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RESEARCH ARTICLE

D. Bernardo · M. Novaes-Ledieu · A. Perez Cabo
F.J. Gea Alegría · C. García Mendoza

Effect of the fungicide Prochloraz-Mn on the cell wall structure of *Verticillium fungicola*

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Abstract The chemical structure of the cell wall of two isolates of *Verticillium fungicola* collected from diseased fruit bodies of the commercial mushroom *Agaricus bisporus* treated with the fungicide Prochloraz-Mn was analyzed. The isolates were obtained during different periods of time and grown in the absence and presence of the LD_{50} values of the fungicide for *V. fungicola*. In addition, another *V. fungicola* isolate collected previous to the routine utilization of Prochloraz-Mn but grown under the same conditions was also analyzed. The overall chemical composition of the cell wall from the three isolates showed detectable differences in their basic components, with a significant decrease in the protein content in fungicide-treated cells. This inhibitory effect was partially compensated by an increase in neutral and/or aminated carbohydrates and was accompanied by appreciable modifications of polysaccharide structure, as deduced after methylation analysis and gas-liquid chromatography-mass spectrometry (GLC-MS). Moreover, differences in hyphal morphology caused by the fungicide were observed by transmission electron microscopy (TEM).

Keywords *Verticillium fungicola* · Cell wall · Partial protein inhibition · Carbohydrate rearrangement · Prochloraz-Mn

Introduction

Verticillium fungicola (Preuss) Hassebr. is the fungal pathogen causing brown spot or dry bubble disease in crops of the commercial mushroom *Agaricus bisporus* (Lange) Imbach. Different macroscopic symptoms during pathogenesis have been described [7, 14,19], and, more recently, electron microscopy studies have demonstrated that the mycopathogen grows both outside and inside the hyphae of *A. bisporus* fruit bodies [3,5]. The infection process seems to be due to the production of extracellular enzymes by *V. fungicola* that lead to necrosis and browning of the mushrooms [10, 12, 17,18]. These enzymes include exo- and endo polysaccharidases and proteases identified by Calonje et al. [3], and some of these are enhanced by the presence of *A. bisporus* fruit-body walls [3].

Control of dry bubble disease caused by *V. fungicola* has been through the use of chemicals, culture conditions, and sanitation. The chemical products utilized to control *V. fungicola* must be effective without damaging the mushroom crop. Since 1985, Spanish mushroom farms have used fungicides containing Prochloraz-Mn as the main active ingredient to effectively control dry bubble disease. However, beginning in 1992 a greater incidence of *V. fungicola* infection was observed in mushroom crops in spite of treatment with this fungicide [8], suggesting the development of resistance to Prochloraz-Mn.

Studies have been carried out on the mode of action of several fungicides, including sterol biosynthesis inhibitors such as Prochloraz-Mn[1, 11, 13, 16]; however, these studies did not exclude more than one mechanism of inhibition (multi-site fungicide). As we have recently described the chemical structure of *V. fungicola* hyphal walls [4], the aim of the present work was to analyze possible changes in the chemical structure and ultrastructure of the cell wall of *V. fungicola* following treatment with the fungicide Prochloraz-Mn.

D. Bernardo · M. Novaes-Ledieu · A. Perez Cabo
C. García Mendoza (✉)
Centro de Investigaciones Biológicas, CSIC,
Velázquez 144, 28006 Madrid, Spain
E-mail: cgm@cib.csic.es
Tel.: +34-915611800
Fax: +34-915627518

F.J. Gea Alegría
Centro de Investigación, Experimentación y Servicios del Champiñón, 16220 Quintanar del Rey, Cuenca, Spain

Materials and methods

Organisms, culture conditions and cell wall preparations

The two *V. fungicola* isolates (nos. 210 and 100) were supplied by the Centro de Investigación, Experimentación y Servicios del

Champiñón (CIES) and were collected from infected sporocarps of Castilla-La Mancha, Spain, mushroom farms during the years 1992 and 2000, respectively. A strain from the Centraalbureau voor Schimmelcultures (CBS 992.69), isolated in 1969 prior to Prochloraz-Mn utilization, was used as reference organism. When necessary, the fungicide Prochloraz-Mn-complex 46% (Sporgon, Schering) was added to Raper medium and used at the corresponding LD_{50} values (Table 1) [15]. LD_{50} evaluation was carried out by culturing the pathogen in fungicide-amended Raper medium at various concentrations. The cultures were filtered through Whatman 3MM paper in a Millipore filtration apparatus and the filters containing mycelia were weighed. Results were expressed as a 50% percentage reduction in mycelial growth.

For cell wall preparation, *V. fungicola* isolates were grown for 5 days statically in 2-l flasks containing 1 l of Raper medium. Fungal biomass was removed by filtration, washed several times with distilled water and freeze-dried until used for fractionation, as described below.

Dry mycelium was first pulverized in a Sorvall omnimixer and then disintegrated in a Fritsch 6 balls-mill [9]. The hyphal wall fragments were purified by repeated washings with distilled water until the material was completely clean (as determined by phase-contrast microscopy and the absence of protein in the water washings) and stored freeze-dried for further analysis.

Fractionation of cell wall material and gel filtration of the soluble saccharide fractions

Dry material (3 g) was extracted with 350 ml of distilled water at 60 °C for 40 h. After centrifugation the residual cell walls were washed twice with distilled water, combining the supernatant and washes. The concentrated and freeze-dried extract constituted fraction F1. The residue from this treatment was extracted with 350 ml of 1 N KOH at 60 °C for 48 h and, after centrifugation, the cell walls were washed twice with 1 N KOH, combining extract and washes. The solubilized material was dialyzed against running tap water. After 24 h of dialysis two fractions were obtained, one insoluble in water (F2I), which was collected by centrifugation and freeze-dried, and the other soluble in water (F2S), which was precipitated overnight at 4 °C with two volumes of 96% ethanol, centrifuged and freeze-dried. The residual cell wall material that was insoluble in alkali was washed with distilled water repetitively until the supernatant was free from alkali and freeze-dried (F3) (Fig. 1).

Samples of F1 and F2S (50–150 mg) were dissolved in 1–3 ml of 0.3 M NaOH and centrifuged at 13,000 *g* for 15 min to eliminate insoluble material. The supernatant of each sample was applied to a (100×2.5 cm) of Sepharose CL-6B column and eluted with 0.3 M NaOH; the flow rate was 18 ml/h. Fractions of 3 ml were collected and monitored for carbohydrate by the phenol-sulfuric acid method [6]. Appropriate fractions were combined, dialyzed and freeze-dried.

Chemical and methylation analyses, and electron microscopy

All the procedures for chemical analysis as well as those for monosaccharide identification and methylation analysis have been described previously [2]. For transmission electron microscopy (TEM), samples of *V. fungicola* cell walls obtained from the three isolates grown in the absence and presence of the fungicide were placed on formvar-coated grids, left to dry, shadowed with Au-Pd and examined in a Philips EM 300 transmission electron microscope.

Results and discussion

The LD_{50} values of the fungicide for the *V. fungicola* isolates ranged from 0.51 ppm (0.58 mg/l) to 3.48 ppm (3.98 mg/l), with resistance levels differing depending on the isolation date [8]. In order to study the effect of the Prochloraz-Mn on the *V. fungicola* cell wall, the two CIES isolates (210 and 100) were selected, with the CBS isolate as the control. The chemical composition of the cell wall of the three isolates grown in the presence or absence of the fungicide is shown in Table 1. The neutral carbohydrate values tended to increase with the use of Prochloraz-Mn, whereas the amount of protein decreased at different rates and the hexosamines remained balanced, showing a more appreciable increase in isolate CIES 210. Cell wall fractionation provided six different fractions (Fig. 1), five of which, F1a, F1b, F2Sa, F2Sb and F3, represented about 75–80% of the total dry weight of the cell wall and were mainly composed of polysaccharides. By contrast, fraction F2I had a very low content of neutral sugars (less than 5%) and consisted mainly of lipids. Carbohydrate analyses of the extracted polysaccharide fractions (Table 2) revealed considerable differences in the percentages of monomers, with glucose decreasing significantly in fractions F1b and F2Sb (glucogalactomannans) of the CBS isolate, and present in a lower proportion in the two CIES *V. fungicola* isolates from farms using Prochloraz-Mn routinely. By contrast, galactose increased according to the effect of the fungicide, mannose remained practically constant. The CBS isolate again showed a clearer effect than the two CIES isolates. However, fractions F1a, F2Sa and F3 of the three isolates were composed only of glucose (glucans) and exhibited no differences whether obtained from cells grown in the absence or presence of Prochloraz-Mn.

The results of the methylation analysis of the cell wall fractions of each isolate are given in Tables 3 and 4, and are in agreement with those previously reported in the same organism [4], although significant differences could be detected after fungicide treatment. Fraction F1a, corresponding to a (1–4)-linked glucan with branch

Table 1. Overall chemical composition of cell walls of three *Verticillium fungicola* isolates treated or nor treated with Prochloraz-Mn. The mean deviation is calculated from the results of at least four experiments. *F* Fungicide Prochloraz-Mn

Component	CBS 992		CIES 210		CIES 100	
	–F	+F	–F	+F	–F	+F
Neutral carbohydrates	45.1 ± 0.5	49.5 ± 0.6	50.5 ± 0.6	53.1 ± 0.6	50.6 ± 0.5	53.7 ± 0.7
Proteins	25.1 ± 0.3	21.0 ± 0.3	18.0 ± 0.2	9.9 ± 0.2	17.8 ± 0.3	16.5 ± 0.3
Hexosamines	15.2 ± 0.2	14.5 ± 0.3	13.9 ± 0.3	20.9 ± 0.4	14.7 ± 0.2	14.2 ± 0.3
Lipids	12.0 ± 0.3	12.6 ± 0.3	14.1 ± 0.4	11.9 ± 0.4	13.8 ± 0.5	13.0 ± 0.4
Ash	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.05	1.3 ± 0.2	0.5 ± 0.1	0.4 ± 0.1

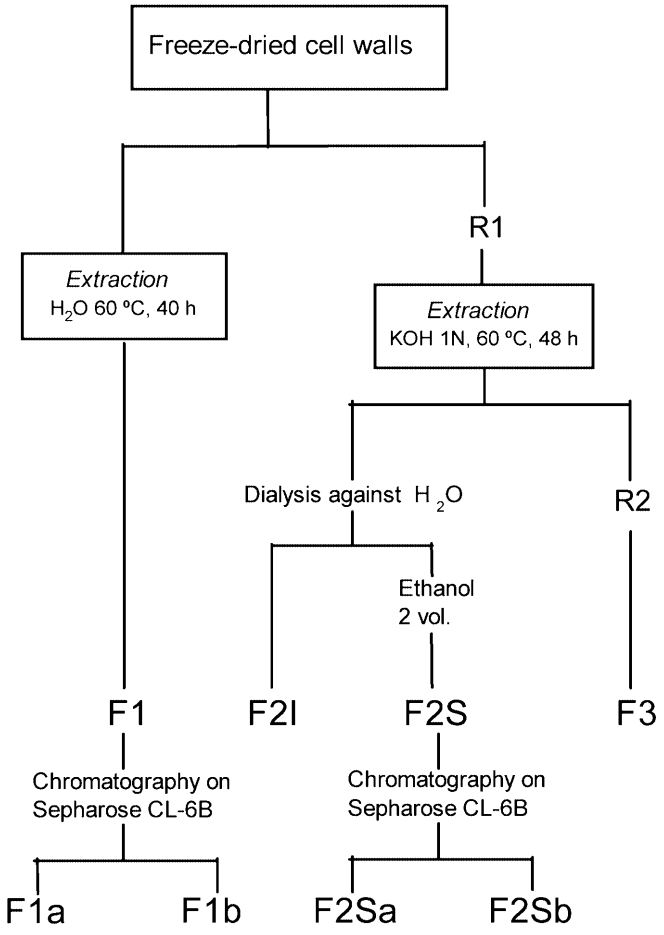

Fig. 1. Fractionation of *Verticillium fungicola* cell walls

points at C-6, became more linear (less branched) with the effect of Prochloraz-Mn (Table 3), whereas fraction F1b, a (1–6)-linked mannan highly branched at C-4 points (glucogalactomannan), showed a significant decrease in (1–4) glucose units in the CBS isolate (Table 4). Fraction F2Sa, another glucan containing principally (1–4)-linked glucosyl residues with branch points at C-6, also lost some of these branch points in the *V. fungicola* 100 isolate in the presence of the fungicide (Table 3). In fraction F2Sb, consisting of another

Table 2. Molar ratio of the neutral sugars detected as alditol acetates by GLC of fractions F1b and F2Sb from *V. fungicola* isolates treated or not treated with Prochloraz-Mn. Mean values are the averages of at least four determinations. *F* Fungicide Prochloraz-Mn

Isolates	Fractions	Glucitol	Galactitol	Mannitol
CBS	F1b	23.1	25.9	51.0
CBS + F	F1b	12.4	35.2	52.2
CIES 210	F1b	14.3	32.4	53.3
CIES 210 + F	F1b	13.5	32.4	54.1
CIES 100	F1b	10.2	32.7	57.1
CIES 100 + F	F1b	9.5	33.9	56.7
CBS	F2Sb	15.6	28.6	55.8
CBS + F	F2Sb	6.6	36.3	57.1
CIES 210	F2Sb	10.0	35.8	55.2
CIES 210 + F	F2Sb	7.1	37.8	55.1
CIES 100	F2Sb	6.0	39.2	54.8
CIES 100 + F	F2Sb	5.4	41.0	53.6

glucogalactomannan (mannose residues linked at (1–6), highly branched at C-4), the (1–3) and (1–4) glucosyl linkages decreased variably in the three isolates in response to Prochloraz-Mn (Table 4). Finally, in fraction F3, consisting of the glucan associated with chitin and containing mainly (1–4)- and (1–3)-linked glucosyl residues with very low branching at C-6, the (1–4) glucosyl linkages increased slightly while the (1–3)-linked glucosyl residues decreased due to the action of the fungicide (Table 3).

Using the shadowing technique followed by TEM, rodlet structures of varying length and organized in bundles could be visualized on the surface of the hyphae (Fig. 2A–C). These typical structures were found in the F1 and F2 cell wall fractions, as was previously demonstrated in the same organism [4]; however, they partially disappeared in Prochloraz-Mn-exposed fungi (Fig. 2D–F) indicating that they might be composed of proteinaceous material.

Taken together, these results show that in *V. fungicola* Prochloraz-Mn acts by a mechanism that is in addition to its known inhibition of sterol biosynthesis [16], i.e., partial inhibition of cell-wall-protein synthesis, as also reported by Siegel [16]. This inhibition of protein synthesis is clearly reflected in the overall chemical composition of the cell wall of *V. fungicola* isolates

Table 3. GLC-MS data for partially methylated alditol acetates from fraction F1a, F2Sa and F3 (glucans) of *V. fungicola* isolates treated or not treated with Prochloraz-Mn. All values are means of at least three replications. *F* Fungicide Prochloraz-Mn

Isolates							
Deduced linkage	Fractions	CBS	CBS + F	CIES 210	CIES 210 + F	CIES 100	CIES 100 + F
Hexp-(1	1 F1a	5.6	4.6	5.1	3.8	5.3	4.9
4)-Glup-(1	1 F1a	88.9	91.2	89.9	92.6	88.5	90.7
4,6)-Glup-(1	1 F1a	5.5	4.2	5.0	3.6	5.2	4.4
Hexp-(1	F2Sa	4.4	4.6	4.5	3.8	5.4	3.9
4)-Glup-(1	F2Sa	92.1	94.2	91.8	93.0	89.5	92.7
4,6)-Glup-(1	F2Sa	3.5	4.2	3.7	3.2	5.1	3.4
Hexp-(1	F3	1.5	1.7	1,9	1,6	1,5	1,5
3)-Glup-(1	F3	58.1	55.5	56.7	54.4	56.5	55.3
4)-Glup-(1	F3	38.7	40.5	39.3	41.6	39.7	41.1
4,6)-Glup-(1	F3	0.8	1.2	1.0	1.1	1.1	1.0
3,6)-Glup-(1	F3	0.9	1.1	1.1	1.3	1.2	1.1

Table 4. GLC-MS data for partially methylated alditol acetates from Fraction F1b and F2Sb (glucogalactomannans) of *Verticillium fungicola* isolates treated or not treated with Prochloraz-Mn. All values are means of at least three replications. *F* Fungicide Prochloraz-Mn

		Isolates					
Deduced linkage	Fractions	CBS	CBS + F	CIES 210	CIES 210 + F	CIES 100	CIES 100 + F
Hexp-(1	F1b	2.6	4.0	5.1	5.2	2.7	2.4
Galp-(1	F1b	26.5	34.0	31.3	32.3	33.9	35.2
2)-Manp-(1	F1b	15.3	12.8	5.4	5.5	7.7	5.9
4)-Glup-(1	F1b	17.5	8.3	8.8	7.9	6.4	5.6
6)-Manp-(1	F1b	2.0	4.8	12.1	10.5	2.3	2.5
2,4)-Hexp-(1	F1b	0.7	1.2	0.5	0.4	0.9	0.9
4,6)-Manp-(1	F1b	33.2	29.9	34.2	36.2	43.7	48.1
2,6)-Manp-(1	F1b	2.0	5.0	3.9	4.0	2.6	2.5
Hexp-(1	F2Sb	3.4	1.8	2.4	1.9	1.8	1.7
Galp-(1	F2Sb	26.3	34.2	33.5	38.5	38.7	39.3
3)-Glup-(1	F2Sb	5.1	2.4	3.1	2.7	2.1	1.9
5)-Galf-(1	F2Sb	1.5	1.3	1.4	1.1	1.3	1.1
4)-Glup-(1	F2Sb	6.4	3.1	5.2	3.0	2.7	2.2
3)-Manp-(1	F2Sb	2.5	2.6	2.9	3.0	2.9	2.7
6)-Manp-(1	F2Sb	6.3	7.6	5.6	5.4	3.0	2.9
4,6)-Manp-(1	F2Sb	45.9	46.3	42.6	42.2	47.1	45.2
2,6)-Manp-(1	F2Sb	3.3	3.0	3.5	3.1	2.9	2.7

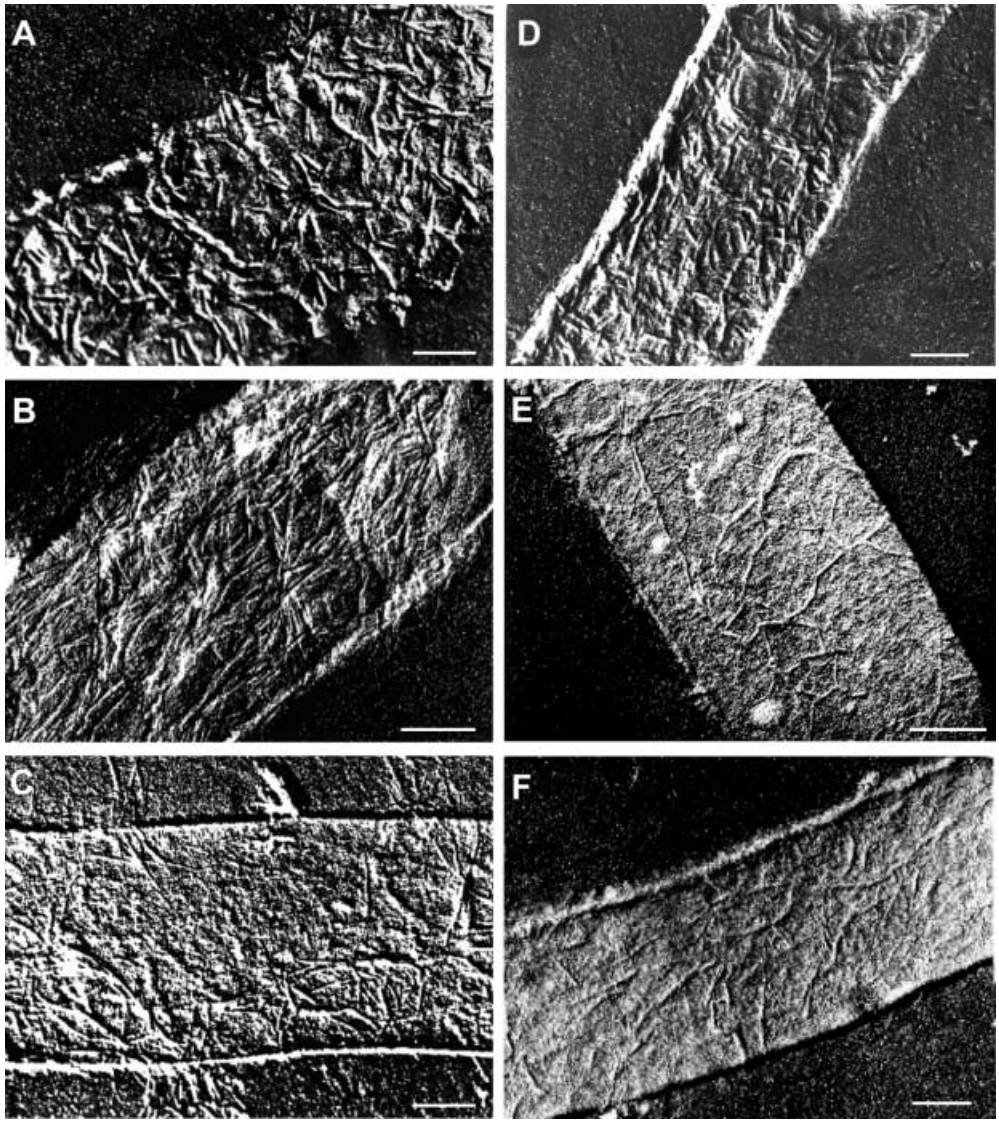

Fig. 2A–F. Shadowed electron micrographs of cell wall surfaces from *V. fungicola* isolates CBS and CIES 210 and 100. *V. fungicola* isolates were grown in the absence and presence of fungicide (*bars* 0.5 μm). **A** Outer surface of the cell wall in the isolate CBS grown without Prochloraz-Mn, showing many rodlet structures. **B, C** Cell wall surfaces (isolates 210 and 100 isolates, respectively), grown without fungicide, exhibiting a less abundant rodlet structure. **D–F** Outer surfaces of cell walls of *V. fungicola* isolates CBS, CIES 210 and CIES 100 grown in the presence of Prochloraz-Mn, showing progressive disappearance of rodlet structures

pretreated (CIES) or not (CBS) with Prochloraz-Mn, and later on in the isolates grown in the absence and presence of the respective LD_{50} concentrations of the fungicide. As a possible effect of the fungicide, while the total neutral carbohydrate values varied to a certain extent, a rearrangement of these carbohydrates was determined, which apparently led to somewhat modified chemical structures. The different degree of response of the three isolates to Prochloraz-Mn could be related to their particular resistance levels. The changes in the cell wall of the CBS strain, isolated prior to fungicide utilization, in response to subsequent Prochloraz-Mn treatment appeared to be more significant than those in the cell wall of the two CIES isolates. These latter isolates were subjected to Prochloraz-Mn for distinct periods of time, so that restructuring of the cell walls occurred progressively. In parallel, the ultrastructural changes detected by TEM on the hyphal surface could be related to the chemical differences in the wall structure produced by the action of the fungicide.

Resistance to sterol inhibitors appears to be multigenic [16], which implies either multiples sites of action and/or more than one mechanism of resistance. This also suggests that *V. fungicola* isolates treated with Prochloraz-Mn are not as pathogenic as the wild-type strain. The chemical and ultrastructural wall modifications caused by the fungicide could in some way affect preinfection mechanisms, such as cellular adhesion and recognition, prior to the development of disease on *A. bisporus* fruit bodies.

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