Fungicide Impact on *in vitro* Germination of Basidiospores of *Puccinia horiana*, the Causal Agent of Chrysanthemum White Rust

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ABSTRACT

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Puccinia horiana is an actionable pathogen that, upon diagnosis, triggers an eradication protocol combining destruction of symptomatic chrysanthemums and a strict fungicide regime for asymptomatic plants. Symptoms typically appear during the fall as growers prepare to ship their crops. To expand the list of effective fungicides and develop fungicide sensitivity baselines, we screened *in vitro* germination of *P. horiana* basidiospores in 0.05% water agar solution amended with varying concentrations of 14 fungicides: azoxystrobin, boscalid + pyraclostrobin, fluoxastrobin, mancozeb, mandestrobin, metconazole, myclobutanil, propiconazole, tebuconazole, triadimefon, trifloxystrobin, trifloxystrobin + triadimefon, and triticonazole. Leaves with pustules ready to sporulate were affixed to petri plate lids over bases containing fungicide-amended agar. After 2 days in the dark, percent basidiospore germination was assessed. Concentrations required for 50% germination (EC_{50}) grouped according to fungicide mode of action. Benzimidazoles exhibited EC_{50} values ranging from 9 to 244 ppm, while strobilurins ranged from 2 to 27 ppb. Mancozeb exhibited an EC_{50} of 7 ppm, and chlorothalonil was 205 ppb. Combinations of strobilurins with other modes of action exhibited EC_{50} values in the same range as the strobilurins. These data provide a baseline for monitoring resistance development to *P. horiana* over time.

INTRODUCTION

Puccinia horiana Henn., causal agent of chrysanthemum white rust, is a serious pathogen of chrysanthemum that is indigenous to eastern Asia (Hiratsuka 1956). First discovered in Japan in 1895 (Hennings 1901), *P. horiana* is an obligate, autoecious, microcyclic basidiomycete, producing copious basidiospores upon germination of teliospores embedded in the abaxial leaf surface (Bonde et al. 2014b). The pathogen and disease have been reported from several continental European countries (Baker 1967), the United Kingdom (Baker 1967), New Zealand (Firman and Martin 1968), South Africa (Firman and Martin 1968), Australia (Exley et al. 1993), and South America (CMI Distribution Maps of Plant Diseases 1989). In 1977, it was discovered for the first time in the United States (Petersen et al. 1978). Although the disease has reappeared on several occasions since 1977 in the United States, each time it was believed to have been eradicated (Bonde et al. 1995). Beginning in 2004, chrysanthemum white rust began to be discovered at increased frequency, primarily in northeastern United States. Because of this observed increased in the number of outbreaks, the suggestion has been made that the pathogen may now be established in the country.

With the possibility of establishment of the disease in the United States, it became apparent that the nearly four-decade policy of exclusion and eradication must be augmented by other appropriate disease management measures. Recently, we began a broad research program to better understand the pathogen and disease. One of the goals was to obtain research information with which to better assess pathogen longevity under specific environmental conditions and determine how *P. horiana* might overwinter in the northeastern United States. We were successful at developing a better and more sensitive method to measure and evaluate teliospore longevity and obtained evidence that *P. horiana* has the capability of systemically infecting chrysanthemum plants and may possibly survive in infected crowns and roots (Bonde et al. 2014b).

Several fungicides have been shown to have protectant or curative properties toward chrysanthemum white rust (e.g., systemics strobilurin and azoxystrobin, contacts chlorothalonil and mancozeb, and systemic DMIs triadimefon and myclobutanil). However, in Europe fungicide resistance has developed toward triazoles and strobilurins (Cook 2001), and it is now evident that additional disease-management tools are necessary to mitigate epidemics in production systems. In this manuscript, we report results of experiments to determine the baseline sensitivities of *P. horiana* to fungicides previously used to manage rust diseases. The method used to determine sensitivities was based on ability to prevent germination of basidiospores in a germination medium. Fungicides were selected to span a range of modes of action as described by the Fungicide Resistance Action Committee (FRAC) (www.frac.info).

SENSITIVITIES TO SPECIFIC FUNGICIDES

Chrysanthemum (*Chrysanthemum* X *morifolium* Ramat.) cv. Vicki was used throughout the study because this cultivar is

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particularly susceptible to *P. horiana* and disease progress is well understood from previous research (Bonde et al. 2014 a, 2014b). *P. horiana* isolates used in the study were received in 2011 from cooperators in Connecticut (CT11-3 and CT11-4) and New York (NY11-1). Each isolate was maintained by periodic inoculation of 1-month-old Vicki plants grown from cuttings. Plants were fertilized with Osmocote 14-14-14 as needed. Throughout the study, all isolates showed similar morphology and aggressiveness.

Seven-week-old plants in 16-cm diameter plastic pots were inoculated by placing them alongside infected donor plants inside a 1.15-m long × 0.84-m wide × 0.95-m high mist chamber that provided a fine mist and RH of 100% (Fig. 1A). The use of the mist chamber with infected donor plants interspersed with recipient plants provided the appropriate amount of moisture and air turbulence for sporulation to occur on infected leaves, distribution of inoculum, and initiation of infection on recipient test plants (Bonde et al 2014b). After 24 h in the mist chamber, inoculated plants were transferred to benches in the greenhouse at 22 to 26°C to be used as sources of spores for the fungicide tests 3 weeks after inoculation.

In order to determine sensitivities to fungicides, a method was developed to measure germination of basidiospores released from germinating teliospores embedded in infected chrysanthemum leaves. Leaves with telia were collected from infected chrysanthemum plants 3 weeks after inoculation and placement in the greenhouse (Fig. 1B, 1C, 1D). The leaves were mounted to the inside surface of Falcon 1007 Petri dish lids with petroleum jelly. Mounted leaves were soaked 10 min with Tween 20 water (1 drop Tween 20 per 100 ml reverse osmosis water), and blotted dry. Lids were placed onto the petri dish bottoms, each bottom containing 7 ml 0.05% water agar solution containing a known concentration of a specific fungicide, and the dishes placed in a plastic box (20 cm wide, 30 cm long, 9 cm high). Three plates were prepared for seven concentrations within each experiment. A minimum of three experiments per fungicide were conducted using isolates from Connecticut and New York, but specific concentrations varied to bracket the linear area of the curve to include germination reduction between 85% and 95%. After being incubated for 2 days in darkness in a growth chamber at 17°C, the dishes were removed from the plastic box. The liquid agar beneath the sporulating leaves was thoroughly mixed with a pasteur pipette and aliquots loaded on a haemocytometer for counting at 100× (Fig.2). Three counts of 100 basidiospores were made for each plate of the fungicide concentration. Basidiospores were considered to be germinated when the germ tube was longer than the diameter of the spore. Each fungicide was tested at a

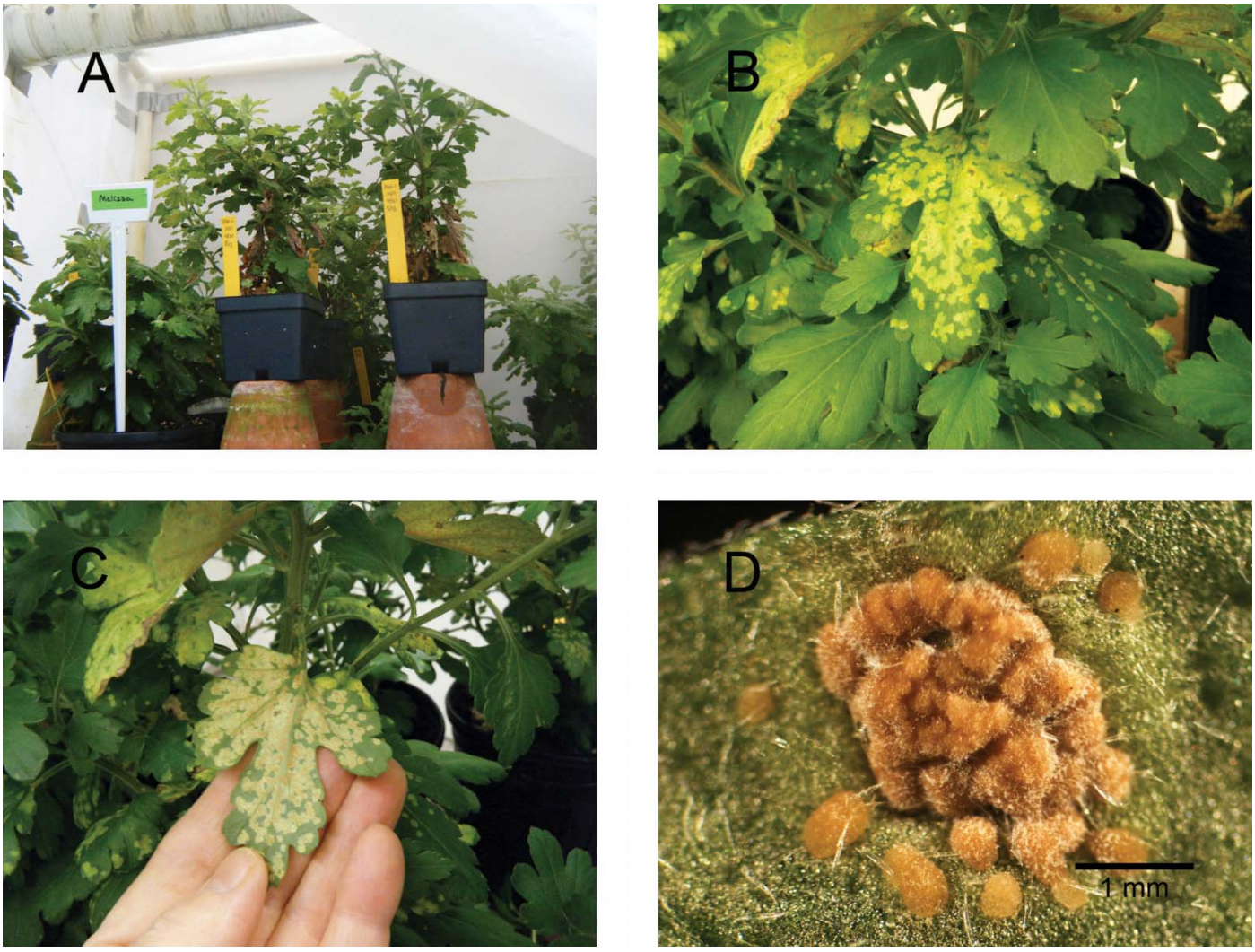

FIGURE 1

Inoculation and symptoms of Chrysanthemum white rust: (**A**) mist chamber with donor plants; (**B**) adaxial leaf surface with chlorotic spots; (**C**) abaxial leaf surface with pustule development; (**D**), 30× magnification of erupting pustule.

gradation of concentrations such that the EC_{50} for germination inhibition could be calculated from regression analyses. Fungicides evaluated for basidiospore germination are listed in Table 1.

Percent germination was normalized as a percent of germinated basidiospores in a non-amended solution (0 ppb) for each experiment. Analysis of variance was conducted using Stata 13.1 MP (StataCorp LP, College Station, TX) at $P < 0.05$. No statistical differences were observed among isolates, so pooled normalized percent germination was regressed against ppb fungicide concentration to calculate EC_{50} and EC_{85} (ppb that prevented germination to 50% or 85%, respectively, of that on non-amended media). The 95% confidence intervals were based upon confidence intervals for the slope and constant in the linear equations. Mean differences were assessed using Scheffe's method at 5% significance.

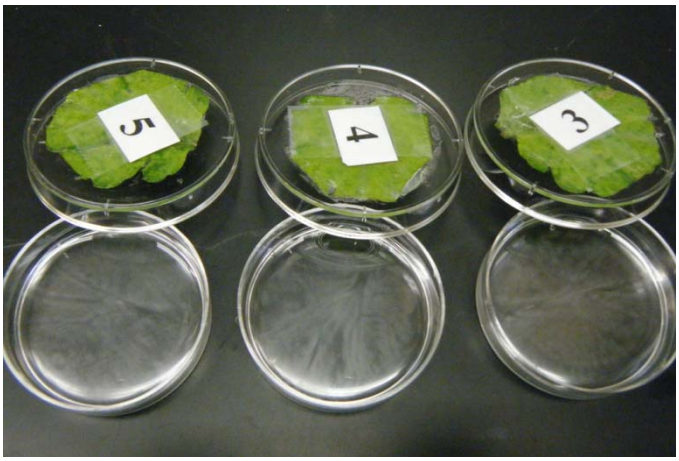

FIGURE 2

Chrysanthemum white rust basidiospores dropped from teliospores on leaves fastened to a petri plate lid after 2 days incubation at 17°C in the dark. Basidiospores were floating on and in 0.05% water agar mixed with fungicide.

In our study, *P. horiana* basidiospore germination was sensitive to fungicides from the DMI (demethylation inhibitor) (FRAC group 3), QoI (quinone-outside inhibitor) (FRAC group 11), and multi-site action (FRAC group M) treatments. Chemicals within FRAC group 11 (trifloxystrobin, fluoxastrobin, and azoxystrobin) in our study were more effective at inhibiting basidiospore germination of *P. horiana* than FRAC group 3 (Table 2). However, FRAC group 11 fungicides are believed to be at "high" risk for developing fungicide resistance (www.frac.info). Fungicides within FRAC group 3 in our study were considerably less effective, by a factor of 10^3 or greater, at preventing germination of *P. horiana* basidiospores, but they are believed to be less likely to promote fungicide resistance in a pathogen. As expected, the fungicides Armada (FRAC groups 11 and 3) and Pageant (FRAC groups 11 and 7) were very effective. FRAC group 7 and FRAC group 11 are both known to inhibit respiration in fungi. Of the multisite fungicides, Daconil (chlorothalonil) and Protect (mancozeb) were intermediate between FRAC groups 11 and 3.

DISCUSSION

Fungal plant pathogens often develop resistance to fungicides when used repeatedly within a cropping season and/or for several consecutive years. As a result, the "Fungicide Resistance Action Committee" (FRAC) was formed to develop a classification scheme with which to group fungicides according to their modes of action (www.frac.info). Information on modes of action toward specific fungal pathogens is extremely useful when developing disease management strategies. For example, knowledge of modes of action for specific fungicides allows mixtures of active ingredients with different modes of action to be used. The use of these fungicides as mixtures, or in sequence, decreases the likelihood that strains of the pathogen will be selected that are resistant to a fungicide.

TABLE 1
Fungicides evaluated for hindering *Puccinia horiana* basidiospore germination.

FRAC MOA	Active ingredients (a.i.)	Products	Label a.i. rate range (ppb)	Screened a.i. rate range (ppb)
M	chlorothalonil	Daconil Weather Stik[a]	1,243,900 – 2,487,800	140 – 2,490
M	mancozeb	Protect T/O[b]	898,400 – 1,796,700	4,500 – 89,000
3	metconazole	Tourney[c]	672,000 – 2,687,800	67 – 671,960
3	myclobutanil	Eagle 20EW[d]	126,800 – 317,000	127 – 25,360
3	propiconazole	Banner Maxx[a]	22,400 – 268,800	2,520 – 126,000
3	tebuconazole	Torque[b]	121,000 – 302,400	121 – 121,000
3	triadimefon	Strike 50WDG[e]	37,500 – 300,000	18,725 – 93,630
3	triticonazole	Trinity[f]	138,800 – 416,500	13,880 – 1,110,640
11	azoxystrobin	Heritage[a]	37,500 – 300,000	0.9 – 37
11	fluoxastrobin	Disarm 480SC[g]	90,600 – 362,500	1.8 – 73
11	mandestrobin	S2200[c]	N/A	0.3 – 344
11	trifloxystrobin	Compass[e]	74,900 – 299,600	0.4 – 7
11 + 3	trifloxystrobin + triadimefon	Armada 50WG[h]	18,700 + 93,800 – 56,200 + 281,300	0.019 + 0.094 – 18.7 + 96.4
11 + 7	pyraclostrobin + boscalid	Pageant[f]	40,000 + 78,800 – 180,000 + 354,4000	0.8 + 1.5 – 6 + 11.8

[a] Syngenta Crop Protection, Greensboro, NC.
[b] Cleary Chemical, Dayton, NJ.
[c] Valent U.S.A. Corporation, Walnut Creek, CA.
[d] Dow AgroSciences, Indianapolis, IN.
[e] OHP Corporation, Mainland, PA.
[f] BASF Corporation, Research Triangle Park, NC.
[g] Arysta, Cary, NC.
[h] Bayer Environmental Sciences, Research Triangle Park, NC.

TABLE 2
Effective fungicide concentrations required to inhibit *P. horiana* basidiospore germination.

		Basidiospore germination (ppb)	
FRAC MOA	Active ingredient	EC50 (95% CI)	EC85 (95% CI)
M	chlorothalonil	205.2 (157.9 - 264.1)	394.1 (328.2 - 476.1)
M	mancozeb	6,576.7 (5,602.0 - 7,697.5)	11,353.3 (10,067.4 - 12,831.9)
3	metconazole	28,276.0 (23,799.0 - 33,499.9)	53,535.3 (47,202.5 - 60,807.4)
3	myclobutanil	9,487.5 (7,144.8 - 12,627.2)	14,928.9 (11,895.7 - 18,994.1)
3	propiconazole	36,551.5 (31,703.9 - 42,083.4)	65,980.6 (59,321.7 - 73,580.9)
3	tebuconazole	26,150.7 (21,119.7 - 32,324.3)	39,054.4 (32,829.3 - 45,693.3)
3	triadimefon	43,549.8 (37,375.0 - 50,669.5)	59,526.5 (52,291.5 - 67,868.6)
3	triticonazole	244,190.1 (203,284.7 - 292,633.6)	461,311.7 (403,513.5 - 529,761.0)
11	azoxystrobin	2.4 (2.0 - 2.8)	3.8 (3.3 - 4.3)
11	fluoxastrobin	10.2 (8.4 - 12.4)	15.8 (13.5 - 18.6)
11	mandestrobin	27.1 (70.4 - 93.8)	44.6 (119.2 - 151.1)
11	trifloxystrobin	2.5 (2.2 - 3.0)	4.2 (3.7 - 4.8)
11+3	trifloxystrobin + triadimefon	1.0 + 5.2 (0.9 + 4.7 - 1.2 + 5.8)	1.6 + 8.3 (1.5 + 7.6 - 1.8 + 9.1)
11+7	pyraclostrobin + boscalid	1.6 + 3.2 (1.4 + 2.8 - 1.8 + 3.6)	2.6 + 5.1 (2.3 + 4.6 - 2.9 + 5.8)

It is hypothesized that within many fungal plant pathogen populations, individuals exist that are resistant, or will become resistant, to a specific fungicide. When that fungicide is applied, the susceptible types are managed, but resistant ones, initially present at very low frequencies, are selected and eventually dominate the population.

In Europe, it is apparent that resistance to a few fungicides has already developed within populations of *P. horiana*. These include both triazole (FRAC group 3) and strobilurin (FRAC group 11) fungicides (Cook 2001). As a result of the loss of effectiveness, additional active ingredients are being tested to determine their potential for controlling chrysanthemum white rust (*K. Heungens, personal communication*). However, susceptibility of *P. horiana* to currently available fungicides for managing rust diseases in the United States is not known.

The U.S. isolates of *P. horiana* were susceptible to the fungicides tested to date as assessed by impact on germination. While most fungicide EC_{85} values were well below label rate by 30% or less, propiconazole and triadimefon were within the labelled rate ranges for Banner Maxx and Strike 50WDG, respectively. The EC_{85} rate for triticonazole exceeded the Trinity maximum label rate. Our findings do not mean these fungicides will not be effective: this study examined only effects on basidiospore germination and not other portions of the life cycle potentially susceptible to fungicide disruption. It is not uncommon that fungicides with different modes of action would impact different life stages. Additional studies *in planta* are warranted along with close monitoring for resistance development in the field. The sensitivities determined in this study for specific fungicides will prove useful in this monitoring.

DISCLAIMER

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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