comparison with standards.

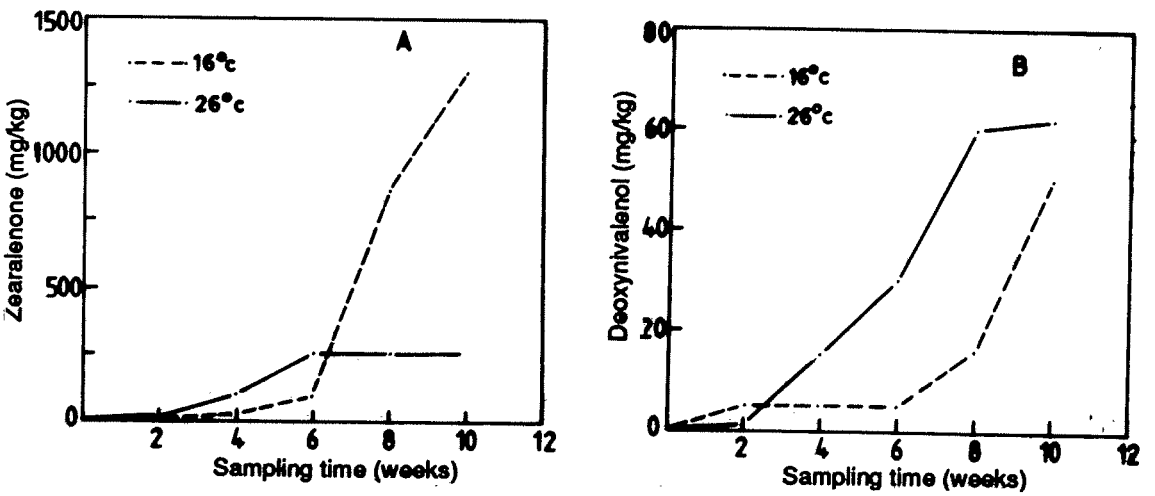

Figure 4. Zearalenone and Deoxynivalenol production by Fusa*rium graminearum* at 16°C and 26°C before transfering to 26°C or 16°C for the next 5 weeks. Samples were assayed every two weeks. TLC quantitation was by visual comparison with standards.

increase at 16°C reaching 1300 mg kg^{-1} after 10 weeks, while stabilising at 250 mg kg^{-1} at 26°C. When the fungus was incubated at 16°C for 5 weeks and then transferred to 26°C for the next 5 weeks, zearalenone production was stimulated after transfer (Fig. 4). There was no significant rise in zearalenone production when the fungus was transferred from 26°C to 16°C.

Deoxynivalenol production was faster when the fungus was grown at 26°C rather than at 16°C (Fig. 4). However, after ten weeks of incubation at 16°C, deoxynivalenol production was nearly the same as that produced at 26°C (50 mg kg^{-1} compared to 62 mg kg^{-1}). When the fungus was first incubated at 16°C for 5 weeks and then transferred to 26°C for the next 5 weeks, deoxynivalenol production was stimulated, reaching a maximum of 137 mg kg^{-1} after ten weeks (Fig. 4). This was much higher than that produced by the fungus incubated at 26°C for the duration of the experiment (62 mg kg^{-1}). When the fungus was first grown at 26°C before being transferred to 16°C the amount of deoxynivalenol produced was nearly the same as that obtained from the fungus grown continuously at 26°C (Fig. 4).

DISCUSSION

Zearalenone and deoxynivalenol occur naturally in Zambian grown maize (Marasas *et al.*, 1977; Siame and Lovelace, 1989) and in this study, like those of Greenhalgh *et al.* (1983), considerably more zearalenone than deoxynivalenol was produced at all times on maize regardless of incubation temperature. The isolate of *F. graminearum* used did not produce detectable amounts of nivalenol. The highest amount of zearalenone was produced in flasks incubated at 16°C, consistent with reports that low temperatures (between 12°C and 16°C) favour production of this toxin (Eugenio *et al.*, 1970; Greenhalgh *et al.*, 1983). The maximum level of zearalenone (1300 mg kg^{-1}) occurred after 10 weeks of incubation at 16°C. For both fungi grown at 16°C for 10 weeks and at 16°C for 5 weeks followed by 5 weeks at 26°C, stimulation of zearalenone production occurred at nearly the same time. It appears that incubation of the fungus at 16°C during the first five weeks of culturing was sufficient to triger production of zearalenone and the subsequent transfer of the fungus to a higher temperature was not important in increasing toxin production.

There was no significant rise in zearalenone production by the fungus incubated at 26°C even when the fungus was later transferred to 16°C. This differs from earlier reports (Eugenio *et al.*, 1970) in which two weeks of incubation at 25°C before transfer to 15°C resulted in stimulation of toxin production. This difference may be attributed to the different species of *Fusarium* used in the two studies, and to the length of time before transfer to a lower temperature.

Deoxynivalenol production was favoured by high temperatures as has been reported already (Vesonder *et al.*, 1982; Greenhalgh *et al.*, 1983). However, the highest amount of the toxin (137 mg kg^{-1}) was produced by the fungus incubated at 16°C for five weeks before transferring to 26°C. This was double the amount produced during the same period by the fungus incubated continuously at 26°C (62 mg kg^{-1}). This suggests that the temperature change may have induced high deoxynivalenol production.

A considerable rise in deoxynivalenol production after the sixth week was observed in the fungus continuously incubated at 16°C and nearly equalled that produced at 26°C by the tenth week. This raises the question as to whether slower fungal growth promotes toxin production.

F. graminearum isolated from Zambian grown maize produced high levels of zearalenone and low levels of deoxynivalenol at both 16°C and 26°C. It seems therefore that Zambian maize invaded by indegenous *F. graminearum* isolates is likely to accumulate high levels of zearalenone regardless of temperature conditions occurring during the growing season. Greater attention should therefore be paid to the reduction of possible adverse effects resulting from use of infected maize grains.

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