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ORIGINAL ARTICLE

Assessment of sugarcane cultivars with stable reaction to *Xanthomonas albilineans* under mechanical inoculation conditions

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Abstract
Leaf scald caused by *Xanthomonas albilineans* in sugarcane is one of the most important diseases around the world since it causes severe losses in both agricultural and industrial yields. In Cuba, resistance to this disease is considered a major selection criterion within the breeding program. The aim of this work was to detect sugarcane cultivars with stable reaction to *X. albilineans* by using both additive main effects and multiplicative interaction (AMMI) and linear general models. For this, 16 cultivars planted simultaneously in 2015, 2016, and 2017 in two locations: Jovellanos (Matanzas) and Florida (Camagüey) in Cuba were mechanically inoculated with a bacterial isolate previously characterized by sequencing its DNA fragment. Disease severity was recorded in plant cane and first ratoon, and results were consistent in both analyses and revealed that L55-5 and C323-68 were the most susceptible cultivars and C1051-73 was the most resistant; however, C1051-73, C275-80, C86-12, C88-382, C89-147, My5514, and Ty86-28 were the most stable across the years and localities evaluated. Results will allow adapting the methodology for the evaluation of the reaction to leaf scald of new sugarcane cultivars.

Keywords AMMI · Sugarcane cultivar · Resistance reaction · *Xanthomonas albilineans*

Introduction

Xanthomonas albilineans (Ashby) Dowson is the causal agent of sugarcane leaf scald. This disease constitutes a threat in more than 65 tropical countries where this crop is grown. It causes severe economic damage due to high losses in tons of cane per hectare and reduced juice quality (Rott and Davis 2000; Alvez et al. 2016).

Symptoms of the disease include the pencil line (one or more long narrow, white stripes, parallel to the midrib with well-defined edges), bleaching, chlorosis, and necrosis. In some cases, the stripes can progress to the sheath and stem, become necrotic, and the necrosis can expand to encompass the whole leaf. Young shoots and stalks may die, and shoots exhibiting leaf symptoms may develop from axillary buds in mature stalks (Hoy and Grisham 1994).

Symptom severity depends on a complex interaction between genetic traits and environmental factors. Stress conditions such as drought, waterlogging, and low temperatures are determinant of disease severity (Ricaud and Ryan 1989).

Leaf scald was first detected in Cuba in 1978, where 14 outbreaks distributed in seven provinces were reported until 1982 and a new one in 2000 (Díaz 2000). Currently, the incidence and distribution of leaf scald in Cuba remain controlled in commercial production areas, according to adopted cultivar management measures. However, in susceptible cultivars, cultural yield was reduced by more than 40% and sugar yield losses exceeded 20% (Pérez Pérez et al. 2019).

In order to control leaf scald different phytosanitary measures such as the exchange of pathogen-free materials between countries, through quarantine procedures and the disinfection of cutting implements to avoid the spread of *X. albilineans* must be adopted (Rott and Davis 2000). However, the use of

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resistant cultivars is considered the most efficient and economical approach, for that reason sugarcane breeding programs has established leaf scald resistance as a major selection criterion.

In resistance assays, the use of control cultivars is important as they represent a guide of the most probable reaction against diseases. These allow the new clones to be classified and provide a measure of the reliability of each test (Magarey et al. 2011). Besides, the moment and the way in which the evaluation is carried out for each of the diseases within the selection scheme of a breeding program depends on the following factors: the easy of the procedure, the economic importance of the disease, the resistance of the plant material, the quality of seed and soil, and the reliability or repeatability of the test (Comstock and Sood 2013).

Traditionally, disease resistance tests to classify the behavior of cultivars were based on the observation of symptoms. Recently, the development of novel diagnostic techniques and the application of statistical models favored the establishment of precise procedures that make the classification of cultivars by resistance level more reliable (Stringer et al. 2013; Persaud and Saravanakumar 2018). Taking into account the high variability observed in the resistance test for sugarcane, statistical methods that allow adjusting results to each evaluation conditions are required. In that sense, the use of additive main effects and multiplicative interaction (AMMI) model constitutes a valuable tool for the differential study of genotypes under different environmental conditions. In addition, it favors the understanding of the genotype×environment interaction (Persaud et al. 2019). Besides, linear general models describe a continuous response variable as a function of one or more predictor variables (Harrell 2001). This model could help to understand and predict the behavior of complex systems and could allow validating AMMI results.

In Cuba, to date, the procedure for evaluating resistance to leaf scald consists of mechanical inoculation with *X. albilineans* and the use of an empirical quantitative scale for the visual diagnosis of symptoms, at the final stages of the selection scheme in the two localities with the highest prevalence of the disease. From these evaluations, all susceptible materials are eliminated (Jorge et al. 2011). However, there are no control cultivars available that represent the different degrees of the evaluation scale. Therefore, the present work aimed to detect genotypes with stable behavior to leaf scald in two localities with high inoculum pressure in order to increase the selection efficiency of the sugarcane breeding program, by using AMMI and linear general models.

Materials and methods

Isolation and identification of *X. albilineans* for inoculum preparation

X. albilineas strain Xa-74 from INICA Collection of Microbial Cultures was used. Its pathogenicity was reactivated on healthy plants of the susceptible cultivar L55-5. It was re-isolated in modified Wilbrink culture medium (Rott 1995) from a sample formed by 2-cm^2 fragments, collected from five + 1 leaves (according to Kuijper's nomenclature) of plants that expressed pencil line symptom.

Total DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega). A 288 base pair (bp) DNA fragment, specific for *X. albilineans*, was amplified with the PGBL1 / PGBL2 primer pair proposed by Pan et al. (1999).

Besides, in order to reconfirm the identity of the bacterial isolate, a 1.3-kb fragment coding for 16S rDNA was amplified with the primer pair p13B: AGGCCCGGGAAG GCGTATTCAC and PCR-1: AGTTTGATCCTGGCTCAG GA (Hung and Annapurna 2004). The amplified product was visualized by 1% agarose gel electrophoresis, followed by staining in GelRed 0.001% (Biotium, USA) and visualization under UV light. Direct sequencing of the 16S rDNA PCR product was performed on an automated sequencer (ABI PRISM® 377, Hitachi, Japan) with the sequencing reagent set (Perkin-Elmer ABI-PRISM Dye Terminator Cycle sequencing kit). The partial consensus nucleotide sequence of the 16-23S rDNA was generated with sequences obtained in both directions. Subsequently, the resulting assembly sequence was analyzed with the BLASTN program (Altschul et al. 1997) to reconfirm the identity of the samples.

The sequence was deposited in the GenBank database (www.ncbi.nlm.nih.gov/genbank.com), and subsequently compared with 25 other sequences corresponding to isolates of *Xanthomonas* sp. (Table 1) with the ClustalX algorithm (Thompson et al. 1997). Its phylogeny was determined by means of MEGA5 program (Tamura et al. 2013), using the near neighbor method with a bootstrap analysis of 1000 repetitions.

Assessment of leaf scald resistance

For the evaluation of resistance to *X. albilineans*, the following sixteen cultivars, B80250, C1051-73, C275-80, C323-68, C86-12, C86-503, C88-382, C89-147, C91-73, Co997, Ja64-19, L55-5, My5514, PR980, SP70-1284, and Ty86-28, were planted in two localities: Jovellanos (Matanzas) and Florida (Camagüey) in Cuba in November of the following 3 years: 2015, 2016, and 2017. Genotypes belong to the germplasm bank of the local sugarcane breeding program; where "B" variety comes from Barbados; "Co" from Coimbatore, India; "L" from Louisiana, USA; "PR" from Puerto Rico; "SP" from Sao Pablo, Brazil; "Ja" of Jaronú; "My" of Mayari and "Ty" of Tayabiyo from Cuba; and "C" also from Cuba. L55-5 is a standard international genotype susceptible to leaf scald and the

Table 1 *Xanthomonas* sp. sequences available at GenBank, used for phylogenetic analysis

GenBank accession number	Description	Origin
AY940629.1	*Xanthomonas albilineans*	Brasil
AF209751.1	*Xanthomonas albilineans*	Brasil
AY940633.1	*Xanthomonas albilineans*	Brasil
AF251154.1	*Xanthomonas albilineans*	EUA
HM181909.1	*Xanthomonas albilineans*	Hawai
JN859042.1	*Xanthomonas arborícola* pv. *juglandis*	Irán
GU144279.1	*Xanthomonas arboricola* pv. *poinsettiicola*	China
AJ936965.1	*Xanthomonas arborícola* pv. *pruni*	Francia
HM469666.1	*Xanthomonas axonopodis*	Hawai
AF442744.1	*Xanthomonas axonopodis* pv. *citri*	EUA
AF442741.1	*Xanthomonas axonopodis* pv. *citrumelo*	EUA
EU203152.1	*Xanthomonas axonopodis* pv. *dieffenbachiae*	EUA
AY576648.1	*Xanthomonas axonopodis* pv. *dieffenbachiae*	EUA
GU144276.1	*Xanthomonas axonopodis* pv. *poinsettiicola*	China
GU144281.1	*Xanthomonas axonopodis* pv. *poinsettiicola*	China
AY940638.1	*Xanthomonas axonopodis* pv. *vasculorum*	Brasil
AY940637.1	*Xanthomonas axonopodis* pv. *vasculorum*	Brasil
AF060175.1	*Xanthomonas campestris* pv. *vitians*	EUA
AF123093.2	*Xanthomonas gardneri*	EUA
GQ461740.1	*Xanthomonas perforans*	Korea
AY940632.1	*Xanthomonas sacchari*	Brasil
AY940636.1	*Xanthomonas* sp.	Brasil
AY940641.1	*Xanthomonas* sp.	Brasil
JQ975047.1	*Xanthomonas* sp.	EUA
FJ827774.1	*Xanthomonas* sp.	India

only control employed in resistance test in breeding programs in Cuba. The other 15 genotypes are commercial varieties, although some of them are no longer used nowadays (C275-80, C86-503, C88-382, C91-73 and PR980).

Seed cane, hydro heat thermo treated at 50.5 °C for 2 h, obtained from the Basic Seed Bank of each locality and certified as pathogen free by specific PCR diagnosis, was used for the trials (30-stem segments with two buds per variety and replicate). A randomized block design with two repetitions was used where each plot was formed by a 5-m row of each cultivar.

The mechanical inoculation of the bacteria was carried out 45 days after sprouting (around April of the following year of plantation), with the *X. albilineans* Xa-74 isolate. The method employed was decapitation, by using a scissors previously submerged in the bacterial suspension (2×10^8 cfu/ mL) (Rott et al. 1997).

Symptoms were monthly observed in the field to evaluate leaf scald in sugarcane, during 2016 to 2019 period. Results were recorded on November of the following 2 years of plantation (plant cane and first ratoon). All the inoculated stems were individually classified, using a scale of severity of symptoms of five classes (from 1 to 5 grades). The mean severity of infection (DS) was determined with the Townsend-Heuberger formula (Townsend and Heuberger 1943) as follows: $DS = [(1 \times FL + 2 \times ML + 3 \times CB + 4 \times N + 5 \times D) / 5 \times T] \times 100$, where FL = number of stems with one or two chlorotic lines on the leaves (grade 1), ML = number of stems with more than two chlorotic lines on the leaves (grade 2), CB = number of stems with chlorosis or generalized discoloration of the leaves (grade 3), N = number of stems with leaf necrosis (grade 4), D = number of dead stems or stems with lateral shoots (grade 5), and T = total number of stems. The disease severity varies between 0 for the most resistant cultivars and 100 for the most susceptible ones.

The disease severity data collected from the 16 cultivars, 3 years of plantations, 2 years of evaluations, and two test locations for resistance to leaf scald were adjusted to the AMMI statistical model described by Gauch (2013). The analysis of variance (ANOVA), principal component analysis, and AMMI biplot procedures were performed with Statictic v8.1 software. Conventional ANOVA first adjusted main additive effects of cultivars and environments (combination of years and locations). Subsequently, the multiplicative effects of the "genotype × environment" interaction were adjusted by means of a principal component analysis.

Besides severity data obtained were fitted to a linear general model as proportion of severity by using InfoStat software (Di Rienzo et al. 2018) and its interface with R-project (R). The model describes the relationship between a dependent or response variable y as a function of one or more independent variables or predictors, Xi. The general formula is $y = \mu + \tau X_i + \varepsilon_i$, where μ represents the general mean, τ represents the treatment effect (environment factor, cultivar factor and their interaction), and ϵ represents the error terms, where ε_i is iid Normal (μ, σ^2); that is, the error terms are independent and identically distributed following a normal distribution with mean μ and variance σ^2. For the analysis cultivar factor, environment (year and locality) factor, "cultivar × environment" interaction, and block were included in the model as fixed effect.

Results

Identification of *X. albilineans*

A fragment of 288 bp was amplified by using PGBL1/PGBL2 primer pair proposed by Pan et al. (1999) from

total DNA isolated from strain Xa-74. The 1.3-kb fragment coding for 16S rDNA amplified with the primer pair p13B/PCR-1 (Hung and Annapurna 2004) was sequenced in both direction, and the consensus sequence was deposited in GenBank with the accession number MT648445. The phylogenetic analysis including other *Xanthomonas* sp. sequences from different geographic regions is shown in Fig. 1. All *X. albilineans* sequences grouped together, including the strain from Cuba, whereas other species of *Xanthomonas* belong to other groups.

Assesment of leaf scald resistance

AMOVA of the severity of leaf scald evaluated between 2016 and 2019 (Table 2) showed significant differences ($p \leq 0.01$) between cultivars, years of evaluation, and localities. Interactions between "cultivar × year," "locality × year," and "cultivar × locality × year" were observed (Table 3). No interaction between "cultivar × locality" was detected, suggesting cultivars do not vary their response according to locality; however, their response is dependent on the conditions of each particular year.

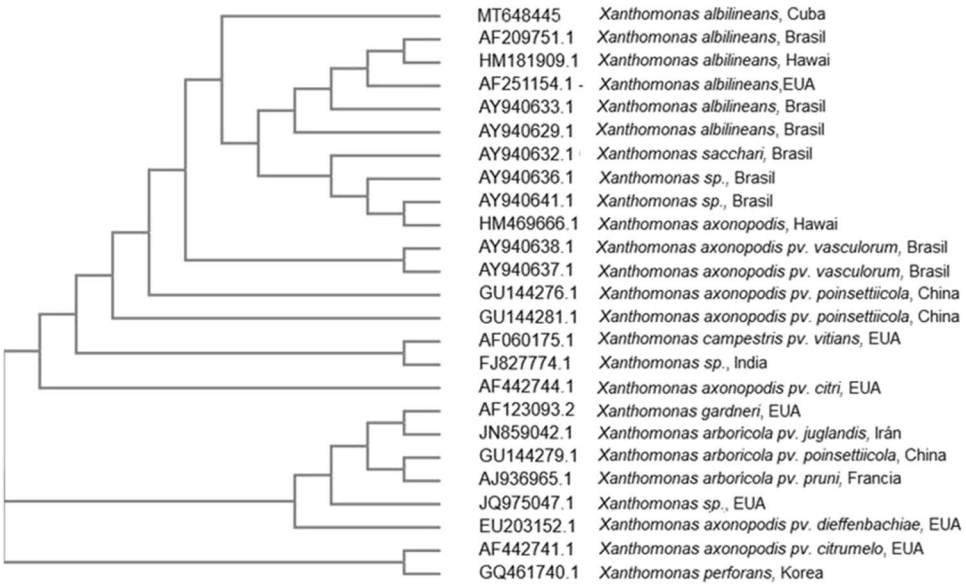


Fig. 1 Phylogenetic tree of 16-23S DNAr of *Xanthomonas* sp. sequences, constructed using the near neighbor method with a bootstrap analysis of 1000 repetitions

Table 2 Mean of disease severity between plant cane and first ratoon of the 16 sugarcane genotypes evaluated under mechanical inoculation conditions against *X. albilineans* for three different years of plantations at two locations in Cuba

Cultivar	Year of plantation					
	2015		**2016**		**2017**	
	Jovellanos	**Florida**	**Jovellanos**	**Florida**	**Jovellanos**	**Florida**
B80250	1.74	37.52	38.03	1.35	1.41	2.88
C1051-73	7.82	7.07	7.07	1.17	1.24	10.03
C275-80	15.02	14.86	16.36	8.61	7.06	17.23
C323-68	1.36	26.08	24.63	42.14	39.66	1.90
C86-12	9.16	10.97	12.47	17.68	14.67	11.40
C86-503	7.29	33.97	32.97	18.11	14.60	9.63
C88-382	5.67	17.58	17.58	8.29	5.80	9.62
C89-147	19.26	12.12	11.12	19.24	17.13	24.73
C91-73	14.74	30.47	31.98	1.29	1.31	17.76
Co997	2.12	1.26	1.27	36.23	34.83	3.13
Ja64-19	1.24	1.49	1.49	26.19	22.67	2.29
L55-5	48.89	13.62	12.62	8.99	6.08	50.78
My5514	16.37	18.18	17.66	9.43	7.94	19.44
PR980	7.05	28.63	26.63	37.62	36.56	7.53
SP70-1284	1.67	15.30	13.80	37.87	34.28	1.82
Ty86-28	14.35	24.39	26.89	14.60	12.21	18.72

The existence of significant interactions allowed the application of the AMMI model. The graphic representation of components 1 and 2, which explain 80.8% of the variance contained in the interaction, reflected the existence of similar severity levels in different years and locations (Fig. 2). This result also reinforced that susceptibility to leaf scald is related to the environmental conditions occurred in each year of evaluation.

The AMMI1 representation showed differences in the resistance responses of the cultivars, as well as in their stability (Fig. 3). The most susceptible cultivars were L55-5, PR980, and C323-68, whereas C1051-73 was the most resistant. In the years and localities evaluated, C86-503, Ty86-28, C89-147, My5514, B80250, C86-12, C275-80, C88-382, and C1051-73 were the most stable cultivars.

When linear model was adjusted to the proportion of severity, the interaction "environment × cultivar" was significant ($p < 0.0001$). The graphic representation of the model (Fig. 4) revealed that the cultivars that showed the highest severity values in at least two environments were L55-5 and C323-68. Both genotypes were among the most susceptible detected by AMMI, whereas C1051-73 was the most resistant cultivar, detected by both models. Cultivars that presented similar values among evaluated environments (those with the most stable behavior against *X. albilineans*) were C1051-73, C275-80, C86-12, C88-382, C89-147, My5514, and Ty86-28. These seven cultivars were among the nine most stable ones detected by AMMI and could be used as controls in resistance tests to leaf scald.

Table 3 Effects of sugarcane cultivars. localities and years over leaf scald severity

Source of variations	DF	SS	MS	F	Statistical significance
Cultivars	15	137.697	9.18	340.7	*
Localities	1	2.856	2.856	106	*
Year	2	15.009	7.505	278.6	*
Cultivars × locality	15	0.548	0.037	1.4	ns
Cultivars × year	30	134.905	4.497	166.9	*
Locality × year	2	29.996	14.998	556.7	*
Cultivars × locality × year	30	438.293	14.61	542.3	*
AMMI1	17	241.06	14.18	525.19	*
AMMI2	27	113.96	4.22	156.32	*
Error	192	5.173	0.027		

DF degrees of freedom, *SS* sum of squares, *MS* mean square, *ns* non significant.

Discussion

Leaf scald of sugarcane, caused by *X. albilineans*, is one of the major diseases of sugarcane (Ricaud and Ryan 1989) since several outbreaks occurred in different countries (Rott et al. 1994).

The molecular methods currently used for *X. albilineans* detection allow its specific identification (Alvez et al. 2016; Ntambo et al. 2019), especially considering the frequent

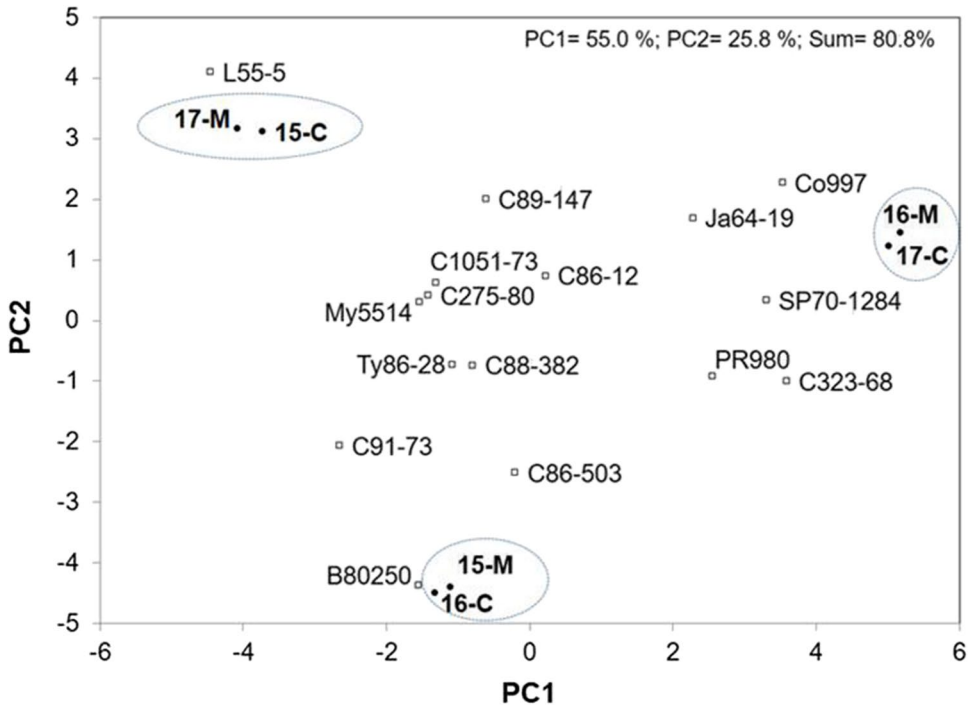
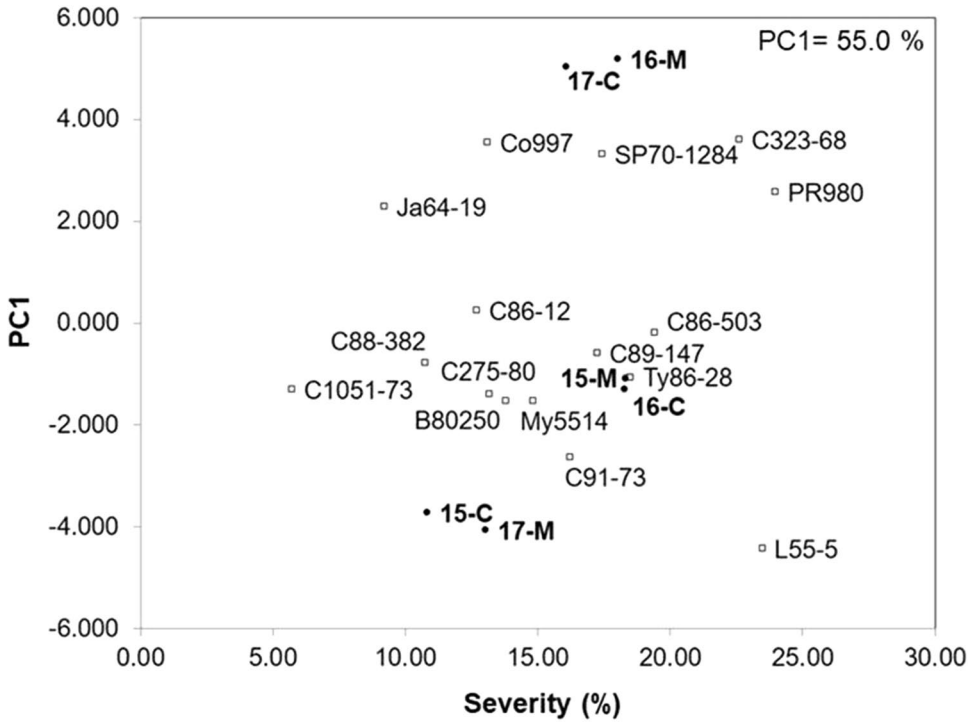


Fig. 2 Biplot of the AMMI2 model that shows similarities and differences between the different cultivars, localities, and years evaluated. C: Camagüey, M: Matanzas, 15: year 2015, 16: year 2016, and 17: year 2017



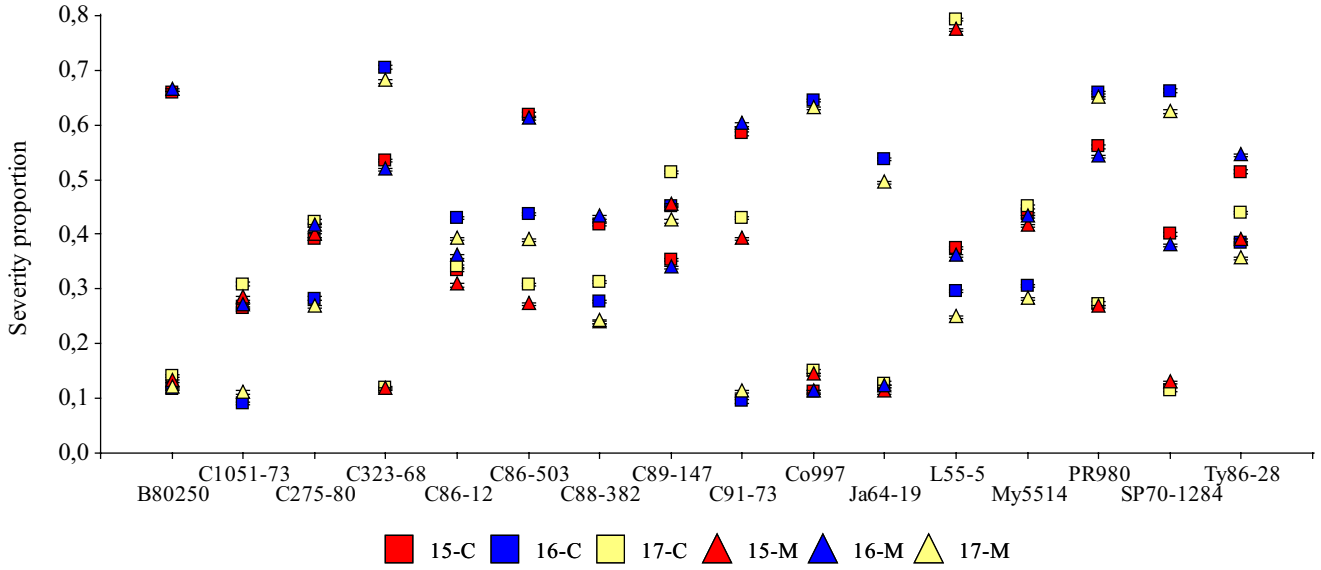


Fig. 3 Principal component 1 (PC1) and severity of leaf scald of 16 cultivars evaluated in two locations and three years. C: Camagüey, M: Matanzas, 15: year 2015, 16: year 2016, and 17: year 2017

Fig. 4 Proportion of severity of leaf scald for the 16 cultivars evaluated in two locations and 3 years (six environments). C: Camagüey, M: Matanzas, 15: year 2015, 16: year 2016, and 17: year 2017

occurrence of latent infections that makes difficult the diagnosis based on visual symptoms. In this work, PCR was effectively used to characterize the bacterial isolate used as inoculum to evaluate the resistance of sugarcane cultivars. In the phylogenetic analysis, *X. albilinenas* isolates clustered together, regardless of their geographical origin.

The inoculation technique employed allowed the symptom expression in the evaluated sugarcane cultivars. Out of the interactions considered, only the "cultivars × locality" interaction did not show significant differences. Environmental conditions associated with each particular year could influence the response of the cultivars. In that sense, another previous research showed a high correlation between

rainfall, populations of the bacteria on the surface of leaves, and the percentage of infected stems (Champoiseau et al. 2009). The opening of the stomata causes an increase in the exchange of substances between the internal and external surfaces of the leaf, favoring the colonization of the tissues by *X. albilineans*.

On the other hand, the choice of appropriate statistical models is crucial before carrying out any study of "genotype × environment" (G × E) interaction and analysis of stability. Due to in sugarcane, proportion of non-linear component of G × E interaction is very high. AMMI model is one of the best models for studying G × E interaction (Bajpai and Kumar 2005). In that sense, several previous studies such as Kulshrestha et al. (2001), Srivastava et al. (2001), Kumar et al. (2009), and Silveira et al. (2013) among others have successfully used AMMI model to study the G × E interaction in the sugarcane. However, this would be the first time where AMMI model is employed to study stability for a given disease under mechanical inoculation conditions. Besides, linear general model validates most of the results obtained by using AMMI. Out of the three susceptible cultivars detected by AMMI, two were also detected by linear general model as susceptible (L55-5 and C323-68). Both models revealed that C1051-73 was the most resistant cultivar. Out of nine cultivars with the most stable behavior against *X. albilineans*, seven were also found by linear general model (C1051-73, C275-80, C86-12, C88-382, C89-147, My5514, and Ty86-28).

For an adequate leaf scald management program, cultivars that show stable and different responses to *X. albilineans* in different environments can be used as controls in mechanical inoculation tests. Until now, highly susceptible cultivars (with a reaction similar to the L55-5 control) are discarded; however, the selection of other control cultivars contributes to a more precise classification of new cultivars. AMMI analysis and biplot visualization facilitated a better understanding of the G x E interaction, as well as of the relationship between disease manifestation and environmental conditions. In addition, the identification of cultivars with stable reaction is useful for breeding programs that select resistant cultivars as an strategy to control leaf scald.

The following cultivars C1051-73, C275-80, C86-503, C88-382, L55-5, My5514, and Ty86-28, evaluated in the present work, correspond to some of the genotypes infected in Cuba during the first outbreaks of the disease (Pérez et al. 2003). Besides, considering the stability of their response to *X. albilineans*, the seven genotypes detected by both models that included some of them (C1051-73, C275-80, C86-12, C88-382, C89-147, My5514, and Ty86-28) could be considered as possible control cultivars in future resistance tests. The tested approach could be used to determine control cultivar for any other important sugarcane disease considered a selection criterion in breeding programs.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by María La O Hechavarría, Yaquelín Puchades Izaguirre, Yosel Pérez Pérez, Gabriela Michavila, Mario A. Casas González, Juana Pérez Pérez, Omelio Carvajal Jaime, Joaquín Montalván Delgado, and Andrea Peña Malavera. The first draft of the manuscript was written by María La O Hechavarría, Yaquelín Puchades Izaguirre, and María Francisca Perera, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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