Pergamon

PII: S0031-9422(96)00766-2

Phytochemistry, Vol. 44, No. 8, pp. 1435–1441, 1997

0031-9422/97 $17.00+0.00

CHARGE DENSITY IN STEM CELL WALLS OF *SOLANUM TUBEROSUM* GENOTYPES AND SUSCEPTIBILITY TO BLACKLEG

PASCALE MARTY,*† BERNARD JOUAN,* YVES BERTHEAU,‡ BRIGITTE VIAN‡ and RENEE GOLDBERG†§

*Laboratoire de Pathologie Végétale, INRA, Domaine de la Motte-au-Vicomte, BP 29, 35650 Le Rheu, France;
†Laboratoire d'Enzymologie en Milieu structuré, Institut Jacques Monod, 2 Place Jussieu, 75251 Paris Cedex 05, France;
‡Laboratoire de Pathologie Végétale, INRA INA P-G, 16 rue Claude Bernard, 75231 Paris Cedex 05, France

(*Received in revised form* 30 *September* 1996)

Key Word Index—*Solanum tuberosum*; Solanaceae; *Erwinia carotovora*; potato; blackleg; cell walls; pectin; pectin methylesterase.

Abstract—Several genotypes belonging to two different *Solanum tuberosum* subspecies were inoculated with *Erwinia carotovora* subsp. *atroseptica* and their susceptibility to subsequent blackleg disease was monitored. The stem cell walls of the resistant genotype were characterized by a higher content of highly methylated and branched water-soluble pectins, although these cell walls were able to de-esterify very rapidly *Citrus* pectin. Numerous pectin methylesterase isoforms were extracted from all cell walls investigated but an acidic isoform, present in the cell walls of the susceptible genotypes, could not be detected in the resistant cell walls. This isoform might therefore be involved in the *in muro* demethylation of the pectins which controls the charge density of the apoplasm. Differences between subspecies were also observed. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Soft rot disease caused by pectinolytic erwinias severely hinder potato production worldwide. *Erwinia carotovora* subsp. *atroseptica* is the most common among the *Erwinia* species isolated from rotting tubers in European countries [1]. These bacteria are able to macerate tubers and stems by producing enzymes, such as pectate lyases (PL), pectin lyase (PNL) and pectin methylesterases (PME), that depolymerize pectin, a major plant cell wall component [2].

Differences in susceptibility are known among potato cultivars. Recent studies investigating resistance mechanisms reported correlations between the resistance of potato tubers and the degree of methylation of their pectins [3, 4]. This parameter can operate at different levels. It is well known that PLs and polygalacturonases can hydrolyse only demethylated or slightly (up to 25%) methylated polygalacturonic acid [5, 6]. Moreover, the degree of methyl esterification is involved in the control of the cell wall charge density [7, 8] and, thus, in the control of apoplasmic pH. Finally, the ionic interactions between cell wall polymers depend also on the number and on the distribution of the methyl groups along the polygalacturonic backbone [9].

In contrast to tuber studies, little information is available on the stem behaviour of potatoes when infected by *E. c. atroseptica*. We attempted, therefore, to assess stem susceptibility to this bacterium of five genotypes from two subspecies of *Solanum tuberosum*, namely *tuberosum* and *andigena*. The latter subspecies was chosen because it can be crossed with common potato cultivars and presents some genotypes resistant to soft rot [10]. A cultivar of *S. t. tuberosum* (Bintje), and four genotypes of *S. t. andigena* (ADG) were selected for this study. Among the *andigena* genotypes, two were tuber-resistant (ADG 88S-262-7 and ADG 88S-412-10) and the two others, tuber-susceptible (ADG 88S-249-3 and ADG 88S-249-4) [10].

The present paper reports some characteristics of pectins extracted from the stem cell walls of *S. tuberosum* genotypes exhibiting different susceptibility to blackleg. We have also investigated cell wall PMEs which might be considered as markers of susceptibility to attack by pectinolytic erwinias, since these activities are supposed to control *in muro* the degree of methylation of pectin and, in turn, the susceptibility of the plant cell walls to pectinolytic enzymes of pathogenic organisms.

RESULTS AND DISCUSSION

Stem susceptibility tests

Reaction of the five genotypes to infection by *E. c. atroseptica* is illustrated in Fig. 1. In all cases, control

§Author to whom correspondence should be addressed.

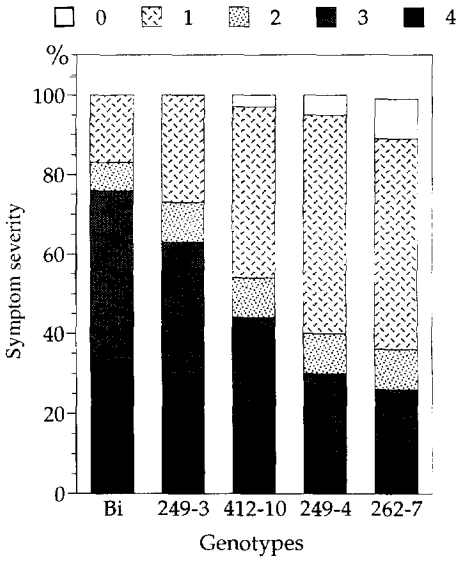

Fig. 1. Distribution of plants in each genotype according to symptom severity noted from 0 to 4 (see Experimental). The number of plants presenting symptoms of a given score is expressed as a percentage of total number of plants from each genotype. Bi, cv. Bintje; 249-3, ADG 88S-262-7; 412-10, ADG 88S-412-10; 249-4, ADG 88S-249-4; 262-7, ADG 88S-262-7.

plants did not exhibit any symptoms. All inoculated genotypes developed disease symptoms, which confirms previous observations [11] reporting the absence of truly resistant genotypes to *Erwinia* among *andigena* or *tuberosum* subspecies. However, obvious differences were observed in the extent of stem rot. More than 60% of inoculated plants from cv. Bintje and the ADG 88S-249-3 genotype were severely affected and developed symptoms reaching levels 3 and 4 (see Experimental). In contrast, necroses did not exceed 1 cm length in *ca* 60% of inoculated plants from *andigena* genotypes 88S-262-7 and 88S-249-4. Therefore, cv. Bintje and ADG 88S-249-3 were defined as susceptible (S) to blackleg, while ADG 88S-262-7 and ADG 88S-249-4, which were reported to be tuber-susceptible [10], were characterized as resistant (R). ADG 88S-412-10, tuber-resistant [10], was intermediate. No correlation was apparent between stem and tuber susceptibilities. The more resistant (ADG 88S-262-7) and the more susceptible (ADG 88S-249-3) *andigena* genotypes and the *tuberosum* genotype (cv. Bintje) were investigated further.

Characterization of pectins extracted from stem cell walls

Cell walls were isolated and their pectins extracted sequentially with boiling water and hot EDTA. The first treatment is known to solubilize highly esterified and branched pectins and, the second one, acidic galacturonan [12, 13]. The susceptible genotypes contained much more acidic galacturonan than the resistant one (Table 1). Moreover, the balance between the neutral sugars and the uronic acids was obviously lower in cell walls isolated from the susceptible genotypes. In these cell walls, the water-soluble pectins represented only 30% of the total pectins. Because of their relatively high content of low methylated pectins, the cell walls of the susceptible genotype could thus be more easily attacked by pectinolytic enzymes, which cannot act on highly methylated galacturonan [14].

The nature of the neutral sugars associated with the water-soluble pectins was investigated. The water-soluble fractions were loaded on a DEAE Sepharose CL 6B column; the highly methylated and branched pectins that did not bind, were eluted with 0.05 M acetate buffer, pH 4.7. These pectic fractions were then hydrolysed. In all cases, the hydrolysis was incomplete, with *ca* 25% of the constitutive monomers being released from pectins of the resistant genotype ADG 88S-262-7 and 47% and 66% from the pectins of ADG 88S-249-3 and Bintje, respectively. The pectins from the resistant genotype were thus less easily hydrolysed than the pectins from the susceptible ones. After hydrolysis, the solubilized sugars were analysed by HPLC (Fig. 2). The sugar composition depended mainly on the subspecies. Galactose was the prevalent sugar in the pectins from both *andigena* genotypes, whereas arabinose represented *ca* 50% of the sugars solubilized from Bintje pectins. In contrast, rhamnose appeared to be related to the resistance because it was detected only in the pectins of the resistant genotype.

Properties of cell wall pectin methylesterases

Cell wall fragments isolated from the three selected genotypes de-esterified *Citrus* pectin very rapidly in the presence of 150 mM NaCl. The bound PMEs could be solubilized with 1 M NaCl. The properties of bound and solubilized cell wall PME activities were investigated in parallel. Figure 3 shows the effects of pH on the reaction rate. Regardless of the pH, the solubilized activities were obviously lower than the bound ones and their pH plots were shifted towards neutral pHs; bound activities exhibited an optimal pH at *ca* pH 8 and solubilized activities at *ca* pH 7. Differences between bound and solubilized enzyme activities have already been reported [15, 17]. Moreover, in all cases, the resistant genotype exhibited higher PME activities than the two susceptible ones. The bell shapes of all pH plots suggest that PME molecules (E) can occur in three ionic states (EH_2, EH and E), with only one of them (EH) being involved in the catalysis. Moreover, for all the investigated fractions, the difference between the apparent pK_a and pK_b values (defined as pH corresponding to the apparent $V_m/2$) was lower than 3.5 pH units, which allowed us to fit the experimental data to the following equation [18]:

$$v_{exp} = v_{theo} \Big/ \left(1 + \frac{10^{-pH}}{10^{-pK_a}} + \frac{10^{-pK_b}}{10^{-pH}}\right).$$

Table 1. Analysis of pectins from stem cell walls. Pectins were sequentially extracted with boiling water and hot EDTA. Uronic acids (UA) were estimated using polygalacturonic acid as a standard, neutral sugars (NS) were estimated using galactose as a standard. Amounts of UA and NS are expressed as μmol mg^{-1} cell walls. DE, degrees of esterification. UA_{ws}, water soluble uronic acids

	'Bintje'	ADG 88S-249-3	ADG 88S-262-7
Water-soluble fraction (WSF)			
UA	0.47 (±0.013)	0.36 (±0.015)	0.45 (±0.003)
DE (%)	56	47	62
NS	0.22 (ε)	0.18 (±0.004)	0.24 (±0.002)
EDTA-soluble fraction			
UA	0.76 (±0.038)	0.79 (±0.012)	0.56 (±0.008)
DE (%)	17.0	10	7.6
NS	ε	ε	0.04 (±0.001)
NS/UA_t	0.15	0.15	0.27
UA_{ws}/UA_t (%)	33	30	43

Results are means (±standard deviation) of triplicates.

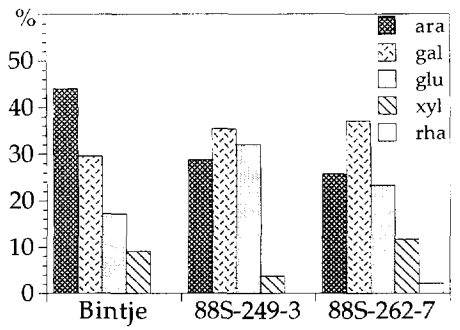

Fig. 2. Sugar composition of methylated pectin fractions. The different neutral sugars released by acidic hydrolysis are expressed as a percentage of the total sugars; arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl) and rhamnose (Rha).

The best fits for ionization constants, pK_a and pK_b, and the theoretical velocity of the reaction (v_{theo}) are described in Table 2. All theoretical values were higher than the experimental ones but the highest theoretical activities were observed with the resistant genotype as were the experimental ones. In addition, it was noted that the bound PMEs of the resistant genotype exhibited a reduced pH range of activity (between pH 7.3 and 9) when compared with the two other bound activities.

PME activities were then assayed in the presence of increasing NaCl and $MgCl_2$ concentrations (Fig. 4). In all cases, regardless of the concentration or the nature of the cation, the resistant genotype exhibited a higher activity than the two susceptible genotypes. As reported for other plant PME activities [19], magnesium ions were more effective than sodium ions. Solubilization also reduced the sensitivity to cations.

The isoform patterns of the PME solubilized from the ionic cell wall extracts were assessed by isoelectric focusing (IEF) (Fig. 5(A)). After detection of PME activities, IEF gels were scanned and analysed lane by lane (Fig. 5(B)). Five groups, A–E were separated, their apparent pI being *ca* pH 5.5, 6.5, 7.5, 8.3 and 9, respectively. The last two groups represented more

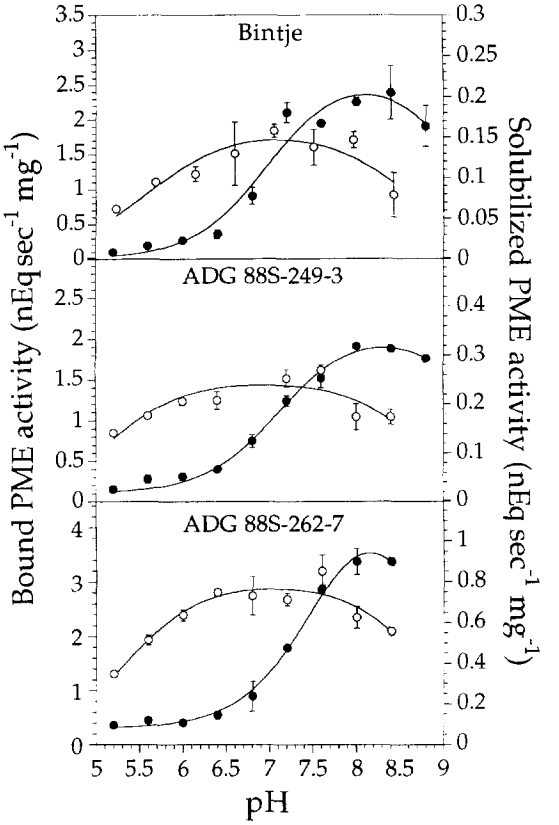

Fig. 3. pH plots of bound (●—●) and solubilized (○—○) PME activities of cell walls isolated from the three *Solanum tuberosum* genotypes. Reaction rates were measured in a pH-stat at various pH values, ranging from 5.2 and 8.4, in the presence of 0.15 M NaCl and under N_2 flux. Activities as nEq H^- sec^{-1} mg^{-1} cell walls. Experimental data are means (±standard deviation) of triplicates.

than 50% of the total PME activity. Differences could be observed between (1) resistant and susceptible genotypes and (2) in the two subspecies. Both susceptible genotypes contained two isoforms in the most acidic group (A), whereas only one isoform could be detected in this group for the resistant genotype.

Table 2. Effect of pH on bound and solubilized PME activities. V theoretical ($v_{theo.}$) and pK values were obtained from the best fits of experimental data (see Fig. 3). V, initial reaction rate, as nEq sec^{-1} mg^{-1} cell walls

	'Bintje' (S)		ADG 88S-249-3 (S)		ADG 88S-262-7 (R)	
	Bound	Solubilized	Bound	Solubilized	Bound	Solubilized
Optimal pH	8.2	6.8	8.4	7	8.2	7
V at optimal pH						
$v_{exp.}$	2.35 (±0.22)	0.14 (±0.01)	1.85 (±0.01)	0.27 (±0.01)	3.35 (±0.14)	0.85 (±0.07)
$v_{theo.}$	2.71 (±0.18)	0.16 (±0.01)	2.04 (±0.08)	0.24 (±0.01)	4.39 (±0.42)	0.79 (±0.02)
pK_a	6.98 (±0.09)	5.51 (±0.12)	7.00 (±0.06)	5.15 (±0.10)	7.32 (±0.09)	5.32 (±0.07)
pK_b	9.22 (±0.19)	8.15 (±0.16)	9.74 (±0.37)	8.72 (±0.14)	9.06 (±0.27)	8.76 (±0.12)

Data are means (± standard deviation) of triplicates.

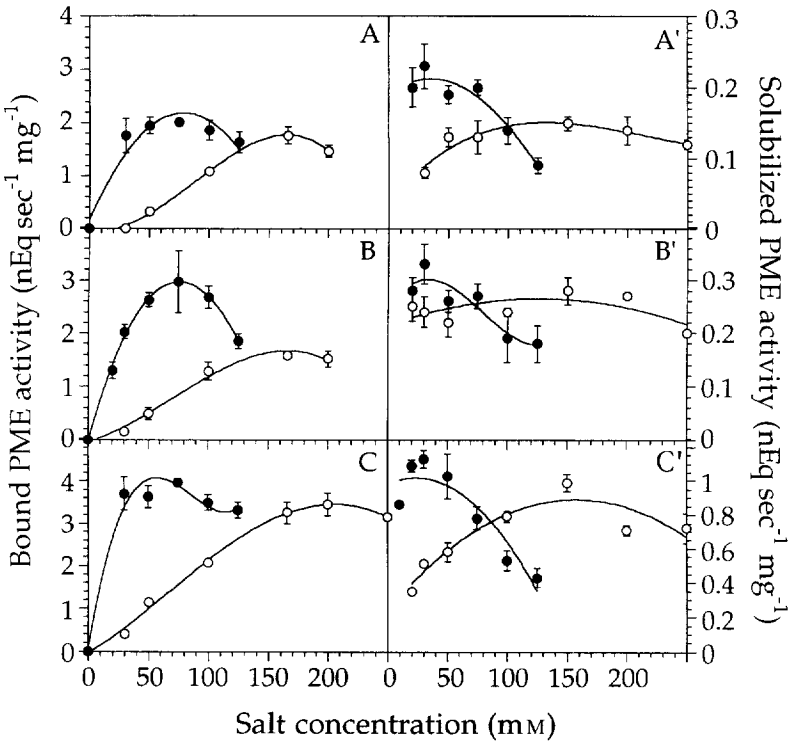

Fig. 4. Effect of increasing NaCl (○—○) and $MgCl_2$ (●—●) concentrations on the de-esterification rate of *Citrus* pectin by bound PME (A, B and C) and solubilized PME (A′, B′ and C′). PME isolated from cv. Bintje (A, A′), ADG 88S-249-3 (B, B′) and ADG 88S-262-7 (C, C′). All measurements were performed at pH 7.6. Experimental data are means (±standard deviation) of triplicates.

Moreover, in group B, the second isoform predominated in the susceptible genotype extract but not in the resistant one. In contrast, both *andigena* genotypes exhibited four isoforms in group C, whereas only two isoforms were present in the cell wall extract from the subspecies *tuberosum*.

CONCLUSIONS

The data suggest a relationship between stem susceptibility and the properties of the pectic fractions. Thus, the proportion of water-soluble pectins in the cell walls is higher in the resistant genotype than in the susceptible ones. Moreover, the degree of esterification of these water-soluble pectins is greater in the resistant genotype. In this case, the amount of neutral sugars associated with the pectins is also higher. The pectins of the resistant genotype might be less easily degraded by *Erwinia* pectinolytic enzymes, which cannot act on branched or highly methylated galacturonans. Conversely, degradation products are known to elicit defence responses. However, the degree of pectin methylation is only one among other factors that control the resistance of a given genotype; accumulation of hydroxyproline rich glycoprotein, production of phytoalexins, lectins and proteinase inhibitors must also be taken into account [11].

Despite the presence of a large amount of methyl-

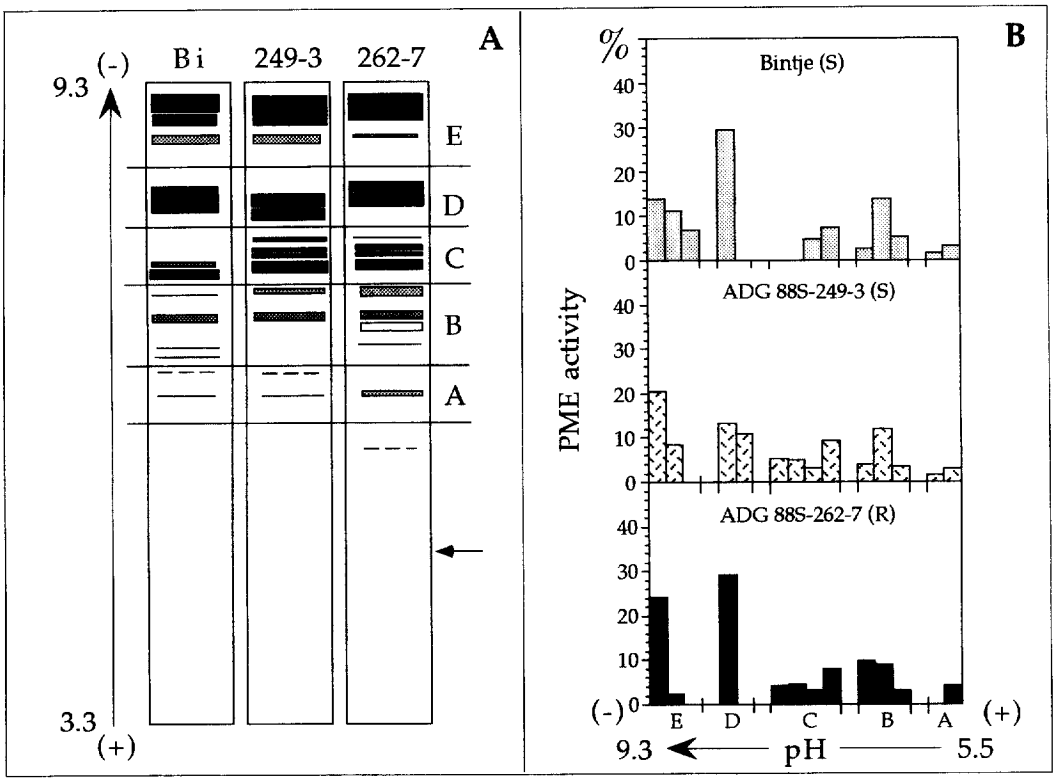

Fig. 5. Distribution of PME activities along the pH gradient after isoelectric focusing (IEF). A, PME activities were detected by the sandwich technique [19]. B, PME activity of each isoform was estimated after scanning and analysis of the gel and expressed as a percentage of the crude extract activity. Arrows represent sample application.

ated galacturonic units, the cell walls of the resistant genotype were still the most effective *in vitro* for the de-esterification of *Citrus* pectin, whatever the concentration or the nature of the cations added in the assays. Taking into account the number of isoforms, it may be suggested that only some of them might function *in vitro*, as already reported for PME from mung bean hypocotyl cell walls [15]. Thus, the acidic isoform (group A), which is missing in the resistant genotype, might represent a marker for susceptibility; if this isoform is especially active *in muro*, the degree of esterification will depend on its presence inside the cell walls. The low amount of methylated galacturonic units in the cell walls of the susceptible genotypes might then result from the activity of this isoform in cell walls. Further work, will be required in order to verify this hypothesis. *In vitro*, the degree of pectin esterification will also depend on several other factors. Thus, the question of the respective localization of the different PME and their substrate must be taken into account. Microdomains differing in their charge density have been detected in the apoplasm [20, 12]; therefore the occurrence of microdomains differing in their isoform composition might also be postulated.

Our results also showed differences between the two investigated subspecies, *tuberosum* and *andigena*, although further investigations with more genotypes are required to confirm these preliminary data. However, in potato stems, the higher amount of methylated and branched pectins, and the isoform pattern of the PME activity, might be considered as potential markers for resistance to blackleg diseases. The availability of plants overexpressing the acidic isoform or with a deleted gene, could address this question more precisely.

EXPERIMENTAL

Plant material. Two subspecies of *S. tuberosum* were investigated. The first belongs to subsp. ssp. *tuberosum* (cv. Bintje), the second to subsp. ssp. *andigena* (ADG). The latter are clones of the INRA collection of the Potato Breeding Station (Kéraïber, 29250 Ploudaniel, France). The genotype ADG 88S-262-7 was obtained from Drs F. Rousselle-Bourgeois and P. Rouselle from the P.I. 281133 accession, the genotypes ADG 88S-249-3 and ADG 88S-249-4 from the PI 280970 accession (Inter-Regional Potato Collection, Sturgeon Bay, WI, U.S.A.) and the genotypes ADG 88S-412-10 from the 007785 accession (German–Dutch Collection, Braunschweig, Germany).

Stem susceptibility. Plants were grown in a greenhouse (23° day, 15° night; photoperiod 16–8 hr), on a sterilized mixt. of soil and sand (2:1). Susceptibility to stem rot was estimated as previously described [21]. Stems of 5-week-old plants (30) were inoculated with strain 86.20 of *E. carotovora* ssp. *atroseptica*, chosen for its high pathogenicity [22]. A toothpick carrying

an undiluted smear of $1-3 \times 10^8$ bacteria cultivated for 24 hr on King B medium or no bacteria for control plants was inserted in the stem (5 mm deep) at the level of the third expanded leaf. After inoculation, rel. humidity was kept above 80% to allow bacterial infection. After 3 weeks, stems were cut longitudinally and the development of the disease was noted as follows: 0, no reaction; 1, brown dry necrosis < 1 cm at the inoculation site, no wilting; 2, brown dry necrosis extending $\geqslant$ 1cm from the inoculation site, no wilting; 3, dark brown necrosis and slimy rot extending for several cm from the inoculation site, wilting and/or chlorosis of the leaves; 4, disease spread through the entire stem, plant collapsed or dead.

Preparation of isolated cell walls. Potato stems were collected from 5-week-old plants grown as described above in the INRA Breeding Station and stored at $-20°$ until further treatment. Stems were ground in liquid N_2 using a pestle and a mortar. The powder was then transferred in 2 mM Na-K Pi buffer (pH 6). The suspension was further ground in Pi buffer using a Polytron homogenizer (2×15 min), then filtered through a cheesecloth (pore size 5 μm). The cell wall residue was stirred with 0.1% Triton X-100 in order to discard membraneous contaminants. The cell wall residue was then thoroughly washed with 6 l of dist. H_2O. All steps were carried out at 4°.

Extraction of pectin fractions. Two pectic frs were sequentially extracted from cell walls, the first with boiling H_2O pH 5.5 (3×1 hr), the second in 1% EDTA (6 hr at 60°). The pectins extracted with EDTA were dialysed against dist. H_2O.

Analyses of pectic fractions. Uronic acids were estimated using the *m*-hydroxydiphenyl method [23] using polygalacturonic acid as standard. Total carbohydrates were detected with anthrone [24]; neutral sugars, defined as the difference between the total amount of carbohydrates and the amount of uronic acids, were estimated using galactose as standard.

Potentiometric measurements. The pectic frs were converted into their H-form by H-exchange chromatography (Amberlite IR 120H) and neutralized at 20° by progressive addition of 10 mM NaOH, under continuous N_2 flux. Measurement of the degree of esterification of the pectic fractions was performed by titration of their free carboxylic groups with 10 mM NaOH before and after chemical de-esterification, as previously described [25].

Carbohydrate composition. Water-soluble pectins from each genotype were chromatographed on a DEAE Sepharose CL 6B equilibrated with HOAC 0.05 M NaOAc buffer, pH 4.7. Highly esterified galacturonans did not bind and were recovered in the void vol. Unesterified galacturonans were then eluted with 1 M NaCl in 0.05 M acetate buffer. Esterified galacturonans were hydrolysed for 2 hr in 2 N H_2SO_4 at 100°. After neutralization with $SrCO_3$, the monomers released were sepd and estimated by HPLC [26] using an NH_2 column. Monomers were eluted with an MeCN–H_2O (4:1) and detected with a RI detector.

Extraction of ionically bound cell wall enzymes. Ionically bound proteins were extracted with 1 M NaCl [15]. The extract was dialysed against H_2O and reduced to a small vol. (*ca* 5 ml) by ultrafiltration at 4° in a stirred Amicon cell with an Amicon PM10 membrane.

Enzymic assay. PME activity was measured titrimetrically by following the increase in free carboxyl groups. The carboxyl groups liberated by PME from *Citrus* pectin (Sigma) in the presence of NaCl were titrated with 10 mM NaOH under N_2 flow, the pH being maintained at chosen values ranging from 5.2 and 8.8 with an automatic titrator. All measurements were performed at 22° and assays contained 15 mg *Citrus* pectin in 6 ml (total vol.).

Electrophoresis. Isoelectrofocusing was performed on ultrathin (0.5 mm) polyacrylamide slab gels (10% acrylamide, 0.26% bis-acrylamide) containing 10% Pharmacia 3–10 pH range ampholines. The IEF was run on a LKB Multiphor at 30 W for 1 hr (ending voltage 2000 V). NaOH (0.01 M) and H_2SO_4 (0.1 M) were used as catholyte and anolyte, respectively. After focusing, PME activity was revealed by the agar–pectin sandwich technique [27] using ruthenium red (0.02%).

Acknowledgements—The authors are indebted to P. Rousselle and F. Rousselle-Bourgeois for providing potato genotypes and to J. P. Dantec for his important contribution to the production of plant material. The authors also thank C. Pasco and J. M. Lucas for their valuable help in susceptibility tests. This work was supported by a grant from INRA-Région Bretagne.

REFERENCES

1. Pérombelon, M. C. M., Proceedings of the 7th International Plant Path Bact, Budapest, 1989, **74**, 745.
2. Kotoujansky, A., *Annual Review of Phytopathology*, 1987, **25**, 405.
3. Weber, J., *Phytopathologische Zeitschrift*, 1983, **108**, 135.
4. MacMillan, G. P., Hedley, D., Fyffe, L. and Pérombelon, M. C. M., *Physiology and Molecular Plant Pathology*, 1993, **42**, 279.
5. Rexova-Benkova, L. and Markovic, O., *Advances in Carbohydrate Chemistry*, 1976, **33**, 323.
6. Favey, S., Bourson, C., Bertheau, Y., Kotoujansky, A. and Boccara, M., *Journal of General Microbiology*, 1992, **138**, 499.
7. Moustacas, A. M., Nari, J., Diamantis, G., Noat, G., Crasnier, M., Borel. M. and Ricard J., *European Journal of Biochemistry*, 1986, **155**, 191.
8. Nari, J., Noat, G., Diamantis, G., Woudstra, M. and Ricard, J., *European Journal of Biochemistry*, 1986, **155**, 199.
9. Jarvis, M. C., *Plant Cell and Environment*, 1984, 7, 153.

10. Rousselle-Bourgeois, F. and Priou, S., *Potato Research*, 1995, **38**, 111.
11. Lyon, G., *Plant Pathology*, 1989, **38**, 313.
12. Marty, P., Goldberg, R., Liberman, M., Vian, B., Bertheau, Y. and Jouan, B., *Plant Physiology and Biochemistry*, 1995, **33**, 409.
13. Goldberg, R., Morvan, C., Hervé du Penhoat, C. and Michon V., *Plant Cell Physiology*, 1989, **30**, 163.
14. de Vries J. A., Voragen A. G. J., Rombouts F. M. and Pilnik W., *Carbohydrate Polymers*, 1981, **1**, 117.
15. Bordenave, M. and Goldberg, R., *Plant Physiology*, 1994, **106**, 1151.
16. Borrego, F., Tari, M., Manjon, A. and Iborra, J. L., *Applied Biochem Biotech.*, 1989, **22**, 129.
17. Ittah, Y., *Journal of Agriculture and Food Chemistry*, 1992, **40**, 953.
18. Segel, I. H., in *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems.* J. Wiley & Sons, Inc, New York, 1975, p. 884.
19. Goldberg, R., Pierron, M., Durand, L. and Mutaftschiev, S., *Journal of Experimental Botany*, 1992, **43**, 41.
20. Roberts, K., *Current Opinions in Cell Biology*, 1990, **2**, 920.
21. Rabot, B., Pasco, C. and Schmidt, J., *Potato Research*, 1994, **37**, 197.
22. Priou S., PhD Thesis, Ecole Nationale Supérieure d'Agronomie de Rennes, 1992.
23. Blumenkranz, N. and Asboe-Hansen, G., *Analytical Biochemistry*, 1973, **54**, 484.
24. Dreywood, R., *Ind. Eng. Anal. Edit.*, 1967, **18**, 499.
25. Goldberg, R., Morvan, C., Hervé du Penhoat, C. and Michon, V., *Plant Cell Physiology*, 1989, **30**, 163.
26. Baïer, M., Goldberg, R., Catesson, A.-M., Liberman, M., Bouchemal, N., Michon, V. and Hervé du Penhoat, C., *Planta*, 1994, **193**, 446.
27. Bertheau, Y., Madgidi-Hervan, E., Kotoujansky, A., Nguyen The, C. and Coleno, A., *Analytical Biochemistry*, 1984, **139**, 383.