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INSOLUBLE PHENOLIC COMPOUNDS AND RESISTANCE OF POTATO TUBER DISC TO *PHYTOPHTHORA* AND *PHOMA*

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This paper is dedicated with great sadness to the memory of Professor Tony Swain, our mentor in phenolic biochemistry and a close friend and colleague for almost 33 years.

Key Word Index—*Solanum tuberosum*; Solanaceae; tubers; *Phytophthora infestans*; *Phoma exigua*; insoluble phenolic polymers; disease resistance.

Abstract—Tuber discs of two potato cultivars, one (Stormont Enterprise) resistant and the other (King Edward) susceptible to a complex race of *Phytophthora infestans* were inoculated with sporangia of the fungus or with mycelial discs of *Phoma exigua*. Penetration by either fungus was impeded faster in Stormont Enterprise than in King Edward; there was less penetration of both cultivars in discs inoculated 24 hr after cutting. Browning of discs, measured by a reflectance method, correlated with the browning of cell walls and their ability to stain with Toluidine Blue. The levels of browning correlated with the resistance of the discs to penetration by either fungus. Treatment of the discs with amino-oxyacetic acid (AOA) before inoculation markedly inhibited browning and permitted both fungi to grow further through the tuber tissue. Amino-oxyacetic acid had little effect on the levels of the two sesquiterpenoid phytoalexins, rishitin and lubimin, in Stormont Enterprise tuber discs. These experiments therefore indicate that accumulation of phenolic compounds in cell walls is more important than phytoalexin accumulation in non-race-specific resistance of tuber discs to *P. infestans* and in resistance to *P. exigua*. The insoluble phenolic material appears to contain polymerized oxidized chlorogenic acid, esterified *p*-coumaric and ferulic acids and either one or both lignin and suberin.

INTRODUCTION

McLauchlin [1, 2] showed that in tubers of potato cultivars varying in non-race-specific resistance to a complex race of *Phytophthora infestans*, browning of discs was correlated with their resistance to the fungus. The ionization difference spectrum of the brown material was similar to that of material made in a model system of tuber cell walls incubated with ferulic or *p*-coumaric acids and hydrogen peroxide and is therefore probably either a suberin-type polymer [3–5] or is lignin-like [6–9]. Resistance of potato tuber tissue to gangrene caused by *Phoma exigua* var *foveata* is also associated with the deposition of suberin and lignin [10, 11].

The experiments reported in the present paper were carried out to determine the rate of deposition of brown phenolic material on cell walls in relation to the spread of both fungi. The effects of α-amino-oxyacetic acid, an inhibitor of phenylalanine ammonia lyase activity [12], on browning and on the rate of penetration of the discs by the two fungi has also been examined. The levels of accumulation of phytoalexin in the two cultivars challenged by both fungi has been determined. The results show that there is a relationship between the rate of browning and the resistance of the tuber discs to the two fungi, and indicate little involvement of phytoalexins in resistance.

Since the completion of this work, Hammerschmidt [13] has reported that lignin in tuber slices is involved in the resistance of potato tuber slices to inoculation with fungi which are not potato pathogens. In addition the nature of the phenolic material has been investigated. These results, which form part of the Ph.D. thesis of Y. A. Ampomah [14], have been briefly reported [15] and also summarized in a review article [16].

RESULTS

Rate of penetration of tuber discs

Although it was possible to see *P. infestans* in tuber discs by light microscopic examination of hand cut sections it was extremely difficult to observe the growth of *P. exigua*. The rate of penetration of tuber discs was therefore measured by an adaptation of the method used by Wilson [17], cutting the discs into four layers, plating samples of each layer on an agar medium, and determining the percentage of infected discs from each layer. Using this method, the rate of penetration of tuber discs of the two varieties by the two fungi was compared. In addition the effect on penetration of ageing the discs for 24 hr before inoculation was determined.

The results are presented in Fig. 1. It will be seen that there is a marked difference in the ease of penetration of discs of the two varieties by the culture of *Phytophthora* used in these experiments. In the case of discs which were inoculated shortly after cutting the differences are apparent in the second millimetre of the discs two days after inoculation; 75% of the King Edward but only 15% of the Stormont Enterprise showed infection. Ageing the

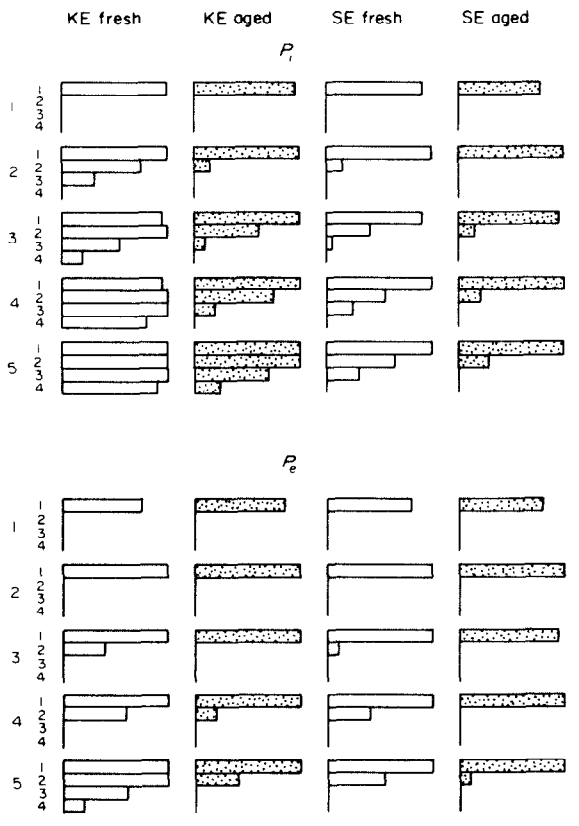

Fig.1. The penetration of the top four mms tuber discs of Stormont Enterprise and King Edward by *Phytophthora infestans* and by *Phoma exigua* and the effects of 'ageing' the discs for 24 hr before inoculation. The bars represent the percentage slices colonized of slices of 1 mm thickness taken from each of 20 discs; details are given in the experimental section. The large numbers are the days after inoculation. The smaller numbers are those of the four slices taken from each disc where No. 1 = top slice, P.i = *P. infestans*, P.e = *P. exigua*.

discs for 24 hr before inoculation slows the rate of penetration of the fungus in both cultivars and the fungus seems to take at least 24 hr longer in the aged discs compared with the fresh discs to reach the same depth. Ageing the discs seemed to exaggerate the differences between the response of the two cultivars to *Phytophthora* and was therefore adopted as a standard procedure.

The data in Fig. 1 also show that *Phoma* penetrates the discs more slowly than *Phytophthora*. Furthermore the differences between the two varieties are less marked for *Phoma*; they become apparent in the 'fresh' discs three days after inoculation but in the aged discs the differences are not really apparent until five days after inoculation.

Microscopic observation of discs after inoculation

Discs of both varieties became brown after inoculation with either fungus; the browning was more intense in Stormont Enterprise than in King Edward discs. Sections, *ca* 1 cell thick, cut perpendicular to the infected surface of the tuber discs, were examined by light microscopy without staining. Twenty-four hours after inoculation by *P. infestans* the first layer of cells were penetrated in both cultivars and there was deposition of brown granular material, more in Stormont Enterprise than in King Edward. Three days after inoculation, in Stormont Enterprise the fungus was mainly confined to the first layer of cells with few cells invaded in the second layer. In Kind Edward extensive hyphal growth, both intercellular and intracellular, was seen in the top two cell layers. At this stage the top two layers of cells appeared brown in both varieties; the browning seemed to be confined to the cell walls. The differences between the two cultivars was most marked on day five after inoculation when there was extensive fungal growth in King Edward coupled with development of aerial mycelium. In Stormont Enterprise fungal growth was limited to the top two cell layers with little aerial mycelium. Browning of cell walls in Stormont Enterprise tissue was more intense than in King Edward.

As indicated earlier, microscopic observation of *Phoma* mycelium in potato tuber tissues was found to be very difficult. Nevertheless, distinct differences in the browning of the cell walls of the two cultivars could be seen, particularly five days after inoculation, when the cell walls in the infected Stormont Enterprise discs were much darker brown than those of infected King Edward.

After staining sections with Toluidine Blue which is supposed to be a specific stain for polyphenolic material [18], cell walls which had orginally appeared brown were now stained blue-green; this colour reaction was observed in tissues inoculated with either *P. infestans* or *P. exigua*. The blue-green colour became more intense at day five and the differences between King Edward and Stormont Enterprise became more discernible. The top layer of cells of Stormont Enterprise stained dark green whereas those of King Edward stained relatively weakly.

The surface layer of cells of uninoculated discs also developed a green colour which was less intense than that of the corresponding inoculated tissue.

Reflectance measurement of browning of discs after inoculation

The visual observation that there was more browning of infected than uninoculated discs was confirmed by the reflectance measurements. The data shown in Figs 2 and 3 show that the browning was more intense in Stormont Enterprise than in King Edward discs and furthermore that there was a greater browning response of both cultivars to *P. infestans* than to *P. exigua*. Reflectance browning measurements thus appeared to be correlated with the observed browning of cell walls in sectioned material.

Effect of AOA

The results of the earlier experiment indicated that there was a correlation between the browning of cell walls and the resistance of discs to fungal penetration. It was therefore decided to examine the effect of AOA on both fungal penetration and browning. It will be seen from Fig. 4 that discs of both cultivars treated with AOA are more easily penetrated by both fungi than are untreated discs. AOA also reduced browning of the discs (Figs 2 and 3) and when sections of AOA-treated discs were examined it was observed that they gave only a very slight reaction with Toluidine Blue.

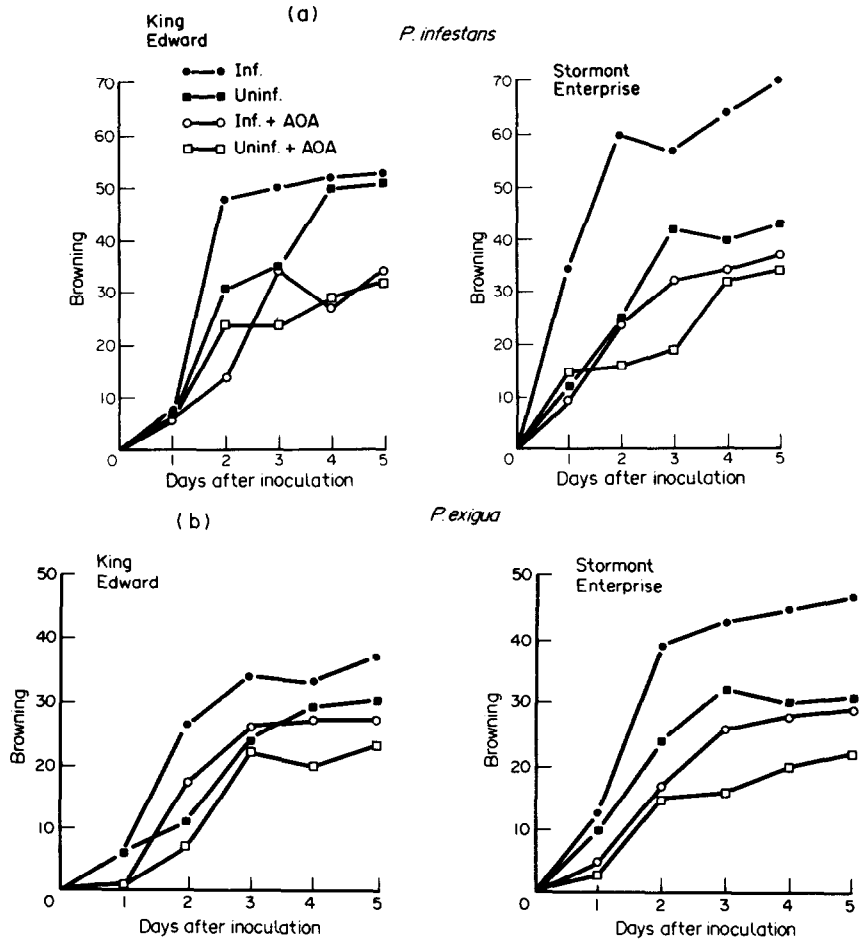

Fig. 2. The browning and the effects of AOA on browning of tuber discs of Stormont Enterprise and King Edward after inoculation by (a) *Phytophthora infestans*, (b) *Phoma exigua*.

The greater penetration of the discs by both fungi in the presence of AOA could have been due to a stimulation of fungal growth by AOA. However addition of AOA in phosphate buffer to either the pea agar or malt agar used for culturing *P. infestans* and *P. exigua* respectively, showed that AOA had no observable effect on the radial mycelial growth of either fungus (Table 1).

Accumulation of rishitin and lubimin

The results in Table 2 show that after inoculation with *P. infestans* there is a detectable difference in the accumulation of both rishitin and lubimin 1 day after inoculation when there is a higher level of both phytoalexins in Stormont Enterprise than in King Edward. The differences between the two cultivars become more pronounced at days two and three.

However in the discs of both cultivars inoculated with *P. exigua* there was only a slow rate of accumulation of both rishitin and lubimin. The only varietal difference seen was the greater level of rishitin in Stormont Enterprise than in King Edward three days after inoculation.

Amino-oxyacetic acid had an inhibitory effect on the production of both rishitin or lubimin in tuber discs of Stormont Enterprise inoculated with *P. infestans*. However the maximum inhibition was only 22%; this was very much smaller than the inhibition of browning by AOA which was almost 50% in Stormont Enterprise three days after inoculation.

Nature of the phenolic material in cell walls of infected tuber discs

After freezing in liquid nitrogen, discs were extracted with methanol–chloroform–water (MCW) under nitrogen to remove soluble phenolic compounds. The MCW residues were then extracted, under nitrogen, with phenol–acetic acid–water (PAW) to give a PAW extract and a PAW residue. The solvent was removed from the PAW extract and the PAW residue respectively by dialysis and by washing with ethanol. The PAW extract and the PAW residue were then tested for the presence of phenolic compounds. The two fractions were separately saponified with 2 M sodium hydroxide under nitrogen at 70°. An ionization difference spectrum, the quinic acid level and the amounts of free phenolic acids (by HPLC) were then determined on the saponification products.

The ionization difference spectra obtained from both the PAW extract and the PAW residue were those characteristic of lignin-like compounds which is probably due to hydroxycinnamic acids, with peaks at 240, 290 and 350 nm. Examination of the ΔA_{350} values (Table 3), for the five day samples, shows that the values were always higher in the infected than in the uninoculated controls.

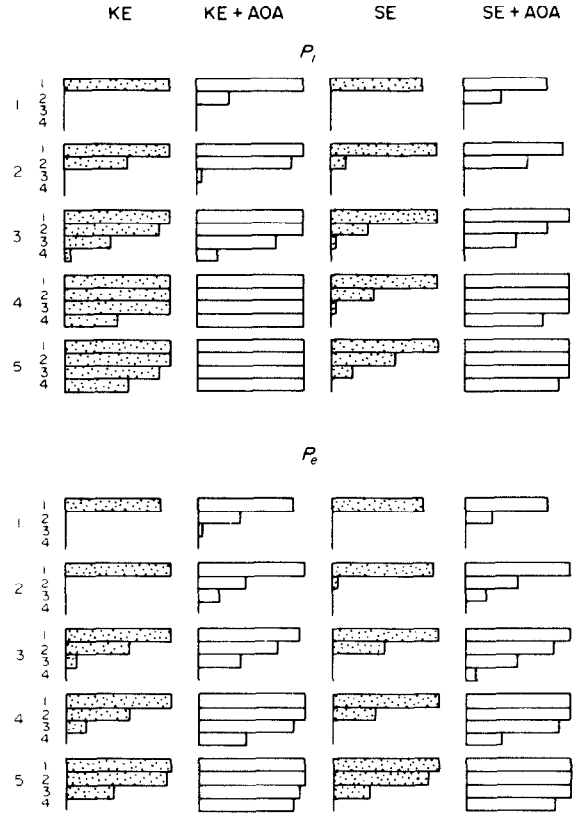

Fig. 3. The effects of AOA on the penetration of the top 4 mm of tuber discs of Stormont Enterprise and King Edward by *Phytophthora infestans* and by *Phoma exigua*. Discs were inoculated 24 hr after cutting; AOA-treated discs were left in AOA solution for this 24 hr period.

Table 1. The effect of AOA on radial growth of *P. infestans* and *P. exigua* on agar media at 18°.

Days after inoculation	Diameter in cm			
	P. infestans		*P. exigua*	
	−AOA	+AOA	−AOA	+AOA
2	0.5	0.5	0.4	0.3
4	1.4	1.2	1.0	0.8
6	2.8	2.6	1.2	1.4
8	5.0	5.2	3.0	2.8
10	7.8	7.5	5.1	5.2

P. infestans was grown on pea agar.
P. exigua was grown on 3% malt extract agar.

Moreover with both fungi the differences (infected minus uninoculated) were always higher in Stormont Enterprise than in King Edward. In all cases (data not shown) extracts from AOA treated tissues had considerably lower values of A_{350}.

Quinic acid, as determined by the TBA reaction, was found only in the PAW extracts, and not in the PAW residues. These results differ from those of McLauchlin [2] who found quinic acid in both the PAW extract and in the PAW residue. The difference can easily be explained since McLauchlin extracted soluble phenolic compounds from tuber discs with methanol, whereas we used the more aqueous solvent MCW. Swain (personal communication) pointed out to us that boiling potato tissue with methanol dehydrates them and under these circumstances some chlorogenic acid will become hydrogen-bonded to cell walls. Results of our own to be reported elsewhere confirm this. Since PAW extracts protein from cell walls [19] we presume that the quinic acid we have estimated is probably derived from the quinic acid side chains of the oxidation product of chlorogenic acid, chlorogenoquinone, bound to protein [20, 21].

HPLC analysis of the saponification product showed the presence of *p*-coumaric and ferulic acids in the PAW residue but not in the PAW extract. This indicates that they were esterified to material of very high M_r such as lignin, suberin or polysaccharide. Since neither acid was found in the PAW extract which is presumed to contain all the protein from the cell wall, we must assume that neither acid was esterified to protein.

The results of the quinic acid (Table 4), *p*-coumaric and ferulic acid (Table 5) determinations show that there is more phenolic material in the infected than in the uninfected samples from both cultivars, irrespective of which fungus is involved. In addition, the differences (infected minus uninoculated) are always higher in Stormont Enterprise than in King Edward. Amino-oxyacetic acid reduces the level of quinic acid in Stormont Enterprise infected with either fungus. Unfortunately the HPLC analysis was not performed on PAW residues from AOA-treated tubers.

Thus the results of all three analyses for phenolic compounds confirm that the brown material which stains with Toluidine Blue is phenolic in nature. Moreover, the amounts of this phenolic material present in both the infected and uninoculated tuber samples correlate with both the visual observation and the reflectance browning measurements. The total amount of insoluble phenolic material, indicated by the $\Delta\varepsilon_i$ measurements, is also reduced by AOA.

DISCUSSION

The experiments reported in the paper show both by reflectance and chemical methods that phenolic material is deposited in cell walls of tuber discs which have been inoculated with either *Phytophthora* or *Phoma*. This material is deposited faster, and reaches a higher level, in Stormont Enterprise than in King Edward and the deposition is inversely correlated with the penetration by the two fungi of the discs of the two varieties. Moreover, both fungi penetrated further into the discs of both cultivars when the discs were pretreated with AOA, which inhibited browning and the ability of the tuber cell walls to stain with Toluidine Blue. AOA had no effect on fungal growth *in vitro*. These two sets of experiments indicate the importance of the deposition of phenolic material in the resistance of Stormont Enterprise to the two fungi.

It is reported elsewhere that the levels of AOA used inhibited phenylalanine ammonia lyase (PAL) activity [14]. The pattern of the results is thus reminiscent of those of Zucker and Hankin [22] who found that addition of cycloheximide to potato discs inhibited PAL activity and suberization and permitted soft-rotting by *Pseu-*

Table 2. Rishitin and lubimin accumulation in potato tuber discs

(a) Inoculated with *P. infestans* 24 hr after cutting and incubated in the dark at 18°

Days after Inoculation	(Values in μg phytoalexin/g fr. wt tuber tissue) Stormont Enterprise		King Edward	
	Rishitin	Lubimin	Rishitin	Lubimin
0	0	0	0	0
1	6.2	4.3	1.0	0.5
2	10.3	12.8	2.1	1.2
3	30.1	27.6	3.6	2.3

(b) Inoculated with *P. exigua* 24 hr after cutting and incubated in the dark at 4°

	Stormont Enterprise		King Edward	
	Rishitin	Lubimin	Rishitin	Lubimin
0	0	0	0	0
1	0.35	0.2	0.36	0.2
2	0.50	0.46	1.5	0.8
3	8.0	0.87	3.1	1.1

(c) Treated with AOA and inoculated with *P. infestans* 24 hr later and incubated in the dark at 18°

	Stormont Enterprise			
	No AOA		+AOA	
	Rishitin	Lubimin	Rishitin	Lubimin
0	0	0	0	0
1	5.0	5.1	4.8	4.6
2	12.8	14.1	10.1	11.0
3	30.1	26.8	26.7	20.8

domonas fluorescens. Walker and Wade [10, 11] have previously shown the importance of lignification and suberization in the resistance of tubers to *Phoma*. Deposition of lignin-like material has previously been shown to be a feature of race-specific resistance of tubers of some R-gene containing potato cultivars to avirulent races of *P. infestans* [23, 24]. In other cultivars the accumulation of terpenoid phytoalexins appears to be an important feature of the resistance reaction [25]; hyphal encasement is also important in some resistance reactions [26].

However, phytoalexin accumulation does not seem to be an important feature of the resistance of Stormont Enterprise tuber discs to either *Phytophthora* or *Phoma*. The levels in the susceptible AOA-treated discs infected with *P.* infestans are still 80% of those in untreated resistant infected discs. Moreover in the discs infected with *P. exigua* the phytoalexin levels are relatively low and at a similar level to those in the more susceptible cultivar, King Edward. Walker and Wade [11] found that there was no consistent correlation of phytoalexin accumulation with resistance of tuber slices to infection with *P. exigua* at 18°; they concluded that suberization followed by lignification was the important factor in resistance of tubers to *P. exigua*.

The analytical methods used to examine the material in both the PAW extracts and residues from the MCW-insoluble fraction of the discs give some indication of its nature. The ionization difference spectra obtained from both fractions indicate that they contain material derived from phenylpropanoid material. Since the PAW extract did not contain any cinnamic acids but did contain quinic acid, it seems most likely that it contained polymerized oxidized chlorogenic acid bound to protein [23, 24]. Although no quinic acid was found in the PAW residues, these residues did contain *p*-coumaric and ferulic acids. These cinnamic acids were probably esterified to a cell wall component which could be lignin, suberin or carbohydrate. Any lignin or suberin would be present in the PAW residue and the structures for suberin proposed by Kolattukudy [3] would be indistinguishable from lignin by the techniques we have used in these experiments.

Walker and Wade [10, 11] used staining techniques to differentiate between lignin and suberin in their studies on the resistance of tubers to *Phoma*. Hammerschmidt [13] suggested that the material in cell walls of tuber slices resistant to non-pathogenic fungi is lignin. It stained for lignin with Toluidine Blue, yielded lignin

Table 3. Total insoluble phenolic material in tuber discs determined from ionization difference spectra from PAW extracts and PAW residues, expressed as ΔA_{350} per 100 mg material

(a) Inoculated with *P. infestans* 24 hr after cutting and incubated in the dark at 18°

Days after inoculation with fungus (I) or distilled water (U)		Stormont Enterprise		King Edward	
		PAW extracts	PAW residues	PAW extracts	PAW residues
3	I	0.60	0.44	0.20	0.30
3	U	0.10	0.26	0.15	0.20
5	I	1.00	0.66	0.60	0.42
5	U	0.55	0.36	0.45	0.26

(b) Inoculated with *P. exigua* 24 hr after cutting and incubated in the dark at 4°

Days after inoculation with fungus (I) or with agar (U)		Stormont Enterprise		King Edward	
		PAW extracts	PAW residues	PAW extracts	PAW residues
3	I	0.55	0.26	0.30	0.18
3	U	0.30	0.14	0.25	0.08
5	I	0.55	0.22	0.45	0.20
5	U	0.25	0.08	0.35	0.10

Table 4. Bound quinic acid levels in PAW extracts from tuber discs (values given are calculated as μmol/g of PAW extract)

(a) Inoculated with *P. infestans* 24 hr after cutting and incubated in the dark at 18°

Days after inoculation with spore suspension (I) or distilled water (U)		Stormont Enterprise	King Edward
0		10.1	8.4
3	I	18.0	10.4
3	U	15.0	8.9
5	I	23.6	17.0
5	U	16.1	14.3

(b) Inoculated with *P. exigua* 24 hr after cutting and incubated in the dark at 4°

Days after inoculation with fungus (I) or with agar (U)		Stormont Enterprise	King Edward
0		10.9	9.1
3	I	15.2	11.2
3	U	13.1	10.4
5	I	20.1	12.6
5	U	14.0	10.4

(c) Effects of AOA–on Stormont Enterprise only

	P. infestans	*P. exigua*
5 days I	19.4	15.0
5 days I and AOA	13.8	12.0

Table 5. Bound *p*-coumaric and ferulic acids in residues after PAW extraction of tuber discs (values given are in μg/g cell wall)

(a) Inoculated with *P. infestans* 24 hr after cutting and incubated in the dark at 18°

Days after inoculation with fungus (I) or distilled water (U)		Stormont Enterprise		King Edward	
		p-Coumaric	Ferulic	*p*-Coumaric	Ferulic
0		2.1	3.1	1.0	1.0
3	I	13.2	8.4	3.7	4.6
3	U	4.8	6.8	3.5	4.8
5	I	15.2	14.6	9.0	12.2
5	U	12.4	9.0	5.8	8.4

(b) Inoculated with *P. exigua* 24 hr after cutting and incubated in the dark at 4°

Days after inoculation with fungus (I) or with agar (U)		Stormont Enterprise		King Edward	
		p-coumaric	Ferulic	*p*-Coumaric	Ferulic
0		0	0	0	0
3	I	9.0	9.1	3.4	3.6
3	U	6.0	9.0	2.1	3.0
5	I	8.1	14.0	6.3	8.5
5	U	5.0	10.1	5.8	7.2

thioglycollic acid, and both *p*-hydroxybenzaldehyde and vanillin were present in cupric oxide oxidation products of alkali-treated tissue. Nevertheless it is difficult to distinguish on the basis of cupric oxide oxidation products between lignin and suberin, since Kolattukudy [3] has proposed that both *p*-coumaric and ferulic acids are present in the aromatic domains in suberin. The possibility that the insoluble phenolic material in potato reacting to pathogens contains hydroxycinnamoyl esters of galactan [7] is supported by the work of Fry [27] who has isolated feruloyl- and *p*-coumaroyl-arabinans and galactans from cell walls of a range of plant cells.

The phenolic material in the cell walls could act as either a chemical or a physical barrier, as proposed earlier [7]. It is interesting to note that both *P. infestans* and *P. exigua* produce galactanases as their major cell wall-degrading enzymes [28–30]. The esterification of cell wall galactans would probably be a sufficient modification to make the galactans less degradable by the fungal galactanases. Lignification and suberisation may well impede the passage of fungal galactanases into the wall.

What is particularly interesting about the present experimental results is that they indicate that the reactions involved in non-race-specific resistance to *P. infestans*, a biotrophic pathogen of potato, appear to be similar to those involved in resistance to necrotrophic pathogens such as *Phoma exigua* and to non-pathogenic fungi.

EXPERIMENTAL

Plant material and pathogens. Stormont Enterprise and King Edward potato tubers were either obtained from Scotland (G. & I. Ltd, Huntley, Aberdeenshire) or grown in the University Botanic Garden. They were stored at 4° in the dark and equilibrated to room temperature 16 hr before use. A complex race of *Phytophthora infestans* race 1,2,3,4,5,6,7,8,9,10,11 was obtained from Dr J. F. Malcolmson (Scottish Crop Research Institute, Pentlandfield). It was maintained on pea agar in the dark at 15° [31]. In order to avoid loss of virulence, it was passaged through Stormont Enterprise tuber tissue every 3 months and re-isolated on antibiotic agar [32]. The isolate of *Phoma exigua* var *foveata* from the Hull University culture collection, was originally obtained as isolate 1/6 from Dr G. J. Jellis (Plant Breeding Institute, Cambridge). It was maintained on malt extract agar at 18°.

Preparation of tuber discs. Potato tubers of similar size were scrubbed to remove adhering soil, then surface sterilized in a 10% solution of Chloros for 10 min, rinsed in ethanol and the parenchymatous tissue cut into discs (either 20 or 14 mm diameter and 5 mm thick). These were mixed, washed with at least 3 changes of sterile distilled water (SDW) and eight discs transferred to a 9 cm petri dish containing either a thin layer of 0.5% agar or a 7.5 cm diameter Watman No. 1 filter paper soaked with 1.5 ml of SDW.

Inoculation of tuber discs. For *P. infestans* the discs were inoculated with 0.1 ml of a suspension of sporangia containing 5 $\times 10^4$ sporangia/ml. Control discs were inoculated with 0.1 ml SDW. The discs were then incubated in the dark at 18°. Since the isolate of *P. exigua* hardly sporulated, mycelial inoculation was used. 14 mm tuber discs were inoculated in the centre of the upper surface with a 10 mm disc of *P. exigua* mycelium cut from the outer edges of 10-14-day-old colonies growing on 3% malt extract agar in petri dishes at 18°. 20 mm tuber discs were inoculated with 20 mm mycelial discs. Controls were inoculated with discs of sterile 3% malt extract agar. The discs were incubated in the dark at 4°.

Browning assay. Tuber disc browning was measured on a purpose-built reflectance scanning system which comprised a video camera connected to a digital integrator [33]. The reflectance values obtained were inversely proportional to browning. The average reflectance from 8 SDW treated control discs was determined and assigned 100% reflectance. The average readings from treated discs were measured and this lower value expressed as a percentage of the control; the difference between the percentage reflectance of control and treated discs was then used as a measure of tuber disc browning.

Rate of penetration of the fungi in potato tuber discs. The method is modified from that of ref. [17]. Samples of 100 discs (20 mm diameter) of each variety were inoculated with either a sporangial suspension of *P. infestans* or mycelial discs of *P. exigua*. 20 discs of each variety were taken daily and the sides cut off to form blocks (15 × 15 mm). Each of the blocks was then sliced into four sections each of 1 mm thick cut parallel to the top surface. The sections were then placed on pea agar medium in petri dishes for discs inoculated with *P. infestans* or on malt agar for discs inoculated with *P. exigua* and incubated at 18° for 10 days. Under these conditions, the fungi grew freely if they were present in the cut potato sections. Results are expressed as the percentage of slices, out of each sample of 20, out of which fungus grew. To ensure that the observed mycelia on the agar surfaces were that of either *P. infestans* or *P. exigua*, samples were taken from the agar media, mounted on slides and compared under the microscope with authentic mycelium of the appropriate fungus.

Application of α-amino-oxyacetic acid (AOA) to discs. 200 20 mm tuber discs of each of the two varieties were prepared and placed in 9 cm petri dishes containing a 9 cm Whatman No.1 filter paper soaked with either 2 ml of 0.05 M Pi buffer pH 7.5 (controls) or 2 ml of 0.05 M Pi buffer pH 7.5 containing 4 mm AOA. 24 hr later the discs were turned upside down and inoculated with either *P. infestans* or *P. exigua* as described above. Controls were respectively inoculated with SDW or a sterile disc of 3% malt agar.

Determination of rishitin and lubimin. The levels of rishitin and lubimin were determined in the top mm of treated discs. Browning measurements were made first and then the top mm of 8 discs were removed and diced with a razor blade. The segments were mixed and duplicate 1 g (±0.05 g) samples weighed into separate boiling tubes containing 10 ml $CHCl_3$–MeOH (2:1). The tissue was macerated for 30 sec with an Ultra-Turrax homogenizer and filtered through a glass sinter. After partial purification of the extracts by TLC [34] the sesquiterpenoids were estimated by GC [35] using methyl stearate as int. standard.

Estimation of insoluble phenolic compounds in tuber discs. The method of extraction used is that of ref. [19]. The discs were first extracted with MeOH–$CHCl_3$–H_2O (2:1:0.8) (MCW) and then with PhOH–HOAc–H_2O (1:1:1) (PAW). The PAW residue was washed with ethanol to remove the PAW and air-dried. The PAW extracts from each sample were dialysed against running tap water overnight to remove the phenol. The contents of the dialysis tubing were centrifuged at 2000 g for 15 min at 1° and the residue washed with EtOH, air-dried and weighed. 20 mg aliquots of the dry ppe were weighed into dry Thunberg tubes. 2 ml of 2 M NaOH was added to the ppe and 2 ml of 0.5 M H_2SO_4 was added to the stopper-receptacle of the tube. The tube was flushed with N_2 and incubated in an oven at 70° for 16 hr to effect alkaline hydrolysis. After the hydrolysis all the precipitate was completely degraded. The tubes were cooled after removal from the oven and the contents of each tube and stopper-receptacle mixed to neutralize and used for the quinic acid assay and to produce ionization difference spectra. The residue left after extraction with PAW was still found to contain some brown material (an indication that some phenolics were probably still left in the residue).

50 mg of the residue was weighed into a dry Thunberg tube and subjected to alkaline hydrolysis as described above. After the hydrolysis and neutralization the contents of each tube were filtered through a No. 1 sintered glass funnel and the tube washed out with 2 ml distilled H_2O. The pH of each filtrate was adjusted to 7.5, the vol. made up to 10 ml and the phenolic compounds in them estimated.

Estimation of phenolic compounds. The method of ref. [36] was used to estimate quinic acid released after alkaline hydrolysis, to estimate the oxidized polymerised chlorogenic acid in the PAW extracts and the alkaline hydrolysate obtained from the cell walls. Ionization difference spectra were obtained using hydrolysed PAW extracts and the residues obtained after PAW extraction using the method described in ref. [23].

Estimation of phenolic acids using HPLC. The methods employed were modifications of refs [37, 38]. The free phenolic acid hydrolysates were isolated by the DEAE cellulose method of ref. [38] and eluted with H_2SO_4. A 25 cm long column of 4.6 mm diameter packed with Hypersil 5 ODS was used in an Applied Chromatography Systems gradient elution apparatus equipped with a variable wavelength UV detector set at 280 nm, and an integrator. The mobile phase was as follows: A. 5% HOAc in 10% MeoH; B. 5% HOAc in 100% MeOH. The gradient was started at an initial concentration of 15% of B in A and completed at 0% B in A.

2 μl quantities of extracts and standards were injected and chromatographed at 2000 psi at a flow rate of 1 ml/min. The column was calibrated frequently using 2 mg of recrystallized samples of standard phenolic acids in EtOAc. Phenolic acids were estimated from calibration curves. The peak area of each of the phenolic acids was linear in the range of 0–2 μg. The phenolic acids in the extracts were identified by comparing their retention times with authentic standards and also by 'spiking' with individual standards.

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