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

Beretning nr. 2250

Differentiation of *Pyrenophora graminea* and *Pyrenophora teres*

II. Antiserum against soluble mycelial proteins

Adskillelse af Pyrenophora graminea *og* Pyrenophora teres
II. Antiserum mod opløselige mycelieproteiner

KAREN HUSTED

Summary

Polyclonal antiserum was raised against total soluble mycelial proteins from the 2 fungi *Pyrenophora graminea* and *P. teres* f. *maculata* respectively. Both antisera showed a high degree of cross-reaction to the other *Pyrenophora* species as well as to other fungi. Different cross-absorption approaches were tried as a way to get more specific antiserum, but none of them succeeded. It is concluded, that the antigenic difference between the 2 species are so small, that an alternative method such as one based on a monoclonal antibody seems to be required for a satisfactory immunological differentiation.

Key words: Antiserum, cross-absorption, *Pyrenophora graminea, Pyrenophora teres,* species differentiation.

Resumé

Polyklonalt antiserum var fremstillet over for opløselige mycelieproteiner fra henholdsvis *Pyrenophora graminea* og *P. teres* f. *maculata.* Begge typer antiserum udviste en høj grad af krydsreaktion med såvel de andre *Pyrenophora*-arter som over for andre svampe. Forskellige krydsabsorptionsforsøg blev, dog uden held, afprøvet som en metode til at opnå mere specifikt antiserum. Det konkluderes, at de antigene forskelle mellem de 2 arter er så små, at en alternativ metode baseret på monoklonale antistoffer synes at være krævet for at få en tilfredsstillende immunologisk påvisning.

Nøgleord: Antiserum, krydsabsorption, *Pyrenophora graminea, Pyrenophora teres,* artsadskillelse.

Introduction

The 2 fungi *Pyrenophora graminea* Ito et Kurib. (anamorph *Drechslera graminea* (Rabenh. ex. Schlecht.) Shoem., syn. *Helminthosporium graminea* Rabenh. ex Schlecht.) and *P. teres* Drechs. (anamorph *Drechslera teres* (Sacc.) Shoem., syn. *Helminthosporium teres* Sacc.) (subdevided in f. *teres* and f. *maculata* Smedeg.) causing barley stripe disease and barley net blotch/leaf spot disease are both serious seedborne pathogens of barley (1). As discussed in another paper (2) the 2 fungi can not be distinguished by morphological caracteristics, but only by a pathogenicity test where the different symptoms are seen. Since a pathogenicity test is cumbersome and laborious there is a high demand for other ways to discriminate between the 2 fungi. By acid phosphatase analysis it has been possible to discriminate between the fungi (2), but the method is not useful for routine tests.

The development of immunologically based methods for the diagnosis of plant pathogenic fungi has become quite common during the last decade, since the first report on the use of ELISA for the detection of fungi appeared in 1979 (3). Detection of fungi on seeds by immunologically based methods has been described in a few cases only (4, 5, 6).

Cross-absorption as a way to get a more specific antiserum against fungal pathogens has only been described in a few cases. *Mohan* (7) tried to get a *Phytophthora fragariae* specific antiserum by passing polyclonal *P. fragariae* antiserum through a column coupled with *P. cactorum* antigens. The resulting antiserum, however, still reacted with all the *Phytophthora* species tested. In another experiment antiserum were affinity purified on a column to which extract from *P. fragariae* infected raspberry roots was coupled. The antiserum eluted from the column still reacted with all the *Phytophthora* species tested, but the cross reaction to other fungi (e.g. *Pythium* species) decreased. The attempt by *Gendloff* et al. (8) to cross-absorb polyclonal antiserum against *Eutypa armeniacae* with a protein fraction from *Phomopsis viticola* was also only partly successful, and cross-absorption of *Verticillium dahlia* polyclonal antiserum by *Fusarium oxysporum* resulted in a more specific serum, but with a lower sensitivity (9).

This paper describes attempts to cross-absorb unspecific polyclonal antiserum against *P. graminea* and *P. teres*, as a way to get a more specific antiserum.

Materials and methods

Preparation of mycelial antigens

Isolates were grown, mycelium harvested, and proteins extracted and the protein concentration of the extract measured as described in (2).

Antiserum production

Polyclonal antiserum was produced by *Johansen* (10). The antiserum was prepared by immunizing 2 rabbits with total soluble mycelial proteins from an isolate of *P. graminea* (Pg 3) and an isolate of *P. teres* f. *maculata* (Pt 1-5A) respectively, mixed 1:1 with Freund's incomplete adjuvant following the immunization scheme of *Harboe & Ingild* (8) using 100 µg protein per kg rabbit. The rabbits were bleeded 14 days after each immunization. Antiserum was stored at 4°C with 0.02 % Na-azide as preservative. In the following the antiserum raised against *P. graminea* will be referred to as anti-P.gr. PAb and the antiserum against *P. teres* f. *maculata* as anti-P.t.m. PAb.

Affinity chromatography

In preliminary investigations cross-absorption of antisera was performed in small vessels by simply incubating protein extracts from different isolates with the antiserum for 1-3 hours at room temperature before using the antiserum in Western blotting. For these cross-absorptions about 2 mg of protein were used per microliter of antisera.

Later on cross-absorptions were performed using affinity chromatography. Antigens from *P. teres* f. *teres* and *P. teres* f. *maculata*, 10 mg from each, were coupled to 1 g Mini-Leak gel matrix according to the manufacturer's instructions (Kem-En-Tec), using a 8.5 % final concentration of PEG, by adding appropriate amount of a buffer consisting of 30 % PEG 20000, and 0.3 M $NaHCO_3$, pH 8.6. Coupling was made overnight by shaking at 4°C. The gel matrix was centrifuged ($2000g_{max}$/5 min.) and the protein concentration in the supernatant was determined as a way to calculate the coupling yield. Excess active groups were blocked with 0.2 M ethanolamine/HCl pH 9.0 for 3-5 hours, and finally the coupled gel matrix was washed on a Büchner funnel using alternately low and high pH solutions (0.1 M acetic acid, 0.5 M NaCl, pH 4.0 followed by 0.2 M ethanolamine/HCl pH 9.0) and finally in K-phosphate-buffer (0.15 M K-phosphate, 0.5 M NaCl, pH 7.2).

The coupled gel matrix was poured into a small

glasstube and polyclonal antiserum (10 µl) against *P. graminea* was added. Cross-absorption was made overnight by shaking at 4°C. The matrix was poured into 5-ml pipettes previously plugged at the tip with glass wool, and 2-2.5 ml cross-absorbed antiserum was collected by adding K-phosphate-buffer (as above) to the column. The column was eluted with 6 M urea in phosphate-buffer and finally phosphate-buffer. 0.02 % Na-azide was added in the final washing as a preservative. In some cases the cross-absorption was repeated by running the cross-absorbed antiserum through the eluted column. The cross-absorbed antiserum was tested in Western blots using standard anti-P.gr. PAb as control.

In another case IgG-type immunoglobulins were purified from *P. teres* f. *maculata* polyclonal antiserum on a protein-A column (12). IgG (0.35 ml, 40 mg/ml) were coupled to 1 g Mini-Leak gel matrix as described above for the coupling of antigens, using a 6 % final concentration of PEG in the coupling solution. Cross-absorption of protein extract from *P. graminea* was performed as described above for cross-absorption of antiserum. In one experiment 2.8 mg protein was added to the matrix and after cross-absorption 10 fractions each of 75 µl were collected. In another experiment only 0.6 mg protein was added and 2 repeated cycles on the column were performed in addition to the first overnight cross-absorption. The final 2.5 ml cross-absorbed

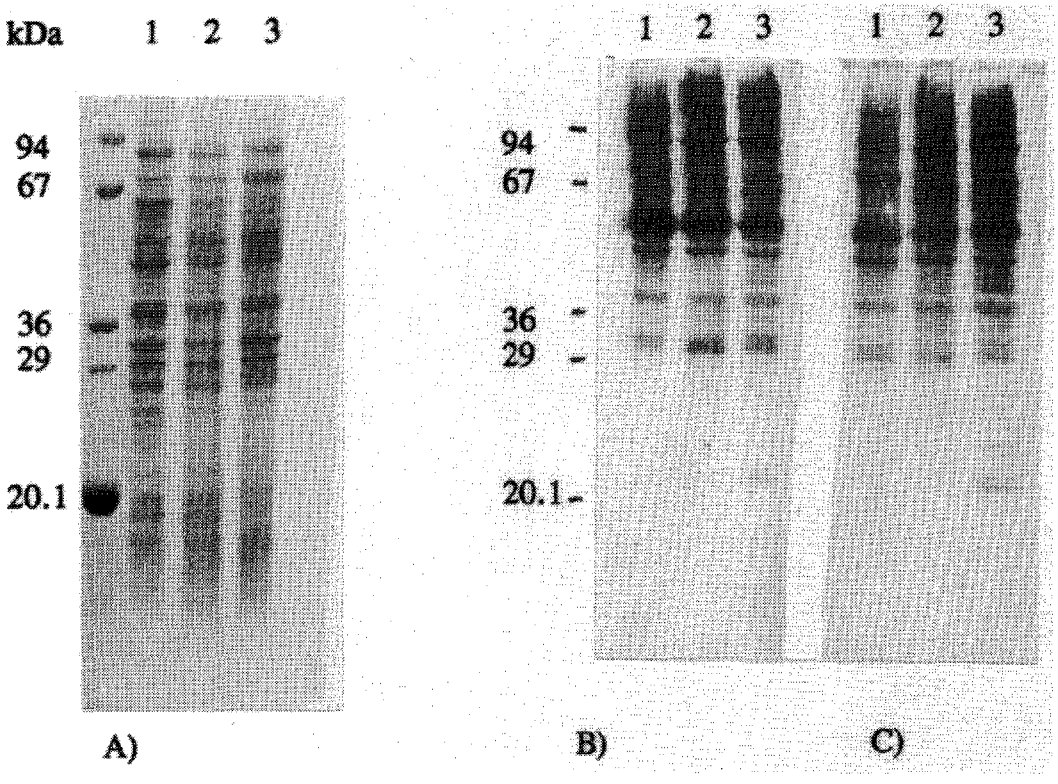

Fig. 1. Protein extract separated by SDS-PAGE and stained with CBB (A) or transferred to a membrane and immunolabelled with *P. graminea* polyclonal antiserum (B) or *P. teres* f. *maculata* polyclonal antiserum (C). Lane 1: *P. graminea;* Lane 2: *P. teres* f. *teres;* Lane 3: *P. teres* f. *maculata.* Molecularweight (kDa) markers are indicated to the left. *Protein ekstrakt adskilt ved SDS-PAGE (natrium dodecyl-sulfat-polyacrylamid-gel elektroforese) og farvet med CBB (Coomassie Brilliant Blue) (A) eller overført til en membran og mærket med* P. graminea *polyklonalt antiserum (B) eller* P. teres *f.* maculata *polyklonalt antiserum (C). Bane 1:* P. graminea; *Bane 2:* P. teres *f.* teres; *Bane 3:* P. teres *f.* maculata. *Molekylvægtsmarkører (kDa) er angivet i venstre side.*

protein extract from the later experiment was precipitated by adding a half volume of 30 % TCA, leaving the solution on ice for one hour, pelletizing by centrifugation at 15.000g_{max} for 10 min., washing in acetone and finally resuspending in 200 μl sample-buffer. The cross-absorbed protein extract was analyzed by SDS-PAGE and immunoblotting as described below.

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) was performed on 12.5 % slab gels according to *Laemmli* (13). Gels were stained for protein with Coomassie Brilliant Blue (CBB) (14) or by the silver staining method of *Morrissey* (15). For immunoblotting the gels were electroblotted as described by *Towbin* et al. (16) except that polyvinylidene difluoride membranes (Immobilon™, Millipore) were used instead of nitrocellulose and the transfer buffer contained only 15 % (v/v) methanol. Excess protein binding capacity of the membranes was blocked with 10 % (v/v) horse serum in TST buffer (0.05 M Tris, 0.5 M NaCl, 0.5 % (v/v) Tween 20, pH 10.2) for 1 h. Immunolabelling was performed by incubating overnight at 4°C with the primary antiserum diluted in TST buffer (1:10.000), washing in 3 changes of TST buffer 10 min. each, incubating for 3-4 h at room temperature with alkaline phosphatase-conjugated secondary antibody in TST buffer and washing as before. The enzyme reaction was developed with 5-bromo-4-chloroindoxyl phosphate as described by *Blake* et al. (17).

Molecular weight markers for SDS-PAGE were from Pharmacia: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), glyceraldehyd 3-phosphat dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa).

Results

Antiserum production

Antiserum was produced against total soluble mycelial proteins from *P. graminea* and *P. teres* f. *teres* respectively (10). As shown in Fig. 1A total soluble proteins from different isolates showed no species-specific banding pattern in SDS-PAGE stained with CBB (see also 2, 10). Tested in Western blots the antisera showed a very high degree of cross-reaction (Figs 1B, 1C) and no species-specific bands were seen for either the *P. graminea* antiserum or the *P. teres* antiserum.

Cross-absorption

In the preliminary investigations it was found that protein extract from other isolates were able to absorb a high amount of antibodies from the sera, although the effectiveness of these cross-absorptions varied (data not shown). Not only extract from *P. graminea* and *P. teres* but also extracts from *P. bromi* and *P. avenae* had a high capacity to cross-absorb the antiserum. Extract from the less closely related *Cocliobulus sativus* cross-absorbed to a minor extent. Cross-absorption of the anti-P.t.m. PAb by extract from *P. teres* f. *teres* resulted in antiserum which no longer gave reaction to *P. teres* f. *maculata,* indicating a very high homology between the 2 forms of *P. teres.* These investigations all together pointed to the application of affinity chromatography as a way to get a more specific antiserum.

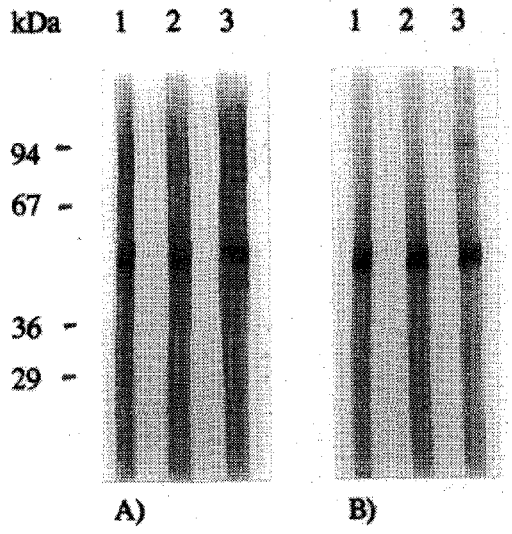

Fig. 2. Protein extract separated by SDS-PAGE, transferred to a membrane and immunolabelled with *P. graminea* polyclonal antiserum (A) or *P. graminea* polyclonal antiserum cross-absorbed with *P. teres* proteins (B). Lane 1: *P. graminea;* Lane 2: *P. teres* f. *teres;* Lane 3: *P. teres* f. *maculata.*

Proteinekstrakt adskilt ved SDS-PAGE, overført til en membran og mærket med P. graminea *polyklonalt antiserum (A) eller* P. graminea *polyklonalt antiserum krydsabsorberet med* P. teres *proteiner (B). Bane 1:* P. graminea; *Bane 2:* P. teres *f.* teres; *Bane 3:* P. teres *f.* maculata.

2 different approaches were tried: 1) Coupling of the antigens to a column and cross-absorption of the common antibodies from the polyclonal antiserum as a direct way to get a more specific antiserum. 2) Coupling of immunoglobulins to a column and cross-absorption of the common antigens from the protein extract. The remaining extract can then be used as immunogen in a new immunization.

Affinity chromatography using *P. teres* mycelial antigens bound to Mini-Leak

The coupling yield for *P. teres* proteins to the Mini-Leak gel matrix was 84 %. This means that a total of 16.8 mg antigens were coupled to the matrix. Tested in Western blots the cross-absorbed antiserum reacted much less than the original anti-P.gr. PAb (Fig. 2) but still it reacted with the 2 forms of *P. teres* along with *P. graminea.*

Affinity chromatography using immunoglobulins bound to Mini-Leak

The coupling yield for the *P. teres* IgG was 87 % which means that 12 mg IgG was coupled to the matrix. SDS-PAGE analysis of the cross-absorbed *P. graminea* protein extract showed a lower protein concentration than in the original extract, but all the protein bands found in the original extract were still detected (results not shown). In Western blots the extract again reacted with both anti-P.gr. and anti-P.t.m. serum in the same way as the original extract (results not shown), indicating that common antigens had not been removed by the cross-absorption.

Discussion

The high degree of cross-reaction found between *P. graminea* and *P. teres* as well as between the 2 forms of *P. teres* using a polyclonal antiserum was not surprising since total soluble protein had shown identical banding pattern for all the isolates in SDS-PAGE. The use of total soluble proteins as immunogen would therefore be expected to produce an unspecific antiserum. Still, it was hoped, that species-specific bands could be identified in Western blots. This was, however, not the case, and several explanations can be suggested: The polyclonal antiserum may not contain any species-specific antibodies at all or their concentration may be too low to be detected, or the species-specific proteins to be recognized have close to the same mobility as some of the unspecific proteins. A combination of these explanations is most likely.

Cross absorption of the *P. graminea* antiserum with *P. teres* antigens obviously removed most of the antibodies in the serum. However, the few remaining antibodies were not specific for *P. graminea.* This lack of specificity in spite of the cross-absorption might have several explanations. The coupling of heterogeneous proteins to the gel matrix under identical conditions might result in sub-optimal coupling of some proteins. In the following cross-absorption the corresponding antibodies are not absorbed, and they will then react with the proteins in Western blots. According to the manufacter Kem-En-Tec certain strongly glycosylated proteins require more than 15 % PEG to couple efficiently. Since glycoproteins have had an important role as immunogens in other cases (18, 19) it is possible that the strong band seen for the cross-absorbed antiserum represents a glycoprotein. A way to avoid this source of error could be to make a number of columns with antigens coupled under different conditions and then run the antiserum through all the columns. Another possible explanation for the lack of specificity of the cross-absorbed antiserum relates to the concentration levels. The composition of the antiserum does not necessarily reflect the composition of the protein extract with respect to concentration (as seen in Fig. 1). Some of the proteins might be very immunogenic, causing a large amount of antibodies to be raised against them. The amount of the protein subsequently coupled to the gel matrix is then too limited compared to the amount of antibodies to be absorbed. This explanation is, however, less likely since antiserum that was recycled 3 times over the column did not loose all the antibodies. A third explanation could be that during the coupling reaction some epitopes are destroyed and thus cannot participate in the cross-absorption.

The lack of cross-absorption of the antigens from *P. graminea* extract by the *P. teres* IgG-coupled gel matrix was surprising, since the coupling seemed to work well. A possible explanation is, that the IgG are coupled in such a way, that the subsequent absorption of antigens by the IgG is impossible. If the concentration of the specific proteins is very limited compared to the concentration of the unspecific proteins, the later might have been diminished substantially without the change being noticed, because of the considerable excess of unspecific proteins initially.

The conclusion of the present work is very much parallel to the one by *Mohan* (7) for the *Phytophthora* species. The antigenic differences between the

2 species are so small, that an alternative method such as one based on a monoclonal antibody seems to be required for a satisfactory differentiation. An alternative to the immunoassay techniques is DNA-hybridization, using species-specific DNA fragments instead of species-specific antibodies. *Husted* (20) has shown that it is possible to distinguish *P. graminea, P. teres* f. *teres* and *P. teres* f. *maculata* by this technique.

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. *Johansen, L.H.; Husted, K.; Olson, L.W. & Heide, M.* 1992. Differentiation of *Pyrenophora graminea* and *Pyrenophora teres*. I. Gel electrophoresis and isozyme analysis of soluble mycelial proteins. Tidsskrift for Planteavl 96, 391-397.
3. *Casper, R. & Mendgen, K.* 1979. Quantitative serological estimation of a hyperparasite: Detection of *Verticillium lecanic* in yellow rust infected wheat leaves by ELISA. Phytopathol. Z. 94, 89-91.
4. *Gleason, M.L.; Ghabrial, S.A. & Ferriss, R.S.* 1987. Serological detection of *Phomopsis longicolla* in soybean seeds. Phytopathology 77, 371-375.
5. *Dewey, F.M.; MacDonald, M.M. & Philips, S.I.* 1989. Development of monoclonal antibody-ELISA, -DOT-Blot and DIP-Stick immunoassays for *Humicola lanuginosa* in rice. J. Gen. Microbiol. 135, 361-374.
6. *Mitchell, L.A.* 1988. A sensitive dot immunoassay employing monoclonal antibodies for detection of *Sirococcus strobilinus* in spruce seed. Plant Dis. 72, 644-667.
7. *Mohan, S.B.* 1989. Cross-reactivity of antiserum raised against *Phytophthora faragariae* with other *Phytophthora* species and its evaluation as a genus-detecting antiserum. Plant Path. 38, 352-363.
8. *Gendloff, E.H.; Ramsdell, D.C. & Burton, C.L.* 1983. Fluorescent antibody studies with *Eutypa armeniacae.* Phytopathology 73, 760-764.
9. *Gerik, J.S.; Lommel, S.A. & Huisman, O.C.* 1987. A specific serological staining procedure for *Verticillium dahliae* in cotton root tissue. Phytopathology 77, 261-266.
10. *Johansen, L.H.* 1987. Adskillelse af *Pyrenophora graminea* og *P. teres* ved gel-elektroforetiske undersøgelser af opløseligt mycelieprotein. Specialeprojekt, Københavns universitet. 82pp.
11. *Harboe, N.M.G. & Ingild, A.* 1983. Immunization, isolation of immunoglobulins and antibody titre determination. p.345-351 in: Handbook of immunoprecipitation-in-gel techniques. Blackwell Scientific Publications. Scand. J. Immunol. vol.17, Supplement 10. ed. by *N.H. Axelsen.*
12. *Hjelm, H.; Hjelm, K. & Sjoquist, J.* 1972. Protein A from *Staphylococcus aureus.* Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobuling. FEBS lett. 28, 73-76.
13. *Laemmli, U.K.* 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
14. *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.
15. *Morrissey, H.J.* 1981. Silver stain for proteins in polyacrylamide gels: A procedure with enhanced uniform sensitivity. Anal. Biochem. 117, 307-310.
16. *Towbin H.; Stachelin, T. & Gordon, J.* 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76,4350-4354.
17. *Blake M.S.; Johnson, K.H.; Russell-Jones, G.J. & Gotchlich, E.C.* 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. 136, 175-179.
18. *Wycoff, K.L.; Jellison, J. & Ayers, A.R.* 1987. Monoclonal antibodies to glycoprotein antigens of a fungal plant pathogen, *Phytophthora megasperma* f.sp. *glycinea.* Plant Physiology 85, 508-515.
19. *Dewey, F.M.; MacDonald, M.M.; Philips, S.I. & Priestley R.A.* 1990. Development of monoclonal antibody-ELISA and -DIP-STICK immunoassays for *Penicillium islandium* in rice grains. J. Gen. Microbiol. 136, 753-760.
20. *Husted, K.* 1992. DNA probes for detection and differentiation of *Pyrenophora teres* and *Pyrenophora graminea.* In preparation

Manuscript received 2 December 1992.