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The growth of aggressive and non-aggressive strains of Ophiostoma ulmi in susceptible and resistant elms, a scanning electron microscopical study

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Abstract

Different wood preparation techniques for a SEM study of the growth of *Ophiostoma ulmi* (Buisman) Nannf. in elm tissue were compared. Critical point drying appeared to be the most suitable method. All observations were made 10–14 cm above the site of inoculation. In the resistant elm, *Ulmus hollandica* cl. 390, inoculated with a non-aggressive strain of *O. ulmi*, no hyphae could be found until 5 days after inoculation. However, when *U. hollandica* cl. 390 was inoculated with the aggressive strain, the fungus could already be observed after 3 days. In the susceptible elm, *U. hollandica* cl. Belgica, whether inoculated with an aggressive or non-aggressive strain, hyphae were found within 2 days after inoculation. The fungus appears only to penetrate the vessel pits and has at no time been seen to penetrate the cell wall directly.

Introduction

The wilting of elms susceptible to *Ophiostoma ulmi* (Buisman) Nannf. (syn. *Ceratocystis ulmi* (Buisman) C. Moreau – De Hoog, 1974), which involves reduction of the sap stream, is generally believed to result from the occlusion of the wood vessels by gum and tyloses (Beckman, 1966; Elgersma, 1969). Ouellette (1960) has pointed to the direct contributions to plugging by fungal growth itself, by debris from the breakdown of fungal cells and to the contents of cells adjoining the vessels. Pomerleau (1970) too, claims to have observed clumping of fungal elements in the vessels, but he did not accept mechanical occlusion of the vessels by the fungus as the sole cause of wilting.

For many years the localization of conidia and developing hyphae of *O. ulmi* in vessels of infected elm trees has been accepted as one of the most important factors involved in the resistance to Dutch elm disease (Elgersma, 1969, 1970; McNabb et al., 1970; Sinclair et al., 1975a). Therefore, as well as a possible delay in tylose formation in susceptible elm clones (Elgersma, 1973), the possibility also exists that the transport of conidia by the sap flow is slower in resistant elms due to the anatomical wood structure. This may be the means to provide sufficient time for localization of the fungus by tylose formation.

Our concept of the resistance mechanism has been complicated by the recent isolation of an aggressive strain of *O. ulmi* (Gibbs et al., 1972). This strain infects and causes the death of many of the so-called 'resistant' elm clones and is causing serious problems in England. Elm clones such as Vegeta and Commelin appear to be sus-

ceptible to this strain, which was first isolated in the Netherlands in 1972, although the clone Groeneveld still demonstrates considerable resistance (Heybroek, 1975). An investigation was conducted therefore into growth differences between an aggressive strain and non-aggressive strain of *O. ulmi* within *Ulmus hollandica* cl. 390 (a clone with high resistance to non-aggressive strains) and *Ulmus hollandica* cl. Belgica (a clone susceptible to both strains).

Although the Dutch elm disease has been investigated for more than 50 years (Kerling and Elgersma, 1970), one of the greatest problems has been the observation of the fungus within elm wood. More recently the transmission electron microscope has been used in the study of *O. ulmi* (MacDonald, 1970; MacDonald and McNabb, 1970; Krause and Wilson, 1972). Although Ishida and Ohtani (1968, 1969) studied tylose structure in 'Haru-nire' (*Ulmus* sp.) with the help of the scanning electron microscope (SEM), King et al. (1974) have been probably the only investigators to utilize this technique in Dutch elm disease research. The SEM was the basic tool in the present investigation because of the large areas of wood surface which could be examined at relatively high magnifications.

Materials and methods

Experiments were conducted during the early growth periods of 1974 and 1975 on 2-year old rooted callus cuttings of *U. hollandica* cl. Belgica, a clone susceptible to the non-aggressive strains of *O. ulmi*, as well as *U. hollandica* cl. 390, a clone which shows a high resistance to the non-aggressive strain but which is susceptible to the aggressive strain of *O. ulmi*. The plants were kept in a greenhouse at about 24 °C for 3 to 4 weeks before inoculation with suspensions of either the aggressive (H6) or the non-aggressive (E2) strains of *O. ulmi* (code of strains according to Gibbs et al., 1975), containing 10^6 conidia per ml. Plants were harvested 2, 3, 4, 5, 7 and 10 days after inoculation. Small pieces of wood (1 cm) were then removed for further treatment from 10 cm above the point of inoculation in the 1974 series and from 12–14 cm above the point of inoculation in 1975 series.

The wood samples collected in 1974 were treated in various ways:

1. Air-dried specimens – fixed in a) Formalin-ethanol-propionic acid or b) according to Laane (1971).
2. Air-dried specimens – first treated in household bleach, diluted 1:5, and then washed.
3. Freeze-dried specimens after placing in liquid nitrogen.
4. Critical point drying. Glutaraldehyde fixation for 4 hours. Dehydration with ethanol series, replaced with amyl acetate.
5. Deep freeze specimens. Stored at –20 °C. A number of these blocks were removed from the deep freeze in 1975 and divided into 2 groups: a) Those left at 24 °C in high humidity for 3 days, b) those only allowed to reach room temperature. Both groups were then fixed and treated for critical point drying (see method used in 1975).

In 1975 wood specimens were treated directly after sampling as follows:

1. Fixation. Blocks (8 × 5 × 2 mm) were first fixed in 1% osmium tetroxide and 2% glutaraldehyde in cacodylate buffer for 1 hour at 0 °C, rinsed several times with cacodylate buffer and fixed in 1% osmium tetroxide for 1½–2 hours at 4 °C.
2. Dehydration. After rinsing dehydration was carried out with increasing concen-

trations of acetone (20%–35%–50%–70%). Specimens could be left in 70% acetone for several days. On the day of critical point drying dehydration was completed using water free acetone.

3. Critical point drying. The acetone was substituted with liquid CO_2 in a pressure chamber (Poloron), by flushing over a period of 5 minutes. During the 1 hour impregnation period the CO_2 was replaced three or four times to ensure the removal of any trace of acetone.

Preparation for scanning electron microscopy:

a) 1974 series. Blocks were refaced with new razor blades during the early dehydration stage. Dried specimens were first coated with a carbon layer (15 nm thick) and then with a gold layer (15 nm thick) at a vacuum of at least 5.10^{-5} Torr.

b) 1975 series. Dried specimens were refaced just before mounting on stubs. The coating was effected by gold sputtering in argon at a vacuum maintained between 5.10^{-2} and 10^{-2} Torr.

Specimens were observed with a Cambridge S4 scanning electron microscope.

Results

All photographs (Fig. 1–14) were made of specimens, from the 1974 and 1975 series, fixed in osmium tetroxide and glutaraldehyde and prepared by the critical point drying technique. Most illustrate a satisfactory fixation of the fungal elements and show only minor problems due to fixative residue. All other techniques used in the 1974 series produced either specimens with an occasional poorly fixed hypha and too many artifacts or in the case of the bleach method, clean wood surfaces devoid of fungal material.

SEM photographs made from the 1974 deep freeze specimens demonstrate hyphal development of two types. Hyphae were found to be thicker (2.5–7 μm) in the blocks removed from the deep freeze and allowed to stand at 24 °C in a humid atmosphere for 3 days before fixation (Fig. 1 and 2) than the hyphae (1–2 μm thick) found in specimens which had only be allowed to reach room temperature (approx. 20 °C) before fixation (Fig. 3 and 4). The aggressive strain (H6) could not be distinguished from the non-aggressive strain (E2) under either of these circumstances. Fig. 3 shows hyphal development 7 days after inoculation close to a region where tylosis appears to have been interrupted. Developing hyphae, 4 days after inoculation, are seen in Fig. 4.

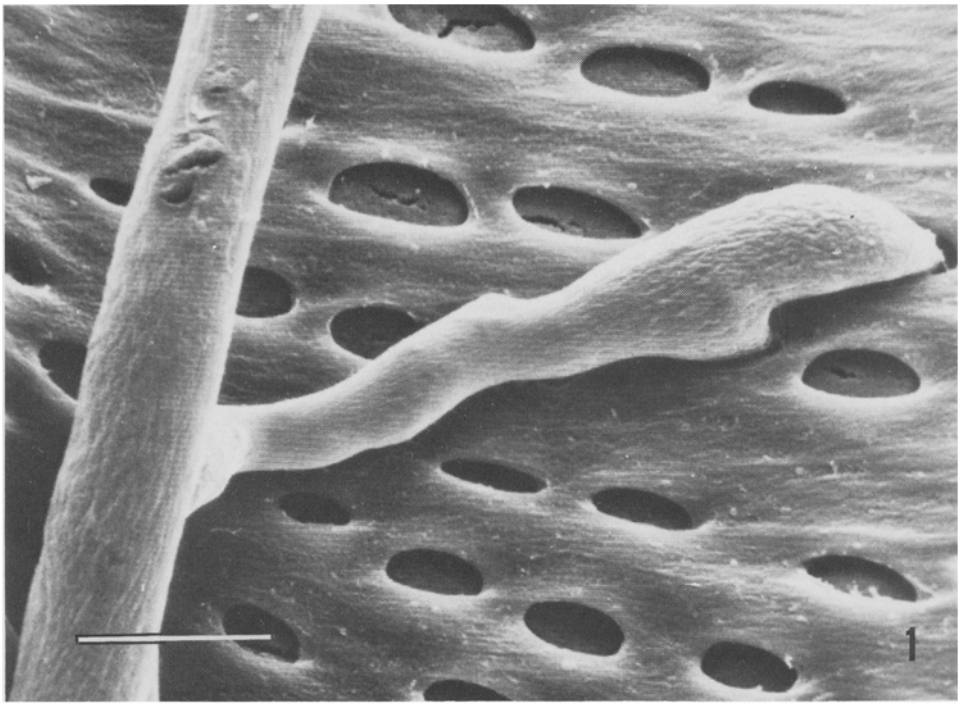
In specimens of the 1975 series of clone 390 inoculated with *O. ulmi* strain E2, no hyphae could be found in the wood vessels until 5 days after inoculation and then only with great difficulty (Fig. 5). However, hyphae were found in clone Belgica within 2 days after inoculation with either strain E2 or strain H6 (Fig. 6). Well-developed hyphae were found in clone 390, 3 days after inoculation with strain H6 (Fig. 7). Fig. 8 shows developing hyphae and conidia which appear to be trapped underneath the vessel perforation rim. These findings are outlined in Table 1. Tyloses were apparent after only 2 days and made observation difficult after 7 days.

The presence of different types of tyloses is illustrated in Fig. 9–13. The types seen in Fig. 9 and 10 are similar and when ruptured produce a dense fibre-like structure (Fig. 11).

The fungus appears only to penetrate the vessel pits.and has at no time been seen

Fig. 1 and 2. Clone 390 – strain E2. Thick hyphae in vessel; deep freeze material after 3 days at 24 °C in a humid atmosphere. Radial surface. Bar represents 10 μm, also in the following figures.
Fig. 3 and 4. Clone 390 – strain H6. Thin hypha in vessel; deep freeze material, fixed after reaching room temperature. Tangential surface. Fig. 3. Seven days after inoculation. Note young tyloses. Fig. 4. Four days after inoculation.
Fig. 5–Fig. 14. Wood specimens fixed directly after sampling.
Fig. 5. Clone 390 – strain E2. Hyphae developing in vessel 5 days after inoculation. Radial surface.
Fig. 6. Clone Belgica – strain E2. Germinating conidium and penetration of pit at 2 days after inoculation.
Fig. 7. Clone 390 – strain H6. Developing hyphae showing frequent penetration of pits at 3 days after inoculation.
Fig. 8. Clone 390 – strain H6. Conidia lodged underneath rim of vessel perforation and hyphae developing in direction of pits at 3 days after inoculation. Radial surface.
Fig. 9. Clone 390 – strain E2. Tyloses 3 days after inoculation. No fungal material present. Tangential surface.
Fig. 10. Clone Belgica – strain H6. Tyloses 3 days after inoculation. Germinating conidia. Tangential surface.
Fig. 11. Clone 390 – strain E2. Fibre-like structure being released from ruptured tylose. Note part of tylose wall at bottom of photo. Radial surface.
Fig. 12. Clone Belgica – strain E2. A different type of tylose showing wart-like structures. Two days after inoculation. Radial surface.
Fig. 13. Clone 390 – strain E2. Smooth thin-walled tyloses. Seven days after inoculation.
Fig. 14. Clone Belgica. Trabecula in vessel. Tangential surface.

Fig. 1 en 2. Kloon Belgica – stam E2. Dikke hyfe in houtvat; diepgevroren materiaal, 3 dagen in een vochtige atmosfeer bij 24 °C gehouden. Radiaal oppervlak. De vergrotingsstreep geeft 10 μm weer, ook in de volgende figuren.
Fig. 3 en 4. Kloon 390 – stam H6. Dunne hyfe in houtvat; diepgevroren materiaal, gefixeerd direct na het bereiken van kamertemperatuur. Tangentiaal oppervlak. Fig. 3. Zeven dagen na inoculatie. Let op pas ontwikkelde thyllen. Fig. 4. Vier dagen na inoculatie.
Fig. 5–Fig. 14. Houtmateriaal direct na het verzamelen gefixeerd.
Fig. 5. Kloon 390 – stam E2. In houtvat ontwikkelende hyfen 5 dagen na inoculatie. Radiaal oppervlak.
Fig. 6. Kloon Belgica – stam E2. Kiemend conidium en doorboring van stippel 2 dagen na inoculatie.
Fig. 7. Kloon 390 – stam H6. Ontwikkelende hyfen, die frequent de stippels doorboren, 3 dagen na inoculatie.
Fig. 8. Kloon 390 – stam H6. Conidiën onder de rand van perforatie plaat en hyfen die in de richting van de stippels groeien op 3 dagen na inoculatie. Radiaal oppervlak.
Fig. 9. Kloon 390 – stam E2. Thyllen 3 dagen na inoculatie. Geen schimmelmateriaal aanwezig. Tangentiaal oppervlak.
Fig. 10. Kloon Belgica – stam H6. Thyllen 3 dagen na inoculatie. Kiemende conidiën. Tangentiaal oppervlak.
Fig. 11. Kloon 390 – stam E2. Vezelachtige structuur, vrijgekomen uit kapotte thyllen. Let op gedeelte van de wand van de thyl aan de onderkant van de foto. Radiaal oppervlak.
Fig. 12. Kloon Belgica – stam E2. Een ander type thyl met wratachtige structuren. Twee dagen na inoculatie. Radiaal oppervlak.
Fig. 13. Kloon 390 – stam E2. Gladde dunwandige thyllen. Zeven dagen na inoculatie. Tangentiaal oppervlak.
Fig. 14. Kloon Belgica. Trabecula in houtvat. Tangentiaal oppervlak.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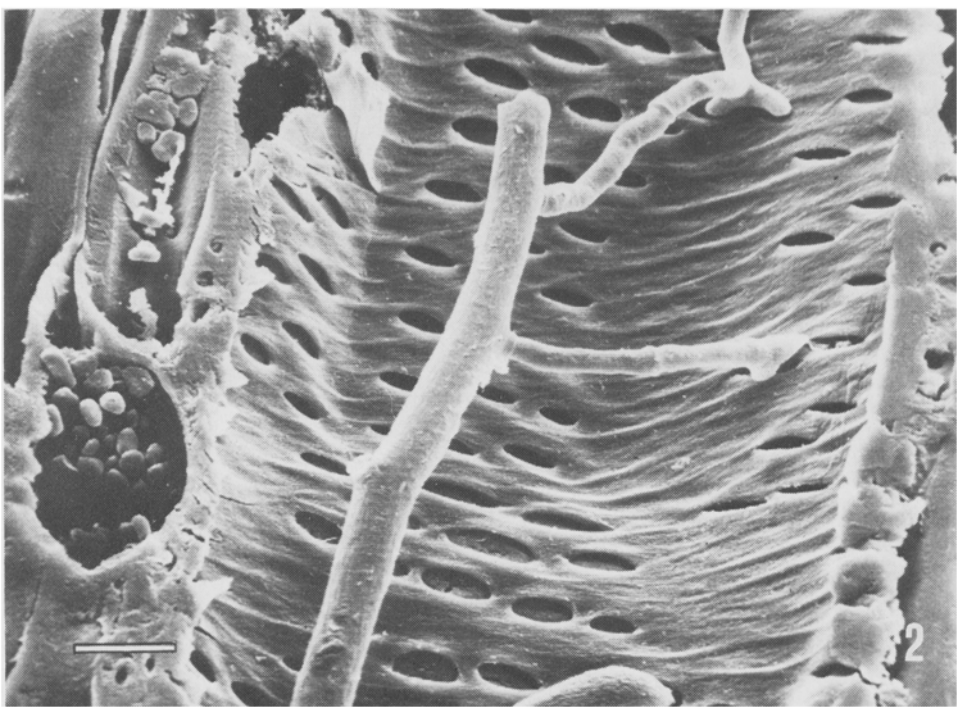
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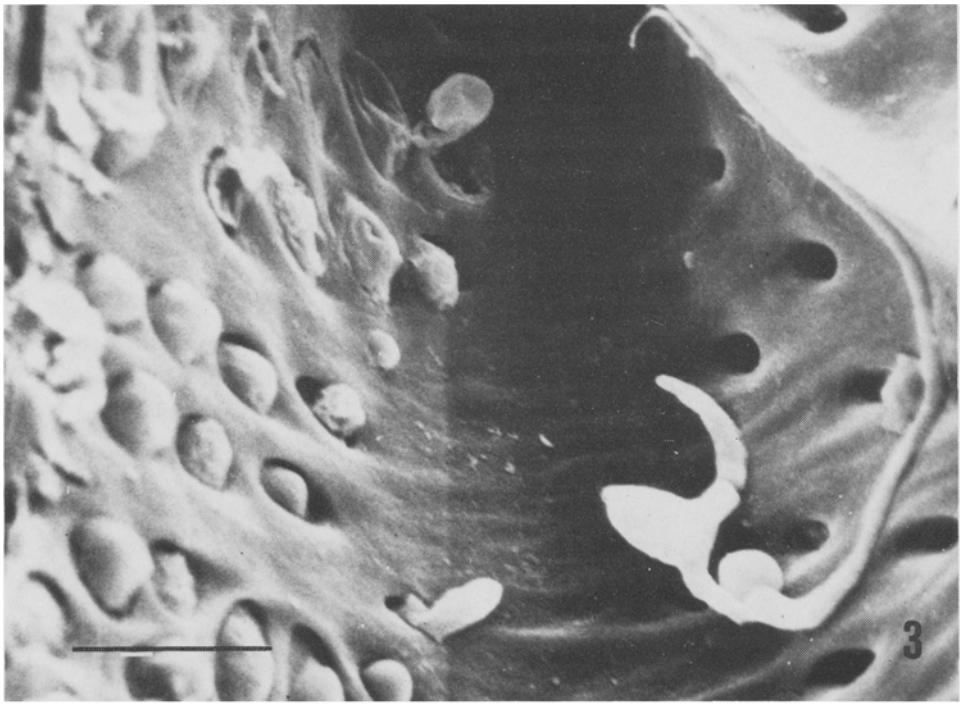
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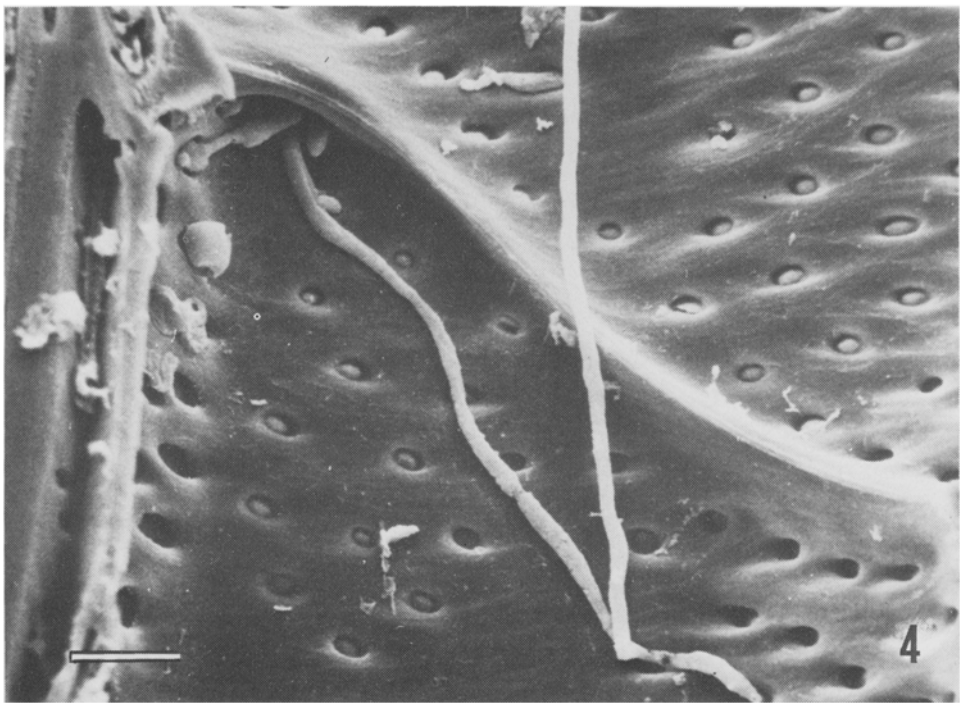
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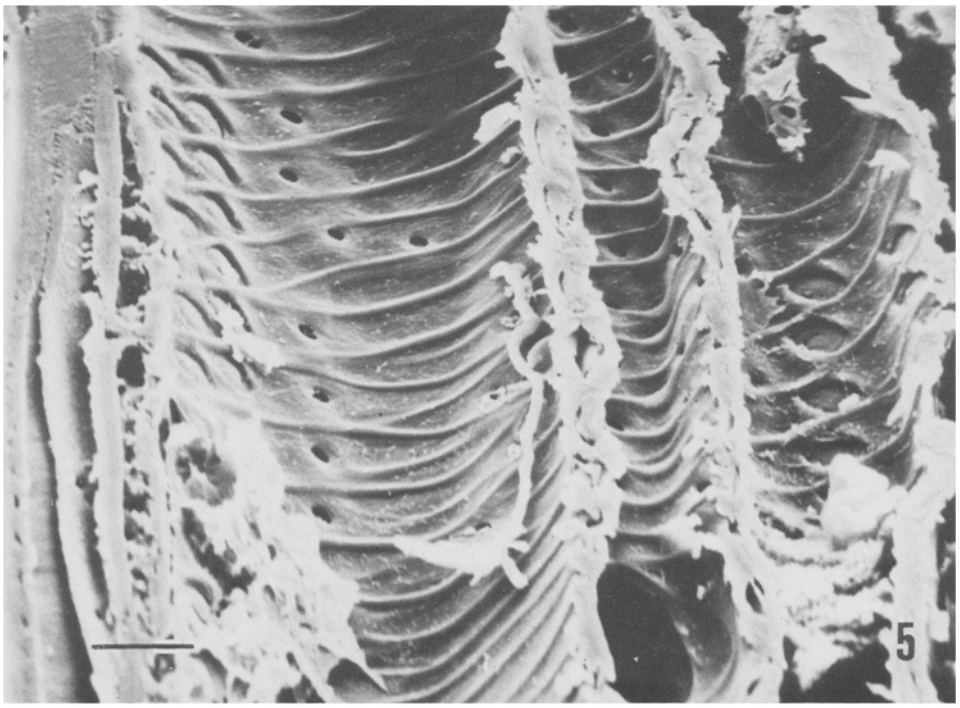
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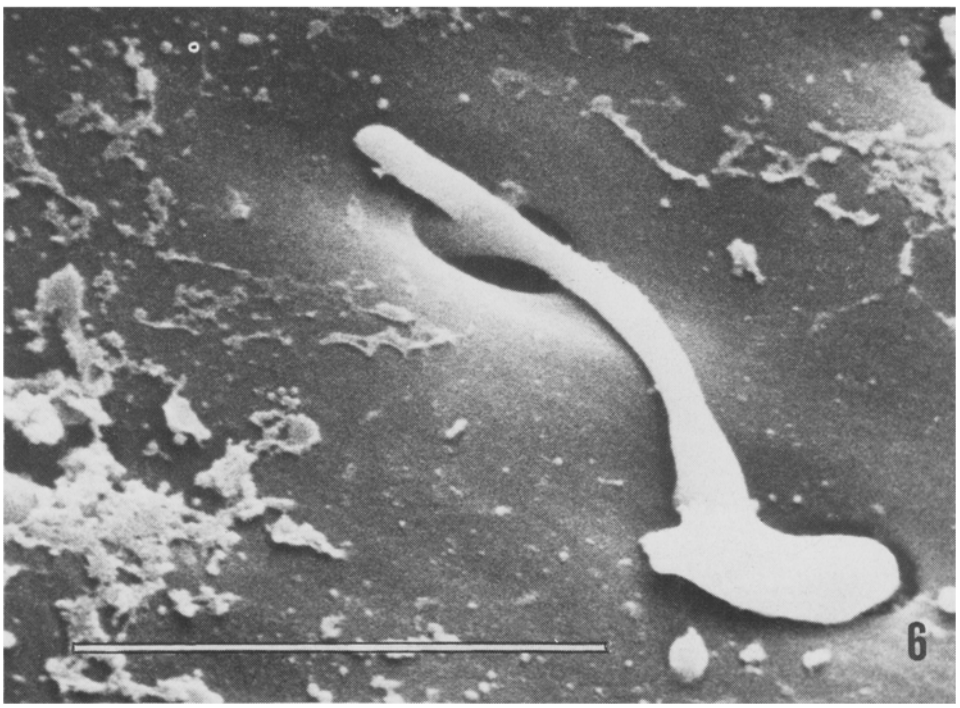
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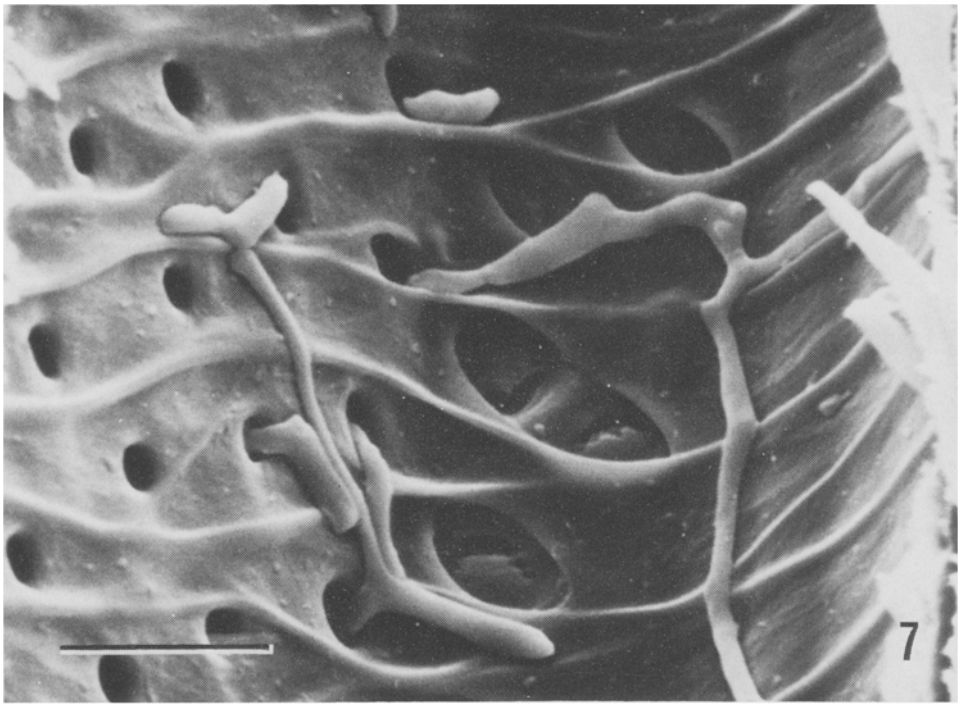
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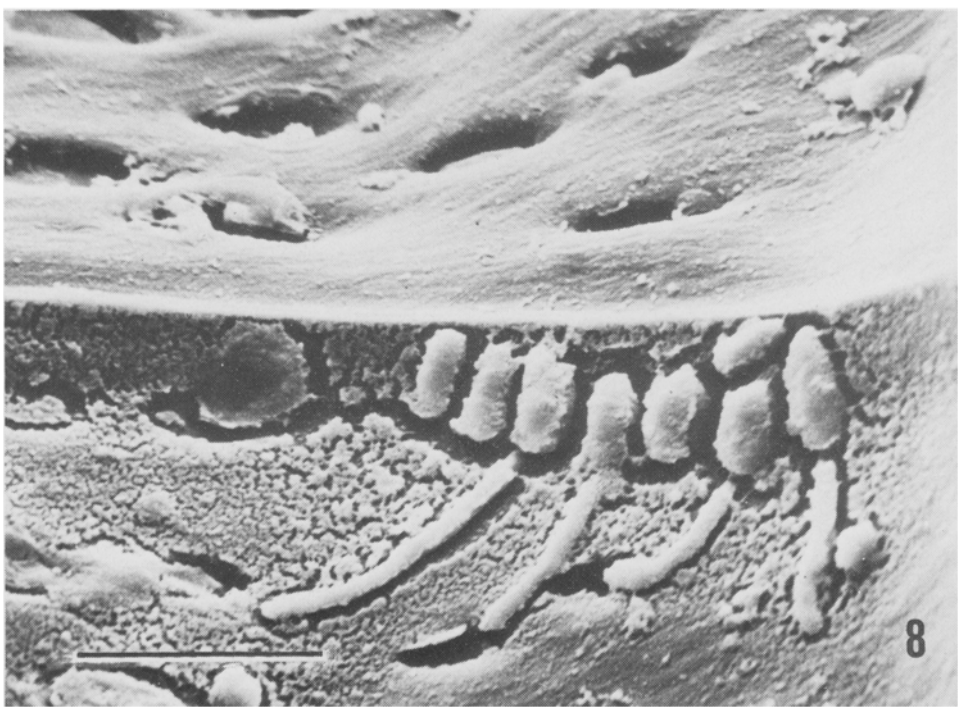
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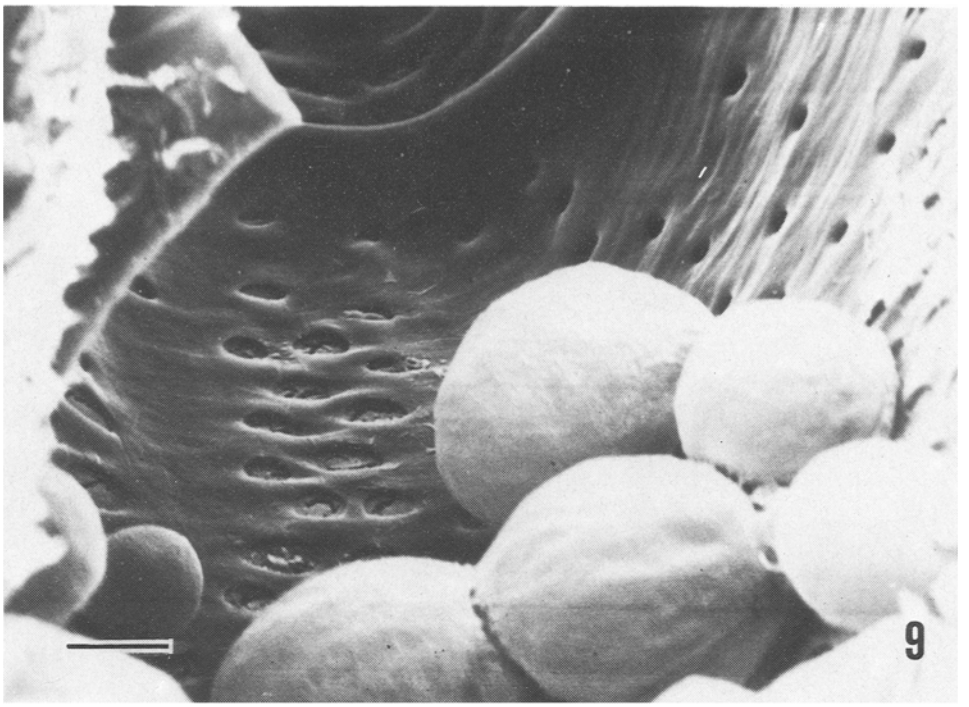
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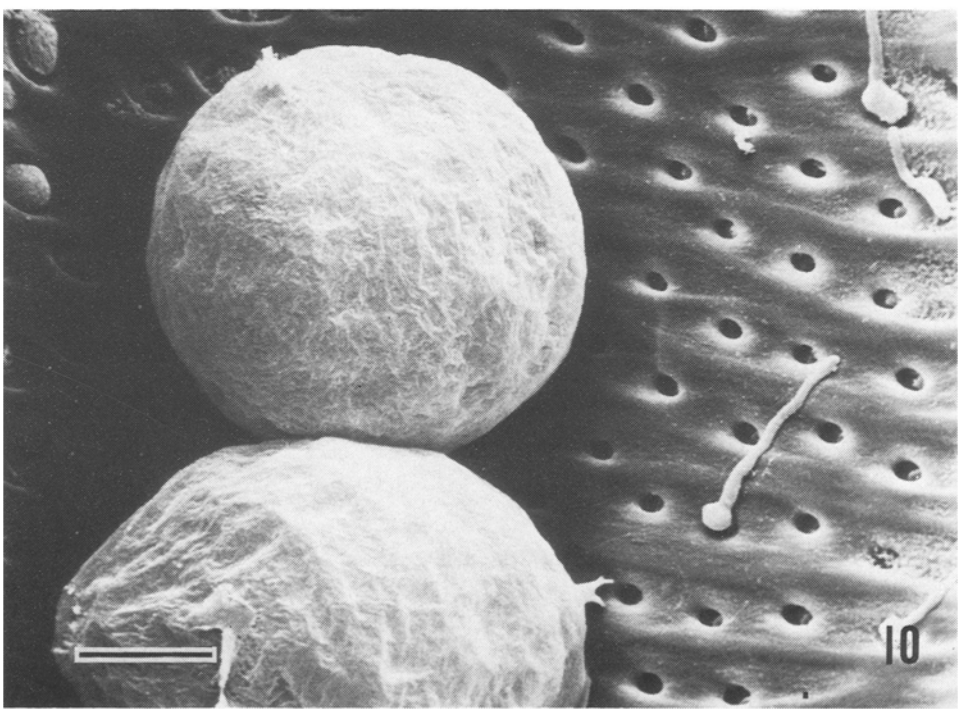
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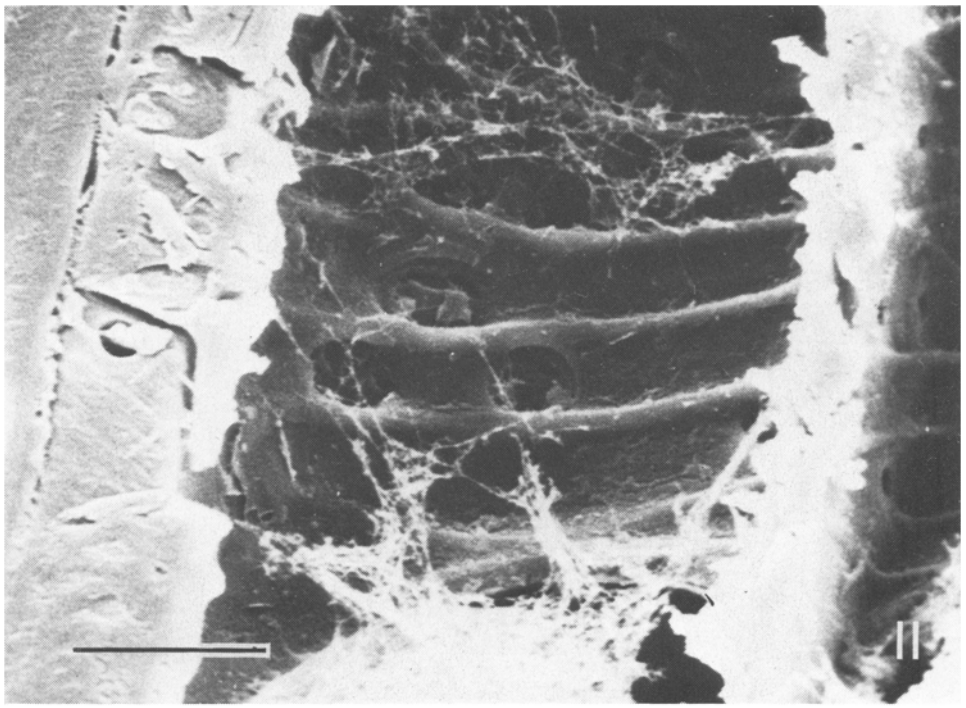
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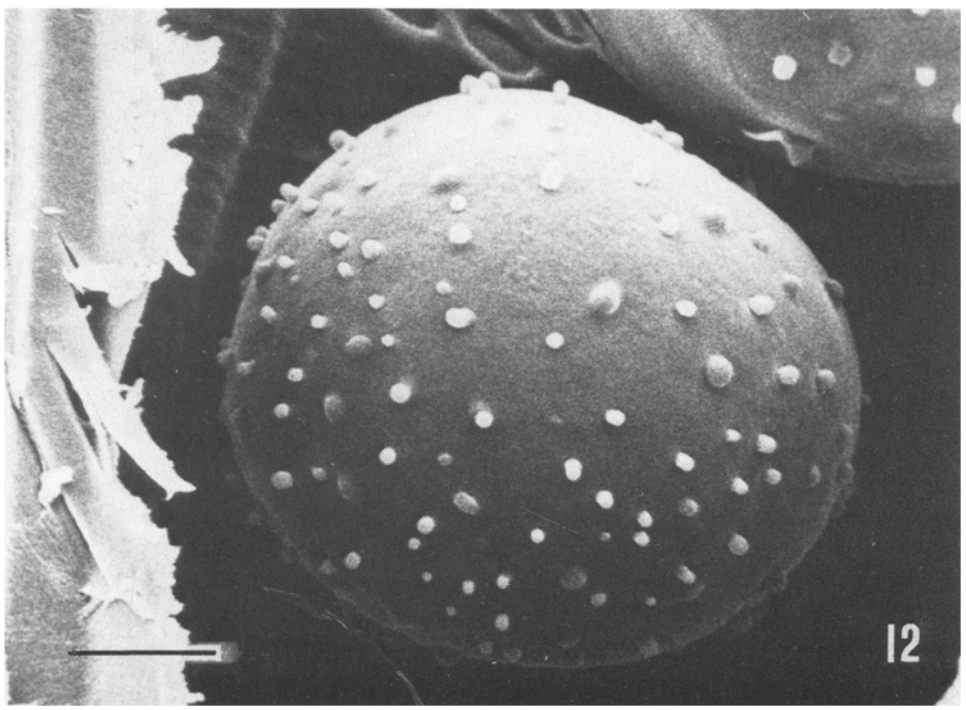
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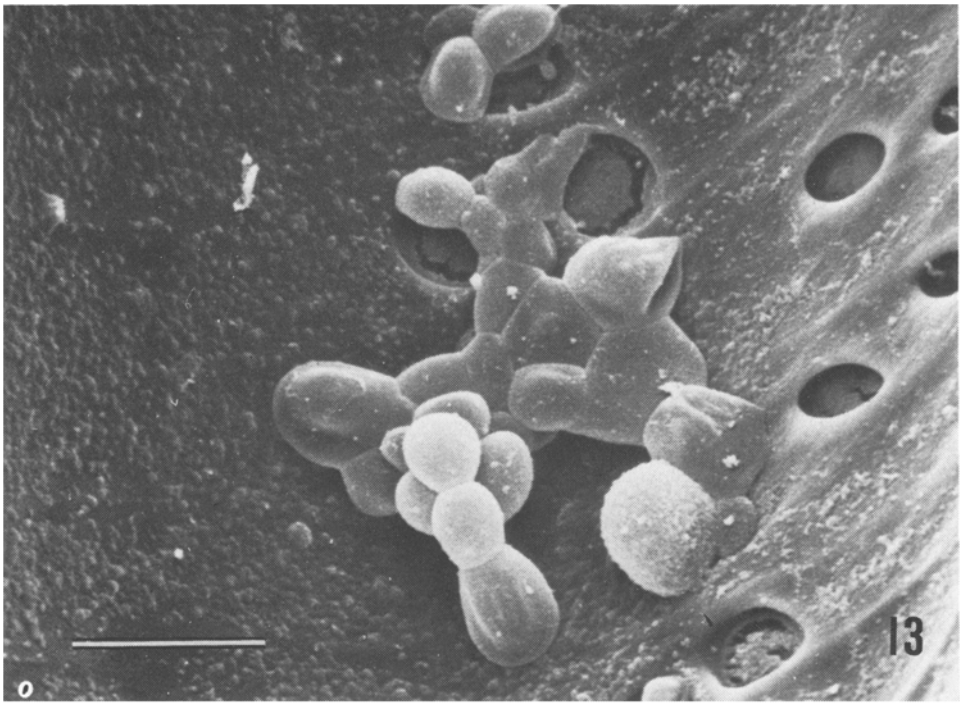
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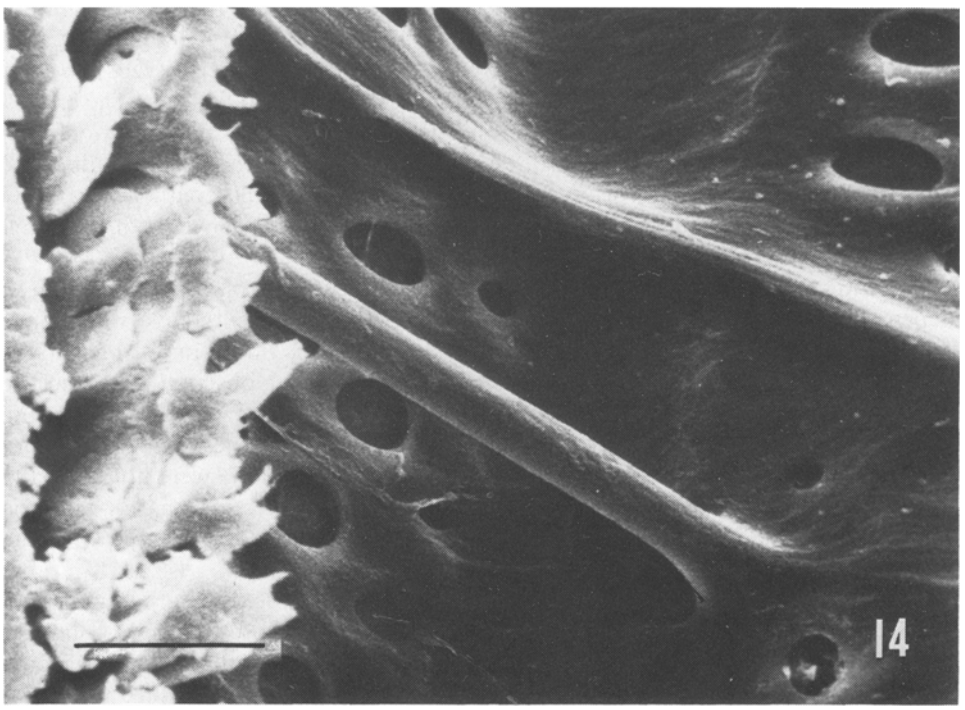
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Table 1. The development or presence of hyphae and/or conidia of *O. ulmi* in the susceptible *U. hollandica* cl. Belgica and the resistant *U. hollandica* cl. 390.

Number of days after inoculation	*U. hollandica* cl. Belgica		*U. hollandica* cl. 390	
	non-aggressive (E2)	aggressive (H6)	non-aggressive (E2)	aggressive (H6)
2	+	+	−	−
3	++	++	−	++
5	+++	+	+	++
7	+++	++	+	+
10	++	++	−[1]	−[1]

− = no conidia or hyphae seen; + = occasional, ++ = few, +++ = several conidia and/or hyphae.

[1] Observations obscured by the abundance of tyloses.

Tabel 1. De ontwikkeling of aanwezigheid van hyfen en/of conidiën van O. ulmi in vatbare U. hollandica kloon Belgica en de resistente U. hollandica kloon 390.

to penetrate the cell wall directly. During the search for possible direct penetration of the cell wall, trabeculae were occasionally found (Fig. 14).

Discussion

In order to overcome the usual difficulty of finding *O. ulmi* in the wood specimens several methods of treating the collected material were tried out before subjection to SEM examination. Most methods proved to be unsatisfactory due to the poor fixation or subsequent loss of fungal material. Storage in the deep freeze, however, did not appear to have an adverse effect on the fungus prior to fixation. The critical point drying method utilizing an acetone dehydration series after fixation with osmium tetroxide and glutaraldehyde, produced the most satisfactory results. The refacing of the block surface prior to mounting on stubs for gold coating apparently exposed an area of wood where loss of fungal material, due to dehydration washing, had been considerably reduced.

Light microscopical examination, generally used in studies of this type, has been dependant on various staining methods, which have also frequently involved numerous dehydration and embedding techniques, as well as the limitation of light itself. Care must be taken in interpreting results obtained from such observations. Quelette (1962), for example, reported mycelium and conidia in quantities sufficient to plug vessels. We assume that the plugging was caused by fibre meshes derived from ruptured tyloses (Fig. 11). Large numbers of hyphae and conidia were not generally found in our material which was studied during the early infection phases. The true number of hyphae and conidia present at later stages may be obscured by large numbers of tyloses.

Prolonged exposure of infected wood samples to room temperature or temperatures optimal for *O. ulmi* produces conditions different from those normally found in living trees. Under more favourable circumstances, especially when nutrients become more readily available, e.g. tissue disruption, or artificially added nutrients,

there is a tendency for *O. ulmi* to produce broad hyphae (Fig. 1 and 2), while thin hyphae were found under more natural conditions (Fig. 3 and 4). The latter hyphal type, whether of the aggressive or non-aggressive strain, was typical in the observations made from the 1975 series. Therefore we regard the use of sterilized sections or blocks, which have been later inoculated and placed on nutrient media (Casagrande and Ouellette, 1971; King et al., 1974), as a source of misleading results.

Several investigators (Elgersma, 1969, 1970; McNabb et al., 1970; Sinclair et al., 1975b) have shown a correlation between the susceptibility of the elm and its anatomy. They report susceptible clones as having wider and longer wood vessel elements than resistant clones, therefore enabling a more effective distribution of fungal material. Further anatomical studies are needed as most studies of this nature have been conducted on two or three year old twigs which undoubtedly show signs of paedomorphosis and produce inaccurate comparisons (Carlquist, 1962; Miller, 1975). The results given in Table 1, however, also show retardation of the spreading of the non-aggressive strain of *O. ulmi* in *U. hollandica* cl. 390 compared to *U. hollandica* cl. Belgica.

The aggressive strain is apparently able to overcome this problem by a more rapid growth and by what appears to be a more active penetration of the vessel pits (Fig. 7 and 8). A faster growth of an aggressive strain in culture and a correlation between cultural characteristics and pathogenicity have been reported (Gibbs and Brasier, 1973). A resistance mechanism as one of more rapid tylose formation in the resistant clone 390 than in the susceptible clone Belgica after inoculation with a non-aggressive strain (Elgersma, 1973), therefore becomes less effective when infection occurs with an aggressive strain.

Although direct penetration of the cell walls was not observed, this method of spreading cannot be excluded, especially in the light of the results of Ouellette (1962), MacDonald and McNabb (1970) and Krause and Wilson (1972). However, Pomerleau (1970) is against this hypothesis and King et al. (1974) were unable to demonstrate direct penetration during their SEM examination of the cell wall. It may be that only in later stages of the infection process cell wall penetration occurs.

The occurrence of different types of tyloses (Fig. 9, 10, 12 and 13) in elm wood has not previously been reported. Any relationship between the different types of tyloses and the infection process still needs to be investigated. Tyloses are known to be of taxonomic value. The origin of trabeculae (Fig. 14) is obscure (Jane, 1970) as they were once believed to have originated from fungal hyphae (McElhanney et al., 1935). Their occurrence too, may be of taxonomic significance. They are known to occur sporadically in coniferous wood and more recently they have also been reported in dicotyledonous woods (Butterfield and Meylan, 1972). They have not previously been reported in elm wood.

Samenvatting

De groei van agressieve en niet-agressieve stammen van Ophiostoma ulmi in vatbare en resistente iepen, een scanning elektronenmicroscopisch onderzoek

Verschillende houtpreparatie-technieken voor bestudering van de groei van *Ophiostoma ulmi* in iepeweefsel met behulp van de SEM werden vergeleken. Alle waarnemin-

gen werden 10–14 cm boven de plaats van inoculatie gedaan. 'Critical point drying' bleek de meest bruikbare methode. In de resistente iep, *Ulmus hollandica* kloon 390, geïnoculeerd met een niet-agressieve stam van *O. ulmi* werden eerst 5 dagen na inoculatie hyfen gevonden in tegenstelling tot de vatbare iep, *U. hollandica* kloon Belgica waarin zowel na inoculatie met de agressieve als met de niet-agressieve stam hyfen konden worden gevonden binnen 2 dagen. Als *U. hollandica* kloon 390 geïnoculeerd werd met de agressieve stam kon de schimmel reeds na 3 dagen worden gevonden. Binnendringen van de schimmel door de vaatwand werd waargenomen, maar alleen via de stippels.

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