POLYGALACTURONASE PRODUCED BY *BOTRYTIS FABAE* AS ELICITOR OF TWO FURANOACETYLENIC PHYTOALEXINS IN *VICIA FABA* PODS

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SUMMARY

Polygalacturonase, produced by *Botrytis fabae* in infected faba bean pods, was separated by isoelectric focusing and tested as possible elicitor of the phytoalexins wyerone acid and wyerone. The isoenzyme profile was characterised by multiple forms within a restricted range of isoelectric points (pI). These were indicated as very acidic, acidic and basic. Using liquid isoelectric focusing, polygalacturonase isoenzymes were separated into four different isoenzyme pools. These pools, tested at a range of concentrations, acted as an elicitor of wyerone acid and wyerone in endocarp pod tissues, the former being more prevalent. The level of phytoalexin accumulation varied depending on the enzymatic pool tested. Time course analysis revealed that phytoalexins accumulated at higher levels when lower enzyme doses were used. Each pool of isoenzymes was found to produce dark-brownish lesions similar to those observed from tissues inoculated with the pathogen. The possible dual role of polygalacturonase isoenzymes from *B. fabae* as putative pathogenicity determinants or defence responses elicitors during broad bean colonisation is discussed.

Key words: *Botrytis fabae*, polygalacturonase, *Vicia faba*, wyerone, wyerone acid.

INTRODUCTION

Chocolate spot disease, caused by *Botrytis fabae* Sard., is a major disease of broad bean (*Vicia faba* L.). *B. cinerea* Pers.: Fr., causing the grey mould of numerous vegetable crops, is a weak pathogen on *V. faba*, producing limited, non-coalescent and non-sporulating lesions (Purkayastha and Deverall, 1965a) whereas *B. fabae* can spread and sporulate from lesions (Purkayastha and Deverall, 1965b; Mansfield and Deverall, 1974a; Harrison, 1988; Hashim *et al.*, 1997). *V. faba* tissues produce, as a post-infection defence response against fungal pathogens, low-molecular-weight secondary metabolites, such as furanoacetylenic phytoalexins (Fawcett *et al.*, 1971; Hargreaves *et al.*, 1977; Ingham, 1982). Two of the most important of these are wyerone acid and its methyl ester wyerone, and their induced accumulation in infected tissues causes inhibition of fungal growth (Letcher *et al.*, 1970; Mansfield and Deverall, 1974b; Hargreaves *et al.*, 1977; Rossall *et al.*, 1980). The predominant broad-bean phytoalexin, wyerone acid, accumulates in *B. cinerea* lesions, whereas in *B. fabae* lesions the phytoalexin starts to accumulate but later tends to decrease. The greater ability of *B. fabae* to colonise broad bean tissues seems to be related to its capacity to detoxify broad bean phytoalexins and to reduce their toxic effects (Mansfield and Deverall, 1974b; Hargreaves and Mansfield, 1975; Hargreaves *et al.*, 1977; Rossall *et al.*, 1980; Rossall and Mansfield, 1984; Madeira *et al.*, 1993).

B. fabae is able to produce pectin degrading enzymes, such as polygalacturonase (PG), during development of chocolate spot (Balasubramani *et al.*, 1971; Harrison, 1988). These enzymes were indicated as the principle cause of plant cell death during lesion development (Mansfield and Richardson, 1981). A recent immunoelectron microscopy study has indicated the central role of pectin-degrading enzymes in *B. fabae* post-penetration processes and host cell wall breakdown (Cole *et al.*, 1998). Pectolytic enzymes have been suggested to be responsible for plant tissue maceration, cell death and putative pathogenicity factors in necrotrophic pathogenesis (Alghisi and Favaron, 1995). However, it has been shown that pectolytic enzymes potentially also play a key role in resistance by releasing plant cell wall-derived elicitors that activate diverse defence responses, such as phytoalexin accumulation (Bruce and West, 1982; Favaron *et al.*, 1988; Davis *et al.*, 1984).

Since PGs as elicitors of broad bean phytoalexins infected by *B. fabae* have yet to be described, we have investigated the ability of separated pools of PG isoenzymes produced by *B. fabae* to induce in faba bean the accumulation of the furanoacetylenic phytoalexins wyerone acid and wyerone.

MATERIALS AND METHODS

Inoculation of pods and extraction of pectolytic enzymes. Broad bean pods (*V. faba* var. *major*) were pre-

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pared and inoculated as previously described (Deverall, 1967). Pods obtained from a local market were cut along the carpel suture line, and the seeds removed to expose the pod cavities. Endocarp tissue was inoculated with *B. fabae* isolate Bf118, using one agar disc (6-mm diameter) per pod cavity cut from the edge of 7-day-old fungal cultures grown on potato dextrose agar (PDA) (Oxoid, Unipath Ltd, Basingstoke, England). Inoculated tissues, incubated at 21°C, 90% RH in the dark, were collected 4 days later, pulverised in liquid nitrogen with a mortar and pestle and extracted using ice-cold 20 mM Tris buffer pH 6.8 (1 g tissue ml^{-1} buffer), containing 0.5% cysteine (Sigma Chemical Co., St. Louis, USA) and 1% insoluble polyvinylpolypyrrolidone (Sigma Chemical Co.). The slurry was strained through three layers of cheesecloth, and the liquid centrifuged at 15,000 *g* for 15 min at 4°C and dialysed against distilled water at 4°C for 24 h.

Measurement of PG activity and isoenzyme patterns. PG activity was determined as the increase in reducing groups over time. Reducing groups were measured by Nelson's method (1944), using D-galacturonic acid (Sigma Chemical Co.) as a standard. Activity was expressed as reducing units (RU). One RU was defined as the amount of enzyme producing 1 µmol of reducing groups min^{-1} at 30°C from 0.25% (w/v) polygalacturonic acid (PGA) (Sigma Chemical Co.) in Na-acetate buffer (0.1 M, pH 5.0). "Endo" or "exo" activity of PGs was established according to Bateman and Basham (1976) using Cannon-Fenske viscometers, size 200, and 5% pectin (Sigma Chemical Co.) in 0.1 M Na-acetate buffer, pH 5.0. Reaction mixtures with heat-inactivated enzyme were used as controls.

PG isoenzymes were separated horizontally on a Multiphor II apparatus (Pharmacia Biotech, Uppsala, Sweden) using 0.4 mm thick polyacrylamide gel containing 5% (v/v) ampholytes (Pharmacia Biotech, Uppsala, Sweden) covering the pH range 3.5-10.0 under conditions previously described (Chilosi and Magro, 1998), followed by incubation at 30°C with ultrathin agarose overlay gel (10 g l^{-1} agarose with 1 g l^{-1} polygalacturonic acid buffered at pH 5.0 with 50 mM Na-acetate) and subsequent 0.5 g l^{-1} ruthenium red staining as described by Ried and Collmer (1985). The pI values of pectolytic isoenzymes were estimated from a regression equation of standard proteins (Pharmacia Biotech, Uppsala, Sweden) versus the distance migrated.

Separation of polygalacturonase isoenzymes. The separation of PG isoenzymes was performed by liquid isoelectric focusing (IEF) in a Rotofor apparatus (Bio-Rad Laboratories Hercules, USA) as previously described (Di Pietro and Roncero, 1996). IEF was carried out in a total volume of 55 ml containing 1.2% (v/v) ampholytes (Pharmacia Biotech) covering the pH range 3.5-10.0. The run was performed at 4°C for 6 h at 12 W constant power. Twenty fractions were collected and analysed for PG activity. Fractions of interest were pooled and dialysed against several changes of distilled water at 4°C for 24 h.

Preparation of enzyme extracts and phytoalexin elicitor assay. Different fractions containing pools of separated PGs were obtained from liquid IEF. Each pool was used as potential phytoalexin elicitor on healthy faba endocarp pod tissues. Each pool was diluted to obtain two PG doses (0.05 and 0.10 RU). Controls consisted of 0.1 RU heat inactivated enzyme. Tissues were treated with enzyme preparations by dispensing 30 ml droplets in each pod seed cavity, followed by incubation at 20°C and high RH, in the dark.

Extraction and detection of wyerone acid and wyerone. The samples were collected at 1, 3 and 5 days after treatment and homogenised in diethyl ether (Fluka, Buchs, Switzerland) in order to extract furanoacetylenic phytoalexins. The ether supernatant was separated from the polar phase and the extraction repeated on the aqueous residue. Pooled extracts were dried by rotary evaporation, adding ethanol to remove residual water, and residues dissolved in HPLC grade methanol (1 ml g^{-1} fresh wt) (Carlo Erba Reagenti, Rodano, Milan, Italy). Stock solutions of the phytoalexins were stored at –20°C. The methanolic samples were analysed by high performance liquid chromatography (HPLC). Isocratic separation was achieved using a Beckman mod. 126 pump system and Beckman mod. 168 diode array detector (Beckman Instruments Inc., Palo Alto, USA). Data were processed by the Beckman Gold Nouveau program. Elution parameters were: column Waters-Spherisorb (Milford, Massachusetts, USA) 15x4.6 mm, stationary phase C18 reverse phase ODS 2; mobile phase acetonitrile (Sigma-Aldrich, Steinheim, Germany): 1% aqueous formic acid (Merck, Darmstadt, Germany) 60:40 v/v, flow rate 1 ml min^{-1}. Elution times and concentrations (peak areas) of wyerone acid and wyerone, were registered and quantified by comparison of peak areas with those of pure standards of known concentration. Phytoalexin standards were recovered from diethyl ether faba pod endocarp tissue inoculated with the weak pathogen *B. cinerea*, in order to obtain significant amounts of wyerone acid and wyerone. Both phytoalexins were separated by preparative thin layer chromatography (TLC) on silica gel 60 (Merck, Darmstadt, Germany) using diethyl ether:methanol (5:1 v/v), as previously described (Hargreaves *et al.*, 1977). Concentrations were measured by ultraviolet spectrophotometry using the following extinction coefficients: wyerone, λ max 350 nm and wyerone acid, λ max 356 nm, ε = 27000 (Fawcett *et al.*, 1968; Letcher *et al.*, 1970). Phytoalexin concentration was indicated as µg g^{-1} fresh weight.

RESULTS

Polygalacturonase activity and PG isoenzyme pattern. Broad bean pod cavities inoculated with *B. fabae* were monitored after inoculation for development of symptoms (dark water-soaked lesions at inoculated sites). Uninoculated broad bean pod cavities were used as control. The fungal isolate produced a consistent amount of PG upon inoculation on faba bean pods, approximately 2.0 RU g^{-1} fresh weight. Pectin lyase (PNL), another pectolytic enzyme produced by several phytopathogenic fungi, was detected at minimal level (data not shown). Control plants treated with sterile PDA blocks remained healthy, with no pectolytic activity in extracts from tissues.

The dialysed extract from four day inoculated pod endocarp tissue was subjected to thin layer polyacrylamide gel IEF and evaluated for PG isoenzymes. A PG pattern of nine PG activity bands was observed; PGs were grouped as very acidic (PG1, PG2 and PG3 with pI <3.5), acidic (PG4, pI 4.7; PG5, pI 5.2; PG6, pI 5.3; PG7, pI 5.7) and basic (PG8, pI 8.0; PG9, pI 8.2) (Fig. 1). Acidic PGs were predominant. No PG bands were detected in extracts from control tissues.

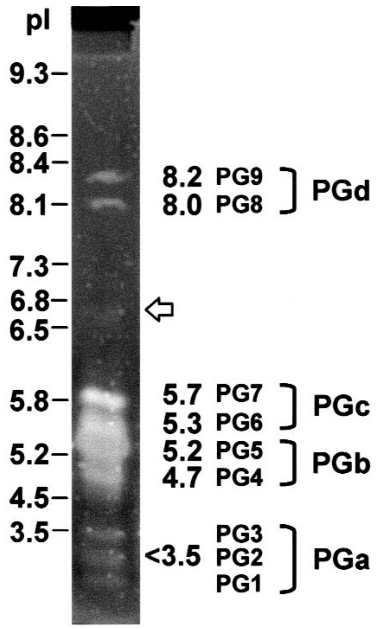

Fig. 1. *Botrytis fabae* polygalacturonase (PG) isoenzyme pattern from broad bean pod endocarp tissues. Samples were separated on an isolectric focusing (IEF) ultrathin gel (pH 3.5-10.0), followed by agarose overlay activity staining. Positions and pI values of polygalacturonase bands are indicated on the right. Positions and pI values of IEF markers are shown on the left. PGa: very acidic isoenzyme pool; PGb and PGc: acidic pool; PGd: basic pool.

Separation of PG isoenzymes by liquid IEF. The protein extracts from infected faba pods were subjected to liquid IEF in the pH range 3.5-10.0. Liquid IEF split PG activity into four peaks (Fig. 2). Peaks with multiple forms (isoenzymes) of PG, were designated PGa (PG1, PG2 and PG3) and PGd (PG8 and PG9) which included very acidic and basic isoenzymes, respectively. Acidic PGs were separated into two pools, PGb (PG4 and PG5) and PGc (PG6 and PG7). PGa and PGd showed mostly endo-activity, PGb had both significant endo- and exo-activity, while PGc displayed prevalent exo-PG activity.

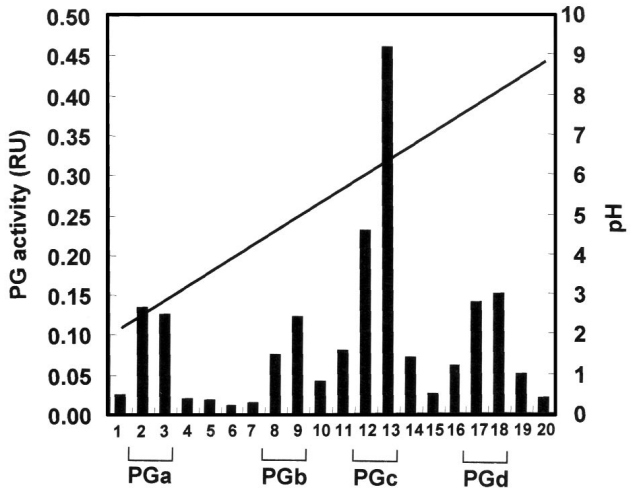

Fig. 2. *Botrytis fabae* polygalacturonase (PG) activity (RU) from broad bean pod endocarp tissues. Samples were separated by liquid isolectric focusing (IEF) on Rotofor apparatus (pH 3.5-10.0). Polygalacturonases were grouped as PGa: very acidic isoenzyme pool; PGb and PGc: acidic pool; PGd: basic pool.

Elicitor activity of PG isoenzymes. Each dialysed pool of PG isoenzymes following liquid IEF was assayed for elicitor activity of wyerone acid and wyerone with two concentrations, 0.05 and 0.10 RU. Each isoenzyme pool produced dark brownish spots at treated sites. No important differences in lesion spot size were observed but 0.1 RU produced generally darker spots. Phytoalexin standards separated by TLC from pods inoculated with *B. cinerea* where essentially wyerone acid and wyerone, whereas the minor peaks observed may be referred to other wyerone derivatives. Elicitor activity was monitored 1, 3 and 5 days after treatment (Table 1a and 1b). Time course analysis revealed that wyerone acid accumulation strongly increased. Wyerone followed essentially a similar pattern, but reached a lower final concentration. High elicitor activity was expressed within 5 days after exposure by PGa, PGb and PGd with enzyme concentration of 0.05 RU. PGc accumulated phytoalexius to lower levels. At the higher concentration of PG pools, less accumulation of phytoalexins was observed. PGd slightly differed since it elicited more of both phytoalexins on the third day after exposure to 0.1 RU, without increase from the third to the fifth day after exposure, for both the phytoalexins. Similarly, PGc did not induce an increase in wyerone production from the third to fifth day after exposure to 0.05 RU. Small amounts of phytoalexins were found in control tissues (Table 1a and 1b).

Table 1a. The accumulation of wyerone acid in pod endocarp tissue following treatment with PG pools a-d.

Pool of isoenzymes (RU) [a]		Wyerone acid [c] (μg g^{-1} fresh weight)		
		1d [d]	3d	5d
PGa				
	control [b]	2.00 ± 0.30 [e]	2.07 ± 0.35	2.05 ± 0.25
	0.05	0.38 ± 0.04	19.85 ± 1.59	51.39 ± 7.36
	0.10	1.18 ± 0.09	6.08 ± 0.52	15.72 ± 2.20
PGb				
	control	1.17 ± 0.33	2.07 ± 0.17	2.05 ± 0.47
	0.05	0.81 ± 0.15	18.20 ± 1.04	24.52 ± 4.56
	0.10	2.57 ± 0.10	5.57 ± 0.48	7.15 ± 1.02
PGc				
	control	0.34 ± 0.20	0.83 ± 0.41	0.82 ± 0.06
	0.05	4.48 ± 1.12	7.68 ± 1.06	8.76 ± 1.48
	0.10	1.24 ± 0.27	4.84 ± 1.05	5.16 ± 0.27
PGd				
	control	2.00 ± 0.24	1.46 ± 0.22	2.05 ± 0.51
	0.05	0.65 ± 0.12	2.62 ± 0.45	38.35 ± 6.82
	0.10	6.22 ± 0.53	13.33 ± 1.07	13.14 ± 1.84

[a] RU = Reducing Units. One RU is the amount of enzyme producing 1 μmol of reducing groups min^{-1} at 30°C from 0.25% polygalacturonic acid in acetate buffer (0.1M, pH 5.0).
[b] Controls consisted of heat inactivated enzyme.
[c] Wyerone extracted from endocarp pod tissue at the site of treatment and quantified by HPLC.
[d] Days after treatments.
[e] Data are the average ± SD of three sets of thirty seed cavities for each treatment.

Table 1b. The accumulation of wyerone in pod endocarp tissue following treatment with PG pools a-d.

Pool of isoenzymes (RU) [a]		Wyerone [c] (μg g^{-1} fresh weight)		
		1d [d]	3d	5d
PGa				
	control [b]	1.03 ± 0.11 [e]	1.39 ± 0.28	1.37 ± 0.36
	0.05	0.20 ± 0.03	6.07 ± 0.49	18.03 ± 2.71
	0.10	0.58 ± 0.05	2.73 ± 0.27	8.12 ± 0.36
PGb				
	control	0.71 ± 0.06	0.94 ± 0.13	1.02 ± 0.26
	0.05	0.28 ± 0.02	2.10 ± 0.26	9.70 ± 0.40
	0.10	0.81 ± 0.05	1.07 ± 0.14	2.12 ± 0.30
PGc				
	control	0.35 ± 0.11	0.52 ± 0.22	0.50 ± 0.28
	0.05	1.54 ± 0.39	3.70 ± 1.04	3.68 ± 1.61
	0.10	0.80 ± 0.02	0.64 ± 0.17	1.12 ± 0.73
PGd				
	control	1.30 ± 0.11	1.39 ± 0.24	1.37 ± 0.36
	0.05	0.12 ± 0.02	0.59 ± 0.05	22.77 ± 2.28
	0.10	1.76 ± 0.25	7.61 ± 0.91	7.33 ± 1.83

For footnotes, see Table 1a.

DISCUSSION

The isolate of *B. fabae* used produced a considerable amount of PG in bean pods. PG activity was previously reported to be produced by this pathogen both *in vivo* and in *vitro* (Deverall and Wood, 1961; Balasubramani *et al*., 1971; Harrison, 1988). The extremely low production of PNL in extracts from infected tissues indicates that this enzyme probably is not involved in cell wall depolymerization during host tissue colonisation. This paper is the first report of expression of a complex pattern of PG isoenzymes during the colonisation of broad bean by *B. fabae*. As indicated for *B. cinerea* (Chilosi and Magro, 1997; Leone and Van Den Heuvel, 1987), the multiple PG isoenzymes produced by *B. fabae* may completely degrade pectic polysaccharides, allowing fungal colonisation.

The different pools of PG isoenzymes differed in their ability to induce phytoalexin accumulation. High elicitor activity was expressed by PGa, PGb and PGd at an enzyme concentration of 0.05 RU. At a higher concentration, accumulation of both phytoalexins in most cases decreased. Generally, wyerone acid appeared to be the predominant phytoalexin elicited by PG, mirroring induction by *B. cinerea* (Hargreaves *et al*., 1977).

Direct addition of pectolytic enzymes to plant tissues induces a wide variety of defence responses such as phytoalexin production or lignification by releasing from plant cell walls oligogalacturonide fragments which serve as endogenous elicitors (Bruce and West, 1982; Davis *et al*., 1984; Favaron *et al*., 1988; Robertsen, 1989). The lower dose tested was more active as elicitor probably because it led to release of a greater number of active oligogalacturonides. In addition, the 0.05 RU dose induced less darkening of the brown spots at treated sites.

The low elicitor activity of PGc in comparison with that of other pools might be due to reduced ability to release galacturonide-type elicitors from plant cell walls, probably consequent of the prevalent *exo*-mode of action of this PG pool. The difference in elicitor activity of the PG pools might also be due to differences in sensitivity of individual PGs toward possible PG inhibiting proteins active in plant tissues (De Lorenzo *et al*., 2001). Browning inducing activity and phytoalexin synthesis of PG pools appears to be opposite and complementary; the shift between the two intrinsic functions of PG (browning factor, phytoalexin inducer) seems therefore to depend on their concentration in the tissue.

During the first day after treatment, control tissues generally expressed a basal level of both phytoalexins without apparent induction of phytoalexin accumulation for most the treatments. This feature might be due to micro-injuries produced during pod preparation, which induced non-specific defence responses.

B. fabae has the specific ability to colonise broad bean tissues soon after penetration. However, it has been shown that host tissues are more susceptible when a high spore inoculum dose is used (Deverall and Wood, 1961). It was suggested that changes in phytoalexin concentration during formation of spreading lesions are a consequence of the ability of *B. fabae* to metabolise and detoxify phytoalexins, preventing the exposure of the hyphae to fungicidal concentrations of wyerone derivatives. By contrast, it was shown that with an inoculum composed by few spores, individual penetrations often develop small non-coalescent lesions restricted to the epidermis; in this case phytoalexins continued to accumulate throughout the course of the experiment, thereby containing fungal growth (Mansfield and Hutson, 1980; Rossall *et al*., 1980; Mansfield and Richardson, 1981; Mansfield, 1982). PGs from *B. fabae* may play a central role in determining the type of lesion during attempted attack by the pathogen. The possible role of polygalacturonase isoenzymes from *B. fabae* as putative determinants of necrosis or defence responses elicitors during broad bean colonisation may be affected mainly by the inoculum concentration.

ACKNOWLEDGMENTS

This research was supported financially by the "Fondi di ricerca di Ateneo (ex 60%)", University of Tuscia.

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Received 20 September 2002
Accepted 5 May 2003