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Light and electron microscopy of Vinca plants infected with mycoplasma-like bodies of the sandal spike disease

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Abstract

Detection of phloem cells containing mycoplasma-like bodies associated with the sandal spike disease was made easier by observing sections from stems of infected *Vinca rosea* plants by means of fluorescence microscopy. The aniline blue-stained cells that contained mycoplasma-like bodies fluoresced yellow in ultraviolet light (366 nm). The presence of mycoplasma-like bodies was confirmed by examination of ultrathin sections obtained from the same stems in the electron microscope.

Introduction

Sandal spike, a disease of *Santalum album*, is of major importance in India. Mycoplasma-like bodies (hereafter called mycoplasmas) are known to occur in the phloem of affected sandal (Dijkstra and Ie, 1969; Hull et al., 1969; Varma et al., 1969), and in that of alternate hosts like *Santalum yassi* (Hull et al., 1970), *Vinca rosea* (Dijkstra and Lee, 1972; Hiruki and Dijkstra, 1973), *Dodonaea viscosa* (Hull et al., 1970 and *Zizyphus onoplea* (Hull et al., 1970).

The mycoplasma was reportedly obtained in culture (Nayar and Ananthapadmanabha, 1970). However, their claim of fulfilling Koch's postulates has been criticized (Davis and Whitcomb, 1971).

A previous anatomical study was unsuccessful in locating mycoplasma by acridin orange staining of phloem tissue of a spike-diseased sandal plant (Dijkstra, unpublished data). Fluorescence microscopy has been used earlier to demonstrate the presence of internal lesions in grapevine plants affected by grapevine flavescence dorée disease (Carle, 1965), the changes in internal phloem and other tissues of plants infected with yellows-type diseases (Cousin and Grison, 1966; Cousin, 1967), and some necroses in Solanaceous plants affected by stolbur (Cousin et al., 1968). Hiruki and Shukla (1973) have demonstrated the presence of fluorescent material in phloem cells of witches' broom-affected plants by staining with water-soluble aniline blue. Some of these cells containing fluorescent material corresponded to those with mycoplasma, as demonstrated by electron microscopy of ultrathin sections of the same plant.

In the present study, further attempts were made to extend the above finding using *Vinca* plants infected with the sandal spike agent. Some of the cyto- and histopatholo-

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gical disturbances induced by the mycoplasma are described, and the utility of fluorescence microscopy in detecting mycoplasma infection is demonstrated by the combined use of electron microscopy and light microscopy.

Materials and methods

Light microscopy. Inoculation of *Vinca rosea* 'Bright Eyes' with mycoplasma from diseased sandal was made with the help of *Cuscuta subinclusa* (Dijkstra and Lee, 1972). Plants were grown in a glasshouse for periods of 8 to 12 weeks. Young stems from affected *Vinca* plants showing typical witches' broom symptoms were sampled. Control samples were obtained from healthy plants. All samples were obtained from potted plants immediately before examination. Free-hand sections, about 20 to 30 μm thick, were made within 5 to 10 min after excising the stem and were fixed immediately by boiling in tap water for 3 to 4 min, then stained in 0.01 % aniline blue in 1/15 M K_3PO_4, pH 9.5, for 20 min. Adjustment of pH was made by adding a dilute NaOH solution. Stained sections from both healthy and infected plants were mounted in a small amount of aniline blue solution on a glass slide at the same time. Observations were made with a Wild microscope with a high pressure mercury vapour lamp (HBO 200 W). Two ultraviolet filters (UG 1) and a 'Rotdämpfungsfilter' (BG 38) with maximum transmission at 366 nm absorbed the visible spectrum. A selective filter (GG 4) was placed in the ocular tube of the microscope. Stem samples, primarily processed for electron microscopy, were also used for comparative observation in ordinary light microscopy by cutting 10 μm thick sections.

Electron microscopy. Stems of *V. rosea*, about 2 to 8 mm from the tip, were excised respectively from healthy and diseased plants that were also used for light microscopy. Tissue pieces of about 2 mm^2 were fixed for 1 h in 3.0 % glutaraldehyde in 0.025 M phosphate buffer, pH 7.4. The tissue was washed twice in the same buffer, postfixed for 1 h in 1 % osmium tetroxide, subsequently washed three times for 20 min each in the buffer, and kept in 0.5 % uranyl acetate in distilled water at 4 °C overnight. The tissue samples were dehydrated in a graded ethyl alcohol series, followed by two changes in propylene oxide. The samples were embedded in Epon-Araldite (Mollenhauer, 1964) and sectioned alternatingly 10 μm and 60 to 90 nm thick with an LKB ultramicrotome III equipped with a glass knife. The thin sections were used for electron microscopy after double staining with uranyl acetate and lead citrate (Reynolds, 1963). The 10 μm sections served as reference in light microscopy study.

Micrographs were taken with a Siemens Elmiskop 101 electron microscope at 60 or 80 kV at magnifications of times 1,400 to 20,000 and enlarged photographically as required.

Results

Light microscopy. With proper precautions, as described under methods, a significant difference between healthy and infected plants was recognized in the area of phloem cells (Fig. 1A and B). In infected stems, four kinds of fluorescent cells were recognizable: sclerenchyma cells in pale yellowish blue, sieve elements (external and internal) in brilliant yellow, other phloem cells in pale and diffused yellow, and xylem cells in

Fig. 1. Fluorescence in ultraviolet light of sections, stained with aniline blue, of young stems from a healthy *Vinca* plant (A), and from a slightly older stem of a plant infected with the sandal spike agent (B).
M = medulla, EP = external phloem, S = sclerenchyma, X = xylem. Bar represents 20 μm.

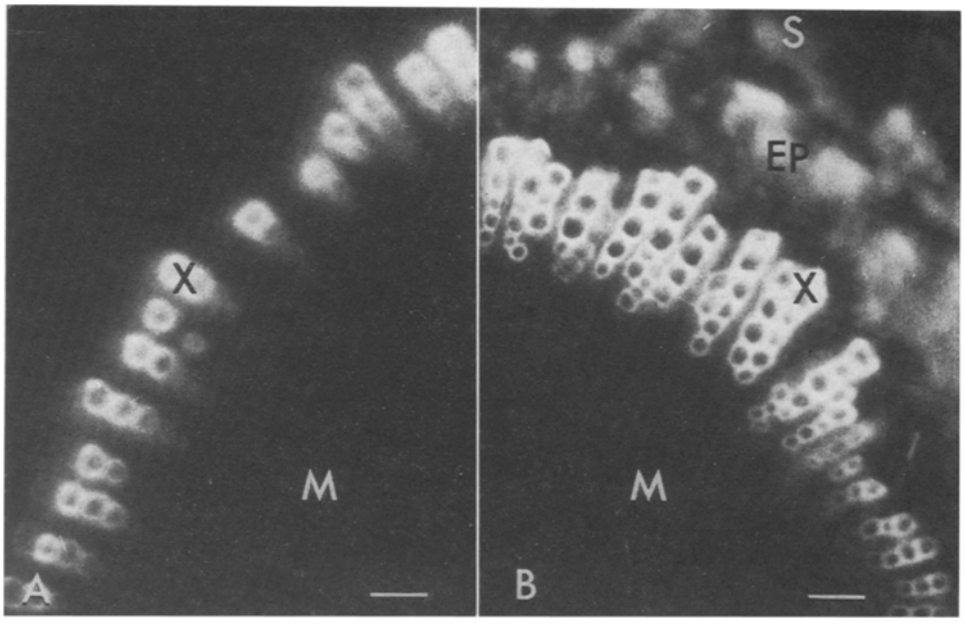

Fig. 1. Fluorescentie in ultraviolet licht van coupes, gekleurd met anilineblauw en afkomstig van jonge stengels van een gezonde Vinca-plant (A) en van een enigszins oudere stengel van een plant geïnfecteerd met het agens van de 'spike'-ziekte van de sandelboom (B).
M = merg, EP = buitenfloëem, S = sclerenchym, X = xyleem. De vergrotingsstreep geeft 20 μm weer.

pale blue. Young tissues often lacked sclerenchyma cells (Fig. 1A) or internal phloem elements (Fig. 1A and B). A few brilliant yellow fluorescent spots appeared not only in the sieve cells of infected stems but also in those of healthy ones (Fig. 2A). In preliminary experiments, the intensity of fluorescence and number of fluorescent spots in sections were found to be influenced by a few factors, viz. the concentration of aniline blue, the time interval between sectioning and fixing the samples, the length of staining time and the time of exposure to ultraviolet light. Fluorescence often appeared as a band covering the zone of phloem in infected stems, but not in healthy stems. The cells in this band were frequently brightly fluorescent and some cells had diffused fluorescence (Fig. 2B, arrow).

In an attempt to identify the fluorescent content of the cells, the parts of the stems that were used for fluorescence microscopy (Fig. 3A) were processed for electron microscopy. A micrograph (Fig. 3B) of a 10 μm thick section of embedded stem served as a reference for electron microscopy of ultrathin sections of the same stem.

Electron microscopy. Examinations of a series of sections yielded sufficient information to permit a comparison of healthy and infected *Vinca* stems as to cyto- and histopathological disturbances caused by mycoplasma (Fig. 4A and B). The drawings

Fig. 2. Fluorescence in ultraviolet light of sections of an older healthy *Vinca* stem (A) and of a stem infected with the sandal spike agent (B).
EP = external phloem, IP = internal phloem, S = sclerenchyma, X = xylem. Bar represents 10 μm.

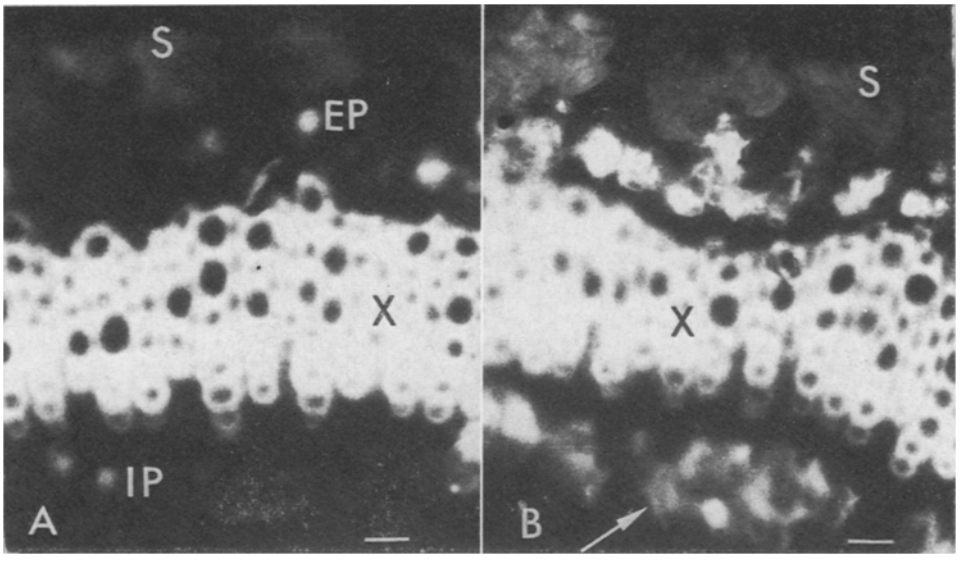

Fig. 2. Fluorescentie in ultraviolet licht van coupes afkomstig van een oudere stengel van een gezonde Vinca-plant (A) en van een stengel geïnfecteerd met het agens van de 'spike'-ziekte van de sandelboom (B).
EP = buitenfloëem, IP = binnenfloëem, S = sclerenchym, X = xyleem. De vergrotingsstreep geeft 10 μm weer.

were made from micrographs of sections viewed at a magnification of times 1,400 at a 2-fold enlargement.

The collapsed sieve elements (arrows), abnormal in size, shape and arrangement, were often filled with anomalous mycoplasmas. No normal-looking (here-after called normal) mycoplasmas were observed in these cells (Fig. 4B). Increased production of the secondary phloem (hyperplasia) was evident and these elements contained abundant normal mycoplasmas (Fig. 5). Hypertrophy was observed not only in the sieve elements but also in the phloem parenchyma (Fig. 4B).

Abnormal accumulation of material, possibly callose, was observed in phloem cells that contained normal mycoplasmas but not in collapsed cells (Fig. 5). In the proximity of collapsed cells 'definitive callose' (Currier, 1957) was observed often in the sieve elements filled with mycoplasmas (Fig. 6). The phloem cells near collapsed cells contained a mixed population of swollen mycoplasma, strongly osmiophilic, small bodies, and empty sac-like membranes (Fig. 7). Abnormal callose depostion on the secondary wall was often found associated with degenerating middle lamella (Fig. 8).

Discussion

The difficulty of locating sieve cells containing mycoplasmas in electron microscopy

Fig. 3. Comparison of a section of a fresh stem, stained and viewed in ultraviolet light (A), and a section of embedded stem, viewed in ordinary light (B). Both samples were obtained from the same diseased stem.
S = sclerenchyma, X = xylem. Bar represents 10 μm.

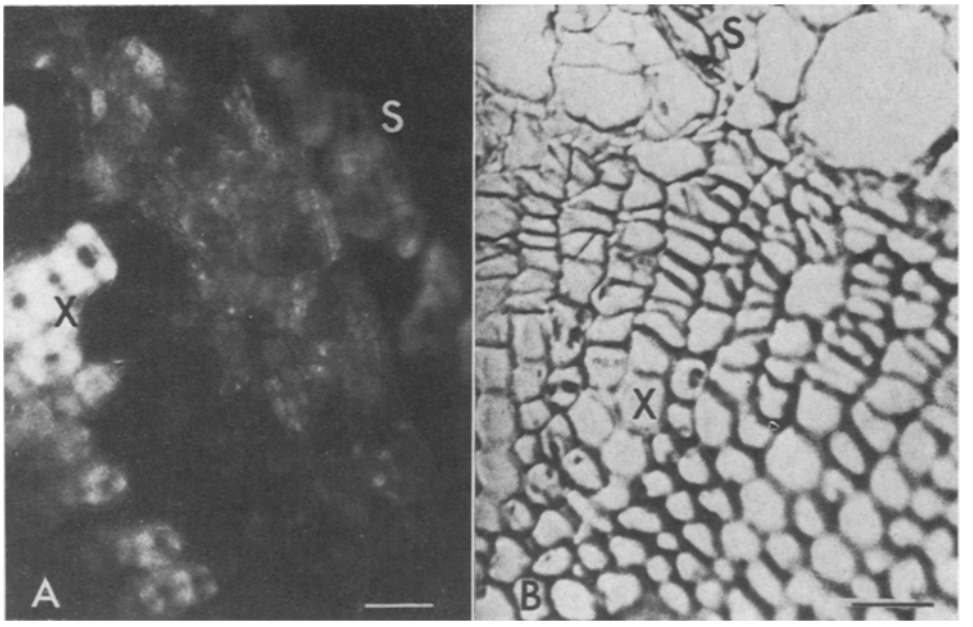

Fig. 3. Vergelijking van een coupe van een verse stengel, gekleurd en bekeken in ultraviolet licht (A) en een ingebedde stengel, bekeken in gewoon licht (B). Beide monsters waren afkomstig van dezelfde zieke stengel.
S = sclerenchym, X = xyleem. De vergrotingsstreep geeft 10 μm weer.

(Horne, 1972) is considerably reduced by combining examinations of thick sections of embedded material with observation of aniline blue-stained sections by means of fluorescence microscopy. Thick sections from the same block were used to locate microscopically a specific phloem area that corresponded to the fluorescing zone. When abundant fluorescence was detected in the light microscope, numerous mycoplasmas were found in ultrathin sections from the same phloem area with the electron microscope. This result strongly suggests a positive correlation between the two phenomena.

Callose is known chemically as β -1,3-D-glucan (Aspinall and Kessler, 1957; Feingold et al., 1958). The aniline blue technique allows precise identification and localization of callose (Eschrich and Currier, 1964). Callose also accumulates on the walls close to the injury by infection with viruses (Wu and Dimitman, 1970; Hiruki and Tu, 1972) and fungi (Aist and Williams, 1971; Hiruki, unpublished data). Carle (1965) found that in grapevine flavescence dorée disease the internal lesions in absence of fluorochrome gave a strong fluorescence and the stain hardly enhanced this effect, whereas callose fluoresces only after staining with aniline blue. Therefore, he concluded that the lesions did not contain accumulated callose. Cousin and Grison (1966) also rejected the possibility of accumulation of callose being the main cause of fluorescence in stems of stolbur-affected plants of *Solanum nigrum* and *Datura*

Fig. 4. Drawings traced from composite electron micrographs of sections of stems from a healthy *Vinca* plant (A) and a plant infected with the sandal spike agent (B). Note the collapsed sieve elements (arrows).
. = anomalous mycoplasma form, o = normal mycoplasma form, ● = a mycoplasma form with dense ribosomes. S = sclerenchyma, X = xylem. Bar represents 10 μm.

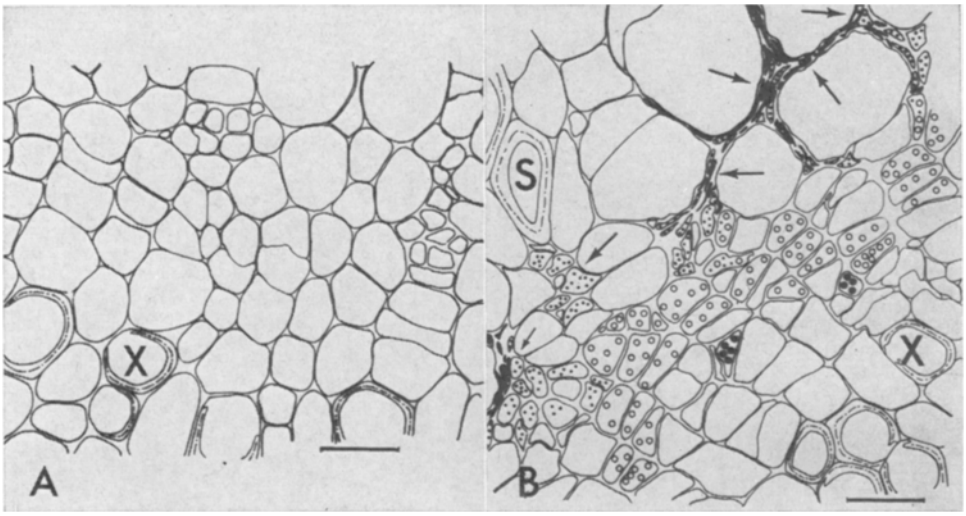

Fig. 4. Tekeningen gemaakt van elektronenmicroscopische foto's van stengelcoupes van een gezonde Vinca-plant (A) en van een plant geïnfecteerd met het agens van de 'spike'-ziekte van de sandelboom (B). Let op de te gronde gegane zeefelementen (pijlen).
. = abnormale mycoplasma-vorm, o = normale mycoplasma-vorm,
● = een mycoplasma-vorm met dichte ribosomen. S = sclerenchym, X = xyleem. De vergrotingsstreep geeft 10 μm weer.

stramonium on the basis of negative reaction with lacmoid. In contrast to these earlier observations, our results clearly show that callose, abnormally deposited on the phloem walls, contributes largely to increased fluorescence as detected in infected stems. In regard to the function of callose, Currier (1957) considered callose as a protective material against leakage from intact wound border cells or as a substance playing a role in constriction of plasmodesmata in pits and sieve area. On the other hand, Müller-Stoll and Lerch (1957) preferred to consider callose as a by-product of disturbed metabolism in the cell wall-cytoplasma region. In any case, it appears that the presence of mycoplasmas greatly influences the accumulation of callose on the phloem walls. This observation agrees with those reported earlier (Hiruki and Shukla, 1973; Hiruki and Dijkstra, 1973).

Degeneration of the middle lamella is another pathological feature found in the phloem cell wall in this investigation. It was closely related to the concomitant accumulation of callose on the walls (Fig. 8). It should be pointed out that the middle la-

Fig. 5. Normal and anomalous forms of mycoplasma in sieve elements of stems from a *Vinca* plant infected with the sandal spike agent. Note abnormal deposition of a substance, possibly callose, (arrows), on the walls.
AM = anomalous mycoplasma, NM = normal mycoplasma. Bar represents 1 μm.

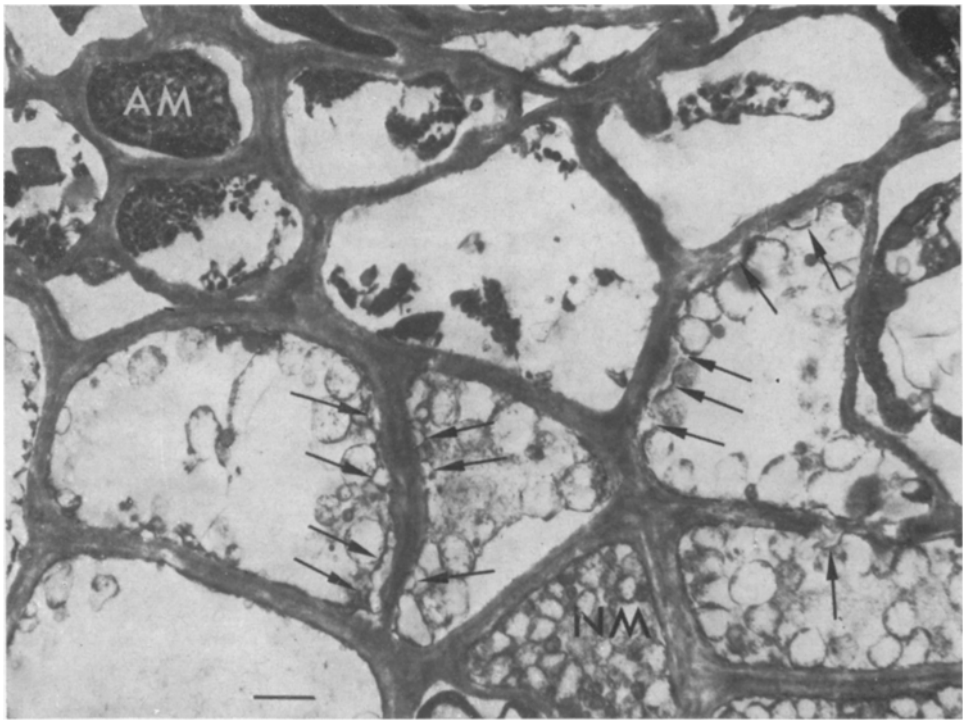

Fig. 5. Normale en abnormale vormen van mycoplasma in zeefelementen van stengels van een Vinca-plant, geïnfecteerd met het agens van de 'spike'-ziekte van de sandelboom. Let op de abnormale afzetting van een stof, vermoedelijk callose, (pijlen) op de wanden.
AM = abnormaal mycoplasma, NM = normaal mycoplasma. De vergrotingsstreep geeft 1 μm weer.

mella when degenerating releases Ca^{++} which stimulates callose formation (Eschrich, 1957).

The second possible source of fluorescence in infected phloem cells are the mycoplasmas themselves. Aniline dyes are widely used for bacteriological and mycological purposes and positively stain some walled or wall-less microorganisms. Unfortunately, the in vitro culture of the sandal spike agent is not in our hands as yet for direct examination to prove or disprove this hypothesis. However, at least one mycoplasma is known to synthesize glucan which may be compared to bacterial slime or capsular polysaccharide (Plackett et al., 1963). It is premature to generalize this finding, but a possibility that some *Mycoplasma* species may react with a fluorochrome (aniline blue) should not be excluded.

Whatever the cause of fluorescence in the phloem cells of infected *Vinca* plants may be, the use of fluorescence microscopy in combination with electron microscopy is a positive advantage in the study of mycoplasma infection.

Fig. 6. Definitive callose accumulation in the sieve area of a *Vinca* plant infected with the sandal spike agent.
DC = definitive callose, M = mycoplasma. Bar represents 1 μm.

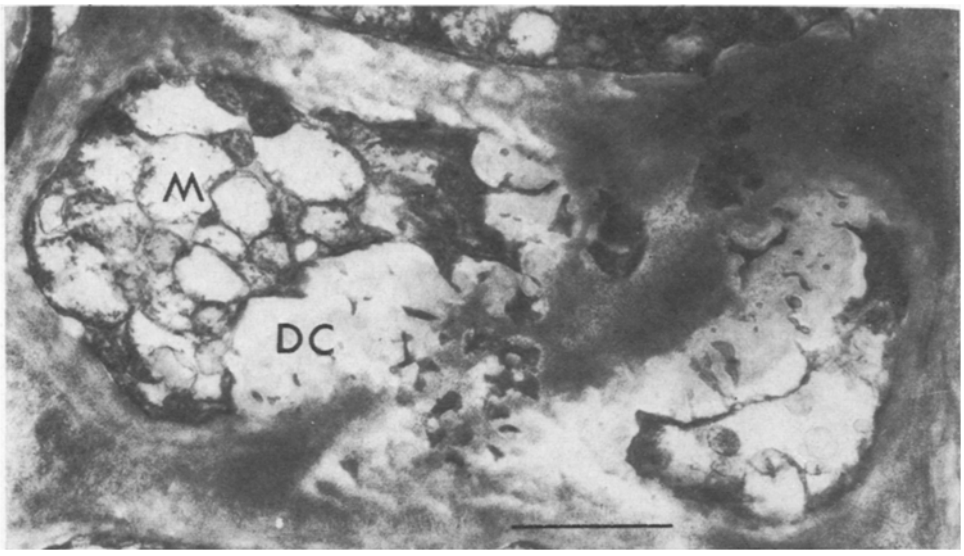

Fig. 6. Ophoping van 'definitive callose' in het zeefgebied van een Vinca-plant, geïnfecteerd met het agens van de 'spike'-ziekte van de sandelboom.
DC = 'definitive callose', M = mycoplasma. De vergrotingsstreep geeft 1 μm weer.

Fig. 7. The occurrence of a mixed population of a swollen form and strongly osmiophilic small form of mycoplasma in a phloem cell near collapsed cells. Bar represents 1 μm.

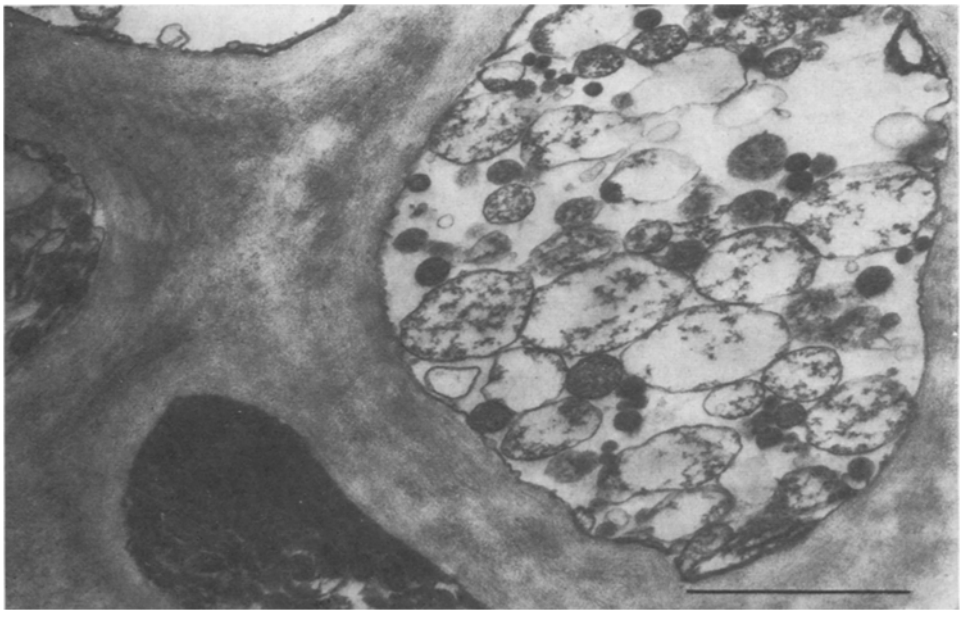

Fig. 7. Het gezamenlijk voorkomen van een gezwollen en een sterk osmiofiele, kleine vorm van mycoplasma in een floëemcel dichtbij te gronde gegane cellen. De vergrotingsstreep geeft 1 μm weer.

Fig. 8. Pronounced callose deposition (arrows) on the degenerating wall of phloem cells of infected *Vinca* stem containing mycoplasmas. Note the degenerating middle lamella.
M = mycoplasma. Bar represents 250 nm.

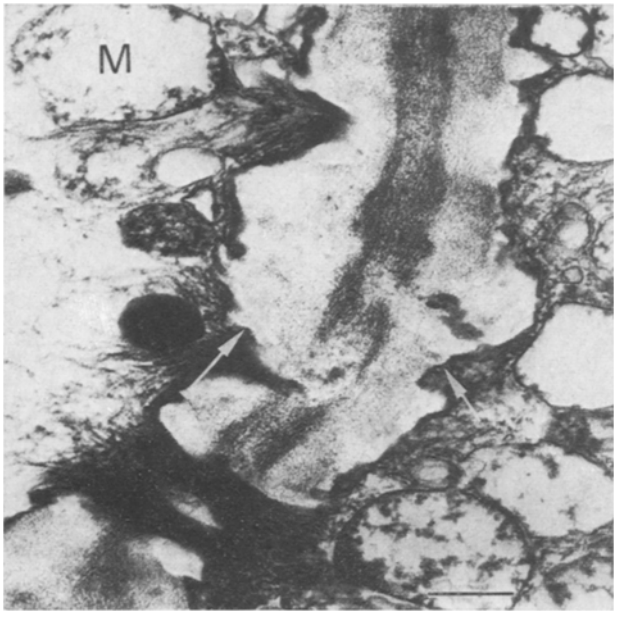

Fig. 8. Sterke calloseafzetting (pijlen) op de degenererende wand van floëemcellen van een geïnfecteerde Vinca-stengel met mycoplasma's. Let op de degenererende middenlamel.
M = mycoplasma. De vergrotingsstreep geeft 250 nm weer.

Samenvatting

Licht- en elektronenmicroscopie van Vinca-planten geïnfecteerd met mycoplasma-achtige lichaampjes van de 'spike'-ziekte van de sandelboom

Het opsporen van floëemcellen met mycoplasma-achtige lichaampjes (verder aangeduid als mycoplasma's) van de 'spike'-ziekte van de sandelboom in *Vinca rosea* werd vergemakkelijkt door bestudering van stengelcoupes van deze plant met behulp van fluorescentiemicroscopie. Met deze laatste techniek, waarbij de coupes werden gekleurd met 0,01 % anilineblauw, waren duidelijke verschillen waarneembaar tussen gezonde en zieke planten (Fig. 1A en B). Enkele heldergeel fluorescerende stippen verschenen niet alleen in de zeefcellen van geïnfecteerde stengels maar ook in die van gezonde (Fig. 2A). In zwaar aangetaste stengels was de fluorescentie gewoonlijk zichtbaar als een band, die zich over het gehele floëemgebied uitstrekte (Fig. 2B).

Teneinde de fluorescerende inhoud van de cellen te identificeren werden gedeelten van de stengels, die gebruikt waren voor fluorescentiemicroscopie (Fig. 3A), verwerkt voor elektronenmicroscopie. Een microfoto (Fig. 3B) van een 10 μm-dikke coupe van de ingebedde stengel diende als vergelijking voor ultradunne coupes van dezelfde stengel.

De tekeningen (Fig. 4A en B) werden gemaakt van samengestelde elektronen-

microscopische foto's van coupes van gezonde en geïnfecteerde stengels teneinde de histo-pathologische verstoringen, als gevolg van mycoplasma-infectie te vergelijken.

De te gronde gegane zeefelementen (Fig. 4B, pijlen), die er wat betreft hun grootte, vorm en rangschikking abnormaal uit zagen, waren vaak gevuld met abnormale mycoplasma's. Er trad een duidelijke extra-produktie op van secundair floëem (hyperplasie) en de cellen hiervan bevatten normaal-uitziende mycoplasma's en abnormale afzetting van een stof, vermoedelijk callose, op de wanden (Fig. 5). Hypertrofie werd niet alleen in de zeefvaten maar ook in het floëemparenchym waargenomen (Fig. 4B). Dichtbij de te gronde gegane cellen werd in de zeefcellen vaak 'definitive callose' (Currier, 1957) aangetroffen (Fig. 6). De floëemcellen in de buurt van de te gronde gegane cellen bevatten een mengsel van gezwollen mycoplasma's, sterk osmiofiele, kleine lichaampjes en lege, zak-achtige membranen (Fig. 7). De abnormale afzetting op de secundaire celwanden ging bij een geïnfecteerde stengel vaak gepaard met het degenereren van de middenlamel (Fig. 8).

We concluderen dat vooral de callose, in abnormale hoeveelheden afgezet op de floëemwanden van geïnfecteerde stengels, verantwoordelijk is voor de sterke fluorescentie. De mogelijkheid is evenwel niet uitgesloten dat mycoplasma's zelf ook reageren met anilineblauw.

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