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Passive transport of microconidia of Fusarium oxysporum f. sp. dianthi in carnation after root inoculation

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Abstract

Root inoculation of susceptible carnations with *Fusarium oxysporum* f. sp. *dianthi* induced characteristic unilateral wilt only if root woundings and use of a microconidial suspension had not been combined at the time of inoculation. The combination, however, induced atypical and sudden stem breaking soon followed by death. In all cases wilt was due to destruction of the xylem. Unilateral wilt appeared to follow sparse natural infection of single roots. Stem breaking was due to destruction of the vascular tissues all around the stem and is ascribed to multilateral infection caused by translocation of microconidia at inoculation through several wounded roots directly into the stem.

Microconidia were carried passively 5-10(-30) cm into stems of susceptible and resistant carnations within 24 h both after immersing cut ends of the roots in a conidial suspension and after pouring a suspension on the soil. Passive spore transport is an inoculation artefact which may severely affect interpretation of experimental results; it seems to be unimportant in natural Fusarium wilt development in carnation.

Additional keywords: Dianthus caryophyllus, inoculation methods.

Introduction

Fusarium wilt threatens the carnation industry world-wide. However, the biology of this disease is not well understood. Colonization and histopathology of carnation stems infected with *Fusarium oxysporum* f. sp. *dianthi* have been studied (Pennypacker and Nelson, 1972; Baayen and Elgersma, 1985; Harling and Taylor, 1985), but how this soil-borne pathogen penetrates and colonizes carnation roots is not known. Nothing is known about the significance of root damage for disease expression or about possible disease resistance in the roots either. The presence of disease resistance (though of unknown nature) in carnation roots was implied by the resistance breeding programme carried out at the Institute for Horticultural Plant Breeding (IVT), Wageningen (L.D. Sparnaaij and J.F. Demmink, personal communication). Therefore, studies on penetration, colonization and histopathology of roots of susceptible and resistant carnation cultivars infected with *F. oxysporum* f. sp. *dianthi* were undertaken. Results on aspects of the infection process are presented here; results on root colonization and histopathology will be published later.

In carnation nurseries, infection presumably proceeds from hyphae (in the vicinity

of host tissue) or from surviving chlamydospores of the pathogen in the soil. In both cases, hyphae may enter the roots by penetration of the epidermal cells or by direct entrance into the xylem vessels if wounds are present. Consequently, root wounding should accelerate infection and symptom expression. For this reason it is applied in inoculations in resistance selection trials (Sparnaaij and Demmink, 1977). However, the use of microconidial suspensions generally employed for these inoculations creates the possibility of passive transport of microconidia through the xylem along with the transpiration stream. Although unlikely at first sight, passive spore transport was indeed suggested by the recovery in selection trials of *F. oxysporum* f. sp. *dianthi* from carnation stems within one day after root inoculation (Rattink, 1985). Passive transport of microconidia was even considered a natural way of infestation of carnation by *Phialophora cinerescens* (Péresse, 1968, 1971, 1977). In the present study, the role of root wounding and passive spore transport in disease development has been further investigated.

Materials and methods

Host material. Rooted cuttings of the susceptible carnation cultivars Early Sam and Lena and the moderately resistant cultivar Barbara were obtained from the IVT, Wageningen (cv. Early Sam) and from Hilverda B.V., Aalsmeer (cvs Lena and Barbara). Cuttings of 'Early Sam' had been rooted in perlite, those of 'Lena' both in perlite and turlite, and those of 'Barbara' in turlite.

Preparation of inocula. A virulent isolate (WCS 816) of *F. oxysporum* Schlecht. f. sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen was cultured in Czapek Dox liquid medium (Oxoid) on a reciprocal shaker. Microconidial suspensions were prepared by filtering five-day-old cultures through sterile glasswool to remove mycelial fragments, washing the resulting conidial suspension twice in sterile tap water by centrifugation and subsequently adjusting it to a concentration of 10^7 conidia ml^{-1}. Infested soil was prepared by mixing 250 ml of two-week-old shake cultures with 10 l of autoclaved soil and incubating the mixture for four weeks at 20 °C. When used for inoculation, the soil contained c. 3.10^5 *F. oxysporum* propagules g^{-1} soil as determined by plating soil suspensions in serial dilutions on malt extract agar (Oxoid) to which 20 μg chlortetracycline ml^{-1} had been added.

Inoculation techniques and disease indexing. One hundred rooted cuttings of 'Early Sam' were inoculated either by planting them in soil infested with *F. oxysporum* f. sp. *dianthi* in 8 cm diameter pots (50 plants; treatment A) or by planting them in steamed soil and subsequently pouring 10 ml conidial suspension on the soil (50 plants; treatment B). Fifty control plants were planted in steamed soil without further treatment.

Another 180 rooted cuttings of 'Early Sam' which had been planted in steamed soil and grown for four weeks in the glasshouse prior to inoculation were treated at the same time. Of these, 100 plants were inoculated by pouring 10 ml conidial suspension on the soil either directly (50 plants; treatment C) or after wounding the roots by five times sticking a scalpel into the soil (50 plants; treatment D). As controls, 50 and 30 plants, respectively, were treated similarly with sterile tap water.

All plants were grown in the glasshouse for nine weeks; wilt was indexed from the

appearance of symptoms onwards on 26, 30, 33, 43, 53 and 64 days after inoculation according to the following scale: 0. no symptoms; 1. first, slight symptoms (one wilted leaf at the stem base, or brown discolouration of the stem base surface); 2. well-developed, characteristic unilateral wilt of otherwise still healthy plants; 3. severe wilt; and 4. complete wilt (death).

Inoculation techniques for determining spore transport distances. In a first series of experiments, 100 cuttings of 'Lena' rooted in perlite were planted in steamed soil and grown in the glasshouse. After three weeks, 50 plants were uprooted and carefully washed to remove most of the soil. After cutting off the roots at 2 cm beneath the stem base, the plants were placed overnight with the terminal 1 cm of their root stumps in a continuously stirred conidial suspension. The next day, the root stumps were removed carefully to avoid contamination of the stem. To determine conidial spread, 1 cm stem segments at 1, 2, 3, 4, 5, 10, 15, 20 and 25 cm height were excised, surface-sterilized in FAPA (Baayen, 1986) for 2 min, dipped in 96% ethanol and flamed. Out of these segments, 2-mm-thick slices were removed aseptically, placed on malt extract agar (Oxoid) to which 20 μg chlortetracycline ml^{-1} had been added, and scored for *F. oxysporum* outgrowth after four days.

After four weeks, 25 plants were uprooted and treated similarly. However, after uprooting the roots were cut off at 7 cm beneath the stem base; after inoculation (again of only the terminal 1 cm of the root stumps) only 2 cm of the root stumps was removed, and conidial spread was determined in two root stumps per plant at 1, 2, 3, 4 and 5 cm above the final cut end as well as in the stem at 1, 5, 10, 15, 20 and 25 cm height. Root stumps were surface-sterilized in FAPA (2 min), washed twice in sterile tap water, cut aseptically into 1 cm segments and placed on malt extract agar as above.

After six weeks, another 25 plants were uprooted and used as controls; the roots were cut off at 7 cm beneath the stem base, and the plants were treated with water instead of a conidial suspension. Presence of *F. oxysporum* was scored for two root stumps per plant at 1, 2, 3, 4 and 5 cm above the cut end and in the stem at 1, 2, 3, 4, 5, 10, 15, 20 and 25 cm height.

In a second series of experiments, 25 cuttings of 'Lena' and of 'Barbara' rooted in turlite were planted in steamed soil and grown in the glasshouse. After four weeks, all plants were inoculated as described above with the roots cut off at 2 cm beneath the stem base. Conidial spread was determined in the stem at 1, 2, 3, 4, 5, 6, 10, 15, 20, 25, 30 and 35 cm height.

In a third series of experiments, 65 cuttings of 'Lena' and of 'Barbara' rooted in turlite were planted in steamed soil and grown in the glasshouse. After four weeks, 50 plants of both cultivars were inoculated by pouring 10 ml conidial suspension on the soil either directly (25 plants of both cultivars) or after wounding the roots by five times sticking a scalpel into the soil (25 plants of each). One day after inoculation, conidial spread was determined as above in the stem at 1, 2, 3, 4, 5, 6, 10, 15, 20, 25, 30 and 35 cm height. Of both cultivars, 15 control plants were treated similarly with sterile tap-water instead.

Results

Disease development in susceptible carnations after inoculation with or without wounded roots and microconidia. The influence of the presence of wounded roots and microconidia at the time of inoculation on disease development was studied on susceptible 'Early Sam' carnations using the four different treatments A – D. Disease symptoms were not noticed during the first three weeks after inoculation in any treatment. Symptom development in plants is shown in Fig. 1 a-d.

Characteristic unilateral Fusarium wilt developed only after treatments A and C.

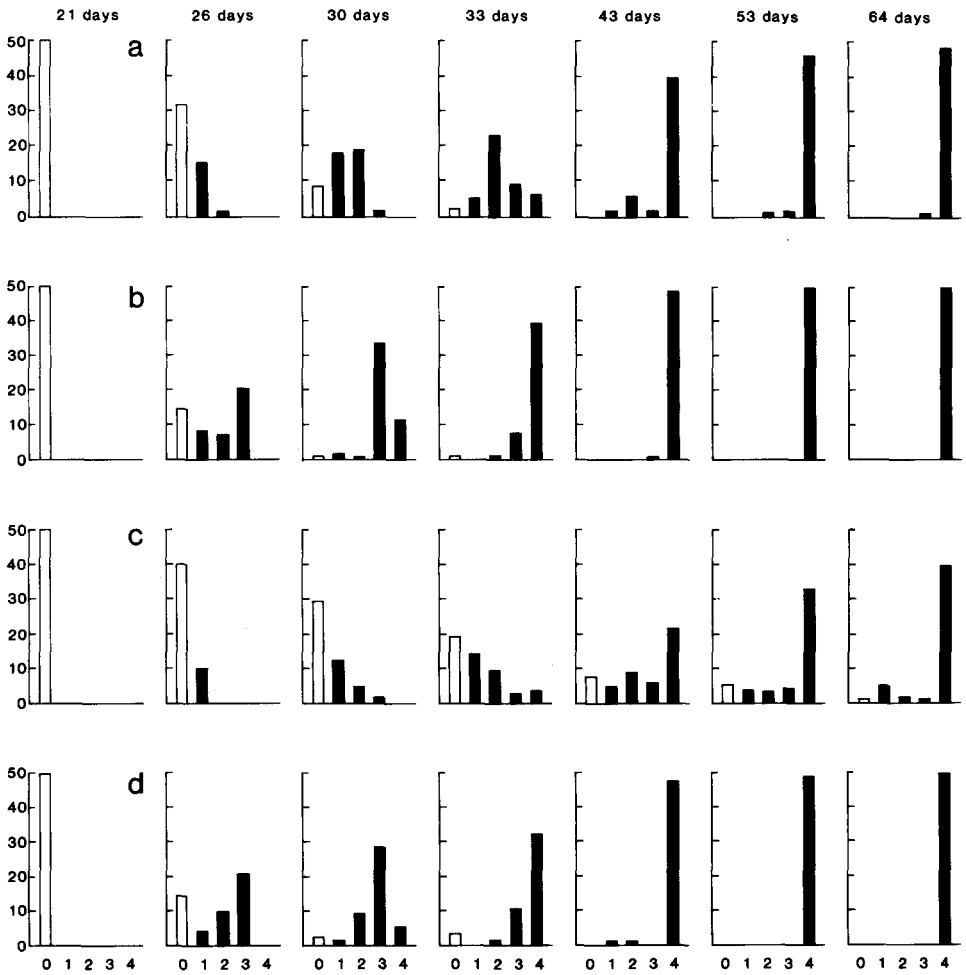

Fig. 1 a-d. Number of 'Early Sam' carnations still healthy (index 0; open bars) or showing disease symptoms (index 1-4; black bars), 21 to 64 days after inoculation by planting rooted cuttings in soil infested with *F. oxysporum* f. sp. *dianthi* (a, treatment A) or in steamed soil on which a conidial suspension was subsequently poured (b, treatment B), or after inoculation of pre-grown carnations with a conidial suspension poured on the soil either directly (c, treatment C), or after wounding the roots with a scalpel (d, treatment D).

In both cases wilt developed slowly and gradually (Fig. 1 a, c). First, one or two wilted leaves appeared at the stem base, preceded or accompanied by a brown longitudinal stripe on the stem base surface. Wilt (without yellowing) of leaves and brown discolouration of the stem surface then slowly spread upwards, resulting in unilateral wilt of otherwise still healthy and often flowering plants. At the affected side, the stem often shrivelled and turned greyish. The base of leaves attached to such a shrivelled part of the stem usually withered completely and turned grey while the rest of these leaves still remained green and turgescent for a while (compare Fig. 2). When symptoms reached the shoot top, crook-necks were observed which were always curved to the affected side. Eventually, wilt also spread to the other sides and then was quickly followed by death of the plants. Wilted plants were dry and yellow, and their stems were often hollow. Wilt development after treatments A and C was reflected in the wilt index, which ran from 0 (no symptoms) through 1 and 2 (unilateral wilt) to 4 (death), whereas few plants with index 3 were observed as severely wilted plants died quickly. Symptoms were first noticed after an ample three weeks; typical unilateral wilt prevailed during the next 2-3 weeks, and plant death was so slow that few plants even survived throughout the experiment.

Deviating and rapid development of disease occurred after treatments B and D (Fig. 1 b, d). Green and healthy-looking, symptomless plants suddenly collapsed; their stems broke somewhere halfway the shoot, while the upper part of the shoot apparently was not diseased at all (Fig. 2). Examination of the stem revealed that it was hollow at the breaking point. Plants exhibiting such symptoms were indexed 3 (severe wilt), as they died soon after collapsing. Regular wilt symptoms (1, 2) were uncommon. The deviating wilt development after treatments B and D was reflected in the wilt index running from 0 (no symptoms) immediately to 3 (collapse) and then to 4 (death). Symptoms were again first noticed after an ample 3 weeks, but plant death generally followed within c. 10 days.

No wilt occurred in the controls, except for natural infection from 53 days onward of two carnations planted in steamed soil and treated directly thereafter with sterile water.

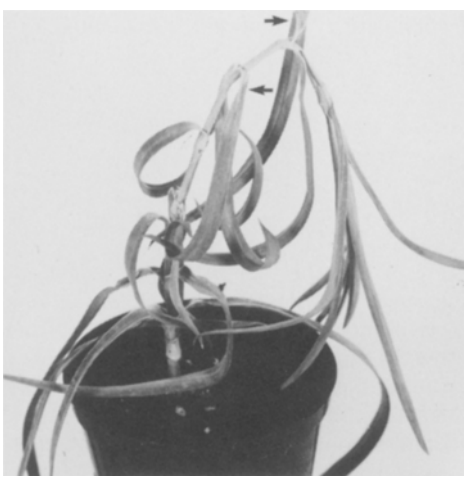

Fig. 2. 'Early Sam' carnation 27 days after inoculation with a conidial suspension poured on the soil and wounding the roots with a scalpel (treatment D), showing a broken stem while the upper part of the shoot still appears healthy. Note the withered bases (arrows) of otherwise green leaves attached to the shrivelled, hollow part of the stem at breaking level.

Passive spore transport in 'Lena' and 'Barbara' carnations after root inoculation. Passive transport of microconidia of *F. oxysporum* f. sp. *dianthi* through roots and stems of susceptible 'Lena' carnations was studied by immersing the terminal one cm of 2- or 7-cm-long root stumps in a conidial suspension for one night and then determining where the fungus was present. In plants with 2-cm-long root stumps, spores had been carried to 1-5(-20) cm height in the stem (Fig. 3) and were maximally transported to (3-)5-12(-22) cm from the cut end (Fig. 4). As expected, spores were spread discontinuously through the stems. In plants with 7-cm-long root stumps, spores had been carried to 0-1(-25) cm height in the stem and were maximally transported to (3-)7-12(-32) cm from the cut end (Fig. 5). No *F. oxysporum* was isolated from control carnations, except for three plants which had apparently been infected naturally, as the fungus was present in the upper parts of roots only (one plant) or in the stems up to 3 cm also (two plants). However, these were exceptions, and *F. oxysporum* was never encountered in the stems of controls above 3 cm height.

Experiments were repeated with susceptible 'Lena' and moderately resistant 'Barbara' carnations with roots cut off at 2 cm beneath the stem base. Both in 'Lena' and 'Barbara', spores were maximally transported (4-)5-10(-17) cm from the cut end confirming the previous findings (data not shown).

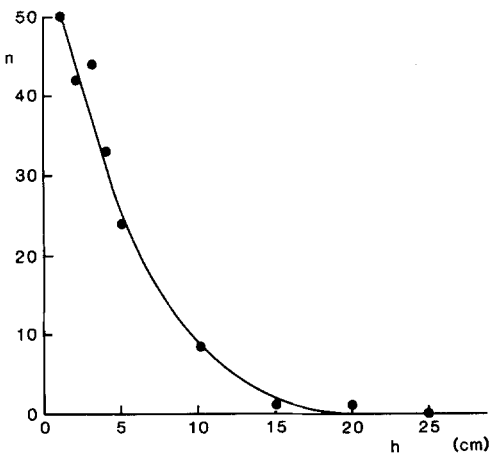

Fig. 3. Number (n) of 'Lena' carnations (out of 50 plants) from the stems of which *F. oxysporum* was isolated at h cm height, one night after cutting off their roots at 2 cm beneath the stem base and immersing the terminal one cm in a suspension of microconidia of *F. oxysporum* f. sp. *dianthi.*

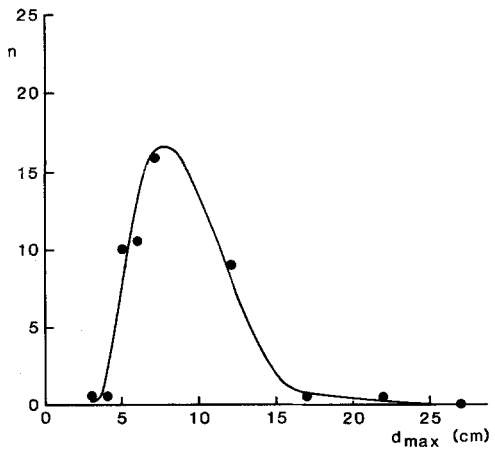

Fig. 4. Number (n) of 'Lena' carnations (out of 50 plants) in which *F. oxysporum* was demonstrated maximally d_{max} cm from the cut end, one night after cutting off their roots at 2 cm beneath the stem base and immersing the terminal one cm in a suspension of microconidia of *F. oxysporum* f. sp. *dianthi.*

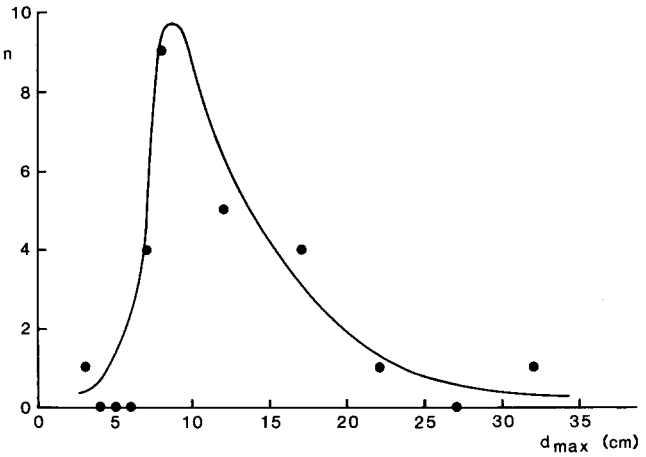

Fig. 5. Number (n) of 'Lena' carnations (out of 25 plants) in which *F. oxysporum* was demonstrated maximally d_{max} cm from the cut end, one night after cutting off their roots at 7 cm beneath the stem base and immersing the terminal one cm in a suspension of microconidia of *F. oxysporum* f. sp. *dianthi.*

Finally, passive spore transport was studied after 'Lena' and 'Barbara' carnations grown for four weeks in steamed soil had been inoculated by pouring a conidial suspension on the soil, both with or without wounding of the roots. In all four cases spores were found to have been carried to 0-6(-10) cm height in the stem. Although considerably fewer spores appeared to have been carried upwards and *F. oxysporum* was not demonstrated in all plants, the distance over which they had been transported may again be assumed to be c. 5-10 cm as here also one or several cm root length must have been passed. Control 'Lena' and 'Barbara' carnations treated similarly with sterile water were found not to contain any *F. oxysporum.* Results are only shown for 'Barbara' carnations not wounded with a scalpel (Fig. 6).

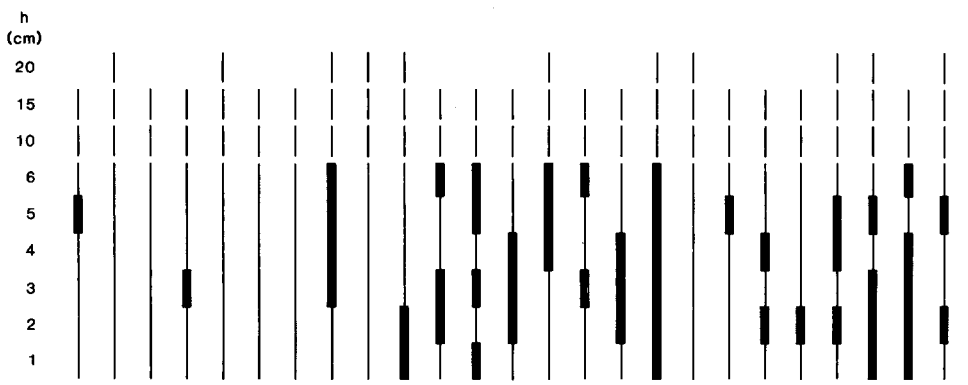

Fig. 6. Segments at h cm height of stems of 25 'Barbara' carnations in which *F. oxysporum* was demonstrated (thick lines) or not (thin lines), one night after pouring a suspension of microconidia of *F. oxysporum* f. sp. *dianthi* on the soil.

Discussion

Unilateral wilt was typical for disease development in 'Early Sam' carnations in the absence of either microconidial suspensions (treatment A) or wounded roots (treatment C). Wilt development corresponded largely with the excellent observations of Hantschke (1961). Treatments with both microconidia and wounded roots (B, D) induced atypical, quick collapse, exceptionally also observed by Hantschke (1961). Wilt of leaves, shrivelling of the stem surface and cavity formation in the stem in all cases were clearly due to destruction of the vascular tissues by the fungus as suggested by Baayen and Elgersma (1985). Toxins apparently were not involved, as no yellowing occurred (however, in other experiments it did sometimes occur; R.P. Baayen, unpublished results).

Breaking of stems (treatments B and D) was caused by destruction of the vascular tissues at the breaking site all around the stem, and therefore indicated multilateral infection. Natural infection of carnation by *F. oxysporum* f. sp. *dianthi,* however, induces unilateral wilt, which indicates that successful colonization of carnation roots occurs only rarely. This seems to be due to vascular resistance in the roots: colonization of the stem only occurs after the fungus overcomes the localization mechanisms operating in the roots (R.P. Baayen and C. van Eijk, unpublished results). Colonization of the stem remains unilateral because lateral and radial spread of the fungus seems to be inhibited by wall appositions and other lateral barriers at the edges of colonized areas (Baayen and Elgersma, 1985; Baayen, 1986; R.P. Baayen, unpublished results). Multilateral infection and quick death of plants, presumably due to multiple break-throughs in the root system, have only been observed under very high infection pressures (Hood and Stewart, 1957; R.P. Baayen and C. van Eijk, unpublished results). In our experiments, unilateral wilt occurred after treatments without root woundings as well as treatments with such woundings, but without microconidia, probably because root wounding per se does not affect the vascular defence mechanism of the roots. However, both treatments combining wounded roots and a microconidial suspension resulted in multilateral destruction of the vascular tissues. This may well have been due to passive transport of the microconidia through wounded roots directly into the carnation stem, thus passing by root resistance, which would explain both the multilateral character and the quick development of the disease seen after these treatments.

Further experiments with 'Lena' and 'Barbara' carnations revealed that passive transport of microconidia of *F. oxysporum* f. sp. *dianthi* indeed occurred both when roots were immersed in a conidial suspension overnight and when the suspension was poured on the soil. Regardless of the cultivar used and the root length involved in the experiment, spores were always transported through the xylem c. 5-10 cm, sometimes even over 30 cm within 24 h. This implies that mean maximal vessel length in carnation is c. 5-10 cm and maximal vessel length c. 30 cm, which is not exceptionally long (Greulach, 1973; Pegg, 1985). As to be expected, spores were spread discontinuously through the stem. However, spore transport also occurred when a microconidial suspension was poured on the soil of pre-grown carnations of which the roots had not deliberately been wounded. Many natural wounds must hence have been present in the involved experiment, as was indeed suggested by the many secondary infections by other fungi present in the roots of control plants from this experiment. Carnation roots

apparently are easily wounded, and plants with uninjured roots difficult to obtain.

Our results confirm those of Péresse (1968, 1971), who reported that conidia of *Phialophora cinerescens* poured on the soil were transported some 5-7 cm into the stem within 48 h. Conidia of *P. cinerescens,* however, are more apt to be transported through the xylem as they are much smaller (c. 2 × 4 μm) than those of *F. oxysporum* (c. 2.5 × 8 μm; Hantschke, 1961). Péresse (1971, 1975, 1977) considered such passive transport to be a natural mode of infection of carnations by *P. cinerescens.* Instead, we believe it is an artefact occurring after artificial inoculation only, as under natural circumstances millions of microconidia presumably do not occur together in the soil. Therefore, one should be careful when interpreting experiments using such an inoculation technique. For instance, the direct penetration of *F. oxysporum* f. sp. *dianthi* into both susceptible and resistant carnation cultivars reported by Rattink (1985) is probably due to such an artefact and no conclusions should be drawn about absence of resistance mechanisms in carnation roots. Similarly, discontinuous colonization of chrysanthemum stems by *F. oxysporum* f. sp. *chrysanthemi* early in the pathogenesis and especially at the base of plants, as reported by Fisher and Toussoun (1983), was in contradiction to the results of earlier studies by Emberger and Nelson (1981) and Stuehling and Nelson (1981). This again may have been an artefact, since inoculation was performed by pouring a conidial suspension on the soil after wounding the roots. Our results imply that screening for resistance by inoculation with a microconidial suspension, which is common practice in carnation breeding for resistance in the Netherlands (Sparnaaij and Demmink, 1977), is likely to overlook any valuable resistance (whether epidermal or vascular) residing in the roots. This may be a considerable disadvantage, especially since carnation breeders search for multiple and polygenic resistance. An alternative may be found in inoculation by planting in soil containing chlamydospores.

Passive spore transport is known to be part of the natural colonization process in several vascular wilt diseases (Hantschke, 1961; Beckman and Halmos, 1962; Péresse, 1975; Pegg, 1985). In elms, resistance to *Ophiostoma ulmi* is even correlated with the presence of shorter and narrower xylem vessel elements (Elgersma, 1970), probably because passive spore transport is part of the infection process in Dutch elm disease (Elgersma, 1967; Banfield, 1968) and because vessels with smaller diameters are more easily blocked by tyloses (Elgersma, 1973; Pegg, 1985). Preliminary investigations do not suggest that resistance of carnation to Fusarium wilt is correlated with special xylem vessel characteristics. Spore transport was not limited in resistant 'Barbara' in comparison with susceptible 'Lena' carnations either. In carnation, passive spore transport probably does not occur in the natural colonization process by *F. oxysporum* f. sp. *dianthi;* conidia appear in the xylem only after the mycelium is well established (Pennypacker and Nelson, 1972), and passive spore transport would imply a much quicker (and discontinuous) upward spread of the pathogen through the plant than observed, especially since spores may easily be carried some 10 cm along with the transpiration stream.

Acknowledgements

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Samenvatting

Passief transport van microconidiën van Fusarium oxysporum f. sp. dianthi in anjer na inoculatie van de wortels

Inoculatie van de wortels van vatbare anjers met *Fusarium oxysporum* f. sp. *dianthi* veroorzaakte de kenmerkende eenzijdige verwelking alleen als wortelbeschadigingen bij de inoculatie niet werden gecombineerd met het gebruik van een suspensie van microconidiën. Die combinatie veroorzaakte namelijk afwijkende symptomen, waarbij de planten plotseling omknakten en vervolgens snel afstierven. De verwelking leek in alle gevallen veroorzaakt te worden door afbraak van het xyleem. Eenzijdige verwelking leek te volgen op spaarzame natuurlijke wortelinfecties. Bij omgeknakte planten bleek het vaatweefsel rondom in de stengel aangetast te zijn, hetgeen toegeschreven wordt aan infectie van verschillende kanten van de stengel als gevolg van passief transport van microconidiën bij de inoculatie door verscheidene beschadigde wortels direct de stengel in.

Microconidiën werden binnen 24 uur 5-10(-30) cm de stengels van vatbare en resistente anjers ingezogen wanneer de wortels afgesneden en met het uiteinde in een sporensuspensie gehangen werden, maar ook wanneer de suspensie op de grond gegoten werd. Passief transport van sporen is een inoculatie-artefact dat echter belangrijke consequenties kan hebben voor de interpretatie van de resultaten van proeven. Bij de natuurlijke verspreiding van *F. oxysporum* in anjers lijkt passief sporentransport van weinig belang.

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Book review

J. Lacey (Ed.), 1985. Trichothecenes and other mycotoxins. Proceedings of the International Mycotoxin Symposium held in Sydney, Australia, August 1984, organized by the Mycotoxicology Committee of the International Society for Plant Pathology. John Wiley & Sons, Chichester. 597 pp. Price £ 55.00.

The importance of mycotoxins in food and feed has gained increasing recognition in recent years and the knowledge in this field is extending rapidly. In 1983, two symposia were devoted to mycotoxins, three weeks after the meeting in Sydney, another was held in association with the Third International Mycological Congress in Tokyo (H. Kurata & Y. Ueno (Eds), 1984. Toxigenic fungi: their toxins and health hazard. Kodansha, Tokyo; Elsevier, Amsterdam). Both meetings contain a diversity of chapters on the present state of the art. In both meetings, emphasis was laid on trichothecene mycotoxins, toxins produced by species of *Fusarium* and *Myrothecium.* In the book from Tokyo, sections cover the ecology and taxonomy of mycotoxin-producing fungi, food and feed mycology, toxicology, and epidemiology of mycotoxins. In the Sydney symposium, chapters are as follows: 1. taxonomy and occurrence of toxigenic fungi (60