

Isolation of a Caffeine-Resistant Mutant of *Aspergillus parasiticus*

ROBERT L. BUCHANAN, LAURA L. ZAIKA, CHARLES A. KUNSCH,
CLEMENT J. PURCELL, JR., and SARAH E. MERTZ

ABSTRACT

A caffeine-resistant mutant of *Aspergillus parasiticus* NRRL 2999 was isolated and subsequently designated strain BCR1. The mutant strain grew in the presence of > 8 mg/mL caffeine, while growth of the parent strain was delayed by 1 mg/mL and inhibited by 2 mg/mL. Strain BCR1 produced abundant amounts of aflatoxin only when cultured in media containing caffeine. Residual caffeine analyses indicated that caffeine-resistance in BCR1 was not due to the metabolic elimination of caffeine.

INTRODUCTION

PREVIOUS INVESTIGATIONS have demonstrated that caffeine (1,3,7-trimethylxanthine) inhibits growth and polyketide mycotoxin production by a variety *Aspergillus* and *Penicillium* species (Buchanan and Fletcher, 1978; Nartowicz et al., 1979; Lenovich, 1981; Buchanan et al., 1982, 1983a, 1983b; Buchanan and Lewis, 1984a). Buchanan et al. (1983b) suggested that the inhibition of growth and aflatoxin production may involve two separate mechanisms. Subsequent investigations (Buchanan and Lewis, 1984a) suggested that the inhibition of aflatoxin production may involve an inhibition of carbohydrate uptake and/or utilization, though the specific loci affected by caffeine were not identified. The objective of the current study was to develop a caffeine-resistant mutant of *Aspergillus parasiticus* that could be used to further elucidate how this naturally occurring compound prevented the formation of aflatoxin. Further impetus for this work was provided by the recent study with green coffee beans by Tsubouchi et al. (1985) who reported the isolation of *Aspergillus ochraceus* strains that grew and produced ochratoxin A in the presence of elevated levels of caffeine.

MATERIALS & METHODS

Microorganism

Aspergillus parasiticus NRRL 2999 was employed as the parent strain. The mold was maintained on Potato Dextrose Agar (PDA) (Difco) slants stored at 4°C. Spore suspensions were prepared as described previously (Tice and Buchanan, 1982) and diluted to contain 10^6 conidia/mL.

Media

Yeast Extract-Sucrose (YES) (Davis et al., 1966), Peptone-Mineral Salts (PMS) (Buchanan and Lewis, 1984b), and Yeast Extract-Glucose (YEG) (Buchanan and Lewis, 1984a) media were prepared as described previously. Plating media were prepared by adding agar at 20 g/L to the respective liquid media. Caffeine was added to the media prior to sterilization by autoclaving (15 min at 15 psi).

Isolation of mutants

Caffeine-resistant mutants were isolated using YES agar containing 4 mg/mL caffeine. Plates were inoculated with 0.5 mL (5×10^5 conidia) of a spore suspension of *A. parasiticus* NRRL 2999 and incubated at 37°C for up to 10 days. The plates were examined daily

for rapidly growing colonies, which were subsequently subcultured and maintained on PDA + 4 mg/mL caffeine slants.

Assessment of caffeine resistance

The effects of caffeine on growth and aflatoxin production by the parent and caffeine-resistant strains were assessed using 125-mL flasks containing 25 mL of YES with 0, 1, 2, 4, or 8 mg/mL caffeine. The cultures were inoculated with 0.5 mL of spore suspension to achieve an inoculum of approximately 2×10^4 conidia/mL. Cultures were incubated at 28°C with or without agitation (0 vs 150 rpm), and analyzed for pH, aflatoxin production, residual caffeine and mycelium dry weight after 3, 6, and 9 days.

The effect of caffeine on aflatoxin production was also evaluated using a modification of the replacement culture technique of Buchanan and Lewis (1984b). YES medium (300 mL in 1000-mL flasks) was inoculated with 2 mL of spore suspension to achieve an inoculum of 6.7×10^3 conidia/mL. Cultures were incubated for 72 hr at 28°C on a rotary shaker (150 rpm). The mycelial pellets were harvested on cheesecloth and subsequently disrupted in a blender. The mycelia were then transferred in 10g portions (wet weight) to 1000 mL flasks containing 300 mL of PMS and incubated for 24 hr at 28°C and 150 rpm. The mycelia were then reharvested on cheesecloth and transferred in 2.0g portions (wet weight) to 50-mL flasks containing 20 mL of YEG with 0, 1, 2, 4 and 8 mg/mL caffeine. After initial mixing, these cultures were incubated at 28°C without agitation for 72 hr and then analyzed for pH, aflatoxin production, residual caffeine concentration and mycelial dry weight.

Analyses

Aflatoxins were extracted, separated by thin-layer chromatography, and quantitated by fluorodensitometry as described previously (Buchanan et al., 1985). Mycelium dry weights were determined gravimetrically after drying the extracted mycelia for 18 hr at 85°C.

Residual caffeine concentrations were determined by high performance liquid chromatographic analysis of medium samples. After clarifying the samples with 0.45 μ m filter and diluting with water to achieve an approximate caffeine concentration of 0.02 mg/mL, caffeine was separated on a reversed phase column (Altex Ultrasphere ODS-C₁₈, 4.6 \times 250 mm). Elution was carried out isocratically with 1% acetic acid + 12% acetonitrile in water at a flow rate of 1 mL/min. Caffeine was detected by monitoring UV absorbance at 254 nm and quantitated by comparison of peak heights against external caffeine standards. Preliminary studies were performed to assure that there were no interfering fungal metabolites or medium components.

Estimation of sporulation

The quantitative effect of caffeine on sporulation was estimated by inoculating YES agar plates containing various levels of caffeine with 2.5×10^4 conidia and incubating the plates at 28°C. After 3, 7, 10, and 14 days, triplicate plates of each caffeine concentration were flooded three times with 3 mL water + Tween 80 (20 μ l/mL), with the conidia being gently dislodged with an inoculating loop. The spore suspensions were brought up to 15 mL, and the conidia enumerated by direct microscopic examination using a counting chamber.

RESULTS

INCUBATION of 172 YES + 4 mg/mL caffeine agar plates (each inoculated with 5×10^5 conidia) yielded six rapidly growing colonies. Subsequent passage on YES agar with and without caffeine confirmed that these rapidly growing isolates had stable caffeine-resistance phenotypes. One isolate (designated strain BCR1) was selected for further characterization.

BCR1 sporulated poorly ($< 2 \times 10^5$ conidia/plate) on YES agar, but there was a dose-related increase in sporulation to moderate levels ($> 10^7$ conidia/plate) on YES agar supplemented with up to 4 mg/ml caffeine. Further increases in the caffeine concentration of YES agar resulted in decreased levels of sporulation. Good sporulation ($> 10^9$ conidia/plate) occurred on PDA with or without caffeine supplementation. Stock cultures of BCR1 were subsequently grown and maintained on PDA + 4 mg/mL caffeine as a precaution against reversion.

The effects of caffeine on growth and aflatoxin production by non-agitated, conidia-initiated YES cultures of NRRL 2999 and BCR1 are presented in Fig 1. Caffeine affected the parent strain as described previously (Buchanan and Fletcher, 1978; Buchanan et al., 1983b) with 1 mg/mL delaying and 2 mg/mL inhibiting growth and aflatoxin formation. The mutant strain grew in the presence of 8 mg/mL caffeine, though the growth rate decreased with increasing caffeine concentration. The extent of growth was equivalent at caffeine levels ≤ 4 mg/mL, but was depressed at 8 mg/mL. Subsequent studies (not shown) indicated that BCR1 grew slowly in media saturated with caffeine. Aflatoxin production by BCR1 was caffeine-dependent. Little aflatoxin production occurred in the absence of caffeine, while the cultures supplemented with 4 mg/mL caffeine produced aflatoxins at levels roughly equivalent to those observed with the parent strain in the absence of caffeine. Similar studies performed with agitated cultures (data not shown) indicated that aeration did not influence the strains' responses to caffeine. BCR1 produced the greatest amount of aflatoxin when cultured in YES adjusted to an initial pH of 6.5 (Table 1); however, changes in initial pH did not greatly affect the overall caffeine-dependent nature of aflatoxin production by the isolate.

The effects of caffeine on aflatoxin production by replacement cultures of *A. parasiticus* NRRL 2999 and BCR1 were also studied to assess toxin formation more directly by mini-

mizing differentials in the rate and extent of growth (Buchanan and Lewis, 1984a, b). After sequential culturing in YES and PMS, 2.0g portions of the pregrown, previously disrupted mycelia (equivalent to 150–200 mg dry weight) were transferred to YEG medium and evaluated at 72 hr for toxin production (Table 2). The parent strain (NRRL 2999) produced abundant amounts of aflatoxins when incubated in YEG without caffeine. Toxin formation was strongly depressed by 1 mg/mL caffeine, and only low levels of toxin production were observed in cultures containing ≥ 2 mg/mL caffeine. Caffeine also prevented the approximate doubling in dry weight observed in the replacement cultures without caffeine. The observed effects of caffeine on aflatoxin production by replacement cultures of NRRL 2999 were similar to those observed with conidia-initiated cultures (Fig. 1) and agree with previous work employing replacement cultures (Buchanan and Lewis, 1984a).

The replacement cultures of strain BCR1 displayed equivalent increases in dry weight with caffeine levels up to 4 mg/mL but were inhibited by 8 mg/mL. Aflatoxin production was dependent on caffeine, with maximal production occurring in the cultures containing 2–4 mg/mL. However, unlike the conidia-initiated cultures, the caffeine-supplemented replacement cultures of BCR1 did not achieve a level of aflatoxin production equivalent to that attained by the parent strain in the absence of caffeine. The reason for this difference is not known; however, since the replacement culture technique involves growing the mold initially in caffeine-free media prior to transferring the mycelia to caffeine-supplemented replacement medium, this suggests that caffeine may affect aflatoxin synthesis in BCR1 by altering the mold's primary metabolism. Further experimentation is currently underway to evaluate this hypothesis.

The residual caffeine content of conidia-initiated YES cultures containing various initial levels of caffeine was monitored to determine if caffeine-resistance in BCR1 was due to the

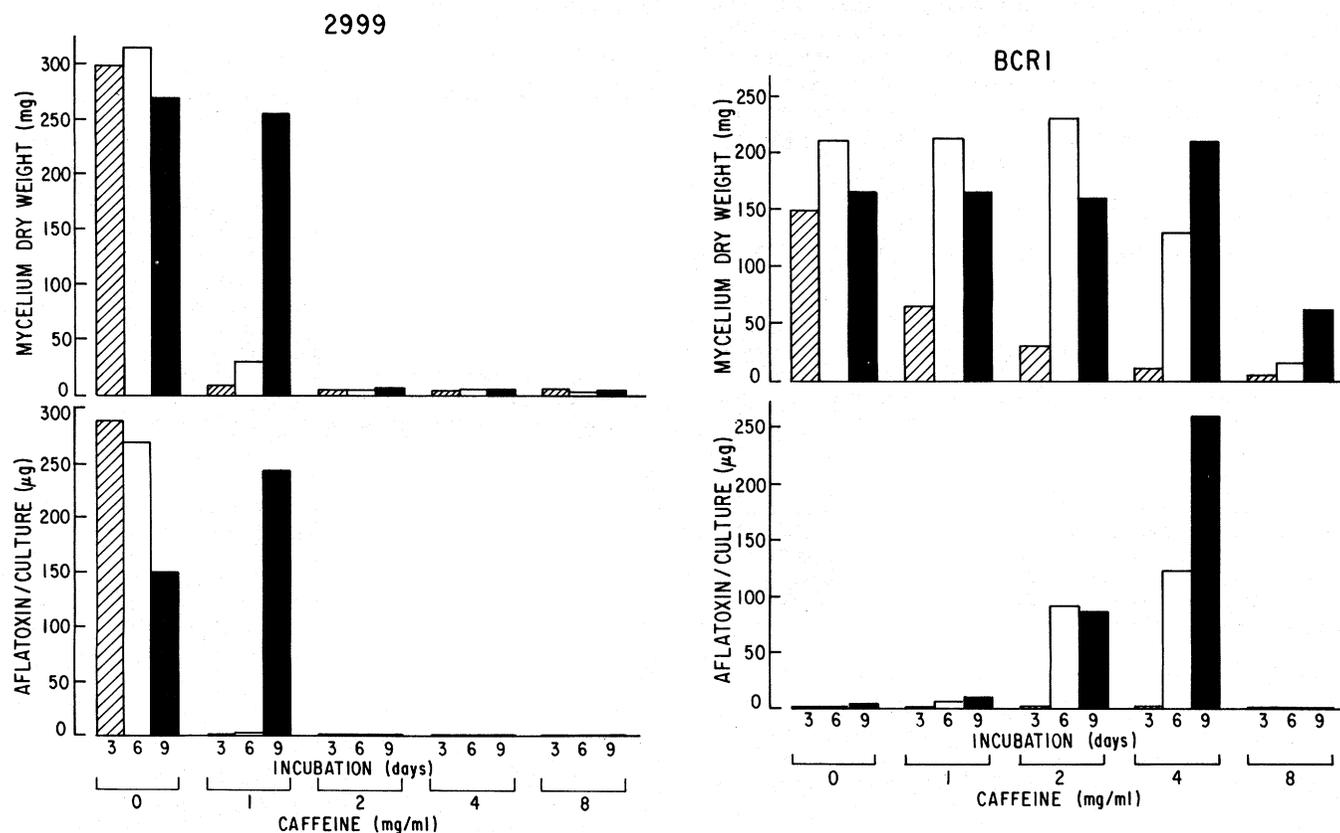


Fig. 1—Effect of caffeine on growth and aflatoxin production by Conidia-initiated YES cultures of *Aspergillus parasiticus* NRRL 2999 (left) and BCR1 (right).

Table 1—Effect of initial pH on growth and aflatoxin production by *A. parasiticus* BCR1 in conidia-initial YES cultures containing 0 and 4 mg/mL caffeine

Caffeine conc. (mg/mL)	Initial pH	Incubation time (days)	Mycelium dry wt (mg) ^a	pH ^a	Aflatoxin	
					culture (μg) ^a	mycelium (ng/mg)
0	4.5	5	199(30)	7.4	0.0	0
		11	139(1)	8.2	0.0	0
	5.5	5	211(5)	7.1	0.0	0
		11	134(2)	8.3	0.0	0
	6.5	5	217(3)	6.9	0.0	0
		11	135(3)	8.3	2.9(0.6)	21
7.5	5	209(4)	6.7	0.3(0.3)	1	
	11	132(3)	8.1	1.7(0.5)	13	
4	4.5	5	48(5)	4.6	0.3(0.1)	63
		11	143(3)	8.1	41.0(5.6)	287
	5.5	5	70(4)	5.2	3.6(0.8)	51
		11	147(7)	8.3	62.2(13.5)	423
	6.5	5	71(8)	5.6	46.6(10.3)	656
		11	140(2)	8.2	90.3(1.3)	644
7.5	5	33(4)	6.3	6.1(1.9)	185	
	11	132(2)	8.1	71.7(6.3)	543	

^a \bar{X} (± SEM), n = 3 replicate cultures.

Table 2—Effect of caffeine on the production of aflatoxin by replacement cultures of *A. parasiticus* NRRL 299 and BCR1^a

Strain	Caffeine conc (mg/mL)	Mycelium dry wt (mg) ^b	pH ^b	Aflatoxin	
				culture (μg) ^b	mycelium (ng/mg) ^b
NRRL 2999	0	428(11)	4.6	712.8(53.0)	1669(136)
	1	350(7)	5.0	15.9(0.6)	44(3)
	2	213(4)	6.0	2.1(0.1)	11(1)
	4	192(5)	6.1	2.6(0.3)	14(2)
	8	178(2)	6.2	1.9(0.1)	11(1)
BCR1	0	382(6)	4.9	6.4(1.3)	17(3)
	1	380(4)	5.2	5.9(1.6)	16(4)
	2	376(16)	5.2	27.4(1.8)	73(5)
	4	377(19)	5.3	21.2(3.1)	57(10)
	8	194(9)	6.0	0.0	0

^a Sequentially cultured in YES and PMS and then transferred to YEG and analyzed after 72 hr of post-transfer incubation.

^b \bar{X} (± SEM), n = 4 replicate cultures.

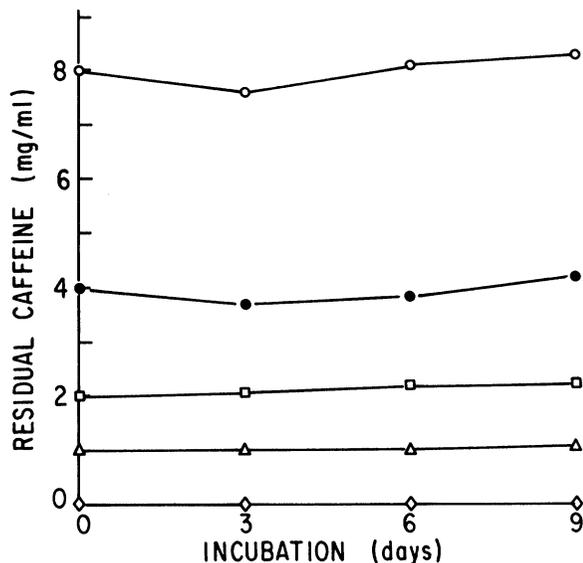


Fig. 2—Residual caffeine content of *Conidia*-initiated YES cultures of *A. parasiticus* BCR1 having an initial caffeine content of 0 (♦), 1 (△), 2 (□), 4 (●), and 8 (○), mg/mL.

strain having acquired the ability to metabolize caffeine (Fig. 2). Caffeine levels in the medium remained constant over the course of the nine day incubation, even though the mold actively grew at all caffeine concentrations. Similar results (not

shown) were obtained with replacement cultures of BCR1 and NRRL 2999 having initial caffeine concentrations of 4 mg/mL, indicating that neither the parent nor mutant strain were capable of metabolizing caffeine. It appeared that caffeine-resistance in BCR1 was not due to detoxification of caffeine.

DISCUSSION

THE PRESENT STUDY demonstrated the possibility of isolating variants of *A. parasiticus* that were able to grow and produce aflatoxins at caffeine concentrations that normally inhibited the mold. In some ways, the caffeine-resistant strain isolated in the current study is similar to the caffeine-resistant strains of *A. ochraceus* reported by Tsubouchi et al. (1985). For example, neither species produced large amounts of their respective mycotoxins unless cultured with a caffeine supplement. However, unlike *A. ochraceus* which completely depleted added caffeine, caffeine-resistance in *A. parasiticus* BCR1 was not associated with metabolic elimination of the compound. The caffeine-dependent nature of both aflatoxin production and sporulation in BCR1 suggested that the mutation involved a bioregulatory locus that influenced multiple developmental processes within the mold.

The mutants isolated in the current study were obtained without employing induced mutagenesis at a mutation rate of 7×10^{-8} . This ready isolation of caffeine-resistant variants suggested that naturally-occurring resistant strains of *A. parasiticus* (and probably *Aspergillus flavus*) were likely to exist in conjunction with caffeine-containing commodities. Confirmation of this supposition will require examination of isolates from commodities such as coffee, tea, and cocoa beans to assess caffeine-resistance and mycotoxigenesis. The results of the current study indicated further that in any assessment of mycotoxigenesis of molds isolated from materials containing a naturally-occurring inhibitor, mycotoxin production should be determined both in the presence and absence of the inhibitory compound. Strain BCR1 would have been classified as only a low-level aflatoxin producer if it had been evaluated only in media without caffeine.

REFERENCES

- Buchanan, R.L. and Fletcher, A.M. 1978. Methylxanthine inhibition of aflatoxin production. *J. Food Sci.* 43: 654.
- Buchanan, R.L., Harry, M.A., and Gealt, M.A. 1983a. Caffeine inhibition of sterigmatocystin, citrinin, and patulin production. *J. Food Sci.* 48: 1226.
- Buchanan, R.L., Hoover, D.G., and Jones, S.B. 1983b. Caffeine inhibition of aflatoxin production: mode of action. *Appl. Environ. Microbiol.* 46: 1193.
- Buchanan, R.L. and Lewis, D.F. 1984a. Caffeine inhibition of aflatoxin synthesis: probable site of action. *Appl. Environ. Microbiol.* 47: 1216.
- Buchanan, R.L. and Lewis, D.F. 1984b. Regulation of aflatoxin biosynthesis: effect of glucose on the activities of various glycolytic enzymes. *Appl. Environ. Microbiol.* 48: 306.
- Buchanan, R.L., Ocker, L.A., and Stahl, H.G. 1985. Effect of 2-deoxyglucose, α -methylglucoside, and glucosamine on aflatoxin production by *Aspergillus parasiticus*. *Arch. Microbiol.* 142: 200.
- Buchanan, R.L., Tice, G., and Mariano, D. 1982. Caffeine inhibition of ochratoxin A production. *J. Food Sci.* 47: 319.
- Davis, N.D., Diener, U.L., and Eldridge, D.W. 1966. Production of aflatoxins B₁ and G₁ in a semisynthetic medium. *Appl. Microbiol.* 14: 378.
- Lenovich, L.M. 1981. Effect of caffeine on aflatoxin production in cocoa beans. *J. Food Sci.* 46: 655.
- Nartowicz, V.B., Buchanan, R.L., and Segall, S. 1979. Aflatoxin production in regular and decaffeinated coffee beans. *J. Food Sci.* 44: 446.
- Tice, G. and Buchanan, R.L. 1982. Regulation of aflatoxin biosynthesis: effect of exogenously-supplied cyclic nucleotides. *J. Food Sci.* 47: 153.
- Tsubouchi, H., Terada, H., Yamamoto, K., Hisada, K., and Sakabe, Y. 1985. Caffeine degradation and increased ochratoxin A production by toxigenic strains of *Aspergillus ochraceus* isolated from green coffee beans. *Mycopathologia* 90: 181.

Ms received 5/23/86; revised 9/8/86; accepted 9/12/86.