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N-CARBOXYMETHYLCHITOSAN INHIBITION OF AFLATOXIN PRODUCTION: ROLE OF ZINC

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ABSTRACT

Aqueous Solutions of N-carboxymethylchitosan (NCMC) suppressed both growth and aflatoxin production by Aspergillus flavus and A. parasiticus in submerged culture (Adye and Mateles A&M). Test media were amended with various concentrations of zinc (15, 30, 45, 60 uM), and NCMC solution (0.62 uM). After 8 days incubation NCMC-treated cultures showed marked reduction of aflatoxin production and fungal growth. Enhanced levels of zinc did not overcome the NCMC-mediated inhibition of fungal growth or aflatoxin production.

INTRODUCTION

Aflatoxins produced by Aspergillus flavus and A. parasiticus, are a group of chemically related secondary metabolites that induce a variety of toxic responses in humans and animals and may often be present in subsrates used for biotechnology process. Attempts to control aflatoxin production by limiting growth of the toxigenic fungus through the application of chemical or microbial agents have had limited success (Cuero et al. l988a). Factors such as zinc concentration affect aflatoxin production in chemically-defined media (Mateles and Adye 1965; Diener and Davis 1969). Cuero et al. (1987) found a relationship between mineral composition of crop soils (zinc, magnesium, iron and calcium) and the presence of toxigenic fungi and aflatoxin in maize kernels on plants grown in the soils. Chelating agents that selectively bind with zinc or other trace metals have been reported to inhibit aflatoxin production (Maggon et al. 1977).

Chitosan is a cationic carbohydrate polymer that is chemically derived by deacetylation of naturally-occurring chitin (Muzzarelli, 1988). Chitosan exhibits chelating activity of Zn, Cu, Co, Mn, Ni, Cd, Pb and Cr (Muzzarelli et al. 1980; Cuero and Lillehoj 1990) and has been identified as a potential control agent of fungi (Hadwiger et al. 1984, Cuero et al. 1988b). The aim of the current study was to acquire information on the role of zinc in chitosan-inhibited control of aflatoxigenic Asperigillus species and concomitant aflatoxin production.

MATERIALS AND METHODS

Cultures: Five hundred ml flasks containing 50 ml of liquid medium (Mateles and Adye 1965) were autoclaved (121° C, 30 min, 20 psi), cooled and inoculated with a O.5 ml spore suspension (10^6 conidia per ml) of *Aspergillus flavus* Link ex Fries (SRRC 167) or *A. parasiticus* Speare (SRRC 255) Isolates.

Fungal Culture Treatments: N-carboxymethylchitosan (NCMC) was produced for the current study from chitin (Protan Lab., Inc; Seattle, WA) by reacting the free amino groups of chitosan with glyoxylic acid to produce a soluble, gel-forming imine and reduction with sodium borohydride at pH 5 (Cuero and Lillehoj l99O). A filter-sterilized aqueous solution (39 uM) of NCMC was added to each culture (total volume = 51 ml) to yield a final level of 0.02 uM. NCMC was added to the medium simultaneously with fungal cells (day 1) in one set of tests. Varios concentrations (15, 30, 45, 60 uM) of zinc ($ZnSO_4 .7H_2O$) were used in the liquid medium to determine the role of zinc in NCMC-mediated control of fungal growth and aflatoxin production. The pH of the culture medium and controls was initially adjusted to 5.5 after the addition of NCMC .

Incubation and Sampling: Fungi were incubated as shake culture (1000 rpm) at 25 C (Psychroterm, New Brunswick Scientific, New Brunswick, NJ). Determination of biomass (dry weight), pH and aflatoxin B^l production were carried out along with microscopic observations (electronic and light compound microscope) at day 8 of incubation

Fungal Biomass and Aflatoxin Determination: After incubation, the contents of each flask were filtered and weighed Whatman 1 filter paper and the filtrate was saved for aflatoxin determination. Mycelia were rinsed, placed in a vacuum-oven at 60°C overnight, and weighed. The filtrate was transferred to a separatory funnel and extracted with 25 ml of chloroform. Chloroform extracts were evaporated to dryness under nitrogen and residues were redissolved in 500 ul of chloroform for analysis by thin layer chromatography (TLC) using silica gel G (250 m) plates (200x 200 x 0.25 mm) and a chloroform:acetone solvent system (Cleveland et al. 1987). Aflatoxin B_1 was quantitated by fluoro-densitometric measurement using aflatoxin standards as reference, and a Shimadzu-CS 930-TLC scanner.

Examination of Fungal Cultures: For light microscopy, samples were water-mounted. For electron microscopy, samples observed under a Cambridge 250 SEM at an accelerating voltage of 15 kv following fixation (Wergin and Stone 1981)

RESULTS AND DISCUSSION

NCMC reduced aflatoxin yield by *A. flavus* and *A. parasiticus* more than 90%, while fungal growth was reduced to less than half (Table 1). There was no direct correlation between fungal growth and aflatoxin production in either NCMC-treated or control fungal cultures. Increased zinc levels did not obviate the NCMC-mediated inhibition of growth or toxin production, but flatoxln yields were less inhibited in NCMC-treated fungal cultures with the highest zinc concentration (60 uM). Therefore, it appears that only part fraction of the NCMC-based inhibition can be attributed to zinc binding, the rest being due to NCMC-mediated variation in fungal metabolism that independently influences growth and toxin production (Ramaut *et al.*, 1970). Microscopic observations of toxigenic fungal cells showed that NCMC presence inhibited spore germination of both *Aspergillus* species and also sporulation of mycelia, which was profuse after 8 days in the non-inhibited cultures (Fig 1 & 2).

TABLE 1 NCMC inhibition of growth and aflatoxin production by A. flavus and A. pararsiticus in submerged culture at 25°C, after 8 days[1].

Treatment[2]	Zinc[3] (uM)	A. flavus Biomass[4] (g/culture)	A. flavus AFLB1[5] (ng/g)	A. parasiticus Biomass (g/culture)	A. parasiticus AFLB1 (ng/g)
I	15	0.34	2.62	0.31	1.12
Control	8	0.59	13.10	0.55	10.00
II	30	0.36	2.60	0.33	1.10
Control	8	0.60	12.00	0.60	9.40
III	45	0.38	2.40	0.35	1.61
Control	8	0.62	12.50	0.66	10.30
IV	60	0.37	5.45	0.35	2.10
Control	8	0.66	13.50	0.63	12.15

1. Shake incubation.
2. Treatment: NCMC (0.62 uM) added with the fungal inoculum at time 0. Control=no NCMC.
3. Zinc sulfate solution added at time 0.
4. Dry weight (g/5l ml culture).
5. Amount of toxin (ng/g); AFLBl = aflatoxin Bl.

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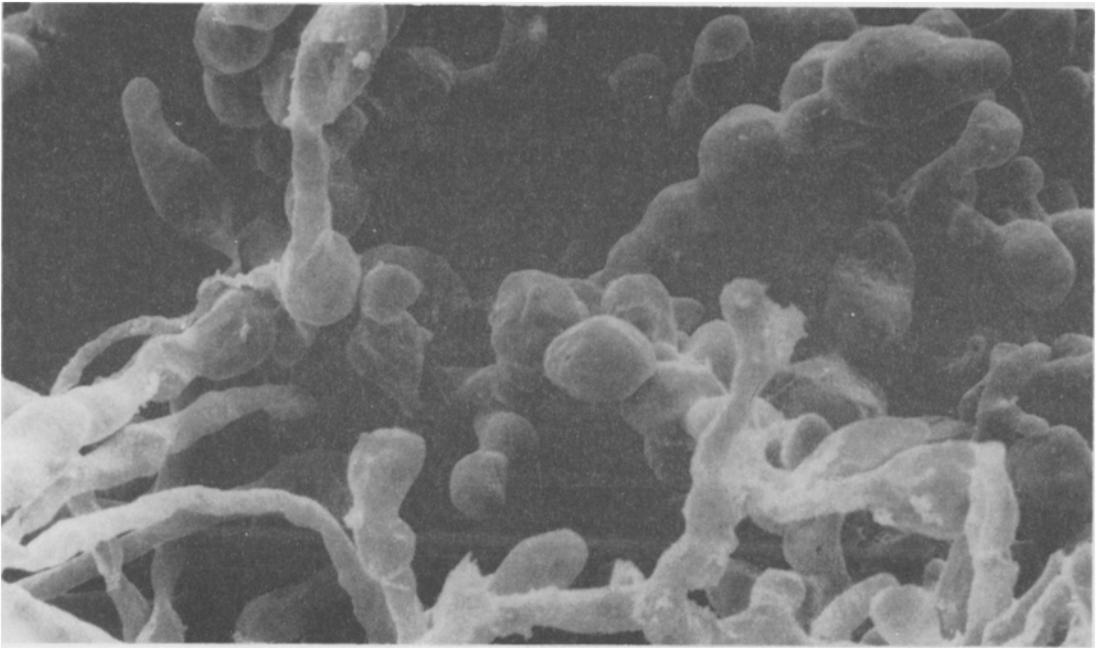

Figure 1. Spore bearing structures of *Aspergillus flavus* mycelia from 8-day culture (no NCMC)

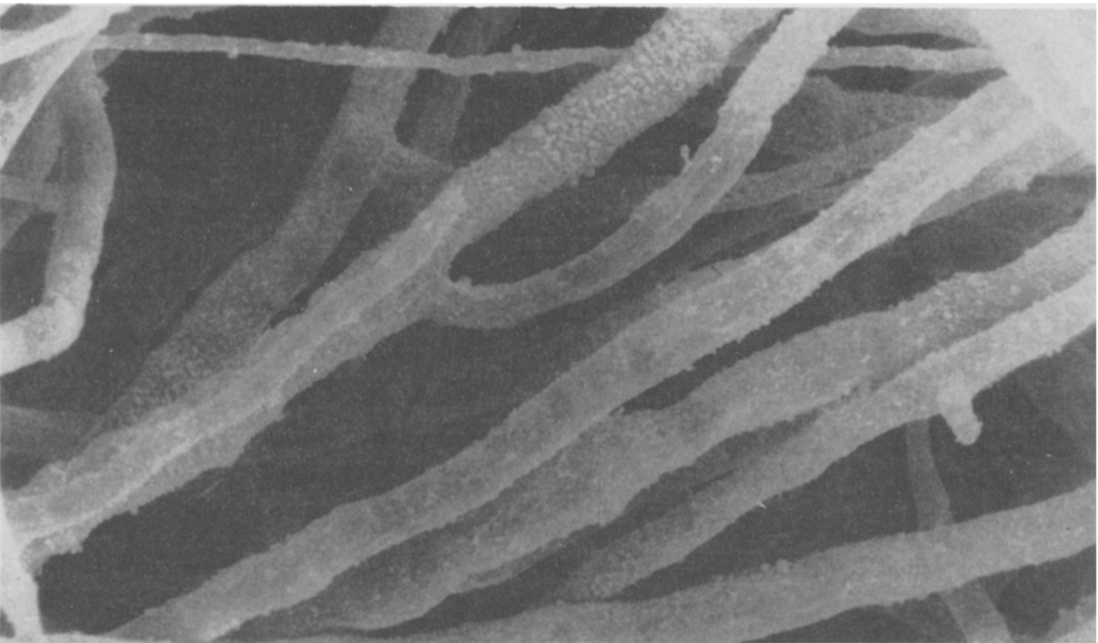

Figure 2. Mycelia of *Aspergillus flavus* from 8-day culture treated with NCMC