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Regeneration of vascular tissues in relation to Fusarium wilt resistance of carnation

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Abstract

Fusarium wilt-resistant 'Novada' carnations responded both to stem inoculation with a conidial suspension of *Fusarium oxysporum* f. sp. *dianthi* or *F. oxysporum* f. sp. *lycopersici* and to root inoculation by planting in soil infected with *F. oxysporum* f.sp. *dianthi* by means of a localization mechanism comprising gel formation in the xylem vessels and hyperplasia of adjacent parenchyma cells. Dye translocation experiments showed that xylem transport was limited by the presence of vascular gels, although wilting did not occur. Overcapacity of the vascular system apparently allowed for sufficient water transport to compensate for local vascular dysfunction. Also, vascular regeneration in the hyperplastic tissue next to occluded xylem vessels created new pathways for water transport to compensate for those lost by occlusion. Regeneration of xylem vessels was eventually followed by regeneration of xylem fibers, xylem parenchyma, cambium, and phloem cells.

'Early Sam' carnations, susceptible to Fusarium wilt, responded to stem inoculation with *F. oxysporum* f. sp. *lycopersici* by similar localization of infection and vascular regeneration. Stem inoculation with *F. oxysporum* f. sp. *dianthi,* however, resulted in colonization of the xylem vessels followed by lysis of the vascular tissues. Vascular gelation, hyperplasia of parenchyma cells, and vascular regeneration did generally not occur. However, if some hyperplasia occurred in attempted defence, some differentiation of hyperplastic cells into single xylem vessel elements was observed which only rarely resulted in complete vascular regeneration next to colonized xylem. In the absence of hyperplasia, differentiation of medulla parenchyma cells bordering destroyed vascular tissue into xylem vessel elements was even more exceptional. Apparently, vascular regeneration in carnation is a normal defence reaction to fungal invasion.

Additional keywords: Dianthus caryophyllus, Fusarium oxysporum f. sp. *dianthi,* hyperplasia.

Introduction

Occlusion of infected xylem vessels by gels appears to play an important role in resistance of carnations to Fusarium wilt (Baayen and Elgersma, 1985; Harling and Taylor, 1985). Generally it is absent in diseased carnations, in contrast to the situation in several other vascular wilt diseases in which widespread occlusion by gels seems to cause the wilting (Beckman and Halmos, 1962; Péresse, 1975; Misaghi et al., 1978). Though vessel occlusion limits the water transport through the xylem in carnation as well, compensation seems to be possible by lateral transport (in carnation, the xylem forms a continuous cylinder). Recent reports indicate that xylem vessel regeneration

may also be important (Harling et al., 1984; Baayen and Elgersma, 1985). However, only early stages of differentiation of parenchyma cells into xylem vessel elements have been reported, and regeneration of functional xylem vessels has been hypothesized only. Both anatomical and physiological evidence of complete vascular regeneration is presented here. Data were obtained from studies on both resistant and susceptible carnations after inoculation with isolates of *F. oxysporum* f. sp. *dianthi*, races 1, 2, 4 and 8 and *F. oxysporum* f. sp. *lycopersici* (R.P. Baayen, J.F. Demmink and L.D. Sparnaaij, unpublished results), and in additional dye transport experiments. Also, the importance of vascular regeneration for Fusarium wilt resistance of carnation was assessed.

Materials and methods

Plant and fungal material. Rooted cuttings of the carnation cultivar Early Sam, susceptible to *F. oxysporum* f. sp. *dianthi*, and the resistant cultivar Novada, obtained from M. Lek & Zonen B.V., Nieuwveen, were planted in steam-sterilized soil (8 cm diameter pots) and grown for 2 weeks in the glasshouse at 20 tot 24 °C prior to stem inoculation. Additional rooted cuttings of 'Novada', obtained from the Institute for Horticultural Plant Breeding, Wageningen, were used for root inoculation experiments.

Isolates of *F. oxysporum* Schlecht. f. sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen race 2 (WCS ('Willie Commelin Scholten') 843, WCS 816) and of *F. oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyder & Hansen (WCS 801) were grown on potato dextrose agar slants.

Plant inoculations. After culturing the fungi in CMC medium (Cappellini and Peterson, 1965) on a reciprocal shaker for 4 days at 20 °C, conidial suspensions (10^7 conidia ml^{-1}) were prepared and used for stem inoculation as described by Baayen and Elgersma (1985). Of each cultivar, 35 plants were inoculated with *F. oxysporum* f. sp. *dianthi* isolate WCS 816, 15 plants with *F. oxysporum* f.sp. *dianthi* isolate WCS 843, 15 plants with *F. oxysporum* f. sp. *lycopersici* isolate WCS 801, and 30 control plants were treated similarly with sterile water.

Additionally, 20 rooted cuttings of 'Novada' were planted in a mixture of steamed soil and soil which was heavily infested with *F. oxysporum* f. sp. *dianthi* isolate WCS 816. After mixing, the soil contained ca. 2.5×10^7 propagules g^{-1} soil as determined by plating out soil suspensions in serial dilutions on malt extract agar (Oxoid) to which 20 μg chlortetracycline ml^{-1} had been added.

Microscopical preparations. Stem segments of three stem-inoculated plants of all six host-pathogen combinations and of two controls were sampled at 3 days and 1, 2, 4 and 8 weeks after inoculation. Stem parts 10 to 15 mm above the inoculation site were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8), dehydrated following Feder and O'Brien (1968), and subsequently embedded in polyethylene glycol methacrylate (JB-4 plastic), sectioned longitudinally or transversely at 3-4 μm, and stained with toluidine blue (Baayen and Elgersma, 1985); other stem parts were sectioned by hand either fresh, or after fixation with FAPA (formaldehyde (40%)-ethanol- water- propionic acid- acetic acid, 5: 45: 45: 2.5: 2.5). Stems of 'Novada'

plants stem-inoculated with *F. oxysporum* f. sp. *dianthi* isolate WCS 816 and of 'Novada' controls treated with sterile water were sampled three months after inoculation and sectioned by hand. Additionally, entire (c. 40 cm long) stems of 'Novada' carnations planted in soil infected with *F. oxysporum* f. sp. *dianthi* isolate WCS 816 were sampled two months after inoculation and sectioned by hand as well. Photographs of sections were made with a Leitz Orthoplan photo-microscope and recorded on Agfapan 25 film.

Dye transport experiments. Ten plants of 'Early Sam' and 'Novada' were cut off at the stem base c. 4 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* isolate WCS 816, put into an 0.1% (aqueous) eosin red solution for 12 h, sectioned by hand, and microscopically examined.

Results

Vascular regeneration in stem-inoculated control plants. Incision and treatment with sterile water of stems of healthy control plants caused local but intense meristematic activity of parenchyma cells close to the incision, which eventually resulted in the formation of a small callus bridging the gap and slightly protruding from the stem surface. Externally, the callus was sealed off by periderm tissue; internally, differentiation of vascular tissues was observed in the callus between both ends of the interrupted vascular system (Fig. 1). Individual, irregularly shaped parenchyma cells situated in the callus differentiated into xylem vessel elements (Fig. 2), eventually forming new xylem vessels on both sides making connection with the regular, cylindrical vessels of

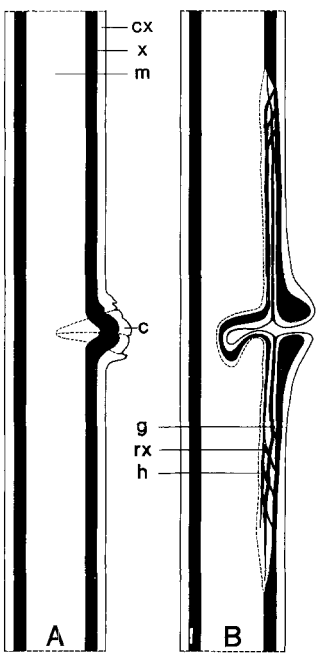


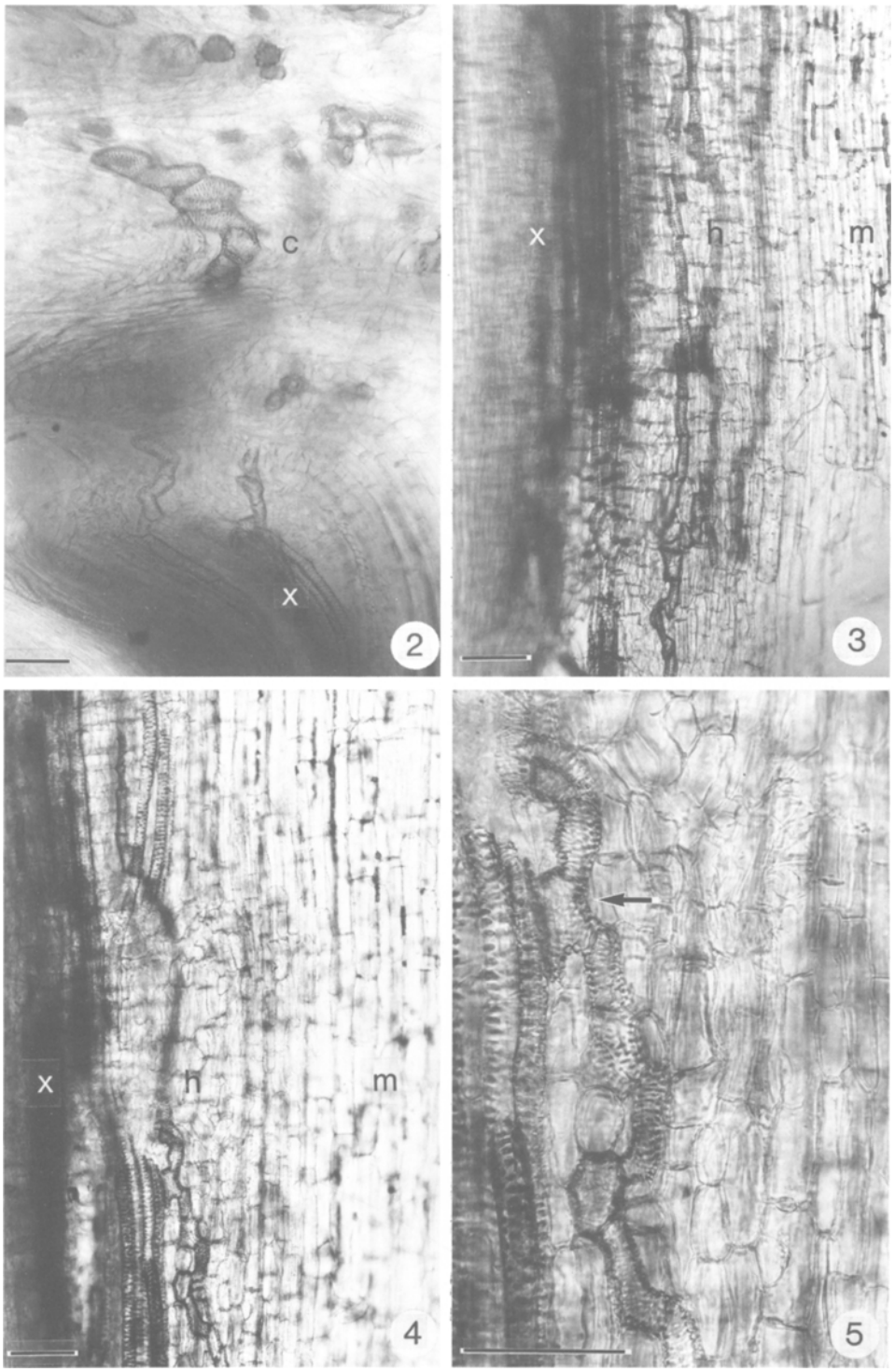
Fig. 1. Diagrammatic longitudinal sections through 'Novada' carnations three months after stem inoculation with sterile water (A) or a conidial suspension of *F. oxysporum* f. sp. *dianthi* race 2 (WCS 816) (B); cx-cortex, x- xylem, m- medulla, c- wound callus, h- hyperplastic tissue, g- zône of xylem vessels occluded by gels, rx- regenerated xylem.

the pre-existing xylem. In several cases, primordia of adventitious roots were seen emerging from the callus.

Vascular regeneration in resistant interactions following stem inoculation. Stem inoculation of 'Novada' carnations with either strain of *F. oxysporum* f.sp. *dianthi* and of both 'Novada' and 'Early Sam' carnations with *F. oxysporum* f. sp. *lycopersici* resulted in localization of infection in the xylem accompanied and probably also caused by vessel gelation and hyperplasia of adjacent xylem parenchyma or rarely medulla parenchyma cells as reported previously (Harling et al., 1984; Harling and Taylor, 1985; Baayen and Elgersma, 1985). At the inoculation site, lytic cavities were formed in the medulla. Xylem vessels occluded by brown gels and bordered by a broad band of hyperplastic tissue were observed up to several cm above, but also below, the inoculation point (Fig. 1). This probably represented the total distance of passive spore transport directly after inoculation. These phenomena were absent in stem inoculated control plants. Eosin translocation experiments showed that vessel occlusion by gels locally inhibited water transport completely, since no dye at all was found in areas with occluded vessels whereas the dye strongly stained unaffected xylem and surrounding tissues. Dysfunction of a significant part of the vascular cylinder was observed two weeks after inoculation in otherwise healthy plants.

In the hyperplastic tissue, individual parenchyma cells were seen differentiating into xylem vessel elements as documented by Harling et al. (1984) and Baayen and Elgersma (1985) within c. 10 days after inoculation, 2 weeks after inoculation forming entirely new xylem vessels running parallel to and terminally making connection to the unaffected parts of the xylem. Regenerated xylem vessels as here described are illustrated in Figs 3-5 for the resistant response occurring below the inoculation site in susceptible 'Early Sam' carnations as discussed below. During the same period, hyperplasia of medulla parenchyma occurred around the cavity formed at the inoculation site, which was also followed by regeneration of xylem vessels, sometimes including so-called circular vessels (Sachs and Cohen, 1982). After 4 weeks, a continuous band of regenerated xylem vessels was being formed (Figs 6-8) connecting both unaffected ends and circumventing the cavity at the inoculation site (Fig. 1). When such plants were cut off at the stem base and put into an eosin solution, the eosin stained the lumina of the regenerated vessels already after a few hours and diffused into the surrounding parenchyma cells, thus proving that the regenerated xylem vessels were functioning. After about 8 weeks, completely regenerated xylem tissue was observed consisting not only of many vessels but also of xylem fibers and xylem

Fig. 2. Part of longitudinal section of 'Novada' three months after stem inoculation with sterile water showing xylem vessel differentiation in wound callus (c) connecting with existing vessels at the severed end of the xylem (x). Freehand section; magnification bar represents 100 μm.
Figs 3-5. Parts of longitudinal sections of 'Early Sam' 2 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 816), exclusively below the inoculation site showing a localization response including the formation in hyperplastic tissue of regenerated xylem vessels which connect with existing vessels (arrow); m- medulla, h- hyperplastic tissue, x- existing xylem. Freehand sections, 0-10 mm below the inoculation site; magnification bars represent 100 μm.
Figs 3,4. General view.
Fig. 5. Detail of Fig. 4.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parenchyma (Figs 9-11). The regenerated xylem was always found only at some distance of the occluded xylem. In the hyperplastic tissue between the existing, affected xylem and the regenerated xylem, regeneration of phloem and cambium was observed (Fig. 11). Both regenerated and original, unaffected vascular tissues eventually formed one continuous band (Fig. 9), resulting in complete restoration of the function of the vascular system after the damage caused by localization of the infection.

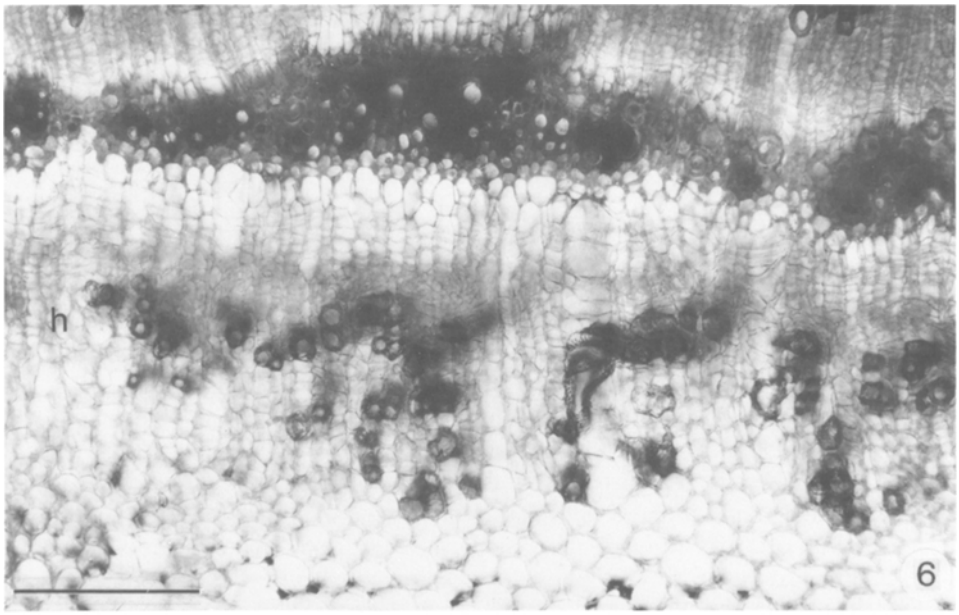
Vascular regeneration in susceptible interactions following stem inoculation. Stem inoculation of 'Early Sam' carnations with either strain of *F. oxysporum* f. sp. *dianthi* resulted in vascular colonization by the fungus. Fungal spread was slow and mainly longitudinal, and radial spread was limited, probably by plant defence reactions such as thickening and suberization of walls of parenchyma cells bordering the colonized tissue (Baayen and Elgersma, 1985; R.P. Baayen, manuscript in preparation). Generally no hyperplasia of parenchyma or vascular regeneration occurred. Sometimes, however, some gel formation and hyperplasia of parenchyma were observed at the inner margin of colonized tissue, which apparently occurred in attempt of defence. Similar observations were reported by Pennypacker and Nelson (1972). In these cases, generally only a narrow band of hyperplastic tissue was formed consisting of irregularly shaped and sometimes even hypertrophic cells. Nevertheless, some differentiation of these hyperplastic cells into single xylem vessel elements was observed. In most cases, only the very first stages of vessel regeneration occurred (Fig. 12), but complete vascular regeneration (Fig. 13), corresponding to the situation in resistant carnations was, be it rarely, observed also. These regenerated, healthy vascular tissues did not become colonized by the fungus. In radial direction, the wall appositions described above apparently function as an efficient barrier between the colonized and regenerated vascular tissues.

Exceptionally, differentiation of medulla parenchyma cells bordering vascular tissue destroyed by the fungus into xylem vessel elements was seen (Fig. 14; photomicrograph from R.P. Baayen, J.F. Demmink and L.D. Sparnaaij, unpublished results). No entire, functional vessels appeared to be formed in this way.

Colonization and disease of susceptible plants as described above generally only occurred from the inoculation site upward. The lower parts often remained unaffected because of localization of infection and vessel regeneration (Figs 3-5).

Vascular regeneration following root inoculation. The calli at the base of healthy carnation cuttings are, like those formed in stem-inoculated controls, composed of meristematic parenchymatous tissue. Differentiation into vascular elements bordering the callus periderm and giving rise to adventitious roots is a normal phenomenon.

Figs 6-8. Parts of transverse sections of 'Novada' 4 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 816) showing regeneration of xylem, phloem and cambium in hyperplastic tissue (h) bordering xylem containing gels. Magnification bars represent 200 μm.
Fig. 6. Three mm above the inoculation site; freehand section.
Fig. 7. Ten mm above the inoculation site; microtome section of plastic-embedded material stained with toluidine blue.
Fig. 8. Detail of Fig. 7.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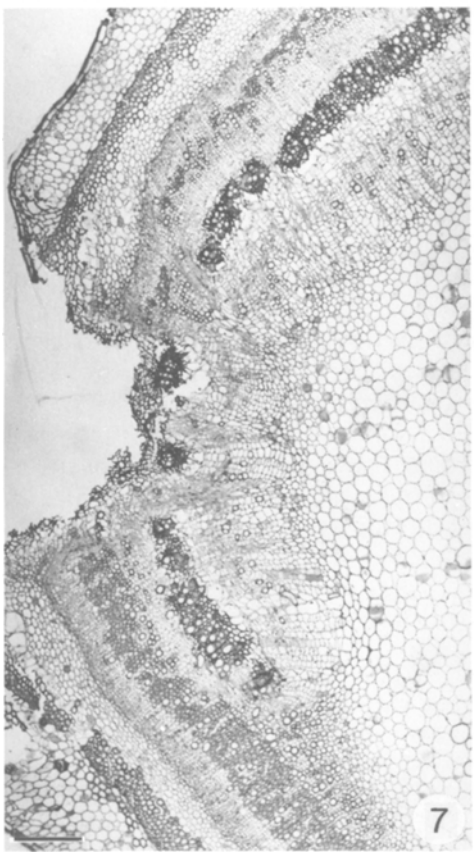
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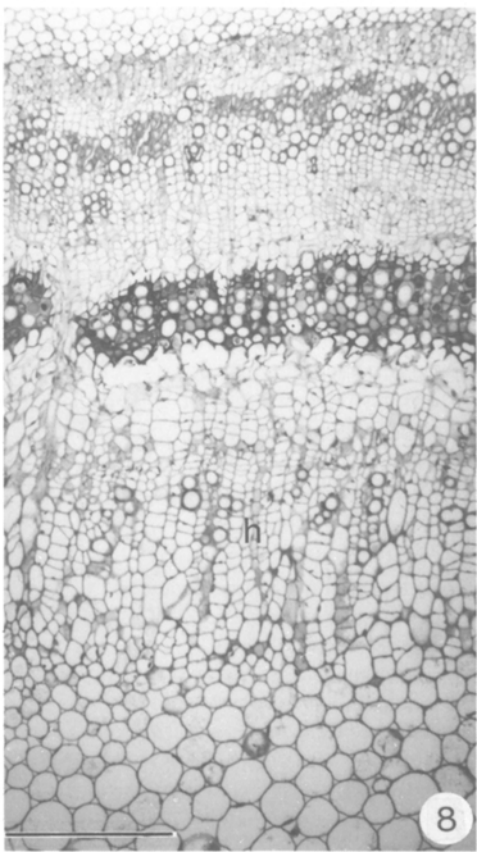
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Brief investigations of the anatomy of root-inoculated 'Novada' carnations revealed the occurrence of the same phenomena as found with stem inoculation, comprising xylem vessel differentiation in hyperplastic parenchyma bordering vessels plugged by gels or – more rarely – colonized by the fungus. Aside from their location parallel to vessels containing gels, however, these regenerated xylem vessels were similar to those normally formed in the calli.

Discussion

This study demonstrates that complete regeneration of functional vascular tissues next to infected xylem is involved in resistance of carnations to Fusarium wilt. The regenerated vascular tissues comprised completely developed xylem, cambium and phloem, and connected both laterally and vertically to healthy vascular tissues, replacing the affected parts. Although regeneration was studied from stem-inoculated carnations, it was also encountered in root-inoculated plants, which proves it to be a natural response to occlusion, not to stem wounding by incision only.

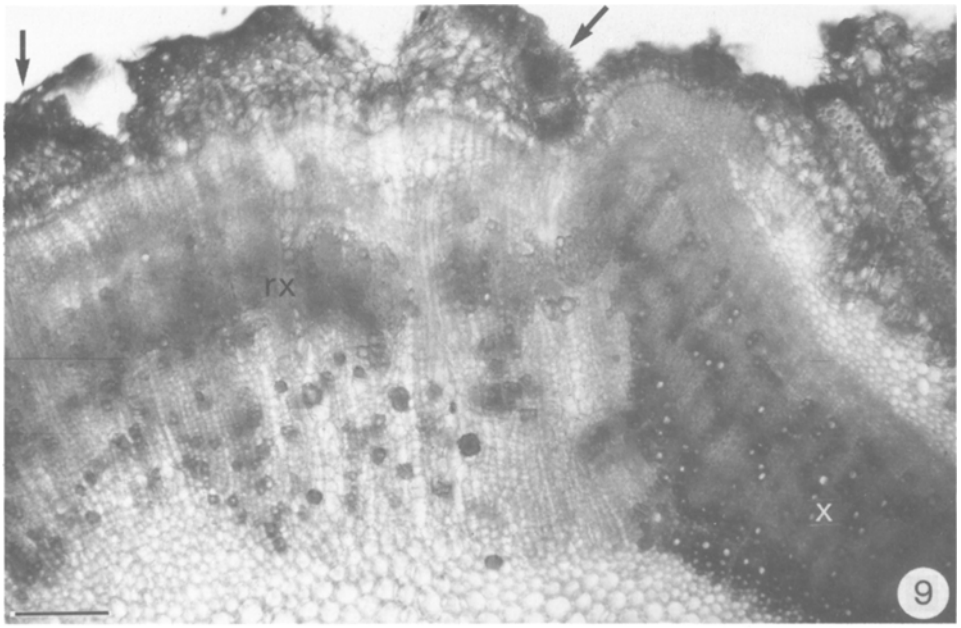
Vascular differentiation and regeneration are well-known phenomena caused by a basipetal flux of auxin through the vascular system from shoots to roots. Vascular dysfunction forces the flux to move through neighbouring parenchyma cells and causes them to differentiate into xylem vessel elements as a result of which they become even better channels for the flux. Because of this positive feedback mechanism, newly formed vessels function as a sink for auxin so that regenerating vessels develop toward and connect with already existing ones (Sachs, 1969, 1981). However, direct connections between regenerated xylem vessels and older vessels of the original xylem as presently found appear to be rare as regenerated vessels generally connect only with xylem vessels last formed by the cambium (Sachs, 1981). Not only xylem vessels, but also phloem and even cambium regeneration are known to be induced by auxin (Sachs, 1981).

In the present study, vascular regeneration was found when a considerable part of the xylem was occluded by gels and therefore no longer functional. On the other hand, destruction of vascular tissues in susceptible interactions normally did not at all incite regeneration. This probably is a consequence of the absence of xylem parenchyma cell divisions (hyperplasia) in susceptible interactions (Baayen and Elgersma, 1985), since maturing parenchyma cells gradually lose their competence to differentiate into vessel elements. This competence can be restored during a certain period under influence of a wound (Sachs, 1981), presumably because of the associated cell divisions (Sinnott

Figs 9-11. Parts of transverse sections of 'Novada' 8 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 843) showing completely regenerated vascular tissues in the hyperplastic area.

Fig. 9. Regenerated xylem (rx) forming one continuous band with the unaffected xylem (x) and extrusion of obstructed vessels (arrow). Three mm above the inoculation site; freehand section; magnification bar represents 200 μm.

Figs 10, 11. Details of hyperplastic tissue containing regenerated xylem vessels (v), fibers (f), and parenchyma (p) (Fig. 10), and regenerated phloem (Fig. 11). Ten mm above the inoculation site; microtome sections of plastic-embedded material stained with toluidine blue; magnification bars represent 20 μm.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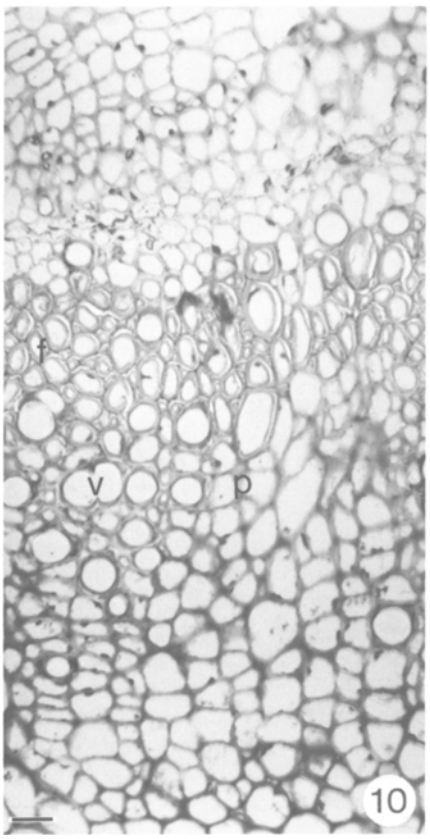
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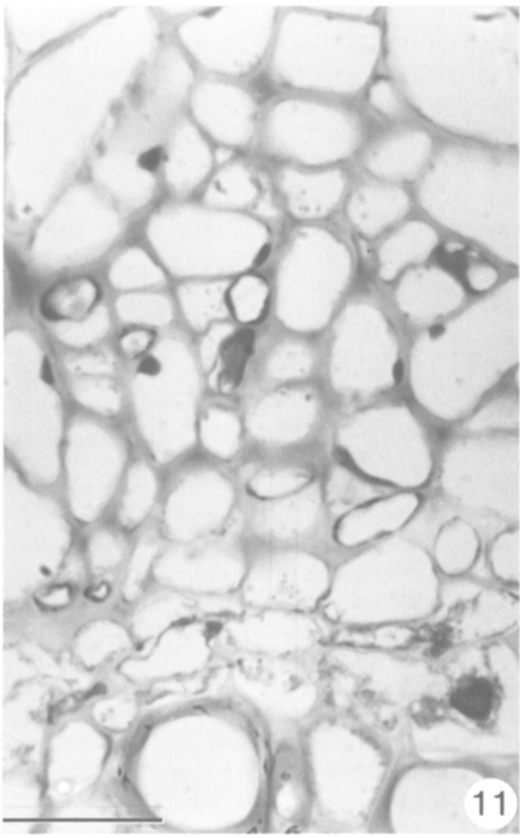
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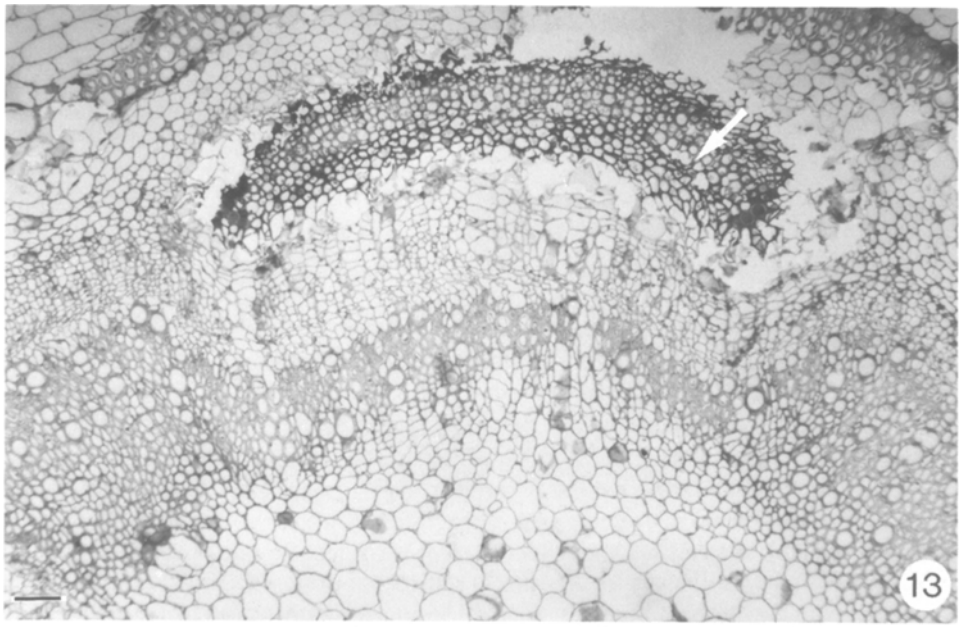
and Bloch, 1945). Only when xylem destruction in susceptible interactions was accompanied by sufficient hyperplasia of xylem parenchyma, regeneration was observed. Without any cell divisions, differentiation of medulla parenchyma cells bordering destructed xylem into xylem vessel elements was extremely rare. Apparently, vascular regeneration is possible as a secondary consequence of the hyperplasia of xylem parenchyma. The hyperplasia itself primarily is a defence reaction associated with periderm formation (R.P. Baayen, manuscript in preparation) and intended for localization of pathogens. Similar situations occur in *Botrytis cinerea* ghost spots of tomato fruits (De Leeuw, 1985) and in coffee resistant to coffee berry disease (Masaba and Van der Vossen, 1982).

Vascular gel formation and related phenomena were described by Harling et al. (1984), Harling and Taylor (1985) and Baayen and Elgersma (1985) for highly resistant carnation cultivars only. However, these phenomena, including vascular regeneration, were found in various resistant reactions such as occur in 'Novada' inoculated with races 1, 2 and 4 of *F. oxysporum* f. sp. *dianthi* (R.P. Baayen, J.F. Demmink and L.D. Sparnaaij, unpublished results) and 'Early Sam' inoculated with a forma specialis of *F. oxysporum* non-pathogenic to carnation. They even occurred after successful localization of the fungus below the inoculation site in susceptible plants of which the upper part of the shoot was diseased. Hence, vascular regeneration per se is not a characteristic of resistant cultivars contrary to susceptible ones, but of resistant reactions or attempts thereof as are most common in resistant cultivars. Therefore, vascular regeneration in carnation should be considered a normal physiological phenomenon, not responsible for but a consequence of Fusarium wilt resistance. However, regeneration contributes to resistance by compensating for the loss of functioning of xylem tissues involved in localization of the pathogen. Exceptionally, vascular regeneration next to colonized xylem may also diminish symptom formation or even prevent wilting in otherwise susceptible interactions, since wilt appears to be a direct consequence of water shortage caused by destruction of the vascular system.

Fig. 12. Part of transverse section of 'Early Sam' 4 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 843) showing both irregular, hypertrophied cells next to xylem undergoing lysis and more regular, hyperplastic cells with some xylem vessel differentiation (arrow) next to xylem containing gels. Ten mm above the inoculation site; microtome section of plastic-embedded material stained with toluidine blue; magnification bar represents 50 μm.

Fig. 13. Part of transverse section of 'Early Sam' 8 weeks after stem inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 843) showing completely regenerated vascular tissue forming one continuous band with the unaffected xylem next to colonized xylem (arrow) with brown margins which are characteristic of disease. Ten mm above the inoculation site; microtome section of plastic-embedded material stained with toluidine blue; magnification bar represents 50 μm.

Fig. 14. Part of transverse section from 'Pallas', 6½ weeks after root inoculation with *F. oxysporum* f. sp. *dianthi* race 2 (WCS 816), showing medulla cells differentiating into xylem vessel elements at the margin of colonized xylem undergoing lysis. Upper part of the stem; microtome section of plastic-embedded material stained with toluidine blue; magnification bar represents 50 μm (photomicrograph from R.P. Baayen, J.F. Demmink and L.D. Sparnaaij, unpublished results).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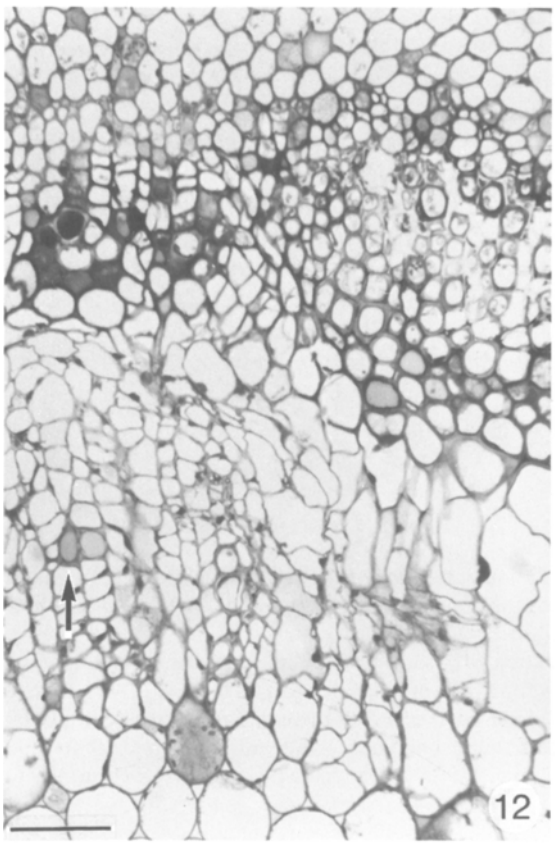
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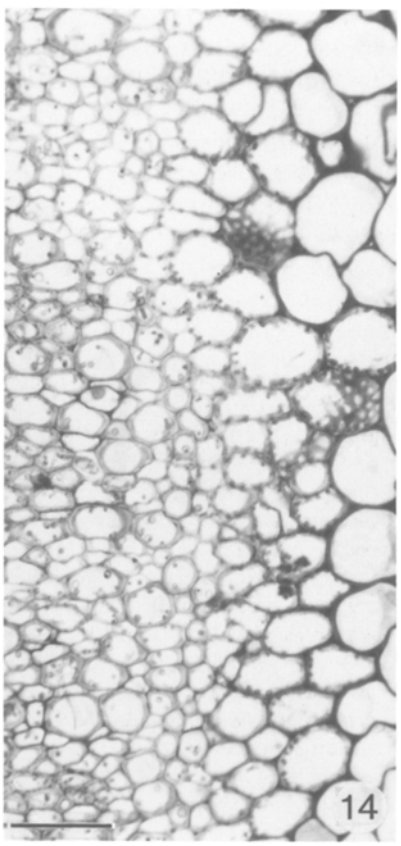
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Samenvatting

Vaatweefselregeneratie bij resistentie van anjer tegen Fusarium-verwelkingsziekte

'Novada' anjers, resistent tegen Fusarium-verwelkingsziekte, reageerden op stengelinoculatie met een conidiënsuspensie van *Fusarium oxysporum* f.sp. *dianthi* of van *F. oxysporum* f.sp. *lycopersici* en op wortelinoculatie door te planten in met *F. oxysporum*f.sp. *dianthi* besmette grond met een lokalisatiemechanisme dat onder meer bestond uit vorming van gommen in de houtvaten en hyperplasie van naburige parenchymcellen. Uit proeven over kleurstoftransport bleek dat de sapstroom door de gomvorming beperkt werd, hoewel dit geen verwelkingssymptomen veroorzaakte. Overcapaciteit van het vaatstelsel zorgde kennelijk voor voldoende compensatie aan watertransport om plaatselijke verstoring van de sapstroom op te vangen. Daarnaast werd het verlies aan functionele houtvaten ook opgevangen door vaatweefselregeneratie in het hyperplastische weefsel grenzend aan door gommen verstopte houtvaten. Na verloop van tijd werden behalve houtvaten ook houtvezels, houtparenchymcellen, cambium- en floeemcellen geregenereerd.

'Early Sam' anjers, vatbaar voor Fusarium-verwelkingsziekte, reageerden op stengelinoculatie met *F. oxysporum* f. sp. *lycopersici* met eenzelfde lokalisatiemechanisme en ook met vaatweefselregeneratie. Stengelinoculatie met *F. oxysporum* f. sp. *dianthi* echter had kolonisatie en vervolgens lysis van het vaatweefsel tot gevolg. Meestal trad er geen gomvorming, hyperplasie van parenchymcellen of vaatweefselregeneratie op. Als echter bij pogingen tot afweer toch enige hyperplasie optrad, bleken sommige hyperplastische cellen wel tot houtvatelementen te differentieren. Dit leidde echter maar zelden tot totale vaatweefselregeneratie parallel aan het gekoloniseerde vaatweefsel. In afwezigheid van hyperplasie differentieerden mergparenchymcellen vlak naast lyserend vaatweefsel slechts bij hoge uitzondering tot houtvatelementen. Vaatweefselregeneratie bij anjer is kennelijk een gewone afweerreactie op besmetting met pathogene schimmels.

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