Techniques

Preparation and Regeneration of Protoplasts from Axenic Mycelia Derived from the Wheat Stem Rust Fungus

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Supported in part by grants from the U.S. National Science Foundation (DCB-8701713) and from the U.S. Department of Agriculture (86-CRCR-1-1937).

Accepted for publication 1 August 1989.

ABSTRACT

Huang, D., Staples, R. C., Bushnell, W. R., and Maclean, D. J. 1990. Preparation and regeneration of protoplasts from axenic mycelia derived from the wheat stem rust fungus. Phytopathology 80:81-84.

Protoplasts were prepared from axenic suspension cultures of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici*, race 126 Anz-5,6,7,11. Protoplasts were released in 3 hr from 3- to 7-day-old mycelia with a mixture of Novozym 234 and cellulase (5 mg each/ml) osmotically stabilized by 0.8 M $MgSO_4$. Protoplast regeneration was detected microscopically within 10 hr after incubation in a nutrient medium containing 0.5 M sorbitol, and colonies usually were visible after a further 3 days of growth. The regeneration frequency ranged from 19 to 39%.

Isolation of protoplasts from rust fungi would make possible a number of studies on these obligate parasites in the areas of genetics and cell biology. Protoplast-based transformation systems, for example, have been developed for certain basidiomycetes, notably *Schizophyllum commune* Fr. (7) and *Ustilago maydis* (DC.) Cda. (11) for the uptake and expression of foreign genes. A quite different need for protoplasts is the study of mechanoreception, the mechanism by which many of the rusts receive information about the location of stomates (3). Studies on mechanoreceptors, carried out using patch clamps (2), require protoplasts as a source of the plasmalemma membrane. Here, we have developed a protocol for producing useful quantities of protoplasts capable of high-frequency regeneration from mycelia of the wheat stem rust fungus *Puccinia graminis* Pers. f. sp. *tritici* Ericks. & E. Henn. grown in axenic culture. For this purpose, we used nonpathogenic strain V1B of the fungus which is adapted to rapid growth in suspension culture (4).

MATERIALS AND METHODS

Fungal strains. Axenic strain V1B was cultured originally from urediospores of *P. g. tritici*, race 126-ANZ-5,6,7,11 (4). It has been used in several investigations requiring large quantities of rust fungal mycelium (5,6).

Culture medium. The basic nutrient medium (BM) consisted of 4 g of Evans peptone (Evans Medical Ltd., Liverpool, England), 4 g of Difco Casamino acids (Difco Laboratories, Detroit, MI), 30 g of dextrose, 100 ml of a 10× stock solution of Czapek's minerals which contained 100 mM sodium citrate, and distilled deionized water to 1 L, pH 6.0. These experiments were carried

out using Evans peptone, but it is no longer manufactured. Peptic peptone (U.S. Biochemical Corp., Cleveland, OH) is an effective substitute.

Isolation of protoplasts. A mycelial colony grown for about 2 wk on BM solidified with 1% Bacto agar was ground in about 10 ml of liquid BM for 5 sec in a sterile Sorvall Omnimixer (OCI Instruments, Waterbury, CT). The mycelial suspension then was transferred to 50 ml of liquid BM and grown 3–5 days at 20 C on an orbital shaker. Confluent mycelium contained in about 40 ml of suspension medium (approximately 1.5 g dry weight) was collected by centrifugation at 10,000 *g* for 20 min. The damp pellet was dispersed in 15 ml of osmotic medium (OM) (0.8 M $MgSO_4 \cdot 7H_2O$, 0.5% MES buffer [Sigma Chemical Co., St. Louis, MO]), 5 mg/ml of Novozym 234 (Novo Biolabs, Wilton, CT) and 5 mg/ml of Cellulase Type V (C-2274, Sigma Chemical Co.), pH 5.5, and enzymatically digested with gentle shaking (80 rpm) at 24 C for 3–4 hr. The protoplasts were filtered first through four layers of sterile cheesecloth and a 20 μm-mesh nylon filter (Spectrum Medical Industries, Inc., Los Angeles, CA), then transferred to a sterile 15-ml centrifuge tube, overlayered with half a volume of SM_1 buffer (0.6 M sorbitol, 0.5 M MES, pH 5.5), and centrifuged at 3,000 *g* for 20 min. The majority of the protoplasts sank to the bottom, but some purified protoplasts accumulated at the interface; for example, $82 \pm 16 \times 10^8$ was at the bottom compared with $29 \pm 12 \times 10^7$ at the step gradient interface. The purified protoplasts at the interface were collected in a pipette, diluted with an equal volume of SM_2 buffer (1 M sorbitol, 0.5% MES, pH 5.5), and centrifuged at 1,500 *g* for 10 min. The protoplasts were washed twice more by resuspension in SM_2 buffer followed by centrifugation at 1,000 *g* for 8 min and 1,000 *g* for 5 min to collect the pellets. The number of protoplasts in the final pellet was determined by counting an aliquot in a hemacytometer.

The protoplasts also can be collected without purification on a step gradient. To do this, the protoplast suspension in OM medium was filtered as described above and diluted with an equal volume of SM_2 medium, and the protoplasts were collected by centrifugation at 3,000 *g* for 20 min. Finally, the pellet was washed three times by dilution with an equal volume of SM_2 buffer, followed by centrifugation at 1,500 *g* for 10 min, 1,000 *g* for 8 min, and 1,000 *g* for 5 min to collect the pellets. The pelleted protoplasts were resuspended in 100 μl of SM_2, and the numbers were determined by counting an aliquot in a hemacytometer.

Regeneration. The protoplasts were added to 10 ml of regeneration medium (RM) (BM + 0.5 M sorbitol) and incubated at 20 C. To observe the regeneration process, samples were withdrawn at intervals and observed using a microscope equipped with phase-contrast optics. The regeneration frequency was the ratio of the number of protoplasts that developed hyphae to the total number of protoplasts.

Fluorescence microscopy. Nuclei in the protoplasts were visualized using the DNA-specific stain mithramycin (Sigma Chemical Co.) (10). Usually 50 μl of the protoplast suspension was added to a 50-μl drop of 1% glutaraldehyde, and the mixture incubated on a microscope slide for 30 min. The liquid then was decanted, and the protoplasts attached to the glass were stained by adding a drop of mithramycin solution (2 mg/ml in 10% $MgCl_2$), covered with a cover glass, and observed using a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY) fitted with an FITC filter kit.

Uvitex (a gift from Ciba Geigy Co., Greensboro, NC) was used to follow wall regeneration by fluorescence microscopy. A 50-μl-volume of protoplasts was diluted with an equal volume of 5% Uvitex in water, covered with a cover glass, and observed by fluorescence microscopy as above.

RESULTS

Choice of enzymes. Novozym 234 was more effective for protoplast isolation from mycelia of *P. g. tritici* than glucuronidase, chitinase zymolyase, or cellulase, and the combination of Novozym 234 and cellulase was more efficient than either alone (data not shown). Because this combination caused protoplasts to be released from both the tip and other parts of the hypha, the protoplasts were variable in size. The average diameter of the protoplasts shown in Figure 1 was 33.6 $\pm$ 7.5 μm ($n = 7$), but their size varies with the medium used for suspension. The protoplasts had two nuclei; however, many of the protoplasts obtained from urediospore germlings of race 32 using this technique had one nucleus (R. Tiburzy, *personal communication*), and nuclear condition may vary with fungal pathovar and axenic condition. Usually $20–30 \times 10^7$ protoplasts was obtained from 1.0 g fresh weight of mycelium after 3–4 hr incubation.

Osmotic stabilizers. Protoplast isolation was strongly dependent upon the presence of osmotic stabilizers (Fig. 2). The optimum yield of protoplasts was obtained using 0.8 M $MgSO_4$ in 0.5% MES buffer. Sorbitol also protected the protoplasts during isolation but not as efficiently as $MgSO_4$. The osmolarities of the various media used here are given in Table 1.

Mycelial age. Protoplast yields were satisfactory from cultures 3 to 7 days old ($29 \pm 12 \times 10^7$/g fresh weight, $n = 5$). Maximum yields were obtained with cultures 5 days old ($50 \pm 3 \times 10^7$/g fresh weight, $n = 4$). Fewer protoplasts were obtained from cultures 9 days old ($3.1 \pm 1 \times 10^7$/g fresh weight, $n = 3$) or older.

Protoplast regeneration. Protoplasts began to regenerate walls about 10 hr after dispersal in liquid RM as observed using Uvitex. Mycelia with septa and branches developed in about 2 days, and

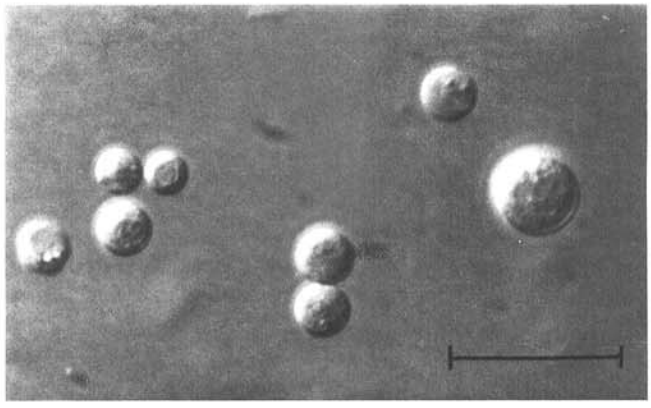

Fig. 1. Differential interference contrast photomicrograph showing protoplasts of *Puccinia graminis* f. sp. *tritici* 2 hr after isolation and suspension in SM_2 buffer. Bar = 100 μm.

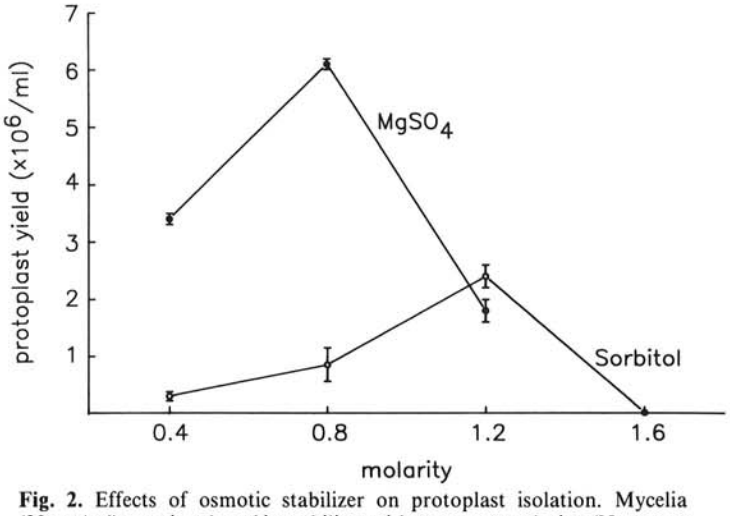

Fig. 2. Effects of osmotic stabilizer on protoplast isolation. Mycelia (20 mg/ml) were incubated in stabilizer with an enzyme solution (Novozym 234, 3 mg/ml + cellulase, 5 mg/ml) in 0.5% MES buffer. Error bars give the standard deviations.

the colonies became visible in about 4 days (Fig. 3). The frequency of regeneration in RM (BM with 0.5 M sorbitol as osmoticum) ranged from 19 to 39%. The osmolarity of RM was 961 mOsm, a value similar to the osmolarity of the OM used to isolate the protoplasts (Table 1). Mycelia did not develop from protoplasts regenerated in BM without sorbitol (334 ± 12 mOsm) or in RM containing more than 0.75 M sorbitol (> 1,202 mOsm). Protoplasts regenerated more quickly in liquid RM than in RM solidified with 0.4% agarose. Once the protoplasts began to regenerate walls (> 10 hr), they could be transferred to BM without an osmotic stabilizer. In several trials, the average frequency of regeneration in BM supplemented with 0.5 M sorbitol (RM) was nearly the same (29 ± 14%) as in BM supplemented with 0.5 M $MgSO_4$ (22 ± 17%). The osmolarities of the solutions were approximately equivalent (Table 1). Polyethylene glycol (1.0 ml of 60% PEG 3350 in 20 mM $CaCl_2$ + 3 ml of RM) increased the average frequency of protoplast regeneration about 10%; however, protoplast fusion also occurred.

DISCUSSION

Culture V1B was selected for use here because it can be grown in liquid suspension culture; the protocol represents a first step towards developing methods for protoplast isolation and regeneration of rust fungi. Most isolates of *P. g. tritici* are difficult to culture axenically, and those that have been cultured grow poorly (4). Although V1B is not pathogenic on wheat and does not sporulate, its identity as *P. graminis* is confirmed by its characteristic high content of sorbitol (5,6). Furthermore, differentiation-specific genes from the bean rust fungus (1) hybridized strongly with DNA from V1B or urediospores of a wild isolate of *P. g. tritici*, whereas DNA from other fungi, that is, *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scribner, *Glomerella magna* Jenkins et Winstead, *Cochliobolus heterostrophus* Drechs., and *Aspergillus nidulans* (Eidam) Wint., hybridized weakly or not at all (Huang et al, *unpublished data*).

As reviewed by Peberdy (9), the release of protoplasts generally depends on three major factors: the lytic enzyme, the osmotic stabilizer, and the physiological status of the organism. The relative importance of these factors varies, depending on the organism. Based on our tests of various factors and their interactions, we have established an efficient method for the isolation of protoplasts from axenic cultures of *P. g. tritici*. The viability and frequency of protoplast regeneration reported here is suitable for these procedures to be used both in transformation protocols and in patch-clamp protocols.

Novozym 234 has been reported to be the most effective lytic enzyme for preparing protoplasts from many filamentous fungi; however for *P. g. tritici*, a combination of Novozym 234 and cellulase gave nearly 10 times the number of protoplasts as did Novozym 234 alone. Finally the nature and concentration of the stabilizer used was found to be a critical factor in both protoplast isolation and regeneration. Results were better with $MgSO_4$ than with sorbitol as stabilizer (Fig. 2). The osmolarities of solutions found to be effective for isolation (OM), washing (SM_2), and regeneration (RM) were in the range 929–964 mOsm (Table 1).

TABLE 1. Osmotic pressure of different protoplast suspension media

Medium	Osmolarity[a]	
	mOsm	Standard deviation
Basic nutrient medium (BM)	334	12
0.8 M $MgSO_4$ + 0.5% MES (OM)	929	65
1 M sorbitol + 0.5% MES (SM_2)	964	72
0.5 M sorbitol + BM (RM)	961	57
0.5 M $MgSO_4$ + BM	1,060	51
1 M sorbitol + BM	1,462	25

[a] Osmolarity was determined using an Advanced Micro-Osmometer, model 3M (Advanced Instruments, Inc., Needham Heights, MA).

Osmolarities that deviated from this range resulted in poor protoplast isolation and regeneration. This contrasts with results from other fungi, for example, *Botrytis squamosa* Walker, the protoplasts of which were stable in media ranging from 800 to 2,000 mOsm (Huang, *unpublished*).

Mycelial age also was an important factor for protoplast isolation. High yields of protoplasts were obtained only from cultures 3–7 days old. These suspension cultures were in a vigorous phase of growth until mycelial growth centers became confluent by 6 to 7 days. Protoplast yields from filamentous fungi are generally high during exponential growth (8).

Use of the step gradient described here was employed to provide a protocol that would remove as much debris as possible from the protoplast suspension; microscopical examination revealed relatively few mycelial fragments at the interface of the gradient. In contrast, omission of the step gradient gave a protoplast pellet in which mycelial contaminants were relatively numerous.

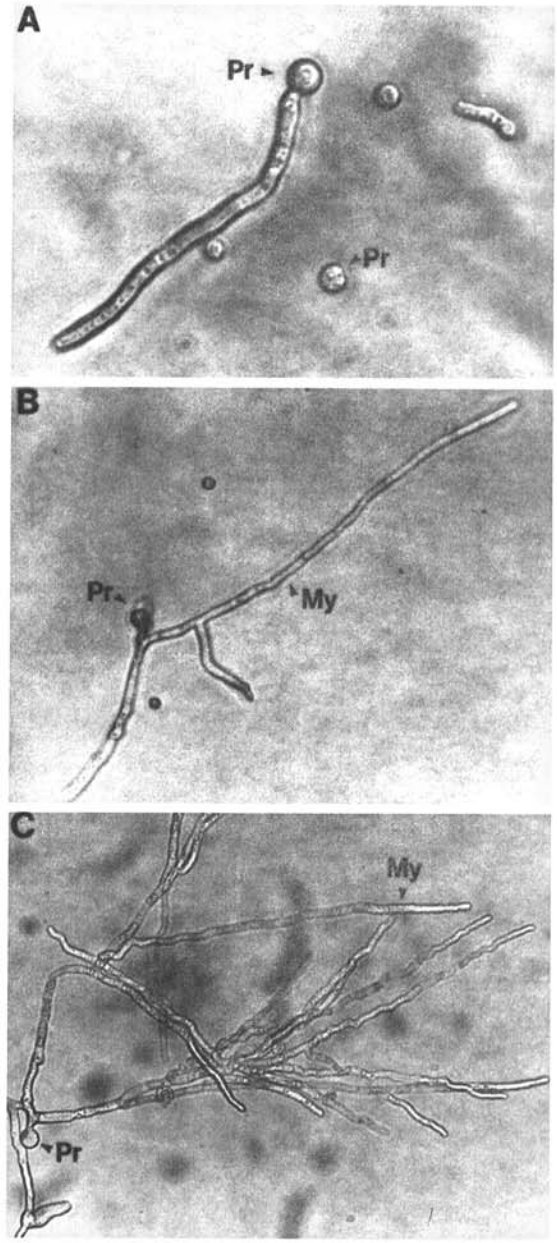

Fig. 3. Early stages in the regeneration of protoplasts of *Puccinia graminis* f. sp. *tritici*. **A,** Short mycelia produced 24 hr after the transfer of protoplasts to regeneration medium (×400). **B,** Branched mycelia observed 48 hr after the start of regeneration (×250). **C,** Young mycelial colonies 3 days after transfer to regeneration medium (×250). Pr = protoplast; My = mycelium.

However, a higher yield of protoplasts was obtained by omitting the gradient. For example, whereas we recovered $29 \pm 12 \times 10^7$ protoplasts from the interface of the gradient, we recovered $82 \pm 16 \times 10^8$ protoplasts in the pellet. The choice of procedure will depend on the degree of purity and yields required.

LITERATURE CITED

1. Bhairi, S. M., Staples, R. C., Freve, P., and Yoder, O. C. 1989. Characterization of an infection structure-specific gene from the rust fungus, *Uromyces appendiculatus*. Gene 81:237-243.
2. Gustin, M. C., Zhou, X.-L., Martinac, B., and Kung, C. 1988. A mechanosensitive ion channel in the yeast plasma membrane. Science 242:762-765.
3. Hoch, H. C., Staples, R. C., Whitehead, B., Comeau, J., and Wolf, E. D. 1987. Signaling for growth orientation and cell differentiation by surface topography in *Uromyces*. Science 235:1659-1662.
4. Maclean, D. J. 1982. Axenic culture and metabolism of rust fungi. Pages 37-120 in: The Rust Fungi. K. J. Scott and A. K. Chakravorty, eds. Academic Press, New York.
5. Maclean, D. J., and Scott, K. J. 1976. Identification of glucitol (sorbitol) and ribitol in a rust fungus, *Puccinia graminis* f. sp. *tritici*. J. Gen. Microbiol. 97:83-89.
6. Manners, J. M., Maclean, D. J., and Scott, K. J. 1984. Hexitols as major intermediates of glucose assimilation by mycelium of *Puccinia graminis*. Arch. Microbiol. 139:158-161.
7. Munoz-Rivas, A., Specht, C. A., Drummond, B. J., Froeliger, E., Novotny, C. P., and Ullrich, R. C. 1986. Transformation of the basidiomycete *Schizophyllum commune*. Mol. Gen. Genet. 205:103-106.
8. Peberdy, J. F. 1979. Fungal protoplasts: Isolation, reversion, and fusion. Annu. Rev. Microbiol. 33:21-39.
9. Peberdy, J. F. 1985. Mycolytic enzymes. Pages 31–44 in: Fungal Protoplasts. J. F. Peberdy and L. Ferenczy, eds. Marcel Dekker, New York.
10. Staples, R. C., and Hoch, H. C. 1982. A possible role for microtubules in the induction of nuclear division in bean rust urediospore germlings. Exp. Mycol. 6:293-302.
11. Wang, J., Holden, D. W., and Leong, S. A. 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 85:865-869.