Suppression of *Aspergillus flavus* in Raisins by Solar Heating During Sun Drying

A. M. Hussein, N. F. Sommer, and R. J. Fortlage

Former graduate research assistant, postharvest pathologist, and research associate, Department of Pomology, University of California, Davis 95616. Present address of first author, assistant professor, Department of Horticulture, University of Alexandria, Egypt.
Based on a portion of a Ph. D. thesis submitted by the first author.
Accepted for publication 31 October 1985 (submitted for electronic processing)

ABSTRACT

Hussein, A. M., Sommer, N. F., and Fortlage, R. J. 1986. Suppression of *Aspergillus flavus* in raisins by solar heating during sun drying. Phytopathology 76:335-338.

Aflatoxin was readily produced in fresh grape berries needle inoculated with conidia of *Aspergillus flavus*, reaching 2,781 μg/kg after 6 days at 25 C. By contrast, aflatoxin reached only 147 μg/kg during the same period when conidia were inoculated into a small lesion caused by *Botrytis cinerea*. Fresh berries inoculated with the same conidial concentration of *A. flavus* and equal numbers of conidia of *B. cinerea* and *Cladosporium herbarum* accumulated an intermediate concentration of aflatoxin. During sun drying in the field, failure of germinating conidia of *A. flavus* to survive internal berry temperatures of 45–50 C or higher may explain the lack of aflatoxin in raisins and inability to reisolate *A. flavus* from test berries. In vitro tests demonstrated that exposure of germinating conidia of *A. flavus* for 30 min at 45 and 50 C reduced survivors to 0.7 and 0.025%, respectively. Internal berry temperatures during sun drying exceeded those temperatures on days with afternoon temperatures of 35 C or higher.

Additional key words: *Aspergillus parasiticus*, mycotoxins, *Vitis vinifera*.

Aflatoxins produced by *Aspergillus flavus* Lk. et Fr. or *A. parasiticus* Speare have been found in many foods or feeds normally stabilized by drying. These include cereal grains, oil seeds, legume seeds, tree nuts, peanuts, and figs (4,12). Aflatoxin contamination follows fungal colonization that occurs before or after harvest. Commodities may be susceptible to *Aspergillus* sp. during drying until moisture becomes limiting, and in storage if they are inadequately dried or inadvertently remoisturized.

The literature has few reports of grapes (*Vitis vinifera* L.) contaminated by aflatoxin. However, Hewitt (3) reported that *A. flavus* was a secondary rot fungus of grapes in California. Schuller et al (10) reported low levels of aflatoxin B_1 in 2 of 33 German wines. Takahashi (13) found aflatoxin in sweet wines imported into the United States. The presence of aflatoxin in wines indicates that grapes had been infected with *A. flavus*.

In the production of raisins, the usual practice is to dry grapes on paper in the vineyard between vine rows. The berries would seemingly be vulnerable to invasion by *A. flavus* and might become contaminated with aflatoxin during the several weeks required to complete drying. Nevertheless, raisins seem to have been free of association with aflatoxin. A survey (12) by the U. S. Food and Drug Administration (FDA) failed to detect aflatoxin in 108 raisin samples.

Understanding why raisins are not contaminated with aflatoxin in the presence of *A. flavus* might assist in the development of methods for avoiding the fungal metabolite in other commodities. This study was designed to determine: whether *A. flavus* is a pathogen of mature grape berries and, if so, whether it would produce aflatoxin in the berries; whether *A. flavus* grows and produces aflatoxin as a mixed infection with other fruit pathogens; whether raisins produced under commercial drying conditions from berries inoculated with *A. flavus* contain aflatoxin; and whether internal berry temperatures during sun drying are lethal to resting and germinating conidia of *A. flavus*?

MATERIALS AND METHODS

Fungus culture. The culture of *A. flavus* used in this study was obtained from a collection maintained by the second author. Cultures were routinely stored on potato-dextrose agar (PDA) slants in a household-type refrigerator at about 4 C. The isolate was found by Buchanan et al (2) to be pathogenic in mature fig fruits and by Sommer et al (11) to be pathogenic in pistachios; high

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

concentrations of aflatoxins were produced in both. Preliminary tests showed that the isolate had retained its ability to produce abundant aflatoxin in vitro. The isolate was highly pathogenic in mature grape berries and aflatoxin was produced.

Conidia of *A. flavus* were produced by growing colonies on 75 ml of sporulation medium (6) in 300-ml Erlenmeyer flasks incubated at 22–24 C. Conidia were harvested from colonies about 3 wk old, as described by Buchanan et al (2). A Tween-80 solution (one drop per 100 ml of sterile distilled water) was used for harvesting and for subsequent spore suspensions. Sterile glass beads and 50 ml of Tween-80 solution were added to the culture flask. The flask was briefly hand-swirled to dislodge and wet the conidia. The suspension was aseptically transferred to a sterilized 50-ml centrifuge tube and conidia were sedimented in a clinical centrifuge. The supernatant was discarded and the conidia were rinsed thrice by resuspending them in a 50-ml aliquot of Tween-80 solution, recentrifuging, and discarding the supernatant.

The concentration of washed conidia suspended in Tween-80 solution was determined with a Bausch and Lomb Spectronic-20 spectrophotometer. Absorbancy was compared with a standard curve for *A. flavus* we previously established by relating absorbancy to conidial concentrations determined by counting with a hemocytometer.

Conidia of *Botrytis cinerea* Pers. ex Fr. and *Cladosporium herbarum* Lk. ex Fr. were grown on PDA but were otherwise handled as described for *A. flavus* above.

Sensitivity to heat. The in vitro sensitivity of resting and germinating conidia of *A. flavus* to temperatures of 45 and 50 C was tested. Heat treatments were done in a water bath equipped with a thermoregulator that maintained temperatures with variations of <0.2 C. Volumetric flasks (25 ml) containing 10 ml of potato-dextrose broth were partially submerged in the water and held for at least 1 hr for temperature equilibration before the tests started. Tests were initiated by adding a 0.1-ml aliquot of spore suspension at approximately 25 C. After completion of the exposure period, the treatment was terminated by removing the test flask from the water bath and adding 10 ml of sterile potato-dextrose broth which had been chilled to 3–5 C. The flask was swirled to quickly lower the temperature. The volume was adjusted to 25 ml by adding cool potato-dextrose broth and the flask was stoppered with a sterile rubber stopper.

An initial conidial suspension of either 2.5×10^5 or 2.5×10^6 conidia per milliliter was prepared before heating. After the dilutions indicated earlier were made, 100 or 1,000 conidia, respectively, were delivered to each plate. The latter concentration was used when the heat treatment was expected, from the results of preliminary tests, to result in $<1\%$ survivors.

Viability of test conidia was determined by incubation on PDA in petri dishes. The medium in the test flask was transferred to a deep petri dish which was frequently swirled to keep spores in suspension. A hand replicator was fashioned to transfer the spores to the surface of the PDA in culture plates. The hand replicator was made by driving 40 finishing nails spaced at equal distances in a circle of about 90 mm diameter into a small wood block ($15 \times 15 \times 2$ cm). When the flame-sterilized replicator was dipped into the spore suspension with nail heads down, and removed, the liquid beaded on them. A total of approximately 0.1 ml of spore suspension was delivered by gently touching the replicator to the agar. Conidia were distributed evenly over the agar surface with a sterile bent glass rod spreader. Ten petri dishes were used for each treatment and they were incubated at 22–25 C for 24–96 hr.

Inoculated berries. Berries of *Vitis vinifera* L. "Thompson Seedless" were grown at the Kearney Horticultural Field Station (KHFS) of the University of California. In tests, a concentration of 10^7 conidia per milliliter was used for inoculating berries which were wounded by stabbing them with a needle contaminated with the spore suspension. In certain tests, mixed inoculum consisted of equal concentrations of conidia of *A. flavus, Botrytis cinerea* Pers. et. Fr., and *Cladosporium herbarum* Lk. ex Fr. In other tests, conidia of *A. flavus* were inoculated into established lesions of *B. cinerea*. Aflatoxin produced in lesions resulting from the mixed inoculum or in lesions caused by *B. cinerea* was compared with that found in lesions inoculated solely with conidia of *A. flavus*. Berries were incubated for 6 days in constant-temperature rooms where temperatures cycled <1 C from the mean temperature of 25 C.

Commercial drying practices were followed in field tests conducted at the Kearney Horticultural Field Station, which is within the commercial raisin-producing area. Grape bunches were harvested and each berry was inoculated as described above. The clusters of berries were then placed between vine rows on kraft paper sheets, 61×91.5 cm. The bunches were turned on the 10th day and were dry by the 21st. The paper sheets were rolled up, with the dried berries in place, and brought to the laboratory where they were separated from stems and other foreign matter by screening.

Reisolation of *A. flavus* from raisins. Samples of raisins, which had been individually needle inoculated before sun drying in the field, were plated to determine whether viable conidia of *A. flavus* were present. These raisins were surface-sterilized with sodium hypochlorite (0.5%) for 2 min. Pieces of tissue from individual raisins were cut with a sterile scalpel and plated aseptically on PDA in a petri dish. Microscopic examination of colonies followed 4–6 days of incubation at 20–24 C.

Berry temperature during drying. Internal temperatures of grape berries were recorded at the Kearney Horticultural Field Station from 31 August to 21 September 1978. Thompson Seedless grape clusters were placed on paper trays for drying. Thermocouples (copper-constantan) were inserted into the middle of six berries and immediately beneath the skin of another six berries which were mostly exposed to sunlight. Berry temperatures were compared with air temperatures in shaded locations. Internal temperatures of berries on a bright, warm day with a maximum shade temperature of 37 C were compared with berry temperatures on an overcast day with the highest shade temperature of 20 C.

Additional tests were conducted at Davis in 1984 to test internal temperatures of berries on the top, middle, and bottom of clusters laid out to dry. Thermocouples were placed in the center of three berries in each of the following locations: top of cluster, constantly exposed to sunlight; in the middle of the cluster, not continuously exposed to sunlight; in the bottom of clusters, mostly shaded, and in the air (to measure ambient temperatures) in a constantly shaded location. Recording started by 0900 hr and was terminated at about 1800 hours after a cooling trend was established.

Air temperatures in the raisin-producing area. Temperature data for the first 21 days of September, during a 14-year-period starting in 1971 and concluding in 1984, were obtained from weather records maintained at the Kearney Horticultural Field Station. An exception was 1978 in which temperatures during the drying period were not recorded due to equipment failure. Data from the nearby Fresno Airport (National Weather Service) are also provided. The number of days during the sun-drying period each year that temperatures equaled or exceeded 30, 32.5, 35, 37.5, and 40 C was determined.

Aflatoxin analyses. Analyses were done as described by Buchanan et al (2). Aflatoxin was confirmed by two procedures: the method of addition wherein samples were chromatographed by high-pressure liquid chromatography with and without the addition of a standard solution of aflatoxins B_1 and G_1; and thin-layer chromatography of the reaction product of trifluoroacetic acid (TFA) with extracts containing aflatoxin as described by Andrellos and Reid (1) and Pohland et al (5).

RESULTS

A. flavus is a wound pathogen of mature grape berries under controlled-environment conditions, and aflatoxin accumulated to over 2,700 μg/kg during a six-day period at 25 ± 1 C (Table 1). Approximately half of the aflatoxin total was aflatoxin B_1 and the remainder was G_1. No more than traces of aflatoxins B_2 and G_2 were found.

When grape berries were inoculated with equal concentrations of conidia of *A. flavus, B. cinerea*, and *C. herbarum*, the level of aflatoxin produced was roughly one-third that found with conidia of *A. flavus* alone. Aflatoxin that accumulated during 6 days at 25 C after conidia of *A. flavus* were inoculated into small, established

lesions caused by *B. cinerea* was only about 5% as much as was found when *A. flavus* colonized healthy tissue. No aflatoxin was detected after inoculated berries were sun-dried for 21 days by the commercial method. Further, repeated attempts failed to reisolate *A. flavus* from raisins produced by drying inoculated berries.

Data in Table 2 show that on a sunny day, in which the shade temperature reached 37 C and ranged from 35 to 37 C for 3 hr, the temperature at the middle of berries exceeded 45 and 50 C for 3 and 1.75 hr, respectively. The temperature of tissues just under the skin reached a maximum of 57 C and was in excess of 55 C for over 1.5 hr. By contrast, on a cool, overcast day, the internal temperatures of berries exceeded the air temperature by no more than about 2 C (Table 2).

At Davis, CA, during July 1984 the effect of exposure to bright sunlight on the internal temperature of berries was further explored. Berries fully exposed to sunlight were compared with those berries in the middle of the cluster and with those on the bottom resting on the paper tray. With thermocouples placed in the center of a berry, the uppermost berries exceeded the ambient temperature by as much as 16 C (Table 3). The maximum temperature differences, compared to the ambient temperature, was 12 C and 10 C for middle and bottom berries, respectively.

TABLE 1. Aflatoxin content of cultivar Thompson Seedless grape berries incubated 6 days at 25 C following inoculation of each berry with a needle contaminated with conidial suspension (10^7/ml) of *Aspergillus flavus*

Treatment	Aflatoxin (μg/kg)		
	B_1	G_1	Total
Inoculated with *A. flavus*	1,416	1,365	2,781[z]
Mixed inoculum-*A. flavus*, *Cladosporium*, and *Botrytis cinerea*	410	481	891
Conidia of *A. flavus* placed in a lesion caused by *B. cinerea*	72	75	147

[z] Means of total aflatoxin between treatments were significantly different, $P = 0.05$, according to Duncan's multiple range test.

TABLE 2. Internal berry temperatures with grapes exposed to direct sunlight on a bright, warm day and on a cloudy day compared to the ambient (shade) temperature during the 1978 raisin-drying season

	Temperature (C)[z] at time (hours)						
	0900	1030	1200	1330	1500	1630	1800
Bright, warm day							
Ambient[z]	12	18	28	35	37	35	22
Berry center	12	24	43	52	50	42	26
Below skin	12	25	43	57	55	46	25
Cloudy day							
Ambient	15	15	17	19	19	20	18
Below skin	15	15	19	20	21	20	19

[z] Temperatures were the average of six readings. Thermocouples were placed either in the center of the berry or immediately below the skin. Data for berry centers on the cloudy day were lost due to instrument malfunction.

TABLE 3. Comparison of ambient (shaded, air) temperatures and internal temperatures of grape berries exposed to sun-drying as measured with thermocouples placed in the centers of berries during the 1984 tests

	Temperature (C)[y] time, (hours)						
	0900	1030	1200	1330	1500	1630	1800
Ambient	26	29	32	36	39	43	41
Berries in full sun[z]	29	41	48	51	53	50	41
Middle berries	27	33	41	47	51	50	41
Bottom berries	29	37	42	44	46	46	41

[y] Temperatures were the average of three readings.

[z] Berries at the top of the layer were in full sun, those in the center were partially shaded, and those at the bottom were mostly shaded, compared with the ambient (shade) temperature.

Records covering a 14-year period (Table 4) show that during the first 21 days of September, a temperature of 35 C or higher was reached on 5–19 days at the Kearney Horticultural Field Station (1978 data are missing) and on 0–17 days at the Fresno Airport.

In vitro tests (Fig. 1) of the sensitivity of ungerminating conidia of *A. flavus* showed that about 2% of 1,000 spores plated survived after being heated for 4 hr at 50 C. Heating for the same period at 45 C caused little or no reduction in survival. By contrast, conidia incubated for 4 or 6 hr at 20 C were more sensitive to heat. After incubation for 4 hr, approximately 2 and 0.2% survived 30 min at

TABLE 4. Days with maximum temperatures at or above that indicated during the first 21 days of September, the usual period for sun drying of raisins

Year	Number of days at or above				
	30 C	32.5 C	35 C	37.5 C	40 C
1971	21 (21)	15 (14)	12 (12)	6 (8)	4 (1)
1972	16 (13)	13 (8)	9 (1)	1 (1)	1 (0)
1973[z]	20 (19)	14 (6)	8 (0)	1 (0)	0 (0)
1974	21 (21)	19 (19)	18 (17)	10 (3)	2 (0)
1975	21 (19)	20 (11)	13 (5)	5 (0)	3 (0)
1976	16 (10)	8 (9)	7 (6)	4 (3)	3 (0)
1977	13 (13)	11 (11)	10 (8)	9 (5)	3 (2)
1978[z]	(6)	(6)	(3)	(2)	(0)
1979	21 (21)	21 (21)	15 (17)	8 (8)	3 (4)
1980	16 (16)	11 (11)	5 (5)	1 (1)	0 (0)
1981	21 (21)	20 (20)	17 (14)	1 (1)	0 (0)
1982	15 (14)	11 (9)	7 (7)	2 (1)	1 (0)
1983	21 (21)	20 (19)	17 (14)	10 (10)	5 (5)
1984	21 (21)	20 (20)	19 (17)	14 (11)	8 (6)
Averages	19 (17)	16 (13)	12 (9)	6 (4)	3 (1)

[z] Data were recorded at the Kearney Horticultural Field Station (KHFS) and at the Fresno Airport (in parentheses). Temperatures data at KHFS are missing for 1 day during the 1973 grape-drying period and were largely missing in 1978 due to instrument failure.

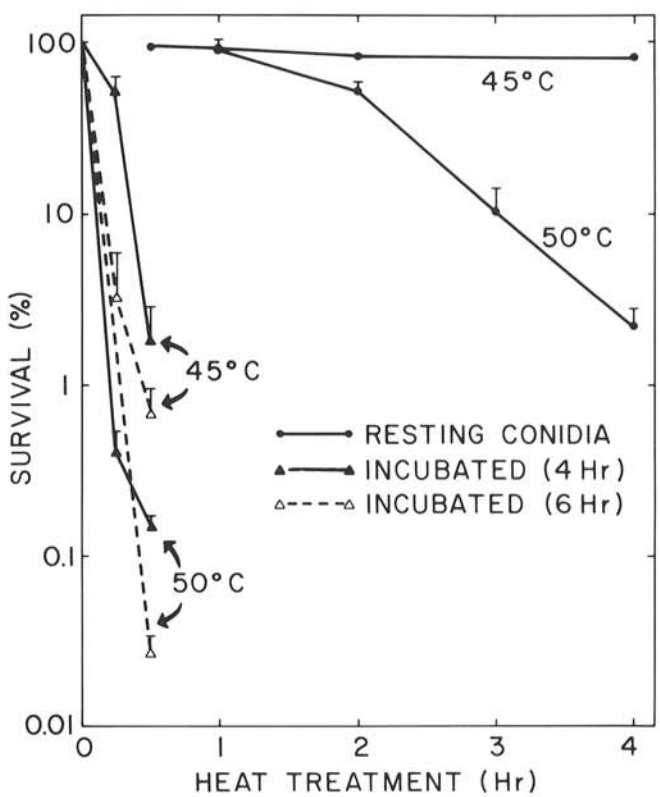

Fig. 1. Survival of resting conidia of *Aspergillus flavus* after exposure to temperatures of 45 or 50 C compared with survival of germinating spores after 4 or 6 hr of incubation at 25 C.

45 and 50 C, respectively. After incubation for 6 hr at 20 C, survival after 30 min at 45 and 50 C was 0.7 and 0.025%, respectively.

DISCUSSION

Aflatoxin is readily produced in inoculated grape berries at concentrations that might cause considerable health concern. Production of aflatoxin was reduced, but not eliminated, when *A. flavus* grew in competition with *C. herbarum* or *B. cinerea* or was growing in a developed lesion caused by the latter fungus. The natural incidence of berry infection by *A. flavus* must be extremely low to have escaped notice in surveys of raisins (12) for aflatoxin.

The absence of infection and subsequent aflatoxin accumulation when berries were inoculated in the field instead of in the constant-temperature laboratory was not readily explainable. We hypothesized that very high temperatures found within berries in direct sunlight might be lethal to conidia of *A. flavus*. Several studies (7–9) have explored the effects of temperatures on growth of the fungus. However, we are unaware of any studies on the possible lethal effects of solar heating. Initial in vitro tests in which we used ungerminated conidia did not readily support our high-temperature inactivation hypothesis. However, the much greater sensitivity of germinating spores suggested that spore inactivation by heat might indeed play an important role in limiting aflatoxin in raisins. It appears likely that the night-day fluctuations in temperature would enhance the lethality of day-time heating. Temperatures at night would be highly favorable for conidia to germinate in fruit wounds. Such conditions would often be followed with afternoon air temperature and solar heating conditions that would heat the berries for several hours to an internal temperature highly lethal to germinated conidia and young mycelia.

During periods of intense sunlight, ultraviolet irradiation was possibly lethal to many conidia of *A. flavus*. Because of poor penetration by ultraviolet rays, however, we believe that they would rarely reach conidia in wounds. Further, although the clusters were in direct sunlight, many of the berries within the cluster were not directly exposed.

Airborne conidia of *A. flavus* and *A. parasiticus* were present at about three viable conidia per cubic meter as determined with an Andersen Particle Sampler in a 1980 and 1981 survey conducted during the grape-drying season (*unpublished*). Consequently, low density of inoculum may contribute to the absence of aflatoxin by limiting infections of wounds in grape berries.

LITERATURE CITED

1. Andrellos, P. J., and Reid, G. R. 1964. Confirmatory tests for aflatoxin B_1. J. Assoc. Offic. Agric. Chem. 47:801-803.
2. Buchanan, J. R., Sommer, N. F., and Fortlage, R. J. 1975. *Aspergillus flavus* infection and aflatoxin production in fig fruits. Appl. Microbiol. 30:238-241.
3. Hewitt, W. B. 1974. Rots and bunch rots of grapes. Calif. Agric. Exp. Stn. Bull. 868. 51 pp.
4. Moreau, C. 1968. Moississures Toxique dans l'Alimentation. 1st ed. Editions Paul Lechevalier. Paris. 371 pp.
5. Pohland, A. E., Cushme, M. E., and Andrellos, P. J. 1968. Aflatoxin B_1 hemiacetal. J. Assoc. Offic. Anal. Chem. 51:907-910.
6. Raper, K. B., and Fennel, D. I. 1965. The genus *Aspergillus*. The Williams and Wilkins Co., Baltimore. 686 pp.
7. Schindler, A. F., Palmer, J. G., and Isenberg, W. V. 1967. Aflatoxin production by *Aspergillus flavus* as related to various temperatures. Appl. Microbiol. 15:1006-1009.
8. Schroeder, C. A. 1965. Temperature relationships in fruit tissues under extreme conditions. Proc. Am. Soc. Hortic. Sci. 87:199-203.
9. Schroeder, C. A., and Hein, H., Jr. 1968. Effect of diurnal temperature cycles on the production of aflatoxin. Appl. Microbiol. 16:988-990.
10. Schuller, P. L., von, Ockuizen, Th., Werringloer, J., and Marquardt, P. 1967. Aflatoxin B_1 und Histamin in Wein. Arzneim.-Forsch. 17:888-890.
11. Sommer, N. F., Buchanan, J. R., and Fortlage, R. J. 1976. Aflatoxin and sterigmatocystin contamination of pistachio nuts in the orchard. Appl. Microbiol. 32:64-67.
12. Stoloff, L. 1976. Occurrence of mycotoxin in foods and feeds. Pages 23-50 in: Mycotoxins and other Fungal Related Food Problems. J. V. Rodricks, ed. Adv. Chem., Ser. 149. 409 pp.
13. Takahashi, D. M. 1974. Thin layer chromatographic determination of aflatoxin in wine. J. Assoc. Offic. Anal. Chem. 57:875-879.