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Effect of glucans from different races of *Phytophthora infestans* on defense reactions in potato tuber

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Abstract

It has been proposed that susceptibility of potato to *Phytophthora infestans* would be a consequence of suppression and /or delaying of defense reactions by a soluble glucan which is released by compatible races of the fungus. In this report, the reaction of potato tuber slices (*Solanum tuberosum* cv. Huinkul) infected with either race I (1,4,7,8,10,11) or C (1,4,10,11) of *Phytophthora infestans* was studied. Race C grew better on slices than race I. Glucans from both races were isolated and their effect on the accumulation of phytoalexins and glucanases in tuber slices was studied. The glucans from the less virulent race (I) did not affect the accumulation of phytoalexins and glucanases in tuber slices infected or elicited with eicosapentaenoic acid, whereas the glucans from race C produced 70% inhibition of phytoalexin accumulation and reduced by 50% the induction of glucanase activities. Purified glucanases from potato degraded the glucans from race C but not from race I. The results reported here show that, at least on this cultivar, glucans from both races affected defense responses in a different manner, which could reflect structural differences between these glucans.

Abbreviations: EPA – 5,8,11,14,17 eicosapentaenoic acid.

Introduction

When plants are attacked by potentially pathogenic microorganisms, they display an active defense response, leading to the formation of physical and chemical barriers, namely the accumulation of pathogenesis-related (PR) proteins and secondary metabolites such as phytoalexins and lignin (Lamb et al., 1989).

In both compatible and incompatible interactions, similar plant responses are observed, but their timing is different. The importance of differences in response time as well as the relative contribution of the various synthesized compounds to the resistance reaction are unknown. It is likely that a combination of compounds, produced at the right time and at the right place, ultimately determines the outcome of the host response. However, it can not be ruled out that other, so far unknown, defense mechanisms are involved (Cuypers and Hahlbrock, 1988; Pieterse et al., 1992).

Although there is abundant information about defense reactions, the processes involved in molecular signalling which lead to compatibility or incompatibility are poorly understood. Plant defense reactions can be triggered by factors from microbial origin, called elicitors, whose significance and modes of action have been reviewed (e.g. Lamb et al., 1989). The majority of elicitors are not race-specific and, therefore, specificity cannot be explained solely by the production of elicitors, but rather determined by substances that are able to interfere with the active plant defense (Bushnell and Rowell, 1981; Heath,

1981; Lamb et al., 1989; Shiraishi et al., 1994). The elicitor of *Mycosphaerella pinodes* induces defense responses in pea such as the accumulation of pisatin, a major phytoalexin in pea, as well as enhancement of the activities of endochitinases and β-1,3-glucanases. These defense responses are markedly suppressed by the concomitant presence of an endogenous suppressor and the elicitor. The endogenous suppressor is active in pea tissue but not in soybean and kidney bean, (Nasu et al., 1995); consequently, this suppressor acts on the defense response in a species-specific manner. Similar results have been obtained with a suppressor from *Mycosphaerella pinodes* (Yoshioka et al., 1992), which suppressed the activation of PR-proteins in pea epicotyls induced by the elicitor, but this suppressor induced the PR-proteins in other leguminous plants like an elicitor.

Potato late blight, caused by the fungus *Phytophthora infestans* (Mont) de Bary (Oomycetes), is one of the most important diseases of potato (*Solanum tuberosum* (L)). For this reason, a considerable amount of research has been dedicated to the potato-*P. infestans* interaction (Pieterse et al., 1992): it has been suggested (Currier, 1981; Doke et al., 1980; Garas et al., 1979) that susceptibility results from the suppression and/or delaying of the production or activation of resistance reactions by glucans (suppressors) released only by compatible races of the fungus.

In this paper we study the effect of glucans from two isolates, differing in virulence, on the accumulation of phytoalexins and glucanase activities in tuber slices. We report that the glucan from the more virulent race (C) is able to suppress phytoalexin accumulation induced by infection with the less virulent race (I) or elicited by EPA. Race C glucans also suppress induction of glucanases in tuber slices infected with race I.

Materials and methods

Plant, fungal races and growth conditions

Potato tubers (*Solanum tuberosum* cv. Huinkul) without known R-genes, were obtained from the Experimental Station of the INTA Balcarce, Argentina. The races: 1,4,7,8,10,11- INTA (**I**) and 1,4,10,11- Córdoba (**C**) of *Phytophthora infestans* were obtained from the Laboratorio de Fitopatología, INTA Balcarce, Argentina. Stock cultures were maintained on V8 agar at 18 °C in the dark. Sporangia were collected in sterile water from 7 – day old cultures and stimulated to release zoospores by incubation at 4 °C for 6–8 h. These suspensions were used for inoculation.

Quantification of mycelial growth

Ten tuber slices of *Solanum tuberosum* cv. Huinkul (4–5 cm diameter $\times$ 1 cm thick) were inoculated with 50 μl of a sporangial suspension of race C or I (approximately 10^6 and 10^3 sporangia$\cdot$ml^{-1}). After 24 h, the slices were turned upside down and the largest diameter of mycelium was measured on the upper surface after seven days of incubation in closed plastic boxes in the darkness, at 18 °C and 90% relative humidity.

Extraction and purification of glucans from P. infestans *cell walls*

Mycelial walls were prepared following the method employed by Ayers et al. (1976). The method described by Keen et al. (1983) for isolation of glucans from *P. megasperma* was used. The dried cell walls (1.5–3 g) were incubated with protease from *Streptomyces griseus*, the solid residue was filtered off, resuspended in distilled water and autoclaved at 120 °C for 3 h. The autoclaved suspension was centrifuged, the supernatant filtered and dialysed against water. The retained material was then chromatographed in DEAE-Sephadex A-25 at pH 7.2 and non-retained material was collected and lyophilyzed. The dry powder (5–10 mg) was resuspended in 0.5 ml of distilled water and chromatographed on a column (2 $\times$ 60 cm) of Bio Gel P-2 equilibrated and eluted with distilled water. The fractions eluting in the void volume were pooled, lyophilyzed and rechromatographed on a column (1 $\times$ 80 cm) of Bio Gel P-4 equilibrated and eluted with distilled water. A carbohydrate-containing peak, which eluted at the first third of the column volume was collected. The range of Molecular Weights was estimated between 2500 and 3000. This material was exclusively composed of glucose, as determined by Gas Chromatography after hydrolysis. It was rechromatographed in FPLC- Fast Desalting column HR 10/10. A_{190} was monitored with a Linear UVIS detector.

Assay of suppressor activity

Suppressor activity was determined by measuring the effect of the glucans on localized browning of tissue, accumulation of phytoalexins and glucanase activities

in potato slices infected with race C or I of *P. infestans*, or treated with an emulsion of 5,8,11,14,17 eicosapentaenoic acid (EPA) as chemical elicitor.

Discs (1.0 cm in diameter, 0.5 mm thick) were cut from the pith medullary tissue of potato tuber. These discs were treated, on the upper surface, either with distilled water or 0.2 μ mole EPA (as emulsion) or 50 μl of sporangia suspension ($10^6 \cdot ml^{-1}$), 6 h after slicing. Glucans (200 μg in 100 μl water) were added 12 h later. The discs were incubated in Petri dishes at 18 °C in the dark (Preisig and Kuc, 1985). Each treatment consisted of 10 discs in three replicates. Controls of the effect of glucans on non-induced (water treated) tissue were included.

For determination of sesquiterpenoids by the method described below, the discs were incubated for 96 h at 18 °C before collecting the upper 1 mm according to the technique described by Preisig and Kuc (1985).

For determination of glucanase activities by the method described below, the discs were incubated for 72 h at 18 °C before processing all tissue from the discs.

Extraction of phytoalexins

Phytoalexins were extracted from tuber tissue as described by Shih et al. (1973). Compounds were separated and detected in thin-layer chromatography (TLC) (plates of non-activated silica gel G, 1 mm thick or plates KODAK 12179, 0.5 mm thick), following the procedure of Bezner and Lund (1975). Bands corresponding to rishitin, phytuberin and lubimin were scrapped from the plates and eluted with a mixture of chloroform and acetone in different proportions (9:1, 3:1, 1:1), and finally with acetone. Each washing was incubated at 40 °C for 10 min. The eluates were pooled and evaporated to dryness.

Quantitative analyses

Sesquiterpene phytoalexins were determined by the colorimetric method described by Shih et al. (1973).

Estimation of phytoalexins by gas-liquid chromatography (GLC): sesquiterpene phytoalexins were identified and measured with a Varian 2440 chromatograph. A stainless steel column packed with HI-EFF-1BP 15% on Chromosorb WAW (Alltech Associates, Inc., Deerfield, IL, USA) was used under the following conditions: column temperature 180 °C; injector temperature: 200 °C; detector temperature: 210 °C; nitrogen flow: 20 $ml \cdot min^{-1}$; hydrogen flow: 20 $ml \cdot min^{-1}$; air flow: 200 $ml \cdot min^{-1}$. Elution: 180 °C, isothermic. The identification and quantitation of each phytoalexin were made by comparison of the retention time and relating peak areas with that of methyl stearate used as internal standard ($2\mu g \cdot \mu l^{-1}$).

Crude enzyme preparations

Healthy potato tuber tissue (200 g) was homogenized in 100 mM sodium-citrate buffer pH. 5.2, 0.1% sodium metabisulfite, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 mM EDTA and 1 mM diisopropylfluorophosphate (DFP), using a Virtis 45 homogenizer (The Virtis Co.; Gardiner, New York, NY). The homogenate was centrifuged for 20 min at 12000 *g*. The supernatant was brought to 60% saturation by adding solid $(NH_4)_2SO_4$. The resultant precipitate was dissolved in 100 mM sodium acetate buffer pH 6.5 and dialyzed overnight against the same buffer. All operations were carried out at 0–4 °C. The same procedure was followed with race I-infected tubers in the experiments of Table 4.

Purification of β-1,3 glucanases

The dialysed crude protein fraction obtained from 48 h wounded tuber was passed through a CM-Sephadex column (2 × 18 cm) equilibrated with 100 mM sodium acetate buffer pH 6.5. After washing with the same buffer, bound proteins were eluted with a linear Na Cl gradient (0–200 mM). β-1,3 glucanase activity eluted at 30 mM NaCl. This fraction appeared as an homogeneous band of 16 kDa on SDS-12% polyacrylamide gels (Laemmli, 1970) stained with Coomassie brilliant blue R-250.

β-1,3-glucanase assay

Glucanase activity was assayed by measuring the rate of reducing sugar production with laminarin from *Laminaria digitata* (Sigma) as the substrate. The reaction mixture consisted of 0.1 ml of 500mM sodium acetate buffer pH 5.2 containing 1% laminarin and 0.1 ml of enzyme extract or 0.1 ml of 100 mM sodium acetate buffer pH 5.2. After 8 h of incubation at 37 °C the enzyme reaction was terminated by heating in boiling water for 2 min and the reducing sugar released was measured according to Ashwell (1957). Glucose was used as a standard. The activity is expressed in units, i.e. nmol glucose $released \cdot hour^{-1} \cdot ml\ enzyme^{-1}$.

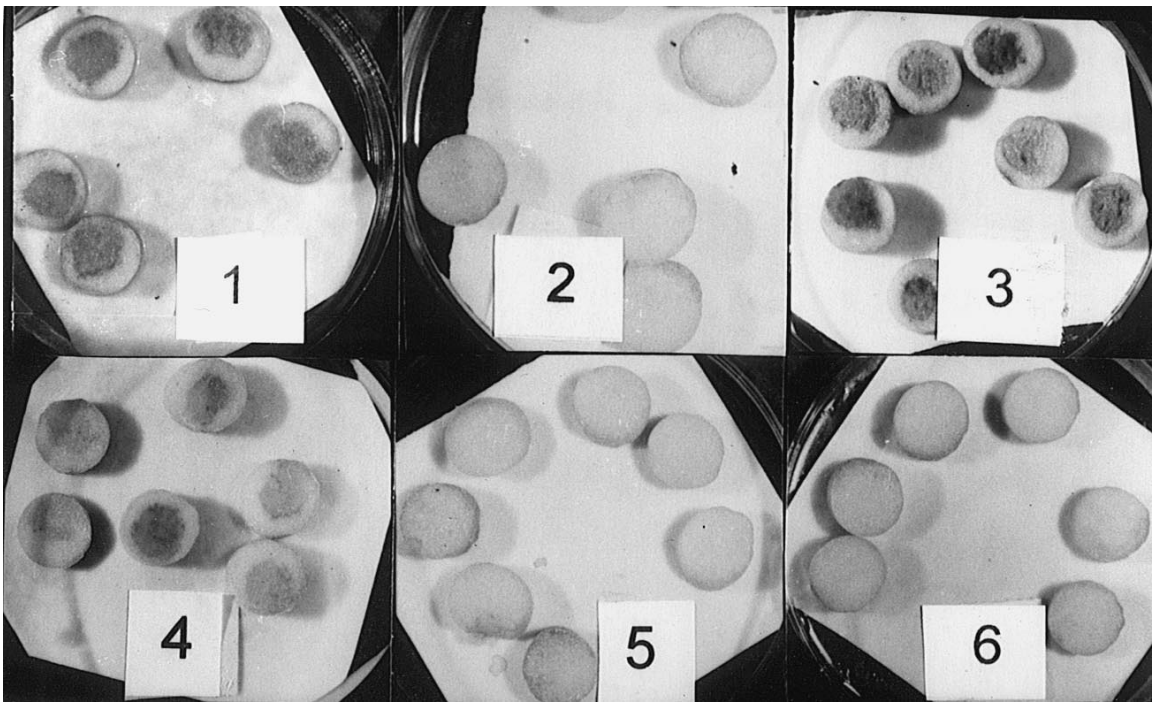

Figure 1. Effect of glucans of *P. infestans* (C, I) on hypersensitive browning in tuber slices elicited by EPA. Discs were sliced and after 6 h either water or EPA emulsion (0.2 μM/disc) was added. Glucans from race I or C were added (200 μg/disk) as designated after 12 h. The discs were treated with: 1, EPA; 2, H2O; 3, EPA + race I glucan; 4, EPA + race C glucan; 5, race C glucan; 6: race I glucan. Photographs were taken 96 h after treatment with EPA.

Experimental design and data analysis

All experiments included at least three replicates per treatment and were performed at least three times, with similar results. Unless otherwise stated, only statistically significant results are discussed. Data were analyzed for significance by two way analysis of variance, followed by Tukey's significant difference test (α=0.05), with SYSTAT software.

Results

Mycelial growth and sesquiterpene phytoalexin accumulation following inoculation with two races of P. infestans

The growth of both races of the fungus on tuber slices of potato cultivar Huinkul was measured 6 days post inoculation. Race C showed a mycelial growth four to five times higher than race I (Table 1). Accumulation of phytoalexins in each condition was studied. Tuber slices inoculated with race I produced four times more phytoalexins than the slices inoculated with race C (Table 2), in inverse relationship with the degree of colonization.

Effect of glucans from the two races of Phytophthora infestans *on the EPA-induced tissue browning and phytoalexin accumulation*

Glucans from both races were purified and applied on tuber slices 12 h after elicitation with EPA. Accumulation of sesquiterpene phytoalexins was accompanied by localized browning of tissue as a result of phenolic oxidation, representing macroscopic evidence of hypersensitive response (Preisig and Kuc, 1985). The slices induced with the chemical elicitor and treated with the glucans from race C showed less browning than the slices (induced and) treated with the glucans from race I. Browning was not observed in slices treated only with glucan from race C or race I (Figure 1). Similar results were obtained when the glucans were added at the same time as EPA (not shown). Considering that, during infection, glucan release would be a late event in signal exchange, further experiments were performed adding the glucans 12 h after EPA.

Table 1. Colonization of tuber tissue slices by two races of *Phytophthora infestans*[a]

Races	Concentration of inoculum (sporangia·ml^{-1})	Diameter growth on slices (cm)
C	10^3	2.5 ± 0.50
I	10^3	0.50 ± 0.10
C	10^6	4.5 ± 0.6
I	10^6	1.1 ± 0.2

[a] Ten tuber slices (1 cm thick) were inoculated with 50 μl of a suspension of *P. infestans* race C or I sporangia (1×10^3 or 1×10^6 sp·ml^{-1}). After 24 h at 18 °C and 90% relative humidity in the dark, the slices were turned upside down and the largest diameter growth of mycelium was measured on the upper surface on the 6th day.

Table 2. Sesquiterpene accumulation in potato tuber tissue resulting from infection with two races of *P. infestans*[1]

	Phytoalexins[2] μg·gFW^{-1}		
Race	Rishitin	Lubimin	Phytuberin
Control	55.7 ± 12.9 b	0.09 ± 0.07 a	0.97 ± 0.38 b
I	593 ± 130 a	1011 ± 92.9 b	958 ± 51.5 a
C	148.0 ± 35.2 b	482.0 ± 21.3 c	0.32 ± 0.27 b

[1] Tuber discs were inoculated with 50 μl of a suspension of sporangia (1×10^6 sp·ml^{-1}) or distilled water (control) 6 h after slicing. Rishitin, lubimin and phytuberin accumulation was determined by GLC 96 h after inoculation with the fungus or water, and expressed as μg×gram of fresh weight^{-1} (μg·gFW^{-1}).
[2] Means in the same column followed by the same letter are not significantly different at P<0.05 according to Tukey's test, least significant difference test (unpooled data).

The addition of the glucans from race C to slices treated with EPA, reduced four times the accumulation of sesquiterpenoid phytoalexins as compared to the slices treated with EPA only (Table 3). In contrast, the addition of the glucans from race I to slices treated with EPA, did not produce significant difference in the accumulation of phytoalexins with respect to the slices treated with EPA. The addition of glucans alone to tuber slices did not produce accumulation of phytoalexins (Table 3).

Effect of glucans from P. infestans *on the induction of glucanase activity*

Because glucanases also may be involved in defense reactions, the effect of glucans on glucanase induction in potato tuber inoculated with the less virulent race I was studied. Water-treated tuber slices already expressed a high glucanase activity as a result of wounding. Values reported in Table 4 represent the increase over the value of this control. Infection by race I stimulated glucanase activity by about 150%. Race C glucans reduced this stimulation by more than 50%. The addition of the glucans from race I to infected slices, did not produce significant difference in the induction of glucanase activity with respect to tuber slices treated with race I sporangia alone. It should be noted that the glucans, when added alone, produced an increase in glucanase activity.

Table 3. Effect of glucans from *P. infestans* on the accumulation of phytoalexins[1]

Treatment	Rishitin + Lubimin + Phytuberin[2] μg·gFW^{-1}		
Addition	H_2O	Glucan C	Glucan I
H_2O	0.040 ± 0.014 a	0.040 ± 0.015 a	0.040 ± 0.012 a
EPA	838 ± 50.3 a	188 ± 11.9 b	811 ± 55.3 a

[1] Tuber slices were treated with distilled water or 0.2 μmole EPA emulsion 6 h after slicing, followed by 200 μg glucan (as glucose equivalents) 12 h later. Rishitin, lubimin, phytuberin accumulation was determined by colorimetric method 96 h after treatment with EPA and expressed as μg·gram of fresh weight^{-1} (μg·gFW^{-1}).
[2] Means in the same row followed by the same letter are not significantly different at P<0.05 according to Tukey's test, least significant difference test (unpooled data).

Degradation of glucans from P. infestans *by glucanases*

Plant glucanases can potentially degrade glucans from fungal cell walls. The activity of a tuber glucanase which is induced by wounding or infection (results not shown) on both glucans was studied. Preparations of partially purified glucanase from healthy, wounded tubers, were able to degrade the glucans from race C but not from race I (Table 5).

Discussion

There were great differences in the degree of colonization in tuber slices of cultivar Huinkul between race C and I of *P. infestans*. Differences were also observed in the accumulation of phytoalexins around sites of infection in the tissue inoculated with the race C or I.

Experimental data on the potato-*P. infestans* interaction have shown that the elicitor of the hypersensitive response (HR) extracted from mycelia was not race specific, and it elicited the response in cultivars with or without R genes for resistance (Lamb,

Table 4. Effect of glucans from *Phytophthora infestans* on the induction of glucanase activities[1]

Treatment	Glucanase activity[2] U·gFW^{-1}
Glucan I	543 ± 28.3 a
Glucan C	610 ± 11.3 a
*P.i.*I + H_2O	746 ± 25.7 a
*P.i.*I + Gl. I	681 ± 10.7 a
*P.i.*I + Gl. C	318 ± 8.3 b

[1] Tuber slices were treated with distilled water or sporangia of race I of *P. infestans* (50μl of 10^6sp·ml^{-1}) 6 h after slicing., followed by 200 μg glucan (as glucose equivalents) 12 h later. The activity of glucanase in $(NH4)_2SO_4$ precipitate was determined 48 h after treatment with the glucans. The activity was expressed in Units·gram fresh weight^{-1} (U·gFW^{-1}): 1 U = 1 nmol glucose·hour^{-1}·ml enzyme^{-1}. Results are expressed as increase over the control (water treated) value (1459± 23.5 U·gFW^{-1}) [2] Means followed by the same letter are not significantly different at P< 0.05 according to Tukey's test, least significant difference test (unpooled data).

Table 5. Activity of glucanases on glucans from *Phytophthora infestans* cell walls[1]

Substrate	Reducing sugar released[2] nmol glucose·hour^{-1}·ml enzyme^{-1}
Glucan C	445 ± 28.9 a
Glucan I	2.34 ± 1.9 b
Laminarin	357 ± 21.9 a

[1] 0.1 ml of purified fraction of glucanase was incubated with 0.1 ml of the glucan C or I (2 mg glucose equivalents·ml^{-1}) or laminarin (10 mg·ml^{-1}), during 8 h at 37 ° C. The activity was measured as indicated in Materials and methods.
[2] Means followed by the same letter are not significantly different at P<0.05 according to Tukey's test, least significant difference test (unpooled data).

1989, Pieterse, 1992). Furthermore, with respect to a PR-protein, Schröder et al (1992) observed that the massive accumulation of β-1,3-glucanases and chitinases in potato leaves infected by different races of *P. infestans* is not involved in determining this specificity. It has been suggested that the virulent type of race contains compounds that specifically suppress the initiation of the HR (Currier, 1981). Although our experimental system is not typical of compatible vs. incompatible specific interactions, a possible explanation of our results is that a compound(s) in race C delays the initiation and affects the magnitude of phytoalexin production and other defense reactions as well.

When the effect of cell wall glucans from both races of *Phytophthora infestans* was studied, it was observed that the glucans from race C were more active in inhibiting browning (Fig 1) and the accumulation of phytoalexins (Table 3). Results reported by Doke et al. (1980) and Garas et al. (1979) on the activity of a suppressor extracted from zoospores in germination or mycelia filtrate on browning and cell death are consistent with our observed suppressor effects of the glucans on browning and on the accumulation of phytoalexins.

The glucans from race C also reduced glucanase activities in tuber slices infected with zoospores of race I (Table 4), whereas the glucans from race I did not. In infected potato tuber, the reduction of activity of a PR-protein by subsequent treatment with mycelial components of *P. infestans* has not been described previously.

The glucans induced glucanase activity when added alone (Table 4). It has been observed previously that the glucans from race I (1,4,7,8,10, 11) increased the levels of gene transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hydroxymethyl glutaryl coenzyme A reductase (HMGR), an enzyme involved in defense reactions (Laxalt et al., 1996). An earlier report showed that glucans enhanced phytoalexin production elicited by fatty acids, but suppressed elicitation by *P. infestans* (Maniara et al., 1984). The enhancer effect was observed regardless of the order of addition of fatty acids and glucans (Preisig and Kuc, 1985). These suppressor-enhancer effects could be explained assuming that suppressors bind to elicitor binding sites, either antagonizing or mimicking the effects of the elicitor (Boller, 1995). Our results cannot be explained in this manner, since glucans are added after EPA or infection. Moreover, it is difficult to assume that a glucan would bind to a fatty acid receptor, if any. In this case, the effect would be exerted later in the signal transduction cascade (Shiraishi et al., 1994).

When both glucans were incubated with purified glucanase, race C glucans were degraded but not race I glucans (Table 5). This result was not expected, but it could be explained considering it together with the effect of glucans on the induction of glucanases: race I glucans, which have no effect on the induction of glucanases, are not degraded by them; race C glucans, which can be degraded by this enzyme, are effective in suppressing its induction. A direct inhibition of glucanase activity by the glucan can not be ruled out.

The activity of the glucans from race C and I on defense responses, namely, the accumulation of phytoalexins and induction of glucanase activities show

that, at least on this cultivar, glucans from different races have differential suppressor effects. This could explain the different aggressiveness of both races on an homogeneous genetic background lacking major resistance genes. Currier (1981) suggested that the race specificity of the glucans that inhibit the hypersensitive response may lie in the degree of polymerization or the number and position of branching. The fact that race C glucans are degraded by glucanase and race I glucans are not, would be a consequence of structural differences between them. Permethylation analysis (data not shown) showed that both glucans have a backbone of 1-4 bound glucose units, 10% of branching at carbon 6 and that glucan C alone has 10% of branching at carbon 3. Although results described here cannot be generalized, due to the restricted system used, they may provide clues to elucidation of the additional mechanisms of resistance and susceptibility to potato late blight.

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G.D. is an established researcher of CIC.

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