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Comparative Responses of *Lilium longiflorum*, cv, 'Ace' Pollen to Aflatoxins B_1, B_2, and G_1

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For the past few years we have been examining the possibility that the growth and development of plants and their organs can be used as bioassay materials for the hepatocarcinogenic aflatoxins (DASHEK & LLEWELLYN 1974, 1977; JONES et al. 1980; DANLEY et al. 1981; DASHEK et al. 1979). One of the plant parts that we have utilized rather extensively is Lilium longiflorum, cv. 'Ace' pollen. The reasons for selecting pollen included the ease with which it can be cultured as well as its commerical availability and relatively large size. The simplicity of quantifying the germination and tube elongation responses of in vitro germinating pollen to exogenous compounds is well known.

The four main aflatoxins are AFB_1, AFB_2, AFG_1 and AFG_2 (Fig. 1). We have been testing the ability of each of these to affect germination and subsequent tube elongation in order to generate dose-response curves, i.e., log percent germination and/or tube length related to medium toxin concentration.

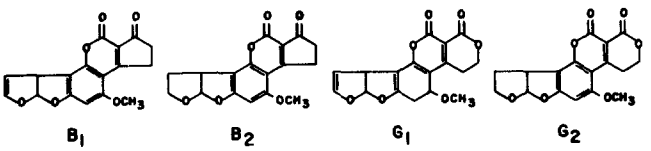

Fig. 1. Structures of AFB_1, AFB_2, AFG_1 and AFG_2.

This paper compares the germination and tube elongation responses of in vitro cultured pollen to AFB_1, AFB_2 and AFG_1.

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EXPERIMENTAL PROCEDURES

Growth conditions. Twenty mg fresh weight lots of L. longiflorum were sown in sterile Petri dishes containing 20-mL aliquots of sterile DICKINSON's (1965) medium without tetracycline. Prior to the addition of medium, AFB_2 or AFG_1 (Applied Sciences Lab, Inc.) was added to the Petri dishes to yield 15 µg/mL stocks of each toxin. The stocks of 10 mL or less were diluted to 20 mL with DICKINSON's medium to yield toxin concentrations of 7.500, 3.750, 1.875, 0.938, 0.468 and 0.234 µg/mL. To obtain AFB_1 (Calbiochem) -media concentrations of 5, 10, 15, 20 and 25 µg/mL, DICKINSON's media containing 30 µg/mL toxin and with or without 3.0 mM KH_2PO_4 were diluted. The toxin which was dissolved in acetone was added to the media which were then autoclaved to remove the solvent. Pollen was germinated at 24 ± 2°C for 2, 4 and 8 h except for pollen sown in media containing either AFB_1 or AFB_2 which were germinated for 4 h.

At each of these times, media aliquots containing germinated pollen were removed from the dishes to shell vials each of which had been prefilled with 100 mL formaldehyde. Then, the vials were maintained at 4°C until pollen germination and tube lengths could be quantified.

Quantification of % germination and tube elongation. Drops were removed from each vial and the % germination and tube lengths were determined with a microscope equipped with an ocular micrometer (JONES et al. 1980; DASHEK et al. 1981).

Chromatography of aflatoxins. AFB_1, AFB_2 and AFG_1 purities were assessed by chromatography on 250-µm thick Adsorbosil-1 thin layer chromatography (TLC) plates (Applied Sciences Lab, Inc.). Plates were developed in 5% chloroform in methanol and then viewed with a UV source to visualize the aflatoxins. To verify media AFB_1, AFB_2 and AFG_1 stock concentrations, fluorescent spots on TLC plates were compared densitometrically to reference samples which were spotted simultaneously with the media samples.

RESULTS AND DISCUSSION

Pollen germination and tube length bioassays of AFB_1. The dose-response curves relating log percent germination and tube length to AFB_1-medium concentration are presented in Fig. 2A and B, respectively. When KH_2PO_4 was withheld from the medium, the log percent germination remained nearly constant from 5-30 µg/mL (Fig. 2A). In contrast, when KH_2PO_4 was included within the medium, the log percent germination did not markedly change until the AFB_1-medium concentration was raised to 20 µg/mL. A 20 % decrease in log percent germination occurred when the AFB_1-medium concentration was changed from 20-30 µg/mL. Linear regression analysis yielded a correlation coefficient of 0.98.

In the absence of KH_2PO_4, log tube length became less

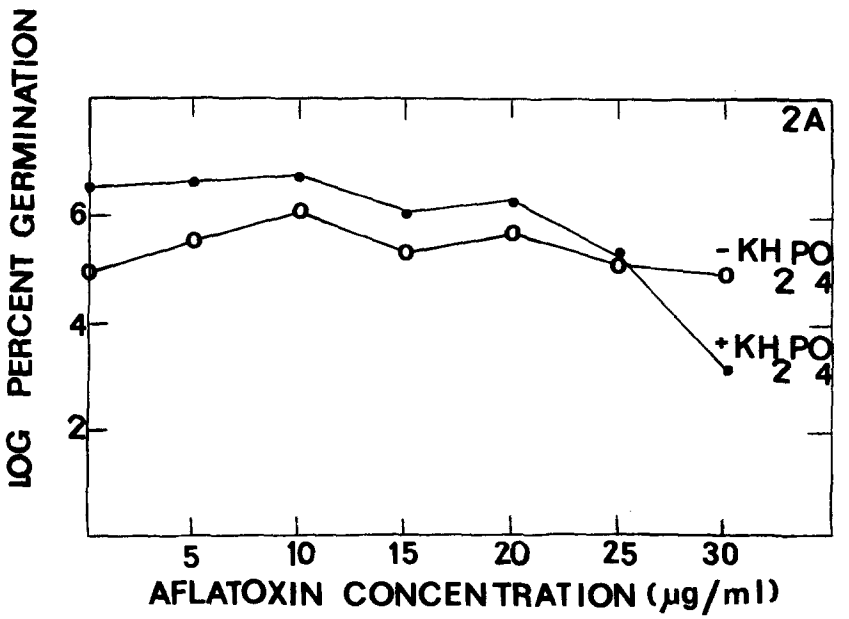

Fig. 2A. Dose-response curve relating log percent germination to AFB_1-medium concentrations.

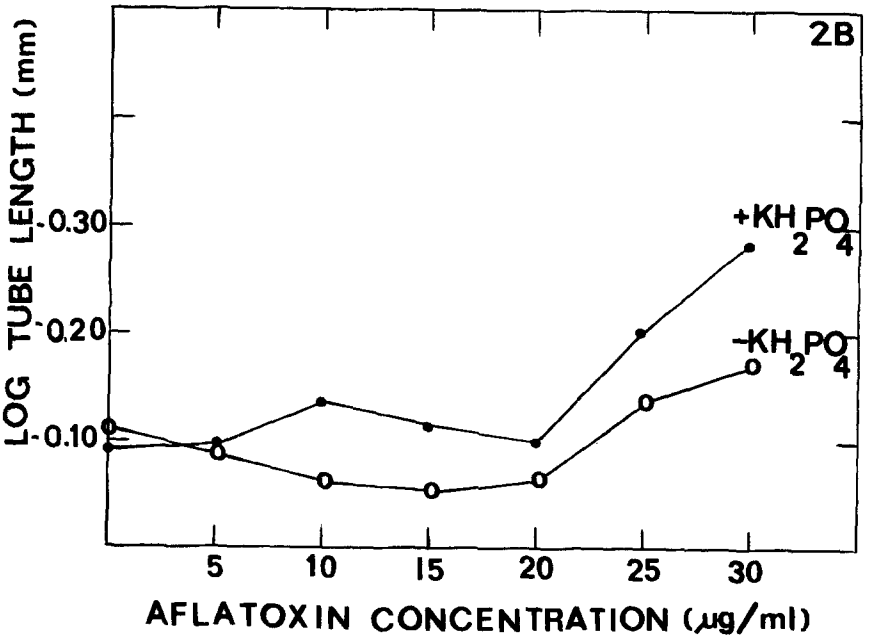

Fig. 2B. Dose-response curve relating log tube length to AFB_1-medium concentrations.

negative (Fig. 2B) as the concentration was raised to 2 µg/mL but more negative as the AFB_1 concentration was elevated from 20-30 µg/mL. A greater AFB_1-induced inhibition of log tube length occurred when tubes were cultured in medium containing KH_2PO_4 than without KH_2PO_4. Correlation coefficients as determined by regression analysis of AFB_1-promoted inhibitions of log tube length between 20-30 µg/mL were 0.99 and 0.98, respectively, for pollen germinated in medium containing and lacking KH_2PO_4. The dose-response curves presented within Fig. 2A relating log percent germination to aflatoxin-medium concentration demonstrate that log percent germination could possibly be developed as an AFB_1 bioassay between 20 and 30 µg/mL provided that 3.0 mM KH_2PO_4 is included within the medium. However, it seems that a greater inhibition of tube elongation occurred when the medium was provided with KH_2PO_4.

Pollen germination and tube length bioassays of AFG_1. The log percent germinations at 0.468, 0.937, 1.875, 3.750, 7.500 and 15.000 µg/mL were 1.74, 1.61, 1.66, 1.19, 1.07 and 1.15 times greater than that of the control for 2 h germinated pollen (Fig. 3A). A correlation coefficient of 0.92 was obtained when a linear regression analysis was performed upon the decrease in log percent germination which occurred between AFG_1-medium concentrations of 0.468 and 7.500 µg/mL.

A 9% increase, 8% decrease, 42% diminution, 20% decline, 34% reduction and 7% enhancement in log tube length from that of the control were observed at medium concentrations of 0.468, 0.937, 1.875, 3.750, 7.500 and 15.000 µg/mL, respectively (Fig. 3B).

At 4 h of culture, the log percent germination increased 1.20 times over that of the control as the AFG_1-medium concentration was raised to 1.875 µg/mL (Fig. 3A). The enhancement was 1.14 when the concentration was elevated to 3.750 µg/mL. At 7.500 µg/mL the log percent germination approximated that of the control, but at 15.000 µg/mL it was 6% less. Linear regression analysis of the decrease in log percent germination which occurred between 1.875 and 15.000 µg/mL resulted in a correlation coefficient of 0.94.

Whereas a 75% decrease in log tube length was seen at 7.500 µg/mL, supplying the germination medium with 0.468, 0.937, 1.875, 3.750 and 15.000 µg/mL promoted tube elongation 2.05, 1.25, 1.07, 1.01 and 1.29 folds, respectively, during a 4 h culture (Fig. 3B).

The log percent germination was 1.18 times greater than that of the control at 0.468 µg/mL but declined to nearly that of the control upon raising the AFG_1-medium concentration to 3.750 µg/mL during an 8 h incubation (Fig. 3A). The log percent germinations were 8 and 15 % less than that of the control at 7.500 and 15.000 µg/mL. A correlation coefficient of 0.93 was obtained when a linear regression analysis was performed for the decline which resulted between 0.468 and 15.000 µg/mL.

A dose-response curve with a clearly defined trend for

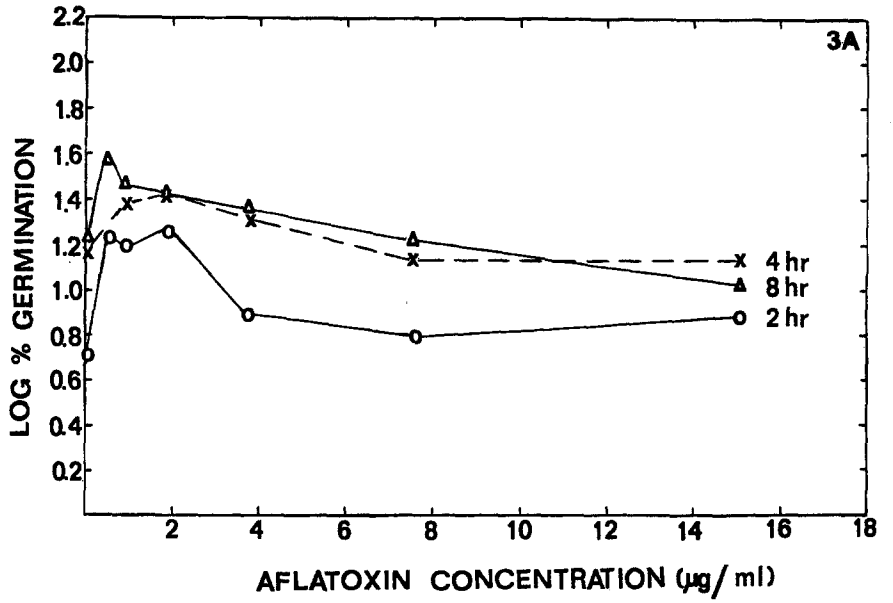

Fig. 3A. Dose-response curve relating log percent germination to AFG_1-medium concentrations.

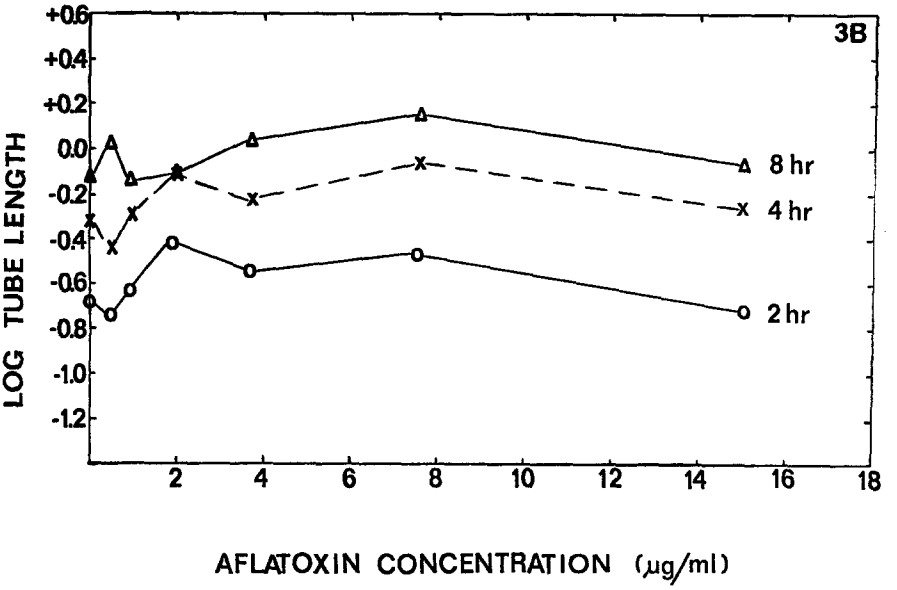

Fig. 3B. Dose-response curve relating log tube elongation to AFG_1-medium concentrations.

concentration-dependent induced effects on log tube elongation was not observed for pollen which was germinated for 8 h. This together with unsatisfactory correlation coefficients (<0.9) preclude the possibility of developing an AFG_1 bioassay which is based upon tube elongation. Although inclusion of 0.468-15.000 µg/mL AFG_1 within the culture medium did not inhibit log percent germination at 2, 4 or 8 h, the linear (r = 0.9) declines (Fig. 3A) in log percent germination from a maximum stimulated level (0.468 µg/mL - 2 and 8 h; 1.875 µg/mL - 4 h) could possibly be developed as bioassays for the detection and quantification of AFG_1.

Pollen germination and tube length bioassays of AFB_2. Fig. 4A demonstrates that the log percent germination remained nearly constant as the medium AFB_2 concentration was enhanced during a 4 h culture precluding the employment of log percent germination as a bioassay for AFB_2, at least over the concentration range of 0.938 - 15.000 µg/mL. In contrast, log tube length exhibited a rise as the medium concentration was brought to 0.938 µg/mL and then decreased from the stimulated level to a level which approximated that of control at 15.000 µg/mL (Fig. 4B). Linear regression analysis of the diminution from the stimulated level yielded a correlation coefficient of <0.90.

Comparison of pollen germination and tube elongation responses to AFB_1, AFB_2, and AFG_2. When the data within Fig. 2A, 3A and 4A are compared, it is apparent that none of the toxins inhibited germination on a log percent germination basis at <15 µg/mL. However, as little as 0.468 µg/mL AFG_1 appeared to stimulate germination. This concentration is considerably less than those which affect the germination of most plant systems (Table 1). The only systems whose germinations appear to be affected by low toxin concentration are Glycine max, cv. 'Essex' seeds (JONES et al. 1980) and Onoclea sensibilis spores (CAHILL et al. 1978) and in those systems the toxin which was tested was AFB_1 rather than AFG_1.

Comparison of the data within Fig. 2B, 3B and 4B reveals that none of the toxins inhibited log tube length at <15 µg/mL. As in the case of germination, AFG_1 concentrations of <2 µg/mL stimulated tube elongation. Such stimulation also occurred with AFB_2. To our knowledge, such low toxin concentrations have not been reported to affect the growth of those plant systems tested thus far (Table 2) for possible aflatoxin-induced alterations of growth and development.

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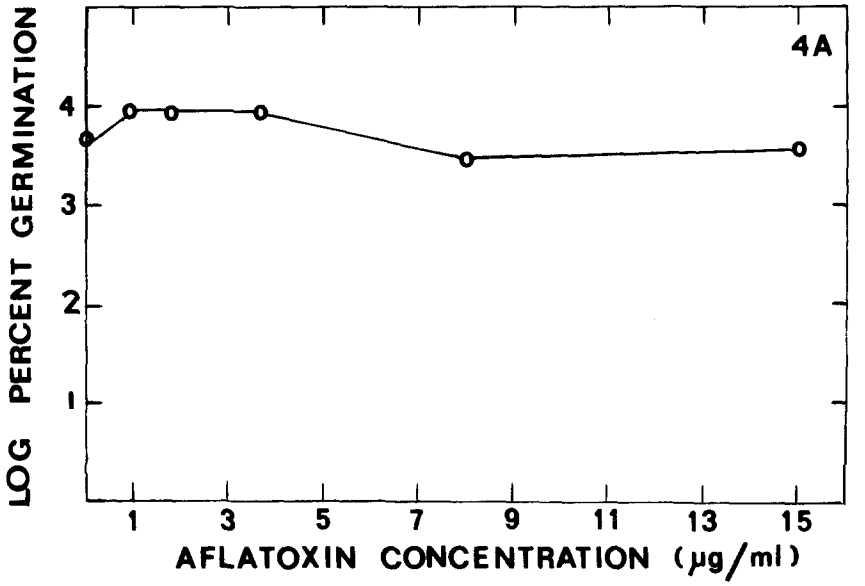

Fig. 4A. Dose-response curve relating log percent germination to AFB_2-medium concentration.

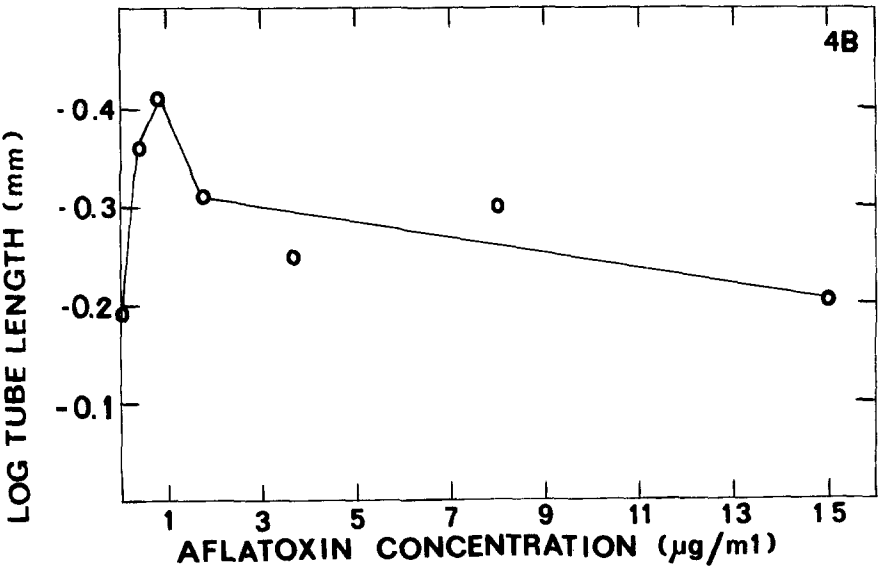

Fig. 4B. Dose-response curve relating log tube elongation to AFB_2-medium concentration.

Table 1. Comparison of Germination of Various Seeds Exposed to Aflatoxin

Seed	Response	Aflatoxin Concentration	Investigator(s)
Arachis hypogaea	No significant effect	31.50 µg/mL mixed aflatoxins for 72, 144, 168 and 240 h	DASHEK et al. (1981)
Avena sativa	No significant effect for cvs. 'Norline', 'Windsor' or 'Moregrain'; 20% inhibition for cvs. 'Coker' and 'Roanoke'	31.50 µg/mL mixed aflatoxins for 65, 89 and 117 h	DASHEK et al. (1981)
Cuminum cyminum	5% inhibiton after 8 days of imbibition	2.50 and 5.00 µg/mL AFB_1	LLEWELLYN et al. (submitted)
Glycine max, cv. 'Essex'	% inhibitions were 5, 20, 40 and 80 or 6, 4, 13, 19 for listed concentrations at 18 and 36 h, respectively	0.38, 2.90, 5.80 and 11.60 µg/mL AFB_1 for 18 and 36 h	JONES et al. (1980)
Hordeum vulgare	No significant effect at 39, 63 and 89 h for cvs. 'Barsoy', 'Volbar' and 'Henry';	31.50 µg/mL mixed aflatoxins for 39, 63, and 89 h	DASHEK et al. (1981)

Table 1. Comparison of Germination of Various Seeds Exposed to Aflatoxin Cont'd.

Seed	Response	Aflatoxin Concentration	Investigator(s)
	17-23% inhibitions for 'Surry' and 'McNair' at 39, 63 and 89 h, respectively		
Lactuca sativa	No inhibition at 1,000 µg/mL in one cv. nor by 100 µg/mL in 29 other cvs.	100-1000 µg/mL AFB_1	CRISAN (1973a)
Lepidium sativum	No impairment;35, 90 and 100% inhibitions, respectively	1,2,5, and 10 µg/mL aflatoxins 25,50 and 100 µg/mL aflatoxins	SCHOENTAL & WHITE (1965)
Lilium longiflorum,	No inhibitory effect; 10.6, 27.3 and 45.1% inhibitions	5-30 µg/mL AFB_1 without 3.0 mM KH_2PO_4	JONES et al. (1980)
		15, 25 and 30 µg/mL AFB_1 with KH_2PO_4	DASHEK et al. (1981)
Onoclea sensibilis	6.7, 7.8, 27.0, 32.6 and 43.8% inhibitions, respectively	0.78, 1.56, 2.34, 3.13 and 3.90 µg/mL	CAHILL et al. (1978)
Pimpinella anisum	<5 % inhibition after 8 days of imbibition	2.50 µg/mL AFB_1	LLEWELLYN et al. (Submitted)

Table 1. Comparison of Germination of Various Seeds Exposed to Aflatoxin Cont'd.

Seed	Response	Aflatoxin Concentration	Investigator(s)
	no inhibition	500 µg/mL AFB_1	
Species of Cruciferae (19 plants belonging to 11 species)	no effect	100 µg/mL AFB_1	CRISAN (1973b)
Variety of seeds	Variable % inhibition (as high as 100% in Phalaris canariensis)	20 µg/mL mixed aflatoxins	JACQUET et al. (1971)
Vigna sinensis	100% inhibiton	50 µg/mL	ADEKUNLE & BASSIR (1973)
Zea mays	No effect	0.35, 0.73, 1.50 and 2.00 µg/mL mixed aflatoxins	DASHEK et al. (1981)
	23 and 25% reductions	5.80 and 11.60 µg/mL mixed aflatoxins	

Table 2. Comparison of Growth Responses of Various Plant Parts to Aflatoxins

Plant	Response	Aflatoxin Concentration	Investigator(s)
Arachis hypogaea	No significant effect	31.50 µg/mL mixed aflatoxins for 72, 144, 168 and 240 h	DASHEK et al. (1981)
Avena sativa	% inhibition of root elongation ranged from 4.3 to 68.8%, respectively	31.50 µg/mL mixed aflatoxins for 65, 89 and 117 h	DASHEK et al. (1981)
Carralluma freri	death and prevention of growth of upper leaves and floral buds	100 and 300 ppm	REISS (1969)
Chlorella pyrenoidosa	similar to nontreated inhibitions of 4 strains	30 ppm 1 µg/mL AFB_1	SULLIVAN & IKAWA (1972)
Cuminum cyminum	% difference from control was 0, 13 and 13, respectively	2.50 µg/mL AFB_1 for 2, 4 8 days	LLEWELLYN et al. (Submitted)
	% difference from control was 0, 0 and 56, respectively	5.00 µg/mL AFB_1 for 2, 4 8 days	

Table 2. Comparison of Growth Responses of Various Plant Parts to Aflatoxins. Cont'd.

Plant	Response	Aflatoxin Concentration	Investigator(s)
Glycine max, cv. 'Essex'	Non-treated, excised roots followed a sigmoidal growth curve with a dry wt. increase from 100% (0 h) to 108.5% (24 h); 4.5% dry wt. decline at 4 h, increase to 101.5% (8 h), and decrease to 99% (12 h)	20 µg/mL AFB_1	YOUNG et al. (1978)
Glycine max, cv. 'Essex'	% inhibition of attached root elongation was 14 (48 h) and 26 (140 h)	2.90 µg/mL AFB_1	JONES et al. (1980)
	% inhibition of attached root elongation was 21 (48 h) and 25 (140 h)	5.80 µg/mL AFB_1	
	% inhibition of attached root elongation was 36 (48 h) and 50 (140 h)	11.60 µg/mL AFB_1	
Hordeum vulgare	% inhibitions of root elongation ranged from 22.4 to 62.2	31.50 µg/mL mixed aflatoxins for 39 and 89 h	DASHEK et al. (1981)

Table 2. Comparison of Growth Responses of Various Plant Parts to Aflatoxins. Cont'd.

Plant	Response	Aflatoxin Concentration	Investigator(s)
Kalanchoe diagremontia	Inhibition of root elongation by approximately 50%	100 µg/mL AFB_1	REISS (1977)
Lepidium sativum	No inhibition of hypocotyl elongation No inhibition of radicle elongation	1 µg/mL AFB_1	REISS (1971)
	14.2 and 58.0% inhibtion of hypocotyl elongation, 23.9% inhibitions of radicle elongation	10 and 100 µg/mL AFB_1	
Lilium longiflorum	23 and 36% inhibitons of pollen tube elongation, respectively	25 and 30 µg/mL AFB_1 with 3.0 mM KH_2PO_4	JONES et al. (1980)
Phalaris canariensis	Radicle elongation impaired by 50%	50 µg/mL mixed aflatoxins	JACQUET et al. (1971)
Pimpinella anisum	% difference from control was 10, 83 and 71, respectively	2.50 µg/mL AFB_1 for 2, 4 and 8 days	LLEWELLYN et al. (Submitted)
	% difference from control was 10, 82 and 82, respectively	5.00 µg/mL AFB_1 for 2, 4 and 8 days	

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