# Evaluation of ACF-SRP Variants for Control of Black Sigatoka

This evaluation was performed in the Dominican Republic, under supervision of MR Agro, and the agronomist and specialist in Black Sigatoka, Mr. Moises Martinez. Two variants of ACF-SRP were used, as follows:

- Fully formulated ACF-SRP (Powdered Variant or PV ), at 3 doses, PV1, PV2, and PV3
- Liquid ACF-SRP (Liquid Variant, or LV), without other formulated chemicals, but with the same bacterial species contained in powdered ACF-SRP, at three doses, LV1, LV2, and LV3.

**Background:**

In this study, ACF-SRP Variants PV and LV were evaluated for control of a fungal infection of banana plants in Santiago, Dominican Republic. Specifics are as follows:

| | |
|---|---|
| Testing Objective: | Control of Mycospharella fijiensis (Black Sigatoka) |
| Producer: | Agro Industrial Filpo Banana Plantation |
| Location: | Hatillo San Lorenzo, Santiago |
| Farm Area: | 2,700 ta (170 ha) |
| Testing area: | 13.2 ta (0.83 ha) |

Black Sigatoka is the main fungal disease affecting banana cropping (Musa paradisiaca) at the global level, in many cases causing productivity loss above 50%. The damage from this disease in not only economic, but also environmental, due to the intensive use of chemicals. As ACF-SRP is non-toxic, it would be a boon to farmers hoping to maintain banana yield while not damaging the environment. This study was designed to evaluate the efficacy of two ACF-SRP variants (fully formulated powder PV plus an aqueous version containing the bacteria only LV, in approximately the same cfu per ml in the liquid as the cfu per gram in the powder).

**Procedures:**

The banana plantation is of Cavendish variety with 2,226 plants per hectare. At the beginning of this trial (first dose), the plants were about three months old and about six-months old at the end of the evaluation. The period from 3 months to 6 months old is considered the most critical time for development of Black Sigatoka (this was also a period of high rainfall which escalates progression of the disease).

The ACF-SRP dosing began on January 30, 2017 and continued for three months.
Applications finished on April 15, 2017 and preliminary assessments were made on the April 30, 2017.

Dosing of the two ACF-SRP variants (PV and LV) were performed in six total parcels with an area of 2.2 tarea (0.14 ta) each. Three different doses of each ACF-SRP variant were used in each parcel, so that a low dose, medium dose, and high dose were performed for both powdered (PV) and liquid (LV) variants.

The tables below show the application rates and bacterial dose per hectare for PV (Fully Formulated ACF-SRP Powder) and LV (Liquid ACF-SRP bacteria without chitin or other organic chemicals in aqueous suspension):

| | Low Dose | Medium Dose | High Dose |
|---|---|---|---|
| **PV (Fully Formulated Powder)** | 226 grams per ha | 454 grams per ha | 904 grams per ha |
| **PV Bacterial Count per Ha** | 2.41E+10 | 4.84E+10 | 9.64E+10 |
| **LV (Bacteria only in Aqueous Suspension)** | 500 ml per Ha | 750 ml per Ha | 1000 ml per Ha |
| **LV Bacterial Count per Ha** | 4.35E+10 | 6.53E+10 | 8.70E+10 |

The PV and LV applications were performed about every two weeks, but rainfall, weather, and availability of personnel dictated actual application dates. Whenever ACF-SRP application was performed, both PV and LV were applied. The date for each application is shown in the table on the next page.

Additionally, the evaluation for efficacy against Black Sigatoka involves a forecasting procedure that requires evaluation and scoring of leaf emission rate and extent of disease progression. The table also includes dates on which the leaf testing and evaluation were performed.

**Application Dates of PV and LV ACF-SRP, and Leaf Evaluation Dates:**

| Date | Leaf Evaluation Performed? | Dosed? |
|---|---|---|
| 1/23/2017 | Tested | No |
| 1/30/2017 | Tested | **Dose** |
| 2/6/2017 | Tested | No |
| 2/13/2017 | Tested | **Dose** |
| 2/20/2017 | No | No |
| 2/27/2017 | Tested | No |
| 3/6/2017 | Tested | **Dose** |
| 3/13/2017 | No | No |
| 3/20/2017 | Tested | No |
| 3/27/2017 | Tested | No |
| 4/3/2017 | No | No |
| 4/10/2017 | Tested | No |
| 4/15/2017 | Tested | **Dose** |
| 4/22/2017 | No | No |
| 4/30/2017 | Tested | No |

The actual procedure for dosing the two ACF-SRP variants was as follows. The treatment was applied with mechanical backpacks of 20 liters (5.3 gallons), so that 20 liters were applied to each 0.14 ha test plot (about 38 gallons per ha).

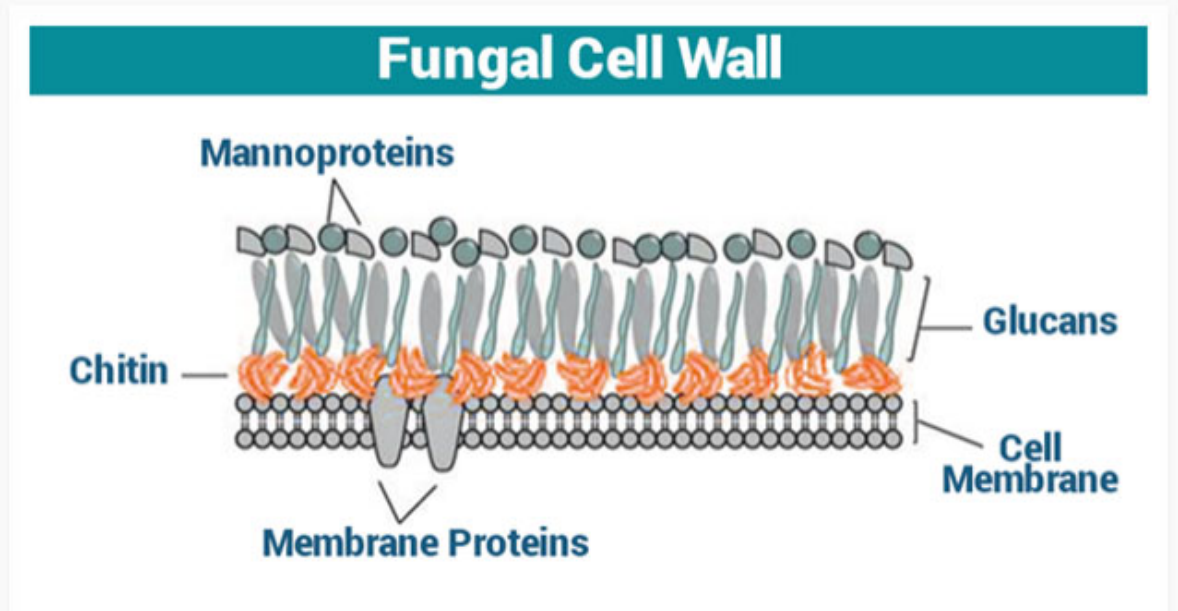
In normal conditions, banana crops are sprayed via air with a consumption of 24 gallons per ha. Actual product used in each 20 liter sprayer was:

| Plot ID | Dose per ha | Dose per plot (0.14 ha) |
|---|---|---|
| PV1 | 226 grams | 32 grams |
| PV2 | 452 grams | 64 grams |
| PV3 | 904 grams | 127 grams |
| LV1 | 500 ml | 70 ml |
| LV2 | 750 ml | 105 ml |
| LV3 | 1000 ml | 140 ml |
| PV = Powdered Variant | | |
| LV = Liquid Variant | | |

# Discussion of Key Parameters –
# Fungal Cell Walls and Effect of Chitinase

Black Sigatoka is a leaf spot disease caused by the ascomycete fungus, *Mycosphaerella fijiensis* (M. fijiensis). M. fijiensis can halve fruit production in affected plantations, as it is easily spread by airborne spores, rain, planting material, irrigation water and packing material used in transporting goods between banana-growing countries.

Mycosphaerella fijiensis is a fungus – a haploid, hemibiotrophic filamentous ascomycete – that causes black leaf streak. Cell walls of fungi contain chitin, as shown in the diagram:




Chitin in the fungal cell wall is the hard substance that facilitates invasion of banana leaf cells, thus promoting infection. In theory, the chitin-dissolving enzyme chitinase would reduce disease transmission and / or the rate of progression by solubilizing the chitin in the cell was, thus reducing the ability of the M. fijiensis to penetrate the plant cells.

For example, Andrew Kiggundu — head of banana biotechnology research at the Uganda's National Agricultural Research Laboratories Institute (NARL) in Kawanda — analysed 19 lines of genetically modified bananas and found promising results in five of them. These researchers inserted genes for chitinase production into genetically modified bananas. Kiggundu said laboratory tests using leaves from transgenic plants showed almost full immunity from Black Sigatoka infection when cultured fungi were applied to the leaves.

The potential biopesticide ACF-SRP is manufactured by TLC Products .

This study was designed to assess the relative performance of fully formulated, powdered ACF-SRP (PV1, PV2, and PV3), compared to liquid suspensions .

Standard banana plantation Black Sigatoka control practice is to forecast the progression of the disease (discussed below). In theory, using the forecasting method the farmer applies a corrective chemical (such as ACF-SRP) at the time that the key parameter (Evolutionary Development) becomes dangerously high. By applying at that time, and not earlier, the farmer not only minimizes use of chemicals / fungicide, but also minimizes the cost of application such as aerial dispersion.

The requirement for rapid farmer reaction (dosing a fungicide) to high level of Evolutionary Development dictates that the curative item (fungicide or ACF-SRP) must be applied immediately or as soon as possible.

The preferred application method of ACF-SRP is to aerate 2 lbs of ACF-SRP in 200 liters of water (using vigorous aeration, at 27 C) for 72 hours.

## Discussion of Key Parameters – Normal Progression of the Black Sigatoka Disease

After initial infection by M. fijiensis, the first symptom, chlorotic specks, generally appear within 2 to 3 weeks post-infection. The disease then proceeds as noted in the table below (Stage 1 through Stage 6):

| Stage | Common Name | Description |
|---|---|---|
| Stage 1 | Initial Speck Stage | Reddish-brown speck on lower surface. |
| Stage 2 | First Streak Stage | Reddish-brown streaks on both sides of the leaf. |
| Stage 3 | Second Streak Stage | Wider streaks. Color starts changing from red to dark brown. |
| Stage 4 | Spot | Dark brown (lower) to black (upper) spots. |
| Stage 5 | Second Spot Stage | Black spot with chlorotic halo. Lesion is slightly depressed. |
| Stage 6 | Third or Mature Spot Stage | Center of spot dries out and becomes whitish to gray. Spot is surrounded by a dark brown to black border and further depressed. |

## Measuring Progression of Black Sigatoka Disease

The industry standard measuring and forecasting system for Black Sigatoka is described in the paper, "A Biological Forecasting System to Control Sigatoka Disease of Bananas and Plantains". Jacky Ganry, Luc De Lapeyre De Bellaire, Xavier Mourich, Fruits, 2008, vol. 63, p. 381–387.

In general, this disease is driven by climatic conditions combined with virulence of the disease. Because of fungal antagonism on old leaves, infection only occurs on the cigar or leaf No. 1. Thus, new attacks are only detected on young leaves.

As the Foliar Emission Rate may be about 1 per week, the progression of the black sigatoka disease is from the top to the bottom of the plant (worst at the top).

Early detection and preventive / curative action is very important, since sporulation starts in necrotic stages of the disease. This is when the rate of infection is at its maximum. Therefore, the method focuses on early detection of new attacks of the disease.

In this method, youngest leaf spotted (virulence) is combined with the foliar emission rate in order to express the stage of evolution of the disease (Evolutionary Development) as a speed value. The greater the Evolutionary Development, the greater the danger of reaching necrotic stage, and extreme crop loss due to sporulation. This method also takes into account that the more vigorous the growth of the banana trees (Foliar Emission Rate). The main factors are explained as follows:

### Youngest Leaf Spotted (Infected)

Virulence is often measured by the rate of Spotting, and is indicated by the age of the youngest leaf spotted. As the "youngest leaf infected" decreases, the virulence is said to increase.

### Foliar Emission Rate

The foliar emission rate is the adjusted generation rate of new leaves per day (taking into account the leaves present in the prior evaluation), and is used as a cofactor to determine the speed of progression of the disease.

### Rate of Disease Progression or Evolutionary Development

The foliar emission rate and the youngest leaf spotted are used together to determine the overall state of the disease progression (Evolutionary Development).

This measurement (Evolutionary Development) is used as a forecasting tool to help time the application of chemical fungicides, particularly in areas where the farmers may be of low income and application when not needed is economically prohibitive. Also, using this forecasting method reduces the environmental impact of chemical fungicide application. As the Evolutionary Development increases, the need for fungicide increases.

Using the method outlined above, the banana farmers in the Dominican Republic use Evolutionary Development of 200 or higher as the trigger point to apply fungicide.

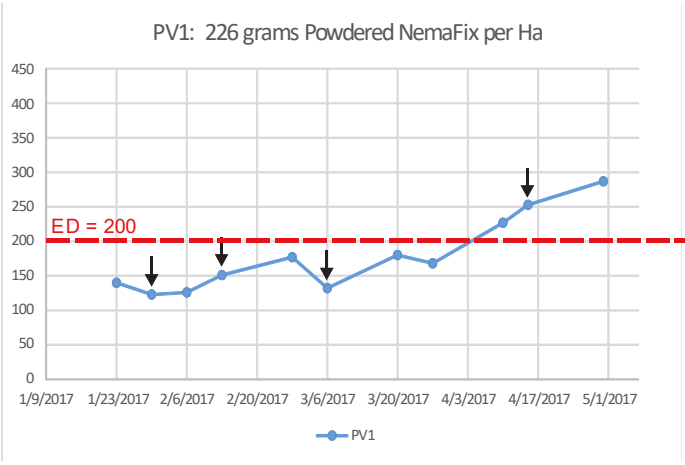
## Results

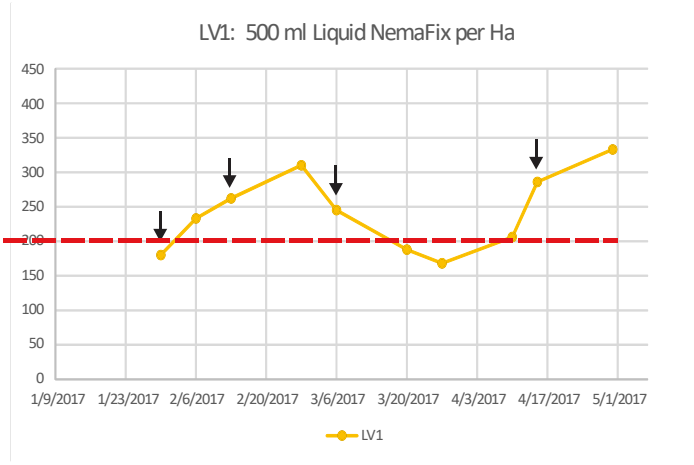
The data were all compiled using standard agronomic procedures, then were input into the standard algorithm to determine "Evolutionary Development of the Disease for Black Sigatoka". Using this index, the farmers are signaled to apply fungicide until the index falls below the threshold of 200. Below 200, the crop is safe and fungicide is not needed. Above 200, the farmer is signaled to apply fungicide.

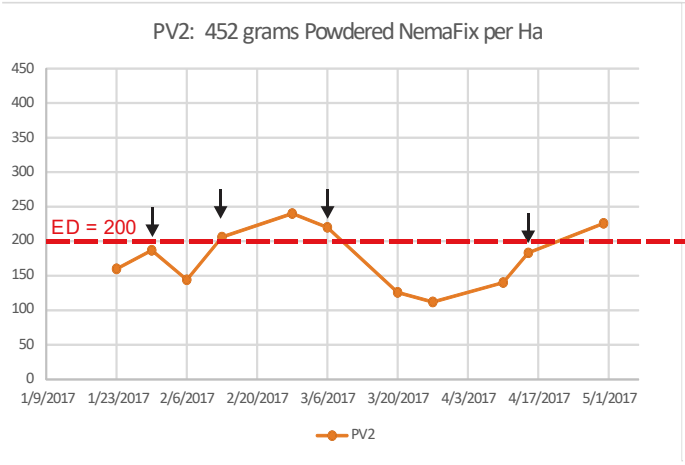
## Evolutionary Development

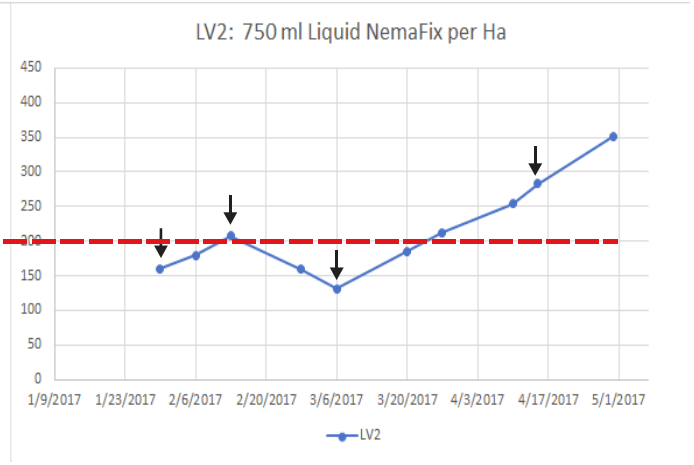
The graph below shows the calculated Evolutionary Development for the Powdered Variants (PV1, PV2, and PV3) on the left hand side of the page, and the Liquid Variants (LV1, LV2, and LV3) on the right hand side of the page. The Y-axis is Evolutionary Development and unitless, and the X-axis is time in weeks, beginning with the first point January 23, 2017 and ending April 30, 2017.

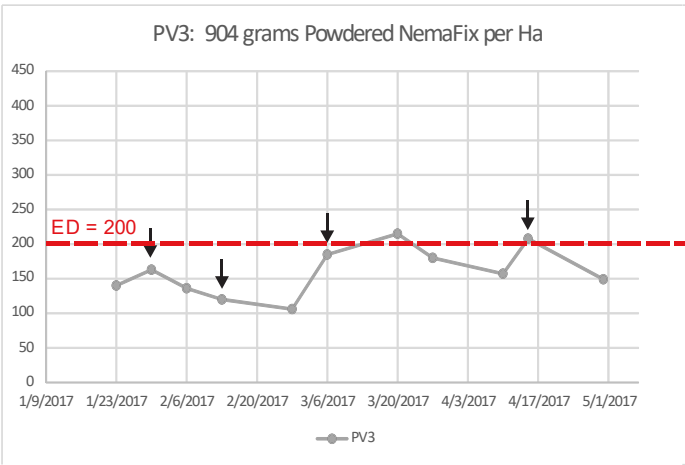
Dose proceeds from low to medium to high from the top graphs to the lower graphs. Arrows indicate ACF-SRP application date. The horizontal line at Evolutionary Development = 200 is the typical fungicide application trigger point.

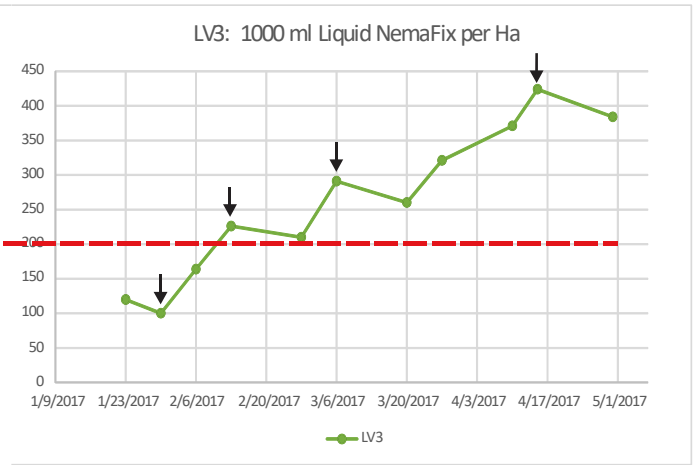
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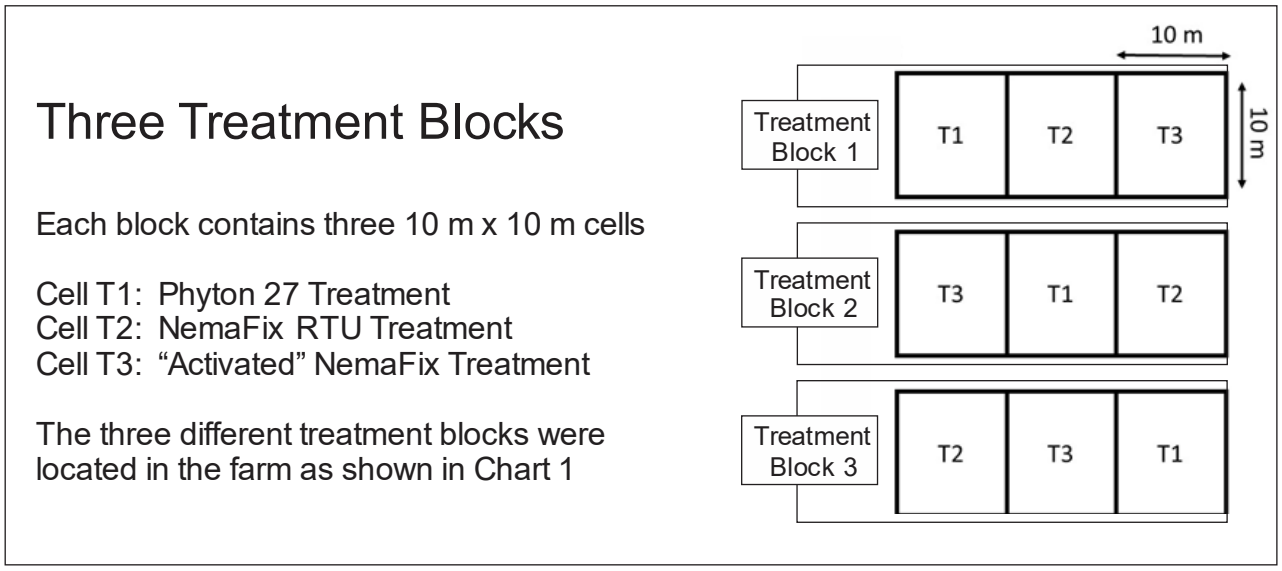
## Comparison of ACF-SRP to Local Fungicide of Choice Phyton 27

Block Design was used to define the trial areas at random. For this, each Block was then then divided into three distinct treatment cells T1, T2, and T3 (see example Chart 2 below). The designated area of each cell (10 m x 10 m) conformed to the recommendations for Black Sigatoka research.

*Chart 2: (Note that NemaFix = ACF-SRP)*




During Phase 2, each cell received three product applications, specifically at 90, 104 and 118 days after planting. Actual dates of dosing were July 22, August 5, and August 19, of the year 2017.

### A. Phyton-27 Dose (T1)

As per the label dose of 1 Liter per hectare, each 10 x 10 cell received a dose of 10 ml Phyton-27. A single 20-liter backpack sprayer was loaded with 54.6 ml Phyton-27 and filled with water.

This backpack sprayer was used to dose Phyton-27 in each 10 x 10 m cell. Each T1 / 10 x 10 m cell was dosed with 3.67 liters of backpack solution. At this aqueous application rate, each cell received 10 ml Phyton-27 per dose (1 Liter per Hectare equivalent)

### B. Ready To Use ACF-SRP (T2)

To achieve the intended dose of 1500 grams powdered ready-to-use ACF-SRP per hectare, 81.7 grams of powdered ACF-SRP were added to 20 liters of water in the backpack sprayer.

Using this backpack sprayer, Each T2 / 10 x 10 m cell was dosed with 3.67 liters of backpack solution. At this aqueous application rate, each T2 cell received 15 grams ready-to-use ACF-SRP per dose (1500 grams per Hectare equivalent)

## C. Activated ACF-SRP (T3)

Activated ACF-SRP was taken from the finished "activation batch" as described in Section IV. A total of 18 liters of well mixed liquid from the finished batch was added to the 20 liter backpack sprayer, and another 2 liters of water was added to fill the backpack sprayer to the 20 liter level. Once diluted this way, the backpack sprayer contained the equivalent of 81.7 grams of ACF-SRP (before activation) in 20 total liters.

Using this backpack sprayer, Each T3 / 10 x 10 m cell was dosed with 3.67 liters of backpack solution. At this aqueous application rate, each T3 cell received the equivalent of 15 grams ready-to-use ACF-SRP per dose (1500 grams per Hectare equivalent). As noted, in T3 treatments, the ACF-SRP was subjected to the "activation" procedure.

Table 3 is a summary of the dose applied to each treatment cell:

*Table 3:*

| Treatment | Description | Doses |
|---|---|---|
| T1 | Conventional – Phyton-27 | 10 ml per 100 m2 cell |
| T2 | Powdered Ready To Use ACF-SRP | 15 grams per 100 m2 cell |
| T3 | Activated ACF-SRP | 15 grams per 100 m2 cell |

## D. T1 / T2 / T3 Application Dates

Applications of Phyton-27, powdered / ready-to-use ACF-SRP, and activated ACF-SRP were made on the following dates (summer of 2017).

*Table 4:*

| Treatments | Total Applications | Treatment Dates |
|---|---|---|
| T1 (Phyton-27) | 3 | Jul. 22,<br>Aug.05,<br>Aug. 19. |
| T2 (Ready To use Powdered ACF-SRP) | 3 | Jul. 22,<br>Aug.05,<br>Aug. 19. |
| T3 (Activated ACF-SRP) | 3 | Jul. 22,<br>Aug.05,<br>Aug. 19. |

## VIII. Evaluation of the Data

The parameters that make up the Evolutionary Development of the Disease are as follows:

- Youngest Infected Leaf
- Foliar Emission Rate
- Evolutionary Development of the Disease

The values of each parameter were obtained or calculated weekly, taking the average of:

- All T1 areas in each of three test blocks
- All T2 areas in each of three test blocks
- All T3 area in each of three test blocks.

Results were as follows:

### A. Youngest Infected Leaf

*Table 5:*

| Date of Evaluation | T1 Phyton-27 | T2 Ready To Use ACF-SRP | T3 Activated ACF-SRP |
|---|---|---|---|
| 7/22/2017 | 6.9 | 7.8 | 7.3 |
| 7/29/2017 | 7.5 | 7.7 | 7.7 |
| 8/5/2017 | 6.9 | 7.5 | 7.9 |
| 8/12/2017 | 7.2 | 7.3 | 7.6 |
| 8/19/2017 | 6.7 | 7.5 | 7.4 |
| 8/26/2017 | 6.9 | 7.5 | 8 |
| **Average** | **7.01** | **7.55** | **7.65** |

As shown in Table 5, in T1 treatment cells (Phyton-27), infected leafs appeared at around 7 days, while the infection was delayed until 7.55 days in T2, and 7.65 days in T3.

### B. Foliar Emmission Rate

The average foliar emission rate for T1 was 1.2, while that for T2 and T3 was 1.3 for each. These data are similar to those of Phase 1, where there was little difference in the foliar emission rate.

C. Evolutionary Development of the Disease

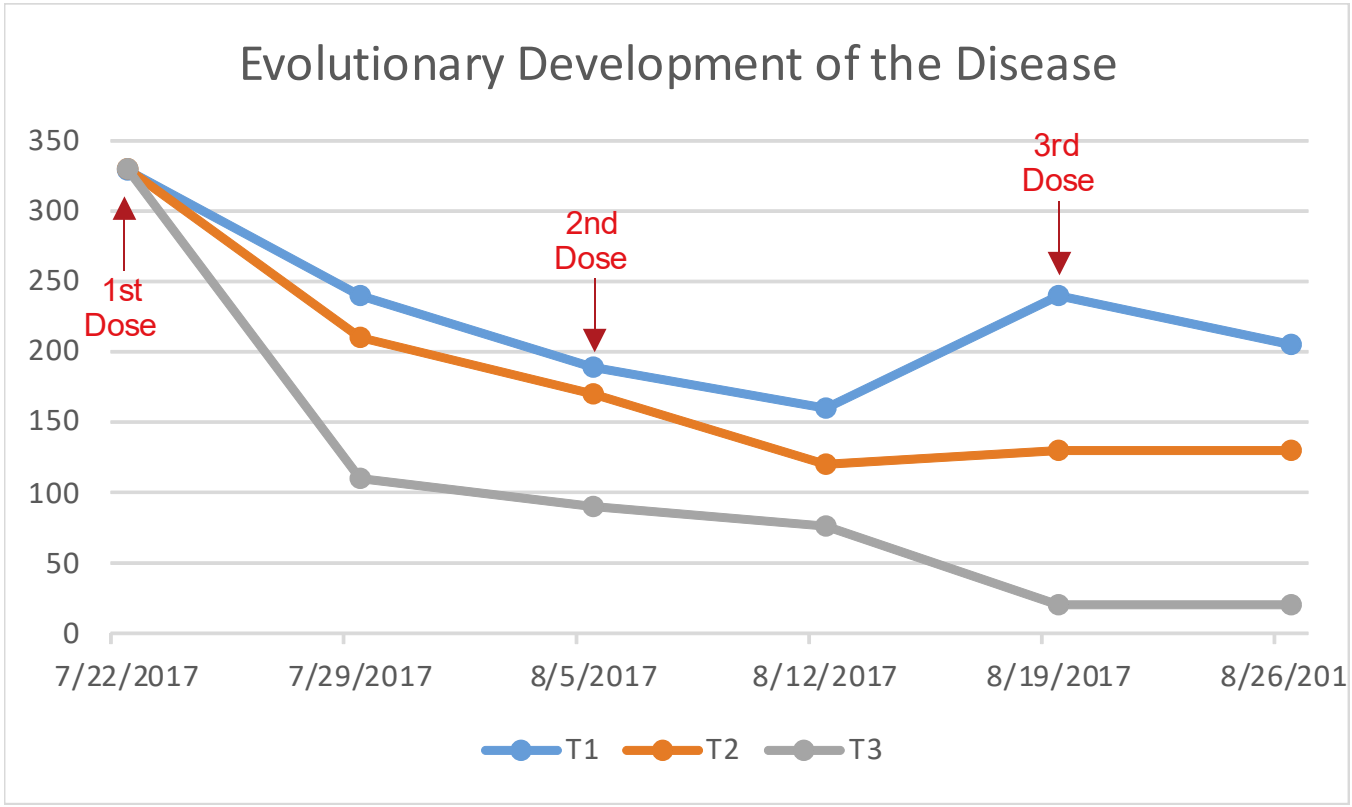
Table 6 below shows the values for Evolutionary Development of the Disease throughout the duration of Phase 2:

*Table 6:*

| Week | T1 Phyton-27 | T2 Ready To Use ACF-SRP | T3 Activated ACF-SRP |
|---|---|---|---|
| 7/22/2017 | 329 | 330 | 330 |
| 7/29/2017 | 240 | 210 | 110 |
| 8/5/2017 | 189 | 170 | 90 |
| 8/12/2017 | 160 | 120 | 76 |
| 8/19/2017 | 240 | 130 | 20 |
| 8/26/2017 | 205 | 130 | 20 |

Data from Table 6 are graphed vs time in Chart 3:

*Chart 3:*



## IX. Conclusions

During Phase 2, brewed (activation of ACF-SRP) was evaluated relative to an equivalent dose of powdered ACF-SRP (without activation) as well as to the local “best practice” application of Phyton-27.

The ACF-SRP dose (both for activated and ready to use variants) was 1,500 grams per ha, as the Evolutionary Development of the Disease was well above the upper threshold value of 200 at the time of the first dose.

As shown by the chart of Evolutionary Development of the Disease, the “best practice” use of Phyton-27 slowed the progression of Black Sigatoka, but the average Evolutionary Development of the Disease was 206 in the five evaluations after the first dose of Phyton-27. Limited control was attained, and the disease remained at or above the danger level.

After the initial dose, the average Evolutionary Development of the Disease was 152 for the powdered ACF-SRP (ready to use), while the average value for brewed, activated ACF-SRP was 63.

The data show that doses of powdered ACF-SRP (without activation) resulted in increased some suppression of Black Sigatoka. However, activation (brewing) of ACF-SRP resulted in superior suppression of Black Sigatoka, outperforming both ready to use, non-activated ACF-SRP, and the local “best practice” Phyton-27.