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Effect of aflatoxin B_1 on germination, respiration and α-amylase in maize[1])

Der Einfluß von Aflatoxin B_1 auf Keimung, Atmung und α-Amylase bei Mais

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Summary

The hepatocarcinogen aflatoxin B_1, a secondary metabolic product of *Aspergillus flavus*, inhibited the germination of maize seeds, with an ED_{50} dose of about 25 µg/ml. The incubation of seeds imbibed with water in cold, with aflatoxin B_1 resulted in the inhibition of respiration with a 7-hour lag period. The activity of α-amylase, which was enhanced 2.5 x by gibberellic acid (10 µM), was inhibited by the toxin and actinomycin D, an inhibitor of DNA-dependent RNA synthesis. Aflatoxin did not inhibit the activity of α-amylase when the toxin was added to the assay mixture. Based on these data and some of our other work, it is proposed that aflatoxin B_1 inhibits respiration most probably by inhibiting the induced synthesis of α-amylase, thus blocking the availability of the carbon source necessary for respiration.

Key words: mycotoxins; aflatoxin; maize; seed germination; respiration; α-amylase

Zusammenfassung

Das lebercarzinogene Aflatoxin B_1, ein sekundäres Stoffwechselprodukt von *Aspergillus flavus*, hemmte die Keimung von Maiskörnern. Die ED_{50} betrug ungefähr 25 µg/ml. Die Inkubation von Körnern, die bei niedrigen Temperaturen in Wasser vorgequollen waren, mit Aflatoxin B_1 führte zur Hemmung der Atmung nach einer Verzögerungsphase von 7 Std. Die Aktivität der α-Amylase, die durch Zugabe von 10 µM Gibberellinsäure um das 2,5-fache erhöht wurde, wurde durch Aflatoxin gehemmt, ebenso auch durch Actinomycin D, einem Hemmstoff für die DNS-abhängige RNS-Synthese. Aflatoxin verursachte keine Hemmung der α-Amylaseaktivität, wenn es direkt dem Meßansatz zugesetzt wurde. Auf Grund dieser und anderer Resultate der Autoren wird angenommen, daß Aflatoxin B_1 die Atmung sehr wahrscheinlich durch Hemmung der induzierten α-Amylase-Synthese blockiert, wodurch die Verfügbarkeit der für die Atmung notwendigen Kohlenstoff-Verbindungen eingeschränkt wird.

Stichwörter: Mykotoxine; Aflatoxin; Mais; Keimung; Atmung; α-Amylase

[1]) Part of the work is from the Ph. D. thesis work of the senior author. The present address of the senior author: Postgraduate Department of Botany, Bhagalpur University, Bhagalpur, India. Reprint requests should be addressed to the second author.

1 Introduction

Aflatoxins are secondary, hepatocarcinogenic metabolites of *Aspergillus flavus* Link ex Fries growing on agricultural commodities. Of the four major aflatoxins and their derivatives, aflatoxin B_1 (AB_1) is the most toxic with the highest hepatocarcinogenicity (DETROY et al. 1971, GOLDBLATT 1969). The biological and biochemical effects of aflatoxins on animal systems have been the subject of extensive research in the recent years (BENJUWICK 1974, DETROY et al. 1971, GOLDBLATT 1969), but comparatively little work has been done on their effects on plants and plant products. The literature on aflatoxins' effects on plants and microorganisms has been recently reviewed by REISS (1978). Aflatoxin B_1 inhibits seed germination in *Lepidium sativum* (SCHOENTHAL and WHITE 1965), cowpea (CRISAN 1973, ADEKUNLE and BASSIR 1973), sorghum (TRIPATHI 1973, 1974) and a few other plants. Although the toxin does not inhibit germination, it strongly inhibits the growth of hypocotyls of many plants, e. g. lettuce (CRISAN 1973), red cabbage (TRIPATHI, personal observations) and many other plants of Cruciferae (CRISAN 1973). Here we report the inhibitiory effect of AB_1 on germination of maize seeds. Our studies to be reported elsewhere (TRIPATHI and MISRA, under preparation) have shown that in the germinating maize seeds AB_1 inhibits the syntheses of RNA, protein and DNA in that order of time sequence and the toxin also inhibited the chromatin directed DNA-dependent RNA polymerase activity. The RNA synthesis was blocked in 2 h and the effects on protein and DNA synthesis were evident in 5 and 6 h, respectively. The DNA-dependent RNA polymerase activity was inhibited 60 % at 50 µg/ml of the toxin, while it was inhibited 92 % by 20 µg/ml actinomycin D. Binding experiments involving equilibrium dialysis, viscosity measurements and difference spectrophotometry indicated that AB_1 formed a complex with purified DNA of germinating maize seeds, suggesting that the inhibition of RNA polymerase activity was most probably due to this binding that would reduce the template availability.

During seed germination in cereals, e. g., maize, starch is hydrolysed to provide simple sugars as carbon source for energy and metabolites necessary for germination. The hydrolysis is effected by the inducible synthesis of α-amylase, the synthesis being stimulated by gibberellic acid (VARNER 1964, GROAT and BRIGGS 1969). The synthesis of this enzyme requires messenger RNA and its synthesis is stimulated by gibberellic acid (HIGGINS et al. 1976). Since aflatoxin B_1 binds with DNA and prevents RNA synthesis, it would be expected that the toxin would prevent the synthesis of α-amylase by interfering with messenger RNA synthesis. The toxin is known to interfere with the synthesis of gibberellic acid-stimulated lipase in cotton seed (BLACK and ALTSCHUL 1965). In this report, we present information on the effects of aflatoxin B_1 on seed germination, the synthesis of α-amylase and respiration.

2 Materials and methods

Aflatoxins were produced by fermentation of rice with *Aspergillus parasiticus* (NRRL 2999) by the method of SHOTWELL et al. (1966). Aflatoxin B_1 was purified from the mixture by column chromatography (PAI et al. 1975) and thin layer chromatography (STUBBLEFIELD et al. 1968). The purity of the toxin was ascertained by thin layer chromatography with standard AB_1 and by ultraviolet absorption characteristics. The AB_1 concentration was determined by an ultraviolet absorption spectrophotometric method of NABNEY and NESBITT (1965). A stock solution of AB_1 was prepared in absolute ethanol (1 mg/ml) and dilutions were made in sterile dist. water. The final concentration of alcohol in the assay mixtures was less than 0.1 %.

Maize seeds (cv. 'Ganga 2') were surface sterilized with 0.1 % sodium hypochlorite and thoroughly washed in sterile distilled water. To see the effect of AB_1 on seed germination, the seeds were germinated on filter papers in Petri plates containing either 10 ml solvent or the toxin solution. The lids of the plates were lined with moist filter papers. The plates, with ten seeds per plate, were incubated at 28°C (± 1°C) with 18 h fluorescent light (1000 lux) and 6 h darkness. On the 6th day the germination counts were taken and the root and shoot lengths were also determined.

For respiration studies, the sterilized and washed seeds were water imbibed by incubating in a moist chamber at 4°C for 8 h. The seeds were then treated with the toxin as given in the results. The respiration was measured by Warburg manometry (UMBREIT et al. 1972). Each Warburg flask contained 0.3 ml of 20 % KOH in the central well, three maize seeds (the volume occupied by seeds was calculated by determining the water replaced by them) and 2.5 ml of test solution containing AB_1, when the effect on untreated, activated seeds was to be studied. In some experiments the seeds were imbibed in water at 4°C along with AB_1 and in such cases no treatment of the toxin was given in the Warburg flask. The flasks were shaken at 28°C at 130 strokes min^{-1} with oscillations of 4 cm. QO_2 (microliters of oxygen consumed h^{-1} g^{-1} dry weight of seeds) was then calculated as an average from 3 flasks.

For α-amylase studies, the hydrated seeds were placed on moist filter paper in Petri plates containing the test chemicals. After 24 h incubation at 28°C in the dark, the seeds were washed with sterile dist. water. The seeds were homogenized and the enzyme preparation was made and assayed by the method of SHUSTER and GIFFORD (1962). The specific activity of the enzyme was expressed as mg of soluble starch hydrolyzed per mg protein in 5 min in the linear portion of the reaction curve. The protein content was measured by the method of LOWRY et al. (1951).

3 Results and discussion

Aflatoxin B_1 inhibited maize seed germination. The increase in inhibition with the toxin concentration was almost linear up to 50 µg/ml at which 98 % inhibition occurred (Fig. 1). The ED_{50} dose for the inhibition was 25 µg/ml. Observations on the seedling growth showed reductions in the shoot length with increasing toxin concentrations. A similar inhibition of germination and shoot length has also been observed in sorghum and barley (TRIPATHI, unpublished).

When AB_1 was added to the untreated seeds in the Warburg flasks, it did not affect respiration up to 7 h. After this time, the toxin progressively inhibited respiration with time. Pretreatment of the seeds with the toxin prior to respiration studies eliminated the lag period and the respiration was inhibited almost from the beginning (Fig. 2). Since AB_1 did not inhibit respiration of untreated seeds up to 7 h it seems that the toxin has no direct effect on respiration. The hydrolysis of starch by α-amylase provides the carbon source necessary for respiration and germination. Evidence exists to show that this enzyme appears during cereal seed germination due to de novo synthesis and not due to activation of preexisting protein (GRABAR and DAUSSANT 1964, VARNER and CHANDRA 1964). This synthesis of the enzyme is influenced by gibberellic acid (GA) (GRABAR and DAUSSANT 1964, VARNER 1964, VARNER and CHANDRA 1964). In barley aleurone layers GA stimulates the synthesis of poly(A)$^+$-messenger RNA specific for α-amylase (HIGGINS et al. 1976). Since AB_1 is known to inhibit the synthesis of GA-stimulated synthesis of α-amylase and lipase in cotton seeds (BLACK and ALTSCHUL 1965), it seemed that in our experiment the toxin might be inhibiting the respiration with a lag period by interfering with some event like the synthesis of α-amylase. There-

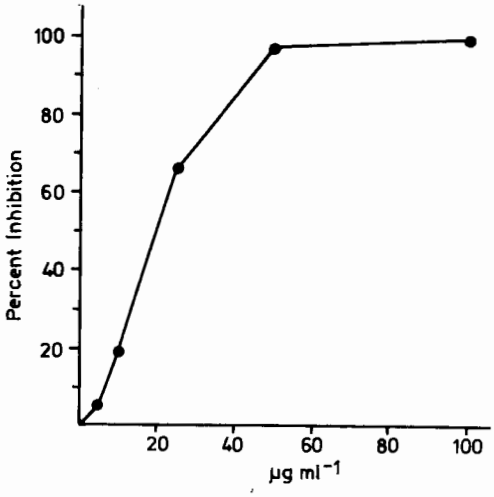

Fig. 1. Inhibition of maize seed germination by aflatoxin B_1.
Abb. 1. Hemmung der Keimung von Maiskörnern durch Aflatoxin B_1.

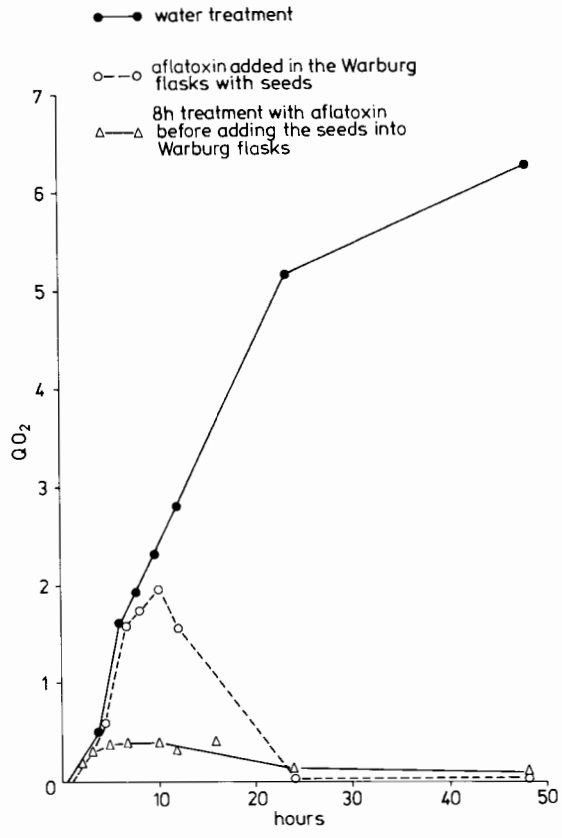

Fig. 2. Respiration by water and aflatoxin B_1 (50 µg/ml) treated maize seeds.
Abb. 2. Atmung von Maiskörnern nach Behandlung mit Wasser oder Aflatoxin B_1 (50 µg/ml).

fore, the effect of AB_1 and actinomycin D, a known inhibitor of DNA-dependent RNA polymerase, on α-amylase formation was studied. Both AB_1 and actinomycin D inhibited α-amylase in germinating maize seeds (Table 1). Since the toxin did not significantly

Table 1. Effect of gibberellic acid, aflatoxin B_1 and actinomycin D on α-amylase of germinating maize seeds. The data are averages of three replications and two independent experiments

Tab. 1. Einfluß von Gibberellinsäure, Aflatoxin B_1 und Actinomycin D auf die α-Amylase keimender Maiskörner

Treatment[1])	Specific activity of α-amylase (mg of starch hydrolysed/mg protein in 5 min)
None	
Gibberellic acid (GA_3), 10^{-5} M	5.3 ± 0.5
Aflatoxin B_1 (50 µg/ml), treatment of seeds, 8 h	13.2 ± 1.5
GA + Aflatoxin B_1	2.5 ± 0.2
GA + Actinomycin D (20 µg/ml)	3.6 ± 0.27
Enzyme preparation from aflatoxin-untreated seeds + Aflatoxin B_1	1.2 ± 0.2
	11.9 ± 1.4

[1]) The seeds were treated with GA and toxins at time zero and incubated for 8 h. For the last treatment the aflatoxin was added in the assay mixture.

affect the reaction catalyzed by α-amylase when the enzyme was obtained from untreated seeds (Table 1), the effect of AB_1 is not on the enzyme activity but rather on the synthesis of the enzyme. In this same maize system we (TRIPATHI and MISRA, under preparation) have shown that AB_1 inhibits the synthesis of RNA, protein and DNA in that time sequence. The chromatin-bound DNA-dependent RNA polymerase activity was also inhibited by AB_1 and actinomycin D. In barley aleurone layers AB_1 inhibits the synthesis of poly $(A)^+$-messenger RNA (TRIPATHI, unpublished). These observations, along with almost complete inhibition of α-amylase synthesis by actinomycin D and AB_1 (Table 1), would suggest that AB_1 inhibited the synthesis by inhibiting the messenger RNA and that the inhibition of respiration was indirectly due to its effect on α-amylase synthesis. However, aflatoxins are also known to affect mitochondrial functions directly, including the electron transport system in animal systems (BABABUNMI and BASSIR 1972; DOHERTY and CAMPBELL 1972; OBIDOA and SIDDIQUI 1978).

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