Evaluation of the Risk of Development of Fluopicolide Resistance in *Phytophthora erythroseptica*

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Abstract

Fluopicolide has shown effective pink rot (*Phytophthora erythroseptica*) control in potato disease management. To efficiently utilize this chemical, the risk of fluopicolide resistance in *P. erythroseptica* needs to be assessed. In this study, 34 isolates of *P. erythroseptica* were obtained from symptomatic potato tubers with pink rot in Maine. The sensitivity of these wild-type isolates to fluopicolide was assessed by culturing them on agar medium amended with fluopicolide at various concentrations. The 50% effective concentration (EC₅₀) of fluopicolide for the inhibition of mycelial growth was determined and used to establish a baseline sensitivity of these *P. erythroseptica* isolates to fluopicolide. The wild-type isolates were sensitive to fluopicolide, with EC₅₀ values ranging from

0.08 to 0.35 µg/ml. By exposing *P. erythroseptica* zoospores to agar medium containing 100 µg/ml fluopicolide, 6 out of the 34 wild-type isolates produced fluopicolide-resistant mutants. The mutants were transferred to fungicide-free V8 medium consecutively for 10 times, and the 10th transfer of mutants was examined for resistance stability and biological fitness. In general, the mutants had similar or slower growth rates compared with their wild-type parents, and the virulence of some mutants was significantly reduced. The results indicated a low to moderate risk of *P. erythroseptica* developing resistance to fluopicolide, and suggested a trade-off between fluopicolide resistance and biological fitness in *P. erythroseptica*.

Pink rot (*Phytophthora erythroseptica*) is an economically important disease of potato (Fiers et al. 2012; Pethybridge 1913; Porter et al. 2007; Taylor et al. 2011) causing significant tuber losses in the field and storage (Al-Mughrabi et al. 2007; Mills et al. 2005; Schisler et al. 2009). Pink rot has been managed chemically (Benson 2008), and it used to be managed effectively with the systemic chemical mefenoxam (Wicks et al. 2000). However, the development of mefenoxam-insensitive populations of *P. erythroseptica* has reduced the efficacy of mefenoxam (Goodwin and McGrath 1995; Lambert and Salas 1994; Taylor et al. 2002). As long as chemical products are used, fungicide resistance is always a concern.

Fluopicolide has been proved to be effective for pink rot control (Zhang et al. 2016), as well as a wide range of oomycete pathogens (Jackson et al. 2010; Lu et al. 2011; Rekanovic et al. 2008). The generic name of fluopicolide is 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl] benzamide (C₁₄H₈Cl₃F₃N₂O). Like other benzamides, fluopicolide acts on cytoskeleton and motor proteins, but it is categorized in B5 group on FRAC (Fungicide Resistance Action Committee) code list with a unique mode of action, delocalizing spectrin-like proteins (FRAC 2018; Toquin et al. 2008). This mode of action differs from other oomycetocides in that it can inhibit isolates that are resistant to phenylamides, strobilurin, dimethomorph, and iprovalicarb (Toquin et al. 2012).

To study the risk of resistance in oomycetes, mutants resistant to fluopicolide have been generated under laboratory conditions. For example, fluopicolide-resistant mutants of *Phytophthora capsici* were selected from zoospores of wild-type isolates on fluopicolide-amended agar media (Lu et al. 2011), and in *Pseudoperonospora cubensis* induced by UV radiation (Wang et al. 2014). However,

the risk of fluopicolide resistance in *P. erythroseptica* remains unknown.

A risk assessment of the likely development of resistance is based on many biological characteristics, such as frequency of mutation, fitness, and virulence. A trade-off between resistance development and biological fitness may occur in plants, bacteria, and fungi (Brown and Rant 2013; Kang and Park 2010; Montarry et al. 2007). Kang and Park (2010) showed that there was a fitness cost to gain antibiotic resistance in bacteria *Acinetobacter* sp. Fluopicolide-resistant *Pseudoperonospora cubensis* mutants showed differences with their wild-type parents in latent period, infection frequency, lesion extension, and sporulation (Wang et al. 2014). However, in most cases, there was no difference between most fluopicolide-resistant *Phytophthora capsici* mutants and their wild-type parents in zoospore production, cyst germination, or virulence (Lu et al. 2011).

The present study aimed to determine the risks of *P. erythroseptica* isolates in developing resistance to fluopicolide and to assess the biological fitness of the resistant mutants. The objectives of this study were to: 1) investigate the baseline sensitivity of wild-type *P. erythroseptica* population to fluopicolide, 2) examine the biology and fitness of fluopicolide-resistant isolates, and 3) test the pathogenicity and virulence of the mutants.

Materials and Methods

Cultures and chemicals. All the experiments were conducted at the University of Maine, Orono. A total of 34 isolates of *P. erythroseptica* were isolated from potato tubers showing pink rot symptoms. The samples were obtained from Maine potato fields (Aroostook County, ME), where there was no history of fluopicolide application. Tuber pieces with partial healthy tissue and rotten lesions were cut (around 1 cm in length) and surface-sterilized in 75% ethanol for 30 s, and then soaked in 0.6% sodium hypochlorite for 5 min, followed by rinsing three times in sterile distilled water. The disinfested tuber pieces were air-dried on sterile filter paper and then placed on a 1.5% water agar plate. The resultant cultures were purified through the single-spore method (Choi et al. 1999). Isolates 1 to 15 were collected prior to 2013 by Dr. David Lambert at the University of Maine. Isolates 16 and 17 were collected in 2014 and isolates 18 to 34 were collected in 2016. All isolates were originally from Maine. The taxonomy of the isolates was confirmed by analysis of DNA sequences on the internal transcription spacer, which was amplified with polymerase chain reaction (PCR) with primers ITS1 and ITS4. Thermal cycler settings were as follows: 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and ended

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with a 7-min extension at 72°C (Villa et al. 2006; White et al. 1990). The sequences of the isolates were analyzed in NCBI database using BLAST algorithm, and confirmed as *P. erythroseptica*, with both coverage and similarity being above 98%. The sensitivity of all *P. erythroseptica* isolates to mefenoxam was determined prior to this study. Among the collected wild-type *P. erythroseptica*, 13 isolates were mefenoxam resistant ($EC_{50} > 10 \mu\text{g/ml}$), including 13A05, 13A07, and 13A39. Five isolates were not determined on mefenoxam sensitivity ($EC_{50} < 10 \mu\text{g/ml}$). The rest (16 isolates) were mefenoxam sensitive, including 13A02, 13A03, and 13A14.

Technical grade of fluopicolide (99.1% active ingredient) was provided by Valent U.S.A. Corporation (Walnut Creek, USA), and mefenoxam was provided by Syngenta (Greensboro, NC). The chemicals were dissolved in dimethyl sulfoxide (DMSO) and adjusted to a stock concentration (10,000 $\mu\text{g/ml}$) and working concentration (1,000 $\mu\text{g/ml}$) for later use.

Sensitivity of *P. erythroseptica* to fluopicolide. The sensitivity of *P. erythroseptica* to fluopicolide was conducted using Autoplate 4000 (Spiral Biotech Inc., Norwood, MA, USA), following the spiral plating method by Förster et al. (2004) with modifications. Briefly, *P. erythroseptica* was incubated on the center of a clarified V8 (200 ml clarified V8 juice, 2.5 g CaCO_3 , 1.5% agar, 800 ml distilled water) plate. Prior to this, cellophane strips (0.5 × 3 cm) were autoclaved in distilled water for 15 min at 121°C, and were then placed on the V8 plate side by side. The cultures were incubated at 25°C in an incubator until all the cellophane strips were covered by mycelia.

To prepare fungicide-amended agar plates, 50 ml potato dextrose agar (PDA) was poured into a 15-mm Petri dish. A 50- μl aliquot of fluopicolide (1,000 $\mu\text{g/ml}$) was distributed to each PDA plate using the autoplate with the exponential mode (Förster et al. 2004). The plates were left in a sterile hood for 30 min to ensure fungicide absorbance. The mycelia-colonized cellophane strips were moved from the V8 plate and placed on the fluopicolide-amended PDA plates, which were arranged radially (Förster et al. 2004; Torres-Londoño et al. 2016). Two replicated strips from each isolate were arranged diagonally on the plate, and strips of 8 isolates were placed on one PDA plate. Two or three days later, the minimum inhibition concentration (MIC, defined as the chemical concentration at which the pathogen growth is significantly reduced) and total inhibition concentration (TIC, defined as the chemical concentration at which pathogen growth is completely suppressed) were recorded (Förster et al. 2004; Torres-Londoño et al. 2016). The ECX package was used to calculate the EC_{50} values (Torres-Londoño et al. 2016). Baseline

sensitivity of *P. erythroseptica* to fluopicolide was determined based on the sensitivities of all *P. erythroseptica* isolates in this study.

Selection of fluopicolide-resistant *P. erythroseptica*. Zoospores of *P. erythroseptica* were produced based on the methods by Al-Mughrabi et al. (2007) and Jiang (2017) with a slight modification. Briefly, *P. erythroseptica* was cultured on 10% V8 agar for 6 days at $22 \pm 1^\circ\text{C}$ in the dark. Mycelial plugs were punched with a 5-mm cork borer and transferred to a Petri plate (10 mm diameter) containing lima bean broth (LBB), with 4 plugs per plate. After being incubated at $22 \pm 1^\circ\text{C}$ under natural light for 3 days, LBB was discarded. The original (10% V8) agar plugs were removed, and the mycelial mats were rinsed with sterilized distilled water three times. The plate with washed mycelial mats was filled with 10 ml of 10% soil extract and incubated at 18°C under continuous fluorescent light. Four days later, the soil extract was discarded and the plate with mycelial mats was triple-rinsed and refilled by 7 ml of chilled (4°C) sterile water. The plate was chilled at 4°C for an hour and then left at 22°C for 30 min. Zoospore concentrations of the suspension were estimated with a hemocytometer.

To obtain fluopicolide-resistant mutants of *P. erythroseptica*, V8 agar was amended with fluopicolide at 100 $\mu\text{g/ml}$, subsequently referred to as F-V8. Zoospores of wild-type *P. erythroseptica* isolates were plated with a sterile spreader on F-V8 medium. The final concentration of DMSO in the medium was adjusted to 0.1%. One milliliter of zoospore suspension (10^4 to 10^5 spores/ml) of wild-type isolates was spread on a F-V8 plate. After the plates were incubated at 25°C in the dark for 5 days, colonies growing on F-V8 plates were considered as fluopicolide-resistant mutants (Lu et al. 2011). Each isolate had 5 replications, and the experiment was conducted twice. Survival/mutation frequency was calculated as the total number of mutant colonies divided by total number of zoospores on the plate. These mutants were used throughout the study.

Morphological observation and characterization of fluopicolide-resistant mutants of *P. erythroseptica*. *Morphology.* The mutants from F-V8 plates were transferred to a fungicide-free clarified V8 agar plate. When the colony grew to the size of a quarter of a Petri plate, mycelia was cut from the colony edge and transferred to a new V8 plate, which was repeated 10 times. The cultures from the first transfer were referred to as the first transfer of resistant isolates/mutants (T1). The morphology of wild-type and mutant isolates were observed under a light microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Fig. 1. Distribution of fluopicolide sensitivity of 34 *Phytophthora erythroseptica* isolates from Maine. EC_{50} : effective concentration of 50% inhibition of mycelial growth.

Mycelial growth of *P. erythroseptica* mutants. The 10th transfer of *P. erythroseptica* mutants was used to investigate the mycelial growth to compare the differences between fluopicolide-resistant and their wild-type parents. An agar plug was cut with a 5-mm cork borer and transferred from each active culture of *P. erythroseptica* isolate onto a clarified V8 agar plate. All the test isolates were incubated at 28°C in the dark. Each isolate had 4 replications. The radius of mycelial growth on each plate was measured at 24, 48, 72, and 96 h of incubation. The original agar plug radius (5 mm) was subtracted before data analysis. Mycelial growth at 24 h was used to compare the growth difference between mutants and wild-type parents in the first 24 h of incubation. Growth curves of the cultures were generated based on the measurements. This assay was repeated once.

Pathogenicity and virulence of *P. erythroseptica* mutants. Disease-free potato ('Russet Norkotah') tubers were used to examine the pathogenicity and virulence of *P. erythroseptica* isolates. The tubers were washed and disinfested with 0.6% sodium hypochlorite followed by rinses in sterile water. The 10th transfer of fluopicolide-resistant *P. erythroseptica* mutants and their wild-type parents was used for inoculation. Three V8 agar plugs of each isolate were placed on tuber eyes and covered by a cap of a 2-ml Eppendorf tube, which was wrapped with a piece of Parafilm. The treated tubers were suspended on a plastic mesh rack above a wet paper towel in an enclosed sterile plastic container. Tubers treated with pathogen-free agar plugs were used for the control. The tubers were incubated at 25°C. A completely randomized design with 4 replications was used in this experiment. Seven days after inoculation, all the tubers were

cut from the point of inoculation, and the depth and width (using the inoculation site as the center) of pink lesions were measured. The pathogenicity was confirmed by the presence of pink rot lesions, and the virulence was indicated by tuber lesion penetration. Tuber lesion penetration was calculated by the equation lesion penetration = (depth + width/2)/2 (Fitzpatrick-Peabody and Lambert 2011).

Statistical analysis. EXC was used in data analysis to calculate EC₅₀ (Torres-Londoño et al. 2016). R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) was used to perform ANOVA and Fisher's LSD (for parametric data) or Wilcoxon each pair analysis (nonparametric data) in fluopicolide sensitivity, mycelial growth, and virulence. Bivariate analysis was used to examine the linear regression fit of mycelial growth rates between 24 and 96 h of incubation (Everitt 1995; Matthews et al. 1990). Significance level α was set at 0.05 for all data analyses.

Results

Baseline sensitivity of *P. erythroseptica* to fluopicolide. Frequency of mutation. All 34 wild-type *P. erythroseptica* isolates were sensitive to fluopicolide, which showed a skewed distribution (Fig. 1). The effective concentration for 50% inhibition of mycelial growth (EC₅₀) of those isolates ranged from 0.08 to 0.35 $\mu\text{g/ml}$, with a mean of 0.18 $\mu\text{g/ml}$ and median of 0.17 $\mu\text{g/ml}$. The standard deviation was 0.05 and the coefficient of variation was 25.93%.

Fluopicolide-resistant *P. erythroseptica* mutants. By picking mycelial colonies on F-V8 plates that were plated with zoospore suspensions, a total number of 7 fluopicolide-resistant mutants was obtained from 6 out of 34 isolates of *P. erythroseptica* (Table 1). There were two types of mutants based on EC₅₀, including type-1 mutants which had EC₅₀ higher but less than 5 times higher than their wild-type parents, and type-2 mutants which had EC₅₀ at least 5 times higher than their wild-type parents. The mutation frequency was between 10⁻⁵ and 10⁻⁴.

Morphology and resistance stability of fluopicolide-resistant *P. erythroseptica* mutants. After transferring to fungicide-free V8 plates, all the mutants grew and formed colonies. However, their morphology was different compared with their wild-type parents. Mutants formed short-branched, swollen septa-free hyphae (Fig. 2), which was different from typical *P. erythroseptica* hyphae and other oomycetes. Some mutants regained normal mycelial growth at T2, but others did not recover from the abnormal growth until

Table 1. Effective concentration of 50% inhibition (EC₅₀, $\mu\text{g/ml}$) of mycelial growth of fluopicolide-resistant mutants and wild-type parents of *Phytophthora erythroseptica*

Type ^y	13A02	13A05	13A06	13A07	13A14	13A39
Wild-type	0.16 a ^z	0.18 a	0.22 a	0.25 a	0.20 a	0.18 a
Mutant-1	-	0.47 b	0.94 b	0.41 b	-	0.44 b
Mutant-2	0.99 b	1.29 c	-	-	>5.31 b	-

^y Mutant-1 indicates the type of mutants having EC₅₀ higher but not more than 5 times higher than wild-type parents. Mutant-2 indicates mutants having EC₅₀ over 5 times higher than the wild-type parents.

^z Mean values not connected by the same letters are significantly different ($P < 0.05$) within each column. "-" indicates no data were available.

Fig. 2. Mycelial morphology of wild-type isolates (left) and the first generation of mutant *Phytophthora erythroseptica* 13A08 (right).

T3 or T4. Some mutants did not produce oospores between T3 and T6, but the oospore reproduction occurred in all the mutants at T8. One mutant stopped producing oospores at T5 and eventually died at T6. Multiple attempts to transfer this mutant from T4 and T5 failed.

Characterization of fluopicolide-resistant mutants of *P. erythroseptica*. Spiral plating assay was conducted using the 10th transfer (T10) and the wild-type parents of all the mutants. EC₅₀ values of mutants at the 10th transfer were significantly different from those of their parents. The type-2 mutants were more resistant/tolerant to fluopicolide than the type-1 from the same parents, and it was observed that the type-2 mutants were not as resilient as type-1 mutants, as type-2 recovered more slowly on V8 plates at T1 (Table 1).

Mycelial growth of *P. erythroseptica* mutants. The mutants grew significantly slower than their wild-type parents in the first 24 h (Table 2). The mycelial growth of each test isolate fit a linear regression model, with $R^2 > 0.9045$. Growth rates of mutants and their parents between 24 and 96 h were similar, except that the growth rate of mutant 13A14-M was lower than its wild-type parent (Table 3).

Pathogenicity of *P. erythroseptica* mutants. All the fluopicolide-resistant mutants were pathogenic to potato tubers, with a disease incidence of 100%, except for mutant 13A14-M, with an incidence of 25% pink rot in the first tuber inoculation experiment. In terms of lesion size, most mutants were not significantly different from their corresponding wild-type parents, with the exceptions that mutants of 13A06-M and 13A14-M caused smaller lesions than the parents, and mutants of 13A07-M had greater lesion penetration than the parents (Table 4).

Discussion

All wild-type isolates of *P. erythroseptica* were sensitive to fluopicolide in this study. This result was not surprising as fluopicolide has not been broadly used in Maine, and the pathogen has not undergone a strong selection pressure. This is similar to the observations of *Phytophthora capsici* populations and *Pseudoperonospora cubensis* populations (Lu et al. 2011; Wang et al. 2014). However, this does not guarantee that there is no risk of resistance development in the future.

The sensitivity of *P. erythroseptica* to fluopicolide varied in a skewed distribution. Mutants were derived only from 6 of 34 wild-type *P. erythroseptica* isolates. The estimated mutation frequency for fluopicolide resistance was approximately 10^{-5} , which was lower than previously reported in other oomycete species (Lu et al. 2011; Wang et al. 2014), suggesting a low to moderate risk of fluopicolide resistance developing in *P. erythroseptica* populations (Brent and Hollomon 2007).

Morphological changes of fluopicolide-resistant *P. erythroseptica* mutants were observed, including misshapen hyphae. This may be associated with the delocalization of spectrin-like proteins (Toquin et al. 2008). Some mutants stopped producing oospores after three transfers (T3).

A trade-off between fluopicolide resistance and biological fitness in *P. erythroseptica* was observed in this laboratory study. Although the mutants were capable of infecting potato tubers, the virulence of mutants was generally reduced compared with their wild-type parents. In fact, the highly resistant (type-2) mutant had lower growth rates and virulence than the moderate resistant (type-1) mutant in the case of 13A05.

Our study of a relatively small number of isolates of *P. erythroseptica* indicated that some laboratory-generated mutants had impaired functions on various biological activities, which reduced their biological fitness, and may lead to reduced risk of fungicide resistance developing in the field. Therefore, the predicted risk of fluopicolide resistance in *P. erythroseptica* should be at a low to medium level. This result provided technical reference in fluopicolide application, which could be an alternative to mefenoxam in pink rot control. As fluopicolide has lower risk of resistance development, it is anticipated that fluopicolide can be broadly used with less concern than with mefenoxam. Regardless, for the best resistance management, fluopicolide should be incorporated in an integrated pest management program to reduce the risk of resistance development.

Table 2. Growth rate (cm/h) of mutants and corresponding wild-type parents of *Phytophthora erythroseptica* measured at 24 h of incubation

Type ^y	13A02	13A05	13A06	13A07	13A08	13A14	13A39
Wild-type	0.96 a ^z	0.85 a	0.75 a	0.81 a	0.88 a	0.82 a	0.91 a
Mutant-1	-	0.64 b	0.60 b	0.59 b	0.61 b	-	0.75 b
Mutant-2	0.53 b	0.38 c	-	-	-	0.20 b	-

^y Mutant-1 indicates the type of mutants having EC₅₀ higher but no more than 5 times that of wild-type parents. Mutant-2 indicates mutants having EC₅₀ over 5 times higher than the wild-type parents.

^z Mean values not connected by the same letters are significantly different ($P < 0.05$) within each column. “-” indicates no data were available.

Table 4. Lesion penetration [(depth + width)/2] on potato tubers inoculated with *Phytophthora erythroseptica* mutants and their wild-type parents

Isolate	Lesion penetration (cm)		
	Wild-type	Mutant-1	Mutant-2
13A02	3.96	-	3.06
13A05	3.15	3.59	2.56
13A06	3.89	2.98 * ^z	-
13A07	3.31	3.60 *	-
13A14	4.06	-	2.04 *
13A39	3.78	3.74	-

^z * Indicates a significant difference between the mutant and its parent ($P < 0.05$) for each isolate. Mutant-1 indicates the type of mutants having EC₅₀ higher but no more than 5 times that of their wild-type parents. Mutant-2 indicates mutants having EC₅₀ over 5 times higher than their wild-type parents.

Table 3. Growth rate^y (from 24 to 96 h incubation) of mutants and their wild-type parents of *Phytophthora erythroseptica* analyzed by linear regression

Isolate	Wild type		Mutant-1 ^z		Mutant-2 ^z	
	Growth rate (cm/h)	R^2	Growth rate (cm/h)	R^2	Growth rate (cm/h)	R^2
13A02	0.0283	0.9972			0.0297	0.9945
13A05	0.0322	0.9860	0.0301	0.9045	0.0291	0.9985
13A06	0.0329	0.9975	0.0283	0.9913		
13A07	0.0295	0.9929	0.0295	0.9929		
13A14	0.0315	0.9981			0.0226	0.9933
13A39	0.0288	0.9899	0.0296	0.9967		

^y Growth rate is the slope of a regression equation generated by bivariate analysis.

^z Mutant-1 indicates the type of mutants having EC₅₀ higher but no more than 5 times that of wild-type parents. Mutant-2 indicates mutants having EC₅₀ over 5 times higher than the wild-type parents.

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