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Fungal polygalacturonase activity reflects susceptibility of carnation cultivars to fusarium wilt

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Abstract

Carnation cultivars with different levels of partial resistance were inoculated with race 2 of *Fusarium oxysporum* f.sp. *dianthi* and monitored for accumulation of host phytoalexins, fungal escape from compartmentalization, production of fungal pectin-degrading enzymes and development of external disease symptoms. Accumulation of phytoalexins, assessed after 10 days in the first 5 cm above the inoculation site, was weakly (methoxydianthramide S) or not (hydroxydianthalexin B) correlated with resistance levels after 12 weeks. Fungal escape from compartmentalization, assessed after 3 weeks as percentages colonized plants at 8 cm above the inoculation site, was highly correlated with expression of susceptibility after 12 weeks. Polygalacturonase (PG) activity, assessed after 4 weeks in the first 5 cm above the inoculation site, was highly correlated to final disease development. Linear increases in disease severity were accompanied by quadratic increases in PG activity. In contrast to water-treated plants, that lacked any PG activity, inoculated plants contained two main groups of fungal PGs, the dominant forms of which had estimated pI values of 7.0 and minimally 9.5, respectively. Compared to those of the first group, enzymes of the second group were produced only in trace amounts in liquid media containing pectin or polygalacturonate as sole source of carbon. On these media, the fungus also produced a pectin methyl esterase (PME) with an estimated pI of 9.3. Besides PMEs of host origin, inoculated plants of susceptible cultivars contained the fungal PME while no more than traces were found in resistant ones.

Assessment of phytoalexin production by the host during defense responses cannot replace monitoring of external symptoms as a resistance test. Assessment of fungal growth, whether by reisolations above the compartmentalization area or by measurement of PG activity, provides a both rapid and reliable prediction of disease development.

Introduction

Resistance of carnation (*Dianthus caryophyllus* L.) cultivars to the fungus *Fusarium oxysporum* Schlecht.: Fr. f.sp. *dianthi* (Prill. & Delacr.) Snyder & Hansen, causal agent of fusarium wilt, is partial and difficult to assess. Current resistance tests are time-consuming as they rely on assessment of disease expression after several months or even an entire growth season (Ben-Yephet et al., 1993). Reproducibility of tests is generally poor, even when experimental conditions are standardized as far as possible (Baayen and Schoffelmeer, 1993), so that tests must be repeated several times. In order to provide breeders with more rapid and reliable resistance tests, we here present an evaluation of possible alternative methods from the literature and a novel approach based on assessment of fungal cell-wall degrading enzymes.

Two components have been described for resistance of carnation to fusarium wilt. The ability of clones (cultivars) to localize the fungus inside infected xylem forms a first component. Permanent containment of the fungus (successful compartmentalization) is observed in most plants of a resistant clone but in fewer plants or none at all of partially resistant or susceptible ones (Baayen and Van der Plas, 1992).

Localization abilities can be estimated from the percentage of stem-inoculated plants in which the fungus has failed to escape from the originally inoculated area (Baayen and Van der Plas, 1992). Failure of plants to restrict the fungus at the site of infection leads to colonization of host tissues, eventually followed by wilt and death. Colonization and symptom expression nevertheless proceed at a slower rate in cultivars with a higher level of partial resistance (Baayen and Van der Plas, 1992). The second component, retardation of fungal growth, likely has a common basis with the first one as both components are closely correlated.

A complex of host reactions including phytoalexin accumulation, gum formation, lignification and suberization are involved in containment of the fungus inside the infected xylem vessels (Baayen, 1988; Baayen and Elgersma, 1985; Niemann and Baayen, 1988; Niemann et al., 1990). Phytoalexins can be quantified by HPLC and have been found to be correlated to resistance (Baayen and Niemann, 1989).

Fungal action in the host is characterized by severe degradation of the vascular tissues, eventually resulting in hollowing-out of stems and wilting of leaves (Baayen and Elgersma, 1985; Baayen et al., 1988). Degradation of host tissues generally begins with production of polygalacturonase (PG) enzymes that hydrolyze pectin, a constituent of the middle lamella and primary cell walls (Cervone et al., 1986; Cooper and Wood, 1975; Jones et al., 1972). The action of pectic enzymes is often already sufficient to cause host tissue maceration and cell death (Brett and Waldron, 1990; Collmer and Keen, 1986). Polygalacturonase production *in vitro* has been reported for several formae speciales of *F. oxysporum* (Cooper and Wood, 1975; Cooper et al., 1978; Pérez Artés and Tena, 1990; Scala et al., 1981; Strand et al., 1976; Suresh et al., 1984) including f.sp. *dianthi* (Scala et al., 1981). The marked degradation of xylem in carnations infected with race 2 of *F. oxysporum* f.sp. *dianthi* (Baayen and Elgersma, 1985; Baayen et al., 1988) prompted us to investigate the involvement of polygalacturonase and related cell-wall degrading enzymes such as pectin methyl esterase (PME) in this process and to evaluate their prospects for the development of novel methods to assess the level of partial resistance in carnation to fusarium wilt. This study presents an evaluation of the prospects of such novel methods compared with those of existing ones.

Materials and methods

Inoculation of plants and disease development. Four-week-old rooted cuttings of twelve carnation cultivars (Novada, IVT 78618-12, Carrier 929, Revada, Niky, Pallas, Elsy, IVT 62093-G, Silvery Pink, Alice, Lena and Early Sam) representing all levels of partial resistance to race 2 of *F. oxysporum* f.sp. *dianthi* (Baayen and Van der Plas, 1992) were kindly provided by the DLO Centre for Plant Breeding and Reproduction Research, Wageningen. Cuttings were planted in steamed soil (8 cm diameter pots) and grown in a glasshouse with the temperature set at 18 to 22 °C for 4 weeks prior to inoculation.

Conidial suspensions of isolate WCS 816 of race 2 of the pathogen were prepared by washing 10-day-old potato dextrose agar (Difco) cultures with sterile water. Mycelial fragments were removed by filtration through glass wool, after which the conidial suspension was adjusted to 10^7 conidia ml^{-1}. Plants were inoculated close to the stem base by depositing 20 μl conidial suspension in a leaf axil, after which the stem was incised through the droplet.

Development of wilt symptoms was monitored using the following index: 0 – no disease symptoms; 1 – weak disease symptoms; 2 – limited local symptoms; 3 – well-developed symptoms on otherwise still healthy-looking plants; 4 – severe wilt and 5 – death. Progress of disease symptoms was determined weekly up to 12 weeks after inoculation. Percentages diseased plants were analyzed with GENSTAT (Payne et al., 1987) using a generalized linear model (GLM) with logit link, where necessary corrected for block effects. Disease indices were analyzed non-parametrically using Wilcoxon's test corrected for ties.

Experiment I. In a first experiment, 40 plants per cultivar (cv. Alice excepted) were stem-inoculated, and 8 plants were treated with the same volume of water. Twenty plants per cultivar were used to evaluate disease development. Twenty inoculated plants and 8 water-treated ones per cultivar were collected four weeks after treatment for extraction of fungal enzymes.

Experiment II. In a second experiment, 128 plants per cultivar (cv. Early Sam excepted) were stem-inoculated, and 32 plants were treated with the same volume of water. Rooted cuttings from the same batch were planted, grown, inoculated and sampled according to a standard protocol in four successive weekly series for obtaining blocks in time, all in two blocks

in space consisting of a single greenhouse compartment each. Inoculations were performed with inoculum raised weekly according to a standard protocol starting from lyophilized cultures from a single batch. Plots allocated to repetitions in time that had not yet begun or had finished before the others were left vacant. Plants were randomized in groups of four per cultivar in equal proportions over all repetitions in time and space, and in a ratio of 1:4 over control and inoculation treatments. Within each group of four, plants were randomized over four monitoring and sampling procedures: assessment of disease development (up to 12 weeks), reisolation of the fungus (after 3 weeks), extraction of phytoalexins (after 10 days) and extraction of fungal enzymes (after 4 weeks). The respective sampling times are optimal for the various procedures (Baayen and Van der Plas, 1992; Baayen and Niemann, 1989; Baayen and Schoffelmeer, 1993; Niemann and Baayen, 1988; Schoffelmeer, unpublished data from preliminary experiments).

Reisolation of the fungus. The first 10-cm segment above the inoculation site of plants selected for this purpose was defoliated and surface-sterilized, after which a 2-mm-thick slice was aseptically removed at 8 cm and placed on potato dextrose agar. Percentages of plants in which *F. oxysporum* was encountered in the stem at 8 cm height above the site of inoculation were analyzed using a GLM with logit link. Colonization at 8 cm height indicates failure of plants in compartmentalizing the fungus within the originally inoculated area (Baayen and Van der Plas, 1992).

Extraction and quantification of phytoalexins. The first 5 cm above the inoculation site of stems of plants selected for this purpose was extracted in acetone and analyzed for phytoalexins by HPLC according to Niemann et al. (1991). Accumulated amounts of hydroxydianthalexine B (HDxB) and methoxydianthramide S (MDS), located by their retention time in comparison with reference samples, were expressed in units absorption g^{-1} fresh weight of extracted stems and analysed using GENSTAT with an analysis of variance (ANOVA) after appropriate transformation in order to stabilize variances. One hundred units absorption correspond to about 3 μg pure compound for both phytoalexins (Baayen and Niemann, 1989).

Extraction of enzymes from plants. The first 5 cm above the inoculation site of stems of plants selected for this purpose was defoliated and weighed. Stem segments were pooled in groups of four, sliced up and pulverized in liquid nitrogen with the aid of a mortar and pestle. Per g fresh weight of stems, 4 ml ice-cold 0.1 M sodium citrate buffer (pH 4.6) supplemented with 0.05 M EDTA, 0.01 M sodium ascorbate and 0.01 M β-mercaptoethanol was added after which the mixture was centrifuged and the pellet discarded. Supernatants were used without further treatments for native horizontal slab gels. PGs appeared to be very stable in this solution, and could be stored at 4 °C up to 3 months without significant loss of activity (Schoffelmeer, unpublished data from preliminary experiments).

Production of enzymes on artificial media. The fungus was cultured at room temperature in the dark in Czapek Dox liquid medium (100 ml, in 300 ml Erlenmeyer flasks) in which glucose had been substituted by 0.1% (w/v) citrus pectin (Fluka), 0.1% (w/v) sodium polygalacturonate (grade II, Sigma) or 0.1% (w/v) sucrose. A spore suspension was added to these media to a final concentration of 10^4 conidia ml^{-1}, after which the cultures were incubated for three days at 22 °C on a reciprokal shaker. Mycelium was removed by filtration through a paper filter (Schleicher and Schuell 604). To this filtrate 0.1 mM sodium azide and 0.1 mM L-cysteine were added prior to evaluation of enzymatic activity.

Reducing group assay for polygalacturonase activity. PG activity was quantified using a spectrophotometric assay for reducing groups that employs 2-cyanoacetamide (Gross, 1982). In order to remove compounds from plant extracts that react with the reagent, samples were filtrated in Eppendorf tubes equipped with a 10000 MW filter (Millipore PLGC membrane) and brought back to the original volume with 10 mM Tris-HCl buffer (pH 7.5) supplemented with 10 mM sodium azide. Reaction mixtures consisting of 160 μl 37.5 mM sodium acetate (pH 4.4), 30 μl 0.2% sodium polygalacturonate (washed with 80% ethanol prior to use) and 10 μl filtered sample were incubated for 6 h at 30 °C. D-galacturonic acid (Merck) was used as a standard. PG activity was expressed in mM reducing groups released h^{-1} ml^{-1} culture filtrate or g^{-1} fresh weight of extracted stems. Data were analysed using ANOVA after appropriate transformation.

Cup-plate assay for polygalacturonase activity. PG activity was quantified as described by Dingle et al.

(1953) with the modifications introduced by Leone and Van den Heuvel (1987). PG activity was expressed in cleared area h^{-1} ml^{-1} culture filtrate or g^{-1} fresh weight of extracted stems. Data were analysed using ANOVA after appropriate transformation.

Electrophoresis. Extracts from carnation stems and culture filtrates were analysed as such by polyacrylamide gel electrophoresis (PAGE) using the method of Cruickshank and Wade (1980) as modified by Van den Heuvel and Waterreus (1985). Samples of 60 μl were analysed on 8% polyacrylamide gels containing 0.09% sodium polygalacturonate and buffered at pH 8.7 with 0.05M Tris – 0.1M glycine. Gels were incubated at 30 °C in 0.1 M malic acid adjusted with Tris to pH 4.2 for 4 h and stained in 0.03% ruthenium red for 30 min (Cruickshank and Wade, 1980).

Samples for isoelectric focusing (IEF) were prepared by precipitation of proteins present in 4 ml stem extract or 15 ml culture filtrate with ammonium sulphate (overnight, 4 °C). Subsequently, proteins were dissolved in 1 ml Tris-HCl buffer (10 mM, pH 7.5) and concentrated by centrifugation over a Millipore PLGC membrane (MW 10000). IEF was performed at 5 °C on a LKB 2117 Multiphore apparatus according to Ried and Collmer (1985). Electrode wicks for the anode and cathode were soaked in 0.04 M aspartic acid and 0.5 M NaOH respectively. A pH gradient was generated in a 5% polyacrylamide gel (0.7 mm thick) containing carrier ampholytes (Pharmalyte pH 3 to 10, 5.9% v/v and Pharmalyte pH 8 to 10.5, 0.075% v/v) by pre-electrophoresis for 30 min at 2.0 W. 15 μl samples were subjected to electrophoresis for 60 min at 4.5 W (constant) at 1000 V. PG and PME were visualized by means of polygalacturonate-agarose and pectin-agarose overlays according to Ried and Collmer (1985). Overlays were incubated for 30 min (polygalacturonate) or 60 min (pectin) at 30 °C and stained in 0.05% ruthenium red. pH Gradients in IEF gels were estimated by cutting a 1-cm-wide strip from the gel over its full length, cutting the strip into 0.5-cm-long pieces, placing the pieces in 1 ml water, and measuring the pH after 24 h.

Results

Quantification of polygalacturonase activity. In a first experiment, PG activity in extracts from inoculated (Table 1) and water-treated plants of 11 carnation cultivars was analysed by a test for reducing groups as well as a cup-plate assay. PG activity was only encountered in extracts from inoculated plants. Activities measured with the first method (*spec*, in mM reducing groups released h^{-1} g^{-1} fresh weight of extracted stems) and those obtained with the second one (*cup*, in mm^2 cleared area h^{-1} g^{-1} fresh weight of extracted stems) were highly correlated ($r = 0.996^{***}$; $cup = -37 + 28 \sqrt{spec}$ for $cup > 0$). Data obtained with the cup-plate assay thus reflect the square root of actual activities.

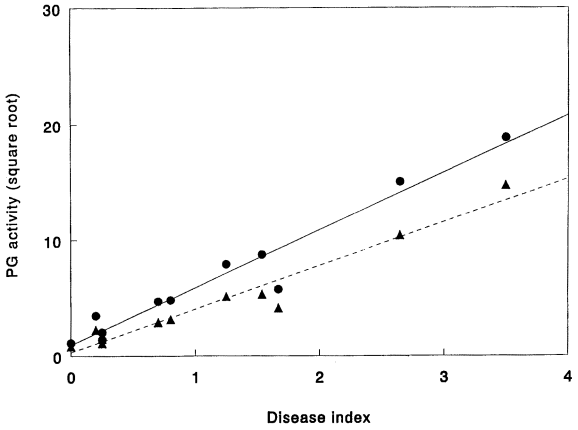

Figure 1. A linear relationship exists between the square root of PG activity (reducing group assay) in stems of 11 carnation cultivars inoculated with *Fusarium oxysporum* f.sp. *dianthi* and the disease index of the cultivars. Data from experiment I, four weeks after inoculation. ▲, PG activity per gram of extracted segments; •, PG activity per 5-cm-long segment.

PG activity in stem extracts and disease development (experiment I). Disease indices of stem-inoculated cultivars after four and eight weeks were reflected in the PG activity in extracts from the first 5 cm of the stem above the inoculation site. Four weeks after stem inoculation, a linear correlation existed between the disease indices of the eleven cultivars tested and the PG activities in stems of such plants as quantified with the cup-plate assay ($r = 0.96^{***}$) or, alternatively, between the disease indices and the square root of the PG activities as quantified with the spectrophotometer ($r = 0.97^{***}$). Notwithstanding minor, although statistically significant, differences between cultivars in stem weight per cm, correlations were unaltered when PG activity was expressed per cm extracted stem instead of per gram fresh weight of extracted stems (Figure 1). It is concluded that, at least during the initial phases of disease development, linear increases in disease development are accompanied by quadratic changes in overall PG activity.

Table 1. Polygalacturonase activity in extracts from stems of eleven carnation cultivars, four weeks after stem inoculation with *Fusarium oxysporum* f.sp. *dianthi*, and the disease indices of these cultivars after four and eight weeks (experiment I). Within columns, values followed by the same letter are not significantly different ($P < 0.05$)

Cultivar	Polygalacturonase activity		Disease index[3]	
	Spectrophotometer[1]	Cup-plate[2]	Four weeks	Eight weeks
Novada	0.7 a	0.0 a	0.00 a	0.05 a
78618–12	1.2 ab	6.1 a	0.25 bc	1.35 b
Revada	2.2 ab	12.4 ab	0.25 ab	0.68 b
Elsy	5.0 abc	30.9 cd	0.20 ab	1.30 b
Niky	9.9 bcd	45.1 cde	0.80 cd	1.45 b
Pallas	8.4 abc	31.8 bc	0.70 cd	2.61 c
Carrier 929	26.3 de	94.8 e	1.25 de	3.14 cde
Lena	28.2 e	93.6 e	1.54 e	3.00 cd
62093-G	16.8 cde	69.4 de	1.67 e	3.42 e
Silvery Pink	107.7 f	257.9 f	2.65 f	3.15 de
Early Sam	216.5 g	382.7 g	3.50 g	4.63 f

[1] Activity in mM reducing groups released per hour per gram fresh weight of extracted stems.
[2] Activity in mm^2 cleared area in a cup-plate assay per hour per gram fresh weight of extracted stems.
[3] Average indices on an ordinal scale from 0 (healthy) to 5 (dead).

Control carnations treated with water instead of a conidial suspension remained healthy throughout the experiment. Extracts from stems of control plants were devoid of PG activity.

PG activity, phytoalexins, fungal escape and disease development (experiment II). Using the same set of 11 cultivars except for cv. Early Sam which had been replaced by cv. Alice, the correlation of PG activity with disease development was compared to that obtained by assessing phytoalexin accumulation or fungal escape from compartmentalization. Percentages of plants in which the fungus had escaped from compartmentalization (as judged by recovery at 8 cm after 3 weeks) were equally strong correlated to final disease levels as were the percentages diseased plants after 8 weeks (Table 2) (correlation coefficients of $r = 0.95^{***}$ and $r = 0.96^{***}$, respectively). PG activities were also strongly correlated to final disease levels ($r = 0.85^{***}$). Accumulation of the phytoalexins MDS and HDxB was at best weakly correlated to final disease levels ($r = -0.55^{*}$ and $r = -0.43^{ns}$, respectively).

Enzymes with PG activity in stem extracts and culture filtrates. Numerous bands with PG activity were detected on polygalacturonate zymograms of inoculated susceptible plants using native gels buffered at pH 8.7 (Figure 2, lane 1). Two groups of PGs were observed, one of which migrated to the cathode and another migrating to the anode. Both groups consisted of a major band accompanied by a series of smaller bands, probably representing enzymes with apparently slightly different charge or size. Assuming that differences in migration were largely due to the charge of enzymes, the enzymes with highest total activity at the anodic and cathodic side of gels were accompanied by others which were slightly more negatively charged (respectively, less positively charged), suggestive of lower pI values. The group of enzymes migrating to the cathode had pI values around 9.5 or higher, as they continued to migrate to the cathode even in gels buffered at pH 9.5–10.0 (not shown).

Using IEF with gel overlays containing sodium polygalacturonate as a substrate, a major band of PG activity was observed at an estimated pI value of 7.0, together with minor bands with slightly lower pI values (Figure 3, lane 1). These enzymes presumably correspond to the major anodic enzyme observed by PAGE and its accompanying bands, respectively. The pI of the cathodic enzymes could not be reliably estimated by IEF, presumably because PGs are no longer active at high pH values and, or, because of the limited pH range of the carrier ampholytes used. A minor band at pI 9.0, detected by IEF, probably corresponds to one of the minor enzymes of the cathodic group on PAGE gels.

The same enzymes as observed by PAGE and IEF in extracts from stems of susceptible plants were also

Table 2. Correlation coefficients between the percentages plants of 11 carnation cultivars inoculated with *F. oxysporum* f.sp. *dianthi* with external disease symptoms after 12 weeks, and other parameters that could possibly be used to predict final disease levels (data from experiment II)

Method	Correlation coefficient
Percentages diseased plants (after 8 weeks)	$r = +0.96^{***}$
Percentages plants with fungal presence at 8 cm (after 3 weeks)	$r = +0.95^{***}$
PG activities in first 5 cm above inoculation site (after 4 weeks)	$r = +0.85^{***}$
Amounts of MDS in first 5 cm above inoculation site (after 10 days)	$r = -0.55^{*}$
Amounts of HDxB in first 5 cm above inoculation site (after 10 days)	$r = -0.43^{ns}$

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{ns} $P > 0.05$ (not significant).

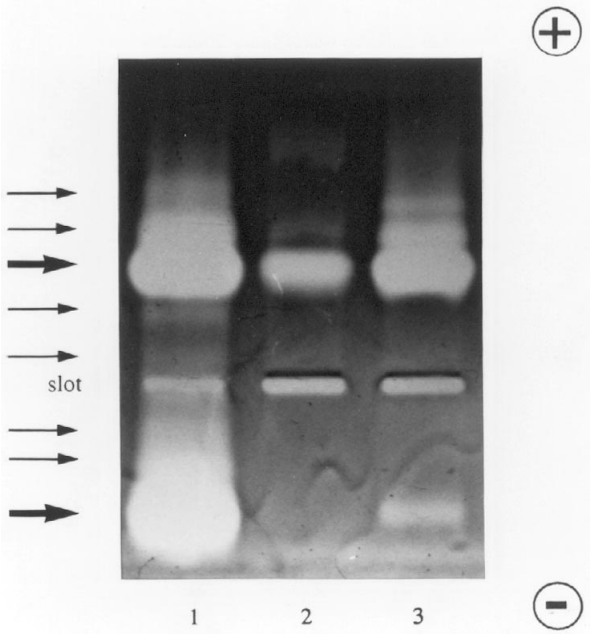

Figure 2. Polygalacturonase zymogram of stem extracts from susceptible cv Early Sam (lane 1), harvested four weeks after stem inoculation with *Fusarium oxysporum* f.sp. *dianthi*, and filtrates from 3-day-old shake cultures containing sodium polygalacturonate (lane 2) or pectin (lane 3) as sole carbon source. Major and minor bands are indicated by thick and thin arrows, respectively. Native polyacrylamide gel (pH 8.7) with sodium polygalacturonate as substrate.

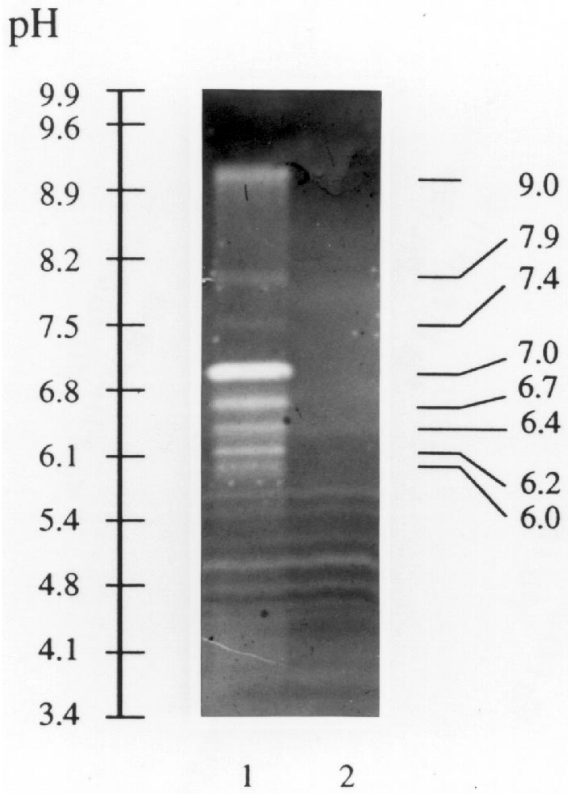

Figure 3. Polygalacturonase zymogram of stem extracts from susceptible cv Lena, harvested four weeks after stem inoculation with *Fusarium oxysporum* f.sp. *dianthi* (lane 1) or treatment with water (lane 2). IEF gel with polygalacturonate-agarose overlay.

observed in extracts from stems of resistant plants, but all in slightly (partially resistant cultivars) or considerably (resistant cultivars) lower amounts (not shown).

Polygalacturonase activity was not detected in extracts from water-treated plants by PAGE (not shown) or IEF (Figure 3, lane 2).

In pectin cultures of *F. oxysporum* f.sp. *dianthi*, the PG enzymes with pI values lower than 8.7 quantitatively dominated over those with pI values above 8.7 (Figure 2, lane 3). The ratio of both groups of PG enzymes thus differed considerably from that in extracts of inoculated plants, where both groups were produced in nearly equal proportions (Figure 2, lane 1). In polygalacturonate cultures, the ratio of both groups of PG enzymes was similar to that in pectin cultures, but PG activity was considerably lower (Figure 2, lane 2). PG activity was not detected when the fungus was cultured on 0.1% sucrose medium (not shown).

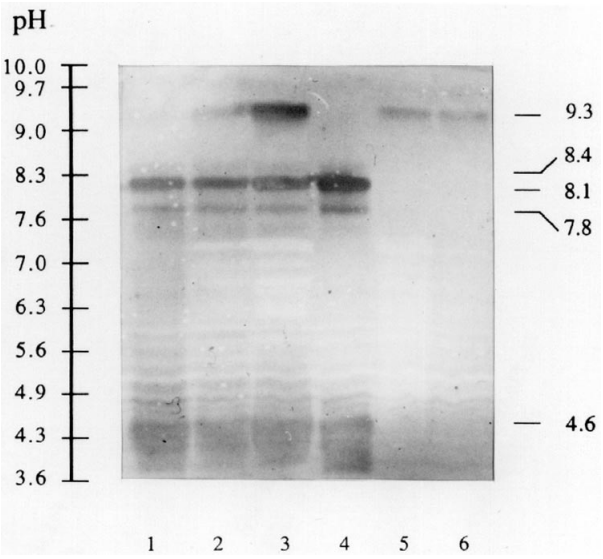

Figure 4. Pectin methyl esterase zymogram of extracts from resistant cv Novada (lane 1), partially resistant cv Pallas (lane 2) and susceptible cv. Lena (lane 3), harvested four weeks after stem inoculation with *Fusarium oxysporum* f.sp. *dianthi* or treatment with water (lane 4, cv Lena), and filtrates from 3-day-old shake cultures containing pectin (lane 5) or sodium polygalacturonate (lane 6) as sole carbon source. IEF gel with pectin-agarose overlay.

Enzymes with PME activity in stem extracts and culture filtrates. Protein samples from infected plants and controls contained PME activity, as indicated by a red smear on native PAGE gels containing pectin (not shown). Using IEF at least four bands representing proteins with PME activity were detected in water-treated plants, with estimated pI values of 8.4, 8.1, 7.8 and 4.6 (Figure 4, lane 4). Stems of infected plants additionally contained a PME with an estimated pI of 9.3 (Figure 4, lanes 2 and 3) that was also produced by the fungus in pectin (Figure 4, lane 5) and polygalacturonate medium (Figure 4, lane 6). The fungal PME was not produced on sucrose medium. Stem extracts from inoculated plants contained more fungal PME in highly susceptible 'Lena' (lane 3) than in moderately resistant 'Pallas' (lane 2), while no fungal PME was encountered in extracts from highly resistant 'Novada' (lane 1).

Discussion

Fungal PG activity in stem-inoculated carnations after 4 weeks, in the early phase of disease expression, proved to be a reliable indicator of disease development after 12 weeks. Correlations were far better than those obtained with two major phytoalexins of carnation, methoxydianthramide S and hydroxydianthalexin B. This confirms previous data on significant but weak correlations between phytoalexin accumulation and partial resistance (Baayen and Niemann, 1989). Presumably, polygenic resistance of carnation to fusarium wilt (Baayen et al., 1991) involves various other resistance components additional to phytoalexins. It is therefore not surprising that resistance is better predicted by measuring the sum of all resistance factors, i.e., the final ability of cultivars to localize the invading fungus in the xylem (Baayen and Van der Plas, 1992). In this study we have assessed the localization ability of the various cultivars by determining the percentage of plants with successful compartmentalization responses. Indeed, our results confirmed that measurement of a cultivar's localization ability provides a rapid (3 weeks) and highly reliable prediction of its level of partial resistance.

Pectolytic activity in stems of infected carnations appeared due to a complex of fungal enzymes with PG activity as well as a single fungal pectin methyl esterase. Highly methyl-esterified pectin is considerably less susceptible to PG than pectin with a low degree of esterification, particularly so for exolytic PGs (Pérez Artés and Tena, 1990). Exolytic as well as endolytic PGs have been reported for *F. oxysporum* (Pérez Artés and Tena, 1990; Strand et al., 1976). In both cases the action of PME prepares the way for fungal PGs (Langcake et al., 1973). Whether the fungal PGs reported here have an endolytic or exolytic mode of action remains to be investigated.

Two major bands of PG activity were encountered in stem extracts, one with estimated pI values of about 7.0 and another with a pI above 9.5. Both were accompanied on PAGE gels by weaker bands, assumedly representing enzymes with slightly lower pI values. Within each group, the occurrence of major and minor bands is probably due to differential glycosylation of one and the same protein as shown for an endo-PG from the closely related fungus *F. moniliforme* by Caprari et al. (1993). In the present system, at least two structurally (and maybe also functionally) different PGs would thus seem to be involved.

PG enzymes with pI values around 7.0 were produced both *in planta* and *in vitro*. Enzymes with high pI values were produced in considerable amounts *in planta* but only in small amounts on pectin medium. Four PGs with pIs from 7.0 to 5.3 were detected in pectin medium cultures of five formae speciales (including

f.sp. *dianthi*) of *F. oxysporum* by Scala et al. (1981). In our hands, the pI values of the enzymes produced on pectin medium were identical or in the same range. Given the large variation in PG profile (pI, molecular weight, mode of activity) among and even within formae speciales of *F. oxysporum* (Pérez Artés and Tena, 1990; Strand et al., 1976; Suresh et al., 1984) and the different methods for PG detection used by the different authors, a comparison of the enzymes encountered in this study with those reported from other formae speciales cannot be made until the presently described enzymes have been further characterized. Furthermore, most of these studies were performed *in vitro* and enzymes with high pI produced *in planta* as presently reported may well have been overlooked.

Extracts from stems of inoculated plants as well as water-treated ones contained PME activity. Various enzymes with PME activity were detected in extracts from inoculated plants, of which only a single one assumedly was of fungal origin. In tomato plants infected with *F. oxysporum* f.sp. *lycopersici* fungal PME accounts for 5 to 10% of total PME activity (Langcake et al., 1973). In the interaction of tomato with *F. oxysporum* f.sp. *radicis-lycopersici*, only PMEs of host origin are found (Chamberland et al., 1990). As mentioned above, pectin methyl esterase may play an important preparatory role in hydrolytic depolymerization of the pectin chain. The postulated role of the present fungal PME in the hydrolysis of pectin is corroborated by the observation that demethylation of pectin occurs in infected carnations (Niemann et al., 1990) and is more severe in susceptible than in resistant cultivars. Given the dominance of host PMEs in carnation stems, however, evaluation of partial resistance to Fusarium wilt by assessing the activity of fungal PME in infected plants will require the use of specific antisera.

The correlation between disease development and PG activity in wilt disease-affected carnations has been substantiated in further experiments, one of them involving 25 different cultivars (unpublished results). Similar results have been obtained for dry rot of potatoes infected with *F. solani* var. *coeruleum* (Olsson, 1989) and cotton balls infected by *Aspergillus flavus* (Cleveland and Cotty, 1991). In all three models PG activity *in planta* is correlated to disease severity in the host, suggestive of a causal relationship. Development of wilt symptoms in inoculated carnations was accompanied by a quadratic increase in PG activity. This coincides with (roughly) quadratic increase in the amount of mycelium present in the stem (Baayen and Elgersma, 1985). As previously suggested for PME and pectin lyase in tomato plants infected with *F. oxysporum* f.sp. *lycopersici* (Langcake and Drysdale, 1985) or *Verticillium albo-atrum* (Cooper and Wood, 1980), PG activity in inoculated carnations is likely to be related to the amount of actively growing fungal mycelium present in the stem. Although the fungal pectin-degrading enzymes clearly contribute to degradation of the xylem of infected plants, PG activity in itself need not be causal, however, to the development of disease symptoms. Regardless of their role in pathogenesis, fungal PGs anyhow provide a reliable (reproducible in time) and rapid (4 weeks) biochemical parameter for monitoring fungal growth and quantifying partial resistance of carnation cultivars to Fusarium wilt.

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