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Comparative effect of the fungicide Prochloraz-Mn on *Agaricus bisporus* vegetative-mycelium and fruit-body cell walls

Summary. Fungicides to control mycopathogens of commercial *Agaricus bisporus*, a mushroom cultivated for human consumption, are a major field of study, since these chemicals are toxic to both the host and its fungal parasites. The fungicide Prochloraz-Mn, used at its LD_{50} for *A. bisporus*, partially inhibited protein biosynthesis in the vegetative mycelial cell walls of this mushroom and caused significant changes in cell-wall polysaccharide structure, as deduced by methylation analysis and gas liquid chromatography-mass spectrometry (GLC-MS). Furthermore, the aggregated mycelial walls showed distinct alterations in their overall chemical composition following the administration of Prochloraz-Mn at the LD_{50} and the LD_{50} ×1000. As expected, GLC-MS studies indicated that the latter dose caused more appreciable differences in polysaccharide structure. The decrease in mushroom crop yields obtained from industrial cultures treated with Prochloraz-Mn to control *V. fungicola* infection depended on the dose of the fungicide employed, whereas fruit-body morphology was only slightly affected at the highest Prochloraz-Mn concentration used. [**Int Microbiol** 2004; 7(4):277-281]

Key words: *Agaricus bisporus* · Prochloraz-Mn · vegetative-mycelial cell walls · fruit-body cell walls · carbohydrate rearrangement

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Introduction

Verticillium disease, or dry bubble disease, caused by *Verticillium fungicola*, affects the cultivated white mushroom *Agaricus bisporus* and is a persistent problem for mushroom farmers. Until now, crop losses have been limited by means of sanitation, culture methods and chemical applications. Although Prochloraz-Mn has been fundamental to preventing *V. fungicola* infection of *A. bisporus*, during the last decade the mycoparasite has become less sensitive to this fungicide [5] due to the development of resistance. Thus far, there has been one study dealing with the effect of Prochloraz-Mn on the cell wall of *V. fungicola* [2], but there are no reports in the literature of studies on the effects of this fungicide on *A. bisporus* vegetative and fruit-body mycelia, which are also exposed to its actions. Moreover, mushroom crop yields of industrial cultures have been shown to be affected to a certain extent by the routine use of Prochloraz-Mn, which is not surprising since both the mycopathogen and the host are fungi.

Previous studies on the mode of action of Prochloraz-Mn [1,6,8,11] have shown sterol biosynthesis inhibition, suggesting at the same time that there is more than one mechanism of inhibition (multi-site fungicide). This hypothesis was recently confirmed by demonstrating partial protein inhibition together with polysaccharide rearrangement in the cell walls of the mycopathogen *V. fungicola* due to the fungicide

[2]. The present work describes a new approach and is a complement to those studies in that it analyzes possible changes in the chemical structure of the cell walls of both vegetative and aggregated mycelia of *A. bisporus* in the presence of Prochloraz-Mn. The consequences that these changes might have on the agronomic characteristics of the mushroom and on crop yields in industrial cultures due to the routine use of this fungicide in the control of verticillium disease have also been evaluated.

Materials and methods

Organism, culture conditions and preparation of hyphal cell walls. *A. bisporus* fruit bodies (Fungisem H 25, a smooth white non-hybrid variety developed by Fungisem S.A., Autol, La Rioja, Spain) were supplied by the Centro de Investigación, Experimentación y Servicios del Champiñón (CIES, Quintanar del Rey, Cuenca, Spain). *A. bisporus* vegetative mycelium was obtained by growing this organism on Raper medium [10] for 2 weeks. When necessary, the fungicide Prochloraz-Mn complex 46% (Sporgon, Schering) was added to the Raper medium and used at the corresponding LD_{50} value obtained (0.34 mg pure product/l). The LD_{50} was measured as described previously [2].

A. bisporus fruit bodies were grown at the CIES in the presence or absence of the fungicide Prochloraz at the LD_{50} calculated for the vegetative mycelium. In another set of experiments, the fruit bodies were grown at a fungicide concentration of LD_{50} ×1000 (340 mg/l), which is close to the recommended agronomic dose (500 mg commercial product /l). In both cases, the aggregated mycelial cell walls were prepared in the same way.

For cell-wall preparations, both vegetative and aggregated mycelia were freeze-dried, pulverized in a Sorvall omnimixer and then disintegrated in a Fritsch 6 balls-mill. 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 analytical techniques. Cell-wall polysaccharides were fractionated as described previously [3], except that fraction FIV was not separated into subfractions a and b. All procedures for chemical analysis, monosaccharide identification and methylation analysis have been described before [2].

Results and Discussion

Before examining the effect of Prochloraz-Mn on the chemical structure of *A. bisporus* vegetative mycelium and fruit-body cell walls, it is important to consider that yields of the mushroom crop in industrial cultures were virtually unaffected by treatment with the LD_{50} of the fungicide, but were somewhat inhibited (2.48-3.14%) by the LD_{50} ×1000. Slight variations of the sporophore surface texture, from smooth to rough, were observed only in response to the highest dose of Prochloraz-Mn used.

Differences in the overall cell-wall composition between *A. bisporus* vegetative and aggregated mycelium were shown previously [3,4]. The presence of the fungicide LD_{50} partially inhibited the synthesis of cell-wall proteins of the vegetative mycelium (Table 1); there was a slight increase in the hexosamine content but the levels of neutral sugars remained unchanged. By contrast, in the aggregated mycelial walls, protein synthesis was not inhibited at concentrations of LD_{50} or even at LD_{50} ×1000. Instead, the amount of protein tended to increase following application of Prochloraz-Mn, accompanied by a progressive decrease in the hexosamine content.

Table 1. Overall chemical composition of vegetative and aggregated mycelial cell walls of *Agaricus bisporus* treated or not treated with Prochloraz-Mn

Components	Vegetative mycelium	Vegetative mycelium + F (LD_{50})	Aggregated mycelium	Aggregated mycelium + F (LD_{50})	Aggregated mycelium + F (LD_{50}×1000)
Neutral carbohydrates	59.5 ± 0.7	57.8 ± 0.6	49.1 ± 0.5	51.8 ± 0.6	56.7 ± 0.7
Proteins	11.4 ± 0.3	**7.5** ± 0.2	6.9 ± 0.2	8.2 ± 0.2	9.3 ± 0.3
Hexosamines	14.3 ± 0.4	**18.9** ± 0.3	40.3 ± 0.5	**36.2** ± 0.4	**30.9** ± 0.4
Lipids	8.6 ± 0.2	8.7 ± 0.2	2.5 ± 0.1	2.3 ± 0.2	2.1 ± 0.1

F, Fungicide Prochloraz-Mn.

The mean deviation is calculated from the results of at least four determinations. Significant differences are shown in bold.

Table 2. Percentages of dry weight and neutral carbohydrates in the fractions of vegetative and aggregated mycelial cell walls of *Agaricus bisporus* treated or not treated with Prochloraz-Mn

	Dry weight (%)				Neutral carbohydrates (%)			
Fraction	Vcw	VcwF	Acw	AcwF	Vcw	VcwF	Acw	AcwF
FI	27.4	28.3	7.0	6.2	61.9	61.4	34.1	**31.3**
FII	11.2	9.5	5.6	5.2	69.7	68.2	70.7	**67.2**
FIII	3.6	3.1	3.8	**5.0**	69.5	68.3	73.3	72.3
FIV	7.5	8.2	9.9	**11.9**	70.7	72.4	67.8	**65.6**
FV	7.2	6.7	20.1	**24.0**	86.8	88.3	73.7	75.5
FVI	25.3	24.9	39.4	37.6	51.7	50.5	18.3	17.7

Vcw, Vegetative mycelial cell walls; VcwF, vegetative mycelial cell walls treated with fungicide Prochloraz-Mn (LD_{50}); Acw, aggregated mycelial cell walls; AcwF, aggregated mycelial cell walls treated with fungicide Prochloraz-Mn (LD_{50}×1000). Average of at least four measurements. Significant differences are shown in bold.

Cell-wall fractionation yielded six different fractions, mainly composed of neutral carbohydrates together with amino sugars and proteins. The percentages of dry weight and neutral carbohydrates of the fractions isolated from the two kinds of walls (Table 2) differed only slightly in their response to Prochloraz-Mn, as in the aggregated walls the small increase in the dry weight of fractions FIII, FIV and FV was offset by the decrease in total neutral sugars of fractions FI, FII and FIV. Carbohydrate analysis of *A. bisporus* vegetative mycelial walls (Table 3) revealed the major presence of glucose in all of the extracted fractions. Nonetheless, treatment with Prochloraz-Mn caused considerable changes in the percentages of monomers (Table 3). For example, a significant increase in the amounts of glucose and galactose and the disappearance of mannose and xylose were observed in fraction FII, whereas in fraction FIII the increase in the percentage of glucose was due entirely to the absence of xylose whereas galactose and mannose remained unchanged. The compositions of the other fractions were not affected by the fungicide.

Regarding aggregated mycelial walls (Table 3), significant increases in the amounts of some monosaccharides in the isolated cell-wall fractions were found following treatment with Prochloraz-Mn, such as mannose in fraction FI, xylose in fraction FII and galactose in fraction FIV. The monosaccharide compositions of the other fractions changed only slightly, but xylose decreased in FIV, and glucose in FII.

The results of methylation analysis of *A. bisporus* vegetative and aggregated mycelial walls are given in Table 4 and are in good agreement with previously reported data on the chemical structure of both types of cell walls [3,4], although significant differences were detected after fungicide treatment. Fraction FI of vegetative mycelial walls (Table 4) consists of mucilage, mainly corresponding to a linear α-(1-4)-linked glucan with low percentages of (1-3) mannose and (1-6) galactose. These monosaccharides became more branched in

Table 3. Molar ratio of the neutral sugars detected as aditol acetates by gas liquid chromatography of fractions isolated from vegetative (Vcw) and aggregated (Acw) mycelial cell walls of *Agaricus bisporus* treated (F) or non treated (–) with Prochloraz-Mn, (LD_{50}) for Vcw and (LD_{50}×1000) for Acw

		Glucitol		Galactitol		Mannitol		Xylitol	
Fraction		–	F	–	F	–	F	–	F
FI	Vcw	93.0	88.1	4.7	5.4	2.3	**6.5**	–	–
	Acw	93.0	90.9	4.4	4.1	2.6	**4.8**	–	0.2
FII	Vcw	70.2	**95.8**	2.3	4.2	13.0	–	14.5	–
	Acw	82.8	**72.5**	–	–	11.5	11.5	5.7	**16.0**
FIII	Vcw	77.4	**92.1**	5.8	5.9	1.9	2.0	14.9	–
	Acw	88.0	90.0	3.2	3.2	2.8	2.1	5.9	4.7
FIV	Vcw	58.8	60.9	19.2	20.3	9.8	6.7	12.2	12.1
	Acw	89.5	88.5	–	2.7	6.5	5.9	4.0	2.9
FV	Vcw	95.1	95.00	2.4	2.8	1.0	2.2	1.5	–
	Acw	100	100	–	–	–	–	–	–
FVI	Vcw	96.8	94.3	–	2.5	3.2	3.2	traces	–
	Acw	98.0	100	2.0	–	–	–	–	–

Average of at least four measurements. Significant differences are shown in bold.

response to Prochloraz-Mn, with the glucose linkages increasing to (1-3,4) and (1-4,6). Fraction FII, a mostly linear α-(1-3) glucan, was clearly affected by the fungicide, which inhibited the formation of xylose and mannose residues, increased (1-3,6) glucose linkages, and caused the introduction of a small amount of galactose into the furanose form. In the presence of Prochloraz-Mn, fraction FIII, composed mainly of a (1-4)-linked glucan, the mannose content was maintained whereas the number of (1-3,4) and (1-4,6) glucose units in xylose increased; also, as in the previous fraction, there was a small amount of galactofuranose. The components of fraction FIV also became more branched, in the form of (1-3,6) glucose linkages, due to the action of the fungicide. Fractions FV and FVI were less affected, but there was also a tendency toward increased branching.

Table 4 shows also the methylation analysis of the fractions isolated from aggregated mycelial walls of *A. bisporus* in the presence and absence of the fungicide. Fraction FI (mucilage) is essentially composed of a linear α-(1-4)-linked glucan, as seen in vegetative mycelial walls, with significant percentages of (1-3) and (1-6) glucose, (1-6) galactose, and (1-3) mannose. These became somewhat more branched in the presence of Prochloraz-Mn, with glucose linkages increasing to (1-3,4) and (1-3,6) and the terminal xylose being eliminated. In fraction FII, comprising mainly α-(1-3)-linked glucosyl residues, the (1-3) and (1-3,4) mannose linkages increased and the (1-6) and (1-2,3) glucose residues decreased in response to the fungicide. Fraction FIII, consisting mainly of (1-6)- and (1-3)-linked glucosyl residues, showed a significant decrease in (1-6) galactose and (1-4) glucose units, which, in Prochhloraz-Mn-treated samples, became more branched due to increased (1-3,4) and (1-3,6) glucosyl linkages. The other three fractions, FIV, FV and FVI, were less affected by the fungicide and showed only small increases in (1-3) mannose (FV) and a slight decrease in (1-6) galactose (FVI).

Preliminary experiments carried out using the shadowing technique followed by transmission electron microscopy

Table 4. Gas liquid chromatography-mass spectrometry data for partially methylated alditol acetales of fractions isolated from vegetative (Vcw) and aggregated (Acw) mycelial cell walls of *Agaricus bisporus* treated (F) or non treated (–) with Prochloraz-Mn, (LD_{50}) for Vcw and (LD_{50}×1000) for Acw

		FI		FII		FIII		FIV		FV		FVI	
		–	F	–	F	–	F	–	F	–	F	–	F
Xylp-(1	Vcw	–	–	9.6	–	0.4	–	3.5	2.5	1.5	0.6	–	–
	Acw	1.9	–	3.8	5.2	2.1	1.6	1.5	1.1	–	–	–	–
4)-Xylp-(1	Vcw	–	–	0.4	–	12.5	–	11.0	12.3	2.6	**1.3**	–	–
	Acw	–	–	2.4	2.3	–	–	–	–	–	–	–	–
Hexp-(1	Vcw	9.4	10.6	2.8	**14.0**	8.2	**17.0**	4.6	4.2	10.4	12.8	13.4	14.0
	Acw	6.0	6.5	0.9	1.3	14.2	16.8	17.5	19.8	6.9	6.4	9.8	9.4
3)-Glup-(1	Vcw	–	–	61.2	55.8	1.0	**4.3**	24.1	19.4	7.0	6.2	9.9	8.7
	Acw	8.8	7.7	78.9	67.5	21.5	22.3	21.1	20.1	7.0	6.9	11.3	11.5
5)-Galf-(1	Vcw	–	–	–	**2.1**	–	**0.5**	–	–	–	–	–	–
	Acw	–	–	–	–	–	–	–	–	–	–	–	–
4)-Glup-(1	Vcw	76.5	64.8	–	–	61.3	53.4	19.3	22.6	62.0	59.6	55.6	53.4
	Acw	53.2	54.3	–	–	2.2	**1.1**	–	–	3.7	4.3	1.6	1.5
3)-Manp-(1	Vcw	1.7	3.1	7.0	–	1.8	1.3	5.3	4.6	1.5	1.9	3.2	3.0
	Acw	1.4	0.9	5.5	**15.0**	1.5	1.7	9.6	7.8	0.4	**1.1**	–	–
6)-Glup-(1	Vcw	–	–	–	–	0.5	–	1.0	–	5.4	3.9	4.0	3.8
	Acw	15.8	13.1	1.3	**0.4**	33.6	34.1	36.4	35.8	74.5	73.2	61.4	63.7
6)-Galp-(1	Vcw	2.4	2.7	1.9	1.5	5.8	4.3	9.2	9.1	2.0	2.4	–	–
	Acw	4.7	6.6	0.6	–	10.0	**0.7**	–	–	0.9	0.5	1.9	**1.0**
3,4)-Manp-(1	Vcw	–	–	7.1	–	–	–	4.3	3.8	–	–	–	–
	Acw	–	–	0.7	2.3	–	–	–	–	–	–	–	–
3,4)-Glup-(1	Vcw	5.1	**12.6**	4.7	4.8	1.4	**4.9**	1.2	2.6	–	1.7	1.4	1.6
	Acw	2.1	2.8	3.4	4.5	1.2	**2.1**	1.1	1.3	–	–	–	–
2,3)-Glup-(1	Vcw	–	–	2.0	1.4	–	–	–	–	–	–	1.3	1.5
	Acw	–	–	1.3	**0.4**	–	–	0.7	0.9	–	–	0.7	0.7
4,6)-Glup-(1	Vcw	4.9	6.2	–	–	5.0	11.7	1.4	0.9	2.8	4.3	2.2	2.4
	Acw	1.2	0.9	–	–	1.7	1.6	0.4	0.9	1.8	2.1	2.2	1.6
3,6)-Glup-(1	Vcw	–	–	3.3	**20.4**	2.1	2.6	5.7	**9.3**	4.8	5.3	9.0	11.6
	Acw	4.9	7.2	1.2	1.1	12.0	**18.0**	11.7	12.3	4.8	5.5	10.1	10.6

Average of at least four measurements. Significant differences are shown in bold.

(TEM) of both types of cell walls in the presence and absence of Prochloraz-Mn did not suggest significant differences in their respective hyphal surfaces, as was reported for the mycoparasite *V. fungicola* treated with the same fungicide [2].

The different effects induced by Prochloraz-Mn on vegetative mycelium and fruit-body *A. bisporus* cell walls could be related to their distinct chemical structures [3,4]. This might explain the greater sensitivity of vegetative mycelial cell walls to the fungicide, and the observation that fruiting bodies only developed from surviving hyphal strands that were not affected by the fungicide. Taken together, the results show a restructuring of both *A. bisporus* vegetative and aggregated mycelial cell walls after exposure to Prochloraz-Mn. Rearrangement of the different carbohydrates led to partially modified chemical structures. However, these modifications apparently did not affect the main agronomic characteristics of the mushrooms. The significance of these changes requires further investigation in light of the fact that the fungicide shows some toxicity not only to the mycopathogen *V. fungicola* [2] but also to its host, *A. bisporus*, which is cultivated for human consumption. New strategies eliminating the use of this chemical must be developed in order to control verticillium disease while avoiding possible secondary effects on human consumers. Note, however, that oral administration of Prochloraz-Mn to rats results in the complete metabolism and excretion of the fungicide [7,9].

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Estudios comparativos del efecto del fungicida Prochloraz-Mn sobre las paredes celulares del micelio vegetativo y de los cuerpos fructíferos de *Agaricus bisporus*

Resumen. Los fungicidas para el control de micopatógenos de *Agaricus bisporus*, un hongo de cultivo comercial para consumo humano, representan un importante tema de estudio debido a que son tóxicos tanto para el huésped como para sus parásitos fúngicos. El fungicida Prochloraz-Mn, empleado a su LD_{50} para *A. bisporus*, inhibe parcialmente la biosíntesis de proteínas en las pared celular del micelio vegetativo de este hongo y provoca cambios significativos en la estructura polisacárida de la pared celular, tal como se observa mediante el análisis de metilación y la cromatografía líquida de gases-espectrometría de masa (GLC-MS). Además, las paredes agregadas del micelio presentan diferentes alteraciones en la composición química global después de la administración de Prochloraz-Mn a la LD_{50} y LD_{50} ×1000. Como cabría esperar, los estudios de GLC-MS, indican que la última dosis causa más diferencias apreciables en la estructura polisacárida. La disminución en la producción del hongo en los cultivos industriales tratados con Prochloraz-Mn para controlar la infección por *V. fungicola*, dependía de la dosis de fungicida empleada, mientras que la morfología del cuerpo fructífero sólo resulta ligeramente afectada a la concentración de Prochloraz-Mn más elevada. [**Int Microbiol** 2004; 7(4):277-281]

Palabras clave: *Agaricus bisporus* · Prochloraz-Mn · paredes celulares del micelio vegetativo · paredes celulares de los cuerpos fructíferos · redisposición de carbohidratos

Estudos comparativos sobre o efeito do fungicida Prochloraz-Mn sobre as paredes celulares do micélio vegetativo e dos corpos de frutificação de *Agaricus bisporus*

Resumo. Os fungicidas usados no controle de micopatógenos de *Agaricus bisporus*, um fungo de cultivo comercial para consumo humano, representam um importante tema de estudo devido ao fato de que são tóxicos tanto para o hospedeiro como para os parasitas fúngicos. O fungicida Prochloraz-Mn, empregado na sua dose LD_{50} para A. bisporus, inibe parcialmente a biossíntese de proteínas da parede celular do micélio vegetativo deste fungo e provoca mudanças significativas na estrutura polissacarídica da parede celular, tal como se observa mediante a análise de metilação e de cromatografia líquida de gases-espectrometria de massa (GLC-MS). Por outro lado, as paredes agregadas do micélio apresentam diferentes alterações na composição química global depois da administração de Prochloraz-Mn nas doses LD_{50} e LD_{50} ×1000. Como esperado, os estudos de GLC-MS, indicaram que a última dose causa diferenças mais significativas na estrutura polissacarídica. A diminuição na produção do fungo nos cultivos industriais tratados com Prochloraz-MN para controle da infeção por *V. fungicola*, foi dependente da dose de fungicida empregada, enquanto que a morfologia do corpo de frutificação resulta ligeiramente afetada somente quando foi usada uma concentração mais elevada de Prochloraz-Mn. [**Int. Microbiol** 2004; 7(4): 277-281]

Palavras chave: *Agaricus bisporus* · Prochloraz-Mn · paredes celulares do micélio vegetativo · paredes celulares dos corpos de frutificação · reposicionamento de carbohidratos