Some Genetic Techniques for *Gibberella zeae*

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ABSTRACT

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Gibberella zeae, an important plant pathogen, is exploited commercially in the production of zearalenone, a fungal sex hormone. Genetically, *G. zeae* is relatively intractable. A variety of mutants induced by ultraviolet irradiation were recovered by using a high-sorbose, filtration-enrichment technique. These mutants included: adenine-, arginine-, and histidine auxotrophs; a non-nutritional, heat-sensitive mutant; and an NADPH-dependent, glutamate dehydrogenase-deficient mutant. Protoplasts were liberated by hyphal digestion with commercially available β-glucuronidase and chitinase. Protoplast fusion, mediated by polyethylene glycol 4000, produced stable heterokaryotic colonies under proper selective conditions. Development of these mutants and techniques lays the groundwork for studying the genetics of *G. zeae*.

Additional key words: auxotrophic mutants, *Fusarium roseum* 'Graminearum,' protoplast fusion.

Gibberella zeae (Schw.) Petch, the perfect state of *Fusarium roseum* (Link emed.) Snyder and Hansen 'Graminearum,' causes stem rot of carnation, stalk and ear rot of maize, and head blight of small grains. Some strains of this fungus are used commercially to make the fungal sex hormone zearalenone (13,32), which is subsequently processed to zearalanol. Zearalanol is marketed as RALGRO® (International Minerals and Chemical Corporation, Terre Haute, IN 47808), an anabolic agent for cattle and sheep (13,23).

Little is known of the basic genetics of *G. zeae*. Its homothallic reproductive system and fastidious requirements for laboratory production of perithecia have steered geneticists to more amenable fungi. In this report, protoplast fusion is proposed as an asexual alternative to the conventional sexual cycle. Techniques are described for auxotroph induction and recovery, for protoplast formation and fusion, and for regeneration of colonies from protoplasts.

MATERIALS AND METHODS

Strains. Four primary strains of *G. zeae* were used: 'Dewar' and 65-338B (two wild types from the IMC/CSC culture collection); 251-15 (a strain freshly isolated from a corn kernel by Dr. J. Tuite, Purdue University); and ATCC 20273 (a patented strain used for zearalenone production [14]). Dewar, 65-338B, and 251-15 are morphologically Type A (7), while ATCC 20273 is morphologically Type B (7).

Media. Bennett's medium (10 g dextrose, 2 g NZ Amine [Type A, Sheffield Products, Norwich, NY 13815], 1 g yeast extract [Difco Laboratories, Detroit, MI], 1 g beef extract [Difco Laboratories, Detroit, MI], 2.5 g NaCl and distilled water to make 1 L) was used whenever a complete medium was required. A modified formulation of Coon's synthetic medium (33) and Neurospora synthetic crossing medium (NSC) (31) were used as the minimal media. NSC sorbose medium (NSC sorbose) contained 0.2% (w/v) dextrose, 0.2% (w/v) fructose, and 6% (w/v) sorbose in place of the normal sucrose. Protoplasting medium (PPM) was modified from that of Miller (17) and consisted of 16 mg cysteine, 2 g sucrose, 14 mg NaH_2PO_4, 300 μg streptomycin sulfate, 500 μg chloramphenicol, 10 mg chitinase (Sigma Chemical Co., St. Louis, MO 63178), 0.75 ml β-glucuronidase (Sigma), and distilled water to make 10 ml. Protoplast regeneration medium (PRM) was described by Acha et al (1). Auxotrophic mutants were grown on a minimal medium supplemented with 200 mg of the required nutrient(s) per liter. Solid media contained 2% agar (w/v) except for Coon's synthetic medium (33), which contained 5% agar (w/v). All media were sterilized by autoclaving at 121 C and 1.02 kPa (15 psi) for 20 min. Enzyme solutions were filter sterilized.

Mutagenesis. Ten milliliters of a macroconidial suspension (10^7–10^8 macroconidia per milliliter) in a 2.5% (v/v) Tween-60 solution were placed in a sterile glass petri dish. Mutations were induced by exposure to ultraviolet irradiation from a new UVSL-25 Mineralight lamp (Ultraviolet Products, Inc., San Gabriel, CA 91778). At a 3-cm distance (44 ergs/sec/mm^2), a 60- to 90-sec exposure gave 80–90% kill.

Auxotroph recovery. A filtration-enrichment procedure similar to those for *Neurospora crassa* Shear and Dodge (3,34,35) and *Cochliobolus heterostrophus* Drechsler (15,35) was used to increase the proportion of auxotrophs in the population examined. The irradiated macroconidial suspension was added to 250 ml NSC sorbose medium in a 500-ml Erlenmeyer flask. This flask was completely wrapped with aluminum foil during the first 24 hr of incubation. After 4–6 hr incubation at 28 C on a rotary shaker (120 rpm), the suspension was filtered through a 105-μm-mesh polypropylene filter (Spectrum Medical Industries #146436; Los Angeles, CA 90054), returned to the flask, and placed back on the shaker. Thereafter the filtration was repeated at 12-hr intervals for 72–96 hr with 30-μm-mesh nylon (Spectrum Medical Industries #146506). The final filtrate was mixed with 250 ml of a double strength, 4% sorbose, complete medium or appropriately supplemented minimal medium with agar that had been held at 48 C. The mixture was poured into sterile petri dishes and incubated at 21 C for 5–8 days. Surviving colonies were tested for temperature sensitivity by growth at 28 C and for nutritional auxotrophy by growth on NSC agar.

Preliminary identification of auxotrophs was by auxanography (18). After colonies were identified as auxotrophic, the specific nutritional requirement was identified by embedding at least 10^5 macroconidia in 20 ml of NSC agar in a sterile petri dish and spotting test substances on the surface. Growth responses could be scored after 72–96 hr.

Formation of perithecia was examined using the techniques of Tschanz et al (27–29), and Wolf and Mirocha (33).

Protoplast formation. Strains were grown at 28 C in a 125-ml Erlenmeyer flask containing 25 ml of appropriately supplemented NSC broth and inoculated with $>10^7$ macroconidia. After 18–36 hr

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the contents were filtered through a coarse sintered glass filter and the retained hyphae were washed twice with sterile distilled water. The washed hyphae were placed in a 50-ml Erlenmeyer flask containing 20 ml of PPM and incubated with gentle shaking for 18–24 hr. Protoplasts were separated from hyphal debris by filtration through two 60-μm-mesh nylon filters (Spectrum Medical Industries #146494) over a coarse sintered glass filter and then washed three times by centrifugation (3,000 *g*, 10 min) in 0.6 M KCl. This procedure yielded 10^5–10^6 protoplasts; the yield varied with the strain. Protoplasts regenerated in 2–6 wk on solid PRM as described by Acha et al (1).

Protoplast fusion. Protoplasts to be fused were prepared as described above. These protoplasts were then mixed in approximately equal numbers, centrifuged (3,000 *g*, 10 min), and resuspended in 1 ml of sterile 90 mM $CaCl_2$ with 25% (w/v) polyethylene glycol (PEG) 4000 (Sigma). After 30 min at 4 C, the mixture was plated on unsupplemented PRM and incubated at 28 C up to 6 wk.

Strain preservations. Cultures of both mutant and wild strains were preserved on carnation leaf disks as described by Fisher et al (10). Nutritional supplementation of the auxotrophic mutants was not required.

RESULTS

Filtration enrichment technique. The high-sorbose filtration technique of Applegate et al (3) and Yoder (35) was modified for *G. zeae*. The efficiency of this modified technique was assessed with 10:1 and 100:1 mixtures of ATCC 20273 and *ade-1* (IMC125).

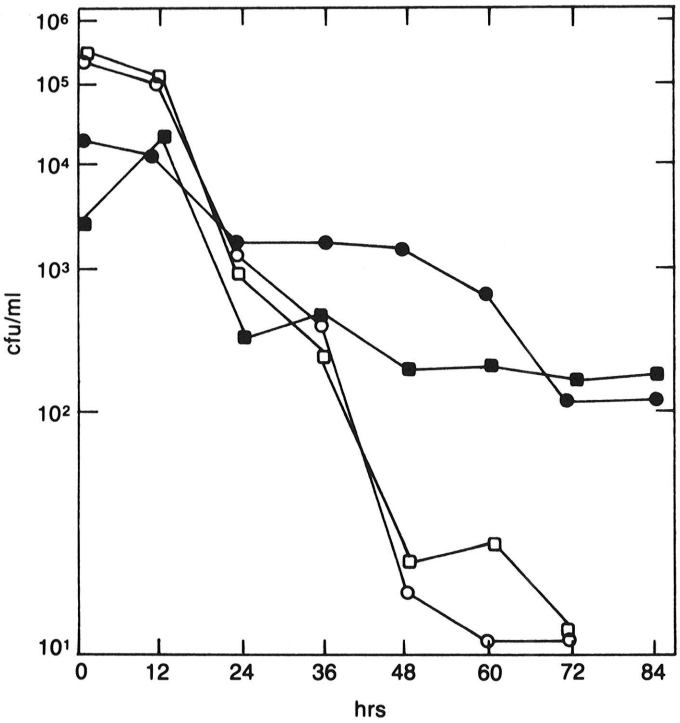

Fig. 1. Efficacy of high sorbose filtration-enrichment using defined inocula. ATCC 20273 and *ade-1* (IMC125) macroconidia were mixed in ratios of 10 ATCC 20273:1 *ade-1*, and 100 ATCC 20273:1 *ade-1*. Each mixture was used to inoculate 250 ml of NSC sorbose medium in a 500-ml Erlenmeyer flask. The filtration enrichment procedure was that described in the text. After each filtering, a sample was removed, diluted, and plated for a viability count on NSC agar and on Bennett's agar. The number of ATCC 20273 colony-forming units (cfu) per milliliter was given by the number of colonies on NSC agar. The number of *ade-1* cfu/ml was obtained by subtracting the number of colonies on NSC agar from the number of colonies on Bennett's agar. Typical results from one experiment are presented, but each experiment was repeated twice. Symbols: □ = 100:1 mixture, ATCC 20273; ■ = 100:1 mixture, *ade-1* (IMC125); o = 10:1 mixture, ATCC 20273; and • = 10:1 mixture, *ade-1* (IMC125).

Samples were collected after each filtration and the number of colony-forming units (cfu) of each type remaining was determined (Fig. 1). In all cases, no detectable ATCC 20273 remained after 72 hr, while at least 100 *ade-1* cfu/ml were retained.

In developing this filtration enrichment a number of important variables were identified. These included: 1) Frequency of filtration—filtering approximately every 12 hr gave the best results. At 24-hr intervals, the accumulated hyphal mass was large enough to plug the filter and retain nongrowing or slowly growing isolates. Filtering more frequently than every 12 hr did not increase the percentage of auxotrophs recovered and increased the chance of contamination. 2) Filter type—both nylon and polypropylene filters were satisfactory. The high surface tension of Teflon® filters greatly retarded the rate of filtering. 3) Filter size—a "large" mesh filter, 100 μm or larger, was required for the first filtration because of the large hyphal mass to be removed. Afterwards, a smaller mesh filter was required for efficient removal of the prototrophic colonies. 4) Molten agar temperature—this temperature should be as low as possible. When the agar temperature exceeded 60 C the number of colonies decreased.

Mutants recovered. Mutants were induced with ultraviolet light and identified by their inability to grow on NSC at 28 C. From nearly 1,000 survivors of 12 independent mutagenesis experiments using ATCC 20273, 24 morphological mutants, 25 auxotrophic mutants, and one non-nutritional temperature-sensitive mutant were identified (5% mutants among the survivors). The morphological mutants were preserved on carnation leaf disks (10) and not studied further. Auxotrophic deficiencies were characterized by auxanography (18). Among the auxotrophs, eight mutants required adenine, one mutant required arginine, one mutant required histidine, one mutant required glutamate, and 14 mutants had undiagnosed requirements. On complete medium, auxotrophs were slower than wild types to produce the characteristic reddish purple pigment. Representative mutant phenotypes, genotypes, and ATCC strain designations are given in Table 1. *Gdh* will be described in more detail elsewhere (Leslie and Kinzel, *unpublished*). Reversion rates of the mutants examined were $<10^{-7}$.

Perithecia formation. *G. zeae* is homothallic and forms perithecia under certain laboratory conditions; some strains are recalcitrant, however. The wild-type strains Dewar, 251-15, and 65-338B usually formed perithecia under the conditions of Tschanz et al (27). Addition of 1–10 ng of zearalenone per leaf disk was stimulatory, and addition of up to 100 ng of zearalenone/leaf disk was not inhibitory, confirming the findings of Wolf and Mirocha (32). Addition of 2% KCl to the agar to increase the osmotic pressure as suggested by Tschanz et al (29) did not affect perithecia formation. None of the strains formed perithecia under the conditions described by Wolf and Mirocha (33).

ATCC strain 20273, which is patented for zearalenone production, formed perithecia on only a few occasions. In each case, the medium was supplemented with zearalenone, and bicelled ascospores were obtained.

Protoplast fusion. Protoplast formation was best with young, 18–24 hr, cultures grown from macroconidia in NSC broth. Both β-glucuronidase and chitinase were required for cell wall digestion; addition of either enzyme alone yielded 1–10% of the protoplasts generated by the combined enzymes. As an osmotic stabilizer, 0.6

TABLE 1. Representative *Gibberella zeae* mutants

Mutant	Allele number	ATCC number	Phenotype
ade-1	IMC125	48063	Requires adenine or inosine
ade-2	IMC140	48065	Requires adenine or hypoxanthine
arg-1	IMC128	48064	Requires arginine or ornithine
gdh	IMC160	48067	Deficient for glutamate dehydrodgenase
his-1	IMC151	48066	Requires histidine
uts-1	IMC103	···	Unknown temperature sensitive (grows at 21 C, but not at 28 C)

M KCl yielded more protoplasts than either 0.8 M mannitol or 10% (w/v) sorbose. The regeneration medium of Acha et al (1), termed PRM, was suitable for the regeneration of protoplasts. Protoplast regeneration required incubation for up to 6 wk.

Protoplast fusion occurred readily in 25% (w/v) PEG 4000; increasing the molecular weight of the PEG to 8000 had no effect on fusion frequency. Addition of 0.2 M $CaCl_2$ to the fusion medium as proposed by Thomas and Davis (25) for *Aspergillus* sp. was detrimental.

Protoplasts from four of the strains were fused in different combinations (Table 2), and phenotypically prototrophic colonies were recovered by plating on unsupplemented PRM. (No prototrophs were recovered when protoplasts of any of the parental auxotrophs were treated with PEG and plated on unsupplemented PRM agar.) Colonies growing on PRM were transferred to NSC agar. Colonies growing on NSC agar displayed morphological and cultural abnormalities. Most of these colonies developed several sectors with different morphologies and variable growth rates. Transfers to Bennett's agar after 1 or 2 days on NSC agar produced unstable colonies that quickly resolved into sectors. Subcultures from Bennett's agar to supplemented and unsupplemented NSC agar showed that the sectors were auxotrophic for one of the two forcing markers. Ten to 14 days after transfer from PRM to NSC agar, sectoring ceased and the outermost sectors appeared morphologically normal. Transfers from these outer sectors to Bennett's agar were morphologically stable. Transfers from the periphery of the colonies growing on Bennett's agar to supplemented and unsupplemented NSC agar showed that in a given transfer only one of the two prototrophic markers remained. Both prototrophic markers were recovered individually from each colony examined, but both prototrophic markers were never recovered concurrently in an individual subculture. Thus the prototrophic colonies generated by protoplast fusion appeared to be heterokaryons that were stable as long as the selective pressure remained.

TABLE 2. Protoplast fusion with auxotrophs of *Gibberella zeae*

Strain 1	Strain 2	Prototrophs[a]/ Regenerates[b]
ade-1(IMC125)	*arg-1*(IMC128)	3/125
ade-1(IMC125)	*his-1*(IMC151)	7/5,245
arg-1(IMC128)	*his-1*(IMC151)	34/4,471
arg-1(IMC128)	*ade-2*(IMC140)	3/425

[a] Number of colonies on unsupplemented PRM agar.
[b] Number of colonies on PRM agar supplemented with adenine, arginine and histidine. The number preceding the slash is the number of prototrophs and the number following the slash is the number of regenerates.

DISCUSSION

Protoplast formation and fusion has recently taken on a new aura of importance. Fungal protoplasts are valuable for the study of cellular ultrastructure and permeability (9,12,24,30), and are frequently required for recombinant DNA studies. Fused protoplasts bypass many natural biological boundaries such as species differences (2,6,22), heterokaryon incompatibility (8), and mutations to sterility (11,26). Secondary metabolite production by the parental strains and by recombinants generated via protoplast fusion may differ both quantitatively and qualitatively (2,20). When suitable commercial strains are available as starting material, protoplast fusion may be preferred over mutation for strain improvement, since the frequency of induced mutations is less than the frequency of protoplast fusion, and since protoplast fusion alone would not induce unknown deleterious mutations.

The protoplasts and auxotrophs described in this report could be used in the transformation of *G. zeae* with foreign DNA. It is possible, for example, that the *gdh* lesion could be complemented by the cloned *am* gene from *Neurospora crassa* (see reference 19) and transformed cultures selected by growth on a minimal medium.

The distribution of mutants isolated is unusual; a large number of adenine mutants (8 of 25 auxotrophs) was collected. Whether the high frequency of these mutants is due to the large number of steps in the pathway, preferential selection in the filtration enrichment, or to a transposable element activated by the ultraviolet light is not known (5,21).

Genetic studies with *G. zeae* may now proceed either sexually or asexually. Exploitation of the sexual route will require one of several innovations. One such innovation would be the development of a synthetic medium suitable for producing fertile perithecia. Auxotrophic markers could then be used to force crosses, a strategy currently unavailable since the auxotrophic mutants grow on carnation leaf disks without nutritional supplementation. Alternatively, complementary meiotic mutants could be used to ensure that both parental nuclei were included in the fertile perithecia. In either case, the meiotic products should be analyzed as tetrads so that any homothallic asci resulting from cross-feeding can be excluded. Without such innovations, working with conventional sexual analysis of *G. zeae* will be difficult, if not impossible.

Using sexual recombination to develop commercial strains does not appear promising. ATCC strain 20273, which is the basis of the commercial fermentation, is at best poorly fertile. This infertility may be attributed to this strain's ability to produce zearalenone in submerged culture. Alternatively, the infertility may be attributed to the increased production of zearalenone, a fungal sex hormone. Increased zearalenone production could influence fertility in two ways. First, excessive amounts of exogenous zearalenone inhibit formation of perithecia (32). Mere overproduction of zearalenone however, is insufficient to explain ATCC 20273's infertility, since this strain produces perithecia when supplemented with zearalenone. Second, and more likely, in the process of mutation and selection for a high zearalenone producer, genes that normally respond to zearalenone were probably altered or inactivated. Since some or all of these genes would normally govern initiation of the sexual process, their dysfunction could lead to this strain's relative infertility. Neither the ability to produce zearalenone in submerged culture nor the ability to produce large amounts of zearalenone could be sacrificed to enhance fertility.

The protoplast fusion system is an attractive, although not perfect, alternative to the conventional sexual cycle. One defect with protoplast fusion, as with other asexual systems, is that the segregation ratios in general do not give information on the number or functional relationship of the genes involved. When complex traits are being studied, this type of information is vital; indeed, it may be the only information available (16). Protoplast fusion could be especially valuable in discerning the regulatory and biosynthetic controls on zearalenone and related secondary metabolite production in a variety of different *Fusaria* (4), since heterokaryons and possibly true recombinants between strains of different species can be generated.

The system described is suitable for analyses when both strains carry markers. The markers tested to date are, unfortunately, all recessive. For efficient commercial strain development and population studies a selectable dominant mutant is needed. (If time and effort are relatively unimportant, then a distinct isozyme could be employed.) Some dominant mutants worth screening for in *G. zeae* include morphological variants and resistance to drugs such as acriflavin and spectinomycin. A doubly marked strain carrying both a dominant and recessive mutation could be fused with an unmarked wild-type or commercially important strain. Hybrids between the two lines could be isolated by selecting for the dominant mutant and against the recessive mutant. These hybrids would be valuable in commercial strain improvement programs and in the study of plant pathogenicity mechanisms.

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