



Multivariate analyses to determine fungicide efficacy on Ecuadorian bananas for consumption

Análisis multivariantes para determinar la eficacia de fungicidas en banano ecuatoriano para consumo

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Abstract

Half maximal effective concentration EC_{50} is considered the main reference for evaluating the efficacy of the products in any plantation using doses and inhibition percentages from laboratory data. However, EC_{50} is not the best representation when other relevant variables and their relationships could be involved. As an agricultural case study, fungicide sensitivity of *Pseudocercospora fijiensis*, the causal agent of black sigatoka, was evaluated on bananas' plantations in three provinces of Ecuador. In this study, multivariate statistical process control was adjusted to a fungicide efficacy evaluation case considering multiple data tables from different locations and years at the same time. The threshold conveyed by inhibition percentages, related to the EC_{50} , along with locations and years allowed the multivariate analyses carried out in the proposal. The multivariate statistical control techniques applied were Multilinear Principal Component Analysis (MPCA) and Dual STATIS-Parallel Coordinates approach (DS-PC). A comparison was developed and showed that both methods discriminate correctly between the normal and anomalous conditions within plantations along years, validating the ability of the novel method DS-PC for exhibiting better signaling of anomalous plantations and performing variable-wise analysis to find out possible causes of this behavior in an easier time-saving graphical framework.

Key words: Bananas, fungicide efficacy, black sigatoka, MPCA, DS-PC.

Resumen

La concentración efectiva media máxima CE_{50} se considera la referencia principal para evaluar la eficacia de los productos en cualquier plantación, utilizando dosis y porcentajes de inhibición a partir de datos de laboratorio. Sin embargo, CE_{50} no es la mejor representación cuando otras variables relevantes y sus relaciones podrían estar involucradas. Como estudio de caso agrícola se evaluó la sensibilidad a los fungicidas de *Pseudocercospora fijiensis*, el agente causal de la sigatoka negra, en las plantaciones de banano en tres provincias del Ecuador. En este estudio, el control estadístico multivariante de procesos se ajustó a un caso de evaluación de eficacia de fungicida al considerar múltiples tablas de datos de diferentes ubicaciones y años al mismo tiempo. El umbral transmitido por los porcentajes de inhibición, relacionados con la CE_{50} junto con las ubicaciones y los años, permitieron los análisis multivariados realizados en la propuesta. Las técnicas de control estadístico multivariantes aplicadas fueron el análisis de componentes principales multilineal (MPCA) y el STATIS Dual-coordenadas paralelas (DS-PC). Se desarrolló una comparación que mostró que ambos métodos discriminan correctamente entre las condiciones normales y anómalas dentro de las plantaciones a lo largo de los años, validando la capacidad del novedoso método DS-PC para exhibir una mejor señalización de plantaciones anómalas y realizando un análisis enfocado sobre variables para descubrir posibles causas de este comportamiento en un marco gráfico que ahorra tiempo.

Palabras clave: banano, eficacia de fungicida, sigatoka negra, MPCA, DS-PC.

Introduction

Cultivated species of the genus *Musa*, bananas and plantains, are among the products of major consumption and food relevance worldwide after rice, wheat and milk. Banana and plantain production areas are located in more than 100 countries in tropical and subtropical regions and cover approximately 5.6 million hectares in Ecuador, Philippines, Costa Rica, Colombia, and Guatemala. In Ecuador, bananas are considered the second main non-petroleum exportation product and aimed mostly to household consumption. Globally, the banana industry generates

around 8 billion dollars per year, with a growing demand and economic impact in third world countries, where it represents 75 % of the monthly income of small-scale farmers (FAO, 2018; Marín et al., 2003).

Banana production is mainly threatened by the ascomycete fungus *Pseudocercospora fijiensis*, which manifests with symptoms such as spots and chlorotic streaks (Marín et al., 2003; Stover, 1980). The most effective disease management is through the application of fungicides according to their different modes of action. The fungicides work by interfering in vital processes of the pathogen (Bolaños, 2006; Luna-Moreno et al., 2019; Martínez-Bolaños et al., 2012; Martínez et al., 2011; Romero & Sutton, 1997).

Chemical control is the main tool for black sigatoka management, including alternating applications of protectant and systemic fungicides or mixtures of the two types of fungicides (Brent & Hollomon, 2007). Protectant fungicides present low or no risk of resistance including mancozeb and chlorothalonil, while systemic fungicides exhibit moderate to high risk of resistance and include benzimidazoles, amines, triazoles, strobilurins, anilinopyrimidines, carboxamides and guanidines. *Pseudocercospora fijiensis* has developed resistance to benzimidazoles, triazoles and strobilurins, which has reduced fungicide effectiveness and limited its use in crops (Guzmán, Orozco-Santos & Pérez Vicente, 2013; Martínez et al., 2011). The development of resistance to the fungicides based on the aforementioned groups has increased the use of amines and anilinopyrimidines, which is seen as a risk, due to the increased selection pressure on the pathogen. Therefore, the use of systemic fungicides in banana must comply with the Fungicide Resistance Action Committee (FRAC) recommendations, which consider a maximum of applications per year: triazoles (8), amines (15), strobilurins (3), anilinopyrimidines (8), benzimidazoles (3), carboxamides (4) and guanidines (6) (Guzmán, Orozco-Santos & Pérez Vicente, 2013; Martínez & Guzmán, 2011; Martínez et al., 2011; Russell, 2004).

Half maximal effective concentration EC_{50} is a standard measurement that has been widely used in sensitivity analyses for chemical products in pathogen control, especially in black sigatoka disease, which belong to the most important annual bioassays for decision makers to keep tested fungicides in market. To examine the effectiveness of a fungicide and changes in sensitivity, *P. fijiensis* response is commonly analyzed under the EC_{50} (Bolaños, 2006; Luna-Moreno et al., 2019; Martínez-Bolaños et al., 2012; Martínez et al., 2011; Romero & Sutton, 1997). Traditional analyses on fungicide efficacy take into account doses and inhibition percentages for a regression model from which a dose response curve is determined. The EC_{50} of a dose response curve represents the fungicide concentration that causes the 50 % spores inhibition or the relative growth of pathogen colonies (Bolaños, 2006; Velásquez et al., 2014).

Conventional screening and disease control studies focus exclusively in the EC_{50} standard for fungicide efficacy without considering its relationship with climate and geographic conditions

that affect the black sigatoka growth and dispersion (Jacome & Schuh, 1992; Pérez-Vicente et al., 2000). In this sense, decisions made on a single number, such as EC_{50} , throw away potentially useful information. Considering multiple numbers (variables) at the same time may give certain insights on the data, unavailable within a univariate analysis. That is where multivariate analysis come into play in many areas of life sciences, which assess multiple variables problems (Ali, 2011; Saed-Moucheshi et al., 2013).

In agriculture, one of the major areas of interest for the improvement of productivity in terms of quality and quantity, is the selection of types of fungicides to apply according to pathogens response (Aguirre, Piraneque & Rodríguez, 2015; Barakat et al., 2017; Godoy et al., 2016; Larsolle & Hamid Muhammed, 2007). Hence, our proposal enriches fungicide efficacy analysis by taking into account EC_{50} among other related variables such as location and time. The suggested multivariate approach allows monitoring all the variables at once and their relationships over a four-year period for the evaluation of three fungicides in different plantations distributed in three provinces of major banana production in Ecuador (Guayas, El Oro and Los Ríos). The multivariate statistical techniques applied in this study are Multilinear Principal Component Analysis (MPCA) and Dual STATIS-Parallel Coordinates (DS-PC) strategy, which aim to combine the available data and conclude a single status about fungicide efficacy in terms of normal (expected) versus abnormal (unexpected) *P. fijiensis* sensitivity to the three products evaluated.

Materials and Methods

The database used in this study was structured by tables with microscope measurements of the germ tube longitude derived from fungicide sensitivity analyses. Three products used to control black sigatoka in banana plantations were evaluated with their corresponding active ingredients: Boscalid, Fenpropimorph and Pyraclostrobin. Assays were performed on several locations from three Ecuadorian provinces (Guayas, El Oro and Los Ríos) in four years from 2014 to 2017 as part of the country surveillance for fungicide efficacy analysis. Due to time, costs and environmental conditions, certain locations were not considered in specific years and less product concentrations were evaluated, registering missing values in data tables.

Fungicide sensitivity data

Samples were obtained from treated areas with a conventional management program (based on chemical fungicides). For each analysis, the method suggested by the FRAC for ascospore germ tube elongation version 1 was applied, with few adaptations specified below.

Sampling: Dry necrotic tissue in grade 6 according to the Stover's scale was collected from one block per farm that was exhibiting high disease severity. The necrotic tissue was cut into small pieces (1-2 cm²) and disposed it into paper bags for its transportation to the laboratory, properly labeled.

Incubation: 5 to 9 pieces of tissue were set using staples, onto filter paper of 9 cm diameter with the lower leaf surface facing up and put in humidity chambers at 26 °C for 48 hours. From every location, a minimum of five filter papers were prepared: one for control (without fungicides), and four corresponding to the product concentrations evaluated in parts per million (C0 = 0, C1 = 0.01, C2 = 0.1, C3 = 1 and C4 = 10).

Culture media: A 2 % water agar was prepared (Bacto Agar Difco® 2%. 20g/L), amended with the different concentrations of fungicides and poured into petri plates.

Ascospore discharge: Each filter paper with incubated ascospores was placed on the top of its corresponding petri plate. One hour was allowed for ascospore discharge (extra time may increase the amount of contaminants), after that, the filter paper was removed and the plates were incubated for 48 hours at 26 °C under dark conditions.

Germ tube measurement: A minimum of 30 ascospores were examined for every fungicide concentration using an inverted microscope with micrometric scale and their germ tube lengths (micrometers) were registered in tables with the structure shown at figure 1.

Location – Year – Product					
Nº	Control	1	2	3	4
1	#	#	#	#	#
2	#	#	#	#	#
3	#	#	#	#	#
4	#	#	#	#	#
...

Figure 1. Germ tube lengths table. Structure of the tables used to register the germ tube lengths in each essay. Concentrations are measured in parts per million (ppm) and longitudes in micrometers (µm). Source: author's own elaboration.

Data from these tables was used to perform univariate fungicide efficacy analysis in every year, considering the mode of action of each evaluated product. Results of these analyses are not part of the investigation subject, therefore, are not shown.

Multivariate statistical analyses

To eliminate the effect over the analysis of ascospores' natural growth from every location, mean inhibition percentages were calculated for each concentration per product by comparing

means of the germinative tube longitudes () with the means of control (). Referred percentages were computed using the following expression:

Locations were coded with the initials of the Province and a number accompanied as shown in Table 1.

For organizational purposes, an “assay set” was defined as the collection of matrices with inhibition data (concentrations and mean inhibition) from the three products evaluated for a specific year and location. Assay sets conducted in the same location along different years were considered as different locations labeled as location-years (LYs). A total of 38 assay sets were defined, indicated in Table 1. Based on empirical criteria, assay set of the locations-years from 1 to 28 were used as reference, 29 and 30 to test normal behavior, and 31 to 38 for anomalous behavior.

Table 1. Locations-Years codes and provinces. Codes are established by the year, with the initial of the province and a number ID

Nº	Code	Province	Nº	Code	Province	Nº	Code	Province
1	2014-EO 01	El Oro	15	2016-LR 05	Los Ríos	29	2015-EO 04	El Oro
2	2014-LR 01	Los Ríos	16	2017-EO 01	El Oro	30	2016-EO 02	El Oro
3	2014-LR 02	Los Ríos	17	2017-EO 04	El Oro	31	2014-EO 03	El Oro
4	2015-EO 02	El Oro	18	2017-EO 02	El Oro	32	2014-LR 12	Los Ríos
5	2015-EO 03	El Oro	19	2017-EO 05	El Oro	33	2014-LR 03	Los Ríos
6	2015-GY 01	Guayas	20	2017-GY 05	Guayas	34	2014-LR 05	Los Ríos
7	2015-GY 02	Guayas	21	2017-GY 06	Guayas	35	2014-LR 13	Los Ríos
8	2015-GY 03	Guayas	22	2017-LR 03	Los Ríos	36	2015-GY 05	Guayas
9	2015-LR 03	Los Ríos	23	2017-LR 07	Los Ríos	37	2015-LR 10	Los Ríos
10	2015-LR 04	Los Ríos	24	2017-LR 08	Los Ríos	38	2017-LR 12	Los Ríos
11	2015-LR 05	Los Ríos	25	2017-LR 04	Los Ríos			
12	2016-EO 04	El Oro	26	2017-LR 09	Los Ríos			
13	2016-GY 04	Guayas	27	2017-LR 10	Los Ríos			
14	2016-LR 06	Los Ríos	28	2017-LR 11	Los Ríos			

Source: author's own elaboration.

Data from every matrix was plotted as sequential dots and connected by straight lines, then a geometric Chaikin smoothing was performed (Chaikin, 1974; Rankin, 2013). This algorithm produces an interpolating curve that allows estimating the mean inhibition in a range with dose values according to the analyzed product, as shown in Table 2. Then, for each product, a vector was generated (Figure 2) with estimated values expected to be around 50 % of inhibition.

Table 2. Ranges of concentrations evaluated by product. Every product is associated to its chemical group. Minimum and Maximum concentrations in the range are presented

Product	Chemical group/Group name	Target site	Minimum	Maximum
P1	Carboxamide	Succinate-Dehydrogenase Inhibitors (SDHIs)	1	2.14
P2	Amine	Sterol Biosynthesis Inhibitors (SBIs)	2	4
P3	Strobilurin	Quinone outside Inhibitors (Qols)	1	2.5

Source: author's own elaboration.

Dose values in the referred ranges, having elements within the interval, were generated as a succession according to the following expression:

$$D = \{d_1, d_2, \dots, d_p, \dots, d_n\}, d_i = \exp(\ln(a) + ((i-1) / (n-1))(\ln(b) - \ln(a)))$$

Binding the — vectors from each product in every assay set, this algorithm creates a matrix of Inhibition vs product, for every location-year (Figure 2). All generated matrices were stacked one after another to conform a three-way data block used for statistical analysis (Figure 2).

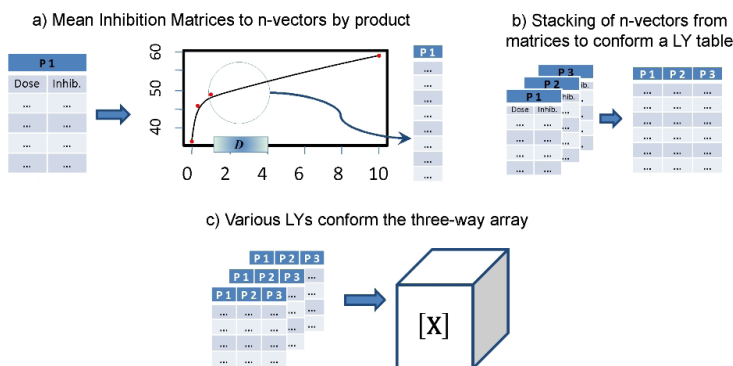


Figure 2. Three-way structuration. Processing of original matrices from the different products creates -vectors of LY tables, which conform the three-way array used in the analysis. Source: author's own elaboration.

MPCA & DS-PC: Both methods are suitable for processing and generating graphic representations of three-way data arrays conformed as sets of tables including mean inhibition values previously computed from the dataset available in the established time frame (2014 to 2017). Every table was preprocessed, first centering with the global mean, then scaling with global standard deviation and normalizing dividing by the number of observations; global mean and standard deviation per variables were calculated from reference information. Later, MPCA and DS-PC methods were computed (Abdi et al., 2012; Inselberg & Dimsdale, 1990; Nomikos & MacGregor, 1994, 1995; Ramos-Barberán et al., 2018; Rousseeuw, Ruts & Tukey, 1999).

Comparison: In order to validate and compare DS-PC strategy, MPCA is used as a reference. In these methods, the three ways often are: observations/times, variables and batches; analogous, the data block in this study has: observations, products and location-years. As mentioned in the multiway structuration, three groups of LYs were conformed: one reference set to state the typical behavior, and the others to represent “normal” and “anomalous” behavior to test the discriminative ability of the methods.

Principal components factor scores for LYs from reference set were computed in MPCA and Interstructure from DS-PC according to Lu et al. (2008) and Ramos-Barberán et al. (2018) respectively. Then, the bagplot-based control charts (CC) were set up as in DS-PC method for both cases; after that, the normal and anomalous LYs were projected to examine their positions in the CCs. Additionally, CCs for each product were defined and LYs were projected using the STATIS Dual intrastructure. Finally, Parallel Coordinates plots for the normal and anomalous LYs were created to confirm the behavior of the LY and to investigate the mean inhibition values by products. All calculations were performed in the open source statistical programming software R.

Results

MPCA

The Principal Components scores plot from MPCA and its control region (Figure 3) shows that every reference and normal LYs are inside; on the contrary, anomalous locations are outside the region. Normal LYs stand for expected *P. fijiensis* sensitivity while anomalous locations refer to unexpected sensitivity due to larger distances to the centroid in comparison to the reference ones.

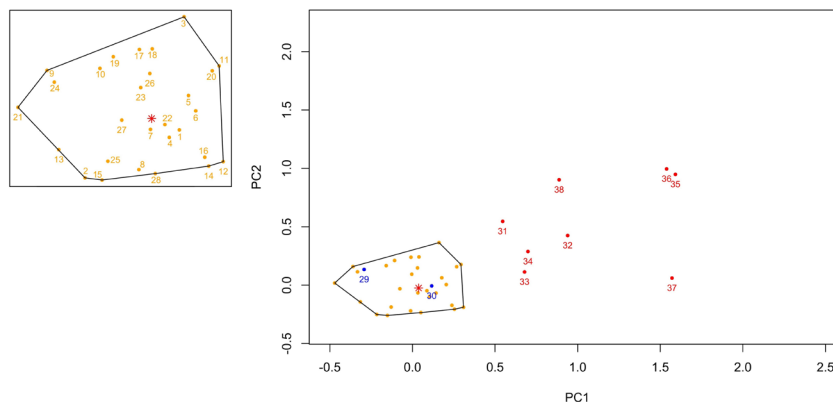


Figure 3. MPCA control chart. Color coding for projected LYs is the following: yellow for reference, blue for normal and red for anomalous. Source: author's own elaboration.

DS-PC strategy

Figure 4 shows DS-PC Interstructure. Anomalous LY are clearly different from the reference ones. The distances to the centroid, are larger than those observed in MPCA. Also, as in MPCA, normal and reference LYs remain inside the control region, while anomalous LYs fall outside. Spatial configuration of anomalous LYs are similar, comparing to MPCA results.

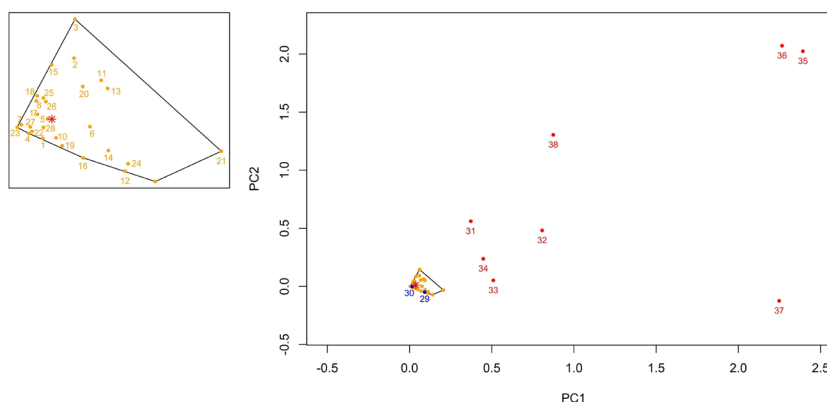
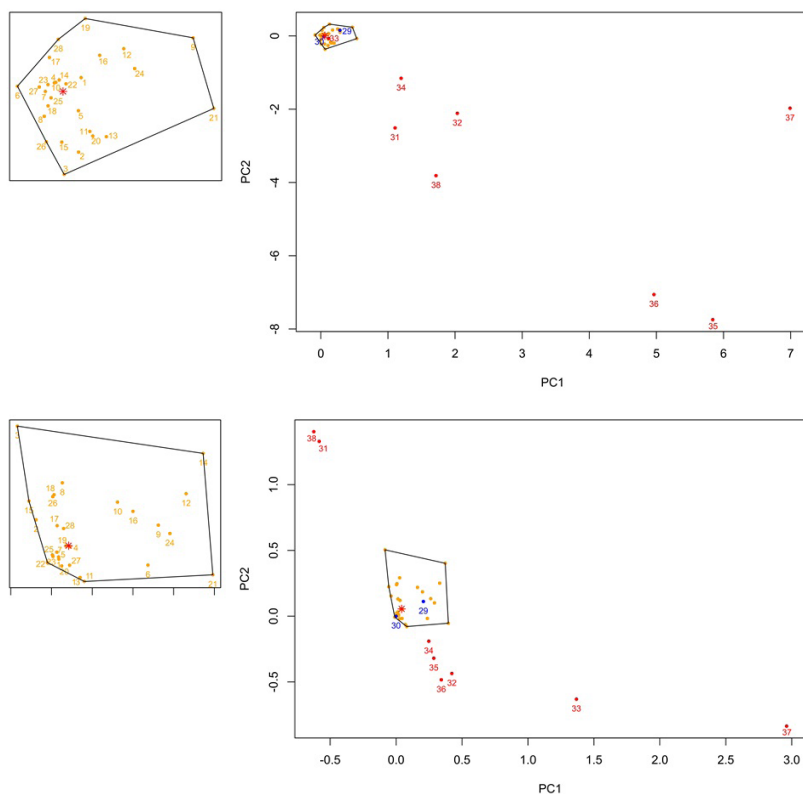


Figure 4. Interstructure control chart. To the left, a zoom image of the reference region. To the right, general view of all the projected LYs; PC1 and PC2 values in the chart from the right is scaled to match with the figure 3. Color coding for projected LYs is the following: yellow for reference, blue for normal and red for anomalous. Source: author's own elaboration.

Most of the LYs outside belong to Los Ríos province, which is likely because of prevailing temperatures between 25–30 °C and relative humidity around 95 % favored the life cycle of *P. fijiensis*, which is strongly determined by weather and microclimate (de Jesus et al., 2008; Marín et al., 2003). Like many foliar fungal pathogens, *P. fijiensis* ascospores are dispersed by wind and require a wet leaf surface or very high relative humidity to germinate and infect the leaf, and the rate of germination and infection during wet or humid periods is governed by temperature (Uchôa et al., 2012). These environmental conditions, associated to location and time, promote an ideal scenario for banana plants development and black sigatoka growth and dispersion (Jacome & Schuh, 1992; Pérez-Vicente et al., 2000).

When it comes to the products CCs (Figure 5), all anomalous LYs remain outside, which means unexpected *P. fijiensis* sensitivity to at least one of the fungicides tested. Nevertheless, the distances to the centroid are different. In P1 CC and P3 CC, LYs 35 and 36 stay far from the centroid, but in P2 CC, stay close, still outside, but relatively close. LY 37 is far in P1 CC and P2 CC, but in P3 CC gets a little closer. In general, anomalous LYs tend to get far in a specific direction. However, in P2 CC, LY 31 and 38 appear in the opposite direction, in comparison to the other anomalous LYs, although still outside.



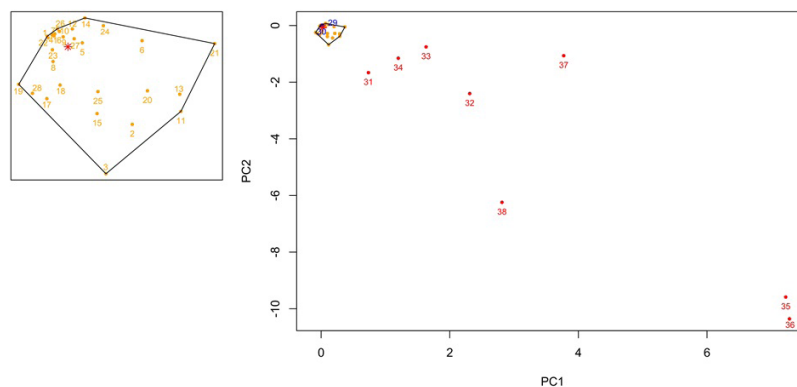


Figure 5. Intrastructure control charts, by product (From top to bottom: P1CC, P2CC, P3CC). All anomalous LYs appear outside the control regions, nevertheless, the distribution observed is different. Color coding for projected LYs is the following: yellow for reference, blue for normal and red for anomalous. Source: author's own elaboration.

Parallel Coordinates show mean inhibition values by product from every LY in black, and reference LYs in gray. A red line connects the global means by product. In figure 9, normal LYs (29 and 30) are inside the gray zone, as expected. LY 29 is above the mean for products P1 and P2; on the other hand, LY 30 is under the mean for product P1.

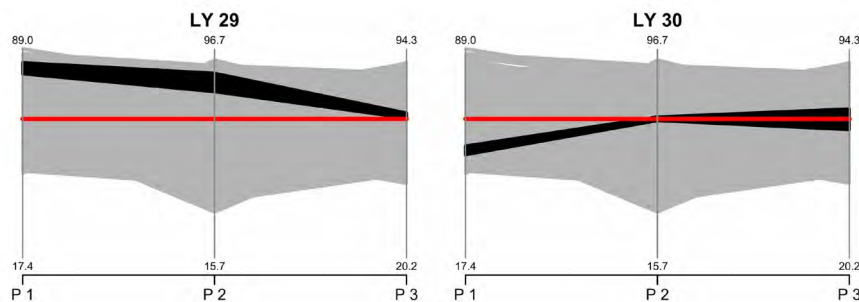


Figure 6. Parallel coordinates for normal LYs. Black lines, which represent normal LYs, stay inside the zone conformed by gray lines of the reference LYs. Source: author's own elaboration.

Anomalous LYs are clearly different, as observed in figure 10. LY 34 has the greater dispersion in product P2, but still stays inside the gray region. Most of the anomalous LYs (except 33 and 37), show normal values for product P2. In addition, LY 31 and 38 have inhibition values above the global mean. For product P1, all except LY 33, present low inhibition values. And all the eight anomalous LYs have low inhibition values for product P3.

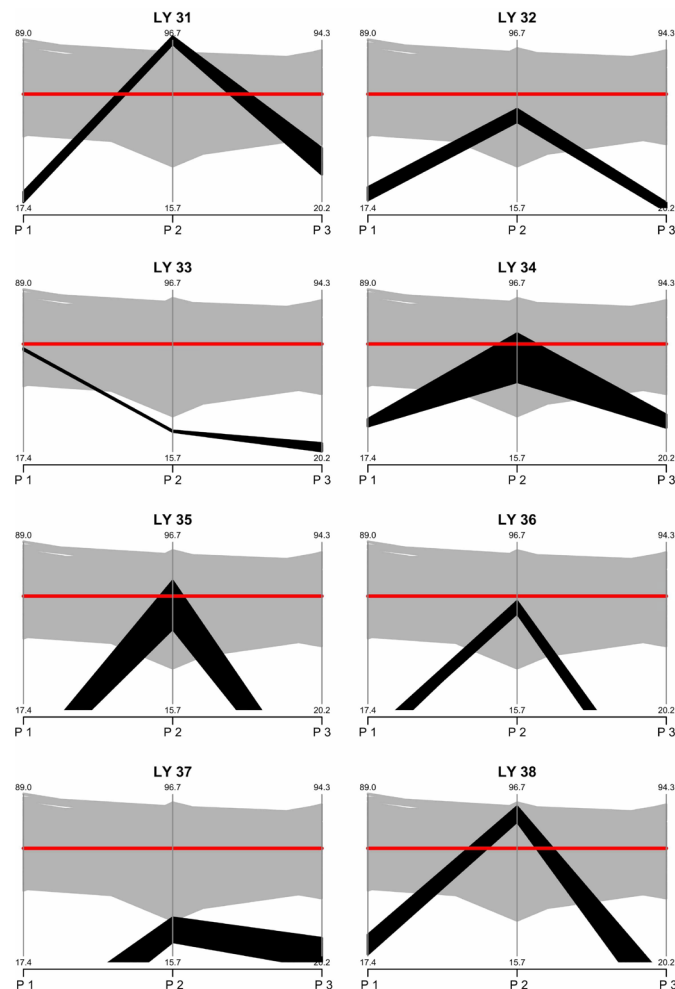


Figure 7. Parallel coordinates for anomalous LYs. Black lines, which represent anomalous LYs, fall outside the zone conformed by gray lines of the reference LYs. Source: author's own elaboration.

Analysis by product (variable-wise analysis from DS-PC strategy) shows LYs more far or close depending on the difference with reference values as shown by Parallel Coordinates, and also, a shift in the direction of projected points related to the kind of differences in the values (higher or lower), which is the case of LYs 31 and 38, whose positions in P2 CC are in a different direction due to the high values presented in product P2. LYs like 35 and 36, which stay closer to the control region in P2 CC, rather than in P1 CC and P3 CC, present the same behavior in Parallel Coordinates, this confirms that the LYs is anomalous, but the product P2 isn't the cause of this anomaly.

Discussion

The threat for resistance development in important pathogens to FRAC groups is one of the biggest concerns in banana crops. The resistance of *P. fijiensis* in banana plantations to fungicides provides compelling evidence that integrated crop management should include fungicide efficacy analysis and other components related to locations and years. Nevertheless, the widely used of EC_{50} allows to evaluate fungicide efficacy only in one dimension, losing other components behaviors needed to develop effective strategies for the management of black sigatoka in banana crops (de Jesus et al., 2008; Guzmán et al., 2013; Marín et al., 2003; Martínez et al., 2011). MPCA and DS-PC performed a correct discrimination of the location-years with normal and anomalous behavior, which validates the capability of the multivariate statistical analyses to monitor black sigatoka in terms of fungicide efficacy within banana plantations. Discriminative ability of DS-PC strategy is better than MPCA, comparing the patterns shown in the interstructure control charts (IS CCs), where normal LYs (expected sensitivity) exhibited a consistent grouping tendency and abnormal LYs (unexpected sensitivity) displayed greater distance from the control region. Although MPCA has shown better performance in variables profiles depending on time, instead of observations, DS-PC analysis possibilities are wider than MPCA in the present study where intrastructure control charts (CO CCs) and parallel coordinates revealed tested fungicides performance for each LY (location-year) (Nomikos & MacGregor, 1994; Ramos-Barberán et al., 2018).

The main advantage of the multivariate approach (MPCA and DS-PC) discussed herein for fungicide efficacy analysis is the possibility of capture the variability between locations, years, products and different observations to conclude a single status of “normal” or “anomalous” (expected versus unexpected *P. fijiensis* sensitivity). Furthermore, the stated use of several inhibition values is more accurate than studying a single estimated score, which leave out the relationships of sensitivity with other associated variables that provide a better understanding of pathogens and diseases’ local dynamics over time. Similarly, it would be like comparing the information provided by the full video versus a frame.

Lack of abnormal trend along the years allows to conclude that anomalous behavior of the fungus can’t be generalized because genetic material affecting banana plantations in Ecuador is continuously renewing. For instance, location LR 10 belonging to Los Ríos province exhibited in 2015 abnormal behavior changing to normal condition 2 years later. Thus, present analysis couldn’t compile enough statistical evidence to assure that low sensibility trend is persistent, therefore, it can’t be said that resistance is showing up in the studied areas. Yet, if we take a deeper look into anomalous location-years through parallel coordinates, in 7 cases carboxamides and strobilurins didn’t perform efficiently, meaning that black sigatoka showed decreased sensitivity to these fungicides, while amines exhibited the expected response. If this trend continues, the behavior stated by previous studies regarding amines would remain similar in Ecuador (Marín et al., 2003; Martínez & Guzmán, 2011; Martínez et al., 2011).

In Ecuador, studies about triazoles and organic fungicides efficacy evaluation have been carried out, by means of percentages of infection and lethal concentration-50 (LC_{50}) (Caicedo, 2015; Chávez, 2012; Diaz-Trujillo et al., 2018; Sabando, 2015), therefore it is not possible to make comparisons with the present study. Monitoring of efficacy can thus be used as an indicator of the possible development of resistance, with any reductions in efficacy below an agreed threshold value (Russell, 2004). For further research, it is necessary that Ecuador, as a major banana exporter, starts collecting evidence of efficacy data to build a baseline in terms of conventional univariate analysis but also, multivariate ones.

Conclusion

In this study, a fungicide sensitivity of *Pseudocercospora fijiensis*, the causal agent of black sigatoka, was evaluated on bananas' plantations in three provinces of Ecuador: Guayas, El Oro, and Los Ríos. This approach took into account the threshold conveyed by inhibition percentages, related to the EC_{50} , along with locations and years allowing the multivariate analyses. Both methods MPCA and DS-PC discriminate correctly between the normal and anomalous conditions within plantations along years. The novel application of these multivariate analyses posed a significant advantage in terms of monitoring and control within plantations. However, the ability of the DS-PC strategy for exhibiting better signaling of anomalous plantations and performing variable-wise analysis was evidenced in an easier time-saving graphical framework.

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