Better understanding epidemiology of Panama disease of banana

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Summary

This study involved traditional and molecular methods to track the movement of *Fusarium oxysporum* f.sp. *cubense* (Foc**)** in the vascular system of banana. Traditional studies were conducted in the field using naturally-infected Lady finger banana plants, and molecular studies are currently being conducted at UQ using banana plants artificially-inoculated with GFP-transformed Foc isolates in the glasshouse.

Panama disease is a polycyclic disease where inoculum causing infection is produced in individual plants infected during the course of the epidemic. This field study clearly demonstrated that the sap produced in such plants will be contaminated with Foc, and will contribute to epidemic build-up if allowed to contaminate the soil. Thus, sap as a source of inoculum is very important when managing disease containment.

The study also suggested that the laticifers are not colonised by Foc and that when a pseudostem is cut, the sap from the laticifers is contaminated by inoculum from severed vascular strands and/or associated necrotic tissues. It is difficult to separate these tissues, but results suggest that mycelial fragments may come from severed vascular strands or xylem fluid, and microconidia from necrotic cells adjacent to the vascular bundles.

It is anticipated that GFP-transformed isolates being used in the experiment at UQ will provide more definitive evidence on the systemic infection process of Foc in banana. It will determine whether movement in the vascular tissue is via mycelial growth or microconidia, and may explain why the incubation and latent periods for the disease are often so long.

Chemical intervention to reduce inoculum levels may be possible but will require much more detailed research.

The production of a volatile chemical (bicyclo(4,2,0) octa-1, 3, 5-triene) detected in this study by race 4 strains of Foc in culture is interesting and presents the opportunity for detection of disease by “sniffer” dogs before external disease symptoms are produced. Whether this chemical is produced in infected plants is yet to be determined.

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Background

This project was initiated in response to an outbreak of Panama disease in Cavendish bananas, caused by *Fusarium oxysporum* f.sp. *cubense* tropical race 4 (Foc TR4), in North Queensland. This is a polycyclic disease, where the inoculum that causes infection is produced in individual plants during the course of the epidemic. The disease presents a sigmoid curve (S – shaped) where management is based on reducing the rate of infection and the production of inoculum which leads to epidemic build-up. As soon as infected plants are detected during an outbreak they, as well as the surrounding healthy plants, are quickly destroyed.

Panama disease is a classical vascular wilt disease, and it has become dogma that the pathogen, *Fusarium oxysporum* f.sp. *cubense* (Foc), moves in the xylem fluid of the vascular tissue of the banana plant via microconidia. Rishbeth (1955) found that a cane knife used to cut the pseudostem of an infected plant became contaminated with 3000 viable microconidia, and a single drop of banana sap contained the same number of conidia. However, Stover (1962) disagreed with this finding, and suggested that in a green infected pseudostem there was only sparse growth of hyphae in diseased vascular strands, and sporulation was sparse or absent. He found that the sap exuding from the cut end of a pseudostem was free of conidia and contained only fragments of hyphae. There is the possibility that Rishbeth (1955) was cutting pseudostems with advanced symptoms of disease, and that the microconidia were not coming from the xylem but from adjacent associated tissues.

Our belief is that spread in the xylem may be solely by hyphal growth and that microconidia and chlamydospores are produced in associated tissues only when the death of the plant is imminent. The reasoning behind the importance of this is that sap released from an infected plant during harvesting or surveillance activities will not be as potent a source of inoculum if movement in the plant is via mycelium rather than microconidia.

For this study we needed to be able to differentiate between sap and xylem fluid. The sap is latex which comes from laticifers. The osmotic potential of the contents of the laticifer is lower (more negative) than the surrounding tissues and so they have a positive turgor. The contents of the laticifers exude when cut, because the cutting sets the turgor pressure of the laticifer to zero, and then the osmotic potential gradient between the latex and surrounding cells causes water to flow into the laticifer along its length. This causes the sap to flow out of the cut surface of the pseudostem. Sap stops flowing when the osmotic gradient becomes zero. Once the laticifers empty, the xylem will start to exude fluid because of root pressure. The laticifer sap is quite milky, whereas the xylem fluid is clear. Foc does not grow in a healthy laticifer. If it were infected it would lose turgor. It is the xylem vessels that harbour the pathogen.

Project Objectives

The aim of this project was to better understand the infection process of Foc in the banana plant to aid in the containment and management of the Panama TR4 epidemic in North Queensland.

It is widely accepted that movement of Foc within banana is via microconidia carried in the xylem vessels. Our belief is that spread in the vascular tissue may be solely by hyphal growth, and microconidia are only produced in associated tissues when death of the host plant is imminent. This study involved traditional and molecular methods to track the movement of the pathogen in the vascular system of the banana. Traditional studies have been conducted in the field using naturally-infected mature plants. Molecular studies, which are currently being conducted at UQ, involve the use of GFP (green fluorescent protein) modified Foc race1 (Foc R1) and subtropical race 4 (Foc STR4), as well as qPCR, on artificially-inoculated glasshouse plants.

Methodology

DAF field studies

All three field investigations were conducted on the property of Peter Molenaar at Mullumbimby (NSW), where Foc R1 and STR4 strains are known to be present in some blocks of Lady finger banana. All trials were conducted in collaboration with Matthew Weinert (NSW DPI) and members of the Panama TR4 diagnostic team (BQ – Wayne O'Neill, Kathy Thomson and Christine Goosem).

Sap trial 1 (9/5/16)

A total of nine Lady finger banana plants were selected for the study: three were judged to have advanced symptoms of Panama disease, three were judged to have early symptoms, and three were considered to be uninfected (selected from an adjacent block).

From each plant, a "window" of pseudostem tissue (approximately 15cm long x 8cm wide x 5cm deep, see Image 1) was cut at two different heights (0.5 and 1.0 m) from the base of the pseudostem. Following removal of the pseudostem tissue, a syringe was used to collect a sample of sap (latex) which initially flowed from where the cuts were made (Image 1). A second sap sample (possibly a mixture of latex and xylem fluid) was collected from the same locations approximately 15 minutes later. Sap samples were transferred to 50 mL falcon tubes and placed in an esky for transport back to the laboratory.

Tissue samples were also collected from each "low" window (i.e. 0.5m from the base of the pseudostem) for later Foc culturing, PCR and VCG (vegetative compatibility group) analysis. These samples were wrapped in paper towel, enclosed in a zip lock plastic bag and placed in an esky for transport back to the laboratory.

The following day after sample collection, sap samples were serially diluted in sterile distilled water (up to a dilution of 10^{-7}), and 20 µL aliquots of each dilution were spread over Nash-Snyder plates (Foc colony numbers counted 3 days later). Isolations were also made from each window tissue sample (discoloured vascular strands) that had been collected on 9/5/16, and plated onto Nash-Snyder agar (Foc colonies growing from each tissue piece counted 6 days later). Foc race was subsequently determined by PCR and VCG analysis. Undiluted sap samples were also examined under the microscope for the presence of Foc microconidia or hyphal fragments.

Sap trial 2 (30/6/16)

Three Lady finger banana plants with external symptoms of Panama disease were selected for this study. Each plant was decapitated at 0.5m above ground, and the sap (latex) which initially exuded from the severed pseudostem of each plant was collected using a 10 mL syringe. Ninety minutes after severing the pseudostem (i.e. allowing time for the laticifers to empty), any remnants of milky exudate which remained on the cut surface of the stem was wiped away with paper towel, and the clear xylem fluid which exuded from the cut surface was collected with a syringe. Tissue samples were also collected from each plant for later Foc culturing.

Serial dilution and plating of sap samples were undertaken as described previously (sap trial 1), except samples were only diluted to 10^{-5}. Tissue isolations were conducted as previously described, although in this instance Foc race identification was done on the basis of cultural characteristics.

Sap trial 3 (18/7/16)

Two Lady finger banana plants with external symptoms of Panama disease were selected for this study. Each plant was decapitated at 0.5m above ground, and the sap (latex) which initially exuded from the severed pseudostem of each plant (0 min sample) was collected as previously described. Remnants of milky exudate which remained on the cut surface of the stem was wiped away with paper towel, and the cut surface sprayed with 70% ethanol to reduce the risk of contamination of subsequent sap samples with Foc present in infected vascular tissue and tissue debris present on the cut surface of the stem. Additional sap samples were collected at 90 and 120 minutes after decapitation of the plant. All sap samples (0, 90 and 120 min) were processed in the laboratory the next day as previously described, except on this occasion it was considered only necessary to plate undiluted sap samples onto Nash-Snyder agar (no serial dilutions required due to relatively low inoculum levels present in previous trials). Tissue samples were also collected (at 0.5 m) for later isolation and identification of Foc race.

Chemical injection trial (30/6/16 and 18/7/16)

In total, 13 Lady finger banana plants with external symptoms of Panama disease were selected for this study. Three plants were injected with the fungicide Sportak® (prochloraz, 450 g/L a.i., diluted 1 in 4), three plants were injected with the growth regular/fungicide Payback® (paclobutrazol 250g/L a.i., diluted 1 in 2), and three plants were injected with the herbicide Round-up® (glyphosate 450 g/L, diluted 1 in 5). Each pseudostem was injected at 3 equidistant points around the circumference of the stem with 12 mL of product/injection point. An attempt was made to locate a further three symptomatic plants to be used as untreated controls, but only one plant was able to be found.

Eighteen days after injection, pseudostem windows were cut at 0.5 and 1.5 m above the base of each treated (and control) plant. Sap which initially exuded from each window was collected and plated as previously described. Tissue samples were also collected from windows cut at both 0.5 and 1.5m for later isolation and identification of Foc race.

UQ laboratory and glasshouse studies

The UQ component of this project involves the use of molecular techniques to track infection of Foc in banana plants artificially inoculated with GFP (green fluorescent protein) modified Foc R1 and STR4 isolates, as well as qPCR. This study forms the basis of a postgraduate research project currently being conducted by Noeleen Warman under the supervision of Assoc. Prof. Elizabeth Aitken, an expert in Panama disease of banana, and is expected to be completed in October 2017. At this stage, Lady finger and Williams plants have been deflasked and will be inoculated early in September with the transformed isolates.

Results and Discussion

Sap trial 1: Isolations from pseudostem vascular tissue (window samples) onto Nash-Snyder agar, and subsequent PCR and VCG analysis of these cultures (data not shown), confirmed the presence of Foc in 5 of the 6 symptomatic plants selected for this study (Table 1). Although isolates from 1 of the 3 plants showing advanced Panama disease symptoms were characterised as Foc STR4 rather than R1, data from this plant was included in this preliminary study as plants infected with either strain would most likely behave in a similar way. As expected, Foc was not recovered from the vascular tissue of asymptomatic plants (Table 1).

Table 2 shows the number of colony forming units of Foc detected in sap collected from severed pseudostems of banana plants with varying degrees of disease severity. As expected, Foc was not detected in the sap of asymptomatic plants at either height above ground, or at either time after excision. Foc was present in sap collected from plants with early or advanced symptoms immediately after excision, but not in sap collected 15 minutes after excision. On average, colony numbers were higher in sap from the low window (0.5 m) compared to the high window (1.5 m) of “early symptom” plants, although this difference was not statistically significant. Colony numbers in the sap of “advanced symptom” plants were on average similar at both heights above ground.

The absence of colonies in the 15 minute flow of sap is highly significant as it clearly shows that Foc has not colonised the laticifers.

Sap trial 2: Isolations from pseudostem vascular tissue onto Nash-Snyder agar confirmed the presence of Foc R1 in the 3 symptomatic plants selected for this study (Table 3). Colony numbers were significantly higher in the sap initially collected from these plants compared to the 90 minute sap samples (Table 3). The 90 minute sap sample would be consist mainly of xylem fluid, unlike the 15 minute sap sample from the previous sample.

Sap trial 3: Isolations from pseudostem vascular tissue onto Nash-Snyder agar confirmed the presence of Foc R1 in the 2 symptomatic plants selected for this study (100 and 70% recovery of Foc from plants 1 and 2 respectively, data not analysed or tabulated). Foc colonies were detected at similar levels in sap collected at both 0 and 90 minutes after excision, but there were no colonies detected at 120 minutes after excision (Table 4). This may indicate that the exudate was entirely composed of xylem fluid at 120 minutes after excision.

Chemical injection trial: Table 5 shows the recovery of Foc from the pseudostem vascular tissue (window samples) for each of the injection treatments. Foc was recovered from all plants in the trial, although was not able to be recovered from tissue taken from the single untreated (but infected) control plant at 1.5 m. At a height of 0.5 m above ground, the untreated control plant had the highest number of Foc colonies detected in sap compared to other treatments, and glyphosate plants had the lowest number of colonies in sap (Table 5). At a height of 1.5 m above ground, the prochloraz treated plants had the highest Foc colony numbers in sap of all treatments. Unfortunately no baseline data was able to be collected at the beginning of the trial in regard to Foc presence in vascular tissue. These trials were preliminary in nature, and further studies are required to clarify the effects of different chemical application methods (e.g. soil drench, injection).

Microscopic examination of banana sap: When samples had discrete vascular discoloration without necrosis of associated tissues, no microconidia were found in the sap. Occasionally hyphal fragments were detected. When the disease was more advanced, with significant necrosis of associated tissue, a few microconidia were detected.

Conclusions

A major conclusion of this work is that the laticifers are not colonised by Foc but the sap from these laticifers can become contaminated with either hyphal fragments or microconidia when pseudostems are cut. No chlamydospores were detected in sap or xylem fluid suggesting that these survival spores are only produced when plant death is imminent. The result also shows that in the previous studies by Rishbeth (1955) and Stover (1962) are both probably correct. We suggest that Stover (1962) was sampling green pseudostems where infection was confined to xylem elements, whereas Risbeth (1955) was possibly sampling plants with more advanced symptoms of the disease. Stover (1962) found only hyphal fragments in his sap samples which one would expect if movement of the pathogen in the host plant is by mycelial growth rather than microconidia. We suggest that the microconidia present in Risbeth's sap samples may have come from associated diseased tissue and not from the xylem itself. The project which is being continued in conjunction with UQ will clarify this further.

Key Messages

When an infected pseudostem is cut the sap is likely to become contaminated with hyphal fragments or microconidia. The amount of cross-contamination is likely to depend on disease severity. Microconidia are more likely to be present where disease symptoms are well advanced. The sap could dribble from the cane knife blade and contaminate the soil. This needs to be taken into account in biosecurity destruction protocols if containment of the pathogen is to be achieved. Cane knives need to be cleaned and sterilised adequately between plants.

The epidemiology of Panama disease has received little attention during the past 60 years, despite it being such a devastating disease. A greater understanding of the epidemiology is required if the disease is to be contained or managed. There are many biological problems associated with this disease that are not well understood. More effective measures are required to suppress inoculum levels in both the plant and soil.

Where to next

We expect that the UQ study using GFP transformed isolates of Foc will give a definitive answer on the movement of the pathogen in the xylem vessels, and support our field studies. Similar studies have been conducted in other countries, but have used unrealistic inoculum levels and juvenile plants (Guo *et al.,* 2015; Xiao *et al*., 2013). The UQ study will involve much older plants and a more natural population of the pathogen.

Understanding the infection process in the banana will allow us to implement intervention strategies to reduce the amount of inoculum produced in an infected plant. Chemical intervention may be a valid strategy.

As banana roots do not contain laticifers it may be easier to examine the xylem tissue there. When a root is severed the cut end will exude xylem fluid. This is caused by the loading of solutes into the stele at the root tip and the subsequent uptake of water.

Although not part of this project, during the current study we located a Lady finger plant infected with Foc STR4. This isolate, along with isolates in Foc TR4, produces the volatile hydrocarbon bicyclo (4,2,0) octa-1,3,5-triene when grown on rice. We will determine if the pathogen produces this compound in infected banana plants. If so biosecurity officers may be able to use "sniffer" dogs to detect banana plants infected with FocTR4 before they become symptomatic. The disease can have a long incubation and latent period before symptoms are expressed.

Budget Summary

Revenue Type	Revenue Budget	Actual Revenue	Difference
Innovation funding	**-$45,435.70**	**-$45,435.70**	**$0.00**
Expense type	**Expense Budget**	**Actual Expense**	**Difference**
Employee expenses • Roger Mitchell • Tony Cooke • On-costs	**$37,835.73**	**$32,104.25** • $12,673.54 • $19,120.76 • $309.95	**$5,731.48**
Supplies & Services • Collaboration with UQ • Laboratory Supplies	**$8000.00**	**$6,269.04** • $5,000.00 • $1,269.04	**$1,730.96**

References

Guo L, Yang L, Liang C, Wang G, Dai Q, Huang J, 2015. Differential colonization patterns of bananas (*Musa* spp.) by physiological race 1 and race 4 Isolates of *Fusarium oxysporum* f.sp. *cubense*. *Journal of Phytopathology*, 163, 807-817.

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Xiao RF, Zhu YJ, Li YD, Liu B, 2013. Studies of vascular infection of *Fusarium oxysporum* f. sp. *cubense* race 4 in banana by field survey and green fluorescent protein reporter. *eSci Journal of Plant Pathology* 02(01), 44-51.

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Tables

Table 1*: Sap trial 1 – recovery of Foc from vascular tissue collected from excised pseudostem windows (0.5 m above ground) in Lady finger banana plants showing external symptoms of Panama disease. Data was collected from 3 plants for each category of disease severity.*

Disease severity	Recovery of Foc from tissue No. of colonies/4 isolation points
Asymptomatic	0.0 b[1]
Early symptoms[2]	2.7 a
Advanced symptoms[3]	4.0 a

[1] Means significantly different using Tukey's test at P=0.05

[2] Foc R1 was isolated from 2 of the 3 plants showing early symptoms of Panama disease. Foc was not isolated from the other plant showing early symptoms.

[3] Foc R1 was isolated from 2 of the 3 plants showing advanced symptoms of Panama disease. Foc STR4 was isolated from the other plant showing advanced symptoms.

Table 2*: Sap trial 1 - number of colony forming units (cfu) of Foc present in sap collected from excised pseudostem windows in Lady finger banana plants showing external symptoms of Panama disease. Sap was collected at 2 heights from the base of the pseudostem (0.5 and 1.5 m), and at 2 times after excision of window tissue (0 and 15 min). Data was collected from 3 plants for each category of disease severity.*

Disease severity	cfu/mL Foc in banana sap			
	0.5 m window 0 min	0.5 m window 15 min	1.5 m window 0 min	1.5 m window 15 min
Asymptomatic	0[1]	0	0	0
Early symptoms	175.0	0	16.5	0
Advanced symptoms	516.5	0	566.3	0

[1] No significant effects of window height on cfu/mL of Foc in sap were found using Tukey's test at P=0.05, but there were significant effects of disease severity and time after excision when averaged over the other 2 factors.

Table 3*: Sap trial 2 - number of colony forming units (cfu) of Foc present in sap collected from decapitated Lady finger banana plants showing external symptoms of Panama disease. Sap was collected at 2 times after excision (0 and 90 min). Data was collected from 3 plants.*

Time after excision (min)	cfu/mL Foc in banana sap	Recovery of Foc from tissue No. of colonies/4 isolation points
0	905.6 a[1]	4[2]
90	55.6 b	4

[1] Treatment means followed by the same letter within columns are not significantly different using Tukey's test at P=0.05

[2] Data not analysed

Table 4*: Sap trial 3 - number of colony forming units of Foc present in sap collected from decapitated Lady finger banana plants showing external symptoms of Panama disease. Sap was collected at 3 times after excision (0, 90 and 120 min). Data was collected from 2 plants.*

cfu/mL Foc in banana sap		
0 min	**90 min**	**120 min**
83.3[1]	125.0	0.0

[1] Data not analysed

Table 5*: Fungicide trial - number of colony forming units of Foc present in sap, and recovery of Foc from excised pseudostem windows, in Lady finger banana plants showing external symptoms of Panama disease and injected with 3 different chemical products. Sap was collected immediately after tissue excision at 2 heights from the base of the pseudostem (0.5 and 1.5 m). Unless otherwise specified, data was collected from 3 plants for each treatment.*

Treatment (injection of pseudostem)	cfu/mL Foc in banana sap		Recovery of Foc from tissue No. of colonies/10 isolation points	
	0.5 m window 0 min	1.5 m window 0 min	0.5 m window 0 min	1.5 m window 0 min
Prochloraz	494.4 ab[1]	561.1 a	7.7 a	6.7 a
Paclobutrazol	516.7 ab	150.0 b	7.7 a	8.0 a
Glyphosate	5.6 b	50.0 b	3.3 a	3.3 a
Untreated control[2]	1133.3 a	16.7 b	4.0 a	0.0 a

[1] Treatment means followed by the same letter within columns are not significantly different using Fisher's test at P=0.05

[2] Data from only one plant was available.

Images

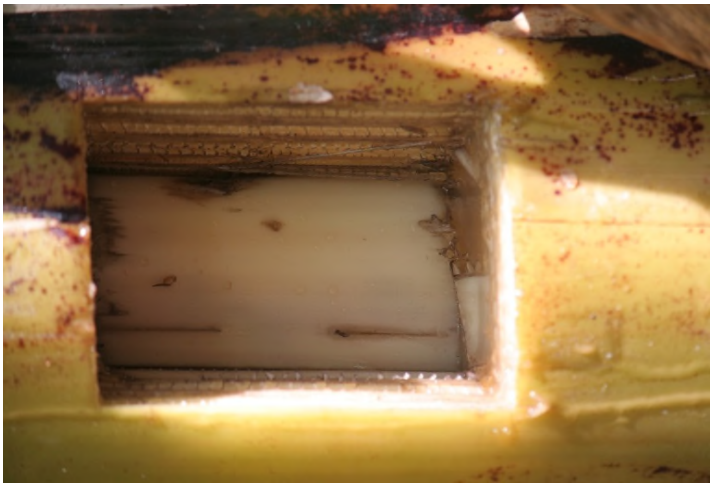
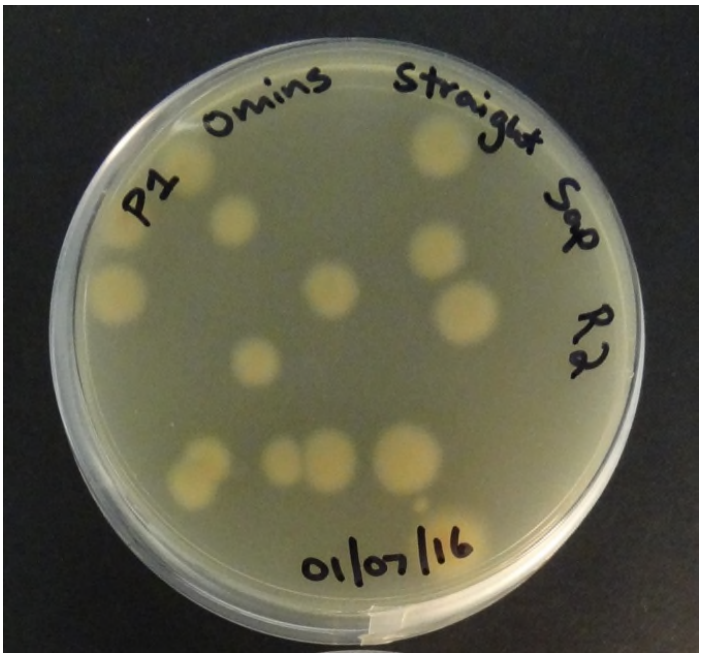





Image 1: Collection of field samples - cutting a window of tissue from a banana pseudostem (top left); discoloured vascular tissue inside pseudostem window (top right); window tissue sample with discoloured vascular strands (centre left); collection of initial sap flow from severed window tissue (centre right); injection of a banana plant (bottom left); collection of sap from a decapitated banana plant (bottom right).



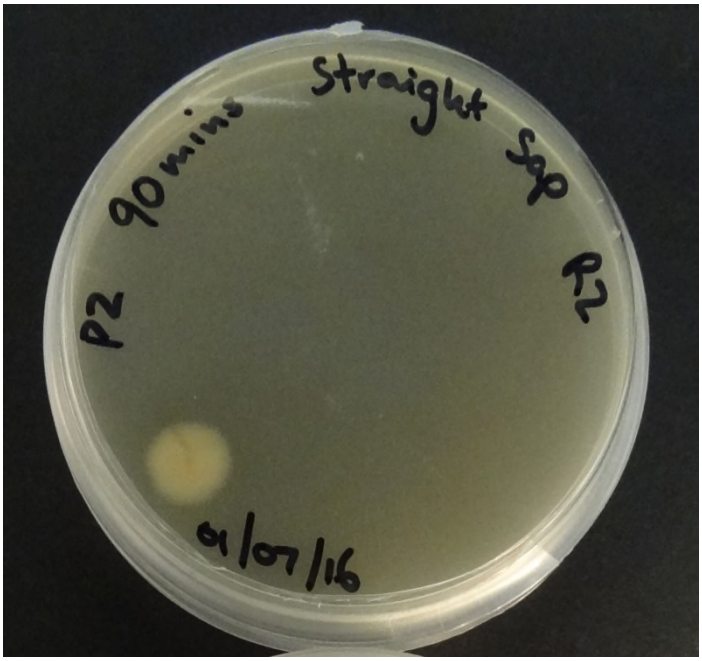


Image 2: Collection of field samples and Foc culturing from sap. Surface sterilisation of severed banana pseudostem with 70% ethanol prior to sap collection (top left); collection of xylem fluid from severed pseudostem (bottom left); Foc colonies (on Nash-Snyder agar) isolated from sap collected at 0 (top right) and 90 (bottom right) minutes after pseudostem severance.