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The involvement of phenolics and phytoalexins in resistance of potato to soft rot

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Summary

Phenolics, for example chlorogenic, caffeic and ferulic acid, and phytoalexins, such as rishitin and phytuberin, were identified in potato tubers cv. Kufri Chandramukhi. The tissue of healthy tubers contained no detectable phytoalexins but did contain phenolics. The levels of these compounds were correlated with soft rot development. The rotting tissue either was free of these groups of compounds or had low concentrations. The wound periderm formed as a result of recovery from injury and infection contained high levels of the compounds. Much higher concentrations were detected at lower storage temperatures when oxygen supply was adequate. Antibacterial properties of the phenolics identified were tested against *Erwinia carotovora*, which was inhibited by chlorogenic, caffeic and ferulic acids. The three phenolics were more effective together, in proportions in which they occurred in wound periderm, than individually. It was observed that none of these phenolics could inhibit pectolytic enzymes of *E. carotovora*.

Introduction

Disease resistance in plants has been generally attributed to the presence of substances which inhibit the growth of pathogens. For example, cinnamic acid derivatives, especially quinones, and phytoalexins induced in potato tubers inhibit certain microbes (Rhodes & Wooltorton, 1978). Antibiotic properties of rishitin and phytuberin, which accumulate in response to *Erwinia carotovora*, have been studied (Lyon & Bayliss, 1975). Unoxidized phenolics and sesquiterpenoid phytoalexins are known to accumulate in wound periderm (Kolattukudy, 1978; Rhodes & Wooltorton, 1978). The present paper describes the role of phenolics and phytoalexins in potato tuber resistance to soft rot, with reference to the influence of environmental conditions on their concentrations. The mechanism of antibacterial activity of phenolic compounds was also studied.

Materials and methods

Organism
E. carotovora var. *carotovora* was isolated from a soft rot lesion of potato.

Potato
Mature and cured tubers of cv. Kufri Chandramukhi were used in these studies.

Inoculation of the tubers
Medium-sized (50-80 g each) sound tubers were washed in running tap-water, air-dried and cut into halves. The organism was grown in nutrient broth for 24 h at 27 °C, and the cells were harvested, washed twice and suspended in sterile distilled water; the suspension was adjusted to contain about 10^8 cells per ml. On each cut tuber surface 0.25 ml of suspension was spread evenly by tilting the tuber, and the surface was allowed to air-dry for 2 h at 25 °C.

The tubers were stored under various conditions of temperature, humidity and oxygen availability (Table 1) for two weeks, except those at 2 °C, which were stored for four weeks. The tubers were stored in open trays, in perforated polythene bags or in sealed polythene bags. The wound periderm formed during storage was removed carefully with a knife; wherever it had not formed, the rotting-tissue samples were collected, freeze-dried to constant weight and stored as powder in air-tight screw-capped bottles at 0 °C.

Extraction of phenolics
Lyophilized powder of wound periderm (10 g) was blended with 50 ml of 95 % methanol for 5 min and the suspension was filtered through a filter paper (Whatman No. 1). The process was repeated three times and all methanol extracts thus obtained were pooled, concentrated in a flash evaporator at 45 °C, dried over anhydrous Na_2SO_4, and stored at -20 °C for analysis (Huang & Agrios, 1979).

Estimation of phenolics
Qualitative analysis of phenolics was carried out by paper chromatography (Hanson & Zucker, 1963) and their quantitative estimation by a method of Clifford & Wight (1976).

High-performance liquid chromatography
Phenolics were estimated by high-performance liquid chromatography (Walter et al., 1979). A diluted portion of the methanol extract, containing about 0.2 g/l (as assessed by spectrophotometric assay), was analyzed by HPLC with a Model K ALC/CPC-202 (Water Associates, Milford, MA 01757, USA) equipped with 6000-A pump, U6K universal injector and a microbondapack C18 column (3.9 mm internal diameter and 30 cm length). The solvent system consisted of 40 ml methanol (double glass-distilled), 60 ml NaH_2PO_4 (0.033 mol/l) and 0.4 ml glacial acetic acid to adjust pH to 3.5, which was delivered isocratically at a flow rate of 0.8 ml/min. The effluent was monitored at 313 nm with an absorbance detector. The peaks were identified by comparison with retention time of authentic phenolic compounds (Sigma). Major cinnamic acid derivatives thus identified were chlorogenic, caffeic and ferulic acid.

Extraction of phytoalexins
Methanolic extracts were prepared from freeze-dried and powdered wound periderm or other potato tissues for the analysis of phytoalexins by the method of Henfling & Kuć (1979).

Separation of phytoalexins by thin-layer and gas-liquid chromatography
For TLC separation, the method of Lyon & Bayliss (1975) was adopted. The phytoalexins were detected by spraying the plates with 50 % H_2SO_4 and heating at 110 °C for

3–5 min and were further identified by comparing the mobilities with those of authentic samples.* Analysis by GLC was performed with a flame ionization detector. The pyrex glass column (3 mm internal diameter, 240 cm length), packed with 5 % OV 17 on 100–120 Supelcoport (Applied Science, USA), was used at a nitrogen flow rate of 30 ml per min. The components separated by GLC were identified by comparing their retention times with those of authentic samples, and the quantitation was done by using methyl stearate as an internal standard (Lyon et al., 1975).

Bacteriostatic and bactericidal action of phenolics

Freshly harvested cells were washed twice, suspended in distilled water (10^8 cells/ml) and then used in an antibiotic cup assay technique with nutrient agar (Booth, 1972) to study the bacteriostatic activity of methanolic solutions (0.1 ml) of cinnamic acid derivatives that included chlorogenic, ferulic and caffeic acids at 3 different concentrations (3.3, 5 and 10 mg/well). To assess the synergistic effect, a mixture of two or three phenolics was employed. The zones of inhibition were measured after incubation of plates for 48 h at 27 °C.

The bactericidal action of various phenolics was assessed by a viable-cell count. The bacterial suspension was added to a final concentration of 5.5×10^5 cells/ml to Seitz filter-sterilized aqueous solutions of the phenolics, incubated for 6 and 24 h and the appropriate dilutions plated on to nutrient agar. Colonies were counted after incubation at 27 °C for 48 h.

Growth inhibitory concentrations of phenolics

Various concentrations (0.5–2.5 g/l) of chlorogenic, caffeic and ferulic acids were added to nutrient broth, which was then inoculated with *E. carotovora* var. *carotovora*. Bacterial growth at 27 °C in shake cultures was monitored for 12 h by measurement of absorbance. Growth inhibition was also measured in mixtures of the three cinnamic acid derivatives in the proportions found in potato.

Results

Identification of cinnamic acid derivative

The phenolic extract separated into four bands by paper chromatography as seen by blue fluorescence under UV light. One, with an Rf value of 0.86, migrated with the authentic sample of caffeic acid, and showed blue fluorescence under UV light before and after NH_3 exposure. On elution with methanol and separation by HPLC, it resolved into two components, caffeic acid and ferulic acid, as judged by comparison of their retention times with authentic compounds. The second band, with an Rf value of 0.76, migrated with authentic chlorogenic acid which also exhibited blue fluorescence turning green on exposure to NH_3 under UV light. On elution and separation by HPLC, this component resolved into three compounds, tentatively identified as 3 chlorogenic acid isomers. The three isomers, chlorogenic acid, isochlorogenic acid and neochlorogenic acid were tentatively identified by two-dimensional paper chromatography also, by using 2 % acetic acid and a butanol/acetone/water mixture (12:3:5) as solvent systems. The spots

*Supplied by Dr G. D. Lyon, Scottish Crop Research Institute, Dundee, Scotland.

turned blue to green with NH_3 under UV light and showed characteristic absorption maxima at 323 nm. Two more bands with much lower Rf values (0.3 and 0.4) could not be identified by HPLC.

Fig. 1 illustrates the pattern of separation of cinnamic acid derivatives by HPLC, which resolved 6 components. The methanolic extract was injected into the column. Peaks 1, 2 and 3 constituted 64–74 % of total phenolics; they were tentatively identified as chlorogenic acid isomers. Peak 4, identified as caffeic acid, and peak 5, identified as ferulic acid, were relatively minor components, amounting to 5–10 % and 10–16 % respectively. Peak 6 remained unidentified as its retention time did not match any of the available authentic phenolic compounds, including catechin, m-coumaric acid, 3,4-dihydroxycinnamic acid, 4-hydroxycinnamic acid, trans-cinnamic acid and quinic acid.

Identification of terpenoid phytoalexins

Fig. 2 shows the pattern of separation of ethyl acetate extracts by TLC. Rishitin and phytuberin were identified by comparison with authentic samples. The identity of these compounds was confirmed by GLC, which gave the resolution pattern as shown in Fig. 3. Peak 1 was identified as phytuberin and peak 2 as rishitin, whereas peaks 3 and 4 remained unidentified.

Phenolic compounds in healthy tuber tissue

Spectrophotometric assay of normal periderm and cortical tissue of healthy tubers detected about 100–120 mg and 40–50 mg (per kg fresh wt. of tissue) respectively of these cinnamic acid derivatives. However, terpenoid phytoalexins were not detected in these healthy tissues.

Influence of oxygen availability on levels of phenolics and phytoalexins

Table 1 shows the concentrations of phytoalexins and phenolics detected in potatoes stored under different conditions. Potato-halves, incubated in open trays with an unrestricted air supply, formed dark, thick wound periderm. In contrast, potatoes either kept in perforated polyethene bags, which had a restricted air supply, or sealed in polythene bags, which had no air supply, failed to produce wound periderm and instead, developed soft rot. Rishitin and phytuberin accumulated only in the tubers kept in open trays, and levels of cinnamic acid derivatives were also higher in these tubers compared to those kept in perforated polythene bags; tubers in sealed bags had none of these compounds. The formation of wound periderm and the observed higher levels of phytoalexins and phenolics seemed to be governed by oxygen availability irrespective of the temperature (15–30 °C).

Effect of temperature on accumulation of phenolics and phytoalexins

The levels of total phenolics in wound periderm formed in response to *E. carotovora* infection at different temperatures, depicted in Fig. 4, shows that their accumulation was higher in the range 10–15 °C; at 10 °C about four times the amount in the range 20–25 °C, detected by HPLC. Table 2 shows that the relative amounts of individual cinnamic acid derivatives were not affected by temperature.

Fig. 5 indicates that rishitin levels were 3 times higher at 10 °C compared to 20–25 °C. The concentration of phytuberin was highest at 20 °C.

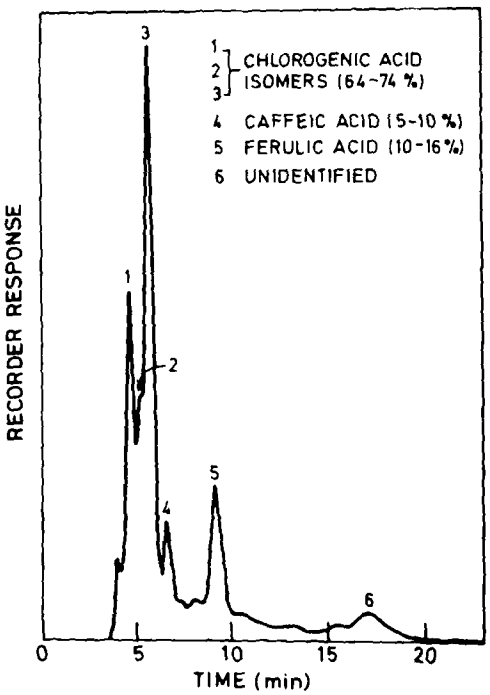

Fig. 1. Separation of phenolics by HPLC.

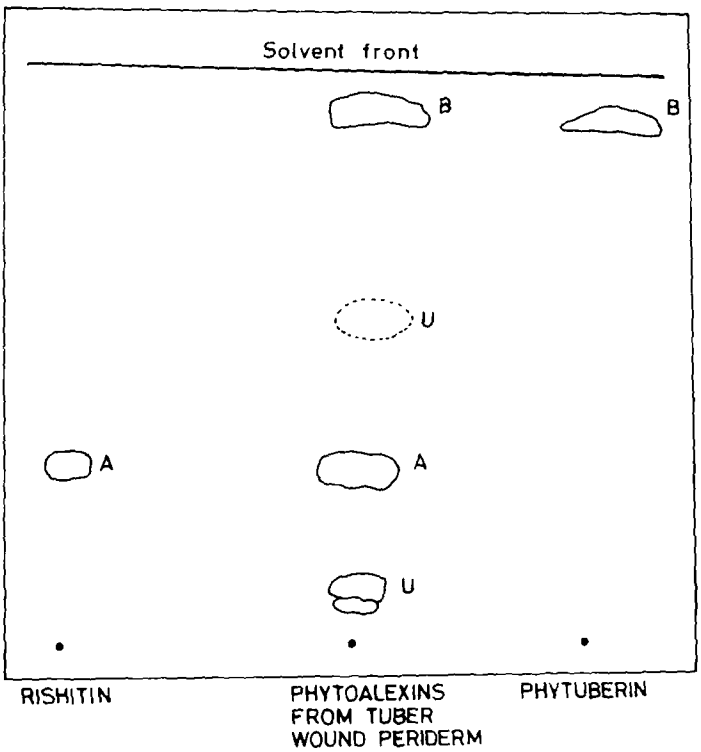

Fig. 2. Separation of phytoalexins by TLC. A: rishitin; B: phytuberin; U: unidentified.

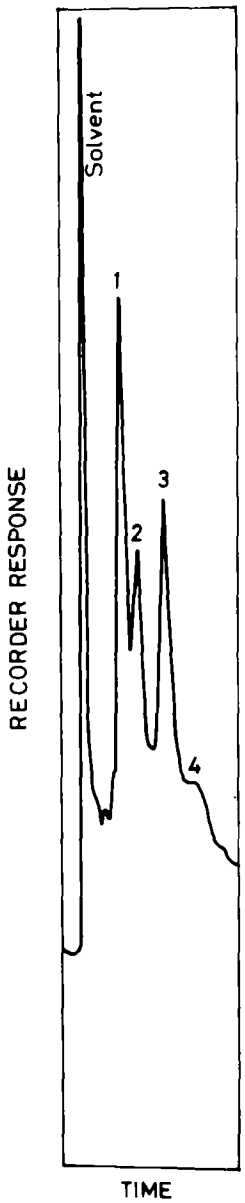

Fig. 3. Separation of phytoalexins by GLC. 1: phytuberin; 2: rishitin; 3 and 4: unidentified.

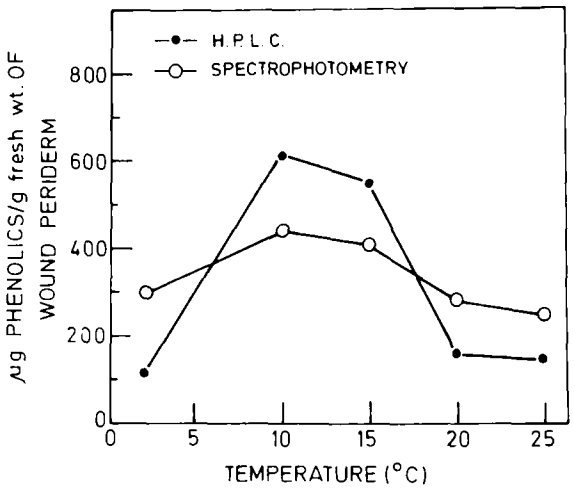

Fig. 4. Influence of storage temperature on concentrations of phenolics in wound periderm formed in response to infection by *Erwinia carotovora*.

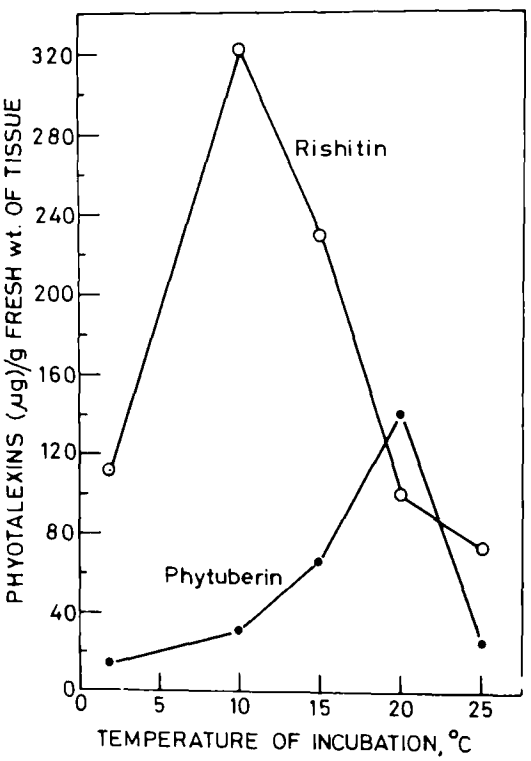

Fig. 5. Influence of storage temperature on levels of phytoalexins in wound periderm formed in response to infection by *Erwinia carotovora*. O—O rishitin; ●—● phytuberin.

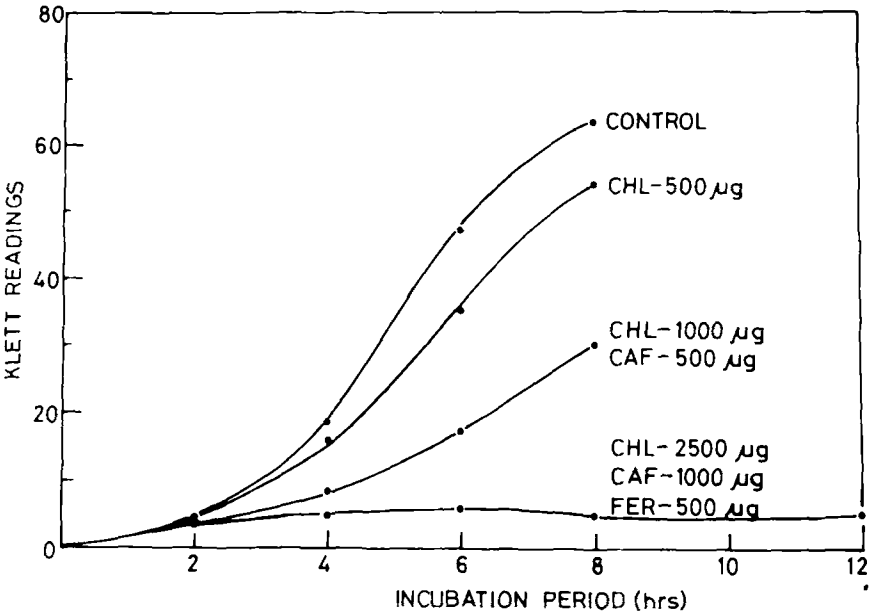

Fig. 6. Inhibition of growth of *Erwinia carotovora* by phenolics. CHL: chlorogenic acid; CAF: caffeic acid; FER: ferulic acid (mg/l).

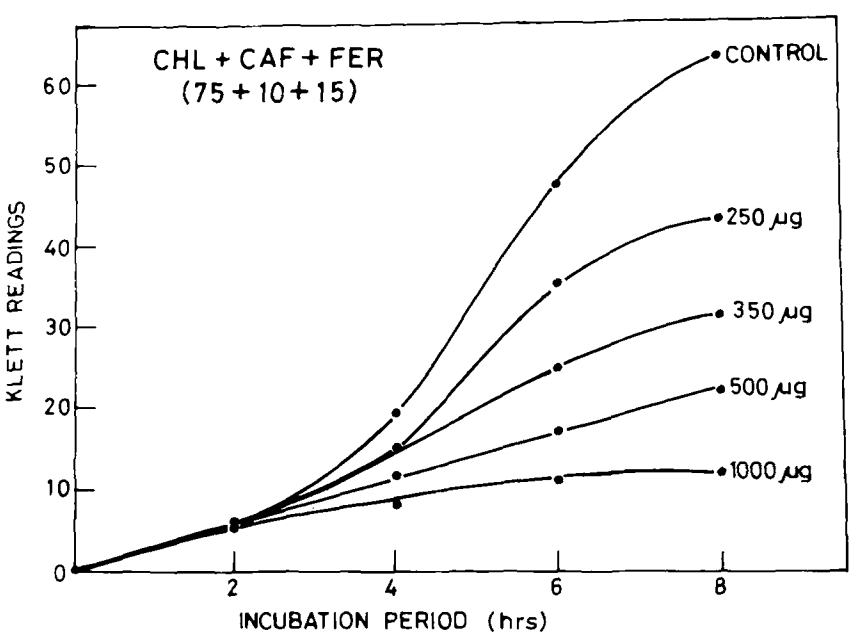

Fig. 7. Inhibition of growth of *Erwinia carotovora* by combination of phenolics. CHL: chlorogenic acid; CAF: caffeic acid; FER: ferulic acid.

Table 1. Influence of oxygen availability on accumulation of antimicrobial compounds in potatoes. [1]

Storage conditions	Nature of host response	Concentration (mg/kg fresh wt. of tissue)								
		rishitin			phytuberin			phenolics		
		15 °C	20 °C	25 °C	15 °C	20 °C	25 °C	15 °C	20 °C	25 °C
Open trays	dark thick wound periderm	230.6	102.4	73.6	67.5	142.0	26.3	407.0	283.3	248.3
Perforated polythene bags	dark rotted tissue	0	0	0	0	0	0	158.0	140.0	128.0
Sealed in polythene bags	white rotted tissue	0	0	0	0	0	0	0	0	0

[1] Details are given in the text.

Table 2. Percentage composition (w/w) of phenolics in potatoes at different storage temperatures after infection by *E. carotovora*.

Phenolic component	Percentage (%)				
	2 °C	10 °C	15 °C	20 °C	25 °C
Total chlorogenic acid isomers	65.6	73.1	68.4	74.2	64.4
Caffeic acid	4.9	5.5	9.1	7.8	10.0
Ferulic acid	14.3	14.6	14.2	10.1	16.5
Unidentified compound	12.3	6.6	9.6	8.8	9.0

Table 3. Bacteriostatic effect of phenolics on *E. carotovora*.

Compound	Quantity (mg)	Diameter of zone of inhibition (mm)
Chlorogenic acid	10	9.0
Caffeic acid	10	9.0
Ferulic acid	10	9.0
Chlorogenic + caffeic acid	5 + 5	11.0
Chlorogenic + ferulic acid	5 + 5	11.5
Caffeic + ferulic acid	5 + 5	12.0
Chlorogenic + caffeic + ferulic acid	3.33 each	16.5

Methanol alone showed no inhibitory activity.

Table 4. Growth rates of *E. carotovora* in presence of cinnamic acid derivatives.

Compound	Concentration (mg/l)	Increase in turbidity (Klett units/h)	Growth rate (% of control)
Control	0	8.0	100
Ferulic acid	150	5.5	68.7
Caffeic acid	100	7.0	87.5
Chlorogenic acid	750	6.0	75.0
In combination[1]	1000	1.5	18.6
In combination	500	2.6	32.5

[1] Mixture of chlorogenic, ferulic and caffeic acid was made in 75:15:10 (by weight) proportion.

Antibacterial properties of cinnamic acid derivatives

Bacteriostatic effect by cup assay

The diameters of the inhibition zones (Table 3) show that the three compounds possessed antibacterial activity against *E. carotovora* and that combination of two or three compounds exhibited synergism.

Growth inhibitory concentrations

Chlorogenic, caffeic and ferulic acids at concentrations of 2500, 1000 and 500 mg/l respectively inhibited growth of *E. carotovora* (Fig. 6). When used as a mixture in the proportions found in potato wound periderm, this acted synergistically and inhibited *E. carotovora* at even lower concentrations (Fig. 7, Table 4), but when tested individually at

concentrations used to form the mixture, they had only a slight inhibitory effect. The pH of the bioassay medium was not appreciably altered when the mixture of the three cinnamic acid derivatives was added. The additive effect was calculated to be 45 %.

The bacterial counts taken at time intervals after holding the cells of *E. carotovora* in the bacteriostatic concentrations of the three cinnamic acid derivatives indicated no bactericidal action. Possible inhibitory effects of these phenolic compounds on pectolytic activity were also assessed. *E. carotovora* was grown in nutrient broth containing 0.5 % Na-pectate for 24 h at 27 °C, and the cell-free supernatant was used as a crude pectolytic enzyme source. Thin (0.5 mm) discs were cut from cylinders of potato tissue aseptically cut from the cortex by using a cork borer (10 mm). They were incubated on a shaker at 27 °C for 24 h in the supernatant to which chlorogenic, caffeic or ferulic acids were added in concentrations up to 10 g/l; none of these compounds had any effect on pectolytic activity.

Discussion

Chlorogenic acid was the most abundant cinnamic acid derivative found in wound periderm, while caffeic and ferulic acids occurred in relatively minor quantities. Caffeic acid (a hydroxy acid) and chlorogenic acid (an ester of quinic acid and caffeic acid) are considered to be the direct precursors of quinones, which are formed as a result of injury or infection and are a defence mechanism in plants (Rhodes & Wooltorton, 1978; Vámos-Vigyázó, 1981). Chlorogenic acids are apparently the storage forms, which can be converted to caffeic acid as and when required. Chlorogenic acids exist in at least three forms in potatoes. The presence of isomeric forms of this compound has been reported in sweet potato, and 3-0-caffeoyl quinic acid ester has been identified as a major component (Walter et al., 1979).

The antimicrobial role of unoxidized cinnamic acid derivatives has so far remained obscure, but results presented here clearly show that the three phenolic compounds exerted antibacterial action against *E. carotovora*, ferulic and caffeic acids being more effective than chlorogenic acid. These phenolics also exhibited synergism; in combination the three compounds were more inhibitory at much lower concentrations than each alone.

The levels of phenolic compounds in wound periderm were higher in the inoculated tubers than in the uninoculated tubers. Rotting occurred when the levels of phenolics were low. This suggests that phenolics have a role in resistance to rotting; thus potatoes in sealed bags, in which phenolics could not be detected, had no resistance to soft rot. According to Rhodes & Wooltorton (1978) and Shirsat & Nair (1981) oxygen is essential for the induced formation of phenylalanine ammonia lyase (PAL), a key enzyme involved in synthesis of phenolics. The levels of phenolic compounds were higher in the tubers kept at lower temperatures (10–15 °C), perhaps accounting for the observed higher resistance of the tubers at lower temperatures.

Unlike phenolics, terpenoid phytoalexins were not present in healthy tissue, but they were found in wound periderm of infected potatoes. Rishitin and phytuberin were the only phytoalexins identified in this study and their levels were influenced by storage conditions; lower temperatures favoured higher levels, especially of rishitin. This terpenoid has been reported to exert a strong antibacterial action on *E. carotovora* at a

concentration in which it occurs in wound periderm (Lyon, 1972; Lyon & Bayliss, 1975; Lyon et al., 1975). Phytuberin, on the other hand, was inactive. It is worth noting that factors influencing the levels of phenolics similarly influenced rishitin levels.

Our results suggest that higher storage temperatures favour decay of the tubers by promoting pathogenesis and suppressing host resistance, while low oxygen availability promotes decay through suppression of host resistance alone.

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