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Media for Identification of *Gibberella zeae* and Production of F-2 (Zearalenone)

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Media are described for the isolation of *Fusarium graminearum* in the perithecial state, *Gibberella zeae*, and for the production of F-2 (zearalenone) by *Fusarium* species. On soil extract-corn meal agar isolation medium, *G. zeae* produced perithecia in 9 to 14 days under a 12-h photoperiod. Species of *Fusarium* were screened for F-2 production on a liquid medium. From strains that produced F-2, the yields, from stationary cultures of *G. zeae* and *F. culmorum* after 12 days of incubation, ranged from 22 to 86 mg/liter. Three strains produced no F-2. Glutamic acid, starch, yeast extract, and the proper ratio of medium volume-to-flask volume were necessary for F-2 synthesis.

The estrogenic syndrome in swine induced by eating *Fusarium*-infected corn was first examined in detail by Stob et al. (12), who crystallized and partially characterized the causative factor, an anabolic uterotrophic compound. Urry et al. (15) determined that the structure was 2,4-dihydroxy-6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-benzoic acid lactone. Under favorable conditions of moisture and temperature, the compound, commonly called F-2 or zearalenone, is produced on grain by *Gibberella zeae* (Schweinitz) Petch, the perfect state of *Fusarium graminearum* Schwabe (12). Several other species of *Fusarium* also can produce F-2 (3). In addition to inducing estrogenic symptoms in swine, F-2 is physiologically active in cattle, rats, mice, guinea pigs, poultry, and plants (11, 12).

Since *G. zeae* is a pathogen of many economically important cereal grains, and many of its isolates are capable of producing F-2 (3), a rapid procedure is needed for identifying and assessing the potential of varions *Fusarium* species to produce F-2. Convenient media now used to isolate and identify the perithecial state require 6 to 12 weeks of incubation (5, 7, 8). Fresh carnation leaf tissue disks were described as a substrate for the rapid production of perithecia (14). However, the rarity of that substrate precludes its use in routine procedures. Two environmental conditions necessary for production of F-2 on natural substrates have been defined, but demonstration of F-2 production by *Fusarium* isolates by that method also required long incubation on corn or other grains (3, 11). Those substrates require long extraction procedures to separate F-2 from interfering compounds and, thus, are satisfactory neither for rapid screening of F-2-producing isolates, nor for studying other basic factors that influence F-2 production. We now report rapid procedures for the isolation of the perithecial state of *F. graminearum*, *G. zeae*, and for the assessment of F-2 production by *Fusarium* species on a semisynthetic liquid medium.

MATERIALS AND METHODS

Culture sources. *G. zeae* strains FR26 and FR29 and *F. culmorum* (Smith) Saccardo strain FR22 were obtained from J. Tuite, Department of Plant Pathology, Purdue University, Lafayette, Ind. These strains were originally isolated from corn and were identified as producers of F-2. Corn samples were provided by C. J. Mirocha, Department of Plant Pathology, University of Minnesota, St. Paul (sample A), and J. Tuite (sample B). Two samples of grain sorghum associated with hyperestrogenism in swine were supplied by R. C. Hatch, Veterinary Diagnostic Assistance Laboratory, College of Veterinary Medicine, University of Georgia, Athens, who determined that the concentrations of F-2 in these two samples were 2 and 5 mg/kg. Additional samples were obtained from local sources (corn samples C and D). Fungi were maintained on soil or on potato-dextrose agar (Difco Laboratories, Detroit, Mich.) at 5°C.

Medium for perithecia. The medium (SECM) used to isolate *G. zeae* consisted of: corn meal agar (Difco), 17 g; soil extract, 1,000 ml; chloroamphenicol, 0.05 mg; and streptomycin sulfate, 0.03 mg. The soil extract was prepared by autoclaving for 30 min 1 kg of commercial potting soil in 1,500 ml of distilled water, filtering the solution through Whatman no. 4 filter paper, and adjusting the volume to 1,000 ml. The pH was adjusted, if necessary, to 6.0 to 6.5 with 1.0 N NaOH or 1.0 N HCl, and the medium was autoclaved for 15 min. Ten commercially available

potting soils were tested, and all gave similar results, except for the high-peat type formulated for African violets. The SECM medium was poured into standard polystrene disposable petri dishes to about one-half the depth of the bottom dish. For comparison of perithecial development, soil extract agar (10) and the synthetic agar of Coons and Strong (5) also were used.

Medium for F-2. Isolates were screened for F-2 production on a starch-glutamate medium, which contained (per liter): soluble starch for iodometry (pH of a 2% solution at 25°C was 5.8, Fisher Scientific Co., Fair Lawn, N.J.), 20.0 g; L-glutamic acid-hydrochloride, 10.0 g; yeast extract (Difco), 1.0 g; $K_2HPO_4 \cdot 3H_2O$, 19.0 g; and sodium citrate $\cdot 2H_2O$, 12.1 g. The initial pH of the medium was 5.6. The medium was boiled for 15 min and autoclaved for 20 min. The effects of carbohydrate and nitrogen sources on F-2 production were determined by replacing starch or glutamic acid with equimolar concentrations of the test compound. All experiments were done in triplicate, and yields were reported as averages.

Cultural conditions. Grain was surface-sterilized by shaking in a 1% solution of NaClO for 1 min and then in 70% ethanol for 1 min, followed by three rinses in sterile distilled water. One kernel was placed in the center of a petri dish of SECM medium. The plates were incubated at 24 to 28°C 30 cm below a bank of two 4-foot (ca. 1.2 m) fluorescent tubes (General Electric Cool White, 40 W) controlled by a time switch set for a 12-h light cycle.

Isolates screened for F-2 production were incubated for 2 weeks in the dark as stationary cultures in starch-glutamate medium (25 ml/500-ml Erlenmeyer flask or 100 ml/2,800-ml Fernback flask at 24 to 28°C). Cultures were shaken briefly during the first 3 days to encourage rapid mycelial growth over the surface of the medium. Inocula for the flasks were 1-cm^2 agar plugs obtained either after subculturing isolates from soil on potato-dextrose agar or directly from SECM medium.

Analysis of F-2. The pH was adjusted to 3.0 to 4.0 with 1.0 N HCL, and each culture was transferred to a blender and homogenized (high speed) with 100 ml of chloroform-methanol (2:1, vol/vol) for 2 min. The homogenate was transferred to a separatory funnel and extracted three times with 50-ml portions of chloroform. The chloroform extracts were filtered through anhydrous Na_2SO_4 and concentrated on a rotory evaporator. The residue was dissolved in 2 ml of chloroform-methanol (1:1, vol/vol). Mycelia mats were collected before extractions, dried at 80°C for 24 h, and then weighed.

Thin-layer chromatography on silica gel (Eastman Chromagram no. 6060 with fluorescent indicator) was used to separate F-2. The extract, 50 μl, was applied as a spot to the thin-layer plates and developed in a solvent system (13) in which the solvent ratio was modified to improve separation. The solvent consisted of petroleum ether-acetone-acetic acid (75:10:5, vol/vol/vol). F-2 was quantitated by the procedure of Mirocha et al. (11). The identity of F-2 was established by co-chromatography with standards, by ultraviolet spectra, and by color reactions with spray reagents (3, 9, 11).

RESULTS

Isolation of *G. zeae*. After 9 days, perithecia of *G. zeae* developed superficially on SECM medium (Fig. 1). The perithecia were dark purple, ovoid to globose, and 96 to 200 μm in diameter; the ascospores produced were viable. Macroconidia were typical of *F. graminearum* and were formed from multibranched conidiophores. Perithecial development varied among isolates and depended on both emergence of the fungus from the kernel and on its growth rate. The fungus completely covered the agar plates in 5 to 7 days, and only then did perithecia develop. Generally, 9 to 14 days were required for perithecial development. Results were the same with grain sorghum.

Under constant darkness or light, perithecia were not produced, even when the cultures were returned to the 12-h light cycle described above. Results were negative after an 8-week incubation when either corn meal or soil extract was incorporated into agar alone. Soil extract and the synthetic agar media (Coons and Strong) were described as useful for isolating *G. zeae* (3, 16). We found, however, that results from those media were inconsistent, and that the incubation times were too long for routine isolation and identification (Table 1).

Production of F-2. A comparative test of F-2 yields showed that high- and low-producing strains of *G. zeae* and *F. culmorum* on corn were similarly high or low producers on the starch-glutamate medium (Table 2). Neither the *G. zeae* strain, *F. tricinctum* (Corda) Saccardo, nor *F. sporotrichioides* Sherbakoff produced F-2 on either corn or starch-glutamate medium. Strains of *F. tricinctum* and *F. sporotrichioides* (synonyms of *Fusarium tricinctum* (Corda) Saccardo emended Snyder and Hansen) were reported to produce F-2 on corn (3). *G. zeae* 163, the highest producer of F-2, was used for culture tests with starch-glutamate medium.

When the concentrations of starch and glutamic acid were increased, the yield of F-2 increased proportionally up to 2 and 1%, respectively (data not shown). F-2 was not produced when yeast extract, starch, and glutamic acid were omitted. The carbohydrate and nitrogen sources in Tables 3 and 4 were tested for their ability to influence the synthesis of F-2. A low yield of F-2 was produced with mannitol but not with other carbohydrates tested. Although the dry weight of mycelium was greater with other carbohydrate and nitrogen sources than with starch and glutamic acid, F-2 was not detected (Tables 3 and 4).

The production of F-2 on starch-glutamate medium was time dependent and followed an

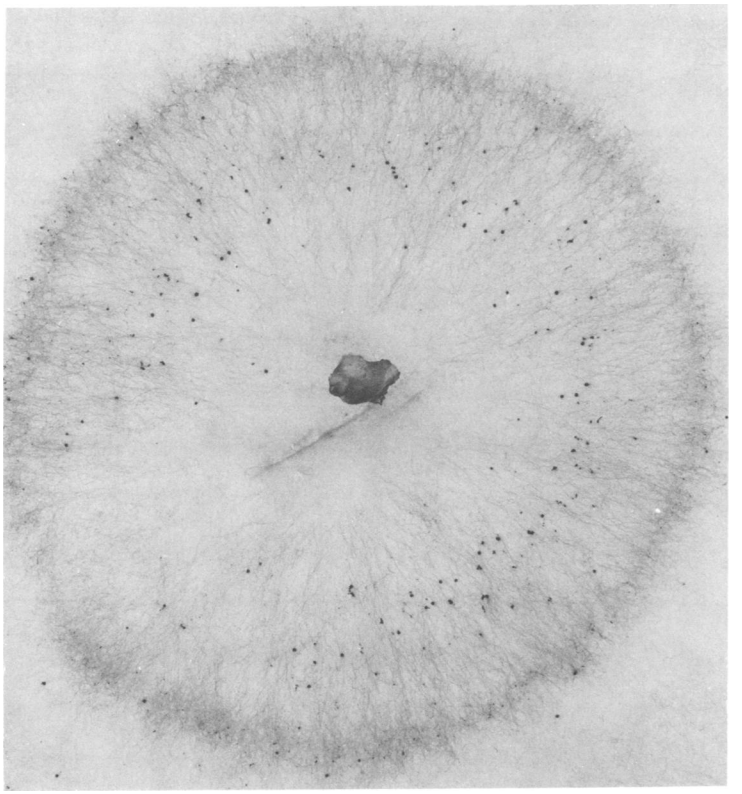

FIG. 1. *Perithecia of G. zeae after 9 days of incubation on SECM agar seeded with a corn kernel.*

TABLE 1. *Effect of isolation medium on determinations of percent infection of corn kernels and sorghum by G. zeae*

Sample[a]	Infection (%)[b]		
	SECM	SE	Coons and Strong
Corn A	40	0	0
Corn B	85	0	0
Corn C	35	1	0
Corn D	0	0	0
Sorghum 1	90	0	0
Sorghum 2	80	0	0

[a] See Materials and Methods for sources of samples.

[b] Percent infection was determined on 50 kernels of each sample type incubated for 10 days on SECM medium and for 8 weeks on SE (soil extract) medium and the synthetic medium of Coons and Strong.

increase in medium pH (Fig. 2). Production was maximum for dry weight and F-2 after 11 and 12 days, respectively, and then decreased for both. F-2 yield was not increased by incubating cultures at 12°C for several time periods as described for natural substrates (3). The fungus did not produce F-2 in shake cultures (25, 50, or 100 ml/500-ml flask) for periods up to 21 days. Effects of medium volume-to-flask volume on F-2 production were investigated. Maximum production of F-2 was 2 mg/25 ml, which occurred only in 500-ml flasks containing 25 ml of medium. The total yield of F-2 was increased by incubating the fungus in 100 ml of medium in 2,800-ml Fernback flasks for 14 days. F-2 was not detected in the other ratios (25 or 50 ml/125-ml flask; 50, 75, or 100 ml/500-ml flask; 100 ml/500-ml flask; and 200 ml/2,800-ml flask) by the method of quantitative analysis (11) used. The limit of sensitivity of this method is 3 μg.

DISCUSSION

For isolation of *G. zeae*, corn kernels were placed directly on SECM-medium and incubated under a 12-h fluorescent light cycle. Isolated *G. zeae* formed perithecia after repeated subculturing on SECM medium; but the number of perithecia often decreased, and the time of development increased to 3 to 4 weeks. If isolates were transferred to potato-dextrose agar or to any other nutrient-rich medium, the ability to form perithecia was progressively lost and was not restored by repeated subculturing

TABLE 2. *Production of F-2 by strains of G. zeae and Fusarium species on starch-glutamate medium and corn*

Species	Strain	Source	F-2	
			Starch-glutamate[a] (mg/liter)	Corn[b] (mg/g)
G. zeae	FR26	Corn	35	0.320
G. zeae	FR29	Corn	40	0.420
G. zeae	163	Corn	86	0.596
G. zeae	165	Corn	30	0.410
G. zeae	166	Corn	0	0
G. zeae	265	Grain sorghum	22	0.090
G. zeae	266	Grain sorghum	76	0.520
F. culmorum	FR22	Corn	25	0.020
F. culmorum	182	Poultry feed	42	0.095
F. tricinctum	161	Corn	0	0
F. sporotrichioides	214	Corn	0	0

[a] Cultures were incubated for 2 weeks.
[b] Fungi were incubated for 8 weeks (11), and F-2 was extracted from corn by the method of Caldwell et al. (3).

TABLE 3. *Effect of substituting carbohydrate sources for starch on F-2 production and mycelial dry weight in cultures of G. zeae 163*

Carbohydrate source[a]	Final pH	Mycelium (mg)	F-2 (mg/liter)
Starch (control)	8.6	348	80
Sucrose	6.0	678	ND[b]
Maltose	5.9	842	ND
Mannitol	6.1	609	9
D-Glucose	5.7	726	ND
D-Sorbitol	6.5	601	ND

[a] Carbohydrate concentration was 20 g/liter, except for glucose and sorbitol, which were 10.55 and 10.67 g/liter, respectively. Incubation was in stationary culture for 2 weeks.
[b] ND, Not detected.

TABLE 4. *Effect of substituting nitrogen sources for glutamic acid on F-2 production and mycelial dry weights in cultures of G. zeae 163*

Nitrogen source	Concn (g/liter)	Final pH	Mycelial dry wt (mg)	F-2[a] (mg/liter)
Glutamic acid (control)	10.00	8.5	371	85
Alanine	6.08	6.2	602	ND[b]
L-Aspartic acid	9.10	8.0	526	ND
L-Methionine	10.20	7.5	422	ND
Casamino Acids	10.00	7.5	776	ND
Peptone	5.63	6.1	622	ND
Urea	4.50	7.4	584	ND
KNO_3	7.60	7.5	726	ND
NH_4NO_3	5.47	8.1	629	ND

[a] F-2 was determined after 12 days of growth.
[b] ND, Not detected.

on SECM medium. Kernels that were inoculated onto rich media did not produce perithecia, which confirmed the similar observation by Booth (2). Macroconidia were always produced on SECM medium, regardless of the inoculum source or number of subcultures. This finding contrasts with earlier reports that isolates of *F. graminearum* do not produce conidia on agar media, especially if they were maintained by mass transfer (2, 4).

F. graminearum is considered predominantly homothallic (8), but heterothallic strains also have been reported (2) and could cause difficulty. In the routine screening of grain from different geographical areas, we have never isolated a heterothallic strain. We did isolate two strains of *G. zeae* that did not form abundant perithecia on SECM medium (less than 0.01% of all strains screened), but they were high producers of F-2 when cultured on starch-glutamate medium or on corn (Table 2, strains 163 and 266). Interesting in this respect are the findings that F-2 is a sex-regulating hormone for *F. graminearum* and that the number of perithecia produced was reduced by high concentrations of F-2 (16). Presumably, high endogenous levels could account for the reduction of perithecia in those two strains.

Prior to this study, F-2 was produced only on cereal products after 8 to 12 weeks of incubation with two temperature shifts, and a solid substrate was considered a prerequisite (11). We have shown that F-2 was produced in a liquid culture medium and that yields were maximum in 12 days; both *G. zeae* and *F. culmorum* produced F-2. Isolates that produced large amounts of F-2 on corn also produced high levels in liquid culture. On a milligram-per-gram of substrate basis, the amount of F-2 produced on starch-glutamate medium was similar to

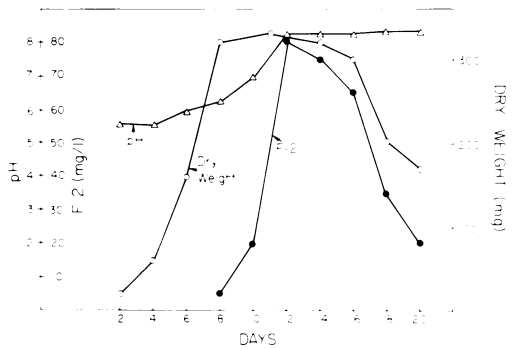

FIG. 2. *Time courses of F-2 production, mycelial dry weight, and pH change in cultures of G. zeae 163.*

that reported from strong and moderate producers on corn (3, 11). However, most strains lost the ability to produce F-2 on the starch-glutamate medium after two generations of transfer onto potato-dextrose agar (data not shown).

F-2 was produced in the medium subsequent to logarithmic-phase growth, but, after continued incubation, production of F-2 and of mycelium declined. The decrease in F-2 might be related to the release of specific enzymes from cellular lysis. Of the substrates tested, starch, glutamic acid, and yeast extract were essential for the production of F-2. That starch was heated in an acid medium suggests that a hydrolysis product might be directly involved in the synthesis of F-2. However, a few hydrolysis products of starch failed to influence the synthesis of F-2 (Table 3). The role played by glutamic acid in the production of fungal metabolites is unknown. Ferreira (6) reported that glutamic acid was indirectly involved in the production of ochratoxin A in shaken culture and that proline could be substituted. A study of the metabolism of glutamic acid during the production of ochratoxin A indicated that portions of it were incorporated into the mycotoxin (1). The production of F-2 followed an increase in pH of the medium, which may represent the deamination of glutamic acid. We did not substitute proline for glutamic acid, but the amino acids tested were not effective substitutes. We are studying the direct involvement of starch, its hydrolysis products, and glutamic acid in the biosynthesis of F-2, since they are found in cereal grain. Another finding revealed that F-2 was produced at only two of the medium volume-to-flask volume ratios tested and was not detected at any other ratios. Cultures grown as described established surface growth more rapidly than those grown in deeper cultures. The relationship of air to F-2 production was not established, since no F-2 was detected in shaken cultures. The media reported reduce the time it takes to identify, produce, and isolate F-2, and thus they are practical for routine screening of F-2 producing isolates of *Fusarium* and for culture studies of that estrogen.

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