

Further Characterization of a Caffeine-Resistant Mutant of *Aspergillus parasiticus*

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ABSTRACT

Studies were performed to characterize further *Aspergillus parasiticus* BCR1, a caffeine-resistant mutant of *A. parasiticus* NRRL 2999, particularly in regard to its caffeine-dependent production of aflatoxins. The enhanced synthesis of aflatoxins by caffeine was highly specific since neither dimethylxanthines nor purines could substitute for the trimethylxanthine. Caffeine's effects were phase dependent and only increased toxin formation if added early in the microorganism's life cycle. The ability of BCR1 to exclude caffeine appeared dependent on the initial levels of caffeine in the growth medium. Respiration and glucose utilization in the wild type strain were inhibited strongly by caffeine, but BCR1 was resistant to these effects. Comparison of glucose uptake kinetics in the wild type and mutant strains indicated that caffeine inhibition of aflatoxin synthesis in the wild type was not due to a disruption of glucose transport.

INTRODUCTION

CAFFEINE (1,3,7-trimethylxanthine) has been proposed to serve several functions in plants and is known to possess significant allelopathic, insecticidal and fungistatic/fungicidal activities. The last effect has been studied extensively, particularly in regard to caffeine's role as a naturally occurring inhibitor of polyketide mycotoxin formation in commodities such as coffee and cocoa beans (Buchanan and Fletcher, 1978; Nartowicz et al., 1979; Lenovich, 1981; Buchanan et al., 1982, 1983a, b; Betancourt and Frank, 1983; Buchanan and Lewis, 1984a; Durakovic et al., 1985a, b).

Tsubouchi et al. (1985) isolated from green coffee beans ochratoxin-producing strains of *Aspergillus ochraceus* that grew over an expanded range of caffeine concentrations. These strains were able to tolerate elevated levels of caffeine due to acquisition of the ability to degrade caffeine. Interestingly, these strains only produced abundant amounts of ochratoxin in the presence of caffeine. Buchanan et al. (1987) were able to isolate aflatoxigenic mutants of *A. parasiticus* that were resistant to caffeine. These resistant *A. parasiticus* strains were similar to the caffeine-resistant *A. ochraceus* strains isolated by Tsubouchi et al. (1985) in that substantially greater amounts of aflatoxins were produced when the mold was cultured in the presence of caffeine. However, unlike *A. ochraceus*, development of caffeine resistance in the *A. parasiticus* isolates was not related to acquisition of a caffeine degrading system. Buchanan and Stahl (1987) found that there was an interaction between caffeine-dependent aflatoxin synthesis and amino acid metabolism in the resistant *A. parasiticus* strains.

Since caffeine resistant mutants of *A. parasiticus* have been isolated only recently, relatively little information is available concerning these strains. Accordingly, the objective of the current study was to characterize more fully various cultural and physiological attributes of a resistant strain, comparing and contrasting it to the wild type parent strain. Particular emphasis was placed on examining the caffeine dependent nature of aflatoxin synthesis in the resistant strain.

MATERIALS & METHODS

Microorganisms

Aspergillus parasiticus BCR1, a caffeine-resistant strain (Buchanan et al., 1987), and *Aspergillus parasiticus* NRRL 2999, the wild type strain, were employed throughout the study. Stock cultures of NRRL 2999 were grown on potato dextrose agar (PDA, Difco) slants incubated at 28°C until well sporulated and then stored at 4°C. BCR1 was handled in a similar manner except PDA + 0.2% caffeine was employed to guard against reversion. Spore suspensions were prepared as described previously (Buchanan and Stahl, 1987) and diluted to contain approximately 1.0×10^6 conidia/mL.

Media

Yeast extract-sucrose medium (YES) (60g sucrose, 20g yeast extract (Difco), 1 L water) of yeast extract-glucose medium (YEG) (60g glucose, 20g yeast extract, 1L water) was used throughout the study. These media were adjusted to an initial pH of 6.0 using 10N HCl. Peptone-mineral salts medium (PMS) was prepared as previously described (Buchanan and Lewis, 1984b) and adjusted to pH 4.5. After being dispensed into appropriately sized flasks, all media were sterilized by autoclaving for 15 min at 15 psi. Caffeine, theophylline, theobromine and xanthine supplements were added directly to the media prior to autoclaving. While adenine and guanine compounds were added as filter-sterilized solutions after autoclaving.

Supplementation Studies

Supplementation studies were performed using 125-mL Erlenmeyer flasks containing 25 mL of appropriately supplemented YES. Each flask received 0.5 mL of spore suspension (BCR1 or NRRL 2999) to achieve an inoculum of 2×10^4 conidia/mL and was then incubated without agitation at 28°C. After designated periods of incubation, replicate cultures were analyzed for pH, aflatoxin production and mycelial dry weight. In experiments requiring the addition of caffeine after initiation of incubation, the compound was added directly to the cultures as a powder.

Respirometry

Oxygen uptake and carbon dioxide production by replacement cultures were monitored using the technique described by Buchanan and Lewis (1984b). After sequential culturing in YES and PMS, 200 mg portions of mycelia were transferred to Warburg flasks containing 3.0 mL of YEG with 0, 2 or 4 mg caffeine/mL. These flasks were incubated without agitation at 28°C. Periodically, the cultures were connected to a respirometer (model IG-20, Gilson International) and oxygen uptake and carbon dioxide release monitored for 20 min.

Determination of residual glucose

The effect of caffeine on glucose utilization by replacement cultures of BCR1 and NRRL 2999 was determined by monitoring residual glucose levels in the medium. After initial agitated culturing (28°C, 200 rpm, 72 hr) (Model G-26 Psycrothem Incubator, New Brunswick Scientific) of NRRL 2999 in YES and BCR1 in YES or YES + 0.2% caffeine, 0.5g portions of mycelial pellets were transferred to 50-mL flasks containing 20 mL of YEG with 0 or 4 mg caffeine/mL. All flasks were incubated without agitation at 28°C. After varying lengths of incubation, triplicate cultures of each caffeine concentration were harvested on cheesecloth and 10 mL samples of the medium collected and subsequently stored at -20°C until analyzed for residual glucose

concentrations. Glucose determinations were performed using a commercially available toluidine reagent (635-6, Sigma Chem. Co.).

Transport of glucose and amino acids

The transport of glucose into BCR1 and NRRL 2999 was initially studied using a modification of the technique of Buchanan and Lewis (1984a) in conjunction with low glucose concentrations (0.01-0.1 mM). The strains were initially grown in YES for 72 hr at 28°C and 150 rpm (Model 6-26 Psychrotherm Incubator, New Brunswick Scientific). The mycelial pellets were harvested and disrupted in a blender. The mycelia were incubated in fresh YES for 24 hr at 28°C and 150 rpm. The mycelia were then transferred in 100 mg portions to test tubes containing 1.0 mL of mineral salts solutions with 0 or 4 mg caffeine/mL. After incubating the tubes at 28°C for 1 hr, 1.0 mL of ¹⁴C(U)-glucose stock solutions was added to the tubes to achieve final glucose concentrations of 0.01, 0.05 and 0.10 mM in conjunction with a specific activity of 0.1 μCi/mL. After rapid mixing, cultures were incubated for 5 min, the mycelia were harvested, rinsed and then dried for 1 hr at 85°C. Uptake of glucose was then determined using a liquid scintillation counter (Model L8100, Beckman Instruments, Inc.).

A second series of glucose uptake determinations was performed using elevated glucose levels equivalent to those that support abundant aflatoxin formation. BCR1 and NRRL 2999 were initially grown in YES or YES + 0.2% caffeine (BCR1 only) for 72 hr at 28°C and 200 rpm (Model G-26 Psychrotherm Incubator, New Brunswick Scientific). The mycelia were transferred in 500 mg portions to sets of quadruplicate 50-mL Erlenmeyer flasks containing 20 mL of YEG with 0, 2, 4 and 8 mg caffeine/mL and incubated for 60 min at 28°C and 200 rpm. Each flask then received sufficient ¹⁴C(U)-glucose to achieve a specific activity of 0.1 μCi/mL. After 15 min incubation, the uptake of labelled glucose was determined as described above. The uptake of an amino acid mixture was determined in the same manner, substituting supplementation with ¹⁴C(U)-amino acid mixture for the radiolabelled glucose.

Uptake of caffeine

After initial culturing (72 hr, 200 rpm, 28°C) of NRRL 2999 in YES and BCR1 in YES or YES + 0.1% caffeine, 1.0g (wet weight) portions of mycelial pellets (0.5 g portions in case of BCR1 in YES + 0.1% caffeine) were transferred to 125-mL flasks containing 25 mL of YES + 4 mg/mL caffeine and incubated at 28°C and 200 rpm. After 0, 1, 2, 4, 6, 24, and 30 hr, triplicate cultures were harvested, rinsed thoroughly, and mycelial dry weights determined.

The intracellular caffeine content of these samples was then determined by transferring the dried samples to individual glass vials (70 × 26 mm) and extracting them overnight with 10 mL of 75% methanol in water. The solvent was then transferred to a 25-mL volumetric flask, and the mycelial sample was extracted with a second 10 mL portion of 75% methanol for 4 hr. This was followed by a third extraction for 4 hr with 4.5 mL of 75% methanol. The three extracts were combined in the volumetric flask and brought to volume with 75% methanol. The extracts were filtered through 0.45 μm nylon 66 filters and then analyzed for caffeine by high performance chromatography as described previously (Buchanan et al., 1987).

Aflatoxin analyses

Aflatoxins (B₁, B₂, G₁, G₂) were extracted (medium + mycelia) with chloroform, separated by thin-layer chromatography and quantified using a fluorodensitometer as described previously (Buchanan and Stahl, 1987). Mycelial dry weights were determined gravimetrically by drying the

Table 1—Growth and aflatoxin production by *Aspergillus parasiticus* BCR1 cultured for 7 days in YES supplemented with various methylxanthines^a

Supplement ^b	Mycelial dry wt (mg)	pH ^c	Aflatoxin/culture (μg)	Aflatoxin/mycelium (ng/mg)
None	197(2)	7.62	4.29(0.46)	21.8
Caffeine	131(4)	5.30	93.94(2.57)	717.1
Theophylline	226(5)	7.45	7.11(1.01)	31.5
Theobromine	243(9)	7.59	3.05(0.08)	12.6
Xanthine	248(4)	7.20	2.82(1.20)	11.4

^a Values = $\bar{X}(\pm\text{SEM})$, n = 4 replicate cultures.

^b Concentration of supplements = 4 mg/mL.

^c Initial pH = 6.0.

Table 2—Growth and aflatoxin production by *Aspergillus parasiticus* BCR1 cultured for 10 days in YES supplemented with various purine compounds^a

Purine ^b	Mycelial dry wt (mg)	pH ^c	Aflatoxin/culture (μg)	Aflatoxin/mycelium (ng/mg)
None	129.3(1.2)	8.6	0.0	0
Caffeine	173.6(9.4)	6.2	15.5(6.3)	89
Adenine	133.4(0.3)	8.6	0.0	0
Adenosine	131.8(2.2)	8.6	0.0	0
AMP	140.6(3.4)	8.6	0.0	0
Guanine	120.6(10.1)	8.4	0.0	0
Guanosine	135.6(0.7)	8.6	0.0	0
GMP	125.8(1.7)	8.6	0.0	0

^a Values = $\bar{X}(\pm\text{SEM})$, n = 3 replicate cultures.

^b Concentration of caffeine = 4 mg/mL, purines = 5 mM.

^c Initial pH = 6.0.

extracted mycelia for 24 hr at 85°C. An exception was the mycelia used for caffeine uptake determinations. In this instance dry weights were determined without prior extraction with chloroform.

RESULTS

THE SPECIFICITY of the caffeine-dependent nature of aflatoxin production in the caffeine-resistant strain BCR1 was assessed by comparing the effects of caffeine to the related compounds, theophylline, theobromine and xanthine (Table 1). Caffeine strongly stimulated aflatoxin formation, which is in agreement with earlier studies (Buchanan et al., 1987; Buchanan and Stahl, 1987). Accompanying this was a decrease in mycelial dry weights and a depression of the pH of the cultures. The other xanthine compounds had little, if any, effect on aflatoxin production, though all three stimulated growth to some degree.

Since the biochemical basis for caffeine's effects in various biological systems has often been associated with purine metabolism, supplementation studies were performed to determine if purine bases, nucleosides or nucleotides could be used in place of caffeine to stimulate aflatoxin production in BCR1 (Table 2). None of the purine compounds affected growth or aflatoxin production; only the caffeine-containing controls had significant toxin formation. The caffeine-containing cultures grew to a greater degree than the caffeine-free controls, which is directly opposite of the results observed in Table 1. This difference in growth responses may be due to the difference in incubation time and the depression of growth rate when BCR1 is grown in the presence of caffeine (Buchanan et al., 1987). However, in subsequent studies on the effect of time of caffeine supplementation (Fig. 1a), there was little difference in the extent of growth of caffeine-free and caffeine-containing cultures after 10 days of incubation. The reason for the variations in growth responses among the experiments is not readily apparent and requires further investigation.

While the time of caffeine supplementation did not affect greatly the extent of growth of BCR1 (Fig. 1a), it did impact significantly caffeine-dependent aflatoxin production (Fig. 1b). Aflatoxin production was greatest when caffeine was present at the time of inoculation and was reduced to some extent if added after one day of incubation. When caffeine supplementation occurred after two or more days, aflatoxin production was equivalent to that of the caffeine-free controls.

The effect of caffeine on respiration in NRRL 2999 and BCR1 was determined using replacement cultures (Fig. 2). Caffeine inhibited strongly both O₂ uptake and CO₂ evolution by the wild type strain, which is in agreement with results reported previously (Buchanan and Lewis, 1984a). Respiration in BCR1 was generally resistant to the effects of caffeine. Peak O₂ uptake in the absence of caffeine was delayed slightly in BCR1 as compared to NRRL 2999. Uptake was similar in the 2 mg/mL caffeine cultures and delayed further in 4 mg/mL cultures. Peak evolution of CO₂ was delayed (but not inhibited) in a dose-related manner by caffeine.

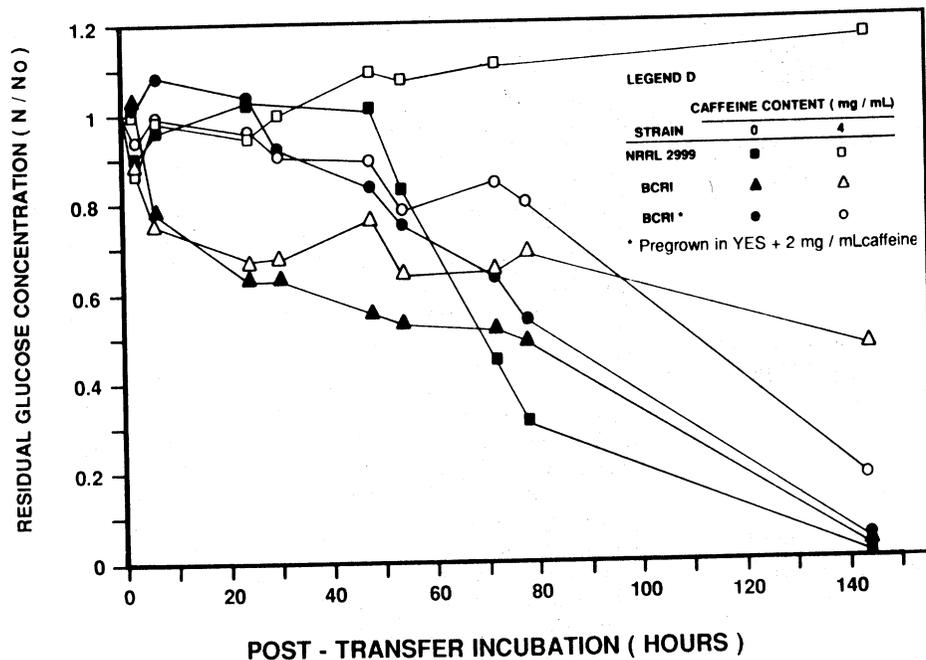


Fig. 3—Effect of caffeine on glucose utilization by replacement cultures of *Aspergillus parasiticus* NRRL 2999 and BCR1.

Table 3—Effect of caffeine on the uptake $^{14}\text{C}(\text{U})$ -glucose by replacement cultures of *Aspergillus parasiticus* NRRL 2999 and BCR1 containing 60 mg/mL glucose^a

Strain	Caffeine in growth medium (mg/mL)	Caffeine in replacement medium (mg/mL)	Glucose uptake ($\mu\text{mol}/\text{min}/\text{g}$ dry mycelia)
BCR1	0	0	3.11(0.90) ^b
		2	3.20(0.58)
		4	3.46(0.61)
		8	3.39(0.44)
	2	0	3.56(0.69)
		2	5.64(0.58)
		4	4.36(0.56)
		8	3.21(0.28)
NRRL 2999	0	0	3.81(0.78)
		2	2.97(0.31)
		4	4.52(0.39)
		8	4.31(0.24)

^a Cultured in YES (72 hr, 28°C, 200 rpm) and then transferred to YEG with 0.8 mg/mL caffeine. Incubated for 1 hr (28°C, 200 rpm) and then supplemented with $^{14}\text{C}(\text{U})$ -glucose to level of 0.1 $\mu\text{Ci}/\text{mL}$. After 15 min of incubation, glucose uptake determined.

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

of caffeine were detected within the mycelia of BCR1 grown initially in YES + 0.1% caffeine. In these cultures there was a transitory increase in caffeine levels immediately after transfer and an elevation in levels after extended incubation (24–30 hr); however, the levels attained were not as great as those observed with the cultures grown without caffeine.

DISCUSSION

THE CAFFEINE-DEPENDENT NATURE of aflatoxin production by the caffeine-resistant isolate, *Aspergillus parasiticus* BCR1, appears highly specific in that dimethylxanthines (theophylline and theobromine) could not substitute for the trimethylxanthine (Table 1). High levels of aflatoxin production in the absence of caffeine could not be restored by supplementation with purine bases, nucleosides or nucleotides (Table 2), suggesting that caffeine dependency does not involve an alteration in purine metabolism. However, this does not rule out the possibility that BCR1 has elevated levels of one or more purine compounds and that caffeine suppresses their production which in turn results in increased aflatoxin

Table 4—Effect of caffeine on the uptake of ^{14}C -amino acids by pregrown mycelia of *Aspergillus parasiticus* NRRL 2999 and BCR1^a

Strain	Caffeine in growth Medium (mg/mL)	Caffeine in replacement medium (mg/mL)	Amino acid uptake (nCi/min/g dry mycelia)
BCR1	0	0	1.13(0.39) ^b
		4	1.72(0.30)
	2	0	0.49(0.08)
		4	0.39(0.14)
NRRL 2999	0	0	0.51(0.04)
		4	0.65(0.12)

^a Cultured in YES (72 hr, 28°C, 200 rpm) and then transferred to YEG with 0 or 4 mg/mL caffeine. Incubated for 1 hr (28°C, 200 rpm) and then received ^{14}C -amino acids to achieve level of 0.1 $\mu\text{Ci}/\text{mL}$. Incubated for 15 min and amino acid uptake determined.

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

formation. The specificity of caffeine-dependent aflatoxin production in BCR1 is reminiscent of the specificity of caffeine inhibition in the wild type strain; NRRL 2999 was unaffected by other methylxanthines (Buchanan and Fletcher, 1978; Buchanan et al., 1978; Buchanan et al., 1983b) and the inhibition of aflatoxin production could not be reversed by purine supplementation (Buchanan et al., 1983b).

As a secondary metabolic process, aflatoxin production is initiated after cessation of exponential growth. Typically, aflatoxin production under conditions similar to those employed in the current study is initiated between 60–72 hr of incubation (Applebaum and Buchanan, 1979; Maggon et al., 1977). In the current study, caffeine stimulation of aflatoxin production in BCR1 was strongly growth phase dependent, and only occurred when the mold was exposed to caffeine early in its growth cycle (Fig. 1). This indicated that caffeine was not directly affecting the aflatoxin biosynthetic pathway, since its effect was greatest prior to the time period when the cultures were capable of synthesizing aflatoxins. Instead, caffeine appeared to be influencing some aspect of the mold's primary metabolism which in turn affected later synthesis of secondary metabolites such as aflatoxins. This is in agreement with Buchanan et al. (1987) who suggested that the mutation producing caffeine resistance involved a bioregulatory locus that influences multiple developmental processes within the mold.

Caffeine strongly impaired both respiration (Fig. 2) and glu-

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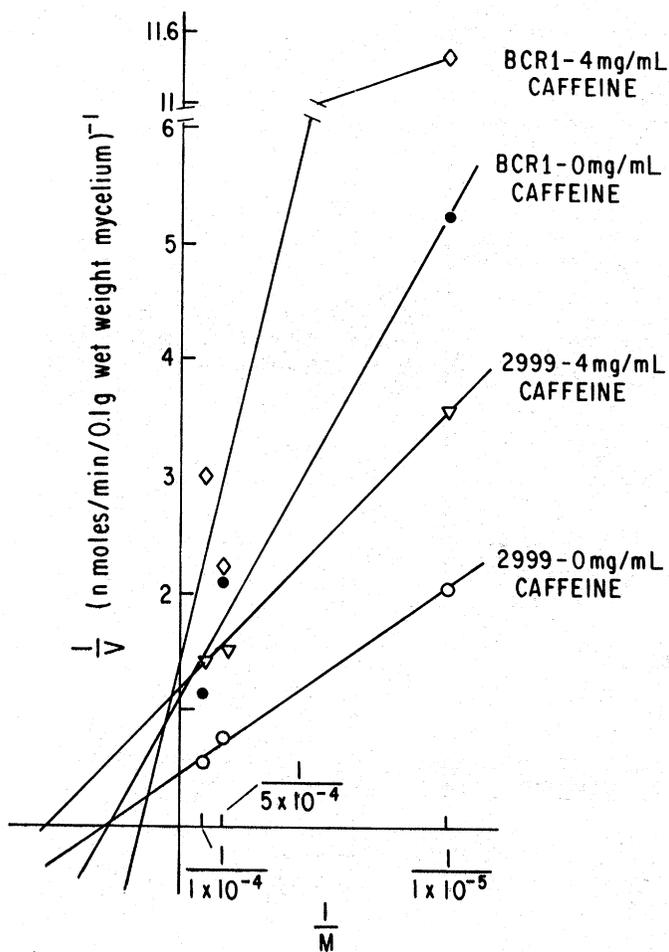


Fig. 4—Lineweaver-Burk plots of glucose uptake by replacement cultures of *Aspergillus parasiticus* NRRL 2999 and BCR1 cultured initially in YES and then transferred to media containing 10-100 μ M glucose and 0 or 4 mg/mL caffeine.

Table 5—Intracellular concentration of caffeine in mycelia of *Aspergillus parasiticus* NRRL 2999 and BCR1 after transfer to YES with 4 mg/mL caffeine

Post-transfer incubation (hr)	Intracellular caffeine (μ mol/g dry mycelium)			
	Initially cultured in YES		Initially cultured YES + 0.1% caffeine	
	NRRL 2999	BCR1	NRRL 2999	BCR1
0	ND ^b	ND	17.0(1.7) ^a	
1	72.2(16.5)	119.9(1.9)	50.1(3.4)	
2	100.6(7.3)	98.7(9.8)	20.6(4.4)	
4	114.3(1.9)	86.7(7.3)	21.9(4.6)	
6	79.6(11.6)	102.8(12.2)	24.4(2.4)	
24	111.9(0.9)	108.0(1.4)	79.0(16.0)	
30	105.3(5.9)	112.2(11.9)	61.5(21.1)	

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

^b None detected.

glucose utilization (Fig. 3) in the caffeine-sensitive wild type strain, which is in agreement with previous investigations (Buchanan and Lewis, 1984a). As might be expected both metabolic processes were active in the presence of caffeine in BCR1, though caffeine still affected this caffeine-resistant strain to some degree as evidenced by delayed peak respiration and depressed glucose utilization. These results suggested that aflatoxin production was not related directly to either respiration or glucose utilization. The respiratory and glucose utilization patterns of BCR1 most closely matched those of NRRL 2999 without caffeine (i.e., producing large amounts of aflatoxins) when the caffeine-resistant strain was also cultured without caffeine. However, aflatoxin production in BCR1 is only abun-

dant when the strain is cultured in the presence of caffeine (Table 1).

Buchanan and Lewis (1984a) hypothesized that caffeine affected aflatoxin production in NRRL 2999 via an inhibition of glucose transport. Similar results were observed in the current study (Fig. 4), indicating that caffeine inhibits glucose uptake by NRRL 2999 under conditions that favor active transport (i.e., low glucose concentrations). However, compared to NRRL 2999, glucose transport in BCR1 occurred at a substantially reduced rate and was inhibited further by caffeine. Considering that aflatoxin production by BCR1 occurs most abundantly in the presence of caffeine, the results suggest that the hypothesis of Buchanan and Lewis (1984a) is incorrect or incomplete. This interpretation is reinforced by the results of the glucose transport determinations performed under conditions more closely resembling cultural conditions associated with abundant aflatoxin production (i.e., unstarved cells exposed to elevated levels of glucose) (Table 3). The lack of effect in both NRRL 2999 and BCR1 suggested that caffeine did not inhibit glucose transport when the concentration of the sugar was such that it entered the cell by facilitated diffusion. This implied that when glucose was present at low concentrations, caffeine inhibited active transport by interfering with energy generating or utilizing processes. Even when glucose was present at high concentrations, it was likely that both sets of nutritional conditions existed simultaneously due to the geometry of the mycelia. Though cells on the exterior of a mycelial pellet or mat would be exposed to the high levels of glucose, it is likely that the cells within the interior would be nutritionally limited. However, this potential nutritional differential does not affect the overall implications of the results; neither caffeine inhibition of aflatoxin synthesis in NRRL 2999 nor caffeine stimulated aflatoxin production in BCR1 could be related directly to caffeine disrupting glucose transport.

The physiological significance of the amino acid transport data (Table 4) is unclear. The lack of effect within strain/culture groups indicated that caffeine did not directly affect amino acid transport in either strain. However, the approximate doubling of transport rates observed with BCR1 when the strain was initially grown without caffeine suggested that the caffeine resistance mutation might include an alteration of amino acid transport. Interestingly, when BCR1 was cultured initially with caffeine, amino acid transport rates dropped back to values equivalent to those of the wild type strain. These were also the cultures where the greatest amount of aflatoxin production was expected due to the time dependent nature of caffeine's stimulation of toxin formation. This suggested a possible relationship between amino acid transport and caffeine dependent aflatoxin production in the caffeine-resistant strain. Previous work has established that abundant aflatoxin production in BCR1 occurs only when cultures contain both caffeine and one or more of a group of specific amino acids (Buchanan and Stahl, 1987).

The mechanism underlying caffeine resistance in BCR1 is not known. Based on the results of residual caffeine determinations, Buchanan et al. (1987) concluded that neither BCR1 nor the wild type strain was capable of metabolizing caffeine to any significant degree, eliminating this as a potential resistance mechanism. Intracellular caffeine determinations in the current study (Table 5) indicated that there were no differences in the levels of caffeine within BCR1 and NRRL 2999 after transfer from a caffeine-free medium to one containing 4 mg/mL. This implied that BCR1 had not acquired an increased ability to exclude caffeine. However, substantially reduced intracellular levels of caffeine were detected when BCR1 was cultured initially in medium containing 1 mg/mL caffeine and then transferred to medium with 4 mg/mL. This suggested that the level of caffeine accumulation within BCR1 may be influenced by the level of caffeine in the environment during an early stage of its growth from conidia. This and other questions concerning the mechanism of caffeine resistance in *A. para-*

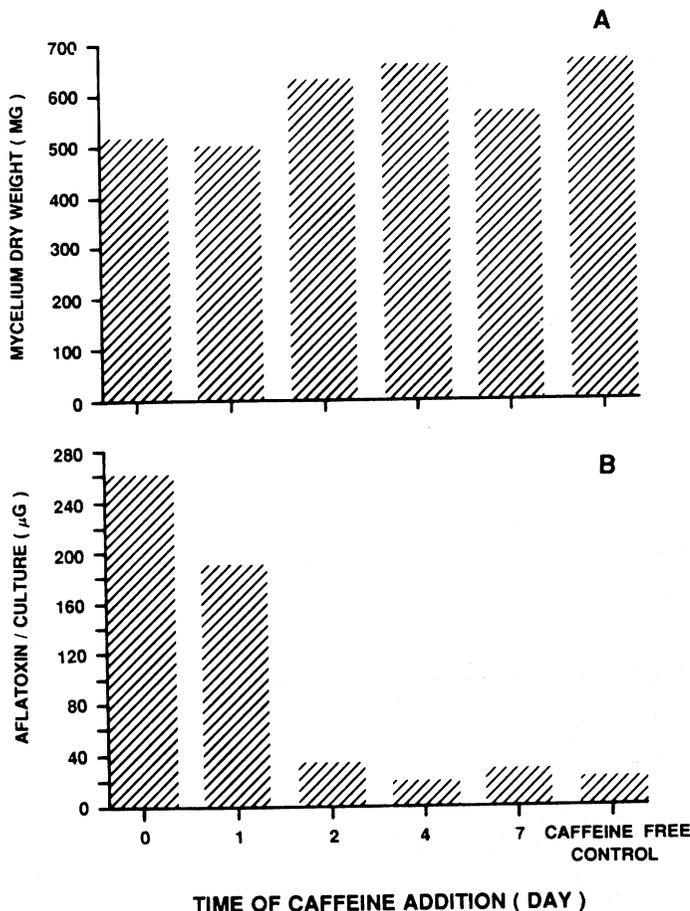


Fig. 1—Effect of the time of caffeine (4mg/mL) addition on growth (A) and aflatoxin production (B) by *Aspergillus parasiticus* BCR1 cultured in YES. All cultures were incubated a total of 10 days.

The effect of caffeine on glucose utilization by the wild-type and mutant strains was assessed by determining the residual glucose concentrations of replacement cultures (Fig. 3). NRRL 2999 depleted (90% utilization) the medium of glucose in the absence of caffeine but did not utilize the sugar when caffeine was present at a concentration of 4 mg/mL. This is in agreement with results reported previously (Buchanan and Lewis, 1984a). BCR1 utilized glucose to an extent equivalent to that of the wild type strain in the absence of caffeine and depleted approximately 50% of the glucose in the cultures containing caffeine. When BCR1 was initially cultured in YES + 0.2% caffeine and then transferred to YES without caffeine, the extent of glucose utilization was again equivalent to that of the wild-type strain and BCR1 initially cultured without caffeine. However, when BCR1 was initially cultured in caffeine-containing medium and then transferred to YES with 4 mg/mL caffeine, the extent of glucose utilization was significantly greater than the corresponding BCR1 cultures grown initially without caffeine.

The effect of caffeine on glucose transport by NRRL 2999 and BCR1 was assessed using two different approaches. Glucose uptake determinations were performed initially using low levels of glucose (0.01–0.1 mM) in conjunction with pre-starved mycelia reverted previously to a filamentous state (Fig. 4). Under these conditions, caffeine inhibited glucose uptake by NRRL 2999 in manner similar to that noted previously by Buchanan and Lewis (1984a). The rate of glucose transport by BCR1 was substantially less than NRRL 2999 in the absence of caffeine and was depressed further by caffeine. The degree of glucose transport inhibition by caffeine in both strains appeared inversely related to the initial glucose concentration.

Glucose transport was reassessed using conditions more

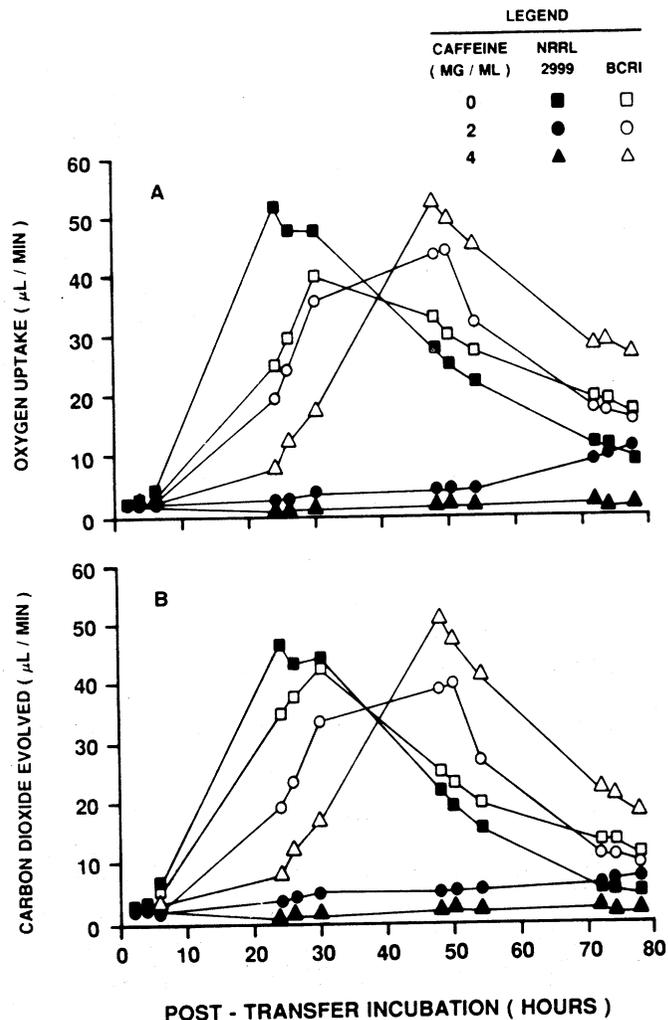


Fig. 2—Effect of caffeine on oxygen uptake (A) and carbon dioxide evolution (B) by replacement cultures of *Aspergillus parasiticus* NRRL 2999 and BCR1.

closely approximating those associated with abundant aflatoxin production (Table 3). This entailed using intact, unstarved mycelial pellets in conjunction with an elevated level of glucose (330 mM). Under these conditions, glucose transport by NRRL 2999 and BCR1 were similar and unaffected by caffeine. Glucose uptake by BCR1 was not affected by initially growing the strain in media containing caffeine.

A similar set of conditions was used to study the effect of caffeine on amino acid uptake by NRRL 2999 and BCR1 (Table 4). Caffeine did not significantly affect amino acid uptake in the wild type strain. Amino acid uptake by BCR1 grown initially in the absence of caffeine was approximately twice that of NRRL 2999, and there was a trend towards increased uptake rates in the presence of caffeine. However, when BCR1 was grown initially in the presence of caffeine (2 mg/mL), the observed transport rate was less than those of NRRL 2999 and was unaffected by caffeine.

Based on residual caffeine determinations, it had been established previously (Buchanan et al., 1987) that neither the wild type strain, NRRL 2999, nor its caffeine resistant derivative, BCR1, metabolized caffeine. The current study examined this further by determining the intracellular concentrations of caffeine (Table 5). When initially grown in YES and then transferred to caffeine-containing medium, caffeine was detected in similar concentrations in washed mycelia of both strains. Uptake appeared rapid, with peak concentrations being achieved within 1-2 hr of transfer. Substantially lower levels

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siticus BCR1 and related caffeine resistant strains await the results of future research.

REFERENCES

- Applebaum, R.S. and Buchanan, R.L. 1979. Intracellular concentrations of cAMP and cGMP in an aflatoxigenic strain of *Aspergillus parasiticus*. *J. Food Sci.* 44: 116.
- Betancourt, L.E. and Frank, H.K. 1983. Bedingungen des mikrobiellen verderbs von grünem kaffee. *Deut. Lebn.-Rund.* 79: 404.
- Buchanan, R.L., Applebaum, R.S., and Conway, P. 1978. Effect of theobromine on growth and aflatoxin production. *J. Food Safety* 1: 211.
- 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. and Stahl, H.G. 1987. Characterization of a caffeine-resistant mutant of *Aspergillus parasiticus*: Role of amino acid metabolism. *J. Food Sci.* 52: 1718.
- Buchanan, R.L., Tice, G., and Mariano, D. 1982. Caffeine inhibition of ochratoxin A production. *J. Food Sci.* 47: 319.
- Buchanan, R.L., Zaika, L.L., Kunsch, C.A., Purcell, C.J., Jr., and Mertz, S.E. 1987. Isolation of a caffeine-resistant mutant of *Aspergillus parasiticus*. *J. Food Sci.* 52: 194.
- Durakovic, S., Durakovic, Z., Beritic, T., Radic, B., Lalic, L.M., and Delas, F. 1985a. Biosynthesis of aflatoxins by *Aspergillus parasiticus* on roasted coffee beans. *Periodicum Biologorum* 87: 503.
- Durakovic, S., Durakovic, Z., Lalic, L.M., Pospisil, O., and Radic, B. 1985b. Influence of selected cultivation parameters on the growth of the toxigenic mold *Aspergillus parasiticus* on coffee beans and the biosynthesis of aflatoxins. *Microbiology (Belgrade)* 22: 1.
- Lenovich, L.M. 1981. Effect of caffeine on aflatoxin production in cocoa beans. *J. Food Sci.* 46: 655.
- Maggon, K.K., Gupta, S.K., and Venkatasubramanian, T.A. 1977. Biosynthesis of aflatoxins. *Bacteriol. Rev.* 41: 822.
- Nartowicz, V.B., Buchanan, R.L., and Segal, S. 1979. Aflatoxin production in regular and decaffeinated coffee beans. *J. Food Sci.* 44: 446.
- 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.

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