Laboratory Efficacy of Selected Fungicides and *Rhododendron catawbiense* Leaf Extracts on the Growth of *Verticillium fungicola*

D P MAMIRO & D J ROYSE
Department of Plant Pathology, 315 Buckhout Laboratory The Pennsylvania State University, University Park, PA 16802, USA. E-mail: dpm204@psu.edu

Abstract: Isolates (105) of *Verticillium fungicola,* collected from 1951 to 2004 from mushroom farms located in various geographical locations were assayed for their sensitivity *in vitro* to five fungicides and one botanical (rhododendron) with fungicidal activity. Potato dextrose yeast extract agar (PDYA) was amended with fungicides (50µg/ml: thiophanate-methyl, chlorothalonil, natamycin, iprodione or 135µg/ml: famoxadone) then inoculated with an agar plug (5 mm diameter) of *V. fungicola* grown on PDYA. Mycelial growth response was rated in one of three categories as follows: no inhibiton (growth > 80% percent of control), moderate inhibition (growth 40-80% of control) and strong inhibition (growth < 40% percent of control). Most isolates were not inhibited by thiophanate-methyl or iprodione, moderately inhibited by natamycin and famoxadone and strongly inhibited by chlorothalonil. Aqueous extracts (50 g fresh leaves/300 ml distilled water) of leaves of *Rhododendron catawbiense* inhibited growth of *V. fungicola in vitro*, while the growth of *Agaricus bisporus* (from spawn grains) was not inhibited. Antifungal compound(s) were present in *R. catawbiense* leaf extracts but further research is required to isolate and characterize the compound(s).

Key words: *Agaricus bisporus*, fungicides, thiophanate-methyl, chlorothalonil, natamycin, iprodione, famoxadone

1 Introduction

Dry bubble disease of the cultivated button mushroom, *A. bisporus* (Lange) Imbach caused by *V. fungicola* (Preuss) Hassebrauk[1] is world wide in distribution. It is the most common and serious fungal disease of the button mushroom crop. Mushroom yield loss due to *V. fungicola* has been minimized by the use of sanitation, cultural methods, chemical applications and biological control.[2-4] Fungicide applications are commonly used as a control measure against dry bubble,[5, 6] However, the use of fungicides does not guarantee total mushroom crop protection due to the occurrence of pathogens with fungicide resistance. Fungicides that are or were used for the control of dry bubble disease of mushrooms in the United States include benomyl (benzimidazole), thiabendazole (benzimidazole) and chlorothalonil (phthalimide).[7] Benomyl is no longer produced.
The search for safer fungicides with less environmental and mammalian toxicity is a major objective.[8] Particularly desirable is the discovery of novel natural antifungal agents representing new fungicide classes with different modes of action. Natural products offer an efficient approach to discovery and optimization of new agrochemicals including fungicides, for disease control.[9-11] Extracts and oils from various higher plants have been found to contain antifungal compounds.[12-14] The discovery and introduction of novel or new fungicides for the control of dry bubble disease is a priority for mushroom growers.

2 Materials and Methods

2.1 Strains

Isolates of *V. fungicola* were obtained from The Pennsylvania State University (PSU), Department of Plant Pathology Disease Culture Collection and from other sources. A list of *V. fungicola* isolates, year of collection and their origin are listed in Table 1. Recently collected (2004) isolates of *V. fungicola* from various mushroom farms in Pennsylvania were included in this study.

Table 1: Strain, year collected, and source of *V. fungicola* isolates used in this study

Strain	Strain #	Year	Source
1	114	1951	BCMF (Bon Chang) Avondale, Pennsylvania
2	116	1969	BCMF (Bon Chang) Avondale, Pennsylvania
3	117	1970	Gaspari Bros.; VGI; Berks Co.
4	120	1958	ML2; Pennsylvania
5	121	1950's	ML3
6	123	1950	ML5; Michigan
7	124	1950's	ML6
8	128	1970	C-7; CPW 707; Chester Co.
9	130		K-2; Korea
10	131	1970's	S-2; Switzerland
11	134	1971	90; Chester Co.; Intermediate to Benomyl
12	138	1978	Giorgio
13	139	1979	Giorgio; J. Cornell
14	140	1979	Dr. Paul Wuest; British Columbia or California
15	142	1979	Slack 1; Slack's Farm; Quebec
16	143	1979	Ohio MR Farm; J. Cornell
17	144	1979	Grocery store Prod.; Chester Co.; J. Cornell
18	145	1979	Diseased Mushrooms; Cathy Harvey; California
19	148	1979?	Salem, Oregon
20	150	1981	from *Pleurotus ostreatus*; Ocean View, California
21	151	1982	DeAngelo Bros.; B7; Berks Co., Pennsylvania
22	152	1982	J. Bonifacino; C3; Chester Co., Pennsylvania
23	153	1982	Ostrom MR Farm; W1; Washington
24	154	1982	C.P.Yeatman; C8; Chester Co., Pennsylvania
25	155	1982	Quirino DiMarco; B4; Berks Co., Pennsylvania
26	156	1982	Ocean View Mushroom Growers; CA1; California
27	157	1982	Chase Mushroom; C10; Chester Co., Pennsylvania
28	158	1982	Antonini Bros.; D1; Delaware
29	159	1982	Sea Lea Farm; B6; Berks Co., Pennsylvania
30	160	1982	Carroll Pratt Farm; C2; Chester Co., Pennsylvania
31	161	1982	Slacks Mushroom Farm; WC2; Waterloo, Canada
32	162	1982	WC4; Toronto, Canada
33	163	1982	Castle and Cooke; Salem, Oregon
34	164	1982	V; Vancouver, British Colombia
35	165	1982	Sno-Top Mushroom Farm; L2; Lawrence Co., Pennsylvania
36	166	1982	Sno-Top Mushroom Farm; L1; Lawrence Co., Pennsylvania
37	167	1982	Giorgio Mushroom Farm; B10-3; Berks Co., Pennsylvania

Strain	Strain #	Year	Source
38	168	1982	Grocery store Prod.; C11; Chester Co. Pennsylvania
39	169	1982	Penn Point Farm; C7; Chester Co., Pennsylvania
40	170	1982	C.P. Yeatman; C9; Chester Co., Pennsylvania
41	171	1982	Parrish Bros., C4; Chester Co., Pennsylvania
42	172	1982	Slacks Mushroom Farm; WC3; Waterloo, Canada
43	173	1982	Giorgio Mushroom Farm; B10-6; Berks Co., Pennsylvania
44	174	1982	Bon Chang Mushroom Farm; C1; Chester Co., Pennsylvania
45	175	1982	Irish Mt. Farm; B5; Berks Co., Pennsylvania
46	182	1986	Leonard North-4; Chester Co., Pennsylvania
47	183	1986	Leonard North-6; Chester Co., Pennsylvania
48	184	1986	Leonard North-7; Chester Co., Pennsylvania
49	185	1986	Leonard North-10; Chester Co., Pennsylvania
50	220	1988	Fiona Davenport
51	221	1988	Fiona Davenport
52	222	1988	Fiona Davenport
53	223	1989	Fiona Davenport
54	224	1989	Fiona Davenport
55	225	1989	Fiona Davenport
56	226	1990	Fiona Davenport
57	227	1990	Fiona Davenport
58	228	1989	Fiona Davenport
59	229	1989	Fiona Davenport
60	253	1993	Dr. Alice Bonnen; MTDF;7/12/93
61	254	1985	V9; Leavers; Canada; Danny Rinker
62	255	1986	V13; Superior; Canada; Danny Rinker
63	256	1986	V15; Castel; Canada; Danny Rinker
64	257	1986	V18; Wentworth Mushroom; Canada; Danny Rinker
65	258	1985	V2; Acton; Canada; Danny Rinker
66	259	1985	V5; A & F; Canada; Danny Rinker
67	260	1986	V12; Superior; Canada; Danny Rinker
68	261	1986	V14; Battista; Canada; Danny Rinker
69	262	1988	V16; Markham Mushroom; Canada; Danny Rinker
70	V-1	1999	Mushroom farm, Pennsylvania
71	V-2	1999	Mushroom farm, Pennsylvania
72	V-3	1999	Mushroom farm, Pennsylvania
73	V-4	1999	Mushroom farm, Pennsylvania
74	V-5	1999	Mushroom farm, Pennsylvania
75	V-6	1999	Mushroom farm, Pennsylvania
76	V-7	1999	Mushroom farm, Pennsylvania
77	V-8	1999	Mushroom farm, Pennsylvania
78	V-10	1999	Mushroom farm, Pennsylvania
79	V-11	1999	Mushroom farm, Pennsylvania
80	V-12	1999	Mushroom farm, Pennsylvania
81	V-13	1999	Mushroom farm, Pennsylvania
82	V-14	1999	Mushroom farm, Pennsylvania
83	V-15	1999	Mushroom farm, Pennsylvania
84	V-16	1999	Mushroom farm, Pennsylvania
85	V-17	1999	Mushroom farm, Pennsylvania

Strain	Strain #	Year	Source
86	V-18	1999	Mushroom farm, Pennsylvania
87	V-19	1999	Mushroom farm, Pennsylvania
88	V-20	1999	Mushroom farm, Pennsylvania
89	V-21	1999	Mushroom farm, Pennsylvania
90	V-28	1999	Mushroom farm, Pennsylvania
91	V-29	1999	Mushroom farm, Pennsylvania
92	V-30	1999	Mushroom farm, Pennsylvania
93	V-31	1999	Mushroom farm, Pennsylvania
94	V-32	1999	Mushroom farm, Pennsylvania
95	V-33	1999	Mushroom farm, Pennsylvania
96	V-34	1999	Mushroom farm, Pennsylvania
97	V-35	1999	Mushroom farm, Pennsylvania
98	V-36	1999	Mushroom farm, Pennsylvania
99	V-37	1999	Mushroom farm, Pennsylvania
100	V-38	1999	Mushroom farm, Pennsylvania
101	V-39	1999	Mushroom farm, Pennsylvania
102	V-40	1999	Mushroom farm, Pennsylvania
103	V-1Mar	2004	Mark Spear, Sylvan, PA mushroom farm
104	V-2Mar	2004	Mark Spear, Sylvan, PA mushroom farm
105	V-3Mar	2004	Mark Spear, Sylvan, PA mushroom farm

2.2 Fungicides

Synthetic fungicides evaluated included thiophanate-methyl (Topsin, 70% active ingredient [a.i.], Elf-Atochem); chlorothalonil (Bravo Ultrex, 82.5% a.i.); iprodione (Rovral 4F, 41.6 a.i., Bayer); natamycin (Natamycin, 50% a.i., Sylvan) and famoxadone (Tanos, 25% a.i., Du Pont). Fungicide concentrations used were 50 µg/ml for thiophanate-methyl, chlorothalonil, iprodione and natamycin; 135 µg/ml for famoxadone.

The *in vitro* sensitivity of *V. fungicola* isolates to the fungicides was determined by using radial growth assays on potato dextrose yeast extract agar (PDYA). Fungicide solutions were added to warm (55°C) PDYA and mixed well before pouring into Petri plates (60 mm x 15 mm). Two replicate plates per fungicide for each *V. fungicola* isolate were used. Amended and non-amended PDYA were center inoculated with inverted 3 mm discs (cork borer) that were taken from non-amended PDYA plates inoculated two weeks earlier with *V. fungicola*. The inoculated plates were incubated on the laboratory bench at room temperature and radial growth was measured when the mycelium on the control medium reached the edge of the plate (10-14 days from the date of inoculation).

2.3 Rhododendron leaf extract

Young, fresh leaves of *R. catawbiense* were collected from the PSU campus. Leaves (50 g) were homogenized (Waring blender) for 60 seconds in 300 ml distilled water. The blended mixture was boiled for 3 hr and then filtered through three-layers of cheesecloth. The filtrate was placed on a hot plate with steady heat (80°C) and the water was allowed to evaporate. Distilled water (10 ml) then was added to the extract to make a concentrated, flowable liquid and the reconstituted solution then was autoclaved at 121°C (15 minutes). This sterile aqueous extract was stored at room temperature until used for radial mycelial growth assays.

An aliquot (5 ml) of rhododendron leaf extract was added to warm (55°C), sterile PDYA (95 ml) and mixed thoroughly before pouring into Petri plates. Radial growth assays were carried out as outlined above except larger plates (90 mm x15 mm) were used. For *A. bisporus* inoculum, a single grain of rye spawn was used to

center inoculate the plates. Radial mycelial growth was measured at the time when mycelium from the control (non-amended) medium reached the edge of the plate (14-21 days from the date of inoculation).

3 Results and Discussion

3.1 Sensitivity to fungicides

Three categories of mycelial growth sensitivity were recognized in this study as follows (reported as percentage of growth based on the controls): 1) 80-100% (insensitive), 2) 40-80% (moderately sensitive), and 3) less than 40% (sensitive). Most isolates were not inhibited by thiophanate-methyl or iprodione, moderately inhibited by natamycin and famoxadone and strongly inhibited by chlorothalonil. Isolates collected prior to 1971 were sensitive to thiophanate-methyl, the year when benomyl was first used in the mushroom industry to control dry bubble disease.

Mycelial growth of isolates of *V. fungicola* (as a percentage of control) on chlorothalonil- amended PDYA (50µg/ml a.i.) was plotted against year of collection (Figure 1). All *V. fungicola* isolates were moderately sensitive to chlorothalonil except one isolate that was collected in 1980. Chlorothalonil was introduced into the mushroom industry in 1988. Thus, isolates that were insensitive to the fungicide existed in the population prior to the introduction of chlorothalonil to the mushroom industry. All isolates collected in 1999 had growth 50-75% (compared to control), while those recently collected (2004) had growth < 65%. Thus, it does not appear that the *V. fungicola* population is developing resistance to chlorothalonil. This may be due to the more broad spectrum mode of action of chlorothalonil compared to thiophanate-methyl.

Chlorothalonil is the only registered fungicide for use in controlling *V. fungicola* on mushrooms in the United States. Since the *V. fungicola* population has not developed resistance to this fungicide in the last 16 years, it is anticipated that chlorothalonil will continue to be useful for control in the future. However, there is a need for other fungicides that control *V. fungicola* while reducing the risk to human health.

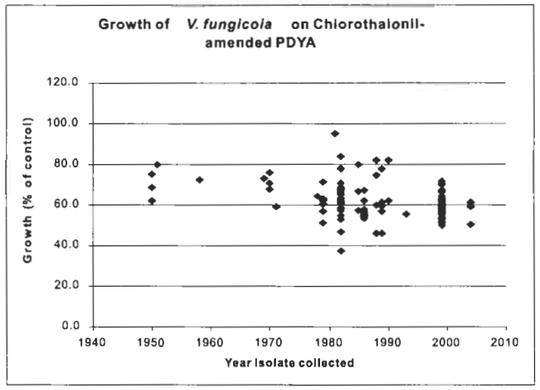

Fig. 1. Growth of *Verticillium fungicola* isolates on chlorothalonil-amended media (50 µg/ml a.i.) versus the year each isolate was collected
Some data points represent more than one isolate.

The growth (as a percentage of control) of each isolate of *V. fungicola* on natamycin- amended PDYA (50 µg/ml a.i.) was plotted against year of collection (Figure 2). Most *V. fungicola* isolates were insensitive to moderately sensitive to natamycin with growth between 55% and 100% of the controls. However, since natamycin has only recently been identified as a potential candidate for use in controlling *V. fungicola*, optimum concentrations for differential inhibition of *V. fungicola* and *A. bisporus* have not been determined. Slightly higher concentrations may be required *in vitro* to inhibit growth of *V. fungicola.*

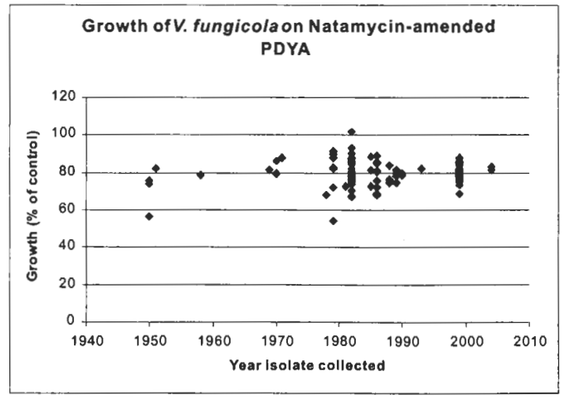

Fig. 2. Growth of *Verticillium fungicola* isolates on natamycin-amended media (50 µg/ml a.i.) versus the year each isolate was collected
Some data points represent more than one isolate.

The growth (as a percentage of control) of each isolate on famoxadone-amended PDYA (50 µg/ml a.i.) is plotted against year of collection (Figure 3). Most *V. fungicola* isolates were moderately insensitive to sensitive to famoxadone with growth between 55% and 105%. Diamantopoulou *et al.*[15] reported significant control *in vivo* when applying famoxadone (135µg/ml a.i.) to the casing surface 24 hr prior to inoculation of the casing with *V. fungicola*. Famoxadone may be more effective at inhibiting spore germination (rather than mycelial growth) or infection at the pin stage of mushroom development. Since split application of famoxadone was found to have similar disease control effect, there is a possibility that the fungicide delayed the development of infection throughout the entire harvest period. Under these circumstances, the disease may be considered controlled. Spores may be more sensitive to the fungicide than the mycelium.

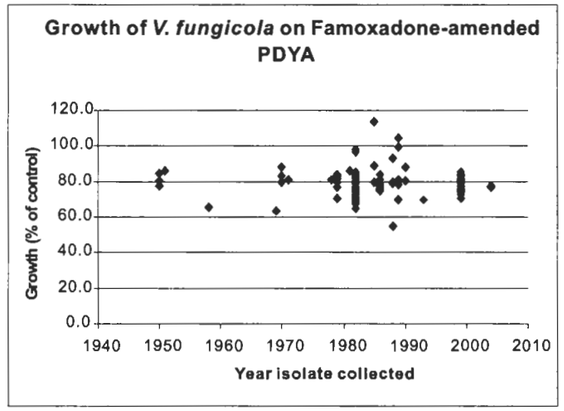

Fig. 3. Growth of *Verticillium fungicola* isolates on famoxadone-amended media (50µg/ml a.i.) versus the year each isolate was collected
Some data points represent more than one isolate.

3.2 Sensitivity to rhododendron leaf extracts

Rhododendron leaf extract inhibited the growth of *V. fungicola* by 94%. On the other hand, mycelial growth of *A. bisporus* appeared to be slightly stimulated. The antifungal compounds contained in rhododendron leaf

extract were water-soluble and heat stable. Thus, it would appear that the fungicidal compound contained in rhododendron leaf extract is neither protein nor volatile. Harbone[16] reported that toxic diterpenes (grayanotoxins) are present in the leaves of many *Rhododendron* and *Kalmia* species. Grayanotoxins may be responsible for the fungicidal effects observed in our study but additional work would be required to more completely characterize the inhibitory substances present. Rhododendron would appear to be a worthy candidate for further study regarding the presence of both fungicidal (to *V. fungicola*) (to *A. bisporus*) of natural origin.

Acknowlegements

We thank the Ford Foundation, the Tanzania Government and The L. F. Lambert Scholarship in honor of James P. Roberts for their support of D. P. Mamiro.

References

[1] Ware WM. A disease of the cultivated mushrooms caused by *Verticillium malthousei* sp. nov. Ann. Botany, 1933, 47:763-784.
[2] De Trogoff H, Ricard JL. Biological control of *Verticillium malthousei* by *Trichoderma viride* spray on casing soil in commercial mushroom production. Plant Dis. Reptr. 1976, 60:677-680.
[3] Fletcher JT, White PF, Gaze RJ. Mushrooms: Pest and Disease Control, 2nd edition. England:Intercept, 1989, 174 pp.
[4] Coles PS, Barber W. Fungal pathogens: pest species biology and control. In: Pennsylvania Mushroom Integrated Pest Management Handbook , 2002, pp 52-60.
[5] Harvey CL, Wuest PJ, Schisler LC. Diseases, weed molds, indicator molds and abnormalities of the commercial mushroom. In: Wuest, P. J. and G. D. Bengtson (eds.). Penn State Handbook for Commercial Mushroom Growers. The Pennsylvania State University, 1982, pp19-20.
[6] Nair NG, Macauley BJ. Dry bubble disease of *Agaricus bisporus* and *A. bitorquis* and its control by prochloraz-manganese complex. New Zealand J. Agric. Res. 1987, 30:107-116.
[7] Bonnen AM, Hopkins C. Fungicide resistance and population variation in *Verticillium fungicola*, a pathogen of the button mushroom, *Agaricus bisporus*. Mycol. Res. 1997, 101:89-96.
[8] Meisterpro. Crop Protection Handbook, 2004.
[9] Varpoorte R. Exploration of nature's chemodiversity: The role of secondary metabolites as leads in drug development. Drug Discovery Today, 1998, 3:232-238.
[10] Wedge DE, Kuhajek JM. A microbioassay for fungicide discovery. SAAS Bulletin, Biochemistry and Biotechnology, 1998, 2:1-7.
[11] Wedge DE, Galindo JCG, Macias FA. Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. Phytochem. 2000, 53:747-757.
[12] Grayer RG, Harborne JB. A survey of antifungal compounds from higher plants, 1982-1993. Phytochem. 1994, 37:19-42.
[13] Wilson CL, Solar JM, El Ghaout A, *et al*. Rapid evaluation of plant extracts and essential oils for antifungal activity against *Botrytis cinerea*. Plant Dis. 1997, 81:204-210.
[14] Hadacek F, Greger H. Testing of antifungal natural products: methodologies, comparability of results and assay choice. Phytochem. Anal. 1997, 11:137-147.
[15] Diamantopoulou P, Philippoussis A, Kastanias MA, *et al*. Effect of timing and rate of application on the selectivity of famoxadone and trifloxystrobin for *Verticillium fungicola* on *Agaricus bisporus*. Mush. Sci. 2004, 16:475-478.
[16] Harbone JB. Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis, 1998, 302 pp.