The Danish Institute of Plant and Soil Science
Biotechnology Group
Lottenborgvej 2
DK-2800 Lyngby

Beretning nr. 2248

Differentiation of *Pyrenophora graminea* and *Pyrenophora teres*

I. Gel electrophoresis and isozyme analysis of soluble mycelial proteins

Adskillelse af Pyrenophora graminea *og* Pyrenophora teres
I. Gel elektroforese og isozym analyser af opløselige mycelieproteiner

LISBETH H. JOHANSEN[1)], KAREN HUSTED[2)], LAURITZ W. OLSON[†3)], & MORTEN HEIDE[4)]

1) Present address: Pajbjergfonden, Gersdorffslundvej 1, Hou, DK-6300 Odder.
2) To whom correspondance should be addressed.
3) Institut for Sporeplanter, University of Copenhagen, Øster Farimagsgade 2 D, DK-1353 Copenhagen K.
4) Present address: Novo Nordisk, Biocontrol, Novo Allé, DK-2880 Bagsværd.

Summary

Soluble mycelial proteins of 46 isolates of *Pyrenophora teres* f. *teres*, 25 isolates of *P. teres* f. *maculata* and 20 isolates of *P. graminea* were compared for species-specific differences by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isozyme analysis. The isolates were of different geografic origin (Europe, North America, Australia and New Zealand). Electrophoretic analysis of total soluble protein by either isoelectric focusing or SDS-PAGE revealed no species-specific banding patterns. Isozyme analysis for acid phosphatase showed species-specific banding patterns, and it was possible to distinguish between the 2 forms of *P. teres*. 9 other isozyme analyses were performed, but no reliable species-specific banding patterns were obtained.

Key words: Isozyme analysis, *Pyrenophora teres*, *Pyrenophora graminea*, species differentiation.

Resumé

Opløselige mycelieproteiner fra 46 isolater af *Pyrenophora teres* f. *teres*, 25 isolater af *P. teres* f. *maculata* og 20 isolater af *P. graminea* blev sammenlignet for arts-specifikke forskelle ved natrium dodecyl sulfat polyacrylamid gel elektroforese (SDS-PAGE) og isozymanalyser. Isolaterne havde forskellig geografisk oprindelse (Europa, Nord Amerika, Australien og New Zealand). Elektro-

foretiske undersøgelser af total opløselige proteiner ved enten isoelektrisk fokusering eller SDS-PAGE viste ingen artsspecifikke båndmønstre. Isozymanalyse for sur fosfatase viste artsspecifikke båndmønstre, og det var muligt at adskille de 2 former af *P. teres*. Der blev udført isozymanalyse for 9 andre enzymer, men i ingen tilfælde fandtes konsistente artsspecifikke båndmønstre.

Nøgleord: Isozymanalyse, *Pyrenophora teres, Pyrenophora graminea,* artsadskillelse.

Introduction

The fungi *Pyrenophora graminea* Ito et Kurib. (anamorph *Drechslera graminea* (Rabenh. ex Schlecht.) Shoem.), syn. *Helminthosporium graminea* (Rabenh. ex Schlecht.) and *Pyrenophora teres* Drechs. (anamorph *Drechslera teres* (Sacc.) Shoem.), syn. *Helminthosporium teres* (Sacc.) are serious seedborne pathogens of barley (1). *P. teres* is the causal agent of net blotch and leaf spot disease. *P. teres* Drechs. f. *teres* induces net-like necrotic lesions on the leaves while *P. teres* Drechs. f. *maculata* Smedeg. induces brown elliptical leaf lesions. *P. graminea* is the causal agent of leaf stripe disease. In contrast to *P. teres, P. graminea* is not capable of infecting barley leaves directly and thus cannot cause epidemic infections.

The only reliable method that discriminates between *P. teres* and *P. graminea* is a pathogenicity test on barley. *P. teres* produces disease symptoms on inoculated barley leaves; *P. graminea* can not infect through the leaves, and leaf stripe disease only develops after seed infection. All other characteristics investigated, such as the morphology of the conidia, the size of the conidia, the mycelial characteristics when growing in vitro (1, 2, 3), and the ability to form a lilac pigment on Kietreibers blotter (4) can not clearly differentiate between these fungi.

Electrophoretic analysis of soluble proteins using unspecific protein or specific isozyme stains have been used to provide qualitative and semi-quantitative measures of the variation among species, tissues, and organs as well as changes due to pathogen invasion (5, 6). A number of papers describe the use of isozyme analysis on fungi (e.g. 7, 8, 9, 10, 11, 12, 13). This analysis can be used in genetic studies because the amino acid sequences of the proteins (enzymes) are dependent on the nucleotide sequences of their coding genes. Thus protein variations reflect variations at the genomic level.

The purpose of this study was to compare soluble proteins extracted from isolates of *P. teres* and *P. graminea* by electrophoretic analysis and to evaluate the potential of this technique for identification and routine testing of the 2 species. The pathogenicity test is time-consuming and requires a large number of test plants, making it cumbersome in routine testing for the presence of *P. teres* and *P. graminea* in barley grain. If electrophoresis of protein extracts can be used to discriminate *P. teres* from *P. graminea* it could be a valuable tool in the development of a rapid and reliable routine test. Such a routine test would be of importance to the export/import certification of barley seeds and the assessment of seed dressing requirements.

Materials and methods

Cultures

46 isolates of *P. teres* f. *teres*, 25 of *P. teres* f. *maculata*, 20 of *P. graminea*, 3 of *Pyrenophora bromi*, 2 of *Pyrenophora avenae*, and 2 of *Bipolaris sorokiniana* (syn. *Helminthosporium sativum)* were included in the experiments. All isolates were maintained in darkness at 20°C on potato dextrose agar (PDA, Difco). The isolates were kindly supplied by: American Type Culture Collection (ATCC), USA; Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands; Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, West Germany; T. Khan, Western Australian Department of Agriculture, Australia; L. Lange, Institute of Seed Pathology for Developing Countries, Denmark; H. Magnus, Norwegian Plant Protection Service, Norway; A. L. Scharen, Montana State University, USA; J. E. Sheridan, Victoria University of Wellington, New Zealand, V. Smedegaard-Petersen, The Royal Veterinary and Agricultural University, Denmark; J. B. Speakman, BASF, West Germany; B. J. Steffenson, University of California Davis, USA; A. Tekauz, Agriculture Canada, Canada and B. Welling, Danish Research Service for Plant and Soil Science, Denmark.

Pathogenicity test

Each of the isolates was grown in 2 Petri dishes containing 12 ml of grass medium (32.5 g pelleted rabbit food dissolved in 1 liter distilled water, which was boiled for 10 min. and agar was then added to 1% (w/v) prior to autoclaving at 120°C for 20 min.). After 14 days growth, (12 hrs. near UV illumination and 12 hrs. in darkness), the mycelium and spores from each Petri dish were scraped into 10 ml double distilled water to which was added 0.05% (w/v) Tween 20, and the material was homogenized for 1 min. in a blender. This suspension was sprayed onto twenty 12-day-old susceptible barley plants of the cultivar Welam.

After inoculation, the seedlings were placed in plastic bags for the first 48 hrs. to maintain high humidity. The inoculated plants were incubated in a greenhouse maintained at 15-20°C. Plants were supplied with artificial light 14 hrs. a day during November through February. Symptoms were recorded 9 days after inoculation.

Production and treatment of mycelium for electrophoresis

Leaves with symptoms of *P. teres* (from the pathogenicity test) were put in a humid chamber for 1-3 days under near UV light (12 hrs. a day) at 20°C, whereby sporulation was promoted. Spores were taken from each isolate and transferred to PDA. This procedure ensures a fresh virulent isolate. For *P. graminea*, which gives no symptoms in the pathogenicity test, this approach was not possible. Here the isolate was used directly from the maintained culture. For a few isolates, however, spores were isolated from leaves taken from plants grown up from infected kernels, so fresh virulent isolates also were ensured in these cases. For mycelium production all fungi were grown on Fries' artificial medium (80 ml in 250 ml flasks) (14). The flasks were seeded with mycelial discs (9 mm diam.) taken from 7-day-old stock cultures, and the cultures were incubated on a rotary shaker in darkness for 14 days at 20-22°C.

Cultures were harvested by vacuum filtration and the mycelium washed with distilled water. Excess water was decanted after centrifugation (13.000×*g* at 4°C for 7 min.) and the mycelium was lyophilized. Lyophilized mycelium was ground to a fine powder in a small mortar, using 0.5 g acid washed sand for 1 g of lyophilized mycelium. Extraction buffer (0.05M Tris, 0.06M sucrose, 0.01 M KCl, 5 mM EDTA, 0.5% glycerol, 0.05% Triton X-100, pH 7.8), 0.7-1.0 ml for each 0.2 g of dried mycelium was added. This paste was mixed for 20 sec. on a vortex mixer and allowed to solubilize for 30 min. at 0°C and then centrifuged at 45.000×*g* for 15 min. at 4°C. The resulting supernatant was used for electrophoretic analysis of total soluble protein. The supernatant was stored at –20°C. Protein determinations were made after the principle of Bradford (15), using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories GmbH, Dachauer Strasse 364/511, D-8000 München 50) and bovine serum albumin as a standard.

Electrophoresis of total soluble protein

Vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous system of *Laemmli* (16) with 12.5% and 3.5% acrylamide in the separation gel and stacking gel, respectively. Gels were loaded with 50-80 μg protein/well. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue (CBB) as described by *Chrambach* et al. (17) or the more sensitive silver staining method of *Morrissey* (18). A low molecular weight standard protein mixture (14,400-94,000 dalton, Pharmacia, Biotechnology AB, Uppsala, Sweden) was used for molecular weight determinations.

Native proteins were analysed using isoelectric focusing on 1% agarose gels (IEF agarose, Pharmacia), on gelbond (Interkemi, MCD, FMC Corporation Rockland, Maine) with a pH gradient of 3-10 (Pharmalytes 3-10, Pharmacia). Gels were loaded with 50-60 μg protein/well. After electrophoresis at 10°C for 2400 Vhrs., the gels were stained with CBB. The IEF procedures were those recommended by Pharmacia and the pH gradient determined by using IEF-MIX 3.5-9.3 (No. I 0630, Sigma).

Isozyme electrophoresis

IEF was used for all isozyme analyses except catalase (CAT 1.11.1.6. E.C.), which was carried out in a discontinuous 3.5-8% polyacrylamide (PAA) gel with Tris/glycine buffer, pH 8.3, as described by *Laemmli* (16), except that SDS was omitted to avoid protein denaturation. The multiple forms of esterase (α-EST 3.1.1.1. E.C.) were visualized by staining with α-naphthylacetate and Fast Blue RR salt (19). Acid phosphatases (ACPH 3.1.3.2. E.C.) were stained by α-naphthylphosphate and Fast Black Salt (20). Alkaline phosphatase (ALPH 3.1.3.1. E.C.) was stained using 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue tetrazolium following the procedures of *Blake* et al. (21). Cat-

alase (CAT 1.11.1.6. E.C.) staining was performed by a modification of the technique of *Woodbury* et al. (22) where rinsing in water for 45 min. was omitted. Malate dehydrogenase (MDH 1.1.1.37. E.C.), phosphoglucomutase (PGM 2.7.5.1. E.C.), glucose phosphate isomerase (GPI 5.3.1.9. E.C.), phosphogluconate dehydrogenase (PGD 1.1.1.44. E.C.), and hexokinase (HK 2.7.1.1. E.C.) were stained using tetrazolium as a proton acceptor as described by *Latner* and *Skillen* (23). NADH diaphorase (DIA 1.6.2.2. E.C.) was stained by NADH and 2.6 dichlorophenolindophenol. After staining, the gels were rinsed with distilled water, pressed, and dried.

Isozyme patterns were evaluated on the basis of the number of bands and their position, intensity, and width.

All isolates were tested for acid phosphatase, whereas only half of them were tested for other isoenzymes.

Results

Pathogenicity test

The pathogenicity test was performed to confirm the identity of the isolates (Table 1). The identification of the isolates corresponded to the information given by the donors except for 5 isolates. These were originally designated as *P. graminea,* but they induced net-forming lesions indicating that they are *P. teres* f. *teres.* A number of *P. teres* isolates produced symptoms which were difficult to identify as either net- or spot-forming lesions. The identification of the *P. graminea* isolates by the pathogenicity test was a 'negative test', in the sense that this species does not infect directly through the leaves. The 2 isolates of *B. sorokiniana* gave spot-forming lesions similar to the symptoms produced by the spot-form of *P. teres.* The 2 isolates from DSM and the one from CBS, which gave no symptoms were originally designated as *P. bromi* and *P. avenae* and *P. bromi,* respectively. Morphological studies confirmed these identifications. Morphological studies also showed, that the isolate from Denmark and the one from Norway which gave no symptoms and gave a different acid phosphatase banding pattern, were *P. bromi* and *P. avenae,* respectively.

Electrophoresis of total soluble protein

SDS-PAGE followed by CBB or silver staining did not reveal any species-specific banding patterns. The patterns of all isolates were very similar with

Table 1. Isolates included in the acid phosphatase analysis. *Isolater inkluderet i sur fosfatase undersøgelserne.*

Origin *Oprindelse*	Phenotype[a] *Fænotype[a]*	ACPH-type[b] *ACPH-type[b]*
ATCC[c]	2 Net	2 ACPH-2
	1 Spot	1 ACPH-3
Australia	1 Net	1 ACPH-2
	1 None	1 ACPH-1
Canada	3 Net	3 ACPH-2
	1 Spot	1 ACPH-3
	1 None	1 ACPH-1
CBS[d]	1 Spot	1 ACPH-3
	1 None	1 Other
Denmark	17 Net	17 ACPH-2
	18 Spot	16 ACPH-3, 2 Other
	4 Spot/net	3 ACPH-2, 1 ACPH-3
	8 None	7 ACPH-1, 1 Other
DSM[e]	1 Net	1 ACPH-2
	3 None	1 ACPH-1, 2 Other
Finland	2 None	2 ACPH-1
New Zealand	4 Net	4 ACPH-2
	2 Spot	2 ACPH-3
	2 None	2 ACPH-1
Norway	5 Net	5 ACPH-2
	7 None	6 ACPH-1, 1 Other
Sweden	2 Net	2 ACPH-2
USA-California	2 Net	2 ACPH-2
USA-Montana	4 Net	4 ACPH-2
	3 Spot	3 ACPH-3
	2 Spot/net	1 ACPH-2, 1 ACPH-3
West Germany	1 Spot/net	1 ACPH-2

a) The phenotype indicates the symptoms found in the pathogenicity test on barley.
b) ACPH-type according to the acid phosphatase isozyme analysis. 'Other' types representing zymograms different from the types 1-3.
c) ATCC: American Type Culture Collection.
d) CBS: Centraal Bureau voor Schimmelcultures, Baarn.
e) DSM: German Collection of Microorganisms and Cell Cultures.
a) Fænotypen indikerer symptomer fundet i patogenitetstest på bygplanter.
b) ACPH-typen svarer til sur fosfatase undersøgelserne. Other svarer til mønstre forskelligt fra de øvrige 3.
c) ATCC: American Type Culture Collection.
d) CBS: Centraal Bureau voor Schimmelcultures, Baarn.
e) German Collection of Microorganisms and Cell Cultures.

minor non-systematic differences. (Fig. 1). IEF of soluble mycelial proteins gave a similar result (data not shown here).

Isozyme analysis of total soluble protein

Isolates were investigated for electrophoretic variations in 10 isozyme systems. The 5 systems malate dehydrogenase, glucose phosphate isomerase, phosphoglucomutase, hexokinase and phosphogluconate dehydrogenase showed no variation among the isolates tested (data not shown here). No reactions were obtained for alkaline phosphatase and NADH diaphorase.

The esterase analyses (data not shown) showed large variations among the isolates, but it was not possible to find species-specific patterns. In the catalase analysis (data not shown) one band was found for all isolates with about the same R_m value. An additional band with a slightly higher R_m value was found in some isolates.

In the acid phosphatase analysis most of the isolates produced 1 of 3 distinctly different banding patterns, in the following denoted ACPH-type 1 to 3 (Fig. 2). Because of the trailing effect it was difficult to locate the individual bands precisely, and it has therefore not been possible to calculate simple matching coefficients (SSM) as described by *Sokal* and *Michener* (24). The overall patterns, however, were clearly different. As shown in table 1 it was found that isolates with a zymogram of ACPH-type 1 corresponded with the isolates giving no symptoms thus being *P. graminea.* Those with a zymogram of ACPH-type 2 and 3 corresponded with the isolates giving net and spot symptoms respectively, thus being *P. teres* f. *teres* and *P. teres* f. *maculata.* By acid phosphatase analysis it has therefore been possible to distinguish between isolates of *P. graminea* and *P. teres* as well as between the two forms of *P. teres.* The isolates of *B. sorokiniana, P. bromi* and *P. avenae* produced patterns differing from the ACPH-types 1 to 3. The zymogram pattern did not show any additional geographic differences.

Based on the grouping of the isolates obtained in the acid phosphatase analysis it appears possible to suggest an identification of the isolates which could not be definitively identified by the pathogenicity test. This means that the isolates which gave a banding pattern of ACPH-type 2 are *P. teres* f. *teres* and the isolates which gave an ACPH-type 3 pattern are *P. teres* f. *maculata.*

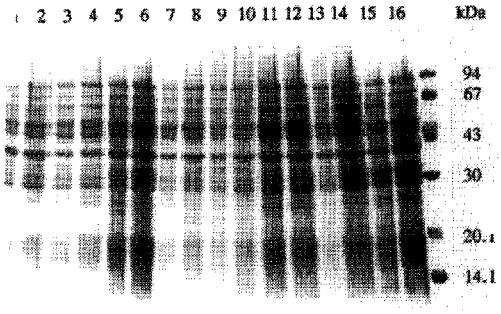

Fig. 1. Electrophoretic separation of total soluble mycelial proteins in 12.5 % sodium dodecyl sulfate-polyacrylamide gel followed by CBB staining. Lanes 1-6: *P. graminea* isolates. Lanes 7-10: *P. teres* f. *maculata* isolates. Lanes 11-16: *P. teres* f. *teres* isolates. The position and molecular weight (kDa) of marker proteins are indicated to the right.
Elektroforetisk adskillelse af total opløselige mycelie proteiner i 12,5% SDS-PAGE, efterfulgt af farvning med Comassie Brilliant Blue (CBB). Bane 1-6: P. graminea *isolater. Bane 7-10:* P. teres *f. maculata isolater. Bane 11-16:* P. teres *f.* teres *isolater. Molekylvægtsmarkører (kDa) er angivet i højre side.*

Discussion

Discrimination of the 3 fungi was obtained for a large number of isolates of different geographic origin. The acid phosphatase banding pattern obtained by isoelectric focusing therefore seems to be a preserved

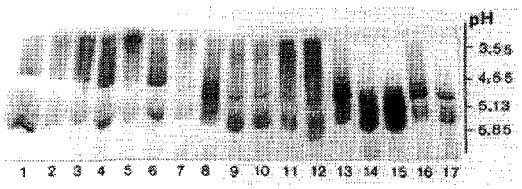

Fig. 2. Isolelectric focusing of soluble mycelial proteins in 1% agarose gel with a pH gradient of 3-10 followed by staining for acid phosphatase activity. Lanes 1-6: *P. teres* f. *teres* (ACPH-type 2). Lanes 7-12: *P. teres* f. *maculata* (ACPH-type 3). Lanes 13-17: *P. graminea* (ACPH-type 1). The pH gradient markers positions are given to the right.
Isoelektrisk fokusering af opløselige mycelie proteiner i 1% agarose gel med en pH gradient fra 3 til 10 efterfulgt af farvning for sur fosfatase. Bane 1-6: P. teres *f.* teres *(ACPH-type 2). Bane 7-12:* P. teres *f.* maculata *(ACPH-type 3). Bane 13-17:* P. graminea *(ACPH-type 1). pH gradientmarkører er markeret til højre.*

characteristic independent of the geographic origin of the isolates.

The 3 distinctly different acid phosphatase patterns found for *P. graminea*, *P. teres* f. *teres*, and *P. teres* f. *maculata* do not support the usual division of these 3 fungi in 2 species, namely *P. graminea* and *P. teres*. The difference between the 2 forms of *P. teres* is comparable to the difference between *P. graminea* and each of the 2 *P. teres* forms. These results indicate that the 3 fungi belong either to the same or to 3 different biological species. Referring to interspecific hybridization experiments performed in 1976 by *Smedegaard-Petersen* (14) and the many morphological and physiological interspecific similarities it could be obvious to suggest that the 3 fungi are in fact 3 forms of the same biological species. The result here, however, show that the 3 fungi have private alleles and the conclusion then is, that there is no gene flow, in spite of sympatry, and thus the fungi are behaving as good biological species in the field. The fact that *Smedegaard-Petersen* was able to cross these species under artificial conditions in the lab does not indicate that interbreeding takes place in the field or that the progeny of such matings are fit relative to within species matings.

As an identification method acid phosphatase analysis has at least 2 advantages compared to pathogenicity test. First of all, the 2 forms of *P. teres* are clearly distinguished by acid phosphatase analysis. This is in general also obtained with the pathogenicity test. There are, however, as shown in table 1, cases where it is difficult to characterize the symptoms as either the net-form *(P. teres* f. *teres)* or the spot-form *(P. teres* f. *maculata)*. This difficulty mainly occurs when the attacks are weak. Secondly, acid phosphatase analysis 'positively' identifies all isolates, whereas the pathogenicity test identifies *P. graminea* in a 'negative' sense, i.e. it gives no symptoms when inoculated onto barley plants. A reliable identification in this way requires very strict inoculum and incubation conditions. Otherwise an isolate could erroneously be identified as a *P. graminea*, e.g. because the inoculum was unable to germinate. If no symptoms appear in the pathogenicity test it may be required to make a kernel infection experiment. *P. graminea* isolates should here give stripe symptoms.

Effective control of the seed-borne pathogens *P. graminea* and *P. teres* requires a rapid and reliable method for routine identification. This method must distinguish between the 2 fungi, since their mode of infection is essentially different. Acid phosphatase analysis provides the required identification, but the procedure described here is not useful as a routine method. It is laborious, time-consuming, and too expensive. By further development it should, however, be possible to obtain a method applicable for routine purposes.

References

1. *Smedegaard-Petersen, V.* 1971. *Pyrenophora teres* f. *maculata* nov. and *Pyrenophora teres* f. *teres* on barley in Denmark. Royal Vet. Agric. Univ. Yearbook, 124-144.
2. *Chidambraram, P.; Mathur, S.B. & Neergaard, P.* 1973. Identification of seed-borne *Drechslera* species. Friesia X (3), 165-207.
3. *Smedegaard-Petersen, V.* 1977. Inheritance of genetic factors for symptoms and pathogenicity in hybrids of *Pyrenophora teres* and *Pyrenophora graminea*. Phytopath. Z. 89, 193-202.
4. *Knudsen, J.C.N.* 1982. Production of pigments by *Pyrenophora graminea* and *Pyrenophora teres* with special reference to the use of this characteristic in seed disease testing. *Seed Sci. & Technol.* 10, 357-363.
5. *Micales, J.A.; Bonde, M.R. & Peterson, G.L.* 1986. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 27, 405-449.
6. *Michelmore, R.W. & Hulbert, S.H.* 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Ann. Rev. Phytopathol. 25, 383-404.
7. *Bonde, M.R.; Peterson, G.L.; Dower, W.M. & May, B.* 1984. Isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildews of maize. Phytopathology 74, 1278-1283.
8. *Chen, W.; Hoy, J.W. & Schneider, R.W.* 1991. Comparisons of soluble proteins and isozymes for seven *Pythium* species and applications of the biochemical data to *Pythium* systematics. Mycological Res. 95, 548-555.
9. *Hwang, B.K.; Yun, J.H. & Kim, Z.S.* 1987. Geographic variation of esterase isozymes in populations of *Alternaria mali* on apple leaves. J. Phytopathology 119, 225-231.
10. *Newman, P.L.* 1985. Variation amongst isozymes of *Rhynchosporium secalis*. Plant Pathology 34, 329-337.
11. *Nygaard, S.L.; Elliott, C.K.; Cannon, S.J. & Maxwell, D.P.* 1989. Isozyme variability among isolates of *Phytophthora megasperma*. Phytopathology 79, 773-780.
12. *Oudemans, P. & Coffey, M.D.* 1991. A revised systematics of twelve papillate Phytophthora species based on isozyme analysis. Mycological Res. 95, 1025-1046.
13. *Wong, A.L. & Willetts, H.J.* 1973. Electrophoretic studies of soluble proteins and enzymes of *Sclerotinia* species. Trans. Br. Mycol. Soc. 61, 167-178.

14. *Smedegaard-Petersen, V.* 1976. Pathogenesis and genetics of net-blotch and leaf stripe of barley by *Pyrenophora teres* and *Pyrenophora graminea.* DSR Forlag, Royal Vet. Agric. Univ., Copenhagen, 176 pp.
15. *Bradford, M. M.* 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.
16. *Laemmli, U.K.* 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
17. *Chrambach, A.; Reisfeld, R.A.; Wychoff, M. & Zoccari, J.* 1967. A procedure for rapid and sensitive staining of protein fractioned by polyacrylamide gel electrophoresis. Anal. Biochem. 20, 150-154.
18. *Morrissey, H.J.* 1981. Silver stain for proteins in polyacrylamide gels: A procedure with enhanced uniform sensitivity. Anal. Biochem. 117, 307-310.
19. *Brewbaker, J.L.; Upadhya, M.D.; Makinen, Y. & MacDonald, T.* 1968. Isoenzyme polymorphism in flowering plants III gel electrophoretic methods and applications. Physiol. Plant. 21, 930-940.
20. *Iype, P.T. & Heidelberger, C.* 1968. Characteristics of murine prostatic acid phosphatase: Comparison with other tissues and species. Archives of Biochemistry and Biophysics 128, 434-441.
21. *Blake, M.S.; Johnston, K.H.; Russell-Jones, G.J. & Gotschlich, E.C.* 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. 136, 175-179.
22. *Woodbury, W.; Spencer, A.K. & Stahmann, M.A.* 1977. An improved procedure using ferricyanide for detecting catalase isozymes. Anal. Biochem. 44, 301-305.
23. *Latner, A.L. & Skillen, A.W.* 1968. Isoenzymes in biology and medicine. Academic Press, New York and London, 190-194.
24. *Sokal, R.R. & Michener, C.D.* 1958. Statistical measure for evaluating systematic relationships. Univ. Kansas Sci. Bull. 38, 1409-1438.

Manuscript received 2 October 1992.