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A histochemical study on sandal (Santalum album) affected with spike disease and its diagnostic value

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Abstract

Fluorescence microscopy of cross sections of young twigs from sandal trees, stained with aniline blue, showed a marked difference in fluorescence in the phloem area of healthy and spike-diseased trees. In sections of twigs from healthy trees fluorescence was restricted to the outer zone of the phloem whereas the phloem zone in spike-diseased trees fluoresced over its total area. Older twigs and leaves, but not roots, showed a similar phenomenon. The diagnostic value of this method is discussed.

Introduction

Fluorescence microscopy has been used to demonstrate changes occurring in plants infected with yellows-type diseases. In some of the studies primary fluorescence of the different tissues was compared (Cousin and Grison, 1966; Cousin, 1967; Cousin et al., 1968). In other cases fluorescence was observed after staining with fluorochromes like thioflavin T and acridin orange (Carle, 1965) and aniline blue (Hiruki and Shukla, 1973; Hiruki and Dijkstra, 1973b).

Aniline blue is a stain which is used in fluorescence microscopy to specifically detect callose in plant tissues and cells (Eschrich and Currier, 1964). This staining technique was introduced by Arens in 1949 and further developed by Fidalgo (1954), Currier and Strugger (1956), Currier (1957) and Hiruki and Tu (1972). It is much more sensitive than that in which ordinary light is used.

Hiruki and Shukla (1973) found in their study on witches' broom disease of *Dicentra spectabilis* that, after staining sections of stems and petioles with aniline blue, the fluorescence was much more intensive in affected phloem elements than in healthy ones. Because of the specific reaction of aniline blue with callose these authors attributed the excessive fluorescence in diseased tissue to abnormal accumulation of callose.

By combined electron microscopy and fluorescence microscopy of *Vinca* plants affected with the sandal spike disease, Hiruki and Dijkstra (1973b) showed that abnormal amounts of callose deposits on the walls of sieve elements contributed largely to the increased fluorescence in the phloem area. When increased fluorescence was detected by light microscopy, numerous mycoplasmas were found in ultrathin sections from the same area with the electron microscope. This suggested a positive correlation between abnormal accumulation of callose in the phloem and the mycoplasmas.

We have now tried to find out whether the difference in fluorescence between healthy and spike-diseased plants after staining with aniline blue also occurs in the sandal tree (*Santalum album*) itself.

Diagnosis of the spike disease in sandal trees has been impossible in an early stage of infection, when external symptoms are not yet visible. Therefore, we also investigated the possible diagnostic value of fluorescence microscopy.

Materials and methods

Trees of sandal were grown from seed and maintained in 30 cm pots in the glasshouse. At the time of investigation they were about 5 years old. In the same pots plants of *Pongamia glabra* or *Acacia farnesiana* were planted on which the sandal could haustorise.

The tree, from which most of the samples were taken, had been grafted with shoots from spike-diseased trees about one year earlier and developed typical spike symptoms in some branches about three months after grafting. At the time of this study the upper part of the tree was completely diseased, whereas some of the lower branches still bore normal leaves and flowers (Fig. 1).

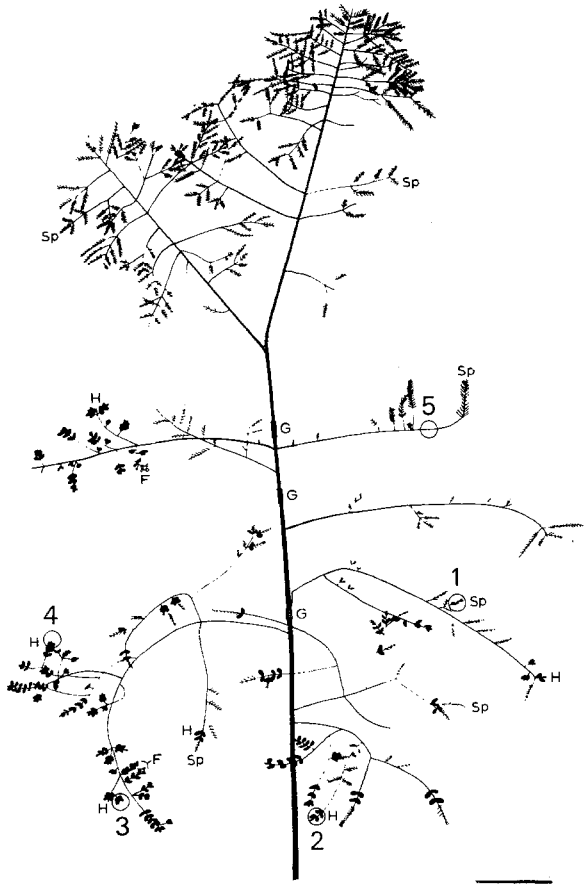


Fig. 1. Drawing of a 5-year-old sandal tree infected with the spike disease after grafting. 1–5: places wherefrom samples were taken. F = flower, G = place where the tree has been grafted, H = healthy-looking leaves, Sp = spike-showing leaves. Bar represents 20 cm.

Fig. 1. Tekening van een 5-jaar-oude sandelboom, na enting geïnfecteerd met de 'spike'-ziekte. 1–5: plaatsen waar monsters zijn genomen. F = bloem, G = plaats van enting, H = gezond-uitziende bladeren, Sp = 'spike'-vertonende bladeren. De vergrotingsstreep geeft 20 cm weer.

Another tree developed spike symptoms about 16 weeks after strands of dodder (*Cuscuta subinclusa*), established on spike-diseased *Vinca* plants, were trained onto it (for more details see Dijkstra and Lee, 1972). After some lower branches had died the rest of the tree did not show any more visible symptoms of the disease at the time of this investigation.

Young and older twigs from healthy trees as well as from healthy-looking and spike-showing parts of diseased trees were sampled. In some cases leaves and roots from both healthy trees and heavily diseased trees were also examined.

The non-woody samples were sectioned at about 20 μm thickness with a microtome designed by Hooker (1967) and slightly improved by the same author in 1970 (unpublished). From woody plant parts also free-hand sections were made.

All sections were prepared within 10 min after collection of the samples and fixed immediately by boiling in tap water for about 8 min. They were then stained in a small amount of aniline blue solution (0.1 % aniline blue in 1/15 M K_3PO_4, pH 12.2) on a microscope slide with a cover glass, and examined after about 20 min.

Observations were made with a Wild microscope with a high pressure mercury vapour lamp (HBO 200 W). One ultraviolet fluorescence exciter filter (UG 1) and a 'Rotdämpfungsfilter' (BG 38) with maximum transmission at 366 nm absorbed the visible spectrum. Moreover, a blue light fluorescence exciter filter (BG 12, 390–440 nm) was added. A barrier filter (GG 13 C) was placed in the ocular tube of the microscope.

Photographs were taken with a Wild attachment (index number 14), using Gevapan 30 plates and Ilford FP 4 film, both 22 DIN (125 ASA).

Results

Transverse sections of a young twig from a spike-showing branch of a heavily diseased tree (Fig. 1 (sample 1) and 2) showed a bright yellow fluorescence throughout the phloem (Fig. 2B). Besides this, dull greyish-yellowish primary fluorescence was visible in sclerenchyma and xylem. Transverse sections of a comparable young twig from a healthy tree (Fig. 3) showed only some fluorescent spots in the outer zone of the phloem (Fig. 3B).

Samples were also taken from still healthy-looking parts at different positions on the diseased tree (Fig. 1, samples 2, 3 and 4). In samples 2 and 3 a marked fluorescence, intermediate between that of healthy and spike-showing twigs, was visible (Fig. 4 and 5, respectively). A cross section of a very young shoot, which had just developed (Fig. 1 (sample 4) and 6), had normal fluorescence only in the outer zone of the phloem (Fig. 6B).

Transverse sections of older, woody twigs with spike-showing leaves (Fig. 1 (sample 5) and 7) exhibited a more widely spread fluorescence (Fig. 7B) than comparable twigs from healthy trees (Fig. 8A and 8B), although the difference was less prominent than in the case of young twigs.

Sections were also made of young and older twigs (Fig. 9 and 10, respectively) with normal-looking leaves from another tree, infected by means of dodder about a year earlier without symptoms after some branches had died. The fluorescence observed (Fig. 9B and 10B, respectively) was comparable to that in twigs with spike-showing leaves.

Fig. 2. Transverse section of diseased, young sandal twig (sample 1, Fig. 1), viewed in ordinary light (A) and in ultraviolet light after staining with aniline blue (B). Note the numerous bright fluorescent spots all over the phloem. P = phloem, S = sclerenchyma, X = xylem. Bar represents 100 μm, also in the following figures.

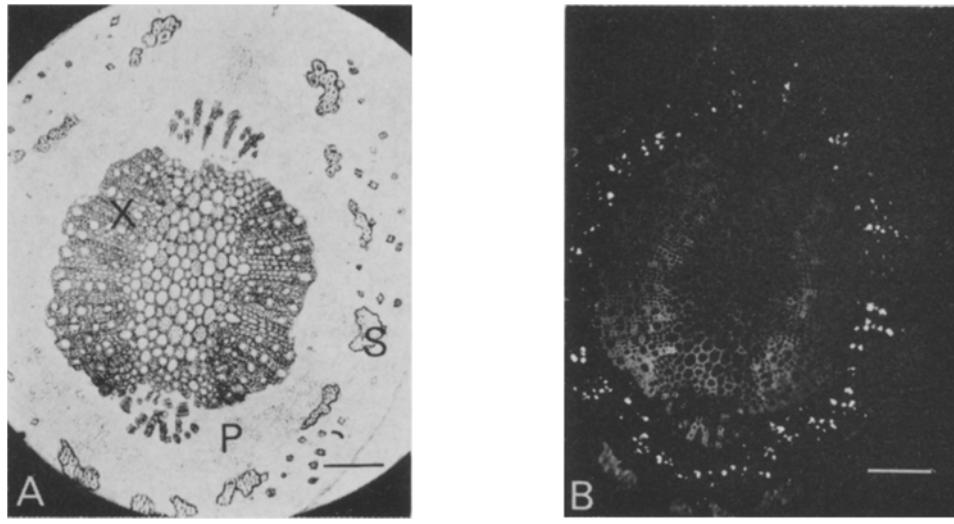


Fig. 2. Dwarsdoorsnede door een ziek, jong takje van een sandelplant (monster 1, Fig. 1), bekeken in gewoon licht (A) en in ultraviolet licht na kleuring met anilineblauw (B). Let op de talrijke, helder-fluorescerende stippen over het gehele floëem. P = floëem, S = sclerenchym, X = xyleem. De vergrotingsstreep geeft 100 μm weer, ook in de volgende figuren.

Fig. 3. Healthy, young sandal twig. The few bright fluorescent spots are restricted to the outer zone of the phloem.

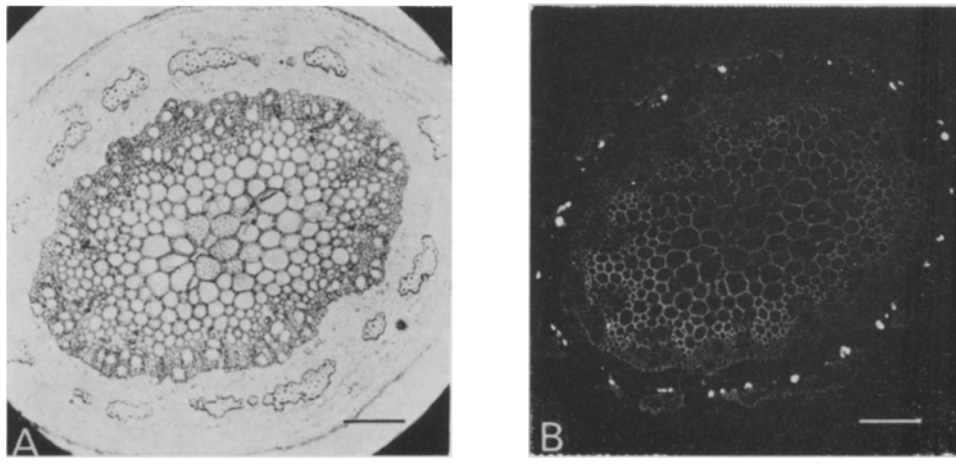
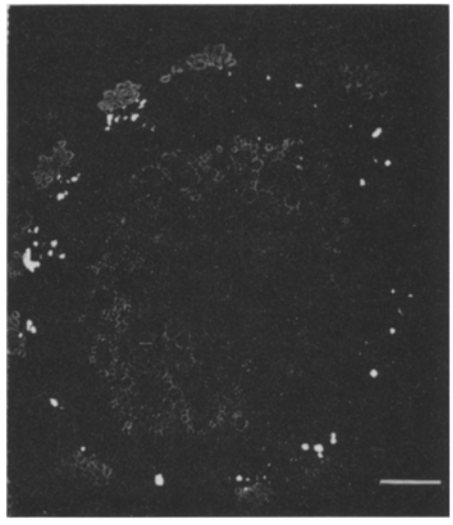


Fig. 3. Gezond, jong takje van een sandelplant. De niet-talrijke helder-fluorescerende stippen zijn beperkt tot de buitenste zone van het floëem.

Fig. 4. (left). Healthy-looking, young sandal twig from a diseased tree (sample 2, Fig. 1). Bright fluorescent spots also occur in the inner zone of the phloem.

Fig. 5 (right). Healthy-looking, young sandal twig from a diseased tree (sample 3, Fig. 1). The numerous bright fluorescent spots also occur in the inner zone of the phloem.



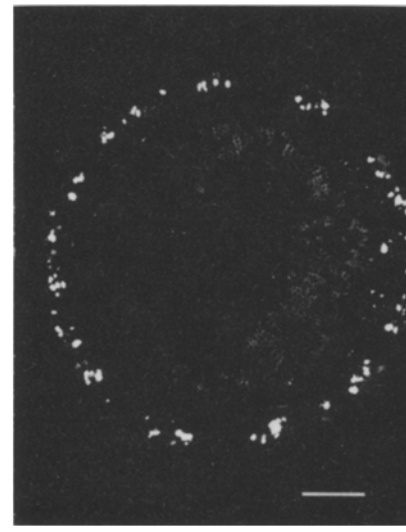
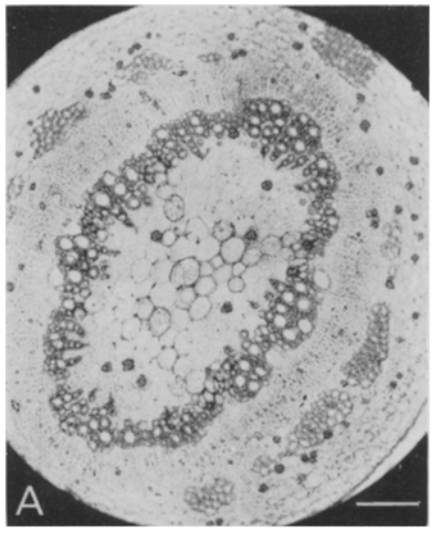


Fig. 4 (links). Gezond-uitziend, jong takje van een zieke sandelplant (monster 2, Fig. 1). Helder-fluorescerende stippen komen ook voor in de binnenste zone van het floëem.

Fig. 5 (rechts). Gezond-uitziend, jong takje van een zieke sandelplant (monster 3, Fig. 1). De talrijke helder-fluorescerende stippen komen ook voor in de binnenste zone van het floëem.

Fig. 6. Healthy-looking, very young sandal twig from a diseased tree (sample 4, Fig. 1). The very small number of bright fluorescent spots are restricted to the outer zone of the phloem.



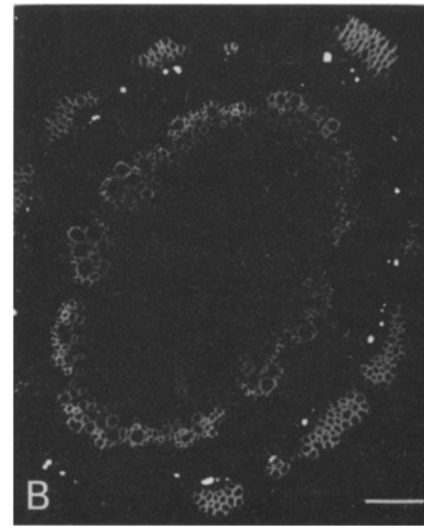


Fig. 6. Gezond-uitziend, zeer jong takje van een zieke sandelplant (monster 4, Fig. 1). Het zeer geringe aantal fluorescerende stippen is beperkt tot de buitenste zone van het floëem.

Fig. 7. Diseased, older (woody) sandal twig (sample 5, Fig. 1). Note the numerous bright fluorescent spots all over the phloem. P = phloem, S = sclerenchyma, X = xylem.

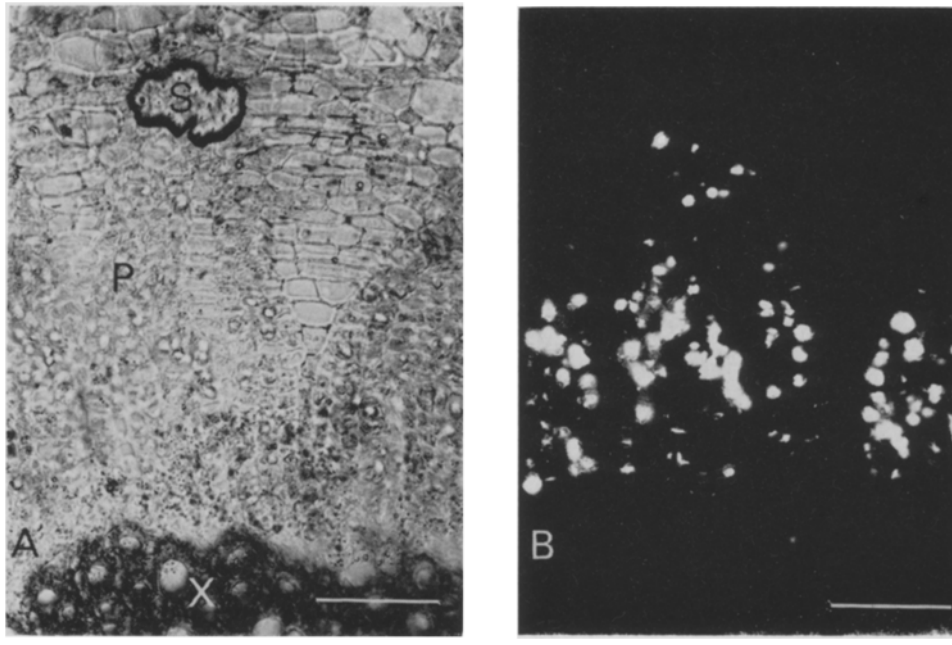


Fig. 7. Ziek, ouder (houtig) takje van een sandelplant (monster 5, Fig. 1). Let op de talrijke, helder-fluorescerende stippen over het gehele floëem. P = floëem, S = sclerenchym, X = xyleem.

Fig. 8. Healthy, older (woody) sandal twig with the same diameter as that in Fig. 7. An appreciable number of fluorescent spots are visible in the phloem.

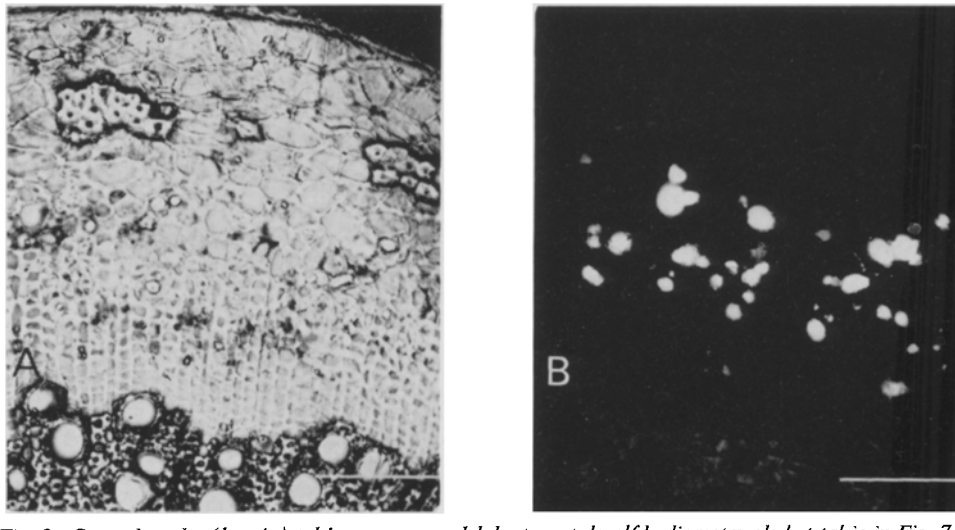


Fig. 8. Gezond, ouder (houtig) takje van een sandelplant, met dezelfde diameter als het takje in Fig. 7. Een aanzienlijk aantal fluorescerende stippen is zichtbaar in het floëem.

Fig. 9. Young twig from a healthy-looking but infected sandal tree. Note the numerous bright fluorescent spots all over the phloem.

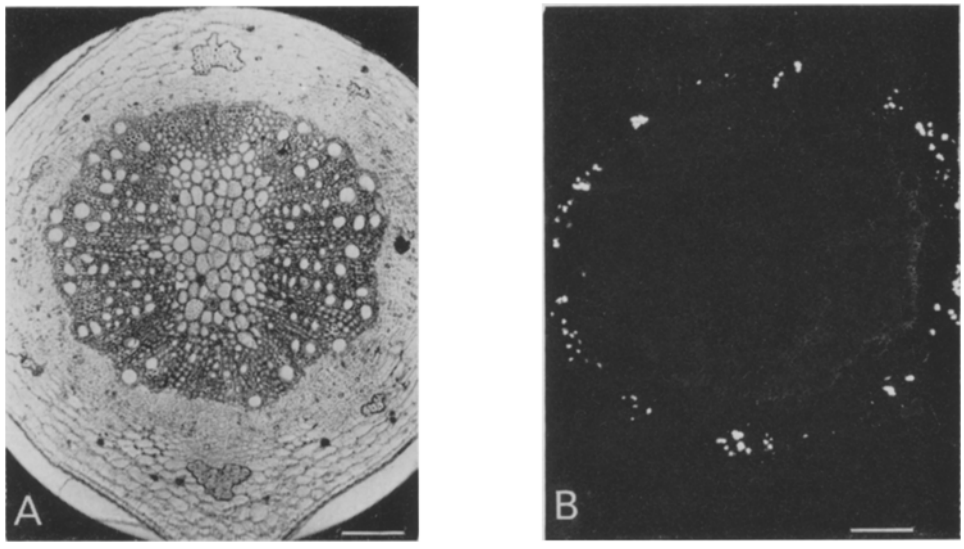


Fig. 9. Jong takje van een gezond-uitziende doch geïnfecteerde sandelplant. Let op de talrijke, helder-fluorescerende stippen over het gehele floëem.

Fig. 10. Older (woody) twig from a healthy-looking but infected sandal tree. The twig had the same diameter as those in Figs. 7 and 8. Note the numerous fluorescent spots all over the phloem.

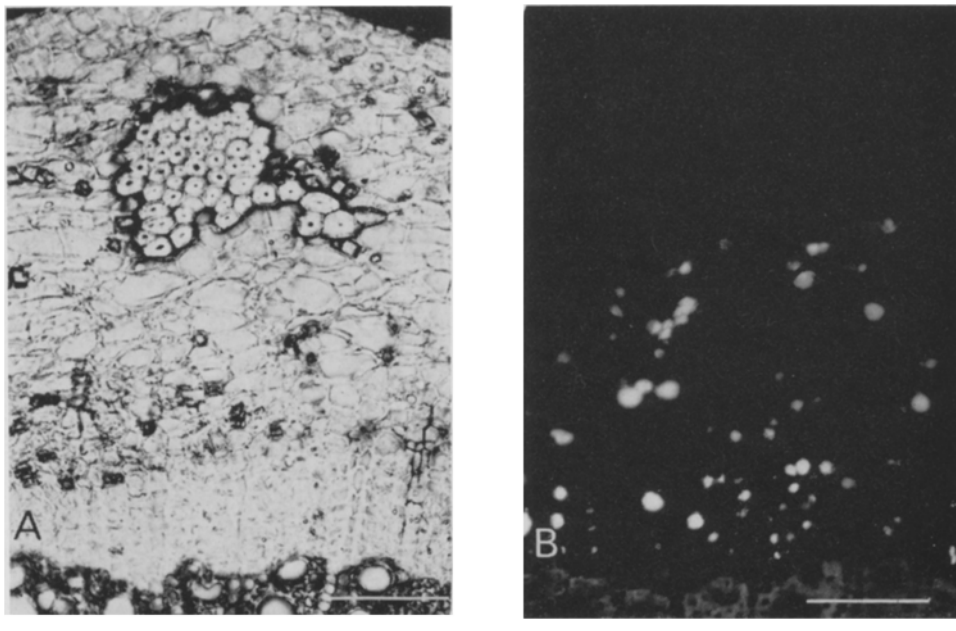


Fig. 10. Ouder (houtig) takje van een gezond-uitziende doch geïnfecteerde sandelplant. Het takje had dezelfde diameter als dat in Fig. 7 en 8. Let op de talrijke, fluorescerende stippen over het gehele floëem.

Sections of leaves from healthy trees (Fig. 11) showed only a minor fluorescence in the outer zone of the phloem in vascular bundles (Fig. 11B), whereas the phloem zone in comparable sections of spike-showing leaves (Fig. 12) fluoresced over a wider area (Fig. 12B).

Fig. 11. Healthy sandal leaf. The very few bright spots are restricted to the outer zone of the phloem of the vascular bundle. P = phloem, X = xylem.

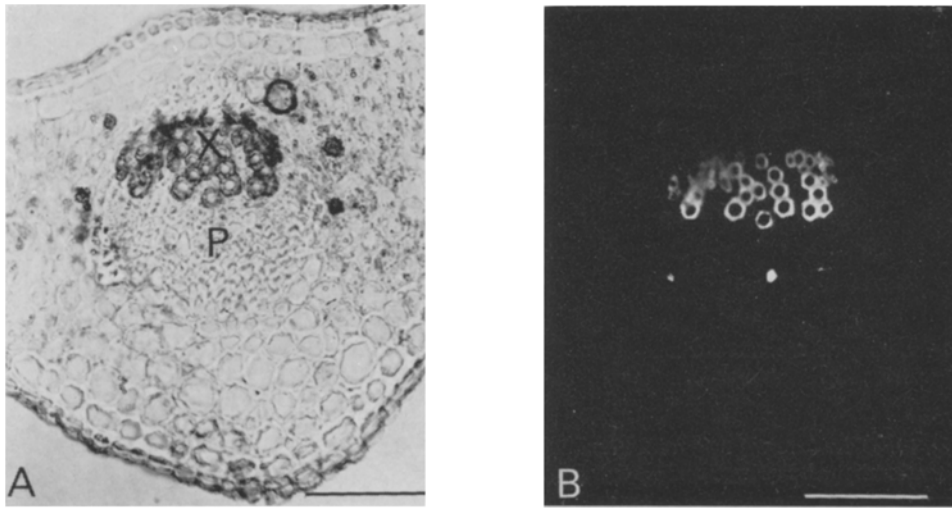


Fig. 11. Gezond sandelblad. De zeer schaarse, helder-fluorescerende stippen zijn beperkt tot de buitenste zone van het floëem van de vaatbundel. P = floëm, X = xyleem.

Fig. 12. Spike-showing sandal leaf. The fluorescent spots are scattered all over the phloem zone.

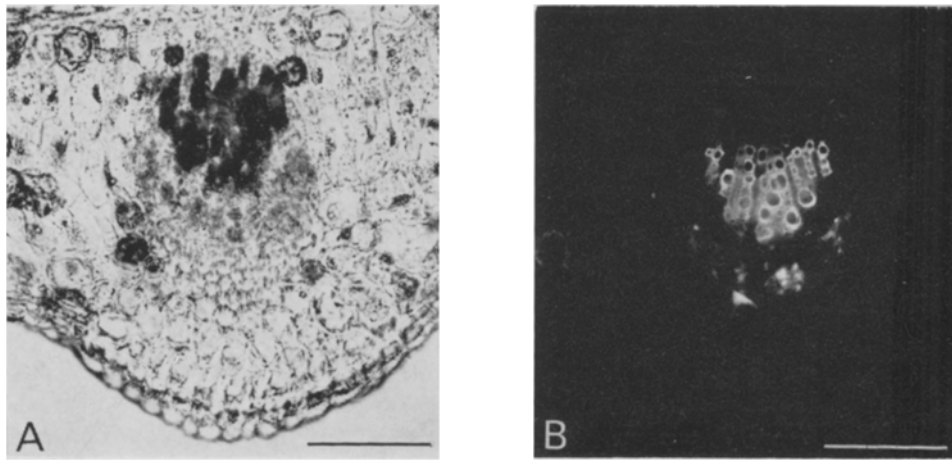


Fig. 12. 'Spike'-vertonend sandelblad. De fluorescerende stippen zijn over het gehele floëem verspreid.

The degree of fluorescence in sections of roots was more or less the same in healthy and diseased plants.

Discussion

Callose formation is known to very rapidly occur within cells in response to injury or penetration of chemicals (Currier and Strugger, 1956; Currier, 1957; Eschrich, 1956, 1963; Evert and Derr, 1964), or infection by viruses (Wu and Dimitman, 1970; Hiruki and Tu, 1972) and fungi (Aist and Williams, 1971). In earlier experiments (Hiruki, unpublished) it has been confirmed that callose is easily formed due to injury. Therefore, the time interval between sampling of the material and fixation by boiling is very critical. Even healthy material may show appreciable fluorescence all over the phloem when sections are not fixed quickly. Having minimised the chances of formation of wound callose in all our specimens by proper precautions, the fluorescence in the outer zone of the phloem in young twigs from healthy plants may be attributed to definitive callose. Definitive callose is one that accumulates on the walls of sieve elements which cease to function (Esau, 1965), and normally occurs in degenerating sieve elements (Evert and Derr, 1964). Eventually it disappears in old, nonfunctioning sieve elements.

In case of older woody twigs the situation is more complicated as the phloem zone is much wider in diseased twigs than in healthy ones of about the same diameter, due to increased production of secondary phloem (hyperplasia) (Fig. 7A and 8A). Moreover, many necrotic cells occur in the phloem and in the cambial layers of old, diseased twigs (Dijkstra and Van der Want, 1970) and stems (Hiruki and Dijkstra, 1973a). Although no abnormal accumulation of callose could be detected in these necrotic cells, sieve elements in the proximity of the latter contained definitive callose (Hiruki and Dijkstra, 1973b). Consequently, more fluorescence could be expected to occur in diseased twigs than in healthy ones, in spite of the fact that the amount of fluorescence in healthy older twigs is appreciable due to a great number of nonfunctioning sieve elements filled with definitive callose.

Mycoplasma-like bodies could not be observed electron microscopically in healthy-looking parts of the diseased trees (Dijkstra and Ie, unpublished), probably due to their low concentration. Nevertheless, the abnormal callose formation in those parts, observed by us and reported here, must have been due to a reaction of the host plant to the mycoplasma-like bodies, even when present at low concentrations.

The fact that green twigs from normal-looking branches of infected trees, even without visible symptoms, showed a marked fluorescence in the phloem, offers prospects for diagnosis. With precautions mentioned earlier, the examination of green twigs (neither woody, nor very young) from different positions on healthy-looking sandal trees, in close vicinity of the diseased trees already showing spike symptoms, may also prove to be valuable in detecting the disease, if present, in an early stage. However, the suggested diagnostic value of this method can be verified only by more extensive, large scale field trials in areas where sandal trees are grown in their natural environment.

Samenvatting

Een histochemisch onderzoek van sandel (Santalum album) aangetast door de 'spike'-ziekte, en zijn diagnostische waarde

Dwarscoupes van jonge takjes van sandelbomen werden gekleurd met 0,1 % aniline-blauw en de fluorescentie in ultraviolet licht met de microscoop bestudeerd.

Van een 'spike'-zieke sandelboom, die in een aantal takken de symptomen duidelijk vertoonde, werd een aantal monsters genomen (Fig. 1, monsters 1 t/m 5). Deze monsters bestonden uit jonge, groene takjes van een 'spike'-vertonende tak (1), gezond uitziende takken (2, 3 en 4), en uit een ouder, verhout takje van een 'spike'-vertonende tak (5). Tevens werd onderzoek verricht aan een sandelboom, die ongeveer een jaar geleden was geïnfecteerd met de ziekte door middel van *Cuscuta*, doch die, na het afsterven van enige takken, op het moment van onderzoek geen symptomen meer te zien gaf. Als controle diende een gezonde sandelboom.

De monsters 1 (Fig. 2), 2 (Fig. 4), 3 (Fig. 5) en 5 (Fig. 7) vertoonden alle fluorescentie over de gehele breedte van het floëem (Fig. 2B, 4, 5 en 7B) in tegenstelling tot dat van de gezonde boom (Fig. 3 en 8) en monster 4 (een heel jong takje) van de zieke boom (Fig. 6).

Takjes van de schijnbaar gezonde boom, die via *Cuscuta* was geïnfecteerd, vertoonden sterke fluorescentie over de gehele breedte van het floëem (Fig. 9 en 10).

Dwarscoupes van bladeren van een gezonde boom (Fig. 11) en van die met 'spike'-symptomen (Fig. 12) vertoonden ook de verschillen in fluorescentie in het floëem van de vaatbundels. In het eerste geval was de fluorescentie weer beperkt tot de buitenste lagen van het floëem (Fig. 11B), terwijl in het tweede geval fluorescentie in het gehele floëem optrad (Fig. 12B).

Dwarscoupes van wortels van gezonde en zieke bomen gaven ongeveer dezelfde hoeveelheid fluorescentie in het floëem te zien.

De toepassing van fluorescentiemicroscopie biedt perspectieven voor de diagnose van de 'spike'-ziekte, wanneer de sandelboom nog geen uitwendige symptomen vertoont.

Acknowledgments

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