Serological Detection of *Plasmodiophora brassicae* by Dot Immunobinding and Visualization of the Serological Reaction by Scanning Electron Microscopy

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ABSTRACT

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An antiserum was made against *Plasmodiophora brassicae*, the causal agent of club root of cabbage. A semipurified suspension of spores of *P. brassicae* was used as antigen, obtained by filtration and Percoll gradient centrifugation of infected roots. Crude root suspension samples were bound to a nitrocellulose membrane and tested by a dot immunobinding assay. The individual steps of the serological procedure were examined with a scanning electron microscope. The surface of the resting spores of *P. brassicae*, race 7, appeared smooth, while the dot immunobinding processed spores had a heavy, irregular coating, which was demonstrated to originate from incubation in the primary antiserum. Normal serum did not give rise to a coating on the resting spores. The antiserum of *P. brassicae* did not react with surface antigens of resting spores of *Polymyxa*. Further, no cross reaction with other common root pathogens such as *Pythium ultimum*, *Rhizoctonia solani*, and *Fusarium oxysporum* was observed. With antiserum prepared against spore surface antigenic determinants the dot immunobinding technique can be used as a routine test for detection of infection of *P. brassicae* in host plants and in bait plants (used as indicators of soil infestation). The sensitivity obtained was within the range permissible for a routine test.

Serological methods were first used in plant pathology for the detection of viruses and later for bacteria (17). Detection of fungal pathogens is a more recent application of these methods (2,5–7,13).

The dot immunobinding technique has been found to be a rapid and sensitive method for detection of viruses in leaves (9,14,15), in seeds (10), and for plant pathogenic bacteria (11,12).

The aim of the present study was to adapt the dot immunobinding technique, developed for plant viruses, for the detection of *Plasmodiophora brassicae* Wor., the causal agent

of cabbage club root. There is an increasing need for rapid and reliable detection of species of *Plasmodiophorales*, including *P. brassicae* but also *Polymyxa graminis, Polymyxa betae*, and *Spongospora subterraneae*, which can act as vectors for transmission of several important plant viruses (3). Detection of these fungi has been based until now on observation of host symptoms and on visual and microscopical examination of host and bait plants, the latter planted to detect the presence of these fungi in a soil sample.

MATERIALS AND METHODS

Fungal isolates. Isolates of *Plasmodiophora brassicae*, *Polymyxa betae,* and *Polymyxa graminis* were obtained from the Danish Research Service for Plant and Soil Science, DK-2800 Lyngby, Denmark. Mature roots of infected white cabbage plants (*Brassica oleraceae* var. *capitata*) and infested soil samples were collected from the locality Studsgård, Jutland, Denmark. The isolates of *Pythium ultimum, Fusarium oxysporum,* and *Rhizoctonia solani* were obtained from Novo BioKontrol, Department of Microbiological Screening, DK-2880 Bagsværd, Denmark.

Antigen preparation. Mature roots of white cabbage (*Brassica oleraceae* var. *capitata*) that were heavily infected by *P. brassicae* were homogenized in a blender in 0.05 M sodium phosphate buffer, 0.14 M sodium chloride, pH 7.6 (1 ml of buffer per gram of tissue). The homogenate was subjected to consecutive filtrations through cheesecloth and 8, 15, and 35 μm mesh nylon filters. The material was washed several times by pouring the suspension onto a Millipore filter, 3 μm pore diameter, which retained the fungal resting spores and allowed host proteins, small cell wall debris, and bacteria to pass through the filter. The material left on the filter was resuspended in a small volume and centrifugated in a continuous 38-ml Percoll density gradient according to company guidelines (Pharmacia) with a mean density of 1.07 g/ml containing 0.14 M NaCl. Each gradient was fractionated after centrifugation into 35 samples (of 1 ml each), which were examined in the light microscope to identify fractions containing the resting spores of *P. brassicae*. Two bands were consistently found: a lower band where the spores were aggregated in cystosori and an upper band of individual resting spores. The upper band with individual resting spores was selected for use as antigen in the preparation of antiserum. Fractions that contained antigen were frozen until needed.

Immunization and preparation of antisera. Two 3-mo-old New Zealand white female rabbits were immunized. Immunization was carried out according to Harboe and Ingold (8) using a mixture of 200 μl of Percoll containing approximately 1.3×10^4 spores of *P. brassicae* and 200 μl of Freund's incomplete adjuvant. Blood was collected from the marginal ear vein and serum was prepared as described by Cooper (4). To the serum was added 0.02% (w/v) sodium azide, and it was kept at 4 C.

Preparation of test samples. Two preparations were used as test samples, one made from club-root symptom bearing white cabbage roots and one made from small plantlets of Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.) that had been infected by *P. brassicae* by growing them as bait plants in infested soil samples. In both cases the material was homogenized in a mortar with a pestle, after an equal volume of phosphate-buffered saline was added. The homogenate was either used directly or filtered through a 35-μm nylon filter and frozen.

Dot immunobinding procedure. The procedure used is a modification of the method described by Lange and Heide (10). Five-microliter samples were applied to nitrocellulose membranes (HWAP 00010, Millipore Corp.), and the membrane was allowed to dry at room temperature. No special fixing of the protein onto the membrane is needed. The sheets were then incubated in a blocking solution consisting of 10%, w/v, skim milk powder in 0.05 M Tris-HCl, 0.5 M NaCl, 0.5% (v/v) Tween 20 buffer (pH 10.3) for 30 min at room temperature. Antiserum (unfractionated) was then added directly to the blocking solution, giving a final concentration of antiserum of 1:1,000. The membranes were incubated for either 3 hr at 37 C or overnight at 4 C. After incubation, the solution was drained, and the sheets were washed gently with running tap water for 3 min.

The membranes were then incubated in alkaline phosphatase-conjugated swine anti-rabbit gamma globulins (Dakopatt, Denmark; diluted 1:2,000 in Tris saline Tween buffer as above containing 5%, w/v, skim milk powder). Conditions for incubation and subsequent washing were as described for the primary antiserum. Staining was done by the addition of a nitro-blue tetrazolium substrate solution diluted in ethanolamine buffer according to Blake et al (1). The staining reaction was terminated after 10–15 min by washing the membranes in tap water.

Light microscopy. The dot formed on the membrane was scored by eye. Photographic reproduction of results was improved by photography through a stereoscopic microscope, illuminated from above and below.

Scanning electron microscopy. Preparations were made by mounting portions of the nitrocellulose membrane with the stained dots on double sticking tape. The specimens were coated with gold and examined in a JEOL 100 cx TEM/Scan microscope at 40 KV in the scanning mode.

RESULTS

When gradient purified resting spores of *P. brassicae* were used in the dot immunobinding reaction, a fine purple-blue ring was formed (Fig. 1A and B). In the scanning electron microscope the reaction zone was seen to contain aggregated resting spores that had settled and bound at the perimeter of the spot during drying (Fig. 2). At higher magnification it could be seen that the surface of the individual resting spores was covered by a dense irregular coating (Fig. 3). Resting spores that had not been subjected to the dot immunobinding procedure had a smooth surface (Fig. 4).

When a root suspension from a white cabbage plant infected by *P. brassicae* was analyzed, the dot immunobinding reaction was similar to that obtained with purified resting spores. Five-microliter aliquots taken directly from a crude root suspension resulted in prominent purple-blue rings on the nitrocellulose (Fig. 5A and B). In the stereoscopic microscope the cluster of the resting spores could be seen as dark colored spots. In the scanning electron microscope the reaction zone was seen to be composed of irregular

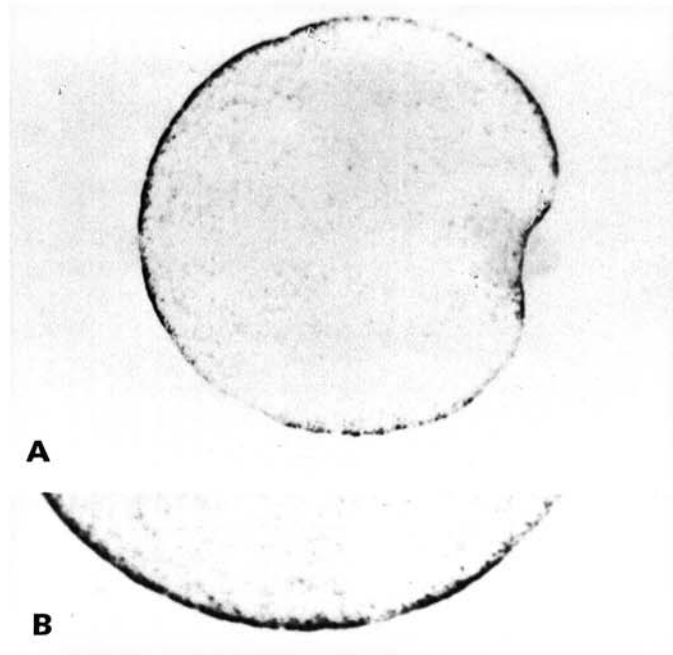


Fig. 1. Dot immunobinding reaction for a gradient purified suspension of resting spores of *Plasmodiophora brassicae*. Stereoscopic microscope photograph. A ×5; B ×10.

aggregates of resting spores, contaminated with other materials, presumed to be remnants of the disrupted roots (Fig. 6). The resting spores seen in Figure 7 (which is a closeup of the reaction shown in Fig. 6) are covered with a thick irregular coating. Scanning electron microscope of membranes spotted with a suspension from a *P. brassicae*-infected cabbage root showed that the surface of the resting spore was smooth (Fig. 8).

Scanning electron microscopical preparations were made of test material processed through the dot immunobinding procedure but interrupted at different steps. The resting spores, after incubation in the blocking solution appeared completely smooth (resembling Fig. 4). Spores that had been exposed to the blocking solution plus the primary antiserum were found to have a heavy irregular coating (resembling Fig. 3). As a control for the specificity of the reaction, normal serum was used instead of the specific antiserum prepared against spores of *P. brassicae*. In such preparations it was observed that the normal serum did not bind to the spore surface. The spores remained smooth (Fig. 9).

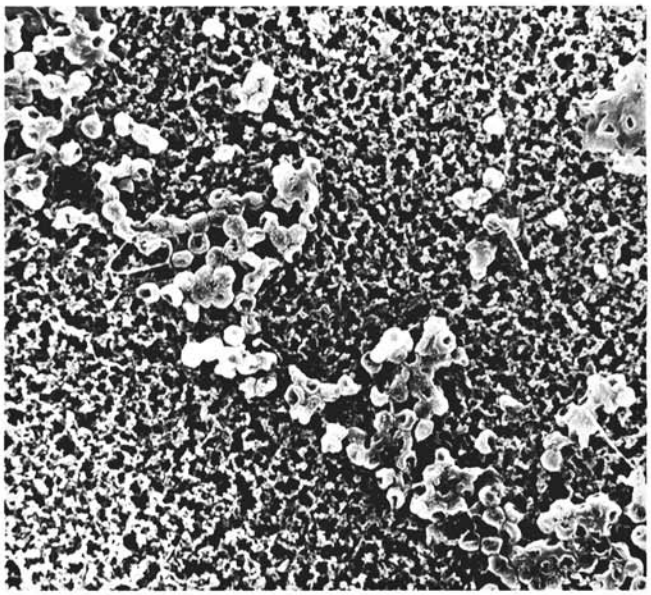


Fig. 2. Scanning electron microscopy micrograph of the periphery of a reaction zone such as the one shown in Figure 1. ×600.

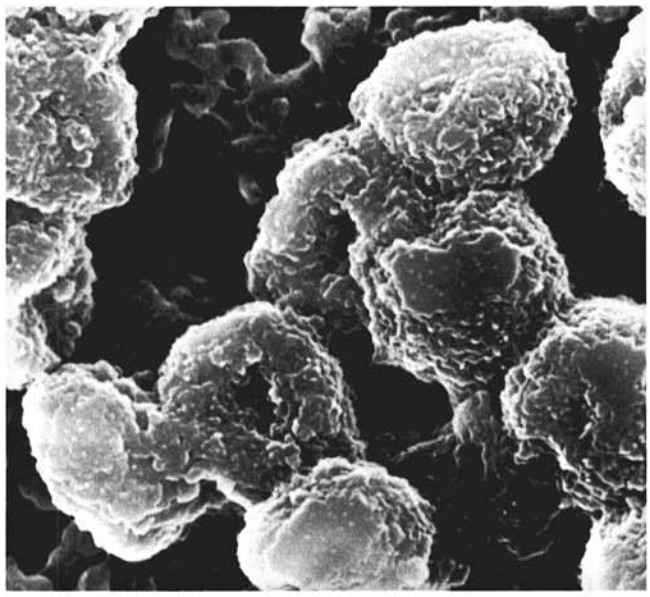


Fig. 3. Higher magnification of Figure 2. The irregular coating on the surface of the spores is composed of components of the serological procedure (primarily antibodies of the antiserum) (compare to Fig. 4). ×5,600.

The specificity of our antiserum was examined for possible cross reaction with other root-inhabiting fungi. For this purpose the dot immunobinding procedure was tested with suspensions of sugar beet roots infected with *Polymyxa betae* and with barley roots infected with *Polymyxa graminis*, both members of the *Plasmodiophorales*. Uninfected roots of Chinese cabbage and roots infected with *P. brassicae* were used as controls. The dot immunobinding procedure showed a color reaction with the samples from *Polymyxa*-infected roots at a level similar to that of the samples for our healthy Chinese cabbage. Scanning electron microscopy examination of the dot immunobinding processed *Polymyxa*-infected material showed that the resting spores of *Polymyxa* had a smooth surface. This indicates that there was

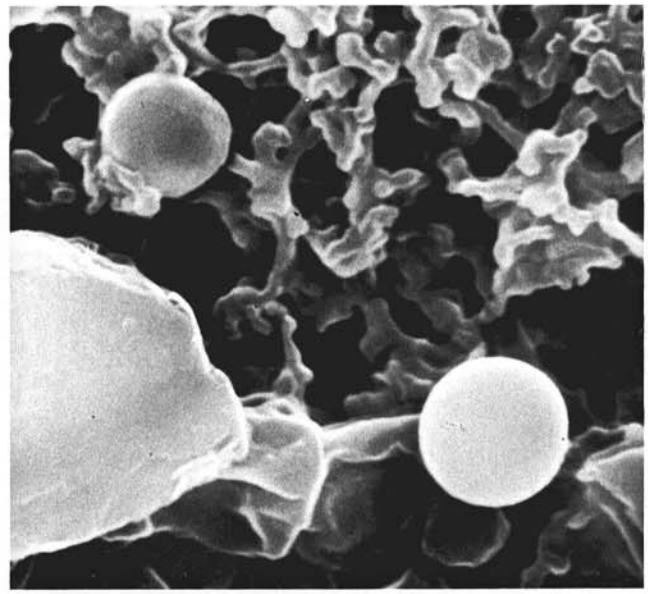


Fig. 4. Nitrocellulose loaded with a gradient purified suspension of *Plasmodiophora brassicae* resting spores. The membrane has not been processed through the serological procedure and the surface of the resting spores appears smooth as compared to the coated spores seen in Figure 3. ×4,500.

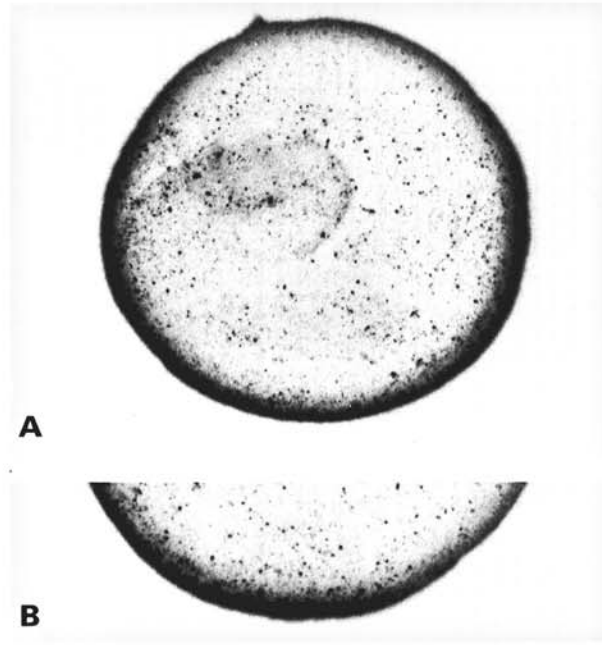


Fig. 5. Dot immunobinding reaction of a suspension made from roots of bait plant seedlings (Chinese cabbage). The positive reaction shows that the roots were infected with *Plasmodiophora brassicae*. A ×5; B ×8.

no cross reaction of our antiserum of *P. brassicae* with *Polymyxa* spores (Fig. 10A and B).

The dot immunobinding with *P. brassicae* specific antiserum was also used for detection of root-inhabiting fungi causing damping off, e.g., *Pythium ultimum, Rhizoctonia solani,* and *Fusarium oxysporum*: The antiserum of *P. brassicae* did not cross react with these fungi.

The resting spores of *P. brassicae* were also studied directly in cells of a mature club root of white cabbage (Figs. 11 and 12). From scanning electron microscopy observations it appeared that the surface of the resting spores in situ are covered with what is presumed to be host cell remnants.

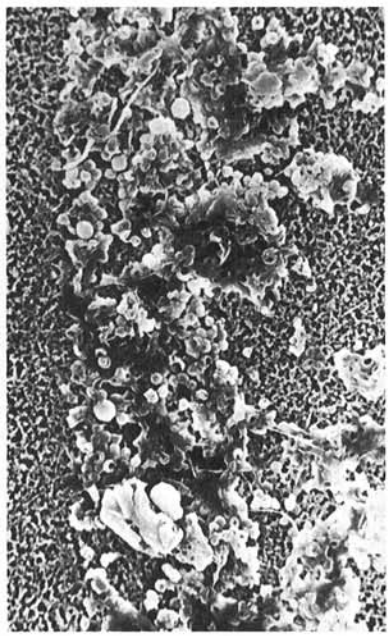
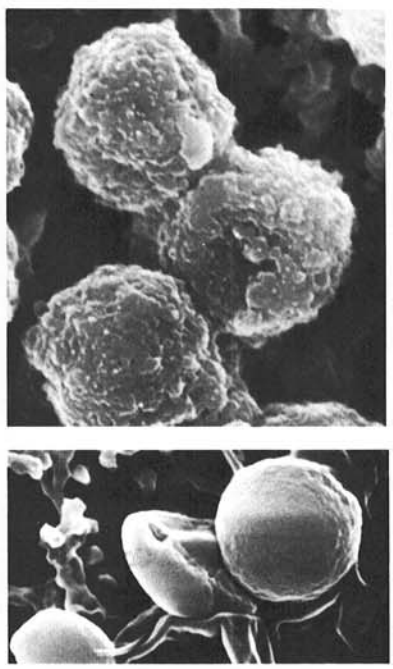


Fig. 6. Scanning electron microscopy micrograph of the periphery of the reaction zone as shown in Figure 5. ×400.



Figs. 7 and 8. 7, (top) Closeup of Figure 6. The irregular coating on the surface of the resting spores is composed of components of the dot immunobinding procedure, indicating a specific serological reaction; compare with **8,** (bottom) a nitrocellulose membrane loaded with a crude root suspension and not processed through the dot immunobinding procedure. 7, ×5,000; 8, ×4,200.

An estimate of the level of the background reaction of the antiserum against *P. brassicae* was made from the experiment shown in Figure 13. Here the membrane was loaded with a series of twofold dilutions of suspensions of infected and uninfected roots of white cabbage (young and mature plants) and Chinese cabbage, i.e., young bait plants. The positive purple-blue reaction in *P. brassicae*-infected white cabbage was still detected at a dilution of 1:256 and in Chinese cabbage at a dilution of 1:2,048.

A brownish reaction, caused by the presence of plant debris, could also be seen in the healthy material of both white and Chinese cabbage to a dilution of 1:16. The brownish color of the host material can easily be distinguished in the laboratory from the purple-blue color of the reaction, but in a black and white photograph (as in Fig. 13) the color distinction is difficult to see.

The sensitivity of the antiserum of *P. brassicae* was estimated by testing serial twofold dilutions of suspensions of infected material diluted with an extract from healthy root material. (A standard 1:16 buffer dilution was selected for all the dilutions, to eliminate most of the host background reaction.) A positive reaction could easily be scored in the preparations where infected and healthy material were mixed in a ratio of 1:64, and a slight positive reaction could be detected at a dilution of 1:128. The corresponding spore counts for these suspensions were 6.3×10^2 for the 1:64 dilution and 3.1×10^2 for the 1:128 dilution.

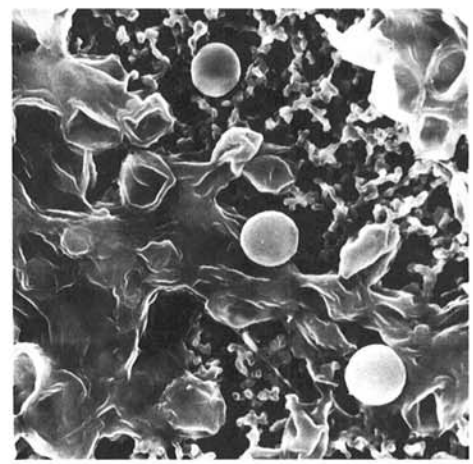


Fig. 9. Resting spores of *Plasmodiophora brassicae* processed through the dot immunobinding procedure using normal serum instead of the specific antiserum. The wall of the resting spores remained smooth. ×5,000.

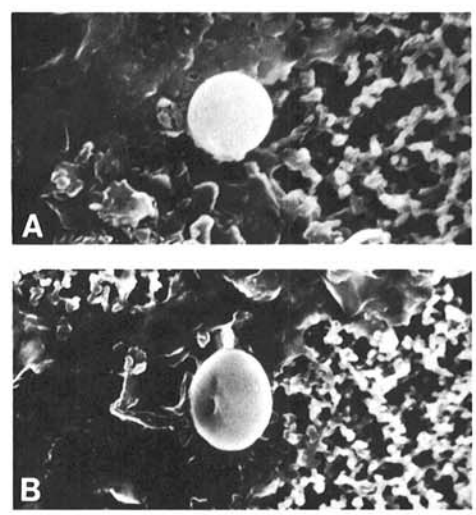
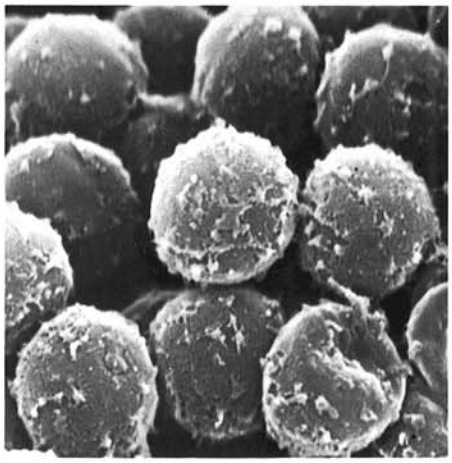


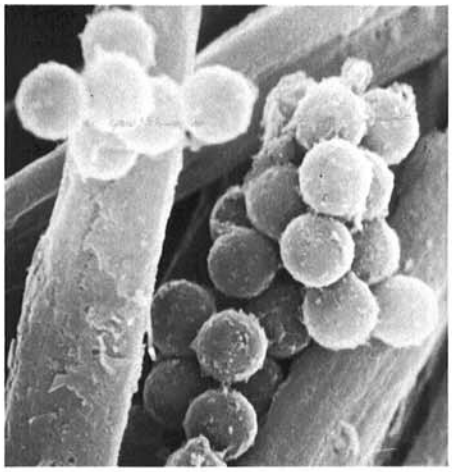
Fig. 10A and B. Resting spores of *Polymyxa betae* processed through the dot immunobinding procedure using the *Plasmodiophora brassicae* antiserum. The spore wall remained smooth also here. ×4,500; ×5,000.

DISCUSSION

Antiserum specificity obtained against fungal pathogens is reported to vary greatly (2,7,16). The antiserum against *P. brassicae* resting spores used in this study showed no cross reaction with other common root pathogens (*Pythium ultimum, Rhizoctonia solani*, and *Fusarium oxysporum*), and did not cross react with resting spores of *Polymyxa betae,* which is also a member of the *Plasmodiophoraceae.*

The specificity of the antiserum against *P. brassicae* resting spores is most probably due to using highly purified resting spores as the antigen for the production of the antiserum. Hardham et al (7) noted that the specificity of the antiserum prepared against *Phytophthora cinnamomi* was much greater when zoospores and spore cysts were used as the antigen rather than using crude mycelial preparations. The species—or at least genus—specific specificity obtained with the antiserum in the present study along with the observation of Hardham et al (7) suggest that careful antigen preparation and selection of the right stage in the life cycle most typical for the species in question are very critical for the specificity of the resulting antiserum. Scanning electron microscopical studies of the cystosori of the resting spores of *P. brassicae* in mature roots of white cabbage show that the individual restings spores in situ have a layer of host cytoplasm remnants covering the surface. This coating is apparently washed off during the dot immunobinding procedure. (Race 7 of *P. brassicae* appears to have smooth resting spore walls.) The stimulation of the antibody response in the rabbit and the specificity of the resulting antiserum may have been enhanced by removal of the host cytoplasm from the resting spores.





Figs. 11 and 12. Mature roots of white cabbage infected with *Plasmodiophora brassicae* (race 7). An uneven layer of what appears to be host cytoplasm is seen on the surface of the resting spores, here in situ, aggregated in a cystosorus. 11 (top), ×6,000; 12 (bottom), ×11,000.

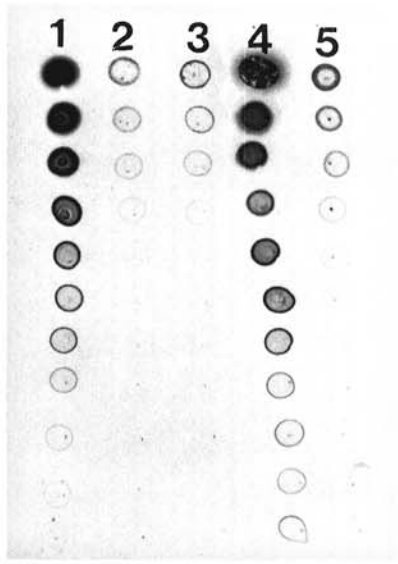


Fig. 13. Examples of the reaction of antiserum against *Plasmodiophora brassicae* as detected by the dot immunobinding procedure on nitrocellulose. Five plant materials were tested: 1) mature roots of white cabbage, infected with *Plasmodiophora brassicae*; 2) mature root of noninfected white cabbage; 3) young root of noninfected white cabbage; 4) young roots of Chinese cabbage, infected with *Plasmodiophora brassicae*; 5) young healthy root of Chinese cabbage. Each of the five samples are diluted (in buffer) in twofold dilutions to 1:2,048. A conspicuous spot (brownish in color) can be seen in columns containing healthy material to a dilution of 1:16 (columns 2, 3, and 5). The specific dot immunobinding reaction (a purple spot) can be detected for white cabbage to a dilution of 1:256 and for the Chinese cabbage seedling roots to a dilution of 1:2,048.

Scanning electron microscopy of the dot immunobinding reaction with antiserum against *P. brassicae* resting spores shows that: 1) in a positive dot immunobinding reaction the resting spores of *P. brassicae* are primarily coated by the primary antiserum, 2) normal serum does not bind to *P. brassicae* resting spores, and 3) the specific antiserum against *P. brassicae* does not bind to resting spores of *Polymyxa betae*, a relative of *P. brassicae*. The antiserum against *P. brassicae* resting spores appears to bind to antigenic sites on the surface of the resting spore.

A brown discoloration of the spots on the nitrocellulose membranes (presumed to be caused by the presence of plant debris) was observed to a dilution of 1:16. The positive purple-blue reaction was observed to a dilution of 1:256 of *P. brassicae*-infected white cabbage and to a dilution of 1:2,048 for infected Chinese cabbage seedlings. The antiserum of *P. brassicae* used here could detect the presence of *P. brassicae* resting spores when infected materials were diluted to 1:64 with uninfected root materials of white cabbage. These results suggest that the dot immunobinding technique can be used as a routine detection of *P. brassicae* infection by pooling samples from 25–50 plants in one test.

Detection of the presence of *P. brassicae* resting spores directly from a soil sample may prove to be possible by the development of a fluorescent antibody technique.

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