A SEROLOGICAL COMPARISON OF THE UREDOSPORES OF UROMYCES PHASEOLI (PERS.) WINT. VAR. PHASEOLI AND PUCCINIA STRIIFORMIS WEST.

by

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ABSTRACT

Uredospores of *Uromyces phaseoli* (Pers.) Wint. var. *phaseoli* were compared serologically with those of *Puccinia striiformis* West. Antisera were obtained from rabbits by subcutaneous injection of soluble uredospore contents emulsified in Freund's incomplete adjuvant. The results of gel diffusion and immunoelectrophoresis tests indicate that the uredospore contents of *U. phaseoli* contain at least two distinct antigens, while those of *P. striiformis* contain three or more. Furthermore, test evidence suggests one of these antigens is common to both species. It is thus possible, based on these observations, to serologically differentiate between the uredospores of the two species of genera.
INTRODUCTION

The serological techniques used in bacterial and viral investigations are not always easily adapted to research involving fungi. For this reason, perhaps, comparatively little serological work has been done with the rust fungi. With this in mind, an attempt has been made to adapt old serological techniques, or develop new ones where appropriate, to a comparison of the serological properties of two species of rusts, *Uromyces phaseoli* (Pers.) Wint. var. *phaseoli* and *Puccinia striiformis* West.

This investigation was undertaken with the primary objectives of ascertaining the antigenicity and serological complexity of the uredospores of these rusts. If found to be sufficiently complex for serological differentiation, subsequent investigation could be undertaken to extend the scope and sensitivity of the techniques applied here, with an ultimate objective of developing a rapid and accurate method of identifying physiological races of rust fungi.

Previous serological investigations of fungi have made use of a variety of antigen sources; mycelial suspensions or extracts, spore suspensions or extracts, or combinations of the above have been used. Mahadevan (11), working in 1964 with both the mycelium and the spores of *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder and
Hansen, showed them to be antigenically similar. Ainsworth (1), in 1952, had demonstrated a similar situation with the mycelial and yeast phases of a human mycotic pathogen, *Histoplasma capsulatum* Darling 1906. In 1964 Madhosingh (10), using only mycelial antigens, serologically distinguished *Fusarium oxysporum* Schlecht., *F. moniliforme* Sheldon, and *F. solani* (Mart.) Appel and Wr., while in 1966 Morton and Dukes (12), also working only with mycelium, were able to differentiate between race 1 and race 2 of *F. oxysporum f. lycopersici*. Burrell et al. (3), finding filamentous fungi generally to be poor antigens, showed via immunofluorescence that the antigens of *Phytophthora* spp. mycelia were localized in the hyphal tips.

In 1961 Buxton, Culbreth, and Esposito, using suspensions of disintegrated spores as antigens, found that forms and races of *Fusarium oxysporum* could be distinguished (4). Gooding and Powers (8), working with aeciospores of *Cronartium fusiforme* Hedge and Hunt ex Cumm, *C. quercuum* (Berk.) Miy. ex Shirai, and *C. ribicola* J.C. Fisher ex Rabh. found that specific antisera could be produced to each. Spores of several *Ceratocystis* spp. were differentiated serologically by Amos and Burrell (2) using agglutination, gel diffusion, and immunofluorescence.

This paper reports the results of a comparison of *Puccinia striiformis* West. and *Uromyces phaseoli* (Pers.)
Wint. var. *phaseoli* in which soluble uredospore contents obtained from disintegrated spores were used as the antigen source.
MATERIALS AND METHODS

Preparation of Antigen

The uredospores of U. phaseoli var. phaseoli, race 33 were provided by Dr. E. K. Vaughan, and uredospores of P. striiformis by Dr. Robert L. Powelson, both of the Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon. Hereafter, Uromyces phaseoli (Pers.) Wint. var. phaseoli race 33 will be referred to as Up and Puccinia striiformis West. as Ps.

Two and a half grams of uredospores of each species were separately disintegrated in a Mickle Cell Disintegrator (Mickle Laboratory Engineering Company, Gomshall, Surry, England) in cold phosphate buffer (pH 6.8, 0.1 M). The spores were placed in vials which contained 100 µ diameter glass beads to approx 1/3 the volume of the vial; several runs were required since the vials, due to their small size (approximately 10 ml capacity), could not hold all the spores at one time. The vials were placed in the vibrators and shaken at maximum vibration for 15 min, then removed and cooled in an ice bath for five min before shaking was resumed. This procedure was carried out three times. Therefore the spores were subjected to the disintegrator for a total of 45 min with two five-min cooling breaks at 15 min intervals. Breakage of spores
achieved was approximately 95%. The resulting homogenate
was removed from the vials and the glass beads rinsed with
phosphate buffer. The combined homogenate and rinses were
brought to .85% NaCl and .005M MgSO₄, then centrifuged at
3000 rpm for 10 min to precipitate the wall fragments and
other particulate matter; the pellet was discarded.

The supernatant, containing the soluble uredospore
contents, was dialyzed against polyethylene glycol (carbo-
wax) to increase the concentration and decrease the volume
of solution. Protein measurements were made by the biuret
method (5) and solutions containing approximately 10 mg/ml
of Ps and Up proteins were prepared.

Preparation of Antiserum

Normal serum was obtained from blood taken from the
ears of three female albino rabbits weighing five to eight
pounds each. The ears were cleaned with 95% alcohol and
rubbed with xylene to distend the central ear vein. Ten
cubic centimeter syringes fitted with 1 in., 20 gauge
needles were used to collect the blood. The needle was
inserted, bevel up, 1/8 to 1/4 in. into the vein. The
blood pressure forced the plunger back, filling the
syringe. Blood collected in this manner was allowed to
stand at room temperature for 30 min, then was placed in a
refrigerator for 30 min more. The serum was then drawn
off the clot and centrifuged at 3000 rpm for 10 min to
precipitate the red blood cells. The clear serum was drawn off, placed in 5 ml serum bottles, and stored by freezing. Blood collected later for antisera was obtained by non-terminal cardiac puncture but processed in the same manner as the normal sera.

A solution of each antigen containing 100 mg protein was emulsified in an equal amount of Freund's incomplete adjuvant. Emulsification was accomplished by placing the adjuvant into the 50 ml cup of an omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) and periodically adding some of the protein solution until it was all thoroughly mixed. The emulsifying procedure was continued until a drop of the emulsion in a beaker of ice cold water showed no tendency to spread. The emulsions were stored in the refrigerator until injection approximately three hr later.

Inoculation of the rabbits was accomplished by injecting a large number of small doses of the antigen subcutaneously. The shots were distributed over the shaven backs of the animals in doses of 0.2-0.5 ml until all the antigen was injected. The total volume of the Up antigen and adjuvant emulsion injected was 24.5 ml, while that of the Ps emulsion was 22.5 ml. A three-week waiting period followed, during which the inoculation sites became swollen, red, and angry in appearance. At the end of the waiting period, test bleedings were accomplished weekly for
three weeks by cardiac puncture. Twenty milliliter vacutainer suction tubes with adapter and 20 gauge needle were utilized in collecting the blood for these tests. The blood obtained was treated in the manner previously described. Titres were determined by tube precipitin test (5, 6) and are shown in Table 1. Six weeks following inoculations, test animals were starved for 12 hr, given 1 ml of Demurol intravenously in marginal ear veins, to render them unconscious, and then exanguinated. The blood was allowed to clot at room temperature for approximately one hr and was then refrigerated at 4 C. Antisera were removed periodically from the collection flasks for three days following bleeding to obtain maximum yields. Antisera were centrifuged at 2000 rpm for 10 min, after which the supernatent was removed, placed in serum bottles, and frozen.

Table 1. Titres obtained from test and final bleedings.

<table>
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<th>Antiserum to</th>
<th>Test bleeding number:</th>
<th>Final bleeding</th>
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<tr>
<td></td>
<td>1 (23)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 (30)</td>
</tr>
<tr>
<td>Uromyces phaseoli</td>
<td>640</td>
<td>1280</td>
</tr>
<tr>
<td>Puccinia striiformis</td>
<td>1280</td>
<td>1280</td>
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<sup>a</sup>Days after inoculation.
Cross-absorption of Antisera

To prepare monospecific antisera to each of the species, antiserum from each species was challenged with antigen of the other species; small amounts of antigen were added until no more precipitate was formed and no cross-reactions occurred in Ouchterlony plates or immunoelectrophoresis slides (6).

A total of 1.65 mg of Up antigen (0.6 ml of 1 mg/ml solution and 0.15 ml of 7 mg/ml solution) was added to 5 ml of Ps antiserum, after which there was no more cross-reactivity. To 3 ml of Up antiserum 4.0 mg of Ps antigen (0.5 ml of 8 mg/ml solution) was added; cross-reaction was then removed.

After the addition of the antigen, the reaction was allowed to proceed for one to two hr at room temperature, refrigerated overnight at 4 C, and centrifuged the next day at 2000 rpm for five min. Supernatents were retained while precipitates were discarded.

Test Methods

Comparison of the antigens involved was accomplished by the Ouchterlony double diffusion (13) and immunoelectrophoresis techniques (7, 9, 14). These techniques were also utilized after cross-precipitation of antisera with heterologous antigen was accomplished.
Into 60 x 15 mm disposable Ouchterlony gel diffusion plates 7 ml of agar was poured. The agar consisted of 0.5% Ionagar-2 in distilled water with 1 ml of 1% sodium azide added per 100 ml to inhibit growth of contaminants. Plates were stored under refrigeration at 4°C until use. Wells were punched in the agar with sizes number 2 and 3 cork borers; agar was removed by a pipette attached to an aspirator hose. The distance between antigen and antibody wells was 7 mm in all cases. Antisera were used at full strength while antigens were used at 1 mg/ml; this ratio was indicated by optimal proportion tests in gel diffusion plates and by the titration tests. Wells were filled once, the plates stored in humidity chambers at room temperature, and observed over the course of one week, after which final readings were made. The gelatin plates were then photographed.

Immunoelectrophoresis was accomplished using barbital buffer, pH 8.6. The gel consisted of Ionagar-2 at 0.75% in buffer and distilled water; electrophoresis lasted one hr at 250 volts and approximately 18 milliamps over two trays of six slides each. Wells on the slides were enlarged from the standard 1 mm diameter to 2.5 mm to accommodate a greater quantity of antigen solution. In the immunoelectrophoresis trials, antigen concentrations of 7 mg/ml for Up and 8 mg/ml for Ps were used to offset the dilution achieved through electrophoretic movement and
immunodiffusion. After the hour-long electrophoresis run, the agar was removed from the troughs on the slides and appropriate antisera were placed in these locations. The trays of slides were then placed in a humid chamber at room temperature for 20 hr, after which the precipitin bands formed were observed and recorded. These slides were then stained and photographed, though in some cases the weaker precipitin bands did not survive the staining procedure.

Each antigen was tested against both its homologous antiserum and against the heterologous antiserum. The same comparisons were made using both Ouchterlony and immunoelectrophoresis techniques, Fig. 1-4.
RESULTS AND DISCUSSION

Among the first tests conducted were tube titration and Ouchterlony gel diffusion experiments in which each antigen was challenged with normal serum and buffered saline control solutions. In no case was any reactivity found.

The results of the Ouchterlony gel diffusion tests showed that $U_p$ produced two precipitin bands in homologous reactions while $P_s$ produced three always, but on at least two occasions appeared to show four. Since, however, four were only occasionally found on immunoelectrophoresis slides, it will be assumed that there are only three major antigens present.

The line drawings, Fig. 1a-i, illustrate typical Ouchterlony gel diffusion results; Fig. 2a-i consists of photographs of these plates.

The reaction shown in Fig. 1a illustrates the typical $U_p$ homologous reaction; one fairly heavy precipitin band near the antiserum well and another, but much more diffuse band, nearer the antigen well. This arrangement is also shown in the 7-well plate pictured in Fig. 1c. A cross-reaction with $P_s$ antigen, though comparatively weak, is observed in Fig. 1a.
Figure 1. Results of Ouchterlony diffusion plates.

Up = *Uromyces phaseoli* (Pers.) Wint. var. *phaseoli* race 33
Ps = *Puccinia striiformis* West.
AS = antiserum
Mx = mixed antiserum
M = monospecific antiserum
Figure 2. Photographs of Ouchterlony diffusion plates.

Up = Uromyces phaseoli (Pers.) Wint. var. phaseoli race 33
Ps = Puccinia striiformis West.
AS = antiserum
Mx = mixed antiserum
M = monospecific antiserum
When Ps antiserum is used instead of Up antiserum, three to four bands are produced in the homologous reaction, though two or three are quite close together (Fig. 1b). In the 7-well homologous reaction (Fig. 1d) only three bands are found, but are quite distinct. However, the heaviest band is not nearest the antiserum well, as in Fig. 1b, but is now nearest the antigen well. As with the Up antiserum, one band is formed near the Ps antiserum well, and it is the presence of this band which indicates cross-reactivity with the other antigen.

When mixed antiserum is placed in the center well of a 7-well plate and challenged with both antigens in alternating wells, fairly predictable results are obtained, as illustrated in Fig. 1e. Ps produces two to three bands, that closest to the antiserum well joining with the heaviest Up band in a reaction of identity. Up produced one other wide, very diffuse band close to the antigen well.

Figures 1f through 1i depict reactions similar in format to those previously described, but in which cross-absorbed antisera were used; antigens from each species were precipitated in the antiserum of the other, producing monospecific antisera.

As shown in Fig. 1f and 1g, in both reactions the cross-precipitation procedure eliminated the heavy band nearest the antiserum wells, as found in Fig. 1a and 1b. Figures 1h and 1i illustrate typical homologous reactions.
in 7-well systems where monospecific Up and Ps antisera were also used. The Up monospecific antiserum produced only a diffuse band close to the antigen wells; absent was the denser line, pictured in Fig. 1a, near the antiserum well. The Ps monospecific antiserum produced precipitin-bands very similar in appearance and number to those produced by the complete antiserum; three bands were consistently formed. The possibility that this may be due to incomplete cross-absorption can be ruled out by the fact that no cross-reaction was found in tests where the monospecific antiserum was challenged by both antigens (Fig. 1g). This event also lends credibility to the contention, stated earlier, that Ps uredospore contents may well contain four or more antigenic components.

The results of the immunoelectrophoresis tests, for the most part, verified those of the Ouchterlony plates. The line drawings (Fig. 3a-i) depict the typical results obtained. The photographs (Fig. 4a-i) were taken of some representative slides; it must be noted, however, that not all precipitin bands mentioned in the text may be found in the photographs, as some did not survive the drying, staining, rinsing, and redrying steps prior to photographing.

In homologous reactions, Up and Ps antigens challenged with their complete antisera showed much the same results as in the Ouchterlony plates. Up normally
Figure 3. Results of immunoelectrophoresis.

Up = Uromyces phaseoli (Pers.) Wint. var. phaseoli race 33
Ps = Puccinia striiformis West.
AS = antiserum
Mx = mixed antiserum
M = monospecific antiserum
Figure 4. Photographs of immunoelectrophoresis slides.

Up = Uromyces phaseoli (Pers.) Wint. var. phaseoli race 33
Ps = Puccinia striiformis West.
AS = antiserum
Mx = mixed antiserum
M = monospecific antiserum
Figure 4—Continued
produced one fairly heavy precipitin band and occasionally a second, but much fainter, band as shown in Fig. 3e. The Ps always produced at least three bands and on four trials out of ten produced four (Fig. 3d).

Figure 3a illustrates the usual result when, after electrophoresis, Up and Ps antigens were challenged with Up antiserum. The Up reaction, as in the typical homologous reaction, produced one heavy band and sometimes a faint second band. The Ps, however, involved here in a heterologous reaction, did not usually produce just one band, as in the Ouchterlony plate cross-reactions, but most often produced two. The reason for this is not clear; it may be that the one cross-reaction band in the Ouchterlony plates, Fig. 1a, is due to two common antigens, with similar diffusion rates but different electrophoretic mobilities. This would explain the occurrence of one band in the gel diffusion plates and two in the immunoelectrophoresis slides.

When both antigens are compared on the same slide against mixed antisera, Fig. 3b, the results are essentially those of the homologous reactions depicted in Fig. 3d and 3e.

If both antigens are again compared on the same slide, but this time challenged with Ps antiserum, bands appear as shown in Fig. 3c. The Ps antigen, involved here in the homologous reaction, typically produces three bands,
though occasionally four are seen. The $Up$ usually produced only one band here, as it is engaged in a heterologous reaction; on two trials in ten, however, it did show an additional, but weak band.

Monospecific antisera were also used to challenge antigens which had been subjected to electrophoresis. In homologous reactions where monospecific antisera were used (Fig. 3h and 3i), $Up$ showed only one relatively weak band, while $Ps$ usually produced three distinct bands. Since $Ps$ also normally produced three bands in reactions with the complete antiserum, it appears there may be another antigen present, very similar, but not identical, to the common antigen already demonstrated. In the reactions in which both antigens were tested against each monospecific antiserum, Fig. 3f and 3g, $Up$ produced the expected one precipitin band while $Ps$ produced the expected three bands. Cross-reactions were lacking, as they should be when antigens are tested against the specific antiserum for a different antigen.

When this investigation was begun, it was planned that another race of *Uromyces phaseoli* var. *phaseoli* would be used in addition to race 33 in an effort to determine whether antigenic differences existed at that level. The other race utilized was race 34; however, perhaps due to a lesser inoculation dose, no antibody response was produced in rabbits. Because of shortage of material, only 40 mg of
race 34 proteins were injected whereas 100 mg of _U. phaseoli_ var. _phaseoli_ race 33 and _P. striiformis_ proteins were used. Tube titrations initially indicated the presence of at least a small amount of antibody, but since no reactions occurred in Ouchterlony plates or immunoelectrophoresis, the comparison of _U. phaseoli_ uredospores at the race level was discontinued. It does not seem logical, however, that one race would be antigenic and another not at all.

In this investigation, a number of problems were encountered which are worthy of brief discussion. One of the first obstacles found was determining the form in which the antigen was to be used. Whole spores, though probably capable of eventually producing antisera, were difficult to work with for at least three reasons.

1. Since the uredospores are quite large (approx. 20-25 µ in diameter) intravenous injection into the rabbit in any quantity is difficult, as they tend to block the veins, preventing further injections; a number of subcutaneous or intramuscular injections would be necessary instead.

2. They do not go readily into suspension; unless a surfactant such as Tween 80 or Triton is added, large numbers of spores will float and adhere to the sides of containers. On addition of the
surfactant, however, the spores then tend to settle out of suspension.

3. Whether or not a surfactant is used, the problem arises as to how antiserum against the spores can be tested for the presence of antibody. The spores will settle out in tubes, whether or not antibody is present, making tube agglutination tests highly subjective, and the spores are far too large to diffuse through agar, thus ruling out the gel diffusion and immunoelectrophoresis tests.

It was for these reasons that initial work with whole spores and fragmented spore walls was abandoned in favor of the more easily handled soluble uredospore contents.

Once it was decided to use only the soluble spore contents, it was necessary to find a rapid and efficient method of fractionating the spores so that the soluble portion would be released into the buffer solution. Since the spores have a tough, thick wall, this was no easy problem. Alternate freezing and thawing appeared slow and of questionable efficiency, while sonification for long periods at high frequency did not damage them. The Mickle cell disintegrator, often used for breaking up yeasts and bacteria, provided both the rapidity and efficiency desired, with simplicity of operation.

In an attempt to purify and concentrate the soluble proteins collected from the spores, the ammonium sulfate
protein precipitation technique commonly used by plant virologists was tried; however, high concentrations of the salt were required to obtain a precipitate and it did not appear to precipitate all protein. Eventually this method of protein concentration was by-passed in favor of dialysis against polyethylene glycol (carbowax) as mentioned earlier.

Another problem was that of choosing a solvent or suspending solution, particularly when the soluble proteins were being released from the uredospores. Considerable variation in this respect is found in the relatively small amount of literature pertinent to fungus serology; perhaps the reason for this can be summed up by stating that Gooding and Powers (8) tested five different solvents and found them all equally effective. The solvent used in this work (0.1 M phosphate buffer, pH 6.8, 0.85% saline, and 0.005 M MgSO\(_4\)) was similar to those tested by Gooding and Powers.

Once the difficulties in preparation of the antigen had been overcome and antiserum had been produced, serological tests were needed which were sensitive and accurate enough to detect any similarities and differences which the two antigens might possess. The Ouchterlony double diffusion technique was chosen because of its simplicity and accuracy; in addition to producing readily observable and reproducible reactions, it separates the
antigens present on the basis of their diffusibility through agar. The other technique used was the immunoelectrophoresis technique, chosen because it measures electrophoretic mobility as well as diffusibility in agar. The combination of the two tests adequately support antigenic comparisons of the two rusts studied.

In the preparation of the monospecific antisera, disproportionate quantities of antigens were required to completely remove common antibodies from heterologous antisera. Preparation of monospecific \textit{Up} antiserum required approximately 1.3 mg of \textit{Ps} antigen per ml of \textit{Up} antiserum, while preparation of monospecific \textit{Ps} antiserum called for approximately 0.33 mg of \textit{Up} antigen per ml of \textit{Ps} antiserum. From these observations it can be assumed that \textit{Up} contains several times the quantity of common antigen as \textit{Ps} even though \textit{Ps} generally appeared to be the better antigen.

The previous discussion has indicated that several difficulties exist which are peculiar to serological studies of fungi. In spite of this, it is firmly believed that serological techniques can profitably be applied to such investigations as clarification of taxonomic relationships of the rust fungi which are presently based on fungus morphology and host range. Serological characterization of races of \textit{U. phaseoli} might well uncover certain similarities, differences, or interrelationships heretofore
unknown, leading to the combining or reclassifying of some races. This relatively untouched area of study appears to have a very promising future.
CONCLUSIONS

The experimental results described in this paper support the conclusions that *Up* uredospores contain at least two soluble antigens, that *Ps* uredospores contain at least three and probably more, and that each has one antigen in common. Even though further investigation and more refined techniques may uncover additional specific or common antigens, the results of this research are believed to provide conclusive evidence of the feasibility of serological methods for differentiation of genera of rust fungi.


