Detection of Cerato-Ulmin on Aggressive Isolates of *Ophiostoma ulmi* by Immunocytochemistry and Scanning Electron Microscopy

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ABSTRACT

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The surface deposition and accumulation of cerato-ulmin by the aggressive isolates of *Ophiostoma ulmi* were demonstrated using polyclonal antiserum directed against cerato-ulmin, 100–150-nm protein A-gold particles, and a scanning electron microscope. The protein A-gold complex was present on the fungal surface in areas containing the toxin cerato-ulmin (CU). The gold label was present on the surface of the vegetative hyphae, synnemata, synnematal spores, perithecia, and ostiolar hairs of the aggressive isolates of *O. ulmi*. The protein A-gold label was either evenly distributed on the fungal surface or in the form of large surface aggregates. The nonaggressive isolate Q412 of *O. ulmi* had a low concentration of protein A-gold label on its fungal structures. When the specific CU antiserum was replaced by preimmune serum, a lack of the protein A-gold label was evident on all fungal structures of the aggressive isolate VA of *O. ulmi*.

Cerato-ulmin, the extracellular toxin of *Ophiostoma ulmi* (Buism.) Nannf., induces morphological and physiological symptoms similar to Dutch elm disease (8,10,12,14). The toxin may be isolated in vitro and in vivo from aggressive isolates of *O. ulmi* (11). This toxin is present in low to negligible quantities in the nonaggressive isolates of *O. ulmi* (11).

Scanning electron microscopy has demonstrated the presence of a fibrous-cottony net or matrix on the surface of vegetative hyphae, conidia, synnemata, spores, perithecia, and ostiolar head fluid of the aggressive isolates of *O. ulmi* (10,13). Takai and Hiratsuka (11) concluded that the absence of these accumulations on the nonaggressive isolates indicated that the surface deposits on the aggressive isolates may represent cerato-ulmin (CU).

Culture filtrates from aggressive strains and water extracts from white elm (*Ulmus americana* L.) demonstrating Dutch elm disease (DED) symptoms were reacted with polyclonal antiserum against CU in a double diffusion test (14). The presence of a positive reaction indicated that the toxin CU was associated with the DED pathogen, and it was present in the culture filtrates of the aggressive isolates (11,14).

The advent of immunocytochemistry may allow the in situ identification of CU on the fungal surface. Antibodies specific for CU would bind to the CU present on the hyphal surface. The highly specific antibody-antigen reaction can be visualized by the attachment of protein A-gold probe to the antibody-antigen complex (1). We have adapted the protein A-gold procedure to enable the use of large gold particles (100–150 nm) and a scanning electron microscope (SEM).

The objective of this study was to determine if the protein A-gold technique may be used in conjunction with SEM to directly identify the matrix deposition present on the surface of the aggressive isolates of *O. ulmi*.

MATERIALS AND METHODS

Fungal cultures. The aggressive isolate VA (7,10), ascocarps from the cross VA × LB098508, and the nonaggressive isolate Q412 (7,10) were used as sources of fungal material. The source and characteristics of each isolate used in this study are provided in Table 1. Isolates VA and MH75 were established as high CU producers by the technique described by Takai (10). The VA × LB098508 mating was on elm sapwood agar (5). Isolates VA and Q412 were cultured on solid modified Zentmyer's medium (10).

Antiserum preparation. Purified CU was provided by S. Takai (Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada)

and used as immunogen in the production of polyclonal antiserum. New Zealand white rabbits (2 kg) were injected subcutaneously with a solution of 2 mg of purified CU dissolved in 2 ml of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO). At 21 days after the primary inoculation a booster shot was administered consisting of 2 mg of CU suspended in 2 ml of Freund's incomplete adjuvant (Sigma). The rabbits were sacrificed 21 days after the secondary inoculation. The serum was separated from clotted blood by centrifugation (10,000 *g*, 30 min) and stored at −70 C.

Gradient gel electrophoresis and electrophoretic blotting. One milligram of purified CU from the aggressive isolate CESS 16K (10) of *O. ulmi* was suspended in 1 ml of sample buffer containing 20% glycerol, 2% SDS (sodium dodecyl sulfate), 2% 2-mercaptoethanol, 0.062 M Tris-HCl (pH 6.8), and 0.001% bromophenol blue, as a tracking dye. Immediately before loading on the gel, the sample mixture was boiled for 1 min. Ten microliters of CU sample was loaded onto the gel. Bio-Rad Laboratories (Richmond, CA) low molecular weight markers served as references.

Polyacrylamide gradient slab gels were prepared by the method described by Jeng and Hubbes (7). The concentration of the gel ranged linearly from 8 to 15%. The electrophoresis was carried out using a Bio-Rad Protean Cell at constant 200 V, until the distance of the bromophenol blue dye was 1 cm from the bottom of the running gel.

On the completion of SDS gel electrophoresis, the slab gel was removed from the glass plates and equilibrated in transfer buffer containing 25 mM Tris (approximate pH 8.3), 192 mM glycine, and 20% methanol for 1 hr. A Bio-Rad nitrocellulose membrane was placed on top of the preequilibrated slab gel and sandwiched between filter papers and fiber pads as described by the manufacturer. Electro-transfer blotting was carried out in a Bio-Rad trans-blot cell containing transfer buffer for 3 hr at 70 V with a Bio-Rad constant voltage power supply (model 250-2.5).

Antiserum reaction with nitrocellulose. The nitrocellulose membrane was stabilized in 20 mM Tris-buffered saline (TBS), pH 7.5, for 10 min. The membrane was subsequently transferred into a blocking solution composed of 3% gelatine in TBS for 1 hr at room temperature with gentle agitation. The membrane was transferred to a solution of 0.05% Tween 20 in Tris-buffered saline (TTBS), pH 7.5, for 10 min. One milliliter of CU antiserum was added to a 100-ml solution containing 1% gelatine in TTBS buffer. The nitrocellulose membrane was immersed in this solution and agitated for 2 hr at room temperature. Following antibody incubation, the nitrocellulose membrane was washed in TTBS for 10 min. Protein A-horseradish peroxidase, 0.5 mg/ml, was diluted 1:10,000 in 1% gelatine in TTBS. The nitrocellulose membrane remained in this solution for 1 hr with gentle agitation at room temperature. Unreacted protein A-HRP was removed by two washes in TTBS buffer for 5 min each and a wash in TBS for 5 min.

Staining of the nitrocellulose membrane was carried out by dissolving 60 mg of peroxidase color development reagent (Bio-Rad) in 20 ml of ice-cold methanol. This solution was kept in darkness because of its light-sensitive nature. Before use, 60 μl of ice-cold 30% hydrogen peroxide and 20 ml of ice-cold methanol, containing the color reagent, were added to 100 ml of TBS. The nitrocellulose membrane was immersed in this staining solution. The presence of a positive reaction was evident by the appearance of a blue-purple band in the position corresponding to the original protein spot position on the SDS gel.

Gold probe preparation. The probe was prepared by the method described by De Mey (6). To 247 ml of boiling distilled water, 2.5 ml of 1% chloroauric acid (Sigma) was added. One milliliter of 1% sodium citrate was added to the boiling chloroauric acid solution. Vigorous mixing of the solution was maintained during the entire procedure, and the solution was refluxed for 30 min until a purple-brown color developed. For every 10 ml of colloidal gold mixture, 0.3 ml of 4 mg/ml of Protein A (Sigma) (dissolved in sterile distilled water) was added. The size of the particles was determined by a transmission electron microscope (Philips 201). The gold colloid particles were estimated to be 100–150 nm in size.

TABLE 1. Source and characteristics of the aggressive and nonaggressive isolates of *Ophiostoma ulmi* used

Isolate	Source	Characteristics
VA	Obtained from S. Takai, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada P6A 5M7. Isolated in United States.	Aggressive field isolate with fluffy white mycelium. Produces black protoperithecia. Rated as high cerato-ulmin producer.
MH75	Formerly known as TOR. Collected from diseased elm in Toronto, Ontario, Canada, by M. Hubbes.	Aggressive field isolate with fluffy white mycelium. Rated as high cerato-ulmin producer.
LB098508	Auxotrophic mutant obtained from isolate MH75 after treatment with MNNG.[a]	Genotype: *ade*2-1 Requirement for adenine. Produces white-beige fluffy mycelium. Rated as low cerato-ulmin producer.
Q412	Collected in Québec, Canada. ATCC 34364.	Nonaggressive field isolate with waxy white mycelium and and negligible to low cerato-ulmin production.

[a] MNNG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

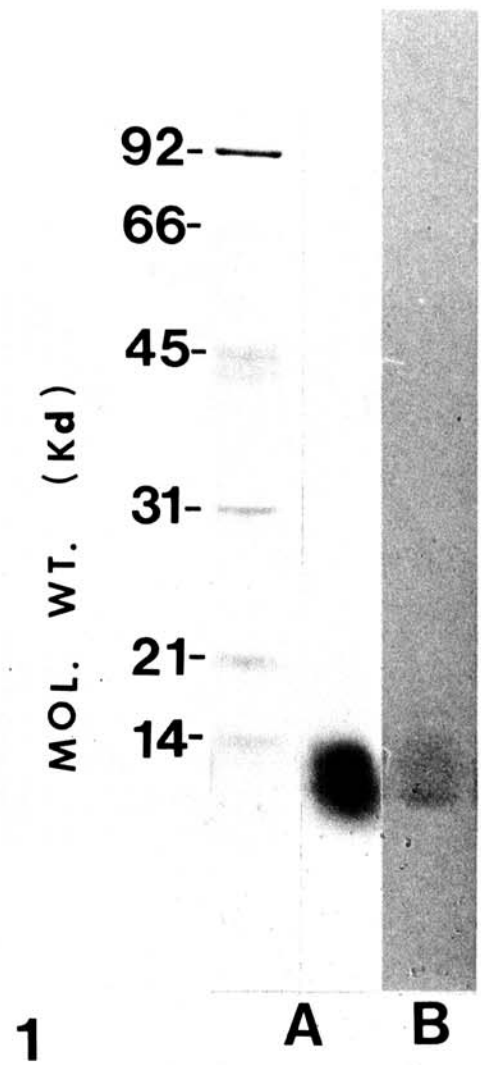
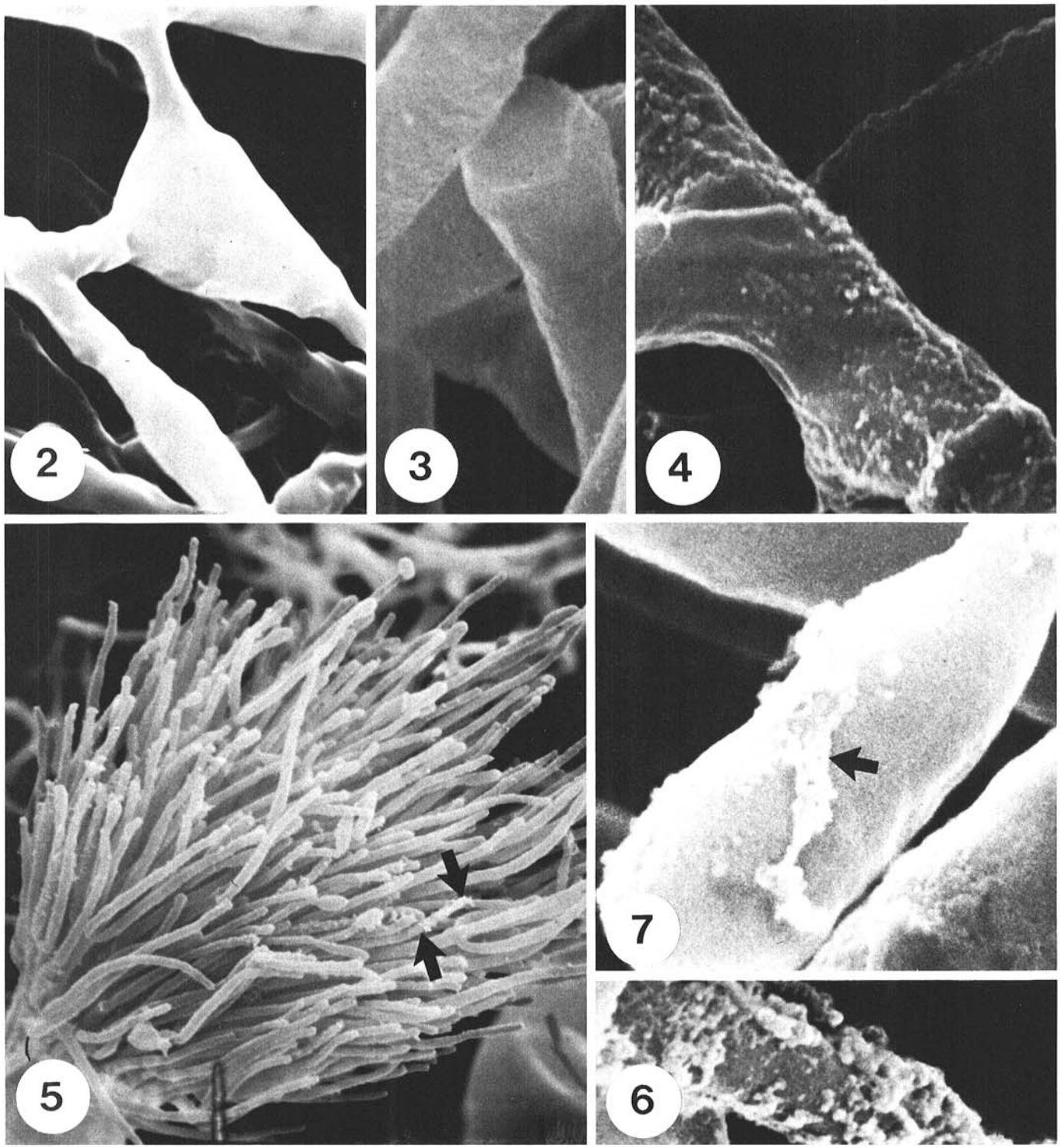


Fig. 1. A, Sodium dodecyl sulfate-gradient electrophoretic gel of purified cerato-ulmin (CU). CU appears as a single band when stained with bromophenol blue. The sample is free of contaminating proteins. **B,** Immuno-electrophoretic trans-blot of the SDS gel in A. The polyclonal antiserum contains antibodies specific to CU.

Antiserum pretreatments. Agar blocks, 0.5 × 0.5 cm, were cut from 15-day-old cultures of *O. ulmi* (isolates VA, Q412, and perithecia from VA × LB098508). Each sample was exposed to two different pretreatments. The first portion of the agar blocks was immediately exposed to the antiserum and then to the protein A-gold solution, freeze-dried overnight, and mounted on SEM specimen stubs. The second portion of each isolate sample was fixed for 2 hr in 4% glutaraldehyde in 0.2 M cacodylate buffer of pH 6.9. Excess glutaraldehyde was removed by three successive washes in distilled water, 10 min each. The blocks were immersed

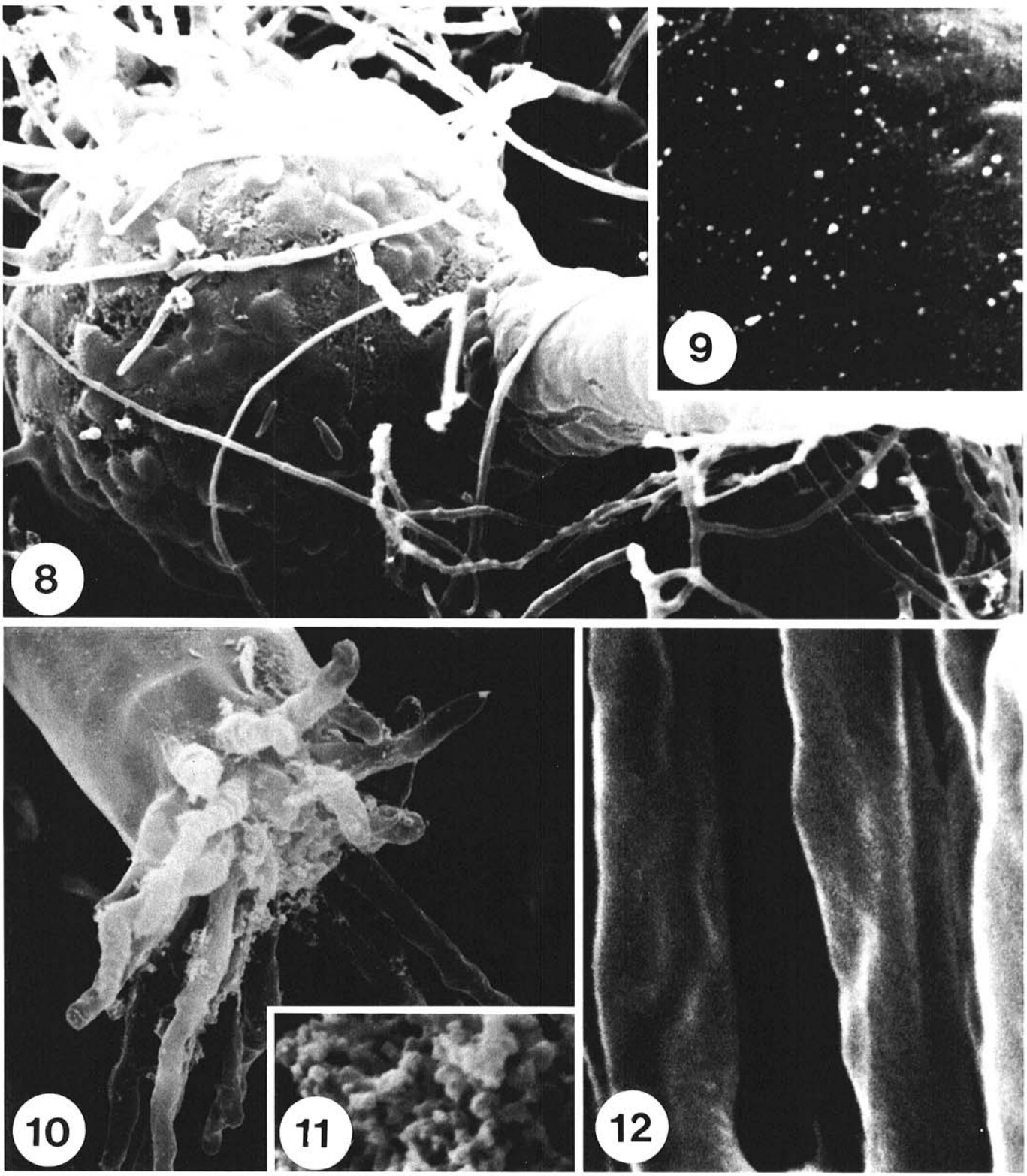


Figs. 2–7. 2, Hyphae of the aggressive isolate VA of *Ophiostoma ulmi* treated with cerato-ulmin (CU) antiserum and protein A-gold. The sample was freeze-dried immediately following antibody-gold treatment. The coating on the hyphal surface did not label. (×6,100) **3,** Hyphae of the nonaggressive isolate Q412 of *O. ulmi*, fixed in glutaraldehyde and washed in 40% ethanol before antibody treatment. The protein A-gold complex was not observed on the surface. (×24,000) **4,** Hypha of the aggressive isolate VA of *O. ulmi* treated with CU antiserum and protein A-gold. The sample was fixed in glutaraldehyde and critical-point-dried following immunological pretreatment. Gold particles cover the hyphal surface indicating the presence of CU. (×24,000) **5,** Synnema of the aggressive isolate VA of *O. ulmi* treated with CU antiserum and protein A-gold. Arrows show large accumulations of gold label. (×3,000) **6,** High magnification of the region defined by arrows in 5. Individual and aggregations of gold particles are evident on the surface. (×30,000) **7,** High magnification of a synnematal spore from isolate VA treated with CU antiserum and protein A-gold. Arrow shows the accumulation of gold label. (×44,000)

for 1–2 min in a solution of 40% ethanol.

Antiserum treatment. The ethanol-washed agar blocks were placed in a 1/20 dilution of CU antiserum for 1 hr at room temperature. Three continuous washes in distilled water removed the unbound antibodies. The washed blocks were submerged in undiluted protein A-gold solution for 1 hr at room temperature. Unbound protein A-gold was removed by three washes in distilled water.

Immunological controls. To check the specificity between the CU antibodies and the CU on the fungal surface, five controls were



Figs. 8–12. 8, Perithecium from the cross VA × LB098508 of *Ophiostoma ulmi* treated with cerato-ulmin (CU) antiserum and protein A-gold. The surface of the perithecium lacks high accumulations of gold label. (×1,600) **9,** High magnification of the perithecial surface in the neck region shows an even covering of individual protein A-gold particles. (×26,000) **10,** The ostiolar hairs situated on the tip of the perithecial neck are heavily labeled with protein A-gold particles. This indicates the presence of a high concentration of CU in this region. (×5,000) **11,** High magnification from a region of the ostiolar hair in 8, shows that the protein A-gold accumulations are composed of individual gold particles. (×35,000) **12,** Synnema of the aggressive isolate VA of *O. ulmi* treated with preimmune serum instead of CU antiserum. The hyphal surface is smooth, lacking the gold label. (×44,000)

carried out. These were: replacement of *O. ulmi* by non-CU producer *Ceratocystis fimbriata* Ell. & Halst. Omission of CU serum in the protocol, replacement of CU serum by preimmune serum, reaction of CU antiserum with pure CU before use in the procedure, and use of colloidal gold particles in place of the protein A-gold in the protocol.

Scanning electron microscopy. The antibody-gold-treated material and the respective controls were fixed in 1% unbuffered osmium tetroxide for 1 hr. Following fixation, the blocks were passed through a graded ethanol series and critical-point-dried.

For each experimental sample, one-half of the sample was coated with carbon (150Å), and the second portion was sputter-coated with palladium-gold (360Å). The specimens were examined using a JEOL JSM-35CF scanning electron microscope at 25 kV. The palladium-gold-coated specimens were scanned using the secondary electron detector. The carbon-coated material was examined with the backscatter detector to confirm the presence of gold particles on the hyphal surface.

RESULTS

Figure 1A shows the protein pattern for purified CU in the SDS-electrophoretic gel. The subsequent transfer of CU onto nitrocellulose by the trans-blot technique and reaction with CU antiserum produce a single band that corresponds to the CU spot on the SDS gel (Fig. 1B).

Unfixed fungal material was directly treated with CU antiserum and protein A-gold solution and freeze-dried. This treatment did not result in the presence of gold label on the mycelial surface (Fig. 2). The hyphae of isolates Q412 and VA were coated with a mucilaginous-like substance that did not react with the CU antiserum. All subsequent treatments included a prefixation in 4% glutaraldehyde and a 40% ethanol wash.

The nonaggressive isolate Q412 of *O. ulmi* had a low concentration of protein A-gold complex on the hyphal surface (Fig. 3). In contrast, the aggressive isolate VA showed a high accumulation of gold particles on the surface of all fungal structures (Fig. 4).

Deposits of protein A-gold particles were observed on the synnemata (Figs. 5 (arrows) and 6), synnematal spores (Fig. 7), perithecia (Figs. 8 and 9), and the ostiolar hairs (Figs. 10 and 11).

The gold label present on the fungal surface varied in appearance. The protein A-gold particles observed on the mycelial surface appeared as either individual spherical particles covering the fungal surface in an even distribution (Figs. 3, 4, and 9) or as clusters of particles (Figs. 5–7, 10, 11).

Synnemata, perithecia, and vegetative hyphae of the high CU producer VA, VA × LB098508 mating, and the non-CU toxin producer *C. fimbriata* were used in a series of five immunological controls. In each of the control experiments an absence of gold label was apparent (all control photos were not included). In Figure 12 preimmune serum replaced CU antiserum in the treatment. The protein A-gold particles were not present on the fungal surface. Treatment of the aggressive isolate VA with protein A-gold, without a prior treatment in the CU antiserum, resulted in the absence of gold label on the fungal surface (Fig. 13). In the related fungal species *C. fimbriata*, treated with CU antibodies and protein A-gold, the gold label was not present on the fungal surface (Fig. 14).

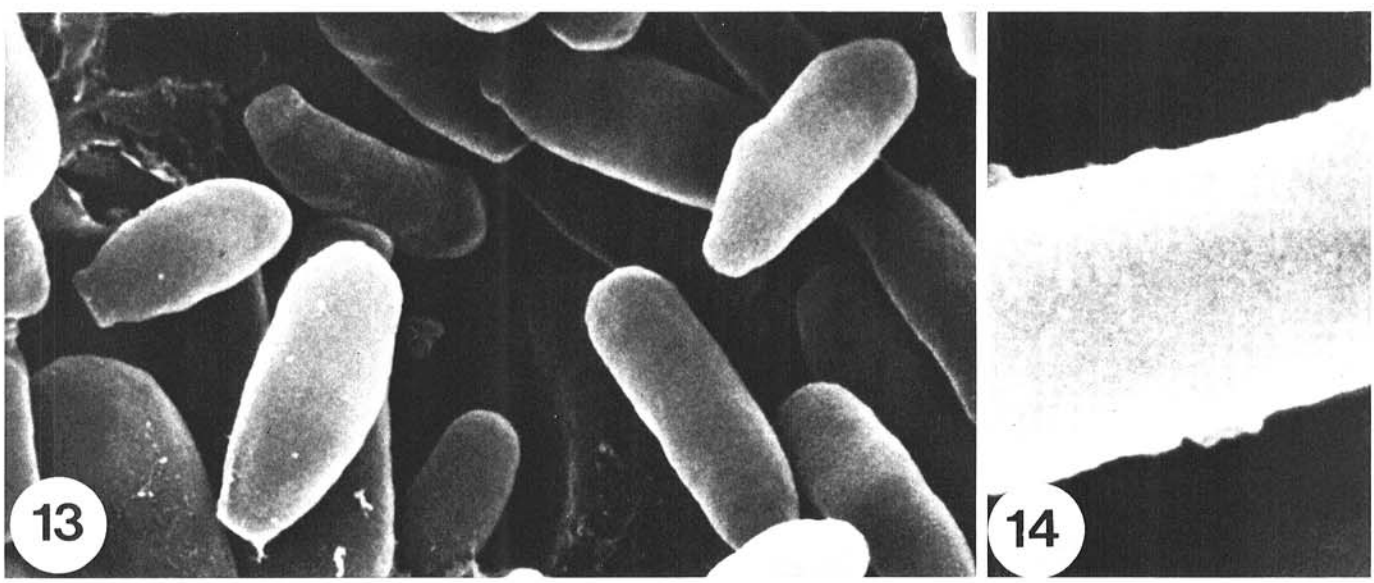
DISCUSSION

The use of large (100–150 nm) protein A-gold particles in conjunction with the SEM provides an excellent method by which to demonstrate the presence of surface proteins (antigens) on the fungal surface. Conventionally, immunocytochemistry has been combined with transmission electron microscopy to demonstrate the presence of various intra or extramycelial antigens (2,3,9). The use of the scanning electron microscope with immunocytochemistry provides an additional investigative tool in the study of surface antigens. The ease and speed of preparation of material for SEM and the examination of large surface areas cannot be ignored as an important aspect of this technique.

The combined use of gradient gel electrophoresis, electrophoretic blotting, and treatment of the nitrocellulose trans-blot membranes with specific anti-CU antiserum demonstrated the specificity of the reaction between the purified antigen and the polyclonal antibody. In this study it was necessary to show that the CU sample used in the production of the antiserum was free of contaminating proteins or any other immunogenic material that may have been retained during the purification process.

The gold label was present in very low concentration on the surface of the nonaggressive isolate Q412. In addition, large mycelial regions were observed without gold label. This supported previous studies (10), which demonstrated that the nonaggressive isolates produce negligent to low concentrations of the exotoxin CU.

The hyphae of the aggressive and nonaggressive isolates, VA and Q412, respectively, were heavily coated with a substance that did not label with CU antibodies. The washing of the hyphae with ethanol before immunological treatment exposed the CU present on the mycelial surface.



Figs. 13–14. Conidiospores of the aggressive isolate VA treated with protein A-gold without a pretreatment in cerato-ulmin (CU) antiserum. (×14,000) **14,** Hypha of *Ceratocystis fimbriata* treated with CU antiserum and protein A-gold. Gold label was not observed on the hyphal surface. (×57,200)

The SEM-protein A-gold technique detected the presence of CU on the vegetative hyphae, synnemata, synnematal spores of the aggressive isolate VA, the perithecia, and ostiolar hairs from the cross VA × LB098508. This confirmed the previous studies (11,13), which proposed the presence of CU on the fungal surface.

The fibrillar or mat-like deposits of CU observed on the surface of aggressive isolates by Takai and Hiratsuka (11) were not observed. This was attributed to the observation that CU is a water soluble protein and therefore large accumulations of CU were washed off the fungal surface in the course of antibody and protein A-gold treatment.

The controls demonstrated that the toxin on the fungal surface was labelled only when the specific CU antibodies and protein A-gold were used.

Examination of the carbon-coated material in the SEM backscatter mode was essential. The backscatter mode confirmed the presence and distribution of the electron-dense gold particles on the mycelial surface.

Little is known of the specific mechanism by which CU acts in the host tissue during DED symptom development. The ability to identify the sites of production and distribution of CU in the infected host, will serve as an important step towards our understanding of the role cerato-ulmin plays in Dutch elm disease development.

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