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Comparison of the interactions of *Erwinia carotovora* ssp. *atroseptica* with *Phytophthora infestans*, *Phoma foveata* and *Fusarium coeruleum* in rotting potato tubers

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Summary

An interaction between *P. infestans* and *E. carotovora* subsp. *atroseptica* (Eca) in rotting tubers was confirmed and the biochemical basis for its occurrence investigated. The pH of tissue infected with *P. foveata* became alkaline whereas tissue infected with *P. infestans* or *F. coeruleum* did not rise above neutrality, which could promote pathogenicity of Eca by maintaining pH closer to the optimum for polygalacturonase activity. Polygalacturonase, pectate lyase and galactanase were detected in cultures of all three fungi grown on media containing cell wall material or pectin from tubers. As *P. infestans* produced more polygalacturonase than the other two fungal tuber rot pathogens the possibility was investigated that oligogalacturonide products of enzymatic degradation of pectin by *P. infestans* stimulates pathogenicity of Eca. However, while tubers soft-rotted after infiltration with supernatant from fungal cultures grown on tuber cell wall material, controls showed that rotting resulted from infiltration rather than the products contained in the infiltrated water.

Introduction

There are many accounts in the literature of an inter-relationship between *Erwinia carotovora* subsp. *atroseptica* (Eca) and fungal pathogens of potato resulting in an increase in blanking and blackleg in the growing crop. The existence of an interaction between *Erwinia carotovora* and *Fusarium* spp., first suggested by Blodgett (1947), has been confirmed since for *F. solani* (Stanghellini & Russell, 1971), *F. coeruleum* (Munzert et al.,1977), *F. sulphureum* and *F. oxysporum* (Zink & Secor, 1982) and *F. sambucinum* (Davis et al., 1983). Likewise Logan & Copeland (1979) indicated that when *Phoma exigua var. foveata* (*P. foveata*) was combined with Eca to inoculate tubers pre-planting there was a five-fold increase in the number of plants that failed to emerge.

These relationships refer to interactions expressed after planting. It is the perception of many potato growers in Northern Ireland that an interaction exists also between Eca and *P. infestans*, but one that is manifest mainly around harvest-time. In

conditions of low humidity *P. infestans* produces a 'dry' blight in tubers with no tissue maceration and no evidence of interaction with other pathogens, but when humidity is high growers consider that the presence of blight greatly pre-disposes tubers to develop bacterial soft rot.

At the onset of rotting *Erwinia* spp. bacteria are already within the epidermal tissue of tubers. So the mechanism of interaction cannot simply be one of *P. infestans* facilitating entry of *Erwinia* through the periderm. As both pathogens produce pectic enzymes (Jarvis et al., 1981; Collmer & Keen, 1986), there is the possibility that degradation products produced by the activity of *P. infestans* enzymes act as elicitors for pectic enzymes secreted by *Erwinia,* so promoting pathogenicity. Thus a comparison was made of the pathogenic interactions in tubers between Eca and the fungi that cause late blight (*P. infestans*), gangrene (*P. foveata*) and dry rot (*F. coeruleum*). The possible role of the production of pectic enzymes, known to be important in the pathogenicity of Eca, *P. infestans* and *F. coeruleum* (Collmer & Keen, 1986; Olsson, 1989; Protsenko et al., 1991), was investigated.

Materials and methods

Bacterial and fungal pathogens. The isolates of pathogens used in the study originated in diseased tubers grown in Northern Ireland. They were maintained on rye agar (*P. infestans*), malt agar (*P. foveata, F. coeruleum*) and nutrient agar (*E. carotovora* ssp. *atroseptica*).

Development of bacterial soft rot in P. infestans inoculated tubers. Minitubers of cv. Russet Burbank, the progeny of microplants grown in peat compost, were bisected lengthways and a hole (5 mm diam., 3 mm deep) punched through the skin in each half. One half of each tuber was inoculated with 20 µl of 3×10^4 ml^{-1} suspension of sporangia of *P. infestans*, the other half with 20 µl sterile distilled water (SDW). After 24 h at high RH and 5, 10, 15, 19, 21, 24 or 27 °C, both halves of eight tubers per temperature received a second inoculation with 20 µl of 2×10^7 c.f.u. ml^{-1} of Eca. One tuber per temperature was inoculated with 20 µl SDW. The scale of bacterial soft rotting was assessed as % tissue rotted 7d after inoculation with Eca. The experiment was repeated three times.

Fungal infection of potato tubers previously infiltrated with Eca. Minitubers of cv. Russet Burbank were immersed under vacuum (Hossain & Logan, 1983) for 5 min in either SDW or 10^6 c.f.u. Eca ml^{-1} from a 24 h-old nutrient agar culture (240 tubers in each). After incubation overnight (10 °C, 90% RH), they were transferred for 5 d to 60–70% RH. Each batch of tubers was then divided into three groups of 80 tubers, from each of which 40 were challenged with a fungal pathogen and 40 were inoculated with SDW.

To challenge with *P. infestans* tubers were sprayed with 6×10^4 sporangia ml^{-1} then incubated at 15 °C and 90% RH until symptoms developed. Control tubers were sprayed with SDW. To challenge with *P. foveata* or *F. coeruleum*, two holes, 5 mm di-

am., 5 mm deep, were made in opposite sides of the surface of each minituber and into each a plug from a fungal culture on Czapek Dox agar (CDA) was placed, then covered with the extracted plug of tuber tissue. As a control, tubers were inoculated with a plug of CDA. All minitubers were incubated in darkness at 10 °C (*P. foveata)* or 15 °C (*F. coeruleum)* for 2–4 wk until symptoms developed. Then they were transferred to 20 °C and high humidity to promote bacterial soft rotting, the development of which was monitored over 2 wk.

pH of tubers inoculated with different fungi. Tubers of cv. Dundrod were surface-sterilised and inoculated with *P. infestans*, *P. foveata* or *F. coeruleum* (10 tubers per fungus) and incubated under the appropriate conditions until symptoms developed. A 15 mm diam. core, extending across both healthy and infected tissue from periderm to central medulla, was then extracted from each inoculated tuber. Cores were divided into discs 2.5 mm thick. Individual discs were macerated in 3 ml SDW and the pH of the macerate measured.

Production of potato tuber cell wall material and pectin. One kg of peeled and chopped potatoes was blended with 1 l of ice-cold acetone for 30 s and filtered through four layers of muslin. The process was repeated with 1 l of acetone, twice with 1 l of 1:1 v/v methanol:chloroform and twice more with 1 l of acetone, filtering the extract between washes. Washed extract was dried until free from acetone, then kept desiccated at room temperature. Normally 1 kg of potatoes produced about 60 g of cell wall material (CWM).

Production of pectin. CWM was boiled in 50 vol. of 0.1 M oxalate-citrate buffer, pH 4.0 for 15 min then centrifuged at 600 g for 90 min. The supernatant was decanted and clarified by filtration through filter paper (Whatman No. 1) then 2 volumes of ethanol were added gradually to precipitate pectin substances. After the precipitate settled, the supernatant was decanted and the remaining liquid removed by centrifugation. The pellet was extensively washed with 70% (v/v) and then 99% (v/v) ethanol, allowed to dry and ground to a fine powder.

The three fungi were grown in Henniger (1963) medium supplemented with 0.5% w/v of either potato tuber CWM or pectin. Five plugs (5 mm diam.) obtained from 7 d-old cultures grown on agar were inoculated into 250 ml flasks containing 150 ml media, pH 5.2. *P. infestans* and *P. foveata* were grown in darkness at 15 °C in still culture, *F. coeruleum* was grown at 20 °C in shake culture (120 rpm). After incubation, cultures were checked for freedom from bacterial contamination, filtered and dialysed overnight at 4 °C against water, then stored at –20 °C until used.

Liquid cultures of fungi were filtered twice through three layers of cheese-cloth then centrifuged at 4000 g for 30 min and the supernatant collected. $(NH_4)_2SO_4$ was slowly added to 20% saturation and stirred for 2 h. After centrifugation at 4000 g for 30 min, $(NH_4)_2SO_4$ was added to 80% saturation and allowed to dissolve overnight. Next day the liquid was centrifuged at 4000 g for 30 min and the pellet retained, dissolved in 2 ml SDW and dialysed against water. All processing was done at 4 °C. Con-

Table 1. The effect of presence of tissue rotted with *Phytophthora infestans* on development of soft rot in tubers subsequently challenged with *Erwinia carotovora* ssp. *atroseptica*.

Incubation of Eca-challenged tubers at (°C)	Tuber flesh (%) soft rotted after 7 d			
	Tubers inoculated, then challenged with			
	Water/ water	Water/ Eca	*P. infestans*/ water	*P. infestans*/ Eca
5	0	0	0	0
10	0	0	0	4.0
15	0	0	0	9.4
21	0	0	0	22.8
24	0	0	0	20.1

L.S.D. ($P \leq 0.05$) 7.3

centrations of pectate lyase, polygalacturonase and galactanase were measured in tuber tissue and pathogen culture filtrate extracts as described by Collmer et al. (1988).

Tubers of cv. Arran Banner naturally contaminated and found, using the procedure of Bain et al. (1990), to carry an average of 5×10^6 c.f.u. Eca per tuber, were vacuum-infiltrated for 5 min with culture filtrates of each of the three fungal pathogens grown on tuber CWM. Filtrates were prepared and dialysed as described above, then divided into two equal portions, one of which was boiled for 30 min to denature the enzymes. As a control, tubers were infiltrated with SDW or left uninfiltrated. All tubers were incubated at 25 °C, in darkness and in high humidity until assessment of the number of rots on each, 5 d later. The experiment was repeated twice.

Data from all tuber experiments were subjected to appropriate multi-factorial statistical analyses.

Results

Development of bacterial soft rot in blighted tubers. There was a highly significant effect of both *P. infestans* infection and the temperature of incubation on the amount of bacterial soft rotting that developed in tubers secondarily challenged with Eca or water (Table 1). No soft rotting occurred in the absence of infection by *P. infestans* or where the second inoculation was with SDW, but when Eca inoculation followed infection by *P. infestans*, extensive soft rotting occurred, especially in tubers incubated at higher temperatures. Similarly, when fungal rot pathogens were inoculated into tubers carrying latent inoculum of Eca, *P. infestans* in combination with Eca produced more soft rot than the sum of the rotting produced by either pathogen alone (Fig. 1). In contrast, no soft rotting occurred in Eca-infiltrated tubers subsequently inoculated with *P. foveata*, but some rot developed after inoculation with *F. coeruleum*. However, there was no indication of a synergistic relationship with Eca as exhibited in tubers infected with *P. infestans*.

All three fungi raised the pH of both healthy and diseased tissue, in rotting tubers (Table 2) and in liquid culture containing tuber CWM or pectin (Fig. 2). *P. foveata*

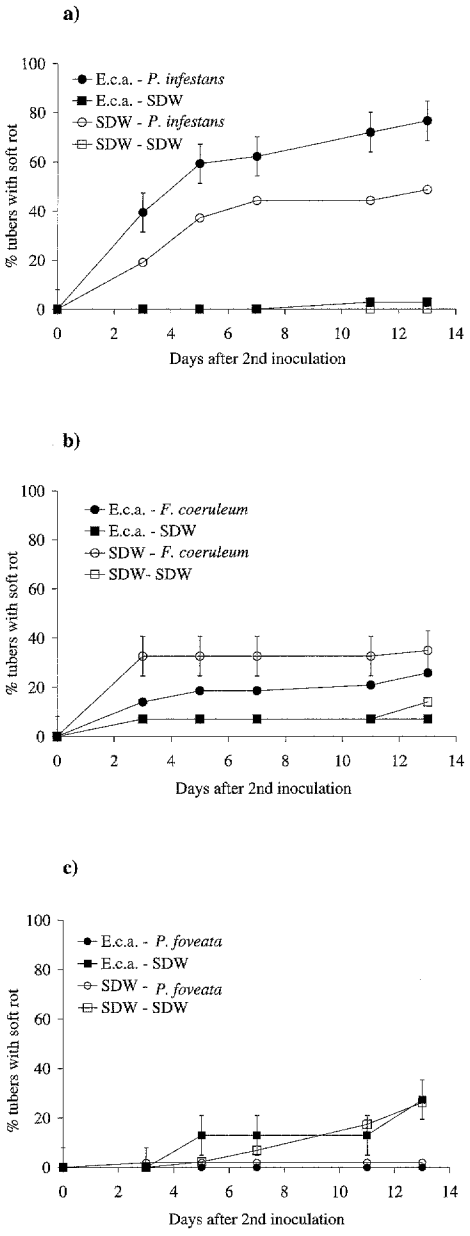

Fig. 1. The effect of inoculation with a) *Phytophthora infestans*, b) *Phoma foveata* or c) *Fusarium coeruleum* on soft rot development in tubers previously infiltrated with *E. carotovora* subsp. *atroseptica*. Vertical bars are SEM.

Table 2. pH of tuber tissue following inoculation with fungal pathogens. Data above the bold line refer to diseased tissue, data beneath the line refer to healthy tissue.

	Tuber tissue pH			
	P. infestans	*P. foveata*	*F. coeruleum*	Uninoculated
Cortical tissue	6.9	7.9	7.0	6.0
Interface of diseased	6.7	7.6	6.9	5.9
and healthy tissue	6.7	6.9	6.6	5.9
		6.5	6.3	
Medullary tissue		6.5	6.2	

affected pH most, raising it to alkalinity, both in tubers and in medium containing pectin as carbon source.

Pectic enzymes. In liquid culture, with tuber CWM or pectin as sole carbon source, *P. infestans* produced the highest quantity of pectic enzymes (Fig. 3). A peak of polygalacturonase and galactanase activity at the beginning of the incubation period was followed by a rise in pectate lyase production, which was slightly greater with CWM as substrate. Neither *P. foveata* nor *F. coeruleum* produced peaks of pectic enzyme activity in the first days of incubation and total expression of pectic enzyme activity was considerably lower than with *P. infestans*.

Reaction products of the degradation of pectin by Eca-secreted pectate lyase and polygalacturonase mediate induction of pectic enzyme expression by Eca (Collmer & Bateman, 1982). Therefore the possibility that the products of fungal-expressed pectic enzymes could do likewise and enhance pathogenicity of Eca was investigated. However, while tubers carrying latent inoculum of Eca did develop soft rots after vacuum infiltration with culture filtrates (Fig. 4), the occurrence of equal numbers of rots after infiltration with boiled filtrate or SDW indicates that it was infiltration of water, rather than the culture products, that promoted pathogenicity of Eca.

Discussion

The experiments confirmed the existence of an interaction between pectolytic bacteria and pathogenic fungi in rotting tubers, in which both the fungus involved and the incubation temperature were important. A clear association between *P. infestans* and Eca was observed in which predisposition to soft rotting occurred both when *P. infestans* was a primary and a secondary pathogen infecting tubers. While the association was most obvious at higher temperatures, it may actually be temperature independent as the progress of soft rotting at low temperatures is normally too slow for it to have become noticeable in the 7 and 13-day incubation periods used. High relative humidity is a more stringent pre-requisite for the association. In preliminary experi-

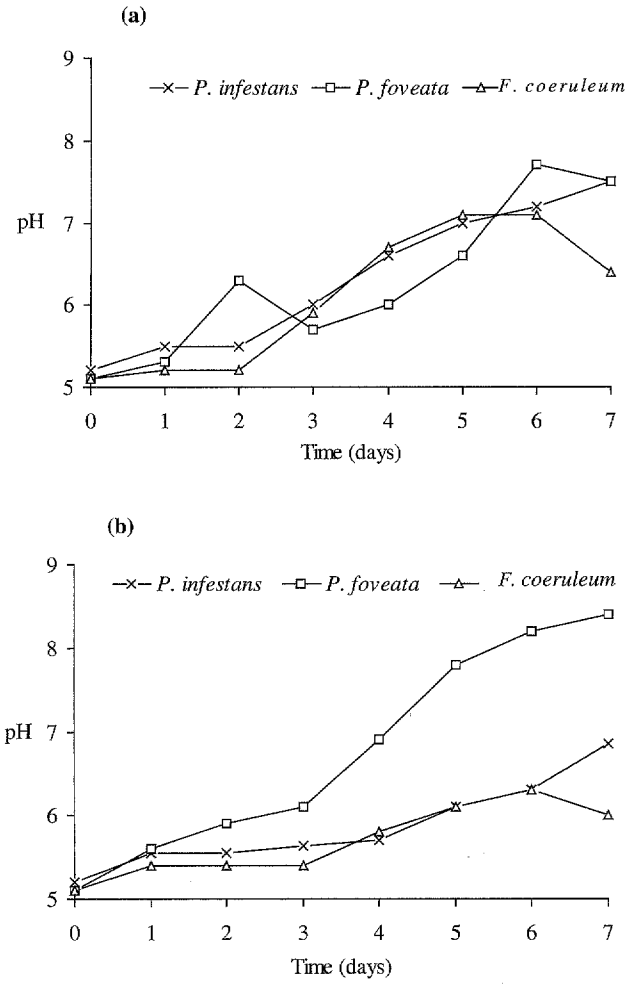

Fig. 2. pH of liquid media in which pathogenic fungi were grown with (a) tuber cell wall material or (b) tuber-sourced pectin, as sole carbon source.

ments Eca did not cause rot in blighted tubers, but when greater care was taken to maintain high RH soft rotting consistently developed.

F. coeruleum was found to share some of the capacity of *P. infestans* to interact with Eca, which is consistent with reports of *Fusarium* spp. in combination with Eca increasing seed piece decay and blackleg (Davis et al., 1983), but its presence did not stimulate bacterial tissue maceration as much as *P. infestans*. In tubers inoculated with *P. foveata*, pectolytic bacteria seemed unable to produce soft rot when the fungus was present. This is consistent with growers' perceptions that *P. foveata* does not

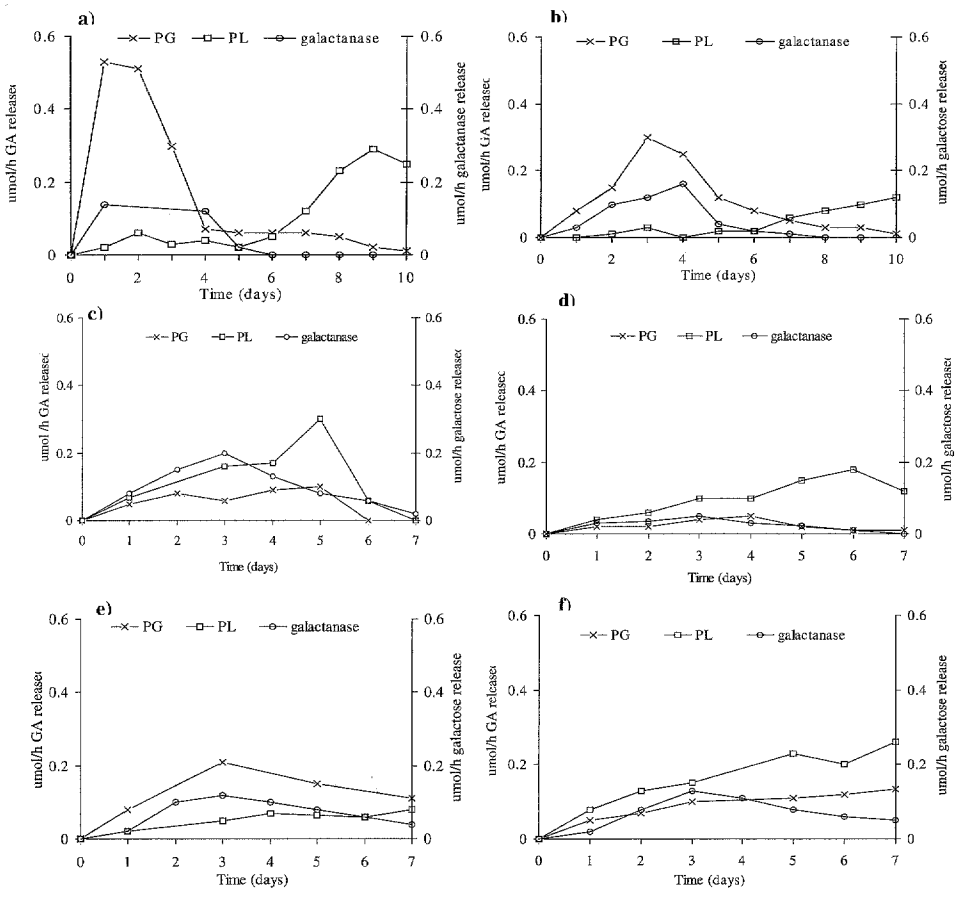

Fig. 3. Pectic enzyme production by *Phytophthora infestans,* (a, b), *Phoma foveata,* (c, d), *Fusarium coeruleum* (e, f) in liquid media with potato cell wall material (a, c, e) or pectin (b, d, f) as a sole carbon source. PG = polygalacturonase, PL = pectate lyase.

normally interact with Eca as much as does *P. infestans* to produce additional soft rotting. In part this may be because the conditions for successful infection and symptom development are different for each fungal pathogen. While *P. infestans* can infect unwounded and unbruised tubers, infection by both *P. foveata* and *F. coeruleum* normally needs wounds or bruises on the surface of the tuber. Water status is important also as *P. infestans* needs high RH for infection of tubers and this also promotes the pathogenicity of Eca (Pérombelon & Kelman, 1980), whereas *P. foveata* and *F. coeruleum* have a less defined RH requirement and can infect largely independent of humidity.

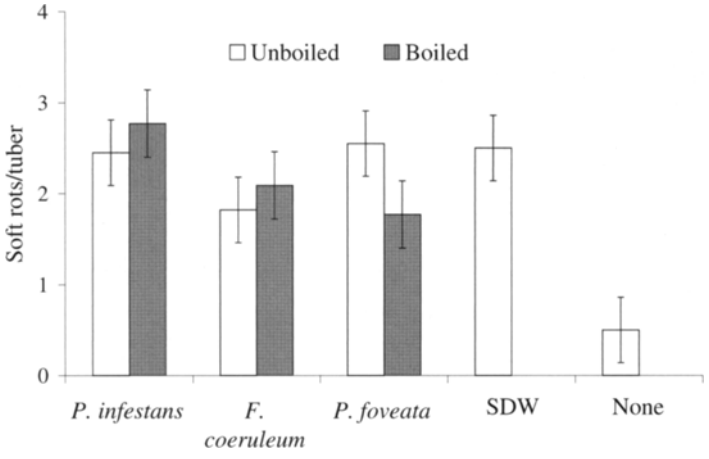

Fig. 4. Development of bacterial soft rot after infiltrating tubers with unboiled and boiled clarified liquid media in which fungal pathogens had been grown using tuber CWM as sole carbon source. Vertical bars are SEM.

In some of the experiments the Eca bacteria that interacted with *P. infestans* were latent in the superficial tissues of tubers when the fungal inoculum was introduced. Thus *P. infestans* must have altered conditions within unwounded tissue to trigger bacterial pathogenicity rather than facilitating entry of Eca through the periderm. Potentially, it could do this in several ways.

One possibility is that infection by the fungus modifies tuber tissue influencing the efficiency of bacterial exoenzymes involved in pathogenicity. As tissue invaded by *P. infestans* has a lower pH than that invaded by the other two fungi it would remain more suited to the degradative activity of bacterial polygalacturonases, which are expressed early in development of soft rotting and have a pH optimum between 5 and 6 (Pagel & Heitefuss, 1990; Collmer et al., 1982).

Alternatively, or additionally, promotion of bacterial soft rotting could result from the fungal pathogens producing substances that induce expression of bacterial pectolytic enzymes. Oligomers and monomers of galacturonic acid released by the basal activities of exopolygalacturonase and pectate lyase degradation of pectin have been shown to be auto-inducers of expression of these enzymes by Eca (Collmer & Bateman, 1982).

P. infestans produced relatively high quantities of polygalacturonase when grown on cell wall material or pectin from tubers and the early peak of production was a distinctive difference between it and the other two tuber fungal pathogens. However, when tubers were infiltrated with culture filtrates that would have contained pectolytic enzymes and their degradation products, including oligo- and mono-saccharides, no more soft rotting was caused than by infiltration with water alone.

The activating effect produced by infiltrated water was greater than that of any in-

ducing products present in the culture liquid. Thus while these investigations did not provide any evidence in favour of a role for substances produced by *P. infestans* in inducing Eca pathogenicity, the possibility cannot be completely discounted.

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