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Bacterial wilt of beans (*Corynebacterium flaccumfaciens* (Hedges) Dowson)

Bønnebakterievisnesyge (Corynebacterium flaccumfaciens (Hedges) Dowson)

Ib G. Dinesen

Summary

The aim of this investigation was to ascertain the movements of *Corynebacterium flaccumfaciens* in the plants and to demonstrate whether special tissues are attacked. In addition to this, the rate of such movements in the plants was investigated. The electron-microscopic analyses aimed, among other things, at establishing what takes place in the plant cell when it is attacked by *C. flaccumfaciens*. When bacteria are injected below the cotyledons in the middle of the stem, a certain propagation takes place in the pith with a subsequent penetration into the oldest primary xylem cells. The movements up into the plant start exclusively in the primary xylem. Bacteria can be observed in the xylem and, besides this the xylem assumes a brown colour. The movement in the plant is relatively slow as it takes between 72 and 96 hours from the time of the injection 2 cm below the cotyledons before bacteria can be isolated from the youngest shoot (the distance being about 6.5 cm).

Electron-microscopic analyses showed a total degradation of the cell wall in the xylem. No clogging material was found, and the wilting of the plant is due to failing transportation of sap caused by the degradation of the xylem.

Key words: *Corynebacterium flaccumfaciens*, bacterial wilt of bean, movement, electron-microscopy, cell wall degradation.

Resumé

Undersøgelsen har til formål at konstatere, hvor bevægelsen af *Corynebacterium flaccumfaciens* finder sted, og om det er specielle væv, der angribes. Desuden undersøges med, hvor stor hastighed fremtrængningen sker i planten. De elektronmikroskopiske optagelser har bl.a. til hensigt at konstatere, hvad der sker i plantecellen ved angreb af *C. flaccumfaciens*. Ved injektion af bakterier under kimbladene i det midterste af stænglen sker der en vis opformering i marven og derefter en indtrængning i de ældste primære xylem-celler. Fremtrængningen op i planten sker udelukkende i det primære xylem. Udover, at der kan iagttages bakterier i xylemet, sker der også en brunfarvning af dette. Fremtrængningen i planten foregår relativt langsomt, idet der går mellem 72 og 96 timer fra injektionen 2 cm under kimbladene, til der kan isoleres bakterier fra det yngste skud (afstanden var ca. 6,5 cm).

Elektronmikroskopiske undersøgelser viste, at der skete en total nedbrydning af cellevæggen hos xylemet. Der konstateredes ingen tilstopningsmaterialer, og nedvisningen af planten skyldes en brist i væsketransporten på grund af det nedbrudte xylem.

Nøgleord: *Corynebacterium flaccumfaciens*, bønnebakterievisnesyge, fremtrængning, elektronmikroskopi, cellevægsnedbrydning.

Introduction

Attacks by *Corynebacterium flaccumfaciens* (Hedges) Dowson on beans (*Phaseolus vulgaris*) were first described i U.S.A. in 1922 (*Hedges*). The disease has not yet been found in Denmark. In a later article (*Hedges,* 1926), the attacks were divided into two types depending on the age of the plant, namely (1) wilting of plantlets, and (2) wilting of older plants. In the case of attacks on young plants, the symptom of an initial attack is a partial dark to bluish-green colouring of the leaves. Then the leaves lose their turgescence and wilt. As a rule, the final result is that the whole plant dies before the first trifoliate leaves have fully developed. In the case of attacks on older plants, the development is, generally, not as drastic as that seen when seed plants are attacked; usually, the older plants survive throughout the growth season and produce seed and, in such cases, only a few shoots or leaves will wilt.

Fig. 1. Bean plant attacked by *Corynebacterium flaccumfaciens*
Bønneplante angrebet af Corynebacterium flaccumfaciens.

Several investigations show that the bacteria may penetrate through injured roots. – At the same time, the bacteria have a certain ability to survive in the soil, which may give rise to new attacks.

Schuster (1959) found that mechanical injuries, nematodes, and wounds may cause sufficient damage to the roots to permit penetration by the bacteria.

The bacteria are unable to force their way into the plant via stomata. Only a wound will permit infection (*Zaumayer,* 1932).

The most important source of infection is infected seed. In bean seeds, the vascular tissue (raphe), which forms a continuation of funiculus, constitutes a part favourable to an internal transmission of the pathogen (*Schuster & Coyne,* 1974) (see fig. 2).

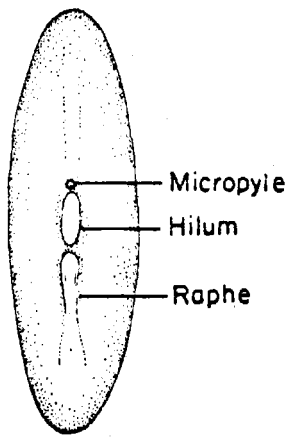

Fig. 2. Bean seed according to *Fahn,* 1974.
Bønnefrø efter Fahn, 1974.

As a rule, the bacteria are found below the avil, but they may also be seen on the avil itself (*Schuster & Christiansen,* 1956). Bacteria in seed have a very great chance of survival. The presence of apparently normal bean plants in a seed field may create problems for the control of *C. flaccumfaciens*. A cool season may prevent the appearance of symptoms, and isolated bacteria from healthy-looking plants gave normal symptoms in glasshouse experiments (*Thomas & Graham,* 1952).

The bacterium may also survive in the soil although the ability of survival is slight there. An investigation made by *Schuster* (1967) showed that straw containing bacteria placed on the surface on the soil was more favourable to transmis-

sion of infection after 10 months than straw placed 10–20 cm below the surface of the soil.

Infected straw from *Amaranthus retroflexus* and *Chenopodium album*, both plants being non-hosts, contained virulent bacteria after 10 months. Even living bacteria were refound after 22 months.

Richard and *Walker* (1965) investigated the influence of various factors on the risk of the occurence of the disease.

The investigation showed that the place of penetration of the bacteria and the growth stage of the plant are decisive for the development of the disease. Although the invasion took place through wounded roots, the disease developed very slowly. When the bacteria were inoculated into the leaves by several injections, they spread to all parts of the plant, but the disease developed rather slowly. Only when the bacteria were injected into the vascular system of the stem, symptoms of wilting quickly appeared. *Richard* and *Walker* (1965) also write that the disease spreads rather slowly in the field. The fact that plants with trifoliate leaves (see fig. 3) showed a much slower development of the disease than plants on which only monofoliate leaves had developed, offers a kind of explanation for the slow spreading. Application of different nutrients made no difference to the intensity of the attacks.

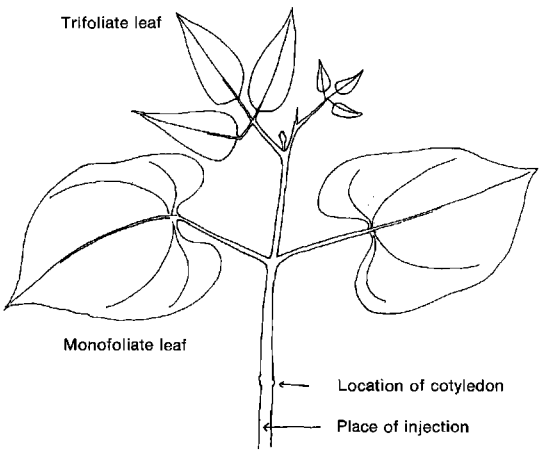

Fig. 3. Bean plant (drawn by *N. Leroul*).
Bønneplante (tegnet af N. Leroul).

All commercial varieties of *Phaseolus vulgaris* are susceptible to *Corynebacterium flaccumfaciens*.

Materials and methods

In the investigation *Phaseolus vulgaris* of the 'Processor' variety was used. The seeds were sown in standard soil (Pindstrup Ready-mix 2) with only one seed per pot. The temperature in the glasshouse was 20–22°C throughout the experiment. Extra light was provided in the experiments started on 28th November, 5th December, and 9th December. The bacteria culture used was *Corynebacterium flaccumfaciens* No. NCPPB 559 from the National Collection of Plant Pathogenic Bacteria, Harpenden, England.

The injection in the plants was made with 48-hour-old *C. flaccumfaciens* cultures. At the time of injection, the bean plants were 14 days old – at the time they had well-developed monofoliate leaves and had reached a height of about 15 cm. The trifoliate leaves had just started appearing. The bacterial concentration was about 10^6 per ml, and the suspension was made in sterile water. The injection was made into the stem, 2 cm below the cotyledons (fig. 3), 0.5 ml suspension per plant being injected. The size of the injection needle was 16 × 5 (25 g × 5/8″). When the injection was to be given, the needle was pushed into the centre of the stem. For control purposes, a set of plants were injected with sterile water.

One week after the injection, internodes were transferred to Petri dishes containing 8 g nutrient broth (Difco) and 20 g agar per 1 litre water. The pieces transferred were taken from the internode between the monofoliate and the trifoliate leaves. They were disinfected in 5 g 8-hydroxy quinoline sulphate in 200 ml water plus 800 ml 96 p.c. alcohol. When the internodes had been immersed therein, the liquid was burned for the purpose of obtaining a surface sterilization as effective as possible.

For the microscopic investigations, internodes were taken from similar parts of the plant from where internodes had been taken for transmission to nutrient broth agar.

A few seeds were inoculated with bacteria un-

der vacuum as it was not possible to procure contaminated seed for a comparison of the movements of the bacteria in the stems of naturally infected plants and the movements in plants in which bacteria had been injected into the stems. The method was that used by *Goth* (1966). A strong suspension of bacteria in sterile water was made. The seeds were added to this suspension and subjected to a vacuum of 500 mm Hg for 2 minutes. After the 2 minutes, the pressure was quickly increased. The seeds were then dried at room temperature.

The determination of the isolated bacteria was made by means of immuno-fluorescence. Further, on the isolated bacteria a Gram's staining were carried out.

The antiserum used was obtained from *A. Trigalet,* I.N.R.A., Angers, France. The antiserum had been produced by injection of killed *C. flaccumfaciens* No. NCPPB 588 in rabbits. 5 injections were made at intervals of 5 days between the injections. Each injection consisted of 2 ml solution.

The immuno-flourescence method is as follows: A bacteria suspension (10^9 bacteria/ml glassdistilled water) is conveyed to a clean microscope slide with two compartments. The slide is airdried and is then covered with 95 p.c. alcohol. After evaporation of the alcohol, the produced antiserum (mid-layer antiserum) is added to one of the compartments. The dilution is 1:320. This is incubated for 30 minutes and then rinsed by the use of a buffer (10.7 g Na_2HPO, 3.9 g NaH_2PO_4, $2H_2O$, and 1,000 ml distilled water – when to be used this solution is diluted 1:9, and 8.5 g NaCl is added), and the microscope slide remains for a further 10 minutes in the buffer. Then plasmaproteinantisera are added with attached fluorescein (dilution 1:400) to both compartments of the slide. A 30-minute treatment is given, and a rinsing with a buffer is carried out in the manner described above. Before observation under fluorescence microscope, the slides are embedded in glycerine, and cover slips are applied.

Injections were made into the stems of 48 plants for this investigation.

Before antiserum was used for the determination, it was investigated for cross-reactions (see table 1).

In the experiment with the rate of movement in the plant, the part of the stem from the youngest internode was used.

The disinfected internodes were macerated in sterile water, and one drop of it was spread on nutrient broth.

The selection took place 24, 48, 72 and 96 hours after the injection. There were 5 plants in each treatment.

Tabel 1. Investigation for cross-reactions
Undersøgelser for krydsreaktioner

Bacteria	Dilution of Antiserum 1:10	1:100	1:400
C. michiganense	–	–	–
C. sepedonicum	–	–	–
C. fascians	–	–	–
C. flaccumfaciens	+	+	+
Xanthomonas phaseoli	–	–	–
Pseudemonas phaseolicola	–	–	–

Experiments were made on November 7 and 28, December 5 and 9, 1977. The distance from the place of injection to the internode from where isolation experiments were made was about 6.5 cm.

The stem parts that were used in the electron-microscopic analyses were taken from artificially infected bean plants of the same origin and by the same method as already described. The plants were selected 68 hours after the injection. The stem part originated from the internode between the cotyledons and the monofoliate leaves.

The fixing was made in Karnowsky fixative for 4 hours at a temperature of 4°C. Refixed in 2 p.c. osmium for 17 hours at 4°C (overnight) and rinsed four times in distilled water, followed by an impregnation for one hour in saturated uranyl acetate and dehydrated through ethanol and propylenoxide (*Begtrup & Thomsen,* 1975), and then imbedded in Spurr resin (*Spurr,* 1969).

The parts were cut in a LKB ultramicrotome,

embedded in formwar-treated grids and stained with uranyl acetate and lead citrate.

A Philips 201S electron-microscope was used for the analyses, and an Eastmen Kodak positive film, type 5302 for the photographing, to be enlarged as desired.

Results

The movement in the plant

From the injected plants, bacteria were isolated. The bacteria colonies were yellow slightly convex, and glistening on the nutrient broth. The isolated bacterium was Gram-positive. These characteristics correspond closely to *C. flaccumfaciens*. That it was a question of *C. flaccumfaciens* was further emphasized by the immunofluorescence investigations which were all positive. One week after the injection in the stem, a vigorous bacterial activity in the xylem was observed in the internode between the monofoliate and trifoliate leaves. The first activity is usually found in the vessels at 1, 2, or 3 (see fig. 4). In certain cases it resulted in a strong brown colouring (see fig. 6). The brown colouring confines itself to the primary xylem, and it started quite clearly in the first-formed cells of the primary xylem.

Two weeks after the injection, the brown colouring and the bacterial activity were found in the secondary xylem, too.

The bacterial activity is mainly limited to the xylem.

Likewise, a good deal of brown-coloured xylem is found in the petioles of monofoliate as well as trifoliate leaves.

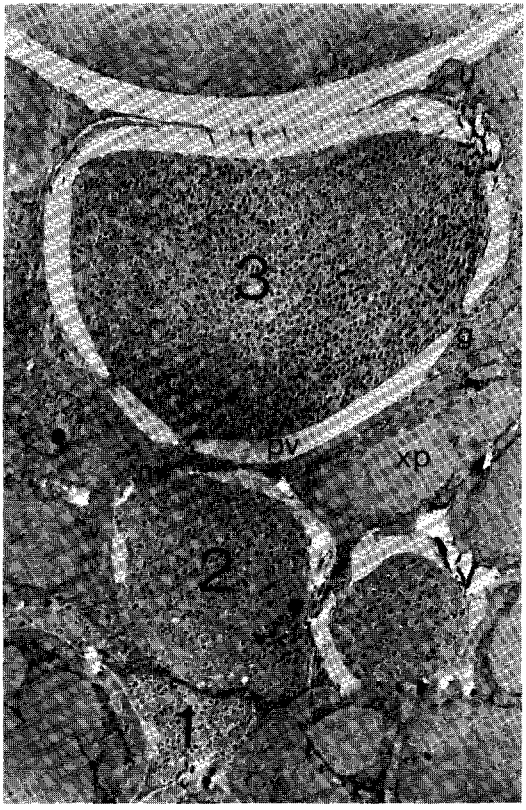

Fig. 4. Xylem attacked by bacteria. In vessel No. 1, the primary wall is completely decomposed; Pv = primary wall; xp = xylem parenchym; ml = middle lamella; g and u decomposition of primary wall. × 2000.
Xylem angrebet af bakterier. Hos celle nr. 1 er den primære væg helt nedbrudt. Pv-primære væg; xp-xylemparenkym; ml-midtlamel; g og u nedbrydning af primær væg. × 2000.

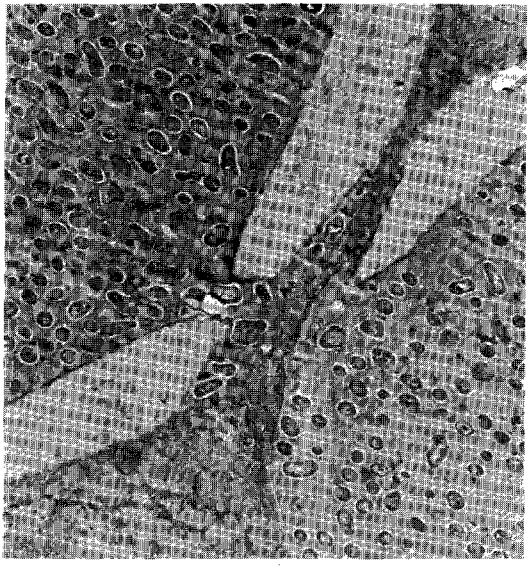

Fig. 5. Enlargement showing details of fig. 4 at ml. × 4470.
Detailbillede af fig. 4 ved ml. × 4470.

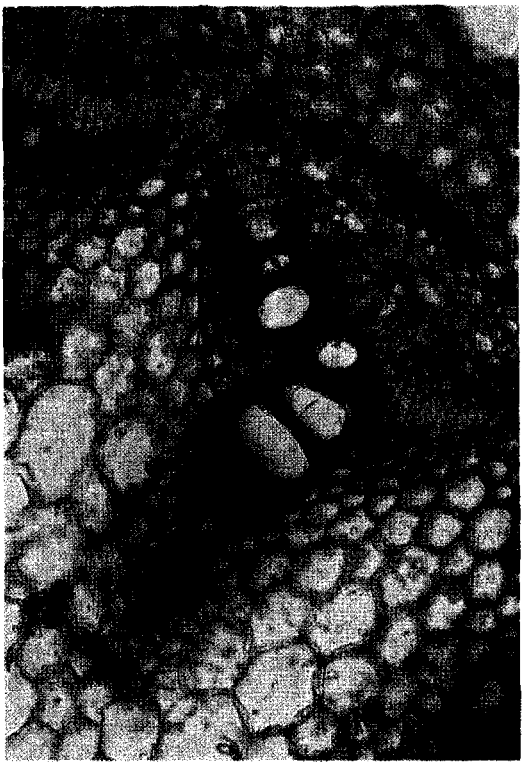

Fig. 6. Xylem attacked by bacteria. Brown colouring caused by the attack. × 580.
Xylem med angreb af bakterier - brunfarvningen er forårsaget af angrebet. × 580.

In the plants where the infiltration took place in the seeds under vacuum, the bacterial activity was found in the same parts as just described above.

The rate of movement in the plant

Table 2. Isolation of bacteria from infected plants
Isolering af bakterier fra inficerede planter

Date	Number of hours after injection 24	48	72		96		Control
7/11	–(5)	–(5)	–(5)		–(2)	+(3)	–(5)
28/11	–(5)	–(5)	–(3)	+(2)	–(4)	+(1)	–(5)
5/12	–(5)	–(5)	–(5)		–(3)	+(2)	–(5)
9/12			+(5)		–(1)	+(4)	–(5)

–( ): Number of plants without isolation of bacteria.
+( ): Number of plants with isolation of bacteria.

The isolated bacteria were in all cases Gram-positive. The immunofluorescence investigations were likewise positive with the *C. flaccumfaciens* antiserum, so the isolated bacteria were *C. flaccumfaciens*.

Beside the isolation of bacteria, the individual plant were subjected to microscopic investigations to ascertain the presence, if any, of bacteria, which gave the following results:

24 hours: No bacterial activity.

48 hours: No bacterial activity in the tips of the youngest shootes or in the internodes between the cotyledons and the monofoliate leaves. Only about 1 cm above the place of injection, bacterial activity was found in the pith.

72 hours: No bacterial activity in the youngest shoots, but in the primary xylem in the internode between the cotyledons and the monofoliate leaves. About 1 cm above the place of injection, the activity began concentrating in the xylem, and only relatively few bacteria were found in the surrounding parenchym cells in the pith.

96 hours: Slight bacterial activity in the primary xylem in the youngest shoots. Vigorous activity in the primary xylem and the start of brown-colouring thereof in the internode between the cotyledons and the monofoliate leaves. The greatest concentration of bacteria was plainly to be found in the xylem and the surrounding parenchym cells (see fig. 7) immediately above the place of injection.

The control plants were all without bacterial activity.

Electron-microscopic investigations

A decomposition of the wall takes place and of the middle lamella as well. Fig. 4 shows the decomposition of the middle lamella. Here some bacteria have penetrated under the primary wall at g and, during the decomposition of the middle lamella, they move upwards to the next xylem cell where a few bacteria can be seen at u, penetrating the primary wall.

In fig. 4, a single xylem cell is almost intact although there are many bacteria. When seen in fig. 8, which shows a later stage of development, it will be observed that the cell walls and middle lamella in all xylem cells have been almost completely decomposed. Further, it is remarkable that no bacteria are found in the surrounding parenchym cells, but are exclusively localized in the xylems. Moreover, no decomposition takes place in the adjacent parenchym cellwalls.

In fig. 9 fibre material from a xylem cell can be seen, and in that cell, the fibre material is all which remains of the cell wall.

Likewise in fig. 4 it may be ascertained that several bacteria are moving forward in the intercellular spaces, from there attacking the cell walls of the xylem cells.

Discussion

These investigations show that the bacteria in the plant move exclusively in the xylem and that the first movement takes place in the first-formed cells in the primary xylem. From there, the bacteria spread to the other xylem cells. Further, a consequence of the attack is that the cells assume a brown colour. No tylosis was found during the investigation, either in the primary or the secondary xylem.

The brown-colouring of the xylem almost invariably takes place at the place of infection, and such colouring is caused by the formation of phenols. An increase of the phenol content is characteristic of bacterial attacks in plants (*Beckman,* 1964).

During the first 48 hours after the injection it is

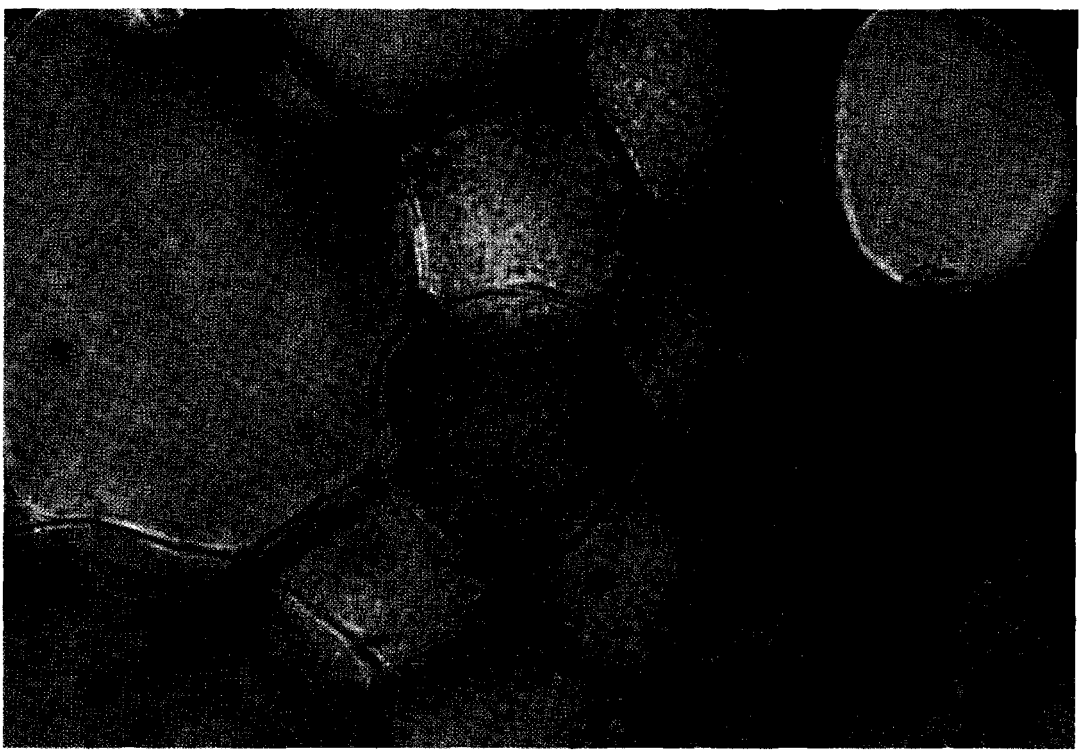

Fig. 7. Xylem and surrounding parenchym cells. The concentration of bacteria increases with increasing proximity to the xylem. × 1900.

Xylem og omkringliggende parenkymceller. Koncentrationen af bakterier er stigende des nærmere xylemet. × 1900.

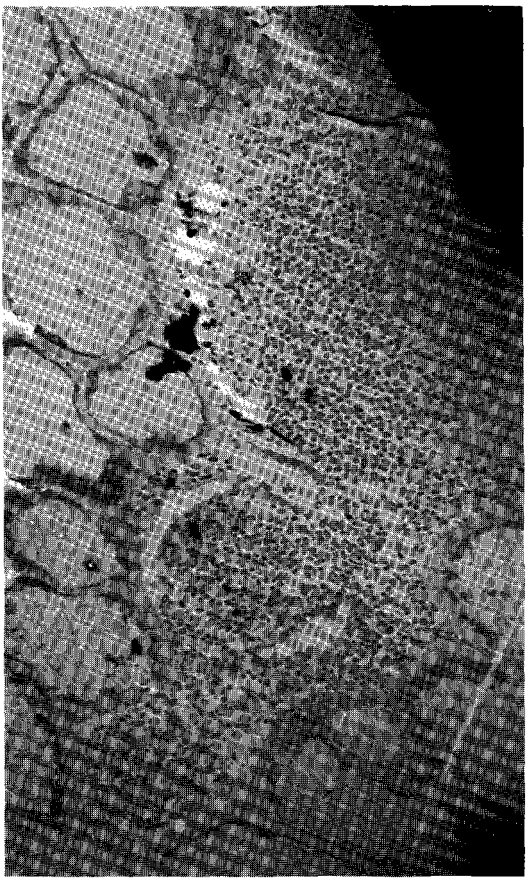

Fig. 8. The same as fig. 4 but at a later time. The primary wall is here almost decomposed. × 3000.
Samme som fig. 4, men på et senere tidspunkt. Primær væggen er her næsten nedbrudt. × 3000.

not possible to isolate bacteria from the youngest internodes. The reason may be; either that there are no bacteria in the internodes or that a certain number of bacteria must be there before it is possible to isolate them from the internode. When the results from the isolating process are compared with microscopic investigations, it seems that there are no bacteria in the youngest shootes after 48 hours. The reason why they have not reached so far could be that it takes a certain period of time for the bacteria to propagate and thereafter penetrate the xylem at the place of injection. This is borne out by the microscopic investigations, showing that the bacterial activity about 1 cm above the place of injection was ascertained in the pith after 48 hours. After 72 hours, the bacterial activity began concentrating in and around the primary xylem situated 1 cm above the place of injection. The greatest concentration was found in the xylem. After penetrating into the xylem, the bacteria can then be transported upwards in the plant by means of the sap flow, and the microscopic investigations after 96 hours did show bacterial activity in the youngest internode, though the activity was not particularly vigorous. This agrees very well with the fact that the first isolations took place in the 72 and 96 hour treatments. These observations agree very well with those made by *Wallis* (1977) in connection with *C. michiganense* and its rate of movement in tomatoes as it took 96 hours before bacteria were isolated in the stem for the first time at a distance of 2.5 cm from the place of inoculation. In the experiment mentioned the distance was 6.5 cm between the place of inoculation and the internode where the isolation took place.

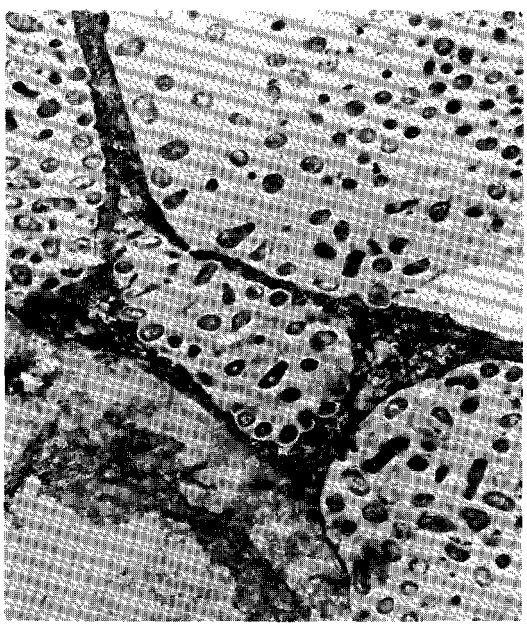

Fig. 9. Fibre material as only remainder from the xylem. × 7735.
Fibermateriale som eneste rest efter xylemet. × 7735.

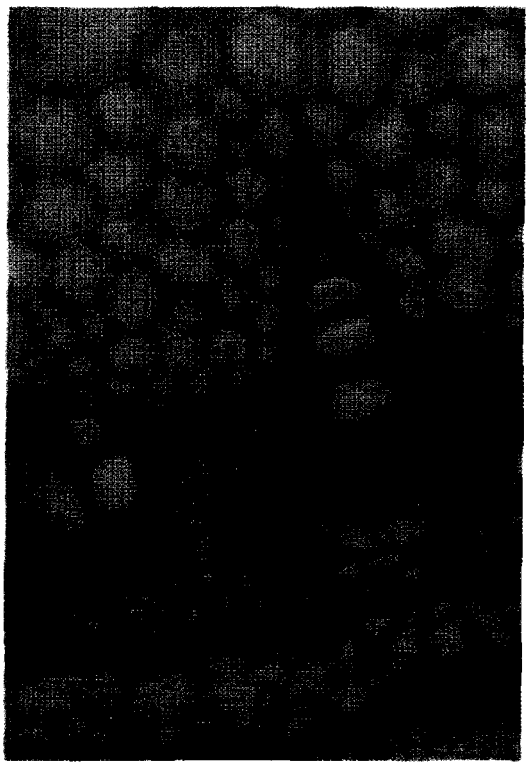

Fig. 10. Xylem without attack by bacteria. × 580.
Xylem uden angreb af bakterier. × 580.

The investigations show without doubt that the bacteria move in the xylem only, and that the surrounding parenchym cells are not invaded. Even after the complete degradation of the cell wall (fig. 8), the bacteria are only found at the place where the xylem had been located.

It is generally agreed that the wilting of a plant in consequence of a bacterial attack can be ascribed to an imbalance in the water economy of the plant, and that the pathogen contributes to this and other abnormalities by liberation of metabolic products.

In certain cases, the xylem will be clogged. Such clogging may be due to the formation of clogging material, or it may originate from the bacteria themselves (*Husain & Kelman,* 1958).

Gorin and *Spencer* (1961) isolated extracellular polysaccharides from *Corynebacterium spp.,* also from *C. flaccumfaciens.*

There has been some discussion about the function of these polysaccharides. *Spencer* and *Gorin* (1961) hold the opinion that, at any rate as far as *C. insidiosum* and *C. sepedonicum* are concerned, the polysaccharides are involved in the rubbery formations produced by the pathogen. These rubbery formations bring about the wilting of the plants.

Strobel and *Hess* (1968) reject this theory and, on the basis of their investigations, they put forward the hypothesis that a toxic glycopeptide indices the wilting of the plant tissue as a consequence of a degradation of the plasma membranes of the cells.

During recent years, the contribution of the various pathogen-syntherized carbohydrates to the wilting of plants has been more appreciated. As the cause of wilting, it is more probable that phytotoxins are only one of several substances which, together, bring about the symptoms (*Durbin,* 1972).

In this case, no special material has apparently been formed, causing a clogging up of the xylem. And the investigations indicate that the wilting was caused by degradation of the water-conducting cells (the xylem) (see fig. 8). As will be seen from fig. 8, the degradation is so complete that there must have been a break-down in the vascular system at that place. Therefore these rudiments of the xylem must have a highly limited function in the sap transportation in the plant. Further, the presence of the numerous bacteria may cause a clogging.

Even though the bacteria are able to degrade the lignified xylem cells, nothing indicates that, within the first two weeks after the injection, the bacteria attack the, likewise lignified sclerenchyma cells adjacent to the primary xylem.

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