Species:	*Mycosphaerella fijiensis* (Banana Black Sigatoka)
Product Class(es):	MBC's, SBI's
Method type described:	Ascospore germ tube elongation
Date of protocol:	03.10.2008
Proven for	All SBI's and QoI's used in bananas
Version	1
Comments	Also adaptable to QoI's

Method:

Leaf sampling procedure
The fastest method of obtaining large quantities of inocula of *Mycosphaerella fijiensis* is by collecting infected leaf tissue in the field. Pieces from the youngest leaves with dry, necrotic, mass-infected tissue should be collected, after 2-3 days without rain. Dry, mass-infected tissue with a greyish-white colour yields the most ascospores. Eliminate any green tissue from the leaf sample. Otherwise, it generates an increase of humidity which enhances tissue decay. The size of each leaf sample should be at least 7 x 7 cm. Collect 10-15 samples per cable from at least 3-4 cables per farm.

Leaf tissue from one plant is considered a single sample. Sampling size will depend on farm size and level of Sigatoka infection. Put the leaf samples from each cable inside a separate manila paper envelope. Be sure the tissue is dry. Identify each paper envelope with the following data: Farm, section, cable, and date of sampling.

Shipment
Send the leaf samples to the monitoring laboratory as soon as possible. Otherwise, a significant reduction of quality and abundance of ascospore discharge may result.

Ascospore discharge from infected leaf tissue
Upon arrival in the laboratory, the tissue should be incubated in plastic bags with moist towelling for 48-96 h at room temperature. This allows immature ascospores to

mature. After incubation, leaf samples are removed from the plastic bag and numbered with ink. From each large piece, small pieces about 2 x 2 cm are cut and numbered the same. The 2 x 2 pieces are attached with staples to a 9 cm filter paper marked with the same numbers as the leaf pieces. Five to nine pieces are attached to each filter paper. Higher and cleaner ascospore discharge is obtained from the upper surface of the infected leaf tissue. The filter paper with attached leaf pieces is then submerged in tap water for 3-5 min. After removal from the water, the filter paper with the leaf pieces is placed inside the top of a Petri dish over 2 % water agar for ascospore discharge. The leaf pieces are allowed to discharge ascospores for one hour. The recommended media is DIFCO Bacto Agar. The dishes are turned upside down and the area of each leaf piece is traced on the bottom of the dish with a wax pencil. Then, the filter paper with the leaf pieces is removed. The agar plates are scanned with the aid of a dissecting or compound microscope to locate where discharged ascospores are deposited. These areas are marked. Leaf pieces with abundant ascospore discharge could be selected and stored dry in plastic bags in a refrigerator for up to 4 weeks with little loss of viability. They could be used later for further monitoring of fungicide resistance.

Monitoring of sensitivity

Following the leaf sampling procedure, collect 10-15 samples per cable, from at least 3-4 cables per farm. Prepare a 1000 ppm active ingredient (a.i.) stock solution or suspension. Pay attention to the concentration of a.i. for each fungicide to be evaluated. Using autoclaved 2 % agar after cooling to 45°C and the active ingredient stock solution or suspension, prepare the required set of concentrations depending on the fungicide to be evaluated. Stir for complete suspension. Pour 16-20 ml of media into duplicate or triplicate Petri dishes labelled for each active ingredient concentration. Allow to solidify. Following the ascospore discharge procedure, allow to discharge for one hour onto active ingredient amended agar (set of concentrations) and control (no fungicide). Incubate plates at 26°C for 48 h to allow ascospores to germinate and germ tube to growth. Incubation time should not vary. Survey the agar surface (traced area) at low magnification, for ascospore deposition. At high magnification (as previously calibrated), evaluate either germination of ascospores and/or measure the length of germ tubes from several microscopic fields per each traced area. Record data accordingly depending on the fungicide to be evaluated. Make the corresponding categorization, calculations (percentage of ascospores in each category, EC_{50}, inhibition or germ tube reduction, resistance factor, etc.) and graphing depending on the fungicide to be evaluated.

Data interpretation

Using Benlate as an example:

Prepare a 1000 ppm benomyl stock suspension by dispersing 0.2 g of Benlate (50%) in 100 ml sterile distilled water. Always prepare a fresh benomyl stock suspension on the same day the agar is prepared. Using autoclaved 2 % agar after cooling to 45°C and the benomyl stock suspension, prepare required concentration as shown in the following table. Stir for complete suspension.

benomyl concentration (ppm)	=	stock suspension (ml)	+	water agar (ml)
0.0		0.0		100.0
0.1		0.01		99.99
1.0		0.1		99.9
5.0		0.5		99.5
10.0		1.0		99.0

Pour 16-20 ml of media into duplicate or triplicate Petri dishes labelled for each benomyl concentration. Allow to solidify. Following the SOP for ascospore discharge, allow to discharge for one hour onto benomyl amended agar (set of concentrations) and control (no fungicide). Incubate plates at 26°C for 48 h to allow ascospores to germinate and germ tube to growth. Incubation time should not vary. Survey the agar surface (traced area) at low magnification, for ascospore deposition. At high magnification, evaluate the germination of at least 100 ascospores from 4-5 microscopic fields per each traced area.

Record data into four categories:
- Normal germination (N) = germ tube length similar to control germ tubes
- Germ tube shorter than normal (S)
- Distorted (D)
- Non-germinated (NG)

Compute the percentage of ascospores in each germination category at each benomyl concentration. Normal germination at 10 ppm benomyl is regarded as the threshold to suspend the use of Benlate.

Author	Dr. Luis H. Jacome Pathotec SA lhjacome@infovia.hn