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Studies on Sensitivity Reduction in Solo and Mixture Treatments and Fungicide-Induced Mutagenesis in *Monilinia fructicola*

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ABSTRACT

Three fungicide-sensitive *Monilinia fructicola* isolates were exposed in weekly transfers of mycelia to a dose gradient of a DMI and a QoI fungicide (azoxystrobin) in solo or mixture treatments and fungicide sensitivity as well as genetic changes were assessed. Isolates showed a faster reduction in sensitivity (higher resistance factors) to azoxystrobin than to SYP-Z048; this process was slower in the mixture treatment. The decrease of fungicide sensitivity was not a heritable trait. Genomic mutagenesis at 8 of 15 microsatellite loci was evidenced in one of three isolates tested after exposure to azoxystrobin. These non-coding regions of the genome either showed single repeat additions or deletions, or large insertions or deletions, suggesting sublethal exposure to azoxystrobin may increase the rate of genomic mutagenesis. Mutagenesis was only observed after exposure to azoxystrobin, which may be dependent on the mode of action of this fungicide, however, more rigorous experimentation is needed before such conclusions can be drawn from these results.

INTRODUCTION

Sublethal exposure of pathogens to fungicides occurs frequently in modern agriculture. For the brown rot fungus of pome and stone fruits, *Monilinia fructicola*, the development of resistance to commonly used fungicides has been well-characterized in field isolates. However, the effect of fungicide dose on the rate of sensitivity reduction or resistance development has not been examined and may be accelerated with repeated sublethal exposure to doses of fungicides. This type of resistance has been shown to occur in bacterial populations in which antibiotic stress was linked with an increase of mutation rates (Cirz et al. 2005; Riesenfeld et al. 1997).

We utilized *Monilinia fructicola*, causal agent of brown rot of pome and stone fruits, to assess the nature and rate of sensitivity reduction to two commercially applied chemical fungicides with differing modes of action (DMI and QoI). Evidence of fungicide-induced mutagenesis was examined by microsatellite analysis. The overall goal of this study was to provide insight into the effect of fungicide dose on the rate of evolution of sensitivity reduction and conduct a preliminary examination into fungicide-induced mutagenesis, which have broad implications for plant disease management.

MATERIALS AND METHODS

Isolates SCDL28, NY9C, and OH6P were subjected to three fungicide treatments consisting of DMI fungicide SYP-Z048 (Chen et *al.* 2012), QoI fungicide azoxystrobin, and a mixture of SYP-Z048 and azoxystrobin. Applications of SYP-Z048 and azoxystrobin were at a rate of 50 µg/ml and 200 µg/ml, respectively, and the mixture contained 25µg/ml SYP-Z048 and 100 µg/ml of azoxystrobin. Potato dextrose agar (PDA) amended with azoxystrobin also contained 100 µg/ml of SHAM (including the mixture treatment). Fungicide stock solution (54.3 µl) was spread on solidified PDA in a Petri dish (15 cm diameter; 50 ml PDA per dish). A spiral plater was used to create a gradient of decreasing fungicide concentration from the center of the plate outward, using the exponential and slow mode settings.

Inoculum for fungicide exposure plates was prepared on 53 x 4 mm Grade P8 filter paper strips as described previously (Förster et *al.* 2004). After 3 days of incubation at 22°C in the dark, the mycelium-covered strips (two for each isolate) were transferred onto PDA amended with a fungicide gradient. Each dish contained six strips with two replicate strips per isolate on opposite sides and three isolates per dish. Aerial mycelium and conidia were collected from the 100% to 50% inhibition zone and used for the subsequent week's inoculation, where the procedure was repeated for a total of 12 consecutive transfers.

Isolates were sub-cultured to unamended plates at the same time as the long-term exposure to fungicides, thus providing a control for random mutations at microsatellite loci that are known to arise via mitotic cell division (Bhargava & Fuentes 2010). Fungicide concentration at the 50% inhibition zone (EC_{50}) was determined using the Spiral Gradient Endpoint software (Spiral Biotech, Norwood, MA; Förster et *al.* 2004). Resistance factor (RF) values were estimated for all isolates and treatments, which is calculated by measuring the EC_{50} at the last transfer and dividing by the EC_{50} value at the initial transfer.

The change in EC_{50} values during transfers was assessed with best model fit and resistance factor estimation. The first step was to define a curvilinear relationship based on a second-order polynomial model: $EC_{50} = \beta_0 + \beta_1 * \text{Transfer} + \beta_2 * \text{Transfer}^2 + \varepsilon$. The β_0 parameter represents the initial EC_{50} value prior to the onset of the study, the β_1 parameter represents the initial change in EC_{50} due to transfer (i.e., the initial slope of the model), and the β_2 parameter represents the change in slope as transfers continue. The parameters of the model were

264

estimated using the method of least squares. Hypothesis tests were conducted to determine if the parameters were statistically significant different from zero.

DNA was extracted from mycelium using a modified Cetyl Trimethylammonium Bromide method (Chen et *al.* 2012). Purified DNA was screened using 15 microsatellite primers developed previously, 13 from Everhart et *al.* (2012) and 2 from Jänsch et *al.* (2012).

RESULTS

In this study we assessed whether the rate of sensitivity reduction to SYP-Z048 and azoxy-strobin can be slowed down in mixture treatments compared to a solo treatment. Reduced sensitivity to SYP-Z048 and azoxystrobin was assessed on dishes amended with single fun-gicides or with a mixture. Statistical analysis revealed no significant difference between iso-lates, therefore the data of all isolates were combined. In regard to SYP-Z048 sensitivity, the initial slopes for both solo and mixture treatments were not significant (test of β_1; $p = 0.08$ and 0.49, respectively) and there was no significant change in the slope as weekly transfers increased (test of β_2; $p = 0.35$, and 0.66, respectively; Figure1A), thus the rate of sensitivity reduction to SYP-Z048 was comparable in early and late transfers. In regard to azoxystrobin sensitivity reduction, there appeared to be slow initial changes followed by rapid subsequent changes. The initial slopes for both solos and mixture treatments were not significant (test of β_1; $p = 0.20$, and 0.35, respectively), but there were significant changes in the EC_{50} slopes in the later transfers (test of β_2; $p<0.05$, and 0.05, respectively; Figure1B) in that the isolates that were exposed to azoxystrobin alone showed a faster reduction in sensitivity as a group, as compared to the cultures that were exposed to the mixture treatment.
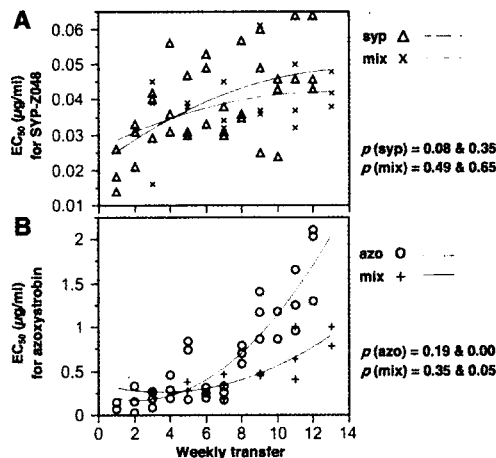

Figure 1   Reduced sensitivity to SYP-Z048 of *Monilinia fructicola* obtained during 12 weekly transfers on medium amended with SYP-Z048 (syp) or the mixture of SYP-Z048 plus azoxystrobin (mix) (**A**); and sensitivity to azoxystrobin during 12 weekly transfers on medium amended with azoxystrobin (azo) or the mixture of SYP-Z048 plus azoxystrobin (mix) (**B**).

After 12 consecutive transfers on PDA amended with the solo or mixture fungicides, the RF to SYP-Z048 were between 2.4 and 3.3 in isolates transferred on SYP-Z048 and between 9 and 29.5 for azoxystrobin in isolates transferred on azoxystrobin (Table 1). The reduction in sensitivity to SYP-Z048 and azoxystrobin following transfer on the mixture of the two fungicides was significantly lower than for transfers on the single fungicides for 1 out of 3 isolates and 3 out of 3 isolates, respectively. Progeny from conidia of all mutants were as sensitive to SYP-Z048 and azoxystrobin as the parental strains indicating that the decreased sensitivity was not a heritable trait (data not shown).

Table 1   Resistance factors (RF) of *Monilinia fructicola* isolates to fungicides SYP-Z048 or azoxystrobin after 12 consecutive, weekly transfers on dishes amended with a single fungicide or a mixture of SYP-Z048 and azoxystrobin

	RF to SYP-Z048[y]		RF to azoxystrobin	
Isolates	Transfers on SYP-Z048	Transfers on mixture	Transfers on azoxystrobin	Transfers on mixture
SCDL28	2.4a[z]	2.7 a	14.8 a	7.1 b
NY9C	2.5 a	1.8 a	29.5 a	14.6 b
OH6P	3.3 a	1.6 b	9.0 a	5.4 b

[y] RF was calculated by dividing the EC50 obtained after the last transfer by the EC50 obtained from the unexposed, parent isolate.

[z] Values followed by the same letter within rows and separately for each fungicide treatment (SYP-Z048 and azoxystrobin) are not significantly different according to ANOVA followed by Fisher's LSD test at $\alpha < 0.05$.

Isolates SCDL28, OH6P, and KAC18 were genotyped and data for isolate SCDL28 are shown. Although the cultures (one culture per isolate) taken following control and SYP-Z048 treatments had the same allelic composition as the parental strain, differences were detected at eight loci in azoxystrobin-treated mycelium of isolate SCDL28. Five loci lost one to three repeats (Mf-SEA, MF-SEF, Mf-SEK, Mf-SEL and Mf-SER), one locus gained six repeats (Mf-SEN), and two loci had fragment size changes not equivalent to multiples of the repeat motif (Mf-SED lost 58 bp and Mf-SEI gained 16 bp). Allelic changes were not detected for either of the other two isolates (data not shown).

Table 2   Allelic composition of microsatellite loci in *Monilinia fructicola* parent isolate SCDL28 and its single, mycelium-derived cultures transferred for 12 weeks on PDA that was either non-amended (negative control), amended with DMI fungicide SYP-Z048, or amended with QoI fungicide azoxystrobin.

	Size of allele at each locus[z]														
	Mf-SEA	Mf-SEC	Mf-SED	Mf-SEE	Mf-SEF	Mf-SEG	Mf-SEI	Mf-SEK	Mf-SEL	Mf-SEN	Mf-SEP	Mf-SEQ	Mf-SER	CHM Fc4	CHM Fc5
Treatment	tetra-	tetra-	tetra-	di-	tetra-	hexa-	tri-	tetra-	tetra-	di-	tetra-	di-	tetra-	septa	di-
Parent	133	173	187	156	127	144	113	252	143	215	261	136	145	231	97
(-) control	133	173	187	156	127	144	113	252	143	215	261	136	145	231	97
SYP-Z048	133	173	187	156	127	144	113	252	143	215	261	136	145	231	97
azoxystrobin	**129**	173	**129**	156	**123**	144	**129**	**248**	**147**	**227**	261	138	**133**	231	97

[z]The primers for each locus were previously published with nucleotide repeat motif (di-, tri-, tetra-, hexa-, or septa-) noted below each locus name (Everhart *et al.* 2012; Jansch *et al.* 2012). Alleles in bold are different from the parent allele size

DISCUSSION

We explored the potential impacts of sublethal fungicide exposure on rate of fungicide sensitivity reduction and on non-coding genomic regions and hypothesize that fungicide-induced mutagenesis may accelerate the emergence of fungicide resistant mutants. Although sublethal exposure to fungicides over the course of 12 generations led to decreased fungicide sensitivities (ie. higher resistance factors) and showed a significant rate increase of EC_{50} for azoxystrobin alone or in a mixture with SYP-Z048, heritable fungicide reduced sensitivity (adaptation) was not observed. The lack of stable development of reduced sensitivity in our mutants indicates that the adjustment to the solo and mixture treatments was transient in nature and may therefore not be relevant to field settings.

Evidence of genomic mutagenesis was evidenced in isolate SCDL28 after exposure to azo-xystrobin. Mutations were observed at 8 of 15 microsatellite loci, where half of these muta-tions were relatively simple, showing a single repeat addition or deletion to the allele size, and the other half showed large fragment gains or losses (insertions or deletions), with the largest being a 58 bp loss at locus Mf-SED. The microsatellite mutations may have been caused directly by the fungicide (DNA-targeted mutagenesis) or by indirect mechanism, such as physiological stress (ie. production of ROS leading to increased polymerase error). The mutation rate of microsatellite loci are thought to vary from 10^{-6} to 10^{-3} mutations per locus per gamete per generation, which is equivalent to 1 mutation per locus per 100 to 1 million generations (Bhargava & Fuentes 2010). Nevertheless, microsatellites are presumed to be fairly stable in fungi grown under laboratory conditions (Kohn et *al.* 2008). Indeed, the negative control in the current study showed no evidence of random mutation as compared to the parental strains. Additionally, no indications of fragment size changes were detected in the DMI treatments. Thus, our evidence suggests that sublethal exposure to azoxystrobin induces mutagenesis in the genome at least in some isolates in the form of increased rate of mutations (as indicated by step-wise increase or decrease in fragment sizes) and random insertions and deletions (indicated by large fragment gains/losses). Alternatively this could be seen as a random event. Thus more thorough studies need to be conducted.

To the best of our knowledge, this study is the first report of fungicide-induced mutagenesis of a fungal plant pathogen. It is interesting to note that a recent study of saprobic *Alternaria* spp. showed a similar response of increased polymorphisms at inter simple-sequence repeat (ISSR) genomic regions after prolonged exposure to isothiocyanates, which are natural fun-gicidal compounds produced by plants in the genus *Brassica* (Troncoso-Rojas et *al.* 2013). However, a study examining variation of microsatellite polymorphisms in *Botrytis cinerea* after growth on media with various amendments (nutrient rich, nutrient poor, antibiotic pyrrolnitrin, or fungicide iprodione) showed no variation in any of the nine microsatellite loci (Ajouz et *al.* 2010). Thus, our current study adds to the evidence accumulating that certain chemical stressors may increase random mutations in genomes, while others may not. It is clear that the scope of potential mutagens and extent of mutagenesis has not been fully explored and more research is needed in the area of fungicide-induced mutagenesis.

Fungicide-induced mutagenesis may play an important role in the emergence of fungicide resistance by providing greater genetic variation of the pathogen population and leading to genotypes with a selective advantage. In bacteria, it is known that stress may lead to increased mutation rates that are thought to play such a beneficial role in the emergence of new bacterial genotypes, some of which may confer a selective advantage within the population and enable accelerated adaptation to stressful conditions (Smith & Romesburg 2007). In our study, mutagenesis was only observed after exposure to azoxystrobin, which may be dependent on the mode of action of this fungicide, however, more rigorous experimentation is needed before such conclusions can be drawn from these results.

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