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The susceptibility of tissue cell walls to *Erwinia* enzymes differs among the potato cultivars

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Summary

In the cell wall matrix of higher plants, cellulose fibrils are embedded in a network of pectic substances, hemicelluloses and structural proteins. But plant pathogenic *Erwinia carotovora* bacteria secrete a mixture of enzymes including pectinases, cellulases and proteases to degrade the cell walls of host plant tissue. Tuber tissue of 31 potato genotypes was exposed to a mixture of *Erwinia* enzymes before the degree of cell wall lysis was determined by a Neutral-red vital staining method. The data indicate that the resistance of cell walls to the action of *Erwinia* enzymes differed significantly among the potato cultivars and clones. The decrease in cell viability caused by the action of enzymes ranged from 7% for cv. Maxilla to >46% for cv. Arnika. An increased rate of N-fertilization coincided with enhanced susceptibility of cell walls to the action of *Erwinia* enzymes. There was a correlation between the amount of cell wall substances in tuber tissue and the resistance of cell walls to enzymic degradation.

Introduction

In the cell wall matrix of higher plants, cellulose fibrils are embedded in a network of pectic polysaccharides, hemicelluloses and structural proteins (Varner & Lin, 1989; Carpita & Gibeaut, 1993). Adjacent primary walls are separated by middle lamellae containing predominantly α-1,4 linked galacturonic acid polymers, either as methylesterified pectins or Ca^{2+}- and Mg^{2+}-pectate salts. Potato cell walls amounting to 1–2% of the tuber tissue fresh mass (Weber, 1976) consist of 40–55% pectic substances (Hoff & Castro, 1969; Weber, 1976). In accordance with the composition of cell walls, the major enzymic activities secreted by *Erwinia carotovora* bacteria causing soft-rot diseases of plants are pectolytic enzymes, predominantly pectate lyases (PL) (Rombouts & Pilnik, 1980; Kotoujansky et al., 1985; Reverchon et al., 1989). The PLs cleave the α-1,4 galacturonide linkage in pectic polysaccharides by ß-elimination. In synergism with additional enzymes secreted by the bacteria, such as cellulases and proteases, the cell wall network of plant tissue is loosened and finally lysed, coincident with the appearance of typical soft-rot symptoms. The destruction of cell walls as 'protecting barriers' in plant tissue is of major importance in the spread of *Erwinia* bacteria invading host plants. This implies that use of potato genotypes showing an increased resistance of their cell walls to the action of *Erwinia* patho-enzymes may reduce the danger of a progressive bacterial soft-rot. Therefore, efficient biochemical methods are needed to characterize the cell wall network in its

complexity comprising the interactions of the wall components and structural variations. For this the application of *Erwinia* enzyme mixtures that cause a measurable lysis of tissue cell walls seems to be more advantageous than extensive chemical analyses of the cell wall composition.

The investigations presented here focus on the susceptibility or resistance of cell walls in potato tuber tissue to *Erwinia* enzymes. Tuber tissue of 14 potato cultivars and 17 different clones from a special breeding programme were exposed to an enzyme mixture of *E. carotovora* subsp. *atroseptica* before the degree of cell wall lysis was determined using the Neutral-red vital staining method (Weber & Wegener, 1986). Furthermore, the effect of nitrogen-fertilization at 40, 80 and 120 kg N/ha on the susceptibility of cell walls to *Erwinia* enzymes was also analysed. For six selected genotypes the correlation between the amount of cell wall substances in tuber tissue and the degree of enzymic cell wall lysis was investigated.

Materials and methods

Potatoes. Seed potatoes of 14 cultivars were obtained from breeders and the 17 clones were from the Institute of Breeding Research on Cultivated Plants, Groß Lüsewitz. Experiments were planted in April 1995 at within-row spacing of 30 cm with 75 cm between rows. Three rates of nitrogen were compared (N1=40; N2=80 and N3=120 kg N/ha) in a split-plot design with two replications, including 40 plants per genotype. Half the N-fertilizer was applied shortly before planting and the remainder soon after planting. Herbicides and fungicides were used according to local practice. Potato tubers used for analyses were stored in controlled environments at 5 °C. The preliminary field-tests were carried out in 1993 using the conditions as detailed before.

Assay of enzymic cell lysis. To determine the decrease in cell viability in tissue caused by the cell wall degrading effect of *Erwinia* enzymes, 20 tissue disks, each of 10 mm diameter and 2 mm thick, were excised from the medulla of potato tubers. The intercellular spaces of tissue disks were infiltrated with a solution of *Erwinia*-derived enzymes containing 0.2 U ml^{-1} of PL activity by applying a vacuum of 200 mbar for 5 min. Following incubation for 30 min at 20 °C the disks were shaken in 20 ml of water for 1 h to generate osmotic stress. Subsequently they were stained for 90 min in a solution of 50 mg ml^{-1} Neutral-red dissolved in 0.2 M sodium-phospate buffer at pH 7.5 containing 0.8 M KNO_3. Finally the disks were rinsed with water, absorbed dye extracted twice for 10 min in 10 ml of 96% ethanol and the volume adjusted to 50 ml with 0.01 M H_2SO4 before OD_{535} was determined (Weber & Wegener, 1986). Control samples were incubated with the thermally inactivated enzyme. Analyses were in triplicate using 20 tubers per trial. Standard deviation (SD) of the test was determined and amounted ≤2%.

Assay of electrolyte leakage. Tissue cylinders, 3 cm diameter and 3 cm long, excised from tubers were infiltrated with an enzyme solution containing 2 U ml^{-1} of PL

activity and incubated for 30 min at 20 °C. To determine the effect of enzymes in the inner region, the core of the cylinder, 1 cm diameter, was excised and cut into 2 mm thick disks. 20 of these disks were shaken in 20 ml distilled water for 60 min before 1 ml of the extract was diluted 30-fold with distilled water and subjected to conductivity measurements using a HI 8788 (Hanna Instruments) apparatus. Control samples were prepared from cylinders incubated with the inactivated enzyme. An increase in conductivity indicates a leakage of electrolytes due to cell rupture. Analyses were in triplicate with SD≤2.5% using 10 tubers per trial. The enzymic cell lysis was determined in the same tissue by vital staining as detailed before after shaking the disks for 1 h in distilled water.

Production of enzymes. E. carotovora subsp. *atroseptica* bacteria were grown in a culture medium containing minerals (Wegener et al., 1989), 0.2% cell sap and 0.02% dried pulp from potatoes (Henniger et al., 1989) in a 10 l Bioreactor LFS 212 (Mytron, Heiligenstadt) at 25 °C for 24 h. Following ultrafiltration of the culture filtrate, the enzymprotein was precipitated by 80% ethanol and lyophilized. One mg of the preparation contained 7.0 U of PL, 2.9 U of endo-1,4-ß-glucanase as well as traces of polygalacturonase and protease activities. Enzyme activities were assayed as detailed by Weber & Wegener (1986).

Assay of the rough cell wall content. 200 g of tissue slices excised from 20 potato tubers were macerated using a Moulinex liquidizer. The cell sap containing starch was removed and the residue resuspended in 200 ml of distilled water, homogenized using an Ultra-Turrax T25 apparatus and filtered through a starch sieve of 90 µm pore size. Subsequently, the residue was washed again with 800 ml of distilled water and the remaining rough cell wall was lyophilized. The rough cell wall preparation of six selected genotypes was resuspended in 80 ml of distilled water and treated in a high pressure homogenizer (Micron Lab 40, APV Gaulin GmbH, Lübeck) at 500 bar. The suspension was filtered through a starch sieve of 70 µm pore size. The remaining cell wall fraction was washed with 500 ml of distilled water and lyophilized. Subsequently, 500 mg of the cell wall preparation was resuspended in 20 ml 0.1 M-acetate buffer at pH 4.75 and incubated for 12 h at 45 °C with α-amylase (5 U) and amyloglucosidase (2 U) enzymes (Boehringer, Mannheim). After incubation, the reaction mixture was adjusted with ethanol (96%) to a concentration of 80%, boiled and centrifuged. The liberated glucose was analysed in the supernatant (Pape et al., 1969) before amounts of starch were determined. The content of cell wall substances in tuber tissue was calculated according to Weber (1976). Analyses were in triplicate.

Assay of pectic substances. Following enzymic starch depolymerization the ethanol-insoluble fraction of the cell wall preparation was resuspended in Teorell-Stenhagen buffer at pH 3.5 and incubated for 12 h at 45 °C with 1.6 U g^{-1} of a pectinase (SERVA, Heidelberg). After filtration the residue was dried at 80 °C, weighed and the content of pectin in the cell wall calculated (Weber, 1976). Anhydrogalacturonic acid was determined using the method of McCready & McComb (1952).

Results

The cell wall degrading effect of enzymes. The effect of enzymes secreted by *E. carotovora* bacteria during invasion of host plants was mimiced by infiltrating potato tuber tissue with an enzyme solution. The lysis of tissue cells caused by the action of enzymes was then determined by the Neutral-red vital staining method. In preliminary tests using tubers of cv. Adretta, the tissue cylinders infiltrated with enzyme solution containing 2 U ml^{-1} of PL activity exhibited in their core a decrease of cell viability of 27% after 5 min of incubation, coinciding with an increase of conductivity of 270 µS in the cell extract. During 30 min incubation, cell viability decreased linearily up to 59% and the conductivity in the extracts reached 1080 µS. The decrease of cell viability combined with an increase in conductivity of cell extracts due to cell lysis is an indication of enzymic degradation of cell walls. To simplify the method used in the preliminary investigations, tissue disks and not cylinders were excised from the medulla of tubers and infiltrated directly with the enzyme solution before incubation. SD of the disk-test was ≤2% for 30 replicates. The method was used to analyse the potatoes grown in 1995.

The effect of enzymes on tissue of different cultivars. The preliminary investigations in 1993 revealed considerable differences in the susceptibility of tuber tissue cell walls to the action of *E. carotovora* enzymes between the 15 cvs investigated (Table 1). Thus a decrease in cell viability by enzyme action of <7% (N1, N2) was recorded for cv. Panda whilst the corresponding values for cv. Arnika and Leyla were >42% (N1

Table 1. Decrease of cell viability in the inner region of tuber tissue cylinders excised from potatoes grown under different rates of N-fertilization after incubation with *Erwinia* enzymes containing 2 U ml^{-1} of PL activity for 30 min at 20 °C. Field-test 1993.

Cultivar	Decrease of cell viability (%)	
	N1 40 kg N/ha	N2 80 kg N/ha
Désirée	21.9	28.6
Adretta	34.9	38.7
Arnika	42.3	45.4
Gloria	14.6	21.2
Karat	27.8	29.5
Rustica	19.5	24.6
Maxilla	15.1	15.5
Granola	32.1	34.2
Panda	4.1	6.8
Christa	31.6	38.9
Leyla	46.8	46.7
Karlena	27.2	35.1
Sissi	37.8	38.0
Likaria	32.7	36.2
Lipsi	23.0	31.3

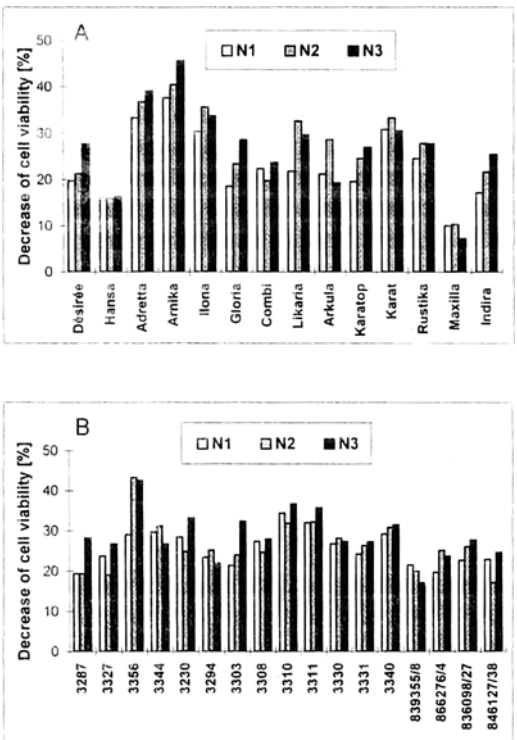

Fig. 1. Decrease in cell viability in tuber tissue disks of different potato cultivars (A) and clones (B) grown under different rates of N-fertilization (N1=40, N2=80, N3=120 kg N/ha) after incubation with *Erwinia* enzymes containing 0.2 U ml^{-1} of PL activity for 30 min at 20 °C.

N2). Similar differences between the cultivars were obtained from potatoes grown in 1995. The decrease in cell viability following enzyme incubation ranged from 7% for cv. Maxilla (N3) to >40% for cv. Arnika (N2, N3) (Fig. 1A). Differences between the 17 potato clones were not as pronounced (Fig. 1B). Values ranged from 17% for clone 84.6127/38 to 43% for clone 3356 using the variant N2 of fertilization and from 17% for clone 83. 9355/8 to 43% for clone 3356 with rate N3. It was notable that only 7 cvs and 7 clones showed a low level of cell lysis of <25% using the medium rate of N-fertilizer (N2).

The influence of N fertilizer on the effect of enzymes. Results of the preliminary studies with potatoes of the 15 cultivars grown in 1993 indicated that an increasing rate of nitrogen fertilizer coincided with an enhanced level of the cell wall degrading effect of the *Erwinia* enzymes on tuber tissue (Table 1). Pronounced differences between N1 and N2 rates were observed for potatoes of cvs Lipsi (+8.3%), Karlena (+7.9%), Désirée (+6.7%) and Gloria (+6.6%). Consequently, further studies

including 31 genotypes were carried out in 1995. The rates N1, N2 to N3 also enhanced the susceptibility of tuber tissue cell walls to the degrading effect of *Erwinia* enzymes (Fig. 1A and B). Results were statistically significant (P>0.001) for comparisons of the N1/N2 rates and also for the N1/N3 rates. While for the N3 rate 4 cultivars and 4 clones were found to have decreased cell viability of <25%, 10 cultivars and 9 clones showed a corresponding low level of enzymic cell lysis using the N1 rate. For example, the cvs Adretta, Arnika, Gloria Karatop, Indira and Désirée showed a linear increase of the cell wall susceptibility due to an enhanced rate of N-fertilizer (Fig. 1A). A similar linear increase was noticed for the clones 3303, 3331, 3340 and 83. 6098/27 (Fig. 1B). Nevertheless, the effect of enzymes on tissue cell walls of cv. Hansa was not influenced by the rate of N-fertilizer. The influence on tissues of cv. Maxilla was negligible.

The amount of cell wall in tuber tissue and the effect of enzymes. Rough cell wall preparations were made from 31 genotypes including the three different rates of N-fertilizer (n=93). Amounts of rough cell wall in tuber tissue differed considerably between the genotypes (Jansen, 1995, unpublished). A weak correlation of r=0.36 was found between the amount of rough cell wall and the degree of enzymic cell lysis for the 14 potato cultivars grown under the three different rates of N-fertilizer (n=42). With the medium rate (N2), the correlation coefficient was r=0.58 (n=14). The rough cell wall preparation contained some starch and proteins which possibly affected the results. Therefore, starch in the rough cell wall preparation of six selected genotypes was completely removed by high pressure homogenizing and a subsequent incubation with α-amylase and amyloglycosidase enzymes. Among the genotypes selected, the highest amount of cell wall was found to be in the tissue of cv. Maxilla (Table 2). The lowest values were recorded in tissue of clone 3356. However. Maxilla was one of the cultivars that was resistant to the action of *Erwinia* enzymes whereas clone 3356 was highly susceptible and similar to cv. Arnika. Therefore, there seemed to be a close correlation between the amount of cell wall substances and the degree of enzymic cell lysis (n=6). The content of pectic substances in the cell wall ranged from 30 to 43%, with the exception of cv. Maxilla which showed a value of 15% (Table 2). Correlations between amounts of pectic substances in the cell wall and the degree of enzymic cell lysis were not as strong as those determined for the amount of cell wall in the tissue. The assay of galacturonic acid in the pectin fraction revealed no pronounced differences between the cultivars investigated (Table 2).

Discussion

Detailed chemical analyses of the cell wall components are time consuming and the results may not be conclusive with respect to the stability of cell walls against biotic and abiotic stresses, such as the attack of pathogens, thermal treatment and mechanical injury. Therefore, biochemical methods are needed to characterize the complex network of cell walls with respect to specific environmental stresses. The application of *Erwinia* enzymes with their capacity to loosen the cell wall network and

Table 2. Decrease of cell viability after incubation of tuber tissue disks with 0.2 U ml^{-1} of PL activity for 30 min at 20 °C, amounts of cell wall and pectic substances in tuber tissue and the content of galacturonic acid in the pectin fraction. Field test 1995; (N3).

Cv / clone	Decrease of cell viability(%)	Amount of cell wall g per 100 g f.wt.	Amount of pectic substances mg per 100 g f.wt.	Galacturonic acid in the pectin fract. (%)
Maxilla	7.2	1.46	220	23.05
Hansa	16.3	1.25	515	19.02
83.9355/8	17.1	1.10	330	22.13
Adretta	39.0	1.10	387	23.52
3356	42.5	0.87	373	24.96
Arnika	45.6	0.90	293	24.55

to lyse it finally was one approach. Soft-rot of potatoes caused by *Erwinia* bacteria is of high economic importance in agriculture (Perombelon & Lowe, 1975; Perombelon & Kelman, 1980). To obtain a natural spectrum of *Erwinia* enzymes, the culture medium used for bacterial enzyme production was supplemented with cell sap of potatoes and pulp containing cell walls. Infiltration of enzymes into the intercellular space of tuber tissue mimiced the spread of enzyme-producing *Erwinia* bacteria in host tissue. Nearly one third of tuber tissue of cv. Adretta was lysed after 5 min of enzyme incubation and half the tissue was degraded after 25 min. Traces of enzyme were needed to reach these measurable effects of cell lysis. The results shown in Fig.1A and B indicate that the susceptibility of cell walls to the action of *Erwinia* enzymes differed considerably between the genotypes. While the enzymes had little effect on tissues of cvs Maxilla and Hansa, they were highly active on those of cvs Adretta and Arnika. Similar differences were noticed for the cultivars investigated in 1993 (Table 1). Weber (1976) showed that amounts of rough cell wall in the tuber tissue differ among potato cultivars. Therefore it was to be expected that cultivar-dependent variations of the enzymic effects are determined by the content of cell walls. This notion is confirmed by the correlation between amounts of cell wall substances in tuber tissue and the degree of enzymic cell lysis determined for the 14 cultivars investigated in 1995. Moreover, detailed analyses of the six selected genotypes recorded a clear correlation between the content of cell walls in tuber tissue and their susceptibility to the action of enzymes. A higher content of cell wall substances seems to be one of the factors supporting the resistance of potato tuber tissue to *Erwinia* enzymes. In this context the pectic substances have a decisive role because they are main constitutents in potato cell walls, as well as being the appropriate substrates of the *Erwinia* pectinases. Weber (1976) showed that the potato cultivars also differ in their content of pectins assembled in the cell walls. In agreement with such results, considerable differences were recorded for the six genotypes investigated in our experiments (Table 2). The high content of cell wall substances in tissue of cv. Maxilla and the low concentration of pectin (Table 2) as substrate of PL enzymes, may be responsible for the enhanced resistance of the tissue

to the effect of *Erwinia* enzymes (Fig. 1A, Table 2). Alternatively, variation in the cell wall susceptibility, noted with potatoes grown in experiments with different rates of N-fertilizer (Fig. 1A and B), may reflect the sensitivity of cell walls to changing growth conditions and/or their dynamic reaction. Nevertheless, the influence of the growth conditions is not as pronounced as that of the individual properties of the cultivar.
We have shown that the Neutral-red vital staining method can be used to define the susceptibility of tissue cell walls to *Erwinia* enzymes and to highlight variations within the potato cultivars as well as those caused by an alteration of the growth conditions. But the method used in these experiments would cover only one of the factors determining the soft-rot resistance of potatoes. Future investigations will show how far the results obtained correspond with tissue resistance to *Erwinia* bacteria and and soft-rot resistance of potato tubers.

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