Wild Type Sensitivity and Mutation Analysis for Resistance Risk to Fluopicolide in *Phytophthora capsici*

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Abstract

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Crown, root, and fruit rot caused by *Phytophthora capsici* is an increasing problem for vegetable growers in Michigan and the United States. The newly registered fungicide fluopicolide is effective to limit crop loss but the potential for *P. capsici* to develop resistance is not well known. A laboratory study assessed the risk of *P. capsici* developing resistance to fluopicolide. Baseline sensitivity to fluopicolide was determined using 126 *P. capsici* Michigan isolates. Values of effective concentrations for 50% inhibition of mycelial growth ranged from 0.08 to 0.24 µg/ml and were distributed as a unimodal curve, indicating that all isolates were sensitive to fluopicolide. Mutants resistant to fluopicolide were obtained from five isolates by screening zoospores on

fluopicolide-amended (5 µg/ml) media at a mutation frequency above 1.0×10^{-7} . The mutant isolates were clustered with either intermediate (resistance factor [RF] = 3.53 to 77.91) or high (RF = 2481.40 to 7034.79) resistance. Resistance was stable through 10 mycelial transfers on fungicide-free medium. All resistant mutants showed similar fitness in zoospore production, cyst germination, and virulence compared with their sensitive parents, with few exceptions. No cross-resistance was detected between fluopicolide and five other fungicides. There could be a moderately high risk of field populations of *P. capsici* developing resistance to fluopicolide, and populations should be monitored.

Phytophthora capsici is an oomycete pathogen infecting more than 50 species of plants (13,24). Synthetic fungicides have been the growers' choice for controlling oomycete diseases in commercial production because crop rotation and other cultural strategies have provided only limited relief. The newly registered fungicide fluopicolide effectively controls oomycete plant pathogens such as *P. infestans* (3,15,25), *P. capsici* (5,14,28), *Pseudoperonospora cubensis* (6,23), and *Plasmopara viticola* (30). However, fungicide resistance among oomycetes has been frequently reported (1,2,7). Accordingly, fluopicolide may face the same risk and the risk of resistance developing in *Phytophthora capsici* needs to be assessed.

Fluopicolide is a systemic fungicide affecting *P. capsici* at all growth stages, including release and motility of zoospores, cyst germination, mycelial growth, and sporangial production (14). Fluopicolide-treated hypha or zoospores of *P. infestans* showed a fast perturbation of a spectrin-like protein (31) but the specific target protein of fluopicolide remains unclear. Based on the mode of action that affects mitosis and cell division, fluopicolide is classified by the Fungicide Resistance Action Committee into group B, which contains benzimidazoles, zoxamide, and several other chemicals (1). However, these chemicals in group B are subgrouped based on their antimicrobial spectrums and other characteristics. Benzimidazoles are broad-spectrum fungicides targeting true fungi (29) and there have been many documented instances of pathogens developing resistance to them (1,20,29). Zoxamide is mainly effective on oomycete pathogens (4), although some true fungi can be inhibited at high doses (32). There have been no reports of resistance to zoxamide with low to intermediate risk (1,33).

Because the registration of fluopicolide in the United States is relatively new, there are no data available on the potential of development of resistance in *P. capsici* or any other oomycete pathogens, although some antiresistance strategies are recommended for *P. infestans* (18). Research on cross resistance in oomycetes to fluopicolide and other fungicides would be invaluable in developing effective and long-sustaining management programs.

Monitoring and managing the development of fungicide resistance in the field requires establishing a baseline sensitivity of the pathogen population (2,26), which requires a large number of *P. capsici* isolates for accuracy (18,19). At present, few reports have described *P. capsici* sensitivity to fluopicolide using a limited number of isolates (14,16). The objectives of this study were to (i) determine baseline sensitivity of *P. capsici* to fluopicolide; (ii) select resistant mutants under laboratory conditions; (iii) assess the risk of fluopicolide resistance by examining resistance level, stability, and fitness components of resistant mutants; and (iv) examine cross-resistance between fluopicolide and several oomycete fungicides.

Materials and Methods

Pathogen isolates and culture conditions. *P. capsici* isolates were all obtained from diseased cucurbitaceous and solanaceous crops in Michigan, except isolate NM13, which was obtained from China and included for comparison. The baseline sensitivity study included isolates SP98 (A2 mating type [MT]), 12889 (A1 MT), and SFF3 (A2 MT) (24), and 123 isolates collected in 2010. For the fluopicolide resistance study, isolates SP98, 12899, SFF3, NM13, MI2, and MI3 were used. MI2 and MI3 were isolated from spaghetti squash (*Cucurbita pepo*) in 2009. Isolates were transferred using a single-zoospore method and MT was determined for all isolates as described by Lamour and Hausbeck (17).

The following protocols were used throughout these studies for in vitro testing, unless otherwise described. Isolates were cultured on potato dextrose agar media (PDA; EMD Chemicals Inc., Gibbstown, NJ) in petri dishes at 28°C in the dark for mycelial growth, and V8 juice agar (21) under constant fluorescent light at room temperature ($22 \pm 1^\circ\text{C}$) for sporangial production. Zoospore sus-

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pensions were prepared by flooding actively sporulating cultures with sterile distilled water and incubating at 4°C for 30 min, followed by 30 min of incubation at room temperature (19). Zoospore concentration was measured and adjusted using a hemocytometer.

Baseline sensitivity of *P. capsici* to fluopicolide. Baseline sensitivity of 126 *P. capsici* isolates (Table 1) was determined based on inhibition of mycelial growth on fluopicolide-amended PDA media. A formulated fluopicolide (Presidio, 39.5% active ingredient [a.i.]; Valent BioScience Co., Libertyville, IL) was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (1×10^4 µg/ml). Fungicide-amended agar plates were prepared by adding various volumes of fluopicolide stock solutions to PDA medium at 50°C before pouring. Final concentrations of fluopicolide were adjusted to 0, 0.08, 0.10, 0.13, 0.15, 0.20, 0.25, and 0.30 µg/ml, and final DMSO concentration was adjusted to 0.1% in PDA. Mycelial plugs (4 mm in diameter) were cut from the edge of actively growing colonies with a cork borer and placed mycelia-side down on amended agar, three plates per concentration. After 4 days of incubation, colony diameters were measured in two perpendicular directions with the original mycelial plug diameter subtracted from this measurement. The averaged values of the two measurements were used for analysis. The experiment was performed two times independently.

Selection of fluopicolide-resistant isolates. Four single-zoospore isolates (SP98, 12889, SFF3, and NM13) and two bulk isolates (MI2 and MI3) of *P. capsici* were used to select fluopicolide-resistant isolates. Zoospore suspensions of *P. capsici* isolates were made as described above. Zoospore concentrations were measured and adjusted to 5×10^6 zoospores/ml, and then 20 µl of the suspension was spread on PDA agar amended with fluopicolide at 5 µg/ml (PDAF) in a 10-cm petri dish. This concentration was approximately 10 times the minimal concentration that completely prohibited growth of sensitive (wild-type) isolates. PDA agar amended with 0.1% DMSO was used as a control and plated with 20 µl of the suspension at 5×10^4 zoospores/ml. After incubation at 25°C in the dark for 5 days, colonies that survived on PDAF (diameters > 10 mm) were considered to be resistant to fluopicolide and the number of these colonies was counted for each plate. Resistant colonies were transferred to new PDA plates for later tests. Mutation frequency was calculated as a ratio of resistant colonies over total number of germinated cysts on control plates.

Characterization of fluopicolide-resistant isolates. *Resistance factor and stability.* Sensitivity of all *P. capsici* isolates resistant to fluopicolide was determined by measuring mycelial growth on PDA plates amended with various concentrations of fluopicolide, as described above. Two series of fluopicolide concentrations (0, 1, 2, 4, 6, 8, and 10 µg/ml and 100, 200, 300, 400, 500, and 600 µg/ml) were used for isolates with low and high values, respectively, for effective concentrations for 50% inhibition of mycelial

growth (EC₅₀). A resistance factor (RF) was calculated as the ratio of EC₅₀ values of a fungicide-resistant isolate relative to its parental isolate. After 10 successive transfers on fungicide-free PDA plates using five sensitive parental isolates and five randomly chosen corresponding resistant progeny isolates, including SP98 versus R8, 12889 versus R24, MI2 versus R56, and NM13 versus R78, the stability of resistant phenotypes was assessed by comparing RFs. The factor of sensitivity change (FSC) to fluopicolide was calculated as the ratio of EC₅₀ values (the isolates from the 10th/1st transfer). The experiment was conducted twice. The five pairs of wild sensitive isolates and their fluopicolide-resistant derivatives were also used in fitness tests, including the effect of temperature, zoospore production and cyst germination in vitro, and virulence in zucchini fruit and pepper seedlings.

Effect of temperature on mycelial growth. Five resistant isolates and their parents (as described above) were incubated on PDA media at 10, 15, 20, 25, and 35°C. Colony diameters were measured after 7 days of incubation in the dark, and two measurements were averaged to represent mycelial growth. There were four replicated plates for each treatment and the experiment was conducted twice.

Zoospore production and cyst germination in vitro. Zoospore production was assayed (19) by excising 10 plugs (4 mm in diameter) from the culture edge and 10 plugs from the area near the initial inoculum plug using a cork borer, and placing all 20 culture plugs in a 15-ml centrifuge tube containing 10 ml of sterile distilled water to produce zoospores as described above. The concentration of zoospores was determined with a hemocytometer. Cystospore germination was measured on 2% water agar medium under a microscope after incubation of zoospore suspensions for 12 h at 25°C in the dark. A germinated cystospore was confirmed if the length of germ tube was longer than the cyst diameter. For each isolate, 100 cysts were examined and two independent experiments were performed.

Virulence on zucchini fruit and pepper seedlings. Virulence of *P. capsici* isolates was determined by inoculation assays on detached zucchini (*C. pepo* 'Black Beauty') fruit and pepper (*Capsicum annuum* 'Sweet Banana') seedlings. Zucchini fruit were inoculated using the method previously described by Granke et al. (12), with slight modifications. Fruit were washed with tap water, disinfested with 10% bleach (0.62% NaClO) for 5 min, rinsed with sterile water, dried at room temperature, and placed in a clear plastic box with moist paper towels placed at the bottom to maintain high relative humidity. Three small superficial wounds were made equidistant with a sterile needle for each fruit. Agar plugs (4 mm in diameter) cut from the margin of actively growing colonies on PDA plates were placed mycelia-side down on the wound. Zucchini were inoculated using PDA plugs (no *P. capsici* culture) as controls. The inoculated zucchini fruit were incubated at room temperature ($22 \pm 1^\circ\text{C}$) for 3 days (16-h photoperiod). The lesion

Table 1. *Phytophthora capsici* isolates collected in Michigan for study of baseline sensitivity to fluopicolide

Location	Host (species)	Number of isolates		
		Mating type A1	Mating type A2	Total
Decatur	Cucumber (<i>Cucumis sativus</i>)	4	1	5
Decatur	Pepper (<i>Capsicum annuum</i>)	2	0	2
Decatur	Squash (<i>Cucurbita pepo</i>)	3	7	10
Decatur	Squash	4	4	8
South Haven	Pepper	15	6	21
Lawrence	Pepper	8	17	25
Newaygo County	Squash	0	1	1
Cass County	Cucumber	3	1	4
Benton	Pepper	6	5	11
Benton	Squash	13	7	20
Hartford	Pepper	6	8	14
Unknown	Squash	2	0	2
Unknown	Pumpkin (<i>Cucurbita maxima</i>)	1	0	1
Unknown	Cucumber	0	1	1
Unknown	Pepper	1	0	1
Total	...	68	58	126

area that developed on zucchini fruit was estimated by measuring the diameter of water-soaked lesions and mycelial colonies perpendicularly.

Pepper seedling inoculation and disease scoring were performed with the method described by Glosier et al. (10), with minor modifications. Five seedlings were grown in each of four pots (10 by 10 cm² on the top). At the six-true-leaf stage, 10 ml of a *P. capsici* zoospore suspension (2×10^4 zoospores/ml) was applied to the soil near the stem for one pot, with three replicated pots for each treatment. Inoculated plants were incubated in a growth chamber (temperature = 23°C, humidity = 65 ± 5%, and photoperiod = 16 h of light). Seven days post inoculation, disease severity for all plants was rated on a scale of 0 to 5 (0 = healthy plant, 1 = leaf yellowing and no stem necrosis, 2 = minor stem necrosis, 3 = moderate stem necrosis and some wilting, 4 = severe stem necrosis and severe wilting, and 5 = dead plant).

To confirm the resistance of the mutant, the zucchini fruit were kept in a fluopicolide solution for 5 min at concentrations of 50, 100, 200, or 500 µg/ml, followed by inoculation with agar plugs of the wild-type isolates (SP98, 12889, MI3 and NM13) and intermediately (R40 and R78) and highly (R8 and R72) resistant mutants on the fruit, as described above. Disease lesions with water soaking or mycelial growth were observed on zucchini fruit after 3 days of incubation.

Cross-resistance to other oomycete fungicides. Six sensitive, six intermediately resistant, and six highly resistant isolates of *P. capsici* were tested for sensitivity to five oomycete fungicides representing different modes of action, using the mycelia growth inhibition method described above. All formulated fungicides were

dissolved in DMSO as described earlier and added to PDA to yield the following final concentrations: cyazofamid (RAMAN, 34.5% a.i.; FMC Corporation, Philadelphia) at 0, 0.01, 0.05, 0.1, 0.5 and 1 µg/ml; mandipropamid (Revus, 23.4% a.i.; Syngenta Crop Protection, Inc. Greensboro, NC) at 0, 0.008, 0.01, 0.012, 0.014, 0.016, and 0.018 µg/ml; mefenoxam (Ridomil Gold, 47.6% a.i.; Novartis Crop Protection, Inc. Greensboro, NC) at 0, 0.005, 0.1, 0.3, 0.6, 1, and 5 µg/ml; zoxamide (Zoxium, 80% a.i.; Gowan Co., Yuma, AZ) at 0, 0.004, 0.008, 0.01, 0.02, 0.04 and 0.06 µg/ml; and azoxystrobin (Heritage, 50% a.i.; Syngenta Crop Protection) at 0, 1, 2, 3, 4, 5, and 6 µg/ml plus a technical grade of salicylhydroxamic acid (99%, Sigma-Aldrich, St. Louis) at 100 µg/ml.

Statistical analysis. Data were analyzed with SAS software (version 9.2; SAS Institute Inc. Cary, NC). EC₅₀ values were calculated using regression analysis (using procedure REG) of percent growth inhibition against the logarithmic values of fungicide concentrations. Procedure GLM was used for an analysis of variance, and Student's *t* test was used for comparisons of means. For cross-resistance tests, EC₅₀ values were log transformed, and then Spearman rank correlation was used to analyze cross-resistance relationships between fluopicolide and each of the other five fungicides with logarithmic transformation of EC₅₀ values.

Results

Baseline sensitivity of *P. capsici* isolates to fluopicolide.

Among the 126 Michigan isolates of *P. capsici*, there were 68 isolates with A1 MT and 58 isolates with A2 MT (Table 1) and no significant differences for EC₅₀ values between two MTs based on the *t* test ($\alpha = 0.05$). EC₅₀ values of the population ranged from 0.08 to 0.24 µg/ml, with a mean of 0.16 µg/ml, and were distributed as a unimodal curve (Fig. 1), suggesting that all wild-type isolates were sensitive to fluopicolide (EC₅₀ ≤ 0.24 µg/ml).

Selection of fluopicolide-resistant mutants of *P. capsici*.

Fluopicolide-resistant mutants were obtained from five of six wild-type isolates regardless of MTs and the geographical source (Table 2). Resistant mutants were derived at frequencies of the same order of magnitude (approximately one resistant colony per 1×10^7 zoospores tested) for single-zoospore and bulk-culture isolates (Table 2).

Characterization of fluopicolide-resistant mutants of *P. capsici*.

RF and stability. Among the fluopicolide-resistant mutants, RFs ranged from 3.53 to 7034.79. The resistance to fluopicolide was considered as intermediately resistant if RF was < 100 and highly resistant if RF was ≥ 100. For isolates with intermediate resistance, RFs ranged from 3.53 to 77.91, corresponding to EC₅₀ values from 0.38 to 9.12 µg/ml (Fig. 2). For highly resistant isolates, RFs ranged from 2481.40 to 7034.79, corresponding to EC₅₀ values from 265.5 to 752.7 µg/ml, respectively (Fig. 2). No mutants fell within the range of 78 to 2,480 RFs, which separated the intermediately and highly resistant groups of mutants distinctively (Fig. 2). After 10 transfers on fungicide-free PDA media, EC₅₀ values of all intermediately resistant isolates increased; for the highly resistant isolates, EC₅₀ values decreased but RFs were all > 1,000 (Table 3).

Effect of temperature on mycelial growth. There was no discernable difference between growth of fluopicolide-resistant mutants

Fig. 1. Distribution of fluopicolide sensitivity, described as effective concentration for 50% inhibition of mycelial growth (EC₅₀) of 126 *Phytophthora capsici* isolates, collected from Michigan vegetable crops in areas without a history of fluopicolide application.

Table 2. *Phytophthora capsici* wild-type isolates and their mutant derivatives resistant to fluopicolide^a

Wild-type isolates	Tested zoospores ($\times 10^7$)	Germination rate (%)	Derived mutants		Total	Mutation rate ($\times 10^{-7}$)
			Intermediately resistant	Highly resistant		
SP98	21.30	70.86	11	9	20	1.32
12889	9.50	83.33	27	8	35	4.42
MI2	13.40	56.79	4	4	8	1.05
MI3	8.33	55.25	4	7	11	2.39
NM13	5.13	79.89	2	3	5	1.22
SFF3	4.40	83.64	—	—	0	—

^a Resistance level to fluopicolide was determined based on resistance factor (RF), which was calculated as the ratio of values of effective concentration for 50% inhibition (EC₅₀) of an isolate relative to its parent. Resistance of *P. capsici* isolates was grouped into intermediate (RF < 100), or high (RF ≥ 100) resistance; — = data not available.

and their parents (Table 4). At 10 temperature points, the parents grew faster than the mutant derivatives but, at 9 temperature points, resistant mutants grew faster than their parents ($\alpha = 0.05$). At the other temperatures, there were no significant differences in mycelial growth between the parents and derived mutants of *P. capsici*

Fig. 2. Distribution of fluopicolide sensitivity, measured as effective concentration values for 50% inhibition of mycelial growth (EC_{50}) of wild-type and mutant isolates of *Phytophthora capsici*. Isolates are grouped by fluopicolide sensitivity, separated by broken horizontal lines: sensitive (S), intermediately resistant (IR), and highly resistant (R). S isolates were all wild-type isolates collected from Michigan (left side of the arrow); IR and R isolates were derived from five of S isolates (right side of the arrow).

(Table 4). For example, resistant mutant R8 grew more slowly than its parent SP98 at 10 and 25°C, R24 grew more slowly than its parent 12889 at 30 and 35°C, R56 grew more slowly than its parent MI2 only at 15°C, and R70 grew significantly more slowly than its parent MI3 only at 15°C. However, R78 grew faster than parental isolate NM13 at 15°C. In general, the growth of most resistant mutants did not differ from that of their parental isolates at tested temperatures (Table 4).

Zoospore production and cyst germination in vitro. There were no differences in zoospore production between resistant mutants and their corresponding sensitive parental isolates, except for isolate R8 (resistant), which produced significantly more zoospores than SP98 (wild-type parent) (Table 5). Three of five resistant mutants had higher cyst germination rates than their corresponding wild-type parental isolate, R78 had a lower germination rate than its parent (NM13), and R24 was similar to its parent (12889) (Table 5).

Virulence on zucchini fruit and pepper seedlings. There was no difference in virulence (lesion and mycelial colony diameters) of all resistant mutants compared with their corresponding parents when tested on zucchini fruit. Disease severity of the mutants tested was not different compared with sensitive parental isolates on pepper seedlings, with the exceptions of R70 versus MI3, in which the mutant R70 had statistically lower disease severity than sensitive parent MI3 (Table 5). Three days after inoculation with R8, a mutant with high RF, the fruit treated in fluopicolide solution showed the same size of symptom compared with the nontreated control, indicating that the infection of these isolates tested was not affected by application of fluopicolide but, when R78, a mutant with lower RF, was used for inoculation, the infection was partially affected (Fig. 3). Results were similar for R40 and R72, respectively (*data not shown*). The infection by all sensitive isolates tested was completely inhibited with fluopicolide at 100 µg/ml (Fig. 3).

Table 3. Resistance stability of fluopicolide-resistant mutants of *Phytophthora capsici*^a

WT isolate ^d	EC_{50} (µg/ml) ^b		Mutants	EC_{50} (µg/ml)		RF ^c		FSC ^e
	T1	T10		T1	T10	T1	T10	
SP98	0.16	0.20	R8	612.82	213.67	3,920.78	1,042.80	0.35
12889	0.11	0.11	R24	2.65	4.73	24.74	42.76	1.79
MI2	0.12	0.12	R56	7.05	7.76	60.25	64.59	1.10
MI3	0.11	0.12	R70	580.72	430.89	5,175.78	3,575.86	0.74
NM13	0.14	0.14	R78	2.23	2.30	15.42	16.05	1.03

^a T1 and T10 = the first and tenth transfers of culture on PDA media.

^b EC_{50} = effective concentration of fluopicolide for 50% inhibition of mycelial growth in *P. capsici*.

^c RF = resistance factor, a ratio of EC_{50} values for an isolate (mutant) relative to the parental isolate.

^d WT = wild type.

^e FSC = factor of sensitivity change for fluopicolide mutants only, a ratio of EC_{50} values of the 1st/10th transferred culture.

Table 4. Effect of temperature on mycelial growth (colony diameter in millimeter) of *Phytophthora capsici* wild-type and fluopicolide-resistant mutants on potato dextrose agar plates

Isolate	Sensitivity ^b	Colony diameter (mm) at each temperature ^a					
		10°C	15°C	20°C	25°C	30°C	35°C
SP98	S ₁	12.1*	19.3	52.1	67.9*	63.6	44.6
R8	R ₁	9.9	33.1*	53.4	63.1	66.6*	47.0
12889	S ₂	5.1	33.3	53.4	62.1	72.3*	47.3
R24	IR ₂	15.4*	33.8	54.3	61.3	65.1	42.8
MI2	S ₃	16.9*	20.9*	53.6	65.4	75.5	57.3
R56	IR ₃	15.4	19.3	52.1	67.9*	76.4	57.0
MI3	S ₄	16.6	27.1*	50.8	63.6	66.1	52.3
R70	R ₄	15.0	21.3	53.6*	68.0*	75.3*	61.0*
NM13	S ₅	16.6	21.3	53.4*	67.3*	79.0*	62.0*
R78	IR ₅	17.4	27.4*	50.3	61.1	66.5	50.3

^a Colony diameter was measured 7 days post inoculation, except at 10°C, when measurement was done 30 days post inoculation due to its slow growth. Student *t* test was performed for each pair (group) of wild-type parent (S) and its derived mutant of *P. capsici* at significance level $\alpha = 0.05$. Symbol “*” indicates a significantly higher value.

^b Sensitivity to fluopicolide was classified as sensitive (S), intermediately resistant (IR, with resistant factor [RF] < 100), and resistant (R, with RF ≥ 100) groups. Subscripted numbers indicate groups of parental and derived mutant isolates (IR or R).

Cross-resistance to other oomycete fungicides in *P. capsici*. In rank correlation analysis for cross-resistance, all *P* values were higher than 0.05, suggesting that there was no correlation between fluopicolide and any of the other five fungicides tested (azoxystrobin, mandipropamid, cyazofamid, zoxamide, and mefenoxam). Therefore, there was no evidence of cross-resistance between fluopicolide and any of the five tested fungicides (Fig. 4).

Discussion

A baseline of fluopicolide sensitivity in *P. capsici* was established using 126 field isolates from Michigan; this information can be used in continued field monitoring of fungicide resistance. The unimodal distribution at low levels of EC₅₀ values demonstrated that fluopicolide resistance has not developed in the field. The population of *P. capsici* isolates used in this study confirmed a similar result of EC₅₀ values of isolates obtained from the southeastern United States (14,16). However, the large number of isolates used in the present study makes it possible to establish a full spectrum of sensitivity distribution with high confidence.

This study is the first to report fluopicolide resistance of *P. capsici* using resistant mutants. The technique of generating mutants by screening zoospores on fungicide-amended media is representative of what could happen naturally in the field. The frequency of resistance to fluopicolide (10⁻⁷) is much higher than that of iprovalicarb (10⁻¹²), an oomycete fungicide belonging to carboxylic acid amides (CAA) (19). Resistant mutants were obtained from both single-spore isolates and bulk isolates in similar frequency, with the exception of isolate SFF3, suggesting that both kinds of isolates are equally effective at generating resistant mutants. It is not clear why resistant mutants were not obtained from SFF3.

In this study, *P. capsici* mutants resistant to fluopicolide retain the resistance persistently without fluopicolide selection pressure, indicating that the resistance is not acquired adaptively but is a result of gene expression, although inheritance of fluopicolide resistance needs to be further studied. Fungicide resistance can be classified into monogenic and polygenic resistance, based on the number of genes involved (8). A field population of a pathogen with monogenic resistance exhibits several distinct subpopulations, and the resistant subpopulation usually has high RF values (>100) and a high inherent risk of developing fungicide resistance, such as to benzimidazoles (20) and phenylamide (7,27). In contrast, a population with polygenic resistance usually shows a continuous distribution of fungicide sensitivity and lower RF values, as evidenced by resistance to demethylation inhibitors (7), and is not likely to result in sudden failures of fungicide efficacy. In the present study, the generated mutants were clustered in two distinctive groups based on RF values. The population of resistant mutants has high RF values (>1,000), suggesting that fluopicolide resistance is controlled monogenically and, therefore, may have a high inherent risk.

Due to the diploid nature of oomycetes, the level of resistance risk is highly affected by the type of inheritance (dominant, semi-dominant, or recessive) when fungicide resistance is controlled monogenically. Phenylamide resistance is controlled by a semi-dominant nuclear gene (27), resulting in a high resistance frequency to metalaxyl (10⁻⁶ under laboratory conditions; 33) and disease control failure with many pathogens (11,13,22). In contrast, it is difficult to develop a resistant mutant population when fungicide resistance is controlled by a single recessive gene (9), such as with CAA fungicides. Isolates resistant to CAAs in the field have been detected only for *Plasmopara viticola* (9) and *Pseudoperonospora cubensis* (34). In this study, both spontaneously intermediate and high resistances were observed, suggesting that fluopicolide resistance may be controlled by semidominant genes and, therefore, *Phytophthora capsici* isolates have a higher possibility to develop resistance in the field. For this situation, sexual recombination is not an issue because dominant or semidominant genes are always expressed.

Fitness of resistant isolates is also an important factor when evaluating the risk of fungicide resistance. Resistant mutants of *P. capsici* showed strong adaptation ability at different stages in the life cycle, including mycelial growth, sporulation, germination, and, more importantly, virulence. This adaptability ensures that the mutants will survive and infect plants successfully with competitive ability. Fluopicolide resistance of these mutants was confirmed by infection of inoculated zucchini regardless of the rate of fluopicolide applied to the fruit, using several isolates. Thus, a fluopi-

Fig. 3. Effect of fluopicolide on *Phytophthora capsici* isolates SP98, NM13 (wild type, fluopicolide-sensitive), R78 (intermediately resistant to fluopicolide), and R8 (highly resistant to fluopicolide). Zucchini fruit were treated in sterile distilled water (non-treated), or fluopicolide solution (100 µg/ml), followed by inoculation with a mycelial plug of each isolate as indicated by arrows.

Table 5. Sporulation, germination, and virulence of fluopicolide-resistant mutants and the corresponding parental isolates of *Phytophthora capsici*^a

Group ^c	Isolate	Sporulation (×10 ⁵ zoospore/ml)	Rate (%) ^d	Virulence (diameter, mm) ^b		
				Lesion	Mycelia	Disease severity ^e
I	SP98	3.81	70.9	38.3	31.8	2.9
I	R8	4.45*	82.4*	39.8	32.3	3.3
II	12889	3.61	83.3	38.7	27.9	3.0
II	R24	3.89	81.8	36.9	27.4	3.1
III	MI2	3.86	56.8	37.1	26.7	3.6
III	R56	3.55	84.2*	33.7	22.3	4.0
IV	MI3	5.53	55.3	32.3	17.9	3.6*
IV	R70	6.61	82.1*	33.4	19.1	2.8
V	NM13	7.85	79.9*	39.7	30.8	3.8
V	R78	8.15	70.9	35.9	27.5	3.5

^a Student *t* test was performed for each pair (group) of mutant (plain text) and parent (bold text) of *P. capsici* at significance level $\alpha = 0.05$; * indicates a significantly higher value.

^b Virulence on zucchini fruit was rated by measuring the lesion size (diameter) and pathogen growth (diameter of mycelial colony).

^c Each group contains a parental (wild type) and derived mutant resistant to fluopicolide.

^d Cyst germination rate.

^e Disease severity in pepper seedlings was evaluated on a scale of 0 to 5 (0 = healthy plant, 1 = leaf yellowing and no stem necrosis, 2 = minor stem necrosis, 3 = moderate stem necrosis and some wilting, 4 = severe stem necrosis and severe wilting, and 5 = dead plant).

colide-resistant subpopulation could establish, reproduce, and dominate once it appears in the field following a certain selection pressure of fluopicolide.

High fungicidal activity, monogenic type of resistance, and high fitness of resistant mutants indicate a high risk of inherent resistance of fluopicolide in *P. capsici*. To reduce the selection pressure, limiting the dose and numbers of applications for each season and applying fluopicolide in a preventive manner will be helpful. Because no cross-resistance has been found in the present study or in a previous report (31), application of fluopicolide alternated and mixed with other oomycete fungicides will help to reduce the resistance risk and corresponding economic losses.

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Fig. 4. Rank correlation analysis for cross resistance between fluopicolide and other oomycete fungicides in *Phytophthora capsici* isolates. EC_{50} = effective concentration of fluopicolide for 50% inhibition of mycelial growth of *Phytophthora capsici*. Values on x and y axes were logarithmic transformation of EC_{50} values. Correlation between fluopicolide and A, azoxystrobin; B, mandipropamid; C, cyazofamid; D, zoxamide; and E, mefenoxam were conducted.

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