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A study of the development of Ophiostoma ulmi in elms with immunological techniques

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Abstract

The indirect immunofluorescence technique (IIF) and the indirect immunoperoxidase technique (IIP) were used to detect specifically *Ophiostoma ulmi* in inoculated elm branches. A strong cross-reaction was observed between the hyperimmune serum to the non-aggressive strain E2 of *O. ulmi* and the aggressive strain H6. No reactions were found with *Verticillium albo-atrum* or *Fusarium oxysporum*.

During pathogenesis *O. ulmi* was never observed outside the vessels. The hyphae of the pathogen penetrated the pit membrane during growth from vessel to vessel; no direct cell wall penetration was observed.

Introduction

Extensive histological studies have been performed on elms infected by *Ophiostoma ulmi* (Buisman) Nannf. by using the light microscope (Ouellette, 1960; Wilson, 1965; Banfield, 1968; Pomerleau, 1970), the transmission electron microscope (MacDonald and McNabb, 1970; Krause and Wilson, 1972) or the scanning electron microscope (King et al., 1974; Miller and Elgersma, 1976). It is still disputed whether the fungus remains confined to the lumina of the xylem vessels during pathogenesis or is also able to invade the surrounding parenchyma cells, ray cells or fibres. Pomerleau (1970) believed that conidia and hyphae found in parenchyma cells and fibres belonged to other fungi. On the other hand, Ouellette (1960), Wilson (1965), Krause and Wilson (1972) claim to have found *O. ulmi* in parenchyma cells. With their techniques, however, it is difficult to discriminate between hyphae and conidia of *O. ulmi* and other fungi in elm tissue. Specific immunological staining techniques were therefore considered to be more appropriate than conventional techniques. Andrews and McCrum (1969) used the fluorescent antibody technique, but nonspecific staining and strong autofluorescence of elm wood was only partially overcome. In this study an attempt was made to solve the controversy by using both the immunofluorescence and the immunoperoxidase technique.

Marerials and methods

Plant material. Four-year-old nursery-grown callus cuttings (Elgersma, 1969) of *Ulmus hollandica* cl. Belgica, susceptible to all strains of *O. ulmi*, was used.

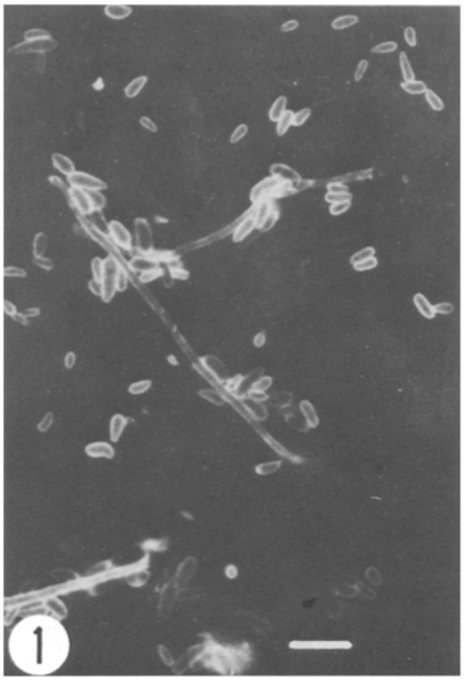
Inoculation and sampling. Inoculations with *O. ulmi* strain E2 were carried out according to Elgersma (1969). Conidial suspensions of 1.6×10^8 conidia per ml were used. At various time intervals after inoculation wood samples upto 9 cm above the site of inoculation were taken and fixed in 96% ethanol.

Antiserum preparation. Antiserum against the non-aggressive strain E2 and the aggressive strain H6 of *O. ulmi* was prepared by subcutaneous and intramuscular injection of rabbits with 300 mg of freeze-dried conidia in Freund's complete adjuvant. For each strain of *O. ulmi* one rabbit was used. These injections were repeated weekly during ten weeks. The antiserum was used in a 1:50 dilution with phosphate-buffered saline (PBS, 0.15 M NaCl containing 0.01 M phosphate, pH 7.2 unless stated otherwise).

Indirect immunofluorescence technique (IIF). Fluorescein isothiocyanate conjugated goat anti-rabbit antiserum (1:10) (Nordic, Tilburg) was used for the indirect labelling technique (Coons et al., 1941; Coons and Kaplan, 1950). Smears of conidia and mycelium of *O. ulmi* on glass slides were air-dried, fixed for at least 10 minutes in 96% ethanol, rinsed for 5 min with PBS, incubated for 30 min with rabbit antiserum, washed twice for 5 min with PBS, incubated 30 min with the conjugate, washed twice for 5 min with PBS and mounted in buffered glycerin (1 part glycerin in 9 parts PBS). Slides were examined by a Leitz Orthoplan microscope with a Ploemilluminator (Ploem, 1969). Cross-sections (15 µm thickness) were stained as described above by transferring through the solutions with a glass rod and finally mounted on glass slides.

Indirect immunoperoxidase technique (IIP). Horseradish peroxidase conjugated to goat anti-rabbit IgG (Inst. Pasteur, Paris) was used for the immunoperoxidase technique (Avrameas, 1969; Avrameas and Ternynck, 1971). Antiserum was used in a dilution of 1:50 and the conjugate in a dilution of 1:200 in PBS. Smears of *O. ulmi* on glass slides were air-dried, fixed for at least 10 min in ethanol 96%, rinsed for 5 min with PBS, incubated for 30 min with antiserum, washed twice for 5 min with PBS, incubated with the conjugate for 30 min, washed twice for 5 min with PBS, incubated for 5 min in diaminobenzidin solution (30 mg diaminobenzidin and 600 mg Tris-(hydroxymethyl) methylamine in 100 ml PBS of pH 7.6) to which 3 drops of H_2O_2 were added per 5 ml, washed twice for 5 min with 0.1% OsO_4, rinsed with

Fig. 1. Smear of *O. ulmi* with positive reaction at the indirect immunofluorescence technique (IIF). Bar represents 20 µm, also in the following figures.
Fig. 2. Smear of *O. ulmi* with positive reaction at the indirect immunoperoxidase technique (IIP).
Fig. 3. Smear of *O. ulmi* treated with normal rabbit serum at the indirect immunoperoxidase technique (IIP).
Fig. 4. Conidia of *O. ulmi* trapped underneath rim of vessel perforation, fifteen minutes after inoculation (IIF).
Fig. 1. Uitstrijkje van O. ulmi met positieve reactie bij de indirecte immunofluorescentietechniek (IIF). De vergrotingsstreep geeft 20 µm weer, ook in de volgende figuren.
Fig. 2. Uitstrijkje van O. ulmi met positieve reactie bij de indirecte immunoperoxidasetechniek (IIP).
Fig. 3. Uitstrijkje van O. ulmi behandeld met normaal serum bij de indirecte immunoperoxidasetechniek (IIP).
Fig. 4. Conidiën van O. ulmi onder de rand van een perforatieplaat, vijftien minuten na inoculatie (IIF).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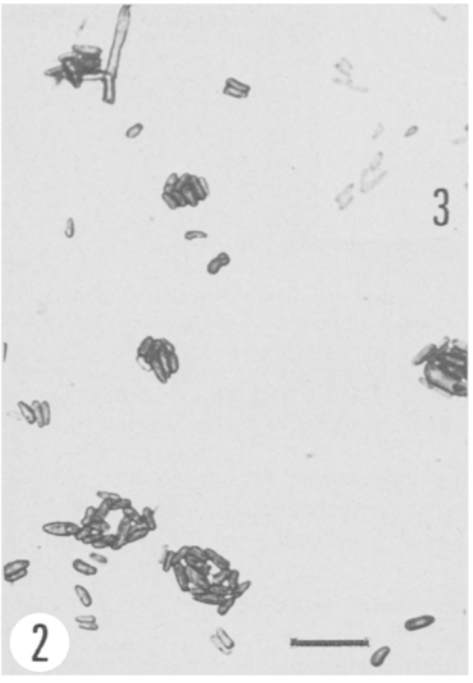
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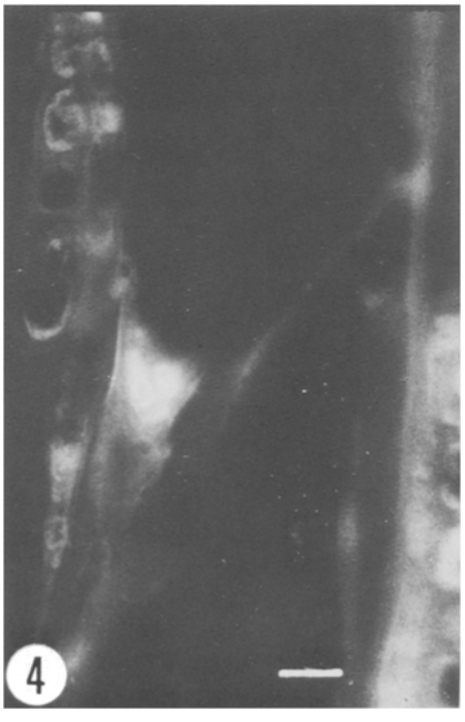
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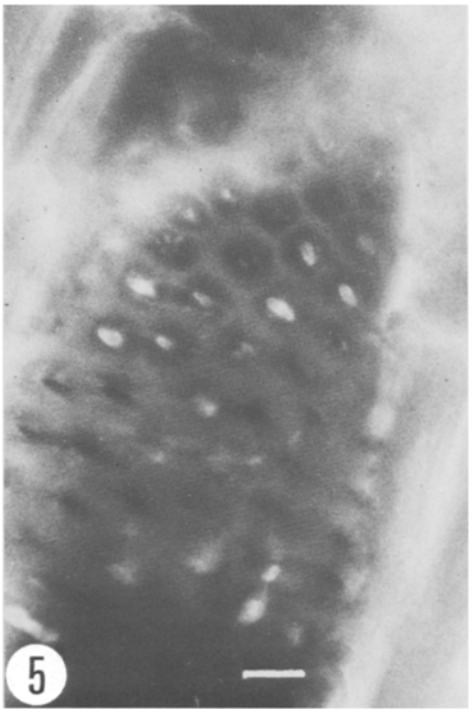
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Table 1. Specificity of the hyperimmune serum to *O. ulmi* strain E2 towards smears of *O. ulmi* strains E2 and H6, *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*, using the IIF and IIP techniques.

Antigen	IIF	IIP
O. ulmi strain E2	++	++
O. ulmi strain H6	++	++
Verticillium albo-atrum	−	−
Fusarium oxysporum f. sp. *lycopersici*	−	±

++, ± and − : Semi-quantitative comparison on fluorescence or brownish precipitation. ++ = a strong reaction; ± = faint brownish precipitation; − = no reaction.

Tabel 1. Specificiteit van het hyperimmuun serum tegen O. ulmi stam E2 tegenover uitstrijkjes van O. ulmi stammen E2 en H6, Verticillium albo-atrum en Fusarium oxysporum f. sp. lycopersici bij gebruik van de IIF- en de IIP-techniek.

distilled water and mounted in polyvinylpyrrolidone. Cross-sections were stained by transferring through the solutions with a glass rod and finally mounted on glass slides. In case of a positive reaction mycelium and conidia showed a brown colour.

Results and discussion

The hyperimmune serum gave a positive reaction with *O. ulmi* on using either the IIF or the IIP techniques (Figs. 1, 2 and 3). Treatment with normal rabbit serum gave no reactions with both techniques. The antibodies evoked by a non-aggressive strain of *O. ulmi* (E2) also reacted strongly with smears of an aggressive strain (H6) (Table 1). Thus, we could not immunologically distinguish between aggressive and non-aggressive strains of the fungus. No reaction occurred between the *O. ulmi* antiserum and smears of *Verticillium albo-atrum* or *Fusarium oxysporum* f. sp. *lycopersici*. Likewise, Andrews and McCrum (1969) did not obtain any agglutination reaction with their antiserum to *O. ulmi* and with nine fungal and five bacterial isolates frequently found as normal inhabitants of healthy elm wood.

In a time sequence study of inoculated elm branches, transport of conidia and growth of hyphae were studied using the IIF and IIP techniques. Special attention was paid to the possible growth of *O. ulmi* into parenchyma cells, ray cells or fibres. Because the observation of the fungus within elm wood is difficult (Miller and Elgersma, 1976) inoculations were done with a high concentration of conidia (1.6×10^8 conidia per ml) of *O. ulmi* strain E2.

Fig. 5. Conidia of *O. ulmi* showing affinity towards pits, six hours after inoculation (IIF).
Fig. 6 and 7. Hyphae of *O. ulmi* growing via pits from vessel to vessel.
Fig. 8. Conidia of *O. ulmi* trapped underneath rim of vessel perforation, fifteen minutes after inoculation (IIP).
Fig. 9. Germinating conidium of *O. ulmi*, six hours after inoculation (IIP).
Fig. 5. Conidiën van O. ulmi, met voorkeur voor de stippels, zes uur na inoculatie (IIF).
Fig. 6 en 7. Hyfen van O. ulmi die via de stippels van vat naar vat groeien.
Fig. 8. Conidiën van O. ulmi onder de rand van perforatieplaat, vijftien minuten na inoculatie (IIP).
Fig. 9. Kiemend conidium van O. ulmi, zes uur na inoculatie (IIP).


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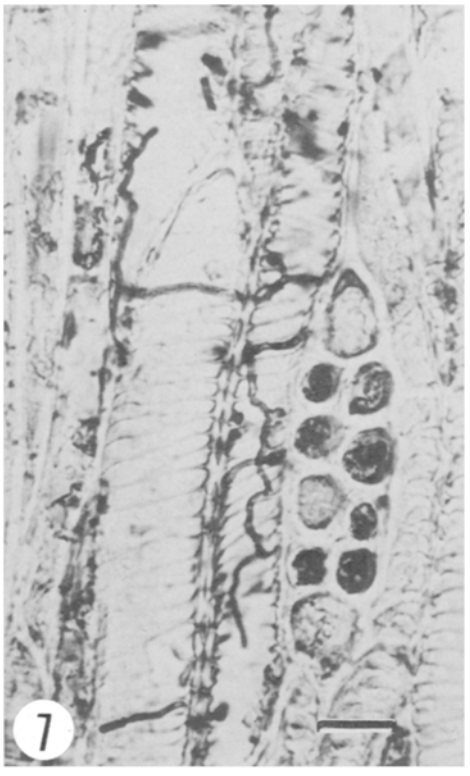
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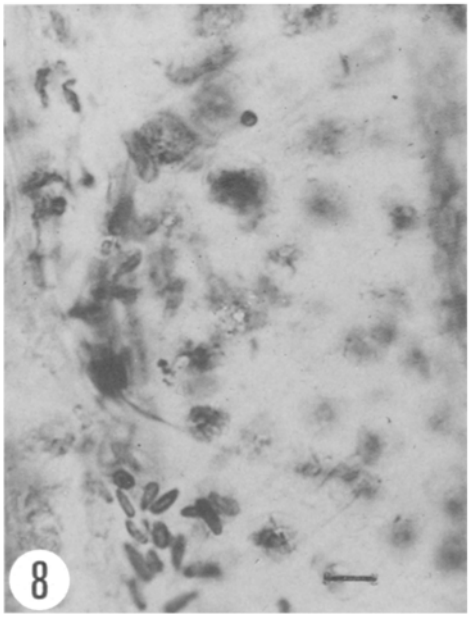
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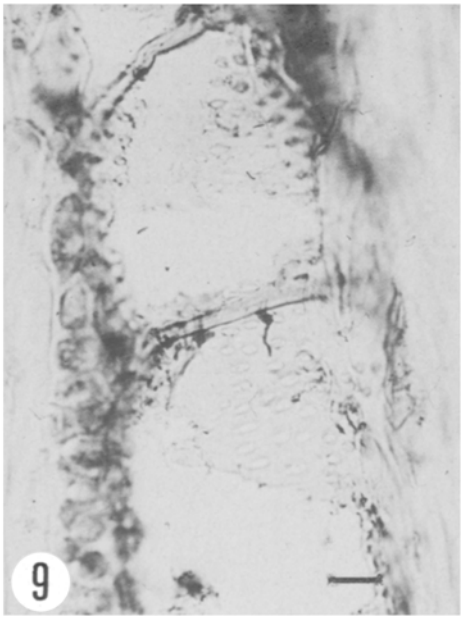
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Non-germinated conidia were found in the vessels within 30 min after inoculation, especially underneath rims of vessel perforations (Figs. 4 and 8). Even one min after inoculation, conidia were found up to 9 cm above the site of inoculation.

Six hours after inoculation conidia with short germ tubes were observed (Fig. 9) and conidia appeared to be localized on pits (Fig. 5). This affinity towards pits may be caused by the water flow through these pits. This phenomenon was observed also by Pomerleau (1970).

Twenty-three hours after inoculation hyphae as well as conidia were found, but only in the vessels.

Between 56 and 74 hours after inoculation little progress in development of the fungus could be detected. Hyphae grew from vessel to vessel via pits. No hyphae or conidia were found in parenchyma cells, ray cells or fibres (Fig. 6 and 7). Even 20 days after inoculation no hyphae or conidia were observed in tissues other than vessels.

As with earlier examinations with the scanning electron microscope (Miller and Elgersma, 1976) we were unable to find direct cell wall penetration. These results are also in agreement with the observations made by Pomerlau (1970).

Both techniques (IIF and IIP) as used in this study can be successfully employed for the examination of *O. ulmi* in elm wood. In contrast to normal light microscopy correct focusing is very critical in UV microscopy (IIF) using incident illumination. This is rather tiring when many cross-sections have to be examined. Nonspecific fluorescence may be advantageous in identifying the tissue elements but too much nonspecific fluorescence prevents good observation of the fungus. The fungus is a little more difficult to locate with IIP than with IIF, but correct focusing is much easier and this enables faster screening of the cross-sections for the pathogen.

Samenvatting

Een onderzoek naar de ontwikkeling van Ophiostoma ulmi in iepen met immunologische technieken

De indirecte immunofluorescentietechniek (IIF) en de indirecte immunoperoxidasetechniek (IIP) werden toegepast om *Ophiostoma ulmi* (Buisman) Nannf. in geïnfecteerd iepehout aan te tonen. Er bleek een sterke kruisreactie op te treden tussen het hyperimmuun serum tegen respectievelijk de niet-agressieve stam (E2) en de agressieve stam (H6) van *O. ulmi*. Geen reactie trad op met *Verticillium albo-atrum* of *Fusarium oxysporum*.

Gedurende de pathogenese kon de schimmel nooit buiten de houtvaten aangetoond worden. Bij de groei van het pathogeen van vat naar vat werd nooit directe celwandpenetratie waargenomen; deze vond altijd plaats via de hofstippels.

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