Use of Cellulose-Acetate Electrophoresis for Rapid Identification of Allozyme Genotypes of *Phytophthora infestans*

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ABSTRACT

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Cellulose-acetate electrophoresis (CAE) provided excellent resolution of allozyme genotypes of *Phytophthora infestans* at the two loci *Glucose-6-phosphate isomerase* (*Gpi*) and *Peptidase* (*Pep*). The cellulose-acetate system has many advantages over traditional starch gel analyses. It is much faster, running in only 15 to 20 min compared to 16 to 18 h for starch gels, and because of the short run times the gels do not need to be cooled during electrophoresis. Cellulose acetate is purchased as precast plates, which eliminates the time required to pour starch gels. Both *Gpi* and *Pep* can be analyzed using a single buffer in the CAE system, whereas four buffers are required to resolve these enzymes using starch gels. Finally, only very small amounts of tissue (e.g., 3,000 sporangia washed from lesions or infected tuber slices) are required for CAE, so it can be useful even when the fungus has not been isolated into axenic culture. These advantages may allow CAE to be useful as a diagnostic tool in field situations, where rapid determination of genotypes could aid disease management strategies. Because populations of *P. infestans* in the United States and Canada currently are highly clonal, mating type and response to metalaxyl are highly correlated with allozyme genotype. Therefore, CAE of allozyme genotypes could provide a rapid, accurate method for predicting mating types and metalaxyl sensitivities of *P. infestans* within fields.

Phytophthora infestans (Mont.) de Bary caused severe late blight epidemics on potatoes and tomatoes in many parts of the United States and Canada during 1992, 1993, and 1994. Much of this disease was caused by new genotypes, recently introduced from Mexico (3,8). Prior to the recent migrations, populations of *P. infestans* in the United States and Canada were dominated by a single clonal lineage, designated US-1 (3,4). US-1 isolates are A1 mating type and so far have been highly sensitive to the phenylamide fungicide metalaxyl (6,9). In contrast, most of the new immigrant genotypes were resistant to metalaxyl (6,9). The occurrence of both metalaxyl-resistant and metalaxyl-sensitive clonal genotypes of *P. infestans* created confusion for growers. Therefore, there is a great need for a rapid, accurate method for determining genotypes. Timely information about mating types and metalaxyl sensitivities of *P. infestans* within a field or seed lot could be extremely useful to growers deciding on disease management strategies, including whether to purchase particular lots of seed.

Since 1992, populations of *P. infestans* in the United States and Canada have been dominated by only four clonal genotypes: US-1, US-6, US-7, and US-8 (3,8) (Table 1). US-1 has been one of the most common genotypes, and it is still highly sensitive to metalaxyl; the other genotypes are resistant (6,9). Each clone can be distinguished on the basis of *Glucose-6-phosphate isomerase* (*Gpi*) genotype alone (Table 1). However, the current method for determining *Gpi* genotype, by using starch gel electrophoresis (SGE), is slow and labor intensive. Although SGE has proven extremely useful for studying the population biology of *P. infestans* worldwide (e.g., 1,15,19,20), starch gels can be difficult to pour, require long run times (16 to 18 h, necessitating cooling during electrophoresis), and work best with tissue grown in axenic culture. These limitations make SGE unsuitable for rapid diagnostic assays.

A method of allozyme analysis using cellulose-acetate plates overcomes many of the limitations of SGE. Cellulose-acetate electrophoresis (CAE) uses precast plates that eliminate the time and labor involved in pouring starch gels. Because the plates are very thin, they run rapidly (in only 15 to 20 min) so require no cooling during electrophoresis. Another advantage of CAE is that very small samples can be analyzed. Typically, only 0.25 to 0.5 µl of a sample is necessary for CAE, compared to a minimum of around 50 µl for SGE; it might be possible to do CAE of *P. infestans* using sporangia washed from single lesions. Because of these advantages, CAE has been recommended as a method for analyzing populations of human parasites in remote locations (12,13,17).

The purpose of the work outlined in this paper was to determine if CAE could be useful for allozyme analysis of *P. infestans*. A secondary goal was to refine the technique so that it could be used on small samples in the field (e.g., sporangia or mycelia from lesions). The eventual goal was to develop a rapid, accurate method for predicting mating types and metalaxyl sensitivities of *P. infestans* in seed potatoes and during epidemics for use in disease management strategies.

MATERIALS AND METHODS

All materials needed for CAE (Table 2) were purchased from commercial sources. Tissue for electrophoresis consisted of small quantities of mycelia grown in pea broth (filtrate from 120 g of autoclaved frozen peas per liter), sporangia washed

Table 1. Characteristics of the four most commonly detected clonal lineages (3,6,8,9) of *Phytophthora infestans* in the United States and Canada from 1992 through 1994

Genotype	Mating type	Allozyme genotype		Response to metalaxyl
		Gpi[a]	*Pep*[b]	
US-1	A1	*86/100*	*92/100*	Sensitive
US-6	A1	*100/100*	*92/100*	Resistant
US-7	A2	*100/111*	*100/100*	Resistant
US-8	A2	*100/111/122*	*100/100*	Resistant

[a] *Glucose-6-phosphate isomerase.*
[b] *Peptidase.*

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from sporulating tissue (tuber slices or foliar lesions), and frozen lyophilized mycelia (5,16). A 5-mm^3 piece of mycelia from broth culture, or two to three wefts of mycelia plucked from a petri plate, were sufficient for CAE. Broth-culture samples were blotted briefly on paper towels to remove excess liquid, then rehydrated with approximately 1 ml of water. Sporangia were washed from leaves or tuber slices in a small amount (1 ml) of water. Tissue samples (mycelia or sporangial washates) were then placed into 1.5-ml microcentrifuge tubes and centrifuged at 13,000 × *g* for 1 min to pellet. Excess supernatant was poured off so that the remaining volume was 100 µl or less and the pellet was ground by hand using a tapered pestle (tissue homogenizer) that closely fit the contours of the microcentrifuge tubes. Samples could also be ground mechanically by attaching the tissue homogenizer to a variable-speed electric drill. Lyophilized mycelia (5,16) could be stored for many months at –80°C with no loss of enzyme activity. For electrophoresis, lyophilized mycelia were quick-frozen in liquid nitrogen, then ground to a fine powder with a mortar and pestle. Samples for electrophoresis were prepared by adding approximately 100 µl (20 mg) of powdered tissue to 300 to 400 µl of water in a microcentrifuge tube and vortexing to mix (3). Samples were centrifuged as above to pellet cell debris. Small aliquots of each sample (regardless of tissue source) were then pipetted from the supernatant into wells on the sample well plate, and the gels were loaded as described in Figure 1. Blue dye (0.25% bromphenol blue, 25% Ficoll, type 400, in H_2O) (14) was spotted along the origin near one side of each gel to monitor the progress of electrophoresis and to provide an orientation index. Dye composed of 0.2% bromphenol blue only could also be used. Gels were stained after 15 to 20 min of electrophoresis using modifications of agar overlays (3) or underlays (12) (Fig. 1). Agar underlays were prepared by pouring the staining solution into the bottom of a 14-cm petri plate. The gels were then placed sample side down on top of the solidified agar and incubated until bands appeared. Agar overlays were poured directly over the gels. Overlays significantly reduce the quantity of chemicals required, lowering costs and minimizing the amount of toxic waste generated. Many of the components of the staining solutions were prepared in dropper bottles (10) for ease of use. Photosensitive chemicals (e.g., MTT, PMS) were prepared in dark brown dropper bottles, or in bottles covered with aluminum foil. The complete CAE protocol for *P. infestans* and a list of all equipment and supplies needed are indicated in Figure 1 and Table 2, respectively. Additional details about CAE can be found in free publications available from Helena Laboratories (Table 2).

Table 2. Materials needed for cellulose-acetate electrophoresis of allozymes of *Phytophthora infestans*

Activity/need	Chemical or supply	Company[a]	Catalog no.[a]
Information	*Methodologies for allozyme analysis…*	Helena[b]	Form K
	The theory of electrophoresis	Helena	Form 56
Sample preparation	Microcentrifuge tubes	Many	…
	Microcentrifuge tube racks	Many	…
	Microcentrifuge	Many	…
	Tissue grinder	Bel-Art[c]	19921-0001
	Pipettors: 2 to 20 µl	Many	…
	20 to 200 µl	Many	…
Electrophoresis	Electrophoresis chamber	Helena	1283
	Blotting paper	S & S[d]	50780
	Trizma base	Sigma[e]	T 1378
	Glycine, free base	Sigma	G 7126
	Bromphenol blue	Sigma	B 6131
	Ficoll, type 400	Sigma	F 4375
	Paper wicks	Helena	5081
	Applicator[f]	Helena	4093
	Sample well plate[f]	Helena	4093
	Aligning base[f]	Helena	4093
	Cellulose-acetate plates	Helena	3024
	Power supply (constant V)	Many	…
Staining	14-cm petri plates	Many	…
	D-Fructose-6-phosphate	Sigma	F 3627
	NAD[g]	Sigma	N 8881
	MTT	Sigma	M 2128
	PMS[h]	Sigma	P 9625
	G-6-PDH[i]	Sigma	G 5885
	Glycyl-leucine	Sigma	G 2002
	Peroxidase	Sigma	P 8250
	O-Dianisidine	Sigma	D 3252
	L-Amino acid oxidase	Sigma	A 5147
	$MnCl_2$	Many	…
	$MgCl_2$	Many	…
	Agar	Many	…

[a] Suppliers and catalog numbers are provided for convenience and to indicate the types and/or formulations of chemicals and/or supplies needed. This is not an endorsement of any particular manufacturer; other sources for these products may be available.
[b] Helena Laboratories, 1530 Lindbergh Drive, P.O. Box 752, Beaumont, TX 77704-0752; phone: 800-231-5663.
[c] Bel-Art Products, Pequannock, NJ 07440-1992.
[d] Schleicher & Schuell, Inc., P.O. Box 2012, Keene, NH 03431-2062; phone: 800-245-4024.
[e] Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178; phone: 800-325-3010.
[f] These can be purchased together in a kit.
[g] Nicotinamide adenine dinucleotide.
[h] Phenazine methosulfate.
[i] Glucose-6-phosphate dehydrogenase.

RESULTS

Cellulose-acetate electrophoresis provided excellent resolution of allozymes of *P. infestans* (Fig. 2). Both *Gpi* and *Peptidase* (*Pep*) could be assayed using a single buffer in the cellulose-acetate system, compared to four buffers for SGE. Adequate resolution of bands was obtained in only 15 to 20 min of CAE. Bands produced by CAE were typically much sharper than those from SGE (Fig. 2); the four most common genotypes of *P. infestans* in the United States and Canada from 1992 through 1994 (Table 1) could be distinguished easily. In addition, samples that were too small for SGE could be analyzed successfully by CAE. Sporangia washed from lesions or tuber slices provided enough tissue for CAE of *P. infestans*; adequate resolution of genotypes was obtained using as few as 3,000 to 5,000 sporangia (Fig. 3). It may be possible to resolve genotypes of *P. infestans* using even fewer sporangia by increasing the number of loadings onto the gel. The CAE system took up very little bench space and thus should be easily transportable to remote locations.

Scoring of genotypes was generally straightforward and easy. Because *P. infestans* is diploid (18), each individual has two copies of each chromosome. Both GPI and PEP are dimeric enzymes in *P. infestans* (18), so two subunits are required to form the active enzyme. Homozygous genotypes produce a single band on a gel, whereas heterozygous genotypes are characterized by three bands: two homodimer bands (when two identical subunits came together to form the active enzyme) and one heterodimer band (when subunits coded by different alleles came together to form the active enzyme). The heterodimer band usually migrates halfway between the two homodimer bands. There should

1. Soak cellulose-acetate gel plates in **electrode buffer**. Lower the plates into buffer in a suitable container (600 ml beaker or equivalent) very slowly so that they become evenly wet without air bubbles. Soak for at least 20 minutes; can be left refrigerated in buffer for several days.

2. Prepare tissue samples. Tissue for electrophoresis can be mycelia: from broth cultures; scraped off plates; or from lesions. Sporangia washed from tuber slices or lesions in about one ml of water can also be used. Place the samples in 1.5 ml microcentrifuge tubes and centrifuge 1-2 minutes at 13,000 × g to pellet. Remove all but 50-100 μl of the supernatant. Homogenize tissue by hand (or mechanically) with a tapered plastic or Teflon pestle that fits inside the microcentrifuge tube. Microcentrifuge 1-2 minutes to pellet cell debris. Tissue extracts should be chilled before loading to prevent enzyme degradation, or can be frozen for later use.

3. Transfer a 5-10 μl aliquot from the supernatant of each sample into a well on the sample well plate. Cover the wells with a glass slide to prevent evaporation of samples while preparing gel plates.

4. Remove a gel plate from the buffer, place between two pieces of blotting paper and blot firmly to remove excess buffer. Put a drop of water on the aligning base to hold the gel plate firm and flat, then position the plate, emulsion side up, using the guides on the aligning base for cathodal application. Remove the glass slide from the sample well plate, position applicator, and depress 3-4 times to pick up samples. Align applicator in guides on base and depress button for several seconds to transfer samples to cellulose acetate. Multiple applications may be made for dilute samples or low activity enzymes (e.g., peptidase). Running dye (0.25% bromphenol blue, 25% Ficoll) can be spotted onto one side of the gel plate if desired to monitor the progress of electrophoresis. Loaded plates should be moved to electrophoresis chamber promptly to prevent drying out.

5. Add 180 ml of **electrode buffer** to each reservoir of the electrophoresis chamber. Position paper wicks over supporting rails in gel chamber. Place gel over moistened wicks, cellulose acetate side down, with origin at the cathodal rail. Press down at rails to ensure good contact. Place top on electrophoresis chamber. Run gels at 175 to 200 V for 15-20 minutes, or until blue dye has migrated to near the end of the gel. Peptidase (PEP) allozymes of *P. infestans* run slightly faster than the dye and 175 V for 15 minutes is the preferred setting if PEP is run alone. Glucose-6-phosphate isomerase (GPI) allozymes run slightly slower than PEP and 200 V for 20 minutes is more appropriate for GPI runs. Cellulose-acetate gels can be cut in half to accommodate fewer samples or to stain both enzymes on the same gel (maximum of six samples per enzyme if on the same gel).

6. Stop electrophoresis. Stain gels using agar underlays or overlays. For agar underlays, pour 20 ml of molten staining solution into the bottom of a 14 cm Petri plate (can be prepared ahead of time if kept in the dark). Place gel, sample side down, on the solidified agar and incubate until bands appear. For agar overlays, pour molten staining solution over gel to cover. Incubate until bands appear. Gels can be incubated at room temperature or at 37 C (bands appear faster at higher temperatures). To prevent overstaining, remove gels from contact with staining solution, rinse and store in cold water until scored. After scoring, gels can be dried at 60 C and saved for future reference.

RECIPES

Buffer concentrate

30 *g* Trizma base
144 *g* Glycine, free base
Water to 1 *l* for buffer concentrate. **Electrode buffer** (0.025 *M* Tris, 0.192 *M* glycine, pH 8.5) is one part buffer concentrate in nine parts water.

GPI underlay

$Tris\text{-}MgCl_2$ (0.1 *M* Tris, 0.1% $MgCl_2$, pH 7.0)	10 ml
Fructose-6-phosphate	50 mg
NAD	20 mg
MTT, 10 mg/ml	1 ml
PMS, 5 mg/ml	0.2 ml
G-6-PDH, 1000 U/ml	40 μl
Agar, 1% (0.1 g / 10 ml) @ 60 C	10 ml

GPI overlay

Tris-HCl, 0.05 *M*, pH 8.0	1.5 ml
Fructose-6-phosphate, 20 mg/ml	5 drops
NAD, 3 mg/ml	1 ml
MTT, 10 mg/ml	5 drops
PMS, 2 mg/ml	5 drops
G-6-PDH, 1 U/μl	3 μl
Agar, 1.6% (160 mg / 10 ml) @ 60 C	2 ml

PEP underlay

TBE buffer (0.045 *M* Tris, 0.025 *M* boric acid, 2.5×10^{-5} *M* EDTA, pH 8.7)	10 ml
Glycyl-leucine	50 mg
o-Dianisidine	15 mg
Peroxidase	20 mg
L-Amino acid oxidase, 0.2 U/mg	5 mg
Agar, 1% (0.1 g / 10 ml) @ 60 C	10 ml

PEP overlay

Tris-HCl, 0.05 *M*, pH 8.0	2 ml
Glycyl-leucine, 15 mg/ml	10 drops
Peroxidase, 1000 U/ml	5 drops
o-Dianisidine, 4 mg/ml	8 drops
$MnCl_2$, 20 mg/ml	2 drops
L-Amino acid oxidase, 10 U/ml	5 drops
Agar, 1.6% (160 mg / 10 ml) @ 60 C	2 ml

Fig. 1. Laboratory protocol for allozyme analysis of *Phytophthora infestans* using cellulose-acetate electrophoresis (modified from 10).

be equal numbers of heterodimer and homodimer molecules. However, because the homodimer molecules are split into two bands, each homodimer band is usually half the intensity of the heterodimer band (e.g., Fig. 2, US-7 genotype). An individual homozygous for the *100* allele (e.g., US-6), will have the *100/100* genotype and produce a single band on an allozyme gel. An individual heterozygous for the *100* and *111* alleles (e.g., US-7) will have the *100/111* genotype and will produce three bands on an allozyme gel corresponding to the 100/100 homodimer, the 100/111 heterodimer, and the 111/111 homodimer.

Both US-1 and US-8 are exceptions to the general rule of diploidy in *P. infestans*; individuals with these genotypes probably have an extra copy of the chromosome containing the *Gpi* locus. US-1 probably has two copies of the *100* allele and one of the *86* allele. In this situation, we expect to see an unbalanced heterozygote pattern, where the ratio of intensities of the bands would be 1:4:4 for the 86/86, 86/100, and 100/100 bands, respectively. (Compare this to the balanced 1:2:1 ratio of intensities for the three bands in the US-7 genotype of Figure 2.) US-8 probably has three different alleles at the *Gpi* locus: *100*, *111*, and *122* (7). This should give a six-banded phenotype on a gel, with three homodimer (100/100, 111/111, and 122/122) and three heterodimer (100/111, 111/122, and 100/122) bands. However, the 111/111 homodimer and the 100/122 heterodimer bands comigrate, so only five bands are resolved on the gels. The ratio of band intensities is approximately 1:2:3:2:1, the middle band being more intense because it is a combination of a heterodimer and a homodimer band (Fig. 2, US-8 genotype).

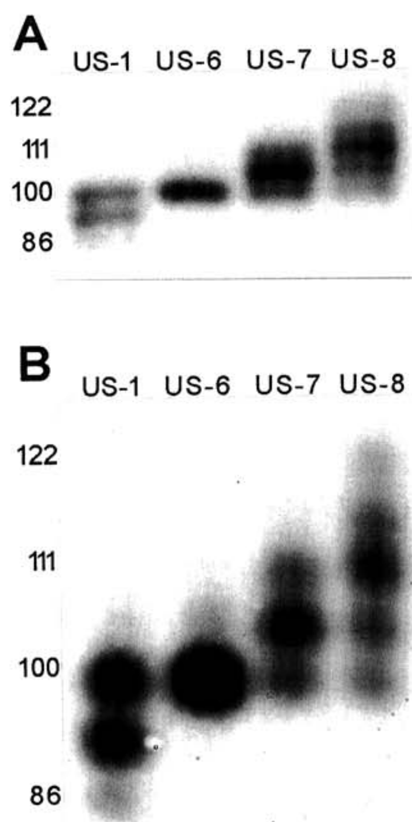

Fig. 2. Comparison of **(A)** cellulose-acetate and **(B)** starch gel electrophoresis of *Glucose-6-phosphate isomerase* allozymes of the four most common genotypes of *Phytophthora infestans* in the United States and Canada from 1992 through 1994. Genotypes shown are (from left to right) *86/100* (US-1), *100/100* (US-6), *100/111* (US-7), and *100/111/122* (US-8). US-1 probably has two copies of the *100* allele (actual genotype *86/100/100*), giving a 1:4:4 ratio of intensities for the 86/86, 86/100, and 100/100 bands, respectively. Locations of the 86, 100, 111, and 122 homodimer bands are indicated on the left. Alleles were originally named on the basis of their migration distance relative to that of the *100* allele (18); thus, for example, the *86* allele migrates approximately 14% slower than the *100* allele, the *111* allele 11% faster.

Occasionally, additional faint bands are visible on allozyme gels (Fig. 3, lanes 1 to 4). These faint bands usually result from contamination of the samples by small amounts of tissue from other organisms: plants, other fungi, or bacteria. Faint bands may also result from degradation of enzymes in old cultures. Best results were obtained with fresh, clean tissue extracts.

DISCUSSION

Populations of *P. infestans* in the United States and Canada currently are highly clonal and are dominated by only four different clonal genotypes (8), each of which can be distinguished on the basis of *Gpi* genotype alone (Table 1). Because of this unusual situation, CAE currently provides a rapid, accurate method for predicting mating types and metalaxyl sensitivities of *P. infestans* within a field. Usually, each lesion contains only one genotype of *P. infestans*. By analysis of sporangia from single sporulating lesions, CAE can provide information about the prevalence of particular genotypes (including mating type and metalaxyl sensitivity) during an epidemic, and about the effectiveness of specific disease management strategies. This information could be extremely important to growers deciding whether or not to spray with metalaxyl. Metalaxyl is still highly effective against the US-1 genotype and therefore will remain an important component of the disease management strategy in fields containing US-1 exclusively. In fields containing both US-1 and one of the A2 genotypes (US-7 or US-8), metalaxyl could reduce the frequency of US-1 and thus minimize opportunities for sexual reproduction. CAE could also provide an early warning system for additional changes in *P. infestans* populations; new gene combinations may indicate that sexual reproduction or additional migrations have occurred. Fields containing new genotypes should be subject to more intensive analyses.

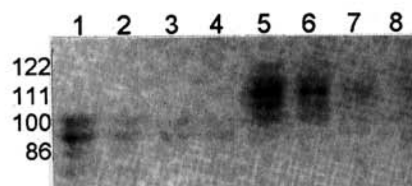

Fig. 3. The lower limit of detection using cellulose-acetate electrophoresis to determine *Glucose-6-phosphate isomerase* allozyme genotypes of *Phytophthora infestans*. Lanes 1 to 4, dilution series of sporangia (US-1 genotype) washed from an infected tuber slice. The approximate numbers of sporangia assayed for lanes 1 to 4 were 14,000, 7,000, 3,500, and 1,750, respectively. Lanes 5 to 8, dilution series of sporangia (US-8 genotype) washed from a single lesion on an infected leaflet. Approximate numbers of sporangia assayed for lanes 5 to 8 were 31,000, 15,500, 7,750, and 3,875, respectively. Sporangia were quantified using a hemacytometer and were diluted in water. After centrifuging, each sporangial pellet was ground by hand in approximately 75 µl of water. Four loadings (0.25 µl each) were made for each lane. Resolution of small samples can be increased by decreasing the volume of water for crushing and/or by increasing the number of loadings. Locations of the 86, 100, 111, and 122 homodimer bands are indicated on the left.

Another use for CAE could be to determine whether metalaxyl-resistant genotypes are present within seed potatoes before planting. This is particularly important because transport of infected seed tubers is one of the primary means by which *P. infestans* moves over long distances. Pieces cut from infected tubers usually produce enough sporulation for CAE after 1 to 2 days of incubation in a moist chamber at 18°C. CAE can be performed using sporangia washed directly from the tuber slices, or the mycelium itself can be picked off and ground for electrophoresis. The ability to rapidly determine genotypes of *P. infestans* in seed tubers could aid growers in deciding whether or not to keep particular lots of seed.

Due to small size and minimal equipment requirements, the cellulose-acetate system could be adapted for use in any remote location with 4 to 5 square feet of bench space and access to an AC power supply. Enzyme staining can be done using either agar overlays or underlays. Because the overlay technique requires only a very small volume, it is more economical and thus is the method of choice in most situations. Underlays may be more appropriate for remote locations where it is necessary to prepare the stain solutions ahead of time. Equipment to set up the system from scratch (Table 2) costs approximately $3,000—only $1,000 if a power supply, microcentrifuge, and associated paraphernalia are already available. Consumable supplies (e.g., chemicals, cellulose-acetate plates) would probably cost an additional

$500 to $1,000, depending on what is already available in the laboratory, the number of assays, and whether peptidase is assayed. Peptidase helps resolve some of the less common genotypes (8; S. B. Goodwin and W. E. Fry, *unpublished*) and increases the ability to identify new gene combinations. Therefore, assaying both *Gpi* and *Pep* is recommended.

Applying CAE to predict mating types and metalaxyl sensitivities of *P. infestans* is possible currently because populations are clonal and so far there has been no selection for metalaxyl resistance within the US-1 clonal lineage in the United States and Canada. However, both mating types are now widely distributed (2,8), and it is probably only a matter of time before sexual recombination eliminates the current correlations between allozyme genotype, mating type, and fungicide sensitivity. Furthermore, resistance to metalaxyl has been detected within the US-1 clonal lineage in Asia (11). The useful life for this particular application of CAE is thus probably only a few years at most. As a component of an integrated disease management strategy, CAE could be very useful to growers until more sensitive DNA-based techniques become available. Even after its utility as a diagnostic tool ends, CAE will continue to be a useful alternative to SGE for analyzing allozyme variation within populations of *P. infestans*.

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