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Electrophoretic and serological comparisons of pathogenesis-related (b) proteins from different plant species

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Abstract

Preparations of pathogenesis-related (b) proteins (PRs) from different *Nicotiana* species, tomato, *Gynura aurantiaca,* bean, and cowpea were compared to each other and to bean chitinase and a constitutive apple agglutinin by electrophoresis in polyacrylamide gels both in the absence and in the presence of SDS, and by serological double diffusion analysis using antisera against tobacco PRs and bean chitinase. PRs from different plant genera displayed a similar but not identical range of relative mobilities in both native and SDS gels, whereas bean chitinase and apple agglutinin were clearly different. None of the antisera reacted with any of the PR preparations from plant genera other than the one from which the antigen(s) had been derived. Whilst PRs within the genus *Nicotiana* are serologically related and can be identical, PRs from different plant genera seem to be sufficiently different to be considered as genus-specific.

Additional keywords: Gynura aurantiaca, Lycopersicon esculentum, Malus sylvestris, amphidiploïd *Nicotiana glutinosa* × *Nicotiana debneyi, Nicotiana sylvestris, Nicotiana tabacum, Phaseolus vulgaris, Vigna sinensis.*

Introduction

The appearance of pathogenesis-related (b) proteins (PRs) has been reported in several plant species following infection or treatment with chemicals. They are recognized as additional protein bands in extracts subjected to polyacrylamide gel electrophoresis and stained with general protein stains. The nomenclature adopted for these new proteins has differed between authors. In the case of *Nicotiana* species where such proteins were first discovered (Van Loon and Van Kammen, 1970; Gianinazzi et al., 1970), they have been called pathogenesis-related proteins (PRs) (Antoniw et al., 1980) or b-proteins (Gianinazzi et al., 1970). In different cultivars of *Nicotiana tabacum,* new protein bands with corresponding relative mobilities have been assumed to be identical. Indeed, PR 1a (b_1), 1b (b_2) and 1 c (b_3) from the tobacco cultivars Xanthi-nc and Samsun NN have similar molecular weights, and the PR 1a (b_1) proteins from the two cultivars have similar amino acid compositions (Antoniw et al., 1980). A genetic analysis of tobacco PRs further supports the similarity of bands with the same relative mobility, not only within *Nicotiana tabacum,* but even in different *Nicotiana* species (Ahl et al., 1982). Moreover, the *Nicotiana* PRs appear to constitute a 'family' of proteins with similar properties (Antoniw et al., 1980; Gianinazzi, 1983).

The occurrence of new proteins in other plant genera, after infection or treatment with chemicals, which migrate to similar positions in polyacrylamide gels as those from *Nicotiana*, raises the question as to how far these proteins from different genera may be related both structurally and functionally. During the workshop an attempt was made to compare the electrophoretic and serological properties of PR preparations from different sources in order to gain insight into the possible biochemical relationship between them. Two other proteins possibly associated with pathological conditions, an inducible chitinase from bean (Boller et al., 1983) and a constitutive agglutinin from apple (Romeiro et al., 1981), were also investigated.

Materials and methods

The origin of protein samples is summarized in Table 1. Most PR preparations were obtained by extraction of leaf tissues at low pH, either in phosphate-citrate buffer pH 3.0 containing 0.5 M NaCl and 0.1% (v/v) 2-mercaptoethanol (Van Loon, 1976), or in phosphate-citrate buffer pH 2.8 containing 0.3% (v/v) 2-mercaptoethanol (Gianinazzi et al., 1977). Extracts were cleared by centrifugation (at least 30 min at 30 000 *g*). Low-molecular-weight contaminants were then removed by gel filtration on Sephadex (G25-G100) or Ultragel LKB AcA54, using Tris-HCl buffer (pH 7.5-8.0) as an eluant. The PR-containing fraction was then dialysed successively against at least 100 vol. of 0.01 M NH_4HCO_3 and of distilled water to remove the buffer salt, and subsequently lyophilized. Individual PRs were further purified according to Antoniw et al. (1980).

In the preparations of total PRs from *N. tabacum* cv. Xanthi-nc, *Phaseolus vulgaris* cvs Prince and Pinto, *Vigna sinensis* cvs Blackeye and Clay and *Gynura aurantiaca,* the gel filtration step was omitted. A PR preparation from *N. tabacum* cv. Paraguay P48 was obtained by grinding lyophilized seedlings in a chilled mortar and pestle in 10 vol. (v/w) of electrophoresis buffer containing 0.5 M sucrose and the homogenate was centrifuged for 30 min at 30 000 *g*. Purified PSs (PRs) from *P. vulgaris* cv. Saxa

(Redolfi, 1983) were obtained by recovering them from 10% polyacrylamide gels in which total PS preparations had been separated by electrophoresis. Tomato p14 was purified from *Lycopersicon esculentum* cv. Rutgers as outlined by Camacho Henriquez et al. (1983). The constitutive agglutinin from *Malus sylvestris* × *M. ranetka* was extracted and purified as described by Romeiro et al. (1981). Bean chitinase was purified according to Boller et al. (1983).

The PR preparations were dissolved in and dialysed overnight against electrophoresis buffer (5 mM Tris-glycine, pH 8.6) containing 0.5 M sucrose. Aliquots of 1-5 µl were then subjected to electrophoresis in discontinuous 10% polyacrylamide gel slabs (1.5 mm thick), using the system of Davis (1964). Gels were stained with the silver stain according to Morissey (1981). Further aliquots (300 µl) were mixed with 100 µl 40 mM Tris-HCl, 4 mM EDTA, 10% (w/v) SDS, 20% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, pH 8.0 and heated for 5 min in a boiling water bath. Samples of 10-100 µl were then subjected to electrophoresis in discontinuous 7.5-17.5% linear gradient polyacrylamide gel slabs in the presence of SDS (Laemmli, 1970). These gels were stained with 0.25% Coomassie Brilliant Blue R250 in methanol : acetic acid : water (5 : 1 : 4, v/v) and destained in methanol : acetic acid : water (5 : 7 : 88, v/v).

Final aliquots were subjected to serological analysis. Double diffusion tests were carried out in Petri dishes containing a 1 mm layer of gel diffusion agar (1% Ionagar, 0.85% (w/v) NaCl, 0.01% (w/v) sodium azide in 0.01 M phosphate buffer pH 7.0. Wells were 3 mm in diameter and spaced 4 mm apart. Antisera against *N. tabacum* cv. Xanthi-nc PR 1a (b_1), *N. tabacum* cv. Samsun NN PRs, and bean chitinase were provided by R.F. White, R.A.M. Hooft van Huysduynen (Department of Biochemistry, University of Leiden, the Netherlands) and T. Boller, respectively. The protein samples were tested undiluted and at a dilution of 1 : 10. All antisera were used at a dilution of 1 : 2. Reactions were scored after 24 h incubation at 20 °C.

Results

Electrophoretic comparison of PRs. PR preparations from different sources contained varying amounts of protein. In cases where no fractionation of the low-pH extracts had been attempted, electrophoresis revealed the presence of many often densely stained proteins in addition to PRs (Figs. 1-4). In the crude preparation from *N. tabacum* cv. Paraguay P48, and in the low-pH extract from *N. tabacum* cv. Xanthi-nc only traces of PRs were discernable (Figs. 1 and 2, lanes 2 and 5, 6, respectively). In contrast, in the fractionated preparations from *N. sylvestris* and *N. tabacum* cv. Samsun NN (Figs. 1 and 2, lanes 3 and 4, respectively) PRs were the dominant components, *N. sylvestris* possessing b_0, b_1 (PR 1a), b_3 (PR 1c) and 'Samsun NN' tobacco showing PR 1a (b_1), 1b (b_2), 1c (b_3), 2 (b_4) and N, O, P, Q, R and S (cf. Fig. 3, lanes 1, 2). In the amphidiploid *N. glutinosa* × *N. debneyi* the major band was $b_{1''}$ (Fig. 1, lane 7). Purified PR 1a (b_1) from 'Samsun NN' tobacco (Figs. 3 and 4, lane 3) moved identically to PR 1a (b_1) from 'Xanthi-nc' tobacco (Figs. 3 and 4, lane 5). Purified PR 1b (b_2), having the same mol. wt (15 000) as PR 1a (b_1), moved as expected to the same position in SDS gels (Fig. 4, lane 4); however, upon electrophoresis in native gels, it was found to be still accompanied by a substantial amount of PR 1c (b_3) (Fig. 3, lane 4). Purified $b_{1''}$ (mol. wt = 13 800) migrated slightly further than PR 1a (b_1) of 'Samsun NN' in SDS gels (Fig. 2, lanes 7 and 4, respectively).

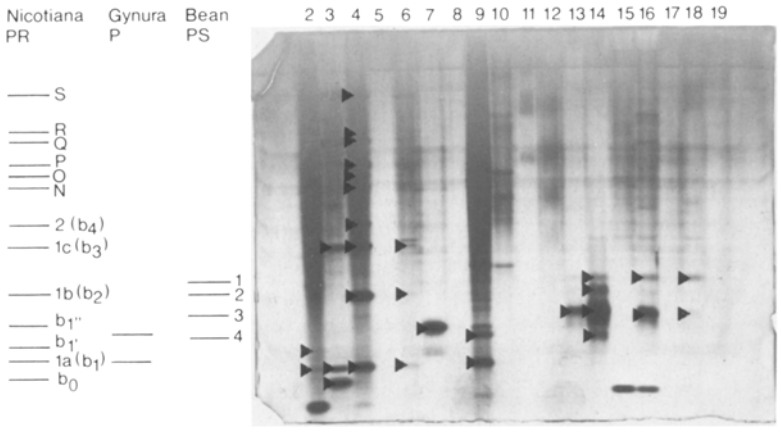

Fig. 1. Electrophoretic patterns in native 10% polyacrylamide gel of PR preparations from (lane 2) tobacco 'Paraguay P48' infected with *Peronospora tabacina,* (3) *N. sylvestris,* infected with tobacco aucuba mosaic virus, (4) tobacco 'Samsun NN' infected with tobacco mosaic virus, (5) tobacco 'Xanthi-nc' treated with aspirin and (6) treated with a culture filtrate of *Stachybotrys chartarum,* (7) amphidiploïd *N. glutinosa* × *N. debneyi,* (8) tomato p14, (9) *Gynura aurantiaca* infected with citrus exocortis viroid, water extract and (10) pH 2.8 extract, (11) apple agglutinin, purified fraction and (12) crude preparation, (13) bean 'Saxa' PS 3 and (14) PS 1-4, (15) bean (control), (16) infected with *Colletotrichum lindemuthianum,* (17) mock-infected and (18) infected with alfalfa mosaic virus, (19) bean chitinase. Lanes 1 and 20 were not used. Gels were stained with silver. The positions of the pathogenesis-related proteins are indicated by arrows as well as represented diagrammatically.

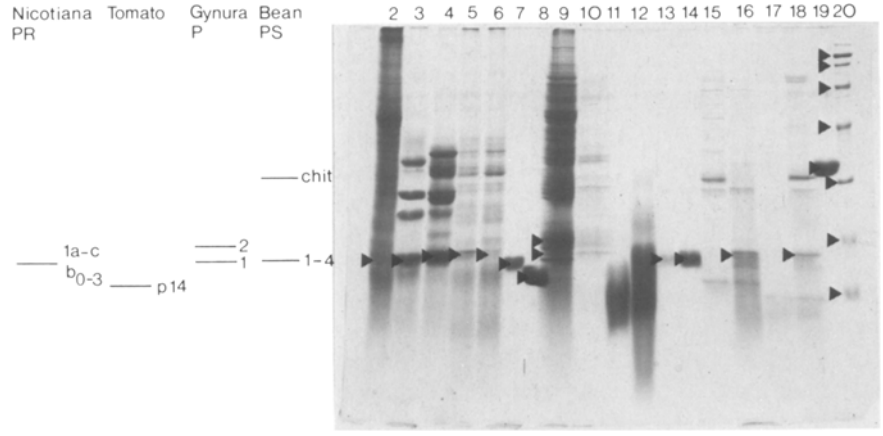

Fig. 2. Electrophoretic patterns in SDS 7.5-17.5% linear gradient polyacrylamide gel of the same PR preparations as shown in Fig. 1, except that the b_{1}'' of the amphidiploïd *N. glutinosa* × *N. debneyi* was purified (lane 7). Lane 20 contains the mol. wt markers β-galactosidase (116 000), phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400). Gels were stained with coomassie blue.

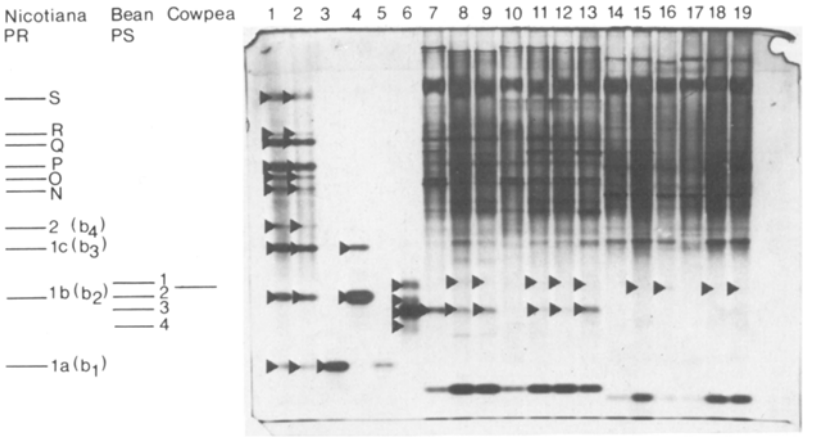

Fig. 3. Electrophoretic patterns in native 10% polyacrylamide gel of PR preparations from (lanes 1 and 2) tobacco 'Samsun NN' infected with tobacco mosaic virus, (3) PR 1a (b_1), (4) PR 1b (b_2) and PR 1c (b_3), (5) tobacco 'Xanthi-nc' b_1 (PR 1a), (6) bean 'Saxa' PS 1-4, (7) bean 'Prince' (control), (8) treated with aspirin and (9) infected with the cowpea strain of southern bean mosaic virus (SBMV), (10) bean 'Pinto' (untouched control), (11) abraded, (12) treated with aspirin, and (13) infected with the bean strain of SBMV, (14) cowpea 'Blackeye' (control), (15) treated with aspirin and (16) infected with the cowpea strain of SBMV, (17) cowpea 'Clay' (control), (18) treated with aspirin, and (19) infected with the cowpea strain of SBMV. Gels were stained with silver.

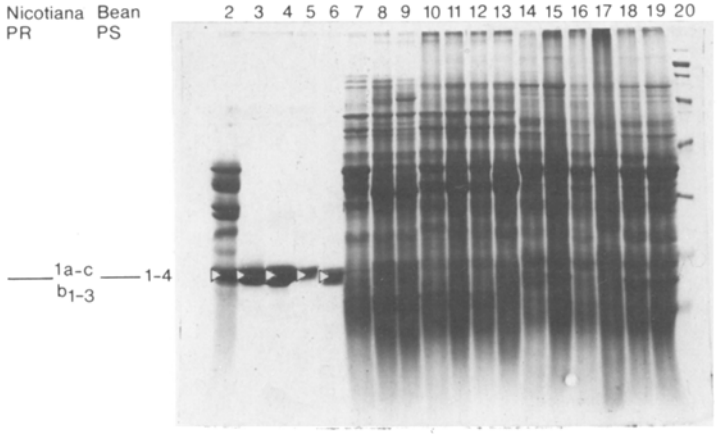

Fig. 4. Electrophoretic patterns in SDS 7.5-17.5% linear gradient polyacrylamide gel of the same PR preparations as shown in Fig. 3. Lane 20 contains the same mol. wt markers as in Fig. 2. Lane 1 was not used. Gels were stained with coomassie blue.

Table 1. Sources of PR preparations and their reactions with antisera against tobacco PRs or bean chitinase[a].

Protein extract from		Origin	Antiserum to		
plant species	inducer		tobacco 'Xanthi-nc' PR-1a (b_1)	tobacco 'Samsun NN' total PRs	bean chitinase
Tobacco 'Paraguay P48'	*Peronospora tabacina*	Coussirat	+	+	–
N. sylvestris	Tobacco aucuba mosaic virus	Gianinazzi	+	–	–
Tobacco 'Samsun NN'	Tobacco mosaic virus	Van Loon	+	–	–
Tobacco 'Xanthi-nc'	(control)	Maiss	–	–	–
Tobacco 'Xanthi-nc'	Aspirin	Maiss	n.t.[b]	n.t.	n.t.
Tobacco 'Xanthi-nc'	Alfalfa mosaic virus	Maiss	–	–	–
Tobacco 'Xanthi-nc'	Tobacco ringspot virus	Maiss	–	+	–
Tobacco 'Xanthi-nc'	*Stachybotrys chartarum*	Maiss	+	–	–
Amphidiploïd *N. glutinosa* × *N. debneyi* ($b_{1\cdot\cdot}$)	–	Ahl	+	+	–
Tomato p14	Potato spindle tuber viroid	Camacho Henriquez	–	–	–
G. aurantiaca (water extract)	Citrus exocortis viroid	Conejero	–	–	–
G. aurantiaca (pH 2.8 extract)	Citrus exocortis viroid	Conejero	–	–	–
Apple agglutinin (purified fraction)	–	Goodman	–	–	–
Apple agglutinin (crude preparation)	–	Goodman	n.t.	n.t.	n.t.
Bean 'Saxa' PS 3	Tobacco necrosis virus	Redolfi	–	–	–
Bean 'Saxa' PS 1-4	Tobacco necrosis virus	Redolfi	–	–	–
Bean	(control)	Abu-Jawdah	–	–	–
Bean	*Colletotrichum lindemuthianum*	Abu-Jawdah	–	–	+
Bean	Alfalfa mosaic virus	Abu-Jawdah	–	–	–
Bean chitinase	Ethylene	Boller	–	–	+
Tobacco 'Samsun NN' PR 1a	Tobacco mosaic virus	Van Loon	+	+	–
Tobacco 'Samsun NN' PR 1b	Tobacco mosaic virus	Van Loon	+	+	–
Tobacco 'Xanthi-nc' b_1	Tobacco mosaic virus	Antoniw	+	–	–

Bean 'Prince'	(control)	Wilson	–	–	+
Bean 'Prince'	Aspirin	Wilson	n.t.	n.t.	n.t.
Bean 'Prince'	Southern bean mosaic virus (cowpea strain)	Wilson	–	–	+
Bean 'Pinto'	(control)	Wilson	–	–	–
Bean 'Pinto'	(abraded)	Wilson	n.t	n.t.	n.t.
Bean 'Pinto'	Aspirin	Wilson	–	–	+
Bean 'Pinto'	Southern bean mosaic virus (bean strain)	Wilson	–	–	+
Cowpea 'Blackeye'	(control)	Wilson	–	–	–
Cowpea 'Blackeye'	Aspirin	Wilson	n.t.	n.t.	n.t.
Cowpea 'Blackeye'	Southern bean mosaic virus (cowpea strain)	Wilson	–	–	–
Cowpea 'Clay'	(control)	Wilson	n.t.	n.t.	n.t.
Cowpea 'Clay'	Aspirin	Wilson	n.t.	n.t.	n.t.
Cowpea 'Clay'	Southern bean mosaic virus (cowpea strain)	Wilson	n.t.	n.t.	n.t.

[a] Protein samples are arranged primarily according to their sequence of depiction in Figs. 1, 2 and 3, 4.
[b] n.t.: not tested.

Purified tomato p14 (mol. wt = 14 000) was found to move slightly further than the tobacco PRs in SDS gels (Fig. 2, lane 8). However, due to its high pI (10.7) it did not enter the native gel (Fig. 1). *Gynura* P_1 and P_2 were clearly revealed when infected leaves had been extracted with water in the presence of 0.25% sodium diethyldithiocarbamate, 0,3% (v/v) 2-mercaptoethanol, 10% (w/w) insoluble polyvinylpyrrolidone (Figs. 1 and 2, lane 9) but were not present in low-pH extracts (Figs. 1 and 2, lane 10). However, in native gels at least one of the PRs comigrated with tobacco PR 1a (Fig. 1, lanes 9 and 4, respectively), and *Gynura* P_1 and tobacco PR 1a had the same mol. wt (Fig. 2). The agglutinin from apple did not resemble any of the PRs, giving a broad, heterogeneous zone both on native and on SDS gels (Figs. 1 and 2, lanes 11, 12).

Purified PS 3 from 'Saxa' bean migrated differently from the tobacco PRs on native gels (Fig. 1, lane 13), but its position on SDS gels was almost identical (Fig. 2, lanes 13 and 4, respectively). All four purified PSs from 'Saxa' bean separated on native gels (Fig. 1, lane 14) moved to the same position in SDS gels (Fig. 2, lane 14; Fig. 4, lane 6). Furthermore, in native gels bean PS 2 had the same relative mobility as tobacco PR 1b (b_2) (Figs. 1 and 3, lanes 14, 4 and 6, 4, respectively). At least PS 1 and 3 were revealed in extracts from bean infected with *Colletotrichum lindemuthianum* (Figs. 1 and 2, lane 16) or alfalfa mosaic virus (lane 18), but not in extracts from uninfected control (lane 15) or mock-inoculated (lane 17) plants. Similar bands were also present in both 'Prince' and 'Pinto' bean upon mechanical abrasion (Fig. 3, lanes 7 and 11), after treatment with aspirin (Fig. 3, lanes 8 and 12) or after infection with the cowpea or bean strain of southern bean mosaic virus (SBMV) (Fig. 3, lanes 9 and 13). Bean chitinase was different from the PSs in that it did not enter the native gel (Fig. 1, lane 19) and showed a substantially higher mol. wt in SDS gels (Fig. 2, lane 19). Although in cowpea two rapidly migrating PRs have been described (Coutts, 1978), their induction in response to aspirin (Figs. 3 and 4, lanes 15, 18) or the cowpea strain of SBMV (Figs. 3 and 4, lanes 16, 19) was less clearly revealed.

Serological comparison of PRs. The reactions of the PR preparations with the antisera against 'Xanthi-nc' tobacco PR 1a (b_1), 'Samsun NN' tobacco total PRs and bean chitinase are summarized in Table 1. Only two concentrations of each protein sample were tested against one dilution of each antiserum and it is possible that some negative reactions could prove otherwise if different concentrations were used in further tests. Thus, the antiserum against tobacco 'Samsun NN' total PRs did not react with the protein extract from tobacco 'Samsun NN' in which PRs were clearly present (Fig. 1, lane 4; Fig. 3, lanes 1 and 2), whereas with the antiserum against tobacco 'Xanthi-nc' PR 1a (b_1) a positive reaction was obtained. The antiserum against 'Xanthi-nc' PR 1a (b_1) reacted only with PR preparations from *Nicotiana* (Fig. 5); however, it proved active not only against itself but also against the related PR 1b (b_2) from 'Samsun NN' tobacco and $b_{1''}$ from the amphidiploid *N. glutinosa* × *N. debneyi.* The antiserum against bean chitinase did not react with purified PSs from bean, but only with certain unpurified extracts (Fig. 6).

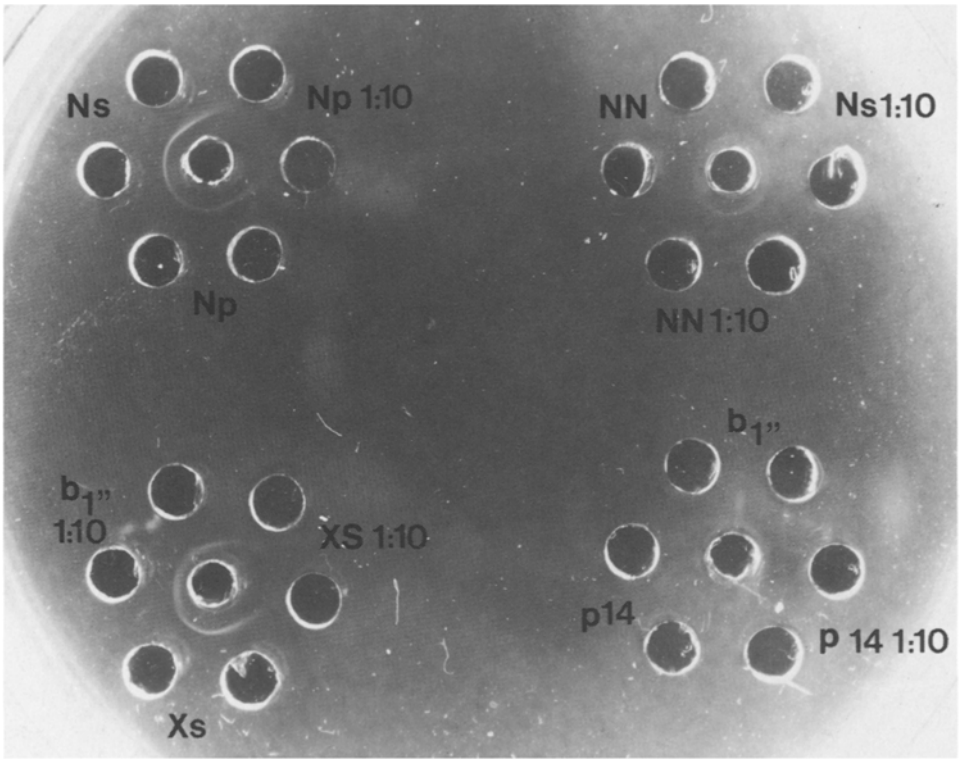

Fig. 5. Reaction patterns to the antiserum against tobacco 'Xanthi-nc' PR la (b_1) (center wells) of protein extracts from tobacco 'Paraguay P48' (Np), *N. sylvestris* (Ns), tobacco 'Samsun NN' (NN), tobacco 'Xanthi-nc' treated with a culture filtrate of *Stachybotrys chartarum* (XS), amphidiploïd *N. glutinosa* × *N. debneyi* ($b_{1\cdot\cdot}$) and tomato p14 (p14). All samples were tested both undiluted and at a dilution of 1 : 10 in two adjacent wells.

Discussion

PRs from *Nicotiana, Phaseolus* and *Gynura* were grouped within a similar range of mol. wts and of relative mobilities in native gels but, apart from one of the PRs from *Gynura* and tobacco PR 1a (b_1), and bean PS 2 and tobacco PR 1b (b_2), their migration in both native and SDS gels was not identical. Tomato p14 and bean chitinase differed from the PRs in *Nicotiana* and bean in having pI's well above 8.6, preventing them from entering native gels during electrophoresis. Unlike PRs from *Nicotiana, Gynura* PRs were extracted at neutral but not at low pH, and the agglutinin from apple migrated as a broad, diffuse zone. Antisera against tobacco PRs did not react with any of the PR preparations from bean or extracts from *Gynura,* thus excluding a close relationship between PRs from tobacco, bean and *Gynura* . Neither did the antisera appear to react with any of the PR preparations from plant species other than the one from which the antigen had been derived.

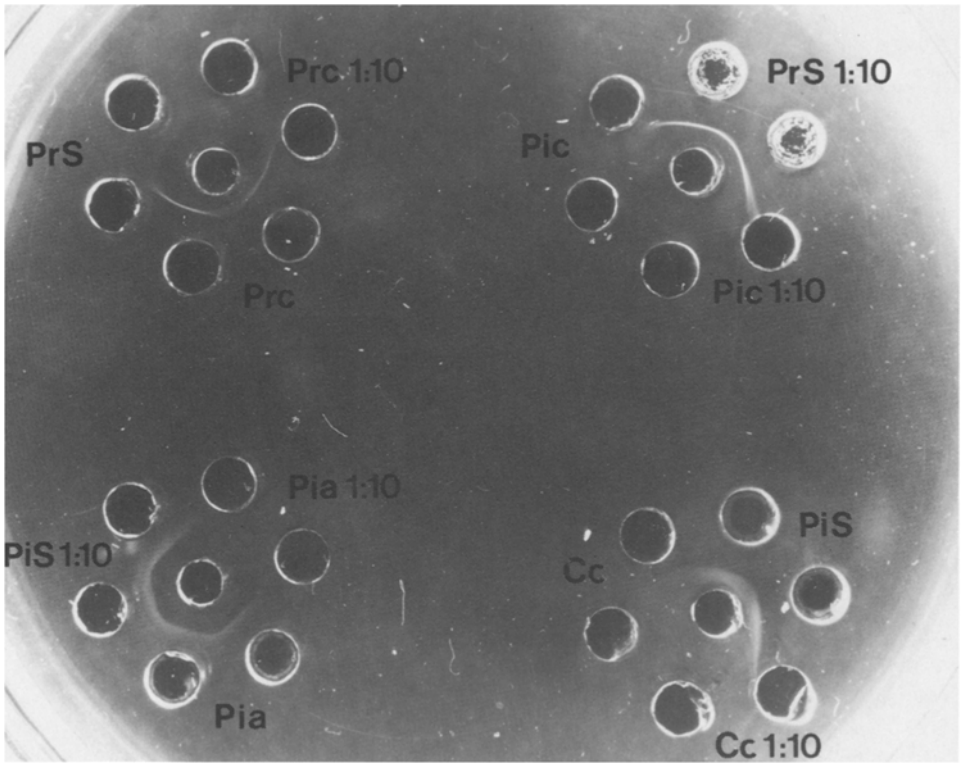

Fig. 6. Reaction patterns to the antiserum against bean chitinase (center wells) of protein extracts from bean 'Prince' (control) (Prc), and infected with the cowpea strain of SBMV (PrS), bean 'Pinto' (control) (Pic), treated with aspirin (Pia) and infected with the bean strain of SBMV (PiS), and cowpea 'Blackeye' (control) (Cc). All samples were tested both undiluted and at a dilution of 1 : 10 in two adjacent wells.

Thus, although PRs from different plant genera may share a number of biochemical properties, they seem to be sufficiently dissimilar to be considered genus-specific.

Acknowledgements

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