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Pectic enzymes production by *Phaeomoniella chlamydospora* (1)

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Summary. Polygalacturonase and polymethylgalacturonase activity was detected in 13 strains of *Phaeomoniella chlamydospora* as well as in filtrates of isolates of some related fungi (*Phaeoacremonium aleophilum*, *P. inflatipes*, *P. rubrigenum*). Tests consisted of growth and colorimetric assays on media designed to evaluate different pectic enzymes. *Phaeomoniella* isolates on the same medium did not differ greatly in their morphological and cultural characters, but pectinolytic activity differed among isolates.

Key words: pectic enzymes, esca, grapevine decline, host colonisation.

Introduction

Enzymes that macerate plant tissue and are able to kill plant cells on their own are known collectively as pectic or pectinolytic enzymes (Alghisi and Favaron, 1995; Annis and Goodwin, 1997). These enzymes may also act as a spreading factor facilitating pathogen spread through the host via pectic connective tissue (Starr, 1961) or vascular tissues (Mann, 1962; Durrands and Cooper, 1988).

Pectic substances are complex structural polysaccharides that occur mainly in the middle lamella and the primary cell walls of higher plants. The pectate network consists of a smooth region with partially methyl-esterified galacturonic acid subunits linked by α-1,4 glycosidic bonds (this compound is a pectin, while the demethylated form is pectic or polygalacturonic acid) and with a hairy region composed mainly of highly branched rhamnogalacturonans (Herron *et al*., 2000). Besides rhamnose, other neutral sugars such as xylose, arabinose and galactose can also be found in this region.

Pectic enzymes fall into two main groups: pectin esterases, which de-esterify pectin by removing methoxyl residues, and depolymerises, which split the D-galacturonic acid main chain. Depolymerases differ: i) in their substrate preference: polygalacturonate and low-methylated pectin (polygalacturonases and pectate lyases) or high-methylated pectin (polymethylgalacturonases and pectin lyases); ii) in their cleavage mechanism: by β-elimination (pectin and pectate lyases) or by hydrolysis (polygalacturonases and polymethylgalacturonases); and iii) in the site of their attack on the polymer: endo or exo (Tardy *et al*., 1997). At least two factors seem to play differential roles in the activity of the various pectic enzymes *in vitro*: the concentration of Ca^{2+} ions and the pH of the

(1) Dedicated to Prof. A. Graniti on the occasion of his 75th birthday

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reaction medium. Hydrolases (polygalacturonases and polymethylgalacturonases) and some pectin lyases (e.g. pectin lyase A from *Aspergillus niger*) have an acidic optimum pH (Mayans *et al*., 1997; van Santen *et al*., 1999; Herron *et al*., 2000) while pectate lyases and pectin lyases (e.g. pectin lyase B from *A. niger)* have their optimum pH near 8.5 (Vitali *et al*., 1998; Herron *et al*., 2000).

As regards the role of calcium ions, Ayers *et al*. (1966) and Cooper *et al*. (1978) reported that concentrations of $CaCl_2$ as low as 0.001 M in the reaction medium consistently decreased the activity of *Rhizoctonia solani* and *Verticillium albo-atrum* polygalacturonases, while the activity of *Fusarium oxysporum* polygalacturonase was unaffected even by higher Ca^{2+} concentrations. Pectate lyases (but not pectin lyases, Mayans *et al*. 1997; Vitali *et al*., 1998), seem to require Ca^{2+} in the reaction medium (Tardy *et al.,* 1997).

Phaeomoniella chlamydospora (*Pch*) (ex *Phaeoacremonium chlamydosporum*), is a tracheomycotic fungus implicated in esca of grapevine and in Petri disease[(1)] (Mugnai *et al*., 1999). It invades the xylem vessels of the vine trunk and travels along them, mostly upwards. In the vessels the mycelium produces conidia, which are carried upwards in the sap stream. The mycelium also advances laterally into adjacent vessels and xylem parenchyma cells. At the same time, tyloses and brown deposits appear in the vessel lumen.

The work presented here studied *in vitro* production by *Pch* of pectic enzymes which help the fungus in colonising host tissues.

Materials and methods

Fungal strains

The fungal strains used in this study (Table 1) were from the collection of the Dipartimento di Biotecnologie Agrarie, Firenze, Italy. For simplicity the reference numbers of the strains in the Table are also those used in the text.

The strains of *Pch* and *Phaeoacremonium aleophilum* (*Pal*) (a fungus which is also associated with esca) were obtained from 10-25-year-old vines showing external symptoms of esca collected in various Italian regions (Piedmont, Lombardy, Friuli, Tuscany, Umbria, Abruzzi, Apulia), from a 6-year-old vine showing Petri disease symptoms (*Pch*55), and from grafted cuttings showing brown wood streaking (*Pal*89). Strain 125 (type strain) of *Phaeocremonium rubrigenum* (*Prubr*) and strain BA5 of *Botrytis cinerea* (*Bc*) were tested for comparison.

All strains were maintained on malt extract agar (MA, Difco Laboratories, Detroit, MI, USA) or as agar plugs in sterile distilled water (SDW) at 4°C.

Media and growth conditions for pectic enzyme production

Solid media: experiment 1

In order to determine whether *Pch* was able to use pectic substances as its sole carbon source, two substrates (Medium 1 and 2) were prepared using Erikksson and Petterson's (1975) mineral medium (EPA), with various modifications, as basal medium. In Medium 1, Sigma's sodium salt of polygalacturonic acid (Sigma, St. Louis, MO, USA) from citrus fruits (0.5% w:v) was employed instead of glucose as the sole carbon source, and low-calcium Oxoid (Oxoid, Basingstoke, England) No. 1 agar (2% w:v) was used as the solidifying agent. $CaCl_2$, usually included in EPA, was omitted from Medium 1. In Medium 2, Sigma citrus pectin (0.5% w:v, methoxy content 8.9%) was employed as the sole carbon source instead of glucose, and calcium-rich Oxoid No. 3 agar (2% w:v) was the solidifying agent. The media were sterilised following the procedures of Ayers *et al*. (1966) and of Durrands and Cooper (1988). After sterilisation the pH was adjusted to 5 and 8 respectively by NaOH 1M or HCl 1N, and the media were poured into 6-cm-diam. plates. All the *Pch, Pal, Prubr*, and *Bc* strains were inoculated using a 5-mm agar plug from 21-day-old MA plates. The diameter of the colony was measured after 7 days of growth at 24°C. For strains *Pch*16, 24 and *Prubr*125 only the diameter was also recorded at 14, 21 and 28 days after inoculation.

Solid media: experiment 2

In order to assess the effect of pH and calcium on the utilisation of citrus pectin, *Pch* strains 16

[(1)] At the general Assembly of the 2nd ICGTD meeting held in Lisbon 2001 it was unanimously decided that the disease variously known as black goo, young grapevine decline, or Petri vine decline will henceforth be called Petri disease.

Table 1. Average diameter (mm) of 7-day-old colonies of *Phaeomoniella chlamydospora* (*Pch*) strains grown on the medium of Erikksson and Petterson (EPA) variously modified and supplemented with sodium salt of polygalacturonic acid (Medium 1) or citrus pectin (Medium 2). The diameter of the colonies of *Phaeoacremonium aleophilum* (*Pal*), *P. rubrigenum* (*Prubr*) and *Botrytis cinerea (Bc)* are also shown. All media were inoculated with a 5-mm agar plug each. Data are average ± SE from 8 replicates. After 7 days of incubation at 25°C the plates were flooded with CTBA and the diameter of the halo produced by the reagent was measured (in mm, including colony diameter).

Fungal species and strain reference No.	Strain code[a]	Medium 1		Medium 2	
		Diameter of the colony (mm)	Diameter of the halo (mm)	Diameter of the colony (mm)	Diameter of the halo (mm)
*Pch*11	229.I.95	12.6 ± 0.18	22.3 ± 0.64	0	0
*Pch*16	389.Z9.95	14.6 ± 0.10	23.3 ± 0.76	0	0
*Pch*17	413.Z7.95	14.2 ± 0.09	21.0 ± 0.28	0	0
*Pch*19	33.20an.95	12.8 ± 0.08	20.9 ± 0.24	0	0
*Pch*24	217.N3.95	11.9 ± 0.13	22.3 ± 0.77	0	0
*Pch*27	1000.95	11.9 ± 0.06	21.1 ± 0.33	0	0
*Pch*28	1026.95	12.3 ± 0.12	21.5 ± 0.51	0	0
*Pch*29	324.R5a.95	14.8 ± 0.43	14.8 ± 0.43	14.0± 0.19	0
*Pch*30	325.R7b.95	13.1 ± 0.13	24.0 ± 0.64	0	0
*Pch*34	Phialo.V.95	11.1 ± 0.06	18.0 ± 0.45	0	0
*Pch*46	981/Ac	11.1 ± 0.09	15.7 ± 1.20	0	0
*Pch*52	56-94 (CBS 229.95)[b]	12.6 ± 0.11	17.7 ± 0.78	0	0
*Pch*55	RV2/GM	13.5 ± 0.76	28.4 ± 1.09	0	0
*Pal*89	327	0	16.2 ± 0.53	14.0 ± 0.31	0
*Pal*72	2220.I.95	25.8 ± 0.46	30.5 ± 0.27	21.4 ± 0.51	0
*Pal*75	373.W.95	25.0 ± 0.41	31.1 ± 0.69	17.9 ± 0.30	0
*Prubr*125	CBS 498.94[b]	16.8 ± 0.64	23.4 ± 1.25	22.2 ± 0.20	28.4 ± 0.36
*Bc*2A5	Bc 2A5	60.0 ± 0	60.0 ± 0	60.0 ± 0	60.0 ± 0

[a] All strains are from the collection of the Dipartimento di Biotecnologie Agrarie, Firenze, Italy, except otherwise indicated.
[b] Type strain deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, NL.

and 52 were grown on 16 solid media based on EPA with different conditions promoting growth. The sole carbon source was either citrus pectin (0.5% w:v), or glucose (0.5% w:v). In 4 of the 8 media containing citrus pectin and in 4 of the 8 media containing glucose, $CaCl_2$ was omitted and low-calcium Oxoid No.1 agar (2% w:v) was added instead of calcium-rich Oxoid No. 3 agar. The media were sterilised as above. After sterilisation, for each combination of 2 carbon sources and 2 calcium levels (citrus pectin/high calcium, citrus pectin/low calcium, glucose/high calcium, glucose/low calcium), 4 pH levels (3, 4, 6 and 8) were obtained by adding sterile NaOH 1M or HCl 1N. The 16 media so obtained were poured into 6-cm-diam. Petri plates, each inoculated with a 5-mm agar plug from 21-day-old Medium 1 plates (see above), and incubated at 24°C. The diameter of the colonies was measured 3, 6, 9 and 15 days after inoculation.

Liquid media

In order to assess hydrolase activity, Richards' solution (Leone and Van Den Heuvel, 1987) with glucose (0.5% w:v) or citrus pectin (0.5% w:v) added as the sole carbon source, or without any carbon, was employed as growth medium. This experiment was performed on strains 16, 24 and 52.

The liquid media were poured into 50-ml Erlenmeyer flasks, 20 ml in each flask, sterilised at 116°C for 10 min. and pH adjusted to 5 with sterile NaOH.

The inoculum was grown on 2% malt extract liquid medium for 7 days. Before inoculation the inoculum was centrifuged twice in SDW at 7000 *g* for 5 min at 15°C and the concentration adjusted to 4.5×10^6 cfu ml^{-1} with SDW. One hundred ml of this suspension was used to inoculate each of the Erlenmeyer flasks, which were then incubated at 24°C. After growth in shake culture (100 rpm) for

6, 15, 19 and 26 days, the liquid cultures were filtered through Whatman No.1 filter paper (Whatman, Maidstone, UK), and through Sartorius filters (0.45 μm, Sartorius, Göttingen, Germany). At each sampling date the pH of each culture was measured and the mycelium dry weight determined.

Enzyme assays

Solid media, experiments 1 and 2

After incubation at 24°C for 7 days on Media 1 and 2 the inoculated plates were flooded with 1% (w:v) cetylmethyl ammonium bromide (CTAB), dissolved in distilled water and heated to 30°C. Enzyme activity was indicated by the appearance after about 5 min of a clear zone around the fungal colonies, against the white/grey background of the precipitated polysaccharides.

Liquid media

Six- to 26-day-old culture filtrates of the 3 *Pch* strains and were assayed for polygalacturonase activity using the colorimetric reaction between reducing sugars resulting from the activity of the enzymes, and p-hydroxybenzoic acid hydrazide in an alkaline solution as reported by Lever (1972) (York *et al*., 1985; Cervone *et al*., 1989). Polygalacturonase activity was expressed as reducing groups units (RGU). One unit of activity is the amount of enzyme that liberates 1 meq of reducing groups per min^{-1} at 30°C using 1% (w:v) polygalacturonic acid as a substrate.

Culture filtrates were also assayed for pectinolytic activity by the radial diffusion cup plate assay (Brown *et al.,* 1992). The media were poured into 60-mm Petri plates, 6 ml per plate, and punched with a cork borer (6- or 8-mm-diam. holes). Each well was filled with 30 or 45 ml of one of the culture filtrates. The plates were incubated for 16 h at 37°C and flooded with 0.05% ruthenium red at 25°C for 30 min. A clear area around the well indicated polygalacturonase activity, while a red zone around the clear area indicated also pectin esterase activity (Brown *et al.,* 1992). Another way to detect pectinolytic activity was by flooding the plates with 1% CTAB at 25°C for 5 min. The reagent was then removed and the assay plates treated with 0.05% ruthenium red at 25°C for 30 min, after which the dye was removed and the plates washed with SDW. A positive result consisted in the rapid appearance (about 5 min) of a clear zone, which became more conspicuous after 5 to 6 h.

Statistical analysis

Differences in the average radial growth of individual *Pch* strains due to pH and calcium concentration of the growth medium were evaluated in a multiple comparison using Tukey's HSD test. Observed differences at each time point among *Pch* strains grown on the same medium were evaluated using Student's t test.

Pch, *Pal* and *Prubr* strains were grouped by the average diameter of the colony and of the halo on Medium 1 after 7 days from inoculation, using the k-Mean clustering algorithm of the software package Statistica 6 (Stat Soft Inc, Tulsa, OK, USA).

Results and discussion

Phaeomoniella chlamydospora used pectic substances as carbon sources for growth *in vitro*, but the extent of such use depended on pH and on the calcium content of the growth medium.

The growth of *Pch* strains on Medium 1 (a traditional medium for detecting polygalacturonase activity) showed that all *Pch* strains tested were able to use non-methylated pectic substances as a carbon source (Table 1). Seven days after inoculation on Medium 1, the average diam. of the colonies of *Pch* strains varied from a minimum of 11.1 mm (*Pch*34 and 46) to a maximum of 14.8 mm (*Pch*29). Differing growth on the solid media was accompanied by a different diam. of the halo around the colonies after treatment with CTAB. Diameters varied from a minimum of 14.8 mm (*Pch*29) to a maximum of 28.4 mm (*Pch*55). All the other fungal species used as controls, *P. aleophilum, P. rubrigenum* and *B. cinerea*, grew on the media except *Pal*89, and they all displayed the typical halo indicating enzyme production. These findings suggest that the strains of *Pch* differ not only in their ability to degrade pectic substances, but also in their uptake and/or in their metabolism, at least *in vitro*, of the products of this degradation.

On the basis of the average colony diameter on Medium 1 and of the corresponding average diameter of the halo (Table 1), the *Pch*, *Pal* and *Prubr* strains were partitioned into 5 clusters with a k-Means clustering algorithm (Fig. 1). (The slope of

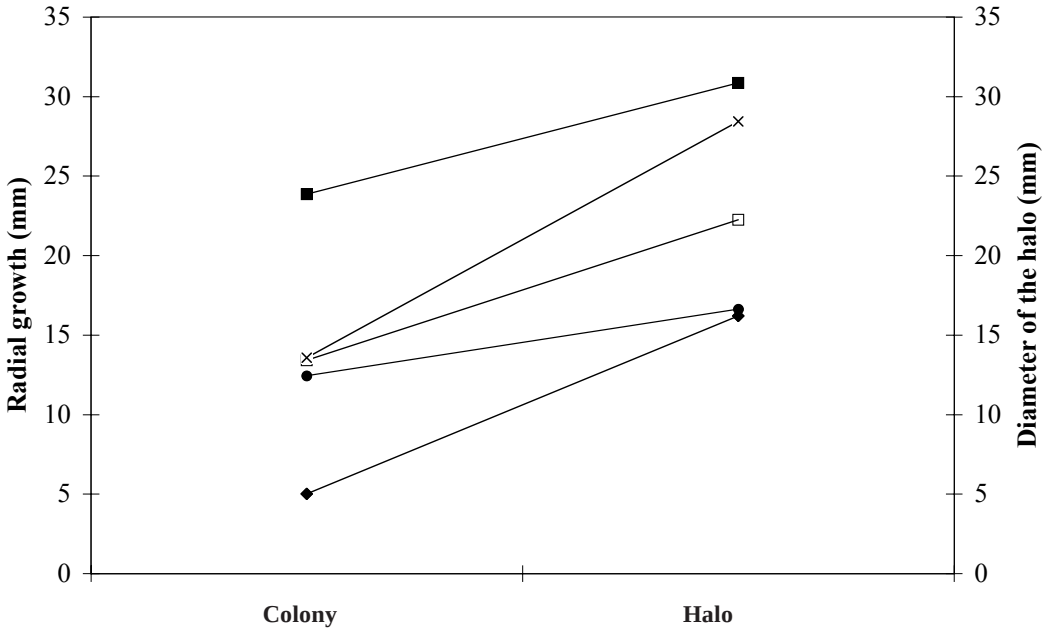

Fig. 1. Clustering of 7 days old colonies of *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *P. rubrigenum* grown on Medium 1 (EPA without $CaCl_2$ and with sodium salt of polygalacturonic acid as sole carbon source, pH 5) considering the mean diameter of the colony (colony) and the mean diameter of the halo (halo) after flooding with 1% CTAB. SE<0.76 mm for all colonies and halos. K-Means Clustering partitioning method was employed with a final required number of clusters equal to five. Colony: F4,12=35.8; P<1.4*10-6; halo: F4,12=56.30; P<1.12*10-7. Cluster 1 (♦): *Pal*89; cluster 2 (●): *Pch*29, 34, 52, 46; cluster 3 (□): *Pch*16, 24, 27, 30, 11, 19, 28, 17 and *Prubr*125; cluster 4 (**x**): *Pch*55; cluster 5 (■): *Pal*72, 75.

the lines in Fig. 1 is related to the magnitude of the ratio between the average diameter of the halo and that of the colony). Two *Pal* strains (72 and 75) showed a similar behaviour, with faster and greater growth than the *Pch* and *Prubr* strains on Medium 1, but the amount of pectic enzymes released into the growth media was relatively small. Interestingly the isolate *Pal*89 was unable to grow on Medium 1 even if a relatively considerable amount of pectic enzymes was released in the substrate. The *Pch* strains differed substantially in enzyme production but not in colony growth. Clusters No. 2 (4 strains), 3 (9 strains) and 4 (1 strain) had very similar radial growth (Fig. 1) but the amount of enzyme(s) released into the medium decreased sharply from group 4 to 2. The behaviour of *Prubr*125 on Medium 1 was similar to that of the *Pch* strains in cluster No. 3.

On Medium 2 (detecting pectin lyase activity) none of the *Pch* strains grew except *Pch*29 (Fig. 2), even after 3 weeks from the inoculation (Fig. 2). Furthermore, only *Botrytis cinerea* and *Phaeoacremonium rubrigenum* produced detectable amounts of pectin lyases (Table 1). To investigate the non-growth of the *Pch* strains on Medium 2, *Pch* strains 16 and 52 were inoculated on EPA with different pH and Ca^{2+} levels (Fig. 3). Both these strains failed to grow at pH 8, and showed significantly higher growth at pH 4 than at pH 3 and pH 6. Growth of these strains was also reduced by the higher calcium concentration in the medium. The growth rate of *Pch*52 was initially higher than that of *Pch*16, but after 9 days on the media with lower calcium and 15 days on the media with higher calcium the growth rate of *Pch*16 equalled or even exceeded that of *Pch*52 (Fig. 4). On MA both strains started to grow between the 3rd and the 6th day after inoculation; the growth rate of *Pch*52 was always significantly higher than that of *Pch* 16 (data not shown).

To establish the time-course pattern of polygalacturonase production, *Pch* strains 16, 24 and 52 were compared using Lever's procedure. After 6 days of culture, *Pch*16 and 24 produced 0.018 and

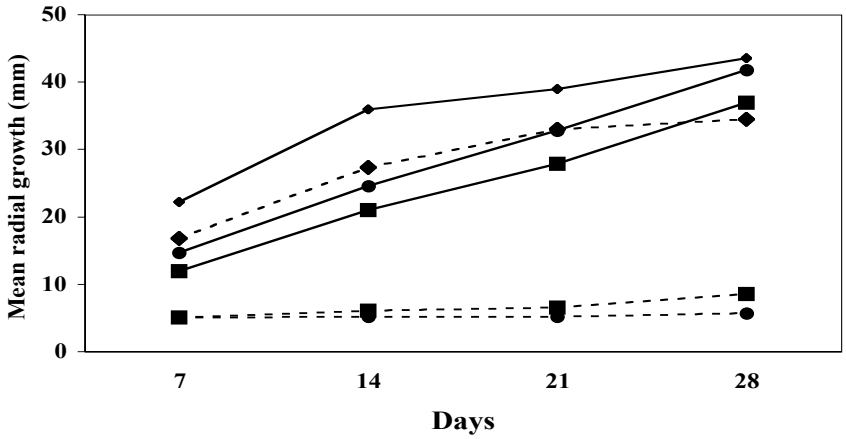

Fig. 2. Radial growth of *Phaeomoniella chlamydospora* (*Pch*) strains 24 (●) and 16 (■), and of *Phaeoacremonium rubrigenum* strain 125 (♦) on Medium 1 (sodium salt of polygalacturonic acid, continuous line) and on Medium 2 (pectin, discontinuous line). Mean results of 8 plates (SE < ± 1.2 mm).

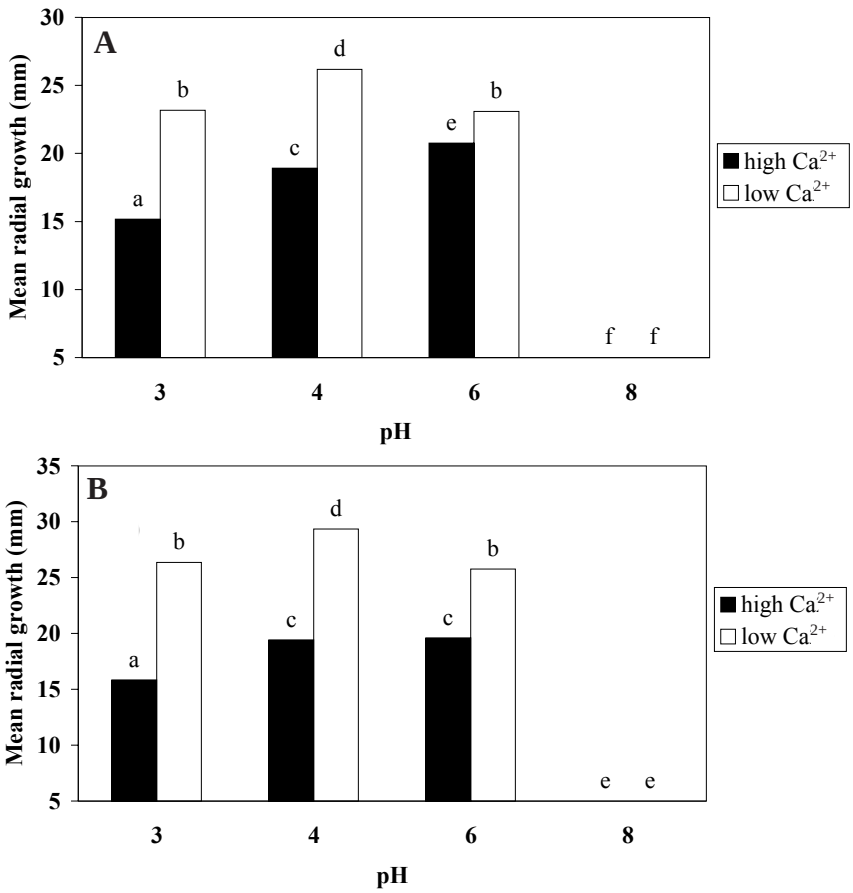

Fig. 3. Effect of pH and of calcium ion concentration on radial growth of *Phaemoniella chlamydospora Pch*52 (a) and Pch16 (b) on EPA containig pectin as the sole carbon source. Mean results of 6 plates. SE< ± 0.7 mm. Radial growth was measured 15 days after incubation at 24°C. The differences among means were evaluated with Tukey's HSD test (P<0.01). Bars with different letters are significantly different (P<0.01).

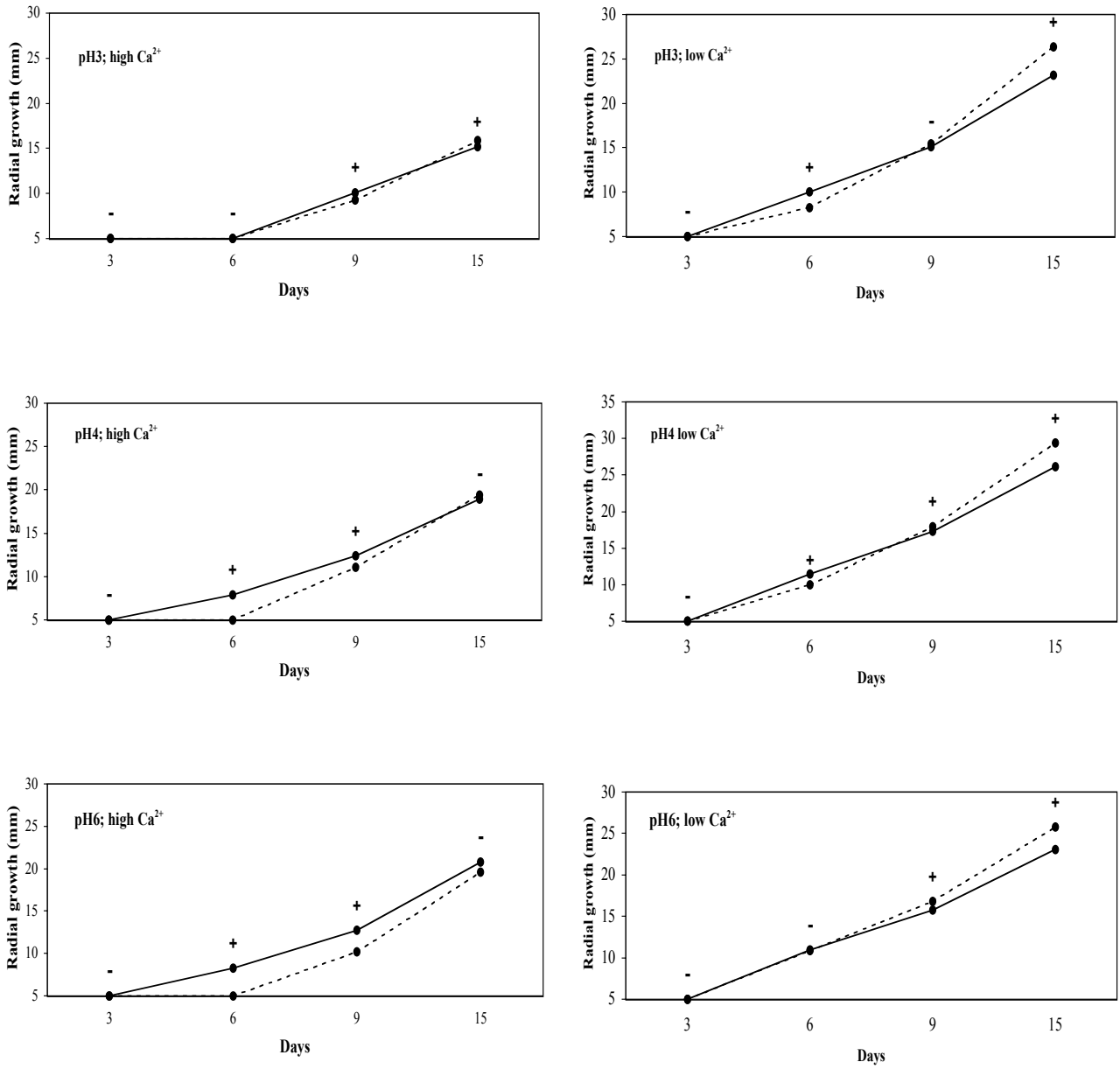

Fig. 4. Differential colony growth of *Phaemoniella chlamydospora* isolate 16 (- -) and 52 (—) on plates containig EPA with pectin as sole carbon source, under the effect of different pH values and higher or lower calcium ions concentration of the media. Mean results of 6 plates. SE < ± 0.7 mm. For each time point the observed differences between the two *Pch* isolates grown in the same condition of pH and calcium level of the media, were evaluated with Student's t test. Significative differences (P<0.01; two tails distribution) are reported on the chart with a "+" sign.

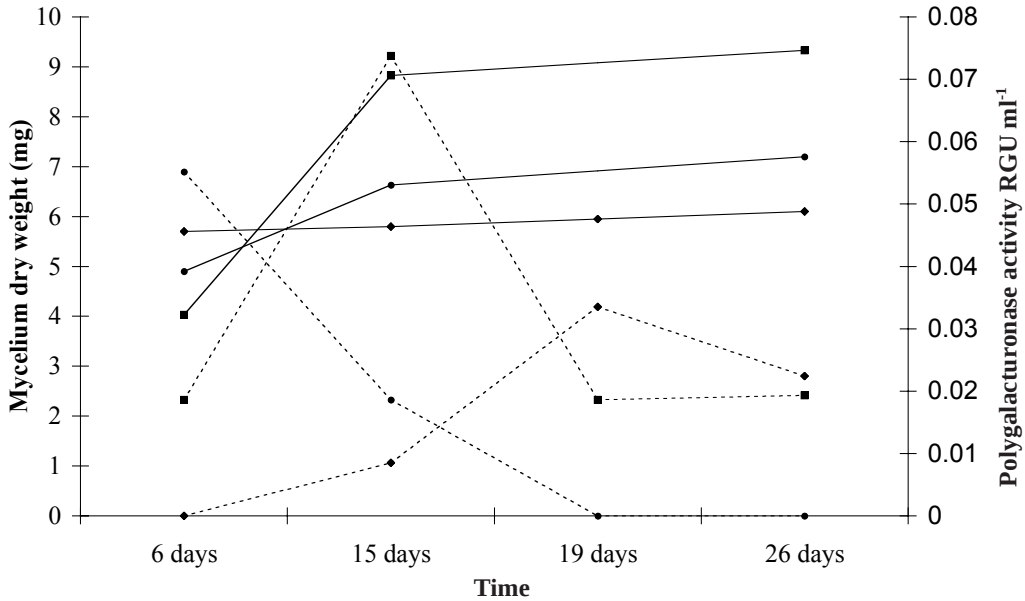

Fig. 5. Growth (—) and polygalacturonase production (- -) by three isolates of *Phaeomoniella chlamydospora* (*Pch*52, ♦; *Pch*16, ■; *Pch*24, ●). Polygalacturonase activity is expressed as RGU ml^{-1}.

0.055 RGU respectively (Fig. 5). Thereafter the pectinolytic activity of culture filtrate from *Pch*16 first went up (0.076 RGU on day 15) then down (0.018 RGU on day 19), but that of the filtrates from *Pch*24 decreased steadily until it vanished altogether by day 19. The enzyme-production pattern of *Pch*52 was different. This strain, as already observed on solid media, grew more rapidly than the other two in the first 6 days (5.7 mg d. wt of mycelium) but did not produce any detectable active filtrates until the enzyme test on day 19 (0.033 RGU). The complete disappearance of polygalacturonase activity in the culture filtrates of *Pch*24, or its decrease in *Pch*16 and *Pch*52 could be due to proteinases in the filtrates.

The production of pectic enzymes of the hydrolase type by *Pch* was confirmed by the diffusion cup plate assay (Fig. 6). All thirteen *Pch* strains displayed both polygalacturonase and pectin esterase activity, since in the tests with ruthenium red the red ring indicating such activity was always obtained (Brown *et al.*, 1992).

A red ring was also produced with *Pal, Prubr* and *Bc* culture filtrates. Clear zones were produced when the plates were flooded with CTAB alone or with CTAB followed by ruthenium red, although the clear zones were more conspicuous with the latter.

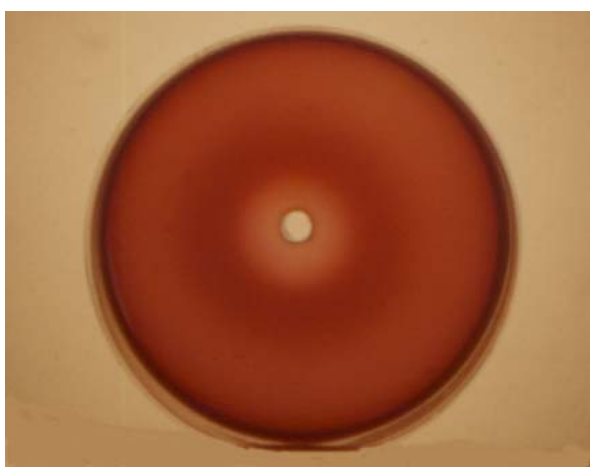

Fig. 6. Diffusion cup plate assay showing the production of hydrolase-type pectic enzymes by *Phaeomoniella chlamydospora* (strain *Pch*16). The clear zone (polygalacturonase activity) and the red ring (pectin esterase activity) was produced when the plate was flooded with CTAB followed by ruthenium red.

Conclusions

Phaemoniella chlamydospora not only cleaved pectic polymers but also used them as carbon sources for growth and energy. Production of pectic enzymes in an acidic medium with low calcium varied among the *Pch* strains. At least two different hydrolase-type pectic enzymes were produced, since the degradation of polygalacturonic acid and of the corresponding methylated form, indicated the production of both polygalacturonase and polymethylgalacturonase. However, the findings did not exclude a pectin lyase-type enzyme, as was reported for *Aspergillus niger* (Mayans *et al*., 1997), which is active in an acidic medium with pectin as the sole carbon source. By contrast, there was no production of pectin lyases active in a basic reaction medium. Most *Pch* strains did not grow at pH 8 with pectin as the sole carbon source, and none showed the typical halo when plates where flooded with CTAB.

In recent years several authors have reported that bacterial and fungal species produce different isoenzyme forms of pectic enzymes. The optimal activity of these isoenzymes depends, sometimes considerably, on medium characteristics such as pH, calcium concentration, degree of methylation, thermal stability and ionic strength. Furthermore, at least in the case of pectate lyases of *Erwinia chrysanthemi*, each corresponding gene is an independent transcriptional unit and can be expressed independently. As several authors have suggested, if pathogens secrete *in vivo* a battery of enzymes which differ in their requirements for optimal activity, then these pathogens might have the potential to act differently depending on even small differences in host tissues during the life cycle and/or between different hosts. It is however very difficult to recreate *in vitro* the conditions to detect the activity of each single molecule.

Pch is a mitosporic fungus which alone or in conjunction with other micro-organisms (*Phaeoacremonium aleophilum*, *Fomitiporia punctata*) is thought to cause the deterioration in vinewood typical of two well-known diseases of grapevine, esca and Petri disease. However, the slow spread of these diseases through vine tissue and the discontinuous course of symptom expression (diseased vines may quite unpredictably not exhibit visual symptoms for one or more years in succession, leaving the vine to all appearances healthy in those years) make it difficult to ascertain the pathogenicity/virulence of the fungi involved. The finding that *Pch* produces not only phytotoxins (Sparapano *et al.,* 2000) but also pectic enzymes may be a step forward in the attempt to elucidate the role of this fungus as a pathogenic agent.

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