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Localization of phytoalexin accumulation and determination of changes in lignin and carbohydrate composition in carnation (Dianthus caryophyllus L.) xylem as a consequence of infection with Fusarium oxysporum f. sp. dianthi, by pyrolysis-mass spectrometry

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Abstract

Minute pieces of xylem and other tissues from stems of healthy and fungus-infected plants of two carnation cultivars Novada and Lena were investigated for lignification (lignin/polysaccharide ratios) and lignin composition by means of pyrolysis mass spectrometry and pyrolysis gas chromatography mass spectrometry. This technique proved also very effective for the localization of dianthramide phytoalexins which accumulate in carnation after infection with *Fusarium oxysporum* f.sp. *dianthi*. The composition of healthy tissues from both cultivars was practically the same. In the resistant cultivar Novada, infection induced a change from guaiacyl-syringyl lignin into a mainly guaiacyl lignin in the gum-occluded parts of the xylem. Considerable amounts of the phenolic amide dianthalexin and of other dianthramide phytoalexins were present in occluded xylem, but not in adjacent phloem, medulla or unoccluded xylem. Xylem from susceptible 'Lena' suffering degradation was characterized by a loss of syringyl groups from the lignin and by demethylation of pectin in an early stage of infection. Small quantities of dianthalexin and other dianthramide phytoalexins were found in 'Lena' when local defense responses (particularly occlusion) had occurred. In both cultivars evidence for degradation of hemicellulose was found.

Additional keywords: cell walls, guaiacyl lignin, syringyl lignin, dianthramides.

Introduction

A major part of the resistance mechanism of carnation to fusarium wilt, caused by *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen f.sp. *dianthi* (Prill. & Del.) Snyder & Hansen (Fod), resides in the physiological response of the host after the fungus has invaded the xylem vessels (Baayen, 1988). In resistant cultivars the vascular parasite becomes localized within the xylem. Barriers are formed by gummosis of affected vessels, by lignification of the primary walls of surrounding cells and by suberization in zones surrounding the infected area (Baayen and Elgersma, 1985; Baayen, 1988). Fungitoxic

phenolic amides, partially wall-bound, are produced (Niemann and Baayen, 1988) which may retard initial fungal growth. To a certain extent similar reactions occur in susceptible cultivars; the barriers formed, however, in this case usually are insufficient and the xylem is colonized and degraded (Baayen and Elgersma, 1985).

The gums seem to be formed in the xylem parenchyma cells adjacent to the infected vessels (Catesson et al., 1976; Baayen and Elgersma, 1985) and are composed of various amounts of polysaccharides encrusted with (poly)phenolic compounds. Phenolics are probably released by the stimulus of infection in these same parenchyma cells. Infusion of phenolics into (or rather their oxidation in) the gums was noticed shortly (about 3 days) after the initial gum formation, as judged from their change in colour from pale yellow to orange and dark brown (Baayen, 1988). Histochemical tests indicated the presence of lignin, but the absence of syringyl (S) constituents in the brown gums. The secondary walls of unaffected xylem vessels contain a mixed guaiacyl-syringyl (G-S) lignin (Niemann et al., 1990); the syringyl groups may be visualised histochemically using Mäule's test (Baayen, 1988). In lignified walls of gum- occluded vessels, however, syringyl groups could often no longer be detected histochemically (Baayen, 1988).

The importance of lignin and carbohydrate cell wall polymers in the defense against fusarium wilt led us to a more extensive investigation of these groups of constituents. Analytical pyrolysis provided a convenient method for the analysis of such high molecular weight material. Thermal dissociation of the complex polymers yields smaller fragments which as such can be analysed by mass spectrometry. Only minute pieces of fresh plant tissue are required, which enabled us to restrict our investigations to visually infected parts of the xylem.

In the course of our investigations pyrolysis-mass spectrometry (PYMS) also proved useful in the detection of the (low molecular) dianthramide phytoalexins of carnation. New data on the restricted location of these compounds could thus be obtained.

A study on lignification and lignin composition in different tissues of stems of healthy carnations, analysed by PYMS, has recently been carried out (Niemann et al., 1990). The effects of infection with *F. oxysporum* f.sp. *dianthi* on resistant and susceptible carnation cultivars are reported here.

Materials and methods

Inoculum. A virulent isolate of *Fusarium oxysporum* f.sp. *dianthi* (WCS 816) was cultured on potato dextrose agar culture slants. Conidial suspensions were obtained by shaking one or more of these cultures with demineralized water. Mycelial fragments were removed by filtration through glasswool and the suspension was adjusted to a concentration of 5×10^6 conidia ml^{-1}.

Plant material and inoculation of the plants. Rooted cuttings of the carnation cultivars Novada (highly resistant to fusarium wilt) and Lena (susceptible) were obtained by courtesy of Dr L.D. Sparnaaij of the Institute for Horticultural Plant Breeding (IVT) at Wageningen. They were planted in steamed soil and allowed to grow in a greenhouse at 22 °C for 3 to 5 weeks prior to inoculation. Stem inoculation was carried out as described previously (Baayen and Elgersma, 1985) with 20 μl inoculum per plant. Control plants of each cultivar remained untreated, or were mock inoculated with water instead of the conidial suspension.

Investigated xylem samples. In a preliminary investigation, occluded and non-occluded parts of the xylem at 5 mm above the inoculation site were removed from three 'Novada' carnations one month after inoculation.

In a second experiment, tissue samples from the stems were obtained three weeks after inoculation at different heights above the inoculation site from three 'Novada' and three 'Lena' carnations. The samples were obtained by carefully dissecting ca 0.2-mm-thick hand-cut transverse sections in a drop of water under a dissecting microscope. Occasionally also a radial section was investigated. Epidermis, cortical parenchyma, the sclerenchymatous fiber sheath, the phloem (including remaining parenchymatous tissue between phloem and fiber sheath), xylem and pith could be pealed off consecutively. Xylem could not always be completely separated from medullary parenchyma, especially in sections closer to the apex, with relatively young xylem. Xylem from infected 'Novada' stems was divided in gum-occluded parts and healthy-looking parts. Xylem from infected 'Lena' stems sometimes also contained small gum-occluded areas; diseased areas were often marked by a central part with whitish loose vessels and markedly brown edges at the inner (medulla) and outer (phloem) side. When present, these parts were dissected separately, as well as healthy-looking xylem from the diseased plants. In other cases diseased xylem was dissected without further subdivision. Only untreated plants were used as controls in this experiment.

In a third experiment, xylem was sampled 1, 3 and 7 days after inoculation at 2-4 mm above the inoculation site from three inoculated, two water-treated and two wholly untreated plants per cultivar per sampling day.

Curie-point pyrolysis methods. Curie-point pyrolysis-mass spectrometry was carried out on the FOMautoPYMS®. The pyrolysis temperature was 510 °C and the total pyrolysis time 0.8 s. The pyrolysis chamber and the expansion chamber were heated to 160 and 200 °C, respectively. To prevent extensive condensation of the pyrolysate, the waiting time before pyrolysis was 10 s. Low voltage electron impact ionization (EI) at about 15 eV was employed to obtain molecular weight distributions of the pyrolysate with a minimum of fragmentation due to the ionization process. The mass range of the mass spectrometer used was m/z 20-250. The scan speed was 10 scan sec^{-1} with a total data acquisition time of 20 s. The PYMS spectra shown are summarized average spectra for the total pyrolysis period calculated from three (sometimes two) analyses per tissue studied.

Pyrolysis-gas chromatography-mass spectrometry (PYGCMS) was used for the identification of the pyrolysis products, and to correlate mass peak-information with molecular structure. A complete list of the hitherto identified fragments in healthy carnation tissues has been given elsewhere (Niemann et al., 1990). Samples of infected xylem were analysed by PYGCMS using a 50 m thick-film (1 μm) apolar fused-silica capillary column, ID 0.32 mm, coated with CPSil-5. During pyrolysis injection, the oven was at room temperature to trap the pyrolysate in the first few centimeters of the column. After injection the oven was first kept at 30 °C for 2 min and then programmed to 325 °C at 4 °C min^{-1}. Only partial total ion current (TIC) profiles from m/z 110-250, which mainly show peaks from pyrolysis products of lignin (and some specific peaks from pyrolysis products of dianthramide phytoalexins) are shown. An extensive description of the method is given by Boon (1989).

Multivariate analysis of PYMS data. Discriminant analysis was performed on the PYMS data files, using a modified ARTHUR package, adapted to PYMS data (Boon et al., 1984). In this method, spectra are considered to be points in a multidimensional space with the mass numbers as coordinate axes. The relative distribution of mass intensities in each spectrum determines the position in the multidimensional space. Similar spectra will cluster as one group. From the file of selected spectra an overall average spectrum ('zero point') is calculated which serves as a reference point for the individual spectra. Mathematically, the differences between the individual spectra are determined by comparison with the zero point spectrum. These data are factor analysed to produce sets of correlated mass peaks (factors). These covariant mass peaks are linearly combined into new independent variables (discriminant functions), which are represented graphically by reconstructed mass spectra. Dissimilarity is quantitatively expressed in discriminant function scores. For multivariate analysis, summarized average PYMS spectra for the total pyrolysis period calculated from each separate analysis were used, thus giving three (sometimes two) locations for each individual sample in the scoreplot.

Results

Analysis of xylem and other tissues of 'Novada'. Fig. 1 shows the PYMS spectra of healthy-looking (A) and gum-occluded (B) xylem dissected at 5 mm above the inoculation site from a carnation which had been inoculated one month earlier. The PYMS spectrum of the healthy-looking xylem is similar to xylem spectra from untreated controls (Niemann et al., 1990), showing a mixed G-S lignin characterized by fragments *m/z* 180, 178, (166), 164, 152, 137 and 124 indicative of guaiacyl (G) lignin and *m/z* 210, 208, 196, 194, 182, 167 and 154 from syringyl (S) lignin. The spectrum of the occluded xylem (Fig. 1B) differs in two major aspects: the mass peaks of S lignin have disappeared or are diminished, and a number of novel peaks (e.g. *m/z* 239, 213, 195, and 105) have emerged or (e.g. *m/z* 167, 149) are increased. The latter mass peaks are derived from dianthalexin (*m/z* 239) and other phytoalexins of carnation as argued below. Mass peaks

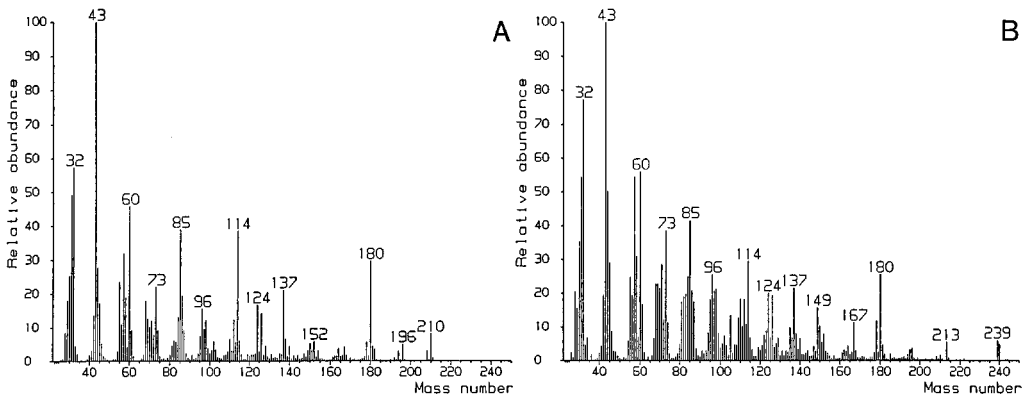
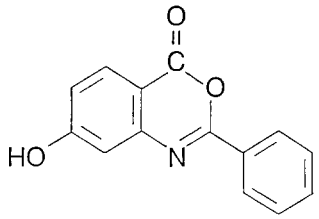
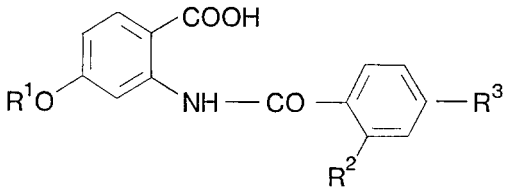


Fig. 1. PYMS spectra of xylem samples from the resistant carnation cultivar Novada, one month after inoculation with Fod. A, healthy-looking xylem; B, gum-occluded xylem.



Dx (dianthalexin)



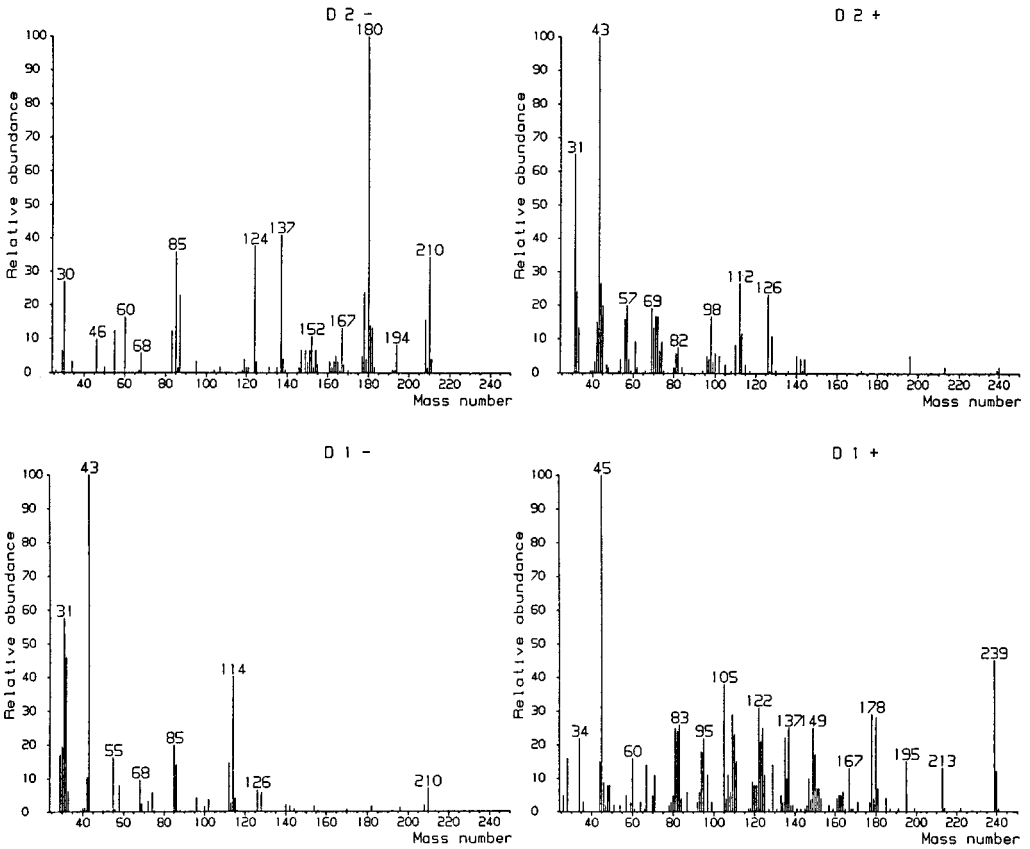
$R^1 = R^2 = R^3 = H$: HDB
$R^1 = Me$, $R^2 = R^3 = H$: MDB
$R^1 = Me$, $R^2 = R^3 = OH$: MDR
$R^1 = Me$, $R^2 = OH$, $R^3 = H$: MDS

Fig. 2. Structures of the main phytoalexins from carnation: dianthalexin (Dx), HDB (hydroxydianthramide B), MDB (methoxydianthramide B), MDR (methoxydianthramide R) and MDS (methoxydianthramide S). B, R and S stand for benzoic, resorcylic and salicylic acid respectively.

m/z 149 and 167 are also indicative of phtalates , usual contamination artifacts, and therefore have to be interpreted with care.

Various phytoalexins are produced by carnations in response to fungal infections (Niemann and Baayen, 1988; Ponchet et al., 1988). The dianthramide derivative dianthalexin (Dx, MW = 239) generally accumulates in highest amount, together with a large number of dianthramides. Of these, hydroxydianthramide B (HDB, MW = 257), methoxydianthramide R, (MDR, MW = 303), B (MDB, MW = 271) and S (MDS, MW = 287) are the major ones. General structures of these phytoalexins are given in Fig. 2. When examining the pure compounds (provided by Dr M. Ponchet, Antibes) we found that both Dx and MDS are fairly stable during pyrolysis with PYMS (low voltage −15 eV −EI) spectra with respectively *m/z* 239 (100%, M^+), 195 (50%, $[M - CO_2]^+$), 162 (14%), 105 (10%, $[ArCO]^+$) for Dx and *m/z* 287 (100%, M^+), 269 (20%, $M - H_2O$), 243 (3%, $M - CO_2$), 167 (80%, M − ArOCO), 150 (30%), 149 (28%) and 121 (14%) for MDS, thus corresponding with a number of the novel mass peaks in Fig. 1B. *M/z* 213 is probably derived from HDB $[M - CO_2]^+$ or MDB $[M - 58]^+$; *m/z* 105 $[ArCO]^+$ could originate from all benzoic acid-derived compounds (Dx, HDB and MDB). Similar fragments from MDS and MDR (*m/z* 121 and 137) were partly masked by lignin and/or polysaccharide fragments, as would the other fragments of these two compounds (*m/z* 167 and 153, $[M - ArOCO]^+$; and *m/z* 149 and 135, $[M - ArOCO - H_2O]^+$).

The possibility to recognize the phytoalexins Dx, HDB and MDS by their molecular ion and/or fragments in the PYMS fingerprint spectra allowed for a precise localization of these compounds in the infected carnations. Thus, the presence or absence of the phytoalexins was determined in occluded xylem and adjacent tissues of four inoculated plants from the second experiment, *viz.* seemingly unaffected xylem from the same stem slices as the occluded xylem; medulla and phloem tissue immediately next to the occluded xylem; apparently unaffected xylem at 5 and 10 cm above the inoculation site (well above the occluded area), and in one plant also newly formed and apparently unaffected xylem in between the occluded area and the cambium. As judged from the PYMS data (not shown), Dx and HDB (and undoubtedly the other dianthramides as well) were almost completely restricted to the visibly occluded parts of



the xylem. Only in one plant small amounts of Dx and HDB were found in seemingly unaffected xylem directly adjacent to the occluded parts, which may have been due to improper dissection. Otherwise, traces of the phytoalexins were not found, neither in phloem or medulla, nor in seemingly unaffected xylem above the occluded area.

Multivariate analysis was carried out on the PYMS spectra of unaffected and occluded xylem from the inoculated plants of the second experiment, including unaffected xylem from four untreated controls. The resulting scoreplot and discriminant functions are shown in Fig. 3. In the scoreplot each individual plant or plant part is presented by three (sometimes two) interconnected symbols; similarly treated and located tissues from different plants received the same character. Discriminant function 1, the one giving highest resolution among the samples, differentiates occluded xylem (the positive discriminant function spectrum, DF1+) from healthy and seemingly healthy xylem (the negative spectrum, DF1−). DF1+ combines the mass peaks *m/z* 180, 178, 164, 152, 137 and 124 of G lignin and *m/z* 239, 213, 195, 122 and 105 of the phytoalexins (probably also including part of *m/z* 181, 167, 149, 137, 135, 123 and 94). DF1- contains *m/z* 210, 208, 182 and 154 from S lignin and a high contribution of pentosans (*m/z* 114). The second discriminant function DF2 gives a division between polysaccharides (mainly in the mass range *m/z* 20-126, + 140 and 144; with 57, 60 and 73 from

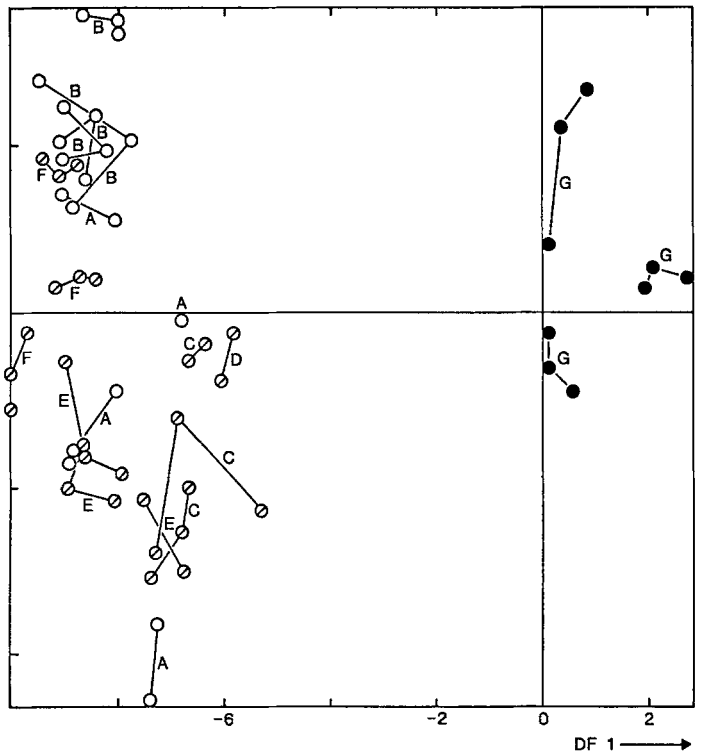


Fig. 3. Score plot and discriminant function spectra for a series of PYMS spectra of 'Novada' xylem samples from healthy and infected plants, three weeks after inoculation. Individual plants or plant parts are represented by three (sometimes two) sample analyses; replicate samples from the same plant are interconnected. D1 −, D1 +, D2 − and D2+ (discriminant function spectra) are the reconstructed mass spectra on which the scoreplot is based (see section *Multivariate analysis of PYMS data*).

o, xylem from untreated plants; ∅, seemingly healthy xylem from inoculated plants; •, occluded xylem from inoculated plants.

A, xylem from the base and B, xylem from the apex of the stem of healthy controls; C and D, seemingly healthy xylem from inoculated plants, found at 5 mm above the inoculation site adjacent to the occluded xylem (C) or newly formed in between the occluded xylem and the cambium (D); E and F, seemingly healthy xylem from inoculated plants at 5 cm (E) and 10 cm (F) above the inoculation site; G, occluded xylem at 5 mm above the inoculation site.

levoglucosan from cellulose, 114 from pentosans from hemicellulose, 126 and 144 from hexosans from cellulose or hemicellulose, and 128 and 144 from rhamnogalacturonans from pectin; not all fragments are present) together with *m/z* 196 (dihydroferulic acid) in positive direction, and G-S lignin in negative direction. The position of the gum-occluded xylem samples in the upper righthand section of the score plot demonstrates the various effects of infection, including phytoalexin accumulation, a change from G-S lignin to G-dominated lignin, and a slightly decreasing lignin-carbohydrate ratio. It appears that xylem from sections close to the apex (samples B and F) is less lignified than xylem close to the stem base (samples A, C, D and E).

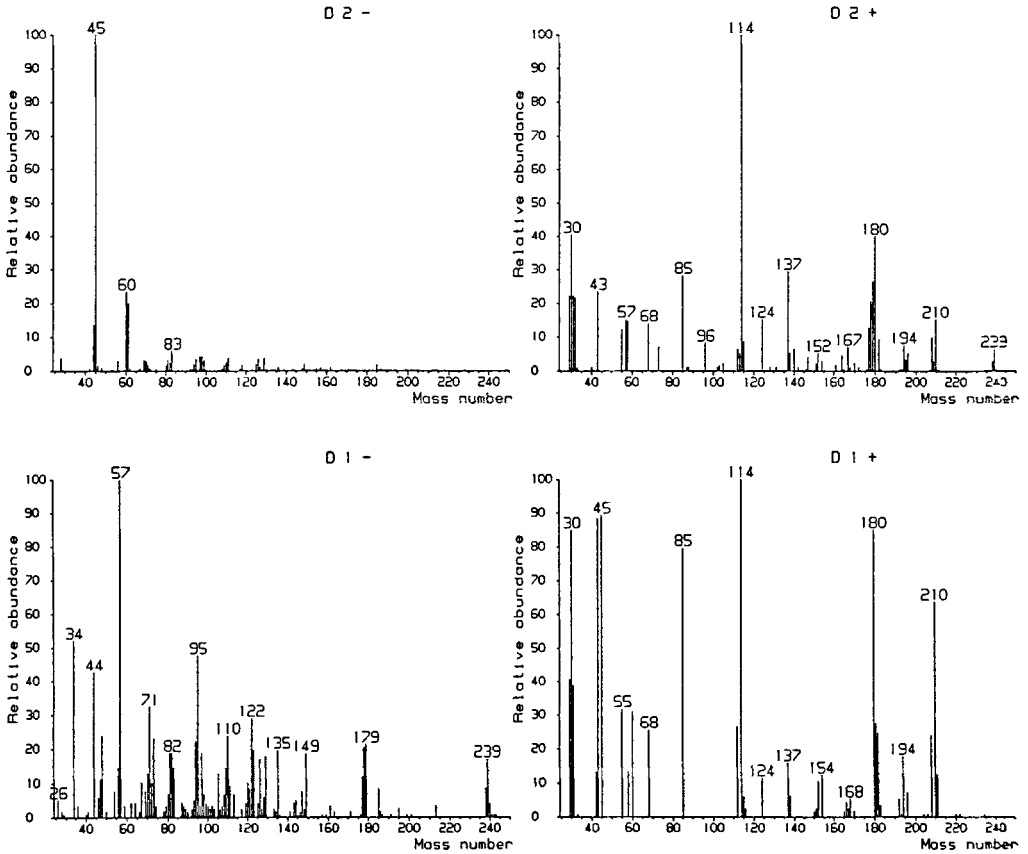


Fig. 4 shows the score plot and disciminant functions for the PYMS data of xylem from inoculated and mock-inoculated plants shortly after treatment, and from untreated controls. The first signs of gummosis of the sampled tissues were detectable by microscopical examination three days after inoculation. DF1− in this case combines the mass peaks of the phytoalexins, protein (with a.o. *m/z* 117 from tryptophan, 92 from phenylalanine, 48 from methionine and 34 from cysteine), adenine (*m/z* 135) and fatty acids (*m/z* 129). The latter might indicate initial suberization, histochemical evidence for suberin formation, however, was only found at a later stage of infection (Baayen, 1988). DF1+ contains the mass peaks of G-S lignin and of hemicellulose pentosans. DF2 gives a further subdivision between hemicellulose with G-S lignin and dianthalexin in DF2+ and acetic acid (*m/z* 60, 45; Pouwels et al., 1987) in DF2−. In the scoreplot, the untreated controls are found at the upper right side. Already one day after inoculation, the spectra of the xylem samples from inoculated plants (G in Fig. 4) shift to the lower middle side due to accumulation of phytoalexins and of some proteins and other signs of metabolic activity (DF1−) and acetic acid (DF2−). Enrichment in polysacharides at one day after inoculation (or wounding) was indicated by the third discriminant function plots (not shown). Phytoalexins have further accumulated at 3 and 7 days after inoculation (H and I). From day 3 to 7 a shift in upper

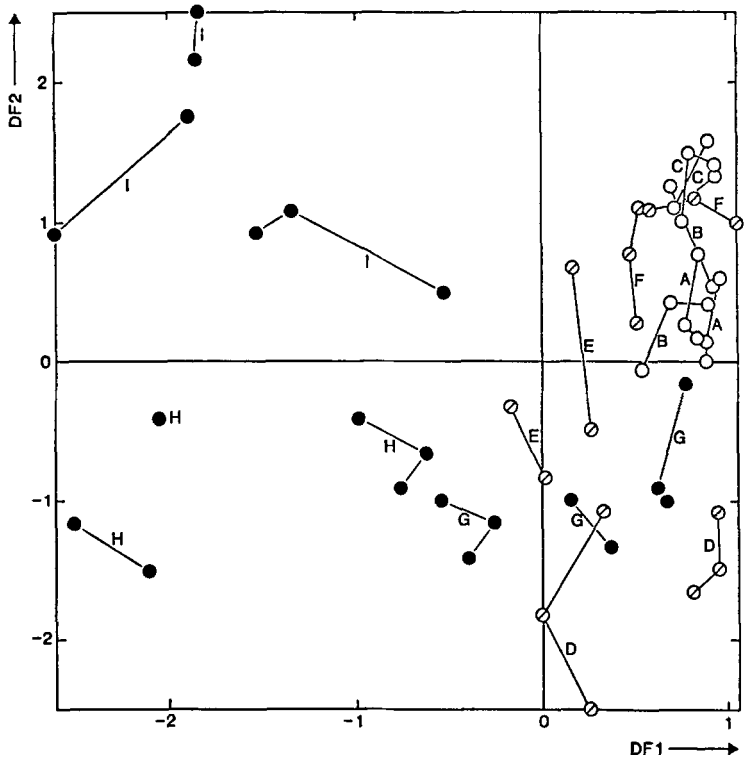


Fig. 4. Score plot and discriminant function spectra for a series of PYMS spectra of 'Novada' xylem samples from untreated (A-C), water-treated (D-F) and Fod-inoculated plants (G-I), dissected 1, 3 and 7 days after treatment at 2-4 mm above the inoculation site. Individual plants or plant parts are represented by three (sometimes two) sample analyses; replicate samples from the same plant are interconnected. D1 −, D1 +, D2 − and D2+ (discriminant function spectra) are the reconstructed mass spectra on which the scoreplot is based (see section *Multivariate analysis of PYMS data*)

o, xylem from untreated plants; ∅, xylem from water-treated plants; •, xylem from inoculated plants.

A, B, C, samples from untreated plants taken after 1, 3 and 7 days, respectively; D, E, F, samples from water-treated plants taken 1, 3 and 7 days after treatment, respectively; G, H, I, samples from inoculated plants taken 1, 3 and 7 days days after inoculation.

direction occurs which is probably due to a change of polysaccarides and increased accumulation of dianthalexin (*m/z* 239 in DF2+). Spectra from xylem of mock-inoculated plants also showed an early shift in lower direction (D, E), mainly due to acetic acid, as a result of wounding. Seven days after the incision (F) the sample spectra are back at the initial position. Acetic acid can be formed from acetylglucuroxyxylan, but also from cellulose (Pouwels and Boon, 1989). Its temporary occurrence shortly after wounding, however, can hardly be explained with these sources.

Analysis of xylem of 'Lena'. Susceptible carnations respond to infection with Fod in various ways, including complete localization of the fungus, colonization and degrada-

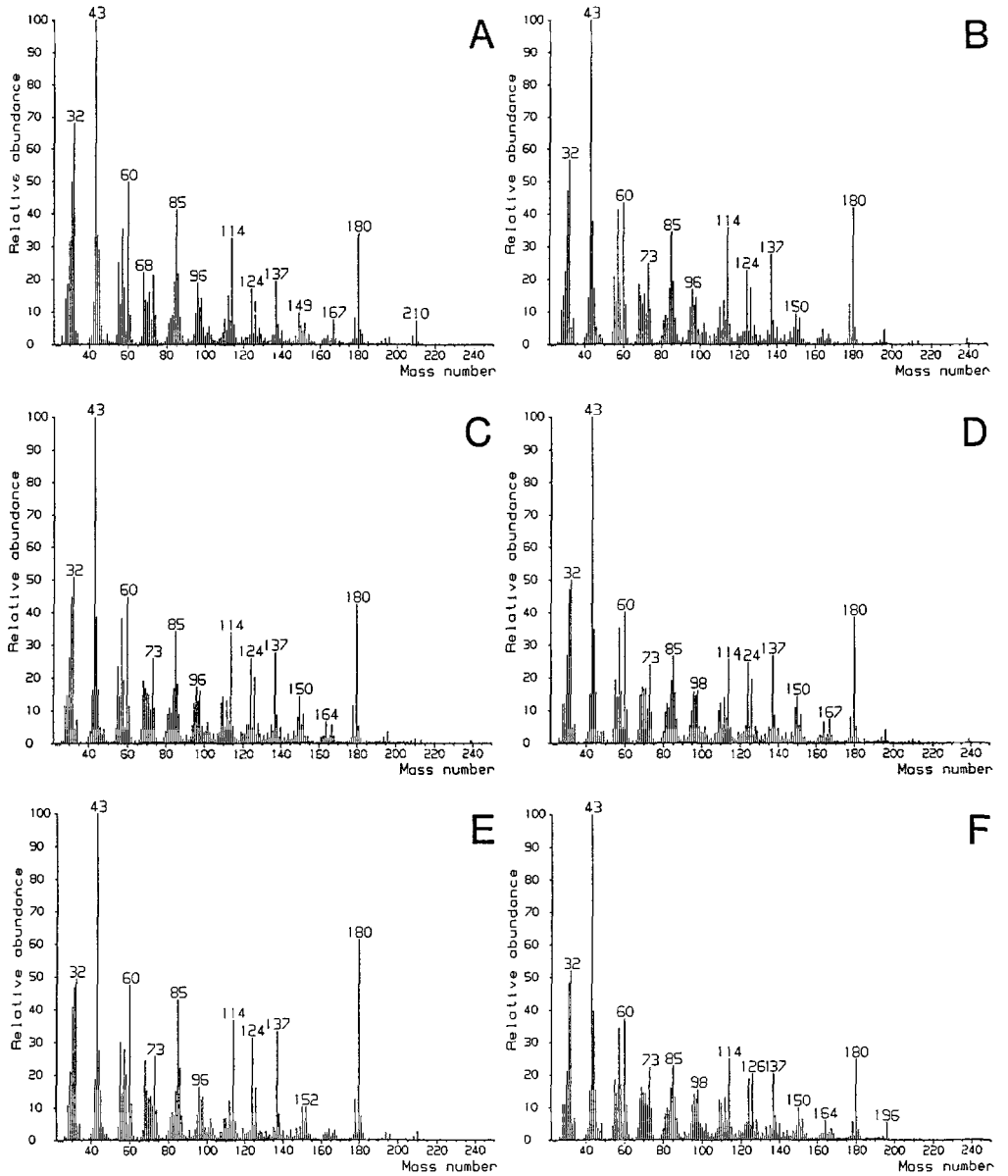
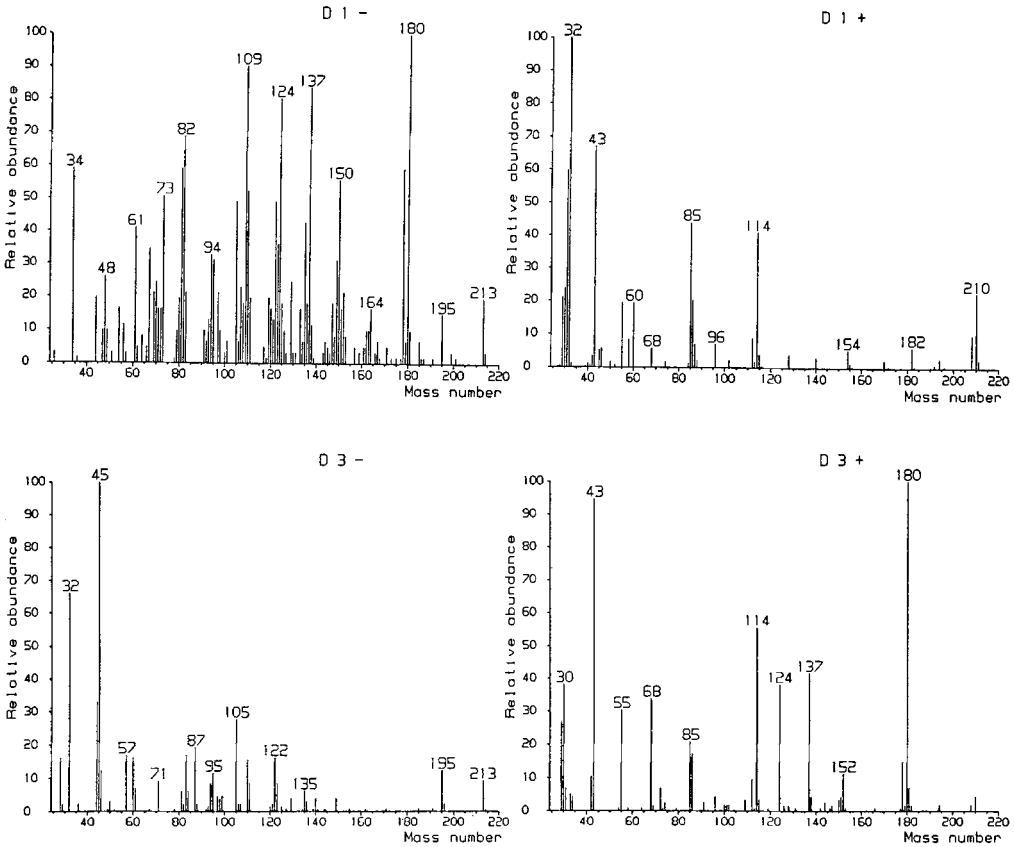


Fig. 5. PYMS spectra of xylem samples taken at 5 mm above the inoculation site from the susceptible carnation cultivar Lena, three weeks after inoculation with Fod.
A, healthy-looking xylem; B, gum-occluded xylem; C, diseased xylem including white core (E) and brown edges (D and F); D, inner brown edge of the diseased xylem, adjacent to the medulla; E, whitish inner core of the diseased xylem, and F, outer brown edge of the diseased xylem adjacent to the phloem.

tion of the xylem by the fungus, or a combination of both, even within one and the same plant (Baayen et al., 1988). When dissecting xylem from the inoculated 'Lena' carnations, most of these responses were encountered, and it proved possible to separately dissect pieces of xylem with such different response types. The PYMS spectra of such different parts of the xylem from one transverse section of a 'Lena' carnation (inoculated three weeks before) are given in Fig. 5. About one third of the xylem from the section seemed healthy, which was confirmed by the PYMS spectrum (Fig. 5A). Part of the remaining xylem was evidently suffering colonization and degradation, resulting in a white inner part with loose, bleached vessels and vessel remnants which was bordered at the sides of phloem and medulla by a narrow layer of tissue with dark brown cell walls known to be lignified (Baayen, 1988). The diseased xylem was either sampled in total, including the brown edges (PYMS spectrum in Fig. 5C) or subdivided into three distinct layers (Fig. 5D, E and F). Locally some xylem could be sampled exhibiting a limited defense response (occlusion; Fig. 5B). Compared with healthy xylem (Fig. 5A), all PYMS spectra of affected xylem contained substantially less syringyl lignin (*m/z* 210, 208, 182 and 154 have disappeared or are decreased). Only minute amounts of the various phytoalexins were detected, mainly in the samples of gum-occluded xylem Fig. 5B) and in still smaller amounts in the inner brown edge of the colonized area (Fig. 5D) and in total diseased xylem (Fig. 5C). Further information on the alterations in infected xylem of 'Lena', three weeks after inoculation, was provided by multivariate analysis of the spectra which is discussed below for a combined scoreplot of data from 'Novada' and 'Lena'.

The alterations in the first few days after inoculation of 'Lena' carnations were analysed as well (scoreplot and discriminant functions not shown). Separation was mainly along the axis of the first discriminant function which contained marker fragments from guaiacyl lignin, phytoalexins, protein, adenine and fatty acids in the positive direction and markers for syringyl lignin, pentosan and methylated pectin in negative direction (Niemann, 1990). All untreated and mock-inoculated plants as well as plants inoculated one day before sampling plotted on the negative side of DF1. Already at three days and still more at seven days after inoculation, significant changes in the positive direction of DF1 occurred which therefore indicate a change from G-S lignin to G lignin, demethylation of pectin and (limited) accumulation of phytoalexin already during the first days after inoculation.

Multivariate treatment of the PYMS data of 'Novada' and 'Lena' xylem. Factor-discriminant analyses were performed on various sample sets from 'Novada' and 'Lena' xylems. Two of the score plots obtained in these analyses are given. One comparison consisted of a data set restricted to xylem samples at 5 cm from the stem base which are therefore considered to be of about the same age (Fig. 6), and another comparison was based on the complete set of data of all xylem samples investigated in the same experiment 2 (Fig. 7). In these figures open circles or triangles represent (seemingly) healthy xylem, the solid ones representing affected xylem. The three (sometimes two) analyses of individual plants are interconnected. Note that the score map in Fig. 6 is based on discriminant functions 1 and 3, whereas that given in Fig. 7 is based on functions 1 and 2. In Fig. 6 discriminant function 1 provides a good separation between (seemingly) unaffected xylem (DF1+) on the one hand and xylem undergoing colonization or occlusion on the other hand (DF1−). The positive function spectrum comprises



S lignin fragments (*m/z* 210, 208, 194, 182 and 154) and a number of other fragments derived from deoxysugars (*m/z* 128), from pentosans (*m/z* 114) and from methylated pectin (*m/z* 85 + (172), 140, 113; Boon et al., 1989). The negative spectrum comprised fragments from G lignin (*m/z* 180, 178, 164, 152, 137, 124), phytoalexins (*m/z* 213, 195, 122, 105; *m/z* 239 is present as well, but not displayed in the spectra, protein (*m/z* 117 from tryptophan; 108 and 94 from tyrosine; 92 from phenylalanine; 81 and 67 from proline; 48 from methionine and 34 from cysteine) and fatty acids (*m/z* 129). On the x-axis of Fig. 6, discriminant function 3 separates occluded xylem (DF3 –) from xylem suffering degradation (DF3 +) on basis of the presence of phytoalexins (negative spectrum) or richness in G-S lignin (positive spectrum). The scoreplot based on DF1 and DF3 shows that, in addition to a change in lignin composition and induced dianthramide accumulation, infection in 'Lena' also causes loss of hemicelluloses and apparently some demethylation of pectin as reported above for the first days after infection. The position of gum-occluded 'Novada' xylem (D in Fig. 6) in the lower left hand corner is determined by enrichment mainly in phytoalexins (D1 – , D3 –), and to a lesser extent probably in polysaccharide content (DF3 –), and by decreasing syringyl (or increased guaiacyl) content of the lignin (DF1 –). Note that gum-occluded xylem from 'Lena' (I in Fig. 6) has an intermediate position between diseased xylem from 'Lena' and gum-

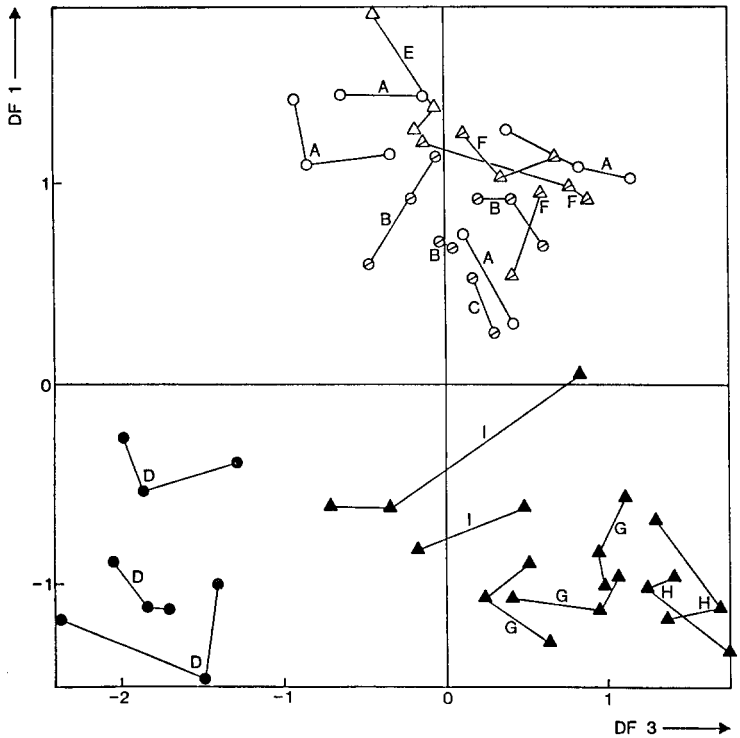
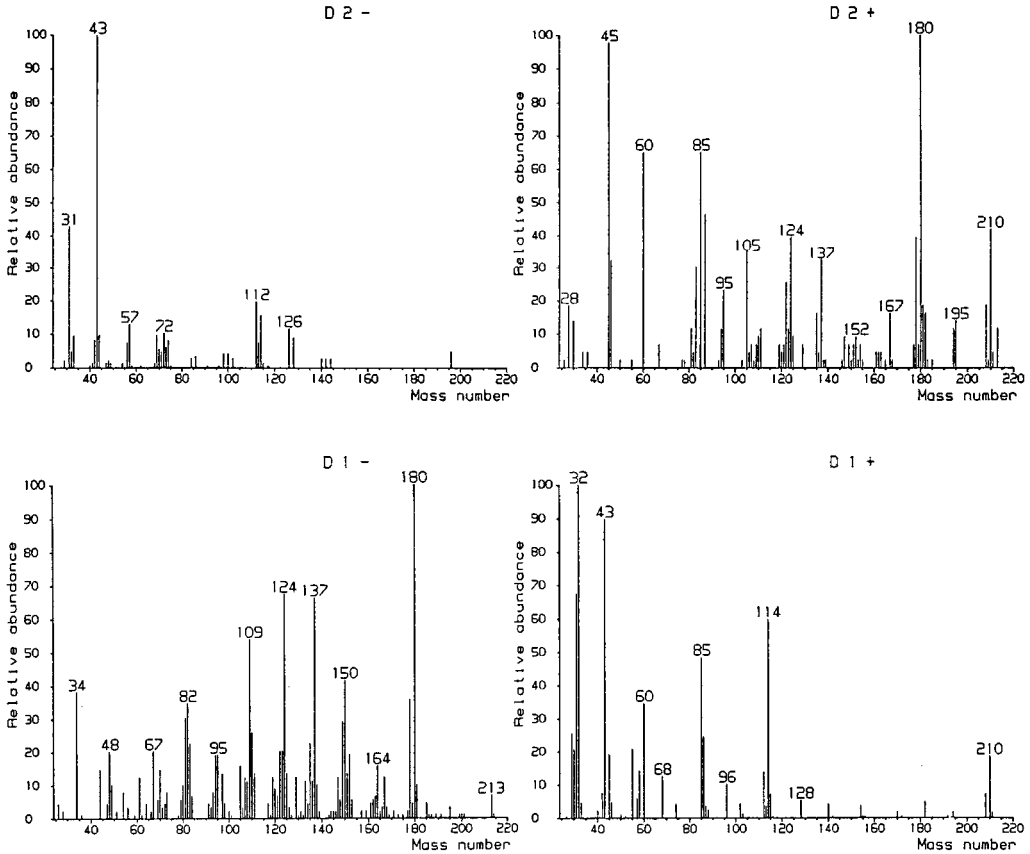


Fig. 6. Score plot and discriminant function spectra for a series of PYMS spectra of xylem samples taken at 5 mm above the inoculation site from healthy and infected 'Lena' and 'Novada' carnations, three weeks after inoculation. Individual plants or plant parts are represented by three (sometimes two) sample analyses; replicate samples from the same plant are interconnected. D1 −, D1 +, D3 − and D3 + (discriminant function spectra) are the reconstructed mass spectra on which the scoreplot is based (see section *Mulivariate analysis of PYMS data).*
o and △, xylem from untreated plants; ∅ and ⧋, seemingly healthy xylem fom inoculated plants; • and ▲, affected xylem from inoculated plants; o, ∅ and •, 'Novada'; △, ⧋ and ▲, 'Lena'. A, xylem from untreated 'Novada' plants; B and C, healthy-looking xylem from inoculated 'Novada' plants, found adjacent (B) to the occluded xylem or newly formed in between the occluded xylem and the cambium (C); D, gum-occluded xylem from inoculated 'Novada' plants; E, xylem from an untreated 'Lena' plant; F, healthy-looking xylem from inoculated 'Lena' plants; G, diseased xylem from inoculated 'Lena' plants, including white core and brown edges; H, whitish inner core of diseased xylem from inoculated 'Lena', and I, gum-occluded xylem from the same inoculated 'Lena' plants.

occluded xylem from 'Novada', again showing the correlation of phytoalexin accumulation (DF3 −) with vessel occlusion, even in susceptible cultivars.

Fig. 7 gives the scoremap for the data set of spectra of xylem samples obtained three weeks after inoculation at different heights above the inoculation site from three 'Novada' and three 'Lena' carnations. In Fig. 7, DF1 is nearly the same as in Fig. 6. DF2 separates the samples based on the guaiacyl-syringyl and dianthramide fragments together in positive direction, and mass peaks indicative of hemicellulose, pectin (*m/z*



140, 128) and dihydroferulic acid (*m/z* 196) in negative direction. The mass spectrum in DF2– is similar to that found for young, non-lignified tissues as reported earlier (Niemann et al., 1990). Both the healthy and the infected xylem samples are thus further classified according to tissue age and other factors affecting the degree of lignification. Indeed, most samples taken close to the stem base are present in the upper half of the score plot, while those taken closer to the apex are present in the lower half of Fig. 7. Slightly affected but still very young xylem (M) was not distinguished from young and unaffected xylem (B and F; note that occlusion in 'Novada' only affected the first 1-3 cm above the inoculation site, E and F therefore being healthy xylem samples). The brown edges of colonized 'Lena' xylem (N) also plotted in the lower half of Fig. 7. This may be due to sampling inaccuracy, some non-lignified phloem and medulla tissue having been sampled along with the xylem edges. On the other hand, enrichment in polysaccharides may also have taken place since the brown wound-gum at the edges of colonized xylem appears similar (Baayen, 1988) to that occluding vessels which, as mentioned above, also appears enriched in polysaccharides.

Apart from the apical samples (M), diseased 'Lena' xylem (K, L and O) plotted in the middle left side of Fig. 7. Gum-occluded 'Novada' xylem (G) plotted in the upper left quadrant, merely due to the high accumulation of phytoalexins. Note that this posi-

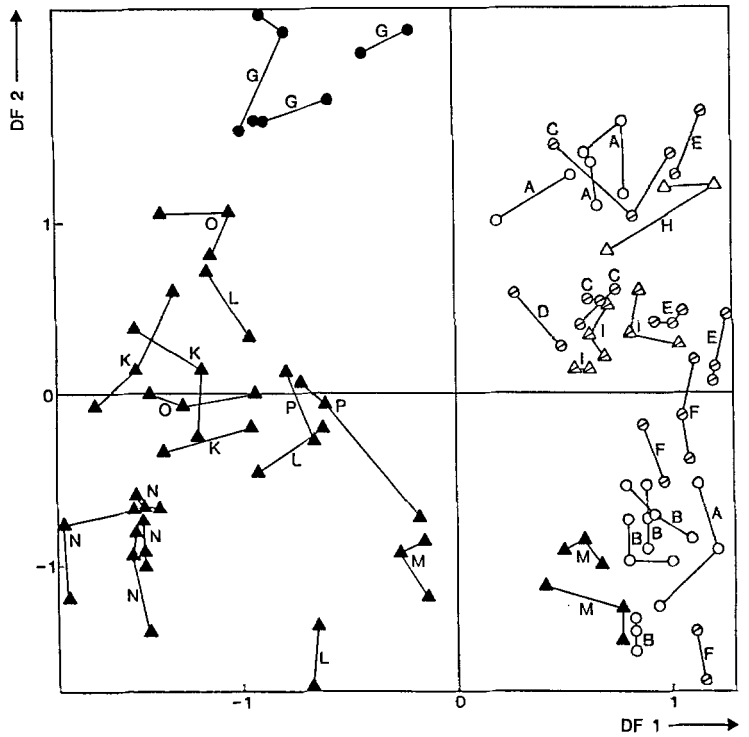
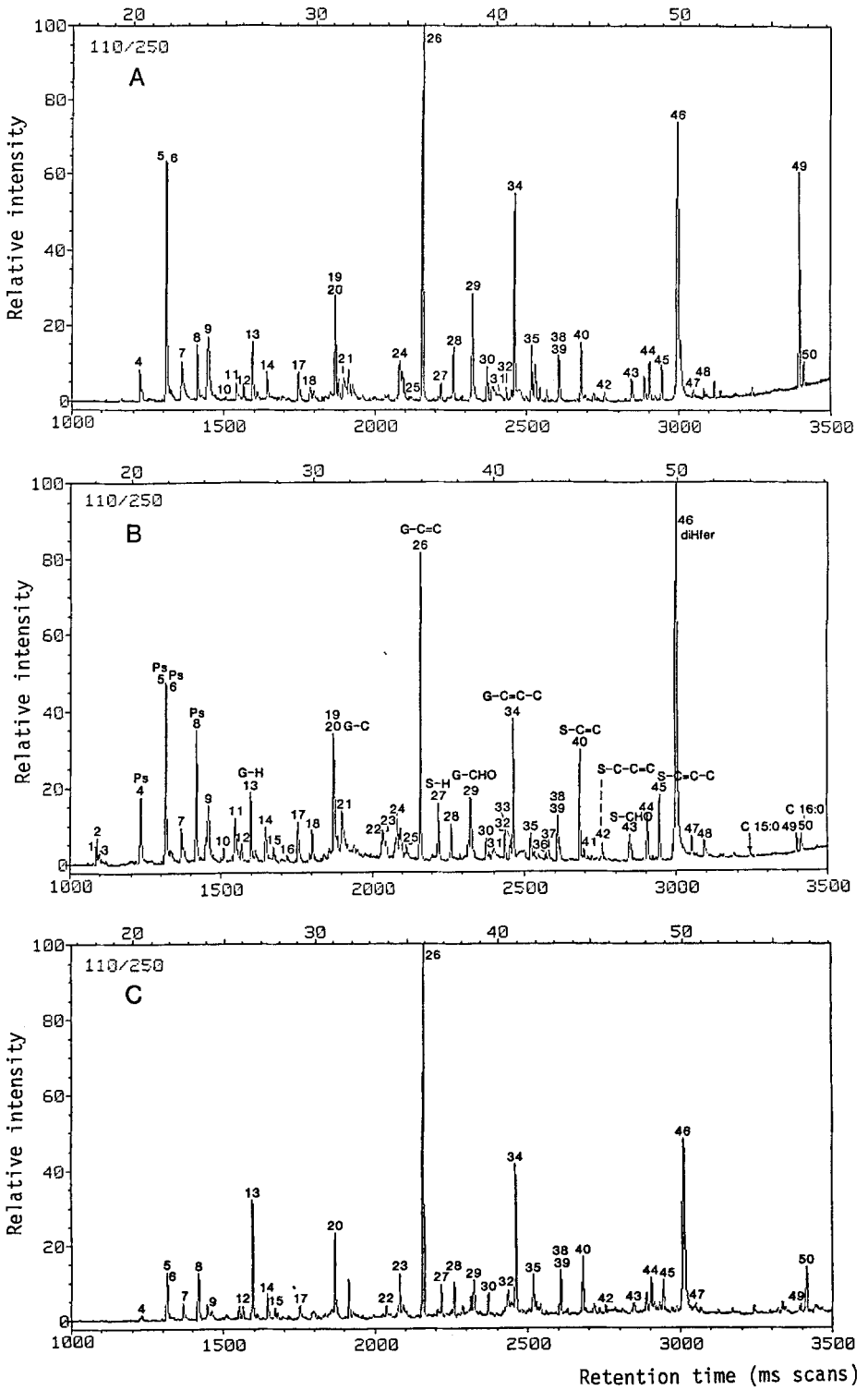


Fig. 7. Score plot and discriminant function spectra for a series of PYMS spectra of xylem samples taken at several heights from the stems of 'Lena' and 'Novada' carnations at three weeks after inoculation. Individual plants or plant parts are represented by three (sometimes two) sample analyses; replicate samples from the same plant are interconnected. D1 −, D1 +, D2- and D2 + (discriminant function spectra) are the reconstructed mass spectra on which the scoreplot is based (see section *Multivariate analysis of PYMS data*).

o and △, xylem from untreated controls; ∅ and ⧋, healthy-looking xylem from inoculated plants; • and ▲, affected xylem from inoculated plants; o, ∅ and •, 'Novada'; △, ⧋ and ▲, 'Lena'.

A, xylem from the base and B, xylem from the apex of the stem of untreated 'Novada'; C and D, healthy-looking xylem from inoculated 'Novada' plants, found at 5 mm above the inoculation site adjacent (C) to the occluded xylem or newly formed in between the occluded xylem and the cambium (D); E and F, seemingly healthy xylem from inoculated 'Novada' plants at 5 cm (E) and 10 cm (F) above the inoculation site; G, occluded xylem from 'Novada' at 5 mm above the inoculation site; H, xylem from the stem base of untreated 'Lena'; I, healthy-looking xylem from inoculated 'Lena', found at 5 mm above the inoculation site adjacent to affected xylem; K-M, diseased xylem (including white core and brown edges) from inoculated 'Lena', taken at 5 mm above the inoculation site (K), halfway up the stem (L) or near the apex (M); N and O, brown edges (N) and whitish inner part (O) of diseased xylem close to the inoculation site (K) from inoculated 'Lena'; P, gum-occluded xylem from the same inoculated 'Lena' plants, at 5 mm above the inoculation site.

tion at the y-axis does not imply that lignification was higher as well: the position on an axis may be determined by single components as well as by combinations of components of the discriminant spectra. Thus, the amount of phytoalexins in gum-occluded


110/250
A
Relative intensity
Retention time (ms scans)
110/250
B
G-C=C
26
46
diHfer
Ps
G-H
G-CHO
S-H
G-C=C-C
S-C=C
S-C-C=C
S-CHO
C 16:0
C 15:0
110/250
C
Relative intensity
Retention time (ms scans)

'Lena' xylem (P) was apparently insufficient in comparison with the degree of lignification for clustering closer to the gum-occluded 'Novada' xylem, notwithstanding the fact that gum-occluded 'Lena' xylem does contain more phytoalexins than colonized 'Lena' xylem (see Fig. 6).

Identification of specific pyrolysis products by pyrolysis-gas chromatography-mass spectrometry. PYMS data are difficult to interpret as far as the identity of individual mass peaks, or their relation with the structure of the polymer or constituent from which they are derived, is concerned. Analysis by pyrolysis-gas chromatography-mass spectrometry (PYGCMS) was therefore used for the identification of pyrolysis products from healthy and Fod-affected xylem of 'Novada' and 'Lena'. Healthy xylem from 'Lena' did not differ essentially from that of 'Novada' which has been investigated previously (Niemann et al., 1990). Fig. 8 therefore only gives the so-called ligninograms (partial ion current profiles for *m/z* 110-250) for healthy (Fig. 8B) and gum-occluded xylem (Fig. 8A) from 'Novada' and diseased xylem from 'Lena' (Fig. 8C). Apart from some minor and as yet unidentified peaks, the main differences were found in the relative concentrations of the different fragments. Compared to healthy xylem (Fig. 8B), both in occluded 'Novada' xylem (Fig. 8A) as well as in diseased 'Lena' xylem (Fig. 8C) G-lignin-derived compounds like 26 and 34 were increased and those of S lignin (27, 40, 42, 43 and 45) decreased in their relative intensity, which confirms the shift from G-S to G lignin. Loss of hemicellulose pentosans, also observed by PYMS (compare page 143), as indicated by the decreased intentities of peaks 5, 6 and 8, is especially obvious in diseased 'Lena'.

In addition to a change in the G/S ratio, the PYGCMS chromatograms in Fig. 8 also seem to indicate a comparative decrease of wall-bound dihydroferulic acid (compound 46, *m/z* 196) in Fod affected xylem. This could, however, not be substantiated from PYMS spectra of various other samples, and seems just incidental. The amounts of esterified dihydroferulic acid apparently are rather variable (Figs. 1 and 5 and spectra not shown), there is no indication of deesterification as a consequence of infection.

Discussion

Healthy xylem from susceptible 'Lena' and resistant 'Novada' was similar in composi-

←

Fig. 8. Partial ion current chromatograms from *m/z* 110-250 ('ligninograms') of xylem samples analysed by PYGCMS. A, gum-occluded 'Novada' xylem; B, healthy 'Novada' xylem; C, diseased 'Lena' xylem. **Ps**, polysaccharide markers; **G** and **S**, guaiacyl and syringyl lignin markers; **diHfer**, dihydroferulic acid. Directly relevant fragment peaks are listed below; for complete identification of the fragments given see Niemann et al. (1990).

13. guaiacol	**G – H**	40. 4-vinylsyringol	**S – C = C**
20. 4-methylguaiacol	**G – C**	42. 4-(prop-1-enyl)syringol	**S – C – C = C**
26. 4-vinylguaiacol	**G – C = C**	43. 4-formylsyringol	**S – CHO**
27. syringol	**S – H**	45. 4-(prop-2-enyl)syringol trans	**S – C = C – C**
29. vanillin [= 4-formyl-guaiacol]	**G – CHO**	46. dihydroferulic acid	**diHfer**
34. 4-(prop-2-enyl)guaiacol trans	**G – C = C – C**		

tion when studied with PYGCMS. Already in early stages of infection with Fod, however, there were differences in response. In both cultivars phytoalexins were found to accumulate, though in 'Novada' always in higher amount (and possibly slightly earlier?) than in 'Lena'. This was accompanied by enrichment in protein, adenin and fatty acid fragments which presumably indicate metabolic activity. Fatty acid mobilization might suggest initial suberization. Judged from microscopic observation, however, at this stage of infection suberin formation only plays a minor role (Baayen, 1988). Selective enrichment in polysaccharides (compared with lignin content) was observed already from the first days after inoculation in 'Novada' only. Changes from G-S to G lignin and demethylation of pectin, however, were encountered in 'Lena' from the first days after inoculation, but were found only later on in 'Novada'. Obviously, in 'Novada' production of pectin methylesterase (Cooper and Wood, 1973) is either inhibited in the early stages of infection or its activity is prevented by early barrier formation. Disappearance (enzymatic degradation?) of hemicellulose pentosan occurred in both cultivars.

Resistant responses of carnation xylem to Fod are characterized by the occlusion of vessels with wound gum and additional lignification of primary walls (Baayen, 1988); degradation is observed only exceptionally. Thus, the enrichment in polysaccharides in 'Novada' may be due to the occlusion response, since the gums react positively to histochemical stains for polysaccharides (Baayen and Elgersma, 1985). Only small amounts of polysaccharides are necessary for such a staining reaction: 1% of pectin in water may already form a firm gel, dependent on the degree of methylation of the pectin. Therefore, minor changes in polysaccharide composition and content as presently observed in 'Novada' could already account for the vessel occlusion phenomenon observed with the light microscope. Phytoalexin accumulation appears in some way correlated with vessel occlusion. Possibly, as recently found for the interaction *Apium graveolens* with *Fusarium oxysporum* f.sp. *apii* (Jordan et al., 1989), polysaccharides and phenolics are synthesized together in the same microbodies. Susceptible interactions are characterized by extensive degradation of middle lamellae, primary walls and in due course also of the lignified secondary walls, occasionally accompanied by defense responses (Baayen et al., 1988). The changes presently observed in 'Lena', for instance the disappearance of S lignin, are therefore more apt to be due to degradation processes.

Changes in composition of the lignin were observed in both cultivars, both with PYGCMS and in the PYMS scoreplots. Using the relative intensity of the G-lignin-derived and S-lignin-derived fragments in the PYGCMS chromatograms as a measure of relative quantity, the ratio G fragments to S fragments changed from ca. 2 in healthy xylem to ca. 4 in diseased 'Lena' and ca. 5 in affected 'Novada'. Increased lignification of primary walls and *de novo* lignification of gums was previously found in Fod-infected 'Novada' carnations (Baayen, 1988); the newly formed lignin did not stain for syringyl groups, however. Xylem vessel walls in occluded areas no longer stained for syringyl groups either. It was therefore assumed that newly formed G lignin masked the former G-S lignin (Baayen, 1988). The phenolic precursors for lignin formation thus seem to be present both in the gums and the cell walls. Mere guaiacyl lignin is also formed in xylem of young carnation tissues (Niemann et al., 1990); syringyl constituents appear only in later stages of development. Possibly, formation of G lignin is due to a limited carbon supply in periods of fast growth or rapid mobilization of lignin precursors in stress situations. However, the PYMS spectra show that the change in the G/S ratio

is not only caused by formation of extra G lignin: part of the syringyl groups originally present seem to have disappeared as well. It is possible that the later formation of syringyl constituents (Niemann et al., 1990) has structural consequences which makes these units more accessible for degradation.

In susceptible 'Lena' vascular degradation must be the cause of the change in the G/S ratio. The selective disappearance of syringyl constituents in this case can only be explained by demethoxylation (or demethylation followed by dehydroxylation) of syringyl units. A similar demethoxylation, preferentially occurring on syringyl lignin units, has been found during early peatification of secondary xylem from *Gordonia* (Stout et al., 1988), while it has also been observed in later stages of wood coalification (Chaffee et al., 1984; Russell and Barron, 1984). In the early stages of vessel colonization, Fod therefore seems to employ a mechanism similar to that of wood-decaying microorganisms. Other microbially induced lignin modifications may also include 3-O-demethylation of guaiacyl units, 4-O-methylation of both guaiacyl and syringyl units and more complex changes of the lignin polymer (Chen and Chang, 1985; Pouwels, 1988; Pouwels and Boon, 1989; Sutherland et al., 1983). It might be of interest to investigate later stages of diseased 'Lena' xylem for similar effects.

PYMS afforded an unexpected possibility to locate dianthramide phytoalexins in affected plants. With HPLC (Niemann and Baayen, 1988), accumulation of Dx was found up to about 90 $\mu g\ g^{-1}$ fresh plant material (that is ca. 30 μg in a 5 cm stem piece) at 10 days after inoculation. For MDS values of 29 $\mu g\ g^{-1}$ (10 μg per stem piece) in non-esterified form and 6 $\mu g\ g^{-1}$ (2.1 μg per stem piece) in wall-bound form were found. When the molecular extinction of the dianthramides is of the same magnitude, this would indicate an estimated total of around 40 μg of these dianthramides per inoculated stem piece. Altogether, ca. 70 μg of phytoalexins (Dx + dianthramides) must have been concentrated in the tiny area of gum-occluded xylem. Assuming an equal specific weight for all tissues (certainly untrue) and an affected area of 3% of the transverse surface (10% xylem with an average occluded part of 30%), spreading 20 mm in upward direction, then 10 days after inoculation more than 1% of the fresh weight of affected xylem must have consisted of dianthramides and dianthalexin. Locally, the differences in phytoalexin concentrations found between cultivars like 'Novada' with a resistant reaction affecting only a small part of the xylem and 'Lena' with susceptible reactions widespread through the xylem, therefore must be much bigger than previously supposed on basis of the values obtained by comparison of extracts of stem pieces of equal length (Niemann and Baayen, 1988).

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Samenvatting

Localisatie van fytoalexinenaccumulatie en bepaling van veranderingen in lignine- en koolhydraatsamenstelling in anjer (Dianthus caryophyllus L.) stengels als gevolg van infectie met Fusarium oxysporum f. sp. dianthi, via pyrolyse-massaspectrometrie

Kleine stukjes xyleem en andere weefsels van de stengels van gezonde en geïnoculeerde planten van een tweetal anjercultivars, Novada en Lena, werden onderzocht op mate van lignificatie (lignine/polysaccharide verhouding) en ligninesamenstelling met behulp van pyrolyse-massaspectrometrie en pyrolyse-gaschromatografie massaspectrometrie. Deze techniek bleek ook bruikbaar voor het localiseren van fytoalexinen van het dianthramide-type, welke in anjer accumuleren na infectie met *Fusarium oxysporum* f.sp. *dianthi*. De samenstelling van gezonde weefsels was bij beide cultivars praktisch gelijk. Bij de resistente cultivar Novada leidde infectie tot een verandering van guaiacyl-syringyllignine in guaiacyl-lignine in door gommen afgesloten delen van het xyleem. Xyleem met gommen bevatte aanzienlijke hoeveelheden van het fenolamide dianthalexine en van de andere fytoalexinen van het dianthramide type. De fytoalexinen werden niet aangetroffen in aangrenzend floeem, merg of gezond ogend xyleem. Bij de vatbare cultivar Lena trad afbraak van het xyleem op, dat daarbij armer aan syringyllignine werd, terwijl tevens in een vroeg stadium pectine werd gedemethyleerd. In 'Lena' werden slechts kleine hoeveelheden fytoalexinen gevonden, met name bij locaal opgetreden afweerreacties zoals gomvorming. Bij beide cultivars werden aanwijzingen voor afbraak van hemicellulosen gevonden.

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