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Biosynthesis of Zearalenone: a Simple and Efficient Method To Incorporate [^{13}C]Acetate Label by Using Solid Cultures

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A suitable method was developed to efficiently incorporate ^{13}C-labeled acetate into zearalenone by using solid cultures. Periodic feeding of the label during the zearalenone production phase significantly increased the label incorporation for the singly labeled acetate.

Incorporation of singly and doubly ^{13}C-labeled acetate into metabolites of fungi by using liquid cultures has been amply described elsewhere (1, 2, 4). Labeled acetate salts, which are soluble in liquids, are efficiently taken up by the microorganism. Therefore, liquid cultures yield metabolites whose incorporation of the label is high enough that they can be directly examined by ^{13}C nuclear magnetic resonance. Isolation and purification of metabolites from liquid culture is also less tedious. For these reasons, liquid cultures have been a method of choice for the study of biosynthesis of mycotoxins. However, elaboration of mycotoxins by a fungus in a liquid medium can be vastly different from that observed in nature, where the fungus grows on grain (solid medium). For instance, isolates of *Fusarium graminearum* produced deoxynivalenol in solid rice cultures; these isolates failed to produce any detectable amount of the toxin in the liquid medium (6). We also observed that strains of *Fusarium roseum* which produce zearalenone and its hydroxy derivatives in solid rice cultures produced significantly lower amounts of zearalenone and virtually no hydroxylated derivatives when grown in liquid cultures (unpublished data). The solid culture mimics the natural growing environment, and often it is necessary to determine the course of biosynthesis or metabolism of mycotoxins under natural conditions.

Obtaining a ^{13}C-labeled metabolite with a ^{13}C-labeled precursor incorporation sufficiently high to carry out nuclear magnetic resonance experiment by the conventional solid culture method as described by Steele et al. (9) may not always be possible. This is primarily because the amount of the ^{13}C-labeled precursor used is several times greater than in a typical ^{14}C-labeling experiment. Furthermore, the mode of administering a labeled substrate to the solid culture (9) precludes adequate distribution of it, which results in poor incorporation of the label. Also, the high concentration of the labeled acetate retards the fungal growth in the areas of the culture where the label is applied.

In this paper, we describe a simple and economical method to efficiently incorporate ^{13}C-labeled acetates into zearalenone by using solid cultures. We believe that this method has a general utility for incorporation of a labeled precursor into metabolites in solid cultures.

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MATERIALS AND METHODS

Source and maintenance of *F. roseum*. The organism used in this study was a subculture of a mass isolate of *F. roseum* 'Graminearum' from moldy corn collected in Minnesota (7). This isolate was maintained on moist, autoclaved soil at 4°C. An inoculum was prepared by transferring spores from the soil to petri plate containing potato dextrose agar medium. The culture was incubated for 5 days at 25°C.

Source of labeled acetates. [1-^{13}C]Sodium acetate (90% enriched) and [1,2-^{13}C]sodium acetate (90% enriched [81% $^{13}CH_3{}^{13}COONa$, 9% $^{13}CH_3COONa$, 9% $CH_3{}^{13}COONa$, 1% CH_3COOH]) were purchased from Stohler/KOR Stable Isotopes, Cambridge, Mass.

Incorporation of [1-^{13}C]acetate. Parboiled rice (2 g; Uncle Ben's converted rice) was placed in each of six vials (8 drams [ca. 29.6 ml]) containing 10 mg of [1-^{13}C]sodium acetate in 2 ml of water, soaked overnight at 4°C, and autoclaved twice on two successive days at 120°C for 1 h. The rice was then inoculated with *F. roseum* 'Graminearum' grown on potato dextrose agar medium for 5 days. The cultures were kept at room temperature, and a 0.2-ml sterilized solution of [1-^{13}C]sodium acetate (10 mg per vial) was layered carefully over the growing surface of the cultures once every 5 days for 25 days before they were harvested.

Incorporation of [1,2-^{13}C]acetate. The cultures in eight vials were prepared by soaking rice in a solution of 10 mg of [1,2-^{13}C]sodium acetate in a manner similar to that described for the singly labeled acetate incorporation. However, the cultures were kept at room temperature for 9 days without any addition of the label and were harvested on day 10.

Isolation of ^{13}C-labeled zearalenone. The cultures were dried and extracted three times with 20-ml portions of ethyl acetate in a blender. The ethyl acetate extract was concentrated and partitioned between equal volumes (20 ml) of acetonitrile and hexane. The acetonitrile layer was concentrated and chromatographed on a thick-layer (1 mm) silica gel G plate using hexane-diethyl ether-glacial acetic acid (70:30:2) to isolate zearalenone (8). The total yields of purified labeled zearalenone were 16.1 and 12.5 mg from [1-^{13}C]acetate and [1,2-^{13}C]acetate incorporation, respectively.

Fourier transformed nuclear magnetic resonance spectra were recorded on a Varian XL-100 spectrometer using acetone-d_6 solutions.

RESULTS AND DISCUSSION

F. roseum 'Graminearum' produces 6,000 to 12,000 ppm (6 to 12 mg/g of dry culture) of zearalenone on rice (7).

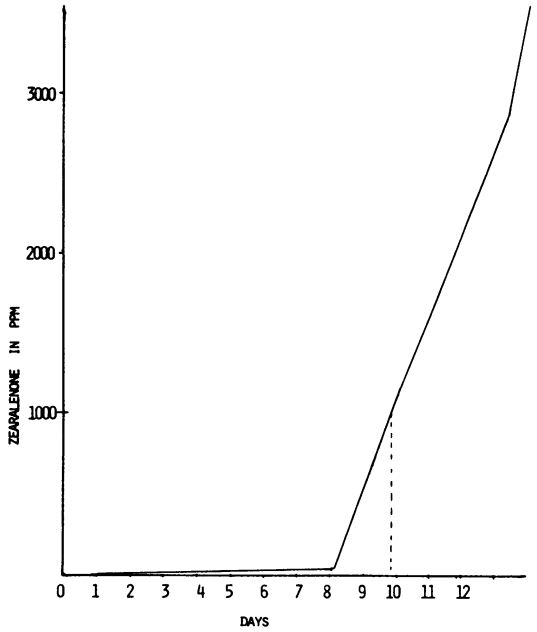

FIG. 1. Zearalenone production on rice cultures by *F. roseum* 'Graminearum'.

Preliminary experiments indicated that the zearalenone production (Fig. 1) during week 1 after inoculation was minimal; it increased moderately to 3,000 ppm the following week. The rate of production of zearalenone in rice culture by this fungus was highest during week 3 after inoculation. The maximum amount of zearalenone isolated from the 3-week-old culture was 12,000 ppm as determined by gas chromatography-mass spectrometry.

One-time feeding of [1-^{13}C]sodium acetate (~0.1% by weight) during the highest zearalenone production period resulted in poor incorporation of the label. Parallel experiments with ^{14}C-labeled acetate indicated that the total incorporation of the label was significantly higher than that reported by Steele et al. (9). We attributed poor ^{13}C-label incorporation to the dilution effect, i.e. lower specific activity. Additions of one-step-higher concentrations of the acetate (>0.1% by weight) retarded the fungal growth and hence the zearalenone production. The dilution effect during the phase of high zearalenone production was alleviated by periodic feeding with the label. Thus, growing cultures periodically fed [1-^{13}C]acetate for 25 days after the culture inoculations produced zearalenone with sufficient (>1%) incorporation of the label, as determined by ^{13}C nuclear magnetic resonance (Table 1).

An inhibition of fungal growth was sometimes noted when [1,2-^{13}C]sodium acetate was added periodically to the cultures, which resulted in poor incorporation of the label. The fungal growth retardation was also noted when the label concentration was lowered to 5 mg for the periodic feedings. Whether this inhibition is related to the total ^{13}C atom concentration in the label is unclear. Further lowering of the label concentration did not improve the incorporation. Rather, consistent incorporation was obtained by seeding the rice, which had been soaked overnight in the aqueous solution of [1,2-^{13}C]sodium acetate and autoclaved twice, with *F. roseum*. The cultures were then harvested on day 10 without any further feeding of the label to produce 600 to 1,000 μg of zearalenone per g of dry culture. The sample of

TABLE 1. Excess ^{13}C at enriched carbons of zearalenone derived from [1-^{13}C]acetate and [1,2-^{13}C]acetate

Carbon no.	Chemical shift (δ)	% Enrichment	
		[1-^{13}C]acetate	[1,2-^{13}C]acetate
1	103.0		
2	165.7	1.4	
3	102.1		0.3
4	162.6	1.9	
5	109.4		0.2
6	144.0	0.8	
1′	133.3		0.2
2′	132.3	1.5	0.2
3′	31.1		0.3
4′	21.0	1.2	0.2
5′	36.1		0.2
6′	209.9	0.7	
7′	42.7		0.3
8′	22.3	1.5	0.2
9′	34.8		0.3
10′	73.6	1.1	0.3
11′	20.3		0.3
12′	171.6	0.4	0.2

zearalenone enriched from the [1,2-^{13}C]acetate yielded a ^{13}C spectrum (5) in which all resonances except those of the quaternary carbons were accompanied by two satellite signals due to one-bond ^{13}C-^{13}C coupling. The entire carbon skeleton of zearalenone is, therefore, assembled from intact pairs of carbon atoms derived from acetate (5, 9). The degree of ^{13}C enrichment at each position was calculated from the intensities of the satellite signals and the central peaks (3). The values indicate uniform incorporation throughout the metabolite.

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