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FUNGICIDE SENSITIVITY OF SELECTED *VERTICILLIUM FUNGICOLA* ISOLATES FROM *AGARICUS BISPORUS* FARMS

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Abstract — Five isolates of *Verticillium fungicola*, isolated from diseased fruiting bodies of *Agaricus bisporus* collected from mushroom farms in Serbia during 2002-2003, were studied. By observing their colony morphology under different growth conditions and their pathogenic characteristics, the isolates were identified as *V. fungicola* var. *fungicola*. The peat/lime casing was the primary source of infection. Testing of sensitivity to selected fungicides showed that all isolates were highly resistant to benomyl (EC50 values were higher than 200.00 mg/l), moderately sensitive to iprodione (EC50 values were between 11.93 and 22.80 mg/l), and highly sensitive to prochloraz-Mn (EC50 values were less than 3.00 mg/l).

Key words: *Verticillium fungicola* var. *fungicola*, fungicides, benomyl, iprodione, prochloraz-Mn, sensitivity

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INTRODUCTION

Agaricus bisporus (Lange) Imbach is the most commonly cultivated mushroom species (Royse, 1996). The production of fruiting bodies is severely afflicted by fungal, bacterial, and viral pathogens that can cause diseases which have an effect on yield and quality. *Verticillium fungicola* (Preuss) Hassebrauk, with two varieties, *fungicola*, widespread in Europe, and *aleophilum*, widespread in North America, is a major *A. bisporus* pathogen and a causal agent of the disease commonly known as "dry bubble". *A. bisporus – V. fungicola* interaction is of an invasive necrotrophic nature. The disease severity and symptoms depend on the stage of mushroom development at the time of infection (North and Wuest, 1993, Grogan et al., 2000), and the symptoms are necrotic lesions with brown colored spots or streaks, stipe blow-out, and undifferentiated structures containing mycelia of both host and pathogen (Savoie and Largeteau, 2004).

In recent years, the usual method of controlling of "dry bubble" disease on farms worldwide is based on the use of fungicides. The most commonly used fungicides on mushroom farms are: benomyl (2,2-diphenyl-1-picrylhydrazyl cineole chamazulene); iprodione (3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide); mancozeb ([1,2-ethanediylbis(carbamodithio)(2-)] manganese ainc salt); and prochloraz-Mn (1-(N-propyl-N-(2-(2,4,6-trichlorophenoxy)ethyl)) carbamoylimiazole) (Gea et al., 1997). However, development of pathogen resistance to fungicides after frequent application (Bonnen and Hopkins, 1997; Gea et al., 1997, 2003; Grogan et al., 2000) and host sensitivity to fungicides (Diamantopoulou et al., 2006) are a serious problems.

The aims of this study were to isolate and identify the causal agent of "dry bubble" in Serbia, and examine variation of the pathogen as evidenced by morphology of its colonies under different growth conditions and their pathogenic characteristics. Sensitivity of the pathogen to benomyl, iprodione and procholoraz-Mn was also tested.

MATERIALS AND METHODS

Isolates and growth conditions

Isolates of *V. fungicola* obtained from diseased fruiting bodies of *A. bisporus* collected during 2002-2003 in Serbia are shown in Table 1. Isolation was done by taking small pieces (2 x 2 x 5 mm) of fruiting bodies with "dry bubble" symptoms, immersing them a 1% sodium hypochlorite solution (for 1 min), and placing them on Potato dextrose agar medium (PDA). The isolates are maintained on PDA at 5°C in the Culture Collection of the Pesticide and Environmental Research Center's Phytopathology Laboratory in Belgrade. *Verticillium fungicola* var. *fungicola* 182 (obtained from the collection of Horticulture Research International, Wellesbourne, UK) and *V. fungicola* var. *aleophilum* DC-170 (obtained from the Plant Pathology Department, Penn State University, USA) were used as controls.

The morphology of colonies of all *V. fungicola* isolates was studied by observing their growth on PDA, malt agar medium (MA), mushroom dextrose agar medium (MDA), water agar medium (WA), and Czapek agar medium (CzA) for 7 days at 20°C. The dimensions of 30 conidia per isolate formed on PDA were measured and compared. Temperature influence was investigated on *V. fungicola* isolates cultivated on the optimal medium for 7 days at 20, 27 and 30°C. Three replicates per treatment and per isolate were used for statistical analysis.

Pathogenicity test

Spawn-run compost (*A. bisporus* Sylvan A 15), produced by Uca & Co., Vranovo, Serbia, was used for the pathogenicity test. Compost bags were encased with a 40 – 50-mm layer of black peat/lime casing ("Makadam" Co., Belgrade), which was artificially inoculated with the studied isolates of *V. fungicola*. The casing inoculation was done by spore

Table 1. Isolates of *Verticillium fungicola.*

Variety	Code of isolates	Origin	Year of isolation
	P_2V_3	Požarevac, Serbia	2002
	VV_2	Vraćevšnica, Serbia	2002
V. fungicola var. *fungicola*	ViV_1	Vinča, Serbia	2003
	RaV_1	Rakovica, Serbia	2003
	BeV_1	Belgrade, Serbia	2003
	182	England, UK	1995
V. fungicola var. *aleophilum*	DC-170	Pennsylvania, USA	1982

suspension spraying (approximately 10^6 conidia/ml) 3 days after encasement. Bags were incubated at 25°C during spawn-running of the casing (for 7 days), after which temperature was lowered to 18°C. The inoculated black peat/lime casing was removed and replaced with new sterile one 30 days after the first encasement.

Testing of sensitivity to selected fungicides

Sensitivity analysis was done with isolates grown on PDA amended with the following fungicides: benomyl (Benfungin WP, 50%, Galenika-Fitofarmacija); iprodione (Kidan EC, 25.5%, Bayer); and prochloraz-Mn (Octave WP, 50%, Bayer). The preliminary concentrations of all selected fungicides were 0.01, 0.10, 1.00, 10.00, 100.00, and 1000.00 mg/l. Testing of sensitivity to iprodione and prochloraz-Mn was repeated with concentrations of 0.1, 0.5, 1.5, 10.0, 50.0, and 100.0 mg/l; and 0.078, 0.156, 0.312, 0.625, 1.250, 2.500, and 5.000 mg/l, respectively.

The plates were inoculated with an inverted mycelial agar disk (10 mm) taken from the edge of 14-day-old culture of *V. fungicola* var. *fungicola* isolates, placed centrally on fungicide-amended and fungicide-free medium (control) and incubated at 20°C. Three replicates per treatment and per isolate were done. Colony diameter was measured after 7 days of growth. Growth of colonies on the fungi-

Fig. 1. Spotting of *Agaricus bisporus* cap artificially induced by *Verticillium fungicola* var. *fungicola* VV2.

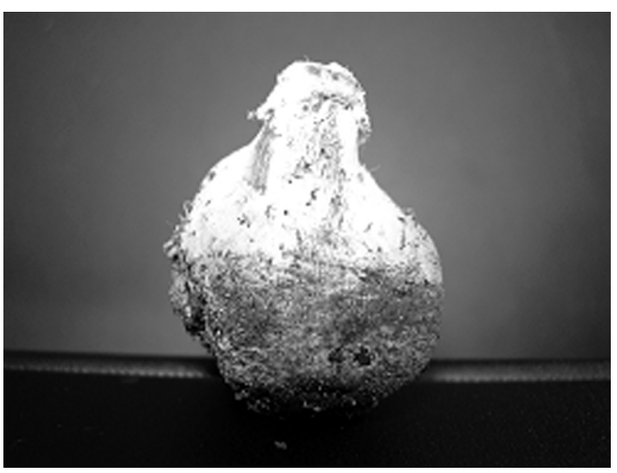

Fig. 2. Deformation of *Agaricus bisporus* fruiting body artificially induced by *Verticillium fungicola* var. *fungicola* VV2

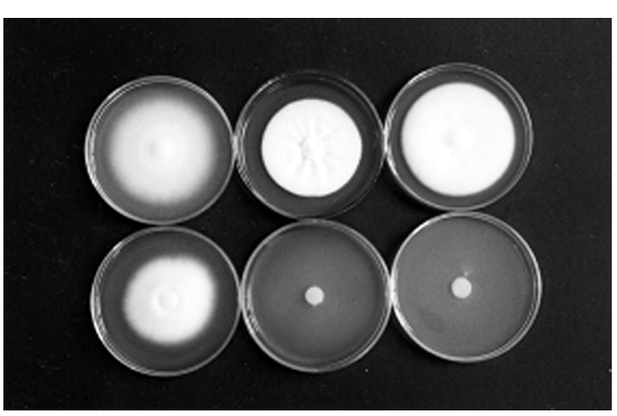

Fig. 3. Influence of medium composition on growth of *Verticilium fungicola* var. *fungicola* VV2. From left to right: upper row - PDA, MA, and MDA; lower row - PDA, CzA, and WA.

cide-amended medium was given as a percentage of the control. EC_{50} and EC_{90} (fungicide concentrations which inhibit mycelial growth by 50 and 90%, respectively) were determined for each isolate by interpolation from computer-generated log-probit plots of fungicide concentration and relative inhibition (Leroux and Gredt, 1972). The effect of fungicides was studied by analyzing means and variance of EC by the multiple range test (Finney, 1964).

RESULTS

Symptoms of "dry bubble" disease

Single or clusters of undifferentiated fruiting bodies of *A. bisporus* with symptoms similar to those caused by *V. fungicola* were observed in screening of mushroom farms in Serbia during 2002-2003. Large brown spots with a fuzzy grayish tint were noticed on fully differentiated fruiting bodies after 12 days (Fig. 1), and undifferentiated fruiting bodies were found 18 days after artificial inoculation of casing with the pathogen (Fig. 2).

Identification of isolates identification

The following morphological characteristics were recorded in the analyzed isolates: dense white aerial mycelia; absence of pigment production; hyaline, erect conidiophores with groups of divergent phialides of verticilliate form; hyaline, cylindrical phialides with slightly inflated base and acute tip; and hyaline, unicellular, ellipsoid to cylindrical conidia produced in a gelatinous matrix. Conidia in all studied isolates measured 2.95-7.38 x 1.97-2.46 µm. After comparing our isolates with control isolates, we concluded that all Serbian isolates were *V. fungicola* var. *fungicola*.

The diameter of colonies of all isolates after 7 days of growth showed significant differences depending on composition of the medium (Fig. 3), ranging between 10.25 mm on MDA (ViV_1) and 14.17 mm on PDA (P_2V_3).

Serbian as well as UK *V. fungicola* isolates grew only at 20°C; growth was absent at 27 and 30°C. However, *V. fungicola* var. *aleophilum* DC-170 grew at all investigated temperatures, the optimal one being 27°C.

Pathogenicity test

Fruiting bodies with split stems and grayish-brown spots on the whole surface of the cap were noticed 12 days after inoculation of the casing with isolates VV_2 and P_2V_3; and 13 days after inoculation with isolates ViV_1, RaV_1, and BeV_1 (Fig. 1).

Undifferentiated masses of fruiting bodies with a dry surface covered with a dusty gray layer of conidia were observed on the 18th day after inoculation with isolates VV_2 and P_2V_3; on the 19th day after inoculation with ViV_1 and RaV_1; and on the 20th day after inoculation with BeV_1 (Fig. 2). However, fruiting bodies with "dry bubble" symptoms were not recorded after replacing the infected casing with a sterile new one.

Testing of sensitivity to selected fungicides

In these *in vitro* investigations of the sensitivity of *V. fungicola* var. *fungicola* isolates to the selected fungicides, all studied isolates showed high resistance to benomyl (EC_{50} values were between 234.55 to 359.95 mg/l); moderate sensitivity to iprodione (EC_{50} values were in the range from 11.93 to 22.80 mg/l); and high sensitivity to prochloraz-Mn (EC_{50} values were in the range from 1.11 to 2.51 mg/l); (Table 2).

DISCUSSION

To judge from taxonomic criteria based on the optimal growth temperature (Gams and Van Zaayen, 1982; Nair and Macauley, 1987), all *V. fungicola* isolates from Serbian *A. bisporus* farms were *V. fungicola* var. *fungicola*. Gams and Van Zaayen (1982) emphasized *V. fungicola* varieties differ significantly with respect to the optimal temperature for mycelial growth (20-24^{0}C for var. *fungicola* and 30^{0}C for var. *aleophilum*). Bonnen and Hopkins (1997) observed a high level of homogenicity in colony morphology, virulence, and fungicide response among analyzed *V. fungicola* isolates from North America and placed them in the same RAPD group. But in investigations of hydrolytic enzyme production and genetic variability between two

Table 2. *In vitro* sensitivity of *Veriticillium fungicola* var. *fungicola* isolates to selected fungicides.

Fungicide	Toxicity parameters	Isolates				
		VV_2	ViV_1	P_2V_3	RaV_1	BeV_1
Benomyl	EC_{50}	357.95	280.85	250.72	359.25	234.55
	Range	246.3-562.0	193.4-398.5	98.0-1432.9	279.6-66.2	147.4-76.0
	EC_{90}	21986.72	8737.72	4179.34	38073.65	20981.54
	Range	8015.83-1638E+1	3526.69-53381.84	2273.47-12347.75	48187.94-16268E+4	7308.98-12560E+1
	b	0.72	0.86	0.72	0.59	0.66
	Range	0.63-0.81	0.71-1.01	0.63-0.81	0.51-0.67	0.57-0.75
Iprodione	EC_{50}	11.93	20.56	18.73	22.80	14.06
	Range	8.6-16.9	15.9-29.1	14.6-26.2	17.6-32.4	11.3-18.4
	EC_{90}	169.70	235.27	231.54	224.22	146.97
	Range	101.26-326.9	122.35-673.3	123.21-630.75	116.8-641.0	84.72-345.9
	b	1.11	1.21	0.72	1.19	1.27
	Range	1.03-1.19	1.06-1.36	0.63-0.81	1.04-1.34	1.12-1.42
Prochloraz-Mn	EC_{50}	1.82	1.70	1.11	1.99	2.51
	Range	1.31-2.76	1.11-2.71	0.87-1.47	1.57-2.66	2.02-3.23
	EC_{90}	87.49	114.72	25.49	26.63	20.01
	Range	35.2-356.69	32.3-1755.3	14.06-58.90	14.3-72.19	12.76-37.8
	b	0.76	0.70	0.94	1.14	1.42
	Range	0.67-0.85	0.57-0.83	0.85-1.03	1.00-1.28	1.29-1.55

EC50 and EC90 expressed in mg/L
b = regression coefficient

V. fungicola isolates originating from North America, Bidochka et al. (1999) showed the presence of 49% divergence in the rDNA sequence of their ITS1, which was confirmed using RAPD and AFLP markers (Juarez del Carmen et al., 2002; Largeteau et al., 2006). Contrary to the situation with var. *aleophilum*, Largeteau et al. (2006) showed the presence of significant differences of physiological and pathogenicity traits among var. *fungicola* isolates.

The noted symptoms on *A. bisporus* fruiting bodies caused by *V. fungicola* var. *fungicola* isolates on Sebian farms were similar to ones previously described on farms worldwide (Nairn and Macauley, 1987; Staunton and Dunne, 1990; North and Wuest, 1993; Savoie and Largeteau, 2004). The results of this study confirm the results of Wong and Preece (1987), and North and Wuest (1993), who showed the peat/lime casing to be the primary source of *V. fungicola*, and that the earliest possible infection occurred during the encasement period, but not before, because conidia which were present in spawned compost were not able to cause development of the disease.

Using electron microscopy, Dragt et al. (1996) showed that *V. fungicola* grows both outside and inside the hyphae of *A. bisporus* fruiting bodies, and emphasized that the pathogen penetrates host chitin cell walls by the combined effect of mechanical pressure and wall-lytic enzymes. Mills et al. (2000) isolated and identified β-1-6-glucanases, chitinases, serine proteinase, stearase, and esterase from culture filtrates of *V. fungicola* grown in the presence of *A. bisporus* cell wall, and Athey-Pollard et al. (2003) isolated the cap-binding protein (eIF4E) from *A. bisporus* and *V. fungicola* and described its gene nucleotide and amino acid composition.

Agaricus species can protect themselves from *V. fungicola* invasion by production of extracellular phenoloxidases, H_2O_2, and antibiotics (Largeteau et al., 2006; Score et al., 1997; Savoie et al., 2004), but efficiency of self-defense depends on the level of resistance of *Agaricus* species (and even strainstrains) to *V. fungicola*, as well as on sensitivity of the pathogen to host metabolites (Savoie and Largeteau, 2004; Dragt et al., 1995; Jaurez del Carmen et al., 2002). Thus, Gea et al. (2003) noted that 26-47% of fruiting bodies on *A. bisporus* farms but only 4-12% on *A. bitorquis* farms were infected, while Savoie and Largeteau (2004) showed that the majority of *V. fungicola* isolates were susceptible to lower H_2O_2 concentration. In recent decades, a common method of pathogen control on farms worldwide is application of various fungicides. For improvement of crop protection and reduction of production costs, the effects of some new fungicides are being tested. However, fungicide efficiency depends on frequency of usage (Bonnen and Hopkins, 1997), as well as on the persistence of fungicides in high concentrations in the casing during cultivation (Grogan and Jekes, 2003).

According to the criteria established by Gea et al. (2003, 2005), *V. fungicola* isolates from Serbian *A. bisporus* farms were highly resistant to benomyl, moderately sensitive to iprodione, and highly sensitive to prochloraz-Mn. Bonnen and Hopkins (1997) also showed absence of benomyl sensitivity in isolates obtained after the year 1979. However, contrary to the situation with Serbian *V. fungicola* isolates, Spanish isolates were resistant to iprodione with EC_{50} values higher than 50.00 mg/L (Gea et al., 1997). In the case of resistance to prochloraz-Mn, the picture is very different from strain to strain of *V. fungicola*. To be specific, 70% of pathogen isolates from Great Britain and Spain were moderately sensitive to prochloraz-Mn with EC_{50} values ranging from 5.0 to 8.0 mg/L (Grogan et al., 2000; Gea et al., 2003), and some farms reported unsatisfactory levels of control by that fungicide, which was explained by the fact that resistance was developed with the passage of time (Gea et al. 2005). Those authors analyzed 105 *V. fungicola* var. *fungicola* isolates from Spanish mushroom crops collected in the period between 1992 and 1999 and demonstrated that their resistance ranged from low (EC_{50} value of 0.8 mg/l) in 1992 to moderate (EC_{50} value of 8.8 mg/l) in 1998. In the case of isolates from 1999, 29.86% were sensitive (EC_{50} value of 5.0 mg/l) and 14% slightly tolerant (EC_{50} values equal to or above 5.0 mg/l), while 60% grew at a fungicide concentration of 50.0 mg/l and 40% at 100.0 mg/l.

Bernardo et al. (2002, 2004) noted that prochloraz-Mn alters structure of the cell wall, as well as the ratio of cell wall components and their structure in both *V. fungicola* and *A. bisporus*, which can be attributed to the fungicide's inhibitory effect on sterol biosynthesis. Diamantopoulou et al. (2006) confirmed the adverse effects of fungicides in testing of tebuconazole, a new fungicide, which caused pileus deformations and severe reduction of total yield at a concentration of 0.8 g/m^2 and deviation in sporophore color at a concentration of 1.2 g/m^2. Owing to everything mentioned above and despite the existence of some efficient fungicides for *V. fungicola* control, special attention is now being paid to genetic reduction of pathogen virulence by generation of mutants with diminished ability to utilize chitin as a carbon source (Amey et al., 2003), as well as to the possibility of using natural products (such as essential oils of different plants) to inhibit pathogen activity (Soković et al., 2006).

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ОСЕТЉИВОСТ НА ФУНГИЦИДЕ ОДАБРАНИХ ИЗОЛАТА *VERTICILLIUM FUNGICOLA* ИЗ ГАЈИЛИШТА *AGARICUS BISPORUS*

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Проучавано је пет изолата *Verticillium fungicola*, изолованих са оболелих плодоносних тела *Agaricus bisporus* сакупљених у гајилиштима Србије у току 2002-2003. На основу морфологије колонија, гајених под различитим условима, и патогених карактеристика, изолати су идентификовани као *V. fungicola* var. *fungicola*. Примарни извор инфекције била је покривка од тресета и креча. Тест осетљивости на одабране фунгициде је показао да су сви изолати високо резистентни на беномил (EC_{50} вредности више од 200.00 mg/l), умерено осетљиви на ипродион (EC_{50} вредности између 11.93 и 22.80 mg/l), и високо осетљиви на прохлораз-Mn (EC_{50} вредности мање од 3.00 mg/l).