A Comparison of Isozymes of *Phakopsora pachyrhizi* from the Eastern Hemisphere and the New World

M. R. Bonde, G. L. Peterson, and W. M. Dowler

Research plant pathologist, biological laboratory technician, and research plant pathologist, respectively, U.S. Department of Agriculture, Agricultural Research Service, Foreign Disease-Weed Science Research, Building 1301, Fort Detrick, Frederick, MD 21701.

Accepted for publication 13 July 1988 (submitted for electronic processing).

ABSTRACT

Bonde, M. R., Peterson, G. L., and Dowler, W. M. 1988. A comparison of isozymes of *Phakopsora pachyrhizi* from the Eastern Hemisphere and the New World. Phytopathology 78:1491-1494.

Eleven isolates of *Phakopsora pachyrhizi* from widely separated areas of Asia and Australia were compared by means of isozyme analysis with one isolate from Puerto Rico and three from Brazil. No differences in isozyme banding patterns were detected among any of the isolates from Asia and Australia, and no differences were detected among any of the four isolates from the New World. However, the maximum coefficient of similarity between isolates from Asia and Australia compared with those from the New World was estimated at 0.07 (7% alleles in common). This low frequency of shared putative alleles indicates that two populations of *P. pachyrhizi* with distinct isozyme polymorphisms are involved in causing rust on soybean, one in Asia and Australia and another in the New World (Brazil and Puerto Rico).

Rust on soybeans (*Glycine max* (L.) Merrill), caused by *Phakopsora pachyrhizi* Sydow, has been known in the Orient since 1914 (1). The disease occurs in many countries from the People's Republic of China (the region of Manchuria) in the north, Japan and Taiwan to the east, Australia to the south, and India to the west (3). Yield losses as high as 70–80% have occurred in individual fields (3). Soybean rust has been described in Taiwan and Thailand as the most economically important fungal disease of soybean (4). In the Americas, the soybean rust pathogen has been reported on a number of leguminous hosts other than soybean since 1913, commonly by its synonym name, *P. vignae* Arthur (4).

In 1976, *P. pachyrhizi* was discovered for the first time in the New World on soybean in the Limani Valley of Puerto Rico (14). The pathogen also was identified on *Dolichos lablab* L., *Phaseolus vulgaris* L., and *P. coccineus* L. Since 1976, the disease also has been reported from Brazil, Colombia, and Costa Rica (4).

Puerto Rican and Brazilian isolates of *P. pachyrhizi* produce smaller uredinia and fewer urediniospores on soybeans than isolates from the Eastern Hemisphere (4). Some legume species, however, are more susceptible to Puerto Rican and Brazilian isolates and support the production of larger uredinia and more urediniospores than isolates from the Orient (14).

Bonde and Brown (2) conducted a morphological comparison of isolates of *P. pachyrhizi* from South Asia with an isolate from Puerto Rico. The only difference discerned among the isolates studied was the appearance of germ pores; germ pores of the Puerto Rican isolate were easier to see by scanning electron microscopy than those of the four Eastern Hemisphere isolates, suggesting that the Puerto Rican isolate may have thinner germ pore plugs. This difference, however, was not sufficient to consider the isolates as taxonomically distinct.

Isozyme analysis is a powerful tool to compare fungal pathogens at the gene level (9). It has been used successfully to distinguish species of *Peronosclerospora* (10), *Endothia* and *Cryphonectria* (11), and *Tilletia* (Bonde, Peterson, and Matsumoto, *unpublished data*). A number of researchers have used isozyme analysis to study the intraspecific variation and genetics of fungal species (9).

The purpose of the present study was to compare isozymes of isolates of *P. pachyrhizi* from several widely separated areas of the Eastern Hemisphere with those produced by isolates from Puerto Rico and Brazil. The information would allow the determination of allelic variation of genes coding for the isozymes in these widely separated regions. Furthermore, the determination of isozyme banding patterns for these pathogenically different cultures could conceivably provide a rapid means of identifying the foreign source of the pathogen should the disease appear in the United States.

MATERIALS AND METHODS

Pathogen cultures. Isolates of *P. pachyrhizi* were obtained from the sources cited in Table 1. Each field collection was either airmailed or hand carried (on dried rusted leaves or cotton swabs) to FDWSRU and increased in the greenhouse on soybean cultivar Wayne after inoculation and incubation overnight in dew chambers at 20 C. Initial inoculations with small quantities of spores were done by rubbing or tapping the spores onto leaves of Wayne, whereas latter inoculations involving greater numbers of

TABLE 1. Isolates of *Phakopsora pachyrhizi* used in the study

Isolate designation	Source (if known)
Eastern Hemisphere	
Australia 72-1	D. E. Byth, Univ. of Queensland, Brisbane Australia; soybean
Australia P-5	...[a]
China 72-1	...
India 73-1	D. N. Thapliyal, Pantnagar, India; soybean
Indonesia 72-1	...
Philippines 77-1	Bureau of Plant Industries, Los Banos; soybean
Taiwan 72-1	Lung-Chi Wu, Nat. Taiwan Univ., Taipei; soybean
Taiwan 80-2	Asian Veg. Res. & Develop. Center (AVRDC), Taiwan; soybean
Taiwan 80-2A	AVRDC, Taiwan; soybean
Taiwan 80-2B	AVRDC, Taiwan; soybean
Thailand 76-1	Udom Pupipat, Farm Suwan, Pak Chang, Thailand; soybean
New World	
Brazil 80-1	J. A. Deslandes, Brazil; lima bean
Brazil 80-2	K. R. Bromfield, Brazil
Brazil 82-1	J. A. Deslandes, Brazil; from lima beans
Puerto Rican composite	K. R. Bromfield, Puerto Rico; from several legume spp. including soybean

[a]... indicates original source not known.

spores were done by means of a spore settling tower (8). Urediniospores for isozyme tests were placed in Nunc cryotubes (Thomas Scientific Co., Swedesboro, NJ) and stored by placing tubes directly into a liquid nitrogen refrigerator (−196 C) until required for isozyme electrophoresis.

Preparation of samples for electrophoresis. Approximately 50 mg of dry urediniospores per sample was germinated on distilled water in 100-× 15-mm petri plates. Three hours after placement on distilled water, germinated spores were collected on a 3-μm Millipore filter, placed in 1.8-ml Nunc cryotubes, and frozen in liquid nitrogen. The frozen samples were removed from the liquid nitrogen and crushed (while still frozen) with the aid of a 6-mm-diameter glass rod, the tip of which had been chilled by dipping in liquid nitrogen. Crushing continued until the sample completely thawed. The crushed samples were transferred to 12- × 75-mm disposable test tubes, brought to 0.25 ml with 0.05 M Tris-HCl buffer, pH 7.5, and centrifuged to remove solid debris.

Gel electrophoresis. Horizontal starch gel electrophoresis and staining were performed as described by Micales et al (9). Enzymes and buffer systems used in the study are listed in Table 2. The experiment was performed three times to determine the repeatability of the results.

Genic nomenclature and treatment of data. The genic nomenclature of May et al (7) was used. Capital-lettered abbreviations with only the first letter capitalized referred to putative loci coding for the enzyme. Alleles at a particular locus were designated by the relative anodal or cathodal mobility from the origin of their protein products. The designation for each allele was relative to the migration of the protein product of one allele (usually the most common) designated 100. Because more isolates from Asia and Australia were included in the study, all designations were relative to the allele present in these isolates. For example, allele Gpi-50 codes for a molecule of glucosphosphate isomerase that migrates 50% as far on a starch gel as one coded by allele Gpi-100. Pgd-129 codes for an enzyme molecule that migrates 29% farther than one coded by Pgd-100.

The coefficients of similarity (CS) between specific pairs of isolates and the average CS between any New World and Eastern Hemisphere isolate were determined according to Rogers (13).

TABLE 2. List of enzymes for which activity was found, abbreviations, and best buffer system for resolution of bands

Name	Abbreviation	Buffer
Acid phosphatase	ACP	R[a]
Diapharase	DIA	C[b]
Esterase	EST	4[c]
Glucose-6-phosphate dehydrogenase	G6PDH	C
Glucose phosphate isomerase	GPI	R
Leucine aminopeptidase	LAP	R
Malate dehydrogenase	MDH	4
Mannose phosphate isomerase	MPI	R
Peptidase with leucylleucylleucine as substrate	PEP-LA (2 loci)	R
Peptidase with leucylleucylalanine as substrate	PEP-LLL (2 loci)	R
Peptidase with phenylalanylproline as substrate	PEP-PAP	R
Phosphogluconate dehydrogenase	PGD	C

[a] Discontinuous buffer according to Ridgway et al (12), 250 V for 3 hr.
[b] Continuous buffer according to Clayton et al (6), 200 V for 3 hr.
[c] Continuous buffer according to Selander et al (15), 170 V for 2 hr.

RESULTS

Enzymes detected in the germinated urediniospores and the best buffer system for resolution of each enzyme are presented in Table 2. Presumed genotypes for each enzyme are presented in Table 3 and specific banding patterns for two enzymes shown in Figure 1, respectively.

All enzymes produced relatively simple banding patterns, and only PEP-PAP and PEP-LLL produced patterns that were interpreted as most likely being coded by more than one genetic locus. With each of these enzymes, the separation of enzymatic activity into two distinctly different zones on the gel was considered the result of two genetic loci. The presence of multiple bands close together when they occurred was interpreted as the result of the presence of secondary enzymes (8).

A total of 13 cultures (10 from Asia or Australia, two from Brazil, and one from Puerto Rico) gave results for most of the 12 enzymes and 14 putative isozyme loci (Table 3). One additional isolate from Australia (P-5) and one from Brazil (80-1) are included for completeness and not for calculating degrees of similarities. Missing bands, where present, for all isolate-enzyme combinations, except for locus PEP-LA-1 for the New World isolates, were probably due to weak samples and are recorded as missing data (Table 3). All isolates displayed only homozygous isozyme loci and no heteromeric (hybrid) isozyme bands were detected with the possible exception of Esterase (EST). EST appeared to be resolved into three bands for the Asian and Australian isolates. Because resolution was questionable, we took the conservative approach and considered the isolates to be homozygous. No differences in isozyme banding patterns were detected among any of the 10 isolates from Asia or Australia. Likewise, no variation occurred among any of the four isolates

TABLE 3. Presumed genotypes of isolates of *Phakopsora pachyrhizi*[a]

	Loci													
Isolate	Acp	Dia	Est	G6pdh	Gpi	Lap	Mdh	Mpi	PepLA-1	PepLA-2	PepLLL-1	PepLLL-2	Pep-Pap	Pgd
Eastern Hemisphere														
Australia 72-1	100	100	···[b]	100	100	100	100	100	100	100	100	100	100	100
Australia P-5	100	100	···	···	100	100	100	100	···	···	···	100	···	100
China 72-1	100	100	100	100	100	···	100	100	100	100	100	100	100	100
India 73-1	100	100	100	100	100	···	100	100	100	100	100	100	100	100
Indonesia 72-1	100	100	100	100	100	···	100	100	100	100	100	100	100	100
Philippines 77-1	100	100	···	100	100	···	100	100	100	100	100	100	···	100
Taiwan 72-1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Taiwan 80-2	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Taiwan 80-2A	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Taiwan 80-2B	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Thailand 76-1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
New World														
Brazil 80-1	···	···	···	···	50	···	···	47	0[c]	95	0	95	···	129
Brazil 80-2	···	···	···	38	50	···	75	47	0	95	0	95	···	129
Brazil 82-1	95	38	108	38	50	100	75	47	0	95	0	95	···	129
Puerto Rican composite	95	38	108	38	50	100	75	47	0	95	0	95	105	129

[a] Genotypes based on the relative distance enzymes moved anodally in starch gels in approximately 3 hr. For example, allele Acp-95 coded for a protein product that migrated 95% as far as a protein coded for by Acp-100. All isolates were interpreted as being homozygous at each isozyme locus.
[b] ··· denotes missing data.
[c] A 0 denotes a null allele that does not code for an active enzyme.

from the New World (Table 3), thus confirming that the populations of *P. pachyrhizi* in Puerto Rico and Brazil are similar if not identical for these traits.

Of 14 putative isozyme loci examined, only Lap (coding for leucine aminopeptidase) had a possible allele in common between Asian-Australian isolates and New World isolates. The average Rogers's coefficient of similarity (CS) among isolates within the Asian-Australian, or within the New World group, was 1.00 (no detectable intragroup variation).

Because results for some loci for a few of the isolates were missing (most likely due to too few urediniospores), the average CS value comparing the Asian-Australian with the New World group was based on 12 isolates (10 Asian or Australian, two New World) for 11 loci. When compared in this manner, the CS value comparing the groups was 0.00 (no similarity). When we included locus Lap, the maximum CS between any Asian or Australian isolate and New World isolate was 0.07.

The enzymes PEP-LA and PEP-LLL each exhibited two putative loci as discussed above. Null alleles were hypothesized for the New World isolates for the Pep-LLL-1 and the Pep-LA-1 isozyme loci (Table 3). The lack of bands was corroborated when we doubled the concentration of urediniospores for the Puerto Rican (a New World) isolate and stained for PEP-LLL and PEP-LA after electrophoresis; again no bands of enzyme activity were detected in those areas on the gels where these loci were expressed with the Asian and Australian isolates. For the purpose of calculating degree of similarity, null alleles were treated the same as other alleles.

The fungal species *Physopella zeae* (Mains) Cumm. and Ramachar, *Puccinia polysora* Underw., *Puccinia carduorum* Jacky, *Colletotrichum coccodes* (Wallr.) Hughes, and *Tilletia indica* Mitra were included in a few experiments, and all were distinctly different from *P. pachyrhizi* in these banding patterns (e.g., Fig. 1).

DISCUSSION

Although the number of isolates used was small, the coefficients of similarity are meaningful because of the wide distribution from which the isolates were obtained. In the Eastern Hemisphere, isolates were obtained from the entire region where rust on soybean has been described. From the New World, only four isolates were available, three from Brazil and one from Puerto Rico. Because few urediniospores of Brazil 80-1 and 80-2 were available, these isolates were not tested for all enzymes. However, it is noteworthy that all isolates from the Eastern Hemisphere were identical for each isozyme locus examined, and likewise the four from the New World were identical at each locus. Yet the two groups were very different. The very low number of possibly shared alleles between the two groups, in spite of similar morphologies, indicates the pathogen inciting soybean rust in Puerto Rico and Brazil as opposed to Asia and Australia is biochemically significantly different. The results confirm that a population of *P. pachyrhizi* with the same, or nearly the same, isozyme phenotype incites rust on soybean in Puerto Rico and Brazil.

As an ancillary study, pathogens other than *P. pachyrhizi* were included in some experiments. There were no similarities between these and either group of isolates of *P. pachyrhizi* (e.g., Fig. 1 top). These results illustrate the usefulness of isozymes for the differentiation of fungal species.

Coefficients of similarity have not been determined for a large number of fungal species. Micales et al (11), in a study of *Cryphonectria cubensis* (Bruner) Hodges isozymes, found the CS intraspecific values between isolates to vary from 0.84 to 1.00. Intraspecific CS values for *Tilletia foetida*, *T. caries*, and *Pyricularia oryzeae* (Bonde and Peterson, *unpublished data*) are all close to 1.00.

In light of the present study, further critical morphological comparison of *P. pachyrhizi* from the Eastern Hemisphere with the

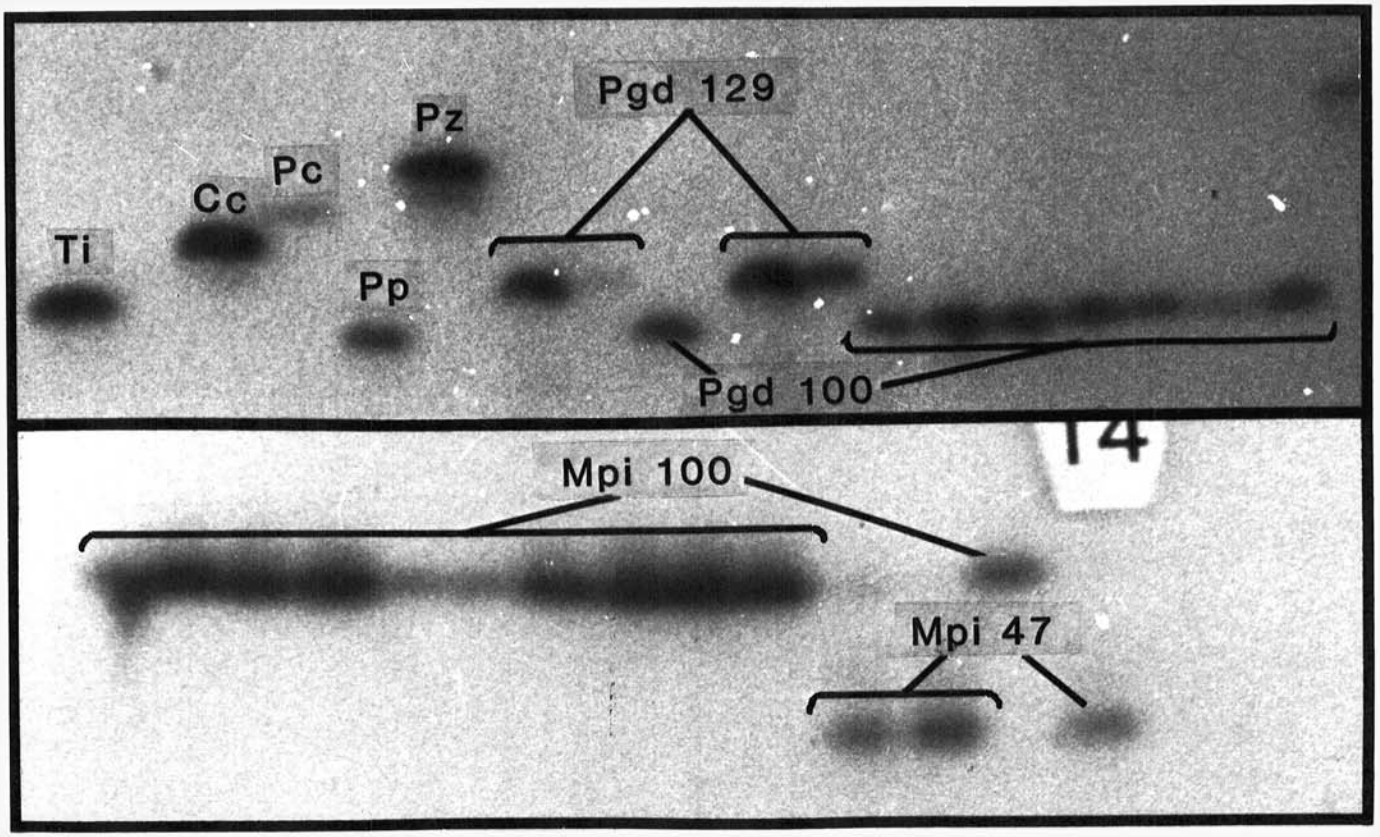

Fig. 1. Top: Putative alleles phosphogluconate dehydrogenase-100 (Pgd-100) (Asian and Australian isolates) and Pgd-129 (New World isolates) evident as isozyme bands. Also shown are bands for enzyme activity produced by *Physopella zeae* (Pz), *Puccinia polysora* (Pp), *Puccinia carduorum* (Pc), *Colletotrichum coccodes* (Cc), and *Tilletia indica* (Ti). Bottom: Mpi-100 is evident for Asian isolates and allele Mpi-47 for New World isolates.

pathogen in the New World certainly are warranted. The relationship of *P. vignae* to *P. pachyrhizi* also should be examined using morphology and isozymes. We believe that at least two pathogen species, and perhaps more, may be involved in causing rust on soybean. It is possible that long-term geographical separation of the pathogen in the two hemispheres has resulted in specialization of the pathogen.

It is premature to speculate on the reason for the apparent lack of isozyme variability within the Asian *P. pachyrhizi* isolates except to say that it may be associated with the lack of an effective sexual cycle. Although teliospores of the pathogen have been observed, germination may be rare or absent (16). Presumably, the presence of an effective sexual cycle would allow a greater chance for isozyme mutations to become combined with appropriate genes that would give the mutations an increased chance of survival. Races of the pathogen, however, have been identified in Asia and Australia, and, therefore, variation in virulence genes exists (4). Burdon and Roelfs (5) demonstrated in the United States with *Puccinia recondita* Robs. ex Desm. f. sp. *tritici*, where there has never been a functioning sexual cycle, a very low level of isozyme diversity yet a large number of polymorphic virulence genes.

Isozyme analysis is a powerful tool that allows a measure of the genetic similarity within and between fungal species. The technique has proven useful for the clarification and delineation of fungal taxa, the identification of fungal cultures to species or subspecies level, and the study of the genetics of a specific fungus (9). The isozyme banding patterns of the two groups of isolates in this study were so distinct that there should be no problem in identifying the hemisphere of origin of the pathogen if soybean rust is found in the continental United States.

LITERATURE CITED

1. Baker, C. F. 1914. The lower fungi of the Philippine Islands. Leafl. Philipp. Bot. 6:2065-2190.
2. Bonde, M. R., and Brown, M. F. 1980. Morphological comparison of isolates of *Phakopsora pachyrhizi* from different areas of the world. Can. J. Microbiol. 26:1443-1449.
3. Bonde, M. R., Melching, J. S., and Bromfield, K. R. 1976. Histology of the suscept-pathogen relationship between *Glycine max* and *Phakopsora pachyrhizi*, the cause of soybean rust. Phytopathology 66:1290-1294.
4. Bromfield, K. R. 1984. Soybean Rust. Monograph No. 11. American Phytopathological Society, St. Paul, MN. 66 pp.
5. Burdon, J. J., and Roelfs, A. P. 1985. Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. Phytopathology 75:907-913.
6. Clayton, J. W., and Tretiak, D. N. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Board Can. 29:1169-1172.
7. May, B., Wright, J. E., and Stoneking, M. 1979. Joint segregation of biochemical loci in Salmonidae: Results from experiments with *Salvelinus* and review of the literature on other species. J. Fish. Res. Board Can. 36:1114-1128.
8. Melching, J. S. 1967. Improved deposition of airborne uredospores of *Puccinia graminis* and *P. striiformis* on glass slides and on wheat leaves by use of a turntable. (Abstr.) Phytopathology 57:647.
9. Micales, J. A., Bonde, M. R., and Peterson, G. L. 1986. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 27:405-449.
10. Micales, J. A., Bonde, M. R., and Peterson, G. L. 1988. Isozyme analysis and aminopeptidase assays within the genus *Peronosclerospora*. Phytopathology 78:1396-1402.
11. Micales, J. A., Stipes, R. J., and Bonde, M. R. 1987. On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. Mycologia 79:707-720.
12. Ridgway, G. J., Sherburne, S. W., and Lewis, R. W. 1970. Polymorphisms in the esterases of Atlantic herring. Trans. Am. Fish. Soc. 99:147-151.
13. Rogers, J. S. 1972. IV. Measures of genetic similarity and genetic distance. Stud. Genet. 7:145-153.
14. Rytter, J. L., Dowler, W. M., and Bromfield, K. R. 1984. Additional alternative hosts of *Phakopsora pachyrhizi*, causal organism of soybean rust. Plant Dis. 68:818-819.
15. Selander, R. K., Smith, M. H., Yang, S. Y., Johnson, W. E., and Gentry, J. B. 1971. IV. Biochemical polymorphism and systematics in the genus Peromyscus. I. Variation in the old-field mouse (*Peromyscus polionotus*). Studies in Genetics VI. Univ. Texas Publ. 7103:49-90.
16. Vakili, N. G., and Bromfield, K. R. 1976. Phakopsora rust on soybean and other legumes in Puerto Rico. Plant Dis. Rep. 60:995-999.