Sensitivity of *Mycosphaerella fijiensis*, Causal Agent of Black Sigatoka of Banana, to Propiconazole

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ABSTRACT

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One hundred monoascosporic isolates of *Mycosphaerella fijiensis* were collected in February and November 1994 from each of two banana (*Musa* spp.) plantations in Costa Rica. Locations at San Pablo and Coopecariari had been sprayed with propiconazole for the past 7 years to control black Sigatoka. One hundred monoascosporic isolates from a third location, San Carlos, with no history of fungicide use, also were tested for sensitivity to propiconazole. Fifty percent effective concentration (EC_{50}) values were calculated for individual isolates by regressing the relative inhibition of colony growth against the natural logarithm of the fungicide concentration. In the February sample, the mean EC_{50} values for San Pablo and Coopecariari populations were 0.06 and 0.05 $\mu\text{g a.i. ml}^{-1}$, re-

spectively, which were not statistically different ($P = 0.05$). The mean EC_{50} value of the population at San Carlos was 0.008 $\mu\text{g a.i. ml}^{-1}$, which was significantly lower ($P = 0.001$) than the mean EC_{50} values obtained at San Pablo and Coopecariari. Frequency distributions of EC_{50} values of isolates from the three populations collected in February showed that 80% of isolates from San Pablo and Coopecariari had EC_{50} values greater than the highest EC_{50} value from San Carlos, indicating a significant shift in reduced sensitivity to propiconazole. Isolates collected in November 1994, after eight treatments of propiconazole at San Pablo and Coopecariari, showed a significant increase in mean EC_{50} values compared with the means observed in February. The high proportion of isolates with reduced sensitivity to propiconazole may account for the unsatisfactory control of black Sigatoka between 1992 and 1993 in the two banana plantations at San Pablo and Coopecariari.

Banana leaf spot or black Sigatoka, caused by *Mycosphaerella fijiensis* Morelet, is the most important disease of bananas worldwide (32). Yield decreases greater than 20% have been recorded in plots with no fungicide treatments, compared with fungicide-treated plots (4; M. Guzmán and R. A. Romero, *unpublished data*). In Costa Rica, the annual cost of black Sigatoka control is approximately \$49 million (1). Black Sigatoka is controlled primarily by fungicides, because nonchemical alternatives do not provide commercially acceptable control. Alternating systemic fungicides with different modes of action, and systemics with protectant fungicides are commonly used methods for controlling black Sigatoka. Benomyl, propiconazole, and tridemorph have been the principle systemic fungicides used in the control of this disease. The availability of benomyl, propiconazole, and tridemorph, which have postinfection activity (10,28), provided an opportunity for the use of an early warning system based on detection of initial stages of symptoms (10,11). Use of this system resulted in a significant decrease in the number of treatments required for black Sigatoka control. Between 1991 and 1992, resistance of *M. fijiensis* to benomyl became widespread, resulting in an increase in propiconazole use.

Propiconazole is a member of the sterol demethylation inhibitor (DMI) fungicides, which are inhibitors of C-14 demethylation of the 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi (17,20). This fungicide has been used in Central America for the control of black Sigatoka of bananas since 1987. Use of propiconazole became widespread in banana plantations because of its high postinfection activity that favored longer intervals be-

tween applications (28,31). Widespread use of propiconazole, however, has raised concerns regarding a decrease in sensitivity to this fungicide. Resistance to DMIs has been reported for other pathogens (6,7,8,24). Resistance of *M. fijiensis* to propiconazole has not been demonstrated conclusively, but studies have indicated a decrease in sensitivity (22,29). The possibility of a gradual increase in the frequency of DMI-resistant isolates in fungal populations (R. Romero, *unpublished data*) and the absence of initial baseline sensitivities to *M. fijiensis* are important limitations for determining the status of sensitivity of *M. fijiensis* to propiconazole in banana plantations.

Unsatisfactory control of black Sigatoka has been observed since 1992 in some banana plantations in Costa Rica. No relationship, however, has been established between unsatisfactory control of the disease and loss of sensitivity to propiconazole in field isolates of *M. fijiensis*. Therefore, this study was initiated to compare the sensitivity of isolates from two populations of *M. fijiensis* treated with propiconazole with a population that had not been treated with fungicides.

MATERIALS AND METHODS

Sampling sites. Three different locations in Costa Rica were sampled in February 1994, prior to the beginning of the rainy season, and again in November 1994, following a 6-month rainy season during which time propiconazole was used frequently. San Pablo and Coopecariari are large commercial plantations of approximately 300 and 400 ha, respectively, located in San Pablo and Cariari, respectively, in the Caribbean coast of Costa Rica. These plantations were planted to the cultivar Valery, which is highly susceptible to black Sigatoka. Each plantation was sprayed approximately eight times annually from 1987 to 1992 with propiconazole, at 100 g a.i. ha^{-1} per application.

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Between 1989 and 1991, the control of black Sigatoka in these two plantations was based on the forecasting system developed by Ganry and Meyer (11), alternating the systemic fungicides propiconazole, benomyl, and tridemorph. During that period, 18 to 23 fungicide applications per year were necessary to achieve satisfactory control of black Sigatoka. However, benomyl use was discontinued in 1992 because of the high proportion of resistance found in the population of *M. fijiensis* in these two plantations (R. A. Romero, unpublished data).

After 1992, as a consequence of the withdrawal of benomyl from the control programs, the number of applications of propiconazole increased to 10 per year. However, because of the unsatisfactory control obtained, the interval between applications of propiconazole was reduced from 18 to 21 days to 12 to 14 days, and mancozeb was reintroduced in the control programs, resulting in an increase in the number of applications per year to an average of 35. The third location, San Carlos, located in the Caribbean coast of Costa Rica, is isolated from the principal banana production areas and was used to collect baseline or reference isolates. Isolates were collected from bananas grown in backyards and small scale farms for local consumption. No fungicides had been used on these bananas; therefore, disease was severe. The cultivars planted in these areas were Valery and Gros Michel, the latter also highly susceptible to black Sigatoka.

Isolate selection. From each sample site, one hundred monoascosporic isolates of *M. fijiensis* were obtained from banana leaf tissue bearing abundant pseudothecia. Leaf tissue was collected from two to three banana plants in each of 20 sample points randomly distributed throughout each location at San Pablo and Coopecariari, using the divisions of banana plantations by cables and sections as reference points for randomization. There are no large banana plantations at San Carlos; thus, 20 small farms or backyards were sampled in a similar manner.

Ascospores were discharged from pseudothecia onto water agar (WA) as described by Stover (27), and single ascospores were transferred immediately to 15 × 100-mm petri dishes filled with potato dextrose agar (PDA). Between eight to ten ascospores from each sample point were placed on each dish with PDA. After 4 days of incubation at 25°C, colonies were visible to the naked eye, transferred to Mycophil agar (Becton Dickinson Microbiology Systems, Cockeysville, MD), and incubated for 15 days at 25°C under continuous fluorescent light with an intensity of 90 $\mu\text{E s}^{-1}\text{m}^{-2}$ for colony growth and conidial production. Five monoascosporic isolates per sample point within each location were tested for sensitivity to propiconazole.

Fungicide sensitivity assay. PDA was amended with propiconazole (Tilt 25EC; Ciba, Basel, Switzerland) at 0, 0.0001, 0.001, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, 0.50, and 1.0 $\mu\text{g a.i. ml}^{-1}$ by adding 1 ml of an aqueous stock suspension of the fungicide to 99 ml of molten PDA at 50°C. Seven milliliters of the amended PDA was dispensed into each 15 × 60-mm petri dish. Inoculum was prepared by vortexing individual colonies grown on Mycophil agar in 1 ml of sterile water in a test tube for 10 s. Two drops of the suspension of conidia and mycelial fragments were transferred to each PDA dish amended with propiconazole using a Pasteur pipette and were spread across the surface of the agar with a sterile glass rod.

Inoculated petri dishes were incubated in the dark at 25°C for 5 days, after which the colonies of the unamended control dishes were approximately 1 to 1.3 mm in diameter. After this time, the diameters of five colonies per concentration were measured with the aid of a microscope with a micrometric scale (4×). At concentrations of the fungicide that caused a strong inhibition in growth, colonies were irregular in shape. In these cases, an average diameter was obtained from two perpendicular measurements of the colonies. A 50% effective concentration (EC_{50}) value was calculated for each isolate by regressing the growth inhibition of each of the five colonies at each concentration as a percentage of the control or unamended dishes against the natural logarithm of

the fungicide concentration. The resulting equation was then used to calculate the concentration of fungicide expected to cause 50% inhibition of growth.

Data analyses. Frequency distributions of EC_{50} values for each of the three populations of isolates were constructed.

To compare the mean EC_{50} value for each distribution among populations by a *t* test using SAS (version 6.01; SAS Institute Inc., Cary, NC), the equality of variances among individual populations was determined by using the *F* criterion (25). If variances for individual populations were different, a pooled variance and pooled degrees of freedom were used to compare the mean EC_{50} values between two populations (25). Also, the range of EC_{50} values for each population was determined. A comparison of the mean EC_{50} value between sample times for each population was also performed, as previously described.

RESULTS

First sampling date. The variances of sensitivity to propiconazole among individual isolates sampled from the populations of San Pablo and Coopecariari in February 1994 (Fig. 1) were similar, but variances from both locations were significantly larger than the variance of the population from San Carlos ($P = 0.05$). Therefore, to compare the mean EC_{50} values between San Pablo

Fig. 1. Sensitivity distribution of three Costa Rican populations of *Mycosphaerella fijiensis* to propiconazole, sampled in February 1994. Isolates were collected from random locations in two banana plantations at San Pablo and Coopecariari, both with a history of fungicide use, and from a population at San Carlos with no history of fungicide use.

TABLE 1. Comparison of sensitivity to the fungicide propiconazole among populations of *Mycosphaerella fijiensis* collected in February 1994 in banana plantations at San Carlos, San Pablo, and Coopecariari, Costa Rica

Population	ln mean EC ₅₀ ^y	Standard deviation of ln EC ₅₀	Range of ln EC ₅₀
San Carlos	-4.8564 (0.008) b ^z	0.6158	-6.60 to -2.38
San Pablo	-2.7596 (0.060) a	0.8827	-4.91 to -0.64
Coopecariari	-2.9795 (0.050) a	0.8194	-4.80 to -0.49

^y EC₅₀ is the concentration of the fungicide that causes 50% inhibition of colony growth of isolates of *M. fijiensis*. Negative values are log transformed. The values in parenthesis correspond to µg ml⁻¹.

^z Means followed by the same letter in the column are not different according to a *t* test at *P* = 0.05.

and Coopecariari, a pooled variance and pooled degrees of freedom were used. To compare EC₅₀ values between San Carlos and Coopecariari and San Pablo, a *t* test was performed using effective degrees of freedom (25). The mean EC₅₀ values of populations of *M. fijiensis* sampled from commercial banana plantations at San Pablo (0.06 µg ml⁻¹) and Coopecariari (0.05 µg ml⁻¹) to propiconazole were similar (*P* = 0.05). Mean EC₅₀ values of isolates from both San Pablo and Coopecariari were seven to eight times larger (*P* = 0.001) than the mean EC₅₀ value of the population of San Carlos (0.008 µg ml⁻¹), the latter of which had no history of propiconazole treatment (Table 1). The range of EC₅₀ values for individual isolates sampled from San Pablo and Coopecariari were very similar. However, both San Pablo and Coopecariari had ranges of EC₅₀ values that were larger than the population of San Carlos (Table 1). The proportion of isolates from San Pablo and Coopecariari with ln EC₅₀ values higher than -2.24 (0.1 µg ml⁻¹; i.e., approximately twice the mean of the populations of San Pablo or Coopecariari) was 30 and 20%, respectively, compared with 1% (one isolate) in the population of San Carlos (Fig. 1). Except for the one isolate in the San Carlos population with an ln EC₅₀ value of -2.38 (0.09 µg ml⁻¹), the proportion of isolates from San Pablo and Coopecariari with an EC₅₀ value that overlapped with the San Carlos population was less than 20% (Fig. 1). In other words, 80% of the isolates in the populations of San Pablo and Coopecariari had EC₅₀ values greater than all but one isolate sampled from San Carlos.

Second sampling date. Compared with the initial sampling date, isolates of *M. fijiensis* collected in the same plantations in November 1994, after eight additional applications of propiconazole, were less sensitive to the fungicide. The distributions of EC₅₀ values from each of the three locations had a greater proportion of isolates with high EC₅₀ values compared with those found in February 1994, including the population from San Carlos. The shifts toward less sensitivity to propiconazole were most significant at San Pablo and Coopecariari, where 40 and 21% of the isolates, respectively, had an ln EC₅₀ greater than -0.5 (0.6 µg ml⁻¹), compared with only 1% in February 1994. The mean EC₅₀ value for isolates of *M. fijiensis* from San Pablo and Coopecariari were significantly larger than those from San Carlos; there was no significant difference among isolates from the San Pablo and Coopecariari populations (Table 2). The mean EC₅₀ value (0.02 µg ml⁻¹) for isolates of *M. fijiensis* from San Carlos was twofold greater than the mean determined in February. Similarly, mean EC₅₀ values (µg ml⁻¹) for isolates of *M. fijiensis* for San Pablo and Coopecariari in November were 4.5- and 3-fold greater than the means obtained in February, respectively. There were several isolates of *M. fijiensis* whose EC₅₀ values could not be determined accurately because they were greater than the highest concentration used. These isolates were grouped and reported as having a ln EC₅₀ of -0.5 (0.6 µg ml⁻¹) (Fig. 2).

DISCUSSION

The method used in this study to determine EC₅₀ values for *M. fijiensis* to propiconazole was based on measuring the diameters

TABLE 2. Comparison of sensitivity to the fungicide propiconazole among populations of *Mycosphaerella fijiensis* sampled in November 1994 in banana plantations at San Carlos, San Pablo, and Coopecariari, Costa Rica

Population	ln mean EC ₅₀ ^y	Standard deviation of ln EC ₅₀	Range of ln EC ₅₀
San Carlos	-4.1776 (0.020) b ^z	1.5197	-6.45 to 0.0
San Pablo	-1.3097 (0.270) a	1.2748	-4.89 to 0.0
Coopecariari	-1.8745 (0.150) a	1.1754	-3.94 to 0.0

^y EC₅₀ is the concentration of the fungicide that causes 50% inhibition of colony growth of isolates of *M. fijiensis*. Negative values are log transformed. The values in parenthesis correspond to µg ml⁻¹.

^z Means followed by the same letter in the column are not different according to a *t* test at *P* = 0.05.

of young colonies and comparing them with diameters of colonies grown on medium not amended with propiconazole. This method is similar to that used for *Venturia inaequalis* by Smith et al. (23). However, unlike Smith et al. (23), we measured colony diameters after 5 days of growth at 25°C, when they were still very small. This enabled us to clearly observe growth inhibition at all concentrations of the fungicide, before colony size was partially obstructed by the production of aerial mycelium. Our method is labor-intensive compared with the measurement of ascospore germ tubes as recommended by the Fungicide Resistance Action Committee (22). However, our method has the advantage of permitting determinations of EC₅₀ values for individual isolates as opposed to a mixture of ascospores, which allows for a more precise analysis of the variation in sensitivities within the population. One limitation of this method is the low production of conidia or the inability of some isolates to produce them in culture, which necessitates the use of mycelial fragments whose size and viability cannot be standardized.

Compared with the San Carlos population, the distributions of propiconazole sensitivity for isolates sampled from San Pablo and Coopecariari populations indicated that a portion of isolates from these populations did not overlap with the distribution of the nontreated population sampled in San Carlos. Twenty and fourteen percent of the San Pablo and Coopecariari isolates, respectively, from the February sample had EC₅₀ values higher than the highest EC₅₀ value found in the isolates sampled in San Carlos (0.09 µg ml⁻¹). Mean sensitivity ratios for *V. inaequalis* (EC₅₀ value of the resistant/EC₅₀ value of sensitive population) between 1.5 and 7 for different DMIs are related to unsatisfactory control of the disease (2,9,15,23). In our study, the sensitivity ratios (µg ml⁻¹) of the mean EC₅₀ values of isolates from San Pablo and Coopecariari divided by the mean EC₅₀ values of isolates from San Carlos were 6 and 7.5, respectively. The unsatisfactory control of black Sigatoka observed between 1992 and 1993 in San Pablo and Coopecariari is likely associated with an increase in the frequency of strains less sensitive to propiconazole, combined with a high proportion of benomyl resistance in the same plantations (R. Romero, unpublished data). In fact, in the last 3 years, the interval between applications of propiconazole has been reduced from 16 to 18 days to 10 to 12 days. In spite of this shorter interval, the control of black Sigatoka achieved is inferior compared with that obtained during the first years of propiconazole use.

The rapid shift toward less sensitivity to propiconazole from February to November 1994 is similar to that described for the disruptive selection model of fungal resistance to fungicides (5,13,14). In disruptive selection, resistance is usually under monogenic control. However, the phenotypic expression of resistance to propiconazole in *M. fijiensis* was characterized by a continuous distribution of EC₅₀ values (Figs. 1 and 2), which indicates that resistance is probably confirmed by more than one gene, as has been reported for other pathogens resistant to DMI fungicides (3,6,18,21). The possibility that both single and multiple gene resistance to a particular DMI fungicide occurs has been suggested (14) and was shown for mutants of *Nectria haematoc*

cocca var. *cucurbitae*, resistant to triadimenol (16). If several genes control the reduced sensitivity to propiconazole, the possibility exists that the least sensitive isolates are also less fit than the sensitive isolates and, under continuous sexual cycles of *M. fijiensis* in the absence of propiconazole use, the least sensitive isolates may revert to sensitive ones. If reduced sensitivity to propiconazole in *M. fijiensis* is controlled by a single gene with no cost on fitness, the reversion toward sensitivity in the population may be less likely. This points out the need to conduct research on the genetics of the resistance to propiconazole in *M. fijiensis*, which would help to design strategies for managing resistance to propiconazole in bananas.

The weather conditions in Costa Rica are highly favorable for the progress of black Sigatoka from May to December each year. Higher concentrations of airborne ascospores are found during this period compared with the period between January and April (12), because of the occurrence of frequent rain and several sexual reproductive cycles of the pathogen. Under such favorable conditions, the selection conditions resulting from eight treatments of propiconazole in 1994 at San Pablo and Coopecariari provided an excellent opportunity for the selection and multiplication of less sensitive isolates and an increase in their frequency in the populations to the levels observed in November 1994 (Fig. 2). This large increase was probably favored by the initially high proportion of less sensitive isolates in February and their ability to cause infections and successfully reproduce in the presence of the fungicide. We estimate that from February to November approximately seven sexual reproductive cycles of the pathogen occurred. As stated previously, it is unknown whether these less sensitive isolates are sufficiently fit to remain in the population if propiconazole use is restricted. Further research is needed to investigate the fitness of propiconazole-resistant isolates of *M. fijiensis* in the absence of the fungicide.

Smith et al. (23) recommended a discriminatory dose for monitoring sensitivity of *V. inaequalis* to DMI fungicides. They suggested that the discriminatory dose should be near the mean EC_{50} value of the baseline population and, additionally, a second dose near the highest level of resistance detected in the baseline population should be tested. Based on our results, the use of a discriminatory dose near the mean EC_{50} value for the San Carlos population would not be useful, because most of the isolates from San Pablo and Coopecariari had shifted toward EC_{50} values much higher than the mean EC_{50} value for San Carlos. Instead, to monitor further shifts in sensitivities in *M. fijiensis*, we recommend the use of $0.1 \mu\text{g ml}^{-1}$, which is slightly higher than both the highest EC_{50} value ($0.09 \mu\text{g ml}^{-1}$) detected in the population of San Carlos and the mean EC_{50} values for the populations of San Pablo and Coopecariari (0.06 and $0.05 \mu\text{g ml}^{-1}$, respectively) (Table 1). A second dose to detect shifts toward very high EC_{50} values in isolates of *M. fijiensis*, such as those observed in the isolates sampled in November 1994 (Fig. 2), should be close to $1.0 \mu\text{g ml}^{-1}$. Nevertheless, in areas where resistance has not been detected or where propiconazole has not been used, a discriminatory dose near the mean EC_{50} value found in the population from San Carlos in February may be effective for monitoring under these conditions.

Ascospores of *M. fijiensis* are airborne and can be dispersed several kilometers (26,30). Thus, the increase in the number of isolates with less sensitivity to propiconazole in the population of San Carlos in November 1994, from February, could be related to long distance dispersal of ascospores from the banana plantations with a high frequency of resistant isolates by tropical storms that usually occur during this period of the year. Changes in sensitivity of this untreated population also could be associated with seasonal shifts in sensitivity, as has been reported for *V. inaequalis* to flusilazole (19). Since immigrants from commercial plantations may enter the population at San Carlos, the absence of a baseline determination of the sensitivity of *M. fijiensis* prior to the introduc-

Fig. 2. Sensitivity distribution of three Costa Rican populations of *Mycosphaerella fijiensis* to propiconazole, sampled in November 1994. Isolates were collected from random locations in two banana plantations at San Pablo and Coopecariari, both with a history of fungicide use, and from a population at San Carlos with no history of fungicide use.

tion of propiconazole on bananas in Costa Rica still limits our ability to determine absolutely the magnitude of the shift with respect to the sensitivity of the initial population. This illustrates the need to establish baseline sensitivities prior to the introduction of DMI fungicides for the control of black Sigatoka in bananas in those countries in Latin America where the disease has recently spread, and to establish appropriate field monitoring programs to relate the effectiveness of disease control strategies with the sensitivity of the pathogen to the fungicides.

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