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Changes in chemical composition related to fungal infection and induced resistance in carnation and radish investigated by pyrolysis mass spectrometry

HARKO STEIJL[1], GERARD J. NIEMANN[1]* and JAAP J. BOON[2]

[1] *Department of Plant Ecology and Evolutionary Biology, Section of Plant Pathology, Utrecht University, P.O. Box 800.84, 3508 TB Utrecht, The Netherlands and* [2] *FOM Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands*

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Pseudomonas fluorescens WCR 417r induces systemic resistance in radish roots challenged by *Fusarium oxysporum* f. sp. *raphani* and, incidentally, in carnation stems challenged by *Fusarium oxysporum* f. sp. *dianthi*. The induced systemic resistance is not associated with accumulation of pathogenesis–related proteins (PRs) or, at least in case of radish, with increased peroxidase activity. We tested whether the induced systemic resistance might be associated with changes in the host cell wall composition. Cell wall degradation, using pyrolysis mass spectrometry, was demonstrated on fungal infection in carnation and probably in radish as well. Lignin was found to be demethoxylated, oxidized and depolymerized. In carnation both syringyl and guaiacyl lignin were broken down. In radish root probably mainly syringyl lignin was degraded. Cellulose and hemicellulose were degraded in carnation and possibly in radish. Bacterization with *P. fluorescens* WCS 417r prior to fungal infection reduced such cell wall degradation, consistent with its resistance inducing action. In the case of the radish roots it seems very improbable that this reduction is a necessary component of the induced fungal resistance. For carnation it remains to be investigated.

Keywords: Cell wall; *Dianthus caryophyllus* L.; *Fusarium oxysporum* f. sp. *dianthi*; *Fusarium oxysporum* f. sp. *raphani*; lignin; polysaccharides; *Pseudomonas fluorescens*; pyrolysis-mass spectrometry; *Raphanus sativus* L.

INTRODUCTION

Both non-pathogenic fungi and bacteria can reduce or retard plant diseases [*13*, *18*, *51*, *52*]. One of the mechanisms of disease suppression recently found for fluorescent pseudomonads is the systemic induction of disease resistance [*31*, *50*, *51*]. Fusarium wilt in carnation and radish caused by the soil-borne pathogens *Fusarium oxysporum* f. sp. *dianthi* and f. sp. *raphani*, respectively, is suppressed by *Pseudomonas fluorescens*. In an attempt to elucidate the mechanism of induced resistance, disease suppression in these host–pathogen combinations was studied. Both hosts react rather differently to invasion by the fungus. In carnation the fungus mainly invades the roots through wounds [*6*], and major defense mechanisms of the host are activated only after the fungus penetrates the vascular cylinder [*3*, *33*, *34*]. In radish, the major defense seems to be restricted to the outer layers of the cortex, once the vascular cylinder is penetrated the fungus invades the plant systemically [*26*].

Chemically, the effects of fungal penetration in carnation are much better understood than those in radish [*30*, *34*]. In an incompatible interaction between carnation and *F. oxysporum* f. sp. *dianthi*, the pathogen is confined to the vascular region of the infection site due to the accumulation of a large array of dianthramide phytoalexins, modification of the walls of surrounding cells, and occlusion of colonized vessels by phenolic-incrusted gums [*3*, *5*, *34*, *35*]. During this process both degradation of (syringyl-) and synthesis of (guaiacyl-) lignin seem to occur at the same time. Using pyrolysis-mass spectrometry it was previously demonstrated that lignin was degraded according to two different pathways, *i.e.* by demethoxylation of syringyl units and by propenyl side chain oxidation [*36*].

The early defense of radish against *F. oxysporum* f. sp. *raphani* has so far eluded clarification. Resistance appears to be of a quantitative nature because cultivars with a range of resistance levels are known [*25*]. Resistance is associated with restricted relative growth rate [*25*],

*All correspondence should be addressed to: Gerard J. Niemann, Department of Plant Ecology and Evolutionary Biology, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, The Netherlands. Fax no: 31-30-2518366. E-mail: G.J.Niemann@bio.uu.nl

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suggestive of a relationship between limited infection and reduced cell expansion potential.

Induced resistance constitutes an enhanced capacity of the plant to defend itself, and appears to be dependent on an earlier and stronger expression of extant resistance mechanisms [*49*]. Along with the increased resistance induced by *Pseudomonas* strain WCS 417r in carnation, upon challenge inoculation an increased accumulation of phytoalexins was found in stems of bacterized plants as compared to non-bacterized ones. No accumulation of these compounds was found in bacterized non-infected plants [*51*]. *Pseudomonas* bacteria added to the roots, later on could not be detected inside the host. Signals, induced by strain WCS 417r at the root system and transported systemically through the plant, must be responsible for the sensitization of defense responses against *F. oxysporum*, such as the synthesis of dianthramide phytoalexins. Whether other defense responses were sensitized as well was not investigated. There is no accumulation of pathogenesis-related proteins upon challenge with *Pseudomonas* and/or *F. oxysporum* f. sp. *dianthi* (S. E. M. van Wees and E. Hoffland, personal communication).

Increased accumulation of phytoalexins alone cannot fully explain the higher resistance found [*34*, *35*]. It rather forms part of a complex of different local reactions which together limit fungal expansion. In an early stage of infection infusion of phenolic-containing material is induced in the primary cell walls and intercellular spaces of the xylem parenchyma. Local increase of cell wall impermeability and cell wall rigidification, therefore, might also play an important role.

No indication of phytoalexin accumulation has been found in the case of radish. Neither is the induced systemic resistance associated with accumulation of pathogenesis–related (PR) proteins [*26*]. In radish roots *Pseudomonas* induces a decrease in activity of ionically bound peroxidases (J. Ton and H. Steijl, personal communication). However, the change occurs comparatively late (after 5 days), and was also produced by Pseudomonads unable to induce systemic resistance. Peroxidases, therefore, seem not to be responsible for the induced systemic resistance either. Induced systemic resistance in radish is expressed as a reduction of the percentage of diseased plants. There is no effect on the severity of disease once the plant has been infected [*26*]. This indicates that infection is prevented at the stage of penetration, an effect which might depend on the composition of the cell wall.

Induction of resistance has previously been associated with changes in cell wall composition. Pathogen-induced resistance in cucumber and tobacco, for instance, was accompanied by increased accumulation of lignin and hydroxyproline-rich proteins in the cell wall after challenge inoculation with different pathogens [*21*, *53*]. In pea root epidermis and outer cortex cells, walls strengthened by appositions of callose and phenolic materials, were observed after challenge inoculation with *Bacillus pumilis* [*8*]. Our investigations, therefore, both for carnation and radish, were focussed on possible changes within the host cell walls.

MATERIALS AND METHODS

Micro-organisms

A virulent isolate of *Fusarium oxysporum* Schlecht. emend Snyder and Hansen f. sp. *dianthi* (Prill. Delacr.) Snyder and Hansen, race 2 (WCS 816) was cultured on potato dextrose agar culture slants. Conidial suspensions were obtained by shaking the cultures with demineralized water. Mycelial fragments were removed by filtration through glass wool, and the suspension was adjusted to a concentration between 5×10^6 and 10^7 conidia ml^{-1}.

Fusarium oxysporum Schlecht. f. sp. *raphani* Kendrick and Snyder {formerly *F. oxysporum* f. sp. *conglutinans* [(Wollenw.) Snyder and Hansen] race Armstrong and Armstrong} strain WCS 600, was isolated from infected radish tubers [*31*] and maintained as microconidia on Microbank beads (Pro-Lab Diagnostics, Wirral, U.K.) at −80 °C. Beads were thawed and the fungus was pre-cultured on potato dextrose agar (PDA). After growth of the fungus in aerated 2% malt extract for 10 days at 22°C, a conidial suspension was prepared by filtering over sterile glass wool. About 2×10^8 washed microconidia in 20 ml of 0·01 M $MgSO_4$ were added to 100 g of sterilized peat (Agrifutur s.r.l., Alfianello, Italy). The inoculum was incubated for 4 days at 22°C before use. The number of colony forming units (cfu) present in the peat mixture was then determined by dilution plating on PDA. Inoculum density in the peat was adjusted by adding sterile peat/quartz sand to a final ratio of 1:9, w/w, prior to inoculation.

Rifampicin-resistant *Pseudomonas fluorescens* strain WCS 417r [*29*] was used as the inducer of systemic resistance [*31*]. After growth of the bacteria on King's medium B agar [*27*] the cells were suspended in 0·01 M $MgSO_4$ and adjusted to 4×10^7 cells ml^{-1}.

Bioassay and determination of resistance level

Rooted cuttings of carnation (*Dianthus caryophyllus* L.) cultivars with different degrees of resistance to *F. oxysporum* f. sp. *dianthi*, Novada (resistant), White Giant (moderately resistant), Pallas (moderately resistant), Elsy (moderately resistant), Silvery Pink (slightly susceptible), Lena (susceptible) and Early Sam (very susceptible) were obtained from Van Staaveren, Aalsmeer, The Netherlands and Lek, Nieuwveen, The Netherlands. They were planted in rockwool cubes (Grodan B.V., Roermond, The Netherlands). Prior to placement in the system, all rockwool

cubes were drenched in a carnation nutrient solution [*13*]. Plants were maintained in a temperature-regulated greenhouse at 19°C during darkness and at 22°C during the photoperiod, at 70% RH. A 16 h photoperiod was maintained using SON-T lamps (Philips, Eindhoven, The Netherlands). Additional nutrient solution was supplied once a week.

The plants were stem-inoculated at the third node from the stem base with 20 μl of the conidial suspension of *F. oxysporum* f. sp. *dianthi* per inoculation site. The suspension was deposited in the leaf axil, after which the stem was incized horizontally through the droplet just into the xylem. Control plants either remained untreated or were mock-inoculated with sterile demineralized water. For spatially separated inoculation of *F. oxysporum* f. sp. *dianthi* and pseudomonads the plant roots were bacterized with 25 ml of a suspension of *P. fluorescens* WCS417r (10^8 cfu ml^{-1}) 7 days prior to the inoculation with *F. oxysporum* f. sp. *dianthi*. From a number of plants, at 3 days after inoculation with *F. oxysporum* f. sp. *dianthi*, hand-cut transverse sections (about 0·2 mm thick) were taken from the stem at 2–4 mm above the inoculation site, and dissected in a drop of sterile demineralized water under a dissecting microscope. Affected parts of the xylem were separated as far as possible from the other tissues and stored frozen (−20 °C) for pyrolysis mass spectrometry. In most cases the xylem could not be completely separated from medullary parenchyma. Prior to platinum filament pyrolysis the samples were homogenized in 200 μl of demineralized water using an all-glass mini mortar. A 5 μl aliquot of the suspension was used for the PyMS analysis.

Numbers of diseased plants were recorded weekly starting 4 weeks after inoculation. Development of Fusarium wilt symptoms was recorded for all plants individually, using the following scale: 0. no disease symptoms; 1. very slight symptoms; 2. limited local symptoms; 3. well-developed symptoms on otherwise still healthy-looking plants; 4. severe wilt and 5. complete wilt and death. The experiment was terminated 14 weeks after inoculation. From other plants 2 weeks after inoculation, 4 successive, 5 cm stem pieces were cut, halved lengthwise and plated on Komada agar, after which the outgrowth of the fungus was recorded.

Radish (*Raphanus sativus* L.) cultivar Saxa × Nova (S & G Seeds B.V., Enkhuizen, The Netherlands), moderately resistant to *F. oxysporum* f. sp. *raphani* was germinated on quartz sand. Five day old seedlings were laid horizontally on compartmentalized rockwool cubes (Grodan B.V., Roermond, The Netherlands) in such a way that spatial separation of the induction treatment and the challenge inoculation of the same root system was ensured [*31*]. Prior to placement in the system, all rockwool cubes were drenched in half strength Hoagland's nutrient solution [*24*], as modified by Leeman *et al.* [*31*]. Plants were cultivated as described for carnation. Additional nutrient solution was supplied once a week.

Subsequent to the transfer of the seedlings to the rockwool system, the induction treatment was carried out by covering the root tips with approximately 10^7 cfu of bacteria in 0·5 g of talcum suspension, prepared by mixing five parts of bacterial suspension in 0·01 M $MgSO_4$ with four parts of talcum powder (Merck, Darmstadt, Germany). In controls, the bacterial suspension was substituted by 0·01 M $MgSO_4$. Two days later the root base was inoculated by covering it with approximately 0·7 g of the peat/sand mixture containing *F. oxysporum* f. sp. *raphani*. For PyMS, for each treatment, 4 mm long root pieces (close to the root base) were harvested from ten individuals at 6 and/or 10 days after inoculation with *F. oxysporum* f. sp. *raphani*. Alternatively, 1 mm pieces were taken from 40 individuals and analysed in sets of four. All samples were stored frozen at −20°C. For PyMS the samples were homogenized in 100 μl demineralized water using an all-glass mortar. The percentage of diseased plants was determined about 20 days after challenge inoculation. The plants were inspected for external (wilting) and internal (browning of the vascular tissue) symptoms, the latter after cutting the main root and the bulb transversely at several places. All plants showing symptoms, either external or internal, were scored as diseased. Disease severity was scored as: 1. healthy/no symptoms; 2. internal vascular browning of the tap root only; 3. internal browning of the tuber; 4. external wilting of leaves or 5. plant completely wilted. At each time point, eight samples of six plants each were examined.

Pyrolysis mass spectrometry method

Pt/Rh filament pyrolysis mass spectrometry was performed on a JEOL DX-303 double focussing mass spectrometer equipped with a platinum-rhodium 90/10 filament in-source pyrolysis probe. Aliquots of 2·5–5 μl of the samples, suspended in water were used for the PyMS analysis. The Pt-Rh filament (0·1 mm diameter, 10% Rh) was attached to an insertion probe and allowed to dry in vacuo before insertion into the ion source (180°C) of the mass spectrometer. To avoid day to day variation in the instrumentation the samples were analysed on the same day. The difference between replicate analyses for the summed abundances of polymers such as protein or lignin, was less than 3%. Instrument conditions were: a mass range of 20–750 m/z, a scan cycle time of 1 s and a source temp of 180°C. A heating range of 16°C s^{-1} up to 800°C was used. To avoid further fragmentation during ionization, low voltage EI at about 16 eV was applied. An extensive description is given by Boon [*9*, *10*] and Van der Hage, Weeding and Boon [*46*].

Quantitative determinations based on relative intensities of ions in PyMS are possible and have been shown

to correlate well with conventional wet chemical methods [*40*, *43*, *44* and their cited references]. Mainly, however, PyMS fragments are used as qualitative and semiquantitative markers. As such they have been used in the present work. The tiny amounts of material recovered under the microscope did not allow normalization other than by volume and total ion current. The total ion current gives a clear indication of the amount of material analysed.

Multivariate data analysis of PyMS data and other statistics

Principal component (PC) and discriminant (DF) analyses were performed on the PyMS data files, using a modified Arthur package, adapted to PyMS data [*12*]. In this method, relative abundances of the 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 numerical reference point for the individual spectra. Mathematically, the differences between the individual spectra are determined by comparison with this zero point spectrum.

These data are factor analysed to produce sets of correlated mass peaks (factors), which can be represented by reconstructed mass spectra of principal components. When multiple analyses are available, discriminant analyses can be performed. The covariant mass peaks are linearly combined into new independent variables (discriminant functions), which are represented graphically by reconstructed mass spectra. Unsimilarity is quantitatively expressed in discriminant function scores. For the multivariate data analysis, summarized average PyMS spectra for the total pyrolysis period (35 s for scans 15–50) calculated from each separate analysis were used.

Statistic validity of the calculated coefficients was determined using Student's *t*-test and/or one-way ANOVA.

RESULTS

Variation in disease incidence

Carnation. Table 1 shows the percentage of diseased control plants for the different carnation cultivars. Disease resistance differed greatly in the various cultivars, reflecting their varying levels of resistance against *F. oxysporum* f. sp. *dianthi*. Internal local reactions such as lignin degradation and phytoalexin accumulation start very soon after infection [*36*] and, therefore, in this combined experiment samples for PyMS were harvested at 3 days after inoculation. No suppression of resistance against *F. oxysporum* f. sp. *dianthi* by *P. fluorescens* WCS417r was observed (Fig. 1). Earlier, suppression was mainly observed for the moderately resistant cultivar Pallas and incidentally for susceptible cv Lena [*13*, *51*].

TABLE 1. *Fusarium wilt in plants of carnation cultivars after inoculation with* Fusarium oxysporum *f. sp.* dianthi. *Percentage of diseased plants (disease index 3–5) at 8 and 13 weeks after inoculation*

Cultivar	8 weeks	13 weeks
Novada	2	35
Pallas	5	32
White Giant	4	20
Elsy	9	40
Silvery Pink	25	62
Lena	63	70
Early Sam	74	95

Radish. With the radish cultivar Saxa × Nova the first internal disease symptoms were observed in some plants at 10 days after inoculation with *F. oxysporum* f. sp. *raphani*. Significant differences in disease incidence between bacterized and non-bacterized plants were found from 17 days after inoculation onwards (Steijl *et al.*, in preparation). The results are in agreement with those of a large number of independent experiments performed in our laboratory [*25*, *26*, *30*, *31*].

Chemical variation

Comparison of the PyMS spectra of the different samples was based on (A) multivariate analyses [principal components (PC) and/or discriminant analysis (DF)] using all mass fragments from m/z 20–500 (or 700) or selected m/zs indicative of lignin fragments, and on (B) (for carnation only) the percentage of the total ion current (% RIC) of some major markers for hexose and pentose polysaccharides, for protein and for lignin.

Carnation

Healthy plants Variation in healthy plants: During previous investigations PyMS spectra of xylem of the stem of carnation cultivars with different levels of resistance to *F. oxysporum* f. sp. *dianthi*, were not found to be essentially different [*36*]. Those spectra, however, were not compared using chemometric procedures. The pyrolysis mass spectrum of healthy xylem of cultivar Pallas is shown in Fig. 2(a). Bacterization gave no mass spectral changes (data not shown).

Infection with F. oxysporum *f. sp.* dianthi The effect of the invasion by *F. oxysporum* f. sp. *dianthi* in the xylem compared to non-inoculated/bacterized or non-bacterized tissues is shown in Figs 2 and 3. For a better understanding of the PyM spectra in these figures we

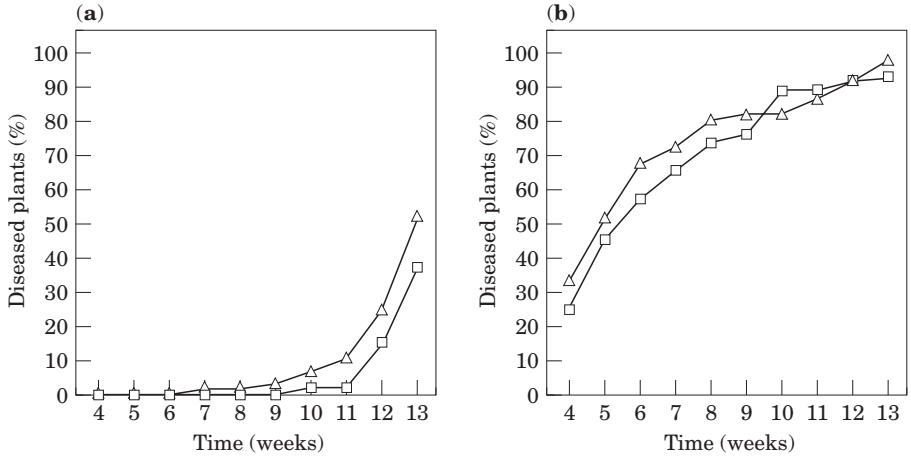

FIG. 1. Percentages of diseased plants with a disease index from 3–5, of the carnation cultivars Novada (**a**) and Early Sam (**b**) after inoculation with *Fusarium oxysporum* f. sp. *dianthi* (□) or after bacterization with *Pseudomonas* sp WCS417r followed by inoculation with *F. oxysporum* f. sp. *dianthi* (△).

insert a short paragraph on the interpretation of such spectra.

Interpretation of PyMS spectra Lists of specific marker fragments for the different plant polymers and other constituents have been presented before [*22*, *37*, *39*, *40*, *43*]. Therefore, only the ions mentioned in the text and those shown in the figures are mentioned, and where necessary their validity as marker ion will be discussed.

Mass peaks m/z 57, 60, 73, 85, 86, 114 and 126 are characteristic for the plant polysaccharides. However, the occurrence of the characteristic masses such as m/z 114 or 126 has to be interpreted with care, as in PyMS only mono- and/or oligomeric units are visible. M/z 114 is an indicator of xylose and/or arabinose and therefore indicates the xylan and xyloglucan fraction of hemicellulose together with the (comparatively low) arabinan and arabinogalactan fraction of pectin, but not of the β(1–3), (1–4)-glucans. M/z 126 indicates hexoses and does not distinguish between different types of glucans, thus this mass represents both cellulose, amylose and the hexose fraction of hemicellulose. Pectin is characterized by rhamnose and methylgalacturonan. Rhamnose, which is also present in homogalacturonan [*17*], has been found to give a series of four dianhydrodeoxysugars with m/z 128 as the molecular ion peak [*11*]. Part of the monoses may also have been derived from glycolipids and/or glycoproteins. Their contribution, however, is comparatively small and, furthermore, in previous work [*40*] percentages of the total ion current of the polysaccharide markers mentioned correlated with the residual (hemi)-cellulose fraction from wet chemical analysis. Mass peaks m/z 31, 32, 43, and a couple of others are non-specific ions for polysaccharides.

For proteins we recently published a number of marker fragment ions [*43*] additional to specific ones such as presented before [*37*, *39*, *40*]. In the present work major changes in proteins were not found.

A recent review on the in-source PyMS of lignin was given by Van der Hage, Weeding and Boon [*46*]. Degradation of lignin by demethoxylation is visualized in the PyMS spectra by a change of the syringyl lignin markers such as m/z 154, 167, 168, 182, 194, 208 and 210 into the guaiacyl ones (m/z 124, 137, 150, 152, 164, 178 and 180) [*36*]. Such a shift, however, can also be explained by selective removal of syringyl-rich secondary cell wall material and retention of guaiacyl-rich middle lamella [*47*]. Oxidation results in a comparative increase of the aldehydes m/z 178 (coniferaldehyde) and 208 (sinapyl aldehyde) and a decrease of the corresponding alcohols m/z 180 and 210. Such an effect may also be the result of a reduced cinnamyl alcohol dehydrogenase activity as induced in transgenic tobacco plants [*20*]. Further oxidation may lead to vanillic aldehyde (m/z 152) and syringic aldehyde (m/z 182) [*36*]. Increased depolymerization leads to an increase in dimer fragments. Dimeric lignin skeletons have tentatively been consigned to: β-5-phenylcoumaran, β-5-phenylcoumarone, 5-5-biphenyl,β-1-stilbene and β-β-resinol types of structures. M/z 272, 302, 332, 358 and 388 are diguaiacyl-, guaiacyl/syringyl- and disyringyl-containing dimeric linin products, respectively [*45*]. Other identified dimeric lignin pyrolysis products are m/z 310, 312, 314, 316, 326, 328, 330, 340, 342, 346, 356, 370, 386, 400 and 418 [*22*, *45*, *48*]. M/z 279 in Fig. 2 is an artifact. M/z 228, 242, 256, 284 and 298 are fatty acids. For multivariate analysis using only lignin maker masses, a number of those masses was randomly chosen.

Fig. 2 gives the PyM spectra of (a) healthy xylem and (b) inoculated xylem of carnation cultivar Pallas. As shown by the total ion current the amount of material in

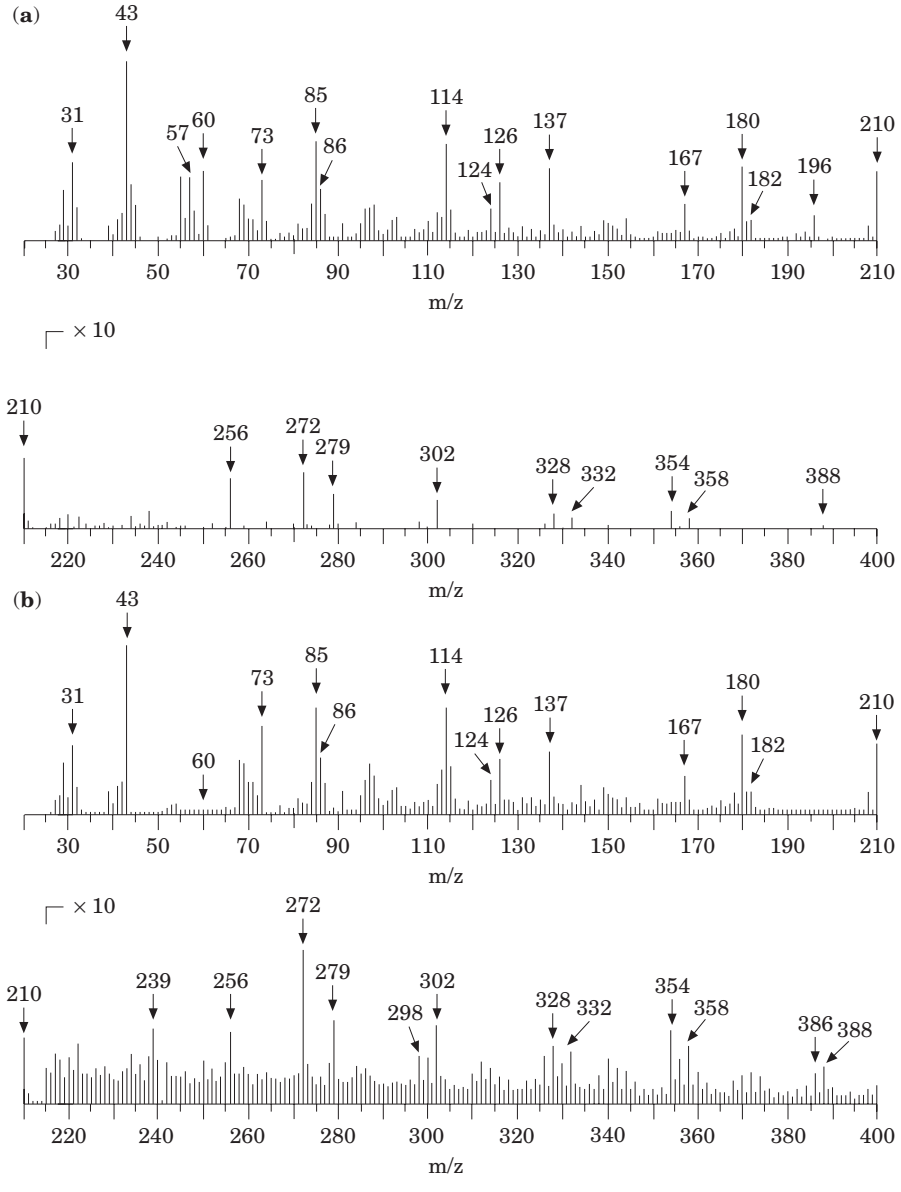

FIG. 2. Mass spectra (16 eV EI) obtained after Pt, Rh filament pyrolysis of homogenized stem xylem material from carnation cultivar Pallas (**a**) healthy xylem and (**b**) affected xylem taken 3 days after infection with *Fusarium oxysporum* f. sp. *dianthi*. It should be noted that for m/z 210–400 enlarged scales, y axis × 10, are used as indicated in the figure. The amount of material used for (**a**) was 2·5 times as much as that for (**b**) which explains the high noise level in the enlarged part of (**b**). Detailed information on fragment ions is given in the section Interpretation of PyMS spectra and in the covering text.

(a) was two and a half times as much as in (b) which explains the high noise level in the enlarged part of (b). As can be seen, there are only very slight differences between spectra (a) and (b). Only in Fig. 2(b) mass m/z 239 shows, which represents the major phytoalexin dianthalexin [*36*]. Spectra of the other cultivars showed a similar behaviour. The spectra of all cultivars together were further analysed by principal component analysis.

In spite of the comparatively small changes shown in Fig. 2, multivariate analysis [Fig. 3(*insert*)], gives a clear separation of the samples derived from three individual healthy plants (o, negative PC1 scores) from all the inoculated xylems (bf/f, positive PC1 scores) in the first function of the principal component analysis. Fig. 3 further shows the principal component functions PC1+ and PC1−. PC1− depicts mass markers of coniferyl alcohol (m/z 124, 137, 180) and sinapyl alcohol (m/z 154, 167, 210) indicative of an intact guaiacyl/syringyl lignin in healthy tissues. M/z 196 is a special carnation marker derived from dihydroferulic acid [*41*]. M/z 167 is compromised because it is also a main ion in the lignin degradation products in PC1+.

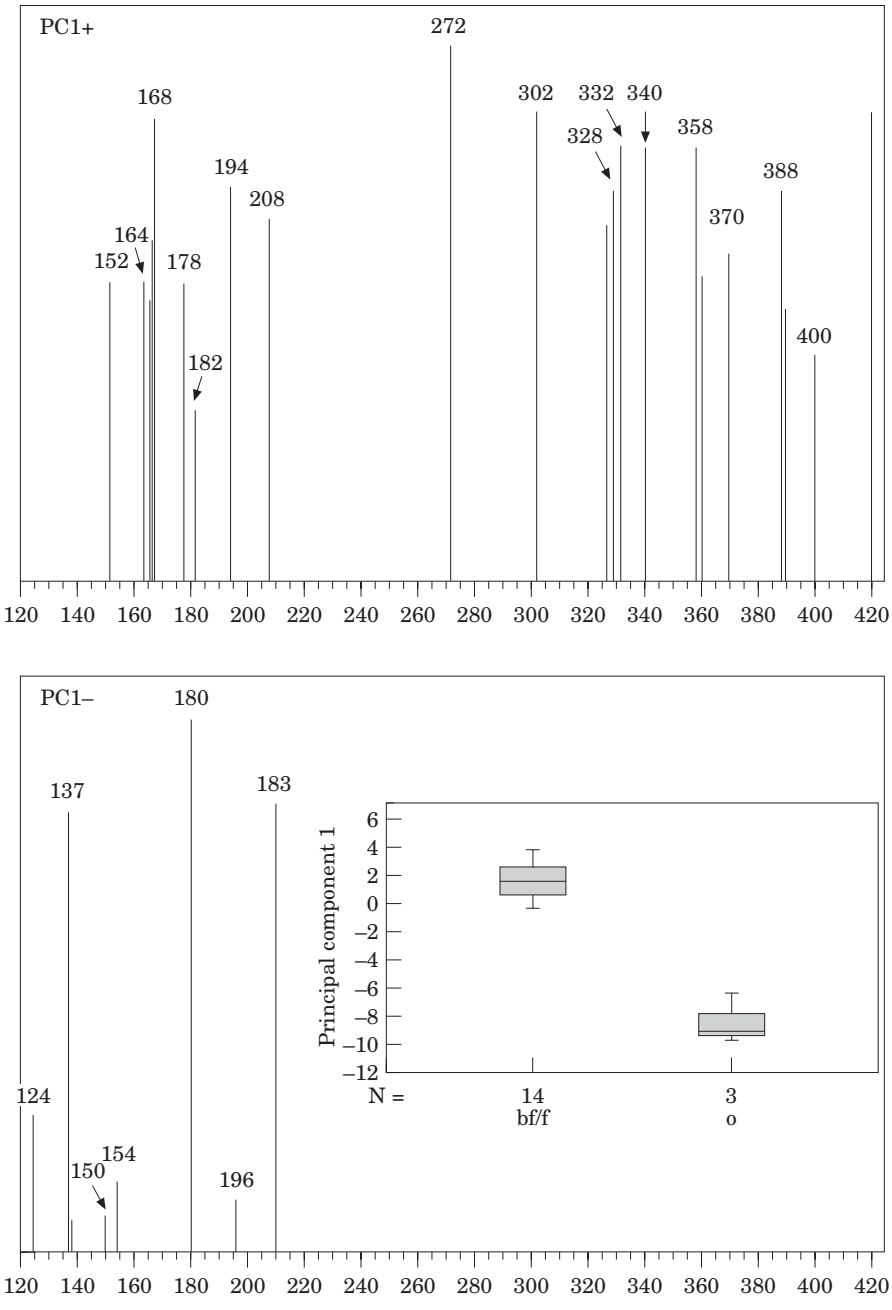

FIG. 3. Separation by principal components (PC) analysis of the pyrolysis low voltage EI mass spectra of xylem of the stem of all carnation cultivars that were either bacterized or non-bacterized with *Pseudomonas* spp. and later inoculated with *Fusarium oxysporum* f. sp. *dianthi* (bf or f) or remained untreated (o) (*insert*) and the principal components spectra of PC1 on which this separation was based, describing 58·7 % of the total variation. Principal components spectra are reconstructed mass spectra which mark the PC1 axis along which the samples were separated (see text). PC1 − shows monomeric markers of both guaiacyl- and syringyl-lignin, whereas PC1 + gives monomers of the oxidized (aldehyde) structures and many dimers. The latter indicates increased depolymerization. Detailed information on fragment ions is given in the section Interpretation of PyMS spectra.

The main peaks in PC1 + are derived from dimers (*m/z* 272–400), and guaiacyl and syringyl compounds indicative of dehydration (*m/z* 164, 194) and oxidation (*m/z* 152, 178, 182 and 208) of the lignin constituents coniferyl- and sinapyl alcohol. Thus, both guaiacyl- and syringyl lignin were degraded by *F. oxysporum* f. sp. *dianthi* activity. Mass peak *m/z* 168 is indicative of vanillic acid, a well known biodegradation product of lignin by soil-borne fungi [*16*] in general and of lignin model compounds by *Fusarium* species in particular [*23*]. The relative increase in dimers in PC1 + is interpreted as the onset of depolymerisation of lignin in the plant tissue.

Xylem from bacterized/inoculated plants For almost all cultivars, bacterized/inoculated xylem (bf) samples

showed higher percentages of the total ion current (% RIC) than the non-bacterized/inoculated xylems (f) for the **G**- and **S**-lignin markers *m/z* 180 and 210, but not for *m/z* 114 (mainly pentose-based hemicellulose), 126 (mainly cellulose) or 117 (protein) (Fig. 4, shown for *m/z* 210 and 126 only). Bacterization seems to inhibit the lignin degradation caused by *F. oxysporum* f. sp. *dianthi*.

Multivariate analysis of the PyMS spectra of the inoculated xylems, for the mass range *m/z* 20–500 (not shown), separates the samples especially by the first three principal components, describing 16·5, 12·2 and 10·5 %, respectively, of the total variation. The first function shows in PC1 + markers for an intact cell wall [pectin, (hemi)cellulose and **G**- and **S**-lignin markers] in the tissue samples and in PC1-markers of phytoalexins (*m/z* 105, 213, 223, 229, 239, 257, 269, 271, 287, 301, 303, 315 [*38*]) and fatty acids. PC2 shows **G**/**S**-lignin (PC2 +) and polysaccharides (PC2 −) and PC3 **S**-lignin (PC3 +) and **G**-lignin (PC3 −). All bacterized/inoculated samples seem to have a more intact lignin than their non-bacterized/inoculated controls. In addition, the non-bacterized samples, in general, appeared to be richer in phytoalexins and/or fatty acids, cv Pallas excepted; the bacterized samples of the two resistant cultivars Novada and White Giant were somewhat richer in sugars. Degradation of the cell wall components pectin and cellulose in the tissue samples seems to occur as well. Variation in lignin composition seems, however, the most prominent process. Multivariate analysis was repeated, therefore, using lignin marker masses only.

Fig. 5 shows the result of multivariate analysis restricted to lignin fragments only. This figure shows a two-dimensional map with principal components 1 and 2 as axes [Fig. 5(a)]. Fig. 5(b) shows the masses responsible for the distances in the score plot, whereas Fig. 5(c and d) represent the variation along the axes. Variation in lignin composition of the samples is based on differences between cultivars and on effects of the treatment. For each cultivar, the bacterized/inoculated (*) sample is clearly distinguished from the non-bacterized/inoculated sample. It appears that for the more resistant cultivars Pallas (P), Novada (N) and White Giant (W) separation occurred mainly by PC1, whereas for the more susceptible Early Sam (ES) and Silvery Pink (S) the samples were separated along PC2. The bacterized/inoculated sample of the intermediate cultivar Elsy (E) was separated from the non-bacterized/inoculated one both by PC1 and 2. Starting in the upper right quadrant and turning clockwise, Fig. 5(b) shows a comparative richness in partly modified (oxidized) **S**-lignin (*m/z* 167, 194, 208; upper right), in intact **S**-lignin (*m/z* 154, 210; lower right), in intact **G**-lignin (lower left) and in modified **G**-lignin (upper left). Thus, combining Fig. 5(a and b), it appears that bacterized samples are either richer in partly modified **S**-lignin (P*, N*, E*) in intact **S**-lignin (W*, ES*) or in intact **G**-lignin (S*). Thus, bacterization seems to retard degradation (demethoxylation) of syringyl units and/or degradation of guaiacyl groups.

Radish cultivar Saxa × Nova. As *F. oxysporum* f. sp. *raphani* grows along the root before penetration, the infected sites on the radish roots were not known and, thus, could not be dissected as such. Consequently, a large area of unaffected tissue was included in the analysis. Only a marginal difference (if any) in chemical composition was expected between the differently treated root segments. Our analyses, therefore, are focussed on a comparison of inoculated vs. non-inoculated and bacterized/inoculated vs. non-bacterized/inoculated root pieces of one radish cultivar only.

Healthy plants As indicated by previous PyMS analysis, slow-growing, resistant radish cultivars contained more cell wall material in the leaves but less in the roots when compared to faster-growing, more susceptible ones [*25*]. The within-variation of the group of root samples of untreated plants of cultivar Saxa × Nova was not analysed separately. The major variation apparent from the spectra of separate individuals was found to be in the comparative contribution of lignin as a marker of cell wall material vs. cytoplasm components (not shown).

Bacterized/inoculated roots In one experiment samples (20 of each treatment) of bacterized/inoculated and non-bacterized/inoculated plants taken at 6 and 10 days after inoculation, were compared. In another experiment both bacterized/- and non bacterized/inoculated and -/non-inoculated samples were included. In the first experiment, neither at 6 or at 10 days after inoculation, bacterized/inoculated roots could be distinguished from non-bacterized/inoculated ones by factor analysis using all masses from *m/z* 20–1000. In the second experiment factor analysis showed a very small overall effect of inoculation ($P = 0{\cdot}05$) in PC1 (describing 21·9 % of the total variation). This indicated an increase in phenolics and fatty acids for the inoculated samples vs. a higher incidence of polysaccharides and guaiacyl- and syringyl lignin for the non-inoculated controls (not shown). Similarly, a possible effect of bacterization ($P = 0{\cdot}05$) showed in PC3 (describing 9·5 %) of the total variation) indicating a higher incidence of polysaccharide markers *m/z* 85, 114 and 126, syringyl lignin markers *m/z* 154, 167, 208 and 210 and especially of the guaiacyl lignin markers *m/z* 124, 137, 178 and 180 for the bacterized samples. Non-bacterized samples plotted closer to PC3 + showing *m/z* 44 of carbon dioxide, possibly derived from phenolic acids [*16*], and markers of phenols and sterols (not shown).

In view of the tendency of group separation in the second experiment, the mass data were further analysed

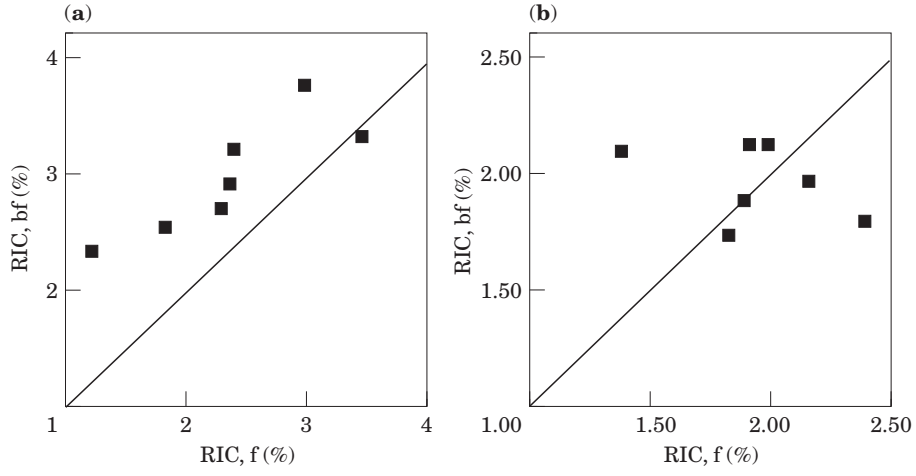

FIG. 4. Comparison of the percentages of the total ion current (% RIC) for the masses m/z 210 (**a**) and 126 (**b**) of bacterized/inoculated carnation xylems (bf) with non-bacterized/inoculated (f) ones. M/z 210 is a prominent marker of syringyl lignin, m/z 126 is the main marker of cellulose.

presuming the occurrence of different categories representing the different treatments. Due to the higher incidence of markers for lignin, the analyses were focussed on such markers. Thus, for the 6 day series, with prior grouping according to treatment, discriminant analysis for the lignin masses alone gave a separation of the bacterized/inoculated samples from the inoculated ones at DF2 (describing 15·9% of the total variation: $P = 0{\cdot}02$). The non-bacterized root samples plot closer to DF2+, which shows the guaiacyl lignin masses m/z 137, (178) 180 and the **G-G** dimer 272 and syringyl m/z 210. The bacterized samples plot closer to DF2- with syringyl lignin masses m/z 154, 167, 168, 182, 194, 196 and 208, and the vanillic aldehyde m/z 152. For the 10 day series the two groups were now separated from each other by DF1 (describing 20·1% of total variation; $P = 0{\cdot}005$). The bacterized samples contained more (partly oxidized) **S**-lignin, the non-bacterized ones were richer in **G**-lignin (not shown).

For the second experiment, prior grouping separated all inoculated samples from the non-inoculated ones in DF1 (describing 36% of the total variation; $P = 0{\cdot}002$; Fig. 6). All bacterized samples were separated from the non-bacterized ones by DF2 (describing 12·2% of the total variation; $P = 0{\cdot}009$; not shown). Regardless of bacterization all inoculated samples plotted closer to DF1- which gives the masses m/z 124, 138, 152, 154, 166, 168, 182, 194, 196 and 208. All these mass peaks, with the exception of m/z 124, 138 and 154, can be interpreted as lignin oxidation products. For example m/z 152 represents vanillic aldehyde, m/z 166 4-ethanalguaiacol, m/z 182 syringic aldehyde, m/z 196 4-ethanalsyringol and m/z 208 sinapyl aldehyde. The bacterized and untreated controls plot closer to DF1+ which gives markers m/z 137, 178 and 180 of guaiacyl lignin and m/z 167 and 210 of syringyl lignin (Fig. 6). In DF2 (representing 12·2% of the total variation, $P = 0{\cdot}009$) a more or less similar separation is seen with all bacterized samples plotting closer to DF2- which is practically equal to DF1+ (not shown).

To summarize, treatment with *F. oxysporum* f. sp. *raphani* tends to lead to lignin degradation, whereas bacterization tends to retard syringyl lignin oxidation caused by fungal infection.

DISCUSSION

For both carnation and radish, various cultivars are known which differ in their level of resistance toward *F. oxysporum* f. sp. *dianthi* and *F. oxysporum* f. sp. *raphani*, respectively. Resistance, therefore, is of a quantitative nature. Induced resistance depends on an earlier and stronger expression of existing resistance mechanisms. In carnation, within one individual plant, both incompatible and compatible responses can be found at different locations in the xylem. The balance between those responses strongly depends on a variety of environmental circumstances. For a resistant response a number of host reactions has to occur more or less simultaneously and sufficiently early after infection. Stronger or earlier expression of only part of these responses is obviously not always sufficient to change the host response from susceptible to resistant. Induction of enhanced resistance in carnation depends not only on the nature of the inductor, but also on a number of other factors such as *e.g.* the virulence of the inoculum and/or the environmental temperature during wilt development. Consequently, expression of induced resistance has been found in only a limited number of experiments [*15*, *51*]. In the present investigation the stimulus of bacterization appeared to be insufficient to affect the balance between susceptible and

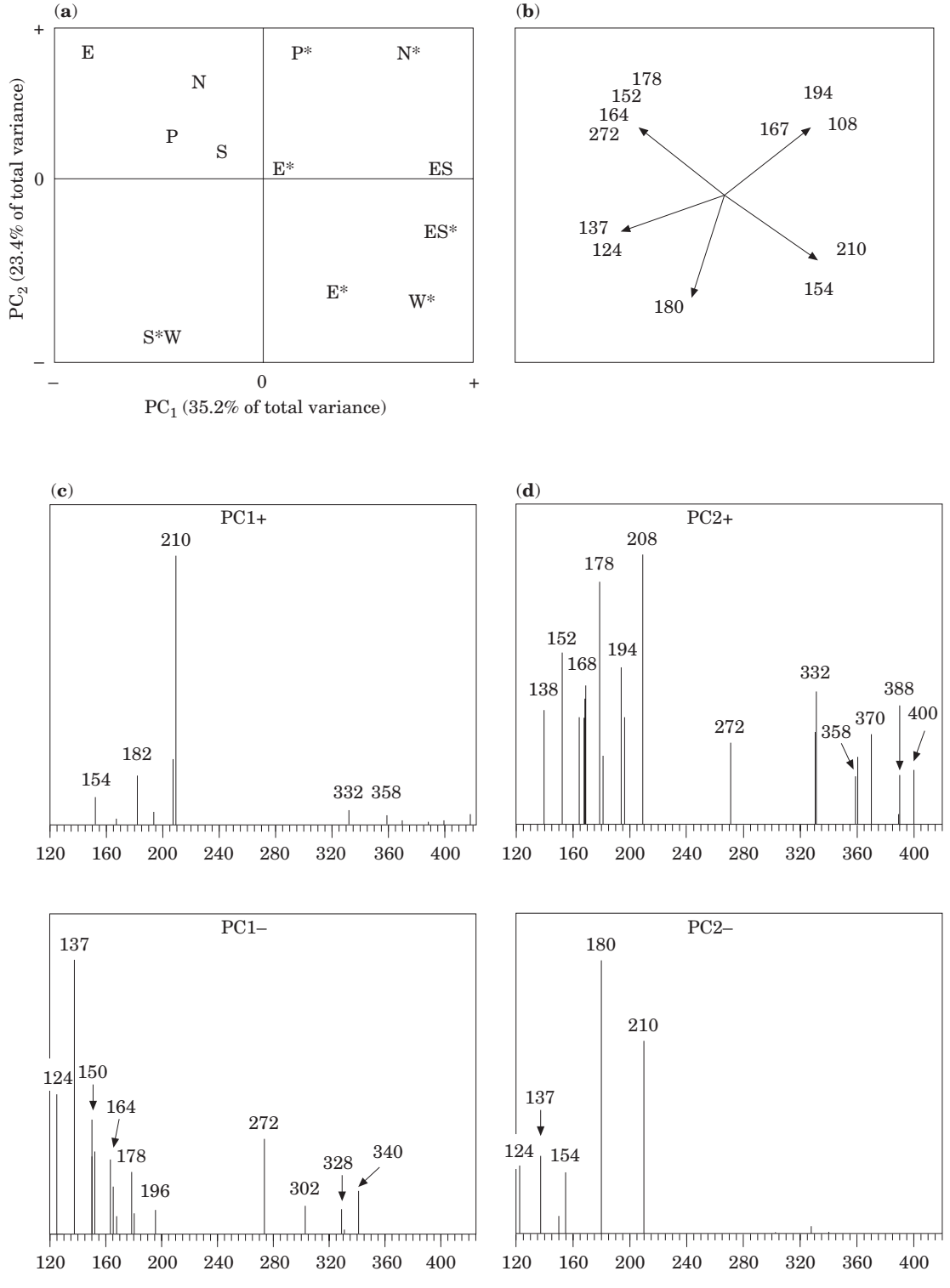

FIG. 5. (**a**) PC1/PC2 plot resulting from multivariate analysis of in-source PyMS spectra of xylem of the stem of different carnation cultivars bacterized (*) or non-bacterized with *Pseudomonas* spp. after inoculation with *Fusarium oxysporum* f. sp. *dianthi*. Carnation cultivars: Pallas (P), Novada (N), Early Sam (ES), Elsy (E), White Giant (W) and Silvery Pink (S). (**b**) Features responsible for the distances between the carnation samples in the multivariate space. (**c**) and (**d**) The principal components spectra PC1 and 2, respectively, on which this separation was based. Principal components spectra are reconstructed mass spectra (see text). PC1+ shows monomeric markers of syringyl-lignin, whereas PC1− gives monomers of the partly oxidized (aldehyde) structures and some dimers of mainly guaiacyl lignin. PC2+ shows an oxidation pattern of both guaiacyl- and syringyl lignin and several dimers. PC2− gives some monomeric markers of both guaiacyl- and syringyl-lignin. The occurrence of dimeric markers indicates increased depolymerization. Detailed information on fragment ions is given in the section Interpretation of PyMS spectra.

resistant interaction in terms of the number of diseased plants. However, this does not imply that chemical changes caused by bacterization could not be an integral part of an (insufficient) resistant interaction.

Information on chemical changes was obtained by pyrolysis mass spectrometry, a method in which the plant polymers are degraded before MS analysis. Changes within the polymeric structures themselves, therefore, would not be noticed. Large compositional differences might also influence the type of fragmentation, especially when large variations in sodium and/or potassium concentration are involved [*28*]. The composition of the plant material investigated is rather similar, however, and for such material qualitative and quantitative determinations based on relative intensities of ions in PyMS [*1*, *40*, *42*, *43*] were shown to correlate well with wet chemical methods.

In carnation, chemical changes as a consequence of fungal development start very soon after infection [*36*]. In the present investigations the major chemical variation related to disease development at 3 days after inoculation was found in the lignin composition. Both syringyl- and guaiacyl-lignin were broken down (Figs 2 and 3). It is known that fungal infection causes a complex of reactions in the host which among others involve both (syringyl- and guaiacyl-) lignin degradation and (guaiacyl-) lignin synthesis in the xylem tissue [*33*, *34*]. The break-down of both syringyl and guaiacyl-lignin was clearly demonstrated in the spectra of Fig. 3. In PC1+ (Fig. 3) aldehydes and dimers of both guaiacyl- and syringyl units were recovered. Therefore, oxidation and depolymerization is a major process of the lignin degradation. It has been shown before [*36*], however, that demethoxylation (or selective degradation [*47*]) occurs as well, even to such an extent that only guaiacyl lignin remains. The fungal lignin degradation process appeared less severe after bacterization (Fig. 5). This might indicate bacterially-induced strengthening of cell walls or suppression of fungal activity based on other mechanisms. At this stage of infection bacterization also somewhat suppressed phytoalexin accumulation in all cultivars except cv. Pallas. In previous experiments [*51*] such an early suppression was also indicated for the susceptible cultivar Lena (after 1 week), whereas for Pallas, 1 week after inoculation, an increase was demonstrated. Later on, also for Lena increased accumulation was found. The initial decrease in phytoalexin accumulation points to retarded fungal activity. All processes are restricted to the affected xylem and phytoalexin synthesis is located in the host cells surrounding the infected area [*3*, *33*]. Increased phytoalexin accumulation after the initial suppression points towards an extra stimulation of host cell metabolism. In addition to phytoalexin accumulation this leads to gum formation and compositional changes in the cell wall. It now appears that changes in host cell wall composition apparently are more prominent or start earlier than an increase in phytoalexin accumulation. As such, these cell wall alterations and/or related phenomena such as increased phenolic turnover, could be responsible for slowing down fungal development. However, it still remains to be proven.

In the case of radish, bacterization led to a significant reduction in the number of diseased plants. These results agree with those of a large number of independent experiments performed in our laboratory [*26*, *30*]. Obviously, the mechanism involved depends much less on small fluctuations in environmental conditions and probably is less complex than that in carnation. It is indicated in radish that induced resistance is effective particularly in the early stages of infection. A failure of the fungus to reach or colonize the vascular tissue is assumed [*26*]. Although the fungus is applied on the main tap root, microscopic observation indicates that the fungus probably mainly penetrates the undifferentiated tops of the side roots (H. Steijl, unpublished). Once infection has occurred the host is unable to check it. The first macroscopic sign of fusarium wilt is a brown discoloration of the root vascular tissues, detectable in transverse sections.

Contrary to carnation, no clear cut chemical variation related to disease development in the radish roots could be detected. In part, this might be due to the fact that, unlike for carnation, infected tissues had to be analysed together with a lot of uninfected tissue. There was a tendency of chemical variation, however, which was found in the lignin composition. For radish roots xylem tissues could not be reliably dissected and the location of the changed lignin, therefore, is not clear. Both the carnation stem xylem and the radish root tissues contain a mixed guaiacyl-syringyl lignin, but in radish the ratio guaiacyl/syringyl seems a bit higher. After inoculation with *F. oxysporum* degradation of lignin was clearly found in carnation stems and indicated in radish stems. However, the type of degradation seems to differ. In the radish PyMS analyses, differentiation between **S**/**G**-lignin markers (control) and oxidized **S**-lignin (after inoculation) points to a chemical change of syringyl lignin only. Possibly, this points to a selective removal of syringyl-rich secondary cell wall material and retention of guaiacyl-rich middle lamella [*cp* 47] in radish. In carnation, probably both secondary wall and middle lamella are being removed, leading to cavity formation in the susceptible interaction [*4*].

As mentioned before, fungal penetration in the radish root tips has been observed (H. Steijl, unpublished). It seems very improbable, therefore, that variation in lignin composition is the cause of the observed reduction in disease incidence. It is more probable that non-lignified tissues such as *e.g.* the exodermis of the root tip, might be induced to a better defense against fungal invasion. As practically no chemical differences were found, a physical

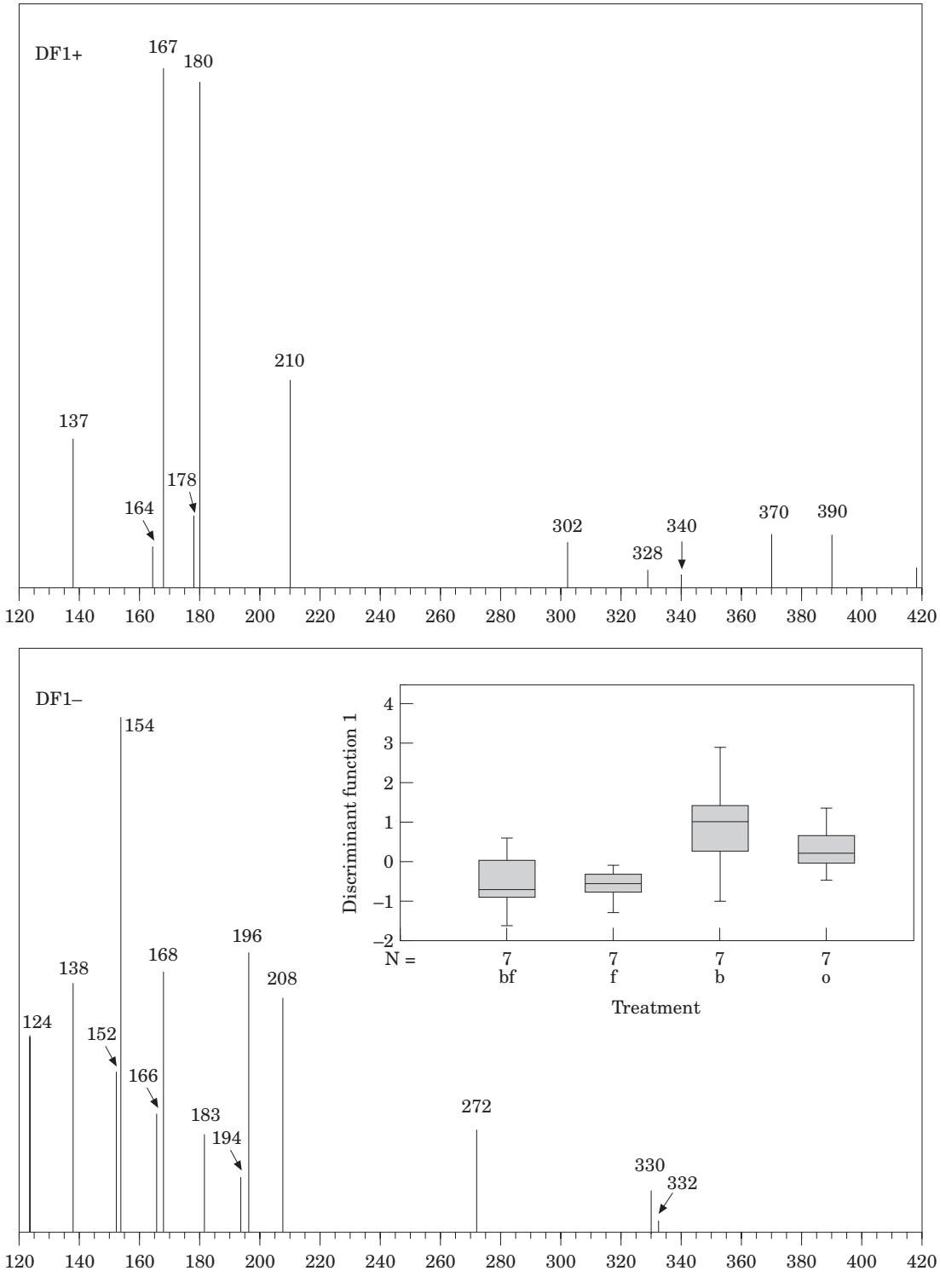

Fig. 6. Separation by discriminant function (DF) analysis of the pyrolysis low voltage EI mass spectra of root segments of individuals of the radish cultivar Saxa × Nova that were inoculated with *Fusarium oxysporum* f. sp. *raphani* with or without previous bacterization with *Pseudomonas* spp. (bf or f), or were only bacterized (b) or remained untreated (o) (insert) and the discriminant function spectra of DF1 on which this separation was based, describing 36 % of the total variation. Discriminant function spectra are reconstructed mass spectra, and predominant ion-signals are explained in the text. Detailed information on fragment ions is given in the section Interpretation of PyMS spectra.

blockade due to a rearrangement of the same chemical components might be indicated.

Direct effects of pseudomonads on plant growth have been demonstrated and were considered to be due to increased availability of minerals or other ions or to microbial production of plant growth regulators [*2*, *19*, *32*]. Transformed pea roots were protected against *F. oxysporum* f. sp. *pisi* after preinoculation with *Bacillus pumilis* [*8*] or *P. fluorescens* strain 63-28R [*7*]. In these studies, root bacterization by itself did not lead to morphological alterations of root tissue. Upon challenge inoculation pathogen growth was restricted to the epidermis and the outer cortex. The walls of these cells were strengthened at the sites of attempted fungal penetration by appositions containing large amounts of callose and phenolic materials, effectively preventing fungal ingres. *P. fluorescens* WCS417r has been reported to induce thickening of cortical cell walls in tomato roots after dense colonization of epidermal or hypodermal cells [*14*]. Preliminary investigations by Steijl (unpublished) with isolated cell walls of radish roots also showed that some small, but consistent, changes were apparent in the polysaccharide composition after bacterization with *P. fluorescens* WCS417r. Such changes were not found in control plants or in plants treated with *P. fluorescens* WCS358, which does not induce resistance.

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