Enzyme Activity and Electrophoretic Profile of Extracellular Protein Induced in *Trichoderma* spp. by Cell Walls of *Rhizoctonia solani*

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(*Received 21 January 1986; revised 8 April 1986*)

1,3-β-D-Glucanase and chitinase activities were induced in *Trichoderma harzianum* when glucose or cell walls of *Rhizoctonia solani* (anastomosis group AG2) were used as sole carbon source. Electrophoresis showed that more proteins were induced by cell walls than by glucose. The composition, as revealed by electrophoresis, of the *T. harzianum* extracellular proteins was similar when grown on *R. solani* AG2 and AG4 cell walls but different for *R. solani* AG1 cell walls. Several major proteins were induced in all strains of *T. harzianum* but there were strain differences in the number and intensity of other proteins. The number of induced proteins was less for four strains of *T. viride* and their composition was different from those of five strains of *T. harzianum*. The results indicate that chitinase, 1,3-β-D-glucanase and a large number of other extracellular proteins may be involved in the degradation of *R. solani* cell walls.

INTRODUCTION

Rhizoctonia solani is an important pathogen of many crop plant species. It exists in soil as a number of different groups based on the ability of isolates to anastomose with the hyphae of others (anastomosis groups, AG). These groups differ in their host range, infectivity and pathogenic habit (Parmeter *et al.*, 1969). Anastomosis groups AG1, AG2 and AG4 are most frequently isolated from soil (Kooistra, 1983).

Recent work indicates that *Trichoderma* spp. may provide an effective method of controlling *R. solani* and other soil borne diseases (Beagle-Ristiano & Papavizas, 1985; Chand & Logan, 1984; Elad *et al.*, 1981*a*, *b*; Wells *et al.*, 1977). However, the control achieved is often more variable than with chemical methods, or large quantities of the antagonist need to be applied to soil (Abd-El Moity & Shatla, 1981; Elad *et al.*, 1980, 1981 *a*, *b*; Wells *et al.*, 1977). By studying more closely the physiological and biochemical features of *Trichoderma* strains antagonistic to plant pathogens their efficiency at controlling plant diseases may be improved. This could be achieved by selecting strains with improved antagonistic features or by altering the formulation of *Trichoderma* spp. to enhance these features.

Trichoderma spp. interact with plant pathogens in a variety of ways. They have been shown to coil around and penetrate fungal hyphae, and to produce toxic metabolites (Dennis & Webster, 1971 *a*, *b*, *c*; Allen & Haenseler, 1934; Elad *et al.*, 1983). They also produce extracellular cell wall degrading enzymes such as 1,3-β-D-glucanases and chitinase (Elad *et al.*, 1982, 1984). There may be a relationship between extracellular enzyme activity and ability to control plant disease (Artigues & Davet, 1984; Elad *et al.*, 1982).

The following work was undertaken to investigate the extracellular enzymes and proteins induced in *Trichoderma* spp. by *R. solani* cell walls. Polyacrylamide gel electrophoresis was chosen as a suitable method for this since it allows high resolution of proteins and allows a number of strains to be compared simultaneously.

Abbreviations: SM, synthetic medium; PDA, potato dextrose agar; GlcNAc, *N*-acetylglucosamine.

METHODS

R. solani cell wall preparation. R. solani strains J363, PW2 and J312 representing AG1, AG2 and AG4 were obtained from the Hull University (Hull, UK) culture collection and grown on potato dextrose agar (PDA; Oxoid) at 25 °C. Macerates of each isolate were prepared by fragmenting about 5 cm^2 from 10 d-old cultures in sterile distilled water (15 ml); they were used to inoculate yeast extract medium (400 ml) in 1 litre flasks (Matsuyama & Kozaka, 1971). The cultures were grown at 25 °C and shaken at 100 cycles min^{-1} for 10 d. For the preparation of crude cell walls the mycelium was filtered through muslin and homogenized in distilled water using a laboratory mixer/emulsifier (Silverson, Chesham, Bucks, UK). The mycelium was refiltered and stored in a lyophilized state.

In one experiment to determine the influence of protein removal on enzyme induction the cell walls were used without further modification. In the remaining experiments the cell walls were purified by the removal of protein. Crude cell walls (about 8 g) were suspended in 1 litre 0·05 M-potassium phosphate buffer pH 7·6 containing ammonium sulphate (60 g) and sodium deoxycholate (5 g) (Riov, 1974). The cell walls were ruptured using an ultrasonic disintegrator (Dawe Soniprobe). The purified cell walls were filtered, resuspended twice in distilled water (2 l), refiltered and lyophilized.

Trichoderma isolates. Three strains of *T. viride* (IMI 298375, IMI 298376, IMI 298377) and four strains of *T. harzianum* (IMI 298371, IMI 298372, IMI 298373, IMI 298374) were isolated from soil. Preliminary studies showed that *T. harzianum* IMI 298372 may be effective for biocontrol and this was used for experiments requiring only one strain. All strains were grown on PDA at 25 °C.

Production of extracellular protein by Trichoderma spp. Five mycelial discs (1·0 cm diameter) from 2 d-old cultures of *T. harzianum* IMI 298372 were inoculated into duplicate 250 ml flasks containing 0·5 g purified *R. solani* PW2 cell walls or 0·5 g glucose and 100 ml synthetic medium [SM, composition (g l^{-1}): $MgSO_4.7H_2O$ (0·2); K_2HPO_4 (0·9); KCl (0·2); NH_4NO_3 (1·0); $FeSO_4.7H_2O$ (0·002); $ZnSO_4.7H_2O$ (0·002); $MnCl_2.4H_2O$ (0·002) (Chet *et al.*, 1967; Elad *et al.*, 1982)]. Uninoculated cell wall and glucose flasks were included as controls. The cultures were grown at 25 °C and shaken at 150 cycles min^{-1}. After 90 h the cultures from duplicate flasks were bulked and filtered (Whatman no. 54), and the protein was concentrated 100-fold by ultrafiltration over a PM10 membrane (Amicon). Samples of the concentrates were stored in a lyophilized state.

Production of extracellular protein by all *Trichoderma* strains was investigated using *R. solani* PW2 cell walls as sole carbon source. In addition to the four strains of *T. harzianum* and the three strains of *T. viride*, *T. harzianum* (WT-6) and *T. viride* (T-1) were also included for comparison (Lewis & Papavizas, 1980; Papavizas & Lewis, 1983). These cultures were grown and the protein was concentrated as described above.

For studying the influence of protein removal from cell walls, *T. harzianum* IMI 298372 was grown as above on purified or crude *R. solani* PW2 cell walls. The extracellular protein was harvested after 45 or 90 h and concentrated as above. For studying the influence of *R. solani* strains on enzyme induction, *T. harzianum* IMI 298372 was grown as above in SM containing purified cell walls from each of the three *R. solani* strains (0·5 g per 100 ml) and extracellular protein harvested after 90 h was concentrated as before.

Protein determination. Protein content of all concentrates was determined using the Biorad protein assay kit with bovine serum albumin as standard.

1,3-β-D-Glucanase activity (EC 3.2.1.6). Protein samples (100 µl) from each experiment were suspended in 3·0 ml McIlvaine citric acid buffer pH 4·8 containing laminarin (3 mg ml^{-1}), and divided into two equal volumes. One of these was boiled for 3 min (blank); the other was incubated at 37 °C for 2 h. The reaction was stopped by boiling. The glucose released was assayed using glucose oxidase reagent (based on the method of Hugget & Nixon, 1957).

Chitinase activity (EC 3.2.1.14). Protein samples (100 µl) were suspended in 3·0 ml 0·066 M-Na_2HPO_4/KH_2PO_4 buffer pH 6·0, and divided into two equal volumes. Purified chitin (3·8 mg, Sigma C3641) was added to both halves and one was then boiled (blank). The other was incubated at 37 °C for 4 h and the reaction stopped by boiling. The amount of *N*-acetylglucosamine (GlcNAc) released was assayed using the method of Reissig *et al.* (1955).

Polyacrylamide gel electrophoresis (PAGE). Anodic discontinuous PAGE was done in slabs (160 × 168 × 1·5 mm) or rods (i.d. 2·7 mm) for two-dimensional electrophoresis as described by Davis (1964). Protein concentrates were suspended in 0·062 M-Tris/HCl stacking gel buffer (30 µl) containing glycerol (12·5%, v/v) and aqueous bromophenol blue (12·5%, v/v). Gels were stained with Coomassie blue R250 (0·115%, w/v). Three types of PAGE were used.

(i) *Homogeneous gel electrophoresis. T. harzianum* IMI 298372 concentrates were electrophoresed in 5, 6, 7, 10 and 15% resolving gels at pH 8·8 with a 4% stacking gel at pH 6·8 (Hames, 1981). Samples were electrophoresed at 30 mA until the bromophenol blue reached the bottom of the gel.

(ii) *Gradient gel electrophoresis.* Gels were prepared (7–20%, w/v) using a gradient mixer (Hames, 1981). Samples were prepared as above and electrophoresed at 30 mA for 2·5 h after the bromophenol blue had reached the bottom of the gel. M_r markers were also included (Sigma).

(iii) *Two-dimensional electrophoresis. T. harzianum* IMI 298372 concentrate (250 µg) was electrophoresed in a 6% tube gel (i.d. 2·7 mm) until the bromophenol blue reached the bottom. The gel was laid onto a 7–20% gradient gel. The protein was re-electrophoresed for 2·5 h after the bromophenol blue had reached the bottom of the gel. Unseparated protein and M_r markers were also included.

Table 1. *Extracellular protein and enzyme activity induced in T. harzianum IMI 298372 grown on glucose or R. solani strain PW2 cell walls (5·0 g l^{-1}) for 90 h*

Treatment	Total protein per 100 ml culture filtrate (μg)	1,3-β-D-glucanase activity: Total activity (glucose released, mmol h^{-1})	1,3-β-D-glucanase activity: Specific activity*	Chitinase activity: Total activity (Glc NAc released, μmol h^{-1})	Chitinase activity: Specific activity*
Growth on glucose					
Non-inoculated (control)	10	0	0	0	0
Inoculated with *T. harzianum*	632	2·2	34·40	24·7	0·39
Growth on cell walls					
Non-inoculated (control)	3800	0·04	0·12	4·52	0·01
Inoculated with *T. harzianum*	2400	1·6	6·64	67·9	0·28

* μmol products released h^{-1} (μg protein)$^{-1}$.

RESULTS

Cell walls of *R. solani* PW2 induced 1,3-β-D-glucanase and chitinase activities in *T. harzianum* IMI 298372. However, these enzyme activities were also induced when glucose was used as sole carbon source (Table 1). The low levels of glucose and GlcNAc detected in uninoculated cell walls may have been due to the release of these sugars directly from the cell walls during the incubation period. Electrophoresis showed that the mixture of proteins was more complex when *T. harzianum* was grown on cell walls (Fig. 1). Certain proteins were particularly intense and were induced only by cell walls.

Extracts from uninoculated cell walls of *R. solani* PW2 also contained protein (Table 1). However, this did not contain any distinct bands when analysed by electrophoresis (Fig. 1). This protein may represent fragments that were released from the cell walls by rotary shaking.

The 1,3-β-D-glucanase and chitinase activities induced by *R. solani* PW2 cell walls varied between strains of *T. harzianum* and *T. viride* (Table 2). When the proteins were analysed by electrophoresis several major bands were present in all strains of *T. harzianum* (Fig. 2). However, there were differences in the total number and intensity of bands between strains. The electrophoretic profiles of induced proteins from *T. viride* strains were different from those of *T. harzianum* strains. In general, the number of distinct bands was fewer for *T. viride*.

The protein induced by crude cell walls is represented mainly by a single streak after 45 h as shown by electrophoresis (Fig. 3). This may correspond to *R. solani* protein being released following the initial lysis of the cell walls, since this feature was absent when purified cell walls were used. After 90 h the electrophoretic profiles were similar from cultures grown on crude or purified cell walls.

The strain of *R. solani* used affected the total extracellular protein produced and the corresponding enzyme activity (Table 3). *R. solani* strain J316 (AG1) induced the highest total 1,3-β-D-glucanase activity whereas strain J312 (AG4) induced the highest chitinase activity. The difference between specific enzyme activities induced by the three *R. solani* strains was less marked.

The results in Tables 1–3 represent single determinations of enzyme activity. When the experiments were repeated, although the levels of enzyme activity were different, the pattern of activities was similar. The actual values of enzyme activity determined are likely to depend on the varying storage times of the proteins but these times were always constant within an experiment. The results should not therefore be regarded as absolute enzyme activities. Each of the experiments should be regarded as unique and not be compared directly with each other.

The anastomosis group of *R. solani* may also affect the electrophoretic profile of extracellular protein induced in *Trichoderma* spp. The profiles of major bands were very similar when grown

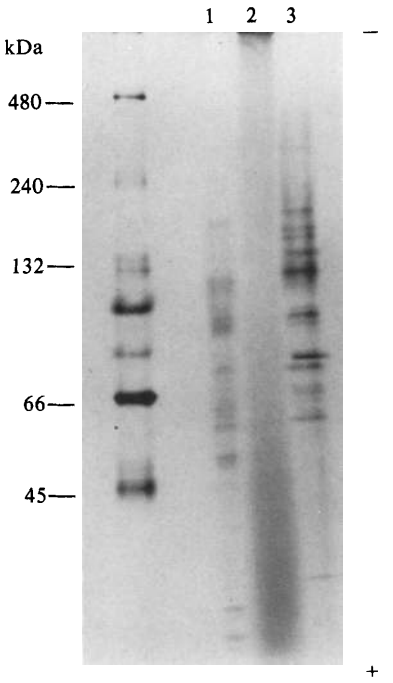

Fig. 1. Separation of extracellular protein induced in *T. harzianum* IMI 298372 using 7–20% gradient PAGE. Lane 1, *T. harzianum* grown on glucose (21·1 μg); lane 2, control *R. solani* PW2 cell wall concentrate (12·7 μg); lane 3, *T. harzianum* grown on PW2 cell walls (80 μg). The marker proteins used were (kDa) Jackbean urease tetramer (480), Jackbean urease dimer (240), bovine serum albumin dimer (132), bovine serum albumin monomer (66) and chicken egg albumin (45).

Table 2. *Extracellular protein and enzyme activity induced in Trichoderma spp. grown on R. solani PW2 cell walls (5·0 g l^{-1}) for 90 h*

Trichoderma strain	Total protein per 100 ml culture filtrate (μg)	1,3-β-D-glucanase activity: Total activity (glucose released, mmol h^{-1})	1,3-β-D-glucanase activity: Specific activity*	Chitinase activity: Total activity (GlcNAc released, μmol h^{-1})	Chitinase activity: Specific activity*
T. viride					
IMI 298375	940	1·2	13·0	90·5	0·96
IMI 298376	1810	4·4	24·4	81·5	0·45
IMI 298377	1710	2·4	13·9	49·7	0·29
T-1 .	3620	4·4	12·2	22·6	0·06
T. harzianum					
IMI 298371	1750	2·3	13·1	136	0·77
IMI 298372	3500	2·1	6·1	280	0·80
IMI 298373	3000	3·3	10·9	398	1·33
IMI 298374	3350	5·1	15·2	239	0·72
WT-6	1090	2·7	24·4	36·2	0·33

* μmol products released h^{-1} (μg protein)$^{-1}$.

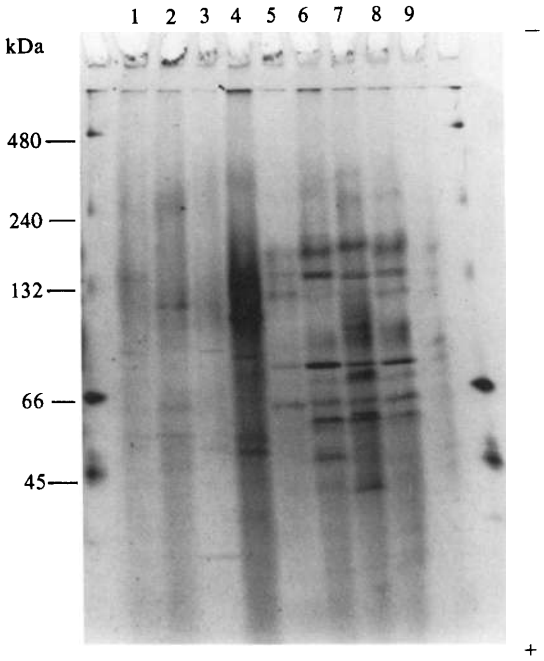

Fig. 2. Comparison of extracellular proteins induced in *Trichoderma* strains by cell walls of *R. solani* PW2 using 7–20% gradient PAGE. Lanes 1–4, *T. viride* IMI 298375 (31 μg), IMI 298376 (60 μg), IMI 298377 (57 μg) and T-1 (121 μg). Lanes 5–9, *T. harzianum* IMI 298371 (58 μg), IMI 298372 (116 μg), IMI 298373 (100 μg), IMI 298374 (112 μg) and WT-6 (36 μg). Marker proteins as in Fig. 1.

Table 3. *Extracellular protein and enzyme activity induced in T. harzianum IMI 298372 when grown on cell walls of three strains of R. solani (5·0 g l^{-1}) for 90 h*

		1,3-β-D-glucanase activity		Chitinase activity	
R. solani strain strain	Total protein per 100 ml culture filtrate (μg)	Total activity (glucose released, mmol h^{-1})	Specific activity*	Total activity (Glc NAc released, μmol h^{-1})	Specific activity*
J363 (AG1)	1600	2·7	16·6	97	0·61
PW2 (AG2)	1200	2·4	19·9	130	1·08
J312 (AG4)	2250	1·5	6·9	217	0·96

* μmol products released h^{-1} (μg protein)$^{-1}$.

on the cell walls of *R. solani* PW2 or J312 (AG2 and AG4) although the protein loading was less for strain PW2 concentrates (Fig. 4). However, the pattern was very different from cultures grown on *R. solani* J363 (AG1).

From the range of homogeneous gels used, 6% gave the optimum separation of proteins. However, the resolution achieved was considerably less than that of the 7–20% gradient gel. The comparatively poor resolution of the 6% gel is illustrated by two-dimensional electrophoresis (Fig. 5). A number of proteins migrated to the same point in the 6% gel but were resolved in the gradient gel. Two-dimensional electrophoresis gives a more accurate representation of the total number of proteins induced. The 7–20% gradient gels shown in Figs 1, 2 and 4 were photographed after separate electrophoresis experiments; these were done at least twice. The band pattern for cell wall concentrates was similar in each experiment.

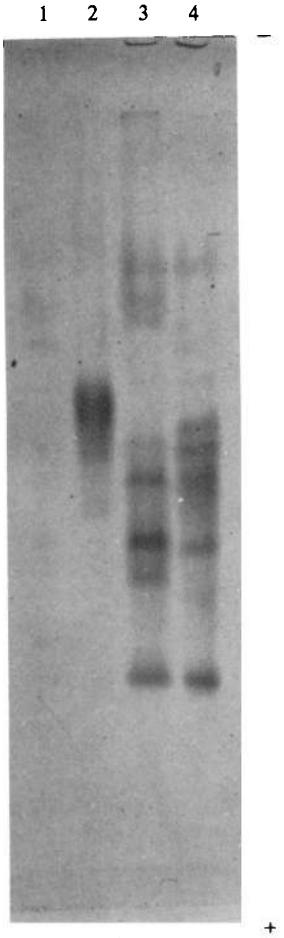

Fig. 3

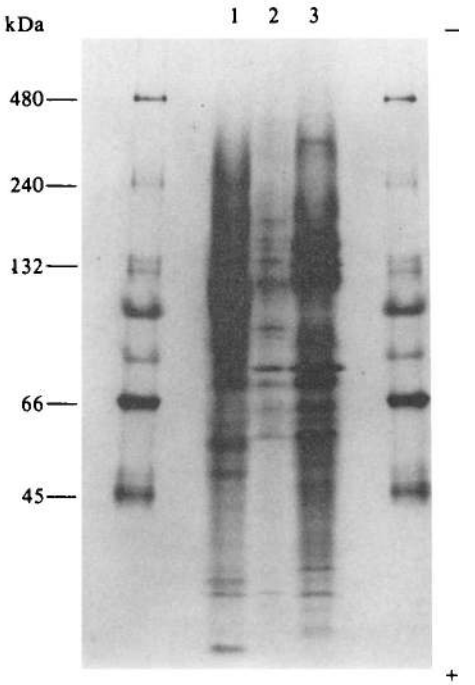

Fig. 4

Fig. 3. Influence of the removal of protein from cell walls of *R. solani* PW2 on the electrophoretic profile of *T. harzianum* IMI 298372 extracellular proteins (6% gel). Lanes 1 and 2, growth on purified and crude cell walls for 45 h (40, 30 μg); lanes 3 and 4, growth on purified and crude cell walls for 90 h (75, 90 μg).

Fig. 4. Influence of *R. solani* strain on protein profile (7–20% gradient PAGE). Lanes 1, 2 and 3, *T. harzianum* IMI 298372 grown on cell walls of *R. solani* J363, PW2 and J312 (160, 120, 225 μg), respectively. Marker proteins as in Fig. 1.

DISCUSSION

Cell walls of *R. solani* induce extracellular 1,3-β-D-glucanase and chitinase in *Trichoderma* spp. These enzymes may be important in the destruction of plant pathogens and could be used as the basis of a screen for potential biocontrol agents (Elad *et al.*, 1982). However, the induction of these enzyme activities may not be specific to *R. solani* cell walls since they were also induced in *T. harzianum* IMI 298372 when glucose was used as sole carbon source. In contrast, there are some extracellular proteins, revealed by electrophoresis, which are induced only by cell walls and not by glucose. Proteins induced specifically by cell walls did not occur in the protein mixture induced by glucose (Fig. 1).

The anastomosis group of *R. solani* used for cell wall production affects the proteins induced in *T. harzianum* IMI 298372. Electrophoretic profiles of major bands were similar when *T. harzianum* IMI 298372 was grown on cell walls of *R. solani* strains from anastomosis groups AG2 and AG4; the profile was different when it was grown on cell walls of an *R. solani* strain from

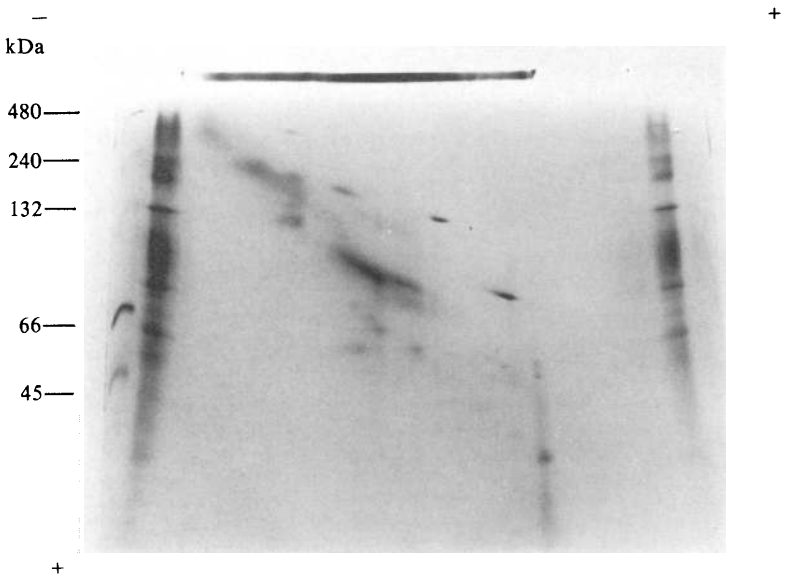

Fig. 5. Two-dimensional electrophoresis of *T. harzianum* extracellular proteins induced by cell walls of *R. solani* strain PW2. *T. harzianum* IMI 298372 protein (250 μg) was electrophoresed in a 6% gel followed by a 7–20% gradient gel. Marker proteins (as in Fig. 1) are shown in the gradient dimension.

AG1. This may reflect differences in the composition or structure of cell walls between anastomosis groups. The anastomosis groups of *R. solani* are known to differ in other biochemical aspects including the composition of cell wall proteins and DNA base sequences (Reynolds *et al.*, 1983; Kuninaga & Yokosawa, 1980).

The M_r of native proteins can only be determined in gradient gels if they are known to reach their pore limit. This depends on their isoelectric point and corresponding migration rate. Proteins with an isoelectric point > 7·0 migrate slowly in basic electrophoretic systems and may only reach their pore limit after extended electrophoresis (Firgaira *et al.*, 1981). Since the isoelectric points of *Trichoderma* proteins are not known, the gradient gel should only be used to improve resolution and not to estimate M_r values.

In addition to the enzymes 1,3-β-D-glucanase and chitinase, a large number of other proteins may be involved in the degradation of *R. solani* cell walls. These can be separated using gel electrophoresis and this may be a useful method for identifying important proteins or comparing *Trichoderma* isolates suitable for biological control.

The award of an SERC CASE studentship to C. J. R. is appreciated. We thank Dr G. C. Papavizas for supplying isolates of *T. harzianum* WT-6 and *T. viride* T-1 and Dr J. M. Whipps for helpful comments.

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