

Effect of Substrate on Metabolite Production by *Alternaria alternata*¹

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Alternariol and alternariol monomethyl ether are commonly associated with weathered grain sorghum. Production of these metabolites and altenuene by isolates of *Alternaria alternata* was evaluated on various sterile grain substrates. At 35% moisture content and 25 C, metabolite yields were highest on rice, intermediate on sorghums, and lowest on wheat and yellow corn. Fourteen- to 21-day cultures on milled rice were best in terms of ease of metabolite recovery, even though yields were higher on 28-day cultures of rough and brown rice. Metabolite production was reduced when rice was supplemented with yeast extract or yeast extract plus Czapek-Dox broth.

Metabolites of *Alternaria alternata* (Fries) Keissler (*A. tenuis* Auct.) were first isolated and characterized by Raistrick et al. from 4- to 5-week cultures on Czapek-Dox liquid medium (13). The same culture medium was used by Rosett et al. (14) and by Thomas (18) for subsequent studies of *A. alternata* metabolites. Pero and Main (10) reported that Czapek-Dox medium modified by adding polished rice and yeast extract (YE) increased production of alternariol monomethyl ether (AME) compared with the original Czapek-Dox liquid medium. The medium used by Pero and Main (10) and, more recently, by Pero and co-workers (11, 12) consisted of 50 g of polished whole rice moistened with 75 ml of nutrient solution in a 1-liter flask. The nutrient solution was 2% (wt/vol) YE in commercial Czapek-Dox broth. Meronuck et al. (9) grew *A. alternata* isolates on a 1:1 mixture of autoclaved, moist corn-rice for 14 to 20 days at 23 to 25 C and extracted AME and tenuazonic acid. The actual moisture content of the medium was not specified.

Based on our identification of alternariol (AOH) and AME in weathered, discolored sorghum grain (16, 17), we initiated a study of metabolite production by isolates of *A. alternata*. To characterize metabolites and to study toxicity, we sought a solid substrate that permitted high yields with a minimum of interfering substances.

MATERIALS AND METHODS

Isolates of *Alternaria*. After testing 20 isolates of *A. alternata* from wheat and sorghum grain, three

were selected as representative of different metabolite production patterns. Isolates RL-671-2 and RL-8442-3 both produced AME, AOH, and altenuene (ALT), and the latter also produced tenuazonic acid. Isolate RL-8442-2 produced a wide range of metabolites: AME, AOH, ALT, altertoxin I, tenuazonic acid, and others yet to be identified. For studies on rice substrates, which required analysis of many samples, we selected RL-671-2 because of its relatively simple metabolite pattern.

Cultures were grown for 6 days on potato dextrose agar plates at 25 C. Each flask of autoclaved substrate was inoculated with two 7-mm disks cut from the edge of a plate culture using a sterile no. 4 cork borer. Noninoculated controls were maintained in each experiment. Flasks were shaken by hand each day to break up grain clumped by fungal growth.

AME, AOH, and ALT analyses. Methanol extracts of 50 g of air-dried, ground substrate were prepared in a Waring blender. After filtering, aqueous ammonium sulfate was added to a portion of the methanol extract, and the resulting aqueous phase was washed with hexane to remove some of the pigments and lipids. AME, AOH, and ALT were extracted from the aqueous phase with two portions of methylene chloride. The compounds were separated by use of Corasil II (Waters Associates, Framingham, Mass.) or Zorbax-Sil (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.) columns and either isoctane-tetrahydrofuran or petroleum ether-tetrahydrofuran with solvent programming. Detection was by simultaneous monitoring of 350-nm absorbance (Varian Instrument Div., Palo Alto, Calif.; photometer modified to 350 nm) and fluorescence (model 1209 FluoroMonitor, Laboratory Data Control, Riviera Beach, Fla.), with the latter usually used for quantitation. Details of the extraction and clean-up procedure and analysis of AME, AOH, and ALT in final extracts by high-pressure liquid chromatography are described elsewhere (15).

Description of experiments. (i) **Effect of substrate MC.** Mixtures of yellow corn and milled rice

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(1:1) were adjusted to 25 or 35% moisture content (MC); 200-g quantities were dispensed into 500-ml Erlenmeyer flasks and autoclaved for 30 min. Each flask was inoculated and incubated for 18 days at room temperature (about 22 C). The three *Alternaria* isolates were used. In all experiments uninoculated substrates were free of *Alternaria* metabolites.

(ii) **Comparison of eight grain substrates.** Two sets of eight substrates were adjusted to 35% MC and autoclaved for 30 min: (i) hard red winter wheat, (ii) white grain sorghum, (iii) red grain sorghum, (iv) milled rice, (v) milled rice + 2% YE, (vi) yellow dent corn, (vii) corn + milled rice (1:1), and (viii) corn + milled rice + 2% YE. One set was inoculated with isolate RL-671-2 and the other with RL-8442-2. Substrates were analyzed for AME and AOH after 14 and 21 days at room temperature.

(iii) **Rough and milled rice with supplements.** Six rice-based media, all adjusted to 35% MC by adding water or Czapek-Dox broth, were inoculated with isolate RL-671-2: (i) rough rice (Colusa variety), (ii) rough rice + 2% YE, (iii) milled rice ("enriched" long grain, from local supermarket), (iv) milled rice + 2% YE, and (v) and (vi) rough and milled rice each with 2% YE + Czapek-Dox broth. Each flask contained 150 g of air-dry rice before moisture content was adjusted. Flasks were incubated at 25 C in an environmental chamber and tested at five intervals. At each sampling, the contents of three replicate flasks of each substrate were air-dried, weighed, ground, and analyzed separately.

(iv) **Raw and parboiled rice.** Rough rice and parboiled rough rice from the same lot (Starbonnet variety) were provided by Uncle Ben's Inc., Houston, Tex. The supplier milled the parboiled rice and enriched a portion with thiamine and iron. Commercially enriched rice is required to have a thiamine level of 4.4 to 8.8 $\mu\text{g/g}$ and an iron level of 29 to 58 $\mu\text{g/g}$ (2). We prepared brown and milled rice from the raw rough rice and brown from the parboiled rough rice. As in experiment (iii) above, the substrates were adjusted to 35% MC, sterilized, inoculated, and incubated. Three replicate flasks of each of the seven treatments were analyzed at four 1-week intervals. Protein, ash, and crude fat were analyzed on duplicate samples of each substrate prior to inoculation.

RESULTS

Effect of substrate MC. After 18 days the amount of metabolite production and visible fungal growth were slight in corn + rice with 25% MC, as compared to 35% MC. Alternariols (AME + AOH) produced by RL-671-2, RL-8442-2, and RL-8442-3 were 0.10, 0.22, and 0.95 $\mu\text{g/g}$ of substrate, respectively, in corn + rice at 25% MC and 40, 140, and 9 $\mu\text{g/g}$, respectively, in the grain mixture at 35% MC.

Comparison of eight grain substrates. Alternariols and ALT produced by two *Alternaria* isolates, each grown on eight substrates, are presented in Table 1. Highest metabolite production by both isolates was in milled rice, followed by white grain sorghum. Production was rather low in wheat, corn, and corn + rice with added YE. Addition of YE to milled rice and to corn + rice generally decreased metabolite production compared to unsupplemented grains. Isolate RL-8442-2 produced more alternariols than did RL-671-2 on each substrate. Yields of alternariols on the best substrates were maximum at 14 days for isolate RL-671-2 and at 21 days for RL-8442-2. Larger amounts of ALT were obtained after 21 days from both isolates.

Rough and milled rice with supplements. Although production of metabolites was lower on rough than on milled rice substrates during the first 10 days, alternariols increased to higher levels on rough than on milled rice and the quantity was twice as high at 21 days (65,500 $\mu\text{g/flask}$) and four times as high at 28 days (136,000 $\mu\text{g/flask}$) (Fig. 1A and B). Metabolite levels elaborated on milled or rough rice fortified with both YE and Czapek-Dox broth (data not shown) were not significantly different from amounts found in media fortified with YE only. Supplementing milled or rough rice with YE or YE plus Czapek-Dox broth decreased production of alternariols by isolate RL-671-2, but ALT production was depressed

TABLE 1. Production (micrograms per gram) of alternariols (AME + AOH) and ALT in eight substrates of 35% MC by two *Alternaria* isolates

Substrate	Isolate RL-671-2				Isolate RL-8442-2			
	AME + AOH		ALT		AME + AOH		ALT	
	14 days	21 days	14 days	21 days	14 days	21 days	14 days	21 days
Milled rice	153	57	10.0	40.0	76	189	0.5	4.9
White grain sorghum	107	64	4.8	29.0	21	166	0.2	14.0
Red grain sorghum	17	50	0.5	1.0	16	68	0.5	11.0
Yellow dent corn	6	30	0.1	0.4	4	58	0.2	0.5
Hard red winter wheat	2	17	0.1	0.9	4	59	0.1	1.3
Corn + milled rice (1:1)	39	46	2.1	13.0	68	154	0.5	4.6
Milled rice + 2% YE	32	37	1.7	34.0	33	106	0.5	8.5
Corn + milled rice (1:1) + 2% YE	4	13	0.3	1.6	3	43	0.2	2.3

more on rough rice than on milled rice. ALT was not detected at 5 days in the milled rice, 10 days in rough rice, or 14 days in rough rice plus YE.

Production of alternariols had reached the maximum level of 30,300 µg/flask on milled rice by 14 days (Fig. 1A). Thereafter, although fungal activity continued, evidenced by continued disappearance of substrate (Fig. 1C), the quantity of metabolites per flask was not increased. ALT production on milled rice reached the maximum level by 21 days (5,110 µg/flask). On rough rice, AME + AOH and ALT increased throughout the 28 days.

After 28 days the average weight of air-dry, fungus-invaded, milled rice per flask was 70 g (Fig. 1C); supplemented milled rice weighed about 12 g more. The three rough rice substrates all weighed about 96 g at 28 days. Even with an allowance for approximately 6% ash in rough rice (Table 2), about 20 g less of available substrate was utilized.

Raw and parboiled rice. Proximate analyses of the substrates used are shown in Table 2.

Among the three milled rice substrates, raw milled rice was the best medium for alternariol production by the selected *Alternaria* isolate (Fig. 2A); production was maximum by 14 days and differed little in two subsequent samplings. The parboiling treatment prior to milling appeared to have some inhibitory effect on alternariol production. Commercial enrichment of

parboiled rice with thiamine and iron further decreased alternariol production. However, fungal activity, as measured by substrate utilization, was greater in parboiled than in plain milled rice (Fig. 2B). At the final sampling of enriched rice, the material in the flasks was black and foul smelling. Filtration and phase separation of the extracts were difficult, and total alternariols per flask had decreased from 21,000 µg at 3 weeks to 6,000 µg.

Alternariol production began much faster on milled and brown rice compared to rough rice. After 7 days of incubation, milled rice contained 30 times more alternariols than did rough rice (Fig. 2A and C). Maximum metabolite levels were reached in 21 days on rough and brown rice, whereas production continued

TABLE 2. Analyses of Starbonnet rice substrates prior to inoculation^a

Rice substrates	Ash (%)	Protein (%)	Crude fat (%)
Raw rough	5.86	8.2	2.61
Raw brown	1.30	9.5	3.01
Raw milled	0.15	8.6	0.56
Parboiled rough	5.88	8.3	2.56
Parboiled brown	1.40	9.5	2.83
Parboiled milled	0.80	8.8	0.54
Parboiled milled, enriched	0.84	8.8	0.39

^a Samples were autoclaved prior to analysis and results are reported on a dry weight basis.

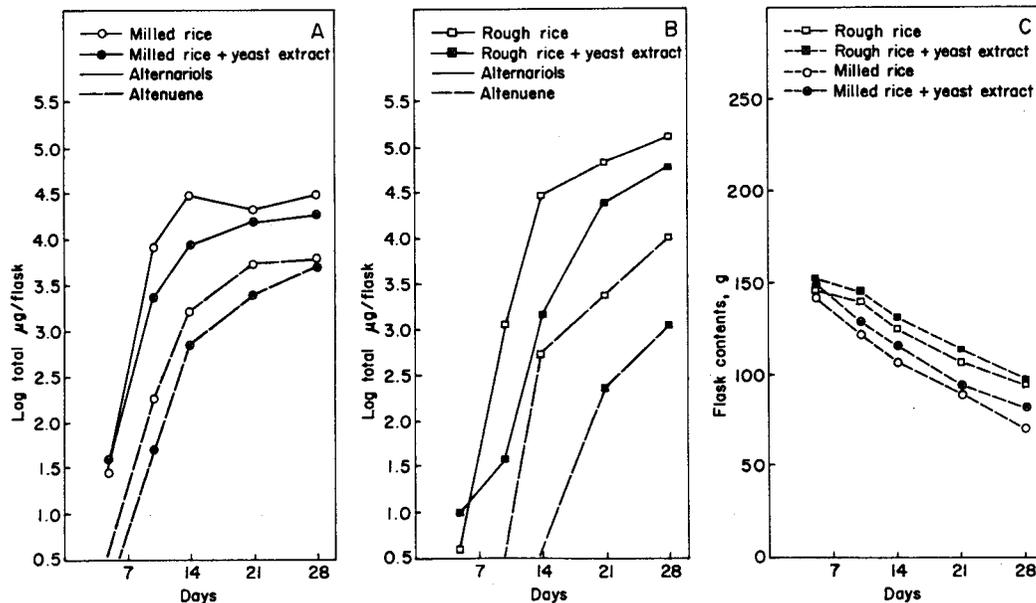


FIG. 1. Production of alternariols (AME + AOH) and ALT on 35% MC milled rice (A) and rough rice (B) with and without 2% YE and utilization of substrate (C) by an isolate of *A. alternata*.

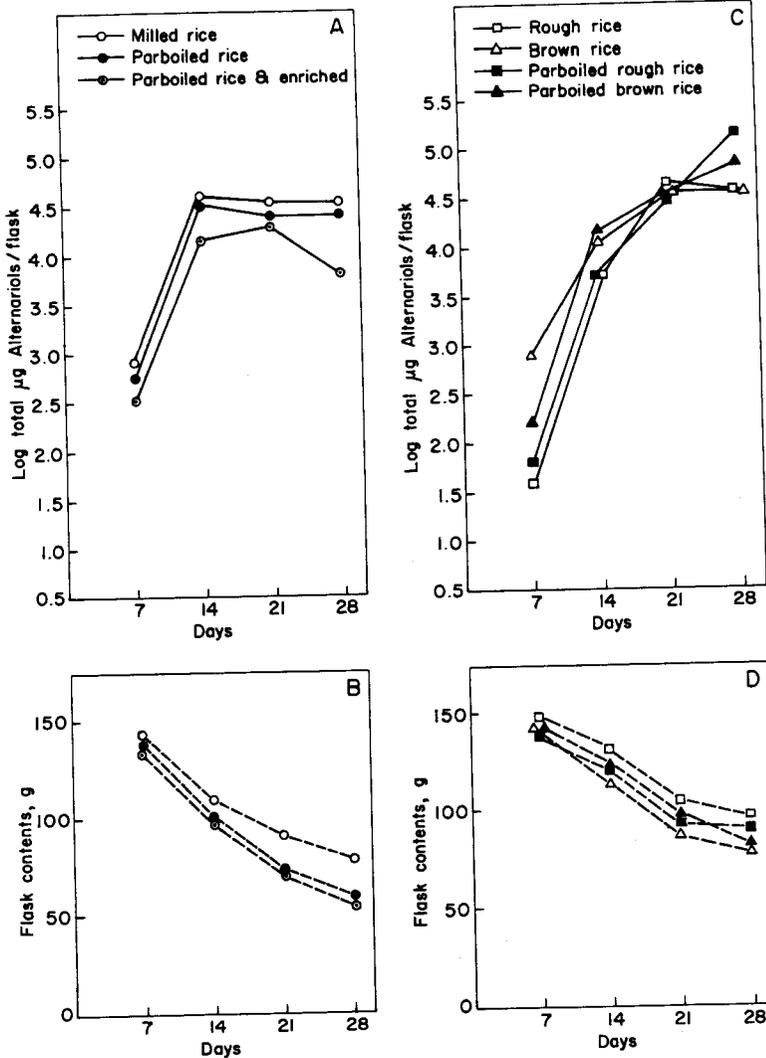


FIG. 2. Production of alternariols (AME + AOH) on milled rice, parboiled rice, and parboiled enriched rice (A) and on rough and brown rice with and without parboiling (C) and utilization of the substrates (B and D) by an isolate of *A. alternata*.

throughout the 4-week period on parboiled rough and parboiled brown rice. Fungal growth or activity continued throughout the 4 weeks on all rice substrates, as measured by weight loss (Fig. 2B and D).

DISCUSSION

MCs that allow rather extensive *Alternaria* growth on sterile grain may not be high enough for appreciable metabolite production. *Alternaria* has been shown to invade 100% of autoclaved sorghum kernels within 8 days at 24% MC (3). In our corn-rice mixture with 25% MC

(above 95% relative humidity), the fungus grew but produced only low levels of alternariols in 18 days; at 35% MC, levels were 10 to 400 times higher, depending on the *Alternaria* isolate. Lindenfelser and Ciegler (7) showed that the initial moisture level was the most critical fermentation condition for ochratoxin production on hard, red winter wheat. Yields were maximum when initial MC was 30 to 31% and were less than initial MC was higher or lower.

Alternariol yields on grain substrates (Table 1) might have been very different had the optimal initial MC for each substrate been known

and used. Wheat and yellow corn at 35% MC are poor media for alternariol production. Hesseltine et al. (6) found both grains unsatisfactory for aflatoxin production in stationary culture when wheat was about 35% MC and corn about 47% MC. Shaking the cultures increased yields markedly. The same workers found that a polished rice substrate (about 40% MC) produced consistently higher aflatoxin yields in stationary culture than did corn, wheat, or yellow sorghum. A larger amount of zearalenone was produced on polished rice than on any other grain substrate tested by Eugenio et al. (5).

Unsupplemented milled rice at 35% MC is a convenient and uniform substrate for production of *Alternaria* metabolites. Good yields of alternariols were extracted after 14 days using isolate RL-672-2 on three different lots of milled rice; 21-day cultures were equally suitable for alternariol recovery and gave a three- or four-fold increase in ALT.

Rough rice is less uniform than milled rice; the proportion and composition of hulls vary considerably among cultivars and with differences in growing conditions (1). This variability probably accounted for the difference in alternariols produced on our two rough rices, Colusa in Fig. 1B and Starbonnet in Fig. 2C. Also, the pigments and oil in rough rice create problems when metabolites are to be extracted and purified.

Proximate analyses (Table 2) of various rice substrates do not adequately explain the differences in metabolite production by *A. alternata*, although ash and crude fat contents vary considerably. Hulls and bran of rough and brown rice contain many constituents that could affect fungal growth and metabolism.

The role of YE as a depressing factor for *Alternaria* metabolite production (Table 1; Fig. 1A and B) is not clear in the results presented. It is generally acknowledged that thiamine is the vitamin most frequently required by fungi, but depressing effects have also been reported (4). We are not aware of any studies of specific requirements of *Alternaria* for B vitamins. The possible involvement of thiamine as a depressing factor is also suggested by the decrease in alternariol production on parboiled rice from that on milled rice and the further reduction on parboiled, enriched rice (Fig. 2A). Approximate levels of thiamine in the three milled rice substrates were 0.07, 0.34, and 0.66 mg/100 g for milled, parboiled, and enriched parboiled rice, respectively (1, 2).

The presence of iron in enriched rice might also affect metabolite production. Marsh et al.

(8) showed a progressive decrease in aflatoxin production by *Aspergillus parasiticus* in a liquid medium when iron was increased from 1 to 25 $\mu\text{g/g}$. Growth of the fungus was not reduced, but metabolite production dropped 10-fold over the range cited.

Increase in ALT production followed the increase in production of alternariols (Table 1; Fig. 1A and B). This is consistent with a recent suggestion by D. Harvan and R. Pero (personal communication) that AME may be a precursor of ALT. Although results described here are based on quantitations of AME, AOH, and ALT, we have noted that substrates that permitted highest yields of these metabolites also permitted highest yields of other methanol-extracted *Alternaria* metabolites, i.e., altertoxin I and others yet to be characterized.

We found that 35% MC sterilized, milled rice was the most suitable substrate for production of *Alternaria* metabolites. The low cost, easy availability, and simplicity of preparation, coupled with low oil content and absence of pigments, outweigh any advantage of greater yield on rough rice. The ease in clean-up of milled rice substrates may be particularly advantageous for the extraction of metabolites that are produced in smaller quantities than alternariols.

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