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Assessment of inoculation techniques for screening sugarcane resistance to red stripe disease caused by *Acidovorax avenae* subsp. *avenae*

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Abstract

The red stripe disease caused by *Acidovorax avenae* subsp*. avenae* in sugarcane, has become a quite relevant issue in Argentina because of its high incidence in the sugarcane growing area. The resistance of host plants is the most promising method for controlling the disease. In that sense, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC) has a Sugarcane Breeding Program, which generates new varieties with higher productivity and good sanitary behavior. The lack of an effective screening technique to select resistant sugarcane genotypes limits the cultivar selection process. To develop a practical and affordable method for achieving the expression of the red stripe disease, three available inoculation techniques were evaluated under controlled conditions over two sugarcane varieties, with a previously adjustment of soil composition and nutrition and relative humidity. They consisted in (i) scrubbing the leaf surface with a cotton ball soaked in the suspension of *A. avenae* subsp. *avenae*; and spraying inoculum under two conditions: (ii) leaves pre-treated with a refined sand scarification and (iii) leaves with no scarification. Fifteen plants were inoculated per cultivar and treatment according to a randomized protocol with three replicates and the severity of the disease was evaluated on a scale of 1- 9 according to the International Society of Sugarcane Technologists. The spray inoculation using a bacterial suspension of *A. avenae* subsp. *avenae* without abrasives was also field tested. These results contribute to sugarcane breeding programs, providing a tool to assess the resistance to red stripe of their materials, overcoming the lack of bacterial pressure or favorable conditions for the disease.

Keywords: *Acidovorax avenae* subsp*. avenae*, disease severity, phenotyping tool, red stripe resistance, spray inoculation.
Abbreviations: *A. avenae* subsp*. avenae*_ *Acidovorax avenae* subsp*. avenae*, AC_ abrasive technique with cotton ball, AS_ abrasive technique with refined sand, CFU_ colony forming units, DGC_ Di Rienzo, Guzmán and Casanoves test, EEAOC_ Estación Experimental Agroindustrial Obispo Colombres, FAA_ foliar area affected, ISSCT_ International Society of Sugarcane Technologists, N_ total number evaluated, RH_ relative humidity, NA_ nutrient agar medium, NB_ nutrient broth, NoA_ non-abrasive technique, OD_ optical density, ON_ overnight, SD_ standard deviation, TRS_ theoretically recoverable sucrose, x_ number of diseased plants.

Introduction

The red stripe, caused by *Acidovorax avenae* subsp. *avenae* (Schaad et al., 2008), is one of the bacterial diseases that affect sugarcane crop. It can significantly reduce theoretically recoverable sucrose (TRS) when its incidence exceeds 25% (Johnson et al., 2016).
Two types of symptoms are associated with the disease: leaf stripe and top rot (Lee and Martin, 1925; Spegazzini, 1895; Tryon, 1923). These symptoms may occur separately or together (Martin and Wismer, 1961) and their expression is favored by high relative humidity (RH), rainfall, high temperature, and low water-holding capacity (Martin and Wismer, 1989). The transmission of the red stripe in the field is due to bacterial exudates on the leaf surface that enter through the stomata (Rott and Davis, 2000); however, the infection can also be aided by leaf injury.
Argentina produces about 1.9 million tonnes of raw sugar (USDA, 2017) and its sugarcane production is spread over two regions: the north (Tucumán, Salta and Jujuy provinces) and the Littoral (Santa Fe and Misiones provinces). The province of Tucumán contributes with 70% of the total production (Centro Azucarero Argentino, 2016; USDA, 2017). The symptoms of the red stripe were detected for the first time in Argentina in 1895 by Spegazzini; however, the presence of the disease was confirmed in Tucumán in 1970, when Ramallo isolated the bacteria and reproduced the symptoms by inoculation. In the past decade, there has been a significant increase in the incidence of red stripe in

Argentina, particularly in the north of the country (Fontana et al., 2013), which has become a problem for Tucumán. The same situation seems to have occurred in other parts of the world since the red stripe, once considered a minor disease of sugarcane, is gaining importance in many recommended varieties as well as in areas with changing climatic conditions (Yonzone and Devi, 2018). Earlier recordings by our research team have shown an increase in the incidence and severity of red stripe in sugarcane fields apparently associated with light soils (with a greater proportion of sand or mulch) and high nitrogen content (120 kg ha-1) (Engineer C. Funes, EEAOC, Argentina, personal communication). In this regard, numerous studies on different diseases of sugarcane have shown that soil properties, such as acidity and phosphorus, sulfur and nitrogen contents, influence the expression of sugarcane diseases, especially brown rust (Anderson et al., 1990; Barrera et al., 2012; Johnson et al., 2007).

This situation has highlighted the need to study the disease in depth taking into account the risk of bacteria affecting susceptible varieties. The best strategy to avoid yield losses due to the red stripe is the use of resistant varieties (Johnson et al., 2016). This means that an effective method for screening for a large number of genotypic reactions against *A. avenae* subsp. *avenae* is needed. In this sense, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC) has a sugarcane breeding program that produces new varieties of higher productivity and good behavior against diseases with significant regional impact.

Although natural infection is the primary means for assessing disease resistance in sugarcane cultivars, the behavior of genotypes cannot be properly determined when natural conditions are not conducive for disease development. In this sense, artificial inoculation solves this problem since it allows the uniform exposure of all plants under disease-favorable conditions to a sufficient concentration of pathogens to induce disease in susceptible genotypes. Although different methods of inoculation have been used to induce red stripe disease, none of them guarantees the success of the proven methodology and does not have the robustness needed to confirm that the phenotype represents the resistant genotype. Among these, inoculation by introducing the bacterial suspension into the growth point of the sugarcane with a plastic syringe or pin (Schaad et al., 2008; Zia-ul-Hussnain et al., 2011; Girard et al., 2014) or using a pressure gun (Chona, 1964; Bourne, 1970) showed severe symptoms, while the spray inoculation method showed only a few symptoms. On the other hand, Fontana et al. (2013) conducted pathogenicity tests on sugarcane by rubbing the leaves with cotton soaked in the bacterial suspension; although they have succeeded in reproducing the red stripe disease, no information on the severity reached by the varieties tested has been shown.

Because the red stripe is widespread in Tucumán, Argentina, the EEAOC Sugarcane Breeding program needs an effective tool for assessing the disease response in a large number of cultivars. In this sense, the objectives of this study were: i) to optimize a practical and affordable method for simulating natural infection, achieving the expression of the red stripe disease and allowing systematic screening of sugarcane genotypes in years or environments in which natural conditions are not conducive to the development of the disease, and ii) to validate the chosen technique in the field, at the appropriate time for the development of the red stripe and with adequate agronomic management.

Results

Acidovorax avenae subsp. avenae´s growth curve

The growth curve of the pathogen was studied in order to determine its exponential phase (Fig. 1); three to four hours of incubation were chosen for subsequent inoculation tests, because at that time the bacteria doubled the number of cells.

Parameters adjustment for Acidovorax avenae* subsp. *avenae inoculation

Relative humidity

In order to reproduce the disease of red stripe under controlled conditions by checking the Koch´s postulates, various parameters were adjusted. The relative humidity (RH) above 80% was reached with vaporizers on for 15 min every two hours. When the vaporizers were turned on for 15 minutes every hour, excessive condensation of water in the infection chamber was observed, while RH of less than 80% was achieved by turning on the vaporizers every two hours for 30 min.

The additional use of plastic bags to cover the inoculated plants favored the appearance of typical symptoms of the disease.

Inoculum preparation

Two inoculum preparations, bacterial culture from solid and liquid media, and three inoculation techniques were tested. They consisting in (i) scrubbing the two leaf surfaces with a cotton ball soaked in the bacterial suspension (Fontana et al., 2013) (cotton as abrasive, AC); and spraying the inoculum on both sides of the leaf under two conditions: (ii) leaves pre-treated with a refined sand scarification (AS) and (iii) leaves with no scarification, i. e. a non abrasive technique (NoA).

No statistical difference was observed between solid and liquid inoculums when two sugarcane varieties were inoculated ($F = 0.18$, $P = 0.6781$) (Table 1).

The moderate resistance (LCP 85-384) and susceptible (TUCCP 77-42) varieties showed leaf stripe symptoms under all tested conditions (Fig 2 a-h). However, they showed statistical differences against red stripe ($F = 161.41$, $P < 0.0001$) (Table 1), which correlated with the known reaction described under field conditions, where TUCCP 77-42 shows severity values between 5 and 9 and LCP 85-384 does not exceed values of 4 (Fig. 3), according to ISSCT's scale of 1 to 9.

The incidence of the disease in the susceptible variety, regardless of the inoculation technique used, was 100% (data not shown). In addition, no “Inoculum source x Sugarcane variety”, “Sugarcane variety x Inoculation technique” or triple interactions were detected (Table 1).

Although all tested techniques were able to reproduce the typical symptoms of the disease, the AS treatment was the most aggressive, since the most severe symptoms had been observed (Fig. 3, mean severity value: 6.5). On the other

hand, an "Inoculum source x Inoculum technique" interaction was observed (Table 1), resulting from the sand injure technique (AS) using a suspension of *A. avenae* subsp. *avenae* from a solid, the only treatment significantly different from the rest ($P < 0.05$) (Fig. 3). The NoA inoculation technique using a bacterial suspension from a liquid culture was chosen because it met the requirements defined above; it is a practical and affordable method that simulates natural infection, achieving the expression of the red stripe disease.

In-field inoculation test

The technique of inoculation by spraying the leaves using a suspension of bacteria from a liquid culture was chosen for its evaluation under field conditions. The symptoms of red stripe disease were observed when the inoculums of *avenae* were used (Fig. 4 a-f), showing statistical differences in incidence ($P < 0.05$) compared to control (Table 2). The highest incidence value was 30.32% for the inoculated treatment, which had both leaf red stripe (severity scale values between 5 and 8) and the death of stem symptoms (value of the severity scale = 9). Koch´s postulates were confirmed since *A. avenae* subsp. *avenae* was reisolated from symptomatic plants.

Discussion

Red stripe disease management

With regard to the management of the sugarcane red stripe disease, the main strategy is to use resistant varieties. In this sense, breeding programs aim not only to obtain new varieties with good agronomic characteristics but also excellent sanitary behavior. To do this, breeders must be able to determine the response of genotypes to specific pathogens before releasing a cultivar. In this sense, the main challenge was to find a method of inoculation that provides some similarity to the natural disease spread in the field, while being practical and economical.

Adjustment of inoculation test under controlled conditions

The present study demonstrated that spraying suspensions of *A. avenae* subsp. *avenae* on sugarcane plants using three inoculation techniques (previous injury due to sand or cotton and no previous injury) made possible to produce the symptoms of red stripe in moderately resistant and susceptible varieties, well correlated with the known reaction under field conditions. In this sense, Bugdee and Sappenfield (1967) analyzed the behavior of cotton cultivars against *Fusarium* spp. and concluded that inoculation techniques should be able to achieve the wilting responses corresponding to the known resistance of cotton cultivars under natural conditions.

The AC technique has been successful in producing the symptoms of the red stripe, as previously reported by Fontana et al. (2013; 2018) when inoculated on the same sugarcane variety TUCCP 77-42. However, the NoA method correctly simulates what happens under natural conditions, where the dispersion of *A. avenae* subsp. *avenae* is caused by wind and rain drops and the entry is mainly produced by the stomata, followed by injuries induced by the friction between the leaves (Rott and Davis, 2000). Moreover, it is a technique that does not interfere with the physical barriers that sugarcane genotypes have structurally. Contrary to our results, in which the susceptible variety reached 100% of the incidence of the disease when the suspension of *A. avenae* subsp. *avenae* was sprayed, Chinea et al. (1978) achieved only 40% incidence of red stripe in the sugarcane susceptible variety using the same inoculation technique, probably because of differences in soil composition, fertilization or relatively humidity. They pointed out that when bacteria enter by stomata without a mechanical agent to break the epidermis and allow it to spread to different parts of the internal tissues, the resulting spread across the plant is visibly low. However, the literature indicates that this is the main mechanism of entry (Rott and Davis, 2000). On the other hand, Ramallo (1970) sprayed the inoculum of *avenae* in the terminal buds of sugarcane without making wounds and observed red stripe symptoms, but no severity of symptoms was mentioned. Bourne (1970) concluded that, as with other grasses, the best results are obtained when a needle is used for inoculation of *A. avenae*, while Dange and Pnyak (1973) have chosen a combination of spray and needle injury inoculation methods under controlled conditions. In our work, inoculation with non-abrasive spraying (NoA) has achieved the expected symptoms, perhaps due to prior adaptation of soil composition and fertilization. In this sense, the fundamental role of optimizing both the inoculation technique and the environmental conditions must be emphasized. According to this, Vesminsh et al. (1973) stated that optimal conditions for the development of the red stripe are high temperatures (30-34°C) and high RH. This is why both parameters were taken into account in our work during the inoculation. It should be emphasized that as no "Source of inoculum x Variety of sugarcane", "Sugarcane variety x Inoculation technique" or triple interaction were detected in the present work, any sources of inoculum and inoculation techniques could be used to evaluate the behavior of sugarcane clones against the red stripe.

Inoculation under field conditions

Once inoculation under controlled conditions was adjusted and the NoA method was considered the best, field tests were performed. The highest incidence value was 30.32% for the inoculated treatment, which is consistent with the known behavior of the susceptible variety TUCCP 77-42, reaching maximum values of 50% and 30%, respectively of the diseased stalks and death stalks in commercial fields (Perez Gómez et al., 2010). The fact that no death stalks were observed during inoculation under controlled conditions was probably due to no true stems was presented in 1 L pots, unlike inoculation in the field. It is important to emphasize that there are few works in which artificial inoculation has been used to cause sugarcane disease in the field. In 2009, Sood et al. evaluated a large number of field-planted sugarcane clones using a small amount of inoculum and a limited number of working hours, thus successfully replicating brown rust disease. On the other hand, Huerta-Lara et al. (2003) evaluated the response of ten sugarcane varieties to *Xanthomonas albilineans* under field conditions by cutting the apical meristem with scissors immersed in the inoculum suspension. Chinea et al. (1995) studied the varietal behavior of sugarcane against *Leifsonia xyli* subsp.

Table 1. *Acidovorax avenae* subsp. *avenae* inoculum source, sugarcane variety, inoculation technique and its interaction effects in a control condition inoculation assay, using linear mixed model with DGC test (α=0.05).

Effects	F-value	P-value
Intercept	534.17	<0.0001
Inoculation technique	2.72	0.093
Inoculum source	0.18	0.6781
Sugarcane variety	161.41	<0.0001*
Inoculum source x Sugarcane variety	0.97	0.3379
Sugarcane variety x Inoculation technique	0.41	0.6727
Inoculum source x Inoculation technique	4.55	0.0251*
Inoculum source x Sugarcane variety x Inoculation technique	1.78	0.1973

*Significant values.

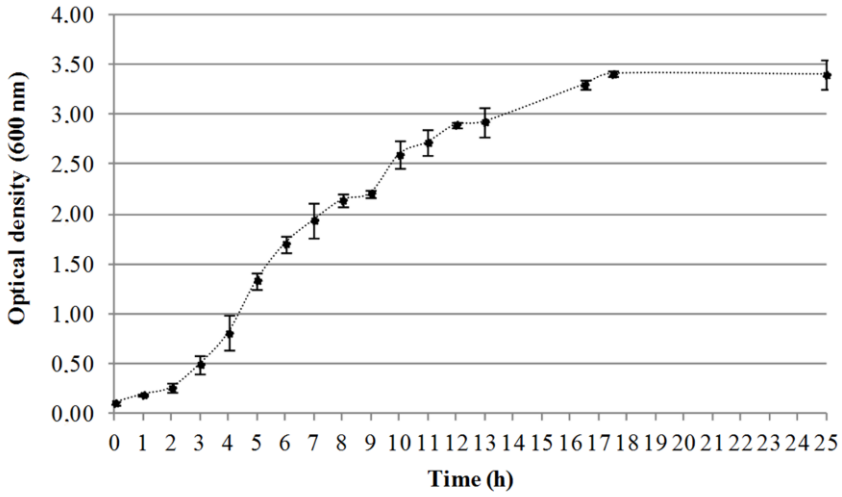

Fig 1. Growth curve of *Acidovorax avenae* subsp. *avenae* in nutrient broth medium at 37°C. Mean and standard deviation (SD) values of optical density (OD) are reported for each time point. The assay was performed in triplicate; each one consisted in two independent bacteria cultures.

Table 2. Red stripe inoculation effect in sugarcane disease incidence (%), using T test for paired samples (α= 0.05).

Obs (1)	Obs (2)	N	Mean (dif)	Mean (1)	Mean(2)	SD (dif)	T	p (Bilateral)
Inoculated	Control	10	18.44	19.08	0.64	7.62	7.65	<0.0001

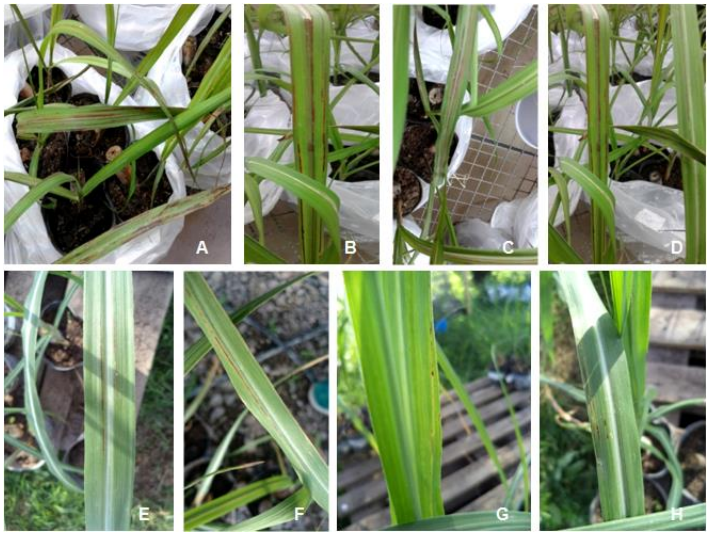

Fig 2. Red stripe symptoms on sugarcane leaves 15 days after artificial infection. **A-D** corresponds to typical symptoms developed in TUCCP 77-42 and **E-H** to LCP 85-384 sugarcane varieties.

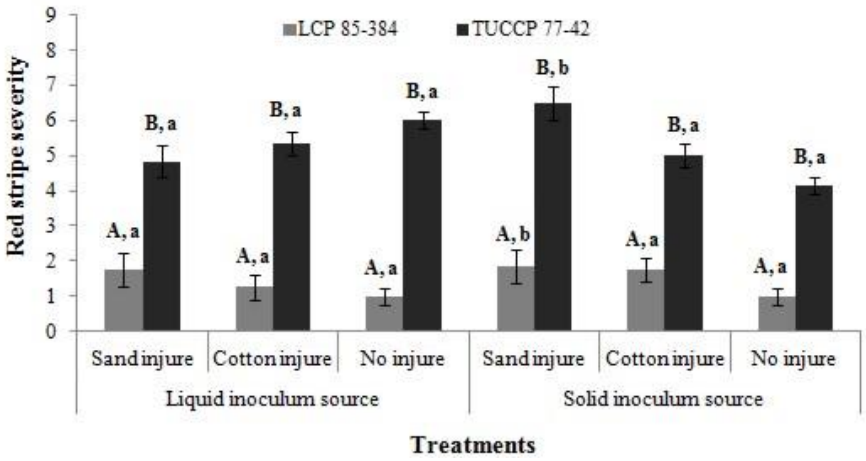

Fig 3. Red stripe severity in the varieties TUCCP 77-42 (susceptible) and LCP 85-384 (moderately resistant), inoculated by spraying *Acidovorax avenae* subsp. *avenae* suspension from a liquid culture and solid culture, using three techniques: previous sand injure, previous cotton injure and without injure (n = 15). Different capital letters indicate statistical differences between the sugarcane varieties for a given treatment, while lower case letters indicate differences between the treatments in a certain variety, using DGC test (*P*<0,05). Bars on treatments indicate mean standard error.

Fig 4. Red stripe symptoms after leaf spray inoculation of TUCCP 77-42 sugarcane variety, with 10^8 CFU/ml of *Acidovorax avenae* subsp. *avenae*, under field conditions (**A-F**).

xyli by inoculation of the seed cane under field conditions, successfully infecting susceptible varieties. The results obtained in this work will enable breeding programs to study the resistance of sugarcane genotypes to the red stripe at different stages of the breeding process, in the months which environmental conditions are favorable for the development of the disease, making evaluation more effective and reducing the costs.

This is probably the first time that a red stripe artificial inoculation method has been tested under field conditions and is working successfully. Since only one susceptible variety has been tested in this work, further field studies should be conducted to evaluate the inoculation technique in commercial sugarcane varieties with different behavior against the red stripe disease, to confirm the usefulness of the technique.

Materials and Methods

Avenae growth curve

The exponential growth phase of *A. avenae* subsp. *avenae* was determined. A bacterial strain isolated from sugarcane

leaves with the typical symptoms of red stripe in Tucumán, Argentina, by the EEAOC Plant Pathology Department, was used. Its identity was confirmed by specie–specific PCR and sequencing the 16S-ITS1-23S ribosomal RNA gene fragment. The fresh culture was initiated from glycerol stock by placing aliquots on nutritive agar (NA) medium (nutrient broth (NB): beef extract, 3 g/l; peptone, 5 g/l; NaCl, 3 g/l; pH: 7 ± 0.2 containing 15 g/l agar and was seeded in 5 ml NB which was incubated at 37°C, 250 rpm overnight (ON). This culture was used to inoculate 25 ml of NB by adjusting the optical density (OD) to 0.1 at 600 nm. This reading was recorded at time "0". The bacterium was incubated at 250 rpm and 37°C and the OD was periodically determined over a day. The test was performed in triplicate; each one consisted of two independent bacterial cultures. The mean values of the OD and the standard deviation (SD) were determined using Infostat software (Di Rienzo et al., 2015) and a standardized growth curve of *A. avenae* subsp. *avenae* was plotted.

Plant material and growing conditions

The plants of red stripe susceptible cultivar TUCCP 77-42 and the LCP 85-384 moderate resistant cultivar (Cuenya et al., 2009) produced from single-bud cuttings in the greenhouse were planted in 1L pots containing a mixture of soil, sand, and mulch, in a proportion 40:40:20 and used in the experiments. The plants were 45 to 70 days old with about four leaves completely lifted at the time of inoculation and were fertilized with 120 kg/ha of Nitrodoble (27-0-0 N-P-K) ten days before inoculation.

Optimization of inoculation under controlled conditions

To evaluate the inoculation methods under controlled conditions, different operating times of the vaporizers were tested in order to keep the humidity above 80% (30 min on every two hours, 15 min on every one or two hours), without causing excessive condensation of water in the infection chamber. Additional coverage of plants inoculated with plastic bags for 24 h after inoculation was probed to promote effective entry of the bacteria.
Two inoculum preparations were tested: bacterial culture from solid and liquid media. In the first case, the bacterium was incubated at 37°C on NA medium. After 48 h, 5 ml of 0.1% Tween 20 was added to the petri dish (Garcés et al., 2014). *Avenae* suspension was diluted with 0.1% Tween 20 to obtain 10^8 colony forming units (CFU)/ml based on OD at 600 nm. In the second case, the bacterium was cultured in NB media at 37ºC, 250 rpm, to the exponential phase. The culture was centrifuged for 10 min at 10,000 rpm and the supernatant discarded. The pellet was rinsed twice in a solution containing distilled water and 0.1% Tween 20 and the concentration was adjusted to 10^8 CFU/ml with 0.1% Tween 20 based on the OD. In addition, three inoculation techniques were used: (i) scrubbing both leaf surfaces with a cotton ball soaked in the bacterial suspension (Fontana et al., 2013) (cotton as abrasive, AC); (ii) spraying the inoculum with a manual atomizer on both sides of the leaf until runoff occurs over leaves pre-treated with a refined sand scarification (AS) and (iii) spraying the inoculum with a manual atomizer on both sides of the leaf until runoff occurs over leaves with no scarification, i. e. a non abrasive technique (NoA).
The control plants were treated with a solution of 0.1% Tween 20. After inoculation, the plants were placed in the greenhouse and covered with a plastic bag during the first 24 h to maintain leaf moisture. During the infection period, the temperature was maintained at 30 ± 2ºC with artificial illumination of 12 h and a RH of 80%. Plants were examined daily for symptom expression up to 15 days post-inoculation to determine symptom progression. The reisolation of *avenae* was performed from symptomatic plants to confirm Koch's postulates. The severity of the disease was evaluated on a scale of 1 [<0.5 % foliar area affected (FAA)] -to-9 (more than 50% FAA) according to the International Society of Sugarcane Technologists (ISSCT).

Experimental design and statistical analysis of inoculation test under controlled conditions

Fifteen plants were inoculated per cultivar and treatment according to a randomized protocol; the experiment was performed three times.
A linear mixed model has been adapted to the severity data using the InfoStat software (Di Rienzo et al., 2015). The Inoculation technique (treatment), the Inoculum source, the Sugarcane variety and their interactions were considered as fixed effects while the repetition of experiments was treated as a random effect. For the relevant significant factors, protected-mean comparisons from all possible pairwise differences in means were tested at $\alpha = 0.05$, using the test of Di Rienzo, Guzmán and Casanoves (DGC).

Inoculation assay under field conditions

The best inoculation technique has been validated in the field. The test was implanted in a commercial field of the variety TUCCP 77-42 (second ratoon), fertilized with 120 kg/ha of nitrogen, in the department of Monteros, Tucumán, Argentina (27°05'25.6"S 65°21'59.0"W) in December 2017. The inoculation treatments were as follows: (1) spraying with a bacterial suspension at 10^8 CFU/ml and (2) spraying with 0.1% Tween 20 (v/v) (control). Only the two central rows of each plot were inoculated with a CO_2 sprayer with two nozzles per row; the pressure was 2.5 atm and the spray volume was 750 ml/two rows. Inoculation was made at the end of the day, towards sunset. During the inoculation and symptom development period, the daily average low and high temperatures ranged from 20.7 to 36.3°C, the RH ranged from 72 to 98%, and the wind speed was from 3.2 to 16.1 km/h. The incidence of the red stripe disease was evaluated and calculated as the proportion of diseased plants ($I = \Sigma x/N$), which corresponds to the number of diseased plants (x) divided by the total number evaluated (N).
The *avenae* was reisolated from symptomatic plants to confirm the Koch´s postulates.

Experimental design and statistical analysis of inoculation test under field conditions

Each plot was represented by four rows of 3 m long with ten repetitions, according to a much paired pattern.
The data were analyzed for statistical differences among treatment means using T test for paired observations by InfoStat software (Di Rienzo et al., 2015).

Conclusion

The results obtained in the present work would be a valuable contribution to sugarcane breeding programs providing a tool to systematically evaluate their materials under controlled conditions, avoiding the problems of lack of pressure of *A. avenae* subsp. *avenae* or conditions not predisposing to the development of the disease or under field conditions with the optimized methodology.

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