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In vitro assessment of the role of carbohydrates on the coffee's resistance against coffee berry disease caused by *Colletotrichum kahawae*

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Abstract

The coffee berry disease (CBD) of *Coffea arabica* caused by *Colletotrichum kahawae* is responsible for 80 % loss of coffee production in Cameroun. In order to assess the possible implication of carbohydrates in the defence of *Coffea arabica* against *Colletotrichum kahawae*, comparative analyses (qualitative and quantitative) of soluble sugars contents was done after inoculation on berries. The berries of two cultivars java and caturra were recorded at 22nd and 25th weeks after flowering (WAF) from the field. The influence of culture conditions of coffee trees *in situ* (full sunlight and under shade), the age of the berries at the time of inoculation were discussed. Additionally, the composition and the content of soluble sugars were analysed. The result showed that the infection rate was significantly high on the berries collected at the 22nd (WAF) compared to those collected at the 25th WAF. Qualitative analyse showed only the presence of glucose. The highest glucose content was obtained from the berries of java variety exposed on full sunlight while the lowest content being observed from the berries of caturra variety under shade (2.96 ± 0.42 mg/g against 0.75 ± 0.03 mg/g respectively). No significant different in sugar content was observed between infected and no infected berries inside the two varieties of coffee. However, the java variety showed a high accumulation of glucose compared to caturra variety. These results support a positive implication of sugar in the interaction between coffee berries and *Colletotrichum hahawae* since a high accumulation of sugar was observed in java variety the resistant cultivar.

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Introduction

Arabica coffee constitutes a livelihood for part of the rural households where the holdings are enhanced by an essentially family labor (ICO, 2007). Estimated production was 25314.96 tonnes in 2018 (OIC, 2018). This production has not increased over several decades in Cameroun due to high disease and pest incidence. Coffee berry disease (CBD) caused by *Colletotrichum kahawae* is one of the major constraints to coffee production (Waller *et al.*, 1993). The symptom is a dark necrotic spots on berries, sometimes with orange-colored acervules. The attacked berries subsequently darken and present a characteristic appearance of an empty bag (Bieysse *et al.*, 2002). Estimated Losses caused by CBD reached up to 80 % of production in Cameroon (Regazzoni *et al.*, 1997; Mouen Bedimo *et al.*, 2012). Previous works showed that the disease severity was higher on coffee trees exposed to full sunlight than those located under the shade (Mouen Bedimo *et al.*, 2007, 2008). Several studies have already been carried out in order to assess the tolerance of varieties to this disease (Bouharmont, 1992; Bella Manga, 1999; Mouen Bedimo *et al.*, 2008). These studies have shown that the susceptibility of berries to CBD is affected by altitude, temperature and humidity (Nicholson, 1992; Mouen Bedimo *et al.*, 2008, 2012; Gadisa *et al.*, 2016). The mastery of the epidemiology of CBD remains a major problem due to changing climatic conditions. The inoculation of detached berries under artificial condition offers the advantage of a direct assessment of *Coffea arabica* and *Colletotrichum kahawae* interaction at the fruit level making possible the characterisation of the resistant component operating under field condition (Pinard *et al.*, 2012). Several studies have shown the implication of sugars as substances that boost plant immunity and plant defenses (Bolouri and Vanden, 2012, 2013; Trouvelot *et al.*, 2014). In addition, during the interaction between *Theobroma cocoa* leaves and *Phythophthora megakarya*, qualitative analyses of sugars revealed the disappearance of sucrose and the persistence of glucose (Djocgoue *et al.*, 2011). Selecting *Arabica* Coffee trees that display lower susceptibility to CBD is therefore become a priority objective for many producing countries. This selection can be achieved using biochemical markers such as phenols, carbohydrates and amino acids (Djocgoue *et al.*, 2011). Ayres *et al.* (1996) reported that disease development is likely to induce substantial changes in the carbohydrate and amino acid contents of host plants, and metabolic alterations that may favour or inhibit fungal development. Herbers *et al.* (1996) reported that hexoses (sucrose) induce the expression of many genes, including plant resistance genes that determine the production of peroxidase and other pathogenesis-related (PR) proteins. The objective of this study was to identify the soluble sugars produced by immature *Coffea arabica* berries infected *in vitro* by *Colletotrichum kahawae*. In particular, this paper investigates the appearance of disease and the effect of *Colletotrichum kahawae* on the accumulation of soluble sugars in two varieties (java and caturra) of Arabica coffee subjected to different lighting modes.

Material and methods

Plant materials

The plant material consisted of immature berries of *Arabica* coffee trees of the java (tolerant) and caturra (sensitive) varieties. Twelve coffee were been selected in the varietal collection of coffee at the Institute of Agricultural Research for Development (IRAD), Foumbot station in West - Cameroon. Three coffee trees of each variety were individually placed in artificial shade using a black shade cloth with regular mesh (Tildnnel EC 50 %, Tilden Industries U.K.). Another three coffee trees of each variety were allowed to full sunlight. The berries were collected at random from these coffee trees at the 22nd and 25th WAF. Two series of artificial inoculation were carried out in 2016 and 2018.

Pathogen and inoculation techniques

The inoculation was made according to the protocol described by Mouen Bedimo *et al.* (2008). The isolate of *Colletotricum kahawae* was obtained from infected berries of caturra coffee trees from the varietal collection at the Institute of Agricultural Research for Development (IRAD) Foumbot station-Cameroun. It was purified using a monoconid culture in a Petri dish

produced on Potato - Dextrose - Agar (PDA) medium. This isolate was characterized by slow radial growth and a dark gray woolly aerial mycelium. Its pathogenicity was previously tested on detached berries of the caturra variety before its use for artificial inoculation. The inoculum consisted of a filtrate of the suspension of conidia obtained by scraping pure cultures of this isolate soaked in sterile distilled water. It was calibrated using the cell of a hematimeter (Malassez cell) at 10^6 conidia per ml before inoculation. The berries taken from each coffee tree were distributed in five Petri dishes containing blotting paper soaked in sterile distilled water, at a rate of 25 berries per Petri dishes. 10µl drop of the inoculum was placed on each of berry using a micropipette. This experiment was carried out at the IRAD phytopathology laboratory in Nkolbisson-Cameroon. The berries were then incubated in a phytotron set at a temperature of 21 °C and a photoperiod of 12 hours in the light and 12 hours in the dark. The influence of shade and the age of berry on the incidence of disease were also evaluated. Qualitative and quantitative analysis of soluble sugar content were made on infected and no infected berries of 22nd WAF.

The observations of the appearance of the disease were carried out over 10 days, starting 24 hours after inoculation. Daily observations consisted in counting the total number of infected berries in each Petri dish.

In vitro evaluation of the incidence of disease

The infection rate of berries (Txinf) per Petri dish was calculated according to the following formulas: Txinf = Bmal * 100/N. Where Bmal represents the total number of diseased berries counted in the Petri dishes on the 10th day of observation. N is the total number of berries inoculated (N = 25).

Analysis of sugars by RI-HPLC

Only the berries of 22nd WAF were used for the analysis of soluble sugars. Analysis of soluble sugars was done following the method described by Tilomirova *et al.* (2016) using refractive index high performance liquid chromatography. Two times 1 g of dried coffee beans were ground in a stainless steel ball homogenizer with 3 balls (MN200, Retsch, Haan, Germany) at a frequency of 1/25 s for 10 min and excess of hexane. The powder was filtered with a paper filter and cold extracted with 25 mL petroleum ether. After vacuum drying, 100 mg of the defatted powder were used for extraction with 1 mL ultrapure water (type I, ELGA purelab, High Wycombe, UK). The suspension was homogenized on vortex for 20 s and incubated on a Thermomixer comfort (Eppendorf, Hamburg, Germany) for 1 h at 1300 rpm and 80 °C. After centrifugation for 10 min at 16060 g (Biofuge pico, Heraeus, Hanau, Germany), 500 µL of the supernatant were collected and 300 mg of PVPP as well as 1.5 mL of ultrapure water were added. The suspension was subjected to an ultrasonic waterbath (Elmasonic S30H, Elma, Singen, Germany) for 10 min and centrifuged for 30 min at 4010 g. The supernatant was filtered through 0.2 µm syringe membrane filter (PES Perfect Flow, Wicom, Heppenheim, Germany) and subjected to HPLC analysis.

Sugars were separated via ligand exchange chromatography on a Rezex RCM-Monosaccharide Ca^{2+}-column with 8 % cross-linking (Phenomenex, Torrance, USA). The separation of 10 µL sample was established at 85 °C using a flowrate of 0.6 mL/min of ultrapure water. For quantification, a set of five standards from 0.02 – 0.1 mg/mL was applied. The HPLC system used consisted of a degaser ERC 3512 (Erma, Tokyo), an autosampler AS-2000A, an intelligent pump L-6200, a column oven L-7350D, and a refraction index detector L-7490 (all Merck Hitachi, Darmstadt, Germany). The software D-7000 HPLC - System - Management HSM version 4. 1, was used to record the data.

Statistical analyses

The analysis of variance (ANOVA) was carried out with the General Linear Model (GLM) procedure of Statistica software version 7.1 to determine the effects of the different factors on disease. The analysis of variance of the rate of diseased berries and of the glucose content *in vitro* was performed following the

f-test. The comparison of the means of diseased berries between the factors studied was carried out with the Newman - Keuls student test at 5 % threshold. The LSD of Fisher test was used to compare the glucose content between the factors studied at 5 %.

Results

Disease development on berries inoculated artificially in the laboratory

Analysis of variance showed that the type of inoculum and the coffee variety had a significant effect at $P \leq 0.05$ on the infection rate of berries. The age of the berries during artificial inoculation and the year of experimentation did not show a significant effect on the infection of the berries (Table 1).

A low infection rate was obtained in 2016 with control coffee trees (0.5 %). However, the infection rate was significantly high on the berries from the caturra coffee tree compared to those from the java coffee trees in 2016 and 2018.

Table 1. *In vitro* analysis of variance of pathogen infection rate.

Sources of variability	DF	F test	Pr > F
Isolates	1	171.36	0.0000 **
Variety	1	5.8938	0.02421**
Age of berries	1	1.4162	0.244776
Shading type	1	1.6519	0.210031
Years of observation	1	0.99	0.326693

**Factors having a significant effect on the infection of berries in vitro at $P \leq 0.05$.

In 2016, a significant difference was noted between the berries collected at the 22nd week and 25th week after flowering. The infection rate was significantly high on the berries at the 22nd week after flowering. In 2018, there was no significant difference between the two series of inoculation. The berries in artificial shade showed a level of disease statistically equivalent to those of berries of coffee trees exposed to the full sunlight in 2016 and 2018 (Table 2).

Five days after incubation the dark necrotic spots as a symptoms (the active form) of CBD was observed on the berries of caturra variety while in java, the symptoms was observed at six days after incubation.

This result confirms the susceptibility to CBD of caturra variety compared to java variety. Inactive lesions (scab form) of disease were not observed. Even if, the results showed the *in vitro* ascending increase of the infection rate at 2016 and 2018, following the time no significant difference was observed at 10th of observation between the two years at P = 0,326695 for the percentage of infected berries (Table 1).

Analysis of sugar content after inoculation

HPLC analysis of soluble sugars showed that, glucose was the main sugar produced by the berries 10 days after inoculation. The variety and types of shade showed a significant effect on the glucose content (Table 3). However, No significant difference was observed inside both varieties between infected berries by *Colletotrichum kahawae* and berries treated with distilled water at P = 0.1822 (Table 3).

The comparison of the means showed a significant difference of the glucose content between the berries sampled under artificial shade and those of the berries sampled under full sunlight in java variety. However, no significant difference was noted between the caturra berries exposed in full sunlight and those under artificial shade.

The highest glucose contents was observed on berries from java variety exposed to full sunlight, while the lowest content being observed on berries from caturra variety under artificial shade (2.96 ± 0.42 mg/g fat free material (ffdm) and 0.75 ± 0.03 mg/g ffdm respectively) (Fig.1).

Table 2. Means of infection level (%) as a function of biotic (isolates, varieties) and abiotic (inoculation, types of shade) factors.

Sources of variability		Average percentages of sick berries *In vitro*	
		Years	
		2016	2018
Type of inoculum	*Colletotrichum Kahawae*	63.50 ± 8.72 [a]	74.50 ± 9.50 [a]
	Distilled water	0.50 ± 0.5 [b]	0.00 ± 00 [b]
Varieties	java	29.00 ± 12.34 [b]	27.50 ± 11.79 [b]
	caturra	35.50 ± 13.08 [a]	47.00 ± 17.90 [a]
Age of berries	22nd WAF	38.50 ± 14.57 [a]	37.00 ± 16.15 [a]
	25th WAF	25.50 ± 10.08 [b]	37.50 ± 15.02 [a]
Shading type	Under shade	35.50 ± 14.11 [a]	40.50 ± 16.34 [a]
	Full sunlight	28.50 ± 11.12[a]	34.00 ± 14.71 [a]

The values with the same letters for each factor are not significantly different at P ≤ 0.05 according to the test of Students Newman–Keul.

No significant difference in sugar content was observed inside both varieties between berries inoculated with sterile distilled water and the inoculated infected berries. Nevertheless, the highest glucose content was obtained in infected berries of both varieties (2.56 ± 0.44 mg/g ffdm and 1 ± 0.77 mg/g ffdm for java and caturra respectively) (Fig.2).

Discussion

Disease incidence

Artificial inoculation of detached berries with *Colletotrichum kahawae*, showed that the type of inoculum and the variety have a significant effect on the infection rate of berries. Infection rate was not affected by the exposition of berries to full sunlight or shade. In fact, it has been shown that the intrinsic sensitivity of berries *in vitro* is observed at the scale of the coffee tree regardless of its position relative to shade (Mouen Bedimo *et al.*, 2008). This result is different to what observed in the field where the spread of disease is reduced in tree placed under shade condition compared to those growing under full sunlight. In this case, shade may act as physical barrier against CBD (Phiri *et al.*, 1999).

There was an ascending trend of the disease infection rate of berries after the incubation period in 2016 and 2018. This shows that the susceptibility of berries to CBD may be observed at the scale of a berry and with respect to its physiological state. Similar results were obtained by Mouen Bedimo (2006) who reported that the sensitivity of Jamaican coffee berries (sensitive variety) to CBD varies according to an ascending gradient regardless of their age. In the present study, the comparison of means showed a significant difference between the infection rate of berries of the 22nd and 25th week after flowering.

This was contrary to 2018 where no significant difference was observed between the infection rates for the two series of inoculation. This result may be explained by the level of ripening of the berries at the time of inoculation. In fact, mature berries are less susceptible to *Colletotrichum kahawae* compared to immature berries (Mouen Bedimo *et al.*, 2008; Garedrew *et al.*, 2017). In general, berries are more susceptibility to CBD during the growth phase of endosperm which is between the 18th and 25th WAF.

The physiological resistance is usually acquired between the 26th and 32nd WAF which correspond to the stage of hardening of endosperm (Garedrew *et al.*, 2017). The infection rate of the berries of the caturra was high compared to that of the berries of the java. Previous *in situ* analysis highlighted the high susceptibility of the caturra coffee berries compared to those from *java* coffee as demonstrated by Bouharmont (1992).

In this study, berries inoculated with distilled water showed less infection rate. This result contrasts with that obtained by Pinard *et al.* (2012) who did no observed any sign of disease on berries inoculated with distilled water.

Table 3. Analysis of variance of glucose content in berries of Arabica coffee.

Sources of variability	DF	F Test	Pr > F
Variety	1	29.8358	0.000***
Shading type	1	8.9651	0.011187***
Treatments	1	2.0044	0.1822
Error		0.27320	

*** Parameters having a significant effect.

The low infection rate observed on our berries inoculated with distilled water may be explained by a latent infection which is quite common in other fruit and suspected in coffee berry (Prusky and Pumbley, 1992).

The symptoms observed during this work were those of the active form of the disease (dark black necrotic spots). This result could be explained by the optimal humidity level in the Petri dishes favorable to the development of *Colletotrichum kahawae* in which, the berries were placed during the experiment. In fact, Relative humidity close to saturation and optimum temperatures of 20 to 22 °C are favor the germination and appressorium formation. The infection hyphae arising from those appressoria observed as black spot while the scam form is usually observed in unfavorable condition (Massaba and Waller, 1992; Mouen Bedimo *et al.*, 2008).

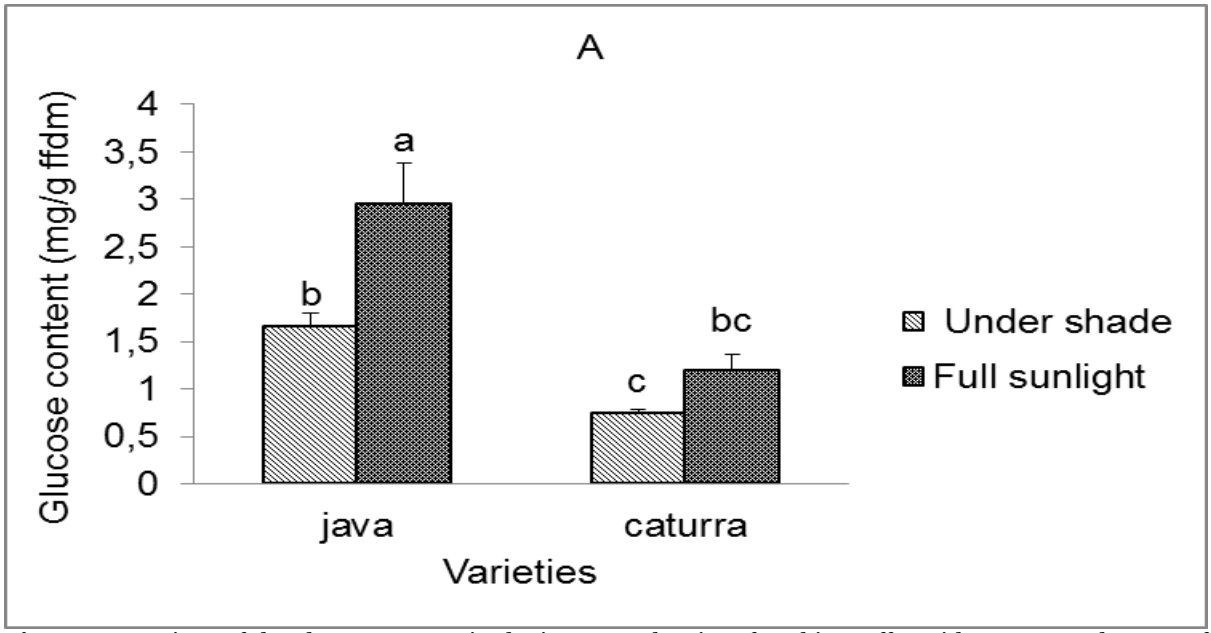

Fig. 1. Comparison of the glucose content in the immature berries of Arabica coffee with respect to the type of lighting and the variety.

The values with the same letters for each factor are not significantly different at P ≤ 0.05 according to LSD test of Fisher.

Sugar content

The study of the soluble sugar composition of the immature berries after infection allowed us to identify the glucose as a single type of sugar produced by berries 10 day after inoculation. Under *in vitro* stress conditions (optimal humidity) and infection, the starch presents in berries is hydrolyzed and give simple sugar such as glucose. Similarly, the accumulation of soluble sugars in tobacco leaves after infection was reported by Shalitin *et al.* (2002). The accumulation of soluble sugars such sucrose, glucose and fructose in *Arabidopsis thaliana* leaves under

stress conditions was also reported by (Hummel *et al*., 2010; Muller *et al*., 2011). In fact, carbohydrates have been reported to act in association with plant defense chemical compound such as polyphenols, phytoalexin, phytoanticipin and lignin in response to stress (Djocgoue *et al*., 2011). The glucose content in the infected inoculated berries and in those of the control coffee trees was not significantly different. This result is similar to those obtained by Andrew *et al*. (2005) where *in vitro* infection of the leaves of Turnip (Brassica rapa subsp. rapa) and *Arabidopsis* did not affect their sugar content. Nevertheless, it has been reported that there is a possible relationship between the content of specific carbohydrates such as sucrose, glucose and galactose and the resistance of the host (Evers *et al*., 2003; Omokolo and Budjeko, 2005; Djocgoue *et al*., 2011).

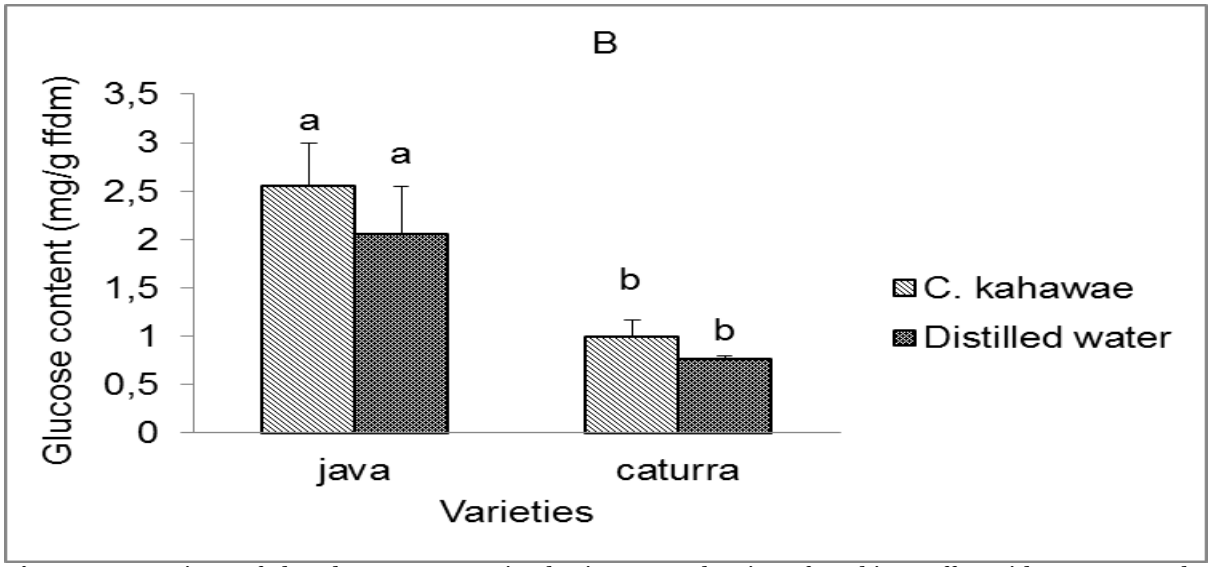

Fig. 2. Comparison of the glucose content in the immature berries of Arabica coffee with respect to the treatments and the variety.

The values with the same letters for each factor are not significantly different at P ≤ 0.05 according to the LSD test of Fisher.

The variety and type of shade showed a significant effect on the glucose content in berries inoculated *in vitro*. The glucose content of coffee berries exposed to the full sunlight was very high compared to those under shade, mainly for the berries of java coffee on the 10th day after inoculation. Previous studies showed that *in vitro* infection rate was not affected by the exposition of berries to full sunlight or shade (Mouen Bedimo *et al*., 2008). The response to environmental stress conditions *in situ* by the java variety may be preserved under stress conditions *in vitro*, which could explained the high glucose content observed *in vitro* in the java berries variety. The results also showed that the highest glucose contents were obtained from infected inoculated berries of both varieties. The glucose seem to be implicated in the induction of the defense mechanisms of Arabica coffee berries against *Colletotrichum kahawae*. Herbers *et al*. (1996) reported that hexoses induce the expression of many genes, including plant resistance genes that determine the production of peroxidase and other pathogenesis-related (PR) proteins. Others authors have reported that soluble sugars are basic substances capable of stimulating plant immunity and defense (Bolouri and Van Den Ende, 2013; Trouvelot *et al*., 2014; Morkunas *et al*., 2014).

The high glucose content observed in berries of java variety may be explained the high tolerance to *Colletotrichum kahawae*. However, it has been reported that the stage of development and the environmental factors to which the plant is subject can influence organ metabolism (Zufferey *et al*., 2012).

Conclusion

This study reported that the degree of maturation of the berries during inoculation has an influence on their susceptibility to coffee berry disease (CBD) *in vitro*. Glucose is the soluble sugar identified in immature berries *in vitro*. The berries of the java (tolerant) coffee gave the glucose content 2.6 times higher than that obtained from the berries of caturra (sensitive) coffee. The highest glucose contents were also obtained from infected inoculated berries of both varieties (java and caturra). The glucose seem to be implicated in the induction of the defense mechanisms of Arabica coffee berries against *Colletotrichum kahawae*. Artificial inoculation on detached berries is a potential approach that can contribute to a better understanding of the epidemiology of coffee berry disease.

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