Protoplasts from *Gibberella fujikuroi*

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ABSTRACT

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Protoplasts from *Gibberella fujikuroi* were formed from early stationary-phase mycelia by treatment with β-glucuronidase. Protoplast formation was significantly enhanced by cysteine and ethylenediamine tetraacetic acid (EDTA) included with the enzyme in osmotic medium. The enhancement by EDTA could be reversed with $MgSO_4$ or KCl. Preincubation of mycelia with thiol compounds such as glutathione, dithiothreitol, or mercaptoethanol also increased protoplast formation. Addition of chitinase with β-glucuronidase, EDTA, and cysteine after preincubation of mycelia with mercaptoethanol and EDTA yielded the greatest number of protoplasts. The latter treatment was also used to obtain protoplasts from *G. fujikuroi* microconidia and macroconidia.

Additional key words: cell wall degradation.

Gibberella fujikuroi (Saw.) Wr. synthesizes gibberellins (GAs), secondary metabolites that induce a variety of plant responses related to growth and development (15,18). The fungus was isolated in 1926 as the cause of the "bakanae" disease of rice in Japan (12). Since that time, the disease has been associated with rice in other countries (24). The fungus also infects maize, sugarcane, tomato, banana, and wheat (4). However, isolates of *G. fujikuroi* from those plants usually lack the ability to synthesize gibberellins (4). Of the 53 naturally occurring GAs, 22 are synthesized by *G. fujikuroi* (19).

Although the metabolic pathway leading to the synthesis of gibberellins has been well studied, very little information is available on the genetic control of gibberellin biosynthesis. Lack of this information may be due to the difficulty in obtaining the sexual stage of the fungus in the laboratory (20,22). Spector and Phinney (22) reported that perithecia of *G. fujikuroi* were obtained when strains of opposite mating types were grown on a citrus stem medium. They detected two genes, g_1 and g_2, involved in gibberellin production. A mutation in gene g_1 inhibited biosynthesis of all the gibberellins. Presumably, an early step in gibberellin production was affected. Alterations in gene g_2 blocked synthesis of GA_3 and GA_1, both end products of GA synthesis, but synthesis of their immediate precursors, GA_4 and GA_7, was not affected. Since Spector and Phinney's (22) original work, no additional information on the genetic control of gibberellin production by the fungus has been reported. The development of a procedure to obtain *G. fujikuroi* protoplasts should facilitate generating this information. Protoplasts can be used to evaluate recombinant products of protoplast fusion (13,16,17,25) or in transformation studies with plasmid or fungal DNA (3,6,14). The protoplast fusion technique may also be an effective procedure to increase the production of gibberellins such as GA_3, GA_4, and GA_7 by *G. fujikuroi.* Fusion products from protoplasts of *Cephalosporium acremonium* synthesized higher amounts of cephalosporin C than either parental strain (17), and improved industrial yeast strains may be obtained by protoplast fusion (23).

A necessary prerequisite for the fusion technique is to develop a procedure for generating sufficient quantities of protoplasts from mycelia or conidia. This report describes techniques for obtaining protoplasts from *G. fujikuroi* and the influence of several thiol compounds, EDTA, and wall-degrading enzymes on the process.

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MATERIALS AND METHODS

Fungus. The culture used in this study was a derivative of *G. fujikuroi* isolate 917 originally obtained from Imperial Chemical

Industries Limited (Cheshire, England). This culture, after several cycles of mutagenesis and selection, produces high yields of three gibberellins: GA_3, GA_4, and GA_7.

Stock cultures were transferred every 2 mo to slants of CYE agar medium (1 g cerelose, 1 g yeast extract, and 15 g purified agar per liter of water), incubated for 2 wk at 27 C, then maintained at 4 C.

Chemicals and enzymes. β-Mercaptoethanol, DL-dithiothreitol, β-glucuronidase, mannitol, chitinase, L-cysteine-HCl, and ethylenediamine tetraacetic acid (EDTA, disodium salt) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Other chemicals were of reagent grade.

Preparation of mycelia for protoplast formation. Fungal growth was completely removed from an agar slant and inoculated into 100 ml of Czapek's broth (Difco) in a 500-ml Erlenmeyer flask. Four flasks were prepared per experiment. Cultures were incubated at 27 C on a rotary shaker at 240 rpm for 18–24 hr. Mycelium was harvested by centrifugation, washed twice with sterile water, and once in either EDTA (5 mM, pH 4.5) for pretreatment experiments or, if no pretreatment was performed, in mannitol-citrate-phosphate buffer, pH 6.1 (0.1 M NaH_2PO_4, 0.1 M Na_3 citrate, and 0.8 M mannitol). Between centrifugations, mycelial suspensions were allowed to stand for 10 min at room temperature. Supernatants were then decanted slowly to prevent loss of mycelia. Dry weights were determined after the final wash.

Mycelium washed by filtration (Whatman No. 1 filter in a Millipore filter apparatus) was also tested in protoplast experiments. The number of protoplasts generated from mycelia washed by filtration was equivalent to that obtained from mycelia washed by centrifugation.

Protoplast formation. For pretreatment conditions, fungal mycelia were incubated for 30 min at 30 C with EDTA (5 mM, pH 4.5) plus either of four different thiol compounds: 0.2% β-mercaptoethanol, 50 mM dithiothreitol, 0.1% glutathione, or 0.1% cysteine. Mycelia were then washed twice in water, once in mannitol-citrate-PO_4 buffer, pH 6.1, and resuspended in equal amounts of the buffer. These mycelia were then incubated in β-glucuronidase enzyme alone to test the effect of various pretreatment conditions. Once an effective pretreatment condition was determined, the mycelia were incubated with various combinations of β-glucuronidase, cysteine, EDTA, and chitinase to test additional effects.

When no pretreatment was performed, washed mycelia were incubated with 10% β-glucuronidase and/or cysteine (0.1%), EDTA (0.1 M), or chitinase (250 μg/ml) in mannitol-citrate-PO_4 medium. All incubations were for 18 hr at 27 C to obtain sufficient protoplast yields. Protoplasts were identified with a phase contrast microscope, and total numbers of protoplasts were determined with a hemacytometer. Viability of protoplasts was determined with methylene blue. Protoplasts that did not retain the methylene blue stain were considered viable. The ability to regenerate in liquid Czapek's broth (Fig. 1) was a confirming test of viability (qualitative measurement).

Isolation of conidia. *G. fujikuroi* was grown at 27 C on CYE agar plates for 2–3 wk. Two milliliters of saline (0.9% NaCl, w/v) was added to each plate. Fungal mycelium and conidia were scraped from agar surfaces and put into large tubes (25 × 150 mm) containing 25 glass beads (5 mm in diameter). Contents from five agar plates were contained in one tube. The suspension was stirred

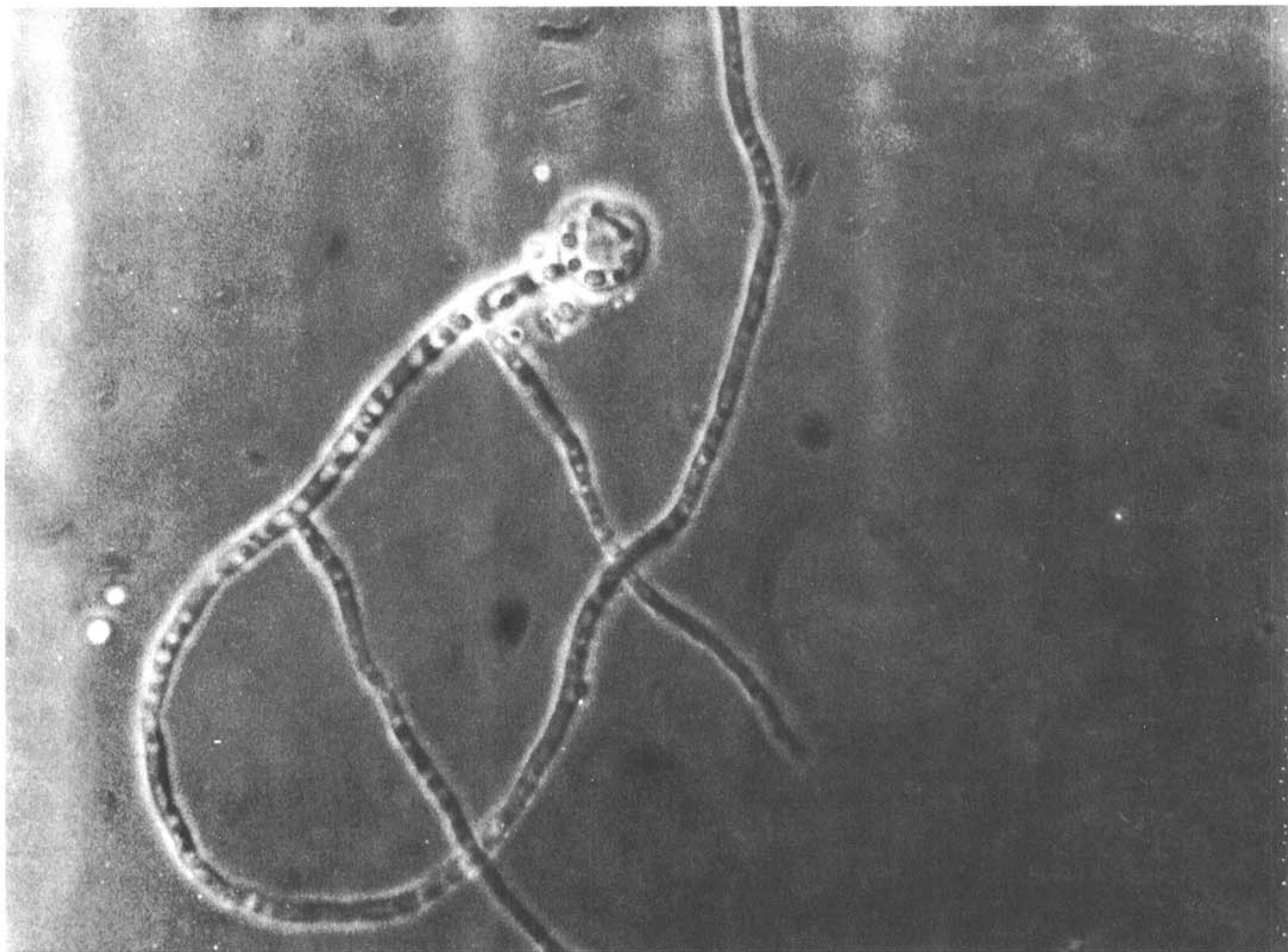

Fig. 1. Regenerated protoplast of *Gibberella fujikuroi* in Czapek's broth (×2,400).

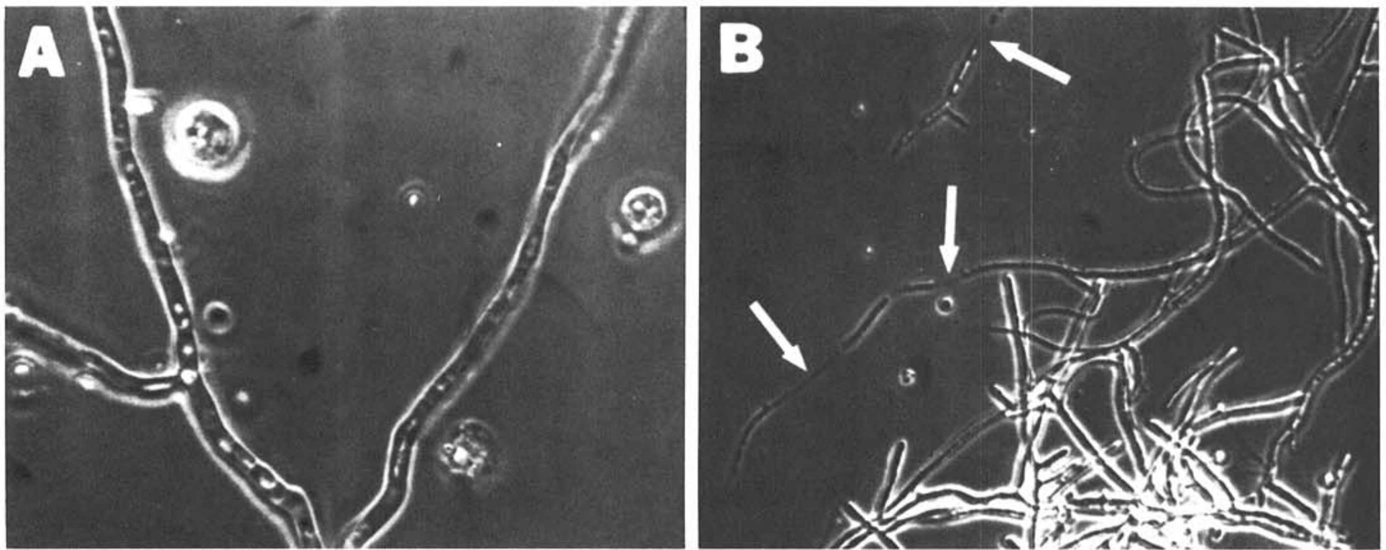

Fig. 2. Phase-contrast micrographs of hyphae and released protoplasts of *Gibberella fujikuroi*. **A,** Fungal protoplasts and parent hyphae. The protoplast contained vacuoles and granules similar to those found in the parent hyphae (×750). **B,** Empty hyphal segments (arrow) in the medium after protoplasts were released following digestion of hyphal walls by β-glucuronidase (×300).

on a vortex mixer for 1 min and filtered through Whatman No. 1 filter paper. Mycelia remained on the filter paper, and macroconidia and microconidia were recovered in the filtrate. Spores were pelleted by centrifugation and resuspended in saline to give an appropriate spore concentration. Frequently, 10% (v/v) glycerol and 50 μg of chloramphenicol per milliliter were added to saline so that spores could be stored at −85 C. Although saline was used to remove spores and mycelia from agar surfaces, water was just as efficient.

Preparation of protoplasts from conidia. Conidia (microconidia and macroconidia) were washed twice in water, resuspended at 5 $\times 10^6$ conidia per milliliter in EDTA (5 mM, pH 4.5) plus 4% β-mercaptoethanol, and incubated for 75 min at 30 C. After incubation, conidia were centrifuged for 15 min at 6,000 rpm in a Sorvall GLC-2B general laboratory centrifuge. The pellet was washed twice in equal volumes of water and once in mannitol-citrate-PO_4 buffer before it was resuspended in mannitol-citrate-PO_4 buffer with 20% β-glucuronidase, 0.2% cysteine, EDTA (0.1 M), and 0.2% chitinase per milliliter. This suspension was incubated for 6 hr at 27 C.

RESULTS

Protoplast formation from mycelia. Early stationary-phase cultures of *G. fujikuroi* (18–24 hr old) in synthetic medium were used for preparation of fungal protoplasts from mycelia. Protoplasts were detected in the medium as early as 30 min after the addition of enzyme and their numbers increased with time. The diameter of protoplasts ranged from 1.8 to 14 μm. Several of the protoplasts contained granular inclusions and vacuoles that were present in the parent hyphae (Fig. 2A). Empty hyphal segments (Fig. 2B) were detected after protoplasts were released into the surrounding medium through openings in the cell walls. Fig. 3 diagrams the release of protoplasts from hyphae. The protoplasts were stable as long as they were maintained in an osmotic environment. Dilution of the protoplast suspension 10-fold with water resulted in lysis. The protoplasts remained viable for at least 6 days at 4 C.

Effect of cysteine and EDTA on the formation of protoplasts. Although protoplasts were produced in osmotic medium containing only β-glucuronidase, the yield was low. Microscopic observation of the mycelia after 18 hr in the enzyme indicated that many of the hyphal filaments were intact. Various compounds were tested for ability to increase protoplast numbers. Table 1 summarizes three different experiments showing the effect of cysteine and EDTA on protoplast formation. A noticeable increase

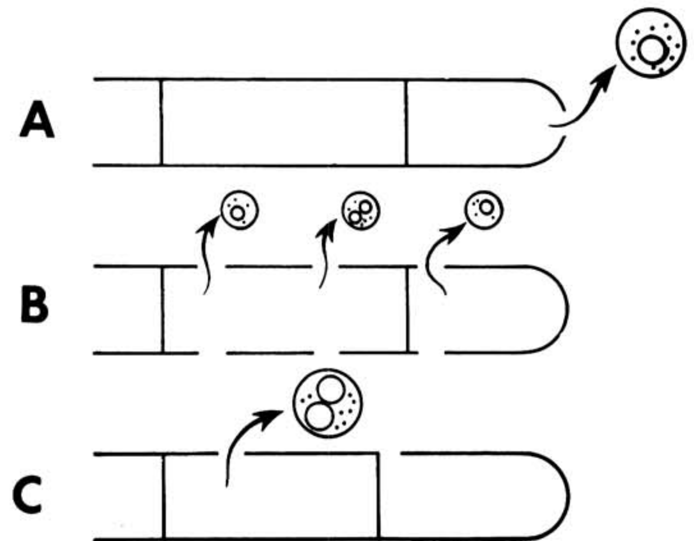

Fig. 3. Diagrams showing release of protoplasts of *Gibberella fujikuroi* from fungal hyphae. **A,** Through openings at the tip end of a hyphae. **B,** As several small protoplasts with two or more protoplasts coming out of one compartment. **C,** As one large protoplast resulting from the entire contents of a compartment being released into the medium. Protoplast formation was slow when only β-glucuronidase was used in osmotic medium. Therefore, all of the above modes of protoplast release could be observed microscopically.

TABLE 1. Effect of EDTA and cysteine on protoplast formation in *Gibberella fujikuroi*

Reaction condition[a]	Protoplasts per ml[b]	Increase in number of protoplasts (× control)
Enzyme (control)	3.8×10^6	···
Enzyme + cysteine	$8.4 (\pm 0.41) \times 10^6$	2.2
Enzyme + EDTA	$5.3 (\pm 0.90) \times 10^6$	1.4
Enzyme + cysteine + EDTA	$1.2 (\pm 0.20) \times 10^7$	3.2

[a] Basal medium contained sodium citrate-phosphate buffer (pH 6.1) with 0.8 M mannitol and 10% β-glucuronidase; either 0.1% cysteine or 0.1 M EDTA was added. Between 40 and 60 mg (dry weight) of mycelia per milliliter was incubated 18 hr at 27 C.

[b] These data are averages of three independent determinations. Numbers of protoplasts were determined with a hemacytometer. Variation in replicates are mainly due to differences in size ranges.

in the yield of protoplasts occurred over control levels when cysteine was added to β-glucuronidase in osmotic medium. EDTA alone with enzyme gave a very slight increase in numbers of protoplasts, but not to the level obtained by using cysteine. Protoplast numbers increased most significantly when cysteine and EDTA were included with enzyme in the osmotic medium. A 3.2-fold increase in numbers of protoplasts was achieved. The increase resulting from EDTA and cysteine was reversed by addition of 10^{-3} M $MgSO_4$ or KCl to the medium. When EDTA alone was added with enzyme in osmotic medium, Mg^{++} or K^+ had no effect. Others (7,8,26) have also reported the EDTA enhancement of protoplast formation with fungi.

Preincubation of mycelia in the presence of thiol compounds. Four thiol compounds were tested for effect on protoplast formation from mycelia. Data are summarized in Table 2. Mycelia were pretreated with a thiol compound plus EDTA, washed, and then incubated with β-glucuronidase.

Pretreatment of mycelia with dithiothreitol or mercaptoethanol resulted in significant increases in numbers of protoplasts with mercaptoethanol generating the highest yield at the concentration of thiol compounds tested. Preincubation with EDTA plus cysteine or glutathione was not effective. Although cysteine had no effect on protoplast formation under pretreatment conditions, cysteine was very effective (Table 1) when used in combination with enzyme directly in the incubation medium.

Chitinase treatment. Although tests for the presence of chitinase in the β-glucuronidase preparation were not made, Torres-Bauza and Riggsby (26) detected "weak chitinase activity" in similar preparations. Since walls of some fungi contain chitin, the effect of chitinase and β-glucuronidase on protoplast formation was tested (Table 3). Chitinase caused an additional increase in numbers of protoplasts. The greatest number of protoplasts were formed after mycelia were pretreated with mercaptoethanol and EDTA with subsequent incubation in β-glucuronidase, cysteine, EDTA, and chitinase.

Protoplast formation from conidia. Protoplasts were generated from both microconidia and macroconidia. After the release of small protoplasts from microconidia, only fungal debris remained in the medium. However, macroconidia appeared to be more stable and remained intact, releasing their protoplasts through openings in the side or tip of the conidium. Some spores did not appear to have been damaged by the treatment and, therefore, 100% release of protoplasts from the spores was not achieved. In a preparation containing macroconidia and microconidia, protoplast sizes ranged from 1.5 to 4 μm.

No protoplasts from conidia were detected after 18 hr of incubation in osmotic medium containing only 10% β-glucuronidase. Either the protoplasts were not generated from spores at all or the quantity was too low to detect. However, protoplasts were obtained when spores were pretreated with 4% mercaptoethanol plus EDTA and then incubated with 20% β-glucuronidase. The total numbers formed appeared to be lower than when all ingredients, β-glucuronidase, cysteine EDTA, and chitinase were used. Approximately 50% of the spores formed protoplasts as a result of this treatment.

DISCUSSION

Gibberella fujikuroi infects a wide variety of plants including rice, corn, sugarcane, and cotton (4). Conditions for generating large numbers of protoplasts from mycelia and conidia of this fungus may be of interest to plant pathologists because protoplasts can be used in genetic and physiological studies. Cell walls of this fungus were sensitive to β-glucuronidase. This is a significant finding since the sensitivity of fungal walls to attack by lytic enzymes varies among fungi, and with a particular fungal species sensitivity can vary from strain to strain (26). Protoplasts of *G. fujikuroi* can be obtained when a combination of mercaptoethanol plus EDTA pretreatment is used with subsequent incubation in the presence of cysteine, EDTA, chitinase, and β-glucuronidase enzymes.

All components seem to play a significant role in protoplast formation. Cysteine may increase the activity of β-glucuronidase, and EDTA has been reported to prevent cell clumping (8). Less clumping of mycelia may permit greater access of hyphal filaments to attack by lytic enzymes. Mercaptoethanol may act directly on cell walls by reducing disulfide bonds in wall proteins, thereby increasing the sensitivity of the walls to β-glucuronidase (1). Enhancement of protoplast formation using chitinase was due to the fungal walls of *G. fujikuroi* containing chitin (27). The successful release of protoplasts from conidia of *Neurospora crassa* (2), *Trichothecium roseum* (11), *Fusarium culmorum* (10), and recently *Aspergillus nidulans* (5), has been reported. In the case of *T. roseum* and *F. culmorum*, Strepzyme, a lytic enzyme extracted from various *Streptomyces* species, was employed. *Oerskovia* lytic enyzme obtained from *O. xanthineolytica* was used with *A.*

TABLE 2. Effect of preincubation with thiol compounds on protoplast formation in *Gibberella fujikuroi*[a]

Preincubation condition[b]	Protoplasts per ml[c]	Increase in number of protoplasts (× control)
EDTA (control)	2.0×10^6	...
EDTA + dithiothreitol	$6.6 (\pm 0.90) \times 10^6$	3.3
EDTA + mercapto-ethanol	$8.4 (\pm 1.8) \times 10^6$	4.2
EDTA + cysteine	$2.2 (\pm 0.49) \times 10^6$	1.1
EDTA + glutathione	$3.0 (\pm 0.93) \times 10^6$	1.5

[a] Basal medium contained sodium citrate-phosphate buffer (pH 6.1) with 0.8 M mannitol and 10% β-glucuronidase. Between 20 and 40 mg (dry weight) of mycelia per milliliter was incubated 18 hr at 27 C.
[b] Basal medium contained 5 mM EDTA (pH 4.5) and either 50 mM dithiothreitol, 25 mM β-mercaptoethanol, 6 mM cysteine, or 6 mM glutathione was added. Incubation was 30 min at 30 C.
[c] These data are averages of three independent determinations. Numbers of protoplasts were determined with a hemacytometer. Variation in replicates are mainly due to differences in size ranges.

TABLE 3. Effect of cysteine, EDTA, and chitinase on protoplast release from hyphae of *Gibberella fujikuroi* after preincubation of mycelia with thiol compounds

Preincubation conditions[a]	Reaction conditions[b]	Protoplasts per ml[c]	Increase in number of protoplasts (× control)
EDTA	Enzyme	2.4×10^6	...
EDTA	Enzyme + cysteine + EDTA	$6.4 (\pm 0.49) \times 10^6$	2.68
EDTA + mercaptoethanol	Enzyme	$1.3 (\pm 0.28) \times 10^7$	5.40
EDTA + mercaptoethanol	Enzyme + cysteine	$1.3 (\pm 0.57) \times 10^7$	5.38
EDTA + mercaptoethanol	Enzyme + cysteine + EDTA	$1.3 (\pm 0.46) \times 10^7$	5.33
EDTA + mercaptoethanol	Enzyme + cysteine + chitinase	$1.6 (\pm 0.21) \times 10^7$	6.88
EDTA + mercaptoethanol	Enzyme + cysteine + chitinase + EDTA	$2.2 (\pm 0.14) \times 10^7$	9.17

[a] Preincubation conditions: Basal medium contained 5 mM EDTA, pH 4.5, with or without 0.2% β-mercaptoethanol.
[b] Reaction conditions: Basal medium contained sodium citrate-phosphate buffer (pH 6.1) with 0.8 M mannitol and 10% β-glucuronidase; either 0.1 M EDTA, 0.1% cysteine, or 250 μg of chitinase per milliliter was added. Between 30 and 40 mg (dry weight) of mycelia per milliliter was incubated 18 hr at 27 C.
[c] These data are averages of two independent determinations. Protoplasts were determined with a hemacytometer. Variation in replicates are mainly due to differences in size ranges.

nidulans. Protoplasts were obtained from conidia of *G. fujikuroi* by increasing the concentrations of all the components required for protoplast formation from mycelia.

Fungal protoplasts can be used in a variety of experiments involving genetic manipulations that may aid in understanding host-parasite interactions. In the case of *G. fujikuroi,* the ability to produce the "bakanae" disease on host plants is dependent on the expression of genes involved in gibberellin synthesis (27). Burrow et al (4) reported that gibberellin production is mainly associated with rice-infecting strains of *G. fujikuroi.* Isolates from other plant sources seldom synthesize gibberellins. This finding suggests a genetic difference between the gibberellin-producing and nonproducing strains possibly initiated by the host plant. Recently, isolates of *G. fujikuroi* that synthesize relatively high levels of gibberellin-like substances have been obtained from maize (Phinney, *personal communication*).

Zak (27) compared the pathogenicity of gibberellin-producing and nonproducing strains of *G. fujikuroi* in oats. The producing strain was more virulent than the nonproducing strain. He concluded that the production of gibberellins enhances the pathogenicity of this fungus. Whether the nonproducing strains lack the ability to synthesize gibberellins due to mutation (chromosomal or extrachromosomal), genetic rearrangement (transposon), or plasmid exclusion, and whether the genetic change can be related to the host plant is not known. The fusion and transformation techniques that involve the use of fungal protoplasts provide an excellent opportunity to investigate the genetics of gibberellin production.

Transformation systems have been developed for *Saccharomyces cerevisiae* (14), *Schizosaccharomyces pombe* (3), and *N. crassa* (6). Fungal protoplasts can be fused to generate recombinant products that can be analyzed by methods used in parasexual cycle analysis (17) or other conventional means (13,16,25). The advantages for using protoplasts over developing the sexual stage, especially for *G. fujikuroi*, are: protoplasts can be used in the protoplast fusion technique, the protoplast fusion technique can be easily performed in the laboratory and is reproducible, there is no requirement for specific mating types to obtain recombinant products (9,13,21), and protoplasts from microconidia (uninucleated spores) can be used to generate fusion products that can be subjected to sexual cycle analysis.

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