Verticillium fungicola var. *fungicola*: comparison of some Mexican and French isolates

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Comparación de algunas cepas mexicanas y francesas de *Verticillium fungicola* var. *fungicola*

Resumen. Se compararon cepas de *Verticillium fungicola* recolectadas en 2002 en el Estado de Veracruz, México, previamente identificadas como *V. fungicola* var. *fungicola*, con cepas francesas para observar su diversidad de polimorfismo genético y algunos aspectos fisiológicos. Los resultados se analizaron tomando en cuenta estudios con un mayor número de cepas europeas de *V. fungicola* var. *fungicola*. Los análisis de RAPD muestran que las cepas mexicanas son genéticamente similares a aquellas colectadas en el Oeste de Europa en el período 1987-2000. El crecimiento micelial, el efecto de antibiosis contra su hospedero *Agaricus bisporus* y la sensibilidad *in vitro* al fungicida chlorothalonil no permiten distinguir las cepas mexicanas de las europeas.
Palabras clave: *Agaricus bisporus*, hongos comestibles, hongos patógenos, diversidad genética, variabilidad fisiológica.

Abstract. Isolates of *Verticillium fungicola* collected in 2002 in the Veracruz State, Mexico, and previously identified as *V. fungicola* var. *fungicola*, were compared to French isolates for genetic polymorphism and diversity in some physiological traits. Results were analysed based on studies of a larger group of European *V. fungicola* var. *fungicola*. RAPD analyses showed that Mexican isolates were genetically similar to those collected in Western Europe over the period 1987-2000. Mycelial growth rate, antibiosis effect against their host *Agaricus bisporus*, and *in vitro* sensitivity to the fungicide chlorothalonil did not distinguish the Mexican isolates from the European isolates studied.
Key words: *Agaricus bisporus*, edible mushroom, fungal pathogen, genetic diversity, physiological variability.

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Introduction

The ascomycete *Verticillium fungicola* (Preuss) Hassebrauk, responsible for dry bubble disease on *Agaricus bisporus* (Lange) Imbach, causes important losses worldwide in the mushroom industry. Two varieties of the pathogen have been identified; currently *V. fungicola* var. *aleophilum* affects mushroom crops in Canada and USA (Collopy *et al*., 2001). The variety *fungicola* is responsible for the disease in the Netherlands, Spain and France (Desrumeaux and Sedeyn, 2001; Gea *et al*., 2003; Largeteau *et al*., 2006). Thus, when we identified Mexican isolates collected in 2002 in the state of Veracruz as *V. fungicola* var. *fungicola* from their ITS1-5,8S-ITS2 region, we theorised that they may have originated from

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Europe (Largeteau *et al*., 2004). The aim of the present study was to compare these Mexican isolates to four French isolates previously characterised for genetic polymorphism (Largeteau *et al*., 2006), some physiological traits and pathogenicity.

Material and Methods

Isolates of *Verticillium fungicola*

All isolates were isolated from bubbles and were identified as V. *fungicola* var. *fungicola* (Largeteau *et al*., 2004, 2006). Isolates VMX1, VMX2 and VMX3 were collected in 2002 in the state of Veracruz (Mexico); isolates VCF, VCTC, VF and VK were collected over the period of 1987-1997 at French mushroom farms. Prior to this study, all fungi were maintained on a malt extract agar medium (1% malt, 1.5 % agar) at 4 °C in darkness.

RAPD analyses

Genomic DNA was extracted from freeze-dried mycelium with the Nucleon Phytopure extraction kit RPN 8510 (Amersham International, Little Chalfort) according to the manufacturer procedure. Amplification was performed in a 25 µl reaction mixture containing 0.1 mM dNTPs, 1X DynAzyme™ fuffer (Finnzymes, Espoo, Finland), 0.8 U DynAzyme™ II polymerase, 0.5 µM decamer primer, 5 ng DNA. The crocodile III Thermal Cycler (Appligene, Illkirch, France) was programmed for one cycle of 6 min at 94 °C; 35 cycles of 1 min at 93 °C, 2 min at 36 °C, 2 min at 72 °C, with a final extension period of 6 min at 72 °C. Primers OPA03, OPA11, OPA18, OPD04, OPD18, OPZ10, OPZ20 (Life Technologies, Cergy-Pontoise, France) and UBC29 (University of British Columbia) were screened. Each RAPD was performed twice. Numeric images of agarose gels were recorded and the presence or absence of RAPD products was scored with the Kodak Digital Science 1D™ analyser.

Mycelial growth rate

All *Verticillium fungicola* isolates were cultivated at 23 °C on a malt extract agar medium (MEA: 10 g l^{-1} malt extract and 15 g l^{-1} agar). All inocula were 10-d old. The colony's growth (two perpendicular diameters) was measured daily, and the growth rate per day was calculated for the period of linear growth. Five replicates were prepared for each strain. The experiment was performed twice.

***In vitro* antibiosis**

Double layer cultures were used to assess the effect of diffusible or volatile compounds produced by *A. bisporus* on the germination and colony extension of *V. fungicola,* and the effect of the pathogen on the development of the colony of the basidiomycete. An off-white commercial strain of *Agaricus bisporus* (2100 Amycel, France) was cultivated on a MEA medium until the colony reached 4 cm in diameter. Afterwards, an agar medium (1.2 g l^{-1} bactopeptone, 6 g l^{-1} sodium pyrophosphate and 15 g l^{-1} agar) containing 10^6 conidia of *V. fungicola*, maintained at 50 °C, was poured over a growing colony of *A. bisporus*. The cultures were incubated at 23 °C for 9 d before the surface area covered by *V. fungicola* colonies was recorded drawing the limits of mycelia on the Petri dish cover. The surface area covered by the mycelium of *A. bisporus* after the supply of the pathogen was also calculated drawing the limits of mycelia on the bottom Petri dish. Drawings were photocopied on tracing paper calibrated at 70 g cm^{-1}, cut off and weighted to determine the surface. Five replicates were prepared for the *V. fungicola* strains. The experiment was performed twice.

***In vitro* susceptibility to chlorothalonil**

The commercial fungicide Banko® (Chlorothalonil, produced in Calliope, Pau, France) was added to the MEA medium just before it was poured into Petri dishes. Cultures of *V. fungicola* on media containing 0, 20, 200, 400, 800 and 4000 ppm chlorothalonil were grown at 23 °C for 19 days. Colonies were measured daily (two perpendicular diameters), and their growth rate as compared to the control (unamended medium) was calculated at the beginning and at the end of the period of linear growth. Two experiements were performed, each with five replicate per isolate and concentration of fungicide. To observe the adaptation of the pathogen to the fungicide, inocula removed from the MEA medium with and without chlorothalonil (20 ppm) were placed on a MEA medium containing 20 ppm of chlorothalonil. The colonies diameters were compared after 19 d at 23 °C. Five replicates were performed for each condition.

Virulence assays

Eight freshly harvested sporophores of studied strain of *A. bisporus* were placed into a moist chamber, and received 20 µl of a conidial suspension of *V. fungicola* (10^6 conidia ml^{-1}) on the cap surface. The diameter and the depth of the necrosis were recorded using a vernier after 5 d of incubation at 20 °C. The experiment was performed twice.

Statistical analyses

Data were analysed using the SAS, Inc PROC ANOVA procedure (SAS Institute Inc., Cary, NC) and the means were separated, if necessary, using the Student-Newman-Keuls test at 95 % level.

Results and discussion

The seven isolates studied had all been previously identified as *V. fungicola* var. *fungicola* (Largeteau *et al.,* 2004). An analysis of their genetic variability was performed at this point. Data of RAPD obtained from eight primers, selected for their ability to discriminate among *V. fungicola* isolates (Largeteau *et al*., 2006), demonstrated that the Mexican isolates are genetically very similar to the isolates collected in France over the period of 1987-2000 (Figure 1). In these previous studies we identified a non polymorphic group of European (France, NL, UK) isolates collected between 1987 and 2000, from which VF and VK were representatives, and found that VCTC and VCF showed polymorphism (presence or absence of a single band) with one and nine primers out of 24, respectively. In the present study, primers OPA03, OPD18, OPZ20 and UBC 29 gave identical RAPD profiles for the Mexican and the French isolates. Three primers (OPA11, OPA18 and OPD04) grouped the three Mexican isolates with VCF, VF and VK and separated them from

Table 1. Classification of the isolates of *Verticillium fungicola* for mycelial development, antibiosis effect and virulence

Isolate	Mycelium growth rate at 23 °C (mm d^{-1}) [1]	Antibiosis		Necrosis	
		V. fungicola (cm^2) [2]	*A. bisporus* (cm^2) [3]	Diameter (cm) [4]	Depth (mm) [5]
VCF	3.5 bcd [6]	23.1 c	17.3 b	1.5 a	0.6 d
VCTC	4.8 a	29.7 ab	6.8 c	1.3 b	3.4 a
VF	3.7 bc	33.9 a	5.1 c	1.5 a	0.4 d
VK	3.4 cd	26.6 b	25.8 a	1.1 c	0.3 d
VMX1	3.8 b	32.1 a	17.4 b	1.0 d	2.1 bc
VMX2	3.3 d	29.6 ab	21.4 ab	0.8 e	1.9 c
VMX3	3.3 d	31.6 a	17.0 b	1.0 d	2.4 b

[1] Mycelium growth rate during the period of linear growth. [2] Surface covered by colonies of *V. fungicola*. [3] Surface covered by *A. bisporus* after the supply of *V. fungicola* conidia. [4] Diameter and [5] depth of the necrosis produced by the deposal of a conidial suspension on sporophore caps. [6] Values within a column followed by the same letter do not differ significantly from the student-Newman-Keuls test ($p = 0.05$).
VCF, VCTC, VF and VK = French isolates. VMX1, VMX2 and VMX3 = Mexican isolates.

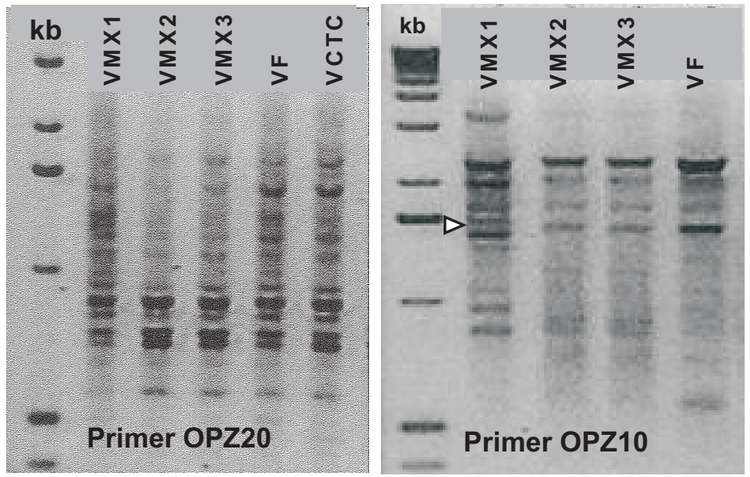

Figure 1. RAPD profiles of Mexican and French isolates of *Verticillium fungicola* using the primers OPZ20 and OPZ10. VCF, VCTC, VF and VK = French isolates. VMX1, VMX2 and VMX3 = Mexican isolates. A white triangle symbol indicates a different band in the Mexican isolate VMX1.

VCTC, which showed a single extra band. With primer OPZ10 only isolate VMX1 showed polymorphism with a different band (Figure 1). Two of the Mexican isolates can be grouped with the non polymorphic group of isolates described previously, and the third isolate, even very close to this group, presents a specific genetic marker. These observations are in agreement with our previous hypothesis of a European origin.

In addition to the genetic polymorphism, phenotypic characterisation of the isolates is of interest. For instance, there was the question of the pathogenic potential of the Mexican isolates. Mycelial growth rate is one of the components of this potential. The mycelium growth rate of isolate VMX1 differed from VMX2 and VMX3 at 23 °C. The French isolate VCTC had maximum growth rate and it was different to all other isolates. However, considering VCTC apart, French and Mexican isolates did not differ on mycelial growth (Table 1), with values close to those observed for other European isolates (Largeteau *et al*., 2006).

The consequences of interspecific interactions on spore germination and mycelial radial growth are another component of the pathogenic power of *V. fungicola.* The mutual effects of host and pathogen were observed *in vitro*. The diffusible or/and volatile compounds produced by *A. bisporus* 2100 had similar effect on the germination and the mycelial growth of all the isolates of *V. fungicola,* except for the VCF strain, which exhibited the lower mycelial extension. The effect of *V. fungicola* on *A. bisporus* mycelial growth was also measured. The Mexican isolates were measured against two French isolates (VCF and VK) for this effect. These five isolates were far less effective than VCTC and VF in reducing the development of the mycelium of *A. bisporus* (Table 1).

The virulence test for estimating the ability of each *V. fungicola* to infest the mushroom was the last component of the pathogenic potential measured. The three Mexican isolates were virulent; however French isolates induced necrosis on the cap of the sporophore having significantly higher diameters, while depth necrosis induced by Mexican isolates was significantly higher than French isolates, except for the most virulent VCTC (Table 1). Taking into account the different data presented here, the Mexican isolates appeared to have a middle pathogenic potential close to most of the French isolates used here as a representative sample of European isolates previously analysed (Largeteau *et al*., 2006). Their studied physiological traits did not differ significantly from the French isolates, except for virulence. However, we have postulated that inoculation directly on the pilei gives information on the virulence, but is more valuable in classifying *A. bisporus* strains for their susceptibility to the disease, than *V. fungicola* isolates for their potential as pathogen.

Comparative pathogenicity tests, including VMX1, were performed in previous studies and gave information on this Mexican isolate. It resulted from these cropping tests that VMX1, VCTC and the Dutch isolate V9503 induced similar percentages of bubbles, spotty caps and stipe blowout (Largeteau *et al*., 2005). This level of agressiveness is relatively high among the *V. fungicola* var. *fungicola*, but it is lower than that of *V. fungicola* var. *aleophilum*. Inoculation of eight strains of *A. bisporus* with five isolates of *V. fungicola* in an other experiment revealed that V9503 and an un-named isolate of *V. fungicola* var. *fungicola* were significantly less aggressive (regardless of symptoms), than three isolates tested of *V. fungicola* var. *aleophilum,* which were representative of the clonal population responsible for the outbreak of the dry bubble disease in Pennsylvania (USA) at the end of the 1990s (Collopy *et al*., 2001).

The increasing resistance of *V. fungicola* to fungicides is well known, and adaptation to such chemicals was proposed as an important factor on the decrease of pathogen variability (Bonnen & Hopkins, 1997). The susceptibility of Mexican and French isolates to chlorothalonil was compared. Data showed that at the beginning of the period of linear growth (d 5), the Mexican isolates differed in susceptibility to 20, 200 and 4000 ppm of fungicide, but whatever the concentration in fungicide, their susceptibility level was statistically the same as at least one French isolate. At the19th day of growth (the end of the period of linear growth), variability in susceptibility was observed both within the Mexican and the French isolates, and both groups of isolates exhibited a similar level of susceptibility irrespective of the concentration in fungicide. Except for VMX3, the susceptibility to concentration in chlorothalonil

Table 2. *In vitro* susceptibility of the isolates of *Verticillium fungicola* to chlorothalonil

Date	Isolate	Percentage of growth for each concentration of fungicide [1]									
		20 ppm		200 ppm		400 ppm		800 ppm		4000 ppm	
5 d	VCF	63.3 c[2]	A[3]	63.8 a	A	53.0 b	BC	57.0 a	AB	46.0 bc	C
	VCTC	73.0 a	A	65.5 a	B	62.1 a	C	58.8 a	D	54.1 a	E
	VF	59.1 d	A	51.5 c	B	52.7 b	B	47.3 b	C	39.7 d	D
	VK	69.0 b	A	62.1 a	B	55.2 ab	C	50.0 b	D	34.7 e	E
	VMX1	75.6 a	A	53.9 bc	B	52.1 b	B	47.6 b	C	54.2 a	B
	VMX2	76.2 a	A	57.9 b	B	55.2 ab	B	48.5 b	C	47.3 b	C
	VMX3	64.3 c	A	52.0 c	B	50.0 b	B	49.2 b	B	43.1 c	C
19 d	VCF	63.9 bc	A	64.4 a	A	59.1 ab	A	60.5 b	A	58.8 b	A
	VCTC	75.9 bc	A	67.7 a	A	63.7 a	A	68.4 a	A	72.5 a	A
	VF	62.0 bc	A	53.5 b	B	52.0 bc	B	52.7 bcd	B	49.5 c	B
	VK	69.0 b	A	70.3 a	A	61.6 a	B	61.0 b	B	52.8 bc	C
	VMX1	72.5 b	A	53.1 b	B	52.8 bc	B	49.6 cd	B	55.9 b	B
	VMX2	84.6 a	A	65.3 a	B	61.0 a	B	54.7 bc	B	58.4 b	B
	VMX3	61.5 bc	A	51.5 b	B	48.5 c	B	44.3 d	C	42.1 d	C

[1] Mycelial growth after 5 and 19 days, as compared to control medium without fungicide. [2] For each date, within a column, values in lower case followed by the same letter do not differ significantly from the student-Newman-Keuls test ($p = 0.05$). [3] On each line values in bold type followed by the same letter do not differ significantly from the student-Newman-Keuls test ($p = 0.05$).
VCF, VCTC, VF and VK = French isolates. VMX1, VMX2 and VMX3 = Mexican isolates.

Table 3. *In vitro* adaptation of the isolates of *Verticillium fungicola* to chlorothalonil

Isolate	Mycelial growth (cm) on MEA medium with 20 ppm chlorothalonil following	
	Preculture without fungicide	Preculture with 20 chlorothalonil
VCF	4.16 b [1]	4.71 a
VCTC	5.91 a	5.05 b
VF	4.19 b	5.16 a
VK	4.56 a	4.56 a
VMX1	3.94 b	4.52 a
VMX2	3.98 a	3.96 a
VMX3	3.92 b	4.63 a

[1] On each line values followed by the same letter do not differ significantly from the student-Newman-Keuls test ($p = 0.05$). VCF, VCTC, VF and VK = French isolates. VMX1, VMX2 and VMX3 = Mexican isolates.

was lower at 19 d and the strains showed a higher percentage of growth (Table 2). Testing for adaptation of *V. fungicola* to the fungicide *in vitro* showed that, compared to a preculture without fungicide, a preculture on a MEA medium, with 20 ppm of chlorothalonil added, had no significant effect on the mycelial growth of isolates VK and VMX2 on a new MEA medium with fungicide. In contrast, the preculture with fungicide induced the adaptation of four isolates (VCF, VF, VMX1 and VMX3) to this concentration in chlorothalonil, and the same treatment increased the susceptibility of VCTC to the fungicide (Table 3). This relatively high resistance to the fungicide tends to indicate that the Mexican isolates were derived from European strains imported after an increasing level of resistance had developed.

The isolates of *V. fungicola* var. *fungicola* collected in the State of Veracruz in 2002 did not differ significantly from isolates of the same variety originating from Western Europe, and can be distinguished from the clonal population of Pennsylvania by both the variety and a lesser aggressiveness (Largeteau *et al*., 2006).

The procedures for *A. bisporus* cultivation in the Mexican region where the *V. fungicola* strains were collected are much closer to the European ones than to the US ones, which could explain why the Mexican isolates are very similar to the French and Dutch isolates. Another explanation is that the physiology traits of *V. fungicola* depend for the major part on the variety. The similarity between the four isolates from Western Europe and the three Mexican isolates supports the European origin we hypothesised for the latter (Largeteau *et al*., 2004). But considering differences among Mexican isolates in the various criteria studied, we question the possibility of several introductions or variability acquired after introduction.

Acknowledgements

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