Black Leaf Streak Disease Affects Starch Metabolism in Banana Fruit

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Ⓢ *Supporting Information*

ABSTRACT: Black leaf streak disease (BLSD), also known as black sigatoka, represents the main foliar disease in Brazilian banana plantations. In addition to photosynthetic leaf area losses and yield losses, this disease causes an alteration in the pre- and postharvest behavior of the fruit. The aim of this work was to investigate the starch metabolism of fruits during fruit ripening from plants infected with BLSD by evaluating carbohydrate content (i.e., starch, soluble sugars, oligosaccharides, amylose), phenolic compound content, phytohormones, enzymatic activities (i.e., starch phosphorylases, α- and β-amylase), and starch granules. The results indicated that the starch metabolism in banana fruit ripening is affected by BLSD infection. Fruit from infested plots contained unusual amounts of soluble sugars in the green stage and smaller starch granules and showed a different pattern of superficial degradation. Enzymatic activities linked to starch degradation were also altered by the disease. Moreover, the levels of indole-acetic acid and phenolic compounds indicated an advanced fruit physiological age for fruits from infested plots.

KEYWORDS: *Musa, starch metabolism, black leaf streak disease, black sigatoka, banana*

■ INTRODUCTION

Black leaf streak disease (black sigatoka, BLSD), the major foliar disease of bananas, is caused by the pathogenic fungus *Mycosphaerella fijiensis* Morelet. This disease is the major limitation in Brazilian banana plantations primarily because of the high susceptibility of Cavendish bananas to this disease.[1] In the Brazilian banana market, two genomic groups are mainly commercialized: Cavendish (AAA) (cvs. Nanicão, Nanica, and Grande Naine) and Prata (AAB).[2]

During the development of banana fruit, large amounts of carbon (12−35% of the fresh weight) are accumulated as starch, which is partially or completely degraded into soluble sugars during ripening (8−20%), depending on the cultivar.[3,4] Heavy infestations of foliar diseases, such as BLSD, can lead to a considerable reduction of the photosynthetic leaf area of the plant. BLSD reduces the chlorophyll content and photosynthetic activity of the leaves. Consequently, the amounts of starch and soluble sugars in the leaves are also reduced by this disease.[5] Once BLSD impairs photosynthetic production, it may lead to lower bunch and fruit weights of bananas compared with those of healthy plants, consequently possibly affecting the synthesis of starch.[6] Additionally, the green life stage, the time between harvest and the initiation of the ripening process, is substantially shortened (33%) in bananas harvested at a standard commercial grade from highly BLSD-infected plants.[6] Recently, Chillet et al.[7] demonstrated that, unlike abiotic stresses, yellow sigatoka (*Mycosphaerella musicola* Leach) directly reduced the green life stage of bananas harvested at identical physiological age in the tropical conditions of Guadeloupe.

Moreover, black sigatoka strongly affects the postharvest quality of the fruit and may cause yield loss.[8,9] Black sigatoka also caused delayed flowering and harvest in addition to premature fruit ripening.[8]

Banana fruits have a typical climacteric period, which begins with the increase in ethylene production and respiration rate followed by ripening-related metabolic changes that include yellowing of the peel and pulp, pulp softening, increased volatile compound production, and conversion of starch into soluble sugars in the pulp.[10,11]

Although an increase in ethylene is associated with ripening initiation, other phytohormones appear to play a role in this process. Indole-3-acetic acid (IAA) has also been correlated with ripening initiation, as free IAA levels have been found to decrease before ripening begins.[12]

Starch degradation provides carbon for the synthesis of sucrose and some volatile compounds, which will produce the flavor of the ripe fruit. There are indications that this process is also responsible for the change in texture in fruits that become softer during ripening.[13]

Although *Mycosphaerella* leaf spot diseases have been shown to heavily influence physiological postharvest behavior in

banana fruit, the biochemical mechanisms that affect the fruit quality remain unknown.

In a previous work,[9] we found a marked effect of this disease on reducing the green life of fruit (by accelerating ripening initiation) and increasing ethylene production, which was observed simultaneously with a fruit respiration disorder (measured as CO_2 production). To evaluate the disease infestation rate between plots, a severity index (SI) was monitored regularly to plot a disease-progress curve and determine the area under the disease-progress curve (AUDPC, described by Jeger and Viljanen-Rollinson,[14] which quantifies the BLSD infestation during the experiment. The disease infestation, evaluated using the AUDPC, was substantially lower in the control plot at approximately 2750, compared to 6260 for the infested plot, as described by Castelan et al.[9] Samples used in the present work were obtained in an identical manner.

In this work, we investigated the influence of BLSD on the starch metabolism of the banana fruit. Total soluble carbohydrates and the amount of starch were monitored during the banana ripening process. The activities of the major enzymes involved in starch degradation, that is, α-amylase (EC 3.2.1.1) and β-amylase (EC 3.2.1.2), were estimated in the soluble fraction of the banana pulp and in the fraction associated with the starch granule. Starch phosphorylase activities were only estimated in the soluble fraction of the pulp because it is already known that this enzyme is not associated with the starch granule.[4] Furthermore, the microscopic characteristics of the starch granules were observed during the entire ripening process, and the major hormonal changes were determined. The results obtained here would provide new insights to further understand how BLSD affects the postharvest quality of Cavendish banana.

MATERIALS AND METHODS

Plant Material. The experiments were conducted with *Musa acuminata* (AAA, cv. Nanicão, Cavendish subgroup).

Experimental Design. Two experiments were conducted in the years 2008 and 2010 to compare high BLSD infestation and low infestation during two different seasons. Both experiments concerned two different plots: a control plot, which was a commercial farm with a low level of BLSD infestation; and a BLSD plot, which was a commercial farm with a high level of BLSD infestation. In both experiments, the plots were selected in a central region of Vale do Ribeira in the state of São Paulo in southeastern Brazil, with identical pedoclimatic conditions (annual rainfall, 1500 mm; yellow latosol soil) in which BLSD has been present since 2004. Cultural practices were very similar for all selected plots (fertilization and density of planting, 1800 plants/ha). The plots were in a third to fifth crop cycle.

In the 2008 experiment, a 2000 m² plot with predominant BLSD infestation was selected as the BLSD plot, and this plot ceased disease control during the experiment. For the control plot, a 2000 m² plot was selected in which leaf spot disease control was performed through the use of aerial fungicides, and this constituted a "healthy plot" with a low disease infestation level.

In the 2010 experiment, a 3000 m² plot with predominant BLSD infestation was selected as the BLSD plot, and in this plot, disease treatment ceased during the experiment. For the control plot, a 3000 m² plot was selected where leaf spot disease control was performed through the use of aerial fungicides, and this constituted a "healthy plot" with a low disease level.

Both experiments were conducted with three harvest replicates, with 20 banana plants selected at the flowering stage in each plot. The selected plants were similar in terms of bunch size and infestation levels of leaf spot disease. Ten bunches were harvested at 700 degree days (dd), representing an early harvest time, and 10 bunches were harvested at 960 dd, representing a complete stage. Except for leaf spot disease control (which was performed only on control farms) through the use of approximately eight treatments/year of aerial fungicide applications [triazole (0.4 L/ha) and strobilurin (0.8 L/ha)], similar crop management strategies were used for all banana plants and included the following: blue polyethylene plastic bags, monthly fertilization, and other phytosanitary treatments in addition to fungicides.

Leaf spot diseases were assessed monthly for 25% of the plants, according to the methods of Stover[15] modified by Gauhl et al.,[16] using the disease SI.

Calculation of the Physiological Age of the Fruit. Temperature probes (Gemini Data Loggers Ltd., Chichester, West Sussex, UK) were placed under a shelter inside the experimental plots. The mean daily temperature was calculated using hourly temperatures. The physiological age of the fruit was expressed in degree days calculated from the sum of the mean daily temperature at the 14 °C threshold during the flowering-to-harvest period.[17]

Carbohydrate Content. The starch content of pulp tissue was determined enzymatically as described by Cordenunsi and Lajolo.[3] Soluble sugars and oligosaccharides were extracted three times with 80% (v/v) ethanol at 80 °C. After centrifugation, supernatants were mixed, and ethanol was evaporated under vacuum using a SpeedVac system. The soluble sugar (i.e., glucose, fructose, sucrose) and oligosaccharide (i.e., maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) content were analyzed by high-pressure liquid chromatography with pulsed amperometric detection (HPLC-PAD; Dionex, Sunnyvale, CA, USA), using a PA1 column (Dionex) in an isocratic run of 18 mM NaOH for 25 min. The total soluble sugar was determined as the sum of the glucose, fructose, and sucrose values. The amylose content was determined using an enzymatic method from Megazyme (kit K-AMYL 04/06, Megazyme International Ireland Ltd., Wicklow, Ireland).

Abscisic Acid (ABA) and Free Indole-3-acetic Acid (IAA). ABA and free IAA were extracted from approximately 0.1 g of dried banana pulp tissue and transferred to 2 mL microcentrifuge tubes. The powdered tissue was mixed with 0.5 mL of isopropanol and acetic acid (95:5 v/v). One microgram of the internal standard [$^{13}C_6$]-IAA (Cambridge Isotopes Inc., Cambridge, UK) and [$^2H_6$]-(+)-ABA (OlChemIm Ltd., Olomouc, Czech Republic) was added, and the mixture was vortexed for 1 min. The tubes were agitated for 2 h (Thermomixer, Eppendorf AG, Hamburg, Germany) at 4 °C, the mixture was centrifuged (10 min, 14000g), and the supernatants were collected. These supernatants were dried, resuspended in methanol, methylated with diazomethane, and analyzed in a gas chromatograph (HP-6890, Palo Alto, CA, USA) equipped with an HP5973 mass selective detector. An HP-1701 column (30 m, 0.25 mm i.d., 0.5 μm film thickness) was used, and the analytes were eluted with helium as the carrier gas at 4 mL/min. The oven temperature program was an initial hold at 160 °C for 2 min followed by an increase to 200 °C at 4 °C/min and a final hold for 10 min. An automatic liquid sampler (Agilent, Palo Alto, CA, USA; model 1100) performed the injections (1 μL) at 250 °C in splitless mode.

IAA ions were monitored at m/z 130 and 189, corresponding to quinolinium and the molecular ion of IAA, respectively, and at m/z 136 and 195, the equivalent ions of the labeled IAA internal standard. The ratio of 130/136 was used to calculate the endogenous amount of IAA, and the ratio of 189/195 was used for confirmation. ABA ions were monitored at m/z 162 and 190, corresponding to endogenous ABA, and at m/z 166 and 194, the equivalent ions of the labeled ABA internal standard. The ratio of 162/166 was used to calculate the endogenous amount of ABA, and the ratio of 190/194 was used for confirmation.

Scanning Electron Microscopy (SEM). Starch granules were isolated from the fruit pulp at two different ripening stages: green and fully ripe (completely yellow fruits with brown spots). Approximately 100 g of banana pulp was homogenized in 300 mL of 100 mM HEPES−KOH, pH 8.0, containing 1 mM EDTA, 5 mM DTT, and 0.05% (v/v) Triton X-100. The homogenate was filtered through a Miracloth membrane (Calbiochem), and the filtrate was decanted

overnight at 4 °C. The major supernatant was discarded, and the aqueous pellet was subjected to a 95% (v/v) Percoll gradient (GE Healthcare, Amersham Biosciences). After 30 min at 4 °C, the solution was centrifuged; the pellet was washed with 500 mM HEPES−KOH, pH 7.0, dried under vacuum (SpeedVac), and stored at 20 °C.

For SEM, dried starch was fixed onto stubs with double-sided tape and coated with a 10 nm thick platinum layer using the Bal-Tec MED-020 coating system. The samples were examined on a Quanta-600 scanning electron microscope (FEI, Eindhoven, The Netherlands). SEM was performed in secondary electron mode at 2−5 kV.

Analysis of Particle Size Distribution. Particle size distribution was determined by laser diffraction spectrophotometry (Malvern Mastersizer S long bed, Malvern, Worcestershire, UK).

Phenolic Compounds. Total phenolic compounds were determined according to the method of Singleton and Rossi.[18] Phenolic compounds were extracted from a 0.5 g sample with methanol (70% v/v) and filtered on filter paper. An aliquot of the extract solution (2.5 mg/mL methanol) was mixed with 0.25 mL of Folin−Ciocalteu reagent (previously diluted with water 1:2 v/v), 0.5 mL of saturated sodium carbonate (Na_2CO_3) solution, and 24 mL of water. The mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 725 nm. The results were expressed as gallic acid equivalents.

Enzymatic Activities. Proteins were extracted from the frozen samples of banana pulp. A 0.1 g sample was homogenized in 2.5 mL of 50 mM HEPES−KOH buffer, pH 7.0, containing 20 mM cysteine, 1% (w/v) polyvinylpyrrolidone MW 40000 (PVP-40) and 1 mM benzamidine. After 15 min of centrifugation (10000g), the supernatant was used immediately in the enzymatic assays.

α-Amylase activity was determined according to the method described by McCleary and Sheehan[19] using the specific reagent Ceralpha-Megazyme, and the developed color was measured at 410 nm. β-Amylase activity was determined according to the method described by McCleary and Codd[20] using the specific reagent Betamyl-Megazyme and was also measured at 410 nm. The activities were expressed as micromoles of p-nitrophenol released by 1 mg of protein in 1 h.

Phosphorylase isoform activities were detected on a polyacrylamide (6% w/v) gel after electrophoresis. Two isoforms were visualized due to their differential affinity toward branched glucans in a native gel containing glycogen (0.4% w/v) according to the method of Sonnewald et al.[21] Gels were incubated for 2 h at 37 °C in citrate−NaOH, pH 6, containing 0.005% (w/v) of soluble starch and 20 mM glucose-1-phosphate. The gels were stained with iodine, and the phosphorylase activity appeared as blue bands. Total proteins were quantified using the method presented by Bradford.[22]

Data Analysis. The average contents of soluble sugars, starch, IAA, and ABA were compared. A normality test (Shapiro−Wilk) and a test for equal variance (Levene) at the 5% significance level followed by Tukey's test (also at the 5% significance level) were performed using the Origin 8.0 software package (Northampton, MA, USA).

■ RESULTS AND DISCUSSION

As previously reported by Castelan et al.,[9] the control fruits were harvested at two different physiological ages, 700 and 960 dd, whereas the fruits from the infested plot could not be harvested at 960 dd because they were already ripe while attached to the plant at that stage. Therefore, the samples were the following: Control-960, Control-700, and BLSD-700. Fruits from Control-700 showed a green life of approximately 25 days, whereas BLSD-700 fruits harvested at an identical physiological age had a green life of nearly half of this period (∼14 days). A shorter green life was also observed in Control-960 fruits (∼16 days). The results reported below were obtained using identical samples as those obtained by Castelan et al.[9]

Free IAA and ABA Contents. Because of the shorter green life of the fruits from the BLSD plot, the IAA and ABA levels were investigated, as their levels appear to be correlated with

the onset of banana ripening.[12,23] Also, ABA has been related with plant responses in phytopathogenic resistance.[24,25] The results reveal that both phytohormones were altered in plants infested by black sigatoka (Table 1). Fruits from BLSD-700 had

Table 1. Phytohormone Levels in Green Stage Bananas[a]

	physiological age (dd)	control	BLSD
IAA (ng g^{-1} FW)	700	9.5 ± 0.2 a	5.7 ± 0.4 b
	960	7.3 ± 1.4 b	NH
ABA (mg g^{-1} FW)	700	68.8 ± 5.7 b	95.7 ± 4.7 a
	960	58.9 ± 2.8 b	NH

[a]Each value is the mean of at least three determinations that are indicated with the standard deviation, respectively. BLSD, black leaf streak disease; IAA, indole-3-acetic acid; ABA, abscisic acid; dd, degree days, NH, not harvested. Means of each phytohormone were compared according to Tukey's test; different letters (a, b) indicate statistically significant differences ($p > 0.05$).

40% less free IAA than Control-700 fruits and 22% less than Control-960 fruits. Moreover, we found greater ABA levels on green bananas harvested from the BLSD plot compared with those harvested from the control plot, independent of the physiological age at harvest. Fruits from Control-700 had 28% less ABA content than fruits from BLSD-700. Fruits from Control-960 had 38% less ABA content than fruits from BLSD-700.

The decreased IAA levels appear to be time correlated with the onset of ripening. It was found that the free IAA levels must be reduced to enable some ripening such as starch degradation and subsequent sweetening of the fruits.[12] We previously demonstrated that high IAA levels delay the activity of β-amylase, suggesting not only the importance of this enzyme on starch degradation but also its regulation by IAA.[26] Because of this correlation between decreasing IAA levels and the onset of ripening, we expected that green fruits from the BLSD plot would have the lower IAA levels than fruits from the control plots harvested at an identical physiological age (700 dd). Indeed, the IAA levels of BLSD-700 fruits were similar to the IAA levels of fruits harvested from the control plot at a completely mature stage (960 dd), indicating an acceleration of fruit maturity for fruits harvested from infected plants (Table 1). Likely, BLSD could harm the foliar meristems, one of the major sites of IAA synthesis, and thus would be the primary cause of low IAA levels in BLSD fruits.

Alternatively, ABA levels on green banana fruit also play a role in ripening initiation and in the sequence of ethylene-mediated ripening events, increasing ethylene sensitivity.[23] Therefore, the higher the ABA levels in green fruit, the faster the onset of ripening initiation, as indeed was observed (Table 1).

Both hormones appear to be linked to the sensitivity of the banana to ethylene, indicating a coordinated balance of these two phytohormones (IAA, ABA) and ethylene in the process of banana ripening. Moreover, their levels in BLSD fruits indicate that the ripening process was advanced by disease in these plants.

Soluble Phenolics. Phenolic compounds are known to be associated with plant defense and may be affected by black sigatoka infection. It was previously shown that this cultivar contains approximately 650 mg gallic acid equiv/100 g pulp, which does not vary during ripening.[27]

Table 2. Phenolics Compound Contents in Green and Ripe Bananas[a]

	green		ripe	
physiological age (dd)	control	BLSD	control	BLSD
700	1231.3 ± 25.7	478.9 ± 49.9	1360.0 ± 4.9	372.1 ± 41.0
960	653.1 ± 4.9	NH	585.4 ± 28.4	NH

[a]Each value is the mean of at least three determinations that are indicated with the standard deviation, expressed as milligrams of gallic acid equivalents per 100 g of dry weight. BLSD, black leaf streak disease; dd, degree days; NH, not harvested.
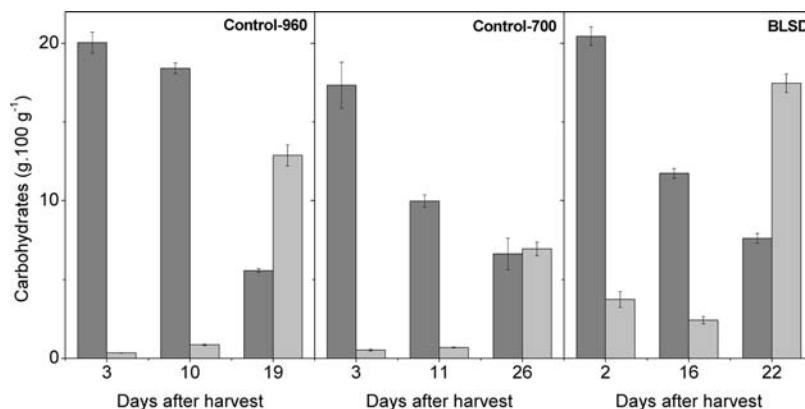
Figure 1. Starch (dark gray) and total soluble sugars contents (light gray) during ripening of Nanicão bananas. BLSD, fruits from black leaf streak disease plot at physiological age 700 degrees days (dd); Control-700 and Control-960, control fruits at 700 and 960 dd, respectively. Columns represent the means (n = 3), and the bars represent the standard deviations.

Table 2 indicates that bananas harvested after full maturity (960 dd) from the control plot contain a similar phenolic content as those previously determined for the same cultivar.[27] We found that Control-700 samples contained higher amounts (2-fold) of phenolic compounds than Control-960 samples. BLSD-700 fruits, similar to the Control-960 fruits, contained lower amounts of soluble phenolic compounds compared to the Control-700 fruits. This result could suggest that the infection by *Mycosphaerella* led to a polymerization of phenolic compounds or that the phenolic synthesis could have been affected by plant disease because fruits from BLSD-700 contained the lowest amount of phenolic compounds of all the samples.

Phenolic compounds involved in plant defense are either preformed (constitutive) or synthesized de novo (postinfectional). Pre-existing phenols are antifungal compounds such as simple phenols, phenolic acids, flavonols, and dihydrochalcones, which act as antimicrobial compounds during defense responses against micro-organisms.[28] Otherwise, phenolic compounds synthesized de novo accumulate in response to plant infection by a pathogen. This defensive response involves the rapid increase of specific phenolic compounds at the infected site, particularly phytoalexins, which can inhibit a broad range of micro-organisms.[28] In some cases, many simple low molecular weight phenolic compounds present in plants may be readily polymerized by oxidation to yield brown tannin-like substances (melanins) containing quinonoid groups.[28] Additionally, an accumulation of soluble phenolics, wall-bound phenolics, and phenolic polymers (lignin and lignin-like polymers) was observed in *Musa acuminata* roots exposed to a cell wall-derived elicitor from the pathogen *Fusarium oxysporum*,[29] indicating that the increased flux through the phenylpropanoid pathway and the incorporation of phenolic compounds into the cell wall fraction are parts of the antimicrobial defenses activated in the root tissue.

The higher content of phenolic compounds in Control-700 fruits and the lower content of these compounds in Control-960 and BLSD-700 fruits suggest an acceleration of the physiological age caused by black sigatoka infection, which could be caused by the shortening of the growth cycle as a consequence of the phytopathogenic stress.

Carbohydrates. Figure 1 presents the content of starch and soluble sugars (glucose, fructose, and sucrose) during the green, intermediary, and ripe stages. The initial levels of starch were similar between BLSD-700 and Control-960 fruits and different from Control-700 fruits, which appeared to still be accumulating starch at that stage.

Fruits from Control-960 demonstrated the typical profile of starch degradation and sugar accumulation as previously described:[3,10,30] during development, approximately 20% of the starch is accumulated in the pulp fruit and is degraded during ripening with concomitant accumulation of large amounts of soluble sugars, with a predominance of sucrose over fructose and glucose.

Moreover, Control-700 fruit accumulated approximately 17% of starch and completed the ripening process with 7% of starch (Figure 1). The Control-700 fruit differed from the Control-960 fruit in the final soluble sugar content, which was below 10%. It appears that the enzymatic apparatus for sucrose synthesis was not ready at that physiological age, as evidenced by a myriad of residual intermediates from the starch-to-sucrose metabolism [i.e., oligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose] that resulted in amounts 5 times higher for Control-700 (0.73 ± 0.10 g G_2-G_7 100 g^{-1} FW) than for Control-960 (0.14 ± 0.06 g 100 g^{-1} FW). Additionally, BLSD-700 (0.22 ± 0.05 g 100 g^{-1} FW) contained oligosaccharide amounts equivalent to those of Control-960.

Fruits from BLSD-700 contained accumulated amounts of starch comparable with those from Control-960 fruits and
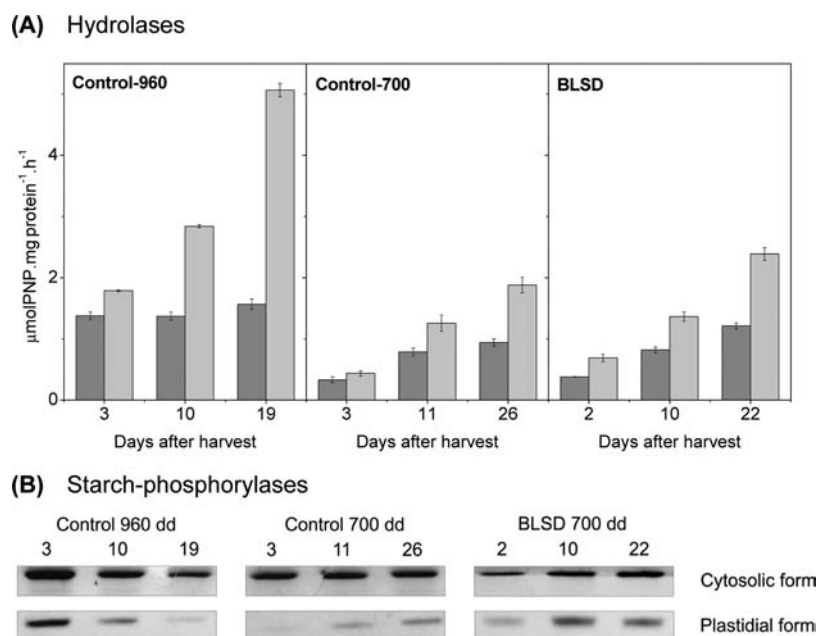
Figure 2. (A) Activity profiles of α-amylase (dark gray) and β-amylase (light gray) in the pulp of bananas during ripening. BLSD, fruits from black leaf streak disease plot at physiological age 700 degrees days (dd); Control-700 and Control-960, control fruits at 700 and 960 dd, respectively. Columns represent the means ($n = 3$), and the bars represent the standard deviations. (B) Native PAGE (6%) with glycogen (25 μg/mL) to detect the starch phosphorylase (cytosolic and plastidial) activity in the pulp of bananas. Numbers represent the days after harvest.

residual amounts similar to those from Control-700 fruits. The high amounts of initial and final soluble sugars in BLSD-700 fruits were unexpected (Figure 1). At the time of harvest, BLSD-700 fruits already contained nearly 4% of soluble sugars. These results indicate that starch degradation initiates before harvest, likely as a result of the shortened life cycle of the plant due to phytopathogenic stress or as a consequence of low free IAA levels.[12]

Starch metabolism during ripening is a fundamental process in banana fruit, being responsible for fruit sweetness. For Cavendish bananas, the starch content in green fruits can reach 35%.[4,31] During ripening, starch must be degraded into soluble sugars to reach 5% or less in ripe fruit, whereas soluble sugar content increases up to 15%.[3] Control-700 samples did not display a normal starch degradation pattern and contained lower amounts of soluble sugars at the ripe stage (Figure 1). This result could be a consequence of early harvest time because the initial starch content was higher in Control-960 fruits, suggesting that Control-700 fruits had insufficient time to accumulate starch at levels identical to those of Control-960 fruits. Moreover, the enzymatic apparatus cannot be entirely operative. For both hypotheses, the absent sugars in the Control-700 sample could exist as intermediate carbohydrates, such as oligosaccharides.

Fruits from Control-700 at the ripe stage contained higher amounts of other sugars, such as oligosaccharides (data shown above), which could be one reason for the lower content of soluble sugars. The larger amounts of oligosaccharides could indicate a dysfunction in the enzymatic starch-to-sucrose apparatus caused by the earlier physiological age, as these sugars are known to have a transitory character.

Enzymatic Activity: α-Amylase, β-Amylase, and Starch Phosphorylases. To clarify the cause for this aberrant carbohydrate metabolism, the enzymatic activity of certain enzymes involved in starch degradation was determined. Figure 2A shows the activity profiles of α-amylase and β-amylase.

Figure 2A reveals that in all of the samples, the β-amylase activity was higher than that of α-amylase, as is typically found in banana ripening,[4] but they differed in their total amounts. The β-amylase activity profiles were similar between BLSD-700 and Control-700 fruits, but their activities were substantially lower (2−4 times) than those of Control-960 fruits.

The activities of the starch phosphorylases (Figure 2B and Supporting Information Figure 6) determined using native PAGE including glycogen demonstrated protein bands with activities corresponding to the cytosolic form in all stages of ripening and treatments (Figure 2B). The cytosolic activities decreased in Control-960, increased in BLSD-700, and remained unchanged in Control-700 during the ripening process. The plastidial form was active at all stages of ripening for all analyzed treatments (Figure 2B). The plastidial isoform of starch phosphorylase was found to decrease in activity during the ripening of Control-960, as we have previously observed for this banana cultivar.[32] The opposite was found for the Control-700 samples, indicating that the starch degradation pathways differed for completely mature fruits (Control-960) and less mature fruits (Control-700).

Starch phosphorylases can use starch as a substrate acting in both directions of synthesis and degradation. As previously shown,[10,33] the activity and expression of starch phosphorylases during banana ripening are responsive to changes in basal levels of ethylene in a negative manner: higher ethylene levels result in lower phosphorylase activities. β-Amylase expression/activity depends on the burst of ethylene that occurs before ripening. It appears that phosphorylases act as the alternative pathway when β-amylase activity is low. Consistent with this, Control-960 fruits demonstrated higher β-amylase activity and decreased starch phosphorylase activity with ripening. Fruits that demonstrated lower β-amylase activity (BLSD-700 and Control-700) demonstrated an increase in starch phosphorylase activity.

Black leaf streak disease infestation in the production field demonstrated obvious effects on the metabolic processes of fruit ripening. The activities of the enzymes related to starch degradation during ripening were different among the samples, although the amount of degraded starch was similar. To understand the influence of the stage of maturity and the disease on the characteristics of the starch granules, we analyzed granules isolated from different stages of ripening from all treatments using SEM.

Scanning Electron Microscopy. Figure 3 and the Supporting Information SEM) shows granules of starch isolated
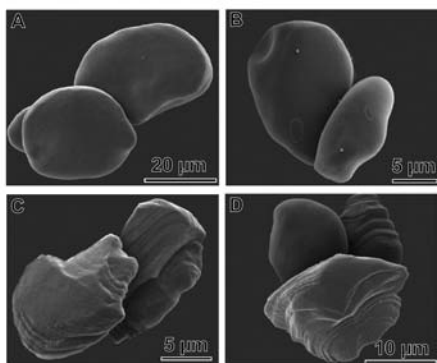
Figure 3. High-magnification scanning electron microscopy image of starch granules isolated from control plot bananas at physiological age 960 degree days. Granules were isolated at green stage (A, B) and ripe stage (C, D).

from the Control-960 fruits at two different ripening stages: green and ripe. The granules in green pulp (Figure 3A,B) had different sizes and shapes, contained predominantly larger granules (90% of the granules were 46.7 μm in size, Table 3), and were elongated in shape with a smooth surface. The granules in ripe pulp (Figure 3C,D) were smaller, showing signs of high degradation, as observed previously.[4,34]

Table 3. Starch Granule Average Diameter and Granule Size Distribution in Green Stage Banana Pulp[a]

|	average diameter (μm)	granule size distribution	
		90% (μm)	10% (μm)
Control-960 dd	26.57	46.73	10.29
Control-700 dd	24.59	42.98	9.75
BLSD 700 dd	27.92	27.92	9.76

[a]BLSD, black leaf streak disease; dd, degree days.

Starch granules of Control-700 fruits had a mean diameter of 24.9 μm with 90% of the granules approximately 43 μm in size (Table 3). The surface differed from the Control-960 fruits by the deposition of a material that resulted in a rough surface (Figure 4A,B), which may be due to the immaturity of the granules still in the formation process or an unknown material that deposits on the granule surface during the granule extraction process. Ripe pulp granules (Figure 4C,D) showed fewer degradation signs than those of the Control-960 fruits (Figure 3C,D).

The granules of starch from BLSD-700 fruits at the green stage were 40% smaller than those from the controls (Table 3). These granules, when compared with the other samples, appeared to still be in the process of layer deposition (Figure
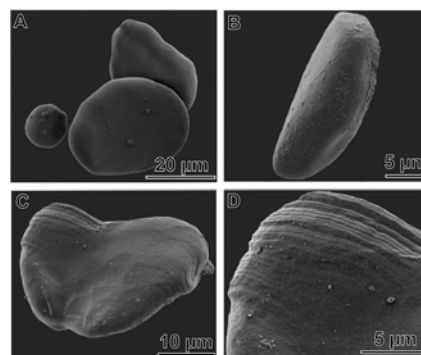
Figure 4. High-magnification scanning electron microscopy image of starch granules isolated from control plot bananas at physiological age 700 degree days. Granules were isolated at green stage (A, B) and ripe stage (C, D).

5A,B). In the ripe stage (Figure 5C,D), granules showed a population of larger granules without signs of degradation and
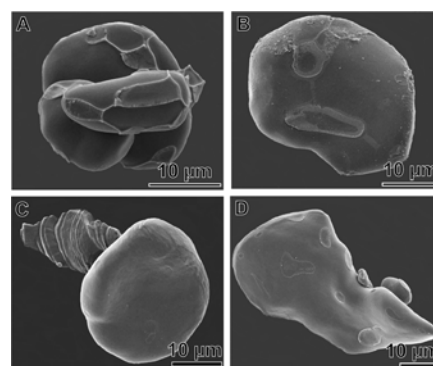
Figure 5. High-magnification scanning electron microscopy image of starch granules isolated from bananas of the black leaf streak disease infested plot at physiological age of 700 degree days. Granules were isolated at green stage (A, B) and ripe stage (C, D).

smaller granules with signs of degradation, suggesting that the latter are more susceptible to degradation, which confirms that smaller granules are more susceptible to degradation as has been shown previously.[4,35] The degradation signs were similar for the starch granules of all treatments, occurring layer by layer and ceasing near the granule axes. The amylose content (Table 4) did not differ between the controls (700 and 960), beginning with nearly 15% of amylose in the green stage, which increased by nearly 19% in the ripe stage. This result indicates that the center of the granules is richer in amylose than the periphery. For BLSD samples, the amylose content was not altered with ripening (15% of amylose, Table 4), an indication that the structure of these granules is different from that of the control samples.

Because smaller granules appear to have less resistance to degradation,[35] larger amounts of smaller granules in BLSD-700 fruits appear to be a strategy to hasten starch degradation and therefore reduce the ripening period. The results from this study revealed a few mechanisms of response to BLSD infection. It appears that the banana plant, as a part of its defense system, when infected, may alter physiological pathways to accelerate fruit formation and maturation. Another possibility is that the short period of time for starch accumulation results in immature granules with smaller size. Nevertheless, the amount of amylose in the interior of the

Table 4. Amylose Content (Percent) in Green and Ripe Bananas[a]

physiological age (degree days)	green		ripe	
	control	BLSD	control	BLSD
700	16.40 ± 0.47	14.78 ± 0.06	19.41 ± 0.23	15.00 ± 0.44
960	15.27 ± 0.84	NH	18.27 ± 0.34	NH

[a]Each value is the mean of at least three determinations that are indicated with the standard deviation. BLSD, black leaf streak disease; NH, bananas not harvested.

granules indicates differences in the network of enzymes that are involved in starch granule synthesis.

In conclusion, fruits from the BLSD-infested plots appear to have a shortened period of maturation, as demonstrated by the length of the green life stage in addition to the levels of hormones and total phenolic compounds. Consequently, the starch-to-sucrose metabolism is altered compared with that of control fruits in terms of both the format and composition of starch granules, as are the enzymes involved in its degradation. The amounts of starch and soluble sugars in the ripe fruit may reflect the quality of the fruit.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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