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Does resistance host modify airborne spore dynamics and leaf infection? Case of Black Leaf Streak Disease

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RESUMEN

Para comprender mejor la epidemiología de la Sigatoka Negra enfermedad causada por el hongo ascomiceto aerotransportado *Pseudocercospora fijiensis*, (i) caracterizamos y cuantificamos los inóculos, y (ii) pusimos de relieve la infección de las hojas. Se colocaron trampas de esporas y bolsas a prueba de esporas en dos variedades de banano en Guadalupe durante un año. Combinamos microscopía, qPCR y análisis de imagen para cuantificar las esporas y las lesiones. Encontramos conidios en el 2,78% y el 4,17% de las muestras de control en el campo y en la variedad Williams. Para utilizar el método qPCR, se creó una curva estándar con un coeficiente de correlación de 0,992. No se encontraron ascosporas.

Palabras clave: *Pseudocercospora fijiensis*, epidemiología, inóculo, allo-infección, auto-infección, Fitosanidad.

ABSTRACT

To better understand Black Leaf Streak Disease epidemiology, caused by the airborne ascomycete fungus *Pseudocercospora fijiensis*, we (i) characterized and quantified the inocula, and (ii) highlighted leaf infection. Spore traps and sporeproof bags were placed in Guadeloupe for a year in two banana varieties, one susceptible and one partially resistant to the disease. We combined microscopy, qPCR and image analysis to quantify spore concentrations and lesions. We found conidia in 2.78% (control) and 4.17% (Williams) samples. Using qPCR method, a standard curve was established with a determination coefficient of 0.992. No ascospore was found. The low spore concentrations were discussed in relation with resistance host and environmental conditions.

Keywords: *Pseudocercospora fijiensis*, epidemiology, inoculum, allo-infection, auto-infection, phytosanity.

INTRODUCTION

Black Leaf Streak Disease (BLSD) caused by the hemibiotroph and heterothallic ascomycete *Pseudocercospora fijiensis* Morelet is the most economically important leaf disease of banana. The disease causes yield and quality losses, ranging from 10 to 100% depending on climatic conditions and market types, due to a reduction of green life duration of fruits (Guzman *et al.*, 2018). In the case of the fruit production for exportation, the early ripening is a key issue for the transport from producing to importing countries.

The majority of exported bananas belongs to the Cavendish subgroup of species (AAA), which is susceptible to the disease. To control BLSD, up to 65 fungicides aerial treatments per year are applied in some countries (Guzman *et al.*, 2018). Deleafing of necrotic tissue on the leaves is also used (Marin *et al.*, 2003). In addition, several breeding programs have been launched globally to create resistant hybrids. Among them, Cirad creates new varieties which are partially resistant to BLSD. One of them named Pointe d'Or (AAA) was created in the 90^{ies}.

BLSD is polycyclic. The fungus has two reproduction modes: an asexual mode with the production of conidia from lesions (streaks lesions, stage 3 according to Fouré (1982)) and a sexual mode with the crossing of two strains with different sexual types leading to the production of ascospores within perithecia, in necrotic tissue stage 6 according to Fouré (1982) (Guzman *et al.*, 2018). Conidia are wind- and water-dispersed and their average dispersal distance is 3.15 meters around the source of emission. Ascospores are mainly wind-dispersed and are dispersed 213.83 meters on average around the source of emission (Rieux *et al.*, 2014). According to empirical knowledge, plant infection is mainly due to the allo-inoculum (defined here as the external inoculum produced outside a given plot, mainly composed by ascospores) whereas the role of the auto-inoculum (defined here as the internal inoculum produced inside a given plot) in the leaf infection would be minor (Stover, 1980).

Knowledge on the inocula quantities and dynamics is important to optimize BLSD management. The studies on the dynamics of the inocula of *P. fijiensis* are scarce and show contradictory results (Burt *et al.*, 1997; Gauhl, 1995). Gauhl (1995) quantified ten times more ascospores than conidia whereas Burt *et al.* (1997) found the opposite. This knowledge is also important to feed epidemiological models parametrized for BLSD. In this study, we propose to (i) characterize and quantify the proportion of conidia and ascospores in the allo- and the auto-inocula and to (ii) identify

the leaves which are infected by each inoculum, both objectives as a function of varieties and microclimate.

MATERIALES Y MÉTODOS

The study was set up in Cirad experimental station, at Neufchateau, Capesterre-Belle-Eau, Guadeloupe in the French West Indies, in June 2023 and is planned to last until June 2024. Two experimental plots are studied (16°04'43''N 61°36'12''W; 16°04'42''N 61°36'08''W). One is cultivated with a hybrid partially resistant to BLSD, named Pointe d'Or (Cirad) since 2008. The other one was planted with a variety susceptible to BLSD, named Williams (Cavendish subgroup), in April 2023. The size of each plot is respectively 0.10 ha and 0.22 ha. Both plots are 70 m distant from each other.

We first characterize and quantify the allo- and the auto-inocula. For that purpose, the air is sampled using multivial volumetric cyclone samplers (Burkard). These are active spore traps, sucking 16.5 L of air per minute into 1.5 µL Eppendorf vials. A total of six traps is used: two traps in each variety and two in plots without banana trees as control. From June to September 2023, two traps were in the Williams plot, one in the control plot and one in the Pointe d'Or plot. The traps are placed on ladders at 3.6 meters height, successively above and under the canopy of banana trees, to catch respectively the allo-inoculum (from June to September 2023) and the auto-inoculum (from November 2023 to August 2024). When the spore traps were above the canopy, a deleafing was made to avoid auto-inoculum. The microclimatic conditions (*i.e.* wind speed, temperature, solar radiation, relative humidity) are monitored in each plot.

The daily air samples are then put into suspension using 100 µL of TWEEN 0.2%. The samples of the same week are pooled together and concentrated thanks to a microcentrifuge (14800 rpm for 1 min) to obtain 100 µL of weekly samples. Two different methods are used to quantify each type of spores per m^3 of air. Conidia of *Pseudocercospora fijiensis*, which are easily identifiable are quantified using a KOVA Glasstic Slide 10 with Grid Chamber (Kova International) under an optical microscope. Ascospores, which are not morphologically distinguishable between *Pseudocercospora* species, *i.e.* non-identifiable microscopically, are quantified after a total DNA extraction (Invitrogen PureLink Genomic DNA mini kit) using ß-tubuline primers (Arzanlou *et al.*, 2007) in a TaqMan quantitative polymerase reaction chain

(qPCR). The number of ascospores is calculated considering the conidia number in the same sample using the equation (1):

$$Asc = \frac{C_{PCR} - (C_{\mu} \times C_{in\ vivo})}{2} \quad (1)$$

With,

Asc, the number of ascospores

C_{PCR}, the total number of cells of *P. fijiensis* found with the qPCR

C_{μ}, the number of conidia counted with the microscope

$C_{in\ vivo}$, the mean number of cells per conidia produced in vivo (*i.e.* on lesions on banana leaves).

$C_{in\ vivo}$ was measured on 600 conidia samples collected on lesions of BLSD on both studied varieties and printed on Potato Dextrose Agar media. The number of cells per conidia was then counted under the microscope. To link the threshold cycle (Ct) obtained with the qPCR and the number of cells of a given sample (C_{PCR}), we established a standard curve based on a range of cells. For that, conidia were produced *in vitro* according to Dita *et al.* (2021) protocol. Seven dilutions using a ten-fold factor from 10^1 to 10^7 conidia per mL were prepared. To ensure the reproducibility of the standard curve, each concentration was replicated 10 times.

Data were analysed using the R studio software (4.1.2.) and after the verification of the conditions to apply an Anova, which were not respected, a Kruskal-Wallis test was used. The second part of the study is to highlight the leaf infection by the allo- and the auto- inocula. For that purpose, we protect leaves from each inoculum using sporeproof bags. The unfurled leaf and the first leaf are protected against the allo-inoculum. The youngest leaf with streaks (*i.e.* the first leaf with stage 3 lesions) is protected against the auto-inoculum, after counting the number of lesions already present. Leaves of 4 banana trees of each variety are protected for a week. As a control, similar leaves (*i.e.* unfurled leaf, first leaf and youngest leaf with streaks) on other banana trees are unprotected. Leaves are checked every day until the first symptoms (*i.e.* rusty brown specks of less than 1 mm on the underside of the leaf (Fouré, 1982)) appear on the unprotected unfurled leaves of the controls. All leaves studied are then cut and photographed. The number of lesions on 20 cm² of the upper left side on the leaf is quantified using a machine learning image analysis (IPSDK Explorer, 2023). The differences between lesions numbers on protected and unprotected leaves allow to quantify each inoculum, depending on the protected leaves.

RESULTS AND DISCUSSION

Our results cover 12 weeks from 19th June to 10th September 2023. During this time, spore traps were above the canopy, catching the allo-inoculum of each plot. We have no results on spore trapping under the canopy yet.

Within all the 12 weekly samples, we counted a total of one conidia per µL in the 36 samples of the control and three in the 72 samples of the Williams plot. No conidia were found in the Pointe d'Or plot in any of the 24 samples. In positive samples, the concentration of conidia per m^3 of air sampled was 0.22 and 0.33, respectively in the control plot and the Williams plot (Table 1).

Table 1. Number of conidia of *P. fijiensis* sampled to assess the allo-inoculum, using Kova Glasstic Slides. No significant differences were found between the treatments in the week concentration of conidia per m^3 of air sampled and in the week concentration of conidia per m^3 of air sampled in positive samplers, according to a Kruskal-Wallis test (letters "a"). There is no positive sample for the Pointe d'Or variety (N/A: not applicable).

	Control (N = 36)	**Banana plot**	
		Susceptible variety (Williams (N = 72))	**Resistant hybrid (Pointe d'Or (N = 24))**
Frequency of conidia (%)	2.78	4.17	0
Week concentration of conidia per m^3 of air sampled	0.0196 [a]	0.0444 [a]	0 [a]
Week concentration of conidia per m^3 of air sampled in positive samples	0.2160 [a]	0.3255 [a]	N/A

The frequency of conidia found in the samples is 1.5 times higher for the susceptible variety compared to the control, and 4.2 times higher compared to the resistant hybrid. However, according to a Kruskal-Wallis test, there is no significant difference in the week concentration of conidia per m^3 of air sampled between the treatments (control, Williams and Pointe d'Or). The concentration in the allo-inoculum quantified in our study is 26 times smaller than in Burt *et al.* (1997). There is also no significant difference in the week concentration of conidia per m^3 of air sampled in the positive samples between the treatments.

These differences could be due to different inoculum pressure between Costa-Rica and Guadeloupe. The low concentration of conidia per m^3 of air measured in Guadeloupe might be due to the type of inoculum sampled (*i.e.* the allo-inoculum) which is dependent on the environment of the plot studied. In Costa-Rica, maybe more uncontrolled BLSD plants were located near the spore traps than in Guadeloupe, creating a higher level of allo-inoculum. The low conidia concentration might be due the impact of the tropical storm Fiona (September 2022) and the year of sampling. In plantations around the experimental plots, the severity of the disease between 2022 and 2023 has decreased (LPG, personal communication). It cannot be excluded, however, that the low frequency of positive samples (*i.e.* containing at least 1 conidia) could be explained by the detection threshold of the counting method of 1 spore per µL.

To quantify the ascospores, we needed to assess the number of cells per conidia ($C_{in\ vivo}$) as described in the Equation (1). 611 and 672 conidia were studied, respectively for the Williams variety and the Pointe d'Or hybrid. The mean number of cells per conidia is 5.22 (Williams) and 4.77 (Pointe d'Or). Means are significantly different between both varieties, using a Kruskal-Wallis test (Figure 1).

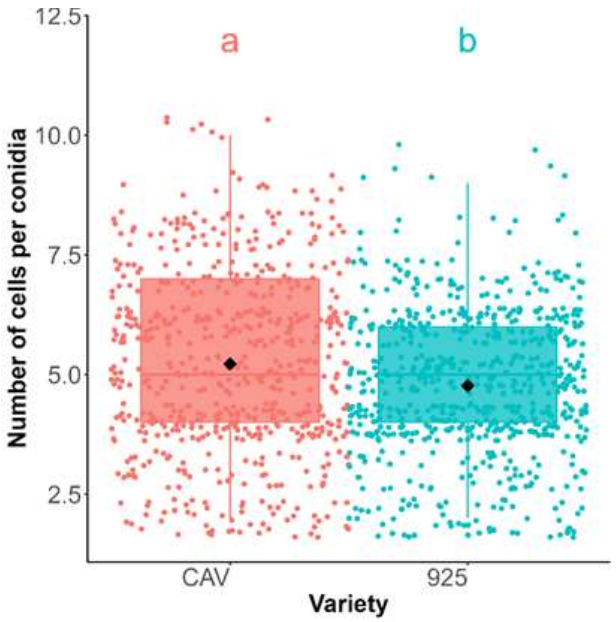

Figure 1. Number of cells per conidia. The mean is 5.22 (Williams) and 4.77 (Pointe d'Or). Letters a and b indicate a significative difference between both varieties, using a Kruskal-Wallis test.

To link the threshold cycle and the quantification of ascospore airborne concentrations, we obtained a standard curve with the following equation: y = 52-2.9 x, where the y-intercept is 52 and x the slope is –2.9 (Figure 2). The determination coefficient R^2 is high with a value of 0.992. Efficiency of the qPCR is high with 119.7%.

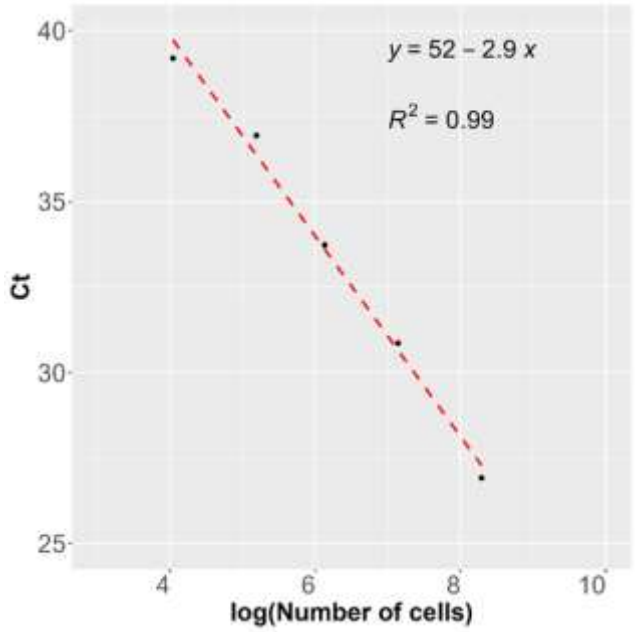

Figure 2. Standard curve of *P. fijiensis* with ten-fold logarithmic dilutions of conidia produced in vitro, with, Ct the threshold cycle, x the slope, and y the intercept. The determination coefficient (R^2) is 0.992.

The qPCR assays for the validation of the standard curve allowed to detect as much as 14 conidia per µL with certainty. The amplification was unreliable with 1 conidia par µL. The limit of quantification is between 4 to 76 cells it is to say 1 and 14 conidia per µL, or between 2 and 28 ascospores.

The Ct of the 132 weekly airborne samples (36 as control, 72 as Williams, 24 as Pointe d'Or) were all higher than 40, meaning unreliable quantification and suggesting that no ascospore could be quantified. This could be explained by a low ascospores concentration below the detection threshold of the qPCR method which limit of quantification is between 38 and 2 ascospores.

CONCLUSIONS

We characterized and quantified the allo-inoculum. We found conidia 1.5 times more often in the susceptible variety (Williams) than in the control plot. No conidia were found in the Pointe d'Or variety. We also found 1.5 more conidia per m^3 of air sampled in the Williams plot compared to the control plot, but this difference was not significative. In order to determine the number of ascospores with the qPCR method, we created a standard curve with a determination coefficient of 0.992. We also showed a significative difference between the number of cells in conidia sampled on Williams lesions (5.22 cells) and on Pointe d'Or (4.77 cells). We found no ascospore in any of the three treatments. These conidia and ascospore airborne concentrations of the allo-inoculum could be due to specific environmental conditions in Guadeloupe. These spore dynamics will be followed within time in the auto-inoculum.

The results on leaf infection by the allo- and the auto-inocula are still ongoing and will be discussed.

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