Black Sigatoka caused by *Mycosphaerella fijiensis* is a devastating disease of bananas, not only in Uganda, but worldwide, affecting all cultivated *Musa* species.

This technical bulletin describes procedures for evaluating the use of botanical extracts as well as bacteria and fungi isolated from the rhizosphere of banana with the aim of managing this economically important disease.

The target group represents individuals working in extension services, at plant disease diagnostic laboratories and certification agencies as well as research workers, teachers and students of plant pathology.

This publication is based on outputs of the Danida-financed research project 09-084LIFE: Saving a precious crop: sustainable management of the Black Sigatoka disease of banana.
Botanicals, endophytes and plant growth-promoting rhizobacteria (PGPR) to control Black Sigatoka disease of banana in Uganda

TECHNICAL BULLETIN

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This technical bulletin is published by the partners of the research project 09-084 LIFE (2009-2013). The project was coordinated by the Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen (UCPH), Denmark.

As the result of the collaboration, UCPH and Makerere University have produced and distributed preliminary outputs from the project. This may lead to a better management of Black Sigatoka of banana, resulting in the production of high quality banana and increased food security.

Cover and back pages photographs: Banana leaf with Black Sigatoka symptoms caused by Mycosphaerella fijiensis (By C. Gumisiriya)
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INTRODUCTION

The disease
Black Sigatoka is among the most damaging and costly diseases affecting banana production in Uganda and the rest of the world. It is caused by the fungus *Mycosphaerella fijiensis* and attacks most banana and plantain cultivars. Under favourable conditions, symptoms develop within three weeks under field and screenhouse conditions.

The disease quickly destroys the photosynthetic capacity of the leaves as they develop and thus prevents accumulation of reserves. As a consequence, the weight of banana bunches are reduced by up to 60% and premature fruit ripening may also occur.

In many African countries, bananas constitute an important part of the diet, especially in Uganda, where matoke is an essential staple food. Both East African Highland bananas (AAA-EA genome group, dominant in East and Central Africa) and plantains (AAB genome group, dominant in West Africa) are susceptible to the disease. Yield losses can lead not only to reduced income to farmers, but also a decrease in country fiscal revenues and eventually a shortage of this popular food item, which is difficult to replace by other plant products.

A drastic reduction of productivity in the traditional banana growing areas of central and south-western Uganda has been reported. Black Sigatoka alone can reduce yields by 30-50%, affecting dessert types such as East African Highland bananas and the disease is considered a major threat to the country’s food security (Gale 2012). Studies conducted in Uganda suggest a mean minimum temperature threshold of 14-15°C for the disease to be established in the field (Tushemereirwe 1996). This suggests that bananas grown in high elevation areas (higher than 1500 metres above sea level) are likely to escape infection by this pathogen.

Black Sigatoka symptoms
The first symptoms of this foliar disease are tiny, chlorotic spots that appear on the lower leaf surface of the 3rd or 4th open leaf. The spots grow into thin brown streaks that are limited by leaf veins (Fig. 1).

![Figure 1. Brown spots on the lower surface of Giant Cavendish leaf. Photo S. N. Kateete.](image1)

The streaks darken, sometimes developing a purple tinge and they become visible on the upper leaf surface. The lesions then enlarge, becoming spindle-shaped or elliptical and darken to give the characteristic black streaking of the leaves (Fig. 2). Adjacent tissue often exhibits a water-soaked appearance, especially under conditions of high humidity.

![Figure 2. Black spots on the upper leaf surface of Giant Cavendish leaf. Typical symptoms include appearance of water soaked lesions (arrow). Photo C. Gumisiriya.](image2)

When the disease severity is high, large sections of the leaf become water-soaked later die with characteristic dark symptoms (Fig. 3). As the lesions mature and dry out, the fungus starts
producing spores that infect new plant tissue. Numerous, tiny, black, spherical fruiting bodies (pseudothecia) containing sac-like structures (asci) filled with ascospores will emerge from the lower side of the leaf (Fig. 4). The spores are ejected from the pseudothecia and are washed along leaf veins giving the characteristic black leaf streaks typical of the disease.

**Figure 3.** Black Sigatoka symptoms in banana plants in Uganda. Photos: C. Gumisiriya and S.N. Kateete.

**Figure 4.** Leaf showing necrotic tissue containing fruiting bodies (arrow). Photos C. Gumisiriya (left) and H.J.L. Jørgensen (right).

**Common names of the disease**
Black Sigatoka, sometimes known as black leaf streak.

**Pathogen**
The asexual form (anamorph) is named *Pseudocercospora fijiensis* (syn. *Paracercospora fijiensis*). The sexual form (teleomorph) is named *Mycosphaerella fijiensis*.

**Host plants**
*Musa* spp., including most dessert bananas, cooking bananas and plantains.

**Distribution**
The disease is found world-wide in the tropics. In Uganda, the disease is endemic and found in nearly all banana-growing regions. The disease is most prominent in the eastern and central districts. All cultivars of the East African Highland banana (AAA-EA genome group) and plantain (AAB genome group) are susceptible. No disease-free areas have been designated in Uganda, although the highland cool regions are less seriously affected.

**Control**
Globally, the control of Black Sigatoka has mainly relied on the use of fungicides that initially yielded positive results in many countries. However, the pathogen easily develops resistance to the products, thus resulting in a demand for new fungicides, which consequently lose efficacy. The high frequency of fungicide applications conducted in commercial plantations (up to 60
sprays per growth season), increases the impact on the environment and on the health of the banana workers. Other management practices such as removal of affected leaves, good drainage, and sufficient spacing have also been used to fight the disease. However, cultural practices are often inefficient since the disease spreads by air. The use of fungicides is not an option in Uganda since fungicides are expensive and beyond the reach of resource-constrained subsistence farmers.

Currently, the main control strategy for Black Sigatoka in Uganda is breeding for resistance and the use of cultural practices involving reduction of moisture level in the crop and removal of infected crop debris. Taking the seriousness of the disease into consideration as well as the difficulties in controlling it at present, there is a demand for development of alternative control strategies in the management of the disease when eradication is not possible.

**Induced resistance**

An obvious possibility for disease control is to utilize the inherent ability of plants to defend themselves by induced resistance (Kloeper et al. 1992). All plants have the ability to defend themselves against disease-causing pathogens. However, disease occurs when the plant discovers too late that it is being attacked or if the reaction is not strong enough to stop the invading pathogen.

Induced resistance describes the phenomenon where the natural defence of the plant is stimulated so it can defend itself faster or to a greater extent than before. The stimulation is exerted by a so-called inducer and there are many different types of inducers including microorganisms (fungi, bacteria), certain chemicals and plant extracts (botanicals). Common for all inducers is that they 'sensitise' the plant, so the defences are put on alert. When a pathogen attempts to infect such a 'sensitised' plant, it can defend itself faster and stronger than before.

An important trait for an inducer for practical disease control is that it evokes a strong protective effect in the plant, but does not harm the plant or the environment. Furthermore, it should give a long-lasting protection to avoid the need for several treatments.

Botanical extracts, an isolate of a plant growth-promoting rhizobacterium (*Pseudomonas fluorescens*) and an endophytic fungus (*Fusarium oxysporum*) were evaluated as inducers against Black Sigatoka in the present studies. Selected plant extracts were applied as foliar sprays to 1-2 months-old plants, whereas soil drenching to banana plants was used for application of *Ps. fluorescens* and *F. oxysporum*.

Ugandan and many other East-African farmers may benefit from application of such alternative disease control agents. However, to implement new disease control strategies, it is necessary to communicate results and procedures to farmers, extension services and control agencies.

The procedures and preliminary results presented here were developed in a cooperative research project 09-084LIFE: ‘Saving a precious crop: sustainable management of the Black Sigatoka disease of banana’. The project was conducted as collaboration between Faculty of Science, University of Copenhagen, Denmark, and Department of Agricultural Production, School of Agricultural Sciences, Makerere University, Uganda.
SCREENING OF ENDOPHYTES, PGPR’S AND PLANT EXTRACTS

Isolation of *Mycosphaerella fijiensis* from infected leaves

In order to perform experiments on induced resistance, it is necessary to be able to inoculate with the pathogen. Isolation of ascospores is done according to Fullerton & Olsen (1995) with minor modifications as follows:

- Collect fresh leaf samples with lesions and observe for the presence of pseudothecia in a stereo microscope.
- Cut sections of the leaf containing pseudothecia (**Fig. 5 left**) and place them in polythene bags with moist cotton wool and incubate them for 24 h.
- Rinse leaf sections by immersion in sterile water for 5 min.
- Staple the rinsed leaf sections onto sterile filter papers.
- Place the rinsed leaf sections previously stapled onto filter papers in the lids of Petri dishes, incubate at 25°C and allow to discharge ascospores over 3% water agar for 3 h.
- Pick up individual ascospores with a sterile fine needle and transfer them to Potato Dextrose Agar (PDA) medium and incubate the plates at 25°C for one month. The fungal colonies formed are then used (**Fig. 5 right**).
- Inoculum for further experiments can also be produced in liquid cultures (Potato dextrose broth or V8 juice broth. Mycelial fragments are added to flasks containing the medium and incubated at around 25°C on a rotary shaker (150 rpm) for 3 weeks (**Fig. 6**).
- After inoculation, plants may develop symptoms in about 21 days (**Fig. 7**).

**Figure 5.**  
**Left:** Fresh leaf piece with lesions containing pseudothecia.  
**Right:** Growth of *Mycosphaerella fijiensis* on PDA medium. Photo. C. Gumisiriya.

**Figure 6.**  
**Left:** inoculum preparation of *Pseudomonas fluorescens*. **Right:** production of inoculum of *Mycosphaerella fijiensis* in liquid culture. Photos S.N. Kateete.
Isolation of *Pseudomonas fluorescens*

*Pseudomonas fluorescens* is an obligate aerobic gram negative bacterium. *Pseudomonas* spp. have been studied mainly because of their widespread distribution in soil, their ability to colonise the rhizosphere of host plants and ability to produce a wide range of compounds inhibitory to a number of serious plant pathogens (Reddy *et al.* 2007).

For isolation of *Pseudomonas fluorescens*, collect samples of root tissue from healthy banana plants.

- Select roots from randomly selected, healthy banana plants and take them to the laboratory for the immediate isolation of the bacteria.
- Remove the soil from the roots and subsequently rinse them with sterile distilled water and cut 2-3 cm sections of banana root tissue using a sterile knife.
- Take 1 g from each root sample and place the material in a sterile conical flask containing 10 ml of sterile distilled water and allow settling for 20 min.
- Take 1 ml of the water and prepare serial dilutions ($10^{-1}$ to $10^{-9}$).
- Take samples of 0.1 μl from each of the serially diluted suspensions and spread with a sterile glass rod onto the surface of Petri dishes containing King’s B medium.
- Incubate the inoculated plates for 3 days at 25 ± 2°C (Reddy *et al.* 2009).
- Observe the production of fluorescent pigment production from the bacterial colonies under near ultraviolet (NUV) light at 365 nm.
- Select distinct colonies producing yellowish green fluorescent pigment under NUV light and purify on nutrient agar plates for 3 days.
- Extract bacterial DNA (Mahuku 2004) and conduct PCR tests with specific primers to confirm that the bacteria belong to the species *Pseudomonas fluorescens* (Charan *et al.* 2011).

**Induced resistance with fluorescent pseudomonads**

Having identified bacteria of potential interest, it is important to establish the isolates can cause significant reduction in disease severity. This is done by applying the bacterium to the plants and subsequently inoculating with the pathogen.

- Conduct tests with young banana plants, which are susceptible to Black Sigatoka.

*Figure 7. Young banana plants showing initial symptoms of infection after inoculation with ascospores of Mycosphaerella fijiensis. Photo: S.N. Kateete.*
• Apply bacterial suspensions (e.g., $10^6$ or $10^7$ cfu/ml) to plant growing in pots by soil drenching and allow them to grow for one week.

• Inoculate the plants with *Mycosphaerella fijiensis* suspensions by spraying $10^5$ pathogen mycelial fragments per ml (Fig. 6) on the lower surfaces of the leaves (Capó et al. 2003), using a paint brush or an atomizer.

• Include plants inoculated with *M. fijiensis*, but without prior bacterial treatment to serve as positive control for the disease-reducing effect of the bacteria.

• Incubate plants in a humidity chamber at approximately 100% relative humidity (RH) for the first 3 days after pathogen inoculation and subsequently at about 70% RH.

• Observe plants for the appearance of disease symptoms and record disease severity once a week and for up to 30 days.

**Induced resistance with *Fusarium oxysporum***
The endophyte *Fusarium oxysporum* strain V5W2 (Fig. 8) used in the study was obtained from the International Institute of Tropical Agriculture (IITA, Uganda, http://www.iita.org/). This strain was previously isolated from banana roots.

• Sub-culture the fungus on half strength Potato Dextrose Agar (PDA) and incubate for 14 days at 25°C (Paparu 2008).

• Prepare *F. oxysporum* inoculum from two-week-old cultures by scraping the surface with a sterile spatula and rinsing it with sterile distilled water.

• Dilute fungal suspension (e.g., to $10^5$ or $10^6$ spores/ml) for inducer treatment.

• Treat young banana plant grown in pots by drenching the soil with *F. oxysporum*

• After, e.g., two weeks, inoculate plants with *M. fijiensis* and observe symptoms as described before.

• Preliminary data showed that plants inoculated with *M. fijiensis* alone (Fig. 9 left) developed disease faster than plants pre-treated with *Fusarium oxysporum* (Fig 9. middle and right).

*Figure 8.* Two 7 day-old culture of *Fusarium oxysporum*, isolate V5W2 growing on PDA. Photo S.N. Kateete.
Figure 9. Reddish streak symptoms on leaves of cv. Kayinja. **Left:** control plant inoculated with *Mycosphaerella fijiensis* alone (no pre-treatment with *Fusarium oxysporum*). **Middle:** plant pre-treated with *F. oxysporum* (5x10^5 cfu/ml) followed by inoculation with *M. fijiensis*. **Right:** plant pre-treated with *F. oxysporum* (5x10^6 cfu/ml) followed by inoculation with *M. fijiensis*. Symptoms recorded 45 days after inoculation with *M. fijiensis*. Observe the development of weakest symptoms on plants inoculated with the highest concentration of *F. oxysporum*.

**Induced resistance with botanical extracts**

Extracts made from a range of plants have shown a remarkable ability to control diseases and pests in plant production. The effect in reducing plant diseases may rely on induced resistance, but the extracts may also be directly toxic to the pathogens and this can be tested in *in vitro* tests.

The preparation of fresh aqueous extracts was done according to Joseph *et al.* (2008) with minor modifications.

- Select plant species which could be anticipated to possess disease-reducing capabilities.
- For experimentation, the same source of material should be used and plants can be established from seeds or cuttings.
- Confirm the identity of each collected plant against authentic herbarium specimens.
- Weigh 20 g of healthy-looking plant parts (*e.g.*, leaves, flower buds, fruits, rhizomes, bark).
- Surface sterilise samples with 10% sodium hypochlorite (NaOCl) for 5 min followed by washing with sterile distilled water to remove all traces of the NaOCl.
- Treat the samples with 70% ethanol for 30 sec and allow to air dry.
- Cut the disinfected tissue into small pieces, add 20 ml sterile distilled water and macerate to pulp with using a sterilised pestle and mortar (**Fig. 10**).
- Allow the crushed plant material to rest for 10 min and remove plant debris by filtration through sterile gauze.
- Collect the supernatant into a clean test tube and filter using a disposable syringe filter (0.22 µm) to remove bacteria.
- The filtrate thus obtained serve as the crude extract (100%) concentration.
Dilute the crude extract (100%) to lower concentrations for experimental use (e.g., 2.5, 5 and 10%) with sterile distilled water.

Test the extracts immediately after preparation for anti-fungal activity against the test fungus *Mycosphaerella fijiensis* (*in vitro* tests).

**In vitro tests**
The evaluation of plant extracts for efficacy was done using a modified procedure of Taiga *et al.* (2008). The effects of the plant extracts on the radial growth of *Mycosphaerella fijiensis* in culture was determined by growing the fungus on PDA plates containing different concentrations of each extract in the medium. Each treatment in the experiment was replicated three times.

- The PDA medium with extract was prepared by spreading 1,000 µl of each extract to form a thin film on the surface of the solidified PDA, using a sterile glass rod. The control plates were treated with 1,000 µl sterile distilled water instead of plant extracts.

- Transfer an 8 mm disc of the pure culture of the isolated fungus, taken aseptically, to the centre of the solidified PDA with extract.

- Incubate plates at 25°C for 21 days.

- Measure the diameter of the radial growth of the fungus in mm at the end of incubation period.

*Figure 10. Preparation of botanical extracts. Photos C. Gumisiriya*

**In planta tests**
Young banana plants in pots are used (Fig. 11 left).

- Spray the lower surface of the two youngest fully expanded leaves with the plant extracts at the selected concentrations. Include control plants which are spray inoculated with sterile distilled water instead of the plant extract.

- Inoculate the abaxial surface of the extract-treated leaves with *M. fijiensis* at 2 days after
application of the extract.

- Incubate plants in a humidity chamber (Fig. 11 right) at approximately 100% relative humidity (RH) for the first 3 days after pathogen inoculation and subsequently at about 70% RH.

- Observe plants for the appearance of disease symptoms and record disease severity once a week and for up to 30 days.

*Figure 11. Left:* plants ready for inoculation. *Right:* plants in a humid chamber after inoculation. Photos C. Gumisiriya.

**Defence response studies**
In order to verify whether induced resistance is involved in the protection by a potential inducing agent, it is necessary to document that defence reactions are activated in the plant by the putative inducer candidate. Several different types of defence responses can be investigated, but all studies should start by microscopy to verify when pathogen growth is arrested and whether this is accompanied by any visible host reactions. Subsequently, a range of biochemical and molecular responses can be investigated to test if any particular reaction is enhanced.

Comparisons of defence responses should take place in:

1) Plants treated with inducer and inoculated with pathogen
2) Plants not treated with inducer and inoculated with pathogen

If defence responses are increased in 1) compared to 2), then and only then, it can be concluded that induced resistance is involved in the protection. This does, however, not exclude that other mechanisms may be involved in the protection (direct toxic effects).

In order to obtain a full picture of the processes occurring in the plant, the following treatments should be included in the studies of defence responses:

1) Plants inoculated with inducer candidate and subsequently with *M. fijiensis*.
2) Plants inoculated with *M. fijiensis* alone (no inducer treatment).
3) Plants inoculated with inducer alone (no *M. fijiensis*).
4) Plants treated with water instead of inducer and no inoculation with *M. fijiensis* (negative control).

To perform gene expression studies:

- Inoculate young banana plants as described earlier.
Collect leaf samples from each treatment at regular intervals, depending on the part of the interaction of interest. Freeze material immediately at -80 °C.

Further studies start with extraction of RNA and require specialised facilities and expertise.

**PRELIMINARY RESULTS**

**Use of bacteria and fungi in control experiments**

- Preliminary results show that both *F. oxysporum* and *Pseudomonas fluorescens* reduced the rate of disease development.

- *Ps. fluorescens* treated plants showed slower symptom development of Black Sigatoka than *F. oxysporum* and control treated plants.

- In both experiments, cv. Kayinja showed the least disease severity compared to other cultivars used.

- Additionally, the plants treated with the highest concentrations of the inducer (either *F. oxysporum* or *Ps. fluorescens*) revealed the least disease severity compared to the lower concentrations of the inducers and the controls.

**Use of botanical extracts in control experiments**

*In vitro tests:*

- All extracts had inhibitory properties on the pathogen compared to the control.

- Only *Capsicum annum* extract was able to completely inhibit radial mycelial growth of the fungus within the period of incubation at 100% concentration.

*In planta tests:*

- Plants of cv. Musakala treated with *Capsicum annum* extract and cv. Giant Cavendish plants treated with *Tephrosia vogelli* extract showed the lowest disease severity as compared to other extracts tested.

- Generally, Giant Cavendish plants showed the highest disease severity compared to other cultivars used.
REFERENCES


APPENDIX

Recipes

*Nutrient agar:*

<table>
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<th>Ingredient</th>
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<tr>
<td>Yeast extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
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<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 l</td>
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Adjust pH to 7 and sterilise by autoclaving at 121 °C for 15 min.

*King’s B agar:*

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</thead>
<tbody>
<tr>
<td>Peptone</td>
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</tr>
<tr>
<td>K$_2$HPO$_4$ (anhydrous)</td>
<td>1.5 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
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Adjust pH to 7 and sterilise by autoclaving at 121 °C for 15 min.

*PDA medium:*

<table>
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<tbody>
<tr>
<td>PDA</td>
<td>39.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 l</td>
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</table>

Adjust pH after dissolving the agar and autoclave at 121 °C for 15 min.
This publication is based on outputs of the Danida-financed research project 09-084LIFE: ‘Saving a precious crop: sustainable management of the Black Sigatoka disease of banana’

Department of Plant and Environmental Sciences, University of Copenhagen, Denmark
University of Copenhagen (http://www.ku.dk/) is one of the largest universities in northern Europe, with 37,000 students, #1 in Scandinavia according to the Shanghai and THES rankings and a member of the International Alliance of Research Universities (http://www.iaruni.org/). UCPH is organized in six faculties, where the Faculty of Science has an applied and business-oriented approach to the natural sciences. The academic staff totals around 3,500, with 9,000 BSc/MSc and over 1,000 PhD students enrolled at the faculty. Department of Plant and Environmental Sciences (http://plen.ku.dk/) with more than 400 employees focuses on research into ecological interactions and agricultural production with a particular focus on climate and environmental effects – molecule, organism and populations to eco-systems.

Department of Agricultural Production, Makerere University, Uganda
Makerere University, Kampala (http://mak.ac.ug/) is Uganda’s largest higher institution of learning and among the 10 highest ranking Universities in Africa. It comprises nine colleges and one school, offering programmes for about 30,000 undergraduates and 3,000 post-graduates. The Department of Agricultural Production offers courses to undergraduate students of Agriculture, Agribusiness management, and Land use management. Graduate programmes leading to MSc and PhD are offered as well as short courses tailored to the needs of specific clientele in animal production.