Original Article

Sensitivity In Vitro Of Isolates Of Moniliophthora Roreri Against Fungicides With Different Modes Of Action

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Abstract

The mush room Moniliophthora roreri, the causative agent of Moniliasis, is the main problem in cocoa cultivation in Ecuador. The objective of this study was to evaluate the sensitivity of 23 strains of M. roreri against fungicides of different modes of action; The strains were isolated from cocoa fruits with symptoms of the disease collected from cocoa plant samples of the Super Tree, National Type and CCN51 ecotypes in the Francisco de Orellana, Loreto and Joya de Los Sachas cantons of the Orellana province. Three systemic fungicides were evaluated: Azoxystrobin, Difenoconazole and Boscalid; and a protectant, copper sulfate pentahydrate 240 g/L-1, at different concentrations (100, 10, 1, 0.1, 0.01 ppm) in PDA culture medium with three replicates for each treatment. The results obtained show that the pathogen is sensitive to systemic fungicides; 82.60% of the evaluated strains are sensitive to Azoxystrobin at a concentration of 10 ppm with a percentage of growth inhibition (PIC) above 50%; On the other hand, 82.60% of the evaluated strains are sensitive to Difenoconazole from the concentration of 1 ppm with a PIC greater than 50%; and, 86.96% of the evaluated strains are sensitive to Boscalid at a concentration of 10 ppm with a PIC above 50%. However, M. roreri It is insensitive to copper sulfate pentahydrate in all concentrations tested. The fungicides Difenoconazole, Boscalid and Azoxystrobin can be used for chemical control in the management of Moniliasis disease in cocoa.

Keywords: Moniliasis, Cocoa, Fungicides, Growth Inhibition, In Vitro.

INTRODUCTION

Thrush caused by *Moniliophthora roreri* It is considered one of the most destructive diseases of the cocoa crop (*theobroma cacao*) in Ecuador (Enríquez, 2010). Currently, it is reported that in the country the damage reaches 80% and in some cases more (Pico *et al*., 2019). In the Amazonian provinces of Sucumbíos, Orellana, and Napo, more than 40% of the production, some 8000 tons of cocoa, can be lost due to the disease (Phillips-Mora and Amores, 2021).

In producing countries, little is reported about the chemical control of Cocoa Moniliasis. In addition, it is argued that if only management is done through agronomic tactics, it is economically unfeasible for small producers due to high labor costs and the instability of cocoa prices (Krauss*et al*., 2010; towers of the cross*et al*., 2013; Tirado-Galician *et al*., 2016). However, it has been shown experimentally that effectively keeping damage levels below the economic threshold requires chemical control and other joint tactics (Krauss*et al*., 2010).

The use of azoxystrobin has been reported to be effective in outbreaks of the disease in Mexico (Torres De La Cruz*et al*., 2013), Costa Rica and Peru (Krauss*et al*., 2003; Bateman *et al*., 2005). Azoxystrobin is a systemic fungicide from the group of strobilurin analogues (Brent and Hollomon, 2007); that has demonstrated its healing properties in the field (Torres De La Cruz*et*

al., 2013). In the case of cocoa, they are efficient in reducing Moniliasis and witches' broom (*Moniliophthora pernicious*) (Tired-Galician *et al*., 2016). Recently, Espinoza-Lozano *et al*. (2022) found a high sensitivity in isolates of *M. roreri* to the fungicide azoxystrobin obtained from cocoa plantations in six provinces of Ecuador.

The previously discussed criteria reveal that currently the fungicide azoxystrobin is the most widely used for the chemical management of Cocoa Moniliasis in several countries around the world, including Ecuador. In other regions of America, a high diversity of strains of *M. roreri* (Diaz-Valderrama *et al*., 2022); which contributes to a loss of sensitivity of emerging strains of the pathogen or using a single fungicide. These considerations reinforce the idea that it is possible to carry out research with other fungicides with different modes of action to promote diversity of options in the management of the disease, especially if one takes into account that a fungicide product, by itself, does not intrinsically induce resistance, but rather acts as a selection agent. The development of resistance occurs when inappropriate practices are followed for the specific crop and pathogen situation (Zurita, 2018).

The objective of this investigation was to evaluate the sensitivity of different isolates of strains of *Moniliophthora roreri* against fungicides of different modes of action determined by the percentage of inhibition of mycelial growth under conditions *in vitro* to have options in the chemical management of Cocoa Moniliasis.

Materials and Methods

Isolation of Strains of M. Roreri and Monosporic Cultures

The strains of *M. roreri* were isolated from cocoa pods with signs of the disease and affected by less than 25%. The samples were collected during 2021 in the Cantons of Loreto, La Joya de Los Sachas and Francisco de Orellana in the Province of Orellana.

After separating the fruits, they were wrapped in paper. During the sampling trips, at night and in the morning of the following day, the wrapping of each fruit was changed to reduce humidity and sporulation of *M. roreri*. Next, the pods were taken to the Basic Sciences-microbiology laboratory of the Orellana Campus of the Escuela Superior Politécnica del Chimborazo (ESPOCH) to be processed (washed, cut into segments of ~0.5 cm^2, disinfected with 70% alcohol for 30 seconds and 0.1% sodium hypochlorite for 2 min, washed three times with sterile distilled water and dried with sterile paper). The segments were then plated on Potato Dextrose Agar (PDA) and incubated in the dark at room temperature (27-30 ^{O}C/4 d). After the incubation time, the mycelial growth of *M. roreri* transferred to fresh PDA incubated for 11 days. As a result, monosporic cultures were obtained according to the recommendations described (Ram, 1989). All the isolates were preserved in 10% glycerol at -20^{O}C.

Sensitivity of M. Roreri to Fungicides with Different Modes of Action

A preliminary test was carried out with two isolates to estimate the appropriate concentrations. The sensibility *in vitro* a Azoxystrobin, Difenoconazole, Boscalide and Copper Sulfate Pentahydrate 240 g/L^{-1} on the mycelial growth of strains of *Moniliophthora* spp., was evaluated at concentrations of 100, 10, 1, 0.1, 0.01 ppm of fungicide in PDA culture medium with three replicates for each treatment. A 6mm disc of the isolated *M. roreri* it was transferred to the PDA culture medium, with incorporated fungicide, and incubated as described above for six days. After the inoculation time, mycelial growth was evaluated as a percentage of growth inhibition PIC= ((R1-R2) /R1) x100, where R1 is the radius of the control pathogen and R2 is the pathogen with fungicide dilutions. The PIC data were processed using the statistical package (STATGRAPHICS Centurion XV).

Analysis and Discussion of Results

In the samplings carried out, the characteristic symptoms of Moniliasis disease in cocoa pods were observed (Fig. 1A). In addition, it was possible to isolate a fungus in a PDA culture medium (Fig. 1B), which was identified by microscopy and taxonomically located by its morphological characteristics in the genus *Moniliophthora* spp.

Figure 1: Characteristic symptoms of moniliasis disease in cocoa pods in the field (A) and isolated from moniliophthora spp. in PDA culture medium (B)

As a result, they were able to isolate under conditions *in vitro* 23 strains of *Moniliophthora* spp. in three cantons of the province of Orellana (Table 1). The largest number of isolated strains (13) were obtained from the Joya de Los Sachas canton and present in cocoa trees of the ecotypes: Super Tree (7) and National Type (6); followed by the canton of Francisco de Orellana with seven strains, all found in the CCN51 ecotype; and the canton of Loreto with only three strains, two of which were collected from the CCN51 ecotype and one from the Super Tree.

Table 1: Strains of moniliophthora spp. isolated from pods with symptoms of moniliasis from the cantons of the province of Orellana sampled during 2021

N° CEPA	CODE	CANTON	ECOTYPE
C1	OL-P-07-A1	Loreto	CCN51
C2	O-FO-05-A3	Francisco Orellana	CCN51
C3	OS-LS-03-A1	Jewel of the Sachas	super tree
C4	OS-UM-03-A1	Jewel of the Sachas	super tree
C5	JJ-EECA-A1	Jewel of the Sachas	National Type
C6	OL-09-A1	Loreto	CCN51
C7	OS-UM-09-A1	Jewel of the Sachas	National Type
C8	OS-SC-03-A1	Jewel of the Sachas	National Type
C9	OS-LS-05-A3	Jewel of the Sachas	super tree
Q10	OL-03-A1	Loreto	super tree
C11	OS-LS-09-A3	Jewel of the Sachas	super tree
C12	OS-LS-05-A2	Jewel of the Sachas	super tree
C13	OS-LS-09-A1	Jewel of the Sachas	super tree
C14	O-FO-09-A2	Francisco Orellana	CCN51
Q15	OS-SC-03-A2	Jewel of the Sachas	National Type
C16	OS-SC-05-A1	Jewel of the Sachas	National Type
C17	OS-SC-09	Jewel of the Sachas	National Type
C18	O-FO-03-A1	Francisco Orellana	CCN51
C19	O-FO-05-A1	Francisco Orellana	CCN51
C20	O-FO-03-A2	Francisco Orellana	CCN51
C21	O-FO-09-A1	Francisco Orellana	CCN51
C22	OS-UM-03-A2	Jewel of the Sachas	super tree
C23	O-FO-MF-0	Francisco Orellana	CCN51

The isolates of strains from different cocoa plantations in the province of Orellana confirmed the presence of the fungus. *M. roreri*, which coincides with previous studies that determined this species as the causative agent Moniliasis (Enríquez, 2010; Pico *et al*., 2019; Espinoza-Lozano *et al*., 2022).

Table 2 shows that strains c11 and c13 presented growth inhibition above 50% with a dose of 0.01 ppm, the rest presented a PIC below 29; Likewise, in the 0.1 ppm dose, the c6, c10 and c12 strains are incorporated, while for the 1 ppm dose, the c7, c9 and c23 are added. In the case of the 10-ppm dose, most of the studied strains show a PIC above 50%, except for c1, c4, c8 and c16, results that are maintained for the use of Azoxystrobin at a rate of 100 ppm, excluding the c4 strain.

Table 2: Percentage of growth inhibition of strains of M. roreri against different doses of the fungicide azoxystrobin

Strains	Azoxystrobin Dose (ppm)/ Percent Growth Inhibition (%)				
	100	10	1	0,1	0,01
c1	48,81	40,31	37,88	32,28	18,41
c2	66,27	61,60	47,49	8,52	8,31
c3	66,02	66,02	64,40	58,37	38,08
c4	51,33	49,47	48,58	38,49	10,10
c5	65,30	65,19	63,78	63,01	47,80
c6	65,21	65,94	68,63	55,14	28,29

c7	100,0	100,0	100,0	46,07	15,47
c8	28,56	21,37	17,68	12,65	0,19
c9	75,67	55,21	52,97	43,42	14,09
c10	100,0	73,34	67,65	50,39	8,72
c11	70,89	74,05	67,36	64,54	50,58
c12	67,79	65,94	58,34	51,15	23,27
c13	100,0	75,05	66,57	65,92	51,86
c14	63,90	58,83	41,57	7,76	2,16
c15	57,04	51,33	38,87	3,04	0,25
c16	49,64	46,93	33,14	7,42	4,02
c17	57,35	52,09	37,10	6,66	6,23
c18	70,18	63,74	48,71	21,61	14,03
c19	65,35	61,98	45,14	5,49	2,58
c20	63,31	54,31	35,73	7,03	2,54
c21	62,93	56,31	41,64	6,96	3,12
c22	56,50	53,46	36,26	14,15	11,38
c23	66,47	62,82	50,92	12,12	5,41

The previous results suggest that 82.60% of the investigated strains present a sensitivity to the fungicide Azoxystrobin from doses of 10 ppm. Four isolates had lower growth inhibition, including c8, which did not exceed 30%. Similar results are shown with this fungicide, which has been used in the management of moniliasis in the country, for example, Espinoza-Lozano*et al*. (2022) found that concentrations of 1 to 0.1 µg/mL^{-1} inhibited the growth of at least 91% of the isolates, but three were less sensitive, two from the provinces of Guayas (Pacific Coast) and one from Sucumbíos (Amazon).

Although four isolates were less sensitive, they can be considered equally susceptible compared to the response of other fungi such as *Phytophthora infesting* (Quin et al., 2016) or *Alternaria you give* (Rogers y Stevenson, 2010).

A high sensitivity of the isolates of *M. roreri* to Azoxystrobin, in this regard the high effectiveness of this fungicide (75/76 isolates) and flutolanil (66/76 isolates) in inhibiting the growth of the fungus in Ecuador has been reported, which leaves a margin of maneuver to include these molecules in an integrated RPF management program, the only way for the disease to be maintained at sustainable levels (Tirado-Gallego et al., 2016; Bailey et al., 2018).

For the different doses of the fungicide Difenoconazole (Table 3), the isolates of *M. roreri* showed little sensitivity to doses of 0.1 and 0.01 ppm, where only isolates c14, c18, c20 and c21 presented PIC between 44 and 47%. While for the 1 ppm dose, four strains presented percentages less than 50%, but above 30%.

Table 3: Percentage of growth inhibition of strains of M. roreri against different doses of the fungicide difenoconazole

Strains	**Difenoconazole Dose (ppm)/ Percent Growth Inhibition (%)**				
	100	**10**	**1**	**0,1**	**0,01**
c1	100,0	69,05	56,03	23,67	15,10
c2	100,0	100,0	67,96	31,40	7,37
c3	100,0	100,0	59,04	26,09	18,53
c4	100,0	100,0	45,41	12,03	0,04
c5	100,0	100,0	37,38	0,55	0,04
c6	100,0	100,0	56,99	20,49	1,07
c7	100,0	100,0	59,15	33,88	12,40
c8	100,0	100,0	62,01	34,94	4,68
c9	100,0	100,0	56,20	26,46	20,68
c10	100,0	75,44	59,57	17,50	0,03
c11	100,0	100,0	69,27	33,40	15,89
c12	100,0	100,0	65,64	32,94	5,79
c13	100,0	100,0	71,11	27,80	0,01
c14	100,0	72,04	67,64	46,66	7,45
c15	100,0	51,00	32,11	9,68	1,10
c16	100,0	66,61	50,33	10,60	0,05
c17	100,0	47,22	38,13	25,65	0,12
c18	100,0	57,74	57,21	46,65	2,65
c19	100,0	73,97	72,36	23,19	6,12
c20	100,0	65,02	55,82	44,36	0,21
c21	100,0	75,51	67,99	46,79	7,93
c22	100,0	76,29	52,47	12,71	0,08
c23	100,0	75,08	69,65	35,08	10,67

The effectiveness of Difenoconazole is demonstrated, in the reduction of growth in conditions *in vitro* of *M. roreri and* other

studies report the efficacy of Difenoconazole (75%) in the control of greasy stain (*Mycosphaerella citri* Whiteside) in orange (*Citrus sinensis* (L.) Osbeck) var. "Valencia" (Guillen *et al*., 2018), as well as in the management of l to rust caused by the fungus *Phakopsora pachyrhizi* under field conditions (Ruiz*et al*., 2022), which belong to the group of Basidiomicetos.

Table 4 shows the effect of the fungicide Boscalida on the percentage of inhibition of strains of *M. roreri*, little sensitivity of the isolates was observed at concentrations of 0.1 and 0.01 ppm with percentages of growth inhibition below 50%. At the 1 ppm dose, an increase in PIC was shown in the studied strains, however, only the isolates c1, c2, c3, c6, c11, c12 and c13 slightly exceed 50%.

However, the dose of Boscalida at a rate of 10 ppm demonstrated the greatest influence on the growth of the isolates under in vitro conditions with percentages of inhibition that exceed 50%, reaching in some cases up to 100%, except for strains c21, c22 and c23 that showed ICP between 30.51 and 47.35% (Table 4).

Table 4: Percentage of growth inhibition of strains of M. roreri against different doses of the fungicide boscalida

Strains	Boscalide Dose (ppm)/ Percent Growth Inhibition (%)				
	100	**10**	**1**	**0,1**	**0,01**
c1	100,0	100,0	52,13	8,70	10,61
c2	100,0	100,0	54,72	1,97	1,40
c3	100,0	100,0	52,30	38,27	24,22
c4	100,0	65,85	34,67	6,18	2,39
c5	100,0	100,0	45,43	17,89	4,59
c6	100,0	100,0	55,07	3,41	0,98
c7	100,0	100,0	34,98	19,31	12,20
c8	100,0	100,0	18,15	2,25	1,67
c9	100,0	100,0	38,14	10,02	1,31
c10	100,0	75,79	49,45	10,00	0,60
c11	100,0	78,88	54,74	12,39	3,51
c12	100,0	71,35	53,58	7,93	0,11
c13	100,0	74,61	50,81	5,91	5,84
c14	100,0	72,67	44,55	12,31	5,46
c15	100,0	52,57	27,00	10,00	0,18
c16	100,0	100,0	32,42	15,12	2,44
c17	100,0	65,43	39,60	17,48	0,13
c18	100,0	69,61	47,82	33,96	0,45
c19	100,0	88,11	47,48	22,23	14,12
c20	100,0	94,06	30,71	25,67	24,78
c21	100,0	47,75	29,14	5,94	0,21
c22	100,0	33,42	24,27	20,16	0,27
c23	100,0	30,51	31,43	27,23	23,84

Concentrations of Copper Sulfate Pentahydrate 240 g/L^{-1} in PDA culture medium, they did not achieve adequate growth inhibition percentages on the isolates obtained, which does not suggest the use of this fungicide for the control of *M. roreri* (data not revealed).

It was evidenced that the fungicide Boscalida allowed the inhibition of the growth of strains of *M. roreri* in conditions *in vitro*, although with a higher dose than Azoxystrobin and Difenoconazole. In this regard, the country tested the rotation of systemic and protective fungicides together with the fortnightly removal of diseased fruits, where the best result with the Tega® 75-Antracol® 70-Silvacur® 300-Antracol® 70 rotation, whose incidence was of 26.7 % and a production of 76.9 % of healthy cocoa, compared to the control, whose incidence was 49.7 % and 57.2 % of healthy cocoa. That is, the incidence reduction achieved by this mixture of three fungicides was only 23 %, with an increase of 19.7 % in the production of healthy fruits (Ayala and Nadia, 2008).

CONCLUSIONS

It can be concluded that the fungicides Difenoconazole and Boscalide can be incorporated together with Azoxystrobin for chemical control in the management of Moniliasis disease in cocoa, however, studies are required to evaluate the efficacy of application of these products individually. or mixtures under field conditions. Additionally, it is necessary to carry out research to determine the genetic diversity of isolates of *M. roreri* in relation to the cocoa ecotypes cultivated in the Amazon region belonging to the province of Orellana.

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